Oncogene (2013), 1–9

www.nature.com/onc

ORIGINAL ARTICLE

Mutations that disrupt PHOXB interaction with the neuronal calcium sensor HPCAL1 impede cellular differentiation in neuroblastoma

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Heterozygous germline mutations in *PHOX2B*, a transcriptional regulator of sympathetic neuronal differentiation, predispose to diseases of the sympathetic nervous system, including neuroblastoma and congenital central hypoventilation syndrome (CCHS). Although the PHOX2B variants in CCHS largely involve expansions of the second polyalanine repeat within the C-terminus of the protein, those associated with neuroblastic tumors are nearly always frameshift and truncation mutations. To test the hypothesis that the neuroblastoma-associated variants exert their effects through loss or gain of protein–protein interactions, we performed a large-scale yeast two-hybrid screen using both wild-type (WT) and six different mutant PHOX2B proteins against over 10 000 human genes. The neuronal calcium sensor protein HPCAL1 (VILIP-3) exhibited strong binding to WT PHOX2B and a CCHS-associated polyalanine expansion mutant but only weakly or not at all to neuroblastoma-associated frameshift and truncation variants. We demonstrate that both WT PHOX2B and the neuroblastoma-associated R100L missense and the CCHS-associated alanine expansion variants induce nuclear translocation of HPCAL1 in a Ca^{2+}-independent manner, while the neuroblastoma-associated 676delG frameshift and K155X truncation mutants impair subcellular localization of HPCAL1, causing it to remain in the cytoplasm. HPCAL1 did not appreciably influence the ability of WT PHOX2B to transactivate the *DBH* promoter, nor did it alter the decreased transactivation potential of PHOX2B variants in 293T cells. Abrogation of the PHOX2B–HPCAL1 interaction by shRNA knockdown of *HPCAL1* in neuroblastoma cells expressing PHOX2B led to impaired neurite outgrowth with transcriptional profiles indicative of inhibited sympathetic neuronal differentiation. Our results suggest that certain PHOX2B variants associated with neuroblastoma pathogenesis, because of their inability to bind to key interacting proteins such as HPCAL1, may predispose to this malignancy by impeding the differentiation of immature sympathetic neurons.

Oncogene advance online publication, 22 July 2013; doi:10.1038/onc.2013.290

Keywords: PHOX2B; HPCAL1; neuroblastoma; neuronal calcium sensor; differentiation; network perturbation

INTRODUCTION

Neuroblastomas are embryonal tumors of the peripheral sympathetic nervous system that typically arise in the sympathetic ganglia and adrenal glands. Although these tumors are primarily sporadic, a genetic predisposition is seen in about 1% of cases.[1] Germline mutations in the anaplastic lymphoma kinase receptor *ALK*, and in the homeodomain (HD) transcription factor *PHOX2B*, account for the majority of familial neuroblastoma cases.[1] Their clinical presentation varies widely, from a symptomless mass to disseminated disease that often becomes resistant to all available therapies. The tumor cells are characterized by a spectrum of differentiation, ranging from undifferentiated, immature cells to partially differentiated cells that have begun the transition to sympathetic neurons. This feature has led to the hypothesis that these tumors develop because of perturbations in genes affecting neuronal differentiation.[2]

PHOX2B is thought to function as a master regulator of sympathetic neuron development.[3] Indeed, loss of *PHOX2B* in mice leads to failure of formation of the sympathetic ganglia and the absence of cells expressing tyrosine hydroxylase and dopamine beta-hydroxylase (DBH), key enzymes in catecholamine biosynthesis[4] and markers of terminal differentiation of sympathetic neurons.[5] Similarly, overexpression of *PHOX2B* promotes the differentiation of avian neural crest and human neuroblastoma cells, the latter in the presence of retinoic acid.[6,7] Heterozygous germline mutations in *PHOX2B* predispose to neuroblastoma and other neurocristopathies, such as congenital central hypoventilation syndrome (CCHS) and Hirschprung's disease,[8–11] characterized by absent or reduced autonomic innervation in the brain and intestine, respectively.

The *PHOX2B* gene encodes a 314-amino-acid protein that includes a HD DNA-binding region and two polyalanine repeats of 9 and 20 alanines within the C-terminal end. Mutations in the majority of CCHS patients are in-frame expansions (from +5 to +13 alanine residues) of the second polyalanine repeat

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Received 3 December 2012; revised 17 April 2013; accepted 26 May 2013

(polyalanine repeat expansion mutations (PARMs)),[8] whereas those associated with tumors of neural crest origin tend to be (i) missense alterations in the HD, (ii) insertions or deletions within the third exon (C-terminus) that alter the reading frame of the gene or (iii) nonsense mutations leading to a truncated protein that lacks the C-terminus (collectively called non-PARMs).[8–13] A detailed understanding of how mutant alleles of the same gene predispose to different diseases has been difficult to acquire. Several studies have shown that the neuroblastoma-associated PHOX2B variants can increase the proliferation of immature sympathetic neurons and inhibit their differentiation both *in vitro* and *in vivo*.[7,14–16] However, even among the spectrum of neuroblastoma-associated PHOX2B variants, it is not clear how missense mutations within the HD vs frameshift and truncation mutations that alter the C-terminus of the protein can predispose to the development of neuroblastoma.

It is well established that proteins do not function in isolation but are typically associated with macromolecular complexes and signaling pathways that are part of highly interconnected interaction networks.[17] Thus, disease-causing mutations in protein-coding regions can lead to either total loss of protein expression or to the expression of mutant proteins that alter one or a few specific interactions.[18–20] Aberrant protein–protein interactions represent a largely unexplored mechanism by which diverse PHOX2B mutations could give rise to different disease phenotypes. Indeed, perturbations of the underlying, wild-type (WT) PHOX2B protein–protein interaction network may explain disease phenotypes that cannot be attributed to mutations, which directly impact the transactivation of key target genes. Although three WT PHOX2B-interacting proteins, all transcription factors or coactivators, have been identified to date,[14,21,22] such studies have not uncovered altered protein–protein interactions involving PHOX2B variants.

