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HIF mediated and DNA damage independent histone H2AX phosphorylation in chronic hypoxia

Abstract: The histone variant 2AX (H2AX) is phosphorylated at Serine 139 by the PI3K-like kinase family members ATM, ATR and DNA-PK. Genotoxic stress, such as tumor radio- and chemotherapy, is considered to be the main inducer of phosphorylated H2AX (γH2AX), which forms distinct foci at sites of DNA damage where DNA repair factors accumulate. γH2AX accumulation under severe hypoxic/anoxic (0.02% oxygen) conditions has recently been reported to follow replication fork stalling in the absence of detectable DNA damage. In this study, we found HIF-dependent accumulation of γH2AX in several cancer cell lines and mouse embryonic fibroblasts exposed to physiologically relevant chronic hypoxia (0.2% oxygen), which did not induce detectable levels of DNA strand breaks. The hypoxic accumulation of γH2AX was delayed by the RNAi-mediated knockdown of HIF-1α or HIF-2α and further decreased when both HIF-αs were absent. Conversely, basal phosphorylation of H2AX was increased in cells with constitutively stabilized HIF-2α. These results suggest that both HIF-1 and HIF-2 are involved in γH2AX accumulation by tumor hypoxia, which might increase a cancer cell's capacity to repair DNA damage, contributing to tumor therapy resistance.

Keywords: DNA damage response; oxygen sensing; tumor hypoxia.

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Introduction: tumor hypoxia and therapy resistance

Hypoxia is a common feature of solid tumors and develops due to inadequate vascularization, tortuous blood vessels and high oxygen consumption. Transient blockage of red blood cell flux, alternating with rapid alleviation, leads to frequent periodical hypoxia/ischemia, followed by reoxygenation (Yasui et al., 2010). Reoxygenation, most likely mediated by the generation of reactive oxygen species (ROS), but not hypoxia can lead to detectable DNA damage (Hammond et al., 2003a,b). Hypoxia is strongly associated with malignant progression, metastatic outgrowth, genetic instability, resistance to radio-and chemotherapy and overall poor patient prognosis in various tumor types (Brown, 1998; Brown and William, 2004; Pouysségur et al., 2006). Therefore, a thorough understanding of the molecular pathways in the hypoxic tumor microenvironment is warranted to develop new strategies for efficient cancer therapy.

Central to the cellular response to hypoxia is the heterodimeric hypoxia-inducible transcription factor HIF, consisting of one of three oxygen-labile α subunits and a common constitutive β subunit (Wenger, 2002; Schofield and Ratcliffe, 2004). HIF activates a large number of oxygen-regulated genes required for the adaptation of normal cells to hypoxia (Wenger et al., 2005). In tumors, HIF-1 is responsible for the generation of new blood vessels through transcriptional regulation of the vascular endothelial growth factor (VEGF), for pH regulation by increasing the expression of carbonic anhydrase (CA) IX and for the aerobically increased glycolytic capacity of cancer cells, also known as the Warburg effect (Seagroves et al., 2001; Minchenko et al., 2002; Svastova et al., 2004). Furthermore, hypoxic tumor cells are able to maintain metabolic functions without an adequate oxygen supply via a switch to anaerobic fermentation (the Pasteur effect), which is facilitated in a HIF-1 dependent manner (Schroeder et al., 2005). Therefore, high HIF-1 levels in the hypoxic tumor microenvironment is a well-established factor for aggressive tumor growth and a negative factor for cancer therapy (Ryan et al., 1998, 2000; Hopfl et al., 2002; Unruh et al., 2003).

