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(54) **NOVEL UBIQUITIN LIGASE AND USE THEREOF**

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(57) **ABSTRACT**

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(2), (4) Date: **Feb. 27, 2012**

Provided is a novel ubiquitin ligase which has linear poly-ubiquitination activity and can be efficiently expressed and purified. It was found out that a complex of

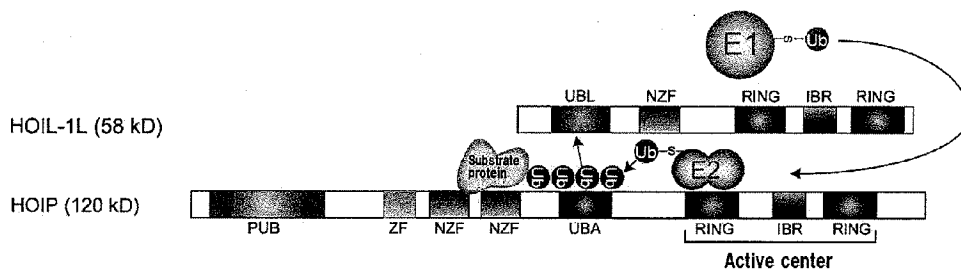
- (a) a protein having a part of HOIP and at least having a UBA region and a RING-IBR-RING region thereof, and
- (b) One or more kinds of proteins which individually form a complex with the above (a)

is a novel ubiquitin ligase which has linear polyubiquitination activity and can be efficiently expressed and purified.

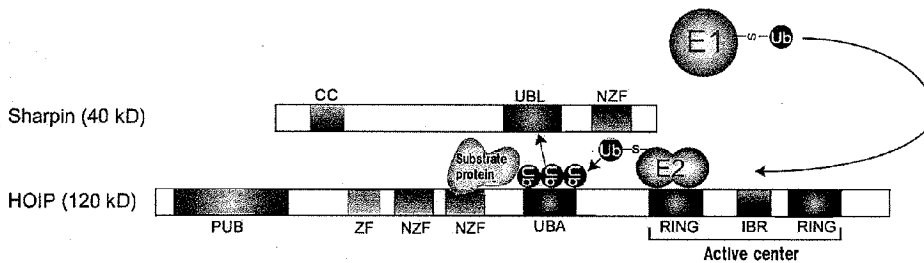
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Aug. 7, 2009 (JP) ..... 2009-184878

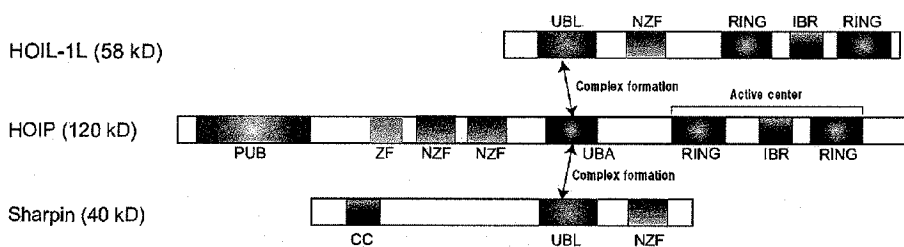
【Fig. 1(a)】



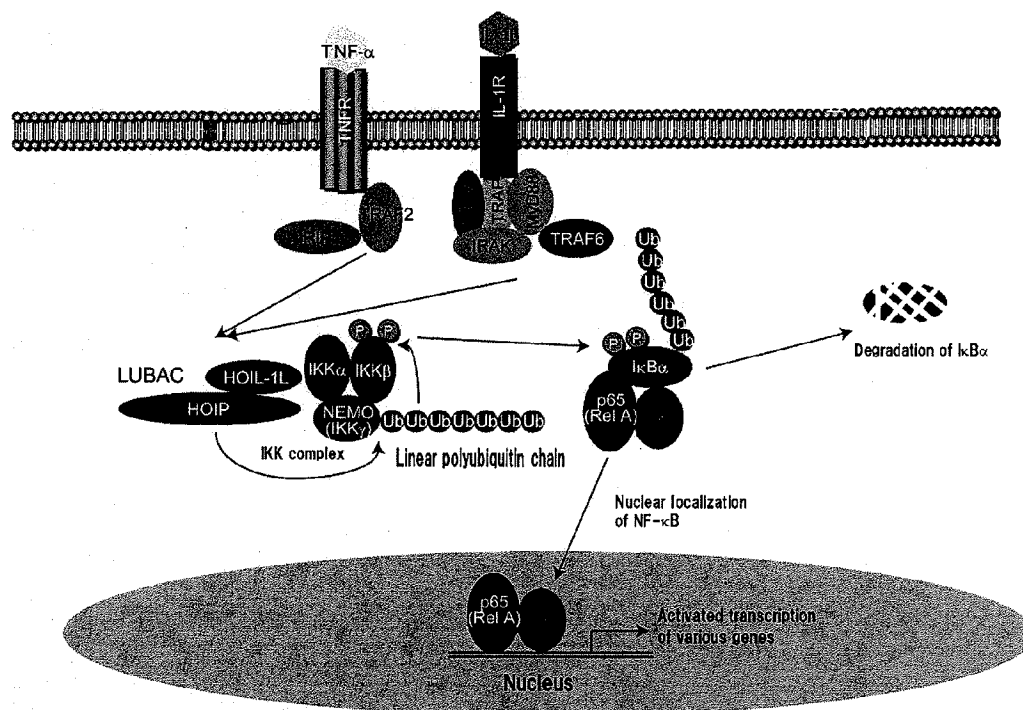
【Fig. 1(b)】



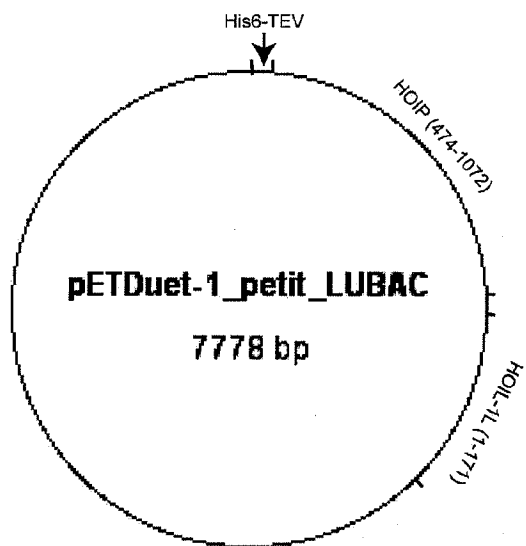
【Fig. 1(c)】



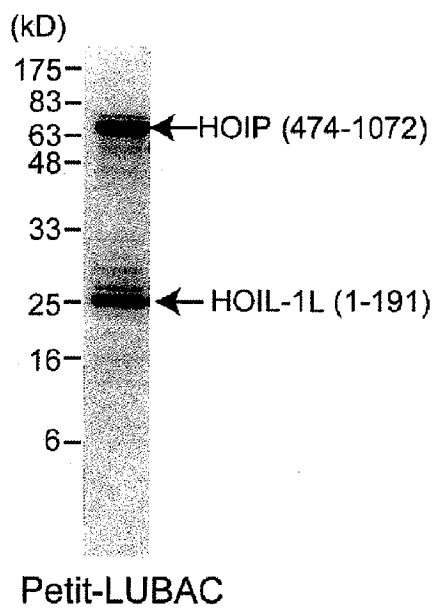
[Fig. 2]



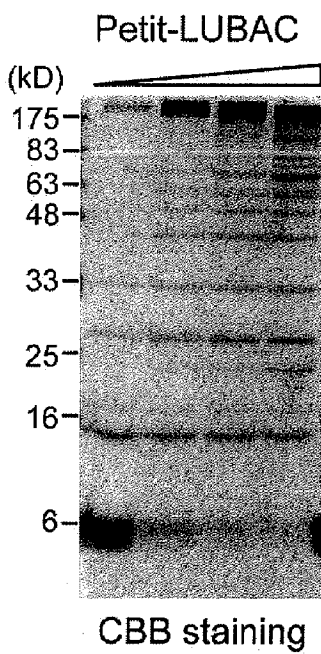
[Fig. 3]



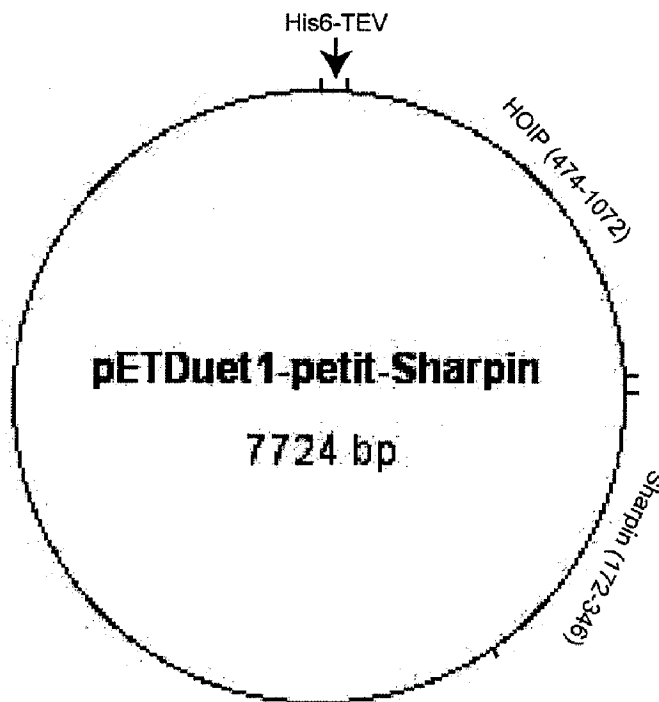
【Fig. 4】



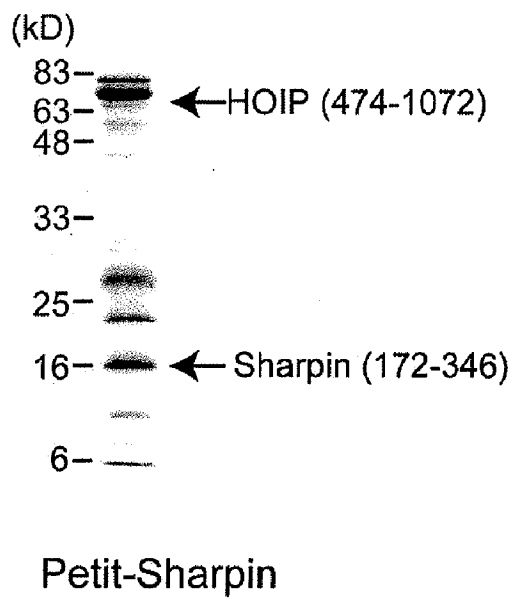
【Fig. 5】



【Fig. 6】

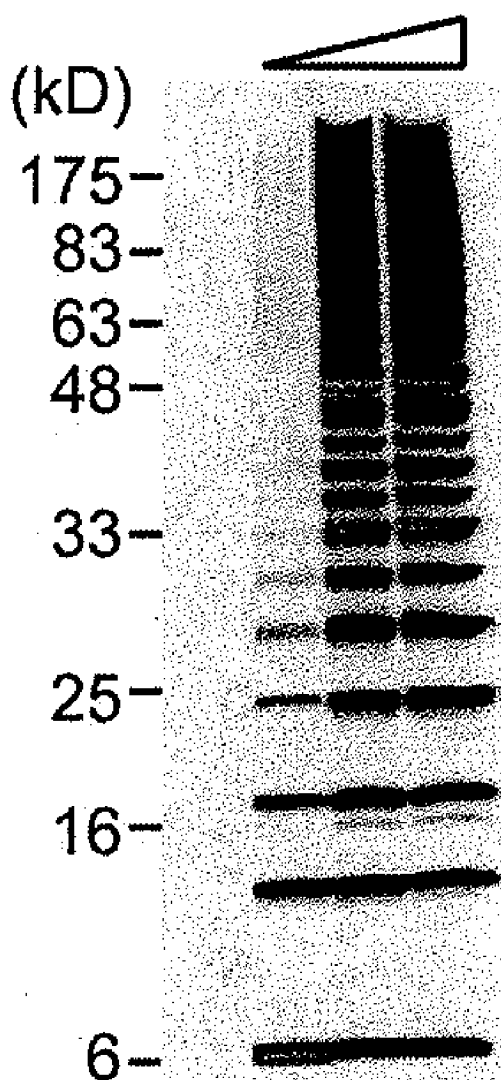


【Fig. 7】



【Fig. 8】

# Petit-Sharpin



Anti-Linear Ub

## NOVEL UBIQUITIN LIGASE AND USE THEREOF

### TECHNICAL FIELD

**[0001]** The present invention relates to a novel ubiquitin ligase and use thereof. In particular, the present invention relates to a novel ubiquitin ligase comprising a complex of plural proteins, an expression vector for a constituent protein of the ubiquitin ligase, a transformant with the expression vector, and a screening method for inhibitors of linear polyubiquitination, the method using the ubiquitin ligase.

### BACKGROUND ART

**[0002]** The ubiquitin conjugation system is a posttranslational modification system. By the function of three kinds of enzymes, a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3), the ubiquitin system conjugates polyubiquitin chains, polymer of ubiquitin, to substrate proteins selectively recognized by E3s and regulates their functions. Although it was originally considered that all polyubiquitinated proteins are led to degradation, the concept of polyubiquitination has been broadened and it is currently understood that polyubiquitination regulates protein functions in various manners. Various polyubiquitin chains with different linkage of ubiquitins are present in the living body, and it is being proved that the regulatory mechanism for polyubiquitinated proteins varies with the kind of the polyubiquitin chain. It has been conventionally considered that polyubiquitin chains are assembled by formation of isopeptide bonds via lysine residues of ubiquitins. In such circumstances, the present inventor is the first in the world to show assembly of a linear polyubiquitin chain via N-terminal methionine, and involvement of the linear polyubiquitination in NF- $\kappa$ B activation.

**[0003]** Specifically, the present inventor found out that a complex of HOIL-1L and HOIP is a ubiquitin ligase which mediates assembly of a linear polyubiquitin chain, and named the complex LUBAC (linear ubiquitin chain assemble complex) (see Non Patent Literature 1). Further, the present inventor clarified that the LUBAC ubiquitin ligase is involved in the classical pathway of NF- $\kappa$ B activation in that the LUBAC ubiquitin ligase mediates linear ubiquitination of NEMO (NF- $\kappa$ B essential modulator), a component of the IKK (I $\kappa$ B kinase) complex, which leads to IKK activation and selective activation of NF- $\kappa$ B (see Non Patent Literature 2).

