# RNA-related nuclear functions of human Pat1b, the P-body mRNA decay factor

Aline Marnef[a], Dominique Weil[b], and Nancy Standart[a]
[a]Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom; [b]UPMC University Paris 06, CNRS-FRE 3402, 75005 Paris, France

**ABSTRACT** **The evolutionarily conserved Pat1 proteins are P-body components recently shown to play important roles in cytoplasmic gene expression control. Using human cell lines, we demonstrate that human Pat1b is a shuttling protein whose nuclear export is mediated via a consensus NES sequence and Crm1, as evidenced by leptomycin B (LMB) treatment. However, not all P-body components are nucleocytoplasmic proteins; rck/p54, Dcp1a, Edc3, Ge-1, and Xrn1 are insensitive to LMB and remain cytoplasmic in its presence. Nuclear Pat1b localizes to PML–associated foci and SC35-containing splicing speckles in a transcription-dependent manner, whereas in the absence of RNA synthesis, Pat1b redistributes to crescent-shaped nucleolar caps. Furthermore, inhibition of splicing by spliceostatin A leads to the reorganization of SC35 speckles, which is closely mirrored by Pat1b, indicating that it may also be involved in splicing processes. Of interest, Pat1b retention in these three nuclear compartments is mediated via distinct regions of the protein. Examination of the nuclear distribution of 4E-T(ransporter), an additional P-body nucleocytoplasmic protein, revealed that 4E-T colocalizes with Pat1b in PML-associated foci but not in nucleolar caps. Taken together, our findings strongly suggest that Pat1b participates in several RNA-related nuclear processes in addition to its multiple regulatory roles in the cytoplasm.**

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## INTRODUCTION

Pat1 proteins are conserved in eukaryotes and play several important posttranscriptional roles in gene expression control. Two Pat1 proteins have evolved in vertebrates, Pat1a and Pat1b, in contrast to the single protein in yeast and invertebrates. Pat1 proteins have recently attracted considerable attention because of their roles in translational regulation, mRNA deadenylation, and 5′–3′ mRNA decay. Nonetheless, Pat1 proteins have no readily identifiable motif or domain sequences (Scheller *et al.*, 2007; Marnef *et al.*, 2010; Marnef and Standart, 2010; Ozgur *et al.*, 2010).

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Address correspondence to: Nancy Standart (nms@mole.bio.cam.ac.uk).

Abbreviations used: 4E-T, 4E-T(ransporter); Act D, actinomycin D; DAPI, 4′,6-diamidino-2-phenylindole; DFC, dense fibrillar compartment; DNC, dark nucleolar cap; EWS, Ewing sarcoma breakpoint region 1 gene; FC, fibrillar center; GC, granular compartment; LNC, light nucleolar cap; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization sequence; Pabp1, poly(A)-binding protein; P-bodies, P(rocessing) bodies; PML, promyelocytic leukemia protein; siRNA, small interfering RNA.



A critical step in 5′–3′ mRNA decay is the transition from an actively translating mRNA to one targeted for degradation. Recent studies strongly indicate that Pat1 proteins participate in this transition. First, the yeast Pat1p is implicated in translation initiation because of its interaction with eIF4E, eIF4G, and PABP1 (poly(A)–binding protein 1) and its association with the 40S–48S ribosomal subunits in polysomal fractions (Bonnerot *et al.*, 2000; Wyers *et al.*, 2000; Tharun and Parker, 2001). Furthermore, yeast Pat1p and *Xenopus laevis* Pat1a proteins are translational repressors (Coller and Parker, 2005; Marnef *et al.*, 2010; Nakamura *et al.*, 2010). Finally, yeast Pat1p, *Drosophila* Hpat, and human Pat1b promote mRNA deadenylation and mRNA decapping, ultimately leading to 5′–3′ mRNA decay (Hatfield *et al.*, 1996; Braun *et al.*, 2010; Haas *et al.*, 2010; Ozgur *et al.*, 2010; Totaro *et al.*, 2010).

To perform their numerous cytoplasmic functions, Pat1 proteins have been proposed to act as scaffold proteins that interact with mRNA and multiple protein factors involved in translational control, as well as in mRNA decapping and deadenylation (Pilkington and Parker, 2008; Braun *et al.*, 2010; Haas *et al.*, 2010; Marnef *et al.*, 2010; Ozgur *et al.*, 2010; Totaro *et al.*, 2010). According to recent reports, the Pat1b N-terminal region binds rck/p54 RNA helicase and Caf1 (a subunit of the Ccr4-Not deadenylation complex), whereas the C-terminal region binds enhancers of decapping and

the decapping factors, including Dcp1a/b, the LSm1-7 complex, Ge-1, and Edc3, and the exoribonuclease Xrn1 (Braun *et al.*, 2010; Marnef and Standart, 2010; Ozgur *et al.*, 2010).

We previously proposed that vertebrates evolved two Pat1 proteins in the germline and soma that function preferentially in either translational repression or mRNA decay, reflecting association with some common and some distinct protein partners (Marnef *et al.*, 2010). Thus maternally expressed Pat1a proteins act as translational repressors rather than mRNA-decapping activators, since decapping activity is observed only after the mid-blastula transition in embryos. Then, Pat1b proteins expression takes over in somatic cells, where they function in mRNA decay. Of interest, translational repression and enhanced mRNA decay are performed by the single Pat1 protein in yeast (Hatfield *et al.*, 1996; Coller and Parker, 2005).

Consistent with their functions in translational repression and mRNA decay, Pat1 proteins localize to processing (P-) bodies in yeast, *Caenorhabditis elegans* embryos, *Drosophila* S2, and human HeLa cells (Sheth and Parker, 2003; Eulalio *et al.*, 2007b; Scheller *et al.*, 2007; Boag *et al.*, 2008; Gallo *et al.*, 2008; Braun *et al.*, 2010; Haas *et al.*, 2010; Marnef *et al.*, 2010; Ozgur *et al.*, 2010). P-bodies are cytoplasmic foci conserved across eukaryotes that harbor repressed mRNAs, 5′–3′ mRNA decay factors, including Dcp1/2, enhancers of decapping such as Edc3, as well as the Xrn1 exonuclease, translational repressors, including rck/p54, CPEB1, and Pat1, microribonucleoprotein components, and only one translation initiation factor, the cap-binding protein eIF4E, and its interacting factor 4E-T(ransporter), but no ribosomes (reviewed by Eulalio *et al.*, 2007a; Kulkarni *et al.*, 2010). Although the role of P-bodies remains to be clarified, studies have shown that silenced mRNAs resident in P-bodies may undergo 5′–3′ mRNA decay or return to translation (Bhattacharyya *et al.*, 2006; Eulalio *et al.*, 2007a, 2007b; Parker and Sheth, 2007; Carbonaro *et al.*, 2011).

Accumulating evidence hints that yeast Pat1p may be a nucleocytoplasmic shuttling protein. For example, Pat1p is found in both nuclear and cytoplasmic fractions (Wyers *et al.*, 2000), and although it is predominantly cytoplasmic in wild-type strains, Pat1p partially relocalizes to the nucleus upon deletion of LSm1, which is part of the decapping activator LSm1-7 complex (Teixeira and Parker, 2007). Moreover, Pat1p also interacts with Crm1p, the nuclear export signal receptor, in a global yeast two-hybrid screen for proteins with nuclear export activity (Jensen *et al.*, 2000). Taken together, these observations suggest that in addition to their roles in posttranscriptional control in the cytoplasm, Pat1p and perhaps other Pat1 proteins may also have a nuclear role.

Of interest, a link between transcription and mRNA decay has started to emerge in yeast (Lotan *et al.*, 2005, 2007; Goler-Baron *et al.*, 2008; Harel-Sharvit *et al.*, 2010). Rpb7 and Rpb4, heterodimer subunits of RNA polymerase II (Lotan *et al.*, 2005, 2007), appear to act as coordinators of several posttranscriptional processes. These two subunits shuttle between the nucleus and the cytoplasm and mediate transcription, stimulate translation, and are also implicated in mRNA decay (Lotan *et al.*, 2005, 2007; Goler-Baron *et al.*, 2008; Harel-Sharvit *et al.*, 2010). Consistent with their roles in mRNA decay, they partially localize to P-bodies, and yeast Rpb4 and Rpb7 interact directly with Pat1p (Lotan *et al.*, 2005, 2007), reinforcing the possibility of Pat1p being a shuttling protein with nuclear roles upstream of the mRNA decay pathway.

Here we show that the P-body component Pat1b is a nucleocytoplasmic shuttling protein, but Pat1a is not, in human tissue culture cells. Pat1b accumulates in splicing speckles and in nuclear foci, which are near or overlap PML bodies. Intriguingly, we show that localization to nuclear foci is mediated via its N-terminus, whereas the C-terminus promotes Pat1b localization to splicing speckles. We also demonstrate that localization of Pat1b to splicing speckles and to nuclear foci requires RNA synthesis. Furthermore, upon inhibition of transcription, Pat1b accumulates in nucleolar caps, which is mediated via its N-terminus. These findings strongly suggest that Pat1b likely perform various nuclear functions upstream of their multiple posttranscriptional regulatory roles in the cytoplasm.

