

RIP140 directs histone and DNA methylation to silence *Ucp1* expression in white adipocytes

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Nuclear receptors control the function of cells by regulating transcription from specific gene networks. The establishment and maintenance of epigenetic gene marks is fundamental to the regulation of gene transcription and the control of cell function. RIP140 is a corepressor for nuclear receptors that suppresses transcription from a broad programme of metabolic genes and thereby controls energy homeostasis *in vivo*. Here we show by analysis of *Ucp1*, a gene which is typically expressed in brown but not white adipocytes, that RIP140 is essential for both DNA and histone methylation to maintain gene repression. RIP140 expression promotes the assembly of DNA and histone methyltransferases (HMTs) on the *Ucp1* enhancer and leads to methylation of specific CpG residues and histones as judged by bisulphite genomic sequencing and chromatin immunoprecipitation assays. Our results suggest that RIP140 serves as a scaffold for both DNA and HMT activities to inhibit gene transcription by two key epigenetic repression systems.

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Introduction

Nuclear receptors are ligand-activated transcription factors that regulate a vast array of biological processes by modulating expression of specific target genes (Yamamoto, 1985; McKenna and O'Malley, 2002a). Their ability to stimulate transcription depends on the recruitment of cofactors that remodel chromatin and the assembly of the basal transcription machinery (Dilworth and Chambon, 2001; Rosenfeld *et al*, 2006). Chromatin remodelling is achieved by enzymes

that catalyse post-translational modifications of histones and the shuttling of nucleosomes by ATP-dependent mechanisms. The histone modifications, which include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, deimination, ADP ribosylation and proline isomerization, lead to alterations in the accessibility of chromatin and allow for regulated transcription of genes (Kouzarides, 2007). These modifications which may take place sequentially or in combination are proposed to constitute a 'histone code' (Strahl and Allis, 2000; Turner, 2000; Jenuwein and Allis, 2001) that dictates the transcription state of genes. In addition to histone modifications, DNA methylation is a key epigenetic mechanism for gene silencing (Bird and Wolffe, 1999). Methylation at cytosine residues of the dinucleotide sequence CpG is essential for animal development (Bird, 2002) and irregular methylation patterns lead to cancer (Yoo and Jones, 2006).

The diverse biological processes regulated by nuclear receptors are attributed to differential recruitment of coactivators and corepressors that function as scaffolds for the recruitment of chromatin remodelling enzymes. In the absence of ligand, certain nuclear receptors recruit corepressors such as SMRT and N-CoR that bind repressive enzymes such as histone deacetylase enzymes (HDACs) (Jepsen and Rosenfeld, 2002) or the histone methyltransferase (HMT) SUV39H1, which specifically methylates histone H3 at lysine 9 (H3K9) (Li *et al*, 2002). In the presence of a ligand, activated receptors recruit coactivators such as the histone acetyltransferases (HATs), HMTs, p160s and CBP/p300 as well as the arginine-specific HMTs PRMT1 and CARM1 to promote gene transcription (Bauer *et al*, 2002; McKenna and O'Malley, 2002b; Stallcup *et al*, 2003). On the other hand, ligand bound nuclear receptors can also bind corepressors such as RIP140 (Cavailles *et al*, 1995) and L-CoR (Fernandes *et al*, 2003) to actively repress gene expression.

RIP140 is highly conserved in vertebrates and widely expressed in tissues. Analysis of mice devoid of the RIP140 gene indicates that the corepressor is essential for ovulation (White *et al*, 2000) and energy homeostasis (Leonardsson *et al*, 2004). Thus, RIP140-null mice accumulate markedly less fat in their adipose tissue, are resistant to high-fat diet-induced obesity and exhibit improved glucose tolerance and insulin sensitivity (Leonardsson *et al*, 2004; Christian *et al*, 2006; Powelka *et al*, 2006). These changes are associated with increased expression of metabolic genes in white adipose tissue (WAT) (Leonardsson *et al*, 2004) and muscle (Seth *et al*, 2007). Among the upregulated genes is uncoupling protein 1 (*Ucp1*) which is expressed in brown adipose tissue (BAT) during adaptive thermogenesis but normally remains silenced in WAT. Further analysis of adipocyte cell lines indicates that RIP140 plays an intrinsic role in suppressing transcription from gene networks responsible for catabolism (Christian *et al*, 2005; Powelka *et al*, 2006). The *Ucp1* gene is stimulated by a number of nuclear receptors that bind to an enhancer region 2.3 kb upstream of the start site of transcription (Kozak *et al*, 1994; Lowell and Spiegelman, 2000).

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We have shown previously that RIP140 is capable of binding to this enhancer and that this is necessary and sufficient to reduce *Ucp1* gene expression (Christian *et al*, 2005).

RIP140 comprises four distinct repression domains (Christian *et al*, 2004) that have been shown to bind HDACs (Wei *et al*, 2000) and carboxy-terminal-binding protein (CtBP) (Vo *et al*, 2001). CtBP has been shown to form a multisubunit protein complex that contains a number of chromatin-modifying enzymes including HDACs, and the HMTs, G9a and EuHMT (Shi *et al*, 2003). To investigate the mechanism by which RIP140 inhibits transcription, we have focused on the *Ucp1* gene and analysed the ability of the corepressor to modify epigenetic marks around its promoter and upstream enhancer. We made use of adipocytes with and without RIP140 (Christian *et al*, 2005) and investigated

whether the corepressor was required to modify histone residues and whether it was involved in DNA methylation. By using *in vitro* DNA methyltransferase (Dnmt) assays as well as chromatin immunoprecipitation (ChIP) experiments, we show that the corepressor controls both DNA and histone methylation of the *Ucp1* enhancer and promoter. Thus, RIP140 serves a role in controlling gene transcription by two key epigenetic repression systems.

Results

RIP140 associates with DNA and HMT activity

It has been shown previously that RIP140 inhibits transcription by means of distinct repression domains that function by HDAC-dependent and -independent mechanisms (Wei *et al*, 2000; Christian *et al*, 2004). In this study, we investigated a potential functional interaction between the corepressor and DNA and HMTs in HeLa cell nuclear extracts. Proteins that bind to RIP140 in glutathione *S*-transferase (GST) pull-down experiments were assayed for DNA and HMT activity by determining the incorporation of [³H]S-adenosyl-L-methionine (SAM) into unmethylated CG-rich oligonucleotide templates or core histones, respectively. As shown in Figure 1A, GST-RIP140 was able to purify Dnmt activity to a similar extent to that purified by GST-G9a, a HMT that interacts with Dnmt1 (Esteve *et al*, 2006). This indicates that the corepressor associates with endogenous DNA methylation activity. This association is specific and significant relative to the background activity purified by GST alone. On the other hand, GST-RIP140 did not purify any HMT activity above the background of GST alone in contrast to GST-CtBP (Figure 1B), a repressor that interacts with the HMTs G9a and EuHMT (Shi *et al*, 2003) as well as with RIP140 (Vo *et al*, 2001).

