1   **Structural basis of PML/RARA oncoprotein targeting by arsenic unravels**

2   **a cysteine rheostat controlling PML body assembly and function**

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ABSTRACT

PML Nuclear Bodies (NBs) are disrupted in PML-RARA-driven acute promyelocytic leukemia (APL). Arsenic trioxide (ATO) cures 70% APL patients, driving PML-RARA degradation and NB reformation. In non-APL cells, arsenic binding onto PML also amplifies NB formation. Yet, the actual molecular mechanism(s) involved remain(s) elusive. Here, we establish that PML NBs display some features of liquid-liquid phase separation and that ATO induces a gel-like transition. PML B-box-2 structure reveals an alpha helix driving B2 trimerization and positioning a cysteine trio to form an ideal arsenic-binding pocket. Altering either of the latter impedes ATO-driven NB-assembly, PML sumoylation and PML-RARA degradation, mechanistically explaining clinical ATO-resistance. This B2 trimer and the C213 trio create an oxidation-sensitive rheostat that controls PML NB assembly dynamics and downstream signaling in both basal state and during stress response. These findings identify the structural basis for arsenic targeting of PML which could pave the way to novel cancer drugs.

50-word statement of significance

Arsenic curative effects in acute promyelocytic leukemia relies on PML targeting. We report a PML B-box-2 structure that drives trimer assembly, positioning a cysteine trio to form an arsenic-binding pocket, mutated in resistant patients. Identification of this ROS-sensitive triad controlling PML dynamics and function could yield novel drugs.

INTRODUCTION

ProMyelocytic Leukemia (PML) protein organizes the formation of stress-sensitive membrane-less organelles (MLO): PML Nuclear Bodies (NBs). PML controls various biological functions, such as apoptosis, senescence or stem cell self-renewal (1,2). PML may elicit these functions by scaffolding the spherical shells of NBs, which subsequently act as hubs of post-translational modifications, in particular sumoylation, for the broad range of proteins trafficking through their inner cores. PML NBs are disrupted in Acute Promyelocytic Leukemia (APL) driven by the PML-RARA oncogenic fusion protein. PML protein expression and NB formation are also downregulated in many other cancers (3-5). The combination of arsenic trioxide (ATO) and retinoic acid treatments drives APL cure by enforcing PML-RARA degradation and PML NB re-assembly, subsequently leading to the activation of a senescence-like program in APL cells (3). ATO, which as single therapy cures 70% of APL patients (6,7), directly binds PML-RARA and PML proteins (8,9). While ATO-driven PML-RARA degradation is biochemically well-understood (10), ATO-enforced NB-assembly remains understudied. Formation of PML intermolecular disulfide bonds was reported in response to ATO (8) and arsenic was proposed to replace a Zn^{2+} ion in the RING finger (9). However, ATO effects on NB assembly remain imperfectly understood.

Biogenesis of MLO has been revisited through the prism of liquid-liquid phase separation (LLPS). Biomolecular condensates may arise by multivalent weak interactions between proteins, or proteins and RNAs, which de-mix from the surrounding media to form spherical structures, maintained as an equilibrium despite dynamic exchange of their components (11,12). LLPS has been proposed to increase kinetics of biochemical reactions among its constituents and could contribute to diseases initiation (13,14). In leukemia, chimeric transcription factors may condensate through LLPS, resulting in aberrant 3D

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98   chromatin structures and contributing to their aberrant transcriptional activity (15,16). *In*

99   *vitro,* chimeric GFP-polySUMO proteins condense in droplets through LLPS when combined

100   with chimeric proteins containing repetitions of SUMO-Interacting motif (SIM) (17). Yet, PML

101   sumoylation is dispensable for PML NB assembly (18,19) and the contribution of LLPS in PML

102   NB assembly still remains elusive.

103   In this study, we elucidate the crystal structure of PML B-box 2 (B2) and identify an

104   α-helix that mediates B2 trimerization through hydrophobic interactions, controlling PML NB

105   assembly and PML dynamics. This α-helix-mediated trimer organizes a triad of free C213

106   cysteines to which trivalent arsenic covalently docks, switching PML NBs from liquid-like to

107   gel-like bodies. B2 trimerization and C213 are mandatory for PML-mediated function, such

108   as oxidative stess response i*n vivo*, as well as ATO-triggered PML-RARA-degradation,

109   uncovering the very first step by which ATO achieves its anti-leukemia cure.

110

111   **RESULTS**

112   **PML NBs switch from liquid-like to gel-like structures upon ATO**

113   PML NBs are spherical membrane-less organelles forming core-shell structures (Fig.

114   1A). To determine whether PML assembly in NBs reflects liquid-liquid phase separation

115   (LLPS), we stably expressed GFP-PML-III in *Pml*^{-/-} mouse embryonic fibroblasts (MEFs) and

116   assessed hallmarks of liquid-like MLO: solubility limit, fusion/relaxation and dynamic

117   exchange. First, we examined whether PML could de-mix from the nucleoplasm above a

118   concentration threshold by live cell imaging following retroviral cell transduction. PML was

119   initially homogeneously distributed in the nucleoplasm, but as GFP-PML expression

120   increased, the first PML NB appeared (Fig. 1B) and NBs then became more numerous, while

6

121 the diffuse fraction remained constant (Supplementary Figure S1A). Similarly, the number of

122 PML NBs increases with PML transcriptional activation upon IFN alpha treatment (20,21).

123 Thus, PML de-mixes from a diffuse nucleoplasmic fraction into NBs above a concentration

124 threshold. Live cell imaging revealed that two PML NBs can fuse and relax in a novel single

125 spherical body, supporting liquid-like behavior of PML NBs (Fig. 1C and Supplementary

126 Movie 1). In these experiments, analysis of the relaxation curves, plotting the size of fusing

127 PML NBs as a function of time, allowed extraction of the diffusion coefficient of PML

128 proteins and a NB capillary viscosity of 10^3 Pa.s (Supplementary Figure S1B) (22), comparable

129 to that of nucleoli or nuclear speckles (23,24). We then quantified the number of events of

130 GFP-PML nucleation from the diffuse nucleoplasmic fraction, PML NB fusions or fissions (Fig.

131 1D). Fusion events occurrence were similar to nucleation ones, maintaining a mean number

132 of 40 PML NBs per nucleus, while fission events were rare. We then assessed the dynamics

133 of PML NB assembly by Fluorescence Recovery After Photobleaching (FRAP). As reported,

134 PML protein dynamically exchanges between the nucleoplasm and NBs, with a recovery $t_{1/2}$

135 of 3.2 min (Fig. 1E) (8,25,26). Taken together, these results establish that PML NBs exhibit

136 hallmarks of liquid-liquid phase separated condensates. However, the residence time of PML

137 at NBs is much longer than other MLO nucleators, such as those driving Cajal bodies,

138 speckles or nucleoli (minutes versus seconds), indicating higher affinity between PML

139 proteins at NBs. Moreover, 30% of the GFP-PML fluorescence bleached at NBs never

140 recovers (Fig. 1E), implying that two PML fractions coexist at NBs, one exchanging with the

141 surrounding nucleoplasm, the other remaining immobile. Together with the existence of the

142 core-shell structure, this challenges a pure LLPS of PML proteins as a simple model of NB

143 assembly.

144   ATO exposure rapidly increased PML NB-associated fraction at the expense of the

145   diffuse one and PML NB assembly dynamics dramatically dropped, so that almost all PML

146   became immobilized (Fig. 1E). Live-cell imaging and super-resolution microscopy showed

147   that PML NBs tend to aggregate without achieving complete fusion, forming PML NB strings,

148   consistent with an increase in the viscoelasticity (Figs. 1F-G and Supplementary Movie 2).

149   Thus, ATO triggers the transition from liquid-like to gel-like PML NBs.

150   PML fusion to RARA altered its distribution in the nucleus, as GFP-PML-RARA

151   localized into many tiny dots with diameter below 100 nm in Pml^{KO} MEFs (8,27) (Fig. 1G). The

152   basal dynamics of GFP-PML-RARA at these microdots was slightly increased compared to

153   PML at NBs, with a $t_{1/2}$ of 1.37 min and, importantly, no immobile fraction (Fig. 1H). Yet, here

154   again, ATO treatment decreased drastically PML-RARA exchange rates, closer to the

155   dynamics of PML. Thus ATO-therapy drives the gel-like transition of both PML- or PML-RARA-

156   driven condensates.

157

158   **PML NB assembly requires the B2-box domain rather than di-sulfide bridges**

159   Arsenic atoms may bind sulfhydryl groups, primarily targeting cysteine residues in

160   cells. Within PML, arsenic was proposed either to directly target cysteines of the RING finger,

161   in which arsenic would replace the structuring zinc atom, or the B-box 2 (B2), another zinc

162   finger domain which contains adjacent cysteines: C212 and C213 (8,9). We first assessed the

163   role of free cysteines in NB formation by using N-ethyl-maleimide (NEM), which alkylates

164   cysteine residues with a freely accessible -SH group. Pre-treatment with NEM blocked both

165   basal PML NB-assembly and ATO-enforced PML NB formation (Fig. 2A). Conversely,

166   treatment with ATO precluded subsequent NEM-induced disorganization of PML NBs. Thus,

167   the cysteine(s) required for baseline NB assembly are accessible, not involved in zinc

168 coordination and likely the same as those targeted by ATO. This does not support the RING

169 finger as being the primary ATO target site.

