

# A DNA-dependent stress response involving DNA-PK occurs in hypoxic cells and contributes to cellular adaptation to hypoxia

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## Summary

DNA-dependent protein kinase (DNA-PK) is involved in DNA double-strand break (DSB) signalling and repair. We report that DNA-PK is activated by mild hypoxia conditions (0.1–1% O<sub>2</sub>) as shown by (1) its autophosphorylation on Ser2056, and (2) its mobilisation from a soluble nucleoplasmic compartment to a less extractable nuclear fraction. The recruitment of DNA-PK was not followed by activation and recruitment of the XRCC4–DNA-ligase-IV complex, suggesting that DSBs are not responsible for activation of DNA-PK. To unravel the mechanism of DNA-PK activation, we show that exposure of cells to trichostatin A, a histone deacetylase inhibitor, leads to DNA-PK autophosphorylation and localisation to DNA. Histone acetylation (mainly H3K14) is increased in hypoxic cells and treatment with anacardic acid, an inhibitor of histone acetyl transferase, prevented both histone modifications and DNA-PK activation in hypoxic conditions. Importantly, in using either silenced DNA-PK cells or cells exposed to a specific DNA-PK inhibitor (NU7026), we demonstrated that hypoxic DNA-PK activation positively regulates the key transcription factor HIF-1 and one subsequent target gene, *GLUT1*. Our results show that hypoxia initiates chromatin modification and consequently DNA-PK activation, which positively regulate cellular oxygen-sensing and oxygen-signalling pathways.

**Key words:** DNA repair, DNA-PK, Hypoxia, Stress response

## Introduction

Hypoxic stress has a pivotal role in normal human development and physiology, including embryogenesis and wound repair, and has been well studied for its importance in several human diseases, namely heart disease, stroke and cancer. The pathways involved in hypoxic adaptation that are required for normal development and are deregulated in disease states are therefore of crucial importance (Brahimi-Horn and Pouyssegur, 2007). Recent reports suggest that hypoxic stress is associated with DNA modifications whose signalling might contribute to the global hypoxic adaptation response. Severe hypoxia induces S-phase arrest, resulting in regions of single-stranded DNA in stalled replication forks and the activation of the Ataxia Telangiectasia and Rad3-related kinase ATR (Hammond et al., 2003a; Hammond et al., 2003b). Loss of ATR results in a further loss of viability in S-phase cells in hypoxic conditions (Hammond et al., 2004). ATR belongs to the phosphoinositide-3-kinase-related kinase (PIKK) family, which in eukaryotes initiates cellular stress responses when genome integrity, mRNA translation or nutrient availability is compromised (Abraham, 2004). Another member of the PIKK family, ATM (Ataxia Telangiectasia mutated), is activated by hypoxia (Bencokova et al., 2009; Gibson et al., 2005). ATM is an essential building block of the DNA-damage-signalling pathway involving the MRN (Mre11–Rad50–Nbs1) complex. Interestingly, ATM is activated in severe hypoxic conditions by an alternative mechanism independently of MRN (Bencokova et al., 2009; Gibson et al.,

2005). The histone variant H2AX (a target of PI3KKs) that participates in DNA-damage response and repair has also been identified as a crucial actor for hypoxia-driven neovascularisation (Economopoulou et al., 2009). The PIKK family also comprises the DNA-dependent protein kinase (DNA-PK), a key player in signalling and repair by the non-homologous end-joining (NHEJ) repair pathway of DNA double-strand breaks (DSBs). DNA-PK comprises the Ku heterodimer, which binds to DNA ends, recruits and activates the DNA-PK catalytic subunit (DNA-PKcs) through a direct interaction. The activated DNA-PK has serine/threonine kinase activity that is necessary for efficient repair (reviewed by Weterings and Chen, 2007). Finally, the XRCC4–DNA-ligase-IV–XLF complex catalyses the final ligation step of the repair process (Weterings and Chen, 2007). In addition to its role in DNA DSB repair, DNA-PK has been associated with several pathways that modulate stress responses, such as apoptosis, telomere homeostasis and specific gene transcription. Interestingly, Um and colleagues have shown that hypoxia-induced accumulation of the transcription factor HIF-1 is reduced in DNA-PK-deficient cells (Um et al., 2004). However, the authors did not show whether DNA-PK is activated under this stress condition.

The hypoxia-inducible factor-1 HIF-1 is the key regulator of the cellular response to oxygen deprivation. It is comprised of a labile oxygen-regulated  $\alpha$ -subunit that is mainly targeted for normoxia-dependent degradation by the proteasomal system, whereas its  $\beta$ -subunit, HIF-1 $\beta$ /ARNT, is constitutively expressed (for reviews,

see Brahimi-Horn and Pouyssegur, 2009; Semenza, 2007; Yee Koh et al., 2008). Therefore, the activity of this complex is exquisitely dependent upon the limiting expression of the  $\alpha$ -subunit. Under hypoxia, HIF-1 $\alpha$  is stabilised, enters the nucleus and heterodimerises with HIF-1 $\beta$ . The heterodimer binds to hypoxia responsive elements (HREs) to transactivate a variety of hypoxia-responsive genes (Yee Koh et al., 2008), therefore contributing to the adaptative response to hypoxic conditions. Our experiments are designed to determine whether DNA-PK is activated by hypoxic stress in human cells, the mechanisms of its activation and the biological consequences for cells of this stress-response pathway. We demonstrate that DNA-PK is activated under hypoxic conditions. This cellular stress response favours hypoxia adaptation by protecting HIF-1 $\alpha$  from degradation. Importantly, our data are consistent with the new hypothesis of a DNA-dependent stress response initiated by chromatin modifications.

