Prohibitin 1 Regulates the H19-Igf2 Axis and Proliferation in Hepatocytes*

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Prohibitin 1 (PHB1) is a mitochondrial chaperone that regulates cell growth. Phb1 knock-out mice exhibit liver injury and hepatocellular carcinoma (HCC). Phb1 knock-out livers show induction of tumor growth-associated genes, H19 and insulin-like growth factor 2 (Igf2). These genes are controlled by the imprinting control region (ICR) containing CCCTC-binding transcription factor (CTCF)-binding sites. Because PHB1 knock-out mice exhibited induction of H19 and Igf2, we hypothesized that PHB1-mediated regulation of the H19-Igf2 axis might control cell proliferation in normal hepatocytes. H19 and Igf2 were induced (8–20-fold) in 3-week-old Phb1 knock-out livers, in Phb1 siRNA-treated AML12 hepatocytes (2-fold), and HCC cell lines when compared with control. Phb1 knockdown lowered CTCF protein in AML12 by ~30% when compared with control. CTCF overexpression lowered basal H19 and Igf2 expression by 30% and suppressed Phb1 knockdown-mediated induction of these genes. CTCF and PHB1 co-immunoprecipitated and co-localized on the ICR element, and Phb1 knockdown lowered CTCF ICR binding activity. The results suggest that PHB1 and CTCF cooperation may control the H19-Igf2 axis. Human HCC tissues with high levels of H19 and Igf2 exhibited a 40–50% reduction in PHB1 and CTCF expression and their ICR binding activity. Silencing Phb1 or overexpressing H19 in the mouse HCC cell line, SAME-D, induced cell growth. Blocking H19 induction prevented Phb1 knockdown-mediated growth, whereas H19 overexpression had the reverse effect. Interestingly, H19 silencing induced PHB1 expression. Taken together, our results demonstrate that the H19-Igf2 axis is negatively regulated by CTCF-PHB1 cooperation and that H19 is involved in modulating the growth-suppressive effect of PHB1 in the liver.

Prohibitin 1 (PHB1) is a ubiquitously expressed mitochondrial chaperone protein with diverse functions (1). Membrane-bound PHB1 regulates downstream signaling pathways, whereas mitochondrial and nuclear PHB1 regulates apoptosis, transcriptional activation, and cell cycle, indicating the important link between its cellular localization and function (2, 3). Studies have demonstrated that PHB1 protects cells from oxidative stress and maintains the functional integrity of the mitochondria (2, 4, 5). Nuclear PHB1 co-localizes with p53, E2F1, and retinoblastoma protein (6–8). In breast cancer and B-cell lymphoma cell lines, Phb1 overexpression induces p53 transcriptional activity, whereas it represses E2F1-mediated transcription via its interaction with the retinoblastoma protein (3, 9). Despite this strong evidence of anti-tumorigenic properties of PHB1, a number of studies have indicated the pro-tumorigenic effects of PHB1. Membrane-associated PHB1 interacts with C-Raf and is indispensable for the activation of the Ras-Raf-MEK-ERK signaling pathway, which may modulate cancer cell survival and migration (10). Also, Phb1 is up-regulated in many cancers such as breast, prostate, ovarian, lung, bladder, thyroid, and gastric cancer (9). One of the reasons for this induction is thought to be the presence of binding sites for c-Myc in the Phb1 promoter (9). However, a direct effect of c-Myc on Phb1 transcription has not been reported so far. It is possible that the controversial role of Phb1 in tumorigenesis and cell survival may be explained by cell type-specific mechanisms, subcellular localization, and even protein post-translational modifications such as phosphorylation (10).

PHB1 was originally cloned from regenerating livers where its expression was nearly absent shortly after two-thirds partial hepatectomy and hence thought to be a tumor suppressor (11). To examine molecular mechanisms of action of Phb1 in the liver, we previously developed the liver-specific Phb1 knock-out (Phb1 KO) mouse model (12). Phb1 KO mice livers develop severe oxidative stress, fibrosis, and liver cancer (12). Microarray analysis of Phb1 KO mice livers revealed a significant up-regulation of the long noncoding RNA, H19 and insulin-like growth factor 2 (Igf2) genes (12). These genes are part of a well characterized cluster that contains the paternally expressed Igf2 and maternally expressed H19. Both genes share enhancers and are asynchronously regulated at a site called the imprinting control region (ICR) (13). Loss of imprinting of both Igf2 and

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The abbreviations used are: ICR, imprinting control region; HCC, hepatocellular carcinoma; CTCF, CCCTC-binding transcription factor; FL, floxed; EV, empty vector.
H19 has been observed in hepatocellular carcinoma (HCC) tissues and cell lines (14, 15). Igf2 is frequently activated in human cancers and in experimental liver carcinogenesis and is known to promote HCC growth (16, 17). H19 expression has been positively correlated with hepatocyte proliferation after partial hepatectomy in rodents and with tumor cell growth (18, 19). Although H19 expression positively correlates with growth, the underlying mechanisms by which H19 may contribute to liver cell proliferation and HCC are not clearly established.

