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HIPK1 interacts with c-Myb and modulates its activity through phosphorylation

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ABSTRACT

The transcription factor v-Myb is a potent inducer of myeloid leukaemias, and its cellular homologue c-Myb plays a crucial role in the regulation of haematopoiesis. In a yeast two-hybrid (Y2H) screening we identified the nuclear kinase HIPK1 as an interaction partner for human c-Myb. The interaction involves a C-terminal region of HIPK1, while a bipartite interaction surface was identified in c-Myb, including regions in its N-terminal DNA-binding domain as well as in its C-terminal region. HIPK1 and c-Myb co-localize in distinct nuclear foci upon co-transfection. c-Myb appears to be phosphorylated by HIPK1 in its negative regulatory domain as supported by both *in vivo* and *in vitro* data. A functional assay revealed that HIPK1 repressed the ability of c-Myb to activate a chromatin embedded target gene, *mim*-1, in haematopoetic cells. Our findings point to a novel link between an important kinase and a key regulator of haematopoiesis.

Introduction

A central theme in signal transduction and control of transcriptional programs is phosphorylation. Many transcription factors are targets of kinases and undergo functional changes which lead to altered gene expression profiles.

HIPK1 (homeodomain-interacting protein kinase 1) is a member of a small family of nuclear serine/threonine kinases, which includes HIPK1, HIPK2 and HIPK3 and also the more distantly related protein HIPK4 [1]. The HIPKs belong to the CMGC group of kinases, first discovered as proteins interacting with the homeodomain protein Nkx-1.2 and acting as co-repressors of NK homeodomain transcription factors [2]. Later these kinases have been found to interact with a range of nuclear proteins including several transcriptional regulators [3]. The best-studied member of the family, HIPK2, is involved in DNA-damage-response, where it signals to p53 by phosphorylating Ser-46, which triggers an apoptotic response through upregulation of pro-apoptotic p53 target genes [4–6]. Another important target for HIPK2 is the co-activator p300, the phosphorylation of which stimulates its histone acetyltransferase and co-activator function [7]. HIPK2 has also been reported to phosphorylate Pax6 and CtBP1 [8,9], and to be part of a Wnt-1 signalling pathway involving TAK1 (TGFβ-activated kinase), HIPK2, and NLK (Nemo-like kinase) [10].

HIPK1 is less studied than HIPK2, but has been implicated in a signalling pathway involving the kinases ASK1 (apoptosis signal-regulating kinase 1) – MEK-JNK [11–13]. Here, the role of HIPK1 appears to be complex, one being downstream of JNK targeting Daxx [14], another involves cytoplasmic translocation of HIPK1 and association with and activation of ASK1 [13]. Cytoplasmic translocation of HIPK1 is caused by SENP1-mediated desumoylation induced by TNFα and is critical for TNFα-induced ASK1-JNK/p38 activation [11,13].

Some functional redundancy obviously exists in this family, since Hipk1 and Hipk2 singly deficient mice were grossly normal, while a double knockout was embryonically lethal, showing developmental defects [7,15,16].

The c-Myb transcription factor plays a central role in the regulation of cell growth and differentiation, in particular during haematopoiesis [17]. It acts as a regulator of stem and progenitor cells in the bone marrow, as well as in colonic crypts and in a neurogenic region of the adult brain [18]. Its basic organization is an N-terminal SANT-related DNA-binding domain (DBD), a central transactivation domain and a C-terminal negative regulatory domain (NRD). Studies in mice have shown that c-Myb is essential for normal haematopoiesis [19]. The transcription factor controls crucial steps at several stages during T- and B-cell development [20,21]. Its key role in haematopoiesis and leukaemogenesis, and the fact that our understanding of molecular mechanisms controlling c-Myb function remains incomplete, motivated us to search for new interaction partners of c-Myb.

