BCAS2 is essential for *Drosophila* viability and functions in pre-mRNA splicing

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ABSTRACT

Here, we show that dBCAS2 (CG4980, human Breast Carcinoma Amplified Sequence 2 ortholog) is essential for the viability of *Drosophila melanogaster*. We find that ubiquitous or tissue-specific depletion of dBCAS2 leads to larval lethality, wing deformities, impaired splicing, and apoptosis. More importantly, overexpression of hBCAS2 rescues these defects. Furthermore, the C-terminal coiled-coil domain of hBCAS2 binds directly to CDC5L and recruits hPrp19/PLRG1 to form a core complex for splicing in mammalian cells and can partially restore wing damage induced by knocking down dBCAS2 in flies. In summary, *Drosophila* and human BCAS2 share a similar function in RNA splicing, which affects cell viability.

Keywords: BCAS2; *Drosophila*; viability; splicing; Prp19 complex

INTRODUCTION

BCAS2 is a 26-kDa nuclear protein that contains two coiled-coil motifs and is expressed ubiquitously in normal human tissue (Kuo et al. 2009). Previously, we found that BCAS2 interacts directly with the tumor suppressor p53 and functions as a negative regulator (Kuo et al. 2009). Knocking down BCAS2 in wild-type p53 cancer cells, such as MCF7, LNCaP, and A549, induces programmed cell death. However, depletion of BCAS2 in p53-null cells (H1299) and p53 mutant (C33A) cells results in growth arrest at G2/M, suggesting that BCAS2 plays an essential role in the control of cell growth, in addition to the regulation of p53 (Kuo et al. 2009).

BCAS2 also was predicted to be a component of the spliceosome (Neubauer et al. 1998). The subspliceosomal complex, containing Prp19 (PSO4), Cef1 (CDC5L), Prp46 (PLRG1), and Snt309 (BCAS2), plays a critical role in yeast and human cells (Supplemental Fig. S1; Ajuh et al. 2000; Ohi and Gould 2002; Ohi et al. 2002, 2005; Grote et al. 2010). The yeast Prp19p-associated complex is required for

stable association of the U5 and U6 snRNP with the spliceosome, after release of U4 snRNP (Chan et al. 2003; Chan and Cheng 2005). Disruption of the yeast BCAS2 ortholog (*Cwf7* and *Snt309*) using genetic approaches results in the accumulation of pre-mRNA (Chen et al. 1998, 1999; Ohi and Gould 2002; Ohi et al. 2002). In addition, the Modifier of *snc1* 4 (MOS4) of *Arabidopsis thaliana* (the hBCAS2 ortholog in plants) has been shown to affect the splicing pattern of the *resistance* gene, *snc1* and *rps4* in plants (Xu et al. 2012). Human Prp19, CDC5L, and PLRG1 are known to be required for splicing (Ajuh et al. 2000, 2001; Grillari et al. 2005), but whether BCAS2 is essential for pre-mRNA processing remains unclear.

In *Drosophila melanogaster*, CG4980 encodes the hBCAS2 ortholog, which is predicted to be a component of the dPrp19 complex (Herold et al. 2009). Therefore, we used the fly as a model system in this study to examine the role of BCAS2 in cell viability, as well as the structure–function relationship in pre-mRNA splicing. Our results show that dBCAS2 and hBCAS2 share a similar function in pre-mRNA splicing. Depletion of dBCAS2 in the whole body leads to larval lethality, and knockdown of dBCAS2 using a tissue-specific promoter results in deformity phenotypes on notum and wings and in increased apoptosis in wing discs, which can be rescued by hBCAS2 with the restored RNA splicing

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function. Therefore, the abnormality caused by depletion of dBCAS2 is, at least in part, a consequence of impaired splicing.

RESULTS

BCAS2 is essential for *Drosophila* viability

A previous report indicated that depletion of hBCAS2 in wild-type p53 cells induces apoptosis, whereas in p53 mutant or null cells, it results in G2 growth arrest (Kuo et al. 2009). Therefore, in addition to the regulation of p53, we hypothesized that BCAS2 is also involved in the basic mechanisms that maintain regular cell growth. We used *D. melanogaster* as an animal model to investigate the function of BCAS2 in vivo. *Drosophila* BCAS2 encodes 278 amino acids (~31 kDa), and BLAST analysis demonstrated that the human and *Drosophila* BCAS2 protein orthologs are 59% identical over the entire sequence (Supplemental Fig. S2). We then used transgenic RNAi flies to examine the role of dBCAS2 using the UAS-GAL4 system. We first used an *Act5C-GAL4* driver to express *UAS-dBCAS2^{dsRNA}* ubiquitously and determined whether dBCAS2 is essential for fly survival. As shown in Table 1, the ratio of offspring with either genotype *Act5C-GAL4/T(2;3)SM6-TM6B* or *UAS-dBCAS2^{dsRNA}/T(2;3)SM6-TM6B* compared with *Act5C-GAL4/UAS-dBCAS2^{dsRNA}* was expected to be 2:1. The majority of third instar larvae *Act5C-GAL4/UAS-dBCAS2^{dsRNA}* died before the wandering stage, and <10% of the expected number survived to the pupae stage at P1–P2. In the end, no viable *Act5C-GAL4/UAS-dBCAS2^{dsRNA}* adult flies were observed, indicating that dBCAS2 is essential for fly viability and that complete knockdown of dBCAS2 is lethal. Because human and *Drosophila* BCAS2 are very similar (Supplemental Fig. S2), we next generated an hBCAS2 transgenic line to investigate whether hBCAS2 can rescue this lethality. We used *UAS-dBCAS2^{dsRNA}, UAS-hBCAS2/T(2;3)SM6-TM6B* crossed to *Act5C-GAL4/T(2;3)SM6-TM6B* and predicted that 310

TABLE 2. hBCAS2 partially rescues lethality caused by the *Act5C-GAL4*-driven *dBCAS2^{dsRNA}* in *Drosophila*

Progeny	<i>Act5C-GAL4/T(2;3)SM6-TM6B</i> or <i>UAS-dBCAS2^{dsRNA}, UAS-hBCAS2/T(2;3)SM6-TM6B</i>	<i>Act5C-GAL4/UAS-dBCAS2^{dsRNA}, UAS-hBCAS2</i>
Predicted ratio	2	: 1
Results (no.)	620	: 91
UAS-dBCAS2 ^{dsRNA} , UAS-hBCAS2/T(2;3)SM6-TM6B was crossed with <i>Act5C-GAL4/T(2;3)SM6-TM6B</i> , and 91 <i>Act5C-GAL4/UAS-dBCAS2^{dsRNA}, UAS-hBCAS2</i> flies survived and showed no obvious defects (data not shown).		

