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(71) Applicant (for all designated States except US): **THE ROCKEFELLER UNIVERSITY** [US/US]; 1230 York Avenue, New York, NY 10065 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ALLIS, C., David** [US/US]; 148 Poe Road, Princeton, NJ 08540 (US). **XIAO, Andrew** [CN/US]; 500 East 63rd Street, 23h, New York, NY 10021 (US).

(74) Agents: **CHILDS, Carissa R.** et al.; Nixon Peabody LLP - Patent Group, 1100 Clinton Square, Rochester, NY 14604-1792 (US).

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(54) Title: WSTF REGULATES THE DNA DAMAGE RESPONSE OF H2A.X VIA NOVEL TYROSINE KINASE ACTIVITY

(57) Abstract: The present invention relates to methods of detecting and regulating cellular DNA damage as well as methods of screening compounds suitable for modulating cellular DNA damage. The methods of the present invention utilize antibodies that selectively bind to a phosphorylated tyrosine residue in a SQEY tyrosine phosphorylation motif sequence or a phosphorylated serine residue in a KENSSQ phosphorylation motif sequence, where the phosphorylated serine residue is closest to the glutamine residue. Also encompassed by the present invention are methods of treating a subject having cancer. These methods involve the administration of an agent that modulates the phosphorylation of the tyrosine residue in a SQEY motif. Suitable agents for modulating the phosphorylation of the tyrosine residue of the SQEY motif are also disclosed.



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**WSTF REGULATES THE DNA DAMAGE RESPONSE OF H2A.X VIA
NOVEL TYROSINE KINASE ACTIVITY**

[0001] This application claims the benefit of U.S. Provisional Patent
Application Serial No. 61/097,672, filed September 17, 2008, which is hereby
5 incorporated in its entirety.

[0002] The subject matter of this application was made with support from the
United States Government under the National Institutes of Health, Grant No. R01-
GM40922. The government has certain rights in this invention.

10 **FIELD OF THE INVENTION**

[0003] The present invention describes methods of identifying and regulating
cellular DNA damage repair and methods of treating a subject having cancer. These
methods involve modulating tyrosine phosphorylation at a newly defined tyrosine
phosphorylation motif. The invention further describes a novel tyrosine kinase
15 involved in regulating DNA damage repair.

BACKGROUND OF THE INVENTION

[0004] One hallmark of the mammalian DNA double-strand break (DSB)
response is the formation of Ionizing Radiation Induced Foci (IRIF), which are
20 composed of compacted chromatin and numerous DNA repair and checkpoint
proteins (Khanna et al., "DNA Double-Strand Breaks: Signaling, Repair and the
Cancer Connection," *Nat Genet* 27:247-254 (2001) and Zhou et al., "The DNA
Damage Response: Putting Checkpoints in Perspective," *Nature* 408:433-439 (2000)).
Despite considerable progress in understanding signaling pathways leading to
25 checkpoint control and DNA repair, the nature of the specialized chromatin structures
at IRIF is not well understood. One of the earliest events occurring at IRIF is the
phosphorylation of H2A.X, a specialized histone H2A variant, at Ser139 (referred to
as γ -H2A.X) by the ATM and ATR kinases (Redon et al., "Histone H2A Variants
H2AX and H2AZ," *Curr Opin Genet Dev* 12:162-169 (2002)). H2A.X-deficient
30 mouse embryonic fibroblasts (MEFs), B and T cells display pronounced levels of
genomic instability, including broken and dicentric chromosomes and random

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translocations (Celeste et al., "Genomic Instability in Mice Lacking Histone H2AX," *Science* 296:922-927 (2002)). Class switch recombination and spermatogenesis are also defective in H2A.X deficient mice, further implying its involvement in DNA damage repair under physiological conditions (Celeste et al., "Genomic Instability in

5 Mice Lacking Histone H2AX," *Science* 296:922-927 (2002); Celeste et al., "H2AX Haploinsufficiency Modifies Genomic Stability and Tumor Susceptibility," *Cell* 114:371-383 (2003); and Reina-San-Martin et al., "H2AX is Required for Recombination Between Immunoglobulin Switch Regions but Not for Intra-Switch Region Recombination or Somatic Hypermutation," *J Exp Med* 197:1767-1778

10 (2003)). Moreover, H2A.X-deficiency accelerates B and T cell lymphoma development in p53-deficient mice (Celeste et al., "H2AX Haploinsufficiency Modifies Genomic Stability and Tumor Susceptibility," *Cell* 114:371-383 (2003); Reina-San-Martin et al., "H2AX is Required for Recombination Between Immunoglobulin Switch Regions but Not for Intra-Switch Region Recombination or

15 Somatic Hypermutation," *J Exp Med* 197:1767-1778 (2003); and Bassing et al., "Histone H2AX: A Dosage-dependent Suppressor of Oncogenic Translocations and Tumors," *Cell* 114:359-370 (2003)). Consistent with these functions in mammalian cells, phosphorylation of the equivalent site on yeast H2A (Ser129) is found at DSB sites and spreads to ~50Kb of the flanking regions (Unal et al., "DNA Damage

20 Response Pathway Uses Histone Modification to Assemble a Double-Strand Break-Specific Cohesin Domain," *Mol Cell* 16:991-1002 (2004)). In mammals this phosphorylation event directly recruits Mdc1 and sets in motion the recruitment of additional factors such as 53BP1, RNF8, and the Brca1 A complex (Harper et al., "The DNA Damage Response: Ten Years After," *Mol Cell* 28:739-745 (2007)).

25 Furthermore, several recent studies have also indicated that ATP-dependent chromatin remodeling complexes are engaged in DNA repair pathways at these sites. The yeast NuA4 and INO80 complexes, for example, are recruited to the damaged chromatin via γ -H2A.X (Morrison et al., "INO80 and Gamma-H2AX Interaction Links ATP-dependent Chromatin Remodeling to DNA Damage Repair," *Cell* 119:767-775

30 (2004); Downs et al., "Binding of Chromatin-Modifying Activities to Phosphorylated Histone H2A at DNA Damage Sites," *Mol Cell* 16:979-990 (2004); and van Attikum et al., "Recruitment of the INO80 Complex by H2A Phosphorylation Links ATP-

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Dependent Chromatin Remodeling With DNA Double-Strand Break Repair,” *Cell* 119:777-788 (2004)).

[0005] Mammalian H2A.X bears significant differences with lower eukaryotes. For example, H2A.X is a minor H2A variant in mammalian cells (1-
5 10%) while the major yeast form of H2A is most similar to H2A.X, because it contains the signature C-terminal sequence of mammalian H2A.X (Redon et al., “Histone H2A Variants H2AX and H2AZ,” *Curr Opin Genet Dev* 12:162-169 (2002)). A *Drosophila* H2A variant, known as H2A.V, is a “hybrid” protein containing signature sequences of mammalian H2A.X and H2A.Z (Redon et al.,
10 “Histone H2A Variants H2AX and H2AZ,” *Curr Opin Genet Dev* 12:162-169 (2002)). The *Drosophila* Tip60 complex exchanges phosphorylated H2A.V with unmodified H2A.V *in vitro* and regulates H2A.V phosphorylation levels *in vivo* (Kusch et al., “Acetylation by Tip60 is Required for Selective Histone Variant Exchange at DNA Lesions,” *Science* 306:2084-2087 (2004)). In mammalian cells,
15 the loss of SWI/SNF chromatin remodeling complex expression leads to defects in the H2A.X DNA damage response. However, it is unclear whether these defects are due to the lack of direct regulation by the SWI/SNF complex or other indirect pathways, since the SWI/SNF complex is involved in a variety of important biological processes (Park et al., “Mammalian SWI/SNF Complexes Facilitate DNA Double-Strand Break
20 Repair by Promoting Gamma-H2AX Induction,” *EMBO J* 25:3986-3997 (2006)).

[0006] Given the unique features of H2A.X in mammalian cells, factors that are directly involved in regulating H2A.X function need to be identified.

SUMMARY OF THE INVENTION

25 [0007] A first aspect of the present invention relates to an antibody or antigen-binding fragment thereof that selectively binds to a phosphorylated tyrosine residue in an SQEY tyrosine phosphorylation motif sequence (SEQ ID NO: 2).

[0008] A second aspect of the present invention relates to an antibody or antigen-binding fragment thereof that selectively binds to the phosphorylated serine
30 residue closest to the glutamine residue in an KENSSQ phosphorylation motif sequence (SEQ ID NO:4).

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[0009] Other aspects of the present invention relate to an isolated polypeptide molecule having an amino acid sequence of SEQ ID NO: 6 or SEQ ID NO: 7, and an isolated nucleic acid molecule encoding the polypeptide.

[0010] Another aspect of the present invention relates to a method of
5 identifying cellular DNA damage in a sample. This method involves providing a cell sample and detecting the presence or absence of a phosphorylated tyrosine residue in an SQEY tyrosine phosphorylation motif (SEQ ID NO:2). Cellular DNA damage in the cell sample is identified based on detecting the presence or absence of tyrosine phosphorylation.

10 [0011] Another aspect of the present invention relates to a method of identifying cellular DNA damage in a sample. This method involves providing a cell sample and detecting the presence or absence of a phosphorylated serine residue at the serine closest to the glutamine residue in an KENSSQ phosphorylation motif sequence (SEQ ID NO:4). Cellular DNA damage in the cell sample is identified
15 based on detecting the presence or absence of serine phosphorylation.

[0012] Another aspect of the present invention relates to a method of screening candidate compounds useful for modulating WSTF kinase activity. This method involves providing a candidate compound and a sample, where the sample contains a WSTF protein or polypeptide having kinase activity, a protein substrate
20 having a SQEY motif sequence, manganese, and ATP. Contacting the candidate compound with the sample and detecting the presence or absence of a phosphorylated tyrosine residue in a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) identifies compounds useful for modulating WSTF kinase activity.

[0013] The present invention is also directed to a method of screening
25 candidate compounds useful for modulating cellular DNA damage repair. This method involves providing a candidate compound and a cell sample and contacting the candidate compound with the cell sample. The presence or absence of a phosphorylated tyrosine residue in a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) in the cell sample is detected, and candidate compounds useful for modulating
30 cellular DNA damage repair are identified based on the presence or absence of tyrosine phosphorylation.

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[0014] Another aspect of the present invention relates to a method of screening candidate compounds useful for treating cancer. This method involves providing a candidate compound and a cell sample and contacting the candidate compound with the cell sample. The presence or absence of a phosphorylated tyrosine residue in a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) in the cell sample is detected and candidate compounds useful for treating cancer are identified based on the presence or absence of tyrosine phosphorylation.

[0015] The present invention is also directed to a method of modulating cellular DNA damage repair. This method involves administering to a cell an agent which modulates tyrosine phosphorylation of a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) under conditions effective to modulate cellular DNA damage repair.

[0016] Another aspect of the present invention is directed to a method of treating a subject having cancer. This method involves administering to the subject having cancer, an agent that modulates tyrosine phosphorylation of a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) under conditions effective to treat the subject having cancer.

[0017] The studies described herein define a novel DNA damage response pathway regulating H2A.X function in mammals that is mediated through the WSTF-SNF2H chromatin remodeling complex and involves the phosphorylation of H2A.X at tyrosine residue 142 ("Tyr142" or "Y142"). Unexpectedly, it was determined that the amino-terminal domain of WSTF, including its WAC domain, exhibits tyrosine kinase activity towards Tyr142 of H2A.X, a novel phosphorylation mark that plays a role in γ -H2A.X maintenance and related downstream chromatin remodeling events. Both the novel tyrosine kinase activity of WSTF and the phosphorylation of H2A.X at Tyr142 are required for eliciting an appropriate downstream DNA damage response in mammalian cells. These findings support an emerging view that the regulatory pathways of chromatin structure brought about by histone covalent modification, chromatin remodeling by ATP-dependent complexes, and the introduction of histone variants are closely interconnected, affecting many DNA-templated processes including the repair of double-strand breaks.

BRIEF DESCRIPTION OF THE DRAWINGS

- [0018] Figures 1A–1B show Tyr142, a novel phosphorylation mark of H2A.X, is regulated by DNA damage signals. Figure 1A is a comparison of extreme C-terminal sequences of H2A.X of various species, demonstrating that Tyr142 is conserved in “multicellular” (mammals and fruit flies) but not in “single-cellular” eukaryotes. As shown in Figure 1A, the SQEY motif of SEQ ID NO:2 is conserved between *H. sapiens* and *M. musculus*; *D. melanogaster* has an SQAY motif (SEQ ID NO: 18); *X. laevis* has an SQEY/F motif (SEQ ID NO: 19); and *S. cerevisiae* has a SQEL motif (SEQ ID NO: 20). In Figure 1B, primary MEF cells were treated with 10 Gy of ionizing radiation (IR) and recovered for the period of time indicated. Acid-extracted histones were separated by SDS-PAGE and subjected to immunoblotting. H2A.X Tyr142 phosphorylation levels in MEFs gradually declined, reaching a minimum at 8 hr. As expected, γ -H2A.X signal was initiated upon damage and maintained up to 16 hours post IR.
- 15 [0019] Figures 2A–2D demonstrate the specific association between the WSTF-SNF2H chromatin remodeling complex and H2A.X nucleosomes *in vivo*. Figure 2A shows the purification scheme of H2A.X-containing mononucleosomes. MEFs reconstituted with Flag-H2A.X (WT or Y142F mutant) were treated with or without ionizing radiation. After hypotonic buffer extraction, nuclear pellets (P1) were further extracted and fractionated into soluble nuclear extracts (S2) and insoluble nuclear pellets (P2) containing primarily chromatin and associated proteins. After Mnase digestion, mononucleosomes were immunoprecipitated with α -Flag to enrich for H2A.X-containing nucleosomes and associated proteins. This approach efficiently and specifically isolated H2A.X nucleosomes (See Figure 6). The immunoprecipitated complexes were separated by SDS-PAGE and silver stained (Figure 2B upper panels). Two polypeptides migrating at 145 and 171 KDa were consistently associated with undamaged WT (Figure 2B, upper left panel), but not the Y142F mutant (Figure 2B, upper right panel) H2A.X mononucleosomes. Mass spectrometry (MS) analyses demonstrated that these polypeptides, WSTF (171KDa) and SNF2H (145 KDa), constitute the mammalian WICH complex (See Figure 7). The third band at 60 KDa was identified as β -actin in the MS analyses; the significance of β -actin is not known. An aliquot of the complex immunoprecipitated
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from the same sample was separated by SDS-PAGE and silver stained for lower molecular weight bands (<20 KDa) (Figure 2B, lower panel). Staining revealed similar levels of H2A.X and other core histones identified by MS analysis. In Figure 2C the association between the WICH complex and undamaged WT H2A.X mononucleosomes was confirmed by IP-western experiments. Note that the S139 phosphorylation level was greatly reduced in the Y142F mutant cells (Figure 2C; also see Figure 4). WSTF knock-down cell lines (WSTF RNAi) were generated by stably expressing short hairpin RNAi constructs specifically targeting the WSTF gene in NIH3T3 cells. Figure 4D shows SDS-PAGE and immunoblot of nuclear extracts from the WSTF RNAi and control 3T3 cells. In WSTF RNAi cells, the expression level of WSTF was significantly diminished and H2A.X Y142 phosphorylation level was significantly reduced.

[0020] Figures 3A–3G illustrate the characterization of the novel kinase domain of WSTF that phosphorylates Tyr142 of H2A.X. Figure 3A is a schematic representation of the domain architecture of the human WSTF protein and a series of recombinant proteins representing portions of WSTF (*i.e.*, the 1-345N construct represents N-terminal amino acids 1 to 345 of WSTF). These constructs were extensively purified through affinity (GST pull down), ion exchange (SP sepharose) and sizing (gel filtration) chromatography. No other proteins at significant level (>2.5%) were co-purified with them, as confirmed by MS analysis. In Figures 3B-F, recombinant WSTF proteins were generated in insect cells and the N- and C-terminal motifs (Figure 3G) were generated in *E. coli*. As shown in Figure 3B, Y142 was phosphorylated by recombinant full length WSTF proteins. WT and Y142F mutant H2A.X nucleosomes were reconstituted with recombinant free histones and DNA molecules *in vitro*. Following *in vitro* kinase assays, reaction mixtures were separated by SDS-PAGE and exposed for radioautography. In Figure 3C, the specific phosphorylation of H2A.X at Tyr142 was also detected by immunoblotting with an α -H2A.X Y142ph antibody. In Figures 3D-G, free H2A.X proteins were used as substrates in *in vitro* kinase assays. The recombinant N-terminal (N-WSTF) and C-terminal portion (C-WSTF) of WSTF were generated from insect cells and tested for kinase activity. Only N-WSTF had kinase activity as shown in Figure 3D. A series of truncated WSTF proteins were generated from insect cells (see Figure 3A) and the

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activity of the 1-340 amino acid construct was much reduced (<50 fold) in comparison to the other constructs (Figure 3E). The C338A point mutant was derived from the WT 1-359 construct and its kinase activity was much reduced compared to WT (Figure 3F). The N-motif and C-motif of WSTF kinase domain were generated either individually or in combination from *E. coli*. The co-expressed N-motif and C-motif had significant kinase activity towards H2A.X, while the N-motif alone had a minimal kinase activity (Figure 3G). The C-motif construct, when expressed alone, was unstable and partially degraded from its C-terminus, however, it was protected when co-expressed with the N-motif.

- 10 [0021] Figures 4A–4J demonstrate how critical WSTF is for the maintenance of γ -H2A.X phosphorylation after DNA damage. In WSTF RNAi cells (as described in Figure 2), γ -H2A.X level maintenance was defective after DNA damage treatment as illustrated in Figure 4A. Western blotting experiments were performed on the histone samples isolated from control and WSTF RNAi cells after 10 Gy of IR
- 15 treatment. The time points labeled indicate the recovery time following the IR treatment. The maintenance of γ -H2A.X foci is also defective in WSTF RNAi cells as shown in Figure 4B. Immunofluorescent staining experiments were performed on control and WSTF RNAi cells fixed at different time points (as labeled) following 10 Gy of IR treatment. As shown in Figures 4C and 4D, phos-ATM and Mdc1
- 20 recruitment is also defective in WSTF deficient cells. Immunofluorescent staining experiments were performed using anti-ATM S1981 phos (Figure 4C) and anti-Mdc1 (Figure 4D) antibodies on control or WSTF RNAi cells 8-hours post 10 Gy of IR treatment. Figures 4E and 4F demonstrates that WSTF kinase activity is also critical for γ -H2A.X and phos-ATM foci maintenance. Figures 4E and 4F are
- 25 immunofluorescent images of WSTF RNAi cells complemented with wildtype or mutant (C338A) kinase domain constructs of WSTF (amino acids 1-359, myc epitope-tagged at N-terminus). Eight hours following treatment with 10Gy of IR, cells were fixed and co-stained with anti-myc (red) and γ -H2A.X (green, Figure 4E) or anti-ATM S1981 phos (green, Figure 4F) antibodies. As shown in Figures 4G and
- 30 4H, the level of γ -H2A.X protein is reduced in Tyr142 mutants. H2A.X null MEFs were reconstituted with WT or Y142 H2A.X mutants (Y to L or Y to F mutants). Histone samples were separated by SDS-PAGE and subjected to Western blotting

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(“V” is vector only) (Figure 4G). Immunostaining experiments with these same cells demonstrated that γ -H2A.X foci (Figure 4H, upper left panel) were observed in WT cells upon DNA damage while they were severely diminished in Tyr142 mutants (upper middle and right panels). Cells were fixed after DNA damage treatment (10Gy
5 2hr) and stained with γ -H2A.X antibodies. In peptide pull-down experiments (Figure 4I), Ser139(ph) peptides specifically interacted with endogenous MDC1, but not BRCA1 from 293T cell nuclear extracts. Either pre-phosphorylation of Y142 (*i.e.*, a doubly phosphorylated (S139(ph) & Y142(ph)) peptide) or replacement of the C-terminal tyrosine to leucine (L) or phenylalanine (F) diminished MDC1 binding
10 significantly in this assay. A recombinant MDC1 BRCT domain specifically bound to Ser139(ph) peptides as demonstrated in Figure 4J. Similarly, pre-phosphorylation or replacement of Y142 diminished binding significantly.

[0022] Figures 5A–5F show the modulation of Y142 phosphorylation level during DNA damage response and the characterization of the α -H2A.X Y142ph
15 antibody. In Figure 5A, α -H2A.X Y142ph antibodies were reacted with *Xenopus* H2A.X in *Xenopus* egg extracts. After incubating with double-strand break (DSB) plasmid DNA (for the time span as indicated), the Y142 phosphorylation significantly decreased while γ -H2A.X increased, reminiscent of mammalian cells. Yeast H2A L132Y mutant strongly reacted with antibodies to mammalian H2A.X Y142
20 phosphorylation (Figure 5B). As expected, WT yeast cells did not have affinity for the anti-H2A.X Y142ph antibody. In contrast, the anti-H2A.X Y142ph antibody reacted with nuclear extracts from untreated H2A L132Y cultures, but did not react with extracts of cells treated with the DNA-damaging agent, MMS. γ -H2A.X (S139ph) antibody reacted strongly with WT and H2A L132Y cells following DNA
25 damage. The equal loading of histone proteins was judged by α -H3 antibodies. In ELISA, α -H2A.X-Y142ph antibody reacted with H2A.X C-terminal peptides (129-142) that contained phosphorylated Tyr142 regardless of the phosphorylation status of Ser139 (Figure 5C, compare (▲) and (x) curves), but not with similar peptides where Tyr142 was not phosphorylated (compare (♦) and (■) curves). In western blot
30 analyses, α -H2A.X Y142ph antibody recognized a single band at the expected molecular weight of H2A.X (~17 KDa in U2OS cells (Figure 5D, left lane)); this band was sensitive to protein tyrosine phosphatase b (Figure 5D, right lane). The signals

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could be competed away only by H2A.X Y142ph peptides, but not unmodified peptides (Figure 5E). In western blot analyses, this antibody only reacted with reconstituted WT H2A.X cells, but not cells expressing a Y142F point mutant (Figure 5F).

5 **[0023]** Figure 6 shows H2A.X-containing nucleosomes enriched in immunocomplexes precipitated with α -Flag. Flag-H2A.X-containing mononucleosomes isolated from reconstituted MEFs (see scheme in Figure 2A) were separated by SDS-PAGE before being subjected to immunoblot analyses or Coomassie blue staining. In western blot analyses (upper panel), α -Flag antibodies
10 recognized a single band at 20 KDa (there was no endogenous H2A.X in these cells and the MW of Flag-tagged H2A.X was larger than H3). Coomassie staining of the immunoprecipitated complexes (lower panel) revealed that Flag-H2A.X (MW 20 KDa) was associated with other core histones at similar stoichiometrical level whereas it was undetectable in input (5%) samples.

15 **[0024]** Figure 7 is an identification of WSTF-SNF2H complex subunits by mass spectrometry. Protein bands (at 171 and 140 KDa, Figure 2B) were isolated from SDS-PAGE gel and subjected to trypsin digestion. Peptide mass fingerprinting (PMF) demonstrated that in the protein band at 171KDa, 19 peptides (SEQ ID NOs: 38–56; selected out of 29 for PMF) were from the mouse WSTF protein, which cover
20 248 out of 1479 amino acids (16.8 %). In the protein band at 140KDa, 10 peptides (SEQ ID NOs: 57–66; out of 35 selected for PMF) were from the mouse SNF2H protein, which cover 109 out of 1052 amino acids (10.4 %). The protein band at 60 KDa was identified as β -actin.

25 **[0025]** Figure 8 demonstrates that alteration of C-terminal Tyr142 in H2A.X does not change the affinity of γ -H2A.X antibodies. In ELISA, γ -H2A.X antibodies reacted with H2A.X C-terminal peptides (amino acid residues 129-142) that contain Phos-Ser139 regardless of the phosphorylation status of Tyr142 or Y142L replacement.

30 **[0026]** Figures 9A–9B show that phosphorylation of H2A.X at Ser139 by the ATM/R kinases upon DNA damage does not require Tyr142 and was not affected by phosphorylation at this site. H2A.X C-terminal peptides (listed in Figure 9B) were incubated with *Xenopus* egg extracts in the presence of DSB DNA (+) or control

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DNA(-) and γ - ^{32} P-ATP (reflective of ATM kinase; see Khanna et al., "DNA Double-Strand Breaks: Signaling, Repair and the Cancer Connection," *Nat Genet* 27:247-254 (2001), which hereby incorporated by reference in its entirety). Incorporation of radioactive ATP was measured by scintillation counting in standard P81 filter paper assays (Khanna et al., "DNA Double-Strand Breaks: Signaling, Repair and the Cancer Connection," *Nat Genet* 27:247-254 (2001), which hereby incorporated by reference in its entirety). Figure 9A is a bar graph showing ATM kinase activity (Ser139 phosphorylation). Ser139 of H2A.X was specifically phosphorylated in the presence of DSB DNA (compare unmodified peptide (UN) in the presence of DSB DNA (+) as compared to control DNA (-) or with pre-phosphorylated Ser139(ph) peptide (DSB+). The status of Tyr142, either in its pre-phosphorylated state (Tyr142(ph)) or a Tyr-to-Leu replacement (Leu142) failed to significantly affect Ser139 phosphorylation in the presence of DSB.

[0027] Figures 10A–10B demonstrate that the *in vitro* kinase activity of WSTF is dependent on Mn^{2+} but not Mg^{2+} . Full length recombinant WSTF proteins isolated from insect cells phosphorylated free H2A.X protein in the presence of Mn^{2+} in the *in vitro* kinase assays (Figure 10A). The linear range of the kinase activity was observed from 50-500 μ M. On the other hand, no kinase activity was observed in the presence of Mg^{2+} . Further increase of Mg^{2+} concentration up to 10mM did not stimulate the kinase activity. Similar Mn^{2+} dependence was observed with recombinant kinase domain (1-359) (Figure 10B).

[0028] Figures 11A–11B show WSTF kinase domain mediated phosphorylation of H2A.X Y142 *in vitro*. Recombinant WSTF kinase domain phosphorylated WT H2A.X histone proteins in an *in vitro* kinase assay, while its activity towards a Y142F mutant was much reduced (Figure 11A). *In vitro*, WSTF kinase domain activity towards H2A.X was much higher than other core histones (Figure 11B).

[0029] Figure 12 shows that the mutant (C338A) WSTF kinase domain has a similar protein fold to its WT counterpart. WT and C338A mutant recombinant proteins were diluted to 10 μ M and their circular dichroism (CD) spectra (260 to 200 nm) were collected at 25 °C every 5 seconds. Spectra shown were averages of three independent measurements.

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[0030] Figures 13A–13C show the two WSTF kinase domain motifs. The recombinant WSTF kinase domain was partially cleaved into several defined polypeptides (Figure 13B). The polypeptides were transferred to a PVDF membrane and analyzed by Edman degradation. Figure 13B shows the sequencing results, which revealed that the recombinant WSTF protein kinase domain consisting of amino acids 1-391 (SEQ ID NO:6) was cleaved into two motifs. Band “a” (SEQ ID NO:8, shown in the bottom of Figure 13B), represents the WSTF kinase domain plus an N-terminal linker sequence and a C-terminal affinity tag. The N-terminal WSTF kinase motif, band “b”, consists of amino acid residues G1–C344 of SEQ ID NO:8. The C-terminal motif, band “d”, consisting of residues K213–H489 of SEQ ID NO: 8 and band “e” consisting of amino acid residues K213–H477 of SEQ ID NO:8, starts from residue K207 of the WSTF kinase domain (SEQ ID NO:6) (band “e” is a further truncated form of band “d”). Band “c” is the co-purified GST affinity tag. The start of N-terminal motif and C-terminal motif of the WSTF kinase domain are underlined in the WSTF kinase domain amino acid sequence of SEQ ID NO:8 shown in Figure 13B. Figure 13C is an alignment of multiple putative kinase regions of WSTF proteins (SEQ ID NOS: 10 to 18) from various vertebrates and invertebrates. Residues conserved in at least 50% of the sequences are shown on black background, and invariant residues are shown on grey background. Sequence abbreviations: hs, human (SEQ ID NO: 9); mm, mouse (SEQ ID NO: 10); gg, chicken (SEQ ID NO: 11); xl, xenopus laevis (SEQ ID NO: 12); dr, zebrafish (SEQ ID NO: 13); cs, ciona savignyi (SEQ ID NO: 14); ac, aplysia californica (SEQ ID NO: 15); lg, lottia gigantea (SEQ ID NO: 16); and pl, paracentrotus lividus (SEQ ID NO: 17).

