Temperature-dependent vitamin D signaling regulates developmental trajectory associated with diapause in an annual killifish

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Edited by David M. Kingsley, Stanford University, Stanford, CA, and approved October 10, 2018 (received for review March 16, 2018)

The mechanisms that integrate environmental signals into developmental programs remain largely uncharacterized. Nuclear receptors (NRs) are ligand-regulated transcription factors that orchestrate the expression of complex phenotypes. The vitamin D receptor (VDR) is an NR activated by 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], a hormone derived from 7-dehydrocholesterol (7-DHC). VDR signaling is best known for regulating calcium homeostasis in mammals, but recent evidence suggests a diversity of uncharacterized roles. In response to incubation temperature, embryos of the annual killifish *Austrofundulus limnaeus* can develop along two alternative trajectories: active development and diapause. These trajectories diverge early in development, from a biochemical, morphological, and physiological perspective. We manipulated incubation temperature to induce the two trajectories and profiled changes in gene expression using RNA sequencing and weighted gene coexpression network analysis. We report that transcripts involved in 1,25$(OH)_2D_3$ synthesis and signaling are expressed in a trajectory-specific manner. Furthermore, exposure of embryos to vitamin D_3 analogs and Δ4-dafachronic acid directs continuous development under diapause-inducing conditions. Conversely, blocking synthesis of 1,25$(OH)_2D_3$ induces diapause in *A. limnaeus* and a diapause-like state in zebrafish, suggesting vitamin D signaling is critical for normal vertebrate development. These data support vitamin D signaling as a molecular pathway that can regulate developmental trajectory and metabolic dormancy in a vertebrate. Interestingly, the VDR is homologous to the daf-12 and ecdysone NRs that regulate dormancy in *Caenorhabditis elegans* and *Drosophila*. We suggest that 7-DHC–derived hormones and their associated NRs represent a conserved pathway for the integration of environmental information into developmental programs associated with life history transitions in animals.

nuclear receptors | life history | dormancy | phenotypic plasticity

There is a growing appreciation for the importance of environmental exposures during development in the determination of a wide range of complex phenotypes beyond classic examples of phenotypic plasticity (e.g., fetal programming in mammalian embryos). Despite their profound influence on form and function, the mechanisms by which phenotypes are modified by environmental factors are largely undefined and unexplored.

The annual killifish *Austrofundulus limnaeus* survives in ephemeral ponds by producing stress-tolerant embryos that survive for months encased in drying mud. Embryos survive by entering into three distinct stages of developmental arrest termed diapause I, II, and III (1–3). Diapause is a state of profound metabolic depression and extreme stress tolerance; diapause II embryos can survive for months without access to oxygen or liquid water (2, 4). Entrance into diapause II (diapause from here forward) is the result of an alternative developmental trajectory that is physiologically, morphologically, and biochemically unique compared with "escape" embryos that develop continuously until hatching or entrance into diapause III (5, 6). Developmental phenotype can be influenced by maternal provisioning and by the embryonic environment. Exposure to increased temperature (30 °C compared with 20 °C to 25 °C) and light promotes the escape trajectory (6, 7). Biochemically, the trajectories diverge early in development, before the formation of the embryonic axis, as evidenced by reduced insulin-like growth factor (IGF) expression in diapause-bound embryos (8). A critical window of development where increased temperature causes irreversible commitment to the escape trajectory occurs during early somitogenesis in embryos possessing 10 to 20 pairs of somites. Importantly, these temperatures are within the bounds experienced by embryos under field conditions. Thus, embryos of annual killifishes offer an unprecedented opportunity to study genome–environment interactions during vertebrate development in an ecologically and evolutionarily relevant context (1, 9, 10).

Here we report that vitamin D_3 synthesis and signaling promotes active development in fish embryos, and inhibition of vitamin D_3 synthesis leads to developmental arrest. In this paper we describe vitamin D_3 signaling as a pathway that regulates developmental trajectory and metabolic dormancy, and allows for the integration of environmental information into the developmental program.

Results

Key Gene Regulatory Networks Are Unique to Each Developmental Trajectory. General results for the RNA sequencing (RNAseq) analysis are provided in *SI Appendix*, Fig. S1 and Table S1 and

Significance

Here we describe a molecular pathway that regulates dormancy in a vertebrate and highlights a mechanism that integrates environmental cues into a developmental program that has clear ecological and evolutionary significance. Further, we provide compelling evidence that vitamin D signaling is critical for normal vertebrate development and can induce a diapause-like arrest in embryos of zebrafish. The vitamin D receptor is homologous to nuclear receptors (NRs) that regulate dormancy in *Caenorhabditis elegans* and *Drosophila*. This conservation of function suggests a conserved role for hormones derived from 7-DHC and their associated NRs in the control of metabolic dormancy and major life history transitions in animals.

