The ARID Family Transcription Factor Bright Is Required for both Hematopoietic Stem Cell and B Lineage Development

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Received 27 September 2010/Returned for modification 20 November 2010/Accepted 21 December 2010

Bright/Arid3a has been characterized both as an activator of immunoglobulin heavy-chain transcription and as a proto-oncogene. Although Bright expression is highly B lineage stage restricted in adult mice, its expression in the earliest identifiable hematopoietic stem cell (HSC) population suggests that Bright might have additional functions. We showed that >99% of Bright−/− embryos die at midgestation from failed hematopoiesis. Bright−/− embryonic day 12.5 (E12.5) fetal liver showed an increase in the expression of immature markers. Colony-forming assays indicated that the hematopoietic potential of Bright−/− mice is markedly reduced. Rare survivors of lethality, which were not compensated by the closely related paralogue Bright-derived protein (Bdp)/Arid3b, suffered HSC deficits in their bone marrow as well as B lineage-intrinsic developmental and functional deficiencies in their peripheries. These include a reduction in a natural antibody, B-1 responses to phosphocholine, and selective T-dependent impairment of IgG1 class switching. Our results place Bright/Arid3a on a select list of transcriptional regulators required to program both HSC and lineage-specific differentiation.

The formation and maintenance of blood throughout fetal and adult life rely on the self-renewal of hematopoietic stem cells (HSCs). Rare HSCs arise in the embryonic yolk sac and aorta-gonad mesonephros AGM, seed the fetal liver, and then circulate in the bone marrow of adult mammals. Fetal and adult HSC progenitors become progressively dedicated to differentiation into erythrocytes, myeloid cells, and lymphocytes. Transcription factors critical for the specification and formation of HSCs cover a wide range of DNA binding protein families. An emerging theme is that many of these same regulators are required later for the differentiation of individual blood lineages, which explains why a number of HSC transcription factors were discovered and originally characterized because of their deregulation in hematopoietic malignancies.

Bright/Arid3a/Drill is the founder of the AT-rich interaction domain (ARID) superfamily of DNA binding proteins (18, 60). Bright, in a complex with Bruton’s tyrosine kinase (Btk) and TFII-I, binds to specific AT-rich motifs within the nuclear-matrix attachment regions (MARs) of the immunoglobulin heavy-chain (IgH) intronic enhancer (E₃) and selected IgH promoters to activate IgH transcription (18, 25, 30, 43, 44, 55, 57, 58). B cell-specific, transgenic overexpression of Bright leads to partial blocks at both the late-pre-B and T1 immature stages, skewed marginal-zone (MZ) B cell development, increased natural IgM antibody production, and intrinsic autoimmunity (49). Transgenic dominant negative (DN) inhibition of Bright DNA binding results in reduced levels of IgM in serum and functional perturbation of IgM secretion by B-1 cells (39, 48). A small pool of Bright cycles from the nucleus into plasma membrane lipid rafts, where it associates with Btk to dampen antigen receptor signaling (48).

While highly B lineage restricted in adult mice, Bright is expressed more broadly during embryonic development and is detectable in the earliest lymphoid progenitors (58). Ectopic overexpression of Bright in mouse embryonic fibroblasts (MEFs) overcomes natural or rasV12-mediated senescence to promote cell cycle entry and in vivo transformation (42). Overexpression in the most highly aggressive subset of human diffuse large-B cell lymphomas (AID-DLBCL) has further implicated Bright as a proto-oncogene (38). The structure of the ARID DNA binding domain of Bright was solved (31) as part of the Human Cancer Protein Interaction Network (HCPIN) human cancer biology theme project (21), whose goal is to provide a three-dimensional (3D) structure database of human cancer-associated proteins.

These data forecasted functions for Bright beyond its established role as a regulator of IgH transcription. Through generation and analyses of null mice, we demonstrated that Bright/Arid3a can be added to the select list of DNA binding factors required for both HSC and lineage-specific differentiation.

MATERIALS AND METHODS

Construction and screening of a Bright/Arid3a null allele. Targeting arms devoid of repetitive sequences and as long as possible were identified within noncoding 5’- and 3’-flanking regions of the genomic Bright locus, allowing construction of a null mutation. A cloning strategy was optimized to ensure spurious expression of the positive selectable marker via cryptic promoter sites within the targeting arms or recombined Bright locus. The 5’-targeting arm consisted of a 1.4-kb Smal fragment cloned into pBluescript to introduce a...
multiple-cloning site to aid engineering of the construct. Similarly, the 3’ arm, a 3.4-kb BamHI fragment, was first cloned into pBluescript. These arms were introduced into a targeting vector, pPNT, containing the neomycin resistance (Neo’; positive selection) and diphtheria toxin (DT-A; negative selection) genes. The incompatable ends between the DT-A marker (XhoI) and the pBluescript-generated EcoRI site of the 5’ arm were ligated after the ends were blunted. The 3.4-kb 3’ arm was excised from pBluescript as a XhoI/NotI fragment and inserted into the equivalent sites of pPNT.