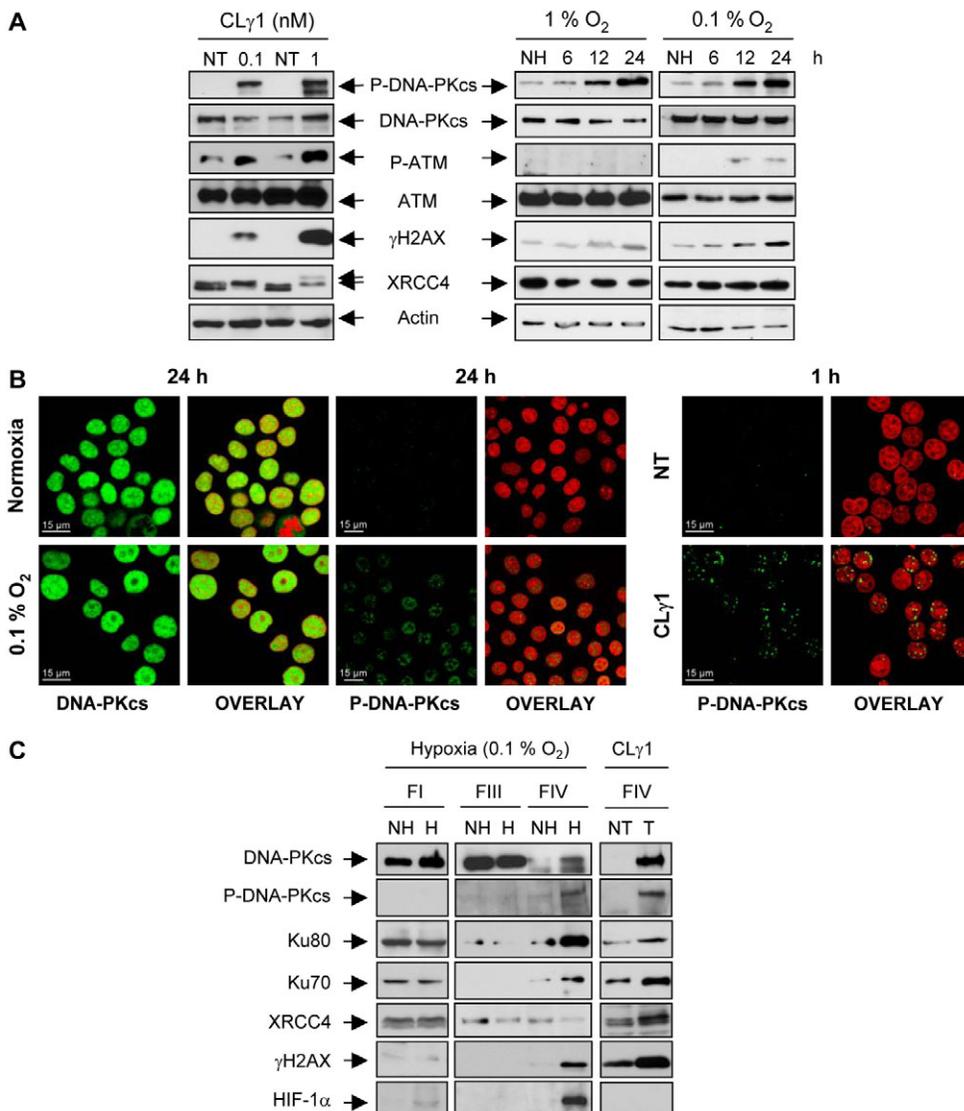
## Results

### DNA-PK is activated in hypoxic cells

During the processing of DNA lesions, DNA-PK is phosphorylated, partly by autophosphorylation. Among the sites identified, Ser2056

appears to be phosphorylated only in response to DNA DSBs in a strictly DNA-PK-dependent manner (Chen et al., 2005). To assess DNA-PK activation, cells were exposed to hypoxia (0.1 or 1% O<sub>2</sub>) at different times. As a control for DNA-PK activation, we used a well-known DNA-strand-breaking agent, calicheamicin gamma-1 (CL $\gamma$ 1) (Fig. 1A, left) (Bouquet et al., 2006).

In the presence of DSBs, we detected a strong and early (1 hour) accumulation of phosphorylated forms of DNA-PKcs (*P*-DNA-PKcs) and ATM (*P*-ATM) (Fig. 1A, left). In hypoxic cells, autophosphorylated DNA-PKcs progressively accumulated during the 24 hours of treatment, whereas the level of DNA-PKcs expression remains constant (Fig. 1A, right panel). By contrast, the autophosphorylation of ATM on Ser1981 was observed only in hypoxic cells exposed to the lowest O<sub>2</sub> concentration and for the later times (12 or 24 hours) in accordance with recently published data (Bencokova et al., 2009). Phosphorylation of the histone variant H2AX ( $\gamma$ H2AX), which is thought to serve as a platform for the assembly of proteins involved in cell cycle checkpoint and DNA repair, was investigated and was progressively detected in hypoxic cells (Fig. 1A, right). In the presence of DSBs, it has been shown that one of the proteins of the ligation complex, XRCC4, is



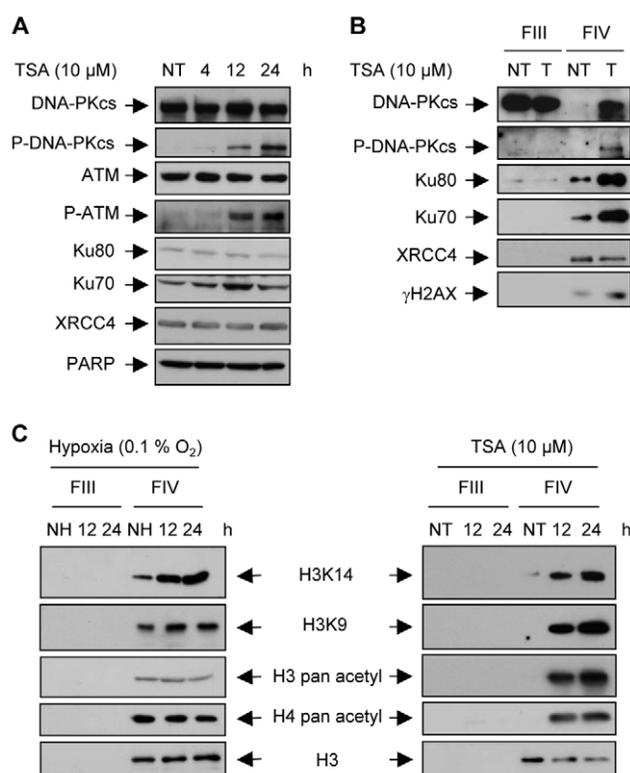
**Fig. 1. DNA-PK is activated in hypoxic cells.** (A) HeLa cells were incubated under non-hypoxic (NH) or hypoxic conditions (1% and 0.1% O<sub>2</sub>). At the indicated times, whole-cell extracts were prepared and used for immunoblot assays with indicated antibodies (right panel). As a control (left panel), cells were exposed for 1 hour to indicated doses of CL $\gamma$ 1 and extracts were prepared at the end of the incubation period. (B) HeLa cells grown on coverslips were exposed to hypoxia (0.1% O<sub>2</sub>, 24 hours) or cultivated in normoxic conditions, fixed, permeabilised and stained with an anti-DNA-PKcs antibody or a phosphospecific antibody directed against Ser2056 of DNA-PKcs, followed by incubation with Alexa-Fluor-488-conjugated secondary antibodies. Similar experiments were performed with CL $\gamma$ 1-treated cells (10 pM, 1 hour). DNA was stained with propidium iodide. (C) HeLa cells were incubated under non hypoxic (NH) or hypoxic conditions (H) (0.1% O<sub>2</sub>, 24 hours). Cells were fractionated and aliquots equivalent to 1/10 volume of the fractions I (pooled FI and FII), III and IV were separated on SDS-PAGE gels and blotted for the indicated antibodies. The FIV fraction of HeLa cells treated (T) or not (NT) with CL $\gamma$ 1 (0.1 nM, 1 hour) is shown as a control.

phosphorylated, retarding its electrophoretic mobility in SDS PAGE (Drouet et al., 2005) (see also Fig. 1A, left). By contrast, XRCC4 migration was not affected in hypoxic conditions (Fig. 1A, right), suggesting that the DNA-damage response induced by hypoxia is different from the one induced by genotoxic agents. To conclude, we highlighted a strong discrepancy between genotoxic insults and hypoxia in regard to activation of DNA repair proteins.

We further investigated the nuclear distribution of DNA-PK during hypoxia treatment. Cells were exposed to either hypoxia or CL $\gamma$ 1 and then stained for total or *P*-DNA-PKcs (Fig. 1B). No redistribution of total nuclear DNA-PKcs was seen upon hypoxia (Fig. 1B). After treatment with CL $\gamma$ 1, cells exhibited discrete, punctuate foci of DNA-PK (Fig. 1B); a similar result was obtained following ionising radiation (IR) (Chen et al., 2005). In hypoxic cells, no cells positive for *P*-DNA-PKcs were seen at 1% O<sub>2</sub> (data not shown), whereas at 0.1% O<sub>2</sub>, almost all cells were positive at later time points (24 hours), with a pattern that appeared more diffuse than that seen in response to CL $\gamma$ 1 (Fig. 1B). In hypoxic cells and in CL $\gamma$ 1-treated cells,  $\gamma$ H2AX foci were present (supplementary material Fig. S1), in accordance with previously published reports (Bouquet et al., 2006; Economopoulou et al., 2009; Hammond et al., 2003a). In response to clastogenic agents, DNA-PK is retained at sites of damaged DNA and as a result, becomes more resistant to biochemical extraction (Drouet et al., 2005). We used this approach to determine whether DNA-PK also became associated with detergent-resistant fractions in hypoxic cells. As expected, activated DNA-PK [including the phosphorylated DNA-PKcs and Ku70–Ku80 complex (Ku70/80)] was recruited to the extraction-resistant fraction (FIV) in cells pretreated with CL $\gamma$ 1 (Fig. 1C). Unmodified and slower-migrating XRCC4 forms were also seen in fraction IV of CL $\gamma$ 1-treated cells (Fig. 1C). When similar experiments were performed with hypoxic-treated cells, we observed that although the amount of DNA-PKcs in an activated form or its regulatory subunit Ku was increased in the latest extraction-resistant fraction (FIV), no increase in the amount of the XRCC4 protein was detected concomitant with retention of DNA-PK (Fig. 1C). It is noteworthy that in our conditions,  $\gamma$ H2AX and HIF-1 $\alpha$  were only present in fraction FIV (Fig. 1C). Therefore, under hypoxic conditions, DNA-PK tethers to detergent-resistant fractions inside a complex that is not related to the NHEJ machinery.

