**Promyelocytic leukemia nuclear body (PML-NB) -free intranuclear
milieu facilitates development of oocytes in mice**

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Running title: Lack of PML-NBs in oocytes

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20 **Abstract**

21 Promyelocytic leukemia (PML) nuclear bodies (PML-NBs), a class of membrane-less
 22 organelles in cells, are involved in multiple biological activities and are present
 23 throughout cells of adult organisms. Although the oocyte nucleus is an active region for
 24 the flux of multiple non-membranous organelles, PML-NBs have been predicted to be
 25 absent from oocytes. Here, we show that the deliberate assembly of PML-NBs during
 26 oocyte growth preferentially sequestered Small Ubiquitin-related Modifier (SUMO)
 27 protein from the nucleoplasm. SUMO not only was involved in the regulation of oocyte
 28 nuclear maturation but also was committed to the response, mediated by liquid droplet
 29 formation, to multiple stressors including nucleolar stress and proteotoxic stresses.
 30 Exogenous assembly of PML-NBs in the nucleus of oocytes affected the efficiency of
 31 the response of SUMO. These observations suggest that the PML-NB-free intranuclear
 32 milieu ensures that a reserve of SUMO remains available for emergent responses in
 33 oocyte development. This work demonstrated a benefit of the PML-NB-free
 34 intranuclear milieu, namely the ability to redirect the flux of SUMO otherwise needed
 35 to control PML-NB dynamics.

36 **Introduction**

37 The nuclei of mammalian interphase cells typically contain dozens of promyelocytic
 38 leukemia (PML) nuclear bodies (PML-NBs), membrane-less organelles that are
 39 formed by phase separation. PML-NBs consist of a shell of PML protein surrounding
 40 an inner core that harbors over a hundred proteins as “client molecules”, notably
 41 including Small Ubiquitin-related Modifier (SUMO), a well-defined posttranslational
 42 modifier (Lallemand-Breitenbach and de The, 2018). The PML protein is the only
 43 resident molecule essential for the formation of PML-NBs (Lamond and Earnshaw,
 44 1998), and polymerized PML undergoes efficient SUMOylation (Wang et al., 2018)
 45 (Li et al., 2019). Six nuclear PML isoforms have been identified in human cells; each
 46 isoform has a different C-terminal region, a distinction that results from alternative
 47 splicing of the PML-encoding primary transcript (Nisole et al., 2013). Five of the 6
 48 isoforms (excepting PML VI) have a SUMO interaction motif (SIM) (Cappadocia et al.,
 49 2015), a domain through which PML can non-covalently interact with SUMO or other
 50 SUMOylated proteins that also often contain SIMs (Sahin et al., 2014). Multivalent
 51 interaction between poly-SUMO and poly-SIM is postulated as a driving force that
 52 endows PML-NBs with liquid-like properties (Banani et al., 2016). The assembly
 53 generated by mixing multivalent molecules generally shows decreased solubility,
 54 thereby promoting phase separation of the assembly; the solubility shift of resident
 55 protein is a critical factor in liquid droplet formation (Banani et al., 2017). It long has
 56 been known that specific binding of arsenic or antimony induces a sharp decline in the
 57 solubility of PML (Müller et al., 1998; Hirano et al., 2015). The membrane-less
 58 properties of PML-NBs may facilitate dynamic interactions among PML-NB client
 59 molecules, which have been shown to be involved in a number of biological processes,

60 including viral infection (Chelbi-Alix et al., 1995; Puvion-Dutilleul et al., 1995;
61 Everett and Chelbi-Alix, 2007), DNA damage response (Louria-Hayon et al., 2003;
62 Bernardi et al., 2004; Bøe et al., 2006), senescence (Ferbeyre et al., 2000; Pearson et
63 al., 2000), and telomere recombination (Draskovic et al., 2009; Flynn et al., 2015).

64 Homozygous *Pml*^{-/-} mice have been shown to exhibit leucopenia (Wang et al., 1998)
65 and compromised innate defense (Lunardi et al., 2011). In contrast, the phenotypes of
66 the *Pml*^{-/-} mice during reproduction and the significance of PML in the meiosis of
67 germ cells have not (to our knowledge) been as thoroughly studied, likely due to the
68 normal fecundity of *Pml*^{-/-} mice (Wang et al., 1998). Indeed, PML has been
69 demonstrated to be dispensable for embryonic development, with *Pml*^{-/-} embryos
70 exhibiting increased resistance to acute oxidative stress compared to wild-type
71 embryos (Niwa-Kawakita et al., 2017). These findings suggest that PML or PML-NBs
72 play an accessory role in embryonic development. Although *Pml* mRNA is present in
73 unfertilized mouse oocytes (Ebrahimian et al., 2010; Cho et al., 2011), the appearance
74 of PML-NBs has not been reported, raising the possibility that *Pml* mRNA may be
75 dormant (i.e., transcripts are stable and left untranslated). Alternatively, although
76 PML-NBs are known to be ubiquitously distributed in adult organisms (Goddard et al.,
77 1995; Bernardi and Pandolfi, 2003), PML protein may not be phase-separated in a
78 manner sufficient to form PML-NBs in oocytes.

79 Mouse oocytes undergo growth in follicles until these cells are ready for
80 hormone-dependent ovulation. In response to hormone signaling, fully grown
81 germinal vesicle (GV) oocytes resume meiotic division characterized by GV
82 breakdown (GVBD) and subsequent polar body extrusion before fertilization with

83 sperm (Racki and Richter, 2006). During oocyte growth at meiosis prophase I, as in
 84 interphase in somatic cells, the nucleus is surrounded by a nuclear membrane and
 85 includes actively transcribing, three-compartmented nucleoli (Fulka et al., 2020). As
 86 oocyte growth proceeds, non-membranous organelles dynamically change their
 87 characteristics and their fates. For instance, processing bodies (P-bodies) disappear
 88 early in oocyte growth, with some P-body components transiently forming subcortical
 89 aggregates (SCAs; storage compartments for maternal mRNAs) in GV oocytes (Flemr
 90 et al., 2010). Decreased transcription in oocytes results in a reduced number of
 91 enlarged nuclear speckles, which appear to retain unspliced pre-mRNAs (Ihara et al.,
 92 2008). The nucleolus loses sub-compartments upon gradual shutdown of rRNA
 93 synthesis, forming nucleolus-like bodies (NLBs) surrounded by heterochromatin in the
 94 nuclei of GV oocytes (Bouniol-Baly et al., 1999). These active fluxes of
 95 non-membranous organelles spatiotemporally regulate maternal RNA metabolism
 96 during oocyte growth.

97 Although the oocyte nucleus is an active region for phase-separated organelles, it
 98 remains unclear why oocytes are devoid of specific membrane-less organelles such as
 99 PML-NBs and Cajal bodies (Fulka and Aoki, 2016). The present study addressed the
 100 question of why the PML-NB-free intranuclear milieu of oocytes facilitates oocyte
 101 growth.