Besides tumor hypoxia, which leads to HIF-α protein stabilization, the loss of tumor suppressor proteins, such as pVHL, p53 or PTEN, or oncogenes, such as v-src, can contribute to high HIF-α levels in cancer cells (Jiang et al.,

1997; Krieg et al., 2000; Ravi et al., 2000; Zundel et al., 2000). Both HIF-1α and HIF-2α are widely overexpressed in many human cancers and are frequently associated with malignancy and a poor prognosis (Birner et al., 2000; Aebersold et al., 2001). Furthermore, high HIF-1α protein levels have been shown to correlate with incomplete responses to chemotherapy and radiotherapy (Aebersold et al., 2001; Koukourakis et al., 2002; Bachtiary et al., 2003; Generali et al., 2006). Hypoxia *per se* affects radiation sensitivity as the radiation-induced DNA damage is dependent on oxygen (Gray et al., 1953). In addition, decreased cell proliferation and lower drug concentrations in the hypoxic tumor areas contribute to the resistance to chemotherapy. However, the underlying molecular mechanisms causing therapy resistance of hypoxic tumor cells are incompletely understood, but it is likely that HIF downstream targets are directly involved in these processes.

Targeting HIF to improve cancer therapy

HIF-1-dependent hypoxic induction of the multidrug-resistance MDR1 gene was one of the first described molecular mechanisms explaining the involvement of HIF-1 in chemotherapy resistance in various tumor cells, including breast carcinoma, gastric cancer, colon cancer and glioma (Comerford et al., 2002; Wartenberg et al., 2003; Zhou et al., 2005; Nardinocchi et al., 2009). Hypoxically dysregulated apoptosis in response to chemotherapy might be another explanation (Erler et al., 2004; Sermeus et al., 2008). The role of HIF-1 in the regulation of apoptosis is very complex and context specific. The involvement of HIF-1 in apoptosis in certain cell types cannot be generalized as cells do not undergo apoptosis under degrees of hypoxia sufficient for HIF-1 induction (Wenger et al., 1998). In primary cells, hypoxia typically leads to cell-cycle arrest and HIF-1-dependent apoptosis in cases of more severe conditions (Greijer and van der Wall, 2004). However, HIF-1 functions as a robust suppressor of apoptosis in most transformed cells. We previously reported that transformed mouse embryonic fibroblasts (MEFs) were more sensitive to chemotherapy as well as to radiotherapy in the absence of HIF-1α due to an impaired DNA double-strand break (DSB) repair capacity (Wirthner et al., 2008). The underlying molecular mechanism involves markedly reduced expression of DNA-PKcs, Ku80 and Ku70, three members of the DNA-dependent protein kinases (DNA-PK), in HIF-1α-deficient MEFs. Our data were supported by a large number of studies that demonstrated reversal of radio- and chemoresistance by targeting HIF-1α in various tumor types (Zhang et al., 2004; Moeller et al., 2005; Williams et al., 2005; Brown et al., 2006; Li et al., 2006a,b; Song et al., 2006; Sasabe et al., 2007). For example, Li et al. (2006a,b) showed that the knockdown of HIF-1α in breast carcinoma cells repressed G_0/G_1-phase accumulation and relieved S-phase block, thereby increasing sensitivity to chemotherapy and attenuating tumor growth (Li et al., 2006a,b). Functional interference with HIF-1α in various tumor cells has been shown to result in enhanced cell death upon treatment with chemotherapeutic agents (Ricker et al., 2004; Peng et al., 2006; Hao et al., 2008; Sermeus et al., 2008; Flamant et al., 2010). However, experimentally increasing HIF-1α enhanced therapy resistance (Ji et al., 2006; Martinive et al., 2006). Of note, HIF-1 in germ cells of *Ceanorhabditis elegans* has recently been reported to antagonize p53-mediated apoptosis due to DNA damage (Sendoel et al., 2010).

The induction of DNA damage by cytotoxic agents has proved to be an effective strategy for cancer therapy (Einhorn, 2002; Agarwal and Kaye, 2003; Pires et al., 2012). Mutations in DNA damage response (DDR) genes can lead to increased frequency and incorrect DNA damage repair, thereby contributing to the genomic instability characteristic for cancer cells (Bolderson et al., 2009). Because HIF-1-mediated therapy resistance was only observed when DSB, but not single-strand break (SSB)-inducing, agents were applied, we suspect that HIF-1 might be involved specifically in DNA-DSB repair (Unruh et al., 2003).