In the present work, we present evidence that c-Myb interacts with HIPK1 as judged from interaction assays in yeast and *in vitro*. Confocal microscopy revealed that c-Myb co-localizes with

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HIPK1. HIPK1 seems to be able to phosphorylate c-Myb as shown by *in vitro* as well as *in vivo* assays. In an endogenous functional assay, HIPK1 causes repression of c-Myb activation.

Materials and methods

Two-hybrid screening and mating assay. The two-hybrid screening was performed as previously described [22]. The interactions were confirmed by transformation of pDBT-hcM, encoding full-length human c-Myb, and rescued interaction candidates in pACT2 into yeast strains of opposite mating type followed by mating. Diploids were grown on yeast minimal medium supplemented with 5 mM 3-aminotriazole and lacking the indicated amino acids or adenine.

cDNA cloning and plasmids. cDNA cloning and plasmids are outlined in Supplementary information.

Cell culture, transfections and RNA isolation. COS-1, CV-1 and HD11 cells were cultured as previously described [23]. Transfections of all three cell types were performed with the FuGENE® 6 Transfection Reagent, Roche Diagnostics.

In vitro pull-down assays. In vitro pull-down assays were performed as previously described [23,24] using total cell extract from COS-1 cells transfected with pCIneo-3Fg-HIPK1.

Immunofluorescence and confocal laser scanning microscopy. CV-1 cells, 1.8×10^4 cells per well, were plated out in 24-well microplates containing cover-slips the day before transfection and transfected with a total of 0.6 mg DNA. Cells were prepared for immunofluorescence transfection as described [24]. Samples were examined using a FluoView laser scanning system from Olympus. Images from the different channels were collected sequentially to prevent bleed through.

Quantitative real-time PCR. Quantitative real-time PCR was performed on a LightCycler rapid thermal cycler system (Roche Diagnostics) as previously described [23]. The amplification specificity of the PCR products was confirmed by melting curve analysis and subsequent gel electrophoresis.

In vitro and in vivo kinase assays. In vitro and *in vivo* kinase assays are outlined in Supplementary information.

Results

c-Myb interacts with HIPK1

In order to identify novel interaction partners for c-Myb, we performed a yeast two-hybrid screening using as bait full-length human c-Myb cDNA expressed from the centromeric pDBT vector [22]. The screening was performed in a mixed cDNA library from human bone marrow and the K562 cell line (1:1). Among the clones that reproducibly activated both the *His3* and LacZ reporters, five were identified as independent, partial human HIPK1 cDNAs. Positive clones were verified by plasmid rescue, retransformation and growth on reporter-selective media (Fig. 1A), thus suggesting HIPK1 as an interaction partner of c-Myb.

A full-length cDNA encoding human HIPK1 was generated using the 5′-RACE (rapid amplification of cDNA ends) technique. Our shortest two-hybrid clone contained only the 324 C-terminal codons of HIPK1 and, thus, overlaps with the proposed p53 interaction domain, but is distinct from the homeodomain interaction domain [2,15]. Based on the full-length cDNA, expression plasmids encoding 3× Flag-tagged full-length HIPK1 as well as a K219A kinase-dead HIPK1 mutant were constructed.

To confirm the interaction between c-Myb and HIPK1 and to map the regions of c-Myb that were important for the interaction, we performed an *in vitro* pull-down assay (Fig. 1B). A lysate from COS-1 cells expressing 3× Flag-HIPK1 was analyzed for binding

