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1 **The N-terminal domain of cGAS determines**
2 **preferential association with centromeric DNA**
3 **and innate immune activation in the nucleus**
4

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21 **Abstract**

22 Cytosolic DNA activates cyclic GMP-AMP (cGAMP) synthase (cGAS), an innate immune sensor
23 pivotal in anti-microbial defense, senescence, auto-immunity and cancer. cGAS is considered to be
24 a sequence-independent DNA sensor with limited access to nuclear DNA because of
25 compartmentalization. However, the nuclear envelope is a dynamic barrier and cGAS is present in
26 the nucleus. Here, we identify determinants of nuclear cGAS localization and activation. We show
27 that nuclear-localized cGAS synthesizes cGAMP and induces innate immune activation of dendritic
28 cells, although cGAMP levels are 200-fold lower than following transfection with exogenous DNA.
29 Using cGAS ChIP-seq and a GFP-cGAS knock-in mouse, we find nuclear cGAS enrichment on
30 centromeric satellite DNA, confirmed by imaging, and to a lesser extent on LINE elements. The
31 non-enzymatic N-terminal domain of cGAS determines nucleo-cytoplasmic localization,
32 enrichment on centromeres and activation of nuclear-localized cGAS. These results reveal a
33 preferential functional association of nuclear cGAS with centromeres.

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36

37 **Introduction**

38 DNA is conserved throughout evolution, posing the problem of distinction of self DNA
39 from pathogen associated or damaged self DNA by the immune system (Schlee and Hartmann,
40 2016). DNA is normally absent from the cytosol, and the presence of cytosolic DNA activates
41 cGAS. Upon DNA binding, cGAS synthesizes the second messenger 2'3'-cyclic GMP-AMP
42 (cGAMP), which binds to Stimulator of Interferon Genes (STING), resulting in activation of NFκB
43 and IRF3, their translocation to the nucleus, activation of a type I IFN response, expression of
44 interferon-stimulated genes (ISGs) and activation of dendritic cells (Li et al., 2013b; Wu et al.,
45 2012).

46 Compartmentalization of DNA in the nucleus and in mitochondria is thought to be essential
47 for avoidance of self-nucleic acid recognition, and this represents the current dogma for cGAS
48 discrimination of self vs non-self DNA (Sun et al., 2013). Accumulation of mitochondrial or
49 nuclear self DNA in the cytoplasm upon damage activates a cGAS-dependent type I IFN response
50 or senescence (Dou et al., 2017; Gluck et al., 2017; Harding et al., 2017; Hartlova et al., 2015; Lan
51 et al., 2014; Mackenzie et al., 2017; Rongvaux et al., 2014; West et al., 2015; Yang et al., 2017).
52 However, cGAS is also required for constitutive (also known as tonic) expression of ISGs,
53 suggesting that a basal level of nucleic acids activate the sensor in the absence of microbial
54 infection or apparent damage (Gough et al., 2012; Schoggins et al., 2014).

55 While mitochondrial integrity is linked to cell survival and its disruption leads to apoptotic
56 cell death (Tait and Green, 2010), the nuclear envelope (NE) is a dynamic barrier in both cycling or
57 differentiated non-dividing cells. In cycling cells, the NE is disassembled and then reassembled
58 during mitosis to ensure DNA segregation in daughter cells after cytokinesis (Guttinger et al.,
59 2009). Nuclear disassembly leaves the nuclear DNA potentially accessible to cytosolic factors.
60 Moreover, confinement of interphase cells, such as during migration in tissues, leads to repeated NE
61 rupture and repair events. During NE rupture, over-expressed cGAS binds to the exposed nuclear
62 DNA (Denais et al., 2016; Raab et al., 2016).

63 Overall, while there is mounting evidence that the cGAS-STING axis can be activated by
64 nuclear DNA released in the cytosol upon damage (Chen et al., 2016; Gluck et al., 2017; Harding et
65 al., 2017; Yang et al., 2017), the regulation and consequences of putative cGAS recruitment into the
66 nucleus is poorly understood. We asked how cGAS recruitment to the nucleus is determined, and to
67 what extent cGAS could be activated in the nucleus itself.

68 **Results**

69 cGAS has been described to be a cytosolic sensor of DNA (Sun et al., 2013), but the
70 localization of the endogenous protein in primary immune cells has not been extensively studied.
71 To exclude interference from the cell cycle, that results in NE disassembly, we examined the
72 localization of endogenous cGAS in primary human monocyte-derived dendritic cells (DCs) that
73 are terminally differentiated and in interphase (Ardehna et al., 2000). Endogenous cGAS protein
74 staining was specific and showed distribution of the sensor in both the cytoplasm and the nucleus
75 (**Fig S1a**). The average cGAS intensity was higher in the nucleus than in the cytoplasmic area of
76 cells (**Fig 1a**). In the nucleus, cGAS displayed a punctate, perinuclear ring of stronger intensity
77 within the DAPI staining (**Fig 1b, S1b**). Biochemical fractionation confirmed the presence of cGAS
78 in the nuclear fraction of DCs at steady state (**Fig 1c, S1c**). Endogenous nuclear cGAS was also
79 present in wild-type (WT) mouse bone marrow-derived DCs and lost in *Cgas*^{-/-} cells (**Fig 1d**). Thus,
80 both a cytoplasmic and a nuclear pool of cGAS are present in DCs.

81 In contrast to endogenous cGAS, GFP-cGAS expressed during interphase localizes mostly
82 to the cytosol of DCs (Raab et al., 2016). Through hematopoietic development, dendritic cells result
83 from a series of mitotic events (Lee et al., 2015). We hypothesized that endogenous nuclear cGAS
84 in interphase could result from interaction with nuclear DNA during NE breakdown that occurred in
85 a previous mitosis. We tracked cGAS localization during the cell cycle using stable expression of
86 GFP-cGAS in a cycling HeLa cell line. In this stable culture, the ratio of nuclear/cytoplasmic mean
87 GFP-cGAS intensity was >1 for 60% of cells and <1 for 40% of cells (**Fig 1e, 1f**). In order to
88 follow the ability of GFP-cGAS to enter the nucleus in mitosis, we tracked cells that contained
89 mostly cytoplasmic cGAS prior to mitosis (**Fig 1e, 1g, Movie S1, Movie S2**). At early metaphase
90 cGAS started to accumulate at the periphery of the nucleus, coinciding with the onset of NE
91 breakdown. After NE breakdown and through mitosis cGAS accumulated on distinctive
92 chromosomes. After cytokinesis, the inherited pool of nuclear cGAS persisted in the nucleus during
93 the next interphase, with a slow decay across several hours, while the cytoplasm, initially devoid of

94 cGAS at the onset of interphase, gradually accumulated cGAS (**Fig S1d, Movie S3**). Importantly,
95 deletion of amino acids regions K173-I220 and H390-C405 in cGAS, that contain DNA binding
96 surfaces of cGAS (cGAS $\Delta_{K173-I220}\Delta_{H390-C405}$) prevented accumulation on chromosomes after NE
97 breakdown (**Fig S1e, Movie S4**) (Li et al., 2013a). Therefore, cGAS is expressed as a cytosolic
98 protein in interphase, but NE breakdown during cell cycle renders the DNA available for cGAS
99 binding and recruitment in the nucleus, and this generates daughter cells with a pool of nuclear
100 cGAS that persists during the next interphase.

101 DCs demonstrate low levels of co-stimulatory molecule expression CD86 at steady state
102 (Gentili et al., 2015), suggesting that endogenous nuclear cGAS is not sufficient to activate innate
103 immunity despite an excess of DNA. We aimed to test if increased levels of nuclear cGAS could
104 lead to innate activation of DCs. DNA damage was recently proposed to induce nuclear
105 translocation of cGAS (Liu et al., 2018). We expressed GFP-cGAS and the DNA damage reporter
106 mCherry-53BP1₁₂₂₄₋₁₇₁₆ (Raab et al., 2016) in DCs. Etoposide induced nuclear 53BP1 foci,
107 indicating DNA damage, but GFP-cGAS did not translocate in the nucleus of DCs (**Fig S1f, S1g**).
108 To enforce nuclear localization of cGAS, we transduced DCs with a GFP control lentivector or with
109 lentivectors coding for GFP-cGAS or GFP-cGAS fused to a Nuclear Localization Signal (NLS)
110 (GFP-NLS-cGAS). We previously showed that CMV-driven GFP-cGAS-coding lentivectors induce
111 CD86 in the absence of vector expression in DCs, due to cGAS expression and activation in
112 lentivector-producing cells, resulting in cGAMP packaging in viral particles and transfer to DCs,
113 which activates STING and interferes with efficient cGAS expression in the DCs (Gentili et al.,
114 2015). To limit cGAS expression in lentivector-producing cells, we developed a lentiviral vector in
115 which the expression of the insert is driven by an inverted HLA-DR α promoter (**Fig 2a**). We next
116 efficiently transduced DCs using Vpx to overcome SAMHD1 restriction (Hrecka et al., 2011;
117 Laguette et al., 2011) (**Fig S2a**). When transduced with GFP-NLS-cGAS, DCs showed exclusive
118 nuclear localization of the sensor, while GFP-cGAS transduction was mainly cytosolic (**Fig 2b**).
119 Monocyte-derived dendritic cells are in G0 phase and do not cycle, hence the cytoplasmic

120 localization of cGAS is consistent with the lack of mitosis in these cells, in contrast to cycling HeLa
121 cells (**Fig 1e**). We measured induction of the costimulatory molecule CD86, a marker of innate
122 immune activation in DCs. DCs transduced in absence of Vpx did not express GFP and did not
123 upregulate CD86 indicating that no significant cGAMP transfer from lentivector-producing cells
124 was occurring (**Fig 2c, 2d, S2a**). DCs transduced with GFP-NLS-cGAS in presence of Vpx
125 upregulated CD86 compared to a control vector (**Fig 2c, 2d**). CD86 upregulation was higher for
126 NLS-GFP-cGAS than GFP-cGAS, despite similar transduction levels (**Fig 2d, S2a**). We conclude
127 that with the inverted HLA-DR α promoter system, cGAMP transfer by viral particles is not
128 implicated, and that NLS-cGAS expression from the lentivector in the transduced DCs induces the
129 CD86 activation marker.

130 Activation of DCs by GFP-NLS-cGAS driven by HLA-DR α -inverted remained limited. To
131 determine if further increasing expression of GFP-NLS-cGAS in DCs could reveal a full activated
132 state, we tested the SFFV promoter (**Fig 2e**). SFFV-driven lentivectors efficiently transduced DCs
133 with Vpx (**Fig S2b, S2c**). In transduced DCs, expression of GFP-NLS-cGAS with Vpx induced
134 CD86 and the interferon stimulated gene SIGLEC1 (**Fig 2f, 2g, 2h, 2i, S2d, S2e**). CD86
135 upregulation was restricted to the GFP-positive fraction of DCs transduced with GFP-NLS-cGAS
136 (**Fig 2j**), while SIGLEC1 was also induced in GFP-negative cells in the same well, which indicated
137 production of soluble type I interferon as a result of GFP-NLS-cGAS expression (**Fig 2k**). CD86
138 and SIGLEC1 induction by GFP-NLS-cGAS expression in DCs required an intact catalytic site in
139 cGAS, indicative of enzymatic activation of cGAS in the nucleus (**Fig S2f, S2g, S2h**). The ISGs
140 *MX1*, *CXCL10*, *IFIT1* and *OAS1* were also upregulated by GFP-NLS-cGAS (**Fig 2l**). To further
141 validate that increasing levels of nuclear cGAS lead to innate immune activation, we performed
142 dose titrations of the lentivectors driven by either promoter, and plotted CD86 over Mean
143 Fluorescence Intensity (MFI) of GFP (**Fig S2i, S2j**). CD86 expression was correlated with the GFP-
144 NLS-cGAS expression level, independently of the type of promoter used. Taken altogether, these
145 results indicate that increasing nuclear cGAS level in DCs results in innate immune activation.

146 To estimate the activity of nuclear cGAS, we reconstituted 293FT cells that are devoid of
147 endogenous cGAS (Gentili et al., 2015). Similar to HeLa cells, stable transduction of cGAS in
148 cycling cGAS-deficient 293FT cells resulted through mitosis in a mixture of cells with either
149 mostly cytoplasmic cGAS, mostly nuclear cGAS, or both (**Fig 3a, S3a**). We also reconstituted
150 293FT with NLS-cGAS, that showed exclusive nuclear localization of the sensor (**Fig 3a, S3a**). We
151 measured cellular cGAMP production using a bioassay (Woodward et al., 2010) (**Fig S3b**). Despite
152 the absence of transfected exogenous DNA, cGAMP was produced endogenously in both stable
153 cGAS- and NLS-cGAS- expressing cells, as measured by cGAMP bioassay (**Fig 3b, 3d**) or cGAMP
154 ELISA (**Fig 3e**). The level of cGAMP produced was similar between cGAS and NLS-cGAS,
155 suggesting that the bulk of cGAMP production by stable cGAS expression was the result of the
156 nuclear pool, and not the cytoplasmic pool of the protein. Strikingly, further supplementing cells
157 with exogenous DNA by transfection of herring testis DNA (HT-DNA) increased cGAMP
158 production in cGAS- and NLS-cGAS-expressing-cells, by 235- and 500-fold, respectively (**Fig 3c,**
159 **3d**). Diploid human cells contain 7 pg of nuclear DNA per cell, while the maximum amount of
160 transfected HT-DNA was 2.5 pg per cell. Therefore the amount of nuclear DNA exceeded the
161 amount of HT-DNA and was not the limiting factor for cGAS activation. We next measured
162 cGAMP concentration in nuclear and cytoplasmic fractions of non-cycling DCs after expression of
163 GFP-cGAS or GFP-NLS-cGAS (**Fig 2b**). While the amount of cytosolic cGAMP was similar for
164 GFP-cGAS and GFP-NLS-cGAS, nuclear cGAMP was more abundant for GFP-NLS-cGAS than
165 GFP-cGAS (**Fig 3f, 3g**). Altogether, we conclude that nuclear-localized cGAS is enzymatically
166 active, and limited by at least 200-fold compared to its activity in the response to transfected DNA.

167 Next, we asked if nuclear entry of cGAS through mechanical nuclear envelope ruptures
168 (Raab et al., 2016), rather than NLS, could similarly lead to activation of the sensor. We used a
169 microfabricated cell confiner to control the extent of NE rupture events in cells (**Fig 4a**) (Le Berre
170 et al., 2014; Raab et al., 2016). We also generated a reporter cell line to simultaneously visualize
171 NE rupture and STING-induced IRF3 nuclear translocation at single cell level by live video

172 microscopy (**Fig 4b**). We used HeLa cells that express a functional endogenous cGAS (Gentili et
173 al., 2015) to activate the STING reporter in response to DNA and monitored NE rupture by
174 assessing localization of a catalytically inactive cGAS (Raab et al., 2016). At steady state, cGAS
175 localized in both the nucleus and the cytoplasm of HeLa cells, while GFP-IRF3 was exclusively
176 cytosolic (**Fig 4c, Movie S5**). Upon HT-DNA transfection, cGAS localized to the transfected DNA
177 and IRF3 translocated to the nucleus, confirming functionality of the single-cell assay (**Fig 4c,**
178 **Movie S5**). Confinement at 3 μ m height induced multiple NE rupture events that increased with
179 time, as revealed by cGAS accumulation on the nuclear DNA (**Movie S6**). Despite multiple NE
180 rupture that recruited cytoplasmic cGAS in the nucleus over 16 hours (Raab et al., 2016), no GFP-
181 IRF3 translocation event was observed (**Fig 4d, 4e, Movie S6**). In contrast, when cells were
182 confined and simultaneously transfected with HT-DNA, IRF3 nuclear translocation was rescued,
183 thus excluding the possibilities that cell confinement interfered with the signaling pathway or that
184 endogenous cGAS protein was in limiting amounts (**Fig 4d, 4f, 4g, Movie S7, Movie S8**).
185 Moreover, catalytically inactive GFP-NLS-cGAS did not inhibit innate immune activation of
186 dendritic cells in response to HIV-1 or HIV-2 infection, a process that requires nuclear cGAS
187 (Lahaye et al., 2018), or to HT-DNA (**Fig 4h, 4i**). Thus, catalytically inactive cGAS was unlikely
188 competing with endogenous cGAS following nuclear envelope rupture. We conclude that in
189 contrast to NLS-mediated entry of over-expressed cGAS, entry of endogenous cGAS through
190 mechanical nuclear envelope rupture is not sufficient to activate STING.

191 We noted that NLS-cGAS was distributed throughout the nucleus, while entry through
192 nuclear envelope rupture produced small and peripheral-localized foci of nuclear cGAS. We next
193 asked if innate immune activation by overexpressed NLS-cGAS in the nucleus resulted from
194 association with spatially-localized, specific DNA elements. While cGAS enzymatic domain has
195 been shown to bind DNA in a sequence-independent manner, we noticed that GFP-NLS-cGAS
196 signal in DCs showed patterns of GFP enrichment (**Fig 5a**). To understand if NLS-cGAS could
197 associate with specific chromatin regions, we performed ChIP-seq analysis on DCs transduced with

198 GFP-NLS-cGAS using GFP-trap on the nuclear fraction (**Fig 5b**). cGAS peaks were distributed
199 along all chromosomes, and were preferentially located on a subset of annotated genomic elements
200 (**Fig S4a**). To determine the enrichment of cGAS on genomic features, we computed the fraction of
201 peaks falling within a given genomic element compared to the expected fraction based on the
202 genomic coverage. cGAS peaks were mostly enriched on the satellite repeat class (**Fig 5c**), mainly
203 on the ALR/Alpha satellites family within this class, and to a lesser extent on LINE elements. α -
204 satellites are the main components of centromeres. cGAS peaks were broadly distributed across the
205 genome, but peaks in close proximity between at least two donors were enriched on centromeres
206 (**Fig 5d**). We computed peaks of CENP-A, the centromeric histone H3 variant, from previously
207 reported ChIP-seq datasets of endogenous CENP-A, that maps to centromeres and pericentromeric
208 heterochromatin (PHC) (Lacoste et al., 2014) (**Fig 5d**). cGAS peaks were associated with CENP-A
209 peaks, as determined by the odds-ratio statistic (**Fig 5e**). Pericentromeric heterochromatin is
210 enriched in Histone 3 Lysine 9 trimethylation (H3K9me3) mark (Muller and Almouzni, 2017).
211 cGAS peaks were also associated with H3K9me3 peaks from the ENCODE database (**Fig 5e**). In
212 contrast, cGAS peaks were not associated with peaks of the Histone 3 Lysine 27 acetylation
213 (H3K27Ac) mark of open chromatin (**Fig 5e**). To assert that centromeric DNA resulted from cGAS,
214 we performed ChIP-seq of GFP-NLS-cGAS in two donors, using GFP-NLS as a control. cGAS-
215 specific peaks were broadly distributed across the genome and were enriched on centromeres (**Fig**
216 **S4b**). Centromeres are bound by CENP-B, which assembles on a 17bp DNA consensus sequence
217 within α -satellites, NTTCGNNNNANNCGGGN, named CENP-B box (Muro et al., 1992). cGAS-
218 specific peaks were enriched in the CENP-B box consensus sequence, but not in telomeric sequence
219 TTAGGG repeats (**Fig 5f**). De novo motif enrichment analysis for cGAS-specific peaks revealed
220 also an enrichment in AATGG and CCATT sequences (**Fig S4c**), which was confirmed by
221 enrichment analysis (**Fig 5f**). (AATGG)_n(CCATT)_n repeat is a characteristic motif of satellite III
222 DNA that is present at centromeres (Grady et al., 1992). cGAS-specific enrichment on satellite was
223 also assessed directly from the sequencing reads. **A global read enrichment of the satellite class was**

224 not detected in GFP-NLS-cGAS ChIP over input. We reasoned that cGAS might be associated to a
225 subset of specific satellite occurrences. We compared the reads abundance of GFP-NLS-cGAS over
226 GFP-NLS (to exclude any non-cGAS specific binding) on individual annotated repeat elements in
227 the genome. To account for the differences in number of individual repeat elements between
228 classes, we sorted the elements by enrichment and computed the fraction of occurrences within rank
229 bins within each class. cGAS-specific enrichment scores could be detected in the repeat classes
230 satellite, LTR, LINE and simple_repeat, and the satellite class ranked highest (**Fig 5g**). To confirm
231 cGAS enrichment on centromeres, we transduced DCs with GFP-NLS and GFP-NLS-cGAS and
232 analyzed GFP intensity surrounding CENP-B protein foci (**Fig S4d**) relative to randomly selected
233 nuclear foci. GFP-NLS-cGAS was significantly enriched at CENP-B foci as compared to free GFP-
234 NLS (**Fig 5h, 5i, 5j, 5k**). Therefore, NLS-cGAS in the nucleus of DCs is preferentially associated
235 with centromeric DNA.

