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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 SSOS-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 archaeal 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<sup>+/−</sup>) relative to wild-type littermates (Ssb1<sup>+/+</sup>) revealed no apparent physiological abnormalities in Ssb1<sup>+/−</sup> mice monitored for up to 2 years. To generate mice with targeted deletion of Ssb1, we intercrossed Ssb1<sup>−/−</sup> breeding pairs, with the expectation that approximately 25 percent of the offspring would be of an Ssb1<sup>−/−</sup> genotype. Interestingly, no viable Ssb1<sup>−/−</sup> mice were detected amongst more than one hundred offspring from these intercrosses genotyped at 12 days post-partum (Table I). 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<sup>−/−</sup> intercrosses at different gestational days, assessed by the presence of a vaginal plug at E0.5. Ssb1<sup>−/−</sup> embryos were recovered at near-Mendelian ratios at E13.5 and E18.5 (Table I), 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<sup>+/+</sup> and Ssb1<sup>−/−</sup> embryos were morphologically indistinguishable, in terms of both body size and body length. Ssb1<sup>−/−</sup> 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<sup>−/−</sup> lethality, we performed caesarian 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<sup>+/+</sup> and Ssb1<sup>−/−</sup> pups established rhythmic breathing, a healthy pink skin color and movement within minutes. However, Ssb1<sup>−/−</sup> pups rapidly became asphyxic and typically died between 10–30 min post caesarian excision, despite taking short, sporadic gasping breaths, suggesting that they could not breathe and oxygenate their blood properly (Figure 1A, Figure S2A). Haematocrit 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<sup>−/−</sup> 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<sup>−/−</sup> embryos, we next sought to determine if their skeletal architecture was altered by performing whole-mount cartilage and mineralized bone staining with alcan blue and alizarin red. Strikingly, we observed a number of defects in formation of both the axial and appendicular skeleton. Most notably, the ribcage of Ssb1<sup>−/−</sup> embryos was poorly formed, small in size, and exhibited an almost complete lack of ossification when compared to control littermates (Ssb1<sup>+/+</sup>, Ssb1<sup>+/−</sup>) (Figure 2A). This defect led to the appearance of “floating ribs”, with no evidence of ossification in all but the four most anterior rib pairs. In addition, the ribcage of Ssb1<sup>−/−</sup> embryos was misshapen, with a lack of curvature in the anterior ribs, and horizontally orientated rib-sternum attachments (Figure 2A, Figure 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 misshapen and poorly developed rib-cage in Ssb1<sup>−/−</sup> 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 (pmx), 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<sup>+/+</sup>, Ssb1<sup>+/−</sup>) (Figure 2C, 2D). The tympanic ring (tr), which supports the cardrum, was also poorly formed in Ssb1<sup>−/−</sup> embryos (Figure 2C). Furthermore, we observed evidence of a variably penetrant cleft palate (n=2 of 5 embryos), which was evident even between Ssb1<sup>−/−</sup> mice of the same litter (Figure S2D–S2F; arrows, arrowheads). Together, these data suggest a spectrum of craniofacial abnormalities in Ssb1<sup>−/−</sup> embryos.
The limb skeleton of Ssb1−/− 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 (**P < 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−/− 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−/− embryos exhibit distal lung differentiation defects

To determine if other causative factors may have contributed to the perinatal lethality in Ssb1−/− 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+/+, Ssb1+/− and Ssb1−/− 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−/− 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−/− embryos (Figure 2F) are smaller and have fewer lobes (Figure 3A). This suggests that Ssb1 is required for proper lung development.