Author contributions: A.L.T.R., E.M.D., J.T.W., and J.E.P. designed research; A.L.T.R., E.M.D., M.M.C., J.T.W., and J.E.P. performed research; A.L.T.R., E.M.D., M.M.C., J.T.W., and J.E.P. analyzed data; and A.L.T.R. and J.E.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The original data sets are available via the National Center for Biotechnology Information's (NCBI) Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra (accession nos. SRX1032655, SRX1032657, SRX1032658, SRX1032664–SRX1032702; BioProject ID PRJNA272154).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1804590115/-/DCSupplemental.

Published online November 16, 2018.

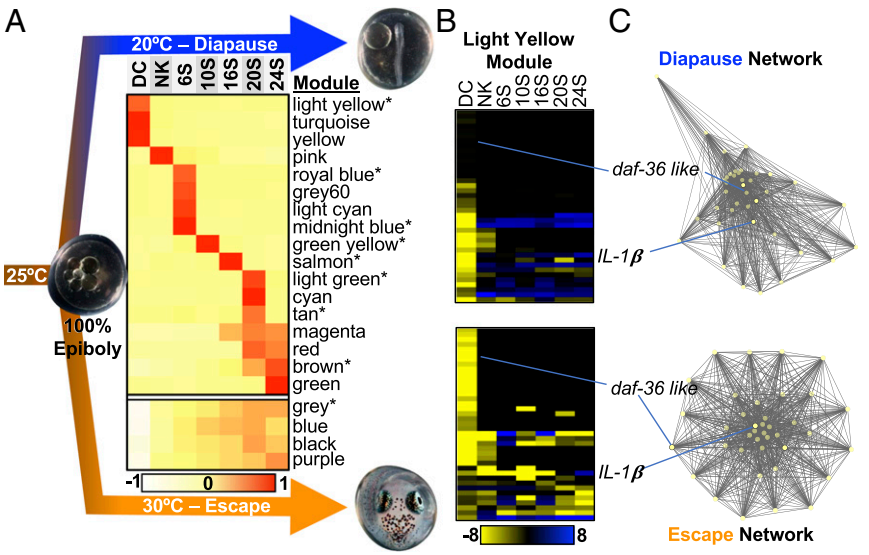

Fig. 1. WGCNA of RNAseq data in embryos of *A. limnaeus* developing along alternative developmental trajectories. To identify trajectory-specific gene expression patterns, embryos were reared 20 °C or 30 °C to induce the diapause or escape trajectories, respectively. Embryos (n = 3 groups of 20 embryos) were sampled at six morphological stages that bracket the temperature-sensitive window where developmental trajectory is determined: DC, dispersed cell stage; NK, neural keel stage; 6S, 6-somite embryo; 10S, 10-somite embryo; 16S, 16-somite embryo; 20S, 20-somite embryo; 24S, 24-somite embryo. (*A*) Heat map of module eigengene (based on weighted average of module gene expression) correlations to each stage of development along the diapause trajectory (positive is red, and negative is white). (*Upper*) Modules are significantly associated ($r > 0.50$; P value $< 10^{-2}$) with a developmental stage. (*Lower*) Modules are not significantly associated. Modules with asterisks are not preserved between diapause and escape networks ($Z_{summary}$ statistic < 10). (*B*) Heat maps of expression level reported as fragments per kilobase of transcript per million mapped reads (FPKM) for the light yellow module that contains a daf-36–like cholesterol desaturase (LOC106533739) and IL-1β as hub genes. (*C*) Cytoscape (version 3.5.1) visualization of the light yellow module, with yellow circles representing the genes and the connecting lines representing similarity strengths (thickness) (44), based on topological overlap determined by WGCNA.

Datasets S1 and S2. Weighted gene coexpression network analysis (WGCNA) was used to construct 21 gene coexpression modules from the more than 16,000 transcripts expressed in embryos developing along the diapause trajectory (11) (*SI Appendix*, Fig. S2). Network topologies of 12 modules were preserved in the escape trajectory and likely represent conserved transcriptional pathways critical to normal development (11, 12). We focused on the remaining nine modules that represent diapause- and escape-specific networks. Based on a measure of intramodular connectivity (kME), we identified 3,034 intramodular "hub" genes (kME > 0.90, $P < 10^{-8}$; Dataset S3) in the diapause trajectory as a means to discover key regulators of each module (11).