25 DETAILED DESCRIPTION OF THE INVENTION

[0031] A first aspect of the present invention relates to an antibody or antigen-binding fragment thereof that selectively binds to a phosphorylated tyrosine residue in an SQEY tyrosine phosphorylation motif sequence (SEQ ID NO: 2). The novel SQEY phosphorylation motif sequence was identified in the histone 2A variant, H2A.X. The full-length amino acid sequence of human H2A.X is provided below as SEQ ID NO:1.

- 13 -

Ser Gly Arg Gly Lys Thr Gly Gly Lys Ala Arg Ala Lys Ala Lys
 1 5 10 15
 5 Ser Arg Ser Ser Arg Ala Gly Leu Gln Phe Pro Val Gly Arg Val His
 20 25 30
 10 Arg Leu Leu Arg Lys Gly His Tyr Ala Glu Arg Val Gly Ala Gly Ala
 35 40 45
 Pro Val Tyr Leu Ala Ala Val Leu Glu Tyr Leu Thr Ala Glu Ile Leu
 50 55 60
 15 Glu Leu Ala Gly Asn Ala Ala Arg Asp Asn Lys Lys Thr Arg Ile Ile
 65 70 75
 20 Pro Arg His Leu Gln Leu Ala Ile Arg Asn Asp Glu Glu Leu Asn Lys
 80 85 90 95
 25 Leu Leu Gly Gly Val Thr Ile Ala Gln Gly Gly Val Leu Pro Asn Ile
 100 105 110
 30 Gln Ala Val Leu Leu Pro Lys Lys Thr Ser Ala Thr Val Gly Pro Lys
 115 120 125
 Ala Pro Ser Gly Gly Lys Lys Ala Thr Gln Ala Ser Gln Glu Tyr
 130 135 140
 35

In a preferred embodiment of the present invention, the antibody or antigen binding
 fragment thereof selectively binds to the phosphorylated tyrosine residue of the SQEY
 40 motif at amino acid position 142 of SEQ ID NO:1.

[0032] A second aspect of the present invention relates to an antibody or
 antigen-binding fragment thereof that selectively binds to the phosphorylated serine
 residue closest to the glutamine residue in an KENSSQ phosphorylation motif
 sequence (SEQ ID NO:4). The novel KENSSQ phosphorylation motif sequence was
 45 identified in the N-terminal region of the WSTF protein. The full-length amino acid
 sequence of the human WSTF protein is provided below as SEQ ID NO:3.

Met Ala Pro Leu Leu Gly Arg Lys Pro Phe Pro Leu Val Lys Pro Leu
 1 5 10 15
 50

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	Pro	Gly	Glu	Glu	Pro	Leu	Phe	Thr	Ile	Pro	His	Thr	Gln	Glu	Ala	Phe	
				20					25					30			
5	Arg	Thr	Arg	Glu	Glu	Tyr	Glu	Ala	Arg	Leu	Glu	Arg	Tyr	Ser	Glu	Arg	
			35					40					45				
10	Ile	Trp	Thr	Cys	Lys	Ser	Thr	Gly	Ser	Ser	Gln	Leu	Thr	His	Lys	Glu	
	50						55					60					
15	Ala	Trp	Glu	Glu	Glu	Gln	Glu	Val	Ala	Glu	Leu	Leu	Lys	Glu	Glu	Phe	
	65					70					75					80	
20	Pro	Ala	Trp	Tyr	Glu	Lys	Leu	Val	Leu	Glu	Met	Val	His	His	Asn	Thr	
					85					90					95		
25	Ala	Ser	Leu	Glu	Lys	Leu	Val	Asp	Thr	Ala	Trp	Leu	Glu	Ile	Met	Thr	
			100					105						110			
30	Lys	Tyr	Ala	Val	Gly	Glu	Glu	Cys	Asp	Phe	Glu	Val	Gly	Lys	Glu	Lys	
			115					120					125				
35	Met	Leu	Lys	Val	Lys	Ile	Val	Lys	Ile	His	Pro	Leu	Glu	Lys	Val	Asp	
	130					135						140					
40	Glu	Glu	Ala	Thr	Glu	Lys	Lys	Ser	Asp	Gly	Ala	Cys	Asp	Ser	Pro	Ser	
	145					150					155					160	
45	Ser	Asp	Lys	Glu	Asn	Ser	Ser	Gln	Ile	Ala	Gln	Asp	His	Gln	Lys	Lys	
					165					170					175		
50	Glu	Thr	Val	Val	Lys	Glu	Asp	Glu	Gly	Arg	Arg	Glu	Ser	Ile	Asn	Asp	
			180						185					190			
55	Arg	Ala	Arg	Arg	Ser	Pro	Arg	Lys	Leu	Pro	Thr	Ser	Leu	Lys	Lys	Gly	
			195					200					205				
60	Glu	Arg	Lys	Trp	Ala	Pro	Pro	Lys	Phe	Leu	Pro	His	Lys	Tyr	Asp	Val	
	210						215					220					
65	Lys	Leu	Gln	Asn	Glu	Asp	Lys	Ile	Ile	Ser	Asn	Val	Pro	Ala	Asp	Ser	
	225					230					235					240	
70	Leu	Ile	Arg	Thr	Glu	Arg	Pro	Pro	Asn	Lys	Glu	Ile	Val	Arg	Tyr	Phe	
					245					250					255		

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	Ile	Arg	His	Asn	Ala	Leu	Arg	Ala	Gly	Thr	Gly	Glu	Asn	Ala	Pro	Trp
				260					265					270		
5	Val	Val	Glu	Asp	Glu	Leu	Val	Lys	Lys	Tyr	Ser	Leu	Pro	Ser	Lys	Phe
			275					280					285			
10	Ser	Asp	Phe	Leu	Leu	Asp	Pro	Tyr	Lys	Tyr	Met	Thr	Leu	Asn	Pro	Ser
		290					295					300				
15	Thr	Lys	Arg	Lys	Asn	Thr	Gly	Ser	Pro	Asp	Arg	Lys	Pro	Ser	Lys	Lys
	305					310					315					320
20	Ser	Lys	Thr	Asp	Asn	Ser	Ser	Leu	Ser	Ser	Pro	Leu	Asn	Pro	Lys	Leu
					325					330					335	
25	Trp	Cys	His	Val	His	Leu	Lys	Lys	Ser	Leu	Ser	Gly	Ser	Pro	Leu	Lys
				340					345					350		
30	Val	Lys	Asn	Ser	Lys	Asn	Ser	Lys	Ser	Pro	Glu	Glu	His	Leu	Glu	Glu
			355					360					365			
35	Met	Met	Lys	Met	Met	Ser	Pro	Asn	Lys	Leu	His	Thr	Asn	Phe	His	Ile
		370					375					380				
40	Pro	Lys	Lys	Gly	Pro	Pro	Ala	Lys	Lys	Pro	Gly	Lys	His	Ser	Asp	Lys
		385				390					395					400
45	Pro	Leu	Lys	Ala	Lys	Gly	Arg	Ser	Lys	Gly	Ile	Leu	Asn	Gly	Gln	Lys
				405						410					415	
50	Ser	Thr	Gly	Asn	Ser	Lys	Ser	Pro	Lys	Lys	Gly	Leu	Lys	Thr	Pro	Lys
				420					425					430		
55	Thr	Lys	Met	Lys	Gln	Met	Thr	Leu	Leu	Asp	Met	Ala	Lys	Gly	Thr	Gln
			435					440					445			
60	Lys	Met	Thr	Arg	Ala	Pro	Arg	Asn	Ser	Gly	Gly	Thr	Pro	Arg	Thr	Ser
		450					455					460				
65	Ser	Lys	Pro	His	Lys	His	Leu	Pro	Pro	Ala	Ala	Leu	His	Leu	Ile	Ala
	465					470					475					480
70	Tyr	Tyr	Lys	Glu	Asn	Lys	Asp	Arg	Glu	Asp	Lys	Arg	Ser	Ala	Leu	Ser
					485					490					495	

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5 Cys Val Ile Ser Lys Thr Ala Arg Leu Leu Ser Ser Glu Asp Arg Ala
 500 505 510

Arg Leu Pro Glu Glu Leu Arg Ser Leu Val Gln Lys Arg Tyr Glu Leu
 515 520 525

10 Leu Glu His Lys Lys Arg Trp Ala Ser Met Ser Glu Glu Gln Arg Lys
 530 535 540

15 Glu Tyr Leu Lys Lys Lys Arg Glu Glu Leu Lys Lys Lys Leu Lys Glu
 545 550 555 560

20 Lys Ala Lys Glu Arg Arg Glu Lys Glu Met Leu Glu Arg Leu Glu Lys
 565 570 575

25 Gln Lys Arg Tyr Glu Asp Gln Glu Leu Thr Gly Lys Asn Leu Pro Ala
 580 585 590

Phe Arg Leu Val Asp Thr Pro Glu Gly Leu Pro Asn Thr Leu Phe Gly
 595 600 605

30 Asp Val Ala Met Val Val Glu Phe Leu Ser Cys Tyr Ser Gly Leu Leu
 610 615 620

35 Leu Pro Asp Ala Gln Tyr Pro Ile Thr Ala Val Ser Leu Met Glu Ala
 625 630 635 640

40 Leu Ser Ala Asp Lys Gly Gly Phe Leu Tyr Leu Asn Arg Val Leu Val
 645 650 655

45 Ile Leu Leu Gln Thr Leu Leu Gln Asp Glu Ile Ala Glu Asp Tyr Gly
 660 665 670

Glu Leu Gly Met Lys Leu Ser Glu Ile Pro Leu Thr Leu His Ser Val
 675 680 685

50 Ser Glu Leu Val Arg Leu Cys Leu Arg Arg Ser Asp Val Gln Glu Glu
 690 695 700

55 Ser Glu Gly Ser Asp Thr Asp Asp Asn Lys Asp Ser Ala Ala Phe Glu
 705 710 715 720

Asp Asn Glu Val Gln Asp Glu Phe Leu Glu Lys Leu Glu Thr Ser Glu

					725					730					735	
5	Phe	Phe	Glu	Leu	Thr	Ser	Glu	Glu	Lys	Leu	Gln	Ile	Leu	Thr	Ala	Leu
				740					745					750		
10	Cys	His	Arg	Ile	Leu	Met	Thr	Tyr	Ser	Val	Gln	Asp	His	Met	Glu	Thr
			755					760					765			
15	Arg	Gln	Gln	Met	Ser	Ala	Glu	Leu	Trp	Lys	Glu	Arg	Leu	Ala	Val	Leu
		770					775					780				
20	Lys	Glu	Glu	Asn	Asp	Lys	Lys	Arg	Ala	Glu	Lys	Gln	Lys	Arg	Lys	Glu
	785					790					795					800
25	Met	Glu	Ala	Lys	Asn	Lys	Glu	Asn	Gly	Lys	Val	Glu	Asn	Gly	Leu	Gly
					805					810					815	
30	Lys	Thr	Asp	Arg	Lys	Lys	Glu	Ile	Val	Lys	Phe	Glu	Pro	Gln	Val	Asp
				820					825					830		
35	Thr	Glu	Ala	Glu	Asp	Met	Ile	Ser	Ala	Val	Lys	Ser	Arg	Arg	Leu	Leu
			835					840					845			
40	Ala	Ile	Gln	Ala	Lys	Lys	Glu	Arg	Glu	Ile	Gln	Glu	Arg	Glu	Met	Lys
		850					855					860				
45	Val	Lys	Leu	Glu	Arg	Gln	Ala	Glu	Glu	Glu	Arg	Ile	Arg	Lys	His	Lys
	865					870					875					880
50	Ala	Ala	Ala	Glu	Lys	Ala	Phe	Gln	Glu	Gly	Ile	Ala	Lys	Ala	Lys	Leu
					885					890					895	
55	Val	Met	Arg	Arg	Thr	Pro	Ile	Gly	Thr	Asp	Arg	Asn	His	Asn	Arg	Tyr
				900					905					910		
60	Trp	Leu	Phe	Ser	Asp	Glu	Val	Pro	Gly	Leu	Phe	Ile	Glu	Lys	Gly	Trp
			915					920					925			
65	Val	His	Asp	Ser	Ile	Asp	Tyr	Arg	Phe	Asn	His	His	Cys	Lys	Asp	His
		930					935					940				
70	Thr	Val	Ser	Gly	Asp	Glu	Asp	Tyr	Cys	Pro	Arg	Ser	Lys	Lys	Ala	Asn
	945					950					955					960

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	Leu	Gly	Lys	Asn	Ala	Ser	Met	Asn	Thr	Gln	His	Gly	Thr	Ala	Thr	Glu	
					965					970					975		
5	Val	Ala	Val	Glu	Thr	Thr	Thr	Pro	Lys	Gln	Gly	Gln	Asn	Leu	Trp	Phe	
				980					985					990			
10	Leu	Cys	Asp	Ser	Gln	Lys	Glu	Leu	Asp	Glu	Leu	Leu	Asn	Cys	Leu	His	
			995					1000					1005				
15	Pro	Gln	Gly	Ile	Arg	Glu	Ser	Gln	Leu	Lys	Glu	Arg	Leu	Glu	Lys		
		1010					1015					1020					
20	Arg	Tyr	Gln	Asp	Ile	Ile	His	Ser	Ile	His	Leu	Ala	Arg	Lys	Pro		
		1025					1030					1035					
25	Asn	Leu	Gly	Leu	Lys	Ser	Cys	Asp	Gly	Asn	Gln	Glu	Leu	Leu	Asn		
		1040					1045					1050					
30	Phe	Leu	Arg	Ser	Asp	Leu	Ile	Glu	Val	Ala	Thr	Arg	Leu	Gln	Lys		
		1055					1060					1065					
35	Gly	Gly	Leu	Gly	Tyr	Val	Glu	Glu	Thr	Ser	Glu	Phe	Glu	Ala	Arg		
		1070					1075					1080					
40	Val	Ile	Ser	Leu	Glu	Lys	Leu	Lys	Asp	Phe	Gly	Glu	Cys	Val	Ile		
		1085					1090					1095					
45	Ala	Leu	Gln	Ala	Ser	Val	Ile	Lys	Lys	Phe	Leu	Gln	Gly	Phe	Met		
		1100					1105					1110					
50	Ala	Pro	Lys	Gln	Lys	Arg	Arg	Lys	Leu	Gln	Ser	Glu	Asp	Ser	Ala		
		1115					1120					1125					
55	Lys	Thr	Glu	Glu	Val	Asp	Glu	Glu	Lys	Lys	Met	Val	Glu	Glu	Ala		
		1130					1135					1140					
60	Lys	Val	Ala	Ser	Ala	Leu	Glu	Lys	Trp	Lys	Thr	Ala	Ile	Arg	Glu		
		1145					1150					1155					
65	Ala	Gln	Thr	Phe	Ser	Arg	Met	His	Val	Leu	Leu	Gly	Met	Leu	Asp		
		1160					1165					1170					
70	Ala	Cys	Ile	Lys	Trp	Asp	Met	Ser	Ala	Glu	Asn	Ala	Arg	Cys	Lys		
		1175					1180					1185					

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	Val	Cys	Arg	Lys	Lys	Gly	Glu	Asp	Asp	Lys	Leu	Ile	Leu	Cys	Asp
	1190						1195					1200			
5	Glu	Cys	Asn	Lys	Ala	Phe	His	Leu	Phe	Cys	Leu	Arg	Pro	Ala	Leu
	1205						1210					1215			
10	Tyr	Glu	Val	Pro	Asp	Gly	Glu	Trp	Gln	Cys	Pro	Ala	Cys	Gln	Pro
	1220						1225					1230			
15	Ala	Thr	Ala	Arg	Arg	Asn	Ser	Arg	Gly	Arg	Asn	Tyr	Thr	Glu	Glu
	1235						1240					1245			
20	Ser	Ala	Ser	Glu	Asp	Ser	Glu	Asp	Asp	Glu	Ser	Asp	Glu	Glu	Glu
	1250						1255					1260			
	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Asp	Tyr	Glu	Val	Ala
	1265						1270					1275			
25	Gly	Leu	Arg	Leu	Arg	Pro	Arg	Lys	Thr	Ile	Arg	Gly	Lys	His	Ser
	1280						1285					1290			
30	Val	Ile	Pro	Pro	Ala	Ala	Arg	Ser	Gly	Arg	Arg	Pro	Gly	Lys	Lys
	1295						1300					1305			
35	Pro	His	Ser	Thr	Arg	Arg	Ser	Gln	Pro	Lys	Ala	Pro	Pro	Val	Asp
	1310						1315					1320			
40	Asp	Ala	Glu	Val	Asp	Glu	Leu	Val	Leu	Gln	Thr	Lys	Arg	Ser	Ser
	1325						1330					1335			
	Arg	Arg	Gln	Ser	Leu	Glu	Leu	Gln	Lys	Cys	Glu	Glu	Ile	Leu	His
	1340						1345					1350			
45	Lys	Ile	Val	Lys	Tyr	Arg	Phe	Ser	Trp	Pro	Phe	Arg	Glu	Pro	Val
	1355						1360					1365			
50	Thr	Arg	Asp	Glu	Ala	Glu	Asp	Tyr	Tyr	Asp	Val	Ile	Thr	His	Pro
	1370						1375					1380			
55	Met	Asp	Phe	Gln	Thr	Val	Gln	Asn	Lys	Cys	Ser	Cys	Gly	Ser	Tyr
	1385						1390					1395			
	Arg	Ser	Val	Gln	Glu	Phe	Leu	Thr	Asp	Met	Lys	Gln	Val	Phe	Thr
	1400						1405					1410			

5	Asn	Ala	Glu	Val	Tyr	Asn	Cys	Arg	Gly	Ser	His	Val	Leu	Ser	Cys
	1415						1420					1425			
10	Met	Val	Lys	Thr	Glu	Gln	Cys	Leu	Val	Ala	Leu	Leu	His	Lys	His
	1430						1435					1440			
15	Leu	Pro	Gly	His	Pro	Tyr	Val	Arg	Arg	Lys	Arg	Lys	Lys	Phe	Pro
	1445						1450					1455			
20	Asp	Arg	Leu	Ala	Glu	Asp	Glu	Gly	Asp	Ser	Glu	Pro	Glu	Ala	Val
	1460						1465					1470			
25	Gly	Gln	Ser	Arg	Gly	Arg	Arg	Gln	Lys	Lys					
	1475						1480								

[0033] In a preferred embodiment of the present invention, the antibody or antigen binding fragment thereof selectively binds to the phosphorylated serine closest to the glutamine residue in the KENSSQ phosphorylation motif at amino acid position 167 of SEQ ID NO: 3.

[0034] The antibodies of the present invention can be monoclonal or polyclonal.

[0035] Procedures for raising polyclonal antibodies are well known in the art. Typically, such antibodies are raised by administering a peptide containing the epitope of interest, *i.e.*, a tyrosine phosphorylated SQEY peptide or serine phosphorylated KENSSQ peptide, subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100 μ l per site at six different sites. Injected material will contain synthetic surfactant adjuvant pluronic polyols, or pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are bled approximately every two weeks after the first injection and periodically boosted with the same antigen three times every six weeks. A sample of serum is collected 10 days after each boost and polyclonal antibodies are recovered by affinity chromatography using the corresponding antigen to capture the antibody. This and other procedures for raising polyclonal antibodies are disclosed in

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ANTIBODIES: A LABORATORY MANUAL (Harlow et al. eds., 1988), which is hereby incorporated by reference in its entirety.

[0036] A monoclonal antibody is obtained from a substantially homogeneous population of antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired activity (See U.S. Patent No. 4,816,567 to Cabilly et al., and Morrison et al., "Chimeric Human Antibody Molecules: Mouse Antigen-Binding Domains with Human Constant Region Domains," *Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984) which are hereby incorporated by reference in their entirety).

[0037] Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler et al., "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity," *Nature* 256:495-7 (1975) or ANTIBODIES: A LABORATORY MANUAL (Harlow et al. eds., 1988), which are hereby incorporated by reference in their entirety. In a hybridoma method, a mouse or other appropriate host animal, is immunized with an immunizing agent to elicit lymphocytes to produce antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*. In accordance with the present invention, the immunizing agent comprises a tyrosine phosphorylated SQEY peptide sequence or a serine phosphorylated KENSSQ sequence, where the serine closest to the glutamine residue is phosphorylated.

[0038] In addition to the traditional approaches for generating monoclonal antibodies, which depend on the availability of purified protein or peptide for use as the immunogen, more recently developed DNA based immunizations are also suitable for generating the antibodies of the present invention. In this approach, DNA-based immunization can be used, where DNA encoding the SQEY or KENSSQ motifs are

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expressed as a fusion protein with human IgG1 and injected into the host animal according to methods known in the art (*e.g.*, Kilpatrick et al., "Gene Gun Delivered DNA-Based Immunizations Mediate Rapid Production of Murine Monoclonal Antibodies to the Flt-3 Receptor," *Hybridoma* 17(6):569-76 (1998) and Kilpatrick et al., "High-Affinity Monoclonal Antibodies to PED/PEA-15 Generated Using 5 Micrograms of DNA," *Hybridoma* 19(4):297-302 (2000), which are hereby incorporated by reference in their entirety. Alternatively, the nucleic acid sequences encoding the SQEY or KENSSQ motifs be can expressed in a baculovirus expression system. The advantages to this system include ease of generation, high levels of expression, and post-translational modifications that are highly similar to those seen 10 in mammalian systems.

[0039] Generally, peripheral blood lymphocytes are used in methods of producing monoclonal antibodies if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The 15 lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (JAMES W. GODING, MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (Academic Press 1986), which is hereby incorporated by reference in its entirety). Immortalized cell lines are usually transformed mammalian cells, including myeloma cells of rodent, bovine, equine, and 20 human origin. The hybridoma cells are cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. Preferred immortalized cell lines (*e.g.*, murine myeloma lines) are those that fuse efficiently and support stable high level expression of antibody by the selected antibody-producing cells. Human myeloma and mouse- 25 human heteromyeloma cell lines have also been described for the production of human monoclonal antibodies (Kozbor et al., "A Human Hybrid Myeloma for Production of Human Monoclonal Antibodies," *J. Immunol.* 133:3001-5 (1984) and MONOCLONAL ANTIBODY PRODUCTION TECHNIQUES AND APPLICATIONS (L.B. Shook ed., 1987), which are hereby incorporated by reference in their entirety). The culture 30 medium in which the hybridoma cells are cultured can be assayed for the presence of monoclonal antibodies directed against the phosphotyrosine of the SQEY motif or the phosphoserine of the KENSSQ motif. Preferably, the binding specificity of

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monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA), or chemiluminescence assays. Such techniques and assays are known in the art, and are fully described in

5 ANTIBODIES: A LABORATORY MANUAL (Harlow et al. eds., 1988), which is hereby incorporated by reference in its entirety.

[0040] After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution or FACS sorting procedures and grown by standard methods. Suitable culture media for this purpose include, for example, Dulbecco's

10 Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

[0041] The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, protein G,

15 hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0042] The monoclonal antibodies of the present invention may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567 to Cabilly et al, which is hereby incorporated by reference in its entirety. DNA

20 encoding the monoclonal antibodies can be readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected

25 into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, plasmacytoma cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells, using the appropriate vectors described herein. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain

30 constant domains in place of the homologous murine sequences or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide (See U.S. Patent No. 4,816,567 to Cabilly et al,

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which is hereby incorporated by reference in its entirety). Optionally, such a non-immunoglobulin polypeptide is substituted for the constant domains of an antibody or substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having
5 specificity for either the phosphotyrosine of the SQEY motif or the phosphoserine of the KENSSQ motif and another antigen-combining site having specificity for a different antigen.

[0043] The antibodies of the present invention may be whole immunoglobulin (*i.e.*, an intact antibody) of any class. Native antibodies are usually heterotetrameric
10 glycoproteins, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a
15 variable domain (V(H)) followed by a number of constant domains. Each light chain typically has a variable domain at one end (V(L)) and a constant domain at its other end. Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of human immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these
20 may be further divided into subclasses (isotypes), *e.g.*, IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2.

[0044] The variable regions of the heavy and light chains form a cleft which comprises the antigen binding domain of antibody. Antibodies of the present invention can be mono-, bi-, or multivalent (*i.e.*, having one, two, or multiple antigen
25 binding domains).

[0045] In addition to whole antibodies, the present invention encompasses chimeric antibodies, hybrid antibodies, and fragments, such as scFv, sFv, F(ab')₂, Fab', Fab and the like, including hybrid fragments. Thus, fragments of the antibodies that retain phosphotyrosine or phosphoserine binding activity are included within the
30 meaning of antibody or antigen binding fragment thereof. Such antibodies and fragments can be made and screened for specificity and activity by techniques known

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in the art (*see* ANTIBODIES: A LABORATORY MANUAL (Harlow et al. eds., 1988), which is hereby incorporated by reference in its entirety).

[0046] Monovalent antibodies can be generated by *in vitro* digestion of whole antibodies to produce fragments, *i.e.*, Fab fragments, using routine techniques known in the art. For instance, digestion can be performed using papain as described in WO94/29348 to Landon, and ANTIBODIES: A LABORATORY MANUAL (Harlow et al. eds., 1988), which are hereby incorporated by reference in their entirety. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields a fragment, called the F(ab')₂ fragment, that has two antigen combining sites and is still capable of cross-linking antigen. Methods for generating stable monovalent antibody fragments for therapeutic utility are further described in WO/2005063816 to Huang et al., which is hereby incorporated by reference in its entirety.

[0047] The Fab fragments produced by antibody digestion contain the constant domains of the light chain and the first constant domain of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain domain including one or more cysteines from the antibody hinge region. The F(ab')₂ fragment is a bivalent fragment comprising two Fab' fragments linked by a disulfide bridge at the hinge region. Other chemical couplings of antibody fragments are also known.

[0048] An isolated immunogenically specific paratope or fragment of the antibody is also provided. A specific immunogenic epitope of the antibody can be isolated from the whole antibody by chemical or mechanical disruption of the molecule. The purified fragments thus obtained are tested to determine their immunogenicity and specificity. Immunoreactive paratopes of the antibody, optionally, are synthesized directly. An immunoreactive fragment is defined as an amino acid sequence of at least about two to five consecutive amino acids derived from the antibody amino acid sequence.

[0049] The antibodies of the present invention can be generated in a non-human species and "humanized" for administration in humans. Humanized forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin

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chains or fragments thereof which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins in which residues of the complementary determining region (CDR) are replaced by residues from a CDR of a non-human species such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Methods for humanizing non-human antibodies are well known in the art as described in U.S. Patent No. 4,816,567 to Cabilly et al.; Jones et al., "Replacing the Complementarity-Determining Regions in a Human Antibody with those From a Mouse," *Nature* 321:522-525 (1986); Riechmann et al., "Reshaping Human Antibodies for Therapy," *Nature* 332:323-327 (1988); and Verhoeyen et al., "Reshaping Human Antibodies: Grafting an Antilysozyme Activity," *Science* 239:1534-1536 (1988), which are hereby incorporated by reference in their entirety.

[0050] Another aspect of the present invention relates to applicants' discovery of a novel kinase domain within the N-terminal region of the WSTF protein. As described herein, WSTF is a component of the WICH ATP-dependent chromatin remodeling complex. The full-length amino acid sequence of the human WSTF protein is set forth *supra* in SEQ ID NO:3. The novel kinase domain of the WSTF is found within amino acids 1-391 of the full length WSTF protein sequence of SEQ ID NO:3 and comprises two motifs, a conserved N-terminal WAC domain and a more divergent C-terminal domain. These two motifs are linked by a long loop region where the ATM phosphorylation site resides (S167 in KENSSQ). Accordingly, another aspect of the present invention relates to an isolated polypeptide having an amino acid sequence of the novel WSTF kinase domain as set forth below as SEQ ID NO:6.

