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RESEARCH ARTICLE

A single KH domain in Bicaudal-C links mRNA binding and translational repression functions to maternal development

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ABSTRACT

Bicaudal-C (Bicc1) is a conserved RNA-binding protein that represses the translation of selected mRNAs to control development. In *Xenopus* embryos, Bicc1 binds and represses specific maternal mRNAs to control anterior-posterior cell fates. However, it is not known how Bicc1 binds its RNA targets or how binding affects Bicc1-dependent embryogenesis. Focusing on the KH domains, we analyzed Bicc1 mutants for their ability to bind RNA substrates *in vivo* and *in vitro*. Analyses of these Bicc1 mutants demonstrated that a single KH domain, KH2, was crucial for RNA binding *in vivo* and *in vitro*, while the KH1 and KH3 domains contributed minimally. The Bicc1 mutants were also assayed for their ability to repress translation, and results mirrored the RNA-binding data, with KH2 being the only domain essential for repression. Finally, maternal knockdown and rescue experiments indicated that the KH domains were essential for the regulation of embryogenesis by Bicc1. These data advance our understanding of how Bicc1 selects target mRNAs and provide the first direct evidence that the RNA binding functions of Bicc1 are essential for both Bicc1-dependent translational repression and maternal vertebrate development.

KEY WORDS: Bicaudal-C, RNA binding, mRNA translation, Repression, Maternal, Post-transcriptional

INTRODUCTION

The conserved Bicaudal-C (Bicc1) protein functions as a cell-fate regulator in metazoans in a variety of biological contexts. *Bicc1* was first identified in *Drosophila* as a maternal gene required for the anterior-posterior patterning of the embryo (Bull, 1966). Subsequently, studies in vertebrates revealed that Bicc1 contributed to the normal formation and function of several organs. For example, the heart, kidneys, and pancreas of *Bicc1*$^{-/-}$ homozygous mutant mice exhibit several abnormalities in structure and function (Maisonneuve et al., 2009; Piazzon et al., 2012; Lemaire et al., 2015). Consistent with these experimental genetic studies, mutations in human *BICC1* are linked to cystic renal dysplasia, a kidney disease (Kraus et al., 2012). While these observations in vertebrate organ systems define roles for zygotic Bicc1, recent loss-of-function analyses of Bicc1 in *Xenopus* embryos establish that maternal Bicc1 is essential for anterior-posterior patterning of vertebrate embryos, indicating that the importance of Bicc1 throughout development is conserved (Park et al., 2016). Based on substantial experimental evidence, particularly (and most recently) from studies of maternal Bicc1 in *Xenopus*, as well as sequence comparisons to known RNA-binding proteins, the crucial biological roles of Bicc1 depend upon its ability to bind directly to target mRNAs and regulate their translation. Indeed, several maternal mRNA targets of *Xenopus* Bicc1 have been defined that encode known regulators of anterior-posterior patterning in vertebrate embryogenesis (Zhang et al., 2013). Thus, the mechanisms by which Bicc1 binds RNA are central to understanding its biological roles, but few experiments have addressed this issue.

The N-terminal region of Bicc1 proteins contain regions with strong sequence conservation to conserved KH (hnRNP-K homology) and KHL (KH-like) domains (Gamberi and Lasko, 2012). KH domains are a prevalent RNA-binding module used by eukaryotic regulatory proteins for post-transcriptional regulatory processes (Dominguez et al., 2018; Nicastro et al., 2015). In depth studies have revealed that the N-terminal region of *Xenopus* Bicc1 is sufficient for binding specific mRNA target substrates both *in vivo*, based on RNA immunoprecipitation experiments (RIP), and *in vitro*, based on electrophoretic mobility shift assays (EMSAs; gel shifts) (Dowdle et al., 2017; Zhang et al., 2013). Although these observations provide compelling evidence that the key RNA-binding function of Bicc1 is encoded by this N-terminal region of the protein, this region contains multiple KH and KHL domains, and it is not possible to predict which ones are important for specific Bicc1 RNA binding. For example, the KH-domain-containing protein ZBP1 (zip code binding protein 1), which functions to localize and translationally repress target mRNAs in the dendrites of neurons, contains four KH domains but only two are crucial for ZBP1-mRNA binding (Nicastro et al., 2017; Farina et al., 2003; Chao et al., 2010). From this and other examples, it is clear that different biologically important multi-KH domain RNA-binding proteins can use KH domains singly or in combination to bind relevant mRNA targets. Thus, direct experimental examination is required to define the mechanism by which a multi KH-domain containing protein such as Bicc1 binds RNA.

In this study, we analyzed how the *Xenopus* Bicc1 protein binds to specific mRNA target substrates, by examining several Bicc1 protein mutants for RNA binding *in vivo* and *in vitro*. Bicc1-RNA interactions could only be observed with the intact multi-KH domain N-terminal region, suggesting that a stable, functional RNA-binding activity required a folded architecture that could only be achieved by the individual KH and KHL domains working together. Using amino acid substitutions to abolish the RNA-binding function of individual KH domains revealed that the KH2 domain was the major determinant of efficient RNA binding. Translation-reporter assays indicated that KH2 was also the only

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domain essential for translational repression, suggesting that target mRNA binding was the primary, and possibly the only, role for this region of Bicc1 in translational regulation. Evolutionary comparison revealed that the KH2 domain and its associated GKGG motif is one of the most highly conserved features of Bicc1 proteins. Finally, a maternal knockdown and rescue assay that we established in a previous study to define the essential maternal role for Bicc1 in vertebrate embryogenesis revealed that canonical KH domain function in RNA binding was essential to the maternal role of Bicc1 in development.

RESULTS

Functional KH domains were required for both mRNA binding and translational repression by Bicc1 *in vivo*

In a previous study, we demonstrated that the N-terminal region of Bicc1 that contains multiple KH and KH-like domains, was sufficient to direct selective target mRNA binding by Bicc1 *in vivo* (Zhang et al., 2013). Because this region of Bicc1 contains multiple domains predicted to be capable of RNA binding, we sought to define whether these KH domains were important for selective target mRNA binding in embryos. We exploited the embryonic expression of HA-tagged Bicc1 protein variants as an *in vivo* assay that we have previously established recapitulates biologically relevant Bicc1-mRNA target interactions (Fig. 1A) (Zhang et al., 2013, 2014; Park et al., 2016). A defining functional feature of KH domains is the presence of a conserved GXXG motif that is essential for KH domains to bind RNA substrates because it makes crucial contacts with the RNA backbone (Hollingworth et al., 2012; Nicastro et al., 2015; Valverde et al., 2008). To begin to analyze the role of its KH domains, we created a Bicc1 variant in which each of the conserved GXXG motifs was changed to GDDG, which has been shown to abolish RNA binding without altering the structure of the domains (Hollingworth et al., 2012) (Fig. 1B; KH1-2-3 GDDG). mRNAs encoding HA-tagged wild type and the KH1-2-3 GDDG variant were injected into the animal cap cells of four- to eight-cell *Xenopus* embryos. After the embryos developed to the blastula stage, extracts were prepared and subjected to anti-HA-directed RNA immunoprecipitation (IP-Bicc1) followed by quantitative RT-PCR to detect specific associated mRNAs (Fig. 1A). Two mRNAs were assessed: Cripto1 and Cyclin B1 (Fig. 1C). The Cripto1 mRNA is a Bicc1 target that was described and validated in previous work, and the Cyclin B1 mRNA is a negative control (Zhang et al., 2013). While the wild-type Bicc1 protein selectively bound endogenous Cripto1 mRNA in embryos, the KH1-2-3 GDDG mutant did not (Fig. 1B,C). The difference in binding was due to activity and not to a difference in expression, as both proteins were efficiently expressed in embryos (Fig. 1D). Therefore, the KH domains were essential for RNA selective binding by Bicc1 in embryos.