236 cGAS is activated by DNA in a length-dependent manner (Andreeva et al., 2017; Luecke et
237 al., 2017). To determine if satellite DNA could preferentially activate cGAS, we transfected DCs
238 with Satellite III DNA AATGG repeats or shuffled sequences of increasing length. While innate
239 immune activation of DCs was similar for both sequences with 12-repeat dsDNA (60nt), a
240 preferential response to the AATGG motif was detected with shortening of the repeats (**Fig 5l, S4e**).
241 With the shortest 4-repeat dsDNA (20nt), CD86, IFN- λ 1, IFN- β and IP-10 expression was
242 significantly increased for the AATGG sequence compared to the shuffled sequence, and a similar
243 trend was observed for SIGLEC1 expression. This suggests that cGAS may be preferentially
244 activated by satellite DNA repeats of smaller length.

245 cGAS protein contains one positively charged N-terminal domain and two positively
246 charged regions in the C-terminal catalytic domain, that can all interact with DNA (Sun et al., 2013;
247 Tao et al., 2017). We wondered if the centromeric association of cGAS was determined in the
248 protein sequence. The C-terminal catalytic domain 161-522 of cGAS is sufficient to recapitulate
249 DNA-binding in a sequence-independent manner and DNA-dependent cGAMP enzymatic activity

250 (Civril et al., 2013; Sun et al., 2013). However, the function of the N-terminal domain 1-212 that
251 also binds to DNA is not fully understood (Du and Chen, 2018; Sun et al., 2013; Tao et al., 2017).
252 To understand whether the N-terminal domain could be a functional determinant in cGAS, we
253 expressed the truncated domains in non-cycling human DCs. To our surprise, GFP-cGAS 161-522
254 showed spontaneous accumulation in the nucleus (**Fig 6a**), while GFP-cGAS 1-212 showed a
255 cytosolic localization. We next examined the intracellular localization of the isolated domain 1-160.
256 In contrast to GFP-cGAS 1-212, GFP-cGAS 1-160 spontaneously accumulated in the nucleus (**Fig**
257 **6b**), similar to GFP-NLS (**Fig 2b**). Thus, amino acids 161-212 in GFP-cGAS 1-212 are essential for
258 cytosolic retention. We conclude that cGAS expressed in interphase is actively retained in the
259 cytosol by the domain 1-212, which counteracts two nuclear-localizing activities in domains 1-161
260 and 161-522.

261 Interestingly, activation of DCs was lost upon deletion of domain 1-160 in cGAS, despite its
262 nuclear localization and an intact catalytic site (**Fig 6c, 6d, S5a**) and its response to transfected
263 cytosolic DNA in a STING-dependent reporter assay (**Fig S5b, S5c**). Adding NLS to GFP-cGAS
264 161-522 did not rescue DC activation, indicating that it was not due to sub-optimal accumulation in
265 the nucleus (**Fig 6c, 6d, S5a**). Human DCs express endogenous cGAS. To confirm the results in the
266 absence of endogenous cGAS, we transduced *Cgas*^{-/-} mouse bone marrow-derived DCs with GFP,
267 GFP-NLS, GFP-cGAS, GFP-NLS-cGAS, GFP-cGAS 1-160 or GFP-cGAS 161-522 lentivectors
268 (**Fig S5d**). GFP-cGAS was transduced at low levels and localized in the cytoplasm (**Fig 6e, S5d**).
269 GFP-NLS-cGAS and GFP-cGAS 1-160 were localized in the nucleus with some detection in the
270 cytoplasm, and GFP-cGAS 161-522 was exclusively detected into the nucleus (**Fig 6e**). Only GFP-
271 cGAS and GFP-NLS-cGAS induced the upregulation of the mouse ISGs *Ifit1*, *Ifit2* and *Oas1* (**Fig**
272 **6f, S5e**). ISG induction by GFP-NLS-cGAS was lost in the presence of reverse transcriptase
273 inhibitors that inhibited lentiviral transduction, showing that it resulted from vector expression and
274 excluding an effect due to cGAMP transfer. In contrast, ISG induction by GFP-cGAS was
275 maintained with the inhibitors, indicative of ISG induction resulting from cGAMP transfer by the

276 lentivector. We hypothesized that domain 1-160 might determine association of nuclear cGAS with
277 centromeres. Indeed, cGAS 161-522 had reduced association of cGAS with CENP-B foci (**Fig 6g**,
278 **S5f**) despite its nuclear localization and irrespective of an ectopic NLS. Catalytic mutations in full
279 length NLS-cGAS had no impact on the association with CENP-B foci. Taken together, these
280 results show that once cGAS is in the nucleus, the N-terminal domain 1-160 is required for
281 association with centromeric DNA and activation of the sensor.

282 Finally, we sought to determine if centromere association also applied to endogenous cGAS.
283 First, we stained endogenous cGAS on metaphase spread chromosomes in a cycling human cell
284 line. CENP proteins remain associated with centromeres in cycling cells (Dunleavy et al., 2005).
285 Staining of endogenous cGAS revealed dispersed cGAS foci across chromosomes, including
286 telomeres and centromeres (**Fig 7a**). On centromeres, cGAS foci were enriched in between pairs of
287 CENP-A and anti-centromere antibodies (ACA, a mix of CENP-A/B/C) foci (**Fig 7a, 7b**), where
288 centromeric DNA is located and stained also by ACA (Dunleavy et al., 2005). cGAS was detectable
289 on all centromeres examined, but the intensity of cGAS staining was variable between centromeres
290 (**Fig 7c**). Next, we examined the DNA associated with endogenous cGAS in the nucleus. Bone
291 marrow-derived macrophages have a constitutive (or tonic) expression of interferon stimulated
292 genes (ISG) expression that requires cGAS (Schoggins et al., 2014). Similar to macrophages, we
293 found that *Ifit1*, *Ifit2* and *Oas1a* were less expressed in bone marrow-derived DCs derived from
294 *Cgas*^{-/-} mice when compared to WT that have a pool of nuclear cGAS (**Fig 1d, 7d**). To identify the
295 DNA associated with endogenous nuclear cGAS in DCs, we generated a GFP-cGAS knock-in
296 mouse (*Cgas*^{KI/KI}) and performed ChIP-seq on *Cgas*^{KI/KI} bone-marrow derived DCs (**Fig 7e, 7f**).
297 Strikingly, endogenous cGAS peaks computed on the mouse genome were mostly enriched on
298 satellite sequences, and to a lesser extent, on LINE elements (**Fig 7g**). Satellites was the most
299 enriched category of genomic features in both human DCs over-expressing GFP-NLS-cGAS and
300 mouse DCs expressing endogenous GFP-cGAS (**Fig 7h**). In contrast to human, mouse centromeres
301 are telocentric and poorly mapped in the reference genome. In particular, minor satellites, which

302 constitute mouse centromeres, are not annotated on mm10. We mapped the reads that failed to map
303 to the mouse genome to a database of repetitive DNA (**Fig 7i**). Endogenous GFP-cGAS reads were
304 again enriched on satellite DNA, and in particular mostly enriched on minor satellites (SATMIN)
305 that are found on centromeres, as compared to major satellites (GSAT_MM) that are found on
306 pericentromeres (Kipling et al., 1991) (**Fig 7i**). We conclude that endogenous cGAS in the nucleus
307 is preferentially associated with centromeric satellite DNA.

308

309 **Discussion**

310 We find that the nuclear pool of cGAS is preferentially associated with centromeric DNA.
311 We provided four distinct pieces of evidence that support this finding. First, ChIP-seq of NLS-GFP-
312 cGAS in human DCs demonstrated a specific enrichment on satellite DNA, using either input DNA
313 or NLS-GFP as controls. Second, immunofluorescence of NLS-GFP-cGAS showed a specific
314 overlap with CENP-B foci. Third, endogenous cGAS was directly observed on the centromeres of
315 metaphase chromosomes. Fourth, ChIP-seq of endogenous murine GFP-cGAS showed that the
316 association is conserved in mouse.

317 We also show that the N-terminal domain of cGAS, which is dispensable for catalytic
318 activity of the recombinant protein and the response to transfected DNA, demonstrates distinct
319 activities according to cGAS localization. When cGAS is cytosolic in interphase, N-terminal
320 domain 1-212 encodes a dominant cytosolic retention activity. When this domain is disrupted in the
321 isolated cGAS fragments 1-160 and 161-522, the presence of nuclear-localization signals is
322 revealed in both fragments. Of note, domain 1-212 does not contain the recently described phospho-
323 Y215 that was suggested to retain cGAS in the cytosol (Liu et al., 2018). When cGAS is nuclear,
324 domain 1-161 is required for association with centromeric DNA and for innate immune activation
325 by the nuclear-localized sensor. Interestingly, the N-terminal domain of cGAS also enhances the
326 enzymatic activity of the sensor in response to short DNA by promoting liquid phase separation (Du
327 and Chen, 2018). Whether this enhancement is functionally linked to subcellular localization
328 remains to be determined. The N-terminal domain of cGAS is also highly variable between species
329 (Wu et al., 2014). The lack of conservation of the N-terminal domain of cGAS could correspond to
330 a functional adaptation for centromeric DNA sequences that are rapidly evolving in eukaryotes
331 (Henikoff et al., 2001). Hence, cGAS could have been tuned by evolution to limit activation by self
332 DNA in the nucleus at steady-state, presumably to minimize the risk of auto-inflammation and auto-
333 immunity, while maintaining responsiveness to DNA in the cytosol or to specific nuclear DNA
334 features such as centromeres via its N-terminal. In accordance with this hypothesis, we find that

335 transfected 4-repeat satellite DNA fragments induce a stronger cellular innate immune activation
336 compared to shuffled sequence, and this difference is lost with increasing numbers of repeats. Since
337 purified cGAS is not active in response to short synthetic dsDNA fragments (Andreeva et al., 2017),
338 cellular factors to be defined may favor the response to short satellite DNA repeats. Additionally,
339 we detected dispersed cGAS ChIP-seq peaks and cGAS foci along chromosomes, an enrichment on
340 LINE elements, and a significant association of human cGAS with H3K9me3, a mark that is not
341 directly associated with CENP-A (Lacoste et al., 2014). We also observed association of
342 endogenous cGAS with telomeres of chromosomes from metaphase spread, which may be the result
343 of preferential association of cGAS with perinuclear chromatin in prometaphase (**Fig 1g**). We did
344 not detect enrichment of telomeric DNA repeats in cGAS peaks by ChIP-seq of non-cycling DCs –
345 but telomere sequences are missing in the reference genome. These findings require further study,
346 and we speculate that additional types of chromosomal DNA also contribute to regulation of nuclear
347 cGAS activity. We recently showed that DNA in the form of purified cellular nucleosomes is a poor
348 substrate for enzymatic activation of cGAS (Lahaye et al., 2018). It will be important to develop
349 assays to determine the contribution of DNA sequences and chromatin proteins in regulating cGAS
350 enzymatic activity for centromeric and non-centromeric DNA.

351 Recent studies have reported that in addition to its localization in the cytosol (Sun et al.,
352 2013), endogenous cGAS is present in the nucleus of primary cells, immortalized cell lines or
353 cancer cells (Dou et al., 2017; Lahaye et al., 2018; Mackenzie et al., 2017; Orzalli et al., 2015; Xia
354 et al., 2018). We find that cGAS accumulates in the cytoplasm during interphase and that its nuclear
355 localization can result from nuclear breakdown in mitosis or nuclear envelope rupture in interphase
356 (**Fig 7j**), in agreement with other studies (Denais et al., 2016; Dou et al., 2017; Harding et al., 2017;
357 Mackenzie et al., 2017; Raab et al., 2016; Yang et al., 2017). Cellular damage that produces
358 cytoplasmic DNA fragments or micronuclei result in cytoplasmic foci with dense accumulation of
359 cGAS, which may overshadow the localization of the remaining cGAS pool in control cells (Dou et
360 al., 2017; Gluck et al., 2017; Harding et al., 2017; Mackenzie et al., 2017). Since monocyte-derived

361 DCs do not divide, our results indicate that endogenous cGAS is maintained in the nucleus for
362 several days. This could result from a sustained retention of endogenous cGAS in the nucleus, or
363 alternatively from replenishment of nuclear cGAS during interphase. The half-life of nuclear cGAS
364 may also vary as a function of cell culture conditions (Yang et al., 2017).

365 We find that nuclear-localized cGAS functionally upregulates cellular innate immune
366 responses. Given that GFP-NLS-cGAS protein and the majority of cellular DNA are present in the
367 nucleus over the cytosol, our data support the notion that nuclear cGAS produces cGAMP in the
368 nucleus, although this conclusion remains limited by the use of an endpoint assay. Diffusion or
369 transport of nuclear cGAMP through nuclear pores would result in activation of STING, which is
370 exclusively cytoplasmic in DCs and macrophages (Lahaye et al., 2018). In the case of GFP-cGAS
371 in DCs, cGAMP was more abundant in the cytosolic fraction, raising the interesting possibility that
372 cGAMP does not freely move across the nuclear pores. Alternatively, we do not exclude that a
373 fraction of cytosolic cGAMP detected in the experiment originated from cGAMP contained in the
374 lentiviral particles used for DC transduction.

375 Our data show that nuclear cGAS activity is restrained by at least four mechanisms. First,
376 over-expressing nuclear-localized cGAS activates innate immunity in DCs, suggesting that the
377 endogenous level of expression of the sensor in DCs is tuned to avoid spontaneous activation.
378 Second, we estimated that nuclear cGAS is at least 200-fold less active towards endogenous nuclear
379 DNA as compared to exogenous DNA transfection. This suggests that enzymatic activation in the
380 nucleus is limited by a yet to be elucidated mechanism. Interestingly, a recent report showed that
381 Zn^{2+} concentration regulates cGAS activity (Du and Chen, 2018). Free Zn^{2+} is not available in the
382 nucleus because it is bound to proteins (Lu et al., 2016) possibly limiting cGAS activity in the
383 nucleus. The circular RNA cia-cGAS was also recently reported to inhibit nuclear cGAS activity in
384 long-term hematopoietic stem cells (Xia et al., 2018). Although cia-cGAS is not expressed in other
385 immune cells including DCs, it remains possible that another circular RNA inhibits nuclear cGAS
386 in DCs. Third, the N-terminal domain of cGAS is crucial to retain the sensor in the cytosol until a

387 NE rupture or NE breakdown occurs. Fourth, where cGAS interacts with nuclear DNA appears to
388 determine cGAS activation: while cGAS is activated after nuclear entry resulting from mitosis or
389 association with a nuclear-localization signal when the sensor is overexpressed, we could not detect
390 endogenous cGAS activation after entry through interphasic NE rupture events. In bone marrow-
391 derived DCs, our results do not allow us to determine if it is the nuclear pool of endogenous cGAS,
392 which we show is associated with self DNA, or the cytosolic pool of endogenous cGAS, whose
393 association with DNA is unknown, that is responsible for tonic ISG expression, but the vast excess
394 of nuclear DNA over cytosolic DNA at steady-state favors the former hypothesis.

395 Our work provides a basis to determine to what extent the roles of cGAS in anti-microbial
396 defense, anti-tumoral immunity, auto-immunity, senescence and DNA damage response, currently
397 attributed to activation by cytosolic DNA (Chen et al., 2016; Gluck et al., 2017; Harding et al.,
398 2017; Yang et al., 2017), implicate the nuclear pool of cGAS. In addition, nuclear cGAS may be
399 endowed with nuclear-specific functions that future work may unveil.

400

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Author contributions

NM and MG designed the study, analyzed data and wrote the manuscript. MG performed most experiments. XL performed experiments with mouse cells, cGAMP ELISA and contributed to experiments with human cells. GN performed parts of experiments with cell confinement and their analysis. EPL performed initial analysis of NGS data on hg19. FN analyzed the NGS data on hg38 and mm10. SH analyzed metaphase chromosomes. EZ performed the chromatin immunoprecipitation experiment. NdS, DR and AB contributed to some experiments, CG and MM to some analyses. AL supervised EPL. MP designed part of the study and supervised GN. SA supervised DR and EZ. DF supervised SH. All authors discussed the results.

425 **Conflict of interest**
426 The authors declare no conflict of interest.
427

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- 568

569 **Figure Legends**

570

571 **Figure 1. cGAS is present in the nucleus as a result of nuclear envelope opening.**

572 (a) Quantification of mean endogenous cGAS intensity in the nucleus (N) or in the cytoplasm (C) of
573 post-mitotic human monocyte-derived dendritic cells (DCs) (n>60 cells for each donor, 3
574 independent donors combined from 2 independent experiments, red lines represent average and
575 black lines represent SD, one-way ANOVA with post-hoc Tukey test, ****P<0.0001).

576 (b) (top) Immunofluorescence staining of endogenous cGAS (red) and DAPI (blue), cGAS staining
577 and (bottom) overlay plots of pixel intensity measured along the yellow line of cGAS (red) and
578 DAPI (blue). For DAPI, refer to Fig S1b. Scale bars are 10µm.

