Number of Nuclear Divisions in the Drosophila Blastoderm Controlled by Onset of Zygotic Transcription

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Summary

The cell number of the early Drosophila embryo is determined by exactly 13 rounds of synchronous nuclear divisions, allowing cellularization and formation of the embryonic epithelium [1]. The pause in G2 in cycle 14 is controlled by multiple pathways, such as activation of DNA repair checkpoint, progression through S phase, and inhibitory phosphorylation of Cdk1, involving the genes grapes, mei4, and wee1 [2–6]. In addition, degradation of maternal RNAs [9] and zygotic gene expression [10, 11] are involved.

The zinc finger Vielfältig (Vfl) controls expression of many early zygotic genes [12, 13], including the mitotic inhibitor frühstart [14, 15]. The functional relationship of these pathways and the mechanism for triggering the cell-cycle pause have remained unclear. Here, we show that a novel single-nucleotide mutation in the 3′ UTR of the RNPII215 gene leads to a reduced number of nuclear divisions that is accompanied by premature transcription of early zygotic genes and cellularization. The reduced number of nuclear divisions in mutant embryos depends on the transcription factor Vfl and on zygotic gene expression, but not on grapes, the mitotic inhibitor frühstart, and the nucleocytoplasmic ratio. We propose that activation of zygotic gene expression is the trigger that determines the timely and concerted cell-cycle pause and cellularization.

Results and Discussion

Embryos from germline clones of the lethal mutation X161 (in the following, designated as mutant embryos) showed a reduced cell number but otherwise developed apparently normally until at least gastrulation stage (Figures 1A and 1B; 24 of 61 embryos). Cell specification along the anterior-posterior and dorsoventral axes proceeded as in wild-type, as demonstrated by the seven stripes of eve expression, mesoderm invagination, and cephalic furrow formation. The reduced cell number can be due to a lower number of nuclear divisions prior to cellularization or to loss of nuclei in the blastoderm. To distinguish these possibilities, we performed time-lapse recordings of mutant embryos in comparison to wild-type (Figure 1C and Table 1). To measure the cell-cycle length, we fluorescently labeled the nuclei in these embryos. We observed three types of embryos: (1) with 13 nuclear divisions with an extended interphase 13 (28 min versus 21 min in wild-type), (2) with 12 nuclear divisions, and (3) with partly 12 and partly 13 nuclear divisions with an extended interphase 13. Because we did not observe a severe nuclear fallout phenotype, we conclude that the reduced cell number in gastrulating embryos is due to the reduced number of nuclear divisions. Consistent with these observations, the number of centromeres and centrosomes was normal in mutant embryos (see Figure S1 available online).

In wild-type embryos, interphase 14 is different from the preceding interphases, in that the plasma membrane invaginates to enclose the individual nuclei into cells. In X161 embryos with patches in nuclear density, furrow markers showed more advanced furrows in the part with a lower number of divisions, indicating a premature onset of cellularization (Figure 1D). Furthermore, in time-lapse recordings, we first measured the speed of membrane invagination, finding no obvious difference between X161 and wild-type embryos (Figure S1). Additionally, we investigated cellularization by live imaging with moesin-GFP labeling F-actin (Figure 1E). Clear accumulation of F-actin at the furrow canals was observed in wild-type embryos after about 20 min in interphase 14, but not in interphase 13. In X161 embryos with 12 nuclear divisions, we observed a comparable reorganization already in interphase 13 about after 25 min. This analysis shows that both the cell-cycle pause and cellularization are initiated in X161 embryos earlier than in wild-type embryos.

To identify the mutated gene in X161, we mapped the lethality and blastoderm phenotype (Figure S2). The X161 gene was separated from associated mutations on the chromosome by meiotic recombination and mapped to a region of four genes by complementation analysis with duplications and deficiencies. Sequencing of the mapped region and complementation tests with two independent RPII215 loss-of-function alleles, RPII215[G0040] [16, 17], and a transgene comprising the RPII215 locus revealed the large subunit of the RNA polymerase II as the mutated gene. We identified a single point mutation in the 3′ UTR of RPII215 about 40 nt downstream of the stop codon. This region in the 3′ UTR is not conserved and does not show any obvious motifs (Figure S2).

