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(54) **Title**: METHYLTRANSFERASES AND USES THEREOF

(57) **Abstract**: The present invention relates to compositions and methods for biomarker screening and research. In particular, the present invention relates to VCP-KMT methyltransferases, antibodies to methylated VCP, and targets of VCP-KMT for screening and research applications.

METHYLTRANSFERASES AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to compositions and methods for biomarker screening and research. In particular, the present invention relates to VCP-KMT methyltransferases, antibodies to methylated VCP, and targets of VCP-KMT for screening and research applications.

BACKGROUND OF THE INVENTION

Malignant tumors (cancers) are the second leading cause of death in the United States, after heart disease (Boring et al., CA Cancel J. Clin. 43:7 (1993)). Cancer is characterized by the increase in the number of abnormal, or neoplastic, cells derived from a normal tissue which proliferate to form a tumor mass, the invasion of adjacent tissues by these neoplastic tumor cells, and the generation of malignant cells which eventually spread via the blood or lymphatic system to regional lymph nodes and to distant sites via a process called metastasis. In a cancerous state, a cell proliferates under conditions in which normal cells would not grow. Cancer manifests itself in a wide variety of forms, characterized by different degrees of invasiveness and aggressiveness.

In attempts to discover effective cellular targets for cancer diagnosis and therapy, researchers have sought to identify polypeptides associated with metastasis. In attempts to discover effective cellular targets for cancer diagnosis and therapy, researchers have sought to identify (1) non-membrane-associated polypeptides that are specifically produced by one or more particular type(s) of cancer cell(s) as compared to by one or more particular type(s) of non-cancerous normal cell(s), (2) polypeptides that are produced by cancer cells at an expression level that is significantly higher than that of one or more normal non-cancerous cell(s), or (3) polypeptides whose expression is specifically limited to only a single (or very limited number of different) tissue type(s) in both the cancerous and non-cancerous state (*e.g.*, normal prostate and prostate tumor tissue). Such polypeptides may remain intracellularly located or may be secreted by the cancer cell. Moreover, such polypeptides may be expressed not by the cancer cell itself, but rather by cells which produce and/or secrete polypeptides having a potentiating or growth-enhancing effect on cancer cells. Such secreted polypeptides are often proteins that provide cancer cells with a growth advantage over normal cells and include such

things as, for example, angiogenic factors, cellular adhesion factors, growth factors, and the like.

There is a great need for additional diagnostic and therapeutic agents capable of detecting and treating cancer.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for biomarker screening and research. In particular, the present invention relates to VCP-KMT methyltransferases, antibodies to methylated VCP, and targets of VCP-KMT for screening and research applications.

Embodiments of the present invention provide compositions, kits, and methods useful in the detection of VCP-KMT methyltransferase activity. Such compositions and methods find use in research, screening (*e.g.*, drug screening), and diagnostic (*e.g.*, screening for the presence of cancer) applications.

In some embodiments, the present invention provides methods of screening compounds for modulation of the methyltransferase activity of a VCP lysine-specific MTase (VCP-KMT) enzyme, comprising: (a) contacting said VCP lysine-specific MTase (VCP-KMT) enzyme with a VCP substrate and a test compound; and (b) detecting the level of methylation of said VCP substrate in the presence and absence of said test compound, wherein the level of methylation of said VCP substrate is indicative of methyltransferase activity of said VCP-KMT enzyme. In some embodiments, the VCP substrate is non-methylated prior to contact with the VCP-KMT enzyme in step (a) and methylation of the previously non-methylated substrate is detected. In some embodiments, the VCP-KMT enzyme is human VCP-KMT. In some embodiments, the VCP-KMT enzyme is *C. elegans* C42C1.13. In some embodiments, the VCP-KMT enzyme is an ortholog of human VCP-KMT. In some embodiments, the VCP substrate is selected from the group consisting of VCP and VCP variants. In some embodiments, the VCP variant is VCPΔD2. In some embodiments, the test compound is a drug. In some embodiments, the drug is a cancer drug.

In some embodiments, the detecting further comprises providing an antigen binding protein that specifically binds to a methylated VCP substrate but not a non-methylated VCP substrate and exposing said VCP substrate to said antigen binding protein to detect methylated VCP substrate. In some embodiments, the methylated

substrate is VCP or VCPΔD2 trimethylated at lysine residue K315.

In some embodiments, the detecting further comprises providing a labeled methyl donor and detecting VCP substrate comprising a labeled methyl group donated from said labeled methyl donor. In some embodiments, the labeled methyl donor is selected from the group consisting of S-[methyl-^{14}C]-SAM and S-[methyl-^{3}H]-SAM.

In some embodiments, the methods further comprise screening a library of test compounds. In some embodiments, the methods further comprise selecting test compounds from said library that modulate methyltransferase activity of said VCP-KMT enzyme. In some embodiments, the methods further comprise clinically testing at least one selected test compound. In some embodiments, the methods further comprise synthesizing a lead compound utilizing at least one selected test compound as a template. In some embodiments, the methods further comprise clinically testing said lead compound. In some embodiments, the methods further comprise providing the test compound or the lead compound for administration to a subject.

In some embodiments, the present invention provides a test compound or lead compound identified by the foregoing methods.

In some embodiments, the present invention provides for the use of a VCP lysine-specific MTase (VCP-KMT) enzyme and a VCP substrate in an assay for identification of one or more compounds that alter the methyltransferase activity of said VCP lysine-specific MTase (VCP-KMT) enzyme.

In some embodiments, the present invention provides for the use of a VCP substrate in an assay to identify one or more compounds that alter the methyltransferase activity of said VCP lysine-specific MTase (VCP-KMT) enzyme.

In some embodiments, the present invention provides a kit, comprising: a) a VCP-KMT enzyme; b) a VCP-KMT substrate; and c) reagents for detection of methylated VCP-KMT substrate. In some embodiments, the VCP-KMT enzyme is human VCP-KMT. In some embodiments, the VCP-KMT enzyme is *C. elegans* C42C1.13. In some embodiments, the VCP-KMT enzyme is an orthologue of human VCP-KMT. In some embodiments, the substrate is VCP or a VCP variant. In some embodiments, the VCP variant is VCPΔD2. In some embodiments, the reagents comprise an antibody that specifically binds to a methylated VCP substrate but not a non-methylated VCP substrate. In some embodiments, the methylated substrate is VCPΔD2 trimethylated at K315. In some embodiments, the reagents comprise a labeled methyl donor.

In some embodiments, the present invention provides an antigen binding protein or antibody, scFV, or fragment thereof that specifically binds to a methylated VCP substrate, preferably methylated (e.g., trimethylated) at a lysine residue corresponding to amino acid residue K315 of wild type human VCP, but not a non-methylated VCP substrate. The VCP substrate may be a wild type VCP, a portion of VCP comprising a lysine residue corresponding to amino acid residue K315 of wild type human VCP, or a variant thereof. In some embodiments, the methylated substrate is a VCP or VCPΔD2 trimethylated at K315.

In some embodiments, the present invention provides a prognostic or diagnostic method comprising contacting a patient sample with the foregoing antigen binding protein or antibody, scFV, or fragment thereof to determine the level of methylation of VCP. In some embodiments, the methods further comprise correlating said level of methylation with a disease, condition, prognosis or outcome for said patient.

In some embodiments, the present invention provides for the use of VCP or VCPΔD2 methylated at K315 or a portion thereof comprising said methylated K315 residue for generating an antibody that specifically binds to methylated VCP or VCPΔD2.

Additional embodiments are described herein.

DESCRIPTION OF THE FIGURES

Figure 1a and 1b. METTL21D is a lysine specific Class I MTase. a, Alignment of METTL21D protein sequences (*H. sapiens*, NP_078834; *A. pisum*, XP_001945214.1; *C. elegans*, NP_001122759.1; *N. vectensis*, XP_001636505.1; *A. thaliana*, NP_973791.1; *C. reinhardtii*, XP_001692568.1). Predicted α-helices and β-strands of human METTL21D are indicated. Hallmark motifs are boxed. Key residues Asp96 and Asp144 are indicated by asterisks. b, METTL21D methylates lysine-containing, positively charged model substrates. Data are represented as means +/– s.d., n=3.

Figure 2a, 2b, 2c, 2d, 2e, 2f, 2g, and 2h. VCP-KMT interacts with and methylates VCP in vitro. a, Structure of hexameric VCP. Three of the protomers have been individually color-labeled and darker color indicates the selected interaction domain (SID) involved in METTL21D interaction. The structure was rendered using symmetry expansion on a published VCP structure [8]. b, Domain structure of VCP showing the N-terminal, D1 ATPase and D2 ATPase domains, and indicating the SID and VCPΔD2. c, VCP-KMT methylates VCP and VCPΔD2. 10 μg of substrate were incubated with

varying amounts of VCP-KMT at pH 7.5. Data are represented as means +/– s.d., n=3. d, VCP-KMT disrupts the hexamer and forms a complex with VCPΔD2. Hexameric VCP or VCPΔD2 were incubated with a twofold molar excess of VCP-KMT, followed by SEC. Left, Elution profiles (absorbance at 280 nm) of protein samples and size standards. Right, Coomassie Blue stained SDS-PAGE of indicated fractions. e, Methylation assay using purified hexameric VCP and VCPΔD2. 10 µg of protein before SEC, hexameric protein purified as shown in panel d, or aggregated protein eluting in the void volume were incubated with 100 pmol VCP-KMT. Error bars indicate range between duplicate samples. f, MS/MS sequencing of Arg-C generated peptide VCPΔD2(288–322)-Kme3 ([M+1H]+ ≙ 4,007.124 Da) showing trimethylation of Lys315 g, Mutational analysis of VCPΔD2 methylation. VCPΔD2 variants with point mutations in the region surrounding Lys315 were incubated with 100 pmol VCP-KMT. Data are represented as means +/– s.e.m., n=4. Four variants (#) showed changed assembly and/or aggregation, see Supplementary Fig. S4. h, Methylation status of VCPΔD2 incubated with varying amounts of VCP-KMT. The methylation status of Lys315 in peptide VCPΔD2(288–322) was quantified by MS.

Figure 3a, 3b, 3c and 3d. VCP-KMT-mediated trimethylation of VCP in vivo. a, MS-analysis of VCP isolated from mouse tissues. Chromatograms for un-, di- and trimethylated Lys315 in Arg-C generated peptide VCP(314–322) are shown. Selective ion settings were m/z = 542.7727, 556.7884 and 563.7962 +/- 6 ppm, z = +2. For each tissue, the intensity is normalized to the Kme3 signal. Expected elution time of the Kme0 peptide (arrows) and irrelevant peaks (*, M+1 isotope peak of an unrelated peptide) are indicated. Monomethylated peptide was not detected. b, Zinc finger nuclease mediated disruption of the VCP-KMT gene in human cell lines. In a schematic representation of the human VCP-KMT gene, boxes indicate the coding (grey) and non-coding (light grey) parts of exons, while solid black lines indicate introns (not to scale). A blow-up of the sequence at the ZFN target site is shown. ZFN cleavage is expected to occur between the two ZFN binding sites (uppercase). The genotypes of selected clones harboring frame shift mutations are shown. U87-MG cells contain only a single copy of the VCP-KMT gene. c, MS-analysis of VCP isolated from wild-type and VCP-KMT-deficient cell lines. Settings as in panel 3a. For each cell line, the chromatograms are normalized to the highest intensity measured. Kme2 peptides (arrows) and irrelevant peaks (*, M+1 isotope peak of an unrelated peptide) are indicated. d, Assessment of VCP methylation by

Western blot. Upper panel, Western blot of immunoprecipitated VCP from wild-type and VCP-KMT-deficient cell lines using pan-Kme3 antibody. Lower panel, similar blot of in vitro methylated VCPΔD2 (positive control). A Ponceau S stain of the membrane is shown as loading control.

Figure 4a, 4b, and 4c. VCP methylation in other organisms. a, Lack of VCP-specific MTase activity of S. cerevisiae YNL024c. VCPΔD2 and the orthologous yeast protein CDC48ΔD2 were incubated with 100 pmol VCPKMT or YNL024c. b, Lack of CDC48 methylation in S. cerevisiae. Chromatograms representing different K325 methylation states for chymotryptic peptide CDC48(313–338) are shown. Selective ion settings were m/z = 742.9026, 749.9104 and 753.4144 +/- 10 ppm, z = +4. Top, CDC48 isolated from S. cerevisiae. Middle, untreated recombinant CDC48ΔD2 (negative control). Bottom, VCP-KMT-treated CDC48ΔD2 (positive control for detection of methylated peptides). c, VCP-KMT activity of C. elegans protein C42C1.13. Human VCPΔD2 and yeast CDC48ΔD2 were incubated with varying amounts of C42C1.13 or human VCP-KMT. Data in a and c are represented as means +/– s.d., n=3.

Figure 5a, 5b, 5c, 5d, 5e, 5f, 5g, and 5h. Effects of VCP-KMT disruption on cellular phenotypes and VCP function. a, Representative growth curves of HeLa (left), 293 T-REx Flp-In (middle) and U87-MG cells (right). An exponential fit to the data is shown and the deduced doubling time τ is indicated. b, Doubling time of wild-type and VCP-KMT-deficient cell lines. Data are represented as means +/– s.e.m., n is indicated. Significant differences between means are indicated (alpha level 0.05, two-tailed independent two-sample Student's t-test) and p-values are shown. c, Migration and invasion of wild-type and VCP-KMT deficient cells. HeLa and U87-MG cells were assayed in a transwell assay with or without Matrigel coating. The percentage of cells having traversed the filter after 24 h is shown. Data are represented as means +/– s.e.m., n=4. Significant differences between means are indicated (alpha level 0.05, two tailed independent two-sample Student's t-test) and p-values are shown. d, VCP content of wild-type and VCP-KMT-deficient cell lines. Whole cell lysate (7.5 µg protein) was separated by SDS-PAGE and subjected to Western blot with VCP- and GAPDH-specific antibodies. e, Localization of VCP in wild type and VCP-KMT-deficient cells. Fixed and permeabilized cells were stained with VCP-specific antibody. A z-projection of a stack of confocal images is shown. Scale bar = 20 µm. f, Size of endogenous VCP-complexes. S100 fractions from HeLa wt and VCP-KMT-deficient cells were separated by SEC and

fractions were analyzed by anti-VCP Western blot. g, ATPase activity of VCP from wild-type and VCP-KMT-deficient cells. VCP was partially purified from wild-type or VCP-KMT deficient HeLa cells by anion exchange chromatography and SEC, and the ATPase activity of SEC fractions was measured. h, Coomassie stained SDS-PAGE of VCP-containing SEC fractions A–D. Co-purified Valyl-tRNA synthetase complex subunits and VCP were identified by MS (arrows).

Figure 6a, 6b, 6c and 6d. Protein MTase activity of additional human Family 16 proteins. a, Sequence alignment of the ten human Family 16 proteins showing the hallmark (D/E)XX(Y/F) motif located downstream of Motif II. b, Automethylation activity of selected MTase Family 16 members. 300 pmol of MT were incubated for 1 h at pH 8.5 with [3H]-SAM. The competitive inhibitor SAH was added at the start or end of the incubation to distinguish between automethylation and SAM-binding. Top, liquid scintillation counting results, data are represented as means +/– s.d., n=3. Significant effects of mutation or SAH addition on mean incorporated radioactivity are indicated (alpha level 0.05, one tailed independent two-sample Student's t-test) and p-values are shown. Bottom, Ponceau S staining and fluorography of duplicate samples separated by SDS-PAGE and blotted on PVDF membrane. c, Lysine-specific automethylation of METTL21C. A mass shift corresponding to monomethylation of tryptic peptide METTL21C(31–47) is detected upon incubation of METTL21C with SAM. d, MS/MS sequencing of peptide METTL21C(31–47)-Kme1 ([M+1H]+ ≙ 1,730.856 Da) showing lysine monomethylation on residue Lys35. The masses of detected b- and y-ions are indicated

Figure 7a and 7b shows the nucleotide (SEQ ID NO:1) and polypeptide (SEQ ID NO:2) sequence of human VCP-KMT (GenBank Acc. No. NM_024558).

