Transcriptome dynamics in early zebrafish embryogenesis

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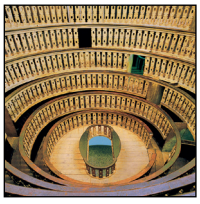
Introduction



Background

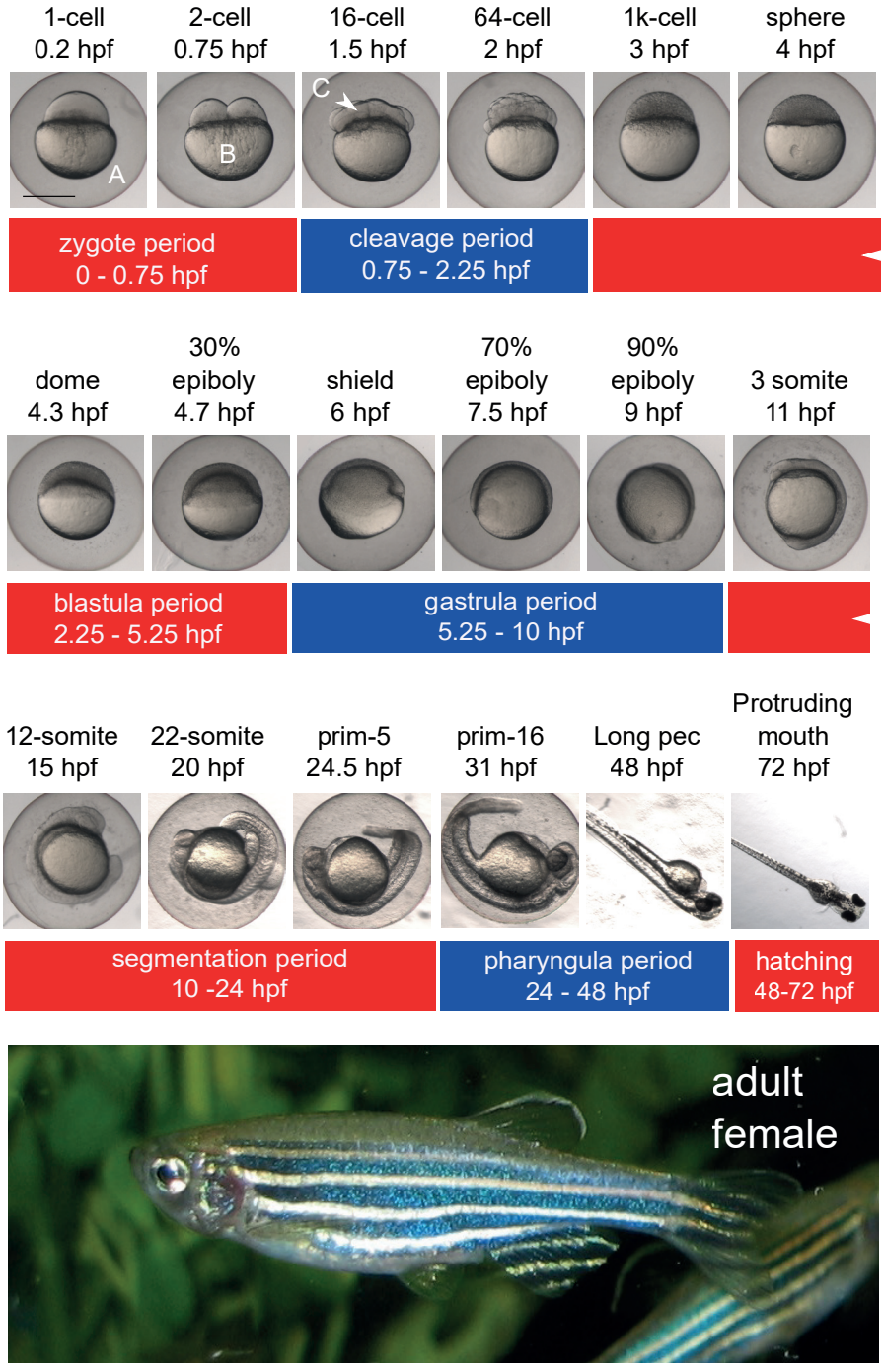
Life in sexually reproducing organisms starts with two highly specialized and fully differentiated cells, the egg and the sperm cell that fuse and become the one cell zygote. This single cell is a totipotent cell or embryogenic stem cell that during development is capable to divide and produce whatever specialized cells are needed in the tissues of the mature organism. In this cell a blueprint for the development of the mature organism is present as is the specific information from the mother and the father that gives rise to individual traits of the newly growing organism. The repertoire of processes and mechanisms in early embryogenesis is enormous and some of these have been elucidated, e. g. a number of genetic mechanisms, epigenetic mechanisms, and regulatory processes on transcriptional, post-transcriptional and translational level. Nevertheless, many questions remain. Even more so, some areas are virtually unmapped territory, such as subtle differences in the developmental program that may exist between individual zygotes, even siblings. Where do such differences originate from, how do they influence the growing organism, how similar is the resulting offspring? It is also unknown how strictly developmental programs are regulated and whether all genes are regulated with the same rigor at the cellular level or at the level of an individual. Also, the timescale at which these developmental processes unfold is mostly still unknown. Basically, these questions are on the dynamics of development. In this thesis, we present several studies, carried out in part-time over a period of 7 years, that, by using advances in transcriptomics technologies and by introducing a new method for single egg and single embryo transcriptome analysis, will answer some of the questions in the area of transcriptome dynamics in early zebrafish embryogenesis.

