Absence of Post-translational Aspartyl \( \beta \)-Hydroxylation of Epidermal Growth Factor Domains in Mice Leads to Developmental Defects and an Increased Incidence of Intestinal Neoplasia

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The BAH genomic locus encodes three distinct proteins: junctin, humbug, and BAH. All three proteins share common exons, but differ significantly based upon the use of alternative terminal exons. The biological roles of BAH and humbug and their functional relationship to junctin remain unclear. To evaluate the role of BAH in vivo, the catalytic domain of BAH was specifically targeted such that the coding regions of junctin and humbug remained undisturbed. BAH null mice lack measurable BAH protein in several tissues, lack aspartyl \( \beta \)-hydroxylase activity in liver preparations, and exhibit no hydroxylation of the epidermal growth factor (EGF) domain of clotting Factor X. In addition to reduced fertility in females, BAH null mice display several developmental defects including syndactyly, facial dysmorphology, and a mild defect in hard palate formation. The developmental defects present in BAH null mice are similar to defects observed in knock-outs and hypomorphs of the Notch ligand Serrate-2. In this work, \( \beta \)-hydroxylation of Asp residues in EGF domains is demonstrated for a soluble form of a Notch ligand, human Jagged-1. These results along with recent reports that another post-translational modification of EGF domains in Notch gene family members (glycosylation by Fringe) alters Notch pathway signaling, lends credence to the suggestion that aspartyl \( \beta \)-hydroxylation may represent another post-translational modification of EGF domains that can modulate Notch pathway signaling. Preliminary work has demonstrated increased levels of BAH in certain tumor tissues and a role for BAH in tumorigenesis has been proposed. The role of hydroxylase in tumor formation was tested directly by crossing BAH KO mice with an intestinal tumor model, APC\( \text{min} \) mice. Surprisingly, BAH null/APC\( \text{min} \) mice show a statistically significant increase in both intestinal polyp size and number when compared with BAH wild-type/APC\( \text{min} \) controls. These results suggest that, in contrast to expectations, loss of BAH catalytic activity may promote tumor formation.

Aspartyl \( \beta \)-hydroxylase (BAH)\(^1\) catalyzes the post-translational hydroxylation of aspartic acid or asparagine residues contained within epidermal growth factor (EGF) domains of proteins. The consensus sequence for aspartyl/asparaginyl hydroxylation has been found in a wide range of proteins including clotting factors, Notch receptors, and their ligands, ligands of the tyro-3/Axl family of receptor tyrosine kinases and structural proteins of the extracellular matrix (1–5). In addition, the gene encoding BAH is conserved from Drosophila to man (6). To date, the biological role for this post-translational modification has remained elusive.

Recent studies on the genomic organization of the BAH locus revealed that BAH contains 24 exons and spans over 200 kilobases of genomic DNA (6). These studies yielded the surprising observation that two additional proteins are produced from this locus. One of these proteins, humbug (junctate), shares an identical NH\(_2\)-terminal half of the protein with BAH, but lacks the entire 52-kDa COOH-terminal catalytic domain (6, 7). Humbug encodes a type II membrane protein with a short putative amino-terminal cytoplasmic domain, a transmembrane domain, and a highly charged lumenal domain. The third protein encoded by this locus, junctin, has been previously described and is known to be an integral part of the ryanodine receptor complex (8, 9). Junctin shares a common NH\(_2\)-terminal end with BAH and humbug that corresponds to the cytoplasmic domain, the transmembrane domain and the first 42 amino acids on the endoplasmic reticulum lumenal side of the membrane (6). Alternative splicing leads to the addition of sequence from an alternative COOH-terminal exon that is specific to junctin (6). Alternative splicing of different exons of the BAH locus also gives rise to minor transcriptional forms of these three proteins.\(^2\)

While a great deal is known about the biochemical activity of BAH (10–16), little is known about the biological role of the hydroxylation of EGF domains. Initial work focused on coagulation cascade proteases that are known to contain varying levels of hydroxylation of their EGF domains (17–20). These studies were unable to demonstrate altered activity of the proteases when the level of hydroxylation was varied. Multiple additional roles for hydroxylase have been postulated. One proposed role is that hydroxylase may modify signaling through the Notch pathway by direct hydroxylation of Notch receptors and/or their ligands, all of which contain multiple EGF domains with consensus hydroxylation sequences (13, 21). There has been no direct proof that BAH plays a role in this pathway or even that these proteins are hydroxylated.

Another proposed role for aspartyl \( \beta \)-hydroxylase, and hum-
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bug, was derived directly from the observation that these two proteins are encoded by the same locus as junctin and share significant coding regions (6, 7). Direct proof of interaction of BAH and humbug with the ryanodine receptor has not been shown, but preliminary experiments suggest that overexpression of humbug in in vitro systems can alter Ca$^{2+}$ movement in cell culture systems (7).

