Title: BCAS2 promotes primitive hematopoiesis by sequestering β-catenin within the nucleus

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Running title: BCAS2 sequesters β-catenin within the nucleus

Abstract

Breast carcinoma amplified sequence 2 (BCAS2), a core component of the hPrP19 complex, plays an important role in RNA-splicing and DNA damage. However, whether BCAS2 has other functions within the nucleus remains largely unknown. Here, we show that BCAS2 is essential for primitive hematopoiesis in zebrafish and mouse embryos. The activation of Wnt/β-catenin signal, which is required for hematopoietic progenitor differentiation, is significantly decreased upon depletion of *bcas2* in zebrafish embryos and mouse embryonic fibroblasts. Interestingly, haploinsufficiency of *bcas2* has no obvious impact on the splicing efficiency of β-catenin pre-mRNA, while significantly attenuating β-catenin nuclear accumulation. Moreover, we find that BCAS2 directly binds to β-catenin via its coiled-coil domains, thereby sequestering β-catenin within the nucleus. Thus, our results uncover a previously unknown function of BCAS2 in promoting Wnt signaling by enhancing β-catenin nuclear retention during primitive hematopoiesis.

Key words: BCAS2, Wnt/β-catenin, coiled-coil domain, nuclear retention, primitive hematopoiesis

Introduction

Hematopoiesis refers to the lifelong process by which all blood cell lineages are generated. It begins at the early stage of embryonic development, providing the growing embryo with sufficient oxygen and nutrients.[1] Evolutionarily conserved across vertebrate species, hematopoiesis consists of two successive and partially overlapping waves: primitive and definitive. In mammals, the first wave of hematopoiesis occurs in the yolk-sac blood islands, producing primitive erythroid, megakaryocyte and macrophage progenitors, which can be observed in mouse embryos as early as embryonic day 7.25 (E7.25).[2-4] In zebrafish, primitive hematopoiesis initiates at around 11 hours post fertilization (hpf), when hemangioblasts emerge from the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM) and later differentiate into both hematopoietic and endothelial cells.[5-7]

Breast cancer amplified sequence 2 (BCAS2), also known as pre-mRNA splicing factor SPF27, is a 26-kDa nuclear protein containing two coiled-coil (CC) domains.[8] It was initially found to be overexpressed and amplified in human breast cancer cell lines.[9-11] Further studies have identified BCAS2 as a vital component of the human Prp19/CDC5L complex, which forms the catalytic ribonucleoprotein (RNP) core of spliceosome and is required for the activation of pre-mRNA splicing.[9,12,13] In Drosophila, the function of BCAS2 in RNA splicing is essential for cell viability.[14] In mouse, disruption of *Bcas2* in male germ cells impairs mRNA splicing and leads to a failure of spermatogenesis.[15] Additionally, BCAS2 also plays an important role in the

DNA damage response through the replication protein A (RPA) complex.[8,16] Interestingly, it has been shown that BCAS2 is a negative regulator of p53 by directly interacting with p53 or modulating alternative splicing of Mdm4, a major p53 inhibitor.[8,17] Zebrafish *bcas2* transcripts were enriched in the sites of both primitive and definitive hematopoiesis during embryonic development.[17] However, a previous study showed that p53 overactivation induced by zebrafish *bcas2* depletion did not affect primitive hematopoiesis, but impaired definitive hematopoiesis.[17] In recent years, several studies have highlighted the importance of regulating the expression and activity of p53 in primitive erythroid cell differentiation in both mouse and zebrafish embryos.[18-20] Thus, it is necessary to reexamine the exact function of BCAS2 in primitive hematopoiesis.

Wnt signaling, usually categorized into canonical and non-canonical pathways, is involved in the process of hematopoiesis.[21-23] Notably, the canonical Wnt signaling pathway, which is dependent on the nuclear accumulation of β-catenin to regulate gene transcription, controls primitive hematopoietic progenitor formation and promotes definitive hematopoietic stem cell (HSC) specification.[24-26] For instance, it has been demonstrated in *Xenopus* that, Wnt4-mediated activation of Wnt/β-catenin signaling plays a critical role in the induction and maintenance of primitive hematopoiesis.[27] Moreover, transient inhibition of canonical Wnt signaling in zebrafish embryos impairs embryonic blood formation.[28] However, previous studies utilizing human pluripotent stem cells revealed an opposite role of Wnt/β-catenin pathway in primitive progenitor

generation.[26,29] Therefore, the impact of Wnt/β-catenin signaling on primitive hematopoiesis remains elusive and even controversial. Moreover, it has been suggested that BCAS2 is important for neural stem cell proliferation and dendrite growth in mice by regulating β-catenin pre-mRNA splicing.[30,31] As a nuclear protein, it is unclear whether BCAS2 can modulate Wnt/β-catenin signaling in a splicing-independent manner.

In this study, we generated two zebrafish *bcas2* mutant lines, both of which exhibited defects in male fertility and embryonic HSC formation, similar to what was previously reported in mice and zebrafish.[15,17] More importantly, loss-of-function experiments suggest that BCAS2 is necessary for primitive hematopoiesis in both zebrafish and mouse embryos. We further find that *bcas2* is dispensable for the survival and proliferation of hematopoietic cells, but plays a crucial role in the differentiation of the hematopoietic lineage from hemangioblasts. Using a comprehensive approach, we reveal that BCAS2 is a nuclear retention factor for β-catenin during primitive hematopoiesis. Subsequent biochemical and functional experiments demonstrate that BCAS2 directly binds to β-catenin and suppresses its nuclear export to promote Wnt signal activation and hematopoietic progenitor differentiation. Furthermore, the CC domains on BCAS2 and the Armadillo (ARM) repeats on β-catenin are responsible for their interaction. Collectively, we have uncovered a novel function of BCAS2 in regulating Wnt/β-catenin signaling by sequestering β-catenin within the nucleus during primitive hematopoiesis.

Methods

Animal models

Our studies including animal maintenance and experiments were performed in compliance with the guidelines of the Animal Care and Use Committee of the South China University of Technology (Permission Number: 2023092). Six strains of zebrafish were used in this study, including Tübingen wild-type, *bcas2* mutant, *Cloche* mutant, *Tg(gata1a:GFP), Tg(kdrl:GFP),* and *Tg(hsp70l:dkk1b-GFP)*. *Cloche* mutant, *Tg(gata1a:GFP)* and *Tg(kdrl:GFP)* lines were provided by Professor Feng Liu (Chinese Academy of Sciences). *Tg(hsp70l:dkk1b-GFP)* strain was purchased from China Zebrafish Resource Center. $Bcas2^{Floxed/Floxed}$ ($Bcas2^{F/F}$) mouse line was generated as previously described.[15] Genotyping of $Bcas2^{F/F}$ mouse and *Flk1*-Cre mouse was performed using primers listed in Supplemental Table 1. The mouse model with *Bcas2* specifically disrupted in the hemangioblasts was derived from mating female $Bcas2^{F/F}$ mice with *Flk1*-Cre transgenic mice. All mouse lines were maintained on a mixed background (129/C57BL/6).

Cell lines and transfection

HEK293T, HeLa, SW480, and L cell lines were obtained from ATCC. Conditional *Bcas2* knockout (*Bcas2*-cKO) mouse embryonic fibroblasts (MEFs) were prepared from $Bcas2^{F/F}$ embryos at E13.5. Cells were cultured in Dulbecco's modified eagle's medium (DMEM, HyClone) supplemented with 10% fetal bovine serum (FBS,

127 HyClone) and 1% penicillin-streptomycin (HyClone) at 37 °C and 5% CO_2. L cell lines
128 expressing Wnt3a were maintained under similar conditions in the presence of 400
129 μg/ml G-418, from which Wnt3a conditioned medium (Wnt3a CM) was generated.
130 Culture medium prepared from L-cells was used as a control. To stimulate Wnt
131 signaling, cells were treated with Wnt3a CM in a 1:1 ratio with normal media. To
132 deplete *Bcas2* expression, *Bcas2*-cKO MEFs were cultured in medium containing 2 μM
133 tamoxifen for 72 h and the knockout efficiency was evaluated using western blot
134 analysis. The same cells cultured without tamoxifen were used as a control. To silence
135 *BCAS2* expression, shRNA constructs in pLL 3.7-GFP plasmid were generated to target
136 the following sequences: shRNA1, GAATGTGTAAACAATTCTA; shRNA2:
137 GAAGGAACTTCAGAAGTTA. Transfection was performed with Lipofectamine
138 2000 (11668019, Invitrogen) according to the manufacturer's instructions.
139

140 **Generation of CRISPR-Cas9-mediated *bcas2* knockout zebrafish**

141 The *bcas2* knockout zebrafish mutants were generated by CRISPR-Cas9 system as
142 previously described.[32] The guide RNA was designed to target the sequences 5′-
143 GGCGCAGCTGGAGCATCAGG-3′ within exon 4 of *bcas2*. Humanized Cas9 mRNA
144 and gRNA were co-injected into wild-type embryos at the 1-cell stage. Embryos or
145 adult fin clips were collected to prepare genomic DNA. To screen for mutant alleles,
146 the genomic regions containing gRNA-targeted sequences were amplified by
147 polymerase chain reaction (PCR) with primers listed in Supplemental Table 1. The PCR

products were sequenced or digested with T7 endonuclease or restriction enzyme FspI for genotyping.

