

## Ret finger protein is a normal component of PML nuclear bodies and interacts directly with PML

Tongyu Cao<sup>1,\*</sup>, Estelle Duprez<sup>2</sup>, Katherine L. B. Borden<sup>2,\*\*</sup>, Paul S. Freemont<sup>2</sup> and Laurence D. Etkin<sup>1,‡</sup>

<sup>1</sup>Department of Molecular Genetics, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston TX 77030, USA

<sup>2</sup>Molecular Structure and Function Laboratory, Imperial Cancer Research Fund, 44 Lincolns Inn Fields, London WC2A 3PX, UK

\*Present address: Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

\*\*Present address: Department of Biochemistry, Faculty of Medicine, Dalhousie University Nova Scotia, Canada

‡Author for correspondence (e-mail: lde@mdacc.tmc.edu)

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### SUMMARY

The ret finger protein (rfp) is a member of the B-box zinc finger gene family many of which may function in growth regulation and in the appropriate context become oncogenic. Members of this family are nuclear proteins that possess a characteristic tripartite motif consisting of the RING and B-box zinc binding domains and a coiled-coil domain. The promyelocytic leukemia gene (PML), another B-box family member, produces a protein product that is detected within punctate nuclear structures called PML nuclear bodies (NBs) or PML oncogenic domains (PODs). These NBs are complex structures that consist of a number of different proteins many of which have yet to be identified. In the disease acute promyelocytic leukemia (APL) a fusion protein, PML-RARA, is produced through the t(15:17) translocation. In APL the morphology of the NBs is altered. We report that rfp co-localizes with PML in a subset of the PML NBs and that it interacts directly with

PML. This interaction is mediated through the rfp B-box and the distal two coils. In contrast, homomultimerization of rfp preferentially involves the B-box and the proximal coil. The association of rfp with the PML NBs is altered by mutations that affect rfp/PML interaction and in NB4 cells that are derived from APL patients. When treated with retinoic acid, rfp reassociates with the NBs in a pattern similar to non APL cells. Additionally, we found that rfp colocalizes with PML-RARA protein produced in APL patients. These results suggest that rfp, along with the other known/unknown components of PML NBs, have an important role in regulating cellular growth and differentiation.

Key words: rfp, PML, Acute promyelocytic leukemia, Differentiation, B-box, RING finger, Zinc finger

### INTRODUCTION

Acute promyelocytic leukemia (APL) is associated with the reciprocal chromosomal translocation t(15:17) (Rowley et al., 1977). This translocation fuses the PML gene located on chromosome 15 with the retinoic acid receptor alpha gene (RARA) on chromosome 17 (Borrow et al., 1990; deThe et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1991; Chang et al., 1992; Kastner et al., 1992). The fusion protein PML-RARA appears to block hematopoiesis at the promyelocytic stage. The PML protein is a member of the B-box zinc finger protein family. Members of this family possess a characteristic tripartite motif consisting of two zinc finger domains, a RING finger and one or two B-box domains, and an  $\alpha$ -helical coiled-coil domain (Reddy and Etkin, 1991; Reddy et al., 1992; Freemont, 1993). In addition to PML, two other B-box family members are oncogenic when their tripartite motifs are fused to protein kinase domains. These include the TIF protein in which the N-terminal RING, B-box and coiled-coil domains recombine with B-raf serine/threonine kinase domain (Le

Douarin et al., 1995) and the ret finger protein (rfp) whose N-terminal tripartite motif recombines with the ret protooncogene (Takahashi et al., 1988). Thus, it is likely that this tripartite motif in concert with other domains from various receptors is somehow involved in initiating oncogenesis.

The Rfp protein is detected in various cell types in mice and humans and is found in specific stages of mouse differentiating sperm cells (Cao et al., 1996; Takahashi et al., 1988). The B-box and coiled-coil domains are involved in rfp homomultimerization, while the RING finger appears to be involved in interactions with components crucial for rfp nuclear localization (Cao et al., 1997) and also in the transforming potential of the recombined oncogene (Hasegawa et al., 1996). In tissues and in various tissue culture cell lines, rfp is detected within nuclei in a punctate pattern (Cao et al., 1996, 1997). This pattern is similar to that observed for PML, which is detected in nuclear structures called ND10, Kr bodies, PML oncogenic domains (PODs), PML nuclear bodies (PML NBs) or PML bodies (Koken et al., 1994; Weis et al., 1994; Dyck et al., 1994; for review see Doucas and Evans, 1996).

PML NBs do not appear to be associated with chromatin, but are tightly bound to the nuclear matrix (Dyck et al., 1994; Weis et al., 1994) and contain several proteins identified mainly through screening autoimmune antisera for immunofluorescence patterns that overlap with that of the PML NBs (Ascoli and Maul, 1991). These include the proteins NDP52 (Zuber et al., 1995), NDP55 (Ascoli and Maul, 1991), NDP65 (Epstein, 1984; Dyck et al., 1994) and ND/KrAg/Sp100 (Szosteki et al., 1990; Xie et al., 1993; Weis et al., 1994) now known as Sp100 (Weis et al., 1994). Three other components of the PML NBs have recently been identified; namely Int-6, which is a dominant negative oncogene present after retroviral infection (Desbois et al., 1996), PIC-1, a protein of unknown function with homology to ubiquitin, which specifically interacts with PML (Boddy et al., 1996), and a ubiquitin-specific protease called HAUSP (Everett et al., 1997). The role of PML NBs is still unknown and the importance of their integrity for PML function is not fully understood. However, evidence suggests that PML NBs are the target for a variety of virus-derived proteins and that subsequent loss of organization of these domains is an important phase of the viral infection cycle (Maul and Everett, 1994; Puvion-Dutilleul et al., 1995; Carvalho et al., 1995; Doucas and Evans, 1996; Ishov and Maul, 1996; Szekely et al., 1996).

In APL patients and in an APL derived cell line NB4, PML NBs are disrupted presumably by the expression of the PML-RARA fusion protein (Lanotte et al., 1991; Perez et al., 1993; Weis et al., 1994; Dyck et al., 1994; Koken et al., 1994). Interestingly, retinoic acid treatment of NB4 cells restores the ability of the cell to differentiate, and also results in a reformation of normal PML NB complexes (Koken et al., 1994). Thus, there seems to be a direct correlation between the presence of PML NBs and the normal growth and differentiation of the promyelocytic cell lineage. However, it was shown that mutants of PML-RARA which do not perturb PML NBs were functionally active in blocking differentiation (Grignani et al., 1996). Therefore, to gain a further understanding of their function in normal promyelocytic differentiation and in APL disease, it is necessary to dissect the components of this multi-protein complex and to determine how they interact with each other.

In the present study, we demonstrate that one of the components of PML NBs is rfp and that the presence of rfp in nuclear bodies is probably due to a direct interaction between PML and rfp. This interaction is mediated by the B-box and distal  $\alpha$ -helical coiled-coil domains of rfp. Also, rfp is found dispersed in microgranules in NB4 cells but reassociates with the PML NBs when these cells are treated with retinoic acid. These results suggest that rfp, along with the other known/unknown components of PML NBs, may have an important role in regulating cellular growth and differentiation.

