Zyxin Is a Critical Regulator of the Apoptotic HIPK2-p53 Signaling Axis

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Abstract

HIPK2 activates the apoptotic arm of the DNA damage response by phosphorylating tumor suppressor p53 at serine 46. Unstressed cells keep HIPK2 levels low through targeted polyubiquitination and subsequent proteasomal degradation. Here we identify the LIM domain protein Zyxin as a novel regulator of the HIPK2-p53 signaling axis in response to DNA damage. Remarkably, depletion of endogenous Zyxin, which colocalizes with HIPK2 at the cytoskeleton and in the cell nucleus, stimulates proteasome-dependent HIPK2 degradation. In contrast, ectopic expression of Zyxin stabilizes HIPK2, even upon enforced expression of its ubiquitin ligase Siah-1. Consistently, Zyxin physically interacts with Siah-1, and knock-down of Siah-1 rescues HIPK2 expression in Zyxin-depleted cancer cells. Mechanistically, our data suggest that Zyxin regulates Siah-1 activity through interference with Siah-1 dimerization. Furthermore, we show that endogenous Zyxin coaccumulates with HIPK2 in response to DNA damage in cancer cells, and that depletion of endogenous Zyxin results in reduced HIPK2 protein levels and compromises DNA damage-induced p53 Ser46 phosphorylation and caspase activation. These findings suggest an unforeseen role for Zyxin in DNA damage-induced cell fate control through modulating the HIPK2-p53 signaling axis. Cancer Res; 71(6); 2350–9. ©2011 AACR.

Introduction

The cellular DNA damage response (DDR) network constitutes an important antitumor barrier that is inactivated during carcinogenesis (1). The DDR is a powerful cellular response counteringacting genomic instability through induction of cell-cycle arrest and DNA repair or cellular senescence and apoptosis (2, 3).

Homeodomain-interacting protein kinase 2 (HIPK2) is a critical regulator of cell fate decisions during development and in response to genomic damage (3–5). In unstressed cells HIPK2 levels are kept low through proteasome-dependent degradation which is facilitated by the ubiquitin ligases Siah-1 and WSB1 (4, 6–8). In response to DNA damage, HIPK2 is stabilized and activated through the ATM/ATR pathway, which involves disruption of the HIPK2–Siah-1 complex through ATM-mediated phosphorylation of Siah-1 (6, 9). If the genome is severely damaged upon UV exposure, ionizing radiation, or chemotherapeutic drug treatment, HIPK2 forms a complex with the tumor suppressor p53 and phosphorylates p53 at Ser46 and thereby stimulates expression of proapoptotic target genes (9–12). In response to UV damage, HIPK2 is corecruited by the promyelocytic leukemia protein (PML) along with p53 to PML nuclear bodies (10, 11, 13), which are nuclear multiprotein domains playing a critical role in the regulation of p53 activity and p53-mediated cell fate upon DNA damage (14–17).

The LIM (Lin-11, Isl-1 and Mecl) domain protein Zyxin localizes to focal adhesion sites and the actin cytoskeleton where it interacts with a number of cytoskeletal proteins and signaling components, thereby regulating actin cytoskeleton dynamics and cell motility and migration (18–20). Blockage of Crm1-dependent nuclear export with leptomycin B (LMB) leads to nuclear accumulation of Zyxin (21, 22). Consistent with these findings, Zyxin was found to shuttle between the cytoplasm and the cell nucleus and to play a role in mechanotransduction and atrial natriuretic peptide signaling. Interestingly, Zyxin has also been implicated in cell death regulation (19, 23–26), but its role in apoptosis regulation is controversial.

Intriguingly, both HIPK2 and Zyxin have been reported to exert tumor suppressive functions: HIPK2 expression is reduced in breast and thyroid carcinoma (27, 28), it is inactivated through mislocalization by the protoprotein HMGA1 (28) and is functionally compromised by mutation in acute myeloid leukemia (AML) and myelodysplastic syndrome (29). Moreover, there is evidence that in mice, HIPK2 acts as haploinsufficient tumor suppressor in the skin (30). For Zyxin, reduced expression has been associated with bladder cancer progression (31), and Zyxin was shown to act as a tumor suppressor in Ewing sarcoma (32) and in prostate cancer cells (33). Although these findings indicate a tumor suppressive
activity of Zyxin, the signaling routes and molecular mechanisms linking Zyxin to tumor suppression remain largely unclear.

