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Expression pattern of *bcar3*, a downstream target of *Gata2*, and its binding partner, *bcar1*, during *Xenopus* development

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Abstract

Primitive hematopoiesis generates red blood cells that deliver oxygen to the developing embryo. Mesodermal cells commit to a primitive blood cell fate during gastrulation and, in order to do so the mesoderm must receive non-cell autonomous signals transmitted from other germ layers. In *Xenopus*, the transcription factor *Gata2* functions in ectodermal cells to generate or transmit the non-cell autonomous signals. Here we have identified *Breast Cancer Antiestrogen Resistance 3* (*bcar3*) as a gene that is induced in ectodermal cells downstream of *Gata2*. *Bcar3* and its binding partner *Bcar1* function to transduce integrin signaling, leading to changes in cellular morphology, motility and adhesion. We show that *gata2*, *bcar3* and *bcar1* are co-expressed in ventral ectoderm from early gastrula to early tailbud stages. At later stages of development, *bcar3* and *bcar1* are co-expressed in the spinal cord, notochord, fin mesenchyme and pronephros but each shows additional unique sites of expression. These co-expression and unique expression patterns suggest that *Bcar3* and *Bcar1* may function together but also independently during *Xenopus* development.

Keywords

Primitive hematopoiesis; non-cell autonomous signals; ectoderm; gastrulation; *Gata2*; *Bcar3*; *Bcar1*; *Xenopus laevis*

1. Introduction

Hematopoiesis, or blood cell production, begins during early development and persists throughout adulthood. Hematopoiesis occurs in two phases. The first phase is primitive hematopoiesis, which occurs only during development, and generates mostly red blood cells

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that function to deliver oxygen. The second phase is definitive hematopoiesis, which begins during embryogenesis but persists in adults. Definitive hematopoiesis generates hematopoietic stem cells that differentiate into both red and white blood cell lineages. These blood cells function to deliver oxygen, form blood clots, and generate proper immune responses.

Primitive blood cells are derived from mesoderm that becomes specified with a hematopoietic fate by the end of gastrulation (Nakazawa et al., 2006; Palis et al., 1999; Smith and Turpen, 1985). It has been shown that the mesoderm needs to receive non-cell autonomous signals from other germ layers to commit to a blood cell fate. In mouse and chick, these signals come from endoderm, while in *Xenopus* they come from ectoderm (Baron, 2003; Belaoussoff et al., 1998; Maeno et al., 1994; Savary et al., 2005). We have shown that the transcription factor Gata2 is required in *Xenopus* ectoderm in order to generate or transmit these non-cell autonomous signals (Dalgin et al., 2007), but targets of Gata2 that mediate these signals have not been identified.

We have used microarray analysis to identify Gata2 target genes that are expressed in ectodermal cells of *Xenopus* embryos during gastrulation. We identified *Breast Cancer Antiestrogen Resistance 3* (*bcar3*, also known as *and-34*) as a gene that is induced in ectodermal cells downstream of Gata2. Bcar3 is an adaptor protein that interacts with Bcar1 (also known as p130 Crk-associated substrate (p130Cas)) to transduce signals downstream of activated integrins (Barrett et al., 2013; Cabodi et al., 2010). Bcar3 downregulates cadherin dependent cell-cell adhesion and promotes cell motility by controlling cytoskeletal remodeling in breast cancer cells (Makkinje et al., 2009; Near et al., 2009; Schrecengost et al., 2007; Sun et al., 2012; Wilson et al., 2013). Changes in cell-cell adhesion and cytoskeletal remodeling are integral components of cell movements that drive gastrulation, which brings the ectoderm and mesoderm into contact. This contact is required for the transmission of non-cell autonomous signals from ectoderm to mesoderm that enable mesoderm to commit to a blood cell fate (Kikkawa et al., 2001). For this reason, we chose to study *bcar3* as a potential key target of Gata2 that might be required in ectoderm to promote primitive hematopoiesis. We show here that *bcar3* is co-expressed with *gata2* in ventral ectoderm from early gastrula through neurula stages. We also expanded our analysis to examine patterns of expression of *bcar3*, and its binding partner *bcar1*, from early development through tailbud stages. We show that *bcar3* and *bcar1* are co-expressed in ectodermal cells during gastrula and neurula stages, consistent with their possible involvement downstream of Gata2 in primitive hematopoiesis, and that they show both unique and overlapping expression patterns in multiple other tissues in later embryos, suggesting involvement in other developmental processes as well.

