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## Hypoxia differently modulates the release of mitochondrial and nuclear DNA

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# 1            **Hypoxia differently modulates the release of mitochondrial and nuclear DNA**

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**Abstract (words: 199/200)**

Background: We investigated the influence of hypoxia on the concentration of mitochondrial and nuclear cell-free DNA (McfDNA and NcfDNA, respectively).

Method: By an ultra-sensitive qPCR-based assay, McfDNA and NcfDNA were measured in the supernatants of different colorectal cell lines, and in the plasma of C57/Bl6 mice engrafted with TC1 tumor cells, in normoxic or hypoxic conditions.

Results: Our data when setting cell culture conditions highlighted the higher stability of McfDNA as compared to NcfDNA, and revealed that cancer cells released amounts of nuclear DNA equivalent to the mass of a chromosome over a 6-hour duration of incubation. In cell model, hypoxia induced a great increase in NcfDNA and McfDNA concentrations within the first 24 hours. After this period, cfDNA total concentrations remained stable in hypoxia consecutive to a decrease of nuclear DNA release, and noteworthy, to a complete inhibition of daily mitochondrial DNA release. In TC1-engrafted mice submitted to intermittent hypoxia, plasma NcfDNA levels are much higher than in mice bred in normoxia, unlike plasma McfDNA concentration that is not impacted by hypoxia.

Conclusion: This study suggests that hypoxia negatively modulates nuclear and particularly mitochondrial DNA releases in long-term hypoxia, and revealed that the underlying mechanisms are differently regulated.

**Keywords:** Hypoxia, Cancer, nuclear cfDNA, mitochondrial cfDNA.

## Background

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Hypoxia plays a key role in tumor progression, cell differentiation and metabolism, as well as in cell death and survival (1–3). The adaptation of cancer cells to the hypoxic tumor microenvironment is critical to their survival and proliferation (4). At the molecular level, cancer cells modify their metabolism, promote aerobic glycolysis, and decrease mitochondrial oxidative phosphorylation, independent of cellular oxygenation levels (5). This process is known as the Warburg effect (6–8). Since that discovery, many studies demonstrated the impact of hypoxia on tumor progression, revealing a number of critical effects on angiogenesis (9), metastatic potential (10), protein synthesis(11), nuclear and mitochondrial DNA replication (12,13). Moreover, the aberrant angiogenesis and rapid cell proliferation create tumor areas with different degrees of hypoxia (14). Studies have shown that hypoxic tumor cells are more resistant to radiotherapy (15) and to many commonly used chemotherapeutic agents (16). In clinical oncology, the presence of hypoxic cells in solid tumors is closely associated with a poor prognosis and survival in patients with many types of cancer (17–19). Epidemiological studies have associated higher mortality and increased cancer aggressivity in patients with intermittent hypoxia due to obstructive sleep apnea (20,21). Moreover, recent studies of the plasma of mice under intermittent hypoxia have shown a correlation between oxygen deprivation in the tumor microenvironment and the presence of circulating cell-free DNA (cfDNA) (22,23). Interestingly, several reports have demonstrated the significant potential of cfDNA analysis in clinical oncology (24). For instance, cfDNA detection can allow the identification of minimal residual disease (25,26), the monitoring of drug efficacy or resistance (27–29), and prognosis of the disease (30,31). The potential of circulating cfDNA analysis was further shown in a recent analysis we performed on a large number of plasma samples taken from metastatic colorectal cancer

68 (mCRC) patients, in which we compared the plasmatic concentrations of circulating cfDNA  
69 of nuclear (NcfDNA) and mitochondrial (McfDNA) origins. This analysis showed  
70 significantly higher NcfDNA concentrations in mCRC patients as compared to healthy  
71 subjects, while McfDNA concentrations were lower (32–35). However, our understanding of  
72 the structure and origins of cfDNA, of their pharmacokinetics, and of the correlation between  
73 DNA release and tumor biology in different physiological states, especially hypoxia, remains  
74 limited (36). The aim of the present work is to study the effect of hypoxia on the release of  
75 DNA of both nuclear and mitochondrial origins. First, we set the appropriate cell culture  
76 conditions for optimizing biological observations, in particular extracellular cfDNA stability  
77 and accumulation with incubation time. Thereafter, we determined the level and kinetics of  
78 the DNA release in normoxic and hypoxic conditions in cancer cell lines. We also used an *in*  
79 *vivo* autologous model to evaluate the amount of circulating NcfDNA and McfDNA in the  
80 plasma of mice engrafted with tumor cells, which were subjected to normoxia or intermittent  
81 hypoxia as previously performed by our group (22).

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## Methods

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### **Cell culture and cell lines**

Human colorectal cancer cell lines (SW620, SW480 and HCT116) were cultured in RPMI-1640 medium (Wako, Osaka, Japan). DiFi and DLD1 cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM Life Technology, UK). All media were supplemented with 10% of fetal bovine serum (FBS, Eurobio) and 100µg/ml of penicillin-streptomycin (Gibco™). For normoxic conditions, the cells were classically incubated at 37°C in humidified atmospheric air with 5% CO<sub>2</sub> addition. For hypoxic incubation, cells were placed in an incubator upon 0.5% O<sub>2</sub>, 94.5% N<sub>2</sub> and 5% CO<sub>2</sub>. Cell lines were routinely tested for mycoplasma contaminations.

### **Tumor animal models**

C57BL/6J male mice (7 weeks old) were acquired from Jackson Laboratories (Bar Harbor, ME). Twelve mice were placed in designed environmental chambers and subjected to intermittent hypoxia (IH), in alternating cycles of 90 seconds (6% FIO<sub>2</sub> followed by 21% FIO<sub>2</sub>, 20 cycles/h) for 12 h/d. A control group (n=9) was exposed to continuous circulating room air (RA). After 2 weeks of exposure, 1.5x10<sup>5</sup> TC-1 cells (ATCC, Manassas, VA 20110 USA) were suspended in 200 µl sterile PBS and injected into the middle right flank of each animal that were then exposed to the same protocol (RA or IH) during 4 weeks. The tumor growth was monitored three times per week using a calibrated caliper. Carbon dioxide (CO<sub>2</sub>) gas was used for euthanasia, and the depth of anesthesia was monitored by toe pinch followed by cervical dislocation as per the institutionally approved ethical protocol. These procedures were always conducted between 9:00-11:00 am in specially designated surgical room within the institutional vivarium. At the time of sacrifice (28 days after tumor injection), blood was

109 collected from the tail, tumors were harvested for volume and weight measurements, and  
110 processed for histologic and biochemical analyses. All experimental procedures were  
111 approved by The Institutional Animal Care and Use Committee of the University of Chicago  
112 (protocol# 2190). Plasma samples were sent to IRCM (France) for blind cfDNA  
113 measurements.