Notably, within 24 h of temperature transfer [dispersed cell (DC) stage embryos], the transcriptomes of the two trajectories diverged. One of the three WGCNA modules associated with this stage (light yellow) was not preserved in the two trajectories ($Z_{summary}$ = 6.2). This module consists of 39 low-abundance transcripts (Fig. 1*B*) with two hub genes of particular interest: interleukin-1β (IL-1β, kME = 0.97, $P < 10^{-12}$) and a daf-36–like cholesterol desaturase (kME = 0.98, $P < 10^{-14}$). These transcripts are escape trajectory-specific with low or undetectable expression in diapause-bound embryos, and there is a dramatic restructuring of the coexpression networks between the two trajectories (Fig. 1*C*). IL-1β is a potent cytokine with unknown roles in fish development (13), but can regulate IGF signaling in mammals (14). The daf-36–like transcript encodes an enzyme that catalyzes the production of 7-dehydrocholesterol (7-DHC), the first step in the synthesis of dafachronic acid hormones that regulate entrance into dauer dormancy in *Caenorhabditis elegans* through the daf-12 nuclear receptor (NR) (15).

Many Dauer Pathway Genes Are Differentially Expressed in Escape- and Diapause-Bound Embryos. There are four signaling pathways that are known to regulate dauer dormancy in *C. elegans* (16), and key genes in each of these pathways are differentially expressed in *A. limnaeus* embryos developing along the two alternate trajectories (*SI Appendix*, Fig. S3). Some of the earliest differences in expression are observed in homologs to genes involved in sensory ciliary signaling, insulin-like signaling, and daf-12 hormonal signaling. Given the identification of the daf-36–like transcript as a hub gene and possible regulator of phenotype, and the key importance of daf-12 signaling for regulating dauer dormancy in *C. elegans*, we decided to focus further on this pathway.

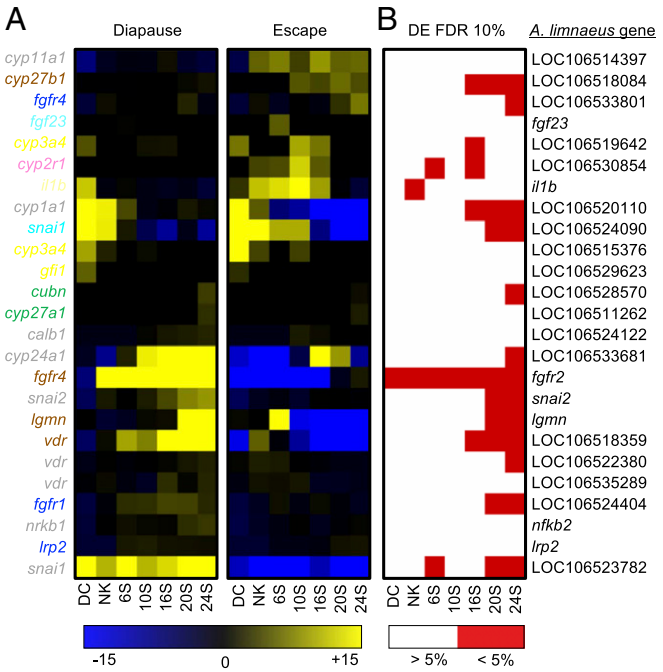

Fig. 2. Vitamin D_3 signaling gene transcripts in *A. limnaeus*. (*A*) Heat maps of expression levels (FPKM) for vitamin D_3 signaling genes in each developmental phenotype. Mammalian homologs are identified by gene symbol on the left. Text color for each gene symbol represents WGCNA module origin. Clustering was accomplished using uncentered Pearson correlation with complete linkage in Gene Cluster 3.0 (45, 46). Heat maps were generated with Java Treeview 1.1.6r4 using median-centered FPKM values (47). (*B*) Heat map showing significant FDR adjusted P values (FDR = 10%, $P < 0.05$, calculated in DESeq2) for transcripts differentially expressed in the two phenotypes; DC, dispersed cell stage; NK, neural keel stage; 6S to 24S indicates the number of somite pairs in each subsequent stage.

Consistent with other vertebrates, the daf-12 homolog in *A. limnaeus* annotates as a vitamin D receptor (VDR) (*SI Appendix*, Fig. S3). Interestingly, IL-1β can induce expression of vitamin D synthetic enzymes in human bone cells (17). Thus, we hypothesized that the vitamin D_3 signaling pathway could be acting to regulate developmental trajectory in *A. limnaeus* in a manner consistent with daf-12 signaling in *C. elegans*.

Expression of Transcripts for Vitamin D_3 Synthesis and Signaling Are Up-Regulated in Escape Embryos. Transcripts for genes that are involved in the synthesis of $1,25(OH)_2D_3$ show trajectory-specific patterns of expression consistent with increased production in escape trajectory embryos (Figs. 2 and 3). The two major cytochrome P450 enzymes known to convert vitamin D_3 to the hormonally active $1,25(OH)_2D_3$ (18) are expressed only in escape trajectory embryos during the critical window when developmental commitment is established (6). Further, levels of $1,25(OH)_2D_3$ are higher in 20- to 24-somite embryos on the escape trajectory compared with those that will enter diapause (Fig. 3).