SM2-129SV mouse embryonic stem (ES) cells were electroporated with the targeting vector, and clones that survived G418 selection were identified by Southern blotting of genomic DNA. To screen for homologous recombination of the 5’ arm, DNA from each clone was digested with DraI, fractionated by electrophoresis through 0.8% agarose gels, transferred to Nitran membranes (Amersham), and hybridized with a 700-bp PstI genomic fragment 5′ of the 5’ arm. Wild-type (WT) ES cells exhibit a 9.0-kb DraI fragment, while Bright-derived ES cells produce the 9.0-kb (WT) DraI fragment and a 3.5-kb (mutant) fragment. For homologous recombination of the 3′ arm, DNA was digested with BgIII and probed with an internal 0.4-kb AecI fragment. This process resulted in a 5.2-kb WT band, while the insertion of Neo’ during recombination results in a larger 6.4-kb band for Bright cells. Correct gene targeting deletes the 8 Bright exons and introns (22.2 kb), leaving the Neo’ gene.

A correctly targeted clone was injected into embryonic day 3.5 (E3.5) C57BL/6 blastocysts, and the resulting chimeric males were mated to wild-type C57BL/6 females for germ line transmission of the altered allele. Because Bright mice were embryonic lethal at E12.5, the strain was maintained by heterozygous breeding. For the studies reported here, Bright mice were backcrossed with C57BL/6 mice for at least four generations. Genotyping of WT and Bright mice was carried out by PCR. The WT allele was identified by the production of a 200-bp PCR product with the Bright-specific primer pair 5′-TGAGTTCCAAAGCTGTGTC-3′ and 5′-GAAGATCG-3′. The Bright null allele was identified by the production of a 408-bp PCR product with Bright-specific (5′-GGAGTGTCAGGTGCTTGAA-3′) and Neo’ cassette (5′-GATCGAGCCCTTCGTCACTA-3′) primers. The samples were heated to 94°C for 2 min (WT) or 5 min (mutant and neo; KO) and subjected to amplification for 35 cycles of 0.5 min at 94°C, 0.5 min at 58°C (WT) or 62°C (KO), and 0.5 min at 72°C, and after the last cycle, an extension at 72°C for 7 min.

Construction and confirmation of Bdp/Arid1b null mice. A Bright-derived protein (Bdp) gene-trapped 129Sv ES cell line, RRJ028 (BayGenomics), was used to target the Bdp gene. The Bdp coding region was flanked by neo arms, including a DraI site of the 5′/H11032 arm, DNA from each clone was digested with DraI, fractionated by agarose gel electrophoresis, and the relative intensities of the PCR products were quantitated using Umini Analyst 3.0 software (Roche, Indianapolis, IN).

Histological procedures. Mice were euthanized under the guidelines of the IACUC and our institutions. Adult tissues and embryos, collected at the number of days postconception indicated in the figures, were fixed in 4% paraformaldehyde for 24 to 48 h, dehydrated through a series of ethanol solutions, and then embedded in paraffin. Hematoxylin and cosin (H&E) staining, in situ hybridization, immunohistochemistry, and terminal deoxynucleotidyltransferase-mediated DUTP-biotin nick end labeling (TUNEL) were performed as previously described (17, 54). Anti-Bright antibody (affinity purified, rabbit polyclonal; 1:2,000) was previously prepared and used (18). Anti-CD3 antibody (mouse monoclonal; 1:100) was purchased from Santa Cruz Biotechnologies and included as negative control.

Cell separation and flow cytometry. Bone marrow or E12.5 fetal liver cells were enriched for lineage-negative cells by incubation with antibodies (BD Pharmingen) to lineage markers, anti-Gr-1 (Ly-6G, RB6-8CS), and anti-CD11b/Mac-1 (M1/70) for myeloid cells, anti-CD19 (1D3) and anti-CD45R/B220 (RA3-6B2) for B lineage cells, anti-CD8 (53-6.7), CD3 (145-2C11), CD43, CD44 (1C10), CD69 (HL2.F3), APC-CD45R/B220 (RA3-6B2), and PerCP-CD45R/B220 (RA3-6B2). Flow cytometry was performed on a FACSCalibur (BD Biosciences). Data were analyzed with either FlowJo (Tree Star, San Carlos, CA) or CellQuest Pro software (BD Biosciences). Gates set in the lower panel of Fig. 3C are described (32), using the following primer pairs: Iγ1 (forward primer, 5′-CTACAATCTCC-3′), VL/HJ-1 (forward primer, 5′-AGAGTGAGC-3′), Vδ1 (reverse primer, 5′-GATCAGCAGCCTCTGTTCCA-3′), and CD7b (reverse primer, 5′-AAGATCG-3′) for T cells, and anti-Ter-119 for erythroid cells, followed by negative selection using the MACS cell separation system (Miltenyi Biotec, Auburn, CA). The same antibodies without CD11b/Mac-1 were used for lineage depletion of fetal liver. Unfractionated and partially lineage-depleted bone marrow and fetal liver cells were stained with fluorescein isothiocyanate (FITC)-antimouse monoclonal antibodies (FITC-antimouse monoclonal antibodies (MAbs) Gr-1; Mac-1 (adult only), Ter-119, and CD45R as well as anti-stem cell MAb’s phycoerythrin (PE)-Sc-1 (Ly6A/E, E13-161.7) and allophycocyanin (APC)-c-kit (2B8). MAb’s were purchased from BD Pharmingen.