In this study, we have identified Zyxin as a critical regulator for the p53 Ser46 kinase HIPK2, which induces apoptosis in response to DNA damage. We demonstrate that Zyxin expression is important to maintain HIPK2 protein stability, as Zyxin depletion results in proteasome-dependent HIPK2 degradation. HIPK2 expression in Zyxin-depleted cells is rescued by proteasome inhibition or by codepletion of the ubiquitin ligase Siah-1. Furthermore, ectopically expressed Zyxin stabilizes HIPK2 and efficiently protects HIPK2 from Siah-1–mediated degradation, presumably by interfering with Siah-1 homodimerization. Depletion of endogenous Zyxin in human hepatocellular carcinoma cells inhibits DNA damage-induced p53 Ser46 phosphorylation and caspase-dependent PARP cleavage, a hallmark of apoptotic cell death. These results suggest Zyxin as a critical regulator of DNA damage–induced cell death induction through regulation of the HIPK2–p53 signaling axis, and thereby propose a potential molecular basis for the tumor suppressive activity of Zyxin.

Materials and Methods

Cell culture and transfections

U2OS, HepG2, MFC7, and 293T cells (all directly obtained from ATCC) were maintained in DMEM/10% FCS/1% (w/v) penicillin/streptomycin/20 mmol/L HEPES. Transient transfections were done using standard calcium phosphate precipitation, Lipofectamine 2000 or HiPerFect (Qiagen).

Expression plasmids and antibodies

HIPK2 and Siah1 expression vectors and antibodies were described previously (10, 34, 43). Zyxin expression vectors were generated by standard PCR reactions and were all verified by DNA sequencing by the DKFZ core facility. Zyxin deletions contained the following regions: dLIM amino acids (aa) 1–380, LIM1/3 a.a. 380–572, LIM1/2 a.a. 380–504, and LIM2/3 a.a. 444–572. The rabbit zyxin antibodies were generated by immunising rabbits with the following KLH-coupled peptides: TLKEVEEQLTQQLM (a.a. 352–367 of human Zyxin). Antibodies were affinity purified against the peptide prior to use. Other antibodies used were flag M2 (Sigma), Myc 9E10 (Santa Cruz), mouse anti-HA 3F10 (Roche), rabbit anti-HIPK2 (10), goat anti-Zyxin (N-19; Santa Cruz), mouse anti-c-Myc 9E10 (Santa Cruz), rat anti-HA (3F10; Roche). The secondary antibodies we used were Alexa 488–coupled goat anti-mouse, Alexa 488–coupled goat anti-rabbit, Alexa 568–coupled donkey anti-goat, Alexa 594–coupled donkey anti-rat, and Alexa 594–coupled goat anti-rabbit (Molecular Probes). Cells were examined using a confocal laser scanning microscope (FluoView 1000; Olympus) with a 60× oil objective using the sequential scanning mode. All images were collected and processed using the FluoView Software (Olympus) and sized in Adobe Photoshop 7.0.

DNA damage induction

For UV irradiation, cell culture medium was removed and cells were exposed to the indicated UV-C dosages using a Stratalinker 1800 (Stratagene). After addition of fresh medium, cells were allowed to recover for the indicated time periods. For Adriamycin treatments, the cells were incubated with fresh culture medium supplemented with 1 μg/mL Adriamycin for the indicated time periods.