The DNA damage response in hypoxia

Upon DNA damage, histone H2AX is rapidly phosphorylated at Serine 139 by ataxia-teleangiectasia-mutated (ATM) kinase, ATM- and Rad3-related (ATR) kinase and DNA-PK (Fernandez-Capetillo et al., 2004; Zhang et al., 2006; Hurley and Bunz, 2007). Previous studies have suggested that severe hypoxia can elicit a DNA damage-like response, implying the activation of the ATR and ATM pathways and subsequent phosphorylation of H2AX (Hammond et al., 2003a,b; Bencokova et al., 2009). More recently, Economopoulou et al. (2009) identified a novel role for histone H2AX in hypoxia triggered angiogenesis. Replication specific γH2AX was found to be induced in an ATR-dependent manner in endothelial cells exposed to milder hypoxia (1% O_2). Whether HIF is involved in the hypoxic induction of γH2AX has not been analyzed so far. Therefore, we investigated a potential role for HIF-1 and

HIF-2 in the phosphorylation of H2AX under chronically hypoxic (0.2% O_2) conditions. Hypoxic γH2AX induction was observed in a range of cancer cell lines, was delayed in HIF-1α-deficient MEFs and, after HIF-1α and HIF-2α knockdown in Hek293 cells, was further decreased when both HIFs were downregulated. *Vice versa*, in 786-0 cells, devoid of pVHL and constitutively expressing HIF-2α, H2AX phosphorylation was increased and could be reversed by pVHL reconstitution. These results suggest that HIF plays a crucial role in the DNA damage response under hypoxia.

Results

γH2AX accumulation in chronic hypoxia

Hammond et al. previously reported that severe hypoxia/anoxia (0.02% O_2) leads to ATR-dependent γH2AX accumulation that was attributed to S-phase arrest (Hammond et al., 2002, 2003a,b). Because an atmospheric oxygen concentration of 0.02% O_2 results in a tissue partial pressure of oxygen that is most likely below the threshold for mitochondrial respiration, we investigated γH2AX induction under physiologically relevant hypoxic conditions. To ensure unimpaired mitochondrial respiration, an atmospheric oxygen concentration of 0.2% O_2 was chosen, corresponding to an oxygen partial pressure of approximately 1.5 mm Hg. Ischemia-like conditions and reoxygenation-induced ROS formation followed by DNA damage was prevented by replacing the cell culture medium every 24 h with pre-equilibrated medium and by harvesting the cells inside of a hypoxic workstation. Several cancer cell lines were exposed to 0.2% O_2 for 3–72 h, followed by analysis of H2AX Serine 139 phosphorylation by immunoblotting. As shown in Figure 1A, γH2AX accumulated in a time-dependent manner in all six cell lines and reached maximal induction after 24–48 h of hypoxic exposure, depending on the cell line. Hypoxic γH2AX induction in wild-type HEK293 cells with normal p53 was similar to SV40 large T antigen immortalized HEK293T cells, suggesting that p53 is not involved in hypoxic H2AX phosphorylation. Only wild-type HEK293 cells were used for subsequent experiments.

We next compared γH2AX accumulation in hypoxia with the effects of the topoisomerase II inhibitor and DSB inducing agent etoposide (Burden and Osheroff, 1998). γH2AX slowly accumulated in hypoxia, reaching a maximum after 48–72 h, and declined after 96 h (Figure 1B). Treatment for 1 h with etoposide in concentrations from 0.25 to 8 μM resulted in a similar, dose-dependent increase in γH2AX levels. Total H2AX levels remained unaffected after both hypoxic exposure and etoposide treatment (Figure 1B).