The hormonal form of vitamin D_3, $1,25(OH)_2D_3$, acts as a potent ligand for the VDR. VDRs are highly conserved across vertebrate taxa, but the role of VDR signaling in development has been only briefly examined (19–22). Consistent with other teleost fishes, there are two VDR paralogs in *A. limnaeus*: VDR-A and VDR-B (23). Two VDR-A genes are expressed at a low and consistent level in both trajectories, while VDR-B exhibits a robust expression with a significant decrease in transcript abundance in escape trajectory embryos with 10 or more pairs of somites (Fig. 2). This divergence in expression coincides with the critical temperature-sensitive window of development when embryos are committed to the escape trajectory, and is consistent with negative feedback regulation of VDR gene expression in

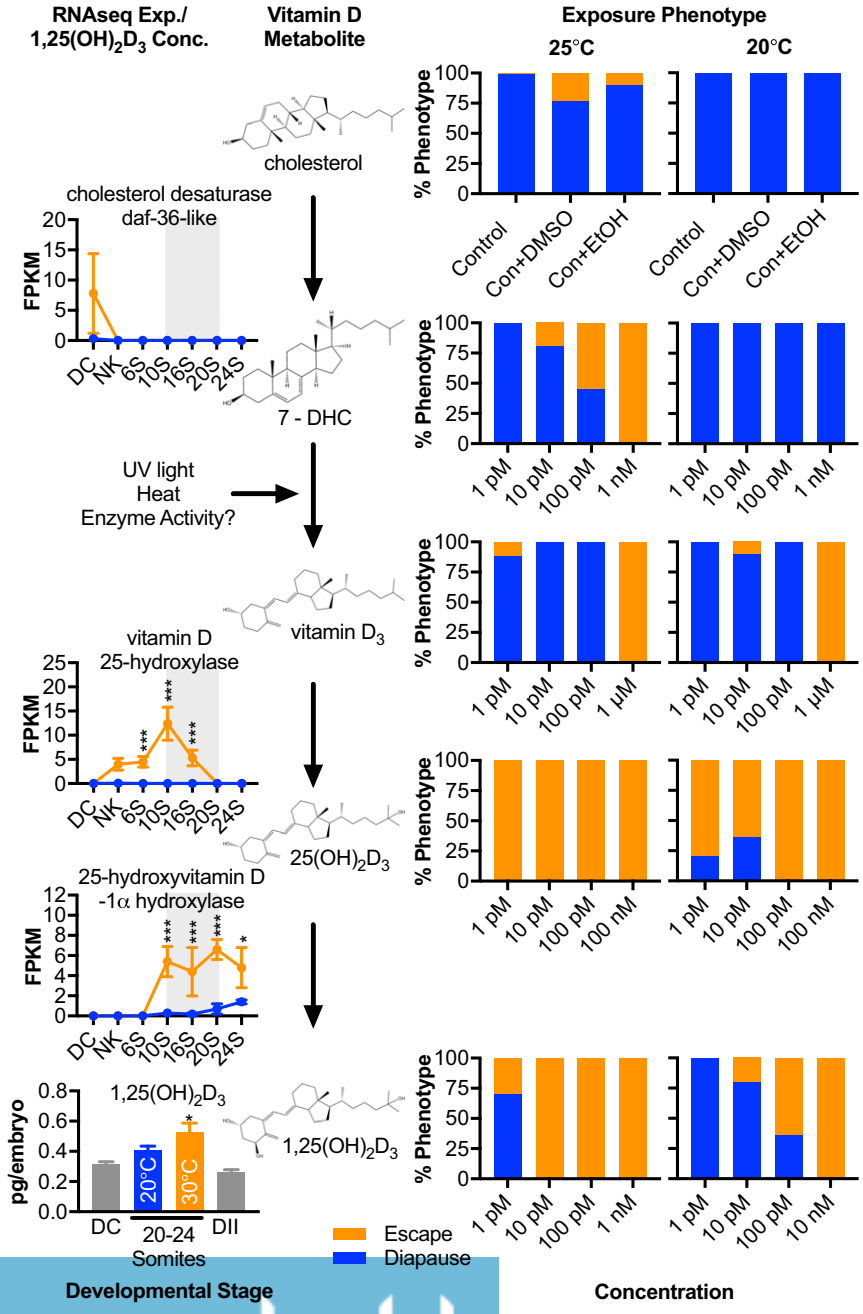

Fig. 3. Vitamin D_3 analogs and their effect on developmental phenotype in embryos of *A. limnaeus*. (*Left*) Line graphs depict the transcript expression level of each enzyme critical for synthesis of active vitamin D_3 (FPKM values ± SEM). Gray shading indicates the critical window of development where temperature affects developmental trajectory. Asterisks identify differentially expressed genes in the two phenotypes (significant P values < 0.05 with FDR: **P*, 10%; ****P*, 1%). (*Lower Left*) The bar chart depicts levels of $1,25(OH)_2D_3$ in 20- to 24-somite embryos developing at 20 °C or 30 °C (n = 3 groups of 20 embryos). The gray bars are from embryos incubated at 25 °C in the dispersed cell stage (DC), or in diapause (DII). (*Middle*) The canonical biochemical pathway for synthesis of $1,25(OH)_2D_3$ is depicted. (*Right*) The bar graphs depict the effect of exogenous treatment of embryos with precursors or analogs of vitamin D_3 on the developmental phenotype of embryos incubated under conditions that should favor entrance into diapause. Embryos were incubated in solution with 1% DMSO or 1% EtOH, depending on the analog used. Control embryos were incubated in embryo medium, or with 1% DMSO, or 1% EtOH. Embryos were scored as diapause or escape by 38 dpf using morphological and physiological criteria as previously described (7); 12 to 24 embryos were used for each treatment and were incubated in individual wells of a 96-well plate with 150 μL of media that was changed daily. Each treatment has been validated several times with different batches of embryos.

mammals and with patterns of daf-12 gene expression in *C. elegans* (24).

The genomic activity of VDR is mediated by the binding of VDR homodimers, or heterodimers with a variety of other NRs, to vitamin D_3 response elements in gene promoters. A rich diversity of NRs is expressed during early development in *A. limnaeus*, many of which exhibit trajectory-specific expression patterns (*SI Appendix*, Fig. S4). The most common partner for VDR is the retinoid X receptor (25). However, partnering with thyroid hormone receptor can alter the DNA binding affinity and transcriptional activation efficiency of VDR (26). The VDR can also partner with a number of transcriptional repressors and chromatin-modifying complexes that determine complex context-dependent transcriptional networks (25). Thus, we hypothesize that VDR signaling regulates developmental trajectory of *A. limnaeus* embryos and orchestrates the expression of the complex phenotype associated with diapause.

