### **Article**

## Transcription in Pronuclei and One- to Four-Cell Embryos Drives Early Development in a Nematode

Jianbin Wang,<sup>1,2</sup> Julianne Garrey,<sup>1,2</sup> and Richard E. Davis<sup>1,\*</sup> <sup>1</sup>Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Aurora, CO 80045, USA

### Summary

**Background:** A long-standing view of development is that transcription is silenced in the oocyte until early divisions in the embryo. The point at which major transcription is reactivated varies between organisms, but is usually after the two-cell stage. However, this model may not be universal.

Results: We used RNA-seq and exploited the protracted development of the parasitic nematode Ascaris suum to provide a comprehensive time course of mRNA expression, degradation, and translation during early development. Surprisingly, we find that  $\sim$ 4,000 genes are transcribed prior to pronuclear fusion and in the one- to four-cell embryos. Intriguingly, we do not detect maternal contribution of many orthologs of maternal C. elegans mRNAs, but instead find that these are newly transcribed in the A. suum zygote prior to pronuclear fusion. Ribosome profiling demonstrates that, in general, early embryonic mRNAs are not stored for subsequent translation, but are directly translated after their synthesis. The role of maternally contributed and zygotically transcribed genes differs between the nematodes A. suum and C. elegans despite the fact that the two nematodes appear to exhibit highly similar morphological patterns during early development.

**Conclusions:** Our study indicates that major transcription can occur immediately after fertilization and prior to pronuclear fusion in metazoa, suggesting that newly transcribed genes appear to drive *A. suum* early development. Furthermore, the mechanisms used for controlling the timing of the expression of key conserved genes has been altered between the two nematodes, illustrating significant plasticity in the regulatory networks that play important roles in developmental outcomes in nematodes.

### Introduction

The transition from the oocyte to embryo development involves transcriptional silencing followed by reactivation of transcription during early cell division, known as zygotic genome, or gene activation. During this period of transcriptional silence, regulation of gene expression is considered to be primarily posttranscriptional, relying on the deposition of maternal mRNAs and proteins from the oocyte [1, 2]. The timing of transcriptional reactivation varies between organisms and has been called the maternal-to-zygotic transition, a period in which maternal mRNAs are degraded and development is dependent on transcription from the zygote. While this model of early development is generally accepted, it remains

<sup>2</sup>These authors contributed equally to this work \*Correspondence: richard.davis@ucdenver.edu to be determined whether transcription is silenced during this period in all organisms.

The maternal-to-zygotic transition has been characterized in many animals, including mice, frogs, fish, fruit flies, sea urchins, and nematodes [1, 2]. Zygotic genome activation is often associated first with a minor wave of transcription, followed by a major wave of transcription. In vertebrates, the first wave of transcription begins as early as the one-cell stage in mice, whereas it occurs at the ~246-cell stage in frogs. The second major wave of transcription in mice and frogs occurs at the two-cell and ~4,000-cell stage, respectively. In invertebrates, the first wave of transcription occurs as early as the one-cell stage in sea urchins and as late as the 64-cell stage in fruit flies. Transcription has been reported to occur in the male pronucleus in zygotes of both sea urchins and mice, but this appears to be limited to only one or a few genes [1, 2]. Thus, in general, major transcription in animals does not occur prior to fusion of male and female pronuclei in the zygote.

The parasitic worm *Ascaris* is a nematode particularly amenable for the analysis of early development. Studies on *Ascaris* have contributed to our understanding of key processes in biology, including the first determinate lineage, the centrosome cycle, meiosis, mitosis, and the continuity of chromosomes [3–7]. Older studies suggested that some transcription might occur in *Ascaris suum* zygotes prior to pronuclear fusion during their maturation in the uterus [8, 9]. In support of this, we recently observed that *A. suum* micro-RNAs are transcribed during the maturation of the zygote as it passes through the uterus [10].

The prevailing view in metazoa is that significant RNA polymerase II (pol II) transcription is absent in zygotes and does not occur until after cleavage initiates [2, 11]. Here, we exploited the protracted and synchronous early development of A. suum to obtain samples that include the extended maturation of the zygote prior to pronuclear fusion and the first embryonic divisions. We used RNA sequencing (RNA-seq) to examine mRNA dynamics during these stages to define and characterize maternal mRNAs, mRNA transcription in zygotes prior to pronuclear fusion and in early embryos, and the maternal-to-zygotic transition. Our study provides a comprehensive analysis of mRNA dynamics during very early stages of development. In addition, it provides a unique molecular and comparative perspective of development between two nematodes, A. suum and C. elegans, that exhibit striking similarity in cell lineage and early development, yet diverged ~400 million years ago [12, 13]. Surprisingly, we find that extensive A. suum mRNA transcription occurs during zygote maturation prior to pronuclear fusion and in the one- to four-cell embryo. Thus, major transcription can start at a stage much earlier than previously thought in metazoa, prior to pronuclear fusion, and before what has historically been considered the time of reactivation of nematode transcription [14-17]. Our data suggest that ongoing transcription, rather than maternal mRNA deposition, drives A. suum early development. These data open the question as to whether significant early transcription also occurs in other organisms during these early periods of development. Our data also indicate that the expression of



conserved and key groups of genes required for early development in related organisms can be rewired to be regulated either by transcriptional or posttranscriptional mechanisms.