Hypoxic γH2AX accumulation is HIF dependent

The involvement of HIF in hypoxic H2AX phosphorylation was investigated by shRNA-mediated stable knockdown of HIF-1α and/or HIF-2α in HEK293 cells. Hypoxic γH2AX accumulation was delayed after shRNA-mediated knockdown of either HIF-1α or HIF-2α, with maximal levels only after 72 h compared to 24–48 h in the parental control (Figure 2A). Total H2AX remained unaffected (Figure 2A). Concomitant HIF-1α and HIF-2α double knockdowns substantially decreased hypoxic phosphorylation of H2AX at all time points (Figure 2B).

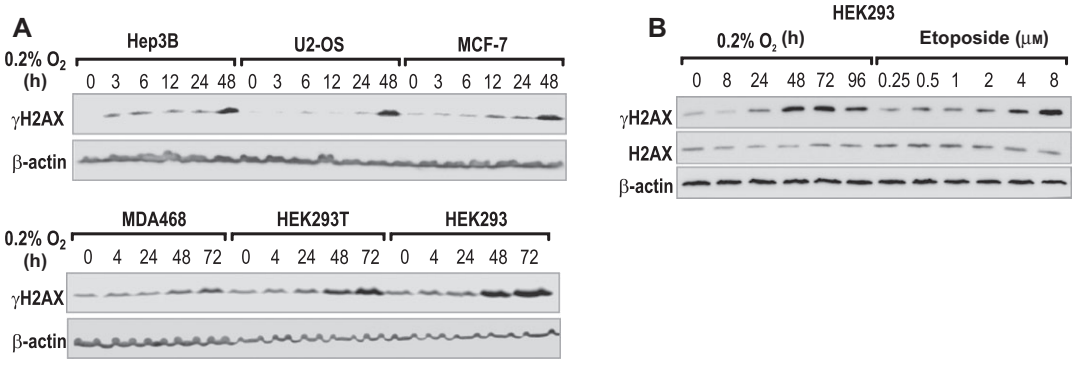

Figure 1 Phosphorylation of H2AX in chronic hypoxia.
(A) The indicated cancer cell lines were cultured under 20% or 0.2% O_2 conditions for up to 72 h, and γH2AX protein levels were analyzed by immunoblotting. β-Actin served as a control for equal loading and blotting. (B) HEK293 cells were exposed to 20% or 0.2% O_2 for up to 96 h or to various etoposide concentrations up to 8 μM for 1 h in normoxia. Phosphorylated and total H2AX were analyzed by immunoblotting.