2. Results

2.1. *bcar3* is a downstream target of Gata2 in ventral ectoderm during gastrulation

We used microarray analysis to find Gata2 targets that might execute Gata2 functions in ectoderm during gastrulation (Mimoto et al., 2015). To perform the microarray, we injected Gata2 MOs or Gata2 RNA into both cells of two cell embryos, dissected ectoderm from early gastrula stage embryos (stage 10), and cultured the ectoderm until sibling embryos

reached stage 12, which is near the end of gastrulation. As an additional method to verify targets, we analyzed gene expression in ectoderm isolated from embryos injected with RNA encoding Friend of Gata (Fog). We have shown that overexpression of Fog dominantly interferes with Gata function, causing disruption of erythropoiesis (Mimoto and Christian, 2012). Genes found to be downregulated in both Gata2 MO and Fog RNA injected embryos and found to be reciprocally upregulated in Gata2 RNA injected embryos, compared to uninjected controls, are expected to be positively transcribed downstream of Gata2. We identified *bcar3* as a putative positive target of Gata2. Specifically, microarray analysis revealed that expression of *bcar3* was reduced 1.8–2 fold in ectoderm from Gata2 morphants or embryos injected with Fog RNA, and was induced 2.4 fold in ectoderm from embryos overexpressing Gata2 (Fig. 1B). Bcar3 is a member of the Novel Src homology 2 (SH2)-containing Protein (NSP) family that has an SH2 domain at the amino terminus, a Serine/Proline-rich domain in the middle and a Guanine nucleotide exchange factor (GEF)-like domain at the carboxy terminus (Cai et al., 1999; van Agthoven et al., 1998). To validate *bcar3* as a Gata2 target gene in explanted ectoderm, we injected embryos with Gata2 MOs or Fog RNA, isolated ectoderm at stage 10, cultured it to stage 12 and then analyzed expression of *bcar3* using real time quantitative PCR (qPCR). As shown in Fig. 1C, expression of *bcar3* was nearly ablated in ectoderm isolated from embryos in which Gata2 expression or function was reduced.

We used three additional analyses to confirm that *bcar3* is a real in vivo target of Gata2 in ectodermal cells of intact *Xenopus* embryos during gastrulation. First, we injected control MOs or Gata2 MOs into both cells of two cell embryos, then dissected ventral ectoderm and ventral mesendoderm at stage 13, and performed qPCR to compare levels of *bcar3* in each sample as illustrated (Fig. 1D). Expression of *bcar3* was reduced 10 fold in ectoderm from Gata2 morphants compared to controls (Fig. 1D). Expression of *bcar3* was not detected in ventral mesendoderm at this stage (Fig. 1D). Second, we injected Fog RNA into one cell at the two cell stage, and examined expression of *bcar3* at stage 15 using whole mount in situ hybridization (WMISH). *bcar3* was expressed throughout the ventrolateral ectoderm, and in a dorsal midline stripe in control embryos (Fig. 1E). Expression of *bcar3* was reduced in ventrolateral ectoderm, in the half of the embryo in which Fog had been overexpressed relative to the uninjected half or to controls (Fig. 1E). Third, we examined whether the reduced expression of *bcar3* in Gata2 morphants was rescued by *gata2* RNA. We injected control MOs, Gata2 MOs alone, or Gata2 MOs together with Gata2 RNA that cannot be recognized by the Gata2 MO (Dalgin et al., 2007) into both cells of two cell embryos, dissected ectoderm at stage 10, cultured the ectoderm until sibling embryos reached stage 12, and then analyzed expression of *bcar3* using qPCR. Compared to control MO injected ectoderm, the expression level of *bcar3* was reduced by 2 fold in ectoderm from Gata2 morphants, and was upregulated 2.4 fold in ectoderm from embryos co-injected with Gata2 MOs and Gata2 RNA (Fig. 1F). Taken together, these data show that expression of *bcar3* in ectodermal cells of gastrula stage embryos requires Gata2.

