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Bach1 Represses Wnt/β-Catenin Signaling and Angiogenesis

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Abstract

Rationale—Wnt/β-catenin signaling has an important role in the angiogenic activity of endothelial cells (ECs). Bach1 is a transcription factor and is expressed in ECs, but whether Bach1 regulates angiogenesis is unknown.

Objective—This study evaluated the role of Bach1 in angiogenesis and Wnt/β-catenin signaling.

Methods and Results—Hind-limb ischemia was surgically induced in Bach1$^{-/-}$ mice and their wild-type littermates and in C57BL/6J mice treated with adenoviruses coding for Bach1 or GFP. Lack of Bach1 expression was associated with significant increases in perfusion and vascular density and in the expression of proangiogenic cytokines in the ischemic hindlimb of mice, with enhancement of the angiogenic activity of ECs (eg, tube formation, migration, and proliferation). Bach1 overexpression impaired angiogenesis in mice with hind-limb ischemia and inhibited Wnt3a-stimulated angiogenic response and the expression of Wnt/β-catenin target genes, such as

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Disclosures

None.

interleukin-8 and vascular endothelial growth factor, in human umbilical vein ECs. Interleukin-8 and vascular endothelial growth factor were responsible for the antiangiogenic response of Bach1. Immunoprecipitation and GST pull-down assessments indicated that Bach1 binds directly to TCF4 and reduces the interaction of β-catenin with TCF4. Bach1 overexpression reduces the interaction between p300/CBP and β-catenin, as well as β-catenin acetylation, and chromatin immunoprecipitation experiments confirmed that Bach1 occupies the TCF4-binding site of the interleukin-8 promoter and recruits histone deacetylase 1 to the interleukin-8 promoter in human umbilical vein ECs.

Conclusions—Bach1 suppresses angiogenesis after ischemic injury and impairs Wnt/β-catenin signaling by disrupting the interaction between β-catenin and TCF4 and by recruiting histone deacetylase 1 to the promoter of TCF4-targeted genes.

Keywords

Bach1 protein; endothelial cells; interleukin-8; ischemia; Wnt signaling pathway

Angiogenesis is a key process involved in vascular development and wound repair, as well as in various pathophysiological situations, such as ischemic circulatory disease and carcinogenesis. The Wnt/β-catenin signaling promotes neovascularization of the retina in patients with diabetic retinopathy and has an important role in angiogenesis[1,2] because it controls the proliferation, migration, and differentiation of vascular cells,[3–5] as well as the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8).[6–9] Canonical Wnt/β-catenin signaling regulates gene transcription by enabling translocation of β-catenin from the cytoplasm into the nucleus. In the nucleus, β-catenin forms a complex with TCF4/LEF-1, which recruits the transcription factors Brg1 and CREB-binding protein to initiate Wnt-targeted gene expression.[10] The binding of β-catenin also displaces transcriptional corepressors, such as C-terminal binding protein,[11] histone deacetylase 1 (HDAC1),[12] and Groucho/TLE[13] from TCF4,[14] and recruits transcriptional coactivators, such as p300/CBP.[15]

BTB and CNC homology 1 (Bach1) is a basic leucine zipper transcription factor[16] that forms a heterodimer with small Maf oncoproteins and binds to Maf recognition elements on the genome, thereby inhibiting the transcription of heme oxygenase-1 (HO-1) and other genes involved in the oxidative stress response.[17] Previous studies have shown that HO-1 expression is elevated in myocardial and smooth muscle cells from Bach1-deficient mice that are resistant to ischemic and proatherosclerotic stresses[18,19]; thus, Bach1 deficiency may protect these mice against oxidative tissue damage.[20–22] Bach1 is also known to inhibit oxidative stress–induced cellular senescence by impeding p53 function in murine embryonic fibroblasts,[23] and some Bach1 target genes are important regulators of cell cycle progression and apoptosis.[24]

We have shown that Bach1 is expressed in human microvascular endothelial cells (HMVECs),[25] but the roles of Bach1 in endothelial function and angiogenesis have not been thoroughly studied, and the molecular targets of Bach1 in the vasculature are mostly unknown. Bach1 may regulate TCF4-targeted genes because the results from a DNA array–based genome-wide analysis of TCF4 chromatin occupancy suggested that TCF4 binding

regions are enriched with Bach1 DNA motifs,[26] but whether Bach1 interacts with the Wnt/β-catenin signaling pathway remains unclear. For the studies described in this report, we manipulated the level of Bach1 expression in cultured ECs and in the limbs of mice with peripheral ischemic injury to determine whether Bach1 has a role in angio-genesis and if so, whether its role is effected through changes in the Wnt/β-catenin signaling.

Methods

All studies were approved by the Ethics Committee of Experimental Research at Fudan University Shanghai Medical College, were consistent with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were performed via standardized protocols. Detailed descriptions of the mice, cells, reagents, and experimental methods are provided in the Online Data Supplement.

Results

Bach1 Suppresses the Angiogenic Response to Peripheral Ischemic Injury

To determine whether Bach1 regulates angiogenesis after ischemic injury, mice heterozygous for a Bach1 deletion mutation were interbred to generate homozygous Bach1-knockout mice (ie, the $Bach1^{-/-}$ group); then, hind-limb ischemia was surgically induced in $Bach1^{-/-}$ mice, in their littermates with wild-type (WT) levels of Bach1 expression (ie, the WT group), and in the C57BL/6J mice. Immediately after hind-limb ischemia induction, the injured limbs of the C57BL/6J mice were injected with adenoviruses coding for GFP (ie, the AdGFP group), for Bach1 (ie, the AdBach1 group), or saline (ie, the NS group; Online Figure IA). Seven and 14 days later, blood flow measurements in the animals' ischemic limbs were significantly greater for $Bach1^{-/-}$ mice than for WT mice and significantly lower for mice in the AdBach1 group than for AdGFP or NS mice (Figure 1A). Bach1 deficiency was also associated with significant increases in capillary density (Figure 1B) and arteriole density (Figure 1C) and in the expression of VEGF (Figure 1D) and a murine functional homologue of IL-8, keratinocyte-derived chemokine (KC) (Figure 1E), whereas the corresponding measurements were significantly lower in AdBach1 mice than in AdGFP mice. Expression of VEGF and KC in the animal's uninjured, contralateral limbs was also greater for the $Bach1^{-/-}$ group than for WT mice, whereas measurements in the uninjured limbs of AdBach1, AdGFP, and NS mice were similar (Figure 1D and 1E). Evaluations of GFP fluorescence and of the expression of the endothelial marker CD31 suggested that the vectors coding for Bach1 expression tended to be located in ECs (Online Figure IB), and when rings of aortic tissues were excised from $Bach1^{-/-}$ and WT mice and cultured, significantly more angiogenic sprouting was observed from the aortic rings of $Bach1^{-/-}$ mice (Online Figure IC).

Bach1 Reduces the Angiogenic Activity of ECs

Bach1 expression was observed in both the nucleus and cytoplasm of human umbilical vein ECs (HUVECs; Online Figure IIA, right) and HMVECs (data not shown) and was upregulated when the ECs were cultured under hypoxic conditions (Online Figure IIB). The in vivo relevance of these observations was shown in WT C57BL/6J mice that exhibited a

7.6-fold increase in Bach1 expression in ischemic hindlimb muscles compared with nonischemic muscles (Figure 1A, right). Furthermore, when the in vivo matrigel plug assay (Online Figure IIIA) was performed with HMVECs that had been transfected with AdBach1 or AdGFP and with HMVECs that had been transfected with Bach1 siRNA (Bach1siRNA) or a control siRNA (ConsiRNA), the higher levels of Bach1 expression were associated with declines in capillary density (Online Figure IIIB) and hemoglobin content (Online Figure IIIC). Thus, we conducted a series of experiments to determine whether the effect of Bach1 on angiogenesis and perfusion in mice with hind-limb ischemia can be attributed, at least in part, to changes in the angiogenic activity of ECs.