BAH overexpression has also been associated with epithelial malignancies. Initial observations suggested that BAH is overexpressed in cholangiocarcinoma and liver cancer (21). These studies measured BAH levels by utilizing both polyclonal antibodies directed against a fusion protein coding for humbug and the monoclonal antibody FB-50, which has now been demonstrated to detect both BAH and humbug (6). Because these antibody reagents recognize both BAH and humbug, it is difficult to tell if one or both of these proteins are up-regulated in these malignancies. Recent studies using NIH 3T3 cells transformed with murine and human BAH suggested a more direct role for BAH in transformation (22).

Because it has been difficult to establish the biological role of BAH by direct biochemical approaches, a genetic approach was taken to assess the role of BAH in vivo. Transgenic mice were generated in which the catalytic activity of BAH was removed by deletion of exons 22 and 23 using homologous recombination. These exons were targeted for removal because they are required for BAH catalytic activity, but are spatially well separated from the coding exons of humbug and junctin. BAH catalytic null animals were evaluated for developmental and reproductive defects. While BAH null animals are viable, they exhibit multiple developmental abnormalities reminiscent of those seen in animals carrying Notch ligand mutations and have altered reproductive capacity. To provide additional insight into a possible role for BAH in tumorigenesis, these mice were crossed with mice carrying the APCmin mutation that leads to multiple intestinal polyp formation. BAH null mice were compared with BAH wild-type animals carrying the APCmin mutation. Analysis of BAH catalytic null animals carrying the APCmin mutation revealed a significant increase in region-specific polyp size and frequency when compared with BAH wild-type animals.

MATERIALS AND METHODS

Producing BAH Knock-out Mice

A region of mouse BAH known to be critical for catalytic activity (14, 15) was targeted for removal by homologous recombination in AB2.2 ES cells. Southern blotting and long range PCR of DNA derived from ES cells were used to confirm correct targeting at this locus (Fig. 1, a and b). Targeted ES cells were injected into donor C57BL/6J blastocysts to produce founder chimeric mice. Founder chimeric mice were mated to produce germ-line transmission of the knock-out.

Production of BAH (−/−) APCmin/−/− and BAH (+/+)

All mice were housed in a fully accredited (by the American Association for the Accreditation of Animal Care International), specific pathogen-free facility at the Bristol-Myers Squibb Pharma Research Labs, Inc. (Wilmingtontine, DE). C57BL/6J/20$^{−}$/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME), stock number 002020, and maintained by crossing APCmin/−/− males with C57BL/6TNc females purchased from Taconic Farms (Germantown, NY), model number B6. APCmin/−/− males were generated by mating APCmin/−/− males with BAH (+/+) or (−/−) females having a mixed strain background (129S6/ScEv × C57BL/6NTac). Male APCmin/−/− males were then crossed with BAH (+/+) females to produce BAH (−/−) APCmin/−/− mice and BAH (+/+). APCmin/−/− mice for study.

Harvesting and Preparation of Intestinal Tissue Samples

Intestines were harvested from 110-day-old BAH (−/−) APCmin/−/−, and BAH (+/+) APCmin/−/− males and female mice after CO₂
euthanasia. For approximately one-third of the BAH (−/−) APCmin/−/− it was necessary to harvest the tissue between days 84 and 108 so as to avoid premature death because of the severity of the disease developing in these animals. Entire intestinal tracts were removed and flushed with phosphate-buffered saline from stomach to cecum, and distally proximal to the cecum. The gut epithelium was dissected out by filling the tract with phosphate-buffered saline-buffered 10% formalin (pH 7.0). The small intestines were divided into three sections as outlined below and polyps were scored along their entire lengths. Proximally, the duodenum included the region 4 cm immediately distal to the stomach. Distally, the ileum encompassed the section 4 cm immediately proximal to the cecum. The middle section (jejunum) connected the duodenum and ileum and varied in length from 10.5 to 30 cm with an average length of 22.1 cm in BAH (+/+) mice and 20.7 cm in BAH (−/−) mice. Polyps were also counted along the entire length of the colon. Each section was opened longitudinally and polyps were counted and measured under a stereomicroscope at ×10–60 magnification.

Polyp Statistics

Tumors from 21 null and 22 wild-type mice were counted and measured by examination at ×30 magnification from four regions of the gastrointestinal tract: duodenum, jejunum, ileum, and the colon. Count data and tumor size data were analyzed using a square root transformation to stabilize the variance.

PCR Genotyping of BAH Catalytic KO and APCmin Mice

APCmin—The three primer pairs used for genotyping APCmin mice were: IMR035 5′ GCCATCCTTCCGTAGT3′, IMR038 5′ TTCCATT- TACTCCCTTGG, and IMR755 5′ TTCTGAAGACAGAACATTA-3′. The PCR conditions used are those recommended by the Induced Mutant Resource of The Jackson Laboratory.