We therefore considered that *PHOX2B* mutations may exert their tumorigenic effects by preventing or modifying the binding of interacting proteins or perhaps by recruiting novel interactors. To test this hypothesis, we performed a high-throughput yeast two-hybrid screen using both WT PHOX2B and representative examples of both neuroblastoma- and CCHS-associated variants. This search identified a neuronal calcium sensor (NCS) protein, HPCAL1 (alternatively known as VILIP-3), which interacts with WT and CCHS-associated variant PHOX2B proteins but only weakly or not at all with the neuroblastoma-associated frameshift and truncation variants. We demonstrate that PHOX2B binding with HPCAL1 is important for the nuclear transport of the calcium sensor and for mediating the effects of PHOX2B on sympathetic neuronal differentiation. These findings implicate mutational disruption of the PHOX2B–HPCAL1 interaction as a contributing factor to neuroblastoma predisposition.

RESULTS

The NCS HPCAL1 is a binding partner of PHOX2B

To identify proteins that interact with PHOX2B, we performed a large-scale high-throughput ORFeome-based yeast two-hybrid (Y2H) screen, using the full-length WT PHOX2B and six different PHOX2B variants (Figure 1a). These included neuroblastoma-associated variants with missense mutations within the HD region (R100L and R141G),[11] truncation of the entire third exon (K155X)[8] and frameshift mutations within the third exon (721del20 and 676delG),[10,23] as well as a +7 alanine repeat expansion mutation associated with CCHS (AlaExp).[24] The WT and mutant *PHOX2B* cDNAs were cloned into Gateway-compatible Y2H destination vectors to generate GAL4 DNA-binding domain (DB) and activation domain (AD) fusion proteins.[25] These clones were screened against the human ORFeome v.3.1 library, which contains approximately 12 000 open reading frames (ORFs) encompassing >50% of the ORFs encoded in the human

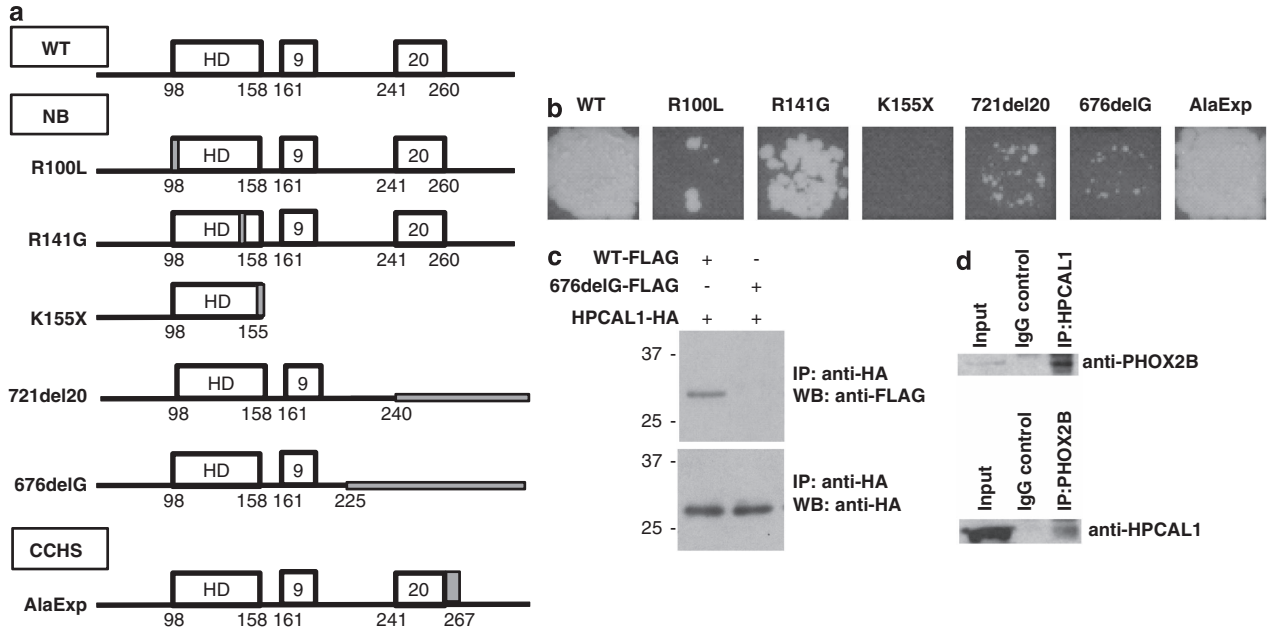

Figure 1. Neuroblastoma-associated PHOX2B variants exhibit impaired binding to HPCAL1. (**a**) Schema of WT and variant PHOX2B proteins used in the yeast two-hybrid (Y2H) screen. All but the AlaExp mutant (CCHS) are neuroblastoma-associated. The 721del20 variant was identified in the SK-N-SH cell line. Locations of the mutations are marked in gray. (**b**) Y2H analysis of interactions between WT and mutant PHOX2B proteins with HPCAL1 as determined by growth on histidine-negative media. (**c**) Verification of protein interactions by overexpression of FLAG-tagged WT (WT-FLAG) and mutant (676delG-FLAG) PHOX2B and HA-tagged HPCAL1 in 293T cells, followed by immunoprecipitation (IP) of the cell extracts with anti-HA antibody and immunoblotting with anti-FLAG and anti-HA antibodies. (**d**) Interaction between endogenously expressed PHOX2B and HPCAL1 in neuroblastoma cells. NGP neuroblastoma cell extracts were immunoprecipitated with HPCAL1 antibodies and immunoblotted with anti-PHOX2B antibodies (upper panel) and vice versa (lower panel). IgG, immunoglobulin; WB, western blotting.

genome.[26] The DB-PHOX2B (WT) and each of the DB-PHOX2B variant-transformed yeast cells were screened against the AD-ORFeome using a mating-based Y2H assay in a 96-well format.[25] In a parallel screen, the reciprocal interactions between AD-tagged PHOX2B preys (WT and each of the mutants) and the DB-tagged ORFeome baits were tested, resulting in 270 potential Y2H hits that were verified through two additional rounds of pairwise mating in yeast cells. Among the verified interactors was the EF-hand NCS protein, HPCAL1, which specifically bound to WT PHOX2B but only weakly to the 721del20 and 676delG frameshift mutants and not at all to the K155X truncation variant (Figure 1b). Interestingly, the HD missense mutants R100L and R141G retained their binding capacity for HPCAL1, although to a reduced degree; while the CCHS-associated PHOX2B alanine expansion variant (AlaExp) showed the same robust binding as WT PHOX2B. Thus, the PHOX2B mutant proteins with the weakest capability to bind to HPCAL1 (K155X, 721del20 and 676delG) were those with significantly altered proximal C-terminal regions, caused by mutations present before the second polyalanine stretch (Figure 1a).

