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► **To cite this version:**

Mélanie Bodnar-Wachtel, Anne-Laure Huber, Julie Gorry, Sabine Hacot, Laetitia Gerossier, et al..
NLRP3 controls ATM activation in response to DNA damage. 2021. hal-03093041

HAL Id: hal-03093041

<https://hal.science/hal-03093041>

Preprint submitted on 5 Jan 2021

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1 **NLRP3 controls ATM activation in response to DNA damage.**

2

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21

22 **Running title:** NLRP3 controls ATM activation

23

24 The authors declare no potential conflict of interest.

25

26 **ABSTRACT**

27

28 The DNA damage response (DDR) is essential to preserve genomic integrity and acts as a
29 barrier to cancer. The ATM pathway orchestrates the cellular response to DNA double strand
30 breaks (DSBs), and its attenuation is frequent during tumorigenesis. Here, we show that
31 NLRP3, a Pattern Recognition Receptor known for its role in the inflammasome complex
32 formation, interacts with the ATM kinase to control the early phase of DDR, independently of
33 its inflammasome activity. NLRP3 down-regulation in human bronchial epithelial cells impairs
34 ATM pathway activation as shown by an altered ATM substrate phosphorylation profile, and
35 due to impaired p53 activation, confers resistance to acute genomic stress. Moreover, we found
36 that NLRP3 is down-regulated in Non-Small Cell Lung Cancer (NSCLC) tissues and NLRP3
37 expression is correlated with patient overall survival. NLRP3 re-expression in NSCLC cells
38 restores appropriate ATM signaling. Our findings identify a non-immune function for NLRP3
39 in genome integrity surveillance and strengthen the concept of a functional link between innate
40 immunity and DNA damage sensing pathways.

41

42

43 **INTRODUCTION**

44

45 Maintenance of genome integrity is crucial for cell survival. Toxic DNA double strand breaks
46 (DSBs) can arise from both exogenous sources, for instance exposure to ionizing radiation (IR),
47 or endogenous sources such as DNA replication stress. If they remain unrepaired or are
48 incorrectly repaired they represent a major risk factor for genome instability, a condition known
49 to favor tumorigenesis. One of the key proteins that orchestrates the rapid cellular response to

50 DSBs is the Ataxia-Telangiectasia Mutated (ATM) kinase. The early molecular mechanism(s)
51 leading to ATM activation upon DSB formation remain elusive. In resting cells, ATM is present
52 as an inactive dimer. Once recruited to DSBs via the action of the MRE11-RAD50-NBS1
53 (MRN) complex, ATM autophosphorylates, monomerizes and initiates a vast cascade of post-
54 translational modifications that are essential for the DNA Damage Response (DDR) ¹.
55 Phosphorylation of the histone variant H2AX on Ser139 (γ H2AX) is one of the earliest events
56 in the DDR and is crucial for an efficient recruitment of DNA repair proteins to strand breaks
57 to form Ionizing Radiation Induced Foci (IRIF) ²⁻⁵. The scaffold protein MDC1 binds to γ H2AX
58 and recruits more ATM to the DNA lesion thus amplifying and maintaining the DNA damage
59 signal ^{6,7}. ATM also phosphorylates many downstream effector proteins including KAP1, p53
60 and CHK2, which induce effector mechanisms such as the activation of cell cycle checkpoints,
61 apoptosis or senescence ⁸. The ATM pathway is tightly regulated and any dysregulation in these
62 protection mechanisms facilitates the progression of cells towards malignancy.

63 NLRP3 belongs to the Nod-Like Receptor (NLR) family, a family of cytosolic Pattern
64 Recognition Receptors (PRRs) involved in innate immunity ⁹. Upon sensing of Pathogen-
65 Associated Molecular Patterns (PAMPs) or Damage-Associated Molecular Patterns (DAMPs)
66 such as nigericin or ATP, respectively, NLRP3 triggers the assembly of a multi-protein
67 complex, the inflammasome, the function of which is to control caspase-1 activation ¹⁰⁻¹².
68 Activated caspase-1 induces the maturation of the pro-inflammatory cytokines IL-1 β and IL-
69 18, and eventually pyroptosis of the cell ^{13,14}. The NLRP3 inflammasome is mostly expressed
70 in macrophages and dendritic cells where its biological functions have been well characterized.
71 Whether NLRP3 exerts functions unrelated to immunity remains unknown ^{15,16}. A previous
72 study reported that in myeloid cells the NLRP3 inflammasome activity relies on the presence
73 of ATM, suggesting a functional link between these two pathways ¹⁷. Here, we investigated
74 whether NLRP3 controls the ATM pathway. We discovered that NLRP3 binding to ATM is

75 instrumental to early ATM activation and to trigger apoptosis in response to DNA DSBs, and
76 we report that NLRP3 expression is down-regulated in NSCLC.

77

78 **RESULTS**

79 **Loss of NLRP3 impairs γ H2AX and P-ATM IRIF formation.**

80 To identify novel NLRP3 functions potentially linked to the regulation of the ATM pathway,
81 we exposed HBEC3-KT cells to IR (2 Gy) and assessed the activation of the DDR pathway in
82 the presence or absence of NLRP3. In control cells, the number of nuclear γ H2AX (Ser139)
83 IRIF peaked around 1 h after IR treatment and then decreased with time as the cells underwent
84 DNA repair (Fig. 1A and Suppl. Fig. 1A). In contrast, in the absence of NLRP3 a significantly
85 lower number of γ H2AX IRIF were initially formed, and detected at all subsequent time points.
86 This difference was greatest 1 h post IR and persisted over the time course of the study. ATM
87 activation by DSBs relies on its monomerization which coincides with autophosphorylation on
88 Ser1981¹. Using the presence of P-ATM(Ser1981) IRIF as an endpoint for ATM activation we
89 observed as early as 15 min post IR, fewer P-ATM foci in the absence of NLRP3, and the
90 difference remained significant at 1 h and 5 h post-irradiation (Fig. 1B). Next, we tested whether
91 the positive amplification loop required for optimal ATM activation was altered in the absence
92 of NLRP3 by assessing the recruitment of MDC1 to DSBs. In siNLRP3-treated cells a
93 decreased number of MDC1 foci were found compared with siCTL-treated cells, clearly
94 illustrating a defect in the recruitment of MDC1 to DSBs, and, as a consequence, a defect in the
95 ability to fully activate ATM (Fig. 1C and Suppl. Fig. 1B). 53BP1 is a DNA repair protein that
96 also forms IRIF in response to DSBs but in an ATM-independent manner¹⁸. The levels of
97 53BP1 foci were similar in the absence or presence of NLRP3 (Fig. 1D and Suppl. Fig. 1C),
98 leading us to conclude that the decrease in the ability to form γ H2AX and P-ATM foci supports

99 a role for NLRP3 in the radiation-induced ATM DSB signaling pathway. This hypothesis was
100 further investigated using mechanistic mathematical modeling (see Methods). Based on our
101 experimental findings that NLRP3 KD reduced the observed number of P-ATM molecules
102 recruited at DSBs, two hypotheses were investigated: A) NLRP3 enhances ATM activation, B)
103 NLRP3 inhibits ATM deactivation (Suppl. Fig. 1D). Hypothesis A achieved a nearly perfect fit
104 to data whereas hypothesis B was not able to reproduce the correct dynamics (SSR_A=0.052,
105 SSR_B=0.29, Figure 1E). The model allowed to predict that the ATM activation rate in
106 siNLRP3 conditions k_{IR} was nearly half of that in siCTL cells as the ratio
107 $\frac{k_{IR}^{control}}{k_{IR}^{NLRP3}}$ was equal to 1.66. Thus, the model A, which best recapitulated our data, supports the
108 notion that NLRP3 enhances ATM activation.

109

110 **NLRP3 is required for optimal ATM activation.**

111 To determine whether NLRP3 played a broader role in the activation of the ATM pathway, we
112 examined the phosphorylation of ATM downstream effectors that regulate cellular outcome in
113 response to different DSB inducers. First, we showed that the absence of NLRP3 resulted in
114 lower levels of KAP1 Ser824 phosphorylation in response to IR (Fig. 2A). Second, we tested
115 the response of HBEC3-KT cells to two chemotherapeutic agents known to induce DSBs,
116 etoposide (Eto), a topoisomerase II inhibitor, and hydroxyurea (HU), that depletes cellular
117 dNTP pools, initially inducing stalled replication forks and, following longer exposure times,
118 fork collapse and DSBs¹⁹. Similarly, the absence of NLRP3 led to lower phosphorylation levels
119 of KAP1 and p53 2 h to 6 h post-treatment with etoposide (Fig. 2B and C) or HU treatment
120 (Suppl. Fig. 2A). NLRP3 down-regulation also resulted in decreased P-ATM irrespective of
121 Eto concentrations (Suppl. Fig. 2B and C), and decreased γ H2AX foci formation upon Eto
122 treatment (Suppl. Fig. 2D). These results suggest that ATM is defective in the absence of
123 NLRP3. Previous studies reported that ATM dysfunction induces ROS production in cells^{17,20}.

124 Consistent with these studies NLRP3-depleted cells displayed enhanced ROS content compared
125 to control cells, which did not increase further by the addition of the ATM inhibitor KU55933
126 (Suppl Fig. 2E). Collectively, these results strongly support the notion that NLRP3 is required
127 for optimal ATM activation in response to DSBs.

128 We then wondered whether NLRP3 was globally required for ATM activation using a
129 heterologous model. Murine bone marrow-derived macrophages (BMDMs) either WT or
130 NLRP3^{ΔΔ} treated with Eto displayed deficient ATM activation, as evidenced by reduced ATM
131 and KAP1 phosphorylation compared to controls (Fig. 2D). As we found A549 and H292 lung
132 cancer cells did not express NLRP3, we re-expressed NLRP3 using a doxycycline-inducible
133 system in these NSCLC tumor cell lines, and evaluated ATM activation after IR exposure by
134 assessing the number of P-ATM and γ H2AX IRIF. NLRP3 re-expression increased the levels
135 of H2AX and ATM activating phosphorylations in both cell lines 1 h post-treatment (Fig. 3 A
136 to D and Suppl. Fig. 3A to D). In addition, upon Eto treatment, the phosphorylation kinetics of
137 the downstream effectors p53 and KAP1 in H292 re-expressing NLRP3 resembled those
138 observed in non-tumoral control cells (Fig. 3E). Similar results were obtained in H520 IR-
139 treated cells reconstituted with NLRP3 (Suppl. Fig. 3E). Thus, NLRP3 re-expression improved
140 ATM activation.

141

142 **NLRP3 controls the ATM pathway independently of its inflammasome activity.**

143 To assess if this new role for NLRP3 in DNA damage signaling is dependent on its well-known
144 inflammasome function, caspase-1 was knocked down using siRNA. The loss of caspase-1 did
145 not alter the level of γ H2AX after Eto treatment (Fig. 4A). In addition, no significant difference
146 in the phosphorylation of H2AX was observed in HBEC3-KT cells treated with the pan-caspase
147 inhibitor z-VAD-fmk or the caspase-1 inhibitor z-YVAD-fmk prior to HU exposure. These
148 results would suggest that neither caspase-1 nor another caspase activity was involved in the

149 activation of the ATM pathway, thus excluding apoptosis as a source of H2AX phosphorylation
150 (Fig. 4B). In addition, DNA damage did not activate the inflammasome as IL-1 β release was
151 barely detectable in cell supernatants after HU or IR treatments, nor could we detect cleaved
152 caspase-1 after Eto treatment (Fig. 4C, D, and E). Thus, DNA DSBs do not activate the catalytic
153 activity of the inflammasome.

