DNA single-strand break-induced DNA damage response causes heart failure

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The DNA damage response (DDR) plays a pivotal role in maintaining genome integrity. DNA damage and DDR activation are observed in the failing heart, however, the type of DNA damage and its role in the pathogenesis of heart failure remain elusive. Here we show the critical role of DNA single-strand break (SSB) in the pathogenesis of pressure overload-induced heart failure. Accumulation of unrepaired SSB is observed in cardiomyocytes of the failing heart. Unrepaired SSB activates DDR and increases the expression of inflammatory cytokines through NF-κB signalling. Pressure overload-induced heart failure is more severe in the mice lacking XRCC1, an essential protein for SSB repair, which is rescued by blocking DDR activation through genetic deletion of ATM, suggesting the causative role of SSB accumulation and DDR activation in the pathogenesis of heart failure. Prevention of SSB accumulation or persistent DDR activation may become a new therapeutic strategy against heart failure.

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Various internal and external stresses induce many types of DNA damage such as chemical change of bases, single-strand break (SSB) and double-strand break (DSB). On DNA damage, the DNA damage response (DDR) signalling is activated to repair damaged DNA. Recruitment and autophosphorylation of ataxia telangiectasia mutated (ATM) kinase are one of the most well characterized DDR, which are usually activated after DNA DSB. Phosphorylated ATM in turn phosphorylates multiple effector proteins including the histone variant H2AX and tumor suppressor p53, and triggers downstream signalling pathways that stop the cell cycle and repair damaged DNA. When the DNA damage is too extensive to be repaired, cells usually undergo apoptosis or stop the cell cycle permanently, which is termed cellular senescence. Activation of DDR is observed not only in mitotic cells but also in post-mitotic cells including cardiomyocytes. Various types of DNA damage including DNA oxidation, SSB and DSB are observed in the infarcted heart and activation of DDR plays an important role in cardiac remodelling after myocardial infarction through inducing cardiomyocyte apoptosis. Activation of DDR is also observed in cardiomyocytes of the patients with end-stage heart failure and the pressure-overload induced heart failure model mice, however, the type of DNA damage and the role of DDR in the pathophysiology of heart failure remain unclear.

In the present study, we show that DNA SSB, but not DNA DSB, is accumulated in cardiomyocytes of pressure-overload induced heart failure model mice. Accumulation of DNA SSB activates persistent DDR and induces inflammatory gene expression in an NF-kB-dependent manner. Cardiomyocyte-specific gene deletion of Xrc1, an essential protein for SSB repair, leads to more severe cardiac inflammation and heart failure after pressure-overload, which is rescued by simultaneous deletion of Atn gene. These results suggest the unrecognized role of DNA SSB in the pathogenesis of pressure overload-induced heart failure.

Results
DNA single-strand break accumulation in heart failure. We performed transverse aortic constriction (TAC) surgery on mice to induce pressure overload-induced heart failure. Left ventricular hypertrophy was observed in 2 weeks, and progressive cardiac dysfunction was observed in 8–10 weeks after the TAC surgery (Supplementary Fig. 1). To elucidate the type of DNA damage in the failing heart, we isolated cardiomyocytes after the TAC surgery and analysed the type of DNA damage by comet assay. Comet tail moment in the alkaline condition (alkaline comet) reflects both DNA DSB and SSB whereas comet tail moment in the neutral condition (neutral comet) reflects only DNA DSB. To optimize the experimental condition of the comet assays, we used cardiomyocytes isolated from doxorubicin-treated mice and confirmed that neutral comet assay could indeed detect DNA DSB in the heart (Supplementary Fig. 2a). We found that the alkaline comet tail moment of cardiomyocytes was increased time dependently after TAC operation whereas neutral comet tail moment was comparable between Sham- and TAC-operated mice (Fig. 1a,b), suggesting that the number of cardiomyocytes with more SSB, but not DSB, is increased in the heart after pressure overload. To further analyse the type of DNA damage in the failing heart, we performed in situ oligo ligation (ISOL), which enables in situ detection of DNA DSB, and immunofluorescent staining for NBS1, which is a component of MRE11-RAD50-NBS1 DSB repair complex and used as a DNA DSB marker. The number of ISOL- and NBS1-positive cardiomyocytes was increased in the heart after myocardial infarction (Supplementary Fig. 2b,c) whereas they were not increased in the heart after TAC operation (Fig. 1c–f), underscoring the results of the comet assay. Notably, the number of ISOL-positive non-cardiomyocytes was increased after TAC operation (Supplementary Fig. 2d). We cannot totally exclude the possibility that the cells with DNA DSB are cleared by phagocytes too quickly to be detected by these assays (neutral comet assay, ISOL and NBS1 staining), or the sensitivity of these assays were not enough to detect DNA DSB in cardiomyocytes, however, it is very likely that the major type of DNA damage that accumulates in cardiomyocytes after pressure-overload is DNA SSB, but not DSB.