## MATERIALS AND METHODS

### Recombinant DNA

The full length mouse rfp cDNA and the human rfp cDNA fragment encoding the RING finger, the B-box, and the coiled-coil domain (RBC) were used as templates to generate mutant constructs by polymerase chain reaction (PCR). Point mutations in the RING finger and the B-box were generated by PCR based mutagenesis and

deletions in the coiled-coil domain were generated by PCR and restriction digestion. We made point mutations in four of the zinc ligands in the rfp RING finger, and two or three of the putative zinc ligands in the B-box.

The PCR products were subcloned and sequenced to ensure there were no point mutations due to PCR errors. The human rfp cDNA inserts were subcloned into pBTM116 (Vojtek et al., 1993) (resulting in fusion proteins between the LexA DNA-binding domain and rfp domains) and pVP16 (Vojtek et al., 1993) (resulting in fusion proteins between the VP16 trans-activation domain and rfp domains) for dimerization studies in the yeast two-hybrid system. The mouse rfp cDNA and human rfp cDNA fragments were subcloned into pMLV-plink2 vector (encoding a fusion protein with a myc tag at the amino terminus) for subcellular localization studies in mammalian cells.

The wild-type and mutant human rfp cDNA in pBTM116 or pVP16 vectors were transformed into *Saccharomyces cerevisiae* strain L40 (Vojtek et al., 1993) to test for their ability to homomultimerize.  $\beta$ -Galactosidase ( $\beta$ -gal) activity of transformed yeast cells was analyzed by either filter lift assay or liquid assay. Procedures were the same as those used by Cao et al. (1997).

### Mammalian cells and transfection

The cDNAs encoding wild-type mouse rfp and mutant human rfp were subcloned into the pMLV-plink2 vector, resulting in a fusion protein with a human c-myc epitope tag at the amino terminus. The human PML cDNA was in the same vector encoding a protein with only PML sequence but not the c-myc epitope (Borden et al., 1996). HeLa cells and mouse A9 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Life Technologies) supplemented with 5% FBS and 5% BCS. Cells grown on coverslips were transiently transfected using LipofectAmine (Life Technologies) as described by Cao et al. (1997).

### Indirect immunofluorescent staining of mammalian cells

HeLa and mouse A9 cells were transfected with constructs encoding wild-type mouse rfp (with c-myc tag), mutant human rfp constructs with a c-myc tag, or wild-type human PML without a c-myc tag. Cells were fixed in 4% paraformaldehyde and incubated in rabbit anti-mouse rfp antiserum (1:300) and mouse anti-human c-myc monoclonal antibody (1:150) (Genosys) or PML monoclonal antibody 5E10 (Stuurman et al., 1992) for 30 minutes at room temperature (Cao et al., 1997). Cells were incubated in goat anti-rabbit IgG (H+L) conjugated with FITC (1:200) (BMB) and goat anti-mouse IgG (H+L) conjugated with rhodamine (1:200) (Pierce). The mouse and human rfp share a high degree of homology and are both recognized by the rabbit anti-mouse rfp antiserum. Cells were then examined by fluorescent microscopy.

NB4 cells were smeared on histological slides using a cytocentrifuge (cytospin, Shandon) and then fixed in acetone/methanol (1:1 v/v) at  $-20^{\circ}\text{C}$  for 2 minutes and allowed to air dry. The slides were incubated with the monoclonal PML antibody (1:5) and a rabbit anti-mouse polyclonal rfp antibody (1:300) in PBS for 1 hour at room temperature and incubated with fluorescein-coupled anti-mouse antibody (Dako) (1:200) and Texas red-coupled anti-rabbit antibody (1:200) (Amersham) for 30 minutes at room temperature. All incubations were followed by three washes in 0.05% Tween in PBS for 10 minutes. Preparations were examined by confocal microscopy. 2C4 cells (human fibrosarcoma, HT1080 derived) were transiently transfected using the calcium phosphate precipitation method. After 40 hours the cells were washed in PBS. For immunofluorescent studies cells were fixed 24 hours after transfection at  $-20^{\circ}\text{C}$  in methanol for 10 minutes. The products of the various constructs were detected using the rabbit anti-mouse rfp antiserum (Cao et al., 1996), PML polyclonal antibody (Boddy et al., 1996), PML 5E10 monoclonal antibody (Stuurman et al., 1992) and the RARA polyclonal antibody (a gift from P. Chambon). Cells were

incubated with secondary antibodies conjugated with FITC and Texas red and examined using confocal microscopy.

### Co-immunoprecipitation of rfp and PML

In co-immunoprecipitation experiments, an anti-human PML antibody mAB 5E10 was covalently attached to Protein A-Sepharose (Pharmacia) by cross-linking with dimethyl pimelimidate (DMP) (Ley et al., 1994; Schneider et al., 1982). Briefly Protein A-Sepharose beads were washed twice in 0.2 M sodium borate (pH 9.0). We used 2  $\mu$ g/ml of antibody for each 1 ml of beads, mixing continuously for 2 hours at room temperature (RT). Beads were washed with sodium borate solution, followed by 0.2 M triethanolamine, pH 8.5. An equal volume of 40 mM DMP in 0.2 M triethanolamine was added and the mixture was gently agitated for 1 hour at RT, centrifuged and supernatant removed. An equal volume of 0.2 M ethanolamine, pH 8.2, was added to the beads for 5 minutes at RT. Beads were washed with the sodium borate solution and stored at 4°C until used. Beads were rinsed in phosphate buffered saline (PBS) prior to use.

Human fibroblast 551 cells were washed two times in serum free medium, trypsinized and transferred to a Falcon tube with medium and serum and pelleted at 800 *g* at RT for 5 minutes. Pellets were washed three times in medium prewarmed to 37°C. Cells were lysed in buffer IPB (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Nonidet P40, 100  $\mu$ M PMSF, and 5  $\mu$ g/ml of the following protease inhibitors: chemostatatin, leupeptin, pepstatin A, aprotinin and antipain). Cells were centrifuged at 800 *g* for 5 minutes at 4°C and the pellets resuspended. They were centrifuged again at 13,000 *g* for 20 minutes at 4°C. Pre-clearing was then carried out according to the method of Harlow and Lane (1988). Supernatants were collected and 20  $\mu$ l of Protein A-Sepharose plus mouse Ig was added. Beads were mixed for at least 30 minutes at 4°C. Samples were centrifuged to remove beads and Protein A-mAB 5E10 beads were added to the supernatant and mixed for 2 hours at 4°C. Beads were then washed three times with modified IPB buffer (0.1% deoxycholate and no nonidetP-40). Beads were spun and collected. An equal volume of reducing sample buffer was added in preparation for SDS-PAGE. Gels were blotted and probed with rfp antibody (Cao et al., 1997).