DEFINITIONS

Unless otherwise explained, all 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. Definitions of common terms in molecular biology can be found in Benjamin Lewin, Genes V, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by

VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "plurality" is used synonymously with the phrase "more than one," that is, two or more. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. The term "comprises" means "includes." The abbreviation, "e.g.," is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g.," is synonymous with the term "for example." Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below.

In order to facilitate review of the various embodiments of this disclosure, the following explanations of specific terms are provided:

As used herein, the term “VCP” refers to Valosin-containing protein or p97. A number of Valosin-containing protein orthologues have been identified including, but not limited to: Human VCP (GenBank Accession No. NP_009057.1 GI:6005942; AAI21795.1 GI:111305821); Rat (Genbank Assession No. NP_446316.1 GI: 17865351); Mouse (Genbank Assession No. AAH49114.1 GI:29144989 (806 amino acids)). As used herein, the term VCP includes proteins that share at least 70%, 80%, 90%, 95%, 97%, 98%, 99% or 100% sequence identity with the human, mouse or rat VCP.

As used herein, the term “VCP lysine-specific MTase” or “VCP-KMT” refers to a methyltransferase that methylates, and preferably trimethylates, VCP at position K315 of the wild type human VCP. In some embodiments, the VCP-KMT enzyme is the *Homo sapiens* VCP-KMT. In other embodiments, the enzyme is a VCP-KMT orthologue from another organism (*e.g.*, a eukaryotic or prokaryotic organism). In some preferred embodiments, the VCP-KMT shares at least 70%, 80%, 90%, 95%, 97%, 98%, 99% or 100% sequence identity with SEQ ID NO:2, human VCP-KMT.

As used herein, the term VCP lysine-specific MTase (VCP-KMT) substrate, or simply VCP substrate, refers to a VCP homolog or fragment thereof that is capable of being methylated, and preferably trimethylated, at a lysine residue corresponding to position K315 of wild type human VCP. For example, the VCP-KMT substrate may be a full-length, wild type VCP, a variant of VCP (e.g., a variant sharing at least 70%, 80%,

90%, 95%, 97%, 98% or 99% sequence identity with human, mouse or rat VCP), or a fragment, such as a truncation mutant, of human, mouse or rat VCP (e.g., a VCP truncation mutant lacking the D2 ATPase domain, wherein the truncated mutant shares at least 70%, 80%, 90%, 95%, 97%, 98% or 99% sequence identity with the corresponding portion of human, mouse or rat VCP).

As used herein, the terms “detect”, “detecting” or “detection” may describe either the general act of discovering or discerning or the specific observation of a detectably labeled composition.

As used herein, the term “subject” refers to any organisms that are screened using the diagnostic methods described herein. Such organisms preferably include, but are not limited to, mammals (*e.g.*, murines, simians, equines, bovines, porcines, canines, felines, and the like), and most preferably includes humans.

The term “diagnosed,” as used herein, refers to the recognition of a disease by its signs and symptoms, or genetic analysis, pathological analysis, histological analysis, and the like.

As used herein, the term "purified" or "to purify" refers to the removal of components (*e.g.*, contaminants) from a sample. For example, antibodies are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind to the target molecule. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to the target molecule results in an increase in the percent of target-reactive immunoglobulins in the sample. In another example, recombinant polypeptides are expressed in bacterial host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant polypeptides is thereby increased in the sample.

As used herein, the term "sample" is used in its broadest sense. In one sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood products, such as plasma, serum and the like. Such examples are not however to be construed as limiting the sample types applicable to the present invention.

The term "biomarker" as used herein in various embodiments refers to a specific biochemical in the body that has a particular molecular feature to make it useful for diagnosing and measuring the progress of disease or the effects of treatment. Major

classes of cancer biomarkers based on clinical utility and application include the following: (1) "diagnostic biomarkers" that are used to: (i) determine if the patient has cancer, and (2) define the type of cancer of the patient. Diagnostic biomarkers can also be used to detect and define recurrent disease after primary therapy. (2) "Prognostic biomarkers" are used to indicate a likely course of the disease. Prognostic biomarkers can reflect, for example, the metastatic state or potential and/or the likely growth rate of the tumor, and are used to estimate patient outcome without consideration of the treatment given. (3) "Predictive biomarkers" are used to identify subpopulations of patients who are most likely to respond to a given therapy. (4) "Pharmacodynamic" or "pharmacological" biomarkers (sometimes referred to as PD biomarkers) can help identify which drug dose to use for an individual. Finally, biomarkers can also be used to monitor a patient's response to treatment. Once a patient begins treatment with a drug, the biomarkers of the present invention can be used to monitor the patient's response, and if necessary, the treatment regiment (drug or dose) can be modified. The biomarkers of the present invention can be used in any of these forms. The present invention specifically encompasses K315 methylated VCP biomarkers as well as VCP-KMT biomarkers.

As used herein the term "immunohistochemistry (IHC)" also known as "immunocytochemistry (ICC)" when applied to cells refers to a tool in diagnostic pathology, wherein panels of antibodies (e.g., monoclonal antibodies) can be used in the differential diagnosis of undifferentiated neoplasms (e.g., to distinguish lymphomas, carcinomas, and sarcomas) to reveal markers specific for certain tumor types and other diseases, to diagnose and phenotype malignant lymphomas and to demonstrate the presence of viral antigens, oncoproteins, hormone receptors, and proliferation-associated nuclear proteins.

As used herein, the term “antigen binding protein” refers to proteins which bind to a specific antigen. “Antigen binding proteins” include, but are not limited to, immunoglobulins, including polyclonal, monoclonal, chimeric, single chain, and humanized antibodies, Fab fragments, F(ab')2 fragments, and Fab expression libraries. Various procedures known in the art are used for the production of polyclonal antibodies. For the production of antibodies, various host animals can be immunized by injection with the peptide corresponding to the desired epitope including, but not limited to, rabbits, mice, rats, sheep, goats, *etc.* In a preferred embodiment, the peptide is conjugated to an immunogenic carrier (*e.g.*, diphtheria toxoid, bovine serum albumin (BSA), or

keyhole limpet hemocyanin (KLH)). Various adjuvants are used to increase the immunological response, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

For preparation of monoclonal antibodies, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used (*See e.g.*, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). These include, but are not limited to, the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, Nature, 256:495-497 (1975)), as well as the trioma technique, the human B-cell hybridoma technique (*See e.g.*, Kozbor *et al.*, Immunol. Today, 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., *pp.* 77-96 (1985)).

According to the invention, techniques described for the production of single chain antibodies (U.S. 4,946,778; herein incorporated by reference) can be adapted to produce specific single chain antibodies as desired. An additional embodiment of the invention utilizes the techniques known in the art for the construction of Fab expression libraries (Huse *et al.*, Science, 246:1275-1281 (1989)) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibody fragments that contain the idiotype (antigen binding region) of the antibody molecule can be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')2 fragment that can be produced by pepsin digestion of an antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of an F(ab')2 fragment, and the Fab fragments that can be generated by treating an antibody molecule with papain and a reducing agent.

Genes encoding antigen-binding proteins can be isolated by methods known in the art. In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western Blots, precipitation

reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, *etc.*), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*) *etc.*

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they bind specifically to a target antigen.

The term "primary antibody" herein refers to an antibody which binds specifically to the target protein antigen in a tissue sample. A primary antibody is generally the first antibody used in an immunohistochemical procedure. In one embodiment, the primary antibody is the only antibody used in an IHC procedure. With regards to ISH, a primary antibody is typically directed to a label, wherein said label (e.g., hapten, etc.) is incorporated into an oligonucleotide probe that is directed to a target sequence. For example, if an oligonucleotide probe is labeled with DIG the primary antibody is an anti-DIG antibody that recognizes and binds the DIG hapten of the oligonucleotide. Further, such a primary antibody used in ISH is typically conjugated to a detection reagent or enzyme which provides a detection means for detecting hybridization events.

The term "secondary antibody" herein refers to an antibody which binds specifically to a primary antibody, thereby forming a bridge between the primary antibody and a subsequent reagent, if any. The secondary antibody is generally the second antibody used in an immunohistochemical procedure.

A "label" is a detectable compound or composition that is conjugated directly or indirectly to another molecule to facilitate detection of that molecule. Specific, non-limiting examples of labels include fluorescent and fluorogenic moieties, chromogenic moieties, haptens, affinity tags, and radioactive isotopes. The label can be directly detectable (e.g., optically detectable) or indirectly detectable (for example, via interaction with one or more additional molecules that are in turn detectable). Exemplary labels in the context of the probes disclosed herein are described below. Methods for labeling nucleic acids, and guidance in the choice of labels useful for various purposes, are discussed, e.g., in Sambrook and Russell, in Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press (2001) and Ausubel et al., in Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Intersciences (1987, and including updates).

The term "kit" or "testing kit" denotes combinations of reagents and adjuvants

required for an analysis. Although a test kit consists in most cases of several units, one-piece analysis elements are also available, which must likewise be regarded as testing kits.

"Fragment" as used herein may mean a portion of a reference peptide or polypeptide or nucleic acid sequence.

"Identical" or "identity" as used herein in the context of two or more polypeptide or nucleotide sequences, may mean that the sequences have a specified percentage of residues or nucleotides that are the same over a specified region. The percentage may be calculated by optimally aligning the two sequences, comparing the two sequences over the specified region, determining the number of positions at which the identical residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the specified region, and multiplying the result by 100 to yield the percentage of sequence identity. In cases where the two sequences are of different lengths or the alignment produces one or more staggered ends and the specified region of comparison includes only a single sequence, the residues of single sequence are included in the denominator but not the numerator of the calculation.

"Variant" as used herein in the context of a nucleic acid may mean a substantially identical or substantially complementary sequence. A variant in reference to a nucleic acid may further mean a nucleic acid that may contain one or more substitutions, additions, deletions, insertions, or may be fragments thereof. A variant may also be a nucleic acid capable of hybridizing under moderately stringent conditions and specifically binding to a nucleic acid encoding the agent. Hybridization techniques are well known in the art and may be conducted under moderately stringent conditions.

A variant in reference to a peptide may further mean differing from a native peptide in one or more substitutions, deletions, additions and/or insertions, or a sequence substantially identical to the native peptide sequence. The ability of a variant to react with antigen-specific antisera may be enhanced or unchanged, relative to the native protein, or may be diminished by less than 50%, or less than 20%, relative to the native peptide. Such variants may generally be identified by modifying one of the peptide sequences encoding an agent and evaluating the reactivity of the modified peptide with antigen-specific antibodies or antisera as described herein. Variants may include those in which one or more portions have been removed such as an N-terminal leader sequence or

transmembrane domain. Other variants may include variants in which a small portion (e.g., 1-30 amino acids, or 5-15 amino acids) has been removed from the N- and/or C-terminal of the mature protein. A variant in reference to a peptide may contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry may expect the secondary structure and hydrophobic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gin, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also contain nonconservative changes. Variant peptides may differ from a native sequence by substitution, deletion or addition of amino acids. Variants may also be modified by deletion or addition of amino acids, which have minimal influence on the immunogenicity, secondary structure, hydropathic, and hydrophobic nature of the polypeptide.

As used herein, the terms “anticancer agent,” “conventional anticancer agent,” or “cancer therapeutic drug” refer to any therapeutic agents (*e.g.*, chemotherapeutic compounds and/or molecular therapeutic compounds), radiation therapies, or surgical interventions, used in the treatment of cancer (*e.g.*, in mammals, in primates, in humans, etc.).

As used herein, the terms “drug” and “chemotherapeutic agent” refer to pharmacologically active molecules that are used to diagnose, treat, or prevent diseases or pathological conditions in a physiological system (*e.g.*, a subject, or *in vivo*, *in vitro*, or *ex vivo* cells, tissues, and organs). Drugs act by altering the physiology of a living organism, tissue, cell, or *in vitro* system to which the drug has been administered. It is intended that the terms “drug” and “chemotherapeutic agent” encompass anti-hyperproliferative and antineoplastic compounds as well as other biologically therapeutic compounds.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for biomarker screening and research. In particular, the present invention relates to VCP-KMT methyltransferases, antibodies to methylated VCP, and targets of VCP-KMT for screening and research applications.

VCP (also called p97) is an essential and highly conserved ATP-dependent chaperone implicated in a wide range of cellular processes in eukaryotes, and mild VCP mutations can cause severe neurodegenerative disease. The present invention demonstrates that mammalian VCP is trimethylated on K315 in a variety of cell lines and tissues, and that the previously uncharacterized protein METTL21D (denoted here as VCP lysine methyltransferase; VCP-KMT) is the responsible enzyme. VCP methylation was abolished in three human VCP-KMT knock-out cell lines generated with zinc finger nucleases. VCP-KMT was recently reported to promote tumor metastasis and VCP-KMT-deficient cells displayed reduced growth rate, migration and invasive potential. Finally, we present data indicating that VCP-KMT, calmodulin-KMT and eight uncharacterized proteins together constitute a novel human protein methyltransferase family. The present work provides new insights on protein methylation and its links to human disease.

Protein methyltransferases (MTases) modify a wide range of cellular proteins, with lysine and arginine being the predominant acceptor sites [1]. Protein methylation can be dynamic and serve regulatory purposes, or it can be static and function as an expansion of the amino acid repertoire. Most MTases use S-adenosyl methionine (SAM) as methyl donor, and the human genome encodes over 200 putative SAM-dependent MTases, most of which remain uncharacterized [2]. The majority of these MTases belongs to either the SET domain family or to the seven β-strand superfamily, also designated "Class I MTases" [2,3]. Most characterized lysine-specific MTases are SET domain proteins acting primarily on histones, but a few lysine-specific protein MTases have also been found in Class I. In humans, these are the histone-specific MTase DOT1L [3] and the calmodulin-specific MTase CaMKMT [4]; in addition, human METTL [10] is a likely ortholog of yeast See1, which methylates elongation factor 1A5. The MTases responsible for many lysine methylations remain elusive [6], and some are likely to be found among the numerous uncharacterized Class I MTases.

VCP is an abundant, essential and highly conserved AAA+ protein (ATPase associated with various cellular activities) found in all eukaryotes [7]. It contains two

ATPase domains, denoted D1 and D2, which form two stacked rings around a central pore in the homohexameric quaternary structure [8]. VCP is involved in a wide range of biological processes, such as cell cycle regulation, membrane fusion, autophagy, and ubiquitin mediated protein degradation, which may be explained by its ability to act as an ATP-dependent chaperone [7,9]. Certain mutations that only modestly affect the biochemical activity of VCP cause the severe autosomal dominant disorder IBMPFD (Inclusion body myopathy with early-onset Paget disease and/or frontotemporal dementia)10, underscoring the importance of fine-tuning of VCP function in humans.

The present invention demonstrates that the putative human Class I MTase METTL21D specifically catalyzes the trimethylation of Lys315 in VCP in vitro, and the enzyme was thus named VCP-KMT. Moreover, this previously unknown VCP modification is present in mouse tissues and human cell lines, and was abolished in the latter by zinc finger nuclease-mediated VCP-KMT disruption. VCP-KMT was in a recent study found to be a promoter of tumor metastasis [11], and accordingly, VCP-KMT-deficient cells showed reduced proliferation, migration and invasive potential. Finally, a combination of biochemical and bioinformatics analyses indicates that VCP-KMT belongs to a novel human protein MTase family with ten members, of which the majority seems to be lysine specific.

The majority of lysine-specific protein MTases (KMTs) belong to the SET-domain family, and until now, only two human Class I KMTs have been described, namely CaM-KMT and DOT1L. Here, by using both in vitro and in vivo strategies, we firmly establish the existence of a third such enzyme, VCP-KMT, which is responsible for the trimethylation of VCP on Lys315.

In a recent study, 119 genes that were up-regulated in a metastatic cancer cell line were subjected to secondary screens, pin-pointing a single gene, denoted NVM-1 (novel metastasis-promoting gene 1), as being particularly important for migratory behavior and metastatic potential [11]. Interestingly, NVM-1 is identical to VCP-KMT. In the present study, we found that genetic ablation of VCP-KMT in human cell lines affected cell growth, migration and invasion, further supporting the notion that VCP-KMT is important for cancer metastasis. VCP-KMT expression may promote cancer cell migration and metastasis by two alternative mechanisms. Conceivably, high expression of VCP-KMT in rapidly growing cancer cells might ensure optimal VCP function through efficient methylation of newly synthesized VCP. Indeed, elevated VCP expression leads

to increased cell proliferation and is correlated with metastasis and cancer [25]. Alternatively, high expression of VCP-KMT may drive cancer metastasis through methylation of proteins other than VCP, i.e. through the methylation of a yet unidentified bona fide substrate, or through aberrant methylation of other proteins. Finally, the formal possibility exists that increased metastasis is mediated by a yet unidentified, methylation independent function of VCP-KMT.