170   ATO exposure induces ROS production due to GSH scavenging and mitochondrial

171 poisoning (28-30). We previously showed that disulfide bonds may link PML monomers,

172 driving their covalent multimerization upon oxidative stress (8). We sought to disentangle

173 the respective contribution of direct arsenic binding or ATO-enhanced disulfide bonds in

174 PML NB assembly. By homology with other TRIM proteins, the C213 residue is likely not

175 involved in zinc coordination (31). We thus focused on both C213 and C389, the latter being

176 an oxidation-sensitive residue implicated in oncoprotein targeting (32,33). PML

177 intermolecular disulfide bonds can be assessed by Western blot analysis in non-reducing

178 conditions, which revealed high molecular weight PML multimers, whose abundance sharply

179 increased upon ATO or H_2O_2 (Fig. 2B, Supplementary Figure S1C) (8), but were lost under

180 reducing conditions (Supplementary Figure S1C,D). C389S mutation impaired ATO-induced

181 disulfides, but only modestly affected those induced by H_2O_2, while C213A decreased PML

182 intermolecular bound species induced by both ATO and H_2O_2. The double C213A/C389S

183 mutation completely abrogated the formation of these PML multimers (Fig. 2B).

184   Following PML NB assembly, PML becomes sumoylated (18,21,34). ATO-induced

185 gel-like NBs are associated with a massive increase in PML sumoylation (Fig. 2B).

186 Interestingly, PML^{C213A} mutants were defective for sumoylation, while PML^{C389S} did not affect

187 basal or ATO-induced SUMO conjugation of PML. We then assessed the localization patterns

188 of wildtype or mutant PML upon those stress. Surprisingly, in contrast to ATO, H_2O_2 did not

189 significantly alter the diffuse nuclear fraction of WT PML, nor increase the NB-associated

190 fraction, suggesting that intermolecular disulfides are not responsible for gel-like transition

191 (Fig. 2C). Critically, C213A or C389S mutations had very different effects on NB formation

9

192   (Fig. 2C). While defective for ATO-induced disulfide, PML^{C389S} NBs and diffuse fraction were

193   identical to those of WT PML, including full ATO sensitivity. In contrast, PML^{C213A} remained

194   mainly diffuse, with a single enlarged body, unaffected by ATO. Taken together, these results

195   indicate that C213 is essential for PML NB assembly, but intermolecular disulfide bonds

196   mediated by C213 or C389 are not, and suggest that C213 may directly contribute to arsenic

197   binding.

198

199   **Crystal structure of PML B2 box reveals an α-helix exposing C213**

200   B-box domains are found in TRIM (TRIpartite Motif) proteins and are often involved

201   in protein-protein interaction (31). We identified a highly conserved short sequence

202   encompassing the adjacent cysteines C212/C213 in all PML-like proteins across evolution

203   (Fig. 3A). Interestingly, this stretch of conserved residues exactly corresponds to the hotspot

204   mutations of PML-RARA or PML in therapy-resistant APL patients (35-38).

205   Despite numerous attempts, no PML B2 crystal structure has been reported to date.

206   We fused MBP to PML B2 to produce a soluble fusion protein from bacteria, and obtain a

207   crystal structure at 2.1 Å resolution (Fig. 3B, Supplementary Figure S2A). In this structure, B2

208   domain adopted a typical B-box-type zinc finger fold, where C3H1 residues coordinated two

209   zinc atoms in a cross-braced conformation and with a conserved β1–β2–α1 arrangement

210   (Figs. 3B-C). Analysis of the crystal packing showed that B2 was sandwiched between two

211   MBP molecules. Indeed, B2 β1 formed an anti-parallel β sheet with MBP β8 through main

212   chain hydrogen bonds, while B2 α1 packed against a hydrophobic patch in the neighboring

213   MBPs (Supplementary Figures S2A-C). Structure alignment demonstrated that PML B2 was

214   similar to other TRIM family B2 domains, such as TRIM5 or TRIM28, although PML B2

215   appears to be more flexible with longer loops (Supplementary Figures S2D-G) (39,40). In the

10

216   first zinc finger, zinc coordination involved C212, but importantly, not C213, which was

217   positioned at the edge of the α1-helix pointing out of the overall B2 structure (Figs. 3B and

218   C). Remarkably, this helix corresponds to the evolutionary conserved primary sequence

219   identified above (Fig. 3A, Supplementary Figures S2D and S2F). In the second zinc finger, the

220   zinc was coordinated by C227 instead of the Alphafold-predicted D219 (Fig. 3B and

221   Supplementary Figures S2D-E). Note that C227 is not conserved in TRIM5 or TRIM28 B2 nor

222   are the residues from the conserved α helix (Supplementary Figures S2D-G). The residues

223   equivalent to PML C227-surrounding sequence in TRIM5 and TRIM28 form a β3 strand - that

224   does not coordinate zinc (Supplementary Figures S2D and F-G). Altogether, we have

225   unraveled the PML B2 structure, highlighting an α-helix that positions outwards the free

226   C213 that is key to NB assembly.

227

228   **PML B2 α1-helix and C213 drive NB assembly and dynamics**

229   To determine the respective roles of PML B2 zinc fingers and the C213-containing

230   helix in NB assembly, we engineered mutations disrupting zinc coordination (C189S or

231   C212S) or point mutations on the α1-helix (C213S or L218G) and stably expressed these GFP-

232   tagged PML mutants in Pml^{KO} MEFs. All these PML mutants exhibited a sharply increased

233   nuclear diffuse fraction with only one to three unusually large bodies, implying that this α1-

234   helix and C213 play key roles in NB formation (Fig. 4A, supplementary Figure S3A).

235   FRAP analysis showed a slight increase in the GFP-PML^{C189S}, GFP-PML^{C212S} or GFP-

236   PML^{C215S} body assembly dynamics, with similar immobile fractions when compared to WT

237   PML (Figs. 4B,C, Supplementary Figure S3B). In contrast, C213S or L218G mutations

238   drastically increased the dynamics of PML NB assembly, with a drop of the half recovery time

239   from 3.2 min to 0.6 or 0.8 min respectively. Moreover, we observed a complete recovery of

240 the photobleached C213S and L218G PML proteins at the remaining body, pointing to a fully

241 liquid compartment. Thus, the α1-helix, including C213, dictates PML dynamics and NB

242 assembly. The crystal structure of MBP-PML B2 with C213A and A216V double mutation

243 (A216V being the hotspot mutation in ATO resistance (35,36)) was very similar to that of WT

244 B2, with an RMSD of 0.26 Å over 42 Cα atoms (Fig. 4D and Supplementary Figure S3C). This

245 supports the idea that the effects of these α1-helix mutations on PML NB assembly dynamics

246 were not due to B2 folding alterations, but rather to loss of its biochemical interactions.

247 Overall, while the zinc fingers contribute to the overall domain structure, the conserved α1-

248 helix is crucial for PML NB assembly and dynamics.

249

Alpha-helix mediated hydrophobic B2 trimerization is key for NB dynamics

251 TRIM5α B2 was proposed to trimerize through a specific α-helix around a

252 tryptophan core *in vitro* (39). We thus looked whether PML B2 monomers could self-

253 assemble into a trimer through Alphafold2 molecular modeling (Fig. 5A, B). In the model, the

254 trimer is predicted to be driven by α1-helix mediated interactions, involving hydrophobic

255 interfaces and in particular, I202, C213, A216, L217 and L218 (Fig. 5A). More specifically,

256 L217 is predicted to play a very central role through its two methyl groups, each bridging two

257 other PML B2 monomer through their own L217, as well as with one I202 on β1 (Fig. 5B).

258 Interestingly, L218 could also interact with I202 of another PML B2, further securing this B2

259 complex (Fig. 5B). Supporting B2 trimerization *in vitro*, SEC-MALS analysis revealed three

260 forms of purified MBP-B2 with molecular weights corresponding to monomer, dimer and,

261 critically, abundant trimer (Fig. 5C).

262 To interrogate the relevance of this model *in cellulo*, we generated point mutations

263 of these potentially interfacing residues. When analyzed by immunofluorescence, L217G

264 substitution had the most drastic effect, completely blocking PML NB assembly (Figs. 5D,

265 quantification in Supplementary Figure S3A). GFP-PML^{I202A} or GFP-PML^{L218G} localization were

266 diffuse in the majority of cells and formed one or two bodies in the remaining ones. A similar

267 defect in NB assembly was observed with the A216V patient-derived mutation (35-37),

268 consistent with our model predicting a constraint environment due to the short distance

269 between A216 and L217 side chain within the trimer (Figs. 5B, 5D). In contrast, the A216G

270 mutation had only minor effects, increasing the diffuse PML fraction, consistent with its

271 predicted modest impairment of trimerization. When analyzed in FRAP, PML^{I202A}, PML^{A216V},

272 or PML^{L218G}, all exhibited highly dynamic exchange with liquid-like behaviors (Fig. 4B,

273 Supplementary Figure S3B). Conversely, increasing hydrophobicity by substituting leucine to

274 residues with aromatic group at position 218 led to the formation of filaments, on which

275 PML exchange was poorly dynamic (Fig. 5E and Supplementary Figures S3D-E). Thus, the α1-

276 helix-mediated hydrophobic interactions between PML monomers through I202, L217 and

277 L218 regulate PML assembly ranging from large liquid-like bodies, standard PML NBs to gel-

278 like filaments.

