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Roles of HIPK1 and HIPK2 in AML1- and p300-dependent transcription, hematopoiesis and blood vessel formation

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Histone acetyltransferases (HATs) p300 and CREB-binding protein (CBP) function as co-activators for a variety of sequence-specific transcription factors, including AML1. Here, we report that homeodomain-interacting protein kinase-2 (HIPK2) forms a complex with AML1 and p300, and phosphorylates both AML1 and p300 to stimulate transcription activation as well as HAT activities. Phosphorylation of p300 is triggered by phosphorylated AML1 as well as by PU.1, c-MYB, c-JUN and c-FOS, and is inhibited by dominant-negative HIPK2. Phosphorylation of p300 and AML1 is impaired in *Hipk1/2* double-deficient mouse embryos. Double-deficient mice exhibit defects in primitive/definitive hematopoiesis, vasculogenesis, angiogenesis and neural tube closure. These phenotypes are in part similar to those observed in p300- and CBP-deficient mice. HIPK2 also phosphorylates another co-activator, MOZ, in an AML1-dependent manner. We discuss a possible mechanism by which transcription factors could regulate local histone acetylation and transcription of their target genes.

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Introduction

Covalent modification of histones (acetylation, phosphorylation, methylation, ubiquitination and sumoylation) is essential for regulating gene expression. These modifications appear to be inter-dependent and represent an evolutionarily conserved 'histone code' that alters the interactions between histones and DNA, as well as marks binding sites on histones for effector proteins that regulate gene expression (Jenuwein and Allis, 2001). Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs and HDACs, directly or through binding to transcription cofactors, interact with transcription factors, which recognize specific DNA sequences in their target genes, to regulate the acetylation of local histones (Marmorstein and Roth, 2001; Thiagalingam *et al*, 2003). The HAT p300 and the closely related CREB-binding protein (CBP) have been shown to function as co-activators for a wide variety of transcription factors and to play central roles in transcriptional control in response to a diverse range of physiological stimuli (Legube and Trouche, 2003).

The *AML1* (*RUNX1*) gene is the most frequent target of leukemia-associated chromosome translocations, which are frequently detected in human leukemia (Look, 1997). AML1 protein forms a heterodimer with CBFβ and binds to a specific DNA sequence to regulate the expression of a number of hematopoietic genes (Meyers *et al*, 1993; Ogawa *et al*, 1993). Both AML1 and CBFβ are essential for the development of all definitive hematopoiesis lineages (Okuda *et al*, 1996; Sasaki *et al*, 1996; Wang *et al*, 1996a, b; Niki *et al*, 1997; Okada *et al*, 1998). AML1 forms complexes with PML and HATs, such as p300/CBP and MOZ, to activate transcription (Kitabayashi *et al*, 1998b, 2001; Nguyen *et al*, 2005). AML1 can act synergistically with other transcription factors, such as AP-1 (Cockerill *et al*, 1996), C/EBPα (Zhang *et al*, 1996), c-Myb (Britos-Bray and Friedman, 1997), PU.1 (Petrovick *et al*, 1998) and Ets-1 (Kim *et al*, 1999). Inducible phosphorylation of AML1 is correlated with increased amounts of MOZ in the AML1 complex during differentiation of M1 myeloid cells (Kitabayashi *et al*, 2001), suggesting that phosphorylation of AML1 is important for regulation of AML1 complex formation and transcriptional activation.

Homeodomain-interacting protein kinases (HIPKs) are nuclear serine/threonine kinases, and three members of the HIPK family (HIPK1, HIPK2 and HIPK3) have been reported (Kim *et al*, 1998). The functional importance of HIPKs has chiefly been studied using HIPK2 and estimated by means of their interacting proteins and phosphorylation targets, including homeoproteins, p53, CtBP1 and Myb (Kim *et al*, 1998; Choi *et al*, 1999; D'Orazi *et al*, 2002; Hofmann *et al*, 2002; Zhang *et al*, 2003; Kanei-Ishii *et al*, 2004). All of these factors represent critical regulators of transcription, suggesting that HIPKs play critical roles in transcriptional regulation.

The activity of CBP or p300 HAT has been shown to be stimulated by a variety of sequence-specific transcription factors, such as HNF1α, HNF4, Sp1, Zta, NF-E2, C/EBPs and phosphorylated Elk1 (Chen *et al*, 2001; Soutoglou *et al*, 2001; Kovacs *et al*, 2003; Li *et al*, 2003; Schwartz *et al*, 2003). Through this stimulation, these sequence-specific transcription factors are thought to increase the acetylation of histones at their target promoters. However, the mechanisms

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regulating the activity of the transcription factor/HAT complexes on their target genes remain unclear. Here, we report that phosphorylation of p300 is induced by phosphorylated (active) AML1 but not by dephosphorylated (inactive) AML1. AML1 interacts with HIPK2 and mediates phosphorylation of p300 to stimulate HAT activity and induction of transcription. HIPK2-dependent phosphorylation of p300 is also induced by other transcription factors, such as PU.1, c-MYB, c-JUN and c-FOS, suggesting that the present novel mechanism of transcriptional regulation is of general relevance. The importance of HIPK1 and HIPK2 for inducible phosphorylation of p300 was revealed in double-deficient mice, which have defective p300 phosphorylation and show in part a similar phenotype to p300- and CBP-deficient mice.

Results

AML1 is phosphorylated at Ser249 and Ser276

Phosphorylation of AML1 is suggested to be important for its function in transcription and cell differentiation. To determine the phosphorylation sites of AML1, a full-length AML1 protein, AML1b, was purified from L-G myeloid cells, in which AML1 is highly phosphorylated. AML1b was then digested with trypsin and subjected to LC/MS/MS analysis. The predicted phosphorylation sites were Ser249 and Ser276. Comparison of these sites and their surrounding sequences among the different species indicated that the phosphorylation sites of AML1 have been evolutionarily conserved (Figure 1A). Mutants in which Ser249 and/or Ser276 were substituted by alanine, migrated faster than wild type (WT) when expressed in L-G cells (Figure 1B). Phosphatase-treated WT AML1b migrated to a similar position as the S249/276A mutant (Figure 1C). These results suggest that Ser249 and Ser276 of AML1 are phosphorylated in L-G cells. Alteration of Ser266, which is specifically phosphorylated by Erk (Tanaka *et al*, 1996), to alanine did not affect migration (Figure 1B).

HIPK2 phosphorylates AML1 at Ser249 and Ser276

It has been reported that AML1b can be phosphorylated by Erk in 3T3 fibroblasts (Tanaka *et al*, 1996). However, treatment of L-G cells with inhibitors of Erk pathways did not affect the phosphorylation of AML1b (Supplementary Figure S1), suggesting the involvement of other kinases in this phosphorylation. We previously purified the AML1b complex from L-G cells (Kitabayashi *et al*, 2001) and identified the serine/threonine kinase HIPK2 as a component of the AML1 complexes (Supplementary Figure S2). Immunoblot analysis confirmed that HIPK2 is present in the purified complex (Figure 1D). To confirm this interaction, 293T cells were transfected with HA-tagged AML1b together with WT or kinase-dead (KD) point mutant FLAG-tagged HIPK2. HIPK2 proteins were immunoprecipitated with anti-FLAG antibodies. On immunoblot analysis, HA-AML1b was co-precipitated with the WT and mutant HIPK2 (Figure 1E).

To determine whether HIPK2 phosphorylates AML1, we tested the phosphorylation of AML1 by HIPK2 *in vivo* and *in vitro*. 293T cells were transfected with WT and mutant AML1 together with HIPK2. Immunoblot analysis demonstrated that co-transfection of HIPK2 leads to retardation of the protein band for WT AML1b (Figure 1F). Note that AML1b phosphorylation levels are low (i.e., its migration is fast) when AML1 is transiently transfected into 293T cells. Substitution of either Ser249 or Ser276 by alanine diminished the changes in band migration caused by HIPK2. In *in vitro* kinase assays, HIPK2 strongly phosphorylated WT AML1 as well as itself (Figure 1G). Mutations in Ser249 and Ser276 strongly inhibited HIPK2-mediated phosphorylation of AML1. These results indicate that HIPK2 is able to phosphorylate AML1 at Ser249 and Ser276.