579 (c) Nuclear/Cytoplasmic fractionation of post-mitotic human DCs and immunoblot for endogenous
580 cGAS (top), Tubulin (middle) and Lamin B1 (bottom). C: cytosolic fraction; N: nuclear fraction.
581 One donor representative of n=4 donors. See Fig S1c for the other donors.

582 (d) Nuclear/Cytoplasmic fractionation of mouse bone-marrow derived DCs from two wild-type
583 (WT) or two cGAS knock-out (*Cgas*^{-/-}) mice and immunoblot for endogenous cGAS (top), Tubulin
584 (middle) and Lamin B1 (bottom). C: cytosolic fraction; N: nuclear fraction (representative of n=3
585 independent mice).

586 (e) Sequential images of cycling HeLa cell stably expressing Histone 2B (H2B)-mCherry (red) and
587 GFP-cGAS (green) before (0 min), at (56-57 min) and after (80 min) nuclear envelope breakdown.
588 Scale bars are 10µm.

589 (f) Nuclear/Cytoplasmic ratio of mean GFP-cGAS intensities in cells as in (e) (n=59 cells combined
590 from 2 independent experiments, red line represent mean, error bars represent SD).

591 (g) Sequential images of one representative HeLa cell as in (e) with GFP-cGAS in the cytosol prior
592 to mitosis. Scale bars are 10µm.

593 See also Figure S1.

594

595 **Figure 2. Nuclear-localized cGAS activates an innate immune response in dendritic cells.**
596 (a) Schematic representation of the lentivector insert with (GFP) under the control of an inverted
597 HLA-DR α promoter. Arrows represent transcription direction from the LTRs in transfected 293FT
598 cells, and from the inverted HLA-DR α promoter in transduced DCs.
599 (b) Confocal microscopy of DCs transduced with GFP (top), GFP-cGAS (middle) or GFP-NLS-
600 cGAS (bottom) lentivectors under the control of the inverted HLA-DR α promoter. GFP is green and
601 DAPI in blue. One representative field from one donor of n=2 donors. Scale bars are 10 μ m.
602 (c) GFP and CD86 expression in DCs after transduction with lentivectors encoding for GFP, GFP-
603 cGAS or GFP-NLS-cGAS, in presence or in absence of Vpx. Representative of n=9 donors in four
604 independent experiments.
605 (d) CD86 expression in DCs transduced as in (c). n=9 donors of four independent experiments.
606 One-way ANOVA with post-hoc Tukey test.
607 (e) Schematic representation as in (a) of the lentivector insert with (GFP) under the control of the
608 SFFV promoter.
609 (f) GFP and CD86 expression in DCs after transduction with GFP-NLS (CTR) or GFP-NLS-cGAS
610 lentivectors in pTRIP-SFFV, in presence or in absence of Vpx. Representative of n=4 donors in two
611 independent experiments.
612 (g) GFP and SIGLEC1 expression in DCs stably transduced as in (f). Representative of n=4 donors
613 in two independent experiments.
614 (h) CD86 expression in DCs transduced as in (f). n=4 donors in two independent experiments. One-
615 way ANOVA with post-hoc Tukey test.
616 (i) SIGLEC1 expression in DCs transduced as in (f) in presence or absence of Vpx. n=4 donors of
617 two independent experiments. One-way ANOVA with post-hoc Tukey test.
618 (j) CD86 expression in dose titration of GFP-NLS or GFP-NLS-cGAS lentivectors in pTRIP-SFFV,
619 within GFP⁺ (green) and GFP⁻ (black) DC populations. Solid lines represent mean, light-colored
620 limits represent SEM. n=4 donors in two independent experiments. One-way ANOVA with post-
621 hoc Tukey test.
622 (k) SIGLEC1 expression of cells transduced as in (j).
623 (l) Expression of *MX1*, *CXCL10*, *IFIT1* and *OAS1* relative to *ACTB*, in DCs transduced with GFP-
624 NLS or GFP-NLS-cGAS lentivectors. n=6 donors combined from two independent experiments.
625 One-way ANOVA with post-hoc Sidak test, on log-transformed data.
626 ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, ns=non-significant.
627 See also Figure S2.
628

629 **Figure 3. Nuclear localization of cGAS results in limited cGAMP production.**
630 (a) Immunofluorescence staining of DAPI (blue) and cGAS (red) in cycling 293FT cells stably
631 transduced with a control (EV) (top), cGAS (middle) or NLS-cGAS (bottom) lentivectors in pTRIP-
632 CMV. Scale bars are 10 μ m.
633 (b) cGAMP quantification by cGAMP bioassay in extracts of cells described in (a). Mean and SEM
634 of n=3 independent experiments. Dilutions are 3-fold.
635 (c) cGAMP quantification in extracts of described in (a) that were stimulated overnight with 1 μ g/ml
636 of HT-DNA. cGAMP was quantified as in (b). Mean and SEM of n=3 independent experiments.
637 Dilutions are 3-fold.
638 (d) cGAMP quantification relative to a cGAMP synthetic standard based on Effective
639 Concentration 50 (EC50) of the cGAMP bioassay curves. Mean and SEM of n=3 independent
640 experiments. One-sample t-test.
641 (e) cGAMP concentrations in cells as in (a) measured by cGAMP ELISA. Mean and SEM of n=3
642 independent experiments. Gray dashed line indicates lower limit of detection, bar shows geometric
643 mean. One-way ANOVA with post-hoc Tukey test, on log-transformed data.
644 (f) Expression of GFP-FLAG-cGAS, GFP-NLS-FLAG-cGAS, Tubulin, Calnexin and Lamin A/C in
645 nuclear (N) and cytoplasmic (C) fraction of DCs transduced with the GFP-NLS or corresponding
646 cGAS lentivectors with SFFV promoter (representative of n=5 independent donors). Reduced
647 material for GFP-FLAG-cGAS samples was associated with activation-induced cell death.
648 (g) cGAMP concentrations in cytoplasmic and nuclear fractions of DCs as in (f), measured by
649 cGAMP ELISA. Gray dashed line indicates lower limit of detection. Mean and SEM of n=5
650 independent donors. Bar shows geometric mean. One-way ANOVA with post-hoc Tukey test, on
651 log-transformed data.
652 *P<0.05, **P<0.001, ns=not significant.
653 See also Figure S3.
654

655 **Figure 4. Confinement-induced nuclear envelope rupture does not activate the cGAS-STING-**
656 **IRF3 axis.**
657 (a) Scheme of the cell confiner.
658 (b) Immunoblot of cGAS, STING and Vinculin in HeLa cells and HeLa cells transduced with
659 mCherry-cGAS E225A/D227A, BFP-2A-STING and GFP-IRF3 (HeLa STING).
660 (c) Sequential images of HeLa STING transfected with 4 μ g/ml of HT-DNA. Transfection was
661 performed at time=0 min. Binding of mCherry-cGAS E225A/D227A to the transfected DNA is
662 shown at time=100 min and accumulates during time. Formation of GFP-IRF3 foci and vesicles in
663 the cytoplasm are shown at time=150 min. GFP-IRF3 translocation peaked at time=165 min. One
664 representative cell for n=2 independent experiments. Scale bars are 10 μ m.
665 (d) Quantification of cells showing GFP-IRF3 nuclear translocation after confinement, confinement
666 and transfection with HT-DNA, or only transfection with HT-DNA. Cycling HeLa cells, that
667 express endogenous cGAS, were stably transduced as in (b) (One-way ANOVA with post-hoc Tukey
668 test. ns=not significant; ****p<0.0001; data pooled from 3 independent experiments).
669 (e) Sequential images of HeLa cells stably transduced as in (b) immediately after confinement at
670 3 μ m (time=0 min; left) and after 6 hours and 45 minutes from confinement (time=345 min; right).
671 Arrows indicate cells with NE ruptures as shown by bright mCherry-cGAS E225A/D227A foci in
672 the nucleus. One representative field. Scale bars are 10 μ m.
673 (f) Sequential images of HeLa cells stably transduced as in (b) subjected to 3 μ m confinement and
674 transfected with 4 μ g/ml of HT-DNA immediately after confinement at 3 μ m (time=0 min; left) and
675 after 6 hours and 45 minutes from confinement (time=345 min; right). Arrows indicate cells with
676 mCherry-cGAS spots in the cytoplasm and with consequent translocation of GFP-IRF3 in the
677 nucleus. One representative field. Scale bars are 10 μ m.
678 (g) Sequential images of HeLa STING cells transfected with 4 μ g/ml of HT-DNA, after transfection
679 (time=0 min) and 345 minutes later. For quantification in (d), only cells with bright GFP-IRF3 foci
680 in the cytoplasm were quantified, to exclude cells in which GFP-IRF3 translocate due to cGAMP
681 transfer via gap junctions. Scale bars are 10 μ m.
682 (h) Expression of GFP-NLS, GFP-FLAG-cGAS E225A/D227A (*), GFP-NLS-FLAG-cGAS
683 E225A/D227A (**), endogenous cGAS (^) and Actin in DCs transduced with the corresponding
684 lentivectors (representative of 2 independent donors).
685 (i) Expression of BFP, CD86 and SIGLEC1 in DCs as in (h) 48 hours after infection with BFP-
686 reporter HIV-1 and HIV-2 viruses and 24 hours after transfection with HT-DNA or cGAMP (n=2
687 independent donors).
688

689 **Figure 5. Nuclear cGAS associated with centromeric satellite DNA.**
690 (a) 3D projection of the nucleus of a DC expressing GFP-NLS-cGAS (green).
691 (b) Experimental scheme for ChIP-seq of GFP-NLS-cGAS stably transduced in DCs.
692 (c) Annotation of the filtered peaks of the ChIP-seq on GFP-NLS-cGAS. Elements with less than
693 10 peaks are grayed out (1 donor representative of 3 independent donors).
694 (d) Circular plot showing the distribution of GFP-NLS-cGAS and of CENP-A peaks and
695 localization of CENP-B box (consensus sequence) on the hg38 genome. The cGAS track represents
696 the fold change (chip over input) of selected filtered peaks (162 regions) from the 3 donors. The
697 CENP-A track represents the density of CENP-A intersection peaks (5977 regions) computed on
698 windows of size 10^7 across the genome. The CENP-B box track reports on the x axis the genomic
699 position of the region (occurrence of CENP-B box consensus sequence) and on the y axis the
700 minimal distance (log10 transformed) of the region to its two neighbouring regions.
701 (e) Association of GFP-NLS-cGAS peaks with public H3K27Ac peaks from GM12878 cells,
702 H3K9me3 peaks from PBMCs, and endogenous CENP-A peaks from HeLa S3 cells. Filtered GFP-
703 NLS-cGAS peaks for donor #1 are used (404 peaks, 1545600 bp) (representative of 3 independent
704 donors).
705 (f) Sequence enrichment in cGAS-specific peaks from GFP-NLS-cGAS ChIP-seq over GFP-NLS
706 ChIP-seq filtered peaks (intersection of peaks from 2 independent donors). Three motifs were
707 assessed: Satellite III DNA motif repeats, $[GGAAT]_{n>3}$; CENP-B box consensus sequence,
708 NTTTCGNNNNANNCGGGN ; and telomeric repeats, $[TTAGGG]_{n>1}$.
709 (g) cGAS-specific read enrichment on repeats. A repeat occurrence is considered if the read count
710 per million (cpm) is ≥ 2 in any sample. Repeats are grouped into $n = 10$ bins according to the ChIP
711 read enrichment over GFP-NLS. For each repeat class R , the fraction of occurrences within the first
712 i bins (corresponding to the top $(100/i)\%$ ranks) is shown as a gradient from white to black. Only
713 repeat classes with at least 10 read occurrences in the genome and that pass the cpm cut-off are
714 considered, and sorted from left to right by decreasing number of occurrences in the top 50% (1
715 donor representative of $n=2$ independent donors).
716 (h) DC stably transduced with GFP-NLS lentivector in pTRIP-SFFV and stained for CENP-B. (left)
717 Z-projection of CENP-B (white) with nuclear mask (yellow) and (right) orthogonal projections
718 (single confocal plane) of CENP-B (red) and GFP-NLS (green). Scale bar is $2\mu\text{m}$.
719 (i) Quantification of GFP intensity in CENP-B foci or random regions in the nucleus, normalized
720 over mean nuclear GFP intensity, in cells transduced as in (h). $n \geq 140$ foci or random regions in 7
721 independent cells. Each dot represents a single CENP-B focus. Mean and standard deviation are
722 represented. One donor representative of $n=4$ donors in 2 independent experiments. Student t-test.
723 (j) DC stably transduced with GFP-NLS-cGAS lentivector in pTRIP-SFFV and stained for CENP-
724 B, shown as in (h). Scale bar is $2\mu\text{m}$.
725 (k) Quantification of GFP-NLS-cGAS intensity in CENP-B foci or random regions in the nucleus
726 as in (i).
727 (l) CD86 and IFN- $\lambda 1$ expression by DCs transfected with synthetic DNA repeats coding for the
728 AATGG satellite motif, the corresponding shuffled sequence, or HT-DNA, at the indicated DNA
729 concentrations (solid line=mean, dotted lines=SEM, independent donors: $n=9$ for CD86, $n=7$ for
730 IFN- $\lambda 1$; two-way ANOVA with Tukey test, on log-transformed data for IFN- $\lambda 1$).
731 **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$.
732 See also Figure S4.
733

734 **Figure 6. cGAS N-terminal domain determines alpha satellites association, cytosolic retention**
735 **and activation in the nucleus**

736 (a) (left) Schematics of cGAS deletions and (right) confocal microscopy of DCs transduced with
737 human full length catalytically inactive cGAS, the N-terminal part of cGAS (cGAS 1-212) or the C-
738 terminal part (cGAS 161-522) fused to GFP in pTRIP-SFFV. GFP channel is shown in black on
739 white. One representative donor of n=4 donors in two independent experiments. Scale bars are
740 10 μ m.

741 (b) (left) Schematics of cGAS deletions and (right) confocal microscopy of DCs transduced with
742 human full length catalytically inactive cGAS or cGAS 1-160 in pTRIP-SFFV. GFP channel is
743 shown in black on white. One representative donor of n=4 donors in two independent experiments.
744 Scale bars are 10 μ m.

745 (c) GFP, CD86 and SIGLEC1 expression in DCs after transduction with a GFP-NLS, GFP-NLS-
746 cGAS, GFP-cGAS 161-522 or GFP-NLS-cGAS 161-522 lentivector in pTRIP-SFFV in presence or
747 absence of Vpx. One representative donor of n=6 donors in three independent experiments.

748 (d) CD86 and SIGLEC1 expression in DCs transduced as in (c). n=6 donors in three independent
749 experiments. One-way ANOVA with post-hoc Tukey test.

750 (e) Confocal microscopy of *Cgas*^{-/-} mouse bone-marrow derived DCs transduced with GFP-NLS,
751 GFP-cGAS, GFP-NLS-cGAS, GFP-cGAS 1-160 or GFP-cGAS 161-522 in pTRIP-SFFV
752 lentivectors. GFP channel is shown in black on white. One representative mouse of n=2. Scale bars
753 are 10 μ m.

754 (f) Expression of *Ifit1* in *Cgas*^{-/-} mouse bone-marrow derived DCs transduced with GFP, GFP-NLS,
755 GFP-cGAS, GFP-NLS-cGAS, GFP-cGAS 1-160 or GFP-cGAS 161-522 in pTRIP-SFFV
756 lentivectors, untreated or treated with reverse transcriptase inhibitors (AZT + NVP), or transfected
757 with cGAMP. n=4 mice combined from 2 independent experiments. Bars represent geometric
758 mean. One-way ANOVA with Sidak test, on log-transformed data.

759 (g) Quantification of GFP intensity in CENP-B foci in the nucleus, normalized over mean nuclear
760 GFP intensity, in DCs transduced with GFP-NLS or the indicated GFP-cGAS lentivectors, in
761 pTRIP-SFFV. n \geq 140 foci per construct were quantified, in 7 or 8 independent cells per construct.
762 Each dot represents a single CENP-B focus. Mean and standard deviation are represented. One
763 representative donor of n=4 donors in two independent experiments. One-way ANOVA with post-
764 hoc Tukey test.

765 ****P<0.0001, ***P<0.001, ns=non-significant.

766 See also Figure S5.

767

768 **Figure 7. Endogenous cGAS associates with centromeres**
769 (a) Representative immunofluorescence images of a metaphase spread of cycling U2OS cells
770 showing endogenous cGAS enrichment at the inner centromere. CENP-A marks centromere
771 position. Yellow arrows point to cGAS localization at an inner centromere. Scale bar is 5µm.
772 (b) (top left) Magnification of a centromere as in (a) showing cGAS enrichment between two
773 CENP-A foci. (top right) Schematic of the expected CENP-A and ACA localization at the inner
774 kinetochore and at the inner kinetochore/centromere, respectively. (bottom) Normalized mean of
775 the fluorescence intensity scan lines of ACA, CENP-A and cGAS along the centromeres. Error bars
776 represent the SEM (standard error of the mean) of 36 centromeres in one cell (representative of 2
777 independent experiments).
778 (c) Heatmaps of ACA, CENP-A and cGAS intensities for individual chromosomes as in (b) after
779 distance normalization (n=22 chromosomes, representative of 2 independent experiments).
780 (d) Baseline expression of the indicated ISGs (*Ifit1*, *Ifit2*, *Oas1a*) in bone marrow-derived DCs from
781 *Cgas*^{-/-} mice or WT littermates. Bars represent mean and error bars are SEM. Each dot represents an
782 individual mouse. n=6 mice per genotype combined from 3 experiments; One-way ANOVA with
783 post-hoc Tukey test; ****P<0.0001.
784 (e) Overview of the GFP-cGAS knock-in locus.
785 (f) Experimental scheme for ChIP-seq of GFP-cGAS mouse DCs from *GFP-cGAS*^{KI/KI} mice.
786 (g) Annotation of the significant peaks of the ChIP-seq on *Cgas*^{KI/KI} mouse DCs over input.
787 Elements with less than 10 peaks are grayed out.
788 (h) Annotation of the significant peaks of endogenous GFP-cGAS over input in mouse DCs (peak
789 intersection of replicates 1 and 2) compared to significant peaks of over-expressed GFP-NLS-cGAS
790 over input in human DCs (donor #1). Elements with less than 10 peaks are not included.
791 (i) ChIP-seq read enrichment on repeats, *Cgas*^{KI/KI} over input in mouse DCs. SATMIN: Mouse
792 minor satellite DNA. GSAT_MM: Mouse gamma-satellite repetitive sequence. IMPB_01:
793 Consensus of repeated region of mouse chromosome 6. SQR1_MM, SQR2_MM, SQR4_MM:
794 Mouse simple repetitive DNA (sqr family). ZP3AR: Satellite from Muridae.
795 (j) Working model of cGAS expression in the cytoplasm in interphase, followed by localization to
796 the nucleus as a result of mitosis.
797

798 **STAR Methods**

799 **CONTACT FOR REAGENT AND RESOURCE SHARING**

800 Further information and requests for reagents may be directed to and will be fulfilled by the Lead
801 Contact, Nicolas Manel (nicolas.manel@curie.fr).