**[0004]** Furthermore, the present inventor found out that Sharpin is also a constituent of the ubiquitin ligase for mediating assembly of a linear polyubiquitin chain. Accordingly, the present inventor decided to refer to, as LUBAC, a ubiquitin ligase complex composed of the three proteins, namely Sharpin, HOIL-1L and HOIP, or composed of two proteins, namely HOIL-1L and HOIP, or Sharpin and HOIP. Regarding Sharpin, it is reported that mice with spontaneous mutation of Sharpin, which are called cpdm mice, present with immune system disorders and the like including chronic dermatitis and absence of Peyer's patches (see Non Patent Literature 3).

### CITATION LIST

#### Non Patent Literature

- [0005]** Non Patent Literature 1:  
**[0006]** Kirisako, T. et al. A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J.*, 25. 4877-4887 (2006)

**[0007]** Non Patent Literature 2:

**[0008]** Tokunaga, F. et al. Involvement of linear polyubiquitination of NEMO in NF- $\kappa$ B activation. *Nature Cell Biol.*, 11. 123-132 (2009)

**[0009]** Non Patent Literature 3:

**[0010]** Seymour, R. E. et al. Spontaneous mutations in the mouse Sharpin gene result in multiorgan inflammation, immune system dysregulation and dermatitis. *Genes Immun.*, 8. 416-421 (2007)

### SUMMARY OF INVENTION

#### Technical Problem

**[0011]** The present inventor has been attempting to establish a LUBAC expression system that enables efficient expression of a recombinant LUBAC, but has not yet succeeded. Accordingly, an object of the present invention is to provide a novel ubiquitin ligase which has linear polyubiquitination activity and can be efficiently expressed and purified, an expression vector for a constituent protein of the ubiquitin ligase, a transformant with the expression vector, and a screening method for inhibitors of linear polyubiquitination, the method using the ubiquitin ligase.

#### Solution to Problem

**[0012]** The present invention includes the following as a solution to the above-mentioned problems.

[1] A ubiquitin ligase comprising a complex of the following (a) and (b).

(a) A protein having a part of HOIP and at least having a UBA region and a RING-IBR-RING region thereof

(b) One or more kinds of proteins which individually form a complex with the above (a)

[2] The ubiquitin ligase according to the above [1], wherein the above (b) is a protein having a region capable of binding to the UBA region of HOIP.

[3] The ubiquitin ligase according to the above [2], wherein the above (b) is the following (1) and/or (2).

(1) HOIL-1L, or a protein having a part of HOIL-1L and at least having a UBL region thereof

(2) Sharpin, or a protein having a part of Sharpin and at least having a UBL region thereof

[4] The ubiquitin ligase according to the above [3], wherein the above (1) is a protein having the amino acid sequence represented by SEQ ID NO: 5, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 5 except for having deletion, substitution or addition of one to several amino acids, and

wherein the above (2) is a protein having the amino acid sequence represented by SEQ ID NO: 12, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 12 except for having deletion, substitution or addition of one to several amino acids.

[5] The ubiquitin ligase according to the above [1], wherein the above (a) is a protein having the amino acid sequence represented by SEQ ID NO: 7, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 7 except for having deletion, substitution or addition of one to several amino acids.

[6] An expression vector containing a polynucleotide encoding the following (A) and/or (B).

(A) A protein which has a part of HOIP and at least has a UBA region and a RING-IBR-RING region thereof and which forms, with the following (B), a complex exhibiting ubiquitin ligase activity

(B) One or more kinds of proteins which individually form, with the above (A), a complex exhibiting ubiquitin ligase activity

[7] The expression vector according to the above [6], wherein the above (B) is a protein which has a region capable of binding to the UBA region of HOIP and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

[8] The expression vector according to the above [7], wherein the above (B) is the following (I) and/or (II).

(I) HOIL-1L, or a protein which has a part of HOIL-1L and at least has a UBL region thereof and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity

(II) Sharpin, or a protein which has a part of Sharpin and at least has a UBL region thereof and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity

[9] The expression vector according to the above [8], wherein the above (I) is a protein having the amino acid sequence represented by SEQ ID NO: 5, or a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 5 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity, and

wherein the above (II) is a protein having the amino acid sequence represented by SEQ ID NO: 12, or a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 12 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

[10] The expression vector according to the above [6], wherein the above (A) is a protein having the amino acid sequence represented by SEQ ID NO: 7, or a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 7 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (B), a complex exhibiting ubiquitin ligase activity.

[11] The expression vector according to the above [9], wherein the above (I) is encoded by a polynucleotide having the base sequence represented by SEQ ID NO: 6, or a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence represented by SEQ ID NO: 6 under stringent conditions and which encodes a protein which forms, with the above (A), a complex exhibiting ubiquitin ligase activity, and

wherein the above (II) is encoded by a polynucleotide having the base sequence represented by SEQ ID NO: 13, or a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence represented by SEQ ID NO: 13 under stringent conditions and which encodes a protein which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

[12] The expression vector according to the above [10], wherein the above (A) is encoded by a polynucleotide having the base sequence represented by SEQ ID NO: 8, or a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence repre-

sented by SEQ ID NO: 8 under stringent conditions and which encodes a protein which forms, with the above (B), a complex exhibiting ubiquitin ligase activity.

[13] A transformant with the expression vector according to any of the above [6] to [12].

[14] A screening method for inhibitors of linear polyubiquitination, the method comprising the steps of: bringing a test substance into contact with the ubiquitin ligase according to any of the above [1] to [5],

measuring the activity level of the ubiquitin ligase, and comparing the above activity level to the activity level of the ubiquitin ligase not brought into contact with the test substance.

#### Advantageous Effects of Invention

**[0013]** According to the present invention, a novel ubiquitin ligase can be provided. In addition, an expression vector for a constituent protein of the ubiquitin ligase and a transformant with the expression vector can also be provided. Use of the expression vector and the transformant of the present invention enables efficient expression and high-yield purification of the ubiquitin ligase of the present invention. By use of the ubiquitin ligase of the present invention, a screening method for inhibitors of linear polyubiquitination can also be provided. The screening method enables selection of substances that selectively inhibit NF- $\kappa$ B activation, and therefore the selected inhibitors can be active ingredient candidates for preventive or therapeutic medicaments for various NF- $\kappa$ B-associated diseases.

#### BRIEF DESCRIPTION OF DRAWINGS

**[0014]** FIG. 1(a) is an explanatory diagram showing the structure of LUBAC composed of HOIL-1L and HOIP and the mechanism of LUBAC-mediated linear ubiquitination.

**[0015]** FIG. 1(b) is an explanatory diagram showing the structure of LUBAC composed of Sharpin and HOIP and the mechanism of LUBAC-mediated linear ubiquitination.

**[0016]** FIG. 1(c) shows the structure of LUBAC composed of three components, namely HOIL-1L, Sharpin and HOIP.

**[0017]** FIG. 2 shows a schematic view of the LUBAC-mediated NF- $\kappa$ B activation mechanism.

**[0018]** FIG. 3 shows the construct of pETDuet-1 petit-LUBAC.

**[0019]** FIG. 4 shows the SDS-PAGE results of petit-LUBAC expressed in *E. coli*.

**[0020]** FIG. 5 shows results confirming the linear polyubiquitination activity of petit-LUBAC.

**[0021]** FIG. 6 shows the construct of pETDuet-1 petit-Sharpin.

**[0022]** FIG. 7 shows the SDS-PAGE results of petit-Sharpin expressed in *E. coli*.

**[0023]** FIG. 8 shows results confirming the linear polyubiquitination activity of petit-Sharpin.

#### DESCRIPTION OF EMBODIMENTS

[LUBAC]

**[0024]** LUBAC (linear ubiquitin chain assemble complex) is a ubiquitin ligase, which the present inventor discovered. As shown in FIGS. 1(a), (b) and (c), LUBAC is a ubiquitin ligase complex composed of three proteins, namely HOIL-1L, HOIP and Sharpin, or composed of two proteins, namely HOIL-1L and HOIP, or Sharpin and HOIP. LUBAC has the activity of assembling a linear polyubiquitin chain.



**[0025]** HOIL-1L is a splicing isoform of HOIL-1 (heme-oxidized IRP2 ligase-1), which has a longer N-terminal sequence compared to HOIL-1, and was discovered as predominant intracellular HOIL-1. As shown in FIG. 1(a), HOIL-1L is a 58-kD protein having a UBL domain (UBL), a Npl4 zinc finger domain (NZF) and a RING-IBR-RING domain in this order from the N-terminus. HOIL-1L is a protein having the amino acid sequence represented by SEQ ID NO: 1, and the base sequence of the HOIL-1L-encoding gene is represented by SEQ ID NO: 2. HOIL-1L is also called RBCK1 or RNF54. The base sequence of the HOIL-1L-encoding gene (RBCK1, transcript variant 2) is registered with DDBJ/GenBank/EMBL as accession number: NM\_031229.

**[0026]** HOIP (HOIL-1L-interacting protein) was identified as a HOIL-1L-associating protein. As shown in FIG. 1(a), HOIP is a 120-kD protein having a PUB domain (PUB) capable of binding to p97/VCP in the N-terminal region, followed by three zinc finger domains (ZF, NZF and NZF), a UBA domain (UBA) and a RING-IBR-RING domain. HOIP is a protein having the amino acid sequence represented by SEQ ID NO: 3, and the base sequence of the HOIP-encoding gene is represented by SEQ ID NO: 4. HOIP is also called RNF31. The base sequence of the HOIP-encoding gene is registered with DDBJ/GenBank/EMBL as accession number: AB265810.

**[0027]** HOIL-1L and HOIP are considered to form a complex and exist as an oligomer in cells. The present inventor found out that a HOIL-1L-HOIP complex has such a ubiquitin ligase activity as to mediate assembly of a linear polyubiquitin chain via N-terminal methionine, not assembly of a polyubiquitin chain by formation of isopeptide bonds via lysine residues of ubiquitins as conventionally known (Non Patent Literature 1).

**[0028]** Furthermore, the present inventor found out that Sharpin (SHANK-associated RH domain-interacting protein) is also a constituent of the ubiquitin ligase for mediating assembly of a linear polyubiquitin chain. In other words, a HOIL-1L-Sharpin-HOIP complex (see FIG. 1(c)) and a Sharpin-HOIP complex (see FIG. 1(b)) each have such a ubiquitin ligase activity as to mediate assembly of a linear polyubiquitin chain via N-terminal methionine, not assembly of a polyubiquitin chain by formation of isopeptide bonds via lysine residues of ubiquitins as conventionally known. As shown in FIGS. 1(b) and (c), Sharpin is a 40-kD protein having a coiled-coil domain (CC), a UBL domain (UBL), a Npl4 zinc finger domain (NZF) and a RING-IBR-RING domain in this order from the N-terminus. Sharpin is a protein having the amino acid sequence represented by SEQ ID NO: 10, and the base sequence of the Sharpin-encoding gene is represented by SEQ ID NO: 11. The base sequence of the Sharpin-encoding gene is registered with DDBJ/GenBank/EMBL as accession number: FJ655995.

**[0029]** As shown in FIG. 1(c), HOIL-1L, Sharpin and HOIP can form a tertiary-protein complex. The present inventor also found out that, in the living body, the tertiary-protein complex is stably present while a complex of HOIL-1L and HOIP and a complex of Sharpin and HOIP are unstable.

[LUBAC-Mediated Selective NF- $\kappa$ B Activation Mechanism]

**[0030]** NF- $\kappa$ B is a transcription factor that can be activated by various stimuli. It is known that NF- $\kappa$ B is associated with cell growth, inflammation, immune response, etc., and that its activity is increased in various cancers including multiple

myeloma. Therefore, selective inhibition of NF- $\kappa$ B activation is thought to be an excellent target for medicaments for rheumatoid/allergic diseases and cancers.

**[0031]** The schematic view of the NF- $\kappa$ B activation mechanism mediated by LUBAC composed of HOIL-1L and HOIP is shown in FIG. 2. The same mechanism is true in the NF- $\kappa$ B activation mediated by LUBAC composed of Sharpin and HOIP, or by LUBAC composed of HOIL-1L, Sharpin and HOIP. As shown in FIG. 2, NF- $\kappa$ B is a hetero-dimeric transcription factor and is present in the cytoplasm in a bound form with an inhibitory protein, I $\kappa$ B $\alpha$  in the absence of stimulation. Activation of the IKK complex by various stimuli results in phosphorylation of I $\kappa$ B $\alpha$  and subsequent degradation thereof. NF- $\kappa$ B released from I $\kappa$ B $\alpha$  enters into the nucleus and activates transcription of various genes. Thus, for understanding of signal-dependent NF- $\kappa$ B activation, the elucidation of stimulation-dependent IKK complex activation mechanism is indispensable, and many studies have been done for this purpose. In the current situation, the conventional dogma is collapsing and consensus has not been reached yet.

**[0032]** The present inventor clarified that the LUBAC ubiquitin ligase selectively binds, in a stimulation (e.g. TNF- $\alpha$ )-dependent manner, to NEMO serving as an IKK complex subunit for activity regulation, and then mediates linear polyubiquitination of NEMO, leading to IKK complex activation, followed by NF- $\kappa$ B activation (Non Patent Literature 2). From the research results so far, it seems that the LUBAC-mediated linear polyubiquitination of NEMO selectively activates NF- $\kappa$ B.

[Novel Ubiquitin Ligase]

**[0033]** The ubiquitin ligase of the present invention at least comprises a complex of the following (a) and (b).

(a) A protein having a part of HOIP and at least having a UBA region and a RING-IBR-RING region thereof

(b) One or more kinds of proteins which individually form a complex with the above (a)

**[0034]** The above (b) is not particularly limited as long as it forms, with the above (a), a protein complex exhibiting ubiquitin ligase activity. The above (b) may be two or more kinds of proteins, and therefore, the ubiquitin ligase of the present invention may be a complex of three or more kinds of proteins including the above (a). For example, preferred as the above (b) is a protein having a region capable of binding to the UBA region of HOIP. More preferred is, for example,

(1) HOIL-1L, or a protein having a part of HOIL-1L and at least having a UBL region thereof, or

(2) Sharpin, or a protein having a part of Sharpin and at least having a UBL region thereof.

**[0035]** The ubiquitin ligase of the present invention more preferably comprises a complex of (a) and (1), a complex of (a) and (2), or a complex of (a), (1) and (2).

**[0036]** The full-length HOIL-1L is a protein having the amino acid sequence represented by SEQ ID NO: 1 as described above, and the UBL region corresponds to a region of residues 70 to 130 of SEQ ID NO: 1. Therefore, in the case where the above (1) is HOIL-1L, a protein having the amino acid sequence represented by SEQ ID NO: 1 can be used. In the case where the above (1) is a protein having a part of HOIL-1L and at least having a UBL region thereof, namely a partial HOIL-1L protein having a UBL region of HOIL-1L, a protein having not the full length of the amino acid sequence represented by SEQ ID NO: 1, but at least a region of residues

70 to 130 thereof can be used. Preferred is a partial HOIL-1L protein having no Npl4 zinc finger domain (NZF), and more preferred is a partial HOIL-1L protein having neither NZF nor a RING-IBR-RING domain (see FIG. 1(a)).

