

# Transcriptional Activation by MEIS1A in Response to Protein Kinase A Signaling Requires the Transducers of Regulated CREB Family of CREB Co-activators\*

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The transcription factor encoded by the murine ecotropic integration site 1 gene (MEIS1) is a partner of HOX and PBX proteins. It has been implicated in embryonic patterning and leukemia, and causally linked to restless legs syndrome. The MEIS1A C terminus harbors a transcriptional activation domain that is stimulated by protein kinase A (PKA) in a manner dependent on the co-activator of cAMP response element-binding protein (CREB), CREB-binding protein (CBP). We explored the involvement of another mediator of PKA-inducible transcription, namely the CREB co-activators transducers of regulated CREB activity (TORCs). Overexpression of TORC1 or TORC2 bypassed PKA for activation by MEIS1A. Co-immunoprecipitation experiments demonstrated a physical interaction between MEIS1 and TORC2 that is dependent on the MEIS1A C terminus, whereas chromatin immunoprecipitation revealed PKA-inducible recruitment of MEIS1, PBX1, and TORC2 on the MEIS1 target genes *Hoxb2* and *Meis1*. The MEIS1 interaction domain on TORC1 was mapped to the N-terminal coiled-coil region, and TORC1 mutants lacking this domain attenuated the response to PKA on a natural MEIS1A target enhancer. Thus, TORCs physically cooperate with MEIS1 to achieve PKA-inducible transactivation through the MEIS1A C terminus, suggesting a concerted action in developmental and oncogenic processes.

The homeodomain is a DNA-binding structure shared by numerous transcription factors throughout eukaryotes, and is most commonly 60 amino acids in length (1). The three-amino acid loop extension class of homeoproteins is so named for an

extra 3 residues in the loop between helices 1 and 2 in the typical homeodomain (2). Members of the three-amino acid loop extension class in mammals include the MEIS, PREP, and PBX families, which participate in relatively complex interactions between themselves and with the products of another group of homeodomain-containing proteins, the HOX family (3). PBX proteins form cooperative DNA-binding heterodimers with MEIS, PREP, or HOX proteins, and coordinate the formation of higher order heterotrimeric complexes of PBX, HOX and MEIS, or PREP (3, 4). DNA-binding PBX homodimers have also been noted, further extending the possible permutations for these partners (5). Targets of PBX·MEIS heterodimers include the bovine *CYP17* gene (6), whereas those of PBX·MEIS·HOX trimers include the *Hoxb1* autoregulatory element (ARE)<sup>5</sup> and *Hoxb2* rhombomere 4 (r4) enhancer (7–9).

The *Meis1* gene was identified near a site of frequent retroviral insertion leading to acute myeloid leukemia in BXH-2 mice (10). It has been further associated with human and mouse leukemias through frequent coordinated up-regulation in these cancers, and through its ability to potentiate the onset of acute myeloid leukemia provoked by *Hoxa7* and *Hoxa9* ectopic expression in mouse bone marrow (11–18). More recently, intronic polymorphisms in MEIS1 have been linked to restless legs syndrome (19, 20).

A C-terminal domain of the MEIS1A isoform is indispensable for its oncogenic properties, however, this function can be entirely rescued by replacement of this C-terminal domain with the potent transcriptional activation domain of VP16, suggesting that the MEIS1A C terminus exerts its oncogenic functions through transcriptional activation of target genes (21, 22).

The transcriptional complexes formed by three-amino acid loop extension and HOX family homeoproteins recruit a variety of coregulators with sometimes opposing functions. HOXD4 and HOXB7 both recruit the histone acetyltransferase coactivator CBP to their N termini, whereas PBX1 N and C termini exert negative effects on transcription by binding corepressor complexes containing NCOR/SMRT and HDAC1 (3, 4, 23–25). A role for PBX and/or MEIS in mediating transcrip-

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<sup>5</sup> The abbreviations used are: ARE, autoregulatory element; TORC, transducers of regulated CREB; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; PKA, protein kinase A; ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline; shRNA, short hairpin RNA; DBD, DNA-binding domain.

tional activation by PKA was first suggested for the bovine *CYP17* gene (6, 26). We have demonstrated that PBX·HOX complexes can be converted from repressors to activators by PKA signaling, and that this is in part due to increased association between HOXD4 and CBP (23). More recently, we have shown that the association of MEIS1A or MEIS1B with PBX and HOX contributes a PKA-inducible and CBP-dependent transcriptional activation domain located in the MEIS1A/B C termini (8). The mapping of this transactivation function to the same domain implicated in MEIS1A-mediated leukemogenesis strongly supports the notion that transcriptional activation is the basis for the oncogenic properties of MEIS1A (21, 27). At least some of the embryonic patterning functions of MEIS family proteins are also achieved by transcriptional activation (4, 28–31).

CREB family transcription factors bind to cAMP response elements (CREs) within target genes, and are targets of PKA (32, 33). Phosphorylation of Ser<sup>133</sup> on CREB provides a high affinity binding site for CBP/p300 and leads to transcriptional activation of CRE-bearing target genes (34, 35). More recently, a parallel PKA response has been described for CREB. In this pathway, PKA provokes the nuclear accumulation of TORC (also known as CREB-regulated transcription co-activator, CRTC) family transcriptional coactivators, which bind to the CREB bZIP DNA-binding domain via a coiled-coil interface in their N termini (36–39). Recruitment of TORCs is not limited to CREB, because the HTLV-1 Tax protein and the AP-1 transcription factor likewise bind TORCs (40–42).

We investigated a possible role for TORC family coactivators in the PKA inducibility of the MEIS1A C-terminal transactivation function. Our results show that PKA signaling to MEIS1A is dependent on TORCs, and that overexpression of TORCs obviates the need for PKA for transcriptional activation through the MEIS1A C terminus. Importantly, MEIS1 physically interacts with TORC1 and TORC2, and TORC2 is found in the nucleus at the regulatory regions of MEIS target genes in association with MEIS1 and PBX1.

## EXPERIMENTAL PROCEDURES

**Plasmid Constructs**—Expression plasmids for MEIS1A, PBX1A, HOXA1, PKA $\alpha$ , GAL4 DNA binding domain (GAL-DBD), GAL-MEIS1A-(335–390), and GAL-MEIS1A-(GQWHYM) have been described previously (8, 23, 43). Both pML5xUAS and pML*Hoxb1*ARE are luciferase reporters, with the former containing five copies of the GAL4 binding site and the latter the 150-bp *Hoxb1* ARE, upstream of the adenovirus major later promoter (8, 23). As for TORC2 and control shRNA plasmids, FLAG-TORC2 and FLAG-TORC2(Wobble) expression plasmids have been previously reported (37, 39). To construct FLAG-TORC1, TORC1 coding sequence was PCR-amplified from template pCMV-SPORT6-TORC1 purchased from Open Biosystems (catalogue number MHS1010–7507865; accession number BC028050), and cloned into BamHI and XhoI sites of pcDNA3.1(+) that had already been inserted with a FLAG tag. FLAG-TORC1-(47–634), FLAG-TORC1-(47–290), and FLAG-TORC1-(148–290) were subcloned as EcoRI-NotI fragments. FLAG-TORC1-(1–431), FLAG-TORC1-(1–518), Flag-TORC1-(1–493), and FLAG-

TORC1-(1–627) were generated by removal of ClaI-XhoI, BsrGI-XhoI, SfiI-XhoI, and BspEI-XhoI fragments, respectively, and ligated following a blunt ending treatment by T4 DNA polymerase (Fermentas).

