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Review

HIPKs: Jack of all trades in basic nuclear activities

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ABSTRACT

Over the past decade several investigators have reported on the physical interaction of serine/threonine kinases of the homeodomain interacting-protein family (HIPKs) with increasing number of nuclear factors and on their localization in different nuclear sub-compartments. Although we are still far from a global understanding of the molecular consequences of HIPK subnuclear compartmentalization, the spatial description of particular interactions and posttranslational modifications promoted by these kinases on key cellular regulators might provide relevant insights. Here we will discuss the possible implications of the HIPK subnuclear localization in the regulation of gene transcription and in the cell response to stress.

The nucleus of eukaryotic cells is delimited by the nuclear envelope and harbors DNA associated activities like gene expression, replication, recombination and repair, as well as RNA processing and ribosome subunit assembly. These events are regulated by the (epi) genome, through histone methylation/acetylation or DNA methylation, and the proteome, through differential expression of specific transcription factors and co-factors. In addition, the complex dynamic, spatial, and temporal organization of proteins and nucleic acids in the nucleus is regarded as a further control layer for nuclear functions [reviewed in [1]. Various nuclear sub-compartments have been described and defined as heterogeneous groups of nucleoplasmic structures, situated within the nuclear matrix, “scaffold”, and distinguished mainly by morphologic (ultrastructural) and antigenic criteria [reviewed in [2]. Nuclear sub-compartments include the nuclear membrane itself with the nuclear pore complexes, nucleolus, perinucleolar and perichromatin regions [3], diverse transcription complexes and replication factories [4] including the AML1 complex [5] and the polycomb group (PcG) bodies [6], nuclear speckles (also named SC35 domains) [7], Cajal-bodies (CBs; also named coiled bodies) [8], and promyelocytic leukemia nuclear bodies [PML-NBs, also named PML oncogenic dots (PODs), ND10, Kr-bodies] [9]. Recently, HIPK nuclear deposition sites (NUDES) or HIPK domains, characterized by the presence of HIPKs and the absence of antigens specific for other nuclear sub-compartments have been also described [10,11]. It is argued that all these structures operate in a coordinated fashion that involves signaling mechanisms using intranuclear kinases and phosphatases, phosphoinositides, and cytoskeletal proteins. The posttranslational modifications of mRNAs and proteins are emerging as important regulatory mechanisms in the biology of nuclear sub-compartments. These modifications can dramatically change the localization of molecules and concomitantly modify the structure/function of nuclear sub-compartments [12].

HIPKs are a family of four serine/threonine kinases (HIPK1–4) highly conserved in vertebrates. HIPK1–3 were originally discovered by their ability to bind homeobox factors [13] while HIPK4 was identified by the human genome sequence based on the high homology with the other three members [14]. An increasing number of screenings for the identification of novel protein–protein interactions and the consequent functional studies have indicated HIPKs, and particularly HIPK2, as a “versatile switchboard” that contributes to the regulation of remarkably diverse nuclear pathways involved in gene transcription, cell survival, proliferation, response to DNA damage, differentiation, and development [15,16]. Interestingly, HIPKs' activities are associated with particular subnuclear distribution of these kinases and of their targets. Here, we will focus our attention on the emerging role(s) played by the HIPK family members in the biology of diverse nuclear sub-compartments. We are far from a clear understanding of the biological activities HIPKs might take part in; however, the increasing number of partners bound, posttranslationally modified, and relocalized within nuclear sub-compartments by HIPKs certainly justifies a revision of our current knowledge on this emerging topic.

1. HIPKs subcellular localization

HIPKs were originally defined, based on the structure of the first three proteins identified (HIPK1, HIPK2, and HIPK3), as nuclear serine/threonine kinases characterized by the presence of a kinase domain in

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their N-terminal region, a homeobox-interacting domain in the central portion of the protein, a speckle-retention signals (SRS) superimposed to a PEST region, and a YH reach region in the C-terminal portion [13,15,16]. The kinase domain and the PEST region are present in all four members of the family while the homeobox-interacting domain is absent in the more recently identified member, HIPK4 (Fig. 1), which is mainly expressed in the cytoplasm, making it possible that HIPK4 is a member of an independent protein kinase family [17]. Thus, for the purpose of this review, we will concentrate on the first three members, HIPK1, HIPK2, and HIPK3, here collectively named HIPKs.