**[0037]** As used herein, HOIL-1L is not limited to a protein having the amino acid sequence represented by SEQ ID NO: 1, and may be a mutant of HOIL-1L as long as the mutant forms, with HOIP, a complex exhibiting ubiquitin ligase activity. Such a mutant HOIL-1L or a partial protein thereof having a region corresponding to the UBL region of HOIL-1L is suitable as the protein of the above (b) (1) according to the present invention. The mutant HOIL-1L is, for example, a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 1 except for having deletion, substitution or addition of one to several amino acids and which forms, with HOIP, a complex exhibiting ubiquitin ligase activity. In addition, a protein having a region equivalent to the UBL region of HOIL-1L, or a partial protein thereof having such a UBL-equivalent region is suitable as the protein of the above (b).

**[0038]** Still more preferred as the protein of the above (b) (1) is a protein having the amino acid sequence represented by SEQ ID NO: 5. The amino acid sequence represented by SEQ ID NO: 5 corresponds to a region of residues 1 to 191 of SEQ ID NO: 1. Needless to say, a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 5 except for having deletion, substitution or addition of one to several amino acids is also suitable as long as the protein forms, with the protein of the above (a), a complex exhibiting ubiquitin ligase activity.

**[0039]** The full-length Sharpin is a protein having the amino acid sequence represented by SEQ ID NO: 10 as described above, and the UBL region corresponds to a region of residues 240 to 300 of SEQ ID NO: 10. Therefore, in the case where the above (2) is Sharpin, a protein having the amino acid sequence represented by SEQ ID NO: 10 can be used. In the case where the above (2) is a protein having a part of Sharpin and at least having a UBL region thereof, namely a partial Sharpin protein having a UBL region of Sharpin, a protein having a part of the amino acid sequence represented by SEQ ID NO: 10 and at least having a region of residues 240 to 300 thereof can be used. Preferred is a partial Sharpin protein having no Npl4 zinc finger domain (NZF), and more preferred is a partial Sharpin protein having neither NZF nor a coiled-coil domain (CC) (see FIG. 1(b)).

**[0040]** As used herein, Sharpin is not limited to a protein having the amino acid sequence represented by SEQ ID NO: 10, and may be a mutant of Sharpin as long as the mutant forms, with HOIP, a complex exhibiting ubiquitin ligase activity. Such a mutant Sharpin or a partial protein thereof having a region corresponding to the UBL region of Sharpin is suitable as the protein of the above (b) (2) according to the present invention. The mutant Sharpin is, for example, a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 10 except for having deletion, substitution or addition of one to several amino acids and which forms, with HOIP, a complex exhibiting ubiquitin ligase activity. In addition, a protein having a region equivalent to the UBL region of Sharpin, or a partial protein thereof having such a UBL-equivalent region is suitable as the protein of the above (b).

**[0041]** Still more preferred as the protein of the above (b) (2) is a protein having the amino acid sequence represented by SEQ ID NO: 12. The amino acid sequence represented by

SEQ ID NO: 12 corresponds to a region of residues 172 to 346 of SEQ ID NO: 10. Needless to say, a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 12 except for having deletion, substitution or addition of one to several amino acids is also suitable as long as the protein forms, with the protein of the above (a), a complex exhibiting ubiquitin ligase activity.

**[0042]** The above (a) is not particularly limited as long as it is a protein having a part of HOIP and at least having a UBA region and a RING-IBR-RING region thereof. The full-length HOIP is a protein having the amino acid sequence represented by SEQ ID NO: 3 as described above, the UBA region corresponds to a region of residues 564 to 615 of SEQ ID NO: 3, the RING-IBR-RING region corresponds to a region of residues 699 to 901 of SEQ ID NO: 3, and the region from the N-terminus of the UBA region to the C-terminus of the RING-IBR-RING region corresponds to a region of residues 564 to 901 of SEQ ID NO: 3. Therefore, as the above (a), a protein having a part of the amino acid sequence represented by SEQ ID NO: 3 and at least having a region of residues 564 to 901 thereof can be used. Preferred is a partial HOIP protein having none of three zinc finger domains (ZF, NZF and NZF), and more preferred is a partial HOIP protein not having any of a PUB domain (PUB) and three zinc finger domains (ZF, NZF and NZF) (see FIGS. 1(a) and (b)).

**[0043]** As used herein, HOIP is not limited to a protein having the amino acid sequence represented by SEQ ID NO: 3, and may be a mutant of HOIP as long as the mutant forms, with HOIL-1L, a complex exhibiting ubiquitin ligase activity. A partial protein of such a mutant HOIP, as long as the partial protein has regions corresponding to the UBA region and the RING-IBR-RING region of HOIP, is suitable as the protein of the above (a) according to the present invention. The mutant HOIP is, for example, a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 3 except for having deletion, substitution or addition of one to several amino acids and which forms, with HOIL-1L, a complex exhibiting ubiquitin ligase activity.

**[0044]** Still more preferred as the protein of the above (a) is a protein having the amino acid sequence represented by SEQ ID NO: 7. The amino acid sequence represented by SEQ ID NO: 7 corresponds to a region of residues 474 to 1072 of SEQ ID NO: 3. Needless to say, a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 7 except for having deletion, substitution or addition of one to several amino acids is also suitable as the protein of the above (a), as long as the protein forms, with the protein of the above (b), a complex exhibiting ubiquitin ligase activity.

**[0045]** As used herein, "having deletion, substitution or addition of one to several amino acids" means having deletion, substitution or addition of an amino acid(s), the number of which is in the range allowed by a known preparation method for mutant peptides, such as site-directed mutagenesis (preferably 10 or less, more preferably 7 or less, and even more preferably 5 or less). Such a mutant protein is not limited to a protein artificially mutated by a known preparation method for mutant polypeptides, and may be a protein isolated and purified from nature. It is well known in the art that modification to some amino acids in the amino acid sequence of a protein can be easily made without any significant effect on the structure or function of the protein. Aside from such artificial modification, it is also well known that there are natural mutants having no significant changes in the structure or function in comparison to wild-type proteins.

**[0046]** A preferable mutant has a conservative or non-conservative amino acid substitution, deletion or addition. More preferred is a silent substitution, deletion or addition, particularly preferred is a conservative substitution, and none of them alters the activity of the protein. Typical examples of the conservative substitution include substitution between two of aliphatic amino acids Ala, Val, Leu and Ile, exchange between hydroxyl residues Ser and Thr, exchange between acidic residues Asp and Glu, substitution between amide residues Asn and Gln, exchange between basic residues Lys and Arg, and substitution between aromatic residues Phe and Tyr.

**[0047]** The ubiquitin ligase of the present invention may comprise an additional peptide. Examples of the additional peptide include epitope peptides for labeling, such as a poly-histidine tag (His-tag), Myc and FLAG.

**[0048]** The ubiquitin ligase of the present invention can be prepared, for example, by a known genetic engineering technique, specifically by separately constructing a recombinant expression vector having an expressible insert of a gene encoding the protein of the above (a), and a recombinant expression vector having an expressible insert of a gene encoding the protein of the above (b), co-transferring these vectors into a suitable host cell for expression of recombinant proteins, and purifying a formed complex from the host cell or a culture medium of the host cell. A recombinant expression vector which can coexpress plural proteins can be also used.

**[0049]** Alternatively, the ubiquitin ligase of the present invention can be prepared, for example, by in vitro coupled transcription-translation system. In this case, a DNA fragment encoding the protein of the above (a) and a DNA fragment encoding the protein of the above (b) can be used with a known in vitro coupled transcription-translation system (for example, a system using cell-free extract of *Escherichia coli*, wheat germ cells or rabbit reticulocytes).

**[0050]** Whether the thus-obtained protein exists in a complex form and has ubiquitin ligase activity can be confirmed by a known method. The complex formation can be confirmed, for example, by analyzing the obtained protein in SDS-PAGE. When the results show plural protein bands, a complex is formed. The ubiquitin ligase activity can be confirmed, for example, by mixing the obtained protein with a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), ATP and ubiquitin for reaction (for example, at 37° C. for about 5 minutes to about 1 hour). The presence or absence of the ubiquitin ligase activity depends on whether linear polyubiquitin chains are assembled or not after the reaction.

#### [Expression Vector]

**[0051]** The expression vector of the present invention at least contains a polynucleotide encoding (A) and/or (B).

(A) A protein which has a part of HOIP and at least has a UBA region and a RING-IBR-RING region thereof and which forms, with the following (B), a complex exhibiting ubiquitin ligase activity

(B) One or more kinds of proteins which individually form, with the above (A), a complex exhibiting ubiquitin ligase activity

**[0052]** As the above (B), the protein of the above (b), which constitutes the ubiquitin ligase of the present invention, can be used. For example, preferred is a protein which has a region capable of binding to the UBA region of HOIP and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity. More preferred is, for example,

(I) HOIL-1L, or a protein which has a part of HOIL-1L and at least has a UBL region thereof and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity, or (II) Sharpin, or a protein which has a part of Sharpin and at least has a UBL region thereof and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

**[0053]** In the above (I), the protein which has a part of HOIL-1L and at least has a UBL region thereof and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity, namely a partial HOIL-1L protein which has a UBL region of HOIL-1L and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity is specifically a protein having a part of the amino acid sequence represented by SEQ ID NO: 1 and at least having a region of residues 70 to 130 of the amino acid sequence. For example, a protein having the amino acid sequence represented by SEQ ID NO: 5 is suitable. Also suitable is a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 5 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

**[0054]** The polynucleotide encoding HOIL-1L is, for example, a polynucleotide having the base sequence represented by SEQ ID NO: 2. In this base sequence, the base sequence encoding the UBL region corresponds to a region of positions 208 to 390. Therefore, in the case where the above (I) is HOIL-1L, suitable as the HOIL-1L-encoding polynucleotide is, for example, a polynucleotide having the base sequence represented by SEQ ID NO: 2. In the case where the above (I) is a partial HOIL-1L protein having a UBL region of HOIL-1L, suitable as the polynucleotide encoding this partial protein is, for example, a polynucleotide having a part of the base sequence represented by SEQ ID NO: 2 and having a region of positions 208 to 390 of the base sequence.

**[0055]** The polynucleotide encoding a protein having the amino acid sequence represented by SEQ ID NO: 5 is, for example, a polynucleotide having the base sequence represented by SEQ ID NO: 6. Also suitable is a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence represented by SEQ ID NO: 6 under stringent conditions and which encodes a partial HOIL-1L protein which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

**[0056]** In the above (II), the protein which has a part of Sharpin and at least has a UBL region thereof and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity, namely a partial Sharpin protein which has a UBL region of Sharpin and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity is specifically a protein having a part of the amino acid sequence represented by SEQ ID NO: 10 and at least having a region of residues 240 to 300 of the amino acid sequence. For example, a protein having the amino acid sequence represented by SEQ ID NO: 12 is suitable. Also suitable is a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 12 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

**[0057]** The polynucleotide encoding Sharpin is, for example, a polynucleotide having the base sequence represented by SEQ ID NO: 11. In this base sequence, the base sequence encoding the UBL region corresponds to a region of

positions 718 to 900. Therefore, in the case where the above (II) is Sharpin, suitable as the Sharpin-encoding polynucleotide is, for example, a polynucleotide having the base sequence represented by SEQ ID NO: 11. In the case where the above (II) is a partial Sharpin protein having a UBL region of Sharpin, suitable as the polynucleotide encoding this partial protein is, for example, a polynucleotide having a part of the base sequence represented by SEQ ID NO: 11 and having a region of positions 718 to 900 of the base sequence.

**[0058]** The polynucleotide encoding a protein having the amino acid sequence represented by SEQ ID NO: 12 is, for example, a polynucleotide having the base sequence represented by SEQ ID NO: 13. Also suitable is a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence represented by SEQ ID NO: 13 under stringent conditions and which encodes a partial Sharpin protein which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

**[0059]** As the above (A), the protein of the above (a), which constitutes the ubiquitin ligase of the present invention, can be used. Preferred is, for example, a protein which has a part of the amino acid sequence represented by SEQ ID NO: 3 and at least has a region of residues 564 to 901 of the amino acid sequence and which forms, with the above (B), a complex exhibiting ubiquitin ligase activity. More preferred is, for example, a protein having the amino acid sequence represented by SEQ ID NO: 7. Also suitable is a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 7 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (B), a complex exhibiting ubiquitin ligase activity.

**[0060]** The polynucleotide encoding HOIP is, for example, a polynucleotide having the base sequence represented by SEQ ID NO: 4. In this base sequence, the base sequence encoding the region from the N-terminus of the UBA region to the C-terminus of the RING-IBR-RING region corresponds to a region of positions 1690 to 2703. Therefore, suitable as the polynucleotide encoding the above (A) is, for example, a polynucleotide having a part of the base sequence represented by SEQ ID NO: 4 and having a region of positions 1690 to 2703 of the base sequence.

**[0061]** The polynucleotide encoding a protein having the amino acid sequence represented by SEQ ID NO: 7 is, for example, a polynucleotide having the base sequence represented by SEQ ID NO: 8. Also suitable is a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence represented by SEQ ID NO: 8 under stringent conditions and which encodes a partial HOIP protein which forms, with the above (B), a complex exhibiting ubiquitin ligase activity.

**[0062]** As used herein, the term "polynucleotide" is interchangeable with the term "gene", "nucleic acid" or "nucleic acid molecule". The polynucleotide of the present invention can be present in the form of RNA (for example, mRNA) or DNA (for example, cDNA or genomic DNA). DNA may be a double strand or a single strand. A single-stranded DNA or RNA may be a coding strand (sense strand) or a non-coding strand (antisense strand). The polynucleotide of the present invention may be ligated to a polynucleotide encoding a labeling tag (a tag sequence or a marker sequence) at the 5'- or 3'-terminus.

**[0063]** Hybridization can be performed according to a well-known method as described in Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory (2001). Usually, as the temperature becomes higher and the salt concentration becomes lower, the conditions of hybridization become more stringent (this means that hybridization becomes harder to achieve), and accordingly, more homologous polynucleotides can be obtained. A suitable hybridization temperature varies with the base sequence and the length thereof, and for example, in the case where an 18-base DNA fragment encoding 6 amino acids is used as a probe, the temperature is preferably 50° C. or lower.