Immunofluorescence microscopy

Cells were washed in PBS. For endogenous protein stainings, preextraction was performed by a 30-minute incubation in CSK buffer (100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L MgCl₂, and 10 mmol/L PIPES, pH 6.8), 2-minute incubation in CSK/0.5% Triton-X-100 and a brief wash in CSK buffer. Cells were fixed with PBS/4% paraformaldehyde for 30 minutes at room temperature. Immunofluorescence stainings were performed as published previously (44). The following primary antibodies were used: mouse anti-Flag (M2; Sigma), rabbit anti-HIPK2 (10), goat anti-Zyxin (N-19; Santa Cruz), mouse anti-c-Myc 9E10 (Santa Cruz), rat anti-HA (3F10; Roche). The secondary antibodies we used were Alexa 488–coupled goat anti-mouse, Alexa 488–coupled goat anti-rabbit, Alexa 568–coupled donkey anti-goat, Alexa 594–coupled donkey anti-rat, and Alexa 594–coupled goat anti-rabbit (Molecular Probes). Cells were examined using a confocal laser scanning microscope (FluoView 1000; Olympus) with a 60× oil objective using the sequential scanning mode. All images were collected and processed using the FluoView Software (Olympus) and sized in Adobe Photoshop 7.0.

RNA interference

For RNA interference the following targeting sequence was inserted into the pSUPER vector to knock-down human Zyxin: 5′-GAAGTTGAGCAGTATTTGAT-3′, or the following dsRNAs synthesised by Drarmacon were used: 5′-GTTTACAAGTGT-GAGGAC-3′ or 5′-GCCCAAAGTGAATCCCTT-3′. The following target sequences were also used: HIPK2 5′-CAT CTA CGA GGT CTT AGA G-3′ and Siah-1 5′-GATAGGAACAGCAGCA-CAA-3′. For the control experiments the following sequence targeting GFL2 firely luciferase 5′-CGTACCCGGAA-TACTTCA-3′ was used (44) either as dsRNA oligo or in pSUPER. All siRNA sequences were verified to confirm their specificity to the respective target mRNA. Stable knock-downs were done by calcium phosphate precipitation of pSUPER-shGFL2 and pSUPER-shZyxin along with pWZL-neo into HepG2 cells and subsequent selection of G418-resistant cell pools.

Immunoprecipitation and immunoblotting

For coimmunoprecipitation experiments, cells were lysed in buffer containing 150 mmol/L NaCl, 50 mmol/L HEPES, pH 7.5, 5 mmol/L EDTA, 0.5% NP-40, and protease inhibitors (Complete without EDTA, Roche; Mg132, Biomol; and PMSF, Applichem) or 250 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 1% NP-40, 25 mmol/L NaF, 10% glycerol, and protease inhibitors. For the HIPK2–Zyxin coimmunoprecipitation, cells were lysed in buffer containing 50 mmol/L Hepes,
pH 7.4, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, and protease inhibitors. Mouse Flag (M2), mouse Myc or mouse HA antibodies were used with protein-A/G–coupled sepharose beads (Santa Cruz). After incubation at 4 °C on a rotating wheel the beads were washed 3 times in NP-40 buffer (150 mmol/L NaCl, 50 mmol/L Hepes, pH 7.5, 5 mmol/L EDTA, 0.5%, NP-40). For total-cell lysates, cells were either lysed in buffer containing 20 mmol/L Tris/HCl, pH 7.4, 1% NP-40, 150 mmol/L NaCl, 5 mmol/L EDTA, 10% glycerol, 25 mmol/L NaF, and 1% SDS, and DNA was sheared by sonication, or cells were lysed in the same buffer containing only 0.1% SDS without sonication. Immunoblotting was done as described (10). Quantifications were done using the ImageJ software.

**GST protein purification, in vitro translations and GST pulldowns**

GST proteins were purified from E. coli BL21 by standard protocols. For Siah and LIM constructs, bacteria were incubated in the presence of 0.5 mmol/L Zinc. HIPK2 and Zyxin were in vitro translated using the TNT coupled Reticulocyte Lysate System (Promega). Pulldowns were incubated for 4 to 6 hours on a rotating wheel at 4 °C in in vitro interaction buffer (0.05% NP-40, 1 mmol/L Na3VO4 and 1 mmol/L PMSF in PBS), washed 3 times for 10 minutes in the same buffer, denatured, resolved by SDS page, and gels were subsequently Coomassie-stained and dried, and the radioactive signal was detected either by autoradiography or by phosphoimager.