Several studies have analyzed the localization of HIPKs upon overexpression of their chimeric forms with different tags, such as GFP, Flag, HA, or Myc in human or mouse cells [11,13,18,19]. These experiments consistently showed a mostly nuclear dotted localization of HIPK1–3, the so called speckles, and a milder and diffuse cytoplasmic staining. The nuclear localization might be regulated by the in silico defined nuclear localization signals (NLS) as well as by the presence of the putative SRS in the C-terminal region of the proteins (Fig. 1). Indeed, in the HIPK2 protein, deletion of the SRS region impairs the nuclear dotted morphology of the kinase without affecting its presence in the nucleus, supporting the involvement of the SRS in the speckled localization [11,13]. Canonical SRSs [20] are required for directing proteins to nuclear speckles or SC35 domains, which are nuclear organelles that are enriched in splicing snRNPs and many other transcription and splicing-related proteins. They also contain speckle-associated proteins, among with ser/thr kinases that by serine phosphorylation of serine/arginine-rich (SR) proteins control both their association with the spliceosome and their recruitment from speckles to active sites of transcription [12]. The amino acid sequence of the HIPK SRS differs among the HIPKs and from the canonical sequences suggesting that the HIPK speckles are different from the SC35 domains. However, direct experiments to verify whether the HIPK speckles co-localize with the SC35 domains have not been reported yet. In addition, we have to take into consideration that the putative HIPKs' SRS overlaps with the region implicated in the HIPKs binding with most of the non-homeobox proteins that have been found thus far to interact with HIPK1–3 and whose co-expression with the kinases in co-transfection experiments promoted the redistribution of HIPK partners in the HIPK-containing speckles (see below for detailed interactions). Based on these results, we cannot exclude that the absence of HIPK localization in the nuclear speckles observed in the absence of the SRS region depends on the loss of protein–protein interaction rather than on the loss of a specific localization signal. Further analyses with more restrained HIPK mutants are required to solve this issue.

