Abstract:

Title: METHOD OF SCREENING FOR ANTICANCER AGENTS

A method of identifying an agent capable of modulating the activity of hSSB1 or hSSB2 is described, the method comprising the steps of contacting a single stranded binding protein (SSB) with a binding partner in the presence of a test agent, and detecting the presence or absence of binding of the SSB and binding partner.
Method of Screening for Anticancer Agents

Field of the Invention
The present invention relates to methods for identifying agents capable of modulating human Single Strand Binding proteins (SSBs), novel agents capable of modulating human SSBs and methods of use of said novel agents, particularly for the therapeutic and/or prophylactic treatment of cancer.

Incorporation by Reference
This patent application refers to the following prior patent application:

This entire content of this application is hereby incorporated by reference.

This patent application refers to the following patent application:

The entire content of this application is hereby incorporated by reference.

The entire content of Richard et al., Nature 2008 May 29; 453 (7195) 677-681 is incorporated by reference.

The entire content of WO2008/1 06709 is incorporated by reference.

Background of the Invention
Previous studies (described in Australian Provisional Application No 2007901166) identified two novel human Single Strand Binding proteins (SSBs), designated hSSB1 and hSSB2. Prior to these studies, Replication Protein A (RPA) was the only identified eukaryotic SSB known to bind single stranded DNA in response to DNA damage. The characterisation of hSSB1 revealed a domain organisation closer to the archaeal SSB than to eukaryotic RPA, implicating a role for the new hSSBs in cellular DNA damage response. Further investigation also showed that depletion of hSSB1 abrogates cellular response to double stranded breaks (DSBs) following DNA damage (e.g. ionising radiation (IR) exposure). Moreover, cells deficient in hSSB1 exhibited increased radiosensitivity, defective checkpoint activation and enhanced
genomic instability coupled with a diminished capacity for DNA repair. This clearly demonstrated that the loss of hSSB1 impairs DNA damage response.

Many DNA damage-signalling proteins are recruited to sites of damage with a hierarchy of proteins involved in the assembly of damage responsive foci (Paull et al., 2000). The present applicants studied the dependency of hSSB1 foci formation on other DNA damage response proteins including histone H2AX, the mediator of DNA damage checkpoint 1 protein (MDC1) and the Mre11-Rad50-Nbs1 (MRN) complex. hSSB1 foci were found to form with apparently normal kinetics in the absence of DNA damage response proteins, suggesting that hSSB1 may function in parallel with or upstream of these proteins. Further, the studies found that hSSB1 interacts with the MRN complex and facilitates the accumulation of this complex and, possibly, other factors to foci at the site of DNA damage. Moreover, hSSB1 was also found to function to promote Rad51-mediated strand exchange and thus contribute to homologous recombination (HR) repair. In addition, the studies found that the loss of functional hSSB1 resulted in the loss of the host cell's ability to initiate DNA damage signalling pathways and initiate HR repair following exposure to DNA damaging agents. This, in turn, results in chromosomal instability, the accumulation of spontaneous mutation and eventually cell death.

As chromosomal aberrations were observed at G1/S phases of cell replication and, as a consequence, were inherited by daughter cells, further studies of the involvement of hSSB1 in tumorigenesis were conducted. As a result of these studies, it was observed that abnormal expression of hSSB1 was associated with cells undergoing transformation, with hSSB1 overexpression present in all tumour and cancer tissues sampled. Further, the cytoplasmic localisation of hSSB1 was found to be associated with worse patient prognosis.

In summary, previous studies identified a novel protein (hSSB1), that behaves in a manner characteristic of DNA DSB "sensors". These studies provide new insights into DNA damage signal transduction and reveal hSSB1 as a key protein in cellular DNA damage responses, including cell cycle checkpoint activation, HR repair and in the maintenance of genomic stability. Further, these studies strongly implicated hSSB1 in tumorigenesis, thus providing a suitable marker for cancer diagnosis, cancer predisposition and the prognosis of existing cancers and tumours.
The present applicant herein describes further studies of hSSB1 elucidating the mechanisms involved in DNA damage signal transduction, to thereby further characterise hSSB1 and, in particular, identify DNA binding regions or active sites of the protein that are involved in signal transduction. Further, the present applicant demonstrates that hSSB1 is phosphorylated by ATM protein kinase (ATM) in response to double stranded DNA breaks and that this phosphorylation event is required for DNA damage-induced stabilisation of hSSB1. In particular, it is shown herein that, upon induction of DNA damage, hSSB1 accumulates in the nucleus, forming distinct foci that co-localise with known repair proteins. However, unlike RPA, hSSB1 was not found to localise to replication foci in S-phase cells and, further, hSSB1-deficiency was not found to influence normal S-phase progression.

Summary of the Invention

The present invention relates to methods for identifying agents that modulate the activity of nucleic acid binding proteins, and to the agents so identified.

In particular, the present invention relates to methods for identifying and producing agents capable of disrupting the interaction of single strand binding proteins with their cognate binding partner(s) (e.g. proteins and nucleic acids) such that the activity of the single strand binding protein is modulated.

Modulation of the activity of the single strand binding protein preferably refers to a down-regulation or up-regulation (but most preferably down-regulation) of the nucleic acid repair activity of the protein. Thus, a cell or system (containing the single strand binding protein) to which the test agent is applied preferably has a reduced capacity for repair of chromosome or other nucleic acid strand breaks. This provides a decrease in the ability of the cell or system to repair genotoxic damage. In the case of treatment of cancers with DNA-damaging irradiation or chemotherapeutic agents that cause DNA damage, an impaired ability to repair the damaged DNA facilitates death of the cancer cell (e.g. through apoptosis) and thereby provides a treatment for the cancer.

In the methods of the present invention the cognate binding partners may be nucleic acids or proteins. In some embodiments the binding partners are preferably proteins, polypeptides or peptides, and are preferably not nucleic acids. In some other
embodiments the binding partners are preferably nucleic acids, e.g. single or double stranded DNA or RNA, and are preferably not proteins, polypeptides or peptides.

In one aspect of the present invention a method is provided, the method comprising the steps of contacting a single strand binding protein (SSB) with a binding partner in the presence of a test agent, and detecting the presence or absence of binding of the SSB and binding partner.

In preferred embodiments the binding partner is a nucleic acid, for example chosen from single stranded DNA, single stranded RNA, double stranded DNA, double stranded RNA, most preferably single stranded DNA.

In other preferred embodiments the binding partner is a protein, polypeptide or peptide.

In a preferred embodiment the binding partner is preferably chosen from:

(i) Ataxia telangiectasia mutated (ATM) kinase,
(ii) MRN complex,
(iii) Proteosomes, and/or
(iv) Histone H2AX,

or a functional fragment of any one of (i)-(iv).

In one preferred embodiment the binding partner is ATM kinase or Fragment 4 of ATM kinase.

In preferred embodiments the SSB is hSSB1 or hSSB2.

Binding of the SSB and binding partner may be detected by detecting formation of a complex of the SSB and binding partner. The complex may optionally comprise additional molecules. In the complex molecules may be associated through covalent bonds or non-covalent bonds, or a mixture of both.

It is not an essential requirement of the invention that a stable complex of SSB and binding partner is detected or detectable. Complexes may be formed transiently, with the interaction of the molecules preferably being sufficient to alter the activity of one or both of them, e.g. through physical, chemical or structural/conformational
modification of one or more of the molecules involved in the complex. Detection of binding may involve indirect detection of the binding event, e.g. by detecting the physical, chemical or structural/conformational modification.

Accordingly, the occurrence of binding may also be detected by detecting a change in one or both of the SSB and binding partner. In some embodiments, this may comprise detecting a change in the phosphorylation state of one or each of the SSB and binding partner. In these embodiments the binding partner may preferably be a kinase. In one preferred embodiment, the occurrence of binding of the SSB and ATM kinase, or a functional fragment thereof, is detected by detecting a change in the phosphorylation state of one or both of said SSB and ATM Kinase.

In some preferred embodiments the binding partner is a fluorescent labelled nucleic acid and binding of the SSB and nucleic acid is detected by detecting a change, preferably an increase, in fluorescent polarisation. The effect (e.g. inhibition) of a test agent on binding of the nucleic acid and SSB may be detected by measuring the change in fluorescent polarisation and comparing the measured change with a control value representing the change in fluorescent polarisation on binding of the SSB and nucleic acid in the absence of the test agent. In some arrangements the change in fluorescent polarisation, if any, would be less in the presence of an inhibitor of SSB-nucleic acid binding.

In some preferred embodiments the binding partner is Allophycocyanin (APC) labelled nucleic acid and binding activity of the SSB is detected by measuring the increase in the ratio between 665nm and 615nm emission following establishment of FRET with the APC labelled nucleic acid and SSB. The effect (e.g. inhibition) of a test agent on binding of the nucleic acid and SSB may be detected by measuring the ratio and comparing the measured ratio with a control ratio value determined in the absence of the test agent.

In some preferred embodiments the binding partner is nucleic acid and binding of nucleic acid to an SSB is measured by competition assay, e.g. in the presence, and optionally in the absence, of a test agent. For example, competition may be between free unlabelled nucleic acid and immobilised nucleic acid. Inhibition of competitive binding may be measured by pre-incubating test agents with the SSB before contacting the nucleic acid.
In some preferred embodiments the binding partner is nucleic acid and a cell proliferation assay is used to determine if a test agent inhibits an SSB. Proliferation of cells, e.g. fibroblasts, may be measured following formation of nucleic acid strand breaks through application of a DNA damaging agent.

In one preferred embodiment the SSB is hSSB1 and binding of hSSB1 with ATM kinase is detected by detecting phosphorylation of hSSB1, e.g. at Threonine 117.

In some aspects of the present invention test agents are assessed for their ability to alter the activity of a single strand binding protein. This may involve the use of an assay of single strand binding protein activity. Preferably, an activity is determined when the assay is conducted in the absence of the test agent, thereby providing a "control activity". The ability of a test agent to modulate the activity of the single strand binding protein is determined by conducting the assay in the presence of the test agent to obtain a "test activity", and comparing the test activity with the control activity.

Accordingly, in one aspect of the present invention a method is provided, the method comprising performing an assay of the activity of a single strand binding protein in the presence of a test agent to determine a test activity of the single strand binding protein, and comparing the test activity to a control activity of the single strand binding protein determined by conducting the assay in the absence of the test agent.

The comparison step may involve comparing the test activity with a standard control activity, e.g. control information provided with instructions for performing the assay (e.g. as part of an assay kit). Optionally the method may include the step of performing the assay in the absence of the test agent in order to provide the control activity.

A change (e.g. increase or decrease) in the activity of the single strand binding protein, as determined in the method, between control and test assays indicates that the test agent is able to modulate the activity of the single strand binding protein. Preferably, the change is statistically significant (preferably p<0.05, more preferably p<0.01, more preferably p<0.005). Optionally the test activity and/or control activity is calculated as the average result of performing the assay more than once (e.g. two, three, four or five times).
The methods of the present invention may identify test agents as modulators of the activity of single strand binding proteins. Owing to their ability to prevent repair of DNA strand breaks, such agents are useful in the treatment of cancer. Accordingly, in further aspects of the present invention an agent identified by a method of the present invention to modulate the activity of a single strand binding protein is provided for use in the treatment of a cancerous condition.

In one aspect of the present invention a pharmaceutical composition or medicament is provided comprising an agent identified by a method of the present invention to modulate the activity of a single strand binding protein, and optionally further comprising a pharmaceutical carrier, adjuvant or diluent.

In another aspect of the present invention an agent identified by a method of the present invention to modulate the activity of a single strand binding protein or pharmaceutical composition or medicament comprising such an agent is provided for use in the prevention or treatment of a cancerous condition.

In another aspect of the present invention the use of an agent identified by a method of the present invention to modulate the activity of a single strand binding protein in the manufacture of a pharmaceutical composition or medicament for use in the prevention or treatment of a cancerous condition is provided.

In another aspect of the present invention a method of treatment of a cancerous condition is provided, the method comprising administering a therapeutically effective amount of an agent identified by a method of the present invention to modulate the activity of a single strand binding protein to a subject in need of treatment.

The agent identified by a method of the present invention to modulate the activity of a single stranded binding protein may be a polypeptide comprising the amino acid sequence of ATM kinase Fragment 4, or having an amino acid sequence having at least 60% (more preferably one of 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%) sequence identity to ATM kinase Fragment 4 (SEQ ID NO: 26). The polypeptide preferably has a length of no more than one of 350, 340, 330, 320, 310, 300, 290, 280, 270, 260, 250, 240, 230, 220, 210 or 200 amino acids, and of more than one of 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, or 330...
amino acids. The polypeptide may consist of the amino acid sequence of ATM kinase Fragment 4 (SEQ ID NO: 26).

The agent identified by a method of the present invention to modulate the activity of a single stranded binding protein may be a short interfering RNA (siRNA) that is capable of repressing or silencing the expression of hSSB1 or hSSB2. For example, the siRNA may be comprise one of \(5'\)-GACAAAGACGGCATGAG-3' (SEQ ID NO: 6) or \(5'\)-GCGCCTGATTCGAGATCCU-3' (SEQ ID NO: 7), or a nucleotide sequence having at least 70% (more preferably one of 80%, 95%, 96%, 97%, 98%, 99% or 100%) sequence identity to one of SEQ ID NOs: 6 or 7.

In aspect "A", the present invention provides a method for identifying an agent which modulates the activity of hSSB1 and/or hSSB2, said method comprising the steps of:

(i) providing a eukaryotic Single Strand Binding (SSB) protein or polypeptide, functional fragment or variant thereof;

(ii) contacting a test agent with said SSB protein or polypeptide, functional fragment or variant thereof; and

(iii) detecting a change in the activity of said SSB protein or polypeptide, functional fragment or variant thereof.

Preferably, the eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, is a human SSB, particularly a hSSB1 or hSSB2 protein or polypeptide, functional fragment or variant thereof.

The test agent may be selected from known and novel compounds, complexes and other substances which may, for example, be sourced from private or publically accessible agent libraries.

In aspect "B", the present invention provides a method for identifying an agent which modulates the activity of hSSB1 and/or hSSB2, said method comprising the steps of:

(i) providing a binding partner for a eukaryotic Single Strand Binding (SSB) protein, or a functional fragment or variant of said binding partner;

(ii) contacting a test agent with said binding partner or functional fragment or variant thereof; and

(iii) detecting binding of the test agent and said binding partner or functional fragment or variant thereof.
Binding partners suitable for use in the method of aspect "B" include ATM, the MRN complex, proteosomes and the histone H2AX.

In aspect "C", the present invention provides a novel agent which modulates the activity of hSSB1 and/or hSSB2 identified in accordance with the method of aspect "A" or "B".

In aspect "D", the present invention provides a method of treating or preventing cancer or tumour growth in a subject, said method comprising administering to said subject an agent which modulates the activity of hSSB1 and/or hSSB2, optionally in combination with a pharmaceutically-acceptable carrier.

Suitable agents for use in the method of the aspect "D" include novel agents identified in accordance with the aspects "A" or "B". Such agents may inhibit or promote the activity of hSSB1 and/or hSSB2.

In aspect "D", the present invention provides a kit for identifying an agent which modulates the activity of hSSB1 and/or hSSB2 comprising:

(i) a eukaryotic Single Strand Binding (SSB) protein or polypeptide, functional fragment or variant thereof; and/or
(ii) a binding partner for a eukaryotic SSB, or a functional fragment or variant of said binding partner.

Description of Preferred Embodiments

The details of one or more embodiments of the invention are set forth in the accompanying description below including specific details of the best mode contemplated by the inventors for carrying out the invention, by way of example. It will be apparent to one skilled in the art that the present invention may be practiced without limitation to these specific details.

In some preferred embodiments methods of the present invention include methods for identifying an agent capable of modulating the activity of a single strand binding protein.
Modulation of activity may include a down-regulation (decrease) in activity or an up-regulation (increase) in activity. The decrease or increase in activity may be determined by reference to a control activity, in which activity of the single strand binding protein is assessed in the absence of the test agent.

In some preferred embodiments methods of the present invention include methods for identifying an agent capable of modulating the binding of a single strand binding protein with a binding partner.

Modulation of binding may include interference in, e.g. complete or partial disruption or inhibition of, the binding interaction.

SSB activity is preferably activity in binding to nucleic acid, e.g. single stranded DNA, or protein, polypeptide or peptide and/or in repairing damage to nucleic acid, for example repairing DNA strand breaks induced by chemical attack or irradiation. SSB activity may be determined by performing one of several assays.

For example, activity in binding nucleic acid, e.g. single-stranded DNA, can be measured and standardized for high-throughput screening using a fluorescence polarization assay. Initial hits for test agents indicated in the assay to be candidate SSB modulators, e.g. inhibitors, can be confirmed using electrophoretic mobility shift (gel shift) assay (EMSA) to detect nucleic acid binding. Leads identified in vitro can be assayed for their ability to mimic the phenotypic aspects of SSB-depleted cells, such as sensitization of cells to DNA damaging agents, interference with homologous recombination-dependent repair of DNA damage and effect on ATM kinase activation and activity.

SSB activity may be determined by measuring SSB binding to nucleic acid, e.g. by measuring changes in fluorescent polarisation of fluorescent labelled nucleic acid or by measuring the change in ratio between 665nm and 615nm emission following establishment of FRET with Allophycocyanin labelled nucleic acid and the SSB. In other embodiments SSB activity may be determined by an assay in which a chemical attack or irradiation is applied to the nucleic acid, preferably causing a single stranded- or double stranded-DNA break. The SSB is allowed to contact the damaged nucleic acid and the ability of the SSB to repair the damage is assessed, e.g. through a cell based proliferation assay. Formation of ssDNA breaks by
application of a DNA damaging agent may also cause accumulation of hSSB1 and other DNA repair proteins within the nuclei of NFFs. This accumulation of protein (foci formation) can be assayed, observed and quantified, for example by immunofluorescence.

