Essential Developmental, Genomic Stability, and Tumour Suppressor Functions of the Mouse Orthologue of hSSB1/NABP2

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Abstract

Single-stranded DNA binding proteins (SSBs) regulate multiple DNA transactions, including replication, transcription, and repair. We recently identified SSB1 as a novel protein critical for the initiation of ATM signaling and DNA double-strand break repair by homologous recombination. Here we report that germline Ssb1+/− embryos die at birth from respiratory failure due to severe rib cage malformation and impaired alveolar development, coupled with additional skeletal defects. Unexpectedly, Ssb1−/− fibroblasts did not exhibit defects in ATM signaling or γH2ax focus kinetics in response to ionizing radiation (IR), and B-cell specific deletion of Ssb1 did not affect class-switch recombination in vitro. However, conditional deletion of Ssb1 in adult mice led to increased cancer susceptibility with broad tumour spectrum, impaired male fertility with testicular degeneration, and increased radiosensitivity and IR–induced chromosome breaks in vivo. Collectively, these results demonstrate essential roles of Ssb1 in embryogenesis, spermatogenesis, and genome stability in vivo.

Introduction

Appropriate and timely repair of damaged DNA is critical for maintaining genomic integrity and tumour suppression [1,2]. DNA double-strand breaks (DSBs) are the most cytotoxic genomic lesions, and can arise from exogenous genotoxic insult, stalled replication forks, or during physiological processes such as meiosis and B and T cell maturation. Organisms have evolved two main pathways for DSB repair: non-homologous end joining (NHEJ) and homologous recombination (HR). In the initial step of HR, DSBs are resected to generate 3′ single-stranded DNA (ssDNA) tails. The ssDNA intermediates are protected from further degradation by ssDNA-binding proteins (SSBs).

The SSB family of proteins are conserved in all three kingdoms of life [3] and are characterised structurally by their oligonucleotide-binding (OB) folds that bind ssDNA. SSB proteins can be subdivided into two sub-groups. First, simple SSBs, typified by the Escherichia coli (E. coli) SSB, contain a single OB-fold. The second sub-group includes the higher ordered replication protein A (RPA), which contains multiple OB-folds and is conserved in yeast and higher eukaryotes [3]. Human RPA is a heterotrimeric polypeptide, widely believed to be a central component of both DNA replication and DNA repair pathways [4,5,6]. Recently, we identified two novel SSB proteins, named SSB1 (also known as OBFC2B, NABP2 or SOSS-B1) and SSB2 (also known as OBFC2A, NABP1 or SOSS-B2) [7], which are conserved in vertebrates but not in lower eukaryotes. These SSBs are more closely related to the bacterial and archaean SSB sub-group than to RPA [3]. Both SSBs encode a conserved single OB-fold followed by a divergent spacer domain and a conserved C-terminal motif, suggesting functional overlap between these proteins. The spacer region is the only significant difference between human SSB1 and SSB2.

Our functional characterization of SSB1 revealed that it is stabilised following exposure of cells to ionizing radiation (IR) forming distinct foci at DSB sites [7]. Depletion of SSB1...
Author Summary

Single-stranded DNA binding proteins (SSBs) play a variety of roles in the cell, regulating transcription, replication, and DNA repair. We recently identified and described a novel SSB, designated SSB1, which was shown to be critical for DNA repair in the cell. In this study we have used a mouse model in which the Ssb1 gene is deleted to further investigate its physiological function. Here, we show that deletion of Ssb1 causes death at birth due to severe respiratory failure, which is caused by an improperly formed rib cage and immature lung development. In addition, we observed multiple additional skeletal defects in Ssb1 deleted mice, indicating that Ssb1 is necessary for proper development of the embryonic skeleton. Furthermore, Ssb1 deletion in the adult mouse caused fertility defects in male mice and led to the development of a variety of tumours. Together, these studies demonstrate a novel and critical role of Ssb1 in embryonic development, in fertility, and in the protection from tumour formation.

Results

Ssb1 deficiency results in perinatal lethality

The murine Ssb1 gene is located on chromosome 10 and spans 7 exons. We engineered a “floxed” Ssb1 allele with unidirectional loxP sites flanking its major protein coding exons 3–6, including the OB-fold domain critical for its DNA binding activity (Figure S1A). Correct targeting was confirmed by Southern blot (Figure S1B) and genotyping PCR (Figure S1C). Evaluation of the growth of Ssb1 heterozygous mice (Ssb1+/−) relative to wild-type littermates (Ssb1+/+) revealed no apparent physiological abnormalities in Ssb1+/− mice monitored for up to 2 years. To generate mice with targeted deletion of Ssb1, we intercrossed Ssb1+/− breeding pairs, with the expectation that approximately 25 percent of the offspring would be an Ssb1−/− genotype. Interestingly, no viable Ssb1−/− mice were detected amongst more than one hundred offspring from these intercrosses genotyped at 12 days post-partum (Table 1). These results suggested that Ssb1 deletion might result in lethality during embryogenesis.

In order to define the time point of embryonic lethality caused by Ssb1 ablation, we collected embryos from Ssb1−/− intercrosses at different gestational days, assessed by the presence of a vaginal plug at E0.5. Ssb1−/− embryos were recovered at near-Mendelian ratios at E13.5 and E18.5 (Table 1), but were significantly growth retarded in terms of both body weight and length at the latter time-point, when compared to wild-type and heterozygous littersates (Figure 1A, 1B; Figure S2A, S2B). Ssb1+/+ and Ssb1−/− embryos were morphologically indistinguishable, in terms of both body size and body length. Ssb1−/− embryos also displayed craniofacial abnormalities, including a recessed mandible (lower jaw) and misshapen snout (Figure 1A, arrowheads; Figure 2C, 2D, Figure S2C). Furthermore, there was a defect in the outgrowth of both fore- and hindlimbs, as well as hindlimb-specific oligodactyly (missing digits) (Figure 1A, arrows). However, these embryos appeared otherwise grossly normal, suggesting that Ssb1 ablation may cause lethality during the perinatal period.

To further investigate the cause of Ssb1−/− lethality, we performed embryonic recovery of embryos at E18.5 or at the time of birth (P0), and stimulated breathing by clearing the facial orifices and gentle stroking of the snout. In the litters examined, all Ssb1+/+ and Ssb1−/− pups established rhythmic breathing, a healthy pink skin color and movement within minutes. However, Ssb1−/− pups rapidly became asphyxic and typically died between 10–30 min post embryonic excision, despite taking short, sporadic gasping breaths, suggesting that they could not breathe and oxygenate their blood properly (Figure 1A, Figure S2A). Haematoxylin and eosin (H&E) staining on these embryos suggested that atelectasis was the primary cause of respiratory failure (Figure 1C). These results suggest that Ssb1−/− embryos survive the entire course of development in utero but die at the perinatal stage.

Ssb1 ablation results in aberrant skeletal patterning

To further investigate the abnormalities we observed in the craniofacial region and hindlimbs of Ssb1−/− embryos, we next sought to determine if their skeletal architecture was altered by performing whole-mount cartilage and mineralized bone staining with alizarin red and alcian blue and alizarin red. Strikingly, we observed a lack of ossification in all but the four most anterior rib pairs. In addition, the ribcage of Ssb1−/− embryos was misshapen, with a lack of curvature in the anterior ribs, and horizontally orientated rib-sternum attachments (Figure 2A, 2B, arrow). The more posterior “floating” ribs in these embryos were also rudimentary and abnormally shaped, contributing to a general decrease in size of the rib-cage (Figure 2A, Figure 2B). The lack of structural support from the missshapen and poorly developed rib-cage in Ssb1−/− embryos would have significantly contributed to the respiratory distress evident in these embryos at birth, and resulted in rapid atelectasis and perinatal death.