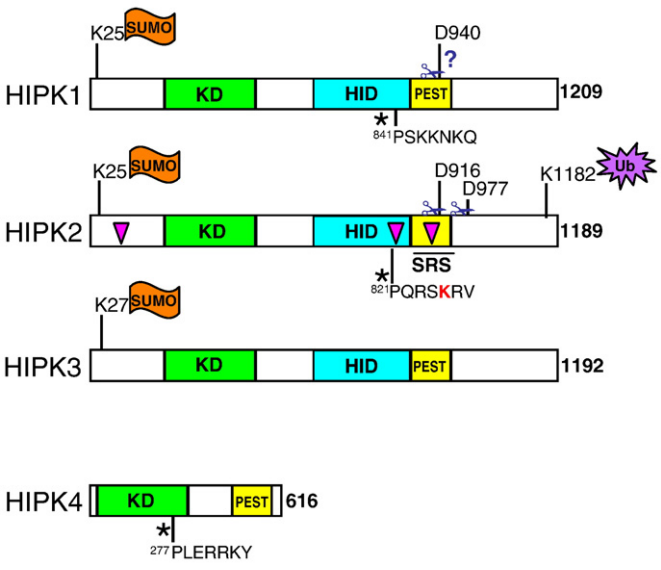

Fig. 1. Schematic representation of HIPK proteins (Swissprot Q9QZR5). KD is the kinase domain; HID is the homeobox-interacting domain; PEST is the PEST-sequence containing region that overlaps with the speckle-retention signal (SRS); D916 and D977 are the caspase-cleavage sites, as indicated by the scissors [49]. A putative caspase-cleavage site, D940, is present in HIPK1 but whether this kinase is cleaved by caspases is still unknown. The sites at the N-terminus of HIPK1–3 sumoylated with SUMO-1 are indicated. K1182 of mouse HIPK2 is ubiquitylated by MDM2 [50]. The C-terminal region of this kinase is also ubiquitylated by WD40 repeat/SOCS box protein WSB-1, but the lysine residue(s) involved is presently unknown [51]. The pick triangles in HIPK2 indicate the putative NLS reported by Kim et al. [13] while the asterisks (*) indicate the sorting signals that predict nuclear localization by the PSORT program [52]. Substitution of K825 (the red K) with alanine did not modify the nuclear localization of HIPK2 (C.R. unpublished results).

The experiments described above were performed with transfected proteins and their results might be artificial. This possible pitfall has been overcome, at least for HIPK1 and HIPK2, since the subcellular localization of their endogenous forms has been studied with specific antibodies in human and mouse cells. The Schmitz's group analyzed the localization of endogenous HIPK2 in human U2OS osteosarcoma cells and observed a speckled distribution of this kinase [11,21]. Other important data on endogenous HIPK1 and HIPK2 were reported by Isono et al., who examined the expression and localization of both kinases by double immunostaining in primary mouse embryo fibroblasts (MEFs) from 12.5 day post-coitus fetuses. HIPK1 and HIPK2 were found co-expressed in the same cells and both proteins had a speckled staining throughout the nucleus apart from the nucleoles. Speckles were composed of a central condensed region surrounded by a less-condensed, foggy area. Interestingly, the condensed speckles for HIPK1 and HIPK2 were mostly separated with limited overlap. This particular distribution was not maintained in overexpressing conditions that rather showed a strong co-localization of the HIPK1 and HIPK2 speckles [22]. The latter result is similar to the observation made by co-transfecting HIPK2 and HIPK3 [11] and strongly suggests the existence of a dynamic redistribution of the HIPKs within the nucleus and among diverse nuclear sub-compartments in different cellular conditions. Probably, overexpression of the kinases is mimicking cell stress and therefore these results should be taken with caution.

2. HIPKs and the PML-NBs

The type of distribution and the shape of HIPK accumulations have stimulated the analysis of their possible co-localization with similar shaped nuclear sub-compartments. The first indication in this regard came from the observation that HIPK2 can bind the tumor suppressor p53 and co-localize with it in the PML-NBs (Fig. 2) [21,23].

The PML-NBs are functionally versatile nuclear structures in which permanently or transiently localize a large number of cellular proteins whose proper assembly is dictated by the PML tumor suppressor protein. These NBs have a “doughnut-like” structure, do not contain detectable nucleic acids but make extensive contacts with chromatin. At the molecular levels, the contribution of PML-NBs has been summarized as follow: i) identification and storage of proteins; ii) posttranslational modification of proteins; iii) transcriptional regulation; iv) chromatin organization. At the functional level, PML-NBs have been implicated in the regulation of several biological events as diverse as DNA-damage response, apoptosis, senescence, and angiogenesis [reviewed in [9].

The p53 tumor suppressor functions as a master regulator of cell response to several types of stress including DNA damage and oncogenic stimuli. To exert these functions, the latent p53 protein, constitutively expressed in an inactive state, is stabilized and activated [24]. These events are thought to depend largely on posttranslational modifications and protein/protein interactions with an increasing array of co-factors that regulate the p53 response at a multiplicity of

