Interaction of PRMT1 with BTG/TOB proteins in cell signalling: molecular analysis and functional aspects

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Abstract

***Background*: Several recent reports have connected protein methylation with differentiation. Furthermore, the BTG/TOB proteins have also been implicated in such control. BTG1 and 2 have been shown to interact with PRMT1 (predominant cellular arginine N-methyltransferase of type I).**

***Results*: First, we have studied the interaction between PRMT1 and the proteins of the BTG/TOB family. We show that boxC, a sequence present only in BTG1 and BTG2, is essential for this association. Using boxC peptide, we have investigated the importance of PRMT1/BTG protein association during type I protein methylation reactions. Finally, we show that the addition of boxC fused to penetratin interferes with the neuronal differentiation of PC12 cells and ES cell-derived neurones.**

***Conclusions*: Taken together, these results indicate that PRMT1/BTG proteins could play a key role in the arginine methylation-mediated signalling pathway as well as in neuronal differentiation.**

Introduction

The BTG/TOB protein family is characterized by a common signature: two well conserved boxes A and B localized in their N-terminal region (Guéhenneux *et al.* 1997). Several members of the BTG/TOB family (BTG1, BTG2/TIS21/PC3, PC3B, BTG3/ANA, TOB,TOB2) are thought to play a role in cell cycle negative control since their RNA expression is associated with different cell cycle arrest processes (differentiation commitment, genotoxic treatment, P53-regulated expression of *BTG2* ...) and their *in vitro* ectopic over-expression is anti-proliferative (Buanne *et al.* 2000; Fletcher *et al.* 1991; Iacopetti *et al.* 1999; Ikematsu *et al.* 1999; Matsuda *et al.* 1996, 2001; Montagnoli *et al.* 1996; Rodier *et al.* 1999; Rouault *et al.* 1992, 1996; Seo *et al.* 1999; Yoshida *et al.* 1998, 2000). Moreover, *BTG2*-mutated ES cells (Embryonic Stem cells) present an altered G2/M block when treated with genotoxic agents (Rouault *et al.* 1996). The mechanisms underlying this inhibition remain to be deciphered, but it was recently proposed that BTG2/TIS21/PC3 over-expression maintained Rb protein in its hypophosphorylated form, thus preventing cell growth (Guardavaccaro *et al.* 2000).

Although the participation of the BTG/TOB proteins in cell cycle control has now been firmly established, the biochemical mechanisms involved remain to be determined. The only significant data currently available on the function of BTG/TOB proteins have been provided by the discovery of their partners. TOB, through its non-BTG/TOB homologous domain, interacts with the growth factor receptor erbB2 and modulates the signal elicited by Epidermal Growth Factor (Matsuda *et al.* 1996). Several recent studies have reported the physical association of BTG/TOB proteins with transcription factors: BTG1 and 2, BTG3/ANA, TOB and TOB2 with CAF1, BTG1 and 2 with HoxB9 and TOB with SMAD proteins (Bogdan *et al.* 1998; Ikematsu *et al.* 1999; Prevot *et al.* 2000; Rouault *et al.* 1998; Yoshida *et al.* 2000, 2001). The exact role of BTG/TOB proteins within these different complexes is not

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completely understood yet. Nevertheless, it should be noted that TOB interferes with the SMAD machinery and inhibits the effects of SMAD, at least during osteoblast formation (Yoshida *et al.* 2000).

It has also been shown that BTG1 and 2 proteins interact with a protein arginine N-methyltransferase (i.e. PRMT1) and positively modulate its activity (Lin *et al.* 1996). This observation is important since arginine methylation by type I arginine N-methyltransferase is a common post-translational protein modification that seems to be catalysed predominantly by PRMT1 (Gary & Clarke 1998; Pawlak *et al.* 2000; Tang *et al.* 2000a).

Type I arginine N-methyltransferase activity promotes the formation of ω-monomethylarginine and asymetric ω-N^G,N^G-dimethylarginine. The function of this modification is not known, but there is evidence suggesting that it may contribute to RNA processing, protein shuttling and to the regulation of transcription (Chen *et al.* 1999; Gary & Clarke 1998; Koh *et al.* 2001; Shen *et al.* 1998; Yoshida *et al.* 2000). However, the inhibition of PRMT1 activity by global protein methylation inhibition prevents the NGF (Nerve Growth Factor)-stimulated differentiation of PC12 cells into neurones and *PRMT1* (–/–) mouse embryos fail to develop (Cimato *et al.* 1997; Pawlak *et al.* 2000). As mentioned above, PRMT1, the first arginine N-methyltransferase characterized, seems to be the enzyme that catalyses most of the type I methylation reactions. Herschman's group showed, using biochemical methods, that the major type I activity could be attributed to PRMT1 (Tang *et al.* 2000a). Furthermore, *PRMT1* mutated cells also display a very reduced type I arginine N-methyltransferase activity (Pawlak *et al.* 2000). PRMT1 binds to the cytoplasmic domain of the IFNAR1 chain of the interferon receptor and is a major effector of interferon antiproliferative signalling through its interaction with STAT1 (Abramovich *et al.* 1997; Mowen *et al.* 2001). Recently, ILF3, poly(A)-binding protein II, hnRNP A2 and HMW-FGF2 (Fibroblast Growth Factor 2) were proposed as PRMT1 substrates (Klein *et al.* 2000; Nichols *et al.* 2000; Smith *et al.* 1999; Tang *et al.* 2000b). Taken together, these results suggest that PRMT1 plays an important role in a number of highly conserved evolutionary pathways.

PRMT1 and some of the BTG/TOB proteins have already been shown to be involved in the negative control of the cell cycle, apoptosis and differentiation. For this reason, we hypothesized that their association might be relevant in this context. In this paper, we first report molecular studies of PRMT1 and BTG/TOB protein interactions, and then analyse their functional consequences.

Results

Molecular analysis of PRMT1 interaction with BTG/TOB proteins leads to the identification of boxC

In this study, we used the two-hybrid interaction system developed by Fields & Song as previously described (Fields & Song 1989; Rouault *et al.* 1998). To screen for BTG proteins which are able to interact with PRMT1, the Y190 pPC86-PRMT1 (PRMT1 fused to GAL4 activation domain) expressing yeast strain was transformed with BTG1, BTG2/TIS21/PC3 and BTG3 pPC62 expressing vectors (BTG proteins fused to GAL4 DNA binding domain). When the two proteins physically interact, the resulting yeast strain grows on L/W/H depleted SC medium and produces a β-galactosidase activity that can be revealed by a yeast colony filter assay (Chevray & Nathans 1992). In this system, BTG1 and BTG2, but not BTG3 associated with PRMT1. BTG1 and BTG2 are very homologous proteins, their only significant difference being a small insertion in the C-terminal regions (Rouault *et al.* 1992). We then gradually deleted the BTG1 in order to delineate the region necessary for this interaction. As shown in Fig. 1A, the required sequence turned out to be a 10 amino acid region juxtaposed to the previously described boxB, which constitutes, along with boxA, the structural signature of the BTG/TOB family (Guéhenneux *et al.* 1997). However, when this peptide (DGSICVLYEA), hence named boxC, was fused to the GAL4 DNA binding domain (pPC62 vector), no interaction with PRMT1 could be detected (Fig. 1A). Sequence similarity searches did not reveal the presence of the boxC peptide in other proteins. However, it should be noted that, among BTG/TOB members, TOB and TOB2 possess a sequence close to their boxB that is partially similar to boxC (GXXXVLYXX) (Matsuda *et al.* 1996; Ikematsu *et al.* 1999).