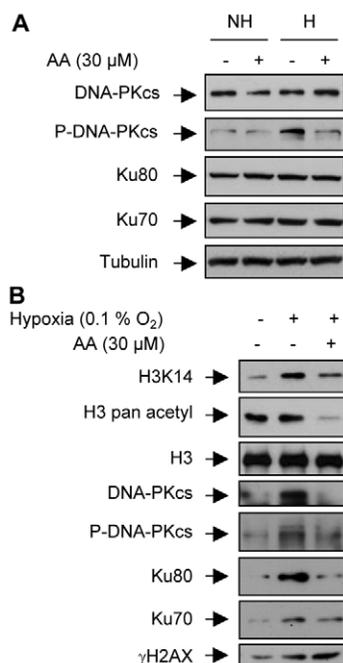
### DNA-PK is activated by chromatin modifications in hypoxic cells

The mechanism contributing to the initiation of DNA-PK activation upon hypoxia remains undetermined. Exposure of cells to the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA) has been shown to induce rapid, diffuse phosphorylation of the ATM protein, which suggests that ATM activation results from changes in chromatin structure (Bakkenist and Kastan, 2003). By contrast, activation of DNA-PK has never been investigated in this context, although it has been demonstrated that histone acetylation facilitates DNA-PK activation in response to DNA DSBs induced by clastogenic agents (Jiang et al., 2006; Sun et al., 2006). As shown in Fig. 2A, we observed that autophosphorylated DNA-PK progressively accumulates during exposure to TSA; we used the detection of autophosphorylated form of ATM as a control. As aforementioned with hypoxic cells (Fig. 1C), DNA-PKcs and Ku70/80 but not XRCC4, are mobilised to the less extractable nuclear fractions after TSA treatment (Fig. 2B). As a control, similar results (activation and redistribution of DNA-PK to



**Fig. 2. DNA-PK is activated by trichostatin A.** (A) HeLa cells were treated or not (NT) with TSA (10  $\mu$ M). At indicated times, whole-cell extracts were prepared and used for immunoblots assays with indicated antibodies. (B) HeLa cells were treated (T) with TSA (10  $\mu$ M, 24 hours) or not treated (NT). Cells were fractionated and aliquots equivalent to 1/10 volume of fractions III and IV were separated on SDS-PAGE gels and blotted for the indicated antibodies. (C) HeLa cells were incubated under non-hypoxic (NH) or hypoxic conditions (0.1% O<sub>2</sub>) or treated with TSA (10  $\mu$ M). At indicated times, cells were fractionated and aliquots equivalent to 1/10 volume of fractions III and IV were separated on SDS-PAGE gels and blotted for the indicated antibodies.

detergent-resistant fractions upon hypoxia or exposure to TSA) were observed in two other cell lines, LS174 and HCT116 (data not shown). Evidence suggests that the epigenetic modifications that occur in response to different types of stress lead to specific signatures of the epigenome. It has been recently demonstrated that long-term exposure to hypoxia (48 hours at 0.2% O<sub>2</sub>) induces a novel signature of chromatin modifications characterised by a slight (32%) decrease of H3K9 associated with a twofold increase in H3K14 acetylation (Johnson et al., 2008). As shown in Fig. 2C (left panel), progressive accumulation of H3K14 was observed in HeLa cells within the first 24 hours of exposure to hypoxic stress, whereas the levels of H3K9 or the global levels of H3 or H4 acetylation remain unchanged during this time course. As expected, TSA induced a global increase in levels of H3 and H4 acetylation, including a significant increase in H3K9 and H3K14 (Fig. 2C, right panel). To further demonstrate that DNA-PK activation in hypoxic cells is associated with histone acetylation, we used anacardic acid (AA), which inhibits histone acetyltransferase (HAT) activity both in vitro and in vivo (Balasubramanyam et al., 2003). After having verified that AA treatment alone had no effect on DNA-PK activation (supplementary material Fig. S2), we then assessed whether exposure to AA efficiently inhibited the increase



**Fig. 3. Hypoxia-induced histone acetylation initiates DNA-PK activation.** (A) HeLa cells were incubated in the presence of 30 μM Anacardic acid (AA), 2 hours before incubation under non-hypoxic (NH) or hypoxic conditions (H) (0.1% O<sub>2</sub>, 24 hours). Whole-cell extracts were prepared and blotted for the indicated antibodies. (B) Cells treated in similar conditions for 24 hours were fractionated. Aliquots equivalent to 1/10 volume of fraction IV were used for immunoblot assays with indicated antibodies.

in H3K14 acetylation, as already observed in fractionated hypoxic cells (Fig. 3B). By contrast, exposure to AA had no effect on H2AX phosphorylation (Fig. 3B). In hypoxic cells, pre-treatment with AA before hypoxia exposure inhibited activation (Fig. 3A) and redistribution to insoluble fractions of both Ku70/80 and DNA-PKcs (Fig. 3B). Taken together, our results suggest that hypoxia induces DNA-PK activation by modifying higher-order chromatin structure and chromatin-remodelling complexes.

#### DNA-PK activation promotes cellular adaptation to hypoxic stress

The biological relevance of DNA-PK activation during hypoxia remains an open question. As stated above, it has been reported that the level of HIF-1α accumulation was decreased in DNA-PKcs-deficient murine cells treated with the hypoxia-mimetic compounds desferrioxamine (DFX) compared with control cells (Um et al., 2004). However, the involvement of DNA-PK kinase activity in this process remains unknown. Moreover, the effects of DNA-PK on cellular oxygen-sensing and oxygen-signalling pathways in truly hypoxic cells remain undetermined. To address these questions, the naturally occurring DNA-PKcs-deficient human cell line (MO59-J/Fus9) and its DNA-PKcs-complemented counterpart (MO59-J/Fus1) were exposed to hypoxia. As shown in Fig. 4A, the increase in HIF-1α accumulation was higher in the DNA-PKcs-proficient cells compared with DNA-PKcs-deficient cells, whereas the level of expression of the HIF-2α isoform (which displays tissue-specific expression) was unaffected by DNA-PKcs status. To ascertain whether the kinase activity of DNA-PK was

required for this effect, cells were exposed to NU7026 (20 μM), a selective inhibitor of DNA-PK (Veuger et al., 2003). In the DNA-PKcs-proficient cells (MO59-J/Fus1), NU7026 decreased hypoxic accumulation of HIF-1α, but not HIF-2α (Fig. 4A). By contrast, in DNA-PKcs-deficient cells (MO59-J/Fus9), exposure to NU7026 had no effect upon the accumulation of either HIF-1α or HIF-2α. Hence, NU7026 abolishes the difference in HIF-1α accumulation between DNA-PKcs-deficient and -proficient cells.

