© 2016. Published by The Company of Biologists Ltd | Development (2016) 143, 864-871 doi:10.1242/dev.131359

RESEARCH ARTICLE

A gradient of maternal Bicaudal-C controls vertebrate embryogenesis via translational repression of mRNAs encoding cell fate regulators

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ABSTRACT

Vertebrate Bicaudal-C (Bicc1) has important biological roles in the formation and homeostasis of multiple organs, but direct experiments to address the role of maternal Bicc1 in early vertebrate embryogenesis have not been reported. Here, we use antisense phosphorothioate-modified oligonucleotides and the host-transfer technique to eliminate specifically maternal stores of both *bicc1* mRNA and Bicc1 protein from *Xenopus laevis* eggs. Fertilization of these Bicc1-depleted eggs produced embryos with an excess of dorsal-anterior structures and overexpressed organizer-specific genes, indicating that maternal Bicc1 is crucial for normal embryonic patterning of the vertebrate embryo. Bicc1 is an RNA-binding protein with robust translational repression function. Here, we show that the maternal mRNA encoding the cell-fate regulatory protein Wnt11b is a direct target of Bicc1-mediated repression. It is well established that the Wnt signaling pathway is crucial to vertebrate embryogenesis. Thus, the work presented here links the molecular function of Bicc1 in mRNA target-specific translation repression to its biological role in the maternally controlled stages of vertebrate embryogenesis.

KEY WORDS: *Xenopus*, Maternal mRNA, Regulated translation, Wnt

INTRODUCTION

Bicaudal-C (Bicc1) proteins are evolutionarily conserved RNA-binding proteins in metazoans with important roles in a variety of biological contexts (Gamberi and Lasko, 2012). The founding member of the Bicc1 family, *BicC*, was identified as a maternal effect gene in *Drosophila melanogaster* (Bull, 1966). Mutations that disrupt maternal *BicC* give rise to fly embryos with anterior-posterior defects (Mahone et al., 1995; Saffman et al., 1998). Recent studies in vertebrates provide clear evidence that Bicc1 functions to control post-maternal stages of development and homeostasis of several organs. For example, homozygous *Bicc1* mutant mice develop into adults but exhibit defects of their kidneys, pancreas and heart (Maisonneuve et al., 2009). Some mutations in the human *BICC1* gene are associated with the kidney disease cystic renal dysplasia, whereas others are associated with defects in bone density (Kraus et al., 2012; Mesner et al., 2014). Collectively, these examples reveal the importance of zygotically expressed Bicc1 to normal vertebrate development and health, but they do not address the role of maternal Bicc1 in vertebrate embryogenesis. Thus, although it is clear that vertebrate oocytes, eggs and embryos express *Bicc1* mRNA, suggesting an important role for Bicc1 in the maternal stages of embryogenesis, no study to date has directly addressed this issue by generating mutants specifically lacking maternal Bicc1 (Wessely and De Robertis, 2000; Wessely et al., 2001; Bouvrette et al., 2010).

Several lines of evidence indicate that Bicc1 proteins function as translational repressors of specific target mRNAs. However, only a few studies present data linking Bicc1-dependent translation repression of relevant target mRNAs to its biological roles (Chicoine et al., 2007; Piazzon et al., 2012; Zhang et al., 2013). In recent studies of maternally controlled stages of embryogenesis of *Xenopus laevis*, we have presented evidence that several maternal mRNAs encoding important cell-fate regulators can bind Bicc1 and are potential targets of Bicc1-mediated translational repression, including most notably the *cripto1* (*tdgf1.3* – Xenbase) mRNA for which we have defined a Bicc1 RNA-binding element in the 3′ untranslated region (3′UTR) (Zhang et al., 2013). However, although these data are consistent with a model in which the translational repression activity of Bicc1 regulates the earliest steps of vertebrate embryogenesis, such as establishing the body axes, experiments that directly address the role of maternal Bicc1 in vertebrates are needed to test this model and establish a connection between the biological and biochemical roles of Bicc1.

In this study, we directly examine the role of maternal Bicc1 in a vertebrate organism by specifically depleting both *bicc1* mRNA and Bicc1 protein from *Xenopus laevis* eggs using two different antisense oligonucleotides in independent experiments and fertilizing these eggs using the host-transfer technique. The Bicc1-depleted embryos exhibited greatly reduced levels of Bicc1 protein, developed with an excess of dorsal-anterior structures and overexpressed several genes that define Spemann's organizer, the organizing tissue that controls cell-fate decisions after zygotic transcription begins (reviewed by Houston, 2012). In previous studies, we identified the *cripto1* and *coco* (*dand5* – Xenbase) mRNAs as potential targets of Bicc1-mediated repression (Zhang et al., 2013). These data along with previous phenotypic studies of *cripto1* and *coco* mutants help link the biological role of Bicc1 in embryogenesis documented here to its biochemical role in translational repression (Tao et al., 2005; Bates et al., 2013). In addition, based on the phenotypes of our Bicc1-depleted embryos, we found that phenotypes of Wnt11b loss-of-function and gain-of-function (overexpression) embryos were consistent with Bicc1 regulation of Wnt11b expression (Tao et al., 2005). Therefore, we hypothesized that *wnt11b* mRNA is a direct Bicc1 target. Here, we provide evidence in support of this hypothesis by showing that Bicc1 could bind to *wnt11b* mRNA in embryos and that this mRNA contains a Bicc1-dependent translation repression element within its 3′UTR. Finally, using antibodies produced against Bicc1 we show

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Received 28 September 2015; Accepted 16 January 2016

Development (2016) 143, 864-871 doi:10.1242/dev.131359

that endogenous Bicc1 protein is present in eggs and embryos and exists in a vegetal-to-animal pole concentration gradient that mirrors Bicc1-mediated translation repression activity. In summary, this study presents the first direct examination of the role for maternal Bicc1 in vertebrates and, together with data from our previous work, begins to establish the connections between this role and the biochemical function of Bicc1 in mRNA-specific translational repression.