### Table 1. Impact of deletion of Ssb1 on embryonic survival in Ssb1+/− intercrosses.

<table>
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<tr>
<td>E13.5 embryos</td>
<td>Expected: 12</td>
<td>24</td>
<td>12</td>
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<td>(48 genotyped)</td>
<td>Observed: 8</td>
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<td>E18.5 embryos</td>
<td>Expected: 9</td>
<td>18</td>
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<td>(36 genotyped)</td>
<td>Observed: 6</td>
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<td>Live Pups (P12)</td>
<td>Expected: 32</td>
<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+/+, Ssb1+/−, and Ssb1−/− offspring. Note the cyanosis in P0 Ssb1−/− pups. Scale = 2 mm. (B) Comparison of weights of E14.5 and E18.5 Ssb1+/+, Ssb1+/− and Ssb1−/− embryos. Data represent the mean ± SEM, n = 3–16 embryos per group from a minimum of 3 litters per timepoint (**P < 0.001; student’s t-test). (C) Haematoxylin and eosin staining of P0 lungs of Ssb1+/+, Ssb1+/− and Ssb1−/− pups delivered by caesarian section. doi:10.1371/journal.pgen.1003298.g001
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,
premaxilla; ppmx, palatal process maxilla; pp, palatal process palatine; p, palatine; tr, tympanic ring. (D) Comparison of Ssb1 control and Ssb1−/− mandibles (Scale=1 mm). (E) Forelimb and (F) Hindlimb of E18.5 Ssb1 control and Ssb1−/− embryos. Sc, scapula; H, humerus; R, radius; U, ulna; F, femur; T, tibia. * designates missing fibula. (G) Quantification of long bone measurements of the forelimb and hindlimb of Ssb1−/− and Ssb1+/+ E18.5 limbs. Data represent the mean ± SEM of bone length (n=3 per condition, **P<0.001, student’s t-test). (H) Whole-autopod (top) and skeletal preparation (bottom) of E18.5 forelimbs (left) and hindlimbs (right) from Ssb1 control and Ssb1−/− embryos. doi:10.1371/journal.pgen.1003298.g002

were consistently smaller than their Ssb1+/+ and Ssb1−/+ counterparts when measured in terms of lobe length and width (Figure 3A, data not shown), although this was in proportion to the overall growth retardation in these embryos. In addition, lungs of Ssb1−/− embryos were correctly lobulated, with four right lobes and a single left lobe flanking the heart, suggesting that early lung development patterning 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 3C–3E, **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 immunohistochemical 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 properly differentiated, we examined levels of Gcl10 (also known as Sgb1a1/Gapj), a marker for secretory Clara cells, as well as Foxj1 (also known as Hfh-4), a marker of ciliated epithelial cells in the proximal epithelium by quantitative real-time PCR (qPCR) in Ssb1 control (Ssb1+/+, Ssb1−/+ n=4) and Ssb1−/− (n=4) lung tissue at E18.5. In addition, we also examined transcript levels of Gd31, a marker of endothelial cells. These analyses revealed no significant differences in the mRNA levels of these markers, suggesting that both proximal airway differentiation and blood vessel formation of Ssb1−/− lungs are intact (Figure SSA). Furthermore, immunohistochemical staining of smooth muscle actin revealed normal bronchi and bronchioli development in these embryos (Figure SSB).

Next, we examined differentiation of the distal sacculles which contain alveolar epithelial type I and type II cells (AECs), responsible for gas exchange and the maintenance of surface tension through surfactant protein secretion, respectively. To determine if Ssb1−/− embryos exhibited defective differentiation in either of these cell types, we performed qPCR on E18.5 control and Ssb1−/− embryos to assess the transcript levels of Aqp5 and Pdlim, as markers of type I AECs, as well as the surfactant protein transcripts Sftp7a, Sftp7b, Sftp7c and Sftp7d, as markers of type II AECs. Although Sftp7a and Sftp7d were unaffected by Ssb1 ablation, we observed a ~2.4 fold change in Sftp7b expression, suggesting aberrant type II AEC differentiation (**P<0.001, Figure 3F). Notably, deletion of Sftp7b in the mouse has been shown to result in severe neonatal respiratory distress syndrome, and is the only surfactant protein that is indispensable for neonatal survival [12,13,14]. In addition to the decrease in Sftp7b levels, we also observed a smaller (~1.25 fold) change in Pdlim, a type I AEC marker (**P<0.01), as well as small, but statistically non-significant decrease in Aqp5, another type I AEC marker (Figure 3F). As type II cells are thought to trans-differentiate to type I cells, this may be a secondary effect of improper type II AEC differentiation [15,16]. Interestingly, we also observed a 1.3 fold increase in Sftp7c mRNA (*P=0.01, Figure 3F). As pro-SPC is expressed from E11.5 to E17.5 in lung epithelial progenitor cells, the relative increase in this transcript may simply represent developmental immaturity of Ssb1−/− lungs [16]. This is in accordance with blinded assessment by an independent pathologist, who observed an increase of immature type II AECs in the lungs of Ssb1−/− P0 embryos. These data indicate that Ssb1 is necessary for proper lung differentiation in the late stages of embryogenesis. Taken together, our results point to an important and novel role of Ssb1 in skeletal and lung differentiation.