**Results**

**Zyxin interacts with HIPK2**

Using the N-terminal part of HIPK2 (encoding amino acids 1–565) as bait in a yeast 2-hybrid screen (34) against a fetal human brain cDNA library, we identified 2 identical cDNA clones encoding the C-terminal region of Zyxin harboring its LIM domains (Fig. 1A). This interaction was confirmed in vitro and further mapped to LIM domains 1 and 2 of Zyxin and the N-terminal 188 amino acids of HIPK2 (Fig. 1B and C). The interaction between Zyxin and HIPK2 could also be detected in intact cells by coimmunoprecipitation experiments (Fig. 1D), which suggests that HIPK2 and Zyxin interact in vivo. These data indicate interaction of HIPK2 and Zyxin in vitro and in vivo (a schematic view is shown in Fig. 1E). We failed to demonstrate interaction of endogenous HIPK2 and Zyxin proteins, which is likely due to complex formation of both factors at particular multiprotein domains, such as cytoskeletal structures and nuclear bodies which are very hard to solubilize under conditions which preserve protein–protein interactions. In fact, immunofluorescence stainings of endogenous HIPK2 and endogenous Zyxin showed colocalization of both factors at cell edges (most likely representing focal contacts) and in a subset of HIPK2-containing nuclear bodies (Fig. 1F).

**Zyxin regulates HIPK2 protein levels**

Therefore, we proceeded to analyze the functional interaction between HIPK2 and Zyxin. Intriguingly, acute knockdown of endogenous Zyxin in various cancer cell lines strongly reduced HIPK2 steady-state protein levels in unstressed cells, whereas HIPK2 mRNA levels remained unchanged (Fig. 2A and B). Stable Zyxin knockdown with a pSuper construct containing an independent Zyxin target sequence also showed striking reduction of the HIPK2 levels (Fig. 2C). Using a gain of function approach, we found that ectopic expression of Myc-tagged Zyxin leads to HIPK2 accumulation in a dose-dependent manner. Interestingly, a Zyxin protein that was predominantly targeted to the nucleus by addition of the SV40 NLS and point mutation of the internal NES sequence (Nuc-Zyxin) was even slightly more efficient in stabilizing ectopically expressed HIPK2 than the wild-type form of Zyxin (Fig. 2D and E), hinting at a role for nuclear Zyxin in this process. Taken together, these results indicate that Zyxin regulates HIPK2 stability.

**Zyxin inhibits HIPK2 degradation in a Siah-1–dependent fashion**

We and others have recently shown that HIPK2 steady-state levels are largely controlled by regulated proteasomal degradation (6–9). Therefore, we examined whether reduced HIPK2 protein levels in Zyxin-depleted cells originate from enhanced HIPK2 protein destabilization. Effective proteasome inhibition (as evident by accumulation of the proteasome target pS3) with the proteasome inhibitor MG-132 fully rescued HIPK2 expression (Fig. 3A), indicating that Zyxin depletion indeed results in increased HIPK2 degradation.

One important mediator of HIPK2 breakdown is the E3 ubiquitin ligase Siah-1 (6, 8, 35). Because Zyxin depletion results in proteasome-dependent HIPK2 degradation, we investigated if Zyxin protects HIPK2 from degradation by antagonizing Siah-1 function. As expected, Siah-1 expression resulted in efficient HIPK2 degradation (Fig. 3B). Remarkably, coexpression of Zyxin rescued HIPK2 levels even in presence of its ubiquitin ligase (Fig. 3B). These findings indicate that Zyxin expression protects HIPK2 from Siah-1–mediated degradation.

Next we investigated whether knock-down of Zyxin may lead to Siah-1–dependent HIPK2 degradation. To this end, we knocked down Siah-1 by RNAi in shZyxin cells. Strikingly, knock-down of Siah-1 specifically rescued endogenous HIPK2 expression in shZyxin cells (Fig. 3C). Taken together, these findings suggest that depletion of Zyxin results in Siah-1–dependent HIPK2 degradation.