Exogenous Vitamin D_3 Analogs Induce the Escape Trajectory Under Environmental Conditions That Favor Induction of Diapause. All analogs of vitamin D_3 were able to induce the escape trajectory under conditions that should favor diapause (20 °C to 25 °C), with 25-hydroxyvitamin D_3 [25(OH)D_3] and 1,25$(OH)_2D_3$ exhibiting potency in the picomolar range (Fig. 3). Surprisingly, 25(OH)D_3 is the more potent form of vitamin D_3. This may be due to higher expression of the 1,25$(OH)_2D_3$-24-hydroxylase enzyme in diapause trajectory embryos (Fig. 2) that inactivates 1,25$(OH)_2D_3$, and/or the need for local conversion to 1,25$(OH)_2D_3$ for maximum potency. Interestingly, 7-DHC was only able to induce the escape trajectory at 25 °C, which may indicate that 20 °C is below the threshold of thermal activity required to convert 7-DHC to vitamin D_3. These data, combined with the higher levels of 1,25$(OH)_2D_3$ in escape trajectory embryos, provide strong evidence of a biosynthetic pathway for synthesis of 1,25$(OH)_2D_3$ in embryos of *A. limnaeus* that promotes active development in a manner parallel to the action of dafachronic acids in *C. elegans*.

In humans, conversion of 7-DHC to vitamin D_3 requires photochemical conversion via UVB radiation and a heat-dependent isomerization (27). However, synthesis of 1,25$(OH)_2D_3$ is possible in a number of species without exposure to UVB, and blue light can induce production of vitamin D_3 in the skin of rainbow trout (28, 29). Further, in poikilotherms, thermal conversion of vitamin D_3 from previtamin D_3 is over a thousand times more efficient in cellular membranes than in solution (30), consistent with a role for the cellular microenvironment, or a possible enzymatic mechanism to facilitate conversion. Environmental activation of vitamin D_3 synthesis is consistent with laboratory data where short (24 h to 48 h) exposures to light and elevated temperature are known to induce the escape trajectory (*SI Appendix*, Fig. S5), and release embryos from dormancy in diapause (4). The potential for solar radiation and increased temperature to induce vitamin D_3 synthesis presents an opportunity for environmental factors to directly affect developmental trajectory.

Hormones That Promote Active Development in *C. elegans* Can Induce the Escape Trajectory in Embryos of *A. limnaeus*. Exposure of *A. limnaeus* embryos to Δ4-dafachronic acid induces the escape trajectory under conditions that favor entrance into diapause (Fig. 4). This effect is likely mediated through the VDR, given the strikingly similar developmental phenotypes observed between embryos treated with dafachronic acid and 1,25$(OH)_2D_3$. Further, we find no evidence that known agonists of closely related NRs (pregnane X, farnesoid X, and liver X) can induce the escape phenotype (*SI Appendix*, Table S2). Dafachronic acids are the major ligands for the daf-12 NR in *C. elegans* that promote active development and prevent entrance into dauer dormancy (15). These data suggest a high degree of functional homology between daf-12 and the vertebrate VDR, and a more general role for hormones derived from 7-DHC in the regulation of life history characters across a deep evolutionary divergence.