Assessments of tube formation, cell migration, and proliferation were significantly greater in lung ECs from Bach1$^{-/-}$ mice than in WT lung ECs (Figure 2A), in Bach1siRNA-HUVECs than in ConsiRNA-HUVECs (Figure 2B; Online Figure IVD and IVE), and in AdGFP-HUVECs than in AdBach1-HUVECs (Figure 2C). Lower levels of Bach1 expression were also associated with significant increases in IL-8, KC, and VEGF expression (Figure 2D), whereas the effect of Bach1 silencing on tube formation was impaired by the inactivation of IL-8 or VEGF (Figure 2E). Bach1 overexpression led to an increase in apoptosis after 48 hours of culture (Online Figure IVB), but proliferation measurements in cultured AdBach1- and AdGFP-HUVECs were similar for (at least) the first 24 hours (Online Figure IVC), which suggests that the effect of Bach1 overexpression on endothelial-cell migration and tube formation (evaluated after 8–12 hours of culture) cannot be explained by declines in cell survival.

Bach1 Suppresses Wnt/β-Catenin Signaling in ECs

Endothelial tube formation, cell migration, and proliferation increased in Bach1siRNA-, ConsiRNA-, AdBach1-, and AdGFP-transfected HUVECs when the cells were cultured with Wnt3a, which is known to stimulate the angiogenic activity of ECs (Figure 2B and 2C). However, the effect of Wnt3a stimulation was abolished by higher levels of Bach1 expression (Figures 2C; Online Figure IVA). Moreover, knockdown of Bach1 expression in HUVECs promoted Wnt3a-stimulated angiogenic response (Figure 2B).

Transcriptome analyses of AdBach1- and AdGFP-HMVECs indicated that Bach1 overexpression reduced the transcription of IL-8 more than the transcription of any other angiogenic cytokine tested (Online Table II). IL-8 is known as Wnt/β-catenin downstream target gene. Canonical Wnt signaling prevents the destruction of β-catenin, which subsequently accumulates in the cytoplasm and translocates into the nucleus, where it functions as a coactivator of TCF/LEF-dependent transcription.[14] The promoter region of IL-8 contains a consensus TCF/LEF binding site from positions −186 to −177. Thus, we generated a series of luciferase reporter constructs containing the IL-8 promoter and then investigated the effect of Bach1 overexpression on the luciferase activity of these constructs. In HEK293T cells, Bach1 overexpression significantly reduced the luciferase activity of all promoter/reporter constructs that contained the TCF/LEF binding site (ie, −1200, −500, and −193) but did not reduce the luciferase activity of the −173 truncation, which lacked the TCF/LEF binding site (Figure 3A); Bach1 overexpression also failed to reduce the luciferase activity of the −193 truncation when the TCF/LEF site was mutated (Figure 3B). When Wnt

signaling was stimulated by culturing the cells in Wnt3a (Figure 3C) or by cotransfecting the cells with a vector coding for β-catenin (Figure 3D), the luciferase activity of the (unmutated) −193 truncation was significantly lower in Bach1-overexpressing cells than in cells with endogenous levels of Bach1 expression. In HUVECs, the luciferase activity of the IL-8 promoter/reporter construct was lower in Bach1-overexpressing cells than in cells with endogenous levels of Bach1 expression (Figure 3E) and in ConsiRNA-HUVECs than in Bach1siRNA-HUVECs (Figure 3F). Higher levels of Bach1 expression also reduced IL-8 promoter activity when the cells were stimulated with Wnt3a, and the expression of 2 downstream targets of Wnt signaling, MMP3 (Figure 3G) and c-myc (Figure 3H), was lower in AdBach1-HUVECs than in AdGFP-HUVECs and in ConsiRNA-HUVECs than in Bach1siRNA-HUVECs.

The results from IL-8 reporter assays were consistent with TOPflash assessments of Wnt signaling. TOPFlash activity increased significantly when HEK293T cells were stimulated with Wnt3a (Online Figure VA) or cotransfected with the β-catenin vector (Online Figure VB), but measurements were significantly lower in Bach1-overexpressing cells than in cells with endogenous levels of Bach1 expression. In HUVECs, TOPflash activity was significantly lower in Bach1-overexpressing cells than in cells transfected with an empty vector and in ConsiRNA-HUVECs than in Bach1siRNA-HUVECs (Online Figure VC); higher levels of Bach1 expression also reduced TOPflash activity when HUVECs were stimulated with Wnt3a (Online Figure VD and VE). Collectively, the results from IL-8 promoter/reporter and TOPflash assays confirm that both endogenous and upregulated levels of Bach1 expression impede Wnt/β-catenin signaling.

Bach1 Interacts With TCF4

The results from chromatin immunoprecipitation assays indicated that Bach1 occupies the region from position −193 to −1 of the IL-8 promoter in HUVECs (Online Figure VI), which also contains the consensus TCF/LEF binding site. Thus, we conducted a series of coimmunoprecipitation and GST-pulldown assays to determine whether Bach1 interacts directly with TC4. TCF4 coprecipitated with Bach1 from the lysate of HUVECs under physiological conditions (Figure 4A), and exogenous TCF4 and Bach1 coprecipitated from the lysate of HEK293T cells that expressed Flag-tagged TCF4 (TCF4-Flag) and V5-tagged Bach1 (Bach1-V5; Figure 4B); GST pulldown assays performed with the lysate from HEK293T cells confirmed that TCF4-Flag interacted directly with GST-tagged Bach1 (Bach1-GST; Figure 4C).

Bach1 contains a BTB/POZ domain, which has been shown to have a role in transcriptional repression, as well as a bZip domain.[16] Thus, we determined which specific regions of Bach1 interacted with TCF4 by performing immunoprecipitation analyses in HEK293T cells that had been transfected with 2 vectors, one coding for TCF4-HA, and the other coding for Flag-tagged versions of the full Bach1 sequence or deletion sequences lacking either the BTB domain or the bZip domain. TCF4 coimmunoprecipitated with the full N-terminal Bach1 sequence and with the sequence that lacked the bzip domain but not with the sequence that lacked the BTB domain (Figure 4D). Thus, Bach1 binds directly with TCF4, and this interaction is mediated by the BTB domain in the N-terminal region of Bach1.

Bach1 Disrupts the Binding of β-Catenin to p300/CBP and Reduces β-Catenin Acetylation

Bach1 did not coprecipitate with β-catenin (data not shown), and Bach1 overexpression did not alter the amount of β-catenin located in the nucleus or in the cytoplasm of HEK293T cells (Online Figure VII). However, Bach1 knockdown increased, whereas Bach1 overexpression reduced, the interaction between exogenous TCF4 and β-catenin in coprecipitation (Figure 5A) and GST-pulldown (Figure 5B) experiments with HEK293T cells. Because the affinity of β-catenin for TCF4 increases when β-catenin is acetylated,[27,28] and β-catenin mediates Wnt signaling by recruiting transcriptional cofactors, such as p300 and CBP,[15] both of which can function as acetyltransferases, we investigated whether Bach1 may disrupt the interaction between β-catenin and p300/CBP and if so, whether this disruption inhibits β-catenin acetylation. When HEK293T cells were cotransfected with HA-tagged β-catenin (β-catenin-HA) and Flag-tagged CBP (CBP-Flag) and with Bach1-V5 or an empty vector, both the amount of β-catenin/CBP coprecipitate (Figure 5C) and the amount of acetylated β-catenin (Figure 5D) were less in the Bach1-overexpressing cells. Bach1 overexpression also reduced the amount of β-catenin/p300 coprecipitate in HEK293T cells that had been cotransfected with β-catenin-HA and Flag-tagged p300 (p300-Flag; Figure 5E).

