Genetic deletion of Nox4 enhances cancerogen-induced formation of solid tumors

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Edited by Navdeep S. Chandel, Northwestern University, Chicago, IL, and accepted by Editorial Board Member Tak W. Mak January 25, 2021 (received for review September 30, 2020)

Reactive oxygen species (ROS) can cause cellular damage and promote cancer development. Besides such harmful consequences of overproduction of ROS, all cells utilize ROS for signaling purposes and stabilization of cell homeostasis. In particular, the latter is supported by the NADPH oxidase 4 (Nox4) that constitutively produces low amounts of H2O2. By that mechanism, Nox4 forces differentiation of cells and prevents inflammation. We hypothesize a constitutive low level of H2O2 maintains basal activity of cellular surveillance systems and is unlikely to be cancerogenic. Utilizing two different murine models of cancerogen-induced solid tumors, we found that deletion of Nox4 promotes tumor formation and lowers recognition of DNA damage. Nox4 supports phosphorylation of H2AX (γH2AX), a prerequisite of DNA damage recognition, by retaining a sufficiently low abundance of the phosphatase PP2A in the nucleus. The underlying mechanism is continuous oxidation of AKT by Nox4. Interaction of oxidized AKT and PP2A captures the phosphatase in the cytosol. Absence of Nox4 facilitates nuclear PP2A translocation and dephosphorylation of γH2AX. Simultaneously AKT is left phosphorylated. Thus, in the absence of Nox4, DNA damage is not recognized and the increased activity of AKT supports proliferation. The combination of both events results in genomic instability and promotes tumor formation. By identifying Nox4 as a protective source of ROS in cancerogen-induced cancer, we provide a piece of knowledge for understanding the role of moderate production of ROS in preventing the initiation of malignancies.

Author contributions: K.S. designed research; V.H., F.F.v.G., N.H., M.M.K., F.R., J.H., I.W., V.M., K.A., and K.S. performed research; N.H., M.M.K., T.S., J.H., I.W., H.H.R., V.M., K.A., A.M.S., S.F., J.B., and R.P.B. contributed new reagents/analytic tools; V.H., F.F.v.G., N.H., M.M.K., T.S., J.H., I.W., V.M., K.A., and K.S. analyzed data; and K.S. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. N.S.C. is a guest editor invited by the Editorial Board.

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The stereotype of ROS produced by NADPH oxidases as cause of malignant diseases persists in a generalized manner. In fact, high levels of ROS formation could be harmful in the context of a disease process. This study demonstrates that loss of the NADPH oxidase Nox4, as a constitutive source of ROS, promotes cancerogen-induced formation of solid tumors. Accordingly, a certain tonic, constitutive low level of Nox4-derived hydrogen peroxide appears to reduce the risk of cancerogen-induced tumor formation.

Significance

Author contributions: K.S. designed research; V.H., F.F.v.G., N.H., M.M.K., F.R., J.H., I.W., V.M., K.A., and K.S. performed research; N.H., M.M.K., T.S., J.H., I.W., H.H.R., V.M., K.A., A.M.S., S.F., J.B., and R.P.B. contributed new reagents/analytic tools; V.H., F.F.v.G., N.H., M.M.K., T.S., J.H., I.W., V.M., K.A., and K.S. analyzed data; and K.S. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. N.S.C. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2020152118/-/DCSupplemental.

Published March 8, 2021.

PNAS 2021 Vol. 118 No. 11 e2020152118
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Results
Loss of Nox4 Promotes Tumor Development. In order to determine the impact of Nox4 on tumor development, two different cancerogen-induced and inflammation-driven cancer models in Nox4 knockout mice were analyzed. When compared with wild-type (WT) mice, Nox4 knockout mice develop twice as many tumors in the azoxymethane/dextran sulfate sodium (AOM/DSS) colon carcinoma model (Fig. 1 A–C). Similar results were obtained in a second model of fibrosarcoma development in response to subcutaneous 3-methylcholanthrene (MCA) injection. In Nox4<sup>−/−</sup> mice, onset of tumor development was earlier and developing tumors grew faster than in WT animals (Fig. 1 D–F). Proliferation in both tumor models was more than doubled in Nox4<sup>−/−</sup> mice when compared with WT mice (Fig. 1 G and H).

Importantly, these effects were specific for Nox4: Deletion of Nox1 rather had an inhibitory effect on tumor development, and deletion of Nox2 was without effect at all (SI Appendix, Fig. S1). In order to exclude unknown effects of adaptation to life-long Nox4 deficiency as a reason for more tumor development, conditional Nox4 knockout mice with tamoxifen-inducible deletion of Nox4 were studied in the MCA model. Although systemic administration of tamoxifen increased the number of Nox4 flox/flox control animals with sarcoma, the relative increase in tumor development in tamoxifen-induced systemic Nox4 knockouts was comparable to Nox4<sup>−/−</sup>. Conditional deletion of Nox4 in macrophages and endothelial cells, in contrast, was without effect on tumor development, suggesting that most likely Nox4 in the transforming cells but not in the tumor stroma mediates the protective effect. Indeed, when we performed similar experiments in fibroblast and enterocyte-specific Nox4<sup>−/−</sup> mice, formation of both kinds of tumors (sarcoma and carcinoma) was enhanced (SI Appendix, Fig. S2). Whether the effect of Nox4 deficiency on proliferation seen in this study is a consequence of an earlier onset of tumor formation or faster proliferation of the tumor cells when missing Nox4 remains elusive (SI Appendix, Fig. S3). The effect of Nox4 on proliferation is discussed controversially (14, 19–21). Conflicting results suggest that unknown differences in the cell culture of different laboratories influence the results. Tumor growth and progression can result from many effects.