Despite our observation that DNA SSB was increased in cardiomyocytes after TAC operation, we found that the level of protein poly(ADP-ribosylation), which is induced by PARP1/2 as an initial process of SSB repair, and the expression levels of SSB repair-related genes were rather decreased in the TAC-operated heart (Fig. 1g–i). Together with the findings that the levels of reactive oxygen species (ROS) were significantly increased (Fig. 1j–l), decreased SSB repair activity as well as increased ROS production may play a role in SSB accumulation in the heart after TAC operation (Supplementary Fig. 3).

Xrc1 deficiency exacerbates heart failure. In mitotic cells, unrepaired SSB usually does not accumulate but develops into DNA DSB and induces catastrophic cell death during mitotic phase. Therefore, accumulation of SSB is supposed to be a unique phenomenon observed only in post-mitotic cells. To examine the causal relation between SSB accumulation and heart failure, we generated the mice with defective SSB repair. X-ray repair complementing defective repair in Chinese hamster cells 1 (XRC1) is a scaffold protein that interacts with various SSB repair enzymes and essential for SSB repair. Since Xrc1 knockout mice exhibit early embryonic lethality due to massive DNA damage and cellular apoptosis, we crossed the mice homozygous for an Xrc1lox allele with transgenic mice expressing Cre recombinase under the control of the α-myosin heavy chain promoter (α-MHC-Cre) and obtained Xrc1lox/lox; α-MHC-Cre and Xrc1lox/lox; αMHC-Cre mice as cardiomyocyte-specific Xrc1 knockout mice. Xrc1lox/lox; αMHC-Cre mice were used as controls. Deletion of Xrc1 in the heart tissue of Xrc1lox/lox; αMHC-Cre mice was confirmed at the DNA, mRNA and protein levels (Supplementary Fig. 4a–c). Cre recombinase mice were born in expected Mendelian ratios, and were viable and fertile. Echocardiographic analysis revealed mild cardiac dysfunction in Xrc1lox/lox; αMHC-Cre mice (Supplementary Fig. 4d), but the mice showed no overt signs of heart failure such as tachypnea, weight loss and pleural effusions beyond 1-year follow-up (Supplementary Fig. 4e), suggesting that deletion of Xrc1 in cardiomyocytes is not sufficient to induce heart failure. After the TAC surgery, however, Xrc1lox/lox; αMHC-Cre mice exhibited more severe left ventricular dilatation and dysfunction (Fig. 2a,b), more severe signs of heart failure (Fig. 2c) and higher mortality (Fig. 2d) compared with Xrc1lox/lox mice.

Comet assay revealed that the level of SSB in cardiomyocytes tended to increase, but statistically comparable between sham-operated Xrc1lox/lox; αMHC-Cre and Xrc1lox/lox mice (Fig. 2e), suggesting that cardiomyocyte-specific deletion of Xrc1 by itself is not sufficient to induce massive SSB accumulation in the non-stressed heart. After the TAC operation, however, the level of SSB in cardiomyocytes was more increased in the heart of Xrc1lox/lox; αMHC-Cre mice compared with Xrc1lox/lox mice (Fig. 2e), whereas the level of DSB in cardiomyocytes was comparable between Xrc1lox/lox; αMHC-Cre and Xrc1lox/lox mice (Supplementary Fig. 5). These results collectively suggest that insufficient SSB repair due to deletion...
of Xrcc1 induces more SSB accumulation and exacerbates cardiac dysfunction in response to pressure overload and also indicate that SSB accumulation is tightly associated with the progression of pressure overload-induced heart failure.