(620/2) flies would express *UAS-dBCAS2^{dsRNA}, UAS-hBCAS2*. As shown in Table 2, we found that 29.4% ($n = 91$, 91/310) of the adult progeny *Act5C-GAL4/UAS-dBCAS2^{dsRNA}, UAS-hBCAS2* were able to survive with no obvious defective phenotype. Expression of *dBCAS2* and *hBCAS2* was confirmed in surviving progeny by RT-PCR (Supplemental Fig. S3). These results suggest that hBCAS2 could compensate for the loss of dBCAS2 function and thus partially rescue the lethality of dBCAS2-depleted flies.

In order to find visible phenotypes induced by knocking down dBCAS2, we chose a tissue-specific *ms1096-GAL4* driver that is expressed in the pouch region of wing discs (Milan et al. 1998) to knock down dBCAS2. All of the progeny carrying *UAS-dBCAS2^{dsRNA}* exhibited growth defects at the notum and wings, such as an irregular scutellum margin (Fig. 1A, panel c) and twisted and shrunken wings (Fig. 1A, panel d). The *apterous-GAL4* (*ap-GAL4*) driver that is expressed in the dorsal compartment of third instar larvae wing discs (Diaz-Benjumea and Cohen 1993) was selected to confirm *UAS-dBCAS2^{dsRNA}*-induced wing deformation. All the flies carrying *UAS-dBCAS2^{dsRNA}* (Supplemental Fig. S4B,H,N) showed defects such as discoloration of the notum; irregular scutellum margin; abnormal number and length of bristles, which appeared similar to *Stubble* (no *Stubble* mutant background) (Supplemental Fig. S4H); and twisted and shrunken wings (Supplemental Fig. S4N). However, flies expressing *hBCAS2* and *dBCAS2^{dsRNA}* under the control of *ms1096-GAL4* had restored phenotypes with full recovery of the scutellum margin (Fig. 1A, panel g) and wing shape, compared with wild-type flies (Fig. 1A, panel a,b) and flies expressing *hBCAS2* (Fig. 1A, panel e,f). But the wing veins in flies expressing *hBCAS2* and *dBCAS2^{dsRNA}* were only partially recovered, the anterior crossvein (ACV) between longitudinal veins L3 and L4 appearing to be absent (Fig. 1A, panel h, arrow) and the posterior crossvein (PCV) between L4 and L5 looking truncated (Fig. 1A, panel h, arrowhead; Blair 2007). But when *UAS-dBCAS2^{dsRNA}, UAS-hBCAS2* was expressed using the *ap-GAL4* driver (Supplemental Fig. S4D, E), we only observed partial rescue (Supplemental Fig. S4D, E, arrow mark) of the wing shape. The different rescue effects

TABLE 1. The *Act5C-GAL4* driven *UAS-dBCAS2^{dsRNA}* in *Drosophila* causes lethality

Progeny	<i>Act5C-GAL4/T(2;3)SM6-TM6B</i> or <i>UAS-dBCAS2^{dsRNA}/T(2;3)SM6-TM6B</i>	<i>Act5C-GAL4/UAS-dBCAS2^{dsRNA}</i>
Predicted ratio	2	: 1
Results (no.)	560	: 0

The genotypes of the parents were *Act5C-GAL4/T(2;3)SM6-TM6B* and *UAS-dBCAS2^{dsRNA}/T(2;3)SM6-TM6B*. The predicted progeny were *Act5C-GAL4/UAS-dBCAS2^{dsRNA}*, *Act5C-GAL4/T(2;3)SM6-TM6B*, and *UAS-dBCAS2^{dsRNA}/T(2;3)SM6-TM6B*. The ratio of offspring with either *Act5C-GAL4/T(2;3)SM6-TM6B* or *UAS-dBCAS2^{dsRNA}/T(2;3)SM6-TM6B* to *Act5C-GAL4/UAS-dBCAS2^{dsRNA}* was expected to be 2:1. The results show an absence of the *Act5C-GAL4/UAS-dBCAS2^{dsRNA}* genotype from the total number of 560 progeny flies.

FIGURE 1. BCAS2 is essential for normal wing development. (A) *hBCAS2* rescues growth defects in notum and wings induced by knocking down *dBCAS2*. Parental genotype (from top to bottom): *ms1096-GAL4* crossed with either (1) *w¹¹¹⁸*, (2) *UAS-dBCAS2^{dsRNA}/T(2;3)SM6-TM6B*, (3) *UAS-hBCAS2*, or (4) *UAS-dBCAS2^{dsRNA}/UAS-hBCAS2/T(2;3)SM6-TM6B*. Scale bar, 0.5 mm. The red arrow indicates the anterior crossvein (ACV) between longitudinal veins L3 and L4, and the arrowhead indicates the posterior crossvein (PCV) between L4 and L5. (B) Expression of *dBCAS2* and *hBCAS2* described above. RNA was isolated from the wing discs of the third instar larvae, and mRNA of *dBCAS2* was measured by quantitative RT-PCR (left panel). (Upper right panel) The mRNA expression of *hBCAS2* was analyzed by RT-PCR. The internal control, *rp49*. (Lower right) Genotypes of each fly.

between these drivers (*ms1096-GAL4*, *ap-GAL4*, and *Act5C-GAL4*) could be accounted for by the strength of the tissue-specific promoter during wing development.

