Published in final edited form as:

Nat Med. 2009 May ; 15(5): 553–558. doi:10.1038/nm.1947.

Histone H2AX is integral to hypoxia-driven neovascularisation

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Introductory paragraph

H2A histone family member X (H2AX, also known as H2AFX) and its C-terminal phosphorylation (γ -H2AX) is important in the DNA damage response and mediates DNA repair(1–6). Hypoxia is a physiological stress that induces a replication-associated DNA damage response(7). Moreover, hypoxia is the major driving force for neovascularisation(8), as the hypoxia-mediated induction of vascular growth factors triggers endothelial cell proliferation(8). We studied here the importance of the hypoxia-induced DNA damage response in endothelial cell function and in hypoxia-driven neovascularisation *in vivo*. Hypoxia induced replication-associated generation of γ -H2AX in endothelial cells *in vitro* and in mice. Consistently, endothelial cell proliferation under hypoxia *in vitro* and in mice was reduced by H2AX deficiency. Whereas developmental angiogenesis was not affected in *H2ax*^{-/-} mice, hypoxia-induced neovascularisation during pathologic proliferative retinopathy, in response to hind limb ischemia, or during tumor angiogenesis was significantly reduced in *H2ax*^{-/-} mice. Together, our findings demonstrate that H2AX functions to help endothelial cells maintain their proliferation under hypoxia and thereby H2AX is important for hypoxia-driven neovascularisation. We identify here a novel crosstalk between the DNA repair response and hypoxia-driven neovascularisation.

Upon DNA damage induction in cells, histone H2AX is rapidly phosphorylated at a conserved SQ-motif in its C-terminus (aa residues 139–140)(3). H2AX can mediate DNA repair by maintaining repair/signalling factors in the proximity of the DNA damage site(3–6). The kinases orchestrating the DNA damage response include the ataxia teleangiectasia mutated (ATM), the ATM-and Rad3-related (ATR) and the DNA dependent protein kinase (DNA-PK) (2,3,9,10). These signalling pathways can be activated by ionizing radiation or by replication-associated stresses, such as hydroxyurea (HU) or aphidicolin(7,11,12). Hypoxia is also a

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replication-type stress. Previous studies suggested that hypoxia, although not necessarily inducing DNA damage to cells, can induce a DNA damage response including the activation of the ATR and/or the ATM pathway as well as the phosphorylation of H2AX(13–15).

Hypoxia is the major trigger of neovascularisation. Upon hypoxia the stabilization of the hypoxia inducible transcription factor (HIF) results in the upregulation of a large number of genes, including the vascular endothelial growth factor (VEGF), thereby stimulating neovascularisation-related endothelial cell functions such as proliferation and sprouting(8). While angiogenesis is important developmentally(8), pathologic conditions in adults may trigger hypoxia-induced neovascularisation(8). This hypoxia-triggered neovascularisation response may be insufficient, such as in ischemic diseases, while it is excessive in proliferative retinopathies such as the retinopathy of prematurity (ROP)(8,16). In ROP, the retinal hypoxia associated with reduced vascularisation after premature birth induces exorbitant abnormal vessel growth that may result in complications and even blindness(17).

At the initial steps of hypoxia-induced neovascularisation, endothelial cells are subject to hypoxia(8,18). As shown in tumor and other cells, hypoxia can induce replication arrest. However, endothelial cells proliferate in response to the hypoxic stimulus due to the increased availability of hypoxia-inducible vascular growth factors. Thus, we reasoned that endothelial cells subject to hypoxia may experience replication-associated stress and hypothesized that an efficient DNA repair response may be a prerequisite for endothelial cell proliferation and neovascularization under hypoxia.

In order to examine the DNA repair response to hypoxia in endothelial cells, we exploited the features of H2AX, since γ -phosphorylation of H2AX at S139 is a very sensitive marker of the DNA damage response(3–6), and a previous study has shown γ -H2AX induction in tumor cells by hypoxia(13). Moreover, H2AX can mediate DNA repair(3,4,5), thus, H2AX-deficient systems(4,19) provide an excellent tool to functionally address the role of DNA repair pathways in hypoxia-induced angiogenesis.