SSB activates DDR and induce inflammation through NF-κB. To elucidate the molecular mechanism by which SSB accumulation in cardiomyocytes exacerbates pressure overload-induced heart failure, we established an in vitro model of cardiomyocytes

![Image]
with SSB accumulation by using alkylating agent methyl methanesulfonate (MMS), which induces only DNA SSB\textsuperscript{20}, and small interfering RNA (siRNA)-mediated knockdown of Xrcc1. To optimize the experimental condition of the comet assays and ISOL staining in \textit{in vitro} cultured cardiomyocytes, we used irradiation to induce DNA DSB and confirmed that DNA DSB can be detected in both assays (Supplementary Fig. 6a,b). We observed concentration-dependent, temporal SSB after 10-min treatment with MMS (Fig. 3a,b) and repetitive treatment with MMS generated unrepaired SSB accumulation in cultured cardiomyocytes (Fig. 3c,d). Knockdown of \textit{Xrcc1} (Fig. 3e) also generated a time-dependent accumulation of SSB in cultured cardiomyocytes (Fig. 3f). DNA DSB was not induced either by MMS treatment or by knockdown of \textit{Xrcc1} (Fig. 3a,b,d,f and Supplementary Fig. 6a--d).

We then analysed how DDR is activated after SSB induction. Single treatment with MMS induced phosphorylation of ATM and H2AX whereas phosphorylation of p53 was not observed (Fig. 4a). Repetitive treatment with MMS or knockdown of \textit{Xrcc1}, however, induced phosphorylation of p53 as well as ATM and H2AX (Fig. 4b,c) that resembles to the DDR after ionizing radiation or doxorubicin, which causes DNA DSB (Supplementary Fig. 7a,b). Persistent activation of DDR usually induces apoptotic cell death or cellular senescence\textsuperscript{21}. Recent reports suggest that senescent cells show inflammatory phenotype, which is termed senescence-associated secretory phenotype (SASP)\textsuperscript{22,23}. Therefore, we tested whether SSB accumulation and persistent activation of DDR induce inflammatory phenotype in cardiomyocytes. Knockdown of \textit{Xrcc1} increased the expression of inflammatory cytokines such as \textit{Il6, Cxcl1, Ccl2} and \textit{Vcam1} in cardiomyocytes suggesting that SSB accumulation leads to acquisition of inflammatory phenotype in cardiomyocytes. Persistent phosphorylation/activation of ATM plays a central role in acquisition of SASP in senescent cells\textsuperscript{4}. Persistent activation of DDR and increased expression of inflammatory cytokines after knockdown of \textit{Xrcc1} were abolished by simultaneous knockdown of \textit{Atm} (Fig. 4d and Supplementary Fig. 7c), suggesting that ATM is essential for SSB accumulation-induced acquisition of inflammatory phenotype in cardiomyocytes.

NF-\kappaB plays a key role during inflammation. Previous report also suggests the involvement of NF-\kappaB in DDR\textsuperscript{24--26}. We also observed that the number of cardiomyocytes with nuclear NF-\kappaB staining was increased after knockdown of \textit{Xrcc1} and was abolished by simultaneous knockdown of \textit{Atm} (Fig. 4e,f). BAY 11-7082, an inhibitor of NF-\kappaB, blocked

**Figure 2** | \textit{Xrcc1} deficiency increase SSB accumulation and exacerbates heart failure. (a) Macroscopic and echocardiographic images of Sham- or TAC-operated \textit{Xrcc1} and \textit{Xrcc1} mice. Scale bar, 2 mm. (b) TAC surgery was performed to \textit{Xrcc1} and \textit{Xrcc1} mice and cardiac function after the operation was assessed by echocardiogram. LVd, LV end-diastolic dimension; LVDs, LV end-systolic dimension; LVPWd, LV posterior wall dimension; LVFS, LV fractional shortening (\textit{Xrcc1} mice: \textit{n} = 80, 22, 30, 11, 10; \textit{Xrcc1} mice: \textit{n} = 85, 28, 40, 13, 9 at each time point, respectively). Statistical significance was determined by Student’s \textit{t}-test at each time point. #\textit{p}<0.05; ##\textit{p}<0.01 versus \textit{Xrcc1} mice.