Because *UAS-dBCAS2^{dsRNA}* and *UAS-hBCAS2* were driven by one set of *GAL4*, it is possible that the activity of the *GAL4* activator was diluted and that the rescue effects resulted from decreased expression of small interfering RNAs. To exclude this possibility, we generated *Drosophila ap-GAL4*, *UAS-mCD8-GFP/UAS-dBCAS2^{dsRNA}*, which contained two *UAS* targeting sites, to drive *UAS-mCD8-GFP* and *UAS-dBCAS2^{dsRNA}* simultaneously. The fly *ap-GAL4*, *UAS-mCD8-GFP/UAS-dBCAS2^{dsRNA}* (Supplemental Fig. S4F,L,R) showed

similar defective phenotypes to those with *UAS-dBCAS2^{dsRNA}* alone (Supplemental Fig. S4B,H,N) indicating that the rescue effect is not due to titration of the RNAi by *hBCAS2*. In addition, the efficiency of *dBCAS2* knockdown in *UAS-dBCAS2^{dsRNA}* (Fig. 1B, left panel, lane 3) was nearly the same as *UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2* (Fig. 1B, left panel, lane 4), as measured by quantitative RT-PCR. Likewise, *hBCAS2* expression in *UAS-dBCAS2^{dsRNA}* and *UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2* was the same as analyzed by RT-PCR (Fig. 1B, right panel, lanes 2,4). The titration effects of *GAL4* can be ruled out by using one or two *UAS* constructs. The *dBCAS2* RNAi line #26676 from VDRC has a potential off target hit against *CG18375* (*dASPP*), which is highly expressed in wing discs, and mutants in *dASPP* demonstrate a wing overgrowth phenotype (Langton et al. 2007). To rule out this off-target effect, we measured the expression of two *dASPP* encoded transcripts (RA and RB) in *dBCAS2*-knockdown flies using quantitative RT-PCR. As shown in Supplemental Figure S5, there was no significant change from the wild type, suggesting that the wing deformation caused by *dBCAS2* RNAi line #26676 is not an off-target effect on the expression of *dASPP*. Taken together, these findings indicate that human and *Drosophila* BCAS2 share a similar function and that human BCAS2 can rescue the defects caused by depleting *Drosophila* BCAS2.

***dBCAS2* is essential for RNA splicing**

Because *dBCAS2* is predicted to be a member of the subsplicingosomal complex, we determined next whether the defective growth of *dBCAS2*-knockdown flies is a consequence of impaired pre-mRNA splicing. To test this hypothesis, RNA extracted from escaper *Act5C-GAL4/UAS-dBCAS2^{dsRNA}* third instar larvae was analyzed by quantitative RT-PCR. We chose the transcripts of *γ-tubulin* and *hpo*, as reported (Andersen and Tapon 2008), to test whether *dBCAS2*-knockdown larvae exhibited abnormal pre-mRNA splicing in vivo. We found that the mRNA of *γ-tubulin* and *hpo* decreased by 30% to 50%, compared with the wild type, and the pre-mRNA of *γ-tubulin* and *hpo* accumulated by 2.7-fold and fourfold, respectively, in *dBCAS2*-knockdown larvae (Fig. 2A, black box). This suggests that lethality (Table 1) and wing deformation (Fig. 1A) induced by depletion of *dBCAS2* may correlate with impaired splicing. We also investigated whether the rescue effect by *hBCAS2* is related to repair splicing efficiency. Therefore, *Act5C-GAL4/UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2* third instar larvae were subjected to RNA splicing analysis. As shown in Figure 2A (gray box), both the pre-mRNA and mRNA of *γ-tubulin* and *hpo* recovered to wild-type levels (white box). This verifies our hypothesis that human and fly BCAS2 share a similar function in RNA processing, which is essential for cell viability. Consistent results of decreased *γ-tubulin*, *hpo*, and *stg* mRNA and accumulated *γ-tubulin* and *hpo* pre-mRNA were obtained from *Drosophila* S2 cells by knocking down

FIGURE 2. hBCAS2 plays a similar role as dBCAS2, which is necessary for pre-mRNA splicing. (A) dBCAS2-knockdown larvae displays impaired pre-mRNA splicing, and hBCAS2 rescues splicing defects. Third instar larvae RNAs were extracted from *Act5C-GAL4/UAS-dBCAS2^{dsRNA}* (black box), *Act5C-GAL4/UAS-dBCAS2^{dsRNA}, UAS-hBCAS2* (gray box), and *Act5C-GAL4/+* (white box). γ -tubulin and *hpo* pre-mRNA and mRNA were analyzed by quantitative RT-PCR. (B) Depletion of dBCAS2 showed impaired pre-mRNA processing in S2 cells. (Upper panel) Knocking down dBCAS2 by dsRNA in *Drosophila* S2 cells. (Lower panel) γ -tubulin, *hpo*, and *stg* mRNA and γ -tubulin and *hpo* pre-mRNA were analyzed by quantitative RT-PCR. Data are shown as means and SD relative to wild-type controls from three independent experiments. The *P*-values estimated by the Student's *t*-test were statistically significant.

dBCAS2 using dsRNA (Fig. 2B, lower panel). However, similar to previous reports, the *stg* pre-mRNA level was not detectable by quantitative RT-PCR; this was suggested to be attributable to low levels or instability of the pre-mRNA (Andersen and Tapon 2008). The decreased expression of dBCAS2 by RT-PCR was shown in the upper panel. Our data confirm that dBCAS2 plays a role in RNA splicing as a component of the spliceosome (Herold et al. 2009).