Examination of the skull of E18.5 embryos revealed normal formation of major bone structures, including the parietal (pr), intraparietal (ip), frontal (fr) and supraoccipital (so) bones. We noted a modest elongation of the premaxillary bone (pmax), consistent with the pointed snout seen in these embryos, and a shortened mandible (micrognathia), which was set at a wider angle than in control embryos (Ssb1+/+, Ssb1+/−) (Figure 2B, 2D). The tympanic ring (tr), which supports the eardrum, was also poorly formed in Ssb1−/− embryos (Figure 2C). Furthermore, we observed evidence of a variably penetrant cleft palate (n=2 of 5 embryos), which was evident even between Ssb1−/− mice of the same litter (Figure S2D–S2F; arrows, arrowheads). Together, these data suggest a spectrum of craniofacial abnormalities in Ssb1−/− embryos.
The limb skeleton of Ssb1<sup>−/−</sup> E18.5 embryos showed a significant decrease in the length of all long bones, including humerus, radius, ulna, femur and tibia, as well as the scapula (Figure 2E–2G), indicating a limb outgrowth defect (**<sup>P</sup> < 0.001, Figure 2G). Overall, this phenotype was more pronounced in the hindlimbs, where we observed varying degrees of abnormalities in these structures, including absent fibulas (Figure 2F). Finally, although the forelimbs of Ssb1<sup>−/−</sup> embryos were properly patterned (albeit smaller in size), hindlimbs displayed aberrant bone mineralization and severe defects in patterning along the anterior-posterior axis, which always manifested as oligodactyly (Figure 2H). Interestingly, this phenotype was variable in penetrance, with between two to a maximum of four digits present, and we often observed variation of patterning defects between the left and right hindlimb autopods within the one embryo. Taken together, these data indicate that Ssb1 is necessary for skeletogenesis and hindlimb digit specification in the embryo, and that it is of particular importance for the later steps of chondrogenesis involving bone ossification. These data highlight a novel and unexpected role for Ssb1 during embryogenesis.

Ssb1<sup>−/−</sup> embryos exhibit distal lung differentiation defects

To determine if other causative factors may have contributed to the perinatal lethality in Ssb1<sup>−/−</sup> embryos, we next performed histological analysis of sagittal sections from E18.5 embryos. We observed grossly normal morphology for major organs including the brain, heart, thymus, intestine, and liver (Figure S3). However, consistent with the respiratory distress phenotype, we observed immature lung morphology in these sections (Figure S3). To more closely examine this, we dissected lungs from E18.5 Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup> and Ssb1<sup>−/−</sup> embryos (Figure 3A) and confirmed complete deletion of the Ssb1 protein by western blot (Figure 3B). Interestingly, we also noted an increase in the protein level of Ssb2 in Ssb1<sup>−/−</sup> lungs (Figure 3B), similar to what has been observed based on siRNA depletion in human cells [8,9,10]. A comparison of the gross morphology of the lungs revealed that the lungs of Ssb1<sup>−/−</sup> embryos

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**Table 1. Impact of deletion of Ssb1 on embryonic survival in Ssb1<sup>+/−</sup> intercrosses.**

<table>
<thead>
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<th>Ssb1&lt;sup&gt;+/+&lt;/sup&gt;</th>
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<td>Observed: 8</td>
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<td>(36 genotyped)</td>
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<td>14</td>
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<tr>
<td>Live Pups (P12)</td>
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<td>64</td>
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<td>(128 genotyped)</td>
<td>Observed: 39</td>
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**Figure 1. Ssb1 deletion causes perinatal lethality due to severe respiratory failure.** (A) Comparison of size and appearance of E14.5 and P0 Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup>, and Ssb1<sup>−/−</sup> offspring. Note the cyanosis in P0 Ssb1<sup>−/−</sup> pups. Scale = 2 mm. (B) Comparison of weights of E14.5 and E18.5 Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup> and Ssb1<sup>−/−</sup> embryos. Data represent the mean ± SEM, n = 3–16 embryos per group from a minimum of 3 litters per timepoint (**<sup>P</sup> < 0.001; student’s t-test). (C) Haematoxylin and eosin staining of P0 lungs of Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup> and Ssb1<sup>−/−</sup> pups delivered by caesarian section.

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Figure 2. *Ssb1* deletion causes multiple skeletal defects. (A) Alcian blue and Alizarin red staining of control (*Ssb1*+/+, *Ssb1*+/−) and *Ssb1*−/− E18.5 ribcages. (B) Butterfly rib-spread of *Ssb1* control and *Ssb1*−/− E18.5 ribcages. (C) Comparison of skull anatomy of *Ssb1* control and *Ssb1*−/− E18.5 embryos. eo, exoccipital; so, supraoccipital; ip, interparietal; pr, parietal; fr, frontal; mx, maxilla; md, mandible; bo, basioccipital; ptg, pterygoid; pmx,
Interestingly, we also observed a 1.3 fold increase in a secondary effect of improper type II AEC differentiation [15,16].

To determine if the higher cell density in lungs of Ssb1−/− embryos were correctly lobulated, with four right lobes and a single left lobe flanking the heart, suggesting that early lung development patternning in these embryos is intact (Figure 3A). However, H&E analysis on coronal sections of these lungs revealed aberrant late-stage lung development, with reduced alveolar lumens and thickened, hypercellular alveolar walls in Ssb1−/− lungs when compared to control (Ssb1+/+ and Ssb1+/−) littermates (Figure SC–EG, **P<0.001). During lung development, regression of the mesenchyme occurs from approximately E15.5 onwards by apoptosis to form the air-blood barrier, necessary for efficient respiration. To determine if the higher cell density in Ssb1−/− lungs results from either a decrease in apoptosis during development or increased proliferation, we performed immunohistological staining on E14.5 and E18.5 lung sections for ApopTag and Ki67, respectively. However, no differences in the levels of Ki67 or ApopTag were observed at these developmental stages (Figure S4).

Perinatal death due to respiratory failure can be caused by impaired differentiation of the proximal and/or distal airway epithelium. To determine if proximal airway epithelium was impaired differentiation of the proximal and/or distal airway epithelium. To determine if proximal airway differentiation and blood vessel formation of Ssb1−/− lungs when compared to control (Ssb1+/+ and Ssb1+/−) littermates (Figure SC–EG, **P<0.001). During lung development, regression of the mesenchyme occurs from approximately E15.5 onwards by apoptosis to form the air-blood barrier, necessary for efficient respiration. To determine if the higher cell density in Ssb1−/− lungs results from either a decrease in apoptosis during development or increased proliferation, we performed immunohistological staining on E14.5 and E18.5 lung sections for ApopTag and Ki67, respectively. However, no differences in the levels of Ki67 or ApopTag were observed at these developmental stages (Figure S4).

Novel Embryonic and Tumour Suppressor Roles of Ssb1

Mouse embryonic fibroblasts (MEFs) from Ssb1+/+ and Ssb1−/− E15.5 embryos were isolated to investigate the role of Ssb1 in DSB repair and signaling in the mouse. Early passage Ssb1+/+ and Ssb1−/− MEFs exhibited similar cell-cycle profiles, but Ssb1−/− MEFs had a slightly diminished proliferative capacity and more rapidly reached the plateau phase when compared with Ssb1+/+ MEFs (Figure S6A, S6B). As we and others had previously described a role of SSB1 in the activation of ATM signaling in response to IR based on siRNA depletion in human cells [7,9,10], we assessed activation of this pathway in MEFs. Although we observed stabilization of Ssb1 in response to IR, interestingly, no attenuation of Atm activation was detected when we assessed autophosphorylation of Atm on serine1987 (serine1981 in human) or phosphorylation of its downstream activation target p33 on serine18 (serine15 in human) (Figure S6C). Similar to what we observed in Ssb1−/− lungs, Ssb2 protein levels were upregulated in Ssb1−/− MEFs. These results suggest that deletion of Ssb1 does not abrogate Atm activation in MEFs, and may highlight potential redundancy between Ssb1 and Ssb2 in these cells. To determine if the response to ionizing radiation was intact in Ssb1−/− cells, we also assessed the dynamics of γ-H2ax foci formation in Ssb1+/+ and Ssb1−/− MEFs by immunofluorescence. These studies revealed no significant differences in the baseline level of γ-H2ax foci induction nor in the clearance of IR-induced γ-H2ax foci (Figure S6D, S6E), indicating that these cells did not exhibit higher levels of endogenous DNA damage and/or defective repair of IR-induced DSBs.