154

155 **NLRP3 forms a complex with ATM.**

156 To determine how NLRP3 regulates ATM activity, we tested whether these two proteins could
157 interact with each other. In HeLa cells, that do not express endogenous NLRP3, co-
158 immunoprecipitation of Flag-ATM pulled down mCherry-NLRP3 (Fig. 5A). Moreover,
159 Flag-NLRP3 co-immunoprecipitated endogenous ATM (Fig. 5B)²¹. Eto treatment dissociated
160 the interaction, suggesting that ATM and NLRP3 formed a complex under basal cell condition
161 (Fig. 5B). We then mapped the NLRP3 domain involved in its binding to ATM. We showed
162 that ATM interacted with the NACHT (domain present in neuronal apoptosis inhibitor protein,
163 the major histocompatibility complex class II transactivator, HET-E and TPI), and the LRR
164 (Leucin Rich Repeats) domains but not with the PYD (Pyrin) domain, which is known to
165 mediate homotypic interactions involved in inflammasome formation (Fig. 5C)¹⁰. NEK7 was
166 used as a positive assay control, as it is a known partner of NLRP3 (Fig. 5C). We next
167 investigated whether NLRP3 was able to translocate to the nucleus. Cell fractionation
168 experiments revealed that endogenous NLRP3 was present in both the cytosolic and nuclear
169 fractions (Suppl. Fig. 4A) supporting earlier findings²². Interestingly, IF labeling of NLRP3
170 domains revealed nuclear localizations for the NACHT and the LRR domains, and a cytosolic
171 localization for the PYD domain, which self-oligomerized as previously described for ASC
172 PYD (Suppl. Fig. 4B)²³. Using this technique, short and full length (FL) NLRP3 were weakly
173 detected in the nucleus (Suppl. Fig. 4B). However, live-imaging of mCherry-NLRP3 revealed

174 its presence in the cell nucleus (Suppl. Fig. 4C). Consistently, co-immunoprecipitation
175 experiments revealed that NLRP3 bound to IPO5 and XPO2 two proteins involved in nuclear
176 import and export, respectively, which we identified by mass spectrometry (Fig. 5C)^{24,25}.
177 Using Proximity Ligation Assay (PLA), we also showed in HeLa cells that Flag-NLRP3 and
178 endogenous ATM formed a complex (Suppl. Fig. 4D). Importantly, we validated that
179 endogenous ATM and NLRP3 interacted in HBEC3-KT cells, and that the complex was
180 dissociated upon Eto treatment (Fig. 5D). We also observed after DNA damage that a smaller
181 fraction of ATM was detected by IF in the nucleus of NLRP3-depleted cells compared with
182 control cells (Suppl. Fig. 5A, B). Collectively, these results establish that under homeostatic
183 conditions NLRP3 forms a complex with ATM, which dissociates upon DSB formation.

184

185 **NLRP3-depleted cells are resistant to genotoxic stress-induced cell death.**

186 Because ATM activity controls cell fate decisions in response to genotoxic stress, we next
187 monitored cell death in response to Eto treatment. In NLRP3-depleted HBEC3-KT cells, less
188 caspase-3/7 activity was detected compared with control conditions, and an increase in the
189 number of viable cells was observed (Fig. 6A and B). These results suggest that decreased
190 NLRP3 expression protects cells from etoposide-induced apoptosis. Indeed, the induction of
191 *PUMA* and *NOXA/PMAIP1*, two p53 apoptosis effector genes, was significantly reduced in the
192 absence of NLRP3 compared to control cells (Fig. 6C and D)^{26,27}. This response was specific
193 to genotoxic stress as the induction of apoptosis via death receptor activation using a
194 combination of TRAIL and MG132 did not result in impaired apoptosis in cells depleted for
195 NLRP3 (Suppl. Fig. 6A and B).

196

197 **NLRP3 is down-regulated in NSCLC.**

198 GWAS studies reported that *NLRP3* is frequently mutated in NSCLC, but we found that A549
199 and H292 cells, isolated from NSCLC patients, do not express NLRP3. To extend this
200 observation, we assembled a panel of NSCLC cell lines, and included 3 HBEC3-KT cell lines
201 for comparison²⁸. Paradoxically, in most of the NSCLC cell lines, including cell lines reported
202 to carry NLRP3 mutations, the NLRP3 protein was barely detectable (Fig. 7A and Suppl. Fig.
203 7A), and very low levels of *NLRP3* mRNA were observed by Q-RT-PCR in comparison with
204 HBEC3-KT cells (Fig. 7B). Among the 3 HBEC3-KT lines, the HBEC3-ET cells did not
205 express NLRP3, and those cells displayed properties of malignant transformation since they
206 were able to grow in an anchorage-independent manner (Suppl Fig. 7B). These results suggest
207 that NLRP3 expression is down-regulated in malignant cells. To validate these observations,
208 we obtained a set of RNA samples from a cohort of patients with primary NSCLC and from
209 adjacent normal lung tissues. As shown in Fig. 7C, *NLRP3* mRNA was detectable in normal
210 lung tissues, while it was significantly down-regulated in NSCLC tissues. Finally, analysis of
211 TCGA data showed that high NLRP3 expression in lung adenocarcinoma (LUAD) was
212 associated with better overall survival and better progression-free interval (Fig. 7D and E).
213 Altogether, these results suggest a down-regulation of NLRP3 in NSCLC, and a positive
214 correlation in LUAD between NLRP3 levels and patient outcome.

215

216

217 **Discussion**

218 Functional links between innate immunity and DNA damage sensing pathways have been
219 described. For instance, ATM was recently shown to be required in macrophages for optimal
220 NLRP3 and NLRC4 inflammasome functions because its inactivation altered the ROS balance
221 and therefore impaired inflammasome assembly¹⁷. It was also suggested that DDR proteins
222 such as RAD50 or BRCA1 are involved in the sensing of nucleic acids from viral pathogens in

223 the cytosol ^{29,30}. However, little is known about the contribution of PRRs to the sensing of stress
224 like DNA damage. Here, we demonstrate that NLRP3 is crucial to reach optimal ATM
225 activation. Under homeostatic conditions, our PLA data showed that NLRP3 forms a complex
226 with ATM in the cytosol, suggesting that NLRP3 binds to the inactive ATM dimer. ATM has
227 already been reported to be found in the cell cytosol ^{20,31}. Upon DNA DSB formation, the
228 complex dissociates to allow ATM relocalization and monomerization onto DNA breaks. In
229 the absence of NLRP3, decreased levels of P-ATMSer1981 foci were observed herein, together
230 with a decreased nuclear ATM pool. These observations suggest that NLRP3 may control either
231 ATM translocation to the nucleus or ATM monomerization. Consequently, the formation of
232 γ H2AX and MDC1 IRIF, which are both essential for the positive ATM amplification loop
233 signaling, were also impaired in NLRP3 KD cells ⁶. This led to a less active ATM as illustrated
234 by the reduced phosphorylation of its substrates KAP-1 and p53, and, importantly, cells became
235 more resistant to apoptosis.

236 No caspase-1 activation and no significant IL-1 β production was detected upon DNA DSB
237 induction. Our findings contrast with those of R. Flavell's group, who demonstrated in mouse
238 models that the severe damage caused to the gastrointestinal tract and the hematopoietic system
239 in response to whole body γ -irradiation are due to the activation of the AIM2 inflammasome by
240 DSBs, which cause massive cell death by caspase-1-dependent pyroptosis in these fast
241 renewing tissues. These observations would suggest that tissue and species-specific differences
242 may exist that clearly warrant further investigation ³².

243 Using different approaches which included restoring NLRP3 expression in NSCLC cell lines
244 displaying low levels of inflammasome proteins, we identified a novel non-inflammasome
245 function for NLRP3 in the DNA damage pathway. This previously unappreciated role for
246 NLRP3 in the ATM pathway may be due to the fact that many common cellular models used
247 in laboratories do not express NLRP3 (e.g. MEF, HeLa, 293, A549). Altogether, our results

248 highlight that NLRP3 is not only a major player in innate immunity but is also a key factor
249 involved in the preservation of genome integrity through the modulation of the ATM signaling
250 pathway in response to DSBs.

251 The DDR is known to be a barrier to cancer in the early phases of tumorigenesis^{33,34}. *TP53* is
252 frequently mutated in cancer, and the ATM pathway is down-regulated in many solid tumors:
253 11% of NSCLC carry somatic mutations in *ATM* and 41% of lung adenocarcinoma have
254 reduced ATM protein expression³⁵⁻³⁸. Although, several cancer genomic studies have reported
255 that *NLRP3* is frequently mutated in NSCLC, our data actually suggest that in NSCLC primary
256 human tissues and cell lines its expression is significantly lower compared to normal tissue.
257 This down-regulation of *NLRP3* expression during malignant transformation may represent an
258 additional mechanism to attenuate ATM and p53 signaling pathways, allowing cells to survive
259 genotoxic stress, despite the presence of genome alterations. Thus, the loss of NLRP3, and the
260 subsequent impairment of the ATM pathway could be an event allowing cells to progress
261 towards malignancy.

262

263

264 **MATERIALS AND METHODS**

265 *Cell culture*

266 HBEC3-KT, HCC15, HCC366, HCC4017 and HCC461 were obtained from J. Minna, NCI-
267 H1703 (H1703), NCI-H292 (H292), NCI-H520 (H520), NCI-H661 (H661), NCI-H358 (H358),
268 NCI-H1792 (H1792), NCI-H441 (H441) and SK-MES-1 from ATCC, and A549 from the PHE
269 culture collection. HBEC3-KT cells were cultured in Keratinocyte-Serum Free Medium
270 (Invitrogen) supplemented with 12.5 mg of Bovine Pituitary Extract (Invitrogen) and 100 ng of
271 epidermal growth factor (Invitrogen). H1792, A549, HCC15, HCC366, HCC4017, HCC461
272 and H441 were cultured in RPMI medium, supplemented with 10% Fetal Bovine Serum (FBS)

273 and 1% penicillin/streptomycin, H1703, H292, H520, H358, H661 in RPMI, 10% FBS, 1 mM
274 sodium pyruvate, 1 mM HEPES and 1% penicillin/streptomycin and SK-MES-1 in RPMI, 10%
275 FBS, 1 mM sodium pyruvate, 1 mM non-essential amino acids and 1 % penicillin/streptomycin
276 (Invitrogen). *NLRP3* mutated cell lines are H661, H358, HCC15, HCC366 and HCC4017.
277 HeLa cells were cultured in DMEM 4.5 g/L of glucose, 10% FCS and 1%
278 penicillin/streptomycin. Treated cells received Etoposide (TEVA santé), HU (Sigma) or γ -ray
279 treatment 24 h post-transfection. For inflammasome activation, cells were primed overnight
280 with 10 μ g/mL poly(I:C) (Invivogen) and treated with nigericin (10 μ M, 6 h), or ATP (5 mM,
281 30 min) (SIGMA). IL-1 β ELISA was purchased from BD. Z-VAD-fmk and z-YVAD-fmk were
282 from Enzo Life Science.

283

284 *Mice*

285 The *NLRP3* flox mice were generated by the “clinique de la souris” Strasbourg. The exon 4
286 was flanked by 2 Lox-P sites in C57BL6 background. *NLRP3*^{flox/flox} mice were bred with
287 Rosa26-Cre-ERT2 mice. Bone marrow-derived macrophages were generated as previously
288 described ³⁹ from +/+; Cre-ERT2 and Flox/Flox; Cre-ERT2 adult mice. To inactivate *Nlrp3*
289 (*Nlrp3* ^{$\Delta\Delta$}), 4OHT (SIGMA) was added at the concentration of 0.5 μ M from day 2 to day 7 of
290 differentiation in both genotypes for control. BMDM were treated with 100 μ M Eto as
291 indicated.