Aim

The primary aim of the studies presented in this thesis is to obtain a deeper insight in transcriptome dynamics during development, by investigating at an omics scale the changing gene-expression profiles during zebrafish embryogenesis.

Approach

For our transcriptome studies we used Zebrafish (*Danio rerio*) as model organism. Zebrafish is a tropical freshwater fish, belonging to the cyprinids (Cyprinidae) family. Its genome contains 25 chromosomes and has a size of 1.4 GBases. In the recent history it has become a popular model organism [1,2], a popularity which is fueled by several characteristics: the short reproduction cycle (6-12 weeks); the applicability of the morpholino technique to easily silence genes in embryogenesis; the transparency of the fertilized egg; the number of eggs per spawn (up to several hundreds); and the convenience of a relatively short developmental time from fertilization up to and including gastrulation (approximately 8 hours, Figure 1). Biology, with protagonists such as Linnaeus and van Leeuwenhoek, has its roots in observation, categorization and classification. This approach is also taken here. In contrast with many contemporary biological studies no genetic or environmental interventions


1-cell
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B
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zygote period
0 - 0.75 hpf
cleavage period
0.75 - 2.25 hpf
dome
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30%
epiboly
4.7 hpf
shield
6 hpf
70%
epiboly
7.5 hpf
90%
epiboly
9 hpf
3 somite
11 hpf
blastula period
2.25 - 5.25 hpf
gastrula period
5.25 - 10 hpf
12-somite
15 hpf
22-somite
20 hpf
prim-5
24.5 hpf
prim-16
31 hpf
Long pec
48 hpf
Protruding
mouth
72 hpf
segmentation period
10 -24 hpf
pharyngula period
24 - 48 hpf
hatching
48-72 hpf
adult
female

have been applied, which makes the design of the studies straightforward and safeguards the experiments from many of the possible pitfalls in omics experimentation [3,4]. Hence, by systematically characterizing the gene expression of the transcriptome in early zebrafish embryogenesis, we were able to better understand the transcriptome dynamics and gene-expression regulation that occur in this stage of the embryogenesis. As we employed an omics approach, we left the detailed unraveling of involved molecular processes and biological pathways to the life-scientists with specific expertise on one or more of the numerous pathways and elements. To support data mining, we made all our data freely, conveniently, and publicly available via a dedicated website: http://genseq-h0.science.uva.nl/shiny/Dr-Browser-v2/.