Ssb1 is not required for the response to DNA double-strand breaks in mouse embryonic fibroblasts or during class switch recombination

Mouse embryonic fibroblasts (MEFs) from Ssb1+/+ and Ssb1−/− E13.5 embryos were isolated to investigate the role of Ssb1 in DSBR 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 p53 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 DSBR repair to interrogate if Ssb1 is necessary for DSBR 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 DSBR 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−/− embryos. (A) Representative image showing the comparison of lung size and morphology of E18.5 Ssb1+/+, Ssb1+/- and Ssb1−/− embryos. Scale bar = 2 mm. (B) Protein level of Ssb1, Ssb2 and Actin in Ssb1+/+, Ssb1+/- and Ssb1−/− 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 Cd19-Cre: Ssb1fl/fl 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 IgG1 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 direct or microhomology-mediated joins in switch region junctions from IL4 plus anti-CD40 stimulated primary Sbb1−/− B cells and wild-type B cells (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 Sbb1−/− mice, we next employed a conditional approach to ubiquitously ablate Ssb1 postnataally using a tamoxifen-inducible Cre system by interbreeding Ssb1fl/fl mice with the Rosa26-CreERT2 strain (Figure S7) [18]. Efficiency of Ssb1 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 Sbb1 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 Sbb1 protein levels were confirmed in multiple tissue samples from tamoxifen induced Rosa26-CreERT2: Sbb1−/− mice, with undetectable levels of Sbb1 protein in splenocytes and thymocytes as early as 10 days after the final tamoxifen induction (Figure S8B). Interestingly, we observed a dramatic up-regulation of Ssb2 in response to Ssb1 ablation in the bone marrow and spleen, but not in the testes and thymus of Rosa26-CreERT2: Sbb1−/− mice (Figure S8C).

**Impaired fertility in conditional Rosa26-CreERT2: Sbb1−/− male mice**

Monitoring of Rosa26-CreERT2: Sbb1−/− mice and control Rosa26-CreERT2: Sbb1+/− 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 abrogation of many DNA repair factors, such as Atm [19], H2ax [20], Mec1 [21] and Mph1/Brht1 [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 Sbb1 deficiency on fertility, we examined ovaries and testes of Rosa26-CreERT2: Sbb1−/− mice six weeks after induction with tamoxifen. Whereas Rosa26-CreERT2: Sbb1−/− ovaries were morphologically normal in females, the testes from Rosa26-CreERT2: Sbb1−/− males were reduced in size (Figure 5A), in terms of both absolute weight \((n = 8, \textit{P}<0.001, \text{Figure } 5B)\) and gonado-somatic index (GSI) [23], an indicator of gonad weight as a proportion of total body mass \((n = 8, \textit{P}<0.001, \text{Figure } 5C)\), when compared to their Rosa26-CreERT2: Sbb1+/− littermates.

**Histological examination of testes from 3-month-old Rosa26-CreERT2: Sbb1−/− 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 ApoTag staining to determine the rate of spermatocyte apoptosis in testes from Rosa26-CreERT2: Sbb1−/− and Rosa26-CreERT2: Sbb1+/− males 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-CreERT2: Sbb1−/− mice.