To test whether the mutation in the noncoding region affects transcript or protein expression, we quantified mRNA levels by reverse transcription and quantitative PCR and protein levels by whole-mount staining and immunoblotting with extracts of manually staged embryos. We found that mRNA levels were not different in wild-type and X161 (Figure 2A and Table S1). In contrast, immunohistology and immunoblotting revealed reduced RPII215 protein levels (Figures 2B and 2C). In summary, our analysis shows that the X161 point mutation within the 3′ UTR affects mainly RPII215 protein levels.

The precocious onset of cellularization raised the hypothesis that the timing of zygotic gene expression may be affected in the X161 embryos. To establish the expression profiles of selected maternal and zygotic genes, we employed nCounter NanoString technology [18] with embryos staged by the nuclear division cycle (Figure S3 and Table S2). Embryos expressing histone 2Av-RFP were manually selected 3 min after anaphase of the previous mitosis or at midcellularization. We first analyzed expression of ribosomal proteins (Figure S3).
They did not change much and were not different in wild-type and mutant embryos, confirming the robustness of the method. Zygotic genes, whose expression strongly increases during the syncytial cycles, showed an earlier upregulation in X161 than in wild-type embryos (Figure 3A). Comparing the profiles by plotting the ratio of the expression levels (Figure 3B), we revealed a clear difference in cycle 12, with a factor of up to ten, indicating that zygotic genes are precociously expressed in X161 embryos. The premature expression of early zygotic genes was confirmed by whole-mount in situ hybridization for slam and frs mRNA (Figure S4).

Next, we analyzed expression profiles of RNAs subject to RNA degradation. We selected transcripts representative for the two classes of degradation, depending on zygotic gene expression (Figure 3C), and on egg activation (Figure S3) [19–23]. Degradation of string, twine, and smaug transcripts in interphase 14 depends on zygotic gene expression. In X161 mutants, the mRNA of these three genes was degraded already in cycle 13, slightly sooner than in wild-type (Figure 3C). The profiles of string and twine RNA were confirmed by RNA in situ hybridization (Figure S4). Consistent with the a clear difference between the profiles of wild-type and X161 mutants. Our data show that zygotic gene expression stars earlier in X161 than in wild-type and that degradation of mRNAs follows zygotic gene expression.

The cell cycle may be paused prematurely by altered levels of maternal factors, such as CyclinB, grapes, and twine, or by precociously expressed zygotic genes, such as frs and trbl [14, 15, 24]. To distinguish these two options, we analyzed mutant embryos with suppressed zygotic gene expression (Figures 4A and 4B). Embryos injected with the RNA polymerase II inhibitor ρ-amanitin develop until mitosis 13 but then fail to cellularize and may undergo an additional nuclear division, depending on injection conditions [10, 25]. Using this assay, we tested whether zygotic genes are required for the reduced number of nuclear divisions in X161 mutants. If the precocious cell-cycle pause were due, for example, to reduced levels of CyclinB mRNA, ρ-amanitin injection should not change the phenotype.

Finally, we analyzed the profile of mRNAs whose degradation depends on egg activation (Figure S4). We did not detect a consistent pattern and precocious RNA degradation in X161, Twine and String protein levels decreased already in interphase 13 of X161 embryos (Figure 3D).

