



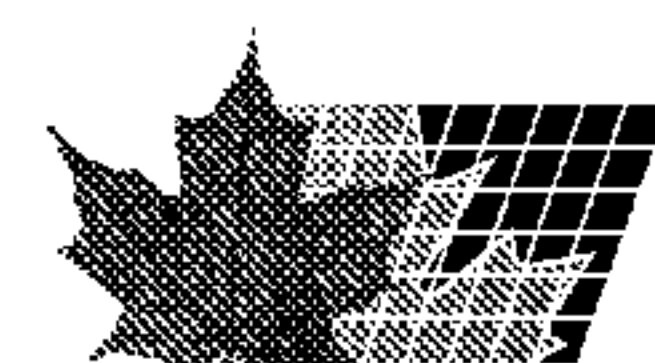
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(54) Titre : MAMMIFERE TRANSGENIQUE PORTANT LE GENE DE LA GANP TRANSFERE DEDANS ET UTILISATION
CORRESPONDANTE
(54) Title: TRANSGENIC MAMMAL CARRYING GANP GENE TRANSFERRED THEREINTO AND UTILIZATION
THEREOF

(57) **Abrégé/Abstract:**

It is an object of the present invention to provide a high affinity antibody effective as a diagnostic or therapeutic for various diseases; a transgenic mammal for producing the high affinity antibody; and a medicine comprising the high affinity antibody or a cell producing the high affinity antibody. According to the present invention, a transgenic mammal carrying a GANP gene transferred thereinto, its progeny, or a part thereof, and a method of producing a high affinity antibody using the same are provided.



ABSTRACT

It is an object of the present invention to provide a high affinity antibody effective as a diagnostic or therapeutic for various diseases; a transgenic mammal for producing the high affinity antibody; and a medicine comprising the high affinity antibody or a cell producing the high affinity antibody. According to the present invention, a transgenic mammal carrying a GANP gene transferred therinto, its progeny, or a part thereof, and a method of producing a high affinity antibody using the same are provided.

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DESCRIPTION

TRANSGENIC MAMMAL CARRYING GANP GENE TRANSFERRED THEREINTO
AND UTILIZATION THEREOF

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TECHNICAL FIELD

The present invention relates to a transgenic mammal carrying a GANP gene transferred thereinto and utilization thereof. More specifically, the present invention relates to a transgenic mammal that expresses a high level of GANP and is capable of producing high affinity antibodies; a method of producing a high affinity antibody using the transgenic mammal; and utilization of the resultant high affinity antibody.

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BACKGROUND ART

The functions of the immune system are classified into the function based on cellular immune responses caused mainly by the effect of T cells and the function based on humoral immunity caused mainly by the effect of antibodies. Actually, these two functions co-operate with each other to perform immune responses. Antibodies are present as cell surface receptors on the surfaces of B cells produced in the bone marrow. It is said that the number of diverse antigens recognized by the first antibody produced in the living body reaches the order of 10^9 to 10^{11} . Such antibodies (antigen receptors) recognize all antigenic determinants that may exist in environments. However, these diverse antigen receptors are generally low in their ability to bind to antigens, and in many occasions, low affinity antibodies are produced. Such antibodies can not cause sufficient immune responses.

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Lymphocytes, especially B cells/immunoglobulins (antibodies) are used in various applications based on their immune responses, e.g. they are used in kits for detecting the antigens of pathogens, or as diagnostics or therapeutics. If an antibody that has high reactivity with antigen is used in such antigen-detecting drugs or various therapeutics, sensitivity to antigen will be excellent and efficacy as a therapeutic at a same dose will be great. However, no means to enhance the affinity of antibodies have been known.

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When pathogens or foreign substances have entered the living body, the body recognizes them as antigens and induces highly frequent somatic mutations in the genes of the V regions of antibodies which bind directly to those antigens. Such changes require stimulation from T cells, and it is considered that stimulation is provided from activated T cells in the germinal center region. Recently, the present inventors have found a molecule designated GANP whose expression increases selectively in activated B cells of this region

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(WO 00/50611). This molecule directly binds to a molecule called MCM (minichromosome maintenance) having DNA helicase activity, and has RNA primase activity. Therefore, it is suggested that this molecule GANP is involved in DNA replication. However, functions of GANP in the immune system have not yet been elucidated.

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DISCLOSURE OF THE INVENTION

It is an object of the present invention to provide a high affinity antibody effective as a diagnostic or therapeutic for various diseases; a transgenic mammal for producing the high affinity antibody; and a medicine comprising the high affinity antibody or a cell
10 producing the high affinity antibody.

As a result of extensive and intensive researches toward the solution of the above-described problems, the present inventor has found that a GANP gene-transferred transgenic animal is capable of producing a high affinity antibody when immunized with an antigen. Thus, the present invention has been achieved.

15 The present invention relates to the following.

(1) A transgenic mammal carrying a GANP gene transferred thereinto or its progeny.

The transferred GANP gene is capable of being expressed in B cells. The transgenic mammal of the invention or its progeny may be generated from GANP
20 gene-infected ES cells. As the mammal, mouse may be given, for example.

(2) A part of the above-described transgenic mammal or its progeny.

(3) A method of producing a high affinity antibody, comprising administering an antigen to the above-described transgenic mammal or its progeny and recovering the antibody from the resultant mammal or progeny.

25 (4) A high affinity antibody obtainable by the method of (3) above, or a fragment thereof.

The antibody of the present invention is 1×10^{-7} M or less as expressed as a dissociation constant. The antibody of the present invention may be either a polyclonal antibody or a monoclonal antibody.

30 (5) A humanized antibody or human antibody, or a fragment thereof, comprising the V region of the above-described antibody or a fragment thereof.

(6) A pharmaceutical composition comprising at least one selected from the group consisting of the above-described antibody or a fragment thereof, and the above-described humanized antibody or human antibody, or a fragment thereof.

35 (7) A high affinity antibody-producing cell which is taken from the transgenic

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mammal as described herein or its progeny, wherein the transgenic mammal or its progeny has been administered an antigen.

In one aspect, the invention relates to a transgenic non-human mammalian cell carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto or a daughter cell thereof carrying the GANP gene.

In another aspect, the invention relates to a method of producing a high affinity antibody which specifically binds to an antigen, comprising (i) administering the antigen to a transgenic non-human mammal carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto, or its progeny carrying a GANP gene, and (ii) recovering the high affinity antibody from the resultant mammal or the progeny.

In another aspect, the invention relates to a method of producing a pharmaceutical composition comprising a high affinity antibody which specifically binds to an antigen, comprising (i) administering the antigen to a transgenic non-human mammal carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto, or its progeny carrying the GANP gene, (ii) recovering the high affinity antibody from the resultant mammal or the progeny, and (iii) preparing a pharmaceutical composition comprising the recovered antibody by including one or more pharmacologically acceptable carriers.

In another aspect, the invention relates to use of a transgenic non-human mammal carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto, or its progeny carrying the GANP gene, for producing a high affinity antibody which specifically binds to an antigen.

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In another aspect, the invention relates to a high affinity antibody-producing cell which is taken from a transgenic non-human mammal carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto, or its progeny carrying the GANP gene, wherein the transgenic mammal or the progeny
5 has been administered an antigen.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of immunohistochemical analyses using anti-GANP monoclonal antibody and ALP-conjugated anti-rat Ig antibody. Scale bar is 100 μ m.

10 Fig. 2 shows the rates of appearance of GANP^{hi} cells in popliteal lymph nodes of female NZB mice. Scale bar is 100 μ m.

Fig. 3 shows the rates of appearance of GANP^{hi} cells in the spleens of female NZB mice. Scale bar is 100 μ m.

15 Fig. 4 shows the results of the staining of plural lineage mice-derived spleen sections with anti-GANP monoclonal antibody. RP: red pulp; F: follicles. Scale bar is 100 μ m.

Fig. 5 shows the identification of GANP^{hi} cells in the spleen red pulp.

Fig. 6 shows the identification of plasma cell markers in GANP^{hi} cells. Scale bar is 100 μ m.

20 Fig. 7 shows the appearance of GANP^{hi} cells in the red pulp region of the spleens of C57BL/6 mice as a result of immunization with TD-Ag. Scale bar is 100 μ m.

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Fig. 8A-C shows somatic mutations in Daudi cell transfectants which are engineered to express mouse GANP stably.

Fig. 9A-C shows an outline of the preparation of a transgenic mouse which is engineered to overexpress GANP in its B cells.

5 Fig. 10 shows the results of analyses of somatic mutations in transgenic (Tg) mice overexpressing GANP and wild-type mice.

Fig. 11A-E shows an outline of the preparation of a B cell-specific GANP deficient mouse (B-GANP^{-/-}).

10 Fig. 12 shows the results of analyses (flowcytometry) of cell surface staining using the B cell-specific GANP deficient mouse (B-GANP^{-/-}).

Fig. 13 shows the results of B cell proliferation assays. Almost no difference was observed, but only the proliferation caused by anti-CD40 antibody stimulation was decreased to about 1/2.

15 Fig. 14 shows antibody titers in the sera from non-immunized Cre-flox/+ mice and B-GANP^{-/-} mice. No difference was observed among the antibody titers of individual isotypes.

Fig. 15 shows the results of measurement of antibody production in B-GANP^{-/-}

mice.

Fig. 16 shows the results of the staining of GC with peanut agglutinin.

Fig. 17 shows the results of measurement of antigen-specific antibody production in B-GANP^{-/-} mice.

5 Fig. 18 shows the results of measurement by differential ELISA of the degrees of maturation of affinity in mice 14 and 35 days after immunization with 100 µg of NP-GC.

Fig. 19 shows the results of flowcytometry on GC-B cells.

Fig. 20A-F shows the results of sequence analyses of V_H186.2 in Cre-flox/+ mice after PCR amplification (sequences continue from A to F in this order).

10 Fig. 20G-L shows the results of sequence analyses of V_H186.2 in Cre-flox/+ mice after PCR amplification (sequences continue from G to L in this order).

Fig. 21 shows the frequencies of IgG1 mutation in Cre-flox/+ mice and B-GANP^{-/-} mice.

15 Fig. 22 shows the frequencies of ³³W to L mutation in V_H186.2 in Cre-flox/+ mice and B-GANP^{-/-} mice.

Fig. 23 shows the results of measurement of activation-induced cell death (AICD) and the results of apoptosis inhibition.

Fig. 24 shows the results of measurement of the apoptosis sensitivities of cells to anti-CD40 and anti-CD95 stimulations.

20 Fig. 25 shows the results of detection of apoptosis cells by TUNEL assay.

Fig. 26 shows the results of detection of apoptosis cells by TUNEL assay.

Fig. 27 shows the RNA expression levels of Bcl-2 family involved in apoptosis inhibition.

25 Fig. 28 shows the results of production of a high affinity antibody using a GANP transgenic mouse.

Fig. 29 shows the results of production of a high affinity antibody using the GANP transgenic mouse-derived hybridoma clones.

Fig. 30 shows association-dissociation curves obtained with Biacore on culture supernatants of the GANP transgenic mouse-derived hybridoma clones.

30 Fig. 31 shows association-dissociation curves obtained with Biacore on culture supernatants of the GANP transgenic mouse-derived hybridoma clones.

Fig. 32 shows an outline of the structure of GANP-GST fusion protein.

35 Fig. 33 shows the results of a pull-down assay for determining the region of GANP which directly binds to MCM. Shown on the left side of each panel are the positions of size standards.

Fig. 34 shows the results of a pull-down assay using *in vitro* translated MCM.

Fig. 35 shows the binding of individual GANP constructs to MCM by immunoprecipitation.

Fig. 36A-B shows the binding of individual GANP constructs to MCM by immunoprecipitation.

Fig. 37 shows an outline of the structures of GANP constructs and their intracellular distributions.

Fig. 38 shows intracellular distributions of GANP constructs.

Fig. 39 shows the nuclear localization of MCM3.

Fig. 40 shows the cytoplasmic localization of MCM3 induced by GANP expression.

Fig. 41 shows a control protein localized in the nucleus.

Fig. 42 shows the effect of GANP construct in the localization of MCM3 mutants.

Fig. 43 shows the nucleus-cytoplasm shuttling of MCM3 detected by a heterokaryon assay.

Fig. 44 shows the localization of GANP during the cell cycle.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinbelow, the present invention will be described in detail.

The present invention has been achieved based on a finding that it is possible to obtain a high affinity antibody by preparing a transgenic animal by transferring a GANP gene into a non-human mammal and immunizing the resultant transgenic animal with an antigen.

1. GANP

GANP which is called "germinal center-associated nuclear protein" is a 210 kDa nuclear protein having homology to yeast Sac3 protein (WO 00/50611). SAC3 is characterized as an inhibitory substance against actin formation. It is known that GANP is selectively up-regulated in germinal center (GC) B cells surrounded by follicular dendritic cells: FDC), has phosphorylation-dependent RNA primase activity, and is involved in the regulation of the cell cycle of B cells (Kuwahara, K. et al., (2000) *Blood* 95: 2321-2328).

In the present invention, the amino acid sequence for mouse GANP protein is shown in SEQ ID NO:2 and the amino acid sequence for human GANP protein is shown in SEQ ID NO: 4. With respect to the gene encoding the GANP protein (hereinafter, referred to as "GANP gene"), the nucleotide sequence for mouse GANP gene is shown in SEQ ID

NO: 1 and the nucleotide sequence for human GANP gene is shown in SEQ ID NO: 3. The above-mentioned amino acid sequences and nucleotide sequences are also described in WO 00/50611.

GANP proteins may be mutant proteins; they may be those proteins which consist of the amino acid sequence as shown in SEQ ID NO: 2 or 4 wherein one or a plurality of amino acids have been deleted, substituted or added and have RNA primase activity. For example, a GANP mutant protein may also be used which consists of the amino acid sequence as shown in SEQ ID NO: 2 or 4 wherein one or a plurality of amino acids (preferably, one or several (e.g. one to ten, more preferably one to five) amino acids) have been deleted, one or a plurality of amino acids (preferably, one or several (e.g. one to ten, more preferably one to five) amino acids) have been substituted with other amino acids, and/or one or a plurality of other amino acids (preferably, one or several (e.g. one to ten, more preferably one to five) amino acids) have been added thereto, and yet has the same RNA primase activity as that of the above-described GANP protein.

“RNA primase activity” means the enzyme activity synthesizing a short primer RNA which will be a starting point for strand elongation when a strand extending opposite to the 5'→3' direction (lagging strand) is synthesized. Usually, a molecule called α primase which binds to DNA polymerase α is used. In germinal center B cells, GANP primase which is the second primase is also induced.

GANP protein includes a protein having the amino acid sequence as shown in SEQ ID NO: 2 or 4, or a mutant amino acid sequence thereof, and a protein having a part of the N-terminal sequence of those sequences (e.g. positions 1-600, preferably 139-566 of the amino acid sequence as shown in SEQ ID NO: 2) or a mutant amino acid sequence thereof.

In the present invention, a GANP gene to be transferred into an animal may be a gene encoding the above-described GANP protein, a part of the N-terminal sequence of the GANP protein, or a mutant GANP protein. Specific examples of such a gene include a gene having the nucleotide sequence as shown in SEQ ID NO: 1 or 3. A gene having only the coding region of the nucleotide sequence as shown in SEQ ID NO: 1 or 3 may also be used. Alternatively, it is also possible to use a gene that has a sequence hybridizable to a complementary sequence to the nucleotide sequence as shown in SEQ ID NO: 1 or 3 under stringent conditions, and encodes a protein having RNA primase activity.

“Stringent conditions” refers to washing conditions after hybridization; specifically, the salt (sodium) concentration is 150-900 mM and the temperature is 55-75°C, preferably salt (sodium) concentration is 250-450 mM and the temperature is 68°C.

Introduction of mutations into a gene may be performed according to known

techniques such as the Kunkel method or the gapped duplex method, using mutation introducing kits utilizing site-directed mutagenesis, such as GeneTailor™ Site-Directed Mutagenesis System (Invitrogen) or TaKaRa Site-Directed Mutagenesis System (Mutan-K, Mutan-Super Express Km, etc.; Takara Bio).

5 Details of mutant genes and methods for obtaining the same are also described in WO 00/50611.

In vitro stimulation of B cells with anti-μ antibody and anti-CD40 monoclonal antibody induces not only the up-regulation of GANP expression but also the phosphorylation of a specific serine residue in the amino acid sequence of GANP protein (e.g. serine at position 502: S502). This reaction is a key reaction for the RNA primase activity of GANP (Kuwahara, K. et al. (2001) *Proc. Natl. Acad. Sci. USA*, 98, 10279-10283). The N-terminal primase domain of GANP protein contains a serine residue whose phosphorylation is catalyzed by Cdk2 *in vitro*. GANP binds to MCM3 replication licensing factor due to its C-terminal domain (Kuwahara, K. et al., (2000) *Blood* 95: 2321-2328; Abe, E. et al., (2000) *Gene* 255: 219-227).

2. Transgenic Mammal Carrying GANP Gene Transferred Thereinto

The present invention relates to a transgenic mammal carrying a GANP gene transferred thereinto. Preferably, the transgenic mammal is capable of expressing the transferred GANP gene in its B cells.

(1) GANP Gene and its Related Molecules

Complexes formed by GANP gene and its related molecules are needed directly or indirectly in the process of induction of mutations in genes. When repairing genetic mutations, GANP protein has the ability to promote induction of mutations in the V region so that high affinity antibodies are obtained. Therefore, the transgenic mammal of the invention carrying the GANP gene or a mutant thereof transferred thereinto is capable of promoting the production of high affinity antibodies of acquired immunity. Further, a transgenic non-human mammal overexpressing this GANP gene is capable of promptly producing an antibody with high binding strength to an antigen. Therefore, by immunizing the above-described transgenic non-human mammal with a specific antigen, it is possible to obtain easily an antibody with a high affinity that has been unachievable by conventional methods. As a result, it becomes possible to obtain polyclonal or monoclonal antibodies capable of eliminating obstinate pathogenic microorganisms or foreign substances. Further, by preparing humanized antibodies using the transgenic mammal of the invention, or by preparing single chain antibodies comprising the V region of the antibody produced by the

transgenic mammal of the invention, it becomes possible to sharply increase the effect of antibody therapy.

Because of the GANP gene or its mutant transferred thereinto, the transgenic mammal of the invention is capable of promoting the production of high affinity antibodies in B cells, and the high affinity antibody-producing cells have resistance to apoptosis induction signals.

In order to confirm that GANP is a molecule functioning in the antibody production in acquired immune responses, the present inventors have created a GANP gene deficient mouse so that GANP is deficient B cell selectively. The results revealed that the deficiency of GANP gene did not influence the development, differentiation and proliferation of cells in the immune system and that no big change is observed in the total yield of antibodies.

It should be noted here that only when B cells have reacted with limited types of antigens, they proliferate and differentiate into antibody-producing cells without T cells. For producing antibodies to ordinary antigens, co-existence of T cells is necessary. Antigens to which antibodies are produced even in the absence of T cells are called T cell-independent antigens. On the other hand, general antigens other than T cell-independent antigens are called T cell-dependent antigens. When B cells have reacted with T cell-dependent antigens, the differentiation of B cells into antibody-producing cells is assisted by helper T cells.

Many of the antigenic determinants (also called antigenic epitopes) of pathogenic viruses are weak in immunogenicity by themselves and activated by the peptide antigens of carrier proteins recognized by T cells.

In the present invention, in order to examine that GANP gene-transferred animals are capable of producing high affinity antibodies highly frequently in those antibody-producing responses to soluble antigens where ordinary animals cannot produce strong antibodies, an antigen designated NP-CG was prepared by coupling a nitrophenyl group (NP group) (which has been extensively analyzed as a hapten) to chicken gamma globulin, followed by examination of responses to T cell-dependent antigen.

It is known that C57BL/6 mice's generate high affinity antibody to NP only when utilized a single V region. This response is dominated by only the V region of IgG heavy chain (called V_H186.2) and lambda 1 light chain of an antibody. With this system, it is possible for antibodies of IgG₁ isotype to examine genetic mutations in high affinity antibodies by analyzing the amino acid sequence of V_H186.2. Furthermore, it is reported that the highest affinity is induced when the amino acid residue tryptophan (W) at position 33 of the amino acid sequence of the heavy chain V region (V_H186.2) has been mutated into

leucine (L) (W³³ to L mutation).

Then, the present inventor examined whether high affinity antibodies could be induced in GANP gene deficient mice and its defect might be associated with W33 to L mutation event or not. As a result, high affinity antibody production was hardly observed in GANP gene deficient mice, compared to the control Cre-flox/+ mice. Therefore, it has been demonstrated that GANP gene has a key function in the production of high affinity antibodies. To investigate this function further, the inventor has created GANP gene-overexpressing mice. Overexpression of GANP gene was achieved by linking a mouse immunoglobulin promoter moiety and a human immunoglobulin gene intron enhancer moiety upstream (5') of GANP gene so that the gene is expressed selectively in B cells.

The GANP-overexpressing mice were born normally, and no particular change was observed in the development, differentiation and proliferation of their lymph tissues. However, a remarkable increase was observed in the high affinity type V region gene (W³³ to L) in responses to NP-CG. Although the functional role of RNA primase activity here has not yet been established, it is believed that the RNA primase activity of GANP gene or the phosphorylation of the 502 serine residue involved in the primase activity is related to the production of high affinity antibodies in view of the following: (i) the phosphorylation of serine residue at position 502 (which is an indicator for the primase activity of GANP molecule) is high in cells present at the region of the germinal center where high affinity B cells are produced (centrocytes), and (ii) the frequency of the mutation at the V region induced by experiments to transfer a *ganp* gene into Daudi cells is high. These results show that high expression of GANP molecule and activation of RNA primase activity are necessary for high affinity antibody production by immune response.

(2) Mammals for Use in GANP Gene Transfer

The term "mammal" used in the present invention means any of non-human mammals such as bovine, horse, pig, goat, rabbit, dog, cat, mouse, rat, hamster and guinea pig. Preferably, mouse, rabbit, rat or hamster is used. Most preferably, mouse is used.

The transgenic mammal of the invention may be prepared by introducing a GANP gene into fertilized eggs, unfertilized eggs, embryonic cells comprising spermatozoa and protocytes thereof, preferably into cells of embryogenesis stage (more preferably, the single cell or fertilized egg cell stage and yet generally before eight-cell stage) in the development of non-human mammals, by a method such as the calcium phosphate method, electric pulsing, lipofection, aggregation, microinjection, the particle gun method, or the

DEAE-dextran method. Further, it is also possible to transfer a GANP gene of interest into somatic cells, organs of the living body, tissue cells, etc. by the above-mentioned gene transfer methods to use the resultant cells, etc. for cell culture or tissue culture. Further, it is possible to create transgenic mammals by fusing these cells with the above-described embryonic cells according to known cell fusion methods.

When a GANP gene is transferred into an animal of interest, it is preferred that the gene be transferred in the form of a gene construct in which the gene is ligated downstream of a promoter capable of directing expression of this gene in cells of the animal of interest. Specifically, a vector in which a GANP gene is ligated downstream of various promoters capable of directing expression of the GANP gene derived from various mammals may be microinjected into fertilized eggs of the mammal of interest (e.g. mouse fertilized eggs) to thereby create a transgenic mammal capable of high expression of the GANP gene of interest.

(3) Expression Vector

Examples of expression vectors for GANP gene include plasmids derived from *Escherichia coli*; plasmids derived from *Bacillus subtilis*; plasmids derived from yeast; bacteriophages such as λ -phage; retroviruses such as Moloney leukemia virus; and animal or insect viruses such as vaccinia virus or baculovirus.

As promoters for regulating gene expression, promoters of viruses-derived genes; promoters of various mammals (such as human, rabbit, dog, cat, guinea pig, hamster, rat and mouse)-derived genes; and promoters of birds (such as chicken)-derived genes may be used.

Examples of promoters of viruses-derived genes include promoters of cytomegalovirus-, Moloney leukemia virus-, JC virus- or breast cancer virus-derived genes.

Examples of promoters of various mammals- and birds-derived genes include promoters of such as albumin, insulin II, erythropoietin, endothelin, osteocalcin, muscle creatine kinase, platelet-derived growth factor β , keratin K1, K10 and K14, collagen type I and type II, atrial natriuretic factor, dopamine β -hydroxylase, endothelial receptor tyrosine kinase, sodium/potassium-dependent adenosinetriphosphatase, neurofilament light chain, metallothionein I and IIA, metalloproteinase I tissue inhibitor, MHC Class I antigen, smooth muscle α -actin, polypeptide chain elongation factor 1 α (EF-1 α), β -actin, α - and β -myosin heavy chains, myosin light chains 1 and 2, myelin basic polypeptide, serum amyloid P component, myoglobin and renin genes.

The above-described vector may have a terminator which terminates the transcription of a messenger RNA of interest in a transgenic mammal. For the purpose of

achieving still higher expression of GANP gene, the splicing signal of each gene, enhancer region, or a part of an intron of an eukaryotic gene may be ligated upstream (5') of the promoter region, between the promoter region and the translation region, or downstream (3') of the translation region, if desired.

5 In a preferred embodiment of the invention, it is possible to allow selective expression of the transferred GANP gene in B cells by ligating the GANP gene downstream of an immunoglobulin promoter or by ligating a human immunoglobulin gene intron enhancer moiety upstream (5') of the GANP gene.

10 (4) Transfer of GANP Gene

The transfer of GANP gene at the fertilized egg cell stage is preferably carried out in such a manner that excessive presence of GANP gene is secured in all the embryonic cells and somatic cells of the mammal of interest. Excessive presence of GANP gene in the embryo cells of the created animal after gene transfer means that all the progeny of that
15 animal has excessive GANP gene in all the embryonic cells and somatic cells. The progeny of this kind of animal which inherited the GANP gene has excessive GANP protein in all the embryonic cells and somatic cells.

In the present invention, first, heterozygotes which have the transferred GANP gene in one of the homologous chromosomes are prepared; then, homozygotes which have the
20 transferred GANP gene in both of the homologous chromosomes are obtained by mating the heterozygotes with each other. Subsequently, by mating female homozygotes with male homozygotes, all the resultant progeny retains the transferred GANP gene stably. After confirmation of the excessive presence of GANP gene, the progeny may be sub-bred in usual breeding environments.

25 Fertilized eggs of a non-human mammal of interest (preferably, mouse) or its ancestor (back-crossing) to be used for transferring a foreign GANP gene different from the endogenous gene of the mammal of interest are obtained by mating allogenic male and female mammals.

Although fertilized eggs may be obtained by natural mating, it is preferred that
30 female mammals after artificial adjustment of their sexual cycle be mated with male mammals. As a method for artificially adjusting the sexual cycle of female mammals, such a method may be used preferably in which follicle-stimulating hormone (pregnant mare serum gonadotropin (PMSG)) and then luteinizing hormone (human chorionic gonadotropin (hCG)) are administered by, e.g., intraperitoneal injection.

35 After the transfer of a foreign GANP gene into the resultant fertilized eggs by the

methods described above, the eggs are artificially transferred/implanted in female mammals. As a result, non-human mammals having a foreign gene-integrated DNA are obtained. In a preferable method, fertilized eggs are transferred/implanted artificially in pseudo-pregnant female mammals in which fertility has been induced by mating with male mammals after administration of luteinizing hormone-releasing hormone (LHRH). As totipotent cells into which a GANP gene is to be transferred, fertilized eggs or early embryos may be used if the mammal of interest is mouse. As a method of gene transfer into cultured cells, DNA microinjection is preferable in view of the production efficiency of transgenic mammal individuals and the transmittance efficiency of the transgene to the subsequent generation.

Subsequently, the gene-injected fertilized eggs are transplanted into the oviduct of a recipient female mammal. Those animals which have developed from the eggs up to individuals and have been successively born are bred under foster parents. Then, DNA is extracted from a part of their bodies (e.g. the tail end in the case of mouse) and subjected to Southern analysis, PCR, etc. Thus, it is possible to confirm the presence of the transgene. Those animals in which the presence of the transgene has been confirmed are designated founder animals. The transgene is transmitted to 50% of their offspring (F1). Further, by mating F1 individuals with wild-type animals or other F1 individuals, F2 individuals which have the transgene in one (heterozygote) or both (homozygote) of the diploid chromosomes can be produced.

Alternatively, transgenic mammals expressing high levels of GANP protein may also be created by introducing the above-described GANP gene into ES (embryonic stem) cells. For example, the GANP gene is introduced into HPRT negative (i.e. lacking hypoxanthine-guanine phosphoribosyltransferase gene) ES cells derived from normal mouse blastocysts. Then, those ES cells in which the GANP gene has been integrated through homologous recombination induced in a mouse endogenous gene are selected by HAT selection. The thus selected ES cells are microinjected into fertilized eggs (blastocysts) obtained from other normal mouse. The resultant blastocysts are transferred into the uterus of other normal mouse as a recipient. Subsequently, chimeric transgenic mice are born from the recipient mouse. By mating these chimeric transgenic mice with normal mice, heterotransgenic mice can be obtained. Further, by mating the heterotransgenic mice with each other, homotransgenic mice can be obtained.

The present invention encompasses not only the above-described transgenic mammal but also its progeny and a part of the transgenic mammal or its progeny in the scope of the invention. As a part of the transgenic mammal, a tissue, organ, cell or the like of the transgenic mammal or its progeny may be enumerated. Specific examples of organs or

tissues include the spleen, thymus, lymph nodes, bone marrow or tonsil; and specific examples of cells include B cells.

The transgenic mammal of the invention may be mated with a mammal that further activates B cells. As a result of such mating, antibodies of still higher affinity can be produced.

Recently, it has been reported that when B cells are activated in peripheral lymph nodes in MRL/lpr mouse, induction of mutations in the V region is further increased in the T cell region after B cells passed through the germinal center. The inventors have also found that non-immunized MRL/lpr mouse shows high expression of GANP equivalent to the GANP expression observed in *ganp* transgenic mouse which was created by ligating a GANP gene downstream of Ig promoter and enhancer. This suggests a possibility that, while high affinity antibodies are not produced against autoantigens normally, high affinity antibodies to autoantigens may be produced in this autoimmune disease mouse because of the abnormal activation of GANP molecule.

Still higher induction of mutations can be expected if such mouse as MRL/lpr, NZB or (NZB x NZW)F1 (all of them are considered as autoimmune disease mice) is used as the above-mentioned animal that still activates B cells.

By creating a GANP transgenic mouse from MRL/lpr mouse utilizing what has been described above, it may be possible to create a super high affinity antibody-producing mouse. In other words, by mating the GANP gene overexpressing transgenic mammal of the invention with various autoimmune disease model animals, it is possible to create mammals capable of producing high affinity antibodies.

3. Preparation of High Affinity Antibodies

The term "antibody" used in the invention means a protein having activity to specifically bind to an antigen, preferably a protein produced by B cells. In the present invention, an antibody having high reactivity with an antigen is called high affinity antibody. The term "high affinity" used herein means that the ability of an antibody to bind to an antigen is high. In the present invention, a high affinity antibody refers to an antibody which has higher ability to bind to an antigen than those antibodies prepared using conventional animals such as mouse, and which is slow in dissociating from that antigen. This means that such an antibody is high and specific in the ability to bind to an antigenic determinant (epitope) sterically and closely. Besides, the binding of such an antibody to the antigenic determinant induces changes not only in the determinant but also the structure of the antigen itself, to thereby show strong activities eventually (e.g. biological activities such

as neutralization of toxicity, prevention of viral infection, deactivation of pathogens, promotion of elimination of pathogens from the body, or induction of denaturation in antigen molecules).

The binding ability of an antibody (i.e. affinity) may be measured as a dissociation constant (KD), dissociation rate constant (Kdiss) or association rate constant (Kass) by Scatchard analysis or with a surface plasmon resonance sensor called Biacore. Biacore systems in which three technologies of sensor chip, microflow system and SPR detection system are integrated are to measure the strength, rate and selectivity of molecular binding. This apparatus enables real time detection of biological molecules and monitoring of interactions among a plurality of molecules without using labels. Specific examples of useful Biacore systems include Biacore 3000, Biacore 2000, Biacore X, Biacore J and Biacore Q (all of them are manufactured by Biacore).

With the above-described Biacore system, parameters showing the affinity of antibodies, i.e. dissociation constant (KD), dissociation rate constant (Kdiss) (1/Sec) and association rate constant (Kass) (1/M.Sec) are measured.

Antibodies with smaller dissociation constant (KD) values are preferable because the smaller the dissociation constant value, the higher the affinity. The binding ability of an antibody (affinity) is determined by the two parameters of Kdiss and Kass, and is represented by the following formula:

$$KD (M) = Kdiss/Kass$$

Although the affinity of the resultant antibody varies depending on a plurality of factor such as the type of the antigen, generally, its KD value is preferably 1×10^{-7} (M) or less. For example, preferable KD values are 1×10^{-8} (M) or less, 1×10^{-10} (M) or less, or 1×10^{-11} (M) or less.

In the present invention, when the resultant antibody reveals any of the above-described effects or natures, the antibody is judged as a "high affinity" antibody.