BAH Catalytic Knock-out—To screen for the BAH catalytic exons 22–23 knockout, a forward primer at the 5′ end outside of the resistance cassette (Spe-2, 5′ TCTGTACTAAATTTGCC3′) and a reverse primer inside the resistance cassette (neo805P, 5′ AGGACATCGTTCACCAA-3′). These primers were used (BAH catalytic exon 22 forward primer, Spe-1, was used in combination with a primer within exon 22 (22-2, 5′ AGTAAAGCACAGGTCTAGGCC3′). The wild-type product is ~800 bp long and the disrupted product is ~400 bp long (Fig. 1a). By evaluating both of these PCR reaction products, the KO status of the mice can be determined. Taq DNA polymerase with associated buffer and MgCl₂ from Invitrogen (Gaithersburg, MD) were used for conventional PCR according to manufacturer’s instructions for both the APCmin and BAH wild-type control PCR. For amplification of the BAH catalytic KO region, Taq DNA polymerase was used with Oligitaka G buffer (Qiogene, Carlsbad, CA).

Real-time PCR

Real-time PCR was performed essentially as described (23). Primers and probes were synthesized and purified by Biosearch Technologies, Inc. (Novato, CA). All probes, with the exception of the 18S probe, were modified at the 5′ end with the reporter dye 6-carboxyfluorescein amidohexylamidite, and at the 3′ end with the quencher dye 6-carboxytetramethyl rhodamine (Biosearch Technologies Inc.). The 5′ end of the 18S probe was modified with VIC (Applied Biosystems, Foster City, CA). For AspH exon 5a (junctin), primers ACCATCACAAAGAGAGCTT- GAA, CCCCTTCCTTATCTTCTCG, and probe CCGCTGCTTCGCTTCTTCATTGCTG were used. For AspH exon 14a (humbug), primers GAATTCAGGGTGATGAGAAAGAC, CGAGTGTATAAGAAGAGGCTCAT, and probe CCACGTTTCGCTTGGCTTCCAA were used. For AspH exon 23 (catalytic domain), primers TGACAGCTCTCCAGGACTGTTG, TTGTATTCTTTCAGTCTGTG, and probe CGGAGACACATACTGGGCTG were used. For 18S rRNA, primers CGATACGACTAAAGACGAA, GCAGACATTGCGGCGCCGA, GCTGGAAATTTCCCGA, and probe TCGTGGCAGACAGATTGCGCCT were used. Total RNA was prepared from murine heart tissue using the RNeasy purification system according to the manufacturer’s (Qiagen, Valencia, CA) instructions. cDNA synthesis was performed using the Advantage RT-PCR kit (CLONTECH, Palo Alto, CA) according to the manufacturer’s instructions. Briefly, 1 μg of total RNA was DNase I-treated and reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase. For Taqman-based real-time PCR expression profiling, 25 ng of each cDNA was added to the Taqman Universal PCR Master Mix (PerkinElmer Life Science, Foster City, CA) along with 900 nM of each primer and 200 nM of probe according to the manufacturer’s instructions. Real-time fluorescence monitoring was performed with the PerkinElmer 7700. Standard curves were generated.
for each transcript using a serial dilution of cDNA. Relative abundance was then determined by comparing the cycle threshold values for each reaction with this standard curve. Abundance levels calculated from negative control reactions performed in the absence of reverse transcriptase were then subtracted from experimental sample abundance. Input amounts were corrected to 18 S ribosomal RNA levels. All expression measurements were performed in quadruplicate using two independently generated cDNA samples.

