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(54) **Title:** GENETICALLY ENGINEERED CELLS AND METHODS FOR IMPROVEMENT OF EXPRESSION AND FUNCTIONAL RESPONSES OF CHEMOSENSORY RECEPTORS

(57) **Abstract:** Described herein are genetically engineered cells (e.g., modified mammalian cells) and methods for improved olfactory receptor expression/ functional response. The genetically engineered cells and methods may adjust levels of chaperone proteins that generally help with cell membrane targeting of the receptors. The genetically engineered cells and methods may also provide a platform for use in studying the functionality of a larger set of chemosensory receptors including taste receptors and TRP channels.

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DESCRIPTION

Title of Invention

GENETICALLY ENGINEERED CELLS AND METHODS FOR IMPROVEMENT OF EXPRESSION AND FUNCTIONAL RESPONSES OF CHEMOSENSORY RECEPTORS

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0001] The contents of the electronic sequence listing (AROM_007_P01US_SeqList_ST26.xml; Size: 35,936 bytes; and Date of Creation: June 12, 2023) are herein incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] Described herein are genetically engineered cells and methods for improved expression and functional responses of olfactory receptors. The engineered cell-lines and methods may adjust levels of chaperone proteins in culture that otherwise interfere with cell membrane targeting of the receptors. The engineered cells and methods may also provide a platform for use in studying the functionality of a larger variety of chemosensory receptors.

BACKGROUND

[0003] Odorants may be detected by chemosensory receptors such as olfactory receptors. Odor detection is mediated by olfactory receptors, which are expressed in the olfactory epithelium and represent the largest multigene family in the human genome. The human nose expresses approximately four hundred different olfactory receptors that function combinatorially to detect and discriminate thousands of different odors. Olfactory and other chemosensory receptors (e.g. taste receptors and TRP channels) are generally trafficked to the plasma membrane of cells through a pathway starting from synthesis in the cytoplasm, translocation into the endoplasmic reticulum (ER), folding and maturation in the ER and Golgi compartments, and then delivery to the membrane through vesicles. This process is supported by a battery of chaperones that help with protein folding as well as enzymes that process oligosaccharide chains and covalent bonds.

[0004] Heterologous cell-based assays using chaperones and accessory factors have partially captured the combinatorial and diverse odor binding properties of olfactory receptors, but large-scale functional studies of olfactory receptors are limited. The primary disadvantage in using currently available laboratory cell lines is that they do not efficiently traffic the receptors, and as a result, olfactory receptor localization on the plasma membrane is often poor, which in turn makes functional assays for odorants extremely challenging.

[0005] However, it was discovered that one chaperone protein called RTP1S (RTP Family Members Induce Functional Expression of Mammalian Odorant Receptors, Saito et al., *Cell* 2004 and Synergism in Accessory Factors in Functional Expression of Mammalian Odorant Receptors, Zhuang and Matsunami, *J. Biological Chemistry*, 2007) could enable robust localization of several murine olfactory receptors in HEK293/HEK293T cells (hereafter "HEK cells"). The discovery spurred many studies on both mouse and human olfactory receptors because with the help of RTP1S, HEK cells became usable as an olfactory receptor expression platform. Subsequently, some studies have established that RTP1S is expressed in almost all olfactory neurons in mice and humans, and that knocking out RTP1S from the mouse genome severely impairs olfactory receptor expression and localization *in vivo* (Olfactory Receptor Accessory Proteins Play Crucial Roles in Receptor Function and Gene Choice, Sharma et al. *eLife,* 2017). However, additional research has found that the RTP1S chaperone protein enables the localization of only a subset of the four hundred human olfactory receptors in HEK cells.

[0006] Accordingly, it would be beneficial to have alternative approaches, cell lines, and methods for improved surface expression and/or functional response of olfactory receptors and other chemosensory receptors.

SUMMARY

[0007] Described herein are genetically engineered cells and methods that may increase trafficking and/or response (e.g., transport) of various chemosensory receptors to the cell membrane. The chemosensory receptors may be olfactory receptors, taste receptors, TRP channels, portions thereof, or combinations thereof. The genetically engineered cells and methods may adjust expression (e.g., reduce expression) of one or more chaperone proteins.

[0008] In general, the genetically engineered cells may exhibit reduced expression of one or more chaperone proteins that are involved in at least folding of a chemosensory receptor being trafficked to a membrane surface of the cell. By reducing (or inhibiting) chaperone protein production, the cell may then exhibit increased transport of the chemosensory receptor to the membrane surface as compared to a control cell.

[0009] The reduced expression of the one or more chaperone proteins may be due to knockdown of one or more genes encoding the one or more chaperone proteins. Exemplary genes may include without limitation, CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB, and RPN1. In some variations, the CANX gene may be a useful gene to knock down.

[0010] Any suitable cell line may be used to reduce the expression of chaperones, but in general mammalian cell lines are chosen. The mammalian cell line may be a kidney cell or a hybrid thereof. In one variation, the kidney cell may be a HEK293/ HEK293T cell.

[0011] Systems for increasing trafficking of a chemosensory receptor to a cell membrane surface are also described herein. The systems may include a plurality of genetically engineered cells and an imaging device or modality configured to detect the presence of the chemosensory receptor on the cell membrane surface. In some variations, the imaging device may be a microscope designed to detect fluorescence.

[0012] Methods for increasing transport of a chemosensory receptor to a membrane surface of a cell are also described herein. The methods may generally include the step of expressing the chemosensory receptor in a cell in which production of one or more chaperone proteins is reduced by knockdown of one or more genes encoding the one or more chaperone proteins. The production of the one or more chaperone proteins may be reduced by about 25% to about 75%, including all values and sub-ranges therein. For example, production of the one or more chaperone proteins may be reduced by about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, or about 75%. The reduced production of the one or more chaperone proteins may be due to knockdown of one or more genes encoding the one or more chaperone proteins.

[0013] The one or more chaperone protein targets may be selected from the group consisting of CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB, and RPN1. One example

of a single knockdown targets CANX and one example of a combination knockdown targets CANX + CALR.

[0014] The methods may employ various cell types. In some variations, the cell may be a mammalian cell. The mammalian cell may be a kidney cell or a hybrid thereof. When a kidney cell is used, it may be HEK293/HEK293T cell.

[0015] Various chemosensory receptors may also be employed in the method. For example, the chemosensory receptors may be olfactory receptors or taste receptors or TRP channels, or portions thereof. The chemosensory receptors may be of mammalian origin and include human and non-human mammalian receptors.

[0016] In some variations of the method, HEK293/HEK293T cells for use in decreasing chaperone protein expression may be prepared by modifying the HEK293/HEK293T cell to knockdown one or more genes encoding the chaperone protein. The one or more genes may be CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB, RPN1, or combinations thereof. The one or more genes may be CANX. In some instances, one or more genes may be a combination of CALR and CANX.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0018] [FIG.1] FIG. 1 is a graph depicting olfactory receptors as well, medium, or poorly localized according to cell membrane expression on HEK293/HEK293T cells. The Y axis represents the percentage of cells showing receptor localization, and each bar along the X axis represents a different olfactory receptor. Bright green represents well localized olfactory receptors, light green represents medium localized receptors, and light grey represents poorly localized receptors.

