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(54) **Title:** TARGETED DESTRUCTION OF PASK BY PEPTIDE-BASED DEGRON

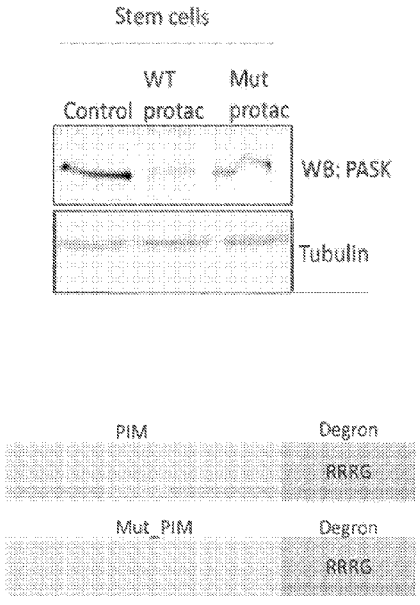

FIG. 8

(57) **Abstract:** The present disclosure concerns identification of a Per-Arnt-Sim kinase (PASK) targeting peptide and the application thereof for construction of degrons to selectively inhibit expression of PASK in cell systems. The PASK targeting peptide or PASK interacting motif (PIM) is derived from a region within PASK that interacts with the PAS domains and can contribute to kinase activity. In addition to inhibiting PASK expression, the PIM peptides of the present disclosure can therefore competitively interact with endogenous PAS domains and inhibit PASK activity without necessarily altering PASK expression in the cell.

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[Continued on next page]

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TARGETED DESTRUCTION OF PASK BY PEPTIDE-BASED DEGRON

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to US Provisional Patent Application 63/510,189, filed June 26, 2023, the contents of which are hereby incorporated by reference in their entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under grant 1R01AR073906-01A1 awarded by the National Institutes for Health. The government has certain rights in the invention.

BACKGROUND

[0003] Mammalian stem cells exhibit two main properties: Self-renewal to preserve lineage identity and differentiation to generate functional cell types. Each of these properties is molecularly and biochemically regulated to safeguard developmental transition, tissue integrity, and long-term tissue function. Conditions that perturb the balance between self-renewal and differentiation are linked with muscle atrophy, cognitive decline, neuromuscular defects, metabolic syndrome, and cancer. Therefore, factors that regulate the balance between self-renewal and differentiation are attractive targets to preserve stem cell function in the diseased state.

SUMMARY

[0004] A 1st aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns a degron peptide comprising a PASK protein or fragment thereof fused to an amino acid sequence of RRRG (SEQ ID NO: 1).

[0005] A 2nd aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 1st aspect, wherein the PASK fragment comprises a PASK interacting motif (PIM) comprising an amino acid sequence with at least 85% sequence identity to SEQ ID NO: 17.

[0006] A 3rd aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 1st or 2nd aspect, wherein the PASK fragment comprises a PASK interacting motif (PIM) comprising an amino acid sequence of $EGX_1X_2X_3GX_4X_5X_6HR$ (SEQ ID NO: 51) or $EGX_1X_2X_3GX_4X_5X_6HRDG$ (SEQ ID NO: 52), wherein X_1, X_3 and X_5 are any amino acid, X_2 is Y or F, X_4 is E, S or N, and X_6 is Y or C.

[0007] A 4th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 3rd aspect, wherein the PIM sequence is selected from on of SEQ ID NOs 18-28 or 42-50.

[0008] A 5th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 3rd aspect, wherein the PIM comprises the amino acid sequence of AEIQEGAYSGSCY (SEQ ID NO: 2) or AEIQEGVYSGSCY (SEQ ID NO: 3).

[0009] A 6th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 5th aspect, wherein the peptide comprises the amino acid sequence of AEIQEGAYSGSCYRRRG (SEQ ID NO: 4) or AEIQEGVYSGSCYRRG (SEQ ID NO: 5).

[0010] A 7th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the any of the 1st through 6th aspects, further comprising a cell permeability sequence.

[0011] An 8th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 7th aspect, wherein the cell permeability sequence comprises the amino acid sequence of YGRKKRRQRR (SED ID NO: 6).

[0012] A 9th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 8th aspect, wherein SEQ ID NO: 6 is fused to at least one of SEQ ID NO: 4 or 5.

[0013] A 10th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 8th aspect, wherein the degron peptide comprises the amino acid sequence of YGRKKRRQRREG$X_1X_2X_3GX_4X_5X_6$HRRRRG (SEQ ID NO: 53) and/or YGRKKRRQRREG$X_1X_2X_3GX_4X_5X_6$HRDGRRRG (SEQ ID NO: 54)), wherein X_1, X_3 and X_5 are any amino acid, X_2 is Y or F, X_4 is E, S or N, and X_6 is Y or C.

[0014] An 11th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 8th aspect, wherein the degron peptide comprises the amino acid sequence YGRKKRRQRRRAEIQEGAYSGSCYRRRG (SEQ ID NO: 7) and/or YGRKKRRQRRRAEIQEGVYSGSCYRRRG (SEQ ID NO: 8) (termed TdXO-P) and/or YGRKKRRQRRRRRG (SEQ ID NO: 9).

[0015] A 12th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns a nucleic acid encoding the degron of any preceding aspect.

[0016] A 13th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns a method for inhibiting PASK activity in a cell comprising administering the degron peptide or nucleic acid encoding the same of any of the 1st through 12th aspects.

[0017] A 14th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 13th aspect, wherein the cell is within a subject.

[0018] A 15th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns the degron peptide of the 14th aspect, wherein the subject is a human.

[0019] A 16th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns a method for treating a stem cell comprising administering to a stem cell the degron peptide or nucleic acid encoding the same of any of the 1st through 12th aspects.

[0020] A 17th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns a method for treating aberrant PASK activity in a subject comprising administering to a subject the degron peptide or nucleic acid encoding the same of any of the 1st through 12th aspects.

[0021] An 18th aspect of the present disclosure, either alone or in combination with any other aspect set forth herein, concerns a method for inhibiting PASK activity in a cell comprising administering to the cell a peptide comprising a PIM motif, wherein the PIM motif comprises an amino acid sequence of $EGX_1X_2X_3GX_4X_5X_6HR$ (SEQ ID NO: 51) or $EGX_1X_2X_3GX_4X_5X_6HRDG$ (SEQ ID NO: 52), wherein X_1, X_3 and X_5 are any amino acid, X_2 is Y or F, X_4 is E, S or N, and X_6 is Y or C.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] FIG. 1A-G show identification of intramolecular interactions in PASK as regulators of PASK nuclear localization.

[0023] FIG. 1A shows domain architecture of human PASK, including two PAS domains (the experimentally verified PAS-A and the PFAM-predicted PAS-B, and the serine/threonine kinase catalytic domain). The PAS737 fragment consists of amino-acids 1-737 and lacks the kinase domain, while Fragment A consists of amino acids 841-1323 and lacks both PAS domains.

[0024] FIG. 1B shows GFP-tagged Fragment A was expressed in HEK293T cells alone, or together with mScarlet-tagged PAS737. 24 hr after transfections, cells were fixed and relative distribution of Fragment A and PAS737 was determined by confocal microscopy. Scale bar = 40 mM.

[0025] FIG. 1C shows WT or kinase-dead (K1028R) version of V5-tagged Fragment A were expressed alone or together with Myc-tagged PAS737 in HEK293T cells, and their interaction was determined by immunoprecipitation.

[0026] FIG. 1D shows domain architecture of C-terminal truncations (Fragments A to D) generated to identify region of PASK that interacts with N-terminal PAS737 region.

[0027] FIG. 1E shows various V5-tagged C-terminal fragments were co-expressed with PAS737-Myc and co-immunoprecipitation was conducted as described in Materials and Methods.

[0028] FIG. 1F shows GFP-tagged Fragments A and D were expressed in HEK293T cells along with mScarlet-tagged PAS737 in HEK293T cells. 24hr after transfections, cells were fixed and relative distribution of fragments (A and D) and PAS737 was determined by confocal microscopy. Scale bar = 40 mM.

[0029] FIG. 1G shows quantification of % cells with nuclear-localized GFP from experiment in Figure 1F.

[0030] FIG. 2A-F show identification of residues Y894 and S897 as critical for intramolecular interactions in PASK.

[0031] FIG. 2A shows primary sequence alignment of PASK from invertebrates and vertebrates. The PAS Interacting Motif (PIM) sequence is depicted by a red line. The yellow and black bars at the bottom of the alignment indicate positional similarity scores.

[0032] FIG. 2B shows fragment A mutants, G891A/G895A (PIM-Mut1) or Y894A/S897A (PIM-Mut2) were co-expressed with PAS737-Myc in HEK293T cells. Co-IP was performed as described in methods and material.

[0033] FIGs. 2C and 2D show WT or Mut2 version of PIM sequence (PASK AA 879-904) was fused with Flag-GFP (PIMtide). The sufficiency of PIM interaction with PAS737 region was evaluated by co-immunoprecipitation of HEK293T co-expressed proteins.

[0034] FIG. 2E shows schematic of PAS737 and Fragment A-WT or Mut2 used in Figure 2F.

[0035] FIG. 2F shows nuclear distribution of GFP-tagged WT or Y894AS897A-mutated Fragment A (Mut2) when expressed in HEK293T cells alone or in combination with mScarlet-tagged PAS737. The %N indicates quantification of % nuclear GFP localization. FIG. 3A-E show mapping N-terminal residues in PAS-A domain that interact with PIM sequence.

[0036] FIG. 3A shows domain truncation analysis of PAS737 region that interacts with Fragment A.

[0037] FIG. 3B shows various fragments of PAS737-Myc were expressed in HEK293T cells and co-immunoprecipitation with V5-LacZ (negative control, lane 1) or Fragment A was conducted as described in Materials and Methods.

[0038] FIG. 3C shows Myc-tagged WT or Mut (PAS737-Mut, K132A/W216A/E223A) PAS737 was co-expressed with V5-tagged Fragment A (lanes 2 and 3) in HEK293T cells. Interaction between proteins was determined by co-immunoprecipitation and western blot using the indicated antibodies as identified in the Figure panel.

[0039] FIG. 3D shows relative nuclear distribution of GFP-tagged Fragment A when co-expressed with either mScarlet-PAS737-WT or mScarlet-PAS737-Mut (K132A/W216A/E223)

[0040] FIG> 3E shows quantification of images in Figure 3D showing % cells with nuclear GFP expression when co-expressed with either mScarlet-PAS737-WT or mScarlet-PAS737-Mut (K132A/W216A/E223A).

[0041] FIG. 4A-E show NMR-based identification of PIM binding site on PAS-A.

[0042] FIG. 4A shows 1H/15N HSQC spectra of 15N-labeled PAS-A (131-237) in the presence of 0.1-2.5 mM unlabeled PIM (879-904), showing ligand-dependent chemical shift perturbations (CSPs) indicating binding.

[0043] FIG. 4B shows residues perturbed by the addition of either PIM (top) or KG-571, a previously-described small molecule ligand (compound 1 in9 (bottom), as identified by CSPs in backbone amide $^{1}H/^{15}N$ chemical shift changes from apo PAS-A. Changes were quantified by using $\Delta\delta(ppm)=(\Delta\delta^2_H+(0.2*\Delta\delta_N)^2)^{0.5}$ solid lines indicate the average level of observed CSPs, while dashed lines indicate the average + one standard deviation of CSPs.

[0044] FIG. 4C shows solution structure of PAS-A (PDB-ID: 1LL89 showing the locations of residues with substantial CSPs (>average + one standard deviation) by the addition of 3.7 mM PIM.

[0045] FIG. 4D shows solution structure of PAS-A showing that the addition of 2 mM KG-571 induces substantial CSPs (>average plus one standard deviation) in residues which cluster around the internal cavity identified in the structure.

[0046] FIG. 4E shows an overlay of the experimental PAS-A structure (blue, PDB-ID: 1LL89and an AlphaFold2 Multimers28 model of PAS-A(131-237) and PIM(879-904) (grey and aqua, respectively), suggesting PIM interacts with PAS-A via regions adjacent to the internal cavity, including portions of the Fa helix, FG loop, Gb strand, and Hb strand. PAS-A residues showing substantial PIM-induced changes in NMR/HDX-MS experiments are shown as pink spheres.

[0047] FIG. 5A-G show PIM interferes with the nuclear import of PAS-A.

[0048] FIG. 5A shows sequence and structural elements of WT or PIM-mutated (PIM-Mut2, Y894AS897A) full-length PASK used in Figure 5B.

[0049] FIG. 5B shows GFP-tagged full-length WT or PIM-mutated (PIM-Mut2, Y894AS897A) full-length PASK were expressed in HEK293T cells, and their nuclear accumulation was observed by quantifying % cells with nuclear GFP (panel to the right).

[0050] FIG. 5C shows depiction of various N and C-terminal fragments used to identify the structural elements regulating PASK nuclear import. NES = mutations in nuclear export sequence (NES, L667SL669SL671S)

[0051] FIG. 5D shows immunofluorescence microscopy of mScarlet-PAS737-NES2 construct co-expressed with indicated GFP-tagged WT or PIM-Mut2 (Y894A/S897A) Fragment A or Fragment D. Scale bars = 20 mM.

[0052] FIG. 5E shows quantification of % cells with nuclear PAS737 expression when co-expressed with WT or Mut2-Fragment A or Fragment D.

[0053] FIG. 5F shows schematic representation of constructs used in Figure 5G.

[0054] FIG. 5G shows GFP-400 (hPASK AA 1-400 fused to GFP) was expressed with or without PIMtide (AA 879-904)-mCherry in HEK293T cells. The GFP nuclear accumulation was observed by quantifying % cells with nuclear GFP (panel to the right). Scale bars = 20 mM.

[0055] FIG. 6A-E show PIM masks a putative nuclear localization motif in PAS-A.

[0056] FIG. 6A shows proposed model describing the relationship between PIM binding and PAS-A domain nuclear import and expected outcome in a co-expression study.

[0057] FIG. 6B shows mScarlet-tagged PAS737-WT or PAS737-NES2 (PASK AA 1-737(L667SL669SL671S)) fragments were co-expressed with WT or Y894A/S897A (PIMtideMut2) mutated GFP-tagged PIMtide. 24 hr after transfection, the relative nuclear levels of mScarlet-PAS737 fragments were visualized by confocal microscopy. Scale bars for all panels = 40 mM.

[0058] FIG. 6C shows quantification of nuclear/cytoplasmic intensities of various PAS737 fragments when co-expressed with WT or PIMtideMut2.

[0059] FIG. 6D shows the location of putative NLS residues in the PAS-A domain of PASK (underlined in red).

[0060] FIG. 6E shows LacZ-mScarlet fusion protein with or without N-terminal NLS from SV40 (PKKKRKR), WT (KRMRQERR) or mutated PASK NLS (ARMRQERR) was expressed in HEK293T cells. 24 hr after transfection, cells were treated with 25 nM Leptomycin B for 2 h.

Relative nuclear levels of various constructs are quantified as % cells with nuclear mScarlet signal onto the right. Scale bars = 40 mM.

[0061] FIG. 7A-G show Signal-regulated import of N and C-terminal domains of PASK.

[0062] FIG. 7A shows mScarlet-PAS737 and GFP-Fragment A were co-expressed in HEK293T cells. Cells were serum-starved for 18 hr. Cells were either refed with serum-free media (0%) or stimulated with 20% serum for 2 hr in the presence of 10 nM of nuclear export inhibitor, LMB. Cells were fixed, and subcellular distribution was visualized by confocal microscopy. Scale bars = 40 mM.

[0063] FIG. 7B shows quantification of % cells with nuclear PAS737 from three independent experiments in Figure 7A.

[0064] FIG. 7C shows quantification of ratio of nuclear vs. cytoplasmic intensities of PAS737 fragment from the experiment in 7A.

[0065] FIG. 7D shows mScarlet-PASNES and GFP-Fragment A were co-expressed in HEK293T cells. Cells were serum and glutamine (Q) starved for 18 hr. Cells were either refed with serum and glutamine-free media (-Q) or stimulated with 2 mM glutamine alone for 2 hr. Cells were fixed, and subcellular distribution was visualized by confocal microscopy. Scale bars = 40 mM.

[0066] FIG. 7E shows quantification of % cells with nuclear PAS737 from three independent experiments in Figure 7A.

[0067] FIG. 7F shows quantification of ratio of nuclear vs. cytoplasmic intensities of PAS737 fragment from the experiment in Figure 7D.

[0068] FIG. 7G shows model depicting a possible mechanism for signaling control of PASK nuclear import mediated by glutamine metabolism that impinges on PAS-PIM interaction. While data here is based on in trans interactions between separated PAS737 and PIM-kinase Fragments, as shown in the Figure, we suggest that the same interactions occur in cis within full-length PASK.

[0069] FIG. 8 shows a western blot for PASK expression in control, WT PIM degron and mutated PIM (Mut_PIM) degron treated stem cells. A tublin blot from the same lanes is also shown as a control.

[0070] FIG. 9 shows muscle stem cells in the presence of 1μM control peptide or TdXO-P for 96 hours. Cells were fixed and stained using anti-Pax7 (green) antibody to detect self-renewing stem cell population and with nuclei staining dye, DAPI. %Pax7+ cells were quantified

from at least five independent experiments. Error bars = ±S.D. Significance was determined using Student's t-test. $P<0.05$ is set as a significance threshold.

DESCRIPTION

[0071] The present disclosure concerns the identification of peptides that target the Per-Arnt-Sim kinase and degrons derived therefrom to target and/or cause the degradation thereof. In some aspects, the present disclosure concerns the identification of degron peptides that target and/or cause the degradation or destruction of Per-Arnt-Sim (PAS) domain-containing Kinase (PASK). PASK is a critical regulator of the transition from self-renewal state to differentiation state. PASK is expressed exclusively in stem cells, and genetic or biochemical manipulation of PASK expression affects stem cell function. The specific degradation or inhibition of PASK in stem cells can therefore allow stem cells to remain in a proliferative state.

[0072] PASK expression is also dysregulated in many human diseases, such as insulin resistance, some types of cancer (hepatocellular carcinoma), and the physiological condition of aging. Unpublished data show that over-expressing PASK in a mouse model shows a decline in muscle stem cell numbers, along with accelerated muscle atrophy. Deletion of the *Pask* gene in mice, results in regenerative defects and stem cell accumulation, confirming a key role for PASK in the ability for stem cells to differentiate.

[0073] PASK inhibition or deletion in adult tissues is linked with improved insulin sensitivity, lowered hepatic lipogenesis, and improvement in hyperglycemia. Thus, PASK inhibition provides a viable strategy for functional tissue maintenance. The current approach to acutely regulate PASK activity in cells relies on a relatively specific PASK inhibitor, BioE-1197 or BioE-1115. These chemical compounds have been effective at inhibiting PASK activity in cells. As a member of the class of ATP competitive inhibitors, BioE-1197 or BioE-1115 suffers from possible off-target inhibition of other protein kinases in cells, reducing its pharmacological precision desirable for clinical usage. Innovation: We have recently identified a unique intramolecular interaction in PASK mediated via Short Linear Interacting Motif (SLIM) binding to PAS domain of PASK. This SLIM-mediated interaction is highly specific and robust in cells and in vitro.

[0074] The present disclosure concerns a modified degron that specifically degrades PASK. In aspects, the degron is attached to an interacting peptide, such as a PASK targeting peptide. In some aspects, the modified degron is also attached to a cell-permeable amino acid sequence which can thereby allow for the administered degron to permeate through the cell membrane.

[0075] In some aspects, fusing a PASK protein or a PASK peptide causes the degradation thereof. In some aspects, the present disclosure concerns a degron with an amino acid sequence of RRRG (SEQ ID NO: 1). In order to develop an approach to successfully implement application of the degron, a modified degron was conceived that renders the degron as a cell-permeable version In some aspects, the degron is modified by attaching a PASK targeting (or PASK binding or PASK interacting) peptide. In some aspects, the degrons of the present disclosure include a cell-permeability peptide and/or a PASK targeting peptide to SEQ ID NO: 1.

[0076] In some aspects, the PASK targeting peptide may also be referred to as a PAS interacting motif or PIM. In some aspects, the PIM is a fragment or region derived from a PASK protein, such as between amino acids 880 and 904, 887 and 902, 888 and 902, 887 and 899, and/or 888 and 899 of human PASK, wherein 880 to 904 is set forth in SEQ ID NO: 17: RGAAGLQREIQEGAYSGSCYHRDGL. In some aspects, a PIM will have at least 85% identity with the amino acid sequence as set forth in SEQ ID NO: 17. In some aspects, the PIM is highly conserved across the metazoan. SEQ ID NOs: 18-27 set forth the corresponding sequences in a representative further number of species as identified in FIG. 2A. In some aspects, the PIM has a sequence of $EGX_1X_2X_3GX_4X_5X_6HR$ (SEQ ID NO: 51) or $EGX_1X_2X_3GX_4X_5X_6HRDG$ (SEQ ID NO: 52) wherein X_1, X_3 and X_5 are any amino acid, X_2 is Y or F, X_4 is E, S or N, and X_6 is Y or C. In some aspects, X_1 is S, T, I, R, A, L, C, or Q, X_3 is H, E, V, D, S, N, A, L, R, or T, and X_5 is C, V, M, G, or A.