In an effort to identify additional substrates, VCP-containing S100 chromatography fraction from VCP-KMT deficient cells (data not shown), or a protein macroarray containing over 3500 denatured proteins including VCP (UniPEx_2, RZPD, Germany), was used as in vitro substrates. No new VCP-KMT substrates could be detected in these experiments; even VCP, although present in both assays, was not identified. Our inability to detect VCP methylation in these experiments is not unexpected, as VCP-KMT is inactive on the intact VCP hexamer (substrate in S100 assay), and requires a folded substrate (proteins immobilized on array are denatured). In line with these results, analysis of histone H3 methylation from wild-type and VCP-KMT-deficient HeLa cells by MS showed no differences in methylation. Taken together, our results indicate that the VCP-KMT activity is highly specific for VCP, although the existence of additional relevant substrates cannot be ruled out.

VCP is a highly conserved and essential protein in eukaryotes and affects many cellular processes through its central function as a molecular chaperone. In contrast, putative VCP-KMT orthologs are only found in certain eukaryotes, including vertebrates, and we found VCP to be unmethylated in the yeast S. cerevisiae, which lacks such an ortholog. Moreover, mutation at Lys315 did not strongly affect VCP function [26]. Accordingly, we found VCP-KMT-deficient human cells to be viable, showing no detectable impairment of the ubiquitin-proteasome system or autophagy, and unmethylated VCP isolated from such cells displayed ATPase activity similar to that of VCP isolated from wild-type cells. Taken together, this indicates that trimethylation at Lys315 is not required for basic VCP function, but may serve a more specialized purpose in VCP-KMT containing organisms, supported by the observed effects of VCP-KMT knockout on cell growth and migration.

Various mutations in VCP have been reported to cause the so-called "VCP disease" IBMPFD, which is the phenotypic overlap of three distinct diseases, namely inclusion body myopathy, Paget's disease of bone and/or frontotemporal dementia.

IBMPFD is a late-onset, dominant disease and the disease-causing mutations are primarily localized in the N-terminal part of VCP, which serves as a binding platform for adaptor proteins, but some mutations are also found in the D1 domain, the region which is subject to methylation. Interestingly, IBMPFD-causing mutant proteins have normal or elevated ATPase activity, and some have been reported to associate more avidly with protein co-factors. Recently, mutations in VCP were also found to cause the severe neurological disorder ALS which, similarly to IBMPFD, is a proteinopathy with aggregation of the protein TDP-43 as a hallmark. Moreover, VCP has been shown to be involved in the formation and clearance of abnormal protein aggregates found in a variety of neurological diseases[29]. Conceivably, VCP methylation may play a role in similar phenotypes.

VCP-KMT belongs to Family 16 of MTases, which is almost identical to what was denoted "Group J" in a recent bioinformatics study, the major difference being that the FAM86 proteins are part of Family 16 but excluded from Group J [2]. Two out of the ten human members of this family have previously been implicated in protein methylation, CaM-KMT [4] and C1orf156 [19]. In the present work, we have detected protein MTase activity for three human Family 16 members, namely VCP-KMT, METTL21A, and METTL21C, and shown that two of these enzymes are lysine-specific. We conclude that all ten human Family 16 members are probable protein MTases, many of which are likely to be lysine specific.

Accordingly, embodiments of the present invention provide compositions, kits, systems and methods for utilizing VCP, VCP substrates, and VCP-KMT in screening and research applications. In some embodiments, compositions and methods utilize *H. sapiens* VCP-KMT, as described herein. In other embodiments, an ortholog of VCP-KMT is utilized (e.g., from an eukaryotic or prokaryotic organism).

For example, in some embodiments, the present invention provides compositions and methods for identifying inhibitors of VCP-KMT methyltransferase activity. In some embodiments, drug screening applications utilize VCP-KMT and VCP or VCP variants (*e.g.*, VCPΔD2).

In some embodiments, the present invention further provides antibodies that recognize methylated VCP and variants thereof (e.g., VCPΔD2). For example, in some embodiments, antibodies recognize tri-methylated VCPΔD2.

Further embodiments of the present invention utilize detection of methylated

VCPΔD2 as a diagnostic or research assay for the presence of cancer and/or neurodegenerative disease (*e.g.*, IBMPFD).

I. Antibodies

In some embodiments, the present invention provides isolated antibodies. In preferred embodiments, the present invention provides monoclonal or polyclonal antibodies that specifically bind to an isolated polypeptide comprised of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 15, 17, 18, 19, or 20 amino acid residues of the methylated form (*e.g.*, trimethylated) VCPΔD2 or to wild type VCP, wherein the polypeptide comprises amino acids flanking the K315 position of wild type human VCP. In some embodiments, antibodies of the present invention specifically bind to VCP or variants (e.g., VCPΔD2) trimethylated at K315 and do not bind to VCP or variants thereof that lack methylation or trimethylation of position K315. The term "specifically binds" refers to, with respect to an antigen such as the trimethylated form of VCP or VCPΔD2, the preferential association of an antibody or other ligand, in whole or part, with the trimethylated form of VCP or a fragment thereof or a cell or tissue bearing that antigen and not to cells or tissues lacking that antigen (i.e., VCP or fragment thereof that is trimethylated at K315). It is recognized that a certain degree of non-specific interaction may occur between a molecule and a non-target protein, cell or tissue. Nevertheless, specific binding can be distinguished as mediated through specific recognition of the antigen. Although selectively reactive antibodies bind antigen, they can do so with low affinity. On the other hand, specific binding results in a much stronger association between the antibody (or other ligand) and cells bearing the antigen than between the bound antibody (or other ligand) and cells lacking the antigen. Specific binding typically results in greater than 2-fold, such as greater than 5-fold, greater than 10-fold, or greater than 100-fold increase in amount of bound antibody or other ligand (per unit time), for example to the trimethylated form of VCP as compared to VCP that is not trimethylated. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats are appropriate for selecting antibodies or other ligands specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow & Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York (1988), for a description

of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

These antibodies find use in the diagnostic, research, and screening methods described herein. In some embodiments, the present invention provides an antigen binding protein or reagent comprising one or complementary determining regions (CDRs) derived from an antibody that specifically binds to VCP or VCPΔD2 trimethylated at K315 and not to wild type or otherwise methylated VCP. Suitable antigen binding proteins include, antibodies and humanized antibodies, fragments thereof such as Fab, F(ab'), F(ab')2 and Fv fragments, dsFvs (disulfide-linked Fv), scFvs (single chain Fv).

An antibody against a protein of the present invention may be any monoclonal or polyclonal antibody, as long as it can recognize the protein. Antibodies can be produced by using a protein of the present invention or a peptide fragment thereof as the antigen according to a conventional antibody or antiserum preparation process.

The present invention contemplates the use of both monoclonal and polyclonal antibodies. Any suitable method may be used to generate the antibodies used in the methods and compositions of the present invention, including but not limited to, those disclosed herein. For example, for preparation of a monoclonal antibody, protein, as such, or together with a suitable carrier or diluent is administered to an animal (*e.g.*, a mammal) under conditions that permit the production of antibodies. For enhancing the antibody production capability, an adjuvant, for example, complete or incomplete Freund's adjuvant may be administered. Normally, the protein is administered once every 2 weeks to 6 weeks, in total, about 2 times to about 10 times. Animals suitable for use in such methods include, but are not limited to, primates, rabbits, dogs, guinea pigs, mice, rats, sheep, goats, llama, chicken, etc.

For preparing monoclonal antibody-producing cells, an individual animal whose antibody titer has been confirmed (*e.g.*, a mouse) is selected, and 2 days to 5 days after the final immunization, its spleen or lymph node is harvested and antibody-producing cells contained therein are fused with myeloma cells to prepare the desired monoclonal antibody producer hybridoma. Measurement of the antibody titer in antiserum can be carried out, for example, by reacting the labeled protein and antiserum and then measuring the activity of the labeling agent bound to the antibody. The cell fusion can be carried out according to known methods, for example, the method described by Koehler and Milstein (Nature 256:495 [1975]). As a fusion promoter, for example, polyethylene

glycol (PEG) or Sendai virus (HVJ), preferably PEG is used.

Examples of myeloma cells include NS-1, P3U1, SP2/0, AP-1 and the like. The proportion of the number of antibody producer cells (spleen cells) and the number of myeloma cells to be used is preferably about 1:1 to about 20:1. PEG (preferably PEG 1000-PEG 6000) is preferably added in concentration of about 10% to about 80%. Cell fusion can be carried out efficiently by incubating a mixture of both cells at about 20°C to about 40°C, preferably about 30°C to about 37°C for about 1 minute to 10 minutes.

Various methods may be used for screening for a hybridoma producing the antibody (*e.g.*, against protein of the present invention). For example, where a supernatant of the hybridoma is added to a solid phase (*e.g.*, microplate) to which antibody is adsorbed directly or together with a carrier and then an anti-immunoglobulin antibody (if mouse cells are used in cell fusion, anti-mouse immunoglobulin antibody is used) or Protein A labeled with a radioactive substance or an enzyme is added to detect the monoclonal antibody against the protein bound to the solid phase. Alternately, a supernatant of the hybridoma is added to a solid phase to which an anti-immunoglobulin antibody or Protein A is adsorbed and then the protein labeled with a radioactive substance or an enzyme is added to detect the monoclonal antibody against the protein bound to the solid phase.

Selection of the monoclonal antibody can be carried out according to any known method or its modification. Normally, a medium for animal cells to which HAT (hypoxanthine, aminopterin, thymidine) are added is employed. Any selection and growth medium can be employed as long as the hybridoma can grow. For example, RPMI 1640 medium containing 1% to 20%, preferably 10% to 20% fetal bovine serum, GIT medium containing 1% to 10% fetal bovine serum, a serum free medium for cultivation of a hybridoma (SFM-101, Nissui Seiyaku) and the like can be used. Normally, the cultivation is carried out at 20°C to 40°C, preferably 37°C for about 5 days to 3 weeks, preferably 1 week to 2 weeks under about 5% CO_2 gas. The antibody titer of the supernatant of a hybridoma culture can be measured according to the same manner as described above with respect to the antibody titer of the anti-protein in the antiserum.

Separation and purification of a monoclonal antibody (*e.g.*, against a cancer marker of the present invention) can be carried out according to the same manner as those of conventional polyclonal antibodies such as separation and purification of immunoglobulins, for example, salting-out, alcoholic precipitation, isoelectric point

precipitation, electrophoresis, adsorption and desorption with ion exchangers (*e.g.*, DEAE), ultracentrifugation, gel filtration, or a specific purification method wherein only an antibody is collected with an active adsorbent such as an antigen-binding solid phase, Protein A or Protein G and dissociating the binding to obtain the antibody.

Polyclonal antibodies may be prepared by any known method or modifications of these methods including obtaining antibodies from patients. For example, in some embodiments, a complex of an antigen and a carrier protein is prepared and an animal is immunized by the complex according to the same manner as that described with respect to the above monoclonal antibody preparation. A material containing the antibody against the immunogen is recovered from the immunized animal and the antibody is separated and purified.

As to the complex of the immunogen and the carrier protein to be used for immunization of an animal, any carrier protein and any mixing proportion of the carrier and a hapten can be employed as long as an antibody against the hapten, which is crosslinked on the carrier and used for immunization, is produced efficiently. For example, bovine serum albumin, bovine cycloglobulin, keyhole limpet hemocyanin, etc. may be coupled to an hapten in a weight ratio of about 0.1 part to about 20 parts, preferably, about 1 part to about 5 parts per 1 part of the hapten.

In addition, various condensing agents can be used for coupling of a hapten and a carrier. For example, glutaraldehyde, carbodiimide, maleimide activated ester, activated ester reagents containing thiol group or dithiopyridyl group, and the like find use with the present invention. The condensation product as such or together with a suitable carrier or diluent is administered to a site of an animal that permits the antibody production. For enhancing the antibody production capability, an adjuvant, for example, complete or incomplete Freund's adjuvant may be administered. Normally, the protein is administered once every 2 weeks to 6 weeks, in total, about 3 times to about 10 times.

The polyclonal antibody is recovered from blood, egg yolk, ascites and the like, of an animal immunized by the above method. The antibody titer in the antiserum can be measured according to the same manner as that described above with respect to the supernatant of the hybridoma culture. Separation and purification of the antibody can be carried out according to the same separation and purification method of immunoglobulin as that described with respect to the above monoclonal antibody.

The polypeptide used herein as the immunogen is not limited to any particular

type of immunogen. For example, in some preferred embodiments, full length VCP or VCPΔD2 or immunogenic portions thereof can be used as the immunogen. Further, fragments of these proteins, methylated or unmethylated, may be used. Fragments may be obtained by any methods including, but not limited to expressing a fragment of the gene, enzymatic processing of the protein, chemical synthesis, and the like.

II. Drug Screening Applications

In some embodiments, the present invention provides drug screening assays (*e.g.*, to screen for anticancer agents). In some embodiments, drug screening assays screen for drugs that inhibit or modulate the methyltransferase activity of VCP-KMT. For example, in some embodiments, the present invention provides methods of screening for compounds that modulate (*e.g.*, inhibit, decrease, block or increase) the expression or activity of VCP-KMT. In some embodiments, candidate compounds are antibodies or small molecules that specifically bind to or interact with VCP-KMT, VCP or a regulator thereof to modulate or inhibit VCP-KMT biological function, e.g., reducing methyltransferase activity of VCP-KMT with respect to the K315 residue of VCP. The compounds or agents may interfere with transcription, by interacting, for example, with the promoter region. The compounds or agents may interfere with mRNA (*e.g.*, by RNA interference, antisense technologies, etc.). The compounds or agents may interfere with pathways that are upstream or downstream of the biological activity of VCP-KMT. In some embodiments, candidate compounds are antisense or interfering RNA agents (*e.g.*, oligonucleotides) directed against VCP-KMT.

In one screening method, candidate compounds are evaluated for their ability to alter VCP-KMT expression by contacting a compound with a cell expressing a VCP-KMT and then assaying for the effect of the candidate compounds on expression. In some embodiments, the effect of candidate compounds on expression of VCP-KMT is assayed for by detecting the level of VCP-KMT expressed by the cell. mRNA expression can be detected by any suitable method.

In some embodiments, the present invention provides assays for screening compounds for modulation of VCP-KMT methyltransferase activity comprising contacting a VCP lysine-specific MTase (VCP-KMT) enzyme with a VCP substrate and a test compound; and detecting the level of methyltransferase activity of said VCP-KMT enzyme in the presence and absence of said test compound. The VCP substrate is

preferably a VCP homolog or fragment thereof that is capable of being methylated, and preferably trimethylated, at a lysine residue corresponding to position K315 of wild type human VCP. For example, the VCP substrate may be a full-length, wild type VCP, a variant of VCP (e.g., a variant sharing at least 70%, 80%, 90%, 95%, 97%, 98% or 99% sequence identity with human, mouse or rat VCP), or a fragment, such as a truncation mutant, of human, mouse or rat VCP (e.g., a VCP truncation mutant lacking the D2 ATPase domain, wherein the truncated mutant shares at least 70%, 80%, 90%, 95%, 97%, 98% or 99% sequence identity with the corresponding portion of human, mouse or rat VCP). In some preferred embodiments, the VCP substrate is non-methylated prior to contact with the VCP-KMT enzyme in assays of the present ivnention and the extent of methylation of the previously non-methylated VCP substrate by VCP-KMT is detected.