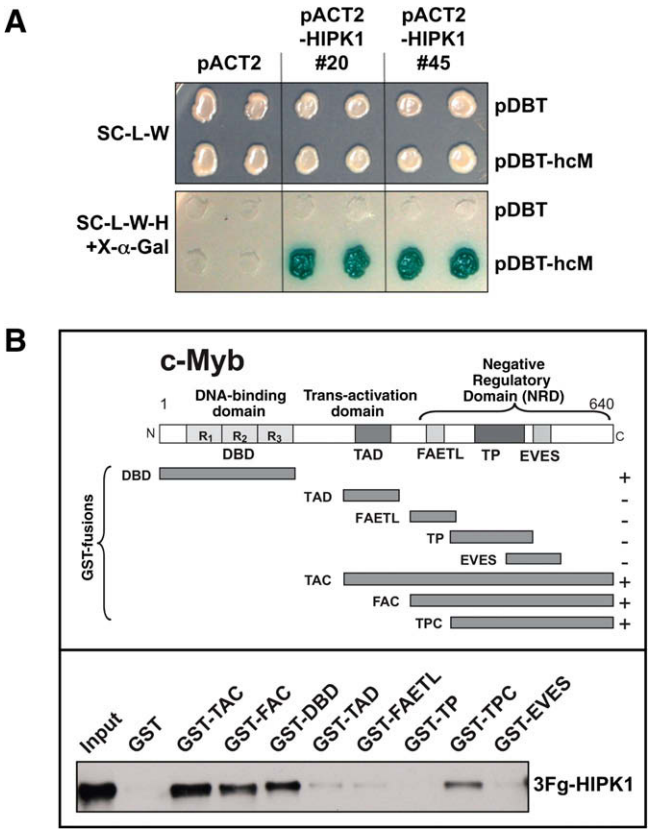

Fig. 1. HIPK1 interacts with c-Myb. (A) The yeast strain AH109 was transformed with the bait vector pDBT or pDBT-hcM, encoding full-length human c-Myb, and mated with Y187 pretransformed with the prey vector pACT2 or pACT2-HIPK1#20 or #45 isolated in the Y2H screening. A control is shown in the top panel with equal growth on medium without tryptophan and leucine (SC-L-W). Interaction was verified by dual activation of the *HIS*3 and the LacZ reporter genes in the lower panel (SC-L-W-H + X-α-Gal). (B) COS-1 cells transfected with a plasmid encoding 3× Flag-tagged full-length human HIPK1 were lysed and incubated with comparable amounts of GST-Myb fusion proteins representing different regions of c-Myb as illustrated in the upper panel. HIPK1 retained on glutathione-Sepharose beads was identified by SDS–PAGE and Western blotting with an anti-Flag antibody.

to a series of different GST-Myb fusion proteins. The extent of HIPK1 binding was assessed by western blotting using an anti-Flag antibody. Significant HIPK1 binding was detected to GST-DBD and to GST-TAC, GST-FAC and GST-TPC, the latter three having in common the C-terminal region of c-Myb. In contrast, only a weak background binding was observed to the internal fragments GST-TAD, GST-FAETL, GST-TP, and GST-EVES, as well as to the GST control. Comparable amounts of GST-fusion proteins were employed in the binding assays (data not shown). We conclude that HIPK1 interacts both with the N-terminal DNA-binding domain and with a C-terminal region of c-Myb.

We reasoned that a significant interaction between HIPK1 and c-Myb *in vivo* would lead to co-localization of the two proteins. Both HIPK1 and HIPK2 have been reported to exhibit punctuate localization in numerous speckles throughout the nucleus apart from the nucleoles [16]. We therefore co-transfected CV-1 cells with expression plasmids for HA-tagged c-Myb and 3× Flag-HIPK1 and analyzed the cells by indirect immunofluorescence and confocal microscopy (Fig. 2). As expected, we found HIPK1 localized mainly in nuclear foci, but with some HIPK1 in the cytoplasm as well. HA-tagged c-Myb was seen both as diffuse nuclear staining and as punctuate forms. Although c-Myb generated fewer foci than HIPK1, they all appeared to co-localize with HIPK1. We conclude that HIPK1 and c-Myb co-localize in the nucleus, consistent with HIPK1 being an interaction partner of c-Myb.