Next, we sought to utilise an in vivo model of DSB repair to interrogate if Ssb1 is necessary for DSB repair in the mouse. Class switch recombination (CSR) involves a programmed Ig heavy gene rearrangement in B-lymphocytes that requires repair of physiological DSBs generated as a result of activation-induced deaminase (AID) catalysed DNA base damage. In B-lymphocytes, the initial secreted antibodies contain heavy chains of the IgM class (or IgD formed via alternative splicing). Upon stimulation of these B-lymphocytes by antigen, the original IgM class heavy chain gene undergoes CSR to encode heavy chains of IgG, IgE, or IgA classes [17]. Several proteins involved in DSB repair including ATM, H2AX and 53BP1 have been suggested to have a role in CSR, to different extents, probably due to their roles in synopsis and/or DNA repair [17]. To assess whether loss of Ssb1 affects CSR, we generated B cell specific conditional Cd19-Cre; Ssb1−/− mice. Western blotting of...
Figure 3. Distal lung differentiation defects in Ssb1<sup>−/−</sup> embryos. (A) Representative image showing the comparison of lung size and morphology of E18.5 Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup> and Ssb1<sup>−/−</sup> embryos. Scale bar = 2 mm. (B) Protein level of Ssb1, Ssb2 and Actin in Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup> and Ssb1<sup>−/−</sup> E18.5 lungs. (C) Haematoxylin and eosin staining showing morphology of alveolar lumen formation and intra-alveolar septae thickness in Ssb1 control.
Whole cell extracts showed loss of Ssb1 protein in B cells from Cal19-Cre; Ssb1+/− mice (Figure 4A) and upregulation of Ssb2 protein levels (Figure 4B), similar to what we observed in Ssb1+/− lungs and MEFs (Figure 3B, Figure S6C). Mice lacking Ssb1 in the B lineage produced normal numbers of mature IgM⁺ lymphocytes in the bone marrow and had spleens of normal size and cellularity. Upon in vitro stimulation of B cells isolated from spleens with anti-CD40 antibody plus IL-4 over 3 days, the extent of IgM to IgGl switching and cell viability in wild-type and Ssb1-deficient B cells was also comparable (Figure 4C, 4D). No difference was found in the percentage and total numbers of double- or microhomology-directed double-strand breaks (Figure 4E). These results suggest that Ssb1 is dispensable for DSB repair by class-switch recombination.

**Conditional Ssb1 gene deletion in adult mice**

Given the perinatal lethality we observed in constitutive Ssb1−/− mice, we next employed a conditional approach to ubiquitously ablate Ssb1 postnatally using a tamoxifen-inducible Cre system by interbreeding Ssh1fl/fl mice with the Rosa26-CreER² mouse strain (Figure S7) [18]. Efficiency of Ssh1 deletion in adult mice (4 weeks old) following a series of tamoxifen injections was confirmed by both PCR for genomic recombination, and western blot analysis for protein depletion in various tissues (Figure S8A–S8C). The floxed Ssb1 allele was efficiently deleted in bone marrow (BM), thymus, spleen, testes and small intestine, partially deleted in lung, kidney, liver and heart, but not in the brain (Figure S8A). Dramatically decreased Ssb1 protein levels were confirmed in multiple tissue samples from tamoxifen induced Rosa26-CreER²; Ssb1−/− mice, with undetectable levels of Ssb1 protein in spleenocytes and thymocytes as early as 10 days after the final tamoxifen induction (Figure S8B). Interestingly, we observed a dramatic up-regulation of Ssh2 in response to Ssb1 ablation in the bone marrow and spleen, but not in the testes and thymus of Rosa26-CreER²; Ssb1−/− mice (Figure S8C).

**Impaired fertility in conditional Rosa26-CreER²: Ssb1−/− male mice**

Monitoring of Rosa26-CreER²; Ssb1−/− mice and control Rosa26-CreER²; Ssb1+/− mice revealed no significant differences in body weight over a period of up to 90 weeks (Figure S9). In addition, histological analysis of all major organs, including the brain, thymus, lung, heart, liver, kidney and small intestine revealed no gross abnormalities. The abnormality of many DNA repair factors, (such as Atm [19], H2ax [20], Mdc1 [21] and Mphh1/Brst1 [22]) has been shown to result in impaired fertility, due to important roles of these proteins in DSB repair during meiosis. To determine the impact of Ssb1 deficiency on fertility, we examined ovaries and testes of Rosa26-CreER²; Ssb1−/− mice six weeks after induction with tamoxifen. Whereas Rosa26-CreER²; Ssb1+/− ovaries were morphologically normal in females, the testes from Rosa26-CreER²; Ssb1−/− males were reduced in size (Figure 5A), in terms of both absolute weight (n = 8, ***P < 0.001, Figure 5B) and gonado-somatic index (GSI) [23], an indicator of gonadal weight as a proportion of total body mass (n = 8, ***P < 0.001, Figure 5C), when compared to their Rosa26-CreER²; Ssb1+/− littermates.

Histological examination of testes from 3-month-old Rosa26-CreER²; Ssb1−/− male mice showed bilateral testicular degeneration with a spectrum of alterations in spermatogenesis. Testicular tubules showed degenerate, sometimes vacuolated, or necrotic spermatogenic cells, the latter with pyknotic nuclei and hypereosinophilic cytoplasm, or apoptotic body formation. Multinucleated giant cells were also frequently present in the lumen, either derived from spermatocytes with arrested development or the coalescence of spermatids (Figure 5D, left panel). Increased apoptosis at approximately the same stage, equivalent to stage IV of the normal seminiferous epithelial cycle has been reported in a number of mutants defective for meiotic recombination and/or meiosis-specific chromosome structures [24]. We performed ApopTag staining to determine the rate of spermatocyte apoptosis in testes from Rosa26-CreER²; Ssb1+/− and Rosa26-CreER²; Ssb1−/− male mice. No difference was found in the percentage and absolute number of positive nuclei in the testes of Rosa26-CreER²; Ssb1+/− and Rosa26-CreER²; Ssb1−/− males, demonstrating that Ssb1 is dispensable for spermatocyte apoptosis. As newly formed spermatozoa are released for passage into the epididymis for further maturation, we examined epididymides of Rosa26-CreER²; Ssb1+/− male mice for developing germ cells that were prematurely sloughed from the seminiferous epithelium and passed into the epididymis. The presence of round germ cells within the lumen of the epididymis (Figure 5E) suggests that, in addition to apoptosis, a significant number of germ cells were being lost via premature sloughing from the supporting Sertoli cells. Taken together, these results reveal a spectrum of testicular degenerations in the Rosa26-CreER²; Ssb1−/− mice.

To further characterize the consequences of Ssh1 ablation on fertility, we interbred induced Rosa26-CreER²; Ssb1−/− mice with wild-type mice. Consistent with the normal physiological appearance of their ovaries, induced female Rosa26-CreER²; Ssh1−/− mice for developing germ cells that were prematurely sloughed from the seminiferous epithelium and passed into the epididymis. The presence of round germ cells within the lumen of the epididymis (Figure 5E) suggests that, in addition to apoptosis, a significant number of germ cells were being lost via premature sloughing from the supporting Sertoli cells. Taken together, these results reveal a spectrum of testicular degenerations in the Rosa26-CreER²; Ssh1−/− mice.