Cell surface phenotypes of lymphocyte populations in adult thymus, spleen (harvested as single-cell suspensions in RPMI medium with 7% fetal calf serum [FCS]), and peritoneal cavity (obtained by lavage with phosphate-buffered saline [PBS]–5% FCS) utilized the additional BD Pharmingen MAb’s: FITC-CD21 (2B8), PE-CD8 (53-6.7), CD13 (145-2C11), CD43, CD44 (1C10), CD69 (HL2.F3), APC-CD45R/B220 (RA3-6B2), and PerCP-CD45R/B220 (RA3-6B2). FITC-IgM, PE-IgD, goat anti-mouse IgM, appropriate isotype controls, and streptavidin-conjugated APC were obtained from BD or Southern-Biotech (Birmingham, AL). Adult splenic bone marrow and thymic subpopulations were identified as described in Shankar et al. (49). Fetal liver and bone marrow hematopoietic populations were defined and measured according to Chen et al. (9).

Cells (~5 × 10^5) were fixed in 0.2% paraformaldehyde overnight and stained as previously described (58). Flow cytometry was performed on a FACS Calibur instrument (Becton Dickinson, Mountain View, CA) or an LSR1 flow cytometer (BD Biosciences, San Jose, CA). Cell-sorting experiments were performed on a FACS Aria cell sorter (Becton Dickinson, Franklin Lakes, NJ). The data were analyzed with either FlowJo (Tree Star, San Carolos, CA) or CellQuest Pro software (BD Biosciences). Gates set in the lower panel of Fig. 3C are specified as low (lo) or high (hi) relative to mean fluorescence intensity.

Adoptive transfer. Bone marrow (~5 × 10^6) cells from Bright’−/− adult mice (4 to 6 weeks old) was injected intravenously into sublethally (500-rad) irradiated C57BL/6 recipients. Four weeks later, bone marrow cells, splenocytes, and thymocytes were harvested from these mice and characterization was confirmed by flow cytometry.

Rag2−/− blastocyst complementation. To obtain Bright’−/− ES lines, blastocysts were flushed out of the horns of 3.5-days-pregnant Bright’−/− females that had been mated with Bright’+/− males (20). Blastocysts were transferred onto STO feeder layers in ES media (Dulbecco’s modified Eagle medium [DMEM] supplemented with 20% fetal bovine serum [FBS], penicillin/streptomycin,
nucleosides, nonessential amino acids, and β-mercaptoethanol) and cultured at 37°C in 5% CO₂ in humidified air for 6 to 7 days without media changes. The inner cell masses were identified, treated with trypsin, disrupted, and then transferred individually and subcultured in 24-well STO feeder plates. Four days later, single-cell clones of compact ES colonies were passaged onto 6-well plates and then split after 2 to 3 generations for confirmation of the Bright null genotype by PCR.

Rag2−/− mice were maintained in a pathogen-free facility, and 4- to 8-week-old females were used as blastocyst donors. Rag2−/− blastocysts were recovered from the uteri of 3.5-days-postcoitum pregnant females and were injected as described previously (8) with 3 Bright−/− and 2 Bright+/+ clones. Injected embryos were then transferred into the uteruses of synchronized pseudopregnant foster mothers. Chimeric offspring were identified by agouti coat color and PCR analysis of tail DNA for the Bright null allele. Reconstituted lymphocytes were verified by fluorescence-activated cell sorter (FACS) analysis of peripheral blood at 4 to 6 weeks.

**Cell culture and in vitro-stimulation assays.** Mouse hematopoietic progenitors from fetal liver were assessed using a methycellulose colony assay. Assays were initiated with 300,000 cells per dish using MethoCult GF M3434 (StemCell Technologies) according to the manufacturer’s directions. Cultures were incubated for a total of 14 days but were checked on day 7 for erythroid burst-forming units (BFU-E) and on day 12 for CFU granulocyte macrophages (CFU-GM) and CFU granulocyte erythocyte monocyte macrophages (CFU-GEMM). Colonies were analyzed visually and were counted using a Nikon TS100 inverted microscope.

Spleenic B cells were T cell depleted using anti-Thy-1 and guinea pig complement. Cells were cultured in suspension by centrifugation through a Ficoll gradient or enriched by exclusion over CD43-coupled magnetic beads (Miltenyi Biotec) as previously described (48, 59). Cells were plated at ~5 × 10⁶ cells/ml in RPMI medium (supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 × 10⁻⁵ M β-mercaptoethanol, and 1 mM sodium pyruvate) alone or with 20 μg/ml LPS (Escherichia coli 0111:B4; Sigma, St. Louis, MO) or anti-IgM (50 μg/ml) or anti-CD40 (20 μg/ml) with interleukin-4 (IL-4; 50 ng/ml). After 72 h, cells and supernatants were harvested for flow cytometry, enzyme-linked immunosorbent assays (ELISAs), or mature and germ line isotype analyses. In some cases, wells were pulsed with 1 μCi of ³H-thymidine for 6 h and harvested, and the incorporation of ³H was measured.