**Antibodies**—Anti-MEIS NT is an affinity purified rabbit polyclonal antibody raised in-house against amino acid residues 1–34 of MEIS1 (8, 44). The anti-MEIS1/2/3 mouse monoclonal antibody was purchased from Upstate Biotechnology (catalogue number 05-779). Goat anti-rabbit IgG conjugated to alkaline phosphatase (catalogue number sc-2007) was used as a nonspecific antibody control in chromatin immunoprecipitation (ChIP) assays, the anti-PBX1 (catalogue number sc-889) and anti-GAL4 (catalogue number sc-577) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology. We purchased the anti-FLAG M2 affinity agarose (catalogue number A2220) as well as the anti- $\beta$ -tubulin (catalogue number T4026) and anti-FLAG (catalogue number F3165) mouse monoclonal antibodies from Sigma. The rabbit polyclonal anti-TORC2 antibody was raised against amino acid residues 454–607 of murine TORC2 (39). The secondary antibodies used in this study were horseradish peroxidase-conjugated goat anti-mouse IgG, F(ab')<sub>2</sub> fragment specific (catalogue number 115-035-072, Jackson ImmunoResearch Laboratories) and mouse anti-rabbit IgG, light chain specific (catalogue number 211-032-171, Jackson ImmunoResearch Laboratories).

**Cell Culture and Transfections**—HEK293 and P19 mouse embryonal carcinoma cells were cultured in Dulbecco's modified Eagle's medium and  $\alpha$  minimal essential medium, respectively, supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. To differentiate P19 cells, cells were aggregated in 100-mm diameter bacterial Petri dishes at a density of 10<sup>5</sup> cells/ml and treated with 0.3  $\mu$ M retinoic acid (catalogue number R2625, Sigma) for 48 h. HEK293 cells were seeded at 75 to 90% confluence in 60-mm diameter tissue culture dishes for immunoprecipitation and in 12-well plates for luciferase assay. The cells were allowed to attach overnight and then transfected by Lipofectamine 2000 reagent (Invitrogen, catalogue number 11668-019). MG132 (Merck, catalogue number 474790) was used at 10  $\mu$ M for 5 h.

**Immunofluorescence**—HEK293T cells were seeded at 1.0  $\times$  10<sup>5</sup> cells/ml in 40 mm-diameter tissue culture dishes lined with ethanol- and acid-washed coverslips and allowed to attach overnight. Medium was changed 6 h after transfection. 24 h after transfection cells were fixed with 4% paraformaldehyde (Sigma) for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 min. Cells were blocked with 10% goat serum (Hyclone) in 0.1% Triton/PBS for 10 min and incubated with primary antibody. The antibody used was goat polyclonal anti-TORC2 (sc-46274) (Santa Cruz Biotechnology, Inc.). Secondary antibody used was anti-goat 488 (Invitrogen). Slides were mounted with Vectorshield 4',6-diamidino-2-phenylindole (Vector Laboratories) before microscopy.

**Luciferase Assay**—For Fig. 1, 10 ng of TORC1, 5 ng of TORC2, and 15 ng of PKA $\alpha$  expression plasmids were transfected into HEK293 cells. For Fig. 2, 800 ng of total DNA consisted of 200 ng of the pML*Hoxb1*ARE luciferase reporter and 100 ng of each expression plasmid. A *lacZ* reporter was co-

## PKA-inducible Transactivation by MEIS1A Requires TORCs

transfected to normalize transfection efficiency. For Fig. 3A, the amounts of pENTR T2i shRNA (TORC2 shRNA) are given within the panel. For Fig. 3B, 25,000 cells per well were plated in 48-well plates and transfected using Lipofectamine 2000 with the following vectors: 40 ng of pML5xUAS, 40 ng of pGAL-DBD or pGAL-MEIS1A(335–390), 40 ng of pRSV-PBS or pRSV-PKA, 40 ng of pENTR U6 (control shRNA) or pENTR T2i shRNA (TORC2 shRNA), and 100 ng of pRSV- $\beta$ -galactosidase. At 48 h post-transfection, cell lysates were prepared using 100  $\mu$ l per well of lysis buffer (1% Triton X-100, 1 mM dithiothreitol, 92.8 mM  $K_2HPO_4$ , pH 7.8, 9.2 mM  $KH_2PO_4$ , pH 7.8) and centrifuged for 5 min at 4 °C. A 20- $\mu$ l aliquot of the supernatant was added to 12.5  $\mu$ l of assay buffer (20 mM ATP, 40 mM  $MgCl_2$ , 0.4 M Tris-Cl, pH 7.8). The mixture was immediately quantified for luciferase activity using a Lumat LB 9507 luminometer (EG&G Berthold) that dispensed 100  $\mu$ l per reaction of luciferin solution, which contains 1 mM D(-)-luciferin (catalogue number 11626353001, Roche) and 0.1 M Tris-Cl, pH 7.8.