CCCTC-binding factor (CTCF) is a ubiquitous transcription factor that acts as both a transcriptional activator and a repressor. On the ICR, it controls allele-specific H19 and Igf2 expression by a DNA methylation-dependent mechanism. CTCF binding to the ICR element negatively regulates Igf2 expression on the maternal allele by blocking the access of Igf2 promoter to a downstream enhancer sequence (13). However, the expression of H19 is retained on the maternal allele. On the paternal allele, the ICR becomes methylated, thereby preventing H19 expression. However, this methylated ICR prevents CTCF from binding and hence allows Igf2 expression from a downstream enhancer (13, 20). To our knowledge, CTCF has not been reported to regulate H19 expression.

The current study was undertaken to investigate the molecular mechanism by which PHB1 may regulate the expression of H19 on liver cell proliferation. Our data demonstrate for the first time that PHB1 negatively regulates H19 and Igf2 levels by acting as a co-repressor with CTCF on the ICR. Depletion of PHB1 in the liver represses CTCF binding activity on the ICR, leading to deregulation of the H19-Igf2 axis, and is associated with increased proliferation. Furthermore, forced expression of H19 promotes liver cancer cell proliferation by negatively regulating Phb1.

Results

Induction of H19 and Igf2 Genes in 3-week-old Phb1 KO Mice—Three-week-old liver-specific Phb1KO mice exhibited ~80% reduction in Phb1 mRNA and ~70% reduction in Phb1 protein levels when compared with age-matched control floxed littermates (Fig. 1, A and B). A 7-fold induction of H19 mRNA level was observed in KO mice livers when compared with control mice (Fig. 1A). Phb1 KO mice also exhibited a 22-fold induction of Igf2 mRNA levels (Fig. 1A) and a 13-fold induction of Igf2 protein levels when compared with floxed controls (Fig. 1B). No change in Ctcf mRNA or CTCF protein levels was observed in these mice (Fig. 1, see original blots in supplemental Fig. 1, A–C). No gender bias was observed with respect to expression of H19, Igf2, or Ctcf in Phb1 KO mice (data not shown).

In Vitro Silencing of Phb1 in AML12 Cells and Human Liver Cancer Cells Induces H19 and Igf2 Expression—Silencing of Phb1 by 70–80% in AML12 cells caused a 2-fold induction of H19 mRNA levels (Fig. 2A) and a 2-fold increase in Igf2 mRNA and protein levels (Fig. 2, A and B) when compared with a negative control siRNA. Ctcf mRNA levels remained unchanged (Fig. 2A), whereas CTCF protein levels decreased by 30% when compared with negative control (Fig. 2B, original blots in supplemental Fig. 2, A–C). Similar findings were observed in human HCC cell lines (HepG2 and Huh7) after PHB1 silencing (supplemental Fig. 5, A and B).

Forced Expression of Ctcf Prevents Phb1 Knockdown-mediated H19 and Igf2 Induction—Ctcf mRNA overexpression in AML12 cells treated with negative control or Phb1 siRNA was 20-fold elevated when compared with empty vector (Fig. 3A). CTCF protein levels increased by 2–3-fold when compared with empty vector (Fig. 3B). Phb1 expression was silenced by 65–70% at both the mRNA and the protein levels (Fig. 3, A and B). Ctcf overexpression did not affect endogenous Phb1 mRNA or Phb1 protein levels in AML12 cells (Fig. 3, A and B, original blots in supplemental Fig. 3, A and B). Ctcf overexpression inhibited basal H19 and Igf2 mRNA levels by 20–30% when compared with empty vector control (Fig. 3C). Silencing of Phb1 enhanced H19 and Igf2 expression in empty vector-treated cells (Fig. 3C). Forced expression of Ctcf in Phb1 knockdown cells suppressed the induction of H19 to levels comparable with Ctcf overexpression alone and partly suppressed Igf2 levels when compared with Phb1 knockdown alone (Fig. 3C).

Silencing Ctcf or Phb1 Alone or in Combination Induces H19 and Igf2 Expression—Ctcf or Phb1 silencing (80–90% knockdown, Fig. 4A) induced H19 and Igf2 mRNA levels by 2-fold when compared with negative control siRNA (Fig. 4B). Co-silencing Ctcf and Phb1 did not have an additive effect on H19 expression.