HIPK2 stimulates cooperation between AML1, MOZ and p300 in transcription activation

In order to clarify the roles of phosphorylation in the function of AML1, we tested the effects of HIPK2 on AML1-mediated transcription. WT/mutant AML1 was co-transfected with p300/MOZ HAT, HIPK2 and MPO-luc or CCP1-luc reporter genes containing AML1-binding sites. As shown in Figure 1H, MOZ and p300 stimulated AML1-dependent transcription, as reported previously (Kitabayashi *et al*, 2001). MOZ-mediated and p300-mediated transcription activation was strongly enhanced by WT HIPK2 and was completely abolished by KD HIPK2, which functions in a dominant-negative manner. These results suggest that HIPK2 plays an important role in AML1-mediated transcription, particularly in its cooperation with MOZ and p300. HIPK2-, MOZ- and p300-induced transcription activation was almost completely abolished by substitution of Ser249 and Ser276 by alanine (S249/276A). In contrast, this activation activity was strongly enhanced when Ser249 and Ser276 were changed to aspartic acid, which mimics phosphorylated amino acids (S249/276D). These results suggest that phosphorylation of Ser249 and Ser276 on AML1 is essential for transcription activation.

Phosphorylation of p300 by activated AML1 and HIPK2

Because the above results suggest that HIPK2 stimulates AML1 cooperation with p300 and MOZ in transcription, we inferred that HIPK2-mediated AML1 phosphorylation stimulates interaction with p300 and/or MOZ. We then tested the effects of HIPK2 on these interactions. Co-immunoprecipitation analysis indicated that co-transfection with WT or KD HIPK2 had no effect on the interaction between AML1 and p300 (Figure 2A). Unexpectedly, however, we observed differences in mobility of the p300 band after transfection with AML1 and HIPK2. As shown in the upper panel of Figure 2A, co-transfection with AML1 resulted in mobility retardation of p300 (lane 2). This shift in mobility was due to phosphorylation, as treatment with phosphatase restored mobility (Figure 2B). A similar shift in mobility was also induced by co-transfection with WT HIPK2 (Figure 2A, lane 3). More importantly, the AML1-induced mobility shift of p300 was completely inhibited by co-transfection with KD HIPK2 (Figure 2A, lane 6). *In vitro* kinase assays revealed direct phosphorylation of p300 by HIPK2 (Figure 2C). These results suggest that AML1 is able to induce HIPK2-mediated phosphorylation of p300.

The mobility shift of p300 was not induced when co-transfection was performed with an alanine mutant (2A = S249/276A), which lacks phosphorylation sites (upper panel in Figure 2D). In contrast, an aspartic acid mutant (2D = S249/276D), which mimics phosphorylated AML1, induced a greater mobility shift than WT AML1. These results suggest that activation of AML1 by phosphorylation is required for AML1-induced phosphorylation of p300. Co-immunoprecipitation experiments indicated that

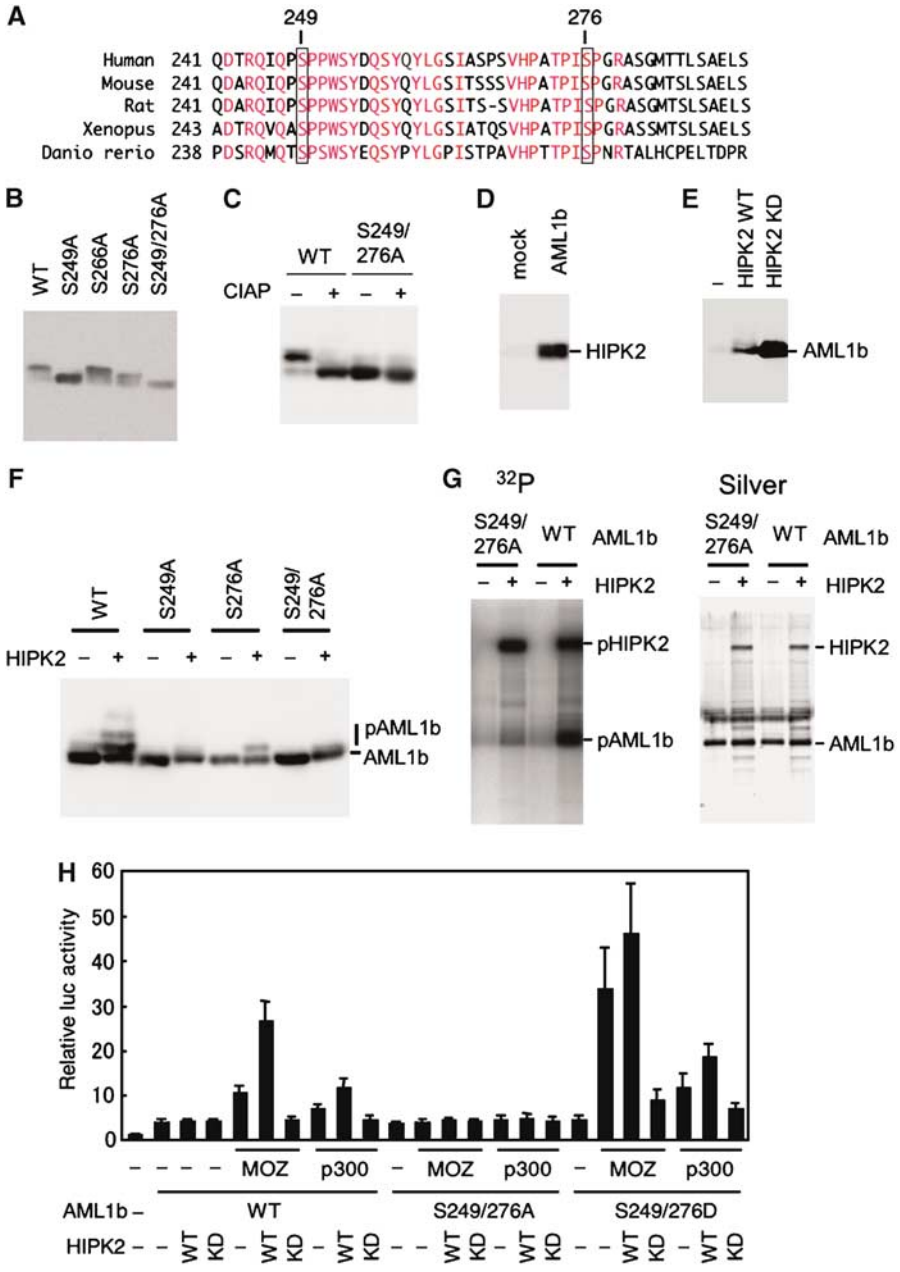

Figure 1 AML1 is phosphorylated at Ser249 and Ser276 by HIPK2. (**A**) Phosphorylation sites are evolutionarily conserved. (**B**) Migration of WT and mutant AML1 in L-G cells. (**C**) Phosphatase treatment of AML1. Purified WT and S249/276A mutant AML1b were treated with (+) or without (−) calf intestine alkaline phosphatase (CIAP) and analyzed by immunobloting with anti-AML1 antibody. (**D**) AML1 complex contains HIPK2. Purified AML1 complexes were analyzed by immunobloting with anti-HIPK2 antibody. (**E**) Interaction of AML1 with HIPK2. 293T cells were transfected with HA-AML1b together with either FLAG-tagged WT or KD HIPK2. Immunoprecipitates with anti-FLAG were analyzed by immunoblotting with anti-HA antibody. (**F**) Phosphorylation of AML1 by HIPK2. Cell extracts from 293T cells expressing WT or mutants of HA-tagged AML1 and HIPK2 were subjected to immunoblot analysis with anti-HA antibody. (**G**) Phosphorylation of AML1 *in vitro*. Purified WT or mutant AML1b proteins were tested for *in vitro* kinase activity. (**H**) HIPK2 activates AML1-mediated transcription. SaOS2 cells were transfected with 50 ng of MPO-luc, 200 ng of LNCX-AML1b, 250 ng of MOZ or p300, 250 ng of WT or KD mutant of HIPK2 and 2 ng of phRL-cmv. Cell lysates were analyzed for luciferase activity at 24 h after transfection.

neither the alanine nor aspartic acid mutation affected the interaction between AML1 and p300 (Figure 2D, lower panel). In addition, neither of these mutations affected the interaction between AML1 and HIPK2 (Supplementary Figure S3).