Enhancement in the affinity of antibody molecules is produced by inducing somatic hypermutations (SHM) in genes of the variable regions (V region) of antibodies. Although specificities of antibodies to antigens are recognized from the beginning of immunization of the living body with antigens, most of early antibodies are IgM class antibodies; their binding affinity to antigens is not high and their ability to remove or deactivate pathogens or foreign substances is low. However, if an antigen is administered to the living body to give several boosters, the binding affinity of antibody to the antigen is enhanced. At this time, B cells need stimulation from T cells, and this activation is considered to take place in the

germinal center region in peripheral lymph tissues. Recently, the RNA editing molecule AID expressed in the germinal center has been reported as a molecule necessary to induce mutations in V region genes. Further, it is reported that uracil DNA glycosidase and, as DNA polymerases necessary for DNA replication, DNA polymerases zeta (ζ) and iota (ι) which easily produce errors are also involved in the above activation. However, the molecule(s) which control(s) these functions has/have not been elucidated. The function of GANP molecule as a novel SHM-inducing molecule has been elucidated. Increase in the expression of this molecule plays a key role in SHM induction. Among all, it has been demonstrated that GANP molecule is important in producing high affinity antibodies.

Antibodies induced by immunizing C57BL/6 mice with nitrophenyl-chicken γ globulin as a hapten carrier antigen have $V_H186.2$ locus as the H chain and $\lambda 1$ as the L chain. In this system, it is known that antibodies obtained after boosters were given are IgG₁ antibodies, and that the mutation induced in the V region sequence of those antibodies with particularly high binding affinity among them is mutation from tryptophan to leucine at position 33. In the Examples of the present specification, this high affinity-type V region mutation is induced highly. This can be said definite evidence at the molecule level showing that high affinity antibodies have been induced.

Therefore, it is possible to obtain high affinity antibodies by administering an antigen to the above-described transgenic mammal or its progeny and letting the resultant mammal or progeny produce antibodies. Briefly, an antigen of interest is administered by conventional methods to an animal that is engineered to express high levels of GANP protein. Then, high affinity antibodies may be prepared from lymphocytes of a tissue such as blood or spleen (not limited to these tissues) of the immunized animal. These high affinity antibodies may be either polyclonal or monoclonal antibodies.

As a method for producing polyclonal antibodies, for example, polyclonal antibodies may be obtained by administering an antigen to the transgenic mammal of the invention, taking blood from the immunized mammal, and then separating and purifying antibodies from the resultant blood.

Methods of immunization are known to those skilled in the art. For example, immunization may be performed by administering an antigen once or more.

The types of the antigen are not particularly limited. All substances which may have a steric structure as an antigenic determinant fall under antigen. In addition to all biological components such as proteins, enzymes, peptides, sugars, lipids, DNAs, RNAs and prions, any substance such as cancer antigens, virus antigens, organic or inorganic synthetic antigens may be used.

The antigen may be administered, for example, two or three times at intervals of 7 to 30 days. The dose may be, for example, about 0.05 to 2 mg of the antigen per administration. The route of administration is not particularly limited. For example, subcutaneous administration, dermal administration, intraperitoneal administration, intravenous administration or intramuscular administration may be selected appropriately. Preferably, the antigen is administered by intravenous, intraperitoneal or subcutaneous injection. The antigen may be used in solution in an appropriate buffer, e.g. a buffer containing conventional adjuvants such as complete Freund's adjuvant or aluminium hydroxide, but the antigen may be used without adjuvant depending on the administration route or other conditions.

After immunized mammals have been bred for a specific period of time, serum samples are obtained from them and antibody titers thereof are measured. When the antibody titer begins to rise, boosters may be given using, for example, 100 µg to 1000 µg of the antigen. One to two months after the final administration, blood is taken from the immunized mammals and subjected to various conventional methods used for protein isolation, e.g. centrifugation, precipitation using ammonium sulfate or polyethylene glycol, and chromatography such as gel filtration chromatography, ion exchange chromatography or affinity chromatography. Thus, polyclonal antibodies may be obtained as polyclonal anti-sera.

As a method for producing monoclonal antibodies, the hybridoma method may be used. First, a peptide constituting an antigen of interest is suspended in an adjuvant. The resultant suspension is administered subcutaneously or intradermally into animals to be immunized (i.e. the transgenic mammal of the invention). The types of the antigen used here are the same as described above. Examples of the adjuvant used here include complete Freund's adjuvant, BCG, trehalose dimycolate (TDM), lipopolysaccharide (LPS), alum adjuvant and silica adjuvant. Preferably, a combination of complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) is used in view of the ability to induce antibodies.

In the production of monoclonal antibodies, preferably, animals which have undergone the first immunization with an antigen are boosted several times; after passage of appropriate number of days, blood samples are taken and antibody titers thereof are measured. Since antibodies produced by the method of the invention are high affinity antibodies, the first immunization may be sufficient without booster. Antibody titers may be measured by known methods such as enzyme-linked immunosorbent assay (hereinafter, referred to as ELISA).

Subsequently, the spleens are removed from the immunization-completed animals to obtain B cells. Obtaining B cells capable of binding to antigens is preferable because it could reduce subsequent screening. The B cells obtained at this point are high affinity antibody-producing cells, which may be used as an immunopotentiator without any processing. It is also possible to obtain V region genes directly from these B cells and to measure somatic hypermutations in the V region.

Subsequently, the resultant B cells are fused with myeloma cells by conventional methods to thereby prepare an antibody-producing hybridoma. For example, if the animal is mouse, the spleen is removed and placed in a solution such as Hanks' balanced salt solution (HBSS). Cells are pushed out with tweezers to obtain spleen lymphocytes (B cells). The resultant spleen lymphocytes are stained with trypanblue or the like to count the number of viable cells, and then fused with myeloma cells to prepare a hybridoma.

The myeloma cell used for the cell fusion is not particularly limited. Known myeloma cells such as P3-X63.Ag8 (X63), P3-X63.Ag8.U1 (P3U1), P3/NS I/1-Ag4-1(NSI) or Sp2/0-Ag14(Sp2/0) may be used. In the selection of the myeloma cell, compatibility with antibody-producing cells should be considered appropriately.

Cell fusion is carried out as described below. Briefly, 1×10^6 - 1×10^7 cells/ml of antibody-producing cells are mixed with 2×10^5 - 2×10^6 cells/ml of myeloma cells (preferable cell ratio of antibody-producing cells to myeloma cells is 5:1) in an animal cell culture medium such as serum-free DMEM or RPMI-1640 and fused in the presence of a cell fusion promoter.

As the method of cell fusion, any of the methods known in the art (the Sendai virus method, the polyethylene glycol method, or the protoplast method) may be selected. Preferably, the polyethylene glycol method is used in view of relatively low cytotoxicity and simple fusion operations. Polyethylene glycol with a mean molecular weight of 1000-6000 daltons may be used as a cell fusion promoter. When production of a large quantity of antibodies is desired, a hybridoma prepared by fusing antibody-producing cells stimulated with a vinyl pyridine derivative with myeloma cells is used preferably.

The resultant hybridoma is cultured in HAT medium (containing hypoxanthine, aminopterin and thymidine) for an appropriate period of time according to conventional methods, followed by selection of hybridoma clones. Subsequently, those hybridoma clones producing an antibody of interest are screened, followed by cloning of the hybridoma clones.

As the screening method, known methods for antibody detection, such as ELISA, radio immunoassay (hereinafter, referred to as RIA), the plaque method, or the aggregation

reaction method, may be used. As the cloning method, known methods in the art, such as the limiting dilution-culture method, the soft agar method or FACS, may be used. The resultant hybridoma is cultured in an appropriate culture broth, or administered into the abdominal cavity of an animal (e.g. mouse) compatible with the hybridoma. From the thus
 5 obtained culture broth or abdominal dropsy, the monoclonal antibody of interest may be isolated and purified by methods such as salting out, ion exchange chromatography, gel filtration or affinity chromatography.

It should be noted that fragments and single chain antibodies of the V region of the above-described antibody are also within the scope of the present invention. A fragment of
 10 the antibody means a portion of the above-described polyclonal or monoclonal antibody. Specific examples of such a fragment include $F(ab')_2$, Fab' , Fab , Fv (variable fragment of antibody), sFv , $dsFv$ (disulphide stabilized Fv) or dAb (single domain antibody). $F(ab')_2$ and Fab' mean those antibody fragments which are prepared by treating an immunoglobulin (monoclonal antibody) with proteolytic enzymes pepsin and papain, respectively, and are
 15 generated through digestion around the disulfide bond present between the two H chains in the hinge region. For example, when IgG is treated with papain, this molecule is cut upstream of the disulfide bond present between the two H chains in the hinge region to yield two homologous antibody fragments in which an L chain consisting of V_L (L chain variable region) and C_L (L chain constant region) and an H chain fragment consisting of V_H (H chain
 20 variable region) and $C_{H\gamma 1}$ ($\gamma 1$ region in H chain constant region) are coupled by a disulfide bond in the C-terminal region. Each of these two homologous antibody fragments is called Fab' . When IgG is treated with pepsin, this molecule is cut downstream of the disulfide bond present between the two H chains in the hinge region to yield an antibody fragment which is slightly larger than the above-described two Fab' fragments ligated at the hinge
 25 region. This antibody fragment is called $F(ab')_2$. A single chain antibody has a structure in which V_L and V_H are linked by a linker.

The high affinity antibody of the invention may be a humanized antibody or human antibody. These human antibodies may be prepared by using mammals whose immune system has been replaced with the human immune system. After immunizing such
 30 mammals, human antibodies may be prepared directly in the same manner as used in the preparation of conventional monoclonal antibodies.

For the preparation of humanized antibodies, reconstructed variable regions consisting of human-derived framework regions and mouse-derived CDRs (complementarity determining regions) is prepared by transferring the CDRs of the variable regions in a mouse
 35 antibody into the human variable regions.

Subsequently, these humanized, reconstructed human variable regions are ligated to human constant regions. Portions derived from non-human amino acid sequences in the finally reconstructed humanized antibody are only CDRs and extremely small parts of FRs. CDRs are composed of hyper-variable amino acid sequences. Since these sequences do not show species specific sequences, it is possible to use humanized antibodies having mouse CDRs. Methods for preparing humanized antibodies are well-known in the art.

Human antibodies may be produced using any animal (e.g. mouse, rat, etc.) in terms of structure, though generally the antigen binding site in the variable region (i.e. hyper variable region) may raise some problem with respect to specificity and binding affinity. On the other hand, it is desirable that the structures of the remaining portion of the variable region and the constant region should be the same as the structures in human antibodies. With respect to genetic sequences common in human, genetic engineering techniques to prepare them have been established.

The isotype of the antibody of the invention is not particularly limited. The antibody of the invention may have any isotype, e.g. IgG (IgG₁, IgG₂, IgG₃, IgG₄), IgM, IgA (IgA₁, IgA₂), IgD or IgE.

4. Use of High Affinity Antibodies

The high affinity antibody of the invention is useful as a drug for diagnosing, treating or preventing diseases.

(1) Diagnosis of Diseases

Diagnosis of various diseases using the antibody of the invention is carried out as described below. Briefly, samples (e.g. sera) taken from subjects suspected of having various diseases are bound to the antibody of the invention by antigen-antibody reaction. Then, the amount of an antigen of interest in the sample is detected from the amount of bound antibody. The detection of the amount of bound antibody may be performed by conventional immunological measuring methods. For example, immunoprecipitation, immunoaggregation, labeled immunoassay, immunonephelometry, immunoturbidimetry, or the like may be used. Labeled immunoassay is especially preferable from the viewpoint of simplicity and high sensitivity. In labeled immunoassay, antibody titers in samples may be expressed directly as the amounts of label detected using a labeled antibody. Alternatively, antibody titers may be expressed relatively using as a standard solution an antibody of known concentration or known titer. Briefly, the standard solution and a sample may be measured simultaneously in the same measuring system, followed by expression of the antibody titer in the sample relatively based on the value of the standard solution.

In labeled immunoassay, any of known measurement methods, such as ELISA, RIA, fluoroimmunoassay, or chemiluminescence immunoassay, may be used. The labeling substance may be appropriately selected depending on the above-mentioned assay method; for example, an enzyme, radioisotope, fluorescent compound, or chemiluminescent compound may be selected. Specific examples of the enzyme useful in the invention include peroxidase, alkaline phosphatase, acid phosphatase and glucose oxidase. Detection sensitivity of the above-mentioned labeling substances may be increased by using avidin-biotin complex. As a specific example of the radioisotope useful in the invention, ^{125}I may be given at first. Specific examples of the fluorescent compound useful in the invention include fluoresceine isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC). Specific examples of the chemiluminescent compound useful in the invention include lophine, luminol and lucigenin. The labeling of antibodies with the above-mentioned substances may be performed according to conventional methods. Hereinbelow, labeled immunoassay using labeled antibodies will be described.

As a method of detection of various diseases according to labeled immunoassay, a method using a known non-competitive reaction system or competitive reaction system may be possible. Non-competitive reaction systems require solid phase (solid phase method). Competitive reaction systems do not necessarily require solid phase (liquid phase method), but use of solid phase is preferable since that will make measuring operations simple. Specific examples of materials for the solid phase include polystyrene, nylon, glass, silicon rubber and cellulose. As the shape of the solid phase, spheres, wells, tubes, sheets, or the like may be enumerated. However, the material and the shape useful in the invention are not limited to those enumerated above. Known materials and shapes used in labeled immunoassay may be used at discretion.

In non-competitive reaction systems, measurement operations are carried out as follows. Briefly, a sample or the antibody of the invention is immobilized on a solid support and then reacted with the antibody of the invention or a sample. Subsequently, a pre-labeled anti-immunoglobulin antibody (secondary antibody) is added to react with the above antibody reacting with the immobilized sample. With the labeling substance of this secondary antibody, it is possible to detect the amount of the antibody bound to the sample. Since the amount of the labeled secondary antibody detected is directly correlated with the amount of the antigen of interest in the sample, the amount of this antigen can be obtained from the amount of the labeled secondary antibody.

In competitive reaction systems, a sample and a specific amount of an antigen of interest are reacted with a specific amount of an antibody. For example, after

immobilization of a sample on a solid support, the sample is reacted with the antibody of the invention which has been pre-reacted with an antigen of interest. Subsequently, the antibody which has reacted with the immobilized sample is reacted with a pre-labeled anti-immunoglobulin antibody (secondary antibody), followed by detection of the amount of the antibody by the labeling substance. The amount of the labeling substance is inversely correlated with the amount of the antigen of interest added. Other types of competitive reaction systems may also be used where the antibody of the invention is immobilized, reacted with a sample, and then reacted with a pre-labeled antigen of interest. The amount of the labeling substance detected is inversely correlated with the amount of GANP protein in the sample bound to the antibody.

As the method of immobilization of an antigen or antibody on a solid support, known methods such as physical adsorption, covalent binding, ionic bonding or crosslinking may be used. Physical adsorption is especially preferable because of its simplicity. As examples of the anti-immunoglobulin antibody (secondary antibody) useful in the invention, anti-IgG antibody or anti-IgM antibody may be given. These antibodies may be used as an entire molecule. Alternatively, antibody fragments Fab, Fab' and F(ab')₂ comprising the antigen binding site obtained by treating antibodies with enzymes may be used. Further, instead of the labeled anti-immunoglobulin antibody, a substance having specific affinity for antibody molecules (e.g. protein A which has specific affinity for IgG) may be labeled and used.

As a preferable example of the above-described labeled immunoassay, ELISA may be given which is an immunoassay using an enzyme as a label. Briefly, a sample or a dilution thereof is placed in 96-well plates or the like and incubated at 4°C to room temperature overnight or at 37°C for about 1-3 hrs so that GANP protein to be detected is adsorbed and immobilized on the plates. Then, the antibody of the invention is reacted. Subsequently, an enzyme-preconjugated anti-immunoglobulin antibody (secondary antibody) is reacted. Finally, an appropriate color-developing substrate reactive with the enzyme (e.g. if the enzyme is phosphatase, p-nitrophenylphosphate or the like) is added to thereby detect the antibody with its color development.

By using the high affinity antibody of the invention, it is possible to evaluate the efficacies of therapeutics for various diseases. The evaluation method using the high affinity antibody of the invention is performed as follows. Briefly, a drug is administered to various disease patients or disease model animals. Then, using the antibody of the invention, the amounts of the antigen (such as virus) in these living bodies are detected. By comparing the amounts, the efficacy of the drug as a therapeutic for various diseases can be

evaluated based on the amounts of the antigen in living bodies.

The high affinity antibody of the invention may be provided in the form of a diagnosis kit for various diseases. This kit may be used in the diagnosis method of the invention or the efficacy evaluation method of the invention. The kit of the invention
5 comprises as least one selected from the following (a) and (b).

(a) the antibody of the invention or that antibody labeled

(b) immobilized reagent in which the antibody or labeled antibody of (a) above is immobilized on a solid support

The "labeled antibody" means an antibody labeled with an enzyme, radioisotope,
10 fluorescent compound or chemiluminescent compound. As the material of a solid support on which the antibody or labeled antibody is immobilized in the kit of the invention, polystyrene, nylon, glass, silicon rubber, cellulose or the like may be used. As the shape of such a solid support, spheres, wells, tubes or sheets may be enumerated. However, the material and the shape useful in the invention are not limited to these ones. Instead of the
15 immobilized reagent, a solid phase and an immobilizing agent may be attached to the kit. As the immobilizing agent, if immobilization by physical adsorption is intended, a coating liquid such as 50 mM carbonate buffer (pH 9.6), 10 mM Tris-HCl buffer (pH 8.5, containing 100 mM sodium chloride) or PBS and, if necessary, a blocking liquid (which is a coating liquid containing 0.5% gelatin) may be enumerated, for example.

20 The antibody contained in the kit of the invention may be in a state of solution in PBS or the like, or in a state where the antibody is linked to a gel (hereinafter, abbreviated to "absorption gel"). This absorption gel may be pre-packed in 0.5-2 ml microcentrifuge-precipitation tubes for absorption by the batch method. Alternatively, the absorption gel may be pre-packed in 0.1-5 ml mini-columns for absorption by the column
25 method.

In addition to the above-described components, the kit of the invention may contain other reagents for carrying out the detection of the invention, e.g. the substrate of an enzyme (color developing substrate, etc.), the substrate in solution, enzymatic reaction-terminating liquid or the like when the labeling substance is an enzyme, and diluents for samples.
30 Specific examples of diluents for samples include 20 mM Tris-HCl buffer (pH 7.4) containing PBS (phosphate-buffered physiological saline, pH 7.4), 137 mM sodium chloride and 3 mM potassium chloride (hereinafter abbreviated to "TBS"); and PBS or TBS containing 0.05% Tween 20 and 0.1-1% BSA. These diluents for samples may be used for diluting other substances such as antibodies.

35 (2) Pharmaceutical Compositions for Treating or Preventing Diseases

When the high affinity antibody of the invention has an effect of neutralizing the activity of an antigen which will become the pathogen of a disease, the antibody of the invention is useful in a pharmaceutical composition for treating or preventing the disease. The pharmaceutical composition of the invention comprises the high affinity antibody of the invention or a fragment thereof as an active ingredient and, is provided, preferably, in the form of a pharmaceutical composition comprising a pharmacologically acceptable carrier.

The "pharmacologically acceptable carrier" used herein includes excipients, diluents, fillers, disintegrants, stabilizers, antiseptics, buffers, emulsifiers, aromatics, coloring agents, sweetening agents, thickening agents, flavoring agents, dissolution aids and other additives. By using one or more of these carriers, various forms of pharmaceutical compositions may be prepared, e.g. tablets, pills, powders, granules, injections, solutions, capsules, troches, elixirs, suspensions, emulsions and syrups. These pharmaceutical compositions may be administered orally or parenterally. Other forms for parenteral administration include solutions for external use which comprise one or more active substances and are prescribed by conventional methods, suppositories for enteric administration, and pessaries.

The dose of the pharmaceutical composition of the invention varies depending on the age, sex, body weight and conditions of the patient, treatment effect, the method of administration, time period for treatment, or the type of the high affinity antibody (the active ingredient) contained in the composition. Usually, the pharmaceutical composition of the invention may be administered to adult patients in the range from 10 μ g to 1000 mg per administration, preferably in the range from 10 μ g to 100 mg per administration. However, the dose is not limited to this range.

For example, in the case of injections, the pharmaceutical composition of the invention may be dissolved or suspended in a pharmacologically acceptable carrier (such as physiological saline or commercial distilled water for injection) so that the concentration of the antibody in the carrier is from 0.1 μ g /ml to 10 mg /ml. The thus prepared injection may be administered to human patients in need of treatment at a rate of 1 μ g -100 mg/kg body weight, preferably at a rate of 50 μ g -50 mg/kg body weight, per administration once to several times per day. The route of administration may be intravenous injection, subcutaneous injection, intradermal injection, intramuscular injection or intraperitoneal injection, for example. Among all, intravenous injection is preferable. Optionally, injections may be prepared in the form of a non-aqueous diluent (e.g. propylene glycol, polyethylene glycol, vegetable oil such as olive oil, alcohol such as ethanol), suspension or emulsion. Sterilization of such injections may be performed by filter-sterilization through a

bacteria removal filter, addition of antiseptics, or irradiation. Injections may take a form that is prepared into an injection at the time of use. Briefly, a solid composition is prepared by lyophilization or the like, and this solid composition may be dissolved in aseptic distilled water for injection or other solvent at the time of use.

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5. Application of the Present Invention

The present inventors have induced overexpression of GANP in B cell tumor strains and analyzed them. As a result, the B cell tumor strains showed that GANP gene transfer has a remarkable effect in inducing somatic hypermutations in V region genes. Since this effect is not observed when a mutant gene in which phosphorylation of serine at position 502 (required for the primase activity of GANP) does not occur is used, it is suggested that RNA primase activity is necessary for the remarkable induction of somatic hypermutations in V region genes. These results demonstrate that GANP has an effect of enhancing the production of specific antibodies as a clinical, supplemental immunopotentiator.

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It is also effective for clinical, supplemental immunopotential to use a retrovirus vector as a vector and a combination of GANP and a stimulation mediated by TNF family molecules such as DC40 or BAFF. Further, by transferring a GANP gene at the bone marrow cell level, induction of high affinity binding in T cells is also expected. It is expected that this gene transfer will manifest an excellent effect in such diseases as AIDS, hepatitis C, adult T cell leukemia or Bovine Spongiform Encephalopathy where high affinity antibodies are not obtained or, even if obtained, the production of high affinity antibodies cannot be maintained because mutations promptly occur in antigens.

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The GANP gene overexpressing mammal of the invention is useful in developing monoclonal antibodies useful in the preparation of biological research reagents and clinical test reagents. For example, the preparation of a monoclonal antibody to a specific signal transduction molecule in a functional domain- or functional motif-specific manner and as a high affinity antibody with high binding ability easily is very widely applicable. Since many antibodies are not screened many times, sometimes it is impossible to use them in Western analysis and immunoprecipitation. When the transgenic mammal of the invention is used for antibody production, high affinity antibody-producing cells may be selected from a relatively small number of clones. Thus, the effect of the present invention in the reduction of cost, time and labor is great. In particular, the preparation of phosphorylated antibodies and specific antibodies to mutated sites of genes is applicable to diagnostics, or the selective injection method for medicines using antibodies. The production of high

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affinity antibodies which selectively bind to a specific gene sequence or nucleotide portion will also become possible.

A part of the steric structure of any substance (such as inorganic substance, carbohydrate, or chemically synthesized substance) is recognized as an antigen motif. Although no high affinity antibodies have been obtained to date, mice created by mating with autoimmune mice are effective for obtaining high affinity antibodies to all antigens. There is a possibility that high affinity antibodies whose binding ability is on the order of 10^{-11} M might be obtained by this method. By introducing the developed technology of ELISA, it is possible to develop a technology to detect trace substances easily.

According to the present invention, it is also possible to provide a gene therapeutic for allergic diseases or autoimmune diseases, comprising an RNA primase inactivated-type GANP gene. The "RNA primase inactivated-type GANP gene" means a GANP gene in which the RNA primase domain is deficient or mutated. Due to mutations of the serine residue at position 502 and neighboring residues in the gene, the structure and function of GANP molecule encoded by this gene has been altered.

The gene therapeutic of the invention may be prepared by combining a recombinant vector comprising an RNA primase inactivated-type GANP gene with a base to be used in the gene therapeutic. As a vector for use in the construction of the recombinant vector, a viral vector such as retrovirus vector, adenovirus vector, adeno-associated vector, vaculovirus vector or vaccinia virus vector may be enumerated. Alternatively, an animal expression plasmid may be used. Preferably, the vector is a viral vector. When an RNA primase inactivated-type GANP gene has been integrated into a viral vector, viral particles containing the recombinant protein may be produced and combined with a base for the gene therapeutic to thereby prepare the gene therapeutic.

Specific examples of the base to be used in the gene therapeutic include those bases conventionally used in injections, e.g. distilled water; solution of sodium chloride or solution of a mixture of sodium chloride and inorganic salt; solution of mannitol, lactose, dextran or glucose; solution of amino acid such as glycine or arginine; mixed solution consisting of organic acid solution or salt solution and glucose solution. Alternatively, injections may be prepared as solutions, suspensions or dispersions by combining those bases with auxiliary agents such as osmoregulator, pH regulator, vegetable oil, surfactant, etc. according to conventional methods well known to those skilled in the art. It is also possible to powder or lyophilize these injections and dissolve them at the time of use.

The gene therapeutic of the invention may be administered systemically by conventional intravenous or intra-arterial administration, or administered locally by local

injection or oral administration. The dose of the gene therapeutic of the invention varies depending on the age, sex, conditions of the patient, the route of administration, the number of times of administration, and the dosage form. Generally, the gene therapeutic of the invention may be administered to adult patients in the range from 1 $\mu\text{g/kg}$ to 1000 mg/kg per day, preferably in the range from 10 $\mu\text{g/kg}$ to 100 mg/kg per day, in the amount of the recombinant gene. The number of times of administration per day is not particularly limited.

EXAMPLES

Hereinbelow, the present invention will be described more specifically with reference to the following Examples which should not be construed as limiting the present invention.

EXAMPLE 1: Expression and Function of GANP in Autoimmune Disease Model Animals

(Materials and Methods)

1. Animals

NZB, NZW, B/WF1, MRL/lpr and BXSB mice were purchased from Japan SLC Co.

C57BL/6 and BALB/c mice were purchased from Charles River Japan. NOD mice were kindly supplied from Dr. Miyazaki, the graduate school of Osaka University.

2. Antibodies and Reagents

Rat monoclonal antibodies to mouse B220 (RA3-6B2), mouse IgM (AM/3) and mouse IgD (CS/15) were purified from hybridoma culture supernatant and labeled with D-biotin-N-hydroxysuccinimide ester (Roche diagnostics, Branchburg, NJ). Biotin-labeled rat anti-mouse Syndecan-1 and anti-mouse CD5 monoclonal antibodies were purchased (BD PharMingen, San Diego, CA). Biotin-labeled peanut agglutinin (PNA) was purchased from Vector Laboratories (Burlingame, CA).

3. Immunization

Trinitrophenyl keyhole limpet hemocyanin (TNP-KLH) and TNP-Ficoll were purchased from Biosearch Technologies (Novato, CA). Briefly, 100 μg of TNP-KLH emulsified in complete Freund's adjuvant or 25 μg of TNP-Ficoll was injected into the

abdominal cavity of the mouse. Fourteen days thereafter, lymph organs were removed and frozen with OCT compound to be used in immunohistological analysis.

4. Immunohistological Analysis

5 Six-micrometer cryosections of organs were fixed in acetone for 5 min, blocked with 3% BSA in PBS for 15 min, and incubated for 1 hr with rat anti-mouse GANP monoclonal antibody (42-23) [Kuwahara, K. et al., 2000, *Blood* 95: 2321-2328] or rat anti-pSer⁵⁰² GANP monoclonal antibody (PG/103) [Kuwahara, K. et al., 2001, *Proc. Natl. Acad. Sci. USA* 98: 10279-10283]. Sections were mounted on slide glasses, which were
10 washed with PBS several times and then incubated with alkali phosphatase (ALP)-conjugated goat anti-rat IgG antibody (ICN Pharmaceuticals, Costa Mesa, CA). Color development was carried out with Vector Blue kit (Vector). Double staining was carried out using biotin-labeled antibodies in combination with horse radish peroxidase (HRP)-conjugated streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD).
15 After color development with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dojin Kagaku), sections were fixed in 1% glutaraldehyde in PBS for 1 min. For mounting, Aquatex (Merck, Darmstadt, Germany) was used. In order to detect cells with proliferation activity *in vivo*, bromodeoxyuridine (BrdU) (Sigma Chemicals Co., St. Louis, MO; 1 mg/mouse) was injected intravenously 2 hrs before slaughter. Cells which synthesize DNA
20 were stained with a combination of anti-BrdU monoclonal antibody (BD PharMingen) and ALP-conjugated goat anti-mouse Ig antibody (sigma), followed by color development with Vector Red (Vector) for detection. PAS staining was carried out as described previously [Jiang, Y. et al., 1997, *J. Immunol.* 158: 992-997].

25 5. Results

(1) Appearance of GANP^{hi} Cells in MRL/lpr Mouse Lymph Nodes

GANP is expressed highly in autoimmune-prone, highly active B cells. High level GANP-expressing lymphocytes (GANP^{hi} cells) appear spontaneously in peripheral lymph nodes of MRL/lpr mice in a non-immunized state.

30 Immunohistochemical analysis was performed on popliteal lymph nodes from autoimmune disease model (MRL/lpr and NZB) female mice and normal C57BL/6 female mice using anti-GANP monoclonal antibody and ALP-conjugated anti-rat Ig antibody.

The results are shown in Fig. 1. While GANP^{hi} cells stained with Vector Blue (ALP substrate) were observed in lymph nodes of MRL/lpr mice at week 7, such cells were
35 not observed in NZB mice of the same age and appeared at week 40 (Fig. 1). In normal

C57BL/6 mice, an extremely small number of GANP^{hi} cells were observed throughout the period of experiment.

Compared to C57BL/6 mice, autoimmune disease model mice revealed a remarkable increase in lymphocytes but showed no GANP^{hi} cells under non-immunized conditions (Fig. 1). The appearance of such GANP^{hi} cells was examined in lymph nodes of NZB mice which develop autoimmune conditions little by little as they get older. While young NZB mice (7 week old) did not have GANP^{hi} cells in their popliteal lymph nodes, aged NZB mice (40 week old) had a great number of GANP^{hi} cells.

It is considered that GANP RNA primase activity may play an important role in the activation and differentiation of B cells. Then, inventor compared the states of phosphorylation of Ser⁵⁰² (which is a key phosphorylation site for RNA primase activity) in NZB mice using anti-pSer⁵⁰² monoclonal antibody.

The expressions of GANP and pSer⁵⁰² GANP were compared in lymph nodes of NZB mice. Briefly, pSer⁵⁰² GANP was detected with anti-pSer⁵⁰² GANP (PG/103) monoclonal antibody (blue) and all sections were stained with biotin-labeled anti-B220 monoclonal antibody, followed by detection with a combination of HRP-conjugated streptavidin and DAB (brown). Representative data obtained from two independent experiments are shown in Fig. 2.

In Fig. 2, the bottom panel (graph) shows the time course of the numbers of GANP^{hi} cells (black column) and pSer⁵⁰² GANP^{hi} cells (column with slant lines) in extrafollicular regions.

GANP expression is remarkable at week 8; GANP^{hi} cells were detected throughout the experiment period up to week 32 (Fig. 2, upper panel). In contrast, pSer⁵⁰² positive cells reached the peak at week 8 and then sharply decreased (Fig. 2, middle panel). The numbers of reactive cells based on peak age obtained by microscopic observation are shown (Fig. 2, bottom panel). From these results, it is understood that GANP expression is accompanied by RNA primase activity at the beginning but this activity is not regulated for a long period of time.

(2) Spontaneous Appearance of GANP^{hi} Cells in the Red Pulp of the Spleen in Autoimmune-Prone Mice

Whether or not the GANP^{hi} cells detected in popliteal lymph nodes of autoimmune-prone NZB mice appear in the spleen under non-immunized conditions was examined.

Immuno-staining was carried out in the same manner as described in (1) above (Fig.

2). Representative data from three independent experiments are shown in Fig. 3.

GANP^{hi} cells appeared in the spleen at week 4. The cell count reached its maximum at week 12 but GANP^{hi} cells disappeared at week 24 (Fig. 3, upper panel). The expression of pSer⁵⁰² GANP was also detected at weeks 8 and 12 (Fig. 3, middle panel). From the results of comparison with relative cell counts in the red pulp, it is understood that the GANP^{hi} cells which had appeared in the spleen moved to peripheral lymph nodes 12 weeks thereafter. The increase of GANP^{hi} cells is proportional to the yield of autoantibody prior to the occurrence of autoimmune disease (Figs. 2 and 3; Theofilopoulos, A.N. et al., 1985, *Adv. Immunol.* 37: 269-390).

The appearance of GANP^{hi} cells may be associated with abnormalities in B cells in autoimmune-prone mice. Therefore, the appearance of GANP^{hi} cells was examined in various autoimmune-prone mice (8 week old) under non-immunized conditions.

The results are shown in Fig. 4. GANP^{hi} cells appeared remarkably in the red pulps of MRL/lpr, NZB and B/WF1 mice.