**Immunizations.** Mice (at least 6 weeks old) were immunized intravenously (i.v.) with trinitrophenol (TNP)-keyhole limpet hemocyanin (KLH) (0.5 μg/ml) in Freund’s complete adjuvant (Sigma, St. Louis, MO) as previously described (49). Mice were boosted on day 7 with the same dose of antigen. Sera were collected at days 0, 7, and 14 postimmunization. Mice were also immunized i.v. with ~1 × 10⁸ heat-killed, pepsin-treated Streptococcus pneumoniae (strain RSA32) cells as previously described (6). Seven days later, sera were collected for ELISAs.

**ELISAs.** A Clonotyping system/AP kit (Southern Biotechnology Associates) was used, according to the manufacturer’s directions, to test for serum isotypes and isotypes generated by in vitro stimulations. Standard curves were generated with isotypes of known concentrations, and Ig levels were quantified using Microsoft Excel software. Antigen-specific antibodies were detected using phospho-specific antibodies were detected using phospho-

**RESULTS.**

**The majority of conventional Bright knockout mice die at midgestation.** A null allele of Bright/Arid3a was constructed and confirmed in 129Sv ES cells (Fig. 1A to C). Following germ line transmission into 129Sv or C57BL/6 mice, Bright heterozygotes had no apparent pathology (data not shown). Greater than 99% of Bright−/− mice generated from multiple chimeras were embryonic lethal in both backgrounds. Death occurred between E11.5 and E13.5 (data not shown). Both Bright null embryos still alive at E12.5 and rare adult survivors were significantly smaller than their littermate controls (Fig. 1D).

**Death of Bright−/− embryos results from failed erythropoiesis.** While Bright mRNA is expressed broadly during early (E5.5 to E8.5) embryogenesis (data not shown), Bright protein is most highly expressed in the fetal liver at E12.5 (Fig. 2A). Although there was no evidence of hemorrhage, subcutaneous edema, or pericardial effusion (Fig. 2 A,B), Bright null embryos and yolk sacs were strikingly pale at E12.5 (Fig. 2B). This pallor was not associated with any apparent defect in cardiac function (data not shown) nor with vascular development, as determined by comparable anti-CD31 immunostaining in control and mutant embryos (Fig. 2C). However, cellularity of the E12.5 mutant livers was dramatically reduced (Fig. 2D). We also noted a reduction in thickness of the myocardial compact zone in E12.5 Bright null embryos (Fig. 2A and data not shown). These features suggested a critical and hitherto unexpected function for Bright/ARID3a in hematopoiesis.

**Primitive erythropoiesis and globin switching are normal in Bright knockout embryos.** In order to determine whether the pallor of Bright−/− embryos is due to failed HSC formation or maintenance, we examined embryos at earlier developmental stages. The extraembryonic yolk sac is the initial site of hematopoiesis and produces primitive blood cells that circulate between the yolk sac and embryo by E8.25. Our analysis of E9.5 embryos and yolk sacs confirmed that there were comparable numbers of erythrocytes in control and mutant littermates (Fig. 2A to d). Moreover, Bright−/− erythrocytes did not undergo aberrant apoptosis at E9.5, as evidenced by a lack of TUNEL staining in mutant blood cells (Fig. 2E to g). Therefore, primitive erythrocyte formation and maintenance appear unaffected by the loss of Bright.

Lethality in Bright null embryos occurs during the time interval in which erythropoiesis shifts in location from the yolk sac to the fetal liver. Primitive erythrocytes produced in the yolk sac express both embryonic and adult globin genes, while definitive erythrocytes produced in the liver express only adult globins. We saw comparable levels of embryonic and adult globin expression in mutant and control erythrocytes at E12.5 (Fig. 2F), indicating that globin switching is unaffected by the loss of Bright.

**Bright knockout impairs embryonic HSC differentiation.** Because the Bright−/− fetal livers showed generally decreased cellularity, we reasoned that fetal liver-derived hematopoietic differentiation in Bright null embryos might be compromised. c-kit is a progenitor cell marker expressed on HSCs, BFU-E, and erythroid CFUs (CFU-E). c-kit+ Ter119+ cells mark proerythroblasts and basophilic erythroblasts, whereas Ter119 single-positive cells are fully differentiated. Flow cytometry indicated that the percentages of mature c-kit+ Ter119+ cells were significantly reduced in Bright−/− fetal livers (Fig. 3A). These data were supported by May-Grunwald Giemsa stains of E12.5 wild-type and Bright null fetal liver cells (Fig. 3B). Of a total of 1,000 cells counted, approximately equal numbers of nucleated cells and nucleated pink cells (which represent mature erythrocytes) were contained in wild-type livers, while approximately half the number of nucleated cells as nucleated cells were contained in knockout livers. These data suggest that a differentiation defect might account for the lower number of erythroid cells in the Bright null fetal livers.

