Chapter 2

Link of Zygotic Genome Activation and Cell Cycle Control

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Abstract

The activation of the zygotic genome and onset of transcription in blastula embryos is linked to changes in cell behavior and remodeling of the cell cycle and constitutes a transition from exclusive maternal to zygotic control of development. This step in development is referred to as mid-blastula transition and has served as a paradigm for the link between developmental program and cell behavior and morphology. Here, we discuss the mechanism and functional relationships between the zygotic genome activation and cell cycle control during mid-blastula transition with a focus on *Drosophila* embryos.

Key words Cell cycle, Mid-blastula transition, Zygotic genome activation

1 Introduction

In most animals, from nematodes to chordates, embryogenesis starts with a series of rapid cleavage cell cycles after fertilization. These fast divisions lead to an exponentially increasing number of cells without an accompanied growth of the embryo. After a species-specific number of divisions, the cell cycle slows down and finally enters a pause. Subsequently, the embryo enters gastrulation with its characteristic morphogenetic movements, loss of symmetry, and cell type-specific differentiation. Mammalian embryogenesis is special in that it begins with differentiation of inner cell mass (ICM) and trophoblast, and the fast embryonic cleavage cycles eventually arise at late blastocyst stage [1–3]. Maternally supplied materials, including proteins, RNAs, and conceivably also metabolites contribute to the initial developmental processes. Maternal products exclusively control development during this first period, as the zygotic genome starts expression only with a delay after fertilization. Following zygotic genome activation (ZGA), both maternal and zygotic factors contribute to developmental control. The switch from maternal to zygotic control is especially prominent in species with large, externally deposited eggs. ZGA coincides with striking changes in cell behavior and molecular processes, including

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cell cycle, DNA replication, maternal RNAs degradation, chromatin structure, metabolite composition, and status of DNA checkpoint. This morphologically visible switch in early development during the blastula stage was first described 120 years ago in sea urchin *Echinus microtuberculat* and *Sphaerechinus granularis*, and later has been referred to as mid-blastula transition (MBT) [4, 5].

1.1 MBT in Model Organisms

Many model organisms are well studied in terms of MBT. Amphibian *Xenopus laevis*, for instance, undergoes 12 short and synchronized cleavage cycles with a lack of gap phases, 35 min each and proceeds with a series of progressively longer and less synchronized divisions from cycles 13 to 15. The transition period is defined as the MBT [5–8]. S phase progressively lengthens, and the cell cycle pauses in G1 or G2 phases during the MBT [9]. Concomitantly, maternal transcripts are deadenylated and degraded. The first zygotic transcripts are detected at cycle 7 and transcription rate increases up to and beyond MBT [10]. During the MBT, developmental control is handed over from maternal to zygotic factors (maternal-zygotic transition, MZT).

In zebrafish *Danio rerio* embryo, 9 rapid cycles with approximately 15 min each are followed by gradually longer cell cycles [11]. MBT begins at cycle 10, and the cell cycle loses synchrony with acquisition of a G1 phase in cycle 11 [12]. Similar to *Xenopus*, ZGA is regulated by the nuclear-cytoplasmic ratio, but DNA damage checkpoint acquisition is independent of zygotic transcription [13]. Maternal factors Nanog, Pou5f1, and SoxB1 are required for de novo zygotic transcription as well as inducing maternal clearance by activating the microRNA *miR-430* expression [14].

In the nematode *Caenorhabditis elegans* (*C. elegans*), zygotic transcription is already activated in the 4-cell stage. Multiple mechanisms and maternal factors, including OMA-1 and OMA-2, are involved and regulated by phosphorylation, nuclear shuttling, and protein destabilization [15, 16]. In contrast to the other species discussed above, cells divide asynchronously and asymmetrically following fertilization in *C. elegans* embryos [17, 18].

1.2 MBT in Drosophila

MBT is observed in embryos of *Drosophila melanogaster* at about 2 h post fertilization. Embryonic development starts with 13 rapid and meta-synchronized nuclear divisions, with extraordinary short S phases and no gap phases [19]. The extraordinary speed of about 10 min per pre-blastoderm cell cycle is achieved by fast replication of DNA and the absence of cytokinesis [20–22]. The syncytial mode of early development is a special feature of insect embryogenesis [23]. Due to the absence of cytokinesis, the early cell cycles are often referred to as nuclear cycles (NC). The onset of the embryonic cell cycle is regulated by *pan gu*, *plutonium*, and *giant nuclei* [24–27]. From NC8 to 9, the nuclei move from the interior of the egg toward the periphery, forming the syncytial blastoderm. From NC10 to 13, nuclei undergo four more divisions at the egg cell cortex, until the nuclei number reaches approximately 6000. Some nuclei remain in the interior egg to differentiate into polyploid yolk nuclei. After mitosis 13, the cell cycle mode changes with the introduction of a long G2 phase, and the embryo enters into cellularization stage [19]. Following NC11, the cell cycle gradually slows down from 10 min in NC11 to 21 min in NC13 and an hour-long G2 pause in interphase 14 (25 °C) [19]. The S phase lengthens and by cycle 14 a difference between early and late replicating euchromatin and the satellite DNA becomes obvious. In addition, the usage of replication origins changes [28].