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### 115 **Sample treatment and cfDNA extraction**

116 All methods were performed according to the pre-analytical guidelines previously established  
117 by our group (37). Supernatants from cultured cancer cell lines or mice plasma were harvested  
118 in Eppendorf tubes, centrifuged to remove all cells (1,200g; 10min), and then frozen until use.  
119 After thawing, samples were centrifuged at 16,000g for 10 minutes at 4°C to remove cellular  
120 debris and organelles (38,39). The supernatants were then transferred to 1.5ml Eppendorf  
121 tubes and immediately extracted using the Qiagen Blood Mini Kit (Qiagen, CA), according to  
122 the manufacturer's protocol. DNA was eluted from the column with 80µL of elution buffer.

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### 124 **Measurement of cfDNA concentration by qPCR and evaluation of MNR and DNA** 125 **integrity.**

126 Table 1 describes the primers used to selectively amplify human and murine DNA sequences  
127 of nuclear and mitochondrial origins. The design of the primer system was performed under a  
128 stringent selective process. Quantifications of human NcfDNA and McfDNA were performed  
129 from the amplification of a 67bp sequence on the *KRAS* gene (24,31) and a 67bp sequence on  
130 the *MT-COX-3* gene (short nuclear and mitochondrial amplicons respectively) (32).  
131 Fragments of 305bp and 296bp (long nuclear and mitochondrial amplicons, respectively)  
132 were amplified to evaluate DNA fragmentation by calculating the DNA Integrity Index (DII)  
133 (the ratio between long and short amplicon quantifications) (40,41). Theoretically, the lower

134 the DII, the more cfDNA is fragmented. The Mitochondrial to Nuclear Ratio (MNR)  
135 corresponds to the ratio of the concentration of the McfDNA to that of NcfDNA, each  
136 calculated via short amplicon quantification. In order to quantify the murine circulating  
137 cfDNA, primers targeting murine *KRAS* (61bp for short amplicon, 146bp for long amplicon)  
138 or murine *COX-1* (114bp for short amplicon) were used for NcfDNA and McfDNA  
139 quantifications respectively. Quantitative PCR (q-PCR) was performed in a final reaction  
140 volume of 25 $\mu$ l, which was composed of 12.5 $\mu$ l of PCR mixture (Bio-Rad iQ SYBR Green  
141 Supermix), 2.5 $\mu$ l of each amplification primer (3 $\mu$ mol/ml), 2.5 $\mu$ l of PCR-quality water, and  
142 5 $\mu$ l of DNA sample. Real-time q-PCR was performed as follows: hot polymerization  
143 activation-denaturation, performed for 3 minutes at 95°C, followed by 40 repeated cycles at  
144 95°C for 10 seconds and then at 60°C for 30 seconds. After amplification, melting curves  
145 were generated by increasing the temperature from 60°C to 90°C in increments of 0.2°C, to  
146 confirm the specificity of the PCR product. Human McfDNA concentrations were calculated  
147 in ng/ml, using an internal standard curve composed of serial dilutions of a 3382bp human  
148 plasmid vector containing a unique MT-CO3 gene sequence (ABM good®). The  
149 concentration and the purity of the vector solution were determined by measuring the optical  
150 density with a BioPhotometer® D30 spectrophotometer (Eppendorf). Human NcfDNA  
151 concentrations were calculated using an internal standard curve composed of serial dilutions  
152 of genomic DNA from human colorectal cancer cells previously quantified by a  
153 BioPhotometer® D30 spectrophotometer, and expressed as ng/ml. The murine genomic DNA  
154 from Promega was used to produce a standard curve. Concentrations of the standard curve  
155 were expressed as ng/ml, and were used to determine the concentration of NcfDNA and  
156 McfDNA in the murine plasma. Mean values were calculated from triplicate reactions, and  
157 internal negative controls with PCR-quality water were routinely used.

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159 **Stability of extracellular cfDNA and evaluation of daily released DNA**

160 Supernatants from DLD1 and SW620 cells, previously cultured during 24h in T25 flasks (1  
161 million cells in 10ml culture medium) were harvested after a 24-hour period of culture,  
162 centrifuged and then placed in an incubator at 37°C for 4 days, to evaluate the intrinsic  
163 stability of extracellular DNA over time independently of the cells. Each day, DNA was  
164 extracted from 400µl of supernatant and McfDNA and NcfDNA concentrations were  
165 evaluated by qPCR as described in previous paragraph. Short and long amplicons were  
166 quantified to measure concentrations of both NcfDNA (67bp and 305bp respectively) and  
167 McfDNA (67bp and 296bp respectively). This allowed us to establish the equation necessary  
168 to estimate the amount of daily released extracellular DNA every 24 hours in the culture  
169 medium:

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$$[\text{cfDNA}]_{\text{neo-released}} = [\text{cfDNA}]_{\text{measured}} - [\text{cfDNA}]_{\text{stable}}$$

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172 **Statistical analysis**

173 Data are expressed as the mean (+/-) standard deviation. A non-parametric statistical Mann-  
174 Whitney U test was used to compare different groups of mice (Graph Pad Prism Software  
175 V7). Correlations were assessed using Spearman's non-parametric method. In all figures,  
176 statistical analyses were performed at the conventional significant p-value (p): \*p ≤ 0.05, \*\*p  
177 ≤ 0.01, \*\*\*p ≤ 0.001, \*\*\*\*p ≤ 0.0001.

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## Results

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### **NcfDNA and McfDNA stability in supernatants from DLD1 and SW620 cell cultures**

As shown in figures 1a (DLD1 cell line) and 1b (SW620 cell line), the concentration of NcfDNA, measured via the detection of the long nuclear amplicon (black circles, full lines), dropped rapidly at D1: only 4% (DLD1 cells, fig.1a) and 7% (SW620 cells, fig.1b) of the concentration measured at D0 could be recovered. On the other hand, NcfDNA, measured via the detection of the short amplicon (black squares, dashed lines), remained relatively stable from D1 to D3 after a loss of 40% between D0 and D1, in DLD1 (fig.1a) or SW620 (fig.1b) supernatants. Likewise, McfDNA concentrations (67bp and 296bp amplicons) decreased between D0 and D1 (40% loss), but then remained stable after D1. This stability was highlighted by the mitochondrial DII (DNA Integrity Index) value, which remained constant at 0.5 (fig.1c, dashed lines), in both cell lines. These profiles should be compared to the significant decrease of nuclear DII from 0.4 at D0 to 0.01 at D3 (fig.1c, full lines). Noteworthy, MNR values, calculated from short amplicons measurements, remained constant throughout the study delay, whatever the cell line (fig.1d). Thus, we show the high stability of short extracellular DNA amplicons of nuclear and mitochondrial origins in cell culture medium in the absence of cells: 60% of NcfDNA and McfDNA were recovered after 24-hour incubation (as compared to D0 of incubation), and 40% of this amount is recovered after 48h of incubation. These results are of importance and will be used to calculate the daily release of DNA in normoxic vs hypoxic conditions.