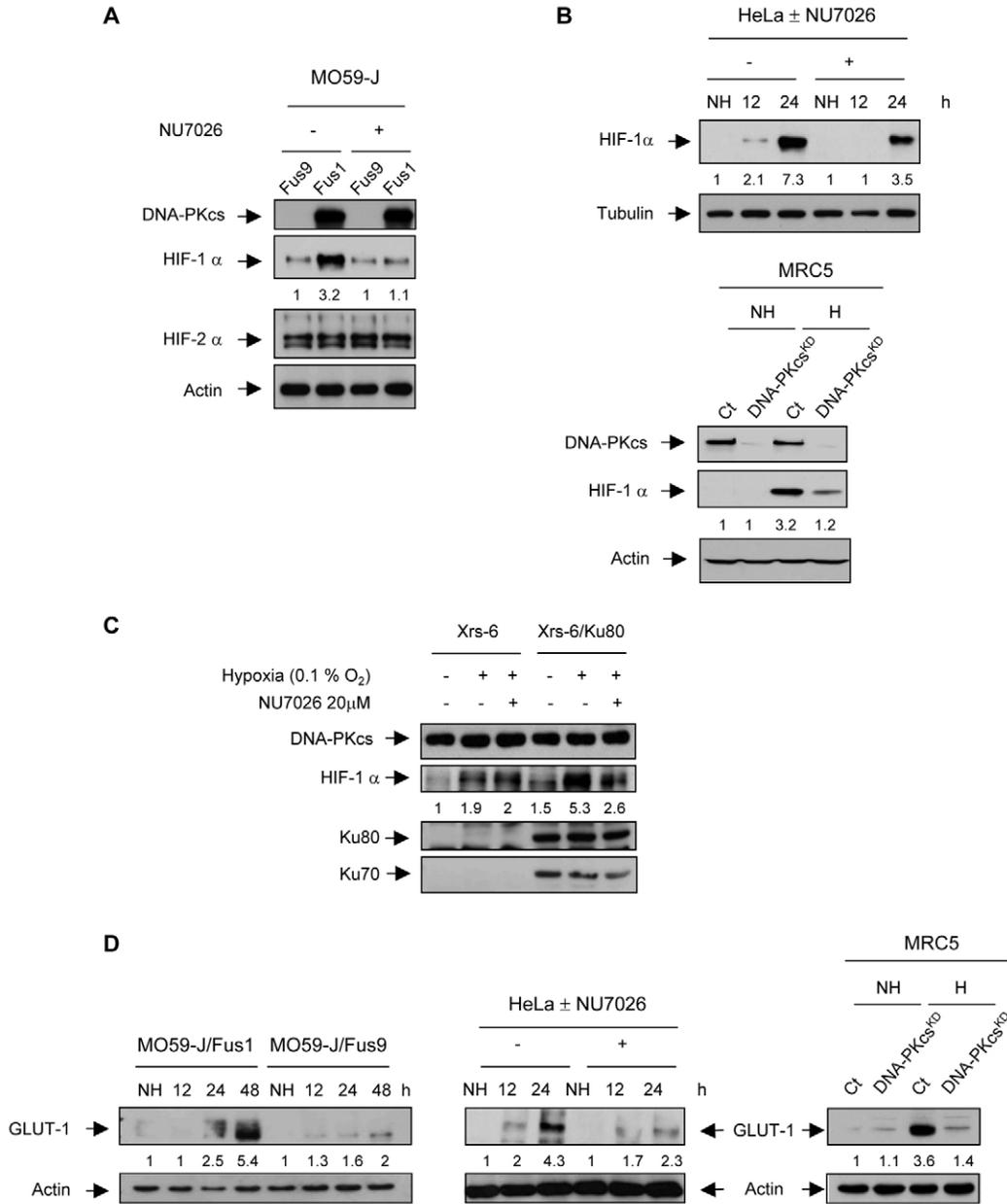
These results are reinforced in three other cell models: (1) HeLa cells exposed to NU7026 exhibited reduced HIF-1α accumulation after hypoxic stress (Fig. 4B, top); (2) MRC5 human fibroblast cells that were transformed with SV40 and stably silenced for DNA-PKcs gene expression, displayed reduced HIF-1α accumulation after exposure to hypoxic conditions (Fig. 4B, bottom); and (3) the Ku80-deficient hamster cell line xrs-6 elicited moderate HIF-1α accumulation upon hypoxia compared with cells transfected with the full-length hamster Ku80 cDNA xrs-6/Ku80 (Fig. 4C). In the latter model, NU7026 had no effect on accumulation of HIF-1α in Ku-deficient cells and its effect was reinstated with xrs-6/Ku80, highlighting again that DNA-PK activation is dependent on Ku in hypoxic conditions.

Furthermore, we sought to demonstrate that DNA-PKcs expression and activity could be directly related to an adaptive response to hypoxia. By using our three cell models (1) MO59-J/Fus1 versus MO59-J/Fus9; (2) HeLa cells treated or not with NU7026; and (3) control MRC5 versus DNA-PKcs<sup>KD</sup> cells, we clearly show that the level of protein expression of the glucose transporter GLUT-1, a HIF-1 gene target (Schofield and Ratcliffe, 2004), was higher in hypoxic DNA-PKcs-proficient cells (Fig. 4D). In addition, we demonstrated that DNA-PK activity slightly but significantly increased both cell survival and lactate production in hypoxic compared with normoxic cells (see supplementary material Fig. S3). These results are in accordance with the role of DNA-PK in the regulation of HIF-1α expression. Finally, experiments using MRC5 human fibroblasts that were stably silenced for DNA-PKcs gene expression showed that DNA-PK activity favoured the cloning efficiency of cells grown under long-term hypoxic conditions (8–10 days) (see supplementary material Fig. S4). Our compelling results highlight that DNA-PK kinase activity, which is strictly dependent on Ku70/80, is able to regulate HIF-1α accumulation and contributes to efficient adaptation to hypoxic conditions.

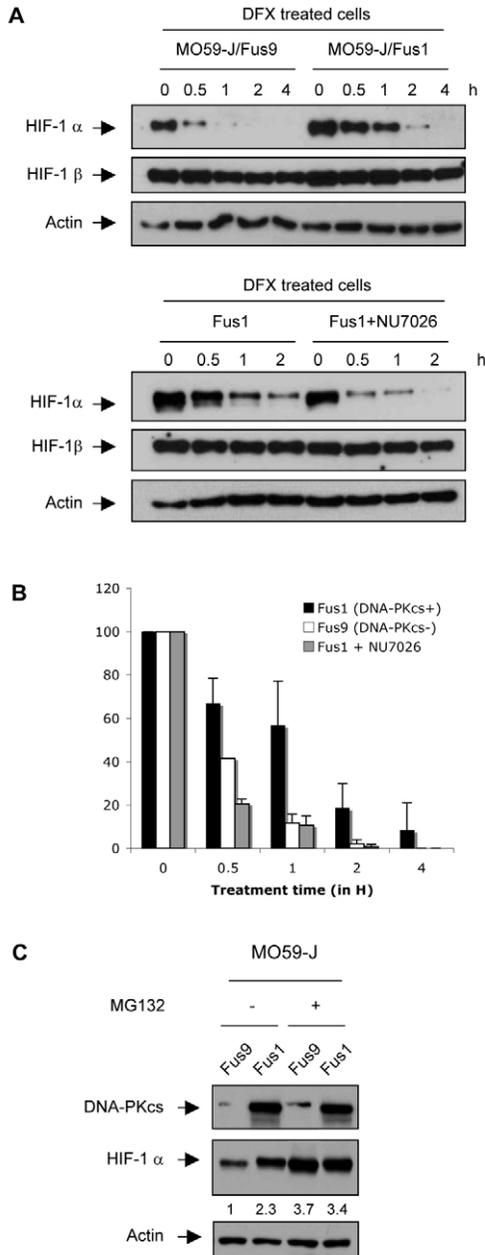
Ultimately, we aimed to understand how HIF-1α could accumulate in hypoxic cells in a DNA-PK-dependent manner. We observed no differences in levels of HIF-1α mRNA or protein between DNA-PKcs-deficient and -proficient cells (see supplementary material Fig. S5). These results strongly favour a role of DNA-PK in the control of HIF-1α protein stabilisation and/or degradation. To test this hypothesis, DFX-treated cells were exposed to the protein synthesis inhibitor cycloheximide to block *HIF1A* mRNA translation. The kinetics of HIF-1α decay in DNA-PKcs-proficient and -deficient cells was measured by immunoblotting. In MO59-J/Fus1 cells exposed to DFX, HIF-1α levels decayed with a half-life of about 90 minutes, whereas in the absence of DNA-PKcs (MO59-J/Fus9), this decay was far more rapid, with a half-life of about 30 minutes (Fig. 5A,B). When DNA-PK kinase activity was inhibited with NU7026, the degradation of HIF-1α decreased with a half-life close to that observed in DNA-PKcs-deficient cells (Fig. 5A,B). These results emphasise that DNA-PKcs participates in HIF-1α accumulation under hypoxia by its ability to interfere with the mechanism(s)

responsible for protein stabilisation and degradation. Hence, in the presence of the proteasome inhibitor MG132 (Meriin et al., 1998), the difference in accumulation of HIF-1 $\alpha$  in DNA-PK- proficient versus -deficient cells was no longer observed (Fig. 5C).