HIPK2 activates p300

In order to investigate the role of phosphorylation by HIPK2 on p300 function, we tested the effects of HIPK2 on p300 HAT and transcription activation activities. To test HAT activity, FLAG-tagged p300 was co-transfected with WT HIPK2 and KD mutant, purified using anti-FLAG antibodies and tested for HAT activity. Intrinsic HAT activity of p300 was activated by WT HIPK2 and AML1b, and was inhibited by KD HIPK2 (Figure 2E). Reporter analysis indicated that WT HIPK2 and AML1 stimulated transcription activation by Gal4-p300 (Figure 2F). These results suggest that HIPK2 stimulates HAT activity and p300-mediated transcription activation.

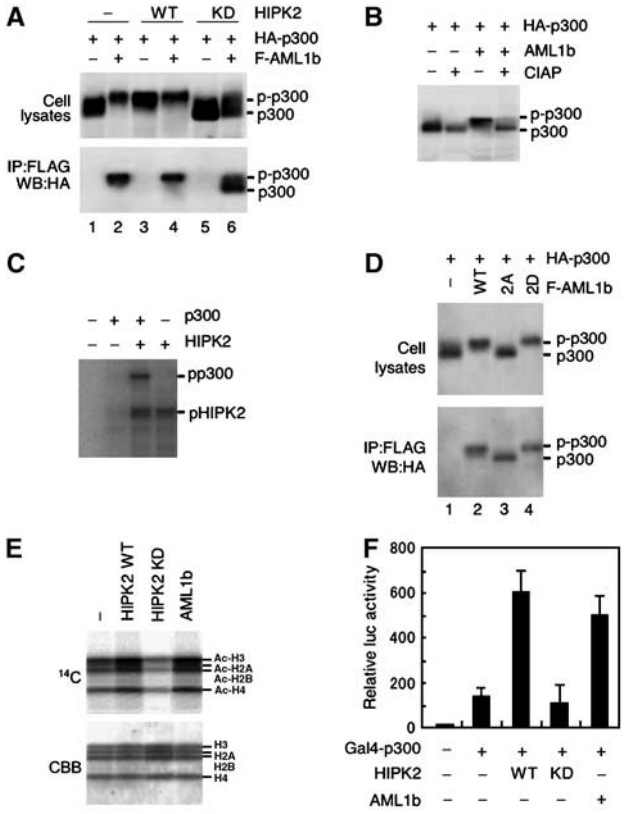

Figure 2 HIPK2 mediates AML1-dependent phosphorylation of p300. (**A**) AML1 induces phosphorylation of p300. 293T cells were transfected with FLAG-AML1b, HA-p300 and HIPK2. Cell lysates were immunoprecipitated with anti-FLAG antibodies. Cell lysates (1%) and immunoprecipitates were then analyzed by immunoblotting with anti-HA antibody. (**B**) CIAP-treatment of AML1–p300 complex. 293T cells were transfected with HA-p300 and FLAG-AML1b. After immunoprecipitation with anti-HA antibodies, immunoprecipitates were treated with CIAP and analyzed by immunoblotting with anti-HA antibody. (**C**) Phosphorylation of p300 *in vitro*. Purified WT p300 protein was incubated with or without HIPK2 in the presence of [γ^{32}P]ATP and was separated by 7% SDS–PAGE. Gels were dried and subjected to autoradiography. (**D**) Phosphorylation of AML1 is required for AML1-induced phosphorylation of p300. 293T cells were transfected with HA-p300 and FLAG-tagged WT, S249/276A (2A) or S249/276A (2D) mutant AML1b. After immunoprecipitation with anti-FLAG antibodies, cell lysates (1%) and immunoprecipitates were analyzed by immunoblotting with anti-HA antibody. (**E**) HIPK2 and AML1 stimulates HAT activity of p300. 293T cells were transfected with FLAG-p300 together with either WT or KD HIPK2, or AML1b. After immunoprecipitation with anti-FLAG antibodies, immunoprecipitates were analyzed for HAT activity. (**F**) HIPK2 and AML1 stimulate transcription activation by a Gal4-p300 fusion protein. 293T cells were transfected with 500 ng of pFR-luc(Gal4-luc), 100 ng of Gal4-p300, 400 ng of WT or KD HIPK2, or AML1b and 2 ng of phRL-cmv. Cell lysates were analyzed for luciferase activity at 24 h after transfection.

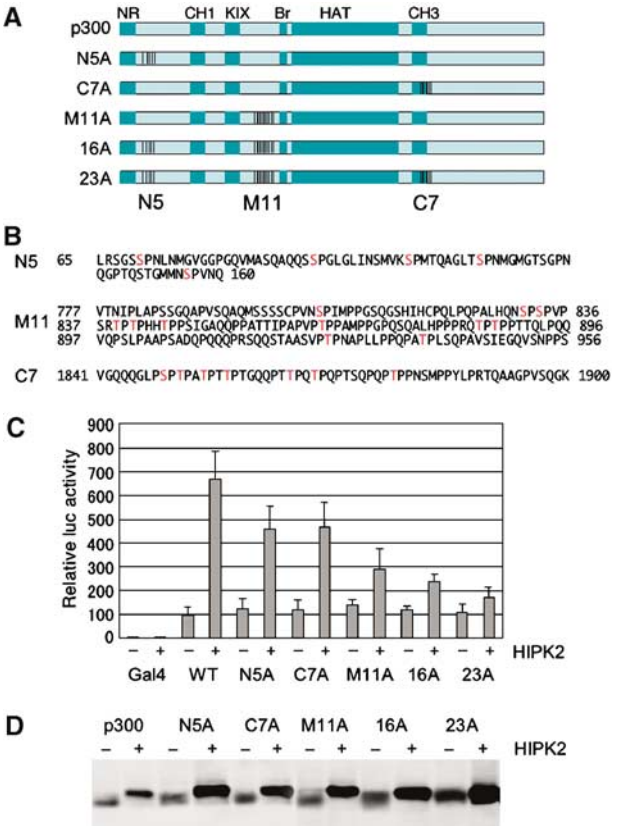

Figure 3 Phosphorylation sites of p300 by HIPK2. (**A**) Schematic representation of p300 mutants used for determining HIPK2-induced phosphorylation sites. (**B**) Amino-acid sequence of putative HIPK2 phosphorylation sites of p300. Serines or threonines shown in red were substituted with alanines. (**C**) HIPK2-induced activation of transcription by Gal4-p300 mutants. 293T cells were transfected with 500 ng of pFR-luc(Gal4-luc), 100 ng of WT or mutant Gal4-p300, 400 ng of HIPK2 and 2 ng of phRL-cmv. Cell lysates were prepared at 24 h after transfection and were analyzed for luciferase activity. (**D**) Phosphorylation of p300 mutants by HIPK2. 293T cells were transfected with WT or mutants of Gal4-p300 together with HIPK2. Cell lysates were subjected to immunoblot analysis with anti-Gal4 antibody.