RNA, morpholinos, and microinjection

Capped mRNAs for zebrafish *bcas2*, human *BCAS2*, *BCAS2 △CC1-2*, and mouse *ΔN-β-catenin* mRNA were synthesized from the corresponding linearized plasmids using an mMESSAGE mMACHINE T7 transcription kit (AM1344, Ambion). Morpholinos (MOs) were designed and purchased from Gene Tools: mismatch MO (cMO 5'-AGCCACTCATCCTGCTCCTCCCATC-3'), and *bcas2* translation-blocking MO (tMO; 5'-AGCGACTGATGCTGGTCCTGCCATC-3'). The mRNAs and morpholinos were injected into embryos at the 1- to 2-cell stage.

Whole-mount *in situ* hybridization

Digoxigenin-labeled and fluorescein-labeled probes were synthesized using a RNA Labeling kit (11175025910, Roche). Whole-mount *in situ* hybridization (WISH) and double fluorescence *in situ* hybridization for zebrafish embryos were performed following previously published methods.[33,34] Anti-digoxigenin-POD (11633716001, Roche) and anti-fluorescein-POD (11426346910, Roche) were used to detect digoxigenin-labeled probes and fluorescein-labeled probes, respectively. After WISH, the stained embryos were embedded in OCT and sections were prepared with a LEICA CM1900. The mouse yolk sac layers were separated as previously described.[35]

o-Dianisidine staining

To evaluate hemoglobin level, embryos were harvested at 36 hpf or 48 hpf, then stained with *o*-dianisidine as previously described.[36]

Proliferation and apoptosis assays

Embryos were incubated with 10 mM bromodeoxyuridine (BrdU) (B5002, Sigma) for 20 min. The incorporated BrdU was detected with anti-BrdU (B2531, Sigma) antibody. TUNEL staining was performed using *In Situ* Cell Death Detection Kit, TMR red (12156792910, Roche) according to the manufacturer's recommendation.

Heat shock treatment

To induce *dkk1* expression, *Tg(hsp70l:dkk1b-GFP)* embryos were subjected to heat shock (42°C) for 10 min at 10 hpf, and then collected at the indicated stage for WISH.

Dual reporter assay

HEK293T cells or MEFs were seeded in 24-well plates and transfected with a Super-TOPflash plasmid containing multimerized TCF-binding elements and a Renilla luciferase plasmid, along with the indicated vectors. Then cells were treated with 100 ng/mL LiCl and/or Wnt3a CM for 12 h and assayed for luciferase activity using the Dual luciferase system (E1910, Promega).

Immunoprecipitation, GST pulldown, and western blotting

For immunoprecipitation, HEK293T cells were transfected with the indicated plasmids and collected 48 h after transfection. Subsequently, HEK293T cells were lysed in a lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA and 0.5% Nonidet P-40) containing protease inhibitors. Immunoprecipitation was performed in accordance with the standard protocols.

For GST pulldown assay, GST, GST tagged β-catenin ARM 1-12 and His tagged BCAS2 were expressed in *Escherichia coli* BL21, then purified using Glutathione-Sepharose 4B beads (71024800-GE, GE Healthcare) and HisPur Ni-NTA beads (88831, Thermo Fisher), respectively. GST and GST-β-catenin ARM 1-12 proteins were immobilized onto Glutathione-Sepharose 4B beads and incubated with purified His-BCAS2 at 4°C for 4 h. Beads were washed three times and analyzed using western blotting.

Cytoplasmic and nuclear extracts were separated with nuclear and cytoplasmic extraction kit (CW0199, CWBIO). Proteins were analyzed by western blot using affinity-purified anti-Flag (F2555, Sigma), anti-HA (CW0092A, CWBIO), anti-β-catenin (M24002, Abmart), anti-BCAS2 (10414-1-AP, Proteintech), anti-Tubulin (CW0265A, CWBIO), anti-GFP (A11120, Thermo Fisher), anti-Histone H3 (ab1791, Abcam), anti-GST (SAB4200237, Sigma), and anti-His Tag (AF5060, Beyotime) antibodies.

Immunofluorescence staining

Cells on coverslips and embryos were processed for immunofluorescence staining as previously described.[37,38] Before fixation, *bcas2*-deficient MEFs were treated with a concentration of 20 μM MG132 or 20 nM leptomycin B (LMB) for 6 h, while *Tg(gata1a:GFP)* embryos were treated with 20 nM LMB from the bud stage to the 10-somite stage. Then the prepared samples were stained with anti-BCAS2 (10414-1-AP, Proteintech), anti-β-catenin (M24002, Abmart), and anti-GFP (A11122, Invitrogen). Meanwhile, 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI, 10236276001, Sigma) was used to label nuclei. Fluorescence imaging was performed using a Nikon A1R+ confocal microscope and all images were captured with the same settings.

Bimolecular fluorescence complementation assay

To construct the plasmids for Bimolecular fluorescence complementation assay (BiFC), BCAS2 was fused to the N-terminal half of yellow fluorescent protein (YN-BCAS2) and β-catenin to the C-terminal half (YC-β-catenin). YN-BCAS2 and YC-β-catenin were either individually or collectively transfected into HeLa cells. Fluorescence was detected 48 h after transfection using a Nikon A1R+confocal microscope.

Fluorescence recovery after photobleaching

BCAS2 and GFP tagged S37A-β-catenin were co-transfected into HeLa cells.

Fluorescence recovery after photobleaching (FRAP) assay was performed according to previously reported methods.[39] Images were acquired by a META Zeiss 510 confocal microscope.

RNA sequencing

Embryos were collected at the 10-somite stage and gently transferred into lysis buffer. Reverse transcription was performed using a SMARTer Ultra Low RNA Kit (Clontech, 634437) directly from the cell lysates. The cDNA library was prepared using an Advantage 2 PCR Kit (Clontech，639206) and then sequenced via the Illumina sequencing platform (NovaSeq 6000). The difference in the number of alternative splicing events between groups was analyzed using rMATS (version 4.1.0).

Reverse transcription PCR

Total RNA was isolated from wild-type and *bcas2* mutant embryos at the 10-somite stage with MicroElute Total RNA kit (OMEGA, R6831-01), followed by reverse transcription using ReverTra Ace qPCR RT Kit (Toyobo, FsQ-101). The cDNA was amplified with primers listed in Supplemental Table 2.

Quantification and statistical analysis

Images were quantified with Image J. Statistical data were analyzed using Graph Pad Prism. Comparisons between experimental groups were done using the Student's *t-test*.

Data are presented as mean ± standard deviation. Differences were considered significant at $P < 0.05$ and marked with "*", very significant at $P < 0.01$ and marked with "**".

Results

BCAS2 is necessary for primitive hematopoiesis

To confirm that *bcas2* is expressed in the posterior intermediate cell mass (ICM) where primitive hematopoiesis occurs in zebrafish, we first examined the spatiotemporal expression pattern of *bcas2* during zebrafish embryo development by performing whole mount *in situ* hybridization (WISH). The results showed that *bcas2* was ubiquitously expressed from 1-cell stage to 10-somite stage (Supplemental Figure 1A). Its expression in the ICM became detectable at 18 hours post fertilization (hpf) and was significantly elevated at 22 hpf (Figure 1A). We further observed that *bcas2* was co-expressed with the primitive erythropoietic marker *gata1* in the ICM at 22 hpf by fluorescence *in situ* hybridization (FISH) (Figure 1B). In contrast, *bcas2* was hardly detectable in the ICM in *cloche*$^{-/-}$ mutants that lack both endothelial and hematopoietic cells (Figure 1C). These results demonstrate a dynamic expression of *bcas2* in the ICM and imply a potential role of this gene in primitive hematopoiesis.

To gain insight into the developmental function of *bcas2*, we employed CRISPR/Cas9 system to generate *bcas2* mutants. Two mutant lines were obtained, designated *bcas2Δ7* (with a 7-base deletion) and *bcas2Δ14* (with a 14-base deletion).

These mutations led to premature translation termination, which resulted in truncated Bcas2 proteins lacking the C-terminal CC domains (Supplemental Figure 2A-B). *bcas2Δ7*$^{+/-}$ and *bcas2Δ14*$^{+/-}$ mutants were identified by T7 endonuclease I assay or restriction enzyme analysis (FspI) (Supplemental Figure 2C). We found that nearly 85% of the embryos derived from crossing *bcas*2$^{+/-}$ males with *bcas*2$^{+/-}$ females did not develop to the cleavage stage (Supplemental Figure 2D). Only 3% of the living embryos were homozygotes. In contrast, embryos obtained by crossing between wild-type males and *bcas*2$^{+/-}$ females were viable and showed normal morphology, with a heterozygosity rate consistent with Mendelian inheritance. This could be explained by male infertility as previously documented in *Bcas2* knockout mice.[15] Combining the above findings, we propose that Bcas2 may have an evolutionarily conserved role in spermatogenesis.

Given the difficulty of obtaining homozygous mutants, embryos lacking one copy of *bcas2* gene were produced from crosses between heterozygous females and wild-type males. We observed a significant decrease of *bcas2* expression in the ICM region in *bcas2Δ7*$^{+/-}$ or *Δ14*$^{+/-}$ mutants, likely resulting from nonsense-mediated RNA decay (Figure 1D). In line with a previous report,[17] a marked reduction in the expression of the HSC marker *cmyb* and T-cell marker *rag1* was found in *bcas2Δ7*$^{+/-}$ or *Δ14*$^{+/-}$ embryos at 5 hpf, indicating an essential role of *bcas2* in definitive hematopoiesis (Supplemental Figure 3A-B).