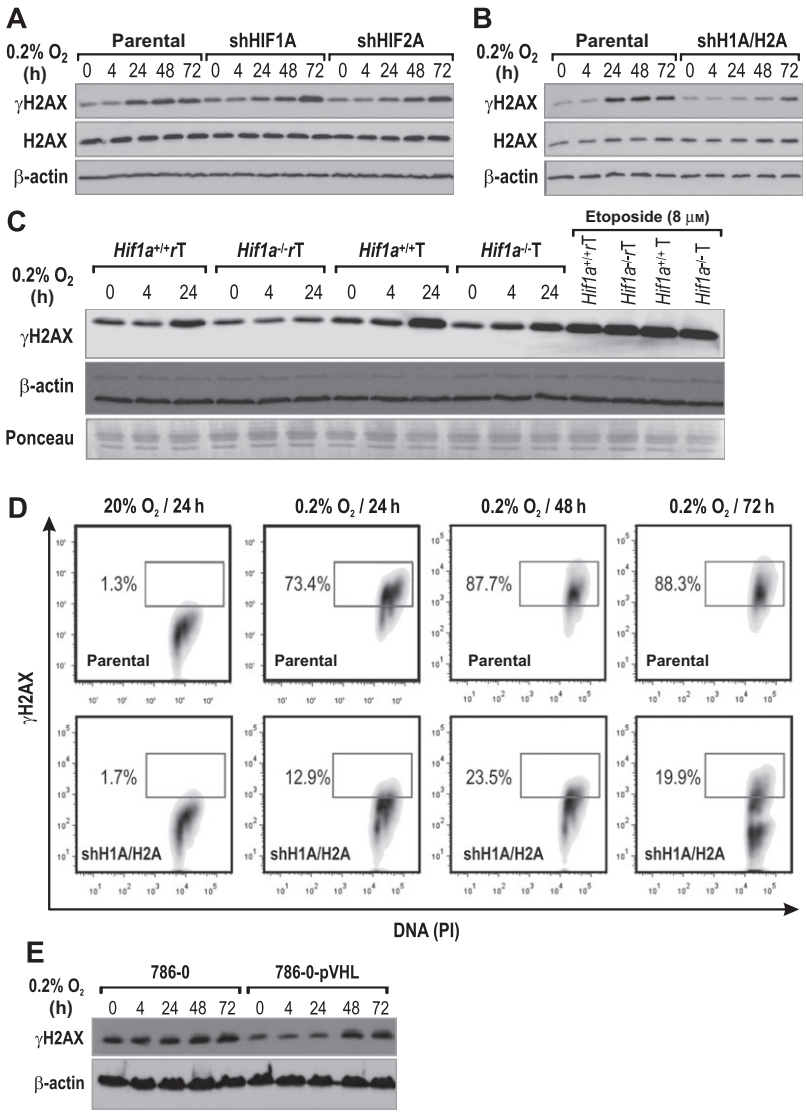

Figure 2 HIFs are required for hypoxic γH2AX accumulation.
(A, B) Parental, shRNA-mediated HIF-1α or HIF-2α knockdown (shHIF1A or shHIF2A, respectively) or HIF-1α/HIF-2α double knockdown (shH1A/H2A) HEK293 cells were grown under 20% or 0.2% O_2 conditions for the indicated time points. Phosphorylated and total H2AX was analyzed by immunoblotting, and β-actin served as a control for equal loading and blotting. (C) MEF-*Hif1a*$^{+/+}$rT, MEF-*Hif1a*$^{-/-}$rT, MEF-*Hif1a*$^{+/+}$T, MEF-*Hif1a*$^{-/-}$T were exposed to 20% or 0.2% O_2 for 4 or 24 h or treated with 8 μM etoposide for 1 h. Ponceau S staining was used as a control for equal extraction and loading of histones. (D) Parental and HIF-1α/HIF-2α double knockdown (shH1A/H2A) HEK293 cells were grown under 20% or 0.2% O_2 conditions for the time points indicated before γH2AX levels were analyzed by FACS. γH2AX positive cells were gated as indicated by the rectangles and quantified relative to the total cell number. (E) 786-0 and 786-0-pVHL cells were grown under 20% or 0.2% O_2 conditions for 24–72 h, and γH2AX and β-actin protein levels were analyzed by immunoblotting.

To corroborate these findings, two different MEF cell lines derived from two different HIF-1α knockout mouse strains were analyzed. These cell lines were either only immortalized by SV40 large T (MEF-*Hif1a*$^{-/-}$T) or immortalized and transformed by H-*ras* (MEF-*Hif1a*$^{-/-}$*r*T), respectively (Feldser et al., 1999; Ryan et al., 2000). Importantly, these MEF cell lines were shown to lack functional HIF-2α protein (Park et al., 2003). Confirming the results obtained with HEK293 cells, γH2AX levels in wt MEFs accumulated after 24 h exposure to 0.2% O_2 but were strongly impaired in MEFs devoid of HIF-1α. Total histone levels remained unaffected, as shown by Ponceau S-staining of the extracted histone fraction (Figure 2C). We previously reported increased susceptibility to DNA damage with enhanced phosphorylation of H2AX in MEF-*Hif1a*$^{-/-}$*r*T upon low dose (0.5–4 μM) etoposide treatment

(Wirthner et al., 2008). However, the HIF dependent difference of γH2AX levels decreased with higher doses of etoposide and was invisible upon treatment with 8 μM (Wirthner et al., 2008). In line with these findings, no HIF-1α-dependent changes in γH2AX induction could be observed after high-dose (8 μM) etoposide treatment, which resulted in γH2AX levels that were only slightly higher than the γH2AX levels in HIF-1α positive MEFs after 24 h of hypoxia (Figure 2C).