Nucleic acid repair may be assessed in a number of ways, such as (i) measuring cell viability (e.g. ability of cells to undergo successful chromosome replication and/or cell division, or cytotoxicity assays such as the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay when used as a measure of the ability of the SSB to repair cytotoxic DNA damage, cell proliferation assays such as BrdU incorporation assays), (ii) determining the presence or absence of cell cycle arrest (e.g. arrest at checkpoints such as G1/S or G2/M), (iii) homologous recombination assay (e.g. determining the ability of a cell or in vitro model system to incorporate nucleic acid).

SSB activity may also be assessed by measuring the ability of the SSB to interact with binding partners involved in the nucleic acid repair process, for example proteins, polypeptides or peptides including ATM kinase, MRN complex, proteosomes, Histone H2AX or functional fragments thereof.

This interaction may be determined by detecting formation of SSB:binding partner complexes, or by detecting phosphorylation of SSB and/or its binding partners.

It is here reported that hSSB1 is phosphorylated by ATM kinase in response to dsDNA strand breaks. Accordingly, in some assays in which hSSB1 or hSSB2, ATM kinase and a test agent are provided the phosphorylation of hSSB1 in response to DNA strand breakage may be measured in order to determine the effect of the test agent on hSSB1 or hSSB2 activity. A block or decrease in phosphorylation of hSSB1 or hSSB2 is indicative of a decrease in SSB activity.

Methods according to the present invention involve contacting components, such as an SSB and binding partner. In order to contact components of the methods a mixture of the components is preferably formed. In some preferred embodiments at least one component (and optionally all components) are provided in liquid or fluid solution. In some preferred embodiments one or more components may be immobilised on a solid or semi-solid support (e.g. a chip, plastics support or column)
and contacted with a solution containing at least one of the other components to allow the components to contact each other.

Complexes may be detected using techniques known to those of skill in the art, for example immunoassays incorporating a detectable label (e.g. fluorescent or radiolabel) such as ELISA or immunoprecipitation. Phosphorylation may be detected using techniques known to those of skill in the art, such as kinase assays (e.g. electrophoresis kinase assays) incorporating radiolabeled phosphorous $^{32}$P).

Methods according to the present invention may be performed in vitro or in vivo, but preferably are performed in vitro. The term "in vitro" is intended to encompass experiments with cells in culture whereas the term "in vivo" is intended to encompass experiments with intact multi-cellular organisms. Where the method is performed in vitro it may comprise a high throughput screening assay. Test agents used in the method may be obtained from a synthetic combinatorial peptide library, or may be synthetic peptides or peptide mimetic molecules. Other test compounds may comprise defined chemical entities, oligonucleotides or nucleic acid ligands.

In some preferred embodiments in vitro assays may be cell based assays. In such assays the SSB and binding partner are components of the cells being used in the assay and the test agent may be added directly to the cell. In other preferred embodiments in vitro assays use cell extracts containing the SSB and binding partner. Cell extracts may be prepared by lysis of cells and centrifugation to extract a desired component, e.g. nuclear or cytoplasmic fraction.

The cells may be non-human cells, e.g. rabbit, guinea pig, rat, mouse or other rodent (including cells from any animal in the order Rodentia), cat, dog, pig, sheep, goat, cattle, horse, non-human primate or other non-human vertebrate organism; and/or non-human mammalian cells; and/or human cells.

The present invention relates, inter alia, to methods of screening agents to identify those that are capable of modulating hSSB1 and/or hSSB2, novel agents capable of modulating hSSB1 and/or hSSB2 and methods of use of said novel agents, particularly for the therapeutic and/or prophylactic treatment of cancer or tumour growth.
Aspect "A" of the present invention provides a method for identifying an agent which modulates the activity of hSSB1 and/or hSSB2, said method comprising the steps of:
(i) providing a eukaryotic Single Strand Binding (SSB) protein or polypeptide, functional fragment or variant thereof;
(ii) contacting a test agent with said SSB protein or polypeptide, functional fragment or variant thereof; and
(iii) detecting a change in the activity of said SSB protein or polypeptide, functional fragment or variant thereof.

Preferably, the eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, is an SSB protein or polypeptide other than eukaryotic Replication Protein A (RPA) or, more preferably, other than eukaryotic RPA or a functional fragment or variant thereof.

More preferably, the eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, is an SSB protein or polypeptide comprising the following amino acid sequence:

```
X^3 L Y X^2 D X^1 E G X^8 C K N X^3 D X^5 F I V L E X^6 G X^7 T X^9 H T K X^1 X^13 E V R X^17 X^18 X^0
```

(SEQ ID NO: 1)

wherein X^3 is selected from F, L and P, X^1 is selected from V and I, X^2 is selected from K and R, X^3 is selected from I and V, X^5 is selected from P and A, X^6 is selected from L and S, X^8 is selected from L and I, X^9 is selected from N and S, X^10 is selected from L, V and I, X^11 is selected from I, L and V, X^12 is selected from T, I and V, X^13 is selected from R and V, X^14 is selected from V and A, X^15 is selected from K and V, X^16 is selected from D and E, X^17 is selected from G and N, X^18 is selected from H and R, X^19 is selected from T, S and N, X^20 is selected from C and F, X^21 is selected from K and R, X^22 is selected from A and G, X^23 is selected from K, R and P, X^24 is selected from T and S, X^25 is selected from G and A, X^26 is selected from S and C, X^27 is selected from N, T and A, X^28 is selected from I and V, X^29 is selected from V and I, X^30 is selected from D and E, X^10 is selected from V, I, L and P, X^11 is selected from N, G, S and K, X^14 is selected from L and F, X^15 is selected from Q and A, X^16 is selected from P and T, X^17 is selected from I and V, X^18 is selected from K and R, X^19 is selected from V, M, L and I, X^14 is selected from F and W, X^15 is selected from K and R, X^16 is selected from G and H, X^14 is selected from T and S, X^15 is selected from R
and K, Xψ is selected from G and N, X15 is selected from D and E, X8 is selected from L and V, X6 is selected from Q and F, X9 is selected from F and Y, X7 is selected from Y and F, X3 is selected from S and N, X is selected from V and S, X5 is selected from P and V, Xκ is selected from F and M, X1 is selected from N and K, X16 is P or is null, X16 is selected from E and D or is null, Xv is selected from Y, L and R, X17 is selected from S, R, N, I, L and A, X18 is selected from T, G, A and E, and X0 is selected from Q and A, or a functional fragment of variant thereof.

Even more preferably, the eukaryotic SSB protein or polypeptide is a mammalian SSB protein or polypeptide comprising the following amino acid sequence:

```
FXIXXDX3X6GKLNX6X5FIVLEX6GVTKTDGHEVRX7KVADX7TGSIX8I
SVWXDX9X10GX11LIQX4ODIIRLTX12GYASX13X14KGCLTLTYRGGX15LQKIG
EFCKVADVSEPFGP46XY17X18QQ (SEQ ID NO: 2)
```

wherein X1 is selected from V and I, X2 is selected from K and R, X3 is selected from I and V, X8 is selected from P and A, X4 is selected from L and V, X5 is selected from 1 and V, X6 is selected from T and I, X7 is selected from T and S, X9 is selected from K and R, X8 is selected from N and T, X9 is selected from D and E, X10 is selected from V and L, X11 is selected from N and G, X12 is selected from P and T, X12 is selected from K and R, X13 is selected from V and M, X14 is selected from F and W, X15 is selected from D and E, X16 is selected from E and D, X17 is selected from S, R and N, and X18 is selected from T and G, or a functional fragment or variant thereof.

Most preferably, the eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, is a human SSB, particularly a hSSB1 or hSSB2 protein or polypeptide, functional fragment or variant thereof.

The eukaryotic SSB protein or polypeptide, functional fragment or variant thereof may be provided by isolating wild-type or variant eukaryotic SSB, preferably hSSB1 or hSSB2 (e.g. from a cell culture, particularly a culture of cells that overexpress hSSB1 (e.g. transformed cell lines such as HeLa or HEK293T cells) or hSSB2), using any of the protein isolation techniques well known to persons skilled in the art such as any one or a combination of ion exchange, chromatography electrophoresis, isoelectric focusing, adsorption chromatography, paper chromatography, reverse-phase chromatography, hydrophobic interaction chromatography, dialysis, ultrafiltration, gel electrophoresis, gel filtration, and ultracentrifugation. Where a
functional fragment is to be provided, the isolated wild-type or variant SSB may be cleaved with a proteolytic enzyme (e.g. trypsin).

Otherwise, the eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, can be provided by expressing recombinant SSB protein or polypeptide (preferably hSSB1 or hSSB2 protein or polypeptide), or a functional fragment or variant thereof. Suitable techniques for expressing recombinant eukaryotic SSB protein or polypeptide, or a functional fragment or variant thereof are well known to persons skilled in the art and include, for example, techniques for expressing recombinant His-tagged SSB from a suitable expression vector or cassette using a suitable host cell (e.g. CHO cells and BL21 cells). Thereafter, the His-tagged expression products can be readily isolated using affinity chromatography (e.g. using an Ni-NTA column (Qiagen Inc., Valencia, CA United States of America)). Where a functional fragment is to be provided, isolated recombinant SSB protein or polypeptide may be cleaved with a proteolytic enzyme (e.g. trypsin).

Optionally, the SSB protein or polypeptide, functional fragment or variant thereof, can be provided by the provision of a host cell which naturally or recombinantly expresses the SSB protein or polypeptide, functional fragment or variant thereof, is provided. In such a case, the method of the aspect "A" utilises isolated whole cell(s) or whole or partly purified cell lysates or preparations. In this regard, normal cell lines such as NFF and lymphoblastoid cells (e.g. C3ABR), may generally be suitable for use in the method of the aspect "A". However, cells that overexpress a eukaryotic SSB, in particular transformed cell lines such as HeLa and HEK293T cells which overexpress hSSB1, may be preferred for embodiments of the method wherein it is desired to identify agents that modulate hSSB1 and/or hSSB2 by inhibiting the activity of hSSB1 and/or hSSB2. Similarly, cells that underexpress a eukaryotic SSB such as L3 cells (lymphoblastoid cells derived from ataxiatelangiectasia) which under express hSSB1, or cells that are defective in stabilization of SSB after DNA damage (such as L3 cells), may be preferred for embodiments of the method wherein it is desired to identify agents that modulate hSSB1 and/or hSSB2 by enhancing or promoting the activity of hSSB1 and/or hSSB2.

Functional fragments of eukaryotic SSB suitable for use in the method of aspect "A" include regions of such proteins or polypeptides that comprise the protein OB fold or which constitute sites at which eukaryotic SSB binds with a binding partner (e.g.
ATM, the MRN complex, proteosomes and the histone H2AX) and which, preferably, include amino acid residue 117, or variants of such regions.

Preferred functional fragments of hSSB1 therefore include fragments comprising the following amino acid sequence:

MTTETFVKDJKLNLILIVFLETGRVTGTKDGHEVRTKAVDKTGSLNSI
WDDVGNIQPGDIIRLATKGYASVFK=GCLTLTGRGDLQKIGECFMVYSEVP
NFSEPN  (SEQ ID NO: 3)
or a variant thereof.

In further preferred embodiments of the method of the aspect "A", the provided eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, is an hSSB1 protein or polypeptide comprising the following amino acid sequence:

MTTETFVKDJKLNLILIVFLETGRVTGTKDGHEVRTKAVDKTGSLNSI
WDDVGNIQPGDIIRLATKGYASVFK=GCLTLTGRGDLQKIGECFMVYSEVP
NFSEPNPEYSTSQAPNKLAVQDNPALSNPSAQPTGSTGSAASPASENQNGS
QAPPGGPPHPHTPSPSTRQPNHTPAGPPGPSSNPVSNGKETR
RSSKR    (SEQ ID NO: 4);
or a variant thereof, or alternatively, an hSSB2 protein or polypeptide comprising the following amino acid sequence:

MNRVNDPLIFRDIKPGKLNLNWFIIVLEIQRVTGTKDGHEVRSCGAVDKTGSLNSI
TISVWDEIGGLIQPGDILLTRGYASWMKGCLTLTGRGDELQKIGECFMVY
SEVNPNSFEPNPDPYRGQQKGAQESEQKNNSMNSNMGTVGTGPGPVNGVHT
GPESREHQFSHAGRSNARGLINESQPLQGTASQTV    (SEQ ID NO: 5);
or a variant thereof.

The step of contacting the test agent with said eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, may be achieved by, for example, simply forming an admixture (e.g. in solution or aqueous phase) of the test agent and the SSB protein or polypeptide, functional fragment or variant thereof, or whole cell(s) (e.g. in a culture medium) or whole or partly purified cell lysate or preparation, as the case may be.

The test agent may be selected from known and novel compounds, complexes and other substances which may, for example, be sourced from private or publically accessible agent libraries (e.g. the Queensland Compound Library (Griffith
University, Nathan, QLD, Australia) and the Molecular Libraries Small Molecule Repository. The test agent may therefore comprise a peptide (e.g. a peptide fragment of hSSB1 or hSSB2) or a mimic thereof (including so-called peptoids and retro-inverso peptides), but more preferably comprise small organic molecules and especially those which comply or substantially comply with Lipinski's Rule of Five for "druglikeness" (Lipinski, CA. et al., 2001). The test agent may also be selected on the basis of structural analysis of known or novel compounds or may otherwise be designed by structural analysis of the binding sites described herein.

The method of the aspect "A" may be adapted for high-throughput screening of large numbers of test agents.

The step of detecting a change in activity of the eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, is preferably conducted using one or more standard enzyme activity assays well known to persons skilled in the art. Such assays may therefore include MTT assays, MRN binding assays, 5-bromodeoxyuridine (BrdUrd) incorporation assays or ATM assays. A change in activity may be observed in such assays by using standard methods including spectrophotometric, fluorimetric, calorimetric or chemiluminescent means preferably providing for the automation or partial automation of the detecting step (e.g. by a microplate reader or use of a flow cytometer).

The method of aspect "A" of the invention may further comprise intermediary steps such as, for example, washing steps or steps for the stabilisation of samples or reagents.

In aspect "B" of the present invention provides a method for identifying an agent which modulates the activity of hSSB1 and/or hSSB2, said method comprising the steps of:

(i) providing a binding partner for a eukaryotic Single Strand Binding (SSB) protein, or a functional fragment or variant of said binding partner;
(ii) contacting a test agent with said binding partner or functional fragment or variant thereof; and
(iii) detecting binding of the test agent and said binding partner or functional fragment or variant thereof.
The provided binding partner, functional fragment or variant thereof may be selected from any protein, polypeptide, peptide, other compounds, complexes or other substances which bind with and interacts with a eukaryotic SSB so as to cause or effect (e.g. bring about) the activity of the eukaryotic SSB. The eukaryotic SSB in this context is preferably one that is other than eukaryotic Replication Protein A (RPA) or, more preferably, other than eukaryotic RPA or a functional fragment or variant thereof.

More preferably, the provided binding partner is selected from any protein, polypeptide, peptide, compound, complex or other substance which binds with and interacts with a eukaryotic SSB so as to cause or effect the activity of a eukaryotic SSB protein or polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or a functional fragment of variant thereof, even more preferably, a mammalian SSB protein or polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or a functional fragment or variant thereof, and most preferably, a human SSB, particularly a hSSB1 or hSSB2 protein or polypeptide, functional fragment or variant thereof.

In a similar manner to the provision of the eukaryotic SSB in the method of aspect "A", the binding partner, functional fragment or variant thereof can be provided by isolation from a suitable source. Further, an isolated binding partner can be cleaved with a proteolytic enzyme, if desired, to produce a functional fragment thereof. Otherwise, where the binding partner, functional fragment or variant thereof is a protein, polypeptide or peptide, the binding partner, functional fragment or variant thereof can be provided by recombinant expression from a suitable host cell. The method of the aspect "B" may, therefore, utilise isolated whole cell(s) or whole or partly purified cell lysates or preparations.

Binding partners suitable for use in the method of the aspect "B" include ATM, the MRN complex, proteosomes and the histone H2AX.

Functional fragments of binding partners suitable for use in the method of the aspect "B" include regions of ATM, the MRN complex, proteosomes and the histone H2AX which bind with a eukaryotic SSB. A particularly preferred functional fragment consists in a protein or polypeptide fragment corresponding to all or an SSB-binding
portion of Fragment 4 of ATM. ATM Fragment 4 consists of amino acid residues 772 to 1102 of the ATM protein.

The step of contacting the test agent with said binding partner or functional fragment or variant thereof, may be achieved by, for example, simply forming an admixture (e.g. in solution or aqueous phase) of the test agent and the binding partner or functional fragment or variant thereof, or whole cell(s) (e.g. in a culture medium) or whole or partly purified cell lysate or preparation, as the case may be.

The test agent may be selected from known and novel compounds, complexes and other substances as described above in connection to the method of the aspect "A". A particularly preferred test agent may be selected from eukaryotic SSB-binding portions of ATM Fragment 4, particularly peptides comprising such binding portions. A test agent selected from mimetics of peptides comprising an SSB-binding portion of ATM Fragment 4 may also be particularly preferred.

As with the method of the aspect "A", the method of the aspect "B" may be adapted for high-throughput screening of large numbers of test agents.