As an alternative approach for investigating the association of DNA and HMT activity with RIP140, we tested immunoprecipitated RIP140 complexes for enzymatic activity. Using a RIP140 monoclonal antibody (see Materials and methods), we were able to purify Dnmt activity from HeLa nuclear extracts (Figure 1C) but not HMT activity (data not shown). As a control, we depleted the cells of RIP140

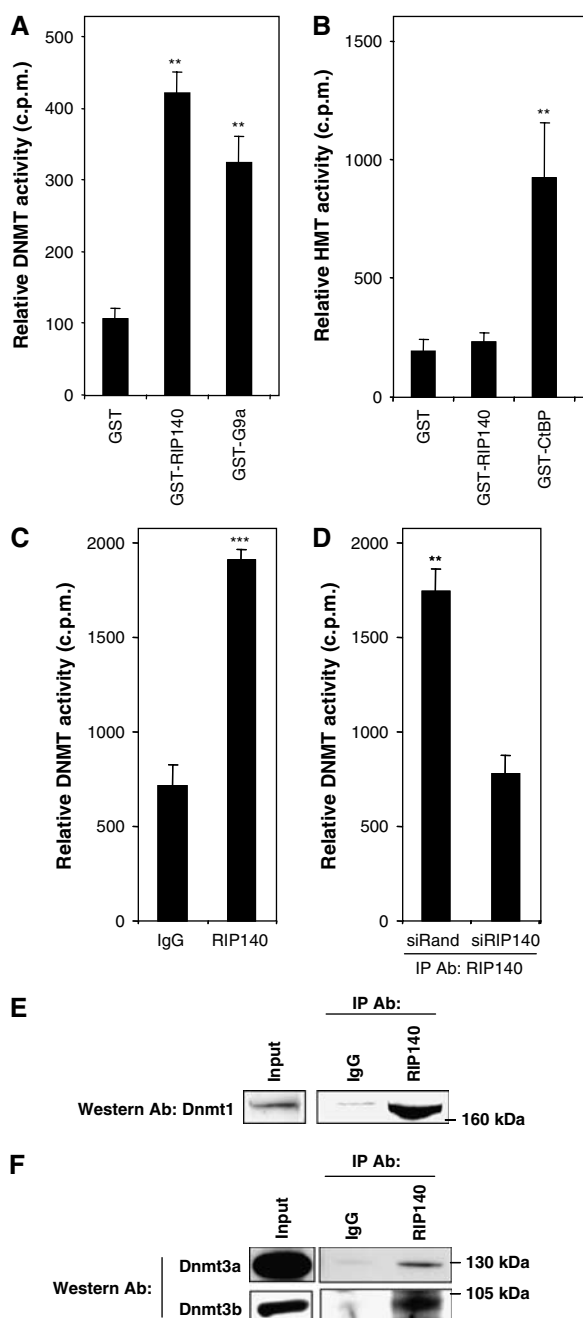


Figure 1 The nuclear receptor corepressor RIP140 associates with DNA methyltransferase (Dnmt) activity. (A) GST-RIP140 was used to purify Dnmt activity from HeLa nuclear extracts. GST alone and GST-G9a were also used as a negative and a positive control, respectively. Relative activity read by scintillation counting as counts per minute (c.p.m.). (B) GST-fused RIP140 and CtBP proteins were used to purify HMT methyltransferase (HMT) activity from nuclear extracts. GST alone was used as a negative control and GST-CtBP as a positive control. (C) Endogenous RIP140 associates with DNMT activity. HeLa cell nuclear extracts were immunoprecipitated (IP) with an antibody (Ab) against RIP140 (P17A6) or IgG and tested for DNMT activity *in vitro*. (D) Similarly, nuclear extracts from HeLa cells treated with siRandom and siRIP140 were immunoprecipitated with an antibody against RIP140 (P17A6) and tested for DNMT activity *in vitro*. The results are averages of three independent experiments with error bars indicating standard error of means (s.e.m.). Statistical significance was determined by a *t*-test (two-tailed) where *P*-value is ** < 0.05 and *** < 0.01. (E) Co-immunoprecipitation of endogenous Dnmt1 with a RIP140 antibody (P17A6), followed by western blotting with a Dnmt1 antibody. (F) Co-immunoprecipitation with a RIP140 antibody of exogenously expressed Dnmt3a and Dnmt3b from HeLa cell extracts, followed by western blotting with Dnmt3a and Dnmt3b antibodies.

prior to the preparation of nuclear extracts using siRNA (Supplementary Figure S1) and demonstrated a marked reduction in the immunoprecipitated DNMT activity (Figure 1D). Furthermore, immunoprecipitated RIP140 purified Dnmt activity from CtBP-null but not from RIP140-null cells, indicating that the association is dependent on RIP140 and not CtBP (Supplementary Figure S2).

We next investigated the ability of RIP140 to associate with Dnmts by performing co-immunoprecipitation/western blotting experiments. We were able to detect an endogenous interaction between RIP140 and Dnmt1 (Figure 1E) but not with Dnmt3a/b (data not shown). This might reflect the relatively low levels of these enzymes in HeLa cells since we were able to detect RIP140–Dnmt3a/b complexes when the proteins were exogenously expressed (Figure 1F).