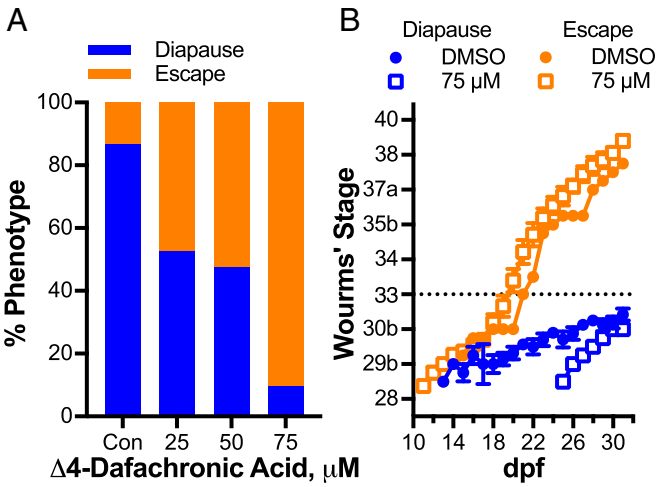

Fig. 4. Δ4-dafachronic acid induces the escape trajectory in *A. limnaeus* embryos. (*A*) Δ4-dafachronic acid causes embryos of *A. limnaeus* to follow the escape trajectory under conditions (25 °C) that should favor entrance into diapause. (*B*) Developmental rate and pattern of escape embryos induced by dafachronic acids are indistinguishable from normal escape embryos. However, embryos that follow the diapause trajectory despite treatment with dafachronic acids have a delayed development. Symbols are means ± SEM (n = 12 to 24). Dashed line at Wourms' stage 33 is diapause II. dpf, days postfertilization.

Blocking Synthesis of 1,25$(OH)_2D_3$ Induces Diapause in *A. limnaeus* and a Diapause-Like State in Zebrafish. Dafadine A, a potent inhibitor of the daf-9 cytochrome P450 enzyme and its mammalian ortholog CYP27A1 (vitamin D_3 25-hydroxylase), can block synthesis of 1,25$(OH)_2D_3$ (31). When *A. limnaeus* embryos are incubated at 30 °C in the presence of 10 μM to 25 μM dafadine A, most embryos enter diapause (Fig. 5). Thus, inhibition of 1,25$(OH)_2D_3$ synthesis results in embryos entering diapause under conditions that should result in active development. Importantly, this phenotype can be rescued by the addition of 100 pM 1,25$(OH)_2D_3$ (Fig. 5*B*). These data provide strong evidence that synthesis of 1,25$(OH)_2D_3$ controls developmental phenotype in embryos of *A. limnaeus*.

Surprisingly, incubation of zebrafish embryos in dafadine A resulted in a developmental and physiological arrest that is strikingly similar to diapause II in *A. limnaeus* (Fig. 6 and *SI Appendix*, Table S3). Recovery from dafadine exposure leads to a resumption of normal morphological and physiological development in most (60 to 100%) embryos (Fig. 6 and *SI Appendix*, Fig. S6). In contrast to embryos of *A. limnaeus*, this phenotype cannot be rescued by addition of up to 25 μM 1,25$(OH)_2D_3$. The reason for this difference is not known, but may be due to zebrafish expressing both VDR-A and B isoforms (32), while *A. limnaeus* expresses mainly the VDR-B isoform (Fig. 2). In fish, the two isoforms have been found to be functionally different in their response and sensitivity to 1,25$(OH)_2D_3$ (32, 33). It is also possible that dafadine A may interact directly with the VDR-A isoform and block the action of 1,25$(OH)_2D_3$, or induce expression of genes that deactivate 1,25$(OH)_2D_3$. Additional studies are needed to explore these possibilities. Despite species-specific differences, it appears that vitamin D_3 signaling promotes active vertebrate development and suggests that diapause-like states may be induced in other fishes through alteration of vitamin D_3 signaling.