The step of detecting binding of the test agent and binding partner or functional fragment or variant thereof, is preferably conducted using any standard binding assay format (e.g. ELISA-based or competition-based assays). Preferably, the test agent will be labelled with a readily detectable label (e.g. a fluorochrome or radioisotope) to allow detection of binding to, for example, an immobilised binding partner or functional fragment or variant thereof.

It is anticipated that test agents found to bind with the binding partner or functional fragment or variant thereof, will modulate the activity of hSSB1 and/or hSSB2 by inhibiting binding between hSSB1 and/or hSSB2 and a binding partner thereof.

The method of aspect "B" of the invention may further comprise intermediary steps such as, for example, washing steps or steps for the stabilisation of samples or reagents.
In aspect "C", the present invention provides a novel agent which modulates the activity of hSSB1 and/or hSSB2 identified in accordance with the method of aspects "A" or "B".

In aspect "D", the present invention a method of treating or preventing cancer or a tumour growth in a subject, said method comprising administering to said subject an agent which modulates the activity of hSSB1 and/or hSSB2, optionally in combination with a pharmaceutically-acceptable carrier.

Suitable agents for use in the method of the aspect "D" include novel agents identified in accordance with aspects "A" or "B". Such agents may inhibit or enhance (i.e. promote) the activity of hSSB1 and/or hSSB2.

Inhibitory agents may particularly include agents which inhibit the activity of hSSB1 and/or hSSB2 by binding to said hSSB1 and/or hSSB2 to thereby prevent binding with and interaction with a binding partner such as ATM, the MRN complex and the histone H2AX, agents which inhibit the activity of hSSB1 and/or hSSB2 by binding to binding partner such as ATM, the MRN complex and the histone H2AX to thereby prevent binding with and interaction with hSSB1 and/or hSSB2, and agents that inhibit the expression of functional hSSB1 and/or hSSB2 (e.g. antisense RNA, ribozymes, DNAzymes and small interfering RNA (siRNA)).

Enhancing agents may particularly include agents that promote hSSB1 and/or hSSB2 activity such as proteosome inhibitors (e.g. MG132).

Particularly preferred agents for use in the method of the aspect "D" include wortmannin or derivatives thereof, and oligonucleotide molecules comprising one of the following oligonucleotide sequences:

- 5'-GACAAAGGACGGGCATGAG-3' (SEQ ID NO: 6) and
- 3'-GCGCCTGATTCGAGATCCU-S' (SEQ ID NO: 7)

or a variant sequence thereof.

The agent may be formulated into any suitable pharmaceutical/veterinary composition or dosage form (e.g. compositions for oral, buccal, nasal, intramuscular and intravenous administration). Typically, such a composition or dosage form will be administered to the subject in an amount which is effective to treat or prevent
cancer or tumour growth, and may therefore provide between about 0.01 and about 100 µg/kg body weight per day of the agent, and more preferably providing from 0.05 and 25 µg/kg body weight per day of the agent. A suitable composition may be intended for single daily administration, multiple daily administration, or controlled or sustained release, as needed to achieve the most effective results.

The method of aspect "D" may be suitable for the treatment of any cancer or tumour (e.g. non-cancerous tumours such as chondromas, fibromas, polyps and gastrinomas). However, preferably, the method is used for the treatment of breast or bowel cancer.

In aspect "E", the present invention provides a kit for identifying an agent which modulates the activity of hSSB1 and/or hSSB2 comprising:

(i) a eukaryotic Single Strand Binding (SSB) protein or polypeptide, functional fragment or variant thereof; and/or

(ii) a binding partner for a eukaryotic SSB, or a functional fragment or variant of said binding partner.

A kit according to aspect "E" may further comprise means for detecting a change in activity of a eukaryotic SSB protein or polypeptide (e.g. as caused by a test agent), means for detecting binding between a binding partner of a eukaryotic SSB and, for example, a test agent, and other components as known to persons skilled in the art including, for example, wash buffers, stabilisation buffers or other reagents.

The term "variant" as used herein in relation to an amino acid sequence, is to be understood as referring to an amino acid sequence encompassing minor variations which do not result in any significant alteration of the biological activity of its derivative protein, polypeptide or peptide. These variations may include conservative amino acid substitutions. Exemplary conservative amino acid substitutions are provided in Table 1 below. Particular conservative amino acids envisaged are: G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkylamino acids.

Table 1

<table>
<thead>
<tr>
<th>Conservative Substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
</tr>
<tr>
<td>Val*, Leu, Nε</td>
</tr>
</tbody>
</table>

*Note: Ala, Val*, Leu, Nε - These are examples of conservative amino acid substitutions. G, A, V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, Nα-alkylamino acids are provided in the text.
Conservative Substitutions

<table>
<thead>
<tr>
<th>Arg</th>
<th>Lys*, Gln, Asn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn</td>
<td>Gln*, His, Lys, Arg, Asp</td>
</tr>
<tr>
<td>Asp</td>
<td>Glu*, Asn</td>
</tr>
<tr>
<td>Cys</td>
<td>Ser</td>
</tr>
<tr>
<td>Gin</td>
<td>Asn*, His, Lys,</td>
</tr>
<tr>
<td>Glu</td>
<td>Asp*, γ-carboxylglutamic acid (Gla)</td>
</tr>
<tr>
<td>Gly</td>
<td>Pro</td>
</tr>
<tr>
<td>His</td>
<td>Asn, Gln, Lys, Arg*</td>
</tr>
<tr>
<td>Ne</td>
<td>Leu*, Val, Met, Ala, Phe, norleucine (Nle)</td>
</tr>
<tr>
<td>Leu</td>
<td>Nle, lie*, Val, Met, Ala, Phe</td>
</tr>
<tr>
<td>Lys</td>
<td>Arg*, Gln, Asn, ornithine (Orn)</td>
</tr>
<tr>
<td>Met</td>
<td>Leu*, lie, Phe, Nle</td>
</tr>
<tr>
<td>Phe</td>
<td>Leu*, Val, lie, Ala</td>
</tr>
<tr>
<td>Pro</td>
<td>Gly*, hydroxyproline (Hyp), Ser, Thr</td>
</tr>
<tr>
<td>Ser</td>
<td>Thr</td>
</tr>
<tr>
<td>Thr</td>
<td>Ser</td>
</tr>
<tr>
<td>Trp</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyr</td>
<td>Trp, Phe*, Thr, Ser</td>
</tr>
<tr>
<td>Val</td>
<td>lie, Leu*, Met, Phe, Ala, Nle</td>
</tr>
</tbody>
</table>

indicates preferred conservative substitutions

Test agents

A test agent (test compound) may modulate or interfere with the interaction of molecules, e.g. of two proteins or of an SSB and a nucleic acid, in one of a number of ways. In one arrangement the agent may directly modulate the interaction by binding to one molecule, masking the site of interaction. Candidate compounds may comprise small molecule, synthetic or naturally occurring, specific inhibitors and may be enzyme active site inhibitors, either competitive or non-competitive. Alternatively, a test agent may comprise a peptide which interacts with the target molecule or an organic compound mimicking the peptide structure.

Other examples of candidate test agents include non-functional homologues of the target molecule, antibodies and antibody products, e.g. monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies and CDR-grated antibodies, which recognise one of the interacting molecules, a complex of bound molecules, or
a domain or region of a protein which is involved in the interaction, e.g. ATM kinase

Suitable agents may result in a change of sub-cellular localisation of a target protein which may result in sequestering of the protein thus preventing the two normally interacting molecules from contacting each other within the cell.

Test agents showing activity in in vitro screens can then be tested in in vivo screens, e.g. in mammalian or insect cells exposed to the candidate agent and tested for susceptibility to cell death following exposure to irradiation or a chemotherapeutic.

The modulating or interfering effect of a test agent may be assayed for by measuring an ability to regulate the cell cycle or to precipitate growth arrest or apoptosis following induction of DNA strand breakage (e.g. by exposure to irradiation or a chemotherapeutic). Such an assay may comprise (a) exposing the cell to DNA damaging agent so as to induce DNA strand breakage, (b) administering the candidate substance to a test cell, preferably a mammalian cell; and (c) determining the effect of the test compound on the cell cycle, e.g. by measuring induction of cell cycle arrest or cell death, e.g. through apoptosis. Apoptosis can be determined by one of a number of techniques known to the person skilled in the art, e.g. the observing of morphological changes such as cytoplasmic blebbing, cell shrinkage, internucleosomal fragmentation and chromatin condensation. DNA cleavage typical of the apoptotic process may be demonstrated using TUNEL and DNA ladder assays.

Interaction
Interaction of substances or molecules, e.g. of compounds, nucleic acids, proteins, peptides or polypeptides, relates to a detectable change, e.g. in a measurable property, or result which is obtained when at least two molecules e.g. a selected compound and SSB and/or binding partner, are brought together, or allowed to contact.

One form of interaction is the binding between one molecule and another, e.g. a molecular partner or target protein in a signal transduction cascade, which may be detected immunologically or by observing a change in size of components of the test sample, e.g. by gel electrophoresis.
Another form of interaction is a phosphorylation or de-phosphorylation which may be detected immunologically, e.g. by using an antibody specific to the phosphorylated or de-phosphorylated form of a protein.

Other interactions may lead to a change in activity, e.g. due to a change of conformation (allosteric activation) during and/or after interaction of the interacting molecules, of one or more of the interacting molecules, e.g. a change in kinase activity detectable by assaying for phosphorylation of a known substrate for the kinase.

**Interference**
Interference of a compound with an interaction relates to the ability of a molecule to interrupt, disrupt or prevent, whether partially or entirely, the normal interaction of the molecules and may be measurable by an altered level of activity of one or more of the normally interacting molecules or by assaying for the presence, absence or partial presence or absence of binding of the normally interacting molecules.

**Modulation**
Modulation describes the ability of a compound to vary the result of an interaction between interacting substances or molecules. Thus, modulation may be detectable by a change (increase or decrease) in the level of an activity, e.g. kinase activity, or apoptosis, or in ability to bind to an interacting partner molecule. Modulating compounds may have an enhancing effect or an inhibiting effect on the relevant activity or binding.

**Activity**
The activity of a given substance or molecule may be measured by assaying for the activity. An activity may be a function of the interaction or binding of the given substance with another molecule.

**Formulating pharmaceutically useful compositions and medicaments**
In accordance with the present invention methods are also provided for the production of pharmaceutically useful compositions, which may be based on a substance or test agent so identified or obtained. In addition to the steps of the
methods described herein, such methods of production may further comprise one or more steps selected from:

(a) identifying and/or characterising the structure of a selected test agent;
(b) obtaining the agent substance or compound;
(c) mixing the selected substance or compound with a pharmaceutically acceptable carrier, adjuvant or diluent.

For example, a further aspect of the present invention relates to a method of formulating or producing a pharmaceutical composition for use in the treatment of a cancerous condition, the method comprising identifying an agent compound or substance that modulates the activity of a single strand binding protein in accordance with one or more of the methods described herein, and further comprising one or more of the steps of:

(i) identifying the compound or substance; and/or
(ii) formulating a pharmaceutical composition by mixing the selected substance, or a prodrug thereof, with a pharmaceutically acceptable carrier, adjuvant or diluent.

Certain pharmaceutical compositions formulated by such methods may comprise a prodrug of the selected substance wherein the prodrug is convertible in the human or animal body to the desired active agent. In other cases the active agent may be present in the pharmaceutical composition so produced and may be present in the form of a physiologically acceptable salt.

In this specification reference to SSB is to a single strand binding protein. SSBs are preferably capable of binding to single-stranded DNA.

Human SSB1 (hSSB1) and human SSB2 (hSSB2) and their homologues are preferred SSBs. hSSB1 is present on chromosome 12q13.3. hSSB2 is present on chromosome 2q32.3. The hSSB1 gene sequence is deposited under Genbank accession no: NM_024068. The hSSB2 gene sequence is deposited under Genbank accession no: NM_001031716.
The hSSB1 sequence is conserved in metazoa and comprises an amino-terminal oligonucleotide/oligosaccharide-binding fold domain followed by a more divergent carboxy-terminal domain.

In this specification, an SSB may be any peptide, polypeptide or protein having an amino acid sequence having a specified degree of sequence identity to one SEQ ID No.:s 1, 2, 3, 4, 5 or 14 or to a fragment of one of these sequences. The specified degree of sequence identity may be from at least 60% to 100% sequence identity. More preferably, the specified degree of sequence identity may be one of at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity.

Replication protein A is another SSB. In preferred embodiments the SSB is not RPA.

**ATM kinase**

In this specification reference to ATM kinase is to ataxia telangiectasia mutated kinase. ATM kinase may be a human or mammalian ATM kinase. The amino acid and nucleotide sequence of one human ATM kinase is deposited under Genbank accession no: NM_000051 GI:71902539.

In this specification, an ATM kinase may be any peptide, polypeptide or protein having an amino acid sequence having a specified degree of sequence identity to the amino acid sequence of ATM kinase deposited under Genbank accession no: NM_000051 GI:71902539 (SEQ ID NO:27) or to a fragment of that sequence (e.g. SEQ ID NO: 26). The specified degree of sequence identity may be from at least 60% to 100% sequence identity. More preferably, the specified degree of sequence identity may be one of at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity.

A functional fragment of ATM kinase is a fragment of ATM that is capable of binding the SSB. One functional fragment comprises, or consists of, the amino acid sequence of ATM Fragment 4, being the amino acid sequence from amino acids 772 to 1102 of ATM kinase (SEQ ID NO: 26).

**MRN complex**
In this specification reference to the MRN complex is to the complex of Mre11-Rad50-Nbs1. Reference to the MRN complex includes the bound complex of Mre11, Rad50 and Nbs1, but also includes the non-complexed individual components of the complex, i.e. Mre11, Rad50 and Nbs1.

Functional fragments
The methods of the present invention may use protein or polypeptide components including the full length amino acid sequence of the respective component. However, in some embodiments functional fragment components may be used, where the functional fragment retains the ability to interact with the other component(s) of the assay, e.g. retains the ability to bind the other component(s) and/or phosphorylate the other component(s). In particular the functional fragment is preferably capable of binding to the SSB protein.

Functional fragments may be truncated or modified forms of the full length amino acid sequence.

Nucleic acid
In this specification reference to nucleic acid includes single stranded and/or double stranded nucleic acids which may be RNA or DNA. Preferred embodiments of the present invention are concerned with repair of breaks in single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA). dsDNA may be chromosomal DNA. Where dsDNA is concerned the break(s) may be in one or both strands.

Nucleic acids may be polynucleotides, e.g. oligonucleotides, and may comprise more than 10, preferably more than 100, nucleotides.

Therapeutic applications
Agents that decrease SSB activity may be useful in sensitising cells to DNA damage to enable selective cell killing.

Agents (including compounds) of the present invention or identified by methods of the present invention may be used in the treatment of tumours and cancer in animals in need of treatment thereof.
The cancerous condition may be any unwanted cell proliferation (or any disease manifesting itself by unwanted cell proliferation), neoplasm or tumour or increased risk of or predisposition to the unwanted cell proliferation, neoplasm or tumour. The cancerous condition may be a cancer and may be a benign or malignant cancer and may be primary or secondary (metastatic). A neoplasm or tumour may be any abnormal growth or proliferation of cells and may be located in any tissue. Examples of tissues include the colon, pancreas, lung, breast, uterus, stomach, kidney, testis, central nervous system (including the brain), peripheral nervous system, skin, blood or lymph.

Methods of treatment of a cancerous condition may comprise administering an agent identified by a method of the present invention to modulate the activity of a single strand binding protein in combination with a DNA-damaging agent as part of concomitant radiotherapy or chemotherapy.

Preferred DNA damaging agents include irradiation (radiotherapy) or chemotherapeutic compounds (chemotherapy). The DNA damaging agent is preferably capable of causing DNA or RNA strand breaks when administered in therapeutic doses. Examples of suitable irradiation include ionising radiation such as X-ray or γ-ray irradiation, or ultraviolet rays especially UV-C rays (~260 nm) and UV-B rays. Examples of suitable chemotherapeutic compounds include alkylating agents such as cyclophosphamides, melphalan, busulfan, chlorambucil, mitomycin, bleomycin; cross-linking agents such as cisplatin; topoisomerase inhibitors such as irinotecan, mitoxantrone; and intercalators such as dactinomycin; as well as compounds producing highly reactive oxygen radicals.

By "combination" is meant administration of the agent identified in the present invention and DNA damaging agent as part of a treatment regime or programme in which administration of the DNA damaging agent is used to induce nucleic acid strand breaks in the cancerous tissue and the agent identified by the present invention is administered to impair repair of the strand break and facilitate death of the cancerous cell(s). The DNA damaging agent and agent of the present invention are not required to be administered simultaneously, although this is permissible, but may be administered separately or sequentially in accordance with a dosage regime to be determined by the prescribing medical practitioner.
Agents of the invention may be formulated as pharmaceutical compositions or medicaments for clinical or veterinary use and may comprise a pharmaceutically acceptable carrier, diluent or adjuvant. Medicaments and pharmaceutical compositions according to aspects of the present invention may be formulated for administration by a number of routes, including but not limited to, parenteral, intravenous, intra-arterial, intramuscular, intratumoural. The medicaments and compositions may be formulated in fluid or solid form. Fluid formulations may be formulated for administration by injection to a selected region of the human or animal body. Injectable formulations may comprise the selected compound in a sterile or isotonic medium.

Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of the disease being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins.

The subject to be treated may be any animal or human. The subject is preferably mammalian, more preferably human. The subject may be a non-human mammal, but is more preferably human. The subject may be male or female. The subject may be a patient. Therapeutic uses may be in human or animals (veterinary use).