**Zyxin colocalizes and physically interacts with Siah-1**

Because of the functional interaction of Zyxin with Siah-1, we next asked whether both molecules physically interact. To this end, we performed coimmunoprecipitation analyses from cell lysates expressing Myc-Zyxin and HA-Siah-1. Interestingly, ligase-deficient Siah-1 (Siah-1C44S) was readily coimmunoprecipitated with Zyxin, in contrast to wild-type Siah-1 (Fig. 4A). Nuc-Zyxin showed a slightly increased binding to Siah-1 in comparison to the wild-type form (Fig. 4B). Similar results were obtained by reciprocal immunoprecipitation analyses (Fig. 4C). Consistent with these findings, indirect immunofluorescence staining and confocal microscopy revealed that

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ligase-deficient Siah-1 as well as wild-type Siah-1 readily colocalized with Nuc-Zyxin (Fig. 4F and H). Siah-1C44S and wild-type Siah-1 showed a comparable subcellular distribution (Fig. 4D). Furthermore, wild-type Zyxin and wild-type Siah-1 colocalized in the cytoplasm (Fig. 4G), however, to a lesser extent than wild-type Zyxin and Siah-1C44S (Fig. 4E). Thus, these findings suggest that Siah-1 and Zyxin form a protein complex, which may occur preferentially in the cell nucleus.

To examine whether Zyxin and Siah-1 may interact directly, we performed GST pulldown assays. In vitro translated 35S-labeled Zyxin was efficiently pulled down by GST-Siah1 Kinase domain, as shown in Fig. 4B and C.

Figure 1. Zyxin and HIPK2 interact and colocalize. A, schematic representation of the Zyxin fragment recovered in a Yeast 2 hybrid screen with the HIPK2 N-terminus as bait. B and C, GST pulldown assays with the indicated GST fusions (B, HIPK2 fragments; C, Zyxin fragments) and 35S-labeled, in vitro translated proteins as indicated. D, Western blot showing precipitated Flag-HIPK2 and coprecipitated Myc-tagged Zyxin. Expression levels in the lysates are shown below. E, schematic representation of the HIPK2/Zyxin interaction domains deduced from the pulldown assays. F, confocal images of immunofluorescence stainings of endogenous HIPK2 (green) and Zyxin (red) in preextracted U2OS cells. Colocalization is shown in yellow. DNA was counterstained with Hoechst (last panels).
suggesting that the interaction between both proteins is indeed direct. Further mapping experiments indicated that the RING domain of Siah-1 (a.a. 1–80) mediates the interaction with Zyxin (Fig. 4J). Taken together, these results indicate that Zyxin physically interacts with Siah-1.

**Zyxin interferes with Siah-1 homodimerization**

Because Siah-1 forms dimers via its substrate binding domain (36, 37), we next assessed whether Zyxin regulates Siah-1 homodimerization. To this end we coexpressed wild-type, ligase-proficient HA-Siah-1 and Flag-Siah-1 proteins in presence or absence of Myc- (Nuc-) Zyxin and investigated Siah-1 homodimerization by coimmunoprecipitation analyses. In the absence of Zyxin, HA-Siah-1 efficiently coprecipitated with Flag-Siah-1 (Fig. 5A). In contrast, in presence of Zyxin the homodimerization of Siah-1 was significantly reduced (Fig. 5A), and again, Nuc-Zyxin showed a slightly stronger effect. A comparable interfering effect of Zyxin on Siah-1C44S homodimer formation was observed (Fig. 5B). Taken together, these data suggest that Zyxin may modulate HIPK2 stability through interference with the assembly of functional active Siah-1 dimers.

**Zyxin accumulates upon DNA damage induced by UV and adriamycin treatment**

To get insight into the functional role of endogenously expressed Zyxin in the DNA damage response, we examined Zyxin protein levels in response to DNA damage in human HepG2 hepatoma cells. Interestingly, lethal UV damage (70 J/m²) (6) resulted in clear Zyxin accumulation