We then performed *in vitro* association assays commonly known as 'pull-down' experiments (Rouault *et al.* 1998). Several purified GST (Glutathione S-Transferase) fusion proteins were incubated with labelled PRMT1; their retention capacity (after extensive washing) was revealed by autoradiography (Rouault *et al.* 1998). A specific retention of S^{35}-methionine labelled PRMT1 was observed with GST-BTG2, but not with GST-BTG3 (Fig. 1B). This confirmed the fact that BTG3 does not interact with PRMT1. Furthermore, GST-boxCdeleted-BTG2 protein, contrary to the native GST-BTG2, did not interact with PRMT1 in this system. These results suggest that boxC is necessary

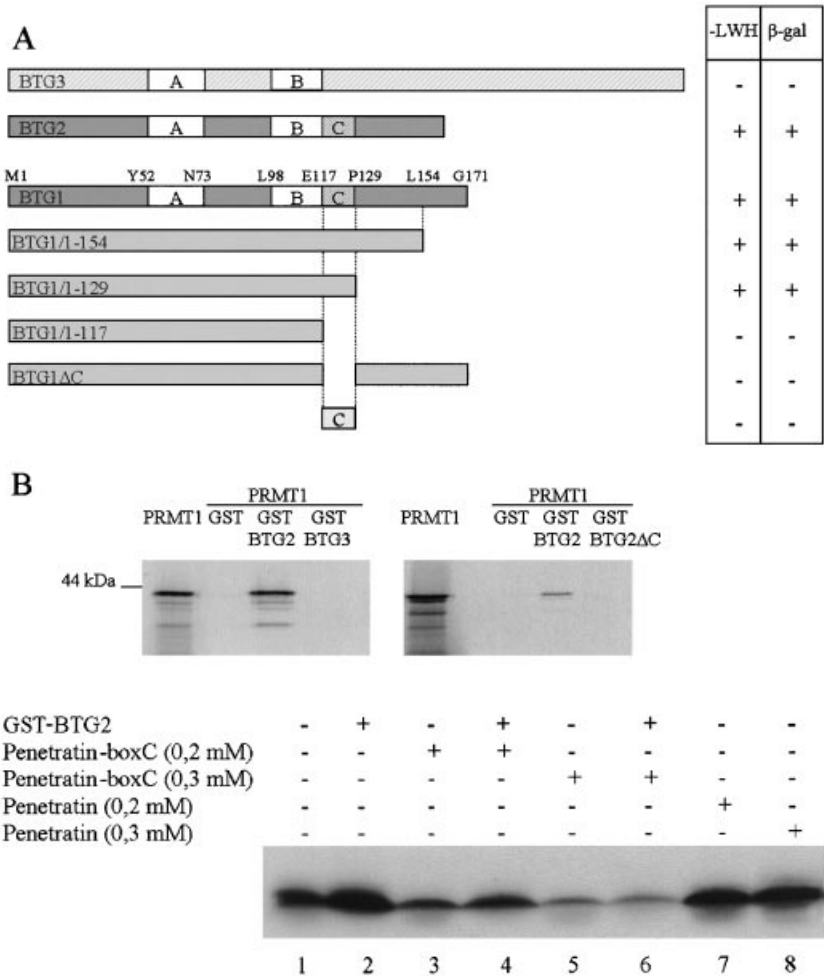

Figure 1 Analysis of PRMT1/BTG family interaction. (A) Yeast strain Y190 was transformed with PRMT1 along with different BTG/TOB expressing vectors. The yeasts were grown on L/W/H depleted SC medium. A yeast colony filter assay was used to determine the β-galactosidase activity in positive clones (+). (B) Pull-down experiments: *in vitro* labelled PRMT1 protein was incubated with different GST proteins bound to gluthatione-Sepharose beads. One quarter of input PRMT1 and the eluted proteins were analysed by SDS-PAGE and visualized by autoradiography.

Figure 2 Histone H2A methylation inhibition by boxC. Histone H2A was used as a substrate for testing the type I methyltransferase activity of PC12 cell extracts. The extracts, as indicated, were incubated with different peptides and then analysed on SDS-PAGE and fluorographed.

although not sufficient for the physical association of PRMT1 with BTG1 and BTG2/TIS21/PC3.

BoxC blocks the stimulating effect of BTG2 on methyltransferase activity

The following experiments were designed to determine the effect of boxC addition on type I arginine methyltransferase activity (Ghosh *et al.* 1988; Lin *et al.* 1996). PRMT1 was recently shown to play a major role in type I activity (90% of total type I activity) (Pawlak *et al.* 2000; Tang *et al.* 2000a). Furthermore, boxC is present only in BTG1 and BTG2/TIS21/PC3 proteins which positively modulate PRMT1 activity (Lin *et al.* 1996). Therefore, it is legitimate to consider that the possible modification of methyltransferase activity by boxC addition will predominantly reflect the effect of BTG on PRMT1 enzymatic activity. Type I arginine methyltransferase activity was determined in a standard reaction mixture using histone H2A as the methyl acceptor substrate, PC12 cytosolic extracts and SAM (S-adenosyl-L-(methyl-^{3}H)methionine) as the methyl donor (Ghosh *et al.* 1988; Lin *et al.* 1996). BoxC was fused with penetratin (see next paragraph) (Derossi *et al.* 1998). Penetratin was used in control experiments. Peptide concentrations (0.2 and 0.3 mM) were determined in the range of previously described competition experiments in methylation assays using synthetic peptides (0.5 mM in this case) (Klein *et al.* 2000). Consistent with the results of Lin and co-workers, the addition of GST-BTG2 protein to the reaction mixture increased arginine methyltransferase activity (Fig. 2, lanes 1 and 2). BoxC inhibited this activity; this inhibition increased in parallel with boxC concentration (lanes 1, 3 and 5). The control peptide (penetratin) used at the same concentration did not modify this activity (lanes 1, 7 and 8). Competition experiments between the boxC peptide and BTG2 showed that BTG2 could still up-regulate type I activity in the presence of a 0.2 mM concentration of boxC (lanes 3 and 4). However, the magnitude of type I

activity up-regulation by BTG2 was inferior to that observed in the absence of boxC (lane 2) or in controls (lane 1). At 0.3 mM, BTG2 could not counteract boxC, and type I activity was nearly abolished (lanes 5 and 6). These results suggest that boxC competitively prevents the enzymatic activation of PRMT1 by BTG proteins. We conclude that the interaction with BTG proteins is important for PRMT1 activity or at least arginine methyltransferase type I activity, since boxC alone strongly inhibits this activity in this system.