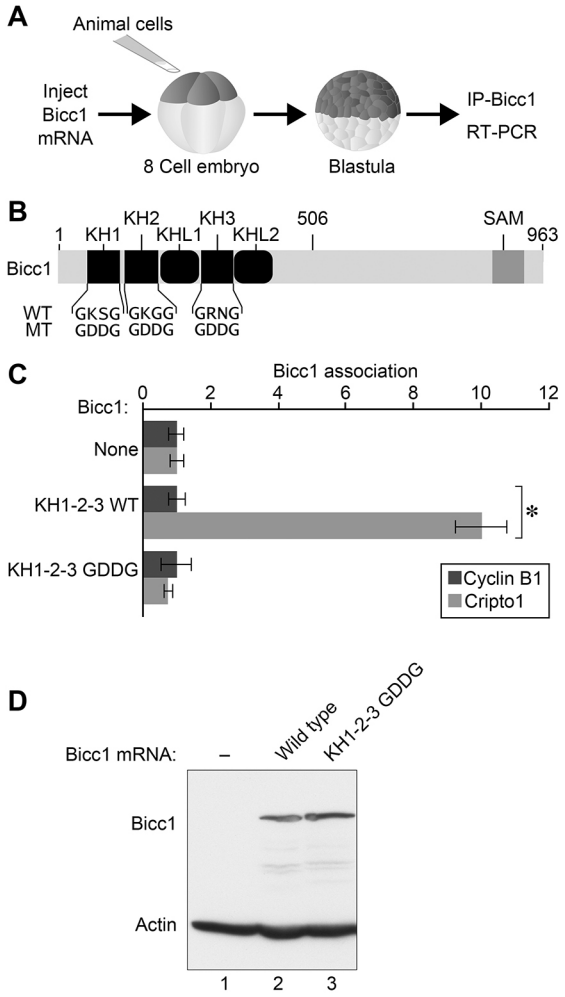

Fig. 1. The Bicc1 KH domains are required for RNA binding. (A) Animal cell assay for *in vivo* Bicc1 binding. Animal cells of eight-cell *Xenopus* embryos were injected with mRNA encoding HA-tagged Bicc1. Some injected samples included luciferase reporter mRNAs. When embryos reached stage 9, Bicc1 was immunoprecipitated with an HA antibody and the associated RNA isolated for analysis (Park et al., 2016; Zhang et al., 2013). RNA samples were reverse transcribed and the cDNA used as template for q-PCR. (B) Diagram of Bicc1 protein showing the three KH domains (KH1, KH2 and KH3) with the wild-type GXXG motif (WT) and the GDDG substitutions (MT) created and analyzed. (C) The Bicc1 protein containing GDDG substitutions (KH1-2-3 GDDG) was expressed in *Xenopus* embryos and analyzed for binding to endogenous mRNAs (see Fig. 1A). The KH1-2-3 GDDG protein was defective for RNA binding in comparison with the Bicc1 wild-type protein. Data are mean±s.e.m. from three separate experiments. **P*<0.05 (*t*-test). (D) Immunoblot analysis with an anti-HA antibody was used to monitor the expression of the Bicc1 proteins expressed in embryos for RNA-binding assays.

Our previous studies established that Bicc1 binding to specific mRNAs, such as Cripto1, targets them for translational repression (Zhang et al., 2013; Park et al., 2016). Therefore, any Bicc1 mutant defective for mRNA binding should also be defective for translational repression. To address this issue, we used a previously established translational reporter assay in which the luciferase coding region is fused to a region of the Cripto1 3′UTR required for both Bicc1 binding and Bicc1-dependent translational repression (Fig. 2A,B) (Zhang et al., 2009, 2013). In this assay, the reporter mRNA was co-injected with an mRNA encoding Bicc1 into animal cells of four- to eight-cell *Xenopus* embryos and the injected embryos were allowed to develop to the blastula stage when embryo extracts were prepared and assayed for luciferase. Bicc1-dependent luciferase expression was defined as the ratio of luciferase activity measured in embryos co-injected with both Bicc1 and the luciferase reporter mRNA to the luciferase activity measured in control embryos that were injected with only the reporter mRNA (Zhang et al., 2009, 2013). These experiments revealed that, while a Bicc1 protein containing the wild-type RNA-binding domain repressed the reporter mRNA fourfold, as expected, the mutant Bicc1 protein in which each KH domain contained a GXXG→GDDG substitution was defective for repression (Fig. 2C). In fact, the KH1-2-3 GDDG mutant was as ineffective at repression as the minus-Bicc1 control (Fig. 2C). Therefore, the KH domains were essential for Bicc1-dependent translation repression.

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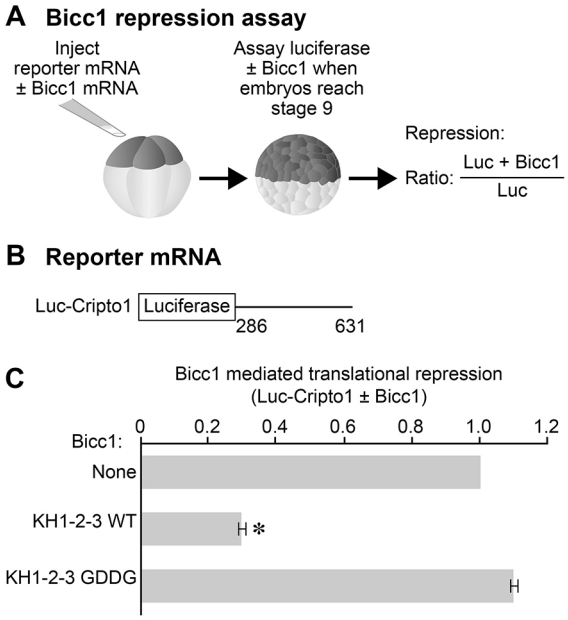

Fig. 2. The Bicc1 KH domains are required for translational repression. (A) Animal cell assay for Bicc1 translational repression. Animal cells of eight-cell *Xenopus* embryos were injected with luciferase reporter mRNAs. Some of the embryos were given a second injection of mRNA encoding full-length *Xenopus* Bicc1 (Zhang et al., 2013). When embryos reached stage 9-10, luciferase assays were performed. Repression, measured by the ratio of luciferase exhibited by a reporter mRNA with and without Bicc1 expression, was calculated and plotted. (B) Diagram of Cripto1 3′UTR fragment incorporated into luciferase reporter mRNAs used to analyze translational repression. (C) The Bicc1 protein containing GDDG substitutions (KH1-2-3 GDDG) was defective for repressing the Luc-Cripto1 reporter mRNA, while the Bicc1 wild-type protein repressed the reporter efficiently. Data are mean± s.e.m. from three separate experiments. **P*<0.05 (*t*-test) wild type compared with no Bicc1 control.