## RESULTS

### Pat1b is a Crm1-dependent nucleocytoplasmic protein

Human Pat1b is a predominantly cytoplasmic protein that localizes to P-bodies in HeLa, HEK293, COS-7, and U2OS cells (Scheller *et al.*, 2007; Braun *et al.*, 2010; Marnef *et al.*, 2010; Ozgur *et al.*, 2010; Totaro *et al.*, 2010). To examine whether Pat1b protein shuttles to the nucleus, HeLa cells were treated for 5 h with 10 mM leptomycin B (LMB), a specific inhibitor of the nuclear export receptor Crm1 (Stade *et al.*, 1997), followed by immunostaining with a previously described affinity-purified Pat1b antibody (Marnef *et al.*, 2010; Figure 1A). On LMB treatment, a distinct redistribution of Pat1b from the cytoplasm and P-bodies to the nucleus was observed, indicating that a large proportion of endogenous Pat1b is retained in the nucleus upon the inhibition of the export pathway (Figure 1A). Partial nuclear retention of Pat1b was observed as early as 1 h after LMB addition, with full retention occurring between 2 and 5 h (data not shown). We confirmed its shuttling by following the redistribution of Pat1b–green fluorescent protein (GFP) to the nucleus upon LMB treatment (Figure 1B). The protein spread diffusely over the nucleoplasm, as well as accumulated in nuclear speckles and foci (see later discussion), and was excluded from nucleoli.

Recent reports suggest that Pat1a proteins, paralogues of Pat1b proteins, are expressed in the germline, where they act as translational repressors (Marnef *et al.*, 2010; Nakamura *et al.*, 2010), whereas the somatic Pat1b proteins function in mRNA decay (Braun *et al.*, 2010; Ozgur *et al.*, 2010; Totaro *et al.*, 2010). Because the maternal Pat1a protein is not expressed in somatic tissues (Scheller *et al.*, 2007; Marnef *et al.*, 2010), we examined the localization of a GFP-tagged version. We observed that, in contrast to Pat1b-GFP, Pat1a-GFP does not shuttle upon LMB treatment (Figure 1C).

Altogether, our results show that Pat1b is a Crm1-dependent nucleocytoplasmic protein, unlike Pat1a, which does not shuttle, at least not via this particular export pathway.

### Identification of the nucleocytoplasmic transport signal in Pat1b

The observation that Pat1b shuttling is sensitive to LMB indicates that Pat1b contains nuclear export sequences (NES) and nuclear localization sequences (NLS), signals commonly found in proteins that traffic through the Crm1 pathway. To identify these signals in Pat1b, we first expressed GFP-tagged truncated versions in HeLa cells (Figure 2A) and analyzed their subcellular distribution with and without LMB treatment (an example of Pat1b-N-terminal half [Pat1b-Nter] is shown in Figure 2B). Previously, we subdivided Pat1b into five domains, based on alignments of vertebrate sequences and predicted secondary structures, as first undertaken for yeast and *Xenopus* proteins (Pilkington and Parker, 2008; Marnef *et al.*, 2010). We deduced that the NES signal(s) lies within regions I and II of Pat1b, since in untreated HeLa cells, Pat1b N-terminal half (Pat1b-GFP-Nter, encompassing regions I–III) and Pat1b-ΔRIII are cytoplasmic, similar to the full-length Pat1b-GFP, whereas the Pat1b C-terminal half protein (Pat1b-GFP-Cter, encompassing regions IV and V) is

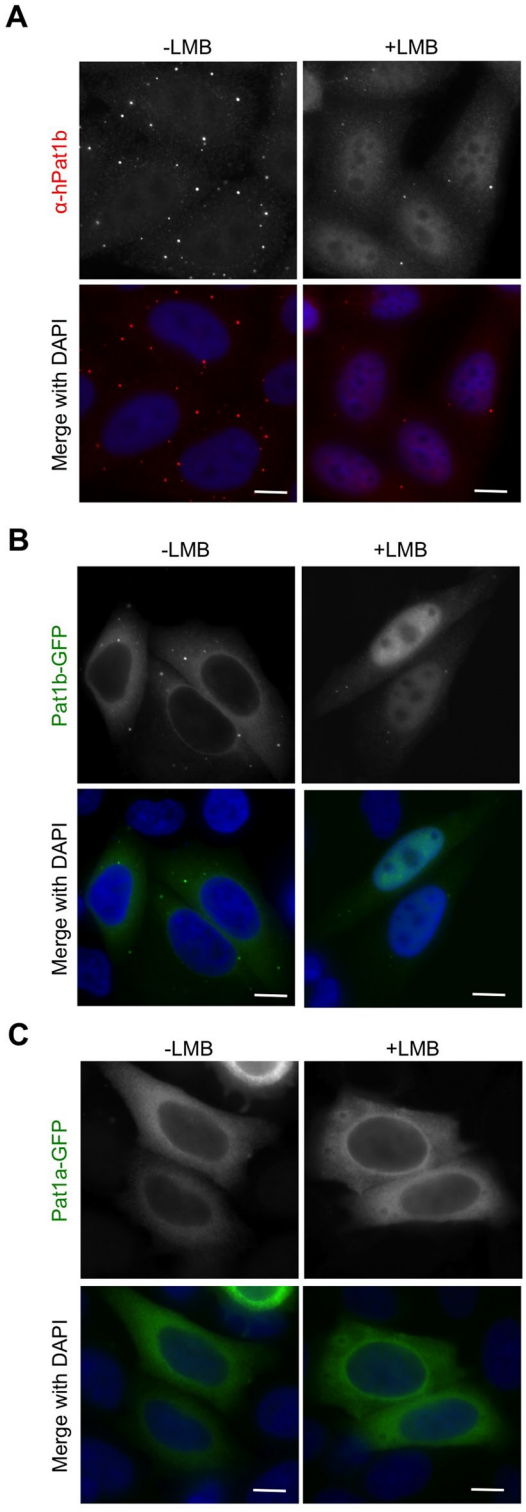


FIGURE 1: Crm1-dependent nuclear accumulation of Pat1b but not Pat1a. Fluorescence imaging of HeLa cells treated with LMB for 5 h (+LMB) or with methanol as a control (−LMB). Cells were also stained with DAPI. Scale bar, 10 μm. (A) Localization of endogenous Pat1b using a α-Pat1b antibody. (B, C) Cells were transfected with GFP-tagged Pat1b (B) or Pat1a (C) and treated 24 h later with LMB. Pat1b-GFP accumulates to the nucleus upon LMB treatment (B), whereas Pat1a-GFP localization remains unchanged (C). Note that the nuclear signal seen with the Pat1b antibody in untreated cells is due to an unrelated cross-reacting protein, as it is unresponsive to Pat1b siRNA (Marnef *et al.*, 2010).

both cytoplasmic and nuclear (Figure 2A). NES sequences are rich in hydrophobic residues (ϕ), and most follow the consensus ϕ-$(x)_{2-3}$-ϕ-$(x)_{2-3}$-ϕ-x-ϕ, where x is an amino acid that is preferentially charged, small, or polar (Kutay and Guttinger, 2005). Three potential NES candidate sequences in regions I and II were tested by replacing the hydrophobic residues with alanine residues. Only one of these sequences, $L_{86}$AER$L$SK$M$V$I_{95}$ (mutated residues underlined), was functional, since this mutant Pat1b-NES*-GFP protein accumulated in the nucleus in the absence of LMB (Figure 2C).

NLS sequences required for import can diverge considerably between proteins (Christophe *et al.*, 2000). The best-characterized NLS sequences are enriched in basic amino acids and may be bipartite (Robbins *et al.*, 1991; Mattaj and Englmeier, 1998). Mutagenesis of the most likely stretch of such residues in region IV ($K_{511}$E$K$QVRD$K$RR$K_{521}$, mutated residues underlined), however, had no effect on the protein location (data not shown). Moreover, Pat1b may contain more than one functional NLS, located in the N-terminal and the C-terminal halves. In fact, Pat1b-C-ter and Pat1b-N-ter are cytoplasmic and nuclear in untreated and LMB-treated cells, respectively (Figure 2A; also see Figure 8 later in the paper).

In summary, we showed here that Pat1b is retained in the nucleus in untreated cells, providing the single N-terminal NES is inactivated. This NES sequence is highly conserved in vertebrate Pat1b proteins (Figure 2D), suggesting that they are all exported through the Crm1 pathway, but is absent from mammalian Pat1a proteins (Scheller *et al.*, 2007), in line with their observed lack of shuttling (Figure 1). However, we were unable to identify a functional NLS signal, indicating that Pat1b is imported by an undefined or multiple sequences or by a partner.

### Only some P-body components shuttle to the nucleus

Next we examined the effect of LMB on additional P-body components, including 4E-T, Dcp1a, rck/p54, Edc3, Ge-1, and Xrn1 (Figure 3A), using immunostaining. Of interest, the data shown in Figures 1 and 3A show that nucleocytoplasmic shuttling, at least using the Crm1 pathway, is not a universal feature of P-body proteins, since of the seven components tested here, only Pat1b and 4E-T are sensitive to LMB. Furthermore, we noted that upon LMB treatment, the number of P-bodies decreases dramatically in HeLa cells, as seen both by immunostaining with antibodies to all these P-body components and with Pat1b-GFP in transfected cells (Figures 1–3). The reduction in P-body number may be an indirect effect of LMB on mRNA metabolism or may reflect the nuclear accumulation of a factor required for P-body formation.