## RESULTS

### A portion of Rfp co-localizes with PML in nuclear bodies

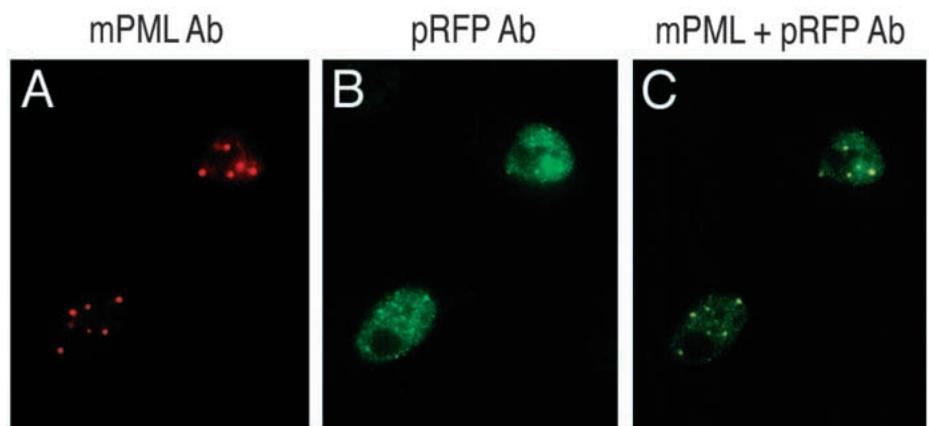
We have recently shown that rfp is localized within nuclei of some mammalian cells in a punctate staining pattern resembling PML NBs (Cao et al., 1997). We therefore wanted to determine if in fact rfp and PML co-localize in these nuclear

structures. To accomplish this, we analyzed HeLa cells by immunostaining with a monoclonal antibody against PML and a polyclonal antibody against rfp. Fig. 1A shows the location of PML nuclear bodies within the HeLa cell nuclei. Fig. 1B shows the location of rfp while Fig. 1C shows the co-localization of PML and rfp. Although a substantial amount of rfp is distributed throughout the nucleus, it is clear that PML and a portion of rfp co-localize in HeLa cells. This suggests that the endogenous rfp protein is a component of PML NBs.

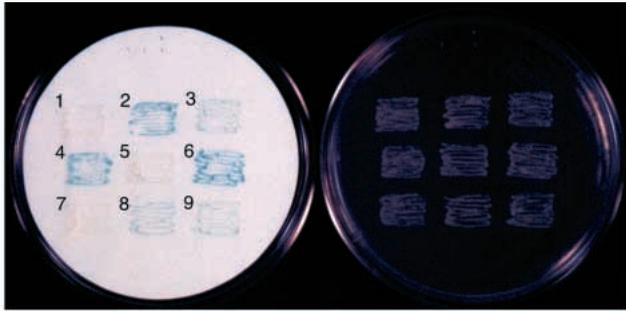
### PML and rfp form multimers which involve the rfp B-box and distal coils

Since the endogenous rfp and PML co-localize to the same nuclear structures, we were interested in determining whether there was direct interaction between the two proteins. To assay for this potential interaction, we used the yeast two-hybrid system and co-immunoprecipitation assays. The cDNA fragment encoding the tripartite motif, including the RING finger, the B-box, and the coiled-coil domain (RBC) from human rfp was subcloned into the yeast two-hybrid DNA binding domain vector forming a LexA-RBC fusion protein. The PML cDNA was subcloned into the transactivation domain vector forming a VP16-PML fusion. After introducing constructs containing RBC and PML into yeast strain L40, we detected high activity of  $\beta$ -galactosidase ( $\beta$ -gal), which was indicative of a strong interaction between PML and rfp (Figs 2, 4C). The rfp and PML did not interact with either the LexA DNA-binding domain or the VP16 trans-activation domain alone (data not shown; Cao et al., 1997). Also, rfp did not interact with lamin C, which was used as a control for non-specific interaction (Fig. 2). Additionally, as shown previously, rfp formed homomultimers when the rfp cDNA was placed in both the activation and DNA binding domain vectors (Fig. 2 and Cao et al., 1997). Fig. 2 also shows that PML interacts with itself. These results demonstrate that rfp and PML interact in the yeast two-hybrid system, suggesting that their co-localization in nuclear bodies in vivo involves direct interaction between the two proteins. Although the experiments above use a truncated form of rfp the full-length rfp also interacts in the two-hybrid system (data not shown).

As further evidence for the interaction of rfp and PML, we showed they can co-immunoprecipitate in extracts from human fibroblast 551 cells. Fig. 3 shows an experiment in which an extract from these cells was prepared and incubated with



**Fig. 1.** Co-localization of rfp and PML to PML nuclear bodies in HeLa cells. Endogenous rfp and PML were detected by indirect immunofluorescent staining using anti-PML monoclonal antibody 5E10 (rhodamine, red) (A) and rabbit anti-rfp antiserum (FITC, green) (B), respectively. Double staining appears yellow due to the overlap of both signals (C).

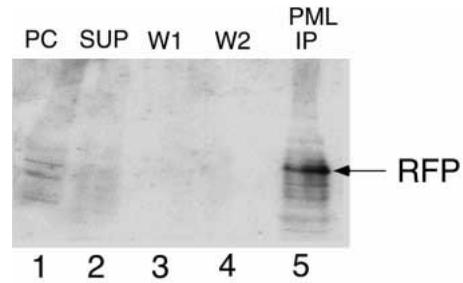


	Fusion with DNA-binding Domain of LexA or GAL4	Fusion with Transactivation Domain of VP16 or GAL4
1	Lamin	RBC
2	RBC	RBC
3	PML	PML
4	RBC	PML
5	$\Delta C_{23}$	PML
6	RING	PML
7	$B_{C^1H^2}$	PML
8	$B_{C^3C^4C^5}$	PML
9	$B_{H^6H^7}$	PML

**Fig. 2.** Interaction of rfp and PML in the yeast two-hybrid system and determination of the rfp domains involved in such interaction. Two constructs are co-transformed to test the interaction between the two fusion proteins they encode. One is a fusion protein with the DNA-binding domain of LexA or GAL4, the other is a fusion protein with the transactivation domain of VP16 or GAL4. The blue coloration on the left is indicative of the activity of reporter gene  $\beta$ -galactosidase. The plate on the right shows normal growth of the transformants.

Protein A-Sepharose beads onto which a monoclonal antibody (mAb 5E10) against PML was covalently attached. The beads were washed, the bound proteins eluted, and analyzed by western blotting using an antibody that recognizes both human and mouse rfp (Cao et al., 1996). It is clear that a major band migrating at 60 kDa was recognized by the antibody. This result provides biochemical evidence for the interaction of endogenous rfp and PML.