The KH2 domain was the most crucial determinant of mRNA binding by Bicc1

The experiments described above set the stage for determining whether all or just a subset of the KH domains were more important for Bicc1 mRNA selective binding and translational repression. To address this issue, individual and pairwise combinations of KH GXXG→GDDG mutant proteins (Fig. 3A) were examined for RNA binding *in vivo* as described above (Figs 1A and 3A). The GXXG→GDDG substitution in the KH2 domain (KH2-GDDG) abolished Bicc1-RNA binding to levels equivalent to that observed in the control embryos (Fig. 3B). In contrast, the KH1 and KH3 domains minimally contributed to RNA binding. Specifically, a GXXG→GDDG substitution in either KH1 (KH1-GDDG) or KH3 (KH3-GDDG) reduced Bicc1 association by about twofold. Combining both substitution mutants into one variant protein (KH1-3-GDDG) caused no further reduction in Bicc1-RNA association (Fig. 3B). In contrast, the GXXG→GDDG substitution in the KH2 domain (KH2-GDDG) abolished Bicc1 RNA binding to levels equivalent to that observed in the control embryos (Fig. 3B). Each Bicc1 variant was expressed similarly in these experiments (Fig. 3C). Thus, the KH2 domain was the most crucial for robust RNA binding by Bicc1.

The same mutants were examined for their ability to direct Bicc1-dependent translational repression as described above (Fig. 2). If substrate RNA binding was an important role for the N-terminal region of Bicc1 in executing translational repression, then the results from the translation reporter assays should mirror the RNA-binding experiments and this result was observed. Consistent with data presented in Fig. 1 and the RNA-binding analyses in Fig. 3B, the GXXG→GDDG KH2 substitution was the only single substitution to reduce Bicc1-dependent translational repression (Fig. 3D). In addition, a mutant containing GXXG→GDDG substitutions in both KH1 and KH3 domains remained capable of substantial Bicc1-dependent translational repression, consistent with the observation that this same mutant remained capable of selective RNA binding (Fig. 3B). Because all variant proteins for translational were expressed to similar levels (Fig. S1) and the reporter mRNA was equally stable in all cases (Fig. S2), the observed effects were caused by defects in Bicc1-RNA interactions. We conclude that the ability of the KH2 domain to interact with RNA is a crucial determinant of the selective mRNA-binding function of Bicc1, and that target mRNA binding is a primary role of the multi-KH domain-containing region of Bicc1 in Bicc1-dependent translational repression.

The KH2 domain was the most crucial determinant of Bicc1-RNA interactions *in vitro*

The embryo experiments demonstrated a pivotal role for the KH2 domain in selective Bicc1-RNA interactions and translational repression *in vivo*. However, they could not rule out the possibility that these effects were facilitated by Bicc1-protein interactions that might occur *in vivo*. Therefore, to test whether the *in vivo* effects could in fact be ascribed to direct Bicc1-RNA binding defects, Bicc1 mutants, expressed as recombinant proteins (Bicc1 RNA binding domain amino acids 1-506), were purified and used to assess RNA binding *in vitro* using protein-RNA gel shift assays (Fig. 4 and Fig. S3). For these experiments, a 32-nucletide region from the Cripto-1 mRNA 3′ UTR that was previously shown to be sufficient for selective Bicc1-RNA binding was used as a substrate (Zhang et al., 2014). The wild-type Bicc1 protein and the two single GXXG→GDDG KH1 and KH3 mutants efficiently bound the Cripto1 RNA substrate in these experiments to efficiently form distinct RNA-protein complexes (Fig. 4A, lanes 1, 2, 4 and 6). In contrast, the triple GXXG→GDDG KH1-2-3 substitution mutant abolished formation of a stable Bicc1-RNA complex, while the single GXXG→GDDG KH2 mutant reduced formation of this complex (Fig. 4A, lane 3). Even the triple GXXG→GDDG KH1-2-3 substitution mutant exhibited some binding to the Cripto1 RNA, as indicated by the diffuse signals in the gel due to RNA-protein complexes dissociating during gel electrophoresis. Thus, although the triple mutant caused a significant RNA-binding defect, it did not abolish RNA-binding activity, indicating that other features of the Bicc1 RNA-binding domain were contributing to the RNA-binding function of Bicc1. In addition, all the RNA-binding activity that was detected showed specificity for Cripto1 RNA substrate: the same proteins exhibited no detectable binding to the negative control CyclinB1 RNA substrate (Fig. 4B). The data from these *in vitro* experiments, together with the data from the *in vivo* experiments, revealed that Bicc1 KH2 was the key domain responsible for efficient RNA binding by Bicc1.

The KH2 domain was not sufficient for RNA binding

For some multi-KH domain proteins, a select subset of their KH domains are both necessary and sufficient for RNA binding (Nicastro et al., 2017, 2015; Valverde et al., 2008; Chao et al., 2010; Farina et al., 2003). Our results described above indicate that the KH2 domain is necessary for Bicc1 binding and we wanted to test whether it was also sufficient. We used the *in vivo* RNA-binding

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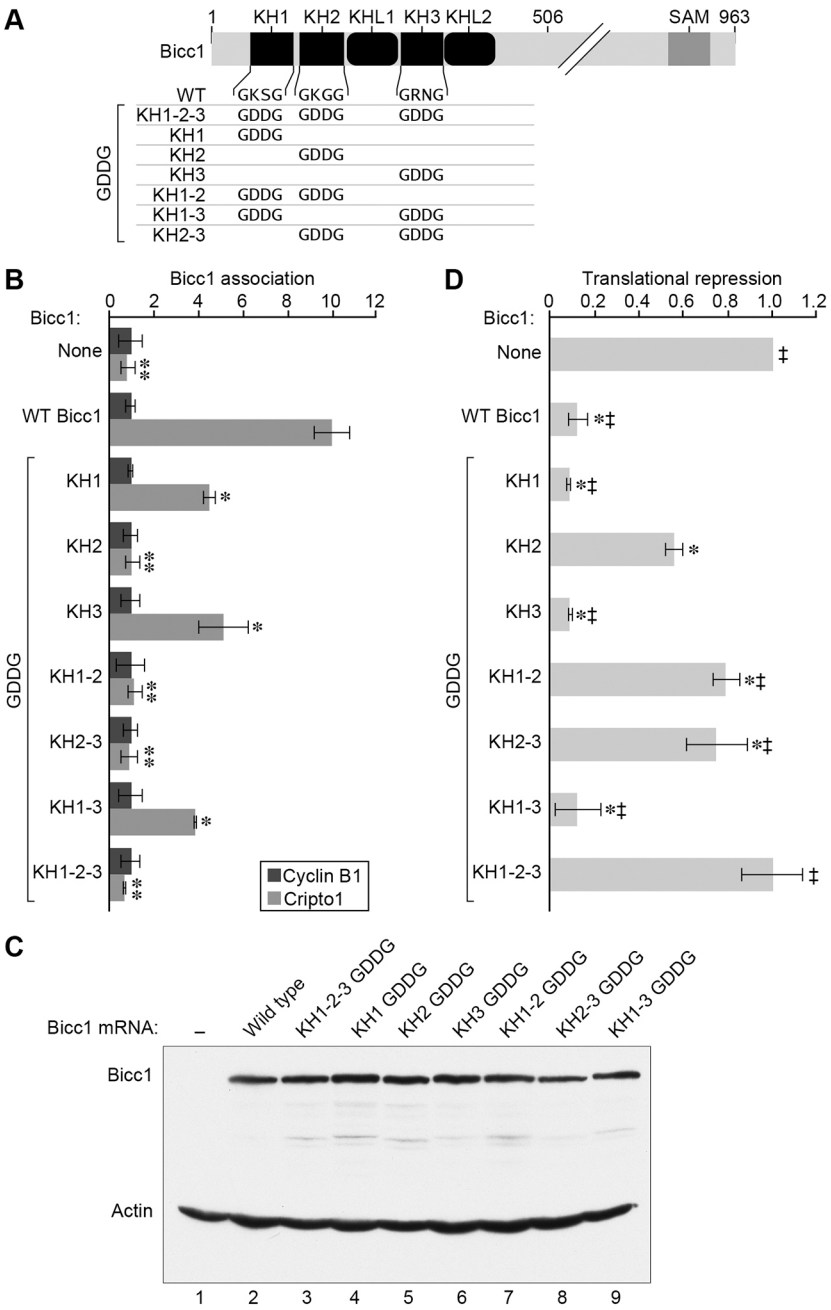