Table 1. Reduced Number of Nuclear Divisions in X161 Mutants

<table>
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<tr>
<th>Genotype</th>
<th>Pause after n</th>
<th>Cell Cycle (Length in Minutes)</th>
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<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
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<td>Wild-type</td>
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<td>18</td>
<td>9.9 ± 1.1</td>
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<td>14.8 ± 1.1</td>
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<td>57.1 ± 4.4</td>
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<td>X161</td>
<td>13</td>
<td>8</td>
<td>10.6 ± 0.9</td>
<td>11.8 ± 1</td>
<td>15.1 ± 1.9</td>
<td>28 ± 2.3</td>
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<td>3</td>
<td>9.7 ± 0.5</td>
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<td>41.8 ± 4.4</td>
<td>48.2 ± 9.5</td>
<td></td>
</tr>
<tr>
<td>X161</td>
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<td>11 ± 1</td>
<td>12 ± 4.5</td>
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<tr>
<td>vfl</td>
<td>13</td>
<td>6</td>
<td>8 ± 1.4</td>
<td>11.6 ± 2.6</td>
<td>13.6 ± 0.9</td>
<td>22 ± 1.4</td>
<td>–^</td>
</tr>
<tr>
<td>vfl</td>
<td>14</td>
<td>2</td>
<td>7</td>
<td>9</td>
<td>17</td>
<td>22</td>
<td>28</td>
</tr>
<tr>
<td>X161 vfl</td>
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<td>6</td>
<td>10.3 ± 1.3</td>
<td>10.5 ± 5.4</td>
<td>15.8 ± 6.7</td>
<td>30 ± 12.8</td>
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<td>13 ± 2.8</td>
<td>10 ± 1</td>
<td>14 ± 1</td>
<td>30 ± 5.2</td>
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</table>

* vfl and X161 vfl embryos do not cellularize and have no zygotically controlled mitosis corresponding to mitosis 14 in wild-type embryos. The given error represents SD.
divisions, comparable to uninjected X161 embryos (Figure 4B and Table 2). This experiment demonstrates that the reduced division number in X161 embryos requires zygotic gene expression.

The expression of many early zygotic genes is controlled by the zinc-finger protein Vfl (also called Zelda) [13]. We tested whether the precocious cell-cycle pause in X161 mutants is mediated by vfl-dependent genes. Analysis of X161 vfl double-mutant embryos revealed that, in contrast to X161 mutants, the cell cycle undergoes at least 13 divisions (Table 2). We further analyzed activation of zygotic gene expression by staining for Vfl and activated RPII215 (Figure S1). We detected staining of both in presyncytial stages of X161 vfl double-mutant embryos (Figure 4E and Table 2). We observed a mixture in the number of nuclear divisions between 12 and 14 in fixed embryos. We even observed embryos containing three patches with nuclear densities corresponding to 12, 13, and 14 nuclear divisions (Figures 4F and 4G). About half of the embryos underwent 12 nuclear divisions, similar to X161 embryos. These data suggest that the activation of grapes depends on the onset of zygotic transcription by analyzing X161 grapes double-mutant embryos (Figure 4D and Table 2). We found that some of the X161 grapes double mutants showed the defects in nuclear envelope formation and chromatin condensation already in interphase 13, indicating that the requirement of grapes for chromatin structure shifted from interphase 14 to 13. These data suggest that the activation of grapes and the DNA checkpoint depends on the onset of zygotic gene expression.

A factor controlling the number of nuclear divisions is the ploidy of the embryo, given that haploid embryos undergo 14 instead of 13 nuclear divisions prior to cellularization [1, 26]. Based on this and on related observations, it has been proposed that the nucleocytoplasmic (N/C) ratio controls the trigger for MBT. To address the functional relationship of X161 and the N/C ratio, we analyzed haploid X161 embryos (Figure 4E and Table 2). We observed a mixture in the number of nuclear divisions between 12 and 14 in fixed embryos. We even observed embryos containing three patches with nuclear densities corresponding to 12, 13, and 14 nuclear divisions (Figures 4F and 4G). About half of the embryos underwent 12 nuclear divisions, similar to X161 embryos. These data suggest that ploidy acts independently of general onset of zygotic transcription, which is consistent with the observation that only a subset of zygotic genes are expressed with a delay of MBT is sensitive to changes in RNA polymerase II activity.