### Results

### Ascaris suum Early Development

The unique biology of A. suum allows us to collect significant amounts of oocytes, zygotes, and discrete stages of early embryos. In particular, the protracted development enables sampling of mRNA levels during zygote maturation (prior to pronuclear fusion) and very early development (Figure 1A). A comparison of RNAs present in mature oocytes ready for fertilization and zygotes as they mature in the uterus enabled us to distinguish maternally contributed mRNAs, characterize their degradation, and identify newly transcribed RNAs in zygotes. As the oocytes (arrested in prophase I of meiosis) pass through the seminal receptacle (spermatheca in C. elegans) and enter the uterus, they are fertilized (Figure 1A). After fertilization, the eggs become impermeable due to the formation of a complex inner layer and an outer chitinous shell and then undergo meiosis I and II as in C. elegans. The zygote, without pronuclear fusion or cell division, moves through 18-25 cm of the uterus over a 12–24 hr period (Figure 1A). During this time, the zygote matures, and this maturation period is required for subsequent development [18]. Importantly, the two pronuclei remain separated during zygote maturation in the uterus. The long uterus allows for the isolation of zygotes from different regions representing different stages of zygote maturation (zygote1-zygote4; see Figure 1A). Zygote4 eggs (0 hr, eggs released from the uterus) will not develop until the appropriate environmental conditions are encountered upon passing from the pig host. These environmental conditions can be reproduced in the lab, allowing for embryo development and subsequent collection of synchronously staged embryos (see Figure 1A). After 24 hr of development at 30°C, the two pronuclei fuse, and the first division occurs at around 36 hr [19]. Subsequent cell divisions occur every  $\sim$  13 hr on average, and development of the L1 and L2 occurs over an extended period of 10 and 21 days, respectively.

# A. suum Early Developmental mRNA Expression Patterns and Differentially Expressed Genes

RNA-seq data were generated from mature oocytes ready for fertilization and 12 discrete stages of *A. suum* development (Figure 1A). We defined mRNA levels in reads per kilobase of mRNA per million reads (RPKM). Of the 15,399 known and predicted *A. suum* genes [20], 7,626 (49.5%) genes are expressed at significant levels during these 13 stages (Figure 1B; see the Supplemental Experimental Procedures).

mRNA poly(A)-tail lengths can change in early development and contribute to differential gene expression [21]. To ensure that our analysis of mRNAs using poly(A)-selected RNA was not biased by short poly(A)-tail length or changes in poly(A)tail length, we compared RNA-seq data from total RNA to polyA-selected RNA. We also carried out quantitative RT-PCR on random primed total RNA (Figures S1A–S1C and Table S1). These analyses suggested that our mRNA profiles are not biased by the poly(A) selection. We further corroborated the RNA-seq data by comparing the developmental expression profiles of 28 genes using RT-PCR (Figures 1C and S1F and Table S1). The RT-PCR results strongly support the RNA-seq data, with 21 (75%) genes demonstrating PCR expression profiles similar to those observed by RNA-seq. The RNA-seq data from five stages were each compared to a corresponding biological replicate used for polysome analysis (RNA-seq data on polysome fractions; see below). An average correlation of R = 0.923 was observed between these replicates (Figure S1D). Furthermore, the expression patterns for 81.5% of the genes are consistent across these five stages (Figure S1E). Although we only have biological replicates for five developmental stages, these five stages all show very high level of consistency, even after a different sample processing procedure (polysome fractionation). Overall, these data illustrate that the RNA-seq expression patterns we obtained are reproducible and unbiased.

The mRNA expression patterns from 7,626 genes were organized into four "supergroups" (A-D) and their groups (1-12) (Figures 1B and S2A-S2C and Table S2). Supergroup A consists of 1,177 genes whose mRNAs are present at the higher levels in oocytes than at any other stage. These maternal mRNAs are rapidly degraded after fertilization. Some of these maternal genes are then transcribed again in zygotes (zygote4) prior to pronuclear fusion, though most are gone before the four-cell stage (Figure 1B, insert). Supergroup B consists of 1,662 genes that are specifically transcribed and expressed in zygotes prior to pronuclear fusion (zygote1zygote4) and degraded before the ten-cell stage (see Figure 1B, insert). Supergroup C consists of 2,962 genes that are transcribed prior to pronuclear fusion, in one- to four-cell embryos, and in 16- to 256-cell embryos (Figure 1B). Supergroup D corresponds to 1,825 genes that are transcribed in the late embryo, during larval development, and in the larvae (Figure 1B). Gene lists for all the groups, their expression levels, and biological functions are available in the Supplemental Information (Figure S2 and Table S2). Further analysis of these gene expression patterns suggested that there were five major developmental transitions (Figure 1D and S1G, Table S2, and the Supplemental Experimental Procedures), including the oocyte to zygote, zygote to early embryo, early to late embryo, late embryo to L1, and L1 to L2 larval stage transitions. Overall, these data demonstrate dynamic expression patterns of mRNA transcription and degradation during A. suum development.

The mRNA expression data demonstrate that gene transcription starts right after fertilization, prior to pronuclear fusion, and remains active through early embryo cleavage. Concordant with the accumulation of new mRNAs during zygote maturation in the uterus, we observed that a number of components of the pol II transcription machinery and a variety of transcription factor mRNAs were synthesized (Table S3). These include the RNA polymerase II subunits, *rpb1* (*ama-1* in *C. elegans*) and *rbp9*. Overall, the significant number of upregulated genes during this very early time point of development provides evidence for some of the earliest, robust, and diverse transcription observed in metazoa.

Our high-resolution RNA-seq data of oocyte and early zygote stages enabled us to define maternal mRNA contributions and transcription prior to pronuclear fusion. mRNA present at a higher level in oocytes than in zygote1 were considered to be maternally contributed (supergroup A). Significant transcription was observed in the one-cell zygotes prior to pronuclear fusion (supergroup B) and early embryos (supergroup C). Thus, in *A. suum*, many mRNAs that would traditionally be considered to be maternal in *C. elegans* (present in zygote4) are newly transcribed immediately after fertilization and prior to pronuclear fusion (groups 4–6;  $\sim$  2,500 mRNAs). The transcription observed in *A. suum* 



### Figure 1. mRNA Profiles during A. suum Early Development

(A) Female reproductive system and timeline for *A. suum* zygote, embryo, and larval development. Samples used for RNA-seq are illustrated. Zygote maturation (red) occurs in the female uterus as the zygotes move toward the vulva. This process takes  $\sim 18$  hr (12–24 hr) to complete and is required for subsequent development. During this process, the male and female pronuclei remain distinct. Early embryonic development (blue) initiates only after the zygote is released from the worm and pig host into a favorable environment. Incubation of zygote4 (0 hr) embryos at 30°C in high humidity leads to relatively synchronous development ( $\sim 85\%$ ) as illustrated. Early embryonic development includes chromatin diminution occurring between 46 and 96 hr of development (four to 16 cells). *A. suum* L2 development (green) occurs within the egg.