Bach1 Increases HDAC Activity and Recruits HDAC1 to the IL-8 Promoter

The results from our coprecipitation experiments also indicated that Bach1 interacts with HDAC1, as reported previously,[23] in HUVECs (Figure 6A) and in HEK293T cells that had been cotransfected with Bach1-V5 and HA-tagged HDAC1 (HDAC1-HA; Figure 6B). Bach1 also increased HDAC activity in nuclear extracts from HUVECs (Figure 6C) and from HEK293T cells (Figure 6D), and this increase was abolished by culturing the cells with the HDAC inhibitor Trichostatin A or by transfecting the HEK293T cells with HDAC1 siRNA (Figure 6E). Furthermore, Trichostatin A treatment increased the transcriptional activity of the IL-8 promoter in Bach1-overexpressing HUVECs (Figure 6F), whereas chromatin immunoprecipitation as-says indicated that HDAC1's occupancy of the IL-8 promoter was ≈2-fold greater in AdBach1-HUVECs than in AdGFP-HUVECs (Figure 6G, left) and ≈3-fold lower in Bach1siRNA-HUVECs than in ConsiRNA-HUVECs (Figure 6G, right). In contrast, the enrichment of β-catenin, H3, and H4 acetylation at these binding sites was decreased in AdBach1-HUVECs and was increased in Bach1siRNA-HUVECs (Figure 6G). Because HDACs most often function as transcriptional repressors, these observations suggest that HDAC1 may be a key component of the mechanism(s) by which Bach1 impedes gene transcription.

The Activation of Wnt Signaling Disrupts the Binding of Bach1 to TCF4 and the Recruitment of HDAC1 to the IL-8 Promoter

We investigated whether the activation of Wnt signaling disrupts the interaction between Bach1 and TCF4. The activation of Wnt signaling in HUVECs significantly increased IL-8 mRNA levels without altering Bach1 mRNA expression (Online Figure VIII). Both Wnt3a treatment (Figure 7A) and β-catenin-HA transfection (Figure 7B) significantly reduced the amount of Bach1/TCF4 coprecipitate in HEK293T cells that had been cotransfected with Bach1-V5 and TCF4-Flag, and GST-pulldown assays confirmed that β-catenin

overexpression reduced the interaction between Bach1-GST and TCF4-Flag (Figure 7C). Chromatin immunoprecipitation as-says also suggested that Wnt3a treatment reduced Bach1 occupancy and HDAC1 occupancy of the IL-8 promoter, and these declines in occupancy were accompanied by increases in the acetylation of histones 3 and 4 and in the β-catenin in HUVECs (Figure 7D).

Discussion

Bach1 is known to be involved in the oxidative stress response,[20] and chromatin immunoprecipitation–coupled DNA microarray analyses suggest that a reasonably high proportion (≈16%) of binding sites for the Wnt signaling effector TCF4 are flanked by ≥1 Bach1 motifs.[26] However, the experiments described in this report are the first to investigate the role of Bach1 in angiogenesis and Wnt/β-catenin signaling pathway. Our findings demonstrate that Bach1 deficiency or lower levels of Bach1 expression are associated with greater perfusion and vascular density in mice with hind-limb ischemia, with increases in the angiogenic activity (eg, migration, proliferation, tube formation, and proangiogenic cytokine production) of ECs, and with higher levels of Wnt/β-catenin downstream target genes (eg, IL-8 and VEGF) in HUVECs. We also show that Bach1 binds directly to TCF4 and reduces the interaction of β-catenin with TCF4. Bach1 occupies the TCF4-binding site of the IL-8 promoter and recruits HDAC1 to the IL-8 promoter in HUVECs. Bach1 also prevents β-catenin from being acetylated by p300/CBP, whereas β-catenin can promote Wnt signaling by disrupting Bach1-TCF4 binding and HDAC1 recruitment. Collectively, these observations suggest that Bach1 suppresses angiogenesis after ischemic injury and impairs Wnt/β-catenin signaling by recruiting HDAC1 to the promoter of TCF4-targeted genes and by functioning as a competitive inhibitor of β-catenin/TCF4 binding (Online Figure IX).

Bach1 is a crucial component of the mechanism by which the chemokine receptor CXCR3-B limits the proliferation of breast-cancer cells,[29] and deficiencies in Bach1 expression have been associated with increases in the survival of acute myeloid leukemia cells.[30] Both of these observations are consistent with the results from our in vitro studies: lower levels of Bach1 expression were associated with increases in the proliferation of HUVECs. However, Bach1 deficiency is associated with premature senescence in murine embryonic fibroblasts under conditions of oxidative stress,[23] which demonstrates that the effect of Bach1 on cell proliferation/survival can differ profoundly among cell types or experimental conditions. Nevertheless, both the experiments performed with murine embryonic fibroblasts and those presented here identify a link between Bach1's role in transcriptional repression and HDAC1 recruitment. Results from the murine embryonic fibroblast studies suggest that Bach1 blocks p53-programmed senescence by recruiting HDAC1 to the promoter of a subset of p53-targeted genes,[23] whereas our results indicate that Bach1 suppresses IL-8 transcription by, at least in part, recruiting HDAC1 to the IL-8 promoter and increasing HDAC activity. Notably, these observations are the first to suggest that Bach1 regulates HDAC activity although the specific intermolecular interactions or conformational changes involved have yet to be determined. HDACs also mediate the transcriptional repression of Raf kinase inhibitory protein by Bach1 in breast-cancer cells[31]; thus, HDAC1 seems to be a common mediator of Bach1-induced transcriptional repression.

Bach1 upregulation increases the level of reactive oxygen species in keratinocytes,[32] and high levels of reactive oxygen species impair neovascularization in the ischemic limbs of mice.[33,34] We also found that Bach1 overexpression enhanced reactive oxygen species production and induced apoptosis in ischemic mouse hindlimbs (unpublished data). Furthermore, the results from one of our previous investigations suggest that Bach1 participates in arsenite-mediated angiogenesis by repressing the expression of HO-1,[25] which has been shown to promote neovascularization in ischemic hearts by upregulating VEGF.[35] The results presented here indicate that Bach1 overexpression in ECs reduces VEGF mRNA levels and that VEGF blockade abolishes the increased angiogenic activity observed in Bach1siRNA–transfected cells. IL-8 is another key mediator of angiogenesis, and the negative correlation between IL-8 (or KC) and Bach1 expression reported here has been previously observed when human ECs were cultured under hypoxic conditions: cellular levels of IL-8 declined, whereas Bach1 levels increased, in response to the activation of hypoxia-inducible factor-1.[36] IL-8 expression has also been linked to increases in the migration and tube formation of ECs,[37] and the results from our current study confirm that the effect of Bach1 silencing on tube formation in HUVECs can be abolished by blocking the activity of IL-8. However, neither HO-1 upregulation nor HO-1 inhibition alters IL-8 levels in human ECs,[36] and Bach1 overexpression inhibits tube formation in ECs from HO-1 knockout mice (unpublished results). Collectively, these observations suggest that Bach1 inhibits angiogenesis through at least 2 independent signaling pathways, one that is mediated by HO-1 and VEGF and another that involves IL-8, as well as by increasing reactive oxygen species production and apoptosis.

TCF/LEF act as transcriptional repressors by binding to Groucho/TLE proteins in the absence of nuclear β-catenin, whereas β-catenin directly displaces Groucho/TLE repressors from TCF/LEF in Wnt-mediated transcription activation.[14] Our results indicate that β-catenin competes with Bach1 in binding to the promoter of IL-8 gene when Wnt signaling is activated. We propose that Bach1 is an intrinsic factor that inhibits TCF4-mediated transcription when cells are quiescent. When the canonical Wnt/β-catenin signaling pathway is activated, β-catenin accumulates in the nucleus and removes Bach1 from the promoters of target genes to initiate gene transcription (Online Figure IX).

