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Alkylglycerol monooxygenase, a heterotaxy candidate gene, regulates left-right patterning via Wnt signaling

Anna R. Duncan, Delfina P. González, Florencia del Viso, Andrew Robson, Mustafa K. Khokha *, John N. Griffin [1]

Pediatric Genomics Discovery Program, Department of Pediatrics and Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT, 06510, USA

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ABSTRACT

Congenital heart disease (CHD) is a major cause of morbidity in the pediatric population yet its genetic and molecular causes remain poorly defined. Previously, we identified *AGMO* as a candidate heterotaxy disease gene, a disorder of left-right (LR) patterning that can have a profound effect on cardiac function. AGMO is the only known alkylglycerol monooxygenase, an orphan tetrahydrobiopterin dependent enzyme that cleaves the ether linkage in alkylglycerols. However, whether AGMO plays a role in LR patterning was unexplored. Here we reveal that Agmo is required for correct development of the embryonic LR axis in *Xenopus* embryos recapitulating the patient's heterotaxy phenotype. Mechanistically, we demonstrate that Agmo is a regulator of canonical Wnt signaling, required during gastrulation for normal formation of the left – right organizer. Mutational analysis demonstrates that this function is dependent on Agmo's alkylglycerol monooxygenase activity. Together, our findings identify Agmo as a regulator of canonical Wnt signaling, demonstrate a role for Agmo in embryonic axis formation, and provide insight into the poorly understood developmental requirements for ether lipid cleavage.

1. Introduction

Ether lipids are present in organisms ranging from bacteria to humans and are fundamental structural and functional components of cell membranes (Hallgren et al., 1974a, 1974b; Watschinger and Werner, 2013b). They play crucial roles in spermatogenesis, optic homeostasis, myelination, immunology, blood brain barrier permeability, and cell signaling (da Silva et al., 2012; Dean and Lodhi, 2018; Erdlenbruch et al., 2000; Gorgas et al., 2006; Haynes et al., 1994). Ether lipids are a unique subclass of glycerophospholipids, in that an alkyl chain is attached to the *sn-1* position by an ether linkage, rather than the more common ester bond, resulting in a higher metabolic stability (Dean and Lodhi, 2018; Watschinger and Werner, 2013b). Alterations in ether lipid biology are associated with numerous human conditions, including cancer, diabetes, and neurological diseases like Alzheimer's (Dean and Lodhi, 2018). However, despite their importance, our understanding of the biological functions, regulation and processing of ether lipids remains superficial, especially in developmental contexts.

Originally identified for its biochemical activity, alkylglycerol monooxygenase (AGMO) is the only enzyme known to cleave the O-alkyl ether bond in alkylglycerols, producing an aldehyde and glycerol derivative (Keller et al., 2010; Tietz et al., 1964; Watschinger et al., 2010, 2012; Watschinger and Werner, 2013a). However, all attempts to purify, identify and structurally analyze the enzyme have thus far failed. Recently AGMO was assigned to the *TMEM195* gene, encoding a 445 amino acid protein that contains 9 conserved membrane-spanning sections and a non-membrane associated helix (Watschinger et al., 2010). When expressed in CHO cells or *Xenopus* oocytes *Tmem195* induces a robust tetrahydrobiopterin-dependent alkylglycerol monooxygenase activity. This effect can be abolished by mutagenesis of conserved catalytic residues, confirming that the *TMEM195* gene encodes AGMO (Loer et al., 2015; Watschinger et al., 2010, 2012). However, while the biochemical function of AGMO is becoming clearer, its physiologic and developmental importance remain undefined.

We previously identified *AGMO* (*TMEM195*), as a candidate disease gene in a genetic analysis of human heterotaxy patients (Fakhro et al., 2011). The proper function of the internal organs of vertebrates relies heavily on their asymmetric development across the left - right body axis (Brueckner, 2007; Sutherland and Ware, 2009); for example, the left and right sides of the heart are anatomically and functionally distinct. Early in

* Corresponding author.
E-mail address: mustafa.khokha@yale.edu (M.K. Khokha).
[1] Present location: School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7 TJ, UK.