### Pat1b is enriched in splicing speckles

In 40 ± 3% of LMB-treated HeLa cells, Pat1b-GFP forms multiple irregular shapes of various sizes in nuclei (Figure 4A), which resemble splicing speckles. These speckles are enriched in poly(A)+ mRNA and pre-mRNA splicing factors, including SR proteins, as well as small nuclear ribonucleoprotein particles, and are often found close to active transcription sites (Spector and Lamond, 2011). They are believed to provide splicing factors to these transcription sites by acting as assembly/storage/modification compartments (Lamond and Spector, 2003). To examine whether Pat1b-GFP localizes to nuclear speckles in LMB-treated cells, we used the speckle marker antibody raised against the SC35 SR pre-mRNA splicing factor (Wansink *et al.*, 1993) and verified by confocal imaging that Pat1b signal significantly overlapped with that of SC35 (Figure 4A). This is not due to LMB treatment, because in 67 ± 4% of untreated cells the Pat1b-NES*-GFP protein also colocalized with SC35 (Figure 4B). These data show that as Pat1b transits through nuclei, it associates

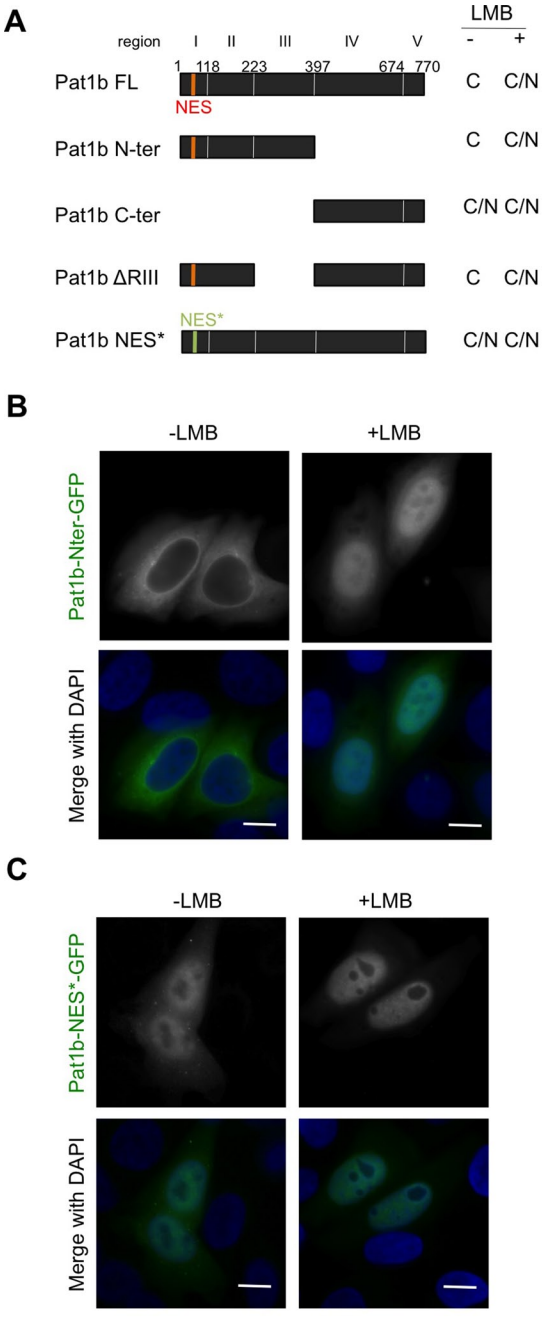


FIGURE 2: Identification of the Pat1b NES sequence. (A) Schematic representation of the localization of different Pat1b truncations tagged with GFP; C and N, cytoplasmic and nuclear localization respectively. The boundaries between the various regions (I–V) are annotated with their corresponding amino acid residues. The NES sequence is highlighted in red, and in green when mutated. (B, C) Fluorescence imaging of HeLa cells treated with LMB for 5 h (+LMB) or with methanol as a control (−LMB). Cells were also stained with DAPI. Scale bar, 10 μm. (B) Shuttling of GFP-tagged Pat1b-N-terminal half (Pat1b-Nter-GFP) upon LMB treatment. (C) Mutation in Pat1b NES (Pat1b-NES*-GFP) causes it to accumulate to the nucleus in the absence of LMB. Four point mutations in Pat1b-NES*-GFP promote its retention to the nucleus in untreated cells. (D) Alignment of six vertebrate Pat1b protein putative NES sequences. Mm1b (*Mus musculus* Pat1b), Rn1b (*Rattus norvegicus* Pat1b), Cf1b (*Canis familiaris* Pat1b), Hs1b (*Homo sapiens* Pat1b), Xl1b (*X. laevis* Pat1b), and Dr1b (*Danio renio* Pat1b). The red amino acids indicate those mutated in human Pat1b NES. Stars indicate conserved hydrophobic residues.

with nuclear speckles, suggesting that it may interact with pre-mRNA splicing factors.

To extend these observations, we tested the effect of inhibiting splicing in HeLa cells using spliceostatin A, previously shown to exert its effects by binding to the splicing factor SF3B (Kaida *et al.*, 2007; Lo *et al.*, 2007). Disruption of splicing was monitored by the reorganization of splicing factors within the nucleus (O'Keefe *et al.*, 1994; Tanackovic and Kramer, 2005; Kaida *et al.*, 2007). Addition of spliceostatin A to HeLa cells rearranged SC35 into larger speckles in the majority of the cells (Figure 4C), as previously described (Kaida *et al.*, 2007). Exploiting the ability of Pat1b-NES*-GFP to be retained in nuclei in the absence of LMB, we assessed its localization in spliceostatin A–treated cells. It is striking that Pat1b-NES*-GFP colocalizes with the enlarged SC35 speckles upon inhibition of splicing (Figure 4C), reinforcing the potential role of Pat1b in splicing-related processes.

### Pat1b also localizes to discrete nuclear foci adjacent to or overlapping with PML bodies

In addition to the cells in which hPat1-GFP localizes to splicing speckles (Figure 4), ~35% display a diffuse nuclear localization and ~25% display small nuclear foci (Figure 5, A and B). These foci do not depend on Pat1b-GFP expression levels; their numbers vary (between 40 and 200, numbers that do not correlate with the nucleus size), they are ~0.2–0.5 μm in diameter, and they are always excluded from the nucleolus. The hPat1-GFP nuclear foci were also observed in human HEK293-T and U2OS cells (data not shown).

To identify the Pat1b nuclear foci, we examined the possible colocalization of characterized proteins that form a similar number and size of foci, including telomeres, centromeres, heterochromatin sites, and PML bodies. To detect telomeres (reviewed in Bhattacharyya *et al.*, 2010), we used an α-TRF1 antibody, but did not observe colocalization of Pat1b nuclear foci with telomeres (Figure 5B). Nor did we observe colocalization between the Pat1b nuclear foci and centromeres, using an α-CPEN-A antibody (Amaro *et al.*, 2010; Figure 5C). We next marked the heterochromatin sites with the HP1-GFP protein (Thiru *et al.*, 2004), but again it did not colocalize with Pat1b nuclear foci (Figure 5D).

On the other hand, using a monoclonal PML antibody (Grimwade *et al.*, 2010), we noted a clear association between PML bodies and Pat1b nuclear foci (Figure 5, E and F). PML bodies are dynamic spherical structures found in most cell lines, which are implicated in a wide variety of processes and contain the promyelocytic leukemia (PML) protein, as well as several seemingly unrelated proteins, including SUMO and CBP. They play roles in sumoylation, transcriptional regulation, and/or nuclear storage (Lallemand-Breitenbach and de The, 2010). In about one-third of Pat1b-GFP foci–containing cells, more than half of the PML bodies (57 ± 2%) were either adjacent to or overlapped Pat1b nuclear foci (Figure 5E), and in a few cases (<5%), the foci and PML bodies