2.2. Expression of *bcar3* overlaps with that of *gata2* in ectoderm during gastrula and neurula stages

Having shown that *bcar3* is a target gene downstream of Gata2 during gastrulation, we next wished to determine if expression of *bcar3* overlaps with that of *gata2* during gastrulation, and we expanded our examination to locate tissues and organs expressing *bcar3* throughout development. We used Northern analysis to examine temporal patterns of expression of *bcar3*, and WMISH to compare spatial patterns of expression of *gata2* and *bcar3*. Northern analysis showed that a single 4 kb *bcar3* transcript was present in eggs, and this persisted throughout development, while a second, more abundant transcript of approximately 2.1 kb was detected at later stages (Fig. 2A). This finding is consistent with the observation that there are multiple *bcar3* transcripts in human and mouse (Vervoort et al., 2007). WMISH analysis of stage 3 embryos confirmed the presence of maternal *bcar3* RNA (Fig. 2E). As previously shown (Kelley et al., 1994; Pieper et al., 2012; Walmsley et al., 1994; Zon et al., 1991), *gata2* was strongly expressed throughout the ectoderm during blastula and early gastrula stages (Fig. 2B–D), and expression became restricted to ventral and lateral ectoderm by late gastrula through neurula stages (Fig. 2M–Q). We found that *bcar3* was also strongly expressed throughout the ectoderm at blastula and early gastrula stages (Fig. 2F–H). At late gastrula stages, *bcar3* was strongly expressed in ventral ectoderm, in neural plate ectoderm and in the notochord (Fig. 2R and 2S). Strong expression of *bcar3* persisted in ventral and lateral ectoderm from stage 17–23 (Fig. 2T–V). Expression of *bcar3* was also detected in the notochord, and in specific stripes within the anterior neural folds during neurula stage (Fig. 2T) and in specific regions of the developing eye and brain at later stages (Fig. 2V, BB). At the tailbud stage, expression of *bcar3* was also detected in the cement gland, cranial ganglia, spinal cord, notochord, abdominal muscle anlagen, pronephros, fin mesenchyme and cloaca (Fig. 2BB–EE).

Our observation that expression of *bcar3* and *gata2* overlap in ectoderm during blastula and gastrula stages is consistent with the possibility that *bcar3* might function downstream of Gata2 to generate signals required for mesoderm to commit to a blood cell fate. In addition, *bcar3* is more widely expressed during later development, suggesting that it may have additional functions in other organs.

2.3. *bcar3* is expressed in all three germ layers during early gastrulation, and is enriched in ventral ectoderm and dorsal mesoderm by the end of gastrulation

One limitation of WMISH is that penetration of probes into deep endodermal tissues is hampered by the large size and high yolk content of cells, especially during gastrulation (Butler et al., 2001). For this reason, we also used semi-quantitative RT-PCR to analyze expression of *bcar3* in ectoderm, dorsal and ventral mesoderm, and endoderm dissected from embryos at stage 10. As controls to demonstrate that dissections were accurate, we used *foxi1* as an ectodermal marker, *t_(xbra)* as a mesodermal marker and *sox17a* as an endodermal marker (Hudson et al., 1997; Smith and Harland, 1991; Suri et al., 2005; Xanthos et al., 2002). This analysis showed that *bcar3* was expressed in all three germ layers at stage 10 (Fig. 3A). Next we examined expression of *bcar3* during gastrulation using WMISH on bisected embryos, which allows for better probe penetration into deep tissues (Fig. 3B–D). We analyzed expression of *foxi1*, *t* and *sox17a* in parallel as controls