Biological perspective

An egg cell faces a unique fate that it shares with a sperm cell. As any other cell they need to stay alive and therefore have metabolic activity, but unlike other cells they must be able to fuse with one another. The egg and sperm cell must undergo a fundamental change of identity from 'belonging to the parent' to 'belonging to itself'. It must become totipotent and at the same time must become the director of its own play. As in mammals, oocytes in teleosts, such as zebrafish, are arrested during their development at the prophase of the first meiotic division. By contact with water, the zebrafish egg is activated and meiosis is completed [5]. In essence, the fertilized egg or zygote, then has to "restart" and unfold its developmental program. Initially this is done by using information and material that stem from the mother and father. Gradually a shift occurs towards the use of information and material that is self-derived, a process, which is called the maternal to zygotic transition (MZT). For zebrafish the zygote genome activation (ZGA), i.e. the period at which the embryo begins to transcribe its first RNAs, generally is positioned at somewhere between cell cycle 7 and 9, which occurs 2 to 2.5 hours post fertilization (hpf) [6–8]. However, already before the 128-cell stage there is abundant mitochondrial gene expression and from the 128-cell stage on nuclear expression has been detected [9]. While all these processes can unfold in more or less variable environments, in the past years it has become clear that environmental conditions can exert a lasting influence on the offspring, in zebrafish, e.g. [10] but also in human, e.g. [11]. Whether these changes are truly transgenerational is, at least for vertebrates under debate [12].

Figure 1. **Images of zebrafish embryonic development**
Stages in the embryonic development of the zebrafish according to Kimmel [42] indicated by hours post fertilization (hpf). Photos embryos: actual samples in our experiments, RB&AB, Leiden, November 2015, Stereo Microscope Leica MZ16 FA. All photos are at the same scale indicated by the bar of 1 mm in the 1-cell zygote. A: Chorion (egg shell), B: Yolk, C: Embryo. Photo Adult Female Zebrafish: Azul, commons.wikimedia.org.

The maternal message.

Here we define the maternal message as all molecules that are either imported from the circulation of the mother into the developing oocyte or are produced within the oocyte during oogenesis. During the primary oocyte growth, intense transcriptional activity takes place [13]. For Bombyx mori [14] and Drosophila melanogaster [15] evidence exists that mRNAs are imported from the circulation of the mother. For fish such information is not yet available. The amount of maternal RNA that is present in an egg is huge compared to the amount of DNA; on average in a zebrafish egg about 400 ng and 10 pg, respectively (cf. Chapter 1), which translates to 25 DNA molecules (if we ignore the polar bodies) and about $250 \cdot 10^9$ RNA molecules. This means that RNA contributes substantially to the genomic information that is conveyed by the mother. It should however be mentioned that the far majority of maternal RNA consists of non-coding RNA, such as ribosomal RNA (rRNA) and transfer RNA (tRNA). For Xenopus a preferential specific oocyte 5S rRNA has been reported [16]. Next to this are also other non-coding RNA such as long non-coding RNAs (lncRNAs), microRNA (miRNA) and Piwi-interacting RNA (piRNA), albeit at relatively low concentrations. The roles of maternal RNA in (early) embryogenesis are only partly known. Bridging the period during early embryogenesis in which the embryo is primarily occupied with DNA replication plus cell division and is mostly transcriptionally silent, is commonly accepted as the most important role for maternal RNA [17,18]. Next to this zebrafish mothers can transfer specific information via maternal RNA to their offspring, such as transcripts that code for Insulin Like Growth Factor 2 Receptor proteins, which are transferred by mothers, experimentally selected to have an above average adult body size [10].

The paternal message

The amount of molecules transferred from the father to its offspring is much smaller than that from the mother: the contents of a zebrafish egg with a diameter of 0.7 mm is approximately 180 thousand times that of a sperm cell of 7.8 μm^3 [19] and for a long time it has been thought that no paternal RNA existed. Nevertheless, like eggs, sperm is loaded with RNA that includes mRNAs, of over 4,000 different genes in human [20], miRNAs, interfering RNAs, antisense RNAs [21] and long non-coding RNAs [22]. However, the function of sperm RNA is largely unknown and mainly regulatory roles in development have been hypothesized, such as via epigenetic reprogramming [22].

Maternal mRNA characteristics

Many maternal mRNAs have a short or no poly(A) tail, which sets them apart from somatic mRNAs[23]. Recently it also has been shown on an omics scale that maternal transcripts can differ from somatic counterparts by their 3'UTR, brought on by a process referred to as alternative polyadenylation (APA). APA is common in eukaryotes and not restricted to embryogenesis. It entails that the sequence surrounding the polyadenylation signal (PAS) is variable and hence the actual site where the poly(A) tail will start (poly(A)-site) can vary as well. In many maternal mRNAs, different poly(A) sites are used in the 3' terminal exon [24,25] or even different 3' terminal exons are used [25,26]. This

results in general in shorter maternal 3'UTRs as compared to their zygotic counterparts. Also the 5' UTRs differ between maternal and zygotic transcripts, probably due to use of different transcription start sites (TSSs) that co-exist in the promoter of maternal genes [27].