**Mimetics**

Agents identified according to the present invention may be peptides or polypeptides. Also provided are peptide or polypeptide mimetics of those agents.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. some peptides may be unsuitable active agents for oral compositions as they tend to be quickly
degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large numbers of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade \textit{in vivo}, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for \textit{in vivo} or clinical testing.

With regard to the present invention, having identified a peptide or peptide mimetic in accordance with the method described, the method may further comprise the step of modifying the peptide structure, optionally followed by repeating the method of the
invention with the modified peptide. This process of modification of the peptide or peptide mimetic may be repeated a number of times, as desired, until a peptide having the desired effect, or level of effect, on SSB protein activity is identified.

The modification steps employed may comprise truncating the peptide or peptide mimetic length (this may involve synthesising a peptide or peptide mimetic of shorter length), substitution of one or more amino acid residues or chemical groups, and/or chemically modifying the peptide or peptide mimetic to increase stability, resistance to degradation, transport across cell membranes and/or resistance to clearance from the body.

RNAi

The present invention also includes the use of techniques known in the art for the therapeutic down regulation of SSB expression. These include the use RNA interference (RNAi). As in other aspects of the present invention, this may be used in the treatment of cancerous conditions.

Small RNA molecules may be employed to regulate gene expression. These include targeted degradation of mRNAs by small interfering RNAs (siRNAs), post transcriptional gene silencing (PTGs), developmentally regulated sequence-specific transcriptional repression of mRNA by micro-RNAs (miRNAs) and targeted transcriptional gene silencing.

A role for the RNAi machinery and small RNAs in targeting of heterochromatin complexes and epigenetic gene silencing at specific chromosomal loci has also been demonstrated. Double-stranded RNA (dsRNA)-dependent post transcriptional silencing, also known as RNA interference (RNAi), is a phenomenon in which dsRNA complexes can target specific genes of homology for silencing in a short period of time. It acts as a signal to promote degradation of mRNA with sequence identity. A 20-nt siRNA is generally long enough to induce gene-specific silencing, but short enough to evade host response. The decrease in expression of targeted gene products can be extensive with 90% silencing induced by a few molecules of siRNA.

In the art, these RNA sequences are termed "short or small interfering RNAs" (siRNAs) or "microRNAs" (miRNAs) depending on their origin. Both types of sequence may be used to down-regulate gene expression by binding to
complementary RNAs and either triggering mRNA elimination (RNAi) or arresting mRNA translation into protein. siRNA are derived by processing of long double stranded RNAs and when found in nature are typically of exogenous origin. Micro-interfering RNAs (miRNA) are endogenously encoded small non-coding RNAs, derived by processing of short hairpins. Both siRNA and miRNA can inhibit the translation of mRNAs bearing partially complimentary target sequences without RNA cleavage and degrade mRNAs bearing fully complementary sequences.

Accordingly, the present invention provides the use of these sequences for down-regulating the expression of SSB.

The siRNA ligands are typically double stranded and, in order to optimise the effectiveness of RNA mediated down-regulation of the function of a target gene, it is preferred that the length of the siRNA molecule is chosen to ensure correct recognition of the siRNA by the RISC complex that mediates the recognition by the siRNA of the mRNA target and so that the siRNA is short enough to reduce a host response.

miRNA ligands are typically single stranded and have regions that are partially complementary enabling the ligands to form a hairpin. miRNAs are RNA genes which are transcribed from DNA, but are not translated into protein. A DNA sequence that codes for a miRNA gene is longer than the miRNA. This DNA sequence includes the miRNA sequence and an approximate reverse complement. When this DNA sequence is transcribed into a single-stranded RNA molecule, the miRNA sequence and its reverse-complement base pair to form a partially double stranded RNA segment. The design of microRNA sequences is discussed on John et al, PLoS Biology, 11(2), 1862-1879, 2004.

Typically, the RNA ligands intended to mimic the effects of siRNA or miRNA have between 10 and 40 ribonucleotides (or synthetic analogues thereof), more preferably between 17 and 30 ribonucleotides, more preferably between 19 and 25 ribonucleotides and most preferably between 21 and 23 ribonucleotides. In some embodiments of the invention employing double-stranded siRNA, the molecule may have symmetric 3' overhangs, e.g. of one or two (ribo)nucleotides, typically a UU of dTdT 3' overhang. Based on the disclosure provided herein, the skilled person can readily design suitable siRNA and miRNA sequences, for example using resources.
such as Ambion's siRNA finder, see http://www.ambion.com/techlib/misc/siRNA_finder.html. siRNA and miRNA sequences can be synthetically produced and added exogenously to cause gene downregulation or produced using expression systems (e.g. vectors). In a preferred embodiment the siRNA is synthesized synthetically.

Longer double stranded RNAs may be processed in the cell to produce siRNAs (see for example Myers (2003) Nature Biotechnology 21:324-328). The longer dsRNA molecule may have symmetric 3' or 5' overhangs, e.g. of one or two (ribo)nucleotides, or may have blunt ends. The longer dsRNA molecules may be 25 nucleotides or longer. Preferably, the longer dsRNA molecules are between 25 and 30 nucleotides long. More preferably, the longer dsRNA molecules are between 25 and 27 nucleotides long. Most preferably, the longer dsRNA molecules are 27 nucleotides in length. dsRNAs 30 nucleotides or more in length may be expressed using the vector pDECAP (Shinagawa et al., Genes and Dev., 17, 1340-5, 2003).

Another alternative is the expression of a short hairpin RNA molecule (shRNA) in the cell. shRNAs are more stable than synthetic siRNAs. A shRNA consists of short inverted repeats separated by a small loop sequence. One inverted repeat is complimentary to the gene target. In the cell the shRNA is processed by DICER into a siRNA which degrades the target gene mRNA and suppresses expression. In a preferred embodiment the shRNA is produced endogenously (within a cell) by transcription from a vector. shRNAs may be produced within a cell by transfecting the cell with a vector encoding the shRNA sequence under control of a RNA polymerase III promoter such as the human H1 or 7SK promoter or a RNA polymerase II promoter. Alternatively, the shRNA may be synthesised exogenously (in vitro) by transcription from a vector. The shRNA may then be introduced directly into the cell. Preferably, the shRNA molecule comprises a partial coding sequence of an SSB. Preferably, the shRNA sequence is between 40 and 100 bases in length, more preferably between 40 and 70 bases in length. The stem of the hairpin is preferably between 19 and 30 base pairs in length. The stem may contain G-U pairings to stabilise the hairpin structure.

siRNA molecules, longer dsRNA molecules or miRNA molecules may be made recombinantly by transcription of a nucleic acid sequence, preferably contained
within a vector. Preferably, the siRNA molecule, longer dsRNA molecule or miRNA molecule comprises a partial sequence of an SSB.

In one embodiment, the siRNA, longer dsRNA or miRNA is produced endogenously (within a cell) by transcription from a vector. The vector may be introduced into the cell in any of the ways known in the art. Optionally, expression of the RNA sequence can be regulated using a tissue specific promoter. In a further embodiment, the siRNA, longer dsRNA or miRNA is produced exogenously (in vitro) by transcription from a vector.

In one embodiment, the vector may comprise a nucleic acid sequence according to the invention in both the sense and antisense orientation, such that when expressed as RNA the sense and antisense sections will associate to form a double stranded RNA. Preferably, the vector comprises the nucleic acid sequences SEQ ID NO:s 6 and 7; or a variant or fragment thereof. In another embodiment, the sense and antisense sequences are provided on different vectors.

Alternatively, siRNA molecules may be synthesized using standard solid or solution phase synthesis techniques which are known in the art. Linkages between nucleotides may be phosphodiester bonds or alternatives, for example, linking groups of the formula P(O)S, (thioate); P(S)S, (dithioate); P(O)NR'2; P(O)R'; P(O)OR6; CO; or CONR'2 wherein R is H (or a salt) or alkyl (1-12C) and R6 is alkyl (1-9C) is joined to adjacent nucleotides through-O-or-S-.

Modified nucleotide bases can be used in addition to the naturally occurring bases, and may confer advantageous properties on siRNA molecules containing them.

For example, modified bases may increase the stability of the siRNA molecule, thereby reducing the amount required for silencing. The provision of modified bases may also provide siRNA molecules which are more, or less, stable than unmodified siRNA.

The term 'modified nucleotide base' encompasses nucleotides with a covalently modified base and/or sugar. For example, modified nucleotides include nucleotides having sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3'position and other than a phosphate group at the
5'position. Thus modified nucleotides may also include 2'substituted sugars such as
2'-O-methyl-; 2”-O-alkyl; 2’-O-allyl; 2’-S-alkyl; 2’-fluoro-; 2’-halo or azido-
ribose, carbocyclic sugar analogues, a-anomeric sugars; epimeric sugars such as
arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, and
sedoheptulose.

Modified nucleotides are known in the art and include alkylated purines and
pyrimidines, acylated purines and pyrimidines, and other heterocycles. These
classes of pyrimidines and purines are known in the art and include
pseudoisocytosine, N4,N4-ethanocytosine, 8-hydroxy-N6-methyladenine, 4-
acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5 fluorouracil, 5-bromouracil, 5-
carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyl uracil,
dihydouracil, inosine, N6-isopentyl-adenine, 1-methyladenine, 1-
methylpsuedouracil, 1-methylguanine, 2,2-dimethylguanine, 2methyladenine, 2-
methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-
methylguanine, 5-methylaminomethyl uracil, 5-methoxy amino methyl-2-thiouracil, -
D-mannosylqueosine, 5-methoxycarbonylmethyluracil, 5methoxyuracil, 2 methylthio-
N6-isopentenyladenine, uracil-5-oxyacetic acid methyl ester, pseueouracil, 2-
thiocytosine, 5-methyl-2 thiouracil, 2-thiouracil, 4-thiouracil, 5methyluracil, N-uracil-5-
oxacyclic acid methylester, uracil 5-oxyacetic acid, queosine, 2-thiocytosine, 5-
propyluracil, 5-propylcytosine, 5-ethyluracil, 5ethylcytosine, 5-butyluracil, 5-
pentyluracil, 5-pentylcytosine, and 2,6 diaminopurine, methylpsuedouracil, 1-
methylguanine, 1-methylcytosine.

Methods relating to the use of RNAi to silence genes in C. elegans, Drosophila,
plants, and mammals are known in the art (Fire A, et al., 1998 Nature 391:806-811;
366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058;

Sequence identity
Percentage (%) sequence identity is defined as the percentage of amino acid residues in a candidate sequence that are identical with residues in the given listed sequence (referred to by the SEQ ID No.) after aligning the sequences and introducing gaps if necessary, to achieve the maximum sequence identity, and not considering any conservative substitutions as part of the sequence identity.

Sequence identity is preferably calculated over the entire length of the respective sequences.

Where the aligned sequences are of different length, sequence identity of the shorter comparison sequence may be determined over the entire length of the longer given sequence or, where the comparison sequence is longer than the given sequence, sequence identity of the comparison sequence may be determined over the entire length of the shorter given sequence.

For example, where a given sequence comprises 100 amino acids and the candidate sequence comprises 10 amino acids, the candidate sequence can only have a maximum identity of 10% to the entire length of the given sequence. This is further illustrated in the following example:

(A) Given seq: XXXXXXXXXXXX (15 amino acids)
Comparison seq: XXXYYYYYYY (12 amino acids)

The given sequence may, for example, be that encoding hSSB1 (e.g. SEQ ID NO: 4).

% sequence identity = the number of identically matching amino acid residues after alignment divided by the total number of amino acid residues in the longer given sequence, i.e. (5 divided by 15) x 100 = 33.3%

Where the comparison sequence is longer than the given sequence, sequence identity may be determined over the entire length of the given sequence. For example:

(B) Given seq: XXXXXXXXXXX (10 amino acids)
Comparison seq: XXXXYYYYYYZZYZZZZZZ (20 amino acids)

Again, the given sequence may, for example, be that encoding hSSB1 (e.g. SEQ ID NO: 4).

% sequence identity = number of identical amino acids after alignment divided by total number of amino acid residues in the given sequence, i.e. (5 divided by 10) x 100 = 50%.

Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways known to a person of skill in the art, for instance, using publicly available computer software such as ClustalW 1.82. T-coffee or Megalign (DNASTAR) software. When using such software, the default parameters, e.g. for gap penalty and extension penalty, are preferably used. The default parameters of ClustalW 1.82 are: Protein Gap Open Penalty = 10.0, Protein Gap Extension Penalty = 0.2, Protein matrix = Gonnet, Protein/DNA ENDGAP = -1, Protein/DNA GAPDIST = 4.

Identity of nucleic acid sequences may be determined in a similar manner involving aligning the sequences and introducing gaps if necessary, to achieve the maximum sequence identity, and calculating sequence identity over the entire length of the respective sequences. Where the aligned sequences are of different length, sequence identity may be determined as described above and illustrated in examples (A) and (B).

The invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.
**Brief Description of the Figures**

Embodiments and experiments illustrating the principles of the invention will now be discussed with reference to the accompanying figures in which:

**Figure 1** (A) shows the nucleotide (SEQ ID NO: 12) and amino acid (SEQ ID NO:4) sequence for the hSSB1 protein, and (B) shows the nucleotide (SEQ ID NO: 13) and amino acid (SEQ ID NO: 14) sequence for the hSSB2 protein, as retrieved using the BLAST algorithm from the NCBI database, while (C) shows an alignment of the hSSB1 and hSSB2 amino acid sequences (designated in the figure as "human 1" and "human 2" respectively) against that of archaeal SSB (*Sulfolobus solfataricus*; SEQ ID NO: 20), the corresponding "mouse 1" (SEQ ID NO: 15) and "mouse 2" (SEQ ID NO: 17) amino acid sequences, as well as the amino acid sequences of the homologues from *Xenopus laevis* (SEQ ID NO: 16), *Danio rerio* (Danio; SEQ ID NO: 18), and *Drosophila melanogaster* (Dros; SEQ ID NO: 19). The alignment indicates that the proteins have a highly conserved N-terminal domain (an oligonucleotide/oligosaccharide-binding (OB-fold) domain) followed by a variable region with no predicted structure and a conserved C-terminal tail.

**Figure 2** shows hSSB1 affinity for various oligonucleotide molecules as determined by gel shift assay. hSSB1 protein concentration is represented on the X axis of the binding curve and percentage DNA bound is represented on the Y axis. The Kds (µM) for each oligonucleotide molecule is also represented. Assays were performed in triplicate.

**Figure 3** shows an hSSB1 immunoblot assay of cell extracts from neonatal foreskin fibroblasts cells (NFFs) following exposure to 6 gray (Gy) ionising radiation (IR) and/or exposure to the proteosome inhibitor MG132 for 2 hours.

**Figure 4** shows hSSB1 expression in IR-treated (6 Gy) extracts of NFF cells treated with wortmannin (20µM). hSSB1 expression was determined by immunoblot analysis.

**Figure 5** shows hSSB1 expression in IR-treated (6 Gy) extracts of NFF cells transfected with ATM siRNA (top panel), and ATM deficient cell lines transfected
with either vector alone or ATM cDNA (bottom panel). hSSB1 expression was determined by immunoblot analysis.

**Figure 6** shows the ATM-dependent stabilisation and phosphorylation of hSSB1 following IR (6 Gy). Lysates were immunoprecipitated using anti-hSSB1 or anti-ATM antibodies or pre-immune serum (NS), and detected by immunoblotting with either anti-ATM or anti-hSSB1 antibodies.

**Figure 7** shows the phosphorylation of hSSB1 by ATM (middle panel), ATM concentration (top panel) and hSSB1 concentration (bottom panel) in a normal cell line (C3ABR) and in L3 cells (AT cell line) with or without prior treatment of cells with 6 Gy IR.

**Figure 8** shows the phosphorylation of wild-type and mutant hSSB1-T117A by ATM in C3ABR cells and L3 cells (AT cell line) with or without prior treatment of cells with 6 Gy IR.

**Figure 9** shows IR-induced stabilisation of wild-type (GFP-hSSB1) and phospho-mutant (GFP-hSSB1-T117A) protein in host cells following irradiation (6 Gy). The cells were analysed at various time points by Western blotting with anti-GFP antibody.

**Figure 10** (A) shows the structural analysis of wild-type hSSB1 and the mutant forms hSSB1-T117A and hSSB1-T117E using circular dichroism (CD) spectroscopy in the far ultraviolet (UV) range (184-260 nm) in 10mM Phosphate pH 7.6 and 150 mM NaF at 20°C, and (B) shows electrophoretic mobility shift assay (EMSA) binding of wild-type hSSB1 and phospho-mutant hSSB1-T117A to an ssDNA substrate (i.e. d30T).

**Figure 11** shows the results of pulldown assays of GST-ATM fragments representing the full length of ATM. Bound proteins were analysed by immunoblotting with anti-hSSB1 antibody (bottom panel) and the levels of GST-ATM fragments were detected by Coomassie staining (top panel).

**Figure 12** shows, by immunoblot analysis, the IR-induced phosphorylation of ATM and its downstream targets in the absence of hSSB1.
**Figure 13** shows IR-induced phosphorylation and foci-formation of γH2AX.

**Figure 14** shows the phosphorylation of p53 and γH2AX using siRNA oligonucleotide molecules targeted to different regions of hSSB1. NFF cells were irradiated (6 Gy) and analysed by Western blot.

**Figure 15** shows the phosphorylation of wild-type hSSB1 and mutant hSSB1T117E by ATM assay.

**Figure 16** shows hSSB1-stimulated Rad51-dependent strand exchange indicated by strand invasion recombination reactions between $^{32}$P-end-labelled linear single stranded DNA (ssDNA) and homologous supercoiled plasmid DNA using Rad51, hSSB1 and RPA as indicated.