[0019] [FIG.2] FIGS. 2A and 2B depict pie charts showing the relative distribution of nine different categories of genes serving different cellular functions. Green slices indicate proportions of transcript representation of protein folding and processing genes among the top

two hundred most expressed genes. In FIG. 2A, the relative distribution of transcript counts in HEK293/HEK293T cells are shown, with the protein folding and processing category representing approximately 11.8% of the top transcripts. In FIG. 2B, the relative distribution of transcript counts in human olfactory epithelial cells are shown, with the protein folding and processing category representing approximately 1.3% of the transcripts in human olfactory epithelial cells.

[0020] [FIG.3] FIGS. 3A-3F depict microscope images of Rho-tagged human olfactory receptors expressed on HEK293/HEK293T cells. FIGS. 3A-3C show olfactory receptor localization in the presence of calnexin knockdown, while FIGS. 3D-3F show olfactory receptor localization in the absence of knockdown.

[0021] [FIGS. 4A-4D and FIG.5] FIGS. 4A-4D and FIG. 5 depict analysis of the images in FIGS. 3A-3F using an image processing program.

[0022] [FIG.6] FIG. 6 provides data showing that single or combination knockdowns of protein folding and processing genes improves surface expression of multiple olfactory receptors. The Y axis represents the control (no knockdown), single, or different combinations of knockdown targets, and the X axis represents human olfactory receptors tested for improvement of surface expression under each condition. The scale bar represents surface localization of an olfactory receptor under each condition relative to its knockdown control.

[0023] [FIGS. 7A and 7B] FIGS. 7A and 7B illustrate an increase in functional response of human olfactory receptors due to a single or combination knockdown of chaperone proteins.

DETAILED DESCRIPTION

[0024] Described herein are genetically engineered cells that may provide increased trafficking of various chemosensory receptors by concurrently adjusting (e.g., reducing) expression of one or more chaperone proteins. The genetically engineered cells and methods may also provide a platform for use in studying the functionality of a larger variety of chemosensory receptors. The genetically engineered cells and methods may be useful for the expression of mammalian olfactory receptors and other mammalian chemosensory receptors. Systems that include a plurality of genetically engineered cells and an imaging device to detect

presence of the chemosensory receptors on the cell membrane surface are also described herein.

[0025] Although gene knockdown is described herein, it is understood that any method or technique for reducing the expression (or inhibiting expression) of one or more chaperone proteins may be used. For example, such methods may include without limitation, siRNA targeting, targeted gene knockout, and knock down or inhibition of a transcription factor. In principle, any molecular biology, cell biology, or selection method may be used to reduce the expression level (or inhibit expression) of single or combinations of chaperone proteins.

[0026] When gene knockdown is employed to adjust (e.g., reduce) chaperone gene expression in comparison to expression in a host cell, it may be accomplished using various techniques. For example, knockdown of a chaperone gene may be achieved by introducing nucleic acid molecules into the cell which hybridize with parts of the mRNA of the gene leading to its degradation (e.g. shRNAs, RNAi, miRNAs), or altering the sequence of the gene in a way that leads to reduced transcription, reduced mRNA stability, or diminished mRNA translation. In one variation, the method for producing a chaperone gene knockdown includes introducing a shRNA into the cell by using lipofectamine mediated transient transfection. The shRNA may target mRNA of human chaperone genes. For example, the shRNA may target mRNA of human genes including, but not limited to, CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB andRPN1. In another variation, modified cell lines may be generated where one or combinations of the above mentioned genes could be targeted for knock down.

Genetically Engineered Cells

[0027] The cells that are to be engineered to reduce expression or production of chaperone proteins may be any type of cell culture (mammalian or non-mammalian). In some variations, the eukaryotic cells are animal cells. The animal cells may be mammalian cells such as that of bovine, canine, feline, hamster, mouse, porcine, rabbit, rat, or sheep. In other variations, the animal cells may be cells of primates, including but not limited to, monkeys, chimpanzees, gorillas, and humans.

[0028] More specifically, the cells that are modified may be fibroblasts, epithelial cells (e.g., renal, mammary, prostate, lung), keratinocytes, hepatocytes, adipocytes, endothelial cells,

hematopoietic cells. In some variations, the cells are adult cells (e.g., terminally differentiated, dividing or non-dividing), or stem cells. In some variations, the cells may be mammalian cells, such as Chinese hamster cells, murine NIH 3T3 fibroblasts, human kidney cells (e.g., HEK293/HEK293T cells), or rodent myeloma or hybridoma cells. In one variation, the genetically engineered cells are HEK293/ HEK293T cells or variants thereof (e.g., HEK293/HEK293T cells). The genetically engineered cells may be used to create a cell line for odorant binding assays (e.g., an odorant binding platform) that include decreased expression of one or more chaperone proteins, as further described in Example 6.

Chemosensory Receptors

[0029] The chemosensory receptors expressed by the genetically engineered cells may include olfactory receptors, taste receptors, or fragments thereof. Trafficking and/or functional responses of these chemosensory receptors may be increased by decreasing production (e.g., expression) of one or more chaperone proteins, as previously stated. Most olfactory receptors are G-protein coupled receptors (GPCRs) that associate with a G-protein for signal transduction after the receptor is activated by an odorant. GPCRs generally have a conserved structural feature of seven α-helical transmembrane regions. Additionally, olfactory receptors are typically about 320±25 amino acids in length. The differences in length may result from variations in the N-terminal and C-terminal regions.

[0030] In one variation, the olfactory receptors described herein are human olfactory receptors. In some variations, the olfactory receptors may be from other mammals such as mice, rats, cats, cows and cattle, horses, goats, pigs, dogs, and bears. In other variations, the olfactory receptors may be hybrid olfactory receptors. Exemplary human olfactory receptors may be from one or more of the following 18 olfactory receptor (OR) families: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 51, 52, 55 and 56. Furthermore, one or more olfactory receptors from any one of the OR families may be expressed in the genetically engineered cells described herein.

[0031] Family OR1 has 21 members: OR1A, OR1B, OR1C, OR1D, OR1E, OR1F, OR1G, OR1H, OR1I, OR1J, OR1K, OR1L, OR1M, OR1N, OR1P, OR1Q, OR1R, OR1S, OR1X, OR1AA, and OR1AB.

[0032] Family OR2 has 41 members: OR2A, OR2B, OR2C, OR2D, OR2E, OR2F, OR2G, OR2H, OR21, OR2J, OR2K, OR2L, OR2M, OR2N, OR2Q, OR2R, OR2S, OR2T, OR2U, OR2V, OR2W, OR2X, OR2Y, OR2Z, OR2AD, OR2AE, OR2AF, OR2AG, OR2AH, OR2AI, OR2AJ, OR2AK, OR2AL, OR2AM, OR2AO, OR2AP, OR2AS, and OR2AT.

[0033] Family OR3 has 3 members: OR3A, OR3B, and OR3D.

[0034] Family OR4 has 21 members: OR4A, OR4B, OR4C, OR4D, OR4E, OR4F, OR4G, OR4H, OR4K, OR4L, OR4M, OR4N, OR4P, OR4Q, OR4R, OR4S, OR4T, OR4U, OR4V, OR4W, and OR4X.

[0035] Family OR5 has 49 members: OR5A, OR5B, OR5C, OR5D, OR5E, OR5F, OR5G, OR5H, OR51, OR5J, OR5K, OR5L, OR5M, OR5P, OR5R, OR5S, OR5T, OR5V, OR5W, OR5AC, OR5AH, OR5AK, OR5AL, OR5AM, OR5AN, OR5AO, OR5AP, OR5AQ, OR5AR, OR5AS, OR5AU, OR5W, OR5X, OR5Y, OR5Z, OR5BA, OR5BB, OR5BC, OR5BD, OR5BE, OR5BH, OR5BJ, OR5BK, OR5BL, OR5BM, OR5BN, OR5BP, OR5BQ, OR5BR, OR5BS, and OR5BT.