for genes known to be restricted to ectoderm, mesoderm and endoderm, respectively (Fig. 3E–M). WMISH of bisected embryos showed that *bcar3* was strongly expressed throughout the ectoderm (blue arrows), and more weakly in dorsal (orange arrows) and ventral (red arrows) mesoderm and endoderm (yellow arrows) at stage 10 and 11 (Fig. 3B and C). By the end of gastrulation (stage 13), expression of *bcar3* became restricted to the inner layer of ventral, but not dorsal ectoderm and to extreme dorsal mesoderm, which will give rise to the notochord (Fig. 3D). These results, showing expression in ventral ectoderm but not ventral mesoderm at stage 13, are consistent with our qPCR results from dissected embryos (Fig. 1D). These results suggest that *bcar3* is ubiquitously expressed until mid-gastrulation, at which time expression becomes restricted to specific tissues.

2.4. *bcar1* and *bcar3* show both unique and overlapping patterns of expression during *Xenopus* embryogenesis

We next examined whether expression of *bcar1* overlaps with that of its binding partner, *bcar3* during embryogenesis, consistent with the possibility that Bcar3 and Bcar1 function in a shared signal transduction pathway in some or all tissues during early development. WMISH showed that *bcar1* was not expressed maternally (Fig. 2I), but zygotic *bcar1* was expressed throughout the dorsal and ventral ectoderm from the blastula (stage 9) through neurula and early tailbud stages (Fig. 2J–L and 2W–AA). *bcar1* was enriched in specific regions of the anterior neural folds during neurula stage (Fig. 2Y) and in regions of the brain at later stages (Fig. 2AA and 2FF–HH). *bcar1* was expressed in somitic mesoderm beginning at stage 20 and in the differentiated somites at stage 34 (Fig. 2Y–AA and 2FF), which is not true of *bcar3*. At stage 34, *bcar1* was also expressed in the eye, branchial arches, spinal cord, notochord, pronephros, fin mesenchyme and ventral blood islands (Fig. 2FF–II). Thus, *bcar3* and *bcar1* are co-expressed in ventrolateral ectoderm throughout gastrulation, in the prospective neural plate at the end of gastrulation, and in the spinal cord, notochord, fin mesenchyme and pronephros during tailbud stages.

3. Discussion

We have identified *bcar3* as a gene that is expressed downstream of Gata2 in *Xenopus* ectoderm during gastrulation. *bcar3* is co-expressed with *gata2* in ventral ectoderm during gastrulation but is more broadly expressed during later developmental stages. We further find that *bcar3* and its binding partner, *bcar1*, show both unique and overlapping patterns of expression in different tissues throughout development.

Our loss of function data demonstrate that Gata2 is required for expression of *bcar3* in ectodermal cells during gastrulation and our current data, together with published data, raise the possibility that Gata2 regulates *bcar3* expression in select tissues later in development as well. For example, *gata2* (Bowes et al., 2010; Kelley et al., 1994) and *bcar3* (Fig. 2) are both expressed in the brain, branchial arches, spinal cord, fin mesenchyme and cloaca during tailbud stages. However, *gata2* is also expressed in the ventral blood island and in cells of the dorsal lateral plate that will give rise to the hemogenic endothelium during tailbud stages (Ciau-Uitz et al., 2013; Ciau-Uitz et al., 2000), whereas *bcar3* is not expressed in these locations. Furthermore, while *bcar3* is expressed in the notochord, pronephric mesoderm,

tailbud and abdominal muscle anlagen during tailbud stages, *gata2* is not expressed in these tissues (Ciau-Uitz et al., 2000).

bcar3 was first identified in a screen for genes whose upregulation induces anti-estrogen resistance in hormone-dependent breast cancer cells (van Agthoven et al., 1998). Bcar3 binds to, and plays a key role in regulating the subcellular localization and signaling activities of Bcar1 (Makkinje et al., 2009; Near et al., 2007; Schrecengost et al., 2007; Sun et al., 2012), which is also upregulated in estrogen resistant breast cancer cells (Brinkman et al., 2000; Gotoh et al., 2000; Wallez et al., 2014). High levels of *bcar3* and/or *bcar1* correlate with poor prognosis and have been implicated in causing cellular acquisition of a more migratory and invasive phenotype (Cabodi et al., 2010).