Interphase 14 corresponds to the MBT in *Drosophila*. Interphase 14 is the stage when the cell cycle pauses in a G2 phase, zygotic transcription strongly increases, and DNA replication switches to a slow replication mode. During interphase 14, visible morphology changes from the syncytial to cellular blastoderm, in a process called cellularization. Cellularization is the first morphological process that depends on zygotic gene products [29, 30].

However, the first signs of MBT are already visible earlier. As mentioned above, the extending interphases in NC11–14 depend on zygotic transcription. The first transcripts and activated RNA polymerase II (Pol II) can be already detected in pre-blastoderm stages. Transcription slowly increases until cycle 12. In cycle 13 many zygotic genes are clearly expressed [31]. Genome-wide analysis showed that gene expression is initiated at different time points throughout early development [32, 33], suggesting that rather than a sharp switch, MZT is likely regulated by multiple and diverse mechanisms [9, 34, 35]. The timing of these multiple and diverse mechanisms depends, to a certain degree, on the ratio of nuclear and cytoplasmic content (N:C ratio). This is further discussed in Subheading 5.

Approximately, two-thirds of all genes are contained in *Drosophila* eggs as maternal mRNAs [34, 36]. A third of all maternal transcripts are eliminated in stages leading to MBT in three ways [36]: First, maternally encoded factors activate mRNA degradation of over 20% of maternal transcripts after egg activation in a ZGA-independent manner [34, 37–39]. The RNA-binding protein Smaug is such a factor, acting together with the CCR4/POP2/NOT deadenylase complex [38, 40, 41]. Another RNA-binding protein, Brain Tumor, functions in a similar way [42]. Second, 15% of maternal mRNAs are eliminated depending on zygotic transcription during MBT [43, 44]. Third, microRNAs induce maternal RNA degradation. More than 100 maternal transcripts are degraded depending on zygotically expressed microRNAs from the *miR-309* cluster, which is activated by the early zygotic transcription factor Vielfältig/Zelda [45–47].

2 Mechanism of Zygotic Genome Activation

Transcription of the zygotic genome only begins shortly after fertilization [48]. The highly dynamic transcription profile was characterized by number of methods, including high-throughput strategies, global run-on sequencing (GRO-seq), and fluorescent labeling of nascent RNA [14, 49–52]. In general, the initiation of low-level zygotic transcription, mostly of signaling and patterning genes, already appears before NC10 ahead of large-scale ZGA [31, 53]. These include small and intron-less genes, as well as genes with TAGteam DNA motif in the control region [36]. A comparable profile is also observed in that of the zebrafish [54]. Full activation of zygotic transcription is observed during MBT, when thousands of genes are transcriptionally activated and transcribed in high levels. Taken together, the activation of the zygotic genome is a gradual process rather than a single sharp switch. This suggests that ZGA is triggered by multiple and diverse events [9, 34, 35].

A contribution to ZGA is intrinsically provided by the division of nuclei and doubling of DNA with every nuclear cycle. Even with a constant activity of the individual zygotic transcription units, the total number of transcripts would exponentially increase. In general, zygotic transcription is quantified in relation to the number of embryos, total mass of embryos (protein or total RNA content), or in comparison to an abundant maternal RNA, such as ribosomal RNA. Most of the older data are based on samples prepared from mixed stages comprising several nuclear division cycles. Alternatively, zygotic transcription may be normalized to the number of nuclei in an embryo. Given recent technological advances, transcription profiling can be conducted with few or even single *Drosophila* embryos, allowing highly accurate staging according to the nuclear division cycle [33, 55]. Such normalization is important to reveal the actual transcriptional activity of a locus.

This hypothesis was tested with normalized transcriptional profiles of selected early zygotic genes (Fig. 1) based on a data set from manually staged embryos [56]. Normalization to the number of nuclei was performed with the assumption of a doubling with every cell cycle. In case of a doubling transcript number from one cycle to the next, this results in a zero value. An increase in transcript number higher than a factor two results in a positive number, whereas an increase less than a factor two, in a negative number (Fig. 1). This simple and exemplary calculation indicates that both the increasing number of nuclei and an increased activity of the transcription units contribute to the overall increase in zygotic transcripts per embryo. There is, however, also transcript-dependent variation. A similar finding was reported recently for dorsoventrally patterning genes [57]. This indicates that depending on the zygotic gene, both an increased activity of individual transcription units and an increased number of transcription units/nuclei contribute to ZGA.