Fig. 3. The KH2 domain is a major determinant of Bicc1 RNA binding and translational repression. (A) Diagram of Bicc1 protein showing the three KH domains (KH1, KH2 and KH3) with the wild-type GXXG motif (WT) and the single and double GDDG substitutions created and analyzed. (B) The Bicc1 protein variants containing GDDG substitutions were expressed in *Xenopus* embryos and analyzed for binding to endogenous mRNAs (see Fig. 1A). The KH2 GDDG, KH1-2 GDDG, KH2-3 GDDG and KH1-2-3 GDDG proteins were defective for RNA binding in comparison with the Bicc1 wild-type protein. Data are mean±s.e.m. from three separate experiments. **P*<0.05, ***P*<0.0005 (*t*-test) when compared with Bicc1 wild-type protein binding to Cripto1 RNA. (C) Immunoblot analysis with an anti-HA antibody was used to monitor the expression of the different protein variants used in RNA-binding assays. (D) The Bicc1 protein variants containing GDDG substitutions were expressed in *Xenopus* embryos and analyzed for translational repression using the Luc-Cripto1 reporter mRNA (see Fig. S2). The KH2 GDDG, KH1-2 GDDG, KH2-3 GDDG and KH1-2-3 GDDG proteins were defective for translational repression in comparison with the Bicc1 wild-type protein. Data are mean±s.e.m. from three separate experiments. **P*<0.05 compared with no protein control (*t*-test). ‡*P*<0.05 compared with KH2 (*t*-test).

assay to test sufficiency. mRNAs encoding HA-tagged variants of the N-terminal region of Bicc1 were injected into four- to eight-cell embryos and binding to specific endogenous mRNAs was assayed as described above (Fig. 1). The KH1-2-3 variant (KH1-2-3 1-506) that contained the entire Bicc1 N terminus was the only protein that showed significant enrichment of the Cripto1 and GRG5 target mRNAs (Fig. 5B), although all variants were expressed at similar levels (Fig. 5C).

To complement these results, we sought to perform *in vitro* RNA-binding studies. We observed that, although the subdomains of the Bicc1 N terminus could be expressed in *E. coli*, they were generally insoluble and difficult to purify. However, two constructs, KH1-KH2 and KH2, could produce sufficient recombinant protein, and these were tested for RNA binding using gel shift assays. The KH1-KH2 protein bound to the Cripto1 RNA, whereas the KH2 protein did not exhibit RNA binding (Fig. 6A). However, the binding activity observed with the KH1-KH2 protein was not specific, as it also bound to the Cyclin B1 RNAs (Fig. 6B). Because the RNA-protein complexes formed with these variants were similar in size to the unbound RNA, we sought to confirm these results with a second assay for RNA binding. We used solution-based fluorescence polarization assays in which the binding of a protein to a fluorescently labeled RNA results in an increase in polarization (Pagano et al., 2011). With this sensitive assay, we also observed that although the KH1-KH2 protein could bind RNA, the binding lacked the specificity of the intact N-terminal region as it bound to both the Cripto1 and Cyclin B1 RNAs (Fig. S4). The solution-based fluorescence assay also demonstrated that the KH1-2-3 GDDG variant Bicc1 protein was defective for RNA binding (Fig. S4), as was observed with gel shifts. The magnitude of the reduction in binding differs with the two assays because the fluorescence assay is solution based and measures both stable and unstable complexes,

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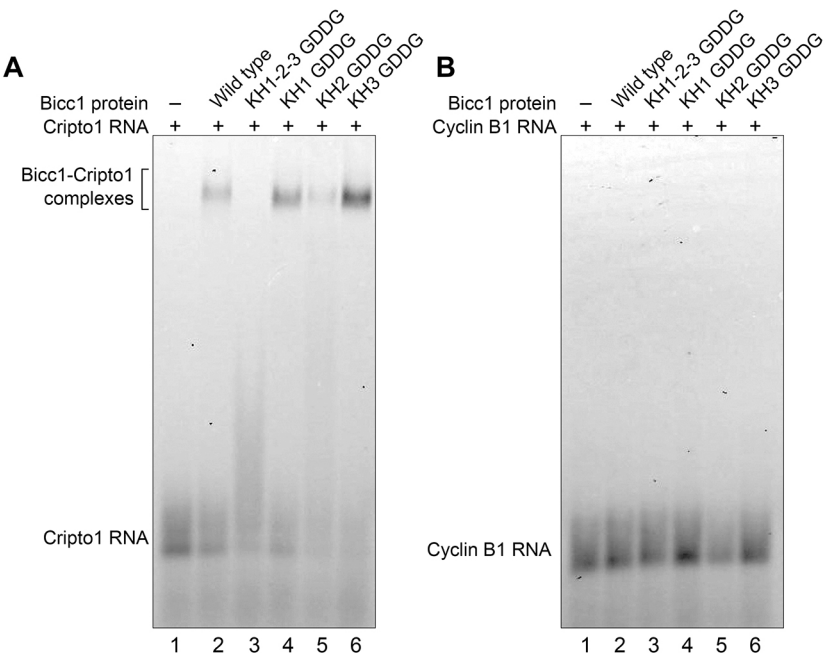