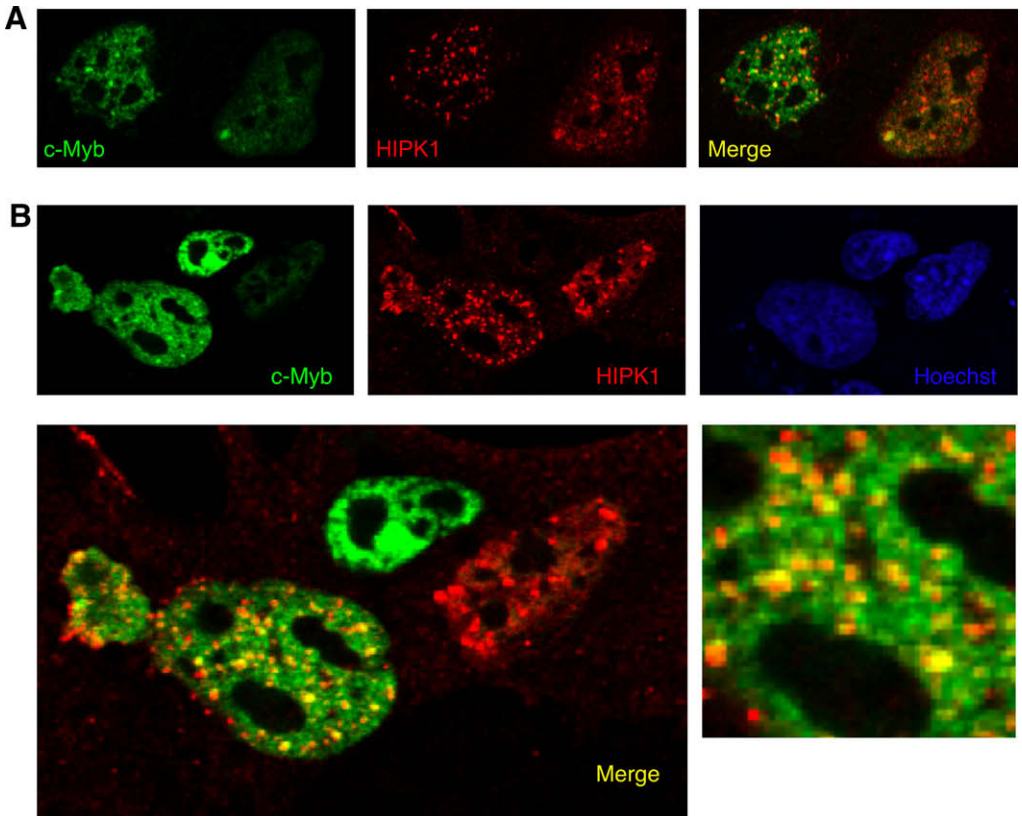

Fig. 2. Co-localization of c-Myb and HIPK1. CV-1 cells were transfected with plasmids encoding HA-tagged c-Myb and/or 3× Flag-tagged full-length human HIPK1 and analyzed by immunofluorescence and confocal microscopy. HA-c-Myb was detected with rabbit anti-HA antibody and Alexa Fluor 488 goat anti-rabbit IgG. FLAG-tagged HIPK1 was detected with mouse anti-FLAG antibody and Alexa Fluor 633 goat anti-mouse IgG1. Merged images are shown in right panel in (A) and in the enlarged panels in (B). (B) Also includes a panel stained with the Hoechst dye to visualize the nuclei.