![Figure 2. Zyxin regulates HIPK2 stability. A and B, Western blot of U2OS (A) or MCF7 (B) cells transfected with the indicated siRNA oligos, probed with the indicated antibodies. Actin serves a loading control. A, knockdown efficiency and HIPK2 mRNA levels are controlled by RT-PCR (bottom). C, Western blot (top) of HepG2 cell pools stably transfected with the indicated shRNA plasmids. Zyxin and HIPK2 mRNA levels are controlled by RT-PCR (bottom). D, HT1080 cells stained for exogenously expressed, Myc-tagged wild-type Zyxin or nuclear-targeted (Nuc-) Zyxin. The cartoons show the respective constructs with mutated residues depicted in red. E, cotransfection of Flag-HIPK2 and increasing amounts of wild-type or Nuc-Zyxin in 293T cells. GFP serves as transfection control.](image-url)
In addition, Zyxin accumulation correlated with HIPK2 stabilization and p53 Ser46 phosphorylation (Fig. 6A). Sublethal UV damage (20 J/m²; ref. 6), however, resulted in weak and transient Zyxin accumulation (Fig. 6A). Furthermore, cells treated with a lethal dose of the DNA-damaging chemotherapeutic drug adriamycin showed elevated Zyxin levels (Fig. 6B), indicating that Zyxin accumulates in response to severe DNA damage, and that Zyxin accumulation correlates with HIPK2 stabilization and p53 Ser46 phosphorylation.

We also addressed the subcellular localization of endogenous Zyxin in U2OS and HepG2 cells before and after UV treatment, using indirect immunofluorescence staining and confocal microscopy. Zyxin predominantly resided in the cytoplasm in unstressed cells. Most cells only showed weak nuclear staining of Zyxin, and no significant changes in the subcellular distribution of Zyxin were detectable after DNA damage (data not shown).

Zyxin depletion inhibits DNA damage–induced p53 Ser46 phosphorylation and apoptotic caspase activation

To examine the functional relevance of Zyxin in DNA damage–induced HIPK2 stabilization and p53 Ser46 phosphorylation, we used HepG2 cells stably depleted of Zyxin (shZyxin) and control cells (shGL2). Cells were exposed to UV light, a well-established HIPK2-activating stimulus (10, 11), and subsequently analyzed by immunoblotting. In accordance with published data (6, 8), HIPK2 was stabilized upon UV and a robust p53 Ser46 phosphorylation was evident in the shGL2 control cell pool (Fig. 6C). In contrast, Zyxin-depleted cells showed a clear reduction in UV-induced HIPK2 stabilization and p53 Ser46 phosphorylation (Fig. 6C). Zyxin-depleted cells showed no signs of caspase activation upon UV damage, whereas the control cell pool showed characteristic caspase-dependent cleavage of the caspase substrate protein PARP (Fig. 6C). Taken together, these findings demonstrate a role of Zyxin in the regulation of DNA damage–induced p53 Ser46 phosphorylation and apoptotic caspase activation.

Discussion

The Ser/Thr protein kinase HIPK2 is an emerging regulator of cell fate decisions in development, morphogenesis, and genotoxic stress (3–5). To activate the cell death response upon chromosomal damage, HIPK2 stimulates various downstream signaling pathways most prominently the tumor suppressor p53. HIPK2-mediated phosphorylation of p53 at Ser46 potentiates the activation of proapoptotic p53 target genes, which finally leads to cell death (10, 11). Activation of HIPK2 in response to genotoxic stress is facilitated through the checkpoint kinases ATM and ATR, which phosphorylation-dependently inhibit HIPK2 proteolysis by the ubiquitin ligase Siah-1 through site-specific phosphorylation of Siah-1 (6).

Our findings here demonstrate that the LIM domain protein and focal adhesion component Zyxin is essential for HIPK2 stabilization, p53 Ser46 phosphorylation and caspase-activation in response to DNA damage. In addition, our data indicate that Zyxin is also essential to maintain the low level steady-state HIPK2 protein expression in unstressed cells, which suggests that HIPK2 may fulfill important, but so far only little investigated functions in cells in the absence of genotoxic...
Figure 4. Zyxin colocalizes and interacts with Siah-1. A–C, Western blots of coimmunoprecipitations of exogenously expressed Myc-Zyxin or Myc-Nuc-Zyxin and HA-Siah-1 or ligase-deficient HA-Siah-1C44S from 293T cells probed with the antibodies indicated. D–H, confocal images of U2OS cells expressing the indicated Zyxin (green) and Siah-1 (red) proteins. Colocalization is shown in yellow. I and J GST pulldown assays were performed with the indicated GST-Siah-1 fusion proteins and 35S-labeled in vitro translated Zyxin.
stress. However, the functional relevance of HIPK2-Zyxin interaction in unstressed cells and DNA-damaged cells remain to be clarified and may pave the way to uncover novel cellular functions of HIPK2 and Zyxin. Moreover, potential functional interplay between HIPK2 and Zyxin in response to DNA damage remains to be elucidated in the future.

