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<td>Authors</td>
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Cite this information as follows: Carrie N. Arnold and Elaine Pirie, Nora G. Smart, Beutler B. Record for busy, updated Sep 19, 2018. MUTAGENETIX (TM), B. Beutler and colleagues, Center for the Genetics of Host Defense, UT Southwestern Medical Center, Dallas, TX. URL: mutagenetix.utsouthwestern.edu
The *busy* mutation was discovered while screening N-ethyl-N-nitrosourea (ENU)-mutagenized G3 mice for aberrant T-dependent and T-independent B cell responses. The index mouse mounted no detectable T-independent immunoglobin M (IgM) response to haptenated ficoll, and a sub-optimal T-dependent IgG response to model antigens encoded by a recombinant suicide vector based on the Semliki Forest Virus (rSFV). Flow cytometry analysis of blood from this mouse revealed a severe reduction in cluster of differentiation (CD) 19\(^+\) cells indicating a lack of peripheral B cells, and significantly increased frequencies of blood CD4\(^+\) and CD8\(^+\) T cells. A male sibling of the *busy* mouse had normal frequencies of peripheral blood lymphocytes and a normal T-dependent IgG response, but a low T-independent IgM response.

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Whole genome sequencing of a homozygous *busy* mouse using the SOLiD technique identified a T to A transversion at base pair 41026881 on Chromosome 19 using NCBI m37 mouse assembly (Build 37.1) located in the *Blnk* gene. The mutation was confirmed using standard Sanger sequencing (Figure 2), and corresponds to nucleotide 1044 of the *Blnk* transcript in exon 7 of 17 total exons using Genbank record NM_008528. Multiple *Blnk* transcripts are displayed on Ensembl.

```
1027  GATAACGATGAAAACCTATATCCATCCCAGAGAA
184   -D--N--D--E--N--Y--I--H--P--R--E--
```

The mutated nucleotide is indicated in red lettering, and converts a tyrosine at amino acid 189 of the encoded protein to a stop codon.

**Protein Prediction**

The *Blnk* gene encodes a 457 amino acid protein known as B cell linker (BLNK), Src homology 2 (SH2) domain-containing leukocyte protein of 65 kDa (SLP-65), or B cell adaptor containing a SH2 domain (BASH) ([1-3](#)), and functions downstream of the pre-B cell receptor (pre-BCR) and the B cell antigen receptor (BCR; see Background). BLNK belongs to the SLP-76 family of adaptor proteins that includes SLP-76, BLNK and cytokine-dependent hematopoietic cell linker (CLNK) [reviewed by (4;5)]. Mouse BLNK shares 85% identity with the 456 amino acid human BLNK, but mice lack the alternatively spliced product found in humans known as BLNK-S and missing residues 203-225 ([1](#)).

The BLNK protein is composed of a basic region comprising the first 45 amino acids, followed by an acidic region at residues 46-110, a central proline-rich region at amino acids 111-345, and a C-
terminal SH2 domain at residues 346-454 (1-3;6)(Figure 3). The basic region is predicted to contain a leucine zipper (LZ) with leucine or isoleucine seven-residue (abcdefg) repeats forming a potential amphipathic α-helix (6). Positions ‘a’ and ‘d’ in each repeat are typically a hydrophobic residue (mainly leucine or isoleucine) and positions ‘e’ and ‘g’ usually are charged amino acids (7). The BLNK LZ is necessary for subcellular localization (see Expression/Localization) and function of the protein. Mutation of residues Leu 18 and Ile 25 abrogated both of these functions (6). The BLNK N-terminal domain has been shown to bind to the chicken proteins BASH N terminus associated protein 1(BNAS1) and BNAS2. Both of these proteins contain LZ domains that likely interact with the BLNK LZ and are either transmembrane (BNAS2) or membrane-associated (BNAS1) proteins localized to the endoplasmic reticulum (ER) (8;9).

Figure 3. The BLNK protein is composed of a basic region, an acidic region, a central proline-rich region, and a C-terminal SH2 domain. The basic region contains a leucine zipper (LZ), and five canonical tyrosine phosphorylation motifs surround Tyr 72, Tyr 84, Tyr 96, Tyr 178, and Tyr 189 within the acidic and proline-rich regions. Phosphorylation of these tyrosines by the spleen tyrosine kinase (Syk) induces associations with SH2 domain-containing molecules. BLNK also binds to Syk through its SH2 domain which also also associates with Ig? and the hematopoietic progenitor kinase (HPK)1. The position of the busy mutation is shown with a red asterisk.

