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REVIEW

Posttranslational modifications regulate HIPK2, a driver of proliferative diseases

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Abstract The serine/threonine kinase homeodomain-interacting protein kinase (HIPK2) is a tumor suppressor and functions as an evolutionary conserved regulator of signaling and gene expression. This kinase regulates a surprisingly vast array of biological processes that range from the DNA damage response and apoptosis to hypoxia signaling and cell proliferation. Recent studies show the tight control of HIPK2 by hierarchically occurring posttranslational modifications such as phosphorylation, small ubiquitin-like modifier modification, acetylation, and ubiquitination. The physiological function of HIPK2 as a regulator of cell proliferation and survival has a downside: proliferative diseases. Dysregulation of HIPK2 can result in increased proliferation of cell populations as it occurs in cancer or fibrosis. We discuss various models that could explain how inappropriate expression, modification, or localization of HIPK2 can be a driver for these proliferative diseases.

Keywords Protein kinase · HIPK2 · Posttranslational modification · Cancer · Fibrosis

Architecture and functions of HIPK2

The activities of enzymes mediating posttranslational modifications (PTMs) such as protein kinases are themselves often regulated by PTMs, thus creating highly wired and complex signaling networks. The systematic analysis of cancer genomes for mutated genes revealed a strong

overrepresentation of kinases [1]. One of the kinases found to be dysregulated in cancer is the homeodomain-interacting protein kinase (HIPK2) [2].

Already, the identification of HIPK2 as an interactor of the homeodomain transcription factor NKx-1.2, suggested its role in transcriptional regulation [3]. Meanwhile, more HIPK2 interaction partners have been found which can be roughly divided in two groups. Group 1 includes a bewildering long and continuously growing list of transcription factors and accessory proteins of the transcriptional machinery. Group 2 consists a heterogeneous group of signaling proteins that have enzymatic functions (e.g., as kinases, acetyl transferases, or ubiquitin E3 ligases) or function as scaffolding proteins (e.g., Han11 and Axin) [4, 5]. Accordingly, HIPK2 serves to augment or to repress gene expression. HIPK2 has been implicated in a perplexingly large group of different signaling pathways. These include the Salvador–Warts–Hippo pathway, BMP, Wnt/Wingless and Notch signaling, Redox regulation, p53 activation, as well as TGFβ and hypoxia signaling [4–6]. All studies share the finding that HIPK2 does not function as a highly connected and essential canonical core component of the respective signaling pathway, but rather as an accessory regulator. The function of HIPK2 could be interpreted as an auxiliary protein that serves to shape the signal output from the signaling cascade and/or as a signal integrator that acts to connect different signaling pathways. The multitude of HIPK2 interactors is also reflected by a complex domain architecture, raising the possibility that this kinase may also function as a scaffold protein. HIPK2 and the other two HIPK family members HIPK1 and HIPK3 share an N-terminal kinase domain with >90 % sequence homology [3]. The C-terminal part contains an interaction domain for the homeodomain transcription factors and a PEST motif that overlap with a region containing a small ubiquitin-like modifier (SUMO)-interacting motif (SIM). These regions are followed by an autoinhibitory domain and a C-terminal region that is rich in short repeats of S, Q, or A.

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Physiological functions of HIPK2

Cell proliferation and survival

Mice lacking the *Hipk2* gene are not born in Mendelian ratios, but approximately 40 % die in the first 3 days after birth. The surviving animals show a reduced weight gain and remain abnormally small [7–9]. These data suggest a role of HIPK2 for cell proliferation, but imply also the occurrence of compensatory mechanisms that allow survival of a fraction of HIPK2$^{-/-}$ mice. These compensatory events could also account for the inconsistent results obtained from the proliferation analysis of HIPK2$^{-/-}$ mouse embryonic fibroblasts (MEFs). While one study revealed an increased proliferation rate of HIPK2$^{-/-}$ MEFs [10], a second study showed a decreased proliferation of HIPK2-deficient MEFs [9]. Since cultured cells can cope with the effects of gene deletions by employing alternative signaling pathways, inducible knockout systems and the use of non-immortalized primary cells will help to clarify this issue. An important function of HIPK2 in cell proliferation is corroborated by a study where shRNA-mediated knockdown of HIPK2 in erythroid precursor cells was shown to inhibit terminal erythropoiesis [11]. In any case, the overexpression of HIPK2 frequently leads to a cell cycle stop and to the induction of cell death [12, 13], showing that the amount of this kinase is of critical importance. A recent study revealed fascinating insights into the mechanisms employed by HIPK2 to control cell division [8]. This study shows that HIPK2 controls cytokinesis and prevents tetraploidization by binding and phosphorylating histone H2B at the midbody, a transient structure connecting two daughter cells at the end of cytokinesis. Depletion of HIPK2 by gene knockout or RNA interference results in absent phosphorylation of H2B at Ser14, a modification that is required for efficient cell cleavage [8].