Maternal mRNA silencing.

As many maternal mRNAs that are produced during oogenesis oocyte are only needed in the developing embryo, maternal mRNAs are silenced in oogenesis [28]. During oocyte maturation and early embryogenesis cytoplasmic modifications of the maternal message take place. On nuclear export mRNAs with a cytoplasmic polyadenylation element (CPE) can be bound to a complex containing a cytoplasmic polyadenylation element-binding protein (CPEB) and several other factors, including the polyadenylation specificity factor (CPSF). CPSF binds to the PAS. This complex shortens or removes the poly(A) tail which has been shown for Xenopus [29], and indirectly also for zebrafish [6,26]. Short poly(A) tails of approximately 20-mers repress translation [30]. In this 'masking' of the transcript for translation the Maskin protein is involved that binds to the cap-binding eukaryotic initiation factor eIF4E and prevents translation initiation factor eIF4G to attach to the complex, effectively blocking translation.

Maternal mRNA organization.

A zebrafish egg is a complex structure [5,31,32]. During oogenesis the oocyte accumulates proteins and maternal RNAs [33] whilst becoming transcriptionally silent. Maternal RNA is present in the vitelline membrane, in the germinal disc in oocytes and in the blastodisc in eggs, but not in yolk RNA [34]. The spatial redistribution of some maternal mRNAs during early embryogenesis, especially those involved in the formation of the primordial germ cells has been studied in depth. This process is spatially and temporally highly organized. Although the movements of individual cells during early embryogenesis are described in detail [35], there is no study yet that characterizes the redistribution of maternal mRNAs in time and space on an omics scale. Furthermore, little is known about the functional relevance of this spatial organization.

Oocyte transcription silencing.

When fertilized early zebrafish embryos are injected with naked plasmid DNA, it is transcribed [7] showing the transcriptional competence of the transcriptionally silent early embryo . In Xenopus injected plasmid DNA also showed transcription, but this transcription was subsequently repressed and only resumed during late blastula. A suppressor complex is proposed that silences oocyte/embryonic DNA until it gets titrated after a number of cell cycles [36]. However, the silencing of other genes seems to be independent of both the ratio of nuclear to cytoplasmic volume and number of cell cycles. Hence, other mechanisms, possibly some kind of developmental timer mechanism must exist [37]. Of interest, transcription silencing applies to the oocyte or early zygote genome, however the mitochondrial genome seems not be silenced at all [9].

Maternal mRNA clearance.
Zebrafish embryonic development starts with a phase of meroblastic cleavages; rapid and synchronous cell divisions of the embryonic cells, whereas the yolk cell does not divide. Around 2.5 to 3 hours after fertilization the cell cycle lengthens and cell divisions become asynchronous. Transcriptome profiling studies show a wave of clearance of maternal (and paternal) RNAs shortly after fertilization and another wave at the maternal to zygotic transition (MZT) which starts around 3.5 hpf [6,38]. An important element in maternal clearance is degradation initiated by miR-430 that induces mRNA degradation of target genes. miR-430 is among the first zygotic genes that are expressed and amongst these genes it is the most abundant one [9]. It is currently unclear whether maternal mRNAs are completely cleared and replaced by zygotically produced mRNA. There are indications that maternal mRNAs are recycled by polyadenylation as zygotic mRNAs.

Zygotic transcription initiation
Over the years there has been quite some confusion as to when zygotic transcription actually starts. Recent findings indicate that zygotic transcription starts with mir430 genes at the 64-cell stage, followed by nuclear protein-coding genes and other non-coding RNAs at the 128- to 512-cell stage [9]. The early transcribed genes are four times shorter than maternal genes, relatively intron poor or intron-less, and evolutionary young; about a quarter of these genes are not expressed as maternal genes [9]. Towards gastrulation, the embryo becomes more and more differentiated. By the mid blastula phase two types of cells can be distinguished, around the 512-cell stage on the margin of the yolk and blastoderm the yolk syncytial layer forms and at gastrulation the endoderm, mesoderm and ectoderm are established. These tissues require localized, synchronized and timely expression of specific transcripts. To this end a plethora of transcriptional and post-transcriptional regulation mechanisms is being used. These mechanisms are not specific to early embryogenesis but provide means for a localized and synchronous regulation, such as the stalling of the polymerase II complex downstream of the transcription start site (Pol II pausing) [39].

