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(71) Applicant: **THE CHILDREN'S HOSPITAL OF PHILADELPHIA** [US/US]; 34th Street & Civic Center Boulevard, Philadelphia, PA 19104 (US).

(72) Inventors: **OLIVER, Paula, M.;** 110 Raynham Road, Merion, PA 19066 (US). **RILING, Chris;** 910 N. Taney Road, Philadelphia, PA 19130 (US).

(74) Agents: **RIGAUT, Kathleen, D.** et al; Dann, Dorfman, Herrell & Skillman, 1601 Market Street, Suite 2400, Philadelphia, PA 19103-2307 (US).

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[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR MODULATION OF IMMUNE FUNCTION

(57) Abstract: Compositions and methods for modulating immune cell function and Nedd-4 family member signaling are disclosed.

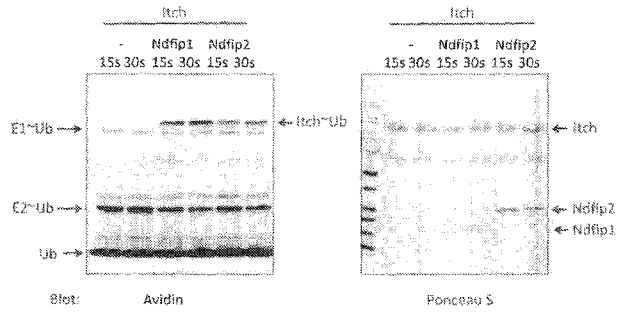


Figure 1A Figure 1B

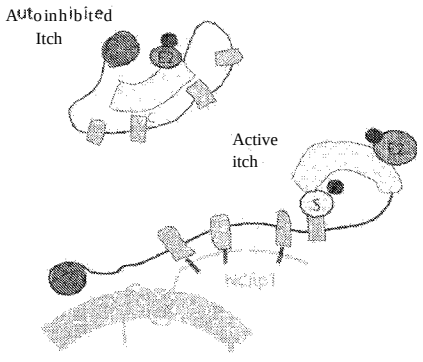


Figure 1C

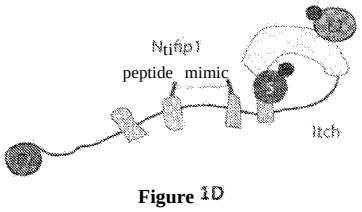


Figure 1D

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KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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Compositions and methods for Modulation of Immune Function

This application claims priority to US Provisional Application No. 61/820,678 filed May 7, 2013, the entire disclosure being incorporated herein by reference as though set forth in full.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has rights in the invention described, which was made with funds from the National Institutes of Health, Grant Number, NIH R01 Al080765.

Field of the Invention

The present invention relates to the fields of protein processing and immune function. More specifically, the invention provides compositions and methods for activating or repressing Nedd4-family E3 ubiquitin ligase action as a means to improve immune function in subjects in need thereof.

Background of the Invention

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated herein by reference as though set forth in full.

Nedd4-family E3 ubiquitin ligases regulate many biological processes including the sodium and iron homeostasis and inflammation. For example, the Nedd4-family members Itch can repress immune function in both innate and adaptive immune cells. Like other Nedd4-family members, Itch normally exists in an auto-inhibited state that prevents their main enzymatic task, transferring ubiquitin to a substrate protein. Thus, to pharmacologically harness Itch activity, one much first determine how auto-inhibition occurs and identify means to "de-repress" its catalytic activity.

Summary of the Invention

In accordance with the present invention, a composition which relieves auto-inhibition of Itch or Nedd4-family members comprising similar motifs, comprising one or more peptides or variants thereof, selected from the group comprising 1) PEQTAGDAPPPYSSITAESAAYFDY; 2) GDAPPPYSSITAESAAYFDYKDESGFPKPPSYNVA; 3)GDAPPPYSSITAESAAYFDYKDESGFPKPPSYNVATTLPSYDEAE in a pharmaceutically acceptable carrier, said peptide optionally being contained within a lipsome is provided. The liposomes may further comprise antibodies immunologically specific for molecules present on the surfact of target cells to facilitate cellular uptake of the peptide. In a preferred embodiment, the peptides are contained within antibodies. In another embodiment, the peptides are operably linked to a cellular penetrating peptide.

In an alternative embodiment of the invention, a composition comprising a plurality of PPPY or PPSY peptide motifs operably linked via flexible linkers in a in a pharmaceutically acceptable carrier, said peptide optionally being contained within a liposome. In one embodiment two, three, four, five or more motifs are operably linked.

In yet another aspect of the invention, a method for reducing inflammation in a patient in need thereof is disclosed. An exemplary method entails administration of an effective amount of the compositions disclosed above which are effective to modulate T cell function in said patient, thereby reducing an undesired immune response. In another embodiment, the patient has a disorder selected from the group consisting of asthma, atopic dermatitis and inflammatory bowel disease.

Brief Description of the Drawings

Figure 1. Ndfipl andNdfip2 promote ubiquitin charging of Itch, A) El, E2, ubiquitin, ATP and Mg++ were incubated together to prepare the E2 for ubiquitin transfer. EDTA was added to prevent further transfer of ubiquitin from E1 to E2. This ensures that only a single ubiquitin will be transferred. Itch was then added to the reaction with or without Ndfipl or Ndfip2. B) Ponceau S stain reveals protein loading for Itch, Ndfipl and Ndfip2. C) Schematic

diagram of auto-inhibition by Itch and activation by Ndfip1. D) Ndfip peptide mimics therapeutically activate

Figure 2. Ndfipl relies on PY motifs to promote Itch activation. A) Itch and Ndfipl constructs used in assay described in panel B. The Ndfipl cytoplasmic domain, including its three PY motifs are shown fused to GST. Mutants of the PY motif are point mutations changing Y~>A. Itch charging was analyzed in the same assay as described in Figure 1, however in this case WT (lane 1) or PY mutants (lanes 2-5) of Ndfipl were added. GST alone was added (lane 6) to assay Itch activity in the absence of Ndfipl.

Figure 3. Itch auto-inhibition relies on HECT and WW domains. A) Itch and Ndfipl constructs used in the assay in panel B. A full length Itch (FL-Itch) and a truncation mutant of Itch (AITCH) that lacks N-terminal regions including the C2 and proline rich motif were generated. B) These were analyzed in a ubiquitin charging assay in the presence or absence of Ndfipl.

Figure 4. Itch autoinhibition is due to an interaction between the HECT and WW domains. Constructs containing WW domain and HECT domain cassettes were generated as shown in panel A. These were used in a ubiquitin charging assay in which equal amounts of the HECT motif were added to the assay with increasing concentrations of the WW domains (B).

Figure 5. Itch contains two PY motifs in Its HECT domain. The sequences of Nedd4-family HECT domains were assessed to determine the source of auto-inhibition. A PY motif (1) was identified in the HECT-domain that is conserved in 4 of 7 Nedd4-family members. A second PY motif (2) was found several amino acids away from the catalytic cysteine (in red) in 5 of 7 members. NEDL1 and 2 lacked these motifs. Itch, and its most related family members WWP1 and WWP2, contain both PY motifs.

Figure 6. WW domains 2 and 3 dominate the Itch autoinhibitory interaction. A) A fret assay was used to determine which WW domains of Itch limited its activity. WW1-4A was used as a positive control. We found that a mutant of Itch with point mutations in WW domains 2 and 3 was active (not auto-inhibited) even in the absence of added Ndfipl . B) An Itch mutant with point mutations in WW2 and 3 was tested for its ability to bind Ndfipl, WT Itch was used as a

positive control. Ndfipl could not bind to Itch when it lacked functional WW2 and WW3 domains.