In conclusion, the results presented here are the first to show that Bach1 suppresses angiogenesis after peripheral ischemic injury and that this function is associated with declines in Wnt/β-catenin target genes. Bach1 impairs Wnt/β-catenin signaling by disrupting the interaction between β-catenin and TCF4 and by recruiting HDAC1 to the promoter of TCF4-targeted genes. Thus, Bach1 may be an appropriate target for angiogenic therapy or for the treatment of other disorders that involve aberrant regulation of Wnt/β-catenin pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ECs	endothelial cells
HDAC1	histone deacetylase 1
HMVECs	human microvascular endothelial cells
HO-1	heme oxygenase-1
HUVECs	human umbilical vein endothelial cells
IL-8	interleukin-8
VEGF	vascular endothelial growth factor
WT	wild-type

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Novelty and Significance

What Is Known?

• Wnt/β-catenin signaling promotes angiogenesis and vascular development.

• The transcription factor BTB and CNC homology 1 (Bach1) is known to suppress oxidative stress responses.

What New Information Does This Article Contribute?

• Loss of Bach1 expression in mice enhances angiogenesis in the ischemic hindlimb.

• Interleukin 8 and vascular endothelial growth factor are responsible for the antiangiogenic effect of Bach1 on human endothelial cells.

• Bach1 suppresses Wnt/β-catenin signaling by disrupting the interaction between β-catenin and TCF4 and by recruiting histone deacetylase 1 to the promoter of TCF4-targeted genes.

Bach1 is a transcriptional repressor of antioxidative enzymes, such as heme oxygenase-1. The role of Bach1 in angiogenesis is unclear. In this study, we found that in mice the lack of Bach1 expression was accompanied by accelerated blood flow recovery from hind-limb ischemia, which was associated with a pronounced increase in angiogenesis. This enhanced neovascularization effect was associated with remarkable increase in the expression of a murine functional homologue of IL-8, keratinocyte-derived chemokine (KC), and vascular endothelial growth factor. In addition, the data from this study demonstrate that Bach1 suppresses Wnt/β-catenin signaling in endothelial cells by recruiting histone deacetylase 1 to the promoter of TCF4-targeted genes and by functioning as a competitive inhibitor of β-catenin/TCF4 binding. Our study lends insight into the important roles of Bach1 in vascular cell biology and suggests that Bach1 may be a potential therapeutic target for angiogenic therapy or for the treatment of other disorders that involve aberrant regulation of Wnt/β-catenin pathway.

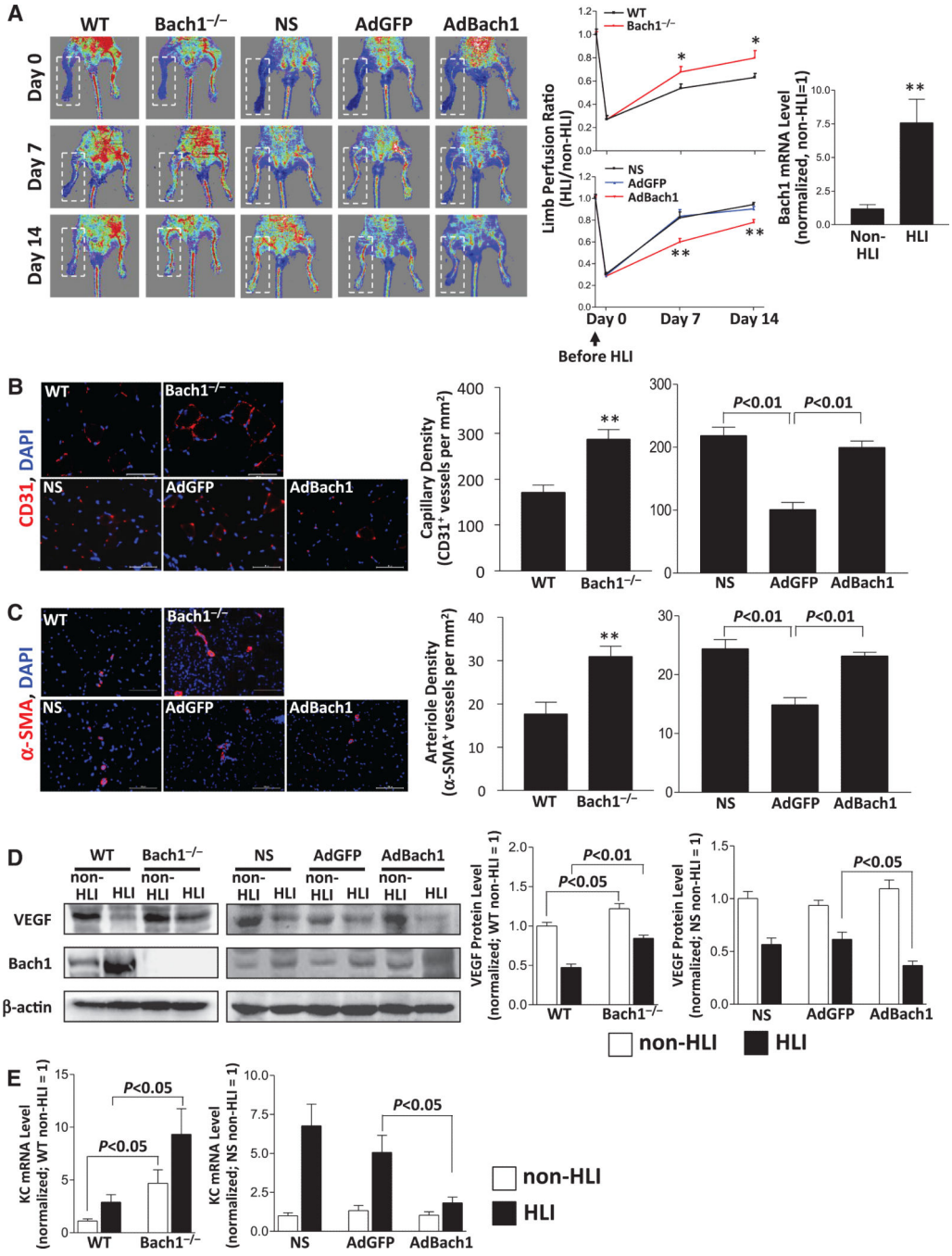

Figure 1. Bach1 impedes angiogenesis after peripheral ischemic injury

Hind-limb ischemia (HLI) was surgically induced in 12-week-old Bach1$^{-/-}$ mice and their wild-type (WT) littermates and in 8-week-old C57BL/6J mice. Immediately after HLI induction, the injured limbs of the C57BL/6J mice were injected with adenoviruses coding for GFP (AdGFP), for Bach1 (AdBach1), or with saline (NS). **A**, On day 0, day 7, and day 14 after HLI, blood flow was evaluated via laser Doppler imaging and quantified for each animal as the ratio of measurements the injured (HLI) and uninjured (non-HLI) limbs (n=5 for Bach1$^{-/-}$ and WT mice, **P*<0.05 vs WT; n=7 for AdGFP, AdBach1, and NS mice,

P<0.01 vs AdGFP; unpaired 2-tailed t test). On day 7, thigh adductor and gastrocnemius muscles were harvested from the limbs of C57BL/6 mice, and then Bach1 mRNA levels were analyzed with real-time polymerase chain reaction (PCR; n=5; **P<0.01 vs Non-HLI; unpaired 2-tailed t test). **B and **C**, Fourteen days after HLI, adductor muscle was harvested from the HLI limb and stained for (**B**) CD31 and (**C**) α-smooth muscle actin (α-SMA) expression. **B**, Capillary density was calculated as the number of $CD31^+$ vessels per mm^2 (n=5 for $Bach1^{-/-}$ and WT mice, **P<0.01 vs WT, unpaired 2-tailed t test; n=6 for AdGFP, AdBach1, and NS mice, P<0.01, 1-way ANOVA; scale bar, 50 μm). **C**, Arteriole density was calculated as the number of α-SMA^+ vessels per mm^2 (n=5 for $Bach1^{-/-}$ and WT mice, **P<0.01 vs WT, unpaired 2-tailed t test; n=6 for AdGFP, AdBach1, and NS mice, P<0.01, 1-way ANOVA; scale bar, 100 μm). **D** and **E**, Seven days after HLI, (**D**) vascular endothelial growth factor (VEGF) protein and (**E**) mRNA levels of the murine functional IL-8 homologue keratinocyte-derived chemokine (KC) were evaluated in HLI and non-HLI limbs via Western blot (protein) and real-time PCR (mRNA; n=5; P<0.05 or P<0.01; 1-way ANOVA).