**Conditional Ssb1 deletion leads to increased radiation sensitivity in vivo**

To assess if conditional deletion of Ssb1 in mice causes a DNA damage response defect in vivo, we challenged Rosa26-CreER²; Ssh1−/−, Rosa26-CreER²; Ssh1+/− and Rosa26-CreER²; Ssh1−/− mice with 8 Gy of total body irradiation (TBI) at 4 weeks post-tamoxifen-induction and monitored them for up to 30 days post-IR (Figure 6A). Although we observed comparable progressive weight loss in all 3 groups within the first few days of radiation exposure, death events started to occur in the group of irradiated Rosa26-CreER²; Ssh1−/− mice by the 10th day. By day 19, 92%
Figure 4. Class switch recombination activity in B-cell specific Ssb1-deleted mice. Ssb1<sup>fl/fl</sup> mice were crossed with a Cd19-Cre transgene expressing C57BL/6J mice to specifically delete Ssb1 in B cells. Splenic B cells were isolated and stimulated for 2, 3, and 4 days using anti-Cd40 antibodies plus IL-4 to induce CSR to IgG1. (A) Western blotting of whole cell extracts showed loss of Ssb1 protein in stimulated B cells from Ssb1 KO mice compared to Ssb1 WT mice. (B) Immunoblot analysis of Ssb2 and Tubulin expression in Exp. 1 and Exp. 2. (C) Flow cytometry analysis of IgG1 expression in AID KO, Ssb1 WT, and Ssb1 KO mice at Day 2, Day 3, and Day 4. (D) Quantification of IgG1+ cells and viability in AID KO, Ssb1 WT, and Ssb1 KO mice. (E) Microhomology-mediated end joining in Ssb1 KO and Ssb1 WT mice. The table shows the number of 1-nt, 2-nt, 3-nt, and 4-nt microhomologies and the total MH. (F) **Legend:** AID KO, Ssb1 KO, Ssb1 WT.
knockout (Ssb1 KO; Cd19Cre+; Ssb1−/−) mice. Equal amounts of stimulated B cell extracts from heterozygous Ssb1 (Ssb1 Het; Cd19Cre+; Ssb1−/+ ) and wild-type Ssb1 (Ssb1 WT; Cd19Cre−; Ssb1+/+) mice were included for comparison. Equal loading was confirmed by probing for Hsp90. (B) Western blotting of Ssb2 levels in B cells from Ssb1 WT and Ssb1 KO mice. (C) FACS analysis of CSR to IgG1 over time in stimulated B cells from mice of the indicated genotypes. Stimulated splenic B cells from AID−/− (AID KO) mice served as a negative control. (D) Summary statistics of CSR activity to IgG1 and viability on day 3 of stimulation. Mean and S.E.M. from three independent experiments are shown. No statistically significant differences (two-tailed unpaired t-test) were found. (E) Switch region junction analysis. Smg1 junctions were amplified from IL4 plus anti-CD40 stimulated primary B cells (day 4) and sequenced. Percentage and total numbers of direct or microhomology-mediated joints are indicated. nt, nucleotides.

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(11 out of 12) of Rosa26-CreERT2: Ssb1−/− mice had died. In contrast, in Rosa26-CreERT2: Ssb1+/− and Rosa26-CreERT2: Ssb1+/+ groups, the first death event occurred on the 13th day and more than 50% of mice survived for at least 30 days after irradiation (Figure 6B). Thus, in vivo radiation sensitivity was significantly increased in Rosa26-CreERT2: Ssb1−/− mice compared to Rosa26-CreERT2: Ssb1+/− or Rosa26-CreERT2: Ssb1+/+ controls based on Kaplan-Meier survival analysis (**P<0.01) (Figure 6B). As injury of the small intestine or bone marrow are the most common causes of death in irradiated mice, we examined these tissues to establish the cause of death in induced Rosa26-CreERT2: Ssb1−/− mice. At 24 h and 3 days post TBI, the histology of the small intestine was comparable across induced Rosa26-CreERT2: Ssb1−/−, Rosa26-CreERT2: Ssb1+/− and Rosa26-CreERT2: Ssb1+/+ mice, as assessed by Haematoxylin and eosin, Ki67 and ApopTag immunohistochemical staining (Figure S10/A and data not shown). However, at 5 days post TBI, we observed some pathological abnormalities in Rosa26-CreERT2: Ssb1−/− mice, including distended crypt lumina lined by attenuated enterocytes and containing desquamated necrotic cellular debris as well as a small increase of cells near deep crypts with apoptotic bodies (Figure 6C).

Further, we performed complete blood count (CBC) analysis on peripheral blood of these mice to assess hematologic radiation toxicity, but no significant difference between the groups was observed (Figure S10/B).

To assess whether Ssb1 deficiency affects radiosensitivity in other tissues, we also isolated and exposed thymocytes to various doses of IR (1–6 Gy). We observed increased radiosensitivity in Ssb1−/− thymocytes as assessed by Annexin V and 7-AAD staining (Figure S11). Taken together, these data indicate that deletion of Ssb1 leads to increased radiosensitivity in vivo and in thymocytes in vitro.

Increased genomic instability in conditional Rosa26-CreERT2: Ssb1−/− mice

To further investigate the increased radiation sensitivity of conditional Ssb1 null mice, we cytologically examined bone marrow metaphase spreads from Rosa26-CreERT2: Ssb1−/−, Rosa26-CreERT2: Ssb1+/− and Rosa26-CreERT2: Ssb1+/+ mice at 24 h after 2 Gy of TBI to assess chromosomal abnormalities. We observed a significant increase in chromosomal breakage, fragmentation and fusion in Rosa26-CreERT2: Ssb1−/− bone marrow metaphases upon irradiation, as assessed by fluorescence in situ hybridization (FISH) analysis (Figure 7). These results provide in vivo evidence that Ssb1 functions to maintain genomic stability.

Broad spontaneous tumour spectrum in conditional Rosa26-CreERT2: Ssb1−/− mice

To assess whether conditional Ssb1 deletion would lead to increased cancer susceptibility, we monitored tumour development in age- and gender-matched long-term survival cohorts of Rosa26-CreERT2: Ssb1−/− (n = 35) and Rosa26-CreERT2: Ssb1+/− (n = 35) mice. No significant difference in body weight was found between Rosa26-CreERT2: Ssb1−/− and Rosa26-CreERT2: Ssb1+/− mice over the 86 week observation period post-Ssb1 deletion (Figure S9). However, during this period, 11 out of 35 (31.4%) Rosa26-CreERT2: Ssb1−/− mice developed tumours, in contrast to only 2 out of the 35 (5.7%) Ssb1+/− mice, revealing a statistically significant difference (**P<0.01) in tumour-free Kaplan-Meier survival analysis (Figure 8A). No tumours were observed in a Cre-negative control group (Ssb1+/+ mice, n = 10) treated with an identical tamoxifen dose or in a vehicle (olive oil: ethanol at 19:1 ratio) treated Rosa26-CreERT2: Ssb1−/− control group (n = 5). In the 11 Rosa26-CreERT2: Ssb1−/− mice that developed tumours, we observed a broad tumour spectrum (Figure 8B) including splenic and metastatic B lymphomas, T cell lymphomas in thymus (Figure 8C), hepatocellular carcinoma, (HCC, Figure 8D) and B or T lymphoblastic leukemia (Figure S12). We also observed p53 stabilization, which is most likely an indication of the presence of mutated p53, in a high proportion of tumours (9 of 11 Ssb1−/− tumours and 2 of 2 Ssb1+/− tumours) when compared with adjacent normal tissue from the same mice (Figure S13 and Figure S14). Moreover, in the two tumours observed in Rosa26-CreERT2: Ssb1−/− mice, the Ssb1 protein was undetectable by immunohistochemical staining, indicating possible loss of heterozygosity (LOH) of the other Ssb1 allele in these tumours (Figure S14). Taken together, these data indicate that Ssb1 prevents tumor formation in vivo.

Discussion

Previous studies using siRNA depletion in human cells have reported a role for SSB1 in the proper co-ordination of DNA repair in response to IR [7,8,9,10]. By disrupting the major protein coding exons of Ssb1 in mice, including the OB-fold domain, we have created mouse models to study the physiological function of Ssb1 in vivo, and describe a wide spectrum of phenotypes upon Ssb1 deletion during embryogenesis and in adult and aged mice.

Major unexpected findings include novel roles of Ssb1 in the regulation of lung and skeletal development, as constitutive germline ablation of Ssb1 resulted in immature alveolar differentiation and multiple skeletal defects encompassing the ribs, craniofacial skeleton, and limbs. Interestingly, a handful of other DNA repair factors have been linked to roles in skeletogenesis: patients with Rothmund-Thompson and Rapaputino syndrome, who have mutations in the DNA helicase RECQ4, exhibit some skeletal defects in the limb [25]; patients with mutations in the repair-associated proteins Cdp-interacting protein (CdDP/ RBBP8), Centrosomal Protein 152 (CEP152), microcephalin 1 (MCPH1), or Ataxia-Telangiectasia Related (ATR) exhibit dwarfism and a characteristic “bird-shaped” face with micrognathia, which is similar to the craniofacial phenotype we observe in Ssb1−/− mice [26,27,28,29]. Similarly, patients with Nijmegen Breakage Syndrome (NBS), who have mutations in the MRN complex protein NBS-1, also have similar craniofacial abnormalities [30]. Previously, we demonstrated an interaction between SSB1 and NBS1, which, in vitro studies, was abrogated by NBS-1 mutations observed in patients [31]; therefore it is tempting to
Figure 5. Testicular degeneration and impaired fertility in conditional Rosa26-CreERT2; Ssb1<sup>+/−</sup> male mice. (A) Representative image of testes from Rosa26-CreERT2; Ssb1<sup>+/−</sup> mice compared with those from Rosa26-CreERT2; Ssb1<sup>+/+</sup> littermates at 10 weeks of age. (B) Testis weight and (C) the gonado-somatic index (GSI) in conditional Ssb1 deleted male mice compared with their littermates. Data represents the mean ± SEM of testis...
speculate that SSB1 may be involved in some of the craniofacial phenotypes of this disorder. However, the broad spectrum of skeletal phenotypes in Ssb1-deficient mice is more pronounced than those reported for any of these human syndromes. This, together with the absence of obvious defects in signalling and repair of IR-induced DNA damage in both MEFs and absence of CSR defects in B cell-specific Sbh1−/− mice, may suggest additional functions of Sbh1 during embryogenesis that are outside of DNA repair.