292

293 *Cell transfection*

294 HBEC3-KT were seeded at 1.5x10⁵ cells per well in 6-well plates and were transfected with
295 non-targeting siRNA (SMART pool Non-targeting siRNA, Dharmacon) or *NLRP3* siRNA
296 (SMARTpool, Dharmacon) using HiPerfect transfection reagent (Qiagen) or INTERFERin
297 (Polyplus transfection) following manufacturer’s instructions. HeLa cells were transfected with

298 Lipofetamin2000™ (Invitrogen). H292 cells were transfected using PEI method. Vectors used
 299 pCR3-Flag-NLRP3 (FL, SHORT, PYD, NACHT, LRR), pcDNA3.1-Flag-His-ATM (Addgene
 300 31985), pcDNA3.1-mCherry-NLRP3. shRNA were from Genecopoeia, hygromycin selection,
 301 *NLRP3*: forward: 5'-TAATACGACTCACTATAGGG-3' Reverse: 5'-
 302 CTGGAATAGCTCAGAGGC-3'; control Forward: 5'-TAATACGACTCACTATAGGG-3'
 303 Reverse: 5'-CTGGAATAGCTCAGAGGC-3'.

304 *Irradiation*

305 Cells were irradiated using a 6-MeV γ -ray clinical irradiator (SL 15 Phillips) at the Léon Bérard
 306 Cancer Centre (Lyon, France) with a dose rate of 6 Gy.min⁻¹ to obtain the required dose.

307

308 *ROS measurement*

309 For intracellular ROS staining, HBEC3-KT cells were incubated with 1 μ M of 2'-7'-
 310 dichlorofluorescein diacetate (CM-H2DCFDA; Invitrogen) for 30 min at 37°C. For a positive
 311 control, cells were pretreated with 5 μ M of ATM inhibitor (ATMi) (KU55933; Selleckchem)
 312 for 5 h prior to staining. Stained cells were collected and analyzed on a BD FACSCalibur, and
 313 data were analyzed using the FlowJo software.

314

315 *Mathematical modeling*

316 ATM dynamics was modeled using the following ordinary differential equation:

$$317 \quad \frac{dA}{dt} = k_{IR} \mathbb{1}_{t \in [IR \text{ exposure}]} - k_{inact} A$$

318 where A is the concentration of activated ATM molecules (expressed in number of foci/cell),
 319 k_{IR} is the activation rate (in number of foci/cell. h⁻¹), only present during irradiation, and k_{inact}
 320 is the inactivation rate (in h⁻¹). A was set at zero at t₀. This model assumed that ATM molecules
 321 were in excess compared to the activated proportion.

322 The model is fitted to experimental data by estimating the optimal values for k_{IR} and k_{inact} for
323 each condition- siCTL or siNLRP3- using a weighted least-square approach⁴⁰. For hypothesis
324 A, i.e. NLRP3 enhances ATM activation, k_{IR} is assumed to be different for both conditions
325 whereas k_{inact} is taken identical. For hypothesis B, i.e. NLRP3 inhibits ATM deactivation,
326 k_{inact} is assumed to vary between siCTL and siNLRP3 conditions and k_{IR} is assumed to remain
327 identical. All computations were done in Matlab (Mathworks, USA).

328

329 *Generation of NLRP3 stably expressing cells*

330 The human *NLRP3* cDNA was inserted into the lentiviral vector pSLIKneo (addgene)
331 containing a TET-ON promoter using the Gateway recombination system (Invitrogen).
332 Sequences of the Gateway shuttle vectors are available upon request. Empty pSLIK vector
333 (without the ccdB containing Gateway recombination cassette) was produced by partial
334 digestion with Xba1 and Xho1 followed by religation. Viral particles were produced by
335 transfecting HEK293T cells with the lentiviral vectors and a second generation packaging
336 system. NCI-H292, NCI-H520 and A549 cells were either transduced with the empty pSLIK
337 control vector or the NLRP3 containing vector. NLRP3 expression was induced by adding 0.5
338 $\mu\text{g/mL}$ doxycycline (Takara Bio) to the cell culture medium.

339

340 *Western blotting*

341 Cells were washed with PBS and detached by trypsinization. Cell pellets were lysed in Laemmli
342 buffer x2 (Tris HCl 0.5 M pH 6.8; 0.5 M DTT; 0.5% SDS) and protein concentrations were
343 determined using the Bradford reagent (Biorad). Protein extracts were separated on SDS-PAGE
344 (8 % or 15 % or 4-15% gradient (vol/vol)) gels. Gels were transferred onto nitrocellulose
345 membranes (GE HealthCare and Biorad) for immunoblotting with the following antibodies:
346 anti-NLRP3 (Cryo-2, 1:1000) and anti-caspase-1 (Bally-1, 1:1000) from Adipogen, anti-ASC

347 (1:2000) from ENZO Life Science, anti- γ H2AX (JBW301, 1:1000), anti-P-Ser15-p53 (1:1000)
348 and anti-ATM Ser1981 (10H11.E12, 1:200) were from Millipore. Anti-P-KAP1Ser824
349 (1:1000), anti-KAP1 (1:1000) and anti-Nek7 (A302-684A) from Bethyl Laboratories, anti-p53
350 (clone DO7 1:2000) and anti-NOXA (114C307, 1:1000) from Santa Cruz, anti-ATM
351 (#ab32420, 1/1000) from Abcam, anti-Flag (F7425 1/5000) from Sigma, anti-XPO2
352 (GTX102005 1/1000) and anti-IPO5 (GTX114515 1/1000) from Genetex and anti-actin (C4,
353 1:100,000) from MP Biomedical.

354 The Fiji and ImageLab programs were used for densitometric analysis of immunoblots, and the
355 quantified results were normalized as indicated in the figure legends.

356

357 *Cell fractionation*

358 HBEC3-KT were fractionated by adapting the method described by Hacot *et al.*⁴¹. The MgCl₂
359 concentration used for the hypotonic buffer was 0.75 mM. Equal amounts of proteins were run
360 by SDS-PAGE.

361

362 *Immunofluorescence*

363 Cells were plated onto sterile glass coverslips and fixed with PBS-PFA 4% (wt/vol) for 15 min
364 at room temperature (RT) and washed twice in PBS. Cells were permeabilized with lysis buffer
365 (sucrose 300 mM, MgCl₂ 3 mM, Tris pH 7.0 20 mM, NaCl 50 mM, Triton X-100 0.5%) for 3-
366 5 min at RT under slow agitation. The following antibodies were diluted in PBS-BSA 4% and
367 applied to the coverslips for 40 min at 37°C: anti- γ H2AX (JBW301, 1:800), P-ATM Ser1981
368 (10H11.E12, 1:200), and 53BP1 (1:500) from Millipore, and anti-MDC1 (1:200) from AbCam.
369 For NLRP3 labeling, cells were fixed with PBS-PFA 4% (wt/vol) for 15 min at RT, washed
370 twice in PBS and permeabilized with 1% triton X100. Anti-flag was diluted in saturation buffer
371 (PBS, 1% BSA; NaCl 0.02%; Tween 20 0.5%; SVF 3%) and incubated on cells for 1 h. Cells

372 were then incubated with Alexa-Fluor 488-conjugated anti-mouse or Alexa-Fluor 555-
373 conjugated anti-rabbit (1:800; Life Technologies) for 20 min at 37°C and in Hoechst (500
374 ng/mL in PBS) for 10 min at RT. Fluorescence microscopy pictures were taken using a Nikon
375 Eclipse Ni-E microscope, and confocal Zeiss LSM 780. The Fiji program was used to analyze
376 fluorescence intensity.

377

378 *Live imaging*

379 mCherry-NLRP3 transfected H292 were imaged using a confocal spinning disk inverted
380 microscope (Leica, Yokogawa). Vital Hoechst was used at 0.5 μ g/mL. The Fiji program was
381 used to analyze fluorescence intensity.

382

383 *Co-immunoprecipitation*

384 HeLa cells were transfected using Lipofectamin2000TM (Invitrogen) according to
385 manufacturer's protocol, and lysed in the following buffer: Tris HCl 100 mM pH 8.0, 10 mM
386 MgCl₂, 90 mM NaCl, 0.1% Triton X-100, Complete® tablet (Roche). Immunoprecipitation was
387 performed using M2-agarose beads (A2220 Sigma) overnight at 4°C.

388

389 *Proximity Ligation Assay*

390 HBECT3-KT and HeLa cells were seeded onto glass coverslips and processed as described by
391 the manufacturer's protocol (Duolink® PLA Technology, Sigma). Antibodies used NLRP3
392 1/500 (ABF23, Millipore), ATM 1/500 (2C1, Abcam). Quantification was carried out using the
393 macro published by Poulard et al ⁴².

394

395 *IL-1 β Luminex Assay*

396 IL-1 β levels in cell supernatants were analyzed using the Magnetic Luminex Screening Assay
 397 according to the manufacturer's protocol (R&DSystems). The samples were read using the
 398 Bioplex-200 from BioRad.

399

400 *Quantitative reverse transcription PCR*

401 Cells were washed and detached by trypsinization. RNA was extracted using NucleoSpin[®] RNA
 402 kit (Macherey Nagel). Five hundred nanograms to one microgram of RNA were reverse
 403 transcribed using SuperScript II reverse transcriptase and oligo(dT) primers (Life technologies)
 404 and RNAsin (Promega). cDNAs were quantified by real-time PCR using a SYBR[®] Green PCR
 405 Master Mix (Applied Biosystems) on a ABI Prism[®] 7000 (Applied Biosystems) or CFX
 406 Connect Real-Time system (BioRad). Sequences of the primers NLRP3 Forward 5'-
 407 GAAGAAAGATTACCGTAAGAAGTACAGAAA; Reverse 5'-
 408 CGTTTGTTGAGGCTCACACTCT; ESD 5'-TTAGATGGACAGTTAC TCCCTGATAA;
 409 Reverse 5'-GGTTGCAATGAAGTAGTAGCTATGAT; HPRT1 Forward 5'-
 410 CATTATGCTGAGGATTTGGAAAGG; Reverse 5'-TGTAGCCCTCTGTGTGCTCAAG;
 411 CBP Forward 5'-CGGCTGTTTAACTTCGCTTC; Reverse 5'-
 412 CACACGCCAAGAAACAGTGA. NOXA Forward 5'-GGAGATGCCTGGGAAGAAG;
 413 Reverse 5'-CCTGAGTTGAGTAGCACACTCG; PUMA Forward 5'-
 414 CCTGGAGGGTCCTGTACAATCTCAT; Reverse 5'-
 415 GTATGCTACATGGTGCAGAGAAAG; ACTIN Forward 5'-
 416 AGCACTGTGTTGGCGTACAG; Reverse 5'-TCCCTGGAGAAGAGCTACGA.

417 NLRP3 mRNA amounts in different NSCLC cell lines were normalized against *ESD* (Fig. 1B)
 418 or in human samples on *CPB* and *HPRT1* (Fig. 1D) mRNA levels or in HBEC-KT on *HPRT1*
 419 (Fig. 5C).

420

421 *Caspase-3/7 assay*

422 Cells were cultured in 96-well plates and treated with 50 μ M of Etoposide for 12 h or with 200
423 ng/mL Trail (Peprotech) and 1 mM MG132 (Sigma). Caspase 3/7 activity was assessed using
424 the Caspase-Glo 3/7 assay reagent (Promega) following the manufacturer's instructions. The
425 luminescence was measured using a TECAN Infinite M200PRO luminometer microplate
426 reader. To normalize the results, a second plate was stained with crystal violet and analyzed as
427 described in the *crystal violet cytotoxicity assay* below.

428

429 *Crystal Violet cytotoxicity assay*

430 Cells were stained with 0.5% crystal violet (Sigma-Aldrich Corp.) in 30% methanol for 20
431 minutes at room temperature. Cells were lysed in a 1% SDS (Sigma-Aldrich Corp.) solution.
432 The absorbance of the solution was measured using a TECAN Infinite M200PRO microplate
433 reader at a wavelength of 595 nm.