FIGURE 1. Induction of H19 and Igf2 genes in 3-week-old Phb1 KO mice. A, liver from 3-week-old Phb1 KO mice and age- and gender-matched floxed (FL) controls was subjected to RNA isolation and real-time RT-PCR as described under “Experimental Procedures.” The relative expression of Phb1, H19, and Igf2 in Phb1 KO livers was compared with FL. Results represent mean ± S.E. from four mice, †, p < 0.005, †, p < 0.001 versus FL B, protein extracted from KO or FL livers was immunoblotted with Phb1, IGF2, CTCF, and actin control antibodies. Representative images and densitometric analysis (mean ± S.E.) from 3–5 mice are shown. +, p < 0.001 versus FL.

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CTCF at these sites (Fig. 5B) demonstrated that PHB1 co-localized with sites with CTCF followed by PHB1 antibody (sequential ChIP, (Fig. 5C)).

The immunoprecipitation with CTCF antibody (Fig. 5B) also confirmed by performing EMSA with supershift of a synthetic CTCF-binding site fragment containing the four CTCF-binding sites of the ICR (see “Experimental Procedures”). Nuclear extracts from Ctcf-overexpression AML12 cells exhibited increased binding and supershift with CTCF antibody (Fig. 6A). Phb1 knockdown extracts exhibited lower CTCF binding and supershift when compared with control. Ctcf overexpression partially recovered the binding of CTCF in Phb1 knockdown cells when compared with both negative control and Phb1 silencing alone (Fig. 6B).

**CTCF and PHB1 Interact and Co-localize on the ICR Regulatory Element Controlling H19 and Igf2 Genes in Normal Mouse Liver**—We next examined the co-occupancy of CTCF and PHB1 to ICR in mouse liver to make sure that the findings in AML12 cells were also true in liver. Both ICR region 1 and ICR region 2 from mouse liver chromatin immunoprecipitated with CTCF antibody (Fig. 7A, top panel). The amplification of ICR region 1 was very similar between the four livers tested, whereas there was variability in the extent of inhibition between different livers, (Fig. 7A, bottom panel). These results suggest that PHB1 and CTCF interact with each other and that PHB1 localizes on the ICR by virtue of its interaction with CTCF.

In **In Vitro Silencing of Phb1 in AML12 Cells Inhibits the Binding of CTCF to the ICR That Is Partly Recovered by Ctcf Overexpression**—We performed ChIP analysis to examine whether PHB1 was required for CTCF binding to the ICR. Phb1 siRNA transfection in AML12 cells lowered the binding of CTCF to ICR regions 1 and 2 by 80% when compared with a negative control siRNA (Fig. 6A), suggesting that PHB1 is required to maintain the binding of CTCF to these regions. Ctcf overexpression induced CTCF binding to the ICR (Fig. 6A). When compared with Phb1 silencing alone, AML12 cells co-expressing Ctcf vector and Phb1 siRNA exhibited partial recovery of CTCF binding to ICR region 1 and complete recovery of CTCF binding to region 2 (Fig. 6A). ChIP analysis was further confirmed by performing EMSA with supershift of a synthetic CTCF-binding site fragment containing the four CTCF-binding sites of the ICR (see “Experimental Procedures”). Nuclear extracts from Ctcf-overexpression AML12 cells exhibited increased binding and supershift with CTCF antibody (Fig. 6B). Phb1 knockdown extracts exhibited lower CTCF binding and supershift when compared with control. Ctcf overexpression partially recovered the binding of CTCF in Phb1 knockdown cells when compared with both negative control and Phb1 silencing alone (Fig. 6B).

**CTCF Binding to the ICR Is Reduced in Phb1 KO Mouse Liver**—An in vivo ChIP assay was designed to evaluate the binding of CTCF to ICR in mouse liver chromatin from Phb1 KO and floxed control mice. Our results showed that the immunoprecipitation of the ICR regions by CTCF antibody was suppressed in Phb1 KO mice when compared with floxed controls (Fig. 8, top panel). However, because agarose gel data on CTCF to ICR binding were very qualitative and there appeared to be variability in the extent of inhibition between different livers, we quantified the exact target site occupancy of CTCF to the ICR by real-time PCR. Our results showed that CTCF occupancy was reduced by 60% in Phb1 KO mice when compared with floxed controls (Fig. 8, bottom panel).
Expression of IGF2 and H19 Negatively Correlates with PHB1 and CTCF Expression and ICR Binding in Human HCC—Next, we analyzed human HCC and normal liver tissues for the expression levels of PHB1, CTCF, H19, and IGF2. A significant 40–50% reduction in the mRNA expression levels of PHB1 and CTCF and a 40–50-fold increase in H19 and IGF2 were observed in HCC tissues when compared with normal liver tissues (Fig. 9A). Importantly, ICR target occupancy of CTCF and PHB1 was significantly reduced in HCC when compared with normal liver tissues (Fig. 9B).