The cell death-regulating function of HIPK2 is mediated by several mechanisms. Classical p53-induced apoptosis is enhanced by the HIPK2-mediated phosphorylation of p53 at Ser46 which allows the subsequent CBP-mediated p53 acetylation and results in induced expression of proapoptotic genes [12, 13]. P53-independent pathways include HIPK2-mediated phosphorylation and degradation of the transcriptional corepressor CtBP which in turn allows upregulation of proapoptotic genes and cell death [14]. The in vivo role of HIPK2 in the control of cell death was also revealed by the characterization of knockout mice. HIPK2-deficient animals have increased expression of antiapoptotic Brn3a target genes and show increased survival of neurons in the trigeminal ganglion [15]. While these studies collectively show a proapoptotic function of HIPK2, the kinase has also opposing functions in specialized cell types. HIPK2-deficient mice exhibit a progressive loss of enteric neurons during

postnatal development, which does not involve apoptosis but rather depends on autophagy [7].

Differentiation

The function of HIPK2 as a regulator of cell proliferation and survival is closely linked to its contribution to cell differentiation. In *Drosophila*, HIPK2 (dHIPK2) regulates eye development in the fly by targeting the corepressor Groucho. DHIPK2-mediated phosphorylation of Groucho has been shown to attenuate its contact to DNA-bound transcription factors and thereby altering gene expression [16, 17]. In this regard, dHIPK2 promotes the Notch pathway which acts at multiple points in eye development by antagonizing the gene repressing activity of Groucho and inactivation of the *Dhipk2* gene results in small, rough eyes and pupal lethality with rare escaper adults [18]. The role of HIPK2 for eye development is also seen in mammals, as HIPK2$^{-/-}$ HIPK1$^{+/-}$ mice often show small eyes with lens deficiency and abnormally thickened and laminated retinas [19]. HIPK2 knockout mice have been also thoroughly characterized for defects in neuronal development. HIPK2 is required for postnatal development of enteric dopaminergic neurons via TGFβ-induced BMP signaling [20]. The analysis of HIPK1/2 double-deficient mouse embryos revealed the occurrence of homoeotic transformations at the axial skeleton and defects in the closure of the dorsal neural tube [19, 21].

The multitude of biological processes employing HIPK2 raises the question how a single kinase can participate in so many different signaling pathways. One widely used principle is the modification of signaling proteins by PTMs, which control the formation of protein/protein interactions and influence the stability, enzymatic activity or localization of a protein.

Regulation of HIPK2 by PTMs

Since PTMs act as reversible molecular switchboards, the function of HIPK2 can only be understood by deciphering these protein-encoded mechanisms and needs mapping of PTM patterns which provide a "protein code" to control numerous functions. Two recent studies describe the modification of HIPK2 by multiple phosphorylations throughout the entire kinase [22, 23]. While mass spectrometric analysis of GST-tagged wild-type HIPK2 revealed 23 phosphorylation sites [23], the analysis of immunoprecipitated Flag-tagged HIPK2 allowed the identification of 17 modification sites [22]. A comparison of these published sites with data retrieved from the public PhosphoSitePlus© database is given in Fig. 1 and shows that 10 of these sites are found in at least two different and independent experimental settings. Both studies show that the vast majority of

phosphorylation sites that were identified in unstimulated cells are mediated by autophosphorylation. What is the function of these modification sites? Detailed information are now available for the activation loop which is contained within the kinase domain and which needs to be phosphorylated at key amino acids to allow correct positioning of catalytic groups [24]. HIPK2 phosphorylation in the activation loop occurs at Tyr354 and Ser357 [22, 23]. Tyr354 is well conserved between HIPK2 and related kinases (Fig. 2) and functional experiments show that this phosphorylation proceeds via an intramolecular mechanism. In contrast to DYRK2 where tyrosine *cis*-autophosphorylation is essential for its kinase activity [25], the tyrosine autophosphorylation of HIPK2 is not an absolute prerequisite for its enzymatic activity. Mutation of Tyr354 rather results in out-of-target phosphorylation activity and cytoplasmic relocalization of the kinase [23]. Only mutation of both phosphorylated activation loop residues at Tyr354 and Ser357 precluded HIPK2 kinase activity and its effects on gene expression [22]. These results imply that the kinase is already produced in a constitutively active form that is independent from upstream activating kinases. This also raises the need to control the activity of HIPK2 by other mechanisms that are discussed below. The possible functions of the phosphorylation sites outside from the activation loop are not known. GST pulldown experiments showed the necessity of HIPK2 phosphorylation for binding to the prolyl isomerase Pin1 [22]. Since Pin1 is also important for HIPK2-mediated phosphorylation of p53 at Ser46 [26], these data suggest that one or several of these phosphorylation sites can control Pin1-dependent regulation of HIPK2 conformation.