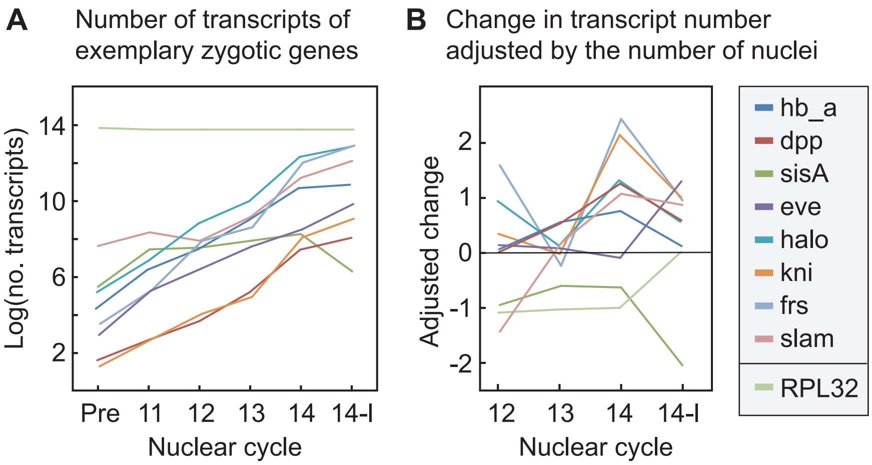

Fig. 1 Zygotic transcription and number of nuclei. (**a**) Number of selected zygotic transcripts based on NanoString analysis with extracts from manually staged embryos plotted on a logarithmic scale. (**b**) The number of transcripts was normalized to the number of nuclei that double with every cycle. Plotted is the difference of log2 of the number of transcripts from one cycle to the previous cycle minus 1. The number of transcripts in pre-blastoderm stages is not included. Transcripts for the ribosomal protein L32 serve as a reference. Staging by the nuclear cycle, pre-blastoderm stage (Pre) and late cellularization (14-l). Data are from Sung et al. [56]

2.1 Vielfältig/Zelda Functions in ZGA Regulation

The zinc-finger protein Vielfältig/Zelda (Vfl/Zld) plays a major role in ZGA. Vfl/Zld specifically binds to TAGteam elements in the early *Drosophila* embryo. The TAGteam CAGGTAG sequence was identified by genome-wide studies as a general *cis*-regulatory element and as the most highly enriched regulatory motif in genes involved in anterior-posterior patterning [36, 58, 59]. Vfl/Zld is an essential transcriptional activator during early zygotic gene expression, as demonstrated by the strongly reduced (but not absent) expression of many early zygotic genes in embryos from females with *Vfl/Zld* mutant germline [60]. *Vfl/Zld* is maternally deposited and uniformly distributed throughout the egg and early embryo. The Vfl/Zld protein levels increase coincidently with the activation of zygotic genome during pre-blastoderm stage, prior to large-scale transcription [49, 61].

Vfl/Zld consists of a cluster of four zinc fingers and a low-complexity activation domain, both of which are required for promoting DNA binding and mediating transcriptional activation [62]. Vfl/Zld binding to promoters is detected already in NC8 for particular genes and roughly a thousand genes during NC10 [63, 64]. The DNA binding is maintained at least until NC14 [49]. During ZGA, Vfl/Zld-binding sites are highly enriched specifically in regions of accessible chromatin, allowing transcription factors to subsequently bind and drive zygotic transcription [63, 64]. Thus, Vfl/Zld acts as a co-activator during MZT. Vfl/Zld also controls the accurate temporal and spatial expression of microRNAs [46].

2.2 RNA Polymerase II Pausing

The binding of Pol II to promotor sequences is the key to transcriptional activation and elongation. Pol II regulates ZGA by three distinct binding statuses: active, no binding, and stalled/paused [65]. Among them, paused Pol II is critical in *Drosophila* ZGA, because approximately 100 genes are bound by active Pol II from NC8 to 12, yet in NC14, over 4000 promotors are occupied by Pol II at the transcription start site (TSS) [55, 66]. Furthermore, compared with NC12, loci with paused Pol II near the TSS show a significant increase in NC13 [67].