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development, symmetry is broken at the left – right organizer (LRO), where motile cilia drive extracellular fluid flow from right to left. This directional fluid flow is detected by sensory cilia on the left side of the LRO and translated into asymmetric expression of genes such as *coco* and *pitx2c*, ultimately producing correct organ situs (Boskovski et al., 2013; McGrath et al., 2003; Tabin and Vogan, 2003; Yoshiba et al., 2012). Impaired development of the embryonic left-right axis can have profound clinical consequences (heterotaxy syndrome), and in particular is often associated with severe forms of congenital heart disease (Brueckner, 2007).

Here we investigate requirements for Agmo in development of the vertebrate left – right body axis. Depletion of Agmo in the frog *Xenopus tropicalis* disrupts gastrulation movements, resulting in impaired formation of the LRO and recapitulation of our patient's heterotaxy phenotype. We identify an unexpected role for Agmo in the regulation of canonical Wnt signaling. Importantly, we also employ a series of catalytically dead mutants to demonstrate this developmental function is dependent on Agmo's ability to cleave ether lipids. Together, our work reveals a critical role for alkylglycerol monooxygenase activity in canonical Wnt signaling during gastrulation and supports a role for AGMO in the pathogenesis of heterotaxy syndrome.

2. Results and discussion

2.1. Agmo is required for left right development

To determine whether Agmo mediates left-right development, we used a translation blocking morpholino (MO^{ATG}) to deplete the protein in *Xenopus tropicalis* embryos. *X. tropicalis* is an ideal model for studying organ situs as their hearts are clearly visible at stage 45 allowing easy examination of cardiac outflow tract looping as a readout of left-right development (Blum et al., 2009; Del Viso et al., 2016; Griffin et al., 2018). In vertebrates, the cardiac outflow tract normally loops to the right (D – loop), while impaired left – right patterning can result in leftward (L - loop) or ambiguous (A - loop) cardiac looping. Depletion of Agmo, by injection of MO^{ATG} at the one cell stage, impaired cardiac