To further confirm the role of HIF in hypoxic γH2AX accumulation, parental and HIF-1α/HIF-2α double knockdown HEK293 cells were grown under 20% or 0.2% O_2 conditions for up to 72 h before γH2AX levels were quantified by FACS analysis. Whereas 88% of parental cells were strongly γH2AX positive after 48 and 72 h of hypoxia, only 20–24% of the HIF-1α/HIF-2α double knockdown HEKs showed similarly elevated γH2AX staining (Figure 2D).

Finally, VHL-deficient 786-0 cells, containing constitutively stabilized HIF-2α (Maxwell et al., 1999) and reconstituted 786-0-pVHL cells were cultured under 20% or 0.2% O_2 conditions for 4–72 h and analyzed by immunoblotting. In line with our findings above, both basal and hypoxic levels of γH2AX were substantially higher in 786-0 cells compared to 786-0-pVHL cells (Figure 2E).

Hypoxic γH2AX accumulation is independent of DNA-DSB formation

Hypoxia has previously been suggested to induce genetic instability associated with increased HIF-1α levels (Bristow and Hill, 2008). However, the previously published lack of detectable DNA damage at 0.02% O_2 suggests that hypoxic γH2AX accumulation might be partially or fully independent of DNA-DSB formation (Hammond et al., 2003a,b). To directly assess DNA-SSB and DNA-DSB formation under 0.2% O_2 conditions, we performed alkaline single-cell electrophoresis (comet assays) in wild-type and HIF-1α-deficient MEFs and concomitantly determined γH2AX protein levels by immunoblotting. As shown in Figure 3A, the emergence of DNA-DSB induced by 1 μM etoposide could be visualized reliably by 'comet halo' formation. Quantification of the median of the tail moment demonstrated a significant ($p<0.0001$) four-fold increase following treatment with 1 μM etoposide for 1 h, but not after up to 24 h of 0.2% O_2 (Figure 3B, upper panel). In contrast, γH2AX levels in HIF-1α wild-type MEFs were even higher after 12 and 24 h of hypoxia than following treatment with 1 μM etoposide (Figure 3B, lower panel). Taken together, these data suggest that DNA-DSB is not a major determinant of hypoxic γH2AX induction.

Discussion

Hypoxic regions in solid tumors result from an imbalance between cellular oxygen consumption and oxygen delivery as a consequence of inefficient tumor vasculature and limited oxygen diffusion (Chitneni et al., 2011). Rapid and frequent variations in red blood cell flux cause temporal and spatial variations in the degree of hypoxia

