BCAS2, a protein enriched in advanced prostate cancer, interacts with NBS1 to enhance DNA double-strand break repair

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BACKGROUND: Breast cancer amplified sequence 2 (BCAS2) plays crucial roles in pre-mRNA splicing and androgen receptor transcription. Previous studies suggested that BCAS2 is involved in double-strand breaks (DSB); therefore, we aimed to characterise its mechanism and role in prostate cancer (PCa).

METHODS: Western blotting and immunofluorescence microscopy were used to assay the roles of BCAS2 in the DSBs of PCa cells and apoptosis in Drosophila, respectively. The effect of BCAS2 dosage on non-homologous end joining (NHEJ) and homologous recombination (HR) were assayed by precise end-joining assay and flow cytometry, respectively. Glutathione-S-transferase pulldown and co-immunoprecipitation assays were used to determine whether and how BCAS2 interacts with NBS1. The expression of BCAS2 and other proteins in human PCa was determined by immunohistochemistry.

RESULTS: BCAS2 helped repair radiation-induced DSBs efficiently in both human PCA cells and Drosophila. BCAS2 enhanced both NHEJ and HR, possibly by interacting with NBS1, which involved the BCAS2 N-terminus as well as both the NBS1 N- and C-termini. The overexpression of BCAS2 was significantly associated with higher Gleason and pathology grades and shorter survival in patients with PCA.

CONCLUSION: BCAS2 promotes two DSB repair pathways by interacting with NBS1, and it may affect PCA progression.

British Journal of Cancer (2020) 123:1796–1807; https://doi.org/10.1038/s41416-020-01086-y

BACKGROUND:
In cells, DNA double-strand breaks (DSB) are a major type of stress during DNA replication or attacks by reactive oxygen species, chemicals, and physical agents, such as UV light and ionising radiation (IR). Incorrectly repaired DSBs can lead to serious consequences, including cell apoptosis and carcinogenesis.1,2 There are two major forms of DNA DSB repair in eukaryotes: homologous recombination (HR) and non-homologous end joining (NHEJ).3 In HR, broken ends are processed by proteins, including BLM, CtIP and the MRE11-RAD50-NBS1 (MRN) complex, to generate 3′-single-stranded DNA (ssDNA) overhangs, which are protected from nucleases by replication protein A (RPA). They are transformed into RAD51-ssDNA nucleoprotein filaments that participate in the formation of D-loop structures and facilitate synapse formation with template homologous sequences for DNA repair.4 In the eukaryote NHEJ pathway, Ku proteins bind to DNA break ends and recruit the DNA–protein kinase catalytic subunit to juxtapose them, which then recruits nucleases, polymerases and ligases to fix the damaged sites, finally collaborating with the XRCC4/Ligase4/XLF complex to resolve and fix the broken ends.5 The MRN complex plays crucial roles in both DNA repair and checkpoint activation.6 Upon DSB induction, the MRN complex binds to free DNA ends and induces the active form of ATM, which phosphorylates γ-H2AX in the nearby DSB-flanking chromatin.7,8 Among the MRN complex proteins, NBS1 plays the roles of recruiter and coordinator in the rapid assembly of the MRN complex at damaged sites. NBS1 is also essential for the nuclear localisation and adequate performance of MRE11 and RAD50. Although NBS1 has no enzymatic activity or DNA-binding ability, it contains a forkhead-associated domain and two adjacent BRCA1 C-terminal domains in its N-terminus. The C-terminus of NBS1 is able to interact with phosphatidylinositol 3-kinase-related kinases, such as ATM and ATR.9,10

