

## Review

# Stemming out of a new PML era?

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The promyelocytic leukaemia protein PML is a growth and tumour suppressor inactivated in acute promyelocytic leukaemia (APL). Recent evidence indicates that PML plays a tumour-suppressive role in cancer of multiple histological origins. However, it is only very recently that PML growth-suppressive functions have been implicated in regulating physiological processes and tissue homeostasis. In particular, it has been shown that PML is one of the key cell-cycle regulators controlling stem cell function in multiple tissues, from the blood to the brain. As a consequence, PML loss has an impact on tissue development and maintenance of stem cell pools. In addition, new data suggest that PML regulates self-renewal in cancer stem cells. Finally, the oncogenic fusion protein PML/RAR $\alpha$ , contrary to the conventional view, appears to hijack growth-suppressive pathways to promote transformation of haematopoietic stem cells and to maintain the APL stem cell niche. Overall, these findings not only represent a change in paradigm in the field of PML/APL research, but also contribute to the understanding of fundamental mechanisms underlying stem cell function *in vivo*. The main objective of this review is to critically discuss the very recent literature on the role of PML in stem cells and tumour-initiating cells. Ultimately, it aims to propose new avenues of investigation. *Cell Death and Differentiation* (2009) 16, 1083–1092; doi:10.1038/cdd.2009.63; published online 12 June 2009

## The Past

**PML regulates cancer development.** The promyelocytic leukaemia protein (PML) gene was found at the t(15;17) translocation of acute promyelocytic leukaemia (APL), which generates the PML/RAR $\alpha$  fusion gene. PML/RAR $\alpha$  is the oncogene of APL and is believed to act mainly by blocking haematopoietic differentiation at the promyelocytic stage thus lending the leukaemic blasts a marked proliferation and survival advantage.<sup>1–3</sup> PML belongs to the tripartite motif (TRIM) family of proteins, which are characterized by a conserved N-terminal RING-B-box-coiled-coil motif and different C termini.<sup>4</sup> C termini determine functional identity of the different isoforms and often regulate the subcellular localization.<sup>4</sup> PML is found associated with a subnuclear structure named the PML nuclear body (PML-NB), of which it is the essential component.<sup>3</sup> The PML-NB is disrupted in APL and loss of PML in an animal model of APL results in increased incidence and decreased onset of the disease, thus implicating PML-suppressive function in APL pathogenesis.<sup>3,5</sup> Furthermore, mutations in the remaining PML allele have been reported in patients with aggressive APL.<sup>3,6–8</sup> Recent studies have highlighted that

PML may bear tumour-suppressive functions also in solid tumours, such as carcinomas of the lung, colon and prostate.<sup>3,9–11</sup> In cancer cells, PML is regulated at the posttranslational level through degradation by the ubiquitin/proteasome system.<sup>10</sup> Nonetheless, additional mechanisms may be in place to regulate PML expression in cancer cells. Overall, these reports indicate that the loss of PML not only contributes to myeloid leukaemias, but it is probably involved in the development of a wider range of tumours.

**How PML works.** PML is believed to interact with an increasingly large number of cellular proteins. However, it is unclear how many of these interactions are truly functional and which of them modulate PML function in *in vivo* settings. This remains one of the key questions in the PML field. We will not list all the reported functional interactions of PML, as this would go beyond the scope of this review. Instead, we will mainly highlight the most recent interactions that have been proposed to contribute to PML growth-suppressive function.

The available literature suggests that PML is functioning as part of a complex tumour-suppressive network. It is well established that PML regulates other tumour suppressors,

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**Abbreviations:** PML, Promyelocytic Leukaemia protein; APL, Acute Promyelocytic Leukaemia; PML/RAR $\alpha$ , PML/Retinoic Acid Receptor alpha; TRIM, Tripartite Motif; RING, Really interesting gene; pRb, Retinoblastoma protein; Mdm2, Murine double minute; PI-3K, Phosphoinositide 3-Kinase; PP2a, Protein Phosphatase 2a; mTOR, Mammalian Target Of Rapamycin; HIF1 $\alpha$ , Hypoxia-inducible factor 1 alpha; PTEN, Phosphatase and Tensin homolog; HSC, Haematopoietic Stem Cells; Gfi-1, Growth factor independent 1; HOXB2, Homeobox B2; GATA-2, GATA binding protein 2; SCA-1, Stem Cell Antigen 1; Lin<sup>–</sup>, Lineage negative; CMR, Complete Molecular Response; CcyR, Complete Cytogenetic Response; ABL, Abelson; BMT, Bone Marrow Transplantation; CML, Chronic Myelogenous Leukaemia; LIC, Leukaemia Initiating Cell; Ara-C, Cytosine Arabinoside; IFN $\alpha$ , Interferon alpha; AML1, Acute Myeloid Leukaemia 1; NPC, Neural Progenitor Cell; VZ, Ventricular Zone; SVZ, Subventricular Zone; DG, Dentate Gyrus; RGC, Radial Glial Cell; BP, Basal Progenitor; Tbr2, T-Box Brain 2; PP1, Protein Phosphatase 1; TEB, Terminal End Bud; ISRE, IFN-stimulated response element; GAS, IFN $\gamma$ -activated site; Stat, Signal transducer and activator of transcription; OSM, Oncostatin M; IL, Interleukin; RNAi, RNA interference; SATB1, SATB Homeobox 1