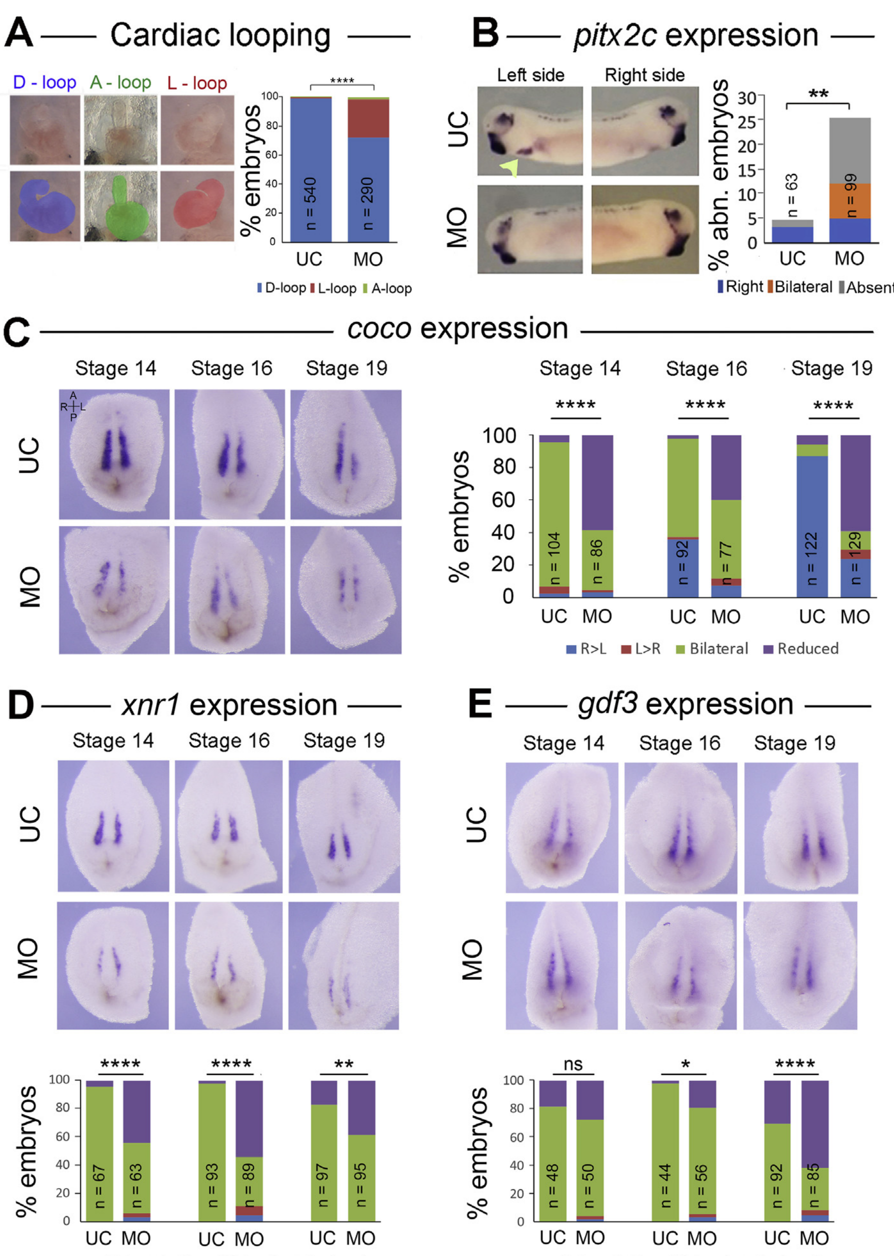

Fig. 1. *agmo* is required for L-R development. A) Depletion of Agmo disrupts cardiac looping. Lower images are pseudo-colored to highlight cardiac looping. B) Expression of *pitx2c,* which is normally only in left side mesoderm (upper row), is impaired in *Agmo* morphants (typically absent, lower row). abn.; abnormal C) *coco* expression is greatly reduced in the LRO from early in its development (before the initiation of flow, stage 14). LROs are dissected out and imaged ventrally. A; anterior, P; posterior, L; left, R; right. D) Expression of *xnr1* and E) *gdf3* is reduced in the left – right organizer of *agmo* morphants. Figure shows three developmental stages: stage 14 (pre-directional fluid flow) and stage 16 and 19. Graphs represent the combined results of six independent experiments for cardiac looping and *xnr1*, three repeats for *pitx2*, five repeats for *coco,* and 3 independent repeats for *gdf3*. UC; uninjected control, MO; morphant, ns; non-significant, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ by Chi-square analysis.

looping in morphants (primarily producing L-loops, Fig. 1A). This phenotype is comparable to that caused by depletion of known heterotaxy genes (Boskovski et al., 2013; Del Viso et al., 2016; Griffin et al., 2018; Kulkarni and Khokha, 2018) and confirms a requirement for Agmo in left – right development.

To better understand Agmo's role in left – right development, we next examined expression of two critical laterality genes, *pitx2c* and *coco* (DAND5 or *Cerl2* in mouse). *coco* is an extracellular nodal antagonist that is initially expressed symmetrically on the left and right sides of the LRO (stage 14–16, pre-flow), but whose expression becomes reduced on the left side downstream of cilia driven fluid flow (stage 19). This inhibition of *coco* allows activation of a downstream signaling cascade that is required for correct organ situs determination, including expression of the homeodomain-containing transcription factor *pitx2c* specifically on the left side of the embryo (Kawasumi et al., 2011; Logan et al., 1998; Schweickert et al., 2010; Vonica and Brivanlou, 2007). Agmo depletion disrupted expression of both *pitx2c* and *coco* (Fig. 1B and C). Importantly, *coco* expression was impaired from the earliest stages of LRO development, prior to the onset of cilia driven fluid flow, suggesting a defect in the initial establishment of the LRO itself (Griffin et al., 2018; Walentek et al., 2012). To confirm this, we examined expression of two additional LRO genes, *nodal1 (xnr1)* and *gdf3* (Hanafusa et al., 2000; Lustig et al., 1996; Schweickert et al., 2010; Vonica and Brivanlou, 2007), at early stages of LRO development (Fig. 1D and E). The reduction in signal for *nodal1* was readily detectable at early and later stages of LRO development while the reduction in *gdf3* expression was less pronounced at early stages compared to later stages. Together, these findings demonstrate that Agmo is required for correct development of the embryonic left-right organizer and that depletion of Agmo recapitulates the patient heterotaxy phenotype in *Xenopus*.