Further analyses of Bright−/− fetal livers indicated that in addition to the numbers of erythrocyte progenitors, the total numbers of lineage-negative (lin−) c-kithi Sca1+ (LSK) cells were substantially reduced. Both lin− c-kit+ Sca1− (myeloid-lineage progenitor; MLP) and lin− c-kit− Sca1+ (common
lymphoid progenitor; CLP) subpopulations were also reduced ~2-fold, consistent with the generally smaller size of the fetal livers from the Bright−/− mice (Fig. 3C). However, colony-forming assays initiated with the same total numbers of wild-type and knockout cells revealed that the hematopoietic potential of knockout cells to yield B lymphocytes, erythromyeloid colonies, and BFU-E was reduced ~80% from that of normal controls (Fig. 3D), suggesting that Bright deficiency results in defective hematopoesis in the fetal liver.

Bright is required for lymphopoiesis and conventional B cell differentiation in adults. The rare survivors (<1%) of Bright−/− lethality, although significantly smaller (Fig. 1D),
showed no gross defects in organogenesis (data not shown). We observed a modest ($P < 0.10$) reduction in splenic cellularity, but this decrease did not result from differences in proliferation or apoptosis (data not shown). Erythrocyte numbers in peripheral blood showed no consistent differences among seven 3- to 9-month-old Bright null survivors and six littermate controls (data not shown).

We reasoned that the erythropoietic compensation in these rare Bright null survivors might be provided by its closely related paralogue, Bdp/Arid3b (60). Bright and Bdp are highly similar across their entire open reading frames, share identical DNA binding properties, form immunoprecipitable protein complexes, and transactivate the IgH locus through the same cis-acting MARs (25, 40). However, upregulation of Bdp was...
not observed in Bright knockout mice (Fig. 1C). Furthermore, Bdp null embryos were lethal at a significantly earlier (E7.5 to E9.5) stage and did not phenocopy Bright−/− HSC defects (Fig. 4) (addressed further in the Discussion). This finding, along with the observation that adult Bdp−/− by Bright+/− compound heterozygotes showed no measurable phenotype, led us to conclude that paralogous redundancy could not account for the occasional survival.

While an unknown mechanism can apparently offset erythropoietic defects, such was not the case for lymphopoiesis. As anticipated from the results shown in Fig. 3, both total cell numbers and frequencies of LSK (c-kit+/Sca1−), MLP (c-kit+ Sca1−), and CLP (c-kit+/ Sca1+ IL7R+) cells were significantly reduced in the bone marrow of 1- to 4-month-old Bright null mice (Fig. 5A). However, further differentiation was perturbed only in B lineages (Fig. 5B): T cell numbers and subset frequencies in these Bright null adults were comparable to those of wild-type controls (Fig. 5B). Strong blocks to conventional (B-2) pro- and pre-B development were observed in Bright−/− bone marrow. Peripheral Bright−/− B cell subsets (e.g., transitional and marginal zone B cells [MZB]), in which Bright is most highly expressed in normal cells (39, 48), were also significantly reduced (Fig. 5B). We observed modest reduction in Bright−/− follicular (FO) and circulating B cell numbers, sub-populations in which Bright expression is normally low, suggesting that homeostatic effects result in compensation of FO B cell numbers in adult spleens (Fig. 5B).

B-1 cell generation and function is impaired in Bright−/− adults. In addition to conventional B-2 cells, which comprise the great majority of the peripheral subsets, bone marrow progenitors of B-1 cells (CD93+ lin−/− Sca1+ CD19+ CD45R−) were also significantly reduced (Fig. 5A). Accordingly, mature B-1 cells (particularly the B-1a subset), which are the predominant B cell population in the peritoneal cavity, were depleted (Fig. 6A).

The B-1a subset is a major producer of natural serum antibody (33, 34). We observed reduced numbers of circulating antibodies, particularly IgM and IgG1, in sera of the surviving knockout mice, and the reductions were sustained over several months (Fig. 6B). B-1b cells have been proposed to be the primary source of T cell-independent antibody production and long-term protection against S. pneumoniae (33, 34). Consistent with this proposition and with our previous data obtained from PC-KLH-immunized DN Bright transgenic mice (39, 48), Bright knockout mice exhibited reduced IgM responses to primary immunization with intact S. pneumoniae serotype RSA32 cell wall-associated PC (Fig. 6C, left panel).

Functional defects in Bright−/− immune responses are B cell intrinsic. Conventional knockout of Bright results in deficiencies in all cell types, compromising conclusions we might draw on intrinsic B cell function. Thus, we generated double-knockout Bright ES cell lines (see Materials and Methods) and transferred them into Rag2−/− 129Sv mice by blastocyst complementation (8). Chimeras generated by injection of Bright null ES cells had relatively normal numbers and percentages of T cells in thymus and spleen but generally had lower levels of splenic B cells than those resulting from the relatively normal reconstitution achieved by wild-type and Bright heterozygous ES cells (Table). 1

As with the conventional knockout mice, Bright−/−/Rag2−/− chimeras exhibited significantly reduced IgM responses to RSA32 cell wall-associated PC (Fig. 6C, lower panel). Mature S107