C-terminal hBCAS2 associates with hPrp19 complex and is necessary for RNA splicing

To determine whether human BCAS2 is a bona fide component of the hPrp19 complex, we used GST-hBCAS2

pull down MCF-7 nuclear extracts and analyzed putative hBCAS2-interacting proteins by mass spectrometry. In addition to the hPrp19/CDC5L complex, we also found numerous RNA processing-related proteins, as shown in Table 3. We then performed immunoprecipitation analysis. As shown in Supplemental Figure S6, both the PLRG1 and hBCAS2 antibodies were able to pull down endogenous CDC5L, hPrp19, and PLRG1 in HEK (human embryonic kidney

TABLE 3. The potential hBCAS2-interacting proteins involved in RNA splicing

Protein name	Accession no. (Swissprot)	No. of peptides ^a	Sequence coverage (%)
hPrp19/CDC5L complex			
CDC5L	Q99459	45	38
hPrp19	Q9UMS4	8	14.3
AD-002	Q9P013	2	4.3
SR and SR-related proteins			
ASF/SF2	Q07955	12	20.6
SRp55 (SFRS6)	Q13247	5	10.1
P54 (SFRS11)	Q6PJY9	3	1.7
U2AF1	Q01081	5	16.7
9G8 (SFRS7)	Q16629	15	26.1
CDC2L5	Q9BVE2	5	8.4
SRp20 (SFRS3)	P84103	16	31.1
hnRNPs			
hnRNP R	Q43390	25	18.8
hnRNP U	Q00839	54	38.3
hnRNP F	P52597	3	8.7
hnRNP H1	P31943	14	21.4
hnRNP H3	P31942	4	9
hnRNP A3	P51991	6	10.8
hnRNP A2/B	P22626	5	12.6
hnRNP core A	P09651	19	31.2
snRNP-associated			
U5 snRNP 100 kD protein	Q9BUQ8	4	2.7
U4/U6 snRNP 90kD protein (Prp3)	Q9Y3B4	12	44
RNA-binding protein			
RNA binding motif protein 25	P49756	34	21.2
RNA binding motif protein 12B	Q8IXT5	7	4.6
RNA binding motif protein 14	Q96PK6	47	30.2
RNA binding motif protein 39	Q14498	16	17.7
RNA binding motif protein 8A	Q9Y5S9	4	14.4
DEAD-box RNA helicase			
DDX 39	B1Q2N1	20	34.4
DDX17	Q92841	29	26.9

Mass spectrometry analysis of GST-hBCAS2 pull down proteins obtained from MCF-7 nuclear extracts shows several putative hBCAS2-interacting proteins involved in RNA processing.

^aNumber of peptides identified for each protein.

epithelial cells) 293 cells (Supplemental Fig. S6, lanes 3,4), confirming that hPrp19, CDC5L, PLRG1, and hBCAS2 form a stable complex in mammalian cells. Because human BCAS2 contains two coiled-coil motifs, which can mediate protein–protein or protein–DNA interactions (Mason and Arndt 2004), in the C-terminal region (Fig. 3A), we used GST-hBCAS2, GST- Δ CC (deletion of two coiled-coil domains), GST-C, His-tagged hPrp19, and CDC5L proteins, generated from *Escherichia coli*, for in vitro protein–protein interactions to investigate in detail their association within the hPrp19 complex. The results showed that His-hPrp19 could bind either full-length hBCAS2 or Δ CC protein (Fig. 3B, lanes 3,4), but not the C-domain protein (Fig. 3B, lane

5). On the other hand, His-CDC5L interacted directly with full-length hBCAS2 and the C-domain protein (Fig. 3C, lanes 3,5). The in vitro binding data are summarized in the right panel of Figure 3A. We conducted further in vivo binding experiments with members of the endogenous hPrp19 complex by transiently expressing Flag-tagged hBCAS2, dCC1, dCC2 (dCC1 and dCC2 plasmid constructs refer to deletion of coiled-coil domain 1 and 2, respectively), Δ CC, and C-domain (Fig. 3D) constructs in HEK 293 cells. The results showed that the full-length hBCAS2 and C-domain protein associated with endogenous CDC5L, hPrp19, and PLRG1 (Fig. 3E, lanes 8,12). However, the coiled-coil deletion mutants Flag-dCC1, Flag-dCC2, and Flag- Δ CC only interacted with hPrp19 and not with CDC5L or PLRG1 (lanes 9–11). These in vivo results (summarized in Fig. 3D, right panel) were consistent with in vitro binding assays (Fig. 3A–C). Taken together, the hBCAS2 N terminus interacts only with hPrp19, whereas its C terminus (containing the coiled-coil domain) is necessary for interaction with all other major components of the hPrp19 complex.

To decipher the structure–function relationship of hBCAS2, the pSV40-CAT (In1) reporter (Fig. 4A, upper panel; Lin et al. 2004) was then used to investigate its function in constitutive splicing. The results showed that overexpressing hBCAS2 increased the pre-mRNA splicing efficiency of the reporter by approximately 1.45-fold, and the C terminus enhanced this by 1.34-fold, whereas the Δ CC protein inhibited splicing to 0.69-fold (Fig. 4A, lower panel). The corresponding hBCAS2 protein expression is shown in Figure 4B. In addition, we then used the adenovirus E1A as a reporter (Fig. 4C, upper panel) to assay alternative splicing regulation of hBCAS2. Selection of the 5' splice sites of E1A produces various amounts of 13s, 12s, and 9s mRNAs that are modulated by a variety of splicing regulatory factors (Lin et al. 2004). The results showed that hBCAS2 and its C terminus increased the ratio of 9s mRNA, but decreased that of 12s mRNA, from the E1A reporter (Fig. 4C,D). In general, when a splicing factor activates a distal 5' splice site (i.e., the 9s site), the level of 9s mRNA is increased with a concomitant reduction of both 12s and 13s mRNAs (Lai et al. 2003; Lin et al. 2004). However, hBCAS2 appeared to have no effect on the 13s mRNA, so that it only reduced 12s and promoted 9s expression, although the mechanism underlying this is not yet clear. Southern blotting also was performed to confirm the RT-PCR products shown in Figure 4A and C (Supplemental Fig. S7A,B). To confirm the effect of hBCAS2 in alternative splicing endogenously, we also chose alternative transcripts associated with cell cycle control, because of the depletion of hBCAS2 causing G2 growth arrest (Kuo et al. 2009). The human Cdc25 phosphatase isoforms Cdc25B and Cdc25C are critical during the transition from G2 to M phase (Boutros et al. 2007). Because our data in Figure 2B (lower panel) also showed that the mRNA level of *stg* (String, *Drosophila* Cdc25 ortholog) was dramatically decreased in dBCAS2-knockdown S2 cells, we determined by RT-PCR

FIGURE 3. The C-terminal coiled-coil region of hBCAS2 associates with the hPrp19 complex. (A) Schematic representation of GST-tagged hBCAS2, Δ CC, and C constructs and summarized data. (B) Full-length and Δ CC of hBCAS2 interacts directly with hPrp19. (C) Full-length and C-terminal hBCAS2 interacts directly with CDC5L. (D) Schematic representation of Flag-tagged hBCAS2, dCC1, dCC2, Δ CC, and C constructs. (Right) Summarized data of in vivo interactions from E. (E) Mapping interaction domain between hBCAS2 and CDC5L, PLRG1, hPrp19 in vivo. 1/20 input was analyzed by Western blot as shown in the left panel (lanes 1–6). Proteins precipitated with Flag-tagged hBCAS2 variants were revealed by blotting using indicated antibodies (right panel, lanes 7–12).