**Figure 17.** ATM-dependent stabilization and phosphorylation of hSSB1 after ionizing radiation, a, Electrophoretic mobility shift analysis showing binding of recombinant hSSB1 to ssDNA substrates, d30T (top), a synthetic replication fork (middle) and dsDNA (bottom). The radiolabel is marked with a black circle, b, Immunoblots of hSSB1 using cell extracts from neonatal foreskin fibroblasts (NFFs) exposed to ionizing (6 Gy) or ultraviolet (20 Jm$^{-2}$) radiation. Cells were harvested at the indicated time points and immunoblotted for hSSB1. c, Western blots of hSSB1 using ionizing radiation-treated (6 Gy) extracts from NFFs transfected with ATM siRNA. d, ATM was immunoprecipitated from mock or ionizing radiation (6 Gy)-treated normal (C3ABR) and A-T (L3) cell lines. In vitro kinase assays were performed using recombinant hSSB1 as a substrate, e, Phosphorylation of hSSB1 (number denotes the position of threonine residue substituted with alanine) by immunoprecipitated ATM kinase.

**Figure 18.** hSSB1 localizes to DNA repair foci after ionizing radiation. a, Irradiated (6 Gy) NFFs were extracted with detergent before fixation and stained with anti-hSSB1 and anti-chH2AX antibodies (top panel; Leica TCS BI-15 microscope). hSSB1 and C-H2AX co-localize at a single DSB induced by the I-Scel restriction enzyme in MCF7 DRGFP cells7 (bottom panel; Olympus BX61 microscope), b, ChIP analysis of hSSB1 on a unique DSB induced by I-Scel in vivo. Real-time PCR on ChIP samples used primers directed at 94-378 nucleotides from...
the DSB8. The enrichment of hSSB1 after induction of the DSB was compared with
that of an IgG control (+/-s.d., n=3). c, hSSB1 and RPA34 foci do not co-localize.
NFFs were irradiated and 2 h later fixed and stained with anti-hSSB1 and anti-RPA34 antibodies. Images were acquired using Deltavision Personal DV. An enlargement of the merged image is displayed, demonstrating proximity of hSSB1 and RPA foci.

Figure 19. hSSB1 inhibition results in defective ionizing-radiation-induced checkpoint activation and defective ATM signalling, a, hSSB1 inhibition results in defective ionizing-radiation-induced G1/S checkpoint. NFFs were mock-treated or irradiated (6 Gy) and labelled with BrdU, stained with anti-BrdU-Alexa488 antibodies and propidium iodide. The percentage of BrdU positive cells (boxed area) was determined by fluorescence-activated cellsorting. b, hSSB1 is required for ionizing-radiation-induced ATM activation and activity. SiRNA-transfected NFFs were treated with ionizing radiation and subjected to immunoblotting with the antibodies indicated. c, hSSB1-T1 17E affects MRN-dependent ATM activity. Purified ATM was incubated with DNA and MRN in the presence of wild-type hSSB1 orT1 17E mutant; phosphorylation of GST-p53 was detected using anti-Ser15 p53 antibody. d, hSSB1-T1 17E induces hyperactivation of ATM. HeLa cells transiently transfected with the indicated constructs were treated with ionizing radiation and immunoblotted with the antibodies indicated.

Figure 20. hSSB1 is required for efficient DNA repair. a, hSSB1-depleted cells are hypersensitive to ionizing radiation. NFFs were treated with siRNAs before treatment with the indicated ionizing radiation doses. Cells were allowed to grow for 36 h before metabolism was measured by the MTT assay (+/- s.e.m., n=3). b, Representative metaphase spreads of irradiated control and hSSB1-deficient cells. Telomeres (red) and centromeres (green) are labelled; chromosome breaks are indicated by arrowheads. Frequencies of spontaneous and ionizing-radiation-induced (2 Gy) chromosome, as well as chromatid, breaks and fragments in siRNA-transfected and AT cells, used as positive control, are indicated (6s.d., n53). Fifty metaphases for each sample were analysed, c, hSSB1 stimulates Rad51-dependent strand exchange. Strand-invasion recombination reactions were performed between 32P-end-labelled linear ssDNA and homologous supercoiled plasmid DNA using Rad51, hSSB1 and RPA as indicated.
Figure 21. hSSB1 specifically interacts with ssDNA. The upper panel shows an
electrophoretic mobility shift experiment with increasing concentrations of hSSB1 (0,
2, 3, 6, 10 μM) incubated with d30T DNA (70 fmol) in gel shift buffer (10 mM Tris pH
7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol and 1
µg/mL BSA) for 30 min at 37 °C. Samples were electrophoresed through a 10%
native polyacrylamide gel and visualised by phosphorimaging. The lower panel
shows the same gel, immunoblotted with a hSSB1 antibody. hSSB1 is present in the
bandshifted protein:DNA complex.

Figure 22. Binding affinity of hSSB1 with longer oligonucleotides, a, Isothermal
Titration Calorimetry (ITC) study of the binding of hSSB1 to poly-dT DNA of varying
lengths in 20 mM HEPES pH 7.3, 100 mM KCl, 1mM MgCl₂. Oligonucleotide (40 μM)
was titrated into a solution of hSSB1 (4 μM). Raw and integrated binding isotherms
are shown in the top panel (inset shows blank titration of buffer into protein).
Titrations are in triplicate ± standard deviation. Binding is exothermic, as seen for
other OB-fold containing SSB proteins. The hSSB1 binding affinity increases with
increasing DNA length. The Kd is indicated at the bottom each panel, b, These data
can also be reproduced in an EMSA with the indicated DNA oligonucleotides. The
trend is the same although binding is weaker due to the nature of the gel shift
experiment and the likely fast on/off rates of the hSSB1:DNA interaction.

Figure 23. Purification, siRNA mediated reduction and antibody characterisation of
hSSB1 a, Purification of recombinant hSSB1. Recombinant His-tagged hSSB1 was
expressed in E.coli and purified initially by nickle-affinity chromatography followed by
heparin column and size exclusion chromatography. b, siRNA mediated reduction of
hSSB1 protein expression. Cells were transfected twice at 24h intervals with control
or hSSB1 siRNA and harvested 48h later. Cell extracts were immunoblotted with
anti-hSSB1 or anti-actin antibodies as a control for equal protein loading. The
hSSB1 antibody specifically recognises a band of 35kDa in cells treated with control
siRNA and this band is significantly diminished in cells transfected with hSSB1
siRNA. c, Anti-hSSB1 antibodies do not recognise hSSB2. Immunoblot of purified
recombinant hSSB1 (lane 1) and hSSB2 (lane 2). d, Coomassie-stained protein gel
showing levels and migration of purified hSSB1 and hSSB2.

Figure 24. IR-induced stabilisation of wild type (GFP-hSSB1) and phospho-mutant
(GFP-hSSB1T1 17A). a, Cells were transfected with the indicated constructs and
mock treated or irradiated (6Gy). Cell extracts were taken and immunoblotted with anti-GFP antibodies, b, MG132-induced stabilisation of wild-type (GFP-hSSB1), T117A and T117E mutants. Cells were treated with MG132 for 2h and processed for western blotting with anti-GFP antibodies.

**Figure 25.** Normal S-phase progression in hSSB1-deficient cells. Cells were transfected with control or hSSB1 siRNA, pulse-labeled with BrdU and harvested at the indicated time-intervals. The cells were stained with anti-BrdU antibodies and PI and analysed by FACS to measure DNA synthesis (BrdU-Alexa 488) and DNA content (PI). Like control siRNA transfected cells, virtually all hSSB1 deficient S-phase cells move to G2 (acquire 4N DNA content) by 8h after labelling with BrdU. The boxes indicate the S-phase population.

**Figure 26.** Defective G2/M checkpoint in hSSB1-deficient cells. NFF cells were treated as indicated, harvested 1 h later and stained with phospho-histone H3 antibodies. The percentage of mitotic cells (boxed area) were determined via FACS.

**Figure 27.** Lack of Cdc25A degradation in hSSB1 siRNA transfected cells. NFF cells were transfected with the indicated siRNA, irradiated (6Gy) and protein was extracted 1h later. Cell lysates were immunoblotted with the indicated antibodies.

**Figure 28.** Clonogenic survival of cells with -50% reduction in expression of hSSB1. Cells were transfected once with the relevant siRNA and irradiated with indicated dose of radiation. Clonogenic survival was determined 13 days following irradiation. Values shown represent the mean results of 3 independent experiments. The A-T cells used are hTERT-immortalised GM5823 fibroblasts.

**Figure 29.** HR repair is reduced in cells transfected with hSSB1 siRNA. 24 h after siRNA transfection MCF7-DRGFP cells were transfected with I-Sce1 plasmid CpCBSCE). Forty-eight hours after pCBSCE transfection FACS analysis was carried out to detect GFP positive cells.

**Figure 30.** hSSB1 regulates Rad51 foci formation, a, SiRNA transfected NFF cells were treated with 6Gy IR and cell extracts immunoblotted with the indicated antibodies, b, Rad51 foci formation after IR exposure. NFF cells were transfected
with control and hSSB1 siRNA and irradiated with 6 Gy IR. Cells were pre-extracted with triton-X-100 before fixation and stained with Rad51 antibodies (Merck). Images were acquired on an Olympus BX61 microscope. The percentage of control and hSSB1-deficient cells showing Rad51 foci after IR. The histogram bars correspond to the average percentage of Rad51 foci positive cells from three independent experiments.

Figure 31. In vivo association of hSSB1 and Rad51. Cells were irradiated with 6 Gy, incubated for 2.5 h and lysates were immunoprecipitated using anti-hSSB1 antibodies or pre-immune serum (NS). Associations were detected via immunoblotting with either anti-Rad51 or anti-hSSB1 antibodies.

Figure 32. (A) Amino acid sequence of ATM kinase fragment 4 (SEQ ID NO: 26); (B) Amino acid sequence of ATM Kinase from Genbank accession no: NM_000051 Gl:71902539 (SEQ ID NO: 27).

Examples

The present invention is hereinafter further described by way of the following, non-limiting examples and accompanying figures.

Example 1

Methods and Materials

Plasmids, recombinant protein purification, cell lines and siRNA

Green fluorescent protein (GFP)-hSSBI fusion protein was expressed from pEGFPd (Clontec). Recombinant His-tagged hSSB1 and hSSB2 was expressed from pET28c and pDEST17 respectively, in BL21 cells (Stratagene, La Jolla, CA, United States of America).

For purification of recombinant protein, BL21 cells were lysed in Ni A buffer (50 mM KCl, 50 mM KH2PO4, 10 mM imidazole, 20 mM β-mercaptoethanol, 10% w/v glycerol, 1 mg/ml lysozyme, 5 mM EDTA, and Complete Mini EDTA-free Protease inhibitor cocktail tablets). The resulting extract was diluted to 1mM EDTA and passed over Qiagen Ni-NTA Superflow resin. The resin was washed with Ni A buffer and bound protein eluted in Ni B buffer (50 mM KCl, 50 mM KH2PO4, 100 mM imidazole, 20 mM
β-mercaptoethanol, 10% w/v glycerol). The eluate was then passed over a HiTrap Heparin HP (GE Healthcare, Waukesha, WI, United States of America) and washed with Buffer A (25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, and 10% w/v glycerol). Protein was then eluted in Buffer A containing 1 M NaCl. 1 ml of the most concentrated fraction was passed over a Superdex 200 column and fractions containing the protein aliquoted and stored at -80 degrees.

Small interfering RNAs (siRNA) were synthesised by Invitrogen (Invitrogen Corporation, Carlsbad, CA, United States of America). The target sequences were:

- **hSSB1** GACAAAGGACGGGCATGAG (SEQ ID NO: 8),
- **ATM** GCGCCTGATTGAGATCCU (SEQ ID NO: 9), and
- **control** UUCUCCGAACGUGUCACGU (SEQ ID NO: 10).

**Antibodies and immunofluorescence**

Antibodies were supplied by Calbiochem (Rad50, Mre11, Rad51), Upstate (γH2AX), Roche (BRDU), Cell Signalling Technologies (pT68-11 Chk2, pS317-Chk1, pS15-p53) and Invitrogen (Alexa secondary antibodies). Sheep antiserum to hSSB1 was raised against full-length recombinant His-tagged hSSB1 using standard methods. Rabbit antiserum was raised against a phosphorylated peptide representing the T117 hSSB1 phosphorylation site (i.e. NPEYSpTQQAPN; SEQ ID NO: 11). This antibody was used to detect hSSB1 by Western blotting and immunofluorescence.

For immunofluorescent staining, cells were pre-permeabilised with 20 mM HEPES, 120 mM KCl, 0.5% NP40 (w/v) for 15 min on ice prior to fixation in 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS) for 10 minutes.

**Assays**

MTT assays were performed 48 hours following ionising radiation (IR) according to methods described by Slavotinek et al. (1994). G1/S checkpoint was measured using the BrdUrd incorporation assay as described by Fabbro, 2004 and G2/M checkpoint measured by labelling cells with anti-phospho-histone H3 antibody (Xu et al., 2001). For analysis of chromosomal aberrations at metaphase, exponentially growing cells were treated with 2 Gy of IR. Colcemid was added at various time points. Cells at metaphase were collected and chromosomal aberrations were scored as described previously (Pandita et al., 2006).
For MRN binding assays, protein complexes containing 50 ng of biotinylated NBS1 were incubated with Promega Streptavidin MagneSphere Paramagnetic Particles in buffer A (25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, 0.1% CHAPS, and 10% w/v glycerol) for 1 hour at room temperature. Beads were then isolated and placed in a fresh 1.5 ml microcentrifuge tube. 130 ng of hSSB1, hSSB1-T117E, or hSSB1-T117A in buffer A was incubated with the MRN bound beads for 30 minutes. The beads were washed three times with buffer A. Bound proteins were eluted with SDS loading buffer and immunoblotted with anti-hSSB1 antibodies. The appearance of ssDNA was detected using a BrdUrd incorporation assay by incubating cells with BrdUrd (10ug/ml) for 30 hours as per Raderschall et al. (1999).

HR was measured by determining the frequency of reconstitution of a green fluorescent protein reporter gene (pDR-GFP) within a chromosomally integrated plasmid substrate in cells, as described previously (Pierce et al., 1999) (Zhang et al., 2005).

For hSSB1 silencing assays, cells were transfected twice with siRNA at a 24 hours interval and processed 48 hours later.

EMSA assays were conducted as previously described (Wadsworth et al., 2000).

Circular Dichroism (CD) spectroscopy
The CD spectra of the purified recombinant proteins were measured in the far UV range (184-260 nm) in 10 mM phosphate pH 7.6 and 150mM NaF at 20°C. A negative mean residue ellipticity (approximately -3 m degree.cm².dmol⁻¹) was observed at 202 nm. The secondary structure of wild-type and mutant hSSB1 was assessed using the CDPro software package, which includes three analysis programs (i.e. CONTIN, CDSSTR, and SELCON3).

Results and Discussion
Structural analysis of hSSB1 and hSSB2
Previous studies revealed the presence of two highly conserved sequence homologues of S. solfataricus SSB (Figure 1), present on chromosomes 2q13.3 and 2q32.3 respectively, which have been designated hSSB1 (i.e. human ssDNA binding protein 1) and hSSB2 (i.e. human ssDNA binding protein 2)(Australian Provisional
Application No 2007901166). On further analysis, both proteins were found to contain a highly conserved N-terminal OB-fold domain, indicating a role in nucleic acid recognition. This domain is followed by a variable region with no predicted structure and a conserved C-terminal tail. Gel filtration data indicated that hSSB1 exists in a dimeric form in solution (data not shown).

Database mining also revealed that homologues for both the hSSB1 and hSSB2 genes exist in other mammals, and single homologues were located in other divergent eukaryotic species (i.e. X. laevis, D. rerio and D. melanogaster) (Figure 1).

hSSB1 binding of ssDNA
Previous studies demonstrated the capacity for hSSB1 to bind ssDNA at the site of enzyme induced double stranded breaks (DSBs), which confirms that hSSB1 functions as a DNA binding protein (Australian Provisional Application No 2007901166). Gel shift experiments (performed in triplicate) show binding curves of hSSB1 using different oligonucleotide molecules. Figure 2 shows that hSSB1 preferentially binds pyrimidines, no binding to poly-A sequences and lower binding with alteration of pyrimidines with dAC. Preferential binding of pyrimidines has also been similarly observed in other binding proteins such as RPA (Kim et al., 1992) further confirming a role for hSSB1 in DNA replication and repair.

Role of hSSB1 in DNA repair
Previous studies investigated the involvement of hSSB1 in cellular responses to DNA damage by monitoring hSSB1 expression following treatment with ionising radiation (IR) or ultraviolet (UV) radiation. A dose dependent expression of hSSB1 was observed with increasing DNA damaging agents until the DNA damage-induced impairment of cell function or cell death. The specific involvement of hSSB1 was also investigated in hSSB1 deficient cells, wherein rapid accumulation of chromosomal aberrations was observed thereby confirming a role of hSSB1 in DNA repair (Australian Provisional Application No. 2007901166).

Cellular clearance of hSSB1
To determine whether clearance mechanisms are involved in regulating cellular hSSB1, cell extracts from neonatal foreskin fibroblasts (NFF) cells were exposed to IR (6 Gy) in the presence or absence of proteosome inhibitor MG132 for 2 hours. Cells were harvested and immunoblotted for hSSB1. The treatment of cells with the
proteosome inhibitor, MG132, led to a similar increase in hSSB1 levels which was not further enhanced by IR treatment (Figure 3). This suggests that hSSB1 levels are controlled, in part, by proteosome-mediated degradation and that, following DNA damage, this process may be disrupted, leading to hSSB1 induction.