Breast carcinoma-amplified sequence 2 (BCAS2) is a member of the spliceosome complex, which also includes the PSO4, CDC5L and PLRG1 proteins. In addition to their roles in pre-mRNA splicing, these proteins also have important functions in DNA repair,11–13 cell-cycle control, apoptosis and adult tissue homeostasis.14 The cellular functions of BCAS2 are of special interest. We previously demonstrated that BCAS2 is a negative regulator of p53, which is involved in cell-cycle arrest and apoptosis.15 BCAS2 also enhances androgen receptor mRNA transcription and protein stability through forming a complex with the HSP90-androgen receptor, thus promoting the proliferation of prostate cancer (PCa) cells.16 We also demonstrated that BCAS2 contributes to pre-mRNA splicing.17 It directly binds with CDC5L and recruits the...
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examined this possibility by the measurement of the cell growth kinetics after treating BCAS2-depleted or non-depleted LNCaP and PC-3 cells with doxorubicin or etoposide, which are two topoisomerase II inhibitors capable of causing DSBs and apoptosis. After 3 days of treatment with 100 nM doxorubicin or 25 μM etoposide, the LNCaP cells (Supplementary Fig. S1b, left panel) with knockdown of BCAS2 showed significantly decreased viability compared with the control group during incubation for 3 days. We observed the same phenomenon in the PC-3 cells (Supplementary Fig. S1b, right panel), and those depleted of BCAS2 were more...
were located at the posterior region of wing discs from wandering third-instar larvae, where Drosophila apoptosis in with the overexpression of BCAS2 had lower levels of IR-induced DNA damage. LNCaP (overexpressing GFP (western blotting. The compared, as described above. Significantly depressed γ-H2AX levels were found in cells overexpressing BCAS2 compared with cells overexpression GFP (n = 4 for c, n = 3 for d; *P < 0.05; Mann–Whitney U test) alleviated the severity of IR-induced apoptosis in Drosophila. Apoptotic cells in wing discs were visualised and quantified by immunofluorescence microscopy using an anti-cleaved caspase-3 (C3) antibody after exposure to 10 Gy γ-radiation followed by a 6-h recovery period. The signals of engrailed-Gal4 driven GFP (green) were located at the posterior region of wing discs from wandering third-instar larvae, where UAS-dBCAS2dna (knockdown of BCAS2) and UAS-dBCAS2 (overexpression of BCAS2) were expressed. The GFP-negative anterior region of the wing discs and the wild-type (w1118) served as controls. Anti-C3 staining (red) revealed apoptotic cells in imaginal wing discs. Merge: merged images. Scale bar = 50 μm. Histograms of the results in (e) show comparisons of C3-positive foci numbers in imaginal wing discs without (f) or with (g) IR. A minor but non-significant increase (2.5-fold, P = 0.13) in C3 foci numbers was found in non-irradiated BCAS2-depleted wing discs compared with controls; in contrast, there was a significant increase (4.1-fold, P < 0.05) in C3 foci numbers in the irradiated BCAS2-depleted wing discs compared with control wing discs (n = 3 per group; ns. non-significant; *P < 0.05, Mann–Whitney U test).

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sensitive to both drugs. In contrast, the BCAS2-overexpressing LNCaP cells (Supplementary Fig. S1c, left panel) and PC-3 cells (Supplementary Fig. S1c, right panel) had significantly increased numbers of viable cells under treatment with doxorubicin or etoposide compared to those without BCAS2 overexpression. These results suggested that BCAS2 expression might diminish DSB-related death in PCa cells.

Drosophila wing discs (dBCAS2) alleviates IR-induced cell apoptosis in Drosophila wing discs in a p53-independent manner

To further examine the possibility that BCAS2 reduces cell death caused by DSBs, we used the Drosophila system because it is a valuable in vivo model in the study of DNA damage repair (DDR) and cell proliferation. The pathways of DSB repair and cell-cycle checkpoints in Drosophila, which are similar to those in humans, are easily manipulated and observed. Here, we examined the role of dBCAS2 in the apoptosis of wing discs of the third-instar larvae triggered by DSBs after exposure to IR (10 Gy), followed by a 6-h recovery period using immunofluorescence microscopy with anti-cleaved caspase-3 (C3) to reveal apoptotic cells. We used engrailed-GAL4 to drive the shRNA for dBCAS2 (UAS-dBCAS2*RNAi, Fig. 1e, middle two columns) and dBCAS2 cDNA (UAS-dBCAS2, Fig. 1e, right two columns) specifically in the posterior part of the wing discs where GFP was also significantly expressed. In contrast to the anterior part of the wing discs, where no obvious difference was observed, there was a significantly higher number (4.1-fold increase, P = 0.0004) of apoptotic foci (Fig. 1e, red foci, red foci, f, g) in the dBCAS2*RNAi wing discs than in the wild-type (w1118) after exposure to IR. However, the overexpression of dBCAS2 (Fig. 1e) significantly reduced the number of IR-related cleaved caspase-3-positive foci in the wing discs compared to the wild-type control (Fig. 1f, g). Furthermore, to determine whether the protective effect afforded by BCAS2 against IR-induced cell apoptosis is dependent on its negative regulatory effect on p53,15 we constructed a wing-specific driver (ms1096-GAL4) in dmp53 null mutant flies to specifically deplete dBCAS2 (Supplementary Fig. S2, dBCAS2*RNAi). The dBCAS2 knockdown effect was confirmed by immunostaining for Delta (Supplementary Fig. S2), a wing-disc-expressing protein that is encoded by a downstream gene of dBCAS2. Immunofluorescence microscopy showed that the number of IR-induced cleaved caspase-3-positive foci in the p53-null and dBCAS2-depleted wing discs was still significantly higher than in the p53-null and dBCAS2 wild-type flies (Supplementary Fig. S2). We also tried to use an antibody to stain γ-H2Av, the Drosophila γ-H2Av homologue, but we failed because the antibody was not suitable for immunostaining (data not shown). Collectively, our results suggest that BCAS2 functions in suppressing apoptosis induced by IR in vivo and that this effect is independent of p53 expression.