Skeletal patterning is a complex process, and involves the spatial and temporal co-ordination of a number of developmental signalling pathways, including Hedgehog (in particular Indian Hedgehog [Ihh] and Sonic Hedgehog [Shh]), Bone Morphogenetic Protein (BMP) and the Transforming Growth Factor Beta (TGF-β) family, Fibroblast Growth Factor (FGF) and Wnt signalling [32,33]. Not surprisingly, a plethora of proteins have been implicated in skeletogenesis. During vertebrate skeletal development, mesenchymal condensations (known as somites) differentiate into the sclerotome and dermomyotome [34,35]. Whilst the sclerotome differentiates into chondrocytes, which form the ribcage and axial skeleton, the dermomyotome further differentiates into the skin (dermatome) and muscle (myotome). Correct outgrowth and differentiation has been shown to be dependent on signalling from each of these compartments [33,34,35].

Interestingly, the rib-cage phenotype we observe in Sbh1−/− skeletons bears striking similarity to that of targeted disruption of the myotome regulator Myf5 [36,37]. In Myf5-deficient mice, a similar lack of ossification in the ribcage and “floating-rib” phenotype is observed, with a partial or complete lack of ossification of the dorsal region of the ribcage, combined with micrognathia [36,37]. Myf5−/− mice also die perinatally, but do not show the same degree of hindlimb defects that we observe in Sbh1−/− mice. Intriguingly, Myf5 is one of the genes hypothesized to have a causal role in cerebro-costo-mandibular syndrome, a rare multiple congenital anomaly syndrome characterized by absent ossification of the posterior ribcage and micrognathia [38,39]. Strikingly, cerebro-costo-mandibular syndrome patients also usually exhibit lung hypoplasia, due to improper development of the lung inside a poorly formed ribcage, and have a poor prognosis for survival [40]. In addition, this disorder has also been associated with hearing defects, variable palate clefting, and sometimes mental retardation [38,39,40]. Although limb-patterning defects have not been described for this disorder, given the striking similarity in other phenotypes, Sbh1 may prove an interesting new candidate gene for this disorder.

Bone development can occur through two major processes, endochondral ossification, where a cartilage precursor template is laid down prior to bone formation, or intramembranous ossification, where mesenchymal cells condense and directly transition to form bone [41,42]. Whilst endochondral ossification is the process responsible for skeletal formation in the majority of the axial and appendicular skeleton, intramembranous ossification is restricted to parts of the skull, including the cranial vault, and maxillo-mandibular bones [41,42]. The skeletal outgrowth and patterning defects observed in Sbh1−/− mice suggest that Sbh1 is important for endochondral ossification. During the preparation of this manuscript, another report of the critical role of Sbh1 in skeletogenesis was published, where the authors had used a similar genetic targeting approach to delete Sbh1 in the mouse [43]. Interestingly, the authors described an almost identical skeletal phenotype to this report, with a similar lack of ossification of the rib-cage, micrognathia, tibial ring malformation and variably-penetrant oligodactyly. In addition, they also reported clefting of the palate, which we observed in two cases but not in others. However, although both mouse models were generated in C57BL/6 mice, craniofacial phenotypes can be heavily affected by sometimes-subtle strain differences [44]. Intriguingly, the role of Sbh1 in skeletogenesis was attributed to p53-dependent apoptosis at E12.5 throughout the somites and limb, and a partial rescue of these phenotypes was observed upon crossing to a p53−/− background. In the case of combined Sbh1 and p53 ablation, however, although the hindlimb digit patterning and ribcage structure was substantially rescued, a distinctive lack of ossification was still evident, particularly in the dorsal extremities of the ribs abutting the vertebrae [45]. This suggests that the Sbh1−/− phenotype cannot be solely attributed to apoptosis, and that some steps in the later stages of endochondral ossification are dependent on Sbh1. Interestingly, the authors did not observe differences in canonical chondrogenic and osteogenic markers by microarray analysis on E18.5 sternum chondrocytes and calvarial osteoblasts [43]. However, the late time point of analysis and tissue origin of these cell lines may have affected the outcome of this study. Indeed, calvarial osteoblasts form through intramembranous, not endochondral ossification [41], and sternum development was not as severely affected as the rest of the rib-cage in Sbh1−/− embryos. It will therefore be of great interest to more rigorously investigate the role of Sbh1 in bone development, and to determine the precise mechanisms that lead to bone-specific apoptosis observed in these mice.

While the development defects in germline Sbh1 knockout mice were surprising, effects of inducible ablation of Sbh1 in adult mice revealed phenotypes more relevant to the proposed role of Sbh1 in maintaining genomic stability, as we observed defects in spermatogenesis, increased radiation sensitivity, increased genomic instability as well as an increased tumour incidence in induced Sbh1−/− mice. Spermatogenesis in the mouse commences postnatally at day 7 and by day 35 post-natal mature sperm can be found within the seminiferous tubules. One round of spermatogenesis takes approximately 28 days and it is a continuous process within the testes. The major phases of spermatogenesis are mitosis, meiosis, and post-meiotic germ cell maturation, which last 11, 10 and 14 days, respectively [45]. We commenced induction of Sbh1 deletion at the age of 28 days, which is at the late stage of meiosis during the first wave of spermatogenesis. We observed a variable degree of testicular degeneration and defective spermatogenesis, which led to smaller...
Figure 6. Conditional Ssb1-deleted mice are sensitive to IR. (A) Schematic diagram of the radiation challenge assay in Ssb1 deleted mice. Rosa26-CreER<sup>2</sup>: Ssb1<sup>+/+</sup>, Rosa26-CreER<sup>2</sup>: Ssb1<sup>+/−</sup> and Rosa26-CreER<sup>2</sup>: Ssb1<sup>−/−</sup> mice were challenged with 8 Gy of total body irradiation (TBI). The acute lethal response of mice to TBI was evaluated over a 30-day observation period. (B) Kaplan-Meier survival analysis of irradiated mice. Kaplan-Meier survival curves compared by log-rank (Mantel-Cox) analysis showed significant difference between Rosa26-CreER<sup>2</sup>: Ssb1<sup>−/−</sup> mice and the other two groups (**P<0.01), while no difference was found between Rosa26-CreER<sup>2</sup>: Ssb1<sup>+/−</sup> and Rosa26-CreER<sup>2</sup>: Ssb1<sup>−/−</sup> groups. (C) Representative images of Haematoxylin and eosin, Ki67 (cell proliferation) and ApopTag (cell death) staining on small intestine sections from mice at Day 5 post 8 Gy of TBI.