RESULTS

Embryos depleted of maternal Bicc1 exhibit enhanced dorsal-anterior structures and organizer-specific gene expression

To address the relevance of maternal Bicc1 to *X. laevis* development, we performed maternal mRNA depletion experiments using antisense oligonucleotides (oligos) (Heasman et al., 1991; Olson et al., 2012). Full-grown *X. laevis* oocytes were injected to identify antisense oligos that would cleave and deplete maternal *bicc1* mRNA. Two different antisense oligos (as9460 and as9463) were effective at reducing *bicc1* mRNA levels as monitored by RNA blot hybridization and qRT-PCR (Fig. 1A,B). Importantly, these oligos targeted both Bicc1 homologs present in the allotetraploid *X. laevis* genome that are predicted to encode the same Bicc1 protein (http://www.xenbase.org). Examination of endogenous maternal Bicc1 protein has not been reported, but assessing Bicc1 protein levels was crucial to interpretation of these experiments. Therefore, we raised an antibody to the N-terminal half of the *X. laevis* Bicc1 protein and used it to examine Bicc1 protein in control and oligo-treated oocytes and eggs by protein immunoblotting (Fig. 1C,D). Bicc1 protein was not detectable in oocytes but accumulated during oocyte maturation, indicating that maternal Bicc1 protein is already present in unfertilized eggs (Fig. 1C). However, oocytes injected with the antisense oligos as9460 and as9463 failed to accumulate Bicc1 protein during maturation (Fig. 1D), indicating that these oligos effectively eliminated the majority of maternal *bicc1* mRNA and Bicc1 protein.

To perform maternal loss-of-function experiments, antisense oligos were synthesized in a phosphorothioate-modified form and injected into oocytes. These oocytes were then matured *in vitro*, transferred into ovulating host females, recovered as eggs, and fertilized to examine the effects of *bicc1* depletion on development (Olson et al., 2012). Depletion with each oligo yielded similar results. *Bicc1*-depleted embryos developed normally up to gastrulation, but were delayed in completing blastopore closure and often formed thickened blastopore lips with deep-set yolk plugs (Fig. 2A). This phenotype is often associated with axis defects. By the tailbud stages, *bicc1*-depleted embryos displayed an excess of dorsal-anterior structures that included enlarged heads, often accompanied by expansion of the cement gland. Dorsoanterior index (DAI) scores ranged from 6 to 7 (Fig. 2B,C,E; Table 1) (Kao and Elinson, 1988). To verify that the phenotypes were due to loss of maternal Bicc1, rescue experiments were performed in which Bicc1-depleted embryos were injected with *in vitro*-synthesized *bicc1* transcript (20 pg) shortly after fertilization. This relatively low dose and post-fertilization injection regimen was necessary, as excess Bicc1 was a highly potent inhibitor of dorsal development and of gastrulation. The addition of the *bicc1* mRNA reduced the proportion of embryos exhibiting exaggerated dorsal-anterior structures and even induced ventralization in some samples, including a fraction of the control samples (Fig. 2E; Table 1). These findings suggest that the concentration and localization of Bicc1, both challenging to control precisely in microinjection experiments, are crucial to normal embryogenesis. Nevertheless, these data indicated that *bicc1* transcript could reverse the dorso-anteriorization associated with Bicc1 depletion and thus indicated the specificity of the antisense oligos for *bicc1* mRNA. Thus, we conclude that Bicc1 depletion and not off-target effects cause the mutant phenotypes.