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such as p53 and pRb.<sup>1,2,12,13</sup> Although p53 can be regulated through direct localization to the PML-NB and changes in its posttranslational modifications, it can also be indirectly stabilized following PML-dependent Mdm2 sequestration to nucleoli on DNA damage.<sup>14</sup> In contrast, PML-mediated regulation of pRb is believed to occur only through direct targeting to the PML-NB.<sup>12</sup> New findings indicate that PML can act as a suppressor of major oncogenic pathways such as the PI3K/Akt pathway. In particular, the laboratory led by Pier Paolo Pandolfi has shown that PML, through its ability to interact with the protein phosphatase PP2a inhibits the nuclear function of Akt, thus leading to suppression of its prosurvival and promotogenic functions.<sup>11</sup> Reduction of *PML* gene dosage results in transition to invasive carcinoma in *PTEN*<sup>+/-</sup> animals, which is accompanied by increased Akt phosphorylation;<sup>11</sup> this suggests a genetic interaction between the two pathways. Nonetheless, it is not clear whether PP2a is delocalized also in *PML*<sup>-</sup> tumours. The same group has demonstrated that another component of the PI3K/Akt pathway, the mTOR kinase can associate with PML-NBs.<sup>15</sup> This, in turn, results in inhibition of mTOR-dependent HIF1 $\alpha$  translation and suppression of tumour angiogenesis *in vivo*.<sup>15</sup> However, it is still unclear to which extent mTOR sequestration acts as regulatory mechanism *in vivo*. A further contribution from the Pandolfi's group provided evidence that PML regulates the function of the phosphatase PTEN, which is a tumour suppressor itself and the main inhibitor of the PI3K pathway. This occurs mainly through inhibition of PTEN deubiquitylation by HAUSP and its nuclear retention.<sup>16,17</sup> In this respect, nuclear exclusion of PTEN has been associated with increased aggressiveness of colon cancer and is predominant in a small number of Cowden disease cases due to a mutation affecting PTEN ubiquitylation. It would be, therefore, interesting to determine whether PML loss in colon and prostate carcinomas<sup>11,16</sup> correlates with PTEN nuclear exclusion. In summary, it appears that PML is able to affect the PI3K pathway at multiple levels. However, it is quite difficult to comprehend how PML could act on the PI3K pathway at so many different levels. One of the potential explanations would be that PML action is dependent on the cell or tumour type. This would imply that cell/tumour-specific modifications of PML or of its targets within the PI3K pathway exist to allow for selective interactions. More, in general, this remains one of the key questions regarding the multifaceted function of PML.

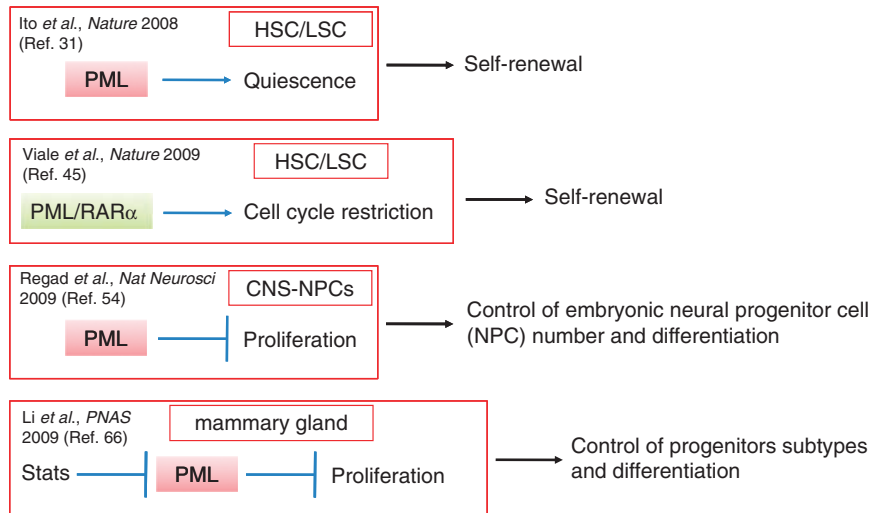
## The Future

### The haematopoietic system

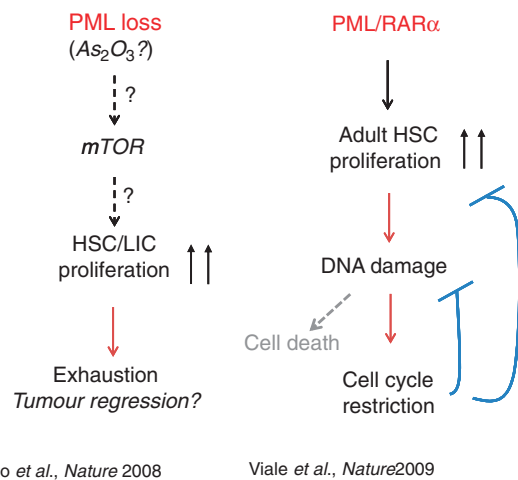
*PML* role in normal and malignant haematopoietic stem cells. Adult haematopoietic stem cells (HSCs) are characterized by a relative quiescence, but can enter cell cycle to either self-renew or differentiate into multiple lineages. Each day the HSC population must give rise to different types of specialized progenitors, which are organized in a hierarchical fashion and can replenish the blood system.<sup>18</sup> The HSC/progenitor pool must also respond efficiently to different forms of stress, such as infection, exposure to cytotoxic agents or blood loss.<sup>19</sup> Efficient self-

renewal is required to avoid depletion of the HSC compartment, and quiescence is key for the maintenance of the undifferentiated, multipotent stem cell pool.<sup>20,21</sup> How HSC quiescence is controlled is one of the main questions in the field of stem cell research. A number of seminal studies suggest that it is likely controlled by both intrinsic and microenvironmental factors in the bone marrow. Several transcription factors have been demonstrated to control HSC fate decisions. For instance, Gfi-1, HOXB2 and GATA-2 control HSC self-renewal and functional integrity.<sup>22-25</sup> In contrast, few factors are known to directly regulate quiescence in HSCs. MEF/ELF4 regulates both self-renewal and quiescence,<sup>26</sup> whereas the role of p21 in regulating quiescence at steady state is probably minimal.<sup>27,28</sup>