In order to determine whether the association of RIP140 with DNA methylation activity was mediated through a direct interaction, we performed GST pull-down experiments. We found that *in vitro* translated (IVT) ³⁵S-labelled Dnmt1, Dnmt3a and Dnmt3b were able to bind to full-length GST-RIP140, suggesting that the enzymes can form a direct interaction with the corepressor (Figure 2A). Using various GST-fused RIP140 fragments containing specific functional domains, we found that the Dnmts interact with both the N-terminal 27–439 fragment that includes repression domain 1

(RD1) and the C-terminal 737–1158 that includes part of RD3 and RD4 but not the middle part of the protein 400–800 (Figure 2B). When we used the individual RDs of RIP140 fused to GST, we found that there is a clear and strong interaction with RD1, whereas RD3 and RD4 alone did not interact. In control experiments, we confirmed that the GST-RIP140 fragments were expressed at similar levels (Supplementary Figure S3) with one exception, namely the 737–1158 fragment that is expressed poorly but nevertheless binds Dnmts very well. Furthermore, we also confirmed the mapping of the interaction by purifying Dnmt activity from HeLa cell nuclear extracts with the N- and C-terminal fragments, but not the region encompassing residues 400–800 (Figure 2C). Thus, we conclude that Dnmt1, Dnmt3a and Dnmt3b associate with RIP140 by interacting with two distinct regions encompassing RD1 and RD3 plus RD4 (Figure 2D).

RIP140 expression results in histone deacetylation and methylation of the *Ucp1* enhancer and promoter

We next focused on the *Ucp1* gene to investigate the influence of RIP140 on the epigenetic state of the promoter, the enhancer and an upstream control region (Figure 3A). We analysed epigenetic marks characteristic of transcriptional activation and repression in RIP140-null adipocytes that express *Ucp1* and RIP140-expressing adipocytes in which the *Ucp1* gene is suppressed (Christian *et al*, 2005). Histone modifications were analysed by ChIP assays (Figure 3B and C). General acetylation of H3 and H4 was decreased in RIP140-expressing adipocytes compared with RIP140-null cells at both the enhancer and promoter of *Ucp1* consistent with reduced expression of the gene, while there were no significant differences in the chromatin state of the upstream control region. Similarly, there was a reduction in the presence of RIP140, of di-methylated histone H3 at lysine 4 (H3K4me2), a mark usually associated with active genes (Santos-Rosa *et al*, 2002). The most striking differences, however, were enrichments in methylation of histone H3 at lysines 9 (H3K9) and 27 (H3K27), marks characteristic of repressed gene promoters (Lachner *et al*, 2003). In particular, H3K9me2 involved in transcriptional repression in euchromatic regions and H3K9me3, which is usually associated with heterochromatin (Peters *et al*, 2003; Rice *et al*, 2003), were increased in RIP140-expressing adipocytes at both the enhancer and promoter. Similarly, there was a marked increase in H3K27me2 and H3K27me3 involved in Polycomb group (PcG) silencing (Cao *et al*, 2002; Kuzmichev *et al*, 2002) at both the enhancer and promoter but not at the upstream control region. We conclude that RIP140 expression in adipocytes results in an enrichment of repressive histone modifications, leading to a reduction of *Ucp1* gene expression.

RIP140 expression results in DNA methylation of the *Ucp1* enhancer and promoter

Since RIP140 is associated with Dnmt activity, we investigated the state of DNA methylation at the *Ucp1* promoter and enhancer. There are 34 CpG dinucleotides at the *Ucp1* proximal promoter region spanning –600 to +200 bp and two in the 200 bp enhancer region. Using bisulphite genomic sequencing, we determined the methylation levels of these CpGs in RIP140-null adipocytes, adipocytes expressing exogenous RIP140 and 3T3-L1 adipocytes (Figure 4A).

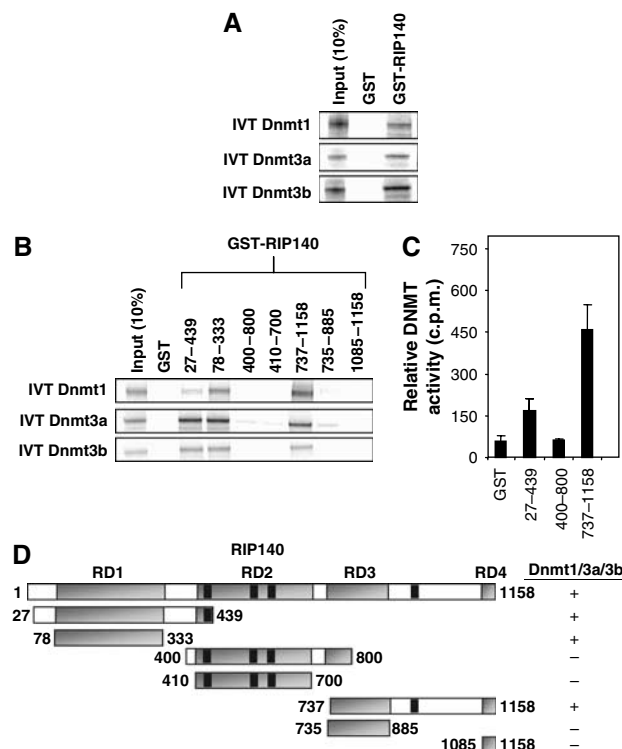


Figure 2 RIP140 directly interacts with Dnmt1, Dnmt3a and Dnmt3b *in vitro*. (A) *In vitro* translated (IVT) full-length Dnmt1, Dnmt3a and Dnmt3b were incubated with full-length GST-RIP140. (B) The interaction was mapped by incubating IVT Dnmts with various GST-RIP140 fragments as indicated. (C) GST-RIP140 fragments were used to purify DNA methyltransferase (Dnmt) activity from HeLa nuclear extracts. (D) The RIP140 protein comprises 1158 amino acids and encompasses four distinct repression domains marked RD1–RD4 shown in grey. Black bars indicate CtBP interaction motifs. (+) Indicates good and (–) no interaction.