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### **Release of DNA in the supernatant of colorectal cancer cell lines**

In order to address the question of the impact of the cell type on DNA release, different colorectal cancer cell lines (DiFi, HCT116 or SW620) were seeded at different concentrations

204 (25,000, 100,000 or 400,000 cells per well). After 6-hour of incubation, culture media and  
205 cells were collected and processed in order to quantify NcfDNA and McfDNA by qPCR. As  
206 shown in figure 2, levels of NcfDNA (fig.2a) and McfDNA (fig.2b) increased with the  
207 number of starting cells, regardless of the cell line tested. The same observation can be made  
208 regarding intracellular DNA (evaluated with the same qPCR protocol as the supernatants),  
209 which showed increased amounts of genomic DNA (fig.2c) and mitochondrial DNA (fig.2d)  
210 in cell pellets. Noteworthy, we also observed that the proportion of cfDNA as compared to  
211 intracellular DNA is fairly constant, whatever the cell line: between 0.6% and 2.8% of nuclear  
212 DNA was released from cells in 6 hours (fig.2e). Lower proportions of mitochondrial DNA  
213 (from 0.14 to 0.22% of total mitochondrial DNA) were found in the supernatants, as  
214 compared to nuclear cfDNA. Finally, we were able to confirm the remarkable stability of the  
215 MNR parameter in cell supernatants, regardless of the number of cells (fig.2f). It is also  
216 interesting to note that the MNR value varied according to cell type: due to a lower  
217 mitochondrial DNA release, the SW620 cell line showed relatively lower MNR values in the  
218 extracellular medium, compared with the DiFi and HCT116 cell lines.

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### 220 **Hypoxia effect on the release of DNA in SW620 cancer cell lines**

221 After seeding SW620 cells for 24 hours, the culture medium was replaced with a fresh  
222 medium and cells were cultured either in normoxic conditions or hypoxic conditions.  
223 Sequential aliquotes of the same supernatant were sampled during 3 days (D1 to D3). In  
224 normoxic condition, the total concentrations of NcfDNA and McfDNA increased during the 3  
225 days of incubation, from 2.5 to 15.4 ng/ml ( $p=0.0002$ ) and from 0.05 to 0.14 ng/ml  
226 ( $p=0.0194$ ) respectively (fig.3a and 3b, normoxia panels). Under hypoxic conditions, total  
227 NcfDNA concentration was significantly higher in the first day of hypoxia (5 times greater  
228 than under normoxic conditions;  $p=0.01$ ), and stabilized over time at 16.2 ng/ml at D3 (fig.3a,

229 hypoxia panel). The total McfDNA concentration increased significantly in the first day of  
230 hypoxia (25 times greater than under normoxia;  $p=0.004$ ), and decreased slightly throughout  
231 the period of the study (1.37 to 0.77 ng/ml; Fig. 3b,  $p=0.0436$ ). When examining DNA release  
232 within one day, different cell behaviors can be distinguished. Indeed, thanks to the results  
233 described in figure 1, we know that the concentrations of cfDNA measured at D2 include 60%  
234 of DNA released during the [0-24h] period, while cfDNA measured at D3 include 60% of  
235 DNA released during the [24h-48h] period and 40% of DNA released during the [0-24h]  
236 period. Thus, the daily release of DNA can be calculated on the basis of the following  
237 algorithm:

$$238 \text{ cfDNA}_{[0-24h]} = \text{cfDNA}_{D1}$$

$$239 \text{ cfDNA}_{[24-48h]} = \text{cfDNA}_{D2} - 60\% \text{ cfDNA}_{[0-24h]}$$

$$240 \text{ cfDNA}_{[48-72h]} = \text{cfDNA}_{D3} - 60\% \text{ cfDNA}_{[24-48h]} - 40\% \text{ cfDNA}_{[0-24h]}$$

241 Consequently, under normoxic conditions, daily nuclear and mitochondrial DNA releases  
242 account respectively for 70% and 65% of the total concentrations measured at D2, and for  
243 80% and 62% of the total concentrations measured at D3 (fig.3c and 3d, normoxia panels).  
244 Under hypoxia, daily release of nuclear DNA decreased down to 50% of the total NcfDNA  
245 concentration at D3, while daily mitochondrial DNA release dropped below 0.1% of the  
246 initial concentration by D3 (fig.3c and 3d, hypoxia panels). This difference of profile between  
247 normoxia and hypoxia is also illustrated in figures 3e and 3f. The nuclear DII remained low  
248 under hypoxia during the 3 days of the study (0.03 to 0.14; black triangles; fig.3e), as  
249 compared to normoxia (0.299 to 0.48; white squares; fig.3e). MNR also progressively  
250 decreased from 0.09 down to 0.04 in hypoxia, while remaining very low in normoxia (from  
251 0.008 to 0.018; fig.3f).

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253 **Hypoxia influences tumor weight and plasma cfDNA levels in grafted mice**

254 Twenty one C57BL/6j mice engrafted with murine TC1 cells were used for *in vivo*  
255 experiment; nine of these mice were exposed to normoxia, while twelve were exposed to  
256 intermittent hypoxia according to the experimental procedure depicted on figure 4a. Plasmatic  
257 circulating cfDNA concentrations were assessed in both conditions. Data showed a significant  
258 increase of the NcfDNA concentration in plasma from mice under intermittent hypoxia as  
259 compared to normoxia (fig.4b,  $p= 0.0142$ ). Conversely, McfDNA concentration was slightly  
260 decreased (but not significantly; fig.4c,  $p= 0.1886$ ). As a result, intermittent hypoxia greatly  
261 reduced the MNR in the plasma of grafted mice (fig.4e,  $p=0.0022$ ). In contrast, no statistical  
262 difference was observed for nuclear DII (fig.4d,  $p= 0.2861$ ).

263 As shown in figure 4f, intermittent hypoxia increased tumor weight ( $p=0.0001$ ). Conversely,  
264 its impact on mice growth (delta body weight, dBWT) was slightly negative (fig.4g;  
265  $p=0.0359$ ). Correlations between tumor weight and other parameters were assessed in the  
266 grafted mice: NcfDNA is positively correlated to tumor weight (supplementary fig.1a;  
267  $r=0.5992$ ;  $p=0.0041$ ), whereas MNR is negatively correlated to tumor weight (supplementary  
268 fig.1d;  $r=-0.5054$ ;  $p=0.0194$ ). McfDNA and nuclear DII are not significantly correlated to  
269 tumor weight (Supplementary fig.1b and 1c respectively).