**[0064]** The procedure for "hybridizes under stringent conditions" means that a filter is incubated in a hybridization solution (50% formamide, 5×SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5×Denhardt's solution, 10% dextran sulfate and 20 µg/ml denatured sheared salmon sperm DNA) at 42° C. overnight and then washed in 0.1×SSC at about 65° C.

**[0065]** Examples of a method for obtaining a polynucleotide used for the expression vector of the present invention include a method using amplification technique such as PCR. For example, based on the 5'- and 3'-terminal sequences of the base sequence of SEQ ID NO: 6 (or their complementary sequences), respective primers are designed, and using these primers and using genomic DNA, cDNA or the like as a template, PCR or the like is conducted to amplify a DNA region flanked by both primers. In this way, a DNA fragment containing a polynucleotide having the base sequence represented by SEQ ID NO: 6 can be obtained in a large amount.

**[0066]** The expression vector of the present invention includes the following expression vectors.

(i) The expression vector containing a polynucleotide encoding the protein of the above (A)

(ii) The expression vector containing a polynucleotide encoding the protein of the above (B)

(iii) The vector containing both of a polynucleotide encoding the protein of the above (A) and a polynucleotide encoding the protein of the above (B)

**[0067]** In the case where the protein of (B) is two or more kinds of proteins, polynucleotides separately encoding three or more kinds of proteins including (A) may be appropriately combined and inserted into a vector for preparation of a coexpression vector containing two or more kinds of polynucleotides.

**[0068]** The expression vector of the present invention is preferably a plasmid vector carrying a recognition sequence for RNA polymerase. In the case where the expression vector contains two or more kinds of polynucleotides, preferred is a plasmid vector carrying two or more recognition sequences for RNA polymerase. Such a plasmid vector can be appropriately selected from known vectors, and is easily available as a commercial plasmid vector.

**[0069]** The preparation method for recombinant expression vectors is not particularly limited, and examples thereof include methods using a plasmid, a phage or a cosmid. The kind of the vector is not particularly limited, and a vector that can be expressed in host cells can be appropriately selected. That is, depending on the kind of the host cell, a promoter sequence is appropriately selected to ensure the expression of a constituent protein of the ubiquitin ligase of the present invention, and the selected promoter sequence and a polynucleotide encoding the constituent protein of the ubiquitin ligase are inserted into any of various plasmids etc. for preparation of the expression vector of the present invention.

**[0070]** The expression vector preferably contains at least one selection marker. Examples of such a marker include a dihydrofolate reductase gene and a neomycin resistance gene for eukaryotic cell culture; and a tetracycline resistance gene and an ampicillin resistance gene for culture of *E. coli* and other bacteria. By use of such a selection marker, it can be confirmed whether the polynucleotide of the present invention has been transferred into host cells and then expressed therein without fail. Also, the polypeptide of the present invention may be expressed as a fusion polypeptide. For example, by use of green fluorescent protein (GFP) derived from *Aequorea coerulea* as a marker, the polypeptide of the present invention may be expressed as a GFP fusion polypeptide.

**[0071]** The host cell described above is not particularly limited, and various known cells can be used preferably. Specific examples of the host cell include bacteria such as *Escherichia coli*, yeasts (budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe*), nematodes (*Caenorhabditis elegans*), *Xenopus laevis* oocytes and animal cells (for example, CHO cells, COS cells and Bowes melanoma cells). The method for transferring the expression vector described above into host cells, i.e., the transformation method, is not particularly limited, and known methods such as electroporation, the calcium phosphate method, the liposome method and the DEAE dextran method can be used preferably.

**[0072]** In the case where the ubiquitin ligase of the present invention is a complex of two kinds of proteins, an expression vector containing two kinds of polynucleotides separately encoding (A) and (B) (coexpression vector) is transferred into a host cell, or both of an expression vector containing a polynucleotide encoding (A) and an expression vector containing a polynucleotide encoding (B) are co-transferred into a host cell.

**[0073]** In the case where the ubiquitin ligase of the present invention is a complex of three or more kinds of proteins, expression vectors each containing a polynucleotide encoding a different constituent protein of the complex are co-transferred into a host cell, or one or more expression vectors each containing a polynucleotide encoding a single protein are appropriately combined with one or more coexpression vectors so that all the polynucleotides encoding different constituent proteins of the complex are transferred into a host cell.

**[0074]** After a host cell into which all the polynucleotides encoding different constituent proteins of the complex have been transferred (transformant) is cultured, cultivated or bred, the ubiquitin ligase of the present invention can be collected and purified from the cultures etc. according to conventional methods (for example, filtration, centrifugation, cell disruption, gel filtration chromatography, ion exchange chromatography, etc.).

#### [Transformant]

**[0075]** The present invention provides a transformant with the expression vector of the present invention. The transformant of the present invention has at least one of the above-mentioned expression vectors of the present invention transferred therein, and does not need to simultaneously have all the polynucleotides encoding different constituent proteins of the ubiquitin ligase of the present invention. As used herein, the term "transformant" encompasses a cell, a tissue and an organ as well as an individual organism. The organism to be transformed is not particularly limited, and examples thereof include various microorganisms, plants and animals mentioned as examples of the host cell in the above.

**[0076]** Among the transformants of the present invention, the transformant with all the polynucleotides encoding different constituent proteins of the ubiquitin ligase of the present invention is suitable for use in production of the ubiquitin ligase of the present invention. It is preferable that the transformant stably expresses the ubiquitin ligase of the present invention, but the transformant may transiently express the same.

**[0077]** Here, as described above, the present inventor has been attempting to establish a LUBAC expression system that enables efficient expression of a recombinant LUBAC, namely a LUBAC expression system that enables efficient expression of a full-length HOIL-1L and a full-length HOIP, or a full-length Sharpin and a full-length HOIP and subsequent complex formation thereof, but has not yet succeeded. Specifically, as described in "Preparation of recombinant proteins" of MATERIALS AND METHODS of Non Patent Literature 1, the present inventor used to obtain LUBAC expressed in bacteria by three-step purification using nickel affinity gel, HiTrapQ and gel filtration. That is, for preparation of a recombinant LUBAC in the expression system described in Non Patent Literature 1, purification requires as many as three steps. From this description, it can be understood that the conventional expression system cannot provide efficient expression of a recombinant LUBAC. On the other hand, by use of the expression vector and the transformant of the present invention, the ubiquitin ligase of the present invention can be easily obtained by single-step purification with a nickel affinity gel, as shown in the Examples described below.

**[0078]** Therefore, the novel ubiquitin ligase, an expression vector therefor and a transformant therefor, each of which is provided by the present invention, are the first to enable efficient expression and high-yield purification of a ubiquitin ligase having linear polyubiquitination activity, and thus are a highly useful invention.

#### [Screening Method]

**[0079]** The screening method of the present invention at least comprises the steps of:

bringing a test substance into contact with the ubiquitin ligase of the present invention,  
measuring the activity level of the ubiquitin ligase brought into contact with the test substance, and  
comparing the above activity level to the activity level of the ubiquitin ligase not brought into contact with the test substance.

**[0080]** The screening method of the present invention enables simple and efficient screening for inhibitors of linear polyubiquitination.

**[0081]** The ubiquitin ligase of the present invention can be brought into contact with a test substance, for example, by dissolving or suspending the test substance in a solution containing the ubiquitin ligase. The contact time and the contact temperature are not particularly limited and can be appropriately selected. Preferably, a control group in which the ubiquitin ligase is not brought into contact with the test substance is prepared for the screening method of the present invention.

**[0082]** The activity level of the ubiquitin ligase can be measured by mixing the ubiquitin ligase to be analyzed, a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), ATP and ubiquitin for reaction (for example, at 37° C. for about 5 minutes to about 1 hour), and quantifying assembled linear polyubiquitin chains. Commercially available E1, E2, ATP and ubiquitin may be also used.

**[0083]** By comparing the activity level of the ubiquitin ligase brought into contact with the test substance to that of the ubiquitin ligase not brought into contact with the test substance, it can be determined whether the test substance is an inhibitor of linear polyubiquitination. When the activity level of the ubiquitin ligase brought into contact with the test substance is lower than that of the ubiquitin ligase not brought into contact with the test substance, the test substance can be determined as an inhibitor of linear polyubiquitination. When the activity level is reduced to preferably 50% or less, and more preferably 25% or less, the test substance is determined as an inhibitor of linear polyubiquitination.

**[0084]** As described above, the present inventor clarified that, in the classical pathway of NF- $\kappa$ B activation, LUBAC ubiquitin ligase selectively binds to NEMO of the IKK complex, and then mediates linear polyubiquitination of NEMO, leading to IKK complex activation, followed by NF- $\kappa$ B activation (see Non Patent Literature 2), and also found out that the LUBAC-mediated linear polyubiquitination of NEMO selectively activates NF- $\kappa$ B. Since the ubiquitin ligase of the present invention is a complex of a partial HOIL-1L protein and a partial HOIP protein, a complex of a partial Sharpin protein and a partial HOIP protein, or a complex of a partial HOIL-1L protein, a partial Sharpin protein and a partial HOIP protein, each complex constituting LUBAC, the screening method of the present invention enables selection of substances that inhibit linear polyubiquitination of NEMO and thus selectively inhibit NF- $\kappa$ B activation. Therefore, the thus-selected inhibitors of linear polyubiquitination are extremely useful as an active ingredient candidate for preventive or therapeutic medicaments for various NF- $\kappa$ B-associated diseases.

**[0085]** Examples of the NF- $\kappa$ B-associated disease include rheumatoid arthritis, atopic dermatitis, inflammatory bowel disease (ulcerative colitis, Crohn's disease), bronchial asthma, malignant lymphoma and multiple myeloma.

## EXAMPLES

**[0086]** Hereinafter, the present invention will be illustrated in detail by examples, but is not limited thereto. In the following Examples, a protein having the amino acid sequence from residues 1 to 191 of HOIL-1L (SEQ ID NO: 7) is called "HOIL-1L (1-191)" and a protein having the amino acid sequence from residues 474 to 1072 of HOIP (SEQ ID NO: 5) is called "HOIP (474-1072)". Also, the ubiquitin ligase of the present invention is called "petit-LUBAC".

### Example 1

#### Construction of Petit-LUBAC Expression Vector

**[0087]** A petit-LUBAC expression vector, pETDuet-1 petit-LUBAC was constructed by inserting a DNA encoding HOIP (474-1072) (SEQ ID NO: 6) downstream of His-Tag of MCS1 of pETDuet-1 vector (a vector designed for coexpression: manufactured by Novagen) and inserting a DNA encoding HOIL-1L (1-191) (SEQ ID NO: 8) into MCS2 thereof (see FIG. 3). The pETDuet-1 petit-LUBAC can express two kinds of proteins, that is, a protein having the sequence of "MRGSHHHHHHSQDPNSENLYFQ" (SEQ ID NO: 9) fused to the upstream (N-terminus) of HOIP (474-1072), and HOIL-1L (1-191).

### Example 2

#### Expression and Purification of Petit-LUBAC

**[0088]** Expression and purification of petit-LUBAC were performed in the following procedures.

(1) BL21 (DE3) RIL was transformed with pETDuet-1 petit-LUBAC and seeded onto LB-ampicillin plates.

(2) One colony was cultured in 2 mL of an LB-ampicillin culture medium at 37° C. overnight.

(3) 1 mL of the culture medium of the above (2) was added to 50 mL of an LB-ampicillin culture medium and the mixture was cultured at 37° C. until the OD600 value reached 0.6.

(4) 50 mL of the whole culture medium of the above (3) was added to 1 L of an LB-ampicillin culture medium and the mixture was cultured at 28° C. until the OD600 value reached 0.6.

(5) IPTG was added at the final concentration of 0.4 mM and the culture was continued at 28° C. for additional 2 hours.

(6) The *E. coli* was collected and then suspended in 80 mL of a sonication medium (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-ME and protease inhibitor).

(7) The *E. coli* was lysed with a sonicator.

(8) The resulting *E. coli* lysate was centrifuged at 15,000 rpm at 4° C. for 20 minutes and the supernatant was collected (*E. coli* extract).

(9) To the *E. coli* extract, imidazole was added at the final concentration of 0.2 mM.

(10) After addition of 1 mL of Ni-NTA beads, the reaction was allowed to proceed at 4° C. for 1 hour.

(11) The beads were washed with wash solution 1 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-ME and 3 mM imidazole).

(12) The beads were washed with wash solution 2 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-ME and 20 mM imidazole).

(13) Elution was performed with an eluent (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-ME and 200 mM imidazole).

(14) The eluate was applied to a PD-10 column for buffer exchange with a buffer containing 20 mM Tris-HCl pH 7.5 and 1 mM DTT, and then preserved at -80° C.

**[0089]** The purified product was subjected to SDS-PAGE. The results are shown in FIG. 4. As is clear from FIG. 4, two bands, that is, the band corresponding to HOIP (474-1072) and the band corresponding to HOIL-1L (1-191) were detected. These results showed that HOIP (474-1072) and HOIL-1L (1-191) form a complex and exist as petit-LUBAC.

### Example 3

#### Confirmation of Linear Polyubiquitination Activity of Petit-LUBAC

**[0090]** The petit-LUBAC obtained in Example 2 was added at four concentration levels (including no addition) to a mixed solution of a ubiquitin activating enzyme (E1), UbCH5c (E2), ATP and ubiquitin, and then incubation was performed at 37° C. The reaction mixtures were subjected to electrophoresis for confirmation of polyubiquitin chain assembly.

**[0091]** The results are shown in FIG. 5. In FIG. 5, the lanes correspond to no petit-LUBAC, and the low, middle and high levels of petit-LUBAC in this order from the left. As is clear from FIG. 5, in the case of addition of petit-LUBAC, the ladder-like bands appear in a manner dependent on the petit-LUBAC concentration, and this means that polyubiquitin chains were assembled. These results showed that petit-LUBAC has linear polyubiquitination activity.

## Example 4

## Construction of Petit-Sharpin Expression Vector

**[0092]** A petit-Sharpin expression vector, pETDuet-1 petit-Sharpin was constructed by inserting a DNA encoding HOIP (474-1072) (SEQ ID NO: 6) downstream of His-Tag of MCS1 of pETDuet-1 vector (a vector designed for coexpression: manufactured by Novagen) and inserting a DNA encoding Sharpin (172-346) (SEQ ID NO: 13) into MCS2 thereof (see FIG. 6). The pETDuet-1 petit-Sharpin can express two kinds of proteins, that is, a protein having the sequence of "MRGSHHHHHHSQDPNSENLYFQ" (SEQ ID NO: 9) fused to the upstream (N-terminus) of HOIP (474-1072), and a protein having an additional methionine fused to the N-terminus of Sharpin (172-346).