Partial Purification of Aspartyl β-Hydroxylase from Animal Tissue

Mouse liver specimens (~30 mg each) were homogenized on ice with 200 μl of buffer A1 (buffer A: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 9.9% Nonidet P-40, 10 mM dithiothreitol, 1 mM EDTA, 1 mg/ml Pefabloc SC, 1 mg/ml aprotinin and 10 mg/ml leupeptin) in a 2-ml tube using a Tissuemizer (Becton Dickinson) running at ~11,000 rpm for 25 s. All subsequent procedures were performed at 4 °C. Cell lysates were diluted by adding an additional 200 μl of buffer A and were then loaded onto QIAshredder spin columns (Qiagen Inc., Valencia, CA) to shear high molecular weight DNA. After centrifuging in microcentrifuge tubes at 14,000 rpm for 15 min, supernatants were transferred to 1.5-ml Eppendorf tubes. The eluates were adjusted to a volume of ~0.5 ml with buffer A and were re-centrifuged at 14,000 rpm for another 30 min. The protein concentrations of the supernatants were determined by the Lowry method. Three mg of total protein (~150–300 μl) from each supernatant was added to 0.5 ml of 50% SP-Sepharose High Performance protein pre-equilibrated with buffer B (buffer B: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.9% Nonidet P-40, 10 mM dithiothreitol, 1 mg/ml Pefabloc SC, 0.1 mg/ml aprotinin, and 1 mg/ml leupeptin). The total volume including resin was adjusted to 1 ml with buffer B. These mixtures were equilibrated on a roller for 40 min and were then centrifuged briefly at ~1000 rpm for 10–15 s. After 4 successive 1-ml washes in buffer B, bound proteins were eluted with 4 successive washes of 0.5 ml of buffer C (buffer C: 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 5% glycerol, 10 mM dithiothreitol, 1 mg/ml aprotinin, 0.1 mg/ml Pefabloc SC, and 1 mg/ml leupeptin) each. All buffers were stored at 4 °C. Each elution was performed by mixing the resin with buffer C on a roller for 5 min. Eluted proteins were pooled to reach a final volume of 1 ml of tissue sample and were stored at ~70 °C awaiting determination of enzymatic activity. To determine the recovery of aspartyl β-hydroxylase catalytic activity during the purification process, a known amount of human 52-kDa aspartyl β-hydroxylase catalytic domain (14) was added to a control preparation prior to homogenization. Based upon the recovery of activity in this sample the recovery of aspartyl β-hydroxylase activity after purification from mouse tissue samples was determined to be 70–80%.

Enzymatic Assay for Aspartyl β-Hydroxylase

Aspartyl β-hydroxylase activity was assayed as described previously (10–12). The first EGF-like domain of human factor IX with aspartic acid at position 18 was used as the substrate. Incubations were carried out in a final volume of 40 μl in 1.5-ml siliconized Eppendorf tubes at 37 °C for 30 min. Final concentrations of reagents were 50 mM PIPES, pH 7.0, 100 mM ferrous ammonium sulfate, 20 mM a-[1-14C]ketoglutaric acid (1-[1-14C]ketoglutaric acid (54.5 μCi/mmol) (PerkinElmer Life Science), 120 μM EGF substrate, and 0.2 μg/ml bovine serum albumin. A linear response was obtained when between 12 and 24 μl of partially purified aspartyl β-hydroxylase was used.

Purification of Factor X

Mouse blood was collected by direct cardiac puncture into anticoagulant (1/10th of the final volume). The anticoagulant consisted of 84 mg of soybean trypsin inhibitor, and 100 units of heparin in a final volume of 10 ml. Plasma was prepared by centrifugation at 3000 x g of wild-type and knock-out tissue proteins were separated by 4–20% SDS-PAGE (Noveg, Invitrogen, Carlsbad, CA). Proteins were transferred onto polyvinylidene difluoride membranes for 2 h at 50 V with an ice pack cooling unit using 10 μg CAPS-NaOH, pH 11.0, 10% methanol as a transfer buffer. Membranes were blocked in 5% milk, phosphate-buffered saline, pH 7.0, for 2 h. Incubation with primary polyclonal antibody was carried out overnight at 4 °C. Antibodies were raised in rabbits against the catalytic domain of human BAH (14). Specific antibodies were purified using a BAH catalytic domain affinity column (25). Detection was carried out using the ECL-PLUS detection system (Amersham Pharmacia Biotech) according to manufacturer’s instructions.

Expression and Purification of Myc-tagged Soluble Human Jagged-1

Myc-tagged soluble jagged was prepared as described (26). Briefly, a Myc peptide (EQKLISEEDL) coding sequence was attached to the COOH terminus of a human jagged-1 coding sequence that encompassed residues 32–1067 of human jagged-1 (accession U73936). The DNA construct was cloned into a CMV-based expression vector. Myc-tagged soluble jagged protein was expressed using transient transfection. Briefly, 1.25 × 106 HEK 293E cells (Invitrogen, Carlsbad, CA) were electroporated with 25 μg of expression vector. Following transfection, cells were transferred to 80 ml of serum-free medium (293 SFM, Invitrogen, Grand Island, NY) and grown in gas permeable tissue culture bags (TC Tech, Minneapolis, MN). After 96 h (cells density of 1.5 × 106 cells/ml), the conditioned medium was recovered and clarified by centrifugation. Western blot analysis (data not shown) indicated that the yield of sJagged per 80-ml bag was ~500 μg.

Myc-tagged soluble jagged was purified from conditioned medium by anti-Myc affinity chromatography with low pH elution, pH 2.5. Fractions containing Myc-tagged jagged-1 were pooled and purified by reverse phase chromatography using a Vydac C18 column with an acetonitrile gradient from 0 to ~100% containing 0.1% trifluoroacetic acid.

