Abstract
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Introduction
Krüppel like factors (KLFs) are highly conserved throughout the animal kingdom and have been implicated in many developmental processes such as differentiation, organ patterning [1], regulation of pluripotency [2], and human diseases [1]. They encode Zinc finger containing transcription factors, which bind DNA and regulate various cellular processes as transcriptional activators or repressors [1]. In evolutionary tree analyses KLF6 clusters with KLF7 [3] and luna is a close homolog in Drosophila and Daphnia [4] [5].

KLF6 is known as a ubiquitously expressed activator associated with proliferation, apoptosis, the hematopoietic system, and various cancers in vertebrates [1]. KLF7, also known as ubiquitously expressed Krüppel like factor (UKLF), is known to regulate sensory neuron development [6] and is involved in fat metabolism [1]. The mouse and zebrafish animal models established a KLF6 function in a developing organism [7,8]. In the mouse, knockout of Klf6 causes developmental arrest due to failure of erythropoiesis and angiogenesis, and Klf6−/− embryonic stem (ES) cells show proliferation defects [7]. In zebrafish, morpholino based knockdown revealed that Klf6/copeb is essential for the proliferation of endoderm derived tissues [8]. KLF7 knockout mice die shortly after birth due to neuronal defects [6].

In Drosophila, early development is characterized by 14 synchronous nuclear divisions in a syncytium, the fertilized egg [9,10]. The first 9 divisions take place before the onset of zygotic transcription at which point the nuclei migrate to the periphery of the embryo. These processes are solely driven by maternal contribution [9,11]. De Graeve and colleagues [4] have shown that RNA interference for luna aborted development in 50% of the animals prior to gastrulation with large vacuoles forming in the egg yolk and hence coined the gene name luna. This approach also affected later developmental stages in Drosophila as did over expression of luna [4]. However these experiments did not address or reveal for which cellular processes and during which time of development luna function was essential.

Here we report the generation of loss-of-function mutants in the Drosophila luna gene and show that independent alleles and RNA interference cause the same phenotypic effect. Phenotypic analyses reveal that luna function is solely required at early developmental stages during the syncytial divisions, prior to cellularization, and is maternally contributed. Most prominently, luna mutants cause DNA separation defects during the early nuclear divisions, while centrosomes proceed their cycling. Hence, we conclude that luna is required for the synchronization of nuclear DNA and centrosome cycles.

Results
Isolation of luna mutants
luna loss-of-function mutants were generated by combining FLP recombinase and FRT bearing insertions [12], which resulted in two independent, precise genomic deletions, specific to CG33473/ luna. Each of these removed distinct coding sequences (Figure 1). Due to the fact that all 3 insertions used to generate the gene

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specific deletions were of the same kind and inserted in the same orientation (Figure 1), standard molecular confirmation of the generated deletions by PCR was not possible, because the recombination generated a 23 kb PBac identical to the two parental ones. Genetic analysis revealed that both alleles were strong loss-of-function mutations, according to their behavior in complementation crosses and phenotypic analyses. No differences of lethal stage or phenotype were observed in embryos from homozygous versus trans-heterozygous luna−/−/deficiency intercrosses (Table 1, Figure 2).

Taken together, we conclude that the two mutant alleles generated are strong loss-of-function alleles.

**Luna function is maternally contributed**

Luna lethality manifests itself “zygotic lethal like”. Progeny from luna mutant stocks and transheterozygous intercrosses are all heterozygous luna+ /CyO animals (Table 1). Examination of lethal embryos derived from luna mutant stocks suggested that luna mutants die at preblastoderm stages since 8–20% lethal embryos were found to have arrested development prior to blastoderm stages (Table 2) and CyO homozygous animals were reported to die at 1st instar stages [13]. Similar numbers were obtained from CyO tort-GFP balanced luna mutant stocks, independent of the paternal genotype (data not shown).

It is unexpected to find such early developmental defects in embryos derived from heterozygous mothers. Furthermore, the zygotic genome is silenced until nuclear cycles 9–10 [14,15]. We thus examined homozygous mutant eggs (via germline clone technology [16], see Materials and Methods for details) to assess if luna function is maternally contributed. Indeed we found that such mutant embryos, lacking the maternal component, died at various early stages of development with up to 20% prior to blastoderm stages, possibly due to defects in the nuclear division cycles and similar to those observed in mutant embryos derived from heterozygous mothers (compare Figures 2 and Figure 3, Table 2). Control germline clones did not show such defects (Tables 3 and 4).