2.3 Epigenetics and ZGA

Epigenetic marks, including histone modifications and chromatin remodeling, dramatically change in early embryogenesis and MBT. Formation of heterochromatin correlates with the emergence of late replication. Heterochromatin Protein 1 (HP1) together with histone modifications on H3K9 and H3K4 is involved in establishing of tightly packed chromatin structure [68, 69]. Modifications of lysine acetylation and methylation in histones H3 and H4 appear during MZT. In zebrafish, a striking change in histone modification correlates with ZGA [70]. An increase in histone methylation during MZT matches high level of zygotic transcription [70, 71]. In *Xenopus* embryo, maternally provided histones H3/H4 and their modification states control the regulation of transcriptional activation and cell cycle lengthening [72, 73]. Similarly, during *Drosophila* early development, genome-wide studies showed that domains of histone methylation H3K4me1, H3K4me3, H3K27me3, and H3K36me3 increased from undetectable to widespread level at NC14 [48, 55, 74]. Levels of acetylation on H3K9 appear correspondingly to methylation marks, whereas H3K18ac, H3K27ac, and H4K8ac levels are evidently precocious at NC12 [48]. These early appearing acetylation marks are strongly correlated with maternal DNA-binding protein Vfl/Zld, demonstrating that Vfl/Zld may regulate transcriptional activation by recruiting histone acetylation, thus allowing opening of genome state [34, 48]. In contrast, the mark H4K5ac, whose level was previously shown to bookmark active transcription in mammalian cells, decreases from NC8 with the slowdown of the cleavage cycles [48, 75]. In addition to histone modifications, remodeling of nucleosomes and linker histones with histone variants may contribute to ZGA. *Drosophila* maternal-specific linker histone H1 dBigH1 is replaced by somatic H1 in early development [76]. dBigH1 seems to suppress ZGA, since increased levels of activated Pol II and expression of zygotic genes are observed in embryos with reduced dBigH1 levels [76].

Both histone modification and Vfl/Zld DNA binding ultimately affect transcriptional activation by altering chromatin accessibility. Highly accessible chromatin regions are locally and globally marked by H3/H4 acetylation and Vfl/Zld enrichment from NC8 to 12 in *Drosophila* [77]. In NC13, however, thousands of enhancers and promotors with nucleosome-free regions accumulate additional transcription factors in a cascade way [48, 78]. This phenomenon has also been observed in zebrafish [79].

2.4 Other Regulators

Drosophila zygotic transcription is modulated by multiple factors including *cis*-regulatory elements. For instance, TATA-dependent promoters, as well as enhancers, are central in transcriptional regulation [80, 81]. Distinct enhancer-core-promoter specificities ensure that developmental and housekeeping genes are activated precisely across the entire genome [81]. Likewise, the post-transcriptional regulation of TATA-binding protein (TBP) affects transcription pattern together with the earliest transcribed genes during the MZT [55]. Smaug may involve ZGA regulation through maternal clearance of transcription factor *tramtrack* mRNA, which is involved in triggering transcription of transcripts depending on the N:C ratio [38, 53].

3 Switch in Cell Cycle Mode During the MBT

The cell cycle switch from a fast syncytial mode to a mode with slow replication and extended G2 phase is the most obvious aspect of MBT in morphological terms. A long-standing question is the functional relationship of the cell cycle switch with ZGA. According to one model, the cell cycle switch allows for the strong increase in zygotic transcription (Fig. 2) [82]. In the opposing model, zygotic

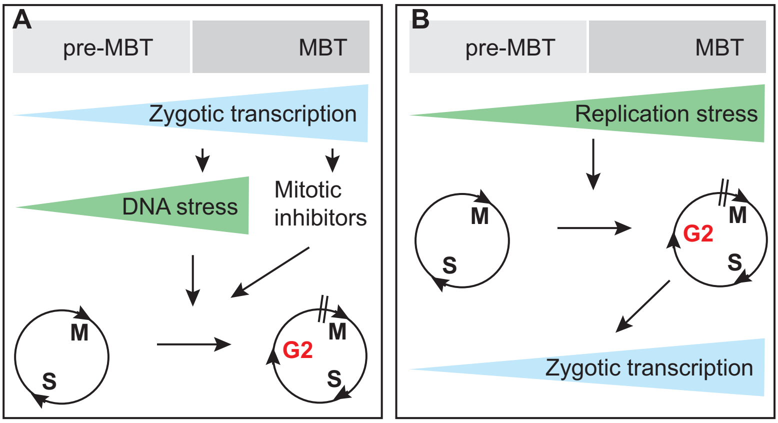

Fig. 2 Models for the control of cell cycle remodeling during MBT. (**a**) The onset of zygotic transcription leads to the activation of the DNA checkpoint due to interference of transcription and replication as well as expression of mitotic inhibitors. These two processes lead to the cell cycle remodeling. (**b**) Activation of the DNA checkpoint, caused by limiting amounts of replication factors, for example, triggers a slowdown and subsequent pause of the cell cycle. The longer interphase promotes zygotic transcription

transcription triggers the remodeling of the cell cycle [56, 67]. Depending on the experimental system, strong experimental evidence speaks in favor of the first or the second model. A synthesis has not been achieved, yet.