Figure 7. Itch activity increases when the protein is mutated in its WW domains. A) WT Itch and the WW2.3 mutant of Itch were transfected into Jurkat T cells. Itch and JunB stability was analyzed by western blot after stimulating cells in the presence or absence of cycloheximide (CHX). B) Itch stability was analyzed in 4 independent experiments and the combined data is shown. C) The impact of Itch levels on JunB stability is shown. The Itch WW2.3 mutant is illustrated by the closed circle. **D)** IL-2 reporter activity (tested using a luciferase assay) is shown. Based on these data, we conclude mutating the binding site in WW domains 2 and 3 makes Itch more active in vitro and reduces the stability of Itch.

Figure 8. Multiple Nedd4-family members likely share this mechanism of auto-inhibition and relief by Ndfipl and Ndfip2. Nedd4 and Itch were expressed, purified and tested in the ubiquitin charging assay in the presence or absence of Ndfipl or Ndfip2 as shown. Neither Itch nor Nedd4 were charged with ubiquitin in the assay when Ndfipl or Ndfip2 were omitted from the assay. These assays were carried out for 30 seconds.

Detailed Description of the Invention

In accordance with the present invention, we have now determined how auto-inhibition Itch is achieved. First, we determined that auto-inhibition does not affect Itch binding to an E2 ubiquitin conjugating enzyme but instead prevents the E2 from transferring ubiquitin to the catalytic cysteine residue in the Itch HECT domain, a process known as ubiquitin charging. As shown by our model in Figure 1C, we have determined that Itch is autoinhibited due to an intramolecular interaction between its HECT and WW domains. It is worth noting that the motifs we have identified that mediate autoinhibition are conserved among other Nedd4-family members, suggesting that these discoveries can be extended to other Nedd4-family E3 ubiquitin ligases. Importantly, we have determined that Nedd4-family interacting protein 1 (Ndfipl) relieves Itch auto-inhibition (see Figure 1C) by binding to the WW domains and releasing the HECT domain to allow ubiquitin charging. By doing this, Ndfipl activates Itch to limit pro-inflammatory cytokine production in T cells and macrophages.

These discoveries provide guidance for the generation and testing of peptides that mimic Ndfipl . We demonstrate that these peptides activate Itch ubiquitin ligase activity in a cell free assay. Specifically, we have generated peptides that span one, two or all three of the motifs in Ndfipl that bind WW domains of Itch. Addition of these peptides to our cell free assay resulted in the ubiquitin charging of Itch and thus Itch activation (modeled in Figure ID). Thus, we now have novel peptides that may be used to activate Itch and possibly other Nedd4-family E3 ubiquitin ligases for therapeutic purposes.

I. Definitions:

The phrase "immune related disorder" refers to disorders characterized by aberrant immune cell function mediated by T and B cells.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it originates. For example, the "isolated nucleic acid" may comprise a DNA or cDNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the DNA of a prokaryote or eukaryote.

With respect to RNA molecules of the invention, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "promoter region" refers to the transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns.

The term "vector" refers to a small carrier DNA molecule into which a DNA sequence can be inserted for introduction into a host cell where it will be replicated. An "expression vector" is a specialized vector that contains a gene or nucleic acid sequence with the necessary regulatory regions needed for expression in a host cell.

The term "operably linked" means that the regulatory sequences necessary for expression of a coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in an expression vector. This definition is also sometimes applied to the arrangement of nucleic acid sequences of a first and a second nucleic acid molecule wherein a hybrid nucleic acid molecule is generated.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, of the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

The phrase "consisting essentially of when referring to a particular nucleotide sequence or amino acid sequence means a sequence having the properties of a given SEQ ID NO:. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application for which the oligonucleotide is used.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including

temperature, source of probe and method of use. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non-complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to act functionally as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product.

The primer may vary in length depending on the particular conditions and requirements of the application. For example, in diagnostic applications, the oligonucleotide primer is

typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able to anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

The term "percent identical" is used herein with reference to comparisons among nucleic acid or amino acid sequences. Nucleic acid and amino acid sequences are often compared using computer programs that align sequences of nucleic or amino acids thus defining the differences between the two. For purposes of this invention comparisons of nucleic acid sequences are performed using the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin. For convenience, the default parameters (gap creation penalty = 12, gap extension penalty = 4) specified by that program are intended for use herein to compare sequence identity. Alternately, the Blastn 2.0 program provided by the National Center for Biotechnology Information(on the world wide web at ncbi.nlm.nih.gov/blast/; Altschul et al., 1990, J Mol Biol 215:403-410) using a gapped alignment with default parameters, may be used to determine the level of identity and similarity between nucleic acid sequences and amino acid sequences.

II. Preparation of Polypeptides which modulate Immune Cell Function

Nucleic acid molecules encoding the peptides of the invention may be prepared by using recombinant DNA technology methods. The availability of nucleotide sequence information enables preparation of isolated nucleic acid molecules of the invention by a variety of means. For example, nucleic acid sequences encoding an Itch modulating peptide may be isolated from appropriate biological sources using standard protocols well known in the art.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in a plasmid cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable E. coli host cell. Alternatively, the nucleic acids may be maintained in vector suitable for expression in mammalian cells. In cases where post-translational modification affects immune function, it is preferable to express the molecule in mammalian cells.

B. Proteins

Polypeptides of the present invention may be prepared in a variety of ways, according to known methods. The protein may be purified from appropriate sources, e.g., transformed bacterial or animal cultured cells or tissues which express peptide encoding nucleic acids, by immunoaffinity purification. The availability of nucleic acid molecules encoding the inventive polypeptides enables production of the same using in vitro expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate in vitro transcription vector, such as pSP64 or pSP65 for in vitro transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocyte lysates. In vitro transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

Alternatively, according to a preferred embodiment, larger quantities of peptide may be produced by expression in a suitable prokaryotic or eukaryotic expression system. For example, part or all of a DNA molecule encoding a peptide for example, may be inserted into a plasmid vector adapted for expression in a bacterial cell, such as E. coli or a mammalian cell such as CHO or Hela cells. Alternatively, in a preferred embodiment, tagged fusion proteins comprising the peptide can be generated. Such peptide-tagged fusion proteins are encoded by part or all of a DNA molecule, ligated in the correct codon reading frame to a nucleotide sequence encoding a portion or all of a desired polypeptide tag which is inserted into a plasmid vector adapted for expression in a bacterial cell, such as E. coli or a eukaryotic cell, such as, but not limited to, yeast and mammalian cells. Vectors such as those described above comprise the regulatory elements necessary for expression of the DNA in the host cell positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression

include, but are not limited to, promoter sequences, transcription initiation sequences, and enhancer sequences.

The peptides of the invention, produced by gene expression in a recombinant prokaryotic or eukaryotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein or nickel columns for isolation of recombinant proteins tagged with 6-8 histidine residues at their N-terminus or C-terminus. Alternative tags may comprise the FLAG epitope, GST or the hemagglutinin epitope. Such methods are commonly used by skilled practitioners.

As discussed above, a convenient way of producing a polypeptide according to the present invention is to express nucleic acid encoding it, by use of the nucleic acid in an expression system. A variety of expression systems of utility for the methods of the present invention are well known to those of skill in the art.

Accordingly, the present invention also encompasses a method of making a polypeptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide (generally nucleic acid). This may conveniently be achieved by culturing a host cell, containing such a vector, under appropriate conditions which cause or allow production of the polypeptide. Polypeptides may also be produced in in vitro systems, such as reticulocyte lysate.

