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Pml represses tumour progression through inhibition of mTOR

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The promyelocytic leukaemia gene PML is a pleiotropic tumour suppressor. We have recently demonstrated that PML opposes mTOR-HIF1α-VEGF signalling in hypoxia. To determine the relevance of PML-mTOR antagonism in tumourigenesis, we have intercrossed Pml null mice with Tsc2 heterozygous mice, which develop kidney cysts and carcinomas exhibiting mTOR upregulation. We find that combined inactivation of Pml and Tsc2 results in aberrant TORC1 activity both in pre-tumoural kidneys as well as in kidney lesions. Such increase correlates with a marked acceleration in tumour progression, impacting on both the biology and histology of kidney carcinomas. Also, Pml inactivation decreases the rate of loss of heterozygosity (LOH) for the wt Tsc2 allele. Interestingly, however, aberrant TORC1 activity does not accelerate renal cystogenesis in Tsc2/Pml mutants. Our data demonstrate that activation of mTOR is critical for tumour progression, but not for tumour initiation in the kidney.

INTRODUCTION

Work from our laboratory has shown that PML opposes the Akt-mTOR pathway at two levels: by promoting Akt dephosphorylation (Trotman et al, 2006) and by inhibiting mTOR downstream Akt (Bernardi et al, 2006). In vivo, loss of Pml leads to increased activity of nuclear Akt and accelerates prostate and colon tumorigenesis in Pten−/− mice (Trotman et al, 2006). However, whether PML exerted tumour suppressive functions by directly antagonizing mTOR remained to be established.

The protein kinase mTOR exists in two complexes: mTORC1 is activated by growth factors and insulin through Akt to control protein synthesis. Activation of mTORC1 is opposed by the Tsc1/Tsc2 complex (Bhaskar and Hay, 2007) and in turn attenuates Akt signalling through a negative feedback loop (Huang and Manning, 2009). Conversely, mTORC2 phosphorylates and activates Akt (Bhaskar and Hay, 2007), and the Tsc1/Tsc2 complex is required for this activity (Huang and Manning, 2009).

On this basis, in order to understand if PML restrains tumourigenesis by regulating mTORC1, we utilized a tumour mouse model proposed to depend on aberrant activation of mTOR, but not Akt. Tsc2+−/− mice develop renal cysts and carcinomas that display Tsc2 loss of heterozygosity (LOH) and increased mTORC1 activity (Kobayashi et al, 1999; Onda et al, 1999), while Akt activity is suppressed (Harrington et al, 2005; Manning et al, 2005). Notably, forced Akt activation by decreased Pten dosage does not accelerate kidney tumourigenesis (Ma et al, 2005; Manning et al, 2005), although it enhances the severity of liver haemangiomas that occur in some strains of Tsc2+−/− mice (Manning et al, 2005). These studies suggested that some tumour types like kidney tumours may be exquisitely sensitive to mTORC1 activation, while others, like prostate tumours (Ma et al, 2005) or liver haemangioma (Manning et al, 2005), may benefit from full activation of the pathway upstream of mTOR.
We crossed \textit{Pml}\textsuperscript{−/−} mice with \textit{Tsc2}\textsuperscript{+/−} mice to assess if: (i) loss of \textit{Pml} impacted on kidney tumourigenesis by promoting further mTORC1 activation; (ii) loss of \textit{Pml} was sufficient to tilt the feedback balance and promote Akt activation in conjunction with mTORC1 activation; (iii) compound \textit{Pml} and \textit{Tsc2} loss would accelerate tumour initiation and/or progression in the kidney. We found that loss of \textit{Pml} in \textit{Tsc2}\textsuperscript{+/−} mice enhances mTORC1 but not Akt activation and accelerates the development of aggressive kidney carcinomas while having little effect on cysts formation. Thus, utilizing a direct genetic approach in the mouse, we demonstrate that \textit{Pml} suppresses tumourigenesis through repression of mTOR, while mTORC1 hyper-activation does not suffice to initiate tumourigenesis in the kidney.

