Published in final edited form as:
Mol Immunol. 2011 October ; 49(1-2): 260–272. doi:10.1016/j.molimm.2011.08.025.

Characterization of a new ARID family transcription factor (Brightlike/ARID3C) that co-activates Bright/ARID3A-mediated immunoglobulin gene transcription

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Abstract

Two members, Bright/ARID3A and Bdp/ARID3B, of the ARID (AT-Rich Interaction Domain) transcription family are distinguished by their ability to specifically bind to DNA and to self-associate via a second domain, REKLES. Bright and Bdp positively regulate immunoglobulin heavy chain gene (IgH) transcription by binding to AT-rich motifs within Matrix Associating Regions (MARs) residing within a subset of V_H promoters and the Eμ intronic enhancer. In addition, REKLES provides Bright nuclear export function, and a small pool of Bright is directed to plasma membrane sub-domains/lipid rafts where it associates with and modulates signaling of the B cell antigen receptor (BCR). Here, we characterize a third, highly conserved, physically condensed *ARID3* locus, *Brightlike/ARID3C*. *Brightlike* encodes two alternatively spliced, SUMO-I-modified isoforms that include or exclude (Δ6) the *REKLES*-encoding exon 6. Brightlike transcripts and proteins are expressed preferentially within B lineage lymphocytes and coordinate with highest Bright expression--in activated follicular B cells. Brightlike, but not BrightlikeΔ6, undergoes nuclear-cytoplasmic shuttling with a fraction localizing within lipid rafts following BCR stimulation. Brightlike, but not BrightlikeΔ6, associates with Bright in solution, at common DNA binding sites *in vitro*, and is enriched at Bright binding sites in chromatin. Although possessing little transactivation capacity of its own, Brightlike significantly co-activates Bright-dependent IgH transcription with maximal activity mediated by the unsumoylated form. In sum, this report introduces Brightlike as an additional functional member of the family of ARID proteins, which should be considered in regulatory circuits, previously ascribed to be mediated by Bright.

Introduction

ARID (AT-Rich Interaction Domain)-containing proteins are distinguished by a highly conserved, helix-turn-helix DNA binding domain (reviewed in Kortschak et al., 2000;

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Wilsker et al., 2005) that binds to the major grove of DNA (Herrscher et al., 1995; Iwahara et al., 2002; Kim et al., 2004). Members of the ARID-superfamily of proteins are found in a broad array of organisms from fungi to mammals and are involved in functions ranging from chromatin remodeling to cell cycle control and are sub-grouped into 7 families (Wilsker et al., 2005). Bright (B cell *r*egulator of IgH transcription) and Bdp (Bright-Dri-like protein) are members of the ARID3 subgroup (Herrscher et al., 1995; Numata et al., 1999).

Bright/ARID3A and Bdp/ARID3B share extended (e) identity beyond their ARID DNA binding domain (Fig. 1), including a domain required for self-association, termed REKLES (named for a hexapeptide within the motif that is conserved among orthologues (Kim et al., 2007; Kortschak et al., 2000]). Sequence alignments allow the REKLES domain to be divided into two sub-domains: A modestly conserved N-terminal REKLES-α and a highly conserved 20 amino acid C-terminal REKLES-β (Fig. 1; Kortschak et al., 2000). REKLES-α and REKLES-β are required for the nuclear import (NLS) and nuclear export (NES) of Bright, respectively (Kim and Tucker, 2006). REKLES-β has also been identified as a region necessary for both homo-and heteromeric eARID interactions (Kim et al., 2007). REKLES-mediated interactions with SUMO-1 E2 and E3 conjugation enzymes (Ubc9 and PIAS-1) led to the finding that a fraction of SUMO-I-modified Bright partitions into PML-nuclear bodies (Zong et al., 2000) and within plasma membrane lipid rafts (Schmidt et al., 2009). The small pool of lipid rafts-localized Bright associates with the BCR to dampen signaling, independent of its nuclear function as a transcription factor (Schmidt et al., 2009).

Bright expression is tightly regulated during B-cell differentiation in normal murine and human lymphocytes (Nixon et al., 2004; Schmidt et al., 2009; Webb et al., 2011; Webb et al., 1998). In association with TFII-I and Bruton's tyrosine kinase, Bright transactivates the IgH locus by binding to specific ATC-rich motifs within the nuclear matrix attachment regions (MARs) flanking the intronic enhancer (Eμ) and upstream of a number of V_H-promoters, including the V_H1 member of the VS107 family (Herrscher et al., 1995; Rajaiya et al., 2005; Rajaiya et al., 2006; Webb et al., 2000). Mutation of the single SUMO-I conjugation site in Bright had no effect on IgH enhancer/promoter DNA binding or transcriptional activities (Schmidt et al., 2009). The SUMO-I-deficient human orthologue of Bright also was shown to bind DNA indistinguishably from Bright (Prieur et al., 2009).

In contrast with Bright, Bdp is ubiquitously expressed exclusively within the nucleus as it lacks the NES encoded in Bright's REKLES-β domain (Kim et al., 2007; Kim and Tucker, 2006, Webb et al., 2011). Initially identified as a putative tumor suppressor that interacts with retinoblastoma protein (Rb) (Numata et al., 1999), Bdp binds to the same IgH MARs as Bright and can form heteromeric complexes with Bright via their REKLES domains (Kim and Tucker, 2006). No significant transactivation alterations have been observed for Bright/Bdp complexes when compared with Bright alone *in vitro* (Kim and Tucker, 2006; Lin et al., 2007). Nor does Bdp compensate for embryonic lethality and hematopoietic deficiencies resulting from the targeted elimination of *Bright* in mice (Webb et al, 2011).

Here, we identify and characterize a compressed genomic locus, which is, in all vertebrate genomes examined, predicted to encode a third *ARID3* gene, *Brightlike/ARID3C*. We show that Brightlike transcripts and proteins are expressed coordinately with Bright with highest levels accumulating in activated B cells. Alternative pre-mRNA splicing generates two Brightlike isoforms, which are differentially expressed in lymphocyte populations as well as sub-cellularly, depending on inclusion or exclusion of a REKLES-β-encoding exon 6. Brightlike and Bright are shown to interact through their REKLES domains but appear to traffic independently of one another under identical BCR activation conditions into and out of lipid rafts. Furthermore, Brightlike binds non-competitively to the same Bright DNA target sequences *in vitro* and *in vivo*, and collaborates with Bright in coactivating V_H1-S107-

MAR-driven IgH transcription. This function of Brightlike is enhanced by loss of sumoylation at a single SUMO-I conjugation motif, conserved with Bright.

Results

The *Brightlike/ARID3C* genomic locus

A genomic search led to the identification of a region that appears to encode a gene paralogous to *Bright/ARID3A* and *Bdp/ARID3B*, which we named *Brightlike/ARID3C* (Wilsker et al., 2005). Clustal-W sequence alignments identified an orthologous gene in all vertebrates examined (data not shown, but further addressed in Discussion). The genomic size of *Brightlike* is compressed when compared to *Bdp* and *Bright* (6kb, as compared to 40–50 kb, respectively; Fig.1A). Large deletions within Brightlike coding exons have compressed its ORF (in human) to 412 amino acids and eliminated the exon 1/exon 2 splice junction shared by *Bright* and *Bdp* (Fig. 1B, Suppl. Fig. 1). Brightlike has retained ~80% identity with both paralogues within the DNA binding (ARID) domains, including extended (e) regions just N- and C-terminal to the ARID (termed eARIDs, Wilsker et al., 2005) which provide contacts critical for highly specific DNA binding (Herrscher et al., 1995) (Fig. 1C; Suppl. Fig. 1). Brightlike has retained a REKLES domain spanning exons 5 and 6 which is more highly conserved with that of Bright (Figs. 1B and C; Suppl. Fig. 1). A SUMO-1 conjugation consensus conserved in Bright but not in Bdp resides in the C-terminus of exon 4 (Fig. 1B and C).

Full-length and alternatively spliced Brightlike isoforms are expressed at low levels in tissues of immunologic interest

Brightlike is flanked on human chromosome 9 (9p13.3) by *dynactin* 3 (2kb telomeric) and the *opioid receptor sigma* (13kb centromeric). These genes are each transcribed in the same direction and are commonly expressed in a wide variety of tissues, based on their high representation in human EST databases. However for Brightlike, only a single porcine *(Sus scrofa*; GenBank accession no. CX065752; tissue not specified) and a single human (Hs. 534549; tonsil) EST had been posted. Our initial RT-PCR efforts (Fig. 2A, “outer primers” panel), as well as a global microarray hybridization and SAGE performed on 24 normal tissues (www.ncbi.nlm.nih.gov/geo) failed to detect Brightlike expression with the exception of the human tonsil (Fig. 2A, lane 11). Thus, Brightlike transcripts are exceedingly rare, suggesting they are induced under rare environmental conditions or in a narrow subset of tissues.

Using a nested RT-PCR amplification scheme with 5’ and 3’ UTR primers flanking the predicted translational start and stop codons, amplicons of both the predicted size and slightly smaller were identified only in murine testes, thymus, and spleen (Fig. 2A “nested primers” panel and data not shown). Cloning and sequencing identified full-length Brightlike as well as a splice variant (termed BrightlikeΔ6), which lacked the C-terminal portion of the highly conserved protein-protein interaction (REKLES-β; exon 6) domain (see Kim et al., 2007 for details on the REKLES-β domain). In confirmation, Southern blotting of the spleen RT-PCR reaction hybridized with an exon 6-specific DNA probe revealed species that differed from the full-length DNA in size by ~90nt (data not shown). The intensity of bands from this experiment along with the sizable enrichment of cDNA for BrightlikeΔ6 suggested that this REKLES-deficient isoform is more abundant, although formal confirmation remains to be determined.

Brightlike and Bright are co-expressed in LPS stimulated splenic B cells

Bright expression is highly B lineage regulated, with optimal levels observed in transitional/immature and in activated follicular (FO) stages (Nixon et al., 2004). Reasoning that

Brightlike might be similarly restricted, we purified murine splenic FO B cells and assayed for changes seen after LPS-induced activation. As shown in Fig. 2B, we detected full length Brightlike only in LPS stimulated FO (CD43-) blasts, where Bright expression is maximal (lanes 3). BrightlikeΔ6 was more abundantly expressed in unstimulated FO as well as in the non-FO (CD43+) splenic fractions (lanes 1 and 2), but was down-regulated in LPS stimulated blasts (lane 3).

These results indicated that Bright and full length Brightlike are co-expressed, at least in a subset of mitogenically stimulated FO B cells, where both Bright levels (Nixon et al., 2004) and Bright DNA binding (Webb et al., 1991; Herrscher et al., 1995) are maximally induced. However, Bright expression is not requisite for Brightlike expression. As shown in Fig. 2C, Brightlike isoforms are differentially expressed within the Bright-negative TcRαβ lineage. Both forms accumulate in DP (lane 2) and CD4 T cells (lane 3), whereas DN only express full length (lane 1) and CD8 only express BrightlikeΔ6 (lane 4).

To detect Brightlike protein, antisera were raised against N- or C-terminal regions, not conserved with either Bright or Bdp, and employed in Western analyses. Brightlike isoforms could not be distinguished because of their modest (30 amino acids) size difference. Consistent with their exceedingly low mRNA levels, Brightlike was only marginally detectable in whole cell lysates (data not shown). However, enrichment by nuclear fractionation allowed detection of an endogenous species of predicted size (~50kD) in some (lanes 2 and 3) but not all human mature B and T cell lines (Fig. 3 and data not shown). Consistent with the RT-PCR data of Fig. 2B, levels of Brightlike in nuclei of 3 day mitogen-activated splenic FO B cell blasts (lane 8) were significantly elevated above untreated controls (lane 7). Stimulation via the BCR ligation following short term culture with anti-μ + anti-CD19 as previously described (Schmidt et al., 2009) had only modest effects on nuclear levels of Bright and Brightlike (compare lanes 5 and 6).