Hypoxia treatment induced distinct γ -H2AX foci in primary cultures of human umbilical vein endothelial cells (HUVEC) (Fig. 1a). As a comparison endothelial cells were subjected to low grade irradiation (2 Gy), which causes double stranded DNA breaks, or treated with hydroxyurea (HU), which induces replication-associated DNA damage (Fig. 1a). While irradiation induced γ -H2AX in all endothelial cells, hypoxia resembled HU in that it induced γ -H2AX formation only in a subpopulation of cells (Fig. 1a). Western blot analysis confirmed the generation of γ -H2AX in HUVEC upon hypoxia (suppl-Fig. 1). Hypoxia-induced γ -H2AX foci were predominantly present in proliferating endothelial cells that stained positive for proliferative cell nuclear antigen (PCNA) (20) (suppl-Fig. 2), and colocalized with replication protein A (RPA) (21) (suppl-Fig. 3), thereby suggesting a replication stress-associated generation of γ -H2AX by hypoxia. We then determined which of the two major signalling pathways, the ATM or ATR pathway, was involved in the generation of γ -H2AX in endothelial cells during hypoxia. Whereas siRNA-mediated knockdown of ATM did not affect the hypoxia-dependent γ -H2AX production, siRNA-mediated knockdown of ATR prevented the hypoxia-induced generation of γ -H2AX (Fig. 1b and 1c). Similar results were observed when different ATR-specific siRNAs were engaged (data not shown). That ATM was not involved in the hypoxia-induced response in HUVEC was also evidenced by the absence of ATM phosphorylation at S1981, which is a marker of ATM activation(9) (data not shown). These data suggest that hypoxia induces replication stress-associated γ -H2AX generation in endothelial cells in an ATR-dependent manner.

We then addressed the functional importance of the DNA repair response for endothelial cell proliferation and hypoxia-induced neovascularisation. To do so, we downregulated the

expression of H2AX in HUVEC by using siRNA (Fig. 1d), as well as we isolated endothelial cells from $H2ax^{+/+}$ and $H2ax^{-/-}$ mice, and studied the effect of loss of H2AX on endothelial cell proliferation under normoxia and hypoxia. Downregulation of H2AX induced a marginal reduction of the FGF2- or FCS-induced HUVEC proliferation under normoxia compared to cells transfected with control siRNA (Fig. 1e). Interestingly, H2AX knockdown significantly reduced the growth factor- or FCS-induced HUVEC proliferation under hypoxic conditions (Fig. 1e). Similar results were observed when the proliferation of H2ax-proficient and H2ax-deficient primary mouse endothelial cell cultures was assessed under normoxia and hypoxia (Fig. 1f). In contrast, other proangiogenic functions of the endothelium, such as extracellular matrix-dependent tube formation were not affected by the absence of H2AX (suppl-Fig. 4). These data indicate that H2AX is important in maintaining endothelial cell proliferation under hypoxic conditions.

The above findings pointed to a role of H2AX in hypoxia-driven neovascularisation. This hypothesis was investigated *in vivo* by engaging $H2ax^{-/-}$ mice that have deficient DNA repair (4,19). $H2ax^{-/-}$ and $H2ax^{+/+}$ mice were studied in physiological developmental angiogenesis in the retina that takes place between postnatal day p1 and p20, and in the mouse model of retinopathy of prematurity (ROP), which is a model for hypoxia-induced retina neovascularisation(17,22,23). In the ROP model p7 old pups are incubated in 75% oxygen for 5 days, which induces regression and obliteration in the developing retina vasculature, resulting in a massive retinal hypoxia after p12 and an increased endothelial cell proliferation and pathological exuberant neovascularisation (proliferative retinopathy)(17,22,23). In pups subjected to the ROP model we found increased generation of γ -H2AX during the hypoxia phase (p13-p15) compared to control mice kept under normoxic conditions (suppl-Fig. 5a). This could be attributed, at least in part, to γ -H2AX generation in endothelial cells during hypoxia-driven neovascularisation, as assessed by immunostaining, whereas little γ -H2AX staining associated with vascular nuclei was observed during normal vascular development (Fig. 1g). Induction of γ -H2AX in retinas of mice subjected to the ROP model was evident predominantly in the nuclei of proliferating retinal vascular endothelial cells including nuclei of pathological neovascular tufts (suppl-Fig. 5b). Thus, hypoxia induces generation of γ -H2AX in the retina vasculature *in vivo*.