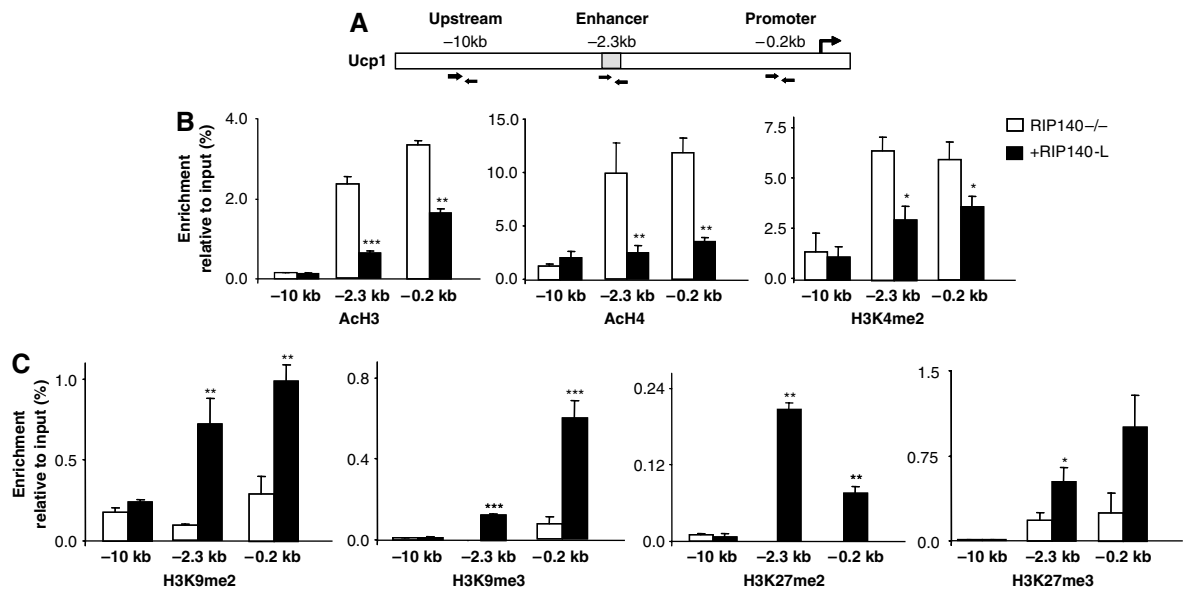


Figure 3 RIP140 expression results in repressive histone modifications at the *Ucp1* enhancer and promoter. (A) Schematic representation of the *Ucp1* gene, arrows indicating ChIP primers designed against the enhancer, proximal promoter and an upstream control region. (B) ChIP experiments using antibodies against active histone marks in the *Ucp1* gene in RIP140^{-/-} (white bars) and +RIP140-L (black bars) adipocytes. (C) ChIP experiments using antibodies against repressive histone marks. Levels presented as enrichment relative to input (%) and corrected for IgG control levels as analysed by quantitative PCR. The results are averages of at least three independent experiments with error bars indicating standard error of means (s.e.m.). Statistical significance was determined by a *t*-test (two-tailed) where *P*-value is **P*<0.1, ***P*<0.05 and ****P*<0.01.

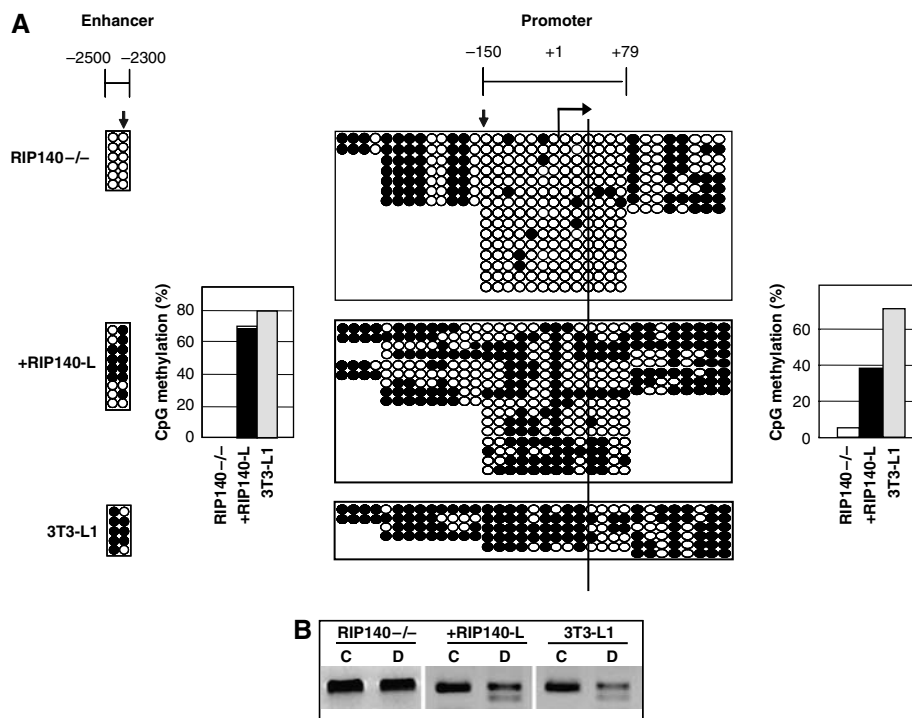


Figure 4 RIP140 expression leads to an induction of DNA methylation at the *Ucp1* enhancer and promoter. (A) The *Ucp1* gene contains two CpGs at the enhancer (-2500 to -2300b) and 34 CpGs at the proximal promoter (-600 to +200b) as indicated by circles. Bisulphite sequencing of CpGs was carried out after isolation of genomic DNA from RIP140^{-/-}, +RIP140-L and wild-type 3T3-L1 adipocytes. Black and white circles indicate methylated and unmethylated CpGs, respectively. Arrows indicate CpGs overlapping with functional CREB-binding sites (Rim and Kozak, 2002). Line indicates CpG targeted for digestion by *TaqI*. (B) Restriction digestion of bisulphite-treated DNA from adipocytes with *TaqI* that digests the sequence TCGA. After the reaction, DNA from control (C) and digested (D) reactions was run on an agarose gel.

In adipocytes devoid of RIP140, both CpGs in the *Ucp1* enhancer were completely unmethylated, whereas RIP140 expression leads to 72–80% methylation. RIP140 expression

also leads to an increase in DNA methylation in the proximal promoter region in the immediate vicinity of the transcriptional start site (-150 to +79 bp). To quantify the level of

methylation at a specific CpG dinucleotide (indicated by line in Figure 4A), we digested bisulphite-treated genomic DNA from the three cell lines with the restriction endonuclease *TaqI*, which only digests methylated DNA (Figure 4B). This confirmed that in the absence of RIP140 this CpG remains unmethylated, whereas RIP140 expression leads to approximately 40% DNA methylation at this site.