To further characterize the consequences of Sbb1 ablation on fertility, we interbred induced Rosa26-CreERT2: Sbb1−/− mice with wild-type mice. Consistent with the normal physiological appearance of their ovaries, induced female Rosa26-CreERT2: Sbb1−/− 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-CreERT2: Sbb1−/− 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-CreERT2: Sbb1+/−, Rosa26-CreERT2: Sbb1−/− and Rosa26-CreERT2: Sbb1−/− 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-CreERT2: Sbb1−/− mice by the 10th day. By day 19, 92%
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. (B) Western blotting of whole cell extracts showed loss of Ssb1 protein in stimulated B cells from Ssb1 KO mice. (C) Flow cytometric analysis showed increased CSR to IgG1 on Day 4 in Ssb1 KO mice compared to Ssb1 WT mice. (D) Bar graph showing percentage of IgG1+ cells in AID KO, Ssb1 WT, and Ssb1 KO mice. (E) Table showing microhomology-mediated end joining (1-nt, 2-nt, 3-nt, 4-nt) in Ssb1 KO and Ssb1 WT mice.
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 Hsp90s. (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 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. Sm-5G1 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 junctions 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 S10A 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 S10B).

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 depletion 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 and 6 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 lymphoma 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 Rapaport 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 Cbp-interacting protein (Cd/P RBBP8), Centrosomal Protein 152 (CEP152), microcephalin1 (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\(^{1/-}\) male mice. (A) Representative image of testes from Rosa26-CreERT2; Ssb1\(^{1/-}\) mice compared with those from Rosa26-CreERT2; Ssb1\(^{+/+}\) 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
weight (n=8, ***P<0.001; student’s t-test) and the GSI (C) from 8 mice in each group. (D) Representative images of testis sections stained with Haematoxylin and eosin (left panels) and ApopTag for the detection of apoptotic cells (right panels). Note the multinucleated giant cells (black arrows, lower panel, right, Scale = 25 μm), (E) Haematoxylin and eosin staining showing epididymides of Rosa26-CreER<sup>T2</sup>: Ssb1<sup>+/−</sup> mice containing prematurely sloughed developing germ cells. The upper panel displays maturing spermatogonia in the wild type epididymis. In contrast, immature germ cells (black arrow) are present in the epididymis of Rosa26-CreER<sup>T2</sup>: Ssb1<sup>−/−</sup> mice (lower panel). Embedded images in the left corners show magnified views of the selected areas (Scale = 50 μm). (F) Litter size from Rosa26-CreER<sup>T2</sup>: Ssb1<sup>+/−</sup> male breeders (n=4) with wild type female mice compared with those from Rosa26-CreERT2: Ssb1<sup>−/−</sup> breeders (n=6, *P<0.05; student’s t-test). (G) Litter interval in Rosa26-CreER<sup>T2</sup>: Ssb1<sup>−/−</sup> male mice (average of 63 days, n=4) and Rosa26-CreER<sup>T2</sup>: Ssb1<sup>−/−</sup> males (average of 27 days, n=6, ***P<0.001, student’s t-test).