[0036] Family OR6 has 21 members: OR6A, OR6B, OR6C, OR6D, OR6E, OR6F, OR6J, OR6K, OR6L, OR6M, OR6N, OR6P, OR6Q, OR6R, OR6S, OR6T, OR6U, OR6V, OR6W, OR6X, and OR6Y.

[0037] Family OR7 has 9 members: OR7A, OR7C, OR7D, OR7E, OR7G, OR7H, OR7K, OR7L, and OR7M.

[0038] Family OR8 has 18 members: OR8A, OR8B, OR8C, OR8D, OR8F, OR8G, OR8H, OR81, OR8J, OR8K, OR8L, OR8Q, OR8R, ORBS, OR8T, OR8U, OR8V, and OR8X.

[0039] Family OR9 has 12 members: OR9A, OR9G, OR9H, OR9J, OR9K, OR9L, OR9M, OR9N, OR9P, OR9Q, OR9R, and OR9S.

[0040] Family OR10 has 29 members: OR10A, OR10B, OR10C, OR10D, OR10G, OR10H, OR10J, OR10K, OR10N, OR10P, OR10Q, OR10R, OR10S, OR10T, OR10U, OR10V, OR10W, OR10X, OR10Y, OR10Z, OR10AA, OR10AB, OR10AC, OR10AD, OR10AE, OR10AF, OR10AG, OR10AH, and OR10AK.

[0041] Family OR11 has 11 members: OR11A, OR11G, OR11H, OR11I, OR11J, OR11K, OR11L, OR11M, OR11N, OR11P, OR11Q.

[0042] Family OR12 has 1 member: OR12D.

[0043] Family OR13 has 11 members: OR13A, OR13C, OR13D, OR13E, OR13F, OR13G, OR13H, OR131, OR13J, OR13K, and OR13Z.

[0044] Family OR14 has 6 members: OR14A, OR14C, OR141, OR14J, OR14K, and OR14L.

[0045] Family OR51 has 21 members: OR51A, OR51B, OR51C, OR51D, OR51E, OR51F, OR51G, OR51H, OR511, OR51J, OR51K, OR51L, OR51M, OR51N, OR51P, OR51Q, OR51R, OR51S, OR51T, OR51V, and OR51AB.

[0046] Family OR52 has 22 members: OR52A, OR52B, OR52D, OR52E, OR52H, OR521, OR52J, OR52K, OR52L, OR52M, OR52N, OR52P, OR52Q, OR52R, OR52S, OR52T, OR52U, OR52V, OR52W, OR52X, OR52Y, and OR52Z.

[0047] Family OR55 has 1 member: OR55B.

[0048] Family OR56 has 2 members: OR56A and OR56B.

[0049] Taste receptors that may be expressed by the genetically engineered cells may detect sweet, bitter, and umami (glutamate) tastes (e.g., G-protein coupled receptors). In some variations, the taste receptors may detect salty and sour tastes (e.g., ion channel receptors or ionotropic receptors).

[0050] Trigeminal receptors (TRP channels) may be expressed by the genetically engineered cells and detect TRP ligands.

Chaperone Proteins

[0051] The expression or one or more chaperone proteins may be adjusted to increase trafficking of a chemosensory receptor to the surface of a cell membrane. As previously described, knockdown of one or more genes may be used to adjust (e.g., reduce) chaperone gene expression in comparison to expression in a host cell. The genes that may be knocked

down include, but are not limited to, CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB, RPN1. In some variations, and as described in Example 3, one or combinations of the following genes may be knocked down: CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB, and RPN1. It is understood that other chaperone genes may be knocked down to increase trafficking of a chemosensory receptor to the cell membrane surface.

Examples

[0052] The following examples are illustrative only and should not be construed as limiting the disclosure in any way.

Example 1: RTPS1 Localization of Human Olfactory Receptors (OR)

[0053] As previously mentioned, some research has found that the RTP1S chaperone protein enables the localization of only a subset of the four hundred human ORs in HEK cells. We co-transfected olfactory receptors with RTP1S in HEK293/HEK293T cells followed by immunostaining and quantification of fluorescence microscope images and found that only about one third of the receptors were well localized, a small number were medium localized, and the vast majority were poorly localized (see FIG. 1). In FIG. 1, the Y axis represents the percentage of cells showing receptor localization, and each bar along the X axis represents a different human olfactory receptor. Bright green represents well localized human ORs (e.g., greater than about 40% of cells showing surface localized ORs), light green represents medium localized human ORs (e.g., about 10% to about 30% of cells showing surface localized ORs), and light grey represents poorly localized human ORs (e.g., about 10% of cells showing surface localized ORs). These observations indicated that RTP1S may be necessary but not sufficient to support trafficking of human ORs for functional studies in cell culture.

Example 2: Comparison of Chaperone and Processing Protein Levels in HEK Cells and Olfactory Epithelial Cells

[0054] Surface expressed receptors are trafficked to the plasma membrane of cells through a pathway starting from synthesis in the cytoplasm, translocation into endoplasmic reticulum (ER), folding and maturation in ER and Golgi compartments, and membrane delivery through vesicles, as previously mentioned. This process may be supported by one or more chaperone proteins that aid protein folding, and one or more enzymes that process oligosaccharide chains

and covalent bonds. The olfactory sensory neurons (OSNs) have an unique and less understood receptor trafficking machinery, compared to cell culture. In this experiment, the transcripts of expressed genes in HEK293/HEK293T cells and OSNs were compared to quantify the differences in chaperone proteins and enzymes involved in protein folding and processing in the trafficking pathway so that the levels of chaperone proteins and enzymes in HEK293/HEK293T cells could be adjusted to match their levels *in vivo*.

[0055] Transcriptome data for HEK293/HEK293T cells and human olfactory epithelial cells from published literature (A Global View of Gene Activity and Alternative Splicing by Deep Sequencing of the Human Transcriptome, Sultan et al., *Science* 2008 and The Human Olfactory Transcriptome. Oleander et al, *BMC Genomics* 2016) was downloaded and the relative gene expression levels of the top 200 most expressed transcripts in each data set were compared. Intergenic region and pseudogene transcripts from human tissue were excluded. Instead, the analysis focused on protein coding genes. Transcripts were categorized according to the functions of the proteins they encode (as listed in the Uniprot data base).

[0056] The analysis found that the broadest categories of transcripts across the two different data sets related to the following functions: 1) gene expression (transcription, RNA processing and translation), 2) ribosomal functions; 3) mitochondrial functions; 4) structural/morphological functions; 5) protein folding; 6) DNA replication and repair; 7) signaling; 8) enzyme functions; and 9) molecular transport. Referring to FIG. 2A, the protein folding and processing category was found to represent approximately 11.8% of the top transcripts in the HEK293/HEK293T cells. In contrast, this category represented only 1.3% of the transcripts in human olfactory epithelial cells, as shown in FIG. 2B. As further studied in Example 3, the difference in receptor trafficking was thought to stem from an overzealous quality control system in the HEK293/HEK293T cells.