Fig. 4. The Bicc1 KH2 domain is required for direct interaction with RNA targets. (A) Electromobility shift assays of Bicc1 wild-type and protein variants, and RNA substrate that is the Bicc1-binding site from the 3′UTR of the *Xenopus* Cripto1 mRNA. Recombinant proteins consisting of the Bicc1 N terminus (amino acids 1-506) were expressed and purified from *E.coli*. Wild-type protein, along with KH1 GDDG, KH2 GDDG, KH3 GDDG and KH1-2-3 GDDG, were generated for analysis. Binding reactions consisting of the different proteins mixed with a fluorescently labeled 32 nucleotide RNA representing a well-characterized Bicc1-binding site derived from the 3′UTR of the *Xenopus* Cripto1 mRNA were analyzed on native polyacrylamide gels electrophoresed horizontally (Dowdle et al., 2017). The KH2 GDDG and KH1-2-3 GDDG proteins were defective for complex formation compared with wild-type Bicc1 and other Bicc1 variants. (B) Electromobility shift assays of Bicc1 wild-type and protein variants, and an RNA substrate derived from the 3′UTR of the *Xenopus* Cyclin B1 mRNA. The Cyclin B1 mRNA is not a Bicc1 target and represents a negative control for binding.

whereas the gel shift assay only measures stable complex formation. Taken together with the results from RNA IP experiments, these data from *in vivo* and *in vitro* approaches provide evidence that subregions of the Bicc1 multi-KH domain do not bind RNA selectively in isolation but only in the context of the intact N-terminal region.

The KH2 domain is a conserved domain feature of vertebrate and invertebrate Bicc1 proteins

Bicc1 proteins share a common architecture: an N-terminal region that contains three predicted KH domains (Gamberi and Lasko, 2012). Comparison of Bicc1 proteins from different species revealed that the N-terminal region is highly conserved (Fig. 7, Fig. S5). In particular, the KH2 GXXG motif (GKGG) and surrounding amino acid residues were the most conserved among animal species conserved in all species, whereas the GXXG motifs for KH1 and KH3 domains were divergent (Fig. 7, Fig. S5). This evolutionary conservation supports our functional analysis and suggests that the KH2 domain is an important feature for RNA binding in all Bicc1 proteins.

The RNA-binding functions of Bicc1 are essential for embryonic patterning

In a recent study, we established the essential embryonic role for maternal Bicc1 using the host-transfer method to generate a maternal knockdown of Bicc1 that depleted maternal sources of Bicc1 protein from eggs prior to fertilization (Park et al., 2016). Embryos significantly depleted of maternal Bicc1 develop abnormally, with an excess of anterior structures, and exhibit an increase in organizer-specific gene expression accompanied by a reduction in ventral-posterior gene expression. Importantly, re-introduction of wild-type Bicc1 into maternal Bicc1 knock-down embryos rescues the anterior-posterior developmental defects. We used this rescue as a functional assay to determine whether the RNA-binding defects caused by the triple GXXG→GDDG KH1-2-3 substitution affected the maternal role of Bicc1 in embryogenesis (Fig. 8). Specifically, as previously described, embryos were substantially depleted of detectable maternal Bicc1 mRNA (Fig. 8A) and protein (Fig. S6) (Park et al., 2016). The Bicc1-depleted embryos (*bicc1*$^{-}$) developed with severe abnormalities compared with untreated control embryos (untreated) or with embryos that were co-injected with wild-type *bicc1* mRNA during the host-transfer experiment (*bicc1*$^{-}$; *+HA-bicc1*) (Fig. 8A-D). If the RNA-binding function of Bicc1 were crucial for the maternal role of Bicc1, as predicted based on the model for how Bicc1 functions in development by acting as an mRNA-selected translational repressor, then an mRNA encoding a mutant Bicc1 defective in mRNA binding (*bicc1*$^{-}$; *+HA-bicc1 KH1-2-3 GDDG*) should fail to rescue embryonic development in a host-transfer/rescue experiment. This result was observed (Fig. 8E,G). Importantly, both the wild-type and mutant *HA-bicc1* mRNAs expressed protein to similar levels (Fig. 8F), indicating that the inability of the *HA-bicc1 KH1-2-3 GDDG* mutant to rescue the maternal knockdown of Bicc1 was not simply the result of reduced stability of the mutant protein in embryos. We conclude that the RNA binding functions of maternal Bicc1 are essential for its control of vertebrate embryogenesis.

DISCUSSION

It is well established that Bicc1 plays crucial roles in metazoan development and it is generally thought that its RNA binding and translational repressor functions are relevant to these roles (Park et al., 2016; Bull, 1966; Mahone et al., 1995; Saffman et al., 1998; Maisonneuve et al., 2009; Yaguchi et al., 2014). However, there is a paucity of mechanistic experiments addressing how Bicc1 directly binds to its relevant mRNA targets and how this binding affects either Bicc1-dependent translational repression or its roles as a cell-fate regulator. Here, we have defined a single KH domain, KH2 of Bicc1, that was critical for the efficient binding of Bicc1 to a key maternal mRNA target of Bicc1, the Cripto1 mRNA. The RNA-binding function provided by this KH2 domain means that it is the only KH domain crucial for Bicc1-dependent translational repression of a luciferase reporter that contained the relevant Cripto mRNA 3′ UTR Bicc1-control region. This provides evidence that the only crucial mechanistic role of the N-terminal region of Bicc1 in translational repression per se is to localize Bicc1 to its

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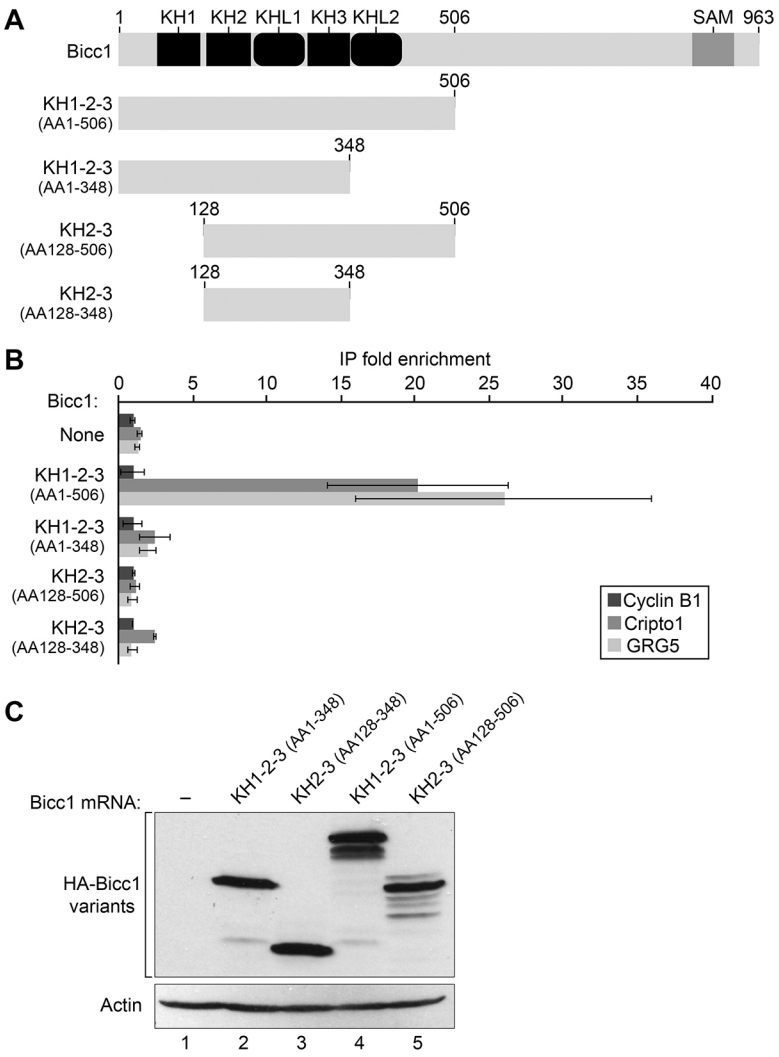