802

803 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

804 **Human subjects**

805 Healthy individuals from Paris area donate venous blood to be used for research. Gender identity
806 and age from anonymous healthy donors was not available. According to the 2016 activity report of
807 EFS (French Blood Establishment), half of donors are under 40 years old, and consist of 52%
808 females and 48% males. The use of EFS blood samples from anonymous donor was approved by
809 the Institut National de la Santé et de la Recherche Médicale committee. EFS provides informed
810 consent to blood donors.

811

812 **Human Cell Lines**

813 Cell lines are described in the Key Resource Table. Female cell lines included 293FT, HeLa and
814 U2OS cells. Male cell lines included HL116, THP-1. Cell lines validation were performed by STR
815 and POWERPLEX 16HS analysis for 293FT and HeLa cell lines. 293FT and HeLa cells were
816 cultured in DMEM with Glutamax supplemented with 10% FBS (GIBCO) and Penicillin-
817 Streptomycin (PenStrep; GIBCO). HeLa cells expressing H2B-mCherry were a kind gift of
818 Matthieu Piel's lab and were previously described (Raab et al., 2016). THP-1 cells were cultured in
819 RPMI medium with Glutamax (GIBCO), 10% FBS (GIBCO) and PenStrep (GIBCO). HL-116 cells
820 were cultured in DMEM medium with Glutamax (GIBCO), 10% FBS (GIBCO), PenStrep
821 (GIBCO) supplemented with 1% HAT (GIBCO). U2OS cells were cultured in DMEM containing
822 10% tetracycline-free fetal bovine serum (Pan Biotech), 100 U/ml penicillin, 100 U/ml
823 streptomycin, and 2 mM L-glutamine. Number of experimental replicates are indicated in the
824 respective figure legends.

825

826 **Primary Human Cells**

827 CD14⁺ monocytes were isolated from peripheral adult human blood as previously described
828 (Lahaye et al., 2013). Monocytes were cultured and differentiated in dendritic cells (MDDCs) in
829 RPMI medium with Glutamax, 10% FBS (GIBCO), PenStrep (GIBCO), 50µg/ml Gentamicin
830 (GIBCO) and 0.01M HEPES (GIBCO) in presence of recombinant human 10ng/ml GM-CSF
831 (Miltenyi) and 50ng/ml IL-4 (Miltenyi). Number of donors and experimental replicates are
832 indicated in the respective figure legends.

833

834 **Mice**

835 All animal procedures were in accordance with the guidelines and regulations of the French
836 Veterinary Department in an accredited animal facility. The animal protocol was approved by the
837 Animal Ethical Committee of Paris Centre (C2EA-59). C57BL/6J-Mb21d1^{tm1d(EUCOMM)Hmgu} (*Cgas*^{-/-}
838) and C57BL/6J and C57BL/6N strains were obtained from The Jackson Laboratory. The GFP-
839 cGAS knock-in is C57BL/6N-Mb21d1^{tm1Ciphe} (GFP-cGAS^{KI/KI}) generated at the Centre
840 d'Immunophénomique, Marseille, France. Age of mice used in experiments was 6-8 weeks (*Cgas*^{-/-})
841 and 8-9 months (*Cgas*^{KI/KI}). Mice used in experiments were females. All mice in each experiment
842 were littermates.

843

844 **Mouse Bone Marrow Isolation and Dendritic Cells Differentiation**

845 Mouse bone marrow derived dendritic cells were differentiated from bone marrow isolated from
846 mouse tibiae. 20 million cells were seeded in gamma-irradiated heavy 14 cm dishes (Greiner Bio-
847 One) in 20ml of BMDCs (bone marrow-derived DCs) medium composed by IMDM, 10% FBS,
848 PenStrep (GIBCO), 50µM β-mercaptonethanol (GIBCO), and granulocyte-macrophage colony

849 stimulating factor (50 ng/mL)-containing supernatant obtained from transfected J558 cells. Cells
850 were split at day 4 and day 7 and harvested at day 10. At day 4 the supernatant was recovered, and
851 the adherent cell were recovered by incubating the dishes in 6ml of PBS (GIBCO) containing 5mM
852 EDTA (GIBCO). Cells were counted and reseeded in BMDCs medium at a concentration of 0.5
853 million cells per ml, 20ml per 14cm dish. At day 7 the culture supernatant was gently discarded and
854 the cells were recovered by incubating the dishes in 6ml of PBS containing 5mM EDTA (GIBCO).
855 Cells were counted and reseeded in BMDCs medium at a concentration of 0.5 million cells per ml,
856 20ml per 14cm dish. At day 10, the culture supernatant was gently discarded and semi-adherent
857 cells were recovered by extensive flushing of the dishes with 10ml of pre-warmed BMDCs
858 medium. The cells were counted and used for further applications. Number of mice and
859 experimental replicates are indicated in the respective figure legend.

860

861 **METHOD DETAILS**

862 **Constructs**

863 The plasmids pSIV3+, psPAX2, pCMV-VSV-G, pTRIP-CMV, pTRIP-SFFV were previously
864 described (Gentili et al., 2015; Lahaye et al., 2013; Raab et al., 2016). pFlap-DeltaU3-HLA-DR α -
865 GFP was obtained from Theravectys. The promoter HLA-DR α and the EGFP sequence were cloned
866 in reverse orientation by PCR and digestion to obtain the backbone pFLAP-DeltaU3-HLA-DR α -
867 inverted GFP. GFP-NLS was previously described (Raab et al., 2016). mTagBFP2 sequence was
868 generated synthetically and was previously described (Gentili et al., 2015). mCherry was cloned by
869 PCR from mCherry-BP1-2 pLPC-Puro, kind gift of Matthieu Piel's lab. Human cGAS WT open
870 reading frame was amplified by PCR from cDNA prepared from MDDCs. Human cGAS
871 E225A/D227A was obtained by overlapping PCR mutagenesis. Human NLS-cGAS or NLS-cGAS
872 E225A/D227A was obtained by addition of the SV40 NLS sequence (PKKKRKVEDP) at the N-
873 terminal of cGAS by overlapping PCR. cGAS 1-212, 1-160, 161-522, 161-522 E225A/D227A,
874 NLS-161-522, 22-522 E225A/D227A, 62-522 E225A/D227A, 94-522 E225A/D227A, 122-522
875 E225A/D227A were obtained by overlapping PCR. FLAG sequence (MDYKDDDDK) was added
876 by overlapping PCR. cGAS $\Delta_{K173-1220}\Delta_{H390-405}$ was generated by deleting amino-acid regions K173-
877 I220 and H390-C405 by overlapping PCR and was previously described (Gentili et al., 2015).
878 Human cGAS WT or $\Delta_{K173-1220}\Delta_{H390-405}$ was cloned in pTRIP-CMV-Puro-2A or pTRIP-CMV or
879 pTRIP-SFFV or pFLAP-DeltaU3-HLA-DR α -GFP inverted and in frame with EGFP to obtain
880 pTRIP-CMV-Puro-2A-cGAS or pTRIP-CMV-EGFP-FLAG-cGAS or pTRIP-CMV-EGFP-FLAG-
881 cGAS $\Delta_{K173-1220}\Delta_{H390-405}$ or pTRIP-SFFV-EGFP-FLAG-cGAS or pFLAP-DeltaU3-HLA-DR α -
882 inverted GFP-FLAG-cGAS. Human cGAS E225A/D227A was cloned in pTRIP-CMV or pTRIP-
883 SFFV or pFLAP-DeltaU3-HLA-DR α -GFP inverted in frame with EGFP or mCherry to obtain
884 pTRIP-CMV-EGFP-FLAG-cGAS E225A/D227A or pTRIP-CMV-mCherry-FLAG-cGAS
885 E225A/D227A or pTRIP-SFFV-EGFP-FLAG-cGAS E225A/D227A or pFLAP-DeltaU3-HLA-
886 DR α -inverted GFP-FLAG-cGAS. Human NLS-cGAS or NLS-cGAS E225A/D227A were cloned in
887 pTRIP-CMV-Puro-2A or pTRIP-SFFV or pFLAP-DeltaU3-HLA-DR α -inverted GFP in frame with
888 EGFP to obtain pTRIP-CMV-Puro-2A-NLS-cGAS or pTRIP-SFFV-EGFP-NLS-FLAG-cGAS or
889 pTRIP-SFFV-EGFP-NLS-FLAG-cGAS E225A/D227A or pFLAP-DeltaU3-HLA-DR α -inverted-
890 GFP-NLS-FLAG-cGAS E225A/D227A. cGAS 1-160, 1-212, 161-522, 161-522 E225A/D227A,
891 NLS-161-522, were cloned in pTRIP-SFFV in frame with EGFP. Human STING WT open reading
892 frame was amplified by PCR from IMAGE clone 5762441 and the H232 residue was mutated to
893 R232 by overlapping PCR mutagenesis and was previously described (Jeremiah et al., 2014).
894 Human STING WT was cloned in pTRIP-SFFV-mTagBFP2-2A and was previously described
895 (Cerboni et al., 2017). Human IRF3 WT open reading frame was amplified by PCR from plasmid
896 obtained from David Levy and cloned in pTRIP-CMV in frame with EGFP. Human STING WT
897 was cloned in pMSCV-Hygro (Clontech) to obtain pMSCV-Hygro-STING and was previously
898 described (Gentili et al., 2015). pTRIP-CMV-mCherry-53BP1 (amino acids 1224-1716 for isoform
899 1) was cloned from mCherry-BP1-2 pLPC-Puro (AddGene #19835). The HIV-1 GFP-reporter virus

900 was NL4-3 Δ vif Δ vpr Δ vpu Δ env Δ nef encoding GFP in nef and the HIV-2 GFP-reporter virus was
901 ROD9 Δ env Δ nef encoding GFP in nef (Manel et al., 2010).

902

903 **Lentiviral particles production in 293FT cells, transductions and infections**

904 Lentiviral particles were produced as previously described from 293FT cells (Gentili et al., 2015).
905 Briefly, lentiviral particles were produced by transfecting 1 μ g of psPAX2 and 0.4 μ g of pCMV-
906 VSV-G together with 1.6 μ g of a lentiviral vector plasmid per well of a 6-well plate. SIV-VLPs were
907 produced by transfecting 2.6 μ g of pSIV3+ and 0.4 μ g of pCMV-VSV-G. HIV-1 and HIV-2 GFP-
908 reporter viruses were produced by transfecting 2.6 μ g of HIV DNA and 0.4 μ g of CMV-VSVG.
909 Medium was changed after 12-14h to 3ml per well of RPMI medium with Glutamax, 10% FBS
910 (GIBCO), PenStrep (GIBCO), 50 μ g/ml Gentamicin (GIBCO) and 0.01M HEPES (GIBCO). The
911 supernatant was harvested 30-32h after medium changed and filtered over 0.45 μ m filters. Lentiviral
912 particles were used fresh for transduction. HIV reporter viral supernatants were stored at -80°C.

913 For 293FT cells transduced with pTRIP-CMV-Puro-2A, pTRIP-CMV-Puro-2A-cGAS, pTRIP-
914 CMV-NLS-FLAG-cGAS, 0.5 million cells were plated in a well of a 6w plate and transduced with
915 2ml of freshly produced lentivirus in presence of 8 μ g/ml of protamine (SIGMA). Cells were
916 selected for one week with 2 μ g/ml of Puromycin (Invivogen). For HeLa cells transduced with
917 pTRIP-SFFV-mTagBFP2-2A-STING WT, pTRIP-CMV-GFP-IRF3 and pTRIP-CMV-mCherry-
918 FLAG-cGAS E225A/D227A, 0.5 million cells were plated in a well of a 6w plate and transduced
919 with 1ml of each freshly produced lentivirus in presence of 8 μ g/ml of protamine. For HeLa cells
920 expressing GFP-FLAG cGAS and GFP-FLAG-cGAS Δ <sub>K173-1220 Δ _{H390-405}, 0.5 million cells were
921 plated in a well of a 6w plate and transduced with 2ml of either pTRIP-CMV-EGFP-FLAG-cGAS
922 or pTRIP-CMV-EGFP-FLAG-cGAS Δ <sub>K173-1220 Δ _{H390-405} freshly produced lentivirus in presence of
923 8 μ g/ml of protamine.</sub></sub>

924 For human monocytes transduction 50,000 monocytes per well were seeded in each well of a 96
925 well plate in 100 μ l of medium and transduced with 100 μ l of freshly produced virus in presence or
926 absence of 50 μ l of SIV-VLPs with protamine at 8 μ g/ml. For experiments with pFLAP-DeltaU3-
927 HLA-DR α -inverted GFP vectors, plates were spinoculated at 1,200 g for 2 hours at 25°C. Cells
928 were analyzed on a FACSVerser cytometer 4 days after transduction. For ChIP-seq experiments and
929 microscopy experiments, 2 million monocytes per well were seeded in a 6 well plate and transduced
930 with 2ml of freshly produced lentiviral particles and 2ml of SIV-VLPs in presence of 8 μ g/ml of
931 protamine.

932 For MDDCs infected by HIV reporter viruses, 3 million monocytes per well were seeded in a 6 well
933 plate and transduced with 3ml of freshly produced lentiviral particles and 3ml of SIV-VLPs in
934 presence of 8 μ g/ml of protamine. Four days after transduction and MDDCs differentiation, cells
935 were harvested, counted and resuspended in fresh media at a concentration of 1 to 0.5 million per
936 ml with 8 μ g/ml protamine, GM-CSF and IL-4, and 100 μ l was aliquoted in round-bottomed 96-well
937 plates. For infection, 100 μ l of media or dilutions of viral supernatants were added.

938 For BMDCs transduction, at day 4 of BMDCs differentiation, the supernatant and adherent cells
939 were recovered, 50,000 cells per well were seeded in each well of a 96 U-bottom well plate in
940 100 μ l of medium and transduced with 100 μ l of freshly produced virus with protamine at 8 μ g/ml, in
941 presence or absence of 25 μ M Azidothymidine (AZT) with 10 μ M Nevirapine (NVP). Plates were
942 spinoculated at 1,200g for 2 hours at 25°C. Cells were analyzed 3 days after transduction in order to
943 estimate the rate of transduction (%GFP⁺ cells) in CD11c⁺CD11b⁺ cells (approximately 90% of the
944 cells).

945

946 **Stimulation of MDDCs**

947 Differentiated MDDCs were harvested, counted and resuspended in fresh media at a concentration
948 of 0.5 million per ml and 100 μ l was aliquoted in round-bottomed 96-well plates. MDDCs were
949 stimulated by transfected 100 μ l of dilutions of 2'3'-cGAMP (Invivogen), HT-DNA (Sigma) or
950 synthetic DNA repeats coding for AATGG satellite motif or shuffled sequence, delivered with

951 Lipofectamine 2000 (Thermo Fisher Scientific). 48 hours after stimulation, cell-surface staining of
952 CD86 and SIGLEC1 were performed. Synthetic dsDNA fragments were obtained from Eurogentec
953 using two steps of purifications (Reverse Phase HPLC (RP-HPLC) and Sephadex G-25) and
954 annealed (sequences are listed in Key Resources Table).

955

956 **Immunofluorescence**

957 293FT cell lines were grown overnight on a 12mm coverslip. MDDCs, transduced MDDCs or
958 transduced BMDCs were adhered on a 12mm coverslip coated with 0.01% (w/v) Poly-Lysine
959 (SIGMA) for 30 minutes in an incubator for MDDCs or overnight for BMDCs. Cells were fixed
960 with 1ml of PFA 2% (Electron Microscopy Sciences) and PHEM Buffer (2X PHEM buffer: 18.14 g
961 PIPES (Euromedex), 6.5 g HEPES (Euromedex), 3.8 g EGTA (Euromedex), 0.99 g MgSO₄ (Carlo
962 Erba Reagenti), pH adjusted to 7.0 with 10M KOH (VWR)) for 20 minutes in an incubator at 37°C.
963 Coverslips were washed 3 times with 1ml of PBS (GIBCO) and quenched with 0.1M Glycine (Life
964 Technologies) for 10 minutes at room temperature (RT). Coverslips were then blocked with 10%
965 goat serum (SIGMA) in PBS (GIBCO), 0.2% (w/v) BSA (Euromedex), 0.05% (w/v) Saponin from
966 quillaja bark (SIGMA) for 30 minutes at RT. Cells were stained with rabbit monoclonal antibody α -
967 cGAS (D1D3G) (CST) at 1:200 (CST Lot #1 – concentration: 17 μ g/ml) or with Normal Rabbit IgG
968 Isotype control (Thermo Fisher Scientific) at corresponding dilution to the primary antibody, or
969 with a mouse monoclonal antibody α -CENP-B (C-10) (Santa Cruz) at 1:50 in PBS (GIBCO), 0.2%
970 (w/v) BSA (Euromedex), 0.05% (w/v) Saponin from quillaja bark (SIGMA) in presence of 10%
971 goat serum (SIGMA) overnight at 4°C in a humid chamber. Coverslips were then washed 5 times
972 every 3 minutes with PBS (GIBCO), 0.2% (w/v) BSA (Euromedex), 0.05% (w/v) Saponin from
973 quillaja bark (SIGMA) and stained with F(ab')₂-Goat α -Rabbit IgG (H+L) Alexa-647 (Thermo
974 Fisher Scientific) for cGAS or F(ab')₂-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary
975 Antibody, Alexa Fluor 555 (Thermo Fisher Scientific) for CENP-B, for 2 hours at RT in the dark in
976 PBS, BSA, Saponin. Coverslips were then washed 4 times every 3 minutes with PBS (GIBCO),
977 0.2% (w/v) BSA (Euromedex), 0.05% (w/v) Saponin from quillaja bark (SIGMA), washed an
978 additional time with DNase/RNase free water (GIBCO) and mounted on slides with DAPI
979 Fluoromont G (eBioscience). Mounted coverslips were dried for 1 to 2h at 37°C. For transduced
980 MDDCs or BMDCs expressing GFP fused constructs, coverslips were directly mounted after
981 fixation and wash. Images were acquired with a Leica DmI8 inverted microscope equipped with an
982 SP8 confocal unit using either a 40X (1.35NA) or 63X (1.4NA) objective.

983

984 **Unfixed chromosome spreads**

985 U2OS cells were grown at 80% confluency on coverslips and treated with colcemid for 2h. Cells
986 were incubated with hypotonic medium (60% medium, 40% water) for 3 min at 37°C and then
987 centrifuged at 1500rpm for 10min. Cells were blocked in KCM buffer (120mM KCl, 20mM NaCl,
988 10mM Tris-HCl pH=7.7, 0.1% Triton-X-100, 0.5 mM EDTA) + 1% BSA for 30 min. Incubations
989 with primary antibodies were conducted in blocking buffer for 1 hour at room temperature using the
990 following antibodies: CENP-A (1:1000; ADI-KAM-CC006-E, Enzo), cGAS (1:200; #15102, CST),
991 ACA (1:500; 15-235-0001, Antibodies Incorporated). Samples were washed in KCM three times
992 and then incubated with the respective secondary antibody (1:500) in blocking buffer for 45 min.
993 Cells were washed in KCM three times and then fixed in 4% formaldehyde for 10 min prior to
994 DAPI staining and slide mounting. Images were acquired on a Fluorescent microscope DeltaVision
995 Core system (Applied Precision) with 100x Olympus UPlanSApo 100 oil-immersion objective (NA
996 1.4), 250W Xenon light source equipped with a Photometrics CoolSNAP_HQ2 Camera. 4 μ m
997 Zstacks were acquired (Z step size: 0.2 μ m).