In some embodiments, the candidate compounds are screened for their ability to modulate, and most preferably at least partially inhibit, VCP-KMT methyltransferase activity. In some embodiments, the effect on VCP-KMT methyltransferase activity is assayed by detecting methylation at the K315 residue of the substrate. The methylation at the K315 substrate can be compared to methylation in the absence of a test compound using the same assay conditions (i.e., comparison to a control or baseline level of K315 methylation). In some preferred embodiments, candidate compounds (e.g., libraries of compounds) are screened for their ability to inhibit VCP-KMT methyltransferase activity. Examples of inhibition include decreasing VCP-KMT methyltransferase activity by at least 10, 20, 30, 40, 50, 60, 70, 80, 90, or 95% as compared to a control reaction in the absence of the candidate compound. In some embodiments, assays utilize VCP-KMT, a VCP-KMT substrate (*e.g.*, VCP or a VCP variant (*e.g.*, VCPΔD2), a candidate compound or library of compounds and reagents for detecting methylated substrates.

In some particularly preferred embodiments, the assays of the present invention are used in high-throughput screening methods. The assays of the present invention can be provided in a multiplate format suitable for high-throughput screening of modulators of VCP-KMT activity. High-throughput formats include, but are not limited to, multi-well plates, capillary systems, beads, and flow cytometry, and in particularly preferred embodiments are automated through the use of robotics.

In some embodiments, libraries of synthetic compounds or tissue extracts are screened for their ability to modulate VCP-KMT methyltransferase activity. Candidate compounds may be obtained from any suitable source. The test compounds of the present

invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone, which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, *e.g.*, Zuckennann *et al.,* J. Med. Chem. 37: 2678-85 [1994]); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are preferred for use with peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam (1997) Anticancer Drug Des. 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.,* Proc. Natl. Acad. Sci. U.S.A. 90:6909 [1993]; Erb *et al.,* Proc. Nad. Acad. Sci. USA 91:11422 [1994]; Zuckermann *et al.,* J. Med. Chem. 37:2678 [1994]; Cho *et al.,* Science 261:1303 [1993]; Carrell *et al.,* Angew. Chem. Int. Ed. Engl. 33.2059 [1994]; Carell *et al.,* Angew. Chem. Int. Ed. Engl. 33:2061 [1994]; and Gallop *et al.,* J. Med. Chem. 37:1233 [1994].

Libraries of compounds may be presented in solution (*e.g.*, Houghten, Biotechniques 13:412-421 [1992]), or on beads (Lam, Nature 354:82-84 [1991]), chips (Fodor, Nature 364:555-556 [1993]), bacteria or spores (U.S. Patent No. 5,223,409; herein incorporated by reference), plasmids (Cull *et al.,* Proc. Nad. Acad. Sci. USA 89:18651869 [1992]) or on phage (Scott and Smith, Science 249:386-390 [1990]; Devlin Science 249:404-406 [1990]; Cwirla *et al.,* Proc. Natl. Acad. Sci. 87:6378-6382 [1990]; Felici, J. Mol. Biol. 222:301 [1991]).

In some embodiments, assays for modulation of VCP-KMT activity utilize an antigen binding reagent (*e.g.*, antibodies as described above) that specifically binds to methylated VCP or VCPΔD2, and in particularly preferred embodiments VCP or VCPΔD2 that is trimethylated at position K315. A variety of methods are known in the art for detecting antigen binding reagent binding to a target molecule such as VCP or VCPΔD2 that is methylated, preferably trimethylated at position K315, including, but not limited to, Western blotting, protein detection chips, bead-based assays, lateral flow devices, and enzyme-linked immunosorbent assays (ELISAs).

In some embodiments, the assays of the present invention use a detectably labeled

antigen binding reagent. In some embodiments, the VCP antigen binding reagent is labeled with a detectable label. The detectable label may be either directly or indirectly detectable. In further embodiments, a primary VCP antigen binding reagent is detected by indirect detection via use of a secondary antibody, such as an antimouse, antirabbit, or antihuman secondary antibody. The secondary antibody may preferably comprise a detectable label that can be directly or indirectly detected.

Suitable labels for direct detection of the VCP antigen binding reagent or second antibody include enzymatic labels, radiolabels, luminescent labels, and fluorescent labels, which may be directly incorporated into or conjugated to the antigen binding reagent or antibody.

In some embodiments, the detectable label may be an enzymatic label, and the assay may be an enzyme-linked immunosorbent assays (ELISA), as is well known in the art. Suitable enzymatic labels include, but are not limited to, horseradish peroxidase, alkaline phosphatase, acid phosphatase, glucose oxidase, β-galactosidase, β-glucuronidase or β-lactamase. Where the detectable label includes an enzyme, a chromogen, fluorogenic compound, or luminogenic compound can be used in combination with the enzyme to generate a detectable signal (numerous of such compounds are commercially available, for example, from Invitrogen Corporation, Eugene Oreg.). Particular examples of chromogenic compounds include diaminobenzidine (DAB), 4-nitrophenylphospate (pNPP), fast red, bromochloroindolyl phosphate (BCIP), nitro blue tetrazolium (NBT), BCIP/NBT, fast red, AP Orange, AP blue, tetramethylbenzidine (TMB), 2,2'-azino-di-[3-ethylbenzothiazoline sulphonate] (ABTS), o-dianisidine, 4-chloronaphthol (4-CN), nitrophenyl-β-D-galactopyranoside (ONPG), o-phenylenediamine (OPD), 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal), methylumbelliferyl-.beta.-D-galactopyranoside (MU-Gal), p-nitrophenyl-α-D-galactopyranoside (PNP), 5-bromo-4-chloro-3-indolyl-.beta.-D-glucuronide (X-Gluc), 3-amino-9-ethyl carbazol (AEC), fuchsin, iodonitrotetrazolium (INT), tetrazolium blue and tetrazolium violet.

In some embodiments, the detectable label may be a radiolabel, and the assay termed a radioimmunoassay (RIA), as is well known in the art. The radioisotope can be detected by a gamma counter, a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are ^{125}I, ^{131}I, ^{35}S, ^{3}H and ^{14}C.

In some embodiments, the detectable label is a fluorophore or fluorochrome.

When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence of the fluorophore. Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Invitrogen, e.g., see, The Handbook--A Guide to Fluorescent Probes and Labeling Technologies, Invitrogen Detection Technologies, Molecular Probes, Eugene, Oreg.). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule or protein such as an antigen binding molecule include, but are not limited to, 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, anthranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumaran 151); cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5',5''-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC (XRITC); 2',7'-difluorofluorescein (OREGON GREEN™); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron™ Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of

sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 nm (Heyduk and Heyduk, Analyt. Biochem. 248:216-27, 1997; J. Biol. Chem. 274:3315-22, 1999), as well as GFP, Lissamine.TM., diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No. 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Invitrogen Detection Technologies, Molecular Probes (Eugene, Oreg.) and including the ALEXA FLUOR™ series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6,130,101 and 6,716,979), the BODIPY series of dyes (dipyrrometheneboron difluoride dyes, for example as described in U.S. Pat. Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOT™ (obtained, for example, from QuantumDot Corp, Invitrogen Nanocrystal Technologies, Eugene, Oreg.; see also, U.S. Pat. Nos. 6,815,064, 6,682,596 and 6,649,138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the bandgap of the semiconductor material used in the semiconductor nanocrystal. This emission can be detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can be coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et. al. (1998) Science 281:2013-6, Chan et al. (1998) Science 281:2016-8, and U.S. Pat. No. 6,274,323.

Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927,069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No. 2003/0165951 as well as PCT

Publication No. 99/26299 (published May 27, 1999). Separate populations of semiconductor nanocrystals can be produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can be produced that emit light of different colors based on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 nm, 655 nm, 705 nm, or 800 nm emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Invitrogen.

In some embodiments, the detectable label is a fluorescent protein. Fluorescent proteins also can be used as a carrier, or can be coupled to a carrier, to facilitate visualization. For example, green fluorescent protein (GFP) was originally isolated from the light-emitting organ of the jellyfish *Aequorea victoria*. Chimeric GFP fusions can be expressed in situ by gene transfer into cells, and can be localized to particular sites within the cell by appropriate targeting signals. Spectral variants with blue, cyan and yellowish-green emissions have been successfully generated from the Aequorea GFP, but none exhibit emission maxima longer than 529 nm. GFP-like proteins have been isolated from Anthozoa (coral animals) that significantly expanded the range of colors available for biological applications. The family of `GFP-like proteins` deposited in sequence databases now includes approximately 30 significantly different members. Fluorescent proteins refers to proteins that can become spontaneously fluorescent through the autocatalytic synthesis of a chromophore. Proteins that fluoresce at red or far-red wavelengths (red fluorescent proteins or RFPs) are known. RFPs can be used in combination with other fluorescent proteins that fluoresce at shorter wavelengths for both multicolor labeling and fluorescence resonance energy transfer (FRET) experiments. Commercially available RFPs are derived from two wild-type GFP-like proteins. DsRed (drFP583) has excitation and emission maxima at 558 nm and 583 nm, respectively. A far-red fluorescent protein was generated by mutagenesis of a chromoprotein that absorbs at 571 nm. HcRed1 (Clontech) has excitation and emission maxima at 588 nm and 618 nm, respectively. The fluorescent protein that emits fluorescence at the longest wavelength (without any mutations being introduced) is eqFP611, cloned from the sea anemone *Entacmaea quadricolor*. This protein absorbs at 559 nm and emits at 611 nm.

In some embodiments, the detectable label is a chemiluminescent compound. The presence of a chemiluminescent-tagged antibody or antigen is then determined by detecting the luminescence that arises during the course of a chemical reaction. Examples

of useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound such as a bioluminescent protein may be used to label the antibody reagent useful in the present invention. Binding is measured by detecting the luminescence. Useful bioluminescent compounds include luciferin and luciferase.

In some embodiments, the detectable label is indirectly detected. In some preferred embodiments, the detectable label is preferably a hapten which may be subsequently detected by a detectably labeled anti-hapten antibody. The detectable labels may be the same as described above. Suitable haptens include, but are not limited to, pyrazoles, particularly nitropyrazoles; nitrophenyl compounds; benzofurazans; triterpenes; ureas and thioureas, particularly phenyl ureas, and even more particularly phenyl thioureas; rotenone and rotenone derivatives, also referred to herein as rotenoids; oxazole and thiazoles, particularly oxazole and thiazole sulfonamides; coumarin and coumarin derivatives; cyclolignans, exemplified by Podophyllotoxin and Podophyllotoxin derivatives; and combinations thereof. Specific examples of haptens include, but are not limited to, 2,4-Dintropheyl (DNP), Biotin, Fluorescein derivatives (FITC, TAMRA, Texas Red, etc.), Digoxygenin (DIG), 5-Nitro-3-pyrozolecarbamide (nitropyrazole, NP), 4,5,-Dimethoxy-2-nitrocinnamide (nitrocinnamide, NCA), 2-(3,4-Dimethoxyphenyl)-quinoline-4-carbamide (phenylquinolone, DPQ), 2,1,3-Benzoxadiazole-5-carbamide (benzofurazan, BF), 3-Hydroxy-2-quinoxalinecarbamide (hydroxyquinoxaline, HQ), 4-(Dimethylamino)azobenzene-4'-sulfonamide (DABSYL), Rotenone isoxazoline (Rot), (E)-2-(2-(2-oxo-2,3-dihydro-1H-benzo[b][1,4]diazepin-4-yl)phenozy)acetamide (benzodiazepine, BD), 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylic acid (coumarin 343, CDO), 2-Acetamido-4-methyl-5-thiazolesulfonamide (thiazolesulfonamide, TS), and p-Mehtoxyphenylpyrazopodophyllamide (Podo). These haptens and their use in probes are described in more detail in co-owned applications US Pat. Publ. Nos. 20080305497, 20080268462, and 20080057513, incorporated herein by reference in their entirety.

In embodiments where the detectable label is a hapten, a labeled antigen binding molecule that binds to the hapten is used in the assay. Examples of suitable antigen binding molecules include, but are not limited to, antibodies, immunoglobulins or immunoglobulin-like molecules (including by way of example and without limitation, IgA, IgD, IgE, IgG and IgM), antibody fragments such as F(ab')2 fragments, Fab'

fragments, Fab'-SH fragments and Fab fragments as are known in the art, recombinant antibody fragments (such as sFv fragments, dsFv fragments, bispecific sFv fragments, bispecific dsFv fragments, F(ab)'2 fragments, single chain Fv proteins ("scFv"), disulfide stabilized Fv proteins ("dsFv"), diabodies, and triabodies (as are known in the art), and camelid antibodies (see, for example, U.S. Pat. Nos. 6,015,695; 6,005,079-5,874,541; 5,840,526; 5,800,988; and 5,759,808).

In other embodiments, modulation of VCP-KMT activity is detected using other suitable methods. Methods for detecting methylation of substrates are known in the art. In some embodiments, the assays of the present invention include a labeled methyl donor. It is contemplated that in the absence of an inhibiting candidate compound VCP-KMT will catalyze transfer of the labeled methyl group from the labeled methyl donor to the VCP-KMT substrate. The labeled substrate can then be detected by any suitable means. Candidate inhibiting compounds are those compounds that cause a decrease in methylation of the VCP-KMT substrate as compared to methylation of the VCP-KMT substrate in the absence of the candidate compound. The present invention is not limited to the use of any particular labeled methyl donor. In some preferred embodiments, the labeled methyl donor comprises a radiolabeled methyl group. Examples include, but are not limited to, detection of a labeled methyl donor (*e.g.*, S-[methyl-^{14}C]-SAM, S-[methyl-^{3}H]-SAM, etc.).

Detection of the detectably labeled reagent or labeled methyl group according to the present invention may be accomplished by a scintillation counter, for example, if the detectable label or group is a radioactive particle emitter, or by a fluorimeter, for example, if the label is a fluorophore. In the case of an enzyme label, the detection is accomplished by colorimetry to measure the colored product produced by conversion of a chromogenic substrate by the enzyme. Detection may also be accomplished by visual comparison of the colored product of the enzymatic reaction in comparison with appropriate standards or controls.

III. Diagnostic and Screening Methods for VCP-KMT/Methylated VCP Biomarkers

As described above, embodiments of the present invention provide diagnostic and research methods that utilize the detection of VCP-KMT, VCP-KMT activity and/or VCP or methylation of VCP. For example, in some embodiments, diagnostic assays screen for

the presence of VCP-KMT activity associated with the presence of cancer (*e.g.*, metastatic cancer) or neurodegenerative disease (*e.g.*, IBMPFD) in a subject. In other embodiments, assays are cellular or *in vitro* and provide information useful in research (*e.g.*, cancer research or research of neurodegenerative disease). VCP-KMT activity or lack thereof can be determined using any suitable method (*e.g.*, those described above). In preferred embodiments, assays are preformed that detect the level methylation of VCP in a sample such as a test fluid or tissue sample, or that detect the level of VCP-KMT activity in a sample such as a fluid or tissue sample.

Accordingly, in some embodiments the present invention provides a biomarker for predicting, detecting, diagnosing or monitoring disease, e.g., cancer or neurodegenerative disease, in a human subject having a biomarker to determine a methylation level of VCP or activity of VCP-KMT, wherein a higher methylation level of VCP, particularly at position K315, or VCP-KMT activity, as compared to a control level is indicative of disease, e.g., cancer or neurodegenerative disease. In one aspect, the biological samples are selected from the group consisting of a tissue sample, a fecal sample, a cell homogenate, a blood sample, one or more biological fluids, or any combinations thereof. In some embodiments, the VCP methylation level or activity of VCP-KMT is determined by, for example, an assay as described in detail above using, for example, VCP antigen binding reagents specific for trimethylated VCP or VCP substrates for detection of VCP-KMT methyltransferase activity. In some embodiments, the assays utilize VCP antigen binding reagents in suitable assays such as immunohistochemistry assays, ELISAs, RIAs or other assays as are known in the art. In some embodiments, the immunohistochemistry assays utilize a detectably labeled VCP antigen binding reagent that specifically binds to the trimethylated form of VCP as described in detail above. In other embodiments, the immunohistochemistry assays utilize a second (or third, fourth, etc.) detectably labeled antibody in conjunction with the VCP antigen binding reagent.