2.2. Agmo regulates gastrulation movements

We next examined preceding steps in formation of the LRO. During gastrulation, the involuting surface mesoderm becomes the gastrocoel roof plate, containing the LRO, on the dorsal side of the embryo (Keller et al., 2003). As such, we next examined gastrulation in our Agmo depleted embryos. Time-lapse imaging revealed that while control embryos smoothly completed gastrulation and proceeded through neurulation (Fig. 2A, upper row), embryos injected with 1 ng of *agmo* MO^{ATG} struggled to gastrulate. In particular, morphants typically required several attempts to close the blastopore and complete gastrulation (Fig. 2A, middle row). Embryos injected with a higher dose of MO (2 ng) were never able to complete blastopore closure, despite multiple attempts (Fig. 2A, lower row). Importantly, the gastrulation phenotype could be rescued by co-injection of 300 pg of wild type human *AGMO* mRNA demonstrating the specificity of our MO^{ATG} knockdown (Fig. 2B and C).

2.3. Agmo depletion impairs canonical Wnt signaling

The canonical Wnt pathway is a highly conserved signaling system that has numerous essential functions in development and disease (Clevers and Nusse, 2012; MacDonald et al., 2009; Moon et al., 2004; Niehrs, 2012). While testing the specificity of our MO in the previous rescue experiment, we noted that over expression of human *AGMO* mRNA in wildtype *Xenopus* embryos resulted in the formation of secondary axes, a phenotype classically associated with ectopic Wnt signaling activity during gastrulation (Fig. 4) (Funayama et al., 1995; Guger and Gumbiner, 1995; McMahon and Moon, 1989). This unexpected result led us to hypothesize that canonical Wnt signaling may be