c-Myb is phosphorylated by HIPK1

To determine whether c-Myb serves as a substrate for HIPK1, we took into consideration that HIPK1 belongs to the CMGC family of protein kinases, being a proline-directed serine/threonine specific kinase. Since the majority of the SP or TP motifs in c-Myb are found in the negative regulatory domain (NRD) of c-Myb, we used this part of c-Myb as a substrate. For practical reasons, this NRD substrate (amino acid residues 410–640) was expressed as a Gal4-DBD fusion protein using the pCIneo-GBD2-hcM-NRD plasmid. This substrate plasmid was co-transfected in CV-1 cells with the expression plasmid pCIneo-3Fg-HIPK1 encoding either wild-type HIPK1 or its kinase-negative mutant, K219A. After cell lysis, aliquots were treated with lambda phosphatase and analyzed by western blotting (Fig. 3A). In the presence of wild-type HIPK1, the Gal4-NRD substrate was seen as a broad band, apparently a double band with a slower migrating component in addition to the main species. This shifted band disappeared after treatment with lambda phosphatase, showing that it represented a phosphorylated form of the substrate. This slower migrating band was much less prominent upon the co-transfection with HIPK1 K219A mutant. This suggested that the c-Myb NRD substrate was being phosphorylated upon co-transfection with HIPK1.

To determine whether the c-Myb-NRD was a direct substrate of HIPK1, we performed an *in vitro* phosphorylation assay (Fig. 3B). To obtain purified HIPK1 kinases, plasmids encoding 3× Flag-HIPK1 wild type and mutant as well as a negative vector control were expressed in COS-1 cells and the kinases were immunopurified on an ANTI-FLAG M2-Agarose Affinity Gel by elution with 3× Flag peptides. As substrate in this assay we used a recombinant GST-hcM-NRD fusion protein, encoding the C-terminal part (amino acid 259–640) of c-Myb ("TAC" in Fig. 1B). This contains all SP/TP motifs in c-Myb. Incorporation of radiolabelled phosphate was observed in two bands in the presence of wild-type HIPK1, one representing autophosphorylated HIPK1 and one being the NRD substrate. Quantification of the scan showed that c-Myb-NRD was clearly phosphorylated *in vitro* by HIPK1 wild-type. We also detected some residual phosphorylation in the presence of the kinase-dead mutant of HIPK1 and to a minor extent for the vector control. This may be caused by the presence of trace amounts of additional kinases following the purification of the kinases from COS-1 cells. We conclude that HIPK1 is able to directly phosphorylate c-Myb *in vitro*.

HIPK1 modulates c-Myb activity transactivation

To examine the functional effects of HIPK1 on c-Myb activity, we took advantage of an established model system for activation of a chromatin embedded Myb target gene, *mim-1*, in HD11 cells. Since these cells are not expressing c-Myb endogenously, the *mim*-1 gene is not activated until c-Myb is expressed ectopically [25,26]. Activation of the *mim-1* gene was monitored by quantitative real-time PCR. As shown in Fig. 4, the *mim*-1 gene expression increased significantly in response to c-Myb, whereas HIPK1 alone did not affect *mim-1* levels. Interestingly, co-expression of c-Myb and HIPK1 produced a substantial decrease in *mim-1*-expression compared to the effect of c-Myb alone. A western control showed that this decrease was not due to reduced c-Myb levels. The HIPK1 mutant also caused a decrease in activation, but significantly less than wild-type HIPK1. Taking the different levels of c-Myb revealed by the western into account, the difference between the effects of wild-type and mutant HIPK1 may be even larger. The residual ef-