Differential conformation of HIPK2 could also explain the observation that HIPK2 phosphorylation is a prerequisite for modification of HIPK2 by SUMOylation [27] and acetylation (M.L.S., unpublished). Attachment of SUMO1 to Lys25 of HIPK2 is promoted by the SUMO E3 ligase Pc2. HIPK2-mediated inducible phosphorylation of Pc2 is required for its ability to increase HIPK2 SUMOylation in response to DNA damage and thus establishes an autoregulatory feedback loop between the kinase and its cognate E3 ligase [27]. SUMO modification of HIPK2 is neither required for its localization in nuclear bodies nor for its recruitment to promyelocytic leukemia nuclear bodies (PML-NBs) [28]. But SUMO proteins cannot only be conjugated covalently to target lysines as they also have the ability to bind proteins noncovalently to SIMs. Also HIPK2 contains a SIM in its C-terminus [29] and mutation or deletion of the SIM precludes localization of the kinase in nuclear speckles and recruitment to PML-NBs [30]. A further study demonstrated the importance of the SIM for the ability of overexpressed HIPK2 to suppress cell proliferation [31].

While phosphorylation of HIPK2 is required for its SUMOylation, attachment of SUMO itself controls the

Phospho residue	PhosphoSite Plus	Saul et al. [22]	Siepi et al. [23]
kinase domain			
S9			x
S111		x	x
T112		x	
S114		x	
S128			x
T134			x
T245			x
T266			x
T353	x		
Y354	x	x	x
S357	x	x	
Y359	x		
Y360	x		
S434		x	x
T443		x	
T475			x
T510		x	x
T559			x
S628			x
S661		x	x
T680			x
T789	x		
S808			x
S819	x	x	
S820	x	x	x
T831	x	x	
S840/1		x	x
SRS T926		x	
S927			x
S948		x	
AID S970		x	
S984			x
S986			x
S1007		x	
S1035			x
S1146			x
S1179			x

Fig. 1 Identification of HIPK2 phosphorylation sites. The structure of HIPK2 is schematically shown at the *left*; the location of the autoinhibitory domain (*AID*) and the speckle retention signal (*SRS*) are indicated. The various basal phosphorylation sites identified in unstimulated cells by published papers [22, 23] and retrieved from the public PhosphoSitePlus© database are displayed

acetylation status [32], thus providing a demonstrative example how PTMs can occur in a hierarchical fashion. SUMOylated HIPK2 is found in constitutive association with the histone deacetylase (HDAC)3, which ensures the deacetylation of HIPK2 in unstressed cells. Elevated levels of reactive oxygen species (ROS) lead to the non-enzymatic deconjugation of SUMO due to reversible and direct inhibition of SUMO-conjugating enzymes [33]. Accordingly, elevated ROS levels lead to the de-SUMOylation of HIPK2 which in turn reduce its association with HDAC3 and allow CBP/p300-mediated acetylation of HIPK2 at 10 lysines. Functional experiments in reconstituted knockout cells showed that cells lacking HIPK2 or harboring the acetylation
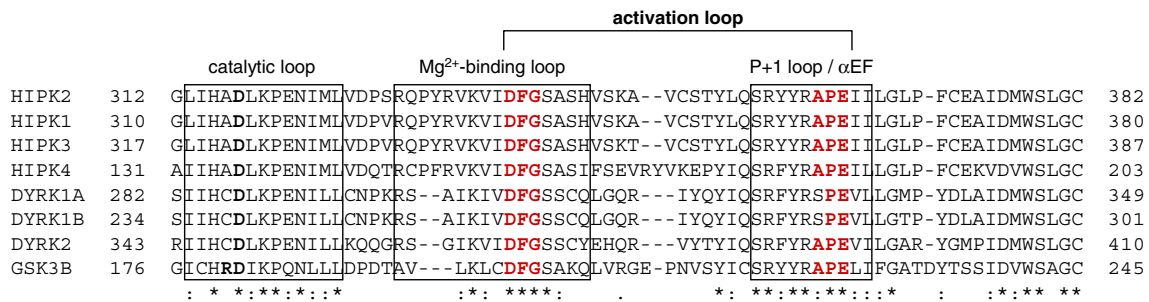
Fig. 2 Sequence alignment of activation loop segments of HIPK2 and related kinases. Amino acid sequence alignment of the indicated kinases was performed using the ClustalW2 program. Identical amino acids are marked with an *asterisk* and similar residues with a *colon*. The conserved catalytic aspartate residue in the catalytic loop is marked. The DFG and APE motifs of the activation loop are shown in *red*