To confirm the maternal requirement and phenotypic features, we used an independent loss-of-function approach, RNA interference experiments with 2 non-overlapping luna sequences expressed via the maternal nanos::VP16-GAL4 driver, active in the unfertilized egg [17]. In both experiments we observed similar phenotypes as mentioned above, whereas control embryos did not show such drastic effects (Figure 4).

To summarize, loss-of-function phenotypes of luna are apparent in mutant preblastoderm embryos derived from heterozygous mothers (Figure 2, Tables 3 and 4), homozygous mutant eggs generated by germline clone technology [16] [see below; Figure 3, Tables 3 and 4] and maternally expressed RNA interference experiments (Figure 4, Tables 3 and 4). We therefore conclude that maternal Luna function is required for embryonic development prior to cellularization and during early gastrulation of the Drosophila embryo and that zygotic loss of luna function might compound the maternal phenotype.

**luna is required for the coordination of DNA and centrosome replication cycles**

During the early nuclear divisions in the syncytial preblastoderm embryo, prior to cellularization, centrioles are essential [18], whereas later embryogenesis and imaginal disc development can proceed normally in the absence of centrosomes [19]. During the syncytial nuclear division stages the DNA replication and centrosomal cycles can be uncoupled by reduction of the cell cycle regulators Cyclin A, B and B3 [20] or by inhibition of DNA replication [21].

When we examined luna loss-of-function animals derived from 3 independent approaches (see above), abnormal division patterns were observed in the pre-blastoderm embryos, at the stage when rapid, synchronized nuclear divisions take place in the syncytium (Figure 2A, D and Figure 3D). Strikingly, the most frequent phenotypic defects observed were non-segregated DNA or DNA “bridges” that remained between chromatin after the completion of nuclear divisions (Figure 2B, C and Figure 3A, B, Table 4): Such structures contained a thick DNA bridge connecting 2 prophase-like nuclei, each associated with two separated centrosomes (Figure 2B and Figures 3A, B, Table 3). In maternal RNAi interference experiments for luna, where all embryos with peripheral nuclei were assessed from a 2 hour collection, about 20% showed these DNA segregation defects (Figure 4).

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**luna** has no requirement during later developmental processes

*luna* is required for the earliest stages of development, precluding a simple analysis of larval and adult stages. However, as it is ubiquitously expressed throughout all developmental stages (flybase 2013) and in the developing eye [4], we generated homozygous mutant clones of the *luna* alleles in order to determine, if there is a requirement at later stages in development, for example during imaginal disc patterning and/or proliferation of imaginal disc cells. Surprisingly, no defects were detected in *luna* mutant tissue in the developing eye and wing, and such mutant clones had a fully wild-type appearance. Specific analyses of cell division or epithelial morphogenesis in imaginal discs, as assayed by the metaphase marker phospho histone H3 or cellular junctional integrity and associated cell adhesion (with anti-DE-cadherin) did not reveal any detectable abnormalities (Figure S1A, B), nor did we observe any defects in adult eye tissue (Figure S1C).

We conclude that *luna* is solely required for the early nuclear division cycles in the syncytial pre-blastoderm embryo and, specifically there, for the coordination of DNA replication and centrosome cycles.

**Luna and KLF6 over-expression can affect many developmental processes**

Luna over-expression has been shown to interfere with normal development at larval stages and to disrupt eye development generally [4]. We wanted to test which specific processes or signaling pathways Luna could interfere with, and targeted Luna and KLF6 over-expression to subsets of cells throughout the *Drosophila* body during various stages of development, in particular to wing cells and to all head/eye cells or a subset of photoreceptor cells (Figure S2B–K). Based on Western blot analyses, expression levels were increased several fold as compared to endogenous Luna levels (Figure S2G). In all cases analyzed, normal development was severely compromised, even though *luna* is not essential at these stages and in these processes, as shown by loss-of-function analyses in the developing eye and wing and the adult eye (Figure S1). For example, in the developing eye, we find that excess levels of Luna and hKLF6 interfere with planar cell polarity establishment (Figure S2D–F) and in the wing over-expression also affects several developmental processes (Figure S2L–K). In conclusion ectopic *luna* and *hKLF6* expression cause similar defects in eye and wing development.