RIP140 targets repressive enzymes at the *Ucp1* enhancer to silence gene expression

Given that RIP140 expression leads to an increase in histone and DNA methylation, we next investigated the binding of repressive histone-modifying enzymes and Dnmts to the *Ucp1* enhancer and promoter in adipocytes, in the presence and absence of RIP140. Using ChIP assays, we demonstrated the presence of HDAC1, HDAC3, CtBP and the H3K9-specific HMT G9a at the enhancer but not the promoter region (Figure 5A). Dnmt1, Dnmt3b and small amounts of Dnmt3a were found at the enhancer, but in addition Dnmt1 and to a much lesser extent Dnmt3a/b could be detected in the vicinity of the proximal promoter (Figure 5B). Importantly, recruitment of these repressive enzymes was entirely dependent on the presence of RIP140. The expression of these

repressive enzymes was not significantly affected by the expression of RIP140 (Supplementary Figure S4), indicating that their recruitment was not a reflection of their relative expression levels. The assembly of RIP140–Dnmt complexes on the *Ucp1* enhancer was then investigated by Re-ChIP experiments using Dnmt antibodies in the first ChIP and a RIP140 antibody in the second ChIP. As shown in Figure 5C, we were able to detect RIP140 together with Dnmt1, Dnmt3a and Dnmt3b at the enhancer, but not at a distal upstream region at –10 kb or the proximal promoter region. Finally, we performed Re-ChIP experiments with CtBP in the first ChIP and RIP140 or Dnmt1 in the second ChIP. Similarly, we were able to detect both CtBP/RIP140 and CtBP/Dnmt1 complexes at the enhancer, but not the distal control or proximal promoter region (Figure 5C). We conclude that RIP140 selectively targets Dnmts, HDACs, CtBP and G9a to the *Ucp1* enhancer resulting in histone and DNA methylation around the enhancer, ultimately leading to parallel changes around the promoter to silence expression of the gene.

Since RIP140 expression results in changes of the epigenetic state of chromatin, we investigated whether it was possible to reactivate *Ucp1* gene expression in RIP140-expressing adipocytes incubated with DNA and histone

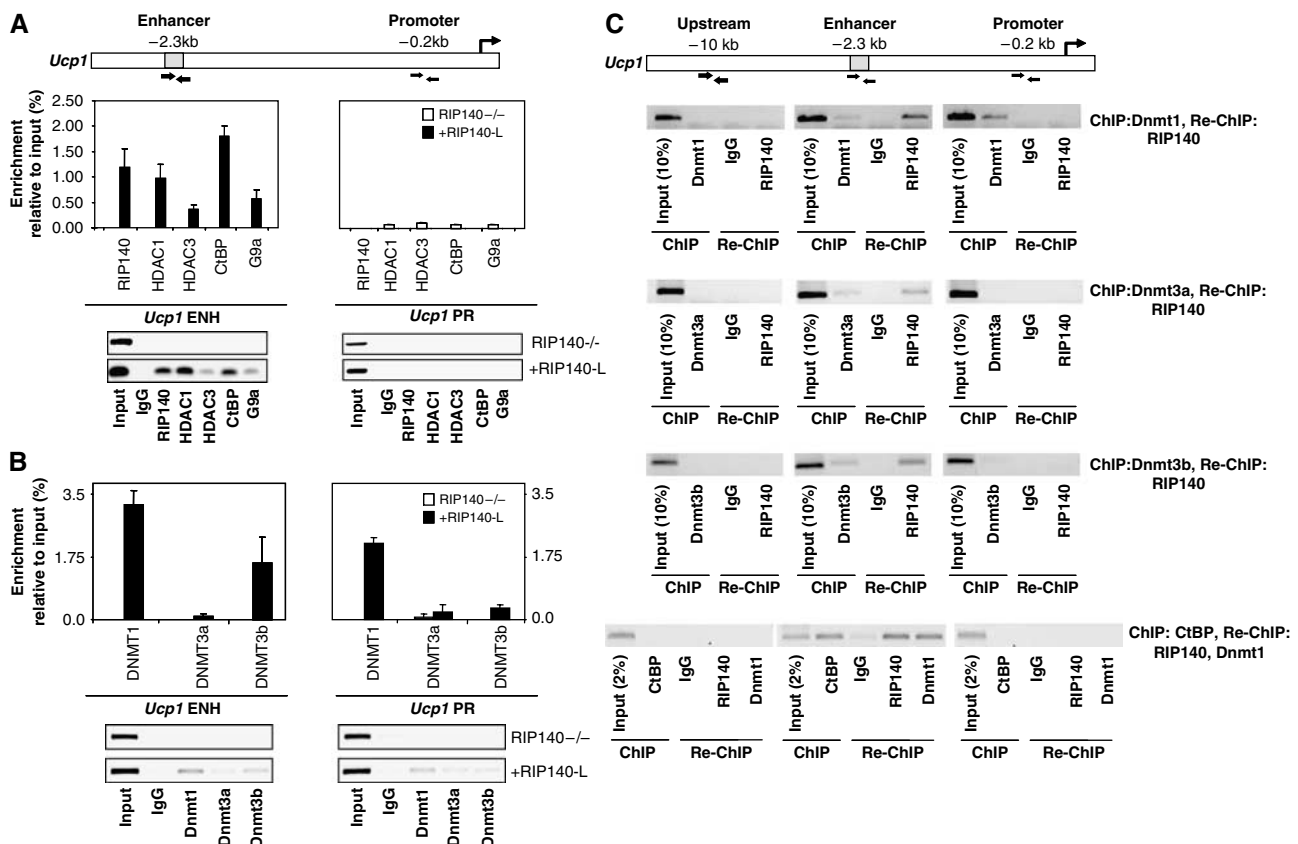


Figure 5 The recruitment of repressive enzymes at the *Ucp1* enhancer is dependent on RIP140. (A) Schematic representation of the *Ucp1* gene with the enhancer (ENH) and proximal promoter (PR) relative to the transcription start site with arrows indicating primers used for ChIPs. ChIP experiments were performed using antibodies against RIP140, HDAC1, HDAC3, CtBP, G9a and (B) Dnmt1, Dnmt3a, Dnmt3b. Their binding was analysed by quantitative PCR in the ENH and PR of the *Ucp1* in RIP140–/– (white bars) and +RIP140-L (black bars) adipocytes. Results are expressed as enrichment relative to input (%) and corrected for IgG control levels. The results are averages of at least three independent experiments with error bars indicating standard error of means (s.e.m.). Results were also analysed by PCR using different primers and a representative gel is shown. (C) Re-ChIP experiments were performed using antibodies against RIP140, Dnmts and CtBP as indicated to identify protein complexes at the upstream control region, the *Ucp1* enhancer and the proximal promoter region. The amount of precipitated DNA from the first ChIP is shown as input.