To explore whether *bcas2* is required for primitive hematopoiesis, we first examined

the expression of primitive erythropoietic markers *gata1* and *hbbe3* in *bcas2Δ7*$^{+/-}$ and *Δ14*$^{+/-}$ embryos at 22 hpf, and observed a marked decrease in the expression of these genes in the mutants (Figure 1E-F). Surprisingly, *o*-dianisidine staining showed similar hemoglobin contents in the *bcas2Δ7*$^{+/-}$ and *Δ14*$^{+/-}$ embryos at 48 hpf compared with control embryos, suggesting that the defect in primitive hematopoiesis induced by haploinsufficiency of *bcas2* was alleviated at later developmental stages. In order to further explore the role of BCAS2 in primary hematopoiesis, we identified several *bcas2Δ14*$^{-/-}$ mutants from about 100 embryos from the mating of *bcas2Δ14*$^{+/-}$ male and female. These homozygous mutants display a severe decrease in hemoglobin (Figure 1G). Similarly, injection of a translation-blocking MO into wild-type embryos to downregulate *bcas2* expression resulted in severe defects in erythropoiesis at 22 hpf and 48 hpf (Supplemental Figure 4A-D). These results indicate that *bcas2* is indispensable for primitive hematopoiesis in zebrafish. In addition, to induce the deletion of Bcas2 in mammalian endothelial/hematopoietic cells, transgenic mice expressing Cre recombinase under the control of the *Flk1* promoter were crossed to *Bcas2*$^{F/F}$ animals. We found that red blood cells were eliminated in the yolk sac of *Bcas2*$^{F/F}$*;Flk1-Cre* mice at E12.5 despite the presence of vessels (Figure 1H). Therefore, Bcas2 has a conserved role in vertebrates to regulate primitive hematopoiesis.

bcas2 deficiency impairs hematopoietic progenitor differentiation

The decrease of primitive hematopoietic cells in *bcas2* deficient animals may be

attributed to a number of possible causes: excessive apoptosis, hampered proliferation of hematopoietic cells, or impaired differentiation of hematopoietic progenitor cells. To shed light on this issue, we first performed terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay in *Tg(gata1:GFP)* embryos at the 10-somite stage to examine DNA fragmentations in apoptotic cells, and found no obvious apoptotic signal in the *gata1*$^{+}$ hematopoietic cells in either *bcas2Δ14*$^{+/-}$ embryos or their wild-type siblings (Supplemental Figure 5A). Meanwhile, BrdU incorporation assay revealed no significant difference in hematopoietic cell proliferation between *bcas2Δ14*$^{+/-}$ mutants and their corresponding wild-types (Supplemental Figure 5B-C). These data suggest that *bcas2* is dispensable for the survival and proliferation of hematopoietic cells.

In the developing embryo, hemangioblasts are derived from the ventral mesoderm at early somite stage, and then differentiate into both hematopoietic and endothelial lineages.[40,41] To test whether *bcas2* functions in cell fate decision during primitive hematopoietic cell development, the expression of hemangioblast markers *npas4l*, *scl*, and *gata2* in *bcas2Δ14*$^{+/-}$ embryos was examined at the 1- to 2-somite stage. As shown in Figure 2A, haploinsufficiency of *bcas2* did not affect the emergence of the hemangioblast population. Then we extended our analysis to include the markers of hematopoietic and endothelial progenitors. Consistent with the decrease in primitive hematopoietic cells in *bcas2* deficient mutants, a marked reduced expression of erythrocyte progenitor markers *gata1* and *hbbe3* was observed in the posterior lateral

337 mesoderm of *bcas2*$^{+/-}$ embryos at the 10-somite stage (Figure 2B). Interestingly, the
338 expression of myeloid progenitor marker *pu.1* was also dramatically decreased (Figure
339 2C). Moreover, overexpression of human BCAS2 enhanced the expression of *gata1* in
340 both wild-type and mutant embryos at the 10-somite stage (Figure 2D). In contrast, the
341 endothelial progenitor marker *fli1a* was expressed at a similar level in *bcas2*$^{+/-}$ embryos
342 as in wild-type animals (Figure 2E). Consistently, blood vessels appeared normal in
343 *bcas2*$^{+/-}$ mutants with *Tg(kdrl:GFP)* background at 54 hpf (Figure 2F). These data
344 provide convincing evidence that *bcas2* is required for the differentiation of the
345 hematopoietic lineage from hemangioblasts during primitive hematopoiesis.
346
347 **BCAS2 functions in primitive hematopoiesis by activating Wnt signaling**
348 Previous studies have shown that Wnt/β-catenin plays a key role in primitive
349 hematopoiesis.[27,28,42] As both BCAS2 and β-catenin-like 1 (CTNNBL1) are members
350 of the Prp19/CDC5L complex, which is a major building block of the spliceosome's
351 catalytic RNP core,[13] we speculate that BCAS2 may be a regulator of Wnt signaling
352 through interaction with β-catenin during hematopoiesis. To test our hypothesis, human
353 BCAS2 was overexpressed in HEK293T cells and MEFs. Ectopic expression of
354 BCAS2 enhanced the Wnt3a-induced expression of the TOPflash luciferase reporter in
355 a dose-dependent manner (Figure 3A-B). Importantly, Wnt3a-induced luciferase
356 activity in HEK293T cells could be effectively reduced by knockdown of *BCAS2* using
357 two shRNAs targeting different regions of human *BCAS2* (Figure 3C-D). Similar results

were also observed in *Bcas2*-cKO MEFs in the presence of tamoxifen (Figure 3E). These findings support that BCAS2 promotes Wnt signaling activation.

To confirm that Wnt signaling was required for zebrafish embryonic hematopoiesis, we induced the expression of canonical Wnt inhibitor Dkk1 by heat-shocking *Tg(hsp70l:dkk1-GFP)*^{w32} embryos at the bud stage.[43] As expected, diminished expression of *gata1* and *hbbe3* was detected in the resulting embryos at the 10-somite stage (Figure 3F-G). To validate that *bcas2* functions in primitive hematopoiesis via Wnt/β-catenin signaling, the expression pattern of β-catenin was examined in *bcas2* morphants with *Tg(gata1:GFP)* background at the 10-somite stage by immunofluorescent staining. The signals of both cytoplasmic and nuclear β-catenin were substantially decreased in hematopoietic progenitor cells (Figure 3H). Moreover, overexpression of ΔN-β-catenin, a constitutively active form of β-catenin, effectively restored the expression of *hbbe3* in *bcas2* morphants and mutants (Figure 3I-J). All these data suggest that BCAS2 functions in primitive hematopoiesis by regulating Wnt/β-catenin signaling.

BCAS2 promotes β-catenin nuclear accumulation independently of protein stability regulation

To investigate how BCAS2 regulates Wnt/β-catenin signaling, HEK293T cells were treated with LiCl, a canonical Wnt agonist that inhibits GSK-3β activity and stabilizes cytosolic β-catenin.[44] The results showed that TOPflash activity was significantly

elevated in LiCl-treated cells (Figure 4A). BCAS2 overexpression further upregulated, whereas shRNA-mediated knockdown of *BCAS2* downregulated LiCl-induced TOPflash activity (Figure 4A-B). Likewise, HEK293T cells transfected with S37A-β-catenin, a constitutively active form of β-catenin that is resistant to GSK-3β-mediated degradation, [45] displayed a much higher level of TOPflash activity, which was reduced by *BCAS2* knockdown (Figure 4C). These results strongly imply that BCAS2 regulates Wnt signaling downstream of β-catenin stability control.

To test the above hypothesis, we evaluated nuclear β-catenin level by performing immunofluorescence staining and immunoblotting experiments. Upon tamoxifen exposure, nuclear accumulation of β-catenin induced by LiCl was greatly inhibited in *Bcas2*-cKO MEFs, while nuclear/cytoplasmic fractionation suggested that cytoplasmic β-catenin level remained relatively unchanged (Figure 4D-E). Similarly, silencing *BCAS2* with shRNA led to reduced nuclear β-catenin in the human colon cancer cell line SW480, in which β-catenin was activated because of mutations in the adenomatous polyposis coli protein (APC), an integral component of the β-catenin destruction complex (Figure 4F).[46] Next, MG132, a proteasome inhibitor, was applied to activate Wnt/β-catenin signaling in *Bcas2*-cKO MEFs by inhibiting β-catenin degradation. In the absence of tamoxifen and MG132, endogenous β-catenin was localized almost exclusively in the cytoplasm; MG132 treatment dramatically triggered β-catenin accumulation in the nuclei (Figure 4G). However, in *Bcas2*-cKO MEFs exposed to tamoxifen, MG132 treatment was not able to induce nuclear accumulation of β-catenin

(Figure 4G). These findings indicate that BCAS2 promotes β-catenin nuclear accumulation in a manner that is independent of β-catenin stability regulation.

BCAS2 sequesters β-catenin within the nucleus

In addition to be affected by protein stability, the nuclear level of β-catenin is also fine-tuned by the opposing actions of nuclear export and import.[47-49] To examine the effect of BCAS2 on the nuclear import and export of β-catenin, fluorescent recovery after photobleaching (FRAP) experiments was carried out in HeLa cells expressing GFP-tagged S37A-β-catenin. After photobleaching the nucleus, no significant difference was found in the recovery of nuclear GFP signals between the cells with and without overexpression of BCAS2 (Supplemental Figures 6A, A', and C), suggesting that BCAS2 does not regulate β-catenin nuclear import. Conversely, after photobleaching the cytoplasm, BCAS2 overexpressed cells showed a much slower recovery of cytoplasmic fluorescence (Supplemental Figures 6B, B', and C), indicating that BCAS2 inhibits β-catenin nuclear export.