**Table 1. Complementation analyses.**

| Parental cross (virgin×male) | F1 Cy (n=) | F1 non Cy (n=) |
|-----------------------------|----------|----------------|
| *luna*Δ2 (###a29) × *luna*Δ1 (###c7) | 89       | 0              |
| *luna*Δ1 (###c10) × *luna*Δ2 (###a23) | 31       | 0              |
| *luna*Δ1 (###b5) × *Df(2R)ED2155* | 12       | 0              |
| *luna*Δ1 (###c7) × *Df(2R)ED2155* | 61       | 0              |
| *Df(2R)ED2155* × *FRT42D luna*Δ1 (###c10) | 250      | 0              |
| *FRT42D luna*Δ2 (###a7) × *Df(2R)Exel6059* | 150      | 0              |
| *Df(2R)Exel6059* × *luna*Δ2 (###a23) | 9        | 0              |
| *FRT42D luna*Δ1 (###c7) × *Df(2R)ED2155* | 250      | 0              |

Individually established stocks are indicated by # in parenthesis.

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Discussion

Here we describe the isolation and characterization of luna loss-of-function mutants in Drosophila. We show that mutant animals die at early embryonic stages likely due to nuclear division defects due to non-segregation of DNA at the syncytial stage, prior to cellularization and before the start of gastrulation. The Drosophila phenotype is reminiscent of the mouse Klf6<sup><s>2</s>2</sup> defects, where under-proliferation of hematopoietic cells in the yolk sac is the cause of early lethality [7]. Also, D’Astolfo et al. show that KLF6 is a positive regulator of cell cycle progression, and an anti-apoptotic factor, by silencing of KLF6 via siRNA in several cultured cell lines [22]. These data are consistent with the Drosophila loss-of-function effects in early embryos, where we observe arrested division cycles. Moreover, Racca et al. describe human KLF6 localization in the syncytium of the human trophoblast in cell culture [23], another example where KLF6 is active in a syncytial tissue, like in the early Drosophila embryo.

luna expression has been shown to be maternally loaded into the egg (flybase 2007, Dmel Release 5.2 and flybase 2013). Since all early regulators of these division cycles stem from maternal contribution [9,10,11], the timing of the luna requirement and phenotype is consistent with that. We show that luna function is required for the synchronization of DNA and centrosome replication, starting in the embryonic syncytium. The most frequent phenotypic defects range from non-segregation of DNA, thick DNA connections between 2 prophase-like structures, which are associated each with two centrosomes that are already primed to initiate the next cycle of nuclear division; Figure 2B, 3A, B) to asynchronous division cycle figures (Figure 2E, 3C), and, more rarely, trespassing mitotic spindles (Figure 2C) and nuclear fall out (Figure 3B, C). In particular, the fused DNA structures associated with 4 centrosomes seem to indicate that DNA replication or segregation is failing, while the centrosomes are ready for the next division cycle in luna mutant embryos. These defects were readily visible in mutant embryos from heterozygous mothers, in homozygous mutant eggs, generated via germline clones in the ovary, and in 20–30% of maternal RNAi treated embryos (Tables 3 and 4, Figure 3).

As luna mutant alleles manifest themselves as “early zygotic lethal like”, we generated tissue patches mutant for luna in developing eyes and wings. Surprisingly, such mutant clones did not display any defects in cell cycle rate or junctional/adhesion property, as assayed by the respective markers (phosphorylated
Table 3. *luna* mutant embryos show severe DNA segregation defects.