102

103 **Experimental Procedures**

104 *Chemicals, reagents, and antibodies*

105 Sodium arsenite (NaAsO₂), Triton X-100, 3-isobutyl-1-methylxanthine (IBMX), and
106 K-modified simplex optimized medium (KSOM) were purchased from Sigma (St.
107 Louis, MO, USA). Paraformaldehyde (PFA), dimethyl sulfoxide (DMSO), bovine
108 serum albumin (BSA), actinomycin D (AcD), and kanamycin were purchased from
109 WAKO (Osaka, Japan). Pregnant mare serum gonadotropin (PMSG) and human
110 chorionic gonadotropin (hCG) were purchased from Sigma and ASKA Pharmaceutical
111 (Tokyo, Japan). Modified human tubular fluid (mHTF) medium was purchased from
112 Kyudo (Saga, Japan). Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) was
113 purchased from Calbiochem (San Diego, CA, USA). Epoxomicin was purchased from
114 Enzo Life Sciences (Farmingdale, NY, USA). α -minimum essential media (MEM)
115 medium, penicillin/streptomycin, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic
116 acid (HEPES) were purchased from Gibco/Thermo Fisher Scientific (Grand Island,
117 NY, USA). Lipofectamine LTX-Plus reagents, TOP10 competent cells, NuPAGE
118 4-12% Bis-Tris gels, lithium dodecyl sulfate (LDS) sample buffer, BoltTM WB
119 systems, and MagicMarkTM XP size standards were purchased from Invitrogen/Thermo
120 Fisher Scientific (Carlsbad, CA, USA). Mineral oil was purchased from Nacalai
121 Tesque (Kyoto, Japan). ML-792 was purchased from MedKoo Biosciences
122 (Morrisville, NC, USA). Hoechst dye was purchased from Dojin Chemical
123 (Kumamoto, Japan). DNase was purchased from Ambion/Thermo Fisher Scientific
124 (Carlsbad, CA, USA). Radioimmunoprecipitation (RIPA) lysis solutions (containing
125 0.1% sodium dodecyl sulfate), protease inhibitor cocktail, phenylmethylsulfonyl

126 fluoride (PMSF), and sodium orthovanadate (SOV) were purchased from Santa Cruz
 127 Biotechnology (Santa Cruz, CA, USA). KOD-FX and KOD-plus Mutagenesis Kits
 128 were purchased from TOYOBO (Osaka, Japan). Fetal bovine serum (FBS) was
 129 purchased from Biowest (Nuaille, France). ECLTM Prime was purchased from GE
 130 Healthcare (Buckinghamshire, UK). The following antibodies were used in this study:
 131 anti- α thalassemia/mental retardation syndrome X-linked protein (ATRX), anti-death
 132 domain-associated protein (DAXX), anti-human PML (sc-966), Alexa 488-conjugated
 133 anti-SUMO2/3, anti-lamin B, horseradish peroxidase (HRP) -conjugated goat
 134 anti-mouse or -rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology),
 135 anti-human PML (A301-167A: Bethyl, Montgomery, TX, USA), anti-mouse PML
 136 (#05-718: Millipore), anti-SUMO1 (#4940), anti-survivin, anti-early endosome
 137 antigen 1 (EEA1) (Cell Signaling Technology, Danvers, MA, USA), anti-multi
 138 ubiquitin (Ub, #D058-3), anti-SUMO2/3 (#M114-3: MBL, Nagoya, Japan),
 139 anti-calcium-responsive transactivator (CREST) protein human antiserum (Immuno
 140 Vision, Springdale, USA), anti-synaptonemal complex protein 3 (SYCP3),
 141 anti-SUMO-conjugating enzyme (Ubc9), anti-proteasome 20S α 1+2+3+5+6+7
 142 (20S) (Abcam, Cambridge, UK), anti-70-kDa heat shock proteins (Hsp70)
 143 (StressMarq Biosciences, Victoria, Canada), anti-splicing component, 35 kDa (SC35)
 144 (S4045: Sigma), and Alexa 488-, 594-, or 647-conjugated secondary antibodies
 145 (Molecular Probes/Thermo Fisher Scientific). The #M114-3 clone of anti-SUMO2/3
 146 antibody also was used to generate additional Alexa 488-conjugated anti-SUMO2/3
 147 antibody, which was labeled using an Alexa FluorTM 488 Antibody Labeling Kit
 148 (Molecular Probes/Thermo Fisher Scientific, Eugene, OR, USA)

149 *Collection and culture of oocytes, zygotes, and embryos*

150 All animal procedures and protocols were in accordance with the *Guidelines for the*
151 *Care and Use of Laboratory Animals* (June 2021 edition) and were approved by the
152 Animal Care and Use Committee of the National Institute for Environmental Studies
153 (NIES) (Approval No. 21-002). C57BL/6J mice were purchased from CLEA-Japan
154 (Kawasaki, Japan). The animals were housed under a 12-hr/12-hr light/dark cycle with
155 free access to food and water. Unless otherwise mentioned, oocytes were cultured in
156 medium for *in vitro* maturation (basal medium: α -MEM medium supplemented with
157 penicillin/streptomycin (100 units/mL and 100 μ g/mL, respectively) and
158 heat-inactivated FBS (10%)). Actively transcribed and meiotically incompetent
159 oocytes collected from postnatal days 12-16 are referred to as “maturing oocytes” in
160 the present study. Subsets of oocytes in the ovary form follicles that then grow
161 (folliculogenesis), a process that initiates just before birth and continues to postnatal
162 days 22-24 (Racki and Richter, 2006; Rodriguez et al., 2019). The status of the
163 full-sized oocytes remains arrested at meiotic prophase with a large nucleus, a
164 structure that is designated the germinal vesicle (GV). The GV oocyte stage can be
165 subdivided into the non- surrounded nucleolus (NSN) stage and the surrounded
166 nucleolus (SN) stage based on maturity. The SN stage is characterized by a
167 transcriptionally inactive heterochromatin rim (i.e., with heterochromatin surrounding
168 the nucleolus-like body (NLB)), wherein the cell is primed for breakdown of nuclear
169 membrane (GVBD), a hallmark of the resumption of meiosis. Except for the oocytes
170 depicted in Fig. S1B-D (which represent samples collected directly from adult ovaries),
171 Metaphase I (MI) and Metaphase II (MII) -stage oocytes were obtained by *in vitro*
172 culture as follows. For collection of fully grown GV oocytes, follicles on the ovarian
173 surface were mechanically ruptured with a pair of forceps. GV oocytes initially were

174 isolated in basal medium supplemented with HEPES (12.5 mM) and IBMX (0.1 mM);
175 IBMX is a phosphodiesterase inhibitor that inhibits/controls spontaneous meiotic
176 resumption. After IBMX was removed by washing with basal medium, oocytes were
177 incubated for 4 hr to allow GVBD to proceed. Development of oocytes was further
178 verified by observation of the extrusion of the first polar body (Pb1; a marker of
179 MII-stage oocytes that have the potential to be fertilized with sperm) after an
180 additional incubation of up to 16 hr. We regarded Pb1-free oocytes with no GV as
181 MI-stage oocytes. To obtain embryos, female mice were initially primed by
182 intraperitoneal injection of PMSG followed 48 hr later by injection with hCG.
183 Superovulated oocytes were collected from the oviducts of euthanized mice by gently
184 teasing apart of the ampulla with a 21-gauge needle (TERUMO, Tokyo, Japan) to
185 release cumulus-oocyte complexes in mHTF medium; these oocytes then were
186 inseminated with pre-capacitated sperm. To assess pronuclear (PN) stages after
187 insemination, we evaluated fertilized oocytes based on the distance between the male
188 and female pronuclei. PN stages were defined as follows: PN3, smaller pronuclei that
189 are distributed distantly; PN5, bigger pronuclei that overlap. After fertilization,
190 embryos were cultured further until the indicated embryonic stages or time points
191 using KSOM medium. Cultures were performed in a drop of medium (30-80 μ L)
192 under a mineral oil overlay at 37 °C in a humidified atmosphere of 5% CO₂
193 (APM-30D; ASTEC, Fukuoka, Japan), except for heat shock (HS) experiments, which
194 were performed at 42 °C in ambient air (FMC-1000; EYELA, Tokyo, Japan).