BCAS2 enhances in vitro and in vivo NHEJ efficiency in DSB repair

Next, we aimed to determine whether BCAS2 is involved in regulating the two major pathways that repair DNA DSBs: the HR pathway and the NHEJ pathway. To demonstrate that BCAS2 is involved in the NHEJ pathway, we performed a cell-free NHEJ assay as previously reported,22,23 in which EcoRI-digested pBSK (+) duplex plasmid DNA was incubated with purified HEK 293T nuclear extracts to ligate the DNA. The assay by agarose gel electrophoresis was followed by staining with a highly sensitive DNA dye to detect the re-joined and catenated DNAs (oligomers), which could be separated from the non-re-joined DNAs on the gel because the former migrated more slowly. The results showed that there were significantly fewer re-joined plasmid DNA oligomers in the nuclear extracts from the BCAS2-depleted HEK 293T cells than in those from the knockdown control (Fig. 2a). We further validated the results and measured the changes in NHEJ activity using a well-established cell-based system,24,25 in which a reporter plasmid pGL3 linearised by endonuclease SphiI was transfected into the HEK 293T cells, and the bioluminescence signal generated by a precise end-joining luciferase gene-containing plasmid was measured to represent NHEJ activity (Fig. 2b). The NHEJ activity in cells co-transfected with shBCAS2 plasmid (either shBCAS2 #1 or #2) was significantly decreased compared to the control, and shBCAS2 #2 plasmid exhibited a dose-dependent effect (Fig. 2b, middle panel). In contrast, the cells co-transfected with the BCAS2 expression plasmid showed a dose-dependent increase in NHEJ activity compared to the cells without BCAS2 overexpression, and they reached a maximum of twofold upregulation (Fig. 2b, right panel). Because cell death and cell-cycle changes may affect DSB repair, we examined such changes in our BCAS2-depleted versus the control HEK 293T cells used in DSB repair experiments. The results (Fig. 2c) showed that neither the apoptosis rate (represented by the percentage of sub-G1 phase, nor the percentage of cells in each cell-cycle phase (G1, S and G2/M) was significantly changed in the cells with BCAS2 knockdown compared to the control cells.

