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Rargb regulates organ laterality in a zebrafish model of right atrial isomerism

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Abstract

Developmental signals determine organ morphology and position during embryogenesis. To discover novel modifiers of liver development, we performed a chemical genetic screen in zebrafish and identified retinoic acid as a positive regulator of hepatogenesis. Knockdown of the four RA receptors revealed that all receptors affect liver formation, however specific receptors exert differential effects. *Rargb* knockdown results in bilateral livers but does not impact organ size, revealing a unique role for *Rargb* in conferring left–right positional information. Bilateral populations of hepatoblasts are detectable in *rargb* morphants, indicating *Rargb* acts during hepatic specification to position the liver, and primitive endoderm is competent to form liver on both sides. Hearts remain at the midline and gut looping is perturbed in *rargb* morphants, suggesting *Rargb* affects lateral plate mesoderm migration. Overexpression of *Bmp* during somitogenesis similarly results in bilateral livers and midline hearts, and inhibition of *Bmp* signaling rescues the *rargb* morphant phenotype, indicating *Rargb* functions upstream of *Bmp* to regulate organ sidedness. Loss of *rargb* causes biliary and organ laterality defects as well as asplenia, paralleling symptoms of the human condition right atrial isomerism. Our findings uncover a novel role for RA in regulating organ laterality and provide an animal model of one form of human heterotaxia.

Keywords

Retinoic acid; BMP; Liver; Laterality; Organogenesis; Zebrafish

Introduction

During embryogenesis, the liver develops asymmetrically across the left–right axis. In human heterotaxic syndromes, misalignment of the visceral organs is often associated with cardiac defects and impaired bile duct formation and typically requires surgical intervention. Liver development originates in the endoderm where hepatic progenitors are specified from

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2012.09.001>.

a multipotent precursor population. Expression of liver-specific transcription factors in a select population of endodermal cells is in part regulated by surrounding mesodermal tissues which provide inductive cues required for proper liver development and positioning. Many of these signaling pathways have been independently characterized, however the sequence and integration of genetic networks regulating liver development have not been well established.

In zebrafish, hepatoblasts are specified in the endodermal rod by 24 hours post-fertilization (hpf), and between 24–28 hpf, the gut begins to loop to the left, concordant with asymmetrical movements of the lateral plate mesoderm (LPM) (Field et al., 2003; Horne-Badovinac et al., 2003; Ober et al., 2003). Although the importance of mesoderm–endoderm signaling during liver development has been highlighted previously (Chung et al., 2008; Ober et al., 2006; Reiter et al., 1999, 2001; Shin et al., 2007), it is not clear how many of these developmental signals intersect during hepatic specification and positioning.

Evidence that retinoic acid (RA) signaling is involved in endoderm formation first surfaced in a study of pancreatic specification in zebrafish (Stafford and Prince, 2002). Global inhibition of zebrafish RARs resulted in reduced or absent expression of endocrine and exocrine pancreas markers as well as *hhex*, a hepatic transcription factor. Analysis of zebrafish *raldh2* mutants (neckless, *aldh1a2^{um22}*, *aldh1a2ⁱ²⁶*) also revealed similar disruptions in endoderm development (Alexa et al., 2009; Stafford and Prince, 2002). Despite these studies, the function of a number of integral RA pathway members, including *raldh3* and *raldh4*, and the impact of individual RARs on liver development remain unknown.

RA signaling has been further suggested to affect organ laterality. Early studies have demonstrated that inhibition of RA signaling or addition of excess RA leads to randomized heart looping in mice (Chazaud et al., 1999), and vitamin A deficient avian embryos develop reversed heart situs (Dersch and Zile, 1993), thereby suggesting a role for RA signaling in patterning organs across the left-right axis. The mechanism by which RA regulates organ positioning has not been elucidated. LPM movements impact visceral organ positioning (Horne-Badovinac et al., 2001; Huang et al., 2008; Yin et al., 2010), suggesting that RA signaling may exert its effect on organ laterality via regulation of LPM migration.

In this study, we define the role of RA signaling during liver specification and positioning. In a large-scale chemical genetic screen, we identified RA as a regulator of normal liver development. We demonstrate that both RA synthesis and receptor-mediated signaling impact stages of hepatic specification, differentiation, and proliferation. We show for the first time that *raldh4* impacts liver development following hepatic specification. *Raraa*, *rarab*, and *rarga* knockdowns lead to smaller livers, whereas *rargb* knockdown results in bilateral livers, demonstrating receptor-specific effects on liver development. The heart and gut remain at the midline in *rargb* morphants, indicative of a left-right patterning defect, however Nodal signaling is unaffected in these embryos. We observe that transient upregulation of Bmp signaling also results in midline hearts and bilateral livers. Inhibition of Bmp signaling rescues the bilateral liver defect in *rargb* morphants, suggesting that RA normally inhibits Bmp signaling during organ laterality determination, and we indeed find that *rargb* knockdown results in elevated levels of phosphorylated Smads 1/5/8 in the developing embryo. *Rargb* morphants also develop bile duct defects and asplenia, and this phenotype parallels the human heterotaxic syndrome right atrial isomerism, or Ivemark syndrome (Ivemark, 1955), in which patients display a midline heart, midline or duplicated livers, biliary atresia, and asplenia. These results suggest that proper RA signaling may be required for *situs solitus* of human organs.

Materials and methods

Zebrafish husbandry

Zebrafish were maintained according to IACUC protocols. *LF2.8-EGFP* referred to as *Lfabp:GFP*, (Her et al., 2003), *cmlc2:GFP* (Huang et al., 2003), *cyp26a1:eYFP* (Hu et al., 2008), *miles apart (mil)* (Kupperman et al., 2000), *hs:bmp2b* (Chocron et al., 2007), *BRE:eGFP* (Laux et al., 2011) and *tp1bglob:eGFP* (Parsons et al., 2009) transgenic and mutant lines have been described previously.

