Arginine methylation of the c-Jun coactivator RACO-1 is required for c-Jun/AP-1 activation

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Introduction

The AP-1 transcription factor is a heterodimeric complex of various Jun, Fos and ATF-2 family members and is induced by a multitude of signals including growth factors, cytokines and extracellular stresses (Davis, 2000). Consequently AP-1 mediates diverse cellular responses ranging from cell proliferation and differentiation to tumourigenesis and cellular apoptosis (Mehtha-Grigoriou et al., 2001; Shaulian and Karin, 2001; Eferl and Wagner, 2003).

c-Jun is essential for cellular proliferation and transformation by controlling the expression of cell cycle regulator genes including cyclinD1 and cdc2 (Johnson et al., 1996; Eferl et al., 1999; Wada et al., 2004). c-Jun-null fibroblasts display a severe proliferation defect, deficiency in cell cycle re-entry after serum withdrawal and an inability to undergo transformation by oncogenic Ras (Johnson et al., 1996; Schreiber et al., 1999).

We have recently described a novel c-Jun coactivator, RING domain AP-1 coactivator-1 (RACO-1), that links growth factor/oncogenic Ras signalling to AP-1 activation. Growth factor signalling stimulates RACO-1 function by increasing RACO-1 protein stability. Mechanistically, RACO-1 stability is controlled by the competition of degradative K48- and non-degradative K63-linked ubiquitylation. RACO-1 is a RING domain-containing ubiquitin E3 ligase and in unstimulated conditions RACO-1 is unstable due to K48-linked autoubiquitylation. Upon activation of the Ras/MEK/ERK pathway, K63-linked ubiquitin chains are attached to the same residues targeted for degradative ubiquitylation, thereby resulting in enhanced protein levels (Davies et al., 2010).

In mammalian cells, protein methylation occurs predominantly on lysine and arginine residues, with distinct families of methyltransferases targeting each amino acid. Despite being identified more than 30 years ago, relatively little is known about protein arginine methylation. Protein arginine methyltransferases (PRMTs) catalyse mono- and dimethylation of the guanidino group of the arginine residue using S-adenosyl methionine (SAM) as a methyl donor. Dimethylation can occur asymmetrically (ADMA) with the two methyl groups placed onto each of the terminal nitrogen groups of the guanidino group, or symmetrically (SDMA) where one methyl group is placed onto one of the terminal nitrogen atoms of the guanidino group. A large number of substrates for arginine methylation are being discovered, many of which are components of the cell cycle, tissue differentiation and development, oncogenesis, apoptosis and inflammation (Bedford and Clarke, 2009). PRMT1 has been shown to be involved in a number of these pathways, acting as a regulator of cell cycle progression, apoptosis and oncogenic transformation. The RING domain-containing ubiquitin E3 ligase and in unstimulated conditions RACO-1 is unstable due to K48-linked autoubiquitylation. Upon activation of the Ras/MEK/ERK pathway, K63-linked ubiquitin chains are attached to the same residues targeted for degradative ubiquitylation, thereby resulting in enhanced protein levels (Davies et al., 2010).

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Results

c-Jun binds to nuclear dimeric RACO-1

RACO-1 is expressed ubiquitously throughout the cell and is present in both the cytoplasm and nucleus (Davies et al., 2010). We have recently described a novel c-Jun coactivator, RING domain AP-1 coactivator-1 (RACO-1), that links growth factor/oncogenic Ras signalling to AP-1 activation. Growth factor signalling stimulates RACO-1 function by increasing RACO-1 protein stability. Mechanistically, RACO-1 stability is controlled by the competition of degradative K48- and non-degradative K63-linked ubiquitylation. RACO-1 is a RING domain-containing ubiquitin E3 ligase and in unstimulated conditions RACO-1 is unstable due to K48-linked autoubiquitylation. Upon activation of the Ras/MEK/ERK pathway, K63-linked ubiquitin chains are attached to the same residues targeted for degradative ubiquitylation, thereby resulting in enhanced protein levels (Davies et al., 2010).
To test whether these two pools of RACO-1 protein may show functional differences, we performed immunoprecipitation (IP) experiments using GST–c-Jun. Notably, recombinant c-Jun specifically interacted with nuclear RACO-1 but not with cytoplasmic RACO-1 protein (Figure 1A). We therefore sought to identify functional differences between cytoplasmic and nuclear pools of RACO-1 protein. In addition to the analysis of RACO-1 post-translational modifications (see below), we also determined whether RACO-1 was capable of dimerisation. Cells were transfected with both Flag- and GFP-tagged RACO-1 followed by Flag IPs. GFP–RACO-1 was co-immunoprecipitated with Flag–RACO-1, implying that dimerisation does occur (Figure 1B). Point mutations of the first two cysteine residues of the RACO-1 RING domain (RACO-1 RM) destroy RING integrity (Davies et al., 2010); however, dimer formation was not affected. Thus, the RING domain is dispensable for dimerisation (Figure 1B). Furthermore, we investigated whether RACO-1 dimerisation was influenced by subcellular localisation, and found that RACO-1 dimerisation occurred specifically within the nucleus (Figure 1C). Next, we investigated RACO-1 dimerisation by biologically crosslinking RACO-1 complexes after immunoprecipitation. Denaturing SDS–polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting for RACO-1 showed that a substantial proportion of RACO-1 migrated at the molecular weight predicted for dimeric RACO-1 after crosslinking (Figure 1D, lane 3). In agreement with this, non-denaturing native gel analysis showed that endogenous RACO-1 protein migrated in two different forms. In addition to the monomeric protein, a second RACO-1 protein form migrating at higher molecular weight was detected. Importantly, depletion of RACO-1 by stable integration of shRNA construct against RACO-1 reduced both RACO-1 protein forms (Figure 1E).