Consistent with weak γ -H2AX generation in the developing retinal vasculature, we observed no difference between WT and $H2ax^{-/-}$ mice when we analysed their normal vascular development in the retina at days p5, p10 and p15 (suppl-Fig. 6). We then compared the hypoxia induced retina neovascularisation at p17 in WT and $H2ax^{-/-}$ mice that were subjected to the ROP model. A significant decrease in hypoxia-driven retina angiogenesis was observed in $H2ax^{-/-}$ mice. Immunohistochemical analysis of retinal sections revealed a 50% reduction in the extent of pathological neovascularisation in $H2ax^{-/-}$ mice (Fig. 2a, 2b). A reduction of pathological neovascular tufts and a more extensive physiological vasculature were observed in retina whole-mounts of $H2ax^{-/-}$ mice as compared to WT mice (Fig. 2c, 2d). The decrease in pathological retinal neovascularisation in $H2ax^{-/-}$ mice was also accompanied by a decrease in endothelial cell proliferation and an increase in endothelial cell apoptosis in $H2ax^{-/-}$ mice as compared to WT mice subjected to the ROP model (Fig. 2d–f). To demonstrate the role of endothelial H2ax in hypoxia-driven pathological angiogenesis and minimize the possibility that systemic H2ax deficiency could account for the observed phenotype, we generated endothelial-specific H2ax-deficient mice, by utilizing the VE-cadherin-Cre system(24) and the $H2ax$ floxed mice(25). Endothelial-specific H2ax-deficient mice displayed significantly reduced neovascularization as compared to H2ax-sufficient mice (Fig. 2g and suppl-Fig. 7). These findings indicate that endothelial H2ax is dispensable during normal vascular development but is functional in the context of hypoxia-driven retina angiogenesis.

The functional role of H2ax in postnatal hypoxia-driven angiogenesis was further studied in the hind limb ischemia model, in which neovascularisation is induced by the acute disruption of the blood supply of the hind limb and the consequent tissue ischemia and hypoxia(26). Consistent with the findings from the ROP model, *H2ax*^{-/-} mice displayed a 60% impairment in the neovascularisation in the hind limb ischemia model (Fig. 3a), which was also associated with a reduction in endothelial cell proliferation (Fig. 3b and 3c).

To provide further evidence for the specific function of H2ax in hypoxia-driven neovascularisation versus neovascularisation that can take place under normoxic conditions, we studied hypoxia-driven tumor angiogenesis in implanted Lewis lung carcinoma and angiogenesis directly induced by the presence of FGF2 in Matrigel. Lewis lung carcinoma cells were injected subcutaneously in WT and *H2ax*^{-/-} mice. To assess tumor angiogenesis, tumor sections were stained for the endothelial specific marker CD31. We observed a significant decrease in vascular density at two different time points (day 21 and day 25) in tumors implanted into *H2ax*^{-/-} mice (Fig. 4a–b). In addition, decreased endothelial cell proliferation and increased endothelial cell apoptosis was found in tumors implanted into *H2ax*^{-/-} mice (Fig. 4c–d), whereas pericyte coverage (NG-2-positive cells) of vessels was similar in tumor vessels of WT and *H2ax*^{-/-} mice (Fig. 4e), suggesting that H2ax deficiency leads to reduced tumor angiogenesis predominantly by affecting endothelial cell proliferation rather than vessel maturation. Associated with impaired tumor angiogenesis, both growth and the final weight of tumors were significantly reduced in *H2ax*^{-/-} mice (Fig. 4f–g). To investigate the role of endothelial H2ax in tumor angiogenesis, we implanted tumors into endothelial-specific H2ax-deficient mice and littermate H2ax-sufficient mice. Tumors implanted into endothelial-specific H2ax-deficient mice displayed significantly reduced neovascularization as compared to tumors implanted into H2ax-sufficient mice (suppl-Fig. 8a). In addition, tumor growth was significantly decreased in endothelial-specific H2ax-deficient mice (suppl-Fig. 8b). These data suggest that endothelial H2ax plays a role in hypoxia-driven tumor angiogenesis. In contrast to tumor angiogenesis, angiogenesis directly induced by FGF2 in the Matrigel model, was not affected by H2ax-deficiency (suppl-Fig. 9).