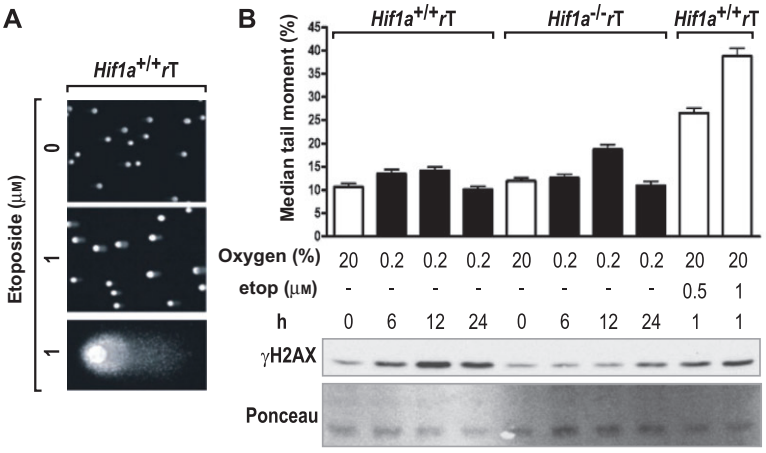

Figure 3 Hypoxia does not induce detectable DNA strand breaks.
(A) Representative example of a comet assay. DNA fragmentation in wild-type MEFs was induced by exposure to 1 μM etoposide for 1 h. DNA was stained with SYBR green, and all images were acquired with fixed exposure times. (B) DNA fragmentation was quantified by determining the median tail moment of at least 150 comets per condition using CometScore software. Data are shown as mean values±SEM. (top panel) Hypoxic induction of H2AX phosphorylation was confirmed by immunoblotting of parallel samples performed as in Figure 2C (bottom panel).

within the same tumor. We found that chronic hypoxia triggers the phosphorylation of the histone variant 2AX in a HIF-dependent manner. In line with previous reports (Hammond et al., 2003a,b), we showed that γH2AX levels after chronic hypoxia were comparable with etoposide treatment. Hypoxia (0.2% O_2) did not lead to detectable DNA damage when analyzed by alkaline single cell electrophoresis. Furthermore, proliferation and cell viability were not altered, even after long-term (3 days) hypoxic exposure (data not shown). However, conditions close to anoxia have been reported to have direct cytotoxic effects and elicit apoptosis (Papandreou et al., 2005). In line with our previous findings (Wirthner et al., 2008), 53BP1 dose dependently accumulated in distinct nuclear foci upon treatment with etoposide and partially overlapped with γH2AX staining (data not shown). These foci are most likely sites of DNA-DSBs. In contrast, in chronic hypoxia, γH2AX did not accumulate in nuclear foci but showed a more diffuse pattern throughout the nucleus (data not shown). A similar granular γH2AX and ATM phospho-S1981 staining has been reported previously to occur in response to severe hypoxia (0.02% O_2) (Hammond et al., 2003a,b; Bencokova et al., 2009). Hammond et al. found that severe hypoxia leads to replication fork stalling and ATR-dependent γH2AX accumulation during S-phase (Hammond et al., 2002, 2003a,b). Moreover, diffuse and pan-nuclear γH2AX staining has been found to occur upon non-ionizing UV-C irradiation, independent of DNA-DSBs (Marti et al., 2006). Infection with inactivated adeno-associated virus has been shown to lead to replication fork stalling and a diffuse γH2AX nuclear staining, which is essential for subsequent cell-cycle arrest in the absence of DNA damage (Fragkos et al., 2009). However, the mechanism behind this diffuse γH2AX distribution pattern as well as its functional relevance are currently unknown.

DNA-DSBs are serious lesions that can lead to genomic instability if improperly repaired, or ultimately to cell death if the repair machinery is saturated. It is essential that the cell closely monitors such stress conditions and initiates signals for an adequate response. Phosphorylation of H2AX on serine 139 is established as a sensitive marker for DNA-DSBs (Bonner et al., 2008). γH2AX is regarded as a key component for DNA repair, even though it seems dispensable for the initial recognition of DNA-DSBs, and H2AX-deficient mice are viable (Celeste et al., 2002, 2003).

The physiologic relevance of hypoxia-induced γH2AX is poorly understood. A recent report showed that hypoxia triggered neovascularization required endothelial H2AX, and γH2AX was induced in an ATR-dependent manner in moderate hypoxia due to replicative stress (Economopoulou et al., 2009). Genetic inactivation of H2AX was sufficient to suppress tumor angiogenesis and growth in xenograft models. However, this study did not address the question of whether HIFs are involved in this effect. In the present work, we were able to show that HIF is an integral factor required for efficient phosphorylation of H2AX under physiologically relevant hypoxic conditions, and that hypoxic γH2AX induction was delayed in the absence of HIF-α. We previously reported that DNA-PK expression was strongly reduced by the absence of HIF-1α under both normoxic and hypoxic conditions (Wirthner et al., 2008), raising the possibility that DNA-PK might be the responsible kinase for H2AX phosporylation in chronic hypoxia. In line with this hypothesis, accumulation of DNA-PKcs, Ku70 and Ku80 following hypoxia and iron chelation have been demonstrated in a number of different cell lines (Ginis and Faller, 2000; Lynch et al., 2001; Um et al., 2004; Bouquet et al., 2011). DNA-PK has been shown to phosphorylate H2AX in different cell lines and *in vivo* in response to DNA damage (Stiff et al., 2004; Koike et al., 2008; An et al., 2010) under hypertonic conditions (Reitsema et al., 2005) and during apoptotic DNA fragmentation (Mukherjee et al., 2006). Of note, the hypoxic DNA-PK activation resulted in increased HIF-dependent gene expression (Bouquet et al., 2011). These data suggest that DNA-PK might be both upstream and downstream of HIF.