In summary, our data support the model that activation of the zygotic genome controls the timing of the MBT. First, onset of MBT is sensitive to changes in RNA polymerase II activity. Second, the changes in zygotic gene expression in X161 embryos occur earlier than the changes in zygotic RNA degradation, Cdc25 protein destabilization, or activation of grapes. Third, the X161 mutant phenotype depends on zygotic transcription and on the transcription factor Vfl, showing that the precocious cell-cycle pause and onset of cellularization

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**Figure 2. Expression of RPII215**

(A) RPII215 mRNA expression by RT-PCR. Error bars show quantification from three independent RNA samples. Expression levels were normalized to levels of 18S rRNA and related to expression in wild-type embryos in stage 1–2. Error bars represent SD.

(B) Fixed wild-type and X161 embryos stained for RPII215.

(C) Immunoblots of extracts from staged embryos as indicated with short and long exposures for RPII215 and β-tubulin. Expression (indicated by the numbers at the bottom) estimated by normalization to the tubulin bands (in a weak exposure film, not shown). Asterisk with arrow marks the activated form of RPII215.
cannot be due to changes in maternal factors, such as higher expression of CyclinB. Although the altered levels of RNA polymerase II in X161 mutants probably affect expression of many genes during oogenesis, these changes seem not to matter in functional terms, given the overall normal morphology and specific mutant phenotype. It is conceivable that transcriptional repressors are expressed or translated in eggs in lower levels. In the embryo, such lower levels of repressors would allow the trigger for onset of zygotic gene expression to reach the threshold earlier than in wild-type embryos. The first signs of zygotic transcription are detected already during the pre-syncytial stages, before nuclear cycle 8/9. This may be the time when the trigger for MBT is activated.

Experimental Procedures

Genetic markers, strains, and genome annotation were according to Flybase (http://flybase.org). X161 was selected from a set of mutations in germline clones with defects in oogenesis and early embryogenesis [28]. Microinjection, RT-PCR, protein analysis, histological procedures, and live imaging were essentially as previously described [29–31]. Gene expression levels in embryos manually staged by the nuclear division was determined by NanoString technology [18].

Supplemental Information

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.12.013.

Acknowledgments

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Figure 4. Reduced Number of Divisions Depends on Zygotic Gene Expression Controlled by vfl

(A) Experimental scheme of the α-amanitin injection experiment. Wild-type embryos injected with α-amanitin undergo 13 or 14 nuclear divisions, depending on conditions.

(B) Number of nuclear divisions is scored in injected wild-type and X161 mutant embryos expressing His2AvGFP. Temperature was 18 °C–20 °C.

(C) Images from time-lapse recordings of embryos from grapes and X161; grapes females injected with labeled histone1 during indicated cell cycle. grapes embryos show abnormal chromatin condensation in interphase 14.

(D) Fixed haploid X161 embryo stained for DNA. Regions with respective nuclear densities are marked.

References


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Figure S1. X161 Phenotype, Vfl Expression and RPII215 Activity
(A and B) Wild type and X161 embryos stained for the centromere protein Cid (green), DNA (blue) (A) or centrosomal protein gamma-tubulin (green) and DNA (blue) (B). Scale bars 10 µm. Insets in A show centromere staining in higher magnification.
(C) Progression of cellularisation in wild type and X161 embryos measured by the length of the furrow.
(D) Fixed wild type embryos and embryos from X161, v/fl and X161 v/fl germline clones as indicated were stained for DNA, Vfl, and RPII215-H5 (CTD phosphorylated form). Wild type embryos were marked with a histone-GFP transgene. Scale bar 50 µm.
(E) Fixed wild type embryos and embryos from X161 germline clones were stained for Vfl and DNA as indicated. Stage by cell cycle number was determined by the nuclear density.
Figure S2. Mapping and Cloning of the X161 Mutation

(A) Image of the X chromosome with the position of the lethal and semi-lethal mutations mapped by meiotic recombination with chromosomes marked with visible markers cv, v, f.