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## Discussion

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DNA molecules of nuclear and mitochondrial origins are found in the extracellular compartment, *in vitro* in the media of cell culture (42,43), and *in vivo* in the physiological fluids (44,45). While the analysis of circulating DNA from plasma is now optimized and standardized (37), only poor experimental works have been performed with using cell culture. To accurately observe the effect of hypoxia on cells, we first carried out a study on standardizing cell culture conditions to avoid any bias. We first examined the stability over time of NcfDNA concentration, as detected via the amplification of a short DNA sequence compared to a long DNA sequence to further estimate the DNA integrity (DII) as it was performed *in vivo* (41). The biphasic shape of the NcfDNA concentration curves suggests that at least two structural entities of different stability coexist in the pool of fragments over 67bp. In contrast, fragments over 305bp highly decrease with time of incubation down to nearly disappear after a few days of incubation. Thus, we can conclude that fragments of NcfDNA over 305bp appear less stable than those higher than 67bp in these *in vitro* conditions (almost 40% being still present following 2 days incubation in cell culture supernatant). We previously showed that targeting a sequence as short as possible enables to improve and consequently to accurately determine cfDNA concentration. The decrease of nuclear DII observed in our stability study, corresponding to the degradation of NcfDNA into fragments between 67bp and 305bp, can be explained by the presence of nucleosomal structures or transcription factor-associated complexes, which constitute 90-98% of the fragments detected in CRC patients (46). Conversely, McfDNA concentration is less impacted by incubation time, regardless of the targeted sequence length. This result indicates structural differences between nuclear and mitochondrial DNA in cell culture supernatant. McfDNA appears more stable as compared to NcfDNA, certainly due to different structural complexes, not yet

297 characterized. Our group has recently demonstrated that cell culture medium could contain  
298 cell-free circulating respiratory-competent mitochondria or cell membrane- or debris-  
299 associated mitochondria (47), what could be one of the reason why McfDNA seems protected.  
300 Noteworthy, the ratio between McfDNA and NcfDNA concentrations, which corresponds to  
301 the MNR, is highly stable and reproducible in cell-free culture medium. This observation is  
302 confirmed in cell model, as MNR, although varying according to the cell line, is constant  
303 regardless of the number of cells. This result indicates that MNR could be considered as a  
304 relevant biomarker, as it appears less influenced by collateral factors such as DNA  
305 conservation or cell number (34).

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307 In cancer cell models, our data revealed a rapid and significant release of DNA into the  
308 culture medium. A rapid release of nuclear DNA was also reported by Wang et al (48) in T47-  
309 D (80ng/ml) and MDA-MB-231 (12ng/ml) breast cancer cells over a short period of time (6  
310 hours of incubation). In addition to this, release of nuclear DNA was also observed during the  
311 first 24 hours of incubation in the culture medium of the human 143 B osteosarcoma cell line  
312 (49). As expected, we observed in our study that NcfDNA and McfDNA concentrations  
313 increased with the number of cells, corresponding to about 2% and 0.1% of the intracellular  
314 content respectively, whatever the cancer cell lines. This difference of proportion may be  
315 explained by different mechanisms implied in either nuclear or mitochondrial DNA release. It  
316 should be noted that this 2% of NcfDNA is approximately equivalent to the extracellular  
317 release of one chromosome in 6 hours, highlighting the high genomic dynamics as well as the  
318 importance of DNA release in extracellular space, which cannot therefore be considered as a  
319 marginal mechanism. From a physiological point of view, these data are of great importance  
320 because extracellular nucleic acids have been described as biologically active in inflammatory  
321 processes, and as potentially active in cell transformation and communication (50).

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323 The study of the DNA release and stability allowed us to provide an *in vitro* experimental  
324 model avoiding bias as much as possible in the following observations. Indeed, we took into  
325 consideration the respective stability of NcfDNA and McfDNA and the accumulation bias by  
326 using an algorithm allowing to delineate the daily rate of DNA release. Hence, our data  
327 revealed that hypoxia has a critical impact on cfDNA concentration in the supernatant of  
328 SW620 human colorectal cancer cell line, as compared to normoxic conditions, within the  
329 first 24 hours of incubation. After this period of time, cfDNA concentrations did not increase  
330 further upon hypoxia. Inversely, cfDNA concentrations increase with normoxia, which  
331 allows a progressive accumulation of cfDNA in the cell supernatant. Furthermore, thanks to  
332 our stability study and the use of our algorithm, we reveal for the first time the different  
333 kinetics of nuclear and mitochondrial DNA release, revealing a biphasic behaviour of the cells  
334 submitted to hypoxia: a high release during the first 24 hours followed by a reduced release at  
335 day 2 and day 3. This effect was much more pronounced for mitochondrial DNA, the release  
336 of which is completely abrogated at day 3. This difference clearly indicates that the  
337 mechanisms implied in nuclear and mitochondrial DNA releases are different from each  
338 other.

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340 The main mechanisms of nuclear DNA release described in the literature are cell death  
341 (apoptosis and necrosis) and active secretion, in particular through microparticles release  
342 (36,51). Several structures such as exosomes, apoptotic bodies, nucleosomes and virtosomes  
343 which may contain extracellular DNA and which have been shown to be positively regulated  
344 by hypoxia, have been described (51) (52,53). Moreover, other studies have shown that  
345 increased autophagy in response to hypoxic stress allows cell survival or death (54,55). In  
346 addition, under hypoxic conditions, the endogenous antioxidant response of cells may be



347 insufficient, resulting in an intracellular increase in reactive oxygen species (ROS) (56), and  
348 the increased release of nuclear and mitochondrial DNA. It may thus be speculated that DNA  
349 release may be increased by hypoxia via exosome production, ROS or autophagy in the early  
350 steps of our study. However, when the amount of ROS of a cell is too high, the necrotic  
351 activity of p53 could be induced, therefore promoting cell death (57,58). Indeed, regulated  
352 necrosis includes multiple cell death modalities, resulting in loss of mitochondrial inner  
353 membrane potential, disruption of ATP production, mitochondrial dysfunction and  
354 consequent necrosis. This could explain our observation that mitochondrial DNA release is  
355 more affected than nuclear DNA by late incubation upon hypoxic conditions (59).  
356 Furthermore, it was shown that mitochondria undergo fission and fusion continually in  
357 response to changes in the extracellular environment: hypoxia promotes the production of  
358 reactive oxygen species (ROS) that cause an increase in mitochondrial fission (60).  
359 Noteworthy, recent studies highlighted modifications in the expression of genes involved in  
360 oxidative phosphorylation (61), linking hypoxia to mitochondrial adaption to this anaerobic  
361 environment. Under hypoxia, glucose consumption increases to maintain ATP production  
362 using less efficient anaerobic glycolysis, what induces major structural and dynamical  
363 changes characterized by impairment of fusion process that leads to mitochondrial  
364 depolarization, and finally loss of mitochondrial DNA (62,63).