Previous reports have linked Zyxin to apoptosis regulation and indicate both pro- and antiapoptotic activities of Zyxin, suggesting tissue and cell-type specific as well as stimulus-dependent functions of Zyxin (23, 25, 26). For example, Zyxin has been shown to exert oncogenic activities by facilitating cell migration in melanoma cells (38, 39), but it can also act as a tumor suppressor in Ewing carcinoma and prostate cancer cells (32, 33). Similar oncogenic versus tumor suppressive activities have been attributed to the ubiquitin ligase Siah-1 (40, 41). Because our results here demonstrate that Zyxin and Siah-1 interact and, furthermore, that Zyxin modulates Siah-1 dimerization and thereby presumably its activity, it is tempting to speculate that increased Zyxin levels, such as in case of DNA damage, contribute to uncoupling the ubiquitin ligase Siah-1 from its substrates. As Zyxin potently regulates HIPK2 stability in a Siah-1-dependent fashion, we propose that Zyxin may control HIPK2 stability through regulation of Siah-1 availability. The resulting stabilization of the Siah-1 target protein HIPK2 then alters the fate of the cells subjected to DNA damage by activating the apoptotic program.

Remarkably, in intact cells exclusively the ligase-deficient Siah-1 point mutant but not the ligase-proficient wild-type form of Siah-1 could readily be shown to interact with Zyxin. This may suggest that the ligase activity of Siah-1 interferes with Zyxin binding, possibly through interaction with the

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**Figure 5.** Zyxin regulates Siah-1 homodimerization. A and B, Western blot of a communoprecipitation experiment. 293T cells were cotransfected with (A) HA- and Flag-Siah-1 or (B) HA- and Flag-Siah-1C44S in the presence of the indicated Zyxin constructs and Flag-Siah-1 was precipitated from the lysates. Quantification of the ratio of coprecipitated HA-Siah-1 to precipitated Flag-Siah-1 was performed using the ImageJ software.

**Figure 6.** Zyxin regulates DNA damage-induced p53 Ser46 phosphorylation. A and B, Western blots of HepG2 treated with UV (A) or adriamycin (B), probed with the indicated antibodies. C, Western blot of stably control- or Zyxin-depleted HepG2 cell pools treated with lethal UV doses, probed with the indicated antibodies. Note the increase in Zyxin levels after DNA damage induction. Caspase-mediated PARP cleavage was used as an indicator of apoptosis induction.
E2 enzyme or Siah-1 autoubiquitination. Nonetheless, the inhibiting effect of Zyxin on the dimerization of the wild-type Siah-1 proteins is quite pronounced, arguing that a weak and transient interaction between both factors may occur. This interpretation is further supported by our finding that wild-type Siah 1 and Zyxin show partial colocalization. Although our data clearly indicate that Zyxin regulates HIPK2 stability through interference with Siah-1 function, it remains to be determined whether direct binding of Zyxin to Siah 1 and/or HIPK2 is required for the observed protection of HIPK2 from Siah-1-mediated breakdown.

Taken together, our findings here indicate a novel function for Zyxin in the regulation of the p53 response and cell fate decision in response to DNA damage. Based on our observations, it appears conceivable to speculate that downregulation of Zyxin, such as described in a subset of human breast cancers (42), might influence the responsiveness to DNA-damaging cancer treatments such as radio- and chemotherapy.

Disclosure of Potentials Conflicts of Interest

No potential conflicts of interest were disclosed.

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