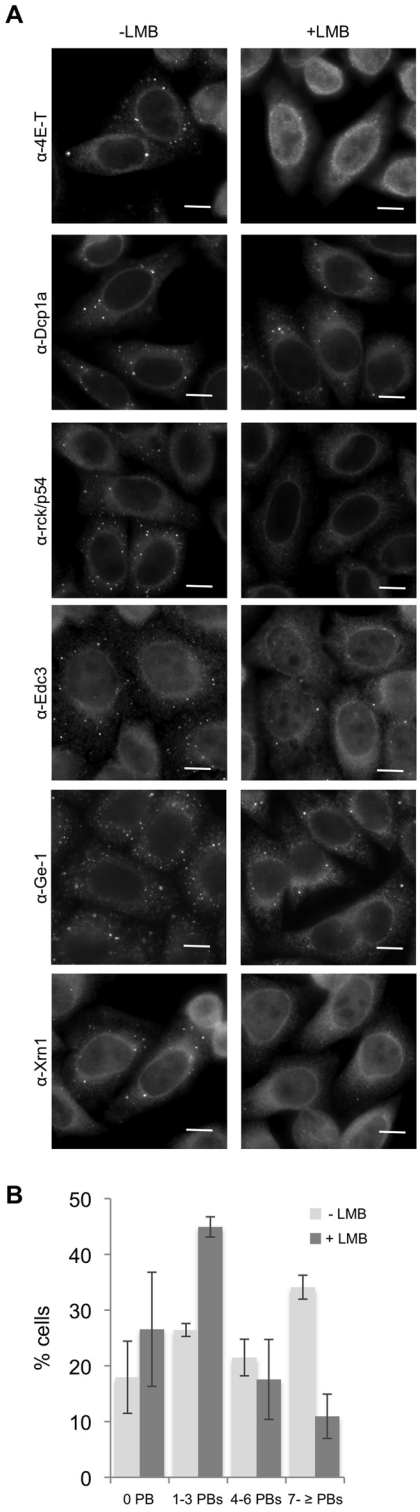


FIGURE 3: Most P-body components are not nucleocytoplasmic proteins. (A) Fluorescence imaging of HeLa cells treated with LMB for 5 h (+LMB) or with methanol as a control (−LMB) stained with antibodies against P-body components as indicated, including 4E-T, Dcp1a, rck/p54, Edc3, Ge-1, and Xrn1. Scale bar, 10 μm. (B) Graph of % of cells containing indicated numbers of P-bodies in the presence (+) or absence (−) of LMB, as seen with α-rck/p54.

colocalized (Figure 5F). Overall, Pat1b nuclear foci showed an association with PML bodies by being adjacent, overlapping, and occasionally colocalizing with them.

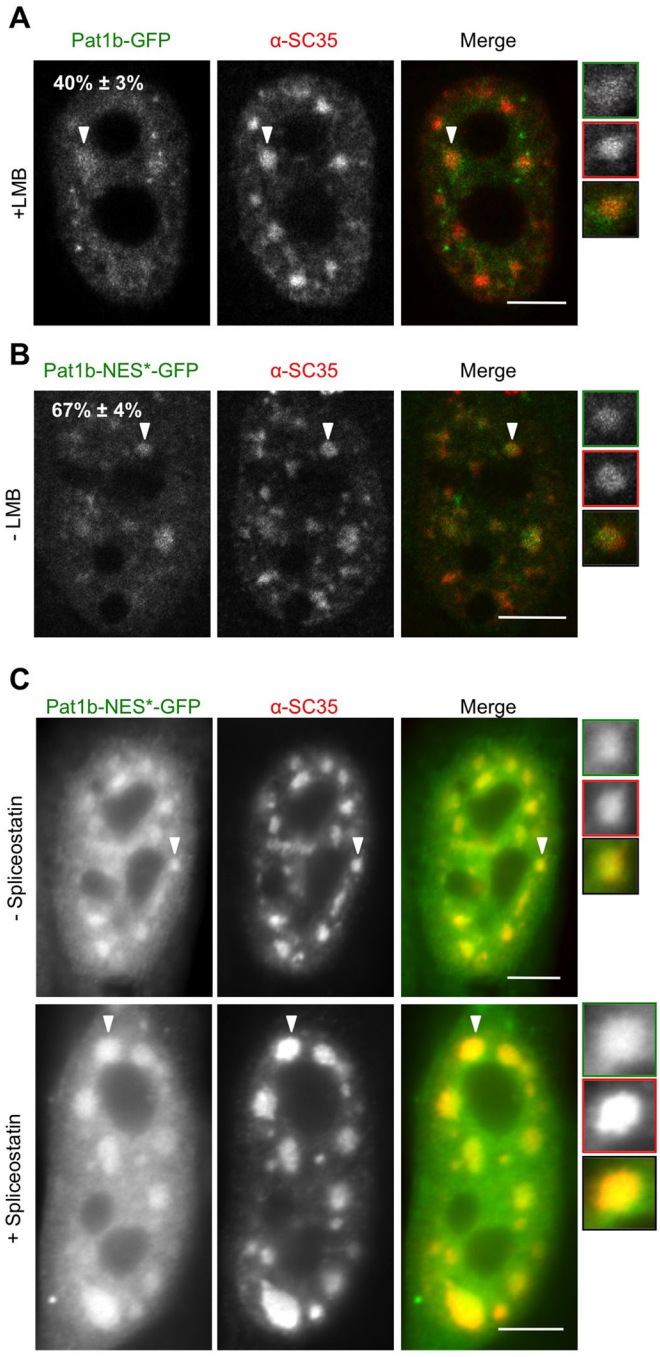


FIGURE 4: Pat1b localizes to nuclear speckles. Confocal imaging of HeLa cells treated with LMB for 5 h (+LMB) or with methanol as a control (−LMB) stained with the speckle marker SC35 (red). Scale bar, 5 μm. (A) Pat1b-GFP significantly colocalizes to nuclear speckles in the presence of LMB in 40 ± 3% of cells. (B) Pat1b-NES*GFP mutant also colocalizes to speckles, as seen with SC35, in the absence of LMB in 67 ± 4% of cells. (A, B) White arrowheads point to speckles zoomed in the box. (C) Fluorescence imaging of HeLa cells transfected with Pat1b-NES*-GFP and treated with 200 nM spliceostatin A for 5 h (+spliceostatin), or with methanol as a control (−spliceostatin) and stained with SC35 antibodies (red). Scale bars, 5 μm.

## Endogenous Pat1b is associated with PML bodies

We then sought evidence for a similar localization of endogenous Pat1b. In HeLa cells, despite an efficient nuclear accumulation of the endogenous Pat1b protein, Pat1b-containing nuclear foci

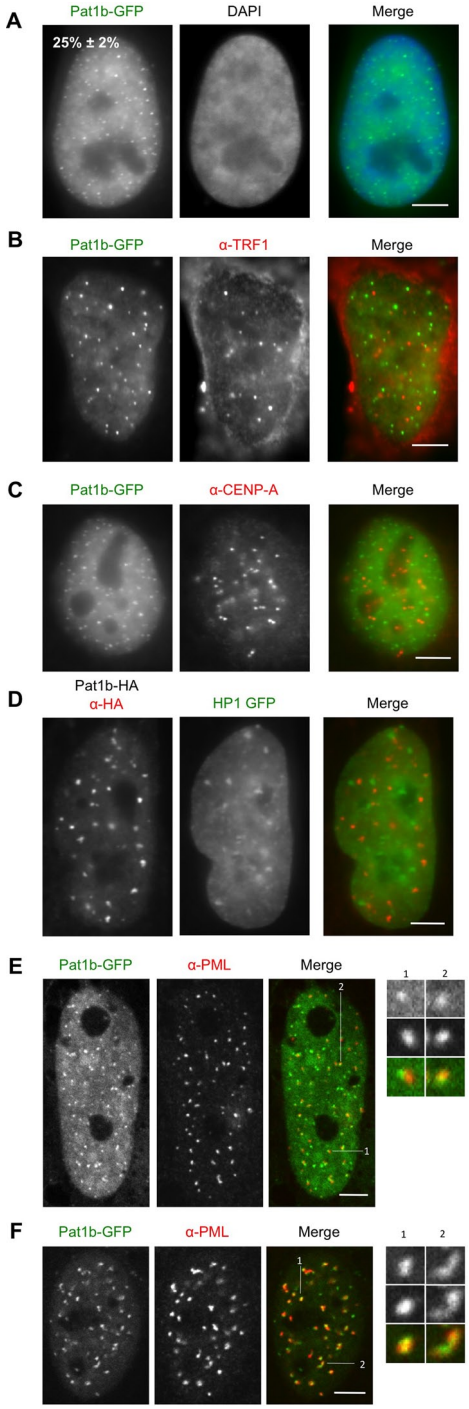


FIGURE 5: Pat1b-GFP localizes to nuclear foci in LMB-treated cells, which associate with PML bodies but not telomeres, centromeres, or heterochromatin. (A–D) Fluorescence imaging of HeLa cells (A, C, and D) or U2OS cells (B) treated with LMB for 5 h (+LMB). Scale bar, 5 μm. (A) Pat1b-GFP localizes to nuclear foci in 25 ± 2% of cells, which were stained with DAPI. (B, C) Pat1b-GFP does not colocalize with telomeres, as seen with α-TRF1 antibody (B), or with centromeres, as seen with α-CENP-A antibody (C). (D) HP1-GFP was cotransfected with Pat1b-HA visualized with α-HA antibody, and Pat1b foci do not colocalize with heterochromatin sites. (E, F) Confocal imaging of HeLa cells treated with LMB for 5 h (+LMB) and stained with a monoclonal α-PML antibody. (E) Pat1b-GFP nuclear foci are adjacent to PML bodies, as seen in example 1, or they overlap with PML bodies, as seen in example 2. (F) Pat1b-GFP can also colocalize with PML bodies, as seen in examples 1 and 2.

were not visible after LMB treatment (Figure 1A, right). We thus turned to the human FOG/SNB19 glioma cell line, which has prominent PML bodies (unpublished data). In untreated cells, the Pat1b antibody detected a weak, diffuse signal throughout the cytoplasm, as well as a diffuse signal in the nucleus (Figure 6A, top), as observed in HeLa cells. However, in contrast to HeLa cells, P-bodies were rarely observed (in <1% of the cells). The absence of P-bodies in this cell line was confirmed with other P-body markers, including rck/p54 or Ge1 antibodies (data not shown). After LMB treatment, some cells contained Pat1b-positive nuclear foci, which were reminiscent of the PML-associated nuclear foci observed with Pat1b-GFP in HeLa cells (Figure 6A, bottom). This was confirmed by coimmunostaining with PML antibodies (Figure 6A, bottom). These foci disappeared following si-Pat1b transfection, confirming the specific detection of endogenous Pat1b (Figure 6B). Confocal imaging showed that the endogenous Pat1b nuclear foci were either adjacent or coalescent with the PML bodies (Figure 6C), just as observed in HeLa cells with Pat1b-GFP (Figure 5E-F).