The p300 sites phosphorylated by HIPK2 were investigated using various mutants of Gal4-p300, in which the putative phosphorylation sites (SP and TP) were substituted with AP, and these mutants were tested for both transcription activation and phosphorylation by HIPK2. Transcription activation and phosphorylation of p300 were markedly reduced by substitution of the 23 SP/TP sites (Figure 3).

Interaction between HIPK2 and p300

Overexpressed HIPK2 was able to phosphorylate p300, even in the absence of AML1, thus suggesting a physical interaction between HIPK2 and p300. To test this interaction, FLAG-tagged HIPK2 or AML1 were co-transfected with HA-p300 or truncated versions thereof as schematically shown in Figure 4A. On IP-Western analysis, p300 was co-precipitated with WT and KD HIPK2 in addition to AML1 (Figure 4B). Reciprocal co-immunoprecipitation experiments also indicated that WT and KD HIPK2 were co-precipitated with p300 (Figure 4C). Deletion analysis of p300 allowed mapping of the HIPK2-binding domain (1304–1571) and AML1-binding domain (142–597) (Figure 4D). Interactions between AML1 and p300 were markedly reduced by deletion of the 142–957 region of p300, but the interaction could be still detected weakly after overexposure (Figure 4D), suggesting an indirect interaction through other factors or another AML1-interacting domain. GST pull-down analysis indicated that HIPK2, which was translated *in vitro*, bound to bacterially produced GST-AML1b and GST-p300(HAT) proteins (Figure 4E), suggesting that HIPK2 directly interacts with AML1 and p300.

Phosphorylation of p300 by various transcription factors

Various transcription factors are known to utilize p300 as a co-activator. It was recently reported that the C/EBP family of

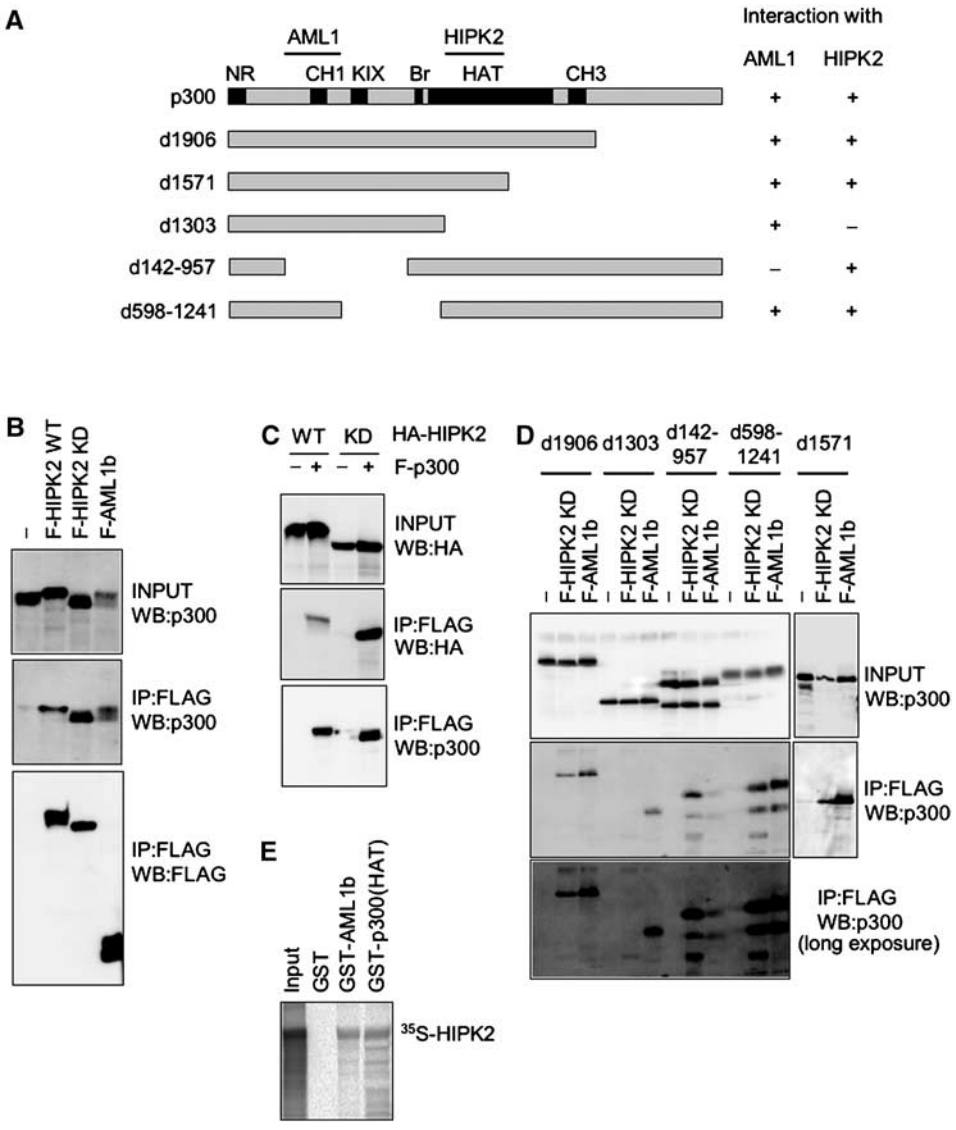

Figure 4 Interaction of p300 and HIPK2. (**A**) Schematic representation of p300 deletion mutants used for determining HIPK2-binding domains. (**B**) Interaction of p300 with HIPK2. 293T cells were transfected with p300 together with FLAG-tagged WT or KD HIPK2, or AML1b. (B–D) Cell lysates (1%) and immunoprecipitates with anti-FLAG antibodies were analyzed by immunoblotting with anti-p300, anti-HA or anti-FLAG antibodies. (**C**) Reciprocal coimmunoprecipitation of HIPK2 and p300. (**D**) Interaction of p300 mutants with HIPK2 and AML1. 293T cells were transfected with p300 mutants as shown together with KD HIPK2 or AML1b. The lower panel represents a longer exposure of the same membrane shown in the middle panel. (**E**) HIPK2 translated *in vitro* and labelled with ^{35}S was tested for binding to bacterially produced GST, GST-AML1b and GST-p300(HAT) proteins as shown. The input lane represents 10% of the material used for binding to the GST fusion protein.

transcription factors induce phosphorylation of p300 and CBP (Kovacs *et al*, 2003; Schwartz *et al*, 2003). To test whether phosphorylation of p300 is induced by other transcription factors that bind to p300, several transcription factors were co-transfected with p300. Mobility shifts of p300 were induced by AML/runx family members, PU.1, C/EBPα, C/EBPε, c-MYB, c-JUN and c-FOS, but were not induced by IRF3, p53, RARα or ATF2 (Figures 5A and C). To determine whether these shifts were mediated by HIPK2, the dominant-negative HIPK2 was co-transfected with these factors. As shown in Figure 5B, the dominant-negative HIPK2 completely inhibited the phosphorylation of p300 induced by AML1/2/3, PU.1, c-MYB, c-JUN and c-FOS, which suggests that HIPK2 mediates p300 phosphorylation by these factors. On the other hand, phosphorylation induced by C/EBPα and C/EBPε was only partially inhibited by the dominant-negative HIPK2, which suggests that other kinase(s) are involved (Figure 5C).