279

280 **Arsenic docks on a C213 tri-cysteine pocket of B2 trimers to drive gel-like**

281 **transition**

282   In APL, ATO and other therapeutic arsenic derivatives provide trivalent arsenic

283 (As(III) oxidation state) at physiological pH and *in vivo*. As(III) can form stable complexes with

284 thiol groups through covalent binding to sulfur and may form complex with three

285 glutathiones in solution (30). Critically, the key C213 residues are organized in a triad

286 positioned at the center of the PML B2 helix trimer (Fig. 6A, supplementary Figures S4A,B), in

287 an enzymatic active center-like configuration. This triad is present at the opposite end of the

288   assembled helices compared to the sites of L217 interactions (Fig. 5B, Fig. 6A). The C213

289   residues are separated by 3.2 Å, fitting with an ideal arsenic docking that would lead to an

290   interacting distance of 2.7 Å between arsenic and the sulfur atoms (Fig. 6A, supplementary

291   Figures S4A-B). We thus hypothesized that ATO could trigger gel-like transition of PML NBs

292   by targeting this triad of C213 residues within the B2 trimer. We first tested whether the

293   effect of ATO on PML NBs depends on arsenic valency. We used monomethyl arsenic

294   (MMA), which may only bind 2 cysteines, as well as antimony (Sb) trioxide (STO) that

295   releases trivalent Sb, the closest chemical to As(III). In contrast to ATO, MMA has no effect

296   on the dynamics of PML NB assembly (Fig. 6B) (41). Conversely, STO had very similar effects

297   to ATO, decreasing the PML diffuse fraction, turning PML NBs in gel-like string bodies (Fig.

298   6B, Supplementary Figure S4C) and subsequently increasing PML sumoylation

299   (Supplementary Figure S4D) (42).

300   For B2 mutants of zinc finger 1 formation (C212S, C189S), or mutants impairing

301   helix-driven trimerization, as well as for C213S, PML nuclear distribution was unaffected by

302   ATO treatment (Fig. 6C). ATO could not drive phase transition of C213S or helix trimer

303   mutant-forming bodies, as supported by FRAP analyses (Fig. 6D, Supplementary Figure S4E).

304   In contrast, A216G mutation only marginally affected ATO-response of PML, as expected

305   from our model (Fig. 6C-D). ReAsH - an arsenic derivative that fluoresces when bound to

306   proteins - labelled the GFP-PML shell and triggered gel-like transition of PML NBs, similar to

307   ATO (Fig. 6E) (8). In contrast, the shell of the body formed by PML[C213S] could not bind ReAsH.

308   Rather, ReAsH accumulated within the NB inner core, an unexpected observation that

309   deserves further studies. Similarly, PML[L218G] failed to bind ReAsH, stressing the fact that

310   efficient arsenic binding depends on both C213 and α1-helix trimerization. Finally, the effect

311   of ATO on PML NBs after 30min-exposure was not reversed by washout experiments after

312 removing ATO, suggestive for covalent binding (Supplementary Figure S4F). Altogether our

313 findings imply that As(III) requires prior B2 helix trimerization to directly and irreversibly

314 targets C213 and trigger gel-like transition.

315 While mutations of C213 removing thiol group (C213S or C213A) and increasing

316 steric hindrance (C213L or C213F) all disrupted basal PML NB assembly, replacing C213 by

317 valine unexpectedly fully rescued the basal NB formation and normal dynamics (Figs. 6D,F).

318 These data suggest that the branched methyl groups of three valines can maintain

319 hydrophobic interactions within the center of the B2 trimer, somehow similar to L217 on the

320 other side of the helix. Strikingly, both PML C213V localization and dynamics were

321 insensitive to ATO, implying that the sensor function was lost (Figs. 6C,D). Accordingly,

322 alkylation by NEM, which disrupted WT NBs, had no effect on PML^{C213V} NBs (Figs. 2A and

323 6G), stressing the unique features of this cysteine residue. Altogether, this suggests that,

324 within the B2 domain, C213 thiol modification controls PML NB assembly dynamics.

325 Basal PML sumoylation was severely impaired in all mutants predicted to destabilize

326 the B2 trimer and alter NB formation (Fig. 6H,I). In contrast, basal PML^{C213V} sumoylation was

327 normal or even enhanced (Fig. 6I), while critically, sumoylation of PML^{C213V} was ATO-

328 insensitive (Fig. 6I, Supplementary Figure S4G,H). As expected, all B2 trimer and other C213

329 mutants were resistant to ATO-enhanced sumoylation. PML sumoylation of K160 drives

330 partner recruitment (18,43). Accordingly, PML^{C213V} efficiently recruited partner proteins,

331 such as Sp100 (Fig. 6J) and the RNF4 SUMO-dependent ubiquitin ligase (Supplementary

332 Figure S4I), while PML^{C213S} did not. As expected, in contrast to PML, ATO did not further

333 promote recruitment of partners into C213V bodies (Fig. 6J). Accordingly, all B2 mutant were

334 resistant to ATO-induced SUMO-triggered degradation (Fig. 6I, Supplementary Figures

335   S4G,H). Thus, C213-mediated trimerization controls PML sumoylation and partner

336   recruitment.

337   PML NBs couple oxidative stress to control of partner sumoylation through

338   recruitment of UBC9 (18,21). We questioned whether impeding α1-mediated B2

339   trimerization would affect partner biochemical modifications. We generated a *Pml*[L222G] mESC

340   line (corresponding to L218G in human PML forming liquid-like bodies), stably expressed

341   His$_{10}$-SUMO2 and we purified SUMO2 conjugates before or after ATO exposure. As we

342   previously described (21), ATO enhanced global sumoylation, as well as that of endogenous

343   mouse PML (mPML) and KAP1 (Fig. 6K). None of these were observed in *Pml*[L222G] mESC (Fig.

344   6K, Supplementary Figure S4J). Thus, α1-mediated B2 trimerization is required for the

345   biochemical function of PML NBs to promote partner sumoylation.

346

C213 mediates PML-RARA ATO-sensitivity

348   We then assessed the role of PML C213 residue in the context of the oncogenic

349   PML-RARA protein, by transducing hematopoietic progenitors (HSPCs) and MEFs with WT or

350   C213 mutant PML-RARA. We first examined the distribution of human PML-RARA and

351   endogenous mouse Pml proteins by immunofluorescence. As previously shown (27,44),

352   PML-RARA exhibited a micro-speckled pattern where it recruited endogenous mPML. In

353   contrast, PML-RARA[C213S] was diffuse in the nucleus, consistently with C213 role in PML-RARA

354   assemblies, but endogenous mPML NB formation was unaffected in both MEFs (Fig. 7A) and

355   HSPCs (Supplementary Figure S5A). PML-RARA[C213V] formed numerous small microdots and

356   also disrupted endogenous mPML localization, similar to PML-RARA[WT]. Thus, C213 controls

357   the efficiency of homo- and hetero-interactions between PML-RARA and Pml within micro-

358   speckles. In ATO-treated primary progenitors or MEFs, PML-RARA and mPML progressively

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359   reassembled into NBs (Fig. 7A, Supplementary Figure S5A). In sharp contrast, PML-RARA[C213S]

360   did not respond to ATO, while endogenous mPML aggregated into fewer NBs (Fig. 7A,

361   Supplementary Figure S5A). Finally, PML-RARA[C213V]-expressing cells had a mixed phenotype,

362   with ATO-aggregated endogenous mPML NBs - particularly in progenitors that express high

363   levels of mPML proteins, but unresponsive PML-RARA micro-speckles. We then examined

364   the effects of ATO on PML-RARA sumoylation/degradation (45). In the context of the

365   oncogenic protein, C213S precluded basal PML-RARA sumoylation, while PML-RARA or PML-

366   RARA[C213V] were both efficiently sumoylated in the basal state (Fig. 7B). Importantly, the two

367   C213 mutants were completely resistant to ATO-triggered hyper-sumoylation and

368   degradation (Fig. 7B quantification in Supplementary Figure S5B), explaining the remaining

369   PML-RARA[C213V] micro-speckles (Fig. 7A, Supplementary Figure S5A). In FRAP analyses

370   performed either in *Pml* proficient (Supplementary Figures S5C,D) or null MEFs (Fig. 7C),

371   PML-RARA[C213V] mutation reduced the basal assembly dynamics of PML-RARA microdots, but

372   blocked ATO-driven gel-like transition, consistent with its inability to bind arsenic.

373   Altogether, these data demonstrate the key role of C213 in ATO-targeted effects on PML and

374   PML/RARA. Interestingly, dynamics of PML-RARA were distinct in *Pml* proficient or null

375   background (Fig. 7C, Supplementary Figure S5C), supporting that PML reduces PML-RARA

376   assembly dynamics and could contribute to its ATO sensitivity.