Exon 6-encoded REKLES-β is required for nuclear export of Brightlike

We have reported elsewhere that Bright shuttles between the nucleus and the cytoplasm in a CRM1-dependent manner (Kim and Tucker, 2006). The NES of Bright has been mapped to the REKLES-β, the domain excluded in BrightlikeΔ6. Based on Bright's REKLES-β dependent nuclear export, we expected Brightlike to be localized in the nucleus and cytoplasm and BrightlikeΔ6 to reside preferentially in the nucleus.

An N-terminally fused GFP-Brightlike was constructed and transfected into NIH 3T3 fibroblasts. As predicted, Brightlike localized within both the cytoplasm and nucleus and the exon 6-deficient form was retained in the nucleus (Fig. 4). These results were confirmed by anti-Bright antibody histochemistry (Fig. 4). The data suggest that Brightlike and Bright are subject to equivalent nuclear import and export cues, as opposed to the totally nuclear residence of Bdp/ARID3B.

Brightlike associates with Bright through its REKLES-β domain

Amino acid residues 521–541 of the REKLES-β subdomain are required for homo- and hetero-interactions of Bright with itself or with Bdp (Kim et al., 2007). This region is highly conserved within exon 6 of Brightlike. This suggested that Brightlike would interact with Bright and Bdp as well as homomerize with itself, whereas REKLES-β-deficient BrightlikeΔ6 would not. The hypothesis was tested by co-immunoprecipitation (CoIP) of the two proteins following their ectopic expression in non-lymphoid cells (HEK-293T, abbreviated as 293T) which do not express their endogenous forms. 293T cells were transfected with combinations of HA-tagged Brightlike, HA-BrightlikeΔ6, GFP-Bright and empty vectors under conditions previously established in non-lymphoid cell lines to detect

Bright-Bright or Bright-Bdp interactions (Kim et al., 2007). Anti-Brightlike and pre-immune sera were used for Co-IP, followed by anti-HA or anti-GFP Western blotting. As shown in Fig. 5A, although inputs of both isoforms of Brightlike were precipitated, co-IP of BrightlikeΔ6 and Bright was not observed (lane 6).

Similar Co-IP experiments to determine whether the Bright-Brightlike interaction demonstrated by over-expression could be observed for the endogenous proteins were unsuccessful, owing at least in part to the low abundance of Brightlike within soluble fractions (data not shown). We reasoned that Bright-Brightlike association might be enriched in the chromatin fraction, where Bright was reported to be enriched (Lin et al., 2007; Schmidt et al., 2009). Cross-linked chromatin, prepared from the AB1.2 murine plasmacytoma line, which expresses Brightlike at reasonable levels (Fig. 3), was subjected to IP with antibodies against Bright, Brightlike, and pre-immune controls. As shown in Fig. 5B, Western blot analysis (Fig. 4B) developed with anti-Bright indicated that anti-Bright (lane 3) and anti-Brightlike (lane 4) precipitated Bright containing chromatin under conditions in which pre-immune sera (lanes 2) did not.

We conclude from these experiments that Bright and Brightlike undergo stable association when over-expressed in nonlymphoid cells and in lymphoid cells following enrichment by chromatin cross-linking. Our data support the notion that the REKLES-β sub-domain mediates the formation of Bright/Brightlike complexes.

Brightlike is sumoylated at a consensus motif conserved in Bright

It is reported that Bright is SUMO-I conjugated at a single site, which may be equivalent to K284 of Brightlike (Fig. 1C; Schmidt et al., 2009; Prieur et al., 2009). K284 of Brightlike falls into a strong sumoylation consensus (http://www.abgent.com/doc/sumoplot), prompting point mutation and further analysis. *In vitro* sumoylation assays were performed using *in vitro* transcribed/translated Brightlike and BrightlikeΔ6 in the presence or absence of purified SUMO E1 (Sae1/2) and SUMO E2 (Ubc9), as described previously (Rangasamy and Wilson, 2000; Rosas-Acosta et al., 2005a; Rosas-Acosta et al., 2005b; Schmidt et al., 2009). In the presence of sumoylation enzymes E1 and E2, slower migrating species, equivalent in size to SUMO-I mono-conjugated forms of Brightlike (lane 2) and BrightlikeΔ6 (lane 6) were observed (denoted by asterisks in Fig. 6A). Substitution of K284R within the SUMO-I consensus motif (283-ΨKxE/D-286) eliminated the SUMO-I conjugated forms of both Brig htlike isoforms (lanes 4 and 8).

To verify these observations, the properties of GFP-tagged mammalian expression versions of the same mutants and SUMO-I were analyzed by forced over-expression in Jurkat T cells. As shown in Fig. 6B, lysates from the wild-type (lane 3), but not the K284R mutant forms of Brightlike (lane 5), contains species corresponding in size and composition to GFP-SUMO-Imono-conjugated Brightlike. These observations indicate that Brightlike is modified *in vivo* with SUMO-1 at K284.

Brightlike is recruited to plasma membrane lipid rafts following B cell antigen receptor stimulation

We have established (Schmidt et al., 2009) that Bright, when localized within plasma membrane lipid rafts, increases the signaling threshold of the BCR in response to BCR ligation in normal B cells and in transformed lymphoblastoid B cell lines. These B cell lines included one Burkett's lymphoma, RAJI, which expressed detectable levels of endogenous Brightlike (Fig. 3C) and which is responsive to strong BCR (anti-IgM + anti-CD19) ligation (Schmidt et al., 2009). Schmidt et al (2009) further reported that discharge of Bright from lipid rafts occurred shortly following BCR ligation. Discharge, at least in part, required

Sumo-I modification and correlated with restoration of BCR signaling. While Bdp/ARID3B is undetectable outside of the nucleus (Kim and Tucker, 2006), we reasoned that the nuclear export and sumoylation properties of Brightlike might engender a Bright phenotype.

RAJI and RAMOS B cells were activated using anti-IgM + anti-CD19 (Methods and Materials and Schmidt et al., 2009), as judged by total phosphotyrosine incorporation (data not shown). Lipid rafts were isolated and confirmed for purity as described (Nagamatsu et al.,1992; Schmidt et al., 2009; Materials and Methods). Proteins were extracted and assayed by Western blotting for the presence of Bright and Brightlike. A lipid raft-restricted protein, Raftlin, whose levels are insensitive to BCR ligation (Saeki et al, 2003; Schmidt et al., 2009), served as a loading control. As shown in Fig. 7A and consistent with previous findings, BCR ligation resulted in Bright discharge from lipid rafts of RAJI (middle panel, compare lanes 3 and 4) and RAMOS (lanes 7 and 8). Brightlike was also detected in lipid rafts, but in contrast to Bright, trafficked in the opposite direction; i.e., accumulating within rafts following BCR ligation (top panel, lanes 3 vs 4 and 7 vs 8). This was a particularly significant enrichment in RAMOS, as Brightlike was hardly detectable in nuclei under relatively similar input concentrations (Fig. 3, lane 4).

Since Bright and Brightlike interact (Fig. 5), raft-localized Brightlike might exist as homomeric Brightlike or as Bright-Brightlike heteromers. We over-expressed Bright by transient transfection in RAJI and RAMOS cells (compare lanes 1 to 4 and 5 to 7 of the middle panel of Fig. 7A) and repeated the above-described analysis. Under these conditions, Brightlike appeared to be titrated from lipid rafts of both B cell lines following BCR stimulation (Fig. 7A, lanes 4 and 8). The most straightforward interpretation of these results (albeit more complicated interpretations cannot be excluded) is that raft-localized Brightlike exists primarily as homomers, which when forced into Bright heteromers by mass action, are discharged from lipid rafts in a manner indistinguishable from Bright monomers.

To address this hypothesis further, we transfected HA-tagged-Brighlike wild type and mutants into BTR fibroblasts, which express neither endogenous Bright nor Brightlike (Schmidt et al., 2009, and data not shown). Ras was used as an endogenous loading control for isolated raft inputs. As shown in Fig. 7B, both Bright (lane 2) and Brighlike (lane 3) accumulated in lipid rafts, suggesting as in previous studies (Schmidt et al., 2009) that B cell-specific factors are not required for initial entry. Consistent with our model, Brightlike mutants which fail to form homomeric complexes (BrightlikeΔ6 and BrightlikeΔ6-K234R, lanes 6 and 7) fail to enter rafts. Unexpectedly, based on our previous results with Bright (Schmidt et al., 2009), Sumo-I deficient but homomeric-competent full length Brightlike-K234R also was excluded from lipid rafts (lane 5). Taken with the reverse trafficking response to BCR ligation observed in B cells (Fig. 7A), these data suggest that, whereas Bright requires sumoylation for rafts exit, sumoylation of Brightlike is required for rafts entry.

Brightlike, but not BrightlikeΔ6, binds to IgH S107 variable region-associated MARs *in vitro* and *in vivo*

Bright is a MAR binding protein (Herrscher et al., 1995; Webb et al., 1991) and may regulate transcription by altering chromatin structure (Kaplan et al, 2001; Lin et al., 2007). Bright and Bdp have been shown to bind MARs that both flank the intronic (Eμ) IgH enhancer and to MARs (termed Tx125 and Bf150) positioned upstream of the V_H1 member of the S107 variable region family (Herrscher et al., 1995; Fig. 8A). The presence of the highly conserved eARID domain in Brightlike suggested that Brightlike might recognize and bind to the same IgH-associated MARs.

Electrophoretic mobility shift assays (EMSAs) were performed to examine Brightlike's MAR binding ability *in vitro* (Fig. 8B). Since REKLES-β (within exon 6) is required for self-association, and thus for Bright to bind to the IgH promoter region (Kim et al., 2007), BrightlikeΔ6 was predicted to not bind to these probes. Radio-labeled DNA fragments containing the Bf150 (or Tx125; see Herrscher et al., 1995 for details) binding site with and without an excess of nonspecific cold competitor DNA were incubated with in vitro translated (IVT) Brightlike or with nuclear extracts prepared from Brightlike-transfected cells. Protein-DNA complexes were resolved by non-denaturing gel electrophoresis. Antibody supershifts were used to confirm specificity of gel retarded complexes. Western blot analysis confirmed that Bright and BrightlikeΔ6 IVT protein inputs were equivalent (data not shown).

Consistent with our prediction, Brightlike but not BrightlikeΔ6 bound to the Bf150 (and Tx125) MARs with relative high affinity (Fig. 8B, lanes 4 and 8 and data not shown). Binding was eliminated (lane 9) by point mutation of a conserved tyrosine in the ARID domain of Brightlike (Y197A), whose paralogous residue in Bright is required for DNA binding (Nixon et al., 2004). Consistent with previous results on Bright (Schmidt et al., 2009) and DRIL1 (Prieur et al., 2009), mutation of the sumoylation conjugation site had no effect on DNA binding (lane 11).

Chomatin immunoprecipitation (ChIP) was carried out to assess *in vivo* recruitment of Brightlike to these same V_H-associated MARs. Cross-linked chromatin immunoprecipitated from the AB1.2 murine plasmacytoma line (Fig. 5B) was reverse cross-linked, and the deproteineized DNA was PCR-amplified with primers previously shown (Rajaiya et al., 2006) to be specific for Bf150 or Tx125. As shown in Fig. 8C, both anti-Bright and anti-Brightlike precipitates showed significant enrichment relative to preimmune controls for both MARs. Indeed, Brightlike recruitment to the more promoter-proximal, TX-125 MAR, was particularly impressive given its modest protein expression levels relative to Bright (Fig. 3).