It has been suggested that the nuclear exit of β-catenin can be either dependent or independent on CRM1, a major nuclear export receptor.[50] To shed light on the mechanism underlying BCAS2 mediated β-catenin nuclear retention, we treated *Bcas2*-cKO MEFs with the CRM1-specific export inhibitor leptomycin B (LMB).[51] Regardless of the presence or absence of endogenous BCAS2, LMB treatment could effectively increase the level of β-catenin in the nucleus (Figure 5A). Consistently,

treatment of LMB was able to rescue the impaired nuclear accumulation of β-catenin in BCAS2-deficient SW480 cells (Figure 5B). Moreover, when *bcas2* morphants in *Tg (gata1:GFP)* background were treated with LMB from bud stage to 10 somite stage, the level of nuclear β-Catenin was partially recovered compared with that of control embryos (Figure 5C). Importantly, the expression of *gata1* was also restored in *bcas2* mutants upon LMB treatment (Figure 5D). Taken together, these findings suggest that BCAS2 negatively regulates CRM1-mediated nuclear export of β-catenin.

BCAS2 directly interacts with β-catenin in the nucleus

To investigate whether BCAS2 inhibits the nuclear export of β-catenin through physical binding, HEK293T cells were co-transfected with Flag-tagged β-catenin and HA-tagged BCAS2 constructs. Co-immunoprecipitation (Co-IP) experiments showed that Flag-β-catenin was precipitated with HA-BCAS2 as well as endogenous BCAS2, indicating an interaction between these two proteins (Figure 6A-B). In addition, the interaction was enhanced upon Wnt3a stimulation (Figure 6C). Given that Wnt ligand stimulation ultimately induces β-catenin nuclear accumulation, this enhanced interaction implies that BCAS2 associates with β-catenin within the nucleus. Therefore, we performed the bimolecular fluorescence complementation (BiFC) assay to visualize the interaction of BCAS2 and β-catenin in living cells. In this assay, the N-terminal fragment of yellow fluorescent protein (YFP) was fused to BCAS2 (YN-BCAS2), while the C-terminal fragment was fused to β-catenin (YC-β-catenin) (Figure 6D). As

expected, the YFP fluorescence was specifically observed in the nucleus (Figure 6E).

Previous studies have divided the β-catenin protein into three distinct domains, including the N-terminal domain (residues 1–133), the central domain with 12 ARM repeats (residues 134–670), and the C-terminal domain (residues 671–781).[52] To identify the BCAS2 binding site, constructs expressing various truncated forms of β-catenin were generated and co-transfected with BCAS2 into HEK293T cells (Figure 6F). Co-IP assays revealed that deletion of the N-terminal or C-terminal domain of β-catenin did not alter the interaction between β-catenin and BCAS2 (Figure 6G). In contrast, when the ARM repeats 1-12 of β-catenin were deleted, the resulting deletion mutant showed virtually no interaction with BCAS2 (Figure 6G). GST pull-down assay also demonstrated a direct interaction between BCAS2 and the ARM repeats of β-catenin (Figure 6H). These results indicate that BCAS2 physically binds to the ARM repeats of β-catenin. Furthermore, we found that the ARM repeats 9-12, but not 1-8, bound to BCAS2 (Figure 6G).

BCAS2 enhances β-catenin nuclear accumulation through its CC domains

To determine which domain of BCAS2 binds to β-catenin, we constructed a series of deletion mutants of BCAS2 (Figure 7A). Notably, we observed that among these truncated mutants, only the one lacking both CC1 and CC2 domains lost the ability to interact with β-catenin (Figure 7B). Moreover, these two CC domains alone or together could interact with β-catenin (Figure 7C). Therefore, we conclude that BCAS2 binds to

β-catenin via its CC domains.

We next examined whether the CC domains is required for BCAS2 to promote Wnt/β-catenin signaling. As shown in Figure 7D, overexpression of BCAS2 without the CC domains failed to increase LiCl-induced TOPflash activity in HEK-293T cells. Likewise, overexpression of the full-length BCAS2, but not BCAS2 lacking the CC domains, could restore the nuclear accumulation of β-catenin in *bcas2* morphants and *gata1* expression in *bcas2* mutants (Figure 7E-F). Collectively, these findings indicate that BCAS2 positively regulates Wnt signaling through sequestering β-catenin within the nucleus via its CC domains during primitive hematopoiesis.

As BCAS2 is involved in the Prp19-CDC5L spliceosome complex that regulates RNA splicing during spermiogenesis, neurogenesis, and definitive hematopoiesis,[15,17] we wondered if this protein participates in primitive hematopoiesis via mRNA alternative splicing. To this end, we performed RNA sequencing of 10-somite stage embryos to identify abnormal events in alternative splicing in *bcas2Δ14*$^{+/-}$ mutants. However, upon haploinsufficiency of *bcas2*, neither the number of five major types of alternative splicing events, nor the typical forms of alternative splicing were significantly affected (Supplemental Figure 7A-B). Additionally, haploinsufficiency of *bcas2* did not result in the alternative splicing of *mdm4* that predisposes cells to undergo p53-mediated apoptosis in definitive hematopoiesis, as reported previously by Yu *et al.* (Supplemental Figure 7C).[17,53] Furthermore, the splicing efficiency of β-catenin pre-mRNA remained almost unchanged in *bcas2Δ14*$^{+/-}$ mutants (Supplemental Figure 7C).

These results demonstrate that the defects in primitive hematopoiesis of *bcas2Δ14*$^{+/-}$ mutants are independent of the regulatory role of Bcas2 in pre-mRNA splicing.

Discussion

BCAS2 is a 26-kDa nuclear protein involved in a multitude of developmental processes, such as Drosophila wing development, dendritic growth, and spermatogenesis.[8,14-16,54,55] In our study, we generated *bcas2* knockout zebrafish. The heterozygotes not only showed male infertility, resembling the phenotype of *Bcas2* germ cell-specific knockout mice reported previously,[15] but also exhibited impaired definitive hematopoiesis, consistent with the earlier study.[17] Importantly, we found a marked decrease in the expression of the primitive erythroid progenitor markers *gata1* and *hbbe3* in these heterozygous mutants, which was rescued by overexpression of BCAS2. Moreover, the defective primitive hematopoiesis in zebrafish mutants was phenocopied in hemangioblast-specific *Bcas2* knockout mice. While the reason(s) for the discrepancy between our data and the observations made by Yu *et al.* regarding the role of *bcas2* in the development of primitive erythroid and myeloid cells remains to be determined,[17] our findings in zebrafish and mouse embryos provide solid evidence that BCAS2 plays a conserved role in primitive hematopoiesis.

As demonstrated in previous studies, BCAS2 is involved in various developmental events by regulating pre-mRNA splicing.[14,15,17,30,31] However, our data showed that haploinsufficiency of *bcas2* did not affect alternative splicing during primitive

hematopoiesis. These results imply that one copy of the *bcas2* gene is sufficient to support mRNA splicing in zebrafish. Instead, we find that Bcas2 promotes primitive hematopoiesis by sequestering β-catenin within the nucleus. It has been reported that the *bcas2* deletion in zebrafish embryos induces alternative splicing of *mdm4* that predisposes cells to undergo p53-mediated apoptosis in HSPCs during definitive hematopoiesis.[17] Intriguingly, we found that the loss of one copy of *bcas2* gene in zebrafish also resulted in severe impairment of HSPCs and their derivatives. It is possible that Bcas2 might also have a role in definitive hematopoiesis independent of its splicing regulatory function.

For the past decades, given the contradictory conclusions obtained from various *in vitro* and *in vivo* studies, the function of Wnt/β-catenin signaling in primitive hematopoiesis remains elusive and controversial.[26-29] In the present study, we have provided several lines of evidence supporting that Wnt/β-catenin signaling positively regulates primitive hematopoiesis: (1) Inhibition of Wnt/β-catenin by overexpression of the canonical Wnt inhibitor Dkk1 disrupts the formation of erythrocyte progenitors at the 10-somite stage. (2) Defects in primitive hematopoiesis in *bcas2* morphants and mutants are readily restored by overexpression of ΔN-β-catenin, a constitutively active β-catenin. (3) Overexpression of the full-length BCAS2, but not the CC domain-deleted BCAS2, restores the formation of the primitive erythroid progenitor in *bcas2* mutants. (4) BCAS2 overexpression enhances the development of primitive blood cells in wild-type embryos. All these data suggest that BCAS2-mediated Wnt/β-catenin signal

activation is necessary for primitive hematopoiesis.

The nuclear translocation of β-catenin is a key event in canonical Wnt signal activation. However, the precise mechanism for the nuclear-cytoplasmic shuttling of β-catenin is not completely understood.[50] It has been demonstrated that β-catenin can transit into and out of nucleus using distinct molecular interactions.[56-58] In some physiological contexts, β-catenin binds to APC and/or Axin proteins, which are actively co-exported from the nucleus by the nuclear export factor CRM1.[59-61] Furthermore, CRM1 can bind directly to and function as an efficient nuclear exporter for β-catenin independently of APC.[62] Our FRAP experiments reveal that BCAS2 is not involved in β-catenin nuclear import, but inhibits the nuclear export of β-catenin. Treatment of LMB, a CRM1-specific export inhibitor, could rescue the defect in β-catenin nuclear accumulation in BCAS2-deficient cells, implying that BCAS2 has an inhibitory role in CRM1-mediated nuclear export of β-catenin. We further show that BCAS2 interacts directly with β-catenin and enhances β-catenin nuclear accumulation through its CC domains. In the future, it will be interesting to investigate whether BCAS2 competes with APC, Axin, and/or CRM1 for binding to β-catenin.