377

378   **B2 trimer and C213 control oxidative stress responses**

379   The role of PML is likely much broader than being just an arsenic binder and PML

380   was repeatedly linked to oxidative stress (46-49). To question any contribution of the C213

381   triad in oxidative stress sensing, we compared the effects of ROS on PML NB assembly

382   dynamics in PML and PML[C213V]. Depending on the dose, H_2O_2 increased or decreased PML

17

383 dynamics assessed by FRAP (Fig. 7D). Remarkably, PML[C213V] was completely insensitive to

384 H_2O_2 (Fig. 7E), suggesting that C213 modification by ROS regulates NB dynamics and could

385 play an important role in oxidative stress-sensing.

386   PML is involved in oxidative stress responses (50,51), notably in hepatocytes (46).

387 To explore any role of B2 trimerization, we engineered a *Pml[A220V]* knock-in mice

388 (corresponding to the A216V hotspot mutation in the human sequence). Similar to *Pml^{-/-}*

389 mice, these animals did not exhibit obvious phenotypes and were fertile. As for stably

390 expressed PML[A216V] mutant (Fig. 5D), rare mPML[A220V] nuclear bodies were observed in

391 hepatocytes and the mutant protein did not undergo detectable sumoylation

392 (Supplementary Figure S5E). Hepatocytes from *Pml[A220V]* or *Pml^{-/-}* animal exhibited an

393 increased number of γH2AX foci (Fig. 7F), which could reflect basal oxidative stress (46) or

394 defects in DNA-repair (52). Transcriptomic studies and GSEA analyses comparing basal gene

395 expression in the livers of *Pml[A220V]* and WT animals revealed major differences (including

396 stress signatures, Fig. 7G), many of which were shared with *Pml^{-/-}* animals (Supplementary

397 Figure S5F). Thus, B2 trimer formation has important consequences in physiological

398 conditions.

399   We then subjected WT, *Pml[A220V]* and *Pml^{-/-}* animals to CCl_4, a classic trigger of

400 oxidative stress, ultimately resulting in fibrotic scars, which are limited by senescence

401 activation (53). We first examined acute response by performing transcriptomic studies of

402 livers 18h post-injection. Remarkably, differentially regulated pathways compared to WT

403 animals were virtually identical between *Pml[A220V]* and *Pml^{-/-}* ones (Fig. 7H), highlighting

404 defects in multiple pathways, including p53, E2F, G2M checkpoint, fatty acid, hypoxia, IFNs,

405 Myc, TNFa or TGFB. They are functionally related to processes regulated by PML, such as

406 apoptosis and senescence, cell growth, metabolism, oxidative stress response, as well as

18

407 inflammatory processes (1,2,50). When we examined liver fibrosis after 5 weeks of

408 treatment, increased fibrosis was noted upon Picro-Sirius red staining in *Pml*^*A220V* and *Pml*^*-/-*

409 animals when compared to WT controls (Fig. 7I), as expected from impaired P53 activation

410 and senescence (53).

411   Altogether our findings support that B2 helix-positioned C213 triads act as rheostats

412 controlling PML NB assembly dynamics, liquid- to gel-like properties, and sumoylation,

413 depending on C213 oxidation states (Fig. 7J). This rheostat controls physiological functions of

414 PML for basal homeostasis and stress adaptation. As(III) highjacks this sensor, turning

415 reversible interactions into high-affinity ones, ultimately driving PML-RARA

416 sumoylation/degradation and yielding APL cure.

417

418 **DISCUSSION**

419   In this study, based on the first determination of PML B2-box crystal structure, we

420 unravel the PML/arsenic interplay on a cysteine trio within a B2 trimer, driving a

421 polymerization-like process of PML NB assembly.

422   Despite repeated claims, there was little available experimental evidence that PML

423 undergoes phase separation. Here, we show that PML can de-mix from the nucleoplasm,

424 forming bodies as a result of a dynamic process of PML exchange between condense and

425 diffuse fractions, with viscosity similar to other LLPS-driven NBs. PML NB biogenesis and LLPS

426 were proposed to rely on SUMOs and SUMO-interacting motifs (17,54). Yet, PML mutants on

427 sumoylation sites can nevertheless de-mix into few bodies, underscoring the dispensable

428 nature of sumoylation for PML condensation (18,19). Our FRAP data unravels that the typical

429 PML NB assembly dynamics is the result of complex PML self-interactions requiring B2

430 trimers rather than a "pure" multi-SUMO/SIM-driven LLPS. PML crystal structure and B2

19

431   trimer model resembles that of TRIM5α (39), with PML-specific tunable central cysteine

432   residues that set PML NB assembly dynamics. Since liquid-like bodies are defective for PML

433   and partner sumoylation, while ATO-induced gel-like condensates promote both of them, we

434   have unraveled tight links between NB biochemical activity and B2-controled PML NBs

435   dynamics.

436   Arsenic may bind three adjacent cysteines on the same polypeptide (55,56). Here,

437   arsenic binding is achieved through a trio of C213 residues from distinct B2 polypeptides,

438   locking a polymeric-like PML complex and driving gel-like transition. *In vivo*, interfering with

439   C213 positioning, with the mouse equivalent of the A216V mutation, induced basal and

440   stress-responsive transcriptional changes similar to those observed in *Pml*^{-/-} cells (47). These

441   included defects in p53, E2F, fatty acid metabolism, hypoxia, Myc, TNFa or TGFB (Fig. 7H),

442   many of which are directly implicated in oxidative stress response and altered in cancers.

443   Thus, in physiological conditions, the oxidation status of the sulfur atoms of C213 trio could

444   be the rheostat of NB biogenesis, accounting for their oxidative stress sensitivity (46,48,51).

445   The biochemical nature of oxidation status changes in the C213 triad remains to be

446   investigated. Distinct from C213, C389-mediated oxidation or disulfide formation may be

447   involved in other functions (33), such as ROS buffering within NBs (21).

448   ATO drives APL remission through PML-RARA degradation and subsequent PML NB

449   reformation (3,57). PML-RARA[C213S] lost the ability to form micro-speckles and delocalize

450   endogenous mouse PML. In this setting, mPML localization was normal and remained fully

451   ATO-sensitive. A similar situation may be observed during clinical resistance observed in

452   PML-RARA B2-mutated APL patients (36,37). Thus, although ATO-induced NB-restoration is

453   required in murine APL models (57), it may be insufficient to initiate response, without

454 concomitant degradation of PML-RARA and the resulting clearance of master genes

455 promoters (58).

456 Identification of the 3D catalytic-like site controlling NB assembly could allow the

457 design of novel arsenic-like molecules to enhance or impede NB formation. These could be

458 of high value for PML NB-targeted therapies (59), or conversely, in cancers where PML is an

459 important survival factor (60,61). Overall, our results elucidate the long-standing question of

460 ATO targeting of PML (62,63) and unravel novel basic regulatory mechanisms which are

461 likely to be important in other physio-pathological settings.

462

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463

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480

Authors contributions

482   PB, QQW and NZ performed all experiments; PB and QQW performed cell biology

483   experiments and generated PML mutations, PB performed live cell imaging, FRAP analyses

484   and transcript extraction from liver. NZ and JZ expressed, purified and crystallized MBP-B2

485   fusion proteins, CZ determined the structures. CW generated the first live cell imaging as

486   well as the PML C389S and C213/389S WB and IF analyses; MAG, CB and MCG provided the

487   data on PML-RARA (WT and mutants generated by PB) in Lin⁻ progenitors and MEFs and PML

488   partner recruitment. JZ performed SEC-MALS experiments, MNK generated and

489   characterized the *Pml^A220V* knock-in mouse and initiated the CCl4 experiments, which were

490   developed by TD, S.Q. analyzed transcriptomics data and SB initiated C213S and C389S

491   mutants and observations on their defects. ZG computed PML NB viscosity. CZ, HN, HdT and

492   VLB designed experiments, interpreted data, with the active contribution of PB, QQW, NZ,

493   who performed critical readings of the manuscript. PB assembled the figures. CZ and HN

494   extensively discussed with VLB and HdT who wrote the paper, which was reviewed and

495   accepted by all co-authors.

496

METHODS

Sequence analysis, cloning and constructs

499   Human PML B2 amino acid sequence was extracted from the NCBI databank

500   (NP_150241.2) and compared to the following sequences: *Macaca mulatta*

22

501   (NP_001035899.1), *Mus musculus* (NP_835188.2), *Molossus molossus* (XP_036113734.1),

502   *Gallus gallus* (XP_004943755.1), *Chrysemys picta bellii* (XP_042707508.1). Amino acid

503   conservation was assessed using the ClustalW2 software (64).

504   PML mutations were performed in pMSCVpuro-HA-PMLIII or pMSCVpuro-GFP-

505   PMLIII using the Q5 site-directed mutagenesis kit (NEB; Cat. n°E0554S) according to the

506   manufacturer's protocol and controlled by sequencing (Eurofins Genomics). PML-RARA was

507   cloned into pMSCVpuro (SfiI/AgeI) from pMSCVpuro-GFP-PMLIII and pMSCV-PML-RARA-neo.