(c) Heart, lung, and body weight of Sham- or TAC-operated \textit{Xrcc1} and \textit{Xrcc1} mice were weighed 8 weeks after the TAC surgery (\textit{n} = 8, 9, 12, 7, respectively). Statistical significance was determined by one-way analysis of variance followed by the Tukey-Kramer HSD test. *\textit{p}<0.05; **\textit{p}<0.01 between arbitrary two groups. (d) Survival curve of \textit{Xrcc1} and \textit{Xrcc1} mice after the TAC surgery (\textit{n} = 26, 33, respectively). Statistical significance was determined by Wilcoxon test. *\textit{p}<0.05 versus \textit{Xrcc1} mice. (e) The type of DNA damage in cardiomyocytes of Sham- or TAC-operated \textit{Xrcc1} and \textit{Xrcc1} mice was assessed by comet assay (Alkaline comet: \textit{n} = 50, 64, 60, 67; Neutral comet: \textit{n} = 31, 57, 42, 50, respectively). Statistical significance was determined by Steel-Dwass test. *\textit{p}<0.01 between arbitrary two groups. Column and error bars show mean and s.e.m., respectively.
SSB accumulation-induced NF-κB activation and inflammatory gene expression in cardiomyocytes (Fig. 4g and Supplementary Fig. 7d,e). These results collectively suggest that SSB accumulation induces inflammatory cytokine expression through persistent activation of DDR and subsequent activation of NF-κB pathway in cardiomyocytes.

Xrcc1 deficiency exacerbates DDR and cardiac inflammation. We then investigated whether our in vitro findings are also observed in vivo. The number of cardiomyocytes positive for phosphorylated H2AX (γH2AX) was increased after TAC operation (Supplementary Fig. 8a,b) and the number was more increased in the heart of Xrcc1<sup>−/−MHC-Cre</sup> mice compared with Xrcc1<sup>f/f</sup> mice (Fig. 5a,b). The amount of ROS in the heart was comparable between Xrcc1<sup>−/−MHC-Cre</sup> and Xrcc1<sup>f/f</sup> mice (Fig. 5c and Supplementary Fig. 9a,b), suggesting that increased number of γH2AX-positive cardiomyocytes in Xrcc1<sup>−/−MHC-Cre</sup> mice was due to defective SSB repair and SSB accumulation but not overproduction of ROS in Xrcc1<sup>−/−MHC-Cre</sup> mice<sup>27</sup>.

Chromatin immunoprecipitation (ChIP) analyses revealed that binding of NF-κB to the Vcam1 promoter was increased twofold in TAC-operated Xrcc1<sup>−/−MHC-Cre</sup> cardiomyocytes compared with Xrcc1<sup>f/f</sup> cardiomyocytes (Fig. 5d). Expression levels of inflammatory cytokines and infiltration of inflammatory cells were increased in cardiomyocytes after TAC operation, and were more increased in TAC-operated Xrcc1<sup>−/−MHC-Cre</sup> mice compared with Xrcc1<sup>f/f</sup> mice (Fig. 5e–g). These in vivo results are consistent with our in vitro observations and strongly support our hypothesis that accumulation of SSB in cardiomyocytes exacerbates heart failure through the activation of persistent DDR and promoting cardiac inflammation.
ATM deletion rescues heart failure in Xrcc1-deficient mice. To further investigate whether SSB accumulation in cardiomyocytes plays a causative role in the pathogenesis of heart failure through activating DDR, we crossed Xrcc1<sup>−/−</sup>MHC-Cre mice with Atm<sup>−/−</sup> mutants<sup>28,29</sup> to block persistent DDR and obtained Xrcc1<sup>−/−</sup>MHC-Cre; Atm<sup>−/−</sup> mice (Supplementary Fig. 10a–d). Mild cardiac dysfunction observed in Xrcc1<sup>−/−</sup>MHC-Cre mice was restored in Xrcc1<sup>−/−</sup>MHC-Cre; Atm<sup>−/−</sup> mice (Fig. 6a,b), suggesting that heterozygous Atm gene deletion indeed suppressed DDR activation which was induced by DNA SSB. Expression levels of cardiomyocytes which tended to be increased in Xrcc1<sup>+/−</sup>MHC-Cre; Atm<sup>−/−</sup> mice (Fig. 6c and Supplementary Fig. 11a,b), however, the number Xrcc1<sup>+/−</sup>MHC-Cre mice remained increased in Xrcc1<sup>−/−</sup>MHC-Cre; Atm<sup>−/−</sup> mice (Fig. 6d,e), suggesting that heterozygous Atm gene deletion indeed suppressed DDR activation which was induced by DNA SSB. Expression levels of...
inflammatory cytokines in cardiomyocytes (Fig. 6f) and infiltration of inflammatory cells (Fig. 6g,h) were mildly increased in the heart of Xrcc1 deficient mice and were attenuated in Xrcc1 homozygous mice, with the exception of Xc3l0 and Vcam1 gene expression. There was an apparent discrepancy between the level of SSB, which tended to increase but did not reach statistical significance, and the other cardiac phenotypes in Xrcc1 deficient mice. This discrepancy may be explained by the presence of threshold between the level of DNA SSB and the downstream events in cardiomyocytes. Just like there is a threshold between the level of DNA damage and cell death (cell death is triggered when the level of DNA damage is above the threshold), it is conceivable to think that persistent DDR and the following inflammatory program are triggered when the level of unpaired SSB in each cell is above the threshold.
We finally performed the TAC surgery to these mice and found that heterozygous Atm gene deletion attenuated the progression of severe left ventricular dysfunction (Fig. 7a,b), alleviated the signs of heart failure (Fig. 7c) and improved the mortality in Xrcc1<sup>−/−</sup>MHC-Cre mice (Fig. 7d). The level of SSB in cardiomyocytes remained high in Xrcc1<sup>−/−</sup>MHC-Cre, Atm<sup>+/−</sup> mice, however, the