BCAS2 upregulates the efficiency of homologous recombination during DSB repair

Next, we examined whether BCAS2 participates in HR by co-transfecting HEK 293T cells with the BCAS2-knockdown (shBCAS2 #1, or #2) or BCAS2-overexpression (FLAG-BCAS2) plasmids and the HR reporter plasmid pmHPRT-DR GFP (Fig. 2d).29 The enzyme-digested upstream GFP, only when it was correctly repaired in vivo
by HR based on the sequence of downstream GFP fragment (Fig. 2d), could be translated into a functional GFP protein and quantified by flow cytometry. The number of GFP-positive cells was significantly diminished (control vs. shBCAS2 #1, 23.3 ± 4.7% vs. 9.5 ± 1.6%, $P < 0.05$) in the BCAS2-depleted HEK 293T cells compared to that in the control cells (Fig. 2e, left panel). The second shBCAS2 construct (shBCAS2 #2) also showed a significant reduction in GFP-positive cell numbers in the cells depleted of BCAS2 compared to the control (control vs. shBCAS2 #2, 24.1 ± 1.4% vs. 16.1 ± 1.8%, $P < 0.05$) (Fig. 2e, right panel). In contrast, in the BCAS2-overexpressing cells, the GFP-positive cell number was significantly higher than in control (control vs. FLAG-BCAS2, 31.8 ± 8.1% vs. 54.7 ± 5.6%, $P < 0.05$) (Fig. 2f). In addition, to evaluate the effect of BCAS2 knockdown on HR efficiency in human cells after...
Fig. 2  BCAS2 positively regulated DSBs repair by enhancing both non-homologous end joining (NHEJ) and homologous recombination (HR) activity. a – c The expression level of BCAS2 was positively correlated with NHEJ ability during DSB repair in vitro and in vivo. a In vitro precise end-joining assay showed repression of NHEJ activity by the downregulation of BCAS2. Linearised or non-linearised pBSK (+) DNA plasmids were assayed for their degrees of ligation by agarose gel electrophoresis followed by staining with GelRed, a highly sensitive fluorescent nucleic acid dye, after incubation with nuclear extracts from HEK 293T cells infected with lentiviruses with controlled shRNA (shscramble) or shRNA against BCAS2 (shBCAS2 #1 and #2) in two different (L: low; H: high) titres. The amounts of joined pBSK (+) dimer, trimer, and multimer were measured by a phosphorimager, and they represented the degree of NHEJ. End-joining efficiency was calculated as the percentage of oligomers (dimer, trimer, and multimer) in all DNAs (namely, the sum of the oligomers divided by the sum of the monomer and oligomers × 100%). A linearised monomer incubated with T4 DNA ligase served as a positive control; a linearised monomer (linearisation) without T4 DNA ligase treatment and a non-linearised plasmid were negative controls. Data on end-joining efficiency were normalised to those of control (shscramble) and presented as mean ± SD (middle panel; n = 3; *P < 0.05; Mann–Whitney U test). Quantification of western blotting results (right panel) demonstrated a significant knockdown effect on BCAS2 (n = 3; *P < 0.05; Mann–Whitney U test). b The overexpression and knockdown of BCAS2 showed a dose-dependent activating and repressive effects, respectively, on the precise end-joining activity in HEK 293T cells. The pGL3 plasmid containing the luciferase reporter gene was linearised by Sphi I and HindIII and then co-transfected into HEK 293T cells with an increasing amount of either the plasmid expressing the shRNAs for BCAS2 (shBCAS2 #1 and #2), or the plasmid expressing the BCAS2 cDNA tagged with three copies of flag sequence (3× FLG-BCAS2). The restored bioluminescence activity represented the in vivo-specific NHEJ activity that occurs in the enzyme-digested luciferase coding domain of pGL3 reporter plasmid. The second luciferase (Renilla) reporter plasmid was co-transfected, and it functioned as an internal control to eliminate differences in transfection efficiency between culture wells. The in vivo precise end-joining activity was measured using the Dual-Luciferase Reporter System, as described in “Methods”. The knockdown of BCAS2 (left and middle panels) showed a significant decrease in the precise end-joining activity in HEK 293T cells. The overexpression of BCAS2 (right panel) revealed the opposite effect. Western blotting confirmed the changes in BCAS2 protein level. Data are presented as means ± SD (n = 3; *P < 0.05; Mann–Whitney U test). c Flow cytometry analysis showed no significant differences (Mann–Whitney U test) in the phase distribution of the cell cycle between the BCAS2-depleted and control HEK 293T cells. Note that the percentage of cells in the sub-G1 phase may reflect the number of apoptotic cells. d–f BCAS2 upregulated HR activity. d Schematic illustration of the reporter plasmid pmHPRT-DR GFP plasmid, I-SceI endonuclease expression vector pCBAcBl and pDsRed plasmid (internal control). The pmHPRT-DR GFP plasmid is composed of two separate, differentially mutated GFP domains, in which the homologous sequences were oriented as direct repeats. The upstream I-SceI-GFP contained the recognition site for the rare-cutting I-SceI endonuclease and two in-frame stop codons, while the downstream sequence enclosed an 812-bp internal GFP fragment (iGFP). When the transfected I-SceI-containing plasmid was expressed and caused a DSB, the downstream internal GFP fragment sequence acted as a donor of the wild-type sequence for the broken I-SceI-GFP gene, which may be repaired through HR and then properly expressed. e, f The HR repair activity, represented by the number of GFP-positive cells, was then measured by flow cytometry in wild-type, BCAS2-depleted (e, shBCAS2 #1 and #2), and BCAS2-overexpressing (f, FLAG-BCAS2) HEK 293T cells. The transfection of pDsRed was used to assess transfection efficiency. The efficiency of HR was calculated as a ratio of GFP+/DsRed+ cells. The changes in BCAS2 protein levels were confirmed by western blotting (lower panels). Data are presented as mean ± SD (n = 3; *P < 0.05; Mann–Whitney U test).

IR, we examined the percentage of cells positive for both γH2AX and RPA2-pS4/8 foci (the latter as a representative of RPA-coated single-stranded DNA seen in the end processing of HR), in both the HEK 293T and PC-3 cells during a time course after IR (10 Gy) or without IR (control). The results showed that the knockdown of BCAS2 caused a significant reduction in the percentage of γH2AX and RPA2-pS4/8 foci double-positive cells in both cell lines at almost each time point of the examination after IR (Supplementary Fig. S3). Together, these results support the hypothesis that BCAS2 plays a positive regulatory role in HR during DSB repair.