Brightlike co-activates Bright-mediated IgH transactivation

Given that Brightlike binds to the S107 V_H1 promoter MARs and interacts with Bright in chromatin, we reasoned that Brightlike would regulate IgH transcription on its own or in combination with Bright. Bright and other nuclear matrix proteins have been shown to most robustly transactivate reporters that are chromatin integrated (Kaplan et al., 2001; Kim and Tucker, 2006). Thus, we utilized NIH3T3 cells that stably maintain a V_H1-S107 distal (Bf150 + Tx125) heavy chain promoter region fused upstream of SV40 promoter-driven firefly luciferase (Fig 9A; Kim and Tucker, 2006; Schmidt et al., 2009). These cells were transiently transfected either with increasing combinations of Bright and Brightlike wild-types and mutants or with constant levels of Bright plus increasing levels of Brightlike wild-types and mutants. Luciferase activities were measured relative to co-transfected Renilla luciferase control and equivalent protein inputs were confirmed by Western blotting (data not shown).

As shown in Fig. 9B, neither full-length Brightlike (BL), BrightlikeΔ6 (BL-Δ6), nor a DNA binding-deficient mutant (BL-Y197A) activated reporter expression. Sumo-I-deficient Brightlike-K284R consistently showed modest (average of ~1.5-fold) trend ($P<.1$) activation. Consistent with previous results (Kim and Tucker, 2006; Schmidt et al., 2009), Bright activated reporter activity 4.5–7 fold. This activity was further stimulated in a dose dependent and statistically significant manner by co-transfection with Brightlike ($p<.01$), with maximal stimulation afforded by the sumoylation deficient form (BL-K284R; $p<.001$). Co-transfections of Bright with either RECKLES-β-deficient (BL-Δ6) or DNA binding-deficient (BL-Y197A) Brightlike achieved no further enhancement over Bright alone.

The results indicate that Brightlike has little transactivation activity on its own but can collaborate with Bright as a co-activator of IgH transcription. Mutant data further indicated that the non-sumoylated Brightlike form is a considerably stronger co-activator and that both interaction with Bright through REKLES-β and with DNA are required.

Discussion

Sequence alignments indicate that *ARID3c* is an ancient gene (Wilsker et al., 2005). Conservation of the 5' UTR of *Brightlike* extends to amphibians and fish. The 3' UTR is less conserved, but it is still highly conserved within mammals. These comparisons support the view that *ARID3c* was created by duplication before the divergence of fish (400 million years ago). Comparison of paralogous sequences flanking the ARID DNA binding domain domains along with the retention by *Brightlike* of REKLES-mediated nuclear export function (Fig. 4) support a more recent divergence of *Brightlike* and *Bright/ARID3A*.

Brightlike transcript amplification required a nested approach, indicative of exceedingly low abundance (Fig. 2). Its absence from EST databases further suggested it was a rare transcript, perhaps only expressed at low levels, in a small subset of cells or after an environmental cue. We identified Brightlike transcripts in murine spleen, thymus, testis, and in human tonsil; i.e., tissues of immunological significance. The majority of cloned amplificons encoded an alternatively spliced form of Brightlike (BrightlikeΔ6) that lacks a highly conserved (RELKES-β) domain, which in addition to NES function endows all ARID3 paralogues the ability for homo/heteromerization and sequence-specific DNA binding (Kim and Tucker, 2006). It cannot be excluded that the apparent dominance of the shorter (by 90 b) BrightlikeΔ6 transcript results from PCR amplification artifact. That caveat notwithstanding, we observed virtually all-or-none accumulation of a single Brightlike isoform in different stages of T cell and B cell differentiation (Fig. 2). Most notable in the context of Bright co-activation, we observed upregulation of the full length form at the near full expense of the RELKES-β-deficient form following activation of follicular B cells by LPS treatment (Fig. 2). It was previously shown that Bright mRNA, protein and DNA binding activity is maximally upregulated in mitogenically stimulated B cells (Webb et al., 1991; Herrscher et al., 1995). However, BrightlikeΔ6 is not predicted to act as a conventional dominant negative, since it neither interacts with Bright (Fig. 5) nor binds DNA (Fig. 8). Choice of μ_m/μ_s heavy chains and CD45 isoforms are well documented examples of pre-mRNA splicing events that play crucial roles in B lineage differentiation events (Hathcock et al., 1992; Bruce et al., 2003). In the Brightlike context, a splicing decision that favors exon 6 exclusion would favor B cell differentiation by increasing the relative levels (Fig. 2) of a Bright transcriptional co-activator—full length Brightlike (Fig. 9). This assumes that mitogenic or antigenic/T cell activation of FO B cells is sufficient to elevate Brightlike to levels required to form functional Brightlike-Bright heteromers. In contrast, the quite dramatic change in Brightlike isoform ratio during T lineage development (Fig. 2) suggests that BrightlikeΔ6 can also function independently of a B cell-specific collaboration with Bright.

MARs in general (Glazko et al., 2003) and IgH-associated MARs in specific (Fernandez et al., 1998) have been linked to chromosomal organization by allowing enhancers to act over large distances via association with the nuclear matrix. Both *in vitro* and *in vivo*, Brightlike binds upstream of the basal IgH promoter at MARs previously shown to bind Bright (Fig. 8; Webb et al., 1991; Herrscher et al., 1995; Rajaiya et al., 2006). BrightlikeΔ6, which lacks critical REKLES-β subdomain functions, fails to bind these MARs. A mutation in a single parologous tyrosine within the eARID domain of Bright (Y330A) or Brightlike (Y197A) disrupts DNA binding function (Nixon et al., 2004; Fig. 8). Because Bright and Brightlike can associate in solution or in chromatin (Fig. 5) and can bind to the same DNA motifs (Fig.

8), it is probable that heterologous Bright/Brightlike/DNA complexes occupy the V_H1 promoter-distal Bf150 and Tx125 MARs. However, co-incubation *in vitro* of various ratios of Bright and Brightlike failed to result in distinct heteromeric complexes in EMSA/antibody super-shift experiments (data not shown). There are several explanations, including, but not limited to, a possible requirement for specific chromatin context, need for additional transacting factors (absent in IVT lysates) or significantly differing affinities, which may preclude formation of heteromeric complexes in the presence of excess probe.

The strongest support for functional Brightlike-Bright promoter interactions came from reporter assays of Fig. 9. We observed that Brightlike, while incapable of transacting expression of V_H1 MARs-driven luciferase on its own, synergized with Bright to significantly increase transcription in NIH3T3 fibroblasts. Brightlike enhancement required both an ability to interact with Bright and an ability to interact with DNA, as mutants in either of these functions were inactive (Fig. 9). While these results provide strong support for a co-activator role for Brightlike via Bright-Brightlike interaction, the situation in B cells is more complicated. A ternary complex of Bright with Bruton's tyrosine kinase (Btk), the defective gene product in X-linked immunodeficiency disease, and with the transcription factor TFII-I is required for maximal Bright-dependent transcription of IgH (Webb et al., 2000; Rajaiya et al., 2006). Our studies in fibroblasts, while demonstrating co-activation in the presence of only two B cell-restricted components, raise fundamental questions as to how Brightlike fits into the ternary complex model in B cells.

Brightlike and BrightlikeΔ6 are sumoylated *in vitro* and *in vivo* (Fig. 6) at a consensus motif conserved in Bright, but not in Bdp/ARID3B (Fig. 1). This indicates that SUMO-I modification differentially diversifies the function of the paralogous members of the ARID3 subfamily. Consistent with what was recently demonstrated for Bright (Prieur et al., 2009; Schmidt et al., 2009), mutation of the sumoylation motif in Brightlike had no effect on nuclear-cytoplasmic localization (data not shown), nor on DNA binding (Fig. 8). However, SUMO-I-deficient Brightlike-K284R was a significantly stronger Bright transcriptional co-activator (Fig. 9). This is in accord with the prevailing model (Gill, 2005) in which sumoylation inhibits transcription factors by relocalizing them to heterochromatin enriched domains.

A second way in which ARID3 function is diversified was uncovered in our analysis of the lipid rafts localization of Brightlike. Antigen receptor stimulation of two responsive human lymphoid lines resulted in discharge of Bright, accumulation of but Brightlike in lipid rafts (Fig. 7). Since Bright discharge is directly correlated with reduced BCR signaling threshold (Schmidt et al., 2009), it is tempting to speculate that the opposite phenotype—an enhanced signaling threshold—results from Brightlike inclusion. We further observed that (1) overexpression of Bright in these same B cell lines eliminated Brightlike inclusion into rafts; (2) that Brightlike was capable of entering lipid rafts in non-B cells in the absence of Bright; (3) but that BrightlikeΔ6 was not. An interpretation of these results that we favor is that the two ARID3 members occupy lipid rafts as homomers. Notably, the SUMO-1-deficient form of Brightlike also is blocked from lipid raft entry (Fig. 7), suggesting that the consequence of Brightlike sumoylation with respect to lipid rafts trafficking is opposite from that of Bright; ie, SUMO-I additional to Bright signals exit and to Brightlike signals entry.

We identify the transcription factor Brightlike as an unsuspected component of the network of ARID transcription factors. Our studies have implicated Brightlike in functions as diverse as a transcriptional coactivator and a potential regulator of early events in BCR signaling. We submit that our findings provide rationale for further investigation of Brightlike localization and function.

Materials and Methods

Computational analysis

ARID3C was identified as an uncharacterized region on mouse chromosome 4 and human chromosome 9 by comparison with Bright and Bdp coding sequences. Genomic sequences were obtained from Ensembl.org. The National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/) was used to compare (BLAST) sequences against the protein and nucleic acid databases. Alignments were generated using ClustalW.

RT-PCR

Tissues or cells were either stored in RNA Later or directly added to Trizol reagent (GibcoBRL). Tissues were homogenized by a polytron homogenizer. Cells were lyzed by repetitive pipetting using a P100. RNA was isolated following Invitrogen's Trizol protocol. RNA was resuspended in DEPC water. After removal of DNA contamination with DNase I for 1 hour at 37°C, the reaction was terminated by adding EDTA (final concentration 2.5 mM) at 70°C for 10 minutes. RNA concentration was determined by using a nanodrop spectrophotometer. SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) was used to make all cDNAs according to manufacturer's protocol. 2–5μg of total RNA was used as template in a 20ul total reaction. Oligo (dT) was used to synthesis all poly-A mRNA transcripts. 2 μl of cDNA was used for Brightlike PCR reactions and 1ul was used for tubulin reactions. For full length amplification an outer PCR reaction was performed using 5' and 3' UTR primers ~50 nucleotides upstream of the start site and stop codon. The UTR reaction was used as a template for the full length open reading frame amplification. Full length primers amplified the open reading frame. Taq polymerase (NEB or Gene Choice) was used with the provided reagents following manufacturer's protocol. Tubulin was used as a positive control in every PCR reaction using cDNA. The PCR products were cloned into Topo 2.1 with the Topo-TA cloning kit (Invitrogen) and the insert was sequenced by the dye termination method (UT sequencing core).

Cell lines

Cell lines were incubated at 37°C and maintained in an atmosphere of 5% CO_2. Adherent cells were cultured in DMEM (GibcoBRL/Invitrogen) supplemented with 10% fetal calf serum (PAA Laboratories), 1mM L-glutamine, 1% non-essential amino acids (GibcoBRL/Invtrogen). Non-adherent cells were cultured in RPMI-1640 (GibcoBRL/Invitrogen) supplemented with, 2 mM L-glutamine, and 10% fetal calf serum. Cells were generally split every 3–4 days.