Modulation of H19 Gene Influences Phb1-mediated Cellular Proliferation in Liver Cancer Cells—So far our results have demonstrated that the H19 and Igf2 genes are induced during Phb1 depletion. PHB1 controls the H19-Igf2 axis by interacting and co-localizing with CTCF on the ICR regulatory region controlling both genes. Our previously published work showed that Phb1 KO mice develop HCC (12). To examine whether Phb1 modulation could lead to tumorigenesis in part via H19, we examined the effect of silencing or overexpressing Phb1 or H19 on proliferation of the liver cancer cell line, S-adenosylmethionine-deficient (SAMe-D), which is derived from the methionine adenosyltransferase 1a KO (Mat1a KO) mouse model of HCC (21). The expression of Phb1 in SAMe-D cells was lower than AML12 hepatocytes (data not shown). Transfection of Phb1 or H19 siRNA in SAMe-D cells caused a 90% knockdown of Phb1 and H19 mRNA levels, respectively (Fig. 10A, left panel). Silencing Phb1 induced endogenous H19 mRNA levels, and H19 silencing induced both endogenous Phb1 mRNA and endogenous PHB1 protein levels (Fig. 10A, left and right panels), indicating a reciprocal regulation between PHB1 and H19.

We further examined whether the cross-regulation between PHB1 and H19 could alter cell growth. SAMe-D cells were transfected with Phb1, H19, or both siRNAs. Silencing Phb1 induced the growth of SAMe-D cells by 1.6-fold when compared with a negative control (Fig. 10B). Silencing H19 caused a 25% decrease in cell growth when compared with negative control (Fig. 10B). Co-silencing of H19 with Phb1 inhibited the inductive effect of Phb1 silencing on SAMe-D proliferation.
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FIGURE 4. Silencing Ctcf or Phb1 alone or in combination induces H19 and Igf2 expression. AML12 cells were transfected with Phb1 siRNA, Ctcf siRNA, or both as described under "Experimental Procedures." Negative control siRNA was used as a transfection control. A, the efficiency of silencing was measured by real-time RT-PCR for Phb1 and Ctcf mRNA. B, the effect of silencing on H19 and Igf2 mRNA expression was assessed by real-time RT-PCR. Results represent mean ± S.E. of 3 experiments. *, p < 0.05, †, p < 0.01, †, p < 0.001 versus negative control siRNA.

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(Fig. 10B). The silencing data on cell proliferation were confirmed by overexpressing Phb1, H19, or both in SAMe-D cells. Phb1 or H19 overexpression in SAMe-D cells induced these genes by 34- and 16-fold, respectively, when compared with empty vector (relative Phb1 mRNA expression: empty vector = 1 ± 0.27, Phb1 vector = *34.35 ± 5.9; relative H19 mRNA expression: empty vector = 1 ± 0.39, H19 vector = †16.25 ± 3.76, *, p < 0.05, †, p < 0.001 versus empty vector). Overexpressing Phb1 suppressed endogenous H19 mRNA levels when compared with empty vector (relative H19 mRNA expression: empty vector = 1 ± 0.39, Phb1 vector = †0.68 ± 0.02, †, p < 0.001 versus empty vector), and H19 overexpression suppressed endogenous Phb1 mRNA levels (relative H19 mRNA expression: empty vector = 1 ± 0.27, Phb1 vector = *0.51 ± 0.16, *, p < 0.05 versus empty vector). Phb1 overexpression inhibited SAMe-D growth by 30% when compared with an empty vector control (Fig. 10C). H19 overexpression caused a 1.6-fold induction in cell growth when compared with empty vector (Fig. 10C). Forced co-expression of H19 with Phb1 reversed the suppressive effect of PHB1 on growth (Fig. 10C), thereby confirming a reciprocal regulation between Phb1 and H19 in SAMe-D cells. The results suggest that the suppressive effect of Phb1 on cell growth may be mediated at least in part by its inhibition of H19 expression, and because silencing H19 induced Phb1 (Fig. 10A), this could also inhibit cell growth (Fig. 9B). Low expression of H19 is associated with high PHB1 level that suppresses cell growth, whereas high expression of H19 is associated with lower PHB1 level that enhances cell growth. These results strongly support the role of H19 gene deregulation in preventing the tumor suppressor activity of Phb1 in liver cancer cells.