We were also interested in determining which domains of rfp were involved in the interaction with PML. We therefore constructed a series of rfp mutants that disrupt the RING finger and B-box zinc finger domain structures and deleted various portions of the coiled-coil domains (Fig. 4A,B). These constructs along with wild-type PML were transformed into yeast cells. Mutations that destroy the rfp RING finger namely  $Cys^3, His^4, Cys^5, Cys^6(R/C^3H^4C^5C^6)$  had no effect on the interaction with PML (Figs 2, 4C). Mutation of the  $Cys^1$  and  $His^2$  ( $B/C^1H^2$ ) of the B-box resulted in a complete loss of interaction; while mutations of the internal Cys residues ( $B/C^3C^4C^5$ ) resulted in a 70% reduction of activity and mutation of  $His^6His^7$  ( $B/H^6H^7$ ) resulted in a significant loss of activity (Figs 2, 4C). Structural predictions indicate that the rfp coiled-coil domain consisted of three subdomains, that we refer



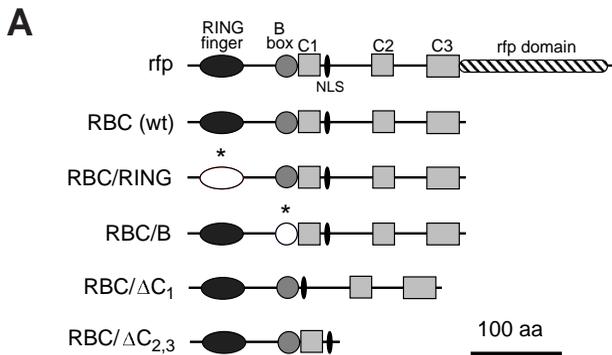
**Fig. 3.** Co-immunoprecipitation of endogenous rfp and PML in cells. Lysates prepared from human fibroblast 551 cells were immunoprecipitated with the PML mouse monoclonal antibody mAb 5E10. Lane 1, preclear from the beads at the pre-clear step (see Materials and Methods); lane 2, supernatant from the co-immunoprecipitation step (SUP); lanes 3 and 4, the last two washes to the beads (washes 5 and 6); lane 5, immunoprecipitate using the PML monoclonal antibody and resolved on western blot probed with the rfp antibody.

to as  $C_1C_2C_3$  from proximal to distal from the B-box (Fig. 4A). Deletion of the proximal coiled-coil domain ( $\Delta C_1$ ) had no effect on rfp-PML interaction, while deletion of the distal two coils ( $\Delta C_2C_3$ ) eliminated all interaction (Figs 2, 4C).

Several conclusions can be drawn from these results. First, the rfp RING finger domain does not function in rfp-PML interaction. Second, a proper B-box zinc finger structure is important in the interaction between rfp and PML. Third, the distal two  $\alpha$  helical coiled-coils are critical while the proximal coiled-coil is dispensable for rfp-PML interaction. Interestingly, while the proximal rfp coil does not function in heteromultimerization of rfp with PML, it is important in rfp homomultimerization (Cao et al., 1997). This indicates that there is specificity in the different coiled-coil domains of rfp with regard to the nature of the interacting proteins.

#### Mutations that affect rfp-PML interaction interfere with their co-localization in nuclear bodies

We have shown that: (1) a substantial portion of rfp and PML co-localize in NBs, (2) rfp and PML interact in the two-hybrid system, and (3) mutations in the B-box and distal coils of rfp interfere with rfp/PML interactions in the two-hybrid system. Therefore, if rfp/PML interaction is necessary for the localization of rfp in NBs, we would anticipate that mutations in the rfp B-box and distal coils will result in loss of one or the other protein in these subnuclear structures. To test this, we transfected HeLa cells with the various rfp mutants that are tagged with the myc-epitope. We then analyzed the transfected cells by indirect immunofluorescence to detect exogenous rfp and the endogenous PML. Fig. 5A-A'' and B-B'' shows that when the B-box was mutated at either the  $Cys^3Cys^4Cys^5$  or  $His^6His^7$  the colocalization of rfp and PML was disrupted. Interestingly, in these cells rfp is detected within nuclear bodies that are distinct from PML NBs, suggesting that the B-box is required for targeting rfp to PML NBs. Additionally, when the distal helical coiled-coils were deleted, rfp also did not co-localize with PML NBs, giving a nuclear diffuse staining pattern (Fig. 5C-C''). It is notable that all three rfp mutants localize to the nucleus and are nucleoli excluded. These results are consistent with the yeast two-hybrid analysis, which



**B**

RING finger:

wildtype: CPVCLQYFAEPMMLDCGHNCCACLARCWGTAEITNVSCPQC  
 conserved: C--C-----C-H--C-C-----C--C  
 1 2 3 4 5 6 7 8

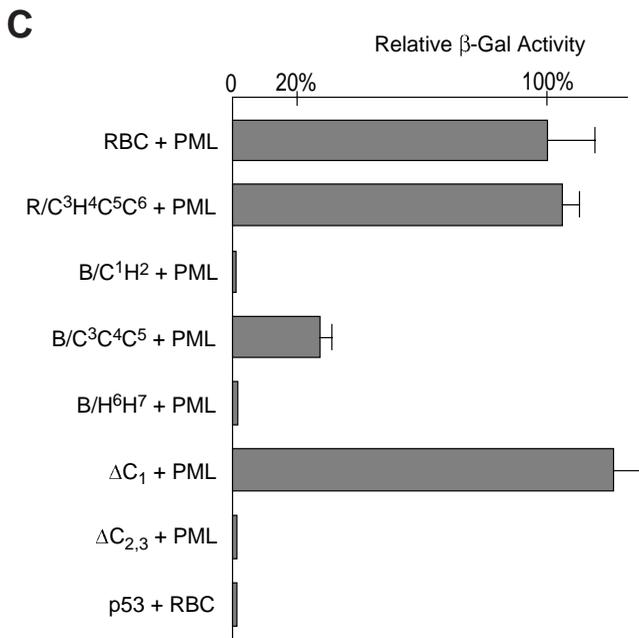
C<sup>3</sup>H<sup>4</sup>C<sup>5</sup>C<sup>6</sup> mt: G N G G

B box:

Xnf7 B box peptide: 1 6 9 17 25 28 34 37 42  
 RPLEKCSSEHDERLKLKLYCKDDGTLSCVICRDSLKHASHNPLPI

rfp B box: GEMGVCEKHREPLKLYCEEDQMPICVVCDSREHRGHSVLP  
 conserved: C--H-----C-----C--C-----H--H  
 1 2 3 4 5 6 7

C<sup>1</sup>H<sup>2</sup> mt: G N  
 C<sup>3</sup>C<sup>4</sup>C<sup>5</sup> mt: G G G  
 H<sup>6</sup>H<sup>7</sup> mt: N N



defines the B-box and  $\alpha$ -helical coiled-coil domains of rfp as being the domains involved in rfp-PML interaction.