Fig. 1. Nox4 knockout promotes tumor development and induces genomic instability. (A–C) AOM-DSS colon carcinogenesis model: Representative macro photo (A), H&E overview (B), and average number of solid carcinoma nodules (C) in the colon induced by AOM/DSS. (n = 11), **P < 0.05 (D–F) MCA fibrosarcoma model. Fibrosarcoma development (D), tumor size (E) (100% equals the maximal tumor size of 1.5 cm, n = 10), and relative tumor growth (F). (G and H) Colon carcinomas and sarcomas stained for phosphorylated histone H3 (pH3) as proliferation marker. The statistics indicate the relative staining intensity (n = 5 to 9). (I and J) Measurement of DNA double-strand breaks in fibrosarcoma cells was performed using comet assay (n = 5). Comet assay of cells treated with or without H₂O₂ (24 h, 5 μM). Original pictures (I) and statistics (J) *P < 0.05; WT vs. Nox4<sup>−/−</sup>; #P < 0.05 CTL vs. H₂O₂ treated. (K) Conditional tamoxifen-inducible knockout mice were treated with tamoxifen for 10 d and 1 wk later cells were isolated from multiple organs and analyzed for DNA fragmentation by Nicoletti assay (n = 3).
without significant individual impact, which nevertheless in vivo sum up to significant differences that highly influence tumor progression.

**Knockdown of Nox4 Results in Genomic Instability.** Genomic instability is an important factor to promote tumor development. Cell lines from WT and Nox4 tumors were established and comet assays for detection of DNA fragmentation were performed. Nox4 knockout tumor cells exhibited a doubling in the number of comets, which indicate DNA double-strand breaks. This effect was reversible, when the tumor cells were treated with H2O2 (5 μmol/L, 24 h) in Nox4−/− tumor cells, whereas comet formation in WT tumor cells remains equal (Fig. 1 I and J). These observations suggest that lack of Nox4-derived H2O2 may promote genomic instability. Indeed, Nicoletti staining of lung, liver, and intestine of healthy Nox4−/− mice demonstrated that DNA strand breaks are more frequent after deletion of Nox4 even in normal tissue (Fig. 1 K).

**Loss of Nox4 Results in Attenuated Recognition of DNA Damage.** Increased DNA fragmentation could be a consequence of more DNA damage or less DNA damage repair. Overwhelming ROS formation can induce DNA damage. However, knockout of Nox4 reduced the level of AmplexRed-detectable H2O2, and no major difference in other ROS was found (SI Appendix, Fig. S4). Total glutathion (GSH) and oxidized glutathion (GSSG) were similar in Nox4−/− and wild-type, while the GSH/GSSG ratio was reduced in Nox4-deficient cells (SI Appendix, Fig. S5). We conclude that DNA fragmentation in Nox4−/− is not a consequence of ROS-induced DNA damage. Despite more DNA fragmentation, phosphorylation of histone 2AX (γH2AX) as a marker of DNA damage decreased in tumors and tumor cells of Nox4−/− mice when compared with WT mice. Accordingly, although DNA fragmentation was enhanced in healthy colon cells and fibroblasts of Nox4−/− mice, γH2AX abundance was lower than in wild-type cells (Fig. 2 A–D). Further, acute exposure of fibroblasts to the carcinogen MCA increased γH2AX less effectively in Nox4−/− cells than in WT cells (Fig. 2D). Expression and activity of the kinase ATM, which phosphorylates H2AX in response to damage and initiates the DNA damage response, was not reduced in Nox4-deficient cells (SI Appendix, Fig. S6). ATM kinase also is a key molecule that stabilizes p53 after DNA damage. Despite a normal function of ATM, but in line with the missing induction of γH2AX, p53 protein and mRNA expression were reduced in Nox4 knockout tumors and cells (Fig. 2 E–H). Treatment with H2O2, the product of Nox4, normalized p53 mRNA expression in Nox4-deficient cells but had no effect in WT cells (Fig. 2F). Importantly, the decreased p53 expression was not a consequence of altered phosphorylation or translation of p53 (SI Appendix, Fig. S7 A–D) nor was the expression of enzymes involved in p53 degradation, like NQO1 and MDM2, increased in Nox4−/− cells (SI Appendix, Fig. S7 E and F). In order to study whether the reduced p53 expression is a consequence of an inappropriate response to DNA damage, healthy lung endothelial cells from WT and Nox4−/− mice were stimulated with the carcinogen MCA. This treatment strongly induced p53 expression in WT but not in Nox4−/− cells (Fig. 2I). These
data indicate a general attenuation of DNA damage response in the absence of Nox4.

**Nox4 Deficiency Enhances Nuclear PP2A Activity.** Steady-state levels of γH2AX are raised by the kinase ATM and reduced by serine-threonine phosphatases of the PP2A family (22, 23). Given the normal ATM expression and activity, we hypothesized that PP2A activity is altered in Nox4−/− cells. Indeed, by proximity ligation assay (PLA) with a pan-C subunit antibody for the PP2A family an increased association of a PP2A member with γH2AX in the nucleus was observed (Fig. 3A). Whereas the total cellular expression of PP2A was similar in WT and Nox4−/− cells, a shift toward more nuclear PP2A was observed in Nox4−/− cells (Fig. 3B). Consequently, global serine-threonine phosphatase activity was decreased in the cytosol and increased in the nucleus of Nox4−/− cells, when compared with the corresponding WT cells (Fig. 3C). In order to verify that an increased nuclear PP2A activity causes reduced γH2AX abundance, experiments with the pan-PP2A inhibitor okadaic acid at a concentration of 1 nM were performed. At this concentration, the inhibitor is highly selective for PP2A, whereas at higher concentrations, other phosphatases are blocked as well (24). Okadaic acid reduced nuclear phosphatase activity in Nox4−/− cells to the level observed in WT cells (Fig. 3C). In line with this, okadaic acid restored the level of γH2AX in Nox4−/− cells to the level of WT cells but had no effect on γH2AX level in WT cells (Fig. 3D). Strikingly, low concentration of H2O2 that mimics Nox4 activity, restored γH2AX and reduced nuclear PP2A in Nox4−/− cells to the level of WT cells (Fig. 3E and F). Collectively, these observations suggest that Nox4-derived H2O2 facilitates accumulation of PP2A in the cytosol. In the absence of Nox4, and thus lack of H2O2, PP2A accumulates in the nucleus, where it dephosphorylates γ2HAX. Consequently, absence of Nox4 attenuates DNA damage response and promotes genomic instability.