Discussion

The tumor modulatory activity of PHB1 is cell type-specific and likely depends on its subcellular localization and post-translational modifications. A strong correlation exists between Phb1 deficiency and conditions leading to rapid liver growth such as following partial hepatectomy, or increased propensity to HCC such as nonalcoholic steatohepatitis and ethanol abuse (11, 22). Despite these findings, the mechanism of action of PHB1 as a cell proliferation control point in the liver is not clearly established. Although liver-specific Phb1 KO mice develop HCC spontaneously, this may have occurred due to the severe liver injury and regenerative response (12). Nevertheless, we observed an inverse relationship between PHB1 expression and growth in the nonmalignant AML12 hepatocyte cell line, suggesting that PHB1 plays a direct growth modulatory role (12). Part of this mechanism may be related to the suppressive effect of PHB1 on E2F trans-activating activity (9). Consistent with this, E2F binding to the cyclin D1 promoter and cyclin D1 expression were higher in liver-specific Phb1 KO livers (12).

However, two tumor growth-associated genes, H19 and Igf2, are also induced in Phb1 KO livers of young 3-week-old mice in both genders (12), which prompted us to hypothesize that Phb1 is involved in regulating the H19-Igf2 axis in normal liver and that this may be another important mechanism controlling liver cell proliferation. In the course of this work, we uncovered a previously unrecognized role of PHB1 in modulating the activity of CTTF as well as the reciprocal regulation between PHB1 and H19.

We probed the factors that were known to regulate the H19-Igf2 gene cluster. One such critical factor, CTTF, negatively regulates the ICR by direct binding, and this CTTF-ICR interaction blocks Igf2 expression on the maternal allele (23). On the other hand, the paternal allele is methylated, and thus CTTF-ICR interaction is prevented, thereby allowing Igf2 to be expressed. H19 expression is regulated by methylation of the ICR (13, 24). Methylation on the paternal allele blocks H19 expression. Hence it is only maternally expressed (13, 24). Deletion of the ICR results in biallelic expression of both Igf2 and H19 (20). A direct effect of CTTF on H19 has not been reported so far. Because CTTF-ICR interaction is a major player in regulating the H19-Igf2 axis and there was deregulation of this axis upon Phb1 depletion, we hypothesized that the CTTF-ICR interaction may be modulated by PHB1 to control this axis in normal cells. To evaluate this hypothesis, we examined whether Phb1 silencing affected Ctcf expression. Phb1 KO mice did not exhibit any change in Ctcf mRNA or protein levels. However, in vitro silencing of Phb1 in normal AML12 hepatocytes did not affect Ctcf mRNA levels but moderately lowered CTTF protein levels. The difference between the in vitro silencing and in vivo deletion may be due to compensatory effects that prevented down-regulation of CTTF expression in vivo. Nevertheless, Phb1 silencing did not alter Ctcf expression dramatically and hence could not be responsible for the increase in H19 and Igf2 expression observed in vitro and in vivo. However, because it is known that CTTF is a regulator of the ICR that controls the
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To examine how the PHB1-CTCF partnership might affect H19 and Igf2 in the liver, we examined interactions of PHB1 and CTCF with the ICR element and with each other both in vitro and in vivo. Sequential ChIP and EMSA analysis demonstrated that PHB1 co-localized with CTCF on the CTCF-binding regions of the ICR. From co-immunoprecipitation studies, we demonstrated that both CTCF and PHB1 interacted with each other. To examine whether PHB1 was required for CTCF ICR binding activity, we examined CTCF binding to the ICR in Phb1-depleted AML12 cells or in Phb1 KO livers. Our results clearly demonstrate that PHB1 depletion substantially reduces the localization of CTCF on the ICR. These data together pro-

H19-Igf2 axis, we examined whether PHB1 could regulate CTCF-ICR interaction to affect the expression of these genes. Ctf overexpression in AML12 cells caused a 20–30% suppression of basal H19 and Igf2 mRNA levels, whereas Ctf silencing induced H19 and Igf2 by 2-fold. Ctf overexpression suppressed induction of H19 and Igf2 caused by Phb1 silencing, indicating that PHB1 and CTCF cooperated with each other to regulate H19 and Igf2 expression. Ctf or Phb1 depletion in AML12 induced both H19 and Igf2 levels. Co-silencing of Phb1 and Ctf did not have an additive effect on H19 and Igf2, further supporting the notion that PHB1 and CTCF act in concert to regulate the H19-Igf2 axis.