Chemical exposures

Zebrafish embryos were exposed to 0.1 mM all-trans retinoic acid (ATRA, Sigma), 1.0 μ M 4-diethylaminobenzaldehyde (DEAB, Sigma), 1.0 μ M AGN193109 (Toronto Research Chemicals), 1.0 μ M MM11253 (Tocris), 25 μ M dorsomorphin, or 0.1 μ M CD1530 (Tocris) during the specified time windows. Stock solutions were diluted in E3 embryo water. Control embryos were concurrently exposed to 0.1% DMSO. After chemical exposure, embryos were washed 3–5 $\times$ in E3 solution then fixed with 4% PFA at the appropriate stages. The chemical genetic screen was performed as described previously (North et al., 2007). Wild type age-matched embryos were arrayed into 48-well plates and exposed to test compounds from 18–72 hpf. Compound libraries used include the NINDS Custom Collection (1040 compounds), SpecPlus Collection (960) and BIOMOL ICCB Known Bioactives (480).

Whole mount in situ hybridization

Zebrafish embryos were fixed in 4% PFA at the specified stages, and *in situ* hybridization was performed according to established protocols (<http://zfin.org/ZFIN/Methods/ThisseProtocol.html>) using the following probes: (Endoderm) *lfabp*, *prox1*, *foxA3*, *shh*; (heart) *cmlc2*, *amhc*, *vmhc*; (spleen) *hox11*; (kidney) *slc20a1a*, *slc12a3*; (thyroid) *nkx2.1a*; (LPM) *hand2*; (Nodal) *spaw*, *pitx2*, *lefty1*, *lefty2*; (Bmp) *smad1*, *smad4*, *smad5*. The number of embryos displaying a particular phenotype is presented as a percentage of the sample size, n.

Whole mount immunostaining

BrdU: Zebrafish embryos were incubated in 10 mM BrdU on ice for 20 min at 24 hpf, returned to warm embryo water for 5 min, then fixed in 4% PFA. Embryos were incubated with anti-BrdU primary (Roche) and goat anti-mouse peroxidase-conjugated secondary (Jackson ImmunoResearch) antibodies and visualized with DAB. BrdU+ cells were counted manually in a defined region of the embryo (n = 5 embryos/chemical treatment).

2F11: Zebrafish larvae were fixed at 5 dpf and incubated in a 1:500 dilution of mouse monoclonal 2F11 primary antibody (Abcam) followed by 1:50 FITC rabbit anti-mouse IgG secondary antibody (Jackson ImmunoResearch).

Morpholino knockdown and mRNA rescue injections

Morpholinos (MOs; GeneTools) were designed against zebra-fish *raldh2* (5' CAACTTCACTGGAGGTCATCGCGTC 3') (100 μ M) and *raldh4* (5' GATATTTTCATGTCTTTTGACATCGC 3') (200 μ M), and previously published MOs were used against RA receptors *raraa*, *rarab*, *rarga*, and *rargb* (400–800 μ M) (Linville et al., 2009). For the RARg rescue experiment, 200 pg mouse *Rarg* mRNA (OriGene Technologies) was co-injected with 400 μ M *rargb* MO at the 1-cell stage.

Flow cytometry analysis

Lfabp:GFP fluorescent embryos were exposed to chemicals or injected with MOs as described above, whole embryos were manually dissociated in $0.9 \times$ PBS, and %GFP+ cells were determined by flow cytometric analysis. 20,000 cells were analyzed per embryo, and 5 embryos were analyzed for each chemical treatment or MO injection using FlowJo software.

Microscopy

Live fluorescence microscopy was performed on *cmlc2:GFP*, *BRE:eGFP*, or *tp1bglob:eGFP* embryos anesthetized in 0.04 mg/ml Tricaine in E3 embryo water using a Zeiss Discovery V8 microscope. Once sorted by phenotype, embryos were washed several times and returned to E3 for further observation and/or until fixation. Embryos used in *in situ* hybridization or BrdU immunostaining experiments were visualized in glycerol. Confocal microscopy of 2F11 immunostained embryos was performed on a Zeiss LSM 510Meta microscope. For each treatment group, images presented are shown at the same magnification. Scale bars represent 100 μ m unless otherwise noted.

Smad Western blots

Protein lysates were isolated at 18 hpf from control, *rargb* morphant, and dorsomorphin-treated embryos by manual disruption in RIPA buffer. 1:1000 anti-pSmad1/5/8 (Cell Signaling Technology) or 1 mg/ml anti-Smad 1/5/8/9 (Cayman Chemical) primary antibody was used, followed by 1:3000 anti-rabbit HRP secondary antibody (Abcam).

Results

A chemical genetic screen identifies retinoic acid signaling as a regulator of liver development

To identify novel regulators of liver development, we conducted a chemical genetic screen in zebrafish (Fig. S1). We exposed wild type or *Lfabp:GFP* fluorescent reporter embryos to a library of known bioactive compounds over the course of liver development (18–72 hpf) and scored embryonic liver size at 72 hpf by *in situ* hybridization or fluorescence microscopy, respectively. Compounds having an effect on liver development were grouped by mechanism of action. Out of 2640 compounds tested, 135 (5.1%) increased liver size, whereas 229 (8.7%) decreased liver size. We identified a number of molecular pathways that impact liver growth, including retinoic acid (RA) signaling. In particular, our screen uncovered eight hits corresponding to six compounds that affect RA signaling: vitamin A, all-trans retinoic acid (ATRA), isotretinoin, beta carotene, and the RA synthesis inhibitors diethylaminobenzaldehyde (DEAB) and disulfiram.

To corroborate our chemical screen findings and to determine the stage at which RA impacts liver development, we treated embryos with RA pathway compounds during specific time windows targeting hepatoblast specification (18–24 hpf), hepatoblast differentiation (24–48 hpf), and hepatocyte proliferation (48–72 hpf). At all time points, embryos treated with ATRA develop bigger livers, whereas embryos treated with DEAB or AGN193109, a pan-RAR inhibitor, develop smaller livers as determined by *Lfabp in situ* hybridization (Fig. 1A – F and Fig. S2(A) and (B)) and GFP expression in live *Lfabp:GFP* reporter embryos (Fig. 1G – I) at 72 hpf. These results suggest that RA signaling is important throughout all stages of hepatic specification, differentiation, and proliferation. RA pathway inhibitors DEAB and AGN193109 were most penetrant during the earliest treatment window (18–24 hpf), with ~50% of embryos developing smaller livers (DEAB: $n = 136$; AGN193109: $n = 25$). To confirm that our drug treatments impact RA signaling in the embryo, we treated *cyp26a1:eYFP* reporter fish with ATRA or DEAB at 18 hpf. Cyp26a1 promotes degradation

of RA and is induced by RA in an auto-regulatory feedback loop (White et al., 1996; Abu-Abed et al., 2001; Sakai et al., 2001; Emoto et al., 2005). Treatment of *cyp26a1:eYFP* embryos with ATRA led to increased fluorescence throughout the embryo, with particularly increased intensity in the brain and liver, whereas DEAB treatment decreased fluorescence in these regions (Fig. 1J–L). These results demonstrate that RA signaling is active in the embryonic liver and is modulated by chemical exposure.