BCAS2 directly interacts with NBS1 through the N-terminus of BCAS2, which is also required for enhancing the efficiency of HR. In comparing cellular proteins pulled down by GST-BCAS2 versus GST using mass spectrometry in our previous published work,15 we found that MRE11 was included in the long list of BCAS2-interacting proteins (Supplementary Table 2). However, because NBS1 functions as an adapter protein and contains two forkhead-associated domains that were reported to interact with a coiled-coil domain,10 we hypothesised that BCAS2 might interact with the MRE11 complex through NBS1, as BCAS2 also contains coiled-coil domains. We then decided to focus on NBS1 first. We produced GST-NBS1 and His-tagged BCAS2 bacterial fusion proteins and performed in vitro reciprocal pull-down assays (Fig. 3a, b). The results showed that His-BCAS2 bound to GST-NBS1 was detected by immunoblotting in the complexes that were pulled down by glutathione-agarose beads (Fig. 3a). Similarly, immunoblotting also identified His-BCAS2-bound GST-NBS1 in the complexes pulled down by the Ni-NTA agarose beads (Fig. 3b). In addition, to demonstrate that BCAS2 and NBS1 interact in vivo, we co-transfected V5-tagged NBS1 and FLAG-BCAS2 plasmids into HEK 293T cells and performed a co-immunoprecipitation assay. The results of immunoblotting indicated that V5-NBS1 was present in the complexes precipitated by an anti-FLAG antibody, and FLAG-BCAS2 was present in the complexes precipitated by an anti-V5 antibody (Fig. 3c). We also conducted confocal microscopy to evaluate the co-localisation status of BCAS2 and NBS1 in U2OS cells 8 h after irradiation. The Z-stack images (Fig. 3d, left panel) and intensity profiles (Fig. 3d, right panel) of the fluorescent signals showed that some, but not all, IR-induced NBS1 and BCAS2 foci overlapped well at the subcellular scale, which suggests that at least some NBS1 and BCAS2 proteins were recruited to the damaged sites and possibly worked in tandem during DDR. We also evaluated the co-localisation of NBS1 and BCAS2 in U2OS cells during a time course by calculating the Manders’ overlap coefficient in double-immunofluorescence microscopy at 2, 8, and 12 h after IR. The results showed that the overall Manders’ overlap coefficient increased significantly at 12 h after IR compared to that at 8 h (Fig. 3e; Supplementary Fig. S4a). To estimate the relative degree of HR after IR during the same time course, we performed immunofluorescence microscopy to detect RPA2-pS4/8, which is a readout for RPA-coated ssDNA. The result demonstrated that U2OS cells at 12 h after IR had a significantly increased percentage of RPA2-pS4/8 positivity compared to those at 2 h or those without IR (Fig. 3f and Supplementary Fig. S4b). In addition, we further map the domains in BCAS2 and NBS1 that are responsible for their mutual interaction, as we first produced a series of deletion proteins of GST-BCAS2 (see Fig. 3g, left panel, schematic representation) in bacteria and incubated each of them with lysates from cells transfected with V5-NBS1. The GST pull-down assay showed that all three C-terminal coiled-coil domain-deleted BCAS2 clones, but not the N-terminus-deleted clone were able to bind NBS1 (Fig. 3g, right panel), which suggests that the N-terminus was required for BCAS2 to interact with NBS1. Reciprocally, we incubated GST-BCAS2 with the lysates from the HEK 293T cells transfected with the full-length or each of the three...
V5-NBS1 deletion clones (Supplementary Fig. S5a, left panel) and performed a GST pull-down assay. The results showed that both the N-terminal and C-terminal V5-NBS1 clones, but not the middle region clone, retained the ability to associate with GST-BCAS2 (Supplementary Fig. S5a, right panel). Congruently, we found that the N-terminus of BCAS2 was required for the upregulation of HR efficiency, and the C-terminus of BCAS2, where coiled-coil domains are located, was dispensable (Fig. 3h). Moreover, we aimed to determine which BCAS2 domain is crucial in enhancing NHEJ by comparing the effect of overexpression of each BCAS2 construct on the precise end-joining efficiency (Fig. 3i).
Fig. 3 Validation of interaction between BCAS2 and NBS1 and identification of the NBS1-interacting domain in BCAS2. a, b In vitro pull-down assay. Bacteria-produced GST-NBS1 (a) and His-BCAS2 (b) proteins were incubated with bead-bound His-BCAS2 and GST-NBS1, respectively. Eluted proteins were analysed by western blotting using anti-His to detect His-BCAS2 or anti-GST antibody to detect GST-NBS1 (upper panels). GST only and GST-MDM2<sup>17</sup> fragments served as negative and positive controls, respectively. The proper expression of GST-NBS1 (a) and His-BCAS2 (b) proteins from bacteria were confirmed by Coomassie blue staining (lower panels). M, protein marker. c Reciprocal co-immunoprecipitation assay. HEK 293T cells were transiently transfected with expression vectors for V5-tagged NBS1 or control vectors, addition to either FLAG-tagged BCAS2 expressing plasmids or FLAG vector. Cell extracts were immunoprecipitated (IP) with anti-V5 and anti-FLAG antibodies and immunoblotted using the indicated antibodies. The levels of β-actin were used as loading controls in the input. d-f Evaluation of the co-localisation status of BCAS2 and NBS1 in U2OS cells. Confocal microscopy analysis (d, left panel) showed subcellular co-localisation of BCAS2 (red) and NBS1 (green) foci in a nucleus in Z-stack images (Y2 and X2). Scale bar = 10 μm. The intensity profiles (right panel) along selected lines (X1–X2, and Y1–Y2) demonstrated the overlapping fluorescence signals for BCAS2 and NBS1. e Quantitative image analysis of co-localisation was also performed using immunofluorescent images of BCAS2 and NBS1 in U2OS cells taken at 2, 8 and 12 h after ionising radiation (IR) (10 Gy). The mean over all Manders’ overlap coefficient of BCAS2 and NBS1 was calculated from 50 to 60 nuclei per coverslip using the software tool implemented in ImageJ via the JACoP plugin. The histogram shows that the mean overall Manders’ overlap coefficient of NBS1 and BCAS2 at 12 h after IR was significantly different (n = 3; *P < 0.05; Kruskal–Wallis test with Dunn’s test) greater than that at 8 h f. The percentages of cells with RPA2-pS4/8-positive foci during a time course after IR or without irradiation. Immunofluorescent microscopy analysis was performed to calculate the percentage of U2OS cells that were positive for phospho-RPA2 (RPA2-pS4/8) foci (the representative images are in Supplementary Fig. S4b) during a time course after IR. The histogram shows that the percentage of cells positive for RPA2-pS4/8 foci at 12 h after IR was significantly higher than that at 2 h or that without irradiation; the percentage at 8 h was also significantly higher than that without irradiation (100 cells per coverslip; n = 6; *P < 0.05, **P < 0.01, ***P < 0.001; Kruskal–Wallis test with Dunn’s test). g Identification of the NBS1-binding domain of BCAS2 and its requirement for homologous recombination (HR) and non-homologous end joining (NHEJ). Domains of BCAS2 were mapped using confocal microscopy (Fig. 3). The results showed that only the full-length BCAS2 possessed the ability to enhance HR activity (vector only). The data are presented as means + SD (n = 3; *P < 0.05; Mann–Whitney U test). cDNA deletion construct in HEK 293T cells. The results showed that only the full-length BCAS2 possessed the ability to enhance NHEJ activity; the loss of either the N-terminus or any coiled-coil domain at the C-terminus negated this ability (Fig. 3i). Together, these results indicated that the BCAS2–NBS1 interaction required the N-terminal domain of BCAS2 and both the N- and C-terminal domains of NBS1. Finally, to determine whether the endogenous BCAS2 and NBS1 proteins interacted with each other, we performed a reciprocal co-immunoprecipitation assay. HEK 293T cells, which confirmed that NBS1 was successfully captured by an anti-BCAS2 antibody, and vice versa (Supplementary Fig. S5b).