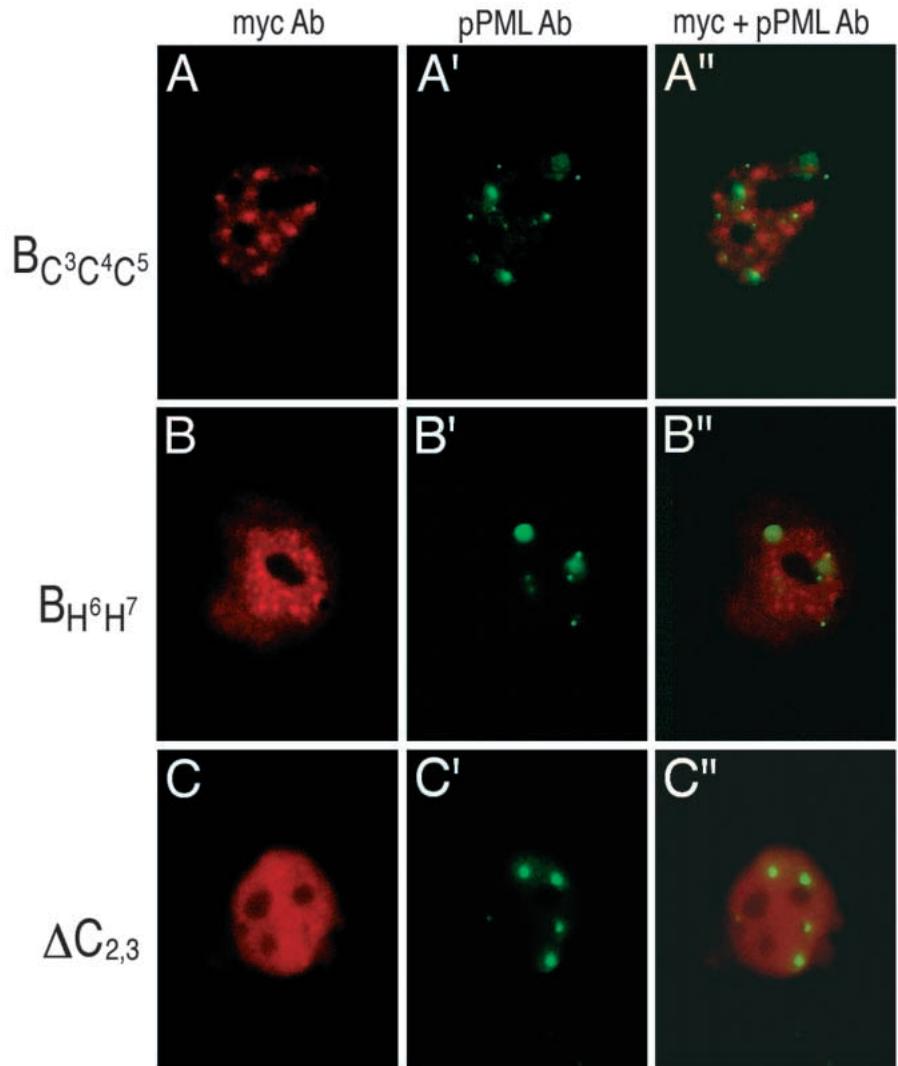
We have previously shown that rfp, while nuclear in HeLa and

**Fig. 4.** Effect of mutations in rfp domains on rfp interaction with PML. (A) Diagram of the rfp mutant constructs used to determine the domains involved in rfp-PML interaction. B-box, B-box zinc finger; NLS, the putative nuclear localization signal; C<sub>1</sub>, C<sub>2</sub>, and C<sub>3</sub> are the three predicted  $\alpha$ -helices in the coiled-coil domain. Point mutations in the RING finger and the B-box are indicated by asterisks. (B) Point mutations in the RING finger and B-box zinc finger used to fine map the sequence involved in rfp-PML interaction. mt, mutant. The numbers in the B-box (1-42) correspond to the amino acid residues of Xnf7 B-box peptide used to solve the B-box structure (Borden et al., 1995). (C) Relative  $\beta$ -galactosidase ( $\beta$ -Gal) activity of yeast cells co-transformed with different RBC mutants and wild-type PML. The  $\beta$ -Gal activity of the yeast transformants was measured by colorimetric method using ONPG as a substrate. The  $\beta$ -Gal activity of wild-type RBC and PML transformant was used as 100% standard, and the bar indicates standard deviation from at least four independent experiments. p53 and RBC co-transformant was used as a negative control for  $\beta$ -Gal activity.

hepatoma cell lines, is predominantly cytoplasmic in mouse A9 and NIH 3T3 cells while PML is localized in nuclear bodies (Cao et al., 1997). In addition, transfected rfp behaves in a similar manner and is retained in the cytoplasm in these cell lines (Cao et al., 1997). In these cells, endogenous rfp is either in low abundance or absent in PML nuclear bodies since the majority of the protein is cytoplasmic. Therefore, we wanted to determine whether exogenous PML and rfp would interact, and if PML could localize exogenous rfp into the nucleus and enter PML NBs when both rfp and PML were overexpressed in A9 cells. Fig. 6A-A'' shows that rfp with a RING finger mutation is cytoplasmic in A9 cells. This is also observed with endogenous and wild-type exogenous rfp (Cao et al., 1997). Fig. 6B-B'', however, shows that when wild-type rfp and PML are overexpressed in A9 cells rfp is detected within PML NBs. Similar results were observed in NIH 3T3 cells in which overexpressed PML dragged rfp into NBs (T. Cao and L. D. Etkin, unpublished observations). In addition, mutation of the RING finger, which did not affect rfp-PML interaction in the two-hybrid system, did not interfere with the co-localization of rfp and PML in nuclear bodies (Fig. 6C-C''). These results suggest that the intermolecular interaction between rfp and PML is strong enough to allow PML to drag rfp into the nucleus, where it participates in NB formation, further supporting the conclusion that rfp and PML interact. The lack of co-localization of the endogenous rfp with PML in these cell lines is curious, and may be due to either the tethering of the endogenous rfp to a cytoplasmic anchor or differences in the posttranslational modifications of the endogenous and exogenous proteins that affect protein-protein interaction. This phenomenon will require further analysis to distinguish these alternatives.

#### Retinoic acid treatment of NB4 cells restores rfp-PML interaction in nuclear bodies

It is well known that in the APL patient-derived cell line NB4, PML is detected in microgranular structures instead of NBs (Daniel et al., 1993; Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994). The disruption of the normal PML NB structures is associated with the expression of the t(15:17) chromosomal translocation product PML-RARA. We therefore analyzed the distribution of rfp in NB4 cells to determine if rfp remained co-localized with PML in this cell type. Fig. 7 shows an



**Fig. 5.** Effect of different rfp mutations on rfp co-localization to PML nuclear bodies with PML. Different rfp mutants were transfected into HeLa cells and detected by indirect immunofluorescence with anti-myc monoclonal antibody (rhodamine, red) (A-C). The PML NBs in the same cells were stained with anti-PML antiserum (FITC, green) (A'-C'). Double staining showed no co-localization of rfp mutants and PML (A''-C'').

immunofluorescence analysis of both rfp and PML in NB4 cells. PML and rfp are distributed in a microgranular pattern and both of these proteins co-localize within a subset of these particles (Fig. 7A,A',A''). When NB-4 cells are treated with all trans retinoic acid (ATRA) the block in differentiation is alleviated and PML NBs are reformed (Koken et al., 1994). We therefore tested the localization of rfp in NB4 cells after ATRA treatment. Fig. 6B,B',B'' shows that rfp co-localizes with PML in many of the newly formed NBs after ATRA treatment.