195 *Western blot analysis for PML and SUMO in CHO-K1 cells*

196 Using Lipofectamine LTX-Plus reagents, CHO-K1 cells were transiently transfected
 197 with plasmids harboring *PmlVI* (human PML transcript variant 5, NM_033244) or a
 198 cDNA encoding a SUMOylation-deficient version of the protein. The former construct,
 199 which we designated v5-hPMLVI, was obtained from OriGene (Rockville, MD, USA).
 200 The latter construct, which we designated v5-hPMLVI (K160, 490R), encodes a
 201 mutant version of the same protein in which the nucleic acid sequences encoding the
 202 K160 and K490 amino acid residues were altered to encode Arg residues (by mutation
 203 using the KOD-plus Mutagenesis Kit according to the manufacturer's instructions).
 204 The K160 and K490 residues correspond to sites known to be required for
 205 SUMOylation (Lallemand-Breitenbach et al., 2008). The transfected cells were
 206 washed with phosphate-buffered saline (PBS, pH 7.1-7.7) and then lysed on ice with
 207 100 μ L of RIPA lysis buffer pre-mixed with protease inhibitor cocktail (1:100,
 208 vol/vol), PMSF (a serine protease inhibitor; 2 mM), and SOV (a phosphatase inhibitor;
 209 1 mM). The lysate was centrifuged at 9,000 x g for 5 min at 4 °C and the resulting
 210 supernatant was designated as the RIPA-soluble fraction. The pellet was washed again
 211 with PBS and then treated with DNase (40 U/mL) to reduce the viscosity. Pellets were
 212 ruptured by ultrasonication at 4 °C for 30 min using a BioruptorTM (UCD-250,
 213 H-amplitude, repeating 15-sec sonication at 45-sec intervals; Cosmobio, Tokyo,
 214 Japan) followed by a 1-hr incubation at 25 °C with intermittent vortexing using a
 215 thermomixer (Eppendorf, Wesseling-Berzdorf, Germany). Aliquots were designated as
 216 the RIPA-insoluble fraction and subsequently mixed with LDS sample buffer and
 217 boiled at 95 °C for 3 min before being stocked frozen at -30 °C. Before loading onto
 218 gels, samples again were boiled at 95 °C for 10 min. Proteins in the samples were
 219 resolved by LDS-polyacrylamide gel electrophoresis (LDS-PAGE) and electroblotted

220 to membranes; the membranes were blocked and then probed with primary antibodies
221 overnight at 4 °C before hybridization with secondary antibodies for 1 hr at room
222 temperature. Signals obtained with ECLTM Prime Chemiluminescent HRP Substrate
223 were detected with an FAS1100 (TOYOBO).

224 *Plasmid or mRNA microinjection*

225 The Emerald Green Fluorescent Protein (EmGFP) -encoding region from Vivid
226 ColorsTM cDNA pcDNATM6.2/N-EmGFP-DEST Vector (Thermo Fisher Scientific)
227 was placed upstream of the coding sequence of *PmlVI* (human PML transcript variant
228 5, NM_033244) as described previously (Hirano et al., 2018). The fusion protein
229 encoded by the resulting construct was designated GFP-hPMLVI. Each plasmid,
230 including v5-hPMLVI and v5-hPMLVI (K160, 490R), was diluted to a concentration
231 of 5 ng/μL with Milli-Q water. mRNA preparation was conducted by BEX (Tokyo,
232 Japan) by *in vitro* transcription from v5-hPMLVI or v5-hPMLVI (K160, 490R) using
233 a HiScribeTM T7 ARCA mRNA Kit with tailing (New England Biolabs, Ipswich, MA,
234 USA) and a MEGAcleanTM Transcription Clean-Up Kit (Thermo Fisher Scientific).
235 Each mRNA was diluted to a concentration of 25 or 100 ng/μL with RNase free water
236 (Jena Bioscience, Jena, Germany). Each solution was loaded into a DNA injection
237 pipette (TIP-DNA [LIC-OD1], NAKA Medical, Tokyo, Japan), and the pipettes were
238 placed in an IM-9B microinjector (Narishige, Tokyo, Japan). MN-4 and MMO-202ND
239 manipulators (Narishige) were adapted to an IX70 inverted microscope (Olympus,
240 Tokyo, Japan) via NO-PIX-4-P (Narishige). Plasmid or mRNA solutions were injected
241 into each oocyte nucleus or cytoplasm (respectively) until adequate swelling of the
242 nuclear or plasma membrane (respectively) was observed. Injection was conducted in

243 the basal medium supplemented with HEPES (12.5 mM) and IBMX (0.1 mM).
 244 Injected oocytes were washed and cultured without HEPES during the interval
 245 indicated for each experiment.

246 *Immunofluorescent (IF) staining*

247 Except for the live imaging of GV oocytes in Figure 2A, oocytes, zygotes, and
 248 embryos at each indicated stage were fixed with 4% phosphate-buffered PFA (pH
 249 7.0-7.4) at room temperature. Cells were permeabilized with 0.5% Triton X-100 in
 250 PBS, blocked with 5% BSA-PBS, and then stained with the indicated primary
 251 antibodies in 1% BSA-PBS. Cells were visualized with Alexa-conjugated secondary
 252 antibodies; Hoechst dye was included to stain DNA. Cells were placed in small drops
 253 (4 μ L each) of 1% BSA-PBS, covered with mineral oil, in a glass-bottomed 35-mm
 254 petri dish (AGC techno glass, Shizuoka, Japan). Images were captured by confocal
 255 microscopy (Leica TCS-SP5; Leica, Solms, Germany).

256 For the detection of nascently translated polypeptides, oocytes or embryos were
 257 cultured with O-propargyl-puromycin (OP-puro) (obtained as part of a Click-iT[®] Plus
 258 OPP Protein Synthesis Assay Kit; Molecular Probes/Thermo Fisher Scientific) at a
 259 concentration of 20 μ M for the indicated time periods, followed by Click reaction
 260 according to the manufacturer's instructions. Images were captured by confocal
 261 microscopy.

262 As a reference for PML-NB staining, bone marrow from euthanized male C57BL/6J
 263 mice was exposed by removing both ends of the femur; bone marrow cells then were
 264 obtained by flushing the marrow with PBS using a 25-gauge needle (TERUMO). The
 265 resulting cells were immediately cytocentrifuged onto slide glass and air-dried. The

cell preparations were used for immunostaining for visualization of endogenous PML-NBs. Images were captured by fluorescence microscopy (ECLIPSE 80i; Nikon, Tokyo, Japan).

Electron microscopic (EM) analysis

GV oocytes were cultured for 20 hr. Metaphase oocytes were fixed in 4% PFA, 0.2% glutaraldehyde, and 0.5% tannic acid in 0.1 M cacodylate buffer, pH 7.4, for 100 min at room temperature. The fixed oocytes then were washed with 0.1 M cacodylate buffer. Further procedures were conducted by Tokai Electron Microscopy, Inc. (Nagoya, Japan). After dehydration, the sample was embedded in resin (LR White; London Resin, Berkshire, UK). The polymerized resin was ultra-thin sectioned at thicknesses of 80 nm, and the sections were mounted on nickel grids. The grids were incubated with the primary antibody (anti-mouse PML, #05-718) in 1% BSA-PBS overnight at 4 °C. The grid-mounted sections subsequently were incubated for 1 hr at room temperature with the secondary antibody conjugated to 20-nm gold particles (EMGMHL20; BBI Solutions, Crumlin, UK). Grids then were placed in 2% glutaraldehyde in 0.1 M cacodylate buffer. Subsequently, the grids were dried and stained with 2% uranyl acetate for 15 min, and then placed in lead staining solution (Sigma) at room temperature for 3 min. The grids were observed by transmission electron microscopy (JEM-1400Plus; JEOL, Tokyo, Japan).

Data analysis

Where indicated, data are presented as means with the standard error of the mean (SEM).