Example 3: Chaperone Gene Knockdown in HEK Cells

[0057] The protein folding gene candidates shown in Table 1 below are over expressed in the HEK cells (compared to the olfactory epithelium) and were selected for knockdown (in HEK cells). The hypothesis tested was that the difference in receptor trafficking between the HEK293/HEK293T cells and human olfactory epithelial cells observed in Example 2 was due to an overzealous quality control system in the HEK293/HEK293T cells. Knockdown was

conducted using RNA interference technology. More specifically, the double stranded small hairpin RNA (shRNA) expressing constructs shown in Table 1 as SEQ ID NOS. 1-40 were purchased from Sigma Aldrich targeting CANX, CALR, HASPA5, HSP90B1, PRKCSH, HYOU1, P4HB and RPN1 (hereafter referred to as "shRNA constructs") were used to produce the modified knockdown cells. Co-expression of the olfactory receptors was also conducted to evaluate if surface expression improved after knockdown of the target genes, as further described below.

Table 1

KNOCK DOWN GENE CANDIDATE	SIGMA ALDRICH TRC NUMBER	TARGET RNA SEQUENCE	SEQ ID NO.
CALR	Category: TRCN0000330928	TCCCGATCCCAGTATCTATG	SEQ ID NO. 1
	Category: TRCN0000019993	CGAGGAGAAAGATAAAGGTTT	SEQ ID NO. 2
	Category:TRCN0000330929	TGGTGCAGTTCACGGTGAAAC	SEQ ID NO. 3
	Category:TRCN0000330853	CGTCTACTTCAAGGAGCAGTT	SEQ ID NO. 4
	Category: TRCN0000330854	GCACGGAGACTCAGAATACAA	SEQ ID NO. 5
HSPA5	Category: TRCN0000231123	GAAATCGAAAGGATGGTTAAT	SEQ ID NO. 6
	Category: TRCN0000218611	AGATTCAGCAACTGGTTAAAG	SEQ ID NO. 7
	Category: TRCN0000218646	GAGCGCATTGATACTAGAAAT	SEQ ID NO. 8
	Category: TRCN0000257180	GGAACCATCCCGTGGCATAAA	SEQ ID NO. 9
	Category: TRCN0000001024	CTTGTTGGTGGCTCGACTCGA	SEQ ID NO. 10
HSP90B1	Category: TRCN0000029426	CGTGGTCTGTTTGACGAATAT	SEQ ID NO. 11
	Category: TRCN0000029425	CCTGTGGATGAATACTGTATT	SEQ ID NO. 12
	Category: TRCN0000276248	GCGAGACTCTTCAGCAACATA	SEQ ID NO. 13
	Category: TRCN0000276247	CCATGATATGATGCCTAAATA	SEQ ID NO. 14
	Category: TRCN0000276250	ATCCTGTGTGGAGAGGGAATG	SEQ ID NO. 15
CANX	Category: TRCN0000029357	GCTGATCGAAGAATAGTTGAT	SEQ ID NO. 16
	Category: TRCN0000029355	CGTGAAATTGAGGACCCAGAA	SEQ ID NO. 17
	Category: TRCN0000029358	CCTGGATCAGTTCCATGACAA	SEQ ID NO. 18
	Category: TRCN0000278235	CGATGATGAAATTGCCAAATA	SEQ ID NO. 19
	Category: TRCN0000029356	GCCAAGAAAGACGATACCGAT	SEQ ID NO. 20
PRKCSH	Category: TRCN0000299872	CGACAAGTTCAGTGCCATGAA	SEQ ID NO. 21
	Category: TRCN0000299739	CCTCACCAATCATCACTTCTA	SEQ ID NO. 22
	Category: TRCN0000299738	CAGCCTTCAAAGATGGGTAAA	SEQ ID NO. 23
	Category: TRCN0000299741	CGATGACTATTGCGACTGCAA	SEQ ID NO. 24
	Category: TRCN0000299808	CGTCTGAAGAAGATCCTTATT	SEQ ID NO. 25
HYOU1	Category: TRCN0000029220	CCTTTGAAGAACGACGAACTA	SEQ ID NO. 26
	Category: TRCN0000029221	GCCTTTAAAGTGAAGCCATTT	SEQ ID NO. 27
	Category: TRCN0000029223	CCCATGGAAATTGTCTTGAAT	SEQ ID NO. 28
	Category: TRCN0000029219	GCCCAGAATATCATGTTCTAT	SEQ ID NO. 29
	Category: TRCN0000029222	CGATTAAGAATCCAAAGGCTA	SEQ ID NO. 30
P4HB	Category: TRCN0000296736	TGCTGTTCTTGCCCAAGAGTG	SEQ ID NO. 31
	Category: TRCN0000296675	AGGTGAAATCAAGACTCACAT	SEQ ID NO. 32
	Category: TRCN0000290651	GTGTGGTCACTGCAAACAGTT	SEQ ID NO. 33
	Category:TRCN0000049193	CGAGTTCTTTGGCCTGAAGAA	SEQ ID NO. 34
	Category: TRCN0000049195	CCAAGGAATATACAGCTGGCA	SEQ ID NO. 35
RPN1	Category: TRCN0000421570	GCCTTTCTCACGCTATGATTA	SEQ ID NO. 36
	Category: TRCN0000072591	CCATTATTTCTACTCTCCCTA	SEQ ID NO. 37
	Category: TRCN0000072590	CCAAGCTATGAGTACCTCTAT	SEQ ID NO. 38
	Category: TRCN0000072589	GCCAAGAACATTGAAATTGAT	SEQ ID NO. 39
	Category: TRCN0000072592	GCTCAAGAAAGACACGTACAT	SEQ ID NO. 40

[0058] In this experiment, HEK293/ HEK293T cells seeded on coverslips were transfected with a human olfactory receptor (OR51D1), RTP1S, and green fluorescent protein (GFP) (as a measure of transfection efficiency), along with a mixture of shRNA constructs targeting calnexin (CANX KD: calnexin knockdown) from Table 1. For comparison, a control was set up with OR51D1, RTP1S, and GFP with no shRNA construct. OR51D1 was selected because

it is not a well-expressed receptor. Following transfection, staining was performed after 48 hours to visualize the Rho-tagged olfactory receptors using an anti-Rhodopsin antibody (clone 4D2, EMD Millipore). Imaging was then performed approximately 24 hours after staining and mounting, and images were acquired at identical exposures for all samples. Representative images of the olfactory receptors transfected with shRNA and no shRNA are shown in FIGS. 3A-3C and FIGS. 3D-3F, respectively. FIGS. 3A-3C show OR51D1 localization in the presence of calnexin knockdown, while FIGS. 3D-3F show OR51D1 localization in the absence of the knockdown. All images were acquired at 1/1.2 second exposure time on a Keyence BZ-X810 microscope.

[0059] Preliminary analysis of the images was performed using ImageJ (https://imagej.nih.gov/ij/), a public domain Java image processing program. Regions containing cells were selected, as shown in FIGS. 4A and 4B, and their grayscale intensities were measured, as shown in FIGS. 4C and 4D, respectively. Referring to FIG. 5, a comparison of the modal intensities of the images for OR51D1 in the presence and absence of calnexin knockdown found that the depletion of calnexin significantly increased the surface expression of OR51D1.

Example 4: Enhanced Surface Expression of Olfactory Receptors Using Knockdowns

[0060] An evaluation of whether more poorly expressed olfactory receptors could be enhanced by targeting either one or combinations of the candidate protein folding and processing genes was performed. In these experiments, HEK293/HEK293T cells were transfected with either single or combinations of shRNA constructs (CANX+HYOU; CANX+HSP; CALR+HYOU; CALR+HSP; CALR+CANX; and CALR+CANX+HSP) along with human olfactory receptors (OR6P1, OR8D1, OR2J3, OR51D1, and OR9Q1).