FIG. 4. Bdp−/− embryos die of neural crest defects and share no phenotype with Bright−/− embryos. (A) Map of wild-type Bdp. Exons are indicated by boxes, with ARID DNA binding domains (green) and REKLES self-association, nuclear import, and nuclear export domains (red and blue). (B) Bdp with the LacZ retroviral insertion (5′ long terminal repeat [LTR], yellow; 3′ LTR, black). Connected horizontal bars above each map indicate positions of primers used to distinguish germ line (A) and LacZ-retroviral integration (B) alleles by PCR. (C) Evidence of germ line transmission of the LacZ integration within Bdp. PCR was performed on tail DNA of first-generation founder mice as detailed in Materials and Methods. (D) Comparison of Bdp+/− (WT, right) and Bdp−/− (KO, left) embryos that survived to E9.5 (the majority die earlier). The KO embryos are developmentally delayed and show prominent levels of LacZ surrogate Bdp expression in neural crest cells of branchial arches and the neural tube. Dotted black lines indicate the general regions of rhombomeres 3 and 5 (r3 and r5) of the neural crest (note area between, where r4 should exist, but which is a neural crest-free region). A dotted red line surrounds the frontonasal prominence where a faint LacZ signal appears.
V_{14}C_{\mu} heavy-chain transcripts that encode dominant (T15) anti-IgM responses to PC were downregulated, whereas those corresponding to natural IgM responses encoded by the 7183, J558, and Af303 VH families were not (Fig. 6C, right panel). These results are consistent with our observations that Bright directly transactivates T15 IgH transcription by binding to DNA consensus motifs within the V_{14} promoter (18, 25, 30, 43, 44, 55, 57, 58).

Bright deficiency results in reduced IgG1 T-dependent responses. Antiprotein responses are typically elicited from FO B cells. Even though the numbers and global proliferation of
FO B cells were only modestly reduced in Bright null mice (Fig. 5B and data not shown), a defect in the immediate BCR signaling pathway was observed (Fig. 7A). This finding suggested potential functional consequences in antiprotein responses, which are typically elicited from FO B cells. Unexpectedly, however, a defect was observed only for IgG1. T-dependent (TNP-KLH) responses of the Bright/H11002/H11002/H11002/Rag2/H11002/H11002/H11002 chimeras (Fig. 7B) as well as of conventional Bright null mice and Bright/H11002/H11002/H11002/Rag2/H11002/H11002/H11002 bone marrow-reconstituted C57BL/6 recipients (data not shown) were normal with the exception of significantly reduced secretion of IgG1. These results were consistent with the reduced natural IgG1 sera levels (Fig. 6B). In line with the in vivo results, FO B cells purified from spleens of Bright⁻/⁻/Rag2⁻/⁻ chimeras (Fig. 7C, left panel) or from conventional Bright⁻/⁻ mice (data not shown) secreted significantly less IgG1 than controls when stimulated with anti-CD40 IL-4 or LPS. Induction of IgG1 class switching, as measured by the expression of \( \lambda 1 \) (Fig. 7D, left panel) and even more strongly in the conventional knockout mice (Fig. 7D, right panel), whereas I region-initiated germ line transcription of other isotypes was unperturbed. This provides an explanation for the selective decrease in the frequency of IgG1-switched B cells in the absence of global proliferative changes (data not shown) and suggests that Bright plays a more critical role in the production of IgG1 than in that of other IgG isotypes.

![Diagram](image-url)
the adult bone marrow as the source of adult hematopoiesis (28). Accordingly, rareBright−/−survivors show parallel deficiencies in LSK and reduced numbers of common myeloid progenitors (CMP) and CLP in their bone marrow. HSCs residual in fetal liver and bone marrow differ in several properties. Consequently, not all hematopoietic transcription factors regulate both stages. For example,Sox17is critical for the generation of fetal, but not bone marrow-derived, HSCs (24).

Differential properties include intrinsic programs regulating growth and multilineage differentiation potential, as well as extrinsic differences in engraftment niches required to support these programs (5). Normal fetal liver HSCs are rapidly cycling (15). That overexpression of Bright can activate E2F1 and cell cycle entry in mouse embryonic fibroblasts (42) provides a plausible pathway deregulated by Bright deficiency in this compartment. However, bone marrow HSCs are largely quiescent (15), making it harder to reconcile an analogous role for Bright in sustaining adult hematopoiesis. This implies that as for the principle hematopoietic regulators studied to date (41), Bright function is highly context dependent.

Transcription factors essential for HSC formation and/or self-renewal (e.g., MLL, Runx1, SCL/tal1) often function later within differentiation of separate blood lineages (41). Conversely, factors initially discovered as lineage-restricted regulators (e.g.,PU.1,Gfi-1, C/EBPα) were later found to perform essential roles in HSC differentiation (41). Bright is similarly deployed as an intrinsic and specific regulator of the adult B lineage, as T cell and erythroid development (at least at the level of resolution employed in this study) were unaffected in Bright null mice. This finding is consistent with the stringently controlled manner of Bright expression, i.e., present in the earliest identifiable HSC progenitors, downregulated in early pro-B cells and the majority of mature quiescent B cells, and upregulated in pre-B, conventional immature/transitional stages, activated B lymphocytes, and B-1 peripheral compartments (47, 48, 58, 59). Bright KO results in B lineage blocking at all post-CLP adult stages, with the exception of the resting FO B cell population and the circulating B cell compartment (which is derived primarily from FO). This can be reconciled by the fact that Bright is normally downregulated in these long-lived compartments (39, 48), which, in addition, are particularly sensitive to homeostatic replenishment even after significant reduction of progenitors following hematopoietic transcription factor knockout (35, 41).