mimicking HIPK2-10KQ are largely protected from H_2O_2-triggered cell death, thus revealing that HIPK2 acetylation protects from ROS-triggered apoptosis. These data also imply that the threshold leading to ROS-triggered cell demise is controlled by HIPK2 acetylation. Since tumor cells frequently show elevated ROS levels, HIPK2 acetylation may ensure their survival even under these adverse conditions as discussed in more detail below. Two acetylated residues are contained in the nuclear localization signal (NLS)1. Accordingly, a modification-specific antibody specifically recognizing the acetylated NLS1 showed that the acetylated kinase does not localize in nuclear speckles but rather in the nucleoplasm [32].

The recent years have provided mounting evidence that the relative abundance of HIPK2 is tightly controlled by degradative ubiquitination. Four different ubiquitin E3 ligases (MDM2, Siah, WSB-1, and Fbx3-formed SCF) ensure the tight limitation of HIPK2 amounts [34–37]. These different E3 ligases can be activated to degrade HIPK2 in different physiological settings. Under conditions of nonsevere DNA damage (where p53 is not phosphorylated at Ser46), activation of p53 allows expression of MDM2 which in turn mediates ubiquitin/proteasome-dependent degradation of HIPK2 [36]. Continuous HIPK2 ubiquitination in unstressed cells is also mediated by Siah1 and WSB-1, thus ensuring efficient restriction of HIPK2 levels under these conditions. Under conditions of genotoxic stress, WSB-1-mediated HIPK2 ubiquitination is blocked by an unknown pathway, while inducible phosphorylation of Siah1 by ATM/ATR leads to the disruption of the HIPK2/Siah1 complex. This in turn results in HIPK2 stabilization, p53 Ser46 phosphorylation, and the induction of cell death [38], as schematically depicted in Fig. 3. Another physiologically relevant setting resulting in HIPK2 degradation is hypoxia. Low oxygen availability leads to the induction of the ubiquitin E3 ligase Siah2 which binds to HIPK2 and causes its efficient polyubiquitination and proteasomal degradation. As HIPK2 is contained in gene-repressing complexes that prevent the expression of specific genes under normoxic conditions,

hypoxia-induced proteasomal elimination of the kinase allows full induction of hypoxia-triggered genes [34]. MDM2-mediated HIPK2 ubiquitination has been mapped to Lys1182 in mouse HIPK2 [36], but the lysines modified by the other ubiquitin E3 ligases are not known and should be identified in the future. Another important future task is the identification of PTMs occurring in primary cells and under conditions known to regulate HIPK2 activity.

Pathophysiological functions of HIPK2 in proliferative diseases

Dysregulation of HIPK2 frequently results in increased proliferation as it is typical in cancer or fibrosis, a process that is characterized by the excessive formation of fibrous tissue [39, 40]. A recently published systematic approach identified HIPK2 as a key regulator of kidney fibrosis [41]. Three different animal models for this disease allowed the identification of this kinase as a driver of kidney fibrosis: HIV-associated nephropathy, unilateral ureteral obstruction, and folic acid-induced renal fibrosis. The starting point for this study was the HIV transgenic mouse model (Tg26), where the proviral transgene recapitulates the renal pathological changes observed in the human disease. The determination of differentially regulated genes was followed by computational analysis of involved transcription factors and protein–protein interaction subnetworks, thus leading to the identification of HIPK2 as a dysregulated candidate kinase. Evidence for a causative role of HIPK2 in kidney fibrosis was revealed by the analysis of knockout transgenic hybrid mice that express the HIV transgene and lack the HIPK2 genes (KO-Tg26 mice). While Tg26 mice develop tubulointerstitial injury and fibrosis and are characterized by proteinuria and lower concentrations of serum urea nitrogen, the KO-Tg26 animals are largely protected from these symptoms [41]. The kidneys of Tg26 mice show upregulated levels of HIPK2 proteins which inversely correlates with the levels of Siah1, one of the ubiquitin E3 ligases, leading to the degradation of