Roles of HIPK family members in phosphorylation and transcription

We generated *Hipk1*- and *Hipk2*-deficient mice by targeted disruption of the corresponding genes (Isono *et al*, 2006). *Hipk1* and *Hipk2* single-deficient mice are grossly normal (Kondo *et al*, 2003; Wiggins *et al*, 2004), but *Hipk1/Hipk2* double homozygotes die between E9.5 and E12.5 (Isono *et al*, 2006). These findings suggest functional redundancy between HIPK1 and HIPK2. We thus addressed the physiological roles of HIPK family proteins by using *Hipk1/Hipk2* double mutants. To investigate the phosphorylation of p300, proteins were extracted from whole embryos at E10.0 and were analyzed by immunoblottng with anti-p300 antibodies. Although significant amounts of highly phosphorylated forms of p300 were observed in *Hipk1*-deficient, *Hipk2*-deficient and WT embryos, highly phosphorylated forms were severely reduced in *Hipk1/Hipk2* double-deficient

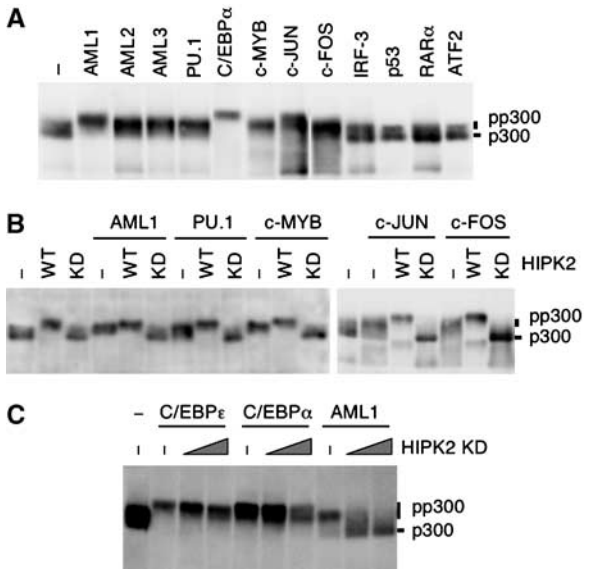

Figure 5 HIPK2 mediates phosphorylation of p300 induced by a set of transcription factors. (**A**) Phosphorylation of p300 by various transcription factors. 293T cells were transfected with HA-p300 together with various transcription factors. Cell lysates were subjected to immunoblot analysis with anti-HA antibody. (**B**) Effects of WT and KD HIPK2 on transcription factor-induced phosphorylation of p300. 293T cells were transfected with HA-p300 and the transcription factors indicated together with WT and KD HIPK2. (**C**) Effects of KD HIPK2 on phosphorylation of p300. 293T cells were transfected with HA-p300 (0.2 μg) and either C/EBPε, C/EBPα or AML1b (0.4 μg) together with increasing amounts (0.2 and 0.4 μg) of KD HIPK2.

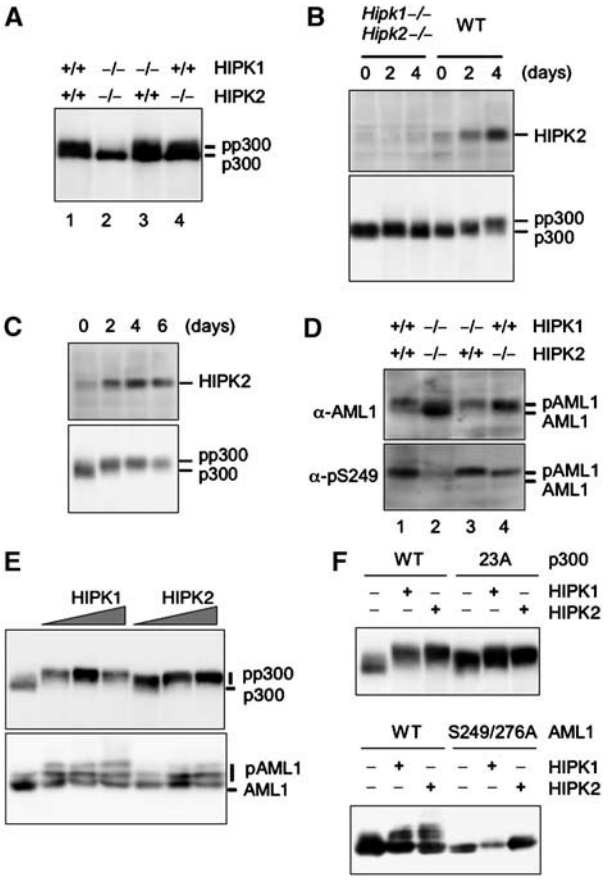

Figure 6 HIPK1 and HIPK2 are essential for phosphorylation of p300 and AML1. (**A**) Phosphorylation of p300 in HIPK1- and/or HIPK2-deficient embryos. Proteins were extracted from WT and mutant E10.0 embryos and were subjected to immunoblot analysis with anti-p300 antibody. (**B**) Expression of HIPK2 and phosphorylation of p300 during differentiation of ES cells. WT and Hipk1/2 double-deficient ES cells were exposed to 1 μM retinoic acid for 2 and 4 days. The cell lysates were subjected to immunoblot analysis with anti-HIPK2 and anti-p300 antibodies. (**C**) Expression of HIPK2 and phosphorylation of p300 during differentiation of myeloid cells. 32Dcl3 cells were exposed to 10 ng G-CSF for indicated number of days. (**D**) Phosphorylation of AML1 in HIPK1- and/or HIPK2-deficient yolk sac. Yolk sac cells from E10.0 embryos were cultured on OP9 cells for 5 days. Cell lysates were subjected to immunoblot analysis with anti-AML1 and phospho-S249-specific AML1 antibodies. (**E**) Phosphorylation of p300 and AML1 by HIPK1 and HIPK2. 293T cells were transfected with HA-p300 or AML1 (0.2 μg) together with increasing amounts (0.05, 0.1, 0.2 μg) of HIPK1 or HIPK2. Cell lysates were subjected to immunoblot analysis with anti-HA antibody. (**F**) 293T cells were transfected with WT or mutant Gal4-p300 or HA-AML1 together with HIPK1 or HIPK2. Cell lysates were subjected to immunoblot analysis with anti-Gal4 or anti-HA antibody.

embryos (Figure 6A). It has been reported that phosphorylation of p300 is stimulated during retinoic acid-induced differentiation of embryonic stem (ES) cells (Kitabayashi *et al*, 1995). To test whether HIPK1 and HIPK2 are required for p300 phosphorylation, we investigated changes in phosphorylation of p300 after retinoic acid-induced differentiation of WT and HIPK1/HIPK2 double knockout ES cells. Phosphorylation of p300 was stimulated and was associated with expression of HIPK2 during differentiation of WT ES cells but not in double knockout ES cells (Figure 6B). Induction of p300 phosphorylation and HIPK2 expression was also observed in G-CSF-induced differentiation of 32Dcl3 cells (Figure 6C).

To confirm the phosphorylation of AML1, which is predominantly expressed in hematopoietic cells, nonadherent cells were prepared from the yolk sac of mutant embryos and were expanded by culture *in vitro* for 5 days. Immunoblot analysis using anti-AML1 antibody indicated that hypophosphorylated forms of AML1 were increased in *Hipk1/Hipk2* double-deficient cells when compared with those in single-deficient cells or WT cells. Immunoblot analysis using phospho-specific antibody (Supplementary Figure S4), which detects AML1 phosphorylated at Ser249, indicated that phosphorylated AML1 was markedly decreased in *Hipk1/Hipk2* double-deficient cells (Figure 6D).

In order to test whether HIPK1 can induce phosphorylation of p300 and AML1, 293T cells were transfected with p300 and AML1 together with HIPK1. Phosphorylation of p300 and AML1 was strongly induced by HIPK1 as well as by HIPK2 (Figure 6E). These phosphorylations were largely inhibited by substitution with alanine at the phosphorylation sites (Figure 6F). Co-immunoprecipitation analysis indicated that HIPK1 as well as HIPK2 interact with AML1 (Supplementary Figure S5).

Reporter analysis indicated that HIPK1 also stimulates transcription activation by Gal4-p300 and by AML1 (Figure 7A and B). Reporter analysis using WT and HIPK1/2 double-deficient mouse embryonic fibroblasts showed an impaired AML1-dependent transcription in HIPK1/HIPK2 double-deficient cells (Figure 7C). Thus, we conclude that both HIPK1 and HIPK2 mediate the phosphorylation of p300 and AML1, and enhance AML1- and p300-dependent transcription.