508   PML-RARA B2 mutants were generated using AvrII/SfiI restriction fragments from

509   pMSCVpuro-GFP-PMLIII mutants. Restriction enzymes and T4 DNA ligase were purchased

510   from NEB. Additional primer sequences used for site-directed mutagenesis can be found in

511   Supplementary table S1.

512   **Cell culture and cell treatments**

513   All reagents and cell sources are indicated in Supplementary tables S2,S3. Large T-

514   transformed Pml^{KO} MEF cells (our laboratory (46)), HEK293T (Platinum-E from Catherine

515   Lavau, Durham, USA), and HeLa cell lines were maintained in DMEM (Thermo Fisher

516   Scientific; Cat. n°41966029) supplemented with 10% Fetal Calf Serum (FCS), 50U/ml

517   penicillin/streptomycin (Thermo Fisher Scientific; Cat n°10378016), 2mM glutamine (Thermo

518   Fisher Scientific; Cat n°25030024), at 37°C with 5% CO_2. Pml^{WT} mESC were originally

519   obtained from P. Navarro (Pasteur Institute, Paris, France) and cultured in the same culture

520   medium as MEFs, supplemented with 1% non-essential amino acid (Thermo Fisher Scientific;

521   Cat n°11140050), 1% Glutamax (Thermo Fisher Scientific; Cat n°35050-038), 1000 units/ml of

522   recombinant Leukemia Inhibitory Factor (LIF, Sigma-Aldrich; Cat n°ESG1107) and 0.1% β-

523   mercaptoethanol (Merck; Cat n°M6250) on gelatin-coated plates. Hematopoietic stem and

524   progenitor cells isolation, culture and transformation with PML-RARA were performed as

23

525   described before (65). *Pml^KO* MEFs were transduced using retroviruses produced by

526   Platinum-E packaging cells, after transfection with Effectene reagent (Qiagen; Cat. n°.

527   301425) with pMSCVpuro-HA-PMLIII or pMSCVpuro-GFP-PMLIII WT or mutants. GFP-positive

528   cells were sorted by flow cytometry (Aria II BD-Bioscience) or selected using 2µg/ml

529   puromycin (Sigma-Aldrich; Cat. n°P8833). HeLa-PML^L218Y cell line was generated by retroviral

530   transduction. pLVX-CMV-GFP-PMLIII L218Y retroviruses were generated by co-transfection

531   into HEK293T cells as above. For colocalization studies between PML and RNF4 or Sp100,

532   Platinum-E cells were transfected with both pMSCVpuro-GFP-PMLIII WT or mutant and

533   pMSCV-Sp100 or pMSCV-Flag-RNF4 and the culture supernatant was used to transduce

534   *Pml^KO* transformed MEFs. All cell lines used were negative for mycoplasma (monthly tested

535   by Eurofins MWG France) and were manipulated separately to avoid any cross

536   contamination during cell passages. The different stable cell lines were at least monthly

537   checked for authentication, based on human or mouse PML expression, sumoylation status

538   and subcellular distribution by immunofluorescence and Western blot analyses. They were

539   frozen before the third passage and kept for 10 to 20 passages, expect for PML/RARA-

540   transduced MEFs or progenitors were less that 5 passages. No karyotype was performed,

541   except for knock in mESCs (see below).

542   Cells were treated with 1µM As2O3 (Sigma-Aldrich; Cat. n° 01969), 10µM N-ethyl-

543   maleimide (Sigma-Aldrich; Cat. n°E3876), 100 or 500µM H_2O_2 (Sigma-Aldrich; Cat. n°516813),

544   2µM Methylarsonic acid (Greyhouse; Cat. n°N-12922), 2µM cacodylic acid (Sigma-Aldrich;

545   Cat. n°C0125), 1µM antimony oxide (Sigma-Aldrich; Cat. n°637173-100G) or 2µM ReAsH

546   (Thermo Fisher; Cat. n° T34562).

547   **Western blot analysis**

548   Cell extracts were obtained using homemade Laemmli buffer devoid of reducing

549   reagents (200mM Tris pH 6,8, 8% SDS, 40% glycerol, 0,2% bromophenol blue), supplemented

550   with 20mM N-ethyl-maleimide (Sigma-Aldrich; Cat. n°E3876) for Western blot analysis in

551   non-reducing conditions, or supplemented with 50mM TCEP, or using direct lysis in

552   commercial Laemmli buffer (Sigma-Aldrich; Cat. n° S3401). SDS-PAGE analyses were

553   performed with 4-12% NuPAGE bis-tris gels (Thermo Fisher, Cat. n°NP0336) and

554   immunoblotted on nitrocellulose membranes (BioTrace Pall Laboratory; Cat. n°732-3031),

555   with antibodies: anti-GFP (1/1000, Roche; Cat. n°11814460001), anti-HA (1/1000, Covance;

556   Cat. n°MMS-101R-1000), anti-Actin (1/1000; Sigma-Aldrich; Cat. n°A2066), homemade anti-

557   hPML. Secondary antibodies from Jackson Immuno-Research: anti-Mouse-HRP (1/5000, Cat.

558   n°115-035-062), anti-Rabbit-HRP (1/5000, Cat. n°111-035-045). Proteins were detected using

559   SuperSignal West Dura or Femto (ThermoFisher; Cat. n°34076 and 34096), using Vilber

560   Fusion-Fx (Vilber). To avoid non-specific signals, membranes were not reproved.

561   **Immunolabelling and microscopy analysis**

562   Cells were fixed with formalin 10% (Sigma-Aldrich; Cat. n°HT501128) for 15 min and

563   permeabilized in PBS 0.5% Triton x-100 (Sigma-Aldrich; Cat. n°T9284) for 15 min. Incubation

564   with antibodies was performed in PBS 0.5% Triton X-100 for 1h. Primary antibodies: anti-GFP

565   (1/1000, Roche; Cat. n°11814460001), anti-HA (1/1000, Covance; Cat. n°MMS-101R-1000),

566   anti-FLAG (11000, Sigma-Aldrich; Cat. n°F7425), homemade anti-hSP100 antibody,

567   homemade anti-hPML and anti-mPML antibodies, anti-SUMO2/3 (1/500, Cell Signaling

568   Technologies; Cat. n° 4971P), γH2AX (Cell Signaling Technologies; Cat n°2577S). Secondary

569   antibodies: goat anti-mouse Alexa Fluor 488 (1/500, Jackson Immuno-Research; RRID:

570   AB_2338840), goat anti-mouse rhodamine red-X (1/500, Jackson Immuno-Research; RRID:

571   AB_2338756), goat anti-rabbit Alexa Fluor 488 (1/500, Jackson Immuno-Research; RRID:

572 AB_2338046), goat anti-mouse rhodamine red-X (1/500, Jackson Immuno-Research; RRID:

573 AB_2338022). Secondary goat anti-mouse antibody (STAR RED (1/250, Abberior; Cat. n°

574 STRED-1001) was used for STED analysis. DAPI (1µg/mL) from Thermo Fisher (Cat. n° 62248).

575 Image acquisitions were performed using a confocal microscope LSM 980 (Carl Zeiss Micro-

576 Imaging) equipped with an Airyscan 2 super-resolution module using a x60 oil-immersion

577 objective or by STED microscopy using an Axio Observer 7 (Carl Zeiss Micro-Imaging)

578 equipped with a STEDYCON module (Abberior) using a x100 oil-immersion objective.

579   For the study of HeLa PML^{L218Y}, stably expressing cells were fixed with 4%

580 paraformaldehyde and permeabilized with 0.1% Triton X-100. Slides were blocked with 2%

581 BSA in PBS, followed by incubation with anti-Flag antibodies (Sigma-Aldrich; Cat. n°F1804)

582 overnight at 4°C. Secondary antibodies (Abbkine; Cat. n°A23210) were incubated at room

583 temperature for 4 h. Slides were mounted using DAPI Fluoromount-G (Southern Biotech;

584 Cat. n°0100-20). The fluorescent signals were visualized under a Zeiss 510 confocal

585 microscope (Carl Zeiss Micro-Imaging).

586 **Live cell imaging and analysis of PML NBs**

587   All live-cell analysis were performed using glass-bottom culture dishes (IBIDI; Cat.

588 n°81156). For the analysis of NB formation Pml^{KO} cells were transduced with the culture

589 supernatant of Platinum-E cells transfected two days prior with a pMSCVpuro-GFP-PMLIII

590 vector. 1mL of anti-evaporation oil was added on top of the supernatant (IBIDI; Cat.

591 n°50051). The acquisition was performed on an Axio Observer video-microscope (Carl Zeiss

592 Micro-Imaging) equipped with a thermostatic chamber (37°C, 5% CO_2). Laser power and

593 exposure time were set at minimum levels to avoid photo-toxicity. Acquisitions were

594 performed every 15 minutes. PML NB formation was tracked in the same cells during the

595 entire experiment.

596   The quantification of PML NB nucleation/fusion/fission events was performed on

597   *Pml^KO* MEFs stably expressing GFP-PMLIII on a spinning disk confocal microscope (Nikon

598   Eclipse Ti, Nikon) equipped with a Coolsnap HQ2 high-sensitivity camera (Photometrics) and

599   a thermostatic chamber (37°C, 5% CO_2). Cells were imaged during two hours every 30

600   seconds. Quantification was performed using the FIJI software on maximum Z projections of

601   n=37 cells from four independent experiments. The magnitude of the effective viscosity of

602   the PML NBs was estimated by quantifying the time required for two NBs to relax in a

603   spherical structure during a fusion event as described before (66). The relaxation time during

604   a fusion event was quantified by measuring the long axis of the two condensates from the

605   beginning of the fusion and was plotted as a function of time for n=21 NBs from three

606   independent experiments. By computing the fusion time as a function of the diameter of the

607   resulting PML bodies (Supplementary Figure S1B) and by assuming that surface tension

608   drives the fusion process whereas viscosity tends to impede it, we determined that PML

609   bodies have an effective viscosity of about 10^3 Pa.s. Analysis of PML NB distribution was

610   performed on confocal images of fixed cells using the FIJI software.