### Pat1b nuclear foci colocalize with the nucleocytoplasmic shuttling protein 4E-T

We next examined the localization of GFP-tagged 4E-T, another P-body component that is also a nucleocytoplasmic shuttling protein, in LMB-treated cells (Dostie *et al.*, 2000a; Andrei *et al.*, 2005; Ferraiuolo *et al.*, 2005). 4E-T-GFP displayed similar nuclear foci in a similar percentage of cells as Pat1b (33 ± 8%) (Figure 7A). Using confocal imaging of cells cotransfected with 4E-T-GFP and Pat1b-HA, we observed that most of the foci colocalized (Figure 7B), confirming that Pat1b and 4E-T nuclear foci represent the same sites.

We additionally tested whether, similar to Pat1b-GFP, 4E-T-GFP would also display a relationship with PML bodies. We observed that, like Pat1b, 4E-T nuclear foci can also be adjacent, overlapping, or colocalizing with PML bodies (Figure 7C).

Taken together, the results indicate that Pat1b and 4E-T are nucleocytoplasmic proteins that localize to the same nuclear foci, which show a close association with PML bodies.

### Pat1b localization to the nuclear foci is mediated via the N-terminus, and the C-terminus promotes Pat1b localization to speckles

To define the regions of Pat1b that promote the nuclear foci or speckle localization, we examined the subcellular localization of two truncations of Pat1b, the N-terminal–half (regions I–III) and C-terminal–half domains (regions IV and V). It is striking that we observed that the Pat1b N-terminal half mediates the formation of the nuclear foci, whereas its C-terminal half promotes Pat1b localization to the speckles (Figure 8, A and B). This difference is not due to differential expression, as verified by Western blotting of GFP-tagged proteins (Figure 8C). We conclude that these two distinct localization patterns reflect the potential of Pat1b to interact with multiple protein partners in the nucleus, similar to its function in the cytoplasm. The distinct localization patterns may also be due to RNA-binding in addition to protein-binding abilities of Pat1b. Previously, we and others showed that yeast and *Xenopus* Pat1 proteins bind RNA in vitro (Pilkington and Parker, 2008; Marnef *et al.*, 2010). Like its vertebrate counterpart, we found that human Pat1b binds RNA largely via the N-terminal region III, although the C-terminal regions IV and V also contribute (Supplemental Figure S1), suggesting that localization to nuclear foci may involve RNA binding.

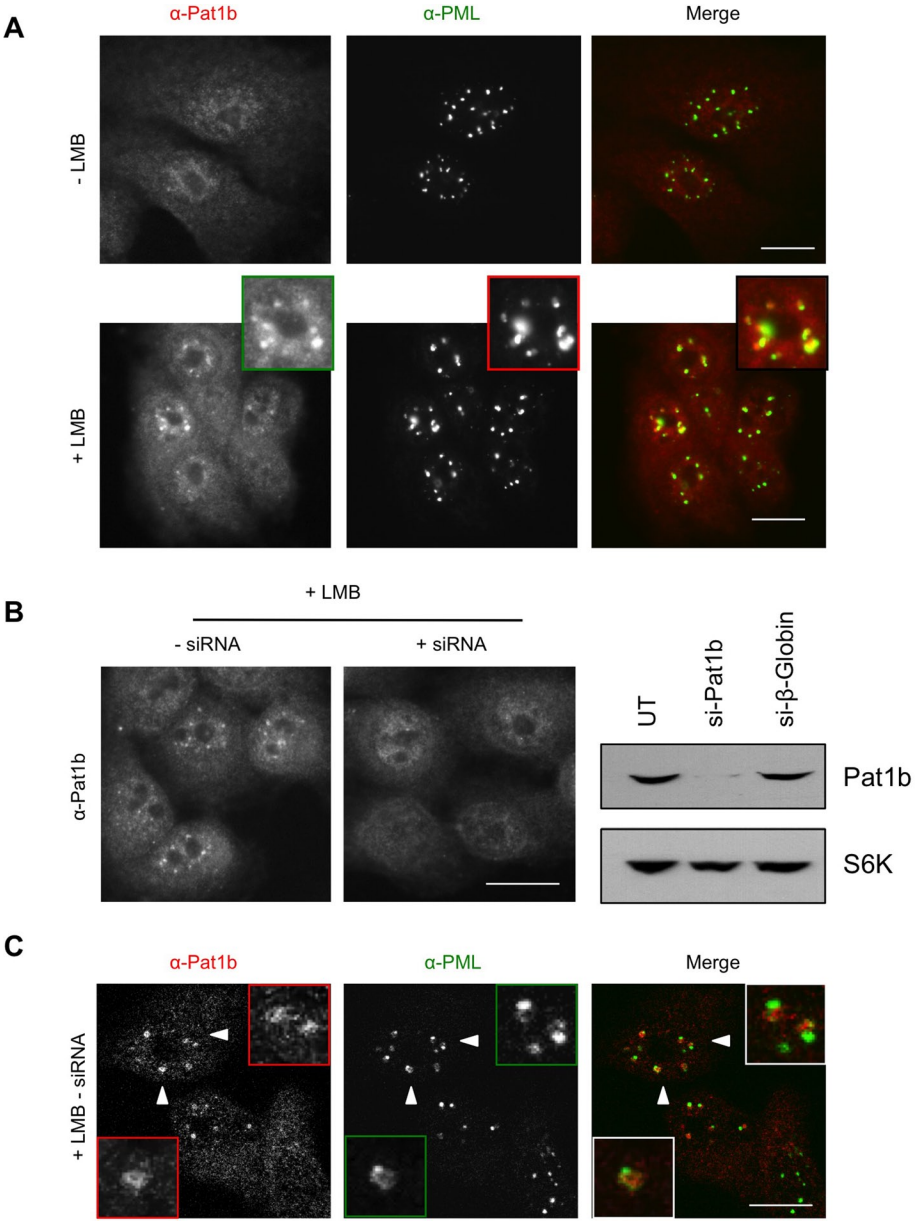


FIGURE 6: Endogenous Pat1b is associated with PML bodies in FOG cells. (A, B) Fluorescence imaging and (C) confocal imaging of FOG cells costained with α-Pat1b (red) and α-PML antibodies (green). Scale bar, 10 μm. (A) Cells were treated with (+LMB) or without LMB (−LMB). (B) Left, si-Pat1b-transfected (+siRNA) and control cells (−siRNA) were compared in the presence of LMB. The zoomed box contains an example of a nucleus containing Pat1b-foci. Right, Western blot of UT (untransfected), si-Pat1b or control si-β-globin, probed with α-Pat1b and α-S6K as a loading control. (C) Pat1b nuclear foci are adjacent (top right, zoomed box) or coalescent (bottom left, zoomed box) to PML bodies.

### Pat1b localization to nuclear foci and speckles requires active transcription

Given that Pat1b is an RNA-binding protein that at steady state is found enriched in cytoplasmic P-bodies, it was of interest to examine whether its traffic to nuclei required ongoing transcription. To this end, cells were treated with actinomycin D (Act D) for up to 24 h, following previous studies of P-bodies (Cougot *et al.*, 2004; Aizer *et al.*, 2008). Inhibition of RNA synthesis did not significantly affect the distribution of Pat1b-GFP or of endogenous Pat1b between the cytoplasm and the nucleus (data not shown and Supplemental Figure S2), indicating that import into nuclei is independent of transcription. Immunostaining with 4E-T antibodies showed that this P-body component also does not enter nuclei upon Act D treatment (Supplemental Figure S2). We did note, however, consistent effects of Act D on P-body size and number. In agreement with previous studies (Cougot *et al.*, 2004; Aizer *et al.*, 2008), cells examined after 5 h of Act D treatment showed a reduction in P-body number, although this conclusion is tempered by our observation that by 24 h many such cells were not viable. It is striking that at 5 h of treatment, as P-body number per cell dropped, their size significantly increased, which correlated with a reduction of diffuse staining and staining in smaller, P-body–like, aggregates. This was true for endogenous P-bodies detected with Pat1b, 4E-T, and rck/p54 antibodies (Supplemental Figure S2). We thus concluded that active transcription is not required for shuttling of either Pat1b or 4E-T but that its inhibition results in fewer and bigger P-bodies.