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

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

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Ser Asp Phe Leu Leu Asp Pro Tyr Lys Tyr Met Thr Leu Asn Pro Ser
 290 295 300
 5
 Thr Lys Arg Lys Asn Thr Gly Ser Pro Asp Arg Lys Pro Ser Lys Lys
 305 310 315 320
 10 Ser Lys Thr Asp Asn Ser Ser Leu Ser Ser Pro Leu Asn Pro Lys Leu
 325 330 335
 15 Trp Cys His Val His Leu Lys Lys Ser Leu Ser Gly Ser Pro Leu Lys
 340 345 350
 20 Val Lys Asn Ser Lys Asn Ser Lys Ser Pro Glu Glu His Leu Glu Glu
 355 360 365
 Met Met Lys Met Met Ser Pro Asn Lys Leu His Thr Asn Phe His Ile
 370 375 380
 25 Pro Lys Lys Gly Pro Pro Ala
 385 390

[0051] The kinase domain of the human WSTF protein of SEQ ID NO:6 is
 30 encoded by the nucleic acid sequence set forth below as SEQ ID NO:5.

atggcgccgc tcttgggccg caagcccttc ccgctggtga agccgttgcc cggagaggag 60
 35 ccgctcttca ccatcccgca cactcaggag gccttccgca cccgggaaga gtatgaagcc 120
 cgcttggaag ggtacagtga ggcgatttgg acgtgcaaga gtactggaag cagtcagcta 180
 acacacaagg aagcctggga ggaagaacag gaagttgctg agcttttgaa ggaggagttt 240
 40 cctgcctggt atgagaagct tgttctggaa atggttcacc ataacacagc ctccttagag 300
 aagttagtag atactgcttg gttggagatc atgaccaa atgctgtggg agaagagtgt 360
 45 gacttcgagg ttgggaagga gaaaatgctc aaggtgaaga ttgtgaagat tcatcctttg 420
 gagaaagtgg atgaagaggc cactgagaag aaatctgatg gtgcctgtga ttctccatca 480
 agtgacaaag agaactccag tcagattgct caggaccatc agaagaagga gacagttgtg 540
 50 aaagaggatg aaggaaggag agagagtatt aatgacagag cacgtagatc gccacgaaaa 600
 cttcctactt cattaaaaaa aggagaaaagg aaatgggctc ctccaaaatt tctgcctcac 660
 55 aaatatgatg tgaaactaca aaatgaagat aagatcatca gtaacgtgcc agcagacagc 720
 ttgattcgta cagagcgccc accaaataag gagatagttc gatactttat acggcataat 780
 gcattacgag ctggtactgg tgaaaatgca ccttgggtcg tagaagatga attggtgaag 840
 60 aaatactctc tgcccagcaa gttcagtgac tttttacttg atccatacaa gtatatgact 900

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ctcaaccctt ctactaagag gaagaatact ggatccccag acaggaagcc ctcaaagaaa      960
tccaagacag acaactcttc tcttagttca ccactaaatc ctaagttatg gtgtcacgta      1020
5  cacttgaaga agtcattgag tggctcgcca ctcaaagtga agaactcaaa gaattccaaa      1080
tctcctgaag aacatctaga agaaatgatg aagatgatgt cgccaataa gctgcacact      1140
10 aactttcaca ttcttaaaaa aggccacact gcc                                1173

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[0052] Accordingly, the present invention also relates to an isolated nucleic acid molecule having a nucleotide sequence of SEQ ID NO:5. In a preferred embodiment, the isolated nucleic acid molecule of the present invention has at least 80% nucleic acid sequence identity to the nucleotide sequence of SEQ ID NO: 5. More preferably, the isolated nucleic acid molecule of the present invention has at least 90% nucleic acid sequence identity to the nucleotide sequence of SEQ ID NO:5. Most preferably, the isolated nucleic acid molecule of the present invention has at least 95% nucleic acid sequence identity to the nucleotide sequence of SEQ ID NO:5.