Contrary to a potential compensatory role,Bdp−/−embryos died earlier with distinctly different phenotypes. Bdp knockouts were developmentally delayed and exhibited aberrant pharyngeal arch development. Bdp expression during this phase of embryogenesis was limited to nascent mesoderm and the neural crest, most prominently within neural crest cells of branchial arches and the neural tube. This is consistent with previous studies that suggested a role for Bdp in craniofacial development and neuroblastoma (27, 52). Thus, it remains unclear why a small percentage ofBrightnull embryos circumvent lethality.

Bright-deficient B cells are intrinsically impaired in mounting primary anti-PC responses. This is due, at least in part, to blocked B-1 development and a selective defect in transcription of the rearranged heavy-chain gene (SI07 V_{H}1) that chiefly encodes PC reactivity (18, 25, 30, 43, 44, 55, 56, 57). For

**TABLE 1. Comparison of lymphocyte reconstitution in chimeric mice**

| Genotype     | No. (%) of cells of indicated type in the spleen (×10^7) | No. (%) of SP + DP cells in the thymus (×10^7) |
|--------------|----------------------------------------------------------|-----------------------------------------------|
| B cells      | T cells                                                  |                                               |
| Bright+/+    | 5.1 (59)                                                 | 13.4 (97)                                    |
| Bright+/−    | 4.2 (54)                                                 | 12.5 (98)                                    |
| Bright+/−    | 4.1 (55)                                                 | 13.1 (98)                                    |
| Rag2−/−      | 0 (0)                                                    | 0 (0)                                        |
| Rag2−/−Bright+/+ | 7.5 (65)                                             | 11.9 (96)                                    |
| Rag2−/−Bright+/+ | 3.5 (38)                                             | 13.0 (93)                                    |
| Rag2−/−Bright+/− | 4.3 (41)                                             | 5.9 (96)                                     |
| Rag2−/−Bright+/− | 4.5 (53)                                             | 8.2 (88)                                     |
| Rag2−/−Bright+/− | 1.9 (32)                                             | 9.9 (94)                                     |
| Rag2−/−Bright+/− | 3.7 (29)                                             | 10.7 (97)                                    |
| Rag2−/−Bright+/− | 2.6 (40)                                             | 8.2 (86)                                     |

* Total numbers were determined by counting with a hemocytometer from single cell suspensions of the indicated organs. Percentages were determined by flow cytometry as B220−IgM− for B cells, CD4+ or CD8+ for single positive (SP) T cells, and CD4+ CD8+ for double positive (DP) T cells.

**DISCUSSION**

Over 20 transcription factors representing a diverse range of DNA binding families have been implicated in hematopoiesis (15, 41). Nearly all of them are associated with hematopoietic malignancy (41). Bright was first described as a B cell-restricted, positive regulator of immunoglobulin gene transcription. Its overexpression in mice, via a B lineage-specific (CD19) promoter-driven transgene, results in enhanced IgM expression and intrinsic B cell autoimmunity but not in cancer. However, Bright ectopic overexpression converts MEFs to tumors in nude mice by bypassing natural or RasV12-induced cellular senescence to promote cellular proliferation via activation of the Rb/E2F1 pathway (42). Bright overexpression, in the absence of locus translocation, correlates with the worst prognosis for patients with the most aggressive form of AID-MLL (38). There is no consistent evidence linking Bright with chromosomal translocation or somatic mutation, such as that observed forPU.1andC/EBPαin myeloid malignancies or forPax5,E2α, andEBFin B-lymphoid malignancies (36). More likely, Bright overexpression leads to lesions in more broadly utilized signaling pathways (e.g., Ras, Rb/E2F1) that regulate hematopoietic lineage decisions. While its oncogenic mechanism remains to be determined, Bright/Arid3a is the first of the 13-member ARID family that fits this unique profile of the major hematopoietic transcription factors.