365

366 Under normoxic conditions, the MNR remains stable over time. This suggests a balance  
367 between cell growth and the release of nuclear DNA and mitochondrial DNA. Conversely,  
368 hypoxia leads to an imbalance in this stability. These results suggest that the regulations of  
369 mitochondrial and nuclear releases could be valuable parameters to follow-up hypoxia in  
370 patients. To date, multiple approaches exist to detect hypoxia either directly or indirectly (64).  
371 Tumor hypoxia was shown to be a prognostic biomarker, as it was shown that extracellular

372 vesicles-associated genes could correlate with hypoxic microenvironment and predict  
373 recurrence in lung adenocarcinoma (65). Evaluating hypoxia could also be indicative for the  
374 selection of patients who would most benefit of chemo-radiotherapy (66).

375

376 The *in vivo* effect of hypoxia on the concentration of circulating NcfDNA and McfDNA was  
377 evaluated in the plasma of mice engrafted with an autologous lung epithelial tumor model  
378 (TC1) cell line. As would be expected from our *in vitro* results, plasmatic NcfDNA  
379 concentration is highly correlated to tumor weight and size, both of which are increased in  
380 mice bred in a hypoxic atmosphere, as has been observed in previous study (23). Noteworthy,  
381 circulating McfDNA is weakly related to hypoxia in the mice model, as its plasmatic  
382 concentration is not significantly different in both groups of mice. As a result, McfDNA is not  
383 correlated to tumor weight. This result is in phase with the *in vitro* model, since we found that  
384 after a rapid and huge increase of its concentration in extracellular medium, mitochondrial  
385 DNA release is then negatively regulated after 24 hours of incubation, and to a higher extent  
386 than nuclear DNA. The lack of difference in McfDNA levels in murine plasma between  
387 hypoxia and normoxia may also be explained by the fact that, due to structural similarities  
388 with bacterial DNA, mitochondrial components, including mitochondrial DNA itself, are  
389 particularly likely to activate the immune system, leading to their degradation (67). This  
390 hypothesis should be tested by the use of immunosuppressed xenografted mice. Nevertheless,  
391 the McfDNA levels can be used to establish the MNR, which permits a more accurate  
392 discrimination between normoxic and hypoxic mice than could be done if using NcfDNA  
393 levels alone. It would therefore appear that McfDNA measurement allows a normalization of  
394 individual differences, highlighting the discriminative power of NcfDNA.

395

396 The results of our study must be interpreted within the context of their limitations and  
397 strengths. Despite colorectal cancer cell lines appear to cover the genetic, transcriptional and  
398 phenotypic constitution of cancer cells, their characteristics may be far from those of *in vivo*  
399 tumors, as most *in vitro* cell lines are derived from rapidly growing, aggressive transplantable  
400 tumor lines. Furthermore, carcinoma cells in *in vivo* models form 3D structures that interact  
401 with the surrounding extracellular matrix (ECM) and cells of the tumor stroma. These  
402 interactions have been shown to be essential for tumor development and progression, which  
403 prominently alter signaling pathways in both the tumor cells and the stromal cells (68,69).  
404 These preliminary results will need to be consolidated by analysis on a larger number of cell  
405 lines and mice. To obtain a more complete picture of these processes, it will be necessary to  
406 identify the release mechanisms in order to establish how and why, in hypoxic conditions, the  
407 high release of mitochondrial DNA is transient and subsequently repressed, while that of  
408 nuclear DNA is much less affected.

409

410 In summary, this is the first study to delineate concomitantly the effect of hypoxia on the  
411 levels of nuclear and mitochondrial cfDNA in cell and murine models. We have clearly  
412 shown that cancer cell lines release high amounts of mitochondrial and nuclear DNA in the  
413 extracellular compartment, and that hypoxia strongly influences the amount and manner in  
414 which these are released. We have also demonstrated that cells are able to regulate  
415 differentially the daily rate of release of DNA according to hypoxic conditions. In the grafted  
416 mice model, we have shown that MNR, which is correlated to tumor size and weight, may  
417 characterize the hypoxic condition of the tumor. While it does not fully recapitulate mCRC in  
418 patients, our *in vivo* study using mice engrafted with TC1 cells does suggest that analysis of  
419 MNR could be relevant in evaluating the degree of hypoxia of human tumors. Moreover, the  
420 amount of NcfDNA in plasma could be indicative of tumor size. Our study is a preliminary

421 one, but could pave the way for the development of blood tests which would evaluate cancer  
422 progression or residual disease.

423

424

### **Additional information**

425

#### **Ethics approval and consent to participate**

427 All experimental procedures on grafted mice were approved by The Institutional Animal Care  
428 and Use Committee of the University of Chicago (protocol # 72190).

429

#### **Consent to publish**

431 All authors agree with the original results described in this study and consent to publish them  
432 in the present manuscript.

433

#### **Data availability**

435 Data are available upon request to the corresponding author, Dr AR Thierry.

436

#### **Conflict of interests**

438 Authors declare no conflicts of interest.

439

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444

#### **Authors' contributions**

446 AO, JDA and ZA performed *in vitro* experiments, analyzed the results and wrote the  
447 manuscript. AK and DG performed *in vivo* experiments. IJ, TF, CP and SC contributed to the

448 design of experiments and to the manuscript writing. ART designed experiments, analyzed the  
449 results, wrote the manuscript and managed the project.

450

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456

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- 637
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639

## Legends

640

### 641 **Figure 1: cfDNA stability in cell culture media**

642 After a 24-hour incubation period of the DLD1 and SW620 cell lines (1 million cells in T25  
643 flask), medium was removed, centrifuged and incubated at 37°C in 6-well plates. The  
644 concentration of cfDNA was monitored every day (D0, D1, D2 and D3) by qPCR, targeting  
645 *KRAS* or *COX3* genes and quantifying amplicons of 67bp and 305bp from nuclear cfDNA,  
646 and quantifying amplicons of 67bp and 296bp from mitochondrial cfDNA. The evaluation of  
647 cfDNA concentrations (in percentage of D0) is shown according to the cell line (a and b); (c)  
648 mitochondrial and nuclear DII; (d) MNR. Average values with (+/-) standard deviations are  
649 represented, results having been reproduced in three independent experiments, each  
650 performed in triplicate. **Abbreviations:** cfDNA: extracellular cell-free DNA; DII: DNA  
651 Integrity Index; MNR: Mitochondrial to Nuclear DNA Ratio; *KRAS*: Kirsten rat sarcoma;  
652 *COX3*: Cytochrome c oxidase subunit 3.