3.1 Cell Cycle Regulation in Drosophila Early Embryogenesis

Cyclin and its partner cyclin-dependent kinase (Cdk) are essential for cell cycle control. In *Drosophila*, cyclin A/B/B3:Cdk1 complexes regulate entry into M phase [20, 83]. The rapid S phases in pre-MBT cycles are maternally controlled, and the catalytic activity level of cyclin:Cdk1 complexes determines the timing for mitotic entry [21, 84]. Distinct mechanisms regulate cyclin:Cdk1 complexes in pre-MBT: First, during each nuclear division, Cyclin A, B and B3 proteins are synthesized in S phase by maternally supplied mRNA [85, 86], and degraded in mitosis by the ubiquitin pathway [87, 88]. Cyclin A, B, and B3 fulfill a redundant but essential function, as RNAi-mediated depletion stops the syncytial cycles [20, 89]. Cyclin B levels also contribute to the cell cycle switch as changes in *cyclin B* gene dose affect the number of nuclear divisions [90]. Second, the inhibitory phosphorylation of T14Y15 sites of Cdk1 are pairwise regulated by maternally supplied kinases Wee1/Myt1 and phosphatase Cdc25/Twine [85, 91–95]. Therefore, Cdk1 is timely activated and inactivated by controlling T14Y15 inhibitory phosphorylation sites [96].

3.2 Cdc25/Twine Degradation at the MBT

In NC14 and to a certain degree already in NC12 and 13, S phase lengthens and a G2 phase is introduced. Central to these changes is the induced inactivation and final degradation of the phosphatase Cdc25/Twine [97, 98] (Fig. 3). *Drosophila* Cdc25/Twine is a dual specificity phosphatase that activates cyclin:Cdk1 complexes by removing inhibitory phosphates from the ATP-binding sites T14 and Y15 [22, 87, 99, 100]. Twine protein is present in high levels during the pre-MBT cycles. Twine protein localization is dynamic with a nuclear accumulation during interphases and uniform dispersal during mitosis [98]. The half-life of Twine was estimated to about 20 min during pre-MBT cycles [98]. Yet with the beginning of NC14, Twine becomes destabilized as indicated by the shortening of its half-life to only about 5 min [98]. Degradation of Twine is required for the cell cycle switch because embryos expressing a more stable version of Twine protein (Twine$^{106\text{-}180}$) undergo an extra mitotic division [98]. The rapid destabilization is the key to the cell cycle switch during MBT, as it depends on the N:C ratio and on zygotic transcription [98].

Prior to MBT, the steady-state level of Twine is relatively stable due to balanced synthesis and degradation. The link of zygotic transcription and the switch-like decrease in the half-life of Twine suggests that zygotic factors may be involved. One of these factors is the pseudokinase Tribbles [101–103], as RNAi-mediated depletion of *tribbles* accelerates Twine degradation [97].

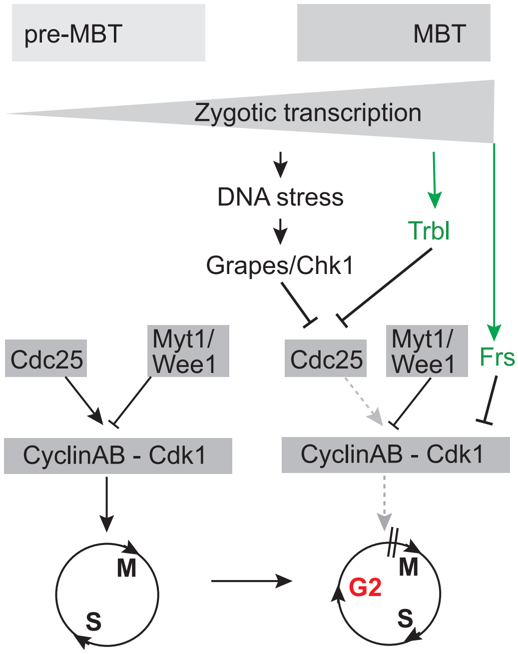

Fig. 3 Model of cell cycle remodeling in Drosophila. Cyclin:Cdk1 is activated by the phosphatase Cdc25 and inactivated by the kinases Myt1/Wee1. In pre-MBT Cyclin:Cdk1 activity is high and promotes fast cell cycles. During MBT the balance of Cyclin:Cdk1 control is shifted toward low activity. Cdc25 is inhibited by the DNA checkpoint, which is activated by DNA stress caused by interference of DNA replication and zygotic transcription. In addition, the zygotic mitotic inhibitors, Tribbles and Frühstart, promote Cdc25 degradation and inhibition of the Cyclin:Cdk1 complexes, respectively