RESULTS

Targeted Disruption of BAH Catalytic Domain Exons Encoding the Active Site—Genomic analysis of the BAH locus reveals a complex structure which encodes at least 3 distinct proteins (BAH, humbug, and junctin) and a series of splice variants that...
can produce different isoforms of the three proteins. Consequently, when designing transgenic knock-out experiments, it is important to remove the activity being tested without altering the transcripts of the other proteins encoded by the BAH locus. To evaluate the in vivo role of aspartyl \( \beta \)-hydroxylase activity, a targeting construct was generated that was designed to specifically delete the coding regions for the active site residues. These residues are contained within exons 22 and 23 located toward the 3' end of the BAH locus. Removal of these exons should not affect the structure or expression of the other two genes, humbug and junctin, encoded by this locus.

Mice with a targeted deletion of exons 22 and 23 of BAH were produced as outlined in Fig. 1a. Targeted ES cell clones were initially identified by Southern analysis through the appearance of a 5.5-kb EcoRI fragment (Fig. 1b). ES cell clones H1, H3, and D2 (not shown) had the correctly sized band for targeting and yielded germ line transmission of the exon 22–23 BAH knockout. Knock-out line H1 was expanded for use in this study in inbred (129/SvEv) and mixed (129/SvEv \( \times \) C57Bl/6) backgrounds.

**Exon 22–23 BAH KO Mice Have No Measurable Aspartyl \( \beta \)-Hydroxylase Protein or Activity**—To assess the level of BAH protein in nulls versus wild-type mice, heart, liver, ovary, brain, and skeletal muscle tissue was harvested from wild-type and BAH catalytic KO mice and analyzed by Western blotting and yielded germ line transmission of the exon 22–23 BAH KO. Partially purified enzyme preparations of liver extracts from each of these mice were tested for their ability to hydroxylate a recombinant EGF domain of Factor IX. Aspartyl \( \beta \)-hydroxylase activity in heterozygous knock-out animals was approximately half of that observed in wild-type mice and BAH null mice exhibited no activity above background (Fig. 2a).

Hydroxylation of a natural substrate in vitro was tested directly by isolation of Factor X from the blood of wild-type and BAH null animals. The quantity of Factor X isolated from the blood of the null animals was not significantly different from the amount purified from wild-type blood. After purification, Factor X was hydrolyzed to amino acids and the relative amount of \( \beta \)-hydroxyaspartic acid (Hya) was determined. The level of Hya detected on Factor X from wild-type mice was 0.53 mol of Hya/mol of Factor X protein. For comparison, the amount of Hya detected for human Factor X is in the range of 0.8–1.0 mol of Hya/mol of Factor X (27). Factor X isolated from BAH null mouse blood contained less than 0.01 mol of Hya/mol of Factor X (Fig. 2c). Taken together, the in vitro and in vivo assessment of hydroxylation indicate that the BAH null mice are devoid of BAH activity. Furthermore, because Factor X isolated from BAH null mouse blood lacks Hya, there is no indication that alternative enzymes exist in mouse liver capable of carrying out this hydroxylation activity. Further studies will be required to extend this observation to other tissues.

**Targeting Exon 22–23 of BAH Does Not Effect the Levels of Expression of Junctin or Humbug**—To determine whether removal of exons 22 and 23 of BAH altered the levels of humbug or junctin mRNA, real-time PCR assays were developed. Primer/probe sets were designed to be specific for humbug (exon 14a), junctin (exon 5a), and BAH (exons 22–23). RNA was isolated from liver, lung, skeletal muscle, and heart of wild-type and null animals and real-time PCR assays were performed. Data is shown in Fig. 3 for heart RNA and similar results were obtained for the other tissues tested. No BAH exon 22–23 RNA was detectable from null animals. In contrast, humbug and junctin RNA levels were similar in both the wild-type and null animals. This demonstrates that targeted removal of exons 22 and 23 of BAH, did not alter abundance of humbug and junctin mRNA.

**BAH KO Mice Have Normal Blood Chemistry, No Major Organ Pathology or Clotting Defect, and Exhibit Normal T-cell Development**—Histopathologic examination of the liver, kidneys, spleen, bladder, stomach, heart, lymph nodes (cervical and mesenteric), salivary glands, pancreas, thymus, brain, skin, ovaries, and uterus from five 12–14-month-old BAH KO mice and two 12–14-month-old wild-type siblings revealed no pathological processes associated with the absence of BAH in the KO mice (data not shown). Fluorescence-activated cell sorter analysis of thymocytes isolated from wild-type and null neonates demonstrated no gross differences in the percentages of alpha-beta and gamma-delta T cells (data not shown). Clotting times (APTT and PT) and a panel of standard blood clinical chemistry values for null and wild-type mice that were within the normal range (data not shown).