III. Uses of Peptide encoding Nucleic Acids and Proteins

Peptide encoding nucleic acids can be used to advantage to produce large quantities of the peptides disclosed herein. Alternatively, they may be inserted into expression vectors suitable for expression of the peptides in human subjects. Peptides produced thereby should be effective to modulate immune cell function in humans and may be used according to this invention, for example, as therapeutic and/or prophylactic agents which modulate T cell or B cell function. The present inventors have discovered that peptides which are effective to increase or repress Nedd4-family E3 ubiquitin ligase activity.

A. Polypeptides

The polypeptides may be used for a variety of purposes in accordance with the present invention. In a preferred embodiment of the present invention, the polypeptides may be administered to a patient via infusion in a biologically compatible carrier. The peptides of the invention may optionally be encapsulated in to liposomes or other phospholipids to increase stability of the molecule. Such liposomes may include an antibody targeting moiety, directing the peptides to a target cell of interest, such a B cell or T cell.

The peptides of the invention may be operably linked to a cellular penetrating peptide. Such peptides are known in the art and include the tat peptide and the MSP peptide. Other CPPs useful in the practice of the invention are disclosed in US Patent 13/264,482 which is incorporated herein by reference.

The peptides may be administered alone or in combination with other agents known to modulate immune function such as immunosuppressive agents or steroids. An appropriate composition in which to deliver the peptides may be determined by a medical practitioner upon consideration of a variety of physiological variables, including, but not limited to, the patient's condition and hemodynamic state. A variety of compositions well suited for different applications and routes of administration are well known in the art and described hereinbelow.

The preparation containing the purified peptide contains a physiologically acceptable matrix and is preferably formulated as a pharmaceutical preparation. The preparation can be formulated using substantially known prior art methods, it can be mixed with a buffer containing salts, such as NaCl, $CaCl_2$, and amino acids, such as glycine and/or lysine, and in a pH range from 6 to 8. Until needed, the purified preparation containing the peptide can be stored in the form of a finished solution or in lyophilized or deep-frozen form. Preferably the preparation is stored in lyophilized form and is dissolved into a visually clear solution using an appropriate reconstitution solution.

Alternatively, the preparation according to the present invention can also be made available as a liquid preparation or as a liquid that is deep-frozen.

The preparation according to the present invention is especially stable, i.e., it can be allowed to stand in dissolved form for a prolonged time prior to application.

The preparation according to the present invention can be made available as a pharmaceutical preparation with immune modulating activity in the form of a one-component

preparation or in combination with other factors in the form of a multi-component preparation.

Prior to processing the purified protein into a pharmaceutical preparation, the purified peptide is subjected to the conventional quality controls and fashioned into a therapeutic form of presentation. In particular, during the recombinant manufacture, the purified preparation is tested for the absence of cellular nucleic acids as well as nucleic acids that are derived from the expression vector, preferably using a method, such as is described in EP 0 714 987.

Peptides useful in the practice of the invention, include for example,

1) PEQTAGP APPPY SSITAESAAYFDY

2) GDAPPPY SSITAESAAYFDYKDESGFPK PPSYNVA

3)GDAPPPY SSITAESAAYFDYKDE SGFPKPPSYNVATTLP SYDEAE

The underlining indicates the motif that is known to bind Nedd4-family E3 ligases. Linked amino acid residues might be irrelevant. While all three of these peptides are effective to relieve Itch auto-inhibition, the minimal motif is predicted to be PPPY or PPSY. Operably linking these two peptides via a flexible linker is predicted to be ideal. Removing lysine (K) residues from the sequence show in 1, 2 and 3 above, is predicted to stabilize the peptides in vivo. Accordingly, such variant peptides are also within the scope of the invention. Methods for modulating immune function include use of one, two or more of the peptide or peptide variants disclosed herein.

These peptides should decrease proinflammatory cytokine production in T cells and in macrophages, dendritic cells and other antigen presenting cells as well as in other innate inflammatory cells. Diseases that could be treated by such peptides include asthma, inflammatory bowel disease, food allergy, and atopic dermatitis. Psoriasis may also be improved by treatment with such peptides. While these peptides are based on sequence of Ndfipl, the peptide from Ndfip2 should also be effective and include the following sequences: PPPY and LPTY. In this case, two peptides (i.e. PPPY and LPTY or two PPPY motifs) connected by a flexible linker should exhibit superior activity in vivo. .

B. Pharmaceutical Compositions

Pharmaceutical preparations comprising the peptide in a suitable biological buffer may be administered to a patient via intravenous bolus infusion. The compositions may be administered alone or in combination with at least one other agent, such as a stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The compositions may be administered to a patient alone, or in combination with other agents which influence the inflammatory response.

In preferred embodiments, the pharmaceutical compositions also contain a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce an immune response harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol, sugars and ethanol. Pharmaceutically acceptable salts can also be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., 18th Edition, Easton, Pa. [1990]).

Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. The

pharmaceutical compositions of the present invention may be manufactured in any manner known in the art (e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes).

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding, free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they may be placed in an appropriate container and labeled for treatment. For administration of the peptides disclosed herein, such labeling would include amount, frequency, and method of administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended therapeutic purpose. Determining a therapeutically effective dose is well within the capability of a skilled medical practitioner using the techniques and guidance provided above. Therapeutic doses will depend on, among other factors, the age and general condition of the subject, the severity of the immune disorder, and the levels of peptide required. Thus, a therapeutically effective amount in humans will fall in a relatively broad range that may be determined by a medical practitioner based on the response of an individual patient to peptide treatment.

The following example is provided to illustrate certain embodiments of the invention. It is not intended to limit the invention in any way.

Example I

Modulation of Nedd4-family E3 ubiquitin ligases as a means to Regulate Immune Function

E3 Ubiquitin ligases are essential in the regulation of many biological processes and have emerged as an exciting new class of "drugable" targets for pharmaceutical intervention. We recently discovered peptides which activate a class of E3 ubiquitin ligases thereby limiting pro-inflammatory cytokine production and inflammation. These peptides can be delivered to cells in vitro and in vivo and their effects on a reduction of the inflammatory response assessed. These agents may have particular utility for reducing inflammation in the gastrointestinal (GI) tract.

The inventive peptides may also be useful for treating asthma, atopic dermatitis and other chronic inflammatory diseases.

We have determined how auto-inhibition of one Nedd4 family member is achieved and have identified the motifs which mediate auto-inhibition. Notably, these motifs are conserved among other family members. Accordingly, the peptides disclosed herein should have utility in modulating the function of most Nedd4-family E3 ubiquitin ligases. Additionally, we have identified a small family of adaptors, Ndfipl and Ndfip2, that relieve auto inhibition and have determined how these adaptors work. This discovery has revealed novel peptides that may be used to activate Nedd4-family E3 ubiquitin ligases for therapeutic purposes. Additionally, these discoveries have revealed ways that small molecules could be used to promote or inhibit the function of Nedd4-family E3 ubiquitin ligases. Our studies have also revealed that in the absence of this regulatory mechanism, i.e. when the two adaptors are not expressed and thus cannot relieve auto-inhibition of Nedd4-family E3 ligases, overt inflammation develops leading to conditions that resemble, lympho-proliferation, asthma, atopic dermatitis and inflammatory bowel disease. Thus, therapeutic regulation of Nedd4-familyE3 ubiquitin ligases could be used to treat patients with these conditions.

Ndfipl and Ndfip2 relieve auto-inhibition of the Nedd4-family E3 ubiquitin ligase Itch.