PML loss was shown to affect myeloid development, but the mechanisms underlying this defect had remained unexplored.<sup>29</sup> In particular, the physiological role of PML in the development and homeostasis of the haematopoietic tissue was still unclear. A recent study from the Pandolfi's group has implicated PML in the regulation of stem cell quiescence in the haematopoietic system (Figures 1 and 2). This work shows that PML expression is enriched in the primitive mouse haematopoietic progenitors (c-Kit + Sca-1 + Lin-KSL), whereas it is reduced in more committed progenitors.<sup>30</sup> Although genetic loss of PML does not affect the number of haematopoietic cells in the peripheral blood, more KSL cells are found in *PML*<sup>-/-</sup> mice. In particular, the number of long-term repopulating HSCs measured as CD34<sup>-</sup> and Thy1<sup>low</sup> KSL cells is higher in *PML*<sup>-/-</sup> mice. Interestingly, in *PML*-deficient animals the number of KSL and CD34<sup>-</sup> KSL cells in G<sub>0</sub> is markedly lower, thus suggesting that the proportion of quiescent cells is reduced in the absence of PML. Consistent with these data, long-term culture of *PML*<sup>-/-</sup> KSL cells results in a reduction in colony-forming cells, thus suggesting that increased cycling associated with PML loss causes HSCs exhaustion. In turn, the repopulating ability of *PML*<sup>-/-</sup> HSCs *in vivo* is also reduced, despite a transient increase in reconstitution. Interestingly, this defect not only affects myeloid cells but also B and T lineages. Age-dependent decrease in bone marrow cellularity is more marked in *PML*<sup>-/-</sup> animals, and *PML*<sup>-/-</sup> HSCs from 18-month-old mice fail to reconstitute recipient animals. Thus, PML contributes to the maintenance of HSC function likely through its ability to regulate their quiescence. Notably, PML is expressed in CD34<sup>+</sup> blasts from chronic myelogenous leukaemia (CML) patients.<sup>30</sup> Paradoxically, CML patients with low PML expression displayed higher complete molecular response (CMR) and complete cytogenetic response (CCyR) compared with those with high PML expression. Moreover, low PML levels correlate with better overall survival in CML, thus suggesting that low PML expression predicts a better clinical outcome. This is quite surprising and is the opposite of what was observed for prostate and other solid tumours, where loss of PML correlates with poor outcome and promotes tumour progression in animal models.<sup>9-11</sup> It remains to be established whether PML is expressed in cancer-initiating cells also in these tumours and if its presence correlates with increased aggressiveness/recurrence. As leukaemia-initiating cells (LICs) are believed to carry similarities with normal



**Figure 1** A new role for PML in the control of stem cell function. A number of recent studies have highlighted a role of PML in stem cells from different tissues, such as the haematopoietic system (reference<sup>31</sup>), the nervous system (reference<sup>54</sup>) and the mammary gland (reference<sup>72</sup>). Paradoxically, the oncogenic fusion protein PML/RAR $\alpha$  triggers cell-cycle restriction in haematopoietic stem cells (HSCs; reference<sup>45</sup>). This pathway is required to permit expression of the APL oncogene and promote self-renewal



**Figure 2** Both PML and PML/RAR $\alpha$  regulate the function of haematopoietic stem cells (HSCs) and leukaemia initiating cells (LICs). PML loss promotes mTOR activation and subsequent exhaustion of the HSC pool (reference<sup>31</sup>). Similarly, PML-deficient LICs are impaired in transplantation potential, thus suggesting that Pml is hijacked by the leukaemic stem cells to promote cell-cycle exit and maintenance of stemness. The APL oncogene PML/RAR $\alpha$  when expressed in HSCs triggers proliferation that results in replication stress (reference<sup>45</sup>). The induction of p21 promotes cell-cycle restriction to allow for DNA repair in the transforming HSCs and is essential for transplantation potential of the LICs

HSCs,<sup>31–33</sup> the authors of this work went on to investigate the function of PML in LICs. Indeed, p210<sup>BRC/ABL</sup>-transduced PML<sup>-/-</sup> bone marrow cells do proliferate more *in vitro* and when transduced in recipient animals (bone marrow transplantation 1, BMT1) promote earlier CML-like disease, which is associated with decreased quiescence.<sup>30</sup> By BMT3, PML<sup>-/-</sup> LICs fail to produce CML-like disease and to display minimal residual disease. In contrast, control LICs generate leukaemia also at BMT4. These results indicate that PML loss results in impairment of LICs maintenance.

Arsenic trioxide, which is known to reduce PML expression and is very effective in treating APL,<sup>34–37</sup> markedly attenuates PML<sup>+/+</sup> HSC function *in vitro* and *in vivo*, but has no effect on PML<sup>-/-</sup> HSCs.<sup>30</sup> Ito *et al.* then show that arsenic trioxide is able to promote proliferation in LICs without detectable effects on cell death, and inhibits LIC maintenance in long-term culture-initiating cell assays (Figure 2). Combination of arsenic with cytosine arabinoside (Ara-C), which targets proliferating cells for cell death, results in complete eradication of LICs. *In vivo* experiments also show that in the second round of BMT, whereas mice transplanted with Ara-C-treated LICs succumb around 20 days after BMT, when donor LICs are treated with both Ara-C and arsenic trioxide CML-like disease is not observed up to 40 days after BMT. However, the authors do not show whether the *in vivo* effects of arsenic trioxide are abrogated when using PML-deficient LICs. This is a key point, as it cannot be excluded that some of the arsenic trioxide effects on LICs are not dependent on PML down-regulation. Expression of a non-degradable mutant of PML in LICs would help to address these questions more conclusively. A recent study by Trumpp and co-workers has shown that interferon- $\alpha$  (IFN $\alpha$ ) promotes activation and proliferation of dormant HSCs and sensitizes them to chemotherapeutic agents.<sup>38</sup> As PML is a target of IFNs, it would be very interesting to determine whether PML plays any part in mediating IFN $\alpha$ -dependent effects and whether there would be any potential disagreement with data by Ito *et al.*