SH2 domains consist of about 100 amino acids and can function as regulatory modules of intracellular signaling cascades by interacting with high affinity to phosphotyrosine-containing target peptides in a sequence-specific manner (10). The acidic and proline domains of BLNK contain five canonical YXXP tyrosine phosphorylation motifs surrounding Tyr 72, Tyr 84, Tyr 96, Tyr 178, and Tyr 189. Phosphorylation of these tyrosines by the spleen tyrosine kinase (Syk) induces associations with SH2 domain-containing molecules such as Vav1-3, which are guanine nucleotide exchange factors (GEFs) for the Rho family of GTP binding proteins, Nck (non-catalytic region of tyrosine kinase adaptor protein), Bruton’s tyrosine kinase (Btk), and phospholipase C ? 2 (PLC-? 2; see the record for queen) (1;2;11-15). Tyr 84, 178 and 189 of BLNK bind PLC-? 2, Tyr 96 binds to Btk, and
Tyr 72 binds to both Vav and Nck (2;14). The proline-rich region of BLNK binds to the SH3 domains of growth factor receptor-bound 2 (Grb2) adaptor protein using an atypical SH3 domain-binding motif (PxxxRxxKP) (1;2;16;17). This motif is missing in the short form of the human protein, which is unable to bind to Grb2 (17). The coordinate binding of these factors to BLNK promotes interactions amongst them and is necessary for the full signaling response downstream of the pre-BCR and BCR (14;15). Recently, phosphorylated Tyr 96 was also found to be critical for binding to and inhibiting the SH2 domain-containing tyrosine kinase Janus kinase 3 (JAK3), while the adjacent tyrosines 84 and 119 had minor roles (18).

In addition to being a Syk substrate, BLNK binds to an autoprophosphorylated tyrosine located near the C-terminus of Syk through its SH2 domain, an association that is required for prolonged Syk activation (19). The SH2 domain of BLNK also associates with the phosphorylated, non-ITAM (immunoreceptortyrosine-based activation motif) Tyr 204 of Ig?, a signal-transducing component of the pre-BCR and BCR (20;21), and the hematopoietic progenitor kinase (HPK)1 (22;23). Like other SH2 domains, the SH2 domain of BLNK contains a central hydrophobic anti-parallel ?-sheet, flanked by 2 short ?-helices (PDB 2EO6)(Figure 4). The loop between strands 2 and 3 in the SH2 domain provides many of the binding interactions with the phosphate group of phosphopeptide ligands, and is known as the phosphate binding loop. The phosphorylated ligand binds perpendicular to the ?-sheet and typically interacts with the phosphate binding loop and a hydrophobic binding pocket that interacts with the phosphorylated tyrosine motif (10).
The phosphorylated tyrosines of BLNK are dephosphorylated by the SH2 domain-containing phosphatase 1 (SHP1; see the record for spin) (24;25). Via Grb2, BLNK is associated with the type II transmembrane protein CD72, which also binds to SHP1 (16). A nine amino acid region located within the proline-rich region of chicken BLNK, corresponding to residues 116-125 in the mouse protein, was found to bind to the activated GTPase H-Ras (26).

Chicken BLNK is phosphorylated on multiple serine and threonine residues. Some of these residues are phosphorylated in resting cells, while others become phosphorylated upon BCR activation. The pattern of BLNK phosphorylation over time is highly complex and dynamic. One of these residues Ser 170 (151 in mouse), was found to be necessary for the activation of the mitogen activated protein (MAP) kinases p38 and c-Jun N-terminal kinase (JNK), which are important in activating the activator protein 1 (AP-1) transcription factor composed of c-Jun and ATF2 (27).
The **busy** mutation causes a truncation of the BLNK protein at one of the PLC-2 binding tyrosines prior to the proline-rich region and the SH2 domain. It is unknown if the premature truncation results in nonsense-mediated protein degradation.