Integrins are major upstream regulators of Bcar1 and Bcar3. Integrin engagement leads to activation of Src, which phosphorylates Bcar1 in a Bcar3 dependent manner to initiate downstream signaling (Sun et al., 2012). Ultimately, Bcar1 converts integrin signals to activation of small GTPases such as Rac and Rap as well as multiple kinases including PI3K, AKT (PKB), ERK and JNK (Barrett et al., 2013; Cabodi et al., 2010). Bcar1 also negatively regulates E-cadherin membrane localization, leading to reduced cell adhesion (Tikhmyanova and Golemis, 2011) and cells overexpressing Bcar3 express high levels of fibronectin, an extracellular matrix (ECM) protein that serves as a marker of epithelial to mesenchymal transition (EMT). Activation of signaling downstream of Bcar3/Bcar1 leads to changes in cell survival, proliferation, morphology and motility that contribute to the invasive program in breast cancer.

Our data show that *bcar3* and *bcar1* are widely expressed throughout early development in *Xenopus*. While *bcar3* and *bcar1* are co-expressed in many tissues and organs including ventral ectoderm during gastrulation, and later in the notochord, fin mesenchyme and pronephros, they also show unique sites of expression, suggesting that the two proteins sometimes function independently of each other. In places where they are not co-expressed, Bcar3 may function in concert with other Cas family members such as NEDD9 (CASL), EFS and CASS4 (HEPL), and Bcar1 may function together with other NSP family members, such as NSP1 (SH2D3A) and NSP3 (SHEP1, SH2D3C) (Wallez et al., 2012), or may act with binding partners outside the NSP family. Consistent with the idea that multiple members of the NSP family can function redundantly to mediate Bcar1 signaling, *bcar1* mutant mice die during embryogenesis due to heart defects (Honda et al., 1998) while mice lacking *bcar3* develop normally but then undergo postnatal rupture of the lens (Near et al., 2009).

bcar3 and/or *bcar1* are expressed in many tissues and cell types in the developing embryo that are undergoing EMT and becoming motile, consistent with studies in cultured cells showing that Bcar3 and/or Bcar1 regulate cell-cell adhesion and cytoskeletal dynamics to promote EMT and cell motility (Near et al., 2007; Vanden Borre et al., 2011; Wilson et al., 2013). For example, cells of the pronephric duct segregate from the lateral plate mesoderm and migrate posteriorly (reviewed in Drawbridge et al., 2003) while the anlagen of the abdominal musculature form as aggregates that detach from the caudal somites and migrate ventrally (Lynch, 1990). Fibronectin is also required for normal gastrulation. Fibronectin

fibrils are one of the major ECM components formed on the basal side of ectoderm during gastrulation, and proper fibrillogenesis is required for mesodermal cell migration (Darribere and Schwarzbauer, 2000; Davidson et al., 2002; Rozario et al., 2009; Winklbauer et al., 1996).

Our finding that expression of *bcar3* is induced downstream of Gata2 in ectoderm and that *gata2*, *bcar3* and *bcar1* are co-expressed in this tissue during gastrulation, raise the possibility that Bcar3/Bcar1 function may be required in ectodermal cells to transduce signals downstream of integrins that are required for mesoderm to form blood. Integrin function is essential for the process of gastrulation, which brings ectodermal and mesodermal cells into direct contact as the mesoderm involutes beneath the ectoderm (Solnica-Krezel and Sepich, 2012). The ectoderm must come into direct contact with mesodermal cells in order relay signals that enable the mesoderm to differentiate into blood. When gastrulation is impaired in *Xenopus*, for example by incubating embryos in high salt solution or by disrupting integrin function or fibronectin fibrillogenesis, the embryos do not form red blood cells (Kikkawa et al., 2001; Marsden and DeSimone, 2001). Thus, it is feasible that Bcar3 function is required downstream of Gata2 in ectodermal cells for normal blood formation. Future loss of function studies will be required to test this possibility.