Notably, arsenic trioxide is active against CML cells and potentiates the effects of Imatinib, which is the frontline therapeutic agent for the treatment of CML.<sup>39,40</sup> More recently, combined treatment with arsenic sulphide and Imatinib has been shown to be extremely effective in a mouse model of CML and to promote cell-cycle arrest and cell death *in vitro*.<sup>41</sup>

Finally, increased activity of the mammalian target of rapamycin (mTOR) is found in PML<sup>-/-</sup> HSCs and rapamycin restores PML<sup>-/-</sup> HSCs function *in vitro* and *in vivo*, but it has

no effect on control HSCs<sup>30</sup> (Figure 2). This is an agreement with a previous work from the same group showing that PML inhibits mTOR function.<sup>15</sup> Rapamycin has similar effects on LICs both *in vitro* and *in vivo*.<sup>30</sup> In view of the well-known feedback role of rapamycin to promote Akt activation,<sup>42</sup> it remains to be established whether the observed biological effects are dependent on mTOR inhibition or whether they can be ascribed to Akt.

In summary, this work represents a change in paradigm in the field of cancer research that highlights the potential double-edged function of a number of growth/tumour suppressors. One of the obvious questions is whether this also applies to other tumour suppressors such as p53 and retinoblastoma protein (pRb), which have been shown to functionally interact with PML.<sup>3</sup> For instance, a recent work from the Nimer's group has shown that p53 regulates HSC quiescence.<sup>43</sup> As p53 is lost or inactivated in most human cancers, it remains to be established whether other p53 family members could play a role in maintenance of cancer stem cells in p53<sup>-</sup> tumours.

*PML/RAR $\alpha$  promotes cell-cycle restriction.* The work by Ito *et al.* has highlighted the importance of quiescence for the maintenance of LICs and tumour development. A recent work from the Pelicci's group in Milan has investigated the role of growth suppression in PML/RAR $\alpha$ -mediated transformation (Figures 1 and 2). The growth suppressor and cycle inhibitor p21 is required for maintenance/self-renewal of PML/RAR $\alpha$  HSCs *in vitro* and BMT experiments.<sup>44</sup> The authors went on to demonstrate that expression of PML/RAR $\alpha$  (and also AML1/ETO) induces DNA damage in HSCs, and this is much more prominent in the absence of p21. Accordingly, PML/RAR $\alpha$  induces p21 expression in HSCs. Based on the model proposed in this study, p21 activates cell-cycle restriction in the presence of DNA damage caused by oncogenic activation (PML/RAR $\alpha$  and AML1/ETO) in HSCs (Figure 2). Thus, p21-mediated cell-cycle arrest may be required to allow for DNA repair caused by replication stress in HSCs. In contrast, in mouse embryo fibroblasts PML/RAR $\alpha$  expression triggers p21-dependent, p53-independent cellular senescence, which is also preceded by DNA damage foci formation and appears irreversible. Therefore, HSCs are able to activate a survival/repair pathway following oncogenic activation, which is different from the classical oncogene-induced senescence observed in primary fibroblasts.<sup>45-47</sup> Furthermore, these findings suggest that HSCs may be more prone to transformation than more committed or fully differentiated cell types. Finally, p21 regulates cell-cycle restriction also in PML/RAR $\alpha$ -expressing LICs, limits DNA damage and prevents their exhaustion. In contrast, p21 is required for AML1/ETO-mediated leukaemogenesis, suggesting that it may play a different role depending on the oncogene involved. This work supports a model, by which (1) oncogene activation can have different endpoints depending on the cell type and the stemness, (2) oncogene-induced DNA damage can be repaired and (3) growth suppressors can be involved in the process of transformation and cancer stem cell maintenance. It remains to be established what triggers DNA damage and which is the repair machinery<sup>23</sup> involved and whether it could

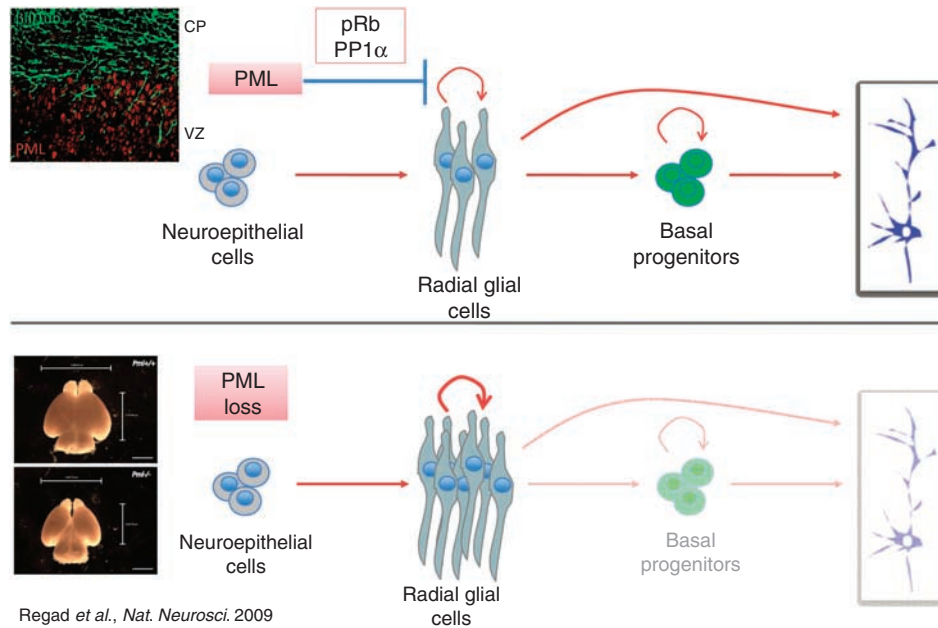
be targeted pharmacologically. One could also hypothesize that not all damage need to be repaired: excessive damage may be corrected but small lesions may be needed to confer selective advantage to stem cells undergoing transformation or to already established cancer stem cells. The relative quiescence state of stem cells and cancer stem cells would confer an advantage in promoting partial tolerance to small DNA lesions.