Although the number of GANP^{hi} cells did not show a remarkable increase in the spleens of BXSB and NOD mice (both are SLE model mice), the number showed an increase when compared to the control mice, i.e. BALB/c mouse (Fig. 4) and C57BL/6 mouse (Fig. 1). Spleen sections showed, as a GC-like structure, or immature association of PNA⁺ B cells. GANP expression in the GC-like region was not high compared to GANP expression in the GC which was created by immunizing normal C57BL/6 mouse and BALB/c mouse with T cell-dependent antigens (TD-Ags). However, GANP^{hi} cells appeared remarkably in the red pulp region in autoimmune-prone mice (fig. 4).

Further, GANP^{hi} cell population was analyzed with markers of lymphoid cells.

Spleen sections from NZB mice were double-stained with biotin-labeled B220 monoclonal antibody, biotin-labeled Syndecan-1 monoclonal antibody, biotin-labeled IgM monoclonal antibody and anti-IgG antibody to thereby identify GANP^{hi} cells.

The results are shown in Fig. 5. The photographs in the left side panel of Fig. 5 show sections when biotin-labeled IgM monoclonal antibody, anti-IgG antibody, biotin-labeled B220 monoclonal antibody and biotin-labeled Syndecan-1 monoclonal antibody were used, respectively. The photographs in the central panel show GANP expression in the same sections as described above. The right side panel is a superposition of the left side panel and the central panel. Those cells which are double-stained in the right side panel indicate that GANP^{hi} cells are B220⁺ Syndecan1⁺ IgM⁺. GANP expression is shown in red when IgM, IgG and B220 antibodies were used, and shown in green when Syndecan-1 antibody was used. Markers are indicated in green when IgM, IgG and B220

antibodies were used, and indicated in red when Syndecan-1 antibody was used.

GANP^{hi} cells show the phenotype of B220⁺ Syndecan-1⁺ and express a large quantity of IgM within cells (Fig. 5). GANP^{hi} cells are negative with respect to CR1, Thy-1, GL-7, CD23 and PNA. From these results, it is shown that GANP^{hi} cells are B-lineage cells of late maturing stage, perhaps plasma cells. In order to examine whether or not these GANP^{hi} cells are proliferative plasmablast cells, BrdU (1 mg/mouse) was intravenously injected into NZB mice, which were then incubated for 2 hrs so that BrdU was taken *in vivo*. Subsequently, spleen sections were prepared from the resultant mice.

Sections were double-stained with anti-GANP monoclonal antibody (blue) and anti-BrdU monoclonal antibody (red). PAS staining was carried out according to conventional methods.

The results are shown in Fig. 6. GC represents germinal center (left panel). GANP singly positive cells are shown with arrows; and PAS singly positive cells are shown with arrow heads (central panel).

Also, sections were stained with biotin-labeled anti-CD-5 monoclonal antibody. The PALS region represents the periaarterial sheath in lymph nodes (right panel). Fig. 6 shows representative data obtained from three independent experiments.

Since GANP^{hi} cells are not positive with respect to BrdU intake (fig. 6), it is suggested that these cells are not proliferative and are more mature than plasmablast stage.

As abnormal differentiation of B-1 cells, Mott cell formation is observed in autoimmune-prone mice. Mott cell is an abnormal morphology of plasma cell; a large number of IgM molecules are accumulated in rough-surfaced endoplasmic reticulum-associated follicles which are detected as intracytoplasmic Russell bodies by PAS staining [Jiang, Y. et al., 1997, *J. Immunol.* 158: 992-997]. GANP^{hi} cells are not stained by PAS staining (Fig. 6), and thus can be distinguished from Mott cells which are B-1 cell-derived plasma cells. Since the GANP^{hi} population in the spleen was negative in CD5 expression (Fig. 6) and peritoneal cells obtained from NZB mice (12 week) were negative with respect to GANP^{hi} cells, it is suggested that B-1 cells are not expressing a large quantity of GANP. From these results, GANP^{hi} cells are classified into highly active B cells of autoimmune state, and it is suggested that this population is of a lineage whose origin is different from the origin of B-1 cells.

(3) Induction of GANP^{hi} Cells in Normal Mice by Immunization with TD-Ag

Whether or not the appearance of GANP^{hi} plasma cells in secondary lymph organs

is limited to autoimmune-prone mice was examined.

Female C57BL/6 mice (7 week old) were immunized intraperitoneally with TNP-Ficoll (TI-2-Ag) or TNP-KLH (TD-Ag). Their spleens were removed on day 14. Those mice immunized with TNP-Ficoll did not show GANP^{hi} cells in the red pulp region when counter-stained with biotin-labeled anti-IgD monoclonal antibody (Fig. 7, left panel). Those mice immunized with TNP-KLH showed the induction of GANP^{hi} cells in the red pulp region (Fig. 7, right panel). In Fig. 7, GANP^{hi} cells are marked with arrows. WP represents the white pulp region.

GANP^{hi} plasma cell population is also induced in the spleens of normal C57BL/6 and BALB/c mice by immunization with TD-Ag, though the number of cells is very small (Fig. 7). Immunization with T cell-independent Ag (TI-Ag) has only a small effect in inducing such cells. The GANP^{hi} cell population showed a phenotype similar to B220^{lo}IgM^{hi}IgD^{lo}GL-7^{lo}PNA^{lo}CD5^{lo}CD40^{lo}, but was Syndecan-1⁺.

These results indicate that the generation of GANP^{hi} plasma cells in autoimmune-prone mice is induced by stimulation similar to the stimulation supplied for immune responses to TD-Ag. Ag-driven B cells which have undergone proliferation and differentiation in the GC may be localized in the red pulp region as the plasma cell stage for a longer period, while expressing GANP.

EXAMPLE 2: Excessive Expression of GANP (Methods)

1. Stable Transfection into Daudi Cells

Ten micrograms of linearized pCXN-2 mouse GANP or GANPS/A502 cDNA was electroporated into Daudi cells with Gene Pulser II (Bio-Rad). After 48 hrs, selection started with G418 (Promega; 1 mg/ml) to thereby obtain Daudi cells which express mouse GANP stably.

2. Analysis of the IgV_H Transcript of Daudi Transfectants

Total RNA was extracted from total cells with Trizol (Invitrogen). cDNA was obtained as described previously (Kuwahara, K. et al., Blood 95, 2321-2328 (2000)). LV_H3-C_H1C_μ transcript was amplified using the following primers and the reaction solution. For amplification, Pfu Turbo (Stratagene) was used.

5'-LV_H3 primer: 5'-CTATAACCATGGACCATGGACATACTTTGTTCC-3' (SEQ ID NO: 5)

3'-XbaI-C_H1-C_μ primer:

5'-TGCATGCATTCTAGAGTTGCCGTTGGGGTGCTGGAC-3' (SEQ ID NO:

6)

Composition of the Reaction Solution:

cDNA	0.5 μl
10x buffer	2.5 μl
10 mM dNTP mix	0.5 μl
5'-LVH3 primer (10 μM)	1 μl
3'-Xba I-CH1-Cμ primer (10 μM)	1 μl
Pfu Turbo	0.5 μl
dH ₂ O	19.5 μl

5

Reaction Conditions:

94°C for 1 min

[94°C for 1 min; 62°C for 1 min ; 72°C for 1 min] x 35 cycles

72°C for 10 min

10 4°C

The resultant PCR product was digested with NcoI and XbaI, purified in a gel, and ligated to a plasmid digested with NcoI-XbaI. After transformation into competent bacterial cells, a small quantity of plasmid DNA was prepared with QIAprep kit (Qiagen).

15 The nucleotide sequence of this plasmid DNA was determined with an automated sequencer (Applied Biosystems).

3. Preparation of GANP-Transgenic (Tg) Mouse

20 A transgene was prepared by inserting a 5.6 kb mouse GANP gene into the XhoI site of pLG vector. This vector having a human immunoglobulin intron enhancer domain (2 kb EcoRI fragment) is a specific vector that directs strong expression in B cells. This gene was linearized and transferred into mice. Briefly, a linearized pLG vector (Koike, M. et al., *Int. Immunol.* 7, 21-30 (1995)) comprising the full-length mouse GANP cDNA was micro-injected into fertilized eggs of C57BL/6 mice. The presence of the transferred gene

25 was screened using genomic DNA obtained from mouse tail vein, the following primers and the reaction solution.

1-5' primer: 5'-TCCCGCCTTCCAGCT GTGAC-3' (SEQ ID NO: 7)

1-3' primer: 5'-GTGCTGCTGTGTTATGTCCT-3' (SEQ ID NO: 8)

Composition of the Reaction Solution:

DNA (50 ng/μl)	1 μl
10x buffer	2.0 μl
2.5 mM dNTP mix	2.0 μl
1-5' primer (10 μM)	0.8 μl
1-3' primer (10 μM)	0.8 μl
Z-Taq DNA polymerase	0.1 μl
dH ₂ O	13.3 μl

Reaction Conditions:

[98°C for 5 sec; 59°C for 5 sec ; 72°C for 10 sec] x 35 cycles

4°C

5

4. RT-PCR

Total RNA was extracted from the spleen or spleen B cells using Trizol (Invitrogen). RT-PCR was performed with two primers (1-5' primer and 1-3' primer) to synthesize cDNA (Kuwahara, K. et al., *Blood* 95, 2321-2328 (2000)). GANP transcript was detected by agarose gel electrophoresis. β-actin transcript was used as a control.

10

5. Results

(1) Somatic Hypermutations (SHMs) in V Region Genes of Daudi Transfectants Expressing GANP Stably

15

A GANP gene was transferred into various human B lymphocytes used in SHM analysis *in vitro* (Rogozin, I. B., et al., *Nat. Immunol.* 2: 530-536 (2001); Kuwahara, K. et al. *Blood* 95: 2321-2328 (2000); and Denepoux, S. et al., *Immunity* 6: 35-46 (1997)). Although a great number of B cell strains were incapable of transfection, it was possible to transfer a GANP gene into Daudi B cells which express AID that usually does not generate SHMs while maintained.

20

The resultant clones showed highly frequent SHMs (5×10^{-4} /bp) in the V regions, compared to wild-type cells and pseudo-transfectants.

V_H3-C_H1C_μ fragment was amplified by PCR and subcloned into a plasmid, followed by sequencing.

25

Schematic diagrams of somatic hypermutations are shown in Fig. 8: A-C. Vertical line “|” represents a silent mutation (where the amino acid is not changed), and the other mark (short vertical line with black circle) represents a mutation where the amino acid is replaced. While Daudi/mock shows few mutations, four clones of Daudi/DANP-14, -15, -17 and -21 more or less show a great number of mutations. The efficiency of inducing

mutations is decreased in the transfectant into which a mutant (GANP S/A) has been introduced; in this mutant, Ser⁵⁰² involved in the control of DNA primase activity is replaced with alanine.

SHMs were not induced in constant region genes (Fig. 8: A-C). The RNA primase activity of GANP is regulated by the phosphorylation of S502, and this phosphorylation can be detected with a specific monoclonal antibody (Kuwahara, K. et al., *Proc. Natl. Acad. Sci. USA* 98: 10279-10283 (2001)). Since both *in vivo* and *in vitro* stimulation of B cells induce the phosphorylation of Ser⁵⁰² (Kuwahara, K. et al., *Proc. Natl. Acad. Sci. USA* 98: 10279-10283 (2001)), whether or not this phosphorylation is involved in the generation of SHMs in Daudi B cells was examined.

When a non-phosphorylated GANP mutant (GANP-S502A) was introduced, SHMs were not induced (Fig. 8A). Therefore, it is suggested that the phosphorylation of S502 is important for the generation of SHMs in GC-B cells.

15 (2) Transgenic Mouse Overexpressing GANP in B Cells

In order to examine the involvement of GANP in immune responses, GANP-transgenic (Tg) mouse which overexpresses GANP under the control of human Ig enhancer and promoter was created (Fig. 9: A-B). Enhancement in GANP mRNA expression was confirmed by RT-PCT.

20 This mouse showed an increase in GANP expression in B cells (Fig. 9C), and showed normal differentiation of B lineage cells in surface marker analysis of bone marrow, spleen and lymph node cells.

In order to investigate into the *in vivo* role of GANP in SHMs, the V_H186.2 region was examined after immunization with NP-CG (which is TD-Ag). Briefly, 50 µg of NP-CG was administered to GANP-overexpressing transgenic (Tg) mice three times at intervals of two weeks. Then, the V_H186.2 region was amplified by PCR, followed by analysis of somatic hypermutations.

The results are shown in Fig. 10. The number of mutations was slightly increased in Tg mice. However, the somatic hypermutation of W³³ to L which indicates high affinity was increased almost 3-fold in Tg mice. “CDR” represents complementarity determining region.

The V_H186.2 locus shows SHMs of a peculiar pattern for high affinity IgG(γ1λ1)NP response. The sequence analysis on the total spleen B cells after immunization with NP-CG revealed that SHM frequency is slightly increased in GANP-Tg mice compared to wild-type mice (Fig. 10).

It has been shown previously that these somatic hypermutations are important for affinity maturation of hapten specific B cells (Allen, D. et al., *EMBO J.* 1995-2001 (1988)).

EXAMPLE 3: Preparation of B Cell Specific GANP-Deficient Mice (B-GANP^{-/-} Mice)

5

(Methods)

1. Establishment of CD19-Cre/+GANP flox Mice

Using genomic DNA encoding GANP, neomycin resistance gene (*neo*) was inserted downstream of exon II to thereby construct a targeting vector. LoxP sites were introduced into the 3' flanking region to neo and the intron between exons I and II, respectively.

Briefly, flox mice in which GANP exon II is sandwiched by two loxP sequences were prepared. These mice were crossed with CD19-Cre mice to thereby establish B cell specific GANP-deficient mice (Fig. 11: A and B).

15

The targeting vector was linearized and electroporated into TT2 ES cells (Yagi, T. et al., *Anal. Biochem.* 214: 70-76 (1993)) for transfection. After selection with G418, ES colonies were picked up and incubated with proteinase K. Homologous recombinants were screened for with the following neo2 primer and CGK3'-2 primer.

neo2 primer: 5'-GCCTGCTTGCCGAATATCATGGTGGAAAAT-3' (SEQ ID NO: 9)

CGK3'-2 primer: 5'-GGCACCAAGCATGCACGGAGTACACAGA-3' (SEQ ID NO: 10)

Homologous recombination was confirmed by analyzing the BamHI-digested DNAs of ES clones by Southern blotting using probe A. Using three positive clones showing a 4 kb band, microinjection into ICR blastocysts was carried out to prepare chimeric first generation mice. The absence of GANP expression in B cells was confirmed by Southern blotting, RT-PCR and cell staining (Fig. 11: C, D and E).

GANP flox/+ mice were backcrossed with C57BL/6 mice at least 10 times. In order to delete GANP gene in B cells, GANP-floxed mice were crossed with CD19-Cre knock-in mice (Rickert, R. C., et al., *Nucleic Acids Res.* 25, 1317-1318 (1997)).

2. FACS Analysis

Lymph organ-derived single cell suspensions were stained with each biotin-labeled monoclonal antibody for 1 hr on ice. After washing with staining buffer, cells were incubated with FITC-conjugated streptavidin (Amersham Bioscience) and PE-conjugated

monoclonal antibody for 1 hr. Lymphocytes were analyzed with FACScan (Becton Dickinson) using Cell Quest software.

3. Purification of B Cells

5 Spleen cells were isolated from Cre-flox/+ mice and B-GANP^{-/-} mice (7 to 8 week old) and treated with 0.15 M ammonium chloride buffer to remove erythrocytes. After incubation at 37°C for 30 min on plastic dishes, unadhered cells were recovered as lymphocytes. Then, T cells were removed therefrom using Dynabeads-anti-mouse Thy1.2 monoclonal antibody (Dyna) according to the protocol attached thereto. The purity of B
10 cells (90% or more) was confirmed by cell surface staining with FITC-conjugated B220 monoclonal antibody (BD Pharmingen).

4. In vitro Proliferation Assay

Purified B cells were incubated in RPMI-1640 medium (with or without cell
15 division promoter) containing 10% thermo-inactivated FCS (JRH Biosciences), 2 mM L-glutamine and 5 x 10⁻⁵ M 2-mercaptoethanol in 96-well microplates at 2 x 10⁵ cells/well for 48 hrs. Cells were recovered after pulsing with [³H]-thymidine at 0.2 µCi/well for 16 hrs. Then, the radioactivity taken up was measured with a scintillation counter.

As the cell division promoter, affinity-purified goat anti-mouse µ-chain-specific
20 antibody (10 µg/ml) [F(ab')₂] (ICN), rat anti-mouse CD40 monoclonal antibody (LB429; 10 µg/ml) and LPS (Sigma; 10 µg/ml) were used.

5. Antigens and Immunization

TNP-KLH, TNP-Ficoll and nitrophenyl-chicken γ globulin (NP-CG) (23:1) were
25 purchased from Biosearch Technologies. Fifty micrograms of TNP-KLH and NP-CG (precipitated with aluminium) or 25 µg of TNP-Ficoll (dissolved in PBS) was injected into the abdominal cavities of Cre-flox/+ mice and B-GANP^{-/-} mice.

6. Measurement of Antigen Specific Antibody Production

30 At day 10 or 14 after the immunization, sera were recovered from immunized mice. ELISA plates were coated with 5 µg/well of TNP-BSA (Biosearch Technology). Each well was blocked with 3% BSA in PBS, and incubated with serially diluted serum. After washing with PBS-0.1% Tween 20, each well was incubated with biotin-conjugated isotype-specific monoclonal antibody and alkaline phosphatase (ALP)-conjugated
35 streptavidin (Southern Biotechnology). Color development was performed in the presence

of substrates.

In order to determine the affinity of NP-binding antibodies in the sera, the ratio of NP2-binding antibody to NP25-binding antibody was calculated by differential ELISA using NP2-BSA (two NPs are bound to BSA per molecule) and NP25-BSA (25 NPs are bound to BSA per molecule) (Biosearch Technology) as coated antigens.

7. Immunohistochemistry

Spleen sections (8 μ m) from immunized mice were fixed lightly in acetone. These samples were blocked with 3% BSA in PBS-Tween 20 and incubated with anti-IgD monoclonal antibody and ALP-conjugated anti-rat IgG (ICN) antibody. The first color development was performed with Vector Blue kit (Vector). The second color development was performed by incubating the sample with biotin-conjugated peanut agglutinin (PNA) (Vector) and horseradish peroxidase-conjugated streptavidin (Kirkegaard & Perry) and then incubating with 3,3'-diaminobenzidine tetrahydrochloride (Dojindo). Samples were fixed with 1% glutaraldehyde in PBS and then mounted with Aquatex (Merck).

8. Sequence Analysis of V_H186.2 Gene

NP-binding IgG1^{dull}CD38^{low} B cells from NP-CG-immunized Cre-flox⁺ and B-GANP^{-/-} mice were fractioned with FACS Vantage (Becton Dickinson Biosciences) using (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP) and incubated with proteinase K at 37°C overnight. Using the resultant lysate, PCR was performed two times as described previously (Takahashi, Y. et al., *Immunity* 14: 181-192 (2001)). The genetic DNA of V_H186.2 was ligated to pBluescript, followed by determination of the sequence with an automated sequencer.

9. Detection of Apoptotic Cells

B cells purified from Cre-flox⁺ and B-GANP^{-/-} mice were stimulated with various reagents for 40 hrs (Watanabe, N. et al., (1998) *Scand. J. Immunol.* 47: 541-547). For detection of AICD, anti- μ antibody (50 μ g/ml) was immobilized on 24-well plates. For detection of other types of apoptosis, purified B cells was stimulated with various stimulants and the incubated with anti-Fas monoclonal antibody (Jo2; BD Pharmingen) for 4 hrs (Wang, J. et al., (1996) *J. Exp. Med.* 184, 831-838). Cells were incubated in propidium iodide (PI) solution (50 μ g/ml PI, 0.1% Triton X-100, 0.1% sodium citrate) at room temperature for 1 hr, and apoptotic cells were calculated (percent) as sub-G₁ area by FACSscan. Further, apoptotic cells were also confirmed microscopically after trypan blue staining.

10. TUNEL Assay

Cre-flox⁺ and B-GANP^{-/-} mice were immunized with SRBC (sheep red blood cells). Spleen cryosections were prepared therefrom and fixed in 4% paraformaldehyde in PBS. Section samples were treated with MEBSTAIN Apoptosis Kit II (MBL) and counter-stained with PI. For use in an experiment conducted together with TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay, section samples were also stained with ant-IgG₁ monoclonal antibody (BD Pharmingen) and Alexa 546-conjugated goat anti-rat IgG antibody (Molecular Probes). Positive signals were detected and the results were confirmed with a fluorescence microscope (BX51; Olympus).

11. Results

(1) The Role of RNA Primase GANP

In order to investigate into the role of RNA primase GANP, B-GANP^{-/-} mice which are deficient in GANP gene in their CD19⁺ B cells were prepared using Cre-loxP system (Fig. 11: A and B). The GANP gene of the B-GANP^{-/-} mice lacked most of exon II (Fig. 11C). B-GANP^{-/-} cells did not express GANP mRNA (Fig. 11D) and, according to immunostaining, expressed little GANP protein (Fig. 11E). B-GANP^{-/-} mice grew normally, showing normal numbers of lymphocytes in the bone marrow, spleen, thymus and lymph nodes. According to flow cytometry, B-GANP^{-/-} mice showed surface marker profiles on cells of the bone marrow, spleen and lymph nodes similar to those observed in the control Cre-flox/+ mice (Fig. 12); there was no difference between B-GANP^{-/-} mice and Cre-flox/+ mice.

The number of mature B cells expressing sIgM^{low}sIgD^{high} (IgM⁺IgD⁺) was decreased in the lymph nodes of B-GANP^{-/-} mice. B cells from B-GANP^{-/-} mice showed normal proliferation responses after *in vitro* stimulation with anti-μ antibody, anti-μ antibody + anti-CD40 monoclonal antibody, or lipopolysaccharide (Fig. 13: white column represents B-GANP^{-/-} and black column represents Cre-flox/+). On the other hand, B cells from B-GANP^{-/-} mice showed a decrease in proliferation activity after stimulation with anti-CD40 monoclonal antibody (5 and 10 μg/ml) (Fig. 13). This indicates that responses to CD40/CD145 interaction are slightly impaired in B cell proliferation in B-GANP^{-/-} mice. The amounts of serum Ig in B-GANP^{-/-} mice were similar to those in Cre-flox/+ mice (Fig. 14).

(2) Antigen Specific Antibody Production in B-GANP^{-/-} Mice

Immunoresponses of B-GANP^{-/-} mice after immunization of TI-Ag or TD-Ag were

examined. At day 14 after immunization with a TI antigen trinitrophenyl (TNP)-Ficoll, anti-TNP antibody titers were measured by ELISA. As a result, TNP-Ficoll induced similar responses in B-GANP^{-/-} mice and Cre-flox/+ mice; no particular difference was observed (Fig. 15).

5 When germinal center (GC) formation was examined, mutant mice showed delayed GC formation in response to TD-Ags such as TNP-keyhole limpet hemocyanin (KLH) or NP-CG, compared to Cre-flox/+ mice.

10 With respect to the peak response on GC formation, Cre-flox/+ mice showed large matured GCs stained with peanut agglutinin (a marker for GC-B cells) at day 10 (arrow marks in Fig. 16). At day 10 after immunization, GC formation in B-GANP^{-/-} mice was slightly less. However, B-GANP^{-/-} mice showed more GC formation than Cre-flox/+ mice at day 14, and they still showed vigorous GC formation even at day 20 (arrow marks in Fig. 16).

15 Since B-GANP^{-/-} mice showed definite GC formation at day 14, their antigen specific antibody responses were measured (Fig. 17). When immunized with TNP-KLH (a TD antigen), B-GANP^{-/-} mice did not show definite GCs until day 10 after the immunization; no difference was observed in antibody titers between B-GANP^{-/-} mice and Cre-flox/+ mice. At day 14, however, B-GANP^{-/-} mice showed gradual increase and expansion of GCs (Fig. 17). Mutant mice showed antibody responses to TNP-KLH similar to those shown by
20 Cre-flox/+ mice.

(3) Obstacles to Affinity Maturation in B-GANP^{-/-} Mice

25 In order to further investigate into the characteristic of the GC in B-GANP^{-/-} mice (i.e. antibody response is of low affinity), antigen specific IgG1⁺ GC-B cells were examined after immunization with NP-CG.

By differential ELISA using conjugates of NP hapten with different molecular weights and a protein, responses to NP2-BSA conjugate were compared to responses to multi-hapten NP25-BSA conjugate.

30 In B-GANP^{-/-} mice, antibody responses to NP2-BSA conjugate were of low affinity (13%) at day 35 after immunization with NP-CG. This value was remarkably lower than the value of Cre-flox/+ mice (42%) (Fig. 18).

35 Further, as shown in Fig. 19, NP-specific IgG1^{dull}CD38^{low} B cells were remarkably decreased in B-GANP^{-/-} mice. Specifically, while the ratio of these B cells was 1,164 cells/10⁶ cells in Cre-flox/+ mice at day 10 after immunization, it was 88 cells/10⁶ cells in B-GANP^{-/-} mice. At day 14, while Cre-flox/+ mice had 879 cells/10⁶ cells, B-GANP^{-/-}

mice had $83 \text{ cells}/10^6 \text{ cells}$. This tendency was unchanged at day 20.

In contrast, $\text{IgG1}^{\text{high}}\text{CD38}^{\text{high}}$ memory B cells were not decreased. These results indicate that the mutation of no GANP expression caused defect in B cell differentiation at the stage of $\text{IgG1}^{\text{high}}\text{CD38}^{\text{low}}$ GC-B cells.

5 In order to confirm the reduced affinity maturation in antibodies of B-GANP^{-/-} mice, the sequence of the V_H186.2 region in spleen B cells was examined after immunization with NP-CG.

Since somatic hypermutations occur at this stage of B cell differentiation, a variety of purified B cells were examined on SHMs in V_H186.2 locus. It should be noted that
10 V_H186.2 locus is used for high affinity IgG ($\gamma 1\lambda 1$) NP-responses (Cumano, A. & Rajewsky, K. (1985) *Eur. J. Immunol.* 15, 512-520). With respect to IgM locus, no difference was observed between B-GANP^{-/-} mice and Cre-flox/+ mice.

Subsequently, B-GANP^{-/-} mice or Cre-flox/+ mice were immunized with NP-CG, followed by sorting for NP-binding IgG₁ weakly positive CD38 weakly positive GC-B cells
15 (i.e. Ag-binding IgG₁ B cells) (Fig. 19). After the sorting, genomic DNA was extracted from the resultant cells. V_H186.2 was amplified by PCR and subjected to sequence analysis. Then, V_H186.2 sequences were compared (Fig. 20: A-L). Panels A-F in Fig. 20 show comparison of V_H186.2 sequences of Cre-flox/+ mice. Panels G-L in Fig. 20 show comparison of V_H186.2 sequences of B-GANP^{-/-} mice.

20 In B-GANP^{-/-} mice, the frequency of mutations in the entire IgV region sequence was 14×10^{-3} , showing a decrease compared to Cre-flox/+ mice (21×10^{-3}) (Fig. 21). Further, the high affinity type mutation of W³³ to L (i.e. mutation of the 33rd amino acid residue tryptophan to lysine, which is observed remarkably in C57BL/6 mice) was 13% (2/15 V regions), showing a remarkable decrease compared to Ce-flox/+ mice (71%, 10/14 V
25 regions) and the lowering of affinity to 1/3 (Fig. 22).

From these results, it was demonstrated that GANP is essential for the affinity maturation of antibodies.

(4) Protective Function from Apoptosis in B-GANP^{-/-} Mouse B Cells

30 It is considered that the decrease in high affinity antibody production in B-GANP^{-/-} mice is caused because B cells after antigen stimulation are unstable. Then, in order to examine the susceptibility of B cells, the apoptosis of B cells *in vitro* was studied.

In normal B cells, activation-induced cell death (AICD) was induced by strongly cross-linked B cell antigen receptor, and this AICD was prevented by stimulation by CD40.
35 In B-GANP^{-/-} B cells, though the susceptibility to AICD stimulation was equivalent to that of

normal B cells (control), inhibition of anti-CD40-mediated apoptosis was inferior to Cre-flox/+ control B cells (Fig. 23). This means that B-GANP^{-/-} mice lack the protective function for antigen-reactive B cells during GC formation.

In GCs, B cells stimulated with Ag and CD40/CD154 interaction induce the surface expression of Fas/CD95 and become susceptible to Fas-induced apoptosis. Then, the inventor measured the susceptibility of B-GANP^{-/-} B cells to anti-CD95 stimulation.

First, spleen B cells were stimulated with anti-CD40 monoclonal antibody (LB429), anti-μ antibody + anti-CD40 monoclonal antibody, IL-4 + anti-CD40 monoclonal antibody, and anti-μ antibody + IL-4 + anti-CD40 monoclonal antibody for 48 hrs, and then anti-CD95 monoclonal antibody was added to the culture medium.

As a result, apoptotic responses of B-GANP^{-/-} mouse B cells were similar to the responses of Cre-flox/+ mouse B cells; no difference was observed between B-GANP^{-/-} mice and Cre-flox/+ mice (Fig. 24).

As described above, the stimulation with anti-CD95 after anti-CD40 (LB429) treatment did not show any difference between B-GANP^{-/-} mice and Cre-flox/+ mice in the induction of expression. This suggests that B-GANP^{-/-} B cells may be susceptible to the apoptotic stimulation normally received by GC-B cells *in vivo*. Therefore, TUNEL assay was carried out using tissue sections from mice immunized with SRBC as TD-Ag.

As a result, TUNEL-positive cells increased in the GC region of B-GANP^{-/-} mice, and most of them also showed IgG₁ expression (Figs. 25 and 26). These results revealed that most of the apoptotic cells of B-GANP^{-/-} mice are GC-B cells (Figs. 25 and 26).

Subsequently, the inventor examined the RNA expression of Bcl-2 family members which are recognized to be the molecules necessary for CD40-mediated inhibition of apoptosis of various malignant lymphoma cells and normal B cells.

Stimulation with anti-μ antibody + IL-4 induced an apparent increase in bcl-2 transcription in Cre-flox/+ B cells, and anti-CD40 mAb further up-regulated this expression (Fig. 27). The B-GANP^{-/-} B cells showed similar up-regulation of bcl-2 transcripts by stimulation with anti-μ antibody, but the response to anti-CD40 mAb (anti-CD40 mAb alone or anti-μ Ab + IL-4 + anti-CD40 mAb) was not as high as the response in Cre-flox/+ B cells (Fig. 27). In other words, the RNA expression levels of Bcl-2 family involved in apoptosis inhibition were decreased in B-GANP^{-/-} B cells compared to the control (Fig. 27).

With respect to *bcl-X_L*, *bax* and *bad* in mutant B cells, the expression levels were equivalent to those in Cre-flox/+ B cells.

These results suggest that GANP regulates the signal transduction of CD40-mediated induction of Bcl-2 expression in GC-B cells, which greatly contributes to

the survival of high-affinity BCR⁺ B cells *in vivo*.

(5) Conclusion

The results obtained from B-GANP^{-/-} mice and GANP-Tg mice demonstrate that GANP is involved in the generation of high affinity B cells after immunization with TD-Ag. As a role of GANP, GANP may mediate efficient recruit and regulation of DNA polymerase in GC-B cells. Once GC-B cells with V-region SHMs have acquired high-affinity BCRs, they should be positively selected and further SHMs in the V regions might be suppressed to thereby guarantee the production of high affinity antibodies *in vivo*. Since AID expression in GC-B cells may generate DNA mutations continuously, regulation of AID activity might be necessary for maintaining high affinity BCRs in B cells. The results obtained from B-GANP^{-/-} mice suggest that GANP is necessary for SHM process.

EXAMPLE 4: Production of High Affinity Antibodies Using GANP Transgenic Mice

1. Comparison of Antibody Titers by Differential ELISA

Each two wild-type (WT) mice and GANP transgenic (Tg) mice were immunized with 100 µg of NP-CG. At day 28 after the immunization, serum samples were taken from them and subjected to ELISA. Briefly, ELISA plates were coated with 20 µg/ml of NP2-BSA or NP17-BSA overnight at 4°C. Then, the plates were blocked with 3% BSA/PBS-0.1% Tween 20 for 1 hr, followed by reaction with the serum for 1 hr. After washing with PBS-0.1% Tween 20 three times, biotin-labeled anti-mouse IgG₁ antibody (Southern Biotechnology) was reacted for 1 hr. Then, after washing with PBS-0.1% Tween 20 three times, alkaline phosphatase-labeled streptavidin (Southern Biotechnology) was reacted for 30 min. After washing with PBS-0.1% Tween 20 three times and with TBS once, color was developed using p-nitrophenyl phosphate as a substrate. Absorbance was measured at 405 nm.

The results are shown in Fig. 28. From these results, it is understood that a high affinity antibody is produced by using GANP transgenic mice.