## Example 5

## Expression and Purification of Petit-Sharpin

**[0093]** Expression and purification of petit-Sharpin were performed in the following procedures.

(1) BL21 (DE3) RIL was transformed with pETDuet-1 petit-Sharpin and seeded onto LB-ampicillin plates.

(2) One colony was cultured in 2 mL of an LE-ampicillin culture medium at 37° C. overnight.

(3) 1 mL of the culture medium of the above (2) was added to 50 mL of an LB-ampicillin culture medium and the mixture was cultured at 37° C. until the OD600 value reached 0.6.

(4) 50 mL of the whole culture medium of the above (3) was added to 1 L of an LB-ampicillin culture medium and the mixture was cultured at 28° C. until the OD600 value reached 0.6.

(5) IPTG was added at the final concentration of 0.4 mM and the culture was continued at 28° C. for additional 2 hours.

(6) The *E. coli* was collected and then suspended in 80 mL of a sonication medium (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-ME and protease inhibitor).

(7) The *E. coli* was lysed with a sonicator.

(8) The resulting *E. coli* lysate was centrifuged at 15,000 rpm at 4° C. for 20 minutes and the supernatant was collected (*E. coli* extract).

(9) To the *E. coli* extract, imidazole was added at the final concentration of 0.2 mM.

(10) After addition of 1 mL of Ni-NTA beads, the reaction was allowed to proceed at 4° C. for 1 hour.

(11) The beads were washed with wash solution 1 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-ME and 3 mM imidazole).

(12) The beads were washed with wash solution 2 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-ME and 20 mM imidazole).

(13) Elution was performed with an eluent (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM 2-ME and 200 mM imidazole).

(14) The eluate was applied to a PD-10 column for buffer exchange with a buffer containing 20 mM Tris-HCl pH 7.5 and 1 mM DTT, and then preserved at -80° C.

**[0094]** The purified product was subjected to SDS-PAGE. The results are shown in FIG. 7. As is clear from FIG. 7, two bands, that is, the band corresponding to HOIP (474-1072) and the band corresponding to Sharpin (172-346) were detected. These results showed that HOIP (474-1072) and Sharpin (172-346) form a complex and exist as petit-Sharpin.

## Example 6

## Confirmation of Linear Polyubiquitination Activity of Petit-Sharpin

**[0095]** The petit-Sharpin obtained in Example 5 was added at four concentration levels (including no addition) to a mixed solution of a ubiquitin activating enzyme (E1), UbCH5c (E2), ATP and ubiquitin, and then incubation was performed at 37° C. The reaction mixtures were subjected to electrophoresis for confirmation of polyubiquitin chain assembly.

**[0096]** The results are shown in FIG. 8. In FIG. 8, the lanes correspond to no petit-Sharpin, and the low, middle and high levels of petit-Sharpin in this order from the left. As is clear from FIG. 8, in the case of addition of petit-Sharpin, the ladder-like bands appear in a manner dependent on the petit-Sharpin concentration, and this means that polyubiquitin chains were assembled. These results showed that petit-Sharpin has linear polyubiquitination activity.

**[0097]** The present invention is not limited to the aforementioned embodiments and examples, and various modifications can be made within the scope of the appended claims. Other embodiments obtainable by suitably combining technical means disclosed in different embodiments of the present invention are also included in the technical scope of the present invention. All the academic publications and patent literature cited in the above description are incorporated herein by reference.

## INDUSTRIAL APPLICABILITY

**[0098]** The ubiquitin ligase of the present invention can be used for screening for active ingredient candidates for preventive or therapeutic medicaments for various NF- $\kappa$ B-associated diseases, and thus is extremely useful.

[Sequence Listing]

## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 13

<210> SEQ ID NO 1

<211> LENGTH: 510

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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1 5 10 15

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Arg Ala Val Ala Gly Gly Asp Glu Gln Val Ala Met Lys Cys Ala Ile  
 20 25 30  
 Trp Leu Ala Glu Gln Arg Val Pro Leu Ser Val Gln Leu Lys Pro Glu  
 35 40 45  
 Val Ser Pro Thr Gln Asp Ile Arg Leu Trp Val Ser Val Glu Asp Ala  
 50 55 60  
 Gln Met His Thr Val Thr Ile Trp Leu Thr Val Arg Pro Asp Met Thr  
 65 70 75 80  
 Val Ala Ser Leu Lys Asp Met Val Phe Leu Asp Tyr Gly Phe Pro Pro  
 85 90 95  
 Val Leu Gln Gln Trp Val Ile Gly Gln Arg Leu Ala Arg Asp Gln Glu  
 100 105 110  
 Thr Leu His Ser His Gly Val Arg Gln Asn Gly Asp Ser Ala Tyr Leu  
 115 120 125  
 Tyr Leu Leu Ser Ala Arg Asn Thr Ser Leu Asn Pro Gln Glu Leu Gln  
 130 135 140  
 Arg Glu Arg Gln Leu Arg Met Leu Glu Asp Leu Gly Phe Lys Asp Leu  
 145 150 155 160  
 Thr Leu Gln Pro Arg Gly Pro Leu Glu Pro Gly Pro Pro Lys Pro Gly  
 165 170 175  
 Val Pro Gln Glu Pro Gly Arg Gly Gln Pro Asp Ala Val Pro Glu Pro  
 180 185 190  
 Pro Pro Val Gly Trp Gln Cys Pro Gly Cys Thr Phe Ile Asn Lys Pro  
 195 200 205  
 Thr Arg Pro Gly Cys Glu Met Cys Cys Arg Ala Arg Pro Glu Ala Tyr  
 210 215 220  
 Gln Val Pro Ala Ser Tyr Gln Pro Asp Glu Glu Glu Arg Ala Arg Leu  
 225 230 235 240  
 Ala Gly Glu Glu Glu Ala Leu Arg Gln Tyr Gln Gln Arg Lys Gln Gln  
 245 250 255  
 Gln Gln Glu Gly Asn Tyr Leu Gln His Val Gln Leu Asp Gln Arg Ser  
 260 265 270  
 Leu Val Leu Asn Thr Glu Pro Ala Glu Cys Pro Val Cys Tyr Ser Val  
 275 280 285  
 Leu Ala Pro Gly Glu Ala Val Val Leu Arg Glu Cys Leu His Thr Phe  
 290 295 300  
 Cys Arg Glu Cys Leu Gln Gly Thr Ile Arg Asn Ser Gln Glu Ala Glu  
 305 310 315 320  
 Val Ser Cys Pro Phe Ile Asp Asn Thr Tyr Ser Cys Ser Gly Lys Leu  
 325 330 335  
 Leu Glu Arg Glu Ile Lys Ala Leu Leu Thr Pro Glu Asp Tyr Gln Arg  
 340 345 350  
 Phe Leu Asp Leu Gly Ile Ser Ile Ala Glu Asn Arg Ser Ala Phe Ser  
 355 360 365  
 Tyr His Cys Lys Thr Pro Asp Cys Lys Gly Trp Cys Phe Phe Glu Asp  
 370 375 380  
 Asp Val Asn Glu Phe Thr Cys Pro Val Cys Phe His Val Asn Cys Leu  
 385 390 395 400  
 Leu Cys Lys Ala Ile His Glu Gln Met Asn Cys Lys Glu Tyr Gln Glu  
 405 410 415



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Asp Leu Ala Leu Arg Ala Gln Asn Asp Val Ala Ala Arg Gln Thr Thr  
                   420                                  425                                  430  
 Glu Met Leu Lys Val Met Leu Gln Gln Gly Glu Ala Met Arg Cys Pro  
                   435                                  440                                  445  
 Gln Cys Gln Ile Val Val Gln Lys Lys Asp Gly Cys Asp Trp Ile Arg  
                   450                                  455                                  460  
 Cys Thr Val Cys His Thr Glu Ile Cys Trp Val Thr Lys Gly Pro Arg  
                   465                                  470                                  475                                  480  
 Trp Gly Pro Gly Gly Pro Gly Asp Thr Ser Gly Gly Cys Arg Cys Arg  
                   485                                  490                                  495  
 Val Asn Gly Ile Pro Cys His Pro Ser Cys Gln Asn Cys His  
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&lt;210&gt; SEQ ID NO 2

&lt;211&gt; LENGTH: 1533

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 2

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ctgagtgtgc aactgaagcc tgaggtctcc ccaacgcagg acatcaggct gtgggtgagc      180
gtggaggatg ctcagatgca caccgtcacc atctggctca cagtgcgccc tgatatgaca      240
gtggcgtctc tcaaggacat ggtttttctg gactatggct tcccaccagt cttgcagcag      300
tgggtgattg ggcagcggct ggcacgagac caggagaccc tgcactccca tggggtgctg      360
cagaatgggg acagtgccta cctctatctg ctgtcagccc gcaacacctc cctcaaccct      420
caggagctgc agcgggagcg gcagctgcgg atgtggaag atctgggctt caaggacctc      480
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cccggacggg ggcagccaga tgcagtcctc gagccccac cgggtggctg gcagtgcccc      600
gggtgcacct tcactcaaaa gccacgcggc cctggctgtg agatgtgctg cggggcgctg      660
cccgagcctc accaggtccc cgcctcatac cagcccagcg aggaggagcg agcgcgctg      720
gctggcgagg aggaggcgtc gcgtcagtag cagcagcgga agcagcagca gcaggagggg      780
aactacctgc agcagctcca gctggaccag aggagcctgg tgctgaacac ggagcccgcc      840
gagtgccccg tgtgtactc ggtgtggcg cccggcgagg ccgtggtgct gcgtgagtgt      900
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gtctcctgcc ccttcattga caaacctac tctgtctcgg gcaagctgct ggagagggag     1020
atcaaggcgc tctgacccc tgaggattac cagcatttc tagaactggg catctccatt     1080
gctgaaaacc gcagtgcctt cagctacatc tgcaagaccc cagattgcaa gggatggtgc     1140
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ctctgcaagg ccatccatga gcagatgaac tgcaaggagt atcaggagga cctggcctg     1260
cgggctcaga acgatgtggc tgcccggcag acgacagaga tgctgaaggt gatgctgcag     1320
cagggcgagg ccatgcgctg cccccagtgc cagatcgtgg tacagaagaa ggacggctgc     1380
gactggatcc gctgcaccgt ctgccacacc gagatctgct gggtcaccaa gggcccacgc     1440
tggggccctg ggggcccagg agacaccagc gggggctgcc gctgcagggt aaatgggatt     1500

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ccttgccacc caagctgtca gaactgccac tga

1533

&lt;210&gt; SEQ ID NO 3

&lt;211&gt; LENGTH: 1072

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 3

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Gln Leu Arg Pro Leu Leu Ala Ser Ser Leu Pro Leu Ala Ala Arg Tyr  
 35 40 45

Leu Gln Leu Asp Ala Ala Arg Leu Val Arg Cys Asn Ala His Gly Glu  
 50 55 60

Pro Arg Asn Tyr Leu Asn Thr Leu Ser Thr Ala Leu Asn Ile Leu Glu  
 65 70 75 80

Lys Tyr Gly Arg Asn Leu Leu Ser Pro Gln Arg Pro Arg Tyr Trp Arg  
 85 90 95

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

Gln Gly Gly Arg Asp Val Leu Arg Leu Tyr Gly Tyr Thr Glu Glu Gln  
 115 120 125

Pro Asp Gly Leu Ser Phe Pro Glu Gly Gln Glu Glu Pro Asp Glu His  
 130 135 140

Gln Val Ala Thr Val Thr Leu Glu Val Leu Leu Arg Thr Glu Leu  
 145 150 155 160

Ser Leu Leu Leu Gln Asn Thr His Pro Arg Gln Gln Ala Leu Glu Gln  
 165 170 175

Leu Leu Glu Asp Lys Val Glu Asp Asp Met Leu Gln Leu Ser Glu Phe  
 180 185 190

Asp Pro Leu Leu Arg Glu Ile Ala Pro Gly Pro Leu Thr Thr Pro Ser  
 195 200 205

Val Pro Gly Ser Thr Pro Gly Pro Cys Phe Leu Cys Gly Ser Ala Pro  
 210 215 220

Gly Thr Leu His Cys Pro Ser Cys Lys Gln Ala Leu Cys Pro Ala Cys  
 225 230 235 240

Asp His Leu Phe His Gly His Pro Ser Arg Ala His His Leu Arg Gln  
 245 250 255

Thr Leu Pro Gly Val Leu Gln Gly Thr His Leu Ser Pro Ser Leu Pro  
 260 265 270

Ala Ser Ala Gln Pro Arg Pro Gln Ser Thr Ser Leu Leu Ala Leu Gly  
 275 280 285

Asp Ser Ser Leu Ser Ser Pro Asn Pro Ala Ser Ala His Leu Pro Trp  
 290 295 300

His Cys Ala Ala Cys Ala Met Leu Asn Glu Pro Trp Ala Val Leu Cys  
 305 310 315 320