Technical perspective

Obviously, the omics era has boosted studies on transcriptome dynamics. Microarray technology and next-generation sequencing allow for time-series experiments as never seen before. However, these technologies always will be completely dependent on the biological experiment, the biological samples. Recently there is an increasing awareness that there are significant differences with respect to transcriptome regulation between individuals and even between single cells. As omics technologies require less input material, the study of these differences becomes increasingly feasible. Nowadays it is straightforward to analyze the transcriptome of a 700 µm zebrafish egg. A perfect example of technology progress is the recently published "genome-wide RNA tomography" in which the RNA from microscopic slices of for instance an embryo can be sequenced independently. The up to about 100 slices over the three axis of the samples allows

the reconstruction of genome-wide 3D expression patterns: in essence the RNA-seq equivalent of in-situ hybridization.

Bioinformatics perspective

An important challenge in whole-transcriptome analysis is the availability, stability of and consensus on the information regarding the zebrafish genome, transcriptome and their annotation. Unfortunately, both genome information and annotation are far from stable. This can be illustrated by a few facts and figures; currently the tenth build of the zebrafish genome assembly is available (1.4 GBase)[40]: this 10th build is 52 million bases longer than build 9, but 99 million bases shorter than build 8. In the current Ensembl annotation (v.84) nearly 25 thousand (25k) coding genes and 6k non-coding genes are annotated [41]. Corresponding numbers for previous assemblies were respectively 26k and 5k for build 9 while 24k coding genes were identified and non-coding genes not annotated in build 8. Bioinformatics data analyses usually start with some form of pre-processing to for instance eliminate the technical noise in an experiment. For this several data normalization procedures have been published. Even after decades of bioinformatics research and omics experimentation, choosing the appropriate normalization procedure often relies on trial and error. This also applies to downstream data analyses approaches. Data analyses rely on finding the relevant genes in biological contrasts and organizing these genes into similar groups, after which a number of standard analyses, usually based on overrepresentation are performed. Understanding the dynamics of complex transcriptomes still requires a lot of creative tinkering to achieve fruitful analysis approaches.

Outline of the thesis

The studies in this thesis focus mainly on zebrafish embryogenesis. To illustrate this, we present the embryogenesis by images from actual experiments done in the context of this thesis (Figure 1). As there are several experiments used in our studies, we have put them in context to each other to visualize the coherence of the studies (Figure 2).

Chapter 1

RNA isolation method for single embryo transcriptome analysis in zebrafish

A zebrafish egg is relatively small: 700 µm in diameter excluding the egg shell (chorion, Figure 1)[42]. The zebrafish embryo, including the yolk, does not substantially increase in size until the somite stages (from 11 hpf onward, Figure 1) [42]. In this chapter the challenge was met how to obtain enough RNA as input for a transcriptomics experiment from a single egg, which is a single cell, or from an embryo.

Chapter 2

Integrating heterogeneous sequence information for transcriptome-wide microarray design; a Zebrafish example.

There are a number of resources that identify or predict, and characterize genomic sequences, such as Ensembl, Vega, Refseq and Unigene. These resources still are incomplete and their information is continuously being updated [43]. In this chapter information from a number of resources is integrated and used to design the microarray that was used in the experiments of chapter 3 and 4.