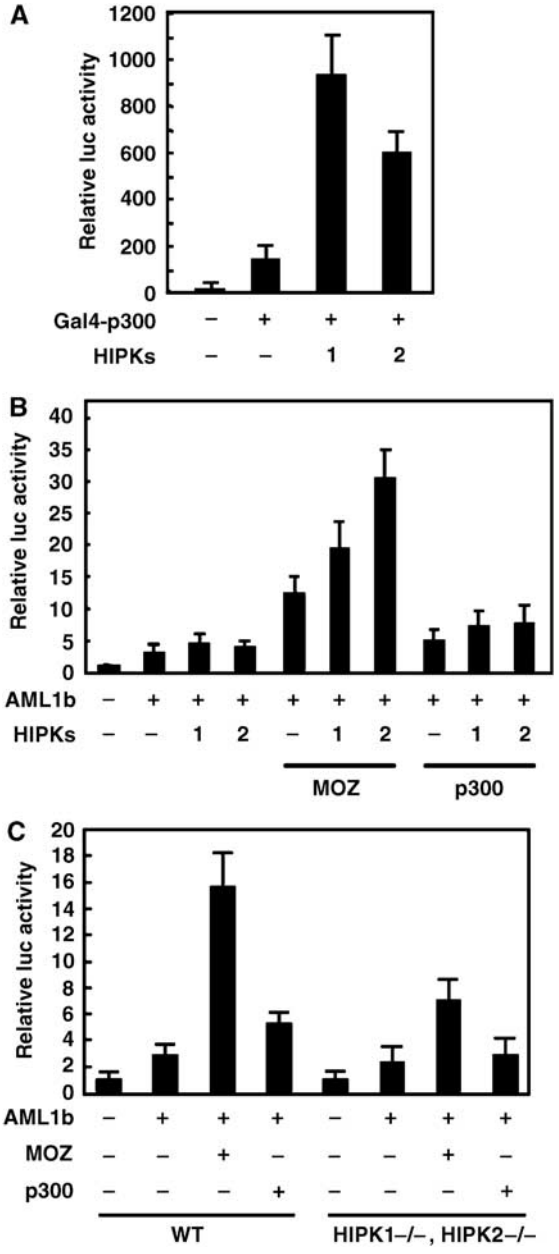

Figure 7 HIPK1 and HIPK2 are involved in p300- and AML1-mediated transcription. (**A**) 293T cells were transfected with 500 ng of pFR-luc(Gal4-luc), 100 ng of Gal4-p300, 400 ng of HIPK1 or HIPK2, and 2 ng of phRL-cmv. (**B**) SaOS2 cells were transfected with 50 ng of MPO-luc, 200 ng of LNCX-AML1b, 250 ng of MOZ or p300, 250 ng of HIPK1 or HIPK2, and 2 ng of phRL-cmv. (**C**) WT and HIPK1/2 double-deficient MEFs were transfected with 50 ng of MPO-luc, 200 ng of LNCX-AML1b, 500 ng of MOZ or p300, and 2 ng of phRL-cmv. Cell lysates were analyzed for luciferase activity at 24 h after transfection.

Hematopoiesis defects in HIPK1/2-deficient mice

Because AML1, p300 and the closely related CBP are essential for hematopoiesis (Okuda *et al*, 1996; Wang *et al*, 1996a; Oike *et al*, 1999; Tanaka *et al*, 2000; Kasper *et al*, 2002; Rebel *et al*, 2002), we analyzed hematopoietic cells in the yolk sac and the paraaortic splanchnopleural (P-Sp) region, in which definitive hematopoiesis is first detected during development. A marked decrease in the total number of cells was observed in *Hipk1−/−Hipk2−/−* yolk sacs (Figure 8A). To investigate

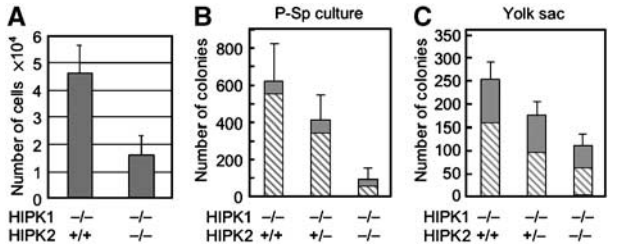

Figure 8 Hematopoiesis in HIPK1/2 mutant embryos. (**A**) Yolk sac cells from embryos at E9.5 were dispersed into single-cell suspensions and the numbers of nucleated erythroid cells were counted. (**B**) Colony-forming cells in P-Sp cultures. P-Sp explants derived from E9.5 embryos obtained by intercrossing HIPK1−/−,HIPK2 +/− mice were cultured on OP9 stromal cells for 7 days and were subjected to colony-formation assay. Dot-filled and oblique-line-filled boxes represent the number of erythroid and myeloid colonies, respectively. (**C**) Colony-forming cells in yolk sac. Yolk sac cells were directly subjected to colony-formation assay.

definitive hematopoiesis, *Hipk1−/−Hipk2 +/−* mice were mated, and the P-Sp region of embryos at 9.5-dpc were dissected and cultured on OP9 cells. After 7 days of culture, colony-forming cells (CFCs) from nonadherent cells were examined on methylcellulose medium. Yolk sac cells were directly tested for CFCs. As shown in Figure 8B and C, the total number of CFCs in the yolk sac and cultured P-Sp cells from double-deficient (*Hipk1−/−Hipk2−/−*) mutants was markedly reduced when compared with those from control littermates (*Hipk1−/−Hipk2 +/−* or *Hipk1−/−Hipk2 +/+*). These results suggest that primitive as well as definitive hematopoiesis is defective in *Hipk1/Hipk2* double-deficient embryos.

Defects in vasculogenesis/angiogenesis in HIPK1/2-deficient mice

Morphologic evaluation of embryos revealed severe defects in vessel formation in *Hipk1/Hipk2* double-homozygous mutant embryos and yolk sacs at E10.0 (Figure 9A and B). Similar defects in vessel formation are also observed in *p300*- and *cbp*-deficient mice (Yao *et al*, 1998; Oike *et al*, 1999; Tanaka *et al*, 2000). To further study the vascular system, whole embryos were stained with anti-PECAM-1 monoclonal antibody, which detects differentiated endothelial cells. *Hipk1/Hipk2* double-deficient embryos failed to form an organized vascular network (Figure 9C). Gross defects in vascular branching were observed, particularly in the head and trunk (Figure 9D and E). To further investigate the defects in the vascular networks in *Hipk1/Hipk2* double mutant embryos, P-Sp regions were dissected from E8.75 embryos obtained by intercrossing *Hipk1−/−Hipk2 +/−* mutant mice, cultured on OP9 stromal cells, and stained with anti-PECAM-1 antibody (Figure 9F). Both sheet (Figure 9G) and network (Figure 9H) formations, which are indicative of vasculogenesis and angiogenesis, respectively, were severely affected in P-Sp cultures from *Hipk1/Hipk2* double-homozygous mutant embryos, while cultures from littermate embryos developed normally. The double-homozygous mutant embryos at E8.75 were comparable in size to their littermates (data not shown). Thus, we concluded that vascular formation in *Hipk1/Hipk2* double-homozygotes is impaired rather than delayed.