In summary, we uncover a novel role of BCAS2 in primitive hematopoiesis through enhancing the nuclear retention of β-catenin. Our study provides new insights into the mechanism of BCAS2-mediated Wnt signal activation during primitive hematopoiesis. Given that BCAS2 and Wnt signaling are well documented to contribute to cancer development,[63-67] it is appealing to further explore whether our findings can be applied

547 to future cancer research.

548

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553

554 **Conflict-of-interest disclosure**

555 The authors declare no competing financial interests.

556

557 **References**

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Figures and figure legends

Figure 1

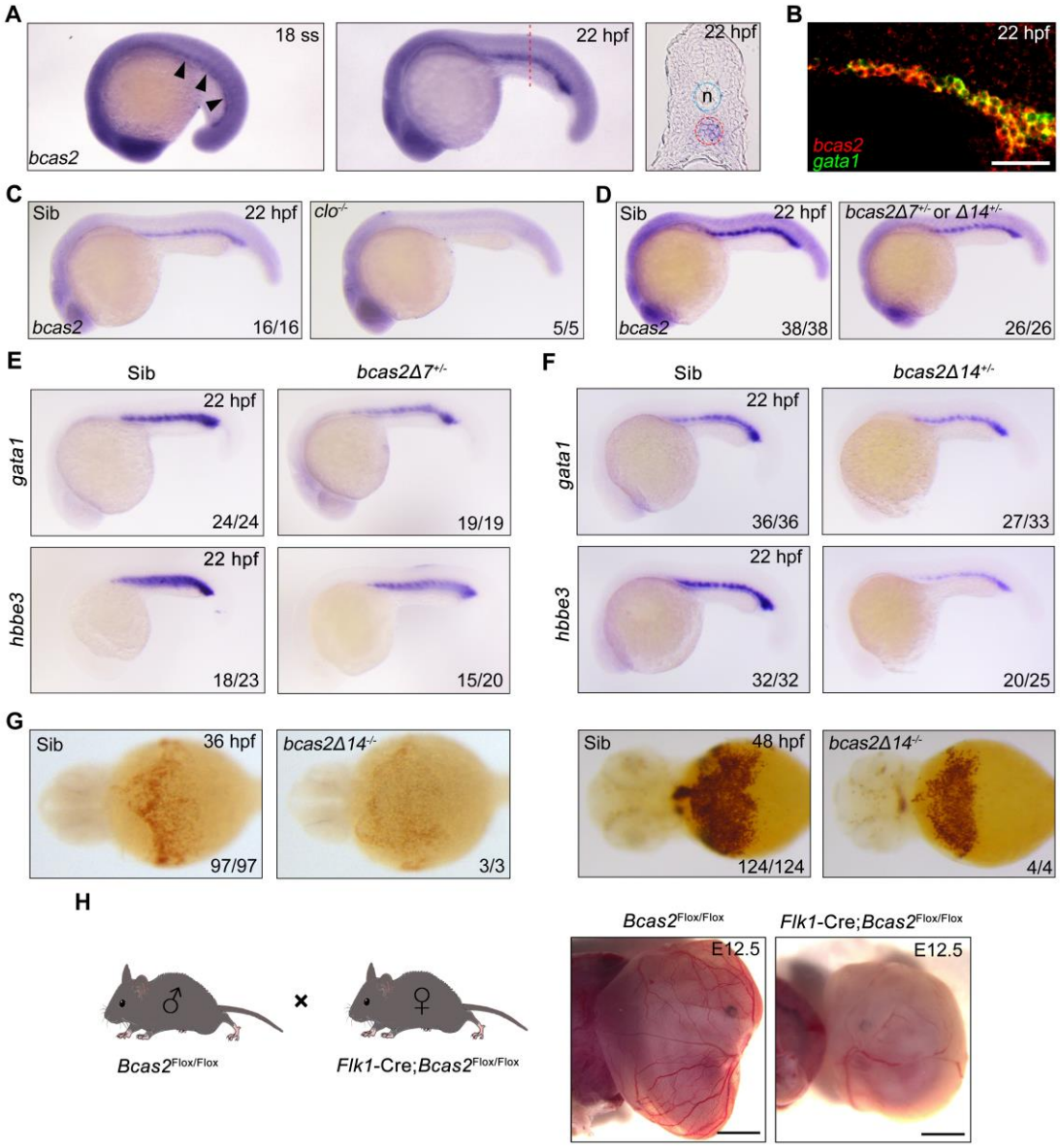
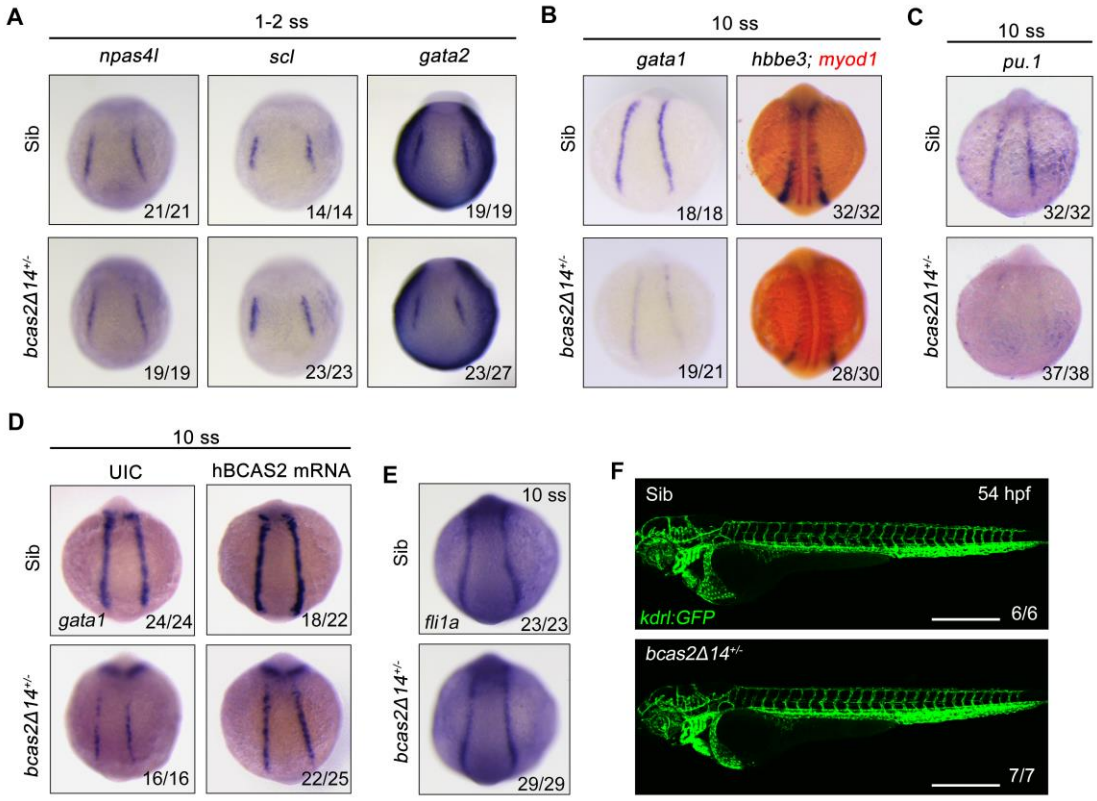


Figure 1. *bcas2* is expressed in the ICM and required for primitive hematopoiesis. (A) WISH assay showing *bcas2* expression in the ICM at the 18-somite stage and 22 hpf. The dotted lines represent the section position and the black arrowheads indicate the ICM region. n, notochord. (B) Double FISH assay showing the expression pattern of *bcas2* and *gata1* in the ICM at 22 hpf. Scale bar, 50 μm. (C-D) Comparison of *bcas2* expression in *cloche* mutants (C) or *bcas2* heterozygous mutants (D) along with their corresponding siblings. (E-F) Expression analysis of *gata1* and *hbbe3* in *bcas2Δ7*$^{+/-}$ and *bcas2Δ14*$^{+/-}$ embryos. (G) Hemoglobin detection using *o*-dianisidine staining in *bcas2* homozygous mutant at 36 and 48 hpf. (H) Representative images of yolk sac from the hemangioblast-specific *Bcas2* knockout mice and their siblings. *Bcas2*$^{F/F}$ females were crossed with *Bcas2*$^{F/+}$*;Flk1-Cre* males to induce the deletion of *Bcas2* in hemangioblasts. Scale bars, 1 mm.