To extend the verified Y2H screening results in mammalian cells, we overexpressed HA-tagged versions of HPCAL1 together with FLAG-tagged WT PHOX2B and a representative variant, 676delG, in 293T cells (which express neither PHOX2B nor the interacting proteins) and examined their binding potential through coimmunoprecipitation (co-IP) assays. Our results revealed that WT PHOX2B immunoprecipitated with HPCAL1, suggesting binding of this protein in these cells. The 676delG frameshift PHOX2B variant did not interact with HPCAL1, verifying the results of our Y2H screen (Figure 1c). To ensure that the interaction between WT PHOX2B and HPCAL1 was also present in human neuroblastoma cells, we performed co-IP experiments on cell lysates from NGP cells that endogenously express both proteins. Again, we noted that endogenously expressed PHOX2B formed a complex with HPCAL1 in these cells (Figure 1d). Together, these results show that HPCAL1 is a *bona fide* interactor of WT PHOX2B in neuroblastoma cells and that this interaction is lost or impaired with certain PHOX2B variants.

HPCAL1 is translocated to the nucleus upon binding to PHOX2B

We next determined whether the interaction between WT PHOX2B and HPCAL1 affects the cellular localization of the two proteins (Figure 2; Supplementary Figure S1). As with other classical transcription factors, WT PHOX2B is localized exclusively to the nucleus as determined by the expression of a green fluorescent protein (GFP)-tagged WT PHOX2B construct in HeLa cells lacking endogenous PHOX2B (Figure 2a). We also determined the subcellular localization of HPCAL1 by expressing this protein fused to the mCherry fluorescent marker in HeLa cells, which also do not endogenously express this gene (reverse transcriptase–PCR analysis, data not shown). mCherry-HPCAL1 localized primarily to the cytoplasm (Figure 2a), but when coexpressed with GFP-tagged WT PHOX2B, its expression was more prominent in the nucleus,

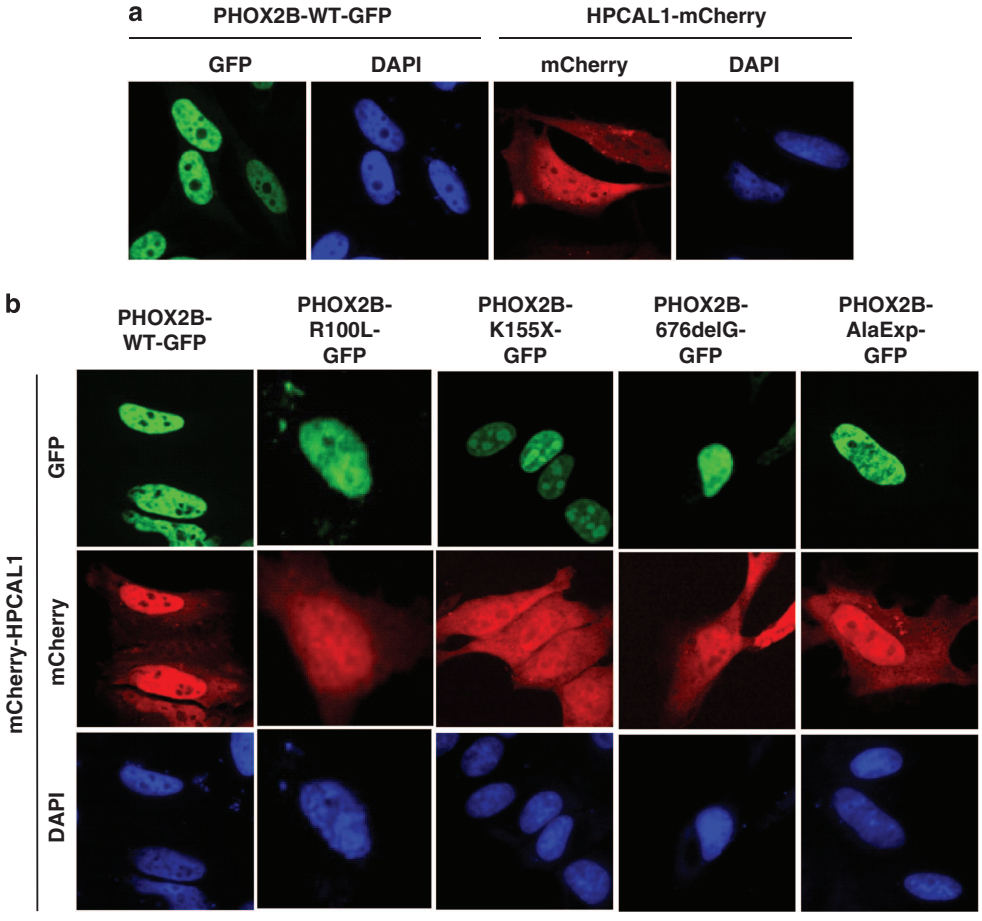

Figure 2. The PHOX2B–HPCAL1 interaction results in nuclear translocation of HPCAL1. (**a**) GFP-tagged WT PHOX2B and mCherry-tagged HPCAL1 were transfected into HeLa cells. Forty-eight hours later, the cells were fixed in 4% formaldehyde in phosphate-buffered saline and mounted on ProLong Gold antifade reagent with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Images were taken on a Nikon Eclipse Ti confocal microscope (Nikon, Melville, NY, USA) and processed with ImageJ software ($\times$ 40 magnification). (**b**) GFP-tagged WT and the indicated mutant GFP-tagged *PHOX2B* genes were cotransfected with *HPCAL1*-mCherry and imaged as in panel (**a**).

suggesting nuclear localization (Figure 2b). This nuclear translocation was not apparent when either the K155X truncation or the 676delG frameshift variants were coexpressed with mCherry-HPCAL1 (Figure 2b and Supplementary Figure S1). By contrast, overexpression of the R100L missense and the CCHS-associated AlaExp mutant allele led to nuclear localization of HPCAL1, similar to results with the WT allele (Figure 2b and Supplementary Figure S1). These data not only confirm the binding of WT PHOX2B to HPCAL1 and the altered binding of distinct neuroblastoma-associated PHOX2B mutants seen in our Y2H and co-IP assays but also suggest that HPCAL1 may regulate PHOX2B function in the nucleus.