BCAS2 regulates DNA repair by facilitating NBS1 recruitment to DSBs. To explain how BCAS2 affects DNA repair on DDR, we hypothesised that BCAS2 regulates the function of NBS1 during DNA repair processes. The reason is that we showed that BCAS2 interacts with NBS1 both in vitro and in vivo. To prove the hypothesis, we performed a time-course experiment (Fig. 4), in which shBCAS2- or mock-transfected U2OS cells were exposed to IR (10 Gy). Immunofluorescence microscopy (Fig. 4a) was performed to compare the change in percentage of NBS1 foci or γ-H2AX foci-positive cells at different time points after IR. The results (Fig. 4a, b) showed that compared to the control, the knockdown of BCAS2 resulted in a significant reduction in the percentage of NBS1 foci-positive cells at 4 h (P < 0.05) and 8 h (P < 0.05) after IR. Moreover, there was a significant increase in the percentage of cells with γ-H2AX foci at 2, 4 and 8 h after IR. Similar results were obtained using the second set of shRNA against BCAS2 (shBCAS2 #2, Fig. 4c and Supplementary Fig. S6). Together, these results suggest that BCAS2 expression facilitates the association of NBS1 with DNA DSBs sites and increases the efficiency of DNA repair. BCAS2 expression increases in aggressive human PCA samples, correlates with β-catenin and NBS1 expression levels and it is associated with shorter survival in PCA patients.