289 **Results**

290 *PML-NBs are not formed during development of oocytes*

291 To understand the location of PML protein in oocytes during and after the resumption
 292 of meiosis, GV oocytes were collected from ovaries and cultured *in vitro*. In the
 293 nucleus of GV oocytes, PML accumulated around chromatin near the nuclear
 294 membrane (**Fig. 1A; Fig. S2C**). The peri-chromosomal distribution of PML protein
 295 also was observed in oocytes in the GVBD, MI, and MII stages (**Fig. 1A**). Immuno-
 296 EM analysis (using anti-mouse PML primary antibody and gold-conjugated secondary
 297 antibody) revealed that gold particles (indicative of PML-positive staining) were
 298 localized peri-chromosomally as amorphous protein aggregate-like structures (**Fig.**
 299 **S1A**). IF analysis of PML, in combination with staining for a chromosomal passenger
 300 complex marker and a kinetochore marker, revealed that PML was arranged in a
 301 pattern extending from the kinetochores with localization along the chromosome arms
 302 (**Fig. S1B,C**). In contrast to mitotic assemblies of PML proteins (MAPPs), which are
 303 tethered to early endosomes in metaphase-stage somatic cells (Dellaire et al., 2006),
 304 PML in oocytes never co-localized with early endosomes (which themselves were
 305 labeled with anti-EEA1 antibodies, as assessed by IF) during metaphase (**Fig. S1D**).
 306 To examine the spatiotemporal distribution of PML protein, oocytes at various meiotic
 307 stages were collected. Among ovarian cells (55 of which were surveyed in detail)
 308 collected from postnatal day 1 mice, cells that stained positively for SYCP3 (a marker
 309 of germ cells in meiotic prophase) exhibited nuclear staining for PML just beneath the
 310 inner nuclear membrane; however, PML was not detected as nuclear bodies (NBs) in
 311 these cells (**Fig. 1B**). In contrast, NBs were seen in 98.1% of the 162 control (bone
 312 marrow) cells examined (**Fig. S1F**). Similarly, in the pronuclei of zygotes as well as

313 fertilized 2-cell embryos, PML protein was observed just beneath the inner nuclear
 314 membrane (**Fig. 1C**), where the heterochromatin is known to be enriched (Bersaglieri
 315 and Santoro, 2019). When a similar analysis was performed on zygotes, no NBs were
 316 observed in the nucleoplasm among the 32 zygotes that were surveyed, (**Fig. 1C**).
 317 During the development of preimplantation embryos, PML-NBs were unambiguously
 318 detected in the nucleus of each blastomere, at least in the morula to early blastocyst
 319 stage (**Fig. S1E**); In post-morula-stage embryos, PML also was slightly enriched
 320 beneath the nuclear membrane, as observed in 2-cell embryos (**Fig. 1C**). PML proteins
 321 in NBs exhibited co-localization with SUMO and with DAXX (**Fig. S1E**), which are
 322 representative clients of the PML-NBs (Lallemand-Breitenbach and de The, 2018).
 323 Thus, it appeared that oocyte PML is associated with the heterochromatin, a
 324 localization that is coordinated with the turnover of nuclear membrane in oocytes
 325 undergoing meiosis.

326

327 *The dynamics of nascent PML-NBs in the nucleoplasm demands SUMO*

328 To address if any possible advantages are present in the PML NB-free intranuclear
 329 milieu of oocytes during development, we attempted the deliberate assembly of NBs
 330 in the nuclei of GV oocytes. For concise interpretation, we injected a plasmid that
 331 encodes GFP-hPML^{VI}, a SUMO interaction motif (SIM) -free variant of PML,
 332 avoiding non-covalent interaction with SUMO or the SUMOylated moieties of
 333 modified proteins that often also contain SIMs. Despite a low success rate of
 334 expression (<10%), presumably due to the transcriptional quiescence of GV oocytes,
 335 injection of this construct resulted in the formation of NBs in the nuclei of GV oocytes

(**Fig. 2A**). In these oocytes, exogenously formed GFP-hPML**VI**-induced PML-NBs did not exhibit colocalization with either the endogenous ATRX or DAXX (**Fig. 2B**), which are known clients of PML-NBs (Lallemand-Breitenbach and de The, 2018). In contrast, GFP-hPML**VI**-induced PML-NBs exhibited colocalization with endogenous SUMO2/3 in a manner that depended on the degree of NB formation. This dependency also was observed in actively transcribing oocytes obtained from mice at approximately postnatal day 12-16 (i.e., maturing oocytes that are meiotically incompetent (De La Fuente, 2006)) (**Fig. 2C**). A higher efficiency of plasmid expression (reaching >70%) was obtained in these maturing oocytes than in GV oocytes. Notably, in the presence of arsenite, even the faintly visible nascent GFP-hPML**VI**-induced PML-NBs efficiently sequestered SUMO (**Fig. 2D**). Given the observed tight association between GFP-hPML**VI**-induced PML-NBs and SUMO, we next assessed the effects of PML-NB formation on molecules involved in the SUMOylation-triggered PML catabolism and nuclear stress responses. Although it has been reported that ubiquitin and proteasomes are recruited to SUMOylated PML when exposed to arsenic (Lallemand-Breitenbach et al., 2008), these two molecules and a related chaperone were not recruited to GFP-hPML**VI**-induced PML-NBs (**Fig. 2E**). Other than the nucleolus, PML-NB also has been reported to act as overflow compartments for misfolded proteins, a process that occurs under conditions of proteotoxic stress and is mediated by the ubiquitin-proteasome system upon in the nuclei of cultured somatic cells (Uozumi et al., 2016; Mediani et al., 2019). The relative paucity of the compartments dealing with the proteinous wastes could be a disadvantage for, PML-NB-free, the oocyte. In practice, we showed that aberrant polypeptides (generated in the presence of the proteasome inhibitor MG132), which

360 were labeled with an analog of puromycin (OP-puro), accumulated only in the
 361 nucleolus, and never in GFP-hPML^{VI}-induced PML-NBs (**Fig. 2F; Fig. S2A**).
 362 Similarly, in post-morula-stage embryos under proteotoxic stress, OP-puro-labeled
 363 aberrant polypeptides did not accumulate in endogenously appearing PML-NBs (**Fig.**
 364 **S2B**). Together, these results suggested that oocyte nucleoplasm can accommodate
 365 PML-NBs that preferentially sequester SUMO.

366 We next used the deliberate assembly of PML-NBs to more thoroughly analyze
 367 SUMO availability in maturing oocytes. First, oocytes were manipulated to limit the
 368 availability of SUMO with which PML could interact. Specifically, we injected
 369 oocytes with a construct (v5-hPML^{VI} (K160, 490R)) that encodes a mutated SIM-free
 370 variant of PML that has decreased SUMOylation efficiency, thereby decreasing the
 371 protein's avidity for SUMO and the SIM moiety of SUMOylated proteins (**Fig. S3A**).
 372 We found that oocytes injected with plasmid v5-hPML^{VI} (K160, 490R) exhibited a
 373 decreased number (2.22 ± 0.36 (mean $\pm$ SEM), across 12 oocytes) of enlarged
 374 v5-hPML^{VI} (K160, 490R) -induced PML-NBs compared to the dozens observed in
 375 oocytes injected with the wild-type PML^{VI}-encoding plasmid (**Fig. 3A,B**). In the
 376 v5-hPML^{VI} (K160, 490R) -injected oocytes, SUMO protein accumulated with the
 377 v5-hPML^{VI} (K160, 490R) -induced PML-NBs, but did not merge with the PML
 378 (K160, 490R) shell of the NB, as if the SUMO protein were physically isolated within
 379 the PML-negative inner core (**Fig. 3B**). While arsenite efficiently promotes
 380 SUMOylation of PML, the SUMOylation-deficient mutant of PML has been reported
 381 to remain biochemically responsive to arsenite (Lallemand-Breitenbach et al., 2001).
 382 We first confirmed this observation by showing (using our system) that arsenite
 383 induces a sharp decline in the solubility (which favors *de novo* phase separation

384 (Banani et al., 2017)) of v5-hPMLVI (K160, 490R) -encoded protein, similar to that
 385 seen with the wild-type v5-hPMLVI -encoded protein (**Fig. S3B**). In oocytes injected
 386 with the plasmid v5-hPMLVI, arsenite treatment altered the spatial relationships
 387 between PML and SUMO from co-localization (**Fig. 3A**) to spherical co-layering (**Fig.**
 388 **3C**). In contrast, we observed the nascent formation of small-dot structures upon
 389 arsenite treatment in the v5-hPMLVI (K160, 490R) -injected oocytes (**Fig. 3D#1,#2,**
 390 **arrows**), and found that these structures frequently were clustered (**Fig. 3D#2,**
 391 **asterisks**). Notably, partial scaffolding of SUMO within the inner cores of
 392 v5-hPMLVI (K160, 490R) -induced PML-NBs assumed crooked shapes, presumably
 393 indicating partial SUMOylation of the v5-hPMLVI (K160, 490R) -encoded protein
 394 (**Fig. 3D#1,#2, arrowheads**).