Interaction between hSSB1 and ATM
ATM activity is essential for cellular signalling in response to DNA breaks via the phosphorylation of several proteins involved in DNA damage response pathways (Khanna et al., 2001, Gatei, M., 2000). An interaction between ATM activity and hSSB1 expression was determined in cell assays utilising the ATM inhibitor wortmannin (Figure 4). Immunoblot analysis of hSSB1 in cell extracts from NFF cells exposed to IR (6 Gy) following prior treatment of with wortmannin (20 µM) showed reduced and delayed hSSB1 expression in response to IR-induced DNA damage, indicating that stabilisation of hSSB1 was reduced and delayed following DNA damage.

Further, immunoblot analysis of NFF cell extracts transfected with ATM siRNA showed that cells depleted of ATM are also impaired in their ability to stabilise hSSB1 (Figure 5). This was confirmed in studies utilising lymphoblastoid cells (L3) encoding a mutant ATM protein kinase from human genetic disorder ataxiatelangiectasia (AT).

To determine whether hSSB1 interacts with ATM, lysates prepared from control (C3ABR, ATM-positive) and AT lymphoblastoid (L3, ATM deficient) cells, with or without prior exposure to 6 Gy of IR, were subjected to immunoprecipitation with anti-hSSB1 antibody. Immunoblot analysis of the anti-hSSB1 immunoprecipitate with an anti-ATM antibody demonstrated an interaction between these two proteins in the control cell line (C3ABR) after exposure to IR but not in the AT cell line (L3) (Figure 6). ATM was detected in hSSB1 immunoprecipitates from irradiated control (C3ABR, ATM-positive) but not ATM-deficient L3 cells (Figure 6). In a reciprocal experiment, constitutive interaction between hSSB1 and ATM was detected which was further increased after exposure of cells to IR (Figure 6).

hSSB1 phosphorylation by ATM
Phosphorylation of hSSB1 by ATM was assessed by in vitro kinase assays on ATM immunoprecipitates from ATM positive control cells and ATM deficient L3 cells with or without prior exposure of cells to irradiation. Assays were performed using full
length recombinant hSSB1 as a substrate (Lisby et al., 2004). ATM was immunoprecipitated from normal cell line (C3ABR) and an ATM deficient cell line (L3) with or without prior treatment of cells with 6 Gy IR. ATM from C3ABR cells was found to phosphorylate hSSB, with an increase in hSSB1 phosphorylation observed following irradiation (Figure 7), whereas immunoprecipitates obtained from L3 cells showed only negligible activity against hSSB1, suggesting that hSSB1 is a substrate of ATM (Figure 7).

Published studies by Kim et al., 1999 and O'Neill et al., 2000 show the consensus target sequence for phosphorylation by ATM to be a serine (S) or threonine (T) followed by a glutamine (SQ/TQ) residue. Analysis of the hSSB1 sequence revealed three possible ATM phosphorylation sites (S134, S182 and T117). Site directed mutagenesis at each of S134, S182 and T117 was conducted to generate hSSB1-S134A, hSSB1-S182A and hSSB1-T117A phospho-mutants. Substitution of T117 with an alanine (A) prevented hSSB1 phosphorylation by ATM as determined by kinase assay (Figure 8). T117 could therefore be positively identified as the site hSSB1 phosphorylation by ATM and, further, confirms the direct interaction of hSSB1 with ATM. On the other hand, substitution at S134, S182 had a negligible effect on hSSB1 phosphorylation.

To evaluate whether this phosphorylation site plays a role in hSSB1 stabilisation following IR, the expression levels of exogenous GFP-hSSB1 wild-type and GFP-hSSB1-T117A mutant protein in HEK293T cells was compared. Cells were transfected with plasmids carrying either the wild-type or T117A mutant hSSB1 protein, and 12 hours after transfection cell extracts from either untreated or irradiated cells were immunoblotted with anti-GFP antibody. After IR exposure, stabilisation was only observed for the wild-type protein (Figure 9). The T117A mutant, which cannot be phosphorylated by ATM, showed no increase in protein levels following IR, confirming the role for ATM in IR-induced stabilisation of hSSB1.

Green fluorescent protein (GFP)-hSSB1 fusion protein was expressed from pEGFPd (Clontech).

To exclude the possibility that the stabilisation defect observed with GFP-hSSB1- T117A mutant is due to incorrect folding, a structural analysis of wild-type hSSB1 and mutants (T117A and E) using circular dichroism (CD) spectroscopy was
conducted. The structural analysis showed similar secondary structural properties for the wild-type and mutant forms of hSSB1. Approximately 30% of the secondary structure was of beta sheet configuration, which is consistent with the presence of an OB fold domain (or barrel) in the protein structure, making up approximately 90-100 of the 211 residues. Of the remaining structure approximately 10% was determined to be of alpha helix configuration, and approximately 25% as turns. These structural properties were present in both the wild-type and mutant forms of hSSBL.

Electrophoretic mobility shift assays (EMSA) of recombinant wild-type and phospho-mutant (T17A) forms of hSSB1 were conducted to confirm the expected functionality of the OB fold domain. The hSSB1 phospho-mutant showed similar binding of ssDNA substrate (d30T) as the wild-type protein. This confirms that the recombinant His-tagged phospho-mutant of hSSB1 retained correct protein structure and ssDNA binding functionality compared to wild-type hSSB1 (Figure 10).

Localisation of site of hSSB1-ATM interaction
To confirm a direct interaction and to map the regions in ATM involved in protein binding, recombinant GST-ATM fragments were utilised in pull down assays (as described in Khanna et al., 1998). Briefly, total cell extracts from unirradiated and irradiated cells were mixed with glutathione agarose beads containing GST-ATM fusion proteins. Bound proteins were analysed by immunoblotting with anti-hSSB1 antibody and levels of GST-ATM fragments were detected by Coomassie staining. These studies showed that the interaction between hSSB1 and ATM was direct and mediated by ATM fragment 4, representing residues 772-1102 (Figure 11).

**hSSB1 required for phosphorylation following DNA damage**
In response to double stranded DNA breaks, cells arrest their cell cycle before progressing into S-phase (G1/S checkpoint) and before committing to mitosis (G2/M checkpoint). These arrests are eventually released when the DNA lesions have been repaired. In previous studies (see Australian Provisional Application No. 200790166), hSSB1 deficiency had little effect on the normal progression of cells from G1 into S phase, however, on irradiation induced DNA damage these cells failed to arrest and progressed into and through S phase. Cells depleted of hSSB1 exhibited a G2/M checkpoint defect and were found to progress into mitosis after IR
treatment.

To gain insights into the mechanism by which hSSB1 mediates both G1/S and G2/M damage-activated cell cycle checkpoints, hSSB1-depleted normal fibroblasts were assessed for their ability to phosphorylate proteins critical for efficient checkpoint activation following irradiation. SiRNA transfected NFF cells were irradiated and left to recover for 30 minutes. Cell lysates were immunoblotted with ATM (GenTex, Inc., San Antonio, TX, United States of America), NBS S343 (Queensland Institute of Medical Research, Herston, QLD, Australia), p53 Ser 5, Chk1 Ser317, Chk2 Thr68 (Cell Signalling Technologies), γH2AX antibodies (Upstate Biotechnology) and control antibodies for actin (Sigma) and hSSB1. As expected, irradiation of cells expressing the control siRNA led to the autophosphorylation of ATM, phosphorylation of p53, Chk1, Chk2 and NBS1 (Figure 12). Notably, exposure of parallel cultures of fibroblasts treated with SSB1-specific siRNA failed to initiate a similar degree of IR induced ATM autophosphorylation, impairing the subsequent phosphorylation of ATM targets (Figure 12). This confirms the dependence of ATM function and the function of its downstream targets on hSSB1 activity.

As hSSB1 controls the phosphorylation of several checkpoint proteins, an examination was conducted to determine whether hSSB1 might have a role in H2AX phosphorylation, γH2AX, one of the first proteins phosphorylated by ATM after DSBs (Burma et al., 2001). siRNA-treated cells were irradiated and γH2AX antibodies were used for immunoblotting and immunofluorescence as described above. Depleting cells of hSSB1 protein significantly affected the rapid IR-induced phosphorylation and foci-formation of H2AX (Figure 13). Similar results were observed with siRNA targeted to a different region of hSSB1. NFF cells were also irradiated (6 Gy) and harvested after 30 minutes for Western blot analysis with indicated antibodies (Figure 14). The significant reduction of H2AX phosphorylation can not be explained solely by the effect on ATM activity since both ATR and DNA-PK can also phosphorylate H2AX, however, H2AX phosphorylation is MDC1 dependent and H2AX foci fail to form in MDC1-deficient cells (Lou et al., 2006).

hSSB1 in nuclear foci formation

The MRN complex localises to nuclear foci upon DSB induction to mediate DSB repair. A short peptide motif in the C-terminus of Nijmegen Breakage protein
(Nbs1) has been shown to mediate recruitment of ATM to sites of DSBs, leading to the activation of ATM (Falck et al., 2005). The phosphorylation of histone H2AX (and γH2AX) is essential for the efficient recognition and repair of double strand breaks (DSBs) (i.e. H2AX becomes rapidly phosphorylated at the site of each nascent DSB; Burma et al., 2001). Previous studies revealed that hSSB1 is rapidly recruited to DNA repair foci following DNA damage by irradiation, indicating a role for the protein in DNA damage response. Thus, hSSB1 was found to co-localise with γH2AX and to be involved in recruiting the MRN complex to foci and for resection of DSBs and HR repair (Australian Provisional Application No 2007901166).

Given the critical role of the MRN complex in the resection of DSBs and HR repair and its regulation of ATM activation and activity (Lee et al., 2005), an investigation was conducted to determine whether hSSB1 affects MRN-dependent regulation of ATM activity. That is, purified ATM was incubated with DNA and MRN in the presence of wild-type recombinant hSSB1 and recombinant T117E mutant and phosphorylation of GST-p53 detected using anti-Ser15 p53 antibody. The wild-type hSSB1 had no effect on ATM activity whereas the phospho-mutant consistently increased activity by 2+ fold (Figure 15), indicating that once ATM is initially activated, a positive feedback loop is initiated through phosphorylated hSSB1, which in turn reinforces ATM activation.

To determine whether phosphorylated hSSB1 exists as a part of complex with MRN, cells were irradiated with 6 Gy of irradiation and incubated for 1 hour, the lysate was immunoprecipitated using anti-hSSB1 antibody and preimmune serum. NS or anti-Mre1 1 antibody and NS and bound protein were analysed by immunoblotting with either anti-Mre1 1, anti-Rad50 or anti-hSSB1 (Figure 16). hSSB1 was found to associate constitutively with the components of the MRN complex as determined by co-immunoprecipitation. The interaction was not stimulated following further exposure of cells to IR (Figure 16).

To further confirm the direct interaction, purified MRN complex containing a biotin-tag on the NBS1 component was bound to streptavidin-coated magnetic beads and incubated with wild-type, T117E, or T117A hSSB1 protein as indicated. Protein bound to the beads was separated by SDS-PAGE, Western blotted, and probed with antibody directed against hSSB1. Pull-down assays of purified hSSB1
incubated with purified human MRN complex revealed a direct interaction that was not altered by the T117 phospho-mutant of hSSB1 (Figure 16).

hSSB1 in homologous recombination repair

To further elucidate the role of hSSB1 in HR repair, the effect of hSSB1 on Rad51-mediated strand exchange was investigated. It is known that Rad51 binds single stranded DNA at resected DSBs to form nucleoprotein filaments that mediate the invasion of ssDNA into homologous duplex DNA (Schlegel et al., 2006). These reactions are stimulated by the presence of the single stranded DNA binding protein RPA (McIlwraith et al., 2000 and Song and Sung, 2000). A comparison was therefore made of the ability of RPA and hSSB1 to stimulate Rad51-mediated strand invasion. It was found that hSSB1 stimulated Rad51-mediated D-loop formation when added after Rad51 (Figure 16 lanes b-e), but not before Rad51 addition (lanes l-o). These results indicate that hSSB1 binds ssDNA and that dissociation by Rad51 may require additional factors such as Rad52 for maximal stimulatory effect. Similar observations have been made with Rad51 and RPA (New and Kowalczykowski, 2002 and Sugiyama and Kowalczykowski, 2002). Combinations of hSSB1 and RPA did not lead to enhancement of stimulation that was seen with either protein alone (lanes q-y). The stimulation of Rad51-mediated strand invasion by hSSB1 is likely to occur by a mechanism similar to that described previously with RPA. Taken together, the data show that hSSB1 performs a dual role in HR. It is required for the aggregation of repair proteins for foci formation, and also in binding displaced ssDNA to promote Rad51-mediated strand invasion.

These studies demonstrate the critical role of hSSB1 in the signal transduction mechanisms involved in initiating normal cellular responses to DNA damage and, more particularly, in DNA repair via its activity as an ssDNA binding protein. hSSB1 has been shown to have a role early in cellular responses to DNA damage, directly amplifying ATM activation and the aggregation of MRN for nuclear foci formation. As a consequence, hSSB1 controls the activation of subsequent repair proteins. Further to this, cellular concentrations of hSSB1 were found to be significantly affected by proteosome clearance.

The direct sites of hSSB1-ATM interaction have been localised to hSSB1 residue 117 and ATM residues 772 to 1102. As the direct interaction of hSSB1 with MRN and proteosomes has also been identified, it is envisaged that the methods described
herein could be adapted to the identification of the sites involved in the interaction of hSSB1 and hSSB2 with these complexes. hSSB1 has also been further characterised structurally, including the localisation of a ssDNA binding region. These sites provide a target(s) to enable the screening of agents that act to modulate hSSB1. As a role for hSSB1 in tumorigenesis has previously been shown (Australian Provisional Patent Application No 2007901166), such agents potentially have a therapeutic and/or prophylactic application in the treatment of cancer and tumours.

Example 2

Single-strand DNA (ssDNA)-binding proteins (SSBs) are ubiquitous and essential for a wide variety of DNA metabolic processes, including DNA replication, recombination, DNA damage detection and repair. SSBs have multiple roles in binding and sequestering ssDNA, detecting DNA damage, stimulating nucleases, helicases and strand-exchange proteins, activating transcription and mediating protein-protein interactions. In eukaryotes, the major SSB, replication protein A (RPA), is a heterotrimer. Here we describe a second human SSB (hSSB1), with a domain organization closer to the archaeal SSB than to RPA. Ataxia telangiectasia mutated (ATM) kinase phosphorylates hSSB1 in response to DNA double-strand breaks (DSBs). This phosphorylation event is required for DNA damage-induced stabilization of hSSB1. Upon induction of DNA damage, hSSB1 accumulates in the nucleus and forms distinct foci independent of cell-cycle phase. These foci colocalize with other known repair proteins. In contrast to RPA, hSSB1 does not localize to replication foci in S-phase cells and hSSB1 deficiency does not influence S-phase progression. Depletion of hSSB1 abrogates the cellular response to DSBs, including activation of ATM and phosphorylation of ATM targets after ionizing radiation. Cells deficient in hSSB1 exhibit increased radiosensitivity, defective checkpoint activation and enhanced genomic instability coupled with a diminished capacity for DNA repair. These findings establish that hSSB1 influences diverse endpoints in the cellular DNA damage response.

Ionizing radiation and anti-cancer drugs can induce DNA DSBs, which are highly cytotoxic lesions. In the S and G2 phases of the cell cycle, homologous recombination can be used to repair DSBs. To initiate homologous recombination, DNA is resected and then bound by RPA, a eukaryotic SSB, to facilitate Rad51 nucleofilament formation and strand invasion. Here we show that, in addition to RPA,
the human genome encodes two further conserved SSB homologues, present on chromosomes 12q13.3 and 2q32.3, which we have named hSSB1 and hSSB2, respectively. The main focus of this study, hSSB1, is highly represented in EST libraries from a variety of tissues. It is conserved in metazoa, comprising an amino-terminal oligonucleotide/oligosaccharide-binding-fold domain, followed by a more divergent carboxy-terminal domain (Fig. 1C). Like RPA, recombinant hSSB1 binds specifically to ssDNA substrates (Fig. 17a and Fig. 21), in particular to polypyrimidines (Fig. 2). The binding affinity increases significantly with the length of the DNA substrate (Fig. 22).

RPA has several functions in the cell, including roles in DNA replication, recombination and repair. To investigate if hSSB1 functions in similar pathways, we analysed the response of hSSB1 to DNA damage. Cells were treated with ionizing or ultraviolet radiation, and hSSB1 was detected by western blotting (see Fig. 23 for protein purification, short interfering RNA (siRNA)-mediated knockdown and antibody characterization). We found that hSSB1 accumulated in the cell in response to DNA damage (Fig. 17b), and that this was due to protein stabilization as treatment with the proteosome inhibitor MG132 led to a similar stabilization (Fig. 3).

ATM kinase activity is essential for cellular signalling in response to DNA breaks. siRNA-mediated depletion of ATM, inhibition of ATM activity (wortmannin treatment) or deficiency of ATM in ataxia telangiectasia (A-T) cells resulted in an inability to stabilize hSSB1 after ionizing radiation (Fig. 17c, Figs 4 and 5). Co-immunoprecipitation and glutathione S-transferase (GST)-ATM fragment pull-down assays indicated that interaction between hSSB1 and ATM was direct and mediated by ATM fragment 4 (amino acids 772-1 102) (Supplementary Figs 6 and 11).