However, *tribbles* is not essential for the cell cycle switch, since embryos deficient for maternal and zygotic *tribbles* do not undergo an extra nuclear cycle [101, 102]. The mechanism for how *tribbles* induces Twine degradation remains unknown, but in other organisms such as yeast, *Xenopus*, and human cells, Cdc25 (or Cdc25C) degradation is induced by phosphorylation due to multiple pathways [56, 104, 105]. In addition to induced destabilization of Cdc25/Twine at NC14, additional mechanisms control pre-MBT levels and activity of Twine. The number of pre-MBT cell cycles is rather insensitive to changes in *twine* gene dose. A tripling of *twine* gene dose to 6×*twine*[+] induces an extra nuclear division in only a few embryos [106], suggesting that mechanisms exist that make Twine protein levels independent of gene dose.

The second *Drosophila* homologue of Cdc25, String, has distinct developmental functions in cell cycle control [84, 107]. String but not Twine is required for mitotic entry in zygotically controlled cycles 14–16. In contrast to these later stages, *string* is not required for progression of the syncytial cell cycles [84]. Premature expression of *string* is sufficient to trigger mitotic entry during later stages of embryonic development but not in pre-MBT stages [84]. Although both *string* and *twine* mRNAs are destructed in interphase 14 [106], String protein stability gradually decreases during syncytial cycle without a sharp switch before MBT [97]. String protein turnover is due to increased checkpoint activity [98].

3.3 DNA Replication Checkpoint at NC13

Before the switch in cell cycle mode in NC14 in *Drosophila*, S phases show a progressive lengthening from 3.4 min in NC8 to 14 min in NC13 [21, 108]. A critical regulator of the slowdown of replication is the *Drosophila* homologue of checkpoint kinase Chk1, Grapes [109]. Grapes starts to inhibit cyclin:Cdk1 activity by promoting the activity of kinases Wee1/Myt1 and suppressing the activity of phosphatase Cdc25, thereby shifting the balance to T14Y15 inhibitory phosphorylation of Cdk1 from NC11 onward [109, 110]. Grapes mediates the DNA replication checkpoint and ensures that cells do not enter mitosis while replication is ongoing. *grapes* mutants prematurely enter mitosis during syncytial divisions, which leads to mitotic catastrophe, as incompletely replicated chromosomes cannot be segregated in anaphase [109, 110]. The checkpoint kinase, ataxia telangiectasia and Rad3-related (ATR, Mei-41 in *Drosophila*), acts upstream and activates Chk1/Grapes similar as in *Xenopus* [111, 112]. *mei-41* mutants show a similar phenotype during syncytial divisions as *grapes*, indicating a functional replication checkpoint is required at the MBT [67].

In Drosophila the DNA checkpoint is triggered by ZGA. Blocking transcription by α-amanitin in *Drosophila* pre-MBT embryos does not suppress lethality of *mei-41* mutant [67]. Nonetheless, embryos from *mei-41 Vfl/Zld* double mutant mothers could partially suppress the mitotic catastrophe, indicating that replication has been finished in time [67]. These observations are consistent with the model that zygotic transcription reduces replication speed and induces DNA stress, leading to DNA checkpoint activation at ZGA [56, 67].

3.4 Other Regulators

In *Drosophila*, cyclin-dependent kinase inhibitor (CKI) Frühstart is another zygotic regulator, which functions to inhibit cyclin:Cdk1 activity by binding the hydrophobic patch of cyclins, thereby interfering with Cdk1 substrate recognition [101, 113, 114]. Together with large-scale ZGA, *frühstart* starts transcription immediately after mitosis 13, and generates a uniform cell cycle pause in cycle 14 [114]. In the absence of Frühstart, embryos enter an extra round of nuclear division especially in embryos with extra copies of *twine*[+] [114]. The expression of Frühstart depends on the N:C ratio, suggesting that Frühstart is involved in the link of N:C with cell cycle regulation [115]. Wee1 and Myt1 kinases are Cdk1 inhibitors that oppose functions to Cdc25 phosphatases [91–93, 116, 117] (Fig. 3). Wee1 can be activated by Grapes, and inhibits Cdk1 activity by adding inhibitory phosphorylation at T14 and Y15 sites [9, 118, 119]. Cyclin:Cdk1 activity is also influenced by some other factors such as mitotic kinase Aurora-A and acquisition of late-replicating heterochromatin domains [95, 120].