To determine whether changes in liver size in chemically treated embryos are related to changes in hepatic cell proliferation, we assessed BrdU incorporation at 24 hpf. We found that proliferation levels correspond to liver size: ATRA-treated embryos have more BrdU+ cells, and DEAB and AGN193109-treated embryos contain fewer BrdU+ cells (Fig. 1M – O). Quantification of BrdU+ cells in the area of the liver primordium (Fig. 1O box, magnified in 1M'–O'') revealed a significant decrease in proliferating hepatic cells in DEAB ($n=5$, *t*-test, $p=0.001$) (Fig. 1P) and AGN193109-treated embryos ($n=5$, *t*-test, $p=0.006$) (Fig. S2(C)) and an increase in BrdU+ cells in ATRA-treated embryos compared to DMSO controls. We verified these changes in hepatic cell proliferation by performing FACS at 72 hpf on *lfabp:GFP* embryos exposed to RA pathway compounds from 18–24 hpf. Quantification of GFP+ cells uncovered significant changes in hepatocyte number in compound-treated embryos compared to controls (DMSO versus ATRA $p=2.0 \times 10^{-5}$, DEAB $p=8.6 \times 10^{-5}$, AGN193109 $p=0.0002$; ATRA versus DEAB $p=4.6 \times 10^{-8}$, AGN $p=1.1 \times 10^{-6}$; DEAB versus AGN $p=0.69$) (Fig. 1Q and Fig. S2(D)). TUNEL analysis confirmed that changes in liver size are not related to hepatic cell death (data not shown). qPCR analysis of embryos exposed to RA pathway compounds further demonstrated that liver size and hepatocyte number correlate with expression levels of the hepatocyte marker *transferrin* at 72 hpf, indicating that the observed changes are not specific for *lfabp* (Fig. 1R) (DMSO versus ATRA: $p=0.02$; versus DEAB: 0.07; ATRA versus DEAB $p=0.01$). Together, these results demonstrate that RA signaling enhances embryonic liver growth.

Retinaldehyde dehydrogenase (Raldh) enzymes are responsible for converting retinaldehyde to RA. The zebrafish expresses three Raldhs - *raldh2*, *raldh3*, and *raldh4* (Begemann et al., 2001; Liang et al., 2008; Pittlik et al., 2008). Raldh2 is expressed in the lateral plate mesoderm during somitogenesis, and zebrafish and medaka *raldh2* mutants display defective liver development (Begemann et al., 2001; Alexa et al., 2009; Negishi et al., 2010). Raldh3 is expressed in the eye and is therefore unlikely to have a role in liver development. Raldh4 is expressed in the developing liver at 48 hpf (Liang et al., 2008), however targeted knockdown of *raldh4* has not been reported. To determine whether Raldh4 is required for normal liver development and whether its temporal requirement is distinct from that of Raldh2, we knocked down *raldh2* and *raldh4* using ATG start site morpholinos (MOs). Liver size was subsequently assessed by *in situ* for *prox1* at 30 and 48 hpf and *lfabp* at 72 hpf. At 30 hpf, 100% of *raldh2* knockdown embryos and 4% of *raldh4* knockdown embryos developed small livers compared to 6% in controls ($n > 25$ /condition) (Fig. 2A–C). Similarly, at 48 hpf, 7% of control embryos, 100% of *raldh2* morphants, and 17% of *raldh4* morphants developed small livers ($n > 18$ /condition) (Fig. 2D–F). When examined at 72 hpf, 0% of control embryos, 80% of *raldh2* morphants, and 82% of *raldh4* morphants developed small livers ($n > 40$ /condition) (Fig. 2G–I). Our results indicate that *raldh2* is required throughout hepatogenesis, whereas *raldh4* is primarily required following hepatocyte specification (after 48 hpf). Additionally, in a separate experiment in which we titrated individual MO doses to prevent toxicity from double MO injection, we found that 0% of uninjected embryos, 31% of *raldh2* morphants, 21% of *raldh4* morphants, and 76% of *raldh2+4* double morphants developed small livers at 72 hpf ($n = 14$ /condition). These data suggest that Raldh2 and 4 enzymes act sequentially and synergistically during hepatogenesis and corroborate our timed chemical exposure studies.

Retinoic acid signaling impacts liver size and laterality by receptor-specific mechanisms

The zebrafish expresses two RA receptor (RAR) α subtypes, *raraa* and *rarab*, and two RAR γ subtypes, *rarga* and *rargb* (Hale et al., 2006). No RAR β has been identified to date. Combinatorial RAR knockdowns result in defects in hindbrain, limb, and pharyngeal arch development (Linville et al., 2009), but receptor-specific roles in liver development have not been described. To elucidate individual receptor contributions to hepatogenesis, we knocked down each receptor using previously published MOs (Linville et al., 2009). Knockdown of *RARs aa*, *ab*, and *ga*, primarily resulted in smaller livers (*raraa*: 43% smaller livers, $n=28$; *rarab*: 15%, $n=34$; *rarga*: 47%, $n=38$) (Fig. 3A – D and G–J), suggesting that these receptors act redundantly during hepatic specification and/or hepatocyte proliferation. Interestingly, we found that knockdown of *rargb* caused embryos to develop bilateral livers (29%, $n=105$) (Fig. 3E and K), suggesting that this receptor regulates liver laterality. Combinatorial knockdown of all four RARs resulted in even smaller (82%), sometimes absent livers (18%, $n=22$), indicating that RARs are required for normal hepatic specification (Fig. 3F and L). These results are corroborated by the fact that single RAR knockout mice are viable, whereas double knockouts display a number of developmental defects (Mark et al., 2006).