ATM phosphorylates several proteins involved in the DNA damage-response pathway. In vitro kinase assays showed that hSSB1 was a substrate of ATM (Fig. 17d) and that the phosphorylation site was mapped to T117 because mutation of the threonine to alanine blocked phosphorylation by ATM (Fig. 17e). To determine the functional relevance of this phosphorylation site in vivo, we compared the expression levels of exogenous GFP-hSSB1 wild-type and T117A mutant in HeLa cells. After ionizing radiation treatment, stabilization was only observed for the wild-type protein (Fig. 24a), confirming the role of ATM in ionizing-radiation induced stabilization. The
stabilization defect is unlikely to be due to incorrect folding because recombinant T117A hSSB1 mutant folds correctly and shows similar ssDNA binding as wild-type hSSB1 (Fig. 10a, b). Furthermore, GFP-hSSB1 and T117Awere rapidly stabilized in the presence of MG132, whereas T117E mutant was not. This indicates that phosphorylation of hSSB1 at T117 prevents its degradation by the proteosome (Fig. 24b).

After exposure to ionizing radiation, many repair proteins, including C-H2AX, localize rapidly to sites of damage. Immunofluorescence showed that hSSB1 localizes to prominent nuclear foci that formed within 30 min of DNA damage and persisted up to 8 h (Fig. 18a). Foci for hSSB1 co-localized with most C-H2AX foci after ionizing radiation and at a single I-Scel-induced DSB (Fig. 18a). Unlike hSSB1, we failed to observe significant colocalization of ionizing-radiation-induced RPA and C-H2AX foci. Chromatin immunoprecipitation revealed that hSSB1 is present close (94-378 base pairs) to the I-Scel-induced DSB, suggesting that it might have a direct role in DSB repair (Fig. 18b).

The observation that hSSB1, like RPA, is recruited to sites of DSBs raises the issue of how these proteins are coordinated. RPA forms foci at sites of DNA replication in unperturbed S phase and after DNA damage both in S- and G2-phase cells. We examined whether hSSB1 exhibited similar foci formation kinetics and cell-cycle dependence. Before exposure to ionizing radiation, 22% of cells were positive for RPA foci, increasing to about 40% 3 h after treatment. By contrast, there were very few cells (less than 5%) positive for hSSB1 foci before exposure to ionizing radiation. However, 30 min after exposure, more than 95% of cells contained hSSB1 foci. Therefore, unlike RPA, hSSB1 foci formation is not cell-cycle dependent. In cells that contained both RPA and hSSB1 foci, we observed little co-localization (less than 5%), although it is interesting to note that about 27% of RPA foci were in close proximity to hSSB1 (less than 50 nm) (Fig. 18c). This proximity may represent different subcompartments within one repair centre or a snapshot of the temporally dynamic composition of foci. The lack of direct co-localization indicates functional differences between these proteins in the repair process, although their proximity might suggest that they function at the same sites of repair.

The localization of hSSB1 to repair foci suggests it may act directly in DNA repair or in ionizing-radiation-induced signalling events. In response to DSBs, the cell cycle is
arrested, before progression into S phase (G1/S checkpoint) or commitment to mitosis (G2/M checkpoint). These arrests are eventually released when the DNA lesions have been repaired. To determine whether hSSB1 was involved in checkpoint activation, we examined the effects of hSSB1 depletion on the activation of G1/S and G2/M checkpoints after ionizing radiation. Unlike RPA, depletion of hSSB1 by siRNA (sihSSB1) did not influence the number of cells in S phase or progression through it (Fig. 25). Although control siRNA-treated cells arrest in G1 and G2 in response to ionizing radiation, hSSB1-depleted cells failed to arrest at either checkpoint (Fig. 19a and Fig. 26). Cdc25a, a marker for rapid G1 arrest, is normally degraded after ionizing radiation, but in hSSB1-deficient cells Cdc25a levels remained stable (Fig. 27). The ATM-mediated phosphorylation of many checkpoint proteins is vital for ionizing radiation-induced checkpoint activation. To determine if this cascade was functional in the absence of hSSB1, we analysed the phosphorylation status of several ATM substrates in hSSB1-deficient cells. As expected, irradiation of control cells led to the autophosphorylation of ATM, phosphorylation of p53, Chk2, Chk1 and NBS1 (Fig. 19b). By contrast, hSSB1-deficient fibroblasts failed to show a similar degree of ATM autophosphorylation and phosphorylation of ATM targets after irradiation (Fig. 19b and Fig. 14). Furthermore, defective phosphorylation of Chk1 also suggests a function of hSSB1 in ATR-dependent signalling.

Like hSSB1, the MRN complex plays a crucial role in the regulation of ATM activation and activity. We therefore determined whether hSSB1 affects MRN-dependent regulation of ATM activity. We found that wild-type hSSB1 had no effect on ATM activity, whereas the phospho-mimic mutant consistently increased activity by more than twofold, 2.3 +/- 0.71 (s.e.m., n=53) (Fig. 19c). hSSB1 itself was not phosphorylated in this assay owing to the vast molar excess of p53. These results suggest that, once ATM is activated, a positive feedback loop is initiated through phosphorylated hSSB1, which in turn reinforces ATM activation. ATM signalling was examined in cells expressing GFP-hSSB1, T117A and T117E. Intriguingly, the expression of T117A suppressed ATM activation, as measured by S1981 phosphorylation, whereas the phospho-mimic T117E enhanced phosphorylation by approximately twofold (Fig. 19d). Together, these results indicate that hSSB1 amplifies ATM-dependent signalling.
Given that ATM is crucial for cell survival after ionizing radiation, we next examined the sensitivity of hSSB1-deficient cells to ionizing radiation. Cells deficient in hSSB1 displayed hypersensitivity to ionizing radiation (Fig. 20a). We were unable to assess cell survival after longer time points because of the severe death phenotype exhibited by ionizing radiation-treated hSSB1-deficient cells. Indeed, we usually observed greater than 90% cell death within 96 h of exposure to very low doses of radiation (0.5 Gy). Furthermore, we found that even depletion of hSSB1 by 50% resulted in ionizing radiation sensitivity (Fig. 28). Cells deficient in hSSB1 also displayed higher frequencies of chromosomal aberrations (chromosome and chromatid breaks, with fragments and telomere fusions) after exposure to ionizing radiation (Fig. 20b). These findings demonstrate that hSSB1 plays a functionally important role in allowing cells to repair genotoxic damage and maintain chromosome stability.

Because SSBs are required for homologous recombination, we determined whether hSSB1 was important for homologous recombination in human cells. Depletion of hSSB1 from the MCF7 cell line, stably integrated with a GFP-based homologous recombination reporter construct resulted in an approximate fivefold reduction in homologous recombination, as measured by GFP-positive cells after I-Sce1 expression (Fig. 29). Rad51 binds ssDNA at resected DSBs to form nucleoprotein filaments that mediate the invasion of ssDNA into homologous duplex DNA. These reactions are stimulated by the presence of RPA. We therefore compared the ability of RPA and hSSB1 to stimulate Rad51-mediated strand invasion. We found that hSSB1 stimulated Rad51-mediated D-loop formation to a similar extent as RPA (Fig. 20c). Wild-type and T117E hSSB1 showed comparable stimulation in this assay, indicating that ATM-dependent hSSB1 phosphorylation does not appear to regulate strand invasion. Like RPA, hSSB1 was unable to stimulate strand invasion when added before Rad51 (data not shown). The stimulation of Rad51-mediated strand invasion by hSSB1 is likely to occur by a mechanism similar to that described previously with RPA. Consistent with these findings, Rad51 was not recruited to foci after ionizing radiation in hSSB1-deficient cells (Fig. 30). Rad51 and hSSB1 were found to co-localize in 12% of cells treated with ionizing radiation, an additional 14% of foci were proximal (less than 50 nm). An interaction between hSSB1 and Rad51 was also detected by co-immunoprecipitation (Fig. 31).

In summary, we have identified a novel protein, hSSB1, that plays a key role in DNA damage response. Before this study, RPA was thought to be the sole functional
homologue of the SSBs in the nucleus of mammalian cells. However, the discovery of a second SSB (hSSB1) in metazoa indicates that higher eukaryotes have preserved this family of proteins in a simpler molecular configuration more closely related to crenarchaeal SSB. Our studies provide new insights into mechanisms of DNA-damage signal transduction and reveal that hSSB1 influences diverse endpoints in the cellular DNA damage response, including cell-cycle checkpoint activation, recombinational repair and maintenance of genomic stability. Our data demonstrate that hSSB1 associates with DNA lesions, and enables the efficient activation of ATM and consequent phosphorylation of downstream proteins. It also promotes Rad51-mediated strand exchange and may thus contribute directly to homologous recombination repair. Finally, as an early participant in the damage response pathway, hSSB1 may also be involved in preventing tumorigenesis and may affect the response of patients and tumours towards radiotherapy and DNA-damaging chemotherapies.

METHODS
Summary
Chromatin immunoprecipitation (ChIP) assays were performed as described previously (Rodrique, A. et al. Interplay between human DNA repair proteins at a unique double-strand break in vivo. EMBO J. 25, 222-231 (2006); Aparicio, O. et al. in Current Protocols in Molecular Biology (eds Ausubel, F. A. et al.) 21.3.1-21.3.1 7 (John Wiley, New York, 2005)). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed essentially as described by Slavotinek et al. with the exception that assays were performed 36 h after ionizing radiation treatment. G1/S checkpoint was measured using bromodeoxy uridine (BrdU) incorporation assay, and G2/M checkpoint by labelling cells with antiphospho-histone H3 antibody. To analyse chromosome aberrations at metaphase, exponentially growing cells were treated with 2 Gy of ionizing radiation, colcemid added at different time points, metaphase cells collected and chromosome aberrations scored as described. The effect of hSSB1 on MRN-dependent activation of ATM was determined as described previously. Homologous recombination was measured by determining the frequency of reconstitution of a green fluorescent protein reporter gene (pDRGFP) within a chromosomally integrated plasmid substrate in cells, as described previously.

Plasmids and siRNA
Full-length hSSB1 was amplified by PCR from HeLa-cell complementary DNA and cloned in the BamHI and Sail sites of bacterial expression His-tag vector pET28c; hSSB2 was cloned into bacterial expression His-tag vector pET19b. GFP-hSSB1 fusion protein was cloned into the HindIII and KpnI sites of pEGFP-C1, and threonine 117 was mutated to alanine using QuickChange site-directed mutagenesis. siRNAs were chemically synthesized (Invitrogen) with a two-nucleotide deoxythymidine overhang at the 39-end. Individual siRNA sequences were as follows: hSSB1 (sense) 5'-GACAAAGGACGGGCAUGAGdTdT (SEQ ID NO: 21), (antisense) 5'-CUCAUGGCCCGUCCUUCGUdTdT (SEQ ID NO: 22), ATM (sense) 5'-GCGCCUGAUUCCAGAUCUdTdT (SEQ ID NO: 23), (antisense) 5'-AGGAUCUGAAUCAGGC GCdTdT (SEQ ID NO: 24). Cells were transfected twice with siRNA at a 24 h interval and processed 48 h later.

The 100-base oligonucleotide used in the D-loop assay was: 5'-GGGCGAATTGGCCGCGACGTGCTGCTCTTGAAGCTCGAGGACATTCCGTAC CCCGGGTTGCAGAAATCGATAAGGTACGTCTCCATTTAAGDGACAAG-3' (SEQ ID NO: 25). The oligo was 5',32P-end-labelled using polynucleotide kinase (New England Biolabs) and [γ-32P]ATP.

**Purification of recombinant** protein.

BL21 cells expressing His-tagged hSSB1 were lysed in buffer A (20mM Tris pH 8.0, 50mM NaCl and 30mM imidazole) with the addition of protease inhibitor cocktail (Complete Mini EDTA-free Protease, Roche) and 0.1% Triton X-100. Clarified soluble E. coli cell lysate pretreated with DNase (30 min, 20 °C) was applied to a Hi-Trap metal chelating column (GE Healthcare) loaded with nickel in buffer A and eluted using a gradient of 0-100% 300mM imidazole. hSSB1 protein isolated by nickel chromatography was diluted 3x in buffer A with 1mM DTT and no NaCl, and loaded onto a heparin column (HiTrap, GE Healthcare). Protein was eluted using a gradient of 0-100% 1M NaCl. hSSB1 was concentrated and re-applied to the gel filtration column (Superdex 200) in buffer B (20mM Tris pH 8.0, 150mM NaCl and 1mM DTT). Fractions were finally pooled, concentrated, snap frozen and stored at -80°C.

**Electrophoretic mobility shift analysis.**

The interaction of hSSB1 with d30T oligonucleotide was investigated using native acrylamide electrophoretic mobility shift analysis. Increasing concentrations of
hSSB1 were incubated with $^{32}$P labelled d30T ssDNA (50 pmol) in buffer (20mM HEPES, pH 7.3, 100mM KCl and 1mM MgCl$_2$, 1mg/µl bovine serum albumin) at 20 °C for 30 min in 10 ml total volume. Reactions were resolved on 10% native acrylamide/TBE gel. Gels were exposed to a phosphorimage plate and the image visualized with a Fuji FLA-5000 Phosphoimager.

Isothermal titration calorimetry.
Calorimetric experiments used a VP-ITC instrument (MicroCal). All solutions were degassed before use. hSSB1 samples were dialysed extensively against 20mM HEPES buffer, pH 7.3, 100mM KCl and 1mM MgCl$_2$. Oligonucleotides were also dissolved in isothermal titration calorimetry (ITC) buffer. Binding experiments were performed in triplicate at 37 °C. A 370-µl syringe, stirring at 300 r.p.m., was used to titrate the oligonucleotide into the sample cell containing approximately 1.4 ml hSSB1. Each titration consisted of a preliminary 1-ml injection followed by up to 25 subsequent 10-ml injections. Heats of dilution were measured in corresponding blank titrations by adding oligonucleotide to ITC buffer and/or ITC buffer to protein, and were found to be similar to heats observed at the end of protein-DNA titrations. ITC-binding isotherms were analysed using a simple single-binding site model with ITC data analysis software (ORIGIN) provided by the manufacturer.

Antibodies and immunofluorescence.
Antibodies used in this study were supplied by Calbiochem (Rad50, Mre11, Rad51), Santa Cruz (Cdc25a), Upstate (cH2AX), Roche (BRDU), Cell Signalling Technologies (pT68-Chk2, pS317-Chk1, pS15-p53), Merck (H3 S10) and Invitrogen (GFP and Alexa secondary antibodies). Sheep antiserum to hSSB1 was raised against full-length recombinant His-tagged hSSB1. For immunofluorescent staining, cells were pre-permeabilized with 20mM HEPES (pH 8), 20mM NaCl, 5mM MgCl$_2$, 1mM ATP, 0.1 mM N$_2$OV, 1mM NaF and 0.5% NP40 for 15 min on ice before fixation in 4% paraformaldehyde (w/v) in PBS for 10 min.

D-loop assay.
For D-loop assay, reactions contained 5'-32P-end-labelled 100-base ssDNA (1 mM), and the indicated concentration of Rad51, hSSB1 or RPA in standard buffer (25mM Tris-acetate pH 7.5, 5mM CaCl$_2$, 2mM ATP, 1mM DTT, 100 mg/µl bovine serum albumin). Proteins were added and mixed in the sequence indicated. After 5 min at 37 °C, excess supercoiled pPB4.3 DNA (0.3mM) was added and incubated for 10
min. The products were deproteinized by the addition of one-fifth volume of stop buffer (0.1M Tris-HCl pH 7.5, 0.1M MgCl₂, 3% SDS and 10 mg/µl proteinase K) followed by electrophoresis, autoradiography and quantification using a Typhoon Trio PhosphorImager (GE Healthcare).

Example 3

SSB Fluorescent polarization (low volume 384)- Bodipy TMR

SSB activity in binding single stranded DNA (ssDNA) can be assayed by measuring the change in polarisation of fluorescently labelled ssDNA. The assay measures the increase in polarisation upon binding of fluorescently labelled single stranded DNA (ssDNA) to the SSB, e.g. hSSB1 or hSSB2.

The effect of a test agent on the ssDNA binding activity of the SSB can be determined by measuring the change in polarisation in the presence of the test agent and comparing against a control value representing the change in polarisation in the absence of the test agent. This assay is suitable for High Throughput Screening (HTS) of test agents.

To assay hSSB1 ssDNA binding activity, suitable reagents include recombinant human hSSB1 with N-terminal 6-HIS tag (final concentration = 94nM) and ssDNA (3OdT) 5' labelled with Bodipy-TMR (final concentration = 2nM). Labelled ssDNA is incubated with hSSB1 for 30 minutes (with or without the test agent) before measurement of fluorescence polarisation (mP).

Example 4

SSB Fluorescent polarization (low volume 384)- Fluorescein

SSB activity in binding single stranded DNA (ssDNA) can be assayed by measuring the change in polarisation of fluorescently labelled ssDNA. The assay measures the increase in polarisation upon binding of fluorescently labelled single stranded DNA (ssDNA) to the SSB, e.g. hSSB1 or hSSB2.

The effect of a test agent on the ssDNA binding activity of the SSB can be determined by measuring the change in polarisation in the presence of the test agent and comparing against a control value representing the change in polarisation in the absence of the test agent. This assay is suitable for High Throughput Screening (HTS) of test agents.
To assay hSSB1 ssDNA binding activity, suitable reagents include recombinant human hSSB1 with N-terminal 6-HIS tag (final concentration = 94nM) and ssDNA (3OdT) 5' labelled with Fluorescein (final concentration = 5nM). Labelled ssDNA is incubated with hSSB1 for 30 minutes (with or without the test agent) before measurement of fluorescence polarisation (mP).