The present invention provides a method for selecting a therapy for a patient diagnosed with cancer or a neurodegenerative disease by determining a methylation level of VCP or activity of VCP-KMT in a biological samples of the subject; and selecting the therapy based on the determination of the presence of increased VCP methylation at position K315 or increased VCP-KMT activity. The present invention also provides a method of performing a clinical trial to evaluate a candidate drug believed to be useful in treating a disease (e.g., cancer or neurodegenerative disease) associated with increased

VCP methylation at position K315 or increased VCP-KMT activity by a) determining the VCP methylation level or VCP-KMT activity level in a biological sample from a the subject, wherein a higher methylation level of VCP, particularly at position K315, or VCP-KMT activity, as compared to a control level is indicative of disease; b) administering a candidate drug to a first subset of the patients, and a placebo to a second subset of the patients; c) repeating step a) after the administration of the candidate drug or the placebo; and d) monitoring a change in the overall VCP methylation level or VCP-KMT activity as compared to the level in the second subset of patients, wherein a statistically significant reduction indicates that the candidate drug is useful in treating said disease state. Yet another embodiment of the invention is a method of using a pharmacodynamic (PD) biomarker for determining a pharmacological response to a treatment of early-onset of colorectal cancer, the method comprising: determining an overall VCP methylation or VCP-KMT activity level in one or more cells obtained from a first biological sample of a subject, wherein a higher methylation level of VCP, particularly at position K315, or VCP-KMT activity, as compared to a control level is indicative of disease; administering a drug to the subject at a first time, repeating the step of determining an overall VCP methylation or VCP-KMT activity level in one or more cells obtained from a second biological sample from the subject at a second time; and comparing the VCP methylation or VCP-KMT activity level at the first and the second time, wherein a statistically significant reduction in LINE VCP methylation or VCP-KMT activity level indicates that the drug is useful in treating said disease state.

IV. Compositions & Kits

In some embodiments, the present invention provides compositions and kits including all components necessary, sufficient or useful for detecting VCP-KMT enzyme activity (*e.g.*, reduced or increased activity). The kits described herein find use in research, therapeutic, screening, and clinical applications.

For example, in some embodiments, kits include VCP-KMT enzymes, substrates (*e.g.*, VCP or VCPΔD2), and reagents for detecting methylation of VCP-KMT substrates (*e.g.*, antibodies that specifically bind to methylated VCP-KMT substrates or labeled methyl donors). In some embodiments, kits further comprise additional reagents for performing assays (*e.g.*, controls, buffers, etc.), instructions, assay vessels, analysis software, etc.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Example 1

A. Methods

Constructs and protein purification

Plasmid 12373: pBD-0001, plasmid 12281: pBD-0005 [26], and plasmid 17229: pQE9-His-p97deltaD2 [27] were obtained from AddGene (URL: addgene.org). Human VCP-KMT (GenBank nucleotide core accession code NM_024558.2 coding for Q9H867-4) was amplified from IMAGE:5197291. Human METTL21A (GenBank nucleotide core accession code NM_001127395.1: 575C>T, rs2551949, coding for Q8WXB1 T192I) was cloned from HeLa cell cDNA. Human METTL21B (GenBank nucleotide core accession code NM_015433.2: rs923829, coding for Q96AZ1) and METTL21C (GenBank nucleotide core accession code NM_001010977.1 43G>C, rs2390760, coding for Q5VZV1 G15R) were cloned from A-704 cell cDNA. The indicated SNPs represent the major alleles in the population cohorts participating in the NHLBI Exome Sequencing project. A variant of human METTL23 (GenBank nucleotide core accession code BC045819.1 with an artificial start codon introduced, coding for AAH45819 Δ1-69, L70M) was cloned from A-704 cell cDNA. C. elegans C42C1.13 (GenBank nucleotide core accession code NM_001129287.1) was amplified from cDNA. S. cerevisiae YNL024c (GenBank nucleotide core accession code NM_001182863.1) was amplified from genomic DNA of strain BY4741. VCP-KMT was cloned into MCS1 of pETDuet-1 (Novagen), other amplificates were cloned into pET28a (Novagen). Site-directed mutagenesis was performed using Quikchange II site-directed mutagenesis kit (Stratagene). Proteins were produced using standard methods [28,29], variations are detailed below.

MTase assays

MTase reactions contained 10 µg of substrate, 100 pmol MTase, and 13 µM of

[^{3}H]-SAM (1.5 Ci mmol^{-1}) in 50 µl of 50 mM Tris-HCl, 50 mM KCl and 5 mM $MgCl_2$, unless otherwise indicated. The pH value was 7.5 at 37 °C for methylation of VCP or 8.5 at 37 °C for automethylation and methylation of pseudosubstrates. After 1 h at 37 °C, the reaction was terminated by adding ice-cold 10 % trichloroacetic acid followed by filtration on GF/C glass microfibre filters (Whatman). Incorporated radioactivity was measured by liquid scintillation counting using Ultima Gold XR (Perkin-Elmer). For MS analysis, [3H]-SAM was replaced with 1.2 mM unlabeled SAM. The reaction was stopped by addition of SDS-PAGE sample buffer and heat denaturation.

Analysis of VCP:VCP-KMT complex formation

Recombinant monomeric VCP-KMT, hexameric VCP and hexameric VCPΔD2 were isolated by SEC in 10 mM Tris pH 8.0, 100 mM NaCl and 1 mM dithiothreitol (DTT) on a Superdex 200 10/300 GL column (GE Healthcare). Following concentration, VCP and VCPΔD2 were mixed with a two-fold molar excess of VCP-KMT, incubated for 30 min at 4°C and then rerun over the Superdex 200 column.

Sample preparation, MS data acquisition and MS data analysis

Protein samples to be analyzed by MS were processed and analyzed by nanoflow on-line liquid chromatographic MS analysis as described [30], using either trypsin (Sigma-Aldrich), chymotrypsin (Roche Applied Science), or Arg-C (Roche Applied Science). Mass spectrometric data were analyzed with the in-house maintained human proteome and human VCP single protein database using SEQUEST™. The mass tolerances of fragment and parent ions were set to 0.05 Da & 7 ppm for HCD, and 0.5 Da & 5 ppm for CID. The following variable (methionine oxidation, Kme1, Kme2, Kme3, K-Ac, Rme1, Rme2, R-Ac, Hme1, Nme1, and Qme1) or fixed (cysteine carbamidomethylation) modifications were selected for analysis. Specific MS^2 spectra were manually inspected with Qual Browser version 2.0.7. Relative quantification of peptides with different methylation states was performed using MaxQuant [31]. Unmethylated peptide was normalized to the sample without enzyme addition and reaction products were normalized to the sum of all reaction products at the given enzyme concentration.

Cell culture and generation of VCP-KMT-deficient cell lines

HeLa, U87-MG and 293 T-REx Flp-In cells (Invitrogen) were cultivated in DMEM high glucose medium (Lonza) supplemented with 10 % FBS, Glutamax I (Invitrogen) and penicillin/streptomycin. The cells were transfected with mRNA encoding a zinc finger nuclease pair targeting exon 1 of human VCP-KMT (Sigma-Aldrich) using Trans-IT mRNA transfection kit (Mirus BIO) and subsequently cloned by limiting dilution. Genomic DNA was prepared and a 789 bp fragment containing the ZFN target site was PCR amplified with primers TGCTACGACTACAGCAGTATAGCTC (SEQ ID NO:27) and TGGTCCCTGGTGACTTTTATCTTC (SEQ ID NO:28). The wild-type sequence targeted by the ZFN pair constitutes a BlpI-cleavage site, enabling primary screening by BlpI-digestion of the PCR product. Uncut PCR product was sequenced to verify the genome editing and select clones with frameshift mutations.

Western blotting and immunoprecipitation

Adherent cells and mouse tissues were lysed in buffer containing 1 % IGEPAL CA-630. A *S. cerevisiae* strain BY4741 cell lysate was prepared with a MicroBeadBeater. Immunoprecipitation of VCP or CDC48 was performed with mouse anti-VCP [5] (Abcam) as described [32]. For western blotting, mouse anti-VCP [5] (Abcam) and mouse anti-GAPDH (Ambion) were used according to manufacturer's suggestions. Rabbit polyclonal anti-VCP trimethylated at lysine 315 (custom methylation-specific antibody to the modification site identified herein; the custom peptide synthesis and immunization was performed by New England Peptide) was used at 1:500 to detect trimethylated VCP in cell lysates.

Expression and purification of recombinant proteins

BL21(DE3) RIPL Codon+ E. coli cells (Stratagene) transformed with pETDuet-1 or pET28a constructs, and TOP10F' cells (Invitrogen) transformed with pQE9 constructs were grown and induced as described[31]. The bacteria were lysed by freeze-thawing and sonication in lysis buffer (50 mM sodium phosphate buffer pH 7.5, 300 mM NaCl, 5 % (v/v) glycerol, 0.5 % IGEPAL CA-630, 3 mM imidazole, 3 mM 2–mercaptoethanol, 1x complete EDTA-free protease inhibitors (Roche Applied Science)) and recombinant methyltransferases with N-terminal hexahistidine tag were purified using TALON immobilized metal affinity chromatography resin (Clontech) according to manufacturer's instructions and buffer exchanged to 20 mM Tris pH 7.3 at 4 °C, 100 mM NaCl, 1 mM

DTT over a Vivaspin 20 ultrafiltration unit (MWCO 10,000) (Sartorius-Stedim). In case of human VCP-KMT, the product was further purified by size exclusion chromatography on Superdex 200 10/300 GL (GE healthcare) in SEC buffer (10 mM Tris pH 8.0 at 4 °C, 100 mM NaCl and 1 mM DTT).

VCP and CDC48 variants were purified as described above with the following modifications: The culture volume was 200 ml and expression induced with 500 µM IPTG. Cells were lysed as described[32] and recombinant proteins with N-terminal hexahistidine tag were bound on 5 ml HisTrap immobilized metal affinity chromatography columns (GE Healthcare) and eluted with a linear gradient from 30 to 250 mM imidazole. Fractions containing the recombinant protein were concentrated and buffer exchanged to 50 mM Tris pH 7.5 at 4 °C, 500 mM NaCl, 5 % (v/v) glycerol and 1 mM DTT over a Vivaspin 20 ultrafiltration unit (MWCO 30,000) (Sartorius-Stedim). For complex formation, VCP and VCPΔD2 were further purified by size exclusion chromatography on a Superdex 200 10/300 GL column (GE healthcare) in SEC buffer and hexameric protein was isolated.

Invasion, migration and proliferation assays

Cells were analyzed in transwell chambers with or without Matrigel coating to distinguish between invasion and migration, respectively, as described previously [33]. The lower chamber contained 10% FBS to attract the cells that were plated in medium containing 1% FBS. The assay was analyzed after 24 h. Cell proliferation assays were performed with the Vybrant MTT cell proliferation assay kit (Invitrogen) according to supplier's instructions. Dilutions of each cell line, ranging from 1,000 to 5,000 cells per well, were plated in triplicates in 96 well plates and the cells were allowed to settle for 15 min at 37 °C (t = 0 h). 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay reagent was added to the cultures at the indicated time points and formazan formation was allowed to continue for three hours. An exponential growth function $y = A \times 2(t/\tau)$ was fitted to the data. Fits with an adjusted R2 value >0.95 were used to derive the doubling time τ for each cell line.

Immunofluorescence microscopy

Cells were fixed for 30 min at 21 °C with 4% formaldehyde in phosphate buffered saline (PBS), and permeabilized for 3 min with 0.1% Triton X-100 in PBS. Cells

were stained with antibody [5] to VCP (Abcam) and Alexa Fluor 488 goat anti-mouse immunoglobulin G (Invitrogen) antibodies diluted in PBS containing 1% bovine serum albumin. Confocal images spaced by 0.5 µm were taken with an Olympus FluoView 1000 confocal laser scanning microscope using a PLAPON 60xO NA 1.42 objective. Average intensity z-projections were generated with ImageJ.

Preparation of S100 cytosolic extract

A detergent-free cytosolic extract was prepared using hypotonic swelling, cell lysis using a Dounce-homogenizer and high speed centrifugation as described [34].

Partial purification of endogenous VCP from human cell lines

HeLa wt or VCP-KMT-deficient cells were grown to ca. 70% confluence and lysed with 0.5% IGEPAL CA-630 in 20 mM Bis-Tris, pH 7.0 at 11 °C, 100 mM NaCl, 2 mM DTT and 1x complete protease inhibitor cocktail (Roche Applied Science). The lysate from 10 dishes per cell line was combined and centrifuged for 1 h at 4,600 g. The cleared lysate was loaded on a RESOURCE Q 6 ml column (GE healthcare). After washing with 7.5 column volumes buffer A [20 mM Bis-Tris, pH 7.0 at 11 °C, 100 mM NaCl, 2 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.2 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)], bound proteins were eluted over 20 column volumes with a linear gradient from 0 to 50% Buffer B (20 mM Bis-Tris, pH 7.0 at 11 °C, 1 M NaCl, 2 mM DTT, 1 mM EDTA and 0.2 mM AEBSF). VCP containing fractions were identified by Western blotting. The majority of VCP eluted between 28 and 32% B. These fractions were combined and concentrated to approximately 75 µl over Vivaspin 20 ultrafiltration units (molecular weight cut off (MWCO) 100,000) (Sartorius- Stedim). The concentrate was applied to a Superose 6 10/300 GL column (GE healthcare) using 150 mM NaCl, 20 mM Tris pH 8.0 at 11 °C, 1 mM MgCl2, 2 mM DTT as running buffer and 1 ml fractions were collected. All steps were performed on ice or in a cool cabinet at 11 °C.

Statistical analysis

The independent two-sample Student's t-test (adjusted for unequal sample size for data shown in Fig. 5b) was used for hypothesis testing, since a Shapiro-Wilk normality test

showed no significant deviation from a normal distribution (alpha level 0.05) for all groups of interest, and equal variance of measurements can be assumed due to identical assay conditions. Tests were one-tailed for reduction of incorporation due to addition of inhibitor or mutation of key residues (Fig. 6b) and two-tailed in all other cases. The comparisons of interest were between means of groups and the alpha level was 0.05.

Results

Bioinformatics analysis of VCP-KMT

Human VCP-KMT, also called METTL21D, belongs to a small subfamily of four highly homologous (30-50 % sequence identity) uncharacterized putative methyltransferases, designated METTL21A-METTL21D. To identify orthologs of VCP-KMT, we used BLAST searches to detect proteins that were highly similar to VCP-KMT, but showed a substantially lower similarity to METTL21A, METTL21B and METTL21C. Plausible orthologs of VCP-KMT are found in a wide range of multicellular eukaryotes, but show a rather scattered distribution. Vertebrates, plants, nematodes and green algae all have a VCP-KMT ortholog, whereas such proteins are absent in the majority of fungi and insects, like in the common model organisms budding yeast (*S. cerevisiae*) and fruit fly (D. melanogaster). Class I MTases contain a Rossmann-fold-like bundle of seven β-strands and display four characteristic conserved sequence motifs denoted I, Post I, II and III [12]. A sequence alignment of putative VCP-KMT orthologs indicates the presence of the characteristic core motifs, and secondary structure prediction further indicates a topology of an archetypical Class I MTase core domain with an added N-terminal elongation harboring three short β-strands (Fig. 1a). This initial bioinformatics analysis consolidates VCP-KMT as a member of the seven β-strand MTase family, and indicates that this enzyme is present in a wide range of multicellular eukaryotes.

Protein MTase activity of VCP-KMT

When testing recombinant VCP-KMT for MTase activity, we were unable to detect any activity on DNA or RNA, but VCP-KMT displayed activity on recombinant histone proteins, in particular on H2B (Fig. 1b). To further analyze the protein MTase activity of VCP-KMT, we tested homopolymers of the L-amino acids lysine (K), arginine (R), histidine (H), asparagine (N), aspartate (D), and glutamate (E), which have all been

shown to be methylated in proteins. Notably, VCP-KMT displayed MTase activity on poly-(L)-K, but not on any of the other homopolymers (Fig. 1b). Protein MTase activity was also observed on a homopolymer of the D-stereoisomer of lysine (not naturally found in proteins), but not on a randomized copolymer of D-glutamate and D-lysine at a ratio of 3:2 (poly-(D)-E-K). No activity could be detected on protamine, which is a highly positively charged chromatin protein like H2B, but contains no lysines. Mass spectrometry (MS) analysis of histone H2B after incubation with VCP-KMT and SAM identified several methylated lysine residues. From the above experiments, we concluded that VCP-KMT is a lysine-specific protein MTase, but that the observed modification of multiple lysine residues within H2B is suggestive of non-specific activity.