VDR and Ciliary Signaling May Regulate Downstream Pathways That Define Developmental Phenotype. To explore the possible transcriptomic effects of VDR signaling, we interrogated the top hub genes associated

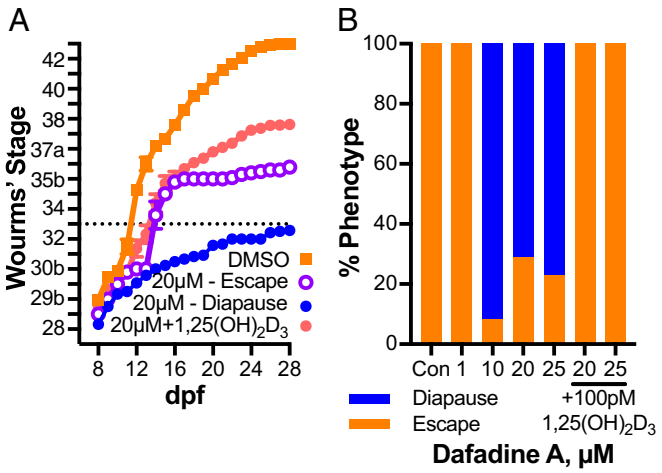

Fig. 5. Dafadine A induces diapause in *A. limnaeus* embryos. (*A*) Dafadine A causes embryos of *A. limnaeus* to enter diapause under conditions (30 °C) that should favor active development. (*B*) The effect of dafadine A on developmental trajectory can be reversed by addition of 100 pM $1,25(OH)_2D_3$, although developmental rate is affected. Symbols are means ± SEM (n = 12 to 24). Dashed line at Wourms' stage 33 is diapause II. dpf, days postfertilization.

with the five WGCNA modules that were not preserved in the two trajectories and were correlated with embryonic stages after the temperature-sensitive window of commitment (identified as salmon, light green, tan, brown, and gray in Fig. 1*A*). Two of these modules can be directly linked to VDR signaling and contain a number of transcripts that are relevant to stress tolerance (salmon and brown), while two others (light green and gray) have hub genes that may play critical roles in the regulation of ciliary signaling (Dataset S3). For a more in-depth discussion of these modules and their potential importance in the determination of the diapause phenotype, please see *SI Appendix*.

Only a Small Number of Stress-Responsive Genes Are Up-Regulated in Diapause-Bound Embryos. Increased and sometimes extreme stress tolerance is an unexplained correlate of metabolic dormancy across all domains of life. Surprisingly, there is not an overall robust transcriptional "stress response" in embryos destined to enter diapause. In fact, for many stress-responsive genes, especially those encoding for heat shock chaperones and antioxidant proteins, expression is higher in the escape trajectory embryos (*SI Appendix*, Fig. S7 *C* and *F*). One exception to this pattern is a small cluster of heat shock proteins and a heat shock factor in the brown module that are strongly up-regulated in diapause-bound embryos (*SI Appendix*, Fig. S7*E*). There are also a small number of antioxidant genes that are much more abundant in diapause-bound embryos (*SI Appendix*, Fig. S7*C*). One clear relationship is a higher expression of genes involved in DNA repair and genome maintenance in diapause-bound embryos (*SI Appendix*, Fig. S7*A*). These expression patterns provide the basis for exploring the molecular underpinnings of stress tolerance associated with diapause.

The Potential Importance of IL-1β in Regulating Developmental Phenotype. Cytokine signaling in early fish development has received very little attention, despite its ubiquitous expression in early zebrafish embryos (13). Please see *SI Appendix* for a brief discussion of a potential role for IL-1β in regulating temperature-dependent phenotypes in *A. limnaeus*.

Discussion

Here we present evidence that VDR signaling regulates developmental trajectory associated with diapause in embryos of *A. limnaeus* in a manner that is consistent with daf-12 regulation of dauer diapause in *C. elegans* (34). We report a role for vitamin D_3 signaling in the determination of life history decisions during vertebrate development, and describe a molecular signaling pathway that regulates developmental trajectory and entrance into metabolic dormancy. Perhaps the most significant implication of this work is the discovery of a conserved function for NRs in the regulation of metabolic dormancy in animals, and the potential to induce diapause-like states in species that do not naturally arrest development. Further, gene expression data in *Xenopus* and Atlantic salmon suggest an unexplored role for VDR in amphibian metamorphosis and salmonid smoltification (35, 36), which suggests a potential broader role for vitamin D_3 signaling in major life history transitions in vertebrates.

The VDR is a member of a family of NRs that are related to the ecdysone receptor in *Drosophila* and the daf-12 NR in *C. elegans* (37). The importance of these receptors in the regulation of life history decisions in nematodes and arthropods is clearly established, and evidence presented in this study extends this role to vertebrates. It is well understood that environmental induction of insulin/IGF1, TGFβ, and cGMP signaling pathways converge on DAF-12 as the key regulator of developmental phenotype in *C. elegans* (34). In *Drosophila*, ecdysone signaling is known to integrate and transduce environmental signals that regulate adult diapause (38, 39). Interestingly, the synthesis of dafachronic acids, ecdysteroids, and vitamin D_3 all depend on 7-DHC as the initial precursor. This suggests that 7-DHC–dependent hormone synthesis could represent an environmental-specific hormonal signaling pathway across all animals, with hormonal chemistry and NR sequence evolving in a concerted response to each organism's natural environment.