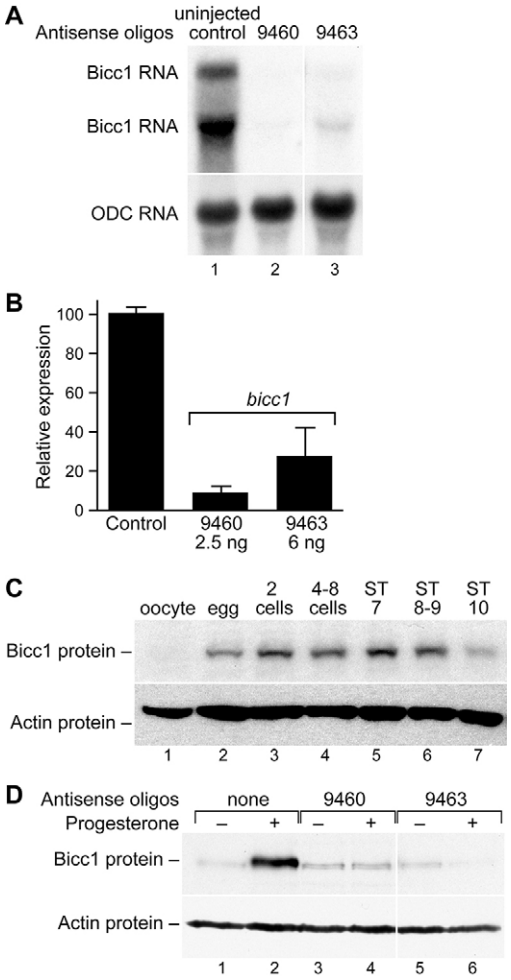

Fig. 1. Identifying antisense oligonucleotides that degrade *Xenopus bicc1* mRNA. (A) Identification of antisense oligonucleotides (oligos) that degrade the *bicc1* mRNA. Oligos (9460 and 9463) were injected into oocytes, then total RNA was isolated from injected cells and analyzed by RNA blot hybridization. The *bicc1* mRNA was detected with a radiolabeled probe generated from the 3′UTR of *bicc1* cDNA. The same filter was hybridized with a radiolabeled probe to detect *ornithine decarboxylase 1* (*odc1*) mRNA as a negative control. (B) In a separate experiment, oligos (9460 and 9463) were injected into oocytes, then total RNA was isolated from injected cells, and analyzed by qRT-PCR. (C) Expression of the Bicc1 protein during early *Xenopus* development. Proteins from an equivalent number or oocytes, eggs or embryos were analyzed by immunoblotting probing with an antibody generated against the N-terminal half of the *Xenopus* Bicc1. The same blot was analyzed with an anti-actin antibody as a loading control. ST, stage. (D) Antisense oligos block accumulation of the Bicc1 protein during oocyte maturation. Oocytes were injected with either the 9460 or the 9463 oligo and incubated for two hours. The injected oocytes were matured overnight with progesterone then the proteins analyzed by immunoblotting and probing with the Bicc1 antibody. Error bars represent s.e.m.

During *X. laevis* development, the formation of dorsal-anterior structures relies on activities of organizer-specific genes, such as *goosecoid homeobox* (De Robertis, 2006; Houston, 2012). The phenotypes of Bicc1-depleted embryos indicated that organizer

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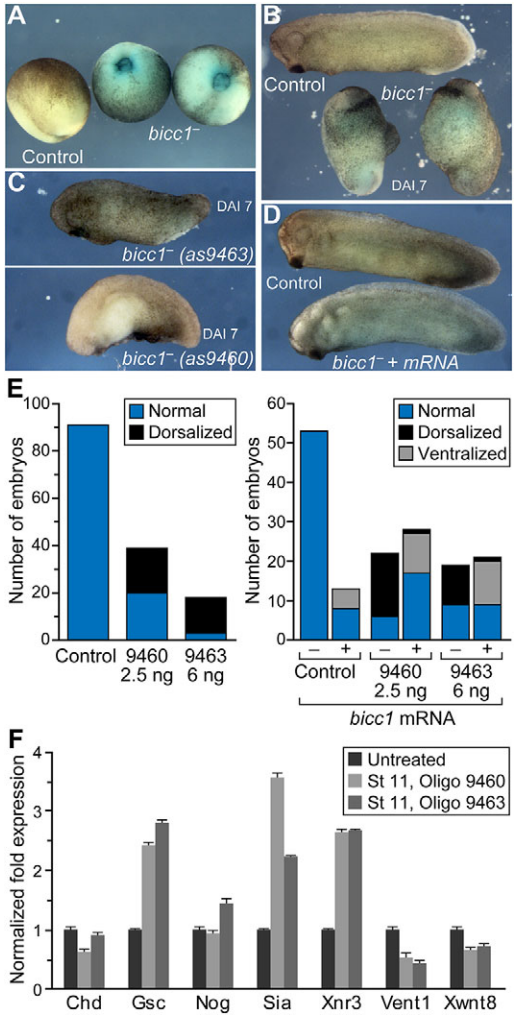

Fig. 2. Embryos depleted of maternal Bicc1 exhibit expanded dorsal-anterior structures and increased organizer-specific gene expression. Phosphorothioate derivatives of the 9460 and 9463 oligos were injected into oocytes and the oocytes matured overnight. Matured oocytes were treated with vital dyes, transferred to an ovulating host female, and the laid eggs from manipulated oocytes were fertilized. (A-C) Phenotypes of control embryos and sibling *bicc1*-depleted embryos. (A) Control (left, green) and maternal *bicc1*-depleted (blue; as9463) embryos at stage 13, showing delayed gastrulation and deep blastopores. (B) Stage 28 embryos depleted of maternal *bicc1* mRNA (as9463) develop expanded dorsal-anterior structures (DAI 7). (C) Stage 30 embryos depleted of maternal *bicc1* mRNA using either the 9460 or the 9463 oligo. Embryos treated with either oligo develop with expanded dorsal-anterior structures (DAI 7). (D) The defects from depleting embryos of *bicc1* can be rescued by injecting *bicc1* mRNA. Embryos depleted of maternal *bicc1* mRNA were injected at the vegetal pole with HA-*bicc1* mRNA (20 pg). (E) Summary of the phenotypes of control and antisense oligo-injected host-transfer embryos. The (+) samples received an injection of HA-*bicc1* mRNA whereas the (–) samples did not. (F) The expression of organizer genes increased in embryos depleted of maternal *bicc1*. Total RNA was isolated from *bicc1*-depleted stage 11 embryos and controls. qRT-PCR was used to analyze the expression of different organizer-specific genes and the *ventx1.2* and *wnt8a* genes, which are markers of ventral-posterior development. The expression changes were significantly different ($P<0.05$, except for *wnt8* in 9463-treated embryos for which $P<0.1$). Error bars represent s.e.m.

function was overactive. To test this directly, we analyzed organizer gene expression at gastrulation (stage 11) using qRT-PCR. Expression of the organizer-specific genes *siamois homeodomain 1* (*sia1*), *goosecoid homeobox* (*gsc*), *nodal3.1* (also known as *Xnr3*) and *noggin* (*nog*) were increased in Bicc1-depleted embryos relative to uninjected controls, whereas the expression of genes expressed by the ventral/posterior cells that flank the organizer, *ventx1.2* and *wnt8a*, were decreased (Fig. 2F). Thus, depletion of Bicc1 led to over-development of the organizer.