To assess whether ongoing transcription was required for Pat1b-GFP and 4E-T-GFP to localize to nuclear foci and speckles, Act D was added at the same time as LMB. We observed that the number of cells displaying Pat1b-GFP and 4E-T-GFP nuclear foci dramatically decreased upon Act D and LMB treatment (reduced to 4 ± 2% compared with 25 ±2 % for Pat1b and to 33 ± 8% for 4E-T in LMB-treated cells). Pat1b-GFP also no longer accumulated in splicing speckles, although endogenous speckles were present and, as expected, were enlarged upon inhibition of transcription (Spector and Lamond, 2011; Figure 9, A–D). Similar observations were also made for Pat1b-Nter and Pat1b-Cter constructs, which did not display nuclear foci or speckles in Act D–treated and LMB-treated cells (Figure 9, C and D). In agreement, endogenous Pat1b also did not accumulate in nuclear foci in FOG cells treated with Act D and LMB (data not shown).

The remaining cells showed Pat1b-GFP diffusely in the nucleoplasm in 32 ± 10% cells, with denser regions, generally in crescent-shaped caps, accumulating around the nucleoli in 64 ± 8% of cells (Figure 9A). Of importance, the Pat1b-NES*-GFP protein also displayed similar localization upon inhibition of transcription and in the absence of LMB (Figure 9B). The nucleolus is composed of three compartments: a fibrillar center (FC) and the dense fibrillar compartment (DFC), both of which are embedded in the granular compartment (GC)—yet these compartments become distinct and juxtaposed upon inhibition of transcription, when the FC and DFC migrate toward the periphery of the nucleolus (Puvion-Dutilleul *et al.*, 1992). These compartments are termed nucleolar caps (Shav-Tal *et al.*, 2005). On inhibition of transcription, the DFC marker fibrillarin localizes to "light nucleolar caps" (LNCs).

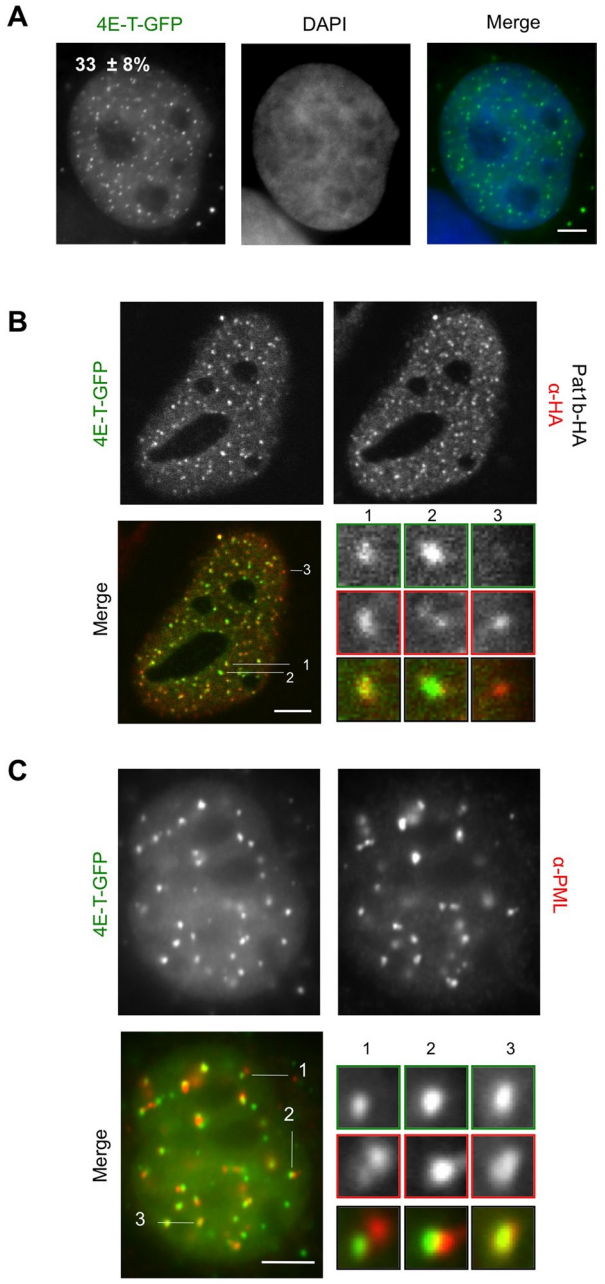


FIGURE 7: Pat1b-GFP nuclear foci colocalize with 4E-T foci in LMB-treated cells. HeLa cells treated with LMB for 5 h. (A) Fluorescence imaging of 4E-T-GFP localizing to nuclear foci; nucleus was stained with DAPI. (B) Confocal imaging of cells cotransfected with HA-tagged Pat1b, visualized using an α-HA antibody, and GFP-tagged 4E-T. Most 4E-T nuclear foci colocalize with Pat1b foci, as seen in example 1. However a few foci do not colocalize, as shown in examples 2 and 3. (C) Fluorescence imaging of 4E-T-GFP showing a relationship with PML bodies, identified with a monoclonal PML antibody. Most 4E-T nuclear foci are adjacent to PML bodies, as seen in example 1, some overlap (example 2), and some colocalize (example 3). Scale bar, 5 μm.

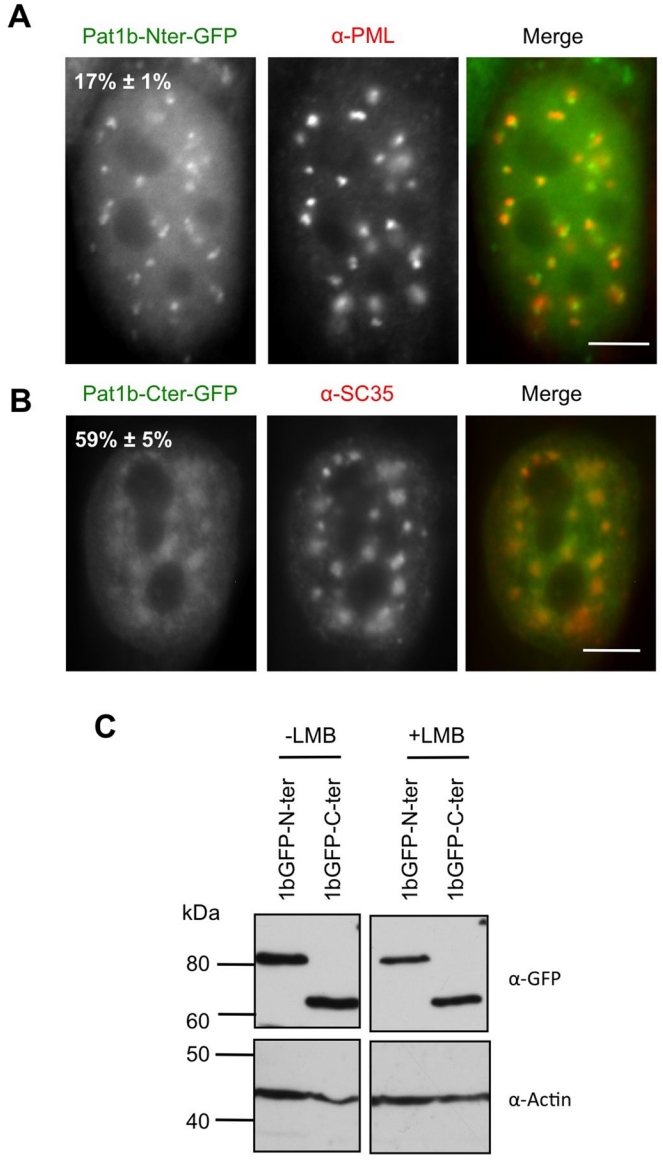


FIGURE 8: Pat1b N-ter mediates its localization to nuclear foci, whereas its C-ter promotes its localization to nuclear speckles. (A, B) Fluorescence imaging of HeLa cells treated with LMB for 5 h. Scale bar, 5 μm. (A) Pat1b N-ter-GFP localizes to nuclear foci in 17 ± 1% cells. These foci may associate with PML bodies, as seen with a monoclonal PML antibody. (B) Pat1b-C-ter displays nuclear speckles in 59 ± 5% of cells, as visualized with antibody to SC35, a speckle marker. (C) Expression levels of GFP constructs transfected in HeLa cells in the presence (+LMB) or absence of LMB (−LMB). Total cell extracts of GFP-tagged Pat1b N-ter and C-ter analyzed by Western blot, using an α-GFP antibody and α-actin antibody (as a loading control).

"Dark nucleolar caps" (DNCs), which are a distinct type of nucleolar cap, are juxtaposed to the LNCs, and also form at the periphery of the nucleolus upon inhibition of transcription (Shav-Tal *et al.*, 2005). Pat1b localization in crescent-shaped regions at the nucleolar periphery is very reminiscent of the DNCs observed by Shav-Tal *et al*. (2005) and is driven by its N-terminal–half domain (Figure 9, C and D). In contrast to Pat1b, 4E-T did not accumulate in nucleolar caps in the presence of Act D, although they are clearly visible in the differential interference contrast (DIC) image (Figure 9E).