(B) Mapping of the lethal mutation by complementation with duplication (blue) and deficiency (red) chromosomes. Chromosomes shown by dashed lines do not uncover, whereas chromosomes shown by solid lines uncover the mutation. The mapped region is marked in yellow and shown in relation to the genome annotation. The green arrow points to the position of the identified nucleotide exchange on the X161 chromosome. The X161 mutation does not complement RPII215[1], a deletion in the 5' region of RPII215 indicated by the red dotted line and RPII215[G0040], a transposon insertion in the 5' untranslated region indicated by the red arrow head. The lethality and embryonic phenotype of X161 is complemented by P[RPII215+], a transgene with a genomic SgrAI fragment comprising the complete RPII215 locus.

(C) Sequencing of the transcribed regions of the X161 and an isogenic (X9) chromosome revealed a single T to A point mutation in the 3' untranslated region of the RPII215 gene.

(D) Alignment by ClustalW of sequences following the stop codon (3' untranslated region) from six Drosophila species. The mutated nucleotide in the X161 allele at position 40 is marked in red.
Figure S3. Gene Expression Profiles by n-Counter NanoString

(A) Wild type and X161 embryos expressing Histone2Av-RFP were individually selected by their nuclear density. Images of embryos at indicated interphases and in cellularisation. X161 embryos in celluarisation were in interphase 13.

(B) Sensitivity of NanoString detection. Total RNA from wild type embryos of indicated stage was analysed for the amount of selected transcripts. Low abundant transcripts reached low readings at an input with 10 ng. Transcript levels in staged embryonic extracts measured by NanoString technology. Pre, presyncytial cycles 1-8, 11, 12, 13, 14, number of interphase. cel. embryos in cellularisation when the furrow is at the basal side of the nuclei in interphase 14 in wild type and interphase 13 in X161 embryos.

(C and D) Pre, presyncytial cycles 1-8, 11, 12, 13, 14, number of interphase. cel. embryos in cellularisation when the furrow is at the basal side of the nuclei in interphase 14 in wild type and interphase 13 in X161 embryos. Transcript levels in wild type embryos are indicated by solid lines, in X161 embryos, by dashed lines. (C) Profiles of genes encoding ribosomal proteins. Y axis, log(2) scale. (D) Profiles of maternal genes, whose degradation depends on egg activation.
Figure S4. Gene Expression by Whole Mount RNA In Situ Hybridization

frs (A and B), slam (C and D), string (E and F), and twine (G and H) transcripts were detected by RNA in situ hybridisation (blue). The cell cycle number was determined by nuclear density as shown by DNA staining. The respective division cycle is indicated by the number in the inset. Progression of cellularization in cycle 14 in wild type and cycle 13 in mutants was determined by staining for Slam protein marking the cellularization front. The outline of the embryos is marked with a dashed yellow line.
### Table S1. Expression of RPII215 by qPCR

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<td>X161</td>
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SD, standard deviation. P student T-test.

### Table S2. Expression Levels by nCounter NanoString

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<td>eau</td>
<td>3733.30</td>
<td>1886.51</td>
<td>735.09</td>
<td>333.57</td>
<td>106.61</td>
<td>50.94</td>
<td></td>
</tr>
</tbody>
</table>

SD, standard deviation, P student T-test.

Embryos were frozen 3 min after anaphase of the preceding mitosis. "Pre" were presynaptic embryos in nuclear cycle 1 to 8. "WT" or "X161" were embryos in cell/embryos when the furrow passed the basal side of the nucleus. For X161 embryos were in interphase 33.