2. Analysis of Antigen-Antibody Binding Affinity Using ELISA and Biacore

Wild-type (WT) mice and GANP transgenic (Tg) mice were immunized with NP-CG. Cells from them were subjected to cell fusion to obtain hybridomas. Using the culture supernatants of positive hybridoma clones, binding curves of antibodies responding to the antigen were obtained by ELISA and with Biacore. The utility of Tg mice was

shown from the resultant binding curves.

(1) Materials

(a) Animals

Wild-type (WT) mice and GANP transgenic (Tg) mice.

5 (b) Antibodies and Reagents

NP16-CG (16 NPs are coupled to CG (chicken immunoglobulin) per molecule), NP2-BSA (2 NPs are coupled to BSA (bovine serum albumin) per molecule), NP17-BSA (17 NPs are coupled to BSA per molecule), HRP-labeled anti-mouse IgG, IgA and IgM were used.

10

(2) Methods and Results

Each five wild-type (WT) mice and GANP transgenic (Tg) mice were immunized with NP16-CG three times at intervals of two weeks. After the 3rd immunization, the mice were exsanguinated, and antibody titers were compared using antisera. The results also confirmed the utility of GANP-Tg mice as the results described in (1) above.

Spleen cells from those mice which showed a high valence among them were fused with P3U1 myeloma cells, and plated at a density of 1×10^5 cells/well based on the numbers of spleen cells from GANP-Tg mice (6.0×10^7) and from WT mice (4.8×10^7). GANP-Tg mice-derived 600 hybridoma clones and WT mice-derived 480 hybridoma clones were cultured in HAT medium.

At day 9 of HAT culture, the culture supernatant was recovered and subjected to ELISA using NP2-BSA (1 $\mu\text{g/ml}$) as an immobilized antigen. Upper 2.5% clones showing production of high affinity antibodies as determined by measurement of absorbance in ELISA were selected from both culture supernatants derived from GANP-Tg mice and WT mice. Then, cloning was carried out using HT medium.

At day 9 of HT culture, culture supernatants were recovered and subjected to ELISA using NP2-BSA (1 $\mu\text{g/ml}$) as an immobilized antigen. As a result, 6 hybridoma clones (G2-6, G2-9, G2-12, G2-14, G2-15 and G2-16) were established from GANP-Tg mice and one hybridoma clone (W2-7) from WT mice.

Individual clones from GANP-Tg mice and WT mice were cultured in RPMI medium, and 1 ml each of culture supernatant appropriate for use in the following experiment was secured. Using this culture supernatant, the following evaluation and examination were carried out.

35 (a) ELISA

For the evaluation of antibody titers, antigens different in nature (i.e. substances different in NP content per CG molecule) were used, and antibody titers were evaluated based on the ratios of ELISA reactivities.

This method is useful for measuring the affinity of NP. It is simple and capable of testing a large number of samples. Therefore, this method is appropriate and reliable as primary screening.

First, NP2-BSA (1 $\mu\text{g/ml}$) and NP17-BSA (1 $\mu\text{g/ml}$) were separately immobilized as antigens at 4°C overnight. The antigen-immobilized plates were washed with PBS-Tween 20 and blocked with skim milk-PBS-Tween 20. After washing further with PBS-Tween 20, RPMI culture supernatants from GANP-Tg mice-derived 6 clones (G2-6, G2-9, G2-12, G2-14, G2-15 and G2-16) and WT mice-derived 1 clone (W2-7) (stock solution to 256-fold dilution) were reacted with the immobilized antigen at room temperature for 1 hr. Subsequently, the plates were washed with PBS-Tween 20. Then, HRP-conjugated anti-mouse IgG, IgA and IgM were reacted at room temperature for 1 hr. After washing with PBS-Tween 20, color was developed with ortho-phenylene diamine (OPD) for 5 min, followed by termination of the reaction with 2N sulfuric acid.

Absorbance was measured with an ELISA reader at 490 nm.

The results of ELISA are shown in Fig. 29. From these results, it is understood that high affinity antibodies are produced by using GANP-Tg mice.

(b) High Affinity Analysis Using Biacore

Using the clone which is predicted to be most high in affinity from the results of ELISA described above, physicochemical binding ability was examined with Biacore.

Analysis with Biacore was performed as described below. Briefly, NP2-BSA (1 $\mu\text{g/ml}$) was bound to Biacore chip as a ligand. As analyte solutions, RPMI culture supernatants from clone Tg (G2-9) which was predicted to be highest in affinity, clone Tg (G2-15) which was predicted to be lowest in affinity, and clone WT (W2-7) were used. Association rate constant (k_{ass}), dissociation rate constant (k_{diss}) and dissociation constant K_D ($K_D = k_{\text{diss}}/k_{\text{ass}}$) that is an indicator of affinity were calculated for each of the above culture supernatants. The smaller the K_D value is, the higher the affinity is evaluated.

As a result, the Biacore pattern of G2-9 (ELISA: 82.9% NP2/NP17 ratio) is shown in Fig. 30. Curves (a) to (e) appearing in Fig. 30 correspond to antibody concentrations of 26.6, 13.3, 6.65, 3.33 and 1.66 nM, respectively. From the above results, the following values were obtained: association rate constant (k_{ass}) = 1.48×10^5 , dissociation rate constant (k_{diss}) = 9.63×10^{-4} , and dissociation constant (indicator of affinity) K_D ($K_D = k_{\text{diss}}/k_{\text{ass}}$)

$= 6.50 \times 10^{-9}$.

On the other hand, the Biacore pattern of G2-15 (ELISA: 33.9% NP2/NP17 ratio) which is predicted to be relatively low in affinity from the results of ELISA is shown in Fig. 31. Curves (a) to (e) appearing in Fig. 31 correspond to antibody concentrations of 23.0,
5 11.5, 5.75, 2.88 and 1.44 nM, respectively.

The following values were obtained: association rate constant (k_{ass}) = 5.33×10^4 , dissociation rate constant (k_{diss}) = 1.56×10^{-2} , and dissociation constant (indicator of affinity) K_D ($K_D = k_{\text{diss}}/k_{\text{ass}}$) = 2.92×10^{-7} . This K_D value was close to the K_D value of 1.67×10^{-7} shown by W2-7 which also showed an equivalent affinity in ELISA (ELISA:
10 31.6% NP2/NP17 ratio).

From what have been described above, it is clear that high affinity antibodies are produced by using GANP transgenic (Tg) mice.

15 EXAMPLE 5: Association of GANP with MCM3, and Shuttling between Nucleus and Cytoplasm during Cell Cycle

1. Outline

In this Example, the present inventor determined the MCM3 binding domain of GANP by using truncated-type mutant GANPs, and characterized the localization of GANP
20 in NIH-3T3 cells using a monoclonal antibody specific to the phosphorylation of serine at position 502 (pSer⁵⁰²) in the GANP specific domain.

The binding of a primase to MCM is a linked function, and the molecular complex resulting from their binding has an action of unwinding the DNA double strand. Therefore, it is believed that if a GANP partial fragment has bound to MCM, that GANP fragment also
25 reveals primase activity and has an action of producing high affinity antibodies.

Then, the localization of GANP and partial fragments thereof, Map80 and MCM3 in the nucleus/cytoplasm compartment was analyzed by cDNA transfection and cell fusion experiment.

The resultant data show that GANP binds to MCM3 and that the localization of
30 GANP is influenced by MCM3 expression. GANP associates with MCM3 by a binding mode different from that by which Map80 associates with MCM3. These results suggest that GANP bound to MCM3 mediates a unique function different from the function of Map80/MCM3AP.

35 2. Materials and Methods

2.1. Cells and Cell Cultures

NIH-3T3, COS7, HeLa and Swiss-3T3 cells were maintained in D-MEM medium (Invitrogen) supplemented with 10% thermo-inactivated FCS (Dainippon Pharmaceutical), 2 mM L-glutamine (Biowhittaker), 100 µg/ml streptomycin, 100 U/ml penicillin and 50 µM 2-mercaptoethanol at 37°C under 5% CO₂ (Takei, Y. et al., (1998) *J. Biol. Chem.* 273, 22177-22180; Sakaguchi, N. et al., (1988) *EMBO J.* 7, 3457-3464, Kimura, H. et al., (1995) *Nucl. Acids Res.* 23, 2097-2104). BAL17 cells were cultured in RPMI-1640 medium (Invitrogen).

2.2. Intracellular Localization of Phosphorylated GANP and MCM3

NIH-3T3 cells were fixed in 3.7% paraformaldehyde in PBS (pH 7.4) for 5 min and made transparent using 0.2% Triton X-100 (Kimura, H. et al., (1994) *EMBO J.* 13, 4311-4320). As primary antibodies, rat anti- pSer⁵⁰² GANP monoclonal antibody (Kuwahara, K. et al., (2001) *Proc. Natl. Acad. Sci. USA* 98, 10279-10283) and rabbit anti-MCM3 antibody (Kimura, H. et al., (1994) *EMBO J.* 13, 4311-4320) were used. As secondary antibodies, Alexa 488-conjugated goat anti-rat IgG antibody (Molecular Probes) was used against GANP and Alexa 546-conjugated goat anti-rabbit IgG antibody (Molecular Probes) was used against MCM3. Counter-staining was carried out using TOTO-3 iodide (Molecular Probes), followed by observation with a confocal laser scanning microscope (FV500; Olympus).

2.3. cDNA Constructs for Expression

pSRα-MCM3-HA is described in the literature (Kimura, H. et al., (1995) *Nucl. Acids Res.* 23, 2097-2104). A vector pECFP-Nuc carrying the three nuclear localization signals (NLSs) of SV40 T-Ag was purchased from Clontech. PCR fragments obtained by using the following combinations of 3' and 5' primers were introduced into pGEX-4T-1 (Amersham). Using the resultant plasmids, different forms of mouse *ganp* cDNAs were expressed as fusion proteins with glutathione-S-transferase (GST).

GANP1-5': 5'-GGGGATCCATACCCGG TGAACCCCTT-3' (SEQ ID NO: 11)
 30 GANP1-3': 5'-GGGTCGACGCGCACAGACTTCCCCTGA-3' (SEQ ID NO: 12)
 GANP2-5': 5'-GGGAATTCTCCCGCCTTCCAGCTGTGAC-3' (SEQ ID NO: 13)
 GANP2-3': 5'-GGGTCGACGTGCTGCTGTGTTATGTCCT-3' (SEQ ID NO: 14)
 GANP3-5': 5'-GGGAATTCCATGAGCT GAGACCCTCAGC-3' (SEQ ID NO: 15)
 GANP3-3': 5'-GGGTCGACTGAGGATGCAGGAGGCGGCT -3' (SEQ ID NO: 16)
 35 GANP4-5': 5'-GGGAATTCTACGTTGGAGAGAGCCTGGC-3' (SEQ ID NO: 17)

GANP4-3': 5'-GGGTCGACCATGCTGTCATCTCCTGTGA-3' (SEQ ID NO: 18)
 GANP5-5': 5'-GGGAATTCGAGAA CCTGGCCAAGGGTCT-3' (SEQ ID NO: 19)
 GANP5-3': 5'-GGGTCGACGAAAAACCGACGGCTGA ACT-3' (SEQ ID NO: 20)
 GANP6-5': 5'-GGGAATTCAAGCCCTTCCAGCCTGCCCT-3' (SEQ ID NO: 21)
 5 GANP6-3': 5'-GGGTCGACCGAGGGAACGTGGTATTTTC-3' (SEQ ID NO: 22)
 GANP7-5': 5'-GGCCCCGGGCC CGTGGGATGACATCATCA-3' (SEQ ID NO: 23)
 GANP7-3': 5'-GGCTCGAGCATGTCCACCATCTC CAGCA-3' (SEQ ID NO: 24)

cDNA constructs were prepared by introducing PCR fragments into pSVEGFP pA to thereby obtain green fluorescence protein (GFP)-tagged Ganp mutants (Kuwata, N. et al.,
 10 (1999) *J. Immunol.* 163, 6355-6359). Subsequently, these constructs were introduced into a mammalian expression vector pCXN2 (Niwa, H. et al., (1991) *Gene* 108, 193-200). Primer sequences were designed as described below so that they encode Ganp.

Gp-gfp-5':

5'-GGGGATCCGAATTCCACCATGGCAGTCTTCAAACCGATA CC-3' (SEQ ID NO:
 15 25)

Gp-gfp-3':

5'-GCAGGGGCTCCTCCTGATCT-3' (SEQ ID NO: 26)

Gsac-gfp-5':

5'-GGGGATC CGAATTCCACCATGTCCGAGGGCCTTGGTTCTTG-3' (SEQ ID NO:
 20 27)

Gsac-gfp-3':

5'-CTGTCTT GTTTCTAAGCCGC-3' (SEQ ID NO: 28)

Gmap80-gfp-5':

5'-GGGGATCCGAATTCCACCATGGAGA ACCTGGCCAAGGGTCT-3' (SEQ ID NO:
 25 29)

Gmap80-gfp-3':

5'-GAGGACTTGTAGATGTTTTAC CATGG-3' (SEQ ID NO: 30)

FLAG-tagged Ganp mutants were prepared by introducing into pCXN2 the cDNA fragments obtained by PCR using the following primers.

30 *FLAG-Gp-5'*:

5'-GGGAATTCCACCATGGATTACAAGGATGACGACGATAAGG
 CAGTCTTCAA CCGATACC-3' (SEQ ID NO: 31)

FLAG-Gp-3':

5'-GGGAATTCCTCCGGGTCTCCCTCAAGTA-3' (SEQ ID NO: 32)

35 *FLAG-Gsac-5'*:

5'-GGGAATTCCACCATGGATTACAAGGATGACGACGATAAGTCCGAGGGCCTTG
GTTCTTG-3' (SEQ ID NO: 33)

FLAGSac-3':

5'-GGGAATTCGCTGTCTTGTCTTAAGCCG-3' (SEQ ID NO: 34)

5 *FLAG-Gmap*-5':

5'-GGGAATTCCACCATGGATTACAAGGATGACGACGATAAGG
AGAACCTGGCCAAGGGTCT-3' (SEQ ID NO: 35)

FLAG-Gmap-3':

5'-GGGAATTCTGAGGACTTG TAGATGTTTT-3' (SEQ ID NO: 36)

10 Internal deletion mutant Gp Δ NLS-GFP and I3 mutant (MCM Δ NLS-HA) were
prepared as described in the literature (Imai, Y. et al., (1991) *Nucl. Acids Res.* 19, 2785-2785).
All of the constructs were sequenced to confirm that they have the proper orientation and that
the reading frame of codons will be correct when they are expressed as tagged fusion
proteins. Thus, their quality was controlled. Expression vectors comprising a mutant
15 RNA/DNA primase domain (PD) are described in the literature (Gp mutant from Ser⁵⁰² to
Ala [GpS502A] or Glu [GpS502E]) (Kuwahara, K. et al., (2001). *Proc. Natl. Acad. Sci. USA*
98, 10279-10283).

2.4. Detection of Transgene Product with Confocal Microscope

20 NIH-3T3 cells were transfected with pCXN2-*ganp-gfp* and/or pSR α -MCM3-HA
using FuGENE 6 (Roche Diagnostics). Sixteen hours before fixation, leptomycin B (LMB)
(Kudo, N. et al., (1999) *Proc. Natl. Acad. Sci. USA* 96, 9112-9117) was added to the medium.
In the co-transfection experiment, rabbit anti-HA antibody (Santa Cruz) and Alexa
546-conjugated goat anti-rabbit IgG antibody were used. In the single transfection
25 experiment, Alexa 488-conjugated goat anti-rabbit IgG antibody (Molecular Probes) was
used. Thus, exogenous MCM3 protein was stained. Nuclear acid was counter-stained
with TOTO-3 iodide in the co-transfection experiment and with propidium iodide (PI;
Sigma) in the single transfection experiment.

30 2.5. GST Pull Down Assay

GST fusion proteins were purified as described in the literature (Kuwahara, K. et al.,
(2000) *Blood* 95, 2321-2328). Various GST fusion proteins (5 μ g each) immobilized on
glutathione-Sepharose beads (Amersham) were incubated with BAL17 lysate prepared with
TNE buffer (10 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10
35 μ g of aprotinin, 1 mM phenylmethyl-sulfonylfluoride [PMSF]). Bound proteins were

separated by 8% SDS-PAGE, transferred onto a nitrocellulose filter and blocked. Subsequently, the filter was incubated with rabbit anti-mouse MCM3 antibody (Kimura, H. et al., (1994) *EMBO J.* 13, 4311-4320) and then with peroxidase-labeled protein A (Amersham) serially. Finally, signals were visualized with ECL detection kit (Amersham).

5 For direct binding assay, radio-labeled MCM3 was prepared with ^{35}S -methionin (Amersham) using *In vitro* Transcription and Translation Binding System (Novagen) according to the manufacturer's instructions. Thus, [^{35}S]-labeled MCM3 was detected by autoradiography.

10 2.6. Immunoprecipitation and Western Blotting of Transgene Product

COS7 cells were transfected with pCXN2-*FLAG-ganp* and/or pSR α -*MCM3-HA* using FuGENE 6. After 26 hrs, cells were lysed in TNE buffer. The resultant lysate was incubated with a combination of protein A-Sepharose (Amersham) and anti-HA antibody. The resultant immunoprecipitates were separated by 8% SDS-PAGE, transferred on a

15 nitrocellulose filter, and blocked. Subsequently, the filter was incubated with anti-mouse FLAG M2 antibody (Stratagene) and then with peroxidase-labeled goat anti-mouse IgG (H+L) antibody (Zymed). For the detection of Gp-GFP and mutants thereof, the blotted filter was probed with rabbit anti-GFP antibody (Santa Cruz) and peroxidase-conjugated protein A (Zymed).

20

2.7. Heterokaryon Assay

HeLa cells were transfected with pSR α -*MCM3-HA* using FuGENE 6. After 20 hrs, transfected HeLa cells and untransfected mouse Swiss-3T3 cells were treated with trypsin and seeded in culture dishes at a ratio of 1:1. After 24 hrs, cells were fused using

25 polyethylene glycol 1500 (Roche Diagnostics) at room temperature for 2 min (Schmidt-Zachmann, M.S. et al., (1993) *Cell* 74, 493-504). The culture dishes were washed with the medium 4 times. Then, cycloheximide-containing medium was added thereto (at a final concentration of 20 $\mu\text{g/ml}$), and the cells were incubated in CO₂ incubator at 37°C for 5 hrs. Subsequently, the cells were fixed with 4% paraformaldehyde in 250 mM

30 HEPES-NaOH (pH 7.4) for 20 min, made transparent using 0.5% Triton X-100 in PBS for 30 min, and washed with PBS. The cells were stained using anti-HA antibody (12CA5; Covence Research Products) and Cy3-conjugated donkey anti-mouse Ig antibody (Jackson). Also, DNA was counter-stained with 100 ng/ml of Hoechst 33342 (Sigma) in PBS for 20 min. Images were collected using Zeiss Axioplan equipped with 100x PlanNeofluar

35 phase-contrast objective lens (NA 1.3) and SpotII CCD.

3. Results and Observations

3.1. Association of GANP with MCM3

The interaction between GANP and MCM3 in B cell lineage has been already
 5 demonstrated by immunoprecipitation (Kuwahara, K. et al., (2000) *Blood* 95, 2321-2328).
 Since a C-terminal domain of GANP is identical with total Map80 protein, it is predicted that
 GANP associates with MCM3 at this domain. In order to determine which domain of
 GANP associates with MCM3, the present inventor performed a pull down assay using GST
 10 fusion proteins containing the various truncated GANP proteins as shown in Fig. 32.
 Briefly, GST was fused to the N-terminus of each of the truncated GANP proteins designated
 1 to 7 and 5-7' in Fig. 32. In the lower panel of Fig. 33, Map80 domain (designated
 GANP5-7') pulled down MCM3 from cell extract as described previously (Kimura, H. et al.,
 (1994) *EMBO J.* 13, 4311-4320).

Surprisingly, GANP1 and GANP2 (which are partial fragments of GANP) also
 15 pulled down MCM3 (Fig. 33: upper and lower panels).

Subsequently, this binding was examined using MCM3 synthesized *in vitro* in a
 reticulocyte lysate system (Fig. 34). GST-GANP1 and GST-GANP2 also pulled down
 [³⁵S]-MCM3 from the *in vitro* translation cocktail.

GST alone (negative control: first lane) or GST fused with an irrelevant protein did
 20 not show any signal. Further, the binding to GST-GANP1 was stronger than the binding to
 Map80 domain (GST-GANP5-7'). This binding was also confirmed in cells by a DNA
 transfection experiment using FLAG-tagged constructs (Fig. 35).

Briefly, COS7 cells were co-transfected with pCXN2-FLAG-Ganp,
 pCXN2-FLAG-Gp and pCXN2-FLAG-Gmap80 in combination with pSRα-MCM3-HA or
 25 pSRα-I3-HA. After immunoprecipitation with anti-HA antibody, Western blotting was
 performed using anti-FLAG monoclonal antibody. The predicted sizes of FLAG-labeled
 proteins are shown in individual lanes with triangle marks. In the left and right panels, the
 migration of bands is similar, but the light exposure for ECL detection was 1 min for the left
 panel and 3 min for the right panel (Fig. 35).

30 FLAG-Ganp, FLAG-Gp and FLAG-Gmap80 bound to wild-type MCM3-HA (HA
 epitope-tagged MCM3) (Fig. 35: left panel). Only FLAG-Gsac did not bind thereto. With
 respect to the binding with I3 mutant MCM3 (MCM3ΔNLS), only FLAG-Gmap80 showed
 a positive result (Fig. 35: right panel). Gp domain carrying the N-terminal NLS associates
 with MCM3 consistently in cells containing a large quantity of MCM3 (Fig. 35: left panel).
 35 These results suggest that GANP associates with the NLS domain of MCM3 through Gp

domain.

The present inventor further examined whether the state of phosphorylation of Ser⁵⁰² in Gp domain influences the binding GANP to MCM3 or not. A GANP mutant lacking primase site (Ganp Δ PD-GFP) and other GANP mutants prepared as GanpS502A and GanpS502E having a mutation at Ser⁵⁰² were fused with GFP (Fig. 36A). Cells were co-transfected with pCXN2-*Ganp-gfp* and pSR α -*MCM3-HA*, and cell lysate was used in immunoprecipitation with anti-HA antibody.

GFP signals were detected with anti-GFP antibody (Fig. 36B: upper panel). This means that GANP has bound to MCM.

Co-transfection using Ganp-GFP mutants was also performed in the same manner. In order to determine the predicted position of each protein, lysates were separated by SDS-PAGE and blotted with anti-GFP antibody in the same manner (Fig. 36B: lower panel).

The non-phosphorylated mutant (GanpS502A-GFP) bound to MCM3 as wild-type Ganp-GFP and GanpS502E-GFP (a mutant much resembling phosphoserine) did (Fig. 36B: upper panel). Interestingly, Ganp Δ PD-GFP does not co-precipitate with MCM3-HA (Fig. 36B: upper panel).

Regardless of the latent binding activity of Map80 domain, GANP molecule as a whole needs RNA primase domain (PD) for its binding to MCM3. Open triangle in Fig. 36B indicates the position of Ganp Δ PD-GFP. The size of Ganp-GFP, which is equal to the sizes of Ganp S502A-GFP and Ganp S502E-GFP, is indicated with filled triangle (Fig. 36B: lower panel). These results suggest that the binding of GANP to MCM3 is mediated by its PD domain, but phosphorylation at Ser⁵⁰² does not influence this binding.

The experiment using truncated constructs revealed the association of GANP with MCM3 in a wide region. However, the association of the entire GANP (involving its N-terminal 600 amino acid region) with MCM3 requires the NLS of MCM3. NLS-deficient MCM3 mutant was unable to effectively associate with entire GANP molecule in cells. Map80 domain bound to NLS-negative MCM3, suggesting that GANP mainly binds to a domain of MCM3 other than the domain required for the interaction with Map80. Although Map80 is considered to be an MCM3 import factor, GANP may play a different role in cooperation with MCM3. It seems that GANP has many potential phosphorylation sites and has many association components in cells (Kuwahara, K. et al., (2000) *Blood* 95, 2321-2328). Therefore, it will be necessary to specify a domain whose state of phosphorylation influences the GANP/MCM3 association and transport between the cytoplasm and the nuclear compartment.

3.2. Intracellular Localization of Map80 and Ganp Mutants Shown by Transfection

GANP has two potential NLSs. One is located in the N-terminal primase domain and the other in the C-terminal Map80 domain. GANP also has two nuclear export signal (NES)-like motifs. One is located between SAC3 homologous domain and Map80 domain and the other within Map80 domain.

NIH-3T3 cells were transfected with pCXN2-*Ganp-gfp* or pCXN2-*Gmap80-gfp*, followed by fixation 48 hrs later. LMB was added 16 hrs before the fixation. Nuclei were pre-stained with PI, and images were collected with a confocal microscope. Representative expression properties are shown in Fig. 37. The numbers of cells were counted by property and expressed in % (Fig. 37).

It was found that Ganp-GFP (almost full-length GANP tagged with GFP) is present in both the cytoplasm and the nuclear compartment, though the ratio of cells showing nuclear dominant expression (N and N>C: 38%) or cells showing cytoplasm dominant expression (C and C>N: 31%) was varied (%from total 500 cells) (Fig. 37: Ganp-GFP, LMB-). In contrast, Gmap80-GFP was found in the cytoplasm for the most part, showing no nuclear dominant expression according to the inventor's classification (N>C, 0%; N=C, 35%; C and C>N, 65%) (Fig. 37: Gmap80-GFP, LMB -). The localization of Ganp-GFP is different from the localization of Gmap80-GFP.

In order to examine whether the N-terminal NLS motif is functional or not, 5' 1-kb DNA fragment comprising RNA/DNA primase domain and the N-terminal NLS (but not NES-like motif) was fused with GFP (Fig. 38: Gp-GFP). Although this Gp-GFP product was present in the nucleus alone (N and N>C: 94%) (Fig. 38), NLS-deficient mutant GpGFP (Gp Δ NLS-GFP; as shown in Fig. 38, amino acids from position 497 to 500 are deleted) was found to be cytoplasmic. Thus, it was confirmed that the N-terminal NLS is involved in the nuclear localization.

The present inventor examined whether or not the mutation of the adjacent Ser⁵⁰² to alanine (GpS502A-GFP; non-phosphorylated type) or to glutamic acid (GpS502E-GFP; phosphoserine-mimic type) influences this localization (Fig. 38). Then, the present inventor observed that these mutations do not alter the localization of Gp. This suggests that the N-terminal NLS is functional regardless of the state of phosphorylation of Ser⁵⁰² (Fig. 38). In contrast, it seems that Gac-GFP having neither N-terminal NLS nor C-terminal NLS is present in the cytoplasm for the most part (N and N>C: 0%; N=C: 3%; C and C>N: 97%) (Fig. 38).

These results suggest that the N-terminal NLS plays a functional role for Ganp to

enter into the nucleus. However, the NLS may not be so strong to maintain GANP expression within the nucleus, because Ganp-GFP is also present in the cytoplasm (Fig. 37). In order to examine this issue further, cells were treated with leptomycin B (LMB) after cDNA transfection in order to inhibit the Crm1-mediated export to the nucleus (Kudo, N. et al., (1999) *Proc. Natl. Acad. Sci. USA* 96, 9112-9117).

In LMB-treated cells, Ganp-GFP localized in the nucleus for most of the transfectants (Fig. 37). The cell fraction showing cytoplasm dominant expression decreased from 31% to 4%, while the cell fraction showing nuclear dominant expression increased from 38% to 81%. Therefore, it appears that the movement of Ganp to the cytoplasm is inhibited by LMB.

The localization of Gmap80-GFP has also changed dramatically after LMB treatment (Fig. 37). The cell fraction showing cytoplasm dominant expression decreased from 65% to 37%, and the cell fraction showing nuclear dominant expression increased from 0% to 41%. These findings were reproduced in other cell systems including COS7 and Ltk⁻ cells, suggesting that the export of GANP from the nucleus to the cytoplasm is regulated by Crm1-dependent pathway. Therefore, GANP and Map80 seem to shuttle between the nucleus and the cytoplasm, and their localization seem to depend on the balance between nuclear import and export mechanisms maintained in cooperation with other molecules.

3.3. Localization of MCM3 and GANP in Cotransfected Cells

Subsequently, the present inventor examined whether or not the movement of GANP is related to MCM3 expression. Mammal MCM3 alters the state of binding with chromatin during cell cycle, but it is present only in the nucleus throughout the interphase (Kimura, H. et al., (1994) *EMBO J.* 13, 4311-4320). NIH-3T3 cells were transfected with pSR α -MCM3-HA or pSR α -I3-HA, fixed, immunolabeled with anti-HA antibody (Alexa 488) and stained with PI.

MCM3-HA in transfected cells agreed with the representative presence of NLS (Kimura, H. et al., (1994) *EMBO J.* 13, 4311-4320, Takei, Y. et al., (1998) *J. Biol. Chem.* 273, 22177-22180) and localized in the nucleus (Fig. 39). This nuclear localization was dependent on the NLS of MCM3, because an MCM3 mutant lacking this NLS (I3; MCM3 Δ NLS-HA) was expressed only in the cytoplasm (Fig. 39: right panel).

Cells were cotransfected with pCXN2-Ganp-gfp or pCXN2-Gmap80-gfp and pSR α -MCM3-HA, fixed and immunolabeled with anti-HA antibody (Alexa 546), and nuclei were pre-stained with TOTO-3 (Fig. 40). Cell counts are shown below the panel (Fig. 40).

Interestingly, when cells were cotransfected with Ganp-GFP, cytoplasmic

localization of MCM3 was induced in 17% of the cells (Fig. 40: marked with white arrows). When cells were cotransfected with Gmap80-GFP or Gp-GFP, such a result was not observed.

In order to prove that the effect of Ganp on MCM3 is specific, expression of ECFP-Nuc which appears in the nucleus was examined before and after transfection using different *ganp-gfp* constructs. Representative images obtained from transfection with Ganp-GFP are shown in Fig. 41. The localization of ECFP-Nuc in the nucleus was not influenced by any cotransfection using Ganp-GFP (Fig. 41) or Gmap80-GFP or Gp-GFP.

Coexpression of Ganp and MCM3 has also altered the localization of GANP. Compared to the transfection with Ganp-GFP alone (38%) or with Gmap80-GFP alone (0%) (Fig. 37), cotransfection using MCM3 raised the nuclear expression levels of Ganp-GFP (74%) and Gmap80-GFP (64%) (Fig. 40). MCM3 retained GANP and Map80 within the nucleus, but overexpression of Ganp alone enhanced the expression of MCM3 in the cytoplasm (Fig. 40: 17% by Ganp-GFP expression). On the other hand, Gmap80 did not enhance the expression of MCM3 in the cytoplasm (4% by Gmap80-GFP expression). The mutation of MDM3 at its NLS (I3; MCM3 Δ NLS-HA) (as a result, MCM3 is present in the cytoplasm) did not induce the accumulation of Ganp-GFP or Gmap80-GFP in the nucleus (Fig. 42).

Unlike wild-type MCM3, I3 mutant (MCM3 Δ NLS-HA) does not associate with Ganp or Ga (Fig. 35). Considering this fact together, it is suggested that the NLS motif of MCM3 is necessary for the functional association with GANP and for the transport of GANP between the nucleus and the cytoplasm.

DNA transfection experiments demonstrated that Ganp-GFP is accumulated in the nuclear compartment when co-introduced with MCM3, suggesting the formation of GANP/MCM3 complex in the nucleus. MCM3 does not contain a definite common NES-like motif recognizable by Crm1. Therefore, the export of MCM3 from the nucleus probably depends on other binding molecules having an NES-like motif or a different export mechanism. The two NES-like motifs on GANP seems to be involved in an LMB sensitive, Crm1 dependent export pathway (Fig. 37). The two NES-like motifs carried by GANP (these might be recognized by Crm1) might possibly be involved in the transport of the complex.

Recently, it was shown that yeast SAC3 carrying a GANP homologous domain is involved in the export of a specific protein from the nucleus and associates with a component of nuclear pore complex (Jones, A.L. et al., (2000) *Proc. Natl. Acad. Sci. USA* 97, 3224-3229). Coexpression with GANP altered the localization of MCM3 in the

cytoplasmic compartment.

The nuclear-cytoplasmic shuttling of MCM3 was examined using cell fusion techniques (Schmidt-Zachmann, M.S. et al., (1993) *Cell* 74, 493-504). HeLa cells were transfected with MCM3-HA and then fused with untransfected mouse Swiss-3T3 cells. After a 5-hour incubation in the presence of cycloheximide to inhibit protein synthesis, cells were fixed and immunolabeled with MCM3-HA. Heterokaryons were examined by Hoechst staining. This staining discriminates mouse nuclei (marked with arrows) with “mottled” heterochromatins from human HeLa nuclei.

As representative images are shown in Fig. 43, MCM3-HA was found in both human nuclei and mouse nuclei in heterokaryons. Unfused mouse cells do not exhibit such staining. This suggests that MCM3-HA has been exported from the HeLa nucleus to the cytoplasm, and then imported into the mouse nucleus.