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Figure 7. Increased genomic instability in irradiated bone marrow metaphases from total body irradiated mice. Five weeks after tamoxifen induction, nine-week-old Rosa26-CreERT2: Ssb1\(^{+/+}\), Rosa26-CreERT2: Ssb1\(^{+/−}\) and Rosa26-CreERT2: Ssb1\(^{−/−}\) mice were given 2 or 6 Gy of TBI and kept for 24 h before bone marrow collection. Metaphases were prepared directly from bone marrow cells of demecolcine-treated mice for fluorescence in situ hybridization (FISH) analysis. (A) Quantification of chromosomal breakage (fragmentation and fusion) in bone marrow metaphase spreads from Rosa26-CreERT2: Ssb1\(^{+/+}\), Rosa26-CreERT2: Ssb1\(^{+/−}\) and Rosa26-CreERT2: Ssb1\(^{−/−}\) mice at 24 h after 2 and 6 Gy of TBI (\(n = 3\) mice per genotype for each condition). (B) Representative images of bone marrow metaphases from mice with indicated genotypes. Red arrows mark some of the chromosomal breakages. Note the presence of DNA debris marked with circles.

doi:10.1371/journal.pgen.1003298.g007
A

![Graph showing tumor-free survival](image)

B

Tumor incidences in a long survival cohort of conditional Rosa26-CreER$^{T2}$ Ssb1 deleted mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mice in the survival cohorts</th>
<th>No. of mice with tumours</th>
<th>Total tumour events</th>
<th>No. of mice with multiple tumours</th>
<th>No. of mice with lymphoma in multiple organs</th>
<th>No. of tumours in indicated subtypes</th>
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<td>1 0 1</td>
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<td>11</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>10 3 2</td>
</tr>
</tbody>
</table>

C

- Thymus (T-cell lymphoma)
- Spleen (B-cell lymphoma)
- Lung (Metastatic B-cell lymphoma)

D

Liver (Hepatocellular carcinoma)
Figure 8. Broad tumour spectrum in conditional Ssb1-deleted Rosa26-CreERT2: Ssb1−/− mice. (A) Long time survival cohort of Rosa26-CreERT2: Ssb1+/− (n = 35) and Rosa26-CreERT2: Ssb1−/− (n = 35) mice were monitored for 86 weeks after Ssb1 deletion for tumour development. (A) Kaplan-Meier survival analysis showed tumour free survival rate of indicated genotypes over 90 weeks (**P < 0.01), (B) Tumour incidence and a spectrum in Rosa26-CreERT2: Ssb1−/− mice compared with Rosa26-CreERT2: Ssb1+/− mice. (C) Representative sections have T lymphoma in thymus (top panel) and B lymphomas in spleen (middle panel), which spread to lung in a Rosa26-CreERT2: Ssb1−/− mice (bottom panel). Representative images of Haematoxylin and eosin stained tissues of tumours in indicated organs are shown. Immunohistochemical staining of Cd3 (top panel, right) confirmed T-cell lymphoma in thymus (Scale bars, i = 40 μm, ii = 120 μm, iii = 20 μm, iv = 40 μm, v = 60 μm, vi = 80 μm). (D) Representative image of liver cancer (hepatocellular carcinoma, HCC) in a Rosa26-CreERT2: Ssb1−/− mouse. Representative images of tumour mass (upper panel) and Haematoxylin and eosin-stained liver section (middle and lower panels) are shown (Scale bars, ii = 100 μm, iii = 140 μm).

doi:10.1371/journal.pgen.1003298.g008

Materials and Methods

Generation of targeting construct

To target the mouse Ssb1 allele, a targeting construct was engineered with unidirectional lox-P sites flanking exons 3-6 of mouse Ssb1, which encompasses the DNA-binding OB-fold domain of the protein. A neomycin resistant cassette (PGK-NEO) necessary for gene targeting in mouse ES cells, was flanked by FRT recombination sites and situated within the lox-P flanked region (Figure S1A). Genomic targeting of the construct was performed in C57BL/6J ES cells using standard homologous recombination and blastocyst manipulation techniques. Gene targeting was confirmed by Southern blot using 5′ and 3′ probes situated outside the targeting vector, in addition to an internal neo probe following restriction digest of genomic DNA using HindIII, SacI or SacII restriction enzymes. Generation of Ssb1 floxed/neo (flneo) mice was a contracted service performed by Ozgene Pty Ltd (Perth, Australia).

Generation of constitutive Ssb1 knockout mice

Ssb1 floxed (fl) mice were generated by first crossing Ssb1 targeted mice against FLPe recombinase transgenic mice to remove the neomycin cassette, and subsequently backcrossed onto a C57BL/6J strain to remove the FLP transgene. To generate constitutive germline deletion of Ssb1, Ssb1fl/+ mice were crossed against CMV-Cre (TgN(CMV-cre)1Cgn) transgenic mice that have been described previously [51]. Offspring containing the Ssb1 null (−) allele were backcrossed to the C57BL/6J strain to segregate the Ssb1−/− null mice and Cre transgene. Ssb1−/− heterozygous mice were crossed intercrossed to generate Ssb1−/− animals. Ssb1+/+ and Ssb1−/− embryos were indistinguishable at the phenotypic level and were used interchangeably for some experiments as explicitly stated in the text.

Generation of conditional Ssb1 knockout mice

To generate conditional and ubiquitous Ssb1−/− mice, Ssb1fl/+, Ssb1fl/fl mice were crossed against Rosa26-CreERT2 transgenic mice (Figure S7) [18,52]. Double transgenic progeny carrying both the floxed and Cre transgenes (Rosa26-CreERT2: Ssb1fl/fl) were subsequently crossed to the Ssb1fl/+, Ssb1fl/+ mouse line to generate experimental animals (Rosa26-CreERT2: Ssb1fl+/+ and Rosa26-CreERT2: Ssb1fl/fl). Induction of Ssb1 knockout was performed by intraperitoneal (IP) injection of 1 mg tamoxifen/mouse for 5 consecutive days into 4 week-old experimental animals. Cre-mediated excision was verified in a number of tissues by both genotyping PCR and western-blot (Figure S8).

To determine if Ssb1 plays a role in class switch recombination (CSR), we crossed Ssb1fl/+, Ssb1fl/fl mice with Cd19-Cre transgenic mice to conditionally delete Ssb1 in B cells [53].

Animal husbandry and ethics

All experimental animals were maintained on a C57BL/6J strain, and were housed at 25°C with a 12 h light/12 h dark cycle.
All experiments were performed in accordance with the Queensland Institute of Medical Research animal ethics guidelines.

**Genotype analysis**

Genotyping was performed using genomic DNA extracted from tails. The sequences of PCR primers for genotyping Rosa26-CreER<sup>T</sup> mice are: 5'-TGTTGAGAGGAGGAGCTAAAG-3' (forward primer) and 5'-CATCAGTGTGCTGCAGAC-3' (reverse primer). As expected, PCR amplification of the 536-bp Rosa26-CreER<sup>T</sup>-specific product reliably identified transgenic mice. Assessment of the Ssb1 gene before and after Cre recombination was performed by PCR designed to detect if the floxed-sequence had been deleted via Cre/loxP recombination. Two different reverse PCR primers were used, together with a common forward primer, result in 482, 360 and 118-bp PCR products, specific for Ssb1+/+ wild-type, null-type, and all alleles, respectively (Figure 2A). The sequences of the common forward primer for Ssb1 wild type, floxed and null allele is: 5'-GCCTTGGCTTCTGGCTTTACCT-3'. The reverse primer for Ssb1+/- type and floxed alleles is 5'-ACAACCTTCTGAAA CACTGAGGC-3' and for the Ssb1 null allele is 5'-GAAATG-GATTCCGAGCTCAA-3'.

**Skeletal preparations**

Alician Blue and Alizarin Red whole-mount skeletal preparations were performed as described previously [54] on E18.5 embryos. Skeletal Preparations were imaged on a Nikon SMZ45 dissecting microscope equipped with a Nikon 5MP colour camera.

**Western blot**

For protein extraction, tissue samples were homogenized in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM PMSF, 1× Roche complete mini protease inhibitor cocktail, 1× Pierce phosphatase inhibitor cocktail). Western blotting was performed as described previously [3] with the following antibodies: sheep anti-SSB1 (Sigma, 1:10,000), mouse anti-phosphorylated-ATM serine1981 (Cell Signaling, 1:1000) and rabbit anti phosphorylated-p53 serine 15 (Sigma, 1:10,000). Detection of the signals with the chemiluminescence reagent (Super Signal West Pico, Pierce) was carried out using the LAS-4000 imaging system (Fujifilm Life Science).

**Alveolar lumen and septa quantitation**

Images of Haematoxylin and eosin stained lung sections from Ssb1 control (Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup>, n = 3) and Ssb1<sup>−/−</sup> (n = 4) were analysed using Image J software (rsbweb.nih.gov/ij/) on four representative images for each lung, with care taken not to include areas with conducting airway. Briefly, images were converted to greyscale and thresholded equally across images from control and Ssb1<sup>−/−</sup> lungs to highlight alveolar septa. The average area occupied by septa and airspace was calculated and subjected to statistical analysis.