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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 Ssb1<sup>−/−</sup> mice, may suggest additional functions of Ssb1 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 Morphogenic 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 Ssb1<sup>−/−</sup> skeletal bears striking similarity to that of targeted disruption of the myotome regulator Myf5 [36,37]. In Myf<sup>−</sup> deficit 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<sup>−/−</sup> mice also die perinatally, but do not show the same degree of hindlimb defects that we observe in Ssb1<sup>−/−</sup> 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 rib-cage 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 rib-cage, 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, Ssb1 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 Ssb1<sup>−/−</sup> mice suggest that Ssb1 is important for endochondral ossification. During the preparation of this manuscript, another report of the critical role of Ssb1 in skeletogenesis was published, where the authors had used a similar genetic targeting approach to delete Ssb1 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, tibiofibular malformation and variably-penetrant oligodonty. 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 Ssb1 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<sup>−/−</sup> background. In the case of combined Ssb1 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 [43]. This suggests that the Ssb1<sup>−/−</sup> phenotype cannot be solely attributed to apoptosis, and that some steps in the later stages of endochondral ossification are dependent on Ssb1. 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 sternal development was not as severely affected as the rest of the rib-cage in Ssb1<sup>−/−</sup> embryos. It will therefore be of great interest to more rigorously investigate the role of Ssb1 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 Ssb1 knockout mice were surprising, effects of inducible ablation of Ssb1 in adult mice revealed phenotypes more relevant to the proposed role of Ssb1 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 Ssb1<sup>−/−</sup> 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 Ssb1 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\textsuperscript{T2}: Ssb1\textsuperscript{+/+}, Rosa26-CreER\textsuperscript{T2}: Ssb1\textsuperscript{+/-} and Rosa26-CreER\textsuperscript{T2}: Ssb1\textsuperscript{-/-} 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\textsuperscript{T2}: Ssb1\textsuperscript{-/-} mice and the other two groups (**P<0.01), while no difference was found between Rosa26-CreER\textsuperscript{T2}: Ssb1\textsuperscript{+/+} and Rosa26-CreER\textsuperscript{T2}: Ssb1\textsuperscript{+/-} 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 demicolcine-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 indicted genotypes. Red arrows mark some of the chromosomal breakages. Note the presence of DNA debris marked with circles.

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A

![Graph showing tumour survival rates with Ssb1 +/+ (n=35) and Ssb1 +/- (n=35).](image)

B

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C

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

D

Liver (Hepatocellular carcinoma)
sized tests and reduced fertility in conditional Ssb1−/− adult male mice. The increased number of apoptotic spermatocytes in testes and premature sloughing of germ cells into the epididymis may be the cause of reduced fertility. The observation of a degree of phenotypic variation between conditional Ssb1−/− mice suggests that the severity of the fertility defects was dependent on the degree of testicular degeneration, which may be correlated with the variation in the residual amount of Ssb1 protein between different mice after Cre-recombination. Further investigation of the function of Ssb1 in spermatogenesis is beyond the scope of this first report, but it would be of great interest to study testicular defects in testis-specific Ssb1-deleted mice.

Aside from meiotic chromosome rearrangement, physiological programmed DSBs are also generated during Class Switch Recombination (CSR) in mature antigen-stimulated B lymphocytes [17]. CSR involves programmed DNA rearrangements situated outside the targeting vector, in addition to an internal neo probe following restriction digest of genomic DNA using HindIII, SacI or ScaI restriction enzymes. Generation of Ssb1 floxed/neo (flneo) mice was a contracted service performed by Ozgene Pty Ltd (Perth, Australia).

**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 ScaI 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 create constitutive germline deletion of Ssb1, Ssb1fl/fl 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 allele and Cre transgene. Ssb1−/− heterozygous mice were 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/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/fl mouse line to generate experimental animals (Rosa26-CreERT2: Ssb1+/− 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 Ssb1+/− 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-CreERT2 mice are: 5’-TGTGGACAGGAGGAGCCATTAGC-3’ (forward primer) and 5’-CATCAGCTGGGGTACGCCAGGC-3’ (reverse primer). As expected, PCR amplification of the 356-bp Rosa26-CreERT2-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, resulting in 402, 360 and 118-bp PCR products, specific for Ssb1 floxed, wild-type, and null alleles, respectively (Figure 2A). The sequences of the common forward primer for Ssb1 wild type, floxed and null allele is: 5’-GCATTGCTTTGTCTTTAACC-3’. The reverse primer for Ssb1 wild-type and floxed alleles is 5’-ACAAGCTTGGACATCGGAG-3’ and for the Ssb1 null allele is 5’-GAAAAGTGATTCCAGAGCTCAGA-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 (Qiagen), followed by Donkey anti-sheep (New England Biolabs) antibody (Millipore) was performed as described previously [7]. For γH2AX antibody (Millipore) was performed as described previously [7]. For γH2Alox foci quantitation, 30 cells for each MEF cell line (n=2 Ssb1+/+, 3 Ssb1−/−) were scored for those containing >10 foci/cell at the indicated timepoints following 2 Gy of gamma-irradiation, across two independent experiments.