**Nox4 Deficiency Retains PP2A in the Cytosol where It Interacts with and Dephosphorylates AKT.** Given the sophisticated regulation of PP2A enzyme complexes and their numerous targets, literature mining was performed to obtain a plausible candidate mechanism. Out of several cytosolic proteins promoting tumor survival, the family of AKT kinases is of outstanding importance (25). AKT is highly abundant in tumors (26) and PP2A family members are responsible for the dephosphorylation of AKT (27, 28). Importantly, in a very different context it was suggested that oxidation of AKT can promote its interaction with PP2A (29). On this basis, we speculated that cytosolic interaction of PP2A and oxidized AKT could cause the reduced nuclear localization

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**Fig. 3.** Nox4 deficiency enhances nuclear PP2A activity, this decreases nuclear γH2AX but increases cytosolic AKT phosphorylation. (A) Interaction between PP2A and γH2AX as determined by proximity ligation assay. Quantification of colocalization relative to the number of cells stained with DAPI in %. (B) PP2A abundance in cytosolic and nuclear fraction as evaluated by Western blot. (C) Serine-threonine phosphatase activity as measured under basal conditions and after treatment with OA in cytosol and nucleus with the aid of NPP as an artificial substrate. (D and E) γH2AX abundance as quantified by immunoblotting with and without 1 nM of the PP2A inhibitor OA overnight (D) or H2O2 (24 h, 5 μM) (E). (F) Nuclear expression of PP2A after treatment with or without H2O2 as determined by Western blot. All experiments were carried out in isolated fibrosarcoma cells (F). Statistics are represented as numbers below the representative Western blots; *P < 0.05 WT vs. Nox4−/−, *P < 0.05 Nox4−/− CTL vs. Nox4−/− OA/H2O2, n = 5. (G) Interaction between AKT and PP2A as determined by PLA. Quantification of colocalization relative to the number of cells stained with DAPI in % (H). Western blot for phosphorylation of AKT at Ser473 in fibrosarcoma tissue, colon carcinoma tissue, and isolated fibrosarcoma cells of WT and Nox4−/− mice.
of PP2A. If this hypothesis was correct, nuclear translocation of PP2A in the absence of Nox4 should result in a decreased association of PP2A with its target AKT, and this was what we found (Fig. 3G). As PP2A dephosphorylates AKT, the reduced association of AKT and PP2A leaves more AKT phosphorylated in tumor tissue and isolated cells (Fig. 3H). Importantly, no evidence for additional phosphorylation of AKT was found. Oxidation, expression, and activity of inducers of AKT phosphorylation such as PTEN or PIP3 were normal (SI Appendix, Figs. S8 and S9). We conclude that in the absence of Nox4, PP2A and AKT associate less effectively, which results in lower AKT dephosphorylation and enhanced nuclear translocation of PP2A.

AKT Is a Redox Target of Nox4. The findings described above render the possibility that Nox4 directly promotes the association of AKT and PP2A. Given that the only known function of Nox4 is to produce H2O2, redox alterations were likely to be the underlying mechanism. Redox-BIAM switch assays were performed in order to visualize oxidation of AKT. Indeed, loss of Nox4 resulted in a significant reduction of AKT oxidation (Fig. 4A). A targeted redox-proteomics approach did not yield sufficient coverage of the peptide sequence of AKT isolated from tissues. Therefore, redox-active cysteines of AKT were mapped from recombinant protein. In a first step, all redox sites of the recombinant AKT were reduced, followed by incubation with H2O2. This redox stimulation forced disulfide bridge formations between cysteine 60 and 77 and between cysteine 296 and 310, the latter being conserved in all AKT homologs (SI Appendix, Fig. S10 and Tables S1 and S2). We conclude that Nox4-dependent H2O2 formation changes the confirmation of AKT by altering disulfide bridge formation. Accordingly, AKT may provide a redox switch responsible for PP2A translocation.

Oxidized AKT Interacts with the PPP2R1A Scaffolding Subunit of PP2A. The redox nature of the interaction of PP2A and AKT was then analyzed in more detail. Immunoprecipitation of AKT followed by proteomics analysis revealed that PPP2R1A coprecipitates with AKT (Fig. 4C and SI Appendix, Table S3). PPP2R1A, also known as PP2A subunit A R1α isoform or PR65α, is a scaffolding molecule that coordinates, besides many others, assembly of the catalytic C subunit and the variable regulatory B subunit (30). Potentially, due to detergent conditions optimized for specificity, the PP2A protein complex was disrupted and neither the B nor the C subunit were detected in that approach. Therefore, regular coimmunoprecipitation experiments followed by Western blots with

**Fig. 4.** The interaction of PP2A and AKT is redox sensitive. (A) AKT redox modification as analyzed by BIAM switch assay in fibrosarcoma cells of WT and Nox4−/− (n = 3). (B) Mapping of the H2O2-dependently formed disulfide bridges on the structure of human AKT1 as obtained from 4EJN (25) (SI Appendix, Tables S1 and S2). (C) AKT1 to 3 proteins were immune-captured and copurified interacting proteins were identified by LC/MS. Label-free quantification (LFQ) values were statistically analyzed using permutation test (FDR < 0.05, 250 randomizations) (SI Appendix, Table S3). Blue and red dots represent significantly enriched proteins. Black and gray dots were enriched in negative control or background, respectively. Marked protein Ppp2r1a was found to be significantly enriched in AKT1 to 3 pull-down samples. (D) Coimmunoprecipitation of AKT followed by Western blotting for PP2A catalytic subunit after overexpression with GFP, the redox-dead mutants for AKT1 to 3 and phospho-dead AKT1 mutant. (E and F) Proximity ligation in wild-type fibrosarcoma cells after overexpression of AKT1 and AKT3 and AKT3 redox-dead mutants (F). Antibodies used for AKT1 were PP2A and HA and for AKT3 PP2A and GFP. Red indicates dots of proximity, while gray represents DAPI staining of nuclei. Results of cells overexpressed with wild-type AKT were set to 100% (E). *P < 0.05 was considered significant.
an antibody directed against both C subunits of PP2A (31) were performed. These experiments demonstrated an interaction of PP2A subunit C with AKT in WT cells, which was impaired in Nox4−/− cells (Fig. 4D). Overexpression of a kinase-dead mutant (T308A/S473A) rescued coprecipitation of PP2A and AKT to the level observed in wild-type cells (Fig. 4D). In contrast, overexpression of redox-dead mutants of AKT did not restore coprecipitation of AKT and PP2A. Accordingly, PLAs demonstrated an attenuated interaction of redox-dead AKT mutants with PP2A even in WT cells (Fig. 4E and F). Collectively, these data suggest an interaction of PP2A and AKT, potentially via PPP2R1A, which requires Nox4-dependent oxidation, but no kinase function of AKT.