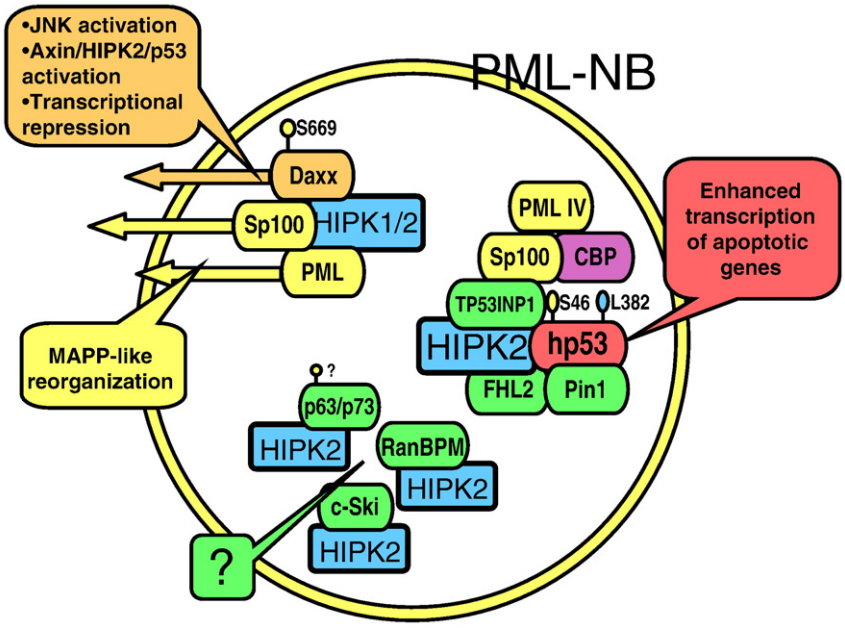

Fig. 2. Schematic representation of the interactions among HIPK2 or HIPK1 and several of their partners, which have been observed to localize to PML-NBs, at least in overexpression. Callouts report the functional outcomes described for the indicated interactions. The arrows indicated the release from the PML-NBs of the indicated proteins.

levels [25], including the localization within the PML-NBs, which are thought to provide a "platform" favoring p53 interaction with specific enzymes [26]. HIPK2 was shown to be one of such enzyme that binds to and activates p53 by specifically phosphorylating it at Ser46 and by promoting the interaction between p53 and the CREB-binding protein (CBP) that acetylates p53 at Lys382 [21,23]. In addition, p53Ser46 phosphorylation was shown to trigger the interaction of p53 with the prolyl-isomerase Pin1, which mediates p53 conformational changes as well as p53 dissociation from the apoptosis inhibitor iASPP [27,28]. All these events are required for efficient loading of p53 on specific target promoters upon stress. Based on these different observations, it has been proposed that HIPK2 might be the trigger of a chain of events that from p53 phosphorylation at Ser46 move to Pin1 modification, CBP and p300 recruitment, p53 acetylation at Lys382, iASPP detachment and specific transcriptional activation [26,28]. Answers in need: up to now, it is not clear whether all these events take place within the PML-NBs, what kind of event activates HIPK2, and where it happens: outside or inside the PML-NBs. What we have learned thus far, is that among the seven PML isoforms, PML IV (previously named PML-3) is capable of binding both p53 and HIPK2 and is required for HIPK2-mediated p53Ser46 phosphorylation and activation of p53-dependent transcription. Indeed, in PML−/− MEFs, overexpression of PML IV, but not of PML III, recruits HIPK2 to PML-NBs and is essential for HIPK2-mediated p53Ser46 phosphorylation and activation of p53-dependent transcription [11].

Although PML IV is required for HIPK2 function at PML-NBs, several other proteins residing within the PML-NBs have been found to play a role in the regulation of HIPK2 and HIPK1 in these NBs. Sp100, one of the permanent residents of PML-NBs, interacts with HIPK2 and partially co-localizes with this kinase in PML-NBs. This interaction contributes to the HIPK2-mediated activation of p53, as shown by depletion of Sp100 expression by RNA interference in human cell lines [29]. The HIPK2-mediated activation of p53 associated with localization to PML-NBs is induced also by proteins, such as the tumor protein p53-induced nuclear protein 1 (TP53INP1) and the LIM domain protein FHL2 (four and half LIM 2). Both proteins were found to interact and co-localize with HIPK2 and enhance HIPK2-mediated, p53-induced transcriptional activity [30,31].