To test the functional significance of the RACO-1 dimer, RACO-1 was biologically crosslinked, which resulted in the

![Figure 1](https://example.com/figure1.png)
formation of covalent RACO-1 dimers with approximately 50% efficiency, that is, roughly half of the total RACO-1 protein was monomeric and the other half was present as a dimer (Figure 1F, lane 2). GST–c-Jun pull-downs showed that recombinant c-Jun bound to dimeric crosslinked RACO-1 with much higher efficiency compared to monomeric RACO-1 (Figure 1F). Taken together, these findings imply that the RACO-1 dimer is a physiologically relevant binding partner of c-Jun.

**Analysis of RACO-1 dimerisation**

Next, we investigated RACO-1 dimerisation. Immuno-precipitated full-length Flag–RACO-1 interacted with two sequential N-terminal deletion constructs (RACO-1 (72–235) and RACO-1 (110–235)) as efficiently as wild-type protein (Figure 2A), implying that the dimer interface resides within the C-terminal portion of the protein. Fine mapping of this C-terminal region using overlapping 60–70 amino acid protein fragments revealed that the C-terminal 72 amino acids (RACO-1 (163–235)) were sufficient for dimer formation with full-length RACO-1 protein (163–235) was generated as a synthetic biotinylated peptide and used for pull-down experiments with cell lysates expressing overlapping GFP-fused RACO-1 fragments encompassing the C-terminal domain (110–235). Biotin–RACO-1 (163–235) strongly interacted with a long C-terminal fragment (GFP–RACO-1 (110–235)) and a shorter protein corresponding to residues 163–235 of RACO-1 (Figure 2C). Thus, RACO-1 dimerisation is mediated by a homodimerisation domain located in the very C-terminus of the protein. However, RACO-1 molecular interactions turned out to be more complex. RACO-1 (1–139), which lacks the C-terminal homodimerisation domain, and RACO-1 (72–139), which in addition lacks the RING domain, were able to interact with full-length RACO-1 as efficiently as wild-type protein (Figure 2D). This suggested the presence of a second interaction domain within the RACO-1 N-terminus. Further fine mapping of additional RACO-1 protein interaction. Lysates from HEK293T cells coexpressing Flag-tagged dimerisation domain deleted RACO-1 (RACO-1 (1–139)) with full-length and various N-terminal GFP-tagged truncated proteins were immunoprecipitated with α-FLAG antibodies and immunoblotted for associated GFP-tagged RACO-1. Schematic below represents constructs used. (F) Flag–RACO-1 (1–139) interacts with a biotinylated RACO-1 peptide corresponding to residues 163–235. Peptide-associated RACO-1 (1–139) was resolved by SDS–PAGE and immunoblotted with α-Flag antibody. Source data for this figure is available on the online supplementary information page.
mapping revealed that the region of RACO-1 identified as the homodimerisation interface (residues 163–235) was also able to interact with the RACO-1 N-terminus (Figure 2E). A biotinylated peptide corresponding to residues 163–235 of RACO-1 was sufficient to interact with Flag–RACO-1 (1–139) (Figure 2F). Therefore, the very C-terminus of RACO-1 is able to interact with two independent domains within RACO-1: a homotypic C-terminal interaction that mediates dimerisation and a second intramolecular interaction between the RACO-1 C- and N-termini.

**RACO-1 is methylated on two arginine residues**

The complex inter- and intramolecular interaction of RACO-1 could represent a mechanism of functional regulation. To investigate this, we performed LC-MS/MS analysis to identify post-translational modifications occurring on RACO-1. We consistently detected the presence of methyl groups located on residues arginine 98 (R98) and arginine 109 (R109). These modifications could be detected using two different forms of enzymatic digestion (Trypsin or Asp-N; Figure 3A and B).