VCP-KMT interacts with and methylates VCP in vitro

To identify substrates of VCP-KMT, a yeast-two-hybrid screen was commissioned (Hybrigenics, Paris, France), using human VCP-KMT as bait with a human placenta cDNA prey library. Interestingly, the majority of the interacting clones (111 of 169) encoded VCP, a much-studied protein which belongs to the family of AAA+ proteins. These clones defined the so-called selected interaction domain (SID), encompassing residues 282-364, as being responsible for the interaction (Fig. 2a,b).

We then tested whether VCP is a substrate for recombinant VCP-KMT in an MTase assay. The SID is located in the D1 ATPase domain and appears inaccessible in the VCP hexamer structure; therefore, a VCP deletion mutant (VCPΔD2) which lacks the D2 ATPase domain and forms a less stable hexamer [13, 14] was also tested (Fig. 2b). Indeed, both VCP and VCPΔD2 were methylated by VCP-KMT *in vitro*, but considerably higher levels of methylation were obtained in the case of VCPΔD2 (Fig. 2c). The activity on VCP/VCPΔD2 was much stronger than that on histone H2B and poly-(L)-lysine; the VCP-KMT concentration required to detect activity was ~300-fold lower and the obtained methylation levels up to ~40 fold higher. Based on the robust activity of VCP-KMT on VCP, we henceforth denote this enzyme as VCP lysine-specific MTase (VCP-KMT).

The observation that VCPΔD2 was a considerably better substrate than VCP suggested that VCP-KMT might be able to disrupt the hexameric structure of VCPΔD2 to exert its MTase activity, and that this could only occur efficiently with the less stable VCPΔD2 mutant. Indeed, incubation of the ~300 kDa homohexameric VCPΔD2 complex

with VCP-KMT led to the formation of heterodimeric VCPKMT: VCPΔD2 that eluted at ~80 kDa in size exclusion chromatography (SEC) (Fig. 2d) and the purified hexameric VCPΔD2 was an efficient substrate for VCP-KMT (Fig. 2e). The more stable full-length VCP hexamer was not disrupted by VCP-KMT (Fig. 2d), and while aggregated VCP contained in the VCP preparation could be methylated, no methylation of intact hexameric VCP was observed (Fig. 2e). In addition, no in vitro methylation of endogenous VCP in a cytosolic fraction from wild-type and VCP-KMT-deficient HeLa cells was detected (data not shown). This suggests that VCP-KMT mediated methylation of VCP occurs prior to hexamer assembly in vivo.

VCP-KMT methylates lysine 315 of VCP

To identify the site(s) of VCP-KMT-mediated methylation in VCP, in vitro methylated VCPΔD2 was analyzed by MS. Amongst peptides covering 88% of VCPΔD2, a single lysine residue, Lys315, was found to be trimethylated in the VCP-KMT-treated sample, but not in the untreated control sample, and no other methylated residues were detected (Fig. 2f). In agreement with these data, the VCP mutant K315L was not subject to METT21D-mediated methylation (Fig. 2g), strongly indicating that Lys315 is the only methylation site.

Substrate and enzyme requirements for VCP-KMT activity

Protein lysine MTases recognize their substrates either through interaction with a short linear target sequence or through binding to a more complex three-dimensional structure. To elucidate the mode of substrate recognition for VCP-KMT, we tested its activity on various VCP-derived substrates. No methylation was observed on glutathione S-transferase (GST)-fusions of two short (13 and 21 aa) peptides representing the methylation site and surrounding residues, regardless of whether the peptides were liberated from GST by site-specific proteolytic cleavage (data not shown). This indicates that a larger portion of VCP is required for productive interaction with VCP-KMT. When three residues at either side of the methylation site in VCPΔD2 were individually replaced by alanine, none of the six mutations affected VCP-KMT-mediated methylation, nor did a simultaneous replacement of all six residues (Fig. 2g). However, a deletion of the amino acid N-proximal to the methylation site (ΔE314) caused aberrant oligomerization and a fourfold reduction in methylation, while a corresponding C-proximal deletion (ΔT316)

had no effect (Fig. 2g). In addition to lysine, other large polar residues can be N-methylated, and we considered the possibility that such amino acids may be methylated by VCP-KMT when placed in an appropriate structural context. We therefore mutated Lys315 of VCPΔD2 into Arg or Gln, but similarly to the K315L variant, these mutants were not methylated (Fig. 2g) although they efficiently formed hexamers (data not shown). In conclusion, our data indicate that VCP-KMT specifically recognizes and methylates a lysine residue placed in a given three-dimensional context, whereas the local sequence surrounding the methylation site is less important for recognition.

To examine the processivity of the methylation reaction, VCPΔD2 was incubated with different amounts of VCP-KMT and the methylation status of Lys315 was analyzed by MS (Fig. 2h). While nearly all VCP was trimethylated at high VCP-KMT concentrations, we observed a mixture of all four lysine methylation states (Kme0, Kme1, Kme2 and Kme3) at intermediate concentrations. This suggests a non-processive reaction mechanism as has been described for DOT115.

Finally, we analyzed mutants of VCP-KMT to pinpoint residues critical to MTase activity (data not shown). A conserved Asp residue in motif Post I is among the most conserved residues in Class I MTases [16] and has been shown to play an important role in coordinating SAM [17]. Accordingly, mutations at this position in VCP-KMT (D96A, D96V) almost completely abolished enzymatic activity. Furthermore, the mutation D144V, which disrupts the (D/E)-X-X-(Y/F) motif present in VCP-KMT and its nine closest homologs (see below and Fig. 6a), also abolished methylation activity.

VCP-KMT-mediated trimethylation of K315 in VCP in vivo

We observed highly efficient and specific VCP-KMT-mediated methylation of K315 in VCP *in vitro*, indicating that the corresponding methylation may also occur *in vivo*. To address this, we isolated VCP by immunoprecipitation from protein extracts of mouse brain, heart, kidney, liver and testicle, and investigated the methylation status of K315 by MS analysis. In all three tissues, VCP was almost completely trimethylated, with only trace amounts of the dimethylated form present (Fig. 3a).

To analyze whether VCP-KMT is the enzyme responsible for methylation *in vivo*, we disrupted the endogenous VCP-KMT gene in three different human cell lines: HeLa (epithelial), U87-MG (glioma) and 293 T-REx Flp-In (kidney). A pair of zinc finger nucleases was designed to specifically target exon 1 of the VCP-KMT gene upstream of

motif I (aa 71-79) (Fig. 3b). When zinc finger nucleases cut a coding sequence, imperfect repair by non-homologous end-joining frequently leads to indel mutations, and a frameshift mutation at the targeted site in the VCP-KMT gene will lead to the loss of almost the entire MTase core domain. Clones harboring frameshift mutations in all VCP-KMT alleles were obtained for all three cell lines (Fig. 3b).

We next analyzed the methylation status of VCP from wild-type and VCP-KMT-deficient cells. Similarly to the observations from mouse tissues, VCP was almost completely trimethylated in all three wild-type cell lines, while no methylation was detected in VCP-KMT-deficient cells (Fig. 3c). The methylation status was further confirmed by immunoprecipitation of VCP followed by immunoblotting with pan-Kme3 antibody (Fig. 3d). Taken together, these results show that VCP is almost completely trimethylated at position K315 in a variety of mammalian cell lines and tissues, and that VCP-KMT is the responsible enzyme.

Organismal distribution of VCP methylation

VCP appears to be present in all eukaryotes, whereas VCP-KMT shows a far more limited distribution. For example, the yeast *S. cerevisiae* has an apparent homolog of the METTL21 proteins (YNL024c), but this protein is more similar to METTL21A than to VCP-KMT. Indeed, recombinant YNL024c failed to show MTase activity on human VCPΔD2 or on the corresponding yeast protein CDC48ΔD2, whereas human VCP-KMT was active on both these substrates (Fig. 4a). Moreover, the peptide covering position K325 (corresponding to K315 in human VCP) was exclusively found in a non-methylated state in CDC48 isolated from yeast cells (Fig. 4b). To address if a putative VCP-KMT ortholog from an organism distantly related to humans also displayed VCP-specific MTase activity, we investigated protein C42C1.13 from the nematode *C. elegans*. Indeed, this protein showed even higher activity than human VCP-KMT on both human VCPΔD2 and yeast CDC48ΔD2 (Fig. 4c). Taken together, the absence of VCP methylation in *S. cerevisiae* and the observed activity of the *C. elegans* protein C42C1.13 on both yeast and human VCP indicate that VCP-methylation can occur in a wide range of eukaryotes but is absent in those that lack a VCP-KMT ortholog.

Functional importance of VCP methylation

Since VCP was non-methylated in VCP-KMT-deficient cells, we used these cells

to address the biological significance of VCP methylation. No appreciable difference between VCP-KMT-proficient and -deficient cells was detected in degradation assays using reporters of the ubiquitin proteosome system or when monitoring autophagy by LC3B processing (data not shown). A recent study reported that VCP-KMT, there denoted NVM-1, was upregulated in a number of metastatic tumors, and that it was required for efficient cell migration and invasion [11]. First, we performed proliferation assays where the various VCP-KMT-deficient cell lines were compared to their wild-type counterparts. These experiments showed a significantly increased doubling time for VCP-KMT-deficient HeLa and 293 T-REx Flp-In cells, but not for the slower growing U87-MG cells (Fig. 5a,b).

We also examined whether the VCP-KMT-deficient cells may have reduced migratory or invasive capacity. A Boyden Chamber assay with uncoated and Matrigel-coated membranes allowed us to distinguish between migration and invasion, respectively (Fig. 5c). Wild-type U87-MG cells showed significantly higher migration and invasion potential than VCP-KMT-deficient U87-MG cells. Wild-type and VCP-KMT-deficient HeLa cells showed similar migratory capacity (Fig. 5c) and no invasive potential. 293 T-REx Flp-In cells were not analyzed due to their limited migration capability.

The growth-related differences between wild-type and VCP-KMT-deficient cells could conceivably be caused by changes in the stability or biochemical properties of VCP. Therefore, we first examined the amount, complex size and subcellular distribution of VCP by Western blotting and immunofluorescence staining, but we did not detect any differences between wild-type and VCP-KMT-deficient cells (Fig. 5d,e,f). Furthermore, we investigated the possible effect of methylation on the ATPase activity of VCP (Fig. 5g). To this end, we used anion exchange chromatography followed by SEC to isolate VCP-complexes from HeLa cells. VCP was the predominant protein in the complex containing fractions, but a co-purifying complex consisting of the Valyl-tRNA synthetase (VARS) and the β, γ and δ subunits of translation elongation factor 1 (EF1) [18] was also detected (Fig. 5h). The complex-containing fractions displayed an expected ATPase activity which showed an elution profile similar to that of VCP, whereas the VARS/EF1 complex eluted somewhat earlier, indicating that the measured activity primarily is due to VCP. However, no appreciable difference in ATPase activity of samples from VCP-KMT-proficient and -deficient HeLa cells was detected, indicating that methylation does not substantially influence the basal ATPase activity of VCP in vitro.

VCP-KMT belongs to a family of novel protein MTases

VCP-KMT belongs to Family 16 of MTases (InterPro: IPR019410; Pfam: PF10294), which has ten human members, and we performed a more detailed bioinformatics analysis of this MTase family. The ten human members form a closed group of proteins that invariably retrieve each other as best hits in BLAST searches, and orthologs of most of these proteins are found in a wide range of eukaryotic species (Table 1). Interestingly, one of these proteins, C2orf34, was recently shown to be the long-sought MTase responsible for trimethylation of Lys115 in calmodulin (CaM-KMT) [4], whereas YIL110W, the closest yeast homolog of human Family 16 member C1orf156, was recently shown to be required for monomethylation of the ribosomal protein Rpl3 on His243 [19]. Notably, CaM-KMT and C1orf156 are among the human Family 16 proteins that display the lowest sequence similarity to VCP-KMT (Table 1).

VCP-KMT orthologs contain a conserved DXXY motif located immediately c-terminal to Motif II (Fig. 1a). A more general expansion of this motif, (D/E)XX(Y/F), is present in all ten human Family 16 members (Fig. 6a), and in their orthologs from other organisms (not shown). A similar, so-called DPPY motif, with consensus (D/N/S)PP(Y/F), is found at the corresponding position in DNA N^6-adenine MTases, DNA N^4-cytosine MTases, and the protein glutamine MTase HemK [20]. Here, the first residue plays an important role in methyl transfer, and mutation at this position leads to a catalytically inactive enzyme. We observed that mutation of the corresponding residue Asp144 in VCP-KMT inactivated the enzyme (data not shown), suggesting that the (D/E)XX(Y/F) motif in the Family 16 proteins may play a similar role as the DPPY motif.

Five out of the ten human Family 16 proteins, VCP-KMT, METTL21A, METTL21B, METTL21C and METTL23, form the distinct subfamily PTHR14614 in the PANTHER database21. To address whether these closest paralogs of VCP-KMT are also protein MTases, in vitro methylation experiments were performed. Unlike VCP-KMT, METTL21A–C and METTL23 failed to show MTase activity towards histones, amino acid homopolymers, or VCP. Some protein MTases display automethylation activity, and with an amino acid specificity identical to that observed for bona fide substrates [22-24]. Indeed, we detected significant automethylation in the case of METTL21A, METTL21C and VCP-KMT (Fig. 6b). The observed automethylation activity was sensitive to the

competitive inhibitor S-adenosyl homocysteine (SAH) when added at the beginning, but not at the end of the incubation period, indicating that the observed activity reflects bona fide methylation, rather than non-covalent association of [^{3}H]-SAM with the enzymes' SAM-binding sites. The automethylation activity of VCPKMT, METTL21A and METTL21C was abrogated by mutations (D96A, D94A and D141A, respectively) of the conserved SAM-binding Asp residue in Motif Post 1, which in the case of VCP-KMT abrogated VCP methylation. The level of automethylation was particularly high in the case of METTL21C, where substantial (~50%) monomethylation at a specific lysine residue, Lys35, was detected by MS (Fig. 6c,d). Our observed protein MTase activity of METTL21A, METTL21C and VCPKMT, taken together with CaM-KMT and C1orf156 already being implicated in protein methylation, clearly suggests that all ten human Family 16 members are protein MTases.

Table 1. Human Family 16 MTases and their putative orthologs

Hs protein	Alt name(s)	% id. to VCP-KMT	Putative orthologs (expect value[b])				Reported activity
			D. melan.	*C. elegans*	*A. thaliana*	*S. cerev.*	
VCP-KMT*	METTL21D C14orf138 NVM-1	100.0		C42C1.13* (3e-23)	AT1G08125 (6e-24)		VCP[c] (K315me3)
METTL21A*	FAM119A HCA557b	38.0			AT1G73320 (4e-20)	YNL024C (1e-15)	Auto[c]
METTL21B	FAM119B HCA557a	33.9					
METTL21C*	C13orf39	29.6					Auto[c] (K35me1)
METTL23	C17orf95	26.7	CG5013 (1e-43)	W06B4.2 (1e-11)	AT1G63855 (9e-22)		
FAM86[a]		24.9	CG7889 (2e-14)	R08D7.4 (2e-9)	At5g27400 (3E-15)	YJR129C (5e-8)	
METTL22	C16orf68	23.4	CG10584 (2e-23)				
CaM-KMT*	C2orf34	22.4	CG10947 (2e-44)	Y48E1C.1 (5e-16)	AT4G35987* (2e-31)		CaM[d] (K115me3)
METTL20	C12orf72	20.1		C37A2.6 (3e-36)			
METTL18	C1orf156 AsTP2	16.0	CG17219 (6e-40)	K01A11.2 (5e-27)	AT4G14060 (5e-27)	YHL110W* (8e-13)	Rpl3[e] (H243me1)

*Experimentally characterized proteins; [a]Four highly similar proteins: FAM86A, FAM86 B1, FAM86B2 and FAM86C; [b]In BLAST searches, using human ortholog as query; [c]Present study; [d]Ref. 4; [e]Ref. 19.