4. Experimental Procedures

4.1. Embryo culture and manipulation

Xenopus embryos were obtained, microinjected, and cultured as described (Mimoto and Christian, 2012). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Ectoderm was dissected with watchmakers' forceps or sharpened tungsten needles and cultured as described previously (Goldman et al., 2006). Ectoderm and mesendoderm were dissected from the ventral half of late gastrula stage embryos as described (Mimoto et al., 2015). Whole mount in situ hybridization assays were performed as described (Harland, 1991) except that BM purple (Roche) was used as a substrate.

4.2. DNA constructs

bcar3 (Image clone ID; 6636021) and *bcar1* cDNAs (Image clone ID; 3398754) were purchased from Thermo Fisher Scientific. MEGAscript SP6, T7 or T3 kits (Ambion) were used to make capped RNA or probes according to the manufacturer's instructions.

4.3. Microarray Analysis

A detailed description of how the microarray was performed and data analyzed is described in Mimoto et al. (Mimoto et al., 2015).

4.4. Analysis of RNA

For Northern blotting, total RNA was isolated from embryos and analysis was performed as described previously (Christian et al., 1991). For qPCR analysis, total RNA was isolated using Trizol (Invitrogen), from which cDNA was generated using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Sciences, Inc.) with a poly d(T) primer according to the manufacturer's instructions. qPCR was performed using a QuantiTect

SYBR Green PCR kit (QIAGEN) and a 7900 HT Sequence Detector (ABI), using a T_m of 60°C. The expression level of *bcar3* was normalized to the housekeeping gene *ornithine decarboxylase* (*odc*). Each experiment was repeated at least three times. For semi quantitative RT-PCR analysis, cDNA was generated as above, amplified by PCR using a C1000 touch Thermal cycler (Bio-rad) as described previously (Nakayama et al., 1998) using the following PCR conditions: 94°C for 5 minutes, followed by a variable number of cycles (determined empirically to be in the linear range for each primer pair), at 94°C for 30 seconds ; 58°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 5 minutes. Primer sequences used for qPCR and semi quantitative RT-PCR are listed in Table 1.

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Highlights

- *Xenopus bcar3* is induced in ectodermal cells downstream of Gata2
- *bcar3*, *bcar1*, and *gata2* are co-expressed in ventral ectoderm during gastrulation
- *bcar3* and *bcar1* show common and unique sites of expression during tailbud stages

Figure 1. Identification of *bcar3* as a target of Gata2 in ectoderm during gastrulation

(A) Schematic of strategy for microarray analysis and target gene identification. Gata2 MO (40 ng), Fog RNA (500 pg) or Gata2 RNA (500 pg) was injected into both cells of two-cell embryos and ectoderm was explanted when embryos reached stage 10. When sibling embryos reached stage 12, RNA was isolated from ectoderm for microarray analysis. Genes showing upregulation in Gata2 MO and Fog RNA injected embryos, and downregulation in Gata2 RNA injected embryos were identified as putative negative targets of Gata2. Genes showing downregulation in Gata2 MO and Fog RNA injected embryos, and upregulation in Gata2 RNA injected embryos were identified as putative positive targets of Gata2. (B) Table showing fold change in *bcar3* expression relative to uninjected controls detected by microarray analysis and schematic diagram of Bcar3 structure, SH2: Src homology 2 domain, S/P-rich: Serine/Proline-rich domain, GEF: Guanine nucleotide exchange factor like domain. (C) qPCR analysis of *bcar3* gene expression in ectodermal explants isolated at stage 10 from embryos that had been injected with Gata2 MO or Fog RNA and cultured to