| Heterozygous mother derived | Maternal genotype | % DNA segregation defect/bridges | n total nuclear figures |
|-----------------------------|-------------------|----------------------------------|------------------------|
| lunaΔ1 or 2                | 55.4%             | 56                               |
| lunaΔ1 or 2                | 46.9%             | 49                               |
| lunaΔ1                     | 84.8%             | 198                              |
| lunaΔ1 or 2                | 19.6%             | 51                               |
| lunaΔ1 or 2                | 12.2%             | 49                               |
| Df(2R);ED2155              | 54.9%             | 133                              |
| Df(2R);ED2155              | 100.0%            | 112                              |
| Df(2R);ED2155              | 98.8%             | 83                               |
| Df(2R);ED2155              | 64.4%             | 45                               |
| Df(2R);ED2155              | 96.8%             | 62                               |
| Df(2R);ED2155              | 96.7%             | 61                               |
| Df(2R);ED2155              | 94.5%             | 55                               |
| Df(2R);ED2155              | 91.8%             | 49                               |
| lunaΔ1                     | 100.0%            | 6                                |
| lunaΔ1                     | 100.0%            | 5                                |
| lunaΔ1                     | 100.0%            | 4                                |
| lunaΔ1                     | 88.9%             | 9                                |
| lunaΔ2                     | 75.7%             | 70                               |
| lunaΔ2                     | 70.1%             | 77                               |
| lunaΔ2                     | 75.0%             | 76                               |
| Germline clone derived     | Allele            |                                  |                        |
| lunaΔ1                     | 40.0%             | 10                               |
| lunaΔ1                     | 53.3%             | 15                               |
| lunaΔ1                     | 95.1%             | 82                               |
| lunaΔ1                     | 32.4%             | 37                               |
| lunaΔ1                     | 27.5%             | 40                               |
| lunaΔ2                     | 100.0%            | 6                                |
| lunaΔ2                     | 60.8%             | 74                               |
| lunaΔ2                     | 88.9%             | 45                               |
| lunaΔ2                     | 63.6%             | 33                               |
| lunaΔ2                     | 23.8%             | 21                               |
| lunaΔ2                     | 36.4%             | 44                               |
| lunaΔ2                     | 64.3%             | 28                               |
| lunaΔ2                     | 64.0%             | 50                               |
| lunaΔ2                     | 90.0%             | 40                               |
| lunaΔ2                     | 42.9%             | 28                               |
| lunaΔ2                     | 70.8%             | 24                               |
| lunaΔ2                     | 59.5%             | 37                               |
| Germline clone derived     | Control           |                                  |                        |
| arm-lacZ/wildtype          | 0.0%              | 303                              |
| arm-lacZ/wildtype          | 20.9%             | 91                               |
| arm-lacZ/wildtype          | 0.0%              | 33                               |
| arm-lacZ/wildtype          | 0.0%              | 43                               |
| arm-lacZ/wildtype          | 0.0%              | 190                              |
| arm-lacZ/wildtype          | 0.0%              | 7                                |
| nos::VP16-GAL4::UAS-luna-IR| IR                |                                  |                        |
| 1                          | 67.2%             | 58                               |
| 1                          | 81.8%             | 132                              |
| 2                          | 97.6%             | 41                               |
| 2                          | 81.4%             | 59                               |
data indicate that morphogenesis, cell fate, or cellular patterning (Figure S2). These changes in Luna protein levels, but no such changes were therefore tested whether PcG heterozygous embryos displayed Polycomb group genes look identical to polyhomeotic (ph) epigenetic regulators of the Polycomb group (PcG) of genes, e.g. maternal effect observed in other mutants/genes acting at that stage? The heterozygous cells [26,27].

Kru¨ppel itself, causes rough eyes and mis-specified photoreceptor expression was several times the endogenous level. Similarly, over-expression of both Luna and hKlf6 interferes with normal development [4] and our studies (Figure S2). This is not unexpected, as KLFs are known transcriptional activators (or repressors) and increasing their levels is likely to interfere with various downstream transcriptional programs and targets. According to our Western blot analysis, Drosophila Luna over-expression was several times the endogenous level. Similarly, misexpression/over-expression of other Klf family members in the fly eye, e.g. the founding member of this transcription factor family Kruppel itself, causes rough eyes and mis-specified photoreceptor cells [26,27].