In the present work we identified that hypoxia-triggered neovascularisation requires the function of H2AX in endothelial cells. We demonstrated that hypoxia induced γ -H2AX in proliferating endothelial cells *in vitro* and in the mouse retina *in vivo* and our findings are in accordance with previous observations that hypoxia induces a replication stress-associated DNA damage response in other cells(7,13,27). The hypoxia-induced DNA repair response may not be the result of induction of DNA damage by hypoxia itself. Low levels of DNA damage happen during replication and the hypoxia-induced replication stress may result in accumulation of this damage(28). Alternatively, hypoxia has been proposed as a direct inducer of DNA damage by repressing genes crucial to DNA repair(29,30). Independent of the nature of its generation, the hypoxia-induced activation of γ -H2AX and an intact DNA repair response are of particular importance in endothelial cells during hypoxia-driven pathological neovascularisation, as this pathway helps the cells to maintain their proliferation under hypoxic conditions. This conclusion is strengthened by our findings that loss of H2AX in endothelial cells markedly compromised endothelial cell proliferation under hypoxia and only marginally under normoxia. Moreover, hypoxia-induced neovascularisation during pathologic proliferative retinopathy(22,23), in tumor angiogenesis or in response to hind limb ischemia (26) were significantly reduced by H2ax-deficiency, whereas neovascularisation that takes place under low levels of hypoxia, such as physiological developmental retinal angiogenesis was not affected. Our findings point to the attractive possibility that designing vascular-targeted inhibitors of H2AX may provide a new strategy for preventing hypoxia-induced pathologic neovascularisation, involved in pathologic conditions such as in tumors, or during ROP and diabetic retinopathy(16,17).

Materials and Methods

Endothelial cell culture and transfections

Detailed methods are described in the supplementary information.

In vitro immunofluorescence

Detailed methods are described in the supplementary information.

Western Blot

Detailed methods are described in the supplementary information.

In vitro endothelial cell proliferation assay

Detailed method is described in the supplementary information.

Mouse hind limb ischemic surgery

Detailed method is described in the supplementary information.

Mice and Hypoxia-induced retinal vascularization (ROP model)

H2ax^{-/-} mice were previously described(19). Mice were subjected to the retinopathy of prematurity model, which was previously described by us and others(22,23,31). At day p17 mice were sacrificed, and eyes were processed for quantification of preretinal neovascular nuclei, as described(22,23). Briefly, 6 μ m paraffin-embedded serial sections were stained with periodic acid Schiff (PAS) and 10 intact sections of equal length, each 18 μ m apart, were evaluated. All retinal vascular cell nuclei anterior to the internal limiting membrane were counted in each section. The mean of the neovascular nuclei was estimated from 10 counted sections. No neovascular cell nuclei anterior to the internal limiting membrane were observed in normal mice kept under normoxic conditions. Animal studies were approved by the NCI Animal Care and Use Committee, Bethesda, MD.

In addition, endothelial-specific *H2ax*-deficient and *H2ax*-sufficient mice were subjected to the ROP protocol. *H2ax*^{flox/flox} mice were previously described(25). We performed deletion of *H2ax* in endothelial cells, by crossing *H2ax*^{flox/flox} mice (kindly provided by Dr. F. Alt, Harvard University, Boston, MA) with *H2ax*^{+/-} mice which carried concomitantly the Cre-recombinase under the control of the *VE-cadherin*-promoter(24) (*VEC-Cre*⁺; *H2ax*^{+/-}). *VEC-Cre*⁺ mice were purchased from the Jackson Laboratory. Mice heterozygous for the floxed and deleted *H2ax* allele (*H2ax*^{flox/-}), which were positive for the *VEC-Cre*^{tg} (*VEC-Cre*⁺ *H2ax*^{flox/-}; endothelial-specific *H2ax*-deficient), as well as littermate control mice that were negative for the *VEC-Cre*^{tg} (*VEC-Cre*⁻ *H2ax*^{flox/-}; *H2ax*-sufficient mice) were generated. Efficient Cre-mediated excision in vascular endothelial cells was assessed by immunohistochemistry analysis (suppl-Fig. 7). Briefly, eyes from endothelial-specific *H2ax*-deficient or *H2ax*-sufficient mice were frozen and sectioned. The sections were fixed in ice cold acetone for 10 min, washed with PBS and blocked with 5% goat serum, 0.1% Triton in PBS. Subsequently they were incubated with a rabbit anti-total H2AX antibody (1:100, Novus) at 4°C overnight. After washing with PBS, the sections were further incubated with an AlexaFluor 568-conjugated goat anti-rabbit-antibody as well as with FITC-conjugated *Bandeirea Simplicifolia* isolectin B4 (Sigma-Aldrich) to visualize vessels, and DAPI. The images were obtained with a Zeiss laser scanning confocal microscope.