Fig. 5. The entire N-terminal region of Bicc1 is required for RNA binding *in vivo*. (A) Diagram of the intact Bicc1 N-terminal region (KH1-2-3 amino acids 1-506) and the different derivatives that lack KHL2 (KH1-2-3 amino acids 1-348), lack KH1 (KH2-3 amino acids 128-506) or lack KH1 and KHL2 (KH2-3 amino acids 128-348). (B) The endogenous *Xenopus* maternal Cripto1 and GRG5 mRNAs were bound by the intact Bicc1 N terminus, but none of the derivatives lacking different regions bound to these mRNAs. None of the proteins bound to the Cyclin B1 mRNA, a negative control for this experiment as Cyclin B1 is not a Bicc1 target. Data are mean±s.e.m. from three separate experiments. (C) Immunoblot analysis with an anti-HA antibody was used to monitor the expression of the different protein variants used in RNA-binding assays.

mRNA target. Using host transfer knockdown and rescue experiments, we show that a Bicc1 mutant containing KH domains defective for contacting RNA failed to provide for the maternal role of Bicc1 in embryonic patterning, providing direct evidence that the ability of Bicc1 to bind RNA through canonical KH domain-RNA interactions is essential for its role in vertebrate development.

Multi-KH-domain containing regions are common features of biologically important RNA-binding proteins, but the precise mechanisms by which they contact their RNA substrates varies between proteins (Gerstberger et al., 2014; Hentze et al., 2018). For some, such as the KSR protein, all four of its KH domains contribute to RNA binding (Hollingworth et al., 2012). For many others, such as the vigilin and ZBP1 proteins, RNA binding is conferred by a specific subset of KH domains (Nicastro et al., 2017; Nicastro et al., 2015; Cheng and Jansen, 2017). Our results provide evidence that the Bicc1 N-terminal region functions similarly to the latter category of multi KH-containing RNA-binding domains. Specifically, although analyses of precise amino acid substitution variants indicated that only the KH2 domain was crucial for Bicc1-RNA contacts, the analyses of subdomains indicated that the KH2 domain was not sufficient to bind RNA. Thus, we suggest that the entire Bicc1-N-terminal region must fold into a structure that allows KH2 to contact Cripto1 RNA to drive stable Bicc1-RNA binding both *in vivo* and *in vitro*. Accordingly, direct RNA contacts by KH1 or KH3, or the KHL domains, are not crucial but rather their interactions with each other in three-dimensional space are required to support the ability of KH2 to contact RNA. Structural work will be required to address this issue.

While the GXXG→GDDG substitution mutants in Bicc1 revealed that the KH2 domain was the crucial domain required for stable Bicc1-Cripto1 RNA interactions, what drives specific Bicc1-target mRNA interactions remains an unresolved issue. Studies of other KH domains establish that the GXXG motif is crucial for contacting the RNA backbone and contributes minimally to sequence-specific RNA binding (Nicastro et al., 2015; Nicastro et al., 2017; Teplova et al., 2011). Consistent with these observations, our analyses of Bicc1-Cripto1 RNA interactions *in vitro*, which were sensitive enough to detect some residual RNA binding by the KH2 GDDG mutant, showed that this residual binding remained specific for Cripto1 mRNA. It is possible that another region of KH2 or other regions of the N-terminal region of Bicc1 provide specificity. In addition, because our previous work provides evidence that Bicc1 has many relevant target mRNAs in addition to Cripto1 mRNA (Zhang et al., 2013), it is possible that the other KH domains are important for binding some of these targets. In other words, the Bicc1 multi KH and KHL domain may define a flexible RNA-binding surface that can bind different target mRNAs using distinct RNA-protein interfaces. Additional target RNA substrate characterization and Bicc1-RNA interaction assays to address these interesting possibilities are under way. Finally,

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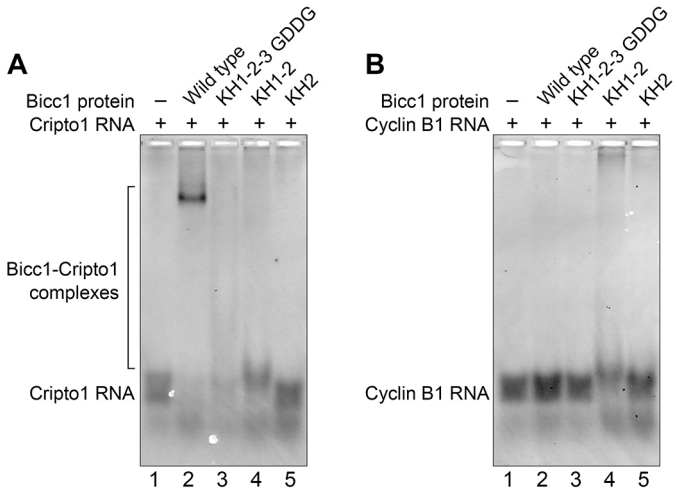

Fig. 6. The KH2 domain is not sufficient for RNA binding.
(A) Electromobility shift assays of Bicc1 wild-type and protein variants. Recombinant proteins consisting of the Bicc1 wild-type N terminus (amino acids 1-506), the KH1-2-3 GDDG protein (amino acids 1-506), KH1-2 protein (amino acids 41-201) and KH2 protein (amino acids 126-201) were expressed and purified from *E.coli*. Binding reactions consisting of the different proteins mixed with a fluorescently labeled 32-nucleotide RNA representing a well-characterized Bicc1-binding site derived from the 3′UTR of the *Xenopus* Cripto1 mRNA were analyzed on native polyacrylamide gels electrophoresed horizontally (Dowdle et al., 2017). (B) Electromobility shift assays of Bicc1 wild-type and protein variants with an RNA substrate derived from the 3′UTR of the *Xenopus* Cyclin B1 mRNA. The Cyclin B1 mRNA is not a Bicc1 target and represents a negative control for binding.

although the GXXD→GDDG KH1 and KH3 substitution variants provide evidence that the canonical RNA-binding activity of these domains is not crucial for Cripto1 mRNA binding or Cripto1-3′ UTR-directed translational repression, it remains possible that these domains are important for mediating protein-protein interactions that are crucial for this translational repression, as KH domains in other proteins have been shown to direct protein-protein interactions (Zheng et al., 2014; Nakel et al., 2015; Du et al., 2007; Teplova et al., 2011). However, although such interactions may be necessary, they are clearly not sufficient because the single GXXD→GDDG KH2 Bicc1 variant was unable to repress translation.