50 ng of total RNA was analysed by NanoString nCounter technology according to the instructions of the manufacturer.
Supplemental Experimental Procedures

Genetics
Genetic markers, strains and genome annotation were according to Flybase, if not otherwise noted. X161 was selected from a set of mutations in germline clones with defects in oogenesis and early embryogenesis (1). For the genomic RPII215 rescue construct a blunt SgrAI fragment from BAC clone CH321-136G02 into the blunt HpaI site of pCasper4. Transgenes were generated by standard procedures. Following mutations were employed vfl[294] (synonymous to zld[294]) (2), grapes[1] (3), RPII215[1], RPII215[G0040] (4, 5). Germline clones were induced with Frt[18E] or Frt[19A] and corresponding ovoD chromosomes by two heatshocks (each 1h, 37°C) to first and second instar larvae. Haploid embryos were generated by crossing females with ms(3)K81 homozygous males (6). Transgenic fluorescent markers were Histone2Av-GFP/ mRFP and sqh-moesin-GFP (7).

Microscopy
Cell cycle lengths were determined by time lapse recordings at about 21-23°C with an inverted Zeiss Axiovert microscope with differential interference contrast (Plan-apochromat 25xoil NA0.5). Fluorescent time lapses were recorded with a Zeiss spinning disc microscope with a Plan Neofluar 40xoil NA1.3 objective. Embryos were dechorionated with 50% bleach for 90s, washed with water, lined up and oriented on a piece of agarose, transfered to a coverslip and covered with halocarbon oil. Fixed and stained embryos were imaged with a Zeiss LSM780 microscope (LCI Plan-neofluar 25xmulti, NA 0.8-apochromat 40xwater NA1.2, Plan-apochromat 63xoil NA 1.4). Images were processed with Fiji/ ImageJ.

Histology
Embryos were dechorionated with 50% bleach, fixed for 20 min with 4% formaldehyde in PBS and stored in methanol at -20°C. For immunostaining rehydrated embryos were blocked with 5% BSA for 1 h, incubated with primary antibodies overnight at 4°C, washed for 1 h, incubated with secondary antibodies for 1 h, washed for 1 h, stained with DAPI (0.2 mg/ l) and mounted in Aquapolymount (Polyscience). Staining/ washing buffer was PBS plus 0.1% Tween20. Following antibodies were used: CID (rabbit, ref. 8), Kugelkern (rabbit, guinea pig, ref. 9), Eve (guinea pig), gamma-Tubulin (GTU88, mouse, 0.2 mg/ l, Sigma), Dlg (4F3, mouse, 0.4 mg/ l, Hybridoma center), RNA polymerase II (clones ANA3 and H5, mouse, Millipore), Slam (rabbit, guinea pig, ref. 10), String (rabbit, S DiTalia), Twine (rat, obtained from S. DiTalia), Vfl (rat, ref. 11). The eve antibody (guinea pig) was raised against recombinant protein expressed from plasmid pAR-eve (obtained from M. Frasch). Secondary antibodies were alexa-coupled goat-anti-rabbit/ mouse/ guinea pig (4 mg/ l, Invitrogen), alkaline phosphatase coupled anti-digoxigenin-Fab fragments (Roche). RNA in situ hybridisation was performed as previously described (12) using digoxigenin labelled probes and detection with alkaline phosphatase. Images were recorded with bright field optics. The RNA antisense probes were prepared from plasmids pCS2-frs, pNB-stg1.8, pSK-twintw (24), pCS2-slam (10). For RNA-protein double staining, RNA staining was developed prior to immunostaining.

Western Blots
Embryos were manually staged according to their morphology in bright field optics, collected in groups of 50 to 100 and frozen in liquid nitrogen. Proteins extracts were prepared by disruption of the embryos with a pistle fitting into a 1.5 ml reaction tube in Laemmli buffer. Protein extracts from about 5-10 embryos were separated by SDS polyacrylamid electrophoresis and transferred to PVDF membrane by wet transfer (110
mA, 4°C, 18hr). Following blocking with 5% non-fat milk in PBT (PBS plus 0.2% Tween20) overnight at 4°C the blot was incubated with primary antibody in PBT plus 1% bovine serum albumin for 2 h, washed with PBT (5x10 min), incubated with peroxidase coupled goat-anti-mouse antibody (Sigma) in PBT with 0.5% bovine serum albumin for 1 h, washed with PBT (5x5min) and developed with the enhanced chemiluminescence reaction (GE healthcare). Primary antibodies were alpha-Tubulin (BS12, mouse, 0.04 mg/l, Sigma), RNA polymerase II 215 subunit (ARN-A-3a, H5, mouse, 1:500, Millipore).