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 γH2AX foci quantitation, 30 cells for each MEF cell line (n=2 Ssb1+/+, 3 Ssb1−/−) 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-CreERT2: 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 = weight of the right testis/2 [23].

Class switch recombination (CSR) analysis

Splenic B cells were stimulated for IgH CSR to IgG1 using anti-CD40 antibodies plus II-4 and analyzed by flow cytometry as described previously [30].

Total body irradiation

Total body irradiation (TBI) was performed using a 137Cs source at 106 cGy/min. Mice were placed in pleixglass 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+/+, 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. 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
(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 makers 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 Sbh1 gene-targeted mice. (A) Schematic diagram showing the Sbh1 gene structure and targeting strategy including the Sbh1 wild-type (wt), Sbh1 targeted (loxP), Sbh1 floxed (fl) and Sbh1 null (null) alleles. (B) Southern blot confirming correct genomic targeting of Sbh1 flox mouse 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 Sbh1 wild type (primer 1; P1 and primer 2; P2 in Sbh1<sup>+/+</sup>), Sbh1 flox (P1 and P3 in Sbh1<sup>loxP/loxP</sup>), Sbh1 floxed (P1 and P2 in Sbh1<sup>fl/fl</sup>) and Sbh1 null (P1 and P4 in Sbh1<sup>−/−</sup>) alleles. (TIF)

Figure S2 Perinatal lethality, growth retardation, micrognathia and cleft palate in Sbh1<sup>−/−</sup> embryos. (A) Sbh1<sup>−/−</sup> P0 embryos exhibit severe respiratory distress and die within 30 minutes of birth. Note the purple colour of Sbh1<sup>−/−</sup> embryos indicating cyanosis. (B) Comparison of crown-rump length of E14.5 and E18.5 Sbh1<sup>+/+</sup>, Sbh1<sup>+/−</sup> and Sbh1<sup>−/−</sup> embryos (n = minimum 3 embryos for E14.5; minimum 6 embryos for E18.5 per genotype) (*P<0.05, **P<0.01 ***P<0.001, student’s t-test). (C) Representative sagittal sections through the heads of Sbh1<sup>+/+</sup> (i–iii) and Sbh1<sup>−/−</sup> (iv–vi) P0 embryos show a misshapen snout and recessed mandible in Sbh1<sup>−/−</sup> embryos. (D) Ventral skull view of E18.5 skeletal preparations with removed mandible showing clefting of the secondary palate in an Sbh1<sup>−/−</sup> embryo. Scale bar = 1 mm. (E) Magnified view of (D). Note the properly fused palatine processes (arrowhead) in Sbh1<sup>−/−</sup> control embryo (left) and lack of palatine process formation in the Sbh1<sup>−/−</sup> embryo (right), exposing the underlying presphenoid bone (arrowhead). Scale bar = 1 mm. (F) Ventral view of P0 Sbh1<sup>+/+</sup> and Sbh1<sup>−/−</sup> heads with removed mandible showing variably penetrant cleft palate between Sbh1<sup>−/−</sup>-littermates. Scale bar = 1 mm. (TIF)

Figure S3 Morphology of Sbh1 control and Sbh1<sup>−/−</sup> embryos. Haematoxylin and eosin staining of sagittal sections through the heads of E18.5 Sbh1<sup>+/+</sup> and Sbh1<sup>−/−</sup> lungs. (A) Quantitation of qRT-PCR for proximal differentiation markers Cx10 (clara cells), Foxj1 (ciliated epithelial cells) and Cd31 (endothelial cells). (B) Immunohistological staining for smooth muscle actin (SMA) in Sbh1 control (Sbh1<sup>+/+</sup>, Sbh1<sup>+/−</sup>) and Sbh1<sup>−/−</sup> E18.5 lungs. Scale bar = 200 μm. (TIF)