BoxC interferes with the PC12 cell differentiation programme

To further investigate the functional relevance of PRMT1/BTG-mediated methylation, we treated PC12 cells with boxC peptide, since we had previously shown that its addition impeded BTG/PRMT1 association and BTG2/TIS21/PC3-controlled methylation. We chose the PC12 cell line because the inhibition of protein methylation had been described as preventing the differentiation of PC12 cells (Cimato *et al*. 1997). Furthermore, PC12 cells enabled the characterization of BTG2/TIS21/PC3 which is up-regulated during NGF-induced differentiation (Bradbury *et al*. 1991). All these data led us to speculate that the BTG/PRMT1 pathway could play a key role in the control of neural differentiation.

We used the penetratin system for intracellular delivery of the peptide boxC (Derossi *et al*. 1998). Penetratin, 16 amino acids corresponding to the third helix of the antennapedia protein homeodomain, can translocate across membranes and be internalized by cells. Penetratin is used as a vector for transferring compounds into cells and thus address their biological activity. We therefore fused boxC with penetratin, and then analysed its *in cellulo* effect.

In a preliminary set of experiments, we checked PC12 cells, either treated or not by NGF, for internalization of the penetratin-boxC peptide. Penetratin and penetratin fused with the 10 amino acids of BTG3, corresponding to the sequence located in the boxC position in BTG1 and 2, were used as controls. The peptides were biotinylated to reveal their internalization through FITC-conjugated streptavidin staining. These experiments showed that the peptides effectively penetrated into PC12 cells at a concentration of 15 μM, close to that used previously by Derossi on neurones (22 μM) (data not shown) (Derossi *et al*. 1996).

Having established that the peptides had entered the recipient cells, we used unbiotinylated peptides in the following experiments. Neither penetratin-boxC nor

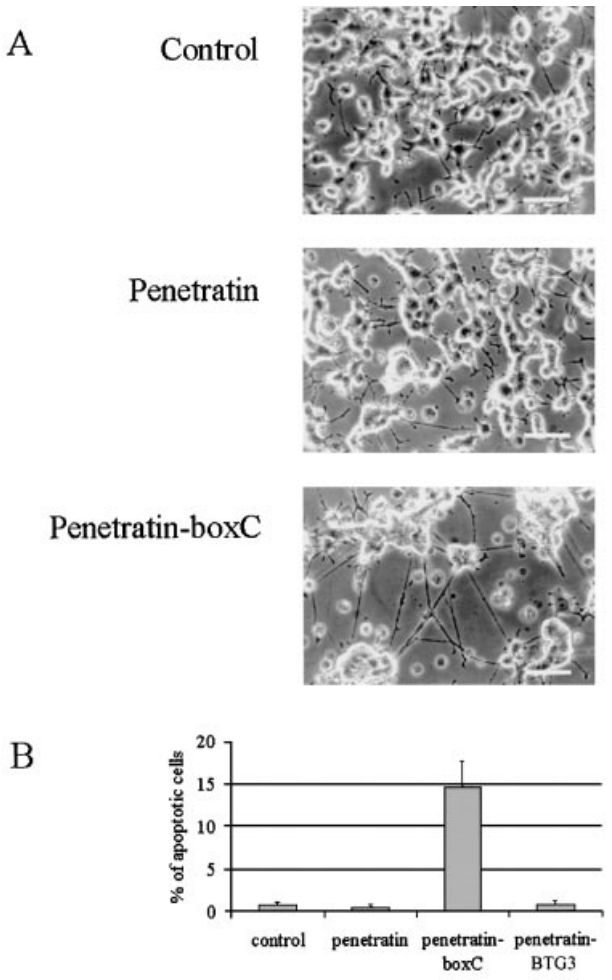

Figure 3 BoxC interference with PC12 cell differentiation. (A) PC12 cells were induced to differentiate by NGF and simultaneously incubated with penetratin or penetratin-boxC. Cells were cultivated for 4 days and photographed (scale bar = 20 μm). (B) Apoptosis of PC12 differentiated cells treated with different penetratin peptides. A TUNEL assay was performed 2 days after the NGF and peptide treatments. Values presented are means of six independent experiments.

controls interfered with the normal growth of PC12 cells in the absence of NGF (data not shown). We then treated PC12 cells with NGF. As shown in the control (Fig. 3A), after 4 days of NGF treatment, PC12 cell differentiation was readily observable and the cells exhibited a large number of neurites. The simultaneous addition of a control peptide did not alter differentiation. In the presence of boxC, cells formed clumps of various sizes and neurite outgrowth was still observed (Fig. 3A). We then hypothesized that this phenomenon could be associated with apoptosis, since interference in differentiation often leads to cellular death, in particular during neuronal

differentiation (Poluha *et al.* 1996). We performed TUNEL assays on the NGF-treated PC12 cells cultured with the different peptides. It appeared that penetratin-boxC addition resulted in an increased rate of apoptosis (Fig. 3B). The magnitude of apoptosis in our experiments (15%) was even greater than that observed in neuroblastoma SH-SY5Y cells, where the activity of the anti-proliferative *P21* gene is prevented by anti-sense oligonucleotides (Poluha *et al.* 1996). These authors reported an apoptosis rate of 10% which they considered highly significant. As these effects only occurred in the presence of NGF, it led us to think that boxC interfered with the control of differentiation. This suggests that the BTG/PRMT1 association (prevented by boxC in these experiments) could be of fundamental importance during the neuronal differentiation of PC12 cells.

We then sought to determine whether the pattern of methylation could be altered during the course of these experiments and thus reveal the molecular consequences of boxC addition. We investigated the endogenous protein methylation of NGF-treated PC12 cells with and without boxC, following procedures described by others (Cimato *et al.* 1997; Tang *et al.* 2000a). The PC12 cell extracts were incubated in a standard reaction, and then analysed for possible discrepancies in protein labelling intensities. It should be noted that only the proteins that are actively methylated in the course of the labelling are detected in these experiments, and that proteins already extensively methylated are not detected, unless they are previously hypomethylated by drugs such as adenosine dialdehyde. Since protein methylation is necessary for PC12 cell differentiation to occur, we chose to focus on newly methylated proteins that were more likely to be involved. The pattern of methylation that we observed was more or less identical to that obtained by Cimato under the same conditions, with high labelling intensities at 20 and 30 kDa (Cimato *et al.* 1997) (Fig. 4). The only significant modification we noticed after boxC addition was the decreased methylation of a 20 kDa protein (Fig. 4). This protein has the same size as a recently characterized and highly arginine-methylated protein (Gu *et al.* 1999). So, boxC addition on PC12 cells prevents the methylation of a 20 kDa protein, presumably by interfering with PRMT1/BTG association.