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All publications, patents, patent applications and accession numbers mentioned in the above specification are herein incorporated by reference in their entirety. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications and variations of the described compositions and methods of the invention will be apparent to those of ordinary skill in the art and are intended to be within the scope of the following claims.

CLAIMS

We claim:

1. A method of screening compounds for modulation of methyltransferase activity of a VCP lysine-specific MTase (VCP-KMT) enzyme, comprising:

(a) contacting said VCP lysine-specific MTase (VCP-KMT) enzyme with a VCP substrate and a test compound; and

(b) detecting the level of methylation of said VCP substrate in the presence and absence of said test compound, wherein the level of methylation of said VCP substrate is indicative of methyltransferase activity of said VCP-KMT enzyme.

2. The method of claim 1, wherein said VCP-KMT enzyme is human VCP-KMT.

3. The method of claim 1, wherein said VCP-KMT enzyme is *C. elegans* C42C1.13.

4. The method of claim 1, wherein said VCP-KMT enzyme is an ortholog of human VCP-KMT.

5. The method of claim 1, wherein said VCP substrate is selected from the group consisting of VCP and VCP variants.

6. The method of claim 5, wherein said VCP variant is VCPΔD2.

7. The method of claims 1 to 6, wherein said test compound is a drug.

8. The method of claim 7, wherein said drug is a cancer drug.

9. The method of claims 1 to 8, wherein said detecting further comprises providing an antigen binding protein that specifically binds to a methylated VCP substrate but not a non-methylated VCP substrate and exposing said VCP substrate to said aantigen binding protein to detect methylated VCP substrate.

10. The method of claim 9, wherein said methylated substrate is VCP or VCPΔD2 trimethylated at lysine residue K315.

11. The method of claims 1 to 8, wherein said detecting further comprises providing a labeled methyl donor and detecting VCP substrate comprising a labeled methyl group donated from said labeled methyl donor.

12. The method of claim 11, wherein said labeled methyl donor is selected from the group consisting of S-[methyl-^{14}C]-SAM and S-[methyl-^{3}H]-SAM.

13. The method of claims 1 to 12, further comprising screening a library of test compounds.

14. The method of claim 13, further comprising selecting test compounds from said library that modulate methyltransferase activity of said VCP-KMT enzyme.

15. The method of Claim 14, further comprising clinically testing at least one selected test compound.

16. The method of Claim 13, further comprising synthesizing a lead compound utilizing at least one selected test compound as a template.

17. The method of Claim 15, further comprising clinically testing said lead compound.

18. The method of Claim 12 or 13, further comprising providing said test compound or said lead compound for administration to a subject.

19. A test compound or lead compound identified by the method of any of Claims 1 to 18.

20. Use of a VCP lysine-specific MTase (VCP-KMT) enzyme and a VCP substrate in

an assay for identification of one or more compounds that alter the methyltransferase activity of said VCP lysine-specific MTase (VCP-KMT) enzyme.

21. Use of a VCP substrate in an assay to identify one or more compounds that alter the methyltransferase activity of said VCP lysine-specific MTase (VCP-KMT) enzyme.

22. A kit, comprising:
a) a VCP-KMT enzyme;
b) a VCP-KMT substrate; and
c) reagents for detection of methylated VCP-KMT substrate.

23. The kit of claim 22, wherein said VCP-KMT enzyme is human VCP-KMT.

24. The kit of claim 22, wherein said VCP-KMT enzyme is *C. elegans* C42C1.13.

25. The kit of claim 22, wherein said VCP-KMT enzyme is an orthologue of human VCP-KMT.

26. The kit of claim 22, wherein said substrate is VCP or a VCP variant.

27. The kit of claim 26, wherein said VCP variant is VCPΔD2.

28. The kit of claim 22, wherein said reagents comprise an antibody that specifically binds to a methylated substrate but not a non-methylated substrate.

29. The kit of claim 22, wherein said methylated substrate is VCPΔD2 trimethylated at K315.

30. The kit of claim 22, wherein said reagents comprise a labeled methyl donor.

31. An antibody, scFV, or fragment thereof that specifically binds to a methylated VCP substrate but not a non-methylated VCP substrate.

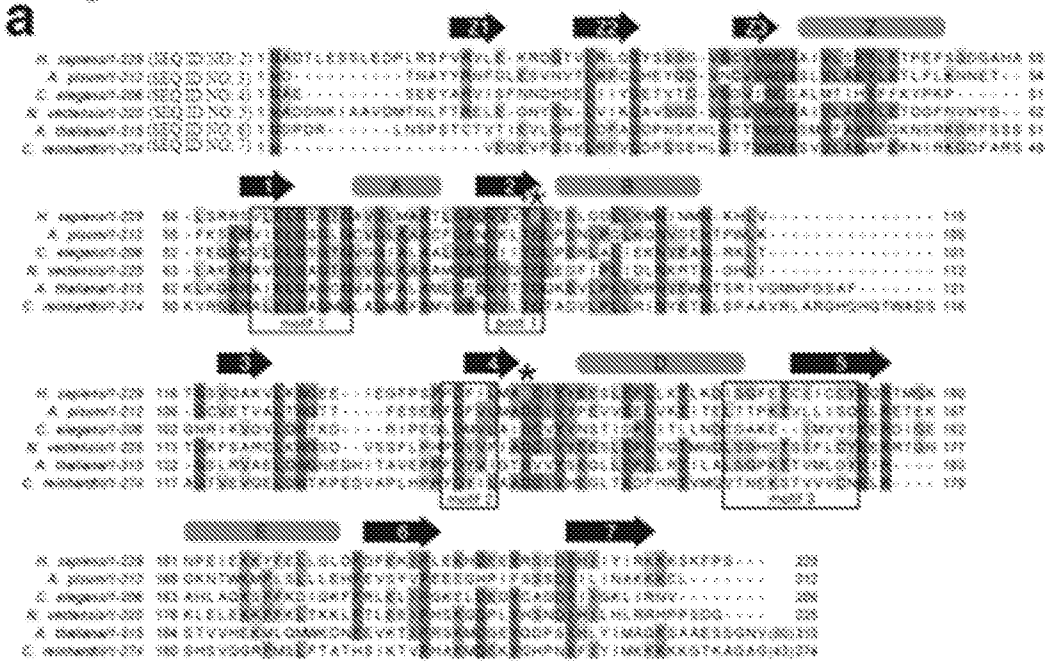
32. The antibody scFV, or fragment thereof of claim 31, wherein said methylated substrate is VCP or VCPΔD2 trimethylated at K315.

33. A prognostic or diagnostic method comprising contacting a patient sample with the antibody of claim 31 to determine the level of methylation of VCP.

34. The method of claim 33, further comprising correlating said level of methylation with a disease, condition, prognosis or outcome for said patient.

35. Use of VCP or VCPΔD2 methylated at K315 or a portion thereof comprising said methylated K315 residue for generating an antibody that specifically binds to methylated VCP or VCPΔD2.

Figure-1


a

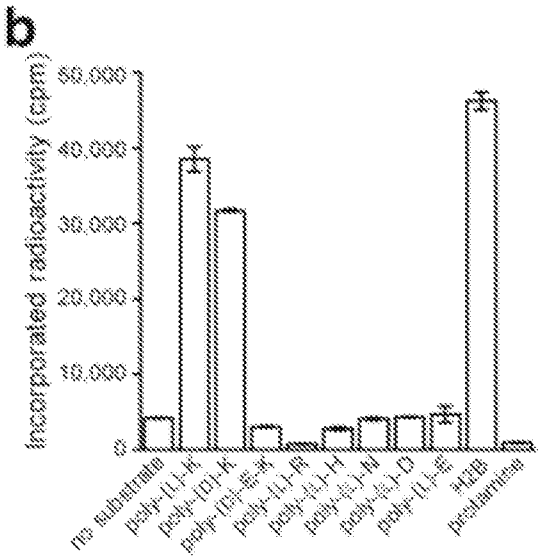

b
Incorporated radioactivity (cpm)
50.000
40.000
30.000
20.000
10.000
0
no substrate
poly-(L)-H
poly-(D)-K
poly-(D)-E,K
poly-(L)-R
poly-(L)-H
poly-E,L-H
poly-(L)-O
poly-(L)-E
HGB
proteinase

Figure-2

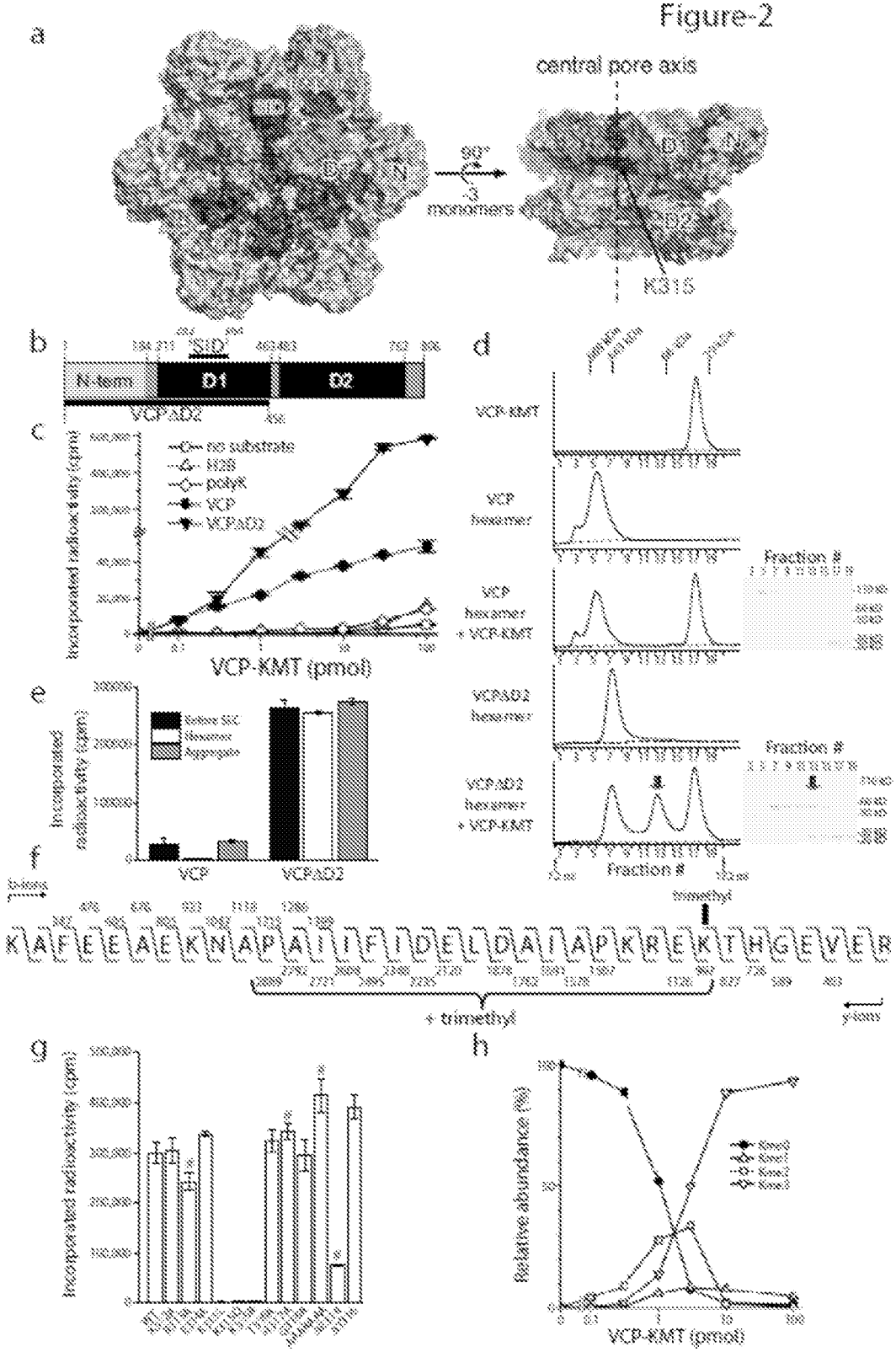

a
central pore axis
90°
3 monomers
D1
D2
N
KG15
b
N-term
D1
D2
SID
VCPAD2
c
no substrate
H2B
polyK
VCP
VCPAD2
incorporated radioactivity (cpm)
VCP-KMT (pmol)
d
VCP-KMT
VCP hexamer
VCP hexamer + VCP-KMT
VCPAD2 hexamer
VCPAD2 hexamer + VCP-KMT
Fraction #
e
incorporated radioactivity (cpm)
VCP
VCPAD2
f
trimethyl
+ trimethyl
g
incorporated radioactivity (cpm)
h
relative abundance (%)
VCP-KMT (pmol)

Figure-3

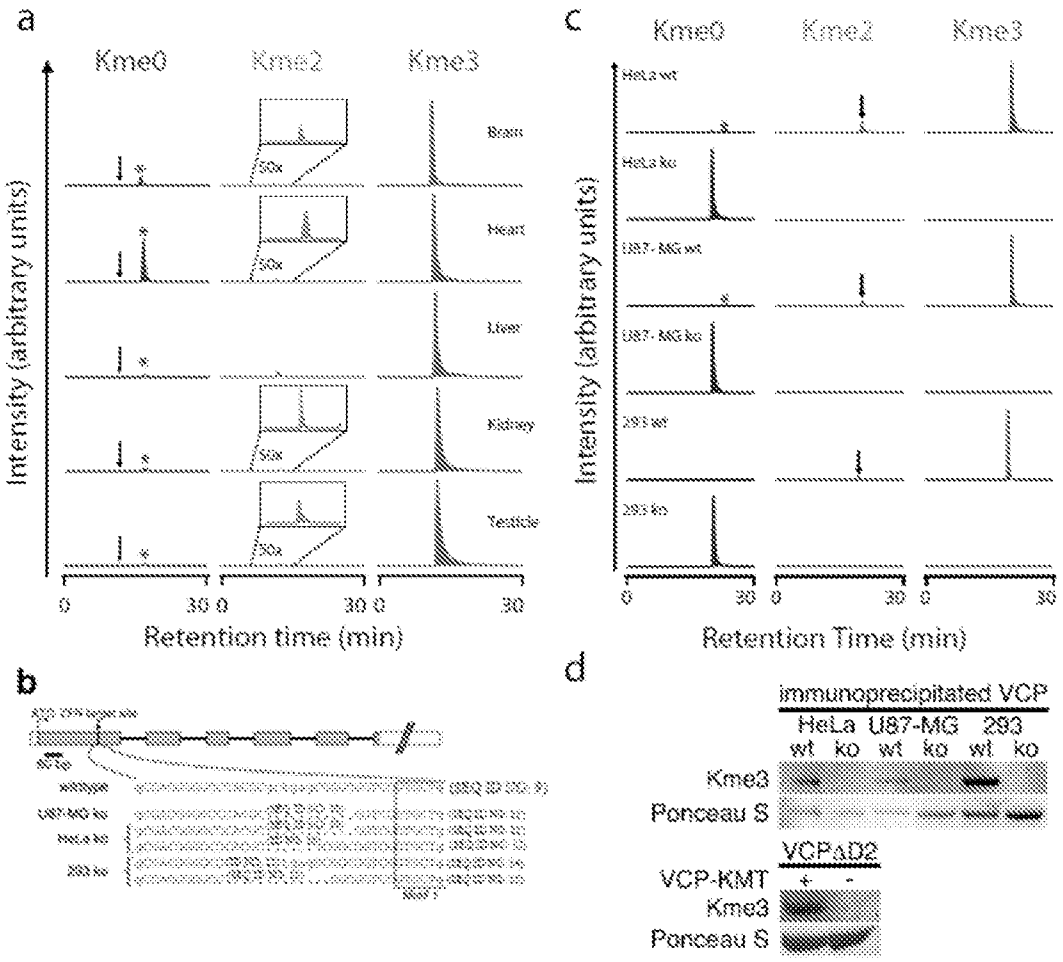

a
Kme0
Kme2
Kme3
Intensity (arbitrary units)
Brain
Heart
Liver
Kidney
Testicle
50x
0
30
Retention time (min)
c
Kme0
Kme2
Kme3
Intensity (arbitrary units)
0
30
Retention Time (min)
b
d
immunoprecipitated VCP
HeLa U87-MG 293
wt ko wt ko wt ko
Kme3
Ponceau S
VCPΔD2
VCP-KMT
+ -
Kme3
Ponceau S