**RESULTS**

\textbf{Pml and Tsc2 cooperate in inhibiting mTORC1 in kidney tubules}

Compound inactivation of \textit{Pml} and \textit{Tsc2} did not affect the histology of adult mouse kidney (Supporting Information Fig 1A), but it affected mTORC1 activity. Phosphorylation of ribosomal protein S6 was low and confined to discrete tubules in the kidney of \textit{wt} and \textit{Tsc2}\textsuperscript{−/−} mice. In \textit{Pml}\textsuperscript{−/−} mice, a small increase in phoso-S6 was found, while a significant increase was observed in compound \textit{Pml}\textsuperscript{+/−}/\textit{Tsc2}\textsuperscript{−/−} and \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{−/−} mice (Fig 1A and B).

We have previously demonstrated that in conditions of hypoxia, mTOR acquires partial nuclear localization and that PML regulates the localization of mTOR (Bernardi et al, 2006). Consequently, mTOR localized to both cytoplasm and nucleus in kidney tubule cells under physiological oxygen concentrations, and in the absence of \textit{Pml}, the number of cells with nuclear mTOR diminished (Fig 1C).

Like \textit{Tsc2} (Onda et al, 1999), \textit{Pml} is expressed in specific kidney tubules (Supporting Information Fig 1B and C) that coexpress markers of distal tubules and collecting ducts (Supporting Information Fig 1D, upper panels). Accordingly, phoso-S6 staining in compound \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{−/−} mice colocalized with the same markers (Supporting Information Fig 1D, lower panels), demonstrating that mTORC1 upregulation in mutant mice occurs in the distal tubules and/or collecting ducts.

Analysis of mTORC1 activity in other organs revealed that total physiological levels of 4EBP1 and S6K are dramatically higher in adult liver than in adult kidney (Supporting Information Fig 2A). Consistently, it was recently observed that mTORC1 activity in mouse kidney is high in the first postnatal days but decreases to minimal levels after P14 (Zhou et al, 2009). In spite of this, increased mTORC1 activity was also observed in livers of compound \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{−/−} mice (Supporting Information Fig 2B and C), indicating the two genes inhibit mTORC1 in the kidney but also in other organs.

In summary, inactivation of \textit{Pml} and, more significantly, compound \textit{Pml} and \textit{Tsc2} inactivation triggers mTORC1 activation in normal tissues.

\textbf{Pml loss in kidney causes increased tumour progression}

We next asked whether increased mTORC1 activity affects tumour initiation. \textit{Tsc2}\textsuperscript{+/−} mice develop kidney cortical cysts and adenocarcinomas with high mTORC1 activity starting at approximately 6 months (Kobayashi et al, 1999; Onda et al, 1999). Surprisingly, despite increased mTORC1 activity in pre-cystic \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{+/−} kidneys, we did not detect a significant increase in the number of cysts and small carcinomas as compared to \textit{Tsc2}\textsuperscript{−/−} mice at 6–8 months (Fig 2A). However, by serial MRI, we observed that older \textit{Tsc2}\textsuperscript{+/−} animals developed more tumour lesions in the absence of \textit{Pml} (Fig 2B), while \textit{Pml}\textsuperscript{+/−} and \textit{Pml}\textsuperscript{−/−} littersmates did not develop kidney tumours.

Tumour size, measured as the number of tumours exceeding 0.5 cm in diameter, was also significantly higher in compound mutants (Fig 2C and D). These data suggest that a twofold increase in mTORC1 activity does not affect cystogenesis but it seemingly affects tumour progression.

Importantly, along with increased tumour size, tumours in \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{+/−} mice showed other features of aggressive carcinomas. Kidney carcinomas from \textit{Tsc2}\textsuperscript{−/−} mice for example display heterogeneous histological phenotypes within the same tumour: a predominant phenotype of regular, cuboidal cells arranged into a papillary architecture (Fig 2E, single arrow) is often flanked by a clear cell histotype (Fig 2E, double arrow). In addition, some tumours show areas of elongated, fibroblast-like cells (Fig 2F), reminiscent of sarcomatoid change that in human renal cell carcinoma correlates with tumour progression and worse prognosis (de Peralta-Venturina et al, 2001). In compound \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{+/−} mice, we detected a small increase in tumours with clear cell morphology (tumours with areas of clear cell morphology: 66% in \textit{Tsc2}\textsuperscript{−/−} mice and 90% in \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{+/−} mice; p = 0.2), and a significant increase in tumours with sarcomatoid changes (Fig 2F), indicating that tumours lacking \textit{Pml} are more aggressive. Consistent with this notion, tumours from compound \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{+/−} mice displayed increased microvessel density and proliferation rates (Fig 3A and B), although they did not metastasize.