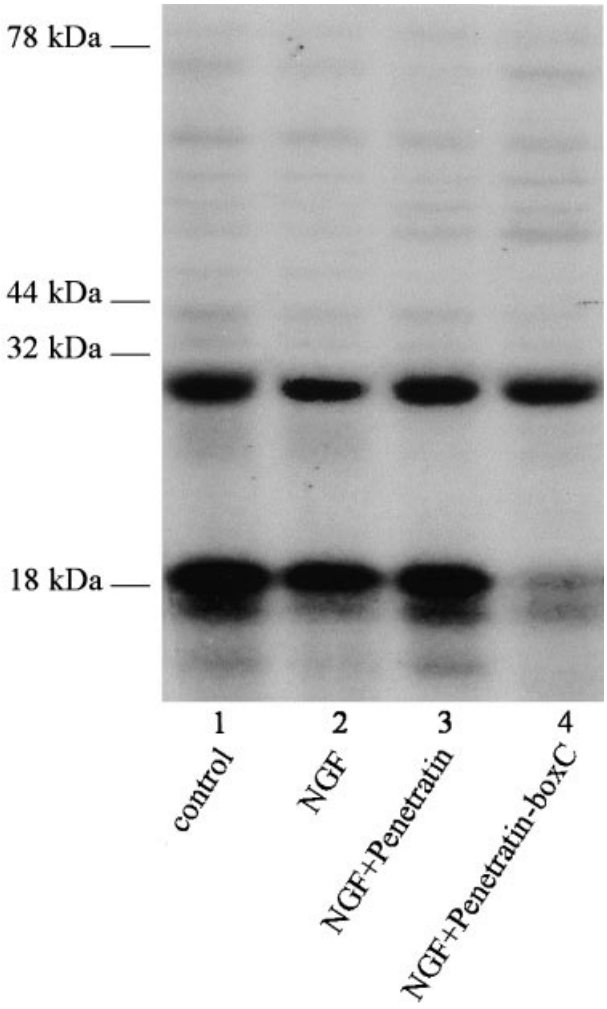

Figure 4 Methylation inhibition of a 20 kDa protein by boxC. Changes in the protein methylation pattern were tested using cytosol extracts from PC12 cells treated as indicated below the autoradiography (4 h). In these experiments, only endogenous methylation was assessed, since no external substrate was added in the cytosolic extracts.

BoxC addition inhibits neuronal cell differentiation

A link between methylation and neuronal differentiation has already been described (first two paragraphs of the Results section and Cimato *et al.* (1997)) and post-mitotic neurones were shown to preferentially express BTG2/TIS21/PC3 (Iacopetti *et al.* 1999). To determine the functional relevance of BTG/PRMT1 association during neuronal differentiation, we examined the boxC effect on ES cells. We chose to work on ES cells because, depending on culture conditions, these cells can differentiate into many lineages *in vitro*. In particular, neuronal cells can be obtained either isolated or as aggregates of various sizes (Fraichard *et al.* 1995).

First, differentiated and undifferentiated ES cells were treated with the biotinylated peptides. In all of the conditions tested, peptides were detected in the cells. It is noteworthy that peptides did not induce any changes in the proliferation rate of undifferentiated ES cells (data not shown). Retinoic acid is known to trigger the differentiation

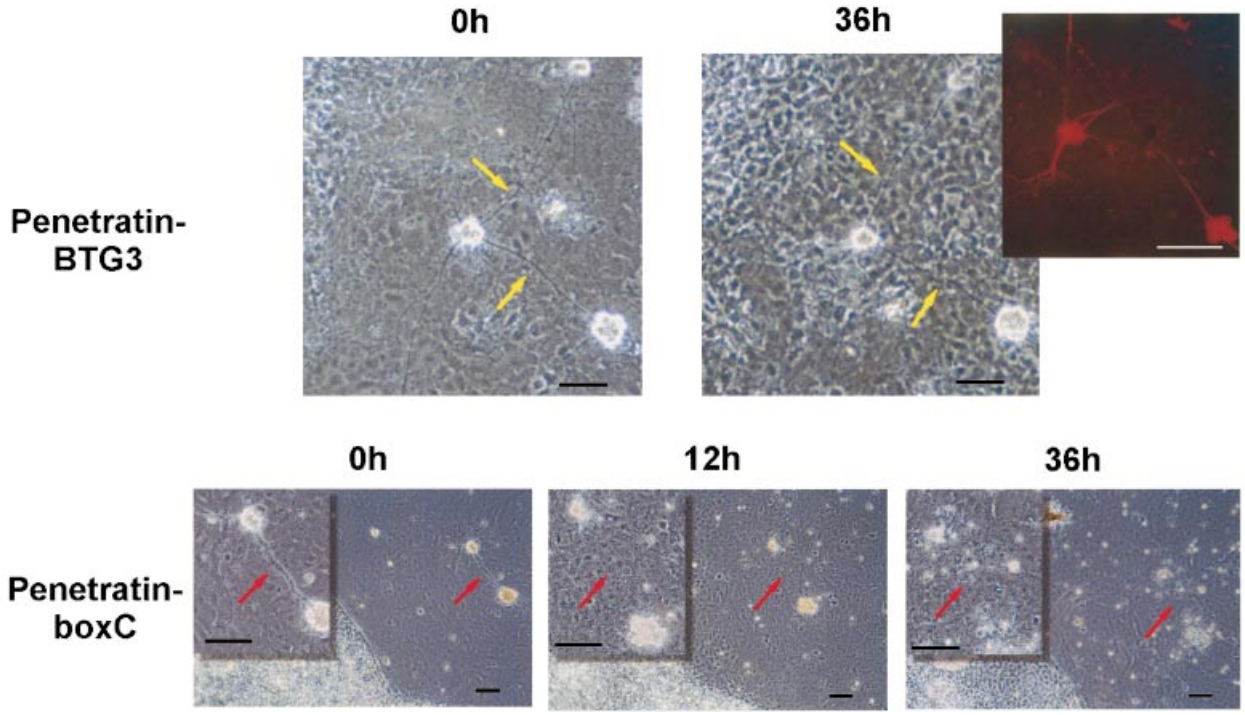

Figure 5 BoxC inhibits ES cell neuronal differentiation. ES cells were triggered to differentiate into various lineages. Different peptides were added to neuronal-like cells at 0 h. The morphology of the cells was analysed at different time points. Yellow arrows indicate Penetratin-BTG3 treated neurites. Red arrows illustrate the disappearance of neurites following Penetratin-boxC treatment. The neuronal phenotype was confirmed by MAP2 staining (upper right part of the figure). Similar results were observed with penetratin-BTG3 and penetratin (scale bar = 20 μm).