(B) Gene expression profiles during *A. suum* development. Using RNA-seq data from 13 developmental stages, we classified 7,626 expressed genes into four supergroups (A–D) with 12 gene expression patterns represented by lines of different color (see Figure S2 and Table S2). The overall expression profiles (legend continued on next page)



### Figure 2. Immunolocalization of Phosphorylated RNA Polymerase II in Early *A. suum* Embryos

One- to eight-cell embryos were stained with DAPI (blue), and immunohistochemistry was carried out with affinity-purified antibodies against the phosphorylated CTD of RNA polymerase II, where Ser2 indicates elongating polymerase and Ser5 indicates activated, promoter-associated polymerase. One-cell embryos exhibit staining in both pronuclei prior to fusion, indicating that early transcription occurs in both pronuclei. Both somatic and germ cell nuclei have significant pol II staining during early development. Other CTD and H5 (Ser2) antibodies produced similar staining patterns (data not shown).

zygotes prior to pronuclear fusion appears to correspond to zygotic gene activation described in other organisms. In addition to transcription in pronuclei, we also observed significant transcription of diverse mRNAs in one- to four-cell embryos, a developmental stage prior to what is traditionally considered the maternal-to-zygotic transition in nematodes (groups 7 and 8, ~1,500 mRNAs) [2, 14].

Our data also demonstrate several distinct periods of mRNA degradation. One period occurs soon after fertilization and leads to rapid degradation of mRNAs (Figure 1B, see groups 1–3). Thus, in *A. suum*, both maternal mRNA turnover and early zygotic transcription begins immediately after fertilization (Figure 1B). In contrast, this is thought to occur at the ~4- to 32-cell stage in *C. elegans* [14, 22]. Interestingly, we did not identify any *A. suum* maternally contributed RNAs equivalent to the *C. elegans* class I maternal RNAs that remain stable throughout early development [23]. At least four distinct additional groups of mRNAs undergo rapid degradation (Figure 1B, see groups 4–7). This suggests discrete control in the specificity and timing of degradation of these mRNAs. Overall, >3,000 mRNA are degraded during the four- to 64-cell stage in *A. suum*.

### Early Transcription Occurs in Both Pronuclei and in

Germline and Somatic Cells in *A. suum* Early Development We carried out immunolocalization studies for phosphorylated RNA polymerase II to determine whether the transcription that we observed by RNA-seq in zygotes prior to pronuclear fusion was derived from one or both *A. suum* pronuclei. We observed significant nuclear staining with antibodies to the unphosphorylated C-terminal domain (CTD; data not shown) and to the phosphorylated CTD ser-2 and ser-5 epitopes (Figure 2), demonstrating that activated and elongating RNA polymerase II (ser-2-P) were present in both pronuclei. Thus, the transcripts identified by RNA-seq are likely derived from both male and female pronuclei. Furthermore, activated and elongated RNA pol II staining (ser-2-P) was also observed in both germ cell and somatic precursor nuclei in early embryos (Figure 2). This differs from what generally has been observed in *C. elegans*, where the germ cell nuclei are transcriptionally inactive and elongating RNA polymerase II is not observed until at least the four-cell stage of development [24].

### Orthologs of *C. elegans* Maternal mRNAs Are Transcribed in *A. suum* Zygotes and One- to Four-Cell Embryos

In C. elegans, a large number of maternal mRNAs and proteins are contributed to the oocyte [25]. After oocyte formation, C. elegans pol II transcription is generally not thought to occur until about the four-cell stage, and development can continue without transcription as far as at least the 120-cell stage [16, 17]. In A. suum, maturation of the zygotes within the uterus is required for subsequent development [18]. During this period, we identified diverse and significant levels of transcription. We found that the steady-state levels of mRNAs corresponding to A. suum genes orthologous to C. elegans maternal genes were significantly higher in developmental stages after oocytes and that transcription of many of these genes occurred during zygote maturation or in the one- to four-cell embryo (Table 1). These include orthologs of C. elegans car-1, mei-1, pal-1, lin-12/glp-1, gld-1, pie-1/oma/ pos-1-like, and members of the puf and par families [25]. These genes are involved in transcription, signaling, polarity, and RNA regulation and are known to play key roles in C. elegans early development. Overall, these data suggest that many orthologs of C. elegans maternal mRNAs are synthesized in A. suum zygotes prior to pronuclear fusion and in early embryos. A more comprehensive comparison of oogenesis-related genes derived from a C. elegans microarray analysis [26] further indicates that for a large number of C. elegans oogenesis/maternal genes, their orthologs in A. suum are not maternally deposited; instead, they are synthesized when needed after fertilization and during early embryogenesis (see the Supplemental Results).

### A. suum mRNA Translation Is Coupled to Newly Transcribed RNAs Rather than Stored RNAs

During early embryonic development, gene expression is often regulated through differential mRNA translation. We examined

for these 12 groups of genes are illustrated as a standard score (*Z* score), with similar colors (as in Figure 1A) indicating the developmental stages in which the genes are primarily expressed. The main plots show changes in mRNA levels as a function of developmental time (x axis, top, shown in days), while the inserts for supergroups A and B show the mRNA changes as a function of developmental stage (x axis). Each gene group is numbered with the number in brackets indicating how many genes are associated with that group.

<sup>(</sup>C) RT-PCR profiles corroborate the RNA-seq data. RT-PCR was performed for selected transcripts corresponding to different gene expression groups (color coding matches transcripts from different stages: pink for oocyte/maternal, red for 1-cell zygotic, blue for early embryonic and green for late embryonic and larval). A strong correlation between the RNA-seq and RT-PCR data was observed (see Figure S1F and Table S1).