wnt11b mRNA is a target of Bicc1 translational repression

Embryos depleted of Bicc1 developed with expanded dorsal-anterior structures (Fig. 2). This phenotype showed similarity to that described for embryos that overexpress the Wnt11b ligand (Tao et al., 2005), raising the possibility that *wnt11b* mRNA was a direct or indirect target of Bicc1 translational repression. Although *wnt11b* mRNA was not identified as a Bicc1 target in our previous studies using an ectopically expressed HA-Bicc1 immunoprecipitation strategy that identified several mRNAs relevant to cell-fate decisions, there were several possible technical reasons that could cause relevant direct targets to be missed (Zhang et al., 2013). Therefore, we tested whether *wnt11b* mRNA encoded elements sufficient to allow Bicc1-mediated repression using a luciferase reporter mRNA assay described in our previous work (Zhang et al., 2013). Specifically, an mRNA containing the luciferase coding region fused to the *X. laevis wnt11b* mRNA 3′UTR was injected into animal cells of 4- to 8-cell embryos, with and without a synthetic mRNA encoding HA-tagged Bicc1 (HA-Bicc1) (Fig. 3A,B). When embryos reached stage 8-9, extracts were prepared and assayed for luciferase activity. The extent of repression by Bicc1 was measured by determining the ratio of luciferase activity for each reporter mRNA in the presence or absence of Bicc1. The reporter mRNA containing the *wnt11b* 3′UTR was repressed by co-expression of HA-Bicc1 to the same extent as the reporter containing the *cripto1* 3′UTR that we previously identified and characterized as a Bicc1-regulated mRNA (Fig. 3C) (Zhang et al., 2009, 2013). The effect was specific, as a reporter mRNA containing the 3′UTR from the *cyclin B1* (*ccnb1*) mRNA, an mRNA not regulated by Bicc1, was not repressed. Additional controls indicated that the effects on luciferase expression were not caused by differences in the amount of HA-Bicc1 expressed or the stability of the mRNA reporters (Fig. 3D,E).

Bicc1 physically associates with the 3′UTR of the *wnt11b* mRNA in embryos

If Bicc1 regulation of *wnt11b* mRNA were direct, then Bicc1 should be capable of physically associating with this mRNA. To test this possibility, an mRNA encoding HA-Bicc1 was injected into animal cells of 8-cell embryos. When the embryos developed to the blastula stage (stage 7) the HA-Bicc1 protein was immunoprecipitated and the bound RNAs were analyzed by qRT-PCR using specific primers (Fig. 4A). *wnt11b* mRNA was present in the HA-Bicc1 immunoprecipitate, as was *cripto1* mRNA, a previously established Bicc1 target (Fig. 4B) (Zhang et al., 2013). The Bicc1 association was specific as the abundant *cyclin B1*, *odc1* or *eif1* mRNAs were not immunoprecipitated. We note that that HA-Bicc1 was less efficient at immunoprecipitating the endogenous *wnt11b* mRNA compared with *cripto1* mRNA. Perhaps this observation explains why *wnt11b* mRNA was not identified in our previous screen for Bicc1 target mRNAs (Zhang et al., 2013). Regardless, these experiments provided evidence that Bicc1 could physically associate with *wnt11b* mRNA in embryos.

Our previous studies of *cripto1* mRNA indicate that its regulation by Bicc1 is mediated via the *cripto1* mRNA 3′UTR. Therefore, we investigated whether the 3′UTR of the *wnt11b* mRNA was sufficient for Bicc1 association by performing HA-Bicc1 immunoprecipitation experiments in embryos co-injected with

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Table 1. Maternal *bicc1* is required for normal dorsoventral development

Experiment	*n*	Normal (%)	Dorsalized (%; DAI 6-7)	Ventralized gastrulation defects (%; DAI≤4)	*P*-value (Fisher's exact test)
Series 1 (three experiments)					
Uninjected	86	86 (100)	0 (0)	N.A.	–
as9460 (2.5 ng)	39	20 (51)	19 (49)	N.A.	$<1\times10^{-12}$
as9463 (6 ng)	18	3 (17)	15 (83)	N.A.	$<1\times10^{-15}$
Series 2 (two experiments)					
Uninjected	53	53 (100)	0 (0)	0 (0)	–
as9460 (2.5 ng)	22	6 (27)	16 (73)	0 (0)	–
as9460 (2.5 ng)+*bicc1* (20 pg)	28	17 (61)	1 (3)	10 (36)	$<1\times10^{-8}$
as9463 (6 ng)	19	9 (47)	10 (53)	0 (0)	–
as9463 (6 ng)+*bicc1* (20 pg)	21	9 (43)	1 (5)	11 (52)	$<1\times10^{-4}$

Summary of phenotypes in *bicc1*-depleted embryos and rescued embryos. Oocytes were injected with indicated oligos and fertilized by host transfer. Phenotypes were assessed at stage 28-30. Dorsoanterior axis index (DAI) was estimated, according to Kao and Elinson (Kao and Elinson, 1988). Fisher's exact test was performed comparing uninjected oocytes with those injected with each oligo (Series 1) or oocytes injected with oligo to those injected with oligo+mRNA (Series 2). N.A., not applicable.

reporter luciferase reporter RNAs containing the indicated 3′UTRs (Fig. 3B). The HA-Bicc1 immunoprecipitates were analyzed by qRT-PCR using primers to the luciferase-coding region. HA-Bicc1 associated with the reporter mRNAs containing either the 3′UTR of *wnt11b* mRNA or of the positive control *cripto1* mRNA. By contrast, HA-Bicc1 did not associate with the negative control reporter mRNA containing the 3′UTR of *cyclin B1* mRNA (Fig. 4C). Together, these results indicate that Bicc1 can associate directly with the *wnt11b* mRNA in embryos and that this interaction is mediated by sequences within the 3′UTR of *wnt11b* mRNA.