[0053] In an alternative embodiment, the isolated polypeptide molecule of the present invention is derived from a WSTF kinase domain consensus sequence. A WSTF kinase domain consensus sequence was generated by aligning the putative kinase regions of WSTF proteins from various vertebrates and invertebrates as shown in Figure 13. The resulting amino acid sequence of the WSTF consensus sequence is set forth below as SEQ ID NO: 7, where X is any amino acid.

```

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Lys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1          5          10          15
30
Xaa Xaa Xaa Xaa Xaa Xaa Phe Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
20          25          30
35
Xaa Xaa Xaa Xaa Glu Glu Tyr Glu Xaa Arg Leu Glu Arg Tyr Xaa Glu
35          40          45
40
Arg Ile Trp Thr Cys Lys Ser Thr Gly Ser Ser Gln Leu Thr His Xaa
50          55          60
45
Xaa Xaa Xaa Xaa Xaa Glu Xaa Glu Val Xaa Glu Leu Leu Lys Glu Glu
65          70          75          80
Phe Pro Xaa Trp Xaa Glu Lys Leu Val Leu Glu Xaa Val His His Asn

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				85					90					95		
5	Thr	Xaa	Ser	Leu	Glu	Lys	Leu	Val	Asp	Xaa	Ala	Trp	Xaa	Glu	Ile	Xaa
				100					105					110		
10	Thr	Lys	Xaa	Ala	Val	Gly	Glu	Xaa	Cys	Asp	Phe	Xaa	Val	Gly	Xaa	Xaa
			115					120					125			
15	Lys	Xaa	Leu	Xaa	Xaa	Lys	Ile	Val	Lys	Xaa	His	Pro	Leu	Xaa	Xaa	Xaa
		130					135					140				
20	Xaa	Xaa	Lys	Glu	Asn	Ser	Ser	Gln	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
					165					170					175	
25	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
				180					185				190			
30	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Lys	Lys	Xaa
			195					200					205			
35	Xaa	Xaa	Lys	Trp	Xaa	Pro	Pro	Lys	Phe	Leu	Pro	His	Lys	Tyr	Asp	Val
		210					215					220				
40	Lys	Leu	Xaa	Asn	Glu	Asp	Lys	Ile	Ile	Ser	Xaa	Val	Pro	Ala	Asp	Xaa
		225				230					235					240
45	Leu	Xaa	Arg	Thr	Glu	Arg	Pro	Pro	Asn	Lys	Glu	Ile	Xaa	Arg	Tyr	Phe
					245					250					255	
50	Ile	Arg	His	Asn	Ala	Leu	Arg	Ala	Gly	Xaa	Gly	Glu	Xaa	Xaa	Pro	Trp
				260					265					270		
55	Val	Val	Glu	Asp	Glu	Leu	Val	Lys	Lys	Tyr	Xaa	Leu	Pro	Ser	Lys	Phe
			275					280					285			
60	Ser	Asp	Phe	Leu	Leu	Asp	Pro	Xaa	Lys	Xaa	Xaa	Xaa	Xaa	Asn	Pro	Ser
		290					295					300				
65	Thr	Lys	Arg	Lys	Xaa	Xaa	Gly	Ser	Pro	Xaa	Xaa	Lys	Pro	Ser	Lys	Lys
		305				310					315					320

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	Xaa	Lys	Xaa	Xaa	Xaa	Xaa	Ser	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
					325					330					335	
5	Trp	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
					340					345					350	
10	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
					355					360					365	

[0054] Using standard recombinant cloning technology and techniques well known in the art, and described in the Examples below, the isolated nucleic acid molecules encoding the WSTF kinase domain of the present invention can be inserted into an expression vector and transformed into a host cell to facilitate, *inter alias*, WSTF kinase domain expression and peptide purification.

[0055] An isolated nucleic acid molecule of the present invention is inserted into an expression system to which the molecule is heterologous. The heterologous nucleic acid molecule is inserted into the expression system or vector in proper sense (5'→3') orientation relative to the promoter and any other 5' regulatory molecules. The preparation of the nucleic acid constructs can be carried out using standard cloning methods well known in the art as described by SAMBROOK AND RUSSELL, MOLECULAR CLONING: A LABORATORY MANUAL (Cold Springs Laboratory Press, 1989), which is hereby incorporated by reference in its entirety. U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference in its entirety, also describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase.

30 **[0056]** Suitable expression vectors include those which contain replicon and control sequences that are derived from species compatible with a chosen host cell. For example, if *E. coli* is used as a host cell, plasmids such as pUC19, pUC18 or pBR322 may be used. As described herein, full length WSTF protein was expressed and purified using a baculovirus system. Therefore, appropriate transfer vectors
35 compatible with insect host cells include, pVL1392, pVL1393, pAcGP67 and pAcSecG2T, which incorporate a secretory signal fused to the desired protein, and pAcGHLT and pAcHLT, which contain GST and 6xHis tags (BD Biosciences, Franklin Lakes, NJ). Other suitable expression vectors are described in SAMBROOK

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AND RUSSELL, MOLECULAR CLONING: A LABORATORY MANUAL (Cold Springs Laboratory Press, 1989), which is hereby incorporated by reference in its entirety. Many known techniques and protocols for manipulation of nucleic acids, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of

5 DNA into cells and gene expression, and analysis of proteins, are described in detail in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Fred M. Ausubel et al. eds., 1992), which is hereby incorporated by reference in its entirety.

[0057] Different genetic signals and processing events control many levels of gene expression (*e.g.*, DNA transcription and messenger RNA (“mRNA”) translation). Transcription of DNA is dependent upon the presence of a promoter, which is a DNA sequence that directs the binding of RNA polymerase, and thereby promotes mRNA synthesis. Promoters vary in their “strength” (*i.e.*, their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters to obtain a high level of transcription and, hence, expression and

15 kinase activity. Therefore, depending upon the host system utilized, any one of a number of suitable promoters may also be incorporated into the expression vector carrying the nucleic acid molecules of the present invention. For instance, when using *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *recA* promoter, ribosomal RNA promoter, the

20 P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5* (*tac*) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene. When

25 using insect cells, suitable baculovirus promoters include late promoters, such as 39K protein promoter or basic protein promoter, and very late promoters, such as the p10 and polyhedron promoters. In some cases it may be desirable to use transfer vectors containing multiple baculoviral promoters.

[0058] Translation of mRNA in prokaryotes depends upon the presence of the proper prokaryotic signals, which differ from those of eukaryotes. Efficient

30 translation of mRNA in prokaryotes requires a ribosome binding site called the Shine-Dalgarno (“SD”) sequence on the mRNA. This sequence is a short nucleotide

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sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, *Methods in Enzymology*, 68:473 (1979), which is hereby incorporated by reference in its entirety.

[0059] Host cells suitable for expressing or propagating the nucleic acid construct encoding the WSTF kinase domain include any one of the more commonly available gram negative bacteria. Suitable microorganisms include *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella gastroenteritis* (typhimurium), *S. typhi*, *S. enteritidis*, *Shigella flexneri*, *S. sonnei*, *S. dysenteriae*, *Neisseria gonorrhoeae*, *N. meningitidis*, *Haemophilus influenzae*, *H. pleuropneumoniae*, *Pasteurella haemolytica*, *P. multilocida*, *Legionella pneumophila*, *Treponema pallidum*, *T. denticola*, *T. orales*, *Borrelia burgdorferi*, *Borrelia spp.*, *Leptospira interrogans*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *P. morganii*, *P. mirabilis*, *Rickettsia prowazekii*, *R. typhi*, *R. rickettsii*, *Porphyromonas (Bacteriodes) gingivalis*, *Chlamydia psittaci*, *C. pneumoniae*, *C. trachomatis*, *Campylobacter jejuni*, *C. intermedius*, *C. fetus*, *Helicobacter pylori*, *Francisella tularensis*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Bordetella pertussis*, *Burkholderia pseudomallei*, *Brucella abortus*, *B. suis*, *B. melitensis*, *B. canis*, *Spirillum minus*, *Pseudomonas mallei*, *Aeromonas hydrophila*, *A. salmonicida*, and *Yersinia pestis*.

[0060] In addition to bacteria cells, eukaryotic cells such as mammalian, insect, and yeast systems are also suitable host cells for transfection/transformation of the expression vector carrying an isolated nucleic acid molecule of the present invention. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. Suitable insect cell lines include those susceptible to baculoviral infection, including *Sf9* and *Sf21* cells. Methods for transforming/transfecting host cells with expression vectors are well-known in the art and depend on the host system selected, as described in SAMBROOK AND RUSSELL, MOLECULAR CLONING: A LABORATORY MANUAL (Cold Springs Laboratory Press,

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1989), which is hereby incorporated by reference in its entirety. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation, and transfection using bacteriophage. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, *e.g.*, vaccinia. For insect cells, the transfer vector containing the nucleic acid construct encoding the WSTF kinase domain is co-transfected with baculovirus DNA, such as AcNPV, to facilitate the production of a recombinant virus resulting from homologous recombination between the WSTF construct in the transfer vector and baculovirus DNA. Subsequent recombinant viral infection of *Sf* cells results in a high rate of recombinant protein production that can be readily purified using standard purification methods known in the art and described in the Examples below.

[0061] Another aspect of the present invention relates to a method of identifying cellular DNA damage in a sample. This method involves providing a cell sample and detecting the presence or absence of a phosphorylated tyrosine residue in an SQEY tyrosine phosphorylation motif (SEQ ID NO:2) in the sample. Cellular DNA damage in the cell sample is identified based on detecting the presence or absence of tyrosine phosphorylation.

[0062] In a preferred embodiment of the invention, the presence or absence of a phosphotyrosine residue in the SQEY motif at amino acid position 142 of SEQ ID NO:1 is detected.

[0063] The presence or absence of the phosphotyrosine residue, in accordance with this aspect of the present invention, can be detected using the antibody or antigen-binding fragment thereof of the present invention that selectively binds to the phosphorylated tyrosine in the SQEY sequence as described *supra*. Other pan-antibodies capable of binding phosphorylated tyrosine residues are well known in the art and commercially available from a number of companies (*e.g.*, Millipore, Billerica, Ma.; Abcam, Cambridge, MA; Santa Cruz, Santa Cruz, CA). In a preferred embodiment, the antibody or antigen binding fragment used for detecting the phosphotyrosine residue selectively binds to the phosphorylated tyrosine residue of the SQEY at amino acid position 142 of SEQ ID NO:1 as described herein.

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[0064] Although the use of a phosphotyrosine specific antibody for detecting a phosphorylated tyrosine residue is a convenient method of detection, alternative methods for detecting a phosphorylated tyrosine of the SQEY motif are known in the art and are suitable for use in this aspect of the invention. For example,

5 phosphorylated amino acids can be detected using *in situ* labeling techniques with [³²P]orthophosphate as described in POST-TRANSLATIONAL PROCESSING: A PRACTICAL APPROACH (Steve Higgins et al., eds., 1999), which is hereby incorporated by reference in its entirety. Alternatively, mass spectrometry based techniques, *e.g.*, liquid chromatography/mass spectrometry/mass spectrometry
10 (LC/MS/MS) can be employed to detect a phosphotyrosine residue in a SQEY motif.

[0065] Upon initiation of the cellular response to DNA damage, the tyrosine residue of the SQEY sequence is dephosphorylated. Therefore, in accordance with this aspect of the present invention, detecting an absence of a phosphorylated tyrosine residue in the SQEY motif indicates the presence of cellular DNA damage and
15 detecting the presence of a phosphorylated tyrosine residue in the SQEY motif indicates a lack of cellular DNA damage in the cell sample.

[0066] Another aspect of the present invention is directed to a method of identifying cellular DNA damage in a sample. This method involves providing a cell sample and detecting the presence or absence of a phosphorylated serine residue at the
20 serine closest to the glutamine residue in an KENSSQ phosphorylation motif sequence (SEQ ID NO:4). Cellular DNA damage in the cell sample is identified based on detecting the presence or absence of serine phosphorylation.

[0067] In a preferred embodiment of the invention, the presence or absence of a phosphorylated serine closest to the glutamine residue of the KENSSQ motif at
25 amino acid position 167 of SEQ ID NO:3 is detected.

[0068] Detecting the phosphorylated serine in the KENSSQ motif can be carried out using an antibody or antigen-binding fragment thereof of the present invention that binds to the phosphorylated serine closest to the glutamine residue in an KENSSQ phosphorylation motif sequence (SEQ ID NO:4), as described *supra*. In a
30 preferred embodiment, an antibody or antigen-binding fragment thereof that selectively binds to the phosphorylated serine closest to the glutamine residue in the KENSSQ motif at amino acid position 167 of SEQ ID NO:3 is used. Non-specific

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phosphoserine antibodies are also suitable for detecting the phosphorylated serine closest to the glutamine residue in the KENSSQ motif. Any of the alternative methods of detecting phosphorylated amino acid residues that are known in the art, and described *supra*, are also suitable.

5 **[0069]** Upon the initiation of DNA damage in a cell, the DNA damage-induced ATM kinase phosphorylates the serine closest to the glutamine residue of the KENSSQ motif in the WSTF amino-terminus. Therefore, in accordance with this aspect of the present invention, detecting the presence of a phosphorylated serine residue at the serine closest to the glutamine residue of the KENSSQ motif indicates
10 the presence of cellular DNA damage, whereas the absence of a phosphorylated serine residue at the serine closest to the glutamine residue of the KENSSQ motif indicates a lack cellular DNA damage.

[0070] Another aspect of the present invention relates to a method of screening candidate compounds useful for modulating WSTF kinase activity. This
15 method involves providing a candidate compound and a sample, where the sample contains a WSTF protein or polypeptide having kinase activity, a protein substrate having an SQEY motif sequence, manganese, and ATP. Contacting the candidate compound with the sample and detecting the presence or absence of a phosphorylated tyrosine residue in the SQEY tyrosine phosphorylation motif (SEQ ID NO:2)
20 identifies compounds useful for modulating WSTF kinase activity.

[0071] In accordance with this aspect of the invention, detecting the presence or absence of a phosphorylated tyrosine residue in the SQEY motif can be carried out using any of the aforementioned techniques and antibodies. In addition, phosphorylation can also be detected indirectly by measuring the depletion of ATP
25 from the reaction as described by Munagala et al., "Identification of Small Molecule Ceramide Kinase Inhibitors Using a Homogeneous Chemiluminescence High Throughput Assay," *Assay Drug Dev Tech* 5:65-73 (2007), which is hereby incorporated by reference in its entirety. Alternatively, the incorporation of labeled ATP (*i.e.*, ³²P-ATP) into the protein substrate, as described herein, can also be
30 measured.

[0072] In a preferred embodiment, the sample used in the method of screening candidate compounds useful for modulating WSTF kinase activity contains an

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isolated WSTF polypeptide of SEQ ID NO:6 or SEQ ID NO:7. In addition, the sample preferably contains an isolated H2A.X protein having the amino acid sequence of SEQ ID NO:1, and phosphorylation of the tyrosine residue in the SQEY motif at amino acid position 142 of SEQ ID NO:1 is detected.

5 **[0073]** The above method of screening candidate compounds useful for modulating WSTF activity can be carried out using a whole cell sample. Any eukaryotic or prokaryotic cell sample expressing a SQEY motif sequence is suitable for use, including mammalian, insect (*e.g.*, *D. melangaster*), amphibian (*e.g.*, *x. laevis*), fungi (*e.g.*, yeast), or bacteria cells. In a preferred embodiment, the cell
10 sample comprises one or more yeast cells containing a genetically modified H2A.X gene. As described herein, a genetically modified *S. cerevisiae* strain containing a leucine to tyrosine mutation in its H2A.X SQEL motif (Figure 1A; SEQ ID NO: 20) is an exemplary cell sample for use in this screening method.

[0074] Detecting the absence of a phosphorylated tyrosine residue in the
15 SQEY motif identifies a compound useful for inhibiting WSTF kinase activity. To identify candidate compounds that mimic WSTF kinase activity, the WSTF protein or polypeptide having kinase activity is excluded from the sample. Alternatively, a cell sample which does not express endogenous WSTF or a cell sample in which endogenous WSTF expression is knocked-down using an siRNA or other
20 biomolecular approach is used. When using a sample void of WSTF kinase activity, detecting the presence of a phosphorylated tyrosine residue in the SQEY motif identifies a compound that mimics WSTF kinase activity.

[0075] The present invention is also directed to a method of screening candidate compounds useful for modulating cellular DNA damage repair. This
25 method involves providing a candidate compound and a cell sample and contacting the candidate compound with the cell sample. The presence or absence of a phosphorylated tyrosine residue in a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) in the cell sample is detected, and candidate compounds useful for modulating cellular DNA damage repair are identified based on the presence or absence of
30 tyrosine phosphorylation.

[0076] Methods for detecting the presence or absence of a phosphorylated tyrosine residue in a SQEY motif are described *supra*. In a preferred embodiment,

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phosphorylation of the tyrosine residue in the SQEY motif at amino acid position 142 of SEQ ID NO:1 is detected.

[0077] In accordance with this aspect of the present invention, detecting the absence of a phosphotyrosine residue in the SQEY motif identifies a candidate
5 compound that induces DNA damage repair. Alternatively, a compound that inhibits DNA damage repair is identified by contacting the candidate compound and cell sample in the presence of a known DNA damaging agent. Any known DNA damaging agent is suitable for use in this method (CASARETT & DOULL'S TOXICOLOGY THE BASIC SCIENCE OF POISONS (Curtis D. Klaassen ed., 1996) which is
10 hereby incorporated by reference in its entirety). Following exposure of the cell sample to a known DNA damaging agent, the detection of a phosphorylated tyrosine residue in the SQEY motif identifies a candidate compound that inhibits the cellular DNA damage repair pathway.

[0078] As described *supra*, any number of cell samples can be utilized in this
15 method (mammalian, insect, yeast, etc.). In a preferred embodiment the genetically modified *S. cerevisiae* H2A L132Y mutant strain is utilized.

[0079] Another aspect of the present invention relates to a method of screening candidate compounds useful for treating cancer. This method involves providing a candidate compound and a cell sample and contacting the candidate
20 compound with the cell sample. The presence or absence of a phosphorylated tyrosine residue in an SQEY tyrosine phosphorylation motif (SEQ ID NO:2) in the sample is detected and candidate compounds useful for treating cancer are identified based on the presence or absence of tyrosine phosphorylation.

[0080] Methods for detecting the presence or absence of a phosphorylated
25 tyrosine residue in an SQEY motif are described *supra*. In a preferred embodiment, phosphorylation of the tyrosine residue in the SQEY motif at amino acid position 142 of SEQ ID NO:1 is detected.

[0081] Preferably, the cell sample is a cancer cell sample. The cancer cell sample can be derived directly from a cancerous tissue (*i.e.*, primary cancer cell) or
30 can be one of the many established cancer cell lines that are well known in the art. Preferably, the cancer cell sample, is a human cancer cell sample, however, any mammalian cancer cell is suitable.

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[0082] Detecting a phosphorylated tyrosine residue in the SQEY motif in the presence of a candidate compound, but not in its absence identifies a compound that is suitable for treating cancer. Such identified compounds are particularly suitable as adjunct therapies to conventional radiotherapy and chemotherapeutic approaches to cancer as described in more detail below.

[0083] In an alternative embodiment, detecting a dephosphorylated tyrosine residue in the SQEY motif in the presence of a candidate compound but not in its absence also identifies compounds that are suitable for treating cancer. Identified compounds are suitable for administration following radiotherapy and chemotherapeutic treatments to promote DNA repair in non-cancerous cells exposed to such therapy. In addition, identified compounds are appropriate for subjects having cancer who are not receiving radiotherapy or chemotherapeutic treatments.

[0084] The present invention is also directed to a method of modulating cellular DNA damage repair. This method involves administering to a cell an agent which modulates tyrosine phosphorylation of a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) under conditions effective to modulate cellular DNA damage repair.

[0085] In a preferred embodiment of this aspect of the present invention, the agent modulates tyrosine phosphorylation of the tyrosine residue in the SQEY motif at amino acid position 142 of SEQ ID NO:1 in the cell. An agent that reduces tyrosine phosphorylation of the SQEY is an agent that enhances DNA damage repair. Agents useful for enhancing DNA damage repair include WSTF kinase domain inhibitors and recombinant phosphatase proteins or peptides.