Quantitative PCR
Embryos were manually staged according to their morphology in bright field optics, collected in groups of 50 to 100 and frozen in liquid nitrogen. Total RNAs was extracted with Trizol (Invitrogen) and analysed with a Bio analyzer (RNA 6000 Nano Kit, Agilent). cDNA was syntheised according to manufactor's instructions (Roche). In brief, 2 µg total RNA was mixed with 1 µM of oligo-dT and 8 µM of 18S rRNA specific primer (HS415 AAC ATG AAC CTT ATG GGA GTG GTG C) in 13 µl. The reaction was started by adding 7 µl with reverse transcriptase (1 unit), dNTP mix (each 1 mM), RNase inhibitor and buffer. For each real-time PCR reaction, cDNA corresponding to 10 ng of original total RNA was mixed with 3 µM of primers and reaction mix containing SyBR Green (iQ SYBR green supermix, Bio-RAD) with a CFX-96 real-time PCR system (Bio-RAD). The amplification curves were analyzed with the comparative CT method using either 18S rRNA as reference genes. The following primer pairs were used: RPII215: HS403 (CCG GTG GAT CGA CAC CGA GC) and HS404 (GCA CTT ACG TGG CGG GGT GG), RPL21: HS386 (AGG CAT ATC ATG GCA AAA CC) and HS397 (GAC CCA TTT GCC CTT CT), RPL32: HS375 (CTG ATC AGC CAG CCT AGC AG) and HS376 (TGG CCA GGG AGT AGC ACT CG). Slam: JG241 (GGT CAT CCA GCT GCA AGC AAT) and JG242 (CGG GCA TTG GAA GTG GGT TAC A), 18S rRNA: HS363 (AGC CTG AGA AAC GGC TAC CA) and HS364 (AGC TGG GAG TGG GTA ATT TAC G).

Expression Analysis by NanoString nCounter
Single dechorionated embryos expressing Histone2Av-RFP were staged on a spinning disc microscope according to nuclear density and cell cycle stage. Embryos (five wild type, three mutant) of a given stage were pooled in vials with heptane on dry-ice. Presyncytial embryos were selected by morphology in bright field optics. Embryos in interphase 11 to 14 were frozen 3 min after anaphase. Embryos in cellularization were frozen when the furrow passed the basal side of the nuclear layer. Total RNA was extracted with Trizol (Invitrogen) and analysed by Bio-analyzer (RNA 6000 Nano Kit, Agilent). The yield was about 50 to 150 ng per embryo. Selected transcripts were quantified by NanoString technology (13) according to the protocol suggested by the manufactorer. Briefly, total RNA (50 ng) was mixed with the code set before adding the capture probe. Hybridization was performed at 65°C for 18 hr. Post-hybridization processing was performed with the nCounter Prep Station. After preparation, the cartridge containing the mRNAs were loaded into the Digital Analyzer and the number of RNA molecules was counted. The number of mRNA was analyzed by nSolver software. Data were corrected using RPL21, RPL32 and RLP2 as reference genes.

Microinjection of Embryos
Microinjection was performed as described as previously described (14). α-amanitin was injected at 500 μg/ml (in water) into presyncytial embryos expressing Histone2Av-RFP. Alexa488 labelled histone1 (2 mg/ml) was injected into early embryos to visualise nuclear dynamics for the embryos grapes and XI61; grapes (7).
Supplemental References