of ES cells into a variety of cell types and, more specifically, into endoderm-like and fibroblast-like cells (Smith 1991). After ES cells had differentiated into embryoid bodies, retinoic acid and the different peptides were added. The morphology of the different cell types did not change notably. However, under these conditions, neuronal-like cells did not develop following boxC addition (data not shown). Furthermore, when boxC peptide was added to a long-term culture (8 days) so as to allow complete differentiation, a neurite size reduction could was noted after 12 h, and a complete disappearance of the soma after 36 h (Fig. 5, lower part). Control peptides (BTG3 and penetratin) did not modify the morphology of these neuronal-like cells (Fig. 5). MAP2 antibody that detects neuronal cells was used (Binder *et al.* 1986). The upper right part of Fig. 5 illustrates this staining on differentiated ES cells. After the boxC treatment, only a few MAP2-positive cell aggregates were present. Furthermore, following boxC treatment the aggregates were surrounded by a reduced number of isolated cells (an average of 29 cells per aggregate vs. 88 cells with control peptides) (Table 1). Therefore, these results confirm and strengthen the idea that the PRMT1/BTG methylation pathway is involved in neurogenesis or at least in maintaining neuronal cells in a differentiated state.

Table 1 Peptides (15 μM) were added to morphologically differentiated ES cells. MAP2-positive isolated neurones, observed around one aggregate of neuronal-like cells were counted 2 days later. 5–10 aggregates were counted per experiment

	Number of neurones
Penetratin	82.4 ± 13.8
	97 ± 19.7
	84.5 ± 16.7
Penetratin-boxC	31.2 ± 13.5
	23.5 ± 0.7
	32.3 ± 22.7
Penetratin-BTG3	99.5 ± 23.5
	97.2 ± 18

To more precisely measure the observed effect, we used ES cell culture conditions leading to the preferential generation of neuronal cells, as described by Lee *et al.* (2000). In this differentiation process, the proliferation of neuronal precursors was sustained by bFGF. During this

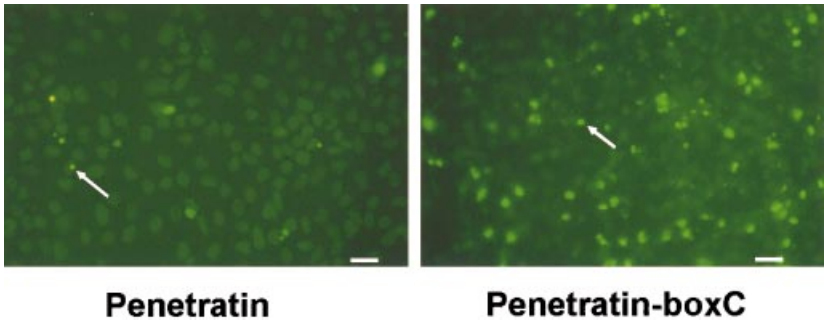

Figure 6 Neuronal cell apoptosis induced by boxC. Illustration of the experiments described in Table 2. Arrows indicate positive apoptotic cells (FITC revelation) (scale bar = 20 μM).

Table 2 BoxC addition and neuronal cell apoptosis

	% of MAP2 positive cells	% of apoptotic cells
Penetratin	35.38 ± 7.52	7.53 ± 3.36
Penetratin-boxC	6.43 ± 0.62	32.01 ± 0.94
Penetratin-BTG3	38.63 ± 3.81	8.23 ± 2.07

ES cells were preferentially differentiated into neuronal cells according to (Lee *et al.* 2000). Different peptides (15 μM) were added during terminal differentiation (after bFGF withdrawal). MAP2 antibody staining and TUNEL assays were performed 2 days later.

phase, the addition of any of the synthetic peptides induced no effect (growth rate, apoptosis, morphological phenotype). When bFGF was withdrawn, the cells stopped proliferating, and then eventually entered terminal differentiation (Lee *et al.* 2000). At this stage, penetratin-boxC addition led to increased rates of apoptosis as well as to the disappearance of MAP2-positive cells (post-mitotic neurones) in the same proportion (TUNEL assay, Fig. 6). Thirty-two per cent of cells treated with boxC were in apoptosis vs. 7% of cells treated with the control peptides (Table 2). These results showed that boxC addition interfered with terminal neuronal differentiation and induced apoptosis in cells engaged in the neuronal lineage.

Discussion

BTG/TOB proteins are highly conserved through evolution and seem to be related with the negative control of the cell cycle, although their precise function remains to be determined. To broaden our knowledge on BTG/TOB proteins and on the signal transduction pathways they might control, partners have actively been sought for. For instance, all BTG/TOB proteins seem to be associated with CAF1 (Bogdan *et al.* 1998; Ikematsu *et al.* 1999; Rouault *et al.* 1998; Yoshida *et al.* 2001). CAF1 belongs to a transcriptional regulatory complex and has recently been shown to be involved in the major cytoplasmic deadenylase in yeast (Tucker *et al.* 2001). However, the actual role of CAF1 remains to be elucidated and this interesting result does not shed any light on the role of the BTG/TOB family in this context. Another relevant study reported that BTG1 and 2 are physically associated with PRMT1 and positively regulate its activity, PRMT1 being the predominant cellular arginine methyltransferase of type I (Lin *et al.* 1996; Pawlak *et al.* 2000; Tang *et al.* 2000a).

Considering the above data, we decided to further analyse the nature and the functional consequences of the interaction between BTG/TOB family members and PRMT1. The present results show that a region of 10 amino acids found only in BTG1 and BTG2, named boxC, is required to achieve the exclusive association between BTG1 and 2 and PRMT1, since other members of the BTG/TOB family, which do not contain this sequence, do not seem to interact with PRMT1. It is noteworthy that CAF1 association with BTG/TOB proteins is mediated by boxB, one of the BTG/TOB family signatures (Guéhenneux *et al.* 1997; Rouault *et al.* 1998).

Lin and co-workers previously suggested that BTG1 and 2 play an important role in regulating PRMT1 methyltransferase activity (Lin *et al.* 1996). We hypothesized that peptide boxC would constitute a good tool for the analysis of this interaction. We studied the effect of boxC addition in a standard reaction using histone H2A as a substrate, a reaction which mostly reflects PRMT1 activity. We showed that boxC addition inhibited the stimulating effect of exogeneously added BTG2. Furthermore, the endogenous methylation activity (without BTG2 addition) was also reduced. These results suggest that boxC competes with BTG1 and 2 and thus prevents PRMT1 activation. These observations confirm the work of Lin and co-workers, who suggested that BTG1 and 2 are the major coactivators of PRMT1 (Lin *et al.* 1996). This means that the enhanced expression of

BTG1 and 2 observed during different processes (cell cycle arrest following stress injuries, differentiation) might stimulate PRMT1 methyltransferase activity and that PRMT1 could thus be a major effector of these phenomena. We suggest that BTG1 and 2/PRMT1 might constitute a new signal transduction pathway that can be inhibited by the addition of boxC.

