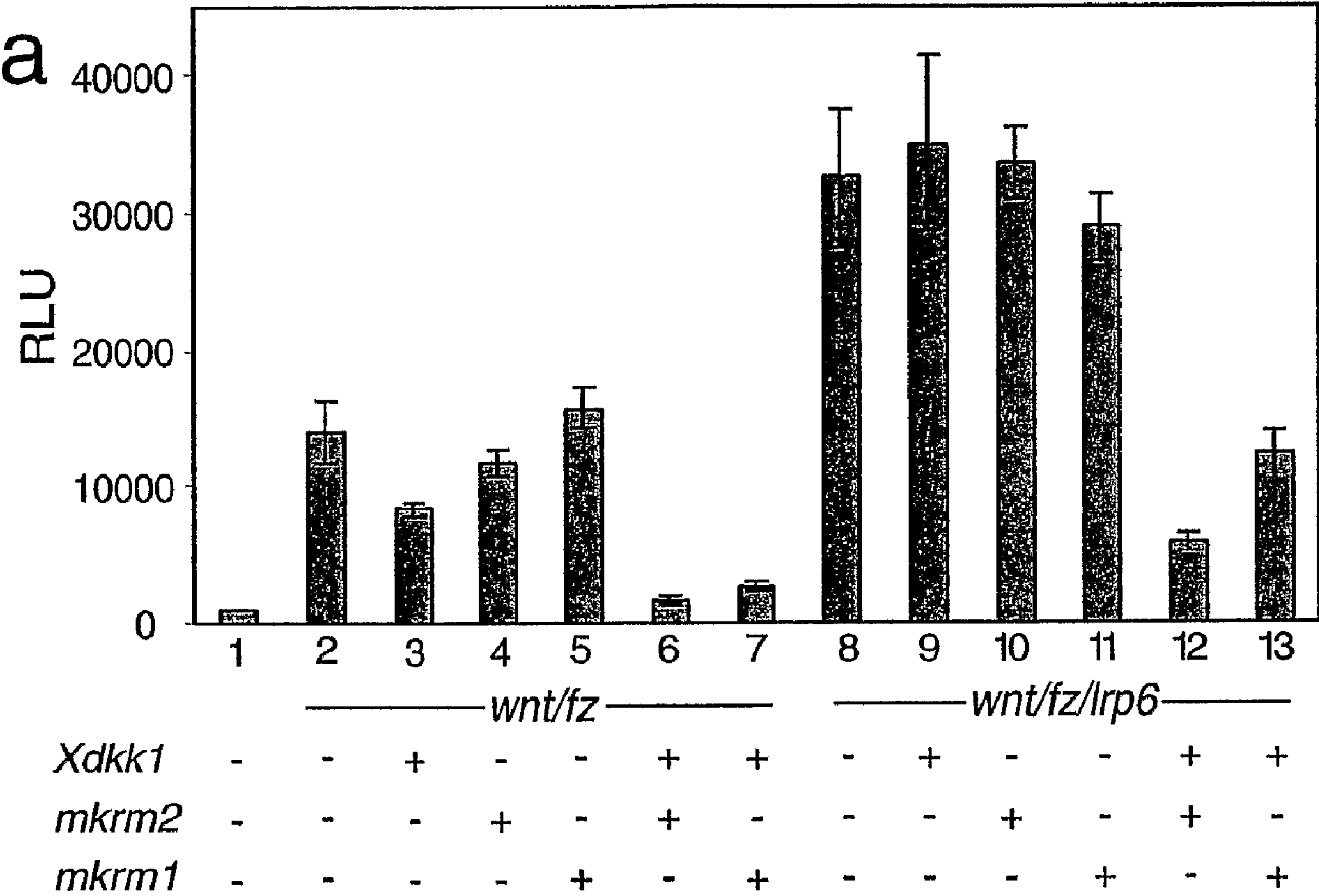




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L'EXPRESSION ABERRANTE DE KREMEN ET/OU WNT  
(54) Title: DIAGNOSTIC AND THERAPEUTIC USE OF KREMEN 1 AND 2, INHIBITORS OF THE CANONICAL WNT-  
SIGNAL TRANSDUCTION



(57) Abrégé/Abstract:  
The present invention relates to a composition useful for the diagnosis and therapy of diseases associated with aberrant expression of the gene encoding the receptor Kremen 1 and/or Kremen 2 e.g. tumors or diseases of the kidneys, bones and eyes, lipid and



(57) **Abrégé(suite)/Abstract(continued):**

glucose metabolism and obesity. The present invention also relates to a pharmaceutical composition containing a compound which is capable of modifying (a) the expression of the gene encoding Kremen 1 and/or Kremen 2 or (b) the activity of the Kremen 1 and/or Kremen 2 receptor.

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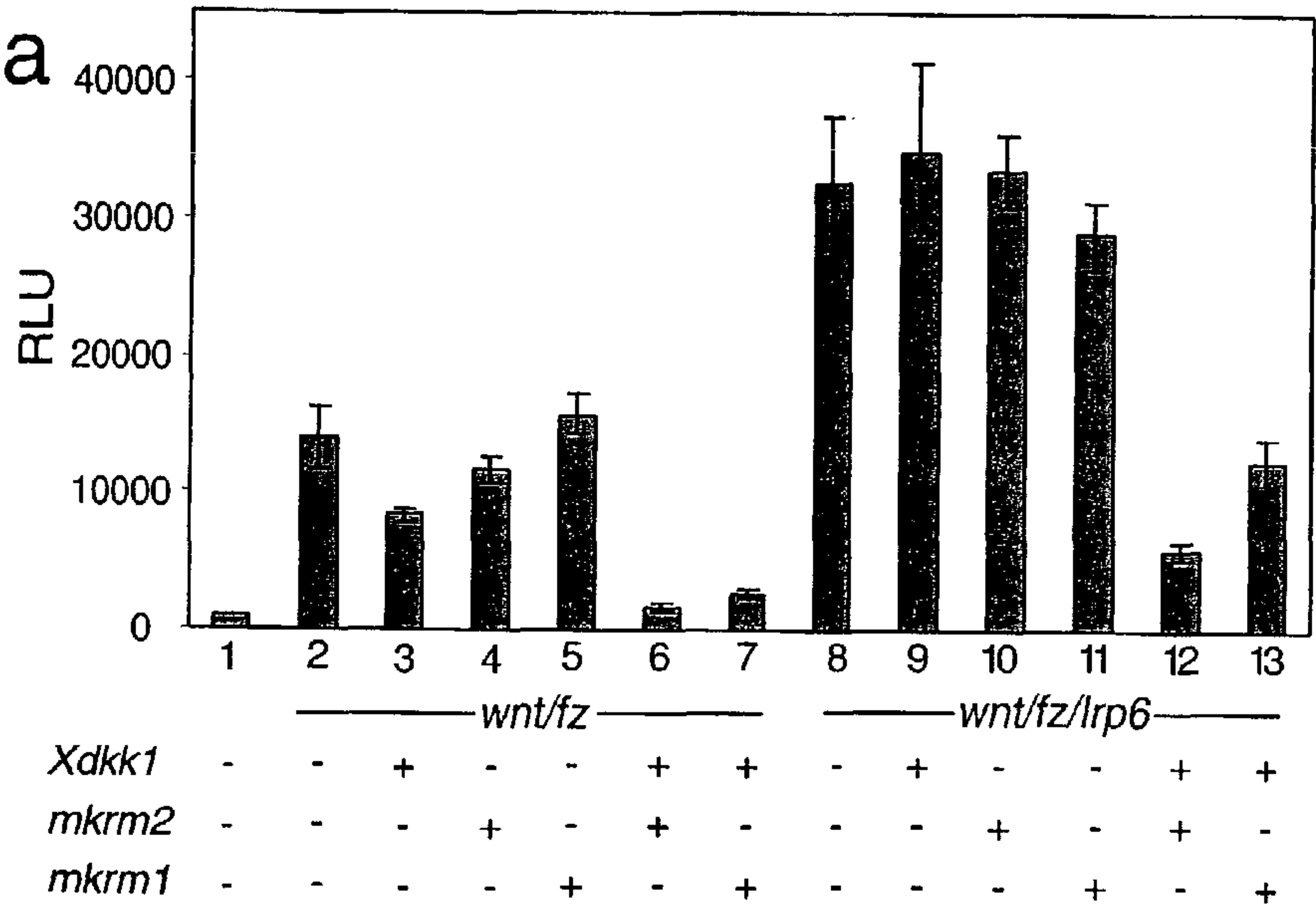
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(54) Title: DIAGNOSTIC AND THERAPEUTIC USE OF KREMEN 1 AND 2, INHIBITORS OF THE CANONICAL WNT-SIGNALTRANSDUCTION



(57) Abstract: The present invention relates to a composition useful for the diagnosis and therapy of diseases associated with aberrant expression of the gene encoding the receptor Kremen 1 and/or Kremen 2 e.g. tumors or diseases of the kidneys, bones and eyes, lipid and glucose metabolism and obesity. The present invention also relates to a pharmaceutical composition containing a compound which is capable of modifying (a) the expression of the gene encoding Kremen 1 and/or Kremen 2 or (b) the activity of the Kremen 1 and/or Kremen 2 receptor.

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## DIAGNOSTIC AND THERAPEUTIC USE OF KREMEN 1 AND 2, INHIBITORS OF THE CANONICAL WNT-SIGNAL TRANSDUCTION

The present invention relates to a composition useful for the diagnosis and therapy of diseases associated with aberrant expression of the gene encoding the receptor Kremen 1 and/or Kremen 2, e.g. tumors, diseases of the kidneys, bones and eyes, lipid and glucose metabolism and obesity. The present invention also relates to a pharmaceutical composition containing a compound which is capable of modifying (a) the expression of the gene encoding Kremen 1 and/or 2 or (b) the activity of the Kremen 1 and/or 2 receptor.

The Wnt signal cascade plays a crucial role as regards regulation of survival, proliferation and differentiation of cells during embryogenesis, and in the adult as shown, e.g., in *Drosophila*, *Xenopus* and mice (Nusse and Varmus, Cell 69 (1992), 1073-1087). Wnt-genes encode secretory glycoproteins which activate a well characterized signal cascade via a Wnt receptor called „frizzled“. In addition to frizzled, Wnts use coreceptors consisting of members of the low density protein receptor related protein (LRP) family, LRP5 and LRP6 to transmit their signals (Zorn, Curr. Biol. 11 (2001), R592-595). LRPs play important roles in various diseases. Most relevant for the present application, in recent studies a new family of proteins, Dkk („Dickkopf“), could be identified acting as inhibitors of Wnt. DKK1 binds and inhibits the Wnt coreceptor LRP 5/6 (Zorn, Curr. Biol. 11 (2001), R592-595) and thus may provide an important tool to diagnose and/or treat LRP5/6 related diseases (WO02092015). Other prominent members of effectors of this signal cascade are beta-catenin as well as the APC tumor suppressor gene (Miller and Moon, Genes Dev. 10 (1996), 2527-2539).