In summary, the switch of the cell cycle from a fast syncytial mode to a slow embryonic mode is controlled on two levels of inhibition: (1) indirectly by interference of zygotic transcription with DNA replication and subsequent activation of the DNA checkpoint, (2) directly by expression of zygotic genes encoding mitosis inhibitors.

4 What Is the Trigger for MBT?

The MBT cell cycle switch depends on ZGA (Fig. 2). First, injection of α-amanitin, a Pol II inhibitor, before MBT induces an extra synchronized mitotic division, indicating that widespread zygotic transcription is required for the cell cycle switch in *Drosophila* [106]. Second, ZGA correlates with DNA stress. About 80% of the RpA-70-GFP-binding sites in early MBT cycles also have RNA Pol II bound [67]. RpA70-GFP marks sites of DNA stress [121]. This indicates that ZGA causes DNA stress and activates the DNA checkpoint [67]. Third, a precocious onset of zygotic transcription is sufficient for an earlier MBT [56]. Fourth, dependent on ZGA, Tribbles and other factors trigger Twine destruction in NC14, resulting in inhibition of Cdk1 activation, thereby pausing the cell cycle [101, 102].

The essential role of the DNA checkpoint for triggering MBT was initially shown by the analysis of the checkpoint mutants, *grapes*/Chk1 and *mei-41*/ATR, in *Drosophila* [109, 111]. Embryos from *grapes* females do not switch the cell cycle mode and do not enter MBT, indicating that the DNA checkpoint is required for MBT in *Drosophila* [67, 109]. Based on the observation that *grapes* embryos would not express zygotic genes, the authors concluded that the checkpoint would be upstream of ZGA [109]. Recent data clearly show, however, that ZGA is normal in checkpoint-deficient embryos and that the initial observation was probably due to technical difficulties in detecting expression of early zygotic genes [67].

An alternative source for checkpoint activation beside interference of replication and transcription are limiting amounts of replications factors. Experiments from mostly *Xenopus* support this model (Fig. 2). In *Xenopus* embryos slowdown of DNA replication has been proposed to be upstream of ZGA [82]. The replications factors Cut5, RecQ4, Treslin, and Drf1 become limiting in MBT, which leads to an activation of the DNA checkpoint, slowdown of the cell cycle, and ZGA [82].

In summary, in vivo and genetic experiments provide strong evidence for the model that ZGA is the trigger for MBT in *Drosophila*. ZGA acts upstream of cell cycle control, including the DNA checkpoint and degradation of Cdc25/Twine. First, ZGA is required for MBT and timely cell cycle pause; second, ZGA is associated with induction of replication stress in time and space (on the chromosome); third, precocious ZGA leads to precocious MBT. In other organisms experimental evidence mainly in *Xenopus* speaks in favor of the alternative model, i.e., that cell cycle control acts upstream ZGA. However not all three criteria are fulfilled in vivo: the mechanism should be necessary, sufficient, and temporally and spatially associated with MBT.

5 What Is the Timer for MBT?

A central unresolved question concerning MBT is the timing mechanism for the associated processes including ZGA and number of pre-MBT cell cycles. Tight control of the cell cycle is important for further embryonic development, since the number of divisions determines the cell number and size. Too few cells may be incompatible with the formation of stripes of pair-rule gene expression, for example, as stripes should be at least one cell wide.

5.1 Molecular Clocks

With the onset of embryonic development, fertilization may trigger a molecular clock, on which MBT and its associated processes may depend. A conceivable mechanism is translation of certain maternal mRNAs, which would lead to a time-dependent accumulation of the product following onset after fertilization. Translational regulators such as FMRP are required for MBT regulation in *Drosophila*, through dynamically regulating RNA metabolism and controlling the availability of specific transcripts, as well as mediating the *frühstart* mRNA activation level [122, 123]. A target for translational regulation may be Vfl/Zld, whose protein level increases during blastoderm concomitantly with activation of zygotic transcription [34, 124].

Maternal RNA degradation may represent a second such a mechanism constituting a molecular clock. A large fraction of these maternal RNAs is degraded following egg activation and independent of zygotic transcription. For some RNAs at least, the degradation proceeds with a constant speed [38, 56], and may in this manner constitute a molecular clock. It has been proposed that the speed of RNA degradation affects the number of nuclear divisions, as expression levels of *smaug* affect the timing of MBT [40, 125]. Distinct from Vfl/Zld, Smaug reaches its peak expression level at NC10, and performs downregulation at the MBT [38, 125]. Smaug is functional to mRNA clearance, and times the ZGA through inducing the destruction of maternal transcriptional inhibitor [27].