653

### 654 **Figure 2: Release of cfDNA by colorectal cancer cells in culture**

655 Culture media and cell pellets from colorectal cell lines (DiFi, HCT116, SW620) were  
656 harvested after 6 hours of incubation at a different number of cells per well (25,000; 100,000  
657 or 400,000). Extracellular and intracellular DNA of nuclear (a and c, respectively) or  
658 mitochondrial origin (b and d, respectively) were measured by qPCR, as described in the  
659 Material and Method section, and estimation was made of the percentage of DNA released in  
660 culture medium as compared to intracellular DNA (e) and of MNR (f). Average values with  
661 (+/-) standard deviations are represented, results having been reproduced in four independent  
662 experiments, each performed in triplicate. **Abbreviation:** MNR: Mitochondrial Nuclear DNA  
663 Ratio.

664

665 **Figure 3: cfDNA release upon normoxic and hypoxic conditions**

666 After seeding SW620 cells for 24 hours, the culture medium was replaced with a fresh  
667 medium and cells were placed in two different incubators: one with normoxic conditions  
668 (21% O<sub>2</sub>), the other with hypoxic conditions (0.5% O<sub>2</sub>). Different aliquotes of the same  
669 supernatant were sampled during 3 days (D1 to D3, days of sample collection). Total  
670 concentrations of nuclear (a) and mitochondrial (b) extracellular cfDNA were quantified by  
671 qPCR. cfDNA[0-24] corresponds to the concentration of released DNA during the first 24  
672 hours; cfDNA[24-48] corresponds to the concentration of released DNA between the 24<sup>th</sup> and  
673 48<sup>th</sup> hour of incubation; cfDNA[48-72] corresponds to the concentration of released DNA  
674 between the 48<sup>th</sup> and 72<sup>nd</sup> hour of incubation. The proportions of nuclear or mitochondrial  
675 DNA newly released during each period, as compared to the total concentration measured  
676 each day, are shown in (c) and (d) respectively; (e) shows the nuclear DII of supernatants  
677 from cells cultured in normoxia (white square, dashed line) or in hypoxia (dark triangle, solid  
678 line); (f) shows the MNR of supernatants from cells cultured in normoxia (white square,  
679 dashed line) or in hypoxia (dark triangle, solid line). Values are presented as mean (+/-)  
680 standard error of four independent wells, each performed in triplicate. **Abbreviations:**  
681 cfDNA: extracellular cell-free DNA; DII: DNA Integrity Index; MNR: Mitochondrial Nuclear  
682 DNA Ratio.

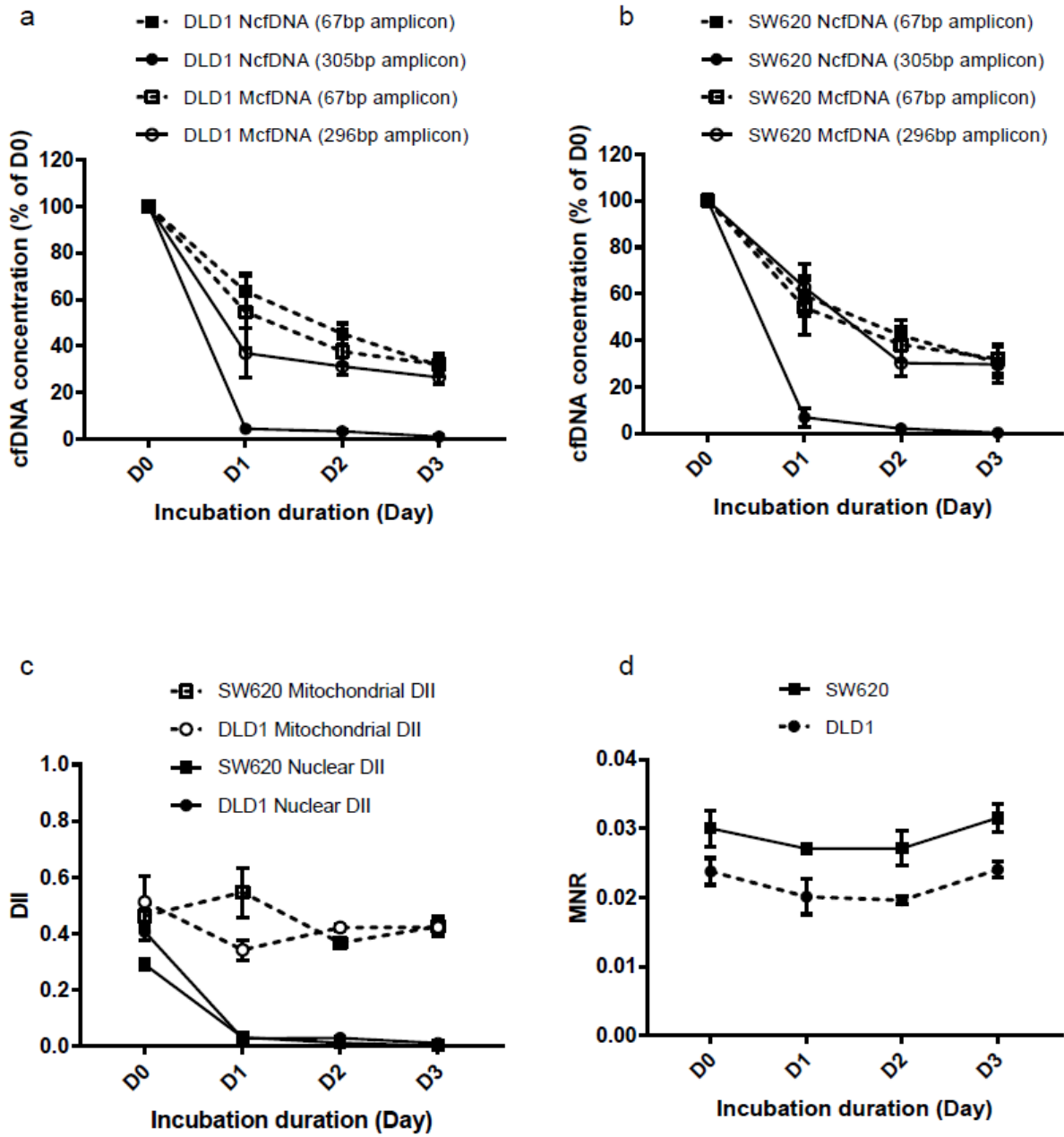
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684 **Figure 4: Effects of intermittent hypoxia**