To determine the physiological relevance of this pathway, we fused boxC with a cargo peptide, penetratin (Derossi *et al.* 1998). We chose PC12 cells as a cellular model. *BTG2/TIS21/PC3* expression is NGF-induced in PC12 cells (Bradbury *et al.* 1991). Furthermore, it has been demonstrated that protein methylation was necessary for PC12 cells to readily differentiate (Cimato *et al.* 1997). BoxC addition on undifferentiated PC12 cells had no effect. By contrast, differences in cell morphology were observed when NGF and boxC were added simultaneously: the differentiated cells formed clumps. We then sought to determine whether this could be due to the induction of apoptosis, since it had been shown that the inhibition of *P21* expression led to the apoptotic cell death of neuroblastoma cells triggered to differentiate (Poluha *et al.* 1996). It is noteworthy that *P21* is the prototype of an anti-proliferative gene involved in numerous processes such as genotoxic response and differentiation. In our model, we observed 15% of boxC-induced apoptosis. This rate is even greater than the 10% observed with *P21* inhibition. *BTG2* expression is induced during PC12 cell differentiation and is a predominant co-activator of PRMT1 (present results and Lin *et al.* (1996)). Therefore, we suggest that BTG1 and 2/PRMT1-driven arginine methylation could partly account for the essential role of protein methylation during PC12 cell differentiation (Cimato *et al.* 1997). Furthermore, even if the methylation of proteins seems to be a constitutive process, these results suggest the existence of a dynamic control. In this context, we noticed that boxC addition on PC12 cells induced the rapid loss of methylation of a 20 kDa protein, which might correspond to the unidentified 20 kDa arginine-methylated protein described by Gu *et al.* (1999). This experiment also indicated that boxC interferes with methyltransferase activity, not only when it is added on cellular extracts but also when it is already present in the cell. The nature of this 20 kDa protein remains to be determined, but it can be postulated that it regulates a process of highest importance during PC12 cell differentiation.

Several studies point to the role of the BTG/PRMT pathway in neuronal differentiation *in vivo*. Iacopetti and co-workers (Iacopetti *et al.* 1999) have shown that BTG2 is expressed in post-mitotic neuronal cells. Studies of *PRMT1*$^{+/-}$ mice reveal a strong expression at early stages of neurogenesis (midline of the neural plate) (Pawlak *et al.* 2000). In order to determine the selective influence of boxC on neuronal differentiation, we used ES cells differentiated into embryoid bodies and treated with retinoic acid, which give rise to different cell lineages (neuronal, haematopoeitic, cardio-muscular, endothelial, etc.). Following boxC treatment, we observed the selective disappearance of cells that expressed MAP2, which corresponded to postmitotic neuronal cells (Binder *et al.* 1986). Therefore, the BTG1 and 2/PRMT1 pathway seems to play a predominant role in the control of neuronal differentiation. We chose to test this hypothesis further using cell culture conditions that resulted in the preferential development of neuronal cells (Lee *et al.* 2000). In this assay, boxC induced a massive apoptosis of terminally differentiated neuronal cells, thus confirming the important role of BTG/PRMT1 in this process. Nevertheless, this work does not enable us to decipher whether arginine methylation mediated by PRMT1/BTG controls an anti-apoptotic signal, or whether it is only necessary for the neuronal differentiation process to be maintained. In the latter case, differentiated neuronal cells would be submitted to an uncomplete signal due to the lack of arginine methylation. They would thus degenerate via apoptosis. It should be noted that BTG proteins have been implicated in both mechanisms. For example, BTG1 has been shown to stimulate myoblast differentiation (Rodier *et al.* 1999), and the P53-induced gene *BTG2* is thought to play a role in G2/M control following genotoxic treatment (Rouault *et al.* 1996).

PRMT1 is thought to play a key role in numerous signal transduction pathways. For instance, PRMT1 is associated with the interferon IFNAR1 receptor and is required for interferon-induced transcription by STAT1 (Mowen *et al.* 2001). Several PRMT1-methylated proteins have also been described. Although the role of this post-translational modification is unknown, these targets could constitute anchors to delineate the controlled pathways. This report focuses on the PRMT1/BTG pathway and on its involvement in neurogenesis. One of the next goals will be to determine the nature and role of the proteins methylated by PRMT1 during differentiation.

Experimental procedures

Two-hybrid experiments

We used pPC62-BTG vectors as previously described (Rouault *et al.* 1998) (Fig. 1A). The *PRMT1* rat cDNA was obtained by RT-PCR using primers PRMT1-353 and PRMT1-1 according to the *PRMT1* sequence U60882 available in GenBank. We

followed the Superscript II (Gibco-BRL) and Promega protocols. The cDNA was then cloned in pPC86 (*Sal*I/*Not*I).

Oligonucleotides:

BTG1-1 aggtcgaCATCCCTTCTACACCCGG
BTG1-17 gctctagaTCCAACAGACTATATC
BTG1-117 catctagactaCTCTCCAATTCTGTAGGACAC
BTG1-129 gctctagacTGGTGAGGCTTCATACAGCAC
BTG1-154 gctctagacGAGAAGTTCCTCCTTACAGCT
boxC tcgacgGATGGCTCCATCTGTGTGCTGTATGAAGCCTCACCAtagt and ctagactaTGGTGAGGCTTCATACAGCACACACAGATGGAGCCATCcg
BTG2-1 aggtcgaACGGGAAGGGAACC
BTG2-158 gctctagaAGGGCCTAGCTGGAGAC
BTG3-1 aggtcgacGAAGAACGAAATTGCGGCT
BTG3-251 gctctagaCTAGTGAGGTGCTAACAT
PRMT1-1 gagtcaccATGGCGGCAGCCGAGGCCGCGAA
PRMT1-353 gagcggccGCCAGCCTGGCACCTCCTCAGC

All plasmids were sequenced using SEQUENASE 2.0 (US Biochemical Corp.).

The two-hybrid experiments were performed as previously described (Rouault *et al*. 1998) except for aminotriazol (20 mM), which was added to L/W/H (Leucine/Tryptophan/Histidine) depleted SC medium.

GST pull-down experiments

The coding sequences of *BTG2* and *BTG3* were inserted into the pGEX-ET expression vector (Pharmacia).

Deletion of boxC in pPC62-BTG2 was performed by mutagenesis using the USE Mutagenesis Kit (Pharmacia), with the following primer:

CCTACCGCATTGGGGAGGCGCCACTGGCCGCC;

The deleted boxC was situated between the underlined nucleotides.

PRMT1 cDNA was subcloned from pPC86-PRMT1 into the *Sal*I/*Not*I sites of pBluescript. *In vitro* [^{35}S]methionine-labelled PRMT1 was produced using the TNT T7 polymerase kit (Promega). GST pull-down experiments were performed as previously described (Rouault *et al*. 1998).