Other proteins have been found to bind to and co-localize with HIPK2 in PML-NBs independently from the HIPK2/p53 interaction. These interactors include the centrosomal protein involved in microtubule nucleation RanBPM [32]; the transcriptional co-repressor c-Ski [33]; the p53 family members p73α and p63α [34]. However, whether the functional outcomes of these interactions depend on their localization at the PML-NBs is still unknown.

The interactions described above, whether demonstrated with the endogenous proteins or observed only upon overexpression, are characterized by a common feature: co-expression with one of the HIPK members promotes/increases the PML-NB localization of the HIPKs' interactors. A different behavior was observed upon the interaction between HIPK1 or HIPK2 and Daxx, a death domain-associated protein. Daxx, originally identified as an interactor of CD95, the fas/APO-1 death receptor, was subsequently shown to localize also in the nucleus, to associate with PML in the PML-NBs and to repress transcription when released from NBs and relocalized to chromatin [reviewed in 35]. It has been shown that HIPK1 and HIPK2 promote Daxx phosphorylation and release from PML-NBs. This process is dependent on the HIPKs' catalytic activity since the kinase-dead HIPK mutants are unable to relocalize Daxx [18,36,37]. In addition, the Daxx-S669A mutant that cannot be phosphorylated by HIPK1, has a stronger repressing activity on specific promoters, suggesting that HIPK1 does not only regulate Daxx localization, but also modulates its transcriptional repressing function [18]. For Daxx interaction with HIPK2, no direct phosphorylation could be detected, and activation of two different pathways has been described. In the first case, HIPK2 and Daxx participate in the transforming growth factor β (TGF-β) signaling pathway and cooperate in the activation of c-Jun NH_2-kinase (JNK) and the induction of apoptosis in a p53-independent manner [37]. In the second case, Daxx was shown to cooperate with the Axin/HIPK2/p53 complex in the induction of a p53-dependent apoptosis [38]. However, it is still unknown whether these two latter pathways are a consequence of the HIPK-induced Daxx release from PML-NBs or whether they rely on the presence of HIPKs in other subcellular compartments.

While the Daxx release from PML-NBs induced by HIPK is probably involved in cell response to stress, it remains puzzling the observation made by Engelhardt et al. upon overexpression of hamster HIPK2 [36]. These authors observed not only the release of Daxx from PML-NBs but a more general reorganization of the entire PML-NBs that resembles the reorganization occurring in the M phase of the cell

cycle, the so called mitotic accumulations of the PML protein (MAPP) [9]. It remains to be elucidated whether this effect is linked to hamster HIPK2 overexpression or it hides a still uncovered function of HIPKs.

3. HIPK-NUDES or HIPK domains

As described above, a portion of the HIPKs speckles observed in the nucleus of human and mouse cells are PML-NBs, and PML IV is required for the recruitment of at least HIPK2 in these NBs. However, the majority of the nuclear HIPK speckles do not co-localize with PML and exist also in PML−/− MEFs, suggesting the presence of HIPKs in other nuclear sub-compartments [11].

Co-localization experiments with nucleolus and Cajal body specific markers have been performed in human U2OS cells carrying an exogenous GFP-HIPK2 protein. Since, in similar conditions, the co-staining with PML revealed a partial co-localization of either HIPK2 or HIPK3 and the PML-NBs, the remaining speckled structures, in the absence of any other marker, were defined as HIPK domains [11]. Of course, this is a temporary definition waiting for further characterization, but it is very useful for reminding us that HIPKs like to have a speckled localization even when they do not belong to PML-NBs.

An initial characterization of the HIPK domains showed that the kinase activity of HIPK2 is dispensable for recruitment into the PML-NBs but is required for speckled localization. Indeed, the kinase-dead HIPK2-K221A mutant is found in the nucleoplasm but does not localize to speckles [11]. Interestingly, no such delocalization was observed for the HIPK1-K219A and HIPK3-K226S kinase-dead mutants indicating a different behavior among the family members [18,19].

The C-terminal region of HIPK2 is also required for the formation of HIPK domains as it is for the presence of HIPK2 in the PML-NBs [10,11,21]. As described above, the HIPK C-terminal region is involved in many of the protein/protein interactions, supporting the hypothesis that the incapacity to aggregate into nuclear sub-compartments might depend on the incapacity to interact with other proteins.