To confirm that RACO-1 was methylated in vivo, we labelled cells with radioactive [3H]-methyl methionine. To exclude incorporation of radioactivity by protein synthesis, protein translation was blocked by addition of cyclohexamide (CHX). CHX treatment was efficient as it prevented incorporation of radioactively labelled 35S into RACO-1 and MBD2, a protein known to be arginine methylated (Tan and Nakielny, 2006) that was used as a positive control for this experiment (Figure 3C). The [3H]-methyl methionine in vivo serves as a precursor of SAM, which is the methyl donor for arginine methylation reactions. RACO-1 and MBD2 were then immunoprecipitated, resolved by SDS–PAGE and exposed to autoradiography film. The [3H]-methyl methionine was incorporated into bands corresponding to the molecular weights of RACO-1 and of MBD2 (Figure 3C). Hence, RACO-1 is methylated in vivo.

**PRMT1 methylates RACO-1**

Mammalian arginine methyltransferases comprise a family of nine highly related proteins. To investigate which protein was responsible for methylating RACO-1, we performed pull-down experiments using recombinant GST–fusion proteins of PRMT1, PRMT3 and CARM1, three of the better-characterised arginine methyltransferases. Transfected RACO-1 interacted with GST–PRMT1 but not GST–PRMT3, GST–CARM1 or GST alone (Figure 4A) and RACO-1 and PRMT1 co-immunoprecipitated from cells (Figure 4B). To verify that RACO-1 is a novel substrate for PRMT1 and not solely a binding partner, we performed in vitro methylation assay using recombinant PRMT1 and RACO-1 in the presence of the methyl donor [3H]-SAM. Recombinant RACO-1 was effectively methylated by recombinant PRMT1 (Figure 4C). We also attempted to generate methyl-specific antibodies directed towards asymmetric di-methyl-R98 and asymmetric di-methyl-R109 of RACO-1; however, neither a polyclonal nor a monoclonal antibody could be successfully produced (data not shown). Subsequently, to further demonstrate RACO-1 arginine methyl transferase in vivo, we generated a stable cell line in which endogenous PRMT1 was silenced by expression of an shRNA construct (Figure 4D). We noted that when Flag–RACO-1 was IP-ed from [3H]-methyl methionine-labelled cells, a single radioactive band was immunoprecipitated (Figure 3C, left panel), implying that RACO-1 is the only protein methylated to a detectable level in the Flag IP. Therefore, we subsequently measured incorporated radioactivity into RACO-1 by scintillation counting. In vivo [3H]-methyl methionine labelling of this cell line after expression of Flag–RACO-1 demonstrated a substantial reduction in the methylation of RACO-1 compared to scrambled shPRMT1-expressing cells (Figure 4D).

As further negative controls, we observed that immunoprecipitated PRMT5 failed to promote the incorporation of [3H]-methyl groups into recombinant RACO-1 (Supplementary Figure 1A). Moreover, in vivo methylation assay in PRMT5-silenced cell lines did not prevent RACO-1 methylation (Supplementary Figure 1B).

Finally, to further validate R98 and R109 as targets for PRMT1-mediated methylation, we compared in vitro methylation of a recombinant wild-type RACO-1 fragment corresponding to residues 72–139 to a mutant RACO-1 form in which the arginine residues R98 and R109 were substituted to lysine (R98/109K), thereby maintaining the positive charge. Mutation of both arginine R98 and R109 reduced methylation of RACO-1 to background levels (Figure 4E). Taken together, these results demonstrate that the arginines at positions 98 and 109 are the predominant methylated residues within RACO-1, and that PRMT1 is the methyltransferase that binds to and methylates RACO-1.

**Arginine methylation stabilises RACO-1**

RACO-1 is a highly labile protein and RACO-1 stability is controlled by the competition of degradative K48- and nondegradative K63-linked ubiquitylation. (Davies et al, 2010). Arginine methylation appeared to counteract RACO-1 degradation, as overexpression of myc–PRMT1 led to a considerable increase in RACO-1 protein levels (Figure 5A) and half-life as determined by CHX chase (Figure 5B). Moreover, mutation of the methylation acceptor arginine residues R98 and R109 to lysines decreased RACO-1 protein levels, an effect that could be partially reverted by proteasome inhibition (Figure 5C). However, mutations of R98 and R109 did not change the subcellular localisation of RACO-1 (Supplementary Figure S2). PRMT1 overexpression increased endogenous RACO-1 protein levels in both the breast cancer cell line BT474 and the lung cancer cell line H727 (Figure 5D), while silencing of PRMT1 reduced RACO-1 levels (Figure 5E).

We have previously shown that K48-linked autoubiquitylation is counteracted by Ras/MEK1-induced K63-linked ubiquitylation (Davies et al, 2010). Given that arginine methylation can affect the deposition of other post-translational modifications on the same protein (Hyllus et al, 2007; Yamagata et al, 2008), we thus investigated the impact of arginine methylation on K63-linked ubiquitylation of RACO-1. Mutation of either R98 or R109 completely suppressed ubiquitylation of RACO-1 induced by constitutively active MEK1 (MEK1 R4F) (Figure 5F), while silencing of PRMT1 reduced MEK1 R4F-induced ubiquitylation (Figure 5G). Moreover, experiments using ubiquitin mutants, which are unable to form specific chains (UbK48R or UbK63R), showed that PRMT1 promoted the formation of K63-linked ubiquitin chains on RACO-1 (Figure 5H). Hence, PRMT1-mediated methylation of RACO-1 at R98 and R109 is a prerequisite for K63-linked ubiquitylation and RACO-1 protein stabilisation.
Arginine methylation mediates RACO-1 dimer formation

The minimal N-terminal region of RACO-1 required for interaction with the RACO-1 C-terminus mapped to amino acids 72–139 and thus includes the methylated arginine residues at positions 98 and 109. We therefore investigated whether arginine methylation plays a role in RACO-1 intramolecular interactions.