How do the Luna loss-of-function defects relate to the phenotypes of other mutants/gens acting at that stage? The heterozygous maternal effect observed in luna was also reported for the epigenetic regulators of the Polycomb group (PcG) of genes, e.g. polyhomeotic (ph), Additional sex combs (Asc), Posterior sex combs (Psc) and Polycomb (Pc) itself [28] and polo, scant double mutants [29] with both sets of genes also affecting the early syncytial division cycles. Whereas embryos from heterozygous mothers of polo, scant double mutants cause a wider array of phenotypes, mutations in the Polycomb group genes look identical to luna loss of function. We therefore tested whether PcG heterozygous embryos displayed changes in Luna protein levels, but no such changes were detectable (data not shown).

Several other genes show similar phenotypes, including mutations in Non-muscle Myosin/Spaghetti squash [30] and xpd [31], but these show full maternal requirement for the early nuclear division cycles. However, the uncoupling of DNA replication and centrosome duplication as observed in luna has been described for microcephalin (MCPH1) [32], except that in MCPH1 mutants centrosomes were also observed to detach leading to monopolar, multipolar or acentrosomal spindles, an effect not seen in luna mutants. Taken together, luna might affect DNA status, which then leads to the secondary effect of uncoupled centrosome cycles. If luna were to affect the centrosomal structure alone, the DNA segregation defects should not be seen, as such phenotypes are not reported for genes essential for integral centrosome function such as centrosomin [33,34,35]. Nevertheless, the fact that luna is only required during the syncytial stages and not later in development indicates that the DNA segregation defect is linked to the centrosomes, since centrosomes are dispensable for later cell divisions [18,19].

Both phenotypes, DNA segregation defects and asynchronous divisions, occur most frequently in luna. We speculate that the formation of DNA bridges is the primary defect and asynchronous divisions arise from unresolved and therefore delayed divisions. Similarly, nuclear fall out, a response to improperly segregated DNA could be a secondary effect [36].

luna mutations do not fully present themselves as dominant female sterile, as stocks can be propagated over a balancer (Table 1) and have 37–50% lethal offspring, with 8–20% lethal at preblastoderm stages (Table 2). Poly comb group mutations in Pc, Psc and ph manifest a similar effect [28].

Many experimental approaches to better understand luna function are precluded because of the syncytial “zygotic lethality behaviour” of luna loss-of-function mutants, likely a compound effect of maternal and zygotic requirements. Further studies at the syncytial blastoderm stage of embryogenesis should be possible via the recently published technique of live imaging of in vitro explants [37] and these could provide insight on the precise connection between luna loss-of-function and the early processes of syncytial nuclear divisions.

Materials and Methods

Fly stocks and crosses

PiggyBac elements PBac{WH}f07504, PBac{WH}f04294 and PBac{WH}f04876 were used to generate precise deletions at the luna locus (Figure 1), according to Parks et al., 2004 [12]. Lethal excision events were selected by complementation analysis to Df(2R)Exel6039, which removes approximately 24 genes. Also Df(2R)ED2153, which removes approximately 89 genes, was used in embryo stainings and complementation analyses in trans-heterozygous combinations.

### Table 3. Cont.

| Heterozygous mother derived | Maternal genotype | % DNA segregation defect/bridges | n total nuclear figures |
|---------------------------|------------------|-------------------------------|------------------------|
| nos::VP16-GAL4::UAS-white-IR | Control          | white                          | 0%                     | 500                    |
|                           |                  | white                          | 0%                     | 194                    |
|                           |                  | white                          | 0%                     | 170                    |
|                           |                  | white                          | 0%                     | 585                    |

Summary of defects quantified from different experimental approaches (first column). “% DNA segregation defect/bridges” represents nuclear figures during division stages, where DNA bridges remain between adjacent nuclei. Note that in the mutant scenarios generally between 50–100% of nuclear figures show bridges, whereas in the control germline clones or RNAi experiment it is mostly at 0%. These phenotypes can also be observed in early division cycles.