Despite the different phenotypes, tumours in \textit{Tsc2}\textsuperscript{+/−} and \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{+/−} mice likely originate from the same kidney structures, as they express similarly gelsolin (Onda et al, 1999). Specifically, a comparable number of cysts showed gelsolin expression (Fig 3C) and in overt tumours, where gelsolin expression varied among tumours areas (Wilson et al, 2005), no significant difference was observed in the absence of \textit{Pml} (Fig 3D, arrow). These data indicate that although cysts in \textit{Tsc2}\textsuperscript{+/−} mice may originate from different structures, or perhaps transdifferentiation processes occur within cysts and tumours, these processes are not modified in the absence of \textit{Pml}.

Taken together, our data indicate that increased mTORC1 activity in the kidney of compound \textit{Pml}\textsuperscript{−/−}/\textit{Tsc2}\textsuperscript{+/−} mice does not modify the incidence of cysts and small carcinomas but accelerates tumour progression, as measured by several parameters that correlate with aggressiveness in human kidney tumours.

Finally, we also evaluated the frequency of liver haemangiomata. \textit{Tsc2}\textsuperscript{+/−} mice bred in our genetic background develop very few liver haemangiomata when older than 18 months.
Although the number of mice analysed was low, absence of Pml did not modify tumour incidence (data not shown).

Cysts and tumours lacking Pml display increased mTORC1 activity and decreased Tsc2 LOH

Next, we measured the phosphorylation status of proteins that are regulated by mTORC1 and mTORC2 complexes. In agreement with higher activation of mTORC1 in pre-cystic kidneys of compound mutants mice (Fig 1A), a twofold increase in mTORC1 activity was also observed in tumours from Pml$^{-/-}$ Tsc2$^{-/-}$ mice (Fig 4A and B). Because loss of Pml causes increased Akt activity (Trotman et al, 2006) while activation of mTORC1 leads to its decrease (Harrington et al, 2005), we asked how loss of Pml would affect Akt status in...
conditions of mTORC1 activation. We did not detect significant change in Akt phosphorylation in kidney or in kidney tumours, either in the presence or absence of Pml or Tsc2 (Fig 4C and Supporting Information Fig 3), indicating that renal Akt activity remains low and not affected by the genetic inactivation of Pml and/or Tsc2 as also documented in other Tsc mouse models (Huang et al., 2009; Pollizzi et al., 2009).

mTORC1 activity was also increased in cysts lacking Pml, because all cysts in Pml−/−Tsc2+/− mice displayed high-phospho-S6 staining as compared to 60% in Tsc2+/− mice.
(Bonnet et al, 2009; Wilson et al, 2006; Fig 4D), further confirming that increased mTORC1 activity observed in pre-cystic kidney and in cysts does not affect tumour initiation.

Finally, we asked if loss of \( Pml \) would surrogate for \( Tsc2 \) LOH, which invariably occurs in human TSC tumours and kidney tumours from \( Tsc2^{+/−} \) mice (Green et al, 1994; Henske et al, 1996; Onda et al, 1999). Polymerase chain reaction (PCR) analysis of large tumours of similar sizes showed that loss of \( Pml \) significantly diminished the rate of \( Tsc2 \) LOH (\( p = 0.006 \); Fig 4E). Thus, the selective pressure to completely eliminate \( Tsc2 \) in order to fully activate mTORC1 is alleviated in the absence of \( Pml \). Moreover, we found that mTORC1 activation is slightly higher, although not significantly, in \( Pml^{−/−}Tsc2^{+/−} \) tumours that have undergone \( Tsc2 \) LOH as compared to those that have not (Supporting Information Fig 4), further confirming that loss of \( Pml \) and \( Tsc2 \) independently lead to...
Figure 4. Kidney tumours from Pml<sup>−/−</sup> Tsc2<sup>+/−</sup> mice have increased mTORC1 activity and decreased Tsc2 LOH.