Figure 6 | Basal characters of Xrcc1<sup>−/−</sup>MHC-Cre and Xrcc1<sup>+/−</sup>MHC-Cre, Atm<sup>+/−</sup> mice. (a,b) Echocardiographic images (a) and cardiac function (b) of Xrcc1<sup>+/−</sup>, Xrcc1<sup>−/−</sup>MHC-Cre and Xrcc1<sup>−/−</sup>MHC-Cre, Atm<sup>+/−</sup> mice. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer HSD test. "P<0.01 between arbitrary two groups. (c) The type of DNA damage in cardiomyocytes of Xrcc1<sup>+/−</sup>, Xrcc1<sup>−/−</sup>MHC-Cre and Xrcc1<sup>−/−</sup>MHC-Cre, Atm<sup>+/−</sup> mice was assessed by comet assay (Alkaline comet: n=50, 76, 77; Neutral comet: n=53, 56, 42, respectively). Statistical significance was determined by Steel-Dwass test. (d,e) Activation of DDR in Xrcc1<sup>+/−</sup>, Xrcc1<sup>−/−</sup>MHC-Cre and Xrcc1<sup>−/−</sup>MHC-Cre, Atm<sup>+/−</sup> mice was assessed by immunostaining for phosphorylated H2AX (d, γH2AX, green, arrowheads). Immunostaining for alpha-actinin (red) was used to label cardiomyocytes. Arrowheads indicate γH2AX-positive cardiomyocytes. Scale bar, 50 μm. The number of γH2AX-positive cardiomyocytes was counted (e, n = 4 each). Statistical significance was determined by one-way ANOVA followed by the Tukey-Kramer HSD test. "P<0.05 between arbitrary two groups. (f) The expression levels of inflammatory cytokines in the isolated cardiomyocytes of Xrcc1<sup>+/−</sup>, Xrcc1<sup>−/−</sup>MHC-Cre and Xrcc1<sup>−/−</sup>MHC-Cre, Atm<sup>+/−</sup> mice was assessed by real-time PCR (n = 7, 7, 6 for each genotype, respectively, technical duplicates). Statistical significance was determined by one-way ANOVA followed by the Tukey-Kramer HSD test. "P<0.05 between arbitrary two groups. (g,h) Heart tissues of Xrcc1<sup>+/−</sup>, Xrcc1<sup>−/−</sup>MHC-Cre and Xrcc1<sup>−/−</sup>MHC-Cre, Atm<sup>+/−</sup> mice were immunostained for CD45 or CD68 (g, green, arrowheads). Immunostaining for alpha-actinin (red) was used to label cardiomyocytes. Arrowheads indicate CD45- or CD68-positive cells. Scale bar, 50 μm. The number of CD45- and CD68-positive cells was counted (h, n = 6, 6, 4 for each genotype, respectively). Statistical significance was determined by one-way ANOVA followed by the Tukey-Kramer HSD test. "P<0.01 between arbitrary two groups. Column and error bars show mean and s.e.m., respectively. LVDb, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; LVPWd, left ventricular posterior wall dimension; LVFS, left ventricular fractional shortening (n = 83, 88, 23 for each genotype, respectively).