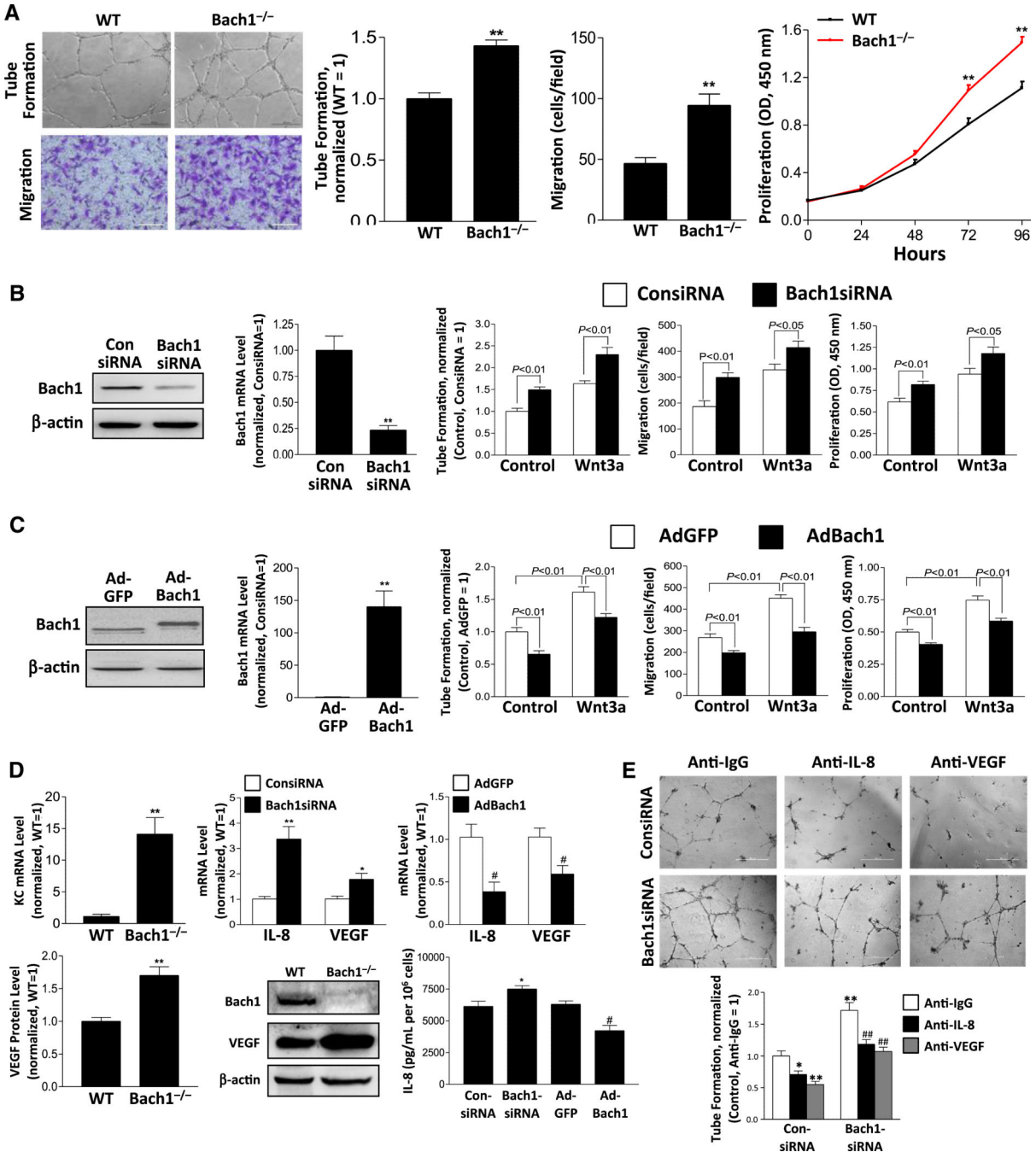

Figure 2. Bach1 reduces the proangiogenic activity of endothelial cells

A–C, Endothelial tube formation in matrigel (measured after 12 hours), cell migration through a membrane (measured after eight hours), and cell proliferation were evaluated in (**A**) mouse lung endothelial cells (MLECs) that had been isolated from Bach1$^{-/-}$ mice and their wild-type (WT) littermates (n=3; ***P*<0.01 vs WT; unpaired 2-tailed *t* test), (**B**) in human umbilical vein endothelial cells (HUVECs) that had been transfected with siRNA against Bach1 (Bach1siRNA) or a control siRNA sequence (ConsiRNA; n=3, ***P*<0.01 vs ConsiRNA, unpaired 2-tailed *t* test; *P*<0.05 or *P*<0.01, 1-way ANOVA), and (**C**) in

HUVECs that had been transfected with adenoviruses coding for GFP (AdGFP) or AdBach1 (n=3; ***P*<0.01 vs AdGFP, unpaired 2-tailed *t* test; *P*<0.01, 1-way ANOVA). For studies with transfected HUVECs (**B** and **C**), Bach1 protein levels were evaluated via Western blot, proliferation was evaluated after 72 hours of culture, and experiments were repeated in the presence of Wnt3a (200 ng/mL). **D**, mRNA or protein levels of interleukin-8 (IL-8), keratinocyte-derived chemokine (KC), and vascular endothelial growth factor (VEGF) were compared in $Bach1^{-/-}$ and WT MLECs (n=3; ***P*<0.01 vs WT; unpaired 2-tailed *t* test), in ConsiRNA- and Bach1siRNA-transfected HUVECs (n=3; **P*<0.05, ***P*<0.01 vs ConsiRNA; unpaired 2-tailed *t* test), and in AdGFP and AdBach1-HUVECs (n=3; #*P*<0.05 vs AdGFP; unpaired 2-tailed *t* test); mRNA levels were evaluated via real-time polymerase chain reaction (RT-PCR) and protein levels were evaluated via Western blot and ELISA. **E**, Endothelial tube formation was evaluated for ConsiRNA- and Bach1siRNA-transfected HUVECs in the presence of an IL-8–neutralizing antibody (Anti–IL-8, 10 μg/mL), a VEGF-neutralizing antibody (Anti-VEGF, 10 μg/mL), or a control anti-IgG antibody (Anti-IgG, 10 μg/mL; n=3; **P*<0.05, ***P*<0.01 vs ConsiRNA+IgG; ##*P*<0.01 vs Bach1siRNA+IgG; 1-way ANOVA; scale bar, 500 μm).