In summary, we showed that the localization of Pat1b and 4E-T to nuclear foci and of Pat1b to speckles is mediated by active transcription. Furthermore, although Pat1b is absent from nucleoli in actively transcribing cells, it partially redistributed to nucleolar caps in the DNCs upon inhibition of RNA synthesis in a process mediated by its N-terminus.

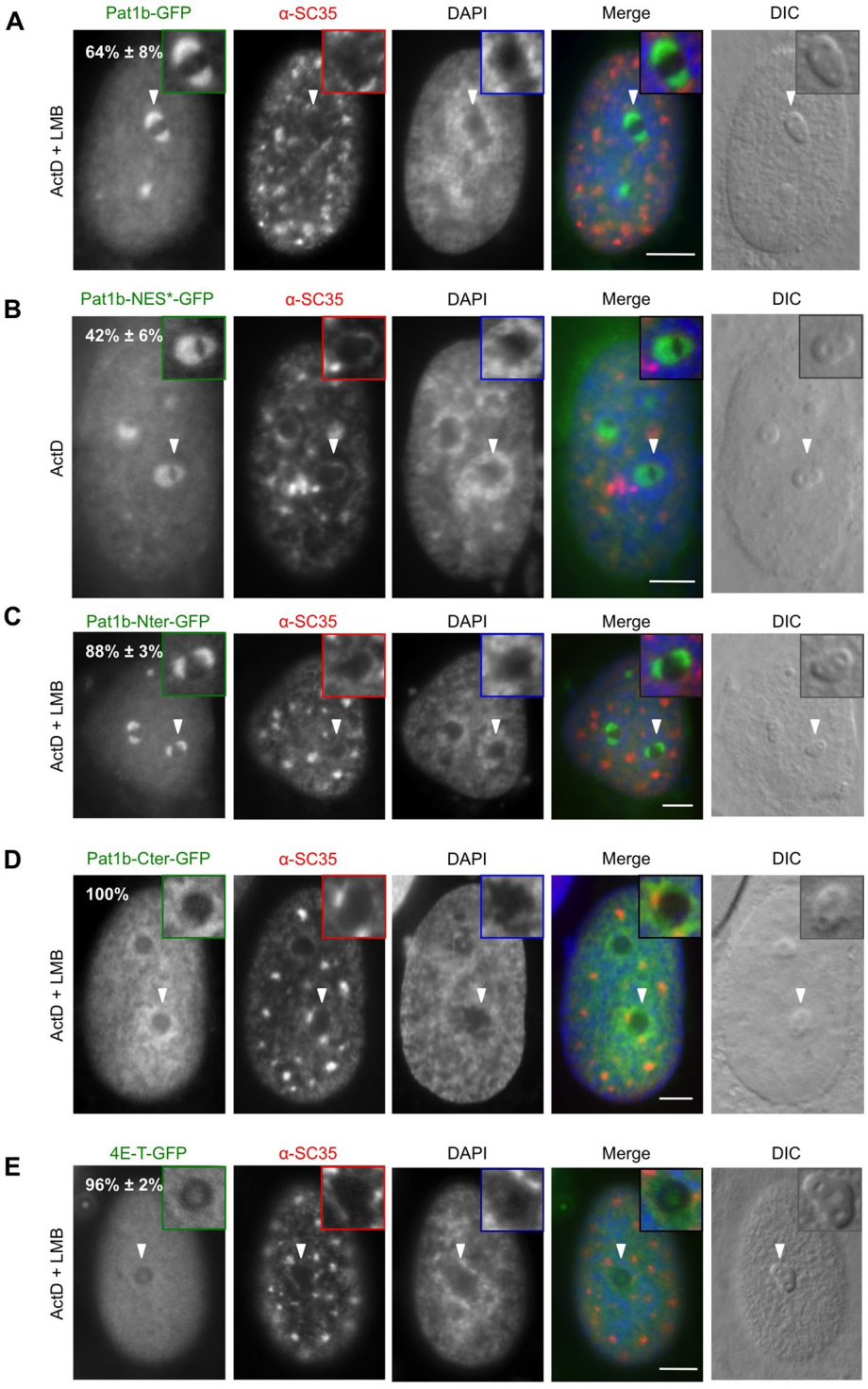


FIGURE 9: Inhibition of transcription leads to the loss of nuclear foci and the redistribution of Pat1b to the nucleolus. Fluorescence imaging of HeLa cells treated simultaneously with Act D and LMB for 5 h (A, C) or with Act D only (B) and stained with DAPI and a α-SC35 antibody. Scale bar, 5 μm. (A) Pat1b-GFP displays a diffuse nucleoplasmic localization in 64 ± 8% of cells treated with both LMB and Act D, with a partial relocalization of Pat1b-GFP to a part of the nucleolus, to nucleolar caps (white arrow and zoomed box). (B) Pat1b-NES*-GFP localizes to the nucleolar cap in 42 ± 6% of cells only treated with Act D. (C) Pat1b-Nter-GFP relocalizes to nucleolar caps in 88 ± 3% of cells, whereas Pat1b-Cter-GFP does not (D). (E) 4E-T-GFP displays a nucleocytoplasmic localization in 96 ± 2% of cells treated with both LMB and Act D.

## DISCUSSION

This study demonstrates that in addition to its previously characterized functions in silencing gene expression in the cytoplasm, Pat1b is a shuttling protein that localizes to several nuclear compartments, including splicing speckles, nuclear foci, and nucleolar caps in mammalian tissue culture cells. Our results lead us to propose that these diverse and distinct sites reflect Pat1b involvement in various nuclear processes, likely to be RNA related, which are mediated by subsets of factors interacting with its N- and C-terminal–half domains, as well as its RNA-binding ability, and that upon exit to the cytoplasm an exchange of factors occurs leading to Pat1b localization and function in mRNA decay in P-bodies.

### Pat1b is a nucleocytoplasmic protein, unlike most P-body components

We showed that by specifically inhibiting the Crm1 nuclear export pathway with LMB, the P-body protein Pat1b is retained in nuclei, suggesting that normally Pat1b shuttles through the nucleus by interacting with the Crm1/exportin1 receptor. This observation is directly supported by the yeast two-hybrid interaction between the yeast proteins Pat1p and Crm1p (Jensen *et al.*, 2000). Moreover, the *Schizosaccharomyces pombe* Pat1 homologue (SPBC19G7.10c) relocalizes from cytoplasmic foci, presumably P-bodies, to the nucleus upon LMB treatment (Matsuyama *et al.*, 2006; *Schizosaccharomyces pombe* Postgenome Database at www.riken.jp/SPD/index.html). Taken together, then, the results indicate that nucleocytoplasmic shuttling is a conserved function of Pat1 proteins.

A canonical NES was identified in Pat1b by mutagenesis of the consensus residues in the Pat1b-NES*-GFP construct, which accumulates in the nucleus in untreated cells. However, Pat1b-NES*-GFP did not fully relocate to the nucleus in such cells, unlike the case of LMB-treated cells, suggesting that Pat1b accumulation in nuclei may rely on other potential NES sequences. We tested the effect of mutating two additional putative NES sequences but found that this triple-mutant construct did not localize to the nucleus any more effectively than the single Pat1b-NES*-GFP protein (data not shown). Alternatively, LMB treatment may favor Pat1b import, for example, by decreasing its retention in the cytoplasm.

We propose that all vertebrate Pat1b proteins shuttle using this NES, since most of its critical residues are conserved (Figure 2D). On the other hand, human Pat1a is not a Crm1-dependent nucleocytoplasmic protein, and, in agreement, mammalian Pat1a proteins lack the N-terminal portion encompassing the Pat1b NES sequence (Scheller *et al.*, 2007). These results further underline the differences

between the functions of these paralogue subfamilies, most homologous in their C-terminal regions (Marnef *et al.*, 2010).

By examining several other endogenous P-body components, we found that not all shuttle into nuclei. Indeed, only Pat1b and 4E-T were sensitive to LMB, but not Dcp1a, rck/p54, Edc3, Ge-1, and Xrn1 (this study; Dostie *et al.*, 2000a). In agreement, the genome-wide analysis of the *S. pombe* ORFeome shows that unlike Pat1, Ste13 (p54/rck), Dcp1 and Dcp2, Edc3, Sum2 (Rap55/Scd6), and Ccr4 do not change localization upon LMB treatment (*Schizosaccharomyces pombe* Postgenome Database). CPEB1, a vertebrate P-body component, also shuttles via Crm1 in *Xenopus* oocytes, HeLa cells, and NIH-3T3 cells (Ernoult-Lange *et al.*, 2009; Lin *et al.*, 2010); in HeLa cells, CPEB1 accumulates in a few foci identified as Crm1 nucleolar bodies (Ernoult-Lange *et al.*, 2009).

In the case of rck/p54, our results agree with the fractionation data of Ernoult-Lange *et al*. (2009), both studies only detecting cytoplasmic rck/p54 protein in LMB-treated HeLa cells. However, previously we noted its striking nuclear import upon fertilization of *Spisula* (clam) oocytes (Minshall *et al.*, 2001), and Smillie and Sommerville (2002) reported its partial nuclear localization in early-stage *Xenopus* oocytes. These cases may rely on import/export mechanisms not involving Crm1 or a more complex mechanism that operates in early development.