[0077] In some aspects, the PASK targeting peptide (PIM) has an amino acid sequence of AEIQEGAYSGSCY (SEQ ID NO: 2). In some aspects the PASK targeting sequence has an amino acid sequence of AEIQEGVYSGSCY (SEQ ID NO: 3). In some aspects, the PASK is fused to the degron, such as AEIQEGAYSGSCYRRRG (SEQ ID NO: 4) and AEIQEGVYSGSCYRRG (SEQ ID NO: 5). In some aspects, one or more additional amino acids may be introduced between the fusion of SEQ ID NO: 2/3 with SEQ ID NO: 1.

[0078] In some aspects, the PIM is a sequence selected from SEQ ID NOS: 18-28. In some aspects, the PIM is selected from EIQEGAYSGSCYHRD (SEQ ID NO: 42), EIQEGTYSGSCYHRD (SEQ ID NO: 43), EIQEGIYSGSCYHRD (SEQ ID NO: 44), EIQEGIFSGSCYHRD (SEQ ID NO: 45), EIQEGTYAGSCYHRD (SEQ ID NO: 46), EIQEGTYTGSCCHRD (SEQ ID NO: 47), EILEGSYSGNCSHRD (SEQ ID NO: 48), SSINGSFVGEAIHAD (SEQ ID NO: 49), SYVDGKYRGEAIHYD (SEQ ID NO: 50). Such can be fused with a degron (e.g. SEQ ID NO: 1) and/or a cell permeability domain (see herein, e.g. SEQ ID NO: 6) and/or a ligase ligand as disclosed herein.

[0079] In some aspects, the degron is further modified through affixing a cell permeability sequence with an amino acid sequence of YGRKKRRQRR (SED ID NO: 6). In some aspects, SEQ ID NO: 6 may be fused with SEQ ID NO: 51 or 52 and with SEQ ID NO: 1, either directly or with one or more amino acids inbetween. In some aspects, the modified degron has an amino acid sequence of YGRKKRRQRREG$X_1X_2X_3GX_4X_5X_6$HRRRRG (SEQ ID NO: 53) and/or YGRKKRRQRREG$X_1X_2X_3GX_4X_5X_6$HRDGRRRG (SEQ ID NO: 54)), wherein X_1, X_3 and X_5 are any amino acid, X_2 is Y or F, X_4 is E, S or N, and X_6 is Y or C.

[0080] In some aspects, SEQ ID NO: 6 may be fused with SEQ ID NO: 2 or 3 and with SEQ ID NO: 1, either directly or with one or more amino acids inbetween. In some aspects, the modified degron has an amino acid sequence of YGRKKRRQRRRAEIQEGAYSGSCYRRRG (SEQ ID NO: 7) and/or YGRKKRRQRRRAEIQEGVYSGSCYRRRG (SEQ ID NO: 8) (termed TdXO-P) and/or YGRKKRRQRRRRRG (SEQ ID NO: 9). As set forth in the examples, the modified degrons as set forth herein are engineered to improve the specificity and strengthen the interaction between SLIM and PAS domain. Further, when expressed in cells, the modified degron eliminates nearly 99% of endogenous PASK from human and mouse stem cells, preventing their differentiation and preserving the self-renewing state of cells. Treatment of cells with 100nM of the modified degron was sufficient to acutely degrade ~80% endogenous PASK in mouse stem cells and prevent precocious differentiation *in vitro*. For example, as shown in FIG. 1, stem cells treated with the WT degron reveal significant decreased presence of PASK protein, whereas a mutated PIM degron does not.

[0081] In some aspects, the PIM may include a sequence of EIQEGTYSGSCYHRD (SEQ ID NO: 12). As set forth herein, mutations include the first tyrosine (Y894) to an alanine (as in all YSGSCY SEQ ID NO: 13 motifs) and/or a first serine (S897) to alanine. Additional degrons therefor may include EIQEGTYSGSCYHRDRRRG (SEQ ID NO: 14) YGRKKRRQRRREIQEGTYSGSCYHRD (SEQ ID NO: 15), and YGRKKRRQRRREIQEGTYSGSCYHRDRRRG (SEQ ID NO: 16).

[0082] It will be appreciated that reference to "degrons" may therefore include any one of SEQ ID NOs: 1-9, as well as derivatives thereof. In aspects, the degron peptides may include additional amino acids at either terminus and/or between the described subunits therein.

[0083] In some aspects, an amino acid spacer of 1 to about 25 amino acids may separate the linked segments, such as with PASK-spacer-degron, permeability-spacer-PASK-degron, permeability-PASK-spacer-degron, permeability-spacer-PASK-spacer-degron, permeability-spacer-degron. In some aspects where the resulting peptide has two or more spacers, it will be

apparent that each spacer can optionally be identical or different in sequence and in length. In some aspects, each resulting peptide of the present disclosure may optionally include an additional amino acid sequence appended at the amino and/or carboxyl terminus.

[0084] In some aspects, the degrons of the present disclosure may be further appended to an E3 ligase ligand to assist or target degradation (see, e.g. Lee *et al.*, Molecules, 2022, 27(19): 6515, doi: 10.3390/molecules27196515). For example, the compound TD-106 can be appended to one or more degrons as set forth herein. The presence of TD-106 can then help to harness proteolysis through the E3 ligase and enlist ubiquitin-dependent proteolysis of the target(s). For example, any one of SEQ ID NOs: 1-9, or 51-54 and the derivatives thereof disclosed herein may be fused and/or appended with an E3 ligase ligand such as TD-106. Furthermore, an E3 ligase ligand, such as TD-106, may be fused and/or appended with YGRKKRRQRRRAEIQEGAYSGSCY (SEQ ID NO: 10) and/or YGRKKRRQRRRAEIQEGVYSGSCY (SEQ ID NO: 11) and/or YGRKKRRQRREGX$_1$X$_2$X$_3$GX$_4$X$_5$X$_6$HR (SEQ ID NO: 55) and/or YGRKKRRQRREGX$_1$X$_2$X$_3$GX$_4$X$_5$X$_6$HRDG (SEQ ID NO: 56). In some aspects, the ligands can be appended to an amino terminus, a carboxy terminus or a side chain of an amino acid along the length of the degrons as described herein.

[0085] In some aspects, the degrons and modified degrons as disclosed herein may feature or include a further appended protein or peptide. The additional protein of peptide may be appended at either terminus or may be inserted between the various assembled fragments of the degron peptide as disclosed herein. The additional peptide or protein may include affinity tags, such as those used for purification, labels, such as fluorescent proteins, antibodies or active fragments thereof, such as the variable domain(s)

[0086] It will be apparent that the degrons and modified degrons as set forth herein can be produced synthetically, such as through solid phase peptide synthesis and solution phase synthesis. The degrons and modified degrons may also be produced recombinantly, such as through introducing a vector or plasmid with a promoter operably linked to a nucleic acid sequence that translates to the amino acid sequence. The degrons may also be produced through a modified viral vector or by transfection of a cell. In some aspects, the degrons may be additionally modified with a tag to allow for purification, such as a hexa-histidine, c-myc, FLAG, and so forth. In some aspects, the degron may be fused with a visual marker, such as a fluorescent protein. Such may allow for visualization of the degron. In some aspects, the degron may be radiolabeled.

[0087] In aspects, the present disclosure concerns the design and engineering of a modified degron attached to the interacting peptide. The peptide was also further engineered to improve the specificity and strengthen the interaction between SLIM and PAS domain. When expressed in cells, this degron eliminates nearly 99% of endogenous PASK from human and mouse stem cells, preventing their differentiation and preserving the self-renewing state of cells. In order to develop an *in vitro* application using this degron, a cell-permeable version of the peptide-containing degron was synthesized (termed TdXO-P). TdXO-P is a novel, bioactive, synthetic molecule not found in nature. As demonstrated herein, treatment of cells with 100nM TdXO-P is sufficient to acutely degrade ~80% endogenous PASK in mouse stem cells and prevent precocious differentiation in vitro.

METHODS

[0088] In some aspects, the degrons of the present disclosure may be used to treat a cell, such as a mammalian cell. In some aspects, the degrons of the present disclosure may be used to treat stem cells. In some aspects, the degrons of the present disclosure may be administered to a subject, such as a human subject. Such may include a pharmaceutically acceptable carrier and/or an excipient. Such are understood in the art. As set forth in the examples, treatment of a stem cell with the degrons of the present disclosure can treat and/or prevent aberrant differentiation. In some aspects, the administration of the degrons of the present disclosure may preserve or maintain a stem cell in a proliferative state. For example, as set forth in the examples, TdXO-P can be effective at preventing aberrant differentiation of isolated stem cells. Because PASK functions in all embryonic and adult stem cells, treatment with the degrons of the present disclosure, such as TdXO-P, can be utilized to preserve the proliferative state of all stem cells *in vitro* or *in vivo* and prevent their differentiation. Many applications, such as regenerative medicine, can therefore benefit from the treatment of stem cells with the degrons of the present disclosure as the treatment maintains a stem cell in a proliferative state. Regenerative medicine, for example, benefits greatly when isolated adult stem cells can be maintained in a proliferative state for extended periods. Furthermore, removing or withdrawing treatment of cells with the degrons of the present disclosure can then allow for the PASK functioning to reinitiate. TdXO-P and the other degrons disclosed herein are therefore a safe, non-toxic, and reversible agent that can preserve stem cell self-renewal.

[0089] In some aspects, the degrons of the present disclosure may be administered to aged stem cells to restore differentiation capabilities thereto. For example, as set forth in the examples,

the PASK inhibitor, BioE-1197, demonstrates that acute PASK inhibition can reverse age-associated loss or diminishment in function in stem cells. Accordingly, as the degrons herein also inhibit PASK activity, administration with the degrons of the present disclosure to aged and/or senescent stem cells can provide an opportunity to restore proliferative activities and/or reverse the aging of the stem cells.

[0090] In aspects, the degrons of the present disclosure may be administered to cells to inhibit PASK activity. As set forth in the examples herein, PASK activity can be upregulated in several known pathophysiological conditions, including Duchesne muscular dystrophy, insulin resistance, and consuming high-fat diet, as well as some types of cancer and/or aberrant cell growth. By administering the degrons to a subject, the degron can negatively impact the over-activity of PASK in such pathophysiological conditions and act as a first or appended line of therpy to treat the subject. In some aspects, the degrons may be administered by any known route, including oral, peritoneal, intravenous, sublingual, transdermal, nasally, intramuscularly, and so forth. In some aspects, the degron may be administered along with a pharmaceutical carrier and/or excipient. The degrons may be administered either alone or in combination with one or more therapeutics known to the administrator.

[0091] In some aspects, the peptides of the present disclosure may be administered to a cell and/or a subject with or without the degron motif attached or included therein. As identified herein, the PIM motif of the present disclosure can interact with the PAS domain(s) of the PASK. In some aspects, administration of the PIM can inhibit endogenous PIM domain(s) within the cell from interacting and/or binding with the PAS domains and impact the resulting kinase activity thereof. In such aspects, the PIM peptides of the present disclosure, either administered alone or in conjunction with a cell permeability peptide can competitively interact with PAS domain over endogenous PIM domains in PASK, thereby negatively impacting kinase activity without triggering degradation of PASK.

EXAMPLES

[0092] Identification of PIM domains

[0093] In proliferating mammalian stem cells, full-length WT PASK is retained in the cytoplasm. At the onset of differentiation, a large proportion of PASK is translocated to the nucleus to catalytically activate the terminal differentiation program. Therefore, it was hypothesized that PASK contains a signal-regulated mechanism for the temporal regulation of PASK nuclear translocation. To evaluate this hypothesis, it was set out to first identify sequence features in PASK that mediate the nuclear import of PASK, which could be targeted by signaling

path-ways during myogenesis. A domain truncation analysis was performed to identify regions in PASK that play important roles in nucleo-cytoplasmic trafficking. Human PASK is 1323 amino acids long and is predicted to contain three clearly identifiable domains: two PAS domains, including PAS-A, (experimentally confirmed between AA 131-237) and PAS-B (predicted only, ca. AA 254-421), and a C-terminal CAMK-type Ser/Thr kinase domain (ex-perimentally confirmed between AA 977-1300) separated from the two PAS domains by a ~600 residue-long unstructured region. Considering these structural landmarks, a series of truncations were generated (Figure 1A, Table 1) in PASK and analyzed their cellular localizations in HEK293T cells (Figure 1B). In contrast to full-length WT PASK, it was noticed that a GFP-tagged C-terminal fragment (Fragment A, AA 841-1323) was localized into the nucleus to varying degrees when expressed individually. In contrast, the N-terminal PAS737 fragment (AA 1-737, including PAS-A) was predominantly localized in the cytosol in HEK293T cells when expressed alone (Figure 1B), likely due to the presence of the recently identified Nuclear Export Sequences (NESs) within its sequence.8

[0094] Co-expression of PASK Fragment A (C-terminal region AA 841-1323) with PAS737 (N-terminal region AA 1-737) resulted in the nuclear exclusion of Fragment A (Figure 1B), raising the possibility of an association between PAS737 and Fragment A, leading to the retention of Fragment A in the cytosol. Indeed, PAS737 and Fragment A co-immunoprecipitate in cell extracts prepared from HEK293T cells (Figure 1C). Since Fragment A contains a kinase domain, it was also asked if PASK catalytic activity was required for binding PAS737; mutation of a key ATP-binding residue (K1028R) did not affect the PAS737:FragmentA co-immunoprecipitation, suggesting that the autophosphorylation activity of PASK is not involved in regulating this association.

[0095] To map the PAS737-interacting residues within Fragment A, a series of truncation analyses from the N-terminal end of Fragment A were performed(Figure 1D). As shown in Figure 1E, strong interactions between PAS737 and Fragments A (AA 841-1323) and B (AA 879-1323) was noticed, but not with the shorter Fragments C (AA 915-1323) and D (AA 949-1323). Thus, these data suggested that residues between 879 and 915 interact with the N-terminal PAS737 Fragment. Consistent with the hypothesis that the PAS domain affects the subcellular distribution of C-terminal fragments by physical association, we noticed that Fragments A and B were retained in the cytosol when co-expressed with the PAS737 fragment in HEK293T and C2C12 cells (Figures 1F, G). In contrast, Fragments C and D, which did not interact with the PAS737 fragment, remained nuclear in PAS737 expressing cells in C2C12 cells. These results suggest that an

intramolecular association in PASK, here emulated by the intermolecular binding of PAS737 with the C-terminal Fragments A and B, might dynamically regulate its nucleo-cytoplasmic shuttling.

[0096] Because PASK is an evolutionarily conserved protein kinase, it was reasoned reasoned that the functionally important residues for Fragment A (841-1323) and PAS737 Fragment interaction would likely be conserved. Indeed, multiple sequence alignment of PASK across various species showed strong sequence conservation between amino acids 879 and 915 (Figure 2A). It was particularly noticed the presence of a GXXXG-like motif (G892-A893-Y894 -S895-G896) and the highly conserved Y894 and S897 residues, which could be targeted by posttranslational modifications. Mutations of either the GXXXG motif with AXXXA (Figure 2B, Table 1, designated as PIM-Mut1) or Y894A and S897A (Table 1, Figure 2B, Table 1, designated as PIM-Mut2) substantially disrupted the co-immunoprecipitations between the mutated Fragment A with PAS737, indicating that these residues are necessary for the intramolecular interaction between PAS737 and Fragment A. Furthermore, a Flag-GFP fusion construct with a peptide comprising the minimal conserved region (Table 1, AA 879-904, termed PIMtideWT) was sufficient to interact with PAS737 (Figure 2C, D); whereas the Y894AS897A mutated PIMtide (Table 1, AA 879-904 (Y894AS897A), termed PIMtideMut2) completely disrupted this association, just as it did in the larger Fragment A context. Functionally, while WT-Fragment A was retained in the cytosol when co-expressed with PAS737,the Y894AS897A mutated Fragment A remained nuclear and unaffected by co-expression of PAS737 fragment (Figures 2E, F). Thus, residues 879-904, which are termed PAS Interacting Motif, or PIM, are necessary and sufficient to mediate an intermolecular interaction between N-terminal and C-terminal regions within PASK that we suspect to also work in an intramolecular context.

Table 1 A list of various truncations and mutations of human PASK used in this study.

Terminology used	PASK	Mutations Type
WT (human PASK) or $PASK^{WT}$	AA 1-1323 (full length)	None
PAS^{737}	AA 1-737	Truncation
PAS^{307}	AA 1-307	Truncation
ΔPAS^{737}	AA 328-737	Truncation
PAS-A	AA 131-237	Truncation
Fragment A	AA 841-1323	Truncation
Fragment B	AA 879-1323	Truncation
Fragment C	AA 915-1323	Truncation
Fragment D	AA 949-1323	Truncation
KD	K1028R	Point mutations
PIM-Mut1	G892A/G896A	Point mutations
PIM-Mut2	Y894A/S897A	Point mutations
NES2-Mut	L667S/L669S/L671S	Point mutations
GFP-400	AA 1-400	Truncation
$PIMtide^{WT}$	AA 879-904	Peptide
$PIMtide^{Mut2}$	AA 879-904 (Y894A/S897A)	Peptide
$PASK^{PIM-Mut2}$	AA 1-1323 (full length) with PIM-Mut2 mutation	None
$PAS^{737-Mut}$	AA 1-737 (K132A/W216A/E223A)	Point mutations
$PAS^{737-NES2}$	AA 1-737 (L667S/L669S/L671S)	Truncation and Point mutations
$NLS1^{WT}$	AA K218-R225	Peptide
$NLS1^{mut}$	AA K218A-R225	Peptide

[0097] Next, it was sought to identify the PIM-binding region in the N-terminal PAS737 fragment. First, truncations in this region were generated (Figure 3A) and measured their interactions with Fragment A (841-1323). The results indicated that the first 307 amino acids (termed PAS307), which contain the PAS-A domain and unstructured flanking regions, are sufficient to interact with Fragment A (Figure 3B). Furthermore, mutation of conserved K132, E223, and W216 in the human PAS737 (K132A/W216A/E223A) abrogated PAS-PIM interaction in a co-immunoprecipitation experiment from cells (Figure 3C). These results revealed the PAS-A domain of PASK to be engaged in a novel short linear interacting motif (SLIM) mediated interaction, which could be targeted for regulation. Finally, it was asked if mutations in PAS-A that disrupted its interaction with PIM in cells (Figure 3D) affect the nuclear accumulation of PASK. GFP-tagged Fragment A and mScarlet-tagged WT or K132A/W216A/E223A-mutated PAS737 (PAS737-Mut) Fragments were expressed. As shown in Figure 3D-E, when co-expressed with mutated PAS737, Fragment A showed increased nuclear levels compared with WT-PAS737.

[0098] A previous solution structure of the PASK PAS-A (131-237) domain identifed that small molecules can bind into a cavity inside of it as part of this work. To confirm the PAS-PIM interaction in solution and determine how that might affect this PAS-A cavity, it was first measured the binding affinity (KD) of PIM (AA 879-904) for the isolated PAS-A (131-237)

domain using protein-detected NMR titrations.10 By selectively monitoring PIM-induced chemical shift changes within uniformly ^{15}N-labeled PAS-A with ^{1}H/^{15}N HSQC spectra across increasing concentrations of PIM (0.1–2.5 mM), measuring an apparent KD of 2.4 mM of PIM for PAS-A (Figure 4A). To identify the residues perturbed by PAS-A:PIM interactions, changes in backbone amide ^{1}H/^{15}N shifts from unliganded PAS-A upon the addition of either PIM or the KG-571 small molecule were compared, which were previously demonstrated to bind inside of the PAS-A cavity (Figures 4B, C). These analyses showed chemical shift changes by either PIM or KG-571 around the PAS-A cavity, including perturbations in the Ab strand, Fa helix, FG-loop, and Gb,Hb,Ib strands. These changes were in complementary groups of residues in the PAS-A solution structure: PIM affected sites adjacent to the cavity, with the largest changes in the FG loop and flanking regions, while KG-571 bound within the cavity consistent with prior observations. Interestingly among the residues mutated above to weaken the PAS-A:PIM interaction, it was observed substantial chemical shift perturbations for W216 (Hb) and E223 (HI-loop) upon the addition of PIM and KG-571, while K132 (Ab) was perturbed only with KG-571, but not PIM, possibly due to the missing N-terminal residues within the NMR constructs which may facilitate transient PAS-A:PIM interactions in the cell-based assay with the longer PAS737 fragment.