395 Next, to understand the clustering of the nascent v5-hPMLVI (K160, 490R) -induced
 396 PML-NBs, we treated maturing oocytes with ML-792 (an inhibitor of a
 397 SUMO-activating enzyme) to limit the nucleoplasmic availability of SUMO (He et al.,
 398 2017). Compared to the nuclei of maturing oocytes treated with vehicle, the nuclei of
 399 maturing oocytes treated with ML-792 lacked general staining with anti-SUMO1
 400 antibody, exhibiting staining only at the nuclear membrane (**Fig. 3E**). Maturing
 401 oocytes injected with the GFP-PML-encoding plasmid and cultured in the presence of
 402 ML-792 exhibited a decreased number (1.96 ± 0.18 ; 27 oocytes cultured for a shorter
 403 period of 22 hr were quantified) of GFP-hPMLVI-induced PML-NBs that clustered
 404 together (**Fig. 3F**). Collectively, these results suggested that maturing oocytes
 405 maintain the dynamics of PML-NBs at the cost of SUMO availability in the
 406 nucleoplasm.

407 *PML-NBs potentially affect the efficiency of the response of SUMO mediated by liquid*
 408 *droplet formation upon exposure to multiple stresses*

409 We noticed that in maturing oocytes, a portion of the SUMO signal was detected as
 410 small drops (**Fig. 4A**) in the nucleolus, which is itself a droplet formed by liquid-liquid
 411 phase separation (Feric et al., 2016). Transcriptional inhibition with actinomycin D
 412 (AcD), an inducer of a nucleolar stress response (Sundqvist et al., 2009), resulted in
 413 dispersion of SUMO drops within the nucleoplasm (**Fig. 4A**). The
 414 nucleoplasm-localized SUMO signal following AcD exposure was sequestered by
 415 GFP-hPMLVI-induced PML-NBs (**Fig. 4B**). These results suggested that formation of
 416 PML-NBs in transcriptionally active maturing oocytes potentially impedes the
 417 SUMOylation typically seen as part of the nucleolar stress response.

418 SUMO localized primarily in the nucleoplasm, with several depositions at sites of
 419 condensed DNA (i.e., heterochromatin) in vehicle-treated NSN-stage GV oocytes (**Fig.**
 420 **4C, arrowheads**). During an examination of the effects of various types of proteotoxic
 421 stress on oocytes (**Fig. 2F; Fig. S2A**), we noticed that upon heat shock (HS;
 422 incubation at 42 °C for 2 hr), SUMO delocalized and exhibited co-localization with
 423 SC35 (a marker of nuclear speckles) -positive droplets (**Fig. 4C, NSN**). In
 424 vehicle-treated SN-stage oocytes, SUMO was highly enriched along the
 425 heterochromatin rim (**Fig. 4C, arrow**), while SC35-positive droplets disappeared.
 426 Upon HS, the SUMO signal formed SUMO droplets that tightly co-localized with
 427 SC35-positive droplets (**Fig. 4C, NSN-SN and SN**). In other experiments, we treated
 428 maturing oocytes with proteasome inhibitors for prolonged intervals. Exposure of
 429 maturing oocytes to MG132 for 39 hr (**Fig. 4D; Fig. S4A**) or to epoxomicin for 45 hr

430 **(Fig. S4D)** resulted in the formation of large SUMO droplets that co-localized with
 431 SC35-positive droplets, consistent with the results of the HS experiments. We next
 432 tested the effect of deliberate assembly of PML-NBs on the response to proteotoxic
 433 stressors, taking into consideration the observation (data not shown) that GV oocytes
 434 were resilient to HS compared to maturing oocytes (in which the whole cells were
 435 easily distorted). However, we were unable to obtain assembly of PML-NBs in GV
 436 oocytes by injecting *hPMLVI* mRNA (a highly effective method for driving gene
 437 expression, used here as an alternative to plasmid injection) unless the recipient
 438 oocytes were exposed to additional stimuli **(Fig. S3C,D)**. Accordingly in maturing
 439 oocytes, when the exposure to a proteotoxic stressor was conducted after deliberate
 440 assembly of PML-NBs by the injection of the GFP-hPMLVI-encoding plasmid,
 441 SUMO was sequestered not with SC35-positive droplets but with
 442 GFP-hPMLVI-induced PML-NBs **(Fig. 4E)**. When considered together with the data
 443 indicating that assembled PML-NBs in oocytes are not involved in the regulation of
 444 misfolded proteins upon proteotoxic stress **(Fig. 2F; Fig. S2A,B)**, these results
 445 suggested that nascent PML-NBs in the nucleoplasm indirectly affect the efficiency of
 446 the SUMO response that is mediated by liquid droplet formation upon exposure to
 447 stressors.

448

449 Discussion

450 We conjecture that if PML proteins were phase separated to form NBs in the
 451 nucleoplasm of oocytes, the available SUMO would be preferentially used for the
 452 maintenance of the dynamics of nascent PML-NBs. We tested this hypothesis by
 453 modifying the availability of SUMO in the nucleoplasm, both from the PML and
 454 SUMO sides. The limited number of enlarged v5-hPML^{VI} (K160, 490R) -induced
 455 PML-NBs (**Fig. 3B**) was consistent with previous work, including studies (a)
 456 conducted in cultured cells expressing PML mutant proteins harboring the K65A
 457 mutation (K65 is another residue involved in SUMOylation, via a process that is
 458 tightly coupled to K160 SUMOylation) (Lallemand-Breitenbach et al., 2008); (b)
 459 testing PML-NBs in SUMO mutant-transfected cells that are deficient in SUMO
 460 conjugation or polymerization (Fu et al., 2005); and (c) in SUMO-conjugating enzyme
 461 (*Ubc9*) -deficient blastocysts (Nacerddine et al., 2005). A recent study employing
 462 polymer physics proposed a model for the organization of paraspeckle, another
 463 core-shell type nuclear body. That report showed that parts of the building domains of
 464 the resident (long non-coding RNAs) are mutually repulsive when positioned at the
 465 surface of paraspeckle, which accordingly determines the dynamics (size, number, and
 466 distribution) of the nuclear body; however, these repulsive domains are not required
 467 for the assembly of paraspeckle itself (Yamazaki et al., 2021). Notably, SUMOylation
 468 is dispensable for the formation of PML-NBs (Sahin et al., 2014). As shown in the
 469 wild-type v5-hPML^{VI} -injected oocytes (**Fig. 3C**), arsenite treatment induces rapid
 470 polymerization of SUMO chains on insolubilized PML (**Fig. S3B**) at the shell, such
 471 that the structure formed spherical co-layer; this observation suggested that the
 472 structure was dominated by minimization of surface tension. The crooked-shaped

shells (**Fig. 3B**), which are composed of mutant PML whose avidity for SUMO is limited, may indicate that the availability of SUMO in the oocyte nucleoplasm is one of the determinants of the surface property (facing the nucleoplasm) of nascent PML-NBs. Tight sequestration of SUMO molecules into the PML-negative inner core (as observed in the v5-hPML^{VI} (K160, 490R) -injected oocytes) is reminiscent of the sequestration of the profluorescent biarsenical dye 4,5-bis(1,3,2-dithiarsolan-2-yl)fluorescein (FIAsH) in the cores of C212/213A-arsenic binding site mutants (Jeanne et al., 2010), and is deserving of further study. In the present work, we utilized a chemical (ML-792) to deplete the availability of SUMO in the nucleoplasm based on a mechanism of covalent SUMO-ML-792 adduct formation within the SUMO E1 enzyme (Brownell et al., 2010; He et al., 2017). We found that newly assembled GFP-hPML^{VI}-induced PML-NBs frequently cluster together in maturing oocytes cultured in the presence of ML-792 (**Fig. 3F**). This aberrant tethering would mechanistically relate (at least in part) to the clustering of nascent v5-hPML^{VI} (K160, 490R) -induced PML-NBs in the nucleoplasm upon arsenite treatment (**Fig. 3D#2**), namely arsenite-dependent boosting of SUMOylation may anyhow be attempted on mutated PML, which has little avidity for SUMO. These results suggested that a NB-free intranuclear milieu is beneficial for maturing oocytes, enhancing the availability of SUMO molecules during oocyte maturation.