Taken together, our results demonstrate that DNA-PK is activated during hypoxic stress and that this stress response favors hypoxia adaptation by protecting HIF-1 $\alpha$  from nuclear degradation. Importantly, the mode of activation of DNA-PK appears to be



**Fig. 4.** DNA-PK activation in hypoxia contributes to HIF-1 $\alpha$  regulation and promotes cellular adaptation to hypoxia. (A) The MO59-J/Fus1 (DNA-PKcs proficient, +) and MO59-J/Fus9 (DNA-PKcs deficient, -) cells were incubated in the presence or absence of 20  $\mu$ M NU7026, 30 minutes before exposure to hypoxia (0.1% O<sub>2</sub>). After 3 hours, cell extracts were prepared and subjected to immunoblot assays; (B) Top panel, HeLa cells were incubated in the presence or absence of 20  $\mu$ M NU7026, 30 minutes before incubation under non hypoxic (NH) or hypoxic conditions (0.1% O<sub>2</sub>). At indicated times, extracts were prepared and used for immunoblots assays with indicated antibodies. Bottom panel, MRC5 cells transfected with a control shRNA (Ct) or with shRNA directed against DNA-PKcs mRNA (DNA-PKcs<sup>KD</sup>) were incubated non hypoxic (NH) or hypoxic conditions. (C) The Ku80-deficient cells xrs-6 and their counterpart transfected with the human full-length cDNA encoding Ku80, xrs-6/Ku80 were incubated with or without NU7026 (20  $\mu$ M), 30 minutes before exposure to hypoxia. After 3 hours, extracts were prepared and aliquots were subjected to immunoblot assays with the indicated antibodies. (D) Expression of GLUT-1 protein levels in the MO59-J/Fus1 and MO59-J/Fus9 cells cultured in hypoxic conditions (0.1% O<sub>2</sub>) for up to 48 hours (left). HeLa cells were incubated in the presence or not of 20  $\mu$ M NU7026, 30 minutes before incubation under non-hypoxic (NH) or hypoxic conditions (0.1% O<sub>2</sub>). At indicated times, extracts were prepared, separated on SDS-PAGE gels and GLUT-1 was evaluated by western blot experiments (middle). Similar experiments were performed with MRC5 cells transfected with a control shRNA (Ct) or shRNA directed against DNA-PKcs mRNA (DNA-PKcs<sup>KD</sup>) (right). Numbers indicate the levels of HIF-1 $\alpha$  expression related to normoxic controls.



**Fig. 5. DNA-PK regulates HIF-1 $\alpha$  degradation.** (A) MO59-J/Fus1 and MO59-J/Fus9 cells were treated with DFX for 3 hours. After 3 hours (T<sub>0</sub>), cells were exposed to cycloheximide to block protein biosynthesis and extracts were prepared at the indicated times. Expression of HIF-1 $\alpha$ , HIF-1 $\beta$  and actin was evaluated as a function of time (top). Similar experiments were performed in MO59-J/Fus1 cells treated with DFX in the presence of NU7026 (20  $\mu$ M) (bottom). (B) Quantification of HIF-1 $\alpha$  expression as a function of time (mean of at least three independent experiments  $\pm$  s.d.). (C) MO59-J/Fus1 and MO59-J/Fus9 cells were incubated under hypoxic conditions (0.1% O<sub>2</sub>) for 3 hours in the presence or absence of MG132, and the levels of HIF-1 $\alpha$  were measured in extracts.

different from that observed after classical genotoxic stress, and our data are consistent with the hypothesis whereby activation of DNA-PK is initiated by chromatin modifications. To reinforce our findings, we also show that treatment of cells with DNA-DSB-inducing agents (either CL $\gamma$ 1 or IR), leading to DNA-PK activation,

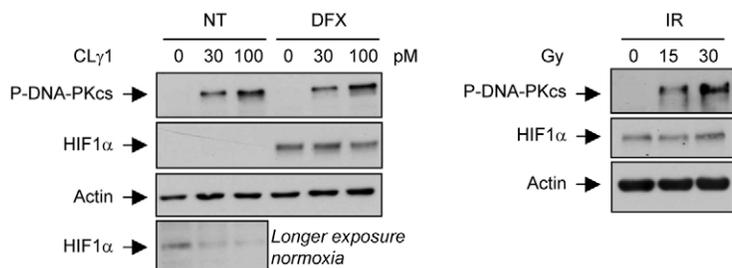
was not able to upregulate HIF-1 $\alpha$  (Fig. 6), even when cells were exposed to DFX to allow HIF-1 $\alpha$  translocation to the nucleus before exposure to CL $\gamma$ 1. DFX was used instead of hypoxia, because CL $\gamma$ 1 activity is dependent on the O<sub>2</sub> concentration (Bouquet et al., 2006).

## Discussion

Compelling evidence, including these results, now demonstrates that hypoxia activates proteins involved in DNA-damage signalling and repair. However, the mechanism(s) involved in this activation and the precise role of this pathway in hypoxia tolerance were poorly understood. Our results show that hypoxia might function as a specific inducer of DNA-PK mainly through modification of higher-order chromatin structure and chromatin-remodelling complexes. We show that DNA-PK acts as an oxygen-dependent regulator of HIF-1 $\alpha$  stability, thereby contributing to the cellular adaptation to hypoxia.

How can DNA-PK be activated during hypoxia? The phosphorylation of DNA-PKcs on S2056 (Fig. 1A) and its redistribution concomitantly with Ku, from soluble to resistant detergent fractions (Fig. 1C) demonstrated a DNA-dependent process because the whole complex was assembled and activated only in tight contact with DNA or chromatin structures (Burma and Chen, 2004; Hammel et al., 2010). In contrast to DNA-damaging agents, the recruitment of DNA-PK was not associated with the activation and recruitment of XRCC4 (Fig. 1A,C). In addition, immunostaining analysis of the nuclear distribution of phosphorylated DNA-PK after either hypoxia or genotoxic treatment strongly suggested the existence of divergent mechanisms of activation (Fig. 1B). Interestingly, induction of DNA-PK activation after a genotoxic insult (either CL $\gamma$ 1 or IR) was not able to upregulate HIF-1 $\alpha$  (Fig. 6), even when cells were exposed to DFX to allow translocation of HIF-1 $\alpha$  to the nucleus before CL $\gamma$ 1 exposure. This confirmed previously published data reporting that ionising irradiation upregulates HIF-1 $\alpha$  expression in vivo, but not in vitro, through reactive oxygen species induced by tumour reoxygenation (Moeller et al., 2004). Hence, our observation highlights a model whereby hypoxia activates DNA-PK by a different pathway to that observed after genotoxic stress. These results are in accordance with a recent study showing that activation of ATM in hypoxic cells arises independently of the MRN complex and is therefore, different to ATM activation after genotoxic injuries (Bencokova et al., 2009). Once activated in hypoxic cells, ATM behaves in a similar manner to damage-activated ATM, in that it phosphorylates downstream targets.