FIGURE 4. The C terminus is indispensable for the function of hBCAS2 in splicing. (A) C-terminal and full-length hBCAS2 enhances the CAT(In1) reporter pre-mRNA splicing. (Upper panel) Schematic representation of the CAT(In1) reporter. The splicing efficiency (lower panel) was calculated from the value of mRNA/(mRNA + pre-mRNA), and the relative splicing efficiency was normalized to the vector control (set as 1). Data are shown as means and SD from three independent experiments. (**P* < 0.05) (B) Western blot analysis with anti-Flag antibody to determine ectopically expressed protein with the reporter in HEK 293 cells. (C) C-terminal and full-length hBCAS2 modulates 5' splice site selection of the AdE1A reporter. Schematic representation of the AdE1A reporter is shown in the upper panel. (Lower panel) AdE1A reporter was cotransfected with either control vector or Flag-hBCAS2 variants into HEK 293 cells and analyzed by RT-PCR to determine 13s, 12s, and 9s RNAs. (D) Quantitative results of alternative splicing. *C* showed one represented experiment. The data of *D* were collected from three independent experiments and shown as means and SD. *P* values were estimated by the Student's *t*-tests and were statistically significant. (E) Depletion of hBCAS2 influences the alternative splicing patterns of Cdc25C variants. The Cdc25C alternative splicing variants were examined by RT-PCR in BCAS2-depleted MCF7 cells. (Left) Knocking down BCAS2 (lane 2) alters the ratio of Cdc25C3/4 to Cdc25C1 variant compared with scramble control (lane 1). (Right) Protein and mRNA expression of BCAS2 and internal control are revealed by Western blot and RT-PCR.

whether hBCAS2 affected the splicing variants of Cdc25C (Pacheco et al. 2006; Albert et al. 2011). Our data revealed that knocking down hBCAS2 changed the ratio of Cdc25C3/4 to C1 variants, compared with the scramble control, in MCF7 cells (Fig. 4E, left panel, lane 2; Supplemental Fig. S7C). The right panel shows that hBCAS2 was efficiently depleted by transient transfection of pSuper-shBCAS2-434 (Fig. 4E, right panel, lane 2) at both the mRNA and protein levels. This verifies the role of hBCAS2 in alternative splicing. In summary, the hBCAS2 C terminus containing the coiled-coil motifs is critical for RNA splicing and association with the hPrp19 complex.

The C terminus of hBCAS2 partially restores defective phenotypes in dBCAS2-depleted flies

Because the C terminus is indispensable for the function of hBCAS2 in RNA splicing (Fig. 5A), we asked the question whether the C-fragment of hBCAS2 rescues defects in dBCAS2-depleted flies in the same way as full-length hBCAS2. We generated an hBCAS2-C transgenic fly that showed no obvious phenotypic change compared with the wild type (data not shown). When *UAS-hBCAS2-C* and *UAS-dBCAS2^{dsRNA}* were expressed with an *ms1096-GAL4* driver, the wing deformation was partially rescued, in that the shrunk and wrinkled wings became partially unfolded and the wing veins could be clearly observed, compared with the dBCAS2-knockdown fly (Fig. 5B, panels b,d); however, the notum still exhibited a similar phenotype (Fig. 5B, cf. panels a and c). Expression of hBCAS2-C mRNA in the wing discs of *ms1096-GAL4*-driven *UAS-dBCAS2^{dsRNA}* and *UAS-hBCAS2-C* flies was confirmed by RT-PCR (Fig. 5C, lane 2). This indicates that the hBCAS2-C fragment can partially rescue the wing deformation of dBCAS2-depleted flies. To confirm that the partial rescue effect of the hBCAS2-C fragment is correlated with the splicing function, we examined next the splicing efficiency in third instar larvae expressing *UAS-*

FIGURE 5. C-terminal hBCAS2 partially rescues dBCAS2-knockdown induced wing and splicing defects. (A) C-terminal hBCAS2 plays a core of hPrp19 complex for RNA splicing. (B) Ectopic expression of hBCAS2-C partially rescues *dBCAS2^{dsRNA}*-induced wing deformation. *UAS-hBCAS2-C* and *UAS-dBCAS2^{dsRNA}* were expressed under the control of *ms1096-GAL4*. Scale bar, 0.5 mm. (C) Expression of hBCAS2-C in *ms1096-GAL4/+;UAS-dBCAS2^{dsRNA}+/+;UAS-hBCAS2-C/+*. RNAs were extracted from wing discs of third instar larvae and subjected to RT-PCR to confirm the expression of hBCAS2-C (lane 2) compared with the wild-type control (lane 1). The internal control, *rp49*. (D) C-terminal hBCAS2 partially rescues impaired splicing in dBCAS2-depleted third instar larvae. *Act5C-GAL4/UAS-dBCAS2^{dsRNA}+/+;UAS-hBCAS2-C/+* (gray box) third instar larvae RNA were extracted and analyzed by quantitative RT-PCR as described in the legend to Figure 2.

dBCAS2^{dsRNA} and *UAS-hBCAS2-C*, driven by *Act5C-GAL4*. As shown in Figure 5D, C-terminal hBCAS2 (gray box) partially rescued the level of mRNA and pre-mRNA of γ -tubulin and *hpo*, compared with *UAS-dBCAS2^{dsRNA}* alone. On the other hand, we also examined the level of viability restored by the hBCAS2-C fragment in the *Act5C-GAL4/dBCAS2^{dsRNA}*. We screened 241 adult flies, and in contrast with the 29.4% restored survival rate of full-length hBCAS2, the progeny expressing *dBCAS2^{dsRNA}* and *hBCAS2-C* had not survived (data not shown). This indicates that the C-frag-

ment appears less potent than the full-length protein in restoring fly viability. Taken together, the rescue effect with hBCAS2-C is weaker than with the full-length protein, yet hBCAS2-C appears as potent as the full-length protein in the mammalian cell splicing assays. The differences of BCAS2 function between fly and mammalian cells are interesting subject for further investigation.