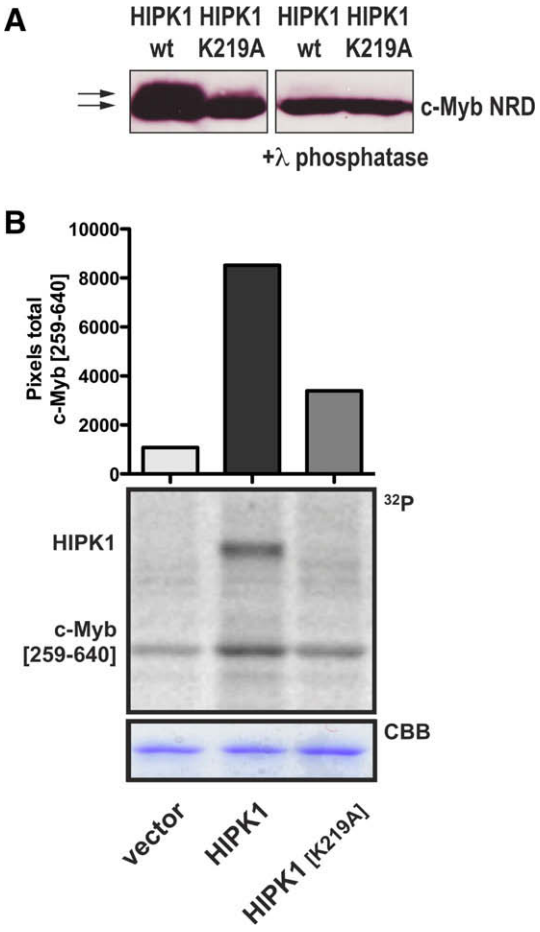

Fig. 3. Examination of the *in vivo* and *in vitro* phosphorylation capacity of HIPK1. (A) Plasmids encoding HIPK1 wild type or the K219A mutant together with plasmids encoding Gal4-c-Myb NRD were co-transfected in CV-1 cells. Aliquots of lysates were treated with lambda phosphatase or mock treated before they were subjected to SDS–PAGE and immunoblot analysis using an anti-HA antibody. The arrows indicate hyperphosphorylated and basal bands in the non-phosphatase treated samples. (B) 3× Flag-tagged HIPK1, wild type and K219A mutant, and negative control (vector only) were expressed in COS-1 cells and immunopurified on ANTI-FLAG M2-Agarose Affinity Gels, and eluted with 3× Flag peptides. As substrate a GST-hcM-NRD fusion protein, encoding the C-terminal part (amino acid residues 259–640) of c-Myb bacterially expressed and purified on glutathione-Sepharose beads, was used. The autoradiogram shown in the lower panel was generated in a Typhoon 9400 scanner and the quantification of the scan shown in the upper panel was performed using the UN-SCAN-IT gel Version 5.3 software.

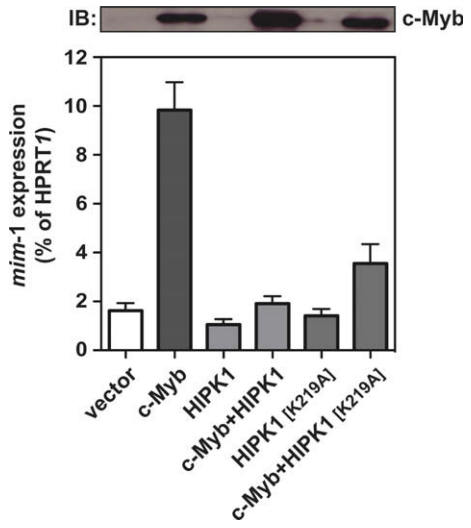

Fig. 4. HIPK1 represses c-Myb induced activation of *mim-1*. Plasmids encoding the indicated proteins were transfected into HD11 cells and total RNA was isolated. Activation of the endogenous Myb target gene, *mim-1*, was measured by quantitative real-time PCR with primers specific for *mim*-1 and *HPRT1* as described in Materials and methods. The *mim*-1 expressions are presented as fractions relative to the *HPRT1* expression ±SD ($n = 4$).

fect of mutant HIPK1 may be caused by other kinases associated with HIPK1 or an inhibitory effect of the interaction between c-Myb and HIPK1 independent of phosphorylation. We conclude that HIPK1 acts as a negative regulator of c-Myb in *mim-1* transactivation.

Discussion

We have in this work revealed a novel link between the transcription factor c-Myb and the nuclear kinase HIPK1. The Y2H screening data suggests an interaction involving the C-terminal region of HIPK1, and the GST-pulldown mapping using different domains of c-Myb indicates a bipartite interaction surface in c-Myb, including regions in its N-terminal DNA-binding domain as well as in its C-terminal region. When co-transfected, HIPK1 and c-Myb co-localize in distinct nuclear foci. c-Myb appears to be phosphorylated by HIPK1 in its NRD region. A functional assay revealed that HIPK1 was acting as a co-repressor of c-Myb.