[0099] To further characterize the structural rearrangements caused by PIM or KG-571 binding to PAS-A, hydrogen–deuterium exchange monitored by mass spectrometry (HDX-MS) was also used, which reports on changes in backbone amide protection from exchange with solvent deuterons. Local HDX-MS was used, where deuterium uptake is monitored at the peptide level to yield information on structural dynamics with more flexible solvent-accessible areas more readily exchanging with deuterons than less solvent-accessible areas. Similar to the NMR results, it was observed higher deuterium exchange around the PAS-A cavity for the apo state compared to those with either PIM or KG-571 added (Figure S2). With PIM or KG-571, it was observed less deuterium exchange (=stabilization) around the Ca, Da,Fa helices, and FG-loop; we also observed PIM-specific in the Ab strand and KG-571-specific stabilization in the Hb-Ib strands. From these results it can be concluded that structural changes within the hydrophobic core in or around the cavity of the PAS-A domain by PIM or ligand binding lead to changes in the structure and/or dynamics of regions of PAS-A involved in intramolecular interactions with other parts of PASK, giving rise to its functional regulation.

[00100] To visualize this interaction, an AlphaFold2 (AF2) Multimers model of the PAS-A and PIM complex was generated. While this model was generated without any direct input from

experimental data, it should be noted that it too suggests PIM binds near the PAS-A cavity (Figure 4E). The model of the PAS-A:PIM complex with the best pLDDT score suggests substantial conformational changes occur upon PIM peptide binding, especially in the FG-loop, Fα,Gb,Hb regions that showed similar changes in NMR and HDX-MS experiments. The PAS-A:PIM interactions were analyzed in the AF2 models with the HADDOCK PRODIGY server which uses contact-based prediction of protein–protein complexes. In this analysis, it was observed that PIM residues Y894 and S897 contacted several points with PAS-A in the AF2 model, corroborating prior co-immunoprecipitation data showing the importance of both residues for PIM interactions with PAS737 (Figure 2). Taken together, these data suggest a model where both ligand and PIM interact around the flexible PAS-A cavity, potentially facilitating allosteric control of inter- or intra-molecular interactions involved in PASK biological functions.

[00101] Based on this *in-cellulo* and *in vitro* studies, the possibility that the disruption of an intramolecular PAS-A and PIM interaction could underline the signal-regulated nuclear trafficking of PASK was considered. However, the mutation of PAS-A binding residues, Y894A/S897A in full-length PASK (PASKPIM-Mut2), did not induce nuclear translocation of full-length PASK (Figure 5A-B). This is not surprising since the identification of two strong Nuclear Export Sequences (NES) located between residues AA 401-410 (NES1) and AA 666-671 (NES2) in PASK (Figure 5C) has been noted, the combination of which is powerful enough retain an engineered PASK in the cytosol even when fused to the strong SV40 NLS.8

[00102] To further understand the functional interplay of the PAS-A:PIM interaction and these nuclear export sequences, PAS737 or NES2-mutated version of PAS737 fragment (PAS737-NES2, PASK 1-737 were co-expressed with L667S/L669S/L671S mutations) (Table 1) along with WT or PIM-Mut2 Fragment A (PASK AA 841-1323 with Y894A/S897A) (Figure 5C-D). As expected, PAS737 was cytoplasmic regardless of the co-expression with either WT or a Y894A/S897S mutated Fragment A, owing to the presence of functional nuclear export sequences. Furthermore, PAS737-NES2 was retained in the cytoplasm when co-expressed with WT-Fragment A. However, co-expression of PAS737-NES2 with PIM-Mut2-Fragment A, which likely disrupted the interaction between PAS-A:PIM, resulted in an increased distribution of PASNES2 construct in the nucleus, along with PIM-mutated Fragment A (Figure 5D-E).

[00103] It was previously shown that a smaller PAS domain-containing fragment, Fragment 1-400 is predominantly nuclear since it lacks both nuclear export sequences in PASK but retains nuclear import capability.8 To determine if interaction with PIM affects the nuclear

import of the 1-400 frag-ment, we expressed GFP-tagged 1-400 fragment (Table 1, termed GFP-400) with or without mCherry-tagged PIMtideWT (mCherry-879-904). Interestingly, co-expression with PIMtideWT reduced the nuclear localization of GFP-400 (Figure 5F-G). These observations raised an intriguing possibility that the interaction between PAS-A and PIM controls the nucleo-cytoplasmic distribution of PASK and that PAS-A and flanking regions can mediate the import of PASK into the nucleus when it is not masked by PIM.

[00104] To directly test this hypothesis, mScarlet-tagged PAS737 or PAS737-NES2 proteins were co-expressed with GFP-tagged PIMtideWT (GFP-AA 879-904) or PIMtideMut2 (GFP-AA 879-904Y894AS897A) plasmids (Table 1, Figure 2C). PAS737 was expected to be excluded from the nucleus under all conditions due to the presence of two functional nuclear export sequences (Figure 6A-B). In contrast, PAS737-NES2 would be excluded from the nucleus if its nuclear localization motif was masked by PIM interaction. Interestingly, while nuclear import and accumulation of PAS737-NES2 were blocked when co-expressed with GFP-PIMtideWT, it was noticed that markedly increased nuclear import and retention of PAS737-NES2 when only when interaction deficient GFP-PIMtideMut2 was co-expressed. These results show that the PIM occupancy in the PAS domain blocks nuclear import pathways (Figure 6B-C).

[00105] Based on analyses, it was hypothesized that the PIM binding to the PAS-A domain masks a Nuclear Localization Sequence (NLS). To test this hypothesis, it was first sought to identify the nuclear localization sequence in the PAS-A domain of PASK. Several bioinformatic analysis algorithms predicted residues K218-R225 (KRMRQERR SEQ ID NO: 38) as a putative monopartite nuclear localization motif within the PAS-A domain (Figure 6D). Additionally, we also observed the segment of PAS-A HI-loop, which includes the NLS sequence (K218-R225), to have chemical shift changes in NMR with KG-571 and PIM (Figure 4B).

[00106] To directly test if residues K218-R225 function as NLS and are sufficient to import a heterologous protein, we generated a LacZ-mScarlet fusion protein (MW = ~100 kDa) with a peptide consisting of AA K218-R225 (218KRMRQERR225 SEQ ID NO: 38) from PAS-A domain (termed NLS1WT), or single point mutant version, NLS1mut (218ARMRQERR225 SEQ ID NO: 39). As a positive control, we fused the SV40NLS (PKRKRRR SEQ ID NO: 41) peptide, which is known to import tagged protein into the cell nucleus with LacZ-mScarlet protein, and nuclear accumulation of LacZ-mScarlet alone was used as a baseline. We tested the ability of these constructs to mediate the nuclear import of LacZ-mScarlet fusion protein. We also used a nuclear export inhibitor, leptomycin B (LMB), to ensure nuclear retention of the imported fusion protein. As shown in Figure 6D, peptide NLS from SV40 or PAS-A domain of PASK (K218-

R225) were comparable at stimulating the nuclear import of LacZ-mScarlet fusion protein in LMB-treated cells. On the other hand, K218A-mutated NLS1 (NLS1mut), was ineffective at promoting the nuclear localization of the LacZ-mScarlet fusion protein (Figure 6D). Thus, these results revealed a unique functional role of the intramolecular interaction between the PAS-A domain and PIM sequence in regulating the PASK nuclear translocation by masking the NLS sequence in the PAS domain.

[00107] PASK is important for the terminal differentiation of embryonic and adult stem cells. While PASK is a predominantly cytoplasmic protein in proliferating stem cells, it is nuclear localized at the onset of myoblast differentiation. This nuclear distribution of PASK is induced by differentiation signaling cues and drives its physical association with its nuclear substrate, Wdr5. In addition, the nuclear export inhibitor, LMB, is ineffective in blocking PASK nuclear export under steady-state conditions. However, under serum-stimulated conditions, LMB effectively blocked the nuclear export of PASK, indicating that the nuclear import of PASK is signal-regulated. Mitochondrial glutamine metabolism induces CBP/p300-induced acetylation of PASK, which drives its nuclear localization. Based on these results, it was hypothesized that signaling pathways target PAS-PIM inter-actions to stimulate its nuclear import at the onset of terminal differentiation. Consistent with this hypothesis, it is found that serum starvation resulted in cytoplasmic retention of PAS737 fragment when co-expressed with WT-Fragment A. Acute serum stimulation, on the other hand, induced strong nuclear localization of both PAS737 fragment and Fragment A (Figure 7A-C). Finally, since mitochondrial glutamine metabolism drives PASK nuclear translocation, it was asked if glutamine metabolism serves to unmask the PAS-PIM interaction to stimulate PASK nuclear translocation. For this experiment, PAS737-NES2 co-expression with WT-Fragment A was used, both of which show cytoplasmic localization (see Figure 5D) under steady-state conditions. The use of PAS737-NES2 allowed for nuclear accumulation of the PAS737 fragment and circumvented the need to use LMB pretreatment. As shown in Figure 7D-F, in glutamine-starved cells, the PAS737-NES2 fragment remained cytosolic when co-expressed with WT-Fragment A. However, acute glutamine stimulation alone (in the absence of serum) was sufficient to increase the nuclear import of PAS737-NES2 and Fragment A.

[00108] Taken together, overall a novel metabolic regulation of PASK nuclear import via disruption of the intramolecular interaction between the N-terminal PAS domain and the C-terminal PAS interacting motif (PIM) is revealed (Figure 7G). These results discovered a

functional role of the PAS domain of PASK in mediating its nuclear import, which could play a crucial role in regulating the transition between self-renewal and differentiation of stem cells.

[00109] The modes by which PAS domains sense and regulate cellular metabolic state are as diverse as the family members it comprises. In this study, a mode by which the PAS domain of PASK regulates the subcellular distribution of PASK in response to a metabolic cue is identified. These studies identified a protein–protein interaction interface in the PASK PAS-A domain that is intramolecularly occupied by a short-linear motif, which is herein termed PIM, situated adjacent to the C-terminal kinase domain. Through combined in situ biochemical and cell biological studies, we revealed the presence of a putative NLS in the PAS-A domain of PASK. Residues surrounding the PAS-A NLS inter-act with PIM, and mutations in PIM that disrupt this binding promote nuclear retention of an N-terminal PASK fragment, which includes the PAS-A domain. A metabolic process likely regulates the dynamic of the PAS-A:PIM interaction since acute glutamine stimulation results in the nuclear import of PASK in a PAS-A:PIM interaction-dependent manner. Thus, this study identified the intramolecular association of PASK as a regulatory feature that could be controlled by metabolic pathways (Figure 7G).

[00110] The discovery of an NLS in the PASK PAS-A domain was surprising since NLS sequences are often associated with unstructured or coiled-coil regions connecting protein domains. However, several literature reports support the notion that intramolecular interactions involving PAS domains can play regulatory roles in controlling the nuclear translocation of PAS-containing proteins. For example, in plant phytochrome B, a NLS is situated in the C-terminal PAS-B domain and is unmasked in a light-dependent manner. Similarly, NPAS4 and PERIOD exhibit control of nucleo-cytoplasmic distribution via intramolecular interac-tion involving PAS domains.15–17 Within PASK, the putative NLS it was identified that (residues K218-R225) can independently function when removed the sur-rounding domain (Figure 6D); the isolated NLS peptide is likely in a random coil conformation. Within the PAS-A domain NMR structure,9 however, it is seen that this sequence is also in an extended conformation that is similar to the one adopted by the SV40 NLS when bound to importin alpha,18 without obvious steric clashes required for the whole PAS-A domain to bind the importin.

[00111] Notably, solution NMR and HDX-MS data suggest that PIM binding to PAS-A masks the NLS, with residues in the Fa helix, FG loop, and Hb strand reporting on this interaction. The unassisted AF2 Multimers model – generated without using any of these data – strongly implies that PIM binding causes conformational changes in PAS-A that cause the FG loop to fold back upon the NLS region, blocking the required interactions with importins required for nuclear

import. These observations from in vitro experiments on minimal fragments are consistent with the cellular data on larger systems (Figures 1-2).

[00112] Excitingly, the *in vitro* data show that these PAS-A:PIM interactions occur adjacent to the internal cavity that were previously identified to bind small molecule ligands, including the artificial KG-571 compound utilized here (Figure 4). This sets up the potential for metabolite binding within the cavity to allosterically control PIM binding, and regulate the overall affinity of the PAS-A:PIM interactions, NLS exposure and PASK nuclear import (Figure 7E). At the same time, in the absence of known physiological ligands, it remains challenging to biochemically assess the relative strength of interaction between PAS-A and PIM-containing fragments in response to signaling cues. Nevertheless, the data in Figures 5 and 6 point to a regulatory role of PAS-A:PIM interactions in situ in mediating the nuclear import of PASK. While further proof of this control requires the identification of physiological ligands for this domain (or alternatively, higher affinity artificial ligands than those currently known), many other PAS domains are well known to regulate comparable PAS:protein interactions via changes in ligand occupancy or configuration.

[00113] Using complementary techniques of *in vivo* and *in vitro* protein–protein interaction and localization assays, a novel functional role of PAS-A domain of PASK as a regulator of its nuclear import machinery is identified. This study suggests a model of N-terminal PAS domain protein/ligand and protein/protein interactions within PASK, building on earlier work demonstrating PAS-A could bind small organic compounds. In this study the analysis is expanded to show that the PAS-A domain engages in intramolecular interactions via a small linear motif, termed PIM that coordinates PASK nuclear import in accordance with metabolic inputs. Specific residues and interaction sites between the PAS-A and PIM fragments arte also identified, which could be key to the overall function of PASK. Taken together, this study could lead to further discovery of PASK biological regulation. As the nuclear import of PASK is critical for the stem cell differentiation program, the analyses of PAS-A: PIM interaction provide a significant mechanistic advance in understanding how metabolic signals drive PASK nuclear import at the onset of terminal differentiation program, which can be targeted to develop therapeutic strategies aimed at balancing stem cell self-renewal and differentiation capabilities.

[00114] Materials and methods

[00115] HEK293T and C2C12 myoblasts were obtained from ATCC and cultured in standard Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum, with glutamine and sodium pyruvate (Gibco, Cat# 119995) and 1% penicillin and streptomycin. For

protein expression, cells were transfected with plasmids as indicated the figure legends using polyethylenimine (PEI) based transfection.

[00116] PCR-based amplification was used to generate C-terminally truncated domains in pCDNA3.1A-V5 vector. Similarly, N-terminal fragments were cloned into pCDNA3.1B-Myc vector following PCR-based amplification. For site-directed mutagenesis, sewing PCR was used using partially overlapping primers with a centrally located mutant nucleotide.

[00117] The protein lysates from overnight transfected HEK293T cells when the cells reached approximately 80% confluency. For that, cells were harvested by scraping and subsequently lysed in native cell lysis buffer. The native cell lysis buffer consisted of 20 mM Tris-HCl pH 7.5, 150mMNaCl,1mMEDTA,1mMNaF,1mM beta-glycerophosphate, and 1% Triton X-100, supplemented with freshly added protease and phosphatase inhibitors including 1 mM PMSF, 1 X protease inhibitor cocktail, and 1 X phosphatase inhibitor cocktail (Sigma). The cell lysates were incubated on ice for 20 min to ensure complete lysis, followed by centrifugation at 15 K RPM X 10mins in refrigerated centrifuge.

[00118] For co-immunoprecipitation, the clarified cell lysates were utilized as described above. Ten percent of each lysate was collected as input, and the remaining lysate was subjected to immunoprecipitation (IP) overnight at 4 °C using either Anti-V5 Agarose Affinity Gel beads (Sigma, A7345) or Anti-FLAG M2 magnetic beads (Sigma, M8823), depending on the target protein. The IP samples were then washed five times on ice using either wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100). Subsequently, the samples were resolved by SDS-PAGE, followed by immunoblot analysis using the specific antibodies as indicated in figure panels.

[00119] For immunofluorescence microscopy, cells were seeded in 24-well plates on glass coverslips. The coverslips were precoated with 0.1% gelatin for HEK293T or C2C12 cells. At the designated experimental time points, the cells were fixed with 4% paraformaldehyde (PFA) in 1 X PBS for 15 min, followed by three washes with 1 X PBS. To visualize fluorescently tagged proteins, coverslips were mounted using ProLong Antifade mounting media containing DAPI (ThermoFisher Sci) and confocal analysis was conducted after mounting media curing. For detection of non-fluorescently labeled proteins, cells were permeabilized using 0.2% Triton X-100 in 1 X PBS for 10 min at room temperature. Subsequently, blocking was performed using 10%normal goat serum in 1 X PBS for 1 hour. Primary antibodies, diluted in 1 X PBS, were added to all wells and incubated overnight at 4 °C in a humidified chamber. Afterward, the wells were washed three times with 1 X PBS. For secondary antibody staining, appropriate secondary

antibodies (Alexa Fluor 488 – green and 568 –red, Thermo Fisher Sci) were added to the wells and incubated for 1 hour at room temperature. Following three washes with 1 X PBS, the coverslips were mounted onto glass slides using ProLong Antifade mounting media with DAPI (Thermo Fisher Sci). The mounted coverslips were allowed to cure overnight at room temperature before imaging was performed using confocal microscopy (Nikon A1R). Analysis and quantification of the acquired images were carried out using calibrated Fiji software.

[00120] The hPASK PAS-A domain (residues 131-237) was expressed as an N-terminal His_6-tagged fusion in *E. coli* BL21(DE3) using $^{15}NH_4Cl$-containing M9 minimal media as previously described. Harvested pellets from 1 L growths were suspended in 40 mL 50 mM Tris-HCl (pH 6.5), 100 mM NaCl, and 25 mM imidazole buffer and sonicated at 4 °C. Lysates were separated by centrifugation at 17500 x g at 4 °C for 45 min, with the resulting supernatants filtered with 0.2-micron filters and subjected to Ni-Sepharose affinity purifi-cation, with the pHis-PAS-A protein obtained by gradient elution with 25–500 mM imidazole in the same buffer. Eluted samples were digested with His_6-TEV protease overnight to specifically cut an engineered TEV protease cleavage site present between the His_6 affinity tag and the PAS-A domain, with the digesting sample dialyzed into 3 L of imidazole-free buffer at 4 °C. The cleaved protein was again applied to Ni-Sepharose affinity chromatography, this time collecting the PAS-A protein in the flow-through fraction. Fractions were concentrated (Amicon Ultra, Millipore) and subjected to a final Superdex 75 size exclusion chromatography step in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, 5 mM DTT, and 6 mM NaN3. Fractions corresponding to monomeric PAS-A were concentrated, and flash frozen in liquid N2 and stored at ~80 °C. The purity of the protein was assessed via SDS-PAGE electrophoresis, with typical yields of 100–200 mg/L.

[00121] All NMR experiments were conducted at 298 K on Bruker Avance III HD NMR spectrometers at 700 or 800 MHz equipped with 5-mm inverse TCI cryoprobes, pulsed-field Z (700 MHz) or XYZ (800 MHz) gradients, and Topspin 3.5 software (Karlsruhe, Germany). NMR experiments of PAS-A (0.1 mM) and PIM (residues 879-902) titration (0.1, 0.3, 0.5, 0.7, 0.9, 1.2, 1.5, 1.7, 2.0, and 2.5 mM) were conducted in 14.7 mM Tris pH 7.4, 35 mM sodium phosphate (pH 7.4), 20 mM NaCl, and 10% D2O. The saturated concentration of artificial ligand KG-571 (2 mM) and PIM (3.7 mM) for NMR chemical shift perturbation data were collected with 225 μM of PAS-A in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl, 5 mM DTT, and 6 mM NaN_3. All NMR data were processed and analyzed with NMRFx and/or NMRViewJ (One Moon Scientific).

[00122] Protein samples for Hydrogen Deuterium Exchange monitored by Mass Spectrometry (HDX-MS) were prepared as described above in 20 mM sodium phosphate (pH

6.5), 50 mM NaCl, 5mMDTT, and 6mM NaN_3. Stock protein concentrations were 100 μM PAS-A, with 2 mM KG-571 or 3 mM PIM added for complex samples (pH of the complexes were corrected for 6.5). Deuterium exchange was initiated at room temperature by diluting 5 μL of sample in 75 μLof D_2O buffer, allowing exchange to continue for 30, 150, 300, 1000, 3000, and 5000 s before being quenched with 80 μL of ice-cold (~3 °C) quench buffer (3 M GdHCl + 3% acetonitrile + 0.8% formic acid (pH ~ 1.9)).

[00123] Quenched protein samples were immediately injected over an Enzymate BEH Pepsin Column (Waters) to digest the protein while desalting the resulting peptides by a 10 μL C8 Opti-lynx II trap cartridge (Optimize Technologies) at a flow rate of 0.12 mL/min using 0.25% formic acid in water as a mobile phase. After three min of injection, peptides were resolved with an analytical C18 column (Hypersil GOLD 1x50 mm, Thermo Scientific) and quickly eluted into a Bruker maXis-II ETD UHR ESI-QqTOF spectrometer at a flow rate of 40 μL/min for mass determination.

[00124] All steps of quenching, digestion, and injection were performed using a LEAP HDX automation robotic system (Trajan Scientific and Medical) with each exchange timepoint repeated twice for statistical validation.