Val Ala Cys Asp Arg Pro Arg Gly Cys Lys Gly Leu Gly Leu Gly Thr  
 325 330 335

Glu Gly Pro Gln Gly Thr Gly Gly Leu Glu Pro Asp Leu Ala Arg Gly  
 340 345 350

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|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Arg | Trp | Ala | Cys | Gln | Ser | Cys | Thr | Phe | Glu | Asn | Glu | Ala | Ala | Ala | Val |
|     |     | 355 |     |     |     |     | 360 |     |     |     |     | 365 |     |     |     |
| Leu | Cys | Ser | Ile | Cys | Glu | Arg | Pro | Arg | Leu | Ala | Gln | Pro | Pro | Ser | Leu |
|     | 370 |     |     |     |     | 375 |     |     |     |     | 380 |     |     |     |     |
| Val | Val | Asp | Ser | Arg | Asp | Ala | Gly | Ile | Cys | Leu | Gln | Pro | Leu | Gln | Gln |
| 385 |     |     |     |     | 390 |     |     |     |     | 395 |     |     |     |     | 400 |
| Gly | Asp | Ala | Leu | Leu | Ala | Ser | Ala | Gln | Ser | Gln | Val | Trp | Tyr | Cys | Ile |
|     |     |     | 405 |     |     |     |     |     | 410 |     |     |     |     | 415 |     |
| His | Cys | Thr | Phe | Cys | Asn | Ser | Ser | Pro | Gly | Trp | Val | Cys | Val | Met | Cys |
|     |     |     | 420 |     |     |     |     | 425 |     |     |     |     | 430 |     |     |
| Asn | Arg | Thr | Ser | Ser | Pro | Ile | Pro | Ala | Gln | His | Ala | Pro | Arg | Pro | Tyr |
|     |     | 435 |     |     |     |     | 440 |     |     |     |     | 445 |     |     |     |
| Ala | Ser | Ser | Leu | Glu | Lys | Gly | Pro | Pro | Lys | Pro | Gly | Pro | Pro | Arg | Arg |
|     | 450 |     |     |     |     | 455 |     |     |     |     | 460 |     |     |     |     |
| Leu | Ser | Ala | Pro | Leu | Pro | Ser | Ser | Cys | Gly | Asp | Pro | Glu | Lys | Gln | Arg |
| 465 |     |     |     |     | 470 |     |     |     |     | 475 |     |     |     |     | 480 |
| Gln | Asp | Lys | Met | Arg | Glu | Glu | Gly | Leu | Gln | Leu | Val | Ser | Met | Ile | Arg |
|     |     |     | 485 |     |     |     |     |     | 490 |     |     |     |     | 495 |     |
| Glu | Gly | Glu | Ala | Ala | Gly | Ala | Cys | Pro | Glu | Glu | Ile | Phe | Ser | Ala | Leu |
|     |     |     | 500 |     |     |     |     | 505 |     |     |     |     | 510 |     |     |
| Gln | Tyr | Ser | Gly | Thr | Glu | Val | Pro | Leu | Gln | Trp | Leu | Arg | Ser | Glu | Leu |
|     |     | 515 |     |     |     |     | 520 |     |     |     |     | 525 |     |     |     |
| Pro | Tyr | Val | Leu | Glu | Met | Val | Ala | Glu | Leu | Ala | Gly | Gln | Gln | Asp | Pro |
|     | 530 |     |     |     |     | 535 |     |     |     |     | 540 |     |     |     |     |
| Gly | Leu | Gly | Ala | Phe | Ser | Cys | Gln | Glu | Ala | Arg | Arg | Ala | Trp | Leu | Asp |
| 545 |     |     |     |     | 550 |     |     |     |     | 555 |     |     |     |     | 560 |
| Arg | His | Gly | Asn | Leu | Asp | Glu | Ala | Val | Glu | Glu | Cys | Val | Arg | Thr | Arg |
|     |     |     | 565 |     |     |     |     |     | 570 |     |     |     |     | 575 |     |
| Arg | Arg | Lys | Val | Gln | Glu | Leu | Gln | Ser | Leu | Gly | Phe | Gly | Pro | Glu | Glu |
|     |     |     | 580 |     |     |     |     | 585 |     |     |     |     | 590 |     |     |
| Gly | Ser | Leu | Gln | Ala | Leu | Phe | Gln | His | Gly | Gly | Asp | Val | Ser | Arg | Ala |
|     |     | 595 |     |     |     |     | 600 |     |     |     |     | 605 |     |     |     |
| Leu | Thr | Glu | Leu | Gln | Arg | Gln | Arg | Leu | Glu | Pro | Phe | Arg | Gln | Arg | Leu |
|     | 610 |     |     |     |     | 615 |     |     |     |     | 620 |     |     |     |     |
| Trp | Asp | Ser | Gly | Pro | Glu | Pro | Thr | Pro | Ser | Trp | Asp | Gly | Pro | Asp | Lys |
| 625 |     |     |     |     | 630 |     |     |     |     | 635 |     |     |     |     | 640 |
| Gln | Ser | Leu | Val | Arg | Arg | Leu | Leu | Ala | Val | Tyr | Ala | Leu | Pro | Ser | Trp |
|     |     |     | 645 |     |     |     |     |     | 650 |     |     |     |     | 655 |     |
| Gly | Arg | Ala | Glu | Leu | Ala | Leu | Ser | Leu | Leu | Gln | Glu | Thr | Pro | Arg | Asn |
|     |     | 660 |     |     |     |     |     | 665 |     |     |     |     | 670 |     |     |
| Tyr | Glu | Leu | Gly | Asp | Val | Val | Glu | Ala | Val | Arg | His | Ser | Gln | Asp | Arg |
|     | 675 |     |     |     |     |     | 680 |     |     |     |     | 685 |     |     |     |
| Ala | Phe | Leu | Arg | Arg | Leu | Leu | Ala | Gln | Glu | Cys | Ala | Val | Cys | Gly | Trp |
|     | 690 |     |     |     |     | 695 |     |     |     |     | 700 |     |     |     |     |
| Ala | Leu | Pro | His | Asn | Arg | Met | Gln | Ala | Leu | Thr | Ser | Cys | Glu | Cys | Thr |
| 705 |     |     |     |     | 710 |     |     |     |     |     | 715 |     |     |     | 720 |
| Ile | Cys | Pro | Asp | Cys | Phe | Arg | Gln | His | Phe | Thr | Ile | Ala | Leu | Lys | Glu |
|     |     |     | 725 |     |     |     |     |     | 730 |     |     |     |     | 735 |     |
| Lys | His | Ile | Thr | Asp | Met | Val | Cys | Pro | Ala | Cys | Gly | Arg | Pro | Asp | Leu |
|     |     | 740 |     |     |     |     |     | 745 |     |     |     |     | 750 |     |     |
| Thr | Asp | Asp | Thr | Gln | Leu | Leu | Ser | Tyr | Phe | Ser | Thr | Leu | Asp | Ile | Gln |

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| 755 |     |     | 760 |     |     | 765 |      |     |     |     |     |     |      |     |     |
|-----|-----|-----|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|------|-----|-----|
| Leu | Arg | Glu | Ser | Leu | Glu | Pro | Asp  | Ala | Tyr | Ala | Leu | Phe | His  | Lys | Lys |
|     | 770 |     |     |     |     |     | 775  |     |     |     |     | 780 |      |     |     |
| Leu | Thr | Glu | Gly | Val | Leu | Met | Arg  | Asp | Pro | Lys | Phe | Leu | Trp  | Cys | Ala |
|     | 785 |     |     |     | 790 |     |      |     |     | 795 |     |     |      |     | 800 |
| Gln | Cys | Ser | Phe | Gly | Phe | Ile | Tyr  | Glu | Arg | Glu | Gln | Leu | Glu  | Ala | Thr |
|     |     |     |     | 805 |     |     |      |     |     | 810 |     |     |      |     | 815 |
| Cys | Pro | Gln | Cys | His | Gln | Thr | Phe  | Cys | Val | Arg | Cys | Lys | Arg  | Gln | Trp |
|     |     |     |     | 820 |     |     |      |     |     | 825 |     |     |      |     | 830 |
| Glu | Glu | Gln | His | Arg | Gly | Arg | Ser  | Cys | Glu | Asp | Phe | Gln | Asn  | Trp | Lys |
|     |     |     |     | 835 |     |     |      |     |     | 840 |     |     |      |     | 845 |
| Arg | Met | Asn | Asp | Pro | Glu | Tyr | Gln  | Ala | Gln | Gly | Leu | Ala | Met  | Tyr | Leu |
|     |     |     |     |     |     |     | 855  |     |     |     |     | 860 |      |     |     |
| Gln | Glu | Asn | Gly | Ile | Asp | Cys | Pro  | Lys | Cys | Lys | Phe | Ser | Tyr  | Ala | Leu |
|     |     |     |     |     | 870 |     |      |     |     | 875 |     |     |      |     | 880 |
| Ala | Arg | Gly | Gly | Cys | Met | His | Phe  | His | Cys | Thr | Gln | Cys | Arg  | His | Gln |
|     |     |     |     | 885 |     |     |      |     |     | 890 |     |     |      |     | 895 |
| Phe | Cys | Ser | Gly | Cys | Tyr | Asn | Ala  | Phe | Tyr | Ala | Lys | Asn | Lys  | Cys | Pro |
|     |     |     |     | 900 |     |     |      |     |     | 905 |     |     |      |     | 910 |
| Glu | Pro | Asn | Cys | Arg | Val | Lys | Lys  | Ser | Leu | His | Gly | His | His  | Pro | Arg |
|     |     |     |     | 915 |     |     |      |     |     | 920 |     |     |      |     | 925 |
| Asp | Cys | Leu | Phe | Tyr | Leu | Arg | Asp  | Trp | Thr | Ala | Leu | Arg | Leu  | Gln | Lys |
|     |     |     |     |     |     |     | 935  |     |     |     |     | 940 |      |     |     |
| Leu | Leu | Gln | Asp | Asn | Asn | Val | Met  | Phe | Asn | Thr | Glu | Pro | Pro  | Ala | Gly |
|     |     |     |     |     | 950 |     |      |     |     | 955 |     |     |      |     | 960 |
| Ala | Arg | Ala | Val | Pro | Gly | Gly | Gly  | Cys | Arg | Val | Ile | Glu | Gln  | Lys | Glu |
|     |     |     |     | 965 |     |     |      |     |     | 970 |     |     |      |     | 975 |
| Val | Pro | Asn | Gly | Leu | Arg | Asp | Glu  | Ala | Cys | Gly | Lys | Glu | Thr  | Pro | Ala |
|     |     |     |     | 980 |     |     |      |     |     | 985 |     |     |      |     | 990 |
| Gly | Tyr | Ala | Gly | Leu | Cys | Gln | Ala  | His | Tyr | Lys | Glu | Tyr | Leu  | Val | Ser |
|     |     |     |     |     |     |     | 1000 |     |     |     |     |     | 1005 |     |     |
| Leu | Ile | Asn | Ala | His | Ser | Leu | Asp  | Pro | Ala | Thr | Leu | Tyr | Glu  | Val |     |
|     |     |     |     |     |     |     | 1015 |     |     |     |     |     | 1020 |     |     |
| Glu | Glu | Leu | Glu | Thr | Ala | Thr | Glu  | Arg | Tyr | Leu | His | Val | Arg  | Pro |     |
|     |     |     |     |     |     |     | 1030 |     |     |     |     |     | 1035 |     |     |
| Gln | Pro | Leu | Ala | Gly | Glu | Asp | Pro  | Pro | Ala | Tyr | Gln | Ala | Arg  | Leu |     |
|     |     |     |     |     |     |     | 1045 |     |     |     |     |     | 1050 |     |     |
| Leu | Gln | Lys | Leu | Thr | Glu | Glu | Val  | Pro | Leu | Gly | Gln | Ser | Ile  | Pro |     |
|     |     |     |     |     |     |     | 1060 |     |     |     |     |     | 1065 |     |     |
| Arg | Arg | Arg | Lys |     |     |     |      |     |     |     |     |     |      |     |     |
|     |     |     |     |     |     |     |      |     |     |     |     |     |      |     |     |

<210> SEQ ID NO 4  
 <211> LENGTH: 3219  
 <212> TYPE: DNA  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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tctctgcccg tagccgcccg ctacctgcag ctggacgccc cagccttgt ccgctgcaac    180
    
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|--|------|
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| aaataaccctg tctttcgcag cacgggtggat gctgtgcagg ggggcccaga tgtgtgcga | 360  |
| ttatatggct acacagagga gcaaccagat ggggtgagct tccccgaagg gcaggaggag  | 420  |
| ccagatgagc accagggtgc tacagtcaca ctggaagtac tgctgcttcg gacagagctc  | 480  |
| agcctgctat tgcagaatac tcatccaaga cagcaggcac tggagcagct gttggaagac  | 540  |
| aaggttgaag atgatatgct gcagctttca gaatttgacc ccctattgag agagattgct  | 600  |
| cctggcccc tcaccacacc ctctgtccca ggctccaactc ctggtcctcg cttcctctgt  | 660  |
| ggttctgccc caggcacact gcaactgcca tccctgtaaac aggcctctgt tccagcctgt | 720  |
| gaccacctgt tccatggaca cccatcccgt gctcatcacc tccgccagac cctgctggg   | 780  |
| gtcctgcagg gtaccacact gagccccagt ttacctgcct cagcccaacc acggccccag  | 840  |
| tegacctccc tgctggccct gggagacagc tctctttctt cccctaatcc tgcaagtgct  | 900  |
| catttgccct ggcactgtgc tgccctgtcc atgctaaatg agccttgggc agtgcctgt   | 960  |
| gtggcctgtg atcggccccg aggctgtaag gggttggggg tgggaactga gggccccaa   | 1020 |
| ggaactggag gcctagaacc tgatcttgca cggggtcggg gggcctgcca gagctgtacc  | 1080 |
| tttgagaatg aggcagctgc tgtgctatgt tccatgatg agcgacctcg gctggcccag   | 1140 |
| cctcccagct tgggtggtgga tccccagat gctggcattt gcctgcaacc ccttcagcag  | 1200 |
| ggggatgctt tgctggcctc tgcccagagt caagtctggt actgtattca ctgtacctc   | 1260 |
| tgcaactcga gccctggctg ggtgtgtgtt atgtgcaacc ggactagtag cccattcca   | 1320 |
| gcacaacatg cccccggcc ctatgccagc tctttgaaa agggaccccc caagcctggg    | 1380 |
| ccccacgac gccttagtgc ccccctgcc agttcctgtg gagatcctga gaagcagcgc    | 1440 |
| caagacaaga tgcgggaaga aggcctccag ctagtgagca tgatccggga aggggaagcc  | 1500 |
| gcaggtgctt gtccagagga gatcttctcg gctctgcagt actcgggcac tgagggtcct  | 1560 |
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| cagcaggacc ctgggctggg tgccctttcc tgtcaggagg cccggagagc ctggctggat  | 1680 |
| cgatcatgga acctgatga agctgtggag gagtgtgtga ggaccaggcg aaggaaggtg   | 1740 |
| caggagctcc agtctctagg ctttggccct gaggaggggt ctctccaggc attgttcag   | 1800 |
| caaggaggtg atgtgtcacg ggcctgact gagctacagc gccaacgcct agagccctc    | 1860 |
| cgccagcgc tctgggacag tggccctgag cccaccctt cctgggatgg gccagacaag    | 1920 |
| cagagcctgg tcaggcggct tttggcagtc tacgcactcc ccagctgggg cgggcagag   | 1980 |
| ctggcactgt cactgctgca ggagacaccc aggaactatg agttggggga tgtggtagaa  | 2040 |
| gctgtgaggc acagccagga cgggcccctc ctgcgccgct tgcttgccca ggagtgtgcc  | 2100 |
| gtgtgtggct gggccctgcc ccacaaccgg atgcaggccc tgacttcctg tgagtgcacc  | 2160 |
| atctgtcctg actgtctccg ccagcacttc acctgcgct tgaaggagaa gcacatcaca   | 2220 |
| gacatgggtg gccctgcctg tggcccctcc gacctaccg atgacacaca gttgctcagc   | 2280 |
| tacttcteta cccttgacat ccagcttcgc gagagcctag agccagatgc ctatgcgttg  | 2340 |
| ttccataaga agctgaccga ggggtgtgctg atgcgggacc ccaagttctt gtggtgtgcc | 2400 |
| cagtgtcctt ttggcttcat atatgagcgt gagcagctgg aggcaacttg tccccagtg   | 2460 |