B cell stimulations

Treatment with LPS (20 μg/ml) was performed for 3 days in complete growth medium as previously described (Herrscher et al., 1995). B cell receptor ligation was achieved following 5 min treatment of ~5×10^8 cells using 500ng of $F(ab')_2$ fragments of anti-IgM (clone JDC-15; Dako) and anti-CD19 (clone HD37; Dako) at 37 °C.

Anti-Brightlike Antibody Production

Anti-Brightlike polyclonal antisera was raised in New Zealand white rabbits against N-terminal (amino acids 4–100) or C-terminal (214–380) portions of BrightlikeΔ6, selected because they lack significant homology to Bright or Bdp. The N- and C-term sequences of Brightlike were cloned into the EcoR1 site of the GST vector pGEX6p1. The fusion proteins were expressed and purified from E. coli, and following 3 immunizations, rabbits were sacrificed and sera collected. Specificity and optimization of anti-Brightlike sera was

confirmed by positive Western signals of NIH-3T3 fibroblasts, transfected with Brightlike and by negative signals when transfected with Bright.

Western blot analysis

Cell lysates were made using RIPA buffer (150mM NaCl, 50 mM Tris pH 7.4, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, 1 mM EDTA) supplemented with protease inhibitors (Roche), incubated on ice for 30 minutes then cleared by centrifugation at 3000 RPM. Bradford (Bio-rad) reagent was used to determine protein concentration of cell lysates. 10 or 12% SDS-PAGE gels were used to separate proteins from cell lysates. 6X SDS loading buffer (300mM Tris, 10% glycerol, 6% SDS, 0.03% bromophenol blue, pH adjusted to 6.8, 30% β-mercapthoethanol) was added to samples and then boiled for 5 minutes before loading proteins into wells. Proteins were transferred to a nitrocellulose membrane (PROTRAN®) using a standard semi-dry transfer apparatus. Membranes were blocked with 5% milk in PBS-T (150mM NaCl, 10mM Tris pH 8, 0.1% Tween-20) at room temperature for 2 hours or overnight at 4°C with agitation. Membranes were incubated with primary antibody for 3 hrs at room temperature or overnight at 4°C with agitation. Membranes were washed twice for 5 minutes and once for 15 minutes with PBS-T (150mM NaCl, 10mM Tris pH 8, 0.05% Tween-20). Membranes were incubated with secondary antibody for 1 hour at room temperature and washed with PBS-T as described above. Blots were developed using ECL Western blotting detection reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The following dilutions were used for each antibody: HA-1:1000, Brightlike- 1:1000, Bright 1:5000, anti-GFP 1:5000, anti-mouse or anti-rabbit 1:8000).

Expression constructs

Brightlike, BrightlikeΔ6, their putative sumoylation or DNA binding mutants were subcloned into PCR3.1 (Invitrogen) and/or pEGFP-C1 (GenBank). Brightlike was PCR amplified with Bgl II flanking primers and cloned into pGEM-t Easy (Promega). The fragment was digested and gel-purified and ligated into the BamH I site of pE-GFP-C1, disrupting both Bgl II and BamH I sites. Digestion with Kpn I was used to determine proper orientation of the insert. 5' Sal I and 3' Not I primers were used to amplify Brightlike from pEGFP-C1 and ligated into PCR3.1-HA cut with Sal I and Not I. DNA sequencing was used to verify all constructs using the dye termination method. Plasmid constructs were grown in appropriate *E. coli* strains and isolated using either Qiagen or Invitrogen minprep kits, according to manufacturer's protocols. DNA was eluted using ddH_2O, warmed to 70°C. Concentrations were obtained using a nanodrop spectrophotometer.

Transient transfections

FuGENE6 (Roche) or Mirus (Mirus Bio Corporation) were used to transfect constructs into fibroblast diploid cell lines (NIH-3T3 and BTR) following manufacturer's protocol. Exponentially growing Jurkat T cells or RAJI and RAMOS B cells (~1.5×10^7) were transfected by electroporation (320V; 975μF) with 20μg of DNA.

GFP fusion protein subcellular localization studies

pEGFP-C1 Brightlike constructs were transiently transfected into NIH3T3 cells grown in either 100mm plates containing coverslips or in 4 well chamber slides. Cells were fed 24 hours post transfection. Images were acquired 48 hours post transfection. Nuclei were visualized by stainig with 1 ng/ml DAPI (4′,6′-diamidino-2-phenylindole) for 5 min at 25°C. Stained sells were mounted with Vectashield mounting media (Vector) containing Hoechst 33342 (final concentration 1μg/ml). Images were acquired using an Olympus IX-70 inverted

microscope, equipped with a 100 watt HBO Hg illuminator and a Diagnostic Instruments Spot RT-KE monochrome cooled CCD camera.

For indirect immunofluorescence analysis, cells (1×10^6) were collected, washed twice with PBS, and then fixed with 500 μl of 4% paraformaldehyde (PFA) for 20 min at 25°C and permeabilized with 0.1% of Triton X-100 in PBS for 15 min at 25°C. After 3 PBS washes, the cells were attached onto slides (Polytech) coated with 1% poly-l-lysine (Sigma), and the slides were immersed first in ice-cold methanol for 5 min and then in ice-cold acetone for 30 s. The cells were blocked with 20% FBS in PBS for 15 min at 25°C and incubated with rabbit anti-Brightlike antiserum (diluted 1:2000). After a washing, the cells were stained with donkey fluorescein isothiocyanate-conjugated anti-rabbit antibody (diluted 1:500; Santa Cruz) for 1 h at 25°C in the dark. Slides were air dried, mounted, and analyzed as described above.

Cell Sorting

Double positive T cells were sorted BD FACSAria using FITC -conjugated anti-CD4 and PE-conjugated anti-CD8 (BD Pharmingen). All remaining thymocytes were labeled as double negative, although other populations of cells remained. Thymuses were taken from 5–6 weeks old C57BL/6 mice. Only sorted cells with higher than 99% purity were used in the experiment. To collect CD4 and CD8 single positive splenocytes, single cell suspensions were generated from pooled spleens from C57B/6 mice, followed by a subsequent magnetic separation of cell populations using anti-mouse CD4 (L3T4) or anti-mouse-CD8a (Ly-2) coated magnetic MicroBeads, according to the manufacturer's instructions (Miltenyi Biotec Inc. Auburn, CA). The resulting CD4 and CD8 cell populations were pelleted and stored at −80 °C until used for RNA isolation. CD43 positive cells were isolated from murine spleen using anti-CD43 MicroBeads, according to manufacturer's protocol (Miltenyi Biotec Inc. Auburn, CA). Unlableled cells were collected as CD43 negative cells. FACS analysis confirmed this fraction to be > 95% B220 positive B cells (data not shown).

Co-Immunoprecipitation

300 mg protein-A beads (Sigma, P3391) were washed in 10 ml of low IPB buffer (25mM Tris pH 7.5, 150mM NaCl, 2mM EDTA, 0.5% NP-40) with 1mg/ml BSA for at least an hour while rotating at room temperature or overnight at 4°C. Beads were washed 2 times in 10 ml low IPB and resuspended in an equal volume of low IPB. Lysates of transfected cells, as indicated in the figures, were pre-cleared with 50% Sepharose A bead slurry. Fresh protease inhibitors (Roche) were added to the 50% slurry before each use. An aliquot of pre-cleared lysate was saved for input lanes. Lysates were precipitated with α-Bright, α-Brightlike, or pre-immune serum over-night at 4°C with the addition of protease inhibitors (Roche) and 12 mM PMSF. Beads were spun down at 3000 RPM and washed with lysis buffer (50 mM HEPES-KOH, pH 7.4, 200 mM KCL, 10% glycerol, 1% NP-40, 1 mM EDTA, 1 mM DTT) 3× 5 minutes. The first 2 washes were supplemented with 1% Trition-X. Beads were resuspended in 30μl of 2X SDS sample buffer (100mM Tris, 25% glycerol, 2% SDS, 0.01% bromophenol blue, pH adjusted to 6.8, 10% β-mercapthoethanol), 10μl extract was used for loading of a lane.

In vitro sumoylation assay

^{35}S-Methionine HA-Brightlike IVT reaction was incubated with or without purified SUMO-1, Ubc9 and SAE1/SAE2 as previously described (Rangasamy and Wilson, 2000; Rosas-Acosta et al., 2005a; Rosas-Acosta et al., 2005b).

Site directed mutagenesis

Stratagene's mutation protocol for the QuikChange II Site-Directed Mutagenesis kit was used to introduce putative sumoylation or DNA binding mutations into pGEM T-easy vectors (Invitrogen) containing Brightlike or BrightlikeΔ6. However we used our own reagents for this standard PCR-based mutagenesis. Phusion DNA polymerase was used with an extension temperature of 72°C for 1 minute/Kb. Pfu HF buffer was used (Stratagene). 10 ng of template was used in the reaction and 125 ng of each Y197A mutant oligonucleotide or 200ng of SUMOK1mutb. Oligonucleotide sequences for the sumoylation mutant (K284R) are: Forward:GCCCGAGCCCAGTAAGGAAAGAGGAGAG; Reverse: CTCTCCTCTTTCCT TACTGGGCTCGGGC and for the DNA binding deficient mutant (Y197A) are: Forward: GAAGTATTTGTACCCAGACGAGTGCGAGACACGGG and Reverse: CCCGTGTCTCGCACTC GTCTGGGTACAAATACTTC. Primers were annealed at 55°C for 30 seconds. 16 cycles were used for the DNA binding mutation reaction and 18 cycles were used for the sumoylation mutation reaction. DNA sequencing by dye termination method (UT sequencing core) was used to confirm the mutations.

In vitro translation

T7 or T3 RNA polymerase was used to synthesize PCR3.1 HA Brightlike or Bright protein, respectively, using Promega's TNT® Quick Coupled Transcription/Translation System according to manufacturer's protocol. The translation reaction was always incubated at 32°C for 2 hours instead of the recommended 30°C. 250ng of PCR3.1-HA Brightlike or Bright constructs were used as a template for the in vitro translation reactions with a total volume of 12.5μl (1/4 of recommended amount) or 1μg in a total volume of 50μl.

Isolation and purity of lipid rafts

500 mg of wet cell pellets of RAJI, RAMOS and BTR cells were washed twice in ice-cold PBS and homogenized in 5ml of 10mM Tris/Cl (pH 7.4), 1mM EDTA, 250mM sucrose, 1mM phenylmethylsulfonyl fluoride and 1μg/ml leupeptin (all from Sigma, St. Louis, Montana) in a tightly fitted Dounce homogenizer using five strokes. The resulting homogenate was centrifuged at 900× g for 10 min. at 4° C, the resulting supernatant was then subjected to centrifugation at 110,000× g for 90 min. at 4° C (Nagamatsu et al, 1992). The membane pellet was resuspended in ice-cold 500μl TNE buffer (10mM Tris/Cl [pH 7.4], 150mM NaCl, 5mM EDTA, 1% Triton X-100 [Sigma], 10× protease inhibitors [Complete tablets, Roche, Indianapolis, Indiana]). Sucrose gradients for the preparation of lipid rafts were assembled exactly as described in an earlier publication (Fuentes-Panana et al, 2005).