Previously, it has been shown that hypoxic cells fail to exhibit DNA DSBs, as evidenced by comet assay experiments (Hammond et al., 2003a). We observed here that the histone variant H2AX is phosphorylated in these hypoxic cells, confirming previous analyses (Economopoulou et al., 2009; Hammond et al., 2003a). However, although histone  $\gamma$ H2AX is an important effector of the DNA-damage-response (DDR) pathway by signalling DSBs and recruiting repair proteins at these damaged sites, it also appears to be tethered to undamaged sites. Initially, the phosphorylation of H2AX was considered to be a surrogate marker of DSBs (reviewed by Kinner et al., 2008). Consequently, DDR pathways that include H2AX phosphorylation were associated with the induction of breaks into the DNA. This seemingly obvious assumption has been challenged by several recent observations in both mammalian and yeast cells. Interestingly, immobilisation of particular repair proteins on DNA was sufficient to activate markers of the DDR pathway in

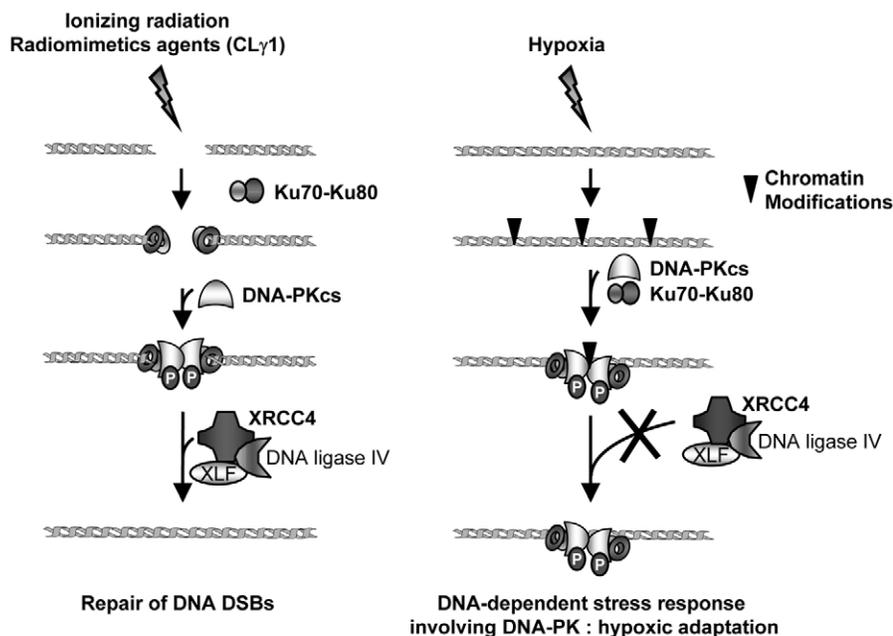


**Fig. 6. Exposure to clastogenic agents does not regulate HIF-1 $\alpha$  accumulation in normoxic or DFX-treated cells.** HeLa cells were exposed to CL $\gamma$ 1 (30 or 100 pM) for 4 hours and extracts were prepared to evaluate HIF-1 $\alpha$  expression (left). As indicated, similar experiments were performed in cells previously treated with DFX for 1 hour, before treatment with CL $\gamma$ 1. U87 cells were exposed to ionising radiation and 1 hour later, cell extracts were prepared and blotted for the indicated antibodies (right).

yeast and mammalian cells (Bonilla et al., 2008; Soutoglou and Misteli, 2008). This is the case for PI3KK transducer kinases, which could be recruited in the absence of DNA lesions (Misteli and Soutoglou, 2009; Pospelova et al., 2009; Soutoglou and Misteli, 2008; Toledo et al., 2008). Activation of DDR pathways involving DNA-PK or ATM could be triggered by stable association of single repair factors with chromatin in the absence of DNA lesions per se (Soutoglou and Misteli, 2008). Hence, the immobilisation of either Nbs1 or MRE11 on the chromatin induced a DDR with phosphorylation of H2AX around the foci formed by these proteins in the absence of DNA breaks (Soutoglou and Misteli, 2008). Consistent with the idea of lesion-independent activation of a DDR, over-expression of a small domain of the ATR-stimulating TopBP1 protein leads to activation of ATR, cell-cycle arrest and senescence in the absence of DNA breaks (Toledo et al., 2008). Furthermore, during senescence, specific DDR markers, including H2AX, are phosphorylated in the absence of detectable DNA breaks (Pospelova et al., 2009). Taken together, these observations raise the intriguing possibility that H2AX phosphorylation is not solely induced by DNA breaks, but possibly also by changes in chromatin structure (Misteli and Soutoglou, 2009; Pankotai et al., 2009). Two compelling pieces of evidence argue for an activation of DNA-PK in hypoxic cells through chromatin modification. First, by using a histone deacetylase inhibitor (TSA), we show that the repair protein complex DNA-PKcs–Ku70/80 did not probe the genome solely for DNA breaks, but also for changes in chromatin

structure (see Fig. 2A,B). This was also observed for ATM after TSA treatment (Bakkenist and Kastan, 2003). Second, although an increase in histone acetylation (mainly H3K14) is observed in hypoxic cells, pre-treatment with a histone acetyltransferase inhibitor (AA) before hypoxia exposure inhibits the activation of Ku and DNA-PKcs and their redistribution into insoluble fractions (Fig. 3A,B). However, we cannot formally exclude the presence of DNA breaks (e.g. replication-induced DSBs) in the genome of hypoxic cells that might, in part, contribute to the activation of DNA-PK. This issue is difficult to address experimentally because it involves demonstration of negative data. In addition, if we consider that  $\gamma$ H2AX is no longer a unique marker of DNA DSBs, there is no alternative sensitive approach to detect low levels of DSBs.

What are the cellular endpoints of DNA-PK activation in hypoxic cells? We showed that active DNA-PK positively regulates HIF-1 $\alpha$  expression under hypoxia in four different cell models, suggesting a canonical pathway (see Fig. 4A–C). The reduced half-life of HIF-1 $\alpha$  in DNA-PK-deficient cells upon hypoxia provided a mechanistic explanation for the observed decrease in HIF-1 $\alpha$  accumulation (see Fig. 5). It is important to note that HIF-1 $\alpha$  entry into the nucleus is not a key event that controls its stability (Berra et al., 2001). A nuclear cytoplasmic shuttle of the von Hippel-Lindau (VHL) protein, which targets the  $\alpha$ -subunits of HIF-1 for ubiquitin-mediated degradation, has been demonstrated (Groulx and Lee, 2002). Therefore, activation of this nuclear stress



**Fig. 7. Mechanisms and consequences of DNA-PK activation following exposure to clastogenic agents and hypoxic conditions.** In the presence of clastogenic agents that create DSBs, the Ku heterodimer, which binds to DNA ends, recruits and activates the DNA-PK catalytic subunit (DNA-PKcs) through a direct interaction. The XRCC4–DNA-ligase-IV–XL-F complex is then recruited to catalyse the final ligation step of the repair process. In hypoxic cells, chromatin modifications initiate DNA-PK recruitment to DNA and activation. This event is not followed by the recruitment of the ligation complex, which might be explained by a different conformational state of DNA-PK depending of its mode of activation. However, we cannot formally exclude that there are no breaks in the genome of hypoxic cells that might, in part, contribute to the activation of DNA-PK. The DNA-PK-activated DNA-damage-response pathway regulates the hypoxic degradation and accumulation of HIF-1 $\alpha$ , therefore contributing to cellular adaptation to hypoxia.

response offers cells additional control to ensure appropriate HIF-1 $\alpha$  expression upon hypoxic conditions within this cellular compartment. One attractive hypothesis is that DNA-PK phosphorylation of HIF-1 $\alpha$  further controls its interaction with phosphorylated VHL in the nucleus, a mechanism that has been demonstrated for the large subunit of RNA polymerase II (Kuznetsova et al., 2003). Preliminary evidence obtained in our laboratory shows that HIF-1 $\alpha$  is not a phosphorylation substrate of DNA-PK in vitro, even when activating DNA containing HRE sequences and also free DNA ends (supplementary material Fig. S6). Using appropriate controls, we did not find any interaction between DNA-PKcs and HIF-1 $\alpha$  (supplementary material Fig. S7), in apparent contradiction to previously published reports (Um et al., 2004). Therefore, these results suggest that DNA-PK modulates the HIF-1 $\alpha$  degradation pathway by means that remain to be identified.