The animal-to-vegetal gradient of Bicc1-dependent translational repression activity reflects a gradient of endogenous Bicc1 protein

mRNAs containing the 3′UTRs of Bicc1 target mRNAs are efficiently repressed in vegetal cells but not in animal cells of *X. laevis* embryos (Zhang et al., 2013). To determine whether this differential repression might be attributed to the distribution of endogenous maternal Bicc1 protein, *X. laevis* blastula stage embryos (stage 7) were dissected into three regions (animal, marginal zone and vegetal) and analyzed for endogenous Bicc1 protein by protein immunoblotting. Vegetal cells contained the highest levels of Bicc1 and animal cells contained the lowest levels, whereas marginal zone cells contained a level of Bicc1 protein intermediate between these two poles (Fig. 5A). By contrast, cytoskeletal actin was uniformly distributed between these three regions of the embryo. Thus, endogenous Bicc1 protein was differentially distributed along the animal-vegetal axis. To address whether the repression activity attributed to Bicc1 followed this same gradient, reporter mRNAs containing Bicc1-responsive 3′UTRs were injected into animal, marginal zone, or vegetal cells of 16-cell-stage embryos. Extracts were prepared from injected embryos after they reached stage 8-9 and assayed for luciferase activity. The luciferase activity of each reporter mRNA introduced into each injection site was normalized to animal cell injections. The repression of *cripto1* and *wnt11b* reporter mRNAs was highest in vegetal cells and lowest in animal cells, with marginal zone cells exhibiting an intermediate level of repression (Fig. 5B). By contrast, the negative control reporter containing the 3′UTR of *cyclin B1* mRNA was translated at similar levels in all three embryonic locations. The differences in luciferase activity observed for the *cripto1* and *wnt11b* reporter mRNAs were due to translational repression and not differences in mRNA stability as all the reporter mRNAs were present in similar amounts in all three regions analyzed as assayed by qRT-PCR (Fig. 5C). Thus, our results strongly suggest a model in which the gradient of endogenous maternal Bicc1 protein establishes the animal-vegetal gradient of Bicc1 translational repression necessary for the maternally controlled stages of *X. laevis* embryogenesis.

In a previous study and in the experiments described above, we have shown that HA-Bicc1 protein generated in animal cells, by injection of HA-*bicc1* mRNA, is sufficient to cause Bicc1-dependent translational repression of reporter mRNAs and to associate with endogenous target mRNAs in animal cells (Fig. 3) (Zhang et al., 2013). These observations provide evidence that Bicc1 protein itself is the limiting component for Bicc1-mediated translational repression in animal cells and that everything else needed for repression must be present (Zhang et al., 2013) (Fig. 3). To test the converse hypothesis, specifically that Bicc1 is present in excess in vegetal cells, the reporter containing the 3′UTR of the *cripto1* mRNA was injected into animal cells or vegetal cells of 8-cell *X. laevis* embryos in the presence or absence of HA-*bicc1* mRNA. Extracts from the injected cells were analyzed for luciferase activity and the amount of Bicc1-dependent repression was determined. As expected, animal cells repressed the *cripto1* reporter mRNA only when additional Bicc1 was provided (Fig. 5D,E). However, and in contrast to these observations, the *cripto1* reporter mRNA was not repressed to any further extent in vegetal cells when additional Bicc1 was provided. Thus, Bicc1 translational repression activity was in excess in vegetal cells, consistent with our observation that the endogenous Bicc1 protein was concentrated in these cells.

DISCUSSION

This study presents the first direct evidence that the maternal Bicc1 translational repressor regulates the earliest maternally controlled patterning events in vertebrates. Specifically, depletion of the maternal *bicc1* mRNA and Bicc1 protein from *X. laevis* embryos caused them to develop with an excess of dorsal-anterior structures. This phenotype is most likely to be caused by defects in the maternal steps required for formation of Spemann's organizer because several organizer-specific genes are overexpressed in the Bicc1-depleted embryos. We examine endogenous maternal Bicc1 protein and show that it forms a concentration gradient across the embryo from high in the vegetal pole to low in the animal pole that mirrors the activity levels of Bicc1-dependent translational repression. Thus, a concentration gradient of maternal Bicc1 protein establishes the gradient of Bicc1-dependent translational repression function.