**Kinase- but Not Redox-Dead AKT Restores Normal Cell Function in Nox4−/− Cells.** In order to verify the in vivo relevance of our findings, subcellular distribution of PP2A as a consequence of AKT oxidation was analyzed. In Nox4 knockout cells, overexpression of any of the three wild-type AKT homologs or kinase-dead versions of AKT restored cytosolic retention of PP2A to the level observed in wild-type cells. In contrast, the redox-dead cysteine mutant of AKT did not have the same effect (Fig. 5A). Consequently, nuclear accumulation of PP2A was reduced upon overexpression of all but the redox-dead AKT (Fig. 5B). According to our model, restoration of the cytosolic level of PP2A should restore DNA damage detection and thus phosphorylation of H2AX. Indeed, overexpression of wild-type AKT restored γH2AX in Nox4−/− cells to the level observed in WT cells. In contrast, overexpression of redox-dead AKT failed to have this effect (SI Appendix, Fig. S11). As a consequence of PP2As cytosolic disposition, overexpression of all but the redox-dead AKT mutants should promote DNA repair. To address this, comet assays were performed in cells overexpressing the different AKT plasmids. Wild-type AKT reduced the number of comets in Nox4−/− cells to the level of WT cells and similar effects were observed in cells overexpressing kinase-dead mutants of AKT (Fig. 5C and D). In contrast, none of the redox-dead versions of AKT was able to reduce the number of comets. Thus, cytosolic retention of PP2A due to its association with Nox4-oxidized AKT stabilized γH2AX in the nucleus, which affords an appropriate DNA damage repair.

**Discussion**

This study provides evidence for genomic instability as a consequence of genetic deletion of the NADPH oxidase Nox4. Nox4 provides a basal tone of H2O2, which oxidizes AKT. Oxidized AKT binds to PP2A and subsequently sequesters this phosphatase in the cytosol. In the absence of Nox4, none-oxidized AKT does not bind PP2A. The phosphatase then translocates into the nucleus and dephosphorylates γH2AX, a main component of DNA damage recognition and repair. Consequently, due to poor recognition, DNA damage remains unrepaired and accumulates to a level higher than normal. Unrecognized DNA damage prevents expression of tumor suppressors such as p53, even upon exposure to DNA damaging carcinogens. Eventually, cancerogens can lead to enhanced tumor formation in Nox4−/− mice.

The role of ROS is ambivalent and especially in diseases like cancer, ROS can have beneficial and harmful effects. ROS activate cancer-associated fibroblasts and on the other hand force apoptosis of damaged cells, for example. A recent review is recommended for further reading (32). Endogenous ROS generator systems, like the NADPH oxidase family, produce ROS in a controlled and inducible manner. Via thiol-based systems those ROS then mediate signal transduction (33). Nox4 is the only member of the NADPH oxidase family that constitutively produces H2O2. Thereby Nox4 provides a basal oxidative tone to the cell, which is protective by preventing inflammation (11) and maintaining the activity of antioxidant systems like KEAP/Nrf2 (12, 34) and protein kinase A (35, 36).

The present work establishes AKT as an additional redox target of Nox4. Oxidation of AKT by Nox4 promoted the interaction of PP2A with AKT, which facilitated AKT dephosphorylation. Accordingly, AKT phosphorylation and potentially its activity rises in the absence of Nox4. Given that AKT promotes survival and proliferation, this finding explains faster proliferation after deletion of Nox4, observed not only in this but also other studies (37). In several tumor cell lines, Nox4 was identified to drive AKT phosphorylation (19, 38–41), while other studies identified AKT as an inducer of Nox4 expression (42–45). These conflicting results are potentially based on unspecific effects of ROS and their random association with Nox4 expression. In fact, any reduction of ROS, like inhibition of mitochondria, Nox1, Nox2, or Nox4, usually leads to a similar protective response in transformed stable tumor cell lines (46–48).

![Fig. 5. PP2A translocation can be rescued by overexpressing AKT. PP2A (catalytic subunit) localization in the cytosol (A) and nucleus (B) was determined by Western blot after nuclear extraction. Cells were overexpressed with the plasmids indicated. *P < 0.05; WT/WT mutated AKT vs. Nox4+/−/Nox4−/− mutated AKT, *P < 0.05 Nox4+/− vs. Nox4−/− AKT OE, n = 5). (C) Comet assay, exemplary photos (C) and statistics (D) of WT and Nox4−/− cells with overexpression of the plasmids indicated *P < 0.05 WT vs. Nox4−/−, *P < 0.05 Nox4 GFP vs. Nox4−/− AKT mutant. n = 5.](https://doi.org/10.1073/pnas.2020152118)
A secondary effect of AKT oxidation by Nox4 and subsequent binding of PP2A to AKT is to deprive other cellular compartments from the phosphatase. After Nox4 knockout AKT is less oxidized, PP2A is released from AKT and translocates into the nucleus, where it dephosphorylates γH2AX (22). γH2AX is H2A, phosphorylated by ATM at Ser139, an effect occurring at sites of DNA double-strand breaks (49) which represents a major prerequisite for a cell’s response to DNA damage (23). The present observation of less γH2AX in the absence of Nox4 verifies a previous report where antioxidants reduced the level of γH2AX in murine lung cancer tumors (5).