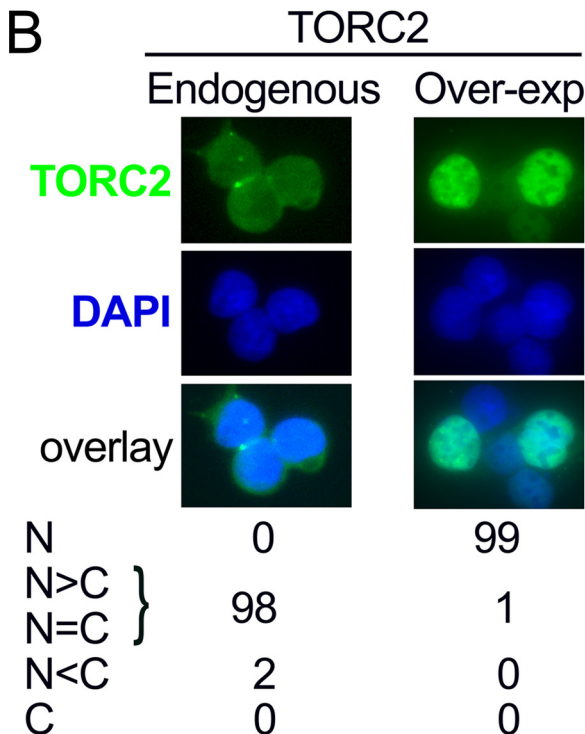
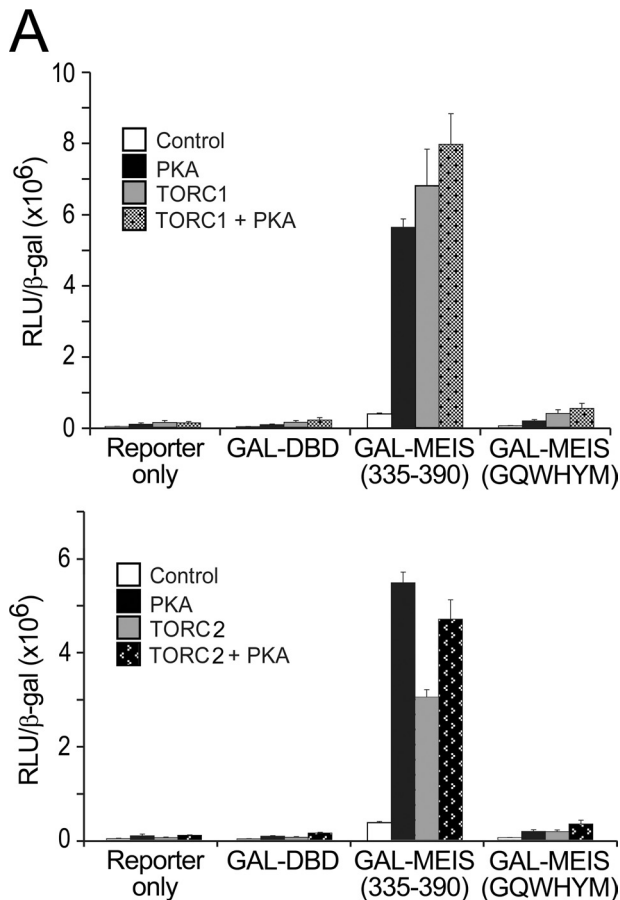
**Immunoprecipitation and Western Blot Analysis**—Cells were washed twice in ice-cold PBS 48 h post-transfection and harvested in 500  $\mu$ l of Buffer B (150 mM KCl, 0.1% Nonidet P-40, 20 mM Tris-Cl, pH 8.0, 5 mM  $MgCl_2$ , 10% (w/v) glycerol) added with protease inhibitor mixture (catalogue number 11873580001, Roche). Following two freeze-thaw cycles, cells were spun down at 4 °C for 10 min. The supernatant was incubated with the appropriate primary antibody from 5 h to overnight at 4 °C, followed by 3 h incubation at 4 °C with 30  $\mu$ l of a 50% slurry of Protein A-agarose (catalogue number 16-156, Upstate Biotechnology), unless the primary antibody was in the form of anti-FLAG M2 affinity agarose. The precipitates were washed three times, each with 500  $\mu$ l of Buffer B. Precipitates of Protein A-agarose were eluted with 1 $\times$  SDS sample buffer and boiling. Elution from anti-FLAG M2 affinity agarose was done by adding a 7.5- $\mu$ g FLAG peptide (catalogue number F3290, Sigma) for 1 h at 4 °C. Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to 0.45- $\mu$ m nitrocellulose membrane. The membranes were blocked with 5% nonfat milk powder in 0.1% Tween 20 in PBS (PBS-T) for 1 h at room temperature to reduce nonspecific background, followed by primary antibody incubation for 3 h at room temperature or overnight at 4 °C. The membranes were then washed four times, 10 min each with PBS-T, and incubated with secondary antibody conjugated with horseradish peroxidase for 45 min at room temperature. Subsequent to three 10-min PBS-T washes, bound antibodies were detected with a chemiluminescent kit (catalogue number KP-54-61-00, Mandel).

**ChIP Assay**—ChIP assays were performed according to the protocol from Upstate Biotechnology with minor changes as reported previously (8, 45). P19 cells induced to differentiate down the neural pathway by aggregation in the presence of retinoic acid (see above) were treated with 20  $\mu$ M forskolin for 2 h, cross-linked with 1% formaldehyde for 10 min at 37 °C, collected, and washed twice with ice-cold PBS containing protease inhibitor mixture. A 200- $\mu$ l aliquot of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl, pH 8.0, protease inhibitor mixture) was added to each 1  $\times$  10<sup>6</sup> cells and incubated on ice for 10 min. The 200- $\mu$ l lysates were sonicated at 4 °C with 10

sets of 10-s pulses at 30% amplitude of a Betatec Sonics Vibra Cell sonicator to an average DNA length of 200 bp and then centrifuged for 10 min at 4 °C. Each 100- $\mu$ l sonicated cell supernatant was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.0, 167 mM NaCl, protease inhibitor mixture) and pre-cleared with 40  $\mu$ l of a 50% slurry of salmon sperm DNA/Protein A-agarose (catalogue number 16-157, Upstate Biotechnology) for 30 min at 4 °C with rotation. After an overnight incubation with anti-MEIS1A, anti-PBX1, anti-TORC2, or anti-rabbit IgG antibodies, 30  $\mu$ l of salmon sperm DNA/Protein A slurry was added for 1 h at 4 °C, along with a no antibody control. To remove non-specific DNA from the protein A-antibody-histone complex, we performed extensive washes with 500  $\mu$ l of each buffer in the following sequences: once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.0, 150 mM NaCl), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.0, 500 mM NaCl), once with lithium chloride buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-Cl, pH 8.0), and twice with TE buffer (1 mM EDTA, 10 mM Tris-Cl, pH 8.0). Each wash was done by first pipetting up and down for 10 times, and then an 8–10-min incubation on a rotating platform at 4 °C. Subsequently, the histone complex was eluted from the antibody by incubating twice with 125  $\mu$ l of elution buffer (1% SDS, 0.1 M  $NaHCO_3$ ) for 15 min at room temperature. Cross-links were reversed at 65 °C for 4 h in the presence of 0.2 M NaCl. DNA was phenol-chloroform-extracted, ethanol precipitated, and resuspended in 40  $\mu$ l of distilled water (catalogue number 15230-147, Invitrogen). Five percent (by volume) of the immunoprecipitated DNA was served as template in quantitative real-time PCR by a SYBR Green JumpStart Taq ReadyMix kit (catalogue number S1816, Sigma) with a Roche LightCycler. The sequences of ChIP primers used in this study were as follows: for *Hoxb1* ARE, 5'-CTCTGGTCCCTTCTTTCC and 5'-GGC-CAGAGTTTGGCAGTC; for *Hoxb2* r4 enhancer, 5'-AGGC-CTTTTAAAGGATATGC and 5'-AGGCCTCAAAGCT-GAAAATGA; for *Meis1* promoter, 5'-TTAGGACTGATTCA-AGGAAAGC and 5'-GCCCCTCAGACCCAACTAC; and for *gapdh*, 5'-AACGACCCCTTCATTGAC and 5'-TCCACGAC-ATACTCAGCAC. The primers for the murine *Meis1* gene flank a consensus PBX-MEIS binding site having the sequence 5'-TGATTGACAG-3'.