998

999 **Nuclear/Cytoplasmic fractionation**

1000 2 million MDDCs at day 4 or day 5 post-differentiation or mouse bone marrow derived dendritic
1001 cells at day 10 post-differentiation were collected, washed with 1ml of PBS, and processed
1002 according to two different fractionation protocols.

1003 Human donor #1, #2 and mouse DCs: Fractionation protocol A. Cells were lysed with 100µl of
1004 Lysis Buffer 1 (LB1) (50mM Tris pH 8.0, 2.5mM EDTA pH 8.0 (Invitrogen), 0.1% NP40
1005 (Euromedex), 10% Glycerol (v/v) (Pharmacia Biotech)) for 5 minutes on ice in presence of
1006 cOmplete EDTA free Protease inhibitor cocktail (Roche). Nuclei were pelleted by centrifuging for 5
1007 minutes at 400g at 4°C. The recovered supernatant represented the cytosolic fraction and were
1008 stored on ice. Nuclei were lysed in 100µl of Lysis Buffer X (LBX) (50mM Tris pH 8.0, 2.5mM
1009 EDTA pH 8.0 (Invitrogen), 0.25% SDS (Euromedex)) in presence of cOmplete EDTA free Protease
1010 inhibitor cocktail (Roche). The lysates were sonicated for 20 minutes at 4°C in a Sonorex Digitec
1011 (model DT100) ultrasonic bath (Bandelin). Both fractions were cleared by centrifugation at 16,000g
1012 for 10 minutes at 4°C. The supernatant from both fractions was recovered and stored at -20°C until
1013 western blotting.

1014 Human donor #3, #4: Fractionation protocol B. Cells were resuspended in 400µl of cold
1015 Cytoplasmic Lysis (CL) buffer (10mM HEPES pH 7.9 (Invitrogen), 10mM KCl, 1.5mM MgCl₂,
1016 1mM NaVO₄, 50mM NaF) in presence of cOmplete EDTA free Protease inhibitor cocktail (Roche)
1017 and centrifuged at 300g for 4 minutes at 4°C. The supernatant was discarded and cells were
1018 resuspended in 40µl of cold CL buffer (10mM HEPES pH 7.9 (Invitrogen), 10mM KCl, 1.5mM
1019 MgCl₂, 1mM NaVO₄, 50mM NaF) in presence of cOmplete EDTA free Protease inhibitor cocktail
1020 (Roche) by gentle flicking for 15 minutes on ice. Cytoplasm was lysed by adding 2.5µl of 10%
1021 NP40 (Euromedex) and gently flicking. Nuclei were pelleted at 16,000g for 5 minutes at 4°C. The
1022 supernatant containing the cytoplasmic fraction was recovered and frozen at -20°C until western
1023 blotting. Nuclei were lysed in 40µl of Nuclear Lysis (NL) buffer (420mM NaCl, 20mM HEPES pH
1024 7.9 (Invitrogen), 1.5mM MgCl₂, 0.2mM EDTA (Invitrogen), 250µl Glycerol (Pharmacia Biotech),
1025 1mM NaVO₄, 50mM NaF) in presence of cOmplete EDTA free Protease inhibitor cocktail (Roche)
1026 on ice for 15 minutes by gentle flicking. Nuclear lysates were vortexed and sonicated for 20
1027 minutes at 4°C in a Sonorex Digitec (model DT100) ultrasonic bath (Bandelin). Nuclear lysate was
1028 cleared by centrifugation at 16,000g for 5 minutes at 4°C and the supernatant was stored at -20°C
1029 until western blotting.

1030

1031 **Western blotting**

1032 For western blotting of 293FT and HeLa cells, 1 million cells were lysed in 100µl of LBX in
1033 presence of cOmplete EDTA free Protease inhibitor cocktail (Roche). The lysates were sonicated
1034 for 20 minutes at 4°C in a Sonorex Digitec (model DT100) ultrasonic bath (Bandelin) and cleared
1035 by centrifugation at 16,000g for 10 minutes at 4°C. The supernatant was stored at -20°C until
1036 blotting. 6X Laemmli buffer (12% SDS (v/v) (Euromedex), 58% Glycerol (v/v) (Pharmacia
1037 Biotech), 375mM Tris HCl pH 6.8, 30% β-mercaptoethanol (v/v) (Pharmacia Biotech), 0.0012%
1038 Bromophenol Blue Before (w/v) (Pharmacia Biotech)) was added to samples to a final
1039 concentration of 1X prior to gel run. Samples were boiled at 95°C for 20 minutes on a thermoblock,
1040 immediately chilled on ice and centrifuged at 16,000g for 5 minutes. 15-40µl of samples were
1041 resolved on 4%-20% SDS-PAGE gels (Biorad) and transferred on nitrocellulose membrane
1042 (Biorad). Membranes were saturated and proteins were blotted with antibodies (listed in Key
1043 Resources Table) in 5% non-fat dry milk, PBS, 0.1% Tween buffer. ECL signal was recorded on a
1044 ChemiDoc Touch Biorad Imager. Data was analyzed with Image Lab (Biorad).

1045

1046

1047 **Live microscopy**

1048 0.15-0.25 million HeLa cells were plated in FluoroDish (World Precision Instruments) one day
1049 before live imaging. One hour prior to imaging, cells were stained with 1µM of SiR-DNA (Tebu
1050 Bio). HeLa cells expressing H2B-mCherry and GFP-cGAS were acquired using an Inverted

1051 Spinning Disk Confocal Roper/Nikon equipped with a 100X objective (1.4NA) and an EMCCD
1052 512x512 QuantEM (pixel size:16 μm) Photometrics. HeLa cells expressing GFP-cGAS Δ_{K173-}
1053 $I220\Delta_{H390-405}$ were acquired with a Leica DmI8 inverted microscope equipped with an SP8 confocal
1054 unit using a 20X (0.75NA) objective. Microscopes were equipped with an on-stage incubation
1055 chamber which maintained the temperature at 37°C and CO₂ concentration at 5% at all times.
1056

1057 **Image processing and analysis**

1058 Images were processed and analyzed with ImageJ Fiji.

1059 *cGAS Nuclear/Cytoplasmic ratio quantification.* A mask of all nuclei was obtained by thresholding
1060 the DAPI channel image and individual nuclei were detected using “analyze particles” function. For
1061 each nucleus, we first measured the mean GFP-cGAS intensity or the mean endogenous cGAS
1062 intensity inside the nucleus. The region of interest corresponding to the nucleus was enlarged by a
1063 1.2 factor to compute the mean cytoplasmic GFP-cGAS or endogenous cGAS intensity at the
1064 periphery of the nucleus. A cytoplasmic mask was obtained by thresholding on the GFP-cGAS or
1065 endogenous cGAS signal and was used to correct the enlarged nuclear mask. For endogenous
1066 cGAS, the average pixel intensity for the nuclear and the peri-nuclear masks were plotted. For GFP-
1067 cGAS we defined an enrichment factor as the ratio between the mean nuclear GFP intensity and the
1068 cytoplasmic one.

1069 *GFP-IRF3 translocation in HeLa.* Percentage of cells with GFP-IRF3 nuclear translocation was
1070 quantified manually. Cells showing persistent GFP-IRF3 nuclear signal were considered as positive.
1071 Bursts of nuclear GFP due to NE ruptures were not considered as positive events, as GFP-IRF3 was
1072 rapidly excluded from the nucleus. For cells transfected with HT-DNA, to avoid overestimation of
1073 translocation events due to cGAMP transfer via gap junctions, only cells showing bright foci of
1074 cytoplasmic GFP-IRF3 were considered as positive. The appearance of such cytoplasmic bright foci
1075 always correlated with translocation of GFP-IRF3.

1076 *GFP enrichment on CENP-B foci or random regions.* DCs were stained as described in the section
1077 “Immunofluorescence”. ZStacks were acquired with a Leica DmI8 inverted microscope equipped
1078 with an SP8 confocal unit using a 63X (1.4NA) objective. Images were oversampled (pixel size of
1079 0.037 μm and 0.15 μm Zstep) and deconvoluted using Huygens Essentials software (Scientific
1080 Volume Imaging). Measurement of GFP enrichment at CENP-B foci was performed on the 3D
1081 deconvoluted stacks using a homemade macro. For each acquired nucleus, a threshold on the α -
1082 CENPB channel was applied and the XYZ positions of each CENP-B focus were measured using
1083 the 3D Object Counter plugin. The mean GFP intensity was measured in a sphere of 0.2 μm radius
1084 around each CENP-B focus position. The mean GFP intensities in CENP-B foci were then
1085 normalized by the nuclear mean GFP intensity, measured from a 3D mask of the nucleus obtained
1086 using the DAPI channel. The same analysis was performed on 20 randomly generated positions in
1087 each nucleus.
1088

1089 **cGAMP bioassay**

1090 cGAMP bioassay was previously described (Gentili et al., 2015). 0.8 million 293FT cells per well
1091 per cell line were plated in a 6 well plate. One plate for each cell line was either untreated or
1092 stimulated with 1 $\mu\text{g}/\text{ml}$ HT-DNA (SIGMA) transfected with Lipofectamine 2000 (Thermo Fisher
1093 Scientific) (1 μg HT-DNA:1 μl Lipofectamine). Cells were harvested 16-20h after wash with PBS
1094 (GIBCO), pelleted and frozen at -80°C until extraction. Cell pellets were thawed and lysed in 500 μl
1095 of MeOH/H₂O (80/20, v/v) and subjected to 5 freeze/thaw cycles in liquid nitrogen. The lysates
1096 were then centrifuged at 16,000g at 4°C for 20 minutes. The recovered supernatants were subjected
1097 to speed vacuum drying in Savant DNA Speed Vac DNA 110 at 65°C for 2 hours. The pellets were
1098 resuspended in 30 μl of RNase-DNase free water (GIBCO). 24 hours prior to the assay, 100,000
1099 THP-1 cells were re-suspended in fresh medium with PMA (Sigma) at 30ng/ml and seeded in 96-
1100 well plate flat bottom. THP-1 cells were washed to removed PMA and gently overlaid with 13 μl of
1101 2X Permeabilization Buffer (100mM HEPES, 200mM KCl, 6mM MgCl₂, 0.2mM DTT, 170mM

1102 Sucrose, 1mM ATP, 2mM GTP, 0.4% BSA, 0.002% Digitonin). The resuspended samples were
1103 diluted in 3-fold serial dilutions and 13µl of each dilution were gently added to cells. Serial
1104 dilutions of synthetic 2'3' cGAMP (InvivoGen) were delivered in 1X Permeabilization Buffer. The
1105 cells were incubated for 30 minutes at 37°C, 5% CO₂ atmosphere, washed with 150µl of cell
1106 medium, 75µl of fresh cell medium were added on the cells and incubated overnight. 50µl of the
1107 supernatant were then transferred on HL-116 cells to measure interferon activity as previously
1108 described (Lahaye et al., 2013).

1109

1110 **cGAMP ELISA**

1111 cGAMP ELISA was performed according to manufacturer's protocol using Cayman Chemical 2'3'-
1112 cGAMP ELISA Kit (Interchim). For 293FT quantification, 10 million cells were harvested, washed
1113 with PBS, pelleted and frozen in 500µl of MeOH/H₂O (80/20, v/v) at -80°C until extraction. For
1114 MDDCs quantification at day 4 post-differentiation and transfection, 4.5 million cells (separated
1115 into three tubes of 1.5 million cells) were washed with PBS and processed according to the
1116 fractionation protocol B. The supernatant containing the cytoplasmic fraction was recovered, one
1117 tube was frozen at -20°C until western blotting. The two others cytoplasmic fractions and two
1118 nuclei pellets were pooled and frozen in a final volume of 500µl of MeOH/H₂O (80/20, v/v) at -
1119 80°C until extraction. The last nuclei pellet was lysed according to the fractionation protocol B and
1120 the supernatant was stored at -20°C until western blotting.

1121 MeOH/H₂O extracts at -80°C were subjected to 5 freeze/thaw cycles in liquid nitrogen. The lysates
1122 were then centrifuged at 16,000g at 4°C for 20 minutes. The recovered supernatants were subjected
1123 to speed vacuum drying in Savant DNA Speed Vac DNA 110 at 65°C for 2 hours. The pellets were
1124 resuspended in 100µl of RNase-DNase free water. cGAMP quantities were normalized to the
1125 number of cells extracted and represented as pg/million cells.

1126

1127 **Flow cytometry**

1128 Cell surface staining was performed in PBS, 1% BSA (Euromedex), 1mM EDTA (GIBCO), 0.01%
1129 NaN₃ (AMRESCO) (FACS Buffer). For MDDCs, the antibodies used were anti-human CD86-PE
1130 (clone IT2.2, eBioscience) and anti-human CD169-APC (SIGLEC1) (clone 7-239, Miltenyi). For
1131 BMDCs, the antibodies used were anti-mouse CD11c-PECy7 (clone N418, eBioscience) and anti-
1132 mouse CD11b-PerCp-Cy5.5 (clone M1170, eBioscience). Cells were stained for 15 minutes at 4°C,
1133 washed for two times in FACS buffer and fixed in 1% paraformaldehyde (Electron Microscopy
1134 Sciences) in FACS Buffer. Data was acquired on a FACSVerser (BD) flow cytometer and analyzed
1135 in FlowJo.

1136

1137 **Cell compression**

1138 0.5 million HeLa cells per well were seeded in a 6 well plate glass bottom in 500µl and then a 3 µm
1139 roof of PDMS was placed on top, as previously described (Le Berre et al., 2014; Liu et al., 2015).
1140 Briefly, silicon wafers were coated with SU8 2005 photoresist (Microchem) 3 µm in height and
1141 holes were made in lithography. To make the PDMS pillars as the 3 µm height spacers, 12 mm
1142 glass coverslips were plasma treated and then placed on top of a PDMS/crosslinker mixture (10/1
1143 w/w) on the wafer containing 3 µm holes. After baking at 95°C for 15 min, coverslips with PDMS
1144 pillars were carefully removed from the wafers using isopropanol and a razor blade. They were then
1145 cleaned with isopropanol, well-dried, plasma activated for 2 min, and treated with 0.1 mg/mL pLL-
1146 g-PEG in 10 mM pH 7.4 HEPES buffer for 1h at room temperature. Coverslips with PDMS pillars
1147 were rinsed and incubated in medium for at least 2 hours before confining the cells. The modified
1148 cover lid of a multi-well plate was used to apply confining slides to cells. In this case, large PDMS
1149 pillars were stuck on the cover lid of the multi-well plate to hold confining slides. The process of
1150 fabrication for these large pillars attached to the 6 well plate lid is as follows: the large PDMS
1151 pillars were fabricated by pouring a PDMS/crosslinker mixture (35/1 w/w) into a custom-made
1152 metallic mold, removing bubbles under vacuum, then baking overnight at 70°C, and getting the

1153 pillars out of the mold with the help of isopropanol. For HT-DNA transfection, cells were pre-
1154 treated for 30 minutes with 4 μ g/ml of HT-DNA with Lipofectamine 2000 (1 μ g HT-DNA:1 μ l
1155 Lipofectamine 2000) and then compressed or left untouched. Time-lapse recordings were acquired
1156 with a 40x objective, 0.95 NA, DIC, using an Eclipse Ti inverted microscope (Nikon) equipped
1157 with a Coolsnap HQ2 camera (Roper Scientific) controlled by MetaMorph software (Universal
1158 Imaging). Microscope was equipped with an on-stage incubation chamber which maintained the
1159 temperature at 37°C and CO₂ concentration at 5% at all times. GFP signal was acquired with an
1160 interval of 5 minutes, while the mCherry signal was acquired every 3 frames of GFP to avoid
1161 phototoxicity mediated cell death.

1162

1163 **Luciferase assay**

1164 45,000 293FT cells were plated in a 24-well plate in 500 μ l of medium. The next day, cells were
1165 transfected in fresh medium with 500ng of total DNA comprising 200ng of IFN β -pGL3 and 150ng
1166 of the empty vector pMSCV-Hygro or or pMSCV-Hygro-STING R232 with TransIT-293 (Mirus).
1167 A master mix of transfection solution was prepared for each condition. 16h hours post transfection
1168 the medium was replaced with fresh medium. 30-36h after medium change cells were washed with
1169 PBS and lysed with 100 μ l of Passive Lysis Buffer (Promega) and 10 μ l of the lysates were used to
1170 perform the Luciferase assay. Luciferase activity was measured using Luciferase Assay Reagent
1171 (Promega). Luminescence was acquired on a FLUOstar OPTIMA microplate reader (BMG
1172 labtech). One well with the same transfection conditions for the assay was lysed for Western
1173 Blotting in 100 μ l LBX.

1174

1175 **Quantitative PCR with Reverse Transcription (qRT-PCR)**

1176 1 million BMDCs from WT or *Cgas*^{-/-} mice were collected at day 10, washed once in PBS (GIBCO)
1177 and lysed in 700 μ l of QIAzol (Qiagen) and froze at -80°C until RNA purification. RNA was
1178 purified with miRNeasy Micro Kit (Qiagen), following manufacturer instructions. 0.25 to 0.5
1179 millions of transduced MDDCs for 4 days or BMDCs for 3 days were collected, washed once in
1180 PBS (GIBCO) and RNA were extracted using Nucleospin RNA II kit (Macherey-Nagel). cDNA
1181 was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) and Random
1182 Primer Mix (NEB) following manufacturer instructions. Quantitative PCR reaction was performed
1183 with LightCycler 480 SYBR Green I Master Mix (Roche) in a LightCycler 480 (Roche) and
1184 analyzed in LightCycler 480 software with the 2^{- Δ Cp} method. The primer pairs are listed in the Key
1185 Resources Table.