Fig. 3 Regulation of HIPK2 stability. In unstressed cells HIPK2 is constantly degraded through complex formation with the ubiquitin E3 ligases Siah1, WSB-1, and Fbx3 which target HIPK2 for proteasomal degradation. In response to genotoxic stress, the checkpoint kinases ATM and ATR are activated and mediate the phosphorylation of Siah1, resulting in its dissociation from HIPK2. Nonsevere DNA damage leads to cell cycle arrest and recovery, whereas HIPK2-mediated phosphorylation of p53 at Ser46 promotes apoptosis
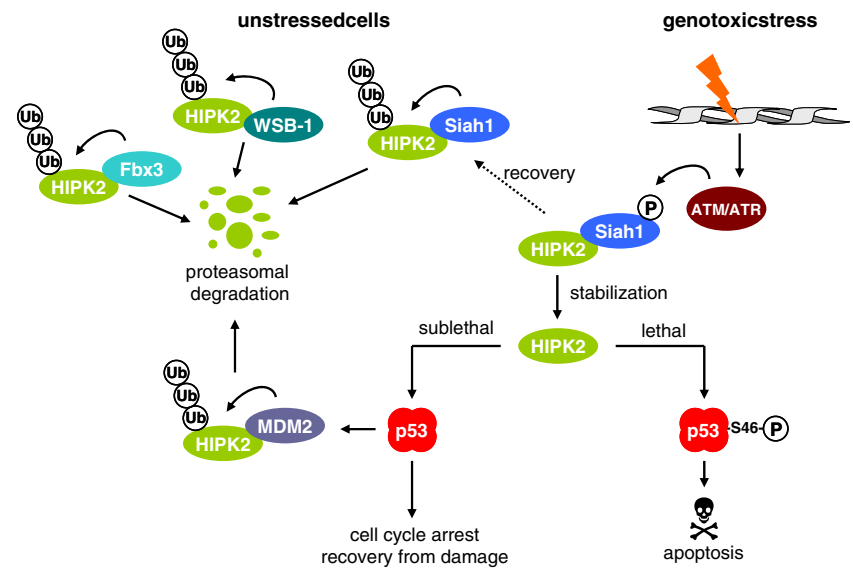

HIPK2. As kidneys of Tg26 mice show elevated levels of ROS, it would be interesting to determine whether also the acetylation status of HIPK2 is changed in this disease model. Another recent study implicated dysregulated HIPK2 levels in idiopathic pulmonary fibrosis (IPF), a disease that is characterized by aberrant proliferation of lung fibroblasts, tissue remodeling, and extracellular matrix deposition [42]. The analysis of HIPK2 expression in IPF samples and in primary fibroblast cell cultures showed the frequent occurrence of loss of heterozygosity (LOH) for the HIPK2 locus at 7q32-34 and concomitantly lower levels of mRNA and protein [42]. While kidney fibrosis was triggered by overexpression of HIPK2, IPF was accompanied by a diminished expression of the kinase.

The role of HIPK2 as a tumor-suppressing kinase was initially revealed by the analysis of HIPK2$^{-/-}$ mice. Although these animals do not develop spontaneous tumors, treatment with a skin carcinogenesis protocol results in elevated formation of skin tumors [10]. Increased tumor formation already appeared in heterozygous mice, suggesting that HIPK2

functions as a haploinsufficient tumor suppressor. The tumor-suppressing function of HIPK2 was also recapitulated in cell culture models, since expression of a constitutively active form of H-ras induced enlarged colonies in HIPK2$^{-/-}$ MEFs [10]. In addition, an unbiased genome-wide screen for genetic alterations in radiation-induced thymic lymphomas generated from p53$^{+/-}$ and p53$^{-/-}$ mice corroborated HIPK2s tumor-suppressing effect and revealed a frequent LOH for the *Hipk2* gene [43]. Of note, point mutations of HIPK2 are only rarely observed [44] and might not even be the cause for tumor development but rather be the consequence of high mutation rates and genomic instabilities as they are typical for cancer cells [45]. A number of studies describe decreased expression levels of HIPK2 in various tumors including thyroid and breast carcinomas [46], papillary thyroid carcinomas, and follicular thyroid carcinomas [47]. These finding are in line with the idea that LOH or impaired expression facilitates tumor growth and accordingly, colon cancers with increased HIPK2 expression have a better outcome than tumors with low expression [48]. In marked contrast, a number of studies