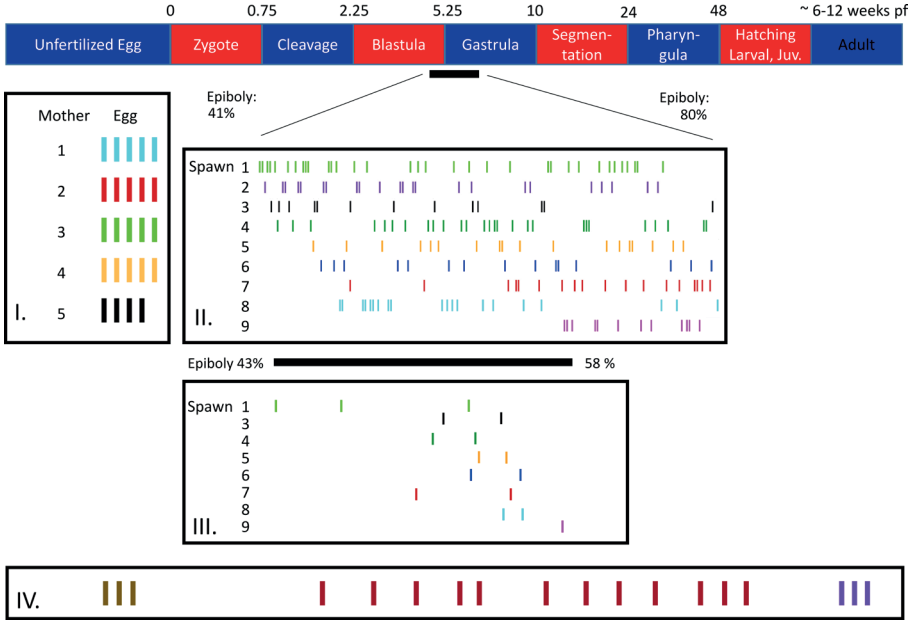

Figure 2. **Sample overview of the transcriptomics experiments in this thesis**
Transcriptomics experiments carried out in thesis to study transcriptome dynamics in early zebrafish embryogenesis. Top bar indicates on a non-linear scale developmental stages according to Kimmel [42] and Parichy [45]. Time indications are in hours post fertilization (hpf) except for the Adult stage (weeks post fertilization). Samples are visualized as vertical bars, spawn is indicated by color. I. Microarray transcriptome analysis of 24 unfertilized eggs that were obtained from five sibling mothers. II. Microarray transcriptome analysis of 179 individual embryos from nine different spawns taken at late blastula to early gastrula (41% to 80% epiboly). III. Small-RNA-sequencing of 16 individual embryos from the same spawns as II. IV. Small-RNA-sequencing of an unfertilized egg (3 technical replicates), 12 individual zebrafish embryos taken at the 64-cell stage to the protruding mouth stage, and an adult sample (3 technical replicates).

Chapter 3A
Mother-Specific Signature in the Maternal Transcriptome Composition of Mature, Unfertilized Zebrafish Eggs.

Maternal mRNA present in mature oocytes plays an important role in the proper development of the early embryo. As the composition of the maternal transcriptome in general has been studied with pooled mature eggs, potential differences between individual eggs are unknown. In this chapter we present a transcriptome study on individual zebrafish eggs from clutches of five mothers in which we study transcriptome dynamics by investigating the differences in maternal mRNA abundance per gene between and within clutches (Figure 2-I)

Chapter 3B
Transcriptome Data on Maternal RNA of 24 Individual Zebrafish Eggs from Five Sibling Mothers.

The data that is produced and the scripts that were developed for the study in chapter 3A are presented with a focus on the bioinformatics procedure that was developed and used to distinguish *'expressed'* and *'non-expressed'* genes.

Chapter 4
Transcriptome dynamics of early zebrafish embryogenesis determined by high-resolution time series analysis

The behavior of transcriptomes in individual embryos has hardly been studied yet. In this chapter we present a high-resolution gene-expression time series with 180 individual zebrafish embryos, obtained from nine different spawns, developmentally ordered and profiled from late blastula to mid-gastrula stage (Figure 2-II). On average one embryo per minute was analyzed. The focus is on identification and description of the transcriptome dynamics of the expressed genes in early embryogenesis. Gene-expression differences over time are analyzed as well as gene-expression differences between individuals.

Chapter 5
Cellular factors influencing transcriptome dynamics in early zebrafish embryogenesis

In this chapter the dynamic expression types that were defined in Chapter 4 are further analyzed, together with the set of genes that show a distinct spawn specificity. We investigate a number of mechanisms that may influence the observed transcriptome dynamics. For this, two small-RNA sequencing experiments using next-generation sequencing [44] are carried out (Figure 2-III and 2-IV) in which we focus on miRNA expression.

Chapter 6
Concluding remarks

In this last chapter our findings are put into perspective; how did our efforts to integrate genomic information work out?; what is the fate of maternal RNA?; what is the significance of the tight regulation of gene expression found in chapter 3 to 5?; and how can we interpret the relative gradual changes in gene expression we observed? We discuss our results and their relation to the studies of others, and give our opinion on possible approaches to deepen our insight into transcriptome dynamics.

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