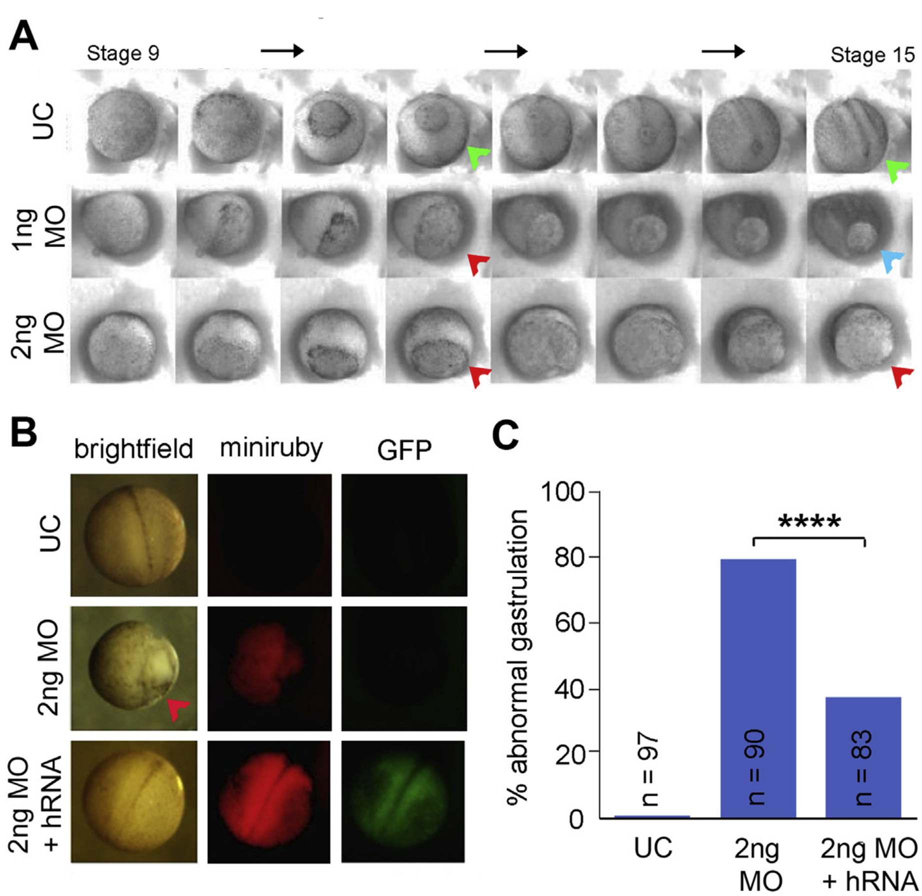

Fig. 2. Depletion of agmo impairs gastrulation. A) Time lapse images of gastrulation in uninjected control (UC, upper row), embryos injected with 1 ng MO^{ATG} (middle row) and embryos injected with 2 ng MO^{ATG} (lower row). UC embryos gastrulate normally, successfully closing the blastopore and forming the neural plate (green arrowheads). 1 ng MO^{ATG} embryos typically require several attempts to fully close the blastopore, and occasionally fail to complete gastrulation (blue arrowhead). 2 ng MO^{ATG} embryos never complete gastrulation despite multiple attempts (red arrowheads). B) The *agmo* morphant gastrulation phenotype can be rescued by co-injection of human *AGMO* mRNA (hRNA). Mini-ruby traces MO^{ATG} injection and GFP traces hRNA. Note that MO^{ATG} only embryos fail to gastrulate while co-injection of hRNA rescues the phenotype. Graph on right displays quantification of the human *AGMO* mRNA rescue. Data represent the combined result of three independent experiments. UC; uninjected control, MO; morphant. ****$p < 0.0001$ by Chi-square analysis.

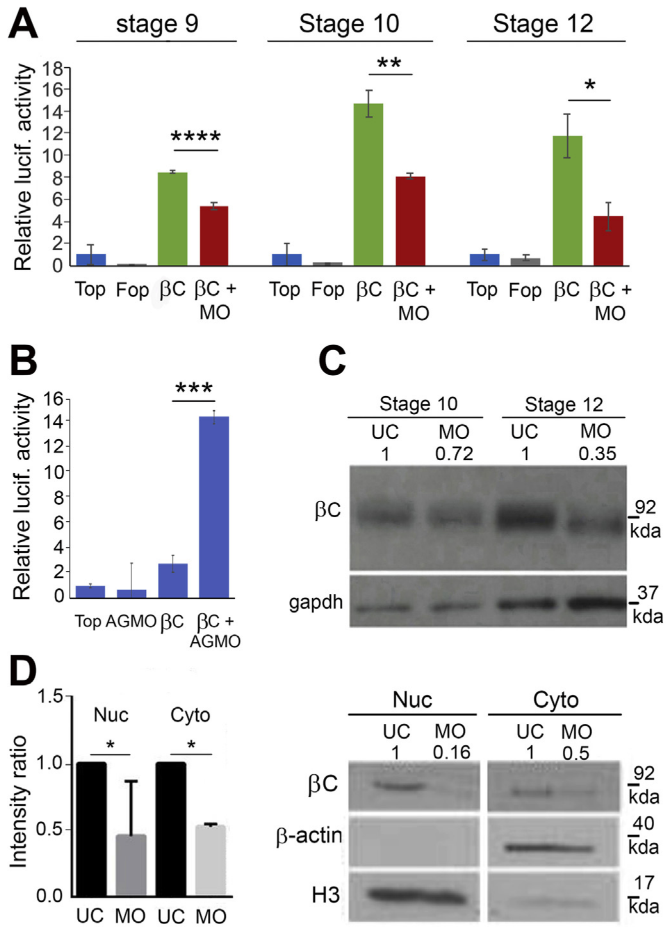

Fig. 3. agmo mediates canonical Wnt signaling. A) TOPflash assays reveal that Wnt driven luciferase production is reduced in *agmo* morphants throughout gastrulation (stages 9, 10, 12). B) Injection of *agmo* mRNA alone had no significant effect on luciferase activity, while co-injection of *agmo* mRNA and β-catenin *mRNA* increased Wnt activity significantly relative to injection of β-catenin alone. In A and B 10 embryos were pooled in each condition, and graphs represent the combined results of three biological and technical replicates. C) β-catenin protein levels are reduced in morphants at stage 10 and 12 as assayed by Western blot. D) Western blot analysis reveals that β-catenin protein levels are reduced in both the cytoplasmic (Cyto) and nuclear (Nuc) fractions of agmo morphants. The graph represents the combined result of four independent experiments. AGMO, AGMO mRNA; Top, TOPFlash reporter plasmid; Fop, FOPFlash negative control; BC, TOPFlash reporter plasmid + *β-catenin* mRNA; BC + MO, TOPFlash reporter plasmid + *β-catenin* mRNA + agmo morpholino; UC, uninjected control. All error bars represent ± sd. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ determined by students T-test.

affected by alterations in Agmo levels. To assess this, we first used a TOPflash assay (Molenaar et al., 1996; van de Wetering et al., 1991, 1997; Veeman et al., 2003) as a readout of canonical Wnt signaling activity in the embryo (Fig. 3A). As expected, injection *of β-catenin* mRNA alone at the one cell stage resulted in a pronounced increase in luciferase activity throughout gastrulation compared to uninjected controls (Fig. 3A, stage 9, 10, 12). Depletion of Agmo significantly reduced this β-catenin driven luciferase activity in stage 10 embryos, while overexpression of AGMO increased it (Fig. 3B), confirming that Agmo does modulate canonical Wnt signaling.