**BAH Null Females Exhibit Decreased Fertility**—A previous study demonstrated that BAH is expressed at high levels in mouse ovary tissue (6). To determine whether reproductive capacity was altered in animals null for BAH activity, mating experiments were performed. An examination of 203 viable offspring in the 129/SvEv purebred background that resulted from heterozygous KO matings revealed a normal Mendelian ratio of 1.0:1.9:1.1 (wild-type:heterozygous:null). Fertility of BAH null mice in an inbred background (129/SvEv) was examined (Table 1). Wild-type mating pairs of 129/SvEv produced average litter sizes of 5.7 pups. Matings of BAH null males with wild-type females produced similar size litters (6.3 pups/litter). In contrast, matings of wild-type males or BAH null males with BAH null females produced significantly smaller litter sizes, 3.2 and 3.7, respectively. This is a reduction in litter size of \( \approx \) 40% when the females are null for BAH activity.

**BAH KO Mice Have Shortened Snouts, Mild Palate Defects, and Syndactyly**—Derivation of mice of 129/SvEv purebred background and mixed background mice (129/SvEv:C57Bl/6) are shown in Fig. 5a. In a sample of mice that resulted from
heterozygous matings in the 129/SvEv purebred background, 11 of 12 null mice were easily identified by gross visual examination prior to genotyping. Compared with wild-type mice, BAH KO mice have shortened snouts (Fig. 4a). Likewise, in a sample of offspring from mixed background (129/SvEv:C57Bl6) animals, 9 of 10 null mice could also be identified by gross visual examination of the face. A careful gross visual inspection of purebred null cadavers was undertaken and defects in the palatal ridges were observed in 4 of 4 inbred null mice examined (Fig. 4b). No such defects were observed in the palatal ridges of 4 wild-type inbred mice examined in parallel. Both forelimbs and hindlimbs of null mice exhibited varying degrees of soft tissue fusions on all 4 paws with high penetrance (Fig. 4c). None of the fusions appeared to be of an osseous nature. This suspicion was verified by alizarin red/safranin blue staining of null embryos (data not shown). An examination of BAH null mice in the mixed background revealed fusion of the digits in 61 of 62 mice. Similarly, in the inbred background, 17 of 17 BAH null mice examined were observed to have digital fusions in one or more paws. Interestingly, one heterozygous BAH KO mouse in the inbred background (of several hundred observed) was observed to have webbing of the digits. The severity of the webbing was similar in the inbred and mixed background animals. A summary of these results is shown in Fig. 5b.

Proteins of the Notch Gene Family Are Hydroxylated—The abnormalities observed in the BAH null animals (syndactyly, craniofacial abnormalities) are reminiscent of similar developmental defects seen with altered forms of Notch signaling proteins. Notch gene family members possess multiple EGF domains, many of which contain consensus sequences for \( \beta \)-aspartyl hydroxylation. To date, there has been no report of hydroxylation of EGF domains in this family of proteins. Human Jagged-1 contains 16 EGF domains of which 10 carry the consensus sequence for hydroxylation. To determine whether Notch gene family members are capable of being hydroxylated, a soluble form of human Jagged-1 (a ligand for Notch receptors and a member of the Notch gene family) was expressed in HEK293 cells. Hydroxylation by the endogenous enzyme or

**FIG. 2.** Aspartyl \( \beta \)-hydroxylase activity in wild-type and null mice and hydroxylation of native proteins. \( a \), a \( \text{CO}_2 \) release assay using crude liver extracts was used to determine the level of aspartyl \( \beta \)-hydroxylase activity in BAH wild-type, heterozygous, and null mice. \( b \), Western blotting of tissues isolated from wild-type (+/+ ) and null (−/−) mice demonstrate no detectable full-length BAH protein (110–120 kDa) in null mouse heart, liver, or ovary. \( c \), Factor X Hya levels in wild-type and null mice and Hya levels in soluble Jagged-1 isolated from 293 cells, transiently transfected with soluble jagged-1 expression construct (\( n = 3 \)).

**FIG. 3.** Expression profiling of juncin, humbug, and the BAH catalytic domain mRNAs in wild-type and BAH null littermates. RNA purified from heart tissue isolated from a representative wild-type mouse (black bars; +/+ ) or an AspH catalytic domain null littermate (gray bars; −/−) was profiled using quantitative real time PCR. Error bars indicate the standard deviations of quadruplicate measurements obtained from two independently generated cDNA samples (\( n = 8 \)).