Figure 7 | ATM gene deletion rescues the cardiac phenotypes of Xrcc1 deficient mice. (a,b) Macroscopic and echocardiographic images (a) and cardiac function (b) of TAC-operated Xrcc1f/f, Xrcc1mHC-Cre, and Xrcc1pMHC-Cre. Atm+/− mice (Xrcc1f/f mice: n = 83, 21, 46, 17; Xrcc1pMHC-Cre mice: n = 88, 28, 60, 13, 16; Xrcc1mHC-Cre, Atm+/− mice: n = 28, 22, 27, 7 at each time point, respectively). Scale bar, 2 mm. (c) Heart, lung, and body weight of TAC-operated Xrcc1f/f, Xrcc1mHC-Cre, and Xrcc1pMHC-Cre. Atm+/− mice were weighed 8 weeks after the surgery (n = 8, 5, 6 for each genotype, respectively). (d) Survival curves of TAC-operated Xrcc1f/f, Xrcc1mHC-Cre, and Xrcc1pMHC-Cre. Atm+/− mice (n = 49, 62, 23, respectively). (e-k) TAC-operated Xrcc1f/f, Xrcc1mHC-Cre, and Xrcc1pMHC-Cre. Atm+/− mice were analysed 4 weeks after the surgery. The type of DNA damage in cardiomyocytes was assessed by comet assay (e, Alkaline comet; n = 50, 76, 77; Neutral comet: n = 53, 56, 42, respectively). Activation of DDR was assessed by immunostaining for phosphorylated H2AX (f, γH2AX, green, arrowheads). Arrowheads indicate γH2AX-positive cardiomyocytes and arrow indicate γH2AX-negative non-cardiomyocytes. Scale bar, 50 μm. The number of γH2AX-positive cardiomyocytes was counted (g, n = 4 each). Expression levels of inflammatory cytokines in the isolated cardiomyocytes were assessed by real-time PCR (h, n = 10, 16, 12 for each genotype, respectively, technical duplicates). ChIP-qPCR analysis of binding of NF-κB to the Vcam1 promoter region. Data is presented as fold enrichment relative to TAC-operated Xrcc1f/f mice (i, n = 4, 5, 5, respectively). Heart tissues were immunostained for CD45 or CD68 (j, green, arrowheads). Arrowheads indicate CD45- or CD68-positive cells. Scale bar, 50 μm. The number of CD45- and CD68-positive cells was counted (k, n = 4 each). Statistical significance was determined by one-way analysis of variance followed by the Tukey-Kramer HSD test for (b) (at each time point), (c,h,i,k), by Wilcoxon test for (d) and by Steel-Dwass test for (e). **P < 0.05; ***P < 0.01 between Xrcc1f/f and Xrcc1pMHC-Cre mice. †P < 0.05; ††P < 0.01 between Xrcc1f/f and Xrcc1mHC-Cre, Atm+/− mice. *P < 0.05; **P < 0.01 between arbitrary two groups. Column and error bars show mean and s.e.m., respectively.
Accumulation of unrepaired oxidative DNA damage and inflammation and heart failure in response to pressure overload. SSB-induced activation of DDR plays a causal role in cardiac function and subsequent activation of DDR in the pathogenesis of heart failure. We found that DDR activation contributes, at least in part, to increased cardiac inflammation and the progression of pressure overload-induced heart failure.