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caccagacct tctgtgtgcg ctgcaagcgc cagtgggagg agcagcaccg aggtcggagc 2520
tgtgaggact tccagaactg gaaacgcatg aacgaccagc aataccaggc ccagggccta 2580
gcaatgtatc ttcaggaaaa cggcattgac tgccccaaat gcaagttctc gtacgccttg 2640
gcccaggagg gctgcatgca ctttactgtg acccagtgcc gccaccagtt ctgcagcggc 2700
tgctacaatg ccttttacgc caagaataaa tgtccagagc ctaactgcag ggtgaaaaag 2760
tccctgcacg gccaccaccc tcgagactgc ctcttctacc tgccgggactg gactgctctc 2820
cggcttcaga agctgtctaca ggacaataac gtcattgttta atacagagcc tccagctggg 2880
gccccggcag tccctggagg cgctgccga gtgatagagc agaaggaggt tcccaatggg 2940
ctcagggacg aagcttggg caaggaaact ccagctggct atgccggcct gtgccaggca 3000
cactacaaag agtatcttgt gagcctcacc aatgcccaact cgctggaccc agccaccttg 3060
tatgaggtgg aagagctgga gacggccact gagcgtacc tgcacgtacg cccccagcct 3120
ttggctggag aggatcccc tgettaccag gcccgcttgt tacagaagct gacagaagag 3180
gtacccttgg gacagagtat cccccgcagg cggaagtag 3219

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<210> SEQ ID NO 5
<211> LENGTH: 191
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 5

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```

Met Asp Glu Lys Thr Lys Lys Ala Glu Glu Met Ala Leu Ser Leu Thr
1          5          10          15
Arg Ala Val Ala Gly Gly Asp Glu Gln Val Ala Met Lys Cys Ala Ile
          20          25          30
Trp Leu Ala Glu Gln Arg Val Pro Leu Ser Val Gln Leu Lys Pro Glu
          35          40          45
Val Ser Pro Thr Gln Asp Ile Arg Leu Trp Val Ser Val Glu Asp Ala
          50          55          60
Gln Met His Thr Val Thr Ile Trp Leu Thr Val Arg Pro Asp Met Thr
          65          70          75          80
Val Ala Ser Leu Lys Asp Met Val Phe Leu Asp Tyr Gly Phe Pro Pro
          85          90          95
Val Leu Gln Gln Trp Val Ile Gly Gln Arg Leu Ala Arg Asp Gln Glu
          100         105         110
Thr Leu His Ser His Gly Val Arg Gln Asn Gly Asp Ser Ala Tyr Leu
          115         120         125
Tyr Leu Leu Ser Ala Arg Asn Thr Ser Leu Asn Pro Gln Glu Leu Gln
          130         135         140
Arg Glu Arg Gln Leu Arg Met Leu Glu Asp Leu Gly Phe Lys Asp Leu
          145         150         155         160
Thr Leu Gln Pro Arg Gly Pro Leu Glu Pro Gly Pro Pro Lys Pro Gly
          165         170         175
Val Pro Gln Glu Pro Gly Arg Gly Gln Pro Asp Ala Val Pro Glu
          180         185         190

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<210> SEQ ID NO 6
<211> LENGTH: 576
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

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&lt;400&gt; SEQUENCE: 6

```

atggacgaga agaccaagaa agcagaggaa atggccctga gcctcaccg agcagtggcg      60
ggcggggatg aacaggtggc aatgaagtgt gccatctggc tggcagagca acgggtgccc      120
ctgagtgtgc aactgaagcc tgaggtctcc ccaacgcagg acatcaggct gtgggtgagc      180
gtggaggatg ctcagatgca caccgtcacc atctggctca cagtgcgccc tgatatgaca      240
gtggcgtctc tcaaggacat ggtttttctg gactatggct tcccaccagt cttgcagcag      300
tgggtgattg ggcagcggct ggcacgagac caggagacct tgcactccca tggggtgctg      360
cagaatgggg acagtgccta cctctatctg ctgtcagccc gcaacacctc cctcaaccct      420
caggagctgc agcgggagcg gcagctgcgg atgctggaag atctgggctt caaggacctc      480
acgctgcagc cgcggggccc tctggagcca ggccccccaa agccccgggt cccccaggaa      540
cccggacggg ggcagccaga tgcagtgcct gagtga                                576

```

&lt;210&gt; SEQ ID NO 7

&lt;211&gt; LENGTH: 599

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 7

```

Gly Asp Pro Glu Lys Gln Arg Gln Asp Lys Met Arg Glu Glu Gly Leu
 1          5          10          15
Gln Leu Val Ser Met Ile Arg Glu Gly Glu Ala Ala Gly Ala Cys Pro
          20          25          30
Glu Glu Ile Phe Ser Ala Leu Gln Tyr Ser Gly Thr Glu Val Pro Leu
          35          40          45
Gln Trp Leu Arg Ser Glu Leu Pro Tyr Val Leu Glu Met Val Ala Glu
          50          55          60
Leu Ala Gly Gln Gln Asp Pro Gly Leu Gly Ala Phe Ser Cys Gln Glu
          65          70          75          80
Ala Arg Arg Ala Trp Leu Asp Arg His Gly Asn Leu Asp Glu Ala Val
          85          90          95
Glu Glu Cys Val Arg Thr Arg Arg Arg Lys Val Gln Glu Leu Gln Ser
          100          105          110
Leu Gly Phe Gly Pro Glu Glu Gly Ser Leu Gln Ala Leu Phe Gln His
          115          120          125
Gly Gly Asp Val Ser Arg Ala Leu Thr Glu Leu Gln Arg Gln Arg Leu
          130          135          140
Glu Pro Phe Arg Gln Arg Leu Trp Asp Ser Gly Pro Glu Pro Thr Pro
          145          150          155          160
Ser Trp Asp Gly Pro Asp Lys Gln Ser Leu Val Arg Arg Leu Leu Ala
          165          170          175
Val Tyr Ala Leu Pro Ser Trp Gly Arg Ala Glu Leu Ala Leu Ser Leu
          180          185          190
Leu Gln Glu Thr Pro Arg Asn Tyr Glu Leu Gly Asp Val Val Glu Ala
          195          200          205
Val Arg His Ser Gln Asp Arg Ala Phe Leu Arg Arg Leu Leu Ala Gln
          210          215          220
Glu Cys Ala Val Cys Gly Trp Ala Leu Pro His Asn Arg Met Gln Ala
          225          230          235          240
Leu Thr Ser Cys Glu Cys Thr Ile Cys Pro Asp Cys Phe Arg Gln His

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| 245 |     |     |     |     | 250 |     |     |     |     | 255 |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Phe | Thr | Ile | Ala | Leu | Lys | Glu | Lys | His | Ile | Thr | Asp | Met | Val | Cys | Pro |
|     |     |     | 260 |     |     |     |     | 265 |     |     |     |     | 270 |     |     |
| Ala | Cys | Gly | Arg | Pro | Asp | Leu | Thr | Asp | Asp | Thr | Gln | Leu | Leu | Ser | Tyr |
|     |     | 275 |     |     |     |     |     | 280 |     |     |     | 285 |     |     |     |
| Phe | Ser | Thr | Leu | Asp | Ile | Gln | Leu | Arg | Glu | Ser | Leu | Glu | Pro | Asp | Ala |
|     | 290 |     |     |     |     | 295 |     |     |     |     | 300 |     |     |     |     |
| Tyr | Ala | Leu | Phe | His | Lys | Lys | Leu | Thr | Glu | Gly | Val | Leu | Met | Arg | Asp |
| 305 |     |     |     |     | 310 |     |     |     |     | 315 |     |     |     |     | 320 |
| Pro | Lys | Phe | Leu | Trp | Cys | Ala | Gln | Cys | Ser | Phe | Gly | Phe | Ile | Tyr | Glu |
|     |     |     |     | 325 |     |     |     |     | 330 |     |     |     |     | 335 |     |
| Arg | Glu | Gln | Leu | Glu | Ala | Thr | Cys | Pro | Gln | Cys | His | Gln | Thr | Phe | Cys |
|     |     |     | 340 |     |     |     |     | 345 |     |     |     |     | 350 |     |     |
| Val | Arg | Cys | Lys | Arg | Gln | Trp | Glu | Glu | Gln | His | Arg | Gly | Arg | Ser | Cys |
|     |     | 355 |     |     |     |     | 360 |     |     |     |     | 365 |     |     |     |
| Glu | Asp | Phe | Gln | Asn | Trp | Lys | Arg | Met | Asn | Asp | Pro | Glu | Tyr | Gln | Ala |
|     | 370 |     |     |     |     | 375 |     |     |     |     | 380 |     |     |     |     |
| Gln | Gly | Leu | Ala | Met | Tyr | Leu | Gln | Glu | Asn | Gly | Ile | Asp | Cys | Pro | Lys |
| 385 |     |     |     |     | 390 |     |     |     |     | 395 |     |     |     |     | 400 |
| Cys | Lys | Phe | Ser | Tyr | Ala | Leu | Ala | Arg | Gly | Gly | Cys | Met | His | Phe | His |
|     |     |     |     | 405 |     |     |     |     | 410 |     |     |     |     | 415 |     |
| Cys | Thr | Gln | Cys | Arg | His | Gln | Phe | Cys | Ser | Gly | Cys | Tyr | Asn | Ala | Phe |
|     |     |     | 420 |     |     |     |     | 425 |     |     |     |     | 430 |     |     |
| Tyr | Ala | Lys | Asn | Lys | Cys | Pro | Glu | Pro | Asn | Cys | Arg | Val | Lys | Lys | Ser |
|     |     | 435 |     |     |     |     | 440 |     |     |     |     | 445 |     |     |     |
| Leu | His | Gly | His | His | Pro | Arg | Asp | Cys | Leu | Phe | Tyr | Leu | Arg | Asp | Trp |
|     | 450 |     |     |     |     | 455 |     |     |     |     | 460 |     |     |     |     |
| Thr | Ala | Leu | Arg | Leu | Gln | Lys | Leu | Leu | Gln | Asp | Asn | Asn | Val | Met | Phe |
| 465 |     |     |     |     | 470 |     |     |     |     | 475 |     |     |     |     | 480 |
| Asn | Thr | Glu | Pro | Pro | Ala | Gly | Ala | Arg | Ala | Val | Pro | Gly | Gly | Gly | Cys |
|     |     |     |     | 485 |     |     |     |     | 490 |     |     |     |     | 495 |     |
| Arg | Val | Ile | Glu | Gln | Lys | Glu | Val | Pro | Asn | Gly | Leu | Arg | Asp | Glu | Ala |
|     |     |     | 500 |     |     |     |     | 505 |     |     |     |     | 510 |     |     |
| Cys | Gly | Lys | Glu | Thr | Pro | Ala | Gly | Tyr | Ala | Gly | Leu | Cys | Gln | Ala | His |
|     |     | 515 |     |     |     |     | 520 |     |     |     |     | 525 |     |     |     |
| Tyr | Lys | Glu | Tyr | Leu | Val | Ser | Leu | Ile | Asn | Ala | His | Ser | Leu | Asp | Pro |
|     | 530 |     |     |     |     | 535 |     |     |     |     | 540 |     |     |     |     |
| Ala | Thr | Leu | Tyr | Glu | Val | Glu | Glu | Leu | Glu | Thr | Ala | Thr | Glu | Arg | Tyr |
| 545 |     |     |     |     | 550 |     |     |     |     | 555 |     |     |     |     | 560 |
| Leu | His | Val | Arg | Pro | Gln | Pro | Leu | Ala | Gly | Glu | Asp | Pro | Pro | Ala | Tyr |
|     |     |     |     | 565 |     |     |     |     | 570 |     |     |     |     | 575 |     |
| Gln | Ala | Arg | Leu | Leu | Gln | Lys | Leu | Thr | Glu | Glu | Val | Pro | Leu | Gly | Gln |
|     |     |     | 580 |     |     |     |     | 585 |     |     |     |     | 590 |     |     |
| Ser | Ile | Pro | Arg | Arg | Arg | Lys |     |     |     |     |     |     |     |     |     |
|     |     | 595 |     |     |     |     |     |     |     |     |     |     |     |     |     |

&lt;210&gt; SEQ ID NO 8

&lt;211&gt; LENGTH: 1800

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 8



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```

ggagatcctg agaagcagcg ccaagacaag atgcgggaag aaggcctcca gctagtgagc 60
atgatccggg aaggggaagc cgcaggtgcc tgtccagagg agatcttctc ggctctgcag 120
tactcgggca ctgaggtgcc tctgcagtgg ttgcgctcag aactgcctca cgtcctggag 180
atggtggctg agctggctgg acagcaggac cctgggctgg gtgccttttc ctgtcaggag 240
gcccggagag cctggctgga tcgtcatggc aacctgatg aagctgtgga ggagtgtgtg 300
aggaccaggc gaaggaaggt gcaggagctc cagtctctag gctttgggccc tgaggagggg 360
tctctccagg cattgttcca gcacggaggt gatgtgtcac gggccctgac tgagctacag 420
cgccaacgcc tagagccctt ccgccagcgc ctctgggaca gtggccctga gcccaccctt 480
tctctgggatg ggccagacaa gcagagcctg gtcaggcggc ttttggcagt ctacgcactc 540
cccagctggg gccgggcaga gctggcactg tcaactgctc aggagacacc caggaactat 600
gagttggggg atgtggtaga agctgtgagg cacagccagg accgggcctt cctgcgccgc 660
ttgcttgccc aggagtgtgc cgtgtgtggc tgggccctgc cccacaaccg gatgcaggcc 720
ctgacttctt gtgagtgcac catctgtcct gactgcttcc gccagcactt caccatcgcc 780
ttgaaggaga agcacatcac agacatggtg tgccctgcct gtggccgccc cgacctcacc 840
gatgacacac agttgctcag ctacttctct acccttgaca tccagcttcg cgagagccta 900
gagccagatg cctatgcgtt gttccataag aagctgaccg aggggtgtgt gatgcgggac 960
cccaagttct tgtggtgtgc ccagtgtcc tttggcttca tatatgagcg tgagcagctg 1020
gaggcaactt gtccccagtg tcaccagacc ttctgtgtgc gctgcaagcg ccagtgggag 1080
gagcagcacc gaggtcggag ctgtgaggac ttccagaact ggaaacgcat gaacgacca 1140
gaataccagg cccagggcct agcaatgtat cttcaggaaa acggcattga ctgccccaaa 1200
tgcaagttct cgtacgcctt ggcccagga ggctgcatgc actttcactg taccagtgcc 1260
cgccaccagt tctgcagcgg ctgctacaat gccttttacg ccaagaataa atgtccagag 1320
cctaactgca gggtgaaaaa gtcctgcac ggccaccacc ctcgagactg cctcttctac 1380
ctgcccggact ggactgtctt ccggcttcag aagctgttac aggacaataa cgtcatgttt 1440
aatacagagc ctccagctgg ggcccgggca gtccttgagg gcggctgccc agtgateagag 1500
cagaaggagg ttcccaatgg gctcagggac gaagcttgtg gcaaggaaac tccagctggc 1560
tatgcccggc tgtgccaggc aactacaaa gagtatcttg tgagcctcat caatgcccac 1620
tcgctggacc cagccacctt gtatgaggtg gaagagctgg agacggccac tgagcgtac 1680
ctgcacgtac gccccagcc tttggctgga gaggatcccc ctgcttacca ggcccgttg 1740
ttacagaagc tgacagaaga ggtacccttg ggacagagta tccccgcag gcggaagtag 1800

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<210> SEQ ID NO 9
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Tag Sequence

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<400> SEQUENCE: 9

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Met Arg Gly Ser His His His His Ser Gln Asp Pro Asn Ser
1           5           10          15