Interaction of PHOX2B with HPCAL1 is not calcium dependent

A subclass of EF-hand-containing calcium-binding proteins that includes HPCAL1 undergo post-translational N-terminal myristoylation. These proteins respond to elevations in cellular calcium (Ca^{2+}) levels by exposing their myristoyl group to the aqueous environment, a conformational change that induces their translocation to specific membrane domains.[27] To test whether the interaction between WT PHOX2B and HPCAL1 is Ca^{2+} dependent, we exposed HeLa cells expressing PHOX2B and HPCAL1 to either the Ca^{2+} chelator BAPTA-AM or calcium chloride ($CaCl_2$) for 48 h, to decrease or increase the intracellular Ca^{2+} concentrations, respectively. We observed that the PHOX2B–HPCAL1 interaction, as measured by co-IP, was not affected by changes in intracellular Ca^{2+}, with robust binding at physiological, increased or decreased Ca^{2+} levels (Figure 3a). Next, we determined whether the nuclear translocation of HPCAL1 complexed with PHOX2B depends on Ca^{2+} concentrations. Compared with untreated cells, the subcellular localization of the WT PHOX2B-HPCAL1 complex was not affected by changes in Ca^{2+} levels (Figure 3b). Moreover, varying the Ca^{2+} level did not alter the impaired nuclear translocation of HPCAL1 seen with the 676delG variant (Figure 3b). Thus, the interaction between WT PHOX2B and HPCAL1 is independent of the cellular Ca^{2+} concentration.

The transactivation potential of PHOX2B is not altered by interaction with HPCAL1 in 293T cells.

PHOX2B directly transactivates its own promoter as well as that of other transcriptional targets, such as the *DBH* gene.[28,29] All of the PHOX2B variants described to date have reduced transactivation potential.[7,14,30] We therefore asked whether the disruption of HPCAL1 binding to PHOX2B might affect the latter protein's ability to activate the regulatory regions of *DBH*. Using a dual-luciferase reporter assay, we analyzed the effect of ectopic expression of WT and mutant PHOX2B on the *DBH* promoter in the presence or absence of *HPCAL1* overexpression (Supplementary Figure S2). Compared with WT PHOX2B, all of the PHOX2B variants demonstrated impaired transactivation of the *DBH* promoter, with

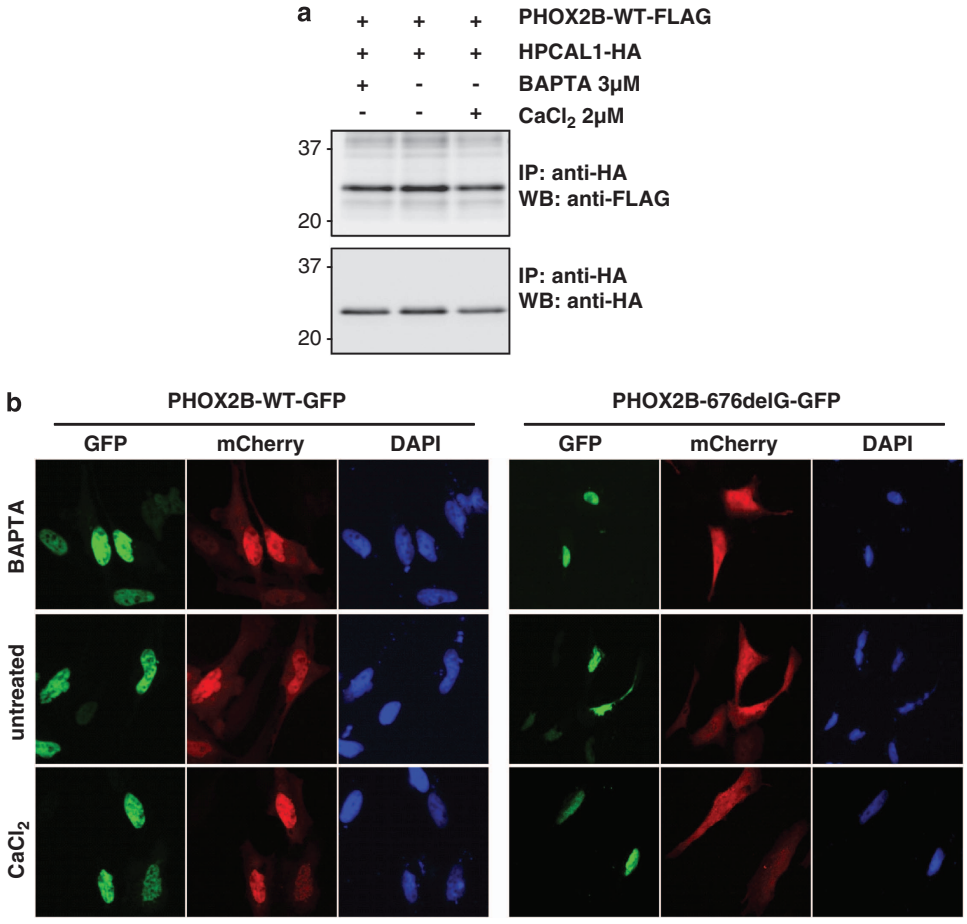

Figure 3. The PHOX2B–HPCAL1 interaction is not calcium dependent. (**a**) *PHOX2B-WT-FLA*G and *HPCAL1-HA* constructs were transfected into 293T cells and harvested 48 h later. Either BAPTA-AM (1,2-Bis(2–aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)) (3 μM) or $CaCl_2$ (2 μM) was added to the cell lysates at the indicated concentrations, immunoprecipitated (IP) with anti-HA antibodies and analyzed by western blotting (WB) with anti-HA and anti-FLAG antibodies. (**b**) *HPCAL1*-mCherry and *PHOX2B*-GFP (both WT and the 676delG mutant) constructs were transiently transfected into HeLa cells and either BAPTA-AM or $CaCl_2$ was added to the media at the indicated concentrations. Cells were fixed and imaged 2 days after transfection as described in Figure 2.