434

435 *Tissues from NSCLC patients*

436 Frozen lung tumor tissues from non-treated patients were obtained from the Biological
437 Resource Centre in Grenoble (Centre de Ressources biologiques de Grenoble) n°BB-0033-
438 00069 and in accordance with the ethical laws. RNA was extracted from regions containing
439 mainly malignant cells using Allprep RNA/DNA kit from Qiagen.

440

441 *TCGA data analysis*

442 RNAseqV2 data of lung adenocarcinoma (LUAD) and corresponding clinical data were
443 downloaded from The Cancer Genome Atlas TCGA data portal. Cox regression model was
444 used to estimate hazard ratio (HR) and 95% confidence intervals (CIs) for overall survival (OS)
445 and progression-free interval (PFI). Survival curve was estimated by the Kaplan–Meier method.

446 Optimal cutoff for NLRP3 expression was chosen based on a maximally selected rank statistic
447 ⁴³.

448

449

450

451 *Statistical analysis*

452 Statistical analysis of the experimental data was performed using Graphpad. Unpaired group
453 comparisons were done using two-tailed Student t-test for most figures, Mann-Whitney test for
454 Figure 5A and B and multiple comparisons for two-way ANOVA for Figure 5C.

455

456 **Acknowledgements:** We thank John Minna for sharing the HBEC3-KT and NSCLC cells, Dr
457 Foray's team and Marine Malfroy for technical help and Agnès Tissier and Pascale Bertrand
458 for helpful discussions. We thank Christophe Vanbelle and Christophe Chamot for their
459 assistance on confocal microscope image acquisition. M.B. was supported by the ANRT, V.P.
460 by the plan Cancer, Ligue Contre le Cancer Comité de l'Ain, the ARC foundation, and the
461 Fondation pour la Recherche Médicale DEQ20170336744, A.L.H. by the ARC foundation and
462 a Marie Skodolvska-Curie grant, N.G. was supported by the CLARA, and B.G. by the ARC
463 foundation. B.P. was supported by the ERC.

464

465 **Author contributions:** M.B.W., A.L.H., J.G., S.H., J.H. and V.P. designed and analyzed
466 experiments. M.B.W., A.L.H., J.G., B.G., S.H., Y.C., L.G. and V.P. performed experiments.
467 F.G., B.B., B.P., S.L., B.P. and N.G. provided reagents. M.B.W., A.L.H., J.G., S.H., J.H. and
468 V.P. contributed to the manuscript writing and figure constructions.

469

470 Competing interests: the authors declare no financial competing interest.

471

472

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592

593 **FIGURE LEGENDS**

594 **Figure 1.** NLRP3 down-regulation impairs ATM-dependent IRIF formation and DNA damage
595 signaling in response to DNA double strand breaks.

596 (A to D) HBEC3-KT transfected with control (siCTL) or NLRP3 siRNA (siNLRP3) were
597 treated with IR (2 Gy). The number of nuclear γ H2AX (A), MDC1 (B), P-ATM (C), and 53BP1
598 (D) IRIF were determined by immunofluorescence at indicated time points. IF of the 1 h time
599 point is shown (x60). Hoechst (blue) was used to stain nuclei. Representative of four (A, C)
600 and two independent experiments (B, D) ($64 \leq n \leq 148$) Scale bars 10 μ m. (E) Mathematical
601 modeling of ATM and NLRP3 interactions. Two hypotheses were investigated A) NLRP3
602 enhances ATM activation, B) NLRP3 inhibits ATM deactivation.

603

604 **Figure 2.** NLRP3 is instrumental for optimal ATM activation.

605 (A) HBEC3-KT transfected with indicated siRNA were IR treated (10 Gy) and collected at
606 different time points and P-KAP1 analyzed by immunoblotting. (B) HBEC3-KT control siRNA
607 and NLRP3 siRNA were exposed to Eto (100 μ M) for indicated time points and P-KAP1 and
608 P-p53 were analyzed by immunoblot. (C) Relative quantification of immunoblot of 3
609 independent experiments as shown in (D). (E) Bone marrow-derived macrophages of wild type
610 NLRP3 or NLRP3-depleted mice were treated with Eto 100 μ M over time and P-ATM and P-
611 KAP1 were analyzed by immunoblotting (representative of 2 experiments). Data represent
612 mean \pm SEM, *** P < 0.001, **** P < 0.0001, ns: not significant (unpaired t-test).

613

614 **Figure 3.** Expression of NLRP3 in NSCLC cell lines improves ATM activation after the
615 induction of DNA DSBs.

616 A549 (**A** and **B**) or H292 (**C** and **D**) cells stably expressing a doxycycline-inducible NLRP3
617 lentiviral vector (pSLIK NLRP3) induced or not with 0.5 $\mu\text{g}/\text{mL}$ doxycycline were irradiated
618 with 2 Gy and P-ATM (**A** and **C**) and γH2AX (**B** and **D**) IRIF assessed 1 h post-treatment. (**E**)
619 H292 cells stably expressing the control or NLRP3 lentiviral vector were induced with
620 doxycycline 24 h before being treated with etoposide over a time course of 16 h. KAP1 and p53
621 phosphorylation was analyzed by immunoblot at the indicated time points. One representative
622 experiment out of 3. **** $P < 0.0001$, *** $P < 0.001$, * $P < 0.05$ (unpaired t-test).

623

624 **Figure 4.** NLRP3 controls the DDR in an inflammasome-independent manner.

625 (**A**) HBEC3-KT control siRNA and caspase-1 siRNA (siCASP1) were treated with Eto 100 μM
626 and H2AX phosphorylation was monitored by immunoblot. (**B**) HBEC3-KT control siRNA
627 were treated with the pan caspase inhibitor z-VAD-fmk (50 μM) or the caspase-1 inhibitor z-
628 YVAD-fmk (50 μM) 30 min before HU treatment (2 mM, 16 h) and H2AX phosphorylation
629 was analyzed by immunoblotting. (**C**) HBEC3-KT transfected with control or NLRP3 siRNA
630 were treated with IR 2 Gy or (**D**) HU 2 mM for the indicated times and IL-1 β was quantified in
631 cell supernatants using a Luminex assay. The line indicates the detection limit. (**E**) HBEC3-KT
632 cells were treated with Eto 100 μM over time and caspase-1 cleavage was analyzed by
633 immunoblot. Actin was used as loading control. These data are from one representative
634 experiment out of two independent experiments. n.d.: not detected

635

636 **Figure 5.** NLRP3 forms a complex with ATM.

637 (**A**) mcherry-NLRP3 and Flag-ATM co-immunoprecipitate in HeLa cells. Immunoprecipitates
638 (IP) and input were analyzed by immunoblotting. (**B**) HeLa cells expressing Flag-NLRP3 were
639 treated or not with Eto for indicated time points. Co-immunoprecipitation of endogenous ATM
640 was analyzed by immunoblotting. (**C**) Different flag-tag NLRP3 domain constructs were

641 transfected in HeLa cells, and Flag-proteins were immunoprecipitated and pull-downed
642 proteins were analyzed by immunoblotting. **(D)** Proximity Ligation Assay was performed in
643 HBEC3-KT cells treated or not with Eto using anti-ATM and anti-NLRP3 (x40). Hoechst (blue)
644 was used to stain nuclei. Scale bars 50 μ m Signal quantification is shown on the graph on the
645 right panel. NT: not treated.

646

647 **Figure 6.** The absence of NLRP3 confers resistance to acute genotoxic stress.

648 **(A and B)** HBEC3-KT transfected with the indicated siRNA were treated with Eto and **(A)**
649 caspase-3/7 activity was then measured by luminometry and **(B)** cell survival by cristal violet
650 cytotoxicity test. **** P < 0.0001, ** P = 0.0046 (unpaired t-test). Results are representative of
651 three experiments. **(C)** *NOXA* and *PUMA* expression were assessed in HBEC3-KT treated with
652 Eto at the indicated time points by Q-RT-PCR relative to *HPRT1* expression. **** P < 0.0001,
653 ** P = 0.0035 (multiple comparisons for two-way ANOVA). **(D)** *NOXA* expression was
654 assesed by immunoblotting. Actin was used as a loading control. Representative of two
655 independent experiments.

656

657 **Figure 7.** NLRP3 expression is reduced in human NSCLC compared to healthy tissue.

658 **(A)** Protein levels of the NLRP3 inflammasome components NLRP3, caspase-1 and ASC were
659 assessed by immunoblotting and **(B)** relative *NLRP3* mRNA by Q-RT-PCR in HBEC3-KT cells
660 and in a panel of NSCLC cell lines. Results are representative of more than three experiments.
661 **(C)** Relative *NLRP3* mRNA levels were determined by Q-RT-PCR in a cohort of non-treated
662 primary tumors from NSCLC patients (n = 20) and the corresponding normal lung tissues (n =
663 10). Data represent mean \pm SEM; *** P < 0.001 (t-test). Kaplan-Meier plots of patients overall
664 survival **(D)** and progression free interval **(E)** in TCGA-LUAD dataset according to NLRP3

665 expression levels; patients were stratified according to the cutoff obtained from maximally
666 selected rank statistic.

667

668 **Supplementary Figure 1.** NLRP3 down-regulation impairs ATM-dependent IRIF formation.

669 **(A)** Immunoblot controlling the efficacy of the siRNA targeting NLRP3 (siNLRP3) against
670 non-targeting siRNA (siCTL) of the HBEC3-KT irradiated cells. Actin was used as a loading
671 control. **(B and C)** Representative pictures of HBEC3-KT cells transfected with control or
672 NLRP3 siRNA and treated with 2 Gy for 1 h to assess MDC1 **(B)** or 53BP1 **(C)** foci formation
673 was analyzed by IF. (x60), Hoechst (blue) was used to stain nuclei. Scale bars 10 μ m. **(D)**
674 Scheme displaying the hypothesis used for mathematical modeling of ATM and NLRP3
675 interactions showing the two hypotheses were investigated A) NLRP3 enhances ATM
676 activation, B) NLRP3 inhibits ATM deactivation.

677

678 **Supplementary Figure 2.** ATM activity is impaired in the absence of NLRP3 in response to
679 DNA damaging agents.

680 **(A)** HBEC3-KT siRNA-transfected cells were treated with 2 mM HU. At indicated time points,
681 cells were lysed and protein extracts analyzed by immunoblotting for NLRP3, γ H2AX (S139),
682 P-p53 (S15), P-KAP1 (S824). Actin was used as a loading control. **(B to D)** HBEC3-KT cells
683 transfected with control or NLRP3 siRNA were treated with **(B)** 0.5 μ M etoposide for 4 h and
684 P-ATM foci were quantified, and **(C)** 100 μ M Eto and mean fluorescence intensity was
685 quantified in the nucleus, **(D)** 0.5 μ M etoposide for 4 h and γ H2AX foci quantified. (x60),
686 Hoechst (blue) was used to stain nuclei. Data represent mean \pm SEM; *** 0.001 < P (unpaired
687 t-test). Scale bar 10 μ m **(E)** ROS measurement was performed on HBEC3-KT cells transfected
688 with control or NLRP3 siRNA using DCFDA probe in presence or in absence of 5 μ M ATMi
689 KU5593. One representative experiment out of 3.

690

691 **Supplementary Figure 3.** NLRP3 re-expression in tumoral cell lines facilitates ATM-
692 dependent DNA damage signaling.

693 A549 (**A** and **B**) or H292 (**C** and **D**) stably expressing a doxycycline-inducible NLRP3 lentiviral
694 vector treated or not with 0.5 μ M of doxycycline were irradiated with 2 Gy for 1 h.
695 Representative pictures of P-ATM (**A** and **C**) and γ H2AX (**B** and **D**) IF staining that was
696 quantified in Figure 3 A to D. (x60), Hoechst (blue) was used to stain nuclei. Scale bars 10 μ m.
697 (**E**) H520 cells stably expressing the NLRP3 or CTL vector were treated with 0.5 μ g/mL of
698 doxycycline 24 h before irradiation with 6 Gy. At indicated time points, cells were lysed and
699 protein extracts analyzed for NLRP3, γ H2AX (S139), P-KAP1 and KAP1 by immunoblotting.
700 Actin was used as a protein loading control.