## RESULTS

**TORC1 and TORC2 Bypass the Need for PKA to Activate Transcription by MEIS1A**—To study the mechanism by which the MEIS1A C terminus responds to PKA signaling, we examined the contribution of TORCs to the transcriptional activity of MEIS1A residues 335–390 fused to the GAL4 DNA-binding domain (DBD). As noted previously (8), co-transfection of expression vectors for GAL-MEIS1A-(335–390) and PKA $\alpha$  in HEK293 cells strongly activates the transcription of a luciferase reporter driven by five tandem copies of the GAL4 DNA binding site (pML5xUAS). By contrast, the GAL-DBD fused to a mutant MEIS1A C terminus bearing alanine substitutions in the last six residues (GAL-MEIS1A-(GQWHYM)) is refractory to stimulation by PKA (Fig. 1A). To test whether TORCs medi-



**FIGURE 1. CREB co-activators TORC1 and TORC2 mediate the MEIS1A C terminus transcriptional activity.** A, HEK293 cells were transfected with expression vectors for GAL-DBD, GAL-MEIS1A-(335–390), or GAL-MEIS1A-(GQWHYM) plus the pML5xUAS luciferase reporter. The effect of the PKA catalytic domain and/or TORC1 on luciferase activity is shown in the upper panel, whereas that of the PKA catalytic domain and/or TORC2 is shown in the

lower panel. RLU, relative luciferase units.  $\beta$ -gal,  $\beta$ -galactosidase. B, TORC2 subcellular localization was quantitated in non-transfected cells and cells overexpressing TORC2. Top panels, green signal corresponds to TORC2. Middle panels, 4',6-diamidino-2-phenylindole-stained nuclei (blue). Bottom panels, overlay. N, exclusively nuclear signal; N>C, nuclear signal stronger than cytoplasmic; N=C, signals in the nucleus and cytoplasm approximately equal; N<C, nuclear signal weaker than cytoplasmic; C, exclusively cytoplasmic signal. Over-exp, images of cells overexpressing TORC2. 100 cells each were scored for the distribution of endogenous versus overexpressed TORC2. The exposure of images showing endogenous TORC2 was increased in Adobe Photoshop to visualize the relatively weak signal.

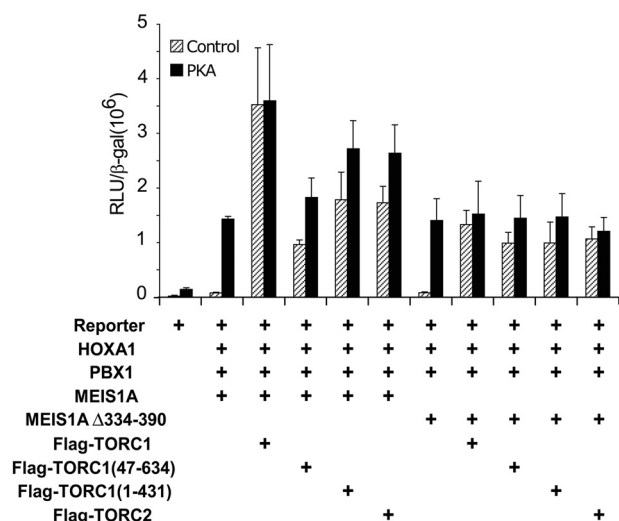
ate MEIS1A C terminus transcriptional activity, we co-transfected TORC1 and pML5xUAS with GAL-DBD, GAL-MEIS1A-(335–390), or GAL-MEIS1A-(GQWHYM) in HEK293 cells both in the presence and absence of PKA $\alpha$ . Both the GAL-DBD and GAL-MEIS1A-(GQWHYM) mutant, which are non-responsive to PKA signaling were also non-responsive to TORC1 (Fig. 1A, upper panel). By contrast, TORC1 was able to bypass PKA $\alpha$  signaling to augment transcription of the luciferase gene by GAL-MEIS1A-(335–390) (Fig. 1A, upper panel). This ability of TORC1 to bypass PKA signaling was mimicked by TORC2 (Fig. 1A, bottom panel), which shares 32% identity with TORC1 (38). The lack of synergistic activation following co-expression of GAL-MEIS1A and TORCs suggests that all of the effects of PKA are mediated by TORCs.

Phosphorylation by SIK2 results in the sequestration of TORC family members in the cytoplasm. PKA inactivates SIK2, leading to the accumulation of TORCs in the nucleus. This suggested that the ability of overexpressed TORCs to bypass PKA for activation through MEIS1A could be due to the forced accumulation of TORCs in the nucleus. To examine the subcellular localization of TORCs expressed from transfected vectors, we performed immunofluorescence experiments using FLAG-tagged TORC constructs. Endogenous TORC2 in HEK293T cells accumulates in both the nucleus and cytoplasm (Fig. 1B). By contrast, following overexpression, TORC2 is strongly concentrated in the nucleus (Fig. 1B). No significant nuclear accumulation was observed for overexpressed TORC1, consistent with previous observations (data not shown).

To evaluate the contribution of TORC1 to MEIS1A transcriptional activity on an authentic MEIS1 target promoter, we used a luciferase reporter vector driven by the 150-bp ARE of the *Hoxb1* gene. As previously observed, the ternary MEIS-PBX-HOX complex strongly activated the luciferase reporter in response to PKA signaling (Fig. 2). As it did for GAL-MEIS1A-(335–390), TORC1 by itself could confer transcriptional activation by MEIS-PBX-HOX to the same extent as PKA $\alpha$  (Fig. 2). This robust transcriptional activity was significantly hampered by the loss of an N-terminal 46-amino acid fragment in TORC1 that contains a conserved coiled-coil domain, and to a lesser degree by the loss of a C-terminal 203-amino acid fragment (Fig. 2). TORC2 also enhanced MEIS-PBX-HOX activation, albeit less vigorously than TORC1 (Fig. 2). Loss of the MEIS1A C terminus (MEIS1A-( $\Delta$ 334–390)) impaired the transcriptional activity of the ternary complex in response to TORC1 and TORC2, suggesting that the MEIS1A C terminus could fulfill its role as a transactivation domain by recruiting TORCs (Fig. 2). Note that deletion of the MEIS1A C terminus would not be expected to eliminate reporter gene

lower panel. RLU, relative luciferase units.  $\beta$ -gal,  $\beta$ -galactosidase. B, TORC2 subcellular localization was quantitated in non-transfected cells and cells overexpressing TORC2. Top panels, green signal corresponds to TORC2. Middle panels, 4',6-diamidino-2-phenylindole-stained nuclei (blue). Bottom panels, overlay. N, exclusively nuclear signal; N>C, nuclear signal stronger than cytoplasmic; N=C, signals in the nucleus and cytoplasm approximately equal; N<C, nuclear signal weaker than cytoplasmic; C, exclusively cytoplasmic signal. Over-exp, images of cells overexpressing TORC2. 100 cells each were scored for the distribution of endogenous versus overexpressed TORC2. The exposure of images showing endogenous TORC2 was increased in Adobe Photoshop to visualize the relatively weak signal.