The Wnt signalling cascade and its components play an

important role in various diseases which makes it desirable to modulate its activity:

i) Cancer

Tumorigenesis represents a complex multistage process in which genetic changes and environmental factors are thought to deregulate the cellular processes that control cell proliferation and differentiation. Several studies indicate that an aberrant Wnt signal cascade is involved in the development of colon cancer, breast cancer and melanoma (Pfeifer, Science, 275 (1997), 1752-1753; Polakis, Genes Dev. 14 (2000), 1837-1851). The first gene encoding a protein of the Wnt signal cascade, *int-1*, was isolated from mouse mammary tumor virus (MMTV) and it could be shown that it is an oncogene. It is thus well established that an aberrant regulation of the activity of Wnt and/or components of the Wnt signal cascade downstream of the Wnt signal, e.g., beta-catenin and APC, are involved in tumorigenesis.

ii) Bone disease

Wnts signals promote bone formation (e.g. Yang, Development, 130(2003), 1003-15; Fischer, J.Biol.Chem. 277 (2002) 30870-30878). Consistent with this notion, a gain-of-function mutation of the Wnt receptor LRP5, that leads to resistance to Dkk1 inhibition, causes high bone disease (Boyden, et al., 346 (2002) N Engl J Med, 1513-21.; Little, et al., 70 (2002) Am J Hum Genet, 11-9.). Conversely, inactivating mutations in LRP5 leads to osteoporosis-pseudoglioma syndrome in humans (Kato, et al., 157 (2002) J Cell Biol, 303-14.; Gong, et al., 107 (2001) Cell, 513-23.).

iii) Eye disease

Inactivating mutation in the Wnt receptor LRP5 lead to pseudoglioma in humans and eye malformations in mice (Kato, et al., 157 (2002) J Cell Biol 303-314; Gong, et al., 107 (2001) Cell, 513-523).



iv) kidney

Aberrant Wnt signalling is involved in renal fibrosis (Surendran, Am J Physiol Renal Physiol 282 (2002) 431-441) and polycystic kidney disease (Saadi-Kheddouci, Oncogene 20 (2001) 5972-5981).

v) Lipid and glucose metabolism, obesity

LRP5 deficiency in mice leads to increased plasma cholesterol levels in mice fed a high-fat diet, because of the decreased hepatic clearance of chylomicron remnants. In addition, when fed a normal diet, LRP5-deficient mice show a markedly impaired glucose tolerance (Fujino, et al., 100 (2003) Proc Natl Acad Sci U S A, 229-234.) Administration of the LRP5 antagonist Dkk1 to mice reduces glucose uptake in various cell line and decreases fat deposition (WO 02/066509).

It is thus clear from the above that Wnt/LRP signalling and antagonism by dkks is involved in a variety of human diseases. Little is known about the mechanism of modulation of the Wnt/LRP signal cascade by inhibitors of the dkk family.

Accordingly, means for the therapy or diagnosis of diseases associated with a dis-regulated signal cascade were not available. Thus, the use of reliable diagnostic molecular markers would be helpful for an understanding of the molecular basis of diseases associated with an aberrant Wnt signal cascade. It can be expected that such markers are also useful for therapy and for the development of novel therapeutic avenues for treatment of Wnt signal cascade dependent diseases, as detailed above.

Thus, the technical problem underlying the present invention is to provide means for diagnosis and therapy of diseases associated with an aberrant Wnt signal cascade.

The solution to said technical problem is achieved by

providing the embodiments characterized in the claims. During the experiments resulting in the present invention two genes, *kremen 1* and *2*, could be identified the products of which bind with high affinity to the polypeptides Dkk1 and Dkk2, which themselves are modulators of the Wnt receptors LRP5 and LRP6. It could be shown that this binding is of physiological relevance since cotransfection of cells with *dkk1* as well as *kremen 1* and *2* results in a synergistic inhibition of activation of the Wnt signal cascade. These data show that Kremen (1 and 2) can be regarded as a receptor for the Dkk polypeptides and that the biological function of Kremen is the mediation of inhibition of the Wnt-LRP signal cascade via Dkk polypeptides. The data obtained provide evidence that the expression of *kremen* is very widespread and that the genes encoding Kremen are involved in a variety of biological functions. Thus, Kremen is useful for the diagnosis and the development of therapies for Wnt-LRP mediated diseases, including but not limited to tumor suppression, bone formation, cholesterol and glucose metabolism (including diabetes), obesity, kidney disease and eye disease. It can be expected that, e.g., the inhibition of the Wnt signal cascade by increasing the expression of *kremen* and/or by stimulating the activity of the polypeptide itself might have a therapeutic effect. Likewise, it can be expected that, e.g., the activation of the Wnt signal cascade by decreasing the expression of *kremen* and/or by repressing the activity of the polypeptide itself might have a therapeutic effect. On the other hand, the Kremen receptor (or the gene encoding it) can be regarded as a drug target allowing the identification of compounds useful for therapy.

#### BRIEF DESCRIPTION OF THE DRAWINGS



Figure 1: Multisequence nucleic acid alignment of cDNAs encoding Kremen1 (krm1) and 2 (krm2) from mouse and human  
 hkrm1 and 2 are deduced from the human genome sequence in public data bases. Identical nucleotides are highlighted in black. All nucleic acid sequences begin with the translation initiator ATG codon.

Figure 2: Multisequence amino acid alignment of Kremen1 and 2 proteins deduced from mouse and human cDNAs (see Figure 1)  
 Identical amino acids are highlighted in black, similar amino acids are in grey.

Figure 3: Kremen is a high affinity receptor for Dkk1 and Dkk2  
 293T cells were transfected with cytomegalovirus (CMV) promoter-driven expression plasmids encoding *mkrm1* (top) or *mkrm2* (bottom) as indicated, incubated with recombinant Dkk1-AP, Dkk2-AP or Dkk3-AP and stained for bound AP activity. **TOP:** Binding curves and Scatchard analysis of Dkk-AP fusion proteins binding to *mkrm2* transfected cells. **Bottom:** Binding curves for Dkk-APs binding to *mkrm1* transfected cells. Dissociation constants ( $K_d$ ) are indicated; **a, c:** Binding curves; **b, d, e:** Scatchard analysis.

Figure 4: Kremen and Dkk1 synergistically inhibit the Wnt signal cascade

293 kidney cells were transfected with the Wnt reporter (TOP-FLASH) with or without the genes indicated. Two days after transfection, the luciferase activity expressed was determined. RLU: relative light units (normalized against cotransfected *Renilla* luciferase). Xdkk1 = *Xenopus* dkk1; mkrm1,2 = mouse kremen 1,2; wnt = mouse wnt1; fz = mouse frizzled8; lrp6 = human lrp6.

Figure 5: Expression of *kremen* in mice

The expression of *kremen 1* and *kremen 2* was analysed by RT-PCR in various tissues of adult mice. The results were normalized

using constitutive histon H4 expression. Abbreviations: -RT= control sample in which reverse transcriptase was omitted; sk muscle= skelletal muscle; mam. gland= mammary gland; H4= Histone 4 as loading control; mkrml,2= mouse kremen 1,2.

The present invention relates to a diagnostic composition comprising(a)at least one nucleic acid molecule which is capable of specifically hybridizing to the nucleotide sequence encoding Kremen 1 as depicted in Figure 1 and/or to the nucleotide sequence encoding Kremen 2 as depicted in Figure 2, or (b) at least one ligand which is capable of specifically binding to a Kremen 1 and/or Kremen 2 polypeptide.

As used herein the term „Kremen 1 polypeptide„ and „Kremen 2 polypeptide„ not only refers to polypeptids encoded by the nucleotide sequence as depicted in Figure 1 and/or 2 but also to polypeptides differing in amino acid sequence due to insertion, deletion and/or substitution of one ore more amino acids and showing at least one biological activity of a Kremen 1 and/or Kremen 2 receptor, e.g. the ability of signal transduction after ligand binding. Preferably, the related polypeptides are polypeptides the amino acid sequence of which shows an identity of at least 40%, in particular an identity of at least 65%, preferably of at least 80% and, particularly preferred, of at least 90% to the amino acid sequences of the polypeptides encoded by the nucleotide sequences shown in Figure 1 or 2.

The nucleic acid molecules useful as probes can be both DNA and RNA molecules, preferably they are single-stranded DNA molecules. They can be isolated from natural sources or can be synthesized according to known methods.



As a hybridization probe nucleic acid molecules can be used, for example, that have a nucleotide sequence which is exactly or basically complementary to a nucleotide sequence as depicted in Figure 1 and 2, respectively, or parts of these sequences. The fragments used as hybridization probe can be synthetic fragments that were produced by means of conventional synthetic methods

As used herein, the term „hybridizing„ relates to hybridization under conventional hybridization conditions, preferably under stringent conditions as described, for example, in Sambrook et al., Molecular Cloning, A Laboratory Manual 2<sup>nd</sup> edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. However, in certain cases, a hybridizing nucleic acid molecule can also be detected at lower stringency hybridization conditions. Changes in the stringency of hybridization and signal detection are primarily accomplished through the manipulation of formamide concentration (lower percentages of formamide result in lowered stringency), salt conditions, or temperature. For example, lower stringency conditions include an overnight incubation at 37°C in a solution comprising 6X SSPE (20X SSPE = 3M NaCl; 9.2M NaH<sub>2</sub>PO<sub>4</sub>; 0.02M EDTA, pH7.4), 0.5% SDS, 30% formamide, 100 µg/ml salmon sperm blocking DNA, following by washes at 50°C with 1 X SSPE, 0.1% SDS. In addition, to achieve even lower stringency, washes performed following stringent hybridization can be done at higher salt concentrations (e.g. 5X SSC). Variations in the above conditions may be accomplished through the inclusion and/or substitution of alternate blocking reagents used to suppress background in hybridization experiments. The inclusion of specific blocking reagents may require modification of the hybridization conditions described above, due to problems with compatibility.



The term „ligand,, as used herein refers to any molecule which is capable of specifically binding to Kremen 1 and/or Kremen 2, thus allowing to determine the level of receptor molecules. Examples of such molecules include antibodies, oligonucleotides, proteins or small molecules. The molecule can be the natural ligand of Kremen, i.e. Dkk1 or Dkk2, or can be closely related to said ligand, e.g., a fragment of the ligand, or a natural substrate, a ligand, a structural or functional mimetic; see, e.g., Coligan, Current Protocols in Immunology 1(2) (1991); Chapter 5. In either case, the molecule can be isolated or rationally designed using known techniques; see also *infra*.

Preferably, the ligand is an antibody. The term „antibody,, preferably, relates to antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations. Monoclonal antibodies are made from an antigen containing fragments of Kremen 1 or Kremen 2 by methods well known to those skilled in the art (see, e.g., Köhler et al., Nature 256 (1975), 495). As used herein, the term „antibody,, (Ab) or „monoclonal antibody,, (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')<sub>2</sub> fragments) which are capable of specifically binding to Kremen. Fab and f(ab')<sub>2</sub> fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody. (Wahl et al., J. Nucl. Med. 24: 316-325 (1983)). Thus, these fragments are preferred, as well as the products of a FAB or other immunoglobulin expression library. Moreover, antibodies of the present invention include chimerical, single chain, and humanized antibodies.

For certain purposes, e.g. diagnostic methods, the nucleic acid molecule used as probe or the ligand, e.g., antibody, can be detectably labeled, for example, with a radioisotope, a

bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, or an enzyme.

The nucleic acid molecules can be used, for example, as probes or primers in the diagnostic assays described below and allow, e.g., the analysis of the expression of *kremen 1* and *2* by determining the mRNA level or the determination of mutations within the coding region or regulatory regions leading to polypeptide molecules with altered, e.g. destroyed, activity, or leading to altered expression. Preferably, the nucleic acid molecules are oligonucleotides having a length of at least 10, in particular of at least 15 and particularly preferred of at least 50 nucleotides. These nucleic acid molecules of the invention can also be used, for example, as primers for a PCR reaction.

The present invention also relates to the use of a nucleic acid molecule or ligand as defined above for the preparation of a diagnostic composition for the diagnosis of a disease associated with (a) aberrant expression of *kremen 1* and/or *kremen 2* and/or (b) aberrant activity of a Kremen 1 and/or Kremen 2 polypeptide.

In a preferred embodiment, the target to which the nucleic acid molecule hybridizes is an mRNA.

The present invention also provides a method of diagnosing a disease associated with (a) aberrant expression of *kremen 1* and/or *kremen 2* and/or (b) aberrant activities or amounts of a Kremen 1 and/or Kremen 2 polypeptide in a subject comprising:

- (a) determining (a) the amount of expression of *kremen 1* and/or *kremen 2* and/or (b) the amount of biologically active Kremen 1 and/or Kremen 2 polypeptide in a biological sample; and
- (b) diagnosing a disease associated with (a) aberrant expression of *kremen 1* and/or *kremen 2* and/or (b) aberrant activities or amounts of a Kremen 1 and/or Kremen 2



polypeptide or a risk for the development of such disease based on an altered amount of expression of *kremen 1* and/or *kremen 2* and/or (b) altered activities or amounts of biologically active Kremen 1 and/or Kremen 2 polypeptide compared to a control.