701

702 **Supplementary Figure 4.** NLRP3 is localized in the cell cytosol and nucleus, but most
703 NLRP3/ATM complexes are present in the cell cytosol.

704 (**A**) HBEC3-KT untreated (0) or irradiated (2 Gy) were separated and proteins from the
705 cytosolic (C) and nuclear (N) fractions were analyzed by immunoblot. Tubulin was used as a
706 marker of the cytosolic fraction and fibrillarin of the nuclear fraction. T is total lysate. (**B**)
707 Confocal images illustrating the cellular localization of the different NLRP3 domains
708 transfected in HeLa cells (x63). Hoechst (blue) was used to stain nuclei. (**C**) NLRP3 is detected
709 in the cytosol and the nucleus compartment in live confocal image of mCherry-NLRP3
710 transfected in H292 cells. (**D**) Proximity Ligation Assay in HeLa cells transfected with an empty
711 vector or a NLRP3-expressing vector using anti-ATM and anti-NLRP3 antibodies (x40).
712 Hoechst (blue) was used to stain nuclei.

713

714 **Supplementary Figure 5.** NLRP3 silencing decreases nuclear ATM.

715 (A and B) HBEC3-KT sh control cells or knocked down for NLRP3 were left untreated (A) or
716 irradiated at 2 Gy for 1 h (B). Total ATM was stained for immunofluorescence and the mean
717 fluorescent intensity was quantified. Results are representative of two independent experiments
718 (x60). Hoechst (blue) was used to stain nuclei. Scale bar 10 μ m.

719

720 **Supplementary Figure 6.** NLRP3 does not control the extrinsic apoptosis pathway.

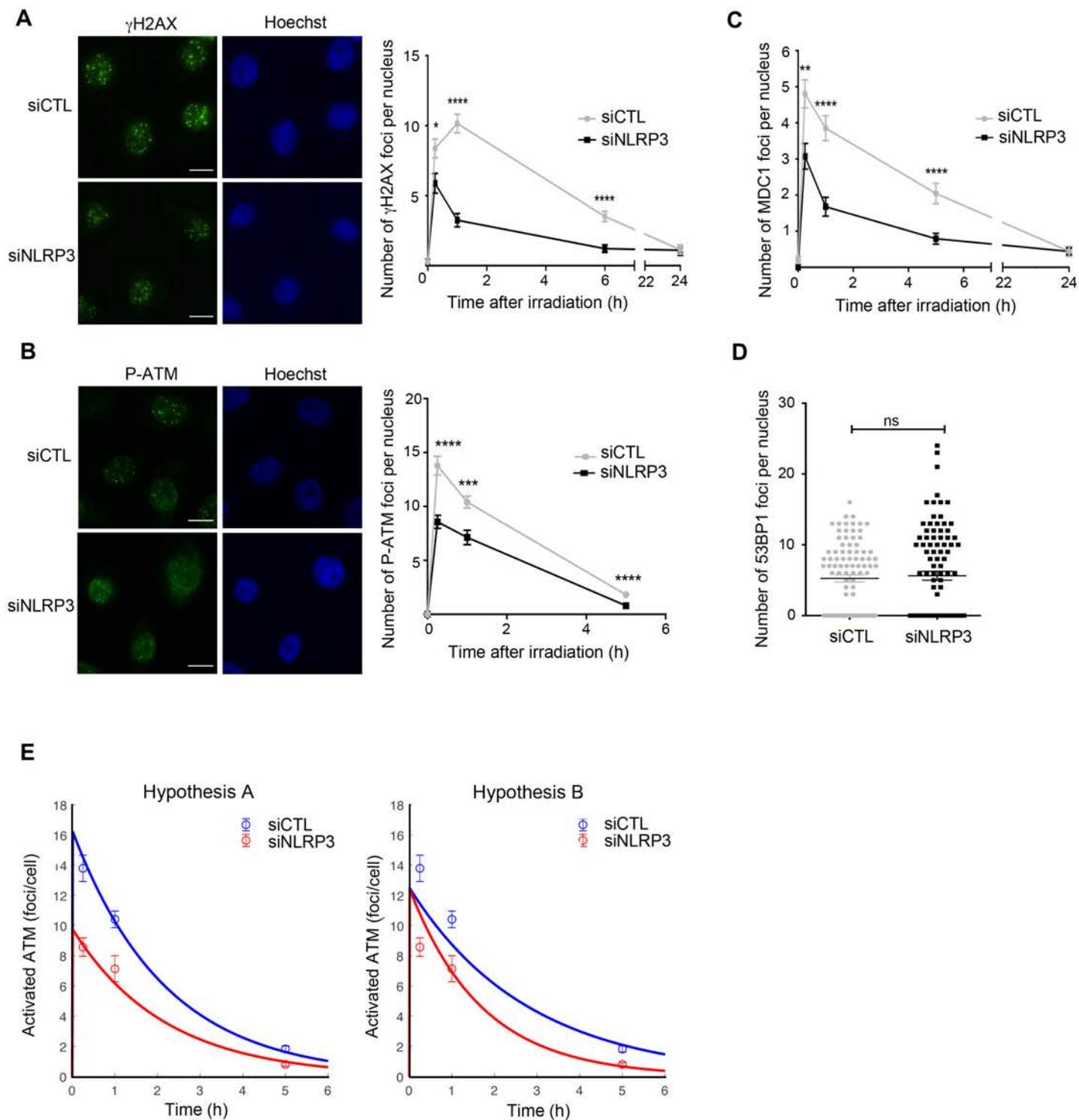
721 (A) HBEC3-KT cells transfected with control or NLRP3 siRNA were treated with Trail 200
722 ng/mL and MG132 1 mM for 12 h to induce death receptor-mediated apoptosis. Data represent
723 mean \pm SEM; ns: not significant (t-test). (B) Model of resistance to genotoxic stress caused by
724 reduced NLRP3 expression. DNA DSBs activate the ATM kinase which phosphorylates many
725 protein substrates involved in the control of the outcome to genotoxic stress. Down-regulation
726 of NLRP3 impairs ATM activation resulting in decreased levels of phosphorylation of several
727 ATM substrates, including p53, thus promoting cell survival.

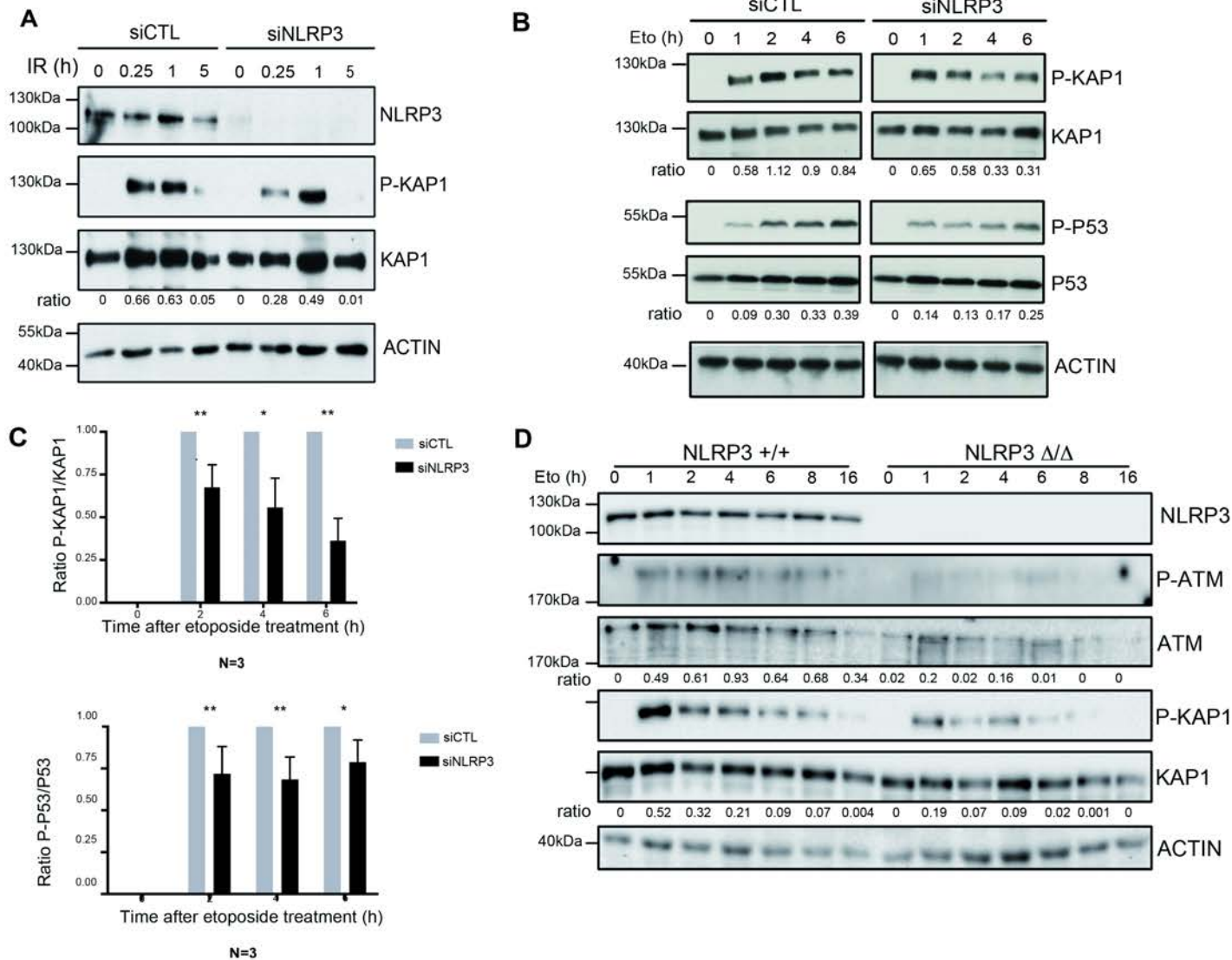
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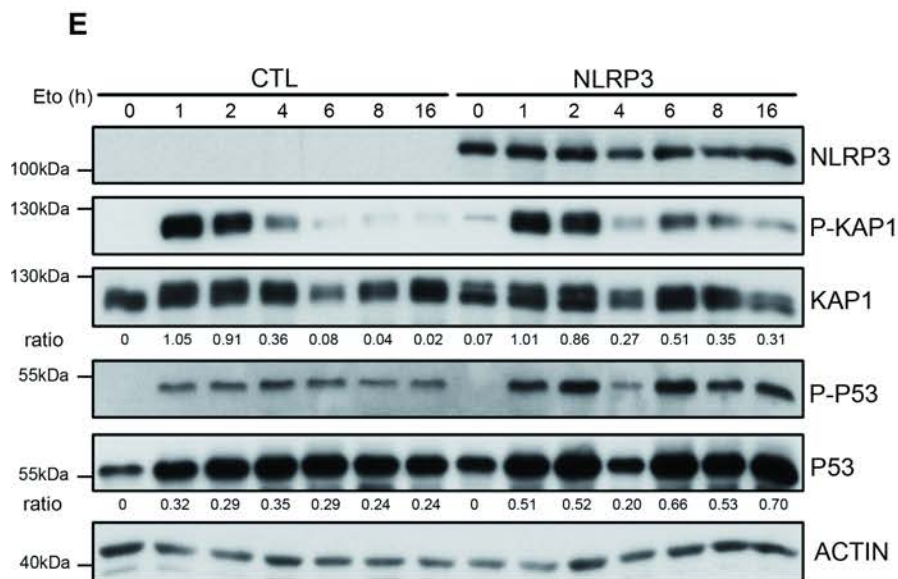
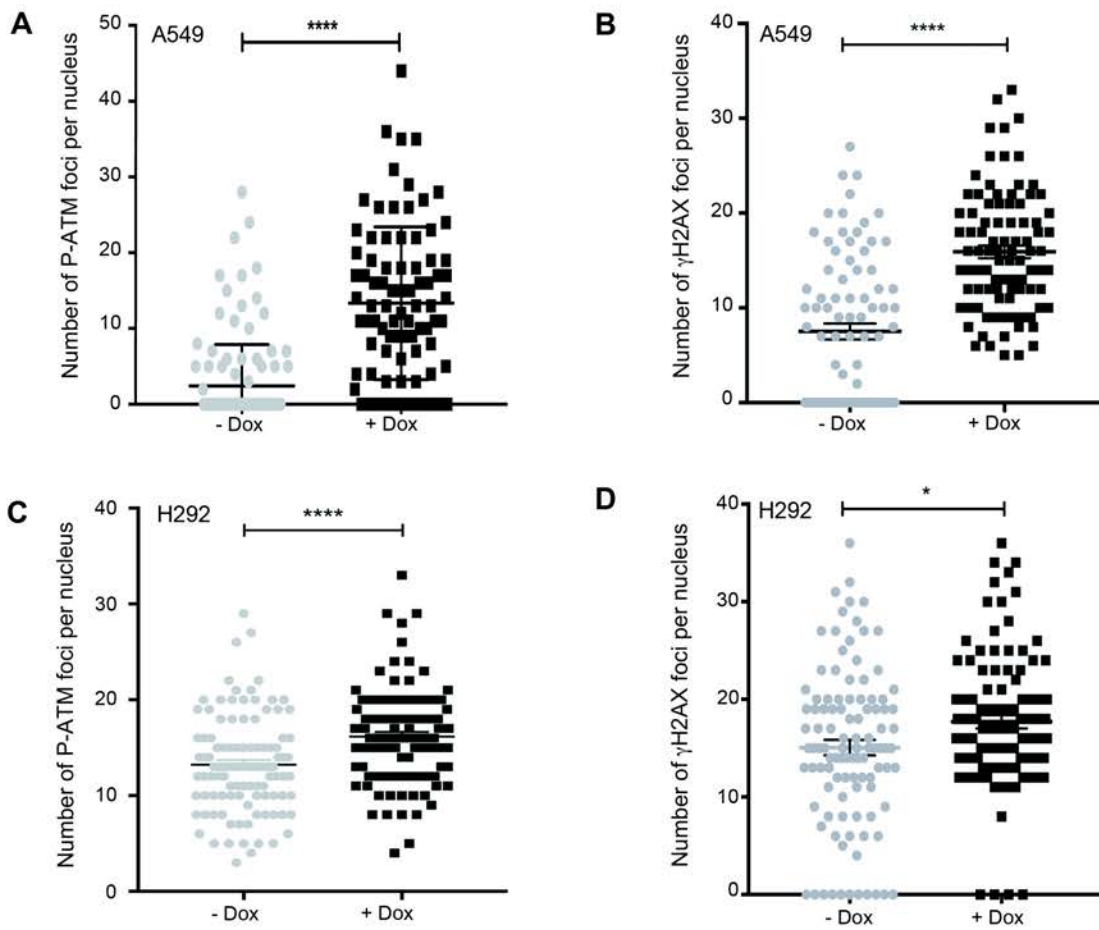
729 **Supplementary Figure 7.** NLRP3 expression is reduced in human NSCLC compared to non-
730 tumoral cells.