**Example 5**

**SSB HTRF assay (low volume 384)**

SSB activity in binding single stranded DNA (ssDNA) can be assayed by measuring the increase in the ratio between 665nm and 615nm emission following establishment of FRET with Allophycocyanin (APC) labelled ssDNA and Eu labelled SSB.

The effect of a test agent on the ssDNA binding activity of the SSB can be determined by measuring the change in ratio in the presence of the test agent and comparing against a control value representing the change in ratio in the absence of the test agent. This assay is suitable for High Throughput Screening (HTS) of test agents.

To assay hSSB1 ssDNA binding activity, suitable reagents include recombinant human hSSB1 with N-terminal 6-HIS tag (final concentration = 48nM); ssDNA (3OdT) 5' labelled with biotin (final concentration = 20nM); anti-6 HIS Eu cryptate (final concentration = 2nM); SureLight™ Allophycocyanin-streptavidin (APC-SA) (final concentration = 50nM). Biotin labelled ssDNA allowed to bind to hSSB1 for 30 minutes. FRET reaction formed by APC-streptavidin binding to biotin ssDNA and the N-terminal 6-HIS tag on hSSB1 captured by anti-6HIS Eu labelled antibody.

**Example 6**

**Biacore SPR assay**

Compound inhibition of ssDNA binding to an SSB measured by competition assay on the Biacore S51. Biotin labelled ssDNA is tethered to a streptavidin (SA) coated chip and the SSB (e.g. hSSB1 or hSSB2) competes for binding in solution to either free unlabelled ssDNA or tethered biotin-ssDNA. Inhibition of competitive binding is measured by pre-incubating test agents with the SSB before contact with the biosensor as described above.
The effect of a test agent on the ssDNA binding activity of the SSB can be determined by measuring the inhibition of competitive binding. This assay is suitable for High Throughput Screening (HTS) of test agents.

**Example 7 - Cell based assay**

*siRNA fibroblast proliferation assay.*

To determine if a test agent inhibits an SSB (e.g. hSSB1 or hSSB2), proliferation of fibroblasts is measured following formation of ssDNA breaks with either ionizing radiation or bleomycin.

Neonatal fibroblasts (NFF) are treated with the test agent(s) for 24 hours before treatment with ionizing radiation (6Gy) or bleomycin (1µM). Cells are then grown for 72 hours and proliferation measured by Alamar blue. Controls used include siRNA knockout of hSSB1 and non-treated NFF (i.e. no ssDNA breaks). If the SSB function is inhibited cells will not proliferate. If no SSB inhibition occurs, NFF cells will recover from treatment and proliferate. Compounds inhibiting proliferation of untreated NFFs are cytotoxic compounds.

**Example 8 - Cell based assay**

*Foci formation*

Formation of ssDNA breaks by either ionizing radiation or bleomycin in NFFs results in accumulation of hSSB1 and other DNA repair proteins within the nuclei of NFFs. This accumulation of protein (foci formation) can be observed and quantified by immunofluorescence.

Inhibition of foci formation by test agents will be determined for both γH2AX and hSSB1. Cells are pre-treated with test agents before formation of ssDNA breaks by either ionizing radiation (6Gy) or bleomycin (1µM). The number and intensity of foci will be measured over a population of cells.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.
All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.
References


Claims:

1. A method of identifying an agent capable of modulating the activity of hSSB1 or hSSB2, the method comprising the steps of
   (i) contacting a single strand binding protein (SSB) with a binding partner in the presence of a test agent, and
   (ii) detecting the presence or absence of binding of the SSB and binding partner.

2. The method of claim 1 wherein the binding partner is a nucleic acid chosen from single stranded DNA, single stranded RNA, double stranded DNA, double stranded RNA.

3. The method of claim 1 wherein the binding partner is a nucleic acid chosen from single stranded DNA.

4. The method of claim 1 wherein the binding partner is a protein, polypeptide or peptide.

5. The method of claim 1 or 4 wherein the binding partner is chosen from:
   (1) Ataxia telangiectasia mutated (ATM) kinase,
   (2) MRN complex,
   (3) Proteosomes, and/or
   (4) Histone H2AX,
   or a functional fragment of any one of (1)-(4).

6. The method of claim 1 wherein the binding partner is ATM kinase or a polypeptide comprising or consisting of the amino acid sequence of Fragment 4 of ATM kinase (SEQ ID NO: 26) or a polypeptide comprising or consisting of an amino acid sequence having at least 70% sequence identity to one of SEQ ID NOs: 26 or 27.

7. The method of any one of claims 1 to 6 wherein the SSB is hSSB1 or hSSB2.

8. The method of any one of claims 1 to 7 wherein step (ii) comprises detecting formation of a complex of SSB and binding partner.
9. The method of any one of claims 1 to 8 wherein step (ii) comprises detecting phosphorylation of the SSB or binding partner.

10. A method of identifying an agent capable of modulating the activity of hSSB1 or hSSB2, the method comprising:
    (a) performing an assay of the activity of a single strand binding protein (SSB) in the presence of a test agent to determine a test activity of the single strand binding protein, and
    (b) comparing the test activity to a control activity of the single strand binding protein determined by conducting the assay in the absence of the test agent.

11. The method of claim 10 wherein step (b) comprises performing the assay in the absence of the test agent in order to provide the control activity.

12. A pharmaceutical composition or medicament comprising an agent capable of modulating the activity of hSSB1 or hSSB2 and identified by the method of any one of claims 1 to 11.

13. The pharmaceutical composition or medicament of claim 12 wherein the agent is a polypeptide comprising the amino acid sequence of ATM kinase Fragment 4 (SEQ ID NO: 26), or an amino acid sequence having at least 70% sequence identity to ATM kinase Fragment 4.

14. An agent capable of modulating the activity of hSSB1 or hSSB2 and identified by the method of any of claims 1 to 11 for use in the prevention or treatment of a cancerous condition.

15. Use of an agent capable of modulating the activity of hSSB1 or hSSB2 and identified by the method of any of claims 1 to 11 in the manufacture of a pharmaceutical composition or medicament for use in the prevention or treatment of a cancerous condition.

16. The agent or use of claim 14 or 15 wherein the agent is chosen from: a polypeptide comprising the amino acid sequence of ATM kinase Fragment 4 (SEQ ID
17. A method of treatment of a cancerous condition comprising administering a therapeutically effective amount of an agent capable of modulating the activity of hSSB1 or hSSB2 and identified by the method of any of claims 1 to 11 to a subject in need of treatment.

18. The method of claim 17 wherein the agent is chosen from: a polypeptide comprising the amino acid sequence of ATM kinase Fragment 4 (SEQ ID NO: 26); or an amino acid sequence having at least 70% sequence identity to ATM kinase Fragment 4.

19. A method for identifying an agent which modulates the activity of hSSB1 and/or hSSB2, said method comprising the steps of:
   (i) providing a eukaryotic Single Strand Binding (SSB) protein or polypeptide, functional fragment or variant thereof;
   (ii) contacting a test agent with said SSB protein or polypeptide, functional fragment or variant thereof; and
   (iii) detecting a change in the activity of said SSB protein or polypeptide, functional fragment or variant thereof.

20. The method of claim 19, wherein the eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, is a human SSB protein or polypeptide, functional fragment or variant thereof.

21. The method of claim 19 or 20, wherein the eukaryotic SSB protein or polypeptide, functional fragment or variant thereof, is a hSSB1 protein or polypeptide, functional fragment or variant thereof.

22. A method for identifying an agent which modulates the activity of hSSB1 and/or hSSB2, said method comprising the steps of:
   (i) providing a binding partner for a eukaryotic Single Strand Binding (SSB) protein or polypeptide, or a functional fragment or variant of said binding partner;
23. The method of claim 22, wherein the binding partner is selected from ATM protein kinase (ATM), the Mre11-Rad50-Nbs1 (MRN) complex, proteosomes and the histone H2AX.

24. A novel agent which modulates the activity of hSSB1 and/or hSSB2 identified in accordance with the method of any one of claims 19 to 23.

25. A method of treating or preventing cancer or tumour growth in a subject, said method comprising administering to said subject an agent which modulates the activity of hSSB1 and/or hSSB2, optionally in combination with a pharmaceutically-acceptable carrier.

26. A kit for identifying an agent which modulates the activity of hSSB1 and/or hSSB2 comprising:

(i) a eukaryotic Single Strand Binding (SSB) protein or polypeptide, functional fragment or variant thereof; and/or
(ii) a binding partner for a eukaryotic SSB, or a functional fragment or variant of said binding partner.
>gi|33875962:135-770 Homo sapiens hypothetical protein MGC2731, mRNA (cDNA clone MGC:2731 IMAGE:2822460), complete cds
ATGACGACGGAGACCTTTGGTGAAGGATATCAAGCCCGCTCAAGGAATCTGAACCTTATCATTTCATTGTC
TGAGACAGGGCAGTGACCAAGACAAAGGACGCGATGAGTTTGAGGACCTGCAAAGTGGCGGACAAAAAC
AGGGACATCAATATCTCTGTCTGGGAGATGTGGCAATCTGATCCAGCCTGGGAGACMTATATCGGGCTC
ACCAGAAGGTAGGGCTCAGTCTAAGTTGACACTATAATATCTGGCCGTTGGGGTGATCAGCAGA
AGATGGAGAAATTCTGTATGGTTATTCTGTAGGTTCTCTAACTCCAGTGAGCCAAACCAGAGTACAGCAC
CCGGACGCAACCAACACAGCGTTGCAAGACAGCAACACCCTCTCAGCTTTCCAGCCTACACTGGGACC
TCTGCTGCCCCTCCACGCTCTGAGAAGCAGAATGGGAATGGACTGATGGCCCAACCGATCCGGGTGTTG
GCCACATCCTCCTCATCTCTCCCCTCCACCACCCGACCACAGGCAATCAGCTGAAAGCCAGGCCCACCACAC
ACGCTGACGGGCGCTTGCCCCTCCGCAAGCCGTTTACAGTAACGGCAAAGAAAAACCGGAGGAGCAGCAAG
AGATAG

>tr|09BQ15 Hypothetical 22.3 kDa protein (Unknown) (protein for MGC:2731) - Homo sapiens (Human).

MTTETFVSDKIKPLNLNMIPVILEGRTKTKGHEVRTCKVADKTGSINISVVWDDVGN
LIQPGDIIIRTNGYASFVGCLLYTGRGGDLQKIGBPCMVSEVPNFSEPNEYSTQQAP
PNKAVQNSNPASQPTTPGSPAASPENQMGNLAPSAPGGPHPPHTPSSHPPSTRIT
RSQPNHTAPGPGSSNPVSNGKETRRSSKR

FIGURE 1 (A)
FIGURE 1 (B)
FIGURE 1 (C)
FIGURE 2

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Apparent $K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d30C</td>
<td>1.5</td>
</tr>
<tr>
<td>d30GT</td>
<td>2.4</td>
</tr>
<tr>
<td>d30AC</td>
<td>7.4</td>
</tr>
<tr>
<td>d30A</td>
<td>no binding</td>
</tr>
</tbody>
</table>

FIGURE 3

MG132 + + - -
IR + - + -

hSSB1

NS loading
**FIGURE 4**

**FIGURE 5**
FIGURE 8

FIGURE 9
FIGURE 10

FIGURE 11
FIGURE 12

FIGURE 13
<table>
<thead>
<tr>
<th>sihSSB1</th>
<th>sicontrol</th>
<th>IR</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
</table>

- p53 Ser15
- H2AX
- hSSB1
- loading

**FIGURE 14**

<table>
<thead>
<tr>
<th>anti-phospho p53$^{ser15}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
</tr>
<tr>
<td>MRN</td>
</tr>
<tr>
<td>DNA</td>
</tr>
<tr>
<td>ATM</td>
</tr>
<tr>
<td>hSSB1</td>
</tr>
<tr>
<td>hSSB1 T117E</td>
</tr>
</tbody>
</table>

**FIGURE 15**
FIGURE 16

FIGURE 17
FIGURE 18
FIGURE 19
FIGURE 20
FIGURE 21
FIGURE 22

FIGURE 23
FIGURE 26

FIGURE 27
FIGURE 30

FIGURE 31
ATM Fragment 4 (SEQ ID NO: 26)
RIGSLRNMQLCTRCLSNCTKSPNKI
ASGFLRLLTSKLMNDIADICKSLASFIKKPFDGEVESMEDDTNGNLMEVEDQSSMN
LFNDYPDSSVDANEPGESQSTIGAINPLAEYLSKQDLFLDMLKFLCLCVTTAQTN
TVSFRAADIRRKLMLDSTLTEPTKSLHLHMYLMLKELPGEEYPLMEDVLELKP
LSNVCSLYRDDQVCKTLNHLVHKNLQSNMDSENTRDAQGQFLTVIGAFWHLTK
ERKYYFSVRMALVNCLTLLEADPYSKWAINVMGKDPVNEVFQFLADNHQVRL
AAESINRLFQDTKG

FIGURE 32A

ATM KINASE (SEQ ID NO: 27; Genbank accession no: NM_000051 GI:71902539)
MSLVLNDLLLCCQLEHDRATERKKEVEKFRLIRDPETIKHD
RHSDSKQGKYLNWDAVFRFLQYIQKETECRIAKPNVSASTQASRQKMKQEMSSLK
YFIKCANRRAAPRLKCQELLNYIMDDTVKQSSNGAIGADCSNILLKDILSVRKYWCEIS
QQOWLFSVYFRLKPSQDHRVLVARIIHAVTKGQCCQTDGLNSKFLDFFSKAIQ
CARQEKSSSGLNHLAALTIFKLTLAVNRIRVCELGDIEILPTLYIWTQHRLNDLKL
EVIEIELFLQIYIHHPKGAKTQEKAGYESTKWSILYNLYDLLVNEISHGSGKYYSS
GFRNIAVKEKIELMADICHQVFNEDTRSLIESQSYTTQRESSDYSVPCKRKKIELG
WEVIKDHQKSSQNDFDLVPAWQLATQISYKAPASLPCELSPLMLSSLQLPPQRHGE
RTPVYLRCLTEVALCQDKRSLLESSQKSDLKLNKWIWCITFRGISESQAENFGG
GAIQGQLVSEVFREWFKLFTGASCAPCPAVCCLALTTSIVPGTVKMGIEQNMCEV
NRSFSLKESIKWLLFYQLEGDLESTVPPILHSNFPHLVEKLVSLTMKCAAM
NFFQSVPECEHHQKDKEELSFEVEELFLQTTFDMDFLTIVREGIEKHOSISGFV
HQLKESLDRLCLGLSELQLNNYSEITNSETLVRCSSLVGVLGCYMGVIAEEEA
YKSELFQKAKSLMQCAGESITLFKNTNEEFRIGSLRNMQLCTRCLSNCTKSPNKI

FIGURE 32B
FIGURE 32B Cont...
FIGURE 32B Cont...
A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N AG1K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
</table>

X Further documents are listed in the continuation of Box C
X See patent family annex

Special categories of cited documents
'A' document defining the general state of the art which is not considered to be of particular relevance
'E' earlier document but published on or after the international filing date
'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
'O' document referring to an oral disclosure, use, exhibition or other means
'P' document published prior to the international filing date but later than the priority date claimed
'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
'X' document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
'Y' document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
'Z' document member of the same patent family

Date of the actual completion of the international search
20 January 2009

Date of mailing of the international search report
09/02/2009

Name and mailing address of the ISA
European Patent Office, P B 5818 Patentlaan 2 NL-2280 HV RIJSWIJK
Tel (+31-70) 340-2040,
Fax (+31-70) 340-3016

Authorized officer
Motrescu-Hateley, E
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>P,X</td>
<td>WO 2008/106709 A (QUEENSLAND INST MED RES [AU]; KHAUNA KIM KIM [AU]; RICHARD DEREK [AU];) 12 September 2008 (2008-09-12) cited in the application page 1, lines 5-10; page 7, lines 21-29; page 23, lines 24-34; page 24, lines 1-8; page 30, lines 1-20; page 31, claim 21.</td>
<td>10,11,26</td>
</tr>
</tbody>
</table>
The present claims 12,14,15,17,24,25 relate to an extremely large number of possible agents. Support and disclosure in the sense of Article 6 and 5 PCT is to be found however for only a very small proportion of the agents claimed (see description page 8, lines 3-10; page 19, lines 10-15; page 20, lines 10-31). The non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search of claims 12,14,15,17,24,25 (PCT Guidelines 9.19 and 9.23).

The search of claims 12,14,15,17,24,25 was restricted to those claimed agents which appear to be supported, namely siRNA, Seq ID Nos: 6 or 7 (see description page 8, lines 3-10), eucaryotic SSB-binding portions of ATM Fragment 4 (see page 19, lines 10-15) and proteasome inhibitors (e.g. MG132) and vortmannin (see page 20, lines 10-31).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2)PCT declaration be overcome.
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

2. [ ] Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   - see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

4. I [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- [ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- [ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- [ ] No protest accompanied the payment of additional search fees.
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CN 101291668 A</td>
<td>22-10-2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2007046426 A1</td>
<td>26-04-2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 101291668 A</td>
<td>22-10-2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 2014675 A1</td>
<td>14-01-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2005019258 A2</td>
<td>03-03-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WO 2008106709 A</td>
<td>12-09-2008</td>
<td>NONE</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2500687 A1</td>
<td>15-04-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1594447 A2</td>
<td>16-11-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2006516089 T</td>
<td>22-06-2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>