To confirm our *rargb* knockdown results chemically, we treated embryos with a selective RAR γ antagonist, MM11253, over the course of hepatogenesis (18–72 hpf). Whereas DMSO controls developed left-sided livers, 24% ($n=33$) of MM11253-treated embryos developed bilateral livers (Fig. 3M and N), confirming our MO knockdown results. Additionally, bilateral livers developed in embryos treated during shorter, early time windows (18–24 and 24–48 hpf) (Fig. 3O and P), suggesting that *Rargb* exerts its effect during hepatic specification. FACS quantification of RAR MO-injected *Lfabp:GFP* reporter fish at 72 hpf demonstrated that *rargb* morphants with two livers have significantly more hepatocytes than their control siblings, whereas *raraa* knockdown embryos have fewer GFP + hepatocytes than control siblings, and combinatorial knockdown embryos fewer still (Fig. 3Q) (*rargb* MO versus control, $p=0.00017$; versus *raraa* MO, $p=0.00023$; versus combo MO, $p=2.40 \times 10^{-6}$). These data illustrate that individual RARs have specific effects on liver specification and laterality.

To further elucidate the timing of *rargb*'s effect on liver laterality, we injected *rargb* MO and assessed hepatic bud formation at 30 hpf using the hepatoblast marker *prox1* and at 48 hpf using the pan-endodermal marker *foxA3*. Whereas control embryos displayed left-sided liver buds at 30 hpf, 26% of *rargb* morphants ($n=23$) developed bilateral populations of hepatoblasts (Fig. 4A and B). This percentage corresponds to the number of bilateral livers seen at 72 hpf (29%). We also observed that some *rargb* morphants display midline liver buds at 30 hpf (21%, $n=23$). We did not observe midline livers at 72 hpf in *rargb* morphants, suggesting that midline populations of hepatoblasts ultimately resolve into unilateral hepatocyte populations or split into two liver buds. These results corroborate our early (18–24, 24–48 hpf) MM11253 treatments, demonstrating that *rargb* knockdown impacts liver laterality during hepatic specification. Examination of *foxA3* expression at 48 hpf further confirmed that *rargb* knockdown leads to bilateral livers (Fig. 4C and D). In control embryos, the pan-endodermal marker *foxA3* marks the left-sided liver and right-sided pancreas. In *rargb* morphants, we identified bilateral livers in 30% of MO-injected embryos ($n=28$). We also discovered that in some cases, embryos with bilateral livers also developed bilateral pancreata (7%, $n=28$). Whereas most bilateral livers occurred in the presence of a normal right-sided pancreas, bilateral pancreata always occurred in conjunction with bilateral livers (Fig. 4D). These results demonstrate that *rargb* knockdown impacts the laterality of the liver and other endoderm derivatives.

Rargb knockdown affects organ laterality

In zebrafish, cardiac progenitors develop bilaterally, coalesce at the midline, then the nascent heart tube jogs asymmetrically to the left of the midline around 1 dpf (Bakkers et al., 2009). In mice, the cardiac mesoderm and septum transversum mesenchyme provide Fgf and Bmp signals to specify hepatic progenitors in the overlying endoderm (Jung et al., 1999; Rossi et al., 2001), and loss of these signals in zebrafish prevents hepatic specification (Shin et al., 2007). It is therefore possible that defective heart positioning and ectopic inductive cues precede the development of bilateral livers. We have observed the coincidence of heart and liver laterality defects in other contexts; double *in situ* hybridization for hepatoblast and cardiac markers *prox1* and *cmlc2*, respectively, revealed that cardia bifida mutants *miles apart (mil)* (Kupperman et al., 2000) also develop bilateral livers (Fig. S3). Accordingly, to determine whether laterality defects in the endoderm are accompanied by heart jogging defects, we examined expression of the pan-cardiomyocyte marker *cmlc2* at 26 hpf. Control embryos developed left-sided heart tubes, whereas 33% of *rargb* morphants displayed midline heart tubes (n=33) (Fig. 5A and D). Knockdowns of RARs *raraa*, *rarab*, and *rarga* did not affect heart jogging (data not shown). Examination of chamber specific markers atrial and *ventricular myosin heavy chain (amhc* and *vmhc*, respectively) revealed that the ventricle is specifically affected by *rargb* knockdown (36%, n=28) (Fig. 5B, C, E and F). We also confirmed our genetic results chemically by treating embryos with the selective RAR γ antagonist MM11253 over the course of cardiac jogging (14–26 hpf) and examining *cmlc2* expression at 26 hpf. MM11253-exposed embryos also displayed heart jogging defects (25%, n=24) (Fig. 5G and H), further indicating that loss of *rargb* perturbs heart laterality.

Just as zebrafish *rargb* morphants develop midline ventricles, dominant negative RAR γ E₃₀₅ chimeric mice exhibit misaligned or unlooped ventricles (Iulianella and Lohnes, 2002), suggesting that RARg functions similarly during vertebrate organogenesis. To demonstrate evolutionary conservation of RAR γ function during cardiac development, we performed a rescue experiment in which zebrafish *rargb* MO was co-injected with mouse *Rarg* mRNA at the 1-cell stage, and *vmhc* expression was evaluated at 30 hpf. Compared to controls (0% midline ventricles, n=20), 35% (n=34) of *rargb* morphants developed a midline ventricle (control versus MO $p=0.0019$), whereas only 9% (n=22) of MO +mRNA co-injected embryos displayed this defect (MO versus MO+mRNA $p=0.0315$) (Fig. 5I). We also examined *lfabp* expression at 72 hpf to determine whether mouse *Rargb* mRNA rescues the *rargb* morphant liver phenotype. All controls developed left-sided livers (n=23), whereas 22% (n=41) of *rargb* morphants (control versus MO $p=0.0208$) and 10% (n=30) of MO +mRNA co-injected embryos had bilateral livers (MO versus MO+mRNA $p=0.218$). Although we consistently see a reduction in the number of bilateral livers in co-injected embryos, we suspect that the rescue is less effective at a later stage due to persistent toxicity from the presence of two exogenous nucleotide constructs. Together, our results demonstrate that RAR γ function is conserved between fish and mammals.