HSC appear to form normally in Bright−/−yolk sacs, but their differentiation into mature erythrocytes is markedly reduced in fetal livers. This results in embryonic death coincident with the shift from primitive to definitive hematopoiesis and the timing of normal Bright expression in fetal liver. Additional work will be required to secure this conclusion, as the contribution of each hematopoietic site (such as the yolk sac and fetal liver) to circulating fetal blood in the fetus or adult has been challenged by recent studies in mice and zebrafish (37, 47). GlobalBrightknockout did not perturb hemoglobin switching, vascularization, or gross organogenesis outside the fetal liver, suggesting a relatively selective role for Bright in HSC expansion and/or differentiation. It is widely accepted that HSCs of the fetal liver circulate to...
FIG. 7. Selective and intrinsic loss of T-dependent immune function in Bright^{-/-} mice. (A) Bright-deficient FO B cells show enhanced proximal signaling prior to and following BCR ligation. Purified FO B cells from wild-type and Bright^{-/-} mice were stimulated with 50 μg of anti-IgM (α-μ) plus 50 μg of anti-CD19 (α-CD19) for 5 min. Western blotting was carried out with antiphosphotyrosine antibody as described previously. (B) Reconstituted Rag2^{-/-}/Bright^{-/-} mice are impaired in IgG1 primary and secondary responses to a protein antigen (TNP-KLH). Groups of 5 Bright^{-/-} (WT) or Bright^{-/-} (KO) mice were primed with 50 μg KLH in adjuvant, and 4 weeks later, they were immunized with TNP-KLH. Sera from these mice were collected 7 days after immunization. Anti-TNP and anti-KLH serum Ig levels were measured by isotype-specific ELISA. Average preimmune serum levels (1:200 serum dilution) are depicted by the solid diamonds and circles. Optical density at 405 nm (OD405) data are shown for each individual mouse from which blood was successfully obtained. (C) Bright deficiency impairs in vitro induction of IgG1 secretion but not induction of class switch recombination. Purified splenic FO B cells from Brigh+/-/Rag2+/- (WT) and Bright^{-/-}/Rag2+/- (KO) chimeras were left untreated (UTX) or were stimulated at 6×10^5/well in vitro for 3 days under the conditions indicated (LPS, 20 μg/ml; anti-CD40, 20 μg/ml; and IL-4, 50 ng/ml). Isotype-specific ELISA was carried out as for panel B. Shown are the average values for 5 mice within each group. (D) Bright-deficient follicular B cells are impaired in induction of heavy-chain-β1 germ line transcripts. FO B cells isolated from splenocytes of Bright^{-/-}/Rag2+/- (left) or Bright^{-/-} (right) mice and their wild-type littermate controls were stimulated in vitro for 3 days with anti-CD40 (20 μg/ml) and IL-4 (50 ng/ml). Primers and conditions for semiquantitative RT-PCR analyses are described in Materials and Methods.
maximum transcriptional activation function, Bright must interact with Btk, an essential transducer of BCR signaling, and TFII-I, a direct phosphorylation substrate of Btk (43, 44). Btk is required for both B1 generation and normal PC responses (11) and acts principally at the checkpoints (pre-B1 and T1) most compromised in both Bright KO and DN mice (13).

These defects were previously observed, albeit less dramatically, in Bright DN transgenic mice (39). The differences in penetrance most likely derive from incomplete DN inhibition, particularly in HSC and early B lineage progenitors, where the transgenic B cell-specific promoter (CD19) is either inactive or significantly weakened (19). Concentration-dependent effects in lineage choice and differentiation are well documented (12, 29, 45). Alternatively, Bright can act outside the nucleus, independently of DNA binding, to dampen signal transduction via association with BCR and Btk in plasma membrane lipid rafts (48). We suspect that this function, which remains unperturbed in DN transgenic B cells, may contribute to the proximal signaling hyperactivity we observed in the knockout FO B cells. Potentially relevant in this regard, Btk can modulate EpoR/c-kit signaling to drive expansion of erythroid progenitors (53). On the other hand, we have recently observed that B cells from both Bright knockout and DN transgenic mice are developmentally plastic (1). Further experiments will be required to more accurately define the mechanism by which Bright deficiency leads to these phenotypic changes.

Bright-deficient FO B cells, while only modestly reduced in number, are intrinsically defective in generating T-dependent IgG1 responses. Selective reduction in IgG1 can be explained, at least in part, by selective reduction in IL-4+ anti-CD40 stimulation of γ1 germ line transcription, a prerequisite for class switch recombination (CSR) and the production of mature γ1 mRNA (50). While these results implicate Bright in CSR, the mechanism underlying the observed γ1 specificity is unclear. One possibility is that a gene critical to the process is a direct target deregulated by the knockout of Bright. For example, particular NF-κB transcription factors can promote germ line transcription in general (16) or specifically promote γ1 germ line transcription (3) by binding to specific I regions and/or to the 3′ enhancer, a CSR control region located downstream of the IgH locus (50). Alternatively, Bright might function at the level of the intergenic switch (S) region sequences that facilitate the recombination step of CSR. Elegant knock-out experiments by Bhattacharya et al. (4) suggest that the switch to γ1 is facilitated in a physiological setting by an as-yet-unidentified IL-4-induced factor that has specific DNA binding for Syl. Bright expression is induced by IL-4+ anti-CD40 (48, 49, 58, 59), and the Syl region is particularly rich in MARs (10), the motif to which Bright binds (18, 60).

Hematopoietic transcription factors operate through diverse mechanisms, but association with chromatin modification proteins is a consistent theme (15, 41). Examples include Ikaros-NuRD in the T lineage (26), EKLF-Brg1 in the erythroid lineage (7), and Gfi-LSD1 in the myeloid lineage control (46). Bright binding to MARs within the IgH enhancer promotes chromatin accessibility (22, 30). Other ARID family members directly remodel chromatin (60). The challenge of future experiments is to identify relevant non-IgH transcriptional targets of Bright and to determine whether Bright acts via chromatin-associated factors to promote HSC differentiation and post-CLP B lineage programming.

ACKNOWLEDGMENTS

We thank S. Ferrell, Chihaya Das, Deborah Surman, and Maya Ghosh for technical assistance. Support was provided by NIH 044215 (C.F.W.), NIH CA31534 (P.W.T.), CPRIT (P.W.T. and M.P.), and the Marie Betzer Morrow Endowment (P.W.T.). We have no financial conflict of interest.

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