Methylation assays

PC12 cell extracts were prepared essentially as previously described (Lin *et al*. 1996), except for the cell disruption, which was performed by freeze-thawing four times in liquid nitrogen.

The 50 μL reaction contained: 25 mM Tris, 1 mM EDTA, 1 mM EGTA at pH 7.4, 5 μCi S-adenosyl-L-(methyl-^{3}H)methionine (SAM) (NEN Dupont, 85 Ci/mmol), 50 μg of cytosol extract, and if required 100 μg of histone H2A (Sigma) as substrate. Ten micrograms of GST-BTG2 or the peptides penetratin or penetratin-boxC were added at different concentrations in some methylation reactions. These peptides were synthesized by Syntem (Nîmes, France). The sequence of penetratin is RQIKIWFQRRMKWKK (Derossi *et al*. 1998) and that of penetratin-boxC is RQIKIWFQRRMKWKK*DGSICVLYEA*.

The reactions were incubated at 30 °C for 45 min, and then stopped by the addition of 10 μL of Laemmli buffer. The samples were run on 10% SDS-PAGE. The gel was fixed (25% isopropanol / 10% acetic acid) for 30 min, soaked in Amplify (Amersham) for 30 min, then dried and exposed.

Cell line and culture

PC12 cells were cultivated essentially as in Cimato *et al*. (1997). The cells were seeded, and then treated with NGF (50 ng/mL) (Sigma). One day after seeding, peptides penetratin and penetratin-boxC (15 μM) were added on to PC12 cells.

Embryonic Stem (ES) cells (ENS cell line (Gauthier *et al*. 1999)) were maintained routinely on feeder cells (40 Gy irradiated primary fibroblasts) in medium (BHK-21, Gibco BRL) supplemented with 10% foetal calf serum, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1% nonessential amino acids, 2 mM glutamine, 0.1 mg/mL penicillin-streptomycin and 2000 U/mL of human leukaemia inhibitory factor. ES cells were induced to differentiate by culture on to nonadherent Petri dishes (10^4 cells in 10 mL of LIF-deprived culture medium) for 5–8 days to allow for the formation of embryoid bodies. Embryoid bodies were then plated on tissue culture dishes coated with 0.1% gelatin, either with 10^{-6} M retinoic acid for 5–20 days, or according to the protocol developed by Lee *et al*. (2000) to select and expand neuronal cells, except that FGF8 was not added to the expansion medium.

Immunocytochemistry

Cells were washed in PBS, then fixed in 70% ethanol for 10 min, washed three times for 5 min each in TBS and dehydrated in 95% ethanol / 5% acetic acid for 15 min at –20 °C, and then washed three times in TBS. Cells were incubated for 20 min with 20% normal goat serum, washed three times in TBS, then incubated for 2 h with MAP2 monoclonal antibody (Sigma ref. M4403, diluted at 1/100 in TBS, 5% glycerol, 0.2% Tween, 2% BSA). MAP2 antibody is raised against microtubule associated proteins A and B, two isoforms that are abundant in the adult mammalian central nervous system. Cells were washed three times in TBS, and then incubated for 2 h with Cy3-conjugated goat anti-mouse antibody (Interchim ref. 115165003, diluted at 1/200 in TBS, 5% glycerol, 0.2% Tween, 2% BSA) and then washed three times in TBS; after 2 min incubation with bis-benzimide (diluted at 0.5 μg/mL in H_2O), cells were washed three times in TBS, and then mounted in Vectashield (Vector Laboratories).

Apoptosis detection

An *in Situ* Cell Death Detection kit (Roche) was used following the manufacturer's instructions for adherent cells except that cells were fixed in 70% ethanol for 10 min. One thousand cells were counted in each experiment.

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References

Abramovich, C., Yakobson, B., Chebath, J. & Revel, M. (1997) A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. *EMBO J.* **16**, 260–266.

Binder, L.I., Frankfurter, A. & Rebhun, L.I. (1986) Differential localization of MAP-2 and tau in mammalian neurons in situ. *Ann. NY Acad. Sci.* **466**, 145–166.

Bogdan, J.A., Adams-Burton, C., Pedicord, D.L., *et al.* (1998) Human carbon catabolite repressor protein (CCR4)-associative factor 1: cloning, expression and characterization of its interaction with the B-cell translocation protein BTG1. *Biochem. J.* **336**, 471–481.

Bradbury, A., Possenti, R., Shooter, E.M. & Tirone, F. (1991) Molecular cloning of PC3, a putatively secreted protein whose mRNA is induced by nerve growth factor and depolarization. *Proc. Natl. Acad. Sci. USA* **88**, 3353–3357.

Buanne, P., Corrente, G., Micheli, L., *et al.* (2000) Cloning of PC3B, a novel member of the PC3/BTG/TOB family of growth inhibitory genes, highly expressed in the olfactory epithelium. *Genomics* **68**, 253–263.

Chen, D., Ma, H., Hong, H., *et al.* (1999) Regulation of transcription by a protein methyltransferase. *Science* **284**, 2174–2177.

Chevray, P.M. & Nathans, D. (1992) Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. *Proc. Natl. Acad. Sci. USA* **89**, 5789–5793.

Cimato, T.R., Ettinger, M.J., Zhou, X. & Aletta, J.M. (1997) Nerve growth factor-specific regulation of protein methylation during neuronal differentiation of PC12 cells. *J. Cell Biol.* **138**, 1089–1103.

Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G. & Prochiantz, A. (1996) Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* **271**, 18188–18193.

Derossi, D., Chassaing, G. & Prochiantz, A. (1998) Trojan peptides: the penetratin system for intracellular delivery. *Trends Cell Biol.* **8**, 84–87.

Fields, S. & Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340**, 245–246.

Fletcher, B.S., Lim, R.W., Varnum, B.C., Kujubu, D.A., Koski, R.A. & Herschman, H.R. (1991) Structure and expression of TIS21, a primary response gene induced by growth factors and tumor promoters. *J. Biol. Chem.* **266**, 14511–14518.

Fraichard, A., Chassande, O., Bilbaut, G., Dehay, C., Savatier, P. & Samarut, J. (1995) In vitro differentiation of embryonic stem cells into glial cells and functional neurons. *J. Cell Sci.* **108**, 3181–3188.

Gary, J.D. & Clarke, S. (1998) RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucl. Acid Res. Mol. Biol.* **61**, 65–131.

Gauthier, K., Chassande, O., Plateroti, M., *et al.* (1999) Different functions for the thyroid hormone receptors TRalpha and TRbeta in the control of thyroid hormone production and post-natal development. *EMBO J.* **18**, 623–631.

Ghosh, S.K., Paik, W.K. & Kim, S. (1988) Purification and molecular identification of two protein methylases I from calf brain. Myelin basic protein- and histone-specific enzyme. *J. Biol. Chem.* **263**, 19024–19033.