Three SUMO isoforms (SUMO1, 2, and 3) are expressed ubiquitously in mammals. SUMO2 is the major isoform expressed in embryogenesis; *Sumo2*-deficient mice die at approximately embryonic day 10.5 (Wang et al., 2014). In contrast, neither SUMO1 nor SUMO3 is essential for embryogenesis. There are three distinct steps to SUMOylation; Ubc9, the sole enzyme required for the second reaction, also is

497 indispensable for embryogenesis. Like embryos lacking SUMO2, *Ubc9*-deficient
 498 embryos die during embryogenesis, at approximately embryonic day 3.5-7.5, that is,
 499 just after implantation (Nacerddine et al., 2005). Mice harboring an oocyte-specific
 500 knockout of the *Ubc9*-encoding gene (*Ube2i-cKO*) show infertility with complete
 501 failure of oocytes to extrude the polar bodies (but no aberrancy in spindle
 502 morphology) (Rodriguez et al., 2019), supporting the notion that SUMOylation is
 503 critical for regulation of molecules involved in the MI-MII transition (Wang et al.,
 504 2010; Yuan et al., 2014; Ding et al., 2018). As has been suggested previously (Ihara et
 505 al., 2008), the *Ube2i-cKO* study indicates that the SUMOylation pathway is required
 506 not only for the resumption of meiosis but also for processes that begin before the GV
 507 stage. Since in the *Ube2i-cKO* study, Rodriguez et al. used a *Gdf-9 Cre* promotor as a
 508 driver (i.e., *Ube2i* deletion would have begun at approximately postnatal day 3), the
 509 observed dysfunction of gonadotropin-primed GV oocytes collected from 3-week-old
 510 mice implies that SUMOylation is required for a step occurring during folliculogenesis,
 511 during which oocytes undergo maturation. Although the specific relevant targets of
 512 SUMOylation currently are unknown, this previous literature suggests the significance
 513 both of SUMOylation and of SUMO availability in the nucleoplasm of maturing
 514 oocytes.

515 Individual maturing oocytes with maximum transcriptional activity collected from
 516 approximately postnatal day 14 gradually achieve a transcriptionally silent state,
 517 rendering these cells meiotically competent SN-type GV oocytes (De La Fuente, 2006).
 518 SN-type GV oocytes are observed from at least postnatal day 16-17 in mice and
 519 increase in proportion with mouse age (Bouniol-Baly et al., 1999; Inoue et al., 2007).
 520 Following global transcriptional repression, oocytes undergo chromosomal

reconfiguration around the nucleolus to acquire the full competence to become embryos. These two events are coordinated in time, but occur independently of each other, given that *nucleoplasmin 2* (*Npm2*) -null oocytes (which fail to remodel the chromosomal configuration) still show global transcriptional repression (De La Fuente et al., 2004), and given that *mixed lineage leukemia 2* (*Mll2*) -null oocytes (which show a normal chromosomal configuration) fail to repress transcriptional activity adequately (Andreu-Vieyra et al., 2010). Oocytes mutated in these genes suffer multiple insufficiencies that affect subsequent development, suggesting that accurate regulation of these events is vital for oocyte/embryo development. In the present study, we found that SUMO, especially SUMO1, was detected as small drops in droplet (the nucleolus) of fresh maturing oocytes in which active ribosomal biogenesis involving rDNA transcription was occurring. Even though global transcriptional repression is essential for development, data from exogenously assembled PML-NBs (induced by injection of a plasmid encoding GFP-hPMLVI) revealed that the SUMO response to AcD was impaired in such injected cells. These results suggested that, in normal oocytes devoid of PML-NBs, SUMO molecules evade sequestration by PML-NBs; this reserve of available SUMO is beneficial in case of the sudden quiescence of transcription (i.e., prematurity).

SUMO forms large droplets with SC35-positive droplets upon exposure to proteotoxic stressors. Although the droplet formation upon HS appears to accompany the arrest of enrichment of SUMO along the heterochromatin rim in SN-type GV oocytes (**Fig. 4C**; also note **Fig. S2C** showing heterochromatin rim in normal SN-type GV oocytes), the significance of this phenomenon remains to be determined. Whereas the enlargement of SC35-positive nuclear speckles also was observed in transcriptional repression (**Fig.**

545 **S4C**), the SUMO dispersion in response to AcD (**Fig. 4A**), as well as the response of
 546 Ubc9 to MG132 (dissimilarly to the response to AcD) (**Fig. S4B,C**, respectively),
 547 indicated that SUMO-SC35 droplet formation upon MG132 treatment is likely
 548 independent of transcriptional repression. Together, these results suggested that
 549 SUMO, in normal oocytes devoid of PML-NBs, is involved in the acquisition of
 550 meiotic competence and participates in the emergency response to multiple stresses.
 551 Notably, the large droplets of SUMO and SC35 were no longer observed in embryos
 552 exposed to HS at approximately the early blastocyst stage, when endogenous
 553 PML-NBs emerge (**Fig. S4E**). We therefore predict that oocytes should exhibit an
 554 interval until the endogenous PML-NB emergence following the completion of oocyte
 555 development.

556 In conclusion, the present study demonstrated time-course-related links of PML
 557 protein to heterochromatin in the development of oocytes, during which PML does not
 558 engage in phase separation to form NBs. The PML-NB-free intranuclear milieu of
 559 oocytes reflects the significance of the reserve of SUMO available for emergent
 560 responses. The insights described here are expected to enhance our understanding of
 561 how the dynamics of membrane-less organelles contributes to cellular events and to
 562 responses to developmental cues.

563

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570

571 **Author Contributions**

572 O.U. performed all experiments, with assistance from A.K-U. O.U. designed the
573 project, analyzed data, prepared figures, and wrote the manuscript. S.H. supervised the
574 project and wrote the manuscript.

575

576 **Declaration of Interests**

577 The authors declare they have no actual or potential competing financial interests.

578

579 **Figure legends**

580

581 **Fig. 1. Promyelocytic leukemia (PML) nuclear bodies (PML-NBs) are not formed**
 582 **during oocyte development.** (A) Subcellular localization of endogenous PML in
 583 oocytes during and after meiotic resumption. Germinal vesicle (GV) oocytes were
 584 cultured to obtain metaphase II (MII) -stage oocytes. Breakdown of the nuclear
 585 membrane (GV breakdown; GVBD), a hallmark of meiotic resumption, was evaluated
 586 by stereomicroscopy during culturing and verified by staining with anti-lamin B
 587 antibody (green). PML was visualized by staining with anti-mouse PML (mPML, red)
 588 antibody. BF, bright-field images of the oocytes. Scale bars, 20 μ m. (B) Representative
 589 image of endogenous PML in oocytes collected from mice at postnatal day 1. The insets
 590 show separate images for each channel. Scale bars, 4.98 μ m. (C) Representative images
 591 of endogenous PML in zygotes at the indicated pronuclear (PN) stages just after
 592 fertilization. The image of the pronuclear stage 5 (PN5) zygote was reconstructed as a
 593 z-stack image. The inner nuclear membrane of the 2-cell embryo also was co-labeled
 594 with anti-lamin B antibody fluorescing in a different color. BF, bright-field images of
 595 the zygotes and the embryo. Scale bars, 20.1 and 20.2 μ m, respectively.