[0086] WSTF kinase domain inhibitors that are suitable for carrying out the present invention include nucleic acid, protein or peptide, or small molecule inhibitors. Nucleic acid inhibitors of the WSTF kinase domain include antisense RNA molecules, short hairpin RNA molecules (shRNA), or small interfering RNA (siRNA) molecules.

[0087] siRNA are double stranded synthetic RNA molecules approximately 20-25 nucleotides in length with short 2-3 nucleotide 3' overhangs on both ends. The double stranded siRNA molecule represents the sense and anti-sense strand of a portion of the target mRNA molecule, in this case a portion of the WSTF mRNA sequence. siRNA molecules are typically designed to a region of the mRNA target

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approximately 50-100 nucleotides downstream from the start codon. Upon introduction into a cell, the siRNA complex triggers the endogenous RNA interference (RNAi) pathway, resulting in the cleavage and degradation of the target mRNA molecule. Various improvements of siRNA compositions, such as the incorporation of modified nucleosides or motifs into one or both strands of the siRNA molecule to enhance stability, specificity, and efficacy have been described and are suitable for use in accordance with this aspect of the invention (*see e.g.*, WO2004015107 to Giese et al., WO2003070918 to McSwiggen et al., WO199839352 to Imanishi et al., U.S. Patent Application Publication No. 2002/0068708 to Jesper et al.; U.S. Patent Application Publication No. 2002/0147332 to Kaneko et al; and U.S. Patent Application Publication No. 2008/0119427 to Bhat et al., which are all hereby incorporated by reference in their entirety).

[0088] Short or small hairpin RNA molecules are longer RNA sequences that make a tight hairpin turn. Like siRNA, they silence gene expression via the cellular RNA interference pathway. shRNA is cleaved by cellular machinery into siRNA. A suitable shRNA molecule that can be used in accordance with the methods of the present invention is provided as SEQ ID NO: 21 below.

tgctgttgac agtgagcgcg gagatacttc gatactttat tagtgaagcc acagatgtaa 60
taaagtatcg aagtatctcc ttgcctactg cctcgga 97

[0089] Alternatively, the WSTF kinase domain inhibitor can be a peptide or protein inhibitor, such as an antibody. In a preferred embodiment, the protein inhibitor of the WSTF kinase domain is an intrabody, which selectively recognizes and binds to the intracellular WSTF kinase domain, comprising amino acids 1-391 of SEQ ID NO:3. A preferred intrabody for use in accordance with the present invention would selectively recognize and bind to an epitope of the WSTF kinase domain comprising amino acids 330-345 of SEQ ID NO:3.

[0090] Intrabodies are generally obtained by selecting a single variable domain from variable regions of an antibody having two variable domains (*i.e.*, a heterodimer of a heavy chain variable domain and a light chain variable domain). Methods for obtaining heavy chain-light chain heterodimers are described by Kohler et al., "Continuous Cultures of Fused Cells Secreting Antibody of Predefined

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Specificity,” *Nature* 256:495-7 (1975); Campbell et al., *Monoclonal Antibody Technology The Production and Characterization of Rodent and Human Hybridomas*, in, *LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY* (Burdon et al., eds., 1985), which are hereby incorporated by reference in their entirety. Single chain Fv fragments which are the minimal recombinant antigen-binding fragments of antibodies are the most commonly used format for intrabody development. However, other suitable formats include Fab fragments, ScFv-Ck fusion proteins, single chain diabodies, V_H-C_H1 fragments, and even whole IgG molecules (see Kontermann R.E., “Intrabodies as Therapeutic Agents,” *Methods* 34:163-70 (2004), which is here by incorporated by reference in its entirety).

[0091] Intrabodies can be bi- or multi-functional. In addition to the antigen binding region, intrabodies can comprise other polypeptide regions defining a bioactive or function domain. Suitable functional domains include an enzyme, *e.g.*, a protease, which can lead to the proteolysis of the WSTF protein. In another embodiment, the intrabody comprises a targeting signal, *e.g.*, ubiquitin, which can target the WSTF protein to a proteasome for subsequent destruction. In yet another embodiment, the intrabody comprises a targeting signal that is capable of retargeting the intrabody bound WSTF protein to another cellular locale. Such a locale may be cytoplasmic, nuclear, lysosomal, plasma membrane-associated, endoplasmic reticulum-associated, peroxisomal, or proteasomal. In addition, the intrabodies or binding molecules of the invention may encompass any art recognized targeting signal for altering the cellular location of a heterologous polypeptide.

[0092] The intrabodies can be obtained from phage display, yeast surface display, or ribosome surface display. Methods for producing libraries of intrabodies and isolating intrabodies of interest are further described in U.S. Published Patent Application No. 20030104402 to Zauderer and U.S. Published Patent Application No. 20050276800 to Rabbitts, which are hereby incorporated by reference in their entirety. Methods for improving the stability and affinity binding characteristics of intrabodies are described in WO/2008/070363 to Zhenping and Contreras-Martinez et al., “Intracellular Ribosome Display via SecM Translation Arrest as a Selection for Antibodies with Enhanced Cytosolic Stability,” *J. Mol. Biol.* 372(2):513-24 (2007), which are hereby incorporated by reference in their entirety.

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[0093] Alternatively, the WSTF kinase domain inhibitor can be a small molecule. ATP analogs, such as ATP- γ -S, ATP- γ -N, AP.PNP, or any of those described in U.S. Patent No. 5,955,447 to Ingall et al. and Bagshaw C., "ATP Analogues at a Glance," *J Cell Science* 114:459-460 (2001) which are hereby

5 incorporated by reference in their entirety, are suitable for use in accordance with this aspect of the invention.

[0094] In an alternative embodiment of this aspect of the present invention, the agent that reduces tyrosine phosphorylation of the SQEY motif is a recombinant phosphatase protein, an active phosphatase polypeptide fragment thereof, or a nucleic acid molecule encoding the recombinant phosphatase protein or active phosphatase polypeptide fragment thereof. An exemplary recombinant phosphatase protein for reducing tyrosine phosphorylation of an SQEY motif is any phosphatase in the family of Eyes Absent (EYA) protein phosphatases, including mammalian EYA-1, EYA-2, EYA-3, and EYA-4, and *Drosophila* EYA proteins. In a preferred embodiment of the present invention, tyrosine phosphorylation of the SQEY motif of SEQ ID NO:1 (H2A.X) is reduced using recombinant EYA-2 or EYA-3 phosphatase proteins as described by Krishnan et al., "Dephosphorylation of the C-terminal Tyrosyl Residue of the DNA Damage-Related Histone H2A.X is Mediated by the Protein Phosphatase Eyes Absent," *J Biol Chem* 284(24):16066-16070), which is hereby incorporated by reference in its entirety. The nucleic acid and amino acid sequences of human EYA protein phosphatases are well known in the art (*see* NCBI Genbank Accession Nos. NG_011735 for EYA1, NG_011673 for EYA-2, and NG_011596 for EYA-4, and NCBI Reference Sequence Nos. NM_001990 and NP_001981 for EYA-3, which are hereby incorporated by reference in their entirety). Methods of making recombinant proteins or peptides are described *supra*.

[0095] A related aspect of the invention involves a method of treating a subject having cancer. This method involves administering to the subject having cancer, an agent that modulates tyrosine phosphorylation of an SQEY tyrosine phosphorylation motif (SEQ ID NO:2) under conditions effective to treat the subject having cancer. In a preferred embodiment, the agent modulates tyrosine phosphorylation of the tyrosine residue in the SQEY motif at amino acid position 142 of SEQ ID NO:1.

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[0096] In accordance with this aspect of the invention, administration of agents that modulate tyrosine phosphorylation of the SQEY motif are selected based on the type and stage of cancer the subject has, and other forms of therapy available to the subject. In one embodiment of this aspect of the invention, it may be desirable to administer agents which reduce tyrosine phosphorylation of the SQEY motif, thereby promoting cellular DNA damage repair mechanisms. Such treatments are suitable following radiation or chemotherapeutic treatments to facilitate DNA repair in non-cancerous cells exposed to such therapy. In addition, agents which promote DNA damage repair are suitable for subjects having cancer who are not receiving radiation or chemotherapy treatment. Agents of the present invention which promote DNA damage repair include WSTF kinase domain inhibitors and recombinant phosphatase proteins or polypeptides that dephosphorylate tyrosine residues of a SQEY motif. Accordingly, any of the WSTF kinase domain inhibitors described *supra*, including WSTF siRNA molecules, intrabodies, or other peptide-based binding molecules or small molecules, are suitable for administration to a subject having cancer. Likewise, a recombinant EYA phosphatase protein or polypeptide, particularly a recombinant EYA-2 or EYA-3 phosphatase protein or polypeptide, as described *supra* are also suitable for administration to a subject having cancer. Pharmaceutical compositions containing WSTF inhibitors or recombinant phosphatase proteins and modes of delivery and administration of such pharmaceutical compositions for the treatment of cancer are described *infra*.

[0097] In another embodiment of this aspect of the invention, it may be desirable to suppress DNA damage repair. Treatments for a wide variety of human solid malignancies and leukemia include the administration of ionizing radiation and chemotherapy. These treatments induce DNA double-strand breaks with the goal of causing cell, in particular, tumor cell lethality. In this context, it is desirable to suppress, rather than enhance, DNA repair mechanisms to avoid therapeutic resistance to such treatments. Agents that impair or suppress DNA damage repair will enhance the efficacy of radiation and chemotherapeutic treatments. Therefore, any agent that induces tyrosine phosphorylation of the SQEY motif is suitable for suppressing DNA damage repair and can be administered to a subject having cancer in combination with the above treatments. One exemplary agent useful for suppressing DNA damage

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repair is any agent that mimics WSTF kinase domain activity or induces endogenous WSTF kinase domain activity. Other agents suitable for suppressing DNA damage repair are EYA phosphatase inhibitors.

[0098] Agents mimicking WSTF kinase domain activity include recombinant proteins or peptides and the nucleic acids encoding such recombinant proteins or peptides. A WSTF kinase domain peptide having an amino acid sequence of SEQ ID NO:6 or SEQ ID NO:7 can be synthesized chemically or produced recombinantly in a host cell using the isolated nucleic acid molecule of SEQ ID NO:5 as described *supra*, in a suitable expression vector.

10 [0099] Agents that inhibit EYA phosphatase proteins, *e.g.*, mammalian EYA-1, EYA-2, EYA-3, or EYA-4, include inhibitory nucleic acid molecules, peptides, and small molecules. Any EYA phosphatase inhibitors known in the art are suitable for use in the methods of the present invention. Exemplary EYA siRNA inhibitor molecules that are useful for inhibiting EYA mediated dephosphorylation of SQEY tyrosine phosphorylation are known in the art (see Krishnan et al.,
15 “Dephosphorylation of the C-terminal Tyrosyl Residue of the DNA Damage-Related Histone H2A.X is Mediated by the Protein Phosphatase Eyes Absent,” *J Biol Chem* 284(24):16066-16070 and Cook et al., “Tyrosine Dephosphorylation of H2AX Modulates Apoptosis and Survival Decisions,” *Nature* 458(2):591-596 (2009), which
20 are hereby incorporated by reference in their entirety). These siRNA molecules are suitable agents for inhibiting DNA damage repair and promoting apoptosis of cancerous cells in a subject.

[0100] Methods of delivering a suitable agent to a subject having cancer, whether it be a WSTF kinase inhibitor, EYA phosphatase, an agent that induces
25 WSTF kinase activity, or an EYA phosphatase inhibitor, will vary depending on the type of agent.

[0101] Intrabodies directed to WSTF or recombinant WSTF polypeptides can be administered via gene therapy. The general approach involves the introduction of a nucleic acid molecule encoding an intrabody or a nucleic acid encoding the WSTF
30 kinase domain (SEQ ID NO:5) into cells such that one or more gene products encoded by the introduced genetic material are produced in the cells to inhibit or mimic WSTF kinase activity.

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[0102] Methods of introducing nucleic acid molecule encoding the polypeptide or intrabody of interest into vectors suitable for gene therapy delivery are described *supra*. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent. No. 5,328,470 to Nabel et al., which is hereby incorporated by reference in its entirety) or by stereotactic injection (see *e.g.*, Chen et al. "Gene Therapy for Brain Tumors: Regression of Experimental Gliomas by Adenovirus Mediated Gene Transfer In Vivo," *PNAS* 91:3054-3057 (1994), which is hereby incorporated by reference in its entirety). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system. Gene therapy vectors typically utilize constitutive regulatory elements which are responsive to endogenous transcriptions factors. In a preferred embodiment, the gene therapy vectors encoding the intrabody or polypeptide is an expression vector derived from a virus that is an adenovirus, adeno-associated virus, retrovirus, lentivirus, or herpes virus. Adenoviral viral vector gene delivery vehicles can be readily prepared and utilized given the disclosure provided in Berkner, "Development of Adenovirus Vectors for the Expression of Heterologous Genes," *Biotechniques* 6:616-627 (1988) and Rosenfeld et al., "Adenovirus-Mediated Transfer of a Recombinant Alpha 1-Antitrypsin Gene to the Lung Epithelium In Vivo," *Science* 252:431-434 (1991), WO 93/07283 to Curiel et al., WO 93/06223 to Perricaudet et al., and WO 93/07282 to Curiel et al., which are hereby incorporated by reference in their entirety. Adeno-associated viral gene delivery vehicles can be constructed and used to deliver a gene to cells as described in Chatterjee et al., "Dual-Target Inhibition of HIV-1 In Vitro by Means of an Adeno-Associated Virus Antisense Vector," *Science* 258:1485-1488 (1992); Walsh et al., "Regulated High Level Expression of a Human Gamma-Globin Gene Introduced Into Erythroid Cells by an Adeno-Associated Virus Vector," *Proc. Nat'l. Acad. Sci.* 89:7257-7261 (1992); Walsh et al., "Phenotypic Correction of Fanconi Anemia in Human Hematopoietic Cells With a Recombinant Adeno-Associated Virus Vector," *J. Clin*

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- Invest.* 94:1440-1448 (1994); Flotte et al., "Expression of the Cystic Fibrosis Transmembrane Conductance Regulator From a Novel Adeno-Associated Virus Promoter," *J. Biol. Chem.* 268:3781-3790 (1993); Ponnazhagan et al., "Suppression of Human Alpha-Globin Gene Expression Mediated by the Recombinant Adeno-Associated Virus 2-Based Antisense Vectors," *J. Exp. Med.* 179:733-738 (1994); and Zhou et al., "Adeno-associated Virus 2-Mediated Transduction and Erythroid Cell-Specific Expression of a Human Beta-Globin Gene," *Gene Ther.* 3:223-229 (1996), which are hereby incorporated by reference in their entirety. *In vivo* use of these vehicles is described in Flotte et al., "Stable in Vivo Expression of the Cystic Fibrosis Transmembrane Conductance Regulator With an Adeno-Associated Virus Vector," *Proc. Nat'l Acad. Sci.* 90:10613-10617 (1993); and Kaplitt et al., "Long-Term Gene Expression and Phenotypic Correction Using Adeno-Associated Virus Vectors in the Mammalian Brain," *Nature Genet.* 8:148-153 (1994), which are hereby incorporated by reference in their entirety. Additional types of adenovirus vectors are described in U.S. Patent No. 6,057,155 to Wickham et al.; U.S. Patent No. 6,033,908 to Bout et al.; U.S. Patent No. 6,001,557 to Wilson et al.; U.S. Patent No. 5,994,132 to Chamberlain et al.; U.S. Patent No. 5,981,225 to Kochanek et al.; and U.S. Patent No. 5,885,808 to Spooner et al.; and U.S. Patent No. 5,871,727 to Curiel, which are hereby incorporated by reference in their entirety.
- 20 **[0103]** Retroviral vectors which have been modified to form infective transformation systems can also be used to deliver nucleic acid encoding a desired protein or polypeptide into a target cell. One such type of retroviral vector is disclosed in U.S. Pat. No. 5,849,586 to Kriegler et al., which is hereby incorporated by reference.
- 25 **[0104]** Regardless of the type of infective transformation system employed, it should be targeted for delivery of the nucleic acid to a specific cell type. For example, for delivery of the nucleic acid into tumor cells, a high titer of the infective transformation system can be injected directly within the tumor site so as to enhance the likelihood of tumor cell infection. The infected cells will then express the desired protein product, for example a WSTF kinase domain, to immolate the cancer cell.
- 30 **[0105]** Non-viral gene delivery vehicles are also a means to effect cell-specific delivery of the therapeutic plasmids for the present invention. These are traditionally

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antibodies or single-chain Fv antibodies that are coupled or fused to DNA complexing agents (see Uherek et al., "Long-Term Gene Expression and Phenotypic Correction Using Adeno-Associated Virus Vectors in the Mammalian Brain," *J. Biol. Chem.* 273:8835-8841 (1998); Foster et al., "HER2-Targeted Gene Transfer," *Human Gene Ther.* 8:719-727 (1997); Chen et al., "Design of a Genetic Immunotoxin to Eliminate Toxin Immunogenicity," *Gene Ther.* 2:116-123 (1995), which are hereby incorporated by reference in their entirety). This class of gene delivery vehicles also includes antibodies or their fragments coupled to liposomes (U.S. Patent Nos. 4,925,661, 4,957,735, and 6,008,202 to Huang, which are hereby incorporated by reference in their entirety).

[0106] Another approach for delivering recombinant proteins or peptides as well as siRNA molecules of the present invention directly into cells involves the use of liposomes. Liposomes are vesicles comprised of one or more concentrically ordered lipid bilayers which encapsulate an aqueous phase. They are normally not leaky, but become leaky if a hole or pore occurs in the membrane, if the membrane is dissolved or degrades, or if the membrane temperature is increased to the phase transition temperature. Current methods of drug delivery via liposomes require that the liposome carrier ultimately become permeable and release the encapsulated drug, in this case recombinant WSTF kinase polypeptide or siRNA molecule, at the target site. This can be accomplished, for example, in a passive manner wherein the liposome bilayer degrades over time through the action of various agents in the body. Every liposome composition will have a characteristic half-life in the circulation or at other sites in the body and, thus, by controlling the half-life of the liposome composition, the rate at which the bilayer degrades can be regulated.

[0107] In contrast to passive drug release, active drug release involves using an agent to induce a permeability change in the liposome vesicle. Liposome membranes can be constructed so that they become destabilized when the environment becomes acidic near the liposome membrane (see *e.g.*, Wang et al., "pH-Sensitive Immunoliposomes Mediate Target-Cell-Specific Delivery and controlled Expression of a Foreign Gene in Mouse," *Proc. Natl. Acad. Sci. USA* 84:7851 (1987), which is hereby incorporated by reference). When liposomes are endocytosed by a target cell, for example, they can be routed to acidic endosomes which will

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destabilize the liposome and result in drug release. Alternatively, the liposome membrane can be chemically modified such that an enzyme is placed as a coating on the membrane which slowly destabilizes the liposome. A liposome delivery system can also be made to accumulate at a target organ, tissue, or cell via active targeting
5 (e.g., by incorporating an antibody or hormone on the surface of the liposomal vehicle). This can be achieved according to known methods.

[0108] Different types of liposomes can be prepared according to Bangham et al., "Diffusion of Univalent Ions Across the Lamellae of Swollen Phospholipids," *J. Mol. Biol.* 13:238-252 (1965); U.S. Patent No. 5,653,996 to Hsu et al.; U.S. Patent
10 No. 5,643,599 to Lee et al.; U.S. Patent No. 5,885,613 to Holland et al.; U.S. Patent No. 5,631,237 to Dzau et al.; and U.S. Patent No. 5,059,421 to Loughrey et al., which are hereby incorporated by reference in their entirety.

[0109] An alternative approach for delivery of proteins or polypeptides involves the conjugation of the desired protein or polypeptide to a polymer that is
15 stabilized to avoid enzymatic degradation of the conjugated protein or polypeptide. Conjugated proteins or polypeptides of this type are described in U.S. Patent No. 5,681,811 to Ekwuribe, which is hereby incorporated by reference in its entirety.

[0110] Yet another approach for delivery of proteins or polypeptides involves preparation of chimeric proteins according to U.S. Patent No. 5,817,789 to Heartlein
20 et al., which is hereby incorporated by reference in its entirety. A chimeric protein suitable for use in the methods of the present invention contains a ligand binding domain and the kinase domain of WSTF (SEQ ID NO:6 or SEQ ID NO:7) or a fragment of variant thereof. The ligand binding domain is specific for cell surface receptors located on a target cell. Thus, when the chimeric protein is delivered
25 intravenously or otherwise introduced into blood or lymph, the chimeric protein will be selectively taken up and internalized by the target cell.

[0111] In practicing the method of the present invention, agents suitable for treating a subject having cancer can be administered using any method standard in the art. The agents, in their appropriate delivery form, can be administered orally,