A biotinylated peptide corresponding to residues 163–235 of RACO-1 interacted with Flag–RACO-1 (72–139). In Figure 3, RACO-1 is arginine methylated in vivo on residues R98 and R109. (A) Identification of arginine modifications on RACO-1 protein. The upper MS/MS spectrum shows the fragment ions of the 2+ charged peptide ion at m/z 757.876 generated from an ‘in gel’ tryptic digest of RACO-1. The lower spectrum indicates the fragment ions of the 2+ charged peptide ion at m/z 793.412 generated from an Asp-N proteolytic digestion of RACO-1. (B) Characterisation of arginine modifications on the RACO-1 protein. The MS/MS fragment ion spectrum of the 2+ charged peptide ion at m/z 588.267 is shown. The peptide was generated by the ‘in gel’ tryptic digestion of RACO-1. (C) RACO-1 is methylated in vivo. Cells were transfected with Flag-RACO-1 or Flag-MBD2 (control) and labelled with [3H]-methyl methionine (left panel) or [35S]-methionine (middle and right panels). Proteins were immunoprecipitated and incorporated methyl groups detected by SDS–PAGE followed by autoradiography. CHX/CAM treatment effectively blocked de novo protein synthesis as shown by the inability of [35S]-methionine to be incorporated into newly synthesised RACO-1 and MBD2 (compare middle and right panels). Source data for this figure is available on the online supplementary information page.

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contrast, mutation of R98 and R109 to lysine within this fragment (Flag–RACO-1 (72–139 R98/109K)) prevented interaction (Figure 6A). Thus, arginine methylation regulates RACO-1 intramolecular binding.

Next, we investigated whether RACO-1 methylation would impact on dimer formation. Disruption of methylation on only one monomer was sufficient to greatly diminish dimerisation (Figure 6B, compare lanes 2 and 3), suggesting that arginine methylation is required in cis for dimer formation, that is, concurrent methylation of both molecules is required for efficient RACO-1 dimerisation. To further support this, we found that in vitro PRMT1-mediated methylation of RACO-1 is required for dimer formation. Nondenaturing gel analysis of endogenous RACO-1 showed that silencing of PRMT1 greatly reduced the abundance of dimeric RACO-1, and increased the prevalence of monomeric RACO-1. This was particularly apparent when lysate concentrations were adjusted to take into account that silencing of PRMT1 reduces RACO-1 protein levels (Figure 6C, compare lanes 2 and 4). Thus, RACO-1 methylation promotes a conformational change that increases RACO-1 protein stability and makes RACO-1 competent for dimerisation.

**PRMT1-mediated RACO-1 arginine methylation is required for c-Jun interaction and AP-1 target gene expression**

Since arginine methylation is required for RACO-1 stabilisation and dimerisation, and dimerisation is a prerequisite for c-Jun binding (Figure 1F), we tested whether RACO-1 methylation might be essential for c-Jun binding. Indeed, mutation of both methylated RACO-1 arginine residues R98 and R109, and also mutation of each single arginine, was sufficient to substantially reduce RACO-1 binding to c-Jun (Figure 6D). Moreover, silencing of PRMT1 markedly reduced RACO-1 interaction with c-Jun, confirming the central role of PRMT1 in the regulation of RACO-1 function (Figure 6E). Strikingly, when wild-type and methylation-defective R98/R109K mutant RACO-1 proteins were co-overexpressed,
c-Jun only interacted with wild-type RACO-1 (Figure 6F). This demonstrates that c-Jun can only interact with dimeric arginine methylated RACO-1, and that both molecules of the RACO-1 dimer need to be methylated, reminiscent of the requirement for arginine methylation in cis for RACO-1 dimer formation (Figure 6B and C).

In order to investigate the biological importance of RACO-1 arginine methylation, we performed microarray analysis on HCT116 cells transfected with PRMT1-silencing oligos (siPRMT1) and compared gene expression of PRMT1-depleted HCT116 cells with the effects of c-Jun (siJun) and RACO-1 knockdown (siRACO-1) (Figure 7A). We have observed previously that of the 88 genes displaying >1.5-fold reduction in expression by siJun, 72 are also downregulated by RACO-1 knockdown and silencing constructs were generated (left panel) and then transfected with GFP–RACO-1 and MEK1-R4F. Ubiquitylated RACO-1 was resolved by Ni²⁺-NTA affinity purification and immunoblotting with GFP antibodies (right panel). Overexpression of PRMT1 induces K63-linked ubiquitylation. HEK293T cells were transfected as indicated and ubiquitylated RACO-1 resolved by Ni²⁺-NTA affinity purification and immunoblotting with GFP antibodies. Source data for this figure is available on the online supplementary information page.