**Quantitative real-time PCR on lungs**

The right lobes of Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup> and Ssb1<sup>−/−</sup> lungs were homogenized and RNA extracted using the RNeasy mini kit (Qiagen), followed by DNase I (New England Biolabs) digestion to remove genomic DNA contamination. 2 µg of RNA was used for first-strand cDNA synthesis using random primers (Life Technologies) and Superscript III reverse transcriptase (Life Technologies). qRT-PCR was performed using Light Cycler 480 Sybr green I mastermix (Roche Applied Science) on a Light Cycler 480 Real-time PCR system (Roche Applied Science). Primer sequences for Cx10, FoxF1, Cd31, Pdpn, Sftpa, Sftpb, Sftpa and Sftpb had been described previously [55,56]. Ssb1<sup>−/−</sup> and β-Actin primer sequences were as follows: Ssb1<sup>−/−</sup>, 5'-CTACGCCCTTCATCACTGAC-3' (forward) and 5'-CCAGAGGATAGCAGCTTACGTGA-3' (reverse); β-Actin, 5'-GGCTGTTATCCTCCTCATCG-3' (forward) and 5'-CCAGATGTTAGACCATGCGTA-3' (reverse). Negative controls with no template and no reverse transcriptase were also included and used in qRT-PCR reactions to ensure no contaminating genomic DNA was present.

**Mouse embryonic fibroblast isolation and 3T3 growth assays**

Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos from Ssb1<sup>+/−</sup> intercrosses as described previously [57]. At least three independent embryos per condition were used for analysis. For 3T3 fibroblast growth assays, Ssb1<sup>+/+</sup> and Ssb1<sup>−/−</sup> cell lines were seeded at passage 2 at a concentration of 0.5×10<sup>4</sup> cells/10 cm dish. Cells were trypsinised, counted and re-seeded every 3 days at the same concentration to monitor relative changes in growth at each passage.

**Immunofluorescence and microscopy**

Cells were plated on glass coverslips and used at approximately 70 percent confluency. Immunofluorescence with the γ-H2AX antibody (Millipore) was performed as described previously [7]. For γ-H2Ax foci quantitation, 30 cells for each MEF cell line (n = 2 Ssb1<sup>+/+</sup>, 3 Ssb1<sup>−/−</sup>) were scored for those containing >10 foci/cell at the indicated timepoints following 2 Gy of gamma-irradiation, across two independent experiments.

**Gonado-somatic index analysis**

The testes from Rosa26-CreER<sup>T</sup>: Ssb1 mice were dissected out and weighed with an analytical balance (Mettler AT261). The gonado-somatic index was determined according to the formula: Gonado-Somatic Index (GSI) = (Gonad weight/total body weight)×100. The testes from Rosa26-CreER<sup>T</sup>: Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup> and Ssb1<sup>−/−</sup> mice were scored for those containing >10 foci/cell at the indicated timepoints following 2 Gy of gamma-irradiation, across two independent experiments.

**Class switch recombination (CSR) analysis**

Splenocytes were stimulated for IgH CSR to IgG1 using anti-CD40 antibodies plus IL-4 and analyzed by flow cytometry as described previously [38].

**Total body irradiation**

Total body irradiation (TBI) was performed using a 137Cs source at 100 cGy/min. Mice were placed in plexiglass cages and irradiated in groups of five simultaneously with the indicated doses.

**Florescence in situ hybridization (FISH) analysis of chromosomal aberrations**

Metaphases were prepared directly from bone marrow cells in demecolcine-treated mice for FISH analysis. Five weeks after tamoxifen induction, nine-week-old Rosa26-CreERT2: Ssb1<sup>+/−</sup>, Rosa26-CreER<sup>T</sup>: Ssb1<sup>−/−</sup> and Rosa26-CreER<sup>T</sup>: Ssb1<sup>−/−</sup> mice were given 2 or 6 Gy of TBI and kept for 24 h before bone marrow collection. Demecolcine (Sigma, 250 µl of a 200 µg/ml solution) was administered by intraperitoneal injection into each mouse 1 h prior to bone marrow collection. Bone marrow was flushed from each femur and tibia with pre-warmed potassium chloride solution (0.06 M). Florescence in situ hybridization

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**Novel Embryonic and Tumour Suppressor Roles of Ssb1**

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(FISH) analysis was performed on metaphases using a biotinylated centromere-specific minor satellite probe. Three mice were analyzed for each genotype per condition and thirty metaphases were analyzed per case for chromosome breaks. Within each spread, the number of chromosomal fragments and fusions (identified by the presence of more than one centromere signals) was determined.

**Lymphocyte surface markers staining**

Lymphocyte surface markers were measured in peripheral blood samples by flow-cytometric analysis. Following lysis with 0.145 M ammonium chloride to remove red blood cells, the remaining lymphocytes were washed and incubated with APC conjugated anti-CD3, PerCP-conjugated anti-CD8, FITC-conjugated anti-CD4, and PE-conjugated anti-CD19 (BD Pharmingen), at 4°C for 30 minutes. Cells were washed, resuspended in PBS, and acquired on a FACs Canto II. Data were analyzed with FlowJo software (Ashland, OR, USA).

**Histopathological analysis and immunohistochemistry**

Tissues were collected and fixed in 10% buffered formalin fixative or 4% Paraformaldehyde, embedded in paraffin blocks, and 5-μm-thick sections were stained with Haematoxylin and eosin for histological examination. Slides were coded and examined in a blinded fashion by an independent veterinary pathologist. Immunohistochemistry staining was performed following standard procedures. Apoptosis was assessed using the ApopTag peroxidase in situ apoptosis detection kit (Chemicon International), according to the manufacturer’s instructions. Stained slides were scanned on Aperio ScanScope XT Slide Scanner and the images were analyzed with Image Scope software.

**Statistical analysis**

Data were analyzed with GraphPad Prism software. The student’s t-test was used for the statistical analysis of embryo weight and length, long bone comparison, qPCR, lung airspace analysis, testis weight, GSI, litter interval, litter size, chromosome breaks and blood cell counting data. Survival curves were plotted using Kaplan-Meier estimates and compared by log-rank (Mantel-Cox) analysis. P values less than 0.05 were considered statistically significant.

**Supporting Information**

**Figure S1** Generation of Ssh1 gene-targeted mice. (A) Schematic diagram showing the Ssh1 gene structure and targeting strategy including the Ssh1 wild-type (wt), Ssh1 targeted (floen), Ssh1 floxed (fl) and Ssh1 null (null) alleles. (B) Southern blot confirming correct genomic targeting of Ssh1 flox mice following ScaI restriction digest. Samples were probed with both an endogenous probe (enP; top) and neomycin probe (neoP; bottom). Neo cont. designates an unrelated neomycin transgenic mouse used as a positive control. (C) PCR genotyping showing Ssh1 wild type (primer 1; P1 and primer 2; P2 in Ssh1+/*), Ssh1 flox (P1 and P3 in Ssh1 floox/fl), Ssh1 floxed (P1 and P2 in Ssh1 flox/fl) and Ssh1 null (P1 and P4 in Ssh1 flox/flox) alleles. (TIF)

**Figure S2** Perinatal lethality, growth retardation, micrognathia and cleft palate in Ssh1−/− embryos. (A) Ssh1−/− P0 embryos exhibit severe respiratory distress and die within 30 minutes of birth. Note the purple colour of Ssh1−/− embryos indicating cyanosis. (B) Comparison of crown-rump length of E14.5 and E18.5 Ssh1+/+, Ssh1+/− and Ssh1−/− embryos (n = minimum 3 embryos for E14.5; minimum 6 embryos for E18.5 per genotype) (**P<0.005, ***P<0.001, **P<0.01, student’s t-test). (C) Representative sagittal sections through the heads of Ssh1+/+ (i–iii) and Ssh1−/− (iv–vi) P0 embryos show a missshapen snout and recessed mandible in Ssh1−/− embryos. (D) Ventral skull view of E18.5 skeletal preparations with removed mandible showing clefting of the secondary palate in an Ssh1−/− embryo. Scale bar = 1 mm. (E) Magnified view of (D). Note the properly fused palatine processes (arrowhead) in Ssh1+/+ control embryos (left) and lack of palatine process formation in the Ssh1−/− embryo (right), exposing the underlying prepharyngeal bone (arrowhead). Scale bar = 1 mm. (F) Ventral view of P0 Ssh1+/+ and Ssh1−/− heads with removed mandible showing variably penetrant cleft palate between Ssh1−/− littersmates. Scale bar = 1 mm. (TIF)