In vivo proliferation and apoptosis in the retina

Detailed methods are described in the supplementary information.

Retina Wholemounts

In order to prepare retina wholemounts, a previously published protocol was used(22,32). Briefly, mice were subjected to the ROP model. At day p16, mice were injected intraperitoneally with BrdU and sacrificed at day p17. The eyes were enucleated and fixed at 4°C overnight. After the retina was extracted and washed, it was permeabilized at 4°C overnight and stained with FITC-conjugated *Bandeirea Simplicifolia* isolectin B4. Some retinas were further stained for BrdU. These retinas were subjected to an antigen presentation with 6N HCl and 0.1% Triton, followed by blocking and incubation with a biotinylated anti-BrdU antibody (1:10, BD Biosciences). The detection of anti-BrdU was performed with AlexaFluor 568-conjugated streptavidin at 4°C for 6 h. Finally, the retinas were flatmounted and coverslipped. The images were obtained with a Zeiss laser scanning confocal microscope and with a Zeiss Axiovert 200M with Axiovision 4.6 software.

Retina Digestion and Immunofluorescence

Detailed method is described in the supplementary information.

Physiologic developmental retina angiogenesis

Detailed method is described in the supplementary information.

In vitro Matrigel tube formation assay

Detailed method is described in the supplementary information.

In vivo Matrigel assay

Detailed method is described in the supplementary information.

Tumor angiogenesis

A previously described protocol was followed(33). Lewis lung carcinoma (LLC) cells (10^6) were injected s.c. at two sites in the back of $H2ax^{-/-}$ or WT mice. Tumor volume and tumor weight after tumor excision was determined as described(33). Implanted tumors after different time points were excised and fixed in 4% PFA solution for 1 h at 4°C and incubated in 15% and 30% sucrose for 16 and 24 h respectively. Tissues were sectioned (8 μ m thick), and slides were blocked with 5% goat serum in 1% BSA and 0.1% Triton in PBS for 1 h at room temperature and stained with a rat anti-CD31 primary antibody (BD Biosciences) overnight at 4°C. After washing with PBS the sections were probed with an AlexaFluor 568-conjugated goat anti-rat antibody (Invitrogen). Tumor vascularity was assessed by measuring 6–10 sections per tumor.

In order to assess vessel proliferation, day 25 tumor sections were stained for CD31 and BrdU using the *in situ* BrdU kit (BD Biosciences) as described under “*In vivo* proliferation and apoptosis in the retina”. The BrdU positive vascular nuclei were counted in 2 random fields / section from 6 different sections / tumor. Apoptosis in the vessels of the tumors implanted in WT or $H2ax^{-/-}$ mice was assessed by staining for CD31 and cleaved caspase 3. The tumors were processed as above and sections were incubated with a rabbit antibody against mouse cleaved caspase 3 (1:300 Cell Signaling Technology) and with a rat anti-CD31 antibody overnight at 4°C. The detection of primary antibodies was performed by the addition of appropriate fluorescent labeled anti-rabbit and anti-rat secondary antibodies. The cleaved caspase 3-positive endothelial cells were counted in 3 random fields / section from 6 different sections / tumor.

For the estimation of pericyte coverage of the tumor vessels in the tumors implanted in $H2ax^{-/-}$ and $H2ax^{+/+}$ mice, we used double staining of CD31 and NG2. Ten μ m sections were

stained for CD31 as described above and were also incubated with a rabbit anti-NG2 (1:100, Millipore), at 4°C overnight. The detection of primary antibodies was performed by the addition of appropriate fluorescent labeled anti-rabbit and anti-rat secondary antibodies. The NG-2 positive cells that covered the vessels were counted in 4 different sections / tumor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by the Intramural Research Program of the NIH, National Cancer Institute. H.F.L. was supported by the German Academy of Sciences (Leopoldina). The authors would like to acknowledge M.E. Kruhlik for the help with confocal microscopy, D. Winkler and S. Kaul for help with genotyping, G. Tosato for help with Matrigel experiments, F. Alt for providing the *H2ax* floxed mice and A. Singer and D.S. Singer for critically reading the manuscript.