To demonstrate that heart and endoderm defects are linked in the same embryo, we injected *rargb* MO into *cmlc2:GFP* reporter fish, sorted embryos by heart phenotype (left or midline) at 30 hpf by fluorescence microscopy, and assessed *lfabp* expression by *in situ* at 72 hpf. If heart and liver defects correlate in the same embryo, this would suggest a unifying underlying cause (e.g., gut looping defect) or that one defect precedes the other (e.g., altered cardiac asymmetry governs liver positioning). We found that heart and liver defects are significantly correlated in the same embryo ($p<0.0001$, Chi-squared analysis). Specifically, whereas only 10% of embryos with a left-sided heart developed bilateral livers (n=71), 74% of embryos with a midline heart developed bilateral livers (n=31) (Fig. 5J). Together, our results demonstrate that *rargb* knockdown impacts heart (ventricle) as well as endoderm laterality and suggest that these phenotypes are linked.

Because *rargb* morphants develop endoderm and cardiac defects, we reasoned that this might be due to an underlying cause such as aberrant LPM migration. Failed gut looping is often associated with bilateral endodermal organs (Chen et al., 2001), and zebrafish mutant for the bHLH transcription factor *hand2* exhibit failed LPM migration and develop gut looping defects and bilateral livers and pancreata (Yin et al., 2010). To assess this possibility, we examined whether gut looping is affected in *rargb* morphants by observing expression of *sonic hedgehog* (*shh*) and *hand2* at 48 hpf. We found that the gut, which normally loops leftward in control embryos, did not loop or remained at the midline in *rargb* morphant embryos (*shh*: 29% midline, 54% weak looping, n=24; *hand2*: 73%, n=22) (Fig. 4E–H). We tested additional markers for the thyroid, hypothalamus, and kidney (*nkx2.1a*, *slc20a1a*, *slc12a3*) and did not note any defects (data not shown), indicating that *rargb* knockdown impacts only a subset of mesendodermal organs.

Nodal signaling is unaffected in *rargb* morphants

Nodal signaling is a master regulator of left-right asymmetry (Shen, 2007). Nodal signals originating in the node propagate anteriorly in the left LPM and impact endoderm and heart asymmetry. To determine whether laterality defects seen in *rargb* morphants are due to altered Nodal signaling, we examined the expression of the zebrafish Nodal signal *southpaw* (*spaw*), downstream effector *pitx2*, and Nodal antagonists *lefty1* and *lefty2* during somitogenesis (18–22 somites). Both control and *rargb* morphant embryos displayed normal left-sided expression of *spaw*, *pitx2*, and *lefty2*, and the molecular midline barrier *lefty1* remained intact (Fig. S4), demonstrating that *Rargb*'s impact on organ laterality is Nodal-independent. We also treated embryos with the RAR γ antagonist MM11253 in two time windows targeting early development, 32 cell–2 somite and 2 somite–20 hpf, and found that Nodal signals remain left-sided in 91% (n = 66) and 92% (n = 51) of these embryos, respectively. The results of these chemical exposures further demonstrate that inhibition of *rargb* during early development does not impact Nodal signaling. It was recently shown that LPM migration defects in *hand2* mutants are not due to Nodal defects alone; in *spaw* morphants, the direction of LPM migration is randomized, whereas in *hand2* mutants, LPM migration does not occur at all (Yin et al., 2010). Furthermore, although previous studies have suggested that early inhibition of RA signaling (prior to the 2 somite stage) affects Nodal signaling, these embryos ultimately develop *situs inversus* rather than duplicated or midline organs (Huang et al., 2011), bolstering our observations that *rargb* morphant defects are likely due to defects in LPM migration rather than misexpression of global laterality signals.

Rargb affects Bmp signaling via altered phospho-Smad levels during organ laterality determination

Bmp4 is expressed asymmetrically in the left LPM between 20–22 somites, and this expression pattern leads to greater activation of phosphorylated Smads on the left side and is correlated with leftward cardiac jogging (Chen et al., 1997; Chocron et al., 2007; Smith et al., 2008). Because Bmp signaling is important for determining heart laterality, we investigated whether ectopic Bmp signaling perturbs liver laterality. We heat shocked inducible *hs:bmp2b* zebrafish embryos at 12 somites, prior to cardiac jogging and hepatic specification, and examined heart and liver development by *in situ* for *cmhc2* (30 hpf) and *lfabp* (72 hpf), respectively. At 30 hpf, 5% (n = 100) of non heat shocked (hs–) control sibling embryos and 72% (n = 79) of heat shocked embryos (hs+) displayed midline hearts (Fig. 6A). Similarly, at 72 hpf, 4% (n = 106) of hs– embryos compared to 33% (n = 111) of hs+ embryos developed bilateral livers (Fig. 6B). These data demonstrate that *rargb* knockdown and *bmp2b* overexpression result in the same phenotype, suggesting interplay between RA and Bmp signaling during laterality determination.

Given that loss of *rargb* or gain of *bmp2b* expression results in bilateral livers and midline hearts, we hypothesized that RA signaling antagonizes Bmp signaling during organ development (or *vice versa*). To determine whether RA inhibits Bmp signaling, we injected *rargb* MO at the 1-cell stage then treated these embryos with the Bmp inhibitor dorsomorphin from 14–17 hpf. At 72 hpf, we assessed whether Bmp inhibition rescued *rargb* morphants' bilateral liver phenotype. Whereas 3% of uninjected/DMSO-treated control embryos (n = 101) and 1% of uninjected/dorsomorphin-treated embryos (n=137) developed bilateral livers, 24% of *rargb* MO-injected/DMSO-treated embryos developed bilateral livers (n=144; p-value < 0.0001 for MO/DMSO *versus* uninjected/ DMSO). In contrast, 17% of *rargb* MO/dorsomorphin-treated embryos (n = 163) developed bilateral livers (Fig. 6C; p-value= 0.1565 for MO/DMSO *versus* MO/dorsomorphin). These results represent six independent experiments in which the same rescue trend was noted in each. Together, these results demonstrate that inhibition of Bmp signaling rescues the *rargb* morphant phenotype and suggesting that Bmp acts downstream of Rargb during laterality determination.

We also performed the reverse experiment by first over-expressing Bmp then treating embryos with a RAR γ agonist, CD1530. *Hs:bmp2b* transgenic embryos were heat-shocked at 12 somites then treated with CD1530 from 16–18 somites, and liver development was assessed at 72 hpf by *lfabp* expression. Whereas *hs-/-CD1530-* and *hs-/-CD1530+* embryos did not display laterality defects, 25% of both *hs+/CD1530-* and *hs+/CD1530+* embryos (n 13/condition) developed bilateral livers (data not shown). These data further indicate that Rargb acts upstream of Bmp signaling to govern organ laterality.