**Expression/Localization**

Human **BLNK** mRNA is predominantly expressed in the spleen, although expression is also detected in liver, kidney, and pancreas. Both human isoforms are expressed at equal levels (1). Expression data from **SymAtlas** shows that **BLNK** mRNA is also highly expressed in human dendritic cells (DCs). In the mouse, **Blnk** mRNA is expressed in the spleen and weakly in the thymus, while no expression was detected in liver, testis, or brain (2). Expression was also seen in multiple human and mouse B cell lines including those representing different developmental stages from the pre-B to the mature B cell stage. **Blnk** mRNA or protein was not detected in T cells or other hematopoietic or nonhematopoietic-derived cell lines (1;2). B cell expression was confirmed by FACS analysis. Analysis of murine bone marrow-derived cells using FACS analysis showed that the highest BLNK expression occurred during early B cell development, with progressively lower expression occurring as the cells matured (28). BLNK is also expressed in murine macrophages (29).

BLNK is mainly cytoplasmic, but some protein is constitutively localized to the membrane via the leucine zipper domain (6). BLNK may also be recruited to the membrane by direct binding of its SH2 domain to the Ig? subunit of the BCR (20;21). Engagement of the pre-BCR or BCR recruits BLNK into lipid rafts, specialized membrane micro-domains that allow the clustering of molecules necessary for signaling (15;30).

**Background**

The SLP-76 family of adaptor proteins is composed of proteins critical for integrating numerous signaling cascades downstream of ITAM-bearing receptors and integrins in hematopoietic cells. BLNK was first identified and cloned based upon its phosphorylation downstream of BCR stimulation (1-3;31). Furthermore, **Blnk** knockout mice display a block specifically in B cell development and defects in BCR signaling, suggesting a B cell specific role (28;32-34). In T cells, the functions performed by BLNK appear to be split between LAT (linker for activation of T cells) and SLP-76 (5). Indeed, BLNK-deficient B cells can be rescued by coexpressing SLP-76 and LAT (35). Similar results are observed when the third member of the SLP-76 family of adaptors, CLNK, is coexpressed with LAT in BLNK-deficient cells (36). CLNK is expressed in activated T cells, NK cells, and mast cells, but is dispensable for normal immune function (37).
Figure 4. Pre-BCR and BCR signaling. Pre-BCR and BCR engagement result in the activation of Syk. In pre-BCR signaling, Syk phosphorylates many substrates and triggers signaling pathways that are involved in both proliferation and differentiation of pre-B cells. Pre-BCR engagement results in the activation of SYK (spleen tyrosine kinase), which together with Src-family kinases (LYN, FYN, BLK), phosphorylates many substrates and triggering signaling pathways that are involved in both proliferation and differentiation of pre-B cells. These include activation of phosphoinositide 3 kinase (PI3K) by phosphorylating the coreceptor CD19 and/or the adaptor protein B-cell PI3K adaptor (BCAP). PI3K activation results in the generation of phosphatidylinosital-3,4,5-triphosphate (PIP$_3$), which recruits plekstrin-homology domain signaling molecules to the membrane including the serine threonine protein kinase B (PKB) and its activating kinase 3- phosphoinositide-dependent protein kinase 1 (PDK1). Signaling through this pathway pathway suppresses recombination-
activating gene 1 (RAG1) and RAG2 expression, blocks Igk (the k chain of the immunoglobin light chain) gene recombination and induces cell proliferation. SYK also phosphorylates SH2-domain containing leukocyte protein of SLP65, resulting in the organization of a molecular complex that includes Bruton's tyrosine kinase (BTK) and phospholipase Cy2 (PLCy2). This complex controls downregulation of l-5, a component of the surrogate light chain (SLC), and upregulates the expression of RAG proteins and the interferon-regulatory factor 4 (IRF4). IRF4 positively regulates Igk recombination. SLP65 also modulates PKB activity either directly or by altering the activity of SYK, CD19, or PI3K. Alternatively, SLP65 may regulate PKB activity by activating lipid phosphatases such as SH2-domain containing inositol-5 phosphate (SHIP) and altering PIP3 levels. Syk-mediated signaling downstream from the BCR regulates cell proliferation, differentiation, and apoptosis as well as the secretion of antigen-specific antibodies. Please see the text for more details on these signaling pathways. This image is interactive. Click on image to view additional mutations.