Together with the work by Ito *et al.*, this study constitutes a change in paradigm as it proposes a new role for growth/tumour suppressors in regulating cancer stem cell maintenance.<sup>30,44</sup> This would imply an unexpected cooperation between oncogenes and tumour suppressors for cancer stem cell maintenance and probably limitation of oncogene-induced DNA damage.

### Beyond the haematopoietic system

*The nervous system.* Correct regulation of cell-cycle progression has been shown to be essential during development of the nervous system (reviewed in references<sup>48-50</sup>), and cell-cycle alterations can contribute to brain tumorigenesis in children.<sup>51</sup> In particular, the neural epithelium lying in the apical area of the developing neocortex generates progenitors, postmitotic neurons and macroglia in a temporally and spatially controlled fashion (reviewed in references<sup>49,50</sup>). How the balance between proliferation and differentiation is maintained during early development and which are the key players involved remains a central question in the field of neuroscience.<sup>49</sup> The role of PML in brain development was completely unexplored. To determine PML function in this context, we took advantage of the *PML* knockout mouse<sup>29</sup> model and focused on the developing brain and in particular the neocortex (Figures 1 and 3). The mammalian neocortex is a complex, highly organized, six-layered structure that contains a large variety of neuronal types and a diverse range of glia.<sup>52</sup> Two major types of neurons are present in the cortex: (1) interneurons, which are generated in the ventral telencephalon and cortical haem; (2) projection neurons, which are generated from progenitors of the neocortical germinal zone located in the dorsolateral wall of the telencephalon, called ventricular zone (VZ).<sup>49,50,52</sup> During the early stages of neocortical development, there is a dramatic expansion of neuroepithelial progenitor/stem cells (hereafter referred as to NPCs) present in the VZ, which then form an additional layer named subventricular zone (SVZ).<sup>49,50,52</sup> NPCs in the VZ and SVZ produce the projection neurons of the different neocortical layers in a tightly controlled temporal order. Newly generated neurons distribute in an inside-out fashion in the developing cortex.

We have found that the expression of PML is restricted to NPCs in the developing neocortex (Figure 3).<sup>53</sup> Few other growth suppressors show such restricted expression pattern. For instance, among cyclin-dependent kinase (Cdk) inhibitors, Ink4c is the only family member whose expression is confined to the VZ.<sup>54</sup> Remarkably, in *PML*<sup>-/-</sup> cortices the overall number of proliferating NPCs is increased (Figure 3). One could hypothesize that the effect of PML loss on neurogenesis is caused by changes in the length of the G<sub>1</sub> phase and, therefore, PML would control cell fate by acting on



Regad *et al.*, *Nat. Neurosci.* 2009

**Figure 3** PML regulates neural progenitor cell (NPC) function in the developing brain. PML expression is confined to the germinal zone of the developing neocortex (inset: Pml is in red, the neuronal marker  $\beta$  III tubulin in green). PML inhibits proliferation at the level of radial glial cells, in part, through its ability to affect PP1 $\alpha$ -dependent phosphorylation of pRb. As a consequence, in PML-deficient cortices the number of radial glial cells is increased, whereas the transition to basal progenitors is impaired. This results in decreased neurogenesis and in smaller brains (inset)

a specific phase of cell cycle. This hypothesis is currently being tested. In this respect, recent studies suggest that, contrary to the conventional view, changes in the length of the G<sub>1</sub> phase of the cell cycle may be a cause, rather than a consequence, of differentiation of NPCs.<sup>55</sup> Three main types of NPCs exist in the neocortex: neuroepithelial stem cells, radial glial cells (RGCs) and basally located intermediated progenitors (BPs), which derive from RGCs via asymmetric division.<sup>52</sup> Interestingly, the transition from RGCs to BPs is impaired in *PML*<sup>-/-</sup> cortices (Figure 2). This, in turn, results in reduced differentiation *in vivo* and *in vitro*, and an overall decrease in brain size and thickness of the cortex wall, although the overall organization of the neocortex is not substantially altered (Figure 3). Interestingly, mice lacking the basal progenitors markers *Tbr2* or *Insm1* display a reduced number of basal progenitors, smaller brains and a thinner neocortex due to impaired neuronal maturation,<sup>56–58</sup> thus suggesting that the phenotype observed in *PML*<sup>-/-</sup> mice may be due to skewing of progenitor subtypes. BPs are not present in lower organisms, and this led to an evolutionary theory by which BPs are responsible for the increased cortical thickness and surface characteristic of the mammalian brain. Unravelling the mechanisms of NPC diversity in the brain has substantial clinical importance, as a number of developmental disorders have been linked to abnormal development of cortical neurons, such as autism spectrum disorder, schizophrenia, fragile X syndrome, Down syndrome and others.<sup>59,60</sup> An increased understanding of the mechanisms underlying NPC function and of how diversity is generated during brain development could also pave the way to cell replacement strategies in both disease and injury states. This will be discussed in the 'Outstanding questions and future directions' section.