Figure 8 | Possible roles of SSB accumulation in pathogenesis of heart failure. Accumulation of DNA SSB in cardiomyocytes induces persistent activation of DDR and subsequent activation of NF-κB pathway, resulting in increased expression of inflammatory cytokines. These mechanisms may contribute, at least in part, to increased cardiac inflammation and the progression of pressure overload-induced heart failure.

Discussion
Accumulation of unrepaired oxidative DNA damage and activation of DDR have been observed in the failing heart, however, their roles in the pathogenesis of heart failure remain elusive. In the present study, we identified the type of DNA damage which triggers DDR in cardiomyocytes and demonstrated that activation of DDR contributes to, at least in part, cardiac inflammation in the failing heart. We found that unrepaired DNA SSB accumulates in cardiomyocytes of pressure overload-induced heart failure model mice. Accumulation of DNA SSB induced persistent DDR and upregulated the expression of inflammatory cytokines through NF-κB pathway. Persistent activation of DDR and upregulation of inflammatory genes may play a role in SSB accumulation and heart failure in response to pressure overload.

In the present manuscript, we showed that accumulation of unrepaired SSB plays a causative role in the pathogenesis of heart failure. Unrepaired SSB develops into DNA DSB during mitosis in mitotic cells, therefore, accumulation of SSB is supposed to be specific to post-mitotic cells such as neurons or cardiomyocytes. Accumulation of unrepaired SSB in neural tissue is observed in spinocerebellar ataxia with axonal neuropathy 1 (SCAN1). Mutation of SSB repair enzyme, Tyrosyl-DNA phosphodiesterase 1, is observed in the patients of SCAN1 and DNA SSB is accumulated in the neural tissue of Tdp1−/− mice. Our findings indicate that SSB accumulation also occurs in stressed, post-mitotic cells and plays pathogenic role in non-genetic disorders. Compared with the pathogenic role of DSB, knowledge about the roles of SSB in human diseases is limited. Future study may reveal the role of SSB in the pathogenesis of various genetic or non-genetic diseases.

ATM becomes activated in response to DNA damage to stop the cell cycle and promote DNA damage repair. Its activation is essentially protective for the organism to maintain homeostasis. In the present study, however, we show that ATM may also play a detrimental role in pressure overload-induced heart failure through NF-κB-dependent induction of inflammatory gene expression. Recent reports also suggested that aberrant activation of ATM plays detrimental roles in myocardial infarction model mice, that is, ATM promotes cardiac inflammation during myocardial ischemia and cardiac remodelling after myocardial infarction are attenuated in ATM knockout mice. Further investigations for the precise molecular action of ATM against chronic DNA damage may provide a novel therapeutic target for heart failure.

On DNA SSB, PARP-1 is rapidly recruited to the damaged site, where it synthesizes branched ADP-ribose polymers (pADPr) on SSB repair proteins including PARP-1, XRCC1 and DNA ligases and facilitates SSB repair. It was surprising for us to see that protein poly(ADP-ribosylation) and the expression levels of the genes that work for SSB repair were decreased in the failing heart, despite the production of ROS and the amount of DNA damage was increased. These results collectively suggest that SSB-induced activation of DDR plays a causal role in cardiac inflammation and heart failure in response to pressure overload.

Methods
Animal models. All animal procedures were approved by the Institutional Animal Care and Use Committee of Osaka University (22-056). C57BL/6 mice were purchased from CLEA JAPAN and H2AX-positve cardiomyocytes was reduced to the level of Xrcc1+/− mice (Fig. 7e-g), suggesting that heterozygous Atm gene deletion suppressed DDR activation also after the TAC operation. The expression level of inflammatory cytokines was also attenuated in MHC-Cretg/+ mice compared with MHC-Cretg/+ mice. These results collectively suggest that SSB accumulation and subsequent activation of DDR in the pathogenesis of heart failure (Fig. 8).

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SSB accumulation
Persistent activation of DDR
Nuclear translocation and activation of NF-κB
Up regulation of inflammatory genes
Cardiomyocyte inflammation
Heart failure progression