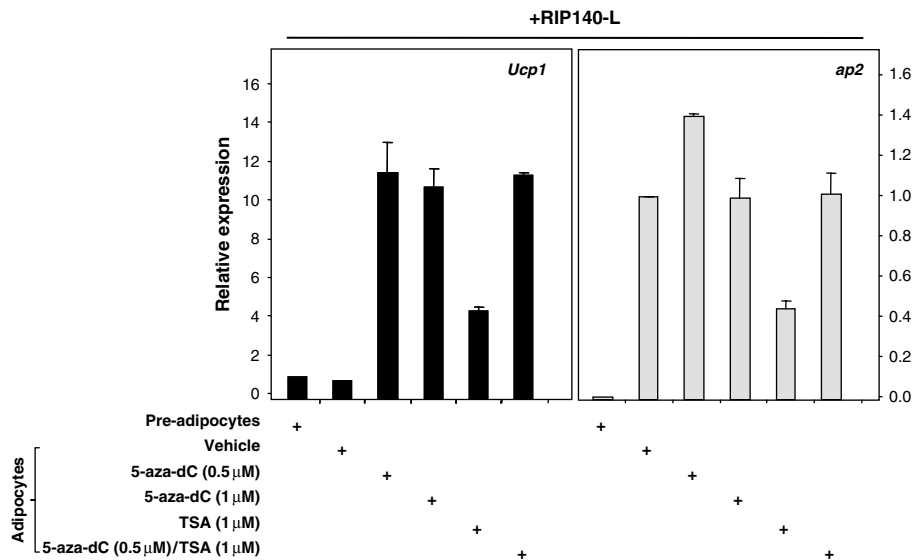


Figure 6 Both DNA methylation and histone deacetylation are required for RIP140 repression of the endogenous *Ucp1* gene. +RIP140-L cells were treated with 5-aza-dC, TSA and 5-aza-dC plus TSA. *Ucp1* and *ap2* expression was determined by real-time PCR. Data were normalized to mitochondrial L19 levels and are expressed relative to vehicle treatments. The results are averages of at least three independent experiments with error bars indicating standard error of means (s.e.m.).

deacetylation inhibitors (Figure 6). Preliminary experiments established that treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine did not interfere with adipogenesis, as judged by the expression of the adipocyte-specific marker *ap2*. Under these conditions, *Ucp1* expression was increased 10–12-fold. Treatment with the HDAC inhibitor, trichostatin A (TSA), did slightly impair adipocyte differentiation as judged by *ap2* expression, nevertheless *Ucp1* expression was increased four-fold. A sequential treatment of 5-aza-dC followed by TSA had no additional effect on *Ucp1* expression compared to 5-aza-dC alone. We conclude that both DNA methylation and histone deacetylation are required for RIP140-mediated *Ucp1* repression.

Discussion

Previous work has clearly established the importance of RIP140 as a corepressor for nuclear receptors and its role in inhibiting metabolic gene expression (Leonardsson *et al*, 2004; Christian *et al*, 2005; Powelka *et al*, 2006; Debevec *et al*, 2007; Seth *et al*, 2007). In this study, we demonstrate that RIP140 targets repressive histone-modifying and DNA methylation activity to the *Ucp1* enhancer to silence gene expression. RIP140 expression in RIP140-null adipocytes leads to an increase in methylation of histone tails at H3-K9 and H3-K27 and CpG dinucleotides in the enhancer/promoter of this gene. Thus it appears that RIP140 provides a scaffold for the assembly of both histone and DNA-modifying enzymes, thereby providing a mechanistic link between two key epigenetic repression systems to regulate *Ucp1* gene expression in adipocytes.

Ucp1 is expressed in BAT during adaptive thermogenesis (Lowell and Spiegelman, 2000), but normally remains silenced in WAT. We have recently demonstrated that the nuclear receptors PPAR α , PPAR γ and ERR α are responsible for the upregulated *Ucp1* expression observed in RIP140-null adipocytes by binding to an upstream enhancer element

(Debevec *et al*, 2007). RIP140 is also recruited to this enhancer which is both necessary and sufficient to mediate repression of the *Ucp1* gene in adipocytes (Christian *et al*, 2005; Debevec *et al*, 2007). Characterization of autonomous repression domains (RDs) within RIP140 indicate that they function by HDAC-dependent and -independent mechanisms (Christian *et al*, 2004). RD1 has been shown to bind HDACs 1 and 3 (Wei *et al*, 2000) and we have shown here that it may also function by recruiting Dnmts. RD2 binds directly to CtBP (Vo *et al*, 2001; Christian *et al*, 2004), which forms a multiprotein repressive complex with HDACs and HMTs (Shi *et al*, 2003). RD3 together with RD4 also seem to recruit DNA methylation activity to the corepressor. Thus, it appears that RIP140 facilitates the binding of a host of repressive enzymes including HDAC1, HDAC3, CtBP, G9, Dnmt1 and Dnmt3a/3b to the *Ucp1* enhancer leading to modification of histones and DNA to prevent gene transcription. It is conceivable that the binding of these enzymes is an indirect consequence of other repressive mechanisms, but we favour a central role for RIP140 for the following reasons: repression of *Ucp1* expression is entirely dependent on RIP140, while the upstream enhancer which binds RIP140 is necessary and sufficient to mediate the repression (Christian *et al*, 2005); the recruitment of repressive enzymes to the enhancer occurs only following RIP140 expression; RIP140 interacts directly with HDACs, CtBP and Dnmts; Re-ChIP experiments demonstrate the simultaneous presence of RIP140, CtBP and Dnmts at the *Ucp1* enhancer and importantly RIP140 purifies DNA methylation activity to methylate DNA templates *in vitro*. It is noteworthy that Dnmts have been shown to be targeted to promoters via their association with sequence-specific binding proteins such as Rb, E2F1 and RP58 (Robertson *et al*, 2000; Fuks *et al*, 2001). We propose that RIP140 functions in a similar manner to target the Dnmts, HDACs, CtBP and G9a to the *Ucp1* enhancer element to induce silencing of the gene.

DNA methylation, histone deacetylation, H3K9 and H3K27 methylation typically coincide in heterochromatin and silent

gene promoters (Bird, 2001; Fuks, 2005). The deposition of H3K9 methylation by G9a at the *Ucp1* enhancer may lead to repression via a number of mechanisms. Both di- and tri-H3K9-methylated peptides form binding sites for HP1 that associates with other repressors including HDACs and RNAs that silence gene expression. This mechanism has been shown to be important in both heterochromatin formation and transcriptional repression (Nielsen *et al*, 2001; Kouzarides, 2002). However, H3K9 methylation and HP1 do not completely colocalize and H3K9me is also sufficient to suppress transcription independent of HP1, through mechanisms involving histone deacetylation (Stewart *et al*, 2005) and by inhibiting phosphorylation of H3S10 (Cheung *et al*, 2000).