<sup>(</sup>D) Differentially expressed genes at five major developmental transitions in *A. suum*. Differentially regulated genes were defined as described in the Supplemental Experimental Procedures using a cutoff of p < 0.01.

Table 1. Exp	ression of Orth	o sbolou	f C. elegaı	as Matema	al Genes du	ring A. suu	m Develop	ment							
A. suum	C. elegans							One Cell	Two Cell	Two to Four	Ten to 26	32 to 64	$\sim 256$ Cell		
Gene ID	Gene Name	Ovary	Oocyte	Zygote1	Zygote2	Zygote3	Zygote4	(24 hr)	(46 hr)	Cell (64 hr)	Cell (96 hr)	Cell (116 hr)	(7 Days)	С1 Г1	2 C. elegans Function
ASU_00037	lin-12/glp-1	2	ю	9	10	14	26	41	17	150	41	6	6	5	3 signaling
ASU_09625	car-1	244	105	315	264	179	356	409	362	183	136	116	91	58 82	2 RNA regulation
ASU_00286	crb-1	œ	0	2	ო	5	7	17	26	19	16	32	75	38	4 signaling
ASU_03524	mei-1	7	ი	4	9	9	8	6	13	6	10	12	10	8	7 meiosis regulation
ASU_04180	pal-1	5	-	9	7	29	30	39	64	66	39	37	30	~	I transcription
ASU_12687	par-1	59	14	8	15	29	18	17	22	38	14	18	35	46 67	7 cellular polarity
ASU_09475	par-3	Ħ	7	2	ი	9	5	24	29	33	27	20	29	16	5 cellular polarity
ASU_03534	par-4	18	14	9	7	9	10	20	33	18	29	26	25	15 28	3 cellular polarity
ASU_05134	gld-1	15	7	12	14	14	19	26	25	13	ю	-	-	-	I RNA regulation
ASU_06726	gld-1	607	45	212	203	285	208	99	30	14	2	-	-	-	D RNA regulation
ASU_05173	pie-1	126	232	364	501	723	851	869	984	705	142	16	1	о 6	0 transcription and translation
ASU_08918	puf-9	20	95	131	177	424	247	185	199	208	65	85	102	51 56	3 RNA regulation
ASU_11218	puf-12	7	9	7	5	7	13	18	22	20	23	18	17	16	5 RNA regulation
ASU_14399	puf-11	23	10	26	30	39	47	27	19	24	5	9	7	9	9 RNA regulation
Values are in	RPKM. See al	lso Table	s S3.												

Т

the translational regulation of A. suum mRNAs to determine whether they were developmentally and differentially recruited to ribosomes for translation. We assessed whether an mRNA is actively translated based on the distribution of the mRNA within a sucrose gradient after centrifugation. Cytoplasmic extracts of 0-, 1-, 3-, 5-, and 7-day A. suum embryos were subjected to sucrose density fractionation to distinguish mRNAs associated with no (free fraction), a few (middle fraction), or many (polysomes; heavy fraction) ribosomes as a measure of their translation (Figure 3A). As the embryos develop, we observed an increase in rRNA subunits in the heavy fractions of the gradients corresponding to polysomes and increased translation from 1- to 7-day embryos (Figure 3A). Treatment of extracts with EDTA or puromycin led to a significant shift in 80S ribosomes to lighter fractions, suggesting that ribosomes in heavier fractions are associated with active mRNA translation (Figure S3A).

RNA-seq libraries corresponding to 0-, 1-, 3-, 5-, and 7-day A. suum embryo mRNAs isolated from free, middle, and heavy (polysomes) regions of sucrose gradients were prepared and sequenced. To confirm the polysome RNA-seq data, we carried out RT-PCR on >20 mRNAs (Figures 3B and S1F and Table S1), and 95% of the expression patterns were consistent with the polysome RNA-seq data. A global analysis of the data indicates that the majority of RNAs are present in the middle fraction at all stages (Figure S3B). The major differences in mRNA distributions are seen in those mRNAs that move to the heavy fraction or polysomes. Unexpectedly, the majority of maternal/one-cell zygote mRNAs are not primarily associated with heavy fraction polysomes and are not developmentally or differentially recruited to ribosomes. Most of these mRNAs are present in the middle fraction, and for most of the developmental stages, less than 25% are found in the heavy fraction (Figure 3C). In contrast, newly transcribed embryo/larva RNAs are more readily recruited to the heavy fraction (Figures 3C and S3B). On average, zygotic genes have  $\sim 30\%$  or more of their mRNAs in polysomes. These data suggest that maternal/one-cell zygote mRNAs are, in general, not developmentally and differentially recruited to polysomes as has been observed for maternal mRNA translation in other organisms [2, 27]. Furthermore, transcripts made during early development appear to be recruited directly to polysomes and are not made and then deposited for future use. These data along with the RNA-seq transcriptome data indicate that the maternal contribution of RNAs and their translation is limited in A. suum; instead, zygotically transcribed mRNAs are made and directly recruited to polysomes as needed during development.

We observed a cluster of 85 genes actively transcribed throughout early development that are poorly recruited to polysomes in 5-day embryos (Figures S3C and S3D and Table S4). Over 80% of these genes encode ribosomal proteins. We identified a UUGUUGU motif present in the 3' untranslated region (UTR) of the majority of these *A. suum* ribosomal protein mRNAs (Figure S3F). However, further analyzes indicate that this sequence motif alone cannot account for the translational regulation of these *A. suum* ribosomal protein mRNAs (see the Supplemental Results).

We also evaluated the role of microRNAs (miRNAs) in the regulation of gene expression during *A. suum* early development. Overall, our analyses (see Figures S4D–S4F, Table S6, and the Supplemental Text) suggest that mRNA levels during this period of development, in general, are not primarily regulated by miRNAs alone.



Figure 3. mRNA Association with Ribosomes during *A. suum* Embryo Development

(A) RNA profiles from *A. suum* embryo sucrose gradient fractionation. Cytoplasmic extracts from 0-, 1-, 3-, 5-, and 7-day embryos were centrifuged through 10%–60% sucrose gradients and fractions collected. Total RNA from each fraction was separated on denaturing agarose gels and visualized with ethidium bromide staining. The prominent bands correspond to rRNAs and tRNAs as labeled. The fractions were pooled into a free fraction (1–7), middle fraction (8–13), and heavy fraction (14–19) for RNA-seq and RT-PCR analyses.

(B) RT-PCR data support the sucrose gradient RNA-seq results. RT-PCR of genes selected to represent different groups identified are shown with their corresponding color (as in Figure 1C). F, free fraction; M, middle fraction; and H, heavy fraction. A strong correlation between RNA-seq and RT-PCR is observed (see Figure S1F and Table S1).

(C) Newly transcribed embryonic mRNAs are readily translated during embryo development.

RNA-seq was used to analyze the association of transcripts in fractions of sucrose gradients for 1-, 3-, 5-, and 7-day *A. suum* embryos. Box-and-whisker plots illustrate and compare the location of transcripts in heavy fractions of the gradient by comparing the amount of each transcript as a percentage of total transcript level. In these and all subsequent box-and-whisker plots (in Figure S3), the top and bottom ends of each box represent the 75<sup>th</sup> and 25<sup>th</sup> percentile, respectively; the middle line represents the median value; the extended lines illustrate the range (highest and lowest value); and dots represent values beyond the extremes of the whisker. \*p < 0.05, \*\*p < 0.01, t test on the five-number summary. See also Figure S3 and Table S4.

# Conservation of Embryonic Gene Expression Patterns between *A. suum* and *C. elegans*

To further understand nematode development and gene expression conservation across nematode evolution, we compared the developmental transcriptomes of *A. suum* to those from three independent studies on *C. elegans* [14, 22, 28] (Figure 4A). Baugh et al. and Levin et al. used handpicked embryos with precise staging, whereas Hillier et al. used either early or late mixed-stage embryos (Figure 4A). For expression comparison, 5,765 orthologous gene pairs (OrthoMCL 1:1 pairs) from *A. suum* and *C. elegans* were used. Data were converted into standard *Z* scores, and expression correlations were evaluated using Pearson's correlation (see the Supplemental Experimental Procedures).

When all these studies are compared, 4,531 (45%) orthologous gene pairs from A. suum and C. elegans show conserved embryonic expression patterns (see Figure 4B and Table S5). This conservation varies for the 12 groups of genes (see Figure 1B). Taking all three studies into consideration, we see an overlap of 297 gene pairs that have very consistent expression patterns. This number increased to 682 when data from the better-matched, handpicked stages of C. elegans (Figure 4B) were used. These numbers are likely underrepresented as (1) different platforms (microarray and RNA-seq) were used for the analysis, (2) synchronicity of matched stages exhibits some variability, and (3) the analysis is based on stringent matching of 1:1 orthologous pairs. However, this set of 682 overlapping genes likely represents a core set of evolutionarily conserved genes necessary for nematode embryonic development and are mainly from gene groups 2, 5, 7, and 11 (Figure 4C). The genes with the most conserved expression between the two nematodes correspond to group 11 (genes that peak at morphogenesis and L1 larvae). Levin et al. [22] reported a similar phenomenon in a study that compared five closely related Caenorhabditis species, where the conserved expression patterns were described as punctuated developmental milestones. Our data extend these observations in

embryo development (four-cell and onward) between nematodes that have diverged  $\sim$  400 million years ago [12].

### Discussion

Here, we describe the early developmental transcriptomes and polysomal RNAs of the parasitic nematode A. suum. The availability of distinct stages of zygote maturation and early embryo development (Figure 1A) enabled us to generate a comprehensive time course of mRNA expression, degradation, and translation in A. suum. Our study provides a unique, high-resolution analysis of maternal mRNAs, the onset of zygotic genome activation, maternal mRNA degradation, and early transcription and mRNA turnover in an organism with protracted early development. Although a variety of previous transcriptome studies have examined early development in model organisms, to our knowledge, none have comprehensively examined the period following fertilization through the two-cell stage at this temporal resolution. Our RNA-seq data demonstrate that significant transcription occurs during zygote maturation prior to pronuclear fusion and in the oneto four-cell embryos of A. suum. This contrasts with the general view in metazoa that transcription is largely quiescent from the oocyte until at least the two-cell stage. Notably, significant transcription occurs prior to pronuclear fusion. In addition, fewer maternally contributed mRNAs are observed in A. suum than in the related nematode C. elegans, and the timing of the maternal-to-zygotic transition occurs much earlier. We found that many orthologs of maternal C. elegans mRNAs are not maternally contributed in A. suum, but are transcribed during A. suum zygote maturation and in the early embryo. Consistent with this, mRNAs made during A. suum early development, in general, do not appear to be stored for subsequent translation, but are directly translated after their synthesis. Overall, these data suggest that the timing for the maternal-to-zygotic transition in A. suum appears to occur soon after fertilization (Figure 5). Our data provide a new



Figure 4. Conservation of Embryonic Gene Expression Patterns between *A. suum* and *C. elegans* 

(A) Early development gene expression studies from C. elegans compared to A. suum developmental stages examined in the current study. Data from Baugh et al. [14] and Levin et al. [22] are derived from microarray experiments using handpicked embryos. Data from Hillier et al. [28] are part of the modENCODE project that used RNA-seq to characterize C. elegans transcriptomes from various mixed developmental stages. (B) Genes with similar expression patterns in both A. suum and C. elegans. Genes with shared expression patterns between A. suum and the other three studies (with Pearson's correlation coefficient R > 0.3) are indicated. Overall, 682 genes have conserved expression patterns with the two sets of handpicked embryos, and 297 genes are conserved from all three studies (see Table S5).

(C) Conservation of gene expression patterns varies during development. Genes with conserved expression between staged *A. suum* and handpicked *C. elegans* embryos (682, see Figure 4B) were compared to the total number of orthologous pairs from the same data sets. Shown are the percentages of the conserved genes (y axis) as a function of the gene groups (see Figure 1B) with distinct expression profiles (x axis). See also Table S5.

perspective for the roles of maternally contributed and zygotically transcribed genes in metazoan early development. In *A. suum*, the maternal contribution is more limited, and newly transcribed genes appear to drive early development. This predominant use of newly transcribed genes may have been enabled by the protracted early development in *A. suum*.

### mRNA Transcription and Turnover during Early *A. suum* Development

Previous studies in sea urchins and mice suggested that there is some, but very limited, transcription in the paternal pronucleus of the zygote [1, 2]. In A. suum, ~2,500 genes are transcribed prior to pronuclear fusion (Figure 5). This represents an unprecedented level of diverse transcription at this early stage of development. These transcripts are likely derived from both male and female pronuclei, as seen from immunohistochemical staining using antibodies against active, elongating RNA polymerase II (ser-2-P-CTD; Figure 2). At least 222 genes show a significant increase in expression from the oocyte to zygote1 (Table S2), indicating transcription occurs immediately following fertilization. We also observed significant miRNA transcription in these stages in a previous study [10]. A number of mRNAs encoding core transcription machinery or specific transcription factors are upregulated during this period, consistent with their requirement for transcription. Many maternal mRNAs are rapidly degraded after fertilization, and all maternal mRNAs and most zygotically transcribed mRNAs are degraded before the four-cell stage. These data suggest that A. suum zygotic gene activation and maternal mRNA degradation occurs very soon after fertilization and prior to pronuclear fusion (Figure 5). Additional waves of transcription occur during early development (Figures 1B, 1D and 5).

Major mRNA degradation occurs in both *A. suum* and *C. elegans* at the four- to 32-cell stage (Figure 5). In *C. elegans*, this developmental period is considered to be

the maternal-to-zygotic transition. The degraded C. elegans mRNAs appear to be exclusively maternal while in A. suum, they are mixed populations of maternal and zygotic mRNAs. Degradation of mRNAs at this time in A. suum, long after the first onset of A. suum transcription and mRNA degradation prior to pronuclear fusion, may suggest that the high mRNA turnover at these stages may not be associated with the maternal-to-zygotic transition per se, but is an integral phenomenon of this developmental transition in nematodes. In A. suum, programmed DNA elimination (chromatin diminution) also occurs during this same time period [20]. Both of these processes may contribute to the mRNA changes we observed. Additional and significant periods of mRNA degradation also occur during the one- to four-cell stage (Figure 5) and between the L1 and L2 larvae (Figures 1B and 1D), suggesting additional dynamic gene regulation during these developmental stages.

### Altered Gene Expression Patterns in *A. suum* Early

**Development Illustrate Molecular Plasticity in Nematodes** Although early cleavages and cell lineage between A. suum and C. elegans are identical [13], we observed significant differences associated with the timing of early transcription and maternal contributions in early development. Significant A. suum transcription occurs during zygote maturation prior to pronuclear fusion and through four-cell development. In contrast, transcription is thought to be absent or very limited during these periods in C. elegans development (Figure 5). Over 60% the mRNAs associated with early C. elegans development are maternally contributed [14, 23]. These maternal mRNAs are divided into two classes [23]. Levels of class I maternal mRNA (70% of the maternal mRNAs) remain the same throughout early development, while class II maternal mRNAs decrease with development (Figure 5). The presence of this large number of maternal mRNAs is thought to drive many aspects of early C. elegans development.



Figure 5. Comparison of mRNA Expression Patterns during *A. suum* and *C. elegans* Early Development

Note that maternal mRNAs are degraded and transcription initiates immediately after fertilization and continues prior to pronuclear fusion in *A. suum* (shaded area). *A. suum* major zygotic transcription also occurs much earlier than in *C. elegans. A. suum* mRNA levels are derived from Figure 1B, with pink, red, blue, and sky blue representing genes from group 1–3, 4 and 5, 6 and 7, and 8 and 9, respectively. *C. elegans* data are obtained from several studies [2, 14, 23]. See also Figure S4 and Table S6.

We hypothesized that genes normally transcribed and stored maternally in oocytes and important for early C. elegans development, might be synthesized in A. suum during the extended period of zygote maturation in the uterus and one- to four-cell stage of the early embryo. In support of this hypothesis, we found that the orthologs of a variety of well-known C. elegans maternal mRNAs that play key roles in development were transcribed in A. suum either in maturing zygotes or one- to four-cell embryos (Table 1 and Table S3). Interestingly, several studies suggest that transcription in some nematodes (Acrobeloides nanus and Rhabditis sp.) is required during the first cleavages [29, 30]. Exposure to α-amanitin to inhibit pol II transcription stops development of A. nanus and Rhabditis sp. embryos at the five-cell stage, whereas similar treatment of C. elegans one-cell embryos results in 120- to 150-cell embryos. These differences in the requirement for transcription in early cleavages have been suggested to be a function of variation in maternal contributions to the embryos in different nematodes [29]. The duration of the C. elegans cell cycle during early development is on average 15-20 min [29, 31], whereas it is  $\sim$ 150 min in A. nanus [29] and ~13 hr in A. suum [19]. In addition, A. suum zygotes undergo an extended period of maturation in the uterus (~12-24 hr). Rapid development and cell division has been suggested to be potentially incompatible with significant transcription, necessitating a reliance on maternal contributions and posttranscriptional gene expression under those circumstances [32-34]. The very slow development in A. suum may have allowed a shift away from the use and

reliance on maternal contributions for early development and enabled transcriptional programs to drive differential gene expression in early development. This was previously suggested for the nematode *A. nanus* [29], and here we provide molecular evidence to support this hypothesis. Therefore, even though *C. elegans* and *A. suum* share identical early developmental patterns, the regulatory mechanisms used for controlling the timing of the expression of key conserved genes have been altered. Our data illustrate significant plasticity in the regulatory networks that play important roles in developmental outcomes in nematodes.

### Onset of Zygotic Gene Activation prior to Cleavage in A. suum

The transition from the oocyte to development involves transcriptional silencing and then reactivation. Zygotic genome activation in animals has been historically observed during early division. Our data indicate that this can occur in the pronuclei of the zygote and prior to pronuclear fusion. This opens the possibility that this strategy of early gene expression may be used in other animals. It is possible that transcription in some other animals may begin as early as we have observed but has not been detected due to the lack of temporal resolution at the appropriate stages or the depth of the analysis. Recent studies in *Drosophila* suggest some transcription occurs in the preblastoderm, earlier than transcription was previously thought to occur [35–38]. In addition, recent studies in mice and humans have described additional transcription at very early stages of development [39].

# Molecular Conservation in *A. suum* and *C. elegans* Early Development

The nematode C. elegans is a widely used genetic model for general developmental studies and is considered a prototypical nematode. However, nematodes are a diverse phylum and studies on early nematode development have shown significant variations [40, 41]. The early cleavages and cell lineage between A. suum and C. elegans are identical despite  $\sim 400$ million years of evolutionary divergence [12, 13]. A comparison of developmental transcriptomes (four-cell stage to L2) between the two demonstrated that  $\sim 40\%$  of orthologous genes have similar expression patterns, and we found 682 genes whose expression patterns are highly conserved (Figure 4 and Table S5). While our study defines major differences in the timing of expression of genes involved in very early development between A. suum and C. elegans, our comparison also revealed conserved nematode gene expression patterns in early development. A similar observation was recently described by Levin et al. [22] for closely related Caenorhabditis sp. and the conserved patterns were defined as developmental milestones. Our data expand this concept to nematodes as divergent as C. elegans and A. suum, suggesting that the expression of these genes represents a core set of important genes for early nematode development.

### Conclusions

In summary, we show that the maternal mRNA contribution to early nematode development in *A. suum* is significantly less than in *C. elegans*. In *A. suum*, significant transcription initiates immediately after fertilization, continues prior to pronuclear fusion, and proceeds in early cell divisions. Overall, these data indicate that significant levels and diversity of zygotic transcription occurs in *Ascaris* much earlier than previously thought in nematodes and metazoa. While there are numerous developmental processes and orthologous genes conserved between *C. elegans* and *A. suum*, our data indicate that there is also flexibility in the mode of regulation for gene expression and that rewiring of the gene expression programs from a primarily posttranscriptional to a transcriptional mechanism can readily be used to generate the appropriate timing of gene expression necessary for early nematode development.

#### Accession Numbers

The sequencing data described in this work have been deposited to NCBI GEO database under accession number GSE46277.

#### Supplemental Information

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, four figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.11.045.

#### Acknowledgments

We thank Richard Komuniecki, Bruce Bamber, Amanda Korchnak, Vera Hapiak, Jeff Myers, and Routh Packing Co. for their support and hospitality in collecting *A. suum* material; David Bentley for generously providing RNA polymerase II ser-2p and ser-5p CTD antibodies; Scott Kuersten of Epicentre for advice and support using Ribo-Zero and TotalScript Kits; Lee Niswander and Tom Evans for their suggestions and comments on the manuscript; Stella Kratzer and Maggie Balas for help in developing immunohistochemistry methods for *Ascaris* embryos; Adam Wallace for help in constructing UTR libraries; and the UC Medical School core facility for sequencing. This work was supported in part by NIH grants to R.E.D. (NIH AI0149558, AI078087, and AI098421).

Received: October 4, 2013 Revised: November 18, 2013 Accepted: November 21, 2013 Published: December 26, 2013

#### References

- Baroux, C., Autran, D., Gillmor, C.S., Grimanelli, D., and Grossniklaus, U. (2008). The maternal to zygotic transition in animals and plants. Cold Spring Harb. Symp. Quant. Biol. 73, 89–100.
- 2. Tadros, W., and Lipshitz, H.D. (2009). The maternal-to-zygotic transition: a play in two acts. Development *136*, 3033–3042.
- Boveri, T. (1887). Ueber Differenzierung der Zellkerne wahrend der Furchung des Eies von Ascaris megalocephala. Anat. Anz. 2, 688–693.
- Boveri, T. (1892). Uber die Entstehung des Gegensatzes zwischen den Geschlechtszellen und den somatischen Zellen bei Ascaris megalocephala. Sitzungsk. Ges. Morphol. Physiol. 8, 114–125.
- Boveri, T. (1899). Die Entwicklung von Ascaris megalocephala mit besonderer Rucksicht auf die Kernverhaltnisse. In Festschr fur C von Kupffer (Jena: Fisher), pp. 383–430.
- Boveri, T. (1910). Die Potenzen der Ascaris-Blastomeren bei abgeanderter Furchung. Zugleichein Beitrag zur Frage qualitativungleicher Chromosomen-Teilung Festschr für R Hertwig, *Volume III* (Jena: G. Fischer), pp. 131–214.
- 7. Van Beneden, E. (1883). Recherches sur la maturation de l'oeuf et la fecondation, Ascarls megalocephala. Arch. Biol. (Liege) 4, 265–640.
- Kaulenas, M.S., and Fairbairn, D. (1968). RNA metabolism of fertilized Ascaris lumbricoides eggs during uterine development. Exp. Cell Res. 52, 233–251.
- Spicher, A., Etter, A., Bernard, V., Tobler, H., and Müller, F. (1994). Extremely stable transcripts may compensate for the elimination of the gene fert-1 from all Ascaris lumbricoides somatic cells. Dev. Biol. 164, 72–86.
- Wang, J., Czech, B., Crunk, A., Wallace, A., Mitreva, M., Hannon, G.J., and Davis, R.E. (2011). Deep small RNA sequencing from the nematode Ascaris reveals conservation, functional diversification, and novel developmental profiles. Genome Res. 21, 1462–1477.
- Zurita, M., Reynaud, E., and Aguilar-Fuentes, J. (2008). From the beginning: the basal transcription machinery and onset of transcription in the early animal embryo. Cell. Mol. Life Sci. 65, 212–227.

- Blaxter, M.L. (2009). Nematodes (Nematoda). In The Timetree of Life, S.B. Hedges and S. Kumar, eds. (New York: Oxford University Press), pp. 247–250.
- Schierenberg, E. (1986). Developmental strategies during early embryogenesis of Caenorhabditis elegans. J. Embryol. Exp. Morphol. Suppl. 97, 31–44.
- Baugh, L.R., Hill, A.A., Slonim, D.K., Brown, E.L., and Hunter, C.P. (2003). Composition and dynamics of the Caenorhabditis elegans early embryonic transcriptome. Development *130*, 889–900.
- Cleavinger, P.J., McDowell, J.W., and Bennett, K.L. (1989). Transcription in nematodes: early Ascaris embryos are transcriptionally active. Dev. Biol. 133, 600–604.
- Edgar, L.G., Wolf, N., and Wood, W.B. (1994). Early transcription in Caenorhabditis elegans embryos. Development 120, 443–451.
- Powell-Coffman, J.A., Knight, J., and Wood, W.B. (1996). Onset of C. elegans gastrulation is blocked by inhibition of embryonic transcription with an RNA polymerase antisense RNA. Dev. Biol. *178*, 472–483.
- Fairbairn, D. (1957). The biochemistry of Ascaris. Exp. Parasitol. 6, 491–554.
- 19. Azzaria, M., and McGhee, J.D. (1992). DNA synthesis in the early embryo of the nematode Ascaris suum. Dev. Biol. *152*, 89–93.
- Wang, J., Mitreva, M., Berriman, M., Thorne, A., Magrini, V., Koutsovoulos, G., Kumar, S., Blaxter, M.L., and Davis, R.E. (2012). Silencing of germline-expressed genes by DNA elimination in somatic cells. Dev. Cell 23, 1072–1080.
- Barckmann, B., and Simonelig, M. (2013). Control of maternal mRNA stability in germ cells and early embryos. Biochim. Biophys. Acta 1829, 714–724.
- Levin, M., Hashimshony, T., Wagner, F., and Yanai, I. (2012). Developmental milestones punctuate gene expression in the Caenorhabditis embryo. Dev. Cell 22, 1101–1108.
- Seydoux, G., and Fire, A. (1994). Soma-germline asymmetry in the distributions of embryonic RNAs in Caenorhabditis elegans. Development 120, 2823–2834.
- Seydoux, G., and Dunn, M.A. (1997). Transcriptionally repressed germ cells lack a subpopulation of phosphorylated RNA polymerase II in early embryos of Caenorhabditis elegans and Drosophila melanogaster. Development 124, 2191–2201.
- Robertson, S., and Lin, R. (2013). The oocyte-to-embryo transition. Adv. Exp. Med. Biol. 757, 351–372.
- Reinke, V., Gil, I.S., Ward, S., and Kazmer, K. (2004). Genome-wide germline-enriched and sex-biased expression profiles in Caenorhabditis elegans. Development *131*, 311–323.
- Farley, B.M., and Ryder, S.P. (2008). Regulation of maternal mRNAs in early development. Crit. Rev. Biochem. Mol. Biol. 43, 135–162.
- Hillier, L.W., Reinke, V., Green, P., Hirst, M., Marra, M.A., and Waterston, R.H. (2009). Massively parallel sequencing of the polyadenylated transcriptome of C. elegans. Genome Res. 19, 657–666.
- Laugsch, M., and Schierenberg, E. (2004). Differences in maternal supply and early development of closely related nematode species. Int. J. Dev. Biol. 48, 655–662.
- Wiegner, O., and Schierenberg, E. (1998). Specification of gut cell fate differs significantly between the nematodes Acrobeloides nanus and caenorhabditis elegans. Dev. Biol. 204, 3–14.
- Edgar, L.G., and McGhee, J.D. (1988). DNA synthesis and the control of embryonic gene expression in C. elegans. Cell 53, 589–599.
- Edgar, B.A., and Schubiger, G. (1986). Parameters controlling transcriptional activation during early Drosophila development. Cell 44, 871–877.
- Kimelman, D., Kirschner, M., and Scherson, T. (1987). The events of the midblastula transition in Xenopus are regulated by changes in the cell cycle. Cell 48, 399–407.
- Shermoen, A.W., and O'Farrell, P.H. (1991). Progression of the cell cycle through mitosis leads to abortion of nascent transcripts. Cell 67, 303–310.
- Ali-Murthy, Z., Lott, S.E., Eisen, M.B., and Kornberg, T.B. (2013). An essential role for zygotic expression in the pre-cellular Drosophila embryo. PLoS Genet. 9, e1003428.
- De Renzis, S., Elemento, O., Tavazoie, S., and Wieschaus, E.F. (2007). Unmasking activation of the zygotic genome using chromosomal deletions in the Drosophila embryo. PLoS Biol. 5, e117.
- Liang, H.L., Nien, C.Y., Liu, H.Y., Metzstein, M.M., Kirov, N., and Rushlow, C. (2008). The zinc-finger protein Zelda is a key activator of the early zygotic genome in Drosophila. Nature 456, 400–403.

- ten Bosch, J.R., Benavides, J.A., and Cline, T.W. (2006). The TAGteam DNA motif controls the timing of Drosophila pre-blastoderm transcription. Development 133, 1967–1977.
- Xue, Z., Huang, K., Cai, C., Cai, L., Jiang, C.Y., Feng, Y., Liu, Z., Zeng, Q., Cheng, L., Sun, Y.E., et al. (2013). Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500, 593–597.
- Schierenberg, E. (2006). Embryological variation during nematode development. WormBook, 1–13.
- Schulze, J., and Schierenberg, E. (2011). Evolution of embryonic development in nematodes. Evodevo 2, 18.