[00125] In addition to the HDX-MS samples noted above, unlabeled samples were run under identical conditions as HDX except in H_2O buffer to provide masses and retention times of undeuterated peptides as reference. The unlabeled run was performed with MS/MS fragmentation to identify peptides using COMPASS DataAnalysis and BioTools software (Bruker). Every matched m/z and retention time pair was assigned to only one peptide sequence based on accurate mass and MS/MS measurements. This resulted in coverage of 100% of the protein sequence. The resulting peptide (sequence, m/z, charge state, and retention time) lists and the exchange rates were analyzed using HDExaminer version 3.3 (Sierra Analytics) software. All peptide matches were manually confirmed after automatic assignment by HDExaminer. At the end of the data analysis, ~90% of the peptides resulted in high confidence coverage for the HDX-MS of the three reported conditions (apo PAS-A, PAS-A/PIM complex, PAS-A/KG-571 complex).

[00126] To generate structural models of PAS-A:PIM complex, AlphaFold2 Multimers were used guided high-accuracy prediction, the hPASK (Uniprot ID: Q96RG2) PAS-A(131-237) and PIM(879-904) sequences. The existing structure of PAS-A (PDB-ID: 1ll8)9 was used as a template for the PAS-A: PIM complex model generation. Out of 10 models generated, the model shown in Figure 4E was the best among these, ranked by the pLDDT confidence measure as

having high accuracy. The model is available in ModelArchive (modelarchive.org) (ID: ma-96ezk [available pre-publication with the accession code MvzBruyYQg]).

[00127] PASK Degron

[00128] Stem cells were treated with the degron as set forth in SEQ ID NO: 8 and/or 9, with a mutant in the PIM used as a control, as well as cells treated with vehicle. Cell lysates were obtained and a western blot was performed for PASK and tubulin (control). The WT degron inhibited PASK expression in the stem cells (see FIG. 8).

[00129] Muscle stem cells were isolated from 8-weeks old C57BL6 mice. Cells were allowed to proliferate in the presence of 1μM control peptide or TdXO-P for 96 hours. Cells were fixed and stained using anti-Pax7 (green) antibody to detect self-renewing stem cell population and with nuclei staining dye, DAPI. %Pax7+ cells were quantified from at least five independent experiments. Error bars = ±S.D. Significance was determined using Student's t-test. $P<0.05$ is set as a significance threshold (See FIG. 9).

[00130] While particular aspects have been illustrated and described herein, it should be understood that various other changes and modifications may be made without departing from the spirit and scope of the claimed subject matter. Moreover, although various aspects of the claimed subject matter have been described herein, such aspects need not be utilized in combination. It is therefore intended that the appended claims cover all such changes and modifications that are within the scope of the claimed subject matter.

[00131] It is appreciated that all reagents are obtainable by sources known in the art unless otherwise specified.

[00132] It is also to be understood that this disclosure is not limited to the specific aspects and methods described herein, as specific components and/or conditions may, of course, vary. Furthermore, the terminology used herein is used only for the purpose of describing particular aspects of the present disclosure and is not intended to be limiting in any way. It will be also understood that, although the terms "first," "second," "third" etc. may be used herein to describe various elements, components, regions, layers, and/or sections, these elements, components, regions, layers, and/or sections should not be limited by these terms. These terms are only used to distinguish one element, component, region, layer, or section from another element, component, region, layer, or section. Thus, "a first element," "component," "region," "layer," or "section" discussed below could be termed a second (or other) element, component, region, layer, or section without departing from the teachings herein. Similarly, as used herein, the singular forms "a," "an," and "the" are intended to include the plural forms, including "at least one," unless the content

clearly indicates otherwise. "Or" means "and/or." As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. It will be further understood that the terms "comprises" and/or "comprising," or "includes" and/or "including" when used in this specification, specify the presence of stated features, regions, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, regions, integers, steps, operations, elements, components, and/or groups thereof. The term "or a combination thereof" means a combination including at least one of the foregoing elements.

[00133] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. It will be further understood that terms such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and the present disclosure, and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

[00134] Reference is made in detail to exemplary compositions, aspects and methods of the present disclosure, which constitute the best modes of practicing the disclosure presently known to the inventors. The Figures are not necessarily to scale. However, it is to be understood that the disclosed aspects are merely exemplary of the disclosure that may be embodied in various and alternative forms. Therefore, specific details disclosed herein are not to be interpreted as limiting, but merely as a representative basis for any aspect of the disclosure and/or as a representative basis for teaching one skilled in the art to variously employ the present disclosure.

[00135] Patents, publications, and applications mentioned in the specification are indicative of the levels of those skilled in the art to which the disclosure pertains. These patents, publications, and applications are incorporated herein by reference to the same extent as if each individual patent, publication, or application was specifically and individually incorporated herein by reference.

[00136] The foregoing description is illustrative of particular embodiments of the disclosure, but is not meant to be a limitation upon the practice thereof. The following claims, including all equivalents thereof, are intended to define the scope of the disclosure.

CLAIMS

1. A degron peptide comprising a PASK protein or fragment thereof fused to an amino acid sequence of RRRG (SEQ ID NO: 1).

2. The degron peptide of claim 1, wherein the PASK fragment comprises a PASK interacting motif (PIM) comprising an amino acid sequence with at least 85% sequence identity to SEQ ID NO: 17.

3. The degron peptide of claim 1 or 2, wherein the PASK fragment comprises a PASK interacting motif (PIM) comprising an amino acid sequence of $EGX_1X_2X_3GX_4X_5X_6HR$ (SEQ ID NO: 51) or $EGX_1X_2X_3GX_4X_5X_6HRDG$ (SEQ ID NO: 52), wherein X_1, X_3 and X_5 are any amino acid, X_2 is Y or F, X_4 is E, S or N, and X_6 is Y or C.

4. The degron peptide of claim 3, wherein the PIM sequence is selected from on of SEQ ID NOs 18-28 or 42-50.

5. The degron peptide of claim 3, wherein the PIM comprises the amino acid sequence of AEIQEGAYSGSCY (SEQ ID NO: 2) or AEIQEGVYSGSCY (SEQ ID NO: 3).

6. The degron peptide of claim 5, wherein the peptide comprises the amino acid sequence of AEIQEGAYSGSCYRRRG (SEQ ID NO: 4) or AEIQEGVYSGSCYRRG (SEQ ID NO: 5).

7. The degron peptide of claim any of claims 1-6, further comprising a cell permeability sequence.

8. The degron peptide of claim 7, wherein the cell permeability sequence comprises the amino acid sequence of YGRKKRRQRR (SED ID NO: 6).

9. The degron peptide of claim 8, wherein SEQ ID NO: 6 is fused to at least one of SEQ ID NO: 4 or 5.

10. The degron peptide of claim 8, wherein the degron peptide comprises the amino acid sequence of $YGRKKRRQRREGX_1X_2X_3GX_4X_5X_6HRRRRG$ (SEQ ID NO: 53) and/or

YGRKKRRQRREGX$_1$X$_2$X$_3$GX$_4$X$_5$X$_6$HRDGRRRG (SEQ ID NO: 54)), wherein X$_1$, X$_3$ and X$_5$ are any amino acid, X$_2$ is Y or F, X$_4$ is E, S or N, and X$_6$ is Y or C.

11. The degron peptide of claim 8, wherein the degron peptide comprises the amino acid sequence YGRKKRRQRRRAEIQEGAYSGSCYRRRG (SEQ ID NO: 7) and/or YGRKKRRQRRRAEIQEGVYSGSCYRRRG (SEQ ID NO: 8) (termed TdXO-P) and/or YGRKKRRQRRRRRG (SEQ ID NO: 9).

12. A nucleic acid encoding the degron of any preceding claim.

13. A method for inhibiting PASK activity in a cell comprising administering the degron peptide or nucleic acid encoding the same of any of claims 1-12.

14. The method of claim 13, wherein the cell is within a subject.

15. The method of claim 14, wherein the subject is a human.

16. A method for treating a stem cell comprising administering to a stem cell the degron peptide or nucleic acid encoding the same of any of claims 1-12.

17. A method for treating aberrant PASK activity in a subject comprising administering to a subject the degron peptide or nucleic acid encoding the same of any of claims 1-12.

18. A method for inhibiting PASK activity in a cell comprising administering to the cell a peptide comprising a PIM motif, wherein the PIM motif comprises an amino acid sequence of EGX$_1$X$_2$X$_3$GX$_4$X$_5$X$_6$HR (SEQ ID NO: 51) or EGX$_1$X$_2$X$_3$GX$_4$X$_5$X$_6$HRDG (SEQ ID NO: 52), wherein X$_1$, X$_3$ and X$_5$ are any amino acid, X$_2$ is Y or F, X$_4$ is E, S or N, and X$_6$ is Y or C.

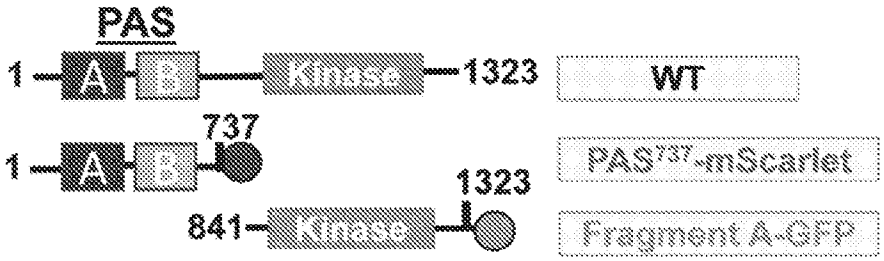


FIG. 1A

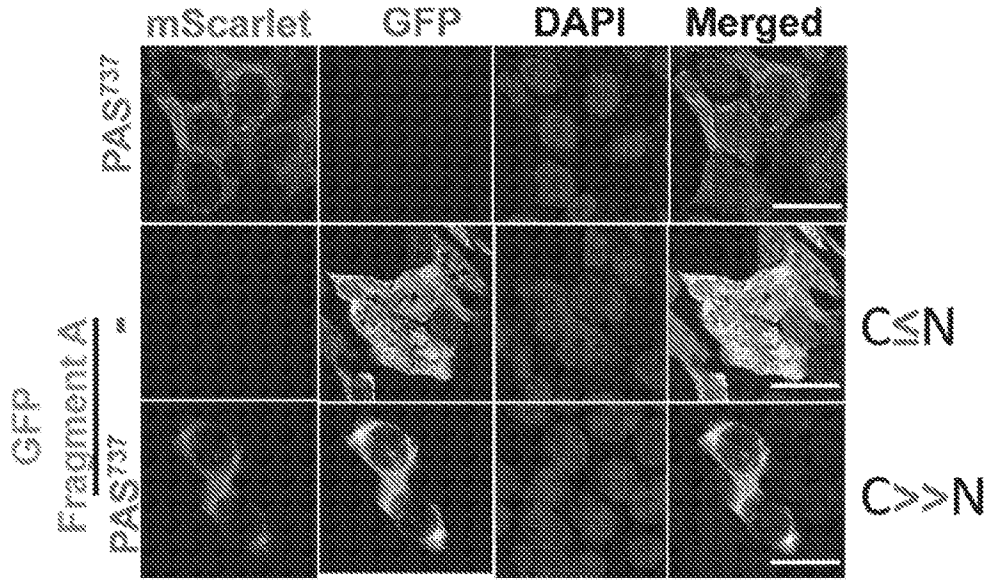


FIG. 1B

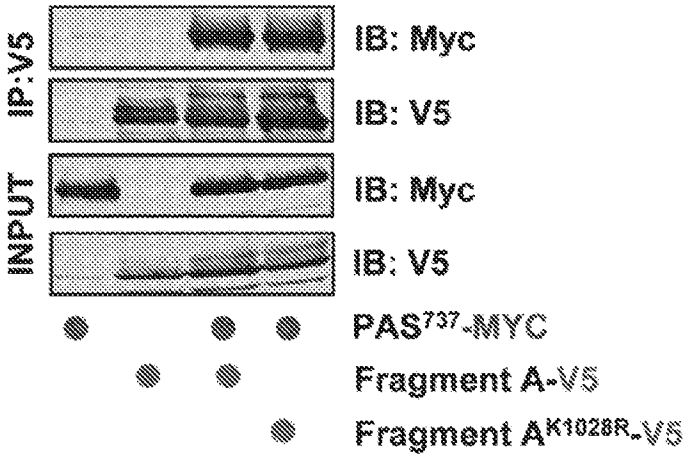


FIG. 1C


841
Kinase
1323
Fragment A
879
Kinase
1323
Fragment B
915
Kinase
1323
Fragment C
949
Kinase
1323
Fragment D

FIG. 1D

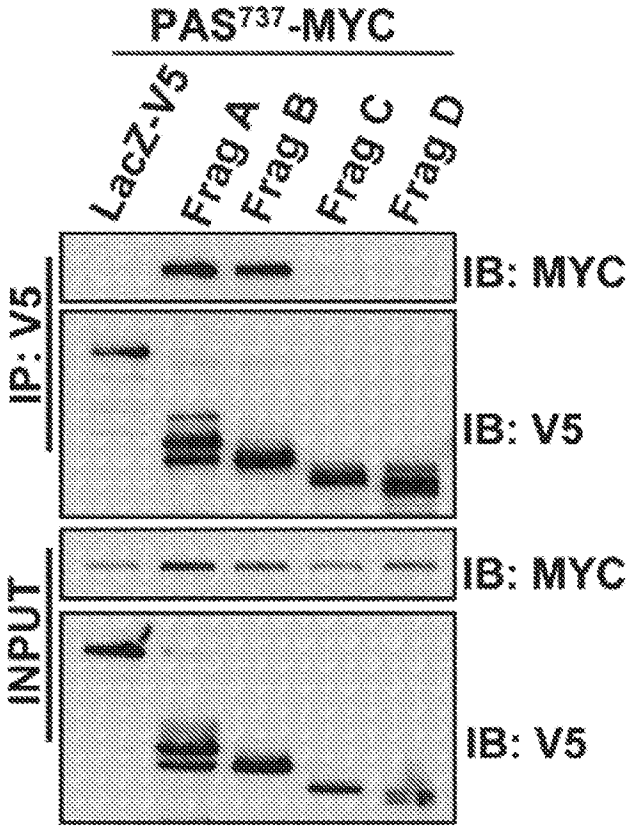

PAS737-MYC
LacZ-V5
Frag A
Frag B
Frag C
Frag D
IP: V5
IB: MYC
IB: V5
INPUT
IB: MYC
IB: V5

FIG. 1E

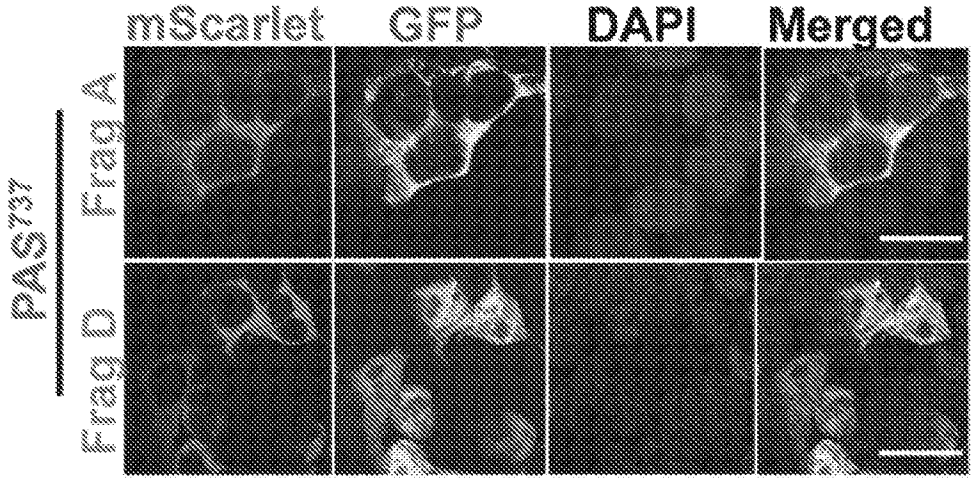

mScarlet
GFP
DAPI
Merged
Frag A
Frag D
PAS737

FIG. 1F

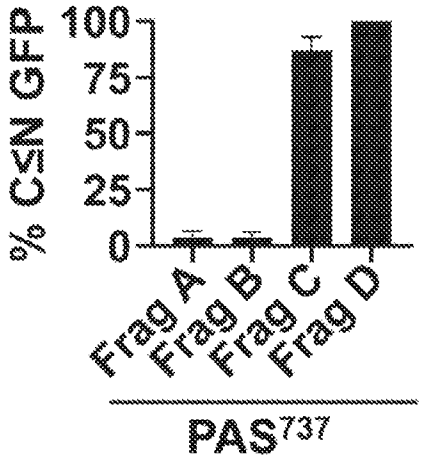
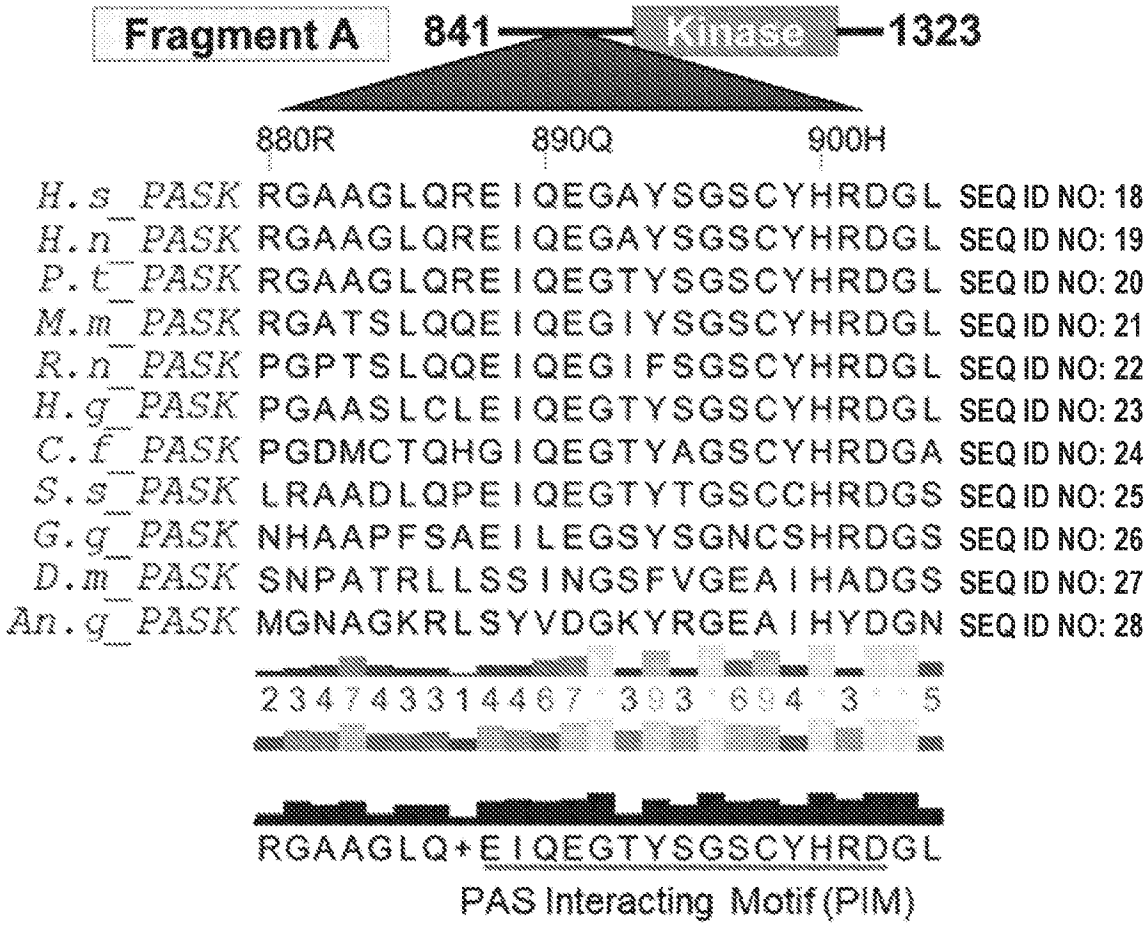

% C≤N GFP
100
75
50
25
0
Frag A
Frag B
Frag C
Frag D
PAS737

FIG. 1G


Fragment A
841
Kinase
1323
880R
890Q
900H
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H.n_PASK RGAAGLQREIQEGAYSGSCYHRDGL SEQ ID NO: 19
P.t_PASK RGAAGLQREIQEGTYSGSCYHRDGL SEQ ID NO: 20
M.m_PASK RGATSLQQEIQEGIYSGSCYHRDGL SEQ ID NO: 21
R.n_PASK PGPTSLQQEIQEGIFSGSCYHRDGL SEQ ID NO: 22
H.g_PASK PGAASLCLEIQEGTYSGSCYHRDGL SEQ ID NO: 23
C.f_PASK PGDMCTQHGIQEGTYAGSCYHRDGA SEQ ID NO: 24
S.s_PASK LRAADLQPEIQEGTYTGSCCHRDGS SEQ ID NO: 25
G.g_PASK NHAAPFSAEILEGSYSGNCSHRDGS SEQ ID NO: 26
D.m_PASK SNPATRLLSSINGSFVGEAIHADGS SEQ ID NO: 27
An.g_PASK MGNAGKRLSYVDGKYRGEAIHYDGN SEQ ID NO: 28
2347433144467*393*6944*3**5
RGAAGLQ+EIQEGTYSGSCYHRDGL
PAS Interacting Motif (PIM)