Gu, H., Park, S.H., Park, G.H., *et al.* (1999) Identification of highly methylated arginine residues in an endogenous 20-kDa polypeptide in cancer cells. *Life Sci.* **65**, 737–745.

Guardavaccaro, D., Corrente, G., Covone, F., *et al.* (2000) Arrest of G(1)-S progression by the p53-inducible gene PC3 is Rb dependent and relies on the inhibition of cyclin D1 transcription. *Mol. Cell. Biol.* **20**, 1797–1815.

Guéhenneux, F., Duret, L., Callanan, M.B., *et al.* (1997) Cloning of the mouse BTG3 gene and definition of a new gene family (the BTG family) involved in the negative control of the cell cycle. *Leukemia* **11**, 370–375.

Iacopetti, P., Michelini, M., Stuckmann, I., Oback, B., Aaku-Saraste, E. & Huttner, W.B. (1999) Expression of the antiproliferative gene TIS21 at the onset of neurogenesis identifies single neuroepithelial cells that switch from proliferative to neuron-generating division. *Proc. Natl. Acad. Sci. USA* **96**, 4639–4644.

Ikematsu, N., Yoshida, Y., Kawamura-Tsuzuku, J., *et al.* (1999) Tob2, a novel anti-proliferative Tob/BTG1 family member, associates with a component of the CCR4 transcriptional regulatory complex capable of binding cyclin-dependent kinases. *Oncogene* **18**, 7432–7441.

Klein, S., Carroll, J.A., Chen, Y., *et al.* (2000) Biochemical analysis of the arginine methylation of high molecular weight fibroblast growth factor-2. *J. Biol. Chem.* **275**, 3150–3157.

Koh, S.S., Chen, D., Lee, Y.H. & Stallcup, M.R. (2001) Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities. *J. Biol. Chem.* **276**, 1089–1098.

Lee, S.H., Lumelsky, N., Studer, L., Auerbach, J.M. & McKay, R.D. (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat. Biotechnol.* **18**, 675–679.

Lin, W.J., Gary, J.D., Yang, M.C., Clarke, S. & Herschman, H.R. (1996) The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. *J. Biol. Chem.* **271**, 15034–15044.

Matsuda, S., Kawamura-Tsuzuku, J., Ohsugi, M., *et al.* (1996) Tob, a novel protein that interacts with p185erbB2, is associated with anti-proliferative activity. *Oncogene* **12**, 705–713.

Matsuda, S., Rouault, J., Magaud, J. & Berthet, C. (2001) In search of a function for the TIS21/PC3/BTG1/TOB family. *FEBS Lett.* **497**, 67–72.

Montagnoli, A., Guardavaccaro, D., Starace, G. & Tirone, F. (1996) Overexpression of the nerve growth factor-inducible PC3 immediate early gene is associated with growth inhibition. *Cell Growth Diff.* **7**, 1327–1336.

Mowen, K.A., Tang, J., Zhu, W., *et al.* (2001) Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. *Cell* **104**, 731–741.

Nichols, R.C., Wang, X.W., Tang, J., *et al.* (2000) The RGG domain in hnRNP A2 affects subcellular localization. *Exp. Cell Res.* **256**, 522–532.

Pawlak, M.R., Scherer, C.A., Chen, J., Roshon, M.J. & Ruley, H.E. (2000) Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable. *Mol. Cell. Biol.* **20**, 4859–4869.

Poluha, W., Poluha, D.K., Chang, B., *et al.* (1996) The cyclin-dependent kinase inhibitor p21 (WAF1) is required for survival of differentiating neuroblastoma cells. *Mol. Cell. Biol.* **16**, 1335–1341.

Prevot, D., Voeltzel, T., Birot, A.M., *et al.* (2000) The leukemia-associated protein Btg1 and the p53-regulated protein Btg2 interact with the homeoprotein Hoxb9 and enhance its transcriptional activation. *J. Biol. Chem.* **275**, 147–153.

Rodier, A., Marchal-Victorion, S., Rochard, P., *et al.* (1999) BTG1: a triiodothyronine target involved in the myogenic influence of the hormone. *Exp. Cell Res.* **249**, 337–348.

Rouault, J.P., Falette, N., Guehenneux, F., *et al.* (1996) Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. *Nat. Genet.* **14**, 482–486.

Rouault, J.P., Prevot, D., Berthet, C., *et al.* (1998) Interaction of BTG1 and p53-regulated BTG2 gene products with mCaf1, the murine homolog of a component of the yeast CCR4 transcriptional regulatory complex. *J. Biol. Chem.* **273**, 22563–22569.

Rouault, J.P., Rimokh, R., Tessa, C., *et al.* (1992) BTG1, a member of a new family of antiproliferative genes. *EMBO J.* **11**, 1663–1670.

Seo, M.S., Lee, M.S. & Lim, I.K. (1999) Expression of rat BTG3 gene, Rbtg3, is regulated by redox changes. *Gene* **240**, 165–173.

Shen, E.C., Henry, M.F., Weiss, V.H., Valentini, S.R., Silver, P.A. & Lee, M.S. (1998) Arginine methylation facilitates the nuclear export of hnRNP proteins. *Genes Dev.* **12**, 679–691.

Smith, A.G. (1991) Culture and differentiation of embryonic stem cellss. *J. Tiss. Cult. Meth.* **13**, 89–94.

Smith, J.J., Rucknagel, K.P., Schierhorn, A., *et al.* (1999) Unusual sites of arginine methylation in Poly(A)-binding protein II and in vitro methylation by protein arginine methyltransferases PRMT1 and PRMT3. *J. Biol. Chem.* **274**, 13229–13234.

Tang, J., Frankel, A., Cook, R.J., *et al.* (2000a) PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. *J. Biol. Chem.* **275**, 7723–7730.

Tang, J., Kao, P.N. & Herschman, H.R. (2000b) Protein-arginine methyltransferase I, the predominant protein-arginine methyltransferase in cells, interacts with and is regulated by interleukin enhancer-binding factor 3. *J. Biol. Chem.* **275**, 19866–19876.

Tucker, M., Valencia-Sanchez, M.A., Staples, R.R., Chen, J., Denis, C.L. & Parker, R. (2001) The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in Saccharomyces cerevisiae. *Cell* **104**, 377–386.

Yoshida, Y., Hosoda, E., Nakamura, T. & Yamamoto, T. (2001) Association of ANA, a member of the antiproliferative Tob family proteins, with a Caf1 component of the CCR4 transcriptional regulatory complex. *Jpn. J. Cancer Res.* **92**, 592–596.

Yoshida, Y., Matsuda, S., Ikematsu, N., *et al.* (1998) ANA, a novel member of Tob/BTG1 family, is expressed in the ventricular zone of the developing central nervous system. *Oncogene* **16**, 2687–2693.

Yoshida, Y., Tanaka, S., Umemori, H., *et al.* (2000) Negative regulation of BMP/Smad signaling by Tob in osteoblasts. *Cell* **103**, 1085–1097.

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