#### Rfp co-localizes with both PML and PML-RARA

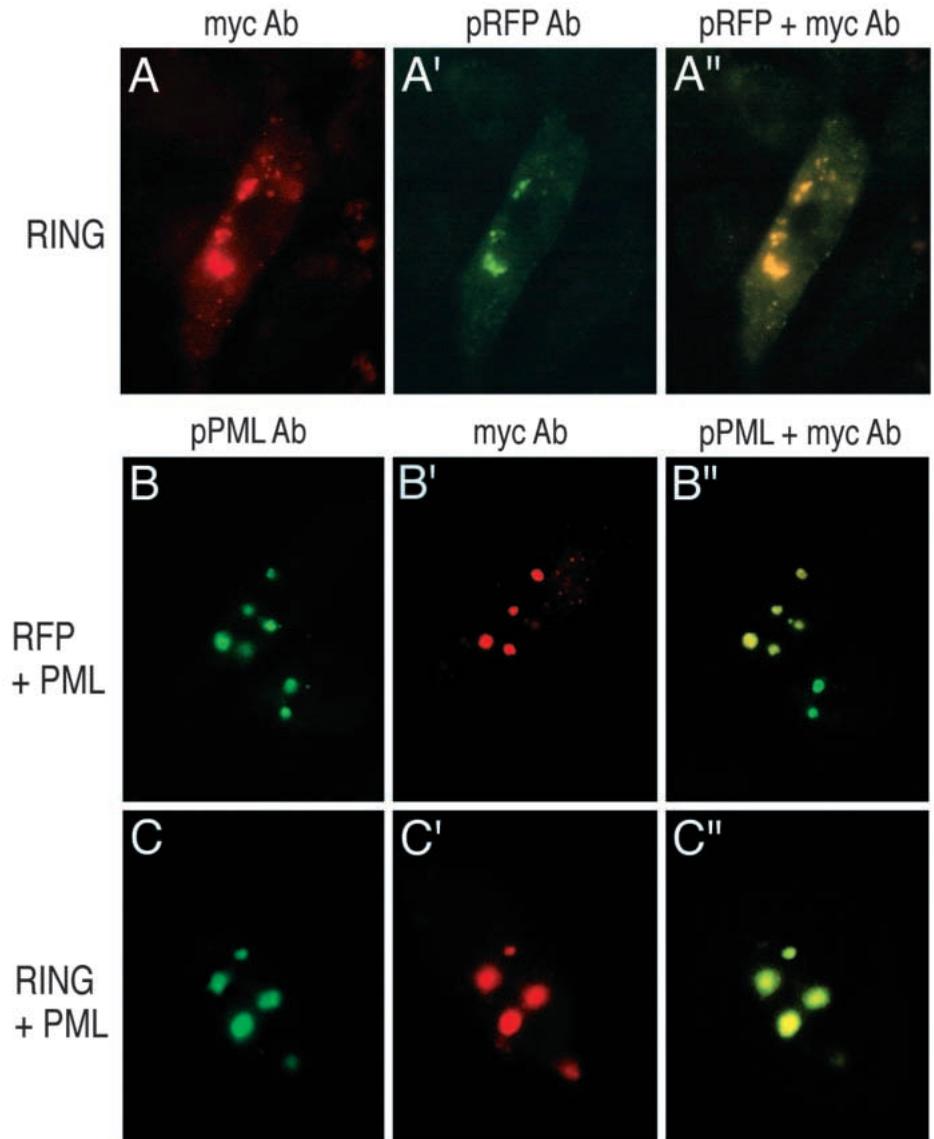
In the previous experiment we could not determine whether or not rfp was co-localizing with the endogenous PML protein and/or with the PML-RARA protein resulting from the t(15:17) translocation. To test this, we performed an experiment in which we transfected human fibrosarcoma HT1080-2C4 cells with constructs containing myc-epitope tagged PML, myc-epitope tagged RFP, and non epitope tagged PML-RARA. Fig. 8A-8A'' shows the results of co-transfection of rfp and PML in which the exogenous proteins were detected with the anti-PML monoclonal antibody 5E10 (A) and the rfp polyclonal antibody (A'). Co-localization of the two proteins

was detected in A''. Fig. 8B-B'' shows co-transfection with rfp and PML-RARA in which the PML-RARA was detected using the PML monoclonal antibody (B), rfp was detected with the rfp polyclonal antibody (B') and their co-localization shown in B''. Fig. 8C-C'' shows cells from the same experiment except the PML-RARA was detected using a polyclonal antibody directed against the RARA portion of the protein (C), rfp was detected with the myc epitope tag antibody (C') and the co-localization of the two proteins is shown in C''. We conclude from this experiment that rfp shows significant co-localization with both the PML protein as well as the recombinant PML-RARA. It is evident, however, that rfp only appears to co-localize with PML and PML-RARA in some of the nuclear bodies. There are numerous examples in which rfp does not co-localize with either the PML or the PML-RARA.