There are 9 members of the Nedd4-family of E3 ubiquitin ligases. These ligases are unusual in that there is a high degree of sequence similarity. All family members are composed of a HECT domain (the catalytic subunit), 2-4 WW domains, and a single C2 domain. Auto-inhibition of family members has been described by others, and different mechanisms of auto-inhibition have been proposed. For example, one family member, Itch, has been shown to be auto-inhibited due to an interaction between the HECT domain and a proline-rich region that sits between the C2 domain and the first WW domain (Gallagher, E et al. PMID 16446428). We have devised a new way to look at auto-inhibition, namely by using a ubiquitin charging assay which is the initial event in activating Nedd4-family E3 ubiquitin ligases. Ubiquitin charging occurs when an E2 ubiquitin conjugating enzyme transfers ubiquitin to the catalytic cysteine of the Nedd4-family E3 ligase. This event is absolutely required for catalytic activity of the E3 ligase. As shown in Figure 2A, when Itch is added to this assay, there is no evidence of a band

indicating ubiquitin charging of Itch, although bands indicating ubiquitin bound E1 and E2 are apparent. In contrast, when Ndfipl or Ndfip2 are added, ubiquitin charging of Itch is observed. These data show that Ndfipl and Ndfip2 promote the transfer of ubiquitin to the Itch catalytic cysteine, i.e., the initial event that results in Itch activation. We have determined that Ndfipl and Ndfip2 are not required for Itch to bind the E2, as association between E2 and Itch occur in the absence of Ndfipl and Ndfip2. E2-Itch binding occurs due to an interaction between the Ll/PPXY (PY) motifs of Ndfipl and the WW domains of Itch. Thus, as shown in Figure 2, when these PY motifs are missing, neither ubiquitin charging of Itch, nor Itch activation, occur.

Itch auto-inhibition is mediated by intramolecular interaction between HECT and WW domains.

Having determined that Ndfipl promotes Itch activation, we decided to focus on how Itch autoinhibition works. We generated a truncation of Itch that contains only the HECT and WW domains and tested this in our ubiquitin charging assay. As seen in Figure 3, Itch auto-inhibition occurs when Itch contains only these domains and this is still relieved by the addition of Ndfipl . Thus, we hypothesized that Itch inhibition is due to an intramolecular interaction between the HECT and WW domains.

To test this, we generated constructs that contain the WW domains (WW 1-4) and the HECT domain as separate cassettes. We then tested auto-inhibition of the HECT domain by adding the WW domains 'in trans'. Additionally, we truncated Itch so that it contained only 4, 2, or 0 WW domains. As seen in Figure 4C and D, as WW domains were removed, Itch auto-inhibition was relieved. Figure 4 panel C shown the constructs containing 4, 2, or 0 WW domains. These were used in the ubiquitin charging assay described. Thus, Itch auto-inhibition is mediated by and interaction between its WW domains and its HECT domain.

Auto-inhibition by this mechanism is not limited to Itch, but rather may be generalized to multiple Nedd4-family members.

WW domains bind PY motifs as well as other motifs that are not well defined. Knowing that auto-inhibition of Itch is mediated by WW domain and HECT domain interactions, knowing that Ndfipl binds to Itch via its PY motifs, and knowing that Ndfipl relieves Itch auto-inhibition of Itch, we hypothesized that the Itch HECT motif might contain PY motifs. We thus surveyed the amino acid sequence or Itch for such motifs and identified two, noted PY1 and PY2 in

Figure 5.

Importantly, not only does Itch contain two such motifs in its HECT domain, but 6 out of 9 Nedd4-family members contain such motifs. Thus we have revealed a means of mediating auto-inhibition of multiple Nedd4-family members. Supporting this, we tested another Nedd4-family member, the prototypic member known as Nedd4. We found that, like Itch, Nedd4 was autoinhibited in the ubiquitin charging assay. Furthermore, this auto-inhibition was relieved by the addition of Ndfipl or Ndfip2.

These data support that auto-inhibition of most Nedd4-family members is due to an interaction between the HECT and WW domains and that interaction with PY motif -containing adaptors is required for such auto-inhibition to be relieved. Based on these data, we propose that peptides that mimic Ndfipl or Ndfip2can be used to promote the activation of Nedd4 family E3 ligases.

In additional experiments, we introduced point mutations to destabilize the two WW domains and observed that Itch was significantly more active. This is most likely because Itch has lost its ability to form an autoinhibitory interaction between these domains and the HECT domain (Figure 6a). Additionally, this mutant was no longer able to bind Ndfipl (Figure 6b).

We then tested whether this was important in vivo by expressing mutant and wild type Itch in human T cells (Jurkat). We found that Itch was much more active when it contained point mutations in WW domains 2 and 3 (Figure 7). Increased Itch activity could be measured by increased degradation of JunB and reduced IL-2 production (two known measures of Itch function.

Mice lacking Ndfipl and Ndfip2 expression develop inflammation at a young age and die of inflammatory related consequences

Ndfipl and Ndfip2 activate Nedd4-family E3 ubiquitin ligases, including Itch, to limit inflammation. Mice lacking Ndfipl or both Ndfipl and Ndfip2 develop conditions reminiscent of food allergy, asthma, atopic dermatitis, and inflammatory bowel disease. Our data support that Ndfipl limits pro-inflammatory cytokines in both adaptive immune cells (T cells) as well as in innate immune cells. Thus peptides or small molecule approaches that relieve auto-inhibition of Itch or other Nedd4family members should have utility for treatment of food allergy, asthma, atopic dermatitis and inflammatory bowel disease.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

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What is claimed is:

1. A composition which relieves auto-inhibition of Itch or Nedd4-family members comprising similar motifs comprising one or more peptides or variants thereof, selected from the group consisting of:

1) PEQTAGP APPPY SSITAESAAYFDY
2) GDAPPPY SSITAESAAYFDYKDESGFPKPPSYNVA
3)GDAPPPY SSITAESAAYFDYKDESGFPKPPSYNVATTLPSYDEAE
in a pharmaceutically acceptable carrier, said peptide optionally being contained within a lipsome. .

2. A composition which relieves auto-inhibition of Itch or Nedd4-family members comprising similar motifs comprising one or more peptides or variants thereof, selected from the group consisting of: two or more PPPY or PPSY peptide motifs operably linked via a flexible linker in a in a pharmaceutically acceptable carrier, said peptide optionally being contained within a liposome.

3. A method for reducing inflammation in a patient in need thereof comprising administration of an effective amount of the composition of claim 1 or claim 2, said composition being effective to modulate T cell function in said patient.

4. The method of claim 3, wherein said composition is contained within an antibody-studded liposome, wherein said antibody is immunologically specific for a target cell of interest.

5. The method of claim 3, further comprising administration of one or more additional anti-inflammatory agents.

6. The method of claim 3, wherein said patient has a disorder selected from the group consisting of asthma, atopic dermatitis and inflammatory bowel disease.