Figure-4

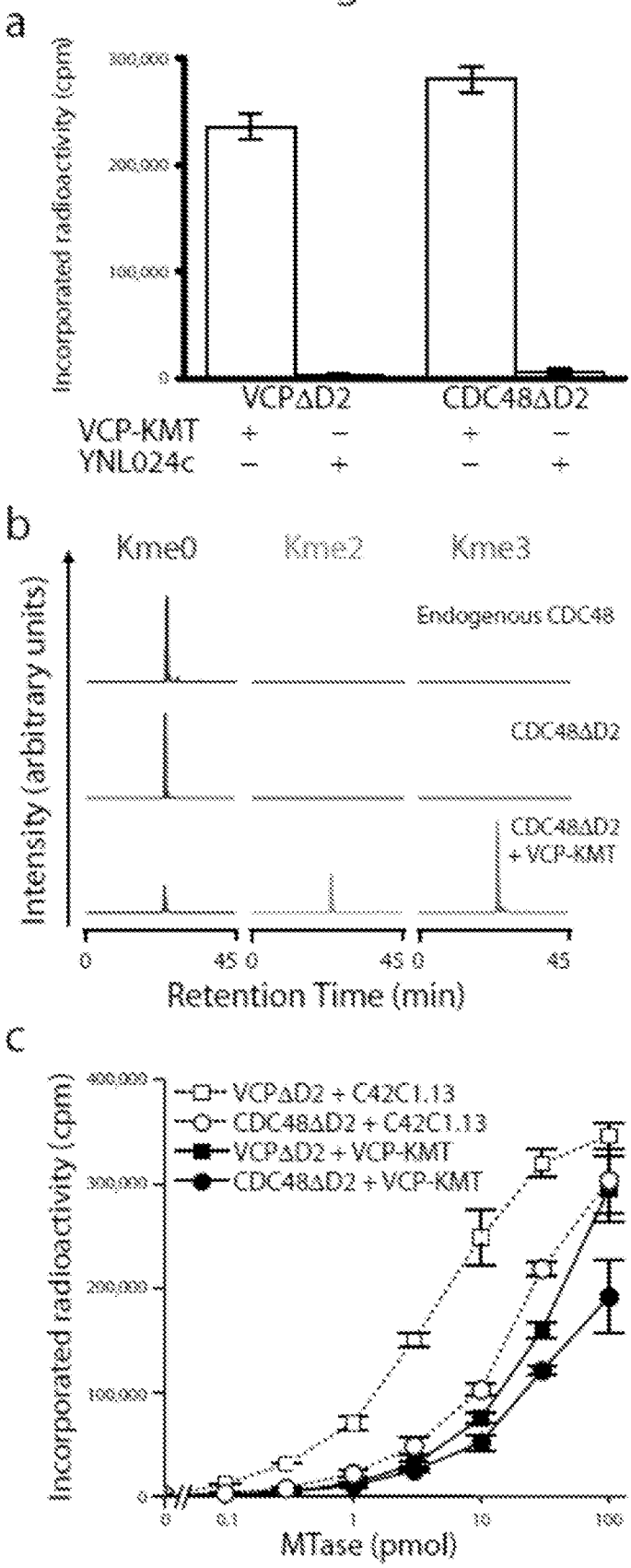

a
Incorporated radioactivity (cpm)
300,000
200,000
100,000
0
VCPΔD2
CDC48ΔD2
VCP-KMT + − + −
YNL024c − + − +
b
Kme0
Kme2
Kme3
Endogenous CDC48
CDC48ΔD2
CDC48ΔD2 + VCP-KMT
Intensity (arbitrary units)
0
45 0
45 0
45
Retention Time (min)
c
Incorporated radioactivity (cpm)
400,000
300,000
200,000
100,000
0
VCPΔD2 + C42C1.13
CDC48ΔD2 + C42C1.13
VCPΔD2 + VCP-KMT
CDC48ΔD2 + VCP-KMT
0
0.1
1
10
100
MTase (pmol)

Figure-5

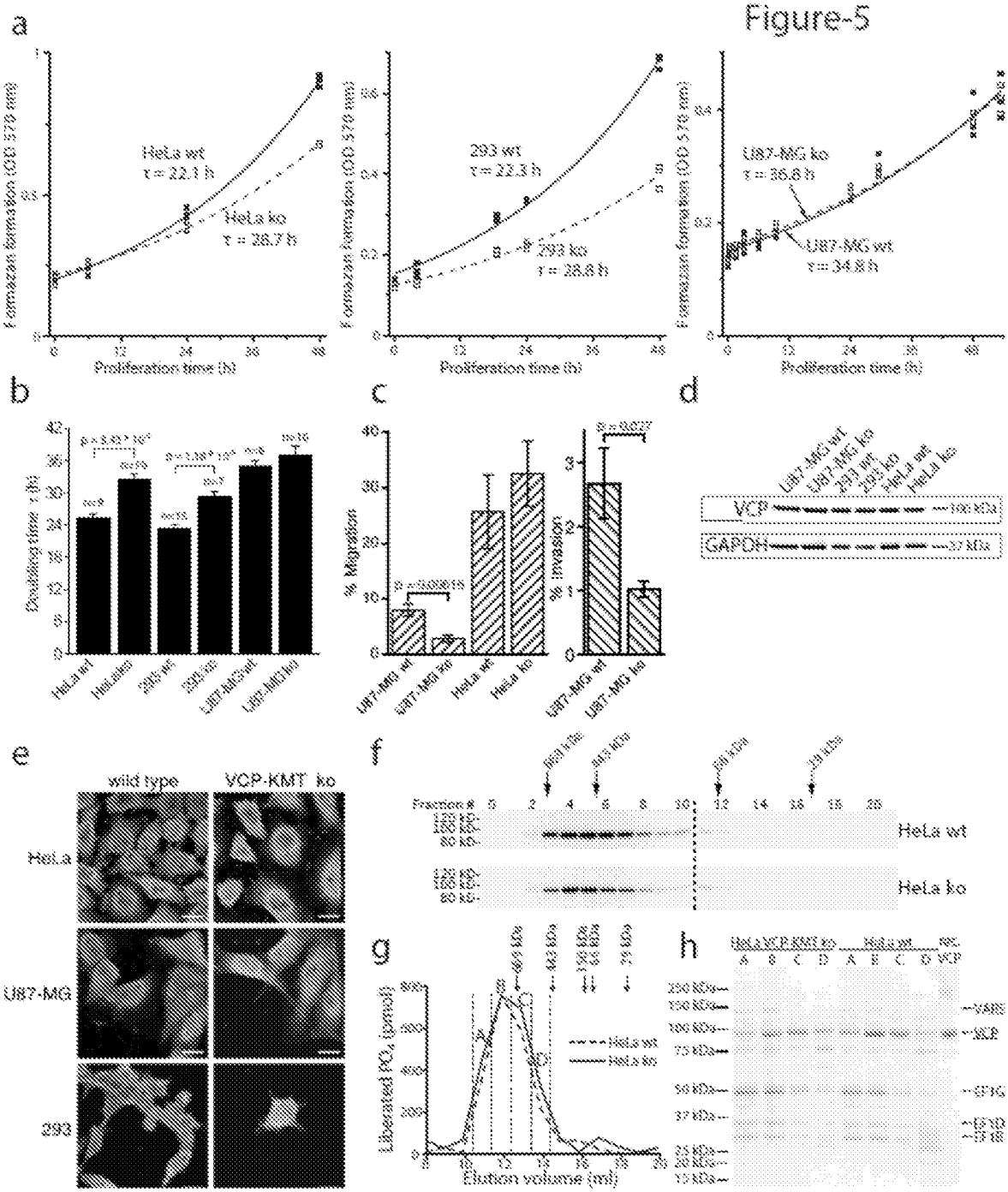

a
HeLa wt
τ = 22.1 h
HeLa ko
τ = 28.7 h
293 wt
τ = 22.3 h
293 ko
τ = 28.8 h
U87-MG ko
τ = 36.6 h
U87-MG wt
τ = 34.8 h
Proliferation time (h)
b
Doubling time (h)
c
% Migration
Invasion
d
VCP
GAPDH
e
wild type
VCP-KMT ko
HeLa
U87-MG
293
f
HeLa wt
HeLa ko
g
Liberated PO4 (pmol)
Elution volume (ml)
h
HeLa VCP-KMT ko
HeLa wt
WC
VCP

Figure-6

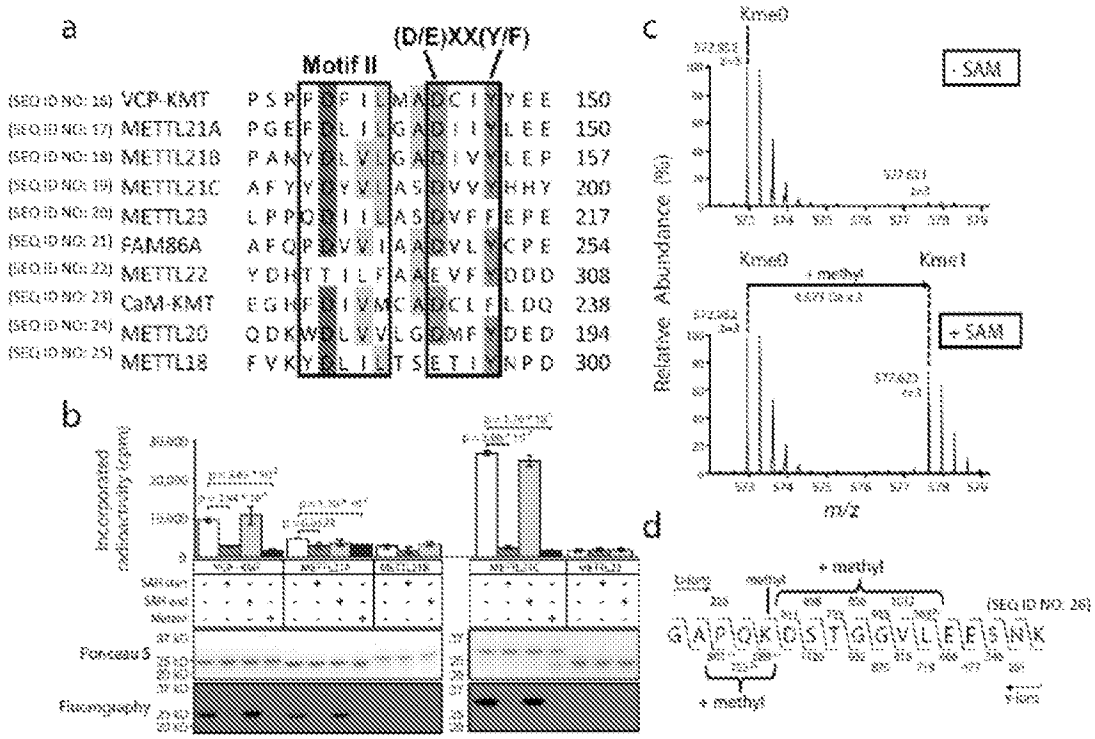

a
Motif II
(D/E)XX(Y/F)
(SEQ ID NO: 16) VCP-KMT P S P F F I M A C I Y E E 150
(SEQ ID NO: 17) METTL21A P G E F L I G A I I L E E 150
(SEQ ID NO: 18) METTL21B P A N Y L V L G A I V L E P 157
(SEQ ID NO: 19) METTL21C A F Y Y Y V L A S V V H H Y 200
(SEQ ID NO: 20) METTL23 L P P D I I L A S V F F E F E 217
(SEQ ID NO: 21) FAM86A A F Q P V V I A A V L C P E 254
(SEQ ID NO: 22) METTL22 Y D H T I L F A A E V F D D D 308
(SEQ ID NO: 23) CaM-KMT E G H F I V M C A C L F L D Q 238
(SEQ ID NO: 24) METTL20 Q D K M L V V L G M F D E D 194
(SEQ ID NO: 25) METTL18 F V K Y L I T S E T I N P D 300
b
Incorporated radioactivity (cpm)
Ponceau S
Fluorography
c
Kme0
- SAM
Kme1
+ methyl
+ SAM
Relative Abundance (%)
m/z
d
+ methyl
(SEQ ID NO: 26)
+ methyl

Figure 7

a. (SEQ ID NO: 1)

```
1 gcgcgccgct ttctgttgcc gggcgcaatg gcggatacgc tggagtcctc gctggaggac
       61 ccactgcgga gctttgtgcg agttttggag aagcgggatg gtacagtgct acgactacag
      121 cagtatagct ccggtggcgt gggttgcgtt gtgtgggacg ctgccattgt cctttctaaa
      181 tacctggaaa cgcccgagtt ttctggcgac ggggcccacg cgctgagccg gcggtcggtg
      241 ctggagctgg gttcgggcac cggggccgtg gggctcatgg ctgctaccct cggggctgat
      301 gttgtagtca ccgatcttga ggaattgcaa gacttgctga agatgaatat taatatgaac
      361 aagcatcttg tcactggttc tgttcaagcc aaggtactga aatgggggga agaaatagaa
      421 ggctttcctt ctccacccga cttcatactg atggccgact gcatatacta tgaagagtct
      481 ttggagccat tgctgaaaac tctaaaagat atcagcggat ttgaaacttg tattatatgt
      541 tgttatgaac aacgaacaat ggggaaaaat ccagaaattg agaaaaaata ttttgagctc
      601 cttcagctag attttgactt tgaaaaaatt cctttggaaa aacatgatga agagtatcga
      661 agtgaagata ttcatattat atacatcaga aagaaaaaat cgaaatttcc atcgtgaagc
      721 ctttaatcat cttacccaag gctctaacaa cctgggtaaa ctaaagatgt gaatagagca
      781 tgtgaataca gcatgggaag attgtgttca cagatttttt tttccggcac gtccttagag
      841 atccgatgta tagatgatga ccaccagggg ctgtctgcaa tacgaaaaat tcctgcttgc
      901 ctgcctgcct gccaatggcc ctgaatccag cttaggttac ttagttcagc atcaagttct
      961 tcttaaatgt tgggaatcag ttattcaaat aaaaattggt attgaggcga ggctgaaatc
     1021 tgccaaaaac agccaacatg atttgactgg acctttttaca ggaagctaga gataaatttc
     1081 tgaagagaac tatctgctat tatataataa tgttttaaat tcaaactagt aaatttttagt
     1141 ttgtcttcag agtttaaaag gttttcattt tgtacataat tatacaatat ttatcatttg
     1201 tattttccac ttcatttctt ttaaaatatc tttaatactg aaatgttctt ttaattttaa
     1261 aaagaactga gatattgtct tgtatactta tattagccaa agttttcttt tcctccacca
     1321 tacatatcta tgtgatttaa tcagtaaatt gtactgtaat gagttgctaa gaagacaaat
     1381 cagaagattg gaggaacaaa agatatcttt acagattttt catttgagca tggagtgaga
     1441 atagaaagac cagtttcagg cttatgcact tacgtggctc atgcacttta tgtatatgtt
     1501 ccaggaaaat ctggcttaaa aatactggta ttgtttacat gaagcagtga aaggtttttg
     1561 aataactaca aatgtagttc tatatgtata taccaaatga atttctgttc tgtgtctctc
     1621 tgttttatgt tatgaagcca ttcgctcata tacaataatc tgtcaaggac tttaatcata
     1681 cttgttccaa agagtagtaa ccggatggaa ttctggtatt tataggcatt ggtgctagat
     1741 ggtacatttt atgtgttaaa ataaacattg tttttgagac cttg
```

b. (SEQ ID NO: 2)

```
MADTLESSLEDPLRSFVRVLEKRDGTVLRLQQYSSGGVGCVVWDAAIVLSKYLETPEFSGDGAHALSRRSVLELG
SGTGAVGLMAATLGADVVVTDLEELQDLLKMNINMNKHLVTGSVQAKVLKWGEEIEGFPSPPDFILMADCIYYEE
SLEPLLKTLKDISGFETCIICCYEQRTMGKNPEIEKKYFELLQLDFDFEKIPLEKHDEEYRSEDIHIIYIRKKKS
KFPS
```