In summary, our data indicate a novel DNA-DSB independent mechanism by which HIF downstream effectors might be involved in histone H2AX phosphorylation during hypoxia and, hence, could contribute to therapy resistance of hypoxic cancer cells.

Materials and methods

Cell culture and lentiviral transduction

All cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, Buchs, Switzerland) as described previously (Stiehl et al., 2006). For chronic hypoxic exposure, cells were grown in a gas-controlled glove box to handle the cells under constant oxygen (Invivo$_2$ 400, Ruskinn Technologies, Leeds, UK). Before medium change, all reagents were pre-equilibrated to the 0.2% O_2-containing gas mixture inside the glove box. Cell number, size and viability were determined by trypan blue exclusion using an automatic cell analyzer (Vi-Cell, Beckman-Coulter, Nyon, Switzerland). Stable knockdown of HIF-1α and HIF-2α in HEK293 cells by RNA interference was achieved by lentiviral transduction of short hairpin (shRNA) constructs. Viral particles were produced in HEK293T human enbryonic kidney cells using the ViraPower lentiviral expression system according to the manufacturer's protocol (Invitrogen, Basel, Switzerland) as described previously (Stiehl et al., 2012).

Immunoblot analysis

Histone immunoblotting was performed as described previously (Wirthner et al., 2008). Primary antibodies used were γH2AX (Millipore, Zug, Switzerland), total H2AX (Millipore), and β-actin (Sigma, Buchs, Switzerland). Horseraddish-peroxidase-coupled secondary anti-mouse and anti-rabbit antibodies were purchased from Pierce (Lausanne, Switzerland). Chemiluminescence detection was performed using Supersignal West Dura (Pierce), and signals were recorded and quantified using a charge-coupled device camera (Lightimager LAS-4000mini, Fujufilm, Dielsdorf, Switzerland). Extracted histones were stained with Ponceau S (Sigma).

Flow cytometry

Single cell suspensions were incubated with an antibody against γH2AX and propidium iodide (PI) according to the manufacturer's instructions. Stained cells were analyzed with a FACSCanto II utilizing FACSDiva software (BD Biosciences, Allschwil, Switzerland).

Single cell electrophoresis (comet assays)

Alkaline single cell electrophoresis was performed as described before (Wirthner et al., 2008). Briefly, MEFs were mixed with 0.5% low-melting-point agarose (Sigma), solidified on microscopy slides and lysed with 1% Triton-X100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 10.0) for 1 h at 4°C in the absence of light. Horizontal electrophoresis (~0.74 V/cm; 300 mA) was performed in 300 mM NaOH, 1 mM EDTA for 30 min. Following SYBR green (Invitrogen) staining, DNA migration was visualized by fluorescence microscopy, and the tail moment (%DNA in the tail multiplied by the tail length) was calculated from >150 cells per condition using the CometScore software package (TriTek, Sumerduck, VA, USA). Quantification of the median tail moments is shown as mean values ± standard error of the mean (SEM). Statistical analysis was performed applying two-tailed Student's t-test using GraphPad Prism version 4.0 (GraphPad Software, Ja Jolla, CA, USA).

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