30 intradermally, intramuscularly, intraperitoneally, intravenously, subcutaneously, or intranasally. The compositions of the present invention may be administered alone or

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with suitable pharmaceutical carriers, and can be in solid or liquid form, such as tablets, capsules, powders, solutions, suspensions, or emulsions.

[0112] The agents of the present invention may be orally administered, for example, with an inert diluent, or with an assimilable edible carrier, or it may be enclosed in hard or soft shell capsules, or it may be compressed into tablets, or they may be incorporated directly with the food of the diet. Agents of the present invention may also be administered in a time release manner incorporated within such devices as time-release capsules or nanotubes. Such devices afford flexibility relative to time and dosage. For oral therapeutic administration, the agents of the present invention may be incorporated with excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, and the like. Such compositions and preparations should contain at least 0.1% of the agent, although lower concentrations may be effective and indeed optimal. The percentage of the agent in these compositions may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of the unit. The amount of an agent of the present invention in such therapeutically useful compositions is such that a suitable dosage will be obtained.

[0113] Also specifically contemplated are oral dosage forms of the agents of the present invention. The agents may be chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the component molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the component or components and increase in circulation time in the body. Examples of such moieties include: polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone and polyproline (A. Abuchowski, *Soluble Polymer-Enzyme Adducts, in ENZYMES AS DRUGS* 367-383 (Holcenberg et al., eds., 1981), which are hereby incorporated by reference in their entirety). Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

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[0114] The tablets, capsules, and the like may also contain a binder such as gum tragacanth, acacia, corn starch, or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose, sucrose, or saccharin. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier such as a fatty oil.

5 [0115] Various other materials may be present as coatings or to modify the physical form of the dosage unit. For instance, tablets may be coated with shellac, sugar, or both. A syrup may contain, in addition to active ingredient, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye, and flavoring
10 such as cherry or orange flavor.

[0116] The agents of the present invention may also be administered parenterally. Solutions or suspensions of the agent can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. Dispersions can also be
15 prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solution, and glycols, such as propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions.
20 Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0117] Pharmaceutical formulations suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must
25 be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.
30

[0118] When it is desirable to deliver the agents of the present invention systemically, they may be formulated for parenteral administration by injection, *e.g.*,

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by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as
5 suspending, stabilizing and/or dispersing agents.

[0119] Intraperitoneal or intrathecal administration of the agents of the present invention can also be achieved using infusion pump devices such as those described by Medtronic, Northridge, CA. Such devices allow continuous infusion of desired compounds avoiding multiple injections and multiple manipulations.

10 [0120] In addition to the formulations described previously, the agents may also be formulated as a depot preparation. Such long acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt

15 [0121] The agents of the present invention may also be administered directly to the airways in the form of an aerosol. This form of administration is particularly suited for siRNA delivery. For use as aerosols, the agent of the present invention in solution or suspension may be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane,
20 or isobutane with conventional adjuvants. The agent of the present invention also may be administered in a non-pressurized form such as in a nebulizer or atomizer.

[0122] Effective doses of the compositions of the present invention, for the treatment of cancer vary depending upon many different factors, including type and stage of cancer, means of administration, target site, physiological state of the patient,
25 other medications or therapies administered, and physical state of the patient relative to other medical complications. Treatment dosages need to be titrated to optimize safety and efficacy.

EXAMPLES

[0123] The Examples set forth below are for illustrative purposes only and are not intended to limit, in any way, the scope of the present invention.

5 Example 1 - Plasmids and Cell Culture

[0124] Tyr142 mutant constructs were derived from WT human H2A.X plasmids (Open Biosystems) using Quickchange kit (Stratagene) and verified by DNA sequencing. Primers: *Y142L forward*: 5'-caagaaggccaccaggcctcccaggagctctaag-3' (SEQ ID NO: 22); *Y142L Reverse*: 5'-cttagagctcctgggaggcctgggtggccttcttg-3' (SEQ ID NO: 23); *Y142F forward*: 5'-caagaaggccaccaggcctcccaggagtctaag-3' (SEQ ID NO: 24); *Y142L Reverse*: 5'-cttagaactcctgggaggcctgggtggccttcttg-3' (SEQ ID NO: 25). These plasmids were amplified by PCR and cloned into pTOPO vectors (Invitrogen). Primers: Forward: 5'-cacctcgggcgcggcaagactg-3' (SEQ ID NO: 26) Reverse: WT: 5'-cttagtactcctgggag-3' (SEQ ID NO: 27); Y142L: 5'-cttagagctcctgggag-3' (SEQ ID NO: 28); Y142F: 5'-cttagaactcctgggag-3' (SEQ ID NO: 29).

[0125] For generating N-terminal Flag tagged constructs, the WT or mutant H2A.X coding region in the pTOPO vectors were cloned in-frame between the BamH1 and Xho1 sites of the pCMV-Tag2A vectors (Stratagene). Tagged or untagged constructs were cloned into MSCV-puro vectors (gifts from Dr. Herr's lab, University of Zurich, Switzerland). To generate constructs for recombinant protein expression in *E. Coli*, the coding regions of the WT or Y142F H2A.X of the untagged vectors (above) were cloned into pET28 vectors (Novagen), in-frame with the N-terminal 6XHis tag.

[0126] MSCV virus production and infection followed standard protocols (Zhou et al., The DNA Damage Response: Putting Checkpoints in Perspective," *Nature* 408:433-439 (2000), which is hereby incorporated by reference in its entirety). In brief, MSCV viruses were packaged in Phoenix cells and used to infect H2A.X +/- MEFs (gifts from Dr. A. Nussenzweig's Lab, NIH, Bethesda, MD), followed by puromycin selection. For each construct, three independent lines were derived. The expression levels of H2A.X were checked by immunoblot and immunofluorescence.

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[0127] The full-length WSTF (human) construct was a gift from Dr. Varga-Wewasz's lab (Babraham Institute, Cambridge, UK). To generate a series of truncated WSTF constructs for insect cell expression, the PCR fragments of the corresponding regions of the WSTF gene were cloned into a modified pFASTBac vector (Invitrogen), which contains an N-terminal GST and a C-terminal His-tag sequence. The C338A mutant (1-359) was derived from the WT 1-359 construct, using the QuickChange site-directed mutagenesis kit (Stratagene) and was verified by DNA sequencing. To generate constructs for protein expression in *E. coli*, the PCR product of the N-motif (1-205) was cloned into pGex6p vector (GE Healthcare) in-frame with the N-terminal GST tag, and the PCR product of the C-motif (208-345) was cloned into a modified pRSFDuet-1 (Novagen) vector in-frame with the N-terminal MBP tag. To generate constructs for mammalian cell expression, the PCR products (the forward primer contains a BamH1 and reverse contains a Xho1 site sequence) of the corresponding fragments of the human WSTF gene were cloned in between the BamH1 and Xho1 sites of pTAG3A vectors (Stratagene), in-frame with the N-terminal Myc epitope tag. The tagged constructs were cloned into MSCV-puro vectors.

[0128] WSTF RNAi cell lines were generated using recombinant pShag-2 vectors containing shRNA constructs targeting the mouse WSTF gene (Clone ID: V2MM_17087, Open Biosystems). Three independent lines were generated by infecting virus carrying these vectors into NIH3T3 cells and propagated under puromycin. In Figure 4, the WSTF RNAi cells were infected with recombinant MSCV viruses carrying the WT (1-359) or the mutant (C338A) constructs.

25 **Example 2 - Purification of H2A.X Containing Mononucleosome and its Associated Protein Factors**

[0129] MEF fractionation and chromatin pellet isolation (Figure 2) were performed as described (Redon et al., "Histone H2A Variants H2AX and H2AZ," *Curr Opin Genet Dev* 12:162-169 (2002), which is hereby incorporated by reference in its entirety). Chromatin pellets were briefly digested with Mnase (Sigma) and the generation of mononucleosomes was monitored by electrophoresis (Celeste et al., "Genomic Instability in Mice Lacking Histone H2AX," *Science* 296:922-927 (2002), which is hereby incorporated by reference in its entirety). Fifty micro-liter FLAG-M2

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agarose beads were used for immunoprecipitating the total mononucleosomes isolated from 2×10^8 cells. The immunocomplex was resolved on 4-12% gradient gels (Invitrogen) and then silver- or coomassie blue stained. The gel bands were isolated and subjected to mass spectrometry.

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Example 3 - Protein Identification by Mass Spectrometry

[0130] Gel-resolved proteins were digested with trypsin, batch purified on a reversed-phase micro-tip, and resulting peptide pools individually analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS) (*UltraFlex TOF/TOF*; BRUKER; Bremen, Germany) for peptide mass fingerprinting (PMF), as described (Celeste et al., "H2AX Haploinsufficiency Modifies Genomic Stability and Tumor Susceptibility," *Cell* 114:371-383 (2003), which is hereby incorporated by reference in its entirety). Selected peptide ions (m/z) were taken to search a "non-redundant" protein database (NR; 3,245,378 entries on 28 January, 2006; National Center for Biotechnology Information; Bethesda, MD) utilizing the PeptideSearch algorithm (Matthias Mann, Max-Planck Institute for Biochemistry, Martinsried, Germany; an updated version of this program is currently available as 'PepSea' from Applied Biosystems/MDS Sciex; Foster City, CA). A molecular mass range up to twice the apparent molecular weight (as estimated from electrophoretic relative mobility) was covered, with a mass accuracy restriction of less than 40 ppm, and maximum one missed cleavage site allowed per peptide. To confirm PMF results, mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples, using the UltraFlex instrument in 'LIFT' mode. Fragment ion spectra were taken to search NR using the MASCOT MS/MS Ion Search program (Reina-San-Martin et al., "H2AX is Required for Recombination Between Immunoglobulin Switch Regions but Not for Intra-Switch Region Recombination or Somatic Hypermutation," *J Exp Med* 197:1767-1778 (2003), which is hereby incorporated by reference in its entirety), version 2.0.04 for Windows (Matrix Science Ltd., London, UK). Any tentative confirmation of a PMF result thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

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Example 4 - Immunoblotting and Immunofluorescence

[0131] Cells at 70-80% confluence were exposed to an ionizing radiation source (Cs) to introduce DNA damage (at 10Gy). Samples were collected at indicated

5 time points post irradiation. Histone extraction, cell fractionation, high salt nuclear extraction and western blotting followed standard protocols (Bassing et al., "Histone H2AX: A Dosage-dependent Suppressor of Oncogenic Translocations and Tumors," *Cell* 114:359-370 (2003) and Unal et al., "DNA Damage Response Pathway Uses Histone Modification to Assemble a Double-Strand Break-Specific Cohesin Domain,"

10 *Mol Cell* 16:991-1002 (2004), which is hereby incorporated by reference in its entirety). For immunofluorescence, cells were grown on cover slips and fixed with 3% paraformaldehyde. Subsequent indirect immunofluorescent staining was achieved following standard protocols (Unal et al., "DNA Damage Response Pathway Uses Histone Modification to Assemble a Double-Strand Break-Specific Cohesin Domain,"

15 *Mol Cell* 16:991-1002 (2004), which is hereby incorporated by reference in its entirety). For WSTF staining, cells were extracted with a high salt buffer for 2 minutes at 4°C before fixing, as described (Harper et al., "The DNA Damage Response: Ten Years After," *Mol Cell* 28:739-745 (2007), which is hereby incorporated by reference in its entirety). Antibodies: γ -H2A.X, α -general H2A.X

20 and α -BRCA1 (Millipore/Upstate); α -WSTF and α -Myc (Sigma); α -BAF53 and α -SNF2H (Rockefeller University, NY, and New York University, NY, respectively).

Example 5 - Recombinant MDC1 Protein Expression and Peptide Pull-Down Assay

25 [0132] GST-MDC1-BRCT protein expression followed manufacturer's protocols (Stratagene). Peptides (unmodified "UN"): N-CPSGGKKATQASQEY-C (SEQ ID NO: 30); S139(ph): N-CPSGGKKATQApSQEY-C (SEQ ID NO: 31); Y142(ph): N-CPSGGKKATQASQEpY-C (SEQ ID NO: 32); S139(ph) & Y142(ph): N-CPSGGKKATQApSQEpY-C (SEQ ID NO: 33); Y142F: N-

30 CPSGGKKATQASQEF-C (SEQ ID NO: 34); Y142L: N-CPSGGKKATQASQEL-C (SEQ ID NO: 35); S139(ph), Y142L: N-CPSGGKKATQApSQEL-C (SEQ ID NO: 36); S139(ph), Y142F: N-CPSGGKKATQApSQEF-C (SEQ ID NO: 37)) were

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conjugated to agarose bead with Sulfolink kit (Pierce). Peptide pull-down experiments were performed as described previously (Morrison et al., "INO80 and Gamma-H2AX Interaction Links ATP-dependent Chromatin Remodeling to DNA Damage Repair," *Cell* 119:767-775 (2004), which is hereby incorporated by reference in its entirety).

Example 6 - Circular Dichroism Measurement

[0133] Circular Dichroism (CD) experiments were performed on an Aviv 62A/DS (Aviv Associates, Lakewood, NJ) spectropolarimeter. Spectra (260nm to 200nm) were collected with a cell path length of 0.1cm and bandwidth of 1nm. Samples were diluted to 10uM with 20mM Tris-HCl, pH 7.5, 100mM KCl, 2mM DTT. Protein concentrations were determined with their 280nm absorbance.

Example 7 - Automated Chemical ('Edman') Protein Sequencing

[0134] Protein samples were analyzed with a Procise 494 instrument from Applied Biosystems (AB) as described (Downs et al., "Binding of Chromatin-Modifying Activities to Phosphorylated Histone H2A at DNA Damage Sites," *Mol Cell* 16:979-990 (2004), which is hereby incorporated by reference in its entirety). Stepwise liberated PTH-amino acids were identified using an "on-line" HPLC system (AB) equipped with a PTH C18 (2.1x220 mm; 5 micron particle size) column (AB).

Example 8 - Preparation of Recombinant WSTF Protein from Insect Cells

[0135] Recombinant baculovirus carrying the full length WSTF (Flag-epitope-tagged at C terminus) was produced using the BaculoGold-BEVS Kit (BD Biosciences). The full length WSTF proteins were purified using a published protocol (van Attikum et al., "Recruitment of the INO80 Complex by H2A Phosphorylation Links ATP-Dependent Chromatin Remodeling With DNA Double-Strand Break Repair," *Cell* 119:777-788 (2004), which is hereby incorporated by reference in its entirety). The viruses that express truncated WSTF constructs were produced with the Bac-to-Bac (Invitrogen) method, all of which were tagged with an N-terminal GST tag and a C-terminal 6XHis tag. Baculoviruses were produced in Sf9 cells, and proteins were expressed in Hi5 suspension cells. For truncated WSTF

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protein purification, harvested Hi5 cells were suspended in 0.4 M KCl, 20mM Tris, pH 7.5, supplemented with the EDTA-free Complete™ Protease Inhibitor Cocktail tablet (Roche). Whole cell lysates were purified through GSTrap columns (GE Healthcare). After removal of the GST tags by PreScission Protease digestion (GE Healthcare), the protein mixtures were purified by HiTrap SP (GE Healthcare). The recombinant WSTF proteins (peaks form at ~200mM KCL, 20mM Tris pH 7.5) were further purified with Superdex 75 (GE Healthcare) in the elution buffer (50mM KCl, 20mM Tris, pH 7.5). The monomeric peaks (~60 KDa) were collected and concentrated for further use. The C338A mutant protein was expressed and purified as the WT protein described above.

Example 9 - Protein Preparation in *E. coli*

[0136] For co-expression of the N- and C-motifs of WSTF, the recombinant plasmid vectors carrying the N- and C-motif constructs (above) were transformed into *E. coli* host strain Rosetta2 (DE3) (Novagen) for protein expression under triple antibiotic selection (ampicillin, kanamycin, and chloramphenicol) in LB medium. After overnight induction by 0.4 mM isopropyl β-D-thiogalactoside at 25 °C, cells were harvested in buffer 0.4 M KCl, 20mM Tris, pH 7.5. Co-expressed proteins were affinity purified using Amylose resin (New England Biolabs, MA) against the MBP tag. Maltose-eluted samples were purified through a Superdex 200 column (GE Healthcare) in the elution buffer (50mM KCL, 20mM Tris, pH 7.5). Most proteins were eluted in a single peak that contains GST-tagged WAC N- and MBP-tagged WAC C-motif with a stoichiometry of 1:1.

[0137] The N- or C-motif were also expressed separately in Rosetta2 (DE3) (Novagen) cells in parallel. The N-motif was purified with GSTrap column and Superdex 200 (GE Healthcare) and C-motif was purified with Amylose resin (New England Biolabs, MA) and Superdex 200 (GE Healthcare). Most proteins were eluted in a monomeric peak (~50 KDa).

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Example 10 - Histone Protein Purification and *In Vitro* Assembly of Histone Octamers and Mononucleosomes

[0138] Individual free histone proteins (H2A.X, H2B, H3 and H4) were expressed and purified using a standard protocol (Kusch et al., "Acetylation by Tip60 is Required for Selective Histone Variant Exchange at DNA Lesions," *Science* 306:2084-2087 (2004), which is hereby incorporated by reference in its entirety). N-terminal 6XHis-epitope-tagged WT and F142 H2A.X proteins were purified with Ni Sepharose6 columns (GE Healthcare). The assembly of the histone octamers followed standard protocols (Kusch et al., "Acetylation by Tip60 is Required for Selective Histone Variant Exchange at DNA Lesions," *Science* 306:2084-2087 (2004), which is hereby incorporated by reference in its entirety). Four recombinant histone proteins were denatured in 8M Guanidine-HCl solutions and dialyzed to 2M NaCl. The crude histone octamers were further purified through a Superdex 75 column (GE Healthcare). Mononucleosomes were assembled as described (Park et al., "Mammalian SWI/SNF Complexes Facilitate DNA Double-Strand Break Repair by Promoting Gamma-H2AX Induction," *EMBO J* 25:3986-3997 (2006), which is hereby incorporated by reference in its entirety). The PCR products of the DNA template (nucleosome positioning sequence (clone 601) (Stewart et al., "MDC1 is a Mediator of the Mammalian DNA Damage Checkpoint," *Nature* 421:961-966 (2003), which is hereby incorporated by reference in its entirety) was initially incubated in a reaction buffer (Park et al., "Mammalian SWI/SNF Complexes Facilitate DNA Double-Strand Break Repair by Promoting Gamma-H2AX Induction," *EMBO J* 25:3986-3997 (2006), which is hereby incorporated by reference in its entirety) supplemented with 2M NaCl and diluted in the same reaction buffer stepwise until the NaCl concentration reached 200mM. The assembly of mononucleosomes was checked by electrophoresis on 5% native PAGE gels.

Example 11 - *In Vitro* Kinase Assays

[0139] Recombinant full-length (50ng-100ng) or truncated WSTF proteins (0.1-0.5ug) were incubated with free histone proteins (1-2μg), histone octamers or nucleosomes assembled *in vitro* (500ng-1μg) in 20mM Tris pH 7.4 and 150mM NaCl supplemented with γ -³²P-ATP (NEN) and 1mM MnCl₂ for 45 minutes. In Figure 10,

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0.1-1mM of MnCl₂ or MgCl₂ was supplemented as indicated. The reaction was stopped by adding 2mM EDTA. The reaction mixtures were separated by SDS-PAGE, stained with Coomassie blue and dried. The dried gels were exposed against phosphorimager screens (FujiFilm). The autoradiography images were scanned in
5 phosphorimager (FujiFilm) and analyzed by Image Reader FLA-5000.

Example 12 - Phosphorylation of H2A.X at Tyr142 is a Novel Phosphorylation Mark That is Regulated in Response to DNA Damage

[0140] A role in the DNA damage response for mammalian H2A.X (γ -
10 H2A.X) is well documented, although its regulation and underlying mechanism of action is only partially understood (reviewed in van Attikum et al., "Recruitment of the INO80 Complex by H2A Phosphorylation Links ATP-Dependent Chromatin Remodeling With DNA Double-Strand Break Repair," *Cell* 119:777-788 (2004), which is hereby incorporated by reference in its entirety). Phosphorylation of S139 in
15 the C-terminal tail of H2A.X is responsible for the recruitment of many proteins to sites of DNA damage and directly recruits Mdc1 (Stewart et al., "MDC1 is a Mediator of the Mammalian DNA Damage Checkpoint," *Nature* 421:961-966 (2003); Goldberg et al., "MDC1 is Required for the Intra-S-Phase DNA Damage Checkpoint," *Nature* 421:952-956 (2003); Lou et al., "MDC1 is Coupled to Activated CHK2 in
20 Mammalian DNA Damage Response Pathways," *Nature* 421:957-961 (2003); Stucki et al., "MDC1 Directly Binds Phosphorylated Histone H2AX to Regulate Cellular Responses to DNA Double-Strand Breaks," *Cell* 123:1213-1226 (2005); Lou et al., "MDC1 Maintains Genomic Stability by Participating in the Amplification of ATM-Dependent DNA Damage Signals," *Mol Cell* 21:187-200 (2006); and Lee et al.,