1186

1187 **Chromatin immunoprecipitation**

1188 *Crosslinking and lysis.* 10 million cells were cross-linked in medium with 1% formaldehyde for 8
1189 min at RT on a slow shaker, quenched with freshly prepared 0.125M glycine, incubated 5min at RT
1190 on a slow shaker, then pelleted at 400g for 5 minutes at 4°C, washed three times with 30ml of ice
1191 cold PBS and then incubated for 20 minutes rotating at 4°C in 1mL of RIPA lysis buffer (10mM
1192 Tris-HCl pH 8.0, 1mM EDTA pH 8.0 (Invitrogen), 140mM NaCl, 1% (v/v) Triton X-100
1193 (Euromedex), 0.1% (v/v) SDS and 0.1% sodium deoxycholate (SIGMA)). Nuclei were pelleted at
1194 1350g for 5 minutes at 4°C, washed for 10 minutes rotating with 1ml of a buffer containing 10mM
1195 Tris, 200mM NaCl, 1mM EDTA (Invitrogen), 0.5 mM EGTA (Euromedex), pelleted and lysed in
1196 buffer containing 0.4% SDS (Euromedex), 10mM EDTA (Invitrogen), 50mM Tris-HCl pH 8.0 for
1197 30min on ice (volume of buffer = 100 μ l/1.6 million cells). Lysates were sonicated on a Bioruptor
1198 Pico (Diagenode) sonication devices (11cycles 30 seconds ON, 30 second OFF) to reach fragments
1199 ranging from 150 to 500bp, and then centrifuged at 10,000g for 10 minutes at 4°C to remove debris.
1200 Samples were then snap-frozen in liquid nitrogen and stored at -80°C until immunoprecipitation.
1201 All buffers contained cComplete EDTA free Protease inhibitor cocktail (Roche).

1202

1203 *Immunoprecipitation.* Lysates were pre-cleared for 15 minutes rotating using 30 μ l of Binding
1204 Control magnetic agarose beads (Chromotek). Chromatin was diluted four-fold in dilution buffer
1205 containing 20mM Tris-HCl pH 8.0, 1% Triton X-100 (Euromedex), 2mM EDTA (Invitrogen),
1206 167mM NaCl. 1% of the diluted lysate was recovered and used as input. For GFP-trap and control
1207 beads, chromatin was incubated for 5 hours in the presence of 0.1% BSA (Euromedex) (30 μ l of
1208 beads (GFP-Trap_MA beads (Chromotek) or Control magnetic agarose beads (Chromotek)/600 μ l
1209 per Eppendorf tube of the diluted lysate). Lysates were washed on a 96 well plate magnet with low
1210 salt washing buffer (140mM NaCl) (5 times), high salt washing buffer (500mM NaCl) (2 times),
1211 high LiCl washing buffer (250mM LiCl) (2 times), TE Buffer (Invitrogen) (1 time). All wash
1212 buffers were diluted in RIPA buffer 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0 (Invitrogen),
1213 140mM NaCl, 1% (v/v) Triton X-100 (Euromedex), 0.1% (v/v) SDS and 0.1% sodium
1214 deoxycholate (SIGMA)) and contained cOmplete EDTA free Protease inhibitor cocktail (Roche).

1215
1216 *DNA purification.* DNA was eluted in elution buffer (1% SDS, 50 mM NaHCO₃) by shaking 2h at
1217 37°C (100 μ l of buffer/tube) with 10 μ g/mL RNaseA (Thermo Fischer), then 4h with 0.2 μ g/ml
1218 proteinase K. Beads were concentrated on the magnet and take out eluate. Samples were
1219 decrosslinked overnight at 65°C. Inputs were treated like ChIP samples. DNA was purified by
1220 phenol/chloroform/isoamyl alcohol (SIGMA) followed by purification on MinElute columns
1221 (Qiagen). DNA was eluted in 50 μ l of H₂O and DNA concentration was measured with a Qubit
1222 fluorometer (Thermo Fischer).

1223
1224 *ChIP-seq.* Traces of high molecular weight fragments were eliminated with SPRiselect beads
1225 (Beckman Coulter). Illumina TruSeq ChIP library prep kit was used to prepare indexed libraries
1226 from IP and Input DNA. Libraries were pooled respecting equimolarity. Sequencing was performed
1227 on Illumina MiSeq sequencer in 150 bp paired-end reads (replicate 1 of human input and GFP-
1228 NLS-cGAS IP; replicate 1 and pooled replicates 2+3 of mouse input and GFP-cGAS IP), 100 bp
1229 single-end reads (replicates 2 and 3 of human input, GFP-NLS and GFP-NLS-cGAS IP).

1231 **ChIP-seq data analysis of overexpressed cGAS in human DCs**

1232 *Mapping and peak calling.* Reads for each replicate were mapped separately to the hg38 primary
1233 assembly (accession: GCF_000001305.14) with Bowtie2 v2.2.9 (Langmead and Salzberg, 2012)
1234 using a seed length of 22bp with at most 1 mismatches (-N 1 -L 22) and keeping the best scoring
1235 alignment per read. Duplicate fragments were identified with MarkDuplicates from picard v1
1236 (<https://broadinstitute.github.io/picard/>). Only non-duplicate, properly paired reads (same reference,
1237 inner-oriented, insert size \leq 500bp; only for replicate 1) with mapping quality \geq 20 were retained,
1238 using samtools v1.3. Alignment files were converted from BAM to BED/BEDPE format using
1239 bedtools bamtobed from bedtools v2.27.1 (<https://bedtools.readthedocs.io/>).

1240 Genome mappability was computed with gemtools v1.7.1 (-l L -m 0.04 -e 0.04 --max-big-indel-
1241 length 15 --min-matched-bases 0.80, where L is the read length)
1242 (<https://github.com/gemtools/gemtools>). The effective genome size was then defined as m/n , where
1243 m is the number of bp with mappability score 1 (*i.e.*, sequences occurring once in the genome) and
1244 n is the genome length.

1245 Peak calling of GFP-NLS-cGAS IP reads on hg38 chromosomes was performed with SICER v1.1
1246 on each replicate separately, using either input or GFP-NLS IP (only for replicate 2 and 3) reads as
1247 background (redundancy threshold 1, window size 200bp, FDR 0.05, effective genome size
1248 estimated with the above procedure). The gap size was set to 600bp for replicate 1 and to 400bp for
1249 replicate 2 and 3. For replicate 1, one read for each pair was used and the fragment size was set to
1250 the average insert size computed from the alignment. Filtered peaks were defined for each replicate
1251 i as the peaks supported by more than M ChIP reads, where M is the median across all peaks for
1252 replicate i . Selected peaks were defined as follows: a peak P_1 from donor i is selected if there is a
1253 peak P_2 from donor j , with $j \neq i$, lying at a distance < 2000 bp from P_1 . Intersection peaks between

1254 replicate 2 and 3 (over GFP-NLS IP) were also identified, across all (non-filtered) peaks, using
1255 bedtools intersect (default parameters) from bedtools v2.27.1.

1256

1257 *Public datasets.* Reads datasets for endogenous CENP-A ChIP-Seq and MNase input in HeLa S3
1258 cells from a previous study (Lacoste et al., 2014) were downloaded from SRA (accessions:
1259 SRR633612, SRR633613, SRR633614, SRR633615). Repeats annotation of hg38 is obtained with
1260 RepeatMasker v4.0.5 on repeats database version 20140131 (downloaded from
1261 <http://www.repeatmasker.org/genomes/hg38/RepeatMasker-rm405-db20140131>).

1262 Peaks for histone marks H3K27ac in GM12878 cells (GEO: GSE29611) and H3K9me3 in PBMCs
1263 (GEO: GSE31755) were from the ENCODE consortium.

1264

1265 *Peak calling of endogenous CENP-A ChIP on hg38.* Peak calling on endogenous CENP-A data was
1266 performed following the approach previously described (Lacoste et al., 2014), with small
1267 modifications. The paired reads sequenced from the same fragment were merged using SeqPrep-1.2
1268 (<https://github.com/jstjohn/SeqPrep>), with prior Illumina adaptors removal, requiring an overlap of
1269 at least 15bp. Fragments shorter than 100bp were filtered out. The first 50bp of each fragment were
1270 mapped separately for each replicate to the hg38 primary assembly (accession:
1271 GCF_000001305.14) with Bowtie v1.2 (<http://bowtie-bio.sourceforge.net/>), allowing up to 3
1272 mismatches (-v 3). Alignment files were converted from BAM to BED format using bedtools v2.27.
1273 The effective genome size is defined as m/n , where m is the number unique 50-mers (downloaded
1274 from https://github.com/biocore-ntnu/epic/blob/master/epic/scripts/effective_sizes/hg38_50.txt),
1275 and n is the genome length. Peak calling of CENP-A-IP reads on hg38 chromosomes was
1276 performed with SICER v1.1 on the two replicates separately, using input reads as background
1277 (redundancy threshold 1, window size 200bp, gap size 400bp, FDR 1e-5, effective genome size
1278 defined as above). Intersection peaks between the two replicates were detected with bedtools
1279 intersect from bedtools v2.27.1, requiring each intersection peak to cover at least 90% of the length
1280 of a peak in either replicate 1 or 2.

1281

1282 *Peak annotation.* The annotatePeaks.pl script from HOMER software v4.9
1283 (<http://homer.ucsd.edu/homer/>) was used to annotate the human cGAS filtered peaks and to
1284 calculate enrichments of the annotated features.

1285

1286 *Association of GFP-NLS-cGAS peaks with public datasets.* Intersection of GFP-NLS-cGAS IP
1287 peaks with publicly available ChIP-Seq datasets (H3K27ac, H3K9me3, endogenous CENP-A) is
1288 computed at the base pair level. Lift-over from hg19 to hg38 is applied for H3K27ac and
1289 H3K9me3, whereas CENP-A peaks on hg38 are computed starting from raw data following the
1290 approach described above. The intersection is computed using bedtools intersect from bedtools
1291 v2.27.1 and the odds-ratio between two datasets A and B is defined as

$$\text{odds-ratio}(A, B) = \frac{x}{a-x} \frac{G - (a + b - x)}{b-x}$$

1292 where G is the genome size, a and b are the cumulative sizes of the regions in A and B , respectively,
1293 and x is the size of the intersection.

1294

1295 *De novo motif discovery and motif enrichment analyses.* This analysis was carried out on all
1296 intersecting peaks of GFP-NLS-cGAS over GFP-NLS in donor #2 and #3. 38 peaks are found. For
1297 *de novo* discovery, the *peak-motifs* pipeline (default parameters) of the software suite Regulatory
1298 Sequence Analysis Tools (RSAT; <http://rsat.sb-roscoff.fr/>) was used. cGAS intersection peaks are
1299 searched for CENP-B box, the consensus (NTTCGNNNNANNCGGGN) and the most common
1300 (NTTCGTTGGAANCGGGA), for Satellite repeats ([GGAAT] n , with $n \geq 4$), and for telomere
1301 repeats ([TTAGGG] n , with $n \geq 2$), using regular expression matching (in-house perl script). Motif
1302 enrichment is computed either over shuffled peaks (same number of peaks, same length) on the

1303 genome (with prior removal of gap regions, gap table from UCSC table browser). 10 peak shuffling
1304 runs are performed using bedtools shuffle from bedtools v2.27.1 and the motif enrichment is
1305 averaged across the 10 runs. The enrichment is defined as the ratio of the motif count on the peaks
1306 over the motif count on the shuffled peaks.

1307

1308 *cGAS reads enrichment on repeats.* Reads for each replicate were mapped separately to the hg38
1309 primary assembly and alignments were filtered as described above. The coverage of each repeat
1310 element x in the genome is defined as the number of reads whose midpoint lies within x (in-house
1311 C++ code). The cGAS read enrichment of region x is defined as

$$enrich(x) = \frac{c_{chip}(x) + 1}{c_{ctrl}(x) + 1} \cdot \frac{N_{ctrl}}{N_{chip}},$$

1312 where c_{chip} and c_{ctrl} are the ChIP and control read count on x and N_{ctrl} and N_{chip} are the library
1313 sizes. All repeat elements in the genome are ranked according to the cGAS read enrichment value
1314 and grouped into n ranking bins, where bin i contains the elements whose percentage ranking ranges
1315 between the top $(100/i)\%$ and the top $(100/(i+1))\%$. The number of elements falling into each bin is
1316 computed for each repeat class R . The first bin represents the bottom $(100/n)\%$ ranks and the n -th
1317 bin represents the top $(100/n)\%$ ranks.

1318

1319 **ChIP-seq analysis of endogenous cGAS in mouse DCs**

1320 *Mapping, peak calling and peak annotation.* Raw reads were aligned to the mouse reference
1321 genome version mm10 (accession: GCA_000001305.2) with Bowtie2 v2.1.0 using a seed length of
1322 22bp with at most 1 mismatch (-N 1 -L 22) and keeping the best scoring alignment per read.
1323 Alignment filtering and peak calling were performed as for the cGAS overexpression dataset
1324 (replicate 1). Intersection peaks between the two replicates were identified using bedtools intersect
1325 (default parameters) from bedtools v2.27.1. For peak annotation, the same procedure as for cGAS
1326 overexpression was followed.

1327

1328 *Mapping to repeats database.* Reads that failed to map to mm10 were aligned against the mouse-
1329 specific repeats from RepBase (<https://www.girinst.org/replib/>). Bowtie2 was run with the same
1330 parameters as above but with soft-clipping option enabled (--local). The read count for each
1331 RepBase sequence was computed from the BAM files with samtools v1.3
1332 (<http://samtools.sourceforge.net/>), and then normalized by the total number of reads mapped to
1333 RepBase. Repeats enrichment in ChIP with respect to Input was computed as the ratio between the
1334 normalized read counts and then log₂-transformed.

1335

1336 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1337 Statistical analyses for all experiments except ChIP-seq were performed in Prism (GraphPad) v7.
1338 For ChIP-seq statistical analysis please refer to the corresponding section in the “Method details”
1339 section. Statistical parameters including the exact value of n , dispersion and precision measures (as
1340 mean \pm SEM) and statistical significance are reported in the Figures and Figure legends. In figures
1341 asterisks denote statistical significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, “ns” =
1342 not significant. Statistical tests used are indicated in the Figure legends.

1343

1344 **DATA AND SOFTWARE AVAILABILITY**

1345 The accession number for the raw data files of the ChIP-seq experiments reported in this paper is
1346 NCBI GEO: GSE125475.

1347

1348

1349

1350 **Supplemental Videos Legends**

1351 **Movie S1. Association of cGAS with chromosomes in mitosis, related to Figure 1E.** HeLa cells
1352 stably expressing H2B-mCherry (red) and GFP-cGAS (green). One cell is going through mitosis.

1353

1354 **Movie S2. Association of cGAS with chromosomes in mitosis, 3D reconstruction, related to**
1355 **Figure 1G.** HeLa cell expressing GFP-cGAS (green) and H2B-mCherry (red) going through
1356 mitosis. Scale bar is 10 μ m.

1357

1358 **Movie S3. Decay of nuclear cGAS after mitosis, related to Figure 1E.** HeLa cells stably
1359 expressing H2B-mCherry (red) and GFP-cGAS (green). One cell is going through mitosis followed
1360 by the next interphase, showing decay of nuclear GFP-cGAS after mitosis. Red indicates nuclear
1361 mask. Blue corona indicates cytoplasmic mask.

1362

1363 **Movie S4. Lack of association of cGAS $\Delta_{K173-1220}\Delta_{H390-C405}$ with chromosomes in mitosis, related**
1364 **to Figure 1E.** HeLa cells stably expressing GFP-cGAS $\Delta_{K173-1220}\Delta_{H390-C405}$ (green) labeled with siR-
1365 DNA (red). Various cells are going through mitosis. Time is hh:mm. Scale bar is 10 μ m.

1366

1367 **Movie S5. Nuclear translocation of GFP-IRF3 upon HT-DNA transfection in HeLa cells,**
1368 **related to Figure 4C.** HeLa cells expressing BFP2A-STING (not shown), GFP-IRF3 (green) and
1369 mCherry-cGAS E225A/D227A labeled with siR-DNA and transfected with 4 μ g/ml of HT-DNA.
1370 Transfection has been performed at time=0 min. Scale bar is 10 μ m.

1371

1372 **Movie S6. Nuclear entry of cGAS but no stable nuclear translocation of GFP-IRF3 upon**
1373 **compression in HeLa cells, related to Fig 4E.** HeLa cells expressing BFP2A-STING (not shown),
1374 GFP-IRF3 (green) and mCherry-cGAS E225A/D227A confined at 3 μ m height. The cells were
1375 confined and the movie was started after compression. Foci of mCherry-cGAS at the nucleus
1376 identify NE ruptures. NE rupture events increase during time. Rapid flashes of GFP-IRF3 in and out
1377 of the nucleus correspond to events of NE rupture. Scale bar is 10 μ m.

1378

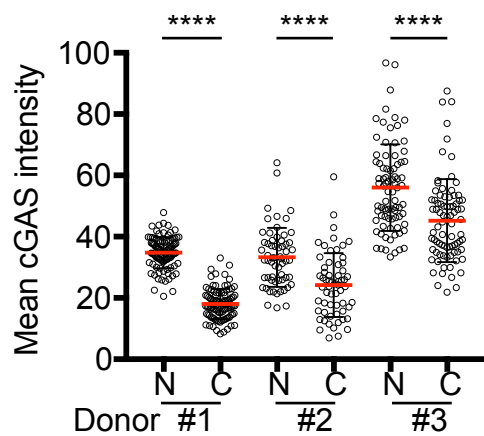
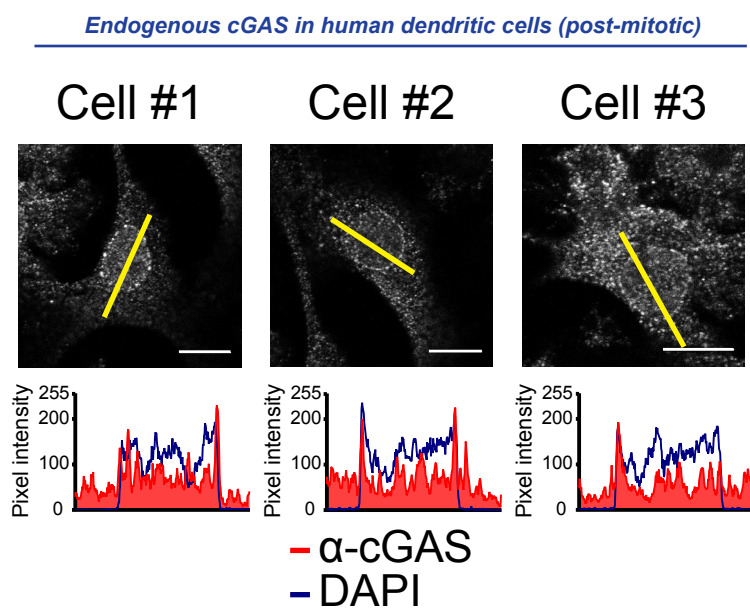
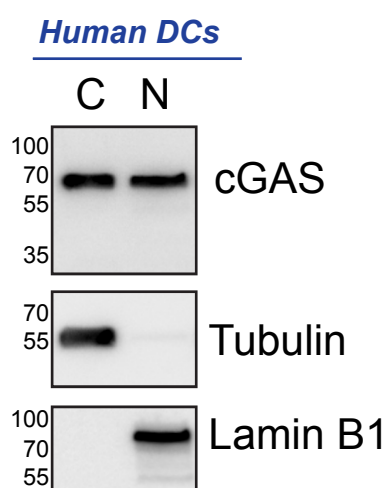
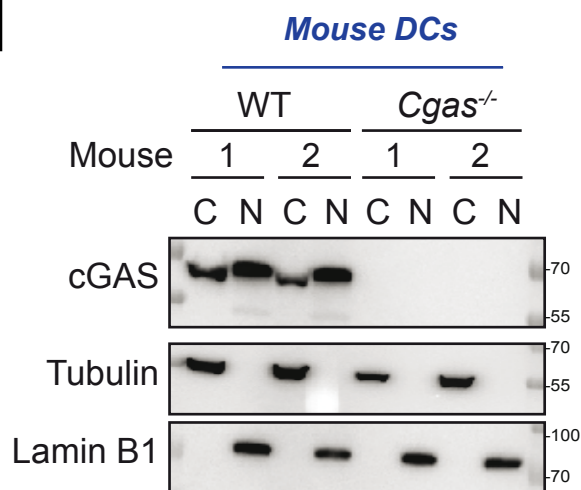
1379 **Movie S7. HT-DNA induces stable nuclear translocation of GFP-IRF3 in compressed HeLa**
1380 **cells that undergo nuclear envelope rupture, related to Figure 4F.** HeLa cells expressing
1381 BFP2A-STING (not shown), GFP-IRF3 (green) and mCherry-cGAS E225A/D227A confined at
1382 3 μ m height and transfected with 4 μ g/ml of HT-DNA. Rapid flashes of GFP-IRF3 in and out of the
1383 nucleus correspond to events of NE rupture. Cells with GFP-IRF3 translocation show bright GFP
1384 foci in the cytoplasm, followed by nuclear translocation. Scale bar is 10 μ m.