## DISCUSSION

### The role of the B-box and coiled-coil domains in protein interactions involving rfp

We have demonstrated that rfp is a normal component of PML



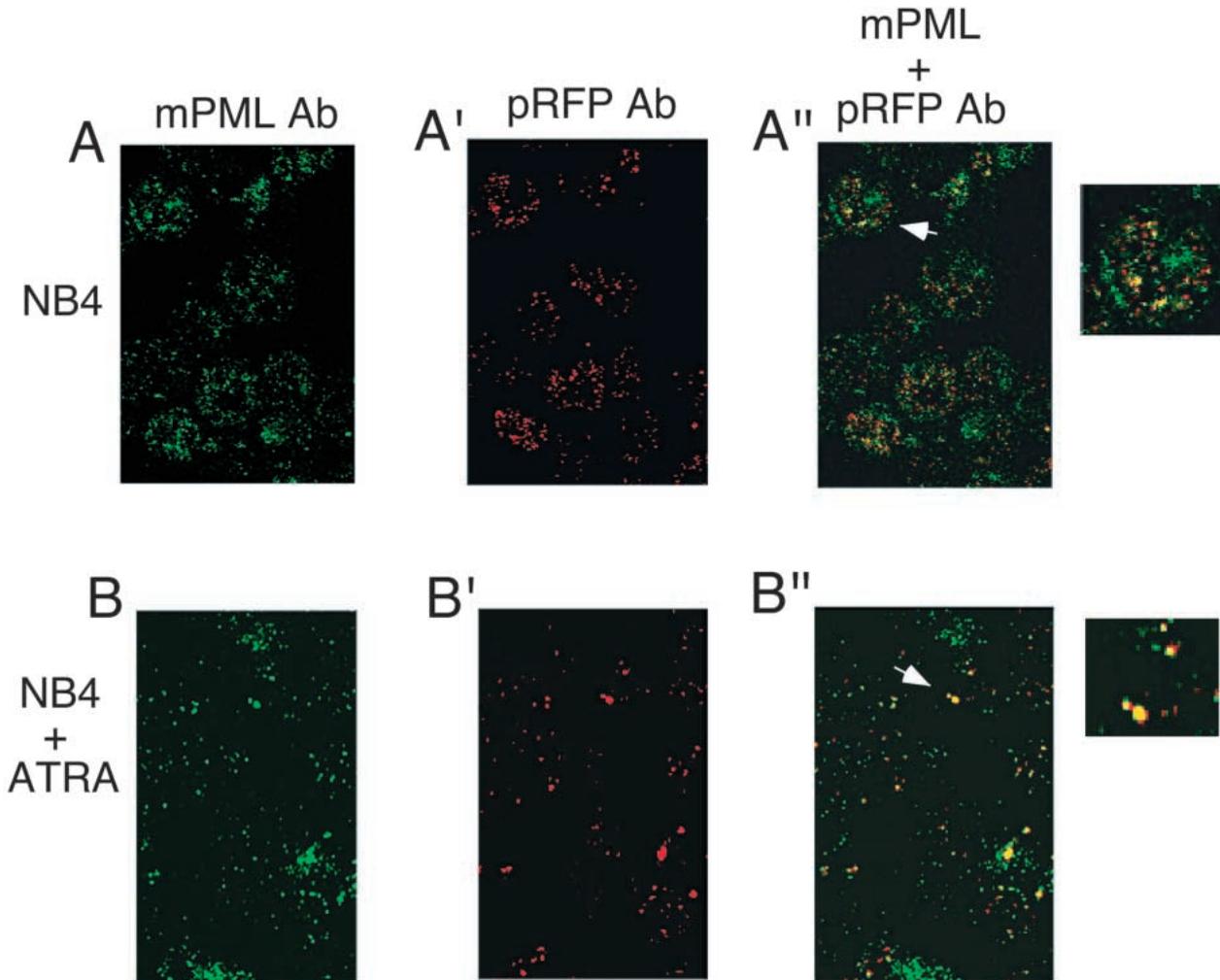
**Fig. 6.** Co-transfection of PML with an rfp RING mutant restores the RING mutant localization to PML nuclear bodies. (A-A'') Human Rfp RING finger mutant is detected in granular structures in the cytoplasm of mouse A9 cells after transfection. Mutant rfp was detected by indirect immunofluorescent staining with anti-myc monoclonal antibody (rhodamine, red) (A) and rabbit anti-mouse rfp antiserum (FITC, green) (A'). Overlapping staining appears yellow (A''). Both human and mouse rfp can be recognized by the rfp antibody. Arrow points to nucleus. (B-B'', C-C'') Co-transfection of PML with wild-type rfp (B-B') or rfp RING finger mutant (C-C'') results in exogenous rfp co-localization to PML NBs. Exogenous PML was detected by anti-PML antiserum (FITC, green) (B,C), and exogenous rfp was detected by anti-myc monoclonal antibody (rhodamine, red; B', C'). Overlapping staining appears yellow (B'',C'').

NBs and that it directly interacts with PML through a specific region of the rfp coiled-coil domain. The interaction through the coiled-coil domain is dependent upon the proper structure of the rfp B-box zinc binding domain but does not involve the RING finger. The B-box zinc binding domain is unusual in that it has seven putative Cys/His metal ligands, which are conserved among all B-box zinc finger proteins (Reddy and Etkin, 1991; Reddy et al., 1992; Freemont, 1993). It was shown that only one zinc atom binds per molecule of B-box peptide (Borden et al., 1993; Bellini et al., 1995). NMR structural analysis showed that Cys<sup>1</sup>, His<sup>2</sup>, Cys<sup>5</sup>, His<sup>6</sup>, and His<sup>7</sup> are the putative zinc ligands and that the most likely residues involved in ligation are Cys<sup>1</sup>, His<sup>2</sup>, Cys<sup>5</sup>, His<sup>6</sup> (Borden et al., 1995). The extra putative zinc ligands Cys<sup>3</sup> and Cys<sup>4</sup> may participate in inter-molecular zinc-binding (Borden et al., 1995, 1996), as observed in the HIV Tat protein (Frankel et al., 1988). Our data, however, show that mutations of either the proposed intra- (Cys<sup>5</sup>, His<sup>6</sup>) or inter- (Cys<sup>3</sup>, Cys<sup>4</sup>) molecular zinc-binding site disrupts the rfp-PML interaction. Furthermore, these mutations

result in exogenous rfp nuclear bodies, which are distinct from PML NBs, suggesting that the integrity of the B-box domain could play a role in targeting rfp to PML NBs.

Within the tripartite motif the B-box leads directly into an  $\alpha$ -helical coiled-coil domain. Although our data suggest that the coiled-coil domain of rfp provides the actual interacting interface for both PML (this analysis) and rfp (Cao et al., 1997), an intact B-box is important for homo- and heteromultimerization and/or targeting rfp to PML NBs. It is therefore possible that the correct folding of the B-box allows the helical coiled-coil domain of rfp to be in a proper orientation for rfp-PML oligomerization and that mutations of the B-box disrupts this interaction. The B-box mutations could even allow rfp to oligomerize with other nuclear proteins which contain coiled-coil domains, possibly explaining the novel exogenous rfp nuclear bodies we observe in the B-box mutants.

Rfp appears to use all three predicted helical coiled-coils in homomultimerization, however, our mutational data suggest that the distal two helical coiled-coils function in



**Fig. 7.** Disruption of rfp localization in APL-derived NB4 cells. (A-A'') Untreated NB4 cells stained for endogenous PML, detected by anti-PML monoclonal (FITC, green) and endogenous rfp detected with the anti-rfp antiserum (Texas red). (B-B'') NB4 cells after treatment with ATRA stained for endogenous PML, detected by anti-PML monoclonal (FITC, green) and endogenous rfp detected with the anti-rfp antiserum (Texas red). Overlapping staining appears yellow (A'',B'') Cells marked with arrows are shown in the insets.