Phosphatases themselves are well-known redox switches as well. Dual-specific phosphatases like the lipid phosphatase PTEN and tyrosine phosphatases (PTPs) are inactivated by H2O2. Nox1 inactivates PTEN (50) and inhibition of Nox1 induces apoptosis by attenuating AKT signaling in cancer cells (51). PTP inactivation by Nox4-derived H2O2 occurs under certain conditions and promotes survival of some cancer cells (47, 52). However, in most cells the tonic activity of Nox4, induces antioxidant defense mechanisms to counteract this process (34, 53, 54). PP2A, the phosphatase of concern in the present work, does not contain a redox-sensitive cysteine but a metal in its active center (55). Therefore, PP2A is not a direct target of Nox4-derived H2O2. Apart from that, PP2A was thought to be a tumor suppressor as okadaic acid and other PP2A inhibitors can serve as chemotherapic agents (56). In contrast, inactivation of PP2A triggers tumor-selective cell death (57) and the PP2A inhibitors cantharidin or okadaic acid induce apoptosis of pancreatic cancer cells through persistent phosphorylation of IKKα and a sustained activation of NFκB (58). Subcellular localization of PP2A activity may be the key to understand the controversial findings. The B subunit determines function and localization of its PP2A complex. In fact, if not retained in the cytosol, a B56c containing PP2A holoenzyme has been described to dephosphorylate γH2AX (59).

**Limitations of the Study.** The role of ROS in cancer is complex, and may vary between different types of cancer, and certainly varies at different stages of tumor initiation, development, and progression (60). This is likely to be the case for Nox4 as well. No evidence for development of spontaneous tumors in Nox4−/− mice was found so far. Accordingly, based on the data presented, Nox4 may not play a role in tumor initiation or progression. Rather the two-hit model of cancer initiation suggests a role for Nox4 in tumor development only in the presence of an applied carcinogen. Several studies report increased expression of Nox4 in cancer (40, 61). Indeed, Nox4 is induced by hypoxia and fibrotic response, both of which are common in tumors. Thus, induction of Nox4 is unlikely to cause tumor formation, but rather represents a cellular defense mechanism due to altered homeostasis and cytokine microenvironment in a growing tumor. Indeed, some studies suggest a protective effect of Nox4 in cancer. A silenced Nox4 promoter promotes hepatocarcinogenesis in rats (62); and in human hepatocellular carcinoma, high Nox1 expression was associated with less favorable outcome, whereas high Nox4 expression was beneficial (63, 64). In a more descriptive study Nox4 siRNA prevented liver cancer in a xenograft model (65). In head and neck cancer cells an inhibitor of epidermal growth factor receptor induced autophagy in a Nox4-dependent way (66). These data underline the ambivalent role of Nox4 in cancer.

Lastly, different to our results, other studies linked NADPH oxidases to an increase in DNA damage. Up-regulation of p22phox contributes to genomic instability in FLT3-expressing leukemia cells (67) and H-Ras transformed cell lines are protected from DNA damage if treated with Nox4 siRNA (68). Additionally Nox1 was associated with DNA damage in cell lines (69). These variable results stress that tumor research on cell lines should be interpreted with caution. Cultured cells often present with overwhelming ROS formation that indeed harms the cells. The selection above, however, illustrates an inter-changeability of individual Nox enzymes, which may not reflect the in vivo situation.

**Conclusion**

The current work establishes Nox4 as an endogenous source of ROS that maintains genomic stability. This work supports and partially explains clinical data showing that antioxidants do not protect from cancer and may even be harmful under certain conditions. We hope our work will strengthen the acknowledgment of the pleiotropic and contextual actions of ROS and NADPH oxidases in cancer and most other diseases.

**Materials and Methods**

Materials. The following chemicals were used: MCA, AOM, NaCl, NiCl2, NaHCO3, Hanks’, BSS without Ca2+ and Mg2+ and trypsin–EDTA solution (T3924) from Sigma-Aldrich, DSS (160011080; MB Biomedicals), Dulbecco’s PBS (Bioco), Hanks’ buffer, SYBR green and Na-EDTA from Applichem, Tris (Carl Roth), and fibronectin (Corning). Collagenase type 2 was purchased from Worthington. The PI3-kinase inhibitor Ly294002 (BML-ST420) was acquired from Enzo Life Science. The following antibodies were used: anti-pH3 (06-570), anti-PP2A, and anti-phospho-histone H2A.X (Ser139) from Millipore, anti-AKT, anti-phospho-AKT (Ser473), p-p53 (Ser15), ATM, HA, and PP2A, Erk, p-Erk, AKT Sepharose bead conjugate and IgG mouse Sepharose bead conjugate from Cell Signaling, anti-p53 (FL-393), anti-p110, NQO1, p-ATM (Ser1981), GFP and anti-topoisomerase 1 (C-15) from Santa Cruz, anti-α-tubulin from Sigma-Aldrich, and anti-pH3 from Abcam (116412) purchased from Boston Biochem. Human recombinant AKT1 (ab116412) was purchased from Abcam.

Animals and Animal Procedures. All animal experiments were approved by the local governmental authorities (approval nos. F28/27, F28/46, FU1194, and FU1235) and performed in accordance with the animal protection guidelines. Nox1+/− mice were generated by targeted deletion of the translation initiation site and of exons 1 and 2 of the Nox4 gene (11), backcrossed into C57BL/6J for more than 10 generations. Tamoxifen-inducible Nox4−/− mice (Nox4lox/loxERT2-Cre+; nox4x−/−) were produced by crossing Nox4lox/loxERT2-Cre; p53+/-; mice to C57BL/6J. Nox4−/− littermates were obtained from C57BL/6J and Nox2y−/− mice were purchased from Charles River. Nox4−/− mice were generated by targeted deletion of the translation initiation site and of exons 1 and 2 of the Nox4 gene (11), backcrossed into C57BL/6J for more than 10 generations. Tamoxifen-inducible Nox4−/− mice (Nox4lox/loxERT2-Cre+; nox4x−/−) were produced by breeding Nox4lox/loxERT2-Cre; p53+/-; mice to C57BL/6J. Nox4−/− mice were obtained by breeding Nox4lox/loxERT2-Cre+; p53−/−; mice to C57BL/6J. Neutrophilic granulocytes were harvested from the bone marrow of C57BL/6J mice. In the cases that tumors reached a diameter of 1.5 cm or 150 d post-tumor induction, mice were killed. Tumor induction in constitutive knockouts and littermates started when the mice reached an age of 7 to 8 wk. In the case of tamoxifen-inducible knockout, tamoxifen administration started at the age of 7 wk, followed by tumor induction. Fibrosarcoma induction was achieved by subcutaneous injection of the chemical carcinogen MCA into the right flank of the mice. In the cases that tumors reached a diameter of 1.5 cm or 150 d after MCA injection, mice were killed and if present the tumor tissue was used for cell isolation and immunohistochemical and biochemical analysis. In order to analyze short-term effects of MCA injection, mice were killed 20 d after MCA injection and skin at the site of injection was used for histological analysis. To analyze the effect of MCA injection on the tumor microenvironment, the mice were killed 20 d after MCA injection and skin at the site of injection was used for histological and biochemical analysis. Colon carcinomas were induced by the following procedure: A single intraperitoneal dose of 10 mg/kg body weight AOM was applied. After 1 wk, drinking water was supplemented with 1.5% or 2% DSS for 1 wk followed by 2 wk of usual drinking water. This procedure was...
repeated two more times. Two weeks after the third DSS cycle, mice were killed and colons were used for further analysis.