K155X, 676delG and 721del20 showing the greatest deficits. Cotransfection with *HPCAL1* did not significantly affect the reduced *DBH* promoter activation seen with the PHOX2B variants (Supplementary Figure S2). The transcription factor *HAND2*, implicated in autonomic neuron development, is another target of PHOX2B. To test the effect of the WT and variant PHOX2B proteins on the *HAND2* promoter, we similarly cooverexpressed the 676delG and K155X mutations with the *HAND2* regulatory region subcloned upstream of a luciferase reporter. Although compared with WT PHOX2B, these mutations led to decreased transactivation of the *HAND2* promoter, there was no significant change induced by the presence of HPCAL1 (data not shown). Thus, overexpression of *HPCAL1* does not alter the transactivation potential of WT or PHOX2B mutant proteins.

HPCAL1 binding is required for the differentiation of neuroblastoma cells

PHOX2B exerts strong antiproliferative activity in primary avian sympathetic[14] and human neuroblastoma cells.[7] More importantly,

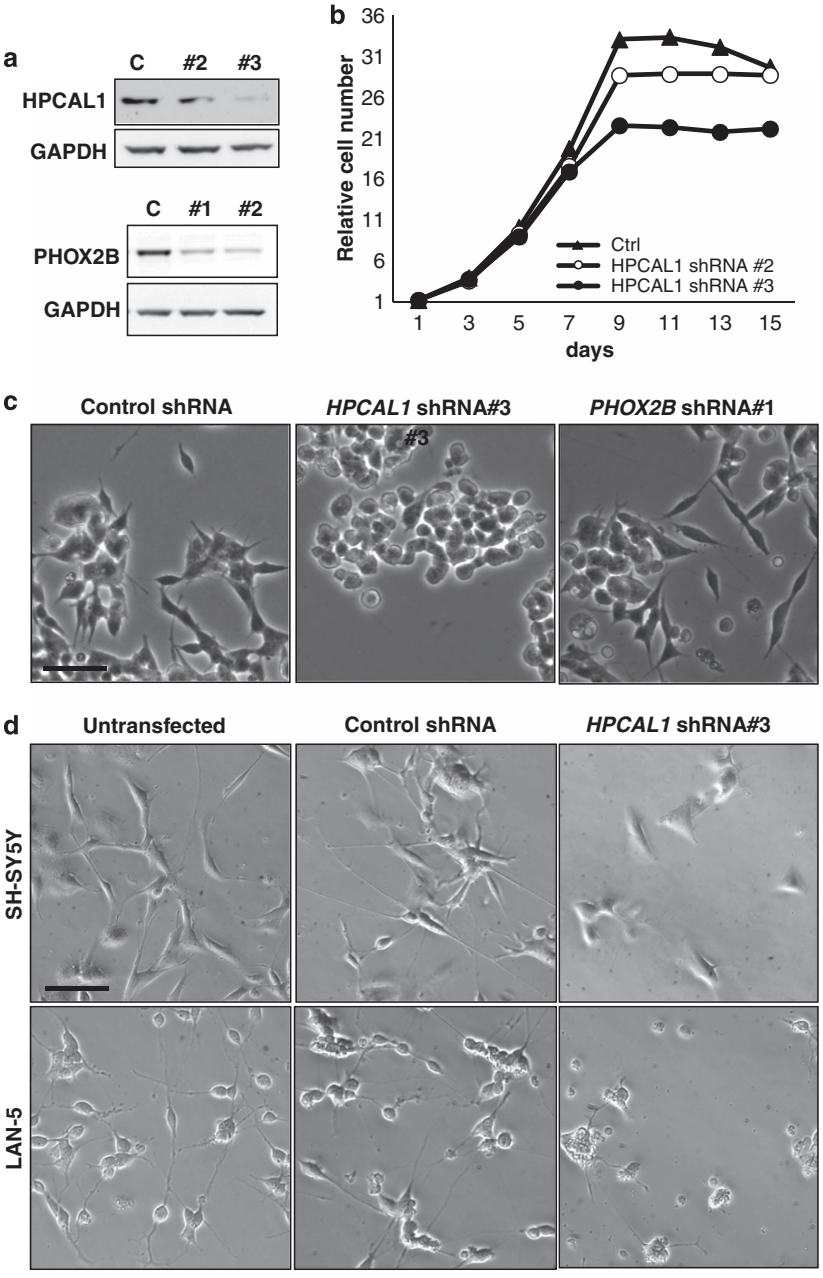

Figure 4. HPCAL1 knockdown (KD) inhibits the differentiation of NGP neuroblastoma cells. (**a**) Western blot analysis of cells in which *HPCAL1* (upper panel) and *PHOX2B* (lower panel) expression was depleted by shRNA KD. NGP cells were infected with lentivirus-expressing shRNAs against *HPCAL1* or *PHOX2B* and selected in 0.5 μg/ml puromycin to obtain stable cell lines. C, control shRNA. (**b**) Growth curve of NGP cells in which *HPCAL1* expression was knocked down. NGP cells stably expressing HPCAL1-shRNA were seeded into 96-well plates in triplicate, and cell growth analyzed by CellTiter-Glo assay (Promega Corp., Madison, WI, USA) every 2 days. Luminescence readings were normalized to day 1. Values represent the mean ± s.d. of at least three independent experiments. (**c**) Phase-contrast micrographs of NGP cells stably expressing *HPCAL1* and *PHOX2B* shRNAs as indicated. Cells were seeded into six-well plates and incubated for 6 days in serum-free media. The same results were obtained for the additional shRNA KD depicted as in panel (**a**). Bar = 25 μm. (**d**) Phase-contrast micrographs of untransfected, control and HPCAL1 shRNA-expressing SH-SY5Y and LAN-5 neuroblastoma cells treated with 10 μM of retinoic acid for 3 days. Bar = 25 μm. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