In NPCs, PML is found in a complex with the tumour suppressor pRb and protein phosphatase 1- $\alpha$  (PP1 $\alpha$ ), and triggers pRb dephosphorylation. Notably, in *PML*-deficient cortices, pRb and PP1 $\alpha$  fail to colocalize and accumulate in both the nucleoplasm and cytoplasm. These data support a model by which PML modulates cell fate during neocortex development by regulating cell-cycle progression in concert with pRb. It would be interesting to assess whether one of the consequences of defective pRb/PML interaction is the shortening of the G<sub>1</sub> phase of cell cycle. Several other questions arise from this study. For example, does PML loss affect the phenotype of *pRb*<sup>+/-</sup> mice and in particular the development of pituitary tumours?<sup>61</sup> This would be an indication of a genetic interaction *in vivo*. In addition, the potential involvement of other Rb family members cannot be excluded. For instance, p107 has been reported to regulate proliferation in the VZ and overlaps PML expression pattern.<sup>62,63</sup> Interestingly, p107 loss results in increased Hes1 expression.<sup>62,63</sup> Hes1 is a known Notch target, essential for the Notch-dependent maintenance of NPCs in the developing brain.<sup>64</sup> The levels of Hes1 oscillate in the developing neocortex to allow for expression of Neurogenin2 and Notch ligand Delta-like 1 that have prodifferentiation activities.<sup>64</sup> In sum, these findings suggest the existence of a crosstalk between the p107 and Notch pathways. It would be worth testing whether Pml expression also undergoes Notch-dependent oscillations in the developing neocortex and if, vice versa, Pml loss affects the Notch signalling.

Overall, our work reveals an unexpected role of PML in controlling the function of NPCs, and, together with the reported loss of PML expression in human tumours of the nervous system,<sup>9</sup> suggests that alterations of PML function

may be involved in the onset of neoplastic disorders originating from the neuroepithelial compartment of the nervous system. Another obvious question is whether PML regulates adult stem cell function and brain function in general. This is currently being addressed in our laboratory.

**The mammary gland.** The mammary gland epithelium is inserted in a stroma rich in adipocytes (called fat pad) and undergoes marked proliferation and differentiation following exposure to steroid hormones. There are three main stages of mammary gland development: embryonic, pubertal and gestational.<sup>65</sup> Distinct epithelial buds can be seen in mouse embryos by E13.5 and then sink into the underlying dermis. In the female, these buds trigger the formation of the mammary mesenchyme and generate a rudimentary structure with few ductules by E18.5. Postnatally, the mammary tree grows in length, and the terminal end buds (TEBs) appear at the tips of the ducts and start to invade the fat pad. Growth ceases when levels of oestrogen start rising at puberty. Proliferation within the TEBs results in ductal elongation and formation of branches. By 10–12 weeks of age, TEBs have disappeared and growth stops. If pregnancy ensues, tertiary branches are formed to generate alveolar buds, and the luminal epithelium proliferates and commit to the secretory alveolar lineage. These cells synthesize and secrete milk. This stage is regulated by two hormones, progesterone and prolactin. Whereas the former triggers side branching and alveologenesi s, the latter promotes differentiation of the alveoli. Finally, once lactation ceases, removal of the excess of alveolar cells is achieved through induction of programmed cell death, and the gland undergoes a profound remodelling process. Approximately 80% of the tissue is eliminated in just 6 days.

The signal transducer and activator of transcription (Stat) family of proteins consist of key transcription factors involved in the response to cytokine signalling and in regulating many different growth, survival and differentiation pathways.<sup>65</sup> Stats play a major role in the development of the mammary gland. For instance, Stat6 loss causes a delay in alveolar development during pregnancy.<sup>66</sup> In contrast, Stat5 promotes functional lobuloalveolar development during late pregnancy and milk production afterwards.<sup>67</sup> Finally, leukaemia inhibitory factor-induced Stat3 is involved in the involution and remodelling processes.<sup>68</sup> PML is a well-known interferon (IFN)-regulated gene through an IFN-stimulated response (ISRE) element and IFN- $\gamma$ -activated site (GAS) in its promoter.<sup>1,69</sup> To add a further level of complexity, PML inhibits IFN- $\gamma$ -induced Stat1 activity.<sup>70</sup>

A study published by the Christine Watson's group this year has shown that PML regulates mammary gland development through the skewing of mammary gland progenitor cells<sup>71</sup> (Figure 4). First, the authors demonstrate that the expression of PML is highly regulated during the mammary gland development.<sup>71</sup> The virgin gland contains high levels of PML protein by both immunoblotting and immunohistochemistry. During gestation and lactation, the amount of PML present in the epithelium declines to almost undetectable levels, whereas it remains high in the surrounding cell types. In contrast, during the early stages of involution, the levels of PML increase again to reach pregestation levels. Suppression

of PML expression was recapitulated in a conditionally immortal murine mammary epithelial cell line, KIM-2. Among the many known PML isoforms,<sup>3</sup> PML isoforms 1 and 2, which represent the main isoforms expressed in the mouse,<sup>72</sup> are the only PML forms found in the mammary gland and appear to be differentially regulated, with isoform 1 being expressed more abundantly in the virgin gland.