The BCR is a multi-subunit complex that is composed of a membrane-bound Ig molecule that binds foreign particles, and the signal transducing Ig? (CD79a)/Ig? (CD79b) heterodimer. Multiple downstream signaling pathways are activated by BCR stimulation and lead to a multitude of cellular responses including BCR cap formation, antigen internalization, T cell-independent B cell proliferation and antibody production, as well as antigen presentation and the T cell-dependent antibody response [reviewed by (38;39)]. Following aggregation and localization of BCR molecules into the cap, the ITAMs located on the cytoplasmic tails of Ig? and Ig? become phosphorylated by Src family kinases (typically Lyn) and by Syk (39;40). These phototyrosines then act as docking sites for the SH2 domains of Syk, resulting in Syk phosphorylation and activation. Syk phosphorylates a number of downstream targets including BLNK, PLC-?2 and protein kinase C ? (PKC?; see the record for Untied). BCR stimulation also activates the p85/p110 form of phosphatidylinositol 3 kinase (PI3K) resulting in the generation of 3?-phosphorylated phosphoinositides. One of these lipids, phosphatidylinositol-3,4,5-triphosphatase (PIP3), binds selectively to the pleckstrin homology (PH) domain of Btk, facilitating membrane recruitment of the kinase. Phosphorylated BLNK also provides docking sites for Btk, as well as PLC-?2, which results in the additional phosphorylation and activation of PLC-?2 by Btk leading to the hydrolysis of phosphatidylinositol-3,4-diphosphatase (PIP2) to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (12). Soluble IP3 and membrane-bound DAG initiate downstream signal transduction pathways involving calcium (Ca2+) mobilization and PKC, respectively. The recruitment of Vav, Nck and Ras by BLNK to the BCR activates MAP kinase cascades such as JNK, p38 and extracellular signal regulated kinase (ERK) [reviewed by (5)]. Together, these signals allow the activation of multiple transcription factors, including nuclear factor of activated T cells (NF-AT), nuclear factor (NF)-? B (see the records for xander and panr2) and AP-1, which subsequently
regulate biological responses including cell proliferation, differentiation and apoptosis, as well as the secretion of antigen-specific antibodies [reviewed by (41)]. Other molecules that play important roles in BCR signaling include Bcl10, mucosa-associated lymphoid tissue translocation gene 1 (MALT1 or paracaspase), and caspase recruitment domain family, member 11 (CARMA1 or CARD11; see the record for *king*), which are involved in NF-κB activation along with PKC? (42-48).