FIGURE 5. CTCF and PHB1 interact and co-localize on the ICR element controlling H19 and Igf2 genes in AML12 cells. A, the position and sequence of the four CTCF-binding sites relative to the H19 promoter (accession ID: AF049091.1). B, the co-localization of CTCF and PHB1 on the ICR region 1 (343-bp amplicon) and region 2 (552-bp amplicon) was assessed by sequential ChIP of chromatin from AML12 cells as described under “Experimental Procedures.” The left panel is a representative agarose gel of the PCR products, and the right panel is the real-time PCR quantification of the target site occupancy of CTCF and PHB1 on the ICR regions. GFP antibody was used as a ChIP control. Results represent mean ± S.E. from 3 experiments in duplicates. C, endogenous PHB1 protein or overexpressed PHB1 (PHB1-DDK) from AML12 extracts was immunoprecipitated (IP) with PHB1 antibody (Ab) and immunoblotted (WB) for CTCF detection (left panel). The same blot was re-probed with PHB1 antibody. Endogenous CTCF or overexpressed CTCF was immunoprecipitated with CTCF antibody and immunoblotted for PHB1 detection. The same blot was re-probed with CTCF antibody (right panel). Actin was used as a loading control. A representative immunoblot from 2 independent experiments is shown.
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In vitro silencing of Phb1 in AML12 cells inhibits the binding of CTCF to the ICR that is partly recovered by Ctf overexpression.

A. AML12 cells were transfected with negative control or Phb1 siRNA in the presence of either EV or Ctf overexpression, and total chromatin was immunoprecipitated with CTCF antibody as described under "Experimental Procedures." The immunoprecipitated DNA was amplified by PCR to detect CTCF binding on ICR regions 1 and 2 (top panel, agarose gel; bottom panel, real-time PCR for target site occupancy quantification). Relative target site occupancy represented as fold over negative control is mean ± S.E. from 3 experiments. * p < 0.05 versus negative control siRNA, † p < 0.001 versus negative control or Phb1 siRNA, †† p < 0.001 versus Phb1 siRNA. A, AML12 cells were transfected with negative control/empty vector, Phb1 siRNA, or Phb1 siRNA/Ctf vector, and EMSA with supershift of nuclear extracts was performed as described under "Experimental Procedures." Lanes 1–4: EMSA with supershift of a biotinylated CTCF-binding site probe with CTCF antibody. Key: p = probe only, EV = empty vector, Neg = Negative control siRNA, siPhb1 = Phb1 siRNA. A representative blot from three experiments is shown.

B. Supershift

Unbound probe

FIGURE 6. In vitro silencing of Phb1 in AML12 cells inhibits the binding of CTCF to the ICR that is partly recovered by Ctf overexpression. A, AML12 cells were transfected with negative control or Phb1 siRNA in the presence of either EV or Ctf overexpression, and total chromatin was immunoprecipitated with CTCF antibody as described under "Experimental Procedures." The immunoprecipitated DNA was amplified by PCR to detect CTCF binding on ICR regions 1 and 2 (top panel, agarose gel; bottom panel, real-time PCR for target site occupancy quantification). Relative target site occupancy represented as fold over negative control is mean ± S.E. from 3 experiments. * p < 0.05 versus negative control siRNA, † p < 0.001 versus negative control or Phb1 siRNA, †† p < 0.001 versus Phb1 siRNA. A, AML12 cells were transfected with negative control/empty vector, Phb1 siRNA, Ctf expression vector, or Phb1 siRNA/Ctf vector, and EMSA with supershift of nuclear extracts was performed as described under "Experimental Procedures." Lanes 1–4: EMSA with supershift of a biotinylated CTCF-binding site probe with CTCF antibody. Key: p = probe only, EV = empty vector, Neg = Negative control siRNA, siPhb1 = Phb1 siRNA. A representative blot from three experiments is shown.

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We have identified a novel mechanism by which PHB1 and CTCF cooperate with each other to control the expression of H19 and Igf2 genes. Loss of this control caused by Phb1 depletion induces the expression of these genes and favors hepatocyte proliferation. Induction of H19, in turn, reverses the growth-suppressive effect of PHB1, in part by reducing PHB1 expression in liver cancer cells.

Experimental Procedures

Materials—All buffers and chemicals were purchased from Sigma and were of molecular biology grade.