Cell Culture. Fibrosarcoma cells were isolated using the tumor dissociation kit for mouse and the gentle MACS Dissociator from Miltenyi Biotec, following the manufacturer’s instructions. In brief, tumor tissue was homogenized enzymatically, erythrocytes were lysed, and eventually cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) + glutaMAX ( Gibco) supplemented with 5% fetal calf serum (FCS) and 1% penicillin (Pen) (50 U/mL) and streptomycin (Strep) (50 μg/mL) in a humidified atmosphere of 5% CO2 at 37 °C. Erythrocyte depletion buffer contained 155 mM NH4Cl, 10 mM NaHCO3, and 100 mM EDTA in double distilled water, with a pH = 7.4.

For isolation of dermal skin fibroblasts from mice 1 cm2 piece of skin was cut into small pieces, transferred into 2.5 mL collagenase solution (1,000 U/mL in Hanks’ without Ca2+/Mg2+) and incubated on a shaker for 10 min at 37 °C. Afterward the solution was resuspended and transferred through a 70-μm nylon filter. Cells were centrifuged and placed on a fibronectin-coated dish. Primary fibroblasts were cultured in DMEM/F-12 ( Gibco, Life Technologies) supplemented with 10% FCS and 1% Pen/Strep in a humidified atmosphere of 5% CO2 at 37 °C. Murine lung endothelial cells were isolated and cultured as described by Schröder et al. (11). Cyst isolation and organoid cultures from murine intestine were achieved as described (72), using the Intesticult System from StemCell Technologies according to manufacturer’s instructions.100 ng/mL murine Wnt3a (Peptope) was added to the medium. A total of 700 crypts were seeded in 50 μL mixture of Matrigel and Intesticult medium in 24-well plates. A total of 400 μL of medium per well was used. Organoids were analyzed by light microscopy and qRT-PCR.

Analysis of the Redox State of Isolated Fibroblasts. In order to analyze for the redox state of the isolated cells, we used the GSH/GSSG-GloTM assay (Promega). We followed the manual provided by the manufacturer: cells were lysed with passive lysis buffer or oxidized glutathione lysis reagent that contain luciferin-NT, the luminogenic GSH probe required for the assay chemistry. After addition of luciferin and incubation for 15 min, luminescence was measured and GSH as well as GSSG were calculated with the aid of a standard curve.

Dihydroethidium (DHE) assays in an HPLC setting were performed as described before (73). Dihydroethidium (DHE, 20 μmol/L) was oxidized by the cells and the resulting products 2-dihydroxyethidium (2EOH) and ethidium (E) from cells and supernatant were separated by HPLC and analyzed by absorbance (350 to 400 nm for DHE) and fluorescence (510 nm/595 nm excitation/emission for 2EOH and E). For fluorescence detection, DHE and its oxidation products were separated by HPLC (Hitachi, Elite Lachrom System L1300 pump) using a C18 column (EC; Nucleosil, 100-5, 250/4.6 Macherey-Nagel), a mobile phase A of H2O:acetonitrile:trifluoroacetic acid (TFA) (9:1.0:1) and phase B of acetonitrile +0.1% TFA. A gradient from 0 to 40% of phase B was achieved within 10 min and to 100% phase B in 20 min with a 0.5 mL/min flow. To estimate 2EOH concentration, potassium superoxide (14 mmol/L) was used as a standard. System control and analytical data analysis were performed using Analyst software 1.6.2.

For Amplex Red measurement, cells were incubated in PBS containing 100 μM Amplex Red and 0.25 units/mL horse radish peroxidase (HRP) (Thermo Fisher). After 30 min, the supernatant was transferred to a 96-well plate, and fluorescence of resorufin as the product of H2O2-dependent oxidation was measured and GSH as well as GSSG were calculated with the aid of a standard curve.

Protein and Western Blot Analysis. For whole cell protein isolation, cells were lysed in containing supernatant. The pellet was further lysed with buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM DTT, PIM, 40 μg/mL PMSF) to obtain the nuclear extract. A Bradford assay was used to determine the protein amount (74). Samples were boiled in sample buffer and transferred on SDS-PAGE followed by Western blotting. Identical amounts of protein from nuclear and cytosolic fractions were loaded. Analysis was performed with an infrared-based detection system using fluorescent-dye-conjugated secondary antibodies from LI-COR Biosciences. Phosphorylation and dephosphorylation of AKT was analyzed according to the manufacturer’s suggestion. Briefly, 50 mM para-nitrophenylphosphate (pNPP) were incubated with lysates of cytosolic or nuclear fractions prepared as described above. After 5 to 10 min the reaction was stopped by addition of NaOH and the amount of the product of the phosphatase reaction, p-nitrophenol, was determined by measuring the absorbance at 405 nm in a microplate reader (Tecan infinite 200 Pro).