From these results, it is concluded that MCM3-HA is a shuttling protein. It should be noted here that proving the movement of an endogenous protein from the nucleus to the cytoplasm with a sensitivity similar to the sensitivity achieved when transgene products are handled is often difficult (Kimura, H., Ohtomo, T. et al., (1996) *Genes Cells* 1, 977-993; Mizuno, T. et al., (1999) *Mol. Cell. Biol.* 19, 7886-7896). That was the case with the results shown in the present Example. It is also difficult to prove the movement of endogenous MCM protein from the nucleus to the cytoplasm in mammal cells with a sensitivity achieved in more primitive cells such as yeast.

However, the results of the present inventor suggest that the nuclear-cytoplasmic shuttling of MCM protein is probably important in untreated cells (though experiments were performed by DNA transfection). To facilitate definite understanding of the nuclear-cytoplasmic shuttling of the MCM complex during cell cycle, discovery of a further component may be necessary.

3.4. Localization of GANP during Cell Cycle

Using a monoclonal antibody specific to the epitope of RNA/DNA primase domain (pSer⁵⁰² GANP) peculiar to GANP, the localization of GANP in NIH-3T3 cells was examined under a confocal laser scanning microscope (Kuwahara, K. et al., (2001) *Proc. Natl. Acad. Sci. USA* 98, 10279-10283). NIH-3T3 cells at different stages of cell cycle were immunostained with anti- pSer⁵⁰² GANP (Alexa 488; green) and anti-MCM3 (Alexa 546; red) antibodies. Nuclei were pre-stained with TOTO-3 iodide (blue). During the interphase, the above-described monoclonal antibody reacted everything within the nucleus except for the nucleolus (Fig. 44).

By the triple labeling with anti-MCM3 antibody and TOTO-3 for staining nucleic acid, the localization of GANP during mitosis was analyzed in detail. As cells proceed from the prometaphase to the metaphase, GANP seems to be dissociated from concentrated chromatin (Fig. 44). The yellow signal in the superimposed image indicates colocalization of GANP and MCM3, but some blue staining shows that GANP alone is also observed in the central part of the prometaphase image. At this stage, GANP and MCM3 are not superimposed with the concentrated chromosome. In metaphase cells, GANP is detected in the spindle region. This signal decreases when chromosomes are separated into two daughter cells in the anaphase.

In the anaphase of mitosis, most of GANP molecules are found in the cytoplasmic compartment until nuclei are formed (telophase). These results suggest that the behaviors of GANP and MCM3 are similar and that they are almost colocalized in the nucleus throughout the interphase. This is consistent with the interassociation of these two molecules. However, as shown by the confocal microscopic examination during mitosis, GANP and MCM3 may be present separately (Fig. 44).

The biological meaning of the nuclear-cytoplasmic shuttling of GANP with respect to the second type RNA/DNA primase remains to be investigated. The shuttling may be associated with the generation of RNA primer at the final stage of DNA repairing. Although the expression level of GANP is low in normal cells, GANP expression is up-regulated in the germinal center where cells rapidly proliferate (Kuwahara, K. et al., (2000) *Blood* 95, 2321-2328; Kuwahara, K. et al., (2001). *Proc. Natl. Acad. Sci. USA* 98, 10279-10283). Further, GANP is expressed at higher levels in certain types of cells having rapid cell cycle. This suggests the possibility that association into MCM complex may stimulate DNA replication (Kuwahara, K. et al., (2001). *Proc. Natl. Acad. Sci. USA* 98, 10279-10283). The expression of GANP having RNA/DNA primase activity, MCM3 binding ability and an acetyltransferase domain (Takei, Y. et al., (2001) *EMBO Rep.* 2, 119-123) may be involved in the regulation of cell cycle progress.

SEQUENCE LISTING FREE TEXT

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SEQ ID NO: 6: primer

SEQ ID NO: 7: primer

SEQ ID NO: 8: primer

SEQ ID NO: 9: primer

SEQ ID NO: 10: primer

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INDUSTRIAL APPLICABILITY

By using the GANP overexpressing mouse of the invention, it is possible to rapidly
 30 prepare antibodies specific to viral antigens and having high affinity therefor, which could
 not be obtained by conventional methods. Therefore, it is expected that specific and potent
 antibodies can be obtained rapidly enough to keep up with the mutations of viral antigens in
 order to prevent the worsening of conditions caused by prolonged infection such as AIDS or
 hepatitis C. Further, with the transgenic animal of the invention, it is possible to prepare
 35 tailored, specific antibodies corresponding to the mutations of viral antigens from infected

patients. The period of immunization necessary for antibody preparation is about only 10 days, and the efficiency of producing antibodies with high affinity mutations reaches almost 60%. High affinity antibody production protocol using bed side patients' samples is expected to become a new immunotherapy that will take the place of vaccine therapy.

5

SEQUENCE LISTING

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UTILIZATION THEREOF

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Phe Asp Ala Lys Ile Asn Asn Glu Asn Met Thr Lys Cys Leu Gln Ser			
	765	770	775
ctg aag gag atg tac cag gac ctg agg aac aag ggt gtt ttt tgt gcc			2765
Leu Lys Glu Met Tyr Gln Asp Leu Arg Asn Lys Gly Val Phe Cys Ala			
	780	785	790
agt gaa gca gag ttt cag ggc tac aat gtc ctg ctt aat ctc aac aaa			2813
Ser Glu Ala Glu Phe Gln Gly Tyr Asn Val Leu Leu Asn Leu Asn Lys			
795	800	805	810
gga gac att ttg aga gaa gtg cag cag ttc cac cct gac gtt agg aac			2861
Gly Asp Ile Leu Arg Glu Val Gln Gln Phe His Pro Asp Val Arg Asn			
	815	820	825
tcc cca gag gtg aac ttc gct gtc cag gct ttt gct gca ttg aac agc			2909
Ser Pro Glu Val Asn Phe Ala Val Gln Ala Phe Ala Ala Leu Asn Ser			

830	835	840	
aat aat ttt gtg aga ttt ttc aaa ctg gtt cag tca gct tct tac ctg			2957
Asn Asn Phe Val Arg Phe Phe Lys Leu Val Gln Ser Ala Ser Tyr Leu			
845	850	855	
aat gcg tgc ctg tta cac tgt tac ttt aat cag atc cgc aag gat gcc			3005
Asn Ala Cys Leu Leu His Cys Tyr Phe Asn Gln Ile Arg Lys Asp Ala			
860	865	870	
ctc cgg gca ctc aat gtt gct tat act gta agc aca cag cgc tct acc			3053
Leu Arg Ala Leu Asn Val Ala Tyr Thr Val Ser Thr Gln Arg Ser Thr			
875	880	885	890
gtc ttc ccc ctg gat ggt gtc gtc cgc atg ctg ctg ttc aga gat agt			3101
Val Phe Pro Leu Asp Gly Val Val Arg Met Leu Leu Phe Arg Asp Ser			
895	900	905	
gaa gag gcg aca aac ttc ctc aat tac cat ggc ctc act gta gct gat			3149
Glu Glu Ala Thr Asn Phe Leu Asn Tyr His Gly Leu Thr Val Ala Asp			
910	915	920	
ggc tgt gtt gag ctg aat cgg tcg gca ttc ttg gaa ccg gag gga tta			3197
Gly Cys Val Glu Leu Asn Arg Ser Ala Phe Leu Glu Pro Glu Gly Leu			
925	930	935	
tgc aag gcc agg aag tca gtg ttt att ggc cgg aag ctg acg gtg tca			3245
Cys Lys Ala Arg Lys Ser Val Phe Ile Gly Arg Lys Leu Thr Val Ser			
940	945	950	
gtt ggg gaa gtt gtg aat gga ggg ccg ttg ccc cct gtt cct cgc cat			3293
Val Gly Glu Val Val Asn Gly Gly Pro Leu Pro Pro Val Pro Arg His			
955	960	965	970
aca cct gtg tgc agc ttc aac tcc cag aat aag tac gtt gga gag agc			3341
Thr Pro Val Cys Ser Phe Asn Ser Gln Asn Lys Tyr Val Gly Glu Ser			

975	980	985	
ctg gct acg gag ctg ccc atc agc act cag aga gct ggt gga gac cca			3389
Leu Ala Thr Glu Leu Pro Ile Ser Thr Gln Arg Ala Gly Gly Asp Pro			
990	995	1000	
gca ggt ggt ggc aga gga gag gac tgt gag gca gag gtg gac ttg			3434
Ala Gly Gly Gly Arg Gly Glu Asp Cys Glu Ala Glu Val Asp Leu			
1005	1010	1015	
cca aca ttg gcg gtc ctc cca cag ccg cct cct gca tcc tca gcc			3479
Pro Thr Leu Ala Val Leu Pro Gln Pro Pro Pro Ala Ser Ser Ala			
1020	1025	1030	
acg ccg gcg ctt cat gtc cag cca ctg gcc cca gcc gca gca ccc			3524
Thr Pro Ala Leu His Val Gln Pro Leu Ala Pro Ala Ala Ala Pro			
1035	1040	1045	
agc ctt ctc cag gcc tcc acg cag cct gag gtg ctg ctt cca aag			3569
Ser Leu Leu Gln Ala Ser Thr Gln Pro Glu Val Leu Leu Pro Lys			
1050	1055	1060	
cct gcg cct gtg tac tct gac tcg gac ctg gta cag gtg gtg gac			3614
Pro Ala Pro Val Tyr Ser Asp Ser Asp Leu Val Gln Val Val Asp			
1065	1070	1075	
gag ctc atc cag gag gct ctg caa gtg gac tgt gag gaa gtc agc			3659
Glu Leu Ile Gln Glu Ala Leu Gln Val Asp Cys Glu Glu Val Ser			
1080	1085	1090	
tcc gct ggg gca gcc tac gta gcc gca gct ctg ggc gtt tcc aat			3704
Ser Ala Gly Ala Ala Tyr Val Ala Ala Ala Leu Gly Val Ser Asn			
1095	1100	1105	
gct gct gtg gag gat ctg att act gct gcg acc acg ggc att ctg			3749
Ala Ala Val Glu Asp Leu Ile Thr Ala Ala Thr Thr Gly Ile Leu			

1110	1115	1120	
agg cac gtt gcc gct gag gaa gtt tcc atg gaa agg cag aga cta			3794
Arg His Val Ala Ala Glu Glu Val Ser Met Glu Arg Gln Arg Leu			
1125	1130	1135	
gag gaa gag aag caa cga gct gag gag gaa cgg ttg aag caa gag			3839
Glu Glu Glu Lys Gln Arg Ala Glu Glu Glu Arg Leu Lys Gln Glu			
1140	1145	1150	
aga gaa ctg atg tta act cag ctg agc gag ggt ctg gcc gca gag			3884
Arg Glu Leu Met Leu Thr Gln Leu Ser Glu Gly Leu Ala Ala Glu			
1155	1160	1165	
ctg aca gaa ctc acg gtg aca gag tgt gtg tgg gaa acc tgc tct			3929
Leu Thr Glu Leu Thr Val Thr Glu Cys Val Trp Glu Thr Cys Ser			
1170	1175	1180	
cag gag cta cag agt gca gta aaa ata gac cag aag gtc cgt gtg			3974
Gln Glu Leu Gln Ser Ala Val Lys Ile Asp Gln Lys Val Arg Val			
1185	1190	1195	
gcc cgc tgt tgt gaa gcc gtc tgt gca cac ctg gtg gat ttg ttt			4019
Ala Arg Cys Cys Glu Ala Val Cys Ala His Leu Val Asp Leu Phe			
1200	1205	1210	
ctt gct gag gaa att ttc cag act gca aaa gag aca ctc cag gaa			4064
Leu Ala Glu Glu Ile Phe Gln Thr Ala Lys Glu Thr Leu Gln Glu			
1215	1220	1225	
ctc cag tgt ttc tgc aag tat cta caa cgg tgg agg gag gct gtt			4109
Leu Gln Cys Phe Cys Lys Tyr Leu Gln Arg Trp Arg Glu Ala Val			
1230	1235	1240	
gca gct cgg aag aaa ttc cgg cgt cag atg cgg gcc ttc cct gca			4154
Ala Ala Arg Lys Lys Phe Arg Arg Gln Met Arg Ala Phe Pro Ala			

1245	1250	1255	
gcg cca tgc	tgt gtg gat gtg aat	gac cgg ctg cag gca	cta gtg 4199
Ala Pro Cys	Cys Val Asp Val Asn	Asp Arg Leu Gln Ala	Leu Val
1260	1265	1270	
ccc agc gca	gag tgc ccc att act	gag gag aac ctg gcc	aag ggt 4244
Pro Ser Ala	Glu Cys Pro Ile Thr	Glu Glu Asn Leu Ala	Lys Gly
1275	1280	1285	
ctt ttg gac	ctg ggc cac gca ggc	aaa gta ggc gtc tcc	tgt acc 4289
Leu Leu Asp	Leu Gly His Ala Gly	Lys Val Gly Val Ser	Cys Thr
1290	1295	1300	
agg ttg agg	cgg ctt aga aac aag	aca gct cac cag ata	aag gtc 4334
Arg Leu Arg	Arg Leu Arg Asn Lys	Thr Ala His Gln Ile	Lys Val
1305	1310	1315	
cag cac ttc	cac cag cag ctg ctg	agg aat gct gca tgg	gca cct 4379
Gln His Phe	His Gln Gln Leu Leu	Arg Asn Ala Ala Trp	Ala Pro
1320	1325	1330	
ctg gac ctg	cca tcc att gtg tct	gag cac ctc ccc atg	aag cag 4424
Leu Asp Leu	Pro Ser Ile Val Ser	Glu His Leu Pro Met	Lys Gln
1335	1340	1345	
aag cga agg	ttt tgg aaa ctg gtg	ctg gtg ttg cct gat	gtg gaa 4469
Lys Arg Arg	Phe Trp Lys Leu Val	Leu Val Leu Pro Asp	Val Glu
1350	1355	1360	
gag cag act	cca gag agt cct ggc	aga ata cta gaa aac	tgg cta 4514
Glu Gln Thr	Pro Glu Ser Pro Gly	Arg Ile Leu Glu Asn	Trp Leu
1365	1370	1375	
aag gtc aaa	ttc aca gga gat gac	agc atg gtg ggt gac	ata gga 4559
Lys Val Lys	Phe Thr Gly Asp Asp	Ser Met Val Gly Asp	Ile Gly

1380	1385	1390	
gat aat gct ggt gat atc cag acc ctc tca gtc ttt aat aca ctt			4604
Asp Asn Ala Gly Asp Ile Gln Thr Leu Ser Val Phe Asn Thr Leu			
1395	1400	1405	
agt agt aaa ggg gat caa aca gtt tct gtc aac gtg tgt ata aag			4649
Ser Ser Lys Gly Asp Gln Thr Val Ser Val Asn Val Cys Ile Lys			
1410	1415	1420	
gtg gct cat ggc acc ctt agt gac agt gcc ctt gat gct gtg gag			4694
Val Ala His Gly Thr Leu Ser Asp Ser Ala Leu Asp Ala Val Glu			
1425	1430	1435	
acc cag aag gac ctg ttg gga acc agt ggg ctc atg ctg ctg ctt			4739
Thr Gln Lys Asp Leu Leu Gly Thr Ser Gly Leu Met Leu Leu Leu			
1440	1445	1450	
ccc ccg aaa gtg aag agt gag gag gtg gca gag gag gaa ctg tcc			4784
Pro Pro Lys Val Lys Ser Glu Glu Val Ala Glu Glu Glu Leu Ser			
1455	1460	1465	
tgg ctg tcg gct tta ctg cag ctc aag cag ctt ctg cag gcc aag			4829
Trp Leu Ser Ala Leu Leu Gln Leu Lys Gln Leu Leu Gln Ala Lys			
1470	1475	1480	
ccc ttc cag cct gcc ctg ccg ctg gtg gtc ctc gtg ccc agc tcc			4874
Pro Phe Gln Pro Ala Leu Pro Leu Val Val Leu Val Pro Ser Ser			
1485	1490	1495	
aga ggg gac tcc gcg ggg agg gca gta gag gac ggt ctg atg tta			4919
Arg Gly Asp Ser Ala Gly Arg Ala Val Glu Asp Gly Leu Met Leu			
1500	1505	1510	
cag gat ttg gtt tca gcc aag ctg att tcc gat tac att gtt gtt			4964
Gln Asp Leu Val Ser Ala Lys Leu Ile Ser Asp Tyr Ile Val Val			

1515	1520	1525	
gag att cct	gac tct gtt aat gat	tta caa ggc aca gtg aag gtt	5009
Glu Ile Pro	Asp Ser Val Asn Asp	Leu Gln Gly Thr Val Lys Val	
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tct gga gca	gtc cag tgg ctg atc	tcc gga tgt cct caa gcc cta	5054
Ser Gly Ala	Val Gln Trp Leu Ile	Ser Gly Cys Pro Gln Ala Leu	
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gac ctt tgc	tgc cag acc ctt gtt	cag tat gtt gag gat ggg atc	5099
Asp Leu Cys	Cys Gln Thr Leu Val	Gln Tyr Val Glu Asp Gly Ile	
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agc cgc gag	ttc agc cgt cgg ttt	ttc cac gac agg aga gag agg	5144
Ser Arg Glu	Phe Ser Arg Arg Phe	Phe His Asp Arg Arg Glu Arg	
1575	1580	1585	
cgc ctg gct	agc ctg ccc tcc cag	gag cct agc acc att att gag	5189
Arg Leu Ala	Ser Leu Pro Ser Gln	Glu Pro Ser Thr Ile Ile Glu	
1590	1595	1600	
ttg ttc aac	agt gtg ctg cag ttc	ctg gcc tct gtg gta tcc tct	5234
Leu Phe Asn	Ser Val Leu Gln Phe	Leu Ala Ser Val Val Ser Ser	
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gag cag ctg	tgt gac atc tcc tgg	cct gtc atg gaa ttt gcc gaa	5279
Glu Gln Leu	Cys Asp Ile Ser Trp	Pro Val Met Glu Phe Ala Glu	
1620	1625	1630	
gtg gga ggc	agc cag ctg ctt cct	cac ctg cac tgg aac tca cca	5324
Val Gly Gly	Ser Gln Leu Leu Pro	His Leu His Trp Asn Ser Pro	
1635	1640	1645	
gag cat cta	gcg tgg ctg aaa caa	gct gtg ctt ggg ttc cag ctt	5369
Glu His Leu	Ala Trp Leu Lys Gln	Ala Val Leu Gly Phe Gln Leu	

1650	1655	1660	
cca cag atg gac ctt cca ccc cca ggg gcc ccc tgg ctc cct gtg			5414
Pro Gln Met Asp Leu Pro Pro Pro Gly Ala Pro Trp Leu Pro Val			
1665	1670	1675	
tgt tcc atg gtc att cag tac acc tcc cag att ccc agc tca agc			5459
Cys Ser Met Val Ile Gln Tyr Thr Ser Gln Ile Pro Ser Ser Ser			
1680	1685	1690	
cag aca cag cct gtc ctc cag tcc cag gcg gag aac ctg ctg tgc			5504
Gln Thr Gln Pro Val Leu Gln Ser Gln Ala Glu Asn Leu Leu Cys			
1695	1700	1705	
aga aca tac cag aag tgg aag aac aag agc ctc tct cca ggc cag			5549
Arg Thr Tyr Gln Lys Trp Lys Asn Lys Ser Leu Ser Pro Gly Gln			
1710	1715	1720	
gag ttg ggg cct tct gtt gcc gag atc ccg tgg gat gac atc atc			5594
Glu Leu Gly Pro Ser Val Ala Glu Ile Pro Trp Asp Asp Ile Ile			
1725	1730	1735	
acc tta tgc atc aat cat aag ctg agg gac tgg aca ccc ccc agg			5639
Thr Leu Cys Ile Asn His Lys Leu Arg Asp Trp Thr Pro Pro Arg			
1740	1745	1750	
ctc cct gtc aca tta gag gcg ctg agt gaa gat ggt caa ata tgt			5684
Leu Pro Val Thr Leu Glu Ala Leu Ser Glu Asp Gly Gln Ile Cys			
1755	1760	1765	
gtg tat ttt ttc aaa aac ctt tta aga aaa tac cac gtt ccc tcg			5729
Val Tyr Phe Phe Lys Asn Leu Leu Arg Lys Tyr His Val Pro Ser			
1770	1775	1780	
tca tgg gaa cag gcc aga atg cag acg cag cgg gaa ctg cag ctg			5774
Ser Trp Glu Gln Ala Arg Met Gln Thr Gln Arg Glu Leu Gln Leu			

1785	1790	1795	
agt cat gga	cgt tcg ggg atg agg	tcc atc cat cct cct	aca agc 5819
Ser His Gly	Arg Ser Gly Met Arg	Ser Ile His Pro Pro	Thr Ser
1800	1805	1810	
act ttt cct	act cca ttg ctt cat	gta cac cag aaa ggg	aag aaa 5864
Thr Phe Pro	Thr Pro Leu Leu His	Val His Gln Lys Gly	Lys Lys
1815	1820	1825	
aag gaa gag	agt ggc cga gag ggg	agc ctc agt aca gag	gac ctc 5909
Lys Glu Glu	Ser Gly Arg Glu Gly	Ser Leu Ser Thr Glu	Asp Leu
1830	1835	1840	
ctg cgg ggg	gct tct gca gaa gag	ctc ctg gca cag agt	ctg tcc 5954
Leu Arg Gly	Ala Ser Ala Glu Glu	Leu Leu Ala Gln Ser	Leu Ser
1845	1850	1855	
agc agt ctt	ctg gaa gag aag gaa	gag aac aag agg ttt	gaa gat 5999
Ser Ser Leu	Leu Glu Glu Lys Glu	Glu Asn Lys Arg Phe	Glu Asp
1860	1865	1870	
caa ctt cag	cag tgg tta tcg caa	gac tca cag gca ttc	aca gag 6044
Gln Leu Gln	Gln Trp Leu Ser Gln	Asp Ser Gln Ala Phe	Thr Glu
1875	1880	1885	
tca act cgg	ctt cct ctc tac ctc	cct cag acg cta gtg	tcc ttt 6089
Ser Thr Arg	Leu Pro Leu Tyr Leu	Pro Gln Thr Leu Val	Ser Phe
1890	1895	1900	
cct gat tct	atc aaa act cag acc	atg gtg aaa aca tct	aca agt 6134
Pro Asp Ser	Ile Lys Thr Gln Thr	Met Val Lys Thr Ser	Thr Ser
1905	1910	1915	
cct cag aat	tca gga aca gga aag	cag ttg agg ttc tca	gag gca 6179
Pro Gln Asn	Ser Gly Thr Gly Lys	Gln Leu Arg Phe Ser	Glu Ala

1920	1925	1930	
tcc ggt tca	tcc ctg acg gaa aag	ctg aag ctc ctg gaa	agg ctg 6224
Ser Gly Ser	Ser Leu Thr Glu Lys	Leu Lys Leu Leu Glu	Arg Leu
1935	1940	1945	
atc cag agc	tca agg gcg gaa gaa	gca gcc tcc gag ctg	cac ctc 6269
Ile Gln Ser	Ser Arg Ala Glu Glu	Ala Ala Ser Glu Leu	His Leu
1950	1955	1960	
tct gca ctg	ctg gag atg gtg gac	atg tag ctgtctgacg	ggagacggat 6319
Ser Ala Leu	Leu Glu Met Val Asp	Met	
1965	1970		
ctctaattca	taatgctttg	tctgtattca	attgtgttat agatgctgtt
			ggaaatgtga 6379
ctattaatta	tgcaaataaa	ctttttgaat	cattccaaaa aaaaaaccat 6429

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 <212> PRT
 <213> Mus musculus

<400> 2

Met	His	Pro	Val	Asn	Pro	Phe	Gly	Gly	Ser	Ser	Pro	Ser	Ala	Phe	Ala
1				5					10					15	

Val	Ser	Ser	Ser	Thr	Thr	Gly	Thr	Tyr	Gln	Thr	Lys	Ser	Pro	Phe	Arg
				20				25						30	

Phe	Gly	Gln	Pro	Ser	Leu	Phe	Gly	Gln	Asn	Ser	Thr	Pro	Ser	Lys	Ser
					35				40					45	

Leu Ala Phe Ser Gln Val Pro Ser Phe Ala Thr Pro Ser Gly Gly Ser
50 55 60

His Ser Ser Ser Leu Pro Ala Phe Gly Leu Thr Gln Thr Ser Ser Val
65 70 75 80

Gly Leu Phe Ser Ser Leu Glu Ser Thr Pro Ser Phe Ala Ala Thr Ser
85 90 95

Ser Ser Ser Val Pro Gly Asn Thr Ala Phe Ser Phe Lys Ser Thr Ser
100 105 110

Ser Val Gly Val Phe Pro Ser Gly Ala Thr Phe Gly Pro Glu Thr Gly
115 120 125

Glu Val Ala Gly Ser Gly Phe Arg Lys Thr Glu Phe Lys Phe Lys Pro
130 135 140

Leu Glu Asn Ala Val Phe Lys Pro Ile Pro Gly Pro Glu Ser Glu Pro
145 150 155 160

Glu Lys Thr Gln Ser Gln Ile Ser Ser Gly Phe Phe Thr Phe Ser His
165 170 175

Pro Val Gly Ser Gly Ser Gly Gly Leu Thr Pro Phe Ser Phe Pro Gln
180 185 190

Val Thr Asn Ser Ser Val Thr Ser Ser Ser Phe Ile Phe Ser Lys Pro
 195 200 205

Val Thr Ser Asn Thr Pro Ala Phe Ala Ser Pro Leu Ser Asn Gln Asn
 210 215 220

Val Glu Glu Glu Lys Arg Val Ser Thr Ser Ala Phe Gly Ser Ser Asn
 225 230 235 240

Ser Ser Phe Ser Thr Phe Pro Thr Ala Ser Pro Gly Ser Leu Gly Glu
 245 250 255

Pro Phe Pro Ala Asn Lys Pro Ser Leu Arg Gln Gly Cys Glu Glu Ala
 260 265 270

Ile Ser Gln Val Glu Pro Leu Pro Thr Leu Met Lys Gly Leu Lys Arg
 275 280 285

Lys Glu Asp Gln Asp Arg Ser Pro Arg Arg His Cys His Glu Ala Ala
 290 295 300

Glu Asp Pro Asp Pro Leu Ser Arg Gly Asp His Pro Pro Asp Lys Arg
 305 310 315 320

Pro Val Arg Leu Asn Arg Pro Arg Gly Gly Thr Leu Phe Gly Arg Thr
 325 330 335

Ile Gln Glu Val Phe Lys Ser Asn Lys Glu Ala Gly Arg Leu Gly Ser
340 345 350

Lys Glu Ser Lys Glu Ser Gly Phe Ala Glu Pro Gly Glu Ser Asp His
355 360 365

Ala Ala Val Pro Gly Gly Ser Gln Ser Thr Met Val Pro Ser Arg Leu
370 375 380

Pro Ala Val Thr Lys Glu Glu Glu Glu Ser Arg Asp Glu Lys Glu Asp
385 390 395 400

Ser Leu Arg Gly Lys Ser Val Arg Gln Ser Lys Arg Arg Glu Glu Trp
405 410 415

Ile Tyr Ser Leu Gly Gly Val Ser Ser Leu Glu Leu Thr Ala Ile Gln
420 425 430

Cys Lys Asn Ile Pro Asp Tyr Leu Asn Asp Arg Ala Ile Leu Glu Lys
435 440 445

His Phe Ser Lys Ile Ala Lys Val Gln Arg Val Phe Thr Arg Arg Ser
450 455 460

Lys Lys Leu Ala Val Ile His Phe Phe Asp His Ala Ser Ala Ala Leu
465 470 475 480

Ala Arg Lys Lys Gly Lys Gly Leu His Lys Asp Val Val Ile Phe Trp
485 490 495

His Lys Lys Lys Ile Ser Pro Ser Lys Lys Leu Phe Pro Leu Lys Glu
500 505 510

Lys Leu Gly Glu Ser Glu Ala Ser Gln Gly Ile Glu Asp Ser Pro Phe
515 520 525

Gln His Ser Pro Leu Ser Lys Pro Ile Val Arg Pro Ala Ala Gly Ser
530 535 540

Leu Leu Ser Lys Ser Ser Pro Val Lys Lys Pro Ser Leu Leu Lys Met
545 550 555 560

His Gln Phe Glu Ala Asp Pro Phe Asp Ser Gly Ser Glu Gly Ser Glu
565 570 575

Gly Leu Gly Ser Cys Val Ser Ser Leu Ser Thr Leu Ile Gly Thr Val
580 585 590

Ala Asp Thr Ser Glu Glu Lys Tyr Arg Leu Leu Asp Gln Arg Asp Arg
595 600 605

Ile Met Arg Gln Ala Arg Val Lys Arg Thr Asp Leu Asp Lys Ala Arg
610 615 620

Ala Phe Val Gly Thr Cys Pro Asp Met Cys Pro Glu Lys Glu Arg Tyr
625 630 635 640

Leu Arg Glu Thr Arg Ser Gln Leu Ser Val Phe Glu Val Val Pro Gly
645 650 655

Thr Asp Gln Val Asp His Ala Ala Ala Val Lys Glu Tyr Ser Arg Ser
660 665 670

Ser Ala Asp Gln Glu Glu Pro Leu Pro His Glu Leu Arg Pro Ser Ala
675 680 685

Val Leu Ser Arg Thr Met Asp Tyr Leu Val Thr Gln Ile Met Asp Gln
690 695 700

Lys Glu Gly Ser Leu Arg Asp Trp Tyr Asp Phe Val Trp Asn Arg Thr
705 710 715 720

Arg Gly Ile Arg Lys Asp Ile Thr Gln Gln His Leu Cys Asp Pro Leu
725 730 735

Thr Val Ser Leu Ile Glu Lys Cys Thr Arg Phe His Ile His Cys Ala
740 745 750

His Phe Met Cys Glu Glu Pro Met Ser Ser Phe Asp Ala Lys Ile Asn
755 760 765

Asn Glu Asn Met Thr Lys Cys Leu Gln Ser Leu Lys Glu Met Tyr Gln
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Asp Leu Arg Asn Lys Gly Val Phe Cys Ala Ser Glu Ala Glu Phe Gln
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Gly Tyr Asn Val Leu Leu Asn Leu Asn Lys Gly Asp Ile Leu Arg Glu
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Val Gln Gln Phe His Pro Asp Val Arg Asn Ser Pro Glu Val Asn Phe
 820 825 830

Ala Val Gln Ala Phe Ala Ala Leu Asn Ser Asn Asn Phe Val Arg Phe
 835 840 845

Phe Lys Leu Val Gln Ser Ala Ser Tyr Leu Asn Ala Cys Leu Leu His
 850 855 860

Cys Tyr Phe Asn Gln Ile Arg Lys Asp Ala Leu Arg Ala Leu Asn Val
 865 870 875 880

Ala Tyr Thr Val Ser Thr Gln Arg Ser Thr Val Phe Pro Leu Asp Gly
 885 890 895

Val Val Arg Met Leu Leu Phe Arg Asp Ser Glu Glu Ala Thr Asn Phe
 900 905 910

Leu Asn Tyr His Gly Leu Thr Val Ala Asp Gly Cys Val Glu Leu Asn
 915 920 925

Arg Ser Ala Phe Leu Glu Pro Glu Gly Leu Cys Lys Ala Arg Lys Ser
 930 935 940

Val Phe Ile Gly Arg Lys Leu Thr Val Ser Val Gly Glu Val Val Asn
 945 950 955 960

Gly Gly Pro Leu Pro Pro Val Pro Arg His Thr Pro Val Cys Ser Phe
 965 970 975

Asn Ser Gln Asn Lys Tyr Val Gly Glu Ser Leu Ala Thr Glu Leu Pro
 980 985 990

Ile Ser Thr Gln Arg Ala Gly Gly Asp Pro Ala Gly Gly Gly Arg Gly
 995 1000 1005

Glu Asp Cys Glu Ala Glu Val Asp Leu Pro Thr Leu Ala Val Leu
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Pro Gln Pro Pro Pro Ala Ser Ser Ala Thr Pro Ala Leu His Val
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Gln Pro Leu Ala Pro Ala Ala Ala Pro Ser Leu Leu Gln Ala Ser
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Thr Gln Pro Glu Val Leu Leu Pro Lys Pro Ala Pro Val Tyr Ser
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Asp Ser Asp Leu Val Gln Val Val Asp Glu Leu Ile Gln Glu Ala
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Leu Gln Val Asp Cys Glu Glu Val Ser Ser Ala Gly Ala Ala Tyr
 1085 1090 1095