685 (a) Flow-chart of the *in vivo* experiment: C57BL/6j mice were preexposed during 2 weeks to  
686 either room air or intermittent hypoxia, and then injected with 10<sup>5</sup> TC1 cells. Blood collection  
687 was performed 4 weeks after the graft. The concentrations of circulating cfDNA in plasma

688 from mice subjected to normoxia (open boxes) or hypoxia (gray boxes) were evaluated by  
689 qPCR by targeting murine *KRAS* (b) or *COX1* (c) genes. This allowed the evaluation of the  
690 nuclear DII (d) and the MNR (e). The effect of hypoxia on tumor weight (f) and mouse body  
691 weight gain (g) are also presented. The line from end to end of the whisker represents the  
692 minimum and maximum values of the data, the line within each box represents the median,  
693 and the lower and upper boundaries of the box indicate the first and third quartiles,  
694 respectively. Significant differences between normoxia and intermittent hypoxia were  
695 estimated using the Mann-Whitney test. P-Values significance: \*P < 0.05, \*\*P < 0.005 and  
696 \*\*\* P < 0.0005. **Abbreviations:** NcfDNA: nuclear cell-free DNA; McfDNA: mitochondrial  
697 cell-free DNA; DII: DNA Integrity Index; MNR: Mitochondrial to Nuclear DNA Ratio;  
698 dBWT: delta body weight.

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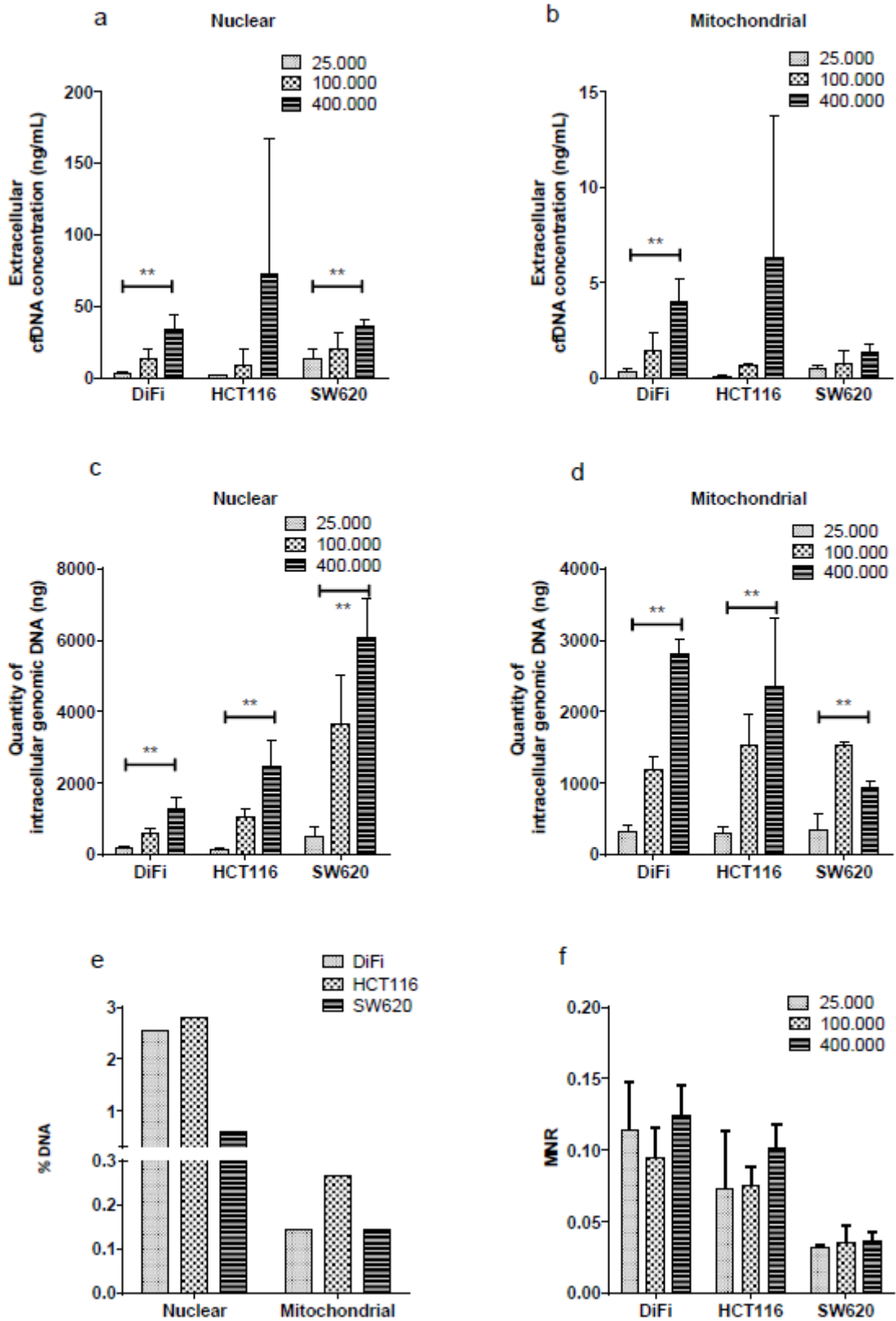
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Figure 1