A. Immunohistochemical analysis of phospho-S6 and phospho-4EBP1 on kidney tumours of the same size from age-matched mice. 20× magnification (scale bar: 50 μM). Higher magnification images of tumour areas are shown in indents.

B. Western blot analysis of phospho and total 4EBP1 and S6 performed on lysates from kidney tumours derived from age-matched mice of the indicated genotype. Graphs at the bottom show mean relative ratios of phospho/total proteins from various western blots ±SD.

C. Western blot analysis of phospho and total Akt, phospho-S6 and phospho-4EBP1 performed on lysates from normal kidneys or kidney tumours derived from age-matched mice of the indicated genotype.

D. Percentage of phospho-S6-positive and -negative kidney cysts from age-matched mice (n = 4 mice/genotype; 8 cysts). p-value obtained comparing mice wt or null for Pml = 0.07.

E. Upper panel: representative image of PCR-amplified wt and null Tsc2 alleles from tails and kidney tumours (tu.) of indicated genotypes. Bottom table: number of tumours with loss of the wt Tsc2 allele. p-values, calculated by Student’s t-test, compare categories indicated with asterisks. All mice in Fig 4 were 14–19-month-old.
The paper explained

PROBLEM:
Aberrant activation of mTORC1 has been reported in a large number of human tumors. In renal carcinoma in particular, this observation has led to the development of novel therapeutic modalities, as mTOR inhibitors have been approved for the treatment of this tumor type. Nevertheless, compelling genetic evidence that the activation of this pathway may contribute to initiation or progression of kidney tumorigenesis is still lacking. Furthermore, genetic mutations in genes regulating the mTORC1 pathway are rarely found in sporadic tumors, and when inherited in the germ line, only lead to hamartomatous syndromes such as Tuberous Sclerosis Complex. Thus, a firm genetic understanding of the role of mTORC1 signaling in kidney tumorigenesis is needed to more accurately guide the clinical use of the ever-growing family of mTOR inhibitors.

RESULTS:
We generated a compound knockout mouse model lacking two tumor suppressors that are known to negatively regulate mTOR. Tsc2 heterozygosity leads to high mTORC1 activity and the development of kidney cysts and, after a longer latency, to carcinomas. Although mTORC1 activity was further elevated in pre-tumoral kidneys of compound Pml-Tsc2 mutant mice, kidney tumor initiation was not affected. A further increase in mTORC1 activity, however, affected tumor progression. Tumors from compound mutant mice were more vascularized, displayed a higher proliferation rate and a more aggressive histological profile. Furthermore, loss of Pml in kidney tumors significantly reduced the rate of loss of heterozygosity (LOH) of the wild-type Tsc2 allele observed in advanced tumoral lesions. These findings provide genetic evidence that Pml and Tsc2 negatively regulate mTORC1 activity, cooperatively suppressing tumor progression in the kidney.

DISCUSSION
Our data indicate that PML is a physiological negative regulator of mTORC1 activity, and that through this regulation, it restrains tumour progression in a mouse model of kidney tumourigenesis initiated by Tsc2 inactivation. Surprisingly, Pml status does not seem to affect Akt activation in this context. This could be explained by a potent negative feedback loop triggered by combined Pml and Tsc2 inactivation that impedes further activation of nuclear Akt by loss of Pml. Alternatively, the role of PML on Akt activation in the nucleus may be tissue specific and less relevant in the kidney than in other organs, such as the prostate (Trotman et al, 2006).

Importantly, we show that PML regulates kidney tumour progression, but not cystogenesis and tumour onset. The other relevant conclusion of our study is that high mTORC1 activity is not sufficient to initiate tumourigenesis in the kidney and may not be the cause of hamartomatous and neoplastic growths in tuberous sclerosis complex (TSC) patients, although it may affect other functions that we have not analysed such as kidney metabolism.