Our *in vivo* data suggest that loss of *rargb* leads to an increase in Bmp signaling, since loss of *rargb* and overexpression of *bmp2b* result in the same laterality defects, and inhibition of Bmp signaling by dorsomorphin rescues the *rargb* morphant phenotype. To corroborate our *in vivo* findings, we examined expression levels of phosphorylated Smad1/5/8 (p-Smads), cytoplasmic effectors of Bmp signaling, in whole embryo lysates at 18 hpf. We found that *rargb* knockdown led to a ~60% increase in p-Smad1/5/8 expression levels compared to control embryos by Western blot analysis (Fig. 6D). Whereas expression patterns of Bmp-related *smads* 1/5 as well as that of co-Smad *smad4* are unchanged at the level of *in situ* hybridization (data not shown), total levels of Smad 1/5/8/9 proteins are also increased in *rargb* morphants as determined by Western blot analysis, indicative of decreased degradation (Fig. S5). Together, our results indicate that Rargb normally inhibits Bmp signaling by reducing levels of active cytoplasmic Bmp effectors p-Smad1/5/8.

In addition to examining p-Smad1/5/8 levels in embryo lysates, we also investigated whether changes in Bmp signaling were visible in the cardiac field of living embryos using transgenic Bmp response element reporter fish *BRE:eGFP* that are responsive to Smad1/5 activity (Laux et al., 2011). In wild type embryos, Bmp signaling is active asymmetrically (Smith et al., 2008), with greater signaling occurring in the left LPM around the 20–22 somite stage, leading to a leftward jogging heart tube by 24 hpf. Compared to control embryos that displayed asymmetric Bmp activity (100%; 6 left, 1 right), 53% of *rargb* morphant embryos (n=15) exhibited symmetrical Bmp activity at 22 somites (Fig. 6E and F). We examined these same embryos at 30 hpf to assess whether changes seen in Bmp signaling at 22 somites ultimately affected heart tube development. Because Bmp remains active in the heart at this stage, the direction of cardiac jogging can be assessed in *BRE:eGFP* embryos. Both control and *rargb* morphant embryos' hearts jogged according to earlier sidedness of Bmp activity. Specifically, all control embryos developed asymmetrically jogged hearts (6 left, 1 right; 100% asymmetrical), whereas 40% of *rargb* morphant embryos developed midline hearts (6 midline, 7 left, 2 right; 60% asymmetrical) (Fig. 6G and H; p-value=0.0225 for control *versus rargb* morphant embryos showing

asymmetrical – left or right – *versus* symmetrical Bmp signaling). Correspondingly, we also found that control embryos displayed normal asymmetric expression of *bmp4* around the cardiac cone, whereas *bmp4* expression is symmetrical in *rargb* morphants as determined by in situ hybridization at 21 somites (Fig. S6(D) and (E)). Together, these results demonstrate *in vivo* that loss of *rargb* affects Bmp activity and ultimately organ laterality.

The zebrafish *rargb* morphant phenotype resembles human right atrial isomerism

Rargb morphant laterality defects are strikingly similar to the human heterotaxic syndromes left or right atrial isomerism in which patients display both a midline heart and a duplicated or midline liver (Sutherland and Ware, 2009; Zhu et al., 2006). Biliary atresia, characterized by blocked or absent hepatic bile ducts, is another hallmark of the human heterotaxic condition (Sutherland and Ware, 2009) so we investigated whether *rargb* morphants also display biliary defects. We first examined tp1bglob:eGFP reporter fish in which the Epstein–Barr Virus TP1 gene, placed upstream of the rabbit β -globin minimal promoter acts as a Notch-responsive element (Parsons et al., 2009). Notch signaling is required for normal bile duct specification and development and highlights the biliary tree as early as 76 hpf (Lorent et al., 2010; Lorent et al., 2004). Compared to control larvae that developed intrahepatic bile ducts with a normal latticework appearance, 56% (n=52) of *rargb* MO-injected larvae exhibited dysmorphic bile duct architecture characterized by reduced intrahepatic branching and connectivity (Fig. 7A and B). In addition to examining bile duct development *in vivo* in Notch reporter fish, we also investigated the expression of the bile duct cell marker 2F11 (Crosnier et al., 2005) by whole mount immunostain in 5 dpf larvae. Confocal projections of control embryos revealed a regular, highly branched network of intrahepatic bile ducts, whereas *rargb* morphant embryos displayed less extensive ductular networks and enlarged cell bodies at duct junctions (84%, n=19) (Fig. 7C – F), defects reminiscent of the human pathophysiology of biliary atresia. The etiology of biliary atresia remains unknown, however our results suggest that loss of *rargb* negatively impacts bile duct formation.

Patients with heterotaxia often exhibit asplenia or polysplenia (Ivemark, 1955; Sutherland and Ware, 2009), so we also investigated whether *rargb* morphants develop spleen defects by examining expression of *hox11* at 5 dpf. 34% (n=58) of *rargb* morphants had a small or absent spleen, compared to only 3% of control embryos (n=33, *p*-value=0.0005), suggesting that loss of *rargb* results in a phenotype similar to human asplenia. Patients with right atrial isomerism, or Ivemark syndrome, typically display asplenia (Ivemark, 1955) whereas patients with left atrial isomerism predominantly develop polysplenia (Zhu et al., 2006), thus the *rargb* morphant phenotype more closely resembles right atrial isomerism. Together, the laterality, biliary, and spleen defects seen in *rargb* morphants implicate RAR γ in human heterotaxic syndromes and suggest that RA signaling is important for *situs solitus* of human organs.

Discussion

In this study, we identified RA as a regulator of zebrafish hepatogenesis and elucidated roles for RA signaling in organ development and positioning. We have shown that RA synthesis by Raldh enzymes and downstream signal transduction by RARs are both important for proper liver development. RAR knockdowns reveal that individual receptors exert specific effects during organogenesis. Loss of *Rargb* results in duplicated livers, midline hearts, and unlooped guts, and epistasis analyses demonstrate that *Rargb* functions upstream of Bmp to affect organ positioning. *Rargb* morphants also develop bile duct defects and asplenia, which in combination with their laterality phenotypes, parallel the human heterotaxic syndrome right atrial isomerism.