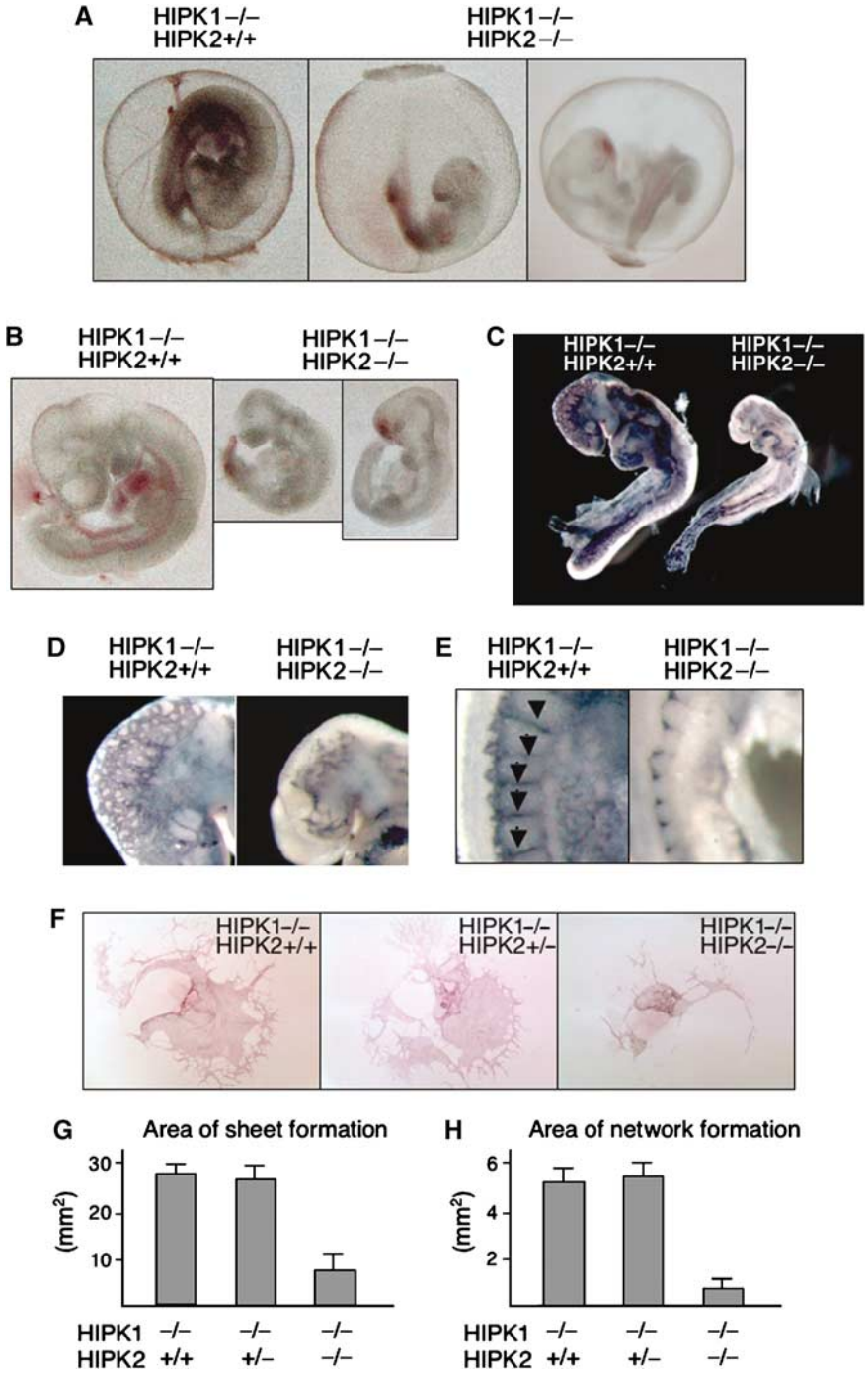

Figure 9 HIPK1/2 mutant embryos showed defective vasculogenesis and angiogenesis. (A, B) Phenotypic comparison of E10.0 *Hipk1−/−*, *Hipk2+/+* and *Hipk1−/−*,*Hipk2−/−* embryos with (**A**) and without (**B**) yolk sac. (**C**) Whole-mount immunohistostaining with anti-CD31 (PECAM-1) mAb of E9.5 *Hipk1−/−*,*Hipk2+/+* and *Hipk1−/−*,*Hipk2−/−* embryos. (**D**) Primary vascular plexus observed in mutant heads. (**E**) Migration of endothelial cells forming intersomitic vessels. (**F**–**H**) Vasculo-angiogenesis in P-Sp culture. P-Sp explants derived from E8.75 embryos obtained by intercrossing *Hipk1−/−*,*Hipk2+/−* mice were cultured on OP9 stromal cells and were stained with anti-CD31 mAb. Representative results are shown in (F). The average values of areas of vascular sheet formation and vascular network formation are shown in (G) and (H), respectively.

Discussion

AML1 stimulates HIPK2-mediated phosphorylation of p300 and activates transcription

In the present study, we found that co-expression of AML1 induced phosphorylation of p300. This effect was inhibited by the dominant-negative form of HIPK2 and was enhanced by WT HIPK2. These results suggest that HIPK2 mediates the AML1-induced phosphorylation of p300. HIPK2-mediated phosphorylation of p300 stimulates its HAT activity and activates p300-dependent transcription. AML1 interacts with both p300 and HIPK2, and thus AML1 induces phosphorylation of p300 by stimulating the physical interaction between the enzyme HIPK2 and the substrate p300.

Interestingly, the alanine mutant AML1-S249/276A, which lacks phosphorylation sites, did not induce p300 phosphorylation, while the aspartic acid mutant AML1-S246/276D, which mimicks the phosphorylated form, induced phosphorylation of p300 more efficiently than WT AML1. Moreover, AML1/p300-mediated transcriptional activation was inhibited by alanine mutations and enhanced by aspartic acid mutations on the phosphorylation sites of AML1. These findings suggest that phosphorylation of AML1 is required for the HIPK2-mediated phosphorylation of p300 and subsequent transcription activation. Thus, AML1 not only recruits HIPK2 to the substrate p300 but also functions as a regulatory subunit for HIPK2 to control p300 phosphorylation, local histone acetylation and transcription of target genes.

HIPK-induced phosphorylation of p300 is common to regulation by various transcription factors

Phosphorylation of p300 was also induced by transcription factors such as the AML1/runx family members, PU.1, c-MYB, c-JUN and c-FOS. These effects were inhibited by co-expression of dominant-negative HIPK2, suggesting that HIPK family members are involved in the phosphorylation of p300 by these transcription factors. It is likely that these transcription factors also regulate transcription by recruiting HIPKs and inducing p300 phosphorylation on their target genes in the same way as AML1. Thus, HIPK-mediated phosphorylation of p300 is a common event in transcriptional regulation by a range of transcription factors. Phosphorylation of p300 was not induced by transcription factors such as IRF3, p53, RARα and ATF2 under the conditions tested, although these transcription factors were able to interact with p300. As these factors are activated in response to a range of physiological stimuli, it is possible that additional modifications or factors induced by these stimuli are required for p300 phosphorylation.

Highly phosphorylated forms of p300 were scarcely detected in *Hipk1/2* double-deficient embryos, suggesting that phosphorylation of p300 is largely regulated by HIPK1 and HIPK2 during embryonic development. A variety of transcription factors, including jun, fos, runx and ets family members, are probably involved in the regulation of p300 phosphorylation during embryogenesis and hematopoiesis. It has been reported that phosphorylation of p300 is stimulated and is associated with expression of c-JUN during retinoic acid-induced differentiation of F9 embryonic carcinoma cells and ES cells (Kitabayashi *et al*, 1995). We found here that the induction of p300 phosphorylation was inhibited in *Hipk1/2* double-deficient ES cells. Thus, the phosphorylation of p300 in this process is likely to be mediated by HIPK1 and/or HIPK2 in combination with transcription factors, including c-JUN.

It has been reported that AML1 activates transcription synergistically with other transcription factors, such as AP-1 (jun/fos), C/EBPα, c-Myb, PU.1 and Ets-1, to activate transcription (Cockerill *et al*, 1996; Zhang *et al*, 1996; Britos-Bray and Friedman, 1997; Petrovick *et al*, 1998; Kim *et al*, 1999). We found here that phosphorylation of p300 is a common effect induced by these transcription factors, thus suggesting that phosphorylated forms of p300 and HIPK2 are involved in the above-mentioned synergistic effects.