Suitable assay formats are well known to the person skilled in the art and, in addition, described below. Suitable positive control samples expressing human kremen 1 and 2 protein are, e.g., HEK 293 cells.

The Kremen 1 or 2 polypeptide or the corresponding mRNA, e.g. in biological fluids or tissues, may be detected directly in situ, e.g. by in situ hybridization or it may be isolated from other cell components by common methods known to those skilled in the art before contacting with a probe. Detection methods include Northern Blot analysis, RNase protection, in situ methods, e.g. in situ hybridization, in vitro amplification methods (PCR, LCR, QRNA replicase or RNA-transcription/amplification (TAS, 3SR), reverse dot blot disclosed in EP-B1 0 237 362), immunoassays, Western Blot and other detection assays that are known to those skilled in the art.

The probe (e.g. a specific antibody or specific oligonucleotide) of the diagnostic composition can be detectably labeled. In a preferred embodiment, said diagnostic composition contains an anti-Kremen 1 or -Kremen-2 antibody and allows said diagnosis, e.g., by ELISA and contains the antibody bound to a solid support, for example, a polystyrene microtiter dish or nitrocellulose paper, using techniques known in the art. Alternatively, said diagnostic compositions are based on a RIA and contain said antibody marked with a radioactive isotope. Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase, and radioisotopes, such as iodine ( $^{125}\text{I}$ ,  $^{121}\text{I}$ ), carbon



( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{112}\text{In}$ ), and technetium rhodamine, and biotin. In addition to assaying Kremen levels in a biological sample, the polypeptide can also be detected in vivo by imaging. Antibody labels or markers for in vivo imaging of protein include those detectable by X-radiography, NMR or ESR. For X-radiography, suitable labels include radioisotopes such as barium or cesium, which emit detectable radiation but are not overtly harmful to the subject. Suitable markers for NMR and ESR include those with a detectable characteristic spin, such as deuterium, which may be incorporated into the antibody by labeling of nutrients for the relevant hybridoma. A protein-specific antibody or antibody fragment which has been labeled with an appropriate detectable imaging moiety, such as a radioisotope (for example,  $^{131}\text{I}$ ,  $^{112}\text{In}$ ,  $^{99}\text{mTc}$ ), a radio-opaque substance, or a material detectable by nuclear magnetic resonance, is introduced (for example, parenterally, subcutaneously, or intraperitoneally) into the mammal. It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of  $^{99}\text{mTc}$ . The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific Kremen polypeptide. In vivo tumor imaging is, e.g., described in S.W. Burchiel et al., „Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments„. (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B.A. Rhodes, eds., Masson Publishing Inc. (1982)).

In a further aspect, the present invention, relates to a method for identifying a binding partner to a Kremen 1 and/or 2 polypeptide comprising:

- (a) contacting said polypeptide with a compound to be screened; and
- (b) determining whether the compound effects an activity of the polypeptide.

The invention also includes a method of identifying compounds which bind to a Kremen 1 and/or Kremen 2 polypeptide comprising the steps of:

- (a) incubating a candidate binding compound with said polypeptide; and
- (b) determining if binding has occurred.

Kremen 1 or 2 polypeptides may be used to screen for proteins or other compounds that bind to Kremen 1 or 2 or for proteins or other compounds to which Kremen 1 and 2 bind. The binding of Kremen 1 or 2 and the molecule may activate (agonist), increase, inhibit (antagonist), or decrease activity of Kremen 1 or Kremen 2 or the molecule bound. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., ligands), or small molecules.

Preferably, the molecule is closely related to the natural ligand of Kremen 1 or 2, e.g., a fragment of the ligand, or a natural substrate, a ligand, a structural or functional mimetic; see, e.g., Coligan, Current Protocols in Immunology 1(2) (1991); Chapter 5.

Preferably, the screening for these molecules involves producing appropriate cells which express Kremen 1 and/or, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, Drosophila, or E. coli. Cells expressing Kremen 1 and/or 2 (or cell membrane containing the expressed polypeptide) are then preferably contacted with a test compound potentially containing the molecule to observe binding, stimulation, or inhibition of activity of Kremen 1 and/or 2.



The assay may simply test binding of a candidate compound to Kremen 1 and/or 2, wherein binding is detected by a label, or in an assay involving competition with a labeled competitor. Further, the assay may test whether the candidate compound results in a signal generated by binding to Kremen 1 and/or Kremen 2. Suitable assays to analyze the activity of kremen 1 and /or 2 include Wnt-inducible luciferase reporter assays in transfected HEK 293 cells, where dkk1 synergizes with kremen 1 and /or 2 to inhibit a Wnt1-induced signal, such as is shown in Figure 4.

Alternatively, the assay can be carried out using cell-free preparations, polypeptide/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate compound with a solution containing Kremen 1 and/or Kremen 2, measuring Kremen/molecule activity or binding, and comparing the Kremen/molecule activity or binding to a standard.

Preferably, an ELISA assay can measure Kremen 1 and/or Kremen 2 level or activity in a sample (e.g., biological sample) using a monoclonal or polyclonal antibody. The antibody can measure Kremen 1 and/or Kremen 2 level or activity by either binding, directly or indirectly, to Kremen 1 and/or Kremen 2 or by competing with Kremen 1 and/or Kremen 2 for a substrate. All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., elimination of a tumor, support of regenerative processes etc.) by modulating, preferably activating the Kremen 1 and/or Kremen 2 molecule. Moreover, the assays can discover agents which may inhibit or enhance the production of Kremen 1 and/or Kremen 2 from suitably manipulated cells or tissues.



Moreover, the invention includes a method of identifying activators/agonists or inhibitors/antagonists of a Kremen 1 and/or Kremen 2 polypeptide comprising the steps of:

- (a) incubating a candidate compound with said polypeptide;
- (b) assaying a biological activity, and
- (c) determining if a biological activity of said polypeptide has been altered.

Suitable assays include analysis of formation of a ternary complex between kremen1 or kremen 2 with recombinant Dkk1 protein and recombinant extracellular domain of LRP6.

In a further embodiment, the present invention relates to method of identifying and obtaining a drug candidate for therapy of diseases associated with (a) aberrant expression of *kremen 1* and/or *kremen 2* and/or (b) aberrant activities or amounts of a Kremen 1 and/or Kremen 2 polypeptide comprising the steps of

- (a) contacting a Kremen 1 and/or Kremen 2 polypeptide or a cell expressing said polypeptide, and optionally the corresponding ligand(s), in the presence of components capable of providing a detectable signal in response to binding to said drug candidate to be screened; and
- (b) detecting presence or absence of a signal or increase of the signal generated, wherein the presence or increase of the signal is indicative for a putative drug.

Suitable assays to analyze the activity of kremen 1 and /or 2 include Wnt-inducible luciferase reporter assays in transfected HEK 293 cells, where dkk1 synergizes with kremen 1 and /or 2 to inhibit a Wnt1-induced signal, such as is shown in Figure 4.

The drug candidate may be a single compound or a plurality of compounds. The term "plurality of compounds" in a method of

the invention is to be understood as a plurality of substances which may or may not be identical.

Said compound or plurality of compounds may be chemically synthesized or microbiologically produced and/or comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating Kremen 1 and/or Kremen 2 polypeptides. The reaction mixture may be a cell free extract or may comprise a cell or tissue culture. Suitable set ups for the method of the invention are known to the person skilled in the art and are, for example, generally described in Alberts et al., Molecular Biology of the Cell, third edition (1994) and in the appended examples. The plurality of compounds may be, e.g., added to the reaction mixture, culture medium, injected into a cell or otherwise applied to a transgenic animal. The cell or tissue that may be employed in the method of the invention preferably is a host cell, mammalian cell or non-human transgenic animal.

If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating a Kremen 1 and/or Kremen 2 polypeptide, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances



of similar chemical and/or physical properties, and most preferably said substances are identical.

Several methods are known to the person skilled in the art for producing and screening large libraries to identify compounds having specific affinity for a target. These methods include the phage-display method in which randomized peptides are displayed from phage and screened by affinity chromatography to an immobilized receptor; see, e.g., WO 91/17271, WO 92/01047, US-A-5,223,409. In another approach, combinatorial libraries of polymers immobilized on a chip are synthesized using photolithography; see, e.g., US-A-5,143,854, WO 90/15070 and WO 92/10092. The immobilized polymers are contacted with a labeled receptor and scanned for label to identify polymers binding to the receptor. The synthesis and screening of peptide libraries on continuous cellulose membrane supports that can be used for identifying binding ligands of the Kremen 1 and/or 2 polypeptides and, thus, possible inhibitors and activators is described, for example, in Kramer, *Methods Mol. Biol.* 87 (1998), 25-39. This method can also be used, for example, for determining the binding sites and the recognition motifs in the Kremen 1 and/or 2 polypeptide. In like manner, the substrate specificity of the DnaK chaperon was determined and the contact sites between human interleukin-6 and its receptor; see Rudiger, *EMBO J.* 16 (1997), 1501-1507 and Weiergraber, *FEBS Lett.* 379 (1996), 122-126, respectively. Furthermore, the above-mentioned methods can be used for the construction of binding supertopes derived from the Kremen 1 or Kremen 2 polypeptide. A similar approach was successfully described for peptide antigens of the anti-p24 (HIV-1) monoclonal antibody; see Kramer, *Cell* 91 (1997), 799-809. A general route to fingerprint analyses of peptide-antibody interactions using the clustered amino acid peptide library was described in Kramer, *Mol. Immunol.* 32 (1995), 459-465. In addition, antagonists of a Kremen 1 and/or Kremen 2 polypeptide can be derived and identified from monoclonal



antibodies that specifically react with a Kremen 1 and/or Kremen 2 polypeptide in accordance with the methods as described in Doring, Mol. Immunol. 31 (1994), 1059-1067.

All these methods can be used in accordance with the present invention to identify activators/agonists and inhibitors/antagonists of a Kremen 1 and/or Kremen 2 polypeptide.