Human Liver Tissue Collection—Human liver tissues were collected by following Institutional Review Board (IRB) protocols approved by the Cedars-Sinai Medical Center and University of Southern California Keck School of Medicine. Written informed consent was obtained from each patient. Normal liver tissue was obtained from five patients with metastatic colon or breast carcinoma. Cancerous liver tissue was obtained from 11 patients undergoing surgical resection for primary HCC. The contamination of HCC samples with noncancerous tissue was less than 5% as determined by histopathology. These tissues were immediately frozen in liquid nitrogen for subsequent experiments.
Animals—Three-week-old liver-specific Phb1 KO mice and corresponding floxed control mice have been described previously (12). Livers from these mice were processed for experiments as described below. All animal procedures were performed by established protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Cedars-Sinai Medical Center, which has been American Association of Laboratory Animal Care (AAALAC)-accredited since 1967. Animals were treated humanely, and all procedures were in compliance with the institution’s guidelines for the use of laboratory animals.

Cell Culture and Transfections—The AML12 normal mouse hepatocyte cell line was purchased from American Type Culture Collection (ATCC; CRL2254) and cultured in DMEM-F12 medium containing 10% serum, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone. SAMe-D cells are derived from Mat1a KO mouse and were cultured in DMEM supplemented with 10% FBS and 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mmol/liter L-glutamine. Pre-validated Silencer® Select siRNAs against Phb1, Igf2, and H19 siRNAs were purchased from Thermo Fisher Scientific. The siRNAs were reverse-transfected into cells at a dose of 20 nM in 6-well plates or 96-well plates using the Lipofectamine RNAiMAX™ transfection reagent (Invitrogen) for 24–72 h. Expression plasmids for Phb1 (encoding for PHB1-DDK tagged protein), H19, and CTCF were purchased from OriGene (Rockville, MD) and forward-transfected at a concentration of 0.5 μg/ml in 6-well plates or 96-well plates using the jetPRIME® reagent (Polyplus-transfection, Radnor, PA) according to the manufacturer’s protocol.

RNA Isolation, Reverse Transcription, and Real-time RT-PCR—For cultured cells, DNA-free RNA was isolated using a column-based purification method according to the manufacturer’s protocol (Quick-RNA™ MiniPrep, Zymo Research, Irvine, CA). For mouse tissues, RNA was first extracted from tissue

FIGURE 7. CTCF and PHB1 interact and co-localize on the ICR regulatory element controlling H19 and Igf2 genes in normal mouse liver. A, the co-localization of CTCF and PHB1 on the ICR region 1 (343-bp amplicon) and region 2 (552-bp amplicon) was assessed by sequential ChIP of total liver chromatin as described under “Experimental Procedures.” The PCR products were electrophoresed on a 1% agarose gel (top panel). GFP antibody was used as a ChIP control. The target site occupancy of CTCF and PHB1 on the ICR regions was measured by real-time PCR (bottom panel). Results represent mean ± S.E. from four normal mouse livers. B, endogenous PHB1 protein from total liver extracts was immunoprecipitated (IP) with CTCF antibody (Ab) using normal IgG as a control and immunoblotted (WB) for PHB1 detection. The same blot was re-probed with CTCF antibody. Actin was used as a loading control. Results from four mouse livers are shown.
homogenized using the TRIsolv reagent (Invitrogen), and then phenol-extracted supernatants were loaded onto Quick-RNA MiniPrep (Zymo Research) to obtain DNA-free RNA. One microgram of total RNA from cells or tissues was reverse-transcribed using 100 units of M-MuLV Reverse Transcriptase (Lucigen Corp., Middleton, WI). Quantitative real-time PCR was performed using TaqMan or SYBR Green-based primer/probes. TaqMan probes for Phb1, CTCF, and H19, and control gene GAPDH, were purchased from Applied Biosystems (Foster City, CA). SYBR Green cDNA-specific primers for Igf2 were: forward, 5'-CTTCAGTTGTCTGGACC-3'; reverse, 5'-TGGACAGTATGCTCT-3'. For TaqMan-based real-time PCR, the thermal profile consisted of initial denaturation at 95 °C for 15 min followed by 45 cycles at 95 °C for 15 s and at 60 °C for 1 min. For SYBR Green-based real-time PCR, the thermal profile consisted of initial denaturation at 95 °C for 3 min, PCR of 45 cycles at 95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Melting curve analysis was as follows: 95 °C for 5 min, 65 °C for 1 min, and 97 °C for 30 min. Relative mRNA levels were calculated from the cycle threshold (Ct value) of target genes normalized to that of GAPDH to obtain the Ct. The relative expression was calculated from the Ct of target genes in experimental condition / Ct of target gene under control condition.