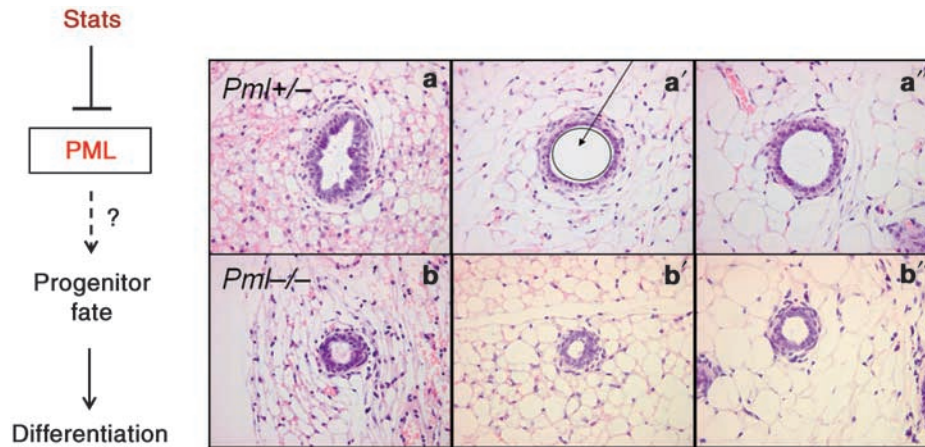
The presence of GAS and ISRE sequences in the PML promoter suggest that PML may be regulated by Stat proteins during mammary gland development. Indeed, Li *et al.* show that Stat proteins are able to affect PML expression in this context. First, treatment with the Stat3 inducer oncostatin M (OSM) in a mammary epithelial cell line downregulates PML expression. Then, prolactin triggers Stat5 activation and subsequent suppression of PML expression. Finally, IL-13, which activates Stat6, downregulates PML. Remarkably, PML expression is markedly upregulated in mammary glands from Stat3 and Stat6 knockout animals, thus confirming the *in vitro* data in *in vivo* settings. What remains to be established is whether Stats directly bind the PML promoter in the developing mammary gland. Chromatin immunoprecipitation studies using whole mammary gland tissue would be required to address this point conclusively.

Next, Li *et al.* analyse the effect of PML deficiency on mammary gland development. Glands from PML<sup>-/-</sup> mice at gestation day 15 contain reduced levels of secreted  $\beta$ -casein and phosphorylated forms of Stat5 and Stat6, suggesting an interplay between PML and Stats during mammary gland development. However, it remains to be determined whether the decreased phosphorylation of Stat5/6 is directly caused by PML loss or if it reflects a reduction in the number of cells positive for phosphorylated Stats. This question could be, in part, addressed by using RNAi of PML in a mammary epithelial cell line. Alternatively, a thorough analysis of Stat phosphorylation in tissues using immunohistochemistry would be required.

Does PML affect gland development? Glands from 6-week-old virgin PML<sup>-/-</sup> mice display significant smaller ducts with a reduced number of branches per duct. The lumen size in ducts from PML<sup>-/-</sup> glands is reduced by an average of 65%, whereas the number of branch points per duct is reduced by 40%. Although the ducts are globally smaller, the cellular organization is not affected. Together, these data uncover a surprising role for PML in the regulation of ductal morphogenesis *in vivo*.

Finally, the authors attempt to determine whether PML loss affects the differentiation of cell subpopulations within the developing gland. Recently, discrete populations of progenitor cells have been identified in the mammary gland, which are capable of growing *in vitro*, repopulating clear fat pads, and differentiate into specific subsets of functionally diverse epithelial cells.<sup>73–75</sup> PML loss affects the generation of Scal<sup>-</sup> luminal progenitors, whereas it leads to an increase in the number of Scal<sup>+</sup> progenitors. Overall, unsorted total epithelial cells from PML<sup>-/-</sup> mice display an increased cloning efficiency, which probably reflects the increase in cloning efficiency by Scal<sup>+</sup> progenitors. Interestingly, PML loss results in decreased expression of the Stat6 target gene GATA-3 in Scal<sup>-</sup> progenitors.

Overall, these results show that PML is a regulator of mammary gland development potentially through interplay



**Figure 4** PML depletion disrupts normal mammary gland development. PML expression is controlled by signal transducer and activator of transcription proteins (Stats). Loss of PML results in skewing of progenitor subtypes and impaired development of the mammary gland. *PML*<sup>-/-</sup> glands from virgin females display smaller ducts compared to control glands (a, a', a'')

with Stat proteins and its ability to affect the generation/maintenance of selected progenitor subtypes. It would be very interesting to determine the mechanisms underlying PML function in epithelial progenitors and in particular whether PML affects cell-cycle progression and cell fate decisions in this context. Finally, it would be key to test whether PML growth-suppressive function plays a role in the development of breast cancer. In this respect, PML expression is lost in primary breast cancer tissue samples and correlates with progression to lymph node metastasis,<sup>9</sup> thus suggesting that its inactivation may cooperate with other oncogenic insults to promote transformation and tumour development in the mammary gland. Furthermore, PML functionally interacts with the chromatin modulator SATB1, which has been shown to play a role in breast cancer pathogenesis.<sup>76,77</sup>

**Outstanding questions and future directions.** The studies highlighted in this review propose a novel role of PML in controlling stem cell function in different tissues and pathological conditions. These findings have the potential to cause a change in direction within the field of PML research and also contribute to the understanding of the mechanisms underlying growth control in progenitor/stem cells.

Several questions remain to be addressed. For instance, it would be crucial to determine Pml involvement in other stem cell niches such as the crypts of the gut. In this respect, recent work from the Hans Clevers group has shown that the protein Lgr5 is a marker for the intestinal stem cell and Lgr5<sup>+</sup> cells very likely represent the cell of origin of intestinal cancer.<sup>78,79</sup> Are Lgr5<sup>+</sup> stem cells also positive for Pml? Does Pml have an impact on the stem cell function in the intestine?

What also remains to be defined is whether the function of PML during tissue development is different from its role in the maintenance of tissue homeostasis in the adult animal. For instance, although PML expression is associated with highly proliferative germinal zones in the developing brain, it is high in quiescent haematopoietic stem cells in the bone marrow. Is PML function different in cycling cells compared to quiescent cells? This leads to another relevant question: is PML growth-suppressive function associated to a particular phase of cell

cycle? Previous studies have proposed that PML promotes accumulation in the G<sub>1</sub> phase of cell cycle,<sup>29,80–82</sup> which are in line with the observed connection with the pRb protein. So it is conceivable that PML may be part of the cellular machinery required for keeping cells in G<sub>1</sub> (quiescent haematopoietic stem cells) or delaying the entrance into S phase (proliferating neural progenitor cells). More questions to come: is the PML/pRb connection relevant in both contexts, that is, embryonic and adult stem cells? Indeed, a number of studies have proposed a role for pRb in erythroid differentiation and a potential connection with PML.<sup>83,84</sup> Therefore, it is conceivable that the PML/pRb functional connection is not restricted to the brain.