Lipid rafts were isolated by flotation on discontinuous sucrose gradients. Membrane pellets were extracted for 30 min on ice in TNE buffer. For the discontinuous sucrose gradient, 1 ml of cleared supernatant was mixed with 1 ml of 85% sucrose in TNE and transferred to the bottom of an ultracentrifugation tube, followed by overlay with 6 ml of 35% sucrose in TNE and 3.5 ml of 5% sucrose in TNE. Samples were spun at 200,000× g for 30h at 4° C; fractions were collected from the top of the gradient and analyzed using for SDS-PAGE/ western blotting. B cell preparations were normalized by re-probing the filter with antibody directed against the lipid rafts-specific marker Raflin. Fibrobast internal normalizations utilized the lipid rafts marker, Ras.

Electrophoretic mobility shift assays

Bf150 or Tx125 probes were digested from 20–5 μg of the following plasmids constructed in our lab: PUC-PCRIL5 or 251-125R by Hind III or EcoR I, respectively. The Erag enhancer used as a negative control was generated by PCR. The PCR fragment (199 bp)

corresponds with Region A of the RAG enhancer (Hu et al., 2006). Digested or PCR products were labeled with γ-ATP^{32} using polynucleotide kinase (New England Biolabs) and gel purified from a 7% polyacrylamide gel. The running buffer for the gel is 1X TBE (90 mM Tris-Borate, 2 mM EDTA). In vitro translated Bright, Brightlike or putative DNA binding mutants were incubated with probes in a binding buffer for 30 minutes at room temperature (20mM HEPES pH 7.9, 40mM KCl, 6mM MgCl2, 1mM DTT, 0.1% NP40, 3mg/ml BSA, 10% glycerol, 2% Ficoll, 50ug/ml of sonicated salmon sperm DNA and protease inhibitor cocktail (Roche)). For super shift reactions anti-HA, anti-Bright or anti-Brightlike was added and incubated on ice for 30 minutes. Pre-immune serum was used as a control. Binding reactions were loaded onto a 4% polyacrylamide non-denaturing gel (6% glycerol in 0.5X TBE) and run overnight at 4°C in 0.5X TBE running buffer. Gels were dried and analyzed by a phosphorimager (Molecular Dynamics).

Chromatin immunoprecipitation (ChIP)

A 20 ml high-density AB1.2 hybridoma culture containing 0.5–0.6 × 10^7 cells was used per IP. Formaldehyde was added directly to culture medium (540 µl added dropwise using a commercial stock solution of 37% HCHO/10% MetOH) and cellular chromatin was crosslinked at RT for 20 minutes with periodic rocking by hand. The reaction was terminated by immediately diluting with a maximal volume of ice-cold PBS supplemented with protease inhibitors. After cell lysis and preparation of nuclei, the chromatin was sonicated with a Branson 250 Sonifier sonicator (90% duty cycle, power setting = 3) using 5 pulses of 15 sec each; the chromatin was incubated 1 min on ice between pulses. An aliquot was reverse crosslinked at 65 °C in the presence of 5M NaCl overnight and analyzed by gel electrophoresis to determine that the chromatin had been successfully sheared to an average size of 200–1000 bp.

Chromatin supernatants were collected and spun at 13,000 rpm at 4 °C for 10 min, and the clarified supernatant was transferred to a new tube and then diluted 1:10 in IP buffer (0.1% SDS, 1% Triton X-100, 1.5 mM EDTA, 150 mM NaCl, 15 mM Tris-HCl pH 8.0, 1× protease inhibitor cocktail, 10 mM NaF, 5 mM Na Butyrate) and were pre-cleared by rocking on a platform with 2 µg sheared salmon DNA and protein A-sepharose (50% slurry in PBS + NaN_3) overnight at 4 °C. The chromatin was immunoprecipitated using 6 µg per IP of affinity-purified rabbit anti-Bright or anti-Brightlike polyclonal antibody or with 6 µg preimmune rabbit IgG obtained from each rabbit as control. Immunoprecipitation was performed overnight at 4 °C.

After washes with sequential buffer solutions, one-third of the beads were removed, spun down, resuspended and boiled in Laemmli loading solution, and run on a 7.5% Western gel to confirm the successful pulldown of Bright or Brightlike. To elute the remaining two-thirds, 250 µl of freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) was added for 15 min at RT on a platform rocker; the elution was repeated once more. Eluates were heated at 65 °C (in the presence of 5M NaCl) for at least 6h to reverse the formaldehyde cross-linking. Chromatin was extracted once with phenol-chloroform, then once more with chloroform only.

A 1.5 µl aliquot (from a total ~ 400 µl) was used for real-time quantitative PCR assays using the following TaqMan primers and probes (Integrated DNA Technologies), previously optimized by Rajaiya et al., 2005, that flanked the ~500bp region spanning the VH1-associated Tx125 and Bf150 MARs: forward: 5′-CTAGATCCACATGTATGATTT-3′; reverse, 5′-GTCTTTCAGACAATAGATTGG-3′); The Cµ ORF was employed as a negative control: forward: 5′-GTATCCAGTGTGAGGTGAAGC-3′; reverse: 5′-GAGCTTCCCATCCTTTAGCCA-3′. Reactions were performed with 96-well plates using the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at

95°C for 15 s and 55°C. Standards were run with every experiment for consistency and quantification of the amplified DNA. Data from triplicate samples were averaged and expressed as fold enrichment using the standardized curve and analyzed using ABI Prism 7700 SDS software (Applied Biosystems).

Luciferase assays

NIH 3T3 cells, stably transfected with Bf150/Tx125-sv40-luciferase (Kim and Tucker, 2006), were plated at 1×10^5 cells per well in 6 well plates. 24 hours later, cells were transiently co-transfected with increasing concentrations (100, 200, and 400 ng) of construct DNAs, with final concentration of 1.5 μg/well achieved by addition of varying amounts of empty vector along with 5 ng of pRL luciferase construct (Promega). Validation of resulting protein concentrations were confirmed by anti-Bright and anti-Brightlike probed western blots employing β-actin as a loading control. The luciferase activity was measured 48 hours post-transfection by using Dual-luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Values were expressed relative to arbitrary 1.00 assigned to the lowest concentration of empty vector after normalization of transfection efficiency, as measured by Renilla luciferase. Values achieved at each DNA concentration were derived from at least 3 independent transfections.

Statistics

For each pair of treatments, a 3-sample t-test was carried out, followed by a multiple testing correction to determine errors. P-values of < 0.05 were considered statistically significant.

HIGHLIGHTS

- We characterized a new transcription factor, Brightlike/ARID3C.
- Two Brightlike isoforms display different functional and localization properties.
- Brightlike stimulates immunoglobulin heavy chain transcription of Bright/ARID3A.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Paul Das for administrative assistance. We thank Chhaya Das, Maya Ghosh June V. Harriss and Melissa Popowski for expert technical assistance. We thank members of our labs for critically reading the manuscript. The work is supported by the Marie Betzner Morrow Endowment and N. I. H. grant CACA31534 (PWT).

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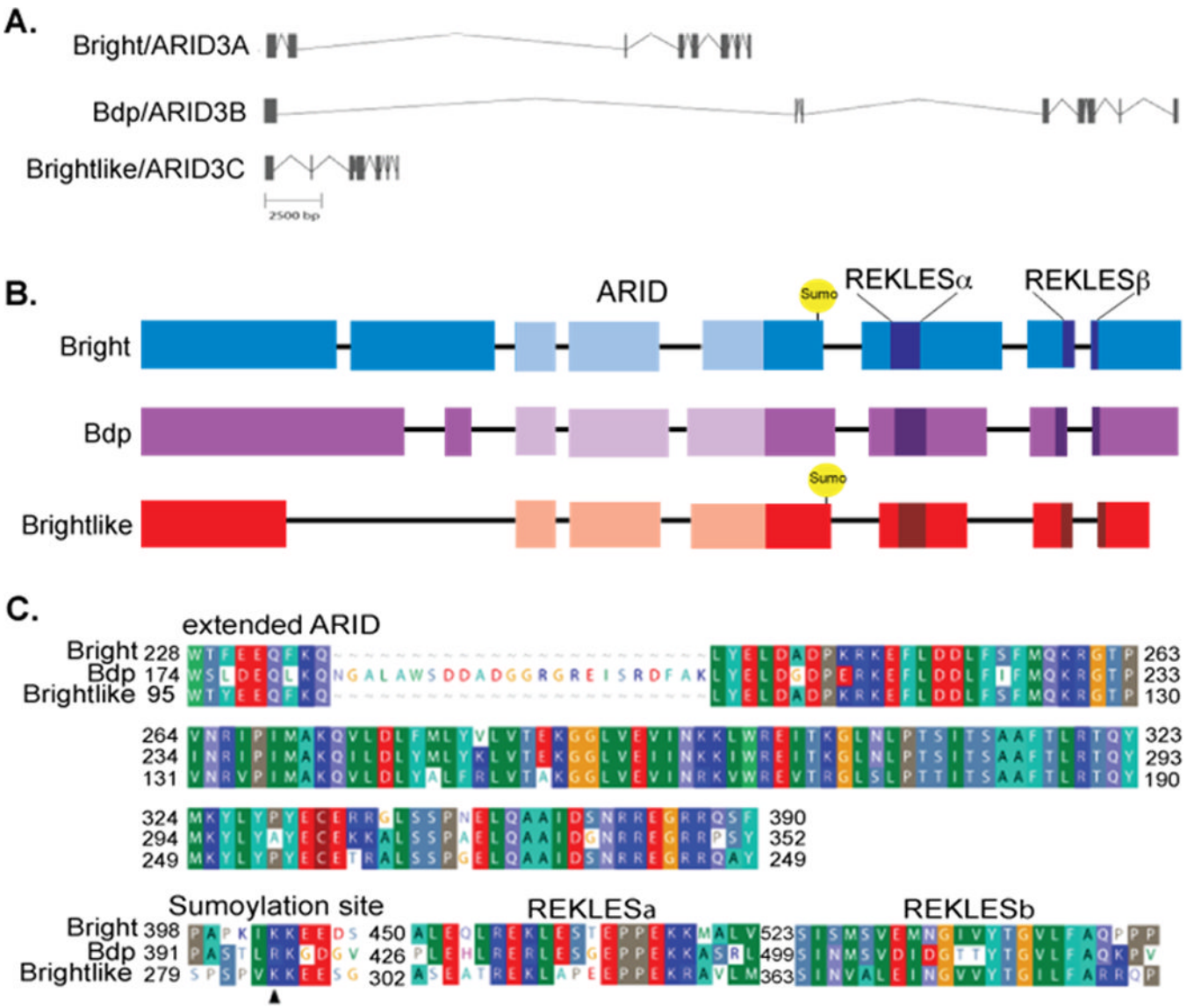

Figure 1. Comparison of ARID3 family genes and domains

(A) Schematic of mouse *ARID3* genomic loci drawn to scale. *Brightlike/ARID3C* is compressed relative to *Bright/ARID3A* and *Bdp/ARID3B*. (B) Alignment (not to scale) of mouse ARID3 exons indicating DNA binding (ARID), SUMO-I conjugation (SUMO) and multifunctional homomerization/nuclear export (REKLES) domains. (C) Amino-acid conservation among ARID3 proteins within essential domains and motifs. Upper panels, ARID domain; lower panel (left), SUMO-I consensus motif with conjugated K (▲); right, REKLES domain, subdivided into NLS-containing (alpha) and NES-containing (beta) regions. Amino acids identities or similarities among paralogues are colored identically.