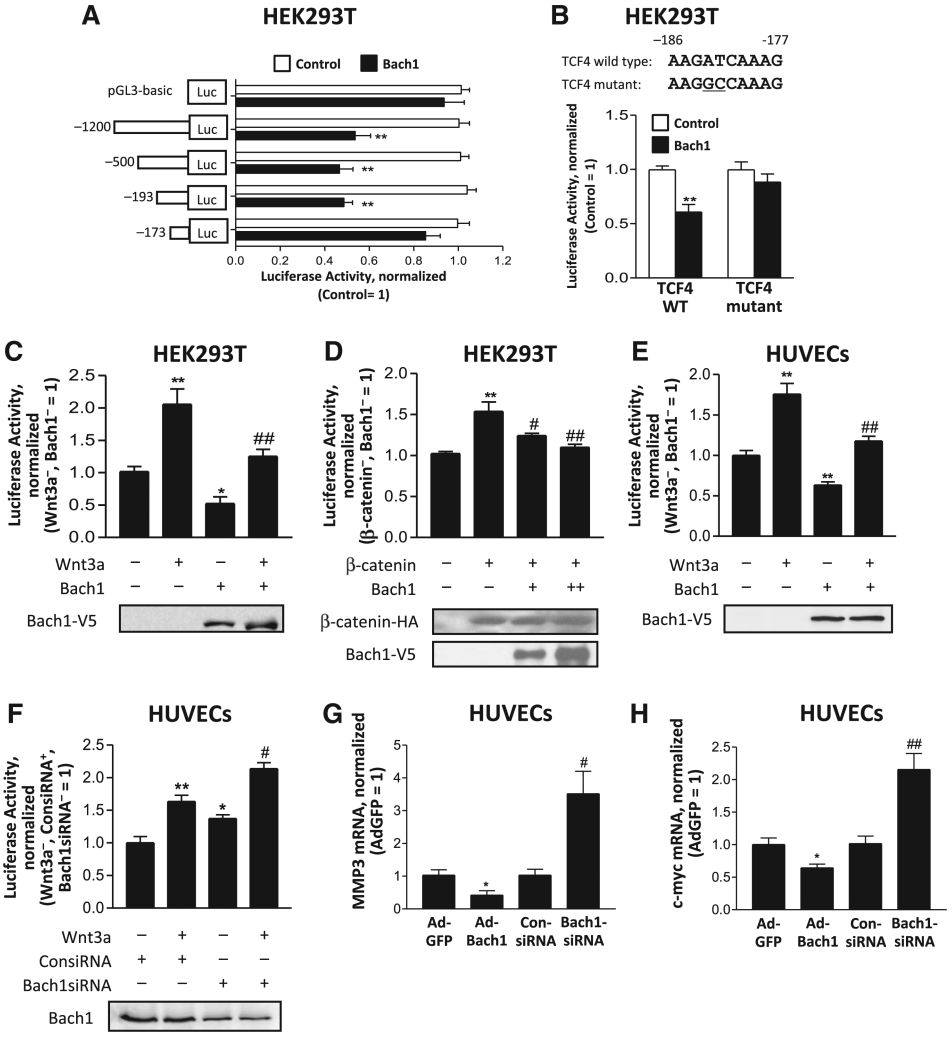

Figure 3. Bach1 suppresses Wnt/β-catenin–induced transcriptional activation of interleukin-8 (IL-8)

A, Luciferase promoter/reporter constructs were created containing the truncated (−1200, −500, −193, and −173) versions of the IL-8 promoter; then, the IL-8 promoter/reporter or a pGL3-basic luciferase reporter and β-gal were cotransfected with a Bach1-coding vector or with an empty vector (control) into HEK293T cells, and luciferase activity was evaluated 36 hours later (n=3; ***P*<0.01 vs control; unpaired 2-tailed *t* test). **B**, Luciferase promoter/reporter constructs containing the wild-type (WT) IL-8(−193) sequence (TCF4 WT) or an IL-8(−193) sequence with a mutation at the TCF/LEF binding site (TCF4 mutant) were cotransfected with a Bach1-coding vector or an empty vector (control) into HEK293T cells, and luciferase activity was evaluated 36 hours later (n=3; ***P*<0.01 vs control; unpaired 2-tailed *t* test). **C** and **D**, Luciferase activity of the IL-8 (−193) promoter/reporter construct was evaluated in (**C**) HEK293T cells that had been transfected with an empty vector or with a Bach1-coding vector for 24 hours and incubated with or without Wnt3a for 12 hours (n=3; **P*<0.05, ***P*<0.01 vs $Wnt3a^{-}/Bach1^{-}$; ##*P*<0.01 vs $Wnt3a^{+}/Bach1^{-}$; 1-way ANOVA), and in (**D**) HEK293T cells that had been transfected with an empty vector, with a vector coding for β-catenin, or with vectors coding for β-catenin and Bach1 (n=3; ***P*<0.01 vs $\beta\text{-catenin}^{-}$/

Bach1^{-}; #*P*<0.05, ##*P*<0.01 vs β-catenin^{+}/Bach1^{-}; 1-way ANOVA). **E** and **F**, Luciferase activity of the IL-8 (−193) promoter–reporter construct was evaluated in (**E**) human umbilical vein endothelial cells (HUVECs) that had been transfected with an empty vector or with a Bach1-coding vector for 24 hours and incubated with or without Wnt3a for 12 hours (n=3; ***P**<0.01 vs Wnt3a^{-}/Bach1^{-}; ##*P*<0.01 vs Wnt3a^{+}/Bach1^{-}; 1-way ANOVA), and in (**F**) HUVECs that had been transfected with Bach1siRNA or ConsiRNA for 24 hours and incubated with or without Wnt3a for 12 hours (n=3; **P*<0.05, ***P*<0.01 vs Wnt3a^{-}/ConsiRNA^{+}/Bach1siRNA^{-}; #*P*<0.05 vs Wnt3a^{+}/ConsiRNA^{+}/Bach1siRNA^{-}; 1-way ANOVA). MMP3 (**G**) and c-myc mRNA (**H**) levels were determined via real-time polymerase chain reaction in HUVECs that had been transfected with adenoviruses coding for GFP (AdGFP), AdBach1, ConsiRNA, or Bach1siRNA (n=3; **P*<0.05 vs AdGFP; #*P*<0.05, ##*P*<0.01 vs ConsiRNA; unpaired 2-tailed *t* test).

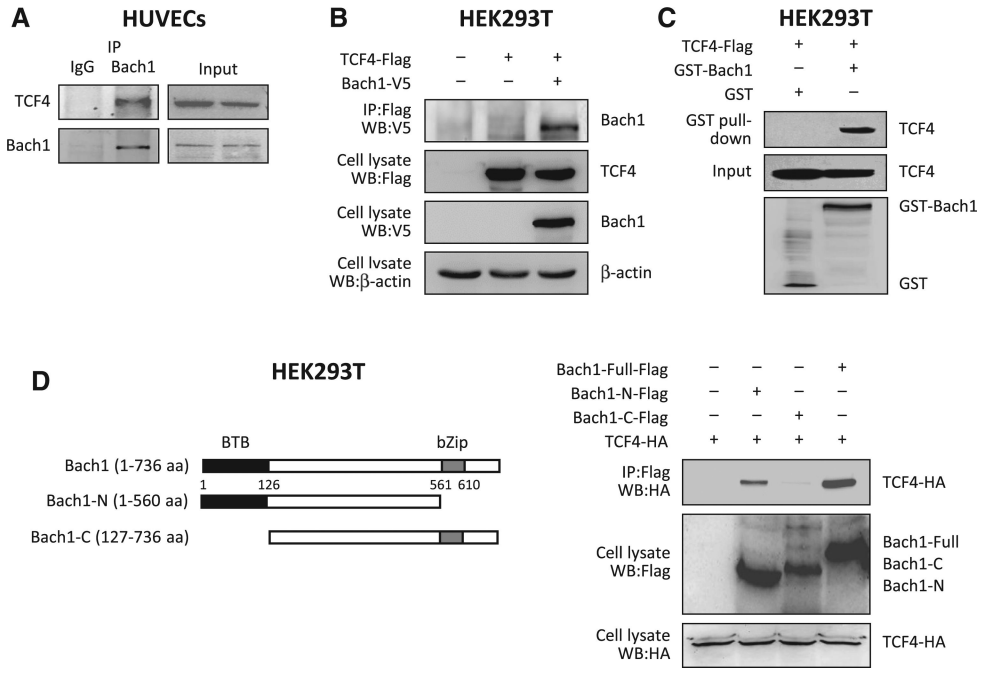

Figure 4. Bach1 interacts directly with TCF4

A, Bach1 was immunoprecipitated from the lysate of human umbilical vein endothelial cells (HUVECs); then, the precipitate was evaluated for the presence of TCF4 via Western blot. **B**, HEK293T cells were cotransfected with V5-tagged Bach1 and an empty vector or Flag-tagged TCF4 and incubated for 48 hours; then, TCF4 was immunoprecipitated with anti-Flag, and the presence of Bach1 in the precipitate was evaluated with an anti-V5 antibody. **C**, HEK293T cells were transfected with TCF4-Flag and lysed; then, the lysate was incubated with GST or GST-tagged Bach1, the GST-bound proteins were eluted, and the presence of TCF4 was evaluated via Western blot with an anti-Flag antibody. **D**, HEK293T cells were transfected with 2 vectors, one coding for TCF4-HA, and the other coding for Flag-tagged versions of the full Bach1 sequence or mutant sequences lacking either the BTB domain or the bZip domain. The Bach1 was immunoprecipitated with an anti-Flag antibody, and TCF4 was detected in the precipitate with an anti-HA antibody.