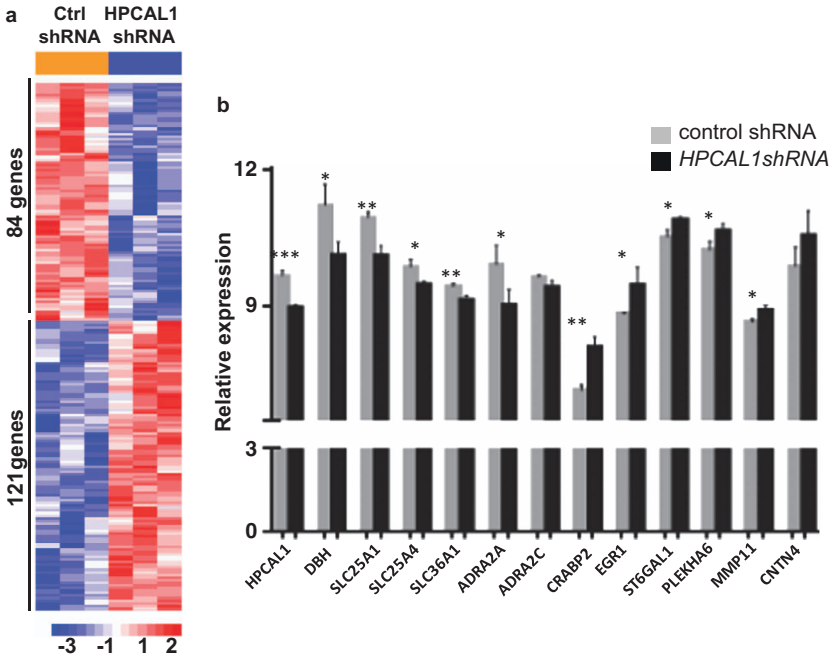

Figure 5. Depletion of *HPCAL1* in neuroblastoma cells expressing WT PHOX2B causes differential regulation of genes involved in sympathetic neuronal differentiation. (**a**) Heat map representation of differentially regulated genes in NGP cells after HPCAL1 knockdown vs control shRNA knockdown. (**b**) Histograms representing the relative expression of the indicated genes that were differentially regulated in cells with HPCAL1 depletion vs those with intact HPCAL1 expression. Data are reported as means ± s.d. ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; $n=3$ for each subgroup).

it regulates sympathetic neuronal differentiation, its absence causing inhibition of terminal differentiation of sympathetic progenitors.[14,16] To investigate the functional role of the PHOX2B–HPCAL1 interaction in neuroblastoma cells and its role, if any, in cell growth and differentiation, we abrogated *HPCAL1* expression in NGP cells using shRNA knockdown and examined the effects on cell growth and differentiation (Figure 4a, upper panel). Compared with findings in control shRNA-expressing cells, a deficit of *HPCAL1* did not affect cell growth (Figure 4b). We next determined the effect of *HPCAL1* knockdown on cell differentiation. NGP cells normally undergo spontaneous differentiation following incubation in serum-free media for 6 days as determined by increased neurite outgrowth (Figure 4c). When we abolished *HPCAL1* expression in these cells, there was no evidence of neurite outgrowth; in fact, they appeared to take on a completely rounded shape (Figure 4c). The lack of neurite outgrowth seen with *HPCAL1* knockdown appeared to be much more prominent than that seen with *PHOX2B* knockdown (Figure 4a, lower panel; Figure 4c). The impaired differentiation on *HPCAL1* depletion was also seen in SH-SY5Y and LAN-5 neuroblastoma cells exposed to all-trans retinoic acid (Figure 4d, Supplementary Figure S3). Together, these results suggest that *HPCAL1* contributes to spontaneous and exogenously induced differentiation in neuroblastoma cells expressing *PHOX2B*.

HPCAL1–PHOX2B interaction is linked to genetic programs mediating neuronal cell differentiation

To elucidate the effects of impaired PHOX2B–HPCAL1 interaction in neuroblastoma cells, we studied the expression profiles of NGP cells in which *HPCAL1* expression was abrogated using shRNA knockdown compared with control cells transduced with shRNA against *GFP* (Figure 5). RNA from three independent biological replicates was processed and hybridized to Affymetrix GeneChip Human Gene 1.0 ST arrays. Supervised analysis revealed 205 probe sets that were differentially expressed on *HPCAL1* knockdown, 121 of which were upregulated (corrected $P<0.01$) (Figure 5a). *HPCAL1* was one of the most differentially expressed genes (Figure 5b), confirming adequate knockdown. Over-represented gene ontology (GO) categories included those involved in the regulation of calcium ion transport, cAMP biosynthetic processes and differentiation of diverse cell types, including T cells, lymphocytes, leukocytes and lung goblet cells (data not shown).

Further scrutiny of the genes involved in cellular differentiation revealed significant downregulation of *DBH*, a major marker of terminal differentiation in sympathetic neuronal and neuroblastoma cells upon *HPCAL1* knockdown (Figure 5b). Other downregulated genes included members of the alpha-2-adrenergic receptor family, *ADRA2A* and *ADRA2C*, which induce neuronal differentiation in PC12 cells.[31] Interestingly, *HPCAL1* knockdown also led to upregulation of the neural cell adhesion molecule, *CNTN4*, whose overexpression has been linked to inhibition of retinoic acid-induced differentiation of neuroblastoma cells[32] (Figure 5b). We also observed involvement of several genes that were present in a recently developed retinoic acid-induced neuroblastoma differentiation signature: namely, *CRABP2*, *EGR1*, *ST6GAL1*, *PLEKHA6*, *MMP11* (upregulated) and *SLC25A1*, *SLC36A1*, *SLC36A4* (downregulated)[33] (Figure 5b). These data suggest that disruption of the HPCAL1–PHOX2B interaction inhibits differentiation in sympathetic neuronal cells.