We demonstrate that the absence of DNA-PK expression or inhibition of its kinase activity negatively regulates the HIF-1 $\alpha$  trans-activating function through expression of its target gene *GLUT1* (see Fig. 4D) and clearly contributes to cellular adaptation to hypoxia (see also supplementary material Figs S3, S4). Therefore, under hypoxic conditions it is important to point out that in contrast to other DNA-repair pathways, which were downregulated, leading to genetic instability (Bristow and Hill, 2008), ATR, ATM and DNA-PK remained expressed and activated. In addition to our present work, a recent paper demonstrated that hypoxia results in ATM-dependent phosphorylation of HIF-1 $\alpha$  on Ser696 and mediates downregulation of mTORC1 signalling (Cam et al., 2010). Taken together, these recent observations clearly open up new pharmacological opportunities to target hypoxic cells within tumours.

To conclude, we propose a model for the activation of DNA-PK under hypoxic conditions whereby hypoxia mainly induces changes in the higher-order chromatin structure, initiating DNA-PK activation and activating an adaptive response to counterbalance oxygen deprivation. In this model, DNA-PK is mainly activated by chromatin changes in the absence of DNA lesions, although we cannot formally exclude that DNA DSBs also partly contribute to DNA-PK activation (see Fig. 7). Therefore, this work enlarges the role of particular DNA repair proteins, which are recruited into DNA structures even in the absence of specific genotoxic attack.

## Materials and Methods

### Cell lines, cell culture, induction of hypoxia

HeLa (cervical adenocarcinoma) and U87 (glioblastoma) cell lines were grown in DMEM medium containing 10% serum. DNA-PKcs-deficient and DNA-PKcs-complemented cell lines (MO59-J/Fus9 and MO59-J/Fus1, respectively, a kind gift from Cordula U. Kirchgessner, Stanford University School of Medicine, Palo Alto, CA) were maintained in DMEM-F12 1:1 medium containing 15% serum. To obtain SV40 transformed MRC5 cells with long term downregulation of DNA-PKcs expression, cells were transfected with EBV-based vectors containing either control or short hairpin RNA coding sequences for human DNA-PKcs as previously described (Despras et al., 2007). The Ku80-deficient *xrs-6* cell line and the *xrs-6*/haKu80 cell line transfected with the hamster Ku80 cDNA were obtained from the European Collection of Animal Cell Culture (Salisbury, UK). For hypoxic conditions, cells were placed in a modular incubator chamber and flushed with a gas mixture, containing 0.1 or 1% O<sub>2</sub>, 5% CO<sub>2</sub> and balanced N<sub>2</sub>, and harvested inside the chamber with equilibrated solutions. When indicated, hypoxic cells were pre-incubated (for 1 hour) in the presence of NU7026 (20  $\mu$ M) or with anacardic acid (30  $\mu$ M) (both from Calbiochem). In indicated experiments, cells were irradiated using a <sup>137</sup>Cs source irradiator (4.4 Gy/minute, Biobeam 8000) or treated with calicheamicin (Calicheamicin  $\gamma$ 1, CL $\gamma$ 1, gift from Philip R. Hamann, Wyeth Research, Pearl River, NY) in the presence or absence of the common hypoxia-mimetic agent desferrioxamine (DFX, 260  $\mu$ M) obtained from Sigma (Saint-Quentin les Ulysses, France). Trichostatin A (Sigma) was used at 10  $\mu$ M and MG132 (Calbiochem) was used at 20  $\mu$ M concentration.

### Cell extracts and western blots

Whole-cell extracts were made as previously described (Monferran et al., 2004) except for the detection of  $\gamma$ H2AX expression, where a different protocol was used (Bouquet et al., 2006). Western blots were performed as previously described (Monferran et al., 2004). Image acquisitions of western blot were scanned at 400 d.p.i. by using an Epson Perfection V10 photo scanner and the Epson scan software.

### Antibodies

Anti-DNA-PKcs (clone 18-2), anti-Ku70 (clone N3H10), anti-Ku80 (clone 111 or S10B1), anti-actin (clone ACTN05) monoclonal antibodies and the anti-GLUT-1 and anti-PARP-1 rabbit antibodies were from Neomarkers (Fremont, CA). Anti-HIF-1 $\alpha$  monoclonal antibody (clone 54) was from BD Biosciences (San Jose, CA, USA). Anti-HIF-2 $\alpha$  (Ab8365), the polyclonal rabbit anti-XRCC-4 antibody and the antibody that specifically recognises the phosphorylated form of DNA-PKcs on S2056 were from Abcam (Cambridge, UK). Anti-ATM (clone 2C1) was from GeneTex (San Antonio, TX) and the antibody that recognises the phosphorylated form of ATM on S1981 was from Calbiochem (clone 10H11.E12, Darmstadt, Germany). The anti- $\gamma$ H2AX (clone JBW 301) and anti-histone H3 (unmodified Lys4) (clone CMA 301) monoclonal antibodies and the polyclonal antibodies anti-acetyl-Histone H3 (Lys9), anti-acetyl-Histone H3 (Lys14) were obtained from Upstate Biotechnology (Molsheim, France).

### Biochemical fractionation

Treated or mock-treated cells in culture dishes were washed twice with ice-cold PBS, collected by scraping and centrifuged. Cell fractionation was carried out by four consecutive extractions. The supernatant was collected at each step and labelled as fraction FI, FII, FIII, FIV. Pellets of about 2 $\times$ 10<sup>6</sup> cells were first resuspended for 5 minutes on ice in 200  $\mu$ l of extraction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 0.2% NP40 supplemented with protease inhibitor (20  $\mu$ g/ml phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin) and phosphatase inhibitors (50 mM NaF, cocktail inhibitor phosphatase I and II, all from Sigma). Following centrifugation at 1000 *g* for 5 minutes, the supernatants were collected (FI), extraction was carried out again and the supernatant was collected (FII) and pooled with the previous one. Therefore, the two pooled first fractions were named FI. The pellets were further resuspended for 40 minutes on ice in 200  $\mu$ l of extraction buffer containing 0.5% NP40 supplemented with protease inhibitor and phosphatase inhibitors. Pellets were clarified by centrifugation at 16,000 *g* for 15 minutes and the supernatants were collected (FIII). Insoluble FIV fractions were resuspended in a Tris-HCl, pH 6.8, buffer supplemented with 2% SDS, 10% glycerol and heated for 10 minutes at 100°C. Equal aliquots of each fraction, derived from equivalent cell numbers, were separated by SDS-PAGE.

### Immunofluorescence

The detection of the DNA-PKcs and its phosphorylated form (DNA-PKcs S2056) by immunofluorescence was performed as previously described (Bouquet et al., 2006) with slight modifications in the fixation procedure (cells were fixed in 1% final formaldehyde directly in the culture medium for 10 minutes at 37°C then neutralised in 50 mM NH<sub>4</sub>Cl for 2 minutes). In addition, for detection of P-DNA-PKcs, cells were incubated overnight with the primary antibody at a 1:100 dilution. Confocal images were obtained by means of a confocal laser microscopy system (Leica, St-Gallen, Switzerland). Images were collected by scanning stained cells sequentially under a 100 $\times$  objective lens. For each sample, over 100 cells were examined in at least three independent experiments.

### Determination of HIF-1 $\alpha$ degradation

HIF-1 $\alpha$  accumulation was induced by treatment for 3 hours with 260  $\mu$ M DFX (T0). Cells were then treated with 100  $\mu$ g/ml cycloheximide (CHX) and extracts prepared at various time points (up to 4 hours) following the addition of CHX, as indicated in the Results. HIF-1 $\alpha$  steady state levels were determined by western blot analysis and normalised against the corresponding actin level.

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/124/11/1943/DC1>

## References

- Abraham, R. T. (2004). PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways. *DNA Repair (Amst.)* **3**, 883-887.
- Bakkenist, C. J. and Kastan, M. B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**, 499-506.