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Fig. 1. Generation of γ -H2AX by hypoxia in endothelial cells and importance of H2AX for endothelial cell proliferation under hypoxia

HUVEC were kept in normoxia, irradiated with 2 Gy, treated with HU, or were exposed to hypoxia for 1 h or 6 h, as indicated. **(a)**, Immunofluorescence staining for γ -H2AX (green) followed by confocal microscopy was performed. Nuclei are shown by DAPI staining. The percentage of cells displaying γ -H2AX foci was $3.1 \pm 0.5\%$, $99.1 \pm 0.4\%$, $19.1 \pm 1.2\%$, $10.9 \pm 0.7\%$ and $17.1 \pm 1.2\%$ for normoxic, irradiated, HU-treated, 1 h hypoxia-treated or 6 h hypoxia-treated cells, respectively (Mean $\pm$ SEM). Representative images are depicted. Bar=10 μ m. **(b)**, Left panel: Representative western blot analysis of the expression of ATR and tubulin in HUVEC transfected with siRNA against ATR or with control siRNA as indicated. Right panel:

Representative western blot analysis of the expression of ATM and tubulin in HUVEC transfected with siRNA against ATM or with control siRNA as indicated. (c), Western blot analysis for γ -H2AX or actin in HUVEC transfected with siRNA against ATR, siRNA against ATM or with control siRNA as indicated, that were kept in normoxia or exposed to hypoxia for 6 h. ATR knockdown decreased the hypoxia-induced γ -H2AX generation. (d), The expression of total H2AX and actin was analysed by Western blot in HUVEC transfected with siRNA against H2AX or with control siRNA as indicated. (e), The proliferation of HUVEC transfected with control siRNA (open bars) or with siRNA targeting H2AX (filled bars) was assessed under normoxic conditions or under hypoxic conditions, as indicated, in the presence of 10% FCS or FGF2 (50 ng/ml). Proliferation of HUVEC is expressed as percent in relation to control. Baseline proliferation under each condition in the absence of stimuli was defined as the 100% control. Data are Mean $\pm$ SEM ($n=4$). *: $P<0.05$. (f), The proliferation of WT and *H2ax*^{-/-} primary mouse lung endothelial cells was assessed under normoxic conditions or hypoxic conditions in the presence of 1% FBS (baseline conditions) or in the presence of 20% FBS together with 1% endothelial cell growth serum (ECGS) (stimulated conditions). Proliferation was assessed by cell counting and is expressed as absolute cell number. Data are Mean $\pm$ SEM ($n=3$). *: $P<0.05$. (g), Mice were subjected to the ROP model. Eyes were removed and retinas were extracted at day p15. Immunofluorescence analysis in retinas for lectin (red) indicating blood vessels, γ -H2AX (green) and DAPI (blue) in mice subjected to the ROP model and in mice kept in room air (non-ROP). In addition, γ -H2AX and DAPI staining were merged. Bar=10 μ m.

Fig. 2. A role for H2ax in retina neovascularisation under hypoxia

Mice were subjected to the ROP model. (a), Retinal neovascularization was quantified on day p17 in WT and *H2ax*^{-/-} mice as described under Materials and Methods. Retinal neovascularization is presented as the number of epiretinal neovascular nuclei per section. Data are Mean ± SEM (*n*=3–5). *: *P*<0.05. (b) Six µm paraffin-embedded axial sections of the retina were stained with periodic acid Schiff and hematoxylin. In the ROP model pathologic neovascularizations are observed anterior to the internal limiting membrane. *H2ax*^{-/-} mice that were subjected to the ROP-model displayed less neovascular regions anterior to the internal limiting membrane as compared with WT mice. The arrows indicate neovascularizations anterior to the internal limiting membrane. Bar=100 µm. (c), Lectin staining of retina whole-