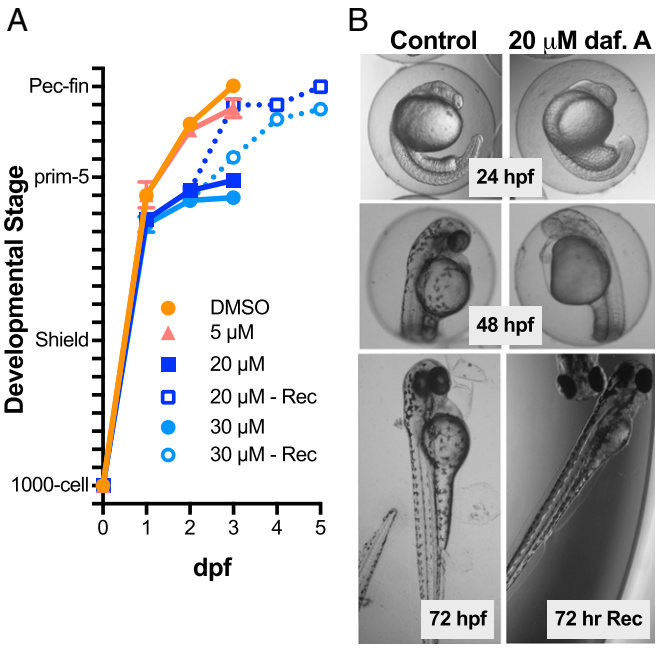

Fig. 6. Dafadine A induces a diapause-like state in zebrafish embryos. (*A*) Dafadine A arrests zebrafish development near the prim-5 stage of development and causes a phenotype that is strikingly similar to that of an *A. limnaeus* diapause embryo. Transfer of embryos treated for 48 h to dafadine-free media leads to a resumption of normal development. Symbols are means ± SEM (n = 3 groups of 20 embryos for normal development and 9 to 12 for recovery). (*B*) Images of zebrafish embryos exposed to control (1% DMSO) or 20 μM dafadine A. Zebrafish embryos were maintained at 28.5 °C and were initially exposed to dafadine A at the 1,000-cell stage. dpf, days postfertilization; hpf, hours postfertilization; Rec, recovery from dafadine A exposure. (Magnification, 40×.)

Materials and Methods

Experimental Design. Adult *A. limnaeus* were housed in the aquatic vertebrate facility at Portland State University (PSU) according to Institutional Animal Care and Use Committee (IACUC)-approved methods established for this species (PSU Protocol #33) (40, 41). Following collection, embryos were maintained for 4 d postfertilization (dpf) at 25 °C in darkness (40). Embryos were transferred to 20 °C and 30 °C at 100% epiboly. Embryos were flash frozen at seven stages as described in the caption of Fig. 1. Zebrafish embryos were obtained from the laboratory of Kim Brown at PSU and used according to PSU IACUC approved protocols (PSU Protocol #33) based on established optimal rearing conditions for zebrafish (42).

Transcriptome and WGCNA Analysis. RNA extraction, sequencing, and processing were performed as described previously (8). Gene counts were normalized as fragments per kilobase of transcript per million mapped reads (FPKM). Count matrices (all genes and sample libraries) were filtered for at least one normalized count across all replicates. Differential gene expression was determined between diapause and escape samples at each stage using DESeq2 in R Bioconductor (43). Significance was determined using a Benjamini–Hochberg false discovery rate (FDR) adjusted P value < 0.10. Coexpression networks were constructed for each trajectory using the WGCNA R software package's default parameters (11). The accession identity of each NCBI biosample used in this study can be found in *SI Appendix*, Table S1.

Vitamin D_3 Analogs, Dafadine A Treatments, and Measurement of $1,25(OH)_2D_3$. Killifish embryos were maintained at 25 °C with minimal light until 4 dpf, when embryos were transferred to experimental conditions. Chemicals were from Sigma-Aldrich (7-DHC, vitamin D_3, dafadine A), Selleck Chemicals [$25(OH)D_3$ and $1,25(OH)_2D_3$] or Cayman Chemicals (Δ4-dafachronic acid). Embryos were monitored for development and staged under dim yellow light using a Leica model DMIRB inverted compound microscope (1, 42). Levels of free $1,25(OH)_2D_3$ were measured using a calcitriol ELISA according to the manufacturer's instructions (Aviva Systems Biology).

ACKNOWLEDGMENTS. We thank Will Patterson for his help supporting the bioinformatics analyses and Andrew McCracken for collecting the data detailing the effects of light and heat on developmental trajectory presented in *SI Appendix*, Fig. S4. This work was supported by National Science Foundation Grant IOS 1354549 (to J.E.P.).

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