To determine whether BCAS2 may serve as a marker of progression and prognosis of human PCA, we performed IHC (Fig. 5a) to stain BCAS2 and β-catenin, which is a potential downstream target of BCAS2, in tissue microarrays that contained benign prostate hyperplasia and PCA tissues. We sought to determine whether BCAS2 protein expression correlated with the expression scores and clinical profiles. The results showed that in general, the BCAS2 IHC scores tended to increase in PCA at higher Gleason grades (Fig. 5b) or higher pathology grades (Fig. 5c). Moreover, the IHC score of BCAS2 showed a moderate and positive correlation (Fig. 5d) with that of β-catenin (Spearman r = 0.4979, P < 0.0001). To complement the results of the IHC study and to evaluate the potential effect of BCAS2 expression on the survival of PCA patients, we performed a survival analysis using the data from The Cancer Genome Atlas. The result showed that compared to those with low BCAS2 mRNA levels, high levels of BCAS2 mRNA in PCA tissues were associated with shorter patient survival (P < 0.05) (Fig. 5e). Furthermore, in agreement with our results at the molecular level, we found a positive correlation (Spearman r = 0.233, P = 0.0001) between the mRNA levels of BCAS2 and NBS1 in PCA tissues (Supplementary Fig. S7a). Based on the mRNA expression level of NBS1, we divided PCA patients with high expressions of BCAS2 into two groups: one with high levels of NBS1 and the other with low levels of NBS1. The survival analysis showed that only the PCA tissues with high mRNA levels of both BCAS2 and NBS1 (Supplementary Fig. S7b, left panel), but not those with high BCAS2 and low NBS1 mRNA levels (Supplementary Fig. S7b, right panel), were significantly associated with shorter patient survival (P = 0.0067) compared with those PCA tissues with low levels of BCAS2 mRNA.
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DISCUSSION

Based on our results, we propose that BCAS2 may play a crucial role in DSB repair and have the potential to stimulate the activities of both major DSB repair pathways through interaction with NBS1, which is a key component in the MRN complex. Because cells need to decide whether DSBs are repaired by NHEJ or HR, and NBS1 has been reported to be involved in HR rather than NHEJ, it seems contradictory that BCAS2, a NBS1-interacting protein, is able to stimulate the activity of both NHEJ and HR. However, this dual ability also exists in the MRN complex itself because it has an enhancing effect on not only HR but also NHEJ. For instance, it was reported that mammalian cells lacking the component of the MRN complex exhibited reduced NHEJ activity or had impaired ability in executing events requiring NHEJ, such as programmed DNA rearrangement and the repair of DNA adducts. This paradox could be explained by the fact that, in addition to the MRN complex, other pathway-specific MRN-interacting proteins are required for cells to make the final decision regarding which pathway to take in DNA repair. The association with different sets of proteins may confer the MRN complex with the ability to enhance the activity of either pathway. Similar explanations may also apply to the situation of BCAS2. We hypothesise that BCAS2 may associate with certain pathway-specific proteins to participate in the decisive step towards or to inhibit those without the BCAS2 knockdown. The knockdown of BCAS2 was confirmed by western blotting (lower panel). Data are presented as means ± SD (50 cells per coverslip; n = 3; *P < 0.05; Mann–Whitney U test). 

The percentages of γ-H2AX foci-positive cells during a time course after irradiation with or without the knockdown of BCAS2. The depletion of BCAS2 significantly increased the percentage of irradiated cells with γ-H2AX foci at 2, 4 and 8 h. The data are presented as means ± SD (50 cells per coverslip; n = 3; *P < 0.05; Mann–Whitney U test).

In initiating either pathway, and is only passively recruited by different upstream master complexes to play a structural or adaptative role in subsequent events after cells have selected a pathway. In both scenarios, BCAS2, either directly or indirectly through NBS1, is able to interact with the proteins other than NBS1. Interestingly, our results showed that the BCAS2-mediated enhancement of NHEJ and HR required different protein domains of BCAS2. Both the in vitro domain mapping and the determination of the domain function in HR both showed that only the N-terminal was indispensable for BCAS2 to interact with NBS1 and to enhance HR. In contrast, the in vivo NHEJ assay showed that the full-length BACs2 possessed the capacity to stimulate NHEJ activity. Together, these results suggest that BCAS2 might use different domains to associate with non-NBS1 factors that may contribute to the enhancement of HR or NHEJ. This possibility is consistent with our hypothesis that through interaction with different pathway-specific factors, BCAS2 may acquire the ability to stimulate the activity of either DSB repair pathway, depending on which pathway-specific factors are predominantly present in the cells at that particular moment.

In this study, we demonstrated that there is a direct interaction between BCAS2 and NBS1, and it is likely that BCAS2 participates in DNA repair through NBS1. However, it remains to be determined exactly how BCAS2 regulates the activity of NBS1 or the MRN complex. We did not observe that BCAS2 increased the phosphorylation of NBS1 on Phos-tag acrylamide gel (data not shown). It is possible that BCAS2 regulates NBS1 by other