mounts at p17 from a WT or a $H2ax^{-/-}$ mouse that were subjected to the ROP model, demonstrating less extensive neovascular tufts in $H2ax$ -deficient mice. For each image, multiple individual images were stitched together. Bar=500 μ m. **(d)**, Lectin (green) and BrdU (red) staining of retina whole-mounts at p17 from a WT or a $H2ax^{-/-}$ mouse that were subjected to the ROP model. Arrows indicate the neovascular tufts. Bar=100 μ m. **(e)**, Endothelial cell proliferation in the retina of mice subjected to the ROP model was assessed by BrdU incorporation as described under Materials and Methods. The number of BrdU-positive endothelial cell nuclei per section was reduced in the retinas of $H2ax^{-/-}$ mice as compared to WT mice. Endothelial cell proliferation is shown as % of control, i.e. endothelial cell proliferation in WT mice represents the 100% control. Data are Mean $\pm$ SEM ($n=3-4$). *: $P<0.05$. **(f)**, The apoptosis in retina vessels was assessed by TUNEL staining, showing that endothelial cells in retinas of $H2ax^{-/-}$ mice displayed increased apoptosis compared to retinas of WT mice. Endothelial cell apoptosis is shown as % of control, i.e. endothelial cell apoptosis in WT mice represents the 100% control. Data are Mean $\pm$ SEM ($n=3$). *: $P<0.05$. **(g)**, Retinal neovascularization was quantified on day p17 in $H2ax$ -sufficient ($VEC-Cre^{-}H2ax^{flox/-}$; open bars) and endothelial-specific $H2ax$ -deficient ($VEC-Cre^{+}H2ax^{flox/-}$; filled bars) mice. Retinal neovascularization is presented as the number of epiretinal neovascular nuclei per section. Data are Mean $\pm$ SEM ($n=5-6$). *: $P<0.05$.

Fig. 3. Reduced angiogenesis in response to hind limb ischemia due to H2ax deficiency
 Recovery ability of *H2ax*^{-/-} mice and WT mice after hind limb ischemic surgery. **(a)**, Doppler scanning monitoring results revealed significant difference between the two groups, with the WT group recovered to 96.6% and 70.7% of *H2ax*^{-/-} group recovered to only 48% and 42.9% (*n*=5). **(b)**, Endothelial cell proliferation in the ischemic muscle in *H2ax*^{-/-} and WT mice: BrdU and CD31 double immunostaining revealed that the CD31⁺/BrdU⁺ ratio of *H2ax*^{-/-} mice was significantly less than the CD31⁺/BrdU⁺ ratio of WT mice. Data are Mean ± SEM. **(c)** Representative images of BrdU (red) and CD31 (green) double immunostaining in ischemic muscles of WT and *H2ax*^{-/-} mice. Blue: DAPI staining to identify nuclei was used. Bars=10 μm. *, *P*<0.05; **, *P*<0.01.

Fig. 4. Reduced tumor angiogenesis and tumor growth due to H2ax deficiency

(a), Tumors implanted in H2ax^{-/-} mice exhibited decreased vascularity. Tumors that were implanted s.c. into WT or H2ax^{-/-} mice, were excised, fixed and stained immunofluorescently for blood vessels (CD31). Tumor angiogenesis is shown as vessels per section at days 21 and 25 and the data are expressed as % of control, i.e. tumor angiogenesis at day 21 or day 25 in WT mice was set as the 100% control, respectively. Data are Mean ± SEM (n=6–10 tumors). *: P<0.05; **: P<0.01. (b), Representative images of CD31 staining from tumors implanted into WT or H2ax^{-/-} mice are shown. Bar=100 μm. (c), Endothelial cell proliferation in tumors excised from WT and H2ax^{-/-} mice was assessed by evaluating the number of BrdU-positive endothelial cell nuclei per field. Endothelial cell proliferation is shown as % of control, i.e.

endothelial cell proliferation in WT mice represents the 100% control. Data are Mean $\pm$ SEM ($n=6-9$ tumors). *: $P<0.05$. **(d)**, Endothelial cell apoptosis in tumors excised from WT and $H2ax^{-/-}$ mice was assessed, by evaluating the number of caspase 3-positive endothelial cells per field. Endothelial cell apoptosis is shown as % of control, i.e. endothelial cell apoptosis in WT mice represents the 100% control. Data are Mean $\pm$ SEM ($n=4$ tumors). *: $P<0.05$. **(e)**, Pericyte coverage of vessels in tumors of WT and $H2ax^{-/-}$ mice was assessed by NG-2 staining. Pericyte coverage is shown as the percentage of vessels that were covered by NG-2-positive cells. Data are Mean $\pm$ SEM ($n=8$ tumors). **(f)**, Tumors implanted into $H2ax^{-/-}$ mice exhibited decreased tumor progression, as assessed by monitoring tumor volume after day 6. Data are Mean $\pm$ SEM ($n=8-10$ tumors). **: $P<0.01$, as compared to the tumor volume of WT mice on the same day. **(g)**, The weight of tumors excised from WT or $H2ax^{-/-}$ at day 25 is shown. Data are Mean $\pm$ SEM ($n=8-10$ tumors). *, $P<0.05$.