Various sources for the basic structure of such an activator or inhibitor can be employed and comprise, for example, mimetic analogs of a Kremen 1 and/or Kremen 2 polypeptide. Mimetic analogs of a Kremen 1 and/or Kremen 2 polypeptide or biologically active fragments thereof can be generated by, for example, substituting the amino acids that are expected to be essential for the biological activity with, e.g., stereoisomers, i.e. D-amino acids; see e.g., Tsukida, J. Med. Chem. 40 (1997), 3534-3541. Furthermore, in case fragments are used for the design of biologically active analogs pro-mimetic components can be incorporated into a peptide to reestablish at least some of the conformational properties that may have been lost upon removal of part of the original polypeptide; see, e.g., Nachman, Regul. Pept. 57 (1995), 359-370. Furthermore, a Kremen 1 and/or Kremen 2 polypeptide can be used to identify synthetic chemical peptide mimetics that bind to or can function as a ligand, substrate or binding partner of said polypeptide(s) as effectively as does the natural polypeptide; see, e.g., Engleman, J. Clin. Invest. 99 (1997), 2284-2292. For example, folding simulations and computer redesign of structural motifs of a Kremen 1 and/or Kremen 2 polypeptide can be performed using appropriate computer programs (Olszewski, Proteins 25 (1996), 286-299; Hoffman, Comput. Appl. Biosci. 11 (1995), 675-679). Computer modeling of protein folding can be used for the conformational and energetic analysis of detailed peptide and protein models (Monge, J. Mol. Biol. 247 (1995), 995-1012; Renouf, Adv. Exp. Med. Biol. 376 (1995), 37-45). In particular,

the appropriate programs can be used for the identification of interactive sites of a Kremen 1 and/or Kremen 2 polypeptide and its ligand or other interacting proteins by computer assistant searches for complementary peptide sequences (Fassina, Immunomethods 5 (1994), 114-120. Further appropriate computer systems for the design of protein and peptides are described in the prior art, for example in Berry, Biochem. Soc. Trans. 22 (1994), 1033-1036; Wodak, Ann. N. Y. Acad. Sci. 501 (1987), 1-13; Pabo, Biochemistry 25 (1986), 5987-5991. The results obtained from the above-described computer analysis can be used for, e.g., the preparation of peptide mimetics of a Kremen 1 and/or Kremen 2 polypeptide or fragments thereof. Such pseudopeptide analogues of the natural amino acid sequence of the protein may very efficiently mimic the parent protein (Benkirane, J. Biol. Chem. 271 (1996), 33218-33224). For example, incorporation of easily available achiral  $\omega$ -amino acid residues into a Kremen 1 or 2 polypeptide or a fragment thereof results in the substitution of amide bonds by polymethylene units of an aliphatic chain, thereby providing a convenient strategy for constructing a peptide mimetic (Banerjee, Biopolymers 39 (1996), 769-777). Superactive peptidomimetic analogues of small peptide hormones in other systems are described in the prior art (Zhang, Biochem. Biophys. Res. Commun. 224 (1996), 327-331). Appropriate peptide mimetics of a Kremen 1 and/or Kremen 2 polypeptide can also be identified by the synthesis of peptide mimetic combinatorial libraries through successive amide alkylation and testing the resulting compounds, e.g., for their binding and immunological properties. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715. Furthermore, a three-dimensional and/or crystallographic structure of a Kremen 1 and/or Kremen 2 polypeptide can be used for the design of peptide mimetic inhibitors of the biological activity of the



polypeptide (Rose, Biochemistry 35 (1996), 12933-12944; Rutenber, Bioorg. Med. Chem. 4 (1996), 1545-1558).

It is also well known to the person skilled in the art, that it is possible to design, synthesize and evaluate mimetics of small organic compounds that, for example, can act as a substrate or ligand to a Kremen 1 and/or Kremen 2 polypeptide. For example, it has been described that D-glucose mimetics of hapalosin exhibited similar efficiency as hapalosin in antagonizing multidrug resistance assistance-associated protein in cytotoxicity; see Dinh, J. Med. Chem. 41 (1998), 981-987.

The nucleic acid molecule encoding a Kremen 1 and/or Kremen 2 polypeptide can also serve as a target for activators and inhibitors. Activators may comprise, for example, proteins that bind to the mRNA of a gene encoding a Kremen 1 and/or Kremen 2 polypeptide, thereby stabilizing the native conformation of the mRNA and facilitating transcription and/or translation, e.g., in like manner as Tat protein acts on HIV-RNA. Furthermore, methods are described in the literature for identifying nucleic acid molecules such as an RNA fragment that mimics the structure of a defined or undefined target RNA molecule to which a compound binds inside of a cell resulting in retardation of cell growth or cell death; see, e.g., WO 98/18947 and references cited therein. These nucleic acid molecules can be used for identifying unknown compounds of pharmaceutical interest, and for identifying unknown RNA targets for use in treating a disease. These methods and compositions can be used in screening for novel or for identifying compounds useful to alter expression levels of polypeptides encoded by a nucleic acid molecule. Alternatively, for example, the conformational structure of the RNA fragment which mimics the binding site can be employed in rational drug design to modify known drugs to make them bind more avidly to the target. One such methodology is nuclear magnetic resonance

(NMR), which is useful to identify drug and RNA conformational structures. Still other methods are, for example, the drug design methods as described in WO 95/35367, US-A-5,322,933, where the crystal structure of the RNA fragment can be deduced and computer programs are utilized to design novel binding compounds.

The compounds which can be tested and identified according to a method of the invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, peptidomimetics, PNAs or the like (Milner, Nature Medicine 1 (1995), 879-880; Hupp, Cell 83 (1995), 237-245; Gibbs, Cell 79 (1994), 193-198 and references cited supra). Furthermore, genes encoding a putative regulator of a Kremen 1 and/or Kremen 2 polypeptide and/or which exert their effects up- or downstream a Kremen 1 and/or Kremen 2 polypeptide may be identified using, for example, insertion mutagenesis using, for example, gene targeting vectors known in the art. Said compounds can also be functional derivatives or analogues of known inhibitors or activators. Such useful compounds can be for example transacting factors which bind to a Kremen 1 and/or Kremen 2 polypeptide or regulatory sequences of the gene encoding it. Identification of transacting factors can be carried out using standard methods in the art (see, e.g., Sambrook, supra). To determine whether a protein binds to the protein itself or regulatory sequences, standard native gel-shift analyses can be carried out. In order to identify a transacting factor which binds to the protein or regulatory sequence, the protein or regulatory sequence can be used as an affinity reagent in standard protein purification methods, or as a probe for screening an expression library. The identification of nucleic acid molecules which encode polypeptides which interact with a Kremen 1 and/or Kremen 2 polypeptide described above can also be achieved, for example, as described in Scofield (Science 274 (1996), 2063-2065) by use



of the so-called yeast "two-hybrid system". In this system the Kremen 1 or Kremen 2 polypeptide or a smaller part thereof is linked to the DNA-binding domain of the GAL4 transcription factor. A yeast strain expressing this fusion polypeptide and comprising a lacZ reporter gene driven by an appropriate promoter, which is recognized by the GAL4 transcription factor, is transformed with a library of cDNAs which will express plant proteins or peptides thereof fused to an activation domain. Thus, if a peptide encoded by one of the cDNAs is able to interact with the fusion peptide comprising a peptide of a Kremen 1 and/or Kremen 2 polypeptide, the complex is able to direct expression of the reporter gene. In this way the nucleic acid molecules encoding Kremen 1 and Kremen 2, respectively, and the encoded peptide can be used to identify peptides and proteins interacting with a Kremen 1 and/or Kremen 2 polypeptide.

Once the transacting factor is identified, modulation of its binding to or regulation of expression of a Kremen 1 and/or Kremen 2 polypeptide can be pursued, beginning with, for example, screening for inhibitors against the binding of the transacting factor to a Kremen 1 or Kremen 2 polypeptide. Activation or repression of a Kremen 1 and/or Kremen 2 polypeptide could then be achieved in animals by applying the transacting factor (or its inhibitor) or the gene encoding it, e.g. in an expression vector. In addition, if the active form of the transacting factor is a dimer, dominant-negative mutants of the transacting factor could be made in order to inhibit its activity. Furthermore, upon identification of the transacting factor, further components in the signal cascade leading to activation (e.g. signal transduction) or repression of a gene involved in the control of a Kremen 1 and/or Kremen 2 polypeptide then can be identified. Modulation of the activities of these components can then be pursued, in order to develop additional drugs and methods for modulating the metabolism of protein degradation in animals. Thus, the

present invention also relates to the use of the two-hybrid system as defined above for the identification of activators or inhibitors of a Kremen 1 and/or Kremen 2 polypeptide.

The compounds isolated by the above methods also serve as lead compounds for the development of analog compounds. The analogs should have a stabilized electronic configuration and molecular conformation that allows key functional groups to be presented to a Kremen 1 and/or Kremen 2 polypeptide or its ligand in substantially the same way as the lead compound. In particular, the analog compounds have spatial electronic properties which are comparable to the binding region, but can be smaller molecules than the lead compound, frequently having a molecular weight below about 2 kD and preferably below about 1 kD. Identification of analog compounds can be performed through use of techniques such as self-consistent field (SCF) analysis, configuration interaction (CI) analysis, and normal mode dynamics analysis. Computer programs for implementing these techniques are available; e.g., Rein, Computer-Assisted Modeling of Receptor-Ligand Interactions (Alan Liss, New York, 1989). Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, Handbook of Organic Chemistry, Springer edition New York Inc., 175 Fifth Avenue, New York, N.Y. 10010 U.S.A. and Organic Synthesis, Wiley, New York, USA. Furthermore, said derivatives and analogues can be tested for their effects according to methods known in the art; see also supra. Furthermore, peptidomimetics and/or computer aided design of appropriate derivatives and analogues can be used, for example, according to the methods described above.

Once the described compound has been identified and obtained, it is preferably provided in a therapeutically acceptable form.



Accordingly, the present invention also relates to a pharmaceutical composition comprising a nucleic acid molecule encoding a Kremen 1 and/or Kremen 2 polypeptide, a Kremen 1 and/or Kremen 2 polypeptide itself, recombinant vector (for examples, see below), antibody, activator/agonist, inhibitor/antagonist and/or binding partner of a Kremen 1 and/or Kremen 2 polypeptide and a pharmaceutically acceptable excipient, diluent or carrier.

Preferably, for therapeutic purposes, the Kremen 1 and/or Kremen 2 polypeptide is recombinantly produced by use of the nucleic acid sequences shown in Figures 1 and 2. Suitable vectors for recombinant expression are known to the person skilled in the art. Preferably, they are plasmids, cosmids, viruses, bacteriophages and other vectors usually used in the field of genetic engineering. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in mammalian cells and baculovirus-derived vectors for expression in insect cells. Preferably, the nucleic acid molecule of the invention is operatively linked to the regulatory elements in the recombinant vector of the invention that guarantee the transcription and synthesis of an mRNA in prokaryotic and/or eukaryotic cells that can be translated. The nucleotide sequence to be transcribed can be operably linked to a promoter like a T7, metallothionein I or polyhedrin promoter. The host cells used for recombinant expression are prokaryotic or eukaryotic cells, for example mammalian cells, bacterial cells, insect cells or yeast cells. The polypeptide is isolated from the cultivated cells and/or the culture medium. Isolation and purification of the recombinantly produced polypeptide may be carried out by conventional means including preparative chromatography and affinity and immunological separations using, e.g., an anti-Kremen 1 or 2 antibody, or, e.g., can be substantially purified by the one-step method described in Smith and Johnson, Gene 67; 31-40 (1988).

Examples of suitable pharmaceutical carriers etc. are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The route of administration, of course, depends on the nature of the disease and the kind of compound contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind and stage of the disease, e.g., tumor, general health and other drugs being administered concurrently.

The delivery of the nucleic acid molecules encoding a Kremen 1 and/or Kremen 2 polypeptide can be achieved by direct application or, preferably, by using a recombinant expression vector such as a chimeric virus containing these compounds or a colloidal dispersion system. Direct application to the target site can be performed, e.g., by ballistic delivery, as a colloidal dispersion system or by catheter to a site in artery. The colloidal dispersion systems which can be used for delivery of the above nucleic acid molecules include macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions (mixed), micelles, liposomes and lipoplexes. The preferred colloidal system is a liposome. Organ-specific or cell-specific liposomes can be used in order to achieve delivery only to the desired tissue. The targeting of liposomes can be carried out



by the person skilled in the art by applying commonly known methods. This targeting includes passive targeting (utilizing the natural tendency of the liposomes to distribute to cells of the RES in organs which contain sinusoidal capillaries) or active targeting (for example by coupling the liposome to a specific ligand, e.g., an antibody, a receptor, sugar, glycolipid, protein etc., by well known methods). In the present invention monoclonal antibodies are preferably used to target liposomes to specific tissues, e.g. tumor tissue, via specific cell-surface ligands.

Preferred recombinant vectors useful for gene therapy are viral vectors, e.g. adenovirus, herpes virus, vaccinia, or, more preferably, an RNA virus such as a Retrovirus. Even more preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of such retroviral vectors which can be used in the present invention are: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV) and Rous sarcoma virus (RSV). Most preferably, a non-human primate retroviral vector is employed, such as the gibbon ape leukemia virus (GaLV), providing a broader host range compared to murine vectors. Since recombinant retroviruses are defective, assistance is required in order to produce infectious particles. Such assistance can be provided, e.g., by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. Suitable helper cell lines are well known to those skilled in the art. Said vectors can additionally contain a gene encoding a selectable marker so that the transduced cells can be identified. Moreover, the retroviral vectors can be modified in such a way that they become target specific. This can be achieved, e.g., by inserting a polynucleotide encoding a sugar, a glycolipid, or a protein, preferably an antibody. Those skilled in the art know additional methods for generating target specific vectors.