[0061] Forty-eight hours after transfection, staining and microscopy were carried out as previously described in Example 3. Fluorescence image analysis was then carried out using internally developed software to quantify the percentage of cells showing human olfactory receptor expression in control and various knockdown conditions. Referring to FIG. 6, it was found that for most conditions of knockdown (single or combinations), increased human olfactory receptor surface expression was achieved. The extent of improvement of surface expression did not show a consistent pattern, but rather appeared to be dependent on the

combination of olfactory receptor and knockdown target(s). However, the data overall showed that either single or combinations of knockdown of protein folding and processing genes are likely to enhance surface expression of human olfactory receptors. In FIG. 6, the Y axis represents the control (no knockdown), single, or different combinations of knockdown targets, and the X axis represents human olfactory receptors tested for improvement of surface expression under each condition. The scale bar represents surface localization of an olfactory receptor under each condition relative to its knockdown control, where localization is indicated by the darkness of the green coloring. More localization is indicated by the darker green color, less localization is indicated by the lighter green color, and no localization is indicated by no color.

Example 5: Functional Assays

[0062] Validation of the chaperone knockdown approach to increasing surface expression of olfactory receptors was completed by performing functional assays. With improved surface expression of olfactory receptors as described in Example 4, it was anticipated that there would be an increased functional response to odor ligands. To test this, HEK293/HEK293T cells were transfected with CALR or CANX+CALR shRNA constructs and a control (no shRNA). Cells were also co-transfected with OR2J3 and OR5L1, signaling elements, and a firefly reporter.

[0063] Forty-eight hours after transfection, cells were stimulated with either "no odor" or pentanol (an odor stimulus of OR2J3 and OR5L1), and receptor activity read at the end of the assay. It was found that OR2J3 showed an increase in signaling in CALR and CANX+CALR as compared to control HEK293/HEK293T cells (see FIG. 7A). OR5L1 showed an increase in signaling under CANX+CALR knockdown (see FIG. 7B). White bars indicate assay readings when no stimulus was applied, and green bars indicate assay readings when pentanol stimulus was applied. Overall, the data showed that either single or combinations of chaperone knockdowns related to protein folding and processing genes is likely to increase functional response of olfactory receptors by increasing their surface expression.

Example 6: Production of Genetically Engineered Cell Lines

[0064] shRNA specifically targeting CALR (calreticulin) was used to generate stable knockdown cell lines to increase surface expression of olfactory receptors. In brief, a short

hairpin loop RNA (shRNA)-encoding plasmid was introduced into HEK293/ HEK293T cells using lipid-mediated transfection, followed by the selection and expansion of cell colonies (polyclonal colonies) that had undergone plasmid integration. This was achieved by exposing the cells to a previously determined concentration of selection antibiotic (puromycin) and choosing the surviving colonies. A total of 10 polyclonal colonies were selected and analyzed for their functional efficacy and confirmation of the knockdown of CALR (calreticulin) mRNA expression levels by quantitative PCR.

[0065] For the next step, 28 single clones were identified by a limiting dilution method and expanded to form monoclonal colonies. The top five clones were assessed for their effectiveness on our cell-based functional assay and were narrowed down to the top two mono clones. These two colonies will be tested for their potential to positively affect the sensitivity and efficacy of our assay as compared to commercially available HEK293/HEK293T cells using various OR:odor pairs.

CLAIMS

1. A genetically engineered cell exhibiting reduced expression of one or more chaperone proteins involved in at least folding of a chemosensory receptor being trafficked to a membrane surface of the cell, wherein the cell exhibits increased trafficking and/or functional response of the chemosensory receptor to the membrane surface as compared to a control cell.

2. The cell of claim 1, wherein the reduced expression of the one or more chaperone proteins is due to knockdown of one or more genes encoding the one or more chaperone proteins.

3. The cell of claim 2, wherein the one or more genes is selected from the group consisting of CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB, and RPN1.

4. The cell of claim 3, wherein the one or more genes is CANX.

5. The cell of claim 1, wherein the cell is a mammalian cell.

6. The cell of claim 5, wherein the mammalian cell is a kidney cell or a hybrid thereof.

7. The cell of claim 6, wherein the kidney cell is a HEK293/HEK293T cell.

8. The cell of claim 1, wherein the chemosensory receptor is an olfactory receptor.

9. The cell of claim 1, wherein the chemosensory receptor is a taste receptor.

10. A system for increasing trafficking of a chemosensory receptor comprising a plurality of any one of the genetically engineered cells of claims 1-9.

11. The system of claim 10, further comprising an imaging device configured to detect expression of the chemosensory receptor on the membrane surface of the plurality of genetically engineered cells.

12. A method for increasing transport of a chemosensory receptor to a membrane surface of a cell comprising expressing the chemosensory receptor in a cell in which production of one or more chaperone proteins is reduced by knockdown of one or more genes encoding the one or more chaperone proteins.

13. The method of claim 12, wherein production of the one or more chaperone proteins is reduced by about 25% to about 75%.

14. The method of claim 12, wherein the reduced production of the one or more chaperone proteins is due to knockdown of one or more genes encoding the one or more chaperone proteins.

15. The method of claim 14, wherein the one or more genes is selected from the group consisting of CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB, and RPN1.

16. The method of claim 15, wherein the one or more genes is CANX.

17. The method of claim 12, wherein the cell is a mammalian cell.

18. The method of claim 17, wherein the mammalian cell is a kidney cell or a hybrid thereof.

19. The method of claim 17, wherein the kidney cell is a HEK293/HEK293T cell.

20. The method of claim 12, wherein the chemosensory receptor is an olfactory receptor.

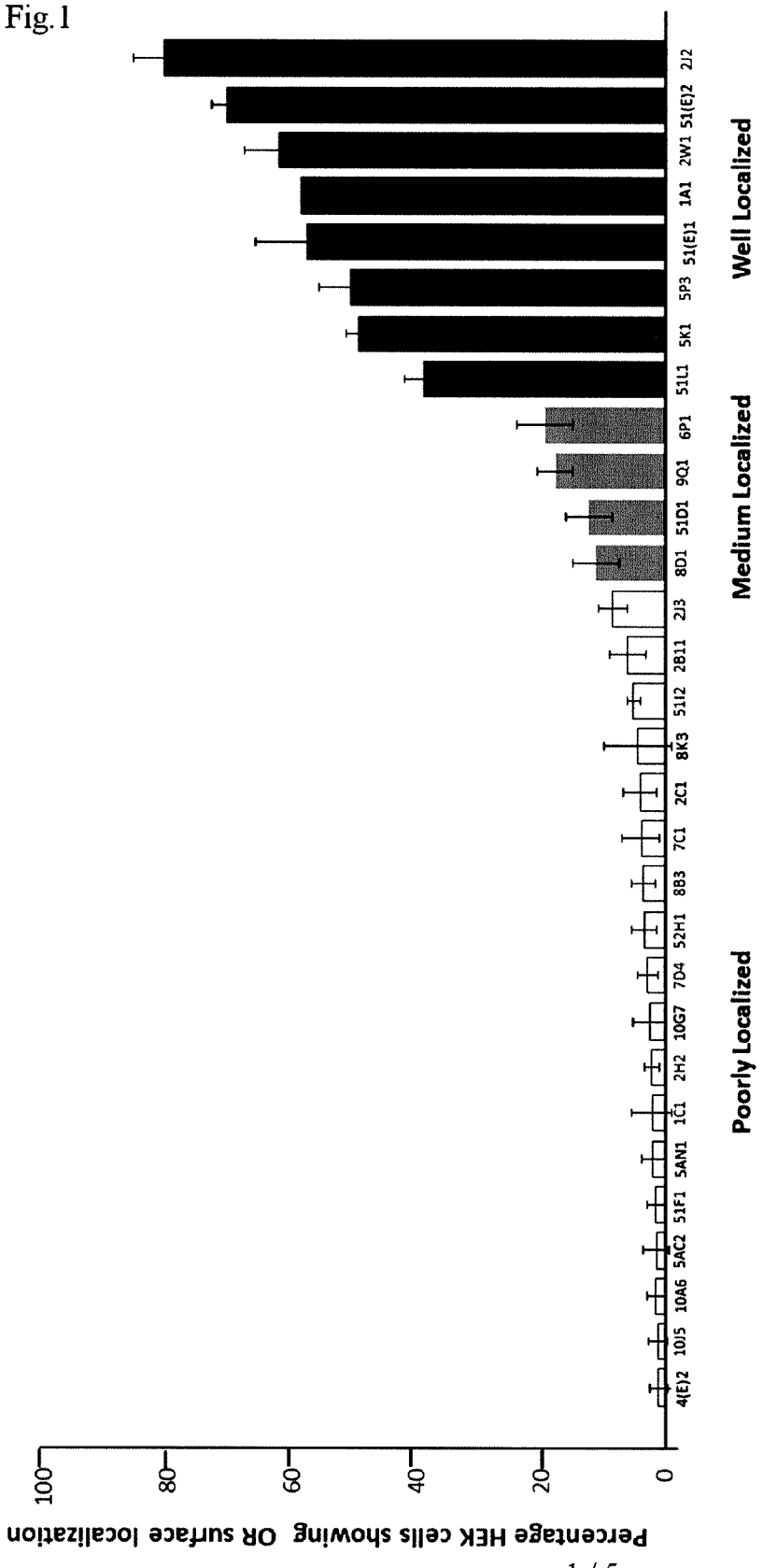
21. The method of claim 12, wherein the chemosensory receptor is a taste receptor.