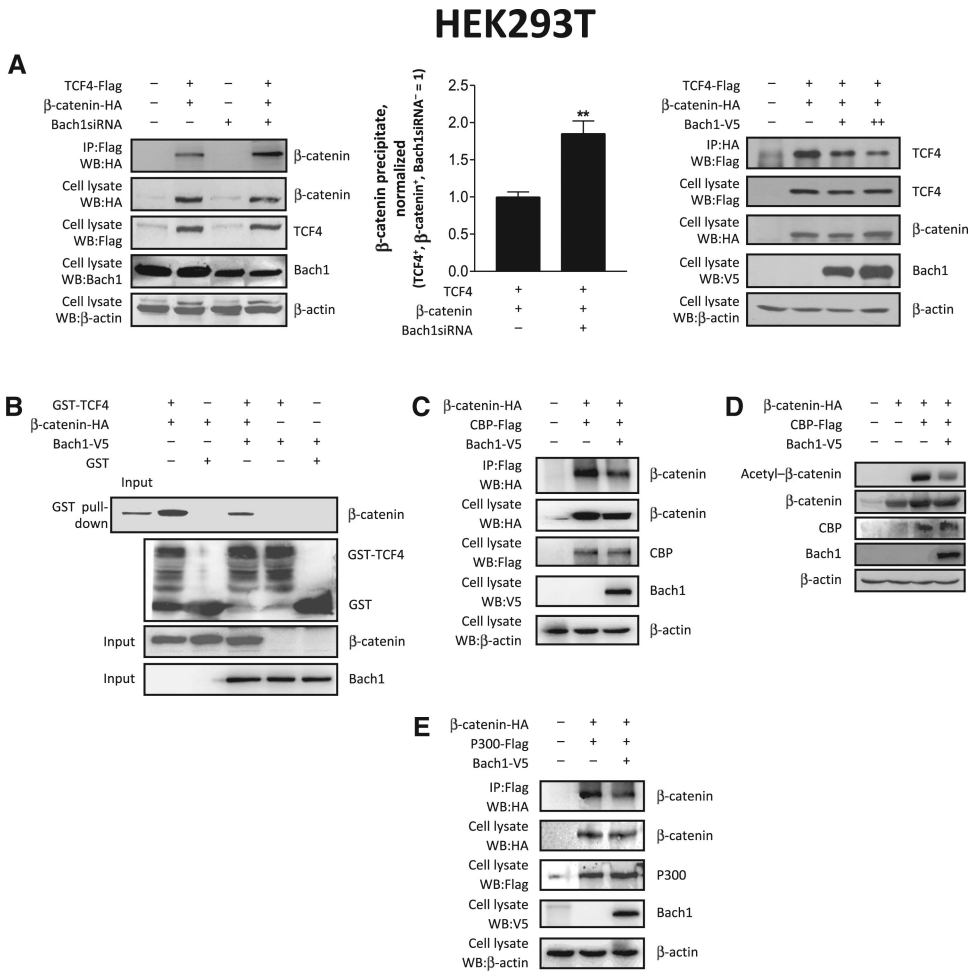

Figure 5. Bach1 disrupts the interaction between β-catenin and p300/CBP

A, left and **middle**, HEK293T cells that expressed β-catenin-HA and TCF4-Flag were transfected with control siRNA or Bach1siRNA and lysed; then, TCF4 was immunoprecipitated with an anti-Flag antibody and β-catenin was detected in the precipitant with an anti-HA antibody (n=3; ***P*<0.01; unpaired 2-tailed *t* test). **A, right**, HEK293T cells that expressed β-catenin-HA and TCF4-Flag were transfected with a control vector or a vector coding for Bach1-V5; then, β-catenin was immunuprecipitated with an anti-HA antibody, and TCF4 was detected in the precipitate with an anti-Flag antibody. **B**, HEK293T cells were transfected with vectors coding for β-catenin-HA or Bach1-V5 and lysed; then, the lysate was incubated with GST or GST-tagged TCF4, the GST-bound proteins were eluted, and the presence of β-catenin was evaluated via Western blot with an anti-HA antibody. **C**, HEK293T cells that expressed β-catenin-HA and CBP-Flag were transfected with an empty vector or a vector coding for Bach1-V5; then, CBP was immunoprecipitated with an anti-Flag antibody, and β-catenin was detected in the precipitant with an anti-HA antibody. **D**, HEK293T cells were transfected for 48 hours with vectors coding for β-catenin-HA, CBP-Flag, and Bach1-V5; then, the cells were lysed, and acetylated β-catenin was detected in the lysate with an antiacetyl-lysine49 β-catenin antibody. **E**, HEK293T cells that expressed β-catenin-HA and p300-Flag were transfected with an empty vector or a

vector coding for Bach1-V5; then, p300 was immunoprecipitated with an anti-Flag antibody, and β-catenin was detected in the precipitant with an anti-HA antibody.

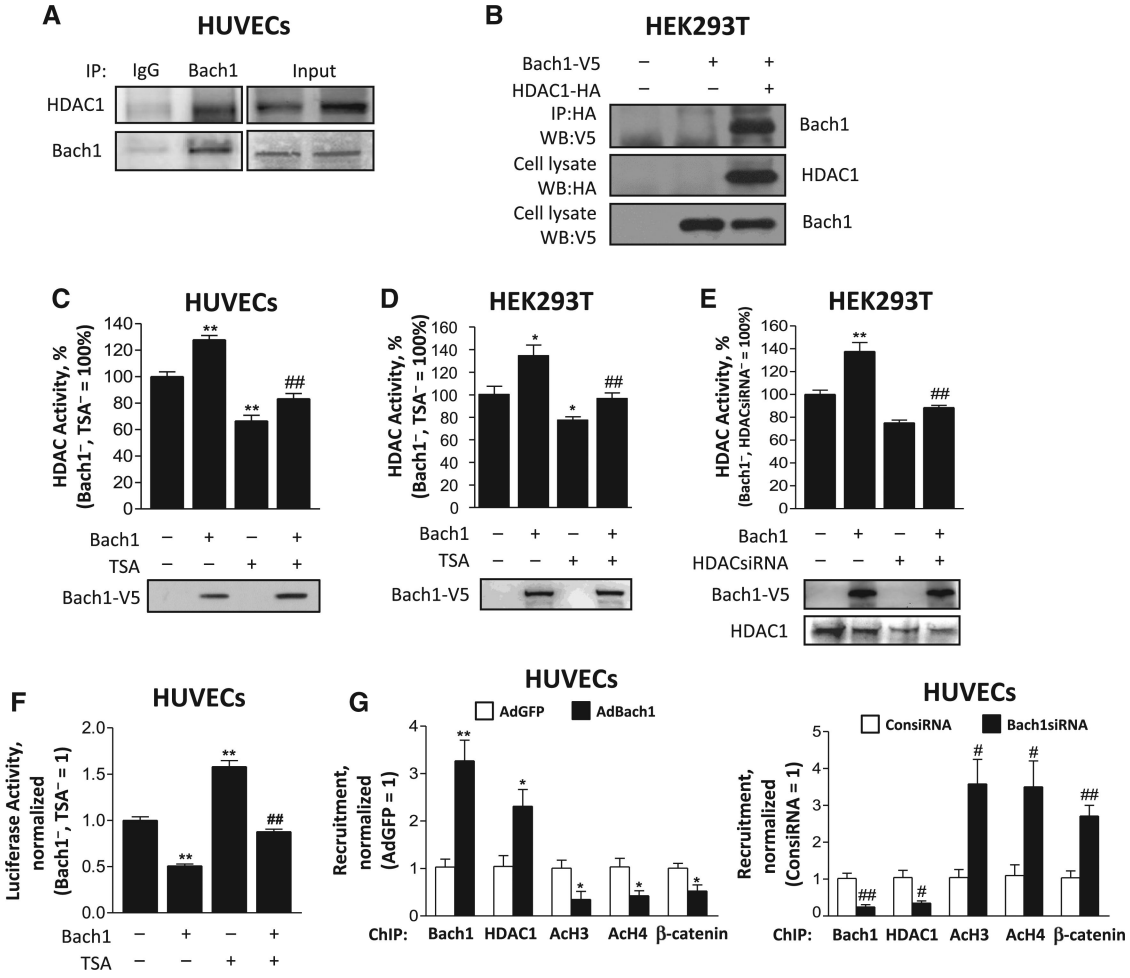

Figure 6. Bach1 recruits histone deacetylase 1 (HDAC1) to the interleukin-8 (IL-8) promoter and increases HDAC activity