5.2 N:C Ratio as a Clock

In contrast to a molecular clock as an absolute timer, more evidence speaks in favor of a regulatory process. The morphologically visible MBT depends on genome ploidy, because haploid embryos undergo one more division and tetraploid embryos, one less division [11]. It has been proposed that the N:C ratio represents the timer for MBT. Nuclear content is determined by the amount of DNA or chromatin, which doubles with every cell cycle, whereas cytoplasmic content remains constant during cleavage divisions. The embryo may measure the N:C in that the increasing amount of chromatin titrates a constant cytoplasmic factor until this becomes rate-limiting [6, 53]. Potential cytoplasmic factors are repressors of transcription, replication, or the cell cycle, for example. In *Xenopus* embryos, DNA content is important for MBT [5, 7]. Injection of purified DNA leads to precocious onset of zygotic transcription, as measured by total transcription rate [7]. However, the amount of DNA seems not to be the only determinant, since an increased or decreased nuclear volume, while keeping the DNA content unchanged, leads to a precocious or delayed MBT including zygotic activation and corresponding cell cycle remodeling [126]. Similar findings come from zebrafish that the timing of ZGA is governed by the N:C ratio [13].

It is unclear what is titrated by the exponentially increasing amount of DNA and chromatin, but maternal histones proteins H3/H4 may be a central factor [72]. Depletion and overexpression of H3/H4 delay the cell cycle switch, and also induce premature transcriptional activation [72]. In *Drosophila* embryos, the maternal form of the linker histone H1 dBigH1 has been implicated in the timing of MBT [76]. Maternal dBigH1 is replaced by the somatic form in early embryogenesis. Embryos with half of the maternal contribution and lacking zygotic expression show increased levels of activated Pol II and zygotic gene expression. However, the link of dBigH1 to MBT remains unclear as mutant defects and embryonic genotypes were not analyzed with sufficiently high temporal resolution and with respect to MBT and ZGA.

The replication factors Cut5, RecQ4, Treslin, and Drf1 have been found to be limiting for replication initiation during MBT in *Xenopus* embryos [82]. Titration of the maternal pool of these replication factors by the exponentially increasing chromatin leads to slower replication initiation, ZGA, longer interphases, and DNA checkpoint activation.

Other cytoplasmic factors may also be titrated, such as metabolites. It has been proposed that deoxynucleotides may serve as a marker for the cytoplasm [127]. The maternal pool may be incorporated in the exponentially increasing amounts of DNA. The existence of such a maternal pool is well known, as inhibition of zygotic synthesis by hydroxyurea (HU), which inhibits the NDP reductase, causes a cell cycle arrest only briefly before MBT [127].

Although it is clear that ploidy determines the number of pre-MBT cell cycles in model organisms, it is much less clear whether all of the MBT-associated processes, including ZGA, cell cycle, RNA degradation, are controlled by the N:C ratio. Haploid *Drosophila* embryos switch the cell cycle mode only after an extra division 14 in NC15 [115, 128]. In contrast, ZGA does not depend on the N:C ratio in *Drosophila*. Although older data indicated a link of ploidy and ZGA in *Drosophila* [53], genome-wide analysis of embryonic transcripts with carefully staged *Drosophila* embryos revealed that the majority of zygotic transcripts (127 out of 215 genes) show an expression profile comparable between haploid and diploid embryos [115]. These data suggest that ZGA timing is controlled by a molecular clock in *Drosophila*. However, a small set of zygotic transcripts (88 out of 215 genes) shows clearly delayed expression in haploid embryos [115]. This small gene set includes genes encoding mitotic inhibitors such as Frühstart [114], which are involved in the MBT-associated remodeling of the cell cycle.

6 Conclusions

Recent years brought striking advances in our understanding of zygotic genome activation and its relation to MBT. This is mainly due to improved technology now allowing to analyze transcriptional activity and chromosome status with high resolution and importantly with very little material, down to single embryos. In this way, the variation and limited temporal resolution of mixtures of many embryos can be overcome. Despite this progress, there is no unifying model for zygotic genome activation, MBT, and cell cycle control. Conclusion on central questions and favored models depend on the experimental system. Strong evidence supports the model that DNA replication onset triggers MBT and ZGA in *Xenopus*. However, the alternative model is supported by convincing experiments from *Drosophila*, where ZGA triggers MBT and cell cycle remodeling. It will be the task for future work to reconcile these opposing views. Having the new technologies available and standardized, we can expect new and surprising findings to come.

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