FIG. 2A

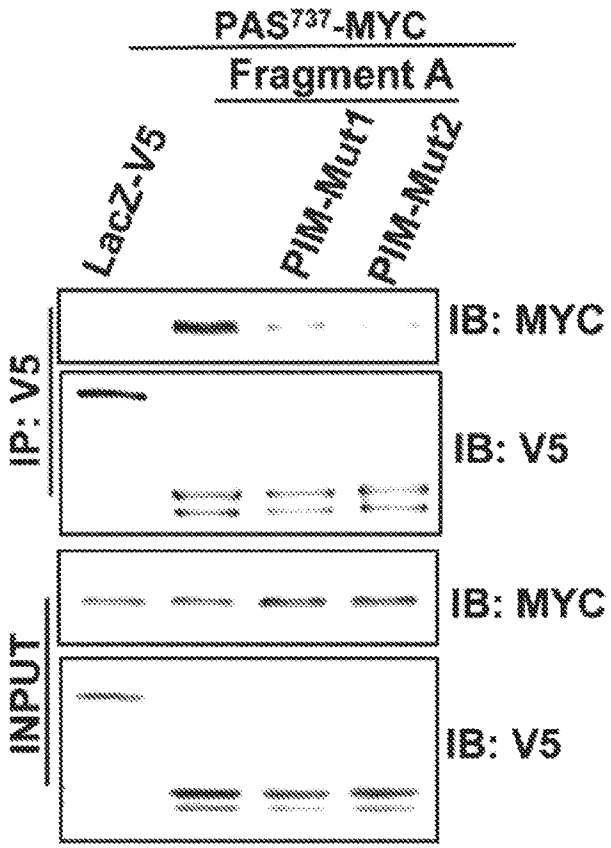

PAS737-MYC
Fragment A
LacZ-V5
PIM-Mut1
PIM-Mut2
IB: MYC
IP: V5
IB: V5
IB: MYC
INPUT
IB: V5

FIG. 2B

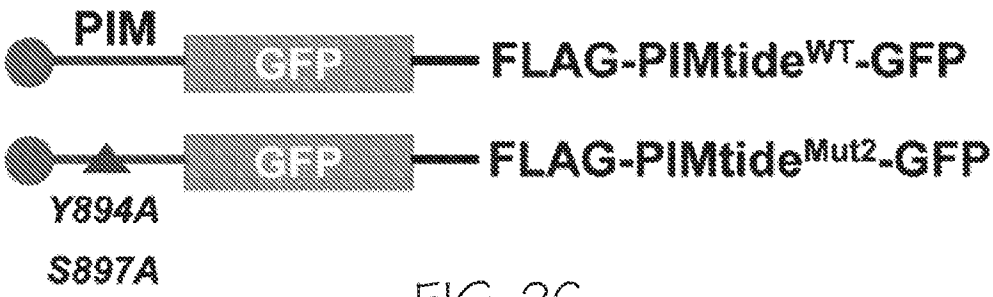

PIM
GFP
FLAG-PIMtideWT-GFP
GFP
FLAG-PIMtideMut2-GFP
Y894A
S897A

FIG. 2C

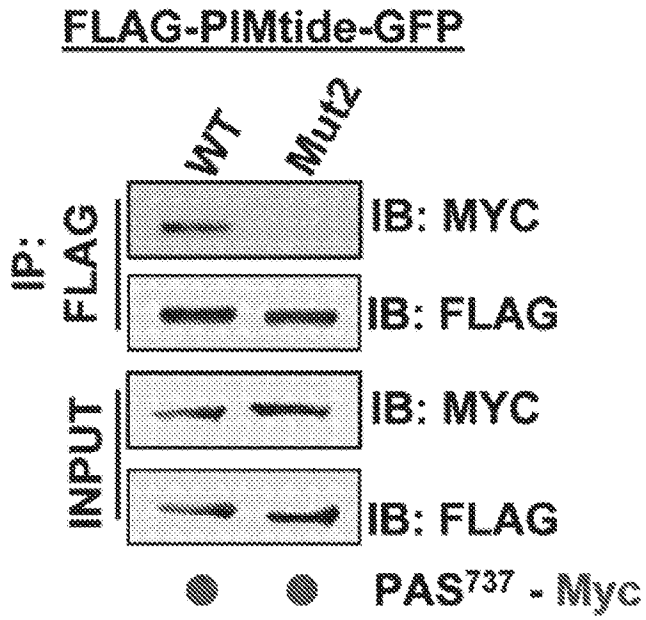

FLAG-PIMtide-GFP
WT
Mut2
IP:
FLAG
IB: MYC
IB: FLAG
INPUT
IB: MYC
IB: FLAG
PAS737 - Myc

FIG. 2D

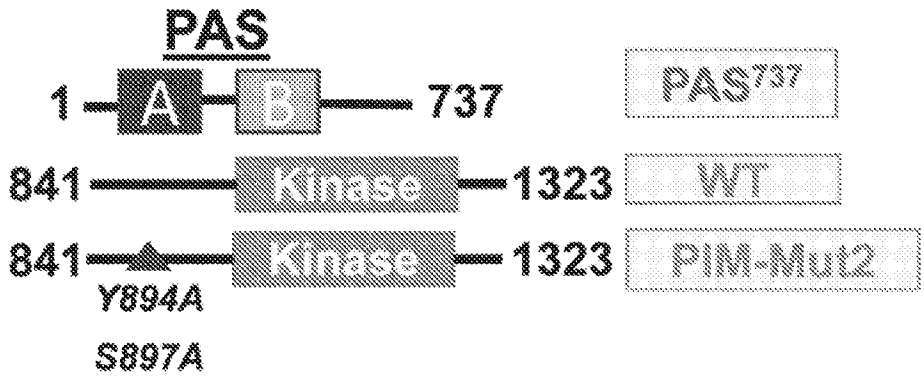

PAS
1
A
B
737
PAS737
841
Kinase
1323
WT
841
Kinase
1323
PIM-Mut2
Y894A
S897A

FIG. 2E

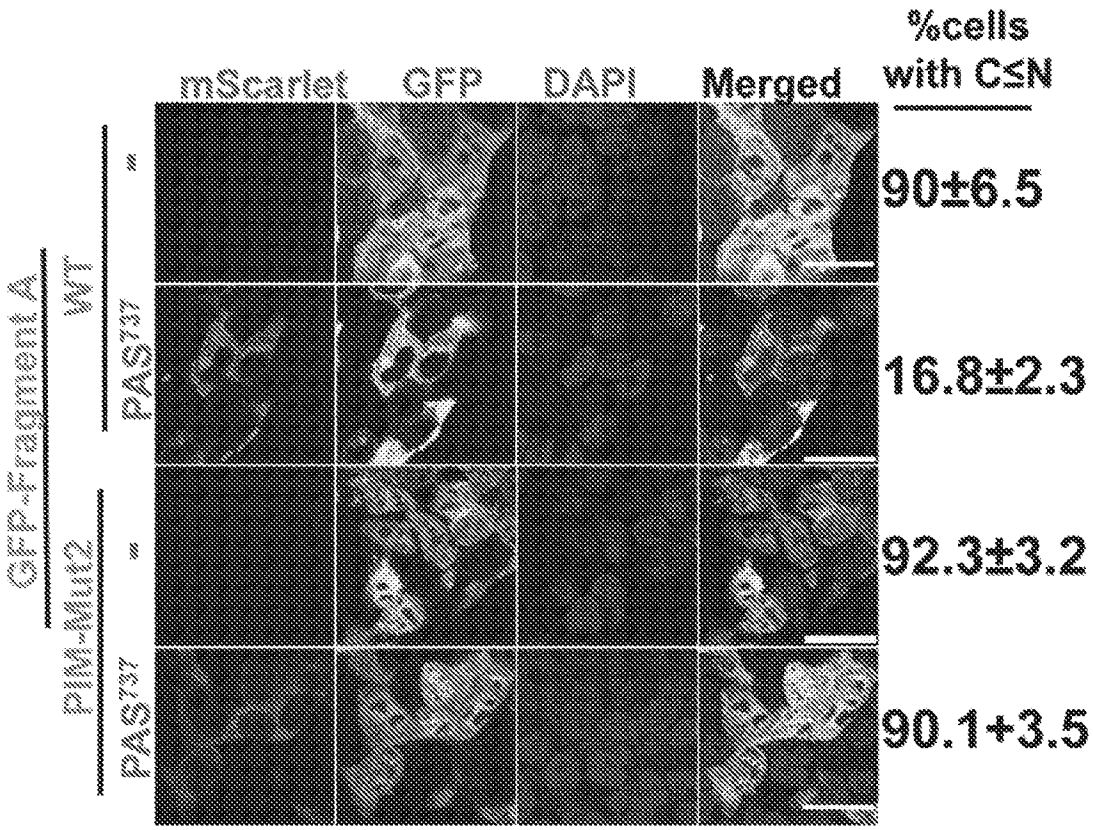

%cells with C≤N
mScarlet
GFP
DAPI
Merged
GFP-Fragment A
WT
PIM-Mut2
-
PAS737
-
PAS737
90±6.5
16.8±2.3
92.3±3.2
90.1+3.5

FIG. 2F

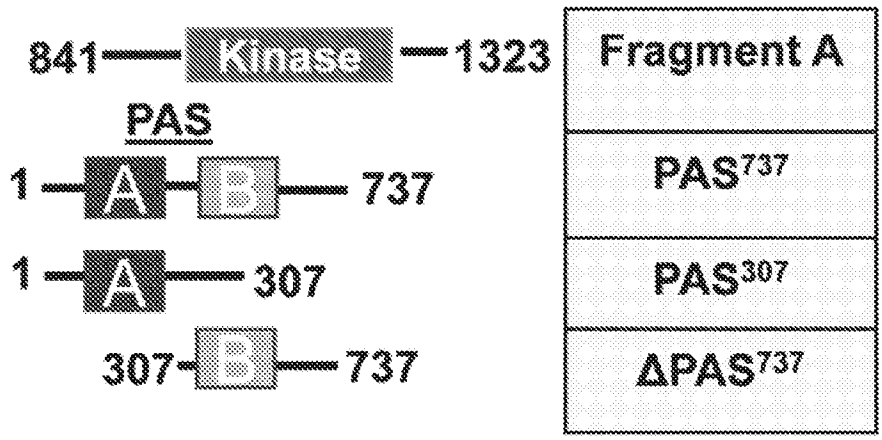

841
Kinase
1323
PAS
1
A
B
737
1
A
307
307
B
737
Fragment A
PAS737
PAS307
ΔPAS737

FIG. 3A

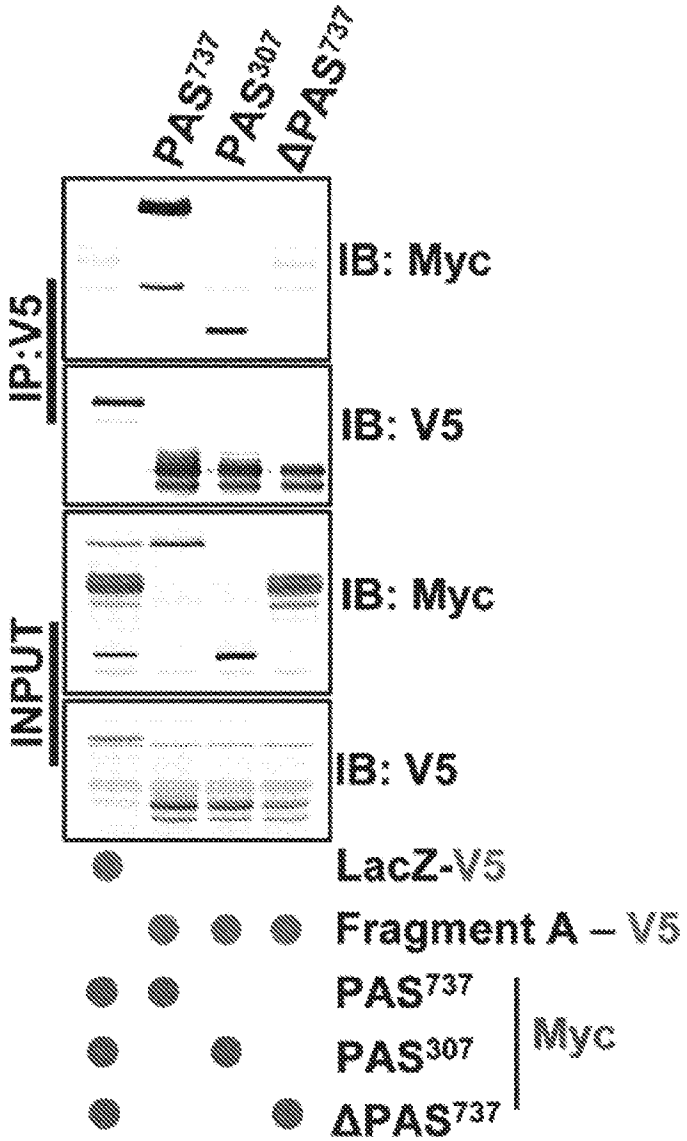

PAS737
PAS307
ΔPAS737
IP:V5
IB: Myc
IB: V5
INPUT
IB: Myc
IB: V5
LacZ-V5
Fragment A – V5
PAS737
PAS307
ΔPAS737
Myc

FIG. 3B

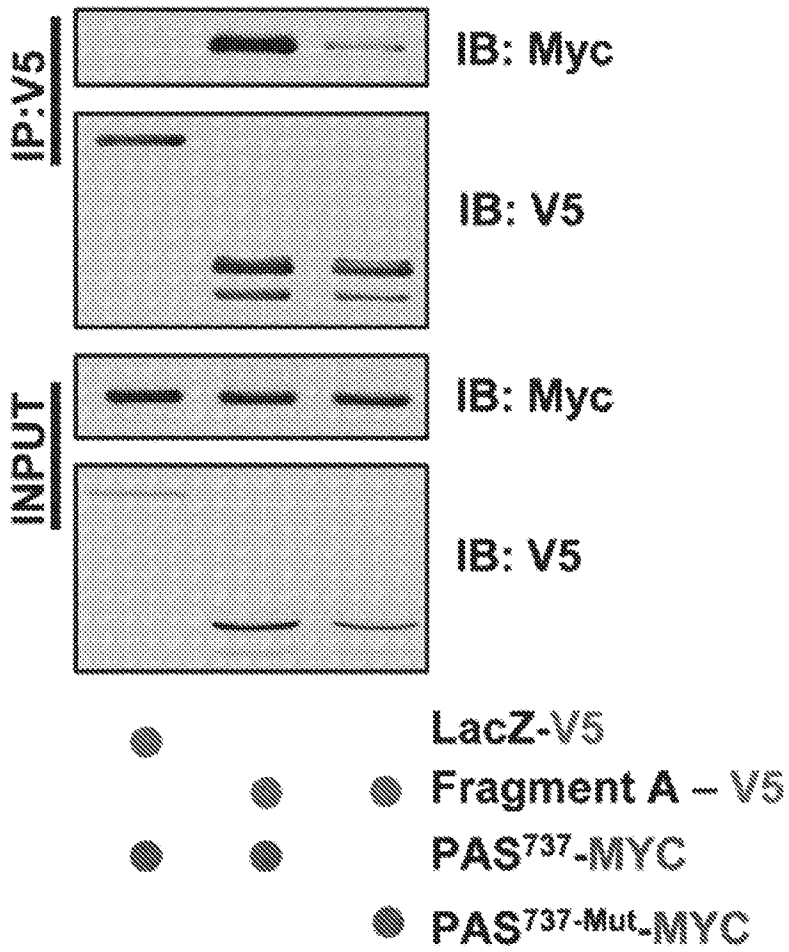

IP:V5
IB: Myc
IB: V5
INPUT
IB: Myc
IB: V5
LacZ-V5
Fragment A – V5
PAS737-MYC
PAS737-Mut-MYC

FIG. 3C

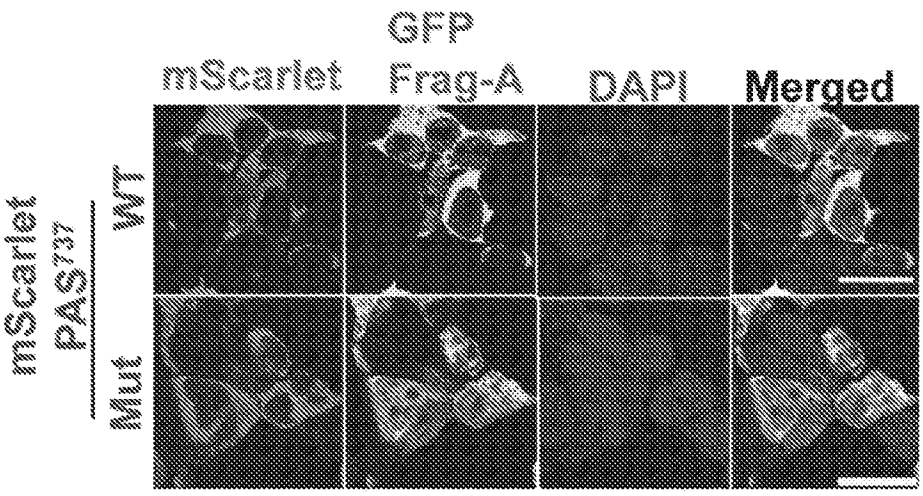

GFP
mScarlet
Frag-A
DAPI
Merged
mScarlet PAS737
WT
Mut

FIG. 3D

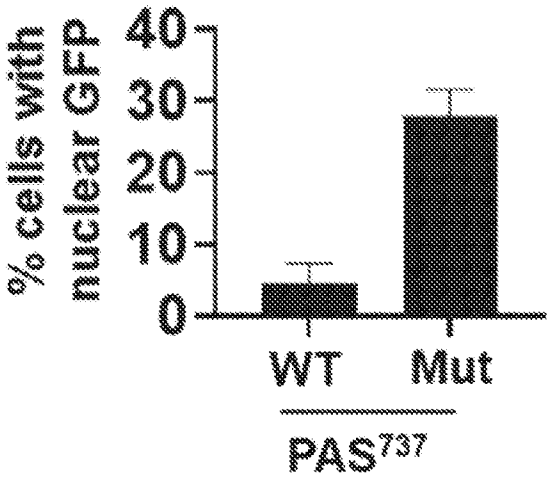

% cells with nuclear GFP
40
30
20
10
0
WT
Mut
PAS737

FIG. 3E

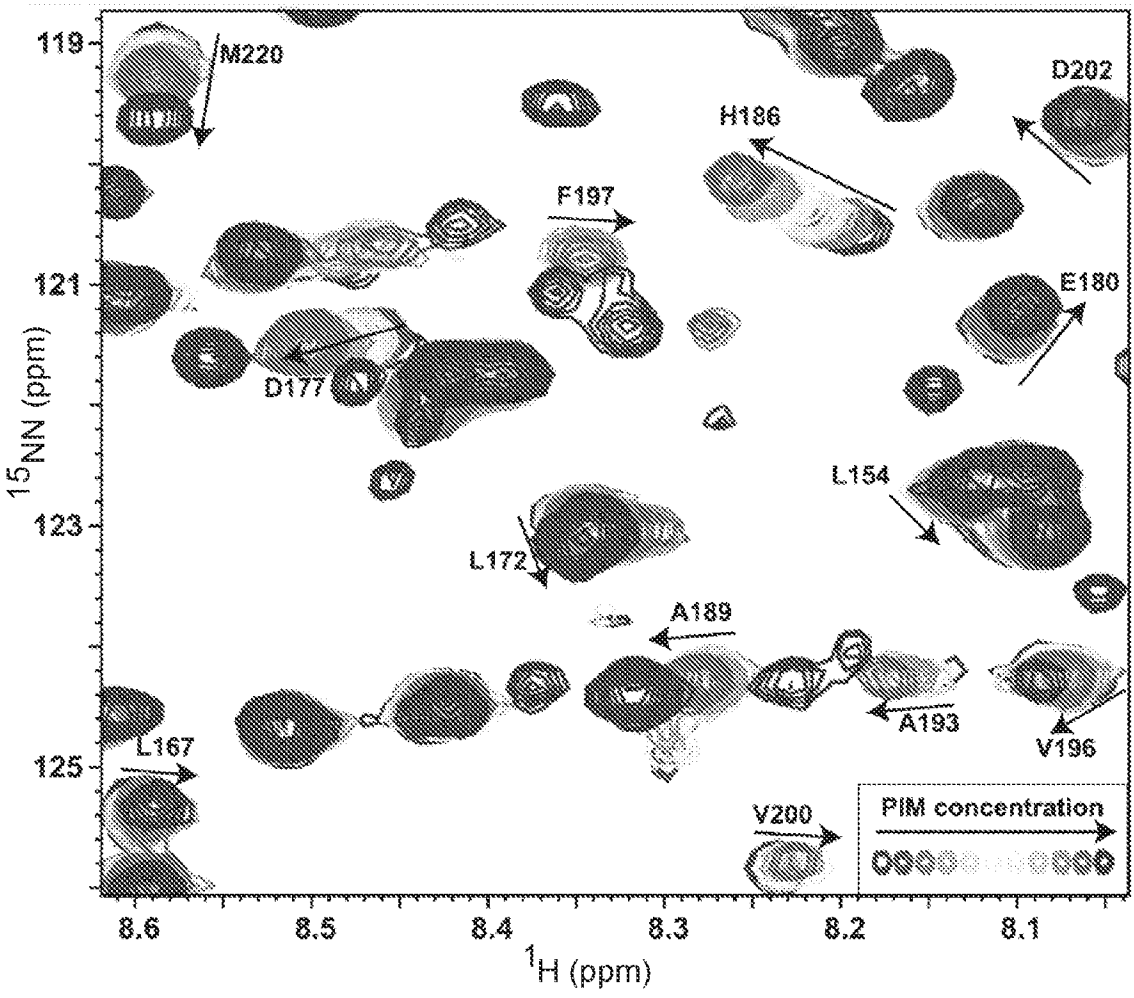

M220
H186
D202
F197
E180
D177
L154
L172
A189
A193
V196
L167
V200
PIM concentration
119
121
123
125
15NN (ppm)
8.6
8.5
8.4
8.3
8.2
8.1
1H (ppm)