An interesting aspect on the HIPK domains regards the HIPK2-mediated activation of the p53 pathway. It has been shown that HIPK2 can phosphorylate p53Ser46, promote p53 acetylation, stimulate p53-mediated transcription and inhibit colony formation only in PML+/+ MEFs, while these functions are completely lost in PML−/− MEFs, despite the maintenance of HIPK domains. These data indicate that, the existence of HIPK domains is not sufficient for activation of the p53-dependent functions of HIPK2 [11]. Although it is tempting to hypothesize that HIPK domains function as a reservoir of inactive HIPKs that can be relocalized to PML-NBs, or other nuclear sub-compartments when required, recent interesting data indicate that at least part of the HIPK domains correspond to the polycomb group (PcG) bodies and are required for transcription repression (see below) [39].

4. HIPKs, SUMO, and the PcG bodies

Small ubiquitin-like modifier-1 (SUMO-1) is a member of a growing family of ubiquitin-related polypeptides. The enzyme conjugation system for SUMO is similar to that of ubiquitin but sumoylation is involved in more diverse functions than ubiquitylation, which is mainly used, though non exclusively, for protein degradations. Indeed, sumoylation was shown to participate in nuclear transport, protein stabilization, transcription regulation, or PML-NBs formation [40,41]. HIPKs are sumoylated with SUMO-1 but not SUMO-2 or SUMO-3 [42] in their N-terminal regions at an evolutionary conserved site (Fig. 1) by the ubiquitin-like protein conjugating (E2) enzyme UBC9 [10] and the polycomb group (PcG) SUMO (E3) ligase Pc2 [39]. The sumoylation *per se* does not change the subnuclear localization of HIPKs [42,43], as originally proposed by Kim et al. based on their observations that HIPK2 deletion mutants not interacting with UBC9 no longer localize to nuclear speckles [10]. At the functional level, sumoylation does not change the ability of HIPK2 to mediate p53 activation but does modify the HIPK2 co-repressor activity with Groucho or the HIPK2-mediated, TGF-β-induced activation of JNK [42–44] suggesting that sumoylation might be involved in conferring specificity to HIPK2 functions. This modulation can be further enriched by the activity of different SUMO deconjugating enzymes, some of which have been already reported to bind and desumoylate HIPK1 and HIPK2 [42,45,46].

Thus far, the most intriguing data on HIPK sumoylation come from the work of Roscic et al. [39]. Starting from a yeast two-hybrid screening for HIPK2 interactors, the authors have shown that the Pc2 SUMO E3 ligase binds to and co-localizes with HIPK2, that HIPK domains largely overlap with PcG bodies and that their formation depends on both HIPK2 sumoylation and kinase activity. Based on the reciprocal localization of HIPK2 and Pc2, three different classes of speckles can be morphologically defined. As summarized in Fig. 3, upon DNA damage, activated HIPK2 phosphorylates Pc2 at multiple sites including Thr495, which is required to increase the ability of Pc2 to sumoylate itself and HIPK2. In turn, this sumoylation increases the HIPK2 activity to repress transcription [15,39]. These data provide an interesting mechanistic clue on the general transcriptional silencing induced by DNA damage and spatially distinguish this event (p53-independent and localized to PcG body) from the transcriptional activation of apoptotic genes (p53-dependent and localized to PML-NB).

5. HIPKs and the AML1 transcription factor complex

AML1 is a transcription factor originally identified at a breakpoint of chromosome 21 translocation present in acute myeloid leukemia (AML). AML1 forms large multiprotein complexes including core binding factor (CBF)β as "core component" and several other chromatin modulators such as CBP/p300, PML, monocytic leukemia zinc finger protein (MOZ), and HIPK2. These complexes bind to specific DNA sequences to activate or repress transcription of genes involved in the development and differentiation of hematopoietic lineages, and a role as chromatin regulatory factories fine-tuned by protein–protein interactions has been proposed [5].