DISCUSSION

Using an unbiased yeast two-hybrid screen, we identified a direct interaction between WT PHOX2B and HPCAL1 that was confirmed in mammalian cells engineered to express both proteins and in human neuroblastoma cells endogenously expressing these two proteins. The interaction was absent or impaired in cells expressing the neuroblastoma-associated truncation and frameshift PHOX2B variants but was retained in those expressing the missense and the CCHS-associated polyalanine expansion mutants. Moreover, HPCAL1 binding to PHOX2B was required for the translocation of the calcium sensor protein to the

nucleus. We also show that binding with HPCAL1 contributes to neurite outgrowth in neuroblastoma cells expressing *PHOX2B*, with disruption of the PHOX2B–HPCAL1 interaction leading to impaired differentiation of these cells. Thus, we report a protein–protein interaction that appears essential for the effects of PHOX2B on sympathetic neuron development, and whose loss may contribute to the neuroblastoma predisposition associated with a range of *PHOX2B* mutations.

Although PHOX2B mutations are seen mainly in hereditary neuroblastoma that accounts for only 1% of cases in this disease, our study provides another example of the newly evolved edgetic perturbation model proposed by Zhong *et al.*,[18] in which distinct alleles of a single gene can have different effects on protein networks, thereby giving rise to varied phenotypes. HPCAL1 is one of the five proteins that form the highly conserved visinin-like (VSNL) subfamily of the EF-hand NCS proteins.[34] It has a restricted expression pattern in the cerebellum, where it localizes to Purkinje and granule cells,[35] as well as in sympathetic ganglia during development (Allen Brain Atlas; http:/www.brain-map.org). VSNL proteins perform specialized functions in membrane trafficking, signal transduction and differentiation in defined subsets of neurons, with each NCS protein having specificity for certain cell types and toward distinct receptors and signaling pathways.[36] Importantly, NCS proteins modulate the function of transcription factors: S100B, which is overexpressed in many tumor cells including melanoma,[37] interacts with the C-terminus of TP53 to inhibit both tetramerization and protein kinase C-dependent phosphorylation, resulting in its inactivation.[38,39] Similarly, the inability of the truncation and frameshift mutations to bind to HPCAL1 suggests that this region is necessary for the requisite protein–protein interaction. By modifying the C-terminus, these particular mutations may alter the protein folding of PHOX2B, accounting for the differences in protein–protein interactions between these neuroblastoma-associated variants and WT PHOX2B. The missense and polyalanine expansion mutations may not cause similar changes in protein conformation, and the binding sites may be preserved. Additionally, the subcellular localization of HPCAL1 correlates with its binding to PHOX2B: both WT PHOX2B and the R100L and the alanine expansion mutants that retained their ability to bind to HPCAL1 caused it to be translocated to the nucleus, whereas HPCAL1 remained in the cytoplasm in the presence of the K155X or 676delG variants. The abrogation of nuclear transport seen with mutant forms of PHOX2B would be expected to have critical effects on PHOX2B function, a prediction that requires further investigation.

The presence of EF-hand calcium-binding motifs, together with a consensus N-terminal myristoylation sequence, enables VSNL proteins to translocate from the cytosolic to subcellular membrane compartments, particularly following elevation of cytosolic Ca^{2+} concentration.[40,41] By contrast, PHOX2B caused HPCAL1 to translocate to the nucleus in a Ca^{2+}-independent manner, suggesting that the HPCAL1 motif which participates in PHOX2B binding is not affected by Ca^{2+}-induced structural changes. HPCAL1 has been shown to localize to the Golgi in a Ca^{2+}-independent manner.[40] Its lower binding affinity for Ca^{2+} could also have a role in its Ca^{2+}-independent activity.[42] Alternatively, these results could also have been influenced by our use of fusion proteins that may have adversely affected Ca^{2+} affinities.[34]

Interaction with HPCAL1 did not appreciably affect the transactivation potential of PHOX2B on either the *DBH* or *HAND2* promoters, nor did the lack of binding with HPCAL1 further influence the already decreased transactivation potential of the neuroblastoma-associated PHOX2B variants in 293T cells. However, our expression microarray data clearly showed that endogenous *DBH* levels are significantly downregulated when HPCAL1 is depleted in neuroblastoma cells expressing both *HPCAL1* and *PHOX2B*. These seemingly discordant results most probably reflect the cell-type specificity of PHOX2B—the former analysis was performed in non-neuronal human embryonic kidney cells while the latter was done in neuroblastoma cells. The inability of PHOX2A, a paralog of PHOX2B, to effectively stimulate transcription from the *DBH* promoter in non-neuronal cultures[43] also attests to the tissue specificity of homeobox proteins. Furthermore, the phenotypic expression of genes encoding catecholamine biosynthetic enzymes, such as *DBH*, is influenced not only by their transcriptional complexes, but also by cell-type-specific signals such as growth factors and neurohormones,[44] which are not likely to be present in 293T cells.

Many NCS proteins have roles in the differentiation of developing neurons.[34] The prototype member of the VSNL family, VILIP-1, was identified in a screen for developmentally regulated proteins that were upregulated during terminal differentiation of the nervous system.[45] Moreover, overexpression of VILIP-1 in SH-SY5Y neuroblastoma cells causes an increase in the number and length of neurites.[46] We report that PHOX2B-expressing neuroblastoma cells in which HPCAL1 was depleted exhibited markedly decreased neurite outgrowth compared with control shRNA-expressing cells. This observation, together with the significantly downregulated expression of *DBH*, one of the major markers of terminal differentiation in sympathetic neuronal cells and neuroblastomas,[5] suggests that HPCAL1 contributes to the role of PHOX2B in promoting sympathetic neuronal cell differentiation. This conclusion is reinforced by the transcriptional profiles of these cells, which showed differential regulation of genes involved in neuronal differentiation upon depletion of *HPCAL1*, such as *CNTN4*.[32]

In conclusion, our study demonstrates that a simple model of 'gene loss' is insufficient to explain complex genotype–phenotype relationships and that allele-specific perturbation of interactions need to be fully understood in the context of protein networks. The observations on the PHOX2B–HPCAL1 interaction described here help to elucidate the phenotypic consequences of *PHOX2B* mutations, leading to the loss of protein-binding affinity and probably subsequent rewiring of the local PHOX2B interaction network as well. As neuroblastoma tumors consist mainly of sympathetic progenitor cells that are either undifferentiated or poorly differentiated, our data suggest that aberrant interactions with HPCAL1, as seen with diverse PHOX2B mutants, could disrupt the normal differentiation of sympathoadrenal cells, which may predispose to neuroblastoma by increasing susceptibility to secondary transforming events.