Role of phosphorylation in p300/CBP function

HIPK1 and HIPK2 double-deficient embryos died between E9.5 and E12.5 and exhibited defects in vasculogenesis, angiogenesis, and primitive and definitive hematopoiesis. The double homozygous mutants exhibited developmental retardation and failed to close the anterior neuropore (Isono *et al*, 2006). These phenotypes were in part similar to those observed in p300- and CBP-deficient mice. Both p300- and CBP-deficient mice show embryonic lethality and die between E9.5 and E12.5 (Yao *et al*, 1998; Oike *et al*, 1999; Tanaka *et al*, 2000). Moreover, double heterozygosity for *p300* and *cbp* is invariably associated with embryonic death, suggesting the overlapping function and overall gene dosage requirement of p300 and CBP. *p300*- and *cbp*-deficient embryos exhibited defective blood vessel formation, defective neural tube closure and apparent developmental retardation, as well as defects in both primitive and definitive hematopoiesis. Phosphorylation of p300 was inhibited in *Hipk1/Hipk2*-double deficient embryos. Taken together these findings suggest that phosphorylation of p300/CBP by HIPK1/HIPK2 is essential for p300 and CBP function in embryonic development. However, we cannot exclude a possibility that HIPK1/HIPK2 may have other substrates whose phosphorylation is required for hematopoiesis and development. p300 null embryos show other defects, in addition to those found in *Hipk1/Hipk2*-double deficient embryos, including anomalous heart formation, turning of the embryo and edema formation (Yao *et al*, 1998). That these defects are not observed in *Hipk1/Hipk2*-double deficient embryos suggests that some of the function of p300 is independent of HIPK1/2-mediated phosphorylation.

HIPK2 regulates transcription via multiple factors

We also found that co-expression of AML1 and HIPK2 induced phosphorylation of MOZ (Supplementary Figure S6). The phosphorylation of MOZ was not efficiently induced by either AML1 or HIPK2 alone, and thus interaction between these three molecules is likely to be required for efficient MOZ phosphorylation. AML1 probably stimulates MOZ phosphorylation by accelerating the physical interaction between HIPK2 and MOZ. Moreover, a highly phosphorylated form of MOZ was preferentially co-precipitated with AML1, suggesting that AML1 interacts with the highly phosphorylated form of MOZ more strongly than with the hypophosphorylated form. Thus, HIPK2 stabilizes the AML1/MOZ complex by phosphorylating MOZ. We generated MOZ mutant mice and found that MOZ homozygous mutant mice die around E15.5 and exhibit severe defects in definitive hematopoiesis (Katsumoto *et al*, 2006). *Hipk1/Hipk2*-deficient mice also show defective definitive hematopoiesis. These defects appear to be more severe than those observed in *cbp*- or *p300*-deficient mice. This is probably due to reduced MOZ activity, in addition to inactive AML1 and p300/CBP, in *Hipk1/Hipk2*-deficient mice.

It was recently reported that the Groucho corepressor is phosphorylated by *Drosophila* HIPK2 and subsequently dissociates from the corepressor complex (Choi *et al*, 2005). TLE, the human homolog of Groucho, also reportedly interacts with AML1 and suppresses AML1-induced transactivation (Imai *et al*, 1998; Levanon *et al*, 1998). Taken together with these findings, the present results suggest that HIPKs play a key role in regulating the transcription of target genes

by relieving Groucho-mediated transcriptional repression as well as by activating transcription factor/HAT complexes.

Materials and methods

Cells and retroviruses

Culture of L-G, BOSC23 and SaOS2 cells were performed as described previously (Kitabayashi *et al*, 2001). Retrovirus infection of cells was performed as described previously (Kitabayashi *et al*, 1998a).

Plasmids

The AML1 expression vectors pLNCX-FLAG-AML1a, pLNCX-FLAG-AML1b, pLNCX-HA-p300, pLNCX-HA-MOZ, mCCP1-luc and MPO-luc were as described previously (Kitabayashi *et al*, 2001). LNCX-FLAG-HIPK2 and LNCX-HA-HIPK2 were generated by subcloning WT and KD HIPK2 (Hofmann *et al*, 2002) into a retrovirus-derived LNCX vector. CMV-HIPK1 was as described by Isono *et al* (2006).

Mass spectrometry

AML1 proteins were purified from L-G cells, digested with trypsin and subjected to LC/MS/MS analysis as described previously (Kitabayashi *et al*, 2001). Phosphopeptides were identified using TurboSEQUEST software.

Immunoprecipitation, immunoblotting and antibodies

Immunoprecipitation and immunoblotting analyses were performed as described previously (Kitabayashi *et al*, 2001). The following primary antibodies were used in the present study: anti-FLAG (M2) antibody (Sigma), anti-HA (3F10) antibody (Roche), anti-AML1 antibody (Kitabayashi *et al*, 1998a), anti-HIPK2 antibody (Hofmann *et al*, 2002) and anti-p300 antibody (N15) (Santa Cruz). The anti-phospho-S249 AML1 antibody was generated by immunizing rabbits with phosphopeptides RQIQPpSPPWSY corresponding to residues 244-254 of human AML1b.

HAT activity and kinase activity in vitro

For HAT activity, 293 cells were transfected with FLAG-tagged p300 together with WT or KD HIPK2. FLAG-p300 was purified by immunoprecipitation as described above and incubated in a 10-μl volume containing 50 mM Tris (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 0.5 μl of [1-^{14}C]-acetyl-CoA (50 μCi/ml, Amersham), and 0.5 μg each of histones H2A, H2B, H3 and H4 at 37°C for 1 h. Reaction mixtures were analyzed by 18% SDS–PAGE, and gels were subjected to autoradiography. Histone acetylation was measured using a BAS2000 imaging analyzer (Fuji).

For kinase activity, FLAG-tagged WT and mutant AML1b, WT HIPK2 and p300 were purified by immunoprecipitation, as described above, and were incubated in 20 mM Tris–Cl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 50 μM ATP, 5 μCi [γ-^{32}P]-ATP at 37°C for 30 min. Reaction mixtures were analyzed by SDS–PAGE, and gels were subjected to autoradiography.

Luciferase assay

Reporter analysis was performed as described previously (Kitabayashi *et al*, 2001).

GST pull-down assay

The GST pull-down assay was performed as described previously (Hofmann *et al*, 2002). GST-AML1b has been described previously (Kitabayashi *et al*, 1998b). GST-p300(HAT) was generated by subcloning of an *Eco*RI–*Stu*I fragment into pGEX.

In vitro culture of P-Sp

P-Sp culture conditions were as described (Takakura *et al*, 2000).

In vitro hematopoietic colony assays

P-Sp cultures from mouse embryos were dispersed into single-cell suspensions and cultured on 1% methylcellulose in Iscove's modified Dulbecco's medium (IMDM) containing 15% fetal bovine serum, 1% bovine serum albumin, 10 μg/ml rh insulin, 200 μg/ml human transferrin, 100 μM 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml rm SCF, 10 ng/ml rmIL-3, 10 ng/ml rh IL-6 and 3 U/ml erythropoietin (MethoCult GF M3434) (Stem Cell Technology Inc., Vancouver, Canada). Cultures were maintained at 37°C under humidified conditions with 5% CO_2. Colonies containing more than 50 cells were counted on day 12, and myeloid CFUs, erythroid burst forming units (BFU-E) and CFU-Mix were defined based on morphology.

Isolation and differentiation of ES cells

Hipk1−/−Hipk2+/− mice were mated and blastocysts were harvested on day E3.5 then individually cultured in ES cell medium (containing 20% fetal calf serum and leukemia inhibitory factor (LIF)) on a feeder layer of mitomycin C-treated embryonic fibroblasts, as previously described (Auerbach *et al*, 2000). ES cells were cultured in ES cell medium containing 10% fetal calf serum plus LIF. Cells were then plated on gelatinized 100-mm plates at a density of 1×10^6 cells/plate in the absence of LIF for 24 h, and then they were treated with 1 μM RA for various periods of time.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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