The presence of HIPK2 in AML1 complexes has been first detected by LC/MS/MS analysis. HIPK2 binds AML1b and p300 and phosphor-

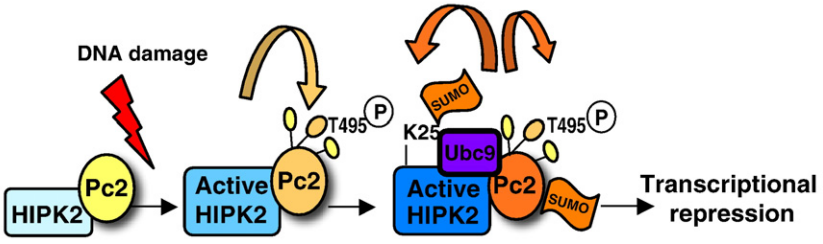

Fig. 3. Schematic representation of the progressive activation of HIPK2 and Pc2 in the PcG bodies. After DNA damage, the first step of HIPK2 activation (still unknown) induces Pc2 phosphorylation which, in turn, promotes its own sumoylation as well as sumoylation of HIPK2 that induces transcriptional repression.

ylates both proteins as well as MOZ, another member of the complex. In the case of AML1b, Ser249 and Ser276 have been identified as direct HIPK2 targets. MOZ phosphorylation requires the presence of both AML1 and HIPK2 and is followed by an increased stabilization of the AML1/MOZ complexes. Finally, HIPK2-induced phosphorylation of p300 depends on AML1 phosphorylation since the non-phosphorylatable AML1-S249A/S276A mutant does not cooperate with HIPK2 in the phosphorylation of p300 and the subsequent transcriptional activation [47]. Interestingly, highly phosphorylated forms of p300 are rare in *Hipk1*/*Hipk2* double knock-out embryos, which die between E9.5 and E12.5 with defects in vascular-genesis, angiogenesis, and hematopoiesis. Similar behaviors were reported in p300 and CBP-deficient mice [47].

Recently, two missense mutations of HIPK2 (R868W and N958I) have been identified in a screening of 50 cases of AML and 80 cases of myelodysplastic syndrome (MDS), a pre-leukemia syndrome [48]. The two mutations are located within the SRS region (Fig. 1) and modify the subnuclear localization of the kinase. Indeed, expression of HA-tagged HIPK2 wild-type and mutant proteins in U2OS cells resulted in a nuclear localization of all three proteins. However, both HIPK2 mutants i) have lost the typical, small speckled distribution; ii) exhibit conical or ring-shaped particles distributed throughout the nucleus; iii) no longer co-localize with AML-1 and p300 although they maintain the capacity to bind and phosphorylate these proteins in vitro; iv) no longer stimulate AML1-mediated, p300-mediated, and p53-mediated transcriptional activity [49]. For the future, it will be interesting to evaluate whether these mutants interfere also with the biology of PML-NBs, whether the reduced p53 transcriptional activity is due to a reduction in p53Ser46 phosphorylation and whether leukemia cells carrying these mutants are more resistant to chemotherapy or radiotherapy.

6. Conclusion

The ten years that have passed since the discovery of the HIPK family have resulted in the identification of a still enlarging body of HIPK's targets and partners. By virtue of protein/protein interaction and phosphorylation of specific serine/threonine residues, HIPKs were shown to be involved in the regulation of gene transcription and response to DNA damage. A diverse and probably dynamic nuclear sub-compartmentalization has added a further level of complexity that we just begin to understand in the activity of these kinases. In the future, immunochemical analysis of the subnuclear redistribution of endogenous HIPKs in diverse cellular conditions in vitro and in vivo, the study of protein composition of the HIPK-containing subnuclear organelles by performing mass spectrometry on biochemically fractionated nuclei, as well as in vivo imaging analyses of inducible GFP-HIPK-chimeras will certainly help in revealing the contribution of HIPKs to the dynamic interactions that govern organization and activities of the nuclear sub-compartments.

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