Further suitable vectors and methods for in vitro- or in vivo-gene therapy are described in the literature and are known to the persons skilled in the art; see, e.g., WO 94/29469 or WO 97/00957.

In order to achieve expression only in the target organ, e.g., a tumor to be treated, the nucleic acid molecules encoding a Kremen 1 and/or Kremen 2 polypeptide can be linked to a tissue specific promoter and used for gene therapy. Such promoters are well known to those skilled in the art (see e.g. Zimmermann et al., (1994) Neuron 12, 11-24; Vidal et al.; (1990) EMBO J. 9, 833-840; Mayford et al., (1995), Cell 81, 891-904; Pinkert et al., (1987) Genes & Dev. 1, 268-76).

The present invention also relates to the use of the above compounds of the invention for the preparation of a pharmaceutical composition for treatment of a disease associated with (a) aberrant expression of *kremen 1*, *kremen 2* and/or genes involved into the Wnt signal cascade, and/or (b) aberrant activities or amounts of a Kremen 1, Kremen 2 and/or a polypeptide involved into the Wnt signal cascade. In a preferred embodiment, said disease is a tumor, preferably breast cancer, a colon carcinoma or a melanoma.

Finally, the present invention relates to the use of a nucleotide molecule encoding a polypeptide having a biological activity of Kremen 1 and/or Kremen 2, a Kremen 1 and/or Kremen 2 polypeptide, an activator/agonist of a Kremen 1 and/or Kremen 2 polypeptide or binding partner of said polypeptide(s) for the preparation of a pharmaceutical composition for inhibiting the Wnt signal cascade which might be useful for supporting regenerative processes in a patient, e.g. growth of tissue like muscle, hair, etc.

The following examples illustrate the invention.



### Example 1

#### Isolation of cDNAs encoding Kremen 1 and 2, respectively

A mouse 13.5 day embryo cDNA library in the expression vector pCMV-SPORT2 (Gibco BRL) was used to prepare pools of about 250 colonies, and plasmid DNA from each pool was transiently transfected into 293T cells in 24-well plates using FuGENE 6 (Roche). After 48 hours cells were incubated with medium containing 1nM Dkk1-alkaline phosphatase (Dkk1-AP) fusion protein (Mao et al., Nature 411 (2001) 321-325) and processed for AP histochemistry. From 1500 pools, 2 positive pools were identified and single clones were isolated by sib selection. Sequencing analysis showed that they represent independent isolates of *mkremen 2*. A full length mouse *kremen 1* clone was isolated from the same library by PCR using published nucleotide sequence data (Nakamura et al, Biochim. Biophys. Acta 1518 (2001), 63-72). The open reading frame of *mkremen 1* and -2 was cloned into pCS2+ to generate pCS2-mkrm1 and -2. pCS-flag-mkrm2 was constructed by inserting a flag epitope after the signal peptide and was used as template to generate the pCS-flag-mkrm2 $\Delta$ WSC by PCR.

### Example 2

#### The binding of Kremen 1 and 2 to Dkk1 and Dkk2 shows high affinity and is physiologically relevant

For binding assays 293T cells were transfected (T) with *mkrm1* or *mkrm2* as indicated, incubated with recombinant Dkk1-alkaline phosphatase fusion protein (Dkk1-AP) or alkaline phosphatase (AP) and stained for bound AP activity. The results are shown in Figure 3.

As shown in Figure 4, luciferase Wnt reporter assays in 293T cells were done in 96 well plates at least in triplicates as described (Wu et al., Curr Biol 10 (2000), 1611-1614). Luciferase activity was normalized against Renilla activity using a commercial kit (Clontech). Xdkk1= Xenopus dkk1 (Glinka, et al. Nature 391, (1998) 357-362); mkrml,2= mouse kremen 1,2; wnt= mouse wnt1; fz= mouse frizzled8; lrp6= human lrp6 (Tamai, et al.. Nature 407 (2000) 530-535); Wnt luciferase reporter TOP-FLASH (Korinek et al. Science 275 (1997)1784-1787).

As shown in Figure 3, the binding of Dkk alkaline phosphatase fusion protein to Kremen 2 and Kremen 1, respectively, shows high affinity. Moreover, it could be shown that only Dkk1 and Dkk2 bind to Kremen but not Dkk3.

In an additional experiment, 293 kidney cells were transfected with the Wnt reporter (TOP-FLASH) with or without the genes indicated. Two days after transfection, the luciferase activity expressed was determined. As shown in Figure 4, cotransfection of Wnt and its receptor, *frizzled* (fz) results in stimulation of the Wnt signal cascade (see Figure 4, lane 1 versus lane 2) and cotransfection of *dkk1* and *kremen 1* and *kremen 2* leads to synergistic inhibition of this activation of the Wnt signal cascade. This effect is even more pronounced if *wnt* has been cotransfected with its receptor *frizzled* (fz) and the co-receptor *lrp6*. A very strong activation of the Wnt signal cascade (lane 8) can be observed. This activation can only inhibited by cotransfection with *dkk1* and *kremen 1,2* (lanes 12 and 13) but not by transfection with the single genes (*dkk1*, lane 9; *kremen 2*, lane 10; *kremen 1*, lane 11).

### Example 3

Determination of the expression profile of kremen 1 and 2 in various tissues of mice



The expression of *kremen 1* and *2* in various tissues of mice was studied by RT-PCR. RNA isolation from adult mouse organs and RT-PCR assays were carried out in the linear phase of amplification and with histone 4 primers as described (Glinka et al., Nature 389 (1997), 517-519) Other primers were: *mkrm1* (f, GTGCTTCACAGCCAACGGTGCA; r, ACGTAGCACCAAGGGCTCACGT); *mkrm2* (f, AGGGAAACTGGTCGGCTC; r, AAGGCACGGAGTAGGTTGC). Cycle no. were *H4*: 26 cycles; *mkrm1*: 35 cycles; *mkrm2*: 32 cycles. The results show that both *kremens* are expressed in all mouse tissues tested, but with varying expression level (Figure 5). Similar results were obtained using *Xenopus* embryos.

## SEQUENCE LISTING

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1428

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&lt;213&gt; Mus musculus

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Pro Pro Pro Leu Thr Gly Thr Ser Lys Thr Ser Asn Lys Leu Thr Ile
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Gln Thr Cys Ile Ser Phe Cys Arg Ser Gln Arg Phe Lys Phe Ala Gly
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**WE CLAIM:**

1. *In-vitro* use of a nucleic acid molecule encoding a Kremen 1 and/or Kremen 2 polypeptide, or a Kremen 1 and/or Kremen 2 polypeptide, for inhibiting the Wnt signal cascade, wherein said Wnt cascade comprises Dkk1/Dkk2, LRP5/LRP6 and frizzled.
2. An *in-vitro* method for identifying a compound for modulating the Wnt signal cascade which is based on identifying a binding partner to a Kremen 1 and/or Kremen 2 polypeptide comprising:
  - (a) contacting said polypeptide with a compound to be screened; and
  - (b) determining whether the compound affects an activity of said polypeptide, wherein said activity is the activity in the Wnt cascade comprising Dkk1/Dkk2, LRP5/LRP6 and frizzled.
3. An *in-vitro* method for identifying a compound for modulating the Wnt signal cascade which is based on identifying a binding partner to a Kremen 1 and/or Kremen 2 polypeptide comprising:
  - (a) contacting said polypeptide with a compound to be screened;
  - (b) determining whether binding of the compound to said polypeptide has occurred; and
  - (c) determining whether modulation of Wnt signalling cascade occurred by measuring Wnt1-induced signal.



4. The method of claim 2 or 3, wherein said activity of the Wnt cascade is measured using a Wnt-inducible luciferase reporter assay.
5. An *in-vitro* method for identifying a compound for modulating the Wnt signal cascade as an activator/agonist or inhibitor/antagonist of a Kremen 1 and/or Kremen 2 polypeptide comprising the steps of:
  - (a) incubating a candidate compound with said polypeptide; and
  - (b) assaying a biological activity, wherein said activity is the activity in the Wnt cascade comprising Dkk1/Dkk2, LRP5/LRP6 and frizzled; and
  - (c) determining if the biological activity of said polypeptide has been altered.
6. The method according to any one of claims 2 to 5, wherein the compound to be screened is an antibody that recognises Kremen 1 and/or Kremen 2.
7. The method according to any one of claims 2 to 5, wherein the compound to be screened is a small molecule.
8. The method according to any one of claims 2 to 5, wherein the compound to be screened is a nucleic acid.
9. The method according to any one of claims 2 to 8, wherein said method is carried out using cells which express Kremen 1 and/or Kremen 2.

10. The method according to any one of claims 2 to 8, wherein said method is carried out using cell-free preparations.
11. A pharmaceutical composition containing an antibody or fragment thereof specifically binding to a Kremen 1 and/or Kremen 2 polypeptide and a pharmaceutically acceptable carrier, for activating the Wnt signal cascade, wherein said Wnt cascade comprises Dkk1/Dkk2, LRP5/LRP6 and frizzled.
12. The pharmaceutical composition of claim 11, wherein said antibody is a monoclonal antibody.
13. The pharmaceutical composition of claim 11, wherein said antibody is a single chain antibody.
14. In-vitro use of an antibody or fragment thereof specifically binding to a Kremen 1 and/or Kremen 2 polypeptide for activating the Wnt signal cascade, wherein said Wnt cascade comprises Dkk1/Dkk2, LRP5/LRP6 and frizzled.
15. The use of claim 14, wherein said antibody is a monoclonal antibody.
16. The use of claim 14, wherein said antibody is a single chain antibody.