Number of γH2AX-positive cardiomyocytes was reduced to the level of Xrcc1+/− mice (Fig. 7e-g), suggesting that heterozygous Atm gene deletion suppressed DDR activation also after the TAC operation. The expression level of inflammatory cytokines was also attenuated in MHC-Cretg/+ mice compared with MHC-Cretg/+ mice. These results collectively suggest that SSB accumulation and subsequent activation of DDR in the pathogenesis of heart failure (Fig. 8).
cardiomyocyte-specific Xrc1 knockout mice. Genotyping PCR was performed as previously described. In brief, deletion of Xrc1 allele in the heart was examined by detection of a 274 bp fragment using primer 5’-TATGCTTG CTGTACAGGGATTTGGCC-3’ and 5’-TGAGCATTGAAAAAAGCTGTGTCGTG-3’. To generate cardiomyocyte-specific Xrc1 knockout mice with an ATM+/− background, Xrc1+/− mice were crossed with ATM+/− mice (Jackson Laboratory; stock#08836, stock name: B6:129S-A.1(Xroctm1(LacZ/Cre))C176/J) and cardiomyocyte-specific Xrc1 knockout mice (ATM−/−×Xrc1−/−) and cardiac Xrc1 knockout mice (ATM−/−×Xrc1−/−) were maintained. Cells were then cultured on Matrigel (BD Biosciences). Cells were then maintained for 24 h under growth conditions and then treated with or without the treatment. Cell viability was determined by the MTT assay at 24 h and 72 h after treatment. Immunofluorescence staining.Cardiomyocytes were cultured on glass slides and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100 in PBS, and blocked with 3% BSA in PBS for 30 min at room temperature. Cells were then incubated with primary antibodies overnight at 4°C and visualized using Alexa Fluor-conjugated secondary antibodies. Primary antibodies included mouse primary antibodies against anti-phospho-ATM (Ser1981) (clone 10H11.E12, Rockland, 1:500), mouse primary antibodies against anti-ATM (H-248, Santa Cruz, 1:500), rabbit polyclonal antibodies against anti-p53 (clone 1C12, Cell Signaling Technology, 1:1,000), rabbit polyclonal antibodies against anti-goat, rabbit polyclonal antibodies against anti-Smad1/5/8 (1:1000, Cell Signaling Technology). The biotinylated oligonucleotide that labels DNA strand breaks with blunt ends was used as a negative control. Western blotting.Immunoblotting was performed using TURBO DNA-free kit (Ambion) or PureLink RNA Mini Kit (Ambion) for cultured cells according to the program provided by Roche. The extracts were centrifuged at 12,000×g for 10 min and the pellet was reserved for further analyses. RNA analysis.Total RNA was extracted using TRIzol reagents (Invitrogen) for tissue homogenisation or PureLink RNA Mini Kit (Ambion) for cultured cells according to manufacturer’s instructions. RNA samples were subjected to DNase treatment to remove genomic DNA using TURBO DNA-free Kit (Ambion) or PureLink RNA Mini Kit (Ambion) and were reverse-transcribed using SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR was performed using PerfeCTa SYBR Green qPCR Mix on a 7300 Real-Time PCR System. Primer sequences and the corresponding UPL numbers were designed with online program provided by Roche.
ChIP-qPCR. Isolated cardiomyocytes were crosslinked with 0.3% formaldehyde at room temperature for 10 min. Samples were sonicated using Bioruptor Plus (Diagenode). After sonication, soluble chromatin was incubated with an anti-NF-xB antibody (5 μg. sc-372X, Santa Cruz). Specific immunocomplexes were precipitated with Dynabeads Protein G (Life Technologies). Immunoprecipitates were washed, reverse-crosslinked and purified by Wizard SV Gel and PCR Clean-Up System (Promega). Extracted DNA was used for quantitative PCR (qPCR). Fold enrichment was determined as the fold change in per cent input (ChIP signal/input signal) at the target region compared to the control region. Primers were designed to amplify the Vcam1 promoter region containing putative NF-xB-binding sites. The control primers were designed against non-conserved and non-repetitive sequence upstream of Vcam1 gene. Quantitative real-time PCR was performed using Universal Probe Library (UPL, Roche) and Light Cycler TaqMan Master kit (Roche). Primer sequences were as follows: target region: 5'-TAGAGGACGGAGGGAATC-3' and 5'-GCTTCTGATGTCCTGTTAGGT-3'; the control region: 5'-GAATACATCATTGTAGTCTGGAA-3' and 5'-AGGGCTTGTGATTCTGCTC-3'.

Statistical analysis. All values are presented as mean ± s.e.m. Two-group comparison was analysed by unpaired two-tailed Student's t-test or Mann-Whitney U-test. Multiple group comparison was performed with one-way analysis of variance followed by the Tukey–Kramer HSD test or Steel-Dwass test for comparison of arbitrary two groups. Survival curves after the TAC surgery were analysed by Kaplan–Meier method and comparison of the two groups was performed with Wilcoxon test. Significant differences were defined as P < 0.05. The optimal sample size (n = 26) to detect a difference of survival rate after pressure overload between XcrCΔ/Δ and XcrCΔC0/MC0 mice was calculated based on our preliminary data that indicate the median survival time of TAC-operated XcrCΔC0/MC0 mice is 60 days. Since the actual median survival time of TAC-operated XcrCΔC0/MC0 mice in the present study was 54 days, we reduced the optimal sample size of XcrCΔC0/MC0 mice (n = 23). We used a power of 80% and a type I error probability of 0.05. Sample size calculations were performed using the software Power and Sample Size Calculations. All the statistical analyses were reviewed by Professor Ayumi Shintani, a statistical expert at Department of Clinical Epidemiology and Biostatistics, Osaka University, Japan.

Data availability. The data that support the findings of this study are available from the corresponding author on reasonable request.

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**Author contributions**
T.H., A.T.N. and I.K. planned the project and designed the experiments. T.H., T.S., M.S., K.G. and A.N. performed the experiments. P.J.M. contributed new reagents/analytical tools. S.N., T.S., S.H., H.A., Y.K., M.I., J.-K.L., I.S., Y.S. and I.K. advised on the experiments. T.H., A.T.N. and I.K. wrote the manuscript.

**Additional information**

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