Naïve B cells are generally divided into three subsets, B-1 B cells, follicular B cells (conventional B-2 cells), and marginal zone (MZ) B cells. B cell development first begins in the fetal liver, but conventional B-2 cells and MZ B cells are produced after birth and replaced in the bone marrow throughout life. The origins of B-1 cells are controversial, but they may derive from progenitors present in the fetal liver and neonatal bone marrow [reviewed by (49)]. While conventional B cells mediate secondary immune responses and secrete immunoglobulin G (IgG), B-1 cells predominantly secrete IgM, IgG3 and IgA (50). The development of B cells in the bone marrow is characterized by the differential expression of marker proteins such as CD45 (B220) (see the record for *belittle*) and CD43, and sequential recombination of immunoglobulin gene loci [reviewed in (38)]. In addition, the development of early B lymphopoiesis is regulated by a network of key transcription factors that include PU.1 (an ets-family member), Ikaros, Bcl11a (a zinc finger transcription factor), E2A (a helix-loop-helix protein), EBF (early B cell factor) and the paired box protein, Pax5 (51), which has been shown to directly regulate Blnk transcription (52). B cell development begins when lymphoid progenitor cells or prepro-B cells receive signals, such as interleukin 7 (IL-7), from bone marrow stromal cells. During the pro-B stage, these cells rearrange their immunoglobulin heavy (IgH) chains in a process known as VDJ recombination mediated by the RAG1 (recombination activating gene 1)-RAG2 complex (see the record for *maladaptive*). The diversity (D) and joining (J) gene segments are first recombined together, followed by the variable (V) segment. Although B lineage cells can undergo VDJ rearrangement at both IgH loci, only one of the IgH alleles is expressed by the B cell, a process known as allelic exclusion. Successful VDJ recombination gives rise to the Ig? chain, two of which combine with the surrogate light chains (SLCs), ?-5 and Vpre5, and the signaling subunits Ig? and Ig? to complete the pre-BCR complex. Large pre-B cells expressing the pre-BCR are competent for pre-BCR signaling, which initiates proliferation, further differentiation, and eventually downregulates expression of the pre-BCR. Subsequently, rearrangement of the immunoglobulin light (IgL) chain by the RAG1-RAG2 complex occurs to form the BCR (or surface IgM) characteristic of immature B cells. Receptor editing through successive rearrangements of Ig genes at this stage is a major mechanism for negatively selecting self-reactive B cells. Transition of the cells into fully mature B cells requires BCR signaling and is associated with migration from the bone marrow to the spleen and lymph nodes (38).
Blocks in B cell development are observed in several mouse mutant models, including animals that are deficient for factors necessary for VDJ recombination, components of the pre-BCR complex, and the pre-BCR signal transduction machinery. While mutations in \textit{Pax5}, \textit{Rag1}, \textit{Rag2}, \textit{Ig?}, \textit{Ig?}, and \textit{Ig?} lead to an absolute block in B cell development during the pro-B stage (38;51;53;54), mutations in the SLC and pre-BCR signal transduction components, including Btk, Syk, BLNK, the p85 subunit of PI3K and PLC-?, result in an incomplete block at the pre-B cell transition (38;55;56). As the pre-BCR signal transduction molecules are also needed for BCR signaling, mice deficient in these proteins display an additional partial or complete block (in the case of Syk-deficient mice) at the immature B cell stage [reviewed by (38;57)]. The partial developmental blocks observed in BLNK-deficient mice suggest that BLNK is dispensable for some aspects of BCR signaling. Indeed, studies of BLNK-deficient primary B cells have shown partially preserved signaling downstream of the BCR including reduced, but not absent, levels of phosphorylated PLC-? 2 and Ca$^{2+}$ flux, and the retention of some MAPK signaling (28;32;58). In addition, BLNK is not needed to establish allelic exclusion in B cells (59), but is necessary for light chain rearrangement and receptor editing (60). One explanation of these phenotypes includes possible partial redundancy with other adaptors or molecules during pre-BCR and BCR signaling. BLNK deficiency, combined with deficiencies in the adaptor molecule LAT, the BCR costimulatory molecule CD19, Btk, or PLC-?, result in much more severe blocks in B cell development than in the single mutants alone (61-63). Although pre-BCR signaling is necessary for pre-B cell proliferation, it is also necessary for the eventual differentiation of these cells. Because of this, many of the mouse models with blocks at the pre-B cell transition display high incidences of pre-B cell lymphoma. 5-15\% of \textit{Blnk} knockout mice develop pre-B cell leukemia and BLNK-deficient pre-B cells display an increased proliferative capacity that is IL-7 and pre-BCR dependent (61;64). IL-7 signaling activates the JAK3/STAT5 (signal transducer and activator of transcription 5) pathway, which is inhibited by BLNK (18).

As in mice, humans with mutations in the \textit{BLNK} gene display a developmental block during B cell development (65;66). This block appears to be more complete in humans with patients displaying normal numbers of pro-B cells, but no pre-B cells or mature B cells (65), although a very small number of peripheral B cells with an immature phenotype have been reported for some patients (66). In humans, mutations in \textit{BTK}, \textit{BLNK}, \textit{?-5}, \textit{Ig?}, \textit{Ig?}, and \textit{Ig?} cause agammaglobulinemia and severe immunodeficiency (OMIM #300755, #601495) [reviewed by (66)]. Many leukemias are also caused by mutations in these genes as well (38), and a subset of childhood pre-B-cell acute lymphoblastic leukaemia (pre-B-ALL) cells lack expression of BLNK, suggesting BLNK may play an important role as a tumor suppressor in this disease (67;68).
Putative Mechanism