**TABLE 1**

| Litter sizes | +/+ Male × +/+ female | −/+ Male × +/+ female | +/+ Male × −/+ female | −/+ Male × −/+ female | Number of litters | Mean litter size ± S.D. | Statistical significance |
|--------------|------------------------|-----------------------|-----------------------|-----------------------|-------------------|------------------------|-------------------------|
| 10           | 14                     | 13                    | 17                    |                       |                   | 5.7 ± 1.89            | 6.29 ± 1.99             | 3.15 ± 2.03             | 3.7 ± 1.9               | \( p < .05 \) for all comparisons of +/+ versus −/+ females |

Asph Knock-out Mice

4c). None of the fusions appeared to be of an osseous nature. This suspicion was verified by alizarin red/safranin blue staining of null embryos (data not shown). An examination of BAH null mice in the mixed background revealed fusion of the digits in 61 of 62 mice. Similarly, in the inbred background, 17 of 17 BAH null mice examined were observed to have digital fusions in one or more paws. Interestingly, one heterozygous BAH KO mouse in the inbred background (of several hundred observed) was observed to have webbing of the digits. The severity of the webbing was similar in the inbred and mixed background animals. A summary of these results is shown in Fig. 5b.

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with the addition of a BAH expression cassette was examined. Human soluble Jagged-1 was purified from the culture medium and amino acid analysis was carried out. These studies revealed that the endogenous human BAH activity adds 1 mol of -hydroxylated aspartic acid/mol of recombinant Jagged-1. This amount increases approximately 2-fold by the transient transfection of a human BAH expression construct into the HEK293 cells (Fig. 2c).

Tumor Size and Number Are Increased in the Small Intestine of APCmin/BAH Null Animals—Previous studies have shown an increased level of expression of BAH/humbug in human tumors which has led to the proposal that BAH may play a role in initiating or maintaining malignancy. This possibility was tested directly by crossing the BAH KO mice with mice carrying the APCmin mutation which leads to the formation of intestinal polyps. Tumors from 21 BAH null mice and 22 BAH wild-type mice, all carrying the APCmin mutation, were counted and measured from four segments of the intestine, duodenum, ileum, jejunum, and colon. Analysis was done to test for BAH related differences in tumor number and tumor size. A significant increase in tumor number, approximately 2-fold, in the duodenum (mean tumor count 10.4, BAH null versus 4.6, BAH wild-type) and jejunum (mean tumor count 91.1, BAH null versus 45.2 BAH wild-type) in the BAH null animals relative to wild-type was observed (p values < 0.01) (Table II). Total tumor count for the intestines as a whole also showed a significant increase in tumor number (mean tumor count 112.5, BAH null versus 58.7, BAH wild-type). A significant increase in tumor size was observed for ileum and jejunum when BAH null animals were compared with BAH wild-type animals (p values < 0.01) (Table III). These results do not support the hypothesis that BAH activity initiates or maintains the malignant state. In contrast, increased tumor size and number are associated with a loss of BAH activity in the APCmin model of neoplasia. Although all BAH wild-type/APCmin mice lived to the predetermined experimental end point (110 days of age), one-third of all BAH null/APCmin mice became morbidly ill prior to the experimental end point and as a result were sacrificed at an average age of 89 days. Therefore, there is a slight downward bias in the polyp size and number estimates in BAH null/APCmin mice in comparison to their BAH wild-type/APCmin controls.

DISCUSSION

BAH domains critical for catalytic activity have significant sequence conservation going back at least as far as Drosophila on the evolutionary scale (6). BAH specifically hydroxylates Asp and Asn residues contained within a consensus sequence of EGF domains found in many important proteins. Despite significant efforts at the biochemical level, the biological role of BAH catalyzed hydroxylation remains unknown. A genetic approach using transgenic mice was undertaken to examine the biological role directly as well as provide additional tools (e.g. cell lines, tissues, and modified proteins) for examining BAH based hydroxylation of EGF domain proteins.