Fig. 4 Depletion of BCAS2 reduced NBS1-related DSB repair activities. a The BCAS2 shRNA (shBCAS2 #1)- or mock-transfected U2OS cells were exposed to 10 Gy ionising radiation (IR) and then harvested in a series of recovery time points (0.5, 1, 2, 4, and 8 h) for immunofluorescence microscopy to visualise NBS1-positive, γ-H2AX-positive and double-positive foci. Scale bar = 10 μm. b Knockdown of BCAS2 significantly decreased the percentage of NBS1 foci-positive cells during a time course after irradiation. U2OS cells containing five or more foci that were positive for both NBS1 and γ-H2AX simultaneously (a, lower panels, merged) in the nucleus were defined as NBS1 foci-positive. The percentages of NBS1 foci-positive cells with the knockdown of BCAS2 were significantly lower at 4 h and 8 h after irradiation than those without the BCAS2 knockdown. The knockdown of BCAS2 was confirmed by western blotting (lower panel). Data are presented as means ± SD (150 cells per coverslip, n = 3; *P < 0.05; Mann–Whitney U test). c The percentages of γ-H2AX foci-positive cells during a time course after irradiation with or without the knockdown of BCAS2. The depletion of BCAS2 significantly increased the percentage of irradiated cells with γ-H2AX foci at 2, 4 and 8 h. The data are presented as means ± SD (50 cells per coverslip; n = 3; *P < 0.05; Mann–Whitney U test).
mechanisms. For example, CtIP endonuclease interacts directly with NBS1, forming a CtIP–MRN complex and translocating to DNA break sites through the DSB sensor ability of MRN. The CtIP–MRN complex activates ATM kinase, which is followed by executing DSB resection to produce RPA-coated ssDNA, leading to cell-cycle checkpoint activation and HR repair. It is possible that BCAS2 facilitates the transition of MRN activities, from DSB sensing to engagement in the DSB repair processing, although BCAS2 does not have the Sae2-like domain of CtIP, which encodes a 5′ to 3′ endonuclease to remove small nucleotides from the ends. Another
Fig. 5: Higher expression of BCAS2 was detected in more aggressive human prostate cancer (PCa) and was associated with the expression of β-catenin. a Representative images of IHC staining for BCAS2 and β-catenin in PCa tissues. Lower expression levels in both BCAS2 and β-catenin were found in the same PCa tissue with a Gleason grade equal to 2 (left panels). Note that the upper and lower panels are from sections that are close to each other. Intermediate expression levels of both BCAS2 and β-catenin were observed in the same PCa tissue with a Gleason grade equal to 3 (middle panels). Higher expression levels in both BCAS2 and β-catenin were detected in the same PCa, where the highest Gleason grade equal to 5 (right panels). Brown, positive IHC signals by diaminobenzidine; light blue, haematoxylin counterstain. Scale bar = 100 μm. b The BCAS2 IHC scores significantly differed between benign lesions and Gleason grade 5 tumours, between Gleason grade 1 and grade 5 tumours, and between Gleason grade 1 and grade 2.5–3 tumours (*P < 0.05; Mann–Whitney U test). c The BCAS2 IHC score of pathology grade 3.5–4 tumours was significantly higher than those of grades 1–1.5, 2, 1–2 or 2–2.5 (P < 0.05; Mann–Whitney U test). d The BCAS2 expression level (represented as the IHC score) correlated with β-catenin expression level. The BCAS2 IHC scores exhibited a significant positive correlation with the β-catenin score (Spearman r = 0.4979, P < 0.001). e i) IHC staining patterns of BCAS2 and β-catenin in high-grade PCa (Gleason grade of 7). ii) Immunoblot showing the expression levels of BCAS2 and β-catenin in PCa tissues. 

In summary, using in vitro and in vivo models, we demonstrate that BCAS2 enhances the efficiency of DNA repair after IR-induced DNA damage. Our results also reveal that BCAS2 promotes the efficiency of both HR and NHEJ, possibly by interacting with and regulating NBS1. Moreover, we demonstrate that the expression of BCAS2 protein significantly increases in human PCa with highly malignant potential. We also show that the overexpression of BCAS2, especially when NBS1 is also overexpressed, is significantly correlated with the probability of shorter survival. Thus, our results may provide a basis for developing a novel strategy for treating human PCa by the genetic and/or pharmacological targeting of BCAS2.

ACKNOWLEDGEMENTS
We thank Dr. June-Tai Wu for providing Drosophila strains, Dr. Sheau-Yann Shieh for providing plasmids and Mr. Ping-Chang Kuo and Mr. Yu-Feng Chien for providing technical help.

AUTHOR CONTRIBUTIONS
L.-P.W. conducted major experiments and wrote the paper. T.Y.C. and C.K.K. performed flow cytometry, NHEJ assays and imaging and co-localisation study. H.-P.H. analysed the IHC staining, performed the statistical analysis and wrote the paper. S.-L.C. initiated and designed the project and edited the paper.

ADDITIONAL INFORMATION
Ethics approval and consent to participate This study was approved by the National Taiwan University Hospital Institutional Review Board (IRB # 201007060R). Most human tissues were provided by US Biomax Inc. and collected under protocols that are approved by The Health Insurance Portability and Accountability Act. Informed consent was obtained from the donors. The study was performed in accordance with the Declaration of Helsinki.

Consent to publish Not applicable.

Data availability The datasets used and/or analysed during this study are available from the corresponding author on reasonable request.
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