Western Blotting and Co-immunoprecipitation Assays—Total cellular protein from AML12 cells, SAMe-D cells, or liver tissue prepared using radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (Sigma) was subjected to SDS-PAGE followed by Western blotting according to standard methods (Amersham Biosciences). Antibodies used for Western blotting were: PHB1 and IGF2 (Abcam, Cambridge, MA), CTCF (Cell Signaling, Danvers, MA), and actin control (Sigma). For co-immunoprecipitation, 500 μg of radioimmunoprecipitation assay extracts of cells or tissues were immunoprecipitated with 2 μg of PHB1 antibody and immunoblotted with CTCF antibody according to previously established protocols (26). Western blots were developed using the chemiluminescence ECL system (Amersham Biosciences). Blots were quantified using the ImageJ densitometry program (National Institutes of Health), and test protein expression was normalized to actin control.

ChIP, Sequential ChIP, and SYBR Green Real-time PCR—The localization of CTCF and PHB1 on the CTCF-binding sites of the ICR was examined by ChIP assay using the EpiTect ChIP OneDay protocol (Qiagen, Valencia, CA). This kit was also used for sequential ChIP to study the co-localization of CTCF and PHB1 on the ICR. Briefly, chromatin immunoprecipitated by CTCF antibody was processed for a second round of immunoprecipitation using PHB1 antibody. The final, purified DNA was detected by PCR analysis. Antibodies used for ChIP were the same as those used in Western blotting. GFP antibody was used as a negative control for ChIP. The ICR region 1 (343 bp) and region 2 (552 bp) upstream of the H19 promoter (accession ID: AF049091.1, Fig. 5A) containing two CTCF-binding sites each were subjected to ChIP-Seq analysis with the following PCR primers: ICR1-forward, 5'-GGACGTCTGCTGCAATCCAGTTGT-3'; ICR1-reverse, 5'-TCAGTTGCAATTCCGTTCAGTTGT-3'; ICR2-forward, 5'-GGCTCATTAGGAAGGCAGTTGT-3'; ICR2-reverse, 5'-CGCGTTGACAGTTGT-3'.
3’; ICR2-reverse, 5’-GTCTGCGAGCAATA-3’. All PCR products were run on 1% agarose gels and stained with SYBR® Green (Thermo Fisher Scientific) for 15–30 min. SYBR® Green-based real-time PCR was performed with the above primers to quantify the target site occupancy of CTCF and PHB1. Real-time PCR Ct values were normalized to that of input DNA according to the formula: target site occupancy = 2^−ΔCt where ΔCt = Ct of immunoprecipitated DNA – Ct of input DNA. In experiments where the relative binding of CTCF was compared between Phb1 silencing and negative control groups, the relative target site occupancy = 2^−ΔΔCt where ΔΔCt = ΔCt of knockdown group − ΔCt of negative control group.

**EMSA with Antibody Supershift Assay**—Nuclear extracts were prepared from AML12 cells according to the NE-PER® nuclear and cytoplasmic extraction protocol (Thermo Fisher Scientific). Extracts were subjected to EMSA following the LightShift® Chemiluminescent EMSA Kit (Thermo Fisher Scientific). Extracts (10 μg) were incubated for 1 h with 1× binding buffer from the kit, 1× PBS, 10% glycerol, 5 mM MgCl₂, 1 mM DTT, 50 ng/μl poly(dI-dC), 0.1% NP-40, and 10 fmol of a 3’-biotin-labeled double-stranded oligonucleotide probe containing CTCF-binding sites: 5’-CCGCGTGCCGCGACGCGCCGCCTGGCGCGGTGGCGCGTG-3’. Following probe binding, supershift of the probe was performed for 1 h using 3 μg of antibodies that were used in the ChIP assay. The samples were electrophoresed on a 5% acrylamide gel and transferred to a Biodyne™ B Nylon Membrane (Thermo Fisher Scientific). Because the available size of Biodyne™ is restricted to 8 by 12 cm, the probe portion of the gel was transferred using a separate membrane but in the same cassette as the rest of the gel (Fig. 6B).

**Cell Proliferation Assays**—To estimate cell proliferation of SAMe-D cells, cells were plated at a density of 1 × 10⁴ cells/well.
of a 96-well plate under knockdown or overexpression conditions as described above. BrDU was added to each well at a dilution of 1:2000 during the last 16 h of knockdown, and its incorporation into DNA (a measure of growth) was measured by absorbance at 450 nm/reference 540 nm using the BrDU Cell Proliferation Assay Kit (Calbiochem).

**Statistical Analysis**—Data are represented as mean ± S.E. from 3–4 experimental groups. Statistical analysis was performed using analysis of variance followed by Student’s t test. Significance was defined as *p < 0.05.

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