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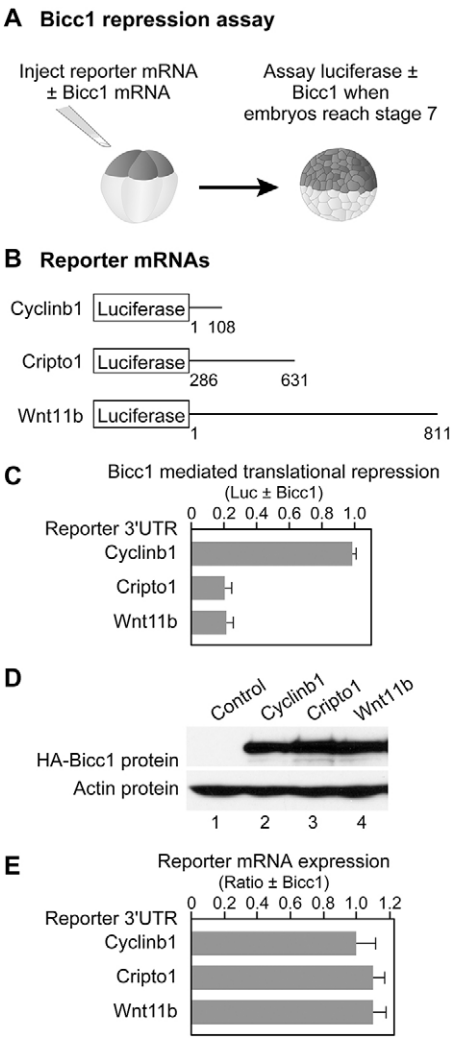

Fig. 3. The *wnt11b* maternal mRNA is a target of Bicc1 repression. (A) Animal cell assay for Bicc1 translational repression. Animal cells of 8-cell *Xenopus* embryos were injected with luciferase reporter mRNAs. Some of the embryos were given a second injection of mRNA encoding full-length *Xenopus* Bicc1. When embryos reached stage 7-8, luciferase assays were performed. Repression, as measured by the ratio of luciferase exhibited by a reporter mRNA with and without Bicc1 expression, was calculated and plotted. (B) Diagram of 3′UTR fragments incorporated into luciferase reporter mRNAs and analyzed for repression. (C) The *wnt11b* maternal mRNA is repressed by Bicc1. Error bars represent the s.e.m. from three separate experiments. (D) Immunoblot analysis of HA-Bicc1 expression in repression assays. (E) qRT-PCR analysis of luciferase reporter mRNAs in repression assays. Error bars represent s.e.m.

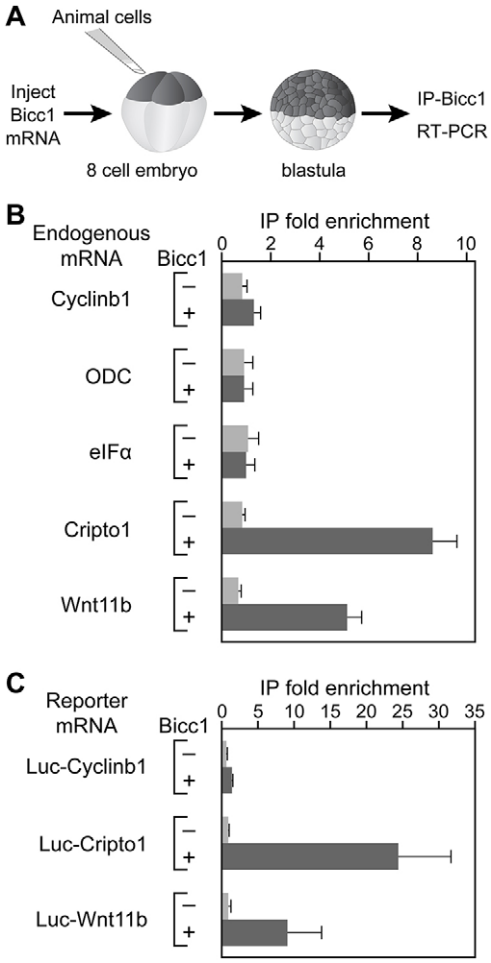

Fig. 4. Bicc1 functions by binding to the 3′UTR of the *wnt11b* mRNA. (A) Animal cell assay for *in vivo* Bicc1 binding. Animal cells of 8-cell *Xenopus* embryos were injected with mRNA encoding HA-tagged Bicc1. Some injected samples included luciferase reporter mRNAs. When embryos reached stage 7-8, Bicc1 was immunoprecipitated with an HA antibody and the associated RNA isolated for analysis. RNA samples were reverse transcribed and the cDNA used as template for q-PCR. (B) The endogenous *Xenopus* maternal *wnt11b* mRNA was bound by Bicc1. The *wnt11b* mRNA was enriched in Bicc1 IP samples, similar to the known mRNA target *cripto1*. (C) The binding of Bicc1 to *wnt11b* mRNA occurs via sequences in the 3′UTR. Embryos were injected with mRNA encoding HA-tagged Bicc1 along with different reporter mRNAs. Bicc1 was immunoprecipitated and the associated RNAs analyzed as described above (Fig. 3A). The reporter mRNAs containing the 3′UTR of the *wnt11b* mRNA associated with Bicc1, as did the positive control reporter mRNA containing the 3′UTR of the *cripto1* mRNA. Error bars represent s.e.m.

We propose that this maternal Bicc1 gradient establishes additional molecular asymmetries of cell-fate regulators, including key signaling proteins that drive embryonic patterning and cell-fate decisions. Finally, the phenotype of Bicc1-depleted embryos led us to identify a new relevant maternal Bicc1 target, *wnt11b* mRNA. Our data provide evidence that Bicc1 attenuated translation of *wntb11* mRNA directly by associating with the 3′UTR of this mRNA. The observations presented here, together with our previous study that identifies *coco* and *cripto1* mRNAs as targets of Bicc1-mediated repression, provide a link between the biological roles of Bicc1 in the maternal stages of vertebrate embryogenesis to its biochemical role as a translational repressor (Zhang et al., 2013). Thus, we propose a model in which Bicc1-specific translational repression helps establish many of the animal-vegetal embryonic asymmetries of cell-fate regulatory proteins, which in turn direct the embryonic patterning and earliest cell-fate decisions of vertebrate development (Sheets et al., 2016).