dBCAS2 knocked down cells have a higher degree of apoptosis

Previous studies have shown that depletion of splicing factors such as dMAFP1 and dPrp38 affects *Drosophila* cell proliferation and induces apoptosis (Andersen and Tapon 2008). We have found that depletion of BCAS2 resulted in splicing defects and wing deformation. Other than the observation of the splicing defect, notum, and wing phenotypes, we examined further cell viability in the wing discs of third instar larvae when knocking down dBCAS2. The *engrailed-GAL4* was used as a driver and is located the posterior part of wing discs where *UAS-dBCAS2^{dsRNA}* (Fig. 6B), *UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2* (Fig. 6C), and *UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2-C* (Fig. 6D) were expressed. As shown in Figure 6, knocking down dBCAS2 significantly increased the number of apoptotic cells, as revealed by staining cleaved caspase 3 (Fig. 6F, red spot), compared with the wild type (Fig. 6E). However, co-expressing hBCAS2 (Fig. 6G) or hBCAS2-C (Fig. 6H) could fully or partially reduce cleaved caspase 3-positive cells in dBCAS2-knockdown wing discs. Because human BCAS2 was reported to be a negative regulator of the tumor suppressor p53 in cancer cell lines (Kuo et al. 2009), it is possible that the apoptosis phenomenon in dBCAS2-knockdown wing discs results from direct effects of dBCAS2 on the *Drosophila* p53 (*dmp53*), rather than splicing defects. To rule out the effect of *dmp53*, we observed caspase 3 activity when knocking down dBCAS2 by *ms1096-GAL4* in *dmp53*-null mutant flies (Sogame et al. 2003). As shown in Supplemental Figure S8C, the cleaved caspase 3 staining in dBCAS2-depleted flies (Supplemental Fig. S8B) was still higher than the control (Supplemental Fig. S8A) in the *dmp53*-null mutant background. In summary, our results provide evidence that the function of BCAS2 in RNA splicing is essential for *Drosophila* cell viability.

DISCUSSION

BCAS2 is essential for *Drosophila* viability and RNA splicing

In this study, we showed that dBCAS2 is essential for *Drosophila* viability via shRNA-mediated depletion in the whole body (Table 1). In addition, conditional knocking down of dBCAS2 using *ms1096-GAL4*, *ap-GAL4*, or *engrailed-GAL4* drivers results in damaged phenotypes and

FIGURE 6. dBCAS2 knock down cells display higher level of apoptosis. Apoptotic cells were visualized by cleaved caspase 3 staining in dBCAS2-knockdown wing discs, and the expression of hBCAS2 or hBCAS2-C reduced cleaved caspase 3-positive staining. *engrailed-GAL4* drives GFP (green, panels A–D) locating the posterior (to the right of discs) region of wing discs where *UAS-dBCAS2^{dsRNA}* (panels B,F,J), *UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2* (panels C,G,K), and *UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2-C* (panels D,H,L) are expressed compared with the wild type (panels A,E,I) from wandering third instar larvae. Anti-cleaved caspase 3 staining (red, panels E–H) revealed apoptotic cells in imaginal wing discs. Merged images (panels I–L). The genotypes of the flies are described in the Materials and Methods.

increased apoptotic cells in wings and imaginal discs, respectively (Figs. 1, 6; Supplemental Fig. S4; Table 1). These deformed phenotypes or lethality could be rescued by human BCAS2 (Figs. 1, 6; Supplemental Fig. S4; Table 2), indicating that hBCAS2 and dBCAS2 share similar functions that affect cell viability. Previously, we found that hBCAS2 is a negative regulator of p53 through direct interaction. Depletion of hBCAS2 increases p53 protein activity and induces p53-targeted gene expression, resulting in apoptosis. (Kuo et al. 2009). However, knocking down human BCAS2 in p53-null and mutant cells induces G2/M arrest, suggesting that the function of BCAS2 in growth control may involve a mechanism other than the regulation of p53. BCAS2 (also named Spf27) was reported to be a component of the multi-protein spliceosome complex by mass spectrometry and EST-database searching, but its role in RNA splicing remains unclear (Neubauer et al. 1998). Here, we confirmed that human BCAS2 is an integral component of the hPrp19 subspliceosomal complex (Supplemental Fig. S6; Table 3), and we

provide evidence that hBCAS2 participates in pre-mRNA splicing (Fig. 4). Furthermore, due to the conservation of human and fly BCAS2, we found depleting *Drosophila* BCAS2 in third instar larvae and in S2 cells caused decreased processing of mRNA and accumulation of pre-mRNA (Fig. 2), and overexpressing hBCAS2 in dBCAS2-depleted flies can restore the defects in RNA processing (Fig. 2), as well as the phenotypes in wings and lethality (Fig. 1; Table 2). Previous reports have illustrated that depletion of spliceosomal factors, such as ASF and dMFAP1, cause G2/M arrest (Bernstein and Coughlin 1998; Li et al. 2005; Andersen and Tapon 2008). Similarly, other members of the hPrp19 complex, hPrp19 and PLRG1, which are both required for RNA splicing, caused lethality at an early stage of embryo development when knocked out in mice (Fortschegger et al. 2007; Kleinriders et al. 2009). These reports support our data that the impairment of RNA splicing when BCAS2 is knocked down in p53-null cells causes lethality or malformations in dBCAS2-depleted flies.