22. A method for preparing a genetically engineered cell comprising modifying a HEK293/HEK293T cell to knock down one or more genes encoding CALR, HSPA5, HSP90B1, CANX, PRKCSH, HYOU1, P4HB, and RPN1.

23. The method of claim 22, wherein the one or more genes encodes CANX.

24. The method of claim 23, wherein the one or more genes encode CALR and CANX.

Fig.1


Percentage HEK cells showing OR surface localization
0
20
40
60
80
100
4(E)2
10I5
10A6
5AC2
51F1
5AN1
1C1
2H2
10G7
7D4
52H1
8B3
7C1
2C1
8K3
51I2
2B11
2J3
8D1
51D1
9Q1
6P1
51L1
5K1
5P3
51(E)1
1A1
2W1
51(E)2
2J2
Poorly Localized
Medium Localized
Well Localized

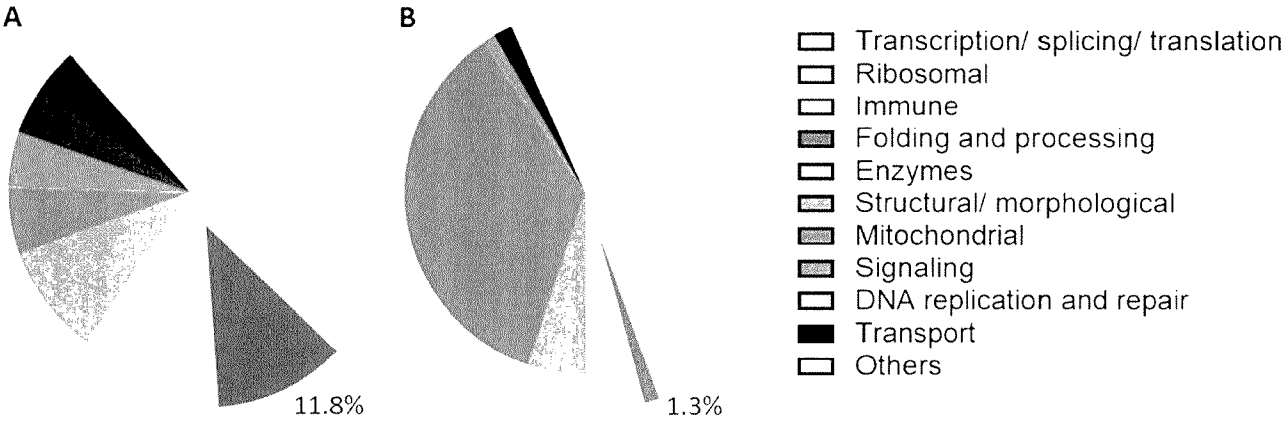

A
B
11.8%
1.3%
Transcription/ splicing/ translation
Ribosomal
Immune
Folding and processing
Enzymes
Structural/ morphological
Mitochondrial
Signaling
DNA replication and repair
Transport
Others