1385

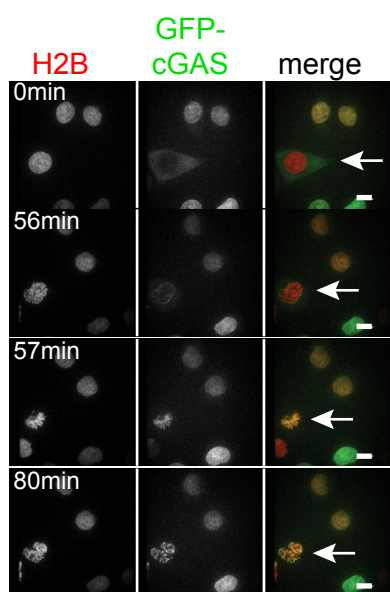
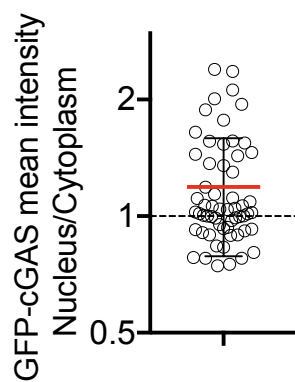
1386 **Movie S8. HT-DNA induces stable nuclear translocation of GFP-IRF3 in control HeLa cells,**
1387 **related to Figure 4G.** HeLa cells expressing BFP2A-STING (not shown), GFP-IRF3 (green) and
1388 mCherry-cGAS E225A/D227A transfected with 4 μ g/ml of HT-DNA. Cells were transfected and
1389 the movie was started. Cells with GFP-IRF3 translocation show bright GFP foci in the cytoplasm,
1390 followed by nuclear translocation. Scale bar is 10 μ m.

1391

Figure 1

a**b****c****d****e**

Stably transduced HeLa (cycling)

**f****g**

Stably transduced HeLa (cycling)

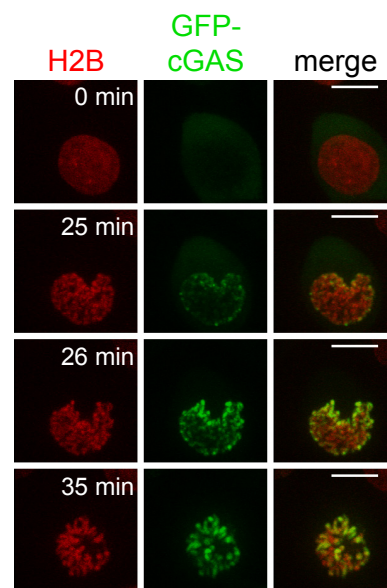


Figure 2

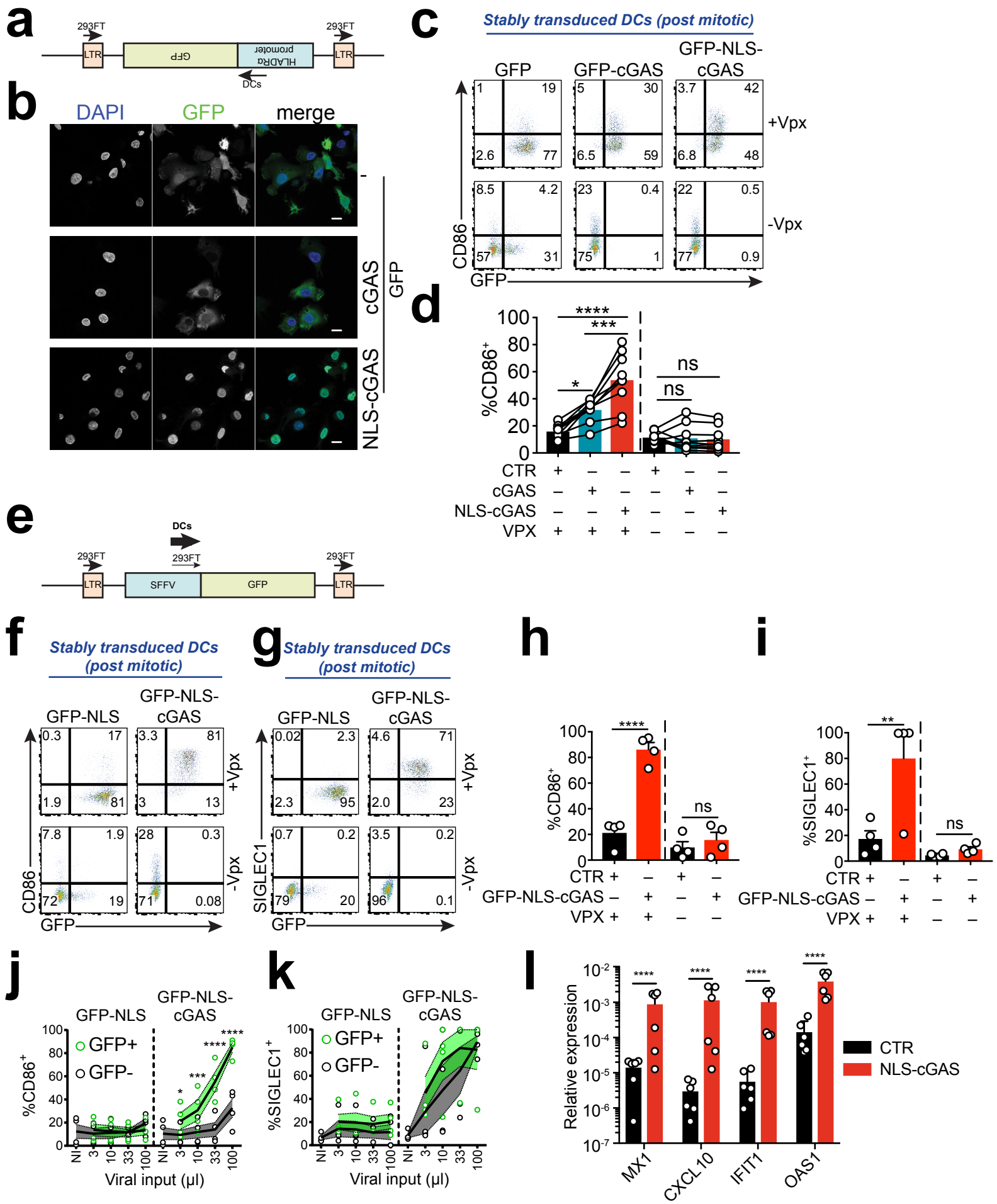


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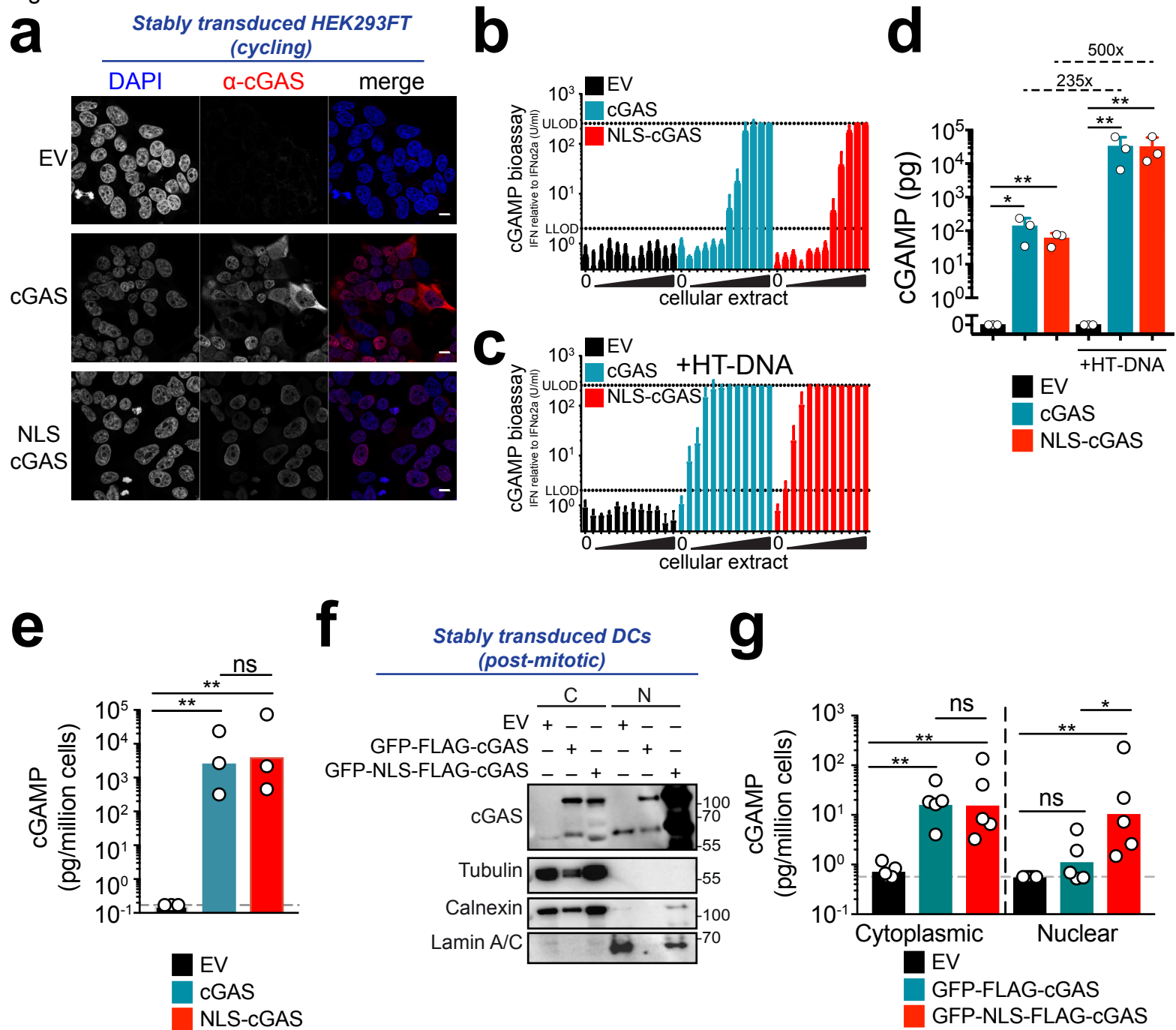


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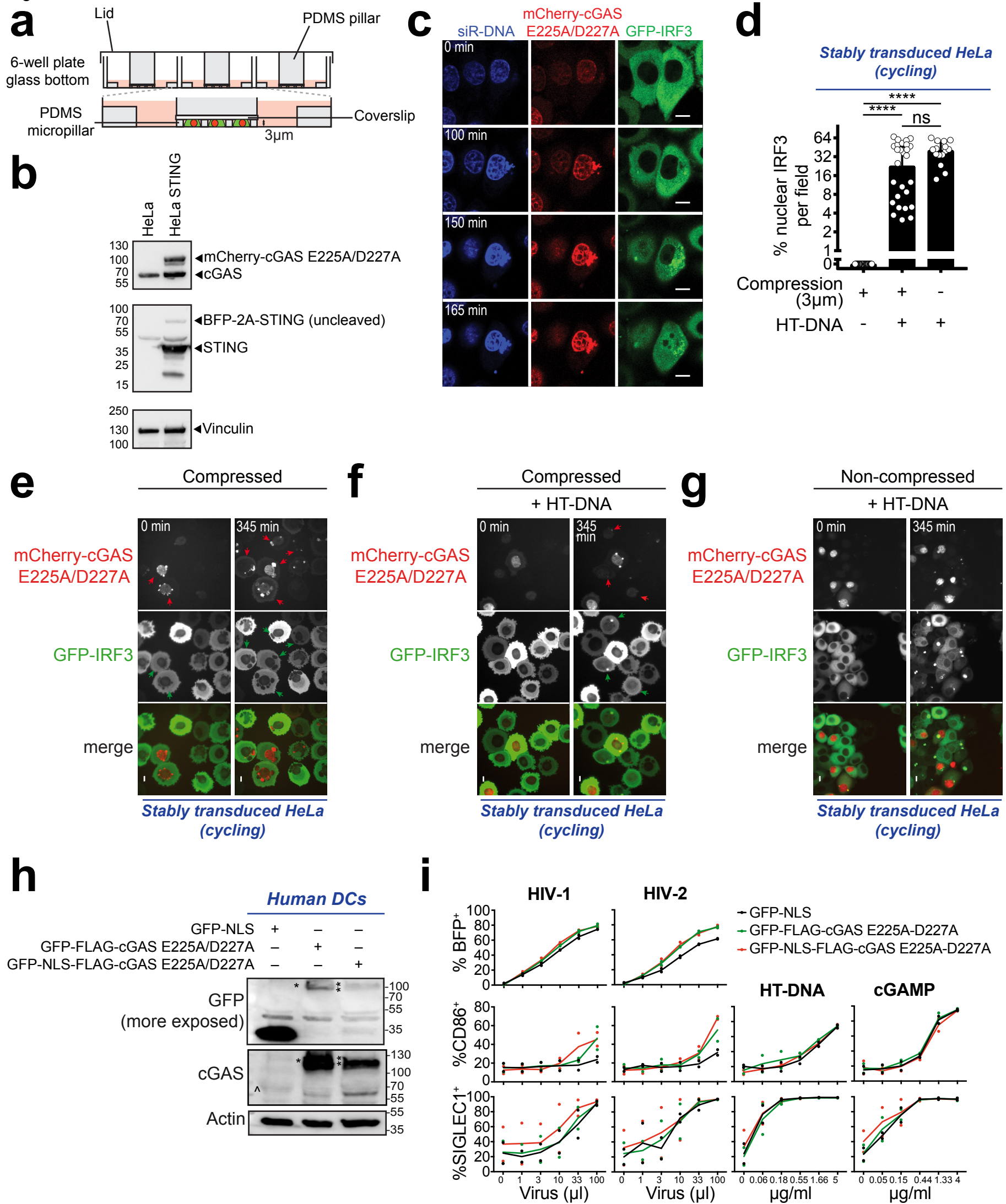


Figure 5

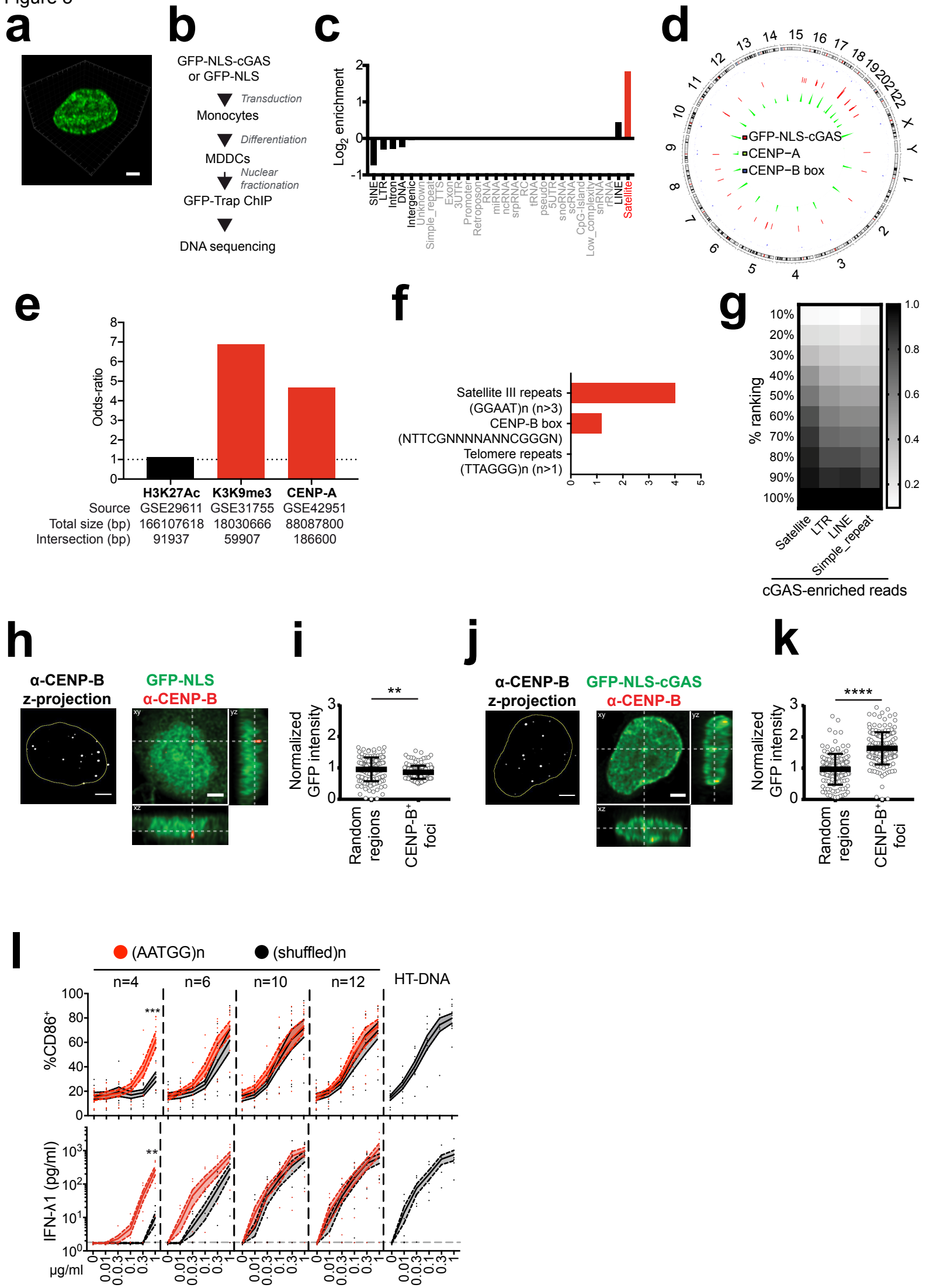
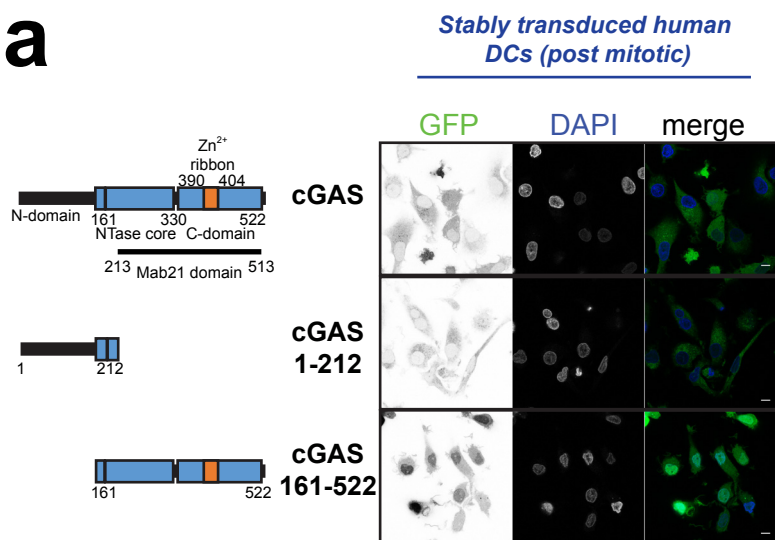
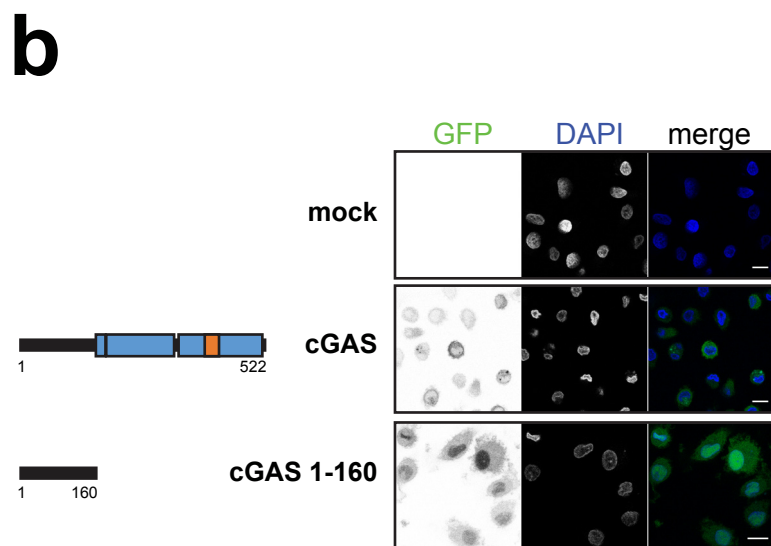