Structure-function relationship of BCAS2

As reported previously (Grote et al. 2010), the amino acid sequences of hBCAS2 and its counterpart Snt309 (*Saccharomyces cerevisiae*-simplyer yeast),

which lacks coiled-coil domains, share only 24% similarity. Although Snt309 is a component of the Prp19 complex, it does not interact directly with Cef1 (CDC5L ortholog) (Ohi et al. 2005). Therefore, we deduce from our data that the C-terminal coiled-coil domains of hBCAS2 contribute primarily to the interaction with CDC5L. Previous reports have shown that depletion of Snt309 protein by anti-Snt309 antibodies does not impair pre-mRNA splicing in vitro (Chen et al. 1998, 1999). Unlike Snt309, human BCAS2 enhances the efficiency of pre-mRNA splicing (Fig. 4). Thus, the coiled-coil domains of hBCAS2 are critical for its splicing function because of its direct interaction with CDC5L.

Moreover, the Cwf7 (BCAS2 counterpart) of the more complex yeast, *Schizosaccharomyces pombe*, contains sequences similar to the C-terminal domain of human BCAS2. Cwf7 can interact with the CDC5L ortholog and can rescue the growth defects of *snt309*-deficient cells (Ohi and Gould 2002; Ohi et al. 2002), suggesting that this BCAS2 ortholog

has evolved to achieve functional complexity. This possibility is supported by the high similarity between *Drosophila*, plant (Palma et al. 2007), and human BCAS2, which all contain coiled-coil domains that can interact directly with CDC5L. In addition, the *Drosophila* Protein Interaction Mapping (DPiM) project has identified putative dBCAS2-interacting proteins, including CG6905 (CDC5L) and Tango4 (PLRG1), which are similar to GST-hBCAS2 pull down proteins (Table 3). Combined with our data (Figs. 3–5), these reports verify the structural and functional conservation between dBCAS2 and hBCAS2.

In this study, we identified a novel function for the constitutive splicing factor human BCAS2 in the 5' splice site selection of an adenovirus E1A reporter (Fig. 4C). We also provide preliminary evidence that depletion of hBCAS2 affects the ratio of Cdc25C alternative transcripts (Fig. 4E), suggesting that BCAS2 has potential functions in the regulation of alternative splicing. Alternative splicing is an important post-transcriptional regulation that diversifies gene expression in higher eukaryotes. Two crucial factors control alternative splicing: the sequence and position of *cis*-acting elements and the local concentration and post-translational status of *trans*-acting factors. In general, *trans*-acting factors that regulate alternative splicing include SR (Ser-Arg) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) particles (Chen and Manley 2009). Recent studies showed that hPrp19 interacts directly with Blom7a, a novel hnRNP K homology domain-containing protein that can modulate the alternative 5' and 3' splice site choices of reporter minigenes (Grillari et al. 2009). In addition, CDC5L/PLRG1 interacts with hnRNP M and affects the selection of alternative splice site (Lleres et al. 2010). It would be interesting to investigate whether BCAS2 regulates alternative splicing by affecting the interaction of POS4-Blom7a or CDC5L/PLRG1-hnRNPM. As BCAS2 only harbors two coiled-coil domains and does not belong to the families of SR proteins or hnRNPs, we still cannot exclude the possibility that the activity of the hBCAS2-C protein in alternative splicing and in restoration of the knockdown phenotype may involve its interaction with other splicing factors (as provided in our GST-hBCAS2 pull down list, Table 3), rather than known factors such as the Prp19 complex. However, it may be possible that BCAS2 affects the overall kinetics of splicing as a result of the reduction or increase of a general splicing factor.

A previous report concluded that the hPrp19 core complex, including hBCAS2 (lack of N-terminal six amino acids), the N-terminal domain of hPrp19, the C-terminal domain of CDC5L, and the N-terminal domain of PLRG1, forms a salt stable bounded mixture and resists a high concentration of the protease subtilisin (Grote et al. 2010). Consistently, our results demonstrate that the C terminus of hBCAS2 (containing the coiled-coil domains) has the capacity to interact directly with CDC5L, which in turn binds with hPrp19 and PLRG1 (Fig. 3) to carry out RNA splicing activities, comparable to full length hBCAS2 (Figs. 3, 4). This indicates that the

capacity for core complex formation is responsible for regular splicing in mammalian cells. But in the *Drosophila* system, flies transgenic for full-length hBCAS2 (Fig. 1A, panel e) and hBCAS2-C (data not shown) show no effects in terms of changes in morphology, perhaps because of endogenous splicing complexes reaching saturation. However, the rescue effects were measured by crossing transgenic full-length or hBCAS2-C flies with dBCAS2-depleted flies, and the restoration of wing-deformation by hBCAS2-C (Fig. 5B, panel d) is weaker than that by the full-length hBCAS2 (Fig. 1A, panel h). The rescue effect of hBCAS2-C is not as potent as full-length hBCAS2, suggesting that there are other roles for BCAS2 in wing shape development, besides RNA splicing. Aside from RNA splicing, BCAS2 also has been reported to interact with estrogen receptor (ER) as a transcriptional co-factor (Qi et al. 2005) and with p53 as a negative regulator (Kuo et al. 2009). ER is known as a growth survivor and p53 as a tumor suppressor, both of these proteins are crucial for cell viability. The detailed roles of BCAS2 regulating the other genes involved in cell growth or development still are being investigated. Moreover, the N fragment of hBCAS2 interacts directly with hPrp19 and seems to have a dominant-negative role in RNA splicing (Fig. 4A,C) in mammalian cells. It would be interesting to investigate further whether the hBCAS2-N fragment binds with hPrp19, resulting in the dilution of the core complex components and impeding RNA splicing in the fly assay.