This work advances our understanding of how Bicc1 contacts its mRNA targets, and establishes that such RNA contacts were indispensable for Bicc1-directed translation repression and linking these functions to the role of Bicc1 as a regulator of anterior-posterior patterning in vertebrate embryos. In addition, the host-transfer rescue experiments used initially to define the maternal role of Bicc1 in embryogenesis, and here to test the importance of the ability of Bcc1 to make direct KH-RNA contacts to this role, provide a powerful context for further examining and refining our understanding of how the biochemical functions of Bicc1 relate to its biological role in cell fate decisions.

MATERIALS AND METHODS

Xenopus laevis oocyte and embryo manipulations

X. laevis oocyte and embryos were obtained and injected as described previously (Sive et al., 2000). Host-transfer experiments were performed using antisense oligodeoxynucleotides (oligos) against *bicc1* (Park et al., 2016). These were synthesized as HPLC-purified phosphorothioate-phosphodiester chimeric oligos (Integrated DNA Technologies) with the following sequences (asterisks indicate phosphorothioate linkages): *bicc1 9460*, 5′-G*C*GTGTTTGTCTCTTC*C*A-3′ (nucleotides 180-162, where the A of the AUG start codon is nucleotide 1); *bicc1 9463*, 5′-T*G*TAACATTGTCTCGAG*C*T-3′ (nucleotides 374-357). Oocytes were injected in the vegetal pole and cultured for 24 h at 18°C before being matured by treatment with 2.0 µM progesterone. Matured oocytes were colored with vital dyes, transferred to egg-laying host females, recovered and fertilized essentially as described previously (Heasman et al., 1991). For rescue experiments, *HA-bicc1* mRNA, which encodes Bicc1 with an N-terminal HA epitope tag, was injected into vegetal cells of *bicc1*-depleted embryos shortly after fertilization. The injected *HA-bicc1*

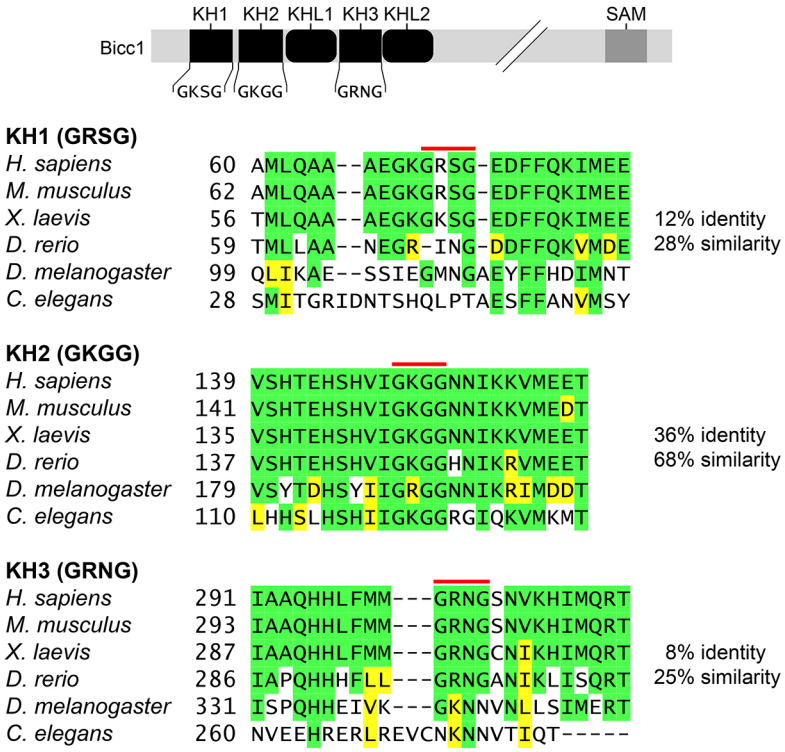

Fig. 7. The KH2 domain is an evolutionary conserved feature of Bicc1 proteins. Amino acid sequences from vertebrate and invertebrate Bicc1 proteins were analyzed with Clustal Omega. The regions surrounding the GXXG motif (red line) of each KH domain are shown. Residues identical to human Bicc1 are highlighted in green, while similar residues are highlighted in yellow. The comparison of full-length Bicc1 proteins is presented in Fig. S4.

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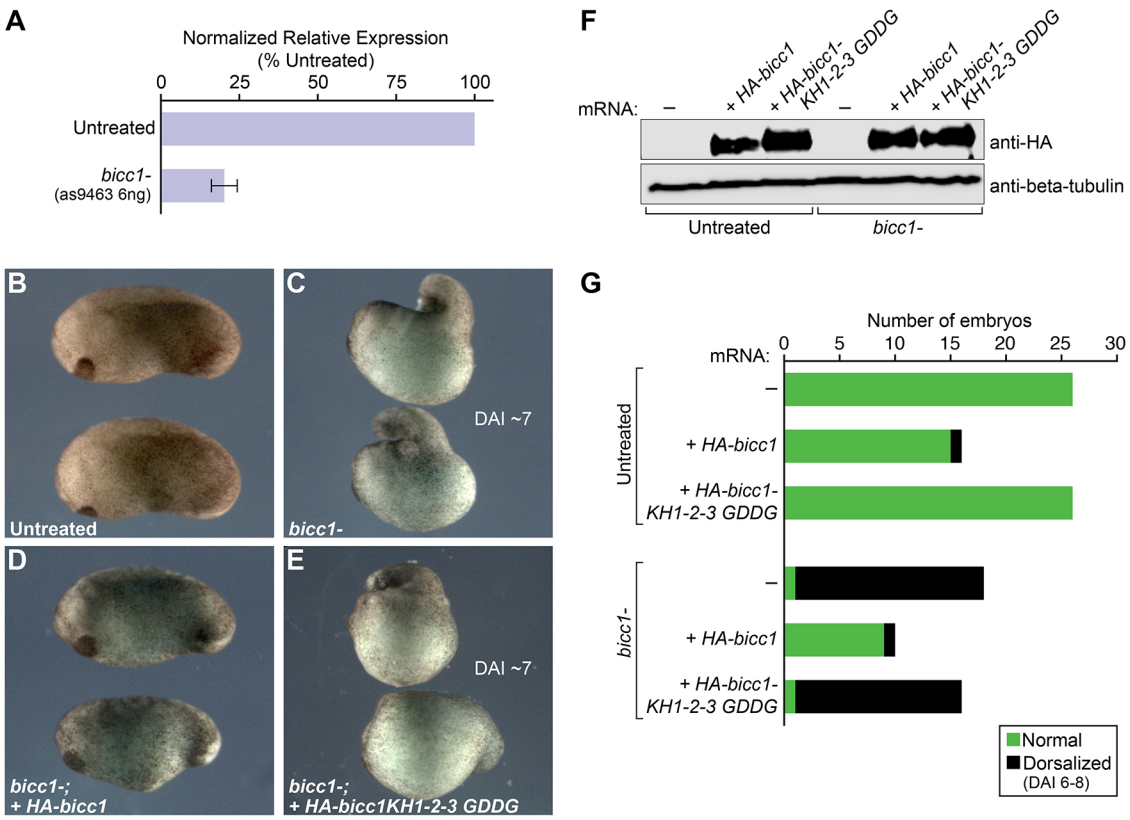