H3K27 methylation is catalysed by the enzymatic activity of the PcG protein EZH2 (Cao *et al*, 2002; Kuzmichev *et al*, 2002). This subsequently leads to repression by facilitating the binding of additional PRC2 and PRC1 repressive PcG multiprotein complexes. Interestingly, Bracken *et al* (2006) have recently identified *Ucp1* as a PcG target gene in human embryonic fibroblasts. In their study, the *Ucp1* promoter showed strong enrichments for the PcG proteins SUZ12, CBX8 and H3K27me₃, while depletion of EZH2 resulted in a significant upregulation of *Ucp1* expression. Therefore, the RIP140-dependent induction of H3K27 trimethylation at the *Ucp1* enhancer and promoter may be mediated by the enzymatic activity of EZH2. EZH2 has been shown to interact directly with Dnmts and is necessary but not sufficient for *de novo* DNA methylation of PcG-repressed genes (Vire *et al*, 2006). Moreover, although EZH2-mediated H3K27 methylation pre-marks genes for silencing in cancer, additional factors are required to promote *de novo* DNA methylation (Schlesinger *et al*, 2007). Our finding that the recruitment of Dnmts for the repression of *Ucp1* is dependent on RIP140, and that H3K27me₃ is increased by RIP140 expression suggests that the corepressor may function as a trigger for EZH2 activation.

We have demonstrated that the RIP140-mediated recruitment of Dnmts leads to a striking increase in DNA methylation at the enhancer and proximal promoter. One mechanism by which DNA methylation is proposed to repress gene expression involves interference with the binding of transcription factors (Bird and Wolffe, 1999). At least two CpG dinucleotides methylated in RIP140-expressing adipocytes overlap functional binding sites for the cAMP-response element-binding protein (CREB), a transcription factor implicated in *Ucp1* regulation (Rim and Kozak, 2002). In particular, cAMP response elements CRE2 and CRE4 (indicated by arrows in Figure 4) bind phosphorylated CREB. A recent genome-wide analysis of CREB promoter occupancy has clearly demonstrated that DNA methylation of CREB response elements is inhibitory to the binding of this transcription factor (Zhang *et al*, 2005). Our results support the notion that RIP140 might suppress *Ucp1* expression by methylation of CREs. It is noteworthy that the methylation levels of these CpGs are significantly higher in WAT than in BAT of wild-type mice, while this difference is less prominent in RIP140-null mice in which there is *Ucp1* expression in WAT (data not shown).

Adipose genes that are subject to repression by RIP140 fall into two groups. One group comprises genes such as *Ucp1* that are generally inactive in WAT but can be derepressed in the absence of RIP140. The ability of RIP140 to direct

repressive histone modifications and DNA methylation provides a mechanism for silencing *Ucp1* and similar genes in WAT that are normally only expressed in BAT. It appears therefore that RIP140 plays a developmental role by epigenetically silencing a subset of genes during adipogenesis that are involved in energy expenditure in brown adipocytes and thereby ensuring that they are not expressed in white adipocytes. On the other hand, the majority of genes repressed by RIP140 are involved in mitochondrial biogenesis, fatty acid oxidation and oxidative phosphorylation and expressed in both brown and white adipocytes. The mechanism by which RIP140 regulates the expression of this second group of target genes remains to be determined.

Taken together, this study highlights a novel role for RIP140 in directing key epigenetic repression mechanisms, namely H3K9, H3K27 and DNA methylation for silencing of a PcG pre-marked gene. These findings provide a mechanistic link between a hormonally regulated nuclear receptor corepressor and essential epigenetic mechanisms. These results also provide an insight into the mechanism of tissue-specific regulation of *Ucp1* and underlie a developmental role for RIP140 in determining the metabolic profile and function of adipose tissue by epigenetically marking relevant genes or gene networks.

Materials and methods

Cell culture

The generation, maintenance and adipocyte differentiation of RIP140^{-/-} and +RIP140-L mouse embryonic fibroblasts (MEFs) have been previously described (Christian *et al*, 2005). All cells were differentiated in the presence of 2.5 μM rosiglitazone.

5Aza-dC and TSA treatments of cells

For 5Aza-dC treatments, cells were allowed to reach confluence and given 5-Aza-2'-deoxycytidine (5Aza-dC; Sigma) (0.5 μM), (1 μM) or DMSO (vehicle) for 48 h changing every 24 h. Then, cells were differentiated into adipocytes for 10 days before RNA was isolated. For Trichostatin A (TSA; Sigma) treatments, cells were differentiated for 8 days and then treated with TSA (1 μM) or DMSO (vehicle) for 48 h changing every 24 h. For combinational treatments, cells were treated for 48 h with 5Aza-dC (0.5 μM) and differentiated for 8 days as before, followed by 48 h of TSA treatments.

Expression analysis

Total RNA from cell lines was extracted using TRIZOL (Invitrogen) according to the manufacturer's instructions and cDNA preparation was performed as described previously (Christian *et al*, 2005). Expression levels for all genes were correlated to the ribosomal coding gene L19. Primer sequences are available on request. Affymetrix gene expression profiling and data analysis have been previously described (Christian *et al*, 2005).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (Christian *et al*, 2005). Cells were treated with Rosiglitazone and insulin for 30 min and then incubated in the protein-protein crosslinking reagent dimethyl adipimidate 2 HCl (Pierce) at 10 mM in DMEM for 30 min followed by 1% formaldehyde in PBS for 10 min at 37°C. The crosslinked cells were lysed, sonicated and immunoprecipitated with Protein A/G PLUS-agarose (Santa Cruz) according to the manufacturer's instructions using the following antibodies: AcH3 (06-599), AcH4 (06-866), H3K9-me² (07-441), H3K27-me² (07-421), H3K27-me³ (07-449), G9a (07-551) (all Upstate), H3K4-me² (ab7766), H3K9-me³ (ab8898), HDAC1 (ab7028), HDAC3 (ab2379), Dnmt3a (ab13888), Dnmt3b (ab2851) (all Abcam), CtBP (sc-11390), Dnmt1 (sc-20701) (both Santa Cruz) and RIP140 (a kind gift from Hongwu Chen). DNA fragments were purified with QIAquick PCR purification kit (Qiagen) and enrichment relative to input was determined by quantitative PCR. End

point PCR was used for representative gel pictures. All quantitative PCR reactions were carried out in duplicates, averaged and expressed relative to the input signal. Primer sequences for quantitative PCR and end point PCR are available on request. For the sequential chromatin immunoprecipitation (Re-ChIP) assay, chromatin was eluted with DTT (10 mM) from the first antibody-protein A/G PLUS-agarose complex.