Val Ala Ala Ala Leu Gly Val Ser Asn Ala Ala Val Glu Asp Leu
 1100 1105 1110

Ile Thr Ala Ala Thr Thr Gly Ile Leu Arg His Val Ala Ala Glu
 1115 1120 1125

Glu Val Ser Met Glu Arg Gln Arg Leu Glu Glu Glu Lys Gln Arg
 1130 1135 1140

Ala Glu Glu Glu Arg Leu Lys Gln Glu Arg Glu Leu Met Leu Thr
 1145 1150 1155

Gln Leu Ser Glu Gly Leu Ala Ala Glu Leu Thr Glu Leu Thr Val
 1160 1165 1170

Thr Glu Cys Val Trp Glu Thr Cys Ser Gln Glu Leu Gln Ser Ala
 1175 1180 1185

Val Lys	Ile Asp	Gln Lys	Val Arg	Val Ala	Arg Cys	Cys Glu	Ala
1190			1195			1200	

Val Cys	Ala His	Leu Val	Asp Leu	Phe Leu	Ala Glu	Glu Ile	Phe
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Gln Thr	Ala Lys	Glu Thr	Leu Gln	Glu Leu	Gln Cys	Phe Cys	Lys
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Tyr Leu	Gln Arg	Trp Arg	Glu Ala	Val Ala	Ala Arg	Lys Lys	Phe
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Arg Arg	Gln Met	Arg Ala	Phe Pro	Ala Ala	Pro Cys	Cys Val	Asp
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Val Asn	Asp Arg	Leu Gln	Ala Leu	Val Pro	Ser Ala	Glu Cys	Pro
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Ile Thr	Glu Glu	Asn Leu	Ala Lys	Gly Leu	Leu Asp	Leu Gly	His
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Ala Gly	Lys Val	Gly Val	Ser Cys	Thr Arg	Leu Arg	Arg Leu	Arg
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Asn Lys	Thr Ala	His Gln	Ile Lys	Val Gln	His Phe	His Gln	Gln
1310			1315			1320	

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Cys Glu	Asp Val Cys Ala His	Leu Val Asp Leu Phe	Leu Val Glu	
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Cys Val	Asp Val Ser Asp Arg	Leu Arg Ala Leu Ala	Pro Ser Ala	
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Leu Gly	His Ala Gly Arg Leu	Gly Ile Ser Cys Thr	Arg Leu Arg	
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Arg Leu	Arg Asn Lys Thr Ala	His Gln Met Lys Val	Gln His Phe	
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Ala Val	Glu Lys Glu Val Glu	Asp Gly Leu Met Leu	Gln Asp Leu	
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gtt tca	gct aag ctg att tca	gat tac act gtt acc	gag atc cct	4651
Val Ser	Ala Lys Leu Ile Ser	Asp Tyr Thr Val Thr	Glu Ile Pro	
1525	1530	1535		
gat acc	att aat gat cta caa	ggt tca act aag gtt	ttg caa gca	4696
Asp Thr	Ile Asn Asp Leu Gln	Gly Ser Thr Lys Val	Leu Gln Ala	
1540	1545	1550		
gtg cag	tgg ctg gtt tcc cac	tgc ccc cat tcc ctt	gac ctc tgc	4741
Val Gln	Trp Leu Val Ser His	Cys Pro His Ser Leu	Asp Leu Cys	
1555	1560	1565		
tgc cag	act ctc att cag tac	gtc gaa gac ggg att	ggc cat gag	4786
Cys Gln	Thr Leu Ile Gln Tyr	Val Glu Asp Gly Ile	Gly His Glu	
1570	1575	1580		
ttt agt	ggc cgc ttt ttc cat	gac aga aga gag agg	cgt ctg ggc	4831
Phe Ser	Gly Arg Phe Phe His	Asp Arg Arg Glu Arg	Arg Leu Gly	
1585	1590	1595		
ggt ctt	gct tct cag gag cct	ggc gcc atc att gag	ctg ttt aac	4876
Gly Leu	Ala Ser Gln Glu Pro	Gly Ala Ile Ile Glu	Leu Phe Asn	
1600	1605	1610		
agt gtg	ctg cag ttc ctg gct	tct gtg gtg tcc tct	gaa cag ctg	4921
Ser Val	Leu Gln Phe Leu Ala	Ser Val Val Ser Ser	Glu Gln Leu	
1615	1620	1625		
tgt gac	ctg tcc tgg cct gtc	act gag ttt gct gag	gca ggg ggc	4966
Cys Asp	Leu Ser Trp Pro Val	Thr Glu Phe Ala Glu	Ala Gly Gly	
1630	1635	1640		

agc cgg	ctg ctt cct cac ctg	cac tgg aat gcc cca	gag cac ctg	5011
Ser Arg	Leu Leu Pro His Leu	His Trp Asn Ala Pro	Glu His Leu	
1645	1650	1655		
gcc tgg	ctg aag cag gct gtg	ctc ggg ttc cag ctt	ccg cag atg	5056
Ala Trp	Leu Lys Gln Ala Val	Leu Gly Phe Gln Leu	Pro Gln Met	
1660	1665	1670		
gac ctt	cca ccc ctg ggg gcc	ccc tgg ctc ccc gtg	tgc tcc atg	5101
Asp Leu	Pro Pro Leu Gly Ala	Pro Trp Leu Pro Val	Cys Ser Met	
1675	1680	1685		
gtt gtc	cag tac gcc tcc cag	atc ccc agc tca cgc	cag aca cag	5146
Val Val	Gln Tyr Ala Ser Gln	Ile Pro Ser Ser Arg	Gln Thr Gln	
1690	1695	1700		
cct gtc	ctc cag tcc cag gtg	gag aac ctg ctc cac	aga acc tac	5191
Pro Val	Leu Gln Ser Gln Val	Glu Asn Leu Leu His	Arg Thr Tyr	
1705	1710	1715		
tgt agg	tgg aag agc aag agt	ccc tcc cca gtc cat	ggg gca ggc	5236
Cys Arg	Trp Lys Ser Lys Ser	Pro Ser Pro Val His	Gly Ala Gly	
1720	1725	1730		
ccc tcg	gtc atg gag atc cca	tgg gat gat ctt atc	gcc ttg tgt	5281
Pro Ser	Val Met Glu Ile Pro	Trp Asp Asp Leu Ile	Ala Leu Cys	
1735	1740	1745		
atc aac	cac aag ctg aga gac	tgg acg ccc ccc cgg	ctt cct gtt	5326
Ile Asn	His Lys Leu Arg Asp	Trp Thr Pro Pro Arg	Leu Pro Val	
1750	1755	1760		
aca tca	gag gcg ctg agt gaa	gat ggt cag ata tgt	gtg tat ttt	5371
Thr Ser	Glu Ala Leu Ser Glu	Asp Gly Gln Ile Cys	Val Tyr Phe	
1765	1770	1775		

ttt aaa aac gat ttg aaa aaa tat gat gtt cct ttg tcg tgg gaa	5416
Phe Lys Asn Asp Leu Lys Lys Tyr Asp Val Pro Leu Ser Trp Glu	
1780 1785 1790	
caa gcc agg ttg cag acg cag aag gag cta cag ctg aga gag gga	5461
Gln Ala Arg Leu Gln Thr Gln Lys Glu Leu Gln Leu Arg Glu Gly	
1795 1800 1805	
cgt ttg gca ata aag cct ttt cat cct tct gca aac aat ttt ccc	5506
Arg Leu Ala Ile Lys Pro Phe His Pro Ser Ala Asn Asn Phe Pro	
1810 1815 1820	
ata cca ttg ctt cac atg cac cgt aac tgg aag agg agc aca gag	5551
Ile Pro Leu Leu His Met His Arg Asn Trp Lys Arg Ser Thr Glu	
1825 1830 1835	
tgt gct caa gag ggg agg att ccc agc aca gag gat ctg atg cga	5596
Cys Ala Gln Glu Gly Arg Ile Pro Ser Thr Glu Asp Leu Met Arg	
1840 1845 1850	
gga gct tct gct gag gag ctc ttg gcg cag tgt ttg tcg agc agt	5641
Gly Ala Ser Ala Glu Glu Leu Leu Ala Gln Cys Leu Ser Ser Ser	
1855 1860 1865	
ctg ctg ctg gag aaa gaa gag aac aag agg ttt gaa gat cag ctt	5686
Leu Leu Leu Glu Lys Glu Glu Asn Lys Arg Phe Glu Asp Gln Leu	
1870 1875 1880	
cag caa tgg ttg tct gaa gac tca gga gca ttt acg gat tta act	5731
Gln Gln Trp Leu Ser Glu Asp Ser Gly Ala Phe Thr Asp Leu Thr	
1885 1890 1895	
tcc ctt ccc ctc tat ctt cct cag act cta gtg tct ctt tct cac	5776
Ser Leu Pro Leu Tyr Leu Pro Gln Thr Leu Val Ser Leu Ser His	
1900 1905 1910	

act att gaa cct gtg atg aaa aca tct gta act act agc cca cag 5821
 Thr Ile Glu Pro Val Met Lys Thr Ser Val Thr Thr Ser Pro Gln
 1915 1920 1925

agt gac atg atg agg gag caa ctg cag ctg tca gag gcg aca gga 5866
 Ser Asp Met Met Arg Glu Gln Leu Gln Leu Ser Glu Ala Thr Gly
 1930 1935 1940

acg tgt cta ggc gaa cga cta aag cac ctg gaa agg ctg atc cgg 5911
 Thr Cys Leu Gly Glu Arg Leu Lys His Leu Glu Arg Leu Ile Arg
 1945 1950 1955

agt tca agg gaa gag gaa gtt gcc tct gag ctc cat ctc tct gcg 5956
 Ser Ser Arg Glu Glu Glu Val Ala Ser Glu Leu His Leu Ser Ala
 1960 1965 1970

ctg cta gac atg gtg gac att tgagcagcct gacctgtggg gagggggtct 6007
 Leu Leu Asp Met Val Asp Ile
 1975 1980

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ttggaaatgt gattaattta atcatgcaga taaaccatth aaatgtc 6114

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20 25 30

Phe Gly Gln Pro Ser Leu Phe Gly Gln Asn Ser Thr Leu Ser Gly Lys
35 40 45

Ser Ser Gly Phe Ser Gln Val Ser Ser Phe Pro Ala Ser Ser Gly Val
50 55 60

Ser His Ser Ser Ser Val Gln Thr Leu Gly Phe Thr Gln Thr Ser Ser
65 70 75 80

Val Gly Pro Phe Ser Gly Leu Glu His Thr Ser Thr Phe Val Ala Thr
85 90 95

Ser Gly Pro Ser Ser Ser Ser Val Leu Gly Asn Thr Gly Phe Ser Phe
100 105 110

Lys Ser Pro Thr Ser Val Gly Ala Phe Pro Ser Thr Ser Ala Phe Gly
115 120 125

Gln Glu Ala Gly Glu Ile Val Asn Ser Gly Phe Gly Lys Thr Glu Phe
130 135 140

Ser Phe Lys Pro Leu Glu Asn Ala Val Phe Lys Pro Ile Leu Gly Ala
145 150 155 160

Glu Ser Glu Pro Glu Lys Thr Gln Ser Gln Ile Ala Ser Gly Phe Phe
165 170 175

Thr Phe Ser His Pro Ile Ser Ser Ala Pro Gly Gly Leu Ala Pro Phe
180 185 190

Ser Phe Pro Gln Val Thr Ser Ser Ser Ala Thr Thr Ser Asn Phe Thr
195 200 205

Phe Ser Lys Pro Val Ser Ser Asn Asn Ser Leu Ser Ala Phe Thr Pro
210 215 220

Ala Leu Ser Asn Gln Asn Val Glu Glu Glu Lys Arg Gly Pro Lys Ser
225 230 235 240

Ile Phe Gly Ser Ser Asn Asn Ser Phe Ser Ser Phe Pro Val Ser Ser
245 250 255

Ala Val Leu Gly Glu Pro Phe Gln Ala Ser Lys Ala Gly Val Arg Gln
260 265 270

Gly Cys Glu Glu Ala Val Ser Gln Val Glu Pro Leu Pro Ser Leu Met
275 280 285

Lys Gly Leu Lys Arg Lys Glu Asp Gln Asp Arg Ser Pro Arg Arg His
290 295 300

Gly His Glu Pro Ala Glu Asp Ser Asp Pro Leu Ser Arg Gly Asp His
 305 310 315 320

Pro Pro Asp Lys Arg Pro Val Arg Leu Asn Arg Pro Arg Gly Gly Thr
 325 330 335

Leu Phe Gly Arg Thr Ile Gln Asp Val Phe Lys Ser Asn Lys Glu Val
 340 345 350

Gly Arg Leu Gly Asn Lys Glu Ala Lys Lys Glu Thr Gly Phe Val Glu
 355 360 365

Ser Ala Glu Ser Asp His Met Ala Ile Pro Gly Gly Asn Gln Ser Val
 370 375 380

Leu Ala Pro Ser Arg Ile Pro Gly Val Asn Lys Glu Glu Glu Thr Glu
 385 390 395 400

Ser Arg Glu Lys Lys Glu Asp Ser Leu Arg Gly Thr Pro Ala Arg Gln
 405 410 415

Ser Asn Arg Ser Glu Ser Thr Asp Ser Leu Gly Gly Leu Ser Pro Ser
 420 425 430

Glu Val Thr Ala Ile Gln Cys Lys Asn Ile Pro Asp Tyr Leu Asn Asp
 435 440 445

Arg Thr Ile Leu Glu Asn His Phe Gly Lys Ile Ala Lys Val Gln Arg
 450 455 460

Ile Phe Thr Arg Arg Ser Lys Lys Leu Ala Val Val His Phe Phe Asp
 465 470 475 480

His Ala Ser Ala Ala Leu Ala Arg Lys Lys Gly Lys Ser Leu His Lys
 485 490 495

Asp Met Ala Ile Phe Trp His Arg Lys Lys Ile Ser Pro Asn Lys Lys
 500 505 510

Pro Phe Ser Leu Lys Glu Lys Lys Pro Gly Asp Gly Glu Val Ser Pro
 515 520 525

Ser Thr Glu Asp Ala Pro Phe Gln His Ser Pro Leu Gly Lys Ala Ala
 530 535 540

Gly Arg Thr Gly Ala Ser Ser Leu Leu Asn Lys Ser Ser Pro Val Lys
 545 550 555 560

Lys Pro Ser Leu Leu Lys Ala His Gln Phe Glu Gly Asp Ser Phe Asp
 565 570 575

Ser Ala Ser Glu Gly Ser Glu Gly Leu Gly Pro Cys Val Leu Ser Leu
 580 585 590

Ser Thr Leu Ile Gly Thr Val Ala Glu Thr Ser Lys Glu Lys Tyr Arg
595 600 605

Leu Leu Asp Gln Arg Asp Arg Ile Met Arg Gln Ala Arg Val Lys Arg
610 615 620

Thr Asp Leu Asp Lys Ala Arg Thr Phe Val Gly Thr Cys Leu Asp Met
625 630 635 640

Cys Pro Glu Lys Glu Arg Tyr Met Arg Glu Thr Arg Ser Gln Leu Ser
645 650 655

Val Phe Glu Val Val Pro Gly Thr Asp Gln Val Asp His Ala Ala Ala
660 665 670

Val Lys Glu Tyr Ser Arg Ser Ser Ala Asp Gln Glu Glu Pro Leu Pro
675 680 685

His Glu Leu Arg Pro Leu Pro Val Leu Ser Arg Thr Met Asp Tyr Leu
690 695 700

Val Thr Gln Ile Met Asp Gln Lys Glu Gly Ser Leu Arg Asp Trp Tyr
705 710 715 720

Asp Phe Val Trp Asn Arg Thr Arg Gly Ile Arg Lys Asp Ile Thr Gln
725 730 735

Gln His Leu Cys Asp Pro Leu Thr Val Ser Leu Ile Glu Lys Cys Thr
740 745 750

Arg Phe His Ile His Cys Ala His Phe Met Cys Glu Glu Pro Met Ser
755 760 765

Ser Phe Asp Ala Lys Ile Asn Asn Glu Asn Met Thr Lys Cys Leu Gln
770 775 780

Ser Leu Lys Glu Met Tyr Gln Asp Leu Arg Asn Lys Gly Val Phe Cys
785 790 795 800

Ala Ser Glu Ala Glu Phe Gln Gly Tyr Asn Val Leu Leu Ser Leu Asn
805 810 815

Lys Gly Asp Ile Leu Arg Glu Val Gln Gln Phe His Pro Ala Val Arg
820 825 830

Asn Ser Ser Glu Val Lys Phe Ala Val Gln Ala Phe Ala Ala Leu Asn
835 840 845

Ser Asn Asn Phe Val Arg Phe Phe Lys Leu Val Gln Ser Ala Ser Tyr
850 855 860

Leu Asn Ala Cys Leu Leu His Cys Tyr Phe Ser Gln Ile Arg Lys Asp
865 870 875 880

Ala Leu Arg Ala Leu Asn Phe Ala Tyr Thr Val Ser Thr Gln Arg Ser
885 890 895

Thr Ile Phe Pro Leu Asp Gly Val Val Arg Met Leu Leu Phe Arg Asp
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Cys Glu Glu Ala Thr Asp Phe Leu Thr Cys His Gly Leu Thr Val Ser
915 920 925

Asp Gly Cys Val Glu Leu Asn Arg Ser Ala Phe Leu Glu Pro Glu Gly
930 935 940

Leu Ser Lys Thr Arg Lys Ser Val Phe Ile Thr Arg Lys Leu Thr Val
945 950 955 960

Ser Val Gly Glu Ile Val Asn Gly Gly Pro Leu Pro Pro Val Pro Arg
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His Thr Pro Val Cys Ser Phe Asn Ser Gln Asn Lys Tyr Ile Gly Glu
980 985 990

Ser Leu Ala Ala Glu Leu Pro Val Ser Thr Gln Arg Pro Gly Ser Asp
995 1000 1005

Thr Val Gly Gly Gly Arg Gly Glu Glu Cys Gly Val Glu Pro Asp
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Pro Glu Pro Val Pro Met Tyr Ser Asp Glu Asp Leu Ala Gln Val
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Val Gly Ser Ala Gly Ala Ala Tyr Ala Ala Ala Ala Leu Gly Val
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Ser Asn Ala Ala Met Glu Asp Leu Leu Thr Ala Ala Thr Thr Gly
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Arg Arg Glu Gln Glu Arg Gln Arg Ala Glu Glu Glu Arg Leu Lys
 1145 1150 1155

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Val Glu Leu Met Glu Arg Val Met Met Glu Phe Val Arg Glu Thr
 1175 1180 1185

Cys Ser Gln Glu Leu Lys Asn Ala Val Glu Thr Asp Gln Arg Val
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Arg Val Ala Arg Cys Cys Glu Asp Val Cys Ala His Leu Val Asp
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Leu Phe Leu Val Glu Glu Ile Phe Gln Thr Ala Lys Glu Thr Leu
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Gln Glu Leu Gln Cys Phe Cys Lys Tyr Leu Gln Arg Trp Arg Glu
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Ala Val Thr Ala Arg Lys Lys Leu Arg Arg Gln Met Arg Ala Phe
 1250 1255 1260

Pro Ala Ala Pro Cys Cys Val Asp Val Ser Asp Arg Leu Arg Ala
 1265 1270 1275

Leu Ala Pro Ser Ala Glu Cys Pro Ile Ala Glu Glu Asn Leu Ala
 1280 1285 1290

Arg Gly Leu Leu Asp Leu Gly His Ala Gly Arg Leu Gly Ile Ser
 1295 1300 1305

Cys Thr Arg Leu Arg Arg Leu Arg Asn Lys Thr Ala His Gln Met
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Lys Val Gln His Phe Tyr Gln Gln Leu Leu Ser Asp Val Ala Trp
 1325 1330 1335

Ala Ser Leu Asp Leu Pro Ser Leu Val Ala Glu His Leu Pro Gly
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Arg Gln Glu His Val Phe Trp Lys Leu Val Leu Val Leu Pro Asp
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Val Glu Glu Gln Ser Pro Glu Ser Cys Gly Arg Ile Leu Ala Asn
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Trp Leu Lys Val Lys Phe Met Gly Asp Glu Gly Ser Val Asp Asp
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Thr Ser Ser Asp Ala Gly Gly Ile Gln Thr Leu Ser Leu Phe Asn
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Ser Leu Ser Ser Lys Gly Asp Gln Met Ile Ser Val Asn Val Cys
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Ile Lys Val Ala His Gly Ala Leu Ser Asp Gly Ala Ile Asp Ala
 1430 1435 1440

Val Glu Thr Gln Lys Asp Leu Leu Gly Ala Ser Gly Leu Met Leu
 1445 1450 1455

Leu Leu Pro Pro Lys Met Lys Ser Glu Asp Met Ala Glu Glu Asp
 1460 1465 1470

Val Tyr Trp Leu Ser Ala Leu Leu Gln Leu Lys Gln Leu Leu Gln
 1475 1480 1485

Ala Lys Pro Phe Gln Pro Ala Leu Pro Leu Val Val Leu Val Pro
 1490 1495 1500

Ser Pro Gly Gly Asp Ala Val Glu Lys Glu Val Glu Asp Gly Leu
 1505 1510 1515

Met Leu Gln Asp Leu Val Ser Ala Lys Leu Ile Ser Asp Tyr Thr
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Val Thr Glu Ile Pro Asp Thr Ile Asn Asp Leu Gln Gly Ser Thr
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Lys Val Leu Gln Ala Val Gln Trp Leu Val Ser His Cys Pro His
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Ser Leu Asp Leu Cys Cys Gln Thr Leu Ile Gln Tyr Val Glu Asp
 1565 1570 1575

Gly Ile Gly His Glu Phe Ser Gly Arg Phe Phe His Asp Arg Arg
 1580 1585 1590

Glu Arg Arg Leu Gly Gly Leu Ala Ser Gln Glu Pro Gly Ala Ile
 1595 1600 1605

Ile Glu Leu Phe Asn Ser Val Leu Gln Phe Leu Ala Ser Val Val
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Ser Ser Glu Gln Leu Cys Asp Leu Ser Trp Pro Val Thr Glu Phe
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Ala Pro Glu His Leu Ala Trp Leu Lys Gln Ala Val Leu Gly Phe
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Gln Leu Pro Gln Met Asp Leu Pro Pro Leu Gly Ala Pro Trp Leu
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 1685 1690 1695

Ser Arg Gln Thr Gln Pro Val Leu Gln Ser Gln Val Glu Asn Leu
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Val His Gly Ala Gly Pro Ser Val Met Glu Ile Pro Trp Asp Asp
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Pro Arg Leu Pro Val Thr Ser Glu Ala Leu Ser Glu Asp Gly Gln
 1760 1765 1770

Ile Cys Val Tyr Phe Phe Lys Asn Asp Leu Lys Lys Tyr Asp Val
 1775 1780 1785

Pro Leu Ser Trp Glu Gln Ala Arg Leu Gln Thr Gln Lys Glu Leu
 1790 1795 1800

Gln Leu Arg Glu Gly Arg Leu Ala Ile Lys Pro Phe His Pro Ser
 1805 1810 1815

Ala Asn Asn Phe Pro Ile Pro Leu Leu His Met His Arg Asn Trp
 1820 1825 1830

Lys Arg Ser Thr Glu Cys Ala Gln Glu Gly Arg Ile Pro Ser Thr
 1835 1840 1845

Glu Asp Leu Met Arg Gly Ala Ser Ala Glu Glu Leu Leu Ala Gln
 1850 1855 1860

Cys Leu Ser Ser Ser Leu Leu Leu Glu Lys Glu Glu Asn Lys Arg
 1865 1870 1875

Phe Glu Asp Gln Leu Gln Gln Trp Leu Ser Glu Asp Ser Gly Ala
 1880 1885 1890

Phe Thr Asp Leu Thr Ser Leu Pro Leu Tyr Leu Pro Gln Thr Leu
 1895 1900 1905

Val Ser Leu Ser His Thr Ile Glu Pro Val Met Lys Thr Ser Val
 1910 1915 1920

Thr Thr Ser Pro Gln Ser Asp Met Met Arg Glu Gln Leu Gln Leu
 1925 1930 1935

Ser Glu Ala Thr Gly Thr Cys Leu Gly Glu Arg Leu Lys His Leu
 1940 1945 1950

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 1955 1960 1965

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59

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gggaattctg aggacttgta gatgtttt

28

75455-2

CLAIMS:

1. A transgenic non-human mammalian cell carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto or a daughter cell thereof carrying the GANP gene.
- 5 2. The transgenic non-human mammalian cell according to claim 1 wherein the transferred GANP gene is expressed in B cells, or a daughter cell thereof carrying the GANP gene.
3. The transgenic non-human mammalian cell according to claim 1 or 2 wherein the mammalian cell has been generated from GANP gene-transfected
10 ES cells, or a daughter cell thereof carrying the GANP gene.
4. The transgenic non-human mammalian cell according to any one of claims 1 to 3 wherein the mammalian cell is from mouse, or a daughter cell thereof carrying the GANP gene.
5. A method of producing a high affinity antibody which specifically binds
15 to an antigen, comprising (i) administering the antigen to a transgenic non-human mammal carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto, or its progeny carrying a GANP gene, and (ii) recovering the high affinity antibody from the resultant mammal or the progeny.
6. The method according to claim 5 wherein the mammal is a mouse and
20 further comprising the step of humanizing the mouse antibody recovered in step (ii) by transferring the CDRs of the variable regions of the mouse antibody into human variable regions so that the reconstructed variable regions consist of human-derived framework regions and the mouse-derived CDRs.

75455-2

7. The method according to claim 5 wherein the immune system of the mammal in step (i) has been replaced with the human immune system so that the high affinity antibody recovered in step (ii) is a human antibody.

8. The method according to any one of claims 5 to 7 wherein the affinity of the high affinity antibody is 1×10^{-7} M or less as expressed as a dissociation constant.

9. The method according to any one of claims 5 to 8 wherein the high affinity antibody is a polyclonal or monoclonal antibody.

10. A method of producing a pharmaceutical composition comprising a high affinity antibody which specifically binds to an antigen, comprising (i) administering the antigen to a transgenic non-human mammal carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto, or its progeny carrying the GANP gene, (ii) recovering the high affinity antibody from the resultant mammal or the progeny, and (iii) preparing a pharmaceutical composition comprising the recovered antibody by including one or more pharmacologically acceptable carriers.

11. Use of a transgenic non-human mammal carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto, or its progeny carrying the GANP gene, for producing a high affinity antibody which specifically binds to an antigen.

12. A high affinity antibody-producing cell which is taken from a transgenic non-human mammal carrying a germinal center-associated nuclear protein (GANP) gene transferred thereinto, or its progeny carrying the GANP gene, wherein the transgenic mammal or the progeny has been administered an antigen.

Application number/numéro de demande: JP03/14221

Figures: 1 to 7, 9B, 9C, 11B to 11E, 16, 25 to 27, 30, 31, 32, 34, 35, 36A,
36B, 37 to

Pages: _____ 44

Drawings - IP

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th Floor)

Documents reçus avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au
10ième étage)