Figure 2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npas4l
scl
gata2
Sib
bcas2Δ14+/-
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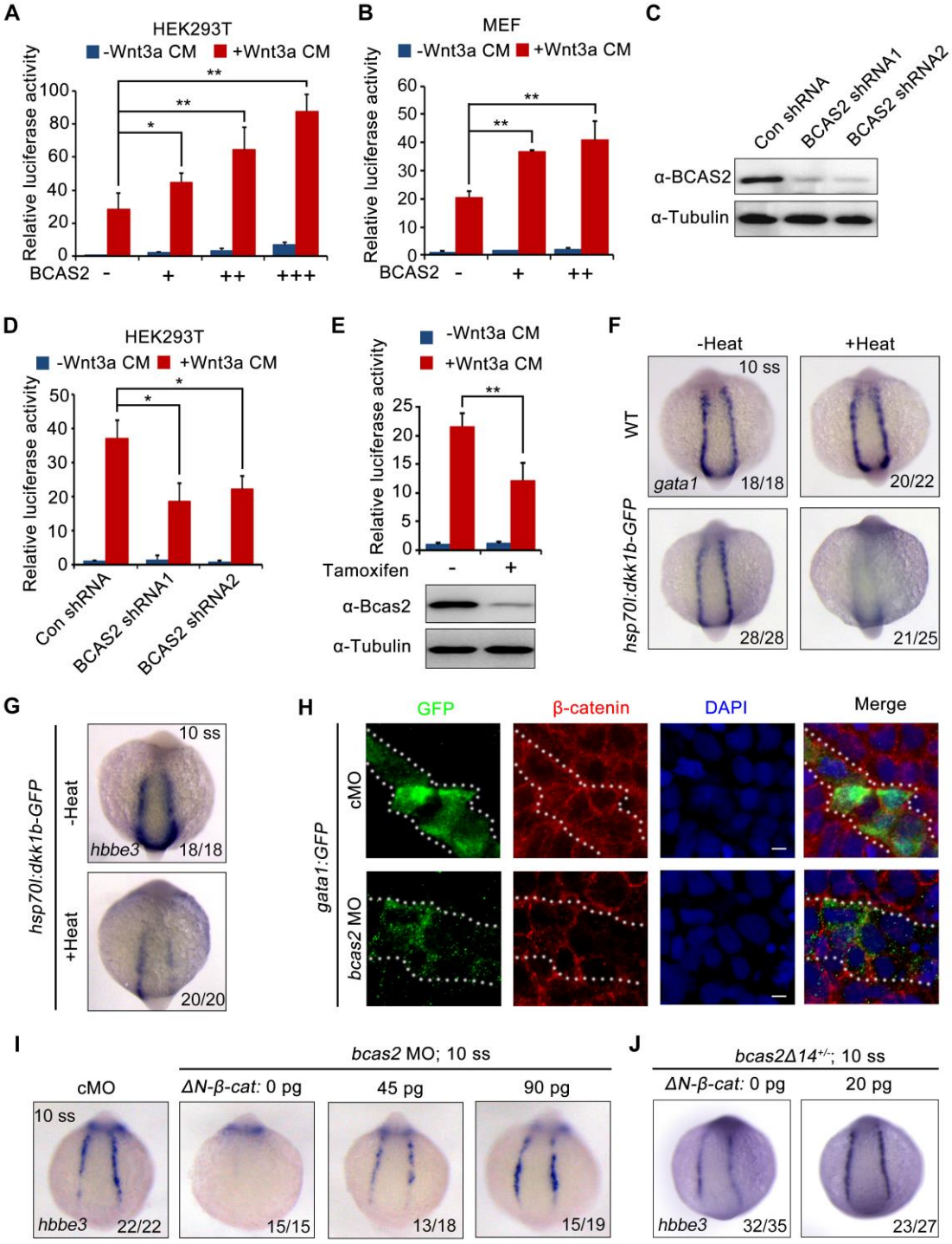
23/27
B
10 ss
gata1
hbbe3; myod1
18/18
32/32
19/21
28/30
C
10 ss
pu.1
32/32
37/38
D
10 ss
UIC
hBCAS2 mRNA
gata1
24/24
18/22
16/16
22/25
E
10 ss
fli1a
23/23
29/29
F
Sib
54 hpf
kdrl:GFP
6/6
bcas2Δ14+/-
7/7

Figure 2. *bcas2* is required for hematopoietic progenitor differentiation. (A-C) Expression analysis of hemangioblast markers *npas4l*, *scl, gata2* (A), erythroid progenitor markers *gata1*, *hbbe3* (B), and myeloid marker *pu.1* (C) in *bcas2Δ14*$^{+/-}$ embryos and their wild-type siblings at indicated stages. (D) Expression changes of *gata1* in *bcas2Δ14*$^{+/-}$ embryos overexpressing BCAS2 at the 10-somite stage. The indicated embryos were injected with or without 300 pg of human BCAS2 mRNA at the one-cell stage. (E) Expression of endothelial marker *fli1a* in *bcas2Δ14*$^{+/-}$ and sibling embryos at the 10-somite stage. (G) Confocal imaging of *bcas2Δ14*$^{+/-}$ and control sibling *Tg(kdrl:GFP*) embryos at 54 hpf. Scale bars, 500 μm.

Figure 3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Relative luciferase activity
BCAS2 - + ++ +++
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Relative luciferase activity
BCAS2 - + ++
C
Con shRNA
BCAS2 shRNA1
BCAS2 shRNA2
α-BCAS2
α-Tubulin
D
HEK293T
-Wnt3a CM
+Wnt3a CM
Relative luciferase activity
Con shRNA
BCAS2 shRNA1
BCAS2 shRNA2
E
-Wnt3a CM
+Wnt3a CM
Relative luciferase activity
Tamoxifen - +
α-Bcas2
α-Tubulin
F
-Heat
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gata1
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20/22
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DAPI
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gata1:GFP
cMO
bcas2 MO
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bcas2 MO; 10 ss
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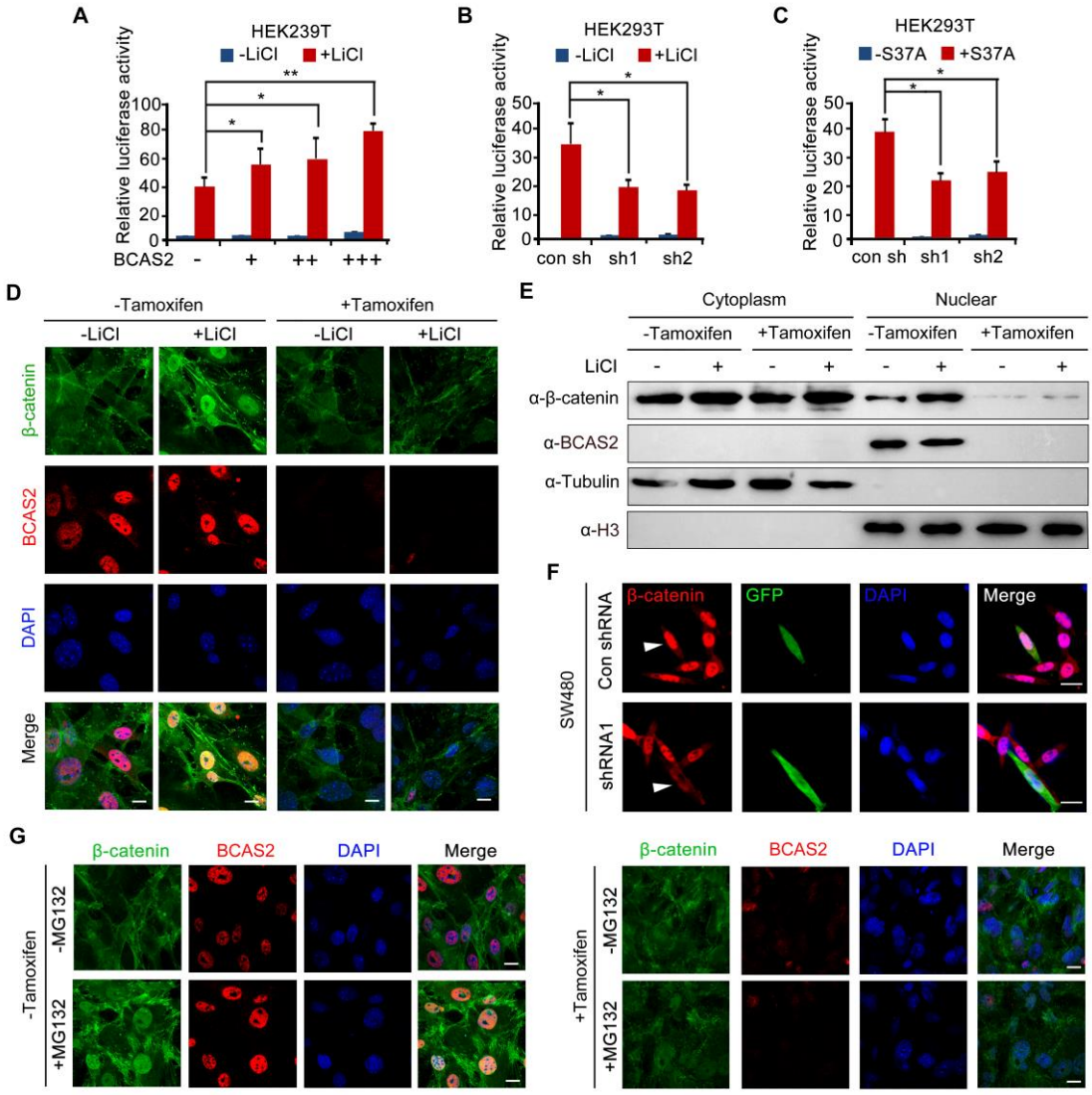
ΔN-β-cat: 0 pg
20 pg
hbbe3
32/35
23/27

Figure 3. BCAS2 promotes primitive hematopoiesis via activating Wnt signaling. (A-B) Overexpression of BCAS2 increases Wnt3a-induced TOPflash activity in HEK293T cells (A) and MEFs (B). Different amounts of plasmid expressing BCAS2 (0, 80, 160, or 320 ng/well) were transfected into cells, together with the super-TOPflash luciferase and Renilla luciferase vectors. After 36 h of transfection, cells were treated with or without Wnt3a CM for 12 h and harvested for luciferase assays. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test). (C-E) The Wnt3a-induced TOPflash activity is decreased in BCAS2-deficient cells. HEK293T Cells were co-transfected with shRNA plasmids, along with indicated plasmids, and harvested for western blot analysis (C) or luciferase reporter assay (D). *Bcas2*-cKO MEFs prepared from *Bcas2*$^{F/F}$ mouse embryos were incubated in medium containing 100 μM tamoxifen for 72 h and then subjected to western blotting and luciferase reporter assay (E). $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test). (F-G) Expression analysis of *gata1* (F) and *hbbe3* (G) in *Tg(hsp70l:dkk1b-GFP)* embryos after heat shock at 16 hpf. (H) Immunofluorescence staining of β-catenin in *Tg(gata1:GFP)* embryos at 16 hpf. The embryos were injected with 8 ng of the indicated MO at the one-cell stage and collected at the 10-somite stage. The dotted lines show the GFP-positive hematopoietic progenitor cells. Scale bars, 5 μm. (I-J) Expression of *hbbe3* in *bcas2* morphants (I) and *bcas2Δ14*$^{+/-}$ mutants (J) overexpressing ΔN-β-catenin. Embryos were injected with the indicated MO together with *ΔN-β-catenin* mRNA at the one-cell stage and harvested at the 10-somite stage for *in situ* hybridization.