Figure 5 Methylation of RACO-1 is required for protein stabilisation. (A) Overexpression of PRMT1 stabilises RACO-1 expression levels and (B) increases protein half-life as determined by cycloheximide (CHX) chase. (C) Mutation of R98 or R109 to lysine reduces protein expression and this can be rescued by proteasome inhibitor treatment (MG132). (D) Overexpression of PRMT1 increases endogenous RACO-1 expression in BT474 and H727 cells, while (E) silencing of PRMT1 decreases endogenous RACO-1 levels in H727 cells. (F) Ubiquitylation of RACO-1 by MEK1-R4F requires R98 and R109. Cells were transfected as indicated and ubiquitylated RACO-1 resolved by Ni²⁺-NTA affinity purification and immunoblotting with Flag antibodies. (G) Silencing of PRMT1 reduces MEK1-R4F induced RACO-1 ubiquitylation. BT474 stable cell lines expressing scrambled or shPRMT1 silencing constructs were generated (left panel) and then transfected with GFP–RACO-1 and MEK1-R4F. Ubiquitylated RACO-1 was resolved by Ni²⁺-NTA affinity purification and immunoblotting with GFP antibodies (right panel). (H) Overexpression of PRMT1 induces K63-linked ubiquitylation. HEK293T cells were transfected as indicated and ubiquitylated RACO-1 resolved by Ni²⁺-NTA affinity purification and immunoblotting with GFP antibodies. Source data for this figure is available on the online supplementary information page.

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Taken together, these results imply that arginine methylation of RACO-1 by PRMT1 is required for efficient expression of c-Jun target genes and identify PRMT1 as an important physiological regulator of c-Jun/AP-1 function.

**Discussion**

We have described RACO-1 as a growth factor-regulated coactivator of c-Jun (Davies et al, 2010). Here we demonstrate an additional level of control of RACO-1 that involves methylation on two arginine residues by PRMT1. This is the first report showing that arginine methylation regulates the AP-1 transcriptional response.

PRMT1 regulates transcription by methylating both histone and nonhistone substrates. PRMT1 stimulates transcription by methylating histone H4 arginine 3 (H4R3), which is read by the scaffolding protein TDRD3 that facilitates gene transcription (Yang et al, 2010). PRMT1 is the main arginine methyltransferase in mammalian cells (Tang et al, 2000), but only a small number of nonhistone substrates involved in transcriptional regulation are known. PRMT1 stimulates transcription by methylating coactivator proteins such as PGC-1α and NIP45 and the nuclear receptor corepressor RIP140 (Mowen et al, 2004; Teysier et al, 2005; Mostaql Huq et al, 2006). The identification of RACO-1 thus extends the rather short list of PRMT1 substrates involved in transcription. PRMT1 recognises substrates with glycine/arginine-rich (GAR) motifs (especially RGG repeats). However, many PRMT1 substrates, including RACO-1, lack this consensus sequence, and it has been suggested that PRMT1 recognises additional binding sites on substrates distal to the methylated residue (Osborne et al, 2007; Wooderchak et al, 2008). This means that the number of PRMT1 substrates may be underestimated by conventional *in silico* analysis, suggesting that the importance of arginine methylation is yet to be fully appreciated.

PRMT1-mediated methylation of arginines 98 and 109 controls several aspects of RACO-1 function. RACO-1 is a highly unstable protein due to autoubiquitylation. It is noteworthy that the three most C-terminal lysines (K195, K196, K197) in RACO-1 are not substrates for PRMT1 (Osborne et al, 2007; Wooderchak et al, 2008). PRMT1 recognises substrates with glycine/arginine-rich (GAR) motifs (especially RGG repeats). However, many PRMT1 substrates, including RACO-1, lack this consensus sequence, and it has been suggested that PRMT1 recognises additional binding sites on substrates distal to the methylated residue (Osborne et al, 2007; Wooderchak et al, 2008). This means that the number of PRMT1 substrates may be underestimated by conventional *in silico* analysis, suggesting that the importance of arginine methylation is yet to be fully appreciated.

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K223 and K224) within RACO-1 are the preferential targets of autoubiquitylation (Davies et al., 2010). Thus, the N-terminal RING domain and the RACO-1 C-terminus, while separated in primary amino acid sequence, may be in proximity in the tertiary structure to allow degradative autoubiquitylation (Figure 8). We hypothesise that methylation of arginines 98 and 109 would enable the C-terminal region to bind to the methylated domain, thus inducing a conformational change that displaces the RING domain from the lysines targeted by autoubiquitylation, thereby facilitating K63-linked ubiquitylation. This stable conformer of RACO-1 would then be competent for dimerisation, and this conformation would also be capable of c-Jun binding. It is worth noting that we cannot discriminate whether the interaction between the RACO-1 C-terminus region and the methylated domain occurs in cis or in trans. However, we favour the hypothesis that this interaction occurs in cis, since the local concentration of the interacting domains is vastly higher for an intramolecular interaction and, secondly, methylation of each dimer partner protein is required for efficient dimer formation and c-Jun binding (Figure 6B and F). Thus, we propose that a conformational change in RACO-1 is the key mechanism that underlies the regulation of RACO-1 by arginine methylation.