## PKA-inducible Transactivation by MEIS1A Requires TORCs

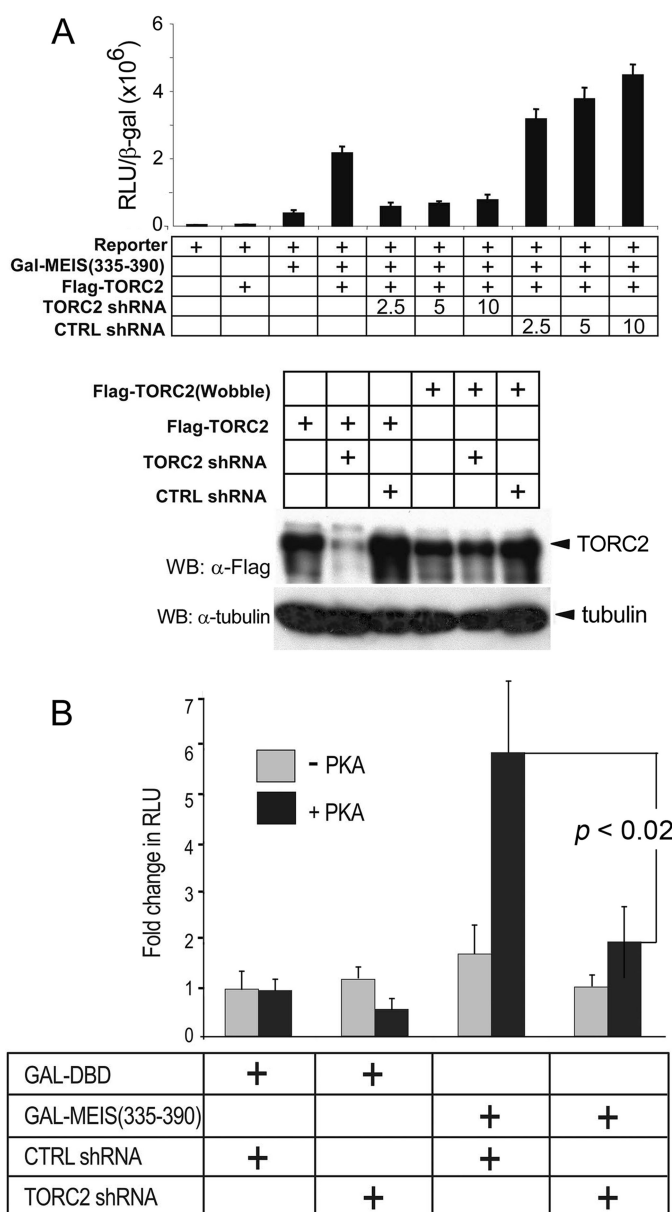


**FIGURE 2. TORCs mediate transcriptional activation through the MEIS1A C terminus at a natural MEIS1 target enhancer.** HEK293 cells were transiently transfected with the pMLHoxb1ARE luciferase reporter and expression plasmids as indicated. Luciferase activities in the absence or presence of the PKA catalytic domain (control or PKA) were measured at 48 h post-transfection.

activation, because endogenous MEIS1 would recruit TORC1 at some level.

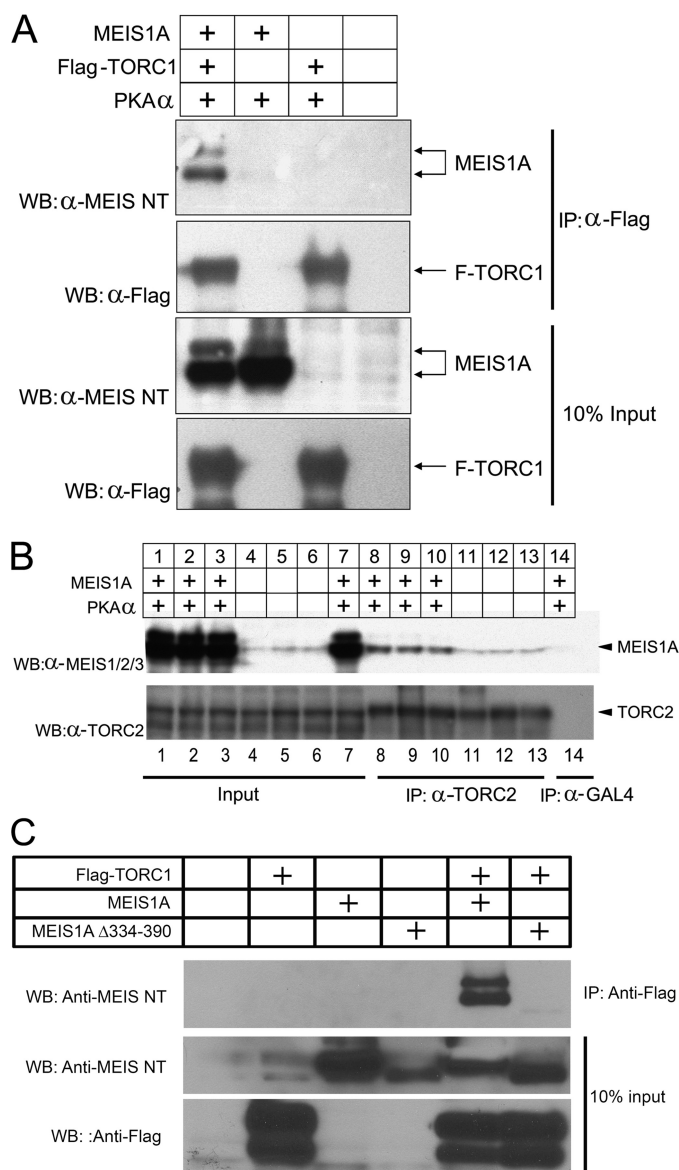
To confirm that TORC mediates MEIS1A transactivation, we performed knockdown studies using an RNA polymerase III-driven hairpin (shRNA) vector against TORC2. When transiently co-transfected with the FLAG-TORC2 expression plasmid in HEK293 cells, TORC2 shRNA plasmid inhibited luciferase transcription by GAL-MEIS1A-(335–390) to a level comparable with that without the co-expression of FLAG-TORC2 (Fig. 3A, upper panel). This abrogation of TORC2 activity correlated with the depletion of FLAG-TORC2 protein levels (Fig. 3A, bottom panel). Increasing amounts of TORC2 shRNA did not further decrease the FLAG-TORC2 activity (Fig. 3A, upper panel). This effect of TORC2 shRNA on FLAG-TORC2 activity was specific because a control shRNA vector had the opposite effect (Fig. 3A, upper and bottom panels). This mild activation by the control shRNA could point to a generalized induction of TORC-mediated activation in response to shRNA. If so, it would argue for even more robust knockdown by the TORC2 shRNA against a background of shRNA-induced activation. An RNA interference-resistant version of FLAG-TORC2 (Flag-TORC2(Wobble)) was not depleted by TORC2 shRNA, further demonstrating the specificity of TORC2 shRNA (Fig. 3A, bottom panel). Importantly, knockdown of endogenous TORC2 likewise impaired the response of the MEIS1 C terminus to PKA signaling (Fig. 3B), demonstrating that the transcriptional effect observed here is mediated by physiological levels of TORC family members.