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Table 4. *luna* mutant embryos show division cycle asynchrony and increased nuclear fall out defects.

| Heterozygous mother derived | Maternal genotype | Asynchrony | Nuclear fall out |
|-----------------------------|-------------------|------------|-----------------|
|                             |                   | stage1 | stage2 | stage3 | % stage1 | % stage2 | % stage3 | n total | n = centrosomes without DNA/areas |
|                             | Df(2R)ED2155      | prophase | metaphase | bridges* | - | - | 100 | - | - | 135 | 17 centrosomes/4 areas |
|                             | Df(2R)ED2155      | metaphase | anaphase | telophase | 23 | 11 | 66 | 379 | 16 centrosomes/4 areas |
|                             | Df(2R)ED2155      | telophase | interphase | bridges* | 16 | 67 | 17 | 555 | 10 centrosomes/4 areas |
| lunaΔ2                     | metaphase | anaphase | telophase | 34 | 41 | 25 | 123 | 0/0 |
| lunaΔ2                     | metaphase | anaphase | telophase | 42 | 45 | 13 | 135 | 4 centrosomes/1 area |
| Germline clone derived     |                   |           |           |           |           |           |           |           |                      |
| Allele                     |                   |           |           |           |           |           |           |           |                      |
| lunaΔ2                     | metaphase | anaphase | telophase | 22 | 41 | 37 | 51 | 0/0 |
| lunaΔ2                     | metaphase | anaphase | - | 32 | 68 | - | 118 | 0/0 |
| lunaΔ2                     | metaphase | anaphase | - | 46 | 54 | - | 74 | 0/0 |
| lunaΔ2                     | metaphase | telophase | - | 10 | 29 | 62 | 21 | 0/0 |
| luna-RNAi2                 | metaphase | interphase | - | 99 | 1 | - | 44 | 0/0 |
| luna-RNAi2                 | metaphase | interphase | - | 96 | 4 | - | 74 | 0/0 |
| luna-RNAi2                 | prophase | - | - | 100 | - | - | 274 | 0/0 |
| luna-RNAi2                 | metaphase | - | - | 100 | - | - | 177 | 7 centrosomes/2 areas |
| Germline clone derived     | Control**         |           |           |           |           |           |           |           |                      |
| Allele                     |                   |           |           |           |           |           |           |           |                      |
| arm-lacZ/wildtype          | anaphase | metaphase | - | 98 | 2 | - | 44 | 0/0 |
| arm-lacZ/wildtype          | anaphase | metaphase | - | 96 | 4 | - | 74 | 0/0 |
| arm-lacZ/wildtype          | prophase | - | - | 100 | - | - | 274 | 0/0 |
| arm-lacZ/wildtype          | metaphase | - | - | 100 | - | - | 177 | 7 centrosomes/2 areas |
| Summary of defects quantified from 3 different experimental approaches (first column). Division stage asynchrony lists the different stages (1–3) and the percentage of nuclear figures found for every stage. Nuclear fallout lists how many individual centrosomes were found without associated DNA and how many such areas occurred in one embryo.

*embryo with DNA segregation defects/bridges.

**note that asynchrony is very rare/almost non-existent in control germline clone or control RNAi cohort (96–100% synchronous).
Embryonic lethality stage was determined by comparing total number of eggs and % of lethal embryos after 2 days from balanced \textit{luna} deletion stocks. Lethal embryos were mounted in mineral oil after dechorionation and examined for developmental stage. 2 classes of dead embryos were found: (1) unpatterned, non gastrulated and (2) segmented, cuticle containing ones. Homozygous \textit{CyO} embryos were found to be late embryonic to first instar lethal [13].

Clones of homozygous mutant \textit{luna} tissue were generated during later stages of development via the MARCM system.

Germline clones were induced in \textit{hsFLP}; \textit{FRT42D luna-}+/\textit{FRT42D wod} or \textit{hsFLP}; \textit{FRT42D arm-lacZ/FRT42D wod} females by heat shock for 1 hour at 37°C for 3 consecutive days from first larval instar stage on. Such females were crossed to \textit{w1118} males and all eggs collected in 7–18 hrs intervals and fixed until females stopped laying. Standard embryo fixation and antibody staining was performed according to Cooley lab protocols. All crosses were performed at 25°C.

For RNA interference during oogenesis freshly eclosed \textit{nanos::VP16-GAL4/+}; \textit{UAS-luna-IR/+} females were crossed to \textit{w1118} males at 29°C for 4 days in well yeasted bottles. Embryos were collected in 2 hour intervals at 29°C and stained as described above. For 2 collections epifluorescence pictures of Hoechst staining were taken, representative of all embryos, where nuclei had reached the cortex. These pictures were categorized according to nuclear division stages and defects for Figure 4. Control embryos were \textit{nanos::VP16-GAL4/+}; \textit{UAS-white-IR/+}.