Figure S4 Apoptosis and proliferation in E14.5 and E18.5 Sbh1<sup>−/−</sup> lungs. (A) Immunohistological staining of Ki67 in E14.5 (top) and E18.5 (bottom) control (Sbh1<sup>+/+</sup>, Sbh1<sup>+/−</sup>) and Sbh1<sup>−/−</sup> lungs to mark proliferating cells. (B) ApopTag TUNEL, immunohistological staining to mark apoptotic cells in Sbh1 control and Sbh1<sup>−/−</sup> E14.5 (top) and E18.5 (bottom) lungs. Scale bar = 50 μm. (TIF)

Figure S5 Proximal Lung Differentiation in E18.5 Sbh1<sup>−/−</sup> lungs. (A) Quantitation of qRT-PCR for proximal differentiation markers Cx10 (clara cells), Foxj1 (ciliated epithelial cells) and Cd31 (endothelial cells). (B) Immunohistological staining for smooth muscle actin (SMA) in Sbh1 control (Sbh1<sup>+/+</sup>, Sbh1<sup>+/−</sup>) and Sbh1<sup>−/−</sup> E18.5 lungs. Scale bar = 200 μm. (TIF)

Figure S6 Sbh1 is not required for the response to DNA double-strand breaks in mouse embryonic fibroblasts. (A) Cell cycle profiles of Sbh1<sup>+/+</sup> and Sbh1<sup>−/−</sup> passage 3 MEFs by propidium iodide staining. (B) T3 proliferation assay showing growth curves for Sbh1<sup>+/+</sup> and Sbh1<sup>−/−</sup> MEFs (n = 3). Data represent mean ± SEM. (C) Western blot showing Atm signalling activation in Sbh1<sup>−/−</sup> and Sbh1<sup>−/−</sup> 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 Sbh1 gene targeting strategy. Sbh1<sup>fl/fl</sup> mice were bred with Rosa26-CreERT2<sup>T2</sup> transgenic mice to enable conditional Sbh1 deletion. Sbh1 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<sup>T2</sup>; Sbh1<sup>fl/fl</sup> mice. (TIF)

Figure S8 Conditional Cre recombination mediated Sbh1 deletion and Sbh2 upregulation in Rosa26-CreERT2<sup>T2</sup>; Sbh1<sup>−/−</sup> mice. (A) PCR genotyping after tamoxifen induced Cre recombination mediated Sbh1 gene deletion. PCR analysis of recombination of the floxed Sbh1 allele in heterozygous Sbh1<sup>lox/lox</sup>-Rosa26-CreERT2<sup>T2</sup> (Rosa26-CreERT2<sup>T2</sup>; Sbh1<sup>fl/fl</sup>) and homozygous Sbh1<sup>lox/lox</sup>-Rosa26-CreERT2<sup>T2</sup> 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 116-bp 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-CreER2; Ssb1+/− and Rosa26-CreER2: 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-CreER2; Ssb1+/− and Rosa26-CreER2: Ssb1−/− mice. (A) Gender distribution comparison of Rosa26-CreER2; Ssb1+/− and Rosa26-CreER2: Ssb1−/− mouse cohorts. (B) Comparison of age of tamoxifen induction between cohorts. (C) Comparison of body weights of Rosa26-CreER2; Ssb1+/− and Rosa26-CreER2: 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-CreER2; Ssb1+/− and Rosa26-CreER2; 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-CreER2 : 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-CreER2 : 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 nuclei (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).

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References


43. Feldhahn N, Ferretti E, Robbiani DF, Callen E, Deroubaix S, et al. (2012) The hSSB1 orthologue Obfc2b is essential for skeletogenesis but dispensable for the DNA damage response in vivo. EMBO J.