Fig.2

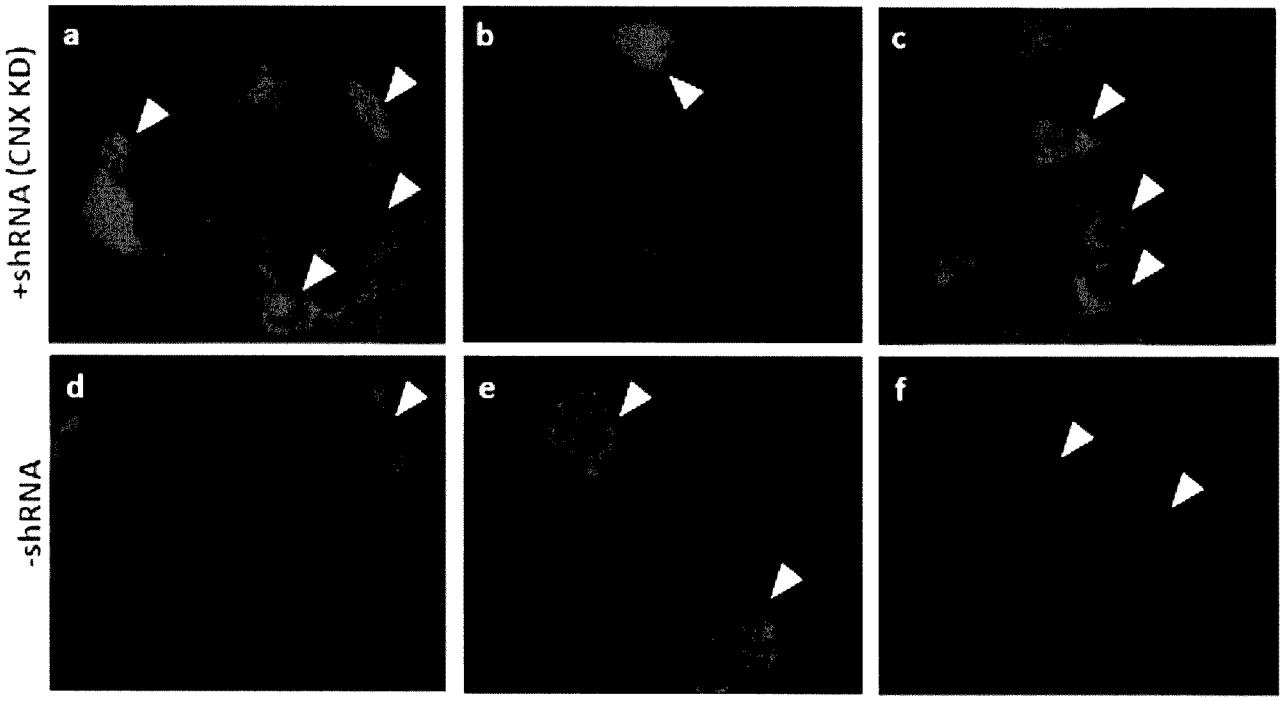

a
b
c
d
e
f
+shRNA (CNX KD)
-shRNA

Fig.3

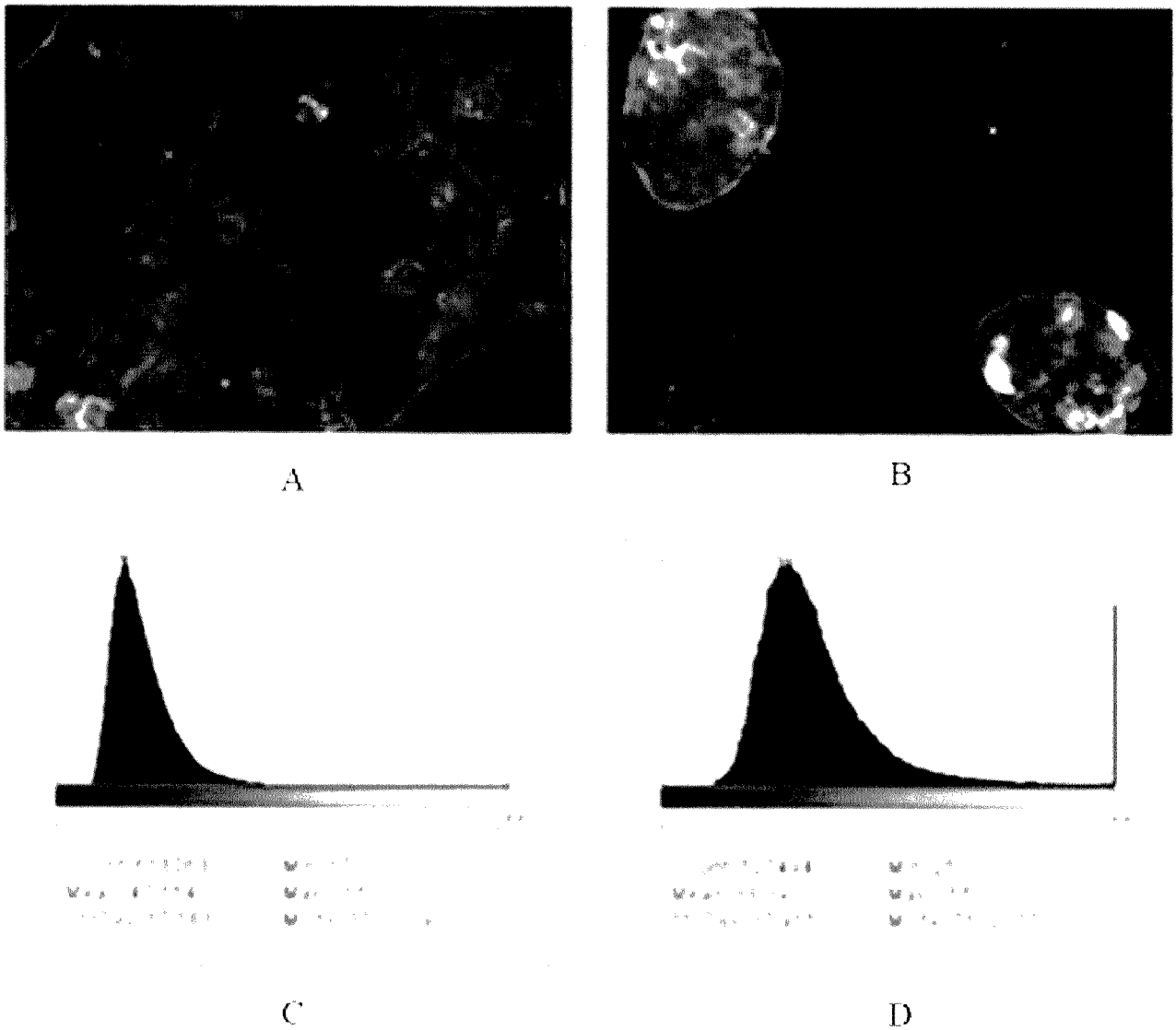


Fig.4

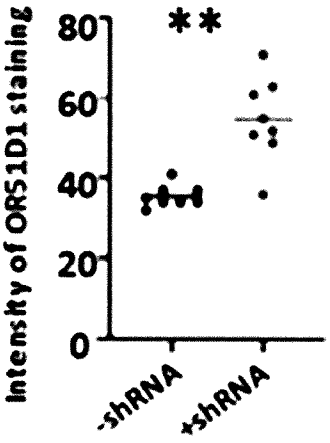

Intensity of OR51D1 staining
80
60
40
20
0
**
-shRNA
+shRNA

Fig.5

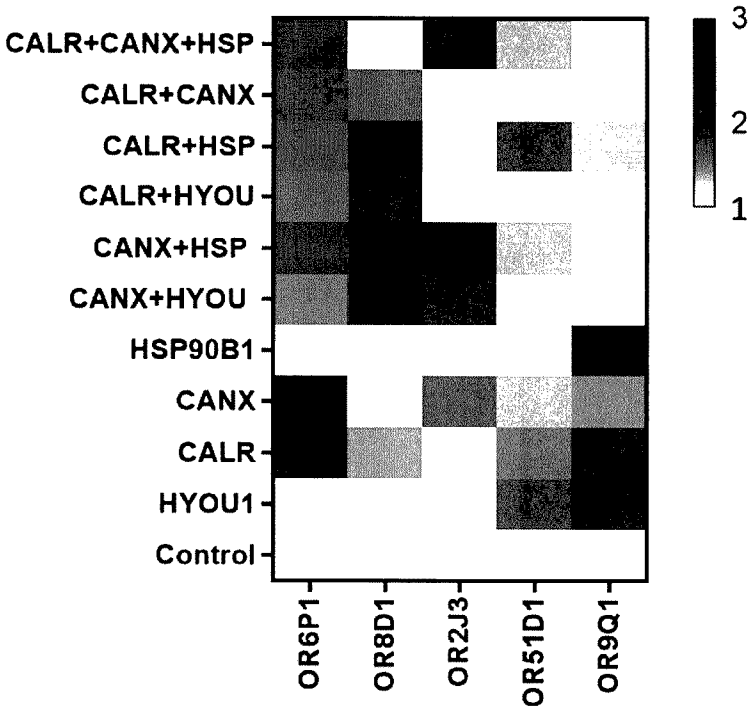
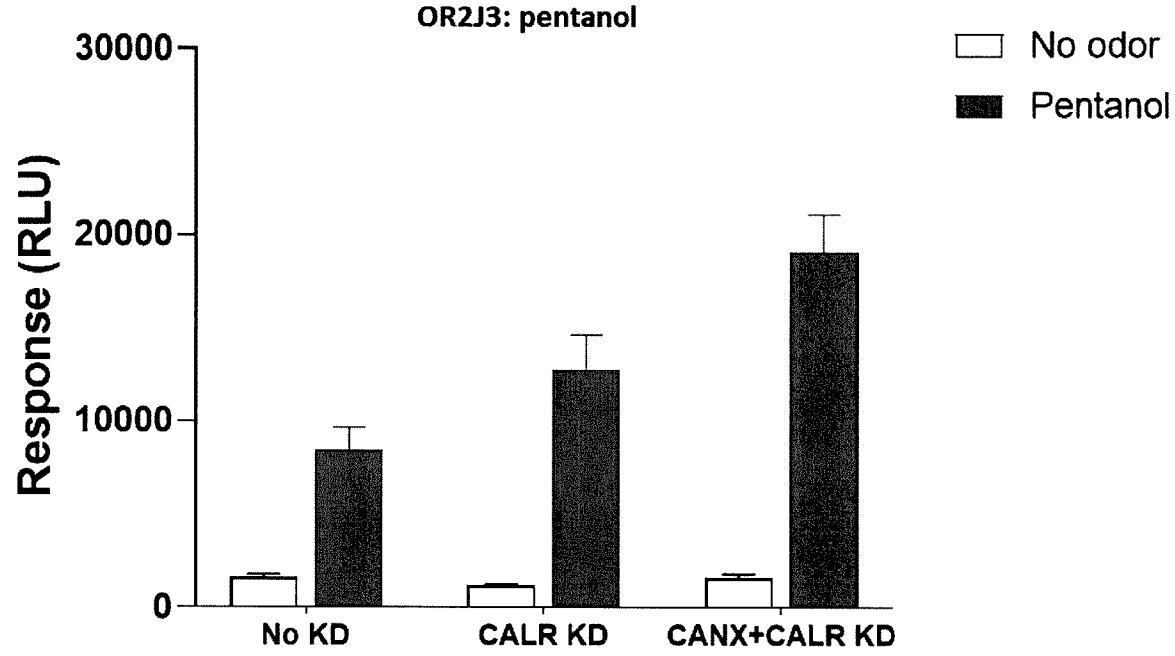
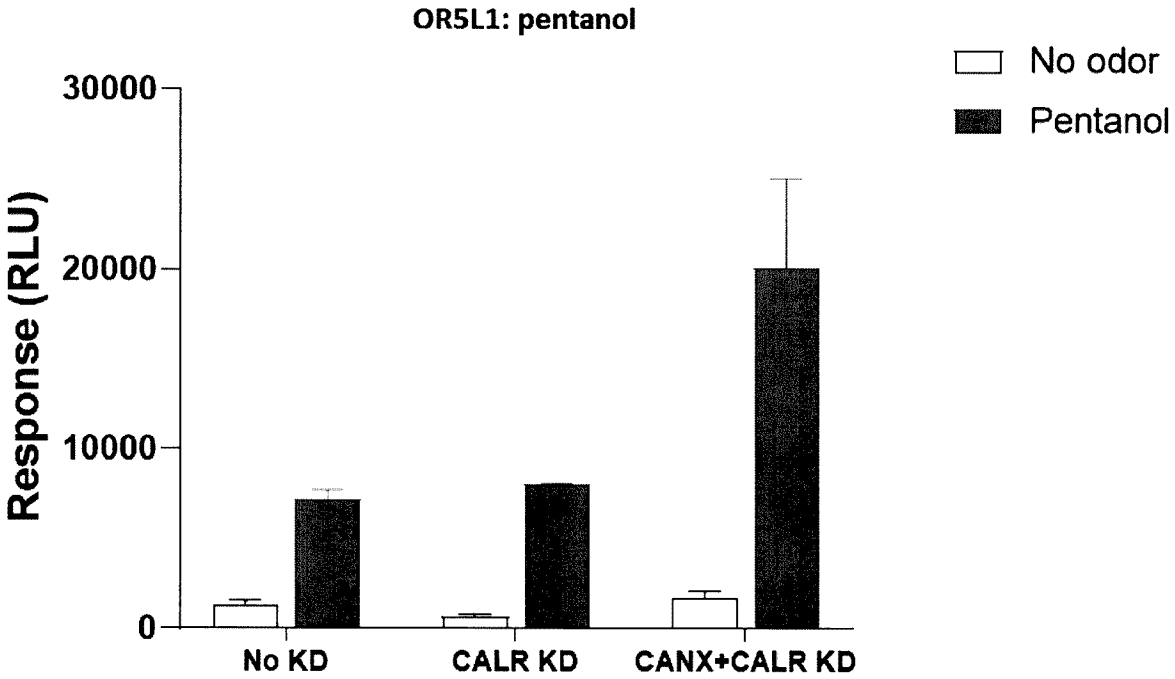

CALR+CANX+HSP
CALR+CANX
CALR+HSP
CALR+HYOU
CANX+HSP
CANX+HYOU
HSP90B1
CANX
CALR
HYOU1
Control
OR6P1
OR8D1
OR2J3
OR51D1
OR9Q1
3
2
1

Fig.6


OR2J3: pentanol
No odor
Pentanol
Response (RLU)
30000
20000
10000
0
No KD
CALR KD
CANX+CALR KD

A


OR5L1: pentanol
No odor
Pentanol
Response (RLU)
30000
20000
10000
0
No KD
CALR KD
CANX+CALR KD

B

Fig.7

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2024/080088

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/47 C12N15/113 C12N15/85 C07K14/705
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/48003 A1 (DEDHAR SHOUKAT [CA]; ST ARNAUD RENE [CA]) 29 October 1998 (1998-10-29) abstract page 7, line 25 - line 31 page 9, line 8 - line 13	1-24
X	WO 2013/082522 A1 (UNIV DUKE) 6 June 2013 (2013-06-06) abstract page 15, paragraph 59 page 23, paragraph 96 - page 24, paragraph 99	1-24

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 2024	11/11/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Grötzinger, Thilo

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2024/080088