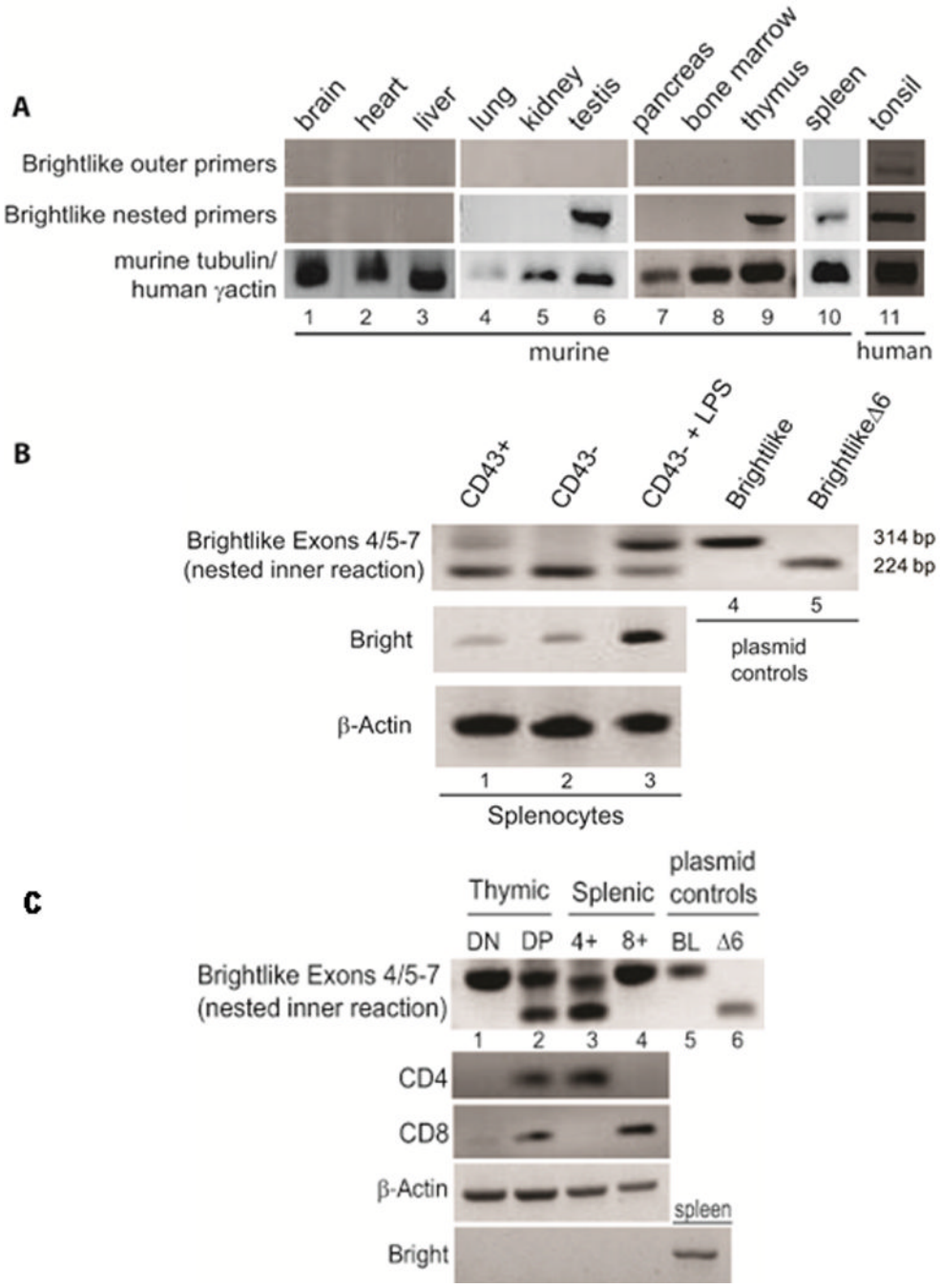

Figure 2. Brightlike is expressed in tissues of immunological significance

(A) Identification of Brightlike mRNA. The open reading frame of Brightlike can be RT-PCR amplified from murine testis, thymus, and spleen and human tonsil (lanes 6, 9, 10, and 11 respectively) after a nested PCR approach. (B) Brightlike expression is upregulated in LPS stimulated murine splenic follicular (FO) B cells. Splenocytes were separated into CD43+ and CD43− populations using CD43 MicroBeads (Miltenyi Biotec Inc). A fraction of CD43− resting FO B cells were cultured for 3 days with LPS. RT-PCR analysis detected BrightlikeΔ6 in all samples (lanes 1–3), whereas Brightlike was upregulated in CD43− splenocytes mitogenically stimulated with LPS (lane 3). (C) Brightlike and BrightlikeΔ6 transcripts are differentially expressed in T cell populations. Double positive (DP) or double

negative (DN) thymocytes were sorted by flow cytometry. Single positive splentocytes were separated using CD4 or CD8 magnetic MicroBeads (Miltenyi Biotec Inc.). cDNA was amplified by RT-PCR using primers that spanned exon 6 to allow size determination between Brightlike and BrightlikeΔ6. Brightlike was amplified in all populations, but BrightlikeΔ6 was only detected in DP thymocytes and CD4+ splenocytes. 1 ng of Brightlike (BL) or BrightlikeΔ6 (Δ6) PCR3.1 expression plasmids served as positive control.

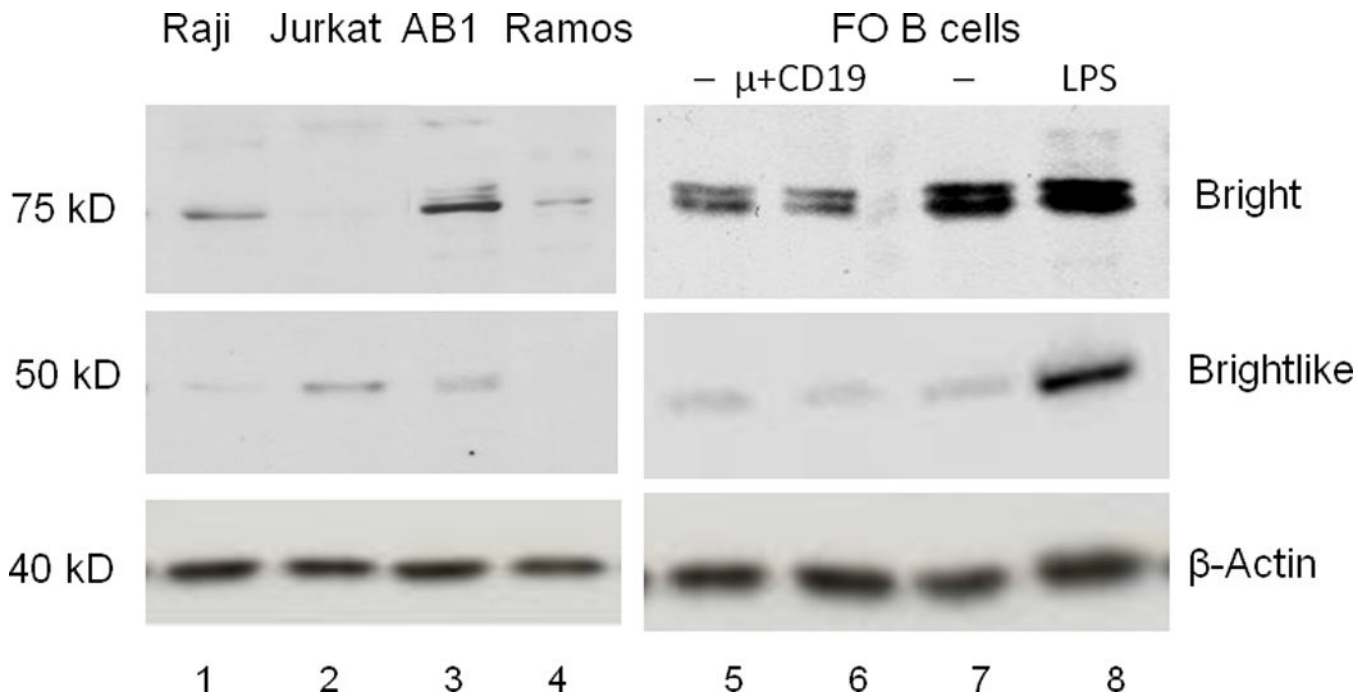

Figure 3. Detection of endogenous Brightlike protein in normal and transformed lymphocytes

Nuclear extracts were prepared from the indicated cell lines (lanes 1–4), and western analysis was performed using polyclonal anti-Brightlike, anti-Bright, or anti β-actin rabbit antisera as described in Materials and Methods. CD43- follicular (FO) B cells were cultured without (lanes 5) or with (lane 6) anti-IgM (α–μ) + anti-CD19 (CD19) or without (lane 7) or with (lane 8) LPS under conditions described in Materials and Methods to activate proliferation and differentiation. Bright and Brightlike are co-expressed in some but not all cell lines, whereas mitogen but not anti-BCR stimulation led to upregulation of both proteins within nuclei of FO B cells.

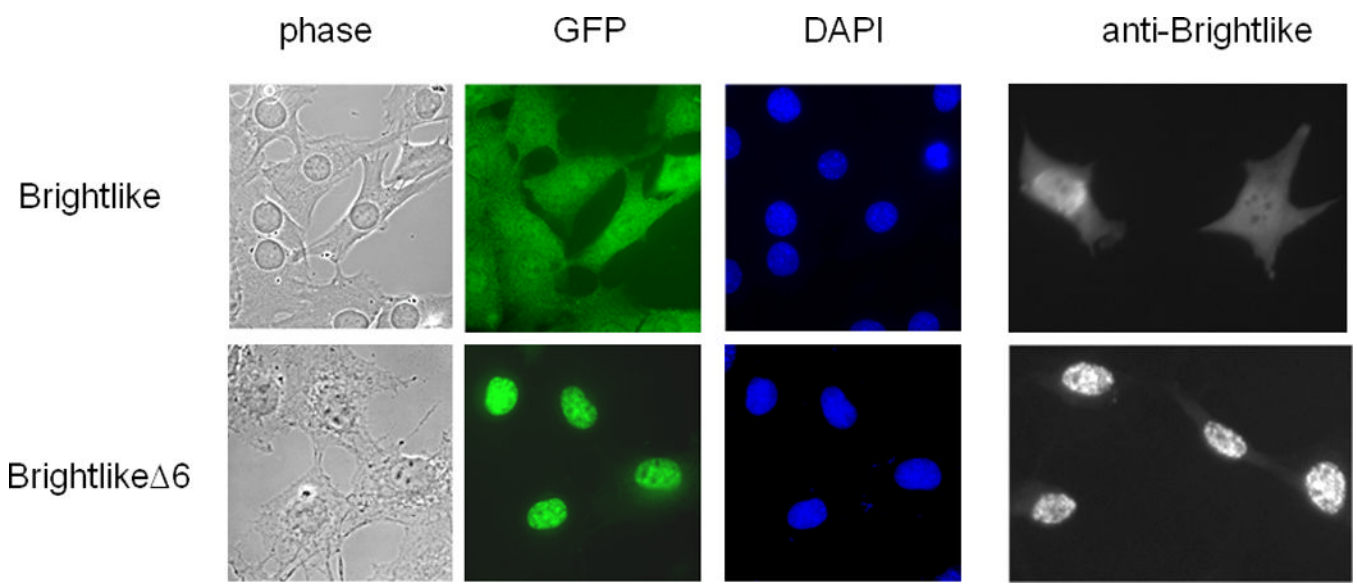

Figure 4. REKLES-β is required for nuclear export of Brightlike

NIH 3T3 cells were transfected with GFP-tagged forms of Brightlike or the REKLES-β-lacking (Δ6) splice variant. 48 hr-post-transfection, fixed cells were analyzed by fluorescence microscopy or by anti-Bright staining. BrightlikeΔ6 was primarily nuclear, consistent with REKLES-β-dependent nuclear export. Brightlike localized to the cytoplasm and nucleus.