25 "Structure of the BRCT Repeat Domain of MDC1 and its Specificity for the Free COOH-terminal End of the Gamma-H2AX Histone Tail," *J Biol Chem* 280:32053-32056 (2005), which are hereby incorporated by reference in their entirety), a critical mediator of DNA damage response. Further inspection of the C-terminus of H2A.X revealed a tyrosine (Tyr142 in mammals) that exists in metazoans, such as human,
30 rodents, and *Drosophila*, but is absent in unicellular eukaryotes such as yeast (Figure 1A). Interestingly, two forms of H2A.X in the *Xenopus* genome, which are different at this residue (the "F" and "Y" form, Figure 1A), are differentially expressed during

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development. Although recent studies suggest a role for Tyr142 in recruiting Mdc1 (Stewart et al., "MDC1 is a Mediator of the Mammalian DNA Damage Checkpoint," *Nature* 421:961-966 (2003); Goldberg et al., "MDC1 is Required for the Intra-S-Phase DNA Damage Checkpoint," *Nature* 421:952-956 (2003); Lou et al., "MDC1 is Coupled to Activated CHK2 in Mammalian DNA Damage Response Pathways," *Nature* 421:957-961 (2003); Stucki et al., "MDC1 Directly Binds Phosphorylated Histone H2AX to Regulate Cellular Responses to DNA Double-Strand Breaks," *Cell* 123:1213-1226 (2005); Lou et al., "MDC1 Maintains Genomic Stability by Participating in the Amplification of ATM-Dependent DNA Damage Signals," *Mol Cell* 21:187-200 (2006); and Lee et al., "Structure of the BRCT Repeat Domain of MDC1 and its Specificity for the Free COOH-terminal End of the Gamma-H2AX Histone Tail," *J Biol Chem* 280:32053-32056 (2005), which are hereby incorporated by reference in their entirety) the *in vivo* function of Tyr142, especially in the regulation of γ -H2A.X, remains unclear (Stucki et al., "MDC1 Directly Binds Phosphorylated Histone H2AX to Regulate Cellular Responses to DNA Double-Strand Breaks," *Cell* 123:1213-1226 (2005), which is hereby incorporated by reference in its entirety).

[0141] Whether Tyr142 is phosphorylated in H2A.X under certain physiological conditions was investigated (Figure 1B). Preliminary studies using a pan anti-phosphotyrosine antibody indicated that H2A.X was phosphorylated prior to DNA damage. To investigate further whether Tyr142 is indeed phosphorylated, an antibody raised against an extreme C-terminal peptide containing phosphorylated Tyr142 of H2A.X was generated and shown to be highly selective for H2A.X-Tyr142ph (hereafter, α -H2A.X-Y142ph; See Figures 5C-F for characterization of antibody). In MEF cells, Tyr142 is constitutively phosphorylated under normal growth conditions and becomes gradually dephosphorylated during the DNA damage response, while γ -H2A.X increases (Figure 1B). Whether this observation is conserved in other organisms was then investigated. It has been reported that H2A.X is highly enriched in *Xenopus* oocytes and eggs (Dimitrov et al., "Remodeling Sperm Chromatin in *Xenopus laevis* Egg Extracts: The Role of Core Histone Phosphorylation and Linker Histone B4 in Chromatin Assembly," *J Cell Biol* 126:591-601 (1994) and Kleinschmidt et al., "DNA-Dependent Phosphorylation of

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Histone H2A.X During Nucleosome Assembly in *Xenopus laevis* Oocytes: Involvement of Protein Phosphorylation in Nucleosome Spacing,” *EMBO J* 10:3043-3050 (1991), which are hereby incorporated by reference in their entirety). Thus, whether its Tyr142 phosphorylation status would be altered in response to DNA damage treatment, in the context of *Xenopus* early embryonic development was examined. Similar to mammalian cells, Tyr142 phosphorylation levels are greatly decreased in response to DNA damage (Figure 5A).

[0142] Since the major form of H2A in yeast carries the “SQEL” motif (Figure 1A and Redon et al., “Histone H2A Variants H2AX and H2AZ,” *Curr Opin Genet Dev* 12:162-169 (2002), which is hereby incorporated by reference in its entirety), whether a point mutant mimicking the mammalian “SQEY” motif would constitute a new phospho-acceptor was investigated. Similar to the mammalian cells, this mutant yeast strain (L132Y) is strongly reactive with the α -H2A.X-Y142ph antibody, with reactivity greatly diminished following DNA damage treatment (Figure 5B). Collectively, these unexpected results raise the intriguing possibility that a conserved phosphotyrosine pathway may regulate H2A.X phosphorylation in mammalian, *Xenopus*, and yeast cells, even though yeast H2A lacks a C-terminal tyrosine naturally. These data provide an early indication that Tyr142 phosphorylation may play a role in regulating the DNA damage response of H2A.X in a wide range of organisms by previously unknown mechanisms.

Example 13 - The WSTF-SNF2H Chromatin Remodeling Complex Interacts with H2A.X

[0143] To investigate novel mechanisms that regulate H2A.X function, protein complexes directly associated with H2A.X *in vivo* were isolated. Primary H2A.X ^{-/-} MEF cells were reconstituted with H2A.X constructs (WT or Y142F mutant), with or without an N-terminal FLAG epitope tag. The FLAG tag did not interfere with typical DNA damage response pathways, including γ -H2A.X. Since the expression of H2A.X at physiological levels in the reconstituted lines is important to study its function (Stucki et al., “MDC1 Directly Binds Phosphorylated Histone H2AX to Regulate Cellular Responses to DNA Double-Strand Breaks,” *Cell* 123:1213-1226 (2005), which is hereby incorporated by reference in its entirety),

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three independent cell lines (WT and mutants) were constructed in which the expression levels were comparable (50-80%) to the control primary MEFs (with two intact endogenous H2A.X alleles) and the majority of the reconstituted cells (~90%) had similar expression levels. To enrich for proteins or complexes that may associate with H2A.X mononucleosomes, an approach that has previously been used to successfully purify such chromatin particles for subsequent biochemical purifications was adapted (Mendez et al, "Chromatin Association of Human Origin Recognition Complex, cdc6, and Minichromosome Maintenance Proteins During the Cell Cycle: Assembly of Prereplication Complexes in Late Mitosis," *Mol Cell Biol* 20:8602-8612 (2000), which is hereby incorporated by reference in its entirety). This method, followed by immunoprecipitation with α -FLAG antibodies, significantly enriched H2A.X containing mononucleosomes and associated proteins (Figure 2A and 6). As shown by silver staining, a small number of polypeptides besides histones were associated with the wild type H2A.X mononucleosomes at a stoichiometric level, the amount of which was decreased after cells were treated with ionizing radiation (Figure 2B). Subsequent mass spectrometry (MS) analysis demonstrated that two of the more prominent proteins are SNF2H (140 KDa), a mammalian homolog of the ISWI ATPase (Fyodorov et al., "The Many Faces of Chromatin Remodeling: SWItching Beyond Transcription," *Cell* 106:523-525 (2001) and Becker et al., "ATP-Dependent Nucleosome Remodeling," *Annu Rev Biochem* 71:247-273 (2002), which are hereby incorporated by reference in their entirety), and WSTF (William-Beuren syndrome Transcription Factor) (171 KDa), a.k.a. BAZ1B, a member of the BAZ/WAL family chromatin remodeling factors (Figure 7) (Ito et al., "ACF Consists of Two Subunits, Acf1 and ISWI, That Function Cooperatively in the ATP-Dependent Catalysis of Chromatin Assembly," *Genes Dev* 13:1529-1539 (1999); Poot et al., "HuCHRAc, a Human ISWI Chromatin Remodeling Complex Contains hACF1 and Two Novel Histone-Fold Proteins," *EMBO J* 19:3377-3387 (2000); Jones et al., "A Novel Family of Bromodomain Genes," *Genomics* 63:40-45 (2000), which are hereby incorporated by reference in their entirety). These proteins constitute the WICH complex (WSTF-ISWI ATP-dependent chromatin-remodeling complex), which mobilizes nucleosomes *in vitro* and is suggested to be involved in the regulation of DNA replication (Poot et al., "The Williams Syndrome Transcription

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Factor Interacts with PCNA to Target Chromatin Remodeling by ISWI to Replication Foci,” *Nat Cell Biol* 6:1236-1244 (2004) and Bozhenok et al., “WSTF-ISWI Chromatin Remodeling Complex Targets Heterochromatic Replication Foci,” *EMBO J* 21:2231-2241 (2002), which are hereby incorporated by reference in their entirety).

5 [0144] Whether other protein factors present in ISWI-containing chromatin remodeling complex, such as CHRAC15 and 17 (Kukimoto et al., “The Histone-Fold Protein Complex CHRAC-15/17 Enhances Nucleosome Sliding and Assembly Mediated by ACF,” *Mol Cell* 13:265-277 (2004), which is hereby incorporated by reference in its entirety), co-purified with H2A.X-containing mononucleosomes at
10 sub-stoichiometric levels was investigated. However, none of these components have been identified by either MS or immunoblotting approaches, leading to the tentative conclusion that only the WICH complex co-purifies with H2A.X-containing mononucleosomes at significant levels *in vivo*. This association was then confirmed by immunoblot analyses of the complexes co-immunoprecipitated with H2A.X-
15 containing nucleosomes. Consistent with the MS results, WSTF and SNF2H were enriched in these complexes (Figure 2C). Interestingly, a significant decrease of γ -H2A.X phosphorylation levels in Y142F cells was observed (Figure 2C and Figure 4). Because of the close proximity between Ser139 and Tyr142 in the C-tail of H2A.X (Figure 1A), the possibility that the mutation of Tyr142 or Tyr142 phosphorylation
20 would influence Ser139 phosphorylation simply due to epitope disruption or impaired ATM/R kinase activity at Ser139 of H2A.X was investigated (See Figures 8 and 9). In addition, the interaction of the WICH complex is greatly reduced with Y142F mutant H2A.X mononucleosomes (Figures 2B and 2C). These results indicate that Y142 phosphorylation plays an important role in regulating H2A.X function during
25 DNA damage.

[0145] To investigate the roles of WSTF in regulating the function of H2A.X, several independent WSTF knock-down NIH3T3 cell lines (WSTF RNAi cells) were generated by infection with a short hairpin WSTF RNAi construct, reducing WSTF protein expression significantly (Figure 2D). Additionally, knockdown of SNF2H
30 using a similar approach was attempted, but SNF2H knockdown resulted in S-phase defects and cell death as described in SNF2H knock-out mice studies (Stopka et al., “The ISWI ATPase Snf2h is Required for Early Mouse Development,” *Proc Natl*

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Acad Sci U S A 100:14097-14102 (2003), which is hereby incorporated by reference in its entirety). Presumably, SNF2H functions in additional chromatin remodeling complexes that are involved in a variety of vital cell functions (Becker et al., "ATP-Dependent Nucleosome Remodeling," *Annu Rev Biochem* 71:247-273 (2002), which is hereby incorporated by reference in its entirety). The specificity of the RNAi knock-down approach was demonstrated by rescuing WSTF expression with the human WSTF gene (the sequences in the WSTF mRNA targeted by the RNAi construct are different between human and mouse) (Figures 4E and 4F). Most interestingly, WSTF deficiency leads to a decrease in Y142 phosphorylation (Figure 2D), indicating that WSTF may also be directly involved in regulating Y142 phosphorylation *in vivo* (see below). These data suggest a novel regulatory mechanisms of H2A.X involves WICH complex and Tyr142 phosphorylation.

Example 14 - WSTF is a Novel Tyrosine Kinase that Phosphorylates Tyr142 of H2A.X

[0146] To further investigate the function of WSTF, purified recombinant, full-length human WSTF proteins were isolated from insect cells (Figure 3A). A single protein band migrating at the expected molecular weight (171KDa) was detected with silver staining (Figure 3B). MS analysis on the purified samples confirmed that this protein was WSTF and did not detect any other proteins except trace amounts of heat shock proteins. The recombinant full length WSTF protein phosphorylated H2A.X-containing nucleosomes (Figure 3B) that were reconstituted *in vitro* using ATP and divalent manganese ion Mn^{2+} (but not Mg^{2+} , see Figure 10A) as cofactors. To address whether WSTF can specifically phosphorylate Y142 of H2A.X, Y142F mutant nucleosomes were also reconstituted. The Y142F mutant does not interfere with histone octamer or nucleosome formation during reconstitution. In *in vitro* kinase assays, WSTF phosphorylated the WT but not Y142F H2A.X, indicating that Y142 is the major site of phosphorylation (Figure 3B). The *in vitro* kinase activity of WSTF towards Y142 of H2A.X was confirmed by immunoblotting using the α -H2A.X Y142ph antibody (Figure 3C).

[0147] Since no detectable proteins other than trace amounts of heat shock proteins were co-purified with WSTF from insect cells (Figure 3B), it was

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hypothesized that WSTF has an intrinsic kinase activity. However, WSTF does not share homology with known kinase domains. Therefore truncated recombinant WSTF proteins were generated (Figure 3A) to investigate which portion of the protein is required for this novel kinase activity. Using this approach, it was determined that the N-terminal portion of WSTF is necessary and sufficient for kinase activity, strongly indicating that the kinase motif resides in its N-terminal portion (Figure 3D). To map the putative kinase domain, a series of protein constructs within the N-terminal regions of WSTF were generated. All these protein constructs were extensively purified (Figure 3A, right), and MS analysis did not identify any contaminating protein kinases. Strikingly, the 1-345 amino acid construct of WSTF had much more potent kinase activity (>50 fold) than the 1-340 construct (Figure 3E). Furthermore, the substrate specificity of the 1-345 construct is similar to the full length WSTF (Figure 11). These results suggest that the amino acid residues in the surrounding regions of 340-345 are critical for the kinase activity of WSTF.

Consistent with these results, the activity of a point mutant at C338 (C338A) was greatly diminished (<50 fold, Figure 3F). The CD spectrum of this mutant is identical to the WT counterpart (Figure 12), indicating this mutation did not alter the global structure of the protein.

[0148] To further exclude the possibility that this activity might come from an undetected kinase co-purified at extremely low levels with WSTF (beyond the detection limit of silver stain and MS), recombinant WSTF proteins were generated in *E. coli* since protein kinases, especially tyrosine kinases, are rare in the *E. coli* genome. The bioinformatical analysis of the kinase domain of WSTF reveals two well-structured motifs (N-motif, containing the conserved WAC domain (Jones et al., “A Novel Family of Bromodomain Genes,” *Genomics* 63:40-45 (2000), which is hereby incorporated by reference in its entirety) and a previously unreported C-motif, Figures 3A and 13). The polypeptides generated in the partial cleavage of the recombinant WSTF kinase domain also support this predication (Figure 13). Based on these findings, recombinant protein constructs representing these two motifs from *E. coli* were generated. Co-expression of both motifs restores the kinase activity (which is perhaps related to the observation that these motifs are strongly associated) while the N-terminal motif alone had much reduced activity. These results strongly

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indicate that the N- and C- motif are required for the optimal kinase activity. Collectively, these results demonstrate that WSTF has a previously unidentified intrinsic kinase activity via a novel kinase domain.

5 **Example 15 - WSTF plays a critical role in the DNA damage foci formation**

[0149] As the functions of the WICH complex, especially WSTF, in the DNA damage response have not been explored, the role of WSTF in this response was further investigated. Upon DNA damage treatment, the initial Ser139 phosphorylation of H2A.X and its foci formation in WSTF RNAi cells (as described in Figure 2) were similar to control cells (up to 1-hour post IR, Figure 4A). However, in control cells, the γ -H2A.X level remained unchanged until 16-hour post IR. In contrast, the level of γ -H2A.X phosphorylation rapidly declined in WSTF RNAi cells 4 hours after IR treatment (Figures 4A). It is well-established that the number and morphology of γ -H2A.X foci undergo significant changes during the DNA damage response. Initially, a number of small foci are formed, while at late stage of the DNA damage response, only a few large foci are usually observed (Aten et al., "Dynamics of DNA Double-Strand Breaks Revealed by Clustering of Damaged Chromosome Domains," *Science* 303:92-95 (2004), which is hereby incorporated by reference in its entirety). As expected, large γ -H2A.X foci were formed in control cells starting 4- hours post IR (Figure 4B, upper panels) and persisting until 12-hours after IR, while the overall level of γ -H2A.X phosphorylation remains relatively constant. However, this morphological progression was not observed in WSTF RNAi cells despite the initial formation of small γ -H2A.X speckles as in the controls (Figure 4B, lower panels). Instead, the amount and intensity of the γ -H2A.X foci were significantly less than in controls (only 16% of the WSTF RNAi retain γ -H2A.X foci 4 hours post IR), and no large foci were observed in WSTF RNAi cells during the entire DNA repair process, even after 12 hours post ionizing radiation (Figure 4B). Since the ATM/R kinases are the major kinase for γ -H2A.X phosphorylation and γ -H2A.X foci maintenance is dependent on sustained recruitment of active ATM to the damage foci via Mdc1, (Stucki et al., "MDC1 Directly Binds Phosphorylated Histone H2AX to Regulate Cellular Responses to DNA Double-Strand Breaks," *Cell* 123:1213-1226 (2005), which is hereby incorporated by reference in its entirety) whether the re-

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localization of ATM and Mdc1 is defective in the WSTF deficient cells was examined. ATM activation during the DNA damage response is reflected by its autophosphorylation of a serine 1981 (referred to as phos-ATM hereafter) (Bakkenist et al, "DNA Damage Activates ATM Through Intermolecular Autophosphorylation and Dimer Dissociation," *Nature* 421:499-506 (2003), which is hereby incorporated by reference in its entirety), although the functional significance of this mark is unclear (Lee et al., "ATM Activation by DNA Double-Strand Breaks Through the Mre11-Rad50-Nbs1 Complex," *Science* 308:551-554 (2005), which is hereby incorporated by reference in its entirety). In control cells, large phos-ATM foci were observed at late stage of DNA damage response (8-hour post 10 Gy of IR) (Figure 4C), while similar foci were not observed in the WSTF deficient cells, indicating that the recruitment and/or the activation of ATM is defective at late stage of DNA damage response in these cells. Similarly, Mdc1 foci formation was also greatly impaired in WSTF deficient cells (Figure 4D). These data strongly suggest that WSTF plays a critical role in the recruitment of active ATM and Mdc1 to the damage sites, both of which are critical for γ -H2A.X foci formation.

[0150] Whether the kinase activity of WSTF is required for its function during the DNA damage response, namely, the maintenance of phos-ATM and γ -H2A.X foci, was next investigated. Since the WSTF mRNA sequences targeted by the short hairpin RNAi constructs are different between human and mouse (see above), WSTF RNAi cells (derived from mouse 3T3 cells) were complemented with wildtype or C338A mutant kinase domain constructs of human WSTF. This approach successfully restored the expression of kinase domain of WSTF (Figure 4E and 4F). The expression of the wildtype WSTF kinase domain rescued the γ -H2A.X and phos-ATM foci formation defects in the WSTF RNAi cells (Figure 4E and 4F). In nearly 100% of the cells complemented with the wildtype construct, γ -H2A.X and phos-ATM foci were observed at least 8 hours after DNA damage treatment (Figure 4E and F). The kinetics and morphology of these foci are indistinguishable from the control 3T3 cells. On the other hand, the phos-ATM and γ -H2A.X foci formation were as defective in cells expressing the C338A mutant as in WSTF RNAi cells containing vector alone (Figure 4E and F). These results demonstrate that WSTF has additional

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roles during the DNA damage response via its novel kinase activity in addition to regulating H2A.X Y142 phosphorylation.

[0151] To explore the *in vivo* role of Tyr142, whether the H2A.X Y142F and Y142L mutations would affect phosphorylation on Ser139 and foci formation were
5 examined. γ -H2A.X levels and foci formation were greatly reduced upon DNA damage in both mutant cells (Figures 4G and 4H), despite similar expression levels of the wildtype and mutant H2A.X proteins. Because of the close proximity between Ser139 and Tyr142 in the C-terminus of H2A.X, the possibility that the mutation of Tyr142 or Tyr142 phosphorylation would influence Ser139 phosphorylation simply
10 due to epitope disruption of the γ -H2A.X antibodies or impaired ATM/R kinase activity at Ser139 of H2A.X was investigated and ruled out (Figures 8 and 9A). These results are consistent with the DNA damage defects observed in the WSTF deficient cells. As recent studies have demonstrated that Tyr142 is critical for the interaction between Mdc1 and the H2A.X phosphorylated at Ser139 (Stewart et al.,
15 "MDC1 is a Mediator of the Mammalian DNA Damage Checkpoint," *Nature* 421:961-966 (2003); Stucki et al., "MDC1 Directly Binds Phosphorylated Histone H2AX to Regulate Cellular Responses to DNA Double-Strand Breaks," *Cell* 123:1213-1226 (2005); and Lee et al., "Structure of the BRCT Repeat Domain of MDC1 and its Specificity for the Free COOH-terminal End of the Gamma-H2AX
20 Histone Tail," *J Biol Chem* 280:32053-32056 (2005), which are hereby incorporated by reference in their entirety), whether mutations on Tyr142 would affect this interaction *in vitro* was tested. Only H2A.X peptides singly phosphorylated on Ser139 interacted with significant levels of Mdc1 from nuclear extracts, while phosphorylation or mutation of Tyr142 in these peptides greatly reduced this
25 interaction (Figure 4I). The recombinant Mdc1 BRCT domain specifically bound to Ser139(ph) peptides, but not to unphosphorylated peptides and either phosphorylation or replacement of Tyr142 greatly reduced the binding (Figure 4J), consistent with the results from nuclear extract pull-downs. Therefore, the observed γ -H2A.X defects in the Tyr142 mutant cells could be due to the impairment of Mdc1 binding to the