MATERIALS AND METHODS

Cell lines and antibodies

NGP, SH-SY5Y and LAN-5 neuroblastoma cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. 293T (human embryonic kidney, 293T) cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Sources of antibodies: anti-FLAG antibody (Sigma-Aldrich, St Louis, MO, USA), mouse anti-HA antibody (Roche Applied Science, Indianapolis, IN, USA), rabbit anti-HA antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA).

Construction of plasmids

PHOX2B mutant constructs were generated with the QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). HPCAL1 cDNA was obtained from CCSB (Center for Cancer Systems Biology, Dana-Farber Cancer Institute, Boston, MA, USA). FLAG and HA fusion constructs were generated by inserting the genes of interest into pcDNA3.1 vectors (Invitrogen, Grand Island, NY, USA) modified with FLAG or HA sequences-engineered C-terminal to the multiple cloning sites, respectively. GFP and mCherry fusion constructs were made by cloning genes of interest into pEGFP-C1 and pmCherry-C1 vectors (BD Biosciences, San Jose, CA, USA), respectively. PHOX2B and its variants were cloned into Y2H destination vectors with GAL4DNA DB or AD tags using Gateway cloning technology (Invitrogen).

Yeast two-hybrid screen

A pairwise mating-based Y2H screen in 96-well high-throughput format was performed to screen yeast cells expressing the AD- or DB-fusion constructs in human ORFeome 3.1, containing ~12 000 ORFs. Briefly, the DB-PHOX2B bait-transformed yeast cells were individually mated with yeast cells expressing human AD-ORF preys; in the reverse screen, the reciprocal pairwise interactions between AD-PHOX2B preys and DB-ORFeome baits were tested. Positive clones from the primary screen were verified through two additional rounds of pairwise retesting in yeast cells to ensure reproducibility and to exclude experimental artefacts. The Y2H phenotypes of candidate Y2H pairs were verified by mating the matching individual *MAT*α Y8930 DB-X yeast strains and *MAT*a Y8800 AD-Y yeast strains on YEPD (yeast extract-peptone-dextrose) media. Growth of the diploid cells on synthetic media lacking histidine (− His) indicated activation of the *GAL1-HIS3* reporter gene. To identify and exclude auto-activators that can spontaneously arise during the Y2H selection process, we used the pDEST-AD-*CYH2* vector carrying the *CYH2* counter-selectable marker, which allows for plasmid shuffling on cycloheximide (CHX)-containing media. Only pairs that gave rise to growth on synthetic media lacking histidine (− His) but not on synthetic media lacking histidine but containing 1mg/l cycloheximide (− His/CHX) in four of the four replicates were considered verified.

Transient transfection and lentiviral infection of shRNA in cultured cells

Transient DNA transfection was performed with the FuGENE 6 Transfection reagent (Roche Applied Science). Briefly, 1 μg plasmid DNA was transfected into cells in each well of a six-well plate, incubated for 48 h and harvested for protein extraction or fluorescence imaging. Lentiviral-based pLKO.1 shRNA constructs were obtained from the RNAi Consortium of the Broad Institute and MIT, Cambridge, MA, USA. Virus was produced by transfecting pLKO.1 shRNA plasmids with packaging plasmid (pCMV-dR8.91) and envelope plasmid (VSV-G/pMD2.G) into 293T cells using Fugene6 (Promega Corp., Madison, WI, USA) and Mirus Transfection Reagent (Mirus Bio LLC, Madison, WI, USA) and harvesting the supernatant after 48 h. For infection, lentivirus in growth medium containing 8 μg/ml polybrene was added to cells on 10-cm dishes and incubated overnight. A final concentration of 1 μg/ml puromycin in regular medium was added to the cells the next day for selection.

Co-immunoprecipitation

Plasmids were transfected into 293T cells and cultured for 48 h before harvest. Cell pellets were lysed in RIPA lysis buffer supplemented with 1 × protease inhibitor cocktail solution and PMSF (Roche Applied Science). In all, 500 μg of total lysate and 1 μg of antibody were mixed and incubated at 4 °C overnight, then 40 μl of protein G Agarose beads (EMD Millipore, Billerica, MA, USA) were added and incubated for 2 h. Beads were washed three times with 1 × TBST buffer, resuspended in 40 μl Laemmli sample buffer and boiled for 5 min before sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis.

Expression array and data analysis

Three independent batches of total RNAs were isolated with the Qiagen RNeasy kit (Qiagen Inc., Germantown, MD, USA) from NGP cells stably infected with an HPCAL1 shRNA and a control GFP shRNA, as described above, and submitted for expression analysis with Affymetrix GeneChip Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). Raw microarray data were normalized using Bioconductor (www.bioconductor.org), and the analyses were performed using the limma (Linear Models for Microarray Data) and biomaRt (Interface to BioMart databases) packages. Gene lists that were normally upregulated and downregulated during cell growth and differentiation were retrieved from public databases. Genes that showed a significant differential effect ($P<0.05$) between control shRNA and HPCAL1 shRNA on cell growth and differentiation were retrieved.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Christo Goridis for the generous gift of the PHOX2B antibody. We thank Stacey Frumm and Dr Kimberly Stegmaier for data regarding the neuroblastoma cell differentiation signature before publication. This study was supported by Alex's Lemonade Stand Foundation (to REG), Abraham Research Fund (to WW), National Cancer Institute Grant R33CA132073 (to MV), National Human Genome Research Grants R01HG001715 and P50HG004233 (to MV and DEH) and The Ellison Foundation (to MV).

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