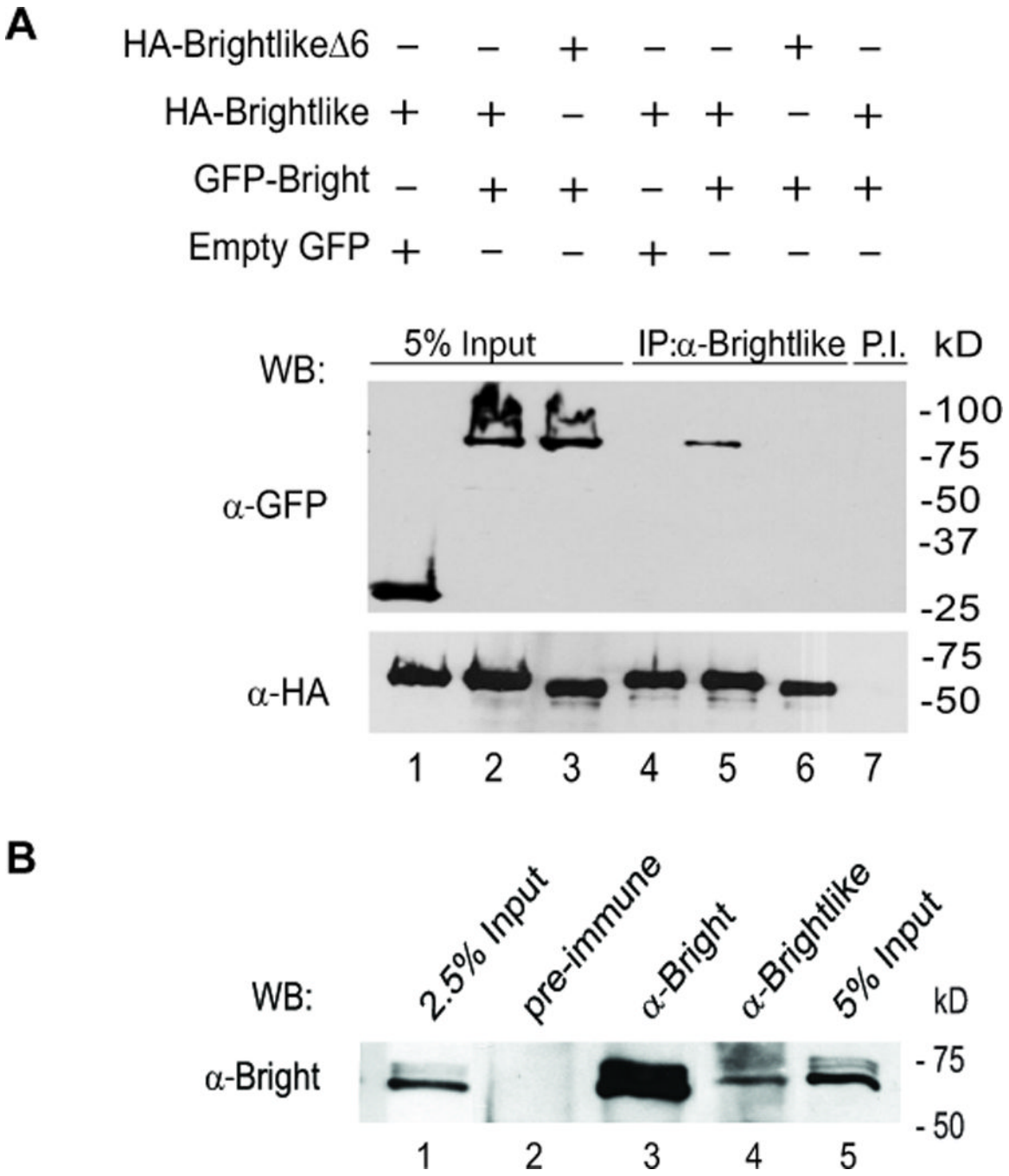

Figure 5. Brightlike, but not BrightlikeΔ6, associates with Bright

(A) 293T cells were transiently co-transfected with GFP-Bright and HA-Brightlike or HA-BrightlikeΔ6. Whole cell lysates were subjected to co-immunoprecipitation (CoIP) with Brightlike antisera or pre-immune serum (P.I.) as a negative control. Western blots were developed with the antibodies indicated at the left. As predicted, Brightlike precipitates Bright (lane 5) and BrightlikeΔ6 does not (lane 6). (B). Chromatin immunoprecipitation of Bright-Brightlike complexes. Chromatin prepared from AB1.2 hybridoma cells, which express high levels of Bright, was cross-linked with formaldehyde and associated proteins were subjected to IP with antibodies (α) against Bright antisera (lane 3), Bright-preimmune antisera (pre-immune, lane 2), or against Brightlike (lane 4). Western blot analysis

developed with anti-Bright indicated that anti-Bright (lane 3) and anti-Brightlike (lane 4) precipitated Bright containing chromatin and pre-immune serum (lane 2) did not.

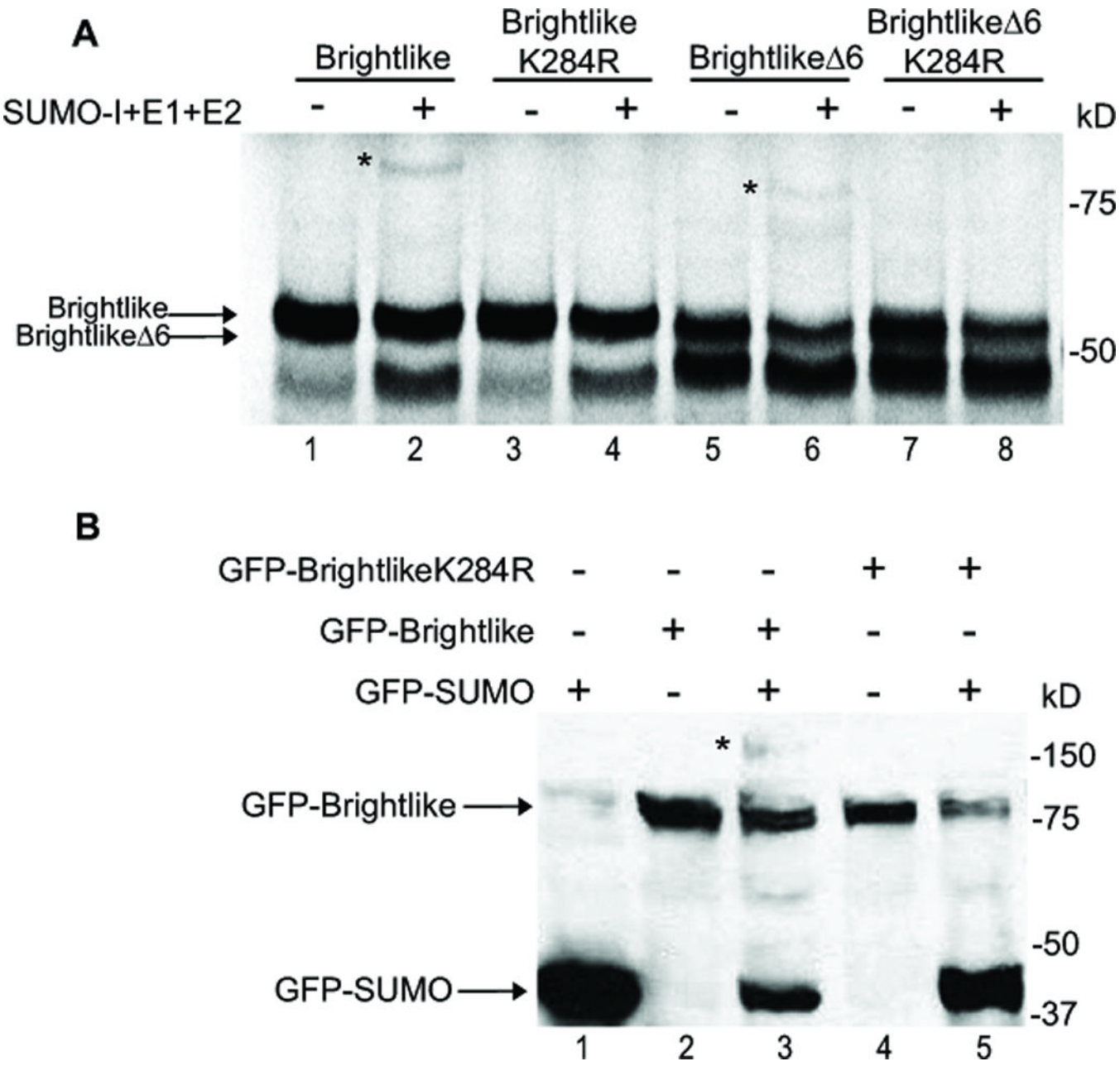

Figure 6. Brightlike is sumoylated within a consensus motif conserved with Bright *in vitro* and *in vivo*

(A) In vitro sumoylation assays were performed using in vitro translated, full length and BrightlikeΔ6 in the presence (+) or absence (−) of SUMO E1 (Sae1/2), SUMO E2 (Ubc9) and Sumo-I as described previously (Schmidt et al., 2009) and detailed in Materials and Methods. Substitution of K284R within the Sumo-I consensus motif (283-ΨKxE/D-286) eliminates the SUMO-I conjugated form (*, lanes 4 and 8). (B) Jurkat cells were transfected with GFP-Brightlike or GFP-BrightlikeK284R +/− GFP-SUMO. Whole cell lysates were analyzed by anti-GFP and anti-Brightlike antibodies. Mutation of the SUMO-I consensus motif (K284R; lane 5) eliminated the SUMO-I conjugated form (*, lane 3).

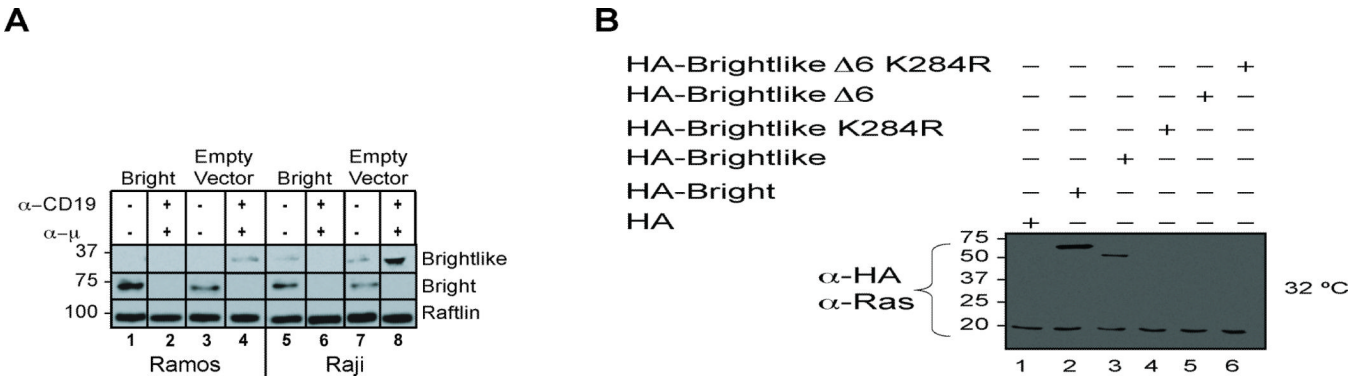

Figure 7. Brightlike accumulates within plasma membrane lipid rafts

(A) B cell antigen receptor ligation drives Brightlike into lipid rafts and Bright out of lipid rafts. RAJI or RAMOS B cells were cultured alone or with anti-IgM (α-μ) + anti-CD19 (α-CD19) with (+ lanes) or without (− lanes) transfection of Bright or with empty vector under conditions previously shown (Schmidt et al., 2009) to stimulate early events of BCR signaling. Lipid rafts were prepared, proteins were fractionated by SDS/PAGE and the membrane was western blotted for the proteins indicated on the right. Loading was normalized to the raflin control. (B) HA-tagged Brightlike, but not HA-BrightlikeΔ6 or mutants deficient in sumoylation (HA-BrightlikeK284R or HA-BrightlikeΔ6K384R) accumulate in lipid rafts of transiently transfected BTR fibroblasts. Endogenous H-Ras was employed as a loading control. Relatively equal levels of transfected proteins were confirmed by Western blots of whole cell lysates (data not shown).