596

597 **Fig. 2. Characterization of nuclear bodies (NBs) deliberately assembled in the**
 598 **nuclei of oocytes.** (A) Representative z-stack image of exogenously formed PML-NBs
 599 in a germinal vesicle (GV) oocyte, a model to gain insight into the benefit of a NB-free
 600 intranuclear milieu in oocytes. Briefly, GV oocytes were injected with a plasmid
 601 harboring an Emerald Green Fluorescent Protein (EmGFP) -encoding sequence
 602 upstream of the sequence of *PmlVI* (human PML transcript variant 5, NM_033244),

603 and cultured for 53 hr. The resulting encoded protein was designated GFP-hPMLVI. BF,
604 bright-field image of the oocyte. Scale bars, 19.9 μ m. (B) Representative images of
605 exogenously formed GFP-hPMLVI-induced PML-NBs and of representative PML-NB
606 markers in GV oocytes. α thalassemia/mental retardation syndrome X-linked protein
607 (ATRX) and death domain-associated protein (DAXX) are representative clients of
608 PML-NBs. Oocytes were visualized by immunofluorescent staining with anti-human
609 PML antibody and by co-staining with anti-ATRX (red, left) or -DAXX (red, right)
610 antibodies. Scale bars, 2.49 and 1.84 μ m, respectively. (C) Representative images of
611 GFP-hPMLVI-induced PML-NBs in the nuclei of actively transcribing maturing
612 oocytes; actively transcribing maturing oocytes were used to obtain a higher efficiency
613 of plasmid expression. Maturing oocytes were injected with a plasmid encoding
614 GFP-hPMLVI and cultured for 26.5 hr. Oocytes were stained with anti-human PML
615 (green) and anti-SUMO1 (red) antibodies. (#1) While GFP-hPMLVI-induced
616 PML-NBs were formed, a lower degree of SUMO sequestration was observed. (#2)
617 GFP-hPMLVI-induced PML-NBs were fully formed and SUMO was well sequestered.
618 Scale bars, 5.03 μ m. (D) Representative z-stack image of the nucleus of a maturing
619 oocyte injected with the plasmid encoding GFP-hPMLVI and cultured for 24 hr
620 followed by treatment with 3 μ M arsenite (As) for 2.5 hr. Oocytes were stained with
621 anti-human PML (green) and anti-SUMO1 (red) antibodies. Note the intensive
622 sequestration of SUMO, even by the faintly visible GFP-hPMLVI-induced PML-NBs.
623 Scale bars, 5.03 μ m. (E) Representative image of the nucleus of a maturing oocyte
624 injected with the plasmid encoding GFP-hPMLVI and cultured for 49.5 hr, then either
625 treated with 3 μ M arsenite or left untreated for another 2.5 hr. Oocytes were stained
626 with anti-multi ubiquitin (Ub), anti-proteasome 20S alpha 1+2+3+5+6+7 (20S), and

627 anti-70-kDa heat shock protein (Hsp70) (red) antibodies. Scale bars, 2.00 μ m. (F) An
 628 examination of whether GFP-hPML VI -induced PML-NBs act as overflow
 629 compartments for misfolded proteins. Representative image of the nucleus of a
 630 maturing oocyte injected with the plasmid encoding GFP-hPML VI and cultured for 50
 631 hr before labeling of newly synthesized aberrant polypeptides with an analog of
 632 puromycin (OP-puro, 20 μ M) (red) combined with proteasome inhibitor (MG132, 10
 633 μ M) for 30 min or 4 hr. Scale bars, 2.00 μ m.

634

635 **Fig. 3. The dynamics of nascent PML-NBs in the nucleoplasm demands SUMO.** (A
 636 and B) Representative images of the nuclei of maturing oocytes injected with plasmid
 637 v5-hPML VI (encoding wild-type human PML VI) (A, z-stack) or plasmid v5-hPML VI
 638 (K160, 490R) (encoding a human PML VI that has limited avidity for SUMO) (B,
 639 z-stack) and cultured for 45 hr or 43 hr, respectively. Oocytes were stained with
 640 anti-human PML (green) and anti-SUMO1 (red) antibodies. (B, inset) Merged images
 641 with Hoechst staining of (B). Scale bars, 2.00 μ m. (C) Representative image of the
 642 nucleus of a maturing oocyte injected with plasmid v5-hPML VI and cultured for 21 hr
 643 followed by treatment with 3 μ M arsenite (As) for 3 hr. Scale bars, 2.00 μ m. Enlarged
 644 images of the inset with diameter information. Scale bars, 1.00 μ m. (D) Representative
 645 images of the nuclei of maturing oocytes injected with plasmid v5-hPML VI (K160,
 646 490R) and cultured for 47 hr (D#1 and 2, z-stack) followed by treatment with 3 μ M
 647 arsenite for 3 hr. Oocytes were stained with anti-human PML (green) and anti-SUMO1
 648 (red) antibodies. Scale bars, 2.00 μ m. Arrows, nascent v5-hPML VI (K160, 490R)
 649 -induced PML-NBs upon arsenite treatment; arrowheads, enlarged v5-hPML VI (K160,
 650 490R) -induced PML-NBs and partial scaffolding of SUMO within the inner cores of

651 v5-hPMLVI (K160, 490R) -induced PML-NBs; asterisks, nascent v5-hPMLVI (K160,
652 490R) -induced PML-NBs that frequently are clustered upon arsenite treatment. (E)
653 Representative z-stack images of the nuclei of maturing oocytes treated with dimethyl
654 sulfoxide (DMSO, vehicle control) or with 20 μ M ML-792, an inhibitor of a
655 SUMO-activating enzyme limiting the nucleoplasmic availability of SUMO, for 4.5 hr.
656 Oocytes were stained with anti-SUMO1 antibody. (F) Representative z-stack images of
657 maturing oocytes injected with a plasmid encoding GFP-hPMLVI and cultured in the
658 presence of 20 μ M ML-792 for 47 hr. Oocytes were stained with anti-human PML
659 (green) antibody. Enlarged images of GFP-hPMLVI-induced PML-NBs that clustered
660 together are shown on the right. BF, bright-field image of the oocytes. Scale bars, 19.9
661 μ m (insets #1 to #4, 2.03 μ m).

662
663 **Fig. 4. PML-NBs potentially affect the efficiency of the response of SUMO**
664 **mediated by liquid droplet formation upon exposure to multiple stresses.** (A)
665 Representative images of the nuclei of maturing oocytes left untreated (cont) or treated
666 with 1 μ M actinomycin D (AcD), a transcriptional inhibitor, for 20 hr. Oocytes were
667 stained with Alexa 488-conjugated anti-SUMO2/3 antibody. (B) Representative image
668 of the nucleus of a maturing oocyte injected with a plasmid encoding GFP-hPMLVI and
669 cultured for 26 hr followed by treatment with 1 μ M AcD for 21 hr. The oocyte was
670 stained with anti-SUMO2/3 antibody (red). (C) Representative images of the nuclei of
671 GV oocytes cultured for 17 hr and then left untreated (cont) or subjected to heat shock
672 at 42 °C for 2 hr. NSN; non-surrounded nucleolus stage, SN; surrounded nucleolus stage.
673 Arrowheads indicate SUMO deposition at sites of condensed DNA in NSN-oocytes.
674 Arrow indicates the heterochromatin rim in SN-oocytes. Oocytes were stained with

675 anti-splicing component, 35 kDa (SC35) (red) and Alexa 488-conjugated
 676 anti-SUMO2/3 (green) antibodies. Scale bars, 2.00 μ m. (D) Representative images of
 677 the nuclei of maturing oocytes cultured for 26 hr and then left untreated (cont) or
 678 subjected to treatment with 10 μ M MG132 for 39 hr. Prolonged exposure to proteasome
 679 inhibitors is proteotoxic to oocytes. Oocytes were stained with anti-SUMO1 (green) and
 680 anti-SC35 (red) antibodies. Scale bars, 2.02 μ m. (E) An examination of whether
 681 exogenously assembled PML-NBs alter the response of SUMO to proteotoxic stress.
 682 Representative image of the nucleus of a maturing oocyte injected with a plasmid
 683 encoding GFP-hPMLVI and cultured for 25 hr followed by treatment with 10 μ M
 684 MG132 for 37 hr. Oocytes were stained with anti-SUMO1 (magenta) and anti-SC35
 685 (red) antibodies. Scale bars, 2.00 μ m.