A, Bach1 was immunoprecipitated from human umbilical vein endothelial cells (HUVECs), and the HDAC1 level in the precipitate was evaluated via Western blot. **B**, HEK293T cells were cotransfected with an empty vector and vectors coding V5-tagged Bach1 or HA-tagged HDAC1, as indicated, and incubated for 48 hours; then, HDAC1 was immunoprecipitated with an anti-HA antibody, and Bach1 was detected in the precipitate with an anti-V5 antibody. HUVECs (**C**) and HEK293T (**D**) cells were transfected with a Bach1-expressing vector or an empty vector for 24 hours; then, the cells were treated with Trichostatin A (TSA; 1 μmol/L) or DMSO (control) for 24 hours, and nuclear extracts were evaluated for HDAC activity (n=3; **P*<0.05, ***P*<0.01 vs Bach1^{-}/TSA^{-}; ##*P*<0.01 vs Bach1^{+}/TSA^{-}; 1-way ANOVA). **E**, HEK293T cells were transfected with an empty vector, with a Bach1-V5–expressing vector, or HDAC1 siRNA or control siRNA as indicated for 48 hours; then, nuclear extracts were evaluated for HDAC activity (n=3; ***P*<0.01 vs Bach1^{-}/HDAC1siRNA^{-}; ##*P*<0.01 vs Bach1^{+}/HDAC1siRNA^{-}; 1-way ANOVA). **F**, HUVECs were transfected with an empty vector or a Bach1-expressing vector, as indicated, and with the IL-8 promoter/reporter construct; then, the cells were treated with or without TSA (1 μmol/L) for 24 hours and luciferase activity was quantified (n=3; ***P*<0.01 vs Bach1^{-}/TSA^{-}; ##*P*<0.01 vs Bach1^{+}/TSA^{-}; 1-way ANOVA). **G**, HUVECs were transfected with

adenoviruses coding for GFP (AdGFP) or AdBach1 (**left**) or with ConsiRNA or BachsiRNA (**right**); then, chromatin immunoprecipitation was performed with antibodies against the indicated proteins. The amount of TCF4 DNA associated with each protein (relative to the total amount of DNA used) was determined via real-time polymerase chain reaction with a primer for the IL-8(−193) promoter (n=3; **P*<0.05, ***P*<0.01 vs AdGFP; #*P*<0.05, ##*P*<0.01 vs ConsiRNA; unpaired 2-tailed *t* test).

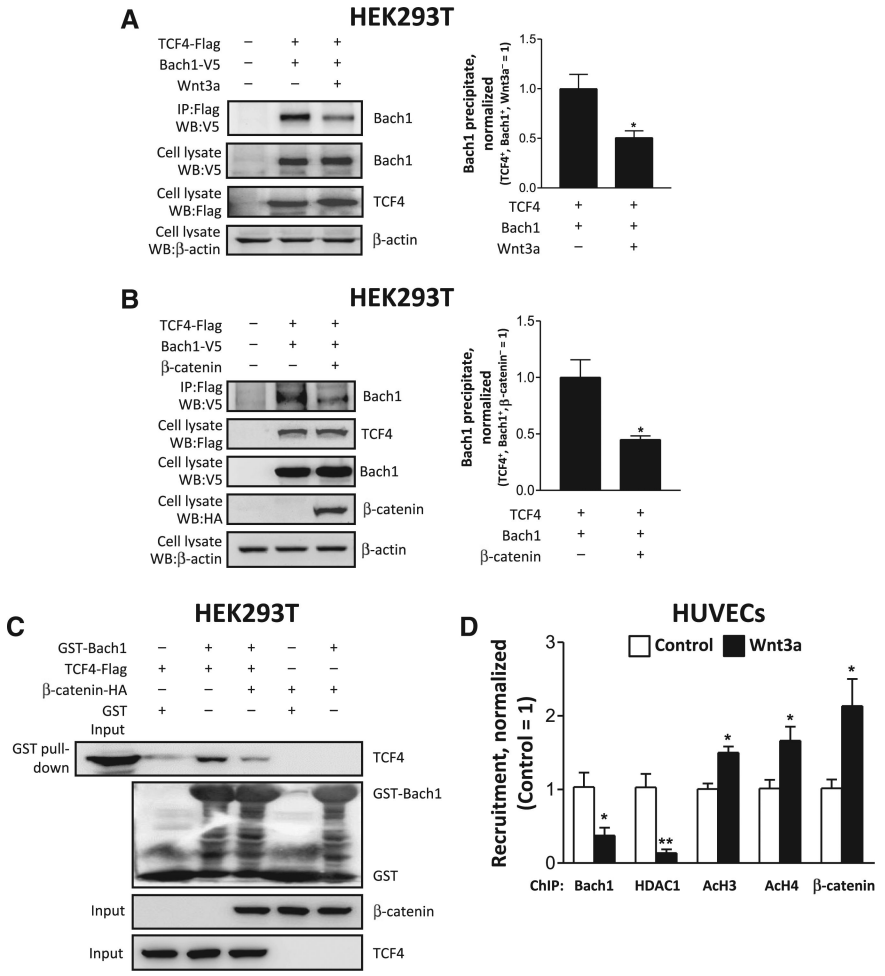

Figure 7. Wnt3a and β-catenin disrupt the Bach1/TCF4 interaction and reduce histone deacetylase 1 (HDAC1) recruitment to the interleukin-8 (IL-8) promoter

A, HEK293T cells were transfected with vectors coding for Bach1-V5 and TCF4-Flag for 24 hours and then incubated with or without Wnt3a for 24 hours; then, the cells were lysed, TCF4 was immunoprecipitated from the lysate with an anti-Flag antibody and Bach1 was detected in the precipitate with an anti-V5 antibody (n=3; **P*<0.05; unpaired 2-tailed *t* test). **B**, HEK293T cells were transfected with vectors coding for Bach1-V5, TCF4-Flag, and β-catenin-HA as indicated; then, TCF4 was immunoprecipitated with an anti-Flag antibody and Bach1 was detected in the precipitate with an anti-V5 antibody (n=3; **P*<0.05; unpaired 2-tailed *t* test). **C**, HEK293T cells were transfected with vectors coding for TCF4-Flag and/or β-catenin-HA as indicated and lysed; then, the lysate was incubated with GST or GST-tagged Bach1, the GST-bound proteins were eluted, and TCF4 was detected with an anti-Flag antibody. **D**, HUVECs were incubated with or without Wnt3a for 24 hours; then, chromatin immunoprecipitation was performed with antibodies against the indicated proteins. The amount of TCF4 DNA associated with each protein (relative to the total amount of DNA used) was determined via real-time polymerase chain reaction with a primer for the IL-8(−193) promoter (n=3; **P*<0.05, ***P*<0.01 vs control; unpaired 2-tailed *t* test).