611   **Analysis by Fluorescence Recovery After Photobleaching (FRAP)**

612   FRAP was performed on *Pml^KO* MEFs stably expressing GFP-PMLIII, GFP-PML-RARA

613   or the indicated B2 mutants. 1µm regions of interest were used for GFP-PMLIII-expressing

614   cells and 2µm ROI were used for GFP-PML-RARA. Acquisitions were performed every 10

615   seconds using a spinning disk confocal microscope (Nikon Eclipse Ti, Nikon) equipped with a

616   Coolsnap HQ2 high-sensitivity camera (Photometrics) and a thermostatic chamber (37°C, 5%

617   CO_2) with a x60 oil-immersion objective. Three images were taken before photobleaching, 15

618   iterations were used to bleach the ROI and fluorescence recovery was followed every 10

619   seconds for 15-30 mins. On the graphs, only the data points pre-bleaching, immediately after

620   bleaching and every 2.5 mins are shown. Fluorescence recovery was analyzed using the FIJI

621   software. For each studied nucleus, the relative fluorescent intensity (RFI) was calculated as

622   previously described using an ImageJ plugin (67). Background correction was applied at

623   every time point and a double normalization was performed to account for global sample

624   bleaching. We assessed that PML recovery was independent of the size of the bleached area

625   by testing several sizes of photobleached areas, showing that interactions between PML

626   proteins at the shell have a predominant role over free diffusion. Then every RFI curve was

627   fitted to an $y=A*[1-\exp(-B/t)]$ model where y is the fluorescence intensity, A the plateau of

628   the intensity (mobile fraction), B the time in seconds and t the half-recovery time ($t_{1/2}$).

629   **Protein expression and purification from bacteria**

630   Escherichia coli Rosetta (DE3) cells (Novagen) were transformed with a pRSFDuet-1

631   vector (Novagen) containing the DNA sequence of human PML B2 domain fused to a MBP

632   tag. 0.2 mM IPTG was added into the culture to induce expression of the recombinant

633   proteins at 18 ºC for 16 hours. Bacteria were harvested by centrifugation (5000 rpm, 8 min),

634   resuspended in a buffer containing 20 mM Tris-HCl pH8.0, 300 mM NaCl, 5% glycerol, 0.3

635   mM TCEP, 1 mM PMSF) and lysed using a cell disruptor (JNBIO). The lysate was loaded onto

636   a pre-equilibrated amylose resin column (NEB). The column was first washed with 5 column

637   volume of lysis buffer and MBP-B2 protein was eluted with a buffer containing 20 mM Tris-

638   HCl pH 8.0, 300 mM NaCl, 5% glycerol, 0.3 mM TCEP, 10 mM maltose. Proteins were

639   concentrated to 20 mg/mL and purified on a Superdex 200 increase gel filtration column

640   (Cytiva) (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.3 mM TCEP). Purified MBP-B2 was

641   concentrated to 15 mg/mL for crystallization.

642   **Crystallization and structure determination**

28

643   Crystals of WT MBP-B2 were grown with 24-well plates using the hanging drop

644   vapor diffusion method by mixing 1.2 µl protein (15 mg/mL) with 1.2 µl crystallization buffer

645   containing 100 mM MES pH 6.5, 250 mM potassium acetate, 22% (w/v) PEG 3350. Crystals

646   grew to full size after incubation at 16°C for 1 week. MBP-B2 C213A and A216V mutant

647   proteins were crystalized in the same conditions. Before X-ray diffraction, crystals were cryo-

648   protected with the reservoir solution supplemented with 25% glycerol and were flash-cooled

649   into liquid nitrogen. Diffraction data were collected at Beamline station BL19U1 of Shanghai

650   Synchrotron Radiation Facility (SSRF, Shanghai, China), integrated and scaled using XDS and

651   the CCP4 program Pointless and Aimless (68-70). The structure of MBP-B2 was determined

652   by molecular replacement using the MBP structure from PDB 1ANF as an initial searching

653   model with Phaser (71). The structural model was built using Coot (72) and refined using

654   PHENIX (73). The structure of MBP-B2 C213A and A216V was determined using WT MBP-B2

655   structure as the initial search model. Figures were generated using PyMOL (The PyMOL

656   Molecular Graphics System, Version 2.0 Schrödinger, LLC). The statistics of the data

657   collection and refinement are shown in Supplementary Table S4. The B2 trimer model was

658   generated with Alphafold 2 (74). "Relevant crystallographic data collection and refinement

659   statistics are provided in Supplementary Table S4. Experimentally-determined 3D structures

660   can be found from the Protein Data Bank (PDB ID : 8J2P)"

661   **SEC-MALS analysis**

662   SEC-MALS were performed using a Superdex 200 increase column (Cytiva)

663   combined with multi-angle laser light scattering using a Wyatt HELEOS-II 18-angle

664   photometer coupled to a Wyatt Optilab rEX differential refractometer (Wyatt Technology

665   Corp). Experiments were carried out at room temperature with a protein concentration of

666   2.0 mg/mL and a flow rate of 0.5 mL/min in 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.3 mM

29

667 TCEP. The data were analyzed using the ASTRA 6.1 software (Wyatt). The molecular mass

668 was determined across the protein elution peak.

CRISPR/Cas9 knock-in mESCs and His pulldown

670   Pml^{KO} cells were generated as described previously (21). To generate Pml^{L222G}

671 mESC, gRNA targeting mouse Pml (5'-G• CCG AGG CTG CGC CAA GCC TCT GTG TTG CAC ATG

672 CGC GCT CGG GGA CCG CAA CCA CAG CCA TCT CCA TTG CGA TAT TGG TG•A• G-3') and

673 recombinant Cas9 protein were synthesized from IDT to form Alt-R CRISPR/Cas9

674 ribonucleoprotein. mESCs were transiently transfected with Alt-R CRISPR/Cas9

675 ribonucleoprotein by using Amaxa^{Tm} mouse ES cell Nucleofector^{Tm} kit (Lonza; Cat n°VPH-

676 1001) and program number A-013 from the nucleofector 2b device (Lonza). After three days,

677 200 mESCs were spread on 150mm cell culture dishes and cultured until forming visible

678 colonies. After isolation and expansion, the DNA was extracted from these clones and

679 genotyping was performed using PCR primers: 5'-GGTTGTCAGACTTGGCTGTG-3' and 5'-

680 GCAGCTGGACTTTCTGGTTCT-3.   Products   were   sequenced   with   primer: 5'-

681 ATCTCTTTGTACTGTCTGG-3' (Eurofins genomics). mESC were transduced using viral particles

682 produced by Platinum-E cells, as described above, after transfection with MSCV-IRES-GFP

683 constructs expressing His10-HA-SUMO2. His10-HA-SUMO2 conjugates were purified using

684 NiNTA agarose beads (Qiagen; Cat L30210) using Guanidium denaturing lysis as described

685 before (21).

Mouse model generation

687   Pml^{A220V} mice were obtained by CRISPR/Cas9 genome edition, performed on

688 BALB/cByJ zygotes, using TAKE methods (75). Briefly, three- to four-week-old BALB/cByJ

689 females were super-ovulated using CARD HyperOva (Cosmo bio, #KYD-010-EX) and human

690 Chorionic Gonadotropin, Sigma; #CG-10) and then mated with males (8-20 weeks) to get

30

691 zygotes. crRNA, TracrRNA, ssDNA and Cas9 nuclease were purchased from IDT and

692 electroporated (NEPA21; Sonidal) to introduce Pml point mutation encoding A220V

693 substitution using Pml target site: 5'-GTGTTGCACATGCGCGCTCC-3' and ssDNA donor

694 sequence:

695 5'CATCTACTGCCGAGGCTGCGCCAAGCCTCTGTGTTGCACATGCGTGCTCCTCGACCGCAACCACAG

696 CCATCTCCATTGCG-3'.   Genotyping   was   performed   using   PCR   pimers: 5'-

697 GGTTGTCAGACTTGGCTGTG-3' and 5'-GCAGCTGGACTTTCTGGTTCT-3 and products were

698 sequenced with primer: 5'-ATCTCTTTGTACTGTCTGG-3' (Eurofins genomics). This study has

699 been carried out in accordance with national (n° 2013-118) and European (n°2010/63/UE)

700 directives for animal experiments. Animals were handled according to the guidelines of

701 institutional animal care committees using protocols approved by the "Comité d'Ethique

702 Experimentation Animal Paris-Nord" (no. 121). Animals were housed at our animal facility

703 (UMS Saint-Louis US53/UAR2030, Institut de Recherche Saint-Louis, Paris, France) in

704 accordance with animal welfare and ethical guidelines (accreditation number B75-10-08).