As regulation of β-catenin levels is key to pathway activation (Clevers and Nusse, 2012; Niehrs, 2012), we next directly examined levels of endogenous β-catenin in *agmo* morphants by Western blot and found that Agmo depletion significantly reduced β-catenin levels compared to controls at stage 10. This reduction became more pronounced as gastrulation proceeded (Fig. 3C). Fractionation revealed that β-catenin levels were reduced in both the cytoplasm and nucleus of morphants, suggesting a reduction in β-catenin stabilization upon pathway activation (Fig. 3D). Together, these results reveal that Agmo is required for canonical Wnt signaling during gastrulation and suggest that dysregulated Wnt signaling may contribute to the morphant phenotype.

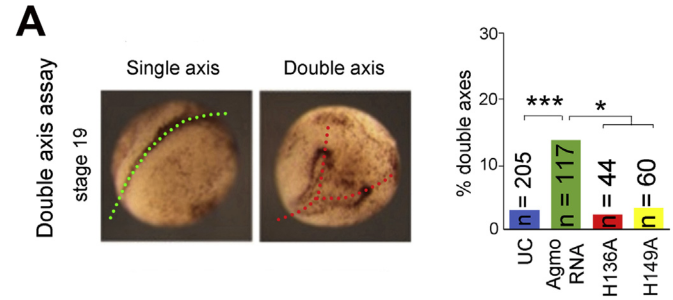

Fig. 4. Agmo mediates Wnt activity via its alkylglycerol monooxygenase activity. A) Injection of *AGMO* RNA alone can induce secondary axes, while injection of the catalytically dead H136A and H149A mutants cannot. Photos are representative examples of single (green dotted line) and *AGMO* induced double axes (red dotted lines) as seen at stage 19. Graph displays percentage of double axes in uninjected control embryos (UC), in embryos injected with wild type *AGMO* mRNA, and embryos injected with the H136A or H149A *AGMO* mutant mRNA. Data represents the combined outcome of three independent experiments. *$p < 0.05$, ***$p < 0.001$ by students T-test.

2.4. AGMO's biochemical activity is essential for normal development

AGMO is an alkylglycerol monooxygenase whose catalytic domain is well characterized (Watschinger et al., 2010, 2012). Our heterotaxy patient harbored a deletion of three exons overlapping the catalytic domain of AGMO, most likely producing an inactive form of the protein (Fakhro et al., 2011). To determine if AGMO activity is required for Wnt signaling, we examined the ability of catalytically dead AGMO mutants (harboring H136A and H149A mutations that ablate alkylglycerol monooxygenase activity) (Watschinger et al., 2010, 2012) to produce double axes. Injection of wild type human *AGMO* mRNA at the one cell stage produced a significant percentage of embryos with secondary axes (Fig. 4A). In contrast, injection of *AGMO* mRNA carrying the H136A or H149A mutations failed to in induce axis duplication (Fig. 4A). The inability of these catalytically dead mutants to produce secondary axes suggest that AGMO's alkylglycerol monooxygenase activity is required for canonical Wnt signaling activity during gastrulation.

Lipids are rapidly emerging as major regulators of the canonical Wnt pathway. For example, Porcupine mediated post-translational palmitoylation of conserved cysteine and serine residues in Wnt proteins at the endoplasmic reticulum (ER) is critical for sorting the ligand into secretory vesicles and cellular release (Biechele et al., 2011; Herr and Basler, 2012; Willert et al., 2003). Lipid modification also plays important roles in selective activation of the different Wnt signaling pathways (via phospholipid binding to the DIX domain of Disheveled) (Capelluto et al., 2002; Sheng et al., 2014; Simons et al., 2009), Wnt receptor endocytosis (Mo et al., 2010; Sato et al., 2010), and intracellular Wnt signal transduction (Lee et al., 2015; Pan et al., 2008). However, the function of Agmo in the canonical Wnt pathway remains unclear. Interestingly, Agmo was previously reported to localize to the ER in CHO cells (Watschinger et al., 2010), perhaps hinting at a role in the post translational modification of a pathway component. Our observation that Agmo depletion counteracts *β-catenin* over expression suggests that it is required at the level of β-catenin regulation or downstream in the pathway. Further investigation of Agmo's subcellular localization, potential ether lipid targets, and the Wnt pathway may help shed light on this issue.