In agreement with our findings, a percentage of cysts from Tsc1+/− and Tsc2+/− mice does not display mTORC1 activation (Bonnet et al, 2009; Wilson et al, 2006). In addition in the Eker rat model, a mutant Tsc2 gene that fails to inhibit mTOR is still able to suppress tumourigenesis (Shiono et al, 2008), and administration of rapamycin reduces the development of macroscopic tumours while having no effect on the number of microscopic precursor lesions (Kenerson et al, 2005). Indeed, the Tsc1/Tsc2 complex has been shown to possess other activities beside inhibiting mTORC1 (Bonnet et al, 2009; Lacher et al, in press), and it has been suggested that renal cystogenesis in TSC and polycystic kidney disease mouse models may be caused by defects in primary cilia and cell polarity via an mTOR-independent pathway (Bonnet et al, 2009). Together with these studies, our work emphasizes that mTOR activation may not be causative of the initial development of the pathological growths that affect TSC patients.

Because many current new regiments for the treatment of TSC patients rely on the use of mTORC1 inhibitors (Sampson, 2009), it is extremely important to determine the role of mTOR in the pathogenesis of TSC. Our study suggests that for at least some aspects of TSC pathology as well as kidney tumourigenesis, mTORC1 inhibitors may antagonize some features of the disease but not others. Further work is thus required to identify additional molecular targets whose modulation may potentiate the efficacy of current treatment modalities.
MATERIALS AND METHODS

Mice
Pml+/− mices were crossed with Tsc2+/− mutant mice to generate all combinations of compound mutant mice. All mice were of mixed 129/Sv and C57BL/6 strains. For tumour onset analysis, five mice per genotype were sacrificed at 6 and 8 months of age for microscopic analysis of the kidneys. Prostate, spleen, liver and lung were also analysed. For assessment of tumour progression, mice were subjected to monthly magnetic resonance imaging (MRI) screening. Imaging analysis and acquisition was performed as previously described (Trotman et al, 2006). Animals were sacrificed when moribund. All mice were cared for according to NIH-approved institutional animal care guidelines and upon approval by the Institutional Animal Care and Use Committee Beth Israel Deaconess Medical Center (IACUC animal protocol 071-2008).

Histopathology and immunohistochemistry
Normal and tumour tissue samples were fixed in 4% paraformaldehyde for 48 h, washed twice with PBS 1X and transferred to 70% ethanol. Samples of kidneys, spleen, liver, lung, lymphonodes and prostate were embedded in paraffin and sections 4–5 mm of thickness were stained with haematoxylin and eosin (H&E) according to standard protocols. For measuring the number of lesions per kidney, whole kidneys was sectioned and H&E staining was performed on 1 every 15 sections. Twelve H&E sections/mouse were analysed by certified pathologists. Immunohistochemical analysis on kidney samples was performed using the following antibodies: anti-PML (Chemicon), anti-Phospho S6 (S235/236) and anti-phospho-4EBP1 (Cell Signaling Technology), anti-mTOR (Cell Signaling Technology), Thiazide-Sensitive NaCl Cotransporter (NCC) (Chemicon), anti-gelsolin antibody was kindly provided by Dr. J. Kwiatkowski, anti-Ki-67 (Novoceastra), CD34 (Dako).

Loss of heterozygosity (LOH) analysis
LOH of Tsc2 allele was performed by PCR as previously described (Ma et al, 2005). Tissues for LOH analysis were obtained from kidney tumours of ≥0.5 cm in diameter. Similar tumour areas (devoid of haemorrhagic or necrotic lesions) were used for this analysis.

Preparation of tissue extracts and immunoblotting
Tissues were dissected and homogenized immediately on ice in RIPA buffer [Tris–HCl, pH 7.8 50 mM, NaCl 150 mM, NP40 1%, sodium deoxycholate 0.5%, SDS 0.1%, protease inhibitor cocktail (Roche), sodium orthovanadate 1 mM, sodium fluoride 1 mM, PMSF 1 mM]. Insoluble material was removed by centrifugation for 20 min at 13,000 rpm at 4 °C. Protein samples were resolved by SDS–PAGE 10% and transferred to a nitrocellulose membrane blocked in 5% nonfat milk and blotted with the following antibodies: mouse anti-α-tubulin (Sigma), anti-phospho-S6 (S235/236), anti-S6, anti-phospho 4E-BP1 (Ser65) and anti-4EBP1 (Cell Signaling Technology).

Author contributions
The experiments were conceived and designed by RB, AP and PPP. Experiments were performed by RB, AP and NC. Immunohistochemistry was performed by AE. Pathological analysis was performed by JTF and SS. The paper was written by RB, AP and PPP.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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