**MEIS1A Associates with the CREB Co-activators TORC1 and TORC2**—To investigate whether TORC1 binds to MEIS1A *in vivo*, we carried out immunoprecipitation experiments using whole cell extracts from HEK293 cells transfected with MEIS1A and/or FLAG-TORC1 expression vectors. Immunoprecipitation of FLAG epitope-tagged TORC1 by anti-FLAG M2-agarose resulted in co-precipitation of MEIS1A (Fig. 4A). Western blot analysis of control M2 immunoprecipitates from



**FIGURE 3. Knockdown of TORCs prevents PKA-mediated activation of the MEIS1A C terminus.** A, upper panel, effect of TORC2 shRNA or non-silencing control (CTRL) shRNA on GAL-MEIS1A-(335–390) luciferase transcription augmented by TORC2. The indicated plasmids were co-transfected with the pML5xUAS reporter in HEK293 cells. Lower panel, knockdown of FLAG-TORC2 protein levels in TORC2 or control shRNA-treated cells was verified by immunoprecipitation with M2 beads followed by Western blot (WB) analysis with an anti-FLAG antibody. Cell extracts were probed for tubulin, confirming equivalent protein concentrations in each sample. FLAG-TORC2(Wobble) served as an RNA interference-resistant control. B, the role of endogenous TORC2 on transcriptional activation through the MEIS1A C terminus. Cells were transfected with the pML5xUAS reporter and expression vectors for either the GAL DBD or GAL-MEIS1A-(335–390), along with a PKA expression vector or empty plasmid. Transcriptional activation by PKA through the MEIS1A C terminus was abrogated by coexpression with the TORC2-specific shRNA but not the control shRNA. The experiment was conducted in triplicate. Error bars are S.D., and *p* signifies the results of the Student's *t* test applied to values for PKA-induced activity in the presence of control shRNA versus TORC2 shRNA. RLU, relative luciferase units.

cells transfected with MEIS1A or FLAG-TORC1 expression vector alone did not yield any sign of MEIS1A co-precipitation (Fig. 4A).



**FIGURE 4. MEIS1A interacts with TORC1 and TORC2.** *A*, co-immunoprecipitation assay of FLAG-tagged TORC1 and untagged MEIS1A in transfected HEK293 cells. Anti-MEIS NT and anti-FLAG Western blot (WB) analyses were performed on FLAG-TORC1 immunoprecipitates (IP) prepared with anti-FLAG M2 affinity agarose. 10% input levels of MEIS1A and FLAG-TORC1 are indicated. *B*, upper panel, Western blot analysis of transfected and endogenous MEIS1A detected in immunoprecipitates of endogenous TORC2 from HEK293 cells. The experiment was performed in triplicate and the results of each assay are shown. Lower panel, anti-TORC2 Western blot analysis showing immunoprecipitated TORC2 by the anti-TORC2 antibody but not the control anti-GAL4 antibody. 10% input levels of MEIS1A and TORC2 are shown. *C*, a MEIS1A mutant lacking the C terminus fails to co-immunoprecipitate with TORC1. HEK293T cells were co-transfected with a FLAG-tagged TORC1 expression vector and a vector encoding either wild-type MEIS1A or a mutant lacking the TORC-responsive C terminus (MEIS1A- $\Delta$ 334-390). On the second day following transfection, cells were treated with the proteasome inhibitor MG132 and cell lysates prepared 5 h later. Immunoprecipitation of TORC1 was performed with an anti-FLAG antibody, and the presence of MEIS1 proteins in the immunoprecipitates was subsequently assessed by Western blotting.

To further examine MEIS-TORC complex formation *in vivo*, we prepared TORC2 immunoprecipitates from HEK293 cells using TORC2 antiserum against the endogenous TORC2 protein (Fig. 4B, lanes 8–13, bottom panel). As shown in Fig. 4B, MEIS1A was recovered from immunoprecipitates of TORC2

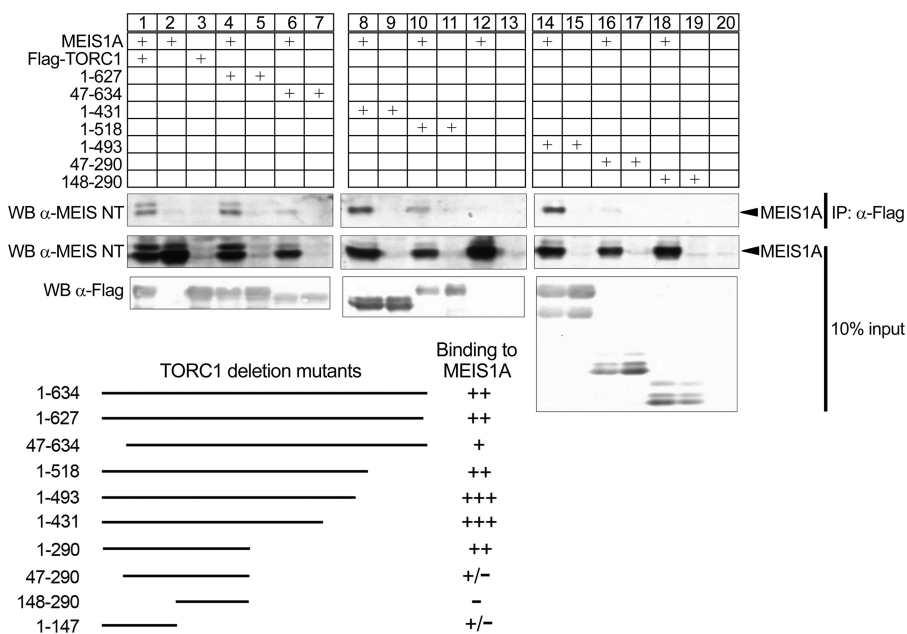
prepared from cells transfected with MEIS1A expression vector (Fig. 4B, lanes 8–10, upper panel). More significantly, ~10% of endogenous MEIS1A protein was found to co-precipitate with endogenous TORC2 (Fig. 4B, lanes 11–13, upper panel). This interaction is specific because no MEIS1A protein was detected from control immunoprecipitates using an anti-GAL4 antibody (Fig. 4B, lane 14).