705 ***In vivo* oxidative stress induction models**

706   For the transcriptomic study, an early oxidative stress model was used. A female mice

707 cohort (8-10 weeks) were treated with one intraperitoneal (IP) injections of 20% Carbon

708 tetrachloride CCl4 (SIGMA; Cat n°289116) diluted in mineral oil (SIGMA; Cat n°M8410) or

709 one IP injection of mineral oil (2,5 µL/g, n=5 for each group). Mice were sacrificed 18h after

710 injections and livers were dissected as follows: left median lobes were stored in RNAlater

711 solution (Invitrogen; Cat n°AM7021) before RNA extraction, right median lobes were frozen

712 in tissue freezing medium (Leica Biosystems; Cat n°14020108926), right lobes were frozen in

713 liquid nitrogen for protein extraction, and left lobes were fixed in AFA fixative solution (VWR;

714 Cat n°11656713) for 24h and embedded in paraffin for histology staining.

31

715   To study a model of longer oxidative stress induction, liver fibrosis was triggered on a

716   female cohort by using 5 weeks treatment of 20% CCl4, two IP injections per week (n=7

717   CCl4-treated, n=3 oil-treated). Mice were sacrificed 48h after the last injection, and livers

718   were dissected as follows: right median lobes were frozen in tissue freezing medium and left

719   lobes were fixed in AFA fixative solution, embedded in paraffin. The protocol was approved

720   and performed in accordance with animal welfare and ethical guidelines (accreditation

721   number B75-10-08).

Picro-Sirius Red Staining and Fibrosis quantification

723   To assess the liver fibrosis, paraffin embedded tissue sections (6 μm) were

724   deparaffinized, rehydrated and stained during 1 hour in a 0,1% Picro-Sirius Red homemade

725   solution : Direct80 (SIGMA; Cat n°365548) diluted in an aqueous solution saturated in picric

726   acid. The stained liver sections were washed in acidified water, dehydrated in several

727   ethanol baths, cleared in SubX (Leica Biosystems; Cat n°3803670E) and mounted with a

728   permanent mounting medium. Each stained slide were scanned using the Zeiss AxioScan 7

729   (Carl Zeiss Micro-Imaging). The red collagen fibers stained by Picro-Sirius Red solution, were

730   quantified using an open source software, QuPath (76).

RNA Extraction and Gene Chip Analysis

732   Total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY, USA)

733   according to the manufacturer's protocol. Triplicate samples were generated for each

734   genotype, from three Pml^{WT}, three PML^{KO}, and three Pml^{A220V} mice. MTA1.0 Affymetrix

735   Protocol was performed as following. RNA quantification and quality control was performed

736   using the HT RNA Reagent Kit (Perkin Elmer; Cat n°CLS960010,), the DNA 5KRNA/CZE

737   LabChip (Perkin Elmer; Cat n°760435) and the Caliper LabChip Microfluidics System (Perkin

738   Elmer; Cat n° GX1133N0432). One hundred nanogrammes of Total RNA was amplified,

739   labeled, and fragmented using GeneChip Plus Reagent Kit (ThermoFisher Scientific; Cat

740   n°902314). Each sample was hybridized onto GeneChip® Mouse Transcriptome Array 1.0

741   (ThermoFisher Scientific; Cat n°902514), washed, and stained using the GeneChip™

742   Hybridization, Wash, and Stain Kit (ThermoFisher Scientific; Cat n°900720) by using the

743   Affymetrix® Fluidics Station 450 (ThermoFisher Scientific; Cat n°60110150). Array Scanning

744   was performed with the Affymetrix® GeneChip Scanner 3000 7G (ThermoFisher Scientific,

745   Cat n°54714280), using the Command Console software (ThermoFisher Scientific) and then

746   analyzed using the Affymetrix® rma-sketch routine (Transcriptome Analysis Console (TAC)

747   software v.4.0.1, ThermoFisher Scientific). Raw microarray data (CEL files) were corrected for

748   background, $\log_2$-transformed and normalized using the standard method RMA (robust

749   multi-array average) implemented in the oligo package (77). Quantile normalization was

750   then applied to mitigate the effects of technical variables using limma package (78) and

751   probe sets were annotated using mta10transcriptcluster.db package. A linear model fitted in

752   Limma package was performed to identify differential gene expression between Pml^{A220V}

753   versus Pml^{WT} or Pml^{KO} versus Pml^{WT} in basal conditions and after 18h of CCl4 treatment.

754   Differentially expressed genes were identified based on the adjusted p-value < 0.05 and

755   filtered with a fold change absolute value > 2. Gene set enrichment analyses (GSEA) were

756   then performed to identify significant hallmark gene sets using the fgsea and msigdbr

757   packages.

758   **Statistical Analysis**

759   Statistical tests performed with Graphpad prism are indicated in the figure legends.

760   Kruskal-Wallis test was used to determine the p-value and look for significant changes in

761   PML dynamics at NBs in Pml^{KO} fibroblasts stably expressing GFP-PML. All data are expressed

762   as mean ± SD of technical or biological replicates as indicated. For all graphs, *P = 0.01-0.05,

763   **P = 0.001-0.01, and ***P < 0.001.

Data availability statement

765   Experimentally-determined 3D structures can be found from the Protein Data Bank (PDB ID:

766   8J2P).

767   Sources for reagents and cells are indicated in Supplementary tables S2,S3.

768   Primary transcriptomic data are accessible on ArrayExpress (accession ID: E-MTAB-13183);

769   (For the reviewers : Login: pierre.bercier@college-de-france.fr; Pswd : nFjSz9JsA!cTTsD)

770   The data generated in this study are available upon request from the corresponding authors.

771

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991

FIGURE LEGENDS

Fig. 1. PML NBs display hallmarks of LLPS and switch to gel-like features upon ATO exposure

A, STED analysis uncovers the typical spherical core-shell PML NB. Scale bar: 1µm. **B,** Time-lapse analysis of PML NB apparition upon GFP-PML expression in $Pml^{-/-}$ MEFs. The white circle indicates the nucleus. Representative data from three independent experiments. Scale bar: 5µm**. C,** PML NBs undergo fusion with spherical relaxation, similar to liquid-like droplets. Representative time-lapse of PML NB fusion (left) and quantification of effective viscosity (right). Mean values ± SD of n=21 fusion events in independent cells from three independent experiments. Scale bar: 1µm. **D,** Quantification of PML NBs nucleation, fusion and fission events over one hour. Mean value ± SD of n=37 cells from five independent experiments. **E,** FRAP analyses of GFP-PML^{WT} dynamics at NBs in control or ATO-treated MEFs (1µM, 30min). Mean value ± SD. NB assessed Ctrl: n=36, ATO: n=28, from at least five independent experiments. **F,** Confocal analysis of PML NBs in GFP-PML^{WT} or GFP-PML-RARA^{WT} MEFs treated with 1µM of ATO for 1h. Scale bar: 5µm. **G,** STED analysis demonstrating incomplete PML NB fusion upon ATO. Scale bar: 1µm. **H,** Comparison between GFP-PML^{WT} and GFP-PML-RARA^{WT} dynamics at NBs by FRAP. ATO treatment (1µM, 30min) induces a liquid- to gel-like transition of GFP-PML-RARA WT. Mean value ± SD. NBs assessed PML: n=36, PML-RARA: n=20, PML-RARA+ATO: n=37, from at least five independent experiments.

Fig. 2. PML NB assembly depends on C213 in B2 zinc finger domain, but not on C389-mediated inter-molecular disulfide bonds

A, Confocal analysis of PML NB formation following cysteine alkylation with NEM (N-ethyl-maleimide, 10µM, 1h) or ATO (1µM, 1h) used alone or sequentially in GFP-PML^{WT} MEFs.

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1016 Scale bar: 5μm. **B,** Western blot analysis (non-reducing conditions) of PML disulfide bond

1017 formation in MEFs expressing HA-PML^WT or PML cysteine mutants, treated or not with ATO

1018 (10μM, 1h) or H_2O_2 (500μM, 1h). Brackets: SUMO-conjugated and intermolecular disulfide-

1019 bound PML. Blue arrowheads: intermolecular disulfide-bound PML. Molecular weight (kDa).