BIAM switch assay was performed to determine the oxidation state of AKT. Briefly, cells were blocked with n-ethylmaleimide (NEM) and scraped in tri-chloroacetic acid (TCA). To block the free thiols, cell pellets were resuspended in NEM-denaturing buffer (containing Tris-HCl pH 8.5, urea, EDTA, and SDS) subsequently followed by aceton precipitation. Reduction of oxidized thiols was performed with DTT-denaturing buffer and labeling of those with biotin-polyethyleneoxide-iiodoacetamide-denaturing buffer followed by aceton precipitation and Triton lysis. Lysates were used for immunoprecipitation (IP) with streptavidin-agarose beads and blotted for AKT.

mRNA Isolation and RT-qPCR. Total mRNA from cells and frozen homogenized tissue was isolated with a RNA-Minikit (Bio-Bioll) according to the manufacturer’s protocol. Random hexamer primers (Promega) and SuperScript III Reverse Transcriptase (Invitrogen) were used for cDNA synthesis. Semi-quantitative real-time PCR was performed with a MX3000P qPCR cycler (Agilent Technologies) using PCR Eva Green qPCR Mix with ROX (Bio-Sell) with appropriate primers. Relative expression of target genes was normalized to eukaryotic translation elongation factor 2 (EF2), analyzed by the delta-delta-Ct method, and given as percentage compared with control experiments. Primer sequences for murine Nox4 were forward 5'-TGGTGG-GCTTAGGATTGTG-3' and reverse 5'-AGGACGCTTCTTGTACTGTCG-3' for p53 forward 5'-AGACGGCCTGACCAAGAAAG-3' and reverse 5'-TTCAGTCGCGAACAGTCTC-3', Cyp1a1 forward 5'-GGCAAGACGATGCTC-3' and reverse 5'-GGGACCTTCTCAGACGCTG-3'; Cyp2b11 forward 5'-GGGACCTTCTCAGACGCTG-3' and reverse 5'-GGGAGTCTTCCTCAGACGCTG-3'; and Cyp2c7 forward 5'-GGGACCTTCTCAGACGCTG-3'.
DNA Damage Detection (Comet and Nicotelli Assays). A suspension of 1 × 10^6 cells/mL was mixed 1:10 with 5% low melting agarose and subjected onto slides coated with 1.5% normal melting agarose. Lysis of the cells was performed for 2 h at 4 °C in lysis buffer (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, pH = 10, 1% Triton X-100, and 10% SDS in double distilled water). Lysis was followed by a 20-min incubation of the slides on ice with the alkaline electrophoresis buffer (300 mM NaOH and 0.5 M EDTA) with subsequent electrophoresis at 25 V for 20 min. Slides were washed three times with PBS and stained with SYBR green. Pictures were taken with confocal microscopy LSM 510 Meta and quantification was performed manually by three independent investigators determining the ratio of cell number/cells with comets. Additionally DNA Fragmentation was determined by analysis of propidium iodide (PI)-stained nuclei using flow cytometry as described previously (75). Cells were lysed and stained for 2 h in a solution of 0.1% trisodium citrate dehydrate and 0.1% Triton X-100 containing 50 μg/mL propidium iodide (Sigma) and analyzed by flow cytometry (FACSCanto II, BD Biosciences).

Immunohistochemistry. Tissue was fixed in 4% paraformaldehyde overnight, dehydrated in ascending ethanol series, and then embedded in paraffin. Paraffin blocks were sliced and slides were dewaxed for further staining in descending ethanol series from 100 to 70%. For antigen retrieval, slides were cooked 10 min in citrate 1× TRS buffer (target retrieval solution; Dako). After cooling down staining procedure started using the Dako CSA system in combination with biotin blocking system (Dako) for 3,3′-diaminobenzidine staining. First, slides were blocked with avidin and biotin for 20 min with the biotin blocking system from Dako, followed by peroxidase and protein blocking from the Dako staining kit. In between washing steps were performed with TBST (Tris-buffered saline with Tween20). After the protein block, the first antibody was applied on the slides overnight at 4 °C. The next day slides were incubated with biotinylated secondary antibody, amplification steps were performed as described in the manual, and diaminobenzidine was used to generate the signal. Counterstaining was performed with hematoxylin.

RNAscope. In situ hybridization by the RNAscope technique was performed according to the manufacturer's instructions (Advanced Cell Diagnostics). Briefly, 4-μm-thick sections were deparaffinized and treated with H2O2 followed by antigen retrieval and protease treatment. Probes for Nox4 (RNAscope LS 2.5 Probe- Mm-Nox4, Cat. No. 457268), and positive/negative controls (peptidylprolyl isomerase B, RNAscope Probe-Mm-Pipbic, Cat. No. 313911-C4) were used. For hybridization to the target RNA sequence, the RNAscope slides were incubated with RNAscope Probe-DapB (Cat. No. 310043) and were cohybridized with RNAscope probe for control. The RNAscope probe was followed by a second gradient to 60% B for 10 min or 15 min, respectively. MS data were recorded by the data-dependent acquisition Top10 method selecting the most abundant precursor ions in positive mode for HCD (higher-energy collisional dissociation) fragmentation. The full MS scan range was 300 to 2,000 m/z with resolution of 70,000, and an automatic gain control (AGC) value of 3 × 10^6 total ion counts with a maximal ion injection time of 160 ms. MS/MS scans were recorded with a resolution of 17,500, an isolation window of 2 m/z, and an AGC value set to 10^6 ions with a maximal ion injection time of 150 ms. Selected ions were excluded in a time frame of 30 s following the fragmentation event.

Mass Spectrometry Measurements of Inositol Lipids. Mass spectrometry was used to measure inositol lipid levels essentially as described previously (77) using a QTRAP 4000 (AB Sciex) mass spectrometer and employing the lipid extraction and derivatization method described for cultured cells (cells isolated from tumors) and whole tissue (tumor tissue), with the modification that initial samples were probe sonicated for 5 seconds (using a microtip) prior to extraction and that final samples were dried in a speedvac concentrator rather than under N2. Measurements were conducted on 1 × 10^5 isolated fibrosarcoma cells or 1 mg wet weight tumor tissue per sample. C16:0/C17:0:10 PI (100 ng) and PIP3 (10 ng) internal standards (ISDs) were added to each sample prior to extraction. Integrated area of lipid species peaks were corrected for recovery against ISD area (giving a response ratio for each lipid) and data expressed as C18:0 C20:4 PI response ratio normalized to C18:0 C20:4 PI response ratio to account for cell input variation. Both endogenous PIP3 and PI were corrected to their own internal standard, and then the one measurement was divided by the other, to get the best estimate of true PIP3/PI.