Fig. 8A

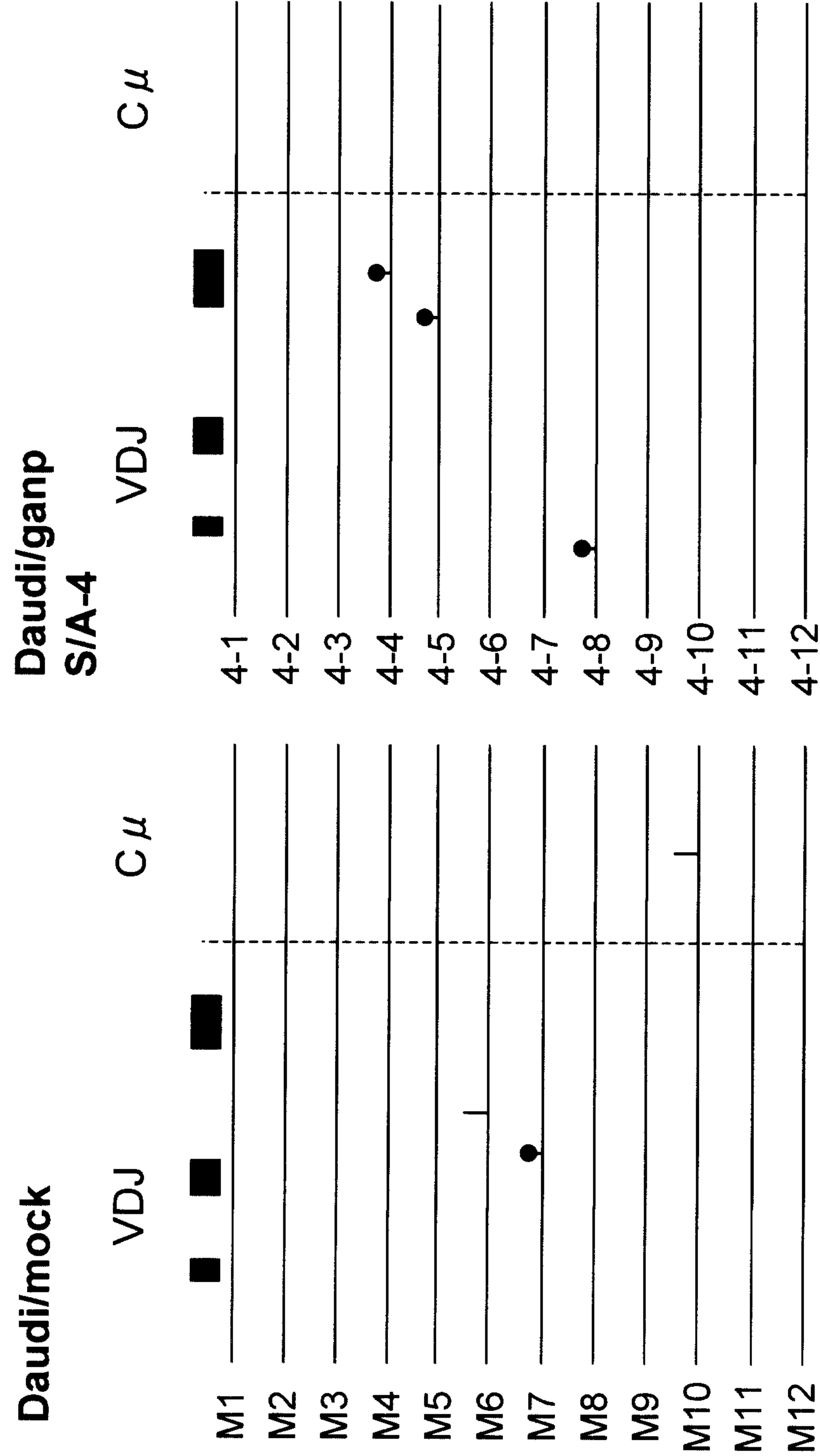


Fig. 8B

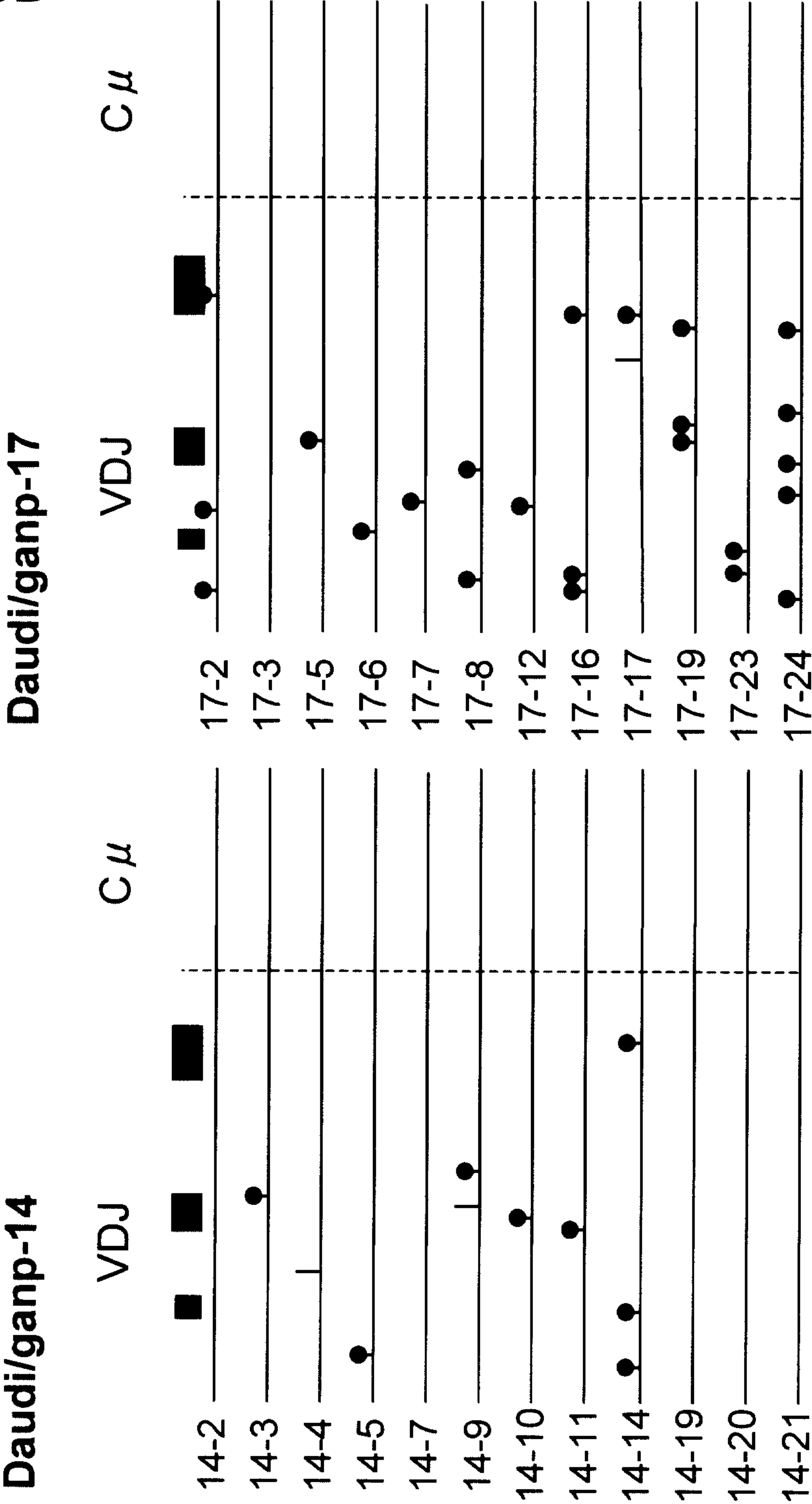


Fig. 8C

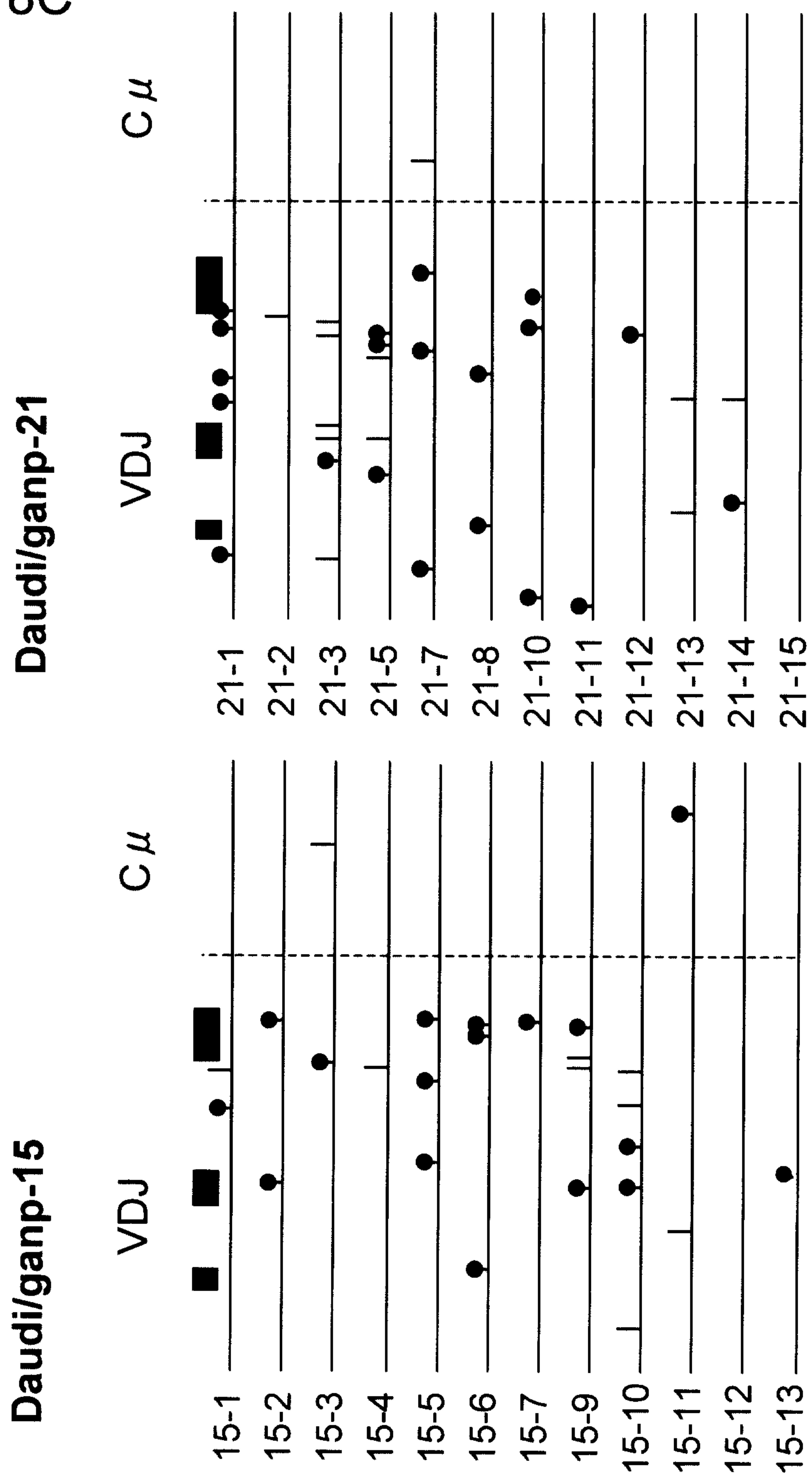


Fig. 9A

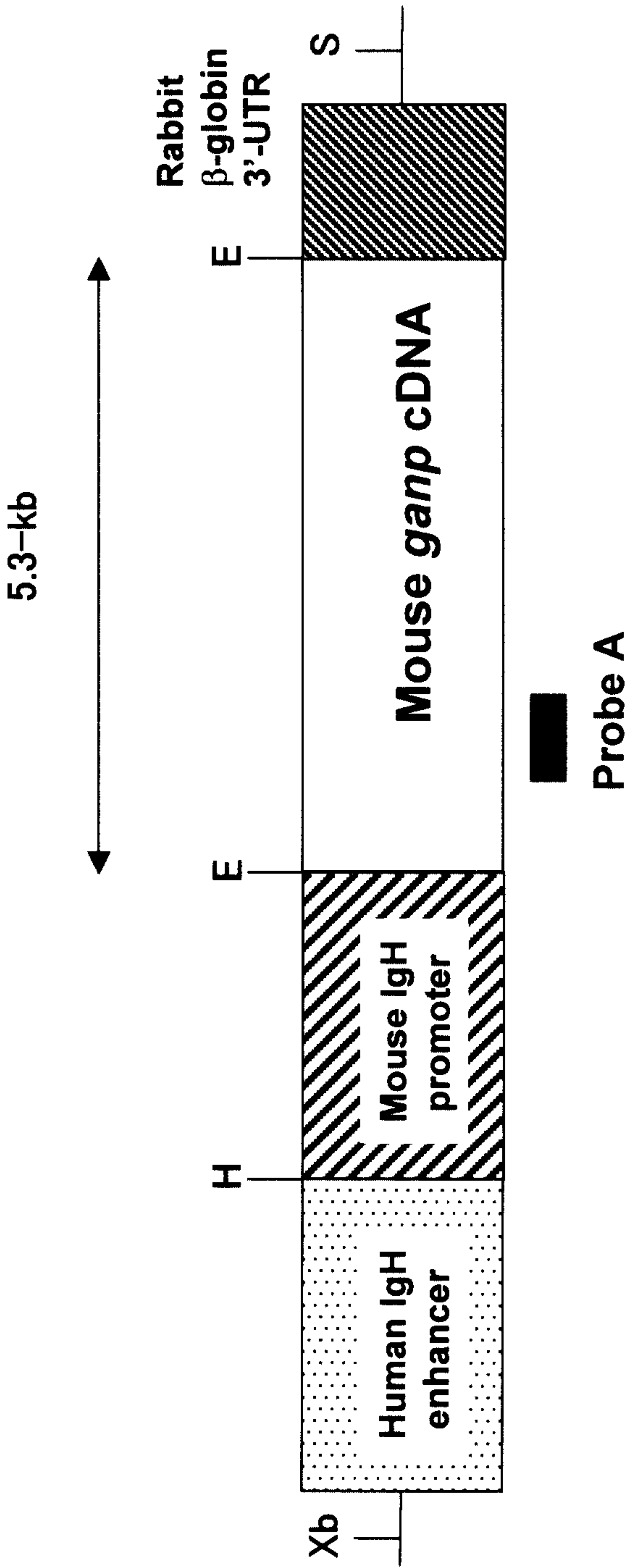


Fig. 10

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Q	V	Q	L	Q	Q	P	G	A	E	L	V	K	P	G	A	S	V	K	L	S	C	K	A	S	G	Y	T	F	T
CAG	GTC	CAA	CTG	CAG	CAG	CCT	GGG	GCT	GAG	CTT	GIG	AAG	CCT	GGG	GCT	TCA	GIG	AAG	CTG	TCC	TGC	AAG	GCT	TCT	GGC	TAC	ACC	TTC	ACC
WT-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	A	T	---	---	S	T	---
WT-10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-14	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-16	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-17	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-18	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-19	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-20	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-21	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
WT-22	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

14/69 WT

Fig. 10 continued 1

CDR2

CDR1

31	S	AGC	TAC	TGG	ATG	CAC	TGG	GTG	AAG	CAG	AGG	CCT	GGA	GGA	GGA	TGG	ATT	GGA	AGG	ATT	GAT	CCT	AAT	AGT	GGT	GGT	ACT	AAG	TAC	AAT	GAG	AAG	TTC	AAG	AGC
32	Y																																		
33	W																																		
34	M																																		
35	H																																		
36	W																																		
37	V																																		
38	K																																		
39	Q																																		
40	R																																		
41	P																																		
42	G																																		
43	R																																		
44	G																																		
45	L																																		
46	E																																		
47	W																																		
48	I																																		
49	G																																		
50	R																																		
51	I																																		
52	D																																		
53	P																																		
54	N																																		
55	S																																		
56	G																																		
57	G																																		
58	T																																		
59	K																																		
60	Y																																		
61	N																																		
62	E																																		
63	K																																		
64	F																																		
65	K																																		
66	S																																		

Fig. 10 continued 3

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Q	V	Q	L	Q	Q	P	G	A	E	L	V	K	P	G	A	S	V	K	L	S	C	K	A	S	G	Y	T	F	T
CAG	GTC	CAA	CTG	CAG	CAG	CCT	GGG	GCT	GAG	CTT	GTC	AAG	CCT	GGG	GCT	TCA	GTC	AAG	CTG	TCC	TCC	AAG	GCT	TCT	GGC	TAC	ACC	TTC	ACC
Tg-3																													
Tg-4									A																				
Tg-5																													
Tg-7																													
Tg-8															S								P						
Tg-9															T								C						
Tg-10																												N	A
Tg-11																													
Tg-12																													
Tg-13															S														
Tg-14															T														
Tg-15																													
Tg-16																													
Tg-17																													
Tg-18																												I	TT
Tg-20																													
Tg-21																													
Tg-23																													

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Fig. 10 continued 5

[illegible]