7. The composition of claim 1 or claim 2, wherein said peptide is operably linked to a cellular penetrating peptide.

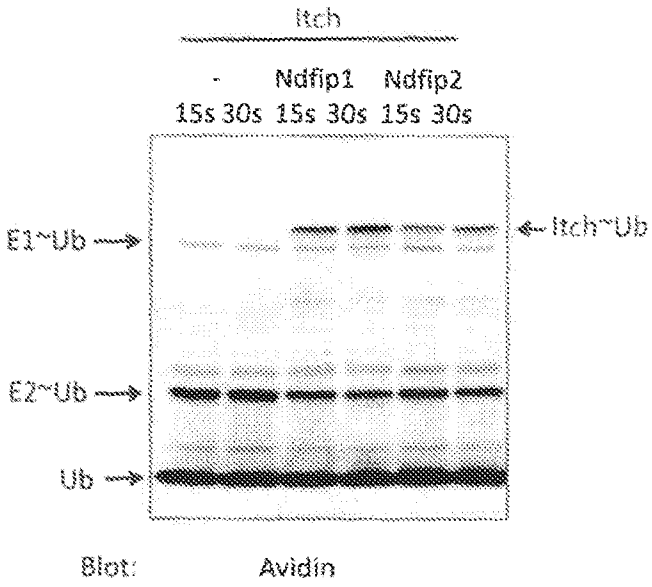


Figure 1A

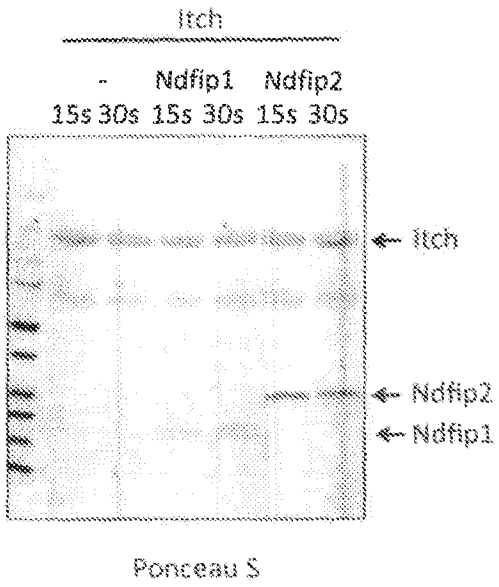


Figure 1B

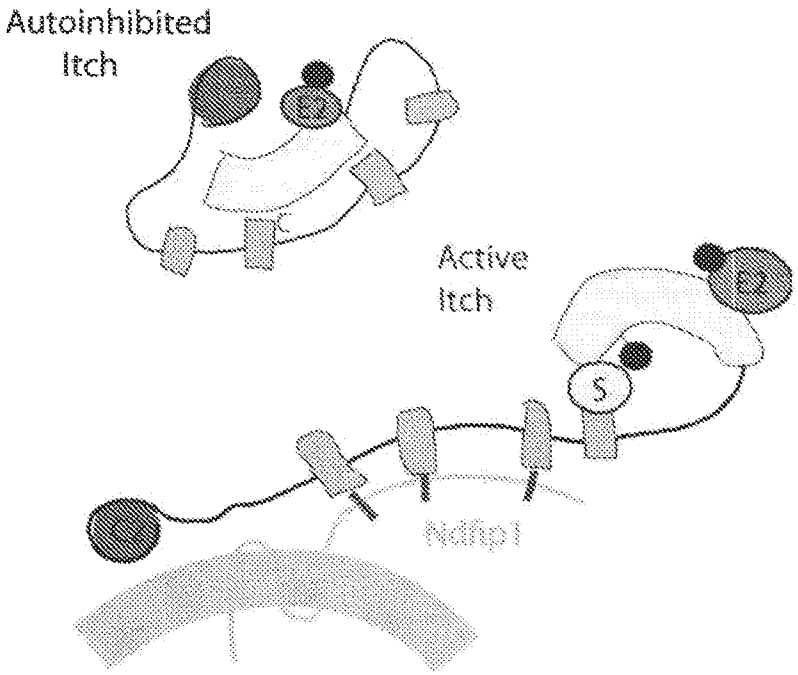


Figure 1C

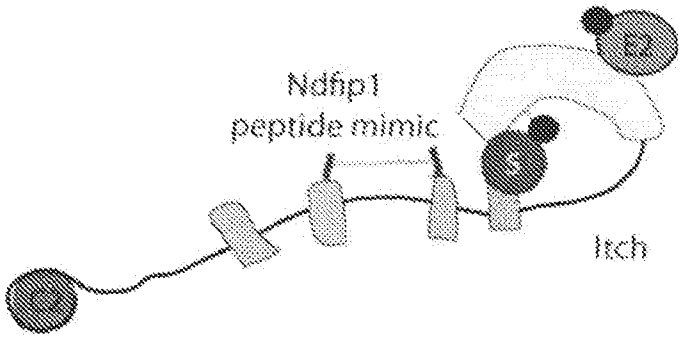


Figure 1D

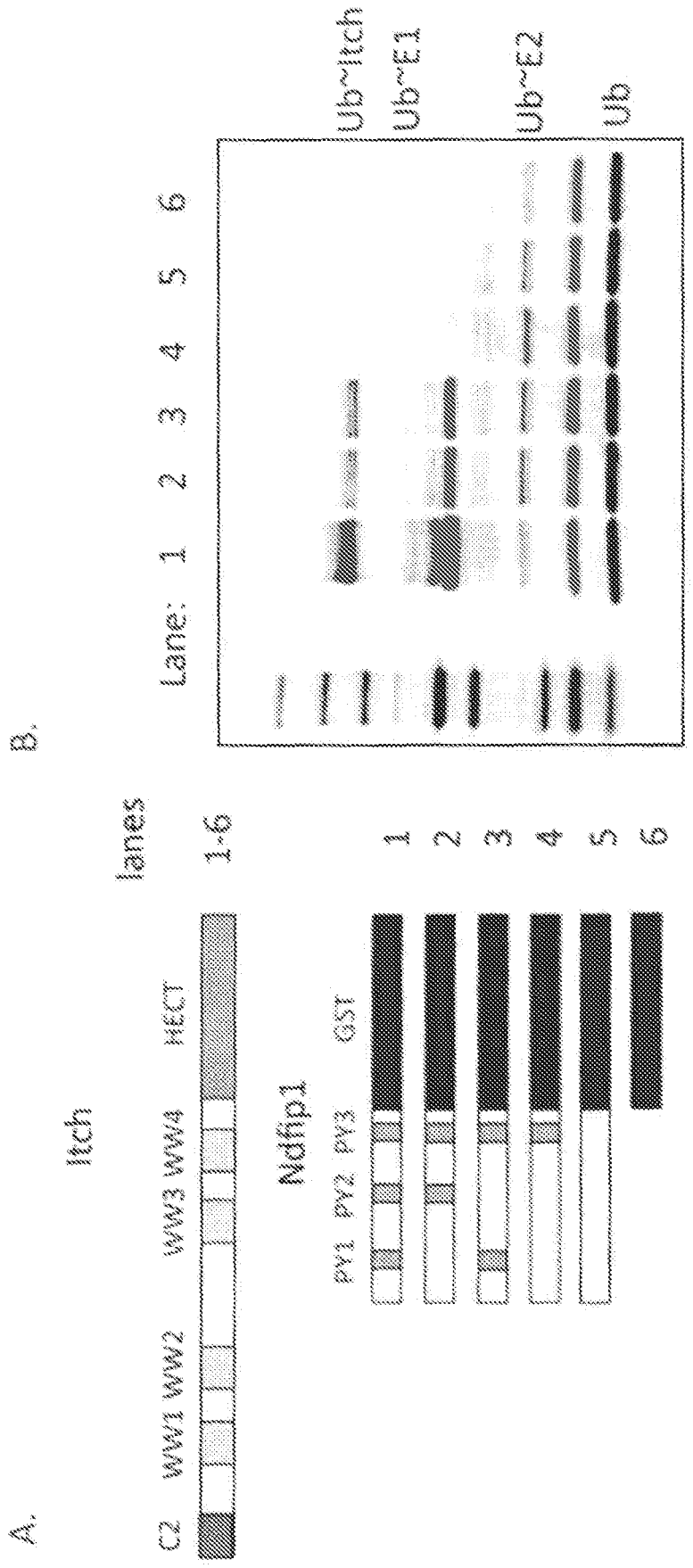

A.
Itch
C2 WW1 WW2 WW3 WW4 HECT
lanes 1-6
Ndfip1
PY1 PY2 PY3 GST
1 2 3 4 5 6
B.
Lane: 1 2 3 4 5 6
Ub~Itch
Ub~E1
Ub~E2