Another outstanding question is why PML-deficient animals do not display a more severe phenotype. For instance, the marked increase in NPC proliferation and substantial decrease in differentiation observed in developing *PML*<sup>-/-</sup> brains is somewhat rescued postnatally. This suggests a degree of compensation occurring in the *PML*<sup>-/-</sup> animals, probably due to the presence of PML-like genes or orthologs. A very recent study by the Knoblich group in Vienna has implicated the TRIM gene TRIM32 in the regulation of neocortex development.<sup>85</sup> TRIM32 is expressed in NPCs as well as in neurons. The authors propose that TRIM32 has a dual function: (1) its N-terminal RING finger promotes the ubiquitin-dependent degradation of c-Myc, whereas (2) its C-terminal NHL domain regulates the function of a number of microRNAs through the interaction with Argonaute. TRIM32 loss results in increased proliferation and impaired differentiation in the developing neocortex, suggesting a degree of functional overlap with PML. Finally, TRIM32 appears to be asymmetrically distributed in mitoses of RGCs, thus suggesting that it could constitute a determinant of differentiation in postmitotic progenitors. Overall, this work, together with our previous study, clearly implicates two members of the TRIM family, PML and TRIM32 in neocortex development, and suggest that there might exist a degree of redundancy among TRIM genes. Whether PML RING domain could also target c-Myc for UPS-dependent degradation remains to be established. Finally, it would be key to determine which TRIMs

are expressed in the bone marrow and mammary gland and if they can play redundant roles also in these tissues.

These discoveries could also have potential therapeutic implications. An incredible research effort is being spent to increase our understanding of stem cell biology and to achieve the necessary knowledge for the use of stem cells in regenerative medicine. Transplantation of stem cells and activation of endogenous stem cells within the brain, have been proposed as future therapies for neurodegenerative diseases, such as Parkinson's disease, stroke, amyotrophic lateral sclerosis (ALS) and Huntington's disease. Work performed in animal models suggests that the integration of functional, stem cell-derived neurons in an existing circuitry is achievable and can to some degree correct an existing damage.<sup>86</sup> Studies in patients with Parkinson's disease (PD) have shown that transplantation of human fetal mesencephalic tissue, rich in postmitotic dopaminergic neurons results in neuronal replacement and clinical benefits.<sup>20,87–91</sup> However, subsequent studies have challenged these results, thus suggesting that cell replacement procedures are far from optimal.<sup>92,93</sup> The use of embryonic stem cells could be an alternative for the generation of dopaminergic neurons for transplantation, as suggested by a number of studies.<sup>86</sup> For this type of approach, maintenance of stemness and genomic stability during the propagation of stem cells are key factors for successful generation of desired neuronal types and efficient grafting. Therefore, it becomes essential to fully understand the molecular mechanisms that regulate proliferation of stem cells and the induction of genomic instability. In this respect, as PML has been shown to be part of cellular checkpoints involved in cell-cycle restriction in stem cells, modulation of its expression/localization could be used to optimize culture conditions and potentially achieve more efficient expansion. One could envision that suppression of PML expression could be desirable in the expansion phase, whereas its increased activity could vice versa contribute to more effective neurogenesis. Screening of small molecule libraries could lead to the identification of agents disrupting PML localization to PML-NBs, or alternatively RNAi approaches could be employed to suppress PML expression.

The studies discussed in this review also propose a provocative view that growth/tumour suppressors may be instrumental in maintaining the cancer stem cell niche, thus proposing an unsuspected 'Janus'-type role. On one hand, p21 seems to be required to permit PML/RAR $\alpha$ -mediated transformation of HSC, through its ability to restrict cell cycle and allow for DNA repair.<sup>44</sup> On the other hand, PML is required for maintenance of CML stem cells, and its expression in CML patients inversely correlates with survival.<sup>30</sup> Whether PML growth-suppressive potential is required to limit genomic instability and permitting DNA repair in CML stem cells remains to be determined. In addition, it is not clear whether p21 and PML pathways are coupled. In this respect, it would be interesting to test whether PML loss could influence p21 expression/activity and vice versa. A previous report has shown that p21 expression is diminished in PML-deficient cells.<sup>29</sup> So, it would be important to test whether PML contributes to PML/RAR $\alpha$ -triggered cell-cycle restriction. Finally, it is known that p53 function is impaired in PML $^{-/-}$  cells<sup>94,95</sup> and that p53 regulates stem cell self-renewal.<sup>43</sup>

However, p21 induction in PML/RAR $\alpha$  expressing HSC is p53-independent, thus suggesting that multiple mechanisms are in place to promote p21-dependent cell-cycle arrest.<sup>44</sup> This a fascinating new area of research that will probably lead to a more complete understanding of how cell-cycle control affects cancer pathogenesis. What also remains to be established is whether similar cell-cycle restriction checkpoints could be activated in normal stem cells for limiting genomic instability during development and in adult tissues. For instance, it would be very interesting to test whether PML loss in the developing mammary gland results in diminished p21 levels and induction of genomic instability.

Overall, these recent contributions have highlighted a novel role of the tumour suppressor PML in the control of cell fate at the level of stem cells. The implications of these findings are several and could lead to a new understanding of both physiological and pathological processes. Finally, they contribute to the idea of a multifaceted function of tumour-suppressive pathways in the control of cell fate decisions.

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