Bisulphite sequencing

Bisulphite genomic sequencing was performed as described previously (Monk *et al*, 2003). Approximately 0.6 µg of DNA in a volume of 32.5 µl was denatured by the addition of 1.1 µl NaOH 10 N for 15 min at 50°C. For bisulphite treatment, 200 µl of approximately 4 M sodium bisulphite, pH 5.0 (Sigma; final concentration 3.5 M), 1.5 µl 75 mM hydroquinone (Sigma, final concentration 0.5 mM), 5 µg glycogen were added and the mixture was incubated for 4 h at 55°C. Desalting was carried out using the QIAquick PCR purification kit (Qiagen), and the eluted DNA (in 50 µl Tris-Cl, pH 7.5) was desulphonated by treatment with 1.6 µl NaOH 10 N. DNA was ethanol-precipitated, resuspended in H₂O, and HotStar DNA polymerase (Qiagen) was used to PCR-amplify CpG island products (annealing temperature of 58°C)-specific primers. The amplified products were either digested with the methylation-sensitive restriction endonuclease *TaqI* or cloned into a TOPO vector and sequenced. The efficiency of C-U conversion in the samples used was determined to be >95%. Primer sequences may be obtained on request.

GST pull-down assays

Dnmt1, Dnmt3a, Dnmt3b and G9a were *in vitro* transcribed-translated from pcDNA3-Myc-Dnmt1, pcDNA3-Myc-Dnmt3a, pcDNA3-Myc-Dnmt3b (a kind gift from F Fuks) and Bluescript SK-G9a (a kind gift from Y Shinkai) using the TNT system (Promega). GST and GST-fusion proteins were expressed in *Escherichia coli* strain BL21 using the pGEX vector system (Pharmacia) and protein was purified from crude bacterial lysates according to the manufacturer's instructions and in the presence of Benzonase (Novagen). GST pull-down assays were carried out essentially as previously described (Christian *et al*, 2004). Input 10% corresponds to 10% of the IVT product used in the reaction.

DNA and HMT assays

DNA and HMT methyltransferase assays were essentially carried out as described previously (Fuks *et al*, 2000; Shi *et al*, 2003). GST and GST fusions were expressed and purified (see GST pull-down assays above), incubated with HeLa nuclear extract overnight at 4°C and the resulting complexes were then tested for *in vitro* methyltransferase activity. Similarly, a RIP140-specific antibody (P17A6) (see Antibody generation below) or IgG-conjugated agarose beads (PIERCE) were incubated with HeLa, RIP140-/- and CtBP-/- nuclear extracts for 4 h, at 4°C and the resulting complexes were then tested for *in vitro* methyltransferase activity. *In vitro* Dnmt activity was assayed in 100 µl reactions containing 1 µg of a 33 bp oligonucleotide (Ramchandani *et al*, 1997), 2 µl [H³]-S-adenosyl-L-methionine (81 Ci/mmol; Amersham), 40 µl 50% glycerol, Tris-HCl (50 mM, pH 7.5), EDTA (5 mM), DTT (5 mM), protease inhibitors and incubated at 37°C for 1 h with shaking. *In vitro* HMT activity was assayed in 50 µl reactions containing core histones (Upstate), 1.6 µl [H³]-S-adenosyl-L-methionine (81 Ci/mmol; Amersham), DTT (5 mM) and protease inhibitors and incubated at 37°C for 1.5 h with shaking. After the reactions, DNA and histones were TCA-precipitated in filter paper and unincorporated nucleides removed by sequential washing with 2 × 10% TCA,

2 × 5% TCA and pure ethanol. Incorporation of radioactive methyl groups was determined by liquid scintillation counting.

Co-immunoprecipitation and western blot

Co-immunoprecipitations were carried out essentially as described previously (Herzog *et al*, 2007). For co-immunoprecipitation of endogenous Dnmt1, 1.5 mg of HeLa cell nuclear extract was incubated with 35 µl anti-RIP140 (P17A6) or anti-IgG-conjugated agarose beads (PIERCE), while whole-cell extracts of transfected HeLa cells with 145 ng of pcDNA3-Myc-Dnmt3a and Dnmt3b were used similarly to precipitate Dnmt3a and Dnmt3b. The following antibodies were used for subsequent western blots of immunoprecipitated proteins, Dnmt1 (ab13537), Dnmt3a (ab23565) and Dnmt3b (ab16049) all by Abcam.

Adenovirus infection and siRNA treatment

Adenoviral vectors expressing siRNA for RIP140 and siRandom have been described previously (Herzog *et al*, 2007). HeLa cells were infected at an MOI of 40 for 1 h before nuclear extracts were prepared. The effect of the siRNA was confirmed by western blot using a RIP140 antibody (6D7) (Herzog *et al*, 2007) and RNA pol II (sc-899, Santa Cruz).

Antibody generation

cDNA encoding amino acids 599–700 of human RIP140 protein was inserted into the pGEX6 vector (Amersham Biosciences). Recombinant protein was expressed and purified from the *E. coli* strain BL21 according to the manufacturer's instruction (Amersham Biosciences). Young Balb/c × B6 F1 crossed mice were immunized by subcutaneous injection with 50 µg of fusion protein emulsified with an equal volume of Titremax Gold (CytRx Corporation). The immunization was repeated three times at 1 monthly intervals, then the mice were rested for 6 months. A further 100 µg of fusion protein in phosphate-buffered saline was injected intraperitoneally at 6 and 3 days prior to killing. Hybridoma lines were then established by fusing splenocytes from the immunized animal with the myeloma line Sp2/0-Ag14 by polyethylene glycol treatment using conventional procedures (Harlow and Lane, 1988). Individual wells were screened for antibody production by a modified dot blot procedure using bacterially expressed fusion protein (Dilworth and Horner, 1993), and single cell cloned a minimum of three times before being grown for antibody isolation. Each monoclonal antibody was screened for specificity by western blotting against total proteins from SDS-derived tissue culture cell lysates. Line (P17A6) was found to be specific for RIP140 (data not shown) in mouse cell lysates.

Supplementary data

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

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