Arginine methylation has been most extensively studied in the context of histone tail modification and the epigenetic regulation of gene expression. PRMT1-mediated methylation of RACO-1 is the first time that arginine methylation has been shown to be important for AP-1 activation. PRMT1 appears to be a major regulator of AP-1-mediated transcription, since a large number of genes whose expression is dependent on c-Jun and RACO-1 are also dependent on PRMT1 (Figure 7A). This suggests that PRMT1 inactivation may substantially...
impair AP-1 function. In support of this, mouse embryonic fibroblasts lacking PRMT1 exhibit growth arrest and accumulate in G2/M phase of the cell cycle (Yu et al, 2009), which are phenotypes also observed in c-Jun-deficient MEFs (Johnson et al, 1993; Schreiber et al, 1999; Wada et al, 2004). c-Jun has important functions in intestinal tumour formation (Nateri et al, 2005; Sancho et al, 2009), and RACO-1 overexpression cooperated with oncogenic Ras and Apc in intestinal tumourigenesis (Davies et al, 2010). Expression of PRMT1 gene variant v1 was shown to be elevated in colorectal tumours and high expression of PRMT1v1 correlated positively with disease progression and aggressiveness (Mathioudaki et al, 2008). Moreover, patients strongly expressing the PRMT1v1 have a higher probability of relapse and lower survival compared with patients with low expression. Therefore, it may be worthwhile to investigate a potential function of PRMT1 in intestinal cancer and AP-1 regulation, especially since pharmacological PRMT1 inhibitors are being developed (Infantino et al, 2010; Dowden et al, 2011).

**Materials and methods**

**Cell lines and antibodies**

Human embryonic kidney (HEK) 293T, HCT116 human colon adenocarcinoma cells, BT474 breast carcinoma cells and H727 lung cancer cells were maintained in DMEM supplemented with 10% FCS. β-Actin, Flag, RACO-1 and HA (rabbit) antibodies were obtained from Sigma, GFP (rabbit) from Abcam (Cambridge, UK), myc (monoclonal clone 9E10) was generated in-house and PRMT1 (rabbit) was obtained from Cell Signalling.

**cDNA constructs and cell transfection**

All mutagenic/fragment constructs generated were confirmed by sequence analysis and primer sequences for all constructs are available on request. Myc–PRMT1 and GST–GAR were a gift from Uta-Maria Bauer (University of Marburg). GFP–PRMT1 was a gift from Mark Bedford (MD Anderson Cancer Center, The University of Texas), GST–PRMT1, GST–PRMT3 and GST–CARM1 were kind gifts from Steven Clarke (UCLA). HEK293T cells were transfected with Lipofectamine Plus (Invitrogen) and HCT116 with Lipofectamine 2000 (Invitrogen). siRNA oligos were transfected using DharmaFECT 1 (Dharmacon).

**RNAi constructs and generation of cell lines with stable knockdown of PRMT1 and RACO-1**

Annealed hairpin oligo siRNA sequences directed towards human PRMT1 (sense strand 5'-AGATTACTAATCTGACACTCC-3'; scrambled PRMT1: sense strand 5'-GCATATATTTCCCAATGT-3') were cloned into pRetroSuper (puro) (OligoEngine). For stable knockdown cell lines, HEK293T, BT474 and H727 cells were transfected and individual clones isolated by puromycin selection (2 μg/ml). Generation of RACO-1 stable knockdown cell lines was performed by lentiviral infection as described previously (Davies et al, 2010). Hairpin sequences (depicted in uppercase characters) were: shRACO-1 (5'-tgTATGCGTAGGAAGAAttcaagagaTTCTTCCTACGGTCCATC-3') and shLUC (5'-tgTATGCGTAGGAATCTTCGAttcaagaga-3'). Nontargeting siRNA and shJun constructs were obtained from Sigma.

**Reporter assay and microarray analysis**

HCT116 cells were seeded in 24-well plates and transfected with 250 ng 2 × AP-1 luciferase reporter construct (Fontana et al, 2012), 50 ng ubiquitin- Renilla, 425 ng of respective shRNA constructs and 50 ng CA-MEK1. After transfection, cells were maintained in serum-free medium for 24 h before cell lysis. The Dual Luciferase reporter assay (Promega) was used according to the manufacturer’s instructions. For microarray analysis, HCT116 cells were transiently transfected by DharmaFECT 1 (Dharmacon) with silencing oligos directed towards RACO-1 (Sigma), c-Jun (Sigma) and PRMT1 (Dharmacon). At 48 h after transfection, cells were harvested for RNA extraction using the RNeasy kit (Qiagen) and subjected to DNase treatment (Ambion) according to the manufacturer’s instructions. Microarray analysis was performed by the Molecular Biology Core Facility, Paterson Institute for Cancer Research, Manchester, UK. Biological pathway enrichment analysis of the genes was carried out using DAVID (Database for Annotation,
Visualization and Integrated Discovery v6.7 (Huang da et al., 2009a, b).