Ub

Figure 2

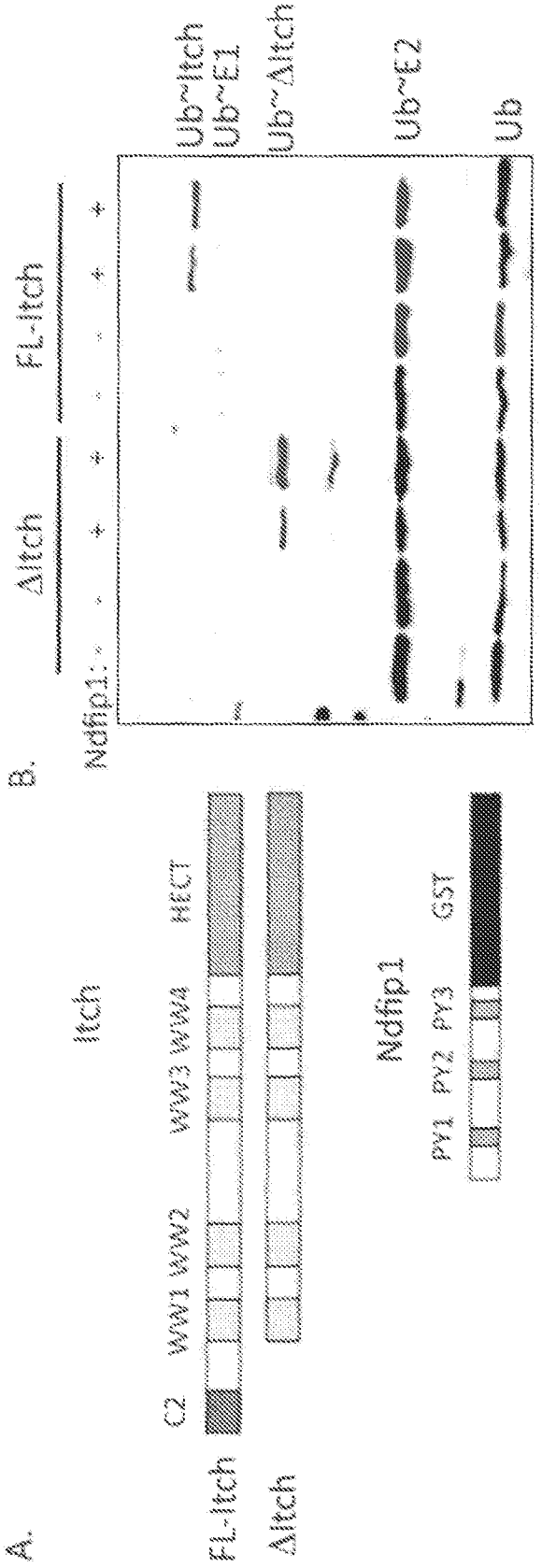

A.
Itch
C2
WW1
WW2
WW3
WW4
HECT
FL-Itch
ΔItch
Ndfip1
PY1
PY2
PY3
GST
B.
ΔItch
FL-Itch
Ndfip1:
Ub~Itch
Ub~E1
Ub~ΔItch
Ub~E2
Ub

Figure 3

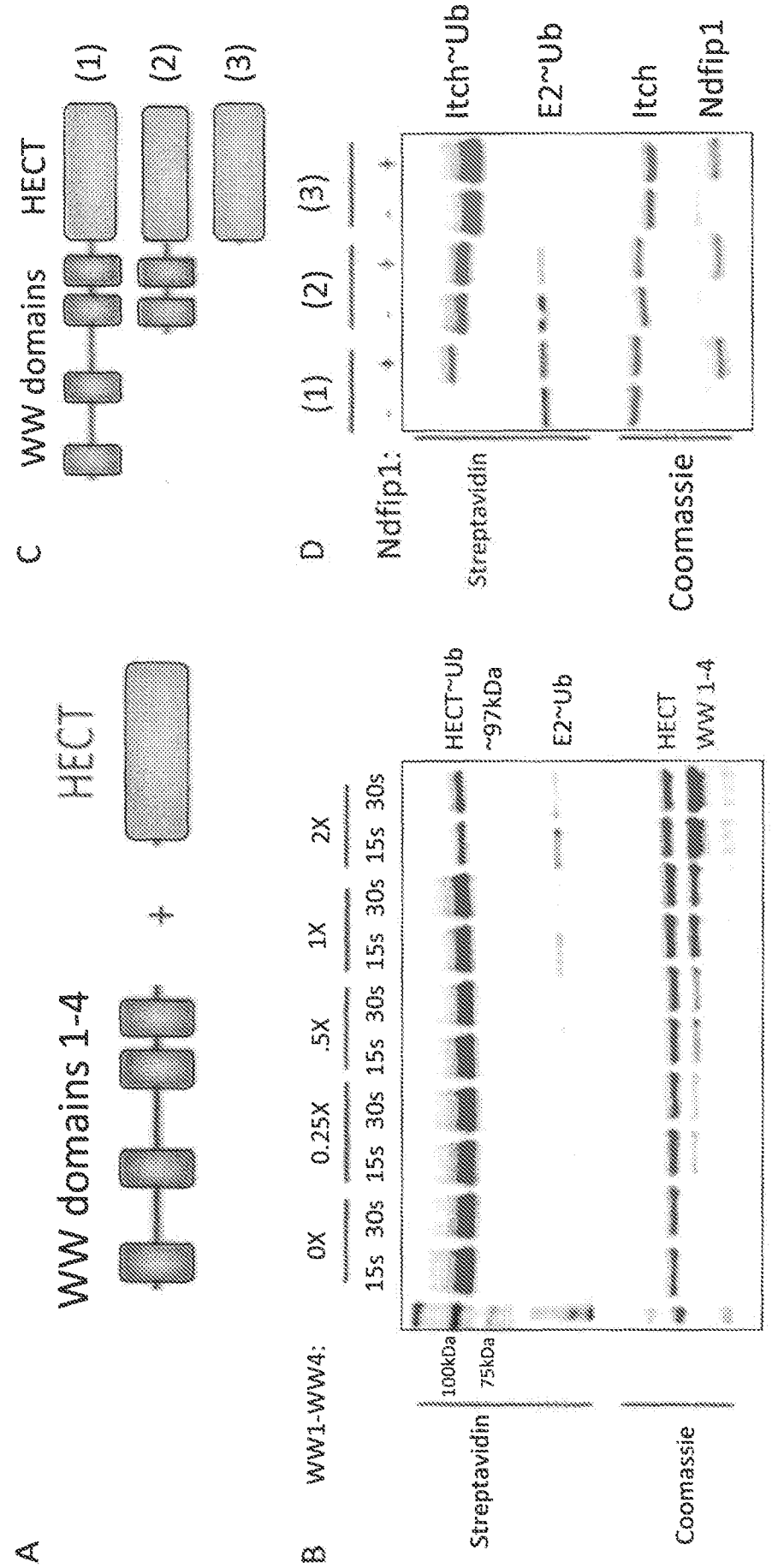

A
WW domains 1-4
HECT
B
WW1-WW4:
0X
0.25X
.5X
1X
2X
15s 30s 15s 30s 15s 30s 15s 30s 15s 30s
100kDa
75kDa
Streptavidin
Coomassie
HECT~Ub
~97kDa
E2~Ub
HECT
WW 1-4
C
WW domains
HECT
(1)
(2)
(3)
D
(1) (2) (3)
Ndfip1:
Streptavidin
Coomassie
Itch~Ub
E2~Ub
Itch
Ndfip1