30 damage sites. Taken together, these data suggest that WSTF plays a critical role in regulating DNA damage foci formation and H2A.X Tyr142 may have multiple functions during the DNA damage response.

Discussion of Examples 1-15

[0152] These studies call attention to a new regulatory mechanism controlling histone H2A.X function mediated by the WSTF-SNF2H (WICH) chromatin remodeling complex. A novel phosphorylation site on H2A.X, Y142, was found that plays a vital role in the DNA damage response. It was also discovered that the amino-terminal domain of WSTF has a previously unrecognized tyrosine kinase activity for this site. WSTF, a gene frequently deleted in the human William Syndrome (WS), has previously been shown to be the regulatory component of the WICH chromatin remodeling complex (Ito et al., "ACF Consists of Two Subunits, Acfl and ISWI, That Function Cooperatively in the ATP-Dependent Catalysis of Chromatin Assembly," *Genes Dev* 13:1529-1539 (1999) and Bozhenok et al., "WSTF-ISWI Chromatin Remodeling Complex Targets Heterochromatic Replication Foci," *EMBO J* 21:2231-2241 (2002), which are hereby incorporated by reference in their entirety). The findings herein, therefore, have identified an unexpected link between a histone minor variant, H2A.X, a histone covalent modification, and an ATP-dependent chromatin remodeling mechanisms that control the mammalian DNA damage response.

[0153] The kinase domain of WSTF is composed of two putative motifs. The N-motif contains the highly conserved WAC domain, which is found in many eukaryotic proteins, while the C-motif is much more divergent. Structural and biochemical studies are underway to determine the molecular basis for the catalytic reaction of WSTF. Eukaryotic kinases often have several downstream targets and important factors in a signaling network may be modified by multiple kinases. Therefore, the outcome of a sophisticated signaling network can be controlled at multiple levels by crosstalk mechanisms (Hunter T., "Signaling--2000 and Beyond," *Cell* 100:113-127 (2000), which is hereby incorporated by reference in its entirety). In light of this notion, WSTF may have other downstream targets given its multiple roles in DNA damage repair, replication, and transcription. William-Beuren syndrome is a well-documented genetic disorder characterized by developmental defects and clinical behavioral phenotypes (Francke U., "Williams-Beuren Syndrome: Genes and Mechanisms," *Hum Mol Genet* 8:1947-1954 (1999), which is hereby incorporated by reference in its entirety), but the molecular mechanisms leading to the

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development of this disease is unclear. The link between the biochemical function of WSTF, including its intrinsic ability to bring about tyrosine phosphorylation of H2A.X and ensuing chromatin remodeling, and the clinical manifestation of WS remains a challenge for future studies.

5 **[0154]** The data demonstrate that WSTF also plays a critical role during the DNA damage response. In WSTF deficient cells, the recruitment of phos-ATM and Mdc1 to the DNA damage foci is compromised and the maintenance of γ -H2A.X foci is also defective. Since ATM and Mdc1 are critical for γ -H2A.X foci formation (Stucki et al., "MDC1 Directly Binds Phosphorylated Histone H2AX to Regulate Cellular Responses to DNA Double-Strand Breaks," *Cell* 123:1213-1226 (2005),
10 which is hereby incorporated by reference in its entirety) it is conceivable that observed γ -H2A.X foci maintenance defects in WSTF RNAi cells may be due to the defective phos-ATM and Mdc1 foci. The kinase activity of WSTF is also required for the foci formation of phos-ATM and in turn, γ -H2A.X, although the underlying
15 mechanisms remain unclear. γ -H2A.X foci formation is defective in Y142 mutant cell lines. This result seems to suggest a critical role for Y142 phosphorylation in H2A.X DNA damage response. On the other hand, it was also found that mutation of Y142 to F or L reduces Mdc1 binding to H2A.X *in vitro*. Therefore, it is possible that H2A.X Y142F or Y142L mutants less efficiently recruit Mdc1 and ATM to the DNA
20 damage sites, which causes γ -H2A.X foci formation defects.

[0155] A clue to how WSTF activity is regulated during the DNA damage response comes from the recent observation that the kinase domain of WSTF is hyper-phosphorylated in response to DNA damage, presumably by the ATM/R kinases (Matsuoka et al., "ATM and ATR Substrate Analysis Reveals Extensive
25 Protein Networks Responsive to DNA Damage," *Science* 316:1160-1166 (2007), which is hereby incorporated by reference in its entirety). This phosphorylation event could modulate the activity of WSTF and provide a mechanism to retain ATM at the damage foci. For example, phosphorylated WSTF may modify yet unknown histone or non-histone substrates, some, if not all, of which may
30 facilitate the retention of ATM. Alternatively, phosphorylated WSTF, together with SNF2H, may remodel the chromatin structure to recruit and stimulate ATM activity as several studies have already implicated chromatin structure in the regulation of

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ATM activity (Bakkenist et al, "DNA Damage Activates ATM Through Intermolecular Autophosphorylation and Dimer Dissociation," *Nature* 421:499-506 (2003) and You et al., "Rapid Activation of ATM on DNA Flanking Double-Strand Breaks," *Nat Cell Biol* 9:1311-1318 (2007), which are hereby incorporated by reference in their entirety). In summary, although the underlying mechanism remains to be explored, WSTF plays a critical role in regulating the DNA damage response. The novel kinase activity may contribute to DNA damage foci formation via multiple pathways, some of which may be independent of H2A.X Y142.

[0156] A number of outstanding issues can be resolved to provide a more complete understanding of the H2A.X-mediated DNA repair pathway. First, the DNA damage induced dephosphorylation of Y142 coincides with S139 (γ -H2A.X foci) phosphorylation, which raises several interesting questions. For example, is Y142 dephosphorylation an obligatory step for γ -H2AX phosphorylation or does γ -H2A.X phosphorylation trigger a Y142 dephosphorylation in response to DNA damage? Identifying and characterizing the function of protein phosphatase(s) for H2A.X Y142 phosphorylation will provide valuable insights. Furthermore, are Ser139 and Y142 simultaneously phosphorylated on the same H2A.X molecule under certain physiological conditions? It is quite possible that the Y142-phosphorylated and doubly phosphorylated (Y142 and S139) H2A.X C-terminus, if indeed it exists *in vivo*, may recruit distinct, yet unknown binding effectors to mediate H2A.X function. Thus, identifying and characterizing these factors will be required to delineate the pathways connecting S139 and Y142 phosphorylation. Third, how Y142 phosphorylation levels are reduced in response to damage remains unclear. Are H2A.X nucleosomes that contain phosphorylated Y142 evicted from sites of damaged chromatin, or are these nucleosomes dephosphorylated *in situ* by a yet unknown phosphatase? Identification of signaling pathways utilizing H2A.X Tyr142 phosphorylation or other physiologically-relevant phosphorylation events regulated by WICH will be of great interest in future studies.

[0157] Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing

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from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

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WHAT IS CLAIMED:

1. An antibody or antigen-binding fragment thereof that selectively binds to a phosphorylated tyrosine residue, wherein the phosphorylated tyrosine residue is located in an SQEY tyrosine phosphorylation motif sequence (SEQ ID NO: 2).
2. The antibody or antigen-binding fragment thereof according to claim 1, wherein the phosphorylated tyrosine residue of the SQEY motif is at amino acid position 142 of SEQ ID NO:1.
3. The antibody according to claim 1, wherein the antibody is monoclonal or polyclonal.
4. The antibody according to claim 1, wherein the antibody is multivalent or monovalent.
5. The antibody according to claim 1, wherein the antibody is humanized.
6. An antibody or antigen-binding fragment thereof that selectively binds to a phosphorylated serine residue, wherein the phosphorylated serine residue is closest to the glutamine residue in an KENSSQ phosphorylation motif sequence (SEQ ID NO:4).
7. The antibody or antigen-binding fragment thereof according to claim 6, wherein the phosphorylated serine residue of the KENSSQ motif is at amino acid position 167 of SEQ ID NO:3.
8. The antibody according to claim 6, wherein the antibody is monoclonal or polyclonal.

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9. The antibody according to claim 6, wherein the antibody is multivalent or monovalent.
10. The antibody according to claim 6, wherein the antibody is humanized.
11. An isolated polypeptide molecule comprising an amino acid sequence of SEQ ID NO:6 or SEQ ID NO:7.
12. An isolated nucleic acid molecule encoding the polypeptide of claim 11.
13. The isolated nucleic acid molecule of claim 12, wherein the nucleic acid molecule has a nucleotide sequence that has at least 80% nucleic acid sequence identity to the nucleotide sequence of SEQ ID NO: 5.
14. The isolated nucleic acid molecule of claim 12, wherein the nucleic acid molecule has a nucleotide sequence that has at least 90% nucleic acid sequence identity to the nucleotide sequence of SEQ ID NO:5.
15. The isolated nucleic acid molecule of claim 12, wherein the nucleic acid molecule has a nucleotide sequence that has at least 95% nucleic acid sequence identity to the nucleotide sequence of SEQ ID NO:5.
16. An expression vector comprising the isolated nucleic acid molecule of claim 12.
17. A host cell containing the expression vector of claim 16.
18. A method of identifying cellular DNA damage in a sample, said method comprising:
providing a cell sample;

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detecting the presence or absence of a phosphorylated tyrosine residue in an SQEY tyrosine phosphorylation motif (SEQ ID NO:2); and
identifying cellular DNA damage in the cell sample based on said detecting.

5

19. The method according to claim 18, wherein the phosphorylated tyrosine residue of the SQEY motif is at amino acid position 142 of SEQ ID NO:1.

20. The method according to claim 18, wherein said detecting
10 comprises contacting the sample with an antibody or antigen-binding fragment thereof that selectively binds to the phosphorylated tyrosine residue in an SQEY tyrosine phosphorylation motif (SEQ ID NO:2).

21. The method according to claim 20, wherein said antibody or
15 antigen-binding fragment thereof selectively binds to the phosphorylated tyrosine residue of the SQEY motif at amino acid position 142 of SEQ ID NO:1.

22. The method according to claim 18, wherein the absence of a
phosphorylated tyrosine residue in the SQEY motif indicates the presence of cellular
20 DNA damage.

23. The method according to claim 18, wherein the presence of a
phosphorylated tyrosine residue in the SQEY motif indicates the absence of cellular
DNA damage.

25

24. A method of identifying cellular DNA damage in a sample,
said method comprising:

providing a cell sample;

detecting the presence or absence of a phosphorylated serine residue at
30 the serine closest to the glutamine residue in an KENSSQ phosphorylation motif
sequence (SEQ ID NO:4); and

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identifying cellular DNA damage in the cell sample based on said detecting.

25. The method according to claim 24, wherein the phosphorylated
5 serine closest to the glutamine residue of the KENSSQ motif is at amino acid position 167 of SEQ ID NO:3.

26. The method according to claim 24, wherein said detecting
comprises contacting the sample with an antibody or antigen-binding fragment thereof
10 that selectively binds to the phosphorylated serine closest to the glutamine residue in an KENSSQ phosphorylation motif sequence (SEQ ID NO:4).

27. The method according to claim 26, wherein said antibody or
antigen-binding fragment thereof selectively binds to the phosphorylated serine
15 closest to the glutamine residue in the KENSSQ motif at amino acid position 167 of SEQ ID NO:3

28. The method according to claim 24, wherein the presence of a
phosphorylated serine residue at the serine closest to the glutamine residue of the
20 KENSSQ motif indicates the presence of cellular DNA damage.

29. The method according to claim 24, wherein the absence of a
phosphorylated serine residue at the serine closest to the glutamine residue of the
KENSSQ motif indicates the absence of cellular DNA damage.

25

30. A method of screening candidate compounds useful for
modulating WSTF kinase activity, said method comprising:
providing a candidate compound;
providing a sample, wherein said sample comprises a WSTF protein or
30 polypeptide having kinase activity, a protein substrate having an SQEY motif
sequence, manganese, and ATP;
contacting the candidate compound with the sample;

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detecting the presence or absence of a phosphorylated tyrosine residue in the SQEY tyrosine phosphorylation motif (SEQ ID NO:2); and
identifying compounds useful for modulating WSTF kinase activity based on said detecting.

5

31. The method according to claim 30, wherein the WSTF protein or polypeptide having kinase activity comprises SEQ ID NO: 6 or SEQ ID NO:7.

32. The method according to claim 30, wherein the phosphorylated
10 tyrosine residue in the SQEY motif is at amino acid position 142 of SEQ ID NO:1 in the sample.

33. The method according to claim 30, wherein the sample is a cell sample.

15

34. The method according to claim 30, wherein detecting the absence of a phosphorylated tyrosine residue in the SQEY motif identifies a compound useful for inhibiting WSTF kinase activity.

20 35. A method of screening candidate compounds useful for modulating cellular DNA damage repair, said method comprising;

providing a candidate compound;

providing a cell sample;

contacting the candidate compound with the cell sample;

25 detecting the presence or absence of a phosphorylated tyrosine residue in a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) in the cell sample; and
identifying candidate compounds useful for modulating cellular DNA damage repair based on said detecting.

30 36. The method according to claim 35, wherein the phosphorylated tyrosine residue in the SQEY motif is at amino acid position 142 of SEQ ID NO:1 in the cell sample.

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37. The method according to claim 35, wherein the cell sample comprises one or more genetically modified yeast cells, wherein the genetically modified yeast cells contain a leucine to tyrosine mutation in their H2A.X SQEL motif.

38. The method according to claim 35, wherein said contacting occurs in the presence of a DNA damaging agent.

39. A method of screening candidate compounds useful for treating cancer, said method comprising;
providing a candidate compound;
providing a cell sample;
contacting the candidate compound with the cell sample;
detecting the presence or absence of a phosphorylated tyrosine residue in an SQEY tyrosine phosphorylation motif (SEQ ID NO:2) in the cell sample; and
identifying candidate compounds useful for treating cancer based on said detecting.

40. The method according to claim 39, wherein the phosphorylated tyrosine residue in the SQEY motif is at amino acid position 142 of SEQ ID NO:1 in the cell sample.

41. The method according to claim 39, wherein the cell sample comprises one or more genetically modified yeast cells, wherein the genetically modified yeast cells contain a leucine to tyrosine mutation in their H2A.X SQEL motif.

42. A method of modulating cellular DNA damage repair, said method comprising:

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administering to a cell an agent which modulates tyrosine phosphorylation of an SQEY tyrosine phosphorylation motif (SEQ ID NO:2) under conditions effective to modulate cellular DNA damage repair.

5 43. The method according to claim 42, wherein said agent modulates tyrosine phosphorylation of the tyrosine residue in the SQEY motif at amino acid position 142 of SEQ ID NO:1 in the cell.

 44. The method according to claim 42, wherein an agent that
10 reduces tyrosine phosphorylation of the SQEY motif enhances cellular DNA damage repair.

 45. The method according to claim 44, wherein the agent is a
15 WSTF kinase domain inhibitor.

 46. The method according to claim 45, wherein the WSTF kinase domain inhibitor is an siRNA molecule comprising SEQ ID NO: 21.

 47. The method according to claim 44, wherein the agent is a
20 recombinant EYA phosphatase protein.

 48. The method according to claim 42, wherein an agent that induces tyrosine phosphorylation of the SQEY motif suppresses cellular DNA damage repair.

25 49. The method according to claim 48, wherein the agent is a nucleic acid molecule encoding a WSTF kinase domain comprising the nucleotide sequence of SEQ ID NO: 5 or the encoded polypeptide comprising an amino acid sequence of SEQ ID NO: 6 or SEQ ID NO:7.

30 50. The method according to claim 48, wherein the agent is an EYA phosphatase protein inhibitor.

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51. A method of treating a subject having cancer, said method comprising:

5 administering to said subject having cancer, an agent that modulates tyrosine phosphorylation of a SQEY tyrosine phosphorylation motif (SEQ ID NO:2) under conditions effective to treat the subject having cancer.

52. The method according to claim 51, wherein said agent modulates tyrosine phosphorylation of the tyrosine residue in the SQEY motif at
10 amino acid position 142 of SEQ ID NO:1.

53. The method according to claim 51, wherein the agent reduces tyrosine phosphorylation of the SQEY motif.

15 54. The method according to claim 53, wherein the agent is a WSTF kinase domain inhibitor.

55. The method according to claim 54, wherein the WSTF kinase domain inhibitor is an siRNA molecule comprising SEQ ID NO: 21.
20

56. The method according to claim 53, wherein the agent is a recombinant EYA phosphatase protein.

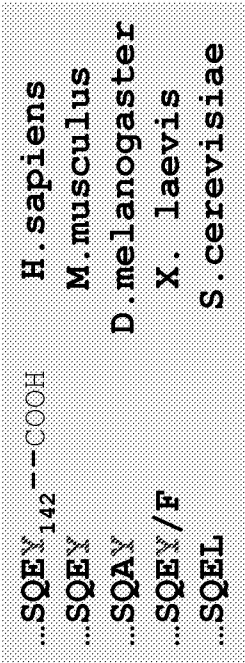
57. The method according to claim 51, wherein the agent induces
25 tyrosine phosphorylation of the SQEY motif.

58. The method according to claim 57, wherein the agent is a nucleic acid molecule encoding a WSTF kinase domain comprising the nucleotide sequence of SEQ ID NO: 5 or the encoded polypeptide comprising an amino acid
30 sequence of SEQ ID NO: 6 or SEQ ID NO:7.

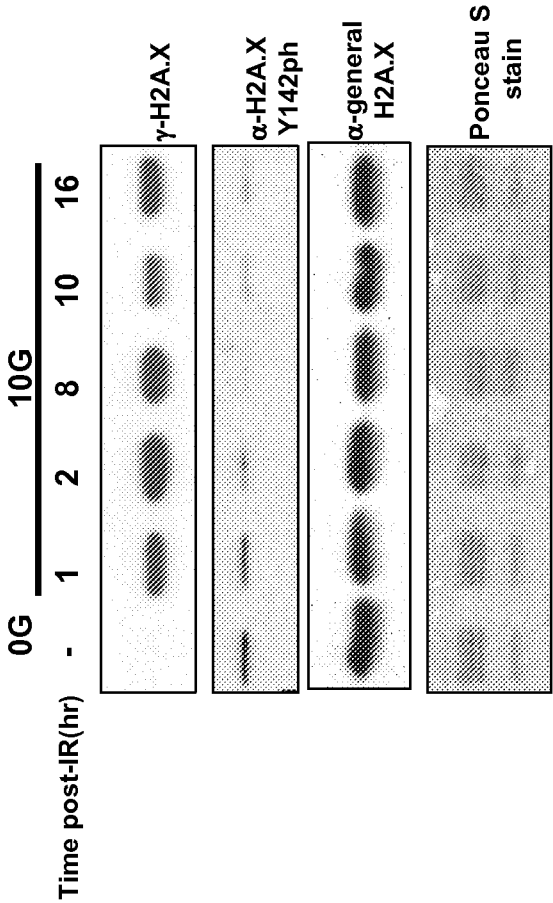
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59. The method according to claim 57, wherein the agent is an EYA phosphatase protein inhibitor.

A



B



Figures 1A-1B

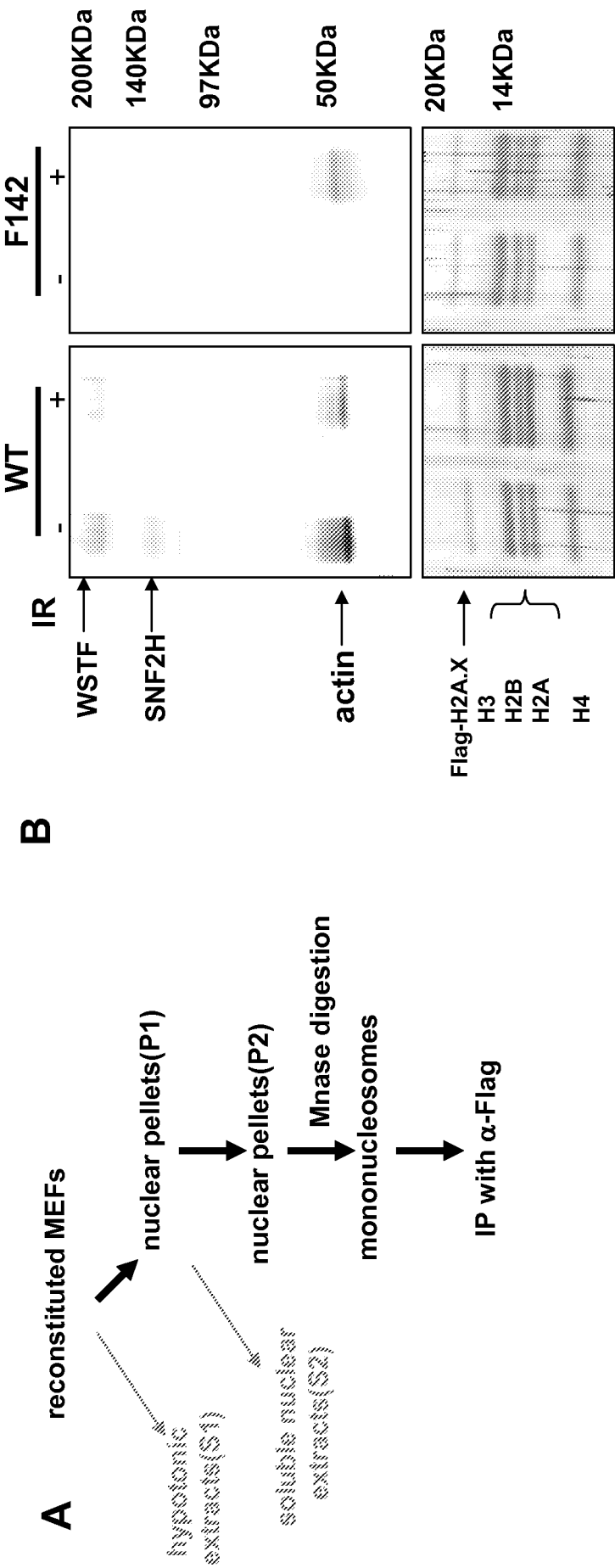


Figure 2A-2B

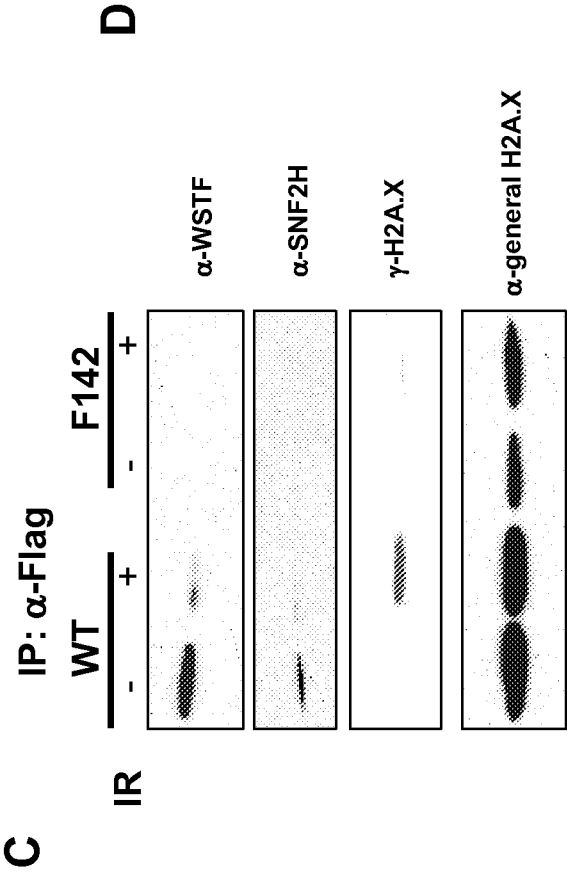


Figure 2C-2D

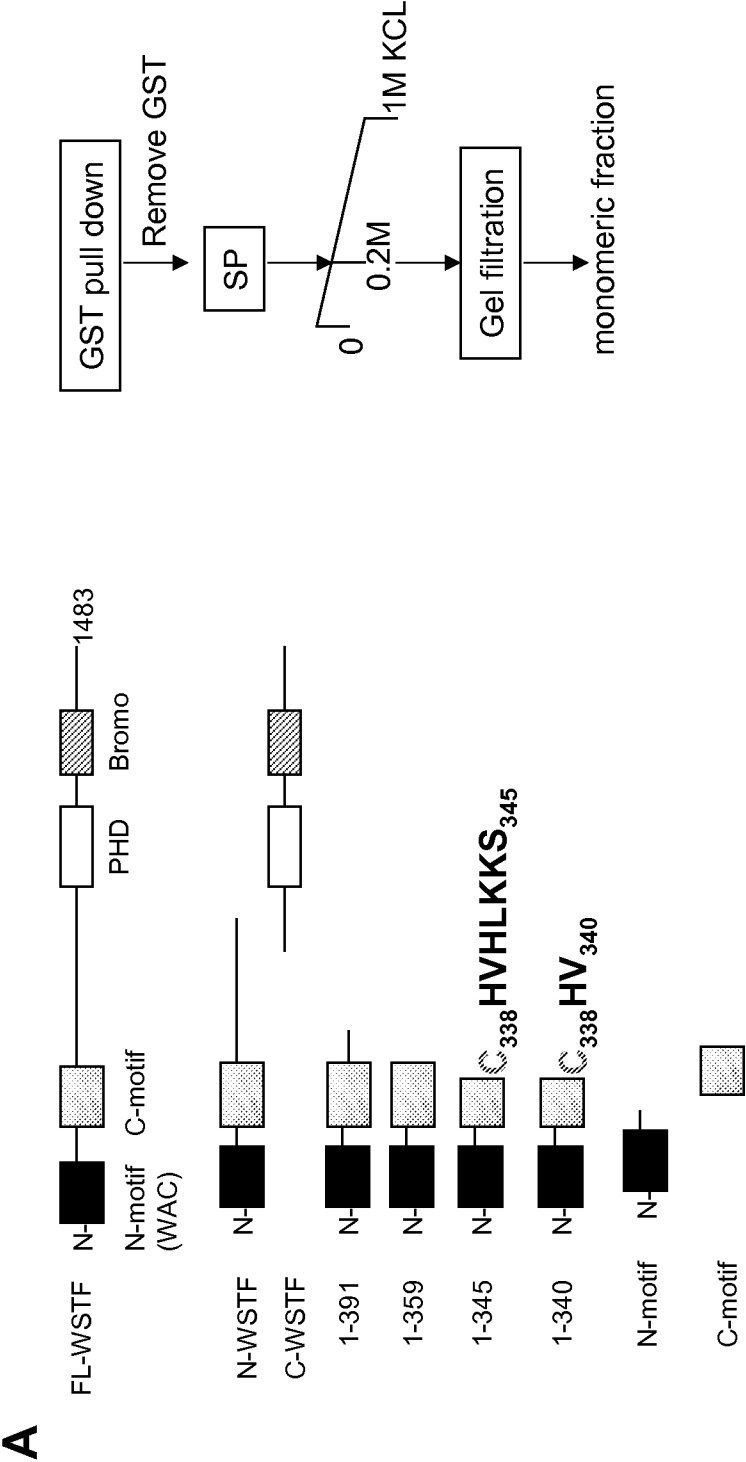
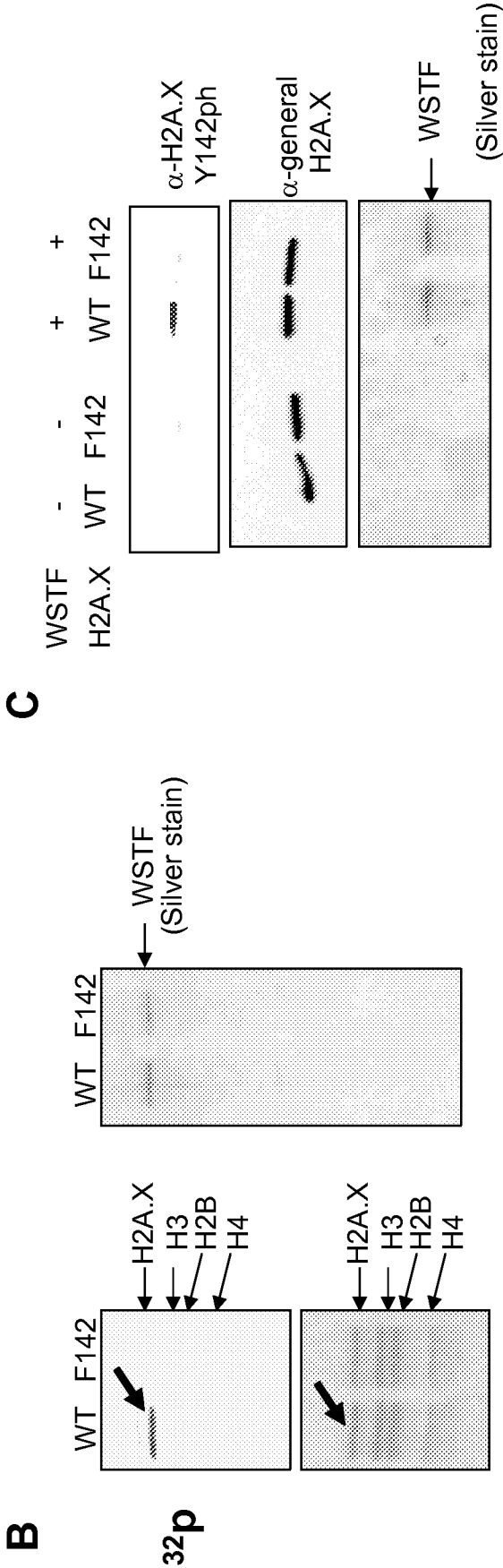
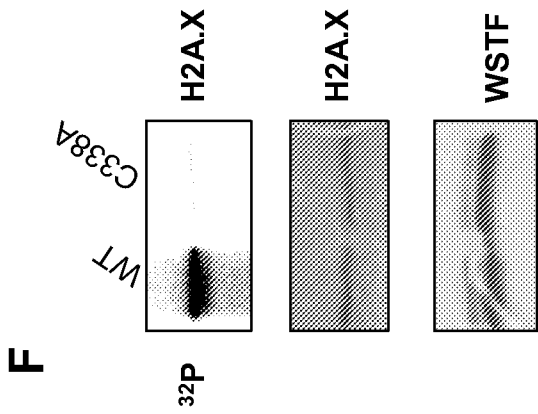
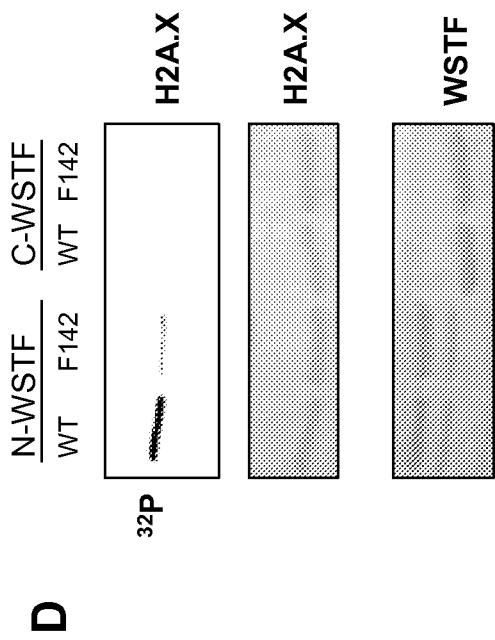
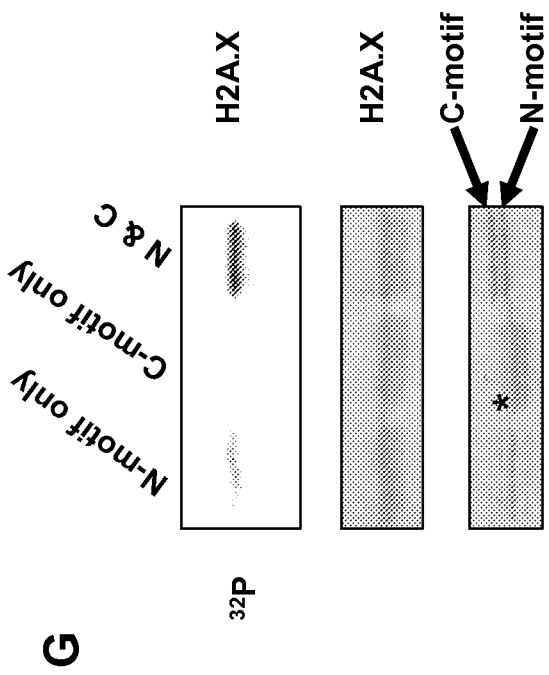
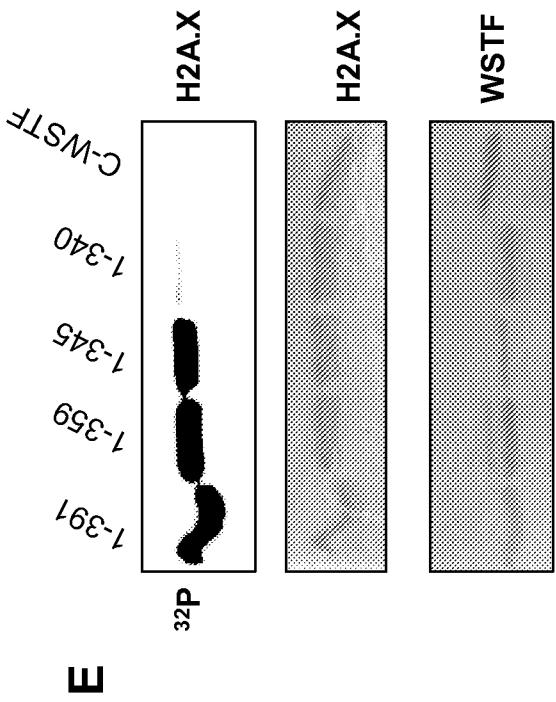


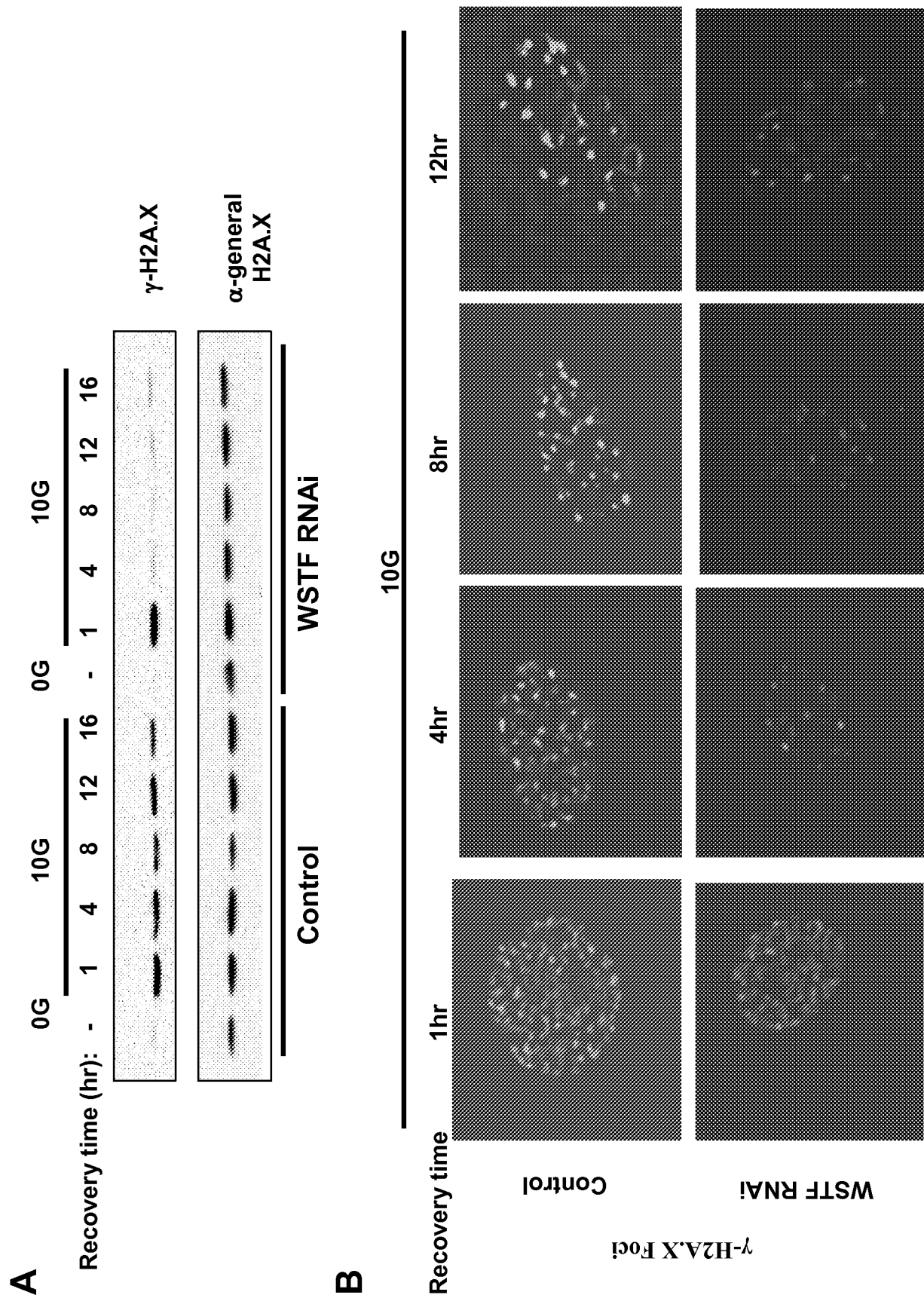
Figure 3A

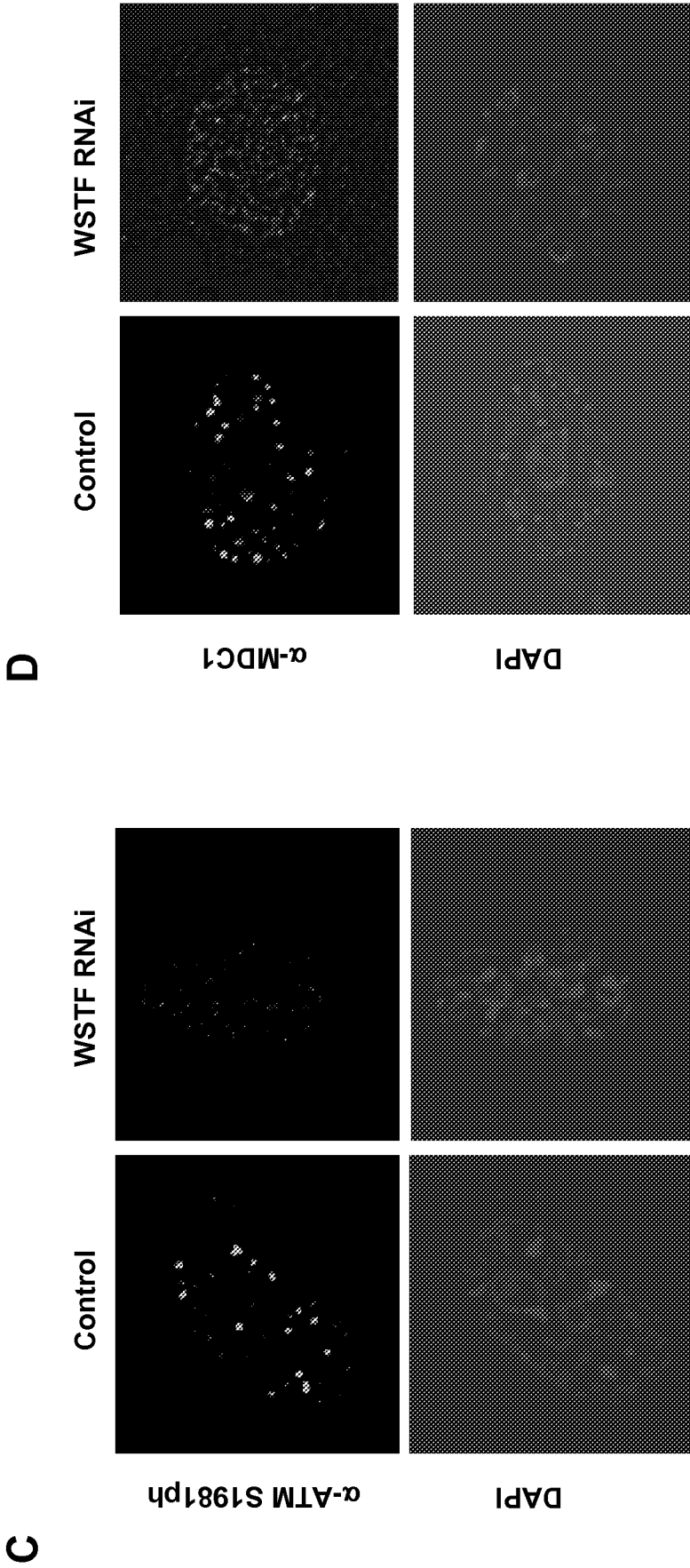


Figures 3B-3C



Figures 3D-3G

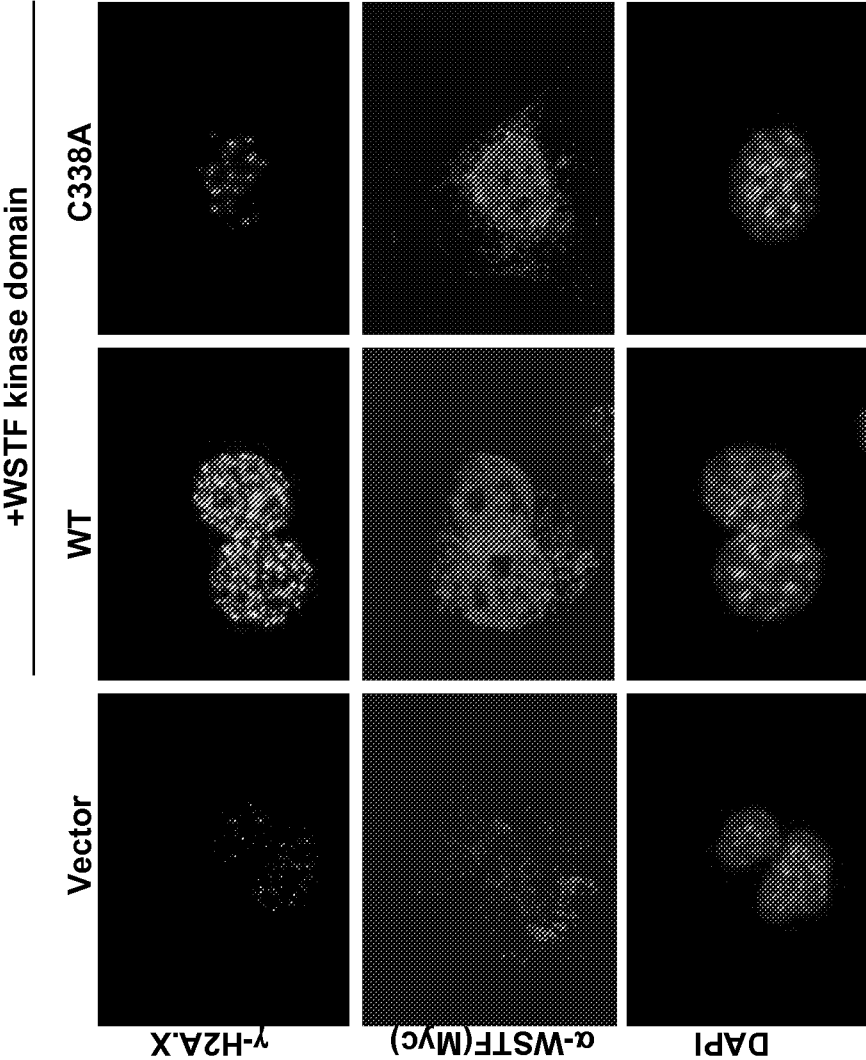




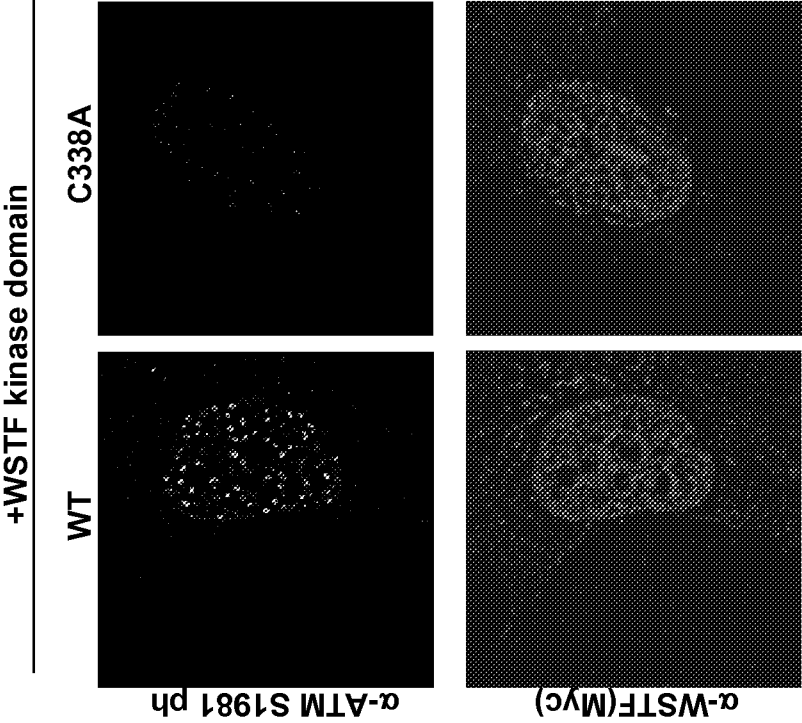
Figures 4C-4D

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E

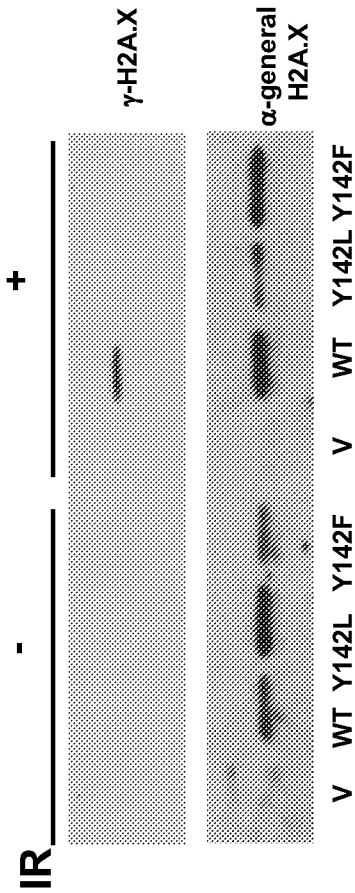


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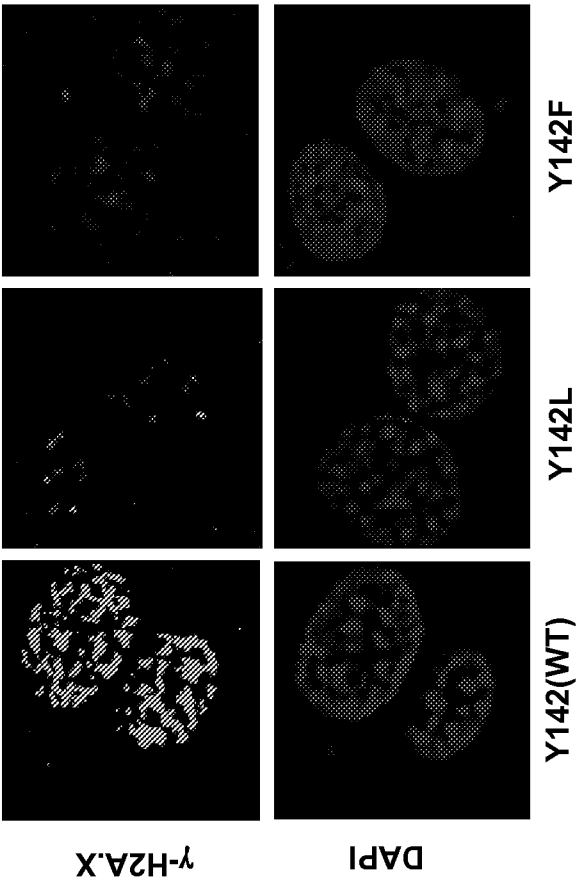


Figures 4E-4F

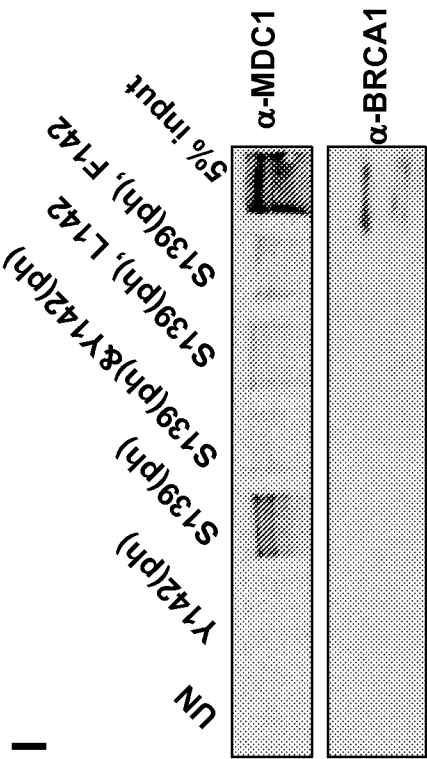
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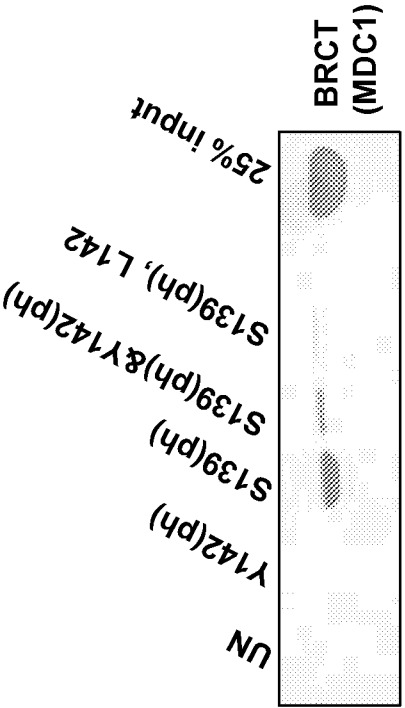
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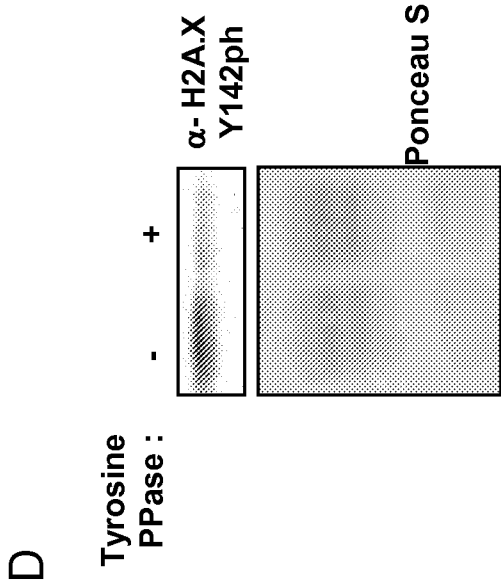
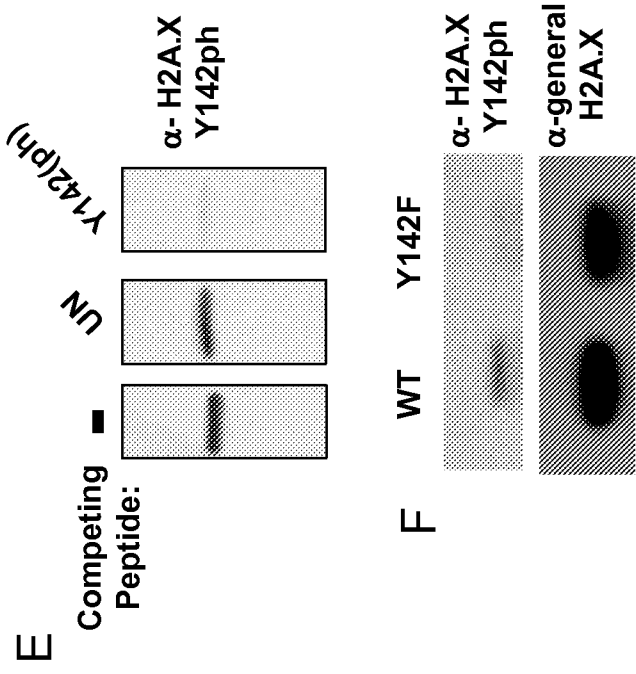
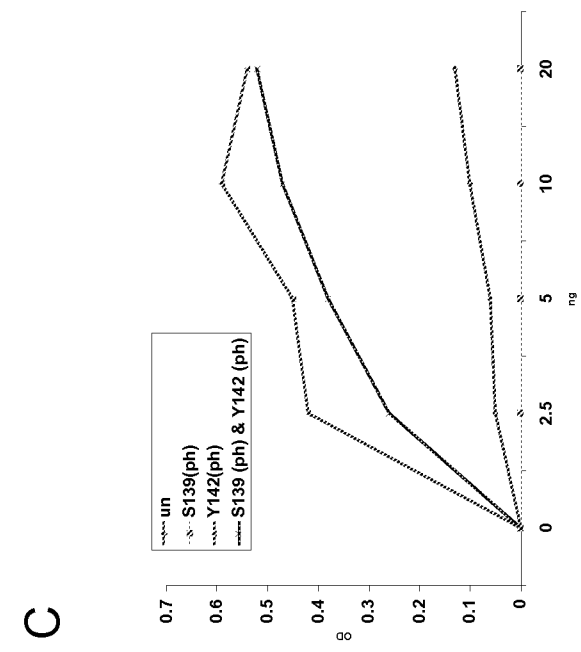
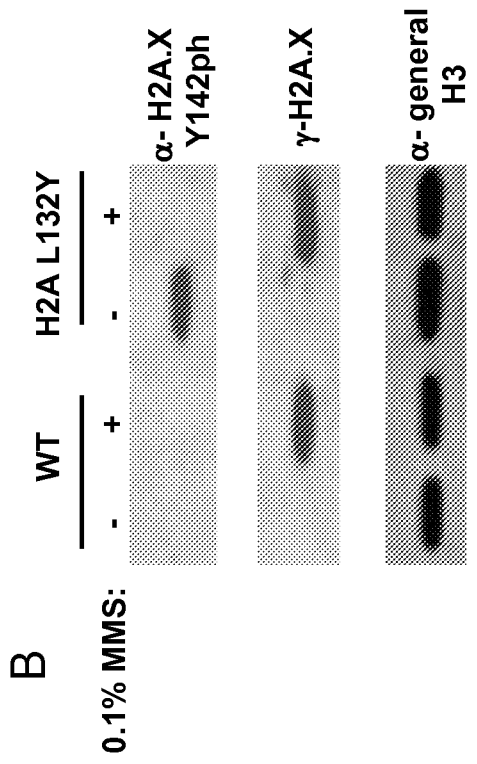
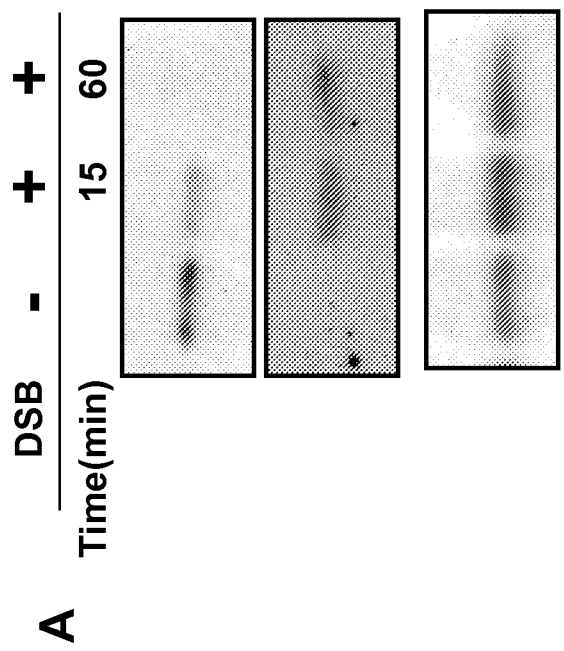
I



J



Figures 4G-4J



Figures 5A-5F

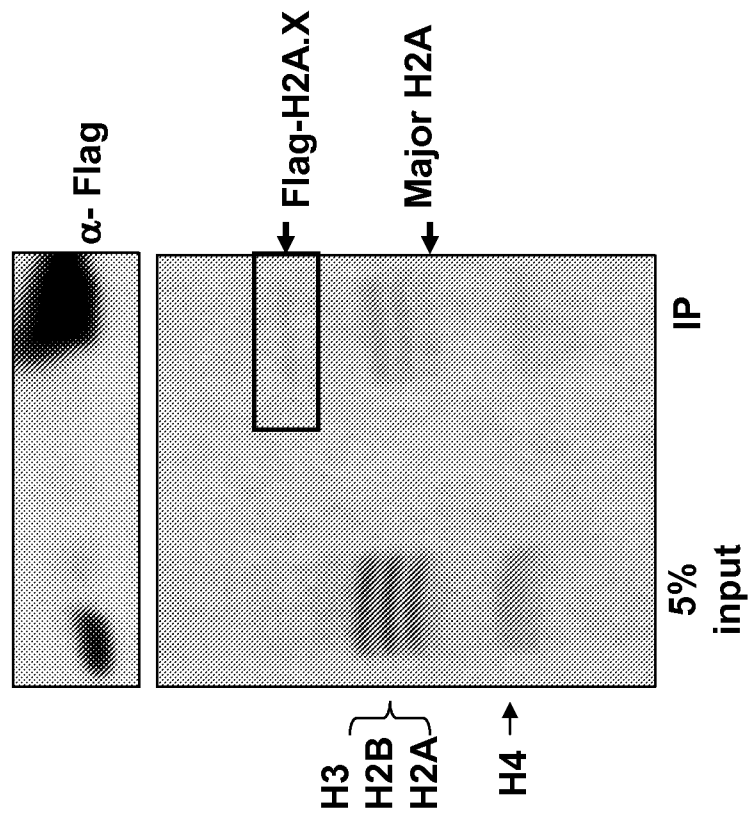


Figure 6

PMF-identification of WSTF (~170kDa); NCBI # 22653671

<u>m/z:</u>	<u>MH+</u>	<u>Amino Acid #</u>	<u>SEQ ID NO:</u>	<u>WSTF Amino Acid Sequence</u>
997.57	997.56	749-756	38	ILTALC _{act} HR
983.57	983.56	513-520	39	ARLPEELR
1003.55	1003.54	1057-1065	40	SDLIEVATR
1075.58	1075.58	1439-1447	41	HLPGHPYVR
1090.54	1090.50	1418-1427	42	GSHVLSCKMEK
1246.59	1246.59	928-937	43	GWVHNSIDYR
1297.74	1297.74	232-243	44	IISNVPADSLIR
1387.72	1387.72	323-335	45	RDSSSLSSPLNPK
1393.73	1393.71	418-430	46	STGNSKSPSKC _{act} VK
1407.71	1407.68	534-544	47	KRWASMSEEQR
1421.74	1421.73	1356-1366	48	FSWPFREPVTIR
1606.88	1606.91	471-484	49	HLPPAALHLIAYYK
1658.95	1658.89	389-404	50	GPAAKKPGKHSKPLK
1940.88	1940.92	1178-1193	51	WDM _{ox} SAENARCKVCRKK
1954.98	1955.00	1315-1331	52	SRPKDDPEVDDLVLQTK
2025.08	2025.09	226-243	53	LQNEDKIISNVPADSLIR
2142.07	2142.09	965-985	54	NASVNAHHGPALEAVETTPK
2677.30	2677.28	1396-1417	55	SVQEFLLTDMKQVFANAELYNCR
2872.50	2872.48	857-878	56	LQILTALCHRLMTYSVQDHMETR

These 19 peptides (out of 29 selected for PMF) cover 248 out of 1479 amino acids (16.8 %)

PMF-identification of SMARC A5 (~140kDa); NCBI # 40254124

<u>m/z:</u>	<u>MH+</u>	<u>Amino Acid #</u>	<u>SMARC A5 Amino Acid Sequence</u>
1115.60	1115.58	303-311	YLVIDEAHR
1153.64	1153.64	88-96	ANRFEYLLK
1159.64	1159.64	927-935	YKAPFHQLR
1223.63	1223.59	606-615	FITDNTVEER
1317.68	1317.66	738-748	KANYAVDAYFR
1365.67	1365.69	644-654	IGKDEM _{ox} LQM _{ox} IR
1397.81	1397.82	624-635	LRLDSIVIQGR
1419.70	1419.72	430-442	DIDILNSAGKMDK
1486.82	1486.81	724-735	IAFTIEWIEPPKR
1619.83	1619.80	767-779	QPNVQDFQFFPPR

These 10 peptides (out of 35 selected for PMF) cover 109 out of 1052 amino acids (10.4 %)

Symbols:

[MH+] : sum of the monoisotopic masses of all residues [M] plus a proton [H+]

m/z : experimental monoisotopic mass (by MALDI-ReTOF mass spectrometry)

C_{act}:
cysteine acrylamide

M_{ox}:
oxidized methionine

Figure 7

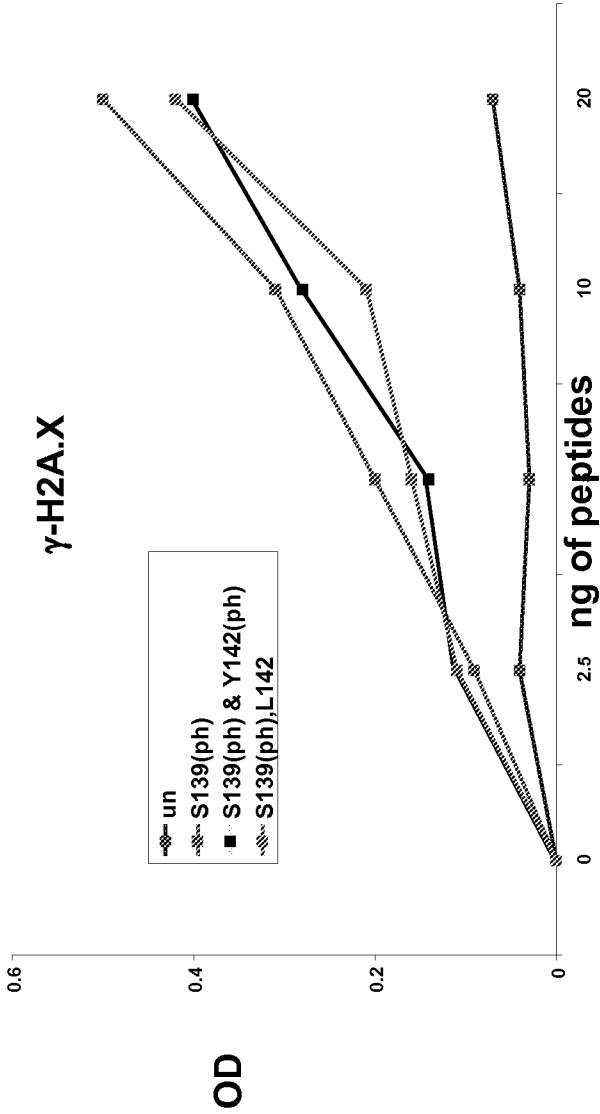
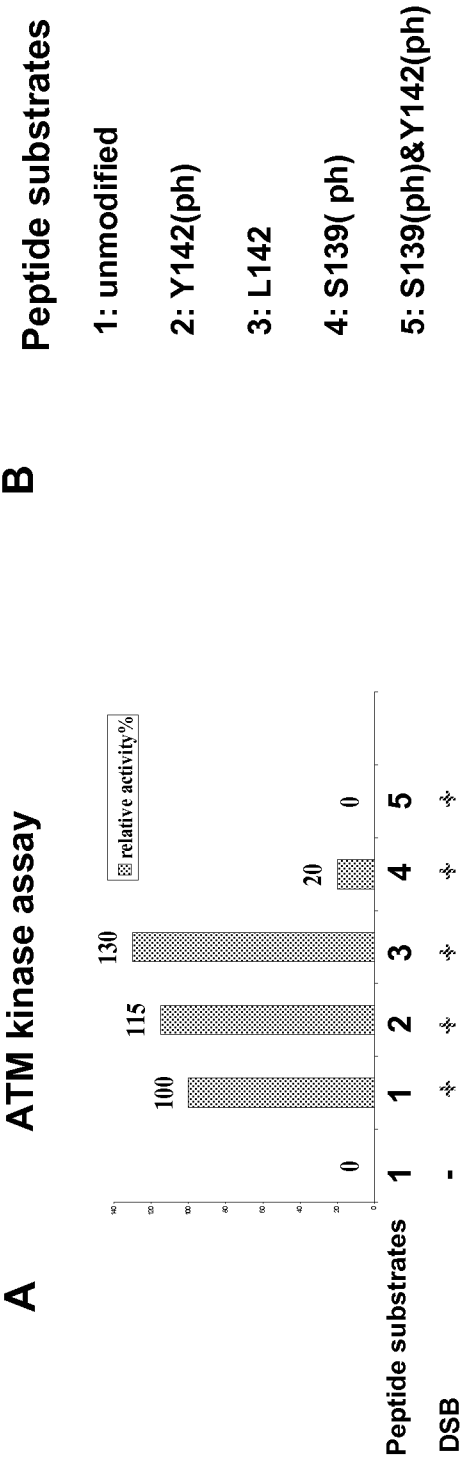
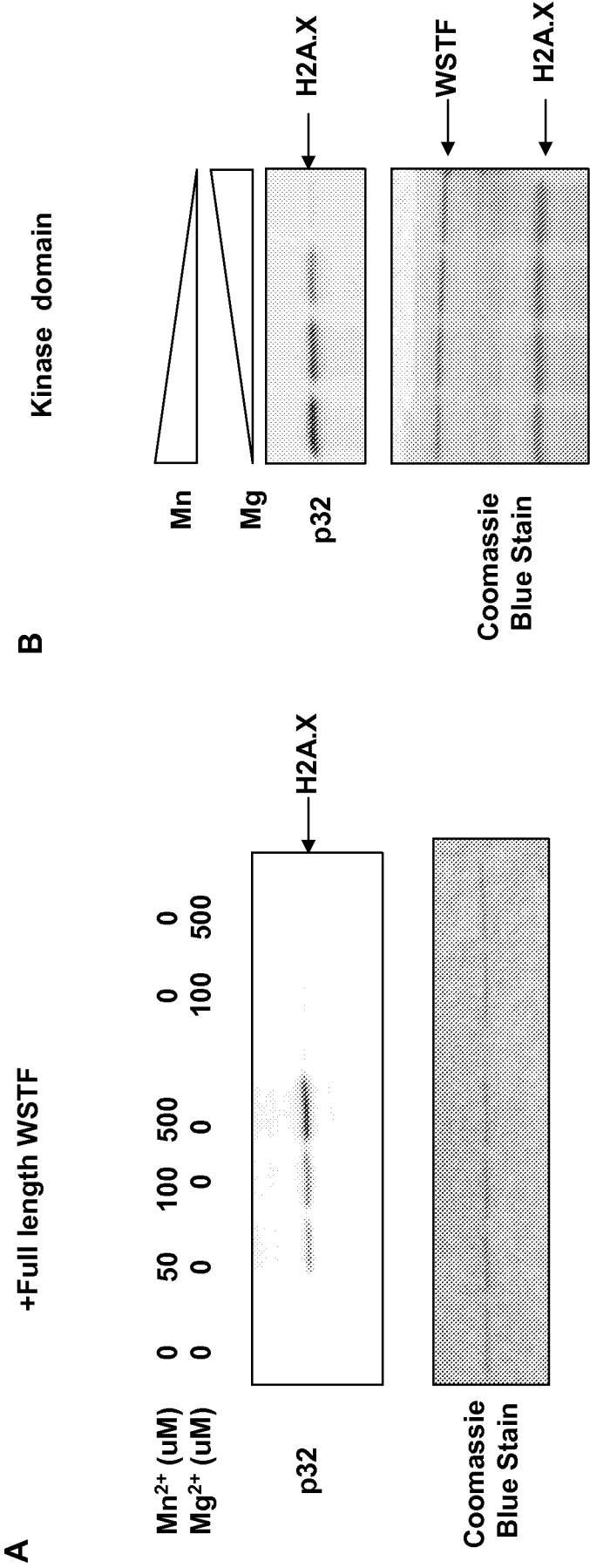


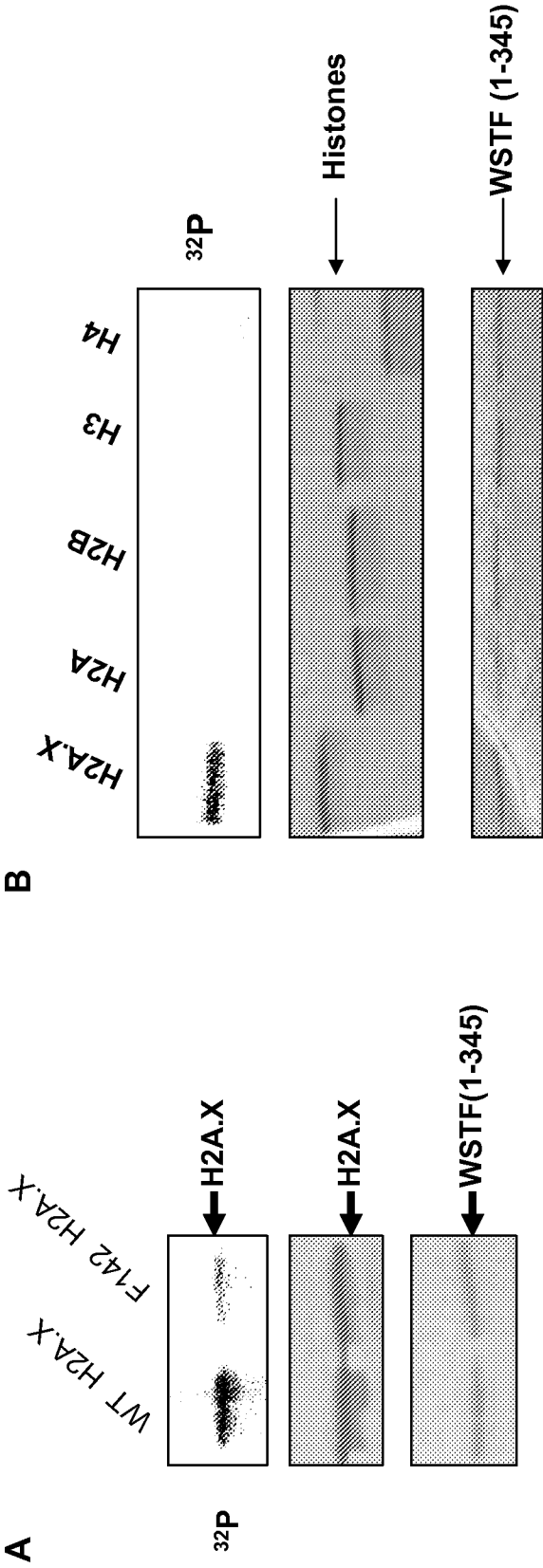
Figure 8



Figures 9A-9B



Figures 10A-10B



Figures 11A-11B

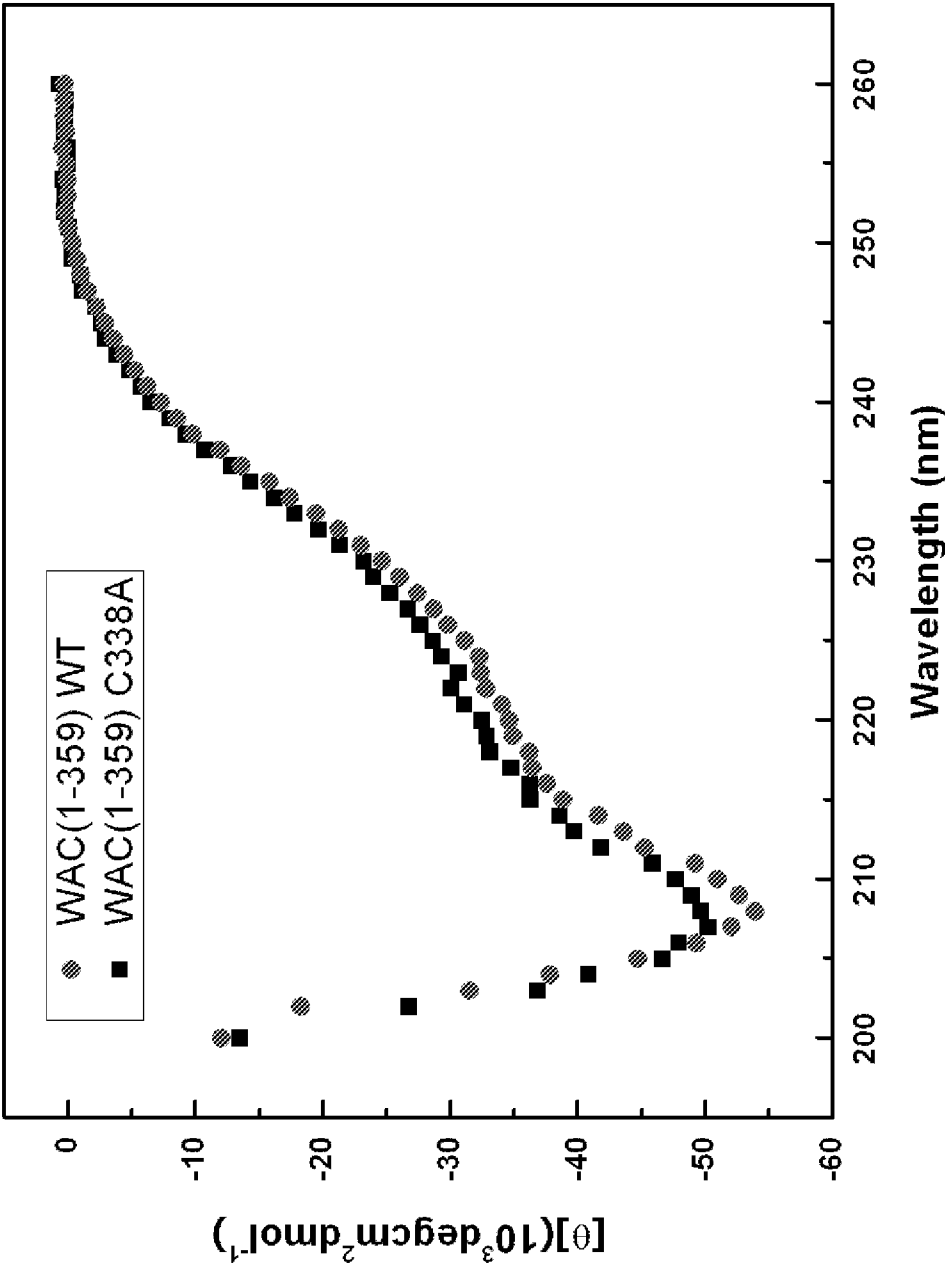
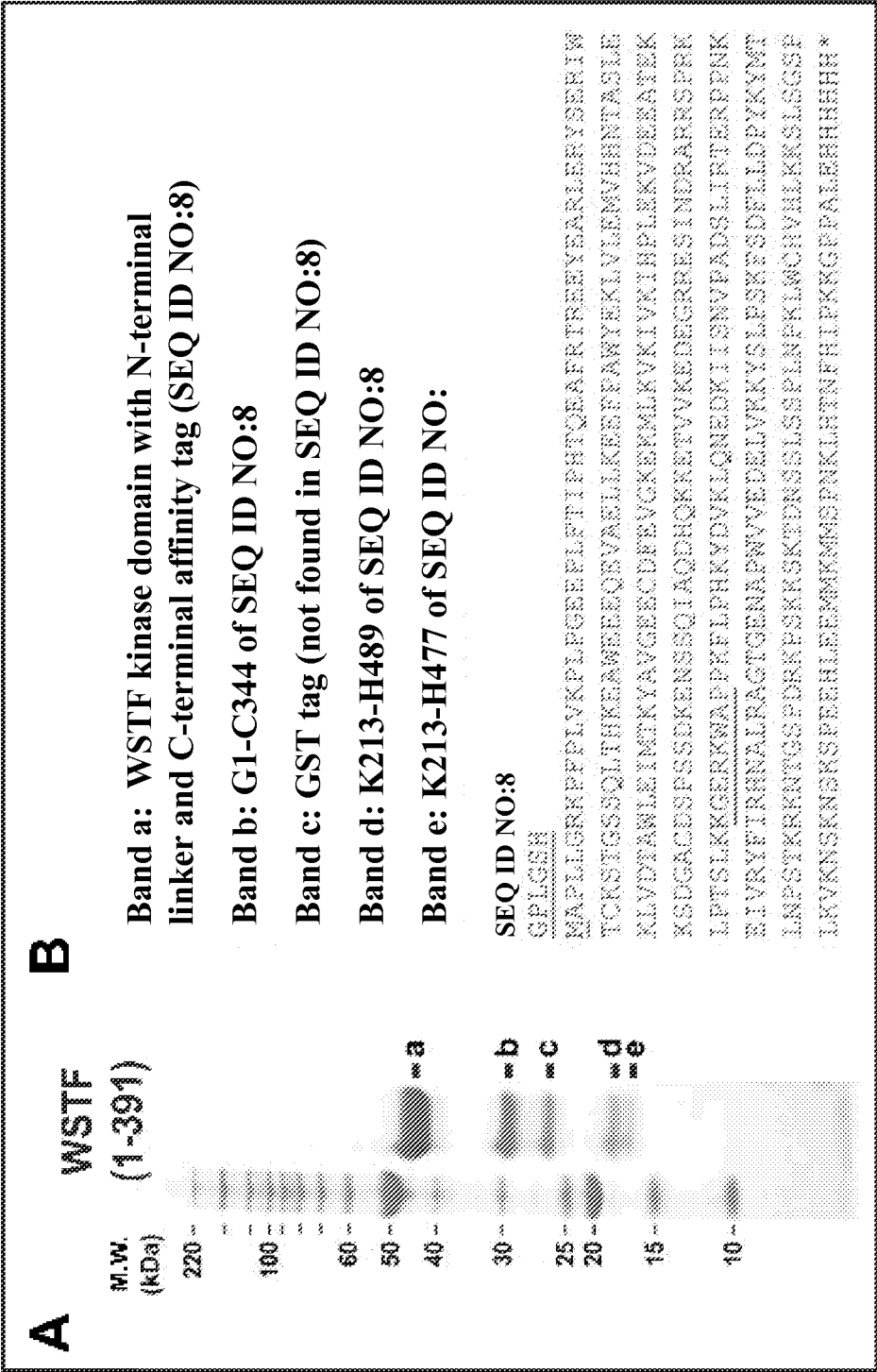


Figure 12



Figures 13A-13B

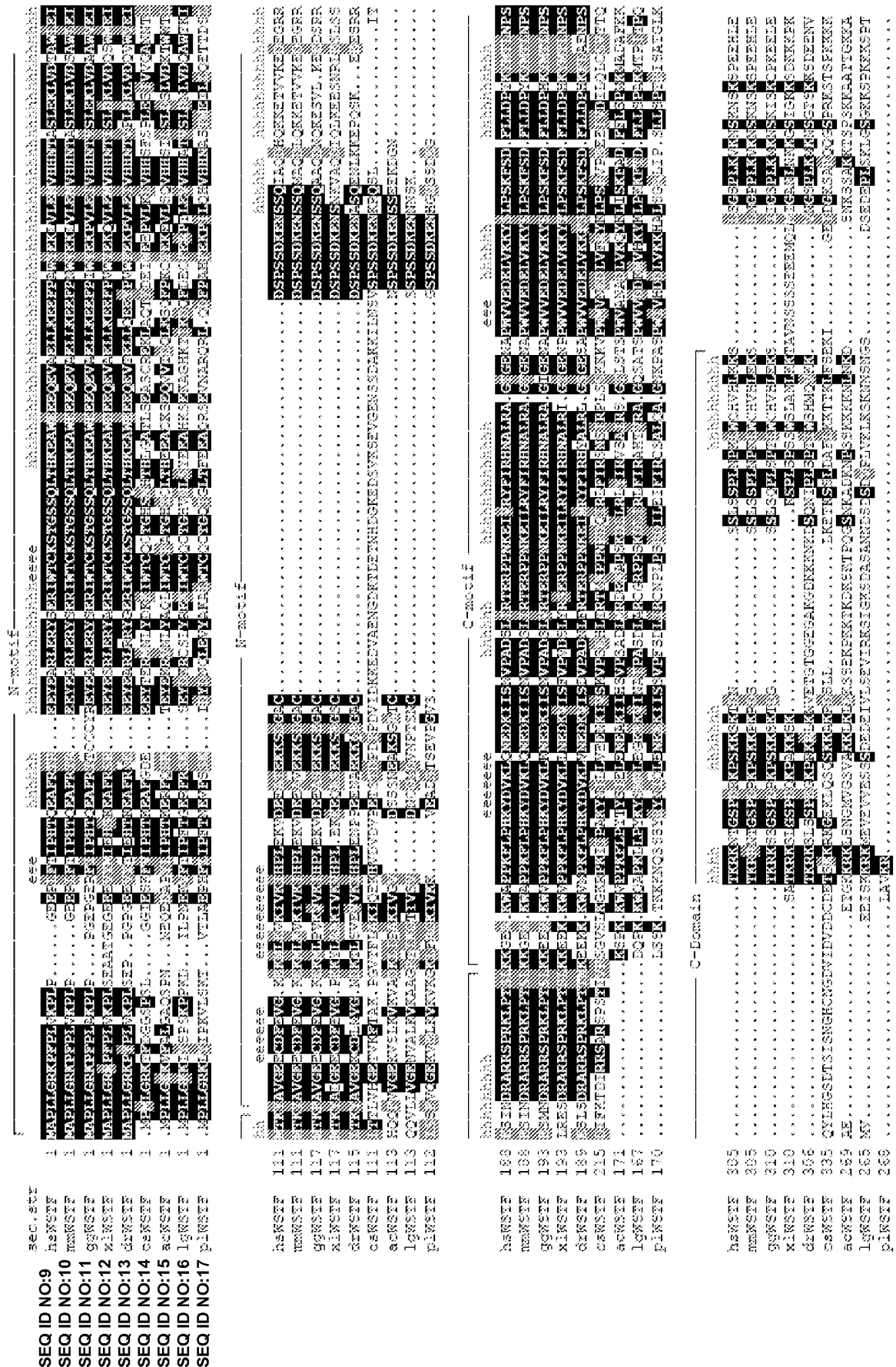


Figure 13C