MATERIALS AND METHODS

Fly strains

Drosophila stocks were kept and crossed at 25°C and supplied with cornmeal medium. *UAS-dBCAS2^{dsRNA}* was purchased from Vienna *Drosophila* RNAi Center (stock no. 26676) and balanced over *T(2;3)SM6-TM6B*, a translocated chromosome 2–3 balancer. *UAS-hBCAS2* and *UAS-hBCAS2-C* were constructed by the insertion of hBCAS2 and hBCAS2-C (278 bp, amino acids 139–225) cDNA from HeLa cells into *pUAST* vectors and randomly inserted into the second and third chromosome of *w¹¹¹⁸*, respectively (kindly provided by the Taiwan Fly Core). *UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2* was established by the recombination of *UAS-dBCAS2^{dsRNA}* with *UAS-hBCAS2* and balanced over *T(2;3)SM6-TM6B*. *UAS-dBCAS2^{dsRNA}* and *UAS-hBCAS2-C* were balanced over *T(2;3)SM6-TM6B* on chromosome II and III, respectively, to generate *UAS-dBCAS2^{dsRNA};UAS-hBCAS2-C/T (2;3) SM6-TM6B*; therefore, *UAS-dBCAS2^{dsRNA}* and *UAS-hBCAS2-C* cosegregate during crosses. *UAS-dBCAS2^{dsRNA}*, *UAS-hBCAS2*, or *UAS-hBCAS2-C* expressing flies could be distinguished by the selectable marker, Tubby. The *dmp53^{ns}* strain was the kind gift of Dr. John M. Abrams (Sogame et al. 2003). *ap-GAL4*, *ms1096-GAL4*, *Act5C-GAL4*, *engrailed-GAL4*, and *w¹¹¹⁸* used in this study were obtained from Bloomington *Drosophila* stock center and maintained by the Fly Core Facility in the College of Medicine, National Taiwan University. Phenotypes of flies were examined 48 h post eclose by stereomicroscope (Leica) after being anesthetized by carbon dioxide.

Wings isolated from adult flies were mounted with Hoyer's mounting medium before imaging. To validate the expression of *UAS-hBCAS2* and *UAS-dBCAS2* driven by *ms1096-GAL4*, total RNAs were extracted from wing discs of third instar larvae and analyzed by RT-PCR.

Cell culture and plasmid constructs

HEK 293 and MCF7 (breast cancer cells) were grown in Dulbecco's modified Eagle's medium and RPMI 1640 medium, respectively, supplied with 10% FBS, L-glutamine, and penicillin/streptomycin at 37°C, 5% CO₂. To knock down dBCAS2 in S2 cells, full-length dBCAS2 cDNA from *Drosophila* S2 cells was used for dsRNA synthesis by in vitro transcription (Promega). dBCAS2 dsRNA was incubated with S2 cells for 5 d before RNA analysis according to the protocol reported previously (Rogers and Rogers 2008). GST-tagged and Flag-tagged hBCAS2 variants were constructed according to the method described previously (Kuo et al. 2009). Primers used in this study are listed in the Supplemental Information.

Protein-protein interaction and antibody

6×His-CDC5L, hPrp19, and GST fusion protein (hBCAS2 variants) were prepared from *E. coli* BL21 (DE3) for in vitro interaction. GST-fusion protein conjugated beads were washed with sonication buffer (50 mM at pH 8.0 NaH₂PO₄, 300 mM NaCl, 20% glycerol, and DTT, PMSF) twice and once with co-IP buffer (20 mM Tris at pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP-40, 5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT) and then incubated with eluted His-tagged proteins. After incubation, samples were washed and eluted for further Western blot analysis. HEK 293 cells transfected with Flag-hBCAS2 variants by calcium phosphate were subjected for in vivo immunoprecipitation (Kuo et al. 2009): anti-hBCAS2, hPrp19, PLRG1 (Bethyl Laboratories); anti-CDC5L (BD, Abcam); anti-GST, Flag M2, Actin (Sigma).

In vivo splicing assays

For detection of mRNA and pre-mRNA, RNA was extracted from three to five third instar larvae of each following genotypes: (1) *Act5C-GAL4/+*, (2) *Act5C-GAL4/UAS-dBCAS2^{dsRNA}*, (3) *Act5C-GAL4/UAS-dBCAS2^{dsRNA},UAS-hBCAS2*, and (4) *Act5C-GAL4/UAS-dBCAS2^{dsRNA},UAS-hBCAS2-C/+*. Two micrograms of RNA was treated with DNase (RQ1, Promega) before reverse transcription (SuperScript III, Invitrogen) and then analyzed by quantitative PCR (KAPA SYBR FAST qPCR, Kapa Biosystems) according to the manufacturer's instructions. Data were collected from technical triplication of each reaction of at least three independent samples. Primers for the detection of mRNA and pre-mRNA of γ -tubulin and *hpo* were based on previous reports (Andersen and Tapon 2008). Details for in vivo constitutive and alternative splicing assays were described in previous reports (Lin et al. 2004). For alternative splicing of endogenous Cdc25C, MCF7 cells were transfected with either pSuper-scramble or pSuper-shBCAS2-434 by electroporation and incubated for 48–72 h before Western blot and RT-PCR analysis. Primers used were following previous reports (Pacheco et al. 2006; Albert et al. 2011).

Immunohistochemistry

Wing imaginal discs were dissected from wandering third instar larvae of each of the following genotypes: (1) *engrailed-GAL4,UAS-GFP/+*, (2) *engrailed-GAL4,UAS-GFP/UAS-dBCAS2^{dsRNA}*, (3) *engrailed-GAL4,UAS-GFP/UAS-dBCAS2^{dsRNA},UAS-hBCAS2*, and (4) *engrailed-GAL4,UAS-GFP/UAS-dBCAS2^{dsRNA},UAS-hBCAS2-C/+*. The discs were then fixed for 20 min in PBS with 4% paraformaldehyde and then blocked in PBS with 0.3% Triton X and 5% BSA (bovine serum albumin) for 30 min at room temperature and incubated with anti-cleaved caspase 3 antibody (1:600; Abcam) overnight at 4°C. After washing with PBS-Triton X three times, they were incubated with Cy3-conjugated goat anti-rabbit IgG (1:1000, Jackson ImmunoResearch Lab) for 1 h at room temperature. The fluorescent image was acquired with a fluorescence microscope (Zeiss AXIO SCOPE A1).

Mass spectrometry analysis of hBCAS2-associated protein

Identification of putative hBCAS2-interacting proteins was performed first by in vitro recombinant GST-hBCAS2 pull down nuclear extracts from MCF7 breast cancer cells then eluted for SDS-PAGE separation and in-gel digestion. The digested peptides were then analyzed by high-resolution and high-mass accuracy nanoflow LC-MS/MS (Liquid Chromatography–Tandem Mass Spectrometry, Thermo Fisher Scientific).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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