FIG. 4A

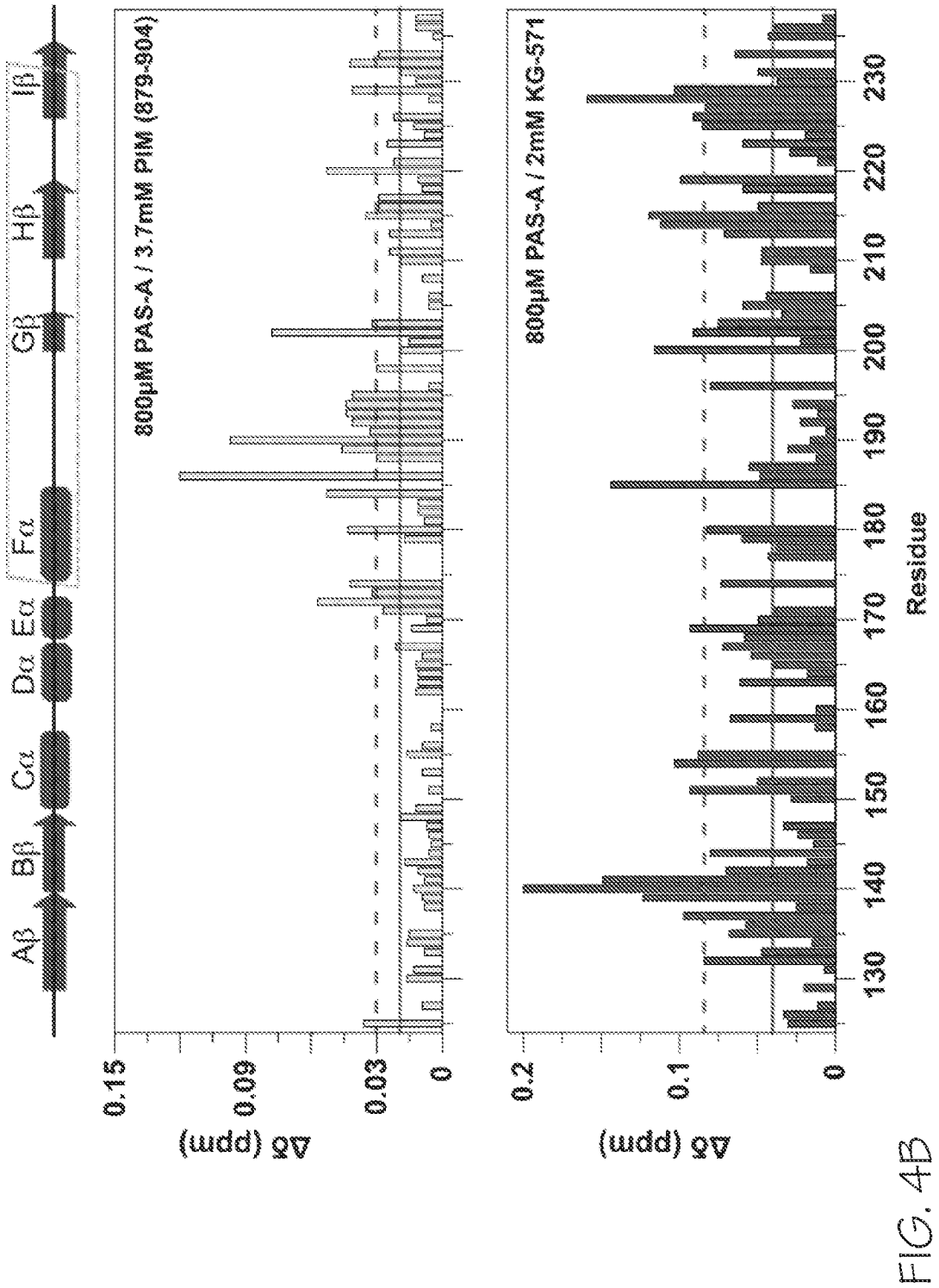

Aβ
Bβ
Cα
Dα
Eα
Fα
Gβ
Hβ
Iβ
800μM PAS-A / 3.7mM PIM (879-904)
800μM PAS-A / 2mM KG-571
Δδ (ppm)
0.15
0.09
0.03
0
0.2
0.1
130
140
150
160
170
180
190
200
210
220
230
Residue

FIG. 4B

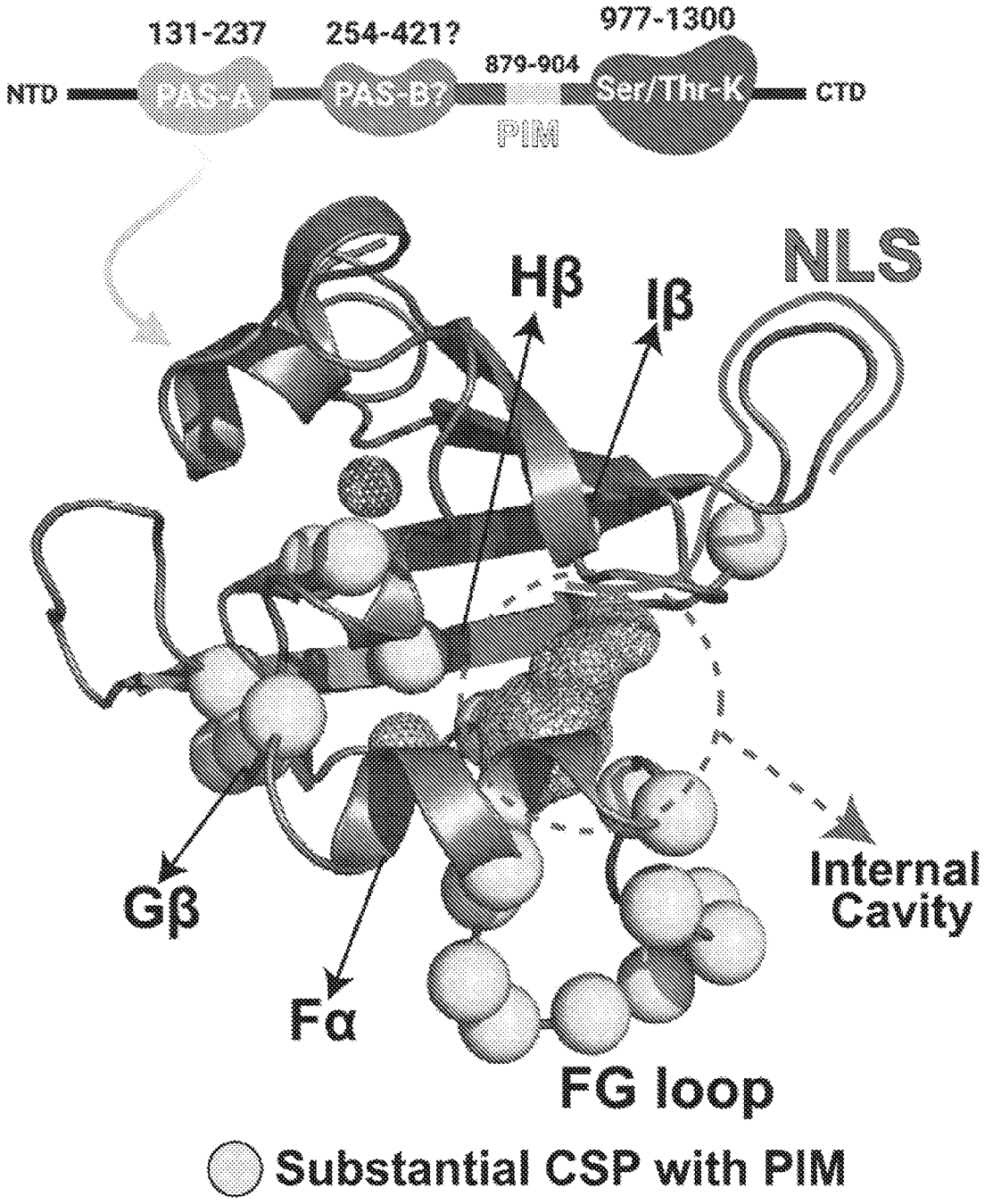

131-237
254-421?
879-904
977-1300
NTD
PAS-A
PAS-B?
PIM
Ser/Thr-K
CTD
Hβ
Iβ
NLS
Gβ
Fα
Internal Cavity
FG loop
Substantial CSP with PIM

FIG. 4C

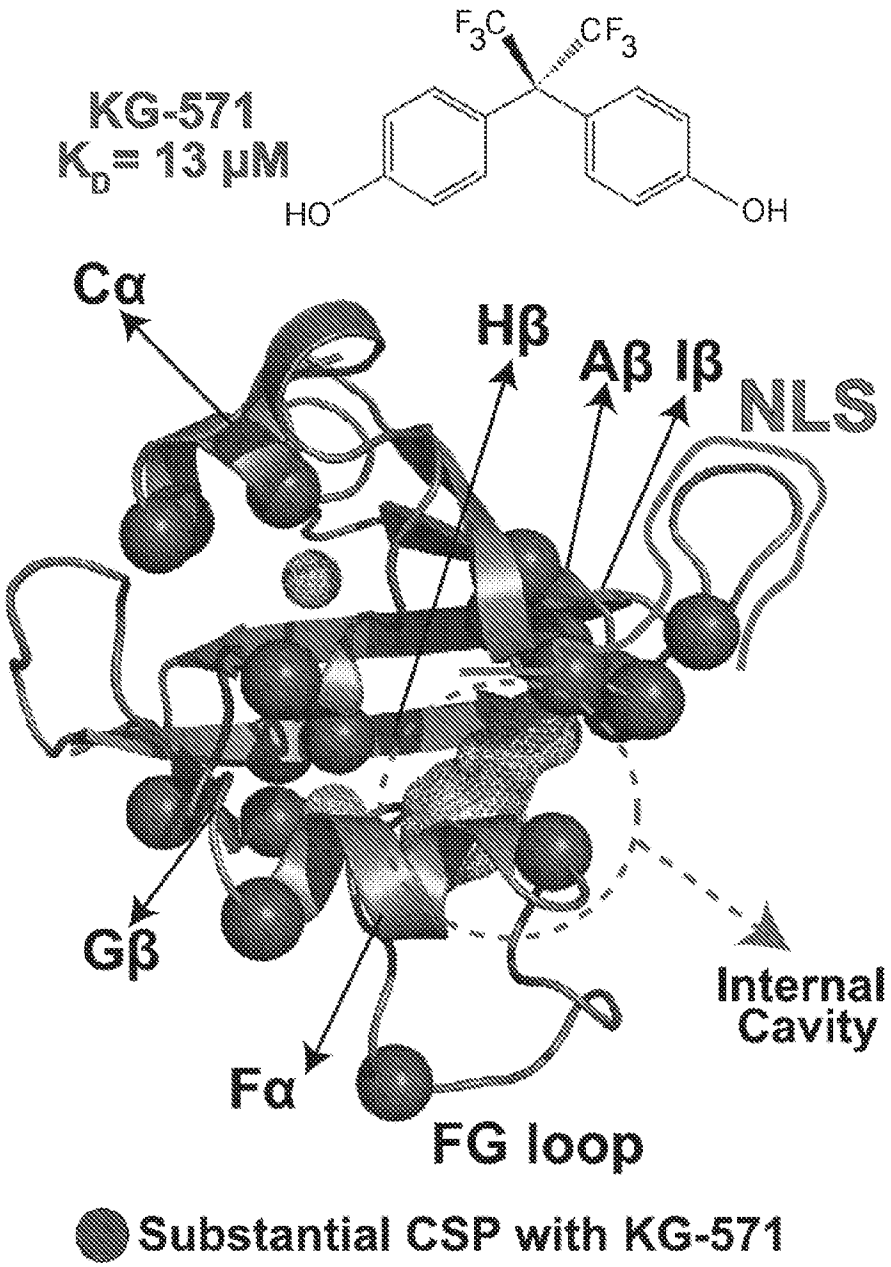

KG-571
K_D= 13 µM
F_3C
CF_3
HO
OH
Cα
Hβ
Aβ Iβ
NLS
Gβ
Internal Cavity
Fα
FG loop
Substantial CSP with KG-571

FIG. 4D

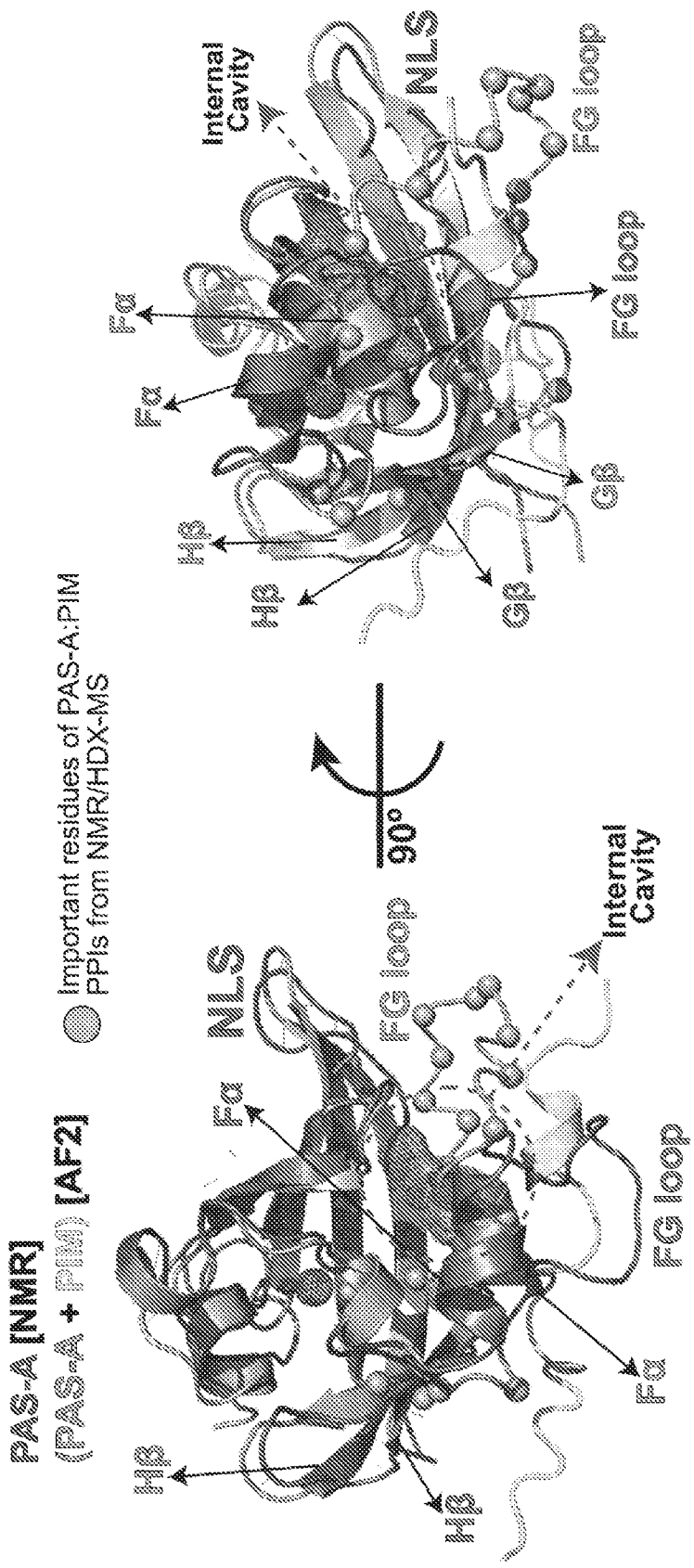

PAS-A [NMR]
(PAS-A + PIM) [AF2]
Important residues of PAS-A:PIM PPIs from NMR/HDX-MS
Hβ
Fα
NLS
FG loop
Internal Cavity
Fα
FG loop
Hβ
Hβ
Fα
Fα
Internal Cavity
NLS
FG loop
FG loop
Gβ
Hβ
Gβ
90°