Sample Preparation and Mass Spectrometry. A total of 2.5 μm recombinant AKT1 (Abcam) was reduced in 100 μM DTT and treated with 300 μM H2O2 for 30 min. Proteins were directly digested overnight with trypsin (sequencing grade, Promega) and analyzed by liquid chromatography/mass spectrometry (LC/MS).

Fibrosarcoma cells of wild-type and Nox4-deficient mice were lysed in buffer A. Lysates were used to trap AKT1 to 3 including interacting proteins using a Sepharose beads immobilized AKT antibody and IgG (Cell Signaling) as negative control. Beads were washed in PBS, resuspended in 50 μ L 8 M urea, 50 mM TrisHCl, pH 8.5, and incubated at 22 °C for 30 min. Thiols were alkylated with 40 mM chloroacetamid and samples were diluted with 25 mM TrisHCl, pH 8.5, 10% acetonitrile to obtain a final urea concentration of 2 M. Proteins were digested with 1 μg trypsin/LysC (sequencing grade, Promega) overnight at 22 °C under gentle agitation. Digestion was stopped by adding trisulfocarboxylic acid to a final concentration of 0.1%. Peptides were loaded and purified on multistop-and-go tip (StageTip) containing six C18 disks (78). Peptides were dried and resolved in 1% acetonitrile, 0.1% formic acid, and analyzed by LCMS.

LCMS-MS was performed on a Thermo Scientific Q Exactive Plus equipped with an ultra-high performance liquid chromatography unit (Thermo Scientific Dionex Ultimate 3000) and a Nanospray Flex Ion-Source (Thermo Scientific). Peptides were loaded on a C18 reversed-phase precolumn (Thermo Scientific) followed by separation on a 2.4 μm Reprosil C18 resin (Dr. Maisch GmbH) in-house packed picotip emitter tip (diameter 100 μm, 15 cm long, New Objectives) using a gradient from mobile phase A (4% acetonitrile, 0.1% formic acid) to 30% mobile phase B (80% acetonitrile, 0.1% formic acid) for 20 min (purified human AKT1) or 90 min (AKT1 enriched from lysate) followed by a second gradient to 60% B for 10 min or 15 min, respectively. MS data were recorded by the data-dependent acquisition Top10 method selecting the most abundant precursor ions in positive mode for HCD (higher-energy collisional dissociation) fragmentation. The full MS scan range was 300 to 2,000 m/z with resolution of 70,000, and an automatic gain control (AGC) value of 3 × 10^6 total ion counts with a maximal ion injection time of 160 ms. MS/MS scans were recorded with a resolution of 17,500, an isolation window of 2 m/z, and an AGC value set to 10^6 ions with a maximal ion injection time of 150 ms. Selected ions were excluded in a time frame of 30 s following the fragmentation event.

Data analysis of purified human AKT1: Xcalibur raw files were analyzed by Peaks7 Studio software for proteomics (http://www.bioinformaticsolutions.com). The enzyme specificity was set to trypsin. Missed cleavages were limited to 3. Monoisotopic precursor mass error tolerance was 3 ppm, and fragment mass accuracy was set to 0.01 Da. The following variable modifications were selected: of methionine (+15.99), disulfide bridge (1.01), dioxidation on cysteines (+31.99), trioxidation on cysteines (+47.98). After de novo sequencing of spectra, StavroX (v3.4.12) was used to identify peptide-spectrum matches with a false discovery rate (FDR) of 1%. For identification of cross-linked peptides by disulfide bridges, a special software StavroX (v3.4.12) was used. A disulfide bridge (−2.02), half of disulfide bridge (−1.01), dioxidation on cysteines (+31.99), and trioxidation on cysteines (+47.98). After de novo sequencing of spectra, the human reference proteome set (download from Uniprot, April 4, 2015; 68,511 entries; https://www.uniprot.org) was used to identify peptide-spectrum matches with a false discovery rate (FDR) of 1%. For identification of cross-linked peptides by disulfide bridges, a special software StavroX (v3.4.12) was used. A disulfide bridge (−2.01565) was used and the search for dipeptides was done using a precise scoring mode. Only dipeptides with the highest scores (>200) were inspected and are shown in SI Appendix, Table S2. Disulfide bridges within the structure of AKT1 (E4EN) (80) were illustrated using Pymol (0.99rev9).

Data analysis of immune-trapped AKT1 to 3: Xcalibur raw files were analyzed by MaxQuant (1.5.2.8) (81) with specificity to trypsin and tolerated missed cleavages of 2. The following variable modifications were selected: of methionine (+15.99), carbamidomethylation on cysteines (+57.02), and acetylation on N terminus (+42.01). Proteins were identified using a mouse proteome set (Uniprot, June 26, 2015, 76,086 entries) with a FDR of 1%. Proteins were quantified using label-free quantification (LFQ) with at least one peptide and further analyzed by Perseus 1.5.4.1 (82). Reverse hits, known contaminants, and identified by side proteins were removed from the list. Missing LFQ values were replaced by a background value generated from the normal distribution to enable calculation of ratios to control. A permutation test was performed to identify interacting proteins on AKT1 to 3.

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Genetic deletion of Nox4 enhances cancerogen-induced formation of solid tumors

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https://doi.org/10.1073/pnas.2020152118
ACKNOWLEDGMENTS. We are grateful to Manuela Sapeth, Sabine Hare, and Sabine Heiden, Musikentwickler für sehr excellent technical support of our study. Furthermore, we thank Tamar Gonen and Beate Fisslthaler for providing AKT mutant plasmids. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SCHR1241/1-1 and SCHR1241/1-2) to K.S., TPH to B.B., and Z1 to I.W. and the Fraunhofer Gesellschaft (Graduate School Translational Research Innovation Pharma [TRIP]) to K.S.

Statistics. All values are means ± SEM. Statistical analysis was performed by ANOVA followed by LSD (least significant difference) post hoc testing or by t-test, as noted. Significant differences were compared by ANOVA for repeated measurements. Densitometry was performed with Odyssey software. A P value of less than 0.05 was considered statistically significant.

Data Availability. All study data are included in the article and/or SI Appendix.
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