Figure 4


A
HEK239T
-LiCl
+LiCl
Relative luciferase activity
BCAS2
-
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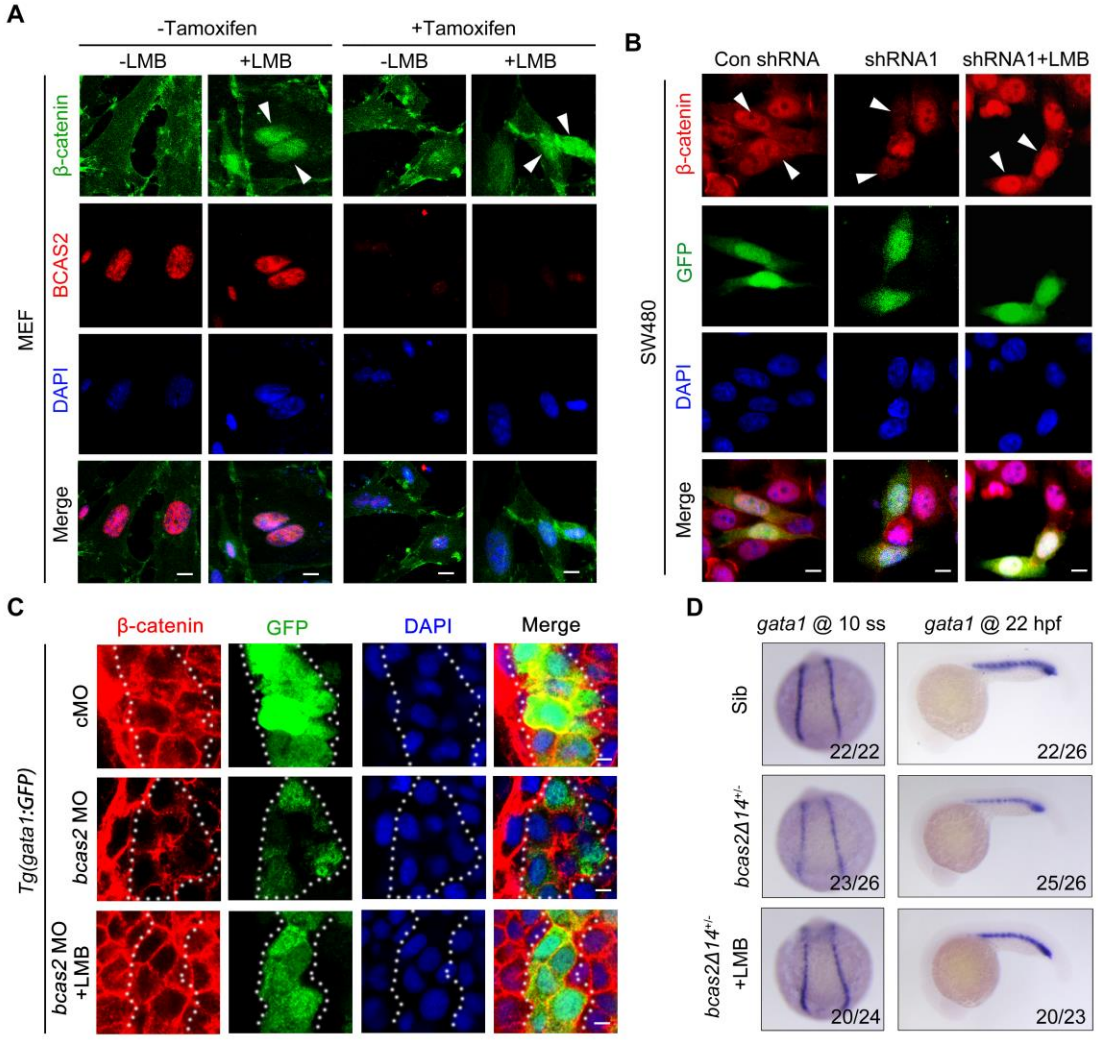
HEK293T
-LiCl
+LiCl
Relative luciferase activity
con sh
sh1
sh2
C
HEK293T
-S37A
+S37A
Relative luciferase activity
con sh
sh1
sh2
D
-Tamoxifen
+Tamoxifen
-LiCl
+LiCl
β-catenin
BCAS2
DAPI
Merge
E
Cytoplasm
Nuclear
-Tamoxifen
+Tamoxifen
LiCl
α-β-catenin
α-BCAS2
α-Tubulin
α-H3
F
SW480
Con shRNA
shRNA1
β-catenin
GFP
DAPI
Merge
G
β-catenin
BCAS2
DAPI
Merge
-Tamoxifen
+Tamoxifen
-MG132
+MG132

Figure 4. BCAS2 is essential for β-catenin nuclear accumulation. (A-C) BCAS2 enhances LiCl-induced TOPflash activity in HEK293T cells. Cells were transfected with BCAS2 expression plasmids (A), shRNA plasmids (B), or S37A-β-catenin expression plasmids (C), together with the TOPflash luciferase and Renilla luciferase vectors. After transfection, cells were subsequently treated with or without 100 ng/ml LiCl for 12 h and assayed for luciferase activity. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t-test*). (D-E) *Bcas2*-cKO MEFs were incubated with tamoxifen for 24 h and then treated with or without 100 ng/ml LiCl. The nuclear accumulation of β-catenin was analyzed using immunofluorescence (D) and western blotting (n=3) (E) Scale bars, 10 μm. (F) SW480 cells were transfected with the indicated shRNA constructs, and the endogenous β-catenin protein was detected using immunofluorescence 48 h after transfection. The expression of GFP served as a transfection control. The arrowheads indicate the cells transfected with indicated shRNA constructs. Scale bars, 10 μm. (G) *Bcas2*-cKO MEFs were cultured in the presence of tamoxifen for 24 h and then treated with 20 μM MG132 for 6 h. The expression of BCAS2 and β-catenin was measured by immunofluorescence. Scale bars, 10 μm.

Figure 5


A
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+Tamoxifen
-LMB
+LMB
-LMB
+LMB
MEF
β-catenin
BCAS2
DAPI
Merge
B
Con shRNA
shRNA1
shRNA1+LMB
SW480
β-catenin
GFP
DAPI
Merge
C
β-catenin
GFP
DAPI
Merge
Tg(gata1:GFP)
cMO
bcas2 MO
bcas2 MO +LMB
D
gata1 @ 10 ss
gata1 @ 22 hpf
Sib
bcas2Δ14+/-
bcas2Δ14+/- +LMB
22/22
22/26
23/26
25/26
20/24
20/23

Figure 5. BCAS2 functions in CRM1-mediated nuclear export of β-catenin. (A) Tamoxifen-treated *Bcas2*-cKO MEFs were incubated with 20 nM LMB for 3 h. The expression of Bcas2 and β-catenin was analyzed using immunofluorescence. The arrowheads show the cells with nuclear β-catenin accumulation. Scale bars, 10 μm. (B) SW480 cells were transfected with the indicated shRNA constructs and then treated with LMB for 3 h before immunostaining. GFP was regarded as a transfection control. The arrowheads indicate the transfected cells. Scale bars, 10 μm. (C) Immunofluorescence staining of β-catenin in *bcas2* morphants with *Tg(gata1:GFP)* background. Embryos were exposed to 20 nM LMB from the bud stage. The dotted lines indicate the GFP-positive hematopoietic progenitor cells. Scale bars, 5 μm. (D) *bcas2Δ14*$^{+/-}$ embryos were treated with 20 nM LMB for 6 h and then subjected to WISH assay to analyze the expression of *gata1* at the indicated stages.