Figure 2



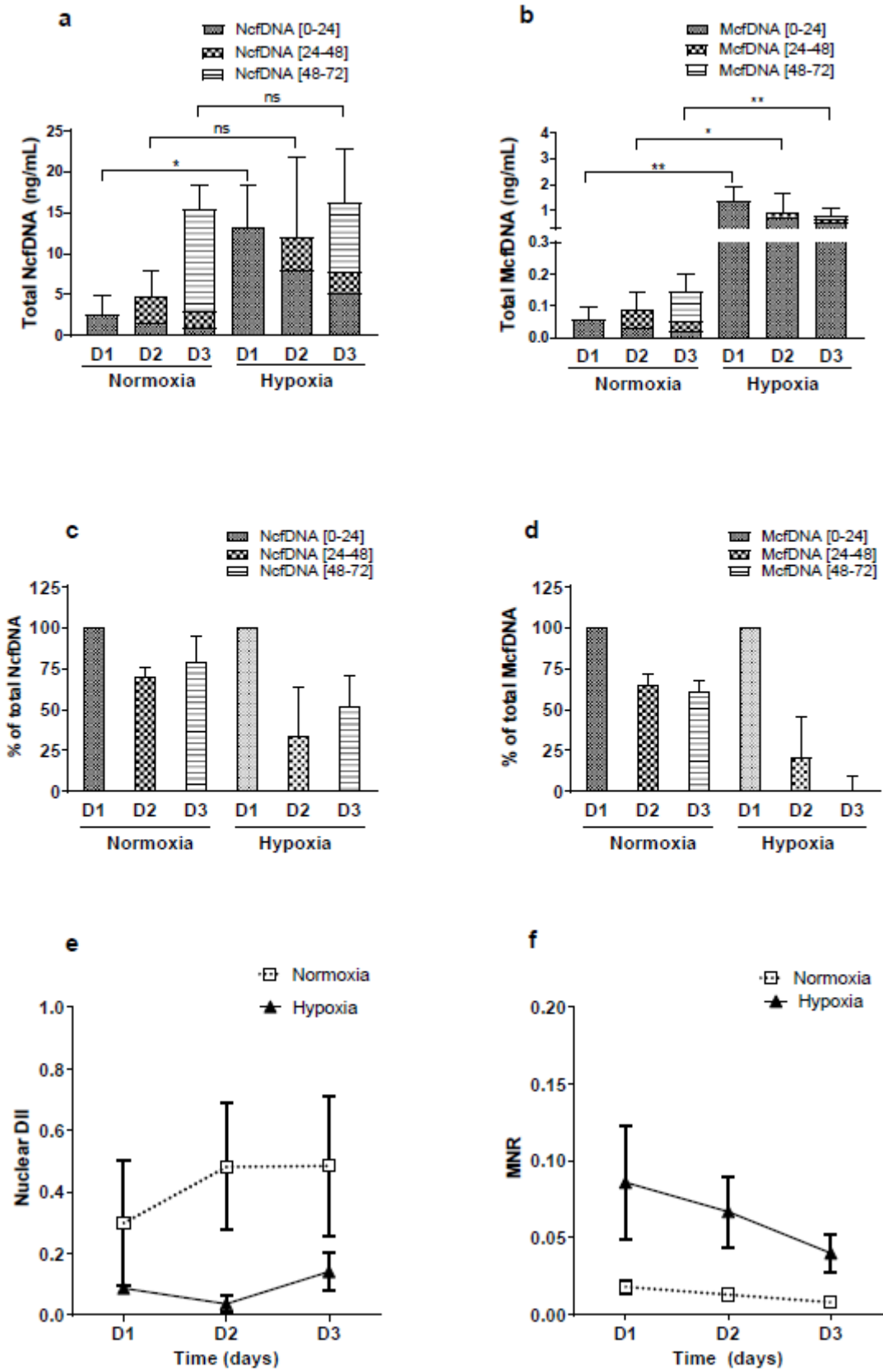
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Figure 2



**Figure 3**

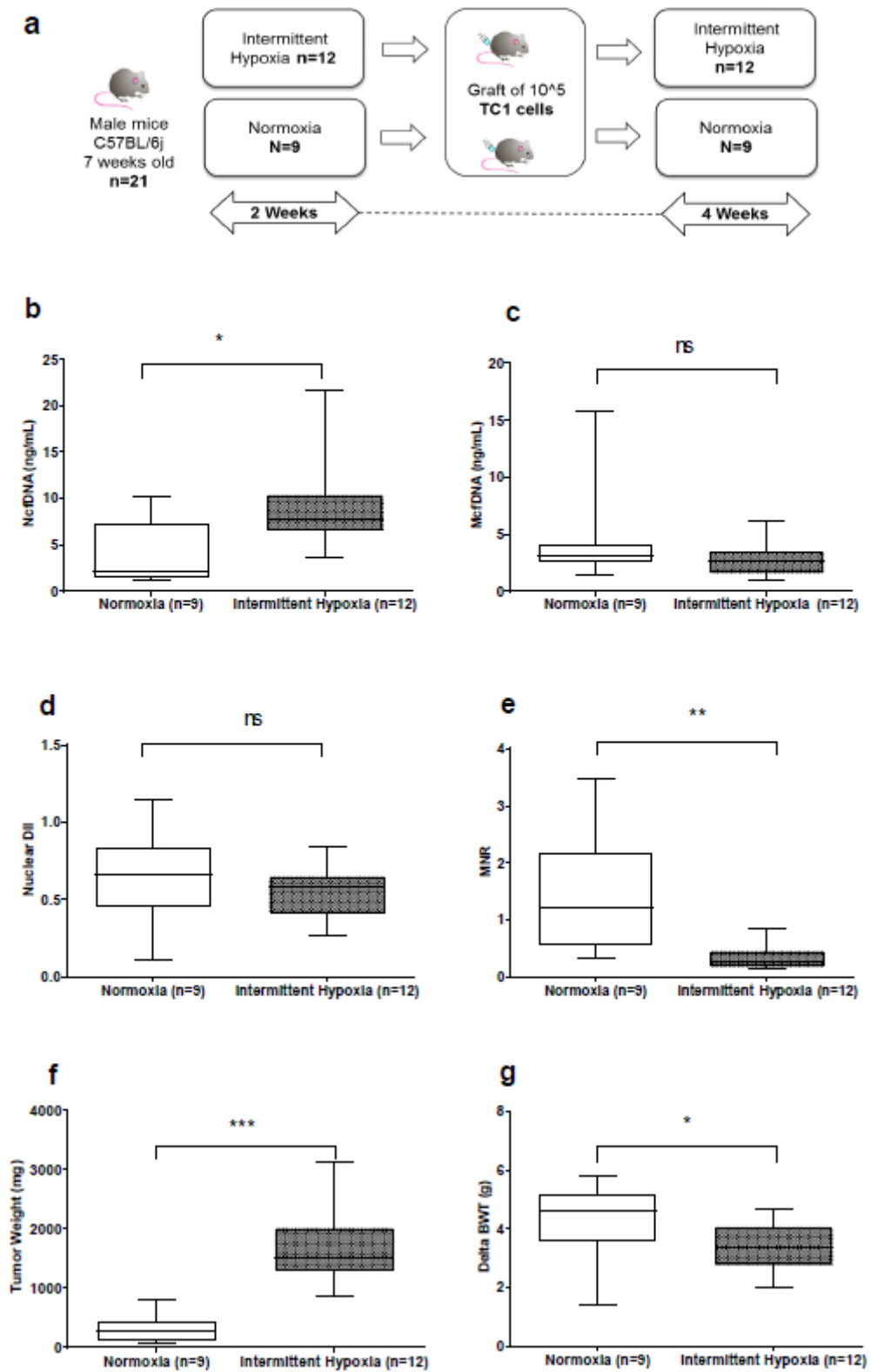


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**Figure 3**

Figure 4



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Figure 4