1/7

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hkrm2	A A T G G G G C T G	A C T A C C G C G G	C C A C C A G A A C	C G G A C T G G C C	157
mkrm1	T G C A . A G G . .	T G G G A A G C C A	T G T C T G T T C T	G G A A C G A G A C	182
hkrm1	T A C A . A G G . .	G G G G A A G C C A	T G T C T G T T T T	G G A A C G A G A C	182
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mkrm1	T T T C C A G C A T	C C G T A C A A C A	C G C T G A A G T A	C C C C A A C G G G	222
hkrm1	T T T C C A G C A T	C C A T A C A A C A	C T C T G A A A T A	C C C C A A C G G G	222
mkrm2	A C A G C A G C A C	A G C T A C A G C A	G C G C C A G C G A	C C C T C A G G G C	234
hkrm2	G C A G C A A C A C	A G C T A C A G C A	G G G C C A G C G A	C C C C C A C G G C	237
mkrm1	G A A G G A G G A C	T G G G G G A G C A	C A A T T A T T G C	A G A A A T C C A G	262
hkrm1	G A G G G G G G C C	T G G G T G A G C A	C A A C T A T T G C	A G A A A T C C A G	262
mkrm2	C G C T G G G G G T	T G G G T G C G C A	T A A C T T C T G T	A G G A A C C C A G	274
hkrm2	C G C T G G G G G C	T G G G C G C G C A	C A A C T T C T G C	C G T A A C C C A G	277
mkrm1	A T G G A G A G G T	G A G C C C T T G G	T G C T A C G T G G	C C G A G C A T G A	302
hkrm1	A T G G A G A G G T	G A G C C C G T G G	T G C T A T G T G G	C A G A G C A C G A	302
mkrm2	A C G G T G A T G T	G C A G C C C T G G	T G C T A C G T G G	C A G A G A C A G A	314
hkrm2	A C G G T G A C G T	G C A G C C G T G G	T G C T A C G T G G	C T G A G A C A G A	317
mkrm1	G G A C G G A G T C	T A C T G G A A G T	A C T G T G A A A T	T C C T G C C T G C	342
hkrm1	G G A T G G T G T C	T A C T G G A A G T	A C T G T G A G A T	A C C T G C C T G C	342
mkrm2	A G A G G G C A T C	T A C T G G G G G T	A C T G T G A T A T	C C C C A C A T G T	354
hkrm2	G G A G G G C A T C	T A C T G G C G C T	A C T G C G A C A T	C C C C T C C T G T	357
mkrm1	C A G A T G C C T G	G A A A C C T T G G	C T G C T A C A A G	G A T C A T G G A A	382
hkrm1	C A G A T G C C T G	G A A A C C T T G G	C T G C T A C A A G	G A T C A T G G A A	382
mkrm2	C A C A T G C C T G	G G T A C C T G G G	C T G C T T C G T G	G A C T C T G G G G	394
hkrm2	C A C A T G C C A G	G C T A C C T G G G	A T G C T T T G T G	G A C T C A G G G G	397
mkrm1	A C C C A C C T C C	T C T C A C G G G C	A C C A G T A A A A	C C T C T A A C A A	422
hkrm1	A C C C A C C T C C	T C T A A C T G G C	A C C A G T A A A A	C G T C C A A C A A	422
mkrm2	C A C C G C C T G C	T C T C A G T G G T	C C C A G T G G C A	C C T C C A C A A A	434
hkrm2	C A C C G C C A G C	C C T C A G C G G C	C C C A G C G G C A	C C T C C A C G A A	437
mkrm1	G C T C A C C A T A	C A A A C C T G T A	T C A G C T T C T G	T C G G A G T C A G	462
hkrm1	A C T C A C C A T A	C A A A C T T G C A	T C A G T T T T T G	T C G G A G T C A G	462
mkrm2	G C T C A C T G T C	C A A G T G T G C C	T T C G A T T C T G	C C G C A T G A A G	474
hkrm2	G C T C A C G G T C	C A G G T G T G C C	T A C G C T T C T G	C C G C A T G A A G	477
mkrm1	A G A T T C A A G T	T T G C T G G G A T	G G A G T C A G G C	T A T G C C T G C T	502
hkrm1	A G G T T C A A G T	T T G C T G G G A T	G G A G T C A G G C	T A T G C C T G C T	502
mkrm2	G G C T A C C A G C	T G G C T G G T G T	G G A G G C T G G T	T A T G C C T G C T	514
hkrm2	G G G T A C C A G C	T G G C G G G C G T	G G A G G C G G G T	T A C G C C T G C T	517
mkrm1	T C T G T G G G A A	C A A T C C T G A C	T A C T G G A A G C	A C G G G G A G G C	542
hkrm1	T C T G T G G G A A	C A A T C C T G A T	T A C T G G A A G T	A C G G G G A G G C	542
mkrm2	T C T G T G G C T C	T G A A A G T G A C	C T G G C C C G C G	G A C G T C C A G C	554
hkrm2	T C T G T G G C T C	T G A A A G C G A C	C T G G C C C G G G	G A C G C C T G G C	557
mkrm1	G G C C A G C A C C	G A G T G C A A T A	G T G T C T G C T T	C G G G G A C C A C	582
hkrm1	A G C C A G T A C C	G A A T G C A A C A	G C G T C T G C T T	C G G G G A T C A C	582
mkrm2	C C C T G C C A C C	G A C T G T G A C C	A G A T C T G T T T	T G G C C A C C C A	594
hkrm2	C C C C G C C A C C	G A C T G T G A C C	A G A T C T G T T T	C G G C C A C C C T	597

Multiple-alignment of mouse and human kremen DNAs (3-1)

Fig. 1 a



2/7

mkrm1	A C G C A G C C C T	G G G G T G G G G A	C G G C A G G A T T	A T C C T C T T T G	622
hkrm1	A C C C A A C C C T	G T G G T G G C G A	T G G C A G G A T C	A T C C T C T T T G	622
mkrm2	G G C C A G C T C T	G T G G A G G C G A	T G G A C G A C T A	G G C A T C T A T G	634
hkrm2	G G A C A G C T G T	G T G G C G G C G A	T G G G C G G C T G	G G C G T C T A T G	637
mkrm1	A C A C T C T C G T	G G G C G C C T G C	G G T G G G A A C T	A C T C A G C C A T	662
hkrm1	A C A C T C T C G T	G G G C G C C T G C	G G T G G G A A C T	A C T C A G C C A T	662
mkrm2	A A G T G T C T G T	G G G C T C C T G C	C A G G G A A A C T	G G T C G C C T C C	674
hkrm2	A A G T G T C G G T	G G G C T C C T G C	C A G G G A A A C T	G G A C A G C G C C	677
mkrm1	G G C A G C C G T G	G T G T A C T C C C	C T G A C T T C C C	T G A C A C C T A C	702
hkrm1	G T C T T C T G T G	G T C T A T T C C C	C T G A C T T C C C	C G A C A C C T A T	702
mkrm2	T C A A G G A G T C	A T C T A C T C C C	C G G A T T T C C C	G G A T G A G T A T	714
hkrm2	T C A G G G C G T C	A T C T A C T C C C	C G G A C T T C C C	G G A C G A G T A C	717
mkrm1	G C C A C T G G C A	G A G T C T G C T A	C T G G A C C A T C	C G G G T T C C A G	742
hkrm1	G C C A C G G G A	G G G T C T G C T A	C T G G A C C A T C	C G G G T T C C G G	742
mkrm2	G G A C C A G A C C	G G A A C T G C A G	C T G G G T A T T G	G G C C A A C T G G	754
hkrm2	G G G C C G G A C C	G G A A C T G C A G	C T G G G C C C T G	G G C C C G C C A G	757
mkrm1	G A G C C T C T C G	C A T C C A T T T C	A A C T T C A C C C	T G T T T G A T A T	782
hkrm1	G G G C C T C C C A	C A T C C A C T T C	A G C T T C C C C C	T A T T T G A C A T	782
mkrm2	G C G C T G T G C .	. . T A G A A C T C	A C C T T C C G C C	T C T T C G A G T T	791
hkrm2	G C G C C G C G C .	. . T G G A G C T C	A C C T T C C G C C	T C T T C G A G C T	794
mkrm1	C A G G G A C T C T	G C A G A C A T G G	T G G A G C T G C T	G G A C G G C T A C	822
hkrm1	C A G G G A C T C G	G C G G A C A T G G	T G G A G C T T C T	G G A T G G C T A C	822
mkrm2	G G C T G A T T C T	C G A G A C C G G C	T G G A G C T A C G	C G A C G T C T . .	829
hkrm2	G G C C G A C C C G	C G C G A C C G G C	T G G A G C T G C G	C G A C G C G G . .	832
mkrm1	A C C C A C C G C G	T C C T G G T C C G	G C T C A G T G G G	A G G A G C C G C C	862
hkrm1	A C C C A C C G T G	T C C T A G C C C G	C T T C C A C G G G	A G G A G C C G C C	862
mkrm2	. C G T C C G G C A	A C C T A C T C C G	T G C C T T C G A C	G G C G C C C A T C	868
hkrm2	. C T T C G G G C A	G C C T G C T C C G	C G C C T T C G A T	G G C G C C C G C C	871
mkrm1	C G C C T C T G T C	T T T C A A T G T C	T C T C T G G A T T	T T G T C A T T T T	902
hkrm1	C A C C T C T G T C	C T T C A A C G T C	T C T C T G G A C T	T C G T C A T C T T	902
mkrm2	C G C C G C C T C C	G G G A C C G G T G	C G C C T G C G C A	C T G C T G C G C T	908
hkrm2	C A C C G C C G T C	C G G G C C G C T G	C G C C T G G G C A	C T G C C G C G C T	911
mkrm1	G T A T T T C T T C	T C T G A T C G C A	T C A A T C A G G C	C C A G G G A T T T	942
hkrm1	G T A T T T C T T C	T C T G A T C G C A	T C A A T C A G G C	C C A G G G A T T T	942
mkrm2	G C T G C T C A C C	T T C C G C A G C G	A G G C A A G A G G	C C A T G . . C T C	946
hkrm2	G C T G C T C A C C	T T C C G A A G C G	A C G C G C G C G G	C C A C G . . C G C	949
mkrm1	G C T G T G T T G T	A C C A A G C C A C	C A A G G A G G A A	C C G C C A C A G G	982
hkrm1	G C T G T T T T A T	A C C A A G C C G T	C A A G G A A G A A	C T G C C A C A G G	982
mkrm2	A A G G C T T C G C	G C T C A C C T A C	C G C G G G C T G C	A G G A T A C A G T	986
hkrm2	A A G G C T T C G C	G C T C A C C T A C	C G C G G G C T G C	A G G A C G C C G C	989
mkrm1	A G A G A C C T G C	T G T C A A C C A G	A C C C T G G C A G	A G G T G A T C A C	1022
hkrm1	A G A G G C C C G C	T G T C A A C C A G	A C G G T G G C C G	A G G T G A T C A C	1022
mkrm2	G G A G G G C A G A	G C A T C T C C A G	A G G A T T . C A A	C T G A G A G T C T	1025
hkrm2	T G A G G A C C C A	G A G G C C C C G G	A G G G C T . C G G	C C C A G A C C C C	1028
mkrm1	C G A G C A A G C C	A A C C T C A G T G	T C A G C G C T G C	C C A C T C C T C C	1062
hkrm1	G G A G C A G G C C	A A C C T C A G T G	T C A G C G C T G C	C C G G T C C T C C	1062
mkrm2	C G C A G G G G A C	C C C G A T G G G G	C T A A C G C G A G	C T G C A G C C C C	1065
hkrm2	C G C G G C G C C C	C T C G A C G G G G	C C A A C G T G A G	C T G C A G C C C C	1068
mkrm1	A A A G T C C T C T	A T G T C A T C A C	C C C C A G C C C C	A G C C A C C C C T C	1102
hkrm1	A A A G T C C T C T	A T G T C A T C A C	C A C C A G C C C C	A G C C A C C C C A C	1102
mkrm2	A A G . . . C C C	G G A G C T G C A C	A G G C T T C G A T	A G G T G C C C C G A	1101
hkrm2	A G G . . . C C T	G G G G C T C C G C	C G G C C G C G A T	T G G G G C C C C G G	1104
mkrm1	C G C A G A C T G C	C C C A G G T A G C	C A T T C C T G G G	C A C C G T C A G T	1142
hkrm1	C T C A G A C T G T	C C C A G G T A G C	A A T T C C T G G G	C G C C A C C C A T	1142
mkrm2	G T C T T C T C C A	C C G T G A C G G C	C T T C T C T G T G	C T G C T G C T G T	1141
hkrm2	G T C T T C T C G A	C G G T G A C G G C	T G T C T C G G T G	C T G C T G C T G C	1144
mkrm1	T G G G G C A A C	A G C C A C A G A G	T G G A A G G A T G	G A C T G T G T A C	1182
hkrm1	G G G G G C T G G A	A G C C A C A G A G	T T G A A G G A T G	G A C A G T C T A T	1182
mkrm2	T G C T C C T G T C	C C T A C T G C G T	T T G C T G C G T C	G A C G G . . . .	1176
hkrm2	T G C T C C T G G G	G C T G C T G C G T	C G G C T G C G C C	G A C G G T G C G G	1184

Multiple-alignment of mouse and human kremen DNAs (3-2)