It has been suggested that RA signaling is involved in endoderm development (Stafford and Prince, 2002; Alexa et al., 2009; Negishi et al., 2010), however roles for specific RARs in liver development and positioning have not been described. Our finding that one receptor, Rargb, impacts organ laterality can be explained by differences in zebrafish RAR expression patterns and divergent RAR phylogenies (Waxman and Yelon, 2007). First, *rargb* is located on a different chromosome than *rarga*, suggesting that these may be different genes rather than splice variants. Furthermore, whereas *rarga* is not expressed maternally, *rargb* is expressed both maternally and zygotically. Phylogenetic analyses have also established that *rargb* is more closely related to mouse and human RARG, supporting our results demonstrating evolutionary conservation of RAR γ function. Knockdown of the RA transport protein Rbp4 results in the formation of two liver buds (Li et al., 2007), corroborating a role for RA signaling in liver progenitor positioning.

Although Nodal is known to be a master regulator of left–right asymmetry (Shen, 2007), we did not find its expression affected by loss of *rargb*. It has previously been shown that blocking RA signaling before the 2 somite stage alters Nodal signaling in zebrafish (Huang et al., 2011), but organs are primarily reversed rather than duplicated or midline in these embryos. Furthermore, studies in other species have suggested that RA signaling can impact organ laterality without disrupting early left–right signals. In *Xenopus*, laterality defects manifest in embryos treated with DEAB after Nodal has established the left–right axis (Lipscomb et al., 2006), and *Raldh2* null mice develop unlooped hearts despite Nodal being unaffected (Niederreither et al., 2001). Characterization of the zebrafish mutant *heart* and *soul* (*has*), which displays failed gut looping and symmetrical visceral organs (Horne-Badovinac et al., 2001), revealed that these laterality defects are accompanied by normal *spaw* and *pitx2* expression (Horne-Badovinac et al., 2003), further suggesting that left–right patterning defects can manifest in the presence of normal Nodal signaling.

RA and Bmp signals intersect in other developmental contexts (Sheng et al., 2010; Shimono et al., 2011; Song et al., 2007; Tehrani and Lin, 2011), so we hypothesized that RA may exert its effect on organ development by regulating Bmp activity. In mice, Bmps generated adjacent to the cardiac mesoderm in the septum transversum mesenchyme induce hepatic progenitors in the nearby endoderm and promote outgrowth of the liver primordium (Jung et al., 1999; Rossi et al., 2001). Studies in mice and chick have demonstrated that RARG inhibits Bmp signaling by promoting degradation of phosphorylated Smad (Sheng et al., 2010; Shimono et al., 2011), further substantiating our finding that RA integrates with Bmp signaling via regulation of Bmp's downstream effectors.

Loss of *rargb* results in laterality defects of the liver, heart, and gut, but it is unclear whether all defects are linked. Gut looping occurs around 30 hpf (Horne-Badovinac et al., 2003), but we observe heart jogging defects as early as 26 hpf; accordingly, abnormal LPM migration can only account for visceral organ but not heart defects. It is possible that aberrant RA signaling affects both LPM migration and local Bmp signaling around the cardiac cone, thereby affecting multiple organs. It has recently been shown that zebrafish RARs primarily act as transcriptional activators rather than repressors (Waxman and Yelon, 2011), so it is also possible that *rargb* may preferentially activate another molecule on the right side that normally acts as a repressor of the hepatic fate. *No tail* (*ntl*), *floating head* (*flh*), and *schmalspur* (*sur*) mutants display midline hearts, loss of gut looping, and duplication of visceral organs, and it has been suggested that because these are loss-of-function mutations, the normally asymmetric liver results from suppression of hepatic activators on the right side (Chen et al., 2001). A previous study suggests that *rargb* is asymmetrically expressed in the neural crest cells of the hindbrain at 18 hpf (Linville et al., 2009), however *rargb* appears to be expressed equally on both left and right sides of the trunk during somitogenesis (Fig. S6(A)), so it remains unclear how *rargb* exerts its affect on visceral organ laterality. It is

possible that *in situ* hybridization is not sensitive enough to detect slight asymmetries in *rargb* expression that may impact downstream signaling.

Organ laterality and bile duct defects exhibited by *rargb* morphants are reminiscent of the human pathological condition right atrial isomerism in which patients display midline hearts, midline or duplicated livers, biliary atresia, and asplenia. Our data suggest that perturbed RA signaling may play a role in human heterotaxia and furthermore, that heterotaxia can result from mutations in genes other than Nodal. We demonstrate that loss of *Rargb* and *Bmp* overexpression result in the same organ laterality defects, where *Rargb* functions upstream of *Bmp* signaling to regulate organ position, and that *Rargb* also impacts biliary development. Biliary abnormalities often result in obstructed bile flow, leading to pancreatitis and jaundice, and necessitate surgical intervention or liver transplantation in infants (Davenport, 2005; Haber and Russo, 2003). The etiology of biliary atresia remains unknown, but the fact that *rargb* morphants and left or right atrial isomerism patients develop organ *situs* and biliary defects suggests a common underlying mechanism. By understanding the role of RA and *Bmp* signaling in organ development and positioning, we may be able to better understand and treat patients with these life-threatening malformations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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MKG devised, executed, and analyzed experiments and wrote the manuscript. CCC, AM, PBK, and JMH performed and analyzed the chemical genetic screen. WG and TEN devised experiments and analyzed data. All authors reviewed and edited the manuscript.

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Fig. 1. Retinoic acid signaling enhances embryonic liver development. (A–F) In situ hybridization for *Ifabp* at 72 hpf reveals differences in liver size after chemical exposure at 18 hpf. ATRA treatment leads to greater *Ifabp* expression, whereas DEAB treatment leads to reduced *Ifabp* expression. First row, lateral view. Second row, dorsal view. (G–I) Fluorescence microscopy of *Ifabp*:GFP reporter fish at 72 hpf confirms *in situ* hybridization results in (A–F). (J–L) Fluorescence microscopy of *cyp26a1*:eYFP reporter fish at 72 hpf demonstrates changes in RA signaling activity in chemically treated embryos. Arrowheads highlight the liver. (M–O) BrdU whole mount immunostaining of 24 hpf embryos demonstrates that ATRA-treated embryos show higher levels of proliferation in the liver primordium (black rectangle) compared to DEAB and DMSO-treated controls. (M'–O') Enlarged images of (M–O) illustrating individual proliferating cells in the liver primordium. (P) Quantification of the number of BrdU+ cells/field (black rectangle in O). (Q) FACS quantification of percent GFP + cells in chemically treated *Ifabp*:GFP embryos reveals that ATRA-treated embryos contain more hepatocytes and DEAB-treated embryos contain fewer hepatocytes than DMSO-treated controls. (R) qPCR analysis reveals differences in the relative expression of the

hepatocyte marker *transferrin* in ATRA and DEAB-treated embryos at 72 hpf compared to DMSO-treated controls (where control expression levels were normalized to 1.0). Scale bars: 100 μ m.