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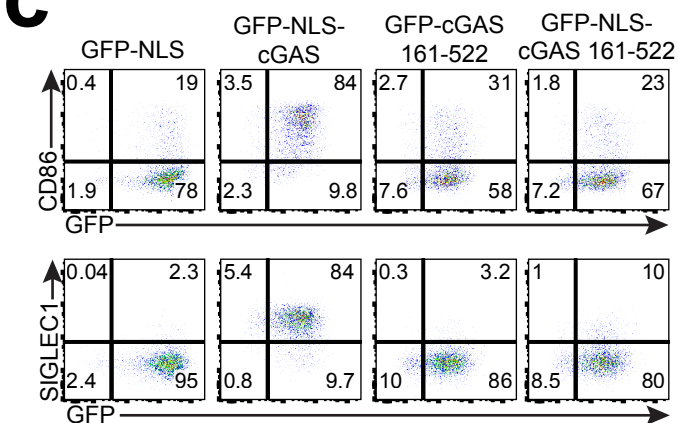
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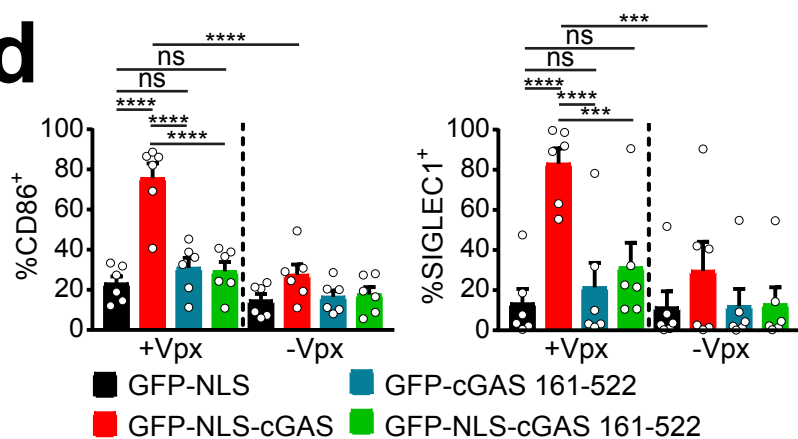
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c

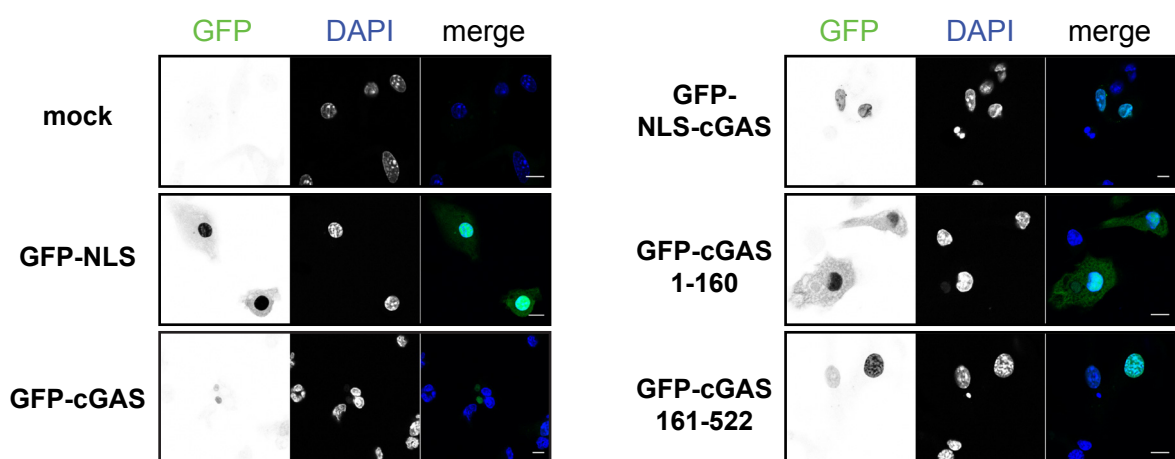


d

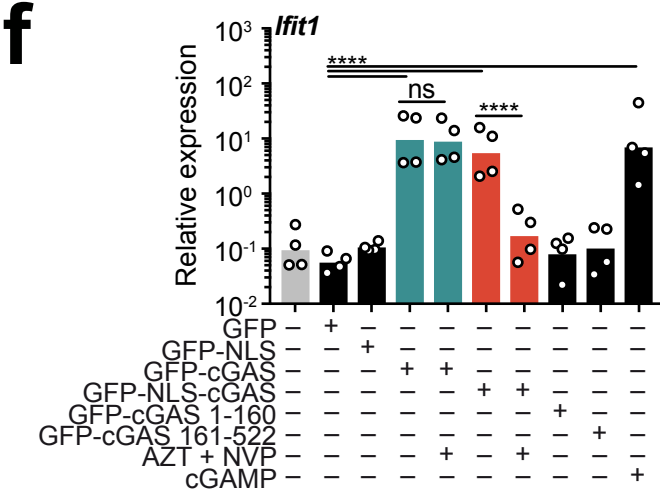


e

Mouse Cgas^{-/-} DCs



f



g

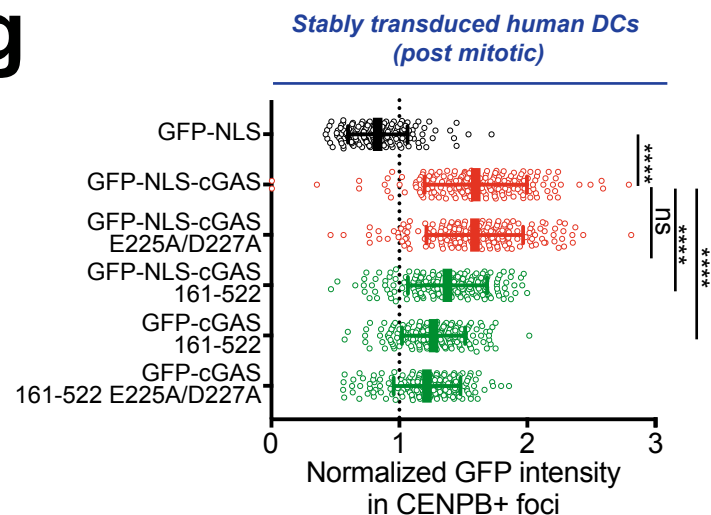
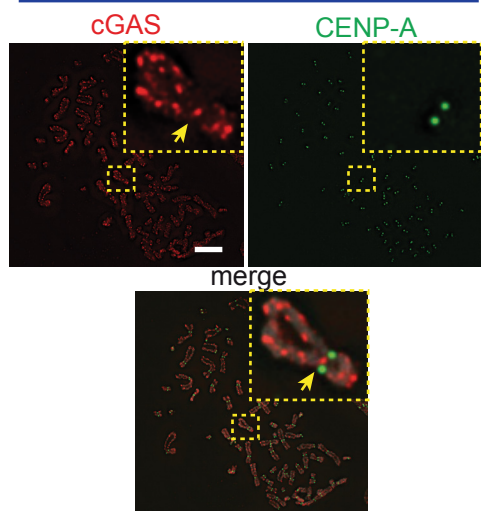
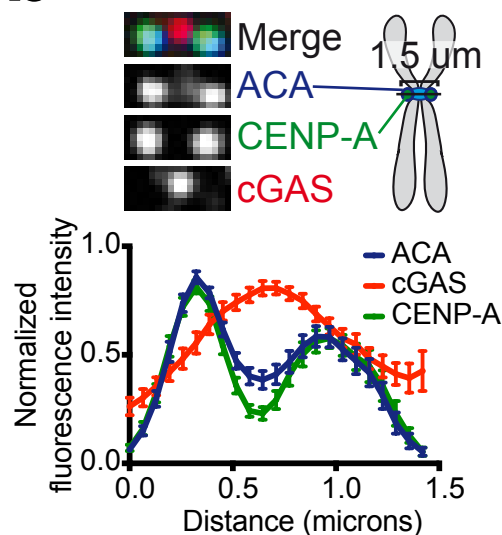


Figure 7

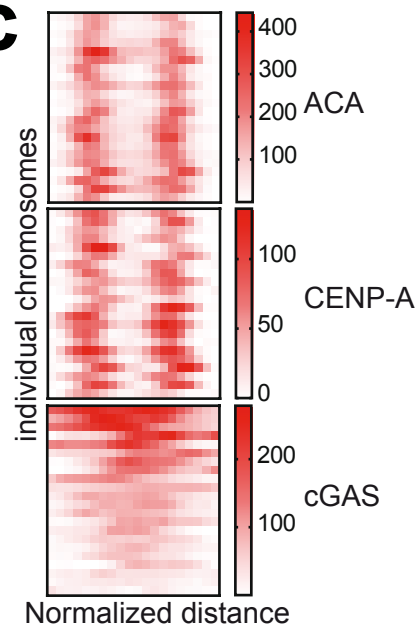
a *endogenous cGAS in U2OS (cycling)*



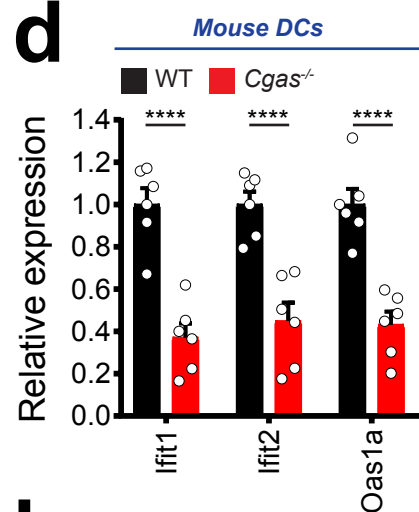
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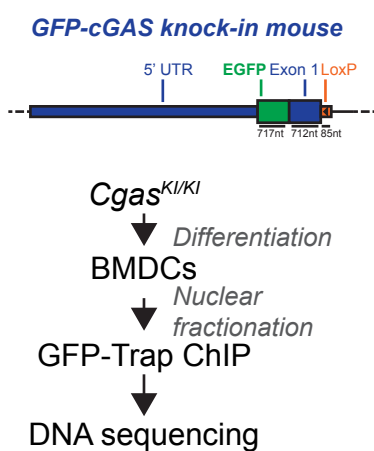
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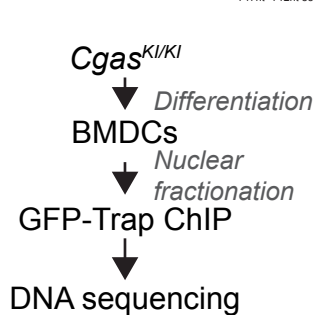
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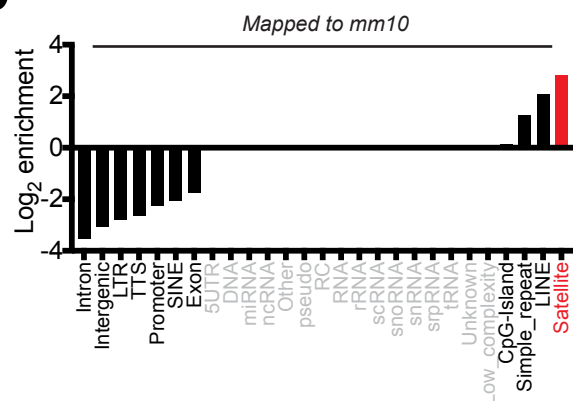
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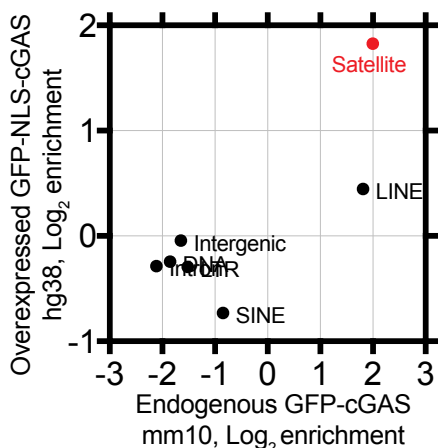
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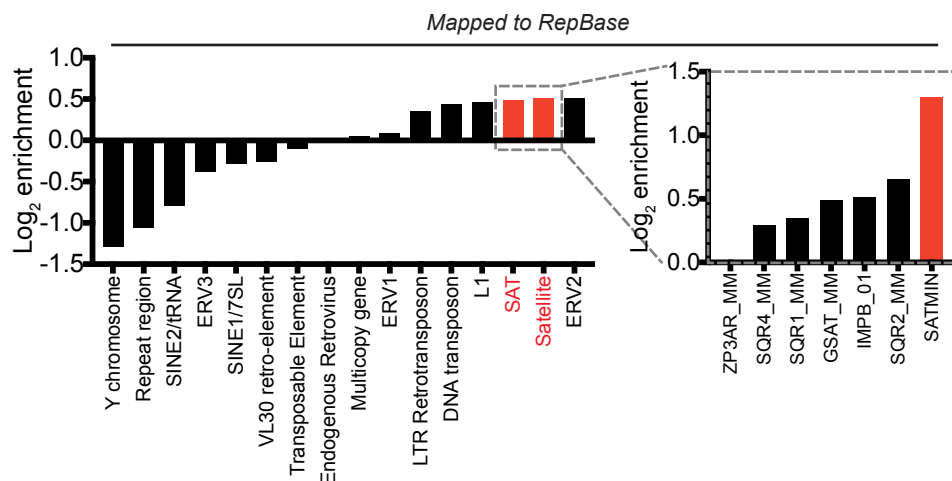
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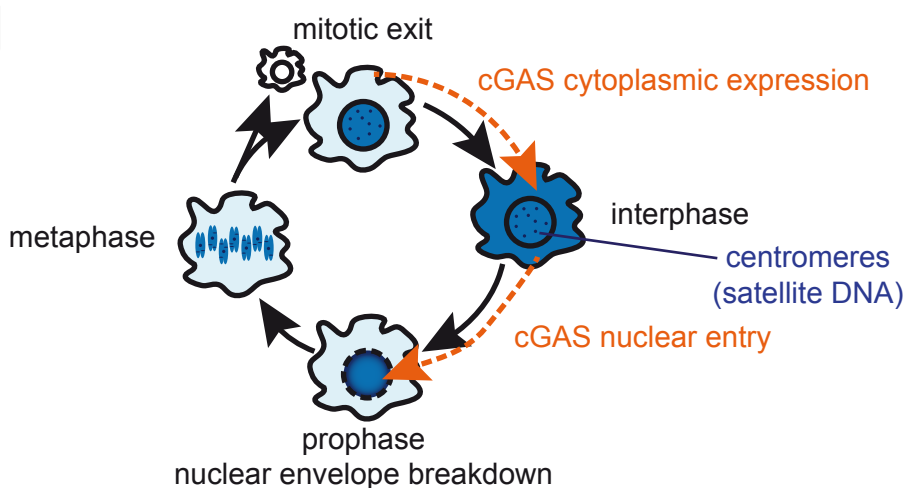
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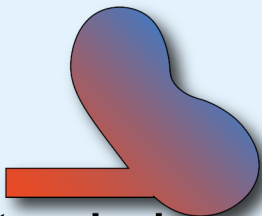
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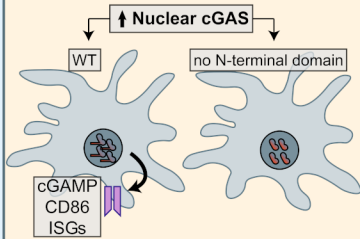


cGAS

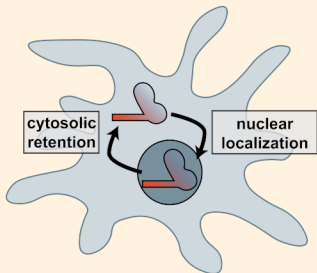


N-terminal domain

Innate immune activation in the nucleus



Nuclear/cytoplasmic localization



Centromeric DNA association