FIG. 4E

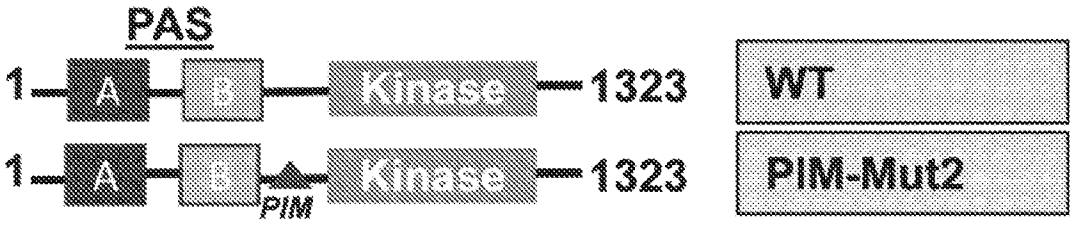


FIG. 5A

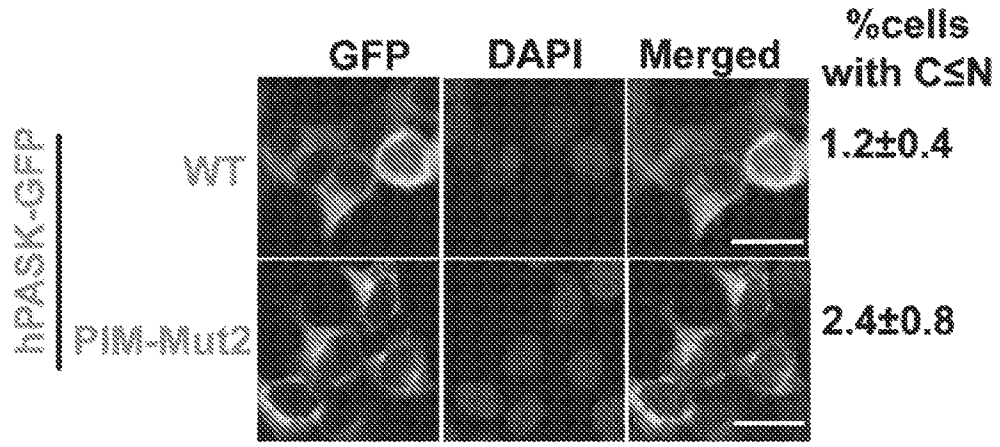


FIG. 5B

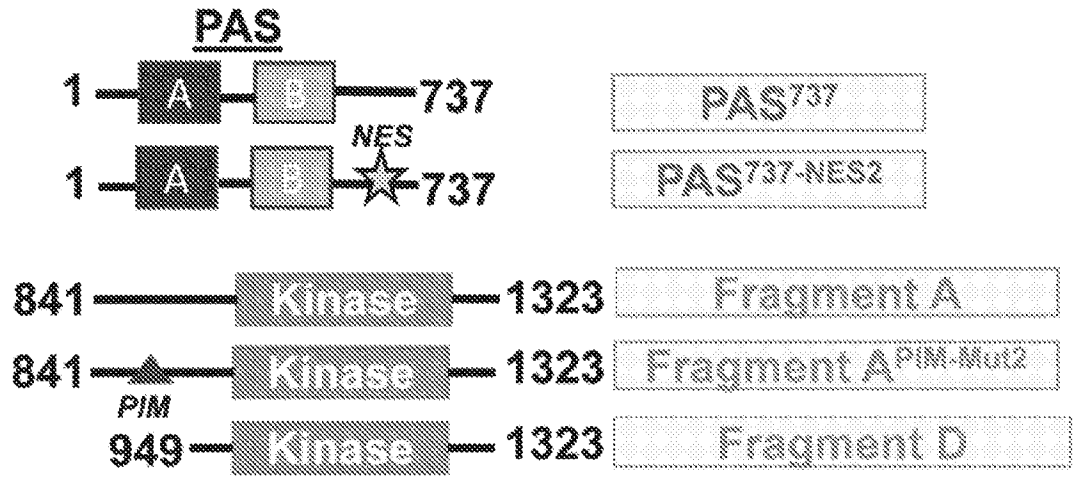


FIG. 5C

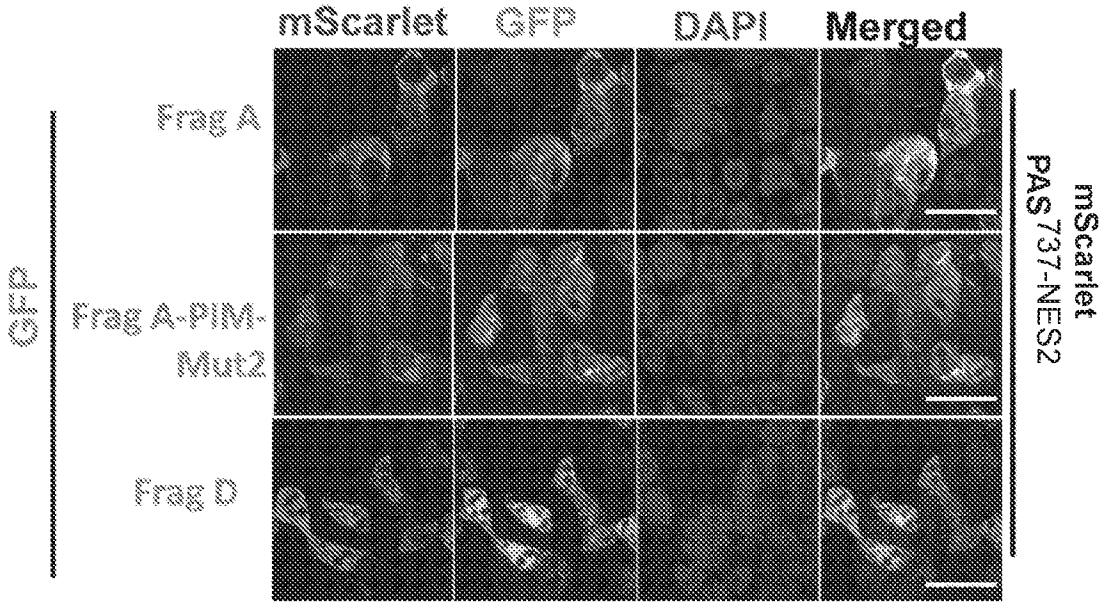


FIG. 5D

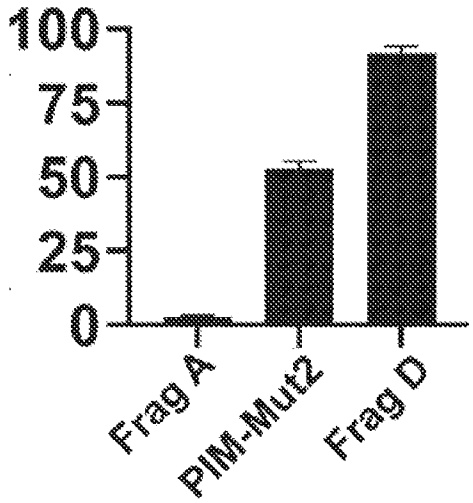


FIG. 5E

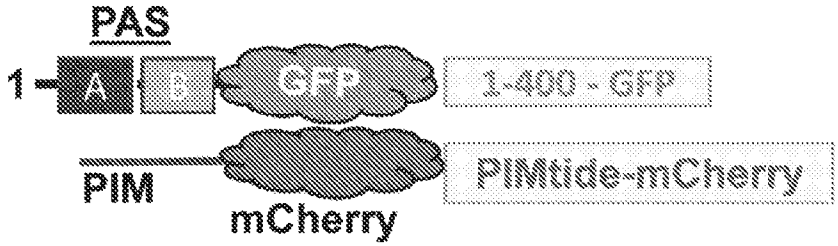


FIG. 5F

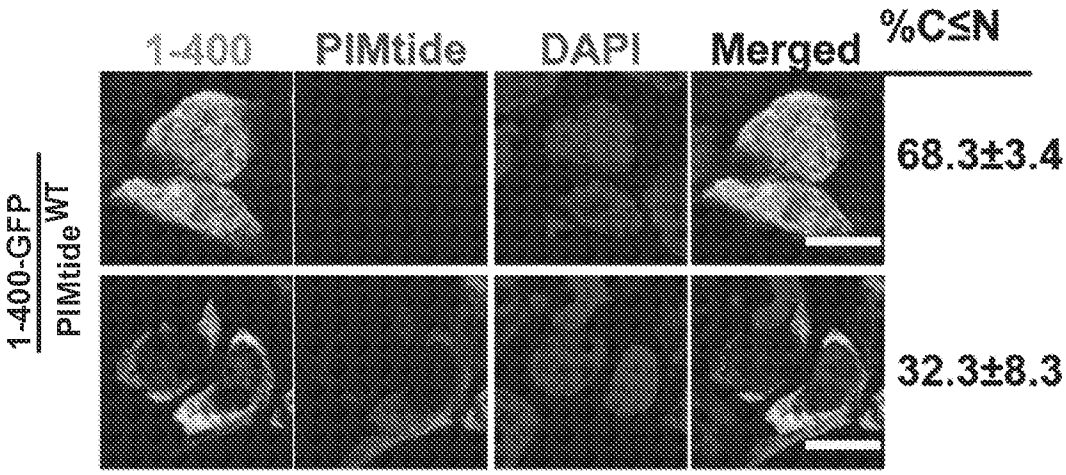

1-400
PIMtide
DAPI
Merged
%C≤N
1-400-GFP
PIMtide^WT
68.3±3.4
32.3±8.3

FIG. 5F

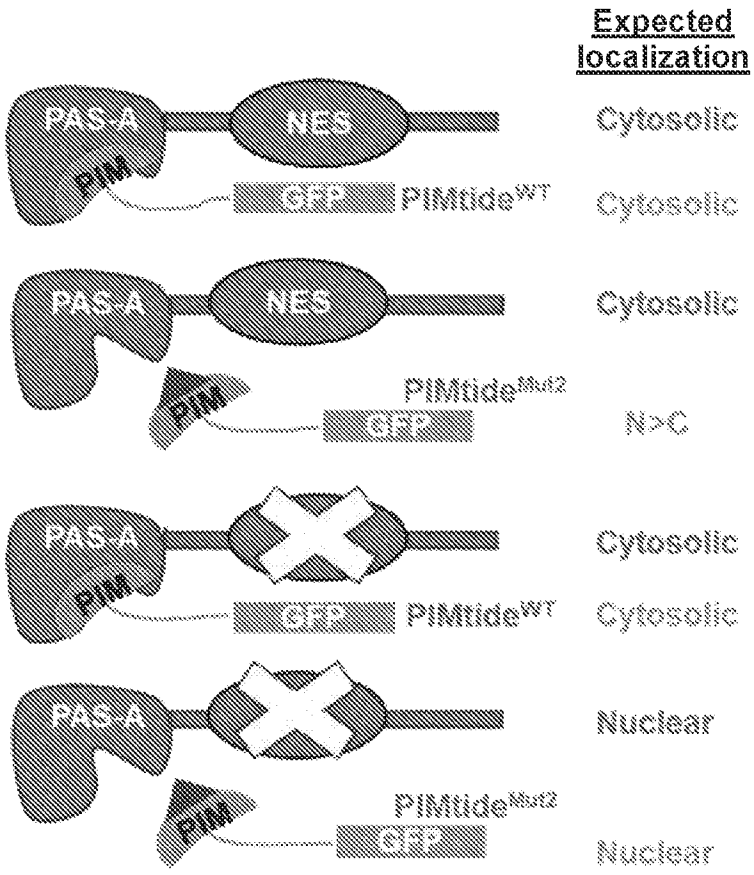

Expected localization
PAS-A
NES
PIM
GFP
PIMtide^WT
Cytosolic
Cytosolic
PAS-A
NES
Cytosolic
PIMtide^Mut2
PIM
GFP
N>C
PAS-A
Cytosolic
PIM
GFP
PIMtide^WT
Cytosolic
PAS-A
Nuclear
PIMtide^Mut2
PIM
GFP
Nuclear

FIG. 6A

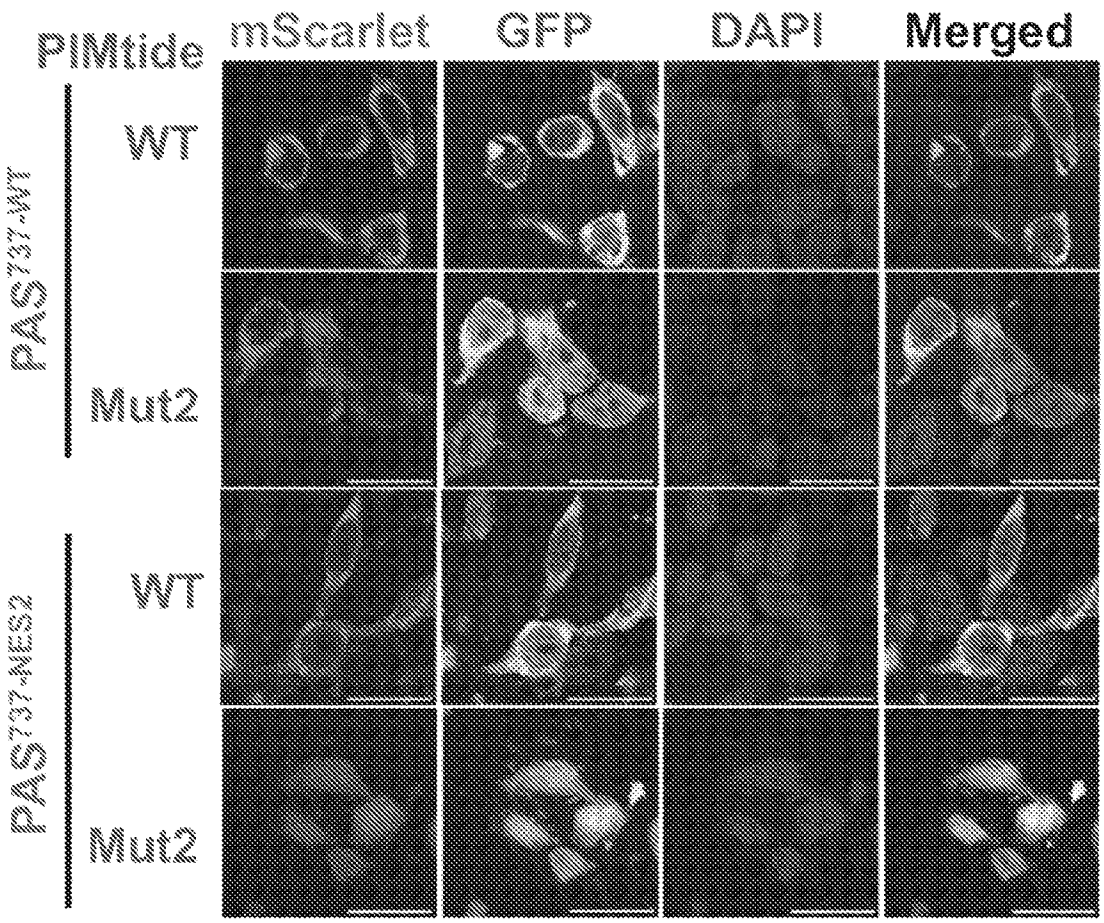

PIMtide
mScarlet
GFP
DAPI
Merged
PAS737-WT
WT
Mut2
PAS737-MES2
WT
Mut2

FIG. 6B

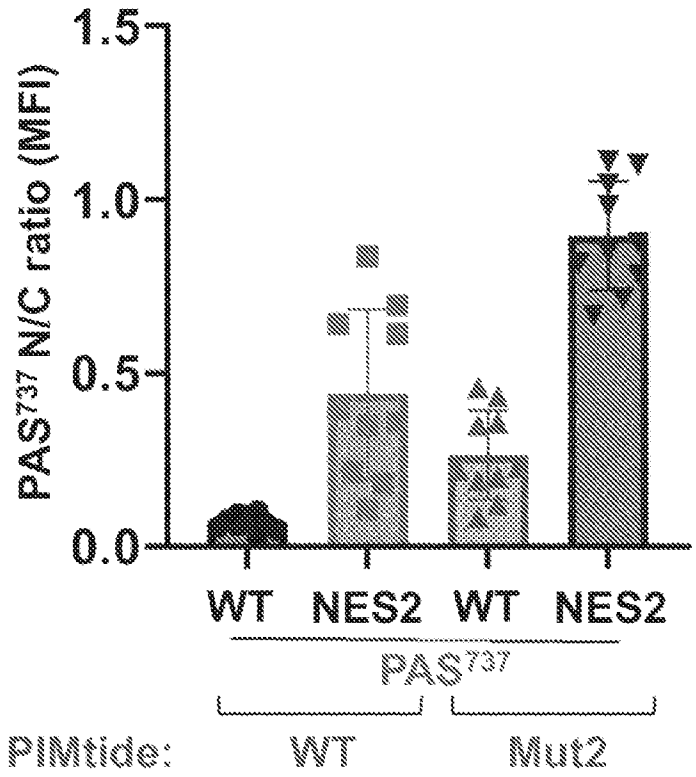

PAS737 N/C ratio (MFI)
1.5
1.0
0.5
0.0
WT
NES2
WT
NES2
PAS737
PIMtide:
WT
Mut2

FIG. 6C

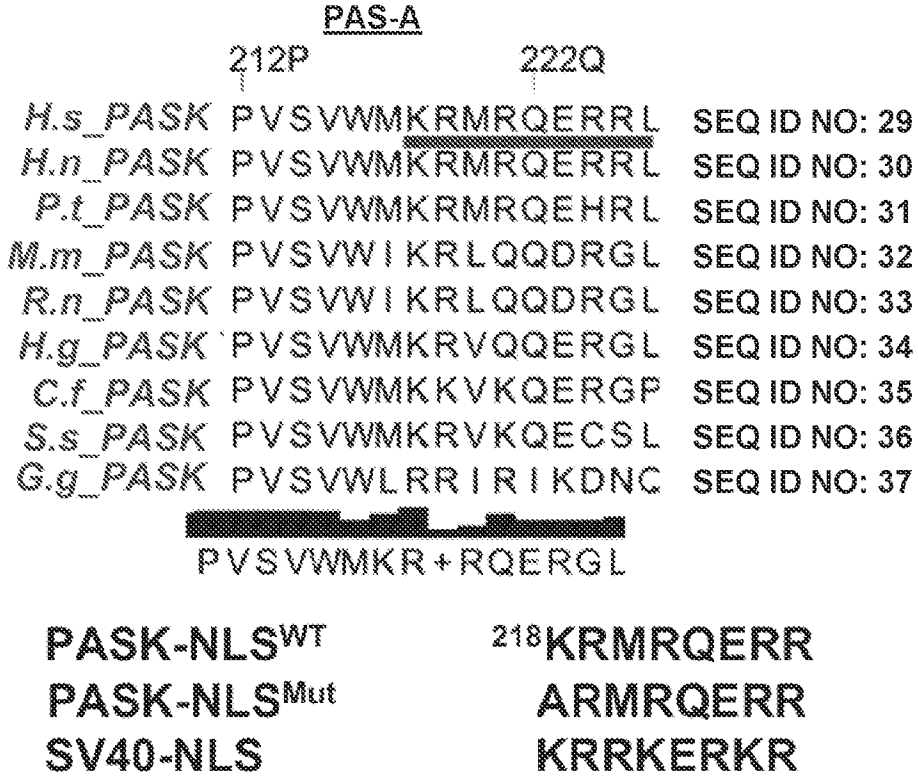

PAS-A
212P
222Q
H.s_PASK PVSVWMKRMRQERRL SEQ ID NO: 29
H.n_PASK PVSVWMKRMRQERRL SEQ ID NO: 30
P.t_PASK PVSVWMKRMRQEHRL SEQ ID NO: 31
M.m_PASK PVSVWIKRLQQDRGL SEQ ID NO: 32
R.n_PASK PVSVWIKRLQQDRGL SEQ ID NO: 33
H.g_PASK PVSVWMKRVQQERGL SEQ ID NO: 34
C.f_PASK PVSVWMKKVKQERGP SEQ ID NO: 35
S.s_PASK PVSVWMKRVKQECSL SEQ ID NO: 36
G.g_PASK PVSVWLRRIRIKDNC SEQ ID NO: 37
PVSVWMKR+RQERGL
PASK-NLSWT 218KRMRQERR
PASK-NLSMut ARMRQERR
SV40-NLS KRRKERKR

FIG. 6D

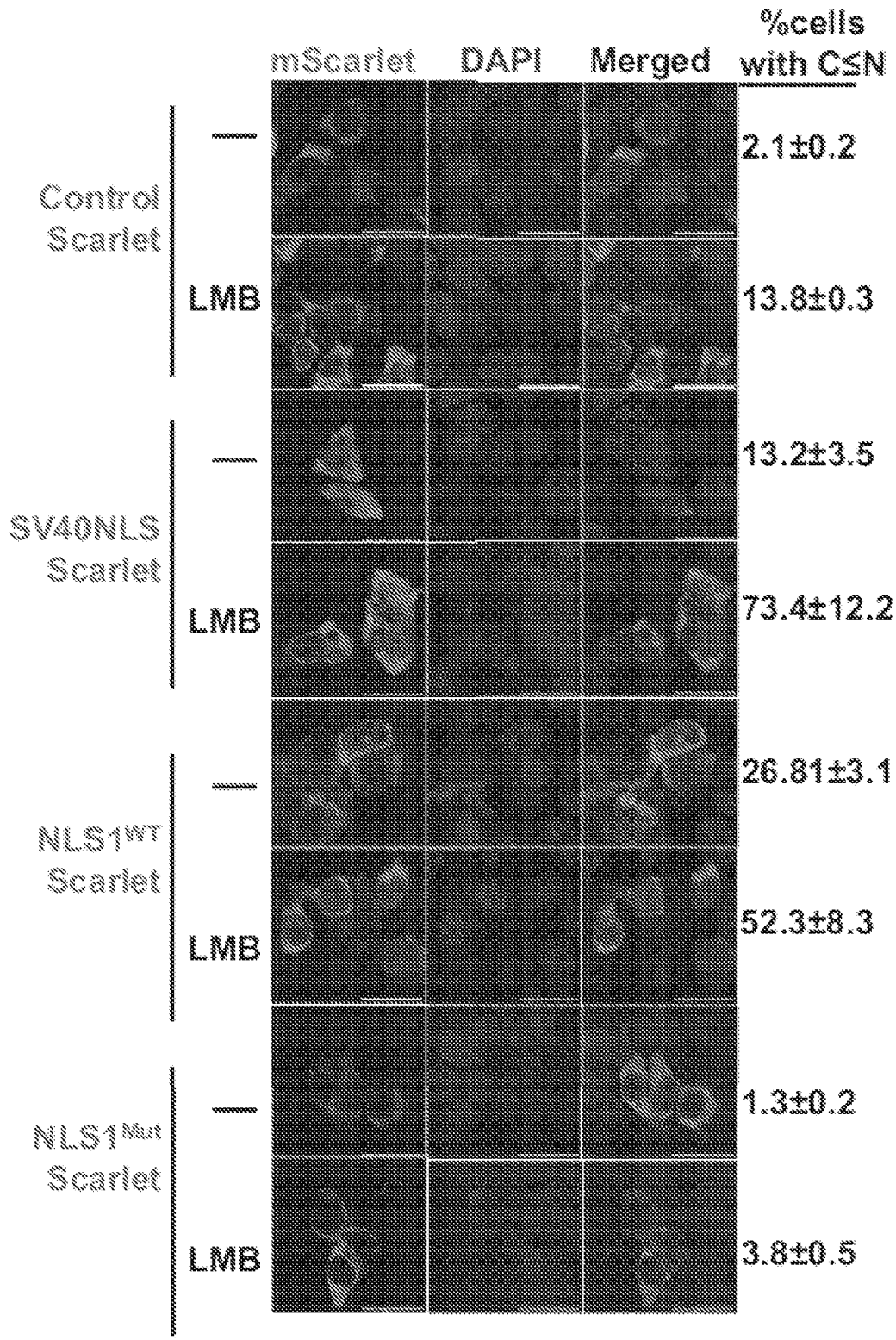

mScarlet
DAPI
Merged
%cells with C≤N
Control Scarlet
—
LMB
2.1±0.2
13.8±0.3
SV40NLS Scarlet
—
LMB
13.2±3.5
73.4±12.2
NLS1WT Scarlet
—
LMB
26.81±3.1
52.3±8.3
NLS1Mut Scarlet
—
LMB
1.3±0.2
3.8±0.5