Fig. 1 b



3/7

mkrm1	G G C C T G G C G A	C C C T C C T C A T	C C T C A C A G T C	A C A G C A G T T G	1222
hkrm1	G G T C T G G C A A	C T C T C C T C A T	C C T C A C A G T C	A C A G C C A T T G	1222
mkrm2	. . . . .	. . . . .	. . . . .	. . . . . A G C T G	1181
hkrm2	G G C G C T G G G G	C A G G G C C T G A	G G G C G G A . C C	G G T G G A G C T G	1223
mkrm1	T C G C A A A G A T	T C T T C T G C A T	G T C A G A T T T A	A A T C T C A T C G	1262
hkrm1	T A G C A A A G A T	A C T T C T G C A C	G T C A C A T T C A	A A T C C C A T C G	1262
mkrm2	T C T G C T G G C T	C C A G G A A A A G	G G T C T C T G G C	C A T G G G A C C T	1221
hkrm2	T C T G C T G G C T	C C G G G A A A A G	G G C C C C G G C	G C T G G G G G C T	1263
mkrm1	A G T C C C T G C A	T C A G G A G A C C	T T A G G G A C T G	T C G T C A G C C T	1302
hkrm1	T G T T C C T G C T	T C A G G G G A C C	T T A G G G A T T G	T C A T C A A C C A	1302
mkrm2	T C C C G G G G C C	C C G G G A G A A G	C T G G G C T G T G	T G G T A C C G C C	1261
hkrm2	T C C A G G G G C C	C C A G G A G A A G	C T G G G C T G T G	T G G T A C C A A C	1303
mkrm1	G G G G C T T C T G	G A G A T A T C T G	G A C C A T T T T C	T A T G A A C C T T	1342
hkrm1	G G G A C T T C G G	G G G A A A T C T G	G A G C A T T T T T	T A C A A G C C T T	1342
mkrm2	G G C C C C G A G G	G G T G G C C C T G	C C C T G T C C C C	C A G G G A C T C	1301
hkrm2	A G C C C C G A G G	G G T G G C C C T G	C C C T G C T C C C	C C G G G A C C C	1343
mkrm1	C C A . . C T A C A	A T C T C C A T C T	T T A A G A A G A A	G C T C A A G G G T	1380
hkrm1	C C A . . C T T C A	A T T T C C A T C T	T T A A G A A G A A	A C T C A A G G G T	1380
mkrm2	T C A G G C T G A G	G G T C C T G C T G	C G G G C T A C C G	T C C C C T G A G T	1341
hkrm2	C C A G G C T G A G	G G T T C T G C C G	C G G G C T A C C G	G C C T C T G A G T	1383
mkrm1	C A G A G T C A A C	A A G A T G A C C G	C A A T C C C C T C	G T G A G T G A C T	1420
hkrm1	C A G A G T C A A C	A A G A T G A C C G	C A A T C C C C T T	G T G A G T G A C T	1420
mkrm2	G C C T C C A G C C	A G A G C T C C T T	G C G C T C G C T C	G T C T C T G C T C	1381
hkrm2	G C C T C C A G C C	A G A G C T C C C T	G C G C T C G C T C	A T C T C C G C T C	1423
mkrm1	G A ~ ~ ~	1422			
hkrm1	A A ~ ~ ~	1422			
mkrm2	T C T G A	1386			
hkrm2	T C T G A	1428			

Multiple-alignment of mouse and human kremen DNAs (3-3)

Fig. 1 c



4/7

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mkrm1 1 MAPPAARLALLSAAALTTLAARPAPGPRS.GP...ECFTANGADYRGTQSWTALQG
hkrm1 1 MAPPAARLALLSAAALTTLAARPAPSPGL.GP...ECFTANGADYRGTQNWITALQG
mkrm2 1 MGTPLHLOGFLLFLFLLLR.LHGASAGSLHSPGLSECFQVNGADYRGHQNYTGPRG
hkrm2 1 MGTQALQGFLLFLFLLLR.LHGASAGSLHSPGLSECFQVNGADYRGHQNRITGPRG

mkrm1 52 .GKPCFLFWNETFQHPYNTLKYPNNGEGGLGEHNYCRNPDGDVSPWCYVAEHEDGVY
hkrm1 52 .GKPCFLFWNETFQHPYNTLKYPNNGEGGLGEHNYCRNPDGDVSPWCYVAEHEDGVY
mkrm2 55 AGRPCFLFDQTQOHSYSSASDPQGRWGLGAHNF CRNPDGDVQ PWCYVAETEEGIY
hkrm2 56 AGRPCFLFDQTQOHSYSSASDPHGRWGLGAHNF CRNPDGDVQ PWCYVAETEEGIY

mkrm1 106 WKYCEIPACOMPGNLGCKYKDHGNPPPLTGTSTKTSNKLTITQTCISFCRSQRFKFAG
hkrm1 106 WKYCEIPACOMPGNLGCKYKDHGNPPPLTGTSTKTSNKLTITQTCISFCRSQRFKFAG
mkrm2 110 WRYCDIPTCHMPGYLGCFVDSGAPPALSGPSGTSTKLTIVQVCLRFECRMKGYQLAG
hkrm2 111 WRYCDIPSCHMPGYLGCFVDSGAPPALSGPSGTSTKLTIVQVCLRFECRMKGYQLAG

mkrm1 161 MESGYACFCGNNPDYWKHGEAASTECSNVCFGDHTQPCGGDGRILFEDTLVGACG
hkrm1 161 MESGYACFCGNNPDYWKYGEAASTECSNVCFGDHTQPCGGDGRILFEDTLVGACG
mkrm2 165 VEAGYACFCGSESDLARGRPAPATDCDQICFGHPGQLCGGDGRLGTYEVS VGSQ
hkrm2 166 VEAGYACFCGSESDLARGRLAPATDCDQICFGHPGQLCGGDGRLGVYEVS VGSQ

mkrm1 216 GNYSAMAAVVYSPDFPDYATGRVCYWTIRVPGASRIHFNF TLFDIRDSADMVEL
hkrm1 216 GNYSAMSSVVYSPDFPDYATGRVCYWTIRVPGASHIHFNF TLFDIRDSADMVEL
mkrm2 220 GNWAPQGVVIYSPDFPDYATGRVCYWTIRVPGASHIHFNF TLFDIRDSADMVEL
hkrm2 221 GNWAPQGVVIYSPDFPDYATGRVCYWTIRVPGASHIHFNF TLFDIRDSADMVEL

mkrm1 271 LDGYTHRVLVRLSGRSRPP.LSFNVSLDFVILYFFSDDRINQAQGFVLYQATKEE
hkrm1 271 LDGYTHRVLARFHHGRSRPP.LSFNVSLDFVILYFFSDDRINQAQGFVLYQAVKEE
mkrm2 274 RDVSSGNLLRAFDGAHPPPGGLRLRTAALLLTFRSDARGHAQGFALT YRGLQDT
hkrm2 275 RDAASGSLLRAFDGARPPPSGGLRLRTAALLLTFRSDARGHAQGFALT YRGLQDA

mkrm1 325 PPQERP AVNQT LAEVITEQANLSVSAAHSSKVLYVITPSPSHPPQTAPGSHSWAP
hkrm1 325 LPQERP AVNQT LAEVITEQANLSVSAARSSKVLYVITPSPSHPPQTVPGSNVSWAP
mkrm2 329 VE.....GRASPEDSTESLAGDPDGAN.....ASCSP.....KPG....AA
hkrm2 330 AE.....DPEAPEGSAQTPAAPLDGAN.....VSCSP.....RPG....AP

mkrm1 380 SVGANSHRVEGWTVYGLATLLILTVTAIVAKILLHVT.....FKSHRV PASG.
hkrm1 380 PMGAGSHRVEGWTVYGLATLLILTVTAIVAKILLHVT.....FKSHRV PASG.
mkrm2 361 QASIGARVFSTVTAFSVLLLLLSLLRLRRRS.....CLLAPGKGS
hkrm2 362 PAAIGARVFSTVTAFSVLLLLLSLLRLRRRCGALGQGLRADRWSCLLAPGKGP

mkrm1 427 .DLRDCRQPGASGDIWTIFYEPSTTISIFKKKLKGQSQ..QDDRNPLVSD~~~~~
hkrm1 427 .DLRDCHQPGTSGEIIWSIFYKPESTTISIFKKKLKGQSQ..QDDRNPLVSD~~~~~
mkrm2 403 LAMGPSRGPGRS...WAVWYRPRGVALPCPPGDSQAEGPAAGYRPLSASSQSSL
hkrm2 417 PALGASRGPRRS...WAVWYQPRGVALPCSPGDPQAEGSAAGYRPLSASSQSSL

mkrm1 474 ~~~~~~
hkrm1 474 ~~~~~~
mkrm2 455 RSLVSAL
hkrm2 469 RSLISAL

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Multiple-alignment of mouse and human Kremen proteins