Fig. 11A

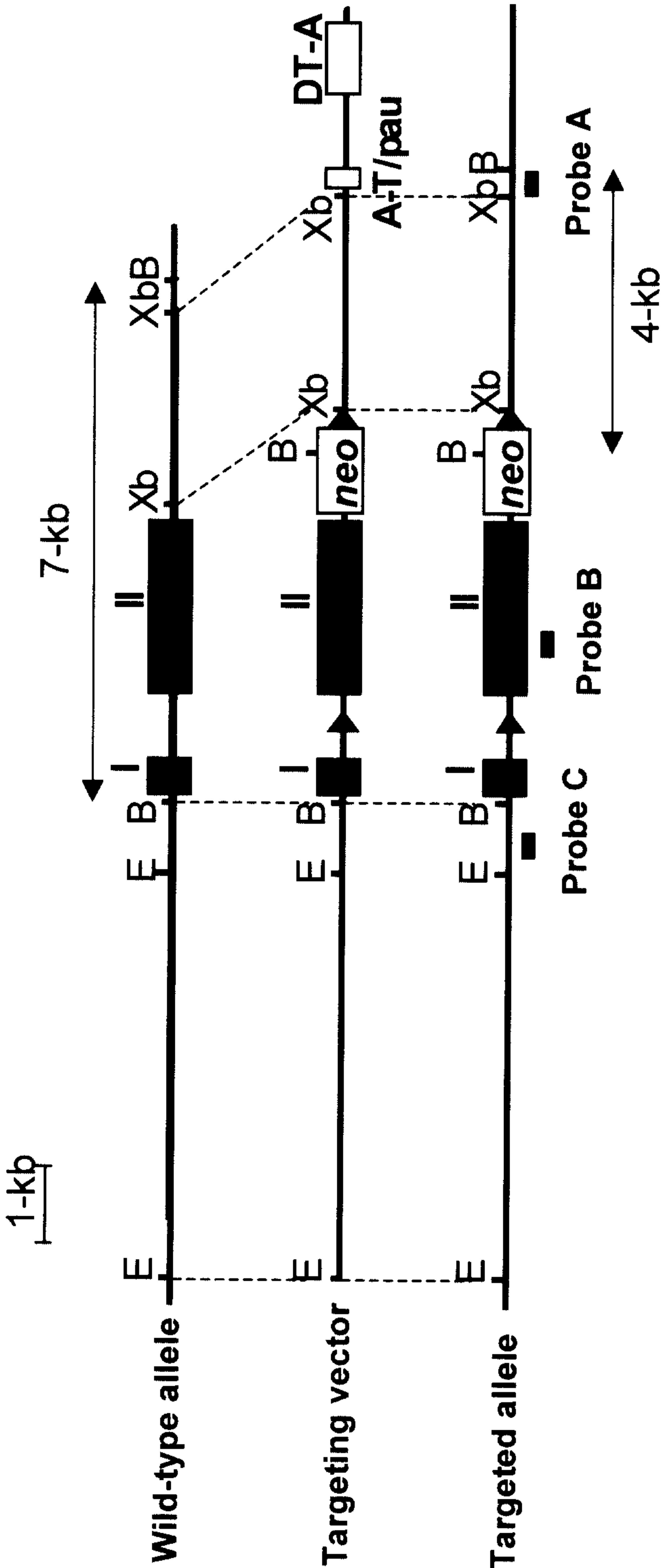


Fig. 12

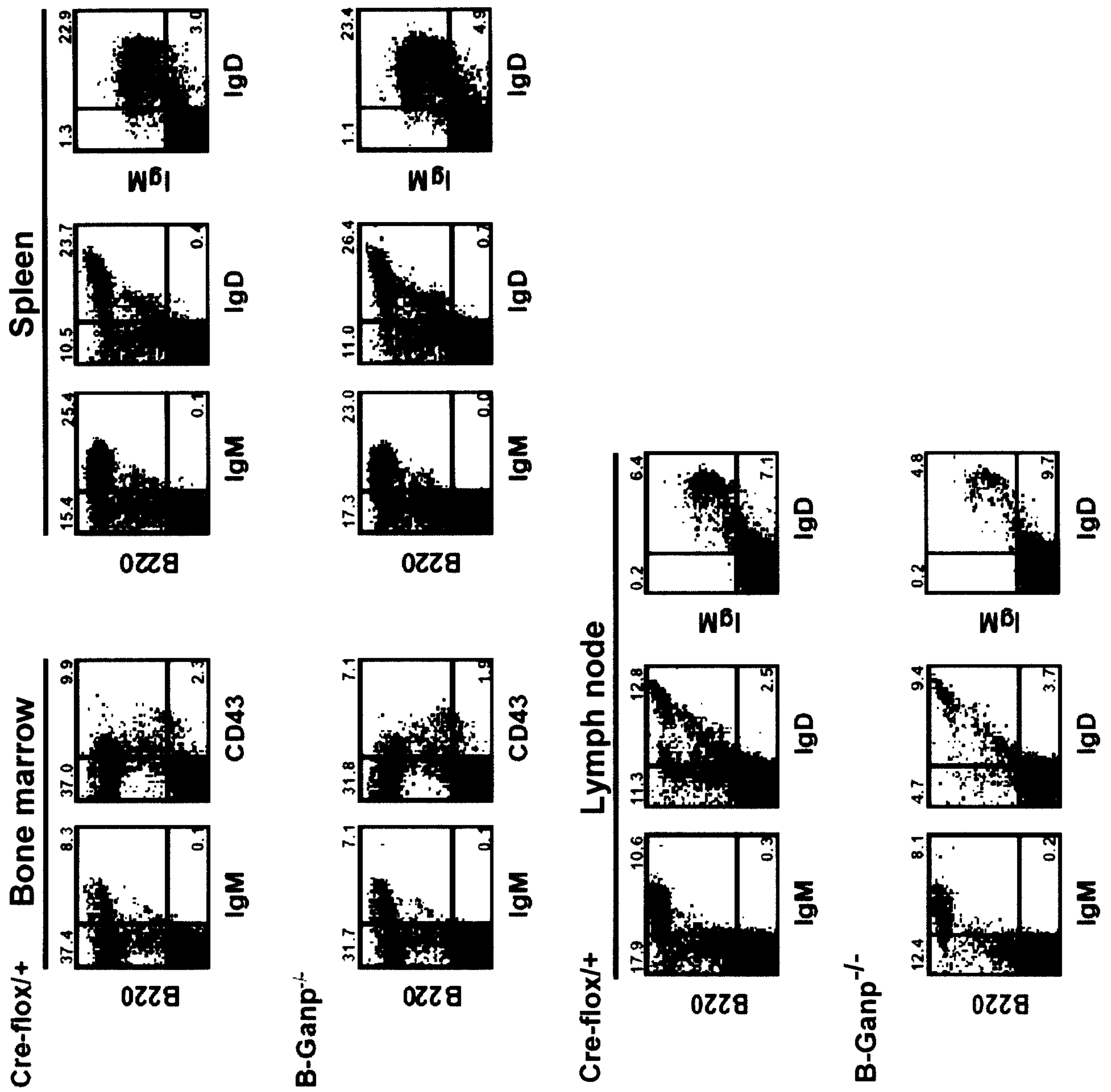


Fig. 13

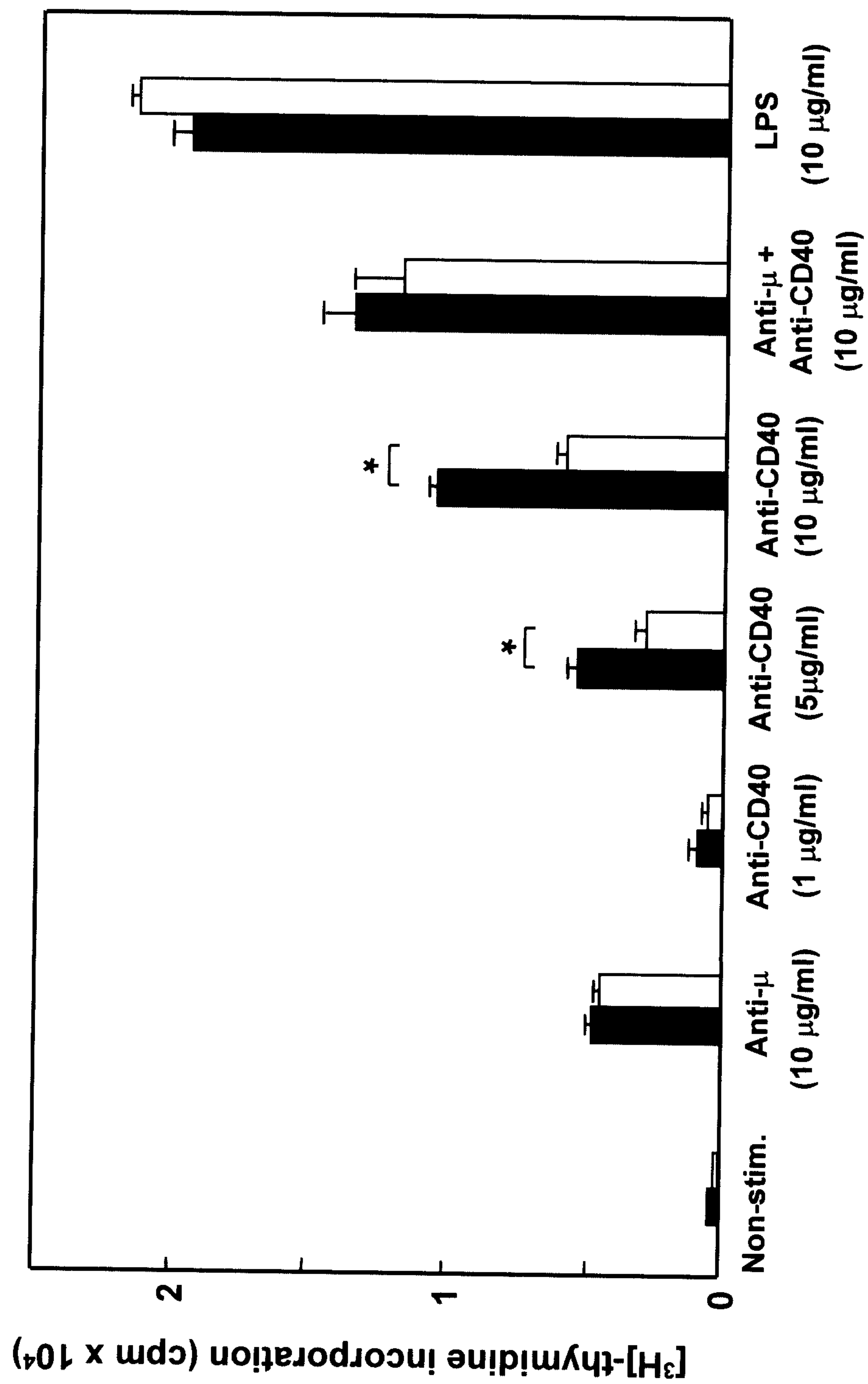


Fig. 14

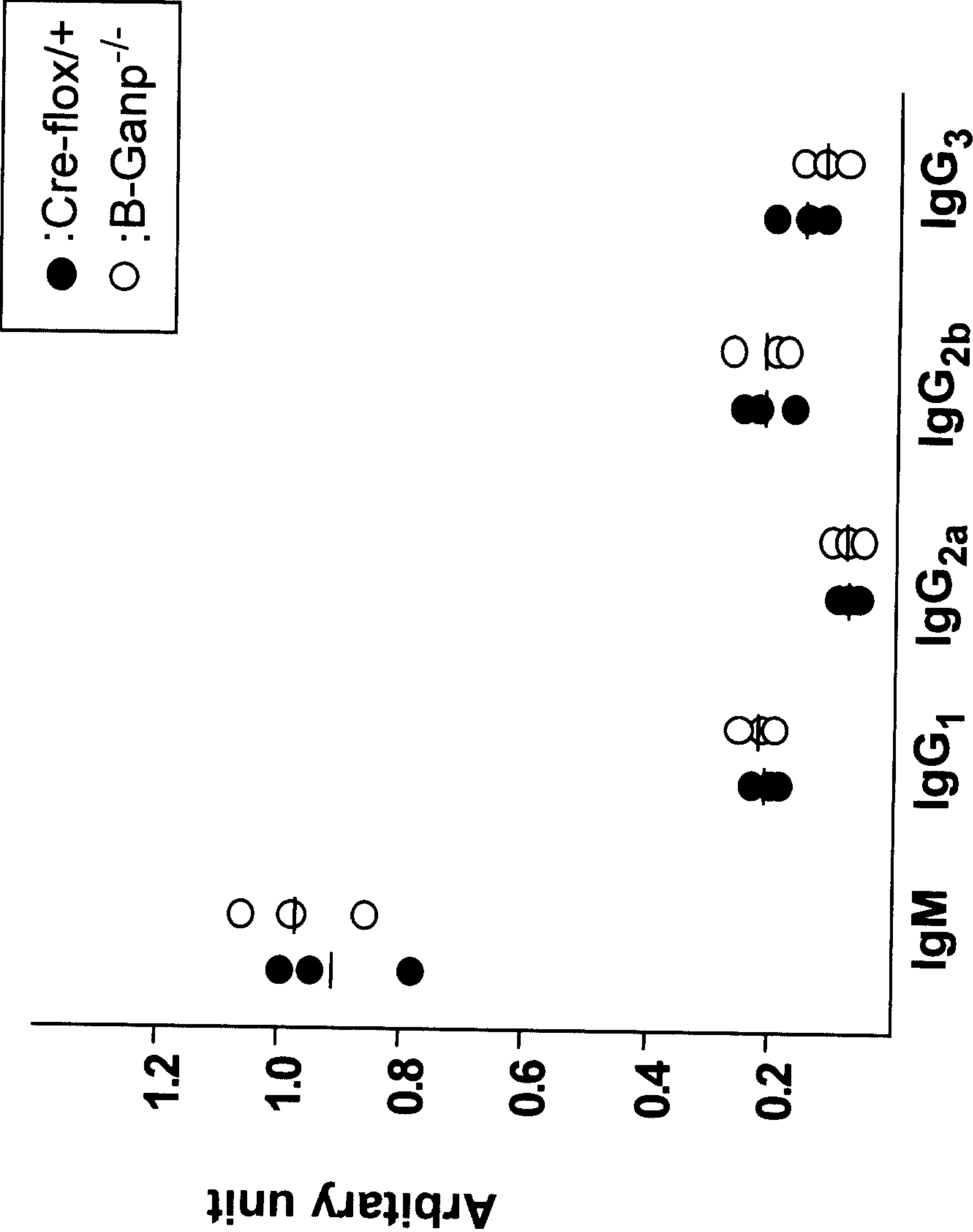


Fig. 15

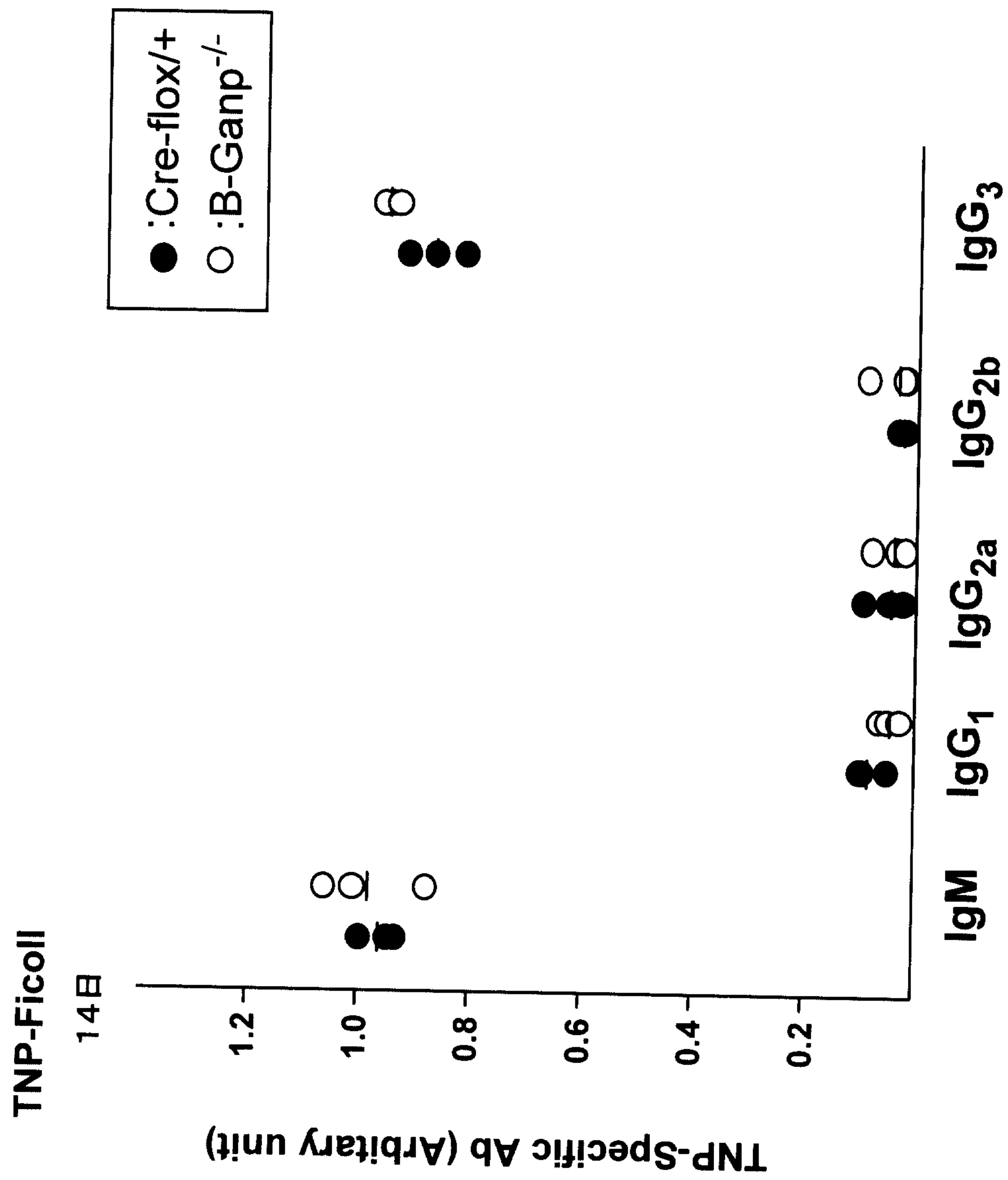


Fig. 17

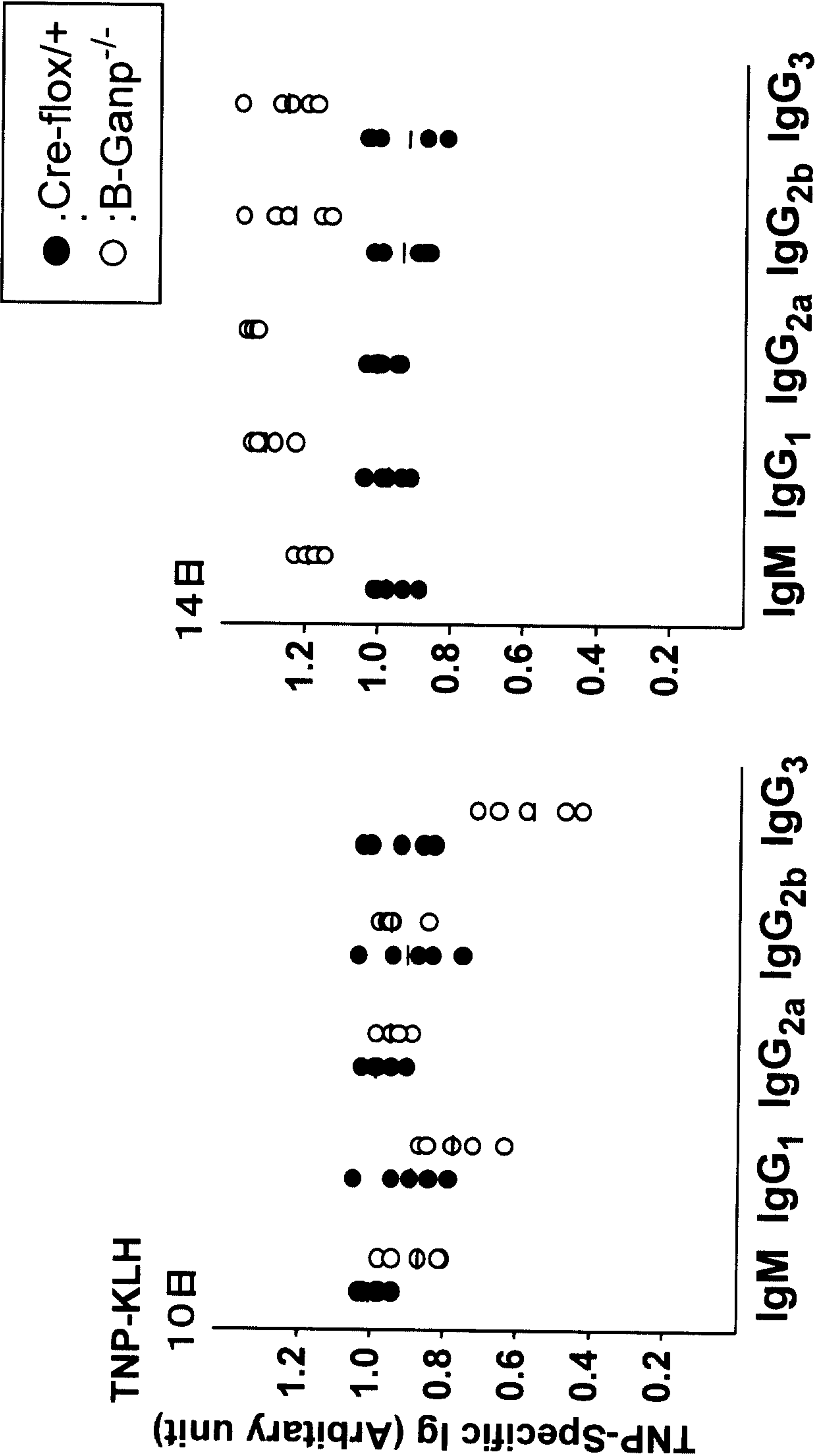


Fig. 18

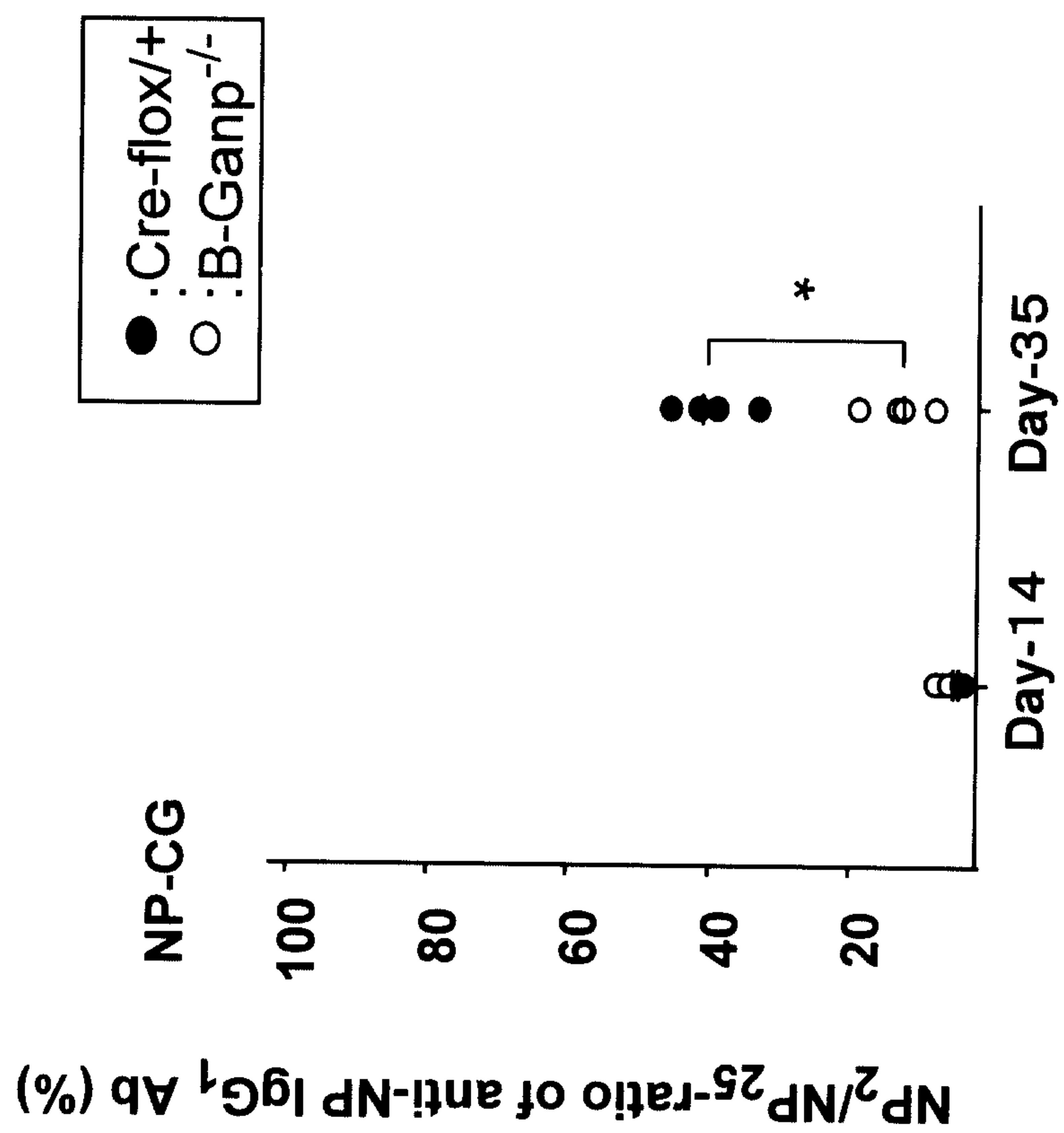


Fig. 19

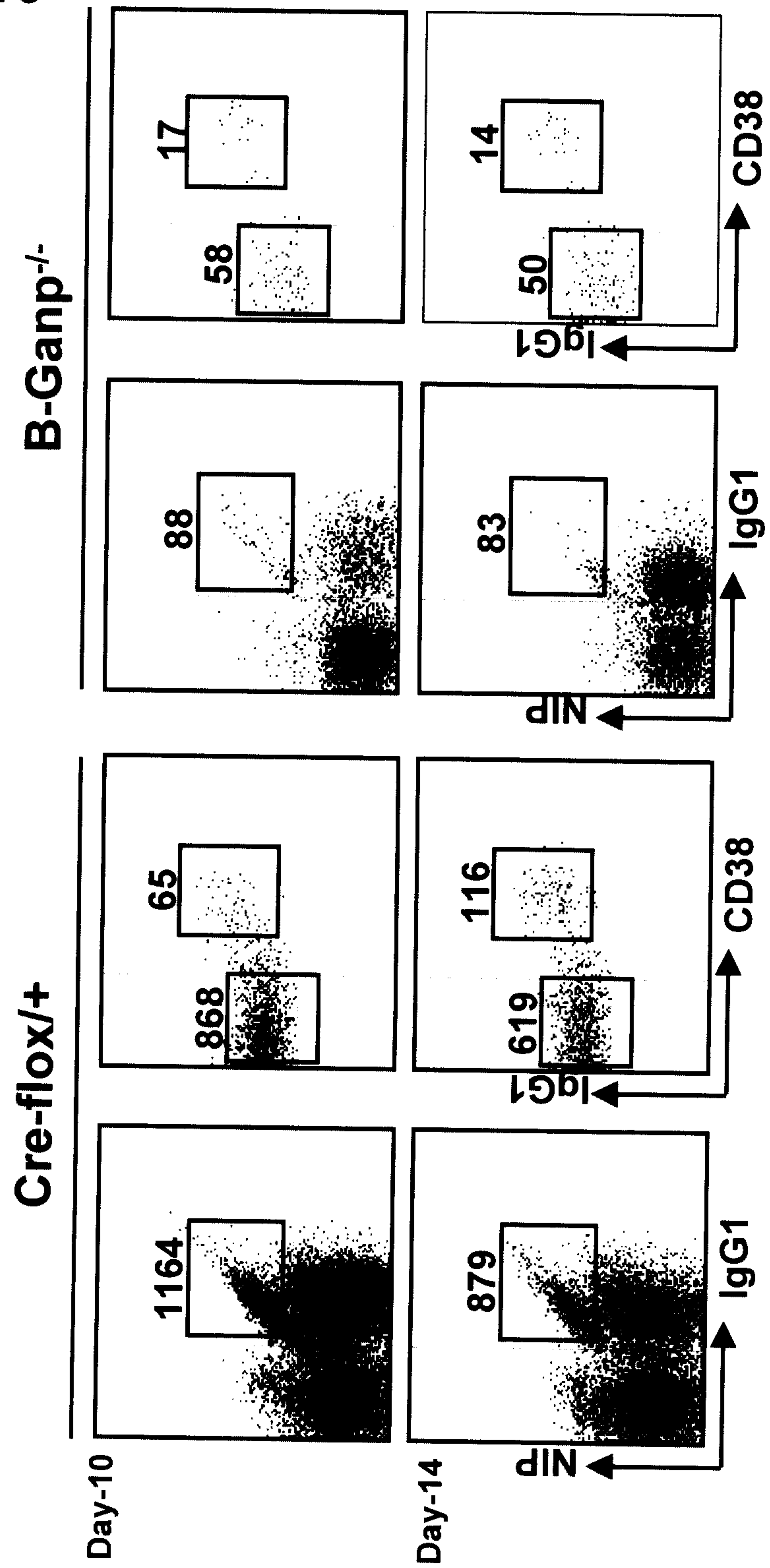


Fig. 20A

Cre-flox/+

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	Q	V	Q	L	Q	Q	P	G	A	E	L	V	K	P	G	A	S
	CAG	GTC	CAA	CTG	CAG	CAG	CCT	GGG	GCT	GAG	CTT	GTG	AAG	CCT	GGG	GCT	TCA
1-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-6	---	---	---	---	---	---	---	---	---	A	---	---	---	---	---	---	---
3-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
3-2	---	---	---	---	---	---	---	---	---	---	---	---	R	---	---	---	---
	---	---	---	---	---	---	---	---	---	---	---	---	G	---	---	---	---
3-3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	G	---
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	G	---
4-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	G	---
4-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4-7	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	G	---
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	G	---
6-1	---	---	---	---	---	---	---	T	A	---	---	---	---	---	---	---	---
6-2	---	---	---	---	---	---	---	---	---	---	A	G	---	---	---	---	---
7-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Fig. 20B

Cre-flox/+

18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
V	K	L	S	C	K	A	S	G	Y	T	F	T	S	Y	W	M	H
GTG	AAG	CTG	TCC	TGC	AAG	GCT	TCT	GGC	TAC	ACC	TTC	ACC	AGC	TAC	TGG	ATG	CAC
	I							A									
---	-TT	---	---	---	---	---	---	-C-	---	---	---	---	---	-T	---	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	R	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	---	-T	A	---	---
---	---	---	---	---	---	---	---	---	---	---	---	---	T	---	L	I	---
---	---	---	---	---	---	---	---	---	---	---	---	---	-C-	---	-T-	-T	---
---	-C	---	---	---	---	---	---	---	I	---	---	---	---	*	---	---	---
---	---	---	---	---	---	---	---	-A	-TT	---	---	---	---	-G	---	---	---
---	---	---	---	---	---	---	---	---	---	L	---	---	T	---	L	---	---
---	---	---	---	---	---	---	---	-T	---	C-	---	---	-C-	---	-TA	---	---
---	---	---	---	---	---	---	---	---	---	L	---	---	T	---	L	---	---
---	---	---	---	---	---	---	---	---	---	C-	---	---	-C-	---	-TA	---	---
---	---	---	---	---	---	---	---	---	---	L	---	---	T	---	L	---	---
---	---	---	---	---	---	---	---	---	---	C-	---	---	-C-	---	-TA	---	---
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---	---	---	---	---	---	---	---	---	---	C-	---	---	-C-	---	-TA	---	---
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---	---	---	---	---	---	---	---	-T-	---	---	---	---	---	---	-T-	---	---
---	V	---	---	---	---	---	---	---	---	---	---	---	---	---	L	---	---
---	G	---	---	---	---	---	---	---	---	---	---	---	---	---	-T-	---	---
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Fig. 20C

Cre-flox/+

36	37	38	39	40	41	42	43	44	45	46	47	48	49
W	V	K	Q	R	P	G	R	G	L	E	W	I	G
TGG	GTG	AAG	CAG	AGG	CCT	GGA	CGA	GGC	CTT	GAG	TGG	ATT	GGA

A

Fig. 20D

Cre-flox/+

	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67
	R	I	D	P	N	S	G	G	T	K	Y	N	E	K	F	K	S	K
	AGG	ATT	GAT	CCT	AAT	AGT	GGT	GGT	ACT	AAG	TAC	AAT	GAG	AAG	TTC	AAG	AGC	AAG
1-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
								D										
3-1	---	---	---	---	---	---	---	-A-	-A-	---	---	---	---	---	---	---	---	---
					M				S	R				Y				
3-2	---	---	---	---	-TG	---	---	-C	-G-	-G-	---	---	---	T-C	---	---	---	---
3-3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
					K							S						
1-8	---	---	---	---	-A	---	---	---	---	---	---	-G-	---	---	---	---	---	---
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1-10	---	G-	---	---	---	-C-	---	---	---	---	---	---	---	---	---	---	---	---
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4-7	---	---	---	---	---	---	---	-A-	---	---	---	---	---	---	---	---	---	---
6-1	---	---	---	---	---	---	---	---	---	---	---	---	---	T	---	---	N	---
													-C-	---	---	-A-	---	
								S									T	
6-2	---	---	---	---	---	---	---	A-	---	---	---	---	---	---	---	---	-C-	---
														T			N	
7-1	---	---	---	---	---	---	---	---	---	---	---	---	---	-C-	---	---	-A-	---

Fig. 20E

Cre-flox/+

[illegible]

Fig. 20F

Cre-flox/+

85	86	87	88	89	90	91	92	93	94	95	96	97	98
S	L	T	S	E	D	S	A	V	Y	Y	C	A	R
AGC	CTG	ACA	TCT	GAG	GAC	TCT	GCG	GTC	TAT	TAT	TGT	GCA	AGA

T D
 A G

Fig. 20G

B-Ganp^{-/-}

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	Q	V	Q	L	Q	Q	P	G	A	E	L	V	K	P	G	A	S
	CAG	GTC	CAA	CTG	CAG	CAG	CCT	GGG	GCT	GAG	CTT	GTG	AAG	CCT	GGG	GCT	TCA
1-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
2-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
2-3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
4-3	---	---	---	---	---	---	---	---	T A	---	A	---	G	---	---	---	---
4-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
6-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
6-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
7-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
8-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
8-2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
9-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
9-3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
9-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Fig. 20H

B-Ganp^{-/-}

18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
V	K	L	S	C	K	A	S	G	Y	T	F	T	S	Y	W	M	H
GTG	AAG	CTG	TCC	TGC	AAG	GCT	TCT	GGC	TAC	ACC	TTC	ACC	AGC	TAC	TGG	ATG	CAC
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											F						
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											F						
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													N		L		
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														T			

Fig. 20 I

B-Ganp^{-/-}

36	37	38	39	40	41	42	43	44	45	46	47	48	49
W	V	K	Q	R	P	G	R	G	L	E	W	I	G
TGG	GTG	AAG	CAG	AGG	CCT	GGA	CGA	GGC	CTT	GAG	TGG	ATT	GGA

[illegible]

Fig. 20J

B-Ganp^{-/-}

	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
	R	I	D	P	N	S	G	G	T	K	Y	N	E	K	F	K
	AGG	ATT	GAT	CCT	AAT	AGT	GGT	GGT	ACT	AAG	TAC	AAT	GAG	AAG	TTC	AAG
					K											
1-1	---	---	---	---	-A-	---	---	---	---	---	---	---	---	---	---	---
1-5	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1-6	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
									S							
2-2	---	---	---	---	---	---	---	---	T	---	---	---	---	---	---	---
2-3	--T	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	N		N							N						
4-3	-AT	---	A	---	---	---	---	---	---	--C	---	---	---	---	---	---
						G										
4-4	---	---	---	---	---	G	---	---	---	---	---	---	---	---	---	---
6-1	---	---	---	---	---	---	---	---	---	---	--T	---	---	E		
													G	---	---	---
6-2	---	---	---	---	---	---	---	---	---	---	--T	---	---	---	---	---
						G					F	D				
7-1	---	---	---	---	---	G	---	---	---	---	-T	G	---	---	---	---
8-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
						G								E		
8-2	---	---	---	---	---	G	---	---	---	--A	---	---	---	-G	---	---
									A							
9-1	---	---	---	---	---	---	---	---	G-C	---	---	---	---	---	---	---
9-3	---	---	---	---	---	---	--C	---	---	---	---	---	---	---	---	---
9-4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Fig. 20K

B-Ganp^{-/-}

[illegible]

[illegible]

Fig. 21

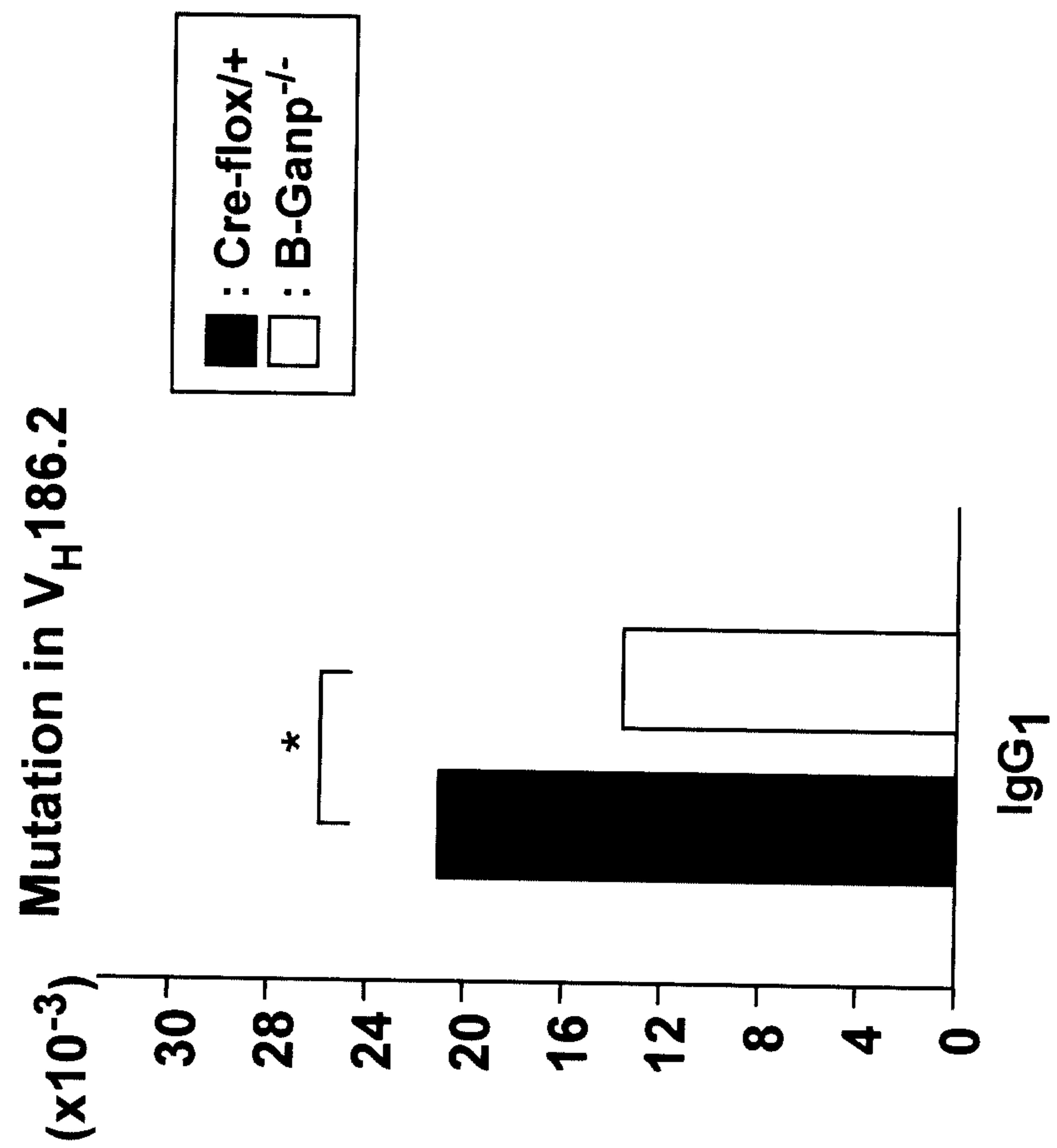


Fig. 22

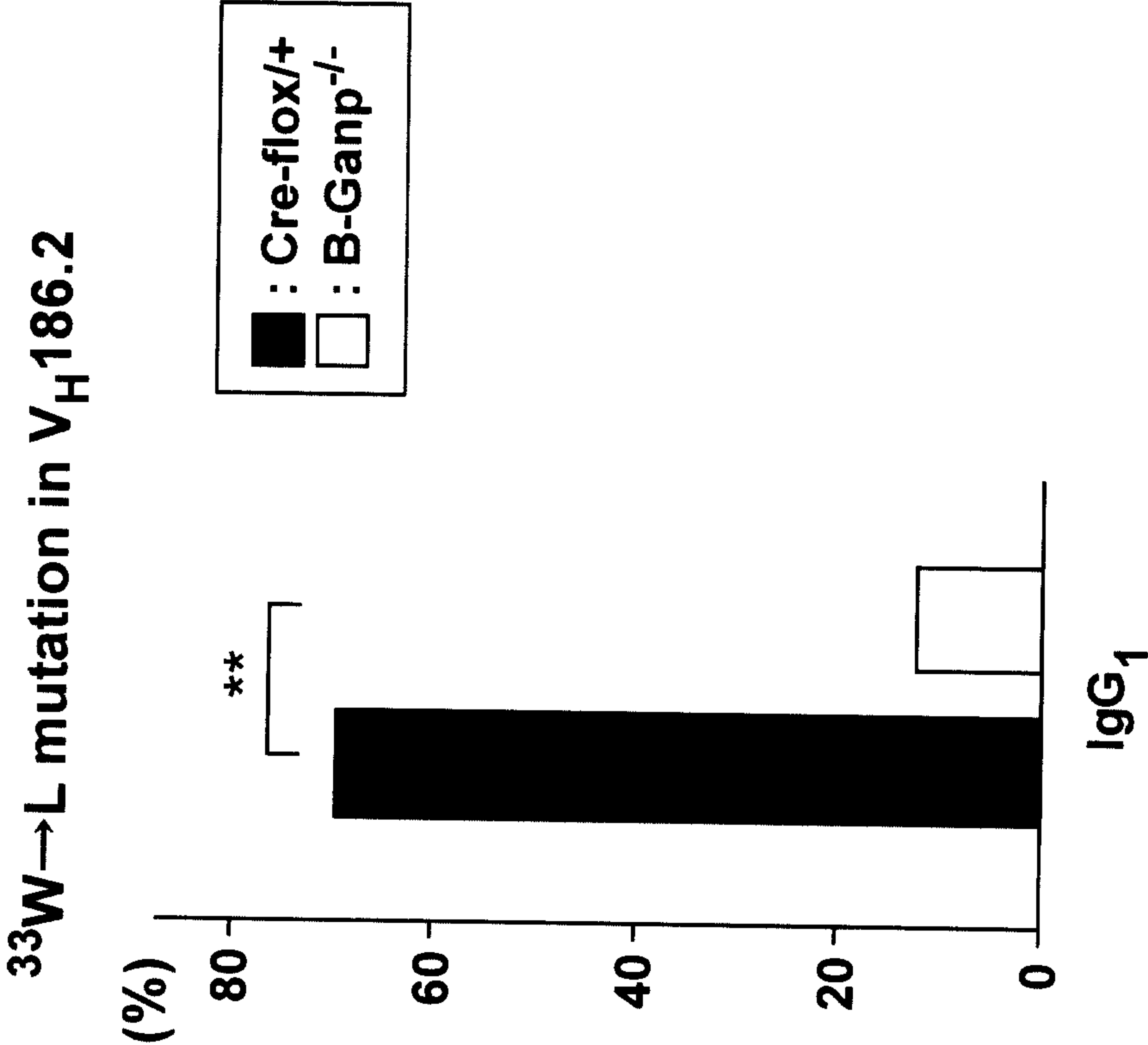


Fig. 23

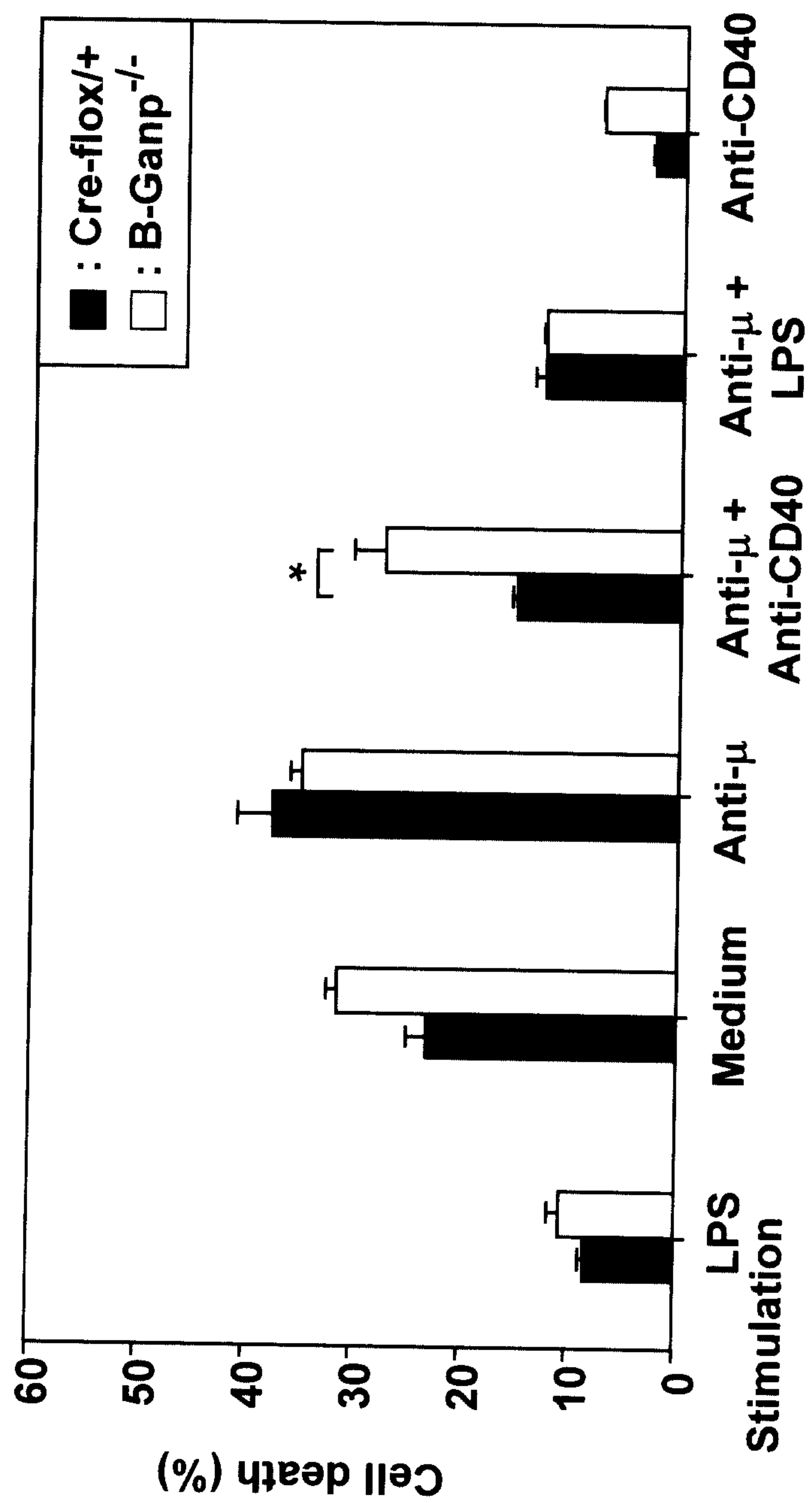


Fig. 24

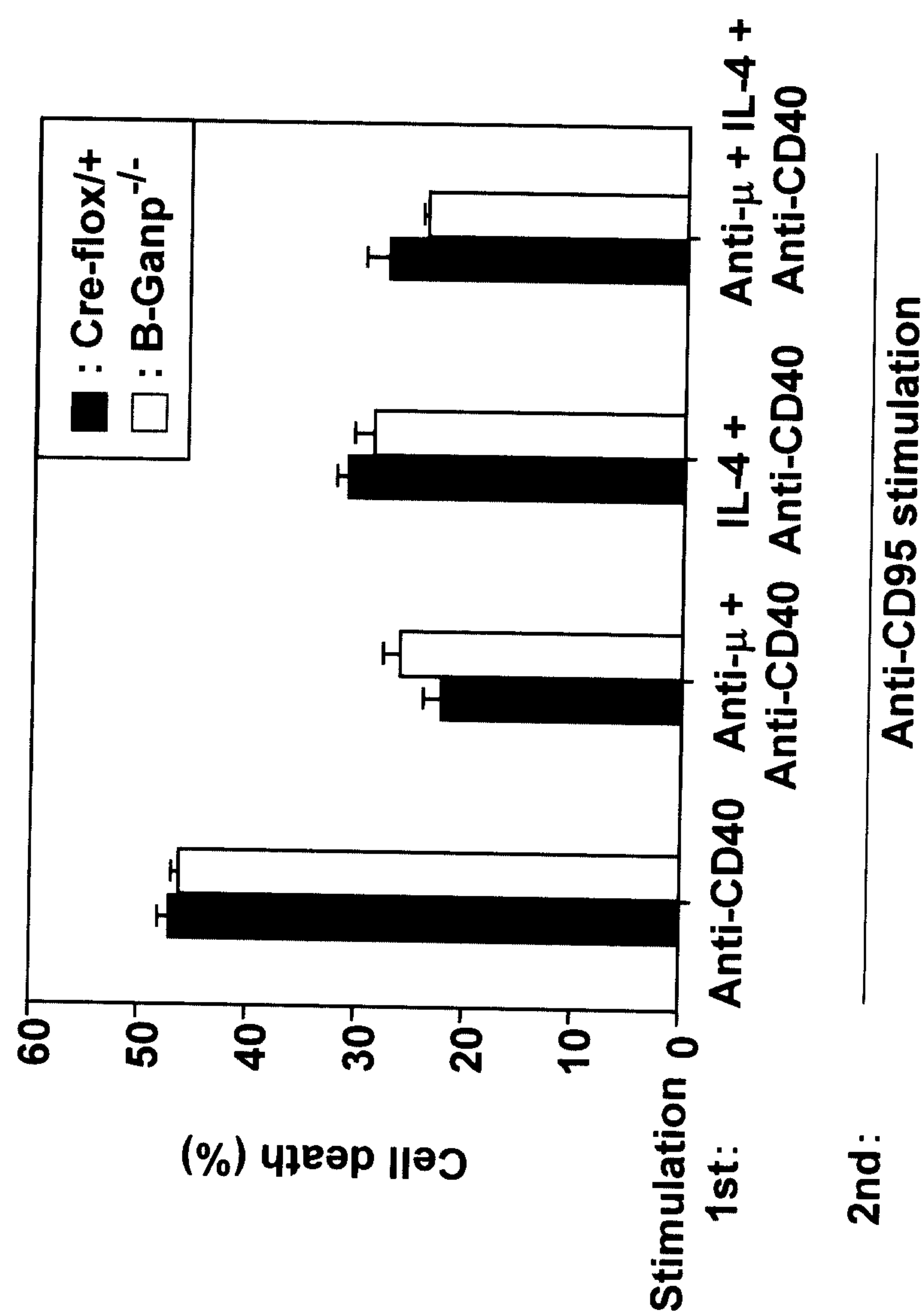


Fig. 29

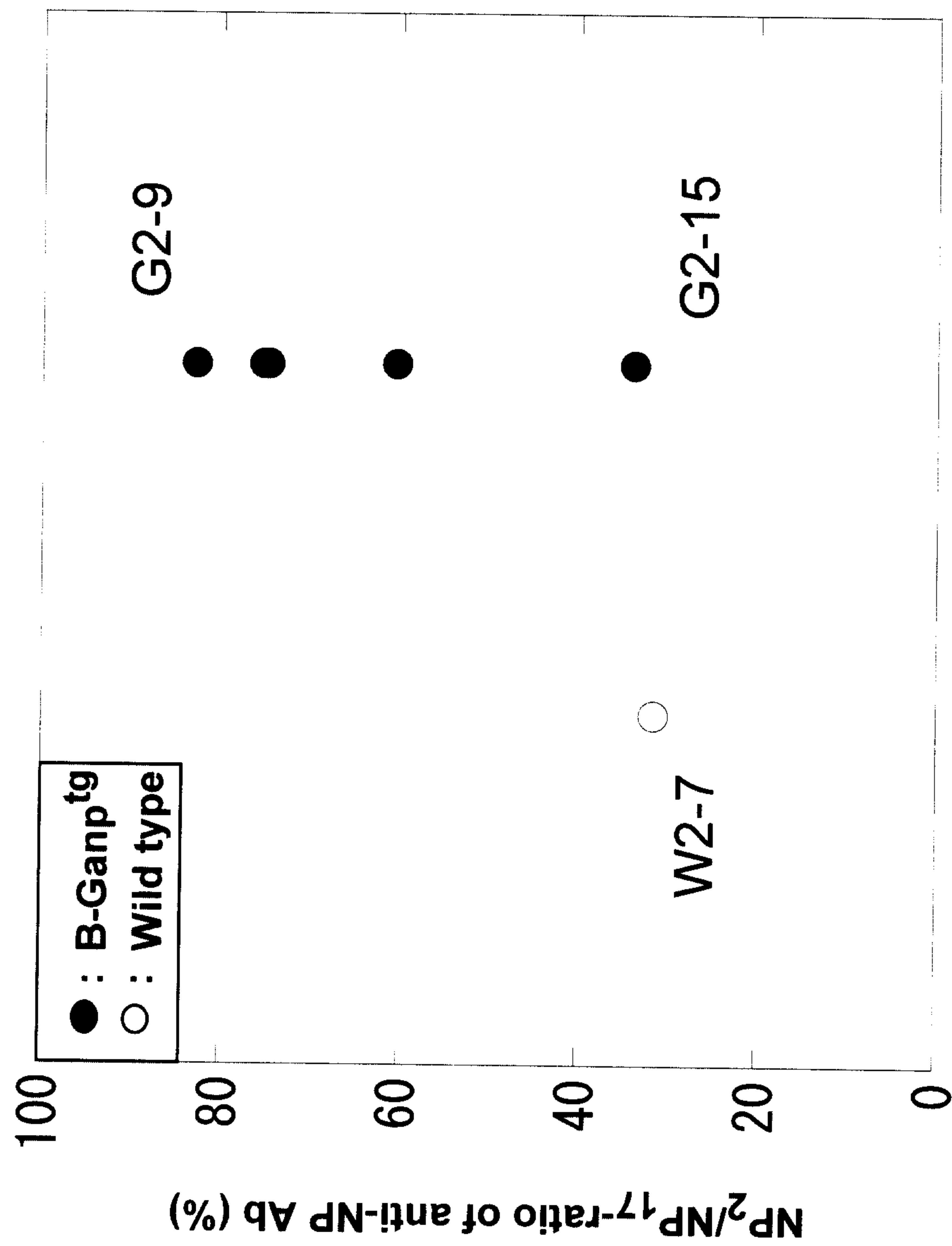


Fig. 33