It has previously been reported that c-Myb interacts with the related kinase HIPK2 [10]. Some differences between our observations on c-Myb and HIPK1 and the reported HIPK2 data are noteworthy. While HIPK2 was previously isolated in a Y2H screening using a mouse embryo library and a c-Myb deletion mutant as bait [10], the screening reported here was performed using a library from human bone marrow plus a human hematopoietic cell line and using full-length wild-type c-Myb as bait. An additional distinction is that the present screening was performed using the bait expressed from a low-copy number centromeric plasmid, which due to lower levels of bait should give more stringent conditions than standard procedures, favouring stronger interactions. We noticed that under these favourable conditions we isolated several independent clones of HIPK1, but none of HIPK2. We therefore believe that HIPK1 may be as relevant a partner for c-Myb as HIPK2 is. Expression data suggest that HIPK1 may be more highly expressed than HIPK2 in hematopoietic cells (http://biogps.gnf.org). Importantly, the HIPKs appear to be crucial for haematopoiesis since Hipk1/Hipk2 double-deficient mice exhibit defects in primitive as well as definitive haematopoiesis [7]. A link between the hematopoietic factor c-Myb and the HIPKs may therefore be of significance for understanding c-Myb function.

According to Kanei-Ishii et al. [10], although HIPK2 binds to the DBD of c-Myb, it did not directly phosphorylate the c-Myb protein. However, HIPK2 was found to act as an activator of the Nemo-like kinase (NLK), which efficiently phosphorylated c-Myb at >10 sites. In our study, we found that HIPK1, like HIPK2, binds to c-Myb DBD, but that HIPK1 in addition also binds to a C-terminal region of c-Myb. Since all SP and TP sites in c-Myb (12 in total) are located C-terminally to the DBD, mostly in the NRD, an interaction surface that involves more than DBD may create a HIPK1–c-Myb complex more favourable for direct phosphorylation. Consistent with this assumption, we did observe a direct *in vitro* phosphorylation of c-Myb-NRD using affinity-purified HIPK1. Future experiments will address which of the 12 candidate S/T-P sites are targeted by HIPK1 and how their phosphorylation lead to reduced gene activation. Another question of interest is to determine whether HIPK1 cooperates with other kinases or are influenced by posttranslational modifications.

We also observed that HIPK1 caused reduced c-Myb dependent gene activation in an assay monitoring the activation of endogenous *mim*-1 expression in HD11 cells. If HIPK1 acted upon c-Myb in the same way as reported for HIPK2, we would have expected the HIPK1-induced loss of activation to occur because of a HIPK-induced degradation of c-Myb. However, our western control showed rather the opposite, that the c-Myb level was slightly enhanced by the coexpressed HIPK1. If this is not due to differences in signalling pathways active in the different cell types used, it might suggest that the two kinases behave differently towards c-Myb. Future experiments are needed to validate this difference and to unravel mechanistic details on how the two kinases act on the transcription factor c-Myb.

We have previously identified three other interaction partners of c-Myb: Ubc9 [22], Mi-2α [23], and FLASH [24], and now HIPK1. Intriguingly, all these interaction partners have in common a link to the SUMO-conjugation system. Ubc9 is an E2 SUMO ligase [27] responsible for sumoylation of c-Myb [22]. Mi-2a has been reported to bind to SUMO, and is recruited to promoters in a SUMOylation-dependent manner [28,29]. FLASH is itself SUMO-conjugated, and its activity is influenced by the SUMO-protease SENP1 [30]. SENP1 is also reported to by involved in a signalling cascade leading to cytoplasmic translocation of HIPK1 [11]. Future work will address how these partners cooperate in regulating c-Myb function.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.139.

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