Fig. 4 Schematical display of three possible mechanisms explaining the functional inactivation of HIPK2. For further details, see text
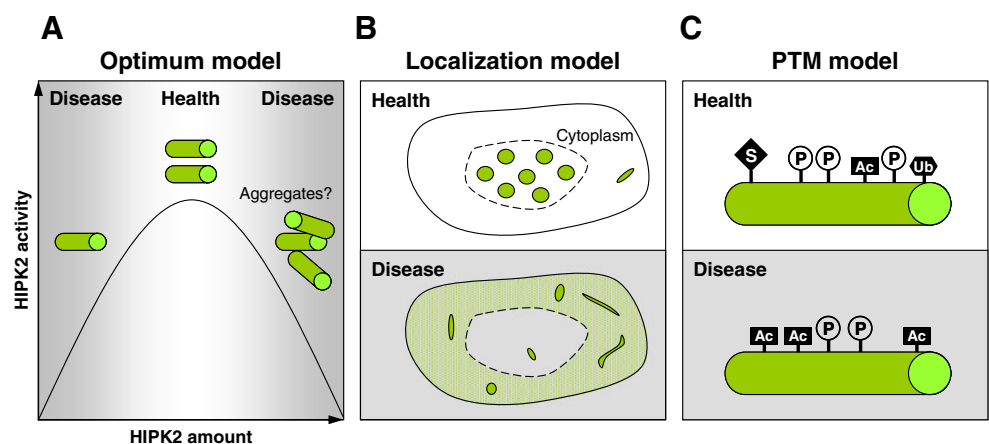
show elevated expression of HIPK2 in tumors such as pilocytic astrocytoma [49, 50] and cervical cancer [51].

Mechanisms mediating pathophysiological inactivation of HIPK2

How can overexpression of a tumor suppressor favor cell proliferation? This situation resembles the situation occurring in fibrosis where both over- and underexpression of HIPK2 were reported. These seemingly contradictory findings might be explained by various models which are discussed here. Although the limited quality of commercially available HIPK2-detecting antibodies may handicap the interpretation of protein expression data in tumor samples, the frequent observation of dysregulated protein amounts could mean that only optimal amounts of HIPK2 are functional. This "optimum model" is schematically depicted in Fig. 4a and implies that higher concentrations of HIPK2 are less active, possibly due to the formation of inactive protein aggregates or misfolded HIPK2. Another important aspect was provided by several studies that revealed the importance of intracellular HIPK2 localization. This "localization model" is schematically shown in Fig. 4b and reflects results from a number of studies showing the delocalization of HIPK2 in cancer. Overexpression of the high-mobility group A1 protein as it frequently happens in tumor cells causes the relocalization of HIPK2 from nucleus speckles to the cytosol [52]. Another example for an aberrant HIPK2 localization comes from AML1(RUNX1)-associated leukemic cells expressing the oncogenic fusion protein PEBP2β-SMMHC. Expression of this fusion protein sequesters HIPK2 to filamentous structures in the cytosol and thus interferes with its functions in the nucleus [53]. The recent progress in the identification and functional characterization of PTMs regulating HIPK2 function enable the development of tools that allow the convenient determination of HIPK2 activity. The "PTM model" (Fig. 4c) reflects the recent findings that modifications of HIPK2 regulate its numerous activities. It will be therefore interesting in future studies to determine the modification status of HIPK2 in patient material. One particular aspect of HIPK2 regulation might be of special relevance in solid tumors such as glioblastomas which are characterized by hypoxic areas. Insufficient vascularization and blood supply is accompanied by low oxygen availability and induction of the ubiquitin E3 ligase Siah2 which binds to HIPK2 and causes its efficient polyubiquitination and proteasomal degradation. HIPK2 downregulation under hypoxic conditions also impairs DNA damage-induced p53 Ser46 phosphorylation [34]. While these findings suggests a molecular mechanism by which hypoxic cancer cells can escape chemotherapeutic drug treatment [34], a confirmatory follow-up study

revealed that HIPK2 expression and p53 Ser46 phosphorylation in hypoxic cells can be rescued by inhibition of the proteasome [54]. Whether also HIPK2 is a suitable target for drugs causing its specific inhibition or activation remains to be studied in the future.

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