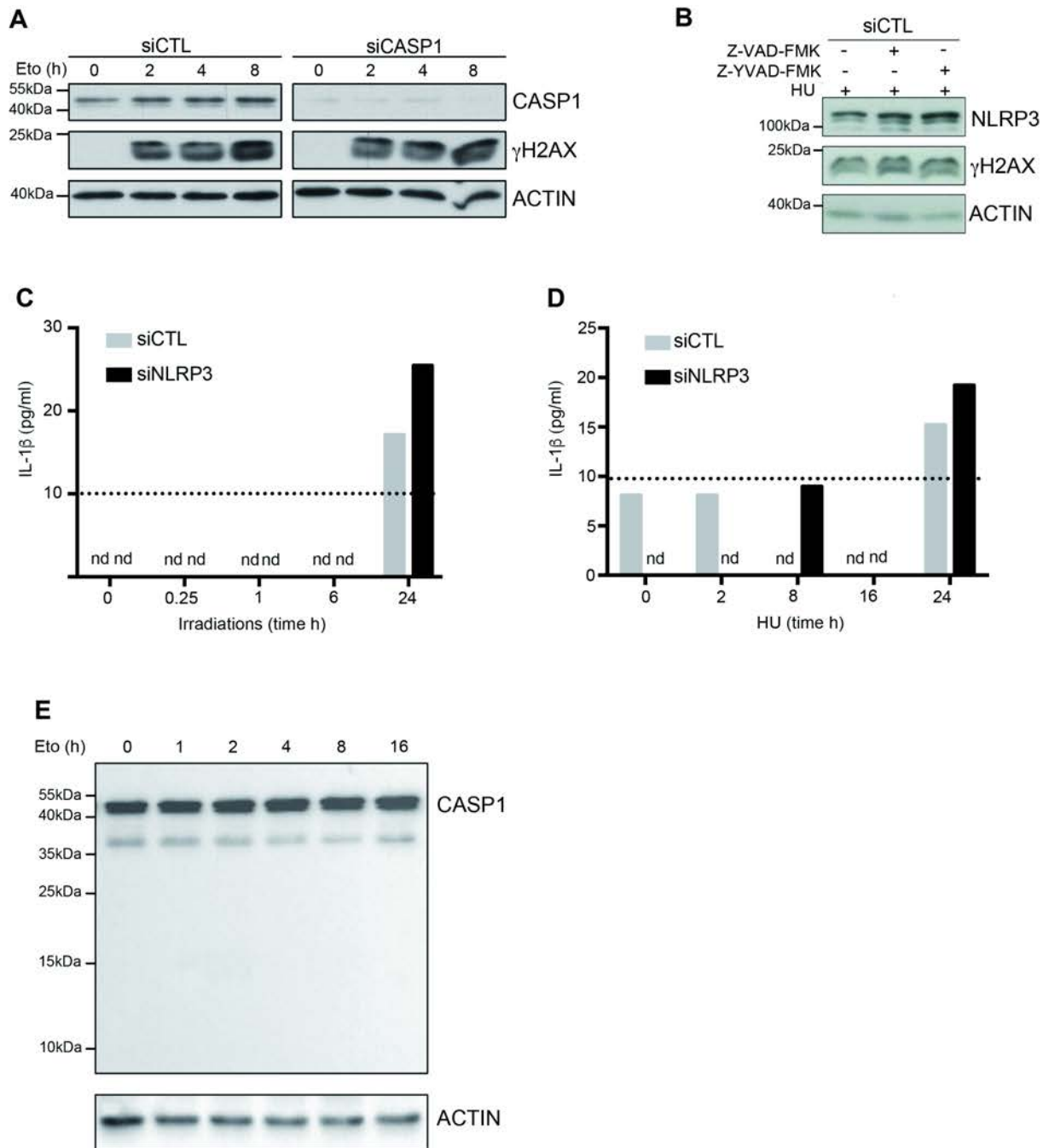
731 (A) NLRP3 inflammasome components, namely NLRP3, caspase-1 and ASC were assessed by
732 immunoblotting in NSCLC cell lines. GAPDH was used as a protein loading control. (B)
733 Anchorage-independent growth ability was assessed in HBEC3-KT cell lines.