Isolating the role of maternal Bicc1 in vertebrate embryogenesis

A challenge for examining maternal effect genes in vertebrates is that the protein might already be present in the egg from maternal stores. An important contribution of this study is that the antisense oligos used to abolish the maternal *bicc1* mRNA in oocytes allowed for the depletion of both *bicc1* mRNA and Bicc1 protein. Using an antibody to detect endogenous Bicc1, we show that substantial amounts of Bicc1 are already present in the egg prior to fertilization, having accumulated during oocyte maturation. Thus, to examine the

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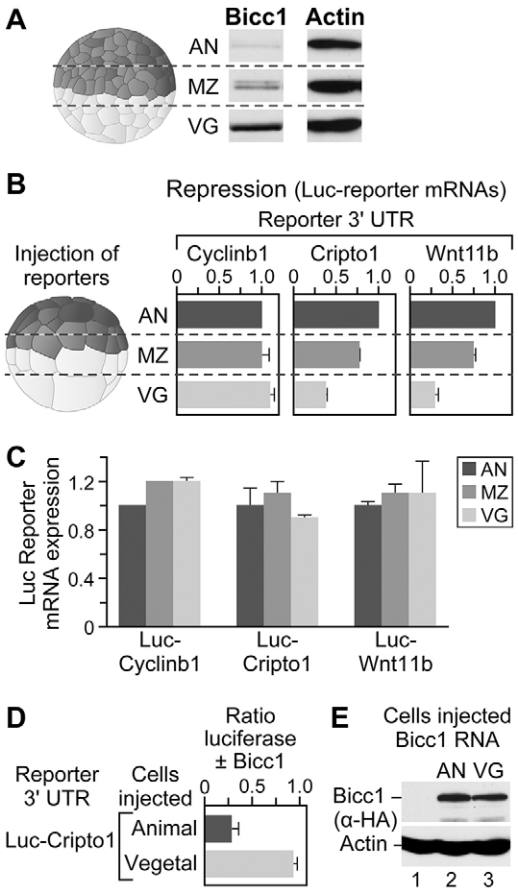

Fig. 5. Bicc1 translational repression activity is present as an animal-vegetal gradient in the embryo. (A) Bicc1 protein is present in an animal-vegetal gradient. Stage 7 embryos were manually dissected into three parts; animal (AN), marginal zone (MZ) and vegetal (VG). The proteins from these regions were analyzed by immunoblotting and probing with a Bicc1 antibody. The same filter was also probed with an antibody recognizing cytoskeletal actin. (B) The Bicc1 repression activity is present in an animal-vegetal gradient. *Xenopus* embryos (16-cell) were injected with luciferase reporter mRNAs containing different 3′UTRs (Fig. 2B): *wnt11b*, *cripto1* and *cyclin B1*. Each reporter was injected into animal cells, marginal zone cells or vegetal cells of separate groups of embryos. When injected embryos reached stage 8-9 they were used to prepare extracts for the assaying of luciferase activity. For each mRNA, the activity when injected into animal cells was set as 1 and used as a reference point for the activities in other cells. (C) Changes in luciferase for each reporter mRNA were due to changes in translation and not changes in mRNA degradation. RNA isolated from embryos from each injection was analyzed for presence of luciferase reporter mRNAs using qRT-PCR. (D) Animal or vegetal cells of 8-cell *Xenopus* embryos were injected with luciferase reporter mRNA containing the 3′UTR from the *cripto1* mRNA. Some of the embryos were given a second injection of mRNA encoding full-length *Xenopus* Bicc1. When embryos reached stage 8-9, luciferase assays were performed. Repression as measured by the ratio of luciferase exhibited by a reporter mRNA with and without Bicc1 expression was calculated and plotted. (E) Immunoblot analysis was used to analyze the expression of HA-Bicc1 in animal and vegetal cells. Error bars represent s.e.m.

maternal role for Bicc1, depleting *bicc1* mRNA from oocytes and then using the host-transfer method to allow for fertilization of the depleted eggs was key. Indeed, a previous study injected morpholinos to disrupt *bicc1* mRNA translation in 2- to 4-cell-stage *Xenopus* embryos and showed that embryos developed to later stages with kidney defects (Tran et al., 2007). These data are fully consistent with studies of homozygous *Bicc1* mutants in mice and support a role for zygotic Bicc1 in normal kidney formation in vertebrates (Maisonneuve et al., 2009; Tran et al., 2010). Importantly, our work shows that by the 2- to 4-cell stage a substantial amount of Bicc1 protein is already present. Thus, the kidney defects from morpholino treatment are probably the result of disrupting zygotic Bicc1 function but leaving maternal Bicc1 function intact.

Bicc1 maternal gradient in the establishment of other essential molecular gradients

The *X. laevis bicc1* maternal mRNA is localized to the vegetal cortex during oogenesis (Wessely and De Robertis, 2000). Here, we show that the *bicc1* mRNA itself is translationally regulated during oocyte maturation; the protein is absent from oocytes, but accumulates during oocyte maturation. Our data also reveal that the Bicc1 protein is distributed in a gradient along the animal-vegetal axis. This protein gradient is likely to be a direct result of *bicc1* mRNA localization achieved during oogenesis. The Bicc1 translational repression activity we measured tracked with this concentration gradient. Thus, mRNA localization during oogenesis sets the stage for a maternal Bicc1 protein gradient that goes on to have a profound impact on embryogenesis by establishing additional molecular gradients via translational repression of cell-fate regulatory mRNAs such as *coco*, *cripto1* and *wntb11* (Sheets et al., 2016). Essentially, a cascade of gradient amplification is achieved through Bicc1-mediated repression, with this initial gradient establishing multiple secondary gradients. Precisely how Bicc1-repression is tailored or fine-tuned for specific targets to achieve the proper combination of cell-fate regulatory gradients remains unknown.