```

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Glu Asn Leu Tyr Phe Gln
20

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<210> SEQ ID NO 10
<211> LENGTH: 387
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

Met Ala Pro Pro Ala Gly Gly Ala Ala Ala Ala Ala Ser Asp Leu Gly
1          5          10
Ser Ala Ala Val Leu Leu Ala Val His Ala Ala Val Arg Pro Leu Gly
20         25         30
Ala Gly Pro Asp Ala Glu Ala Gln Leu Arg Arg Leu Gln Leu Ser Ala
35         40         45
Asp Pro Glu Arg Pro Gly Arg Phe Arg Leu Glu Leu Leu Gly Ala Gly
50         55         60
Pro Gly Ala Val Asn Leu Glu Trp Pro Leu Glu Ser Val Ser Tyr Thr
65         70         75         80
Ile Arg Gly Pro Thr Gln His Glu Leu Gln Pro Pro Pro Gly Gly Pro
85         90         95
Gly Thr Leu Ser Leu His Phe Leu Asn Pro Gln Glu Ala Gln Arg Trp
100        105        110
Ala Val Leu Val Arg Gly Ala Thr Val Glu Gly Gln Asn Gly Ser Lys
115        120        125
Ser Asn Ser Pro Pro Ala Leu Gly Pro Glu Ala Cys Pro Val Ser Leu
130        135        140
Pro Ser Pro Pro Glu Ala Ser Thr Leu Lys Gly Pro Pro Pro Glu Ala
145        150        155        160
Asp Leu Pro Arg Ser Pro Gly Asn Leu Thr Glu Arg Glu Glu Leu Ala
165        170        175
Gly Ser Leu Ala Arg Ala Ile Ala Gly Gly Asp Glu Lys Gly Ala Ala
180        185        190
Gln Val Ala Ala Val Leu Ala Gln His Arg Val Ala Leu Ser Val Gln
195        200        205
Leu Gln Glu Ala Cys Phe Pro Pro Gly Pro Ile Arg Leu Gln Val Thr
210        215        220
Leu Glu Asp Ala Ala Ser Ala Ala Ser Ala Ala Ser Ser Ala His Val
225        230        235        240
Ala Leu Gln Val His Pro His Cys Thr Val Ala Ala Leu Gln Glu Gln
245        250        255
Val Phe Ser Glu Leu Gly Phe Pro Pro Ala Val Gln Arg Trp Val Ile
260        265        270
Gly Arg Cys Leu Cys Val Pro Glu Arg Ser Leu Ala Ser Tyr Gly Val
275        280        285
Arg Gln Asp Gly Asp Pro Ala Phe Leu Tyr Leu Leu Ser Ala Pro Arg
290        295        300
Glu Ala Pro Ala Thr Gly Pro Ser Pro Gln His Pro Gln Lys Met Asp
305        310        315        320
Gly Glu Leu Gly Arg Leu Phe Pro Pro Ser Leu Gly Leu Pro Pro Gly
325        330        335
Pro Gln Pro Ala Ala Ser Ser Leu Pro Ser Pro Leu Gln Pro Ser Trp
340        345        350
Ser Cys Pro Ser Cys Thr Phe Ile Asn Ala Pro Asp Arg Pro Gly Cys
355        360        365

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-continued

Glu Met Cys Ser Thr Gln Arg Pro Cys Thr Trp Asp Pro Leu Ala Ala  
 370 375 380

Ala Ser Thr  
 385

<210> SEQ ID NO 11  
 <211> LENGTH: 1164  
 <212> TYPE: DNA  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

```

atggcgccgc cagcgggccc ggcggcggcg gcggcctcgg acttgggctc cgccgcagtg    60
ctcttggtctg tgcacgccgc ggtgaggccg ctgggcggcg gccagacgc cgaggcacag    120
ctgcggaggc tgcagctgag cgcggaccct gagaggcctg ggcgcttcgg gctggagctg    180
ctgggcggcg gacctggggc ggttaatttg gaggggcccc tggagtcagt ttcctacacc    240
atccgaggcc ccacccagca cgagctacag cctccaccag gagggcctgg aaccctcagc    300
ctgcacttcc tcaaccctca ggaagctcag cggtagggcag tcctagtccg aggtgccacc    360
gtggaaggac agaatggcag caagagcaac tcaccaccag ccttggggccc agaagcatgc    420
cctgtctccc tgcccagtc cccggaagcc tccacactca agggccctcc acctgaggca    480
gatcttccta ggagccctgg aaacttgacg gagagagaag agctggcagg gagcctggcc    540
cgggctattg caggtggaga cgagaagggg gcagcccaag tggcagccgt cctggcccag    600
catcgtgtgg ccctgagtgt tcagcttcag gaggcctgct tcccacctgg ccccatcagg    660
ctgcaggta cacttgaaga cgtgcctct gccgcacccg ccgcgtctc tgcacacgtt    720
gccttcagc tccaccccc ctgcaactgt gcagctctcc aggagcagg gttctcagag    780
ctcggtttcc cgcagcccg gcaacgctgg gtcacggac ggtgcctgtg tgtgcctgag    840
cgcagccttg cctcttaagg ggttcggcag gatggggacc ctgctttcct ctacttgctg    900
tcagctctc gagaaagccc agccacagga cctagccctc agcaccccc gaagatggac    960
ggggaacttg gacgcttggt tccccatca ttggggctac cccagggccc ccagccagct   1020
gcctccagcc tgcccagtc actccagccc agctggtcct gtccttctg caccttcac   1080
aatgccccag accgcccctg ctgtgagatg tgtagcacc agaggccctg cacttgggac   1140
ccccttgctg cagcttccac ctag                                     1164

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<210> SEQ ID NO 12  
 <211> LENGTH: 176  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

```

Met Arg Glu Glu Leu Ala Gly Ser Leu Ala Arg Ala Ile Ala Gly Gly
  1          5          10          15
Asp Glu Lys Gly Ala Ala Gln Val Ala Ala Val Leu Ala Gln His Arg
  20          25          30
Val Ala Leu Ser Val Gln Leu Gln Glu Ala Cys Phe Pro Pro Gly Pro
  35          40          45
Ile Arg Leu Gln Val Thr Leu Glu Asp Ala Ala Ser Ala Ala Ser Ala
  50          55          60
Ala Ser Ser Ala His Val Ala Leu Gln Val His Pro His Cys Thr Val

```

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|   |     |     |     |
|---|-----|-----|-----|
| 65  | 70  | 75  | 80  |
| Ala Ala Leu Gln Glu Gln Val Phe Ser Glu Leu Gly Phe Pro Pro Ala | 85  | 90  | 95  |
| Val Gln Arg Trp Val Ile Gly Arg Cys Leu Cys Val Pro Glu Arg Ser | 100 | 105 | 110 |
| Leu Ala Ser Tyr Gly Val Arg Gln Asp Gly Asp Pro Ala Phe Leu Tyr | 115 | 120 | 125 |
| Leu Leu Ser Ala Pro Arg Glu Ala Pro Ala Thr Gly Pro Ser Pro Gln | 130 | 135 | 140 |
| His Pro Gln Lys Met Asp Gly Glu Leu Gly Arg Leu Phe Pro Pro Ser | 145 | 150 | 155 |
| Leu Gly Leu Pro Pro Gly Pro Gln Pro Ala Ala Ser Ser Leu Pro Ser | 165 | 170 | 175 |

&lt;210&gt; SEQ ID NO 13

&lt;211&gt; LENGTH: 531

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 13

```

atgagagaag agctggcagg gagcctggcc egggtattg caggtggaga cgagaagggg    60
gcagcccaag tggcagccgt cctggcccag catcgtgttg ccctgagtgt tcagcttcag    120
gaggcctgct tcccacctgg ccccatcagg ctgcaggtea cacttgaaga cgctgcctct    180
gcgcacatcg ccgctgctc tgcacacgtt gccctgcagg tccaccccca ctgcaactgt    240
gcagctctcc aggagcaggt gttctcagag ctcggtttcc cgccagccgt gcaacgctgg    300
gtcatcggac ggtgcctgtg tgtgcctgag cgcagccttg cctcttacgg ggttcggcag    360
gatggggacc ctgctttcct ctacttgctg teagctcctc gagaagcccc agccacagga    420
cctagccctc agcaccacca gaagatggac ggggaacttg gacgcttgtt tccccatca    480
ttggggctac ccccagggcc ccagccagct gctccagcc tgcccagttg a          531

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**1-14.** (canceled)

**15.** A ubiquitin ligase comprising a complex of the following (a) and (2), or a complex of the following (a), (1) and (2).

(a) A protein having a part of HOIP and at least having a UBA region and a RING-IBR-RING region thereof

(1) HOIL-1L, or a protein having a part of HOIL-1L and at least having a UBL region thereof

(2) Sharpin, or a protein having a part of Sharpin and at least having a UBL region thereof.

**16.** The ubiquitin ligase according to claim **15**, wherein the above (1) is a protein having the amino acid sequence represented by SEQ ID NO: 5, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 5 except for having deletion, substitution or addition of one to several amino acids, and

wherein the above (2) is a protein having the amino acid sequence represented by SEQ ID NO: 12, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 12 except for having deletion, substitution or addition of one to several amino acids.

**17.** The ubiquitin ligase according to claim **15**, wherein the above (a) is a protein having the amino acid sequence repre-

sented by SEQ ID NO: 7, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 7 except for having deletion, substitution or addition of one to several amino acids.

**18.** An expression vector for use in preparation of the ubiquitin ligase according to claim **15**, the expression vector containing a polynucleotide encoding at least one kind of protein selected from the group consisting of the following (A), (I) and (II).

(A) A protein which has a part of HOIP and at least has a UBA region and a RING-IBR-RING region thereof and which forms, with the following (I) and/or (II), a complex exhibiting ubiquitin ligase activity

(I) HOIL-1L, or a protein which has a part of HOIL-1L and at least has a UBL region thereof and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity

(II) Sharpin, or a protein which has a part of Sharpin and at least has a UBL region thereof and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity

**19.** The expression vector according to claim **18**, wherein the above (I) is a protein having the amino acid sequence represented by SEQ ID NO: 5, or a protein which has an

amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 5 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity, and

wherein the above (II) is a protein having the amino acid sequence represented by SEQ ID NO: 12, or a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 12 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

20. The expression vector according to claim 18, wherein the above (A) is a protein having the amino acid sequence represented by SEQ ID NO: 7, or a protein which has an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 7 except for having deletion, substitution or addition of one to several amino acids and which forms, with the above (I) and/or (II), a complex exhibiting ubiquitin ligase activity.

21. The expression vector according to claim 19, wherein the above (I) is encoded by a polynucleotide having the base sequence represented by SEQ ID NO: 6, or a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence represented by SEQ ID NO: 6 under stringent conditions and which encodes a protein which forms, with the above (A), a complex exhibiting ubiquitin ligase activity, and

wherein the above (II) is encoded by a polynucleotide having the base sequence represented by SEQ ID NO: 13, or a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence represented by SEQ ID NO: 13 under stringent conditions and which encodes a protein which forms, with the above (A), a complex exhibiting ubiquitin ligase activity.

22. The expression vector according to claim 20, wherein the above (A) is encoded by a polynucleotide having the base sequence represented by SEQ ID NO: 8, or a polynucleotide which hybridizes with a polynucleotide having a base sequence complementary to the base sequence represented by SEQ ID NO: 8 under stringent conditions and which encodes a protein which forms, with the above (I) and/or (II), a complex exhibiting ubiquitin ligase activity.

23. A transformant with the expression vector according to claim 18.

24. A screening method for inhibitors of linear polyubiquitination, the method comprising the steps of:

bringing a test substance into contact with the ubiquitin ligase according to claim 15, measuring the activity level of the ubiquitin ligase, and

comparing the above activity level to the activity level of the ubiquitin ligase not brought into contact with the test substance.

25. A ubiquitin ligase comprising a complex of the following (a) and (1').

(a) A protein having a part of HOIP and at least having a UBA region and a RING-IBR-RING region thereof

(1') A protein having a part of HOIL-1L containing a UBL region thereof but neither NZF nor a RING-IBR-RING region thereof.

26. A method for preparing a ubiquitin ligase, the method comprising:

a protein expression step of making proteins of the following (a) and (1'), proteins of the following (a) and (2), or proteins of the following (a), (1) and (2) expressed in bacteria as recombinant proteins, and

a complex purification step of purifying a complex of the following (a) and (1'), a complex of the following (a) and (2), or a complex of the following (a), (1) and (2) from the bacteria or a culture medium of the bacteria,

the method enabling collection of the complex by single-step purification using a tag sequence fused to at least one kind of recombinant protein expressed in the bacteria.

(a) A protein having a part of HOIP and at least having a UBA region and a RING-IBR-RING region thereof

(1) HOIL-1L, or a protein having a part of HOIL-1L and at least having a UBL region thereof

(1') A protein having a part of HOIL-1L containing a UBL region thereof but neither NZF nor a RING-IBR-RING region thereof

(2) Sharpin, or a protein having a part of Sharpin and at least having a UBL region thereof.

27. The method according to claim 26, wherein the above (1) or (1') is a protein having the amino acid sequence represented by SEQ ID NO: 5, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 5 except for having deletion, substitution or addition of one to several amino acids, and

wherein the above (2) is a protein having the amino acid sequence represented by SEQ ID NO: 12, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 12 except for having deletion, substitution or addition of one to several amino acids.

28. The method according to claim 26, wherein the above (a) is a protein having the amino acid sequence represented by SEQ ID NO: 7, or a protein having an amino acid sequence the same as the amino acid sequence represented by SEQ ID NO: 7 except for having deletion, substitution or addition of one to several amino acids.

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