**qRT–PCR on cell lines**

RNA was extracted from cells using the Qiagen RNeasy mini kit, DNase treated (Ambion) and cDNA synthesised using Superscript III (Invitrogen) according to the manufacturer’s instructions. qPCR was performed using a ABI Prism 7900HT Sequence Detection System with SYBR Green incorporation. qPCR primers were designed.

Human c-Jun forward: 5′-ccaaagcgaggccgagtgtt-3′ and reverse: 5′-gcgccctctcaagctgtatgctgac-3′; human CyclinD1 forward: 5′-aagctgcttgctcgggagca-3′ and reverse: 5′-aagctgctgtaaatggccggtgct-3′; human cdc2 forward: 5′-gtgagtcaggataaatgctgatc-3′ and reverse: 5′-gtgagtcaggataaatgctgatc-3′; human Actin forward: 5′-tgctgagccaagcagggattc-3′ and reverse: 5′-gcgctgagcccagcagggattc-3′. All primer pairs generated a single product as determined by dissociation curve analysis.

**GST fusion protein expression**

c-Jun (1–285), lacking the leucine zipper motif, was cloned by PCR into pGEX-6P1. DNA was transformed into BL21 (DE3) bacteria and IPTG for 4 h at 37 °C. Purified recombinant GST fusion protein expression was generated a single product as determined by dissociation curve analysis.

**Isolation of cytoplasmic and nuclear extracts and Ni2⁺-NTA-agarose purification**

Cytoplasmic and nuclear extracts were isolated as described previously (Davies et al., 2010). For co-immunoprecipitations, volumes of nuclear and cytoplasmic lysates were made up to 1 ml with Buffer A (20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2% NP-40, protease inhibitor cocktail (Sigma), 100 mM PMSF, 1 mM Na3VO4, 50 mM NaF and 1 mM β-glycerophosphate) before immunoprecipitation. For 4 h before lysis, 25 μM of the proteasome inhibitor MG132 (Calbiochem) was added to cells. For in vivo ubiquitination assays, His-Ubi was affinity purified with Ni⁺⁺-NTA-Agarose beads as described previously (Campanero and Fleming, 1997).

**In vivo [3H]-methyl methionine and [35S]-methionine labelling**

Transfected cells were plated in complete DMEM (methionine free) media and incubated with cycloheximide (100 µg/ml) and chloramphenicol (40 µg/ml) for 1 h to inhibit de novo protein synthesis. [3H]-Methyl methionine (specific activity 70–85 Ci (2.59–3.145 TBq)/mmol, PerkinElmer) or EasyTag [35S]-methionine (specific activity 1000 Ci (37.0 TBq)/mmol, PerkinElmer) was then added to cultures for a further 4 h. Cells were harvested and lysed in Buffer A and Flag–RACO-1 immunoprecipitated overnight with 5-Flag-agarose beads (Sigma). After four washes with Buffer A, immunoprecipitates were denatured, resolved by SDS–PAGE and proteins transferred to nitrocellulose membrane. To verify equal loading of expressed and endogenous RACO-1, protein expression was verified by immunoprecipitation of transfected plasmids, one-tenth of the immunoprecipitated proteins transferred to nitrocellulose membrane. To verify equal protein synthesis, one-tenth of the immunoprecipitated proteins transferred to nitrocellulose membrane. To verify equal protein synthesis, one-tenth of the immunoprecipitated proteins transferred to nitrocellulose membrane.

**In vitro methylation assay**

Purified recombinant GST–PRMT1 was incubated with 10 μg His-SUMO–RACO-1 and 2 μl S-[methyl-3H]-adenosyl-l-methionine (SIGSAM) (specific activity: 35–85 Ci/mmol, PerkinElmer) in a total volume of 55 µl supplemented with 100 mM sodium phosphate (pH 7.5) buffer. Reactions were incubated at 37 °C for 1 h and denatured protein resolved by SDS–PAGE. Protein was transferred onto PVDF membrane, the tritium signal was enhanced by treating membranes with EN3HANCE (PerkinElmer) and was exposed to autoradiography film for at least 1 month at –80 °C.