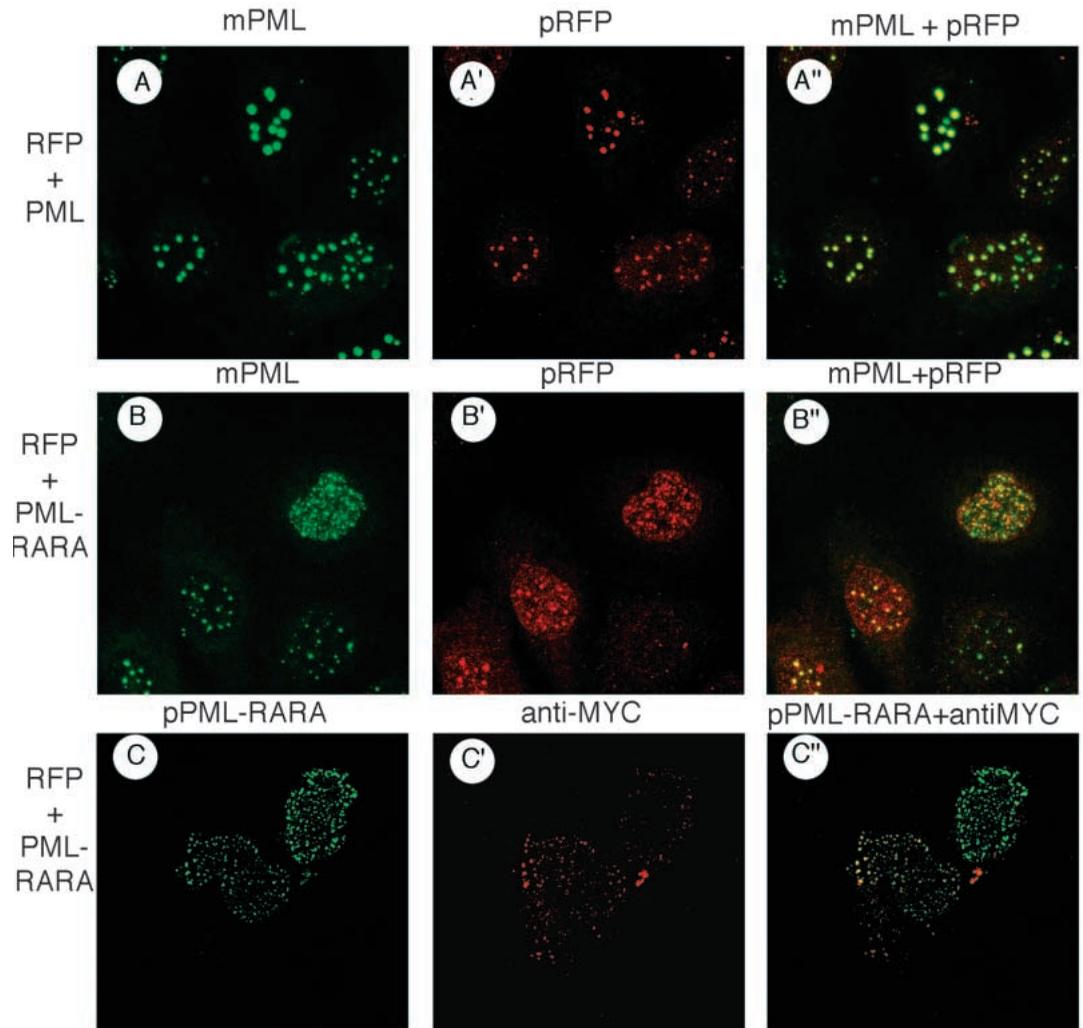
heteromultimerization with PML. This suggests that there is specificity in the function of the different subdomains of the coiled-coil region of rfp allowing both homo- and hetero-oligomeric interactions. It is interesting to speculate that this could be a general function for the helical coiled-coil domain within the tripartite family, which is often observed as comprising sub-domains of predicted helical regions with distinct breaks between them (N. Boddy and P. Freemont, unpublished observations).

#### Rfp as a component of PML nuclear bodies

A number of different proteins have been identified that co-localize with PML in nuclear bodies (for review see Doucas and Evans, 1996; deThe, 1996). These include the proteins NDP52 (Zuber et al., 1995; Korioth et al., 1995), NDP55, (Ascoli and Maul, 1991), NDP65 (Epstein, 1984; Dyck et al., 1994), ND/KrAg/Sp100 (Szosteck et al., 1990; Xie et al., 1993; Weis et al., 1994) now known as Sp100 (Weis et al., 1994), Int6 (Desbois et al., 1996) and PIC1 (Boddy et al.,

1996). In normal somatic and sperm cells, rfp is also detected within nuclear structures that are similar to PML NBs (Cao et al., 1996). Here we provide strong evidence to show that rfp is another endogenous component of PML NBs through a direct interaction with PML via the rfp helical coiled-coil domain. Furthermore, in APL-derived NB-4 cells that are refractile to differentiation, rfp is dispersed into microgranules but maintains its co-localization with PML and/or PML-RARA. Upon treatment with ATRA, which results in terminal differentiation of promyelocytes and complete remission in APL patients, rfp and PML co-localize together with the other components in the reformed PML NBs. These results demonstrate that rfp is a normal component of the PML NBs, although its association with the PML NBs can vary between different cell types. Given that we have demonstrated a direct interaction between PML and rfp and that rfp behaves like PML in ATRA treated NB4 cells, one could suggest that rfp, along with other components of PML NBs, may play an important role in regulating cellular growth and differentiation.

**Fig. 8.** Rfp interacts with both PML and PML-RARA. (A-A'') Co-expression of PML and RFP. (A) PML is detected by the 5E10 PML monoclonal antibody (green); (A') RFP is detected by the rfp polyclonal antibody (red); (A'') co-localization of the PML and RFP signals (yellow). (B-B'') Co-expression of PML-RARA and RFP. (B) transfected PML-RARA was detected with the 5E10 PML monoclonal antibody (green); (B') RFP was detected with the rfp polyclonal antibody (red); (B'') co-localization of PML-RARA and RFP (yellow). (C-C'') Co-expression of PML-RARA and RFP using the PML-RARA antibody. (C) transfected PML-RARA was detected by the PML-RARA polyclonal antibody (green); (C') RFP was detected with the 9E10myc monoclonal antibody (red); (C'') co-localization of the RFP with PML-RARA (yellow).



We have observed that while there are many PML NBs in cells, rfp appears to associate with only a limited subset of these structures. This suggests that the interaction of rfp with PML is not the only factor in the association of rfp with NBs. One possibility is that the PML NBs are dynamic structures that change their composition depending on various physiological factors such as cell cycle, growth and differentiation of the cell. Perhaps proteins, such as rfp, only associate with those NBs that are active in performing a specific function.

### Rfp and oncogenicity

Both PML and rfp have transforming activity when their tripartite motifs are recombined with other sequences. PML was found fused with the retinoic acid receptor  $\alpha$  resulting from the t(15;17) chromosomal translocation observed specifically in patients with APL (Dyck et al., 1994; Kastner et al., 1992; Weis et al., 1994). Rfp was found recombined with the tyrosine kinase domain of c-ret to form a transforming fusion protein rfp-ret (Takahashi et al., 1988; reviewed by Cao, 1995). It will be interesting to determine in rfp-ret transformed cells whether the recombined rfp-ret product is localized in nuclear bodies or, like PML-RARA, is dispersed in the cytoplasm.

It is clear that differentiation therapy by treatment of APL with retinoic acid is an important clinical strategy (Huang et al., 1988; Warrell et al., 1991). Recent studies have shown that ATRA treatment causes a change in the half life of the PML-RARA fusion protein, resulting in a more rapidly degraded protein product (Raelson et al., 1996). This suggests that the newly reformed PML NBs after ATRA treatment contain primarily wild-type endogenous PML. In this study we have shown that rfp is also relocalized to these newly formed mainly endogenous PML NBs. It is therefore tempting to speculate that both rfp and PML, and perhaps other PML NB components, are either direct or indirect targets of this treatment. Therefore, analysis of the rfp-PML interaction and their interaction/s with other components in PML NBs could perhaps help us understand how this treatment works and also elucidate the contribution, if any, of delocalized rfp to the APL disease phenotype.

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rfp-ret fusion gene. We also thank Graeme Carlile for performing the immunoprecipitation experiments.

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