Fig. 2



5/7

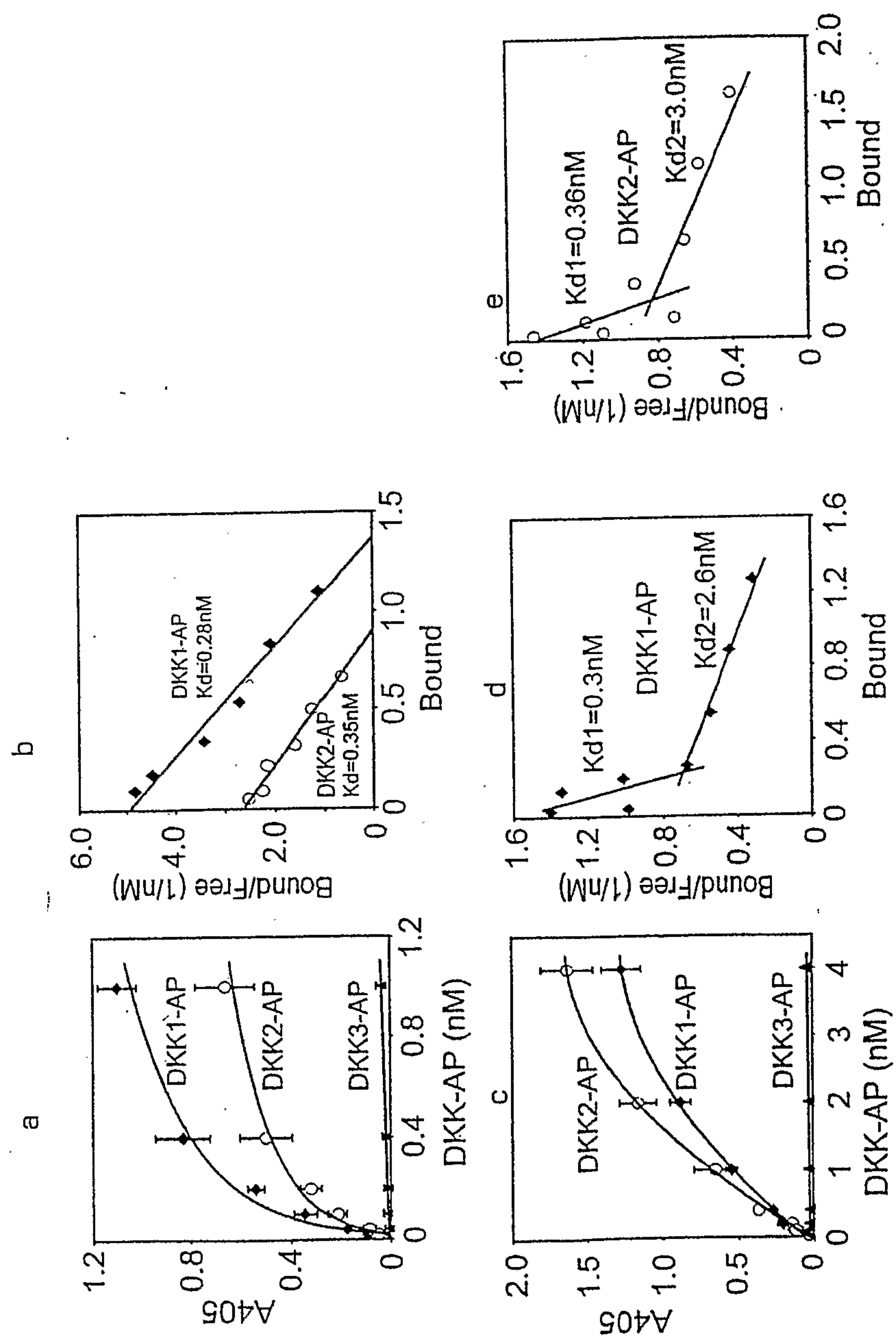


Fig. 3

6/7

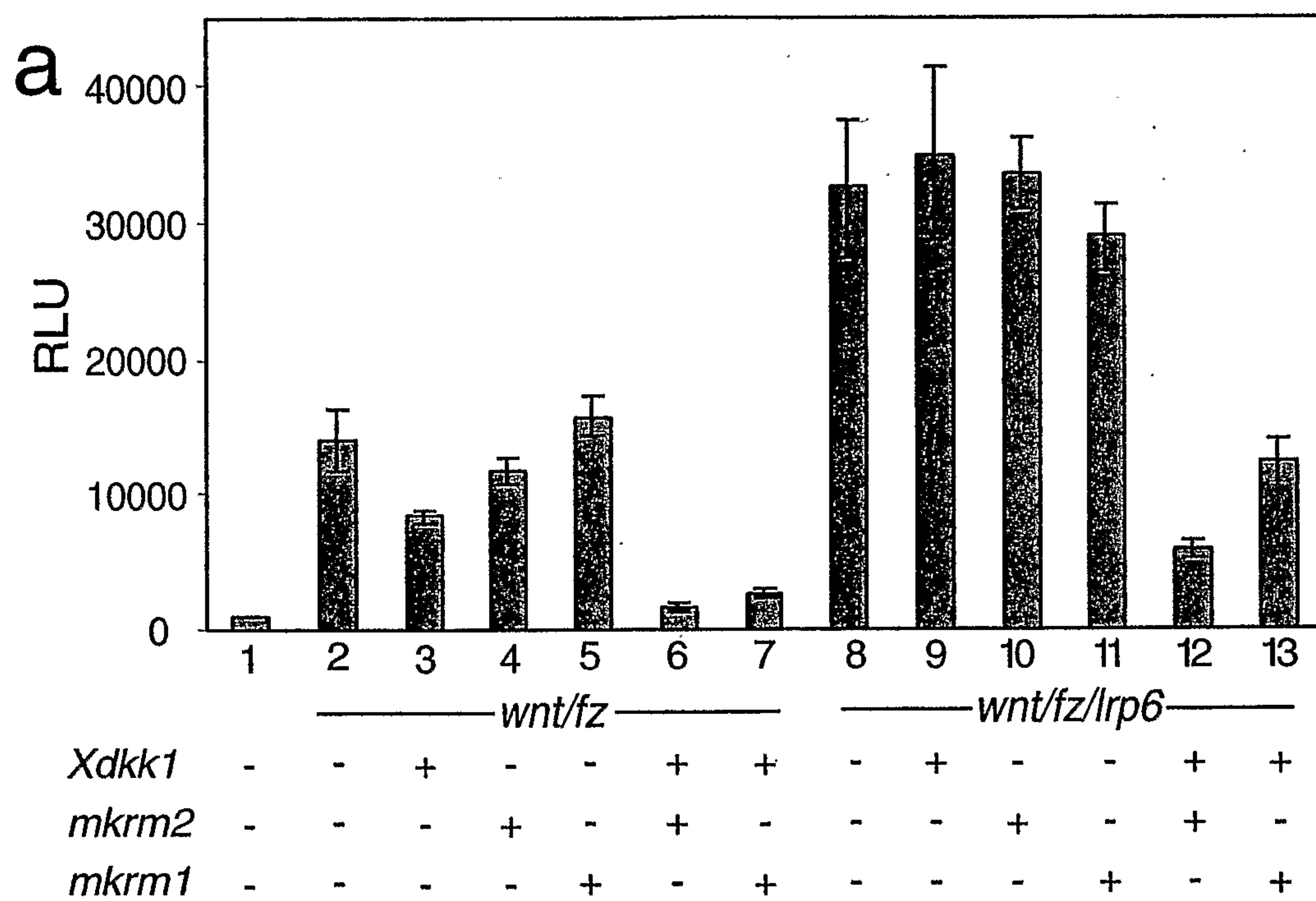


Fig. 4



7/7

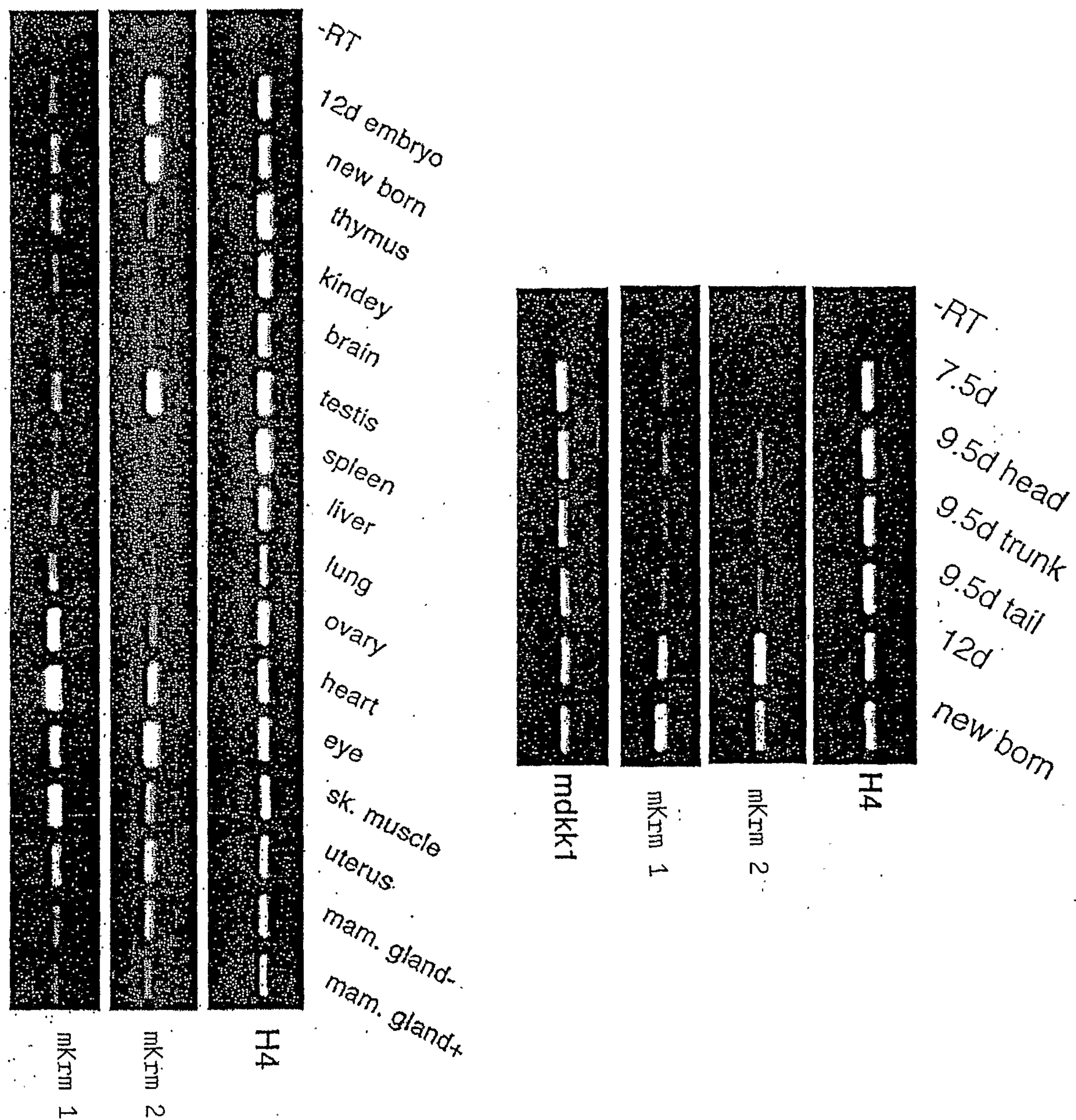
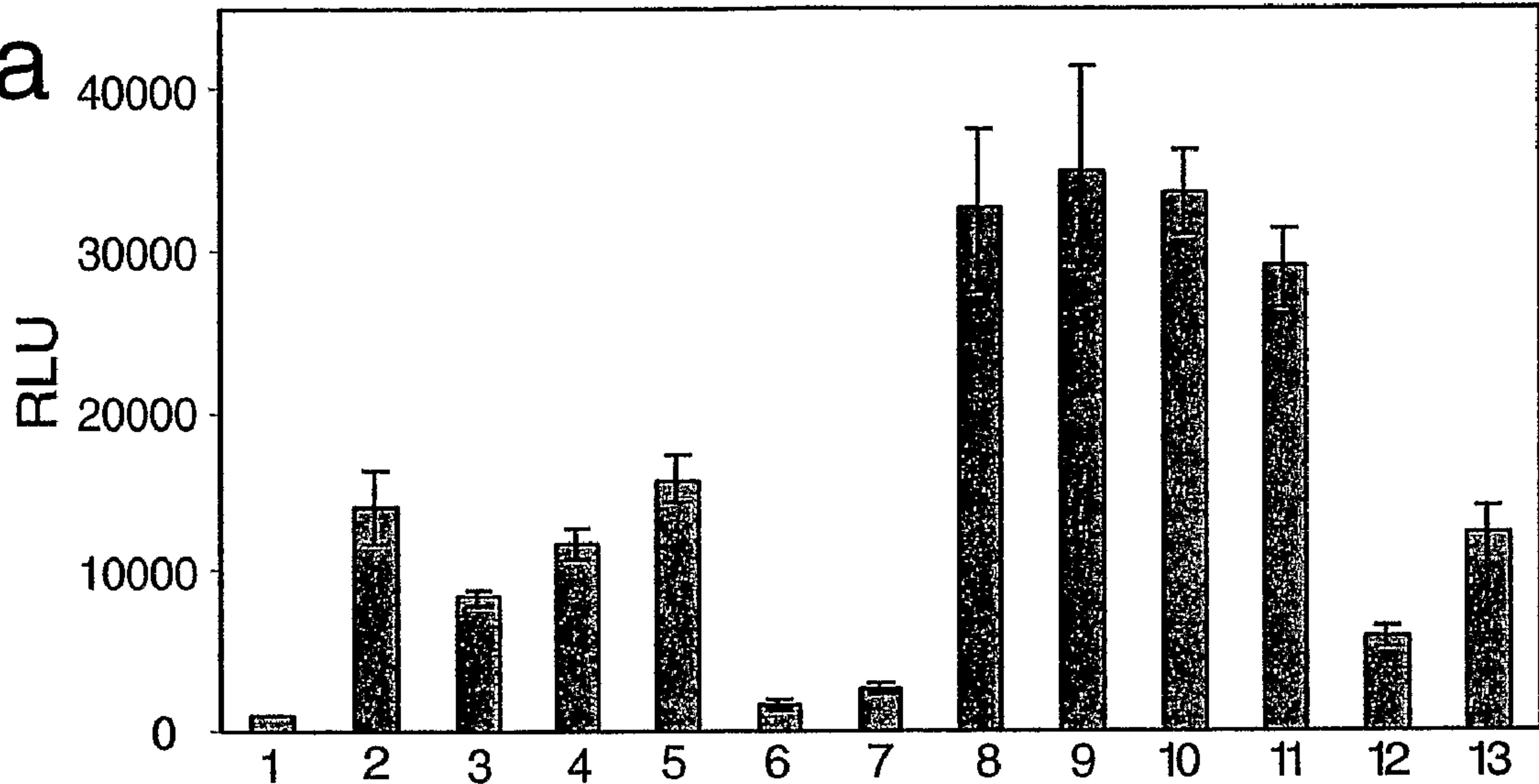


Fig. 5

a



	<i>wnt/fz</i>							<i>wnt/fz/lrp6</i>					
<i>Xdkk1</i>	-	-	+	-	-	+	+	-	+	-	-	+	+
<i>mkrm2</i>	-	-	-	+	-	+	-	-	-	+	-	+	-
<i>mkrm1</i>	-	-	-	-	+	-	+	-	-	-	+	-	+