Figure 6

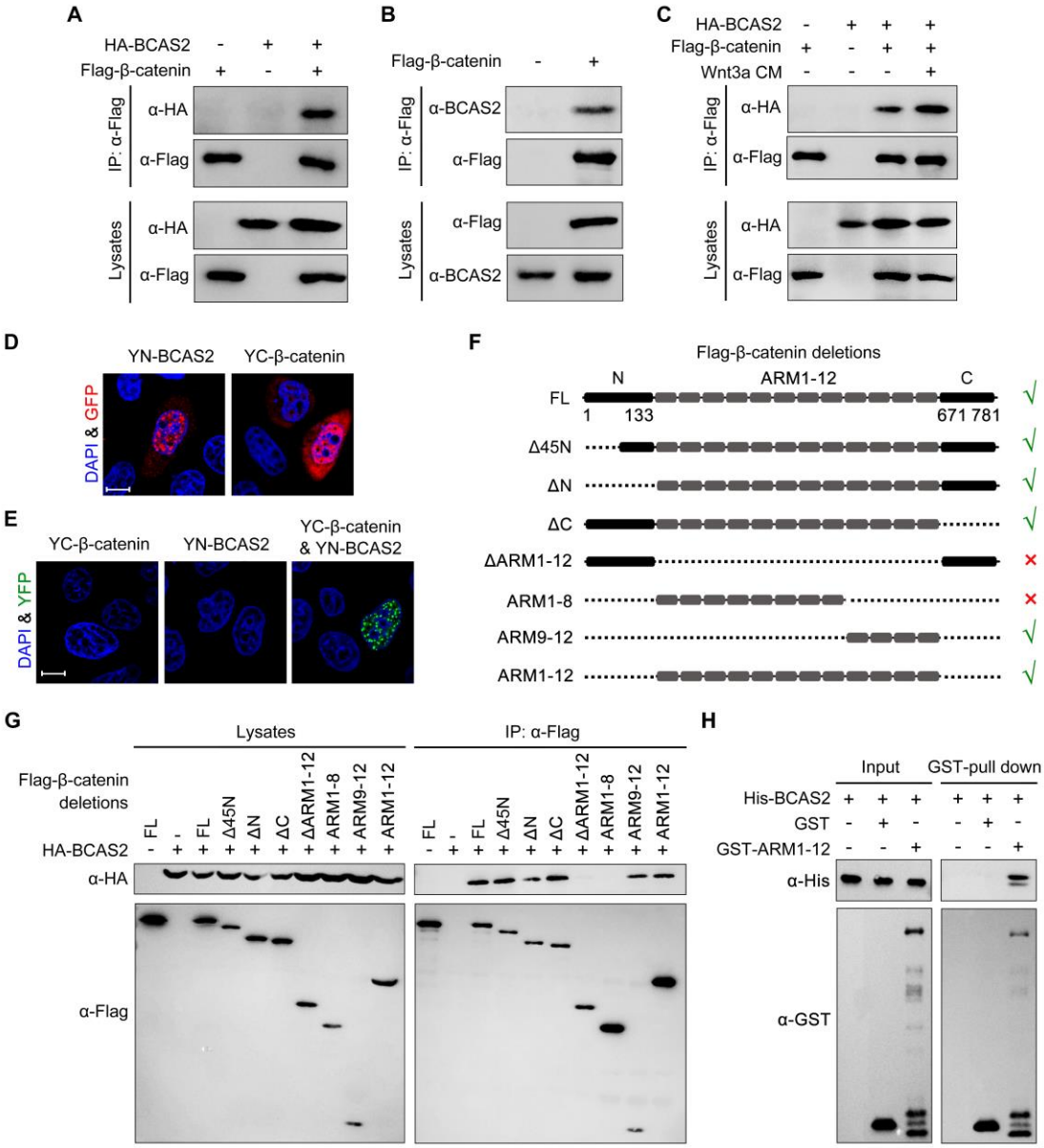
A
HA-BCAS2 - + +
Flag-β-catenin + - +
IP: α-Flag
α-HA
α-Flag
Lysates
α-HA
α-Flag
B
Flag-β-catenin - +
IP: α-Flag
α-BCAS2
α-Flag
Lysates
α-Flag
α-BCAS2
C
HA-BCAS2 - + + +
Flag-β-catenin + - + +
Wnt3a CM - - - +
IP: α-Flag
α-HA
α-Flag
Lysates
α-HA
α-Flag
D
YN-BCAS2
YC-β-catenin
DAPI & GFP
E
YC-β-catenin
YN-BCAS2
YC-β-catenin & YN-BCAS2
DAPI & YFP
F
Flag-β-catenin deletions
N
ARM1-12
C
FL
1
133
671 781
Δ45N
ΔN
ΔC
ΔARM1-12
ARM1-8
ARM9-12
ARM1-12
G
Lysates
IP: α-Flag
Flag-β-catenin deletions
HA-BCAS2
FL
Δ45N
ΔN
ΔC
ΔARM1-12
ARM1-8
ARM9-12
ARM1-12
α-HA
α-Flag
H
Input
GST-pull down
His-BCAS2
GST
GST-ARM1-12
α-His
α-GST

Figure 6. BCAS2 interacts with β-catenin. (A-C) Flag-tagged β-catenin was co-transfected with or without HA-tagged BCAS2 into HEK293T cells. Cell lysates were immunoprecipitated using anti-Flag antibody. Eluted proteins were analyzed by western blotting using indicated antibodies. In panel C, for Wnt signaling activation, cells were treated with Wnt3a CM for 5 h before harvest. (D-E) YN-BCAS2 and YC-β-catenin were either individually or collectively transfected into HeLa cells. The expression of YN-BCAS2 and YC-β-catenin was analyzed with anti-GFP antibody (D). The reconstituted YFP fluorescence in living cells was detected by confocal laser scanning microscopy with excitation at 488 nm (E). Scale bars, 10 μm. (F) Schematics of full-length and deletion mutants of β-catenin. (G) HEK293T cells were transfected with HA-tagged BCAS2 and Flag-tagged deletion mutants of β-catenin. Cell lysates were then immunoprecipitated using anti-Flag antibody followed by western blot analysis. (H) GST pull-down assays were performed using bacterially expressed GST, GST-ARM1-12, and His-BCAS2.

Figure 7

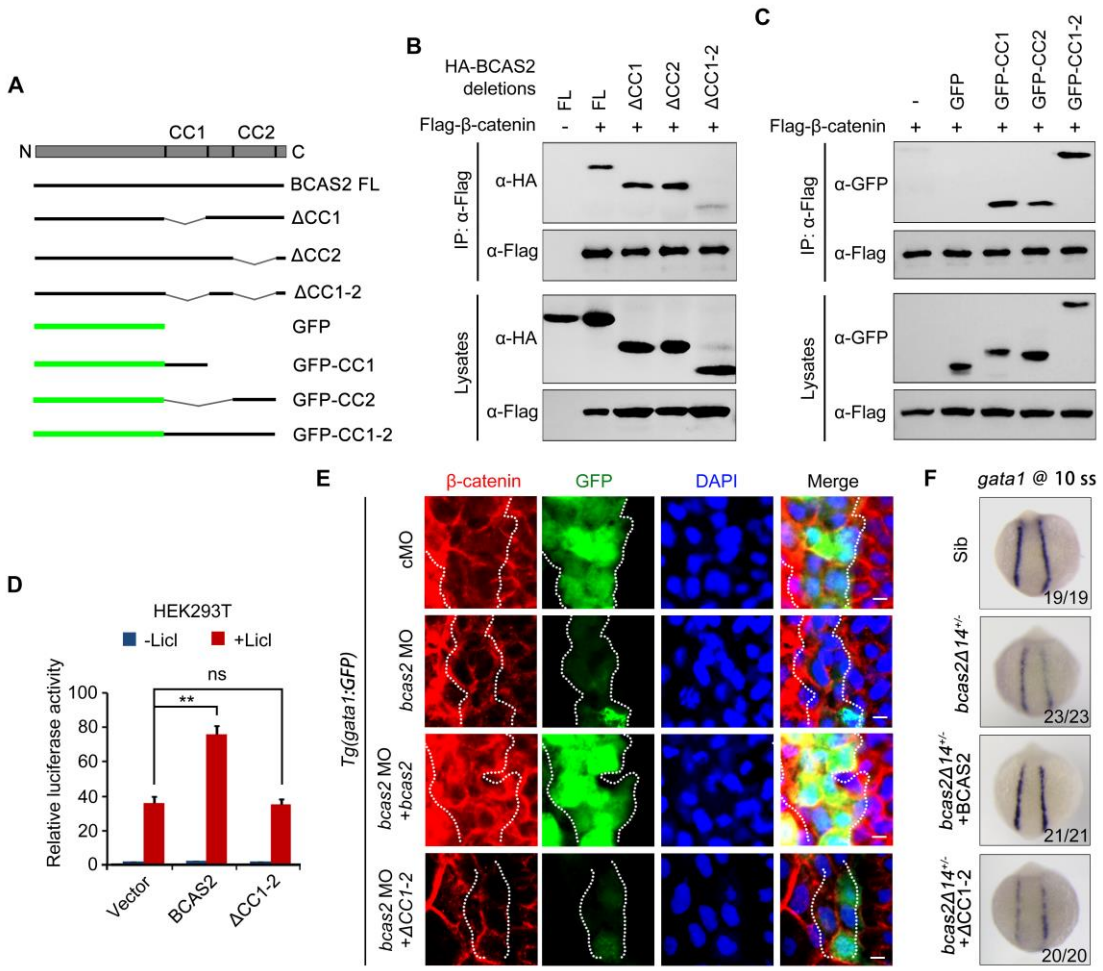

Figure 7. BCAS2 sequesters β-catenin in the nucleus via its CC domains. (A)

Schematics of full length and deletion mutants of BCAS2. (B-C) HEK293T cells were transfected with Flag-β-catenin and indicated deletion mutants of BCAS2. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody. Eluted proteins were immunoblotted using anti-HA (B) or anti-GFP antibodies (C) for BCAS2 detection. (D) HEK293T cells transfected with the indicated plasmids were treated with 100 ng/ml LiCl for 12 h, and then subjected to luciferase assay. Note that overexpression of BCAS2 without the CC domains failed to increase LiCl-induced TOPflash activity. Data represent the mean ± SD of three independent experiments. ns, not significant; $^{**}P < 0.01$ (Student's *t-test*). (E) Immunofluorescence staining of β-catenin in *Tg(gata1:GFP)* embryos injected with 8 ng *bcas2* MO and 300 pg of full length *BCAS2* mRNA or *ΔCC1-2* mRNA at the one-cell stage. Scale bars, 5 μm. (F) Transcripts of *gata1* were evaluated by WISH in *bcas2Δ14*$^{+/-}$ embryos injected with 300 pg of *BCAS2* mRNA or *ΔCC1-2* mRNA.