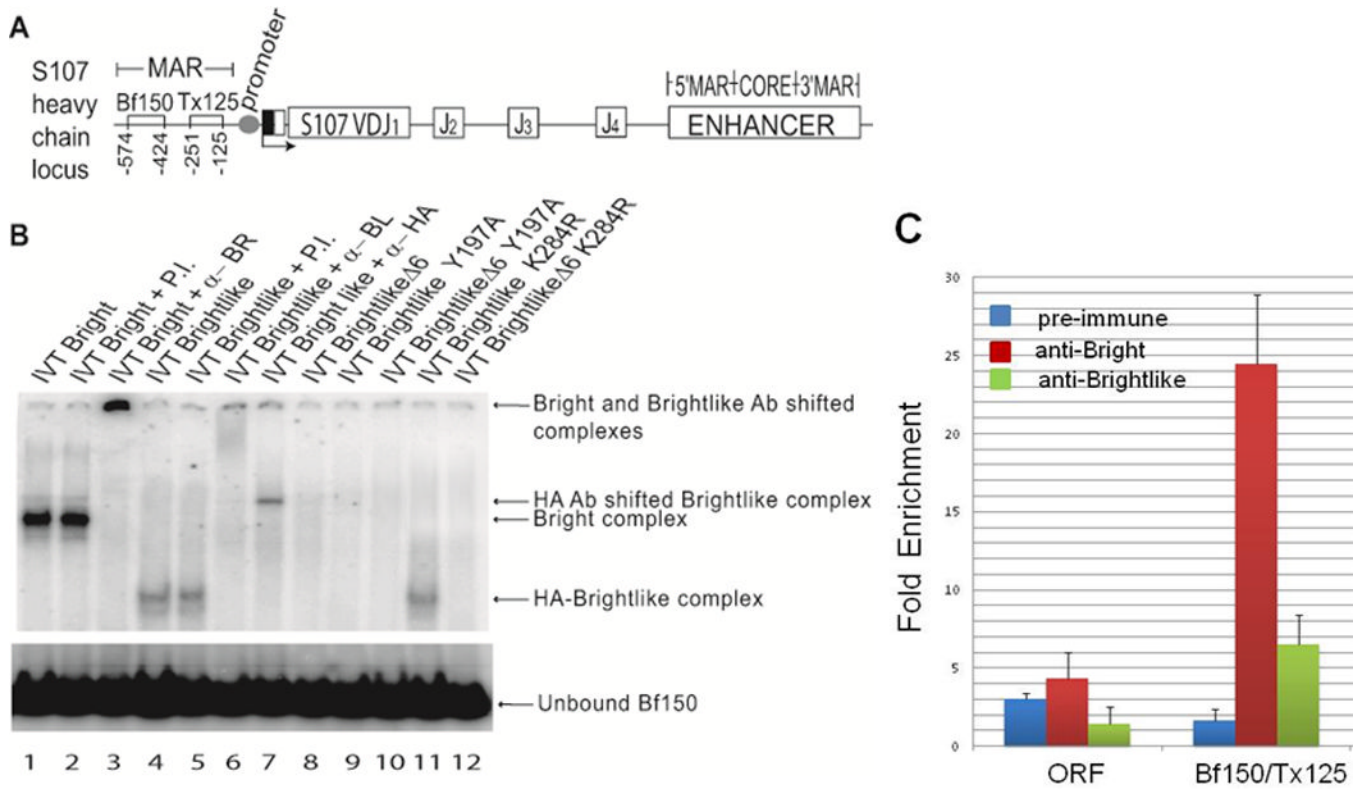

Figure 8. *In vitro* and *in vivo* recruitment of Brightlike to heavy chain variable region-associated MARs

(A) Schematic of the rearranged V_H1-IgH S107 locus (adapted from Herrscher et al., 1995) denotes MARs positioned upstream of the proximal promoter (Tx125 and Bf150) previously shown to bind specifically to Bright and Bdp (Webb et. Al., 1991; Herrscher et al., 1995; Kim and Tucker, 2006). (B). Full length Brightlike, but not mutants lacking a conserved ARID domain residue (Y197A) or deletion mutants lacking a REKLES-β domain, binds to a V_H1-associated MAR *in vitro*. *In vitro* translated Bright, full length, HA-tagged Brightlike, BrightlikeΔ6 or mutants carrying substitutions in an essential DNA binding residue (Y197A) were incubated with Bf150 and subjected to an electrophoretic mobility shift analysis (EMSA). Bright and Brightlike formed protein/DNA complexes that could be super-shifted with anti-Bright (lane 3) or anti-Brightlike (lane 6) or anti-HA (lane 7) antibody, whereas BrightlikeΔ6 (lane 8) and Y197A mutants (lanes 9, 10) did not. (C) Chromatin immunoprecipitation detects recruitment of Bright and Brightlike to V_H1-associated MARs *in vivo*. Cross-linked chromatin, prepared from AB1.2 hybridoma cells, which co-express Bright and Brightlike (Fig. 3), was immunoprecipitated with either anti-Bright (red), anti-Brightlike (anti-BL, green), or the corresponding pre-immune sera (blue). Cross links were reversed, the precipitated DNA was sheared to ~1kbp, and then subjected to quantitative PCR analysis using primers flanking the Bf150-Tx125 promoter region or the Cμ open reading frame (ORF). Highly significant enrichment ($P<.01$ for 3 independent replicas) was observed for both Brightlike and Bright across the Tx125 and Bf150 MAR region but not within the ORF.

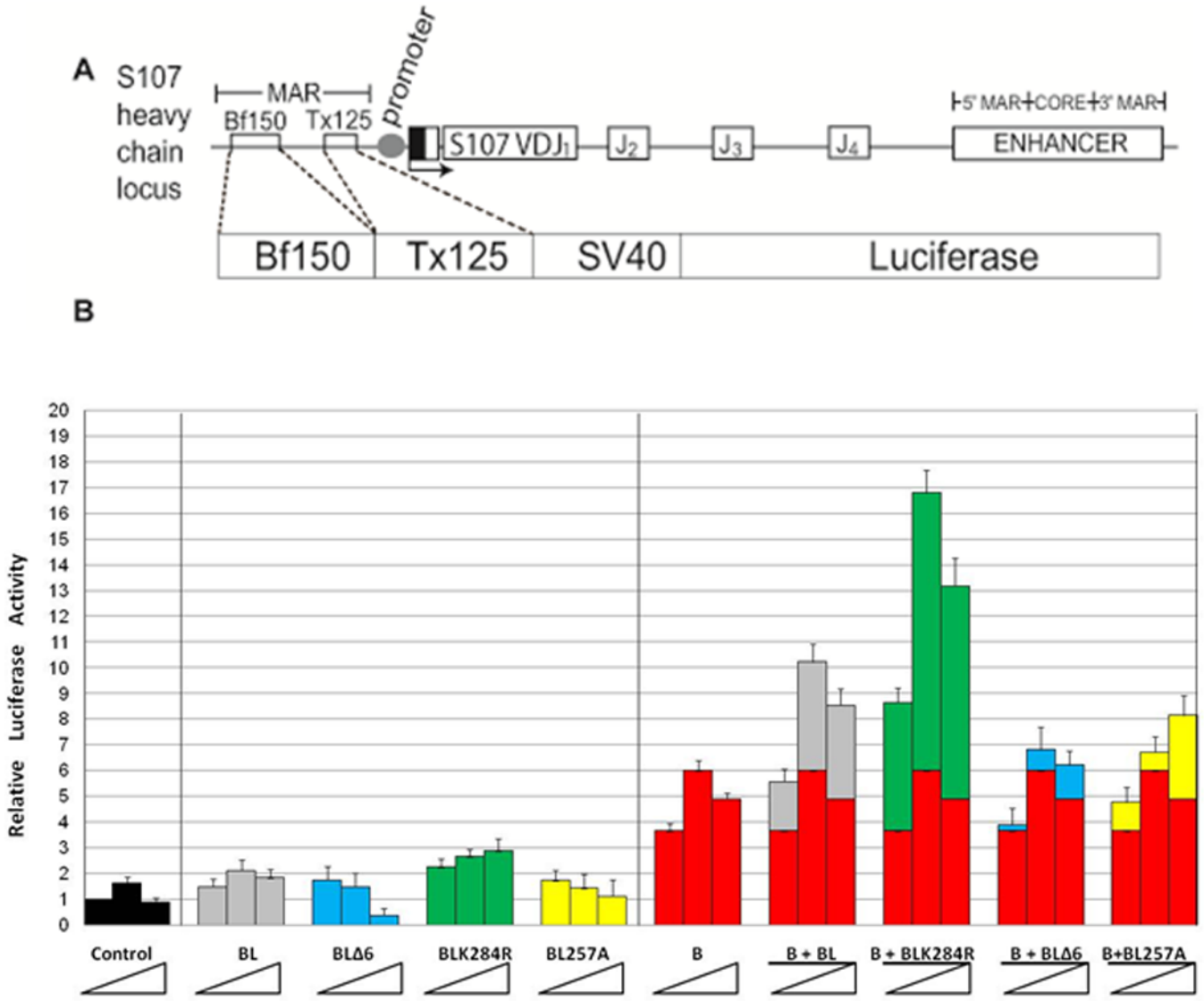

Figure 9. Brightlike enhances Bright transactivation driven by the S107 V_H1 heavy chain promoter-associated MARs

(A) Schematic of luciferase reporter (Kim and Tucker, 2006) containing MARs previously shown (Herrscher et al. 1995) to contain specific Bright binding sites upstream of the S107 IgH variable region. (B) Loss of SUMO-I modification increases Brightlike enhancement of Bright-mediated IgH promoter activity. NIH3T3 cells stably transfected with the luciferase reporter construct of (A) were transiently co-transfected with Renilla luciferase and with increasing DNA concentrations (indicated by triangles and detailed in Materials and Methods) of empty expression vector (Control, back bars), Brightlike (BL, gray bars), BrightlikeΔ6 (BL-Δ6, blue bars), the Sumo-I-deficient mutant, Brightlike-K284R (BL-K284R, green bars), Bright (B, red bars) or a DNA-binding deficient Bright mutant (B-Y257A, yellow bars). To test the potential combined effect of Bright and Brightlike, the above transfections were repeated with the addition in each case of a Bright at the lowest DNA concentration (indicated by the straight lines + the triangles). Western blotted confirmed that increasing transfected DNA increased Bright and Brightlike protein levels accordingly (data not shown). Firefly luciferase activities were measured and normalized to Renilla luciferase activities. Values are plotted relative to 100% for the empty vector control

at lowest DNA concentration. Corresponding bar colors indicate enhancements. Results and error bars are representative of 3 independent experiments for each DNA concentration. Bright consistently activated reporter expression at levels previously documented for this system (Kim and Tucker, 2006). Neither Brightlike, BrightlikeΔ6, Brightlike-K284R, nor Brightlike-Y257A showed statistically significant luciferase activity above control alone, but Brightlike ($P<.01$) and Brightlike-K284R ($P<.001$) significantly co-activated Bright levels.