Figure 4

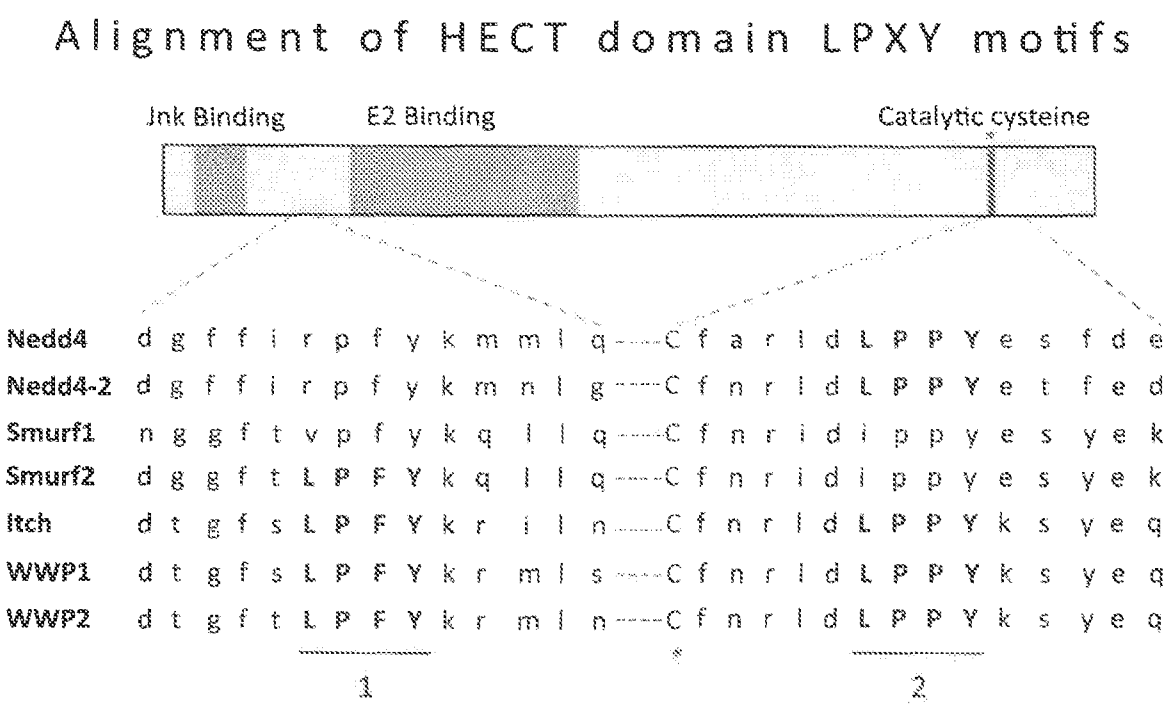


Figure 5

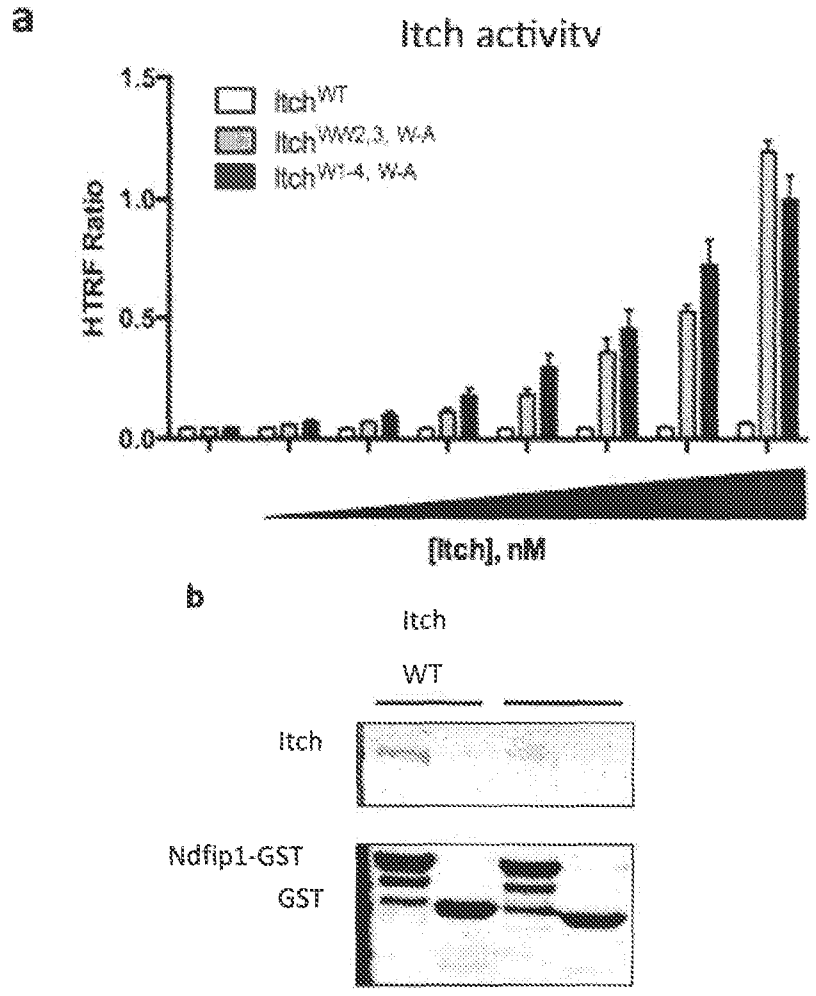


Figure 6

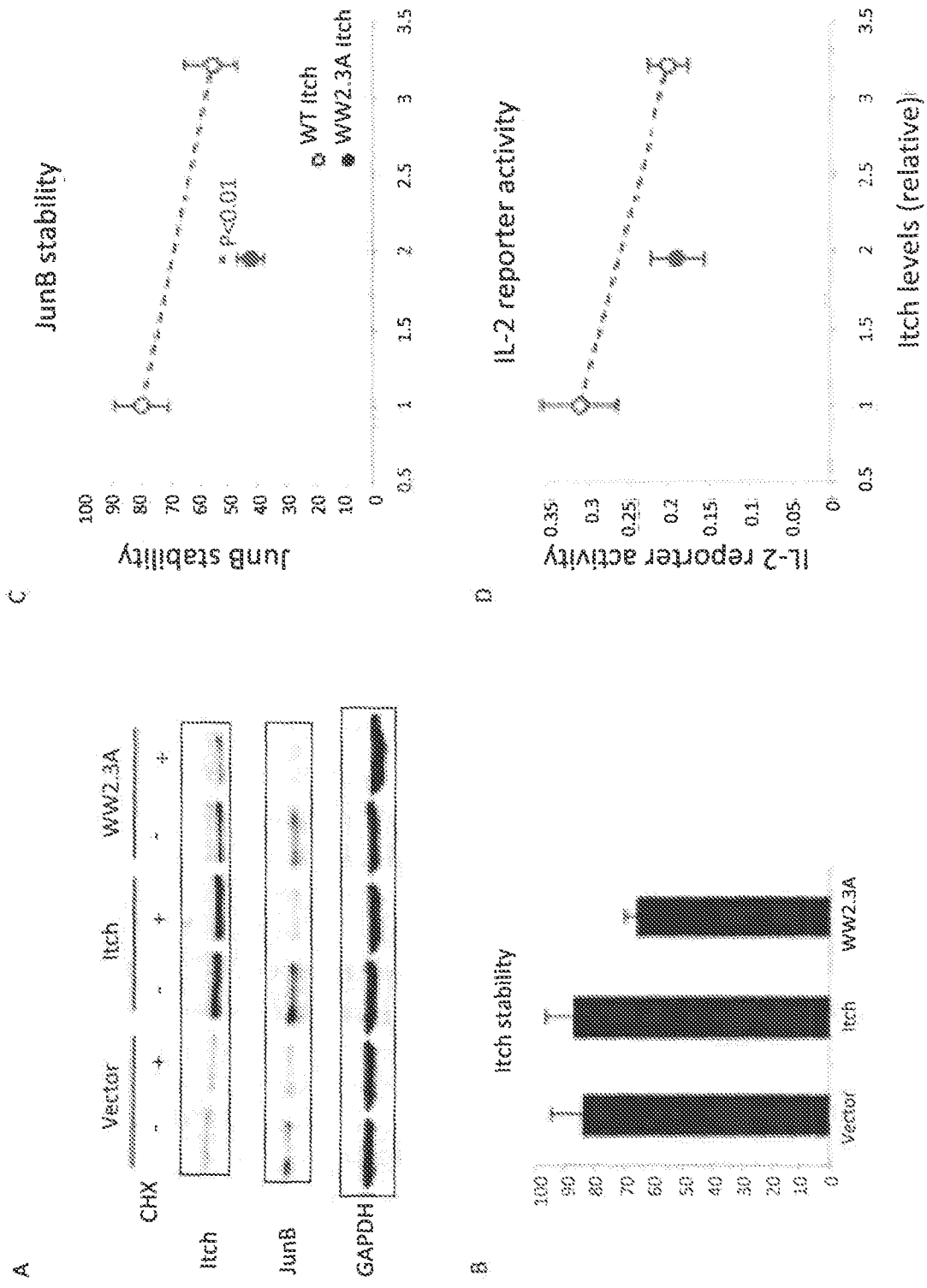

A
Vector
Itch
WW2.3A
CHX
- + - + - +
Itch
JunB
GAPDH
B
Itch stability
100 90 80 70 60 50 40 30 20 10 0
Vector
Itch
WW2.3A
C
JunB stability
JunB stability
100 90 80 70 60 50 40 30 20 10 0
0.5 1 1.5 2 2.5 3 3.5
P<0.01
WT Itch
WW2.3A Itch
D
IL-2 reporter activity
IL-2 reporter activity
0.35 0.3 0.25 0.2 0.15 0.1 0.05 0
0.5 1 1.5 2 2.5 3 3.5
Itch levels (relative)

Figure 7

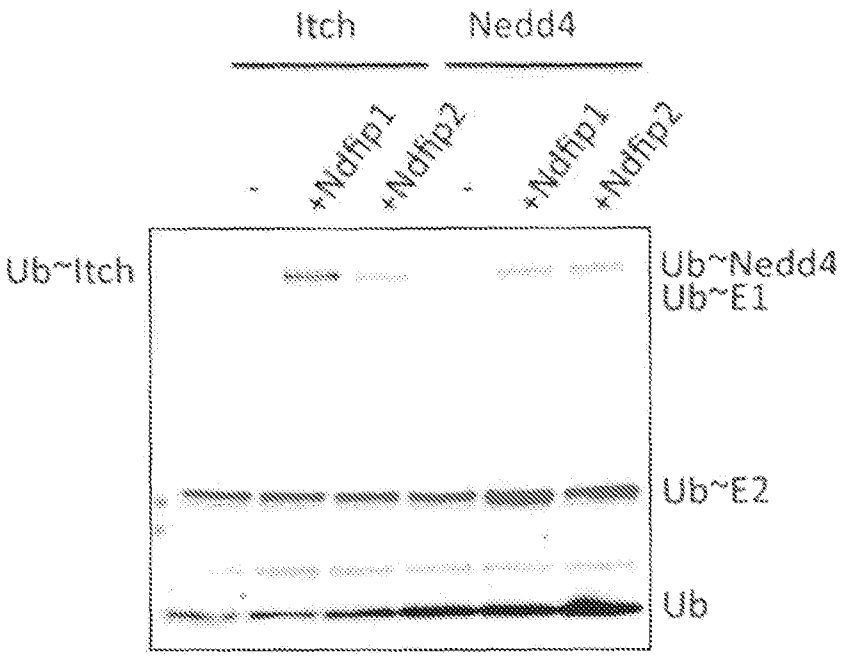

Itch
Nedd4
-
+Ndfip1
+Ndfip2
-
+Ndfip1
+Ndfip2
Ub~Itch
Ub~Nedd4
Ub~E1
Ub~E2
Ub

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/37126

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/395, 9/127; C07K 16/00 (2014.01)
CPC - A61 K 38/00, 9/127, 9/1272

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 39/395, 9/127; C07K 16/00 (2014.01)
CPC: A61K 38/00, 2039/505, 9/127, 9/1272, 9/1271; C07K 2317/24

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C.B, DE-A, DE-T, DE-U, GB-A, FR-A); TotalPatent (US. EP, WO, CN, JP, KR, DE, FR, GB, CA); PubMed/PubMed Central/NCBI Blast; ProQuest Dialog; Google; Google Scholar: itch, 'AIP4,' 'NEDD4,' reduce, relieve, release, 'auto-inhibition,' 'auto-ubiquination,' 'PPPY,' 'PPSY,' motif, 'peptide motif,' linker, connector, bridge, pharmaceutical, medicament

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOLLIFFE, CN et al. Identification Of Multiple Proteins Expressed In Murine Embryos As Binding Partners For The WW Domains Of The Ubiquitin-Protein Ligase Nedd4. Biochem J. 01 November 2000, Vol. 351, Pt. 3; pages 557-565; page 558, first column, first paragraph; page 560, Table 1; Genbank Supplement, pages 1-3; PMID: 11042109.	1-7