**Mass spectrometry**

pR8E2-HA-RACO-1 was transfected into 293T cells (4 × 10⁶ dishes per condition) and 24 h later treated with the proteasome inhibitor MG132 (25 μM, Calbiochem) for 4 h before lysis with Buffer A. After sonication and clarification, cell lysates were incubated with HA–agarose beads (Sigma) for 2 h and then washed 4 × with Buffer A. Immunoprecipitations were resolved on a 4–12% Bis/Tris gradient gel (NuPage, Invitrogen) and stained without fixing using a methanol-free colloidal coomassie blue stain (Protoc) Safe Blue, National Diagnostics). Polyacrylamide gel slices (1–2 mm) containing purified RACO-1 were prepared for mass spectrometric analysis using the iodoacetamide liquid handling system (PerkinElmer, UK). Briefly, the excised protein gel pieces were placed in a well of a 96-well microtitre plate and destained with 50% v/v acetonitrile and 50 mM ammonium bicarbonate, reduced with 10 mM DTT, and alkylated with 55 mM iodoacetamide. After alkylation, proteins were digested with either 6 ng/μl Trypsin (Promega, UK) or 6 ng/μl endoprotease Asp-N (Roche Diagnostics, Germany) overnight at 37 °C. The resulting peptides were extracted in 1% v/v formic acid, 2% v/v acetonitrile. The digest was analysed by nano-scale capillary LC-MS/MS using a nanoAcquity UPLC (Waters, UK) to deliver a flow of 300 nl/min. A C18 Symmetry 5 μm, 180 μm × 20 mm μ-Precolumn (Waters) trapped the peptides prior to separation on a C18 BEH130 1.7 μm, 75 μm × 100 mm analytical UPLC column (Waters). Peptides were eluted with a gradient of acetonitrile. The analytical column outlet was interfaced with a Triversa nanomate microfluidic chip for mass spectrometric analysis (Advion, UK). Mass spectrometric information was obtained using an orthogonal-acceleration quadrupole-time-of-flight mass spectrometer (SYNAPT HDMS, Waters). Data-dependent analysis was carried out, where automatic MS/MS was acquired on the eight most intense, multiply-chargeur ion precursors in the m/z range 400–1500. MS/MS data were acquired over the m/z range 50–1995. LC/MS/MS data were then searched against a protein database (Uniprot KB, release 15.5 or an in-house sequence database) using the Mascot search engine program (Matrix Science, UK) (Perkins et al., 1999). The experiment was repeated eight times.

**In vitro chemical crosslinking assay**

HEK293T cells were transfected for 6 h using Lipofectamine PLUS reagent with Flag–RACO-1. After 24 h, cells were lysed in Buffer A and immunoprecipitated for 2 h using α-Flag-agarose beads (Sigma). After three washes in Buffer A, 100 mM sodium phosphate buffer (pH 7.5), associated proteins were eluted with two rounds of Flag peptide elution (Sigma, 0.2 mg/ml, 10 min shaking at 25 °C). Of the pooled eluate, 50% was left untreated while the remainder was chemically crosslinked with glutaraldehyde (0.5%, RT from 30 min). Reaction mixtures were terminated with 100 mM Tris–HCl, pH 7.5, resolved by SDS–PAGE and immunoblotted with α-Flag antibody. In some experiments, after chemical crosslinking, reactions were further subdivided and incubated with GST or GST-c-Jun for 2 h at 4 °C with rotation. Pull-downs were washed with Buffer A and resolved by SDS–PAGE and immunoblotted with anti-Flag antibody.

**Biotin pull-down assays**

Biotin-labelled RACO-1 peptides were synthesised in-house and immobilised onto streptavidin-coated DynaBeads (Dynal) as follows: biotinylated peptide (1 mg) was incubated with Dynabeads M280 streptavidin in buffer containing 100 mM sodium phosphate buffer (pH 7.5), associated proteins were eluted with two rounds of Flag peptide elution (Sigma, 0.2 mg/ml, 10 min shaking at 25 °C). Of the pooled eluate, 50% was left untreated while the remainder was chemically crosslinked with glutaraldehyde (0.5%, RT from 30 min). Reaction mixtures were terminated with 100 mM Tris–Cl, pH 7.5, resolved by SDS–PAGE and immunoblotted with α-Flag antibody. In some experiments, after chemical crosslinking, reactions were further subdivided and incubated with GST or GST-c-Jun for 2 h at 4 °C with rotation. Pull-downs were washed with Buffer A and resolved by SDS–PAGE and immunoblotted with anti-Flag antibody.

**Nondenaturing PAGE analysis**

H727 cells, with stably integrated control shRNA or shRNA against RACO-1, were plated in Buffer A. Cell extracts were dissolved in gel loading buffer (375 mM Tris–Cl, pH 8.8, 10% Glycerol and 0.01% Bromphenol Blue) and loaded onto 8% continuous nondenaturing PAGE (375 mM Tris–Cl, pH 8.8). Gel
was run using electrophoresis buffer (25 mM Tris and 192 mM Glycine, pH 8.3) at 4 °C. Analysed samples were immunoblotted using anti-RACO-1 antibody.

**Supplementary data**

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

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**Author contributions:** CCD and AC performed the experiments, compiled data, generated figures and co-wrote the manuscript, MED generated shRACO1 stable cell lines, MS performed MS analysis and AB directed the project and wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