Fig. 2. Raldhs 2 and 4 temporally regulate liver development. (A–I) Wild type embryos were injected with *raldh2* or *raldh4* MO at the 1-cell stage, and liver development was assessed over the next 72 h. *In situ* hybridization for the hepatoblast marker *prox1* at 30 hpf (A–C) and 48 hpf (D–F) and hepatocyte marker *lfabbp* at 72 hpf (G–I) demonstrates distinct temporal requirements for Raldh2 and Raldh4 during liver development. Arrowheads denote the liver primordium, and the dotted lines outline the liver. Scale bars: 100 μ m.

Fig. 3. Retinoic acid receptors differentially impact liver development and laterality. (A–L) *Lfabp* expression at 72 hpf in RAR morphant embryos. Loss of RARs *raraa*, *rarab*, and *rarga* leads to a reduction in liver size (A–D and G–J), whereas loss of *rargb* causes embryos to develop bilateral livers (E, K and K'). Embryos simultaneously injected with a lower dose of all four RAR MOs develop small livers or none at all (F and L). (M–P) Embryos treated with the RAR γ antagonist MM11253 over the time points given develop bilateral livers, confirming genetic knockdown data. (Q) FACS quantification of percent GFP+ cells in *Ifabp:GFP* RAR morphant embryos reveals that *rargb* morphant embryos with bilateral livers contain significantly more GFP+ hepatocytes than control siblings. Embryos injected with *raraa*, *rarab*, *rarga*, and *rargb* in combination have roughly half as many GFP+ hepatocytes as control embryos. Scale bars: 100 μ m.

Fig. 4.

Rargb knockdown impacts the left-right asymmetry of visceral organs. (A and B) *Prox1* expression demonstrates that hepatic progenitors normally migrate to the left of the midline at 30 hpf (A, black arrowhead). Loss of *rargb* results in bilateral (B) or midline (B') hepatoblast populations. (C–D) In situ hybridization for the panendodermal marker *foxA3* at 48 hpf demonstrates that loss of *rargb* results in bilateral livers (black arrowheads) that are sometimes accompanied by bilateral pancreata (white arrowheads). (E and F) *Rargb* morphants develop gut looping abnormalities (arrow), as demonstrated by *shh* expression at 48 hpf (E and F). (G and H) *Hand2* expression at 48 hpf demonstrates aberrant LPM migration in *rargb* morphants (H). Insets in (G and H) highlight the differences in LPM phenotypes. Scale bars: 100 μ m.

Fig. 5. Midline hearts and bilateral livers correlate in *rargb* morphants. (A–F) Control embryos develop leftward jogging hearts as shown by the pan-cardiomyocyte marker *cmlc2* at 26 hpf (A) and chamber-specific markers *vmhc* and *amhc* at 30 hpf (B and C), whereas *rargb* morphants develop midline ventricles (D and E). (G and H) Embryos treated with the RAR γ antagonist MM11253 develop midline hearts, confirming genetic knockdown data. (I) RAR γ function is evolutionarily conserved. The midline heart phenotype of *rargb* morphant embryos is rescued by injection of mouse *Rarg* mRNA. (J) Midline hearts and bilateral livers correlate in the same embryo. *Cmlc2*:GFP reporter fish injected with *rargb* MO were

sorted by heart phenotype at 30 hpf, then liver laterality was assessed at 72 hpf by *lfabp* expression. Scale bars: 100 μ m.

Fig. 6.

Rargb negatively regulates Bmp activity and localization *in vivo*. (A and B) Overexpression of Bmp in heat shock inducible *hs:bmp2b* embryos results in midline hearts (A) and bilateral livers (B), paralleling the phenotype of *rargb* morphants. (C) Treatment of *rargb* morphant embryos with the Bmp inhibitor dorsomorphin results in a reduction in the number of bilateral livers compared to DMSO-treated control *rargb* morphants. (D) Western blot for Bmp effectors phospho-Smads 1/5/8 in uninjected control (Un) and *rargb* MO-injected embryos (MO) at 18 hpf. Band density values normalized to Actin demonstrate that loss of *rargb* results in increased expression of p-Smads 1/5/8. Embryos treated with the Bmp inhibitor dorsomorphin (DM) show reduced p-Smad 1/5/8 expression compared to DMSO-treated controls. (E–H) Fluorescence microscopy of Bmp response element reporter fish BRE:eGFP. At 20 hpf, control embryos display greater Bmp activity on the left side (E), whereas *rargb* morphants display symmetrical Bmp activity (F). Altered Bmp localization at 20 hpf corresponds to heart laterality defects at 36 hpf in *rargb* morphants (H). White dotted lines denote the midline (E and F) or outline the heart tube (G and H). Scale bars: 100 μ m.

Fig. 7.

Rargb morphants develop biliary defects. (A and B) Fluorescence microscopy of Notch reporter fish *tp1bglob:eGFP* at 5 dpf. *Rargb* morphant embryos develop defective bile duct architecture (B, arrows) compared to the normal latticework appearance of the control embryo's biliary tree (A). (C–F) Confocal microscopy of 2F11 whole mount immunostained embryos at 5 dpf. *Rargb* morphant embryos display enlarged cell bodies (D and E, white arrowheads) and reduced branching of intrahepatic bile duct networks (F) compared to control embryos (C). White dotted lines outline the liver. Images are taken at the same magnification. (G–I) *In situ* hybridization for *hox11* at 5 dpf. *Rargb* morphants display small

(H) or absent (I) spleens compared to control embryos (G). Black arrowheads denote the spleen. Scale bars: 50 μm .