A	WANG, J et al. Calcium Activates Nedd4 E3 Ubiquitin Ligases By Releasing The C2 Domain-Mediated Auto-Inhibition. J Biol Chem. 16 April 2010, Vol. 285, No. 16; pages 12279-12288; abstract; page 12281, first paragraph; DOI: 10.1074/jbc.M1 09.086405.	1-7
A	OLIVER, PM et al. Ndfipl Protein Promotes The Function Of Itch Ubiquitin Ligase To Prevent T Cell Activation And T Helper 2 Cell-Mediated Inflammation. Immunity. December 2006, Vol. 25, No. 6; pages 929-940; page 9, third paragraph; Genbank Supplement, pages 1-3; DOI: 10.1016/j.immuni.2006.10.012.	1-7
A	US 2003/0104521 A1 (WHITTAKER, PA) June 5, 2003; paragraphs [0008], [0043], [0075]; Claims 1, 12	1-7
P, X	ALTIN, JA et al. Ndfipl Mediates Peripheral Tolerance To Self And Exogenous Antigen By Inducing Cell Cycle Exit In Responding CD4+ T Cells. Proc Natl Acad Sci USA. 11 February 2014, Vol. 111, No. 6; pages 2067-2074. DOI: 10.1073/pnas.132273911 1.	1-7
A	EASTERDAY, MC et al. Neural Progenitor Genes Germinal Zone Expression And Analysis Of Genetic Overlap In Stem Cell Populations. Dev Biol. 15 December 2003, Vol. 264, No. 2; pages 309-322. DOI: 10.1016/j.ydbio.2003.09.003.	1-7
A	GAY, DL et al. Cbl- And Nedd4-Family Ubiquitin Ligases: Balancing Tolerance And Immunity. Immunol Res. 2008, Vol. 42, Nos. 1-3; pages 51-64. DOI: 10.1007/s12026-008-8034-0^	1-7

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 Septemberf 2014 (23.09.2014)	17 OCT 2014

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273^3201	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774