For over-expression, \textit{UAS-luna}[DG], \textit{luna-EP} insertions, \textit{UAS-hKLF6} and \textit{GAL4} drivers \textit{sevenless} (\textit{sex-GAL4}), \textit{engrailed} (\textit{en-GAL4}) and \textit{scalloped} (\textit{sd-GAL4}) were used.

**Figure 4. DNA segregation and division asynchrony are the most prominent \textit{luna} phenotypes in RNAi knock-down experiments.** Graph of embryonic phenotype evaluation of 2 independent maternal \textit{nos::VP16-GAL4/+/FRT42D luna-} females performed at 25°C was performed according to Cooley lab protocols. All crosses were stopped laying. Standard embryo fixation and antibody staining were taken, representative of all embryos, where nuclei had reached the cortex. These pictures were categorized according to nuclear division stages and defects for Figure 4. Control embryos were \textit{nanos::VP16-GAL4/+}; \textit{UAS-white-IR/+}.

**Imaging and histology**

Embryos were collected from \textit{luna-} /\textit{CyO} stocks or \textit{luna-} /\textit{CyO} and \textit{Df(2R)Exel5659/CyO} or \textit{Df(2R)ED2155} intercrosses, germline clone crosses (see above) or from \textit{nanos::VP16-GAL4/+}, \textit{UAS-luna-RNAi-1} or \textit{2/+} mothers at 29°C and stained for DNA with Hoechst, phosphorylated histone H3, gamma-tubulin or centrosomin, and cylin B.

Confocal microscopy was performed on a Zeiss Meta LSM 510. Images are projections of several consecutive grazing sections.

Antibodies and dilutions used:

- rat anti-Elav (1:50 from Developmental Studies Hybridoma Bank/DSHB),
- rabbit anti-phospho histone H3 (1:200, Upstate Biotechnology),
- rat anti-DExad (1:20 from DSHB),
- rabbit anti-Cnn (1:100, kindly provided by Tom Kaufmann),
- mouse anti-gamma tubulin (1:500 from Sigma),
- mouse anti-cycE (1:5 from DSHB),
- Hoechst 33342 (1:500 from Sigma)
- mouse anti-cycB (1:5 from DSHB),
- Hoechst 33342 (1:500 from Sigma)
- rabbit anti-hKLF6 (1:400 for tissue and 1:1000 for Western from Santa Cruz).

Phenotypic analysis was based on confocal images covering half to 2/3 of the embryo cortex and ImageJ was used to count division figures and organelles for Tables 3 and 4.

**Constructs and molecular analysis**

\textit{UAS-KLF6} was cloned by PCR, amplifying the KLF6 coding sequence with forward primer containing 5’ -ttgagccgtcccctgcgct 3’ sequences and reverse primer 5’ -tcaagaggctctctattgg 3’. The resulting PCR product was cloned as EcoRI fragment into the pUAST vector. The final construct was confirmed by DNA sequencing in both orientations.

\textit{UAS-lunaRNAi} constructs were generated as previously described [38]. Primer sets were designed using the Heidelberg eRNAi prediction site (www.dkz.de/signaling2/e-rnai).

\textit{UAS-lunaRNAi-2: FWD-RNAi-31071 5’ CCTAGGACGAGTAGTAGCGGCTGGTG 3’ and REV-RNAi-31071 5’ GGATCCATCGGAGTGCTAAAAATGCT-3’},

\textit{UAS-lunaRNAi-1: FWD-RNAi-31812 5’ CCTAGGACGCTTGGCCATTGTCCCTC 3’ and REV-RNAi-31812 5’ GATGCTTTGCATCATGAAACGCGCTCTGA 3’}.

Flanking AvrII and BamHI restriction sites were added (underlined). PCR amplified sequences were cloned via the DNA topoisomerase I technique. Constructs were sequenced in both orientations.

Western and \textit{in vivo} Klf6/Luna stainