The maternal-zygotic transition and zygotic activation of the *Mnemiopsis leidyi* genome occurs within the first three cleavage cycles


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Funding information
National Science Foundation, Grant number: IOS-1558061; National Human Genome Research Institute (USA); National Institutes of Health (USA)

The maternal-zygotic transition (MZT) describes the developmental reprogramming of gene expression marked by the degradation of maternally supplied gene products and zygotic genome activation (ZGA). While the timing and duration of the MZT vary among taxa, little is known about early-stage transcriptional dynamics in the non-bilaterian phylum Ctenophora. We sought to better understand the extent of maternal mRNA loading and subsequent differential transcript abundance during the earliest stages of development by performing comprehensive RNA-sequencing-based analyses of mRNA abundance in single- and eight-cell stage embryos in the lobate ctenophore *Mnemiopsis leidyi*. We found 1,908 contigs with significant differential abundance between single- and eight-cell stages, of which 1,208 contigs were more abundant at the single-cell stage and 700 contigs were more abundant at the eight-cell stage. Of the differentially abundant contigs, 267 were exclusively present in the eight-cell samples, providing strong evidence that both the MZT and ZGA have commenced by the eight-cell stage. Many highly abundant transcripts encode genes involved in molecular mechanisms critical to the MZT, such as maternal transcript degradation, serine/threonine kinase activity, and chromatin remodeling. Our results suggest that chromosomal restructuring, which is critical to ZGA and the initiation of transcriptional regulation necessary for normal development, begins by the third cleavage within 1.5 hr post-fertilization in *M. leidyi*.

KEYWORDS
chromatin remodeling, ctenophora, gene expression, maternal transcript degradation, RNAseq

INTRODUCTION

The maternal-zygotic transition (MZT) describes the developmental reprogramming of gene expression marked by the degradation of maternally supplied gene products and zygotic genome activation (ZGA). A number of molecular mechanisms drive the variable duration and timing of the MZT among plants and animals, shaping...
maternal and zygotic transcriptional dynamics and thus regulation of organisational development (Lee, Bonneau, & Giraldez, 2014). Across metazoans (animals) there is minimal coupling between the MZT and cleavage-cycle stages. For example, among bilaterian species such as the urchin Strongylocentrotus purpuratus and the mouse Mus musculus, ZGA occurs at the single-cell stage: onset of this event begins almost immediately after fertilization in the sea urchin (Pocchia, Wolff, Kragh, & Williamson, 1985), but does not take place until approximately 10 hr post-fertilization (hpf) in the mouse (Bouniol, Nguyen, & Dekeyser, 1995). In the zebrafish Danio rerio, ZGA begins around the seventh cleavage cycle, about 2 hpf (Heyn et al., 2014), while zygotic expression in the frog Xenopus laevis begins midway through blastula stages, about 6 hpf (Newport & Kirschner, 1982a, 1982b). In the ecdysozoan nematode Caenorhabditis elegans, ZGA begins by the second cleavage cycle, less than 2 hpf (Baugh, Hill, Slonim, Brown, & Hunter, 2003; Edgar, Wolf, & Wood, 1994), while in the crustacean Parhyale hawaiensis, MZT occurs at the 32-cell stage after germ layer specification (Nestorov, Battke, Levesque, & Gerberding, 2013). In the non-bilaterian cnidian Nemastostella vectensis, the MZT begins during formation of the embryonic blastula, between 2 and 7 hpf (Helén, Siebert, Turlin, Smith, & Dunn, 2013). Presumably the lack of temporal uniformity in zygotic transcription and chromosomal restructuring marking the onset of ZGA during early development among animals is attributable to diverse life histories and to the differential deployment of mechanisms responsible for the MZT.

The molecular drivers of maternal transcript degradation and ZGA have been extensively studied for over 30 years (reviewed by Lee et al., 2014; Schier, 2007; and Tadros & Lipshitz, 2000). Deadenylation of mRNA transcripts (Semotok et al., 2005) and microRNA (miRNA) activity (Bushati, Stark, Brennecke, & Cohen, 2008; Giraldez et al., 2006) are critical for maternal mRNA destabilization and degradation, and both can be linked to maternally supplied and zygotically transcribed factors (Bashirullah et al., 1999). Transcriptional repression of the zygotic genome preceding ZGA is often a result of chromatin-mediated silencing (Newport & Kirschner, 1982a, 1982b; Ruzov et al., 2004), lack of transcriptional machinery (Kikyo & Wolff, 2000; Prioleau, Huet, Sentenac, & Mechali, 1994), or mitosis-induced transcript abortion in rapidly dividing embryos (Shermon & O’Farrell, 1991).

Mechanisms responsible for lifting transcriptional repression and subsequent ZGA include titration of transcriptional repressors by increasing amounts of chromatin, via the nucleocytoplasmic ratio model (Newport & Kirschner, 1982a, 1982b); transcriptional activation following initiation of gene regulatory cascades at fertilization, via the maternal-clock model (Howe, Howell, Hunt, & Newport, 1995); and large-scale chromatin remodeling that can alter transcription rates in different chromosomal regions (Iwafuchi-Doi & Zaret, 2014; Kikyo & Wolff, 2000; Prioleau et al., 1994; Stancheva & Meehan, 2000). These mechanisms play a critical role in shifting the balance of chromatin accessibility and transcriptional complex assembly as the developing embryo transitions from dependence on maternal transcripts to activation of gene expression from the zygotic genome. Given the temporal and mechanistic variation associated with the MZT, investigating this critical early developmental period in non-bilaterarians can reveal evolutionarily conserved aspects of the MZT as well as highlight potentially novel, lineage-specific aspects of MZT regulation (Helm et al., 2013).

Ctenophora is a non-bilaterian phylum in which the MZT and early developmental transcriptional dynamics remain largely unexplored. Ctenophores, or comb jellies, are gelatinous marine animals characterized by the presence of eight ciliated comb (ctene) rows used for locomotion (Dunn, Leys, & Haddock, 2015; Pang & Martindale, 2008a). Ctenophora represent one of the earliest branching extant metazoan lineages (Dunn et al., 2008; Hejnol et al., 2009; Moroz et al., 2014; Ryan et al., 2013; Shen, Hittinger, & Rokas, 2017; Whelan, Kocot, Moroz, & Halanych, 2015), evolving independently from other animal lineages for well over 600 million years (Hedges, Blair, Venturi, & Shoe, 2004; Wang, Kumar, & Hedges, 1999). A number of recent studies have focused on some of the unique and derived features of ctenophore biology, such as a highly derived mitochondrial genome (Kohn et al., 2012; Pett et al., 2011), a lack of genes required for mesoderm specification, and a nervous system missing key neurotransmitters (Moroz et al., 2014; Ryan et al., 2013).

Mnemiopsis leidyi is one of the most well-studied ctenophore species, and has emerged as a model system for ctenophore biology (e.g., Martindale & Henry, 1999; Pang & Martindale, 2008b; Presnell et al., 2016; Schnitzler et al., 2012; Vandepas, Warren, Amemiya, & Browne, 2017; Yamada, Martindale, Fukui, & Tochinai, 2010). M. leidyi is a pelagic lobate ctenophore and, like most ctenophore species, is simultaneously hermaphroditic. M. leidyi embryonic development is rapid, reaching a juvenile cyclidipid stage less than a day after fertilization. Identification and analyses of the MZT in ctenophores should offer additional insight into the evolution of early embryonic developmental mechanisms related to the MZT, including maternal gene product degradation and ZGA in animals. For example, miRNAs are known to play a role in maternal transcript degradation (Bushati et al., 2008; Giraldez et al., 2006); however, the M. leidyi genome appears to lack both miRNAs and the necessary processing machinery for miRNA biogenesis (Maxwell, Ryan, Schnitzler, Browne, & Baxevanis, 2012). Subsequent genomic and transcriptomic analyses of the ctenophore Pleurobrachia bachei have corroborated this absence of the canonical miRNA pathway in ctenophores, while also highlighting the expression of many ctenophore-specific genes during early embryogenesis (Moroz et al., 2014).

Embryonic lineage tracing in M. leidyi suggested differential cell fate potential as early as the four-cell stage (Martindale & Henry, 1995), and revealed a clear segregation of developmental potential at the eight-cell stage (Martindale & Henry, 1999). Subsequent cell lineage analyses paired with pharmacological reagents inhibiting transcription, protein synthesis, and DNA synthesis have confirmed that specification of some cell fates in M. leidyi are governed by a cleavage clock in which determinants are spatially segregated at the eight-cell stage into the E- and M-blastomeres (Fischer, Pang, Henry, & Martindale, 2014). An additional conclusion drawn from this
pharmacological study is that ZGA in M. leidyi initiates just prior to gastrulation, at the 60-cell stage.

Here, we present the results of RNA-sequencing based analyses of single- and eight-cell samples from M. leidyi embryos spanning the first 1.5 hr of development. These data were used to explore differential transcript abundance during the initial segregation of developmental potential during M. leidyi embryogenesis (Figure 1a). In contrast to previous embryological and pharmacological studies, our transcriptomic approach provides a new perspective of the MZT and the initiation of ZGA in ctenophores. Specifically, our analyses identified gene activity associated with the MZT and the initiation of new gene expression at the eight-cell stage during early M. leidyi development.

2 | RESULTS

2.1 | Assembly of reference transcriptome and read counts

Twelve paired-end RNA-seq samples were used to assemble a reference transcriptome, including four single-cell, three complete eight-cell, three eight-cell M-blastomere, and two eight-cell E-blastomere samples. After trimming adapters from the sequences and evaluating the sequences using appropriate quality control measures (see section 4.2), a de novo reference transcriptome was assembled using Trinity (Grabherr et al., 2011), as implemented within Agalma (Dunn, Howison, & Zapata, 2013).
TABLE 1  Transcriptome summary statistics

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<th>Raw reads</th>
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<th># Contigs &gt;1 kbp</th>
<th>15,910</th>
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<tr>
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<td>N10</td>
<td>4,601 bp</td>
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<td>Smallest contig</td>
<td>301 bp</td>
<td>N50</td>
<td>2,819 bp</td>
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<tr>
<td>Largest contig</td>
<td>21,725 bp</td>
<td>N70</td>
<td>1,283 bp</td>
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<tr>
<td>Mean contig length</td>
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<td>N90</td>
<td>565 bp</td>
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<tr>
<td>GC content</td>
<td>41.6%</td>
<td># Contigs with ORF</td>
<td>17,706</td>
</tr>
</tbody>
</table>

Transcriptome summary statistics generated by Transrate (Smith-Unna et al., 2016). The term "contig" is used rather than "gene" to distinguish between the method of de novo transcriptome assembly and referencing the Mnemiopsis genome during RNA-sequencing read alignment. ORF, open reading frame.

The de novo transcriptome assembly approach was chosen to maximize contig assembly, read mapping, and subsequent read counting, thereby providing a comprehensive analysis of mRNA abundance and transcript variation. On average, 94.6% of the reads mapped to the de novo transcriptome, whereas only 53.0% of the reads mapped to the reference genome (Table S1). The depressed mapping rates to the reference genome are partially a reflection of sequence heterogeneity between the M. leidyi population used for this study and the reference genome sequence, as well as incomplete annotation of the reference genome gene models. Our de novo transcriptome includes 682 contigs containing 562 unique genes with significant BLAST (Basic Local Alignment Search Tool) hits (Altschul, Gish, Miller, Myers, & Lipman, 1990) (E-value ≤ 1 × 10^{-5}) to the Swiss-Prot protein database (UniProt, 2015) that do not align to any M. leidyi gene models (File S1).

We additionally scanned the de novo transcriptome for contamination using an alien index (Gladyshev, Meselson, & Arkhipova, 2008), as implemented in alien_index (Ryan, 2014); this process recovered minimal expression of non-animal transcripts across all samples (File S2).

Alignment of reads from the 12 individual samples to the de novo reference transcriptome identified a total of 28,122 expressed contigs encompassing 6,216 unique M. leidyi gene models (File S3). Downstream analyses were carried out at the contig-level to highlight transcript diversity and to maximize read mapping rates not attainable by directly referencing the M. leidyi genome assembly (see previous paragraph). Read counts were further normalized for between-sample variation using RUVseq (Risso, Ngai, Speed, & Dudoit, 2014) (Figures 2a and 2b). Principal component analyses (PCA) distinguished single-cell expression profiles from eight-cell expression profiles, with the first two principal components accounting for 47% of the variation, indicating substantial variation between single-cell and eight-cell stages (Figures 2c and 2d).

Despite capturing significant variation, PCA analysis does not discriminate between the occurrence of maternal mRNA degradation and/or zygotic transcription, as either process could produce different expression profiles at the single- and eight-cell stage. One way to gain insight into the roles of these developmental mechanisms was to closely examine the eight-cell samples in the PCA (Figure 2c). Complete eight-cell embryos, isolated eight-cell M-blastomere, and isolated eight-cell E-blastomere samples did not group as distinct sample types in the analysis, as reflected in less-robust pairwise differential abundance between these three sample groups. Importantly, the eight-cell samples did group at the individual specimen level, regardless of sample type (i.e., complete, M-, or E-blastomere specific; Figure 2c). One possible explanation for this result is that by the eight-cell stage, sequencing identified the differential timing of maternal transcript degradation and/or ZGA cascades at the individual-specimen level. Thus, gene expression patterns associated with a specimen slightly further along or behind in development are reflected in read-frequency data as being slightly asynchronous between individuals, but highly correlated within samples from a given individual. As a result, the differential timing of large-scale transcript degradation pathways or the initiation of zygotic transcription can magnify between-sample transcriptional dynamics within the 1.5-hpf period examined. Two plausible scenarios still remain: either maternal transcript degradation alone or a combination of transcript degradation and zygotic transcription could be occurring at this stage of development. Differential abundance analyses were carried out in an attempt to distinguish between these two possibilities.

2.2 | Differential abundance analysis

Noise in the differential abundance analysis was reduced by requiring a minimum mapping threshold of at least five reads in two or more samples to identify reference contigs for inclusion in our analysis using edgeR (Robinson, McCarthy, & Smyth, 2010). We generated a final set of 23,326 reference contigs for differential abundance analysis. We defined differentially abundant contigs as having an absolute log2 fold change ≥ 2 between sample groups supported by a false-discovery rate ≤ 0.05 (Figure 3; File S4). We identified 1,908 contigs as significantly differentially abundant between single-cell (0 hpf) and complete eight-cell samples (Figures 3a and 3b; Figure S1). Of these contigs, 1,208 had a higher representation in the maternally loaded single-cell stage, whereas 700 had a higher representation at the eight-cell stage (Figure 3b), which is consistent with initiation of the MZT. Critically, 217 contigs are exclusively present in the single-cell stage and 267 contigs are exclusively present in the eight-cell stage (Figures 3b and 3c). The presence of 267 contigs in the eight-cell samples that are completely absent in single-cell samples rules out the possibility that all 700 contigs with higher representation at the eight-cell stage are due solely to the differential processing of maternally supplied mRNAs. Taken together, these results confirm both the MZT and ZGA have initiated by the third cell cycle in M. leidyi at ~1.5 hpf.

Turnover of transcript abundance can be visualized as a heat map of log counts per million (Figure 3c; Figure S1). Many contig clusters
decrease in relative abundance from single-cell to eight-cell stages (Figure S1), reflecting either the degradation of maternally loaded mRNAs, the dilution of maternal transcripts by nascent zygotic transcription, or possibly both processes. Conversely, a number of contig clusters also increase between single- and eight-cell stages, supporting zygotic transcription (Figure S1). Expression dynamics at the individual-level uncovered some variable expression changes associated with contig clusters between samples, lending additional support to the idea that the differential abundance analyses are sensitive to slight differences in timing of gene expression changes between individuals (Figure 3c; Figure S1). Despite some noise detected in expression counts, contig annotation revealed common MZT processes at work.

We also carried out three pairwise comparisons between complete eight-cell, isolated eight-cell E-blastomeres, and isolated eight-cell M-blastomeres in an attempt to identify transcriptional differences associated with the segregation of developmental potential between E- and M-blastomeres at the eight-cell stage. We found...
157 differentially abundant contigs between complete eight and isolated E-blastomere samples; 77 differentially abundant contigs between complete eight and isolated M-blastomere samples; and 7 differentially abundant contigs between isolated E- and isolated M-blastomere samples (File S4). The seven differentially abundant genes between E- and M-blastomere samples included a Na\(^+\)/K\(^+\)/Ca\(^{2+}\) exchanger (ML261714a), Exportin2 (ML29681a), Actin-binding LIM protein 3 (ML435828a), three lineage-specific genes (ML12703a, ML065736a, ML15402a), and one contig that did not map to the reference genome (contig29091). Two possible explanations for the
limited differential abundance observed between the E- and M-blastomeres are the masking of differential abundance signal due to stress-induced gene expression from mechanical separation and isolation of E- and M-blastomeres and/or variability in gene expression between individuals. Alternatively, maternally deposited proteins rather than maternal or zygotic mRNAs may be responsible for the fate determination of these cell populations. This analysis, along with the findings of Fischer et al. (2014), supports the idea that maternal proteins and their differential partitioning during the first three cleavage cycles, rather than nascent translation of mRNA, drives early cell fate specification of blastomeres during *M. leidyi* embryogenesis.

### 2.3 Annotation of assembled reference contigs

Using the reference de novo transcriptome contigs as the query, BLAST identified 22,648 statistically significant hits against *M. leidyi* protein models and 11,718 statistically significant hits against the Swiss-Prot protein database (*E*-value ≤ 1 × 10^{-5}) (File S5). In addition, 14,106 contigs could be assigned one or more gene ontology terms (Ashburner et al., 2000) (File S6). Contig sequence counts of some of the most highly represented Biological Process and Molecular Function terms across the entire transcriptome, as well as broad terms that are specifically related to MZT mechanisms, are listed in Figure 4. These include regulating maternal transcripts for degradation (serine/threonine/tyrosine kinase activity); silencing and activating the zygotic genome; large-scale chromatin structural modifications; and regulation of DNA-templated transcription.

We recovered a number of contigs containing genes thought to play important roles during the MZT. Each is among the top 20% most-abundant contigs across both single- and eight-cell stage samples. The serine-threonine kinase SMAUG (SMG), for example, recruits the deadenylase complex CCR4/POP2/NOT to mediate maternal transcript degradation (Benoit et al., 2009; Semotok et al., 2005; Tadros et al., 2007); we recovered expressed contigs for both the kinase *Smg1* (contig12703) and the deadenylase complex subunit *Cnot4* (contig2682). Chromatin-mediated silencing represses zygotic transcription early in development, and depletion of the methyltransferase DNMT1 results in early zygotic transcription in *Xenopus* through

![Gene ontology (GO) identifiers, description, and sequence counts of the most highly represented GO terms as well as terms that are broadly related to MZT/ZGA processes. (a and b) Biological Process and (c and d) Molecular Function. GO identifiers from each respective table correspond to the x-axis labels](Image)
decreased CpG methylation (Stancheva & Meehan, 2000); we recovered a contig for Dnmt1 (contig9358) during the first three cell cycles, in addition to the related methyltransferase Dnmt3a (contig2787, contig2788, contig7017). Chromatin remodelers are crucial for both zygotic transcriptional repression as well as activation. BRG1 (Brahma-related gene 1; also called SMARCA4 [Switch/sucrose non-fermentable-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4]) is a catalytic subunit of the chromatin remodeling SWI/SNF (Switch/sucrose non-fermentable-related)-related complexes that function in gene expression reprogramming during M. musculus ZGA (Bultman et al., 2006). Smc4 (contig6831), a gene highly similar to Brg1, was also expressed in single- and eight-cell samples.

The OSKM transcription factor genes, Oct4 (also called Pou5f1), Sox2, Klf4 (Krüppel-like factor 4), and c-Myc, participate in chromatin remodeling during pluripotency-related cell reprogramming and are associated with ZGA in vertebrates (Lee et al., 2013; Leichsenring, Maes, Mössner, Driever, & Onichtchouk, 2013; Soufi, Garcia, Jaroszewicz, Osman, & Pellegrini, 2015; Zeng & Schultz, 2005). During the first ~1.5 hr of M. leidyi development, we recovered expressed contigs for homologs to the OSKM cellular reprogramming circuit. The POU class homeodomain containing gene family includes Oct4 (Ryan & Rosenfeld, 1997). We detected significant expression of MlePou2a (contig16781, contig16782), which contains a POU-specific domain upstream of a POU class homeodomain (Ryan et al., 2010). The Sox gene family is characterized by the presence of a high mobility group (HMG) DNA-binding domain, and the Sox2 transcription factor, along with other members of the Sox gene family, play key roles in regulating cell fate during development in metazoans (Sarkar & Hochedlinger, 2013). During the first three cell cycles, we detected expression of a single Sox gene, MleSox2 (contig22607, contig22608), a member of the SoxC group (Schnitzler, Simmons, Pang, Martindale, & Baxevanis, 2014). Members of the Krüppel-like factor and specificity protein (KLF/SP) gene family are characterized by the presence of a highly conserved triple C2H2 zinc finger DNA-binding domain and play key roles in essential biological processes, including balancing stem cell proliferation and differentiation (Bialkowski, Yang, & Mallipattn, 2017; Presnell, Schnitzler, & Browne, 2015). We detected significant expression for both MleKlf5a (contig2761, contig16159, contig16164, contig26148) and MleKlf5b (contig4663, contig27551) (Presnell et al., 2015). The MYC transcription factor contains a highly conserved basic-helix-loop-helix leucine zipper (bHLH-LZ) DNA-binding domain, and plays a central role in cell growth and proliferation (Grandori, Cowley, James, & Eisenman, 2000; Kress, Sabb, & Amati, 2015; Young et al., 2011). We detected significant expression of a Myc ortholog (contig20351) during the first three cell cycles. Expression of these and other chromatin remodelers suggests that large-scale chromatin restructuring involved in the repression and activation of zygotic gene expression likely occurs within the first 1.5 hr of M. leidyi development.

Predicted protein-coding sequences from the M. leidyi genome assembly include a large fraction of expressed lineage-specific genes (7,798/16,548) (Ryan et al., 2013), defined as having an identity derived from the Mnemiopsis reference genome (Moreland et al., 2014) but no orthology to genes in other organisms. Transcript profiling in the ctenophore Pleurobrachia bachei also identified a significant number of ctenophore-specific genes that are differentially expressed during early development (Moroz et al., 2014). Our results in M. leidyi similarly identified differential abundance of a number of lineage-specific genes within the first 1.5 hr of development. For example, ~13% of contigs exclusively expressed in both single- and eight-cell stage embryos were lineage-specific (29 and 35 contigs respectively; File S4). Most contigs exclusively detected at the single-cell stage were characterized by highly variable expression between samples, and may reflect non-uniform loading of maternal transcripts (Figure 3c). In contrast, a number of uniformly expressed contigs exclusively detected at the eight-cell stage encode homeodomain-containing transcription factors. Among these are three contigs (contig8798, contig31508, contig10728) that respectively correspond to the ctenophore-specific SINE (Sine oculis) class genes, Mlesix59a, Mlesix13a, and Mlesix13f (Ryan et al., 2010). We also detected differential activation at the eight-cell stage of three divergent, unclassified homeodomain genes—Mleh107c (contig14607, contig14733), Mleh11a (contig25571), Mleh11b (contig23590)—and a single ANTP (Antennapedia) class HOX-like gene—Mleantp03c (contig23663) (Ryan et al., 2010).

3 | DISCUSSION

The results of this study demonstrate that molecular components essential for carrying out the MZT are present within the first three cleavage cycles of the ctenophore M. leidyi. ZGA has commenced by the eight-cell stage, ~1.5 hpf. Expression profiles of single- and eight-cell samples reflect a large turnover between maternally supplied and zygotic transcripts, and our differential abundance analyses definitively rule out the possibility that maternal gene products alone account for this difference between samples. Furthermore, annotation of the expressed transcripts is consistent with maternal transcript degradation and large-scale chromatin remodeling activity involved in both zygotic genome silencing and activation.

The early onset of ZGA in M. leidyi is consistent with a rapid, highly deterministic cleavage program that promotes development into a juvenile cypidid within 24 hpf. Recent RNA-sequencing analysis of development in another non-bilaterian lineage, the cnidarian Nemastella vectensis, also uncovered molecular players involved in the MZT and identified initiation of the MZT between 2 and 7 hpf, followed by a major wave of zygotic transcription between 7 and 12 hpf (Helm et al., 2013). Our study in M. leidyi provides an additional example of the variable timing of the MZT and ZGA among metazoans. Additionally, our results highlight the deep conservation of molecular mechanisms underlying MZT and ZGA, including factors associated with the OSKM cellular pluripotency reprogramming circuit.

In contrast to a recent study using actinomycin D drug treatment to inhibit transcription (Fischer et al., 2014), RNA-sequencing afforded a fine-scale granular analysis of early developmental transcriptional dynamics in M. leidyi. We found that ZGA initiates much earlier in M. leidyi than previously reported and, as in other animals, a temporal
overlapping between maternal transcript degradation and zygotic transcription exists—that is, MZT checkpoints are not occurring in a sequential on/off fashion. Thus, our results highlight that the potential of maternally loaded transcripts to support the early cleavage program through gastrulation does not necessarily imply that zygotic transcription has not commenced prior to this stage of development, explaining why actinomycin D treatment alone was unable to accurately define the onset of ZGA. The temporal dynamics of these developmental mechanisms result, in part, from both mRNA transcript destabilization pathways and chromatin restructurung.

Additional fine-scale RNA-seq analyses in M. leidyi paired with assessments of chromatin states (open/closed) will offer more biological context to early genome-wide remodeling associated with ZGA. Importantly, proteomic analyses of ctenophore development may provide much needed insight into the identification and partitioning of maternally loaded proteins responsible for the remarkably stereotyped early-cleavage fate-specification program (Freeman, 1976; Martindale & Henry, 1999) and the results of our differential abundance analysis. Here, we provided a new and revised perspective on the MZT in ctenophores that reflects the deep conservation of this critical early developmental event in metazoans.

4 MATERIALS AND METHODS

4.1 Collection, RNA extraction, and sequencing

Adult M. leidyi individuals were collected in Biscayne Bay, Miami, FL (25°28′05″N 80°12′45″W). Individual adult animals were induced to spawn following a light-dark cycle. Single- and eight-cell embryos were collected after fertilization. For eight-cell embryo collections, synchronized spawns were monitored until the third cleavage. The egg envelopes from a subset of eight-cell embryos were removed using fine-tipped forceps and sharpened tungsten wire needles. For each individual spawn, the E- and M-blastomeres were then separated by hand with glass needles and pooled.

RNA was extracted and isolated from all 12 pooled samples using TRIzol. RNA quantity was assessed via Qubit fluorometer (Invitrogen, Carlsbad, CA), and RNA quality was assessed via Bioanalyzer (Agilent Technologies, Santa Clara, CA). Paired-end sequencing for each sample was carried out on an Illumina HiSeq 2000 platform (Husman Institute of Human Genomics, Miami, FL). Sequenced RNA reads included four single-cell samples (0 hpf), three complete eight-cell samples (∼1.5 hpf), and five mechanically separated E- and M-blastomere-specific samples from matched eight-cell samples (∼1.5 hpf). Raw sequencing files can be obtained from the National Center for Biotechnology Information (NCBI) SRA repository (NCBI BioProject ID PRJNA396768).

4.2 Raw read preparation

Before transcriptome assembly, a series of trimming and quality control measures were carried out on the 12 RNA-sequencing samples. Reads were subject to Trimmomatic processing, including cropping the hexamer primers and sequencing adaptors from each read; removing low quality reads (reads with four consecutive bases averaging a sequencing score less than 15); clipping leading or trailing undefined (N) bases; and discarding reads less than 36 bases in length (Bolger, Lohse, & Usadel, 2014). A total of 374,560,885 reads resulted from this filtering step. Next, forward and reverse reads from all 12 samples were concatenated into two separate files, and each set of forward and reverse reads were digitally normalized (kmer length: 20) using the khmer-2.2 software package to decrease sample variation and discard redundant data (Brown, Howe, Zhang, Pyrkoz, & Brom, 2012; Crusoe et al., 2015). Resultant reads with quality scores less than 33 (Andrews, 2010) were removed, and ribosomal RNAs were identified and removed. A master reference transcriptome was then assembled with Trinity-r20140413p1 (Grabherr et al., 2011) (File S7). Transcriptome preparation and assembly was executed with the Agalma-0.5.0 workflow (Dunn et al., 2013) and Bioilite (Howison et al., 2012), and summary statistics were generated with Transrate v1.0.3 (Smith-Unna, Boursnell, Patro, Hibberd, & Kelly, 2016). Identification of putative non-metazoan gene expression contamination was carried out with the alien_index program (Ryan, 2014) (File S2).

4.3 Read counts, count normalization, differential abundance

Sample reads were aligned to the assembled transcriptome and the reference genome using Bowtie2-2.2.6 (Langmead & Salzberg, 2012), and RSEM-1.2.9 (Li & Dewey, 2011) was implemented to generate counts from each sample’s reads against the reference transcriptome (File S3 displays a table of raw read counts). Bioconductor (Gentleman et al., 2004) RUVseq package (Risso et al., 2014) was then used to normalize between-sample count variation in an effort to mitigate batch effects and to remove lowly expressed contigs (>5 reads in 2 or more samples were kept).

PCA of the normalized reads used for differential abundance analysis was carried out in R through the RUVseq package. Differential abundance analysis of the remaining 23,326 contigs was accomplished at the contig level using the generalized linear model (GLM) approach in edgeR (Robinson et al., 2010): dispersion was estimated with estimateGLMCommonDisp and estimateGLMTagwiseDisp functions; glmFit and glmLRT were used to fit the negative binomial GLM for each tag and to carry out a likelihood-ratio test, respectively. Significantly differentially abundant contigs were defined in this analysis as having a false-discovery rate ≤0.05 and an absolute log2 FC > 2 (File S4). The heat map of log-counts per million (logCPM) of contigs was created using the mixOmics package (Le Cao, Gonzalez, & Dejean, 2009) in R. The Venn diagram summarizing results of pairwise differential abundance analyses was generated with the VIB/UGent Venn diagram web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

4.4 Annotation and enrichment analyses

Contigs within the assembled transcriptome were annotated for significant hits (E-value ≤1 × 10−5) against the Mnemiopsis genome.
protein models (https://research.nhgri.nih.gov/mnemiopsis) and Swiss-Prot protein database (UniProt, 2015) (File S5). Blast2GO (Conesa et al., 2005) was implemented to assess and compile these annotations and to assign gene ontology terms (Ashburner et al., 2000) (File S6). Manual screening of the transcriptome was conducted for contigs of interest, including ctenophore-specific genes and genes potentially critical to the MZT, as identified from the literature.

ACKNOWLEDGMENTS

This work was supported by startup funds from the University of Miami College of Arts and Sciences and Provost Research Award to W.E.B. This work was also supported, in part, by the Intramural Research Program of the National Human Genome Research Institute, National Institute of Health. J.Q.H. (J.J.H.) was supported by National Science Foundation grant IOS-1558061. We thank the Center for Computational Science at the University of Miami for the fellowship provided to P.L.D. We thank the University of Miami’s John P. Hussman Institute for Human Genomics sequencing core for data generation.

AUTHORS’ CONTRIBUTIONS

Conceptualization, WEB; Methodology, PLD, BJK, CES, ADB, and WEB; Formal Analysis, PLD, BJK, CES, ADB, and WEB; Investigation, PLD, BJK, CES, JQH, MQM, ADB, and WEB; Writing—Original Draft, PLD, and WEB; Writing—Review & Editing, PLD, BJK, CES, JQH, MQM, ADB, and WEB; Visualization, PLD, and WEB; Resources, ADB and WEB.

CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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**How to cite this article**: Davidson PL, Koch BJ, Schnitzler CE, et al. The maternal-zygotic transition and zygotic activation of the *Mnemiopsis leidyi* genome occurs within the first three cleavage cycles. *Mol Reprod Dev*. 2017:84:1218–1229. [https://doi.org/10.1002/mrd.22926](https://doi.org/10.1002/mrd.22926)