FIG. 6E

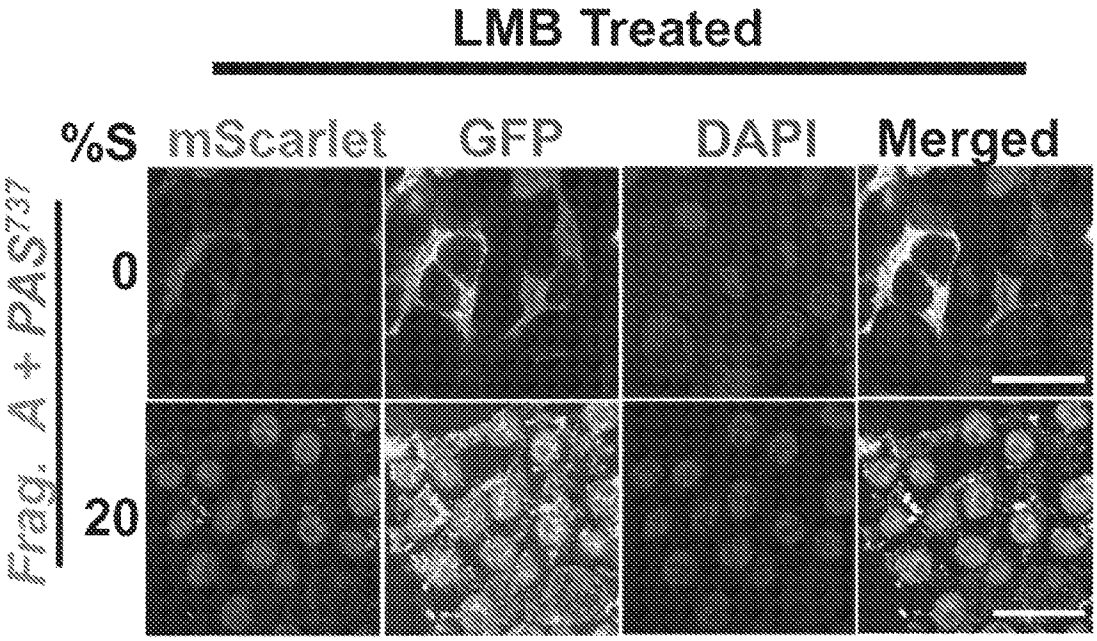


FIG. 7A

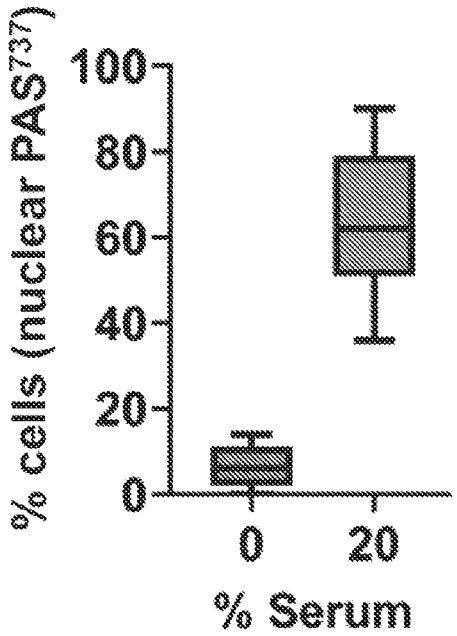


FIG. 7B

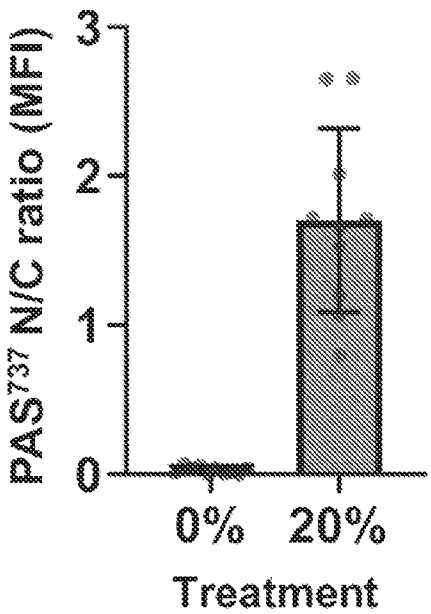


FIG. 7C

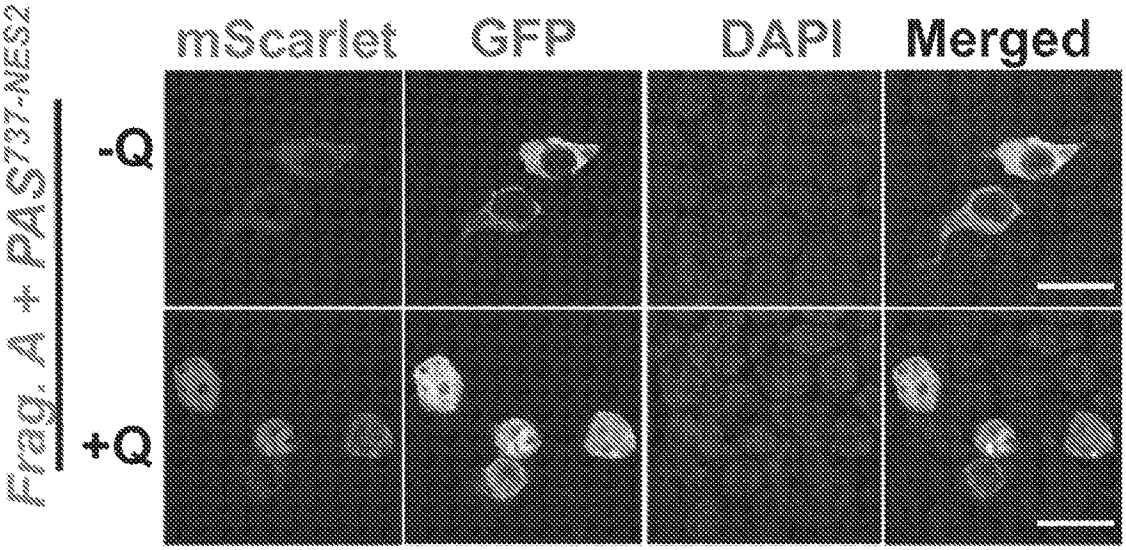


FIG. 7D

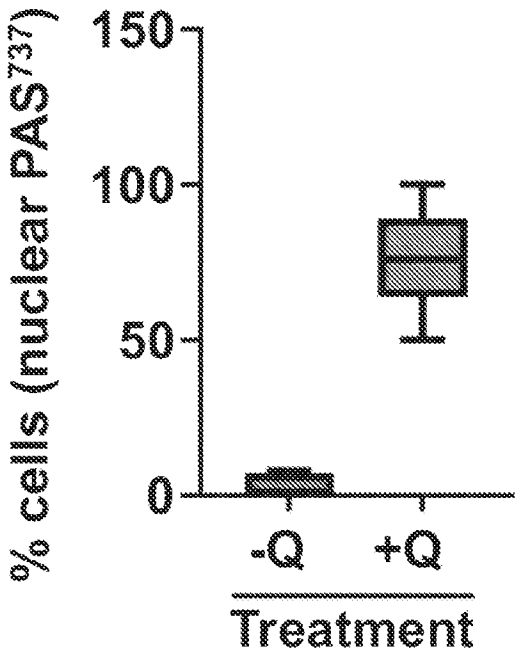


FIG. 7E

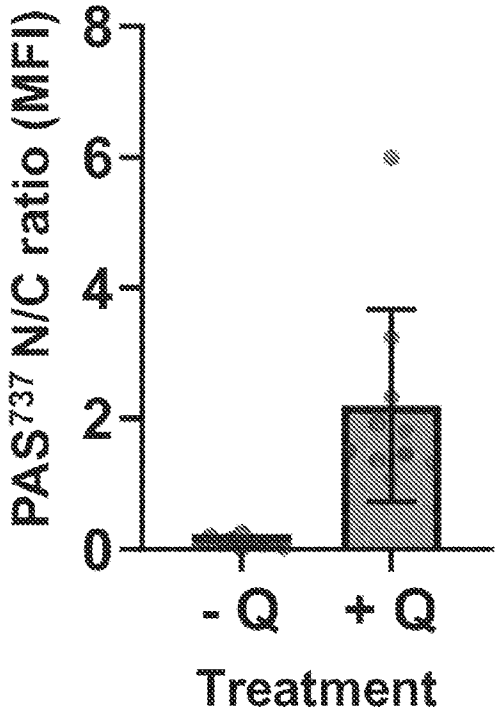


FIG. 7F

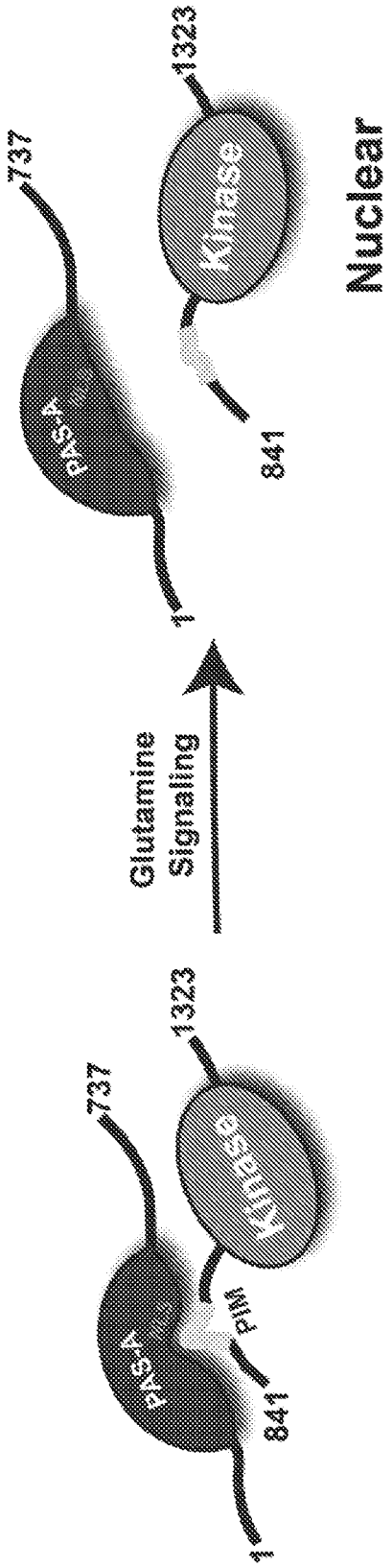

1323
737
Kinase
PAS-A
841
1
Nuclear
Glutamine
Signaling
737
1323
Kinase
PAS-A
PIM
841
1

FIG. 7G

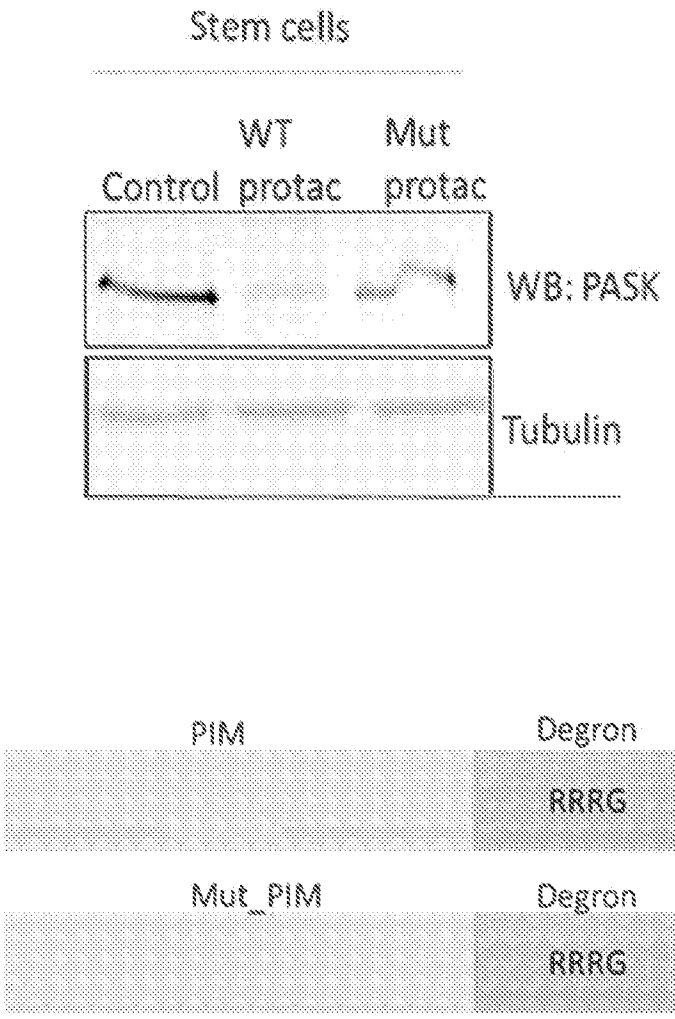
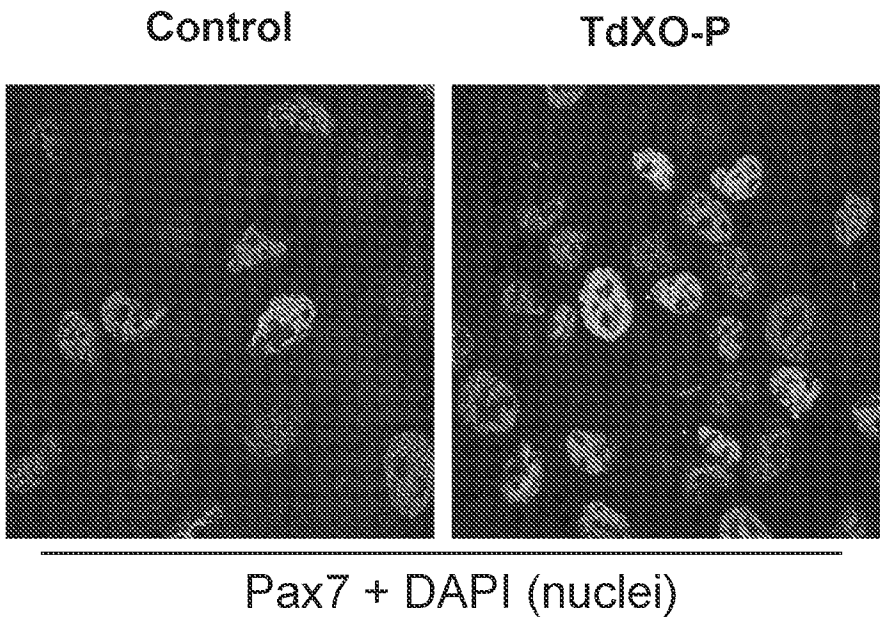

Stem cells
WT
Control
protac
Mut
protac
WB: PASK
Tubulin
PIM
Degron
RRRG
Mut_PIM
Degron
RRRG

FIG. 8


Control
TdXO-P
Pax7 + DAPI (nuclei)

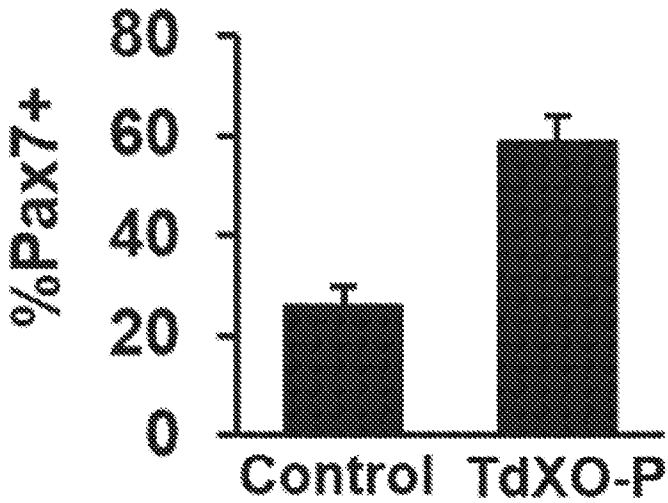

%Pax7+
80
60
40
20
0
Control
TdXO-P

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/035569

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C07K 19/00*** (2024.01); ***C12N 9/12*** (2024.01); *C12N 15/62* (2024.01); *C12N 5/00* (2024.01)

CPC: ***C07K 19/00; C12N 9/12; C12Y 207/11001;*** *C07K 2319/95; C12N 15/62; C12N 5/0607*

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)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2022/155153 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 21 July 2022 (21.07.2022) entire document	1-3
Y	US 2006/0234344 A1 (PLOWMAN et al.) 19 October 2006 (19.10.2006) entire document	1-3, 18
Y	WO 2018/057782 A1 (UNIVERSITY OF UTAH RESEARCH FOUNDATION) 29 March 2018 (29.03.2018) entire document	18

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

"D" document cited by the applicant in the international application

"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
23 August 2024 (23.08.2024)	**23 October 2024 (23.10.2024)**

Name and mailing address of the ISA/US	Authorized officer
COMMISSIONER FOR PATENTS **MAIL STOP PCT, ATTN: ISA/US** **P.O. Box 1450** **Alexandria, VA 22313-1450 UNITED STATES OF AMERICA**	**TAINA MATOS**
Facsimile No. **571-273-8300**	Telephone No. **571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/035569

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. ☐ 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

International application No.
PCT/US2024/035569

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☑ Claims Nos.: **7-17**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-6 and 18 are drawn to degron peptides, and methods for inhibiting PASK activity in a cell.

The first invention of Group I+ is restricted to a PIM comprising SEQ ID NO: 17, degron peptides comprising the same, and methods for inhibiting PASK activity in a cell comprising the same. The first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. Specifically, the first named invention was selected based on the first listed compound species presented in the claims (see claim 2). It is believed that claims 1-3 and 18 read on this first named invention and thus these claims will be searched without fee to the extent that they read on a PIM comprising SEQ ID NO: 17, degron peptides comprising the same, and methods for inhibiting PASK activity in a cell comprising the same.

Applicant is invited to elect additional PIMs to be searched by paying an additional fee for each election. An exemplary election would be a PIM comprising SEQ ID NO: 18, degron peptides comprising the same, and methods for inhibiting PASK activity in a cell comprising the same. Additional PIMs will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element responsible for PIM activity requiring the selection of alternative amino acid sequences "wherein the PASK fragment comprises a PASK interacting motif (PIM) comprising an amino acid sequence with at least 85% sequence identity to SEQ ID NO: 17," or "wherein the PASK fragment comprises a PASK interacting motif (PIM) comprising an amino acid sequence of EGXX2X3GX4X5X6HR (SEQ ID NO: 51) or EGXIX2X3GX4X5X 6HRDG (SEQ ID NO: 52), wherein X1, X3 and X5 are any amino acid, X2 is Y or F, X4 is E, S or N, and X6 is Y or C," or "wherein the PIM sequence is selected from one of SEQ ID NOs 18-28 or 42-50."

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

Additionally, even if Groups I+ were considered to share the technical features of a degron peptide comprising a PASK protein or fragment thereof fused to an amino acid sequence of RRRG (SEQ ID NO: 1); and a method for inhibiting PASK activity in a cell comprising administering to the cell a peptide comprising a PIM motif, wherein the PIM motif comprises an amino acid sequence of EGX1X2X3GX4X5X6HR (SEQ ID NO: 51) or EGX1X2X3GX4X5X6HRDG (SEQ ID NO: 52), wherein X1, X3 and X5 are any amino acid, X2 is Y or F, X4 is E, S or N, and X6 is Y or C, these shared technical features do not represent a contribution over the prior art.

Specifically, WO 2022/155153 A1 to The Regents Of The University Of California teaches a degron peptide comprising a kinase protein or fragment thereof fused to an amino acid sequence of RRRG (SEQ ID NO: 1) (the target protein may contain a caged degron, Pg. 15, Ln. 21; The target protein may be any protein. However, in some embodiments, the target protein may be… an intracellular kinase, Pg. 17, Lns. 11-12; A particularly potent C-terminal minimal degron motif of the sequence RRRG (Arg-Arg-Arg-Gly; also referred to as the "Bonger" motif) was used, Pg. 64, Lns. 12-13).

Further, US 2006/0234344 A1 to Plowman et al. (hereinafter, "Plowman") teaches PASK or a fragment thereof (By a "kinase polypeptide" is meant 10 (preferably 20, more preferably 40, most preferably 75) or more contiguous amino acids set forth in an amino acid sequence selected from the group consisting of those set forth in… SEQ ID NO:156, Para. [0019]; Additional characteristics may be found, inter alia, in the tables, namely… Table 4, shown below, Para. [0131]; Table 4 discloses SEQ ID NO: 156 comprises a Pas kinase); and a PIM motif comprises an amino acid sequence of EGX1X2X3GX4X5X6HR (SEQ ID NO: 51) or EGX1X2X3GX4X5X6HRDG (SEQ ID NO: 52), wherein X1, X3 and X5 are any amino acid, X2 is Y or F, X4 is E, S or N, and X6 is Y or C (By a "kinase polypeptide" is meant 10 (preferably 20, more preferably 40, most preferably 75) or more contiguous amino acids set forth in an amino acid sequence selected from the group consisting of those set forth in… SEQ ID NO:156, Para. [0019]; SEQ ID NO:156 of Plowman comprises 100% sequence identity to instant SEQ ID NO: 51 when X1 is A, X2 is Y, X3 is S, X4 is S, X5 is C, and X6 is Y).

Further, WO 2018/057782 A1 to University Of Utah Research Foundation teaches a method for inhibiting PASK activity in a cell comprising administering to the cell a peptide comprising a PIM motif (A method of preventing differentiation of a stem cell, the method comprising: contacting a stem cell with a PAS domain containing protein kinase (PASK) inhibitor, Claim 1; The method of claim 1, wherein the PASK inhibitor is BioE-1197, Claim 2).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/035569

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-3, 18**

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.