Importantly, there is growing evidence to link canonical Wnt signaling to establishment of the left - right axis and heterotaxy. For example, numerous investigators have demonstrated the importance of canonical Wnt signaling in gastrulation and formation of dorsal tissues, including the LRO (De Robertis et al., 2000; Harland and Gerhart, 1997; Khokha et al., 2005; Niehrs, 2004), and so it is not surprising that impaired Wnt signaling would have knock on effects on left-right development. Furthermore, we have previously shown that depletion of the heterotaxy gene *rapgef5* impairs canonical Wnt signaling in the gastrulating embryo, which in turn results in a mispatterned LRO and abnormal left-right development (Griffin et al., 2018). Others have shown that genes such as *Foxj1* (a master regulator of motile cilia formation), and *coco* in the LRO itself are targets of canonical Wnt signaling (Caron et al., 2012; Kitajima et al., 2013; Stubbs et al., 2008; Walentek et al., 2012). Thus, impaired canonical Wnt signaling can likely impact numerous steps in establishment of the left – right body axis, including patterning of the LRO. Here, we found that Agmo depletion impairs expression of *coco, xnr1* and *gdf3* from early stages of LRO development, likely disrupting the embryo's ability to produce, interpret and/or transduce asymmetric signals.

While we know relatively little of AGMO's requirements in development and homeostasis, it has been implicated in several human disease states. Numerous genomic studies have linked DGKB/TMEM195 (*AGMO)* variants to impaired glucose homeostasis, obesity and diabetes (Boesgaard et al., 2010; Dupuis et al., 2010; Fujita et al., 2012; Goodarzi et al., 2013; Tarnowski et al., 2017). Others have associated *AGMO* polymorphisms with microcephaly and autism (Alrayes et al., 2016; Sebat et al., 2007). Interestingly, *AGMO* has also been linked to increased risk of colorectal cancer (Jung et al., 2017), a disease with a well establish connection to the Wnt pathway. We previously identified *AGMO* as a candidate heterotaxy gene (Fakhro et al., 2011) and here demonstrate that it is a potent regulator of canonical Wnt signaling, gastrulation, and left – right development. While the relationships between lipid metabolism, Wnt signaling and disease remains far from clear, we note that aberrant canonical Wnt signaling can contribute to each of the *AGMO* associated conditions (Buchman et al., 2011; Caron et al., 2012; Grant et al., 2006; Griffin et al., 2018; Harada et al., 1999; Jung et al., 2017; Kwan et al., 2016; Walentek et al., 2012; Welters and Kulkarni, 2008) and suggest that future studies assess the possible contribution of Wnt signaling to AGMO linked phenotypes.

We have demonstrated a novel role for Agmo in canonical Wnt signaling and embryonic development. Agmo is the only enzyme known to cleave the ether bond of the biologically important alkylglycerol ether lipids. As such, further investigation of the gene has great potential to elucidate the intriguing but poorly understood relationship between ether lipid biochemistry, development and disease.

3. Materials and methods

3.1. Xenopus

Xenopus tropicalis were housed and cared for in our aquatics facility following Yale University Institutional Animal Care and Use Committee and IACUC protocols. Embryos were produced and raised as previously described (del Viso and Khokha, 2012).

3.2. Antisense morpholino knockdown and mRNA injections

Embryos were injected with either a translation blocking morpholino for *agmo* (5′ GGCCTGTGAAACCCCCCATGTTTGC 3′ from Gene tools) or mRNA at the one cell stage. 1 ng of MO was injected for the low dose experiments and 2 ng was injected for the higher dose gastrulation experiments. The MO was traced with mini ruby dye (Invitrogen). Capped mRNAs for injection were produced by *in vitro* transcription of GFP-beta-catenin (Addgene #16839), AGMO-GFP, AGMO-H136A-GFP, and AGMO-H149A-GFP (generous gifts from the Werner lab, Innsbruck Medical University, Austria) using the mMessage machine kit (Ambion) following the manufacturer's instructions.