B cell responses are classified as T-dependent (T-D) or T-independent (T-I) based on their requirement for T cell help in antibody production. T cell-dependent antigens are processed and presented to helper T cells via the MHC class II molecules, and induce a long-lasting immune response that includes the formation of memory B and T cells, and the production of high-affinity antibodies of multiple isotypes. T-I antigens are divided into type I and type II. The former are mitogenic stimuli such as lipopolysaccharide (LPS), CpG DNA, or poly-IC (a double-stranded RNA mimic) that elicit polyclonal B cell activation via Toll-like receptors (TLRs), while the latter are polysaccharides that cannot be processed and presented by MHC molecules. These antigens are often expressed on the surface of pathogens in an organized, highly repetitive form that can activate specific B cells by cross-linking of antigen receptors. The formation of antigen receptor clusters can recruit and activate multiple Btk molecules, resulting in long-term mobilization of intracellular ionized Ca\(^{2+}\), gene transcription and B cell activation and proliferation. T-I type II antigens elicit robust antigen-specific primary and memory responses. The T-D B cell response is mediated by conventional B-2 cells, while T-I B cell responses are mediated by peritoneal B-1 and MZ B cells [reviewed by (69;70)].

BLNK-deficient animals are reported to have a severe defect in their primary antibody response with severely reduced levels of serum IgM and IgG3, but a fairly normal secondary response with only mildly affected levels of IgG1 and IgG2 (28;32-34). Furthermore, these mice are unable to mount T-I type II responses, but can respond to T-dependent antigens (32;34). These results are likely due to the complete absence of B-1 cells reported in these animals, while the presence of some peripheral, albeit immature, B-2 cells allows the development of a T-D response. Further analysis of the T-D response in Blnk knockout mice revealed a defect in the primary antibody response with suboptimal antibody formation, but normal affinity maturation and memory B cell generation (71). These findings are consistent with the phenotypes observed in busy mice, which completely lack a T-I response but display a suboptimal T-D response. B cell proliferation and the generation of the T-I, but not the T-D antibody response, depends upon the activation of many of the BCR signaling molecules as mice deficient in PLC-?2, Btk, the p85 subunit of PI3K, and PKC\(^{\gamma}\) all displayed a defective T-I, but fairly normal T-D response (57). In addition, splenic B cells from mice carrying a targeted mutation of Ig\(?\), in which the BLNK-binding tyrosine Tyr204 has been mutated, display a specific defect in response to T-I antigens, but have normal T-D responses (72). It is unclear if BLNK is necessary for B cell proliferation in response to T-I type I stimuli, or plays a role in upregulating MHC class II molecules as both defective and normal responses have been reported for BLNK-deficient B cells (28;32-34;...
A diminished capacity to upregulate MHC class II molecules may partially explain the suboptimal T-D response seen in busy and other BLNK-deficient mouse models.

The busy mutation causes a premature truncation of the BLNK protein. It is likely that this protein is non-functional as it is missing most of the proline-rich region and the SH2 domain, which have been shown to be critical in transducing BLNK-dependent signals downstream of the BCR (17; 19-21). However, the truncated protein retains the N-terminal LZ domain important in BLNK localization, as well as most of the tyrosines necessary for binding to many of the molecules involved in BCR signaling, raising the possibility that the busy allele is hypomorphic rather than functionally null.

**Genotyping**

*Busy* genotyping is performed by amplifying the region containing the mutation using PCR, followed by sequencing of the amplified region to detect the single nucleotide change.

**Primers**

Busy (F): 5’- TAAGGGACTCCAGCAGCCATGAAC -3’
Busy (R): 5’- ACCATTCTGAGTGAAGGCAGCG -3’

**PCR program**

1) 95°C  2:00  
2) 95°C  0:30  
3) 56°C  0:30  
4) 72°C  1:00  
5) repeat steps (2-4) 29X  
6) 72°C  7:00  
7) 4°C  8

**Primers for sequencing**

Busy_seq(F): 5’- GCCATGAACTTAGCATTCAAGG -3’
Busy_seq(R): 5’- AAGCAGGTGCTTCCCTAGTC -3’

The following sequence of 547 nucleotides (NCBI Mouse Genome Build 37.1, Chromosome 19, bases 41,026,604 to 41,027,150) is amplified:

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(Generated on Oct 12, 2018)
Primer binding sites are underlined; sequencing primer binding sites are highlighted in gray; the mutated T is indicated in red.

References

BNAS1 Regulates Antigen-Receptor Signal Transmission in B Cells. *Int. Immunol.* 18, 545-553.


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Mutagenetix Phenotypic Mutation 'busy'


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(Generated on Oct 12, 2018)