**Figure S3** Morphology of Ssh1 control and Ssh1−/− embryos. Haematoxylin and eosin staining of sagittal sections of E18.5 lungs to mark proliferating cells. (B) ApopTag TUNEL immunohistochemical staining to mark apoptotic cells in Ssh1 control and Ssh1−/− E18.5 lungs. Scale bar = 50 μm. (TIF)

**Figure S4** Apoptosis and proliferation in E14.5 and E18.5 Ssh1−/− lungs. (A) Immunohistological staining of Ki67 in E14.5 (top) and E18.5 (bottom) control (Ssh1+/+, Ssh1−/−) and Ssh1−/− lungs to mark proliferating cells. (B) ApopTag TUNEL immunohistological staining to mark apoptotic cells in Ssh1 control and Ssh1−/− E14.5 (top) and E18.5 (bottom) lungs. Scale bar = 200 μm. (TIF)

**Figure S5** Proximal Lung Differentiation in E18.5 Ssh1−/− lungs. (A) Quantitation of qRT-PCR for proximal differentiation markers Gz10 (clara cells), Foxj1 (ciliated epithelial cells) and Cd31 (endothelial cells). (B) Immunohistological staining for smooth muscle actin (SMA) in Ssh1 control (Ssh1+/+, Ssh1−/−) and Ssh1−/− E18.5 lungs. Scale bar = 200 μm. (TIF)

**Figure S6** Ssh1 is not required for the response to DNA double-strand breaks in mouse embryonic fibroblasts. (A) Cell cycle profiles of Ssh1+/+ and Ssh1−/− passage 3 MEFs by propidium iodide staining. (B) T3 proliferation assay showing growth curves for Ssh1+/+ and Ssh1−/− MEFs (n = 3). Data represent mean ± SEM. (C) Western blot showing Atm signalling activation in Ssh1−/− and Ssh1−/− MEFs following 6 Gy of ionizing radiation with indicated antibodies. (D) Immunofluorescence imaging and (E) quantitation for γ-H2ax foci after 2 Gy of ionizing radiation at the indicated timepoints. Data represent mean ± SEM. (TIF)

**Figure S7** Schematic diagram of the conditional Ssh1 gene targeting strategy. Ssh1floox mice were bred with Rosa26-CreERT2 transgenic mice to enable conditional Ssh1 deletion. Ssh1 gene deletion was induced by intraperitoneal injection (I.P.) of 1 mg mouse tamoxifen daily for 5 consecutive days into 4-week-old Rosa26-CreERT2; Ssh1floox mice. (TIF)

**Figure S8** Conditional Cre recombination mediated Ssh1 deletion and Ssh2 upregulation in Rosa26-CreERT2; Ssh1−/− mice. (A) PCR genotyping after tamoxifen induced Cre recombination mediated Ssh1 gene deletion. PCR analysis of recombination of the floxed Ssh1 allele in heterozygous Ssh1-flox::Rosa26-CreER T2 (Rosa26-CreER T2; Ssh1floox/+; Rosa26-CreER T2; Ssh1floox/+ mice and homozygous Ssh1-flox::Rosa26-CreER T2; Rosa26-CreER T2; Ssh1floox/− mice was performed ten days after the final tamoxifen injection. The efficacy of gene interruption in indicated tissues is shown. The PCR products
of floxed (β), wild type (wt) and deletion (null) alleles of Ssb1 were detected as 402–360 and 118–hp bands, respectively. (B) Western blot analysis of Ssb1 protein in tissue extracts from mice following Cre recombination. Ssb1 protein levels were analyzed in the indicated tissues ten days after the final tamoxifen injection by immunoblotting with an antibody specific for Ssb1 and β-actin as a loading control. (C) Western blot analysis of Ssb1 and Ssb2 protein in indicated tissues prepared from Rosa26-CreER T2: Ssb1+/− and Rosa26-CreER T2: Ssb1−/− mice ten days after Cre recombination. Ssb1+/− and Ssb1−/− mice were subjected to 6 Gy of total body irradiation (TBI). Indicated tissues were extracted 6 h post irradiation, and Ssb1 and Ssb2 protein levels were analyzed by immunoblotting. Immunoblotting of β-Actin was used as a loading control.

Figure S9 Comparison of body weights of Rosa26-CreER T2: Ssb1+/− and Rosa26-CreER T2: Ssb1−/− mice. (A) Gender distribution comparison of Rosa26-CreER T2: Ssb1+/− and Rosa26-CreER T2: Ssb1−/− mouse cohorts. (B) Comparison of age of tamoxifen induction between cohorts. (C) Comparison of body weights of Rosa26-CreER T2: Ssb1+/− and Rosa26-CreER T2: Ssb1−/− mice after tamoxifen injection (n = 33).

Figure S10 Histological analysis and complete blood count of mice at 24 h post total body irradiation (TBI). (A) Representative images of Haematoxylin and eosin, Ki67 (cell proliferation) and ApopTag (cell death) staining on small intestine sections from mice at 24 h post 8 Gy of TBI. (B) Complete blood count (CBC) analysis on peripheral blood from mice at 24 h post 8 Gy of TBI. Whole blood samples were processed for counts using Beckman Coulter ACT whole blood counter. Numbers of white blood cell (WBC), red blood cell (RBC), hemoglobin (Hgb), and platelets (Plt) were assessed. Scale = 100 μm.

Figure S11 Assessment of radiosensitivity of thymocytes from Rosa26-CreER T2: Ssb1+/− and Rosa26-CreER T2: Ssb1−/− mice. Thymocytes were isolated from mice with indicated genotype and exposed to 1, 3 and 6 Gy of irradiation. (A) Percentage of cell death (Annexin V+7-AAD+) of Ssb1+/− and Ssb1−/− thymocytes at indicated doses of irradiation (n = 3, **P < 0.01, ***P < 0.001; student’s t-test). (B) Percentage of apoptotic cells (Annexin V+7-AAD−) at indicated conditions (n = 3, **P < 0.01, ***P < 0.001; student’s t-test).

Figure S12 B-cell leukemia identified in a Rosa26-CreER T2: Ssb1−/− mouse. (A) Representative flow cytometric analysis on lymphoblasts from peripheral blood (PB). Lymphoblasts were stained as CD19 (B cell) positive lymphomas. (B) Wright’s stain on PB smears showing lymphoblast cluster from a Rosa26-CreER T2: Ssb1−/− mouse (ii) compared with a healthy control littermate (i).

Leukocytes featured as large-sized undifferentiated haematopoietic cells with a small basophilic cytoplasm and visible nucleoli (Scale = 20 μm). (C) Lymphocytic leukemia involving the liver. Representative images of Haematoxylin and eosin stained sections (upper panel) and immunohistochemical staining of B220 or Cd3 (lower panel) showing periportal infiltration by B-lymphocytes (Scale = 50 μm). (D) Effacement of the lymph node architecture. Low-power (upper panel) and enlarged views (lower panel) show a periportal lymphocytic infiltrate in the lymph node (Scale = 50 μm). (TIF)

Figure S13 Representative images of p53 immunohistochemistry staining on tumour sections from Rosa26-CreER T2: Ssb1−/− mice. Tumours developed in indicated organs from Rosa26-CreER T2: Ssb1−/− mice. Two tumours observed in Rosa26-CreER T2: Ssb1−/− mice were stained with p53 antibody (bottom panel) and compared with adjacent normal tissue from the same mice (top panel), Scale = 100 μm.

Figure S14 Representative images of Ssb1 and p53 immunohistochemistry staining on tumour sections from Rosa26-CreER T2: Ssb1−/− mice. Two tumours observed in Rosa26-CreER T2: Ssb1−/− mice were stained with Ssb1 and p53 antibodies. Left panel is the control staining of Ssb1 on the respective organs from littermate control of Rosa26-CreER T2: Ssb1−/− mice. Middle panel is Ssb1 staining from the Rosa26-CreER T2: Ssb1−/− mice which developed tumours. Right panel is p53 staining of the tumour sections. Scale = 100 μm.

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Conceived and designed the experiments: WS ALB BS FA-E CS LW HC UH MK YM MJ WJ CW KJS FWA KKK. Performed the experiments: WS ALB BS FA-E CS LW HC UH MK YM MJ WJ CW KJS FWA KKK. Contributed reagents/materials/analysis tools: ALB BS FA-E JS LW HC MSM MK MW. Analyzed the data: WS. Wrote the paper: WS ALB BS FWA KKK.

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