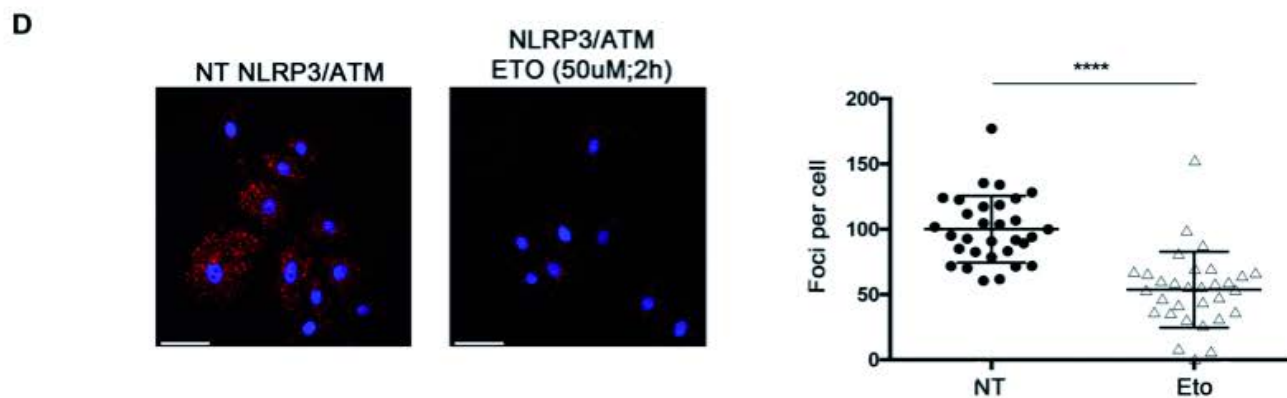
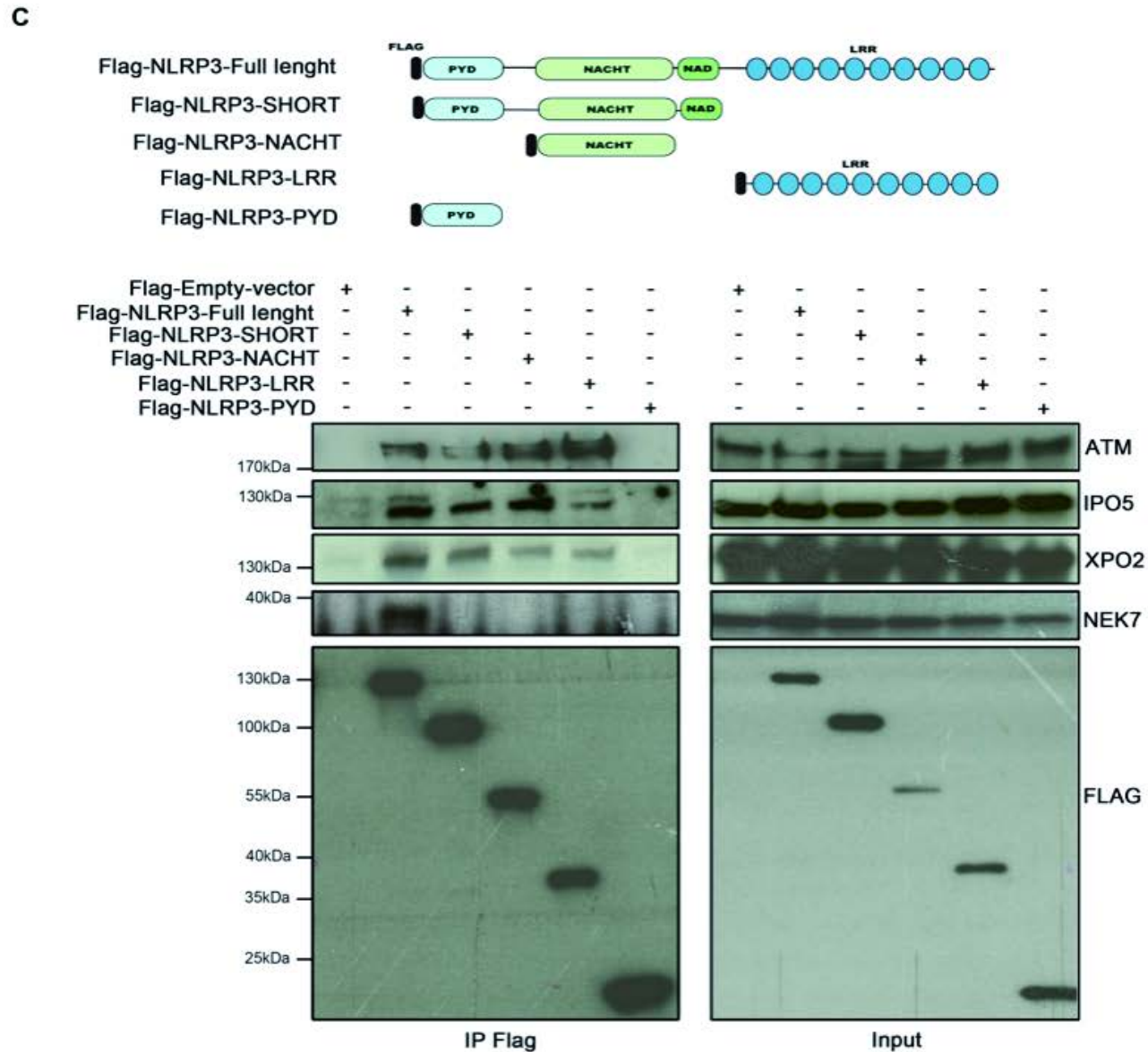
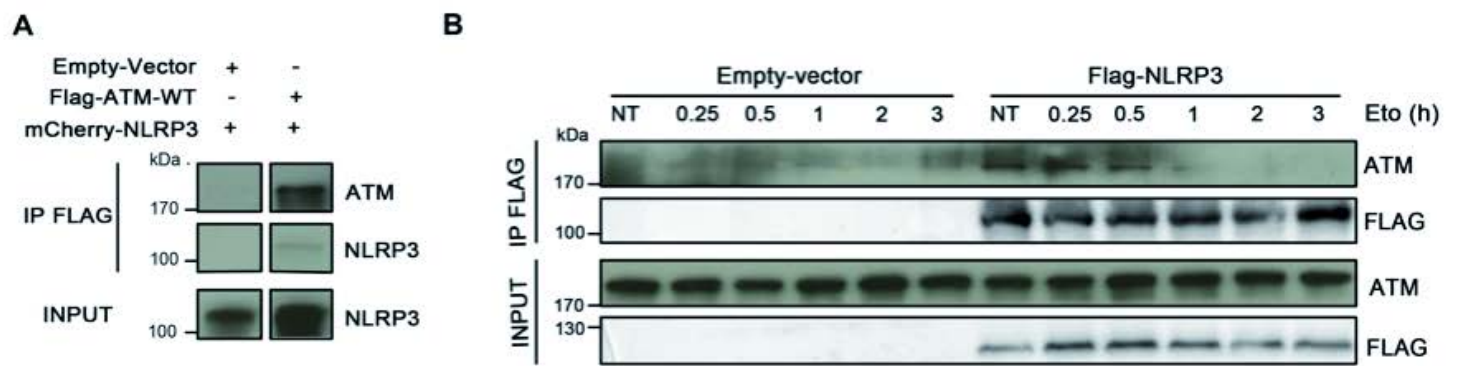
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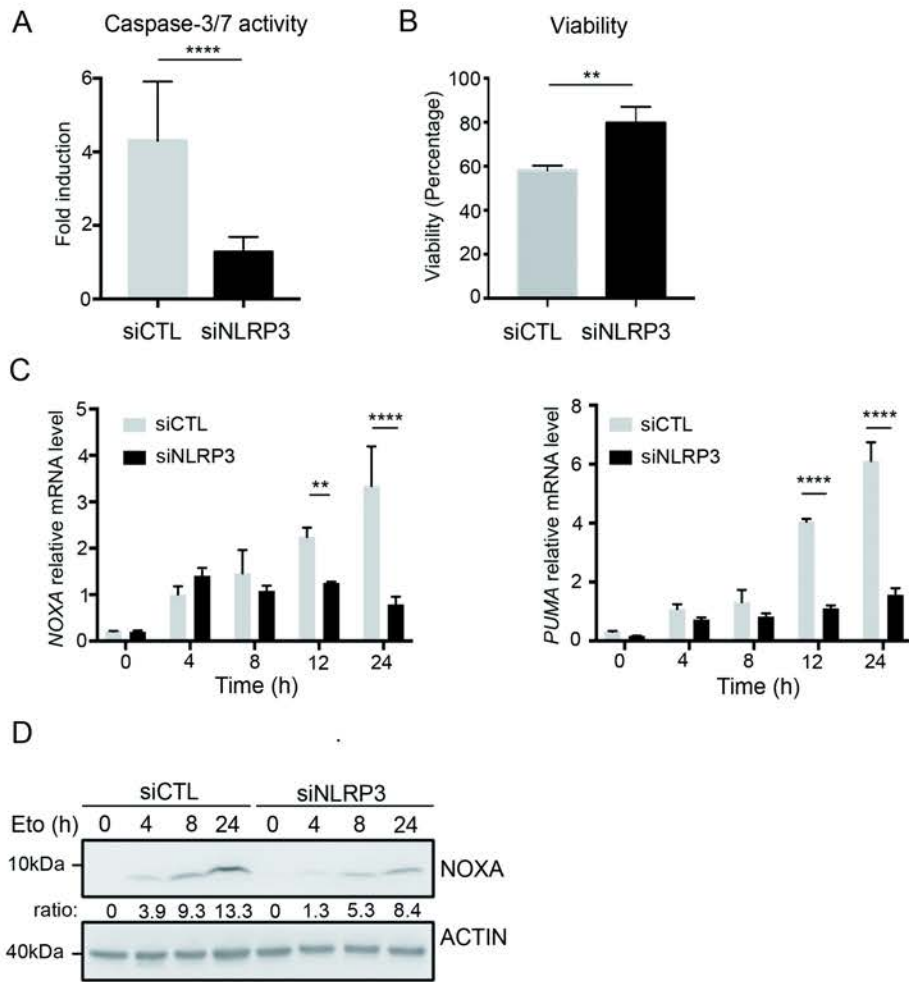


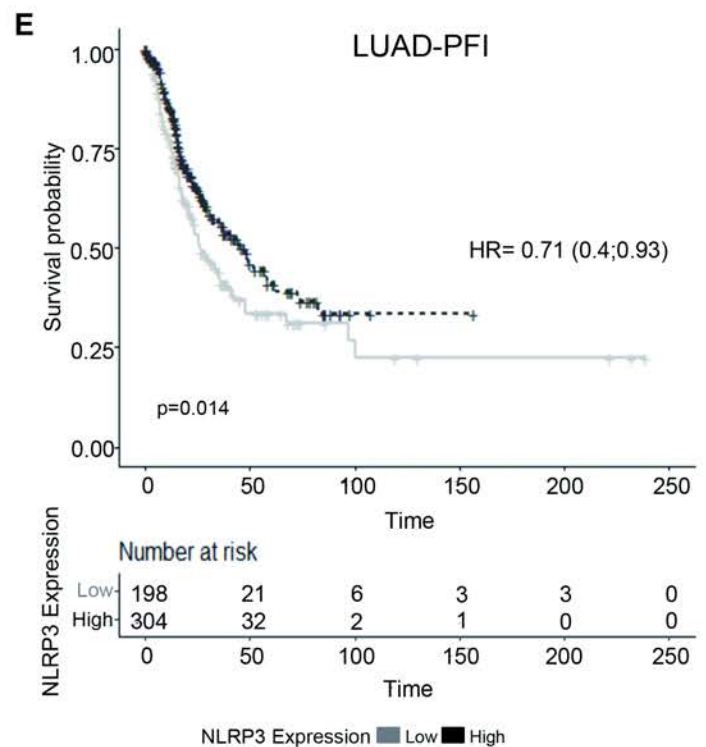
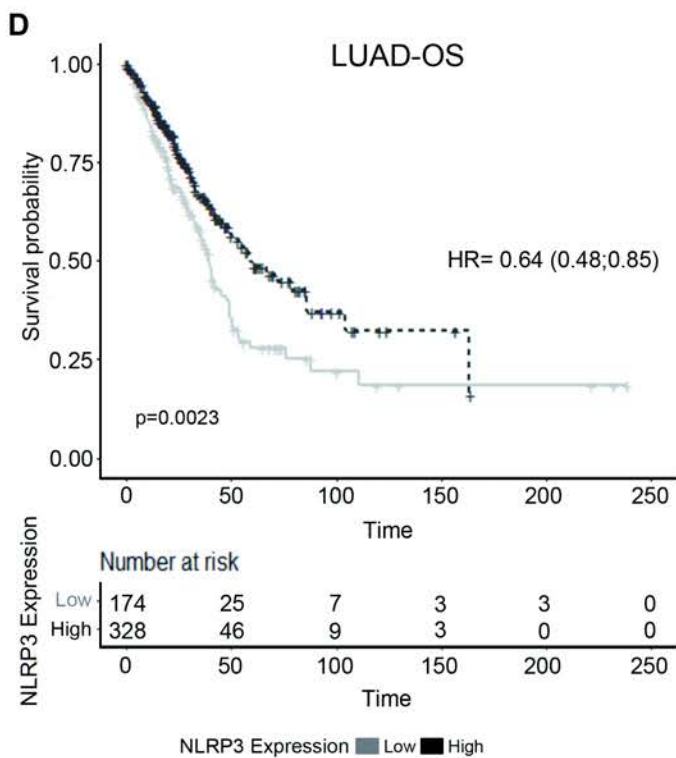
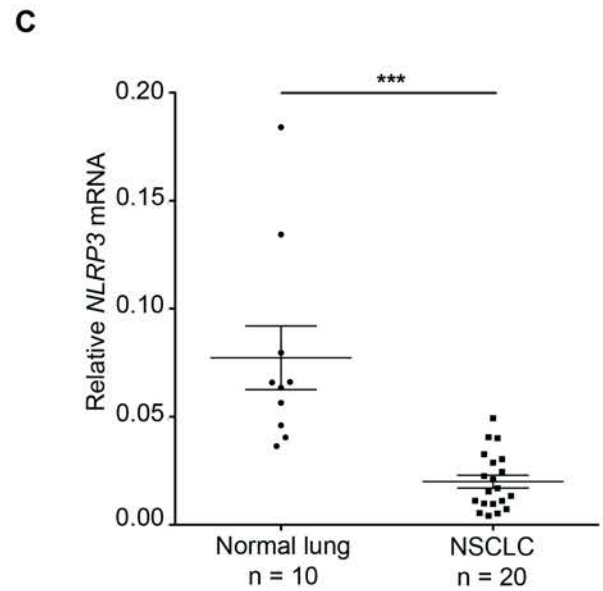
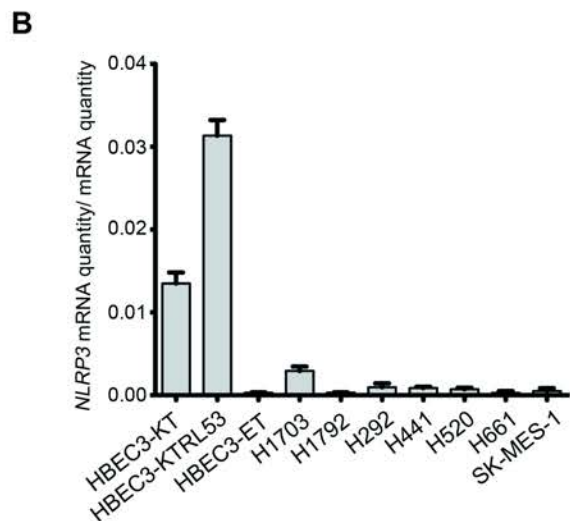
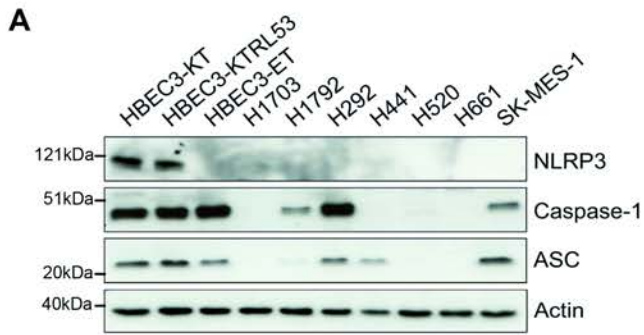