### RNA-related nuclear functions of Pat1b

Nuclear Pat1b is found diffusely in the nucleoplasm and in addition localizes to splicing speckles and PML-associated nuclear foci in a transcription-dependent manner, as well as to dark nucleolar caps when RNA synthesis is inhibited. Pat1b accumulation in speckles suggests that it may function in mRNA splicing. Indeed, our observation that spliceostatin A–mediated inhibition of splicing, which leads to rearrangement of SC35 speckles (Kaida *et al.*, 2007), similarly affects Pat1b-NES*-GFP supports a possible role in splicing processes.

In addition, Pat1b may be linked to transcription, as nuclear speckles are believed to supply protein factors to transcription sites (Lamond and Spector, 2003) and contain active and inactive forms of RNA polymerase II (Xie *et al.*, 2006). In yeast, among a wide variety of P-body components involved in deadenylation and mRNA decay, only Pat1p (and weakly LSm2) bind the Rpb7/4 RNA polymerase II subunits (Lotan *et al.*, 2005; 2007) that link transcription and mRNA decay to translation (Harel-Sharvit *et al.*, 2010).

Pat1b also localized to nuclear foci, shown to be associated with PML bodies, as did 4E-T. PML bodies have roles in posttranslational modifications and in the regulation of DNA replication, transcription, senescence, and apoptosis, although detailed understanding of their function remains elusive (Lallemand-Breitenbach and de The, 2010). The fact that the nuclear foci are adjacent to, and in some cases colocalize with, PML bodies suggests that proteins may be exchanged between the two structures. The translation initiation factor eIF4E partitions between the cytoplasm and the nucleus, where it has been observed in nuclear speckles and PML bodies (Dostie *et al.*, 2000b; Lai and Borden, 2000). Moreover, since eIF4E is transported to the nucleus via 4E-T (Dostie *et al.*, 2000a), we speculate that 4E-T accumulation in nuclear foci permits eIF4E retention in PML bodies.

We consistently noted that Pat1b is present in speckles in ~40% of transfected cells, whereas ~25% cells contain Pat1b in foci, but we do not understand the underlying reason. Possibly this distribution reflects a cell cycle stage–dependent fluctuation in speckles or foci or in the levels of Pat1b-binding factors that influence its localization. Furthermore, Pat1b localization to speckles is LMB independent, whereas its accumulation in nuclear foci requires LMB, as Pat1b-NES*-GFP does not form nuclear foci in untreated cells (data not shown). This suggests that nuclear foci may only exist transiently and are stabilized by LMB or that a partner of Pat1b (which is also sensitive to LMB) is required for the anchoring of a complex containing Pat1b to the nuclear foci.

When examining the functions of speckle proteins that redistribute to nucleolar caps upon Act D treatment, we found that most are implicated in alternative splicing, microRNA processing, or both. Proteins with these properties include EWS (product of the Ewing sarcoma breakpoint region 1 gene), heterogeneous nuclear ribonucleoproteins F, H, and K, p68 RNA helicase, TLS, and CBP20 (Saitoh *et al.*, 2004; Shav-Tal *et al.*, 2005; Iwamoto *et al.*, 2008). Moreover, primary microRNAs have recently been observed to accumulate in speckles (Pawlicki and Steitz, 2009).

### Conclusions and Perspectives

Pat1 proteins have been at the forefront of several recent studies due to their multiple roles in cytoplasmic gene regulation. Here we show that Pat1b displays diverse subnuclear localizations, including nuclear speckles, PML-associated foci, and nucleolar caps, likely reflecting on its ability to interact with various protein partners, as well as with RNA. Most P-body components do not share these localization patterns, indicating that Pat1b plays distinct roles at these sites, and future studies investigating its nuclear partners will be critical to our understanding of Pat1b function. Such studies are likely to be particularly challenging, as components of these nuclear sites are involved in transcription, alternative splicing, microRNA processing, and sumoylation to ubiquitinylation, among other roles. Similar to its scaffold function in the cytoplasm (Braun *et al.*, 2010; Ozgur *et al.*, 2010), we speculate that Pat1b, via its distinct N- and C-terminal–half domain partners, may link these diverse nuclear processes.

## MATERIALS AND METHODS

### Cell culture and transfections

HeLa, HEK293-T (kind gift of John McCafferty), U2OS (a kind gift of Stephen Jackson), and FOG/SNB19 glioma (a kind gift of Marie-France Poupon; Miccoli *et al.*, 1998) cells were routinely grown in DMEM and supplemented with 10% fetal calf serum. To inhibit Crm1-dependent export, LMB (in methanol) (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 10 nM for 5 h. Spliceostatin A (in methanol) was used at a final concentration of 200 nM (Kaida *et al.*, 2007) for 5 h. Addition of the same volume of methanol to the cells was used as a control for these inhibitor experiments. For transcription inhibition of RNA polymerases I and II, 4 μM Act D was used. Transient transfections were performed in 24-well plates using 1 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 0.5 μg of plasmid constructs, or in six-well plates using 5 μl of Lipofectamine 2000 and 3 μg of small interfering RNA (siRNA). The Pat1b and control β-globin siRNAs were described in Marnef *et al*. (2010). The cells were fixed and stained with the appropriate antibody 24 h after plasmid transfection. For siRNA transfection, cells were split into thirds 24 h after transfection and fixed and stained 53 h after transfection.

### Plasmids and mutagenesis

The human Pat1b (Marnef *et al.*, 2010) and the human Pat1a (kind gift of Georg Stoecklin) open reading frames (ORFs) were cloned into the EGFPC1 vector (Clontech, Mountain View, CA) via the *Xho*I and *Xma*I restriction sites. Pat1bNES*-GFP mutant was created using a site-directed mutagenesis kit (QuikChange; Stratagene, Santa Clara, CA), resulting in the mutation of Leu86Leu90Met93Iso95 all

to Ala. Pat1b-Nter-GFP and Pat1b-C-ter-GFP were generated by cloning the ORF encoding 1–396 and 397–770 amino acids, respectively, in the *Xho*I/*Xma*I restriction sites of the EGFPC1 vector. The HA-Pat1b construct was obtained by cloning the Pat1b ORF into the *Eco*RI and *Not*I sites in the pCI-neo vector to which a hemagglutinin (HA) tag was added upstream of the *Eco*RI site (a kind gift of Witek Filipowicz). The human 4E-T-GFP cDNA was based on h4E-T-YFP, a gift of Reinhard Luhrmann, and the HP1-GFP construct was a gift from Natasha Murzina.

### Immunofluorescence and microscopy

Cells were plated on 13-mm glass coverslips in 24-well plates. All cells except FOG were fixed in 4% paraformaldehyde for 20 min, washed three times with phosphate-buffered saline (PBS), and permeabilized in PBS with 0.5% Triton X-100 for 4 min 30 s, followed by three PBS washes. FOG cells were fixed in methanol for 6 min at −20°C. Cells were incubated with primary antibodies for 1 h using rabbit Pat1b (1:100), mouse SC35 (1:500; Sigma-Aldrich), rabbit rck/p54 (1:1000; Bethyl Laboratories, Montgomery, TX), rat HA (1:200; Roche, Indianapolis, IN), goat 4E-T (1:200; Abcam, Cambridge, MA), rabbit Dcp1a, Edc3, Ge-1, and Xrn1 (all at 1:200; kind gifts of Jens Lykke-Andersen), mouse monoclonal PML (hybridoma supernatant diluted 1:5 for FOG cells and used neat for HeLa cells; a kind gift of Lizz Grimwade), and rabbit TRF1 (1:250) and mouse CENP-A (1:1000) (kind gifts of Stephen Jackson) antibodies. The cells were then washed three times in PBS, followed by the incubation of the secondary antibodies conjugated to rhodamine or Alexa 488 used at a 1:1000 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. After rinsing three times with PBS, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1.25 µg/ml) for 10 s. The coverslips were mounted in Citifluor medium (Citifluor, London, United Kingdom). All steps were performed at room temperature. Cells were either observed under a Leica SP1 inverted confocal microscope (Leica, Heidelberg, Germany) with a 100× 1.32 oil immersion objective or with an Axioimager M1 fluorescence microscope (Zeiss, Thornwood, NY) with a Plan-Apochromat 100/1.4 oil DIC objective. At least 100 cells were counted in each indicated experiment, and three independent repeats were performed to obtain the average number of cells with a particular phenotype.

### Protein gel electrophoresis and Western blotting

SDS–PAGE gels and Western blotting were performed as described by Marnef *et al*. (2010) and Huang *et al*. (2011). A total of 40 µg of protein per lane was loaded. Primary antibodies and dilutions used were as follows: rabbit Pat1b affinity purified (1:500) (Marnef *et al*., 2010), mouse S6 kinase (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit actin (1:2000) (Sigma-Aldrich), and mouse GFP (1:1000) (Santa Cruz Biotechnology). Western blots were subsequently detected by enhanced chemiluminescence.

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