- Balasubramanyam, K., Swaminathan, V., Ranganathan, A. and Kundu, T. K. (2003). Small molecule modulators of histone acetyltransferase p300. *J. Biol. Chem.* **278**, 19134-19140.
- Bencokova, Z., Kaufmann, M. R., Pires, I. M., Lecane, P. S., Giaccia, A. J. and Hammond, E. M. (2009). ATM activation and signaling under hypoxic conditions. *Mol. Cell Biol.* **29**, 526-537.
- Berra, E., Roux, D., Richard, D. E. and Pouyssegur, J. (2001). Hypoxia-inducible factor-1 alpha (HIF-1 alpha) escapes O(2)-driven proteasomal degradation irrespective of its subcellular localization: nucleus or cytoplasm. *EMBO Rep.* **2**, 615-620.
- Bonilla, C. Y., Melo, J. A. and Toczyski, D. P. (2008). Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage. *Mol. Cell* **30**, 267-276.
- Bouquet, F., Muller, C. and Salles, B. (2006). The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage. *Cell Cycle* **5**, 1116-1122.
- Brahimi-Horn, M. C. and Pouyssegur, J. (2007). Oxygen, a source of life and stress. *FEBS Lett.* **581**, 3582-3591.
- Brahimi-Horn, M. C. and Pouyssegur, J. (2009). HIF at a glance. *J. Cell Sci.* **122**, 1055-1057.
- Bristow, R. G. and Hill, R. P. (2008). Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. *Nat. Rev. Cancer* **8**, 180-192.
- Burma, S. and Chen, D. J. (2004). Role of DNA-PK in the cellular response to DNA double-strand breaks. *DNA Repair (Amst.)* **3**, 909-918.
- Cam, H., Easton, J. B., High, A. and Houghton, P. J. (2010). mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1alpha. *Mol. Cell* **40**, 509-520.
- Chen, B. P., Chan, D. W., Kobayashi, J., Burma, S., Asaithamby, A., Morotomi-Yano, K., Botvinick, E., Qin, J. and Chen, D. J. (2005). Cell cycle dependence of DNA-dependent protein kinase phosphorylation in response to DNA double strand breaks. *J. Biol. Chem.* **280**, 14709-14715.
- Despras, E., Pfeiffer, P., Salles, B., Calsou, P., Kuhfittig-Kulle, S., Angulo, J. F. and Biard, D. S. (2007). Long-term XPC silencing reduces DNA double-strand break repair. *Cancer Res.* **67**, 2526-2534.
- Drouet, J., Deltail, C., Lefrancois, J., Concannon, P., Salles, B. and Calsou, P. (2005). DNA-dependent protein kinase and XRCC4-DNA ligase IV mobilization in the cell in response to DNA double strand breaks. *J. Biol. Chem.* **280**, 7060-7069.
- Economopoulou, M., Langer, H. F., Celeste, A., Orlova, V. V., Choi, E. Y., Ma, M., Vassilopoulos, A., Callen, E., Deng, C., Bassing, C. H. et al. (2009). Histone H2AX is integral to hypoxia-driven neovascularization. *Nat. Med.* **15**, 553-558.
- Gibson, S. L., Bindra, R. S. and Glazer, P. M. (2005). Hypoxia-induced phosphorylation of Chk2 in an ataxia telangiectasia mutated-dependent manner. *Cancer Res.* **65**, 10734-10741.
- Groulx, I. and Lee, S. (2002). Oxygen-dependent ubiquitination and degradation of hypoxia-inducible factor requires nuclear-cytoplasmic trafficking of the von Hippel-Lindau tumor suppressor protein. *Mol. Cell Biol.* **22**, 5319-5336.
- Hammel, M., Yu, Y., Mahaney, B. L., Cai, B., Ye, R., Phipps, B. M., Rambo, R. P., Hura, G. L., Pelikan, M., So, S. et al. (2010). Ku and DNA-dependent protein kinase dynamic conformations and assembly regulate DNA binding and the initial non-homologous end joining complex. *J. Biol. Chem.* **285**, 1414-1423.
- Hammond, E. M., Dorie, M. J. and Giaccia, A. J. (2003a). ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J. Biol. Chem.* **278**, 12207-12213.
- Hammond, E. M., Green, S. L. and Giaccia, A. J. (2003b). Comparison of hypoxia-induced replication arrest with hydroxyurea and aphidicolin-induced arrest. *Mutat. Res.* **532**, 205-213.
- Hammond, E. M., Dorie, M. J. and Giaccia, A. J. (2004). Inhibition of ATR leads to increased sensitivity to hypoxia/reoxygenation. *Cancer Res.* **64**, 6556-6562.
- Jiang, X., Sun, Y., Chen, S., Roy, K. and Price, B. D. (2006). The FATC domains of PIKK proteins are functionally equivalent and participate in the Tip60-dependent activation of DNA-PKcs and ATM. *J. Biol. Chem.* **281**, 15741-15746.
- Johnson, A. B., Denko, N. and Barton, M. C. (2008). Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. *Mutat. Res.* **640**, 174-179.
- Kinner, A., Wu, W., Staudt, C. and Iliakis, G. (2008). Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res.* **36**, 5678-5694.
- Kuznetsova, A. V., Meller, J., Schnell, P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, J. W., Conaway, R. C. and Czyzyk-Krzeska, M. F. (2003). von Hippel-Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination. *Proc. Natl. Acad. Sci. USA* **100**, 2706-2711.
- Meriin, A. B., Gabai, V. L., Yaglom, J., Shifrin, V. I. and Sherman, M. Y. (1998). Proteasome inhibitors activate stress kinases and induce Hsp72. Diverse effects on apoptosis. *J. Biol. Chem.* **273**, 6373-6379.
- Misteli, T. and Soutoglou, E. (2009). The emerging role of nuclear architecture in DNA repair and genome maintenance. *Nat. Rev. Mol. Cell Biol.* **10**, 243-254.
- Moeller, B. J., Cao, Y., Li, C. Y. and Dewhirst, M. W. (2004). Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer Cell* **5**, 429-441.
- Monferran, S., Paupert, J., Dauvillier, S., Salles, B. and Muller, C. (2004). The membrane form of the DNA repair protein Ku interacts at the cell surface with metalloproteinase 9. *EMBO J.* **23**, 3758-3768.
- Pankotai, T., Hoffbeck, A. S., Boumendil, C. and Soutoglou, E. (2009). DNA damage response in the absence of DNA lesions continued. *Cell Cycle* **8**, 4025-4026.
- Pospelova, T. V., Demidenko, Z. N., Bukreeva, E. I., Pospelov, V. A., Gudkov, A. V. and Blagosklonny, M. V. (2009). Pseudo-DNA damage response in senescent cells. *Cell Cycle* **8**, 4112-4118.
- Schofield, C. J. and Ratcliffe, P. J. (2004). Oxygen sensing by HIF hydroxylases. *Nat. Rev. Mol. Cell Biol.* **5**, 343-354.
- Semenza, G. L. (2007). Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci. STKE* **2007**, cm8.
- Soutoglou, E. and Misteli, T. (2008). Activation of the cellular DNA damage response in the absence of DNA lesions. *Science* **320**, 1507-1510.
- Sun, Y., Jiang, X., Chen, S. and Price, B. D. (2006). Inhibition of histone acetyltransferase activity by anacardic acid sensitizes tumor cells to ionizing radiation. *FEBS Lett.* **580**, 4353-4356.
- Toledo, L. I., Murga, M., Gutierrez-Martinez, P., Soria, R. and Fernandez-Capetillo, O. (2008). ATR signaling can drive cells into senescence in the absence of DNA breaks. *Genes Dev.* **22**, 297-302.
- Um, J. H., Kang, C. D., Bae, J. H., Shin, G. G., Kim, D. W., Kim, D. W., Chung, B. S. and Kim, S. H. (2004). Association of DNA-dependent protein kinase with hypoxia inducible factor-1 and its implication in resistance to anticancer drugs in hypoxic tumor cells. *Exp. Mol. Med.* **36**, 233-242.
- Veuger, S. J., Curtin, N. J., Richardson, C. J., Smith, G. C. and Durkacz, B. W. (2003). Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1. *Cancer Res.* **63**, 6008-6015.
- Weterings, E. and Chen, D. J. (2007). DNA-dependent protein kinase in nonhomologous end joining: a lock with multiple keys? *J. Cell Biol.* **179**, 183-186.
- Yee Koh, M., Spivak-Kroizman, T. R. and Powis, G. (2008). HIF-1 regulation: not so easy come, easy go. *Trends Biochem. Sci.* **33**, 526-534.