stage 12. The level of expression of *bcar3* was normalized to that in ectoderm from uninjected controls. (D) Gata2 MO (40ng) or control MO (40 ng) were injected into both cells of two-cell embryos and ventral ectoderm and ventral mesendoderm were dissected at stage 12–13. RNA was extracted from tissue from 10 pooled explants in three independent experiments and *bcar3* gene expression was analyzed by qPCR. The level of expression of *bcar3* was normalized to that in ectoderm from control MO injected embryos. VE: ventral ectoderm, VM: ventral mesendoderm, ND: not detected. (E) Fog RNA (500 pg) was injected into one cell of two-cell embryos and expression of *bcar3* was examined at stage 15 using WMISH. Uninjected embryos were used as controls. In each row, dorsal, lateral and ventral views of the same embryo are shown. Brackets indicate the Fog RNA injected side. (F) Gata2 MO (40ng), control MO (40 ng), or Gata2 MO (40ng) and Gata2 RNA (1ng) together was injected into both cells of two-cell embryos and ectoderm was explanted when embryos reached stage 10. When sibling embryos reached stage 12, RNA was isolated from ectoderm for qPCR analysis. The level of expression of *bcar3* was normalized to that in ectoderm from control MO injected embryos.

Figure 2. Expression patterns of *gata2*, *bcar3* and *bcar1* during *Xenopus* development

(A) Northern analysis showing *bcar3* RNA transcripts. *odc* was used as a loading control. All lanes are from the same blot, aligned following removal of intervening lanes (following the 2nd and 3rd lanes) using Photoshop. N/F: Nieuwkoop and Faber stages. (B–II) WMISH analysis of expression of *gata2* (B–D and M–Q), *bcar3* (E–H, R–V and BB–EE) and *bcar1* (I–L, W–AA and FF–II) during development. For each stage, the same embryo is shown in animal, vegetal, lateral, dorsal or ventral views. The embryos in panels CC and GG were mounted in Murray's clear to visualize internal structures. Brown is pigment. All other embryos were photographed in PBS to visualize superficial structures. (DD, EE, HH and II) Transverse sections of previously stained st. 34 embryo at the level shown in panels CC and

GG respectively. Np: neural plate, n: notochord, anf: anterior neural folds, b: brain, sm: somitic mesoderm, e: eye, cg: cement gland, g: cranial ganglia, sc: spinal cord, fm: fin mesenchyme, tb: tail bud, ma: abdominal muscle anlagen, p: pronephros, c: cloaca, ba: branchial arches, vbi: ventral blood island

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Figure 3. *bcar3* is ubiquitously expressed at the onset of gastrulation but becomes restricted to prospective notochord and ventral ectoderm by the end of gastrulation

(A) Semi quantitative RT-PCR was used to analyze expression of *bcar3*, *foxi1*, *t*, *sox17a* and *odc* (as a loading control) in equivalent amounts of RNA from whole embryos (WE) or from ectoderm (EC), dorsal mesoderm (DM), ventral mesoderm (VM) or endoderm (EN) dissected from embryos at stage 10. Reverse transcriptase was omitted from one set of samples (-RT) as a control for genomic contamination. (B-M) Expression of *bcar3*, *foxi1*, *t* and *sox17a* was analyzed by in situ hybridization of probes to bisected embryos at stage 10, 11 and 13. Stage 10 and 11 embryos are oriented with dorsal to the right and stage 13 embryos are oriented with dorsal to the top. Arrows indicate ectoderm (blue), dorsal mesoderm (orange), ventral mesoderm (red) and endoderm (yellow).

Table 1

Primer sequences used for qPCR and semi quantitative RT-PCR

Primer Name	Sequence
Bcar3 F	AAC CAC CTG CAA ACA GCT CG
Bcar3 R	CCA CAG CAG TCG CAT CTG AAA
Foxi1 F	AGT AGG TCA GTT CCA CTT GG
Foxi1 R	AAG GAC TTT GTC GTG ACT GC
t F	TTC TGA AGG TGA GCA TGT CG
t R	GTT TGA CTT TGC TAA AAG AGA CAG G
Sox17 α F	GGA CGA GTG CCA GAT GAT G
Sox17 α R	CTG GCA AGT ACA TCT GTC C
ODC F	TGC AGA GCC TGG GAG ATA CT
ODC R	CAT TGG CAG CAT CTT CTT CA