Post-transcriptional control of Wnt signaling

A previous study of mouse embryos revealed that Bicc1 affected development at least in part through the Wnt signaling pathway (Maisonneuve et al., 2009). Thus, it was particularly notable that in this study we identified *wnt11b* mRNA as a direct target of Bicc1. Our earlier work identified several potential maternal mRNA targets for Bicc1 making it likely that the Bicc1-repression mechanism fine-tunes the expression of multiple signaling pathways (Zhang et al., 2013). However, it is interesting to note that some of the other Bicc1 mRNA targets we identified in our previous studies have also been implicated as modulators of Wnt signaling. For example, we have shown that *cripto1* mRNA is a Bicc1 target, and the Cripto1 protein affects multiple signaling events, including those of the Wnt pathway (Nagaoka et al., 2012, 2013; Klauzinska et al., 2014). We also identified *ddx5* mRNA as a Bicc1 target (Zhang et al., 2013), and Ddx proteins are documented modulators of Wnt signaling (Cruciat et al., 2013). These observations raise the possibility that Bicc1 is a major regulator of Wnt signaling localization and strength via its attenuation of expression of multiple proteins that regulate this pathway. Whether the bulk of Bicc1 regulation of maternal development can ultimately be explained by Wnt pathway modulation or instead involves modulation of other independent signaling pathways remains an open question.

MATERIALS AND METHODS

Xenopus laevis oocyte and embryo manipulations

X. laevis oocyte and embryos were obtained and injected as described (Sive et al., 2000). Host-transfer experiments were performed using antisense oligonucleotides (oligos) against *bicc1*. 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);

Development (2016) 143, 864-871 doi:10.1242/dev.131359

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 (Heasman et al., 1991; Olson et al., 2012). 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* mRNA used for rescue was not affected by the antisense oligos because the oligos are degraded a few hours after injection.

Luciferase reporter mRNA plasmids and mRNA synthesis

Firefly luciferase reporter mRNAs were generated that contained the translational control element of the *cripto1* mRNA 3′UTR, the 3′UTR of the *cyclin B1* mRNA or the 3′UTR of the *wnt11b* mRNA (Sheets et al., 1994; Fritz and Sheets, 2001; Zhang et al., 2009). All plasmids were linearized with appropriate restriction enzymes and used to generate capped mRNAs as described (Sheets et al., 1994; Fritz and Sheets, 2001; Zhang et al., 2009, 2013).

mRNA injections and luciferase assays

Each reporter mRNA was diluted to 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 (Sheets et al., 1994; Fritz and Sheets, 2001; Zhang et al., 2009).

RNA blot hybridization

Total RNA from embryos injected with reporter mRNAs was analyzed by RNA blot hybridization (Sheets et al., 1994; Zhang et al., 2009) using a radiolabeled probe to detect the *bicc1* mRNA.

Immunoblotting

The analysis of proteins by immunoblotting was performed as described (Zhang et al., 2013) using mouse monoclonal anti-HA-tag antibody and anti-Actin antibody. Anti-Bicc1 polyclonal antibodies were raised in rabbits (Harlan Labs, Madison, WI, USA) immunized with a GST-Bicc1 fusion protein encoding the N-terminal 486 amino acids of *X. laevis* Bicc1 expressed in *Escherichia coli*. Antibodies were affinity purified using recombinant MBP-Bicc1 fusion protein (the N-terminal 486 amino acids; Fig. S1) and used at 1:2000 for immunoblotting.

qRT-PCR

Quantitative RT-PCR to analyze mRNAs associated with Bicc1 was performed as described (Park et al., 2011). Primers used to analyze changes in gene expression have been previously published (Kofron et al., 2004; Heasman et al., 1991; Luxardi et al., 2010).

Immunoprecipitations

Embryos were injected with mRNA encoding Bicc1 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 5000 rpm, 2700 ***g***) and the supernatant incubated with anti-HA antibody coupled to protein-G agarose (2 h, 4°C). The beads were collected (1 min, 3000 rpm, 960 ***g***) and washed 4 times in 1 ml TNMEN-150 buffer. For each wash, the beads were incubated in buffer at 4°C for 5 min, spun at 3000 rpm (960 ***g***) for 1 min and supernatant removed. RNA was isolated from the washed beads for analysis.

Acknowledgements

We thank Catherine Fox (University of Wisconsin-Madison) for useful discussions and edits on the manuscript. We also thank Mary Lou King (University of Miami) for providing a plasmid containing the Wnt11b 3′UTR and Laura Vanderploeg for preparing the figures.

Competing interests

The authors declare no competing or financial interests.

Author contributions

S.P. and M.D.S. were involved in all phases of the work, including planning and executing and analyzing most of the experiments, and writing the manuscript. M.A.M. and D.W.H. planned and performed the maternal loss-of-function experiments and assisted in writing relevant sections of the manuscript. S.B. performed some experiments and assisted in the writing of the manuscript. All of the authors have read the manuscript and agree with its content.

Funding

This work was supported by National Science Foundation grant [1050395 to M.D.S.]; the University of Iowa (D.W.H.); and by a grant from the National Institutes of Health [R01GM083999 to D.W.H.]. Deposited in PMC for release after 12 months.

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