Fig. 8. The Bicc1 KH domains are required for the function of Bicc1 in embryonic patterning. (A) Validated Bicc1 antisense phosphorothioate oligonucleotide (oligo 9463) was injected into oocytes and the oocytes matured overnight. Matured oocytes were treated with vital dyes, transferred to an ovulating host female and the laid eggs from manipulated oocytes were fertilized. (B-D) Phenotypes of control and sibling experimental *Xenopus* embryos. Summary presented in G. (B) Control embryos (stage 22). (C) Stage 22 embryos depleted of maternal *bicc1* mRNA. The maternal knockout embryos develop with expanded dorsal-anterior structures (DAI 7). (D) The defects from depleting embryos of *bicc1* were rescued by wild-type *bicc1* mRNA. Embryos depleted of maternal *bicc1* mRNA were injected at the vegetal pole with wild-type *HA-bicc1* mRNA (20 pg). (E) The defects from depleting embryos of *bicc1* were not rescued by *KH1-2-3 GDDG bicc1* mRNA. Embryos depleted of maternal *bicc1* mRNA were injected at the vegetal pole with *KH1-2-3 GDDG bicc1* mRNA (20 pg). (F) The wild-type and KH1-2-3 GDDG Bicc1 proteins were expressed at comparable levels. Proteins from maternal knockout embryos injected with the different mRNAs were analyzed by immunoblotting and probing with an HA antibody. (G) Summary of the phenotypes from control, antisense oligo-injected host-transfer embryos and antisense oligo-injected host-transfer embryos co-injected with either mRNA encoding wild-type HA-Bicc1 or mRNA encoding HA-Bicc1 KH1,2,3 GDDG. The (+) samples received an injection of *HA-bicc1* mRNA or *HA-bicc1 KH1,2,3 GDDG* mRNA whereas the (–) samples did not.

mRNA used for rescue was not affected by the antisense oligos because the oligos are degraded a few hours after injection.

Bicc1 variants

Xenopus Bicc1 variants were created via PCR and cloned into a pCS2+ plasmid as fusions with a 3×HA epitope tag at their N-terminal end. All plasmids were verified by sequencing.

mRNA synthesis

Capped mRNAs encoding HA-tagged full-length Bicc1, HA-tagged Bicc1 variants and Firefly luciferase reporter mRNAs that contained the TCE of the *cripto1* mRNA 3′UTR or the 3′UTR of the *cyclinb1* mRNA were synthesized as described previously (Fritz and Sheets, 2001; Zhang et al., 2009, 2013; Sheets et al., 1994; Zhang et al., 2013).

Luciferase reporter mRNAs

Reporter mRNAs were diluted to a concentration of 2.5 nM and 5 nl (12.5 amol) was injected into embryonic cells. When injected embryos reached the appropriate stage, extracts were prepared and analyzed for luciferase activity (Fritz and Sheets, 2001; Zhang et al., 2009; Sheets et al., 1994).

Immunoblotting

The analysis of proteins by immunoblotting was carried out as described previously (Zhang et al., 2013) using rat anti-HA monoclonal antibody (1:2500; Clone 3F10, Roche, 11867423001), anti-actin monoclonal antibody (1:5000; Developmental Studies Hybridoma Bank, JLA20) and Bicc1 polyclonal antibody (1:2500; polyclonal antibody to *Xenopus* Bicc1 generated by the Sheets laboratory; Park et al., 2016).

Immunoprecipitations and qRT-PCR

Embryos were injected with mRNA encoding Bicc1 variants fused at the N terminus with an HA epitope tag (Ha-Bicc1) (Zhang et al., 2013). When injected embryos reached the appropriate blastula stage (stage 7), injected embryos were lysed in 100 µl of TNMEN-150 buffer (Cooke et al., 2010). The lysate was centrifuged (4°C, 10 min at 2400 ***g***) and the supernatant incubated with anti-HA antibody coupled to protein-G agarose (2 h, 4°C). The beads were collected (1 min, 850 ***g***) and washed four times in 1 ml TNMEN 150 buffer. For each wash, the beads were incubated in buffer at 4°C for 5 min, spun at 850 ***g*** for 1 min and supernatant removed. RNA was isolated from the washed beads for analysis by qRT-PCR (Park et al., 2016). Quantitative RT-PCR to analyze reporter mRNAs and endogenous mRNAs associated with Bicc1 was performed as described previously (Park et al., 2011). For each mRNA analyzed, the Q-PCR signal from HA-Bicc1 immunoprecipitates using the HA antibody was compared with the Q-PCR signal from embryos not expressing HA-Bicc1 and plotted. The signal for the Cyclin B1 mRNA was always very low, but detectible in each sample. To facilitate the comparison between samples, we normalized the values for the Cyclin B1 mRNA and compared the measurements of the other mRNAs to that value.

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Bicc1 protein expression and purification

Bicc1 variants were cloned into pET28b bacterial expression vectors as N-terminal fusions with a His-6 SUMO tag (Malakhov et al., 2004). Cultures of *E. coli* cells containing each plasmid were grown to an OD600 of 0.6 and induced with 1 mM IPTG at 25°C overnight. The cells were collected and lysed in B-PER reagent (bacterial protein extraction reagent, Thermo Fisher, 78248), 1/2×TBS, 2.5 mM $MgCl_2$, 2.5% glycerol, 1 mM BME, 10 mM imidazole, 200 mM NaCl, 1 mM ATP and protease inhibitors. The soluble lysate was applied to a nickel chromatography resin and allowed to bind overnight. The resin was then washed five times with 1×TBS, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 20 mM imidazole, 400 mM NaCl and 1 mM ATP, followed by one wash under the same conditions with no ATP. The proteins were then eluted with 450 mM imidazole and dialyzed in 1×TBS.

EMSA

Recombinant SUMO-Bicc1 N-terminal fusion proteins were expressed and purified as described above. The Cripto1 and CyclinB1 3′-fluorescein-labeled RNA substrates were purchased from IDT. Binding reactions (50 µl) contained SUMO-Bicc1 protein, 10 mM HEPES (pH 7.5), 1 mM EDTA, 50 mM KCl, 0.02% Tween 20, 0.1 mg/ml yeast tRNA, 100 µg/ml BSA, 2 mM DTT and 10 nM fluorescent RNA. Reaction products were analyzed on 7.5% (1×TBE) native polyacrylamide gels (Dowdle et al., 2017). The gels were then scanned at 473 nm using a fluorimager.

Fluorescent polarization assays for RNA binding

Binding reactions as described above were assembled into individual wells of a 96-well black round-bottomed plate. The reactions were scanned using a plate reader with an excitation wavelength at 485 nm and an emission wavelength of 528 nm in the parallel and perpendicular direction (Pagano et al., 2011). The data were analyzed using Gen5 software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.E.D., S.P., M.D.S.; Validation: S.B.I., D.W.H.; Formal analysis: M.E.D., S.P., M.D.S.; Investigation: M.E.D., S.P., S.B.I., D.W.H., M.D.S.; Writing - original draft: M.E.D., S.P., C.A.F., M.D.S.; Writing - review & editing: M.E.D., S.P., C.A.F., D.W.H., M.D.S.; Supervision: M.D.S.; Project administration: M.D.S.; Funding acquisition: M.D.S.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.172486.supplemental

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