686
 687 **Fig. S1, related to Fig. 1. Subcellular localization of endogenous PML in**
 688 **metaphase-stage oocytes.** (A) Representative electron micrograph image of
 689 peri-chromosomal localization of endogenous PML visualized with primary anti-mouse
 690 PML (mPML) antibody and gold particle-conjugated (Gold) secondary antibody.
 691 Germinal vesicle (GV) oocytes were cultured *in vitro* for 20 hr. A magnified electron
 692 micrograph is shown on the right. (B-D) Representative fluorescent images of oocytes
 693 collected from the ovaries of adult mice. The chromosome arm of the metaphase II
 694 (MII) -stage oocyte (B, z-stack), the kinetochore of the GV breakdown (GVBD)-
 695 metaphase I (MI) -stage oocyte (C, z-stack), and the early endosome of the MII-stage
 696 oocyte (D) were labeled with anti-survivin antibody, calcium-responsive transactivator
 697 (CREST) antiserum, and anti-early endosome antigen 1 (EEA1) antibody, respectively.
 698 BF, bright-field image of the oocyte. (E) Representative image of an embryo at

699 approximately the early blastocyst stage. (Upper) Subcellular localization of
 700 endogenous PML (red) and SUMO2/3 (green). Scale bars, 975 nm. (Lower)
 701 Co-localization of SUMO2/3 (green) with DAXX (red), a representative client of
 702 PML-NBs. Images were reconstructed as z-stack images. Scale bars, 20.2 μ m. (F)
 703 Representative image of endogenous PML-NBs (red) in control bone marrow-derived
 704 cells.

705

706 **Fig. S2, related to Fig. 2. An examination of whether exogenously assembled and**
 707 **endogenously appearing PML-NBs act as overflow compartments for misfolded**
 708 **proteins.** (A) Representative image of the nucleus of a maturing oocyte injected with a
 709 plasmid encoding GFP-hPMLVI and cultured for the indicated time. Before the end of
 710 culturing, newly synthesized aberrant polypeptides were labeled with 20 μ M OP-puro
 711 (red) combined with 100 μ M MG132 for the indicated time. BF, bright-field images of
 712 the oocytes. Scale bars, 2.00 μ m. (B) (Left) Representative image of an embryo cultured
 713 for 76 hr before labeling of newly synthesized aberrant polypeptides with 20 μ M
 714 OP-puro (red) combined with 10 μ M MG132 for 4 hr (80 hr post-insemination, 80
 715 h.p.i.). Endogenously appearing PML-NBs in the nucleus of each blastomere at the
 716 morula to early blastocyst stage were visualized by immunofluorescent staining with
 717 anti-mouse PML (mPML, green) antibody. BF, bright-field image of the embryo. Scale
 718 bars, 19.9 μ m. (Right) Enlarged images of the nuclei of representative blastomeres.
 719 Scale bars, 2.00 μ m. (C) Representative image of the nucleus of a germinal vesicle
 720 (GV) oocyte treated with dimethyl sulfoxide (DMSO, vehicle control) for 24 hr.
 721 Endogenous PML was visualized by staining with anti-mouse PML (mPML, red)
 722 antibody. The oocyte was further stained with Alexa 488-conjugated anti-SUMO2/3

723 antibody.

724

725 **Fig. S3, related to Fig. 3. Preparations for modifying the SUMO availability in**

726 **oocytes.** (A) Schematic domain structures of wild-type human PMLVI and a mutant

727 version of the same protein that has limited avidity for SUMO; these proteins were

728 encoded by the plasmids v5-hPMLVI and v5-hPMLVI (K160, 490R), respectively.

729 RING: Really Interesting New Gene domain, with conserved cysteine. B1 and B2:

730 zinc-binding boxes. CC: coiled-coil domain. Two SUMOylation sites are indicated by

731 aqua blue circles labeled with an S. These sites were mutated from lysine to arginine in

732 the SUMOylation-deficient mutant protein encoded by the v5-hPMLVI (K160, 490R)

733 construct. (B) Expression of constructs and biochemical response of their products to

734 arsenite. Immunoblot analysis of CHO-K1 cells transiently expressing wild-type human

735 PMLVI or the SUMOylation-deficient mutant protein. The fractions soluble or

736 insoluble to radioimmunoprecipitation (RIPA) lysis buffer were immunoblotted (IB)

737 with the indicated antibodies. The cells were exposed to 3 μ M arsenite (As) for 3 hr.

738 Green asterisk indicates the biochemical response of the v5-hPMLVI (K160, 490R)

739 -encoded mutant protein to arsenite (i.e., corresponding to a shift into the

740 detergent-resistant nuclear matrix (Lallemand-Breitenbach et al., 2008)), which was

741 comparable to that seen with the unmutated protein encoded by the v5-hPMLVI

742 construct. (C and D) Representative images of the nuclei of GV oocytes injected with

743 the mRNA transcribed *in vitro* from plasmid v5-hPMLVI (encoding wild-type human

744 PMLVI) (C) or v5-hPMLVI (K160, 490R) (encoding human PMLVI with limited

745 avidity for SUMO) (D) and cultured for 46 hr or 48 hr, respectively, and then left

746 untreated (cont) or subjected to treatment with 3 μ M arsenite for 2 hr. Oocytes were

747 stained with anti-human PML (red) and Alexa 488-conjugated anti-SUMO2/3 (green)
748 antibodies. NSN; non-surrounded nucleolus stage, SN; surrounded nucleolus stage.
749 Scale bars, 2.00 μm .

750

751 **Fig. S4, related to Fig. 4. Characterization of SUMO- SC35 droplets. (A and B)**

752 Representative images of the nuclei of maturing oocytes treated with dimethyl
753 sulfoxide (DMSO, vehicle control) or 20 μM ML-792 or 10 μM MG132 for the
754 indicated time. (A) Oocytes were stained with anti-SC35 (red) and Alexa
755 488-conjugated anti-SUMO2/3 (green) antibodies. (B) Oocytes were stained with
756 anti-Ubc9 antibody. (C) Comparison of the response of SC35 droplets with and
757 without transcriptional inhibition. Representative images of the nuclei of maturing
758 oocytes treated with DMSO or 1 μM actinomycin D (AcD) for 16 hr. Oocytes were
759 stained with anti-SC35 (red) and anti-Ubc9 (green) antibodies. Scale bars, 2.00 μm .
760 (D) Representative z-stack image of a maturing oocyte cultured in the presence of 1
761 μM epoxomicin (Epox), another proteasome inhibitor, for 45 hr. Oocytes were stained
762 with anti-SUMO1 antibody. An enlarged image of the nucleus is shown at the bottom.
763 BF, bright-field image of the oocyte. Scale bars, 19.9 μm . (E) Representative images
764 of post-morula-stage embryos. Embryos at 80 hr post-insemination (80 h.p.i.)
765 subsequently were left untreated (cont) or exposed to heat shock at 42 °C for 2 hr.
766 Embryos were stained with anti-SC35 (red) and Alexa 488-conjugated anti-SUMO2/3
767 (green) antibodies. BF, bright-field images of the embryos. Scale bars, 19.9 μm .

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Fig. 1

Fig. 2

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