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. DEY ET AL: "Calreticulin chaperones regulate functional expression of vomeronasal type 2 pheromone receptors", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 108, no. 40, 4 October 2011 (2011-10-04), pages 16651-16656, XP055070882, ISSN: 0027-8424, DOI: 10.1073/pnas.1018140108 abstract page 16652, right-hand column, last paragraph; figure 2 page 16653, left-hand column, paragraph 3 - right-hand column, paragraph 2 -----	1-24
X	LIU YI-SHI ET AL: "N-Glycan-dependent protein folding and endoplasmic reticulum retention regulate GPI-anchor processing", THE JOURNAL OF CELL BIOLOGY, vol. 217, no. 2, 18 December 2017 (2017-12-18), pages 585-599, XP093015074, US ISSN: 0021-9525, DOI: 10.1083/jcb.201706135 Retrieved from the Internet: URL:http://rupress.org/jcb/article-pdf/217/2/585/1378085/jcb_201706135.pdf> abstract page 586, right-hand column, paragraphs 2,3 page 588, right-hand column, paragraph 1 -----	1-24
X	GUODONG LI ET AL: "Heat Shock Protein 90B1 Plays an Oncogenic Role and is a Target of microRNA-223 in Human Osteosarcoma", CELLULAR PHYSIOLOGY AND BIOCHEMISTRY, vol. 30, no. 6, 1 January 2012 (2012-01-01), pages 1481-1490, XP055633955, CH ISSN: 1015-8987, DOI: 10.1159/000343336 abstract page 1484, last paragraph page 1485; figure 1 -----	1-24

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2024/080088

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/JP2024/080088

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9848003 A1	29-10-1998	AU 7022898 A	13-11-1998
		CA 2287198 A1	29-10-1998
		EP 0977831 A1	09-02-2000
		JP 2002501380 A	15-01-2002
		KR 20010020238 A	15-03-2001
		US 6461865 B1	08-10-2002
		WO 9848003 A1	29-10-1998
WO 2013082522 A1	06-06-2013	US 2014323348 A1	30-10-2014
		WO 2013082522 A1	06-06-2013