The BAH locus encodes three distinct proteins, junctin, humbug, and BAH, that arise from different splicing and exon use. To ensure that studies of BAH loss in transgenic mice can be directly attributed to the loss of BAH catalytic activity, it is important to knock-out BAH without disturbing junctin or humbug expression. Transgenic mice were generated in which BAH exons 22 and 23 of this 24 exon gene were removed. Exons 22 and 23 were removed because they encode the regions of BAH critical for catalytic activity and are spatially well separated from junctin and humbug coding regions. The mice that were generated were evaluated both for loss of BAH activity...
and for maintenance of junctin and humbug mRNA abundance. RNA analysis demonstrated that BAH null mice lacked RNA containing exons 22 and 23 as expected, but that RNA levels for junctin and humbug were similar between wild-type and BAH null animals. Loss of BAH protein and activity was evaluated in three ways. First, Western analysis failed to detect any BAH protein from BAH null tissue. Second, using a partial protein purification scheme, no BAH activity was seen in protein fractions purified from BAH null animals. Third, Factor X was purified from the blood of BAH wild-type and null animals and assayed for the presence of Hya. Although approximately half of all Factor X isolated from wild-type mice was found to be hydroxylated, no Hya modification of Factor X was detected in null animals. Taken together, these data demonstrate that hydroxylated was provided by transfection experiments using the gene encoding a truncated, soluble form of recombinant human Jagged-1 and subsequent analysis of the isolated Jagged-1 protein. In mammals, Notch signaling occurs in response to binding of one of two families of conserved Notch ligands, jagged and delta. Signaling through a Notch receptor can be modulated at multiple steps along the signaling pathway (29–32). It is interesting to note that one of the modifiers of Notch signaling, Fringe, has recently been demonstrated to encode a glycosyltransferase that specifically post-translationally modifies specific EGF domains in Notch by adding N-acetylgalactosamine to an O-linked fucose (33). Fringe modification of Notch ligands modulates the response of Notch signaling (31). Specifically, Fringe action potentiates Delta1 mediated signaling through Notch1 and down-regulates Jagged-1 mediated signaling. In those EGF domains in Notch and Notch ligands that contain a consensus sequence for both β-hydroxylation and glycosylation by Fringe, modification of EGF domains by Fringe occurs two amino acids from the site of EGF domain putatively hydroxylated by BAH. Future experiments directed at understanding a role for BAH hydroxylation of EGF domains on Notch signaling and alteration of Fringe glycosylation of EGF domains can be carried out directly using cells derived from the BAH null animals.

In a previous study, mouse ovary was identified as a tissue that expressed high levels of BAH mRNA. Based on this observation, mating studies were carried out with the BAH KO animals. BAH heterozygote matings revealed normal Mendelian inheritance (wild-type:heterozygote:null; 1:0:1.9:1.1) suggesting that the BAH null animals were not compromised during embryogenesis. When female BAH null animals were mated with wild-type or male BAH null animals, there was a significant reduction in litter size when compared with wild-type matings. This reduction was not seen when male BAH null animals were mated with wild-type females, suggesting that the litter size reduction is only sensitive to the genotype of the female animal. Additional studies are needed to understand the role that BAH plays in reproduction.

Previous studies have linked the expression of BAH to malignant transformation. Specifically, BAH and/or humbug has been shown to be expressed at elevated levels, relative to normal tissue, in both hepatocellular and cholangiocarcinoma (21). A subsequent study described experiments where NIH-3T3

| Tumor location | n (number of animals in group) | Mean counts | Standard deviation |
|----------------|-------------------------------|-------------|-------------------|
|                | −/−  | +/+  | −/−  | +/+  | −/−  | +/+  |
| Total          | 21   | 22   | 112.5 | 58.7 | 53.8 a | 56.7 | 35.4 |
| Duodenum       | 21   | 22   | 10.4  | 4.6  | 5.8 a a | 5.2  | 4.9  |
| Jejunum        | 21   | 22   | 91.1  | 45.2 | 45.9 a a | 46.9 | 29.9 |
| Ileum          | 21   | 22   | 9.2   | 7.1  | 2.1   | 5.5  | 6.4  |
| Colon          | 21   | 22   | 1.8   | 1.6  | 0.2   | 2.7  | 1.5  |

* Significant at 0.01 level.

| Tumor                         | Mean     | Standard deviation |
|-------------------------------|----------|-------------------|
| Duodenum                      | 1.71     | 1.62              |
| Ileum                         | 1.47     | 1.10              |
| Colon                         | 1.67     | 2.51              |
| Total                         | 1.05     | 1.02              |
| Difference                    | 0.09     | 0.57              |
| Difference                    | 0.37     | 0.88              |
| Difference                    | 0.19     | 1.67              |
| Difference                    | 0.81     | 0.52              |

* Significant at 0.01 level.
fibroblasts transfected with both human and mouse BAH genes led to a malignant phenotype including transformed foci, growth in soft agar, and tumor development in mice (22). If BAH plays a causal role in malignancy, then tumor development may be blocked or retarded in BAH KO transgenic mice. To evaluate this possibility, the BAH KO mice were bred with mice carrying the APCmin mutation. APCmin mice develop intestinal polyps that can be assessed by direct visual examination of the intestinal tract. If BAH is causally associated with a decrease in overall tumor height as well (34). These observations suggest that the factors that control polypl width and height may be related. These studies evaluate the role of BAH in a single tumor model. Additional animal models can be studied to examine the relationship of BAH activity to the malignant process.

To clarify the biological role of BAH along with the two additional proteins encoded by the same locus, humbug and Asph, models can be studied to examine the relationship of BAH and the two initial glimpse of the role of this protein. Additional studies with these animals as well as similar transgenic approaches for humbug and Asph should prove useful in furthering our understanding of these proteins.

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