1020 **C,** Confocal analysis of PML NBs upon ATO (1μM, 1h) or H_2O_2 (500μM, 1h) exposure in HA-

1021 PML^WT- or cysteine mutant-expressing MEFs. Arrow: single NB in HA-PML^C213A-expressing

1022 MEFs. Scale bar: 5μm.

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1024 **Fig. 3. Uncovering PML B2 structure**

1025 **A,** Conservation of PML B2 box through evolution. Top part: putative position of the two zinc

1026 fingers intertwined in a cross-brace. Bold: amino acids implicated in putative zinc

1027 coordination. The additional cysteine in position 213 is highlighted in blue. Predicted

1028 secondary structures of PML B-box-2 are depicted, arrow: beta-sheet; loop: α1-helix; Stars:

1029 evolutionary conserved amino acids (bold: identical, custom: 1/6 variation only). The highly

1030 conserved α helix is boxed in blue. Bottom part: PML-RARA mutations from therapy-

1031 resistant APL patients. **B,** Crystal structure of PML B2 monomer shown in a cartoon

1032 representation. PML B2 folds around two zinc fingers organized in a cross-brace. The C3H1

1033 zinc coordination leaves the C213 free. α1-helix (C213 to L218) exposes C213 towards the

1034 outside of the structure. Spheres: zinc atoms, blue bold font: C213. **C,** (Left) Composite omit

1035 map contoured at 2 σ of a segment from PML B2 near zinc finger 1 at 2.1 Å. C213 appears as

1036 a free cysteine (blue). (Right) Composite omit map contoured at 2 σ of a segment from PML

1037 B2 near Zinc finger 2 at 2.1 Å. C227, not D219, coordinates Zn^{2+}. Cyan spheres, zinc atoms.

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1039 **Fig. 4. PML B2 α1-helix controls PML assembly and dynamics**

A, Representative PML NB formation in MEFs expressing PML B2 mutants in Zn^{2+}-coordinating cysteines or α1-helix (underlined in blue) residues. Scale bar: 5μm. **B,** FRAP analyses of GFP-PML dynamics at NBs in WT- or mutant-expressing MEFs. Mean value ± SD. R.F.I.: relative fluorescence intensity. NBs assessed PML[WT]: n=36, PML[C189S]: n=25, PML[C212S]: n=42, PML[C213S]: n=24, PML[L218G]: n=20, from at least five independent experiments. **C,** Plot representation of **B**, with half recovery time (left) and immobile fraction (right) of PML mutants harboring mutations on the α1 -helix or zinc finger. Each dot represents an individual NB. Median ± 95% confidence interval, statistical significance by comparison with GFP-PML, Kruskal-Wallis test. *** P ≤ 0.001 are displayed. P-value for Zn-finger mutants, C189S=0,0097, C212S=0,0115, C213S=0,028. **D,** Superposition of PML B2 crystal structure (cyan) with that of PML[C213A/A216V] mutant (orange) demonstrates absence of misfolding.

Fig. 5. PML B2 mediates PML trimerization and is required for biological functions

A, Modelling of a PML B2 trimer. B2 monomers are colored in green, grey and pink. Exponents refer to affiliation to a specific monomer. **B,** Close-up view of the PML B2 trimer model depicting hydrophobic interactions between the α1-helices. Residues involved in these hydrophobic interactions are shown in blue, linked by dashed lines. Distance between these key hydrophobic residues are in Å. **C,** SEC-MALs analysis showing a mix of monomeric, dimeric and trimeric PML B2 (left). The C213A mutant shifts towards monomeric and dimeric states (right). MBP-B2 MW: 46.2 kDa. **D,** Representative PML NB formation in PML B2 mutants predicted to impair trimerization expressed in MEFs. Scale bar: 5μm. **E,** GFP-PML[L218F] yields filaments (left). Scale bar: 5μm. FRAP analysis of the exchange rates of GFP-PML[L218F] mutant compared to GFP-PML (right). Mean value ± SD. NBs assessed WT: n=36, PML L218F: n=24, from at least five independent experiments.

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1065 **Fig. 6. ATO binding to B2 trimer is responsible for ATO-induced phase transition**

1066 **A,** Modelling of PML B2 trimer around an arsenic atom. The key C213 residue are indicated

1067 in blue and the arsenic atom is shown as an orange sphere. The predicted distance between

1068 the arsenic atom and C213, represented by the dashed line is 2.7 Å. **B,** FRAP analyses of GFP-

1069 PML dynamics at NBs in untreated cells or cells treated with trivalent metalloid oxides: ATO

1070 (1µM, 30min) or STO (1µM, 30min) or divalent arsenic (MMA, 1µM, 30min). Mean value ±

1071 SD., assessed NBs Ctrl: n=36, MMA: n=21, ATO: n=28, STO: n=33, from at least 5

1072 independent experiments. **C,** Representative confocal analyses of PML NB formation in $Pml^{-/-}$

1073 MEFs stably expressing GFP-PML B2 mutants, +/- ATO (1µM, 1h). Scale bar: 5µm. **D,** FRAP

1074 analyses of GFP-PML mutant-expressing MEFs treated or not with ATO (1µM, 30min). Mean

1075 value ± SD. NBs assessed PML^{C212S} Ctrl: n=42, PML^{C212S} ATO: n=28, PML^{C213S} Ctrl: n=24,

1076 PML^{C213S} ATO: n=27, PML^{L218G} Ctrl: n=20, PML^{L218G} ATO: n=26, PML^{A216G} Ctrl: n=40, PML^{A216G}

1077 ATO: n=28, PML^{A216V} Ctrl: n=43, PML^{A216V} ATO: n=43, PML^{C213V} Ctrl: n=32, PML^{C213V} ATO:

1078 n=31, from at least 5 independent experiments. **E,** Representative images of red fluorescent

1079 arsenic (ReAsH) localization to NBs in GFP-PML or mutant MEFs. Scale bar: 1µm. **F,**

1080 Representative PML NB formation in PML C213 mutant-expressing MEFs. Scale bar: 5µm. **G,**

1081 Confocal analysis of PML NB formation following cysteine alkylation with NEM (N-ethyl-

1082 maleimide 10µM, 1h) in GFP-PML or PML^{C213V} MEFs. Scale bar: 5µm. **H,** Western blot analysis

1083 of basal PML sumoylation in GFP-PML^{WT}- or mutant-expressing MEFs. **I,** Western blot

1084 analysis of PML sumoylation in GFP-PML^{WT} or mutant MEFs treated or not with ATO (1µM,

1085 1h). **J,** Confocal analysis of PML and Sp100 localization in GFP-PML- or C213 mutant-

1086 expressing MEFs treated or not with ATO (1µM, 1h). Scale bar: 5µm. **K,** Pulldown of HIS_{10}-

1087 $SUMO_2$ conjugates from Pml^{WT} or Pml^{L222G} mESCs treated or not with ATO (1µM, 30min).

1088   ATO-treated non-transduced Pml^{WT} mESCs are shown as controls. Western blot with anti-

1089   SUMO$_{2/3}$ (left), anti-mPML (right, top) or anti-KAP1 antibodies (right, bottom). Sumoylated

1090   species are indicated. Representative data of n=3 independent experiments.

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1092   **Fig. 7. PML-RARA ATO-sensitivity requires C213 and B2 controls *in vivo* responses to ROS**

1093   **A,** Confocal analysis of $Pml^{+/+}$ MEFs stably expressing human PML-RARA or its B2 mutants

1094   treated or not with ATO (1μM, 6h). Scale bar: 5μm. Different display settings for each

1095   genotype. **B,** Western blot analysis of PML-RARA sumoylation in Lin$^-$ hematopoietic

1096   progenitors expressing PML-RARA or the indicated mutants, treated or not with ATO (1μM,

1097   1h). Representative data from three independent experiments. **C,** FRAP analysis of GFP-PML-

1098   RARA dynamics at NBs in GFP-PML-RARA^{WT}- or GFP-PML-RARA^{C213V}-expressing $Pml^{-/-}$ MEFs

1099   treated or not with ATO (1μM, 30min). Mean value ± SD. NBs assessed PML-RARA^{WT}: n=20,

1100   PML-RARA^{WT}+ATO: n=37, PML-RARA^{C213V}: n=42, PML-RARA^{C213V}+ATO: n=25, from at least 5

1101   independent experiments. **D,** FRAP analysis of GFP-PML dynamics at NBs in MEFs treated or

1102   not with H_2O_2 (30min). Mean value ± SD. NB assessed PML: n= 35, H_2O_2 100μM: n=27, H_2O_2

1103   500μM: n=32, from at least 5 independent experiments. **E,** Same as **D** with GFP-PML^{C213V}.

1104   Mean value ± SD. NB assessed PML^{C213V}: n= 32, H_2O_2 100μM: n=24, H_2O_2 500μM: n=22, from

1105   at least 5 independent experiments. **F,** Box plot representing γH2AX dots counts assessed by

1106   immunofluorescence on liver tissues from three untreated mice. Pml^{WT}: n=633, PML^{KO}:

1107   n=527, Pml^{A220V}: n=333. *** P ≤ 0.001, unpaired t-test. **G,** GSEA analysis of differentially

1108   expressed genes from liver samples from untreated Pml or Pml^{A220V} (equivalent of human

1109 PML^{A216V}) mice. Key pathways are boxed. Livers from n=3 Pml or Pml^{A220V} mice. **H,** GSEA of

1110   differentially expressed genes in liver samples from Pml or Pml^{A220V} in comparison to WT

1111   after a 18h CCl_4 treatment. Key pathways are boxed. Livers from n=3 mice of each genotype.

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1112   **I,** Liver fibrosis following a 5 weeks CCl$_4$ treatment detected by Picro-Sirius Red staining (left).

1113   Quantification of the fibrotic areas (right). Livers from n=7 *Pml*, *Pml^{KO}* or *Pml^{A220V}* mice. Scale

1114   bar: 400µm. ** P ≤ 0.01, Mann-Whitney test. **J,** Model of PML NB liquid to gel-like transition

1115   controlled by the C213-containing B2 α1 helix hijacked by ATO. Hydrophobic-mediated

1116   trimerization of PML B2 (grey disk), regroups the three C213 in the center of the structure.

1117   The latter is impaired in α-helix mutants. In the physiological states, this C213 triad behaves

1118   as a rheostat depending on their oxidation state (asterisks), fine-tuning the interaction

1119   within the trimer (light and dark grey disk). Arsenic binding crosslinks these cysteines,

1120   highjacking the ROS-rheostat to yield polymerization-induced gel-like transition.

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Bercier et al., Figure 1

Bercier et al., Figure 2

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