To further implicate the MEIS1A C terminus in interaction with TORCs, we performed co-immunoprecipitation experiments with wild-type MEIS1A and a MEIS1A mutant lacking all residues C-terminal to the homeodomain. We observed that overexpression of TORC1 destabilizes the MEIS1A mutant. This loss of mutant MEIS1A protein is the result of proteasome-mediated degradation, because the mutant protein is recovered by addition of the proteasome inhibitor MG132 to the culture medium (data not shown). In the presence of MG132, the wild-type and mutant MEIS1A proteins accumulate to similar levels, but the mutant is strongly impaired for interaction with TORCs (Fig. 4C). This result confirms the importance of the MEIS1A C terminus for interaction with TORC family members.

To characterize the MEIS1A binding domain on TORC1, we tested multiple TORC1 deletion derivatives for their association with MEIS1A *in vivo*. Immunoprecipitation experiments were performed using whole cell lysates from HEK293 cells co-transfected with plasmids expressing untagged MEIS1A along with FLAG-tagged TORC1 deletion mutants. MEIS1A associated with FLAG-TORC1-(1–627) (Fig. 5, lane 4), FLAG-TORC1-(1–518) (lane 10), FLAG-TORC1-(1–493) (lane 14), and FLAG-TORC1-(1–431) (lane 8), indicating that the TORC1 C terminus is dispensable for MEIS1A binding activities. Although deletion of the N-terminal 46-amino acid fragment in TORC1 still allowed the formation of a TORC1·MEIS1A complex, the loss of this conserved coiled-coil domain considerably reduced TORC1 interaction with MEIS1A relative to the full-length TORC1 (Fig. 5, compare lanes 1 and 6). As seen in lane 16 of Fig. 5, the TORC1 fragment spanning residues 47 to 290 was sufficient for weak binding to MEIS1A. Further deletion of the N-terminal 101-amino acid residues of FLAG-TORC1-(47–290) generated FLAG-TORC1-(148–290) that failed to bind MEIS1A (Fig. 5, lane 18). Additional deletion constructs show that residues 1 to 147 mediate weak binding to TORC1, whereas maximal binding requires residues 1–290 (Fig. 5, lower panel, and data not shown). Region 1–290 includes, but is more extensive than, a coiled-coil domain spanning residues 1–45 in the TORC1 N terminus (37).

*MEIS1, PBX1, and TORC2 Are Recruited to MEIS1 Targets—*The importance of TORCs for transcriptional activation by the MEIS1A C terminus prompted us to assess the recruitment of MEIS1, TORC2, and PBX1 to known MEIS1 targets *in vivo*. We performed ChIP assays on neurally differentiating mouse P19 embryonal carcinoma cells either untreated or treated with forskolin for 2 h. Real-time PCR was carried out on immunoprecipitated DNA using primers spanning the *Hoxb1* ARE, *Hoxb2* r4 enhancer, and *Meis1* promoter. Values obtained from the LightCycler quantification were normalized against the corre-

## PKA-inducible Transactivation by MEIS1A Requires TORCs



**FIGURE 5. A MEIS1 interaction domain maps to a coiled-coil region at the TORC1 N terminus.** Upper panel, co-immunoprecipitation between untagged MEIS1A and full-length FLAG-tagged TORC1 (Flag-TORC1) or its deletion derivatives in transfected HEK293 cells. MEIS1A proteins co-precipitated with FLAG-TORC1 derivatives prepared using anti-FLAG M2 affinity agarose were revealed by Western blot (WB) analysis with anti-MEIS NT antibody. The bottom two panels show inputs of MEIS1A and FLAG-TORC1 derivatives, respectively. Lower panel, schematic diagram of TORC1 constructs and their MEIS1A binding activities. WB, Western blot; IP, immunoprecipitation. The plus and minus signs below *Binding to MEIS1A* correlate with the extent of binding to MEIS1A by the various TORC1 mutants.

sponding input and nonspecific IgG antibody, and expressed as relative occupancy.

The *Hoxb1* ARE, *Hoxb2* r4 enhancer, and *Meis1* promoter were effectively recovered from both the immunoprecipitates of MEIS1 and PBX1 (Fig. 6). In comparison to their untreated counterparts, immunoprecipitates of forskolin-treated cells revealed a greater recruitment of MEIS1 and PBX1 to the *Meis1* promoter, the *Hoxb2* r4 enhancer, and the *Hoxb1* ARE (Fig. 6, A–C). Strikingly, TORC2 was also recruited to the *Hoxb2* r4 enhancer and *Meis1* promoter under forskolin-treated conditions (Fig. 6). Forskolin-induced recruitment of TORC2 to the *Hoxb1* ARE was not observed. The specificity of our ChIP analysis was validated based on “no antibody” and nonspecific IgG precipitation controls (used to normalize the values presented in Fig. 6), and on the lack of differential recruitment to the housekeeping gene *gapdh* (Fig. 6D). These results demonstrate that TORC2 is indeed recruited and present with MEIS1 on some MEIS1 target genes *in vivo*, strongly supporting physical and functional associations between these transcriptional regulators.

## DISCUSSION

Our previous study established that the MEIS1A C terminus has a transcriptional activation domain that responds to PKA signaling. Supporting this work, two studies using mouse models of HOXA9-induced leukemia mapped a conserved transcriptional function to the MEIS1A C terminus required for accelerating leukemogenesis (21, 22). Here we show that a mechanism by which the MEIS1A C terminus achieves its transcriptional function involves CREB coactivators TORC1 and TORC2.

*TORCs Mediate PKA Signaling by Physical Association with MEIS1A*—We have shown that two members of the recently identified CREB co-activator family, TORC1 and TORC2, bypass the need for PKA stimulation to induce MEIS1A transcriptional activity both in a heterologous GAL4 reporter system and an authentic MEIS1 target promoter (Figs. 1A and 2).