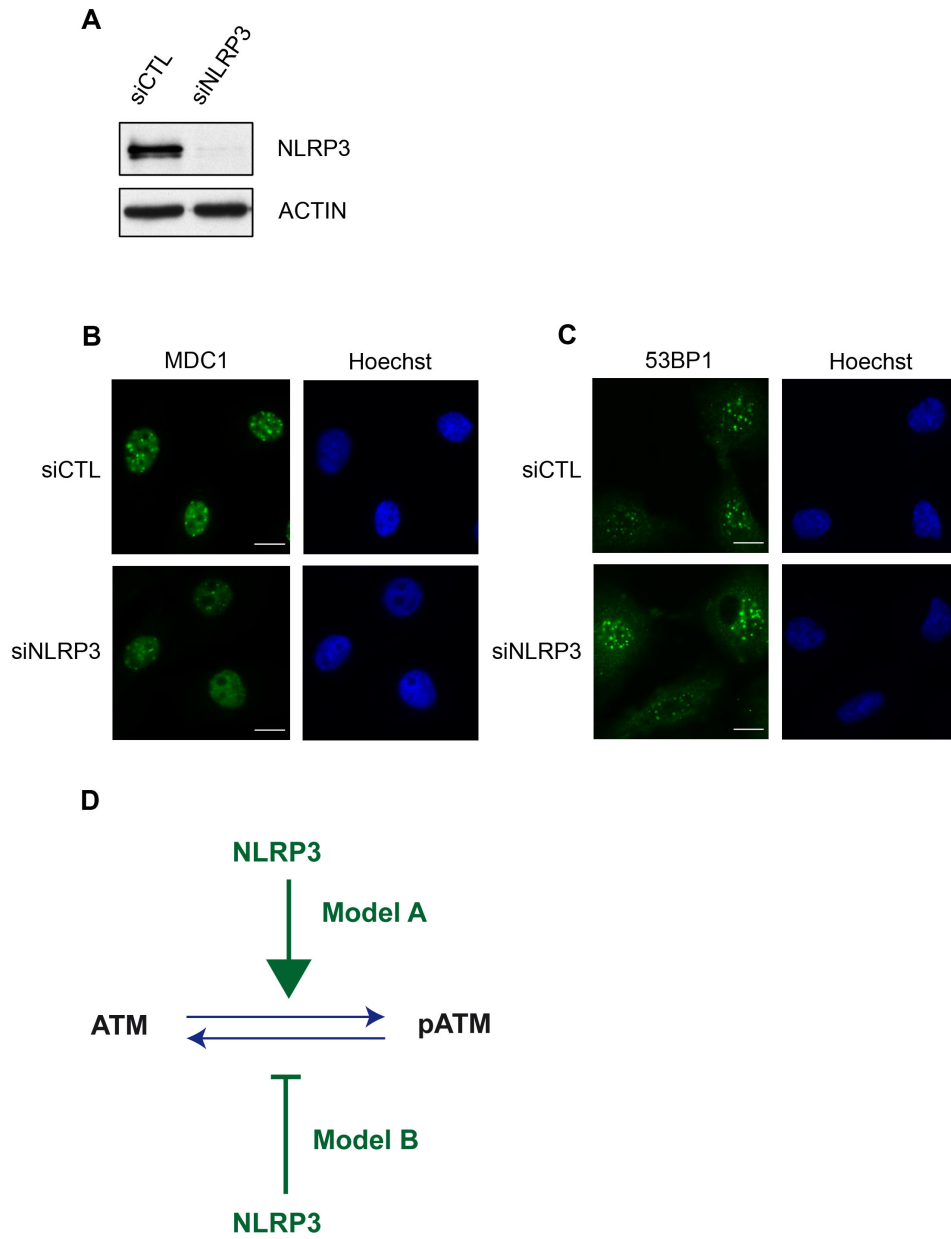


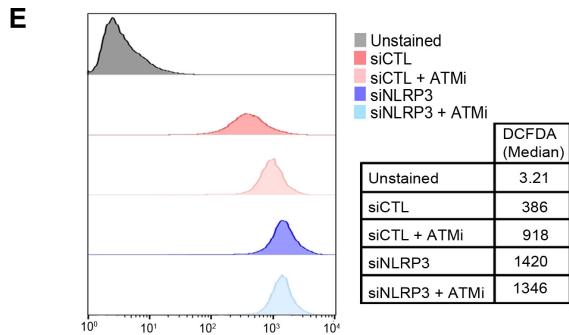
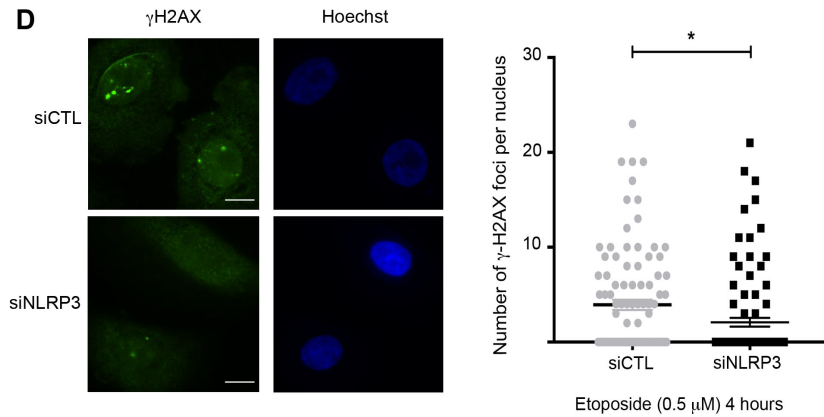
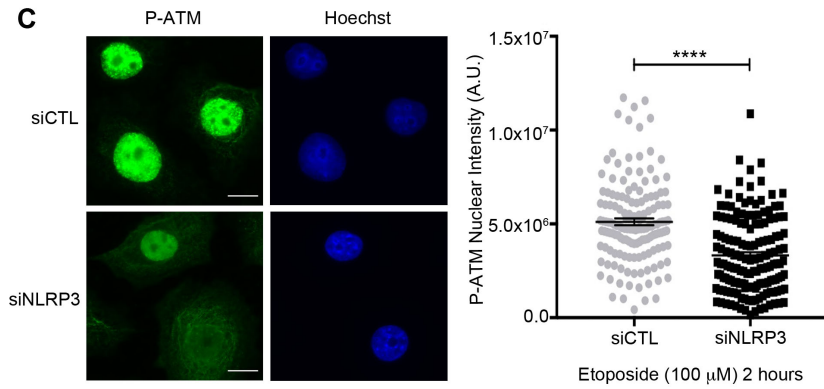
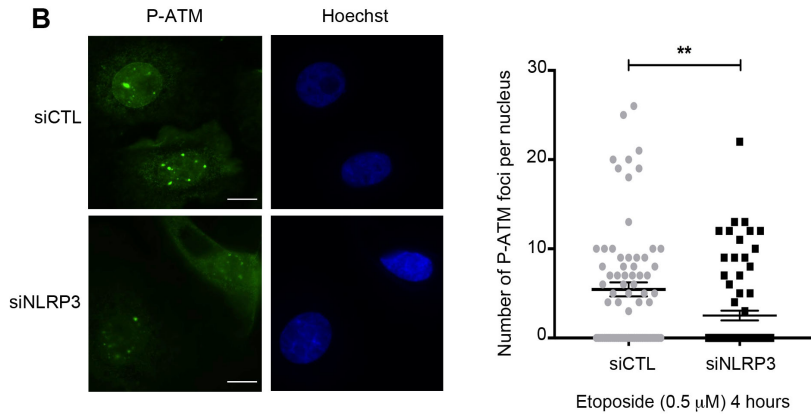
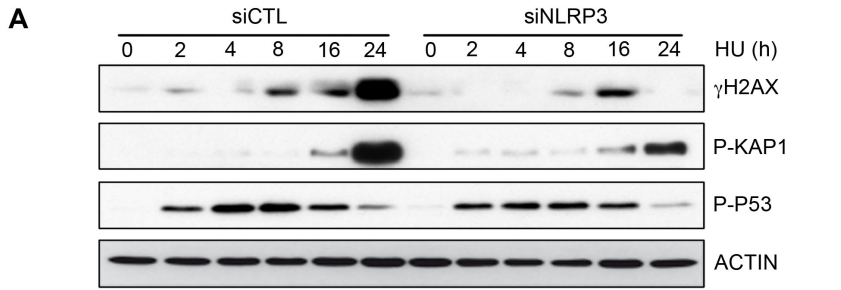




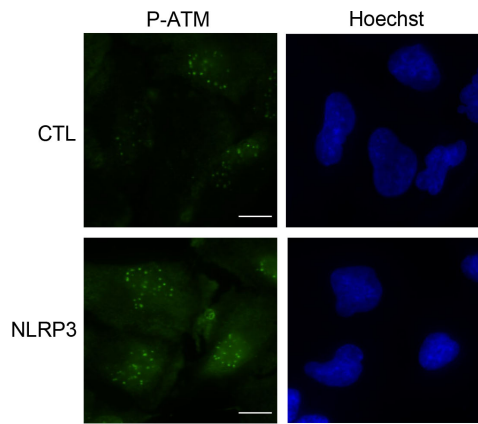




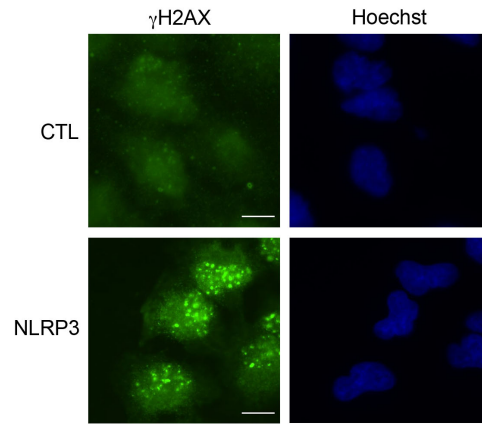




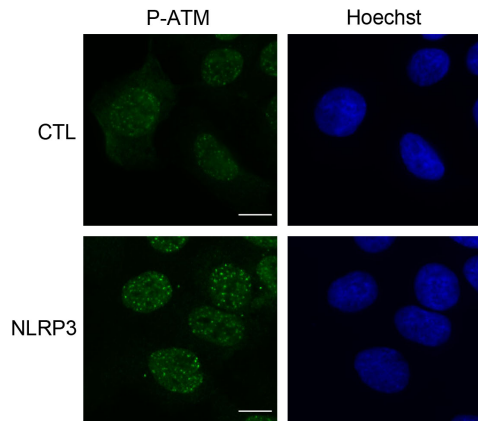
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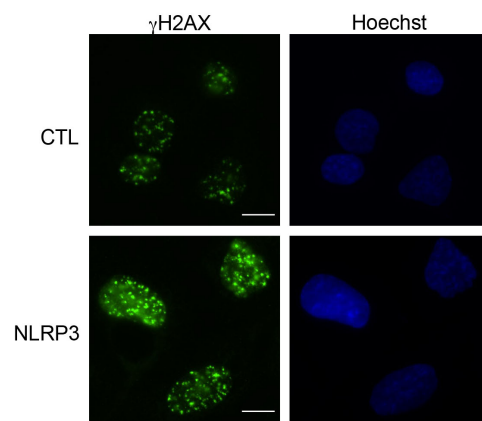
B A549



C H292



D H292



E