3.3. Cardiac looping assay

Xenopus embryos were injected with MO at the 1 or 2 cell stages and raised to stage 45 in order to examine cardiac looping. Embryos were treated with benzocaine and scored ventrally using a Zeiss Discovery V8 Stereo-microscope. A D-loop was defined as the outflow tract going to the right, an L-loop was to the left, and an A-loop was midline. The combined results of six independent experiments are shown in Fig. 1.

3.4. Whole mount in-situ hybridization

In-situ hybridization was performed as previously described (Khokha et al., 2002). Digoxingenin labeled anti-sense RNA probes for *coco*; TEgg007d24, *xnr1*; TGas124h10, *gdf3*; Tgas137g21 and *pitx2c*; TNeu083k20 were made using a T7 mMessage mMachine transcription kit (Ambion #AM1344). The expression data shown in Fig. 1 represents the combined results of three independent experimental repeats for *pitx2c,* five repeats of *coco*, six repeats of *xnr1* and 3 repeats of *gdf3*.

3.5. Secondary axis assay

Embryos were injected with AGMO-GFP, AGMO-H136A-GFP, or AGMO-H149A-GFP at the one cell stage. Embryos were collected at stage 22 and scored for the presence of either a single or double axis.

3.6. Protein extraction, fractionation and western blotting

Western blots were carried out as previously described (Griffin et al., 2015). Embryos were collected at stages 9–12, and homogenized in 1x RIPA buffer (100 μL per 10 embryos). Lysates were centrifuged at 4 degrees for 20 minutes. The middle layer was removed, and this was centrifuged for 10 minutes more at 4 degrees in order to separate out lipids. The middle layer of lysate was once again removed and retained for western blotting. Nuclear/cytoplasmic fraction was performed exactly as described in (Griffin et al., 2018).

Western blots were performed using 4–12% Tris-Bis gels and semi-dry transfer module from Invitrogen according to Invitrogen's established protocols. PVDF membranes were blocked with 5% milk or BSA for 1 hour followed by primary antibody incubation at 4 degrees overnight. Primary antibodies used include anti-Beta-catenin H-102 (Santa Cruz sc-7199) at 1:1000, anti-Gapdh (Ambion #4300) at 1:2000. Membranes were then washed in 1x TBST and incubated in secondary for 1 hour at room temperature. Secondary antibodies used included Affinipure Donkey Anti-mouse 1gG (Jackson ImmunoResearch laboratory #715-035-150) at 1:10,000 and Mouse Anti-rabbit IgG (Jackson ImmunoResearch laboratory #211-032-171) at a 1:15,000 concentration. Membranes were then washed in 3 x TBST and developed in a dark room using Amersham ECL Western Blot Detection Reagents (GE Healthcare). Western blots were quantitated using NIH's ImageJ software.

3.7. TOPflash assays

TOPFlash assays were carried out using the Dual Luciferase Reporter Assay System (Promega #E1910). All embryos were injected with 20 pg of Renilla plasmid (as an internal injection control) and with 100 pg of either the TOPFlash plasmid (containing TCF binding sites upstream of a minimal reporter and the firefly luciferase gene) or FOPFlash plasmid (negative control with mutated TCF binding sites) at the one cell stage. Subsets of the TOPFlash plasmid injected embryos were co-injected with 200 pg of GFP-WT-β-catenin, 1 ng *agmo* translation blocking morpholino, or AGMO-GFP. Pools of 10 embryos were collected and lysed in 100 μl passive lysis buffer. Firefly and Renilla luciferase activity were measured using a Promega Glomax luminometer and the Dual Luciferase Reporter Assay System (Promega #E1910).

3.8. Statistical analysis

Embryos were randomly selected from a fertilized population and utilized for injections, scoring or collection. We estimated 20–25 samples per experimental condition were necessary for statistical significance given the magnitude of the changes expected, and sample size is reported for each experiment. Each experiment was performed a minimum of 3 times (the precise number of repeats is indicated in the figure legends). The statistical significance of each experiment in Fig. 1 was examined using a Chi-square test, while experiments in Figs. 2–4 where analyzed using a student's T-test. In all figures and results, statistical significance was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Author contributions

ARD, JNG and MKK conceived and designed the experiments. ARD, DPG, FdV, JNG and AR performed the experiments. ARD, JNG, DPG, FdV and MKK analyzed the data. JNG and MKK wrote the paper, which all authors approved.

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