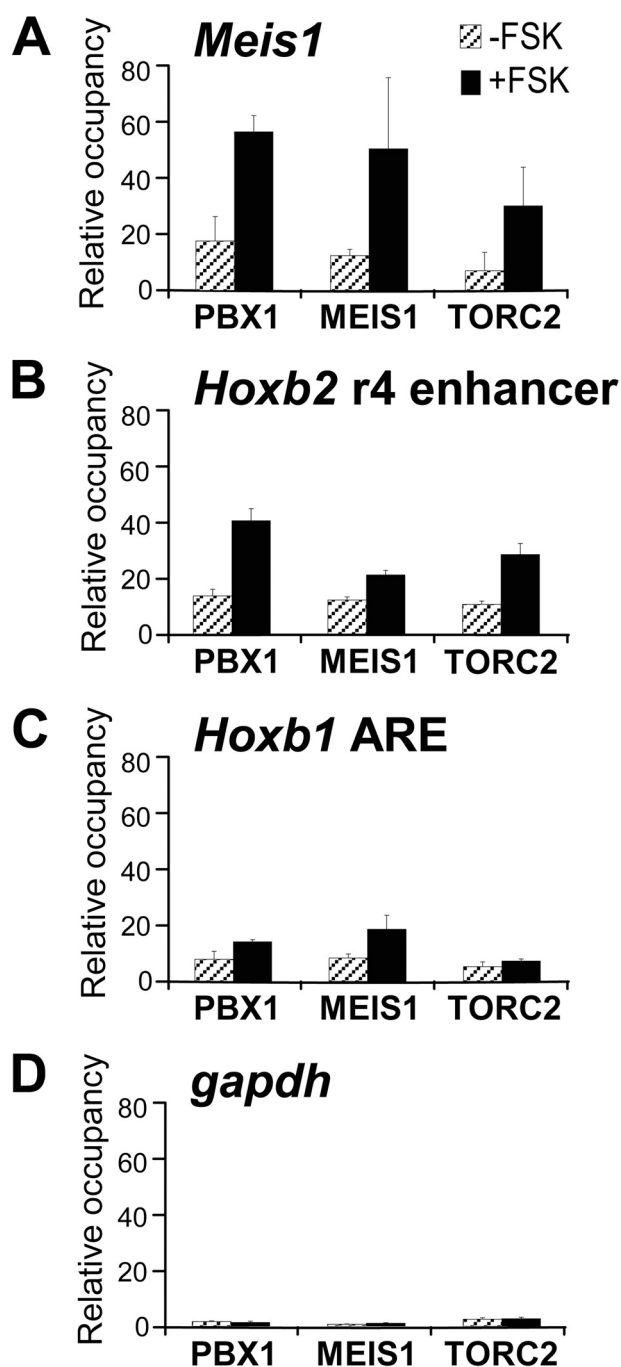
PKA redirects TORC proteins from the cytoplasm to the nucleus through inhibitory effects on SIK2 and related kinases. We showed that overexpression of TORC2 likewise results in strong nuclear accumulation, explaining how PKA is bypassed under these conditions (Fig. 1B). However, we were unable to show similar nuclear localization following overexpression of TORC1 (data not shown). This may be explained by low-level nuclear localization of TORC1 being sufficient for reporter activation. For

example, TORC1 could be tenaciously bound to GAL-MEIS1A at UAS elements in the luciferase reporter, whereas overall nuclear levels of TORC2 are kept low.

Using truncated versions of TORC1, the inherent functions of two regions were found to contribute to the TORC1 effect on MEIS1A. The N-terminal 46 residues of TORC1 encompass part of a highly conserved coiled-coil domain required for tetramerization and association with CREB (37), the disruption of which is expected to interfere with TORC1 function. In addition, the TORC1 C-terminal 203 residues that overlap a transcriptional activation domain and were previously proposed to coordinate the assembly of the transcriptional apparatus (37) were also required for optimal MEIS1A activity (Fig. 5).

TORC2 shares 32% identity with TORC1 (38) and mimicked TORC1 in potentiating MEIS1A activity (Figs. 1 and 2). However, TORC2 was notably weaker than TORC1, consistent with the findings of Conkright *et al.* (37). Depletion of overexpressed and endogenous TORC2 using TORC2-specific shRNA confirmed a specific effect on the MEIS1A C terminus (Fig. 3).

Our co-immunoprecipitation results demonstrate a direct or indirect physical interaction between MEIS1 and overexpressed TORC1, and between purely endogenous MEIS1 and TORC2 in HEK293 cells (Fig. 4). The MEIS1A interaction domain on TORC1 spans residues 1 to 290, and includes the TORC1 coiled-coil domain. TORC1 lacking this domain was unable to induce transcriptional activation via the MEIS1A C terminus (Fig. 5). We were unable to map the TORC1 interaction domain on MEIS1A in reciprocal immunoprecipitates (data not shown). This could be because our anti-MEIS1 antibody occludes the TORC1 binding domain. In the absence of this evidence, four observations argue that TORCs interact



**FIGURE 6. MEIS1, PBX1, and TORC2 are recruited to endogenous MEIS1 targets.** The results of ChIP assays in untreated (–FSK) or forskolin-treated (+FSK) mouse P19 cells are presented. Values obtained by LightCycler quantification were normalized against the corresponding input and nonspecific IgG antibody, and expressed as relative occupancy. The data are presented as the mean  $\pm$  S.E. of three experiments. *A*, the *Meis1* promoter, which harbors a consensus PBX–MEIS binding site. *B*, the *Hoxb2* r4 enhancer. *C*, the *Hoxb1* ARE. *D*, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) served as an internal control.

with MEIS1A via its C terminus. First, the C-terminal 56-residue fragment of MEIS1A fused to the GAL4 DNA-binding domain was sufficient to activate transcription in the presence of TORC1 and/or TORC2 (Fig. 1). Second, in GAL fusions, mutations within the MEIS1A C terminus abolished activation by TORC1 and TORC2 (Fig. 1). Third, by comparison to full-length, unfused MEIS1A, a C-terminal truncated protein

(MEIS1A- $\Delta$ (334–390)) was impaired for activation of a *Hoxb1* ARE reporter in response to TORC1 and TORC2 (Fig. 2). Fourth, a MEIS1A mutant lacking the C terminus does not co-immunoprecipitate with TORC1 (Fig. 4C).

In addition to MEIS1A, the C-terminal divergent MEIS1B isoform might also interact with TORCs because it was also found to cooperate with TORC1 and TORC2 to potentiate transcription from the *Hoxb1* ARE (data not shown). On the basis of previous studies and results presented here, the TORC component of a TORC·MEIS complex may promote the assembly of a transcriptional initiation complex by recruitment of the TFIID-associated factor TAF<sub>II</sub>130 and CBP (37, 46).

**TORCs Are Recruited to MEIS1 Target Enhancers in Vivo**—Our ChIP studies demonstrate co-occupancy of endogenous MEIS1, PBX1, and TORC2 on the *Hoxb2* r4 enhancer and *Meis1* promoter upon elevation of cAMP levels, confirming the biological relevance of our findings (Fig. 6). Together with our *in vitro* tests of HOX- and MEIS-responsive enhancers, our results imply that HOX, MEIS, PBX, and TORC will collaborate to induce a subset of genes involved in embryonic development, and normal hematopoiesis or leukemogenesis. With regard to the latter, the *FLT3* promoter recruits MEIS1 in acute myeloid leukemia-initiating progenitors (21, 27) and possesses conserved cAMP-responsive elements that are occupied by CREB *in vivo* (47). We speculate that TORC, which binds both CREB and MEIS, may provide the means by which these two transcription factors respond cooperatively to PKA signaling on the *FLT3* promoter.

MEIS and TORC are evolutionarily conserved, and *Drosophila* TORC strongly activates transcription through cAMP-responsive reporters (38), making it likely that the regulatory interactions reported here are used across species. PKA signaling regulates embryonic patterning and morphogenesis as demonstrated by the action of hedgehog family members in dorso-ventral patterning and skeletogenesis (48). The broad expression of TORC, MEIS, and PBX family members and their control of HOX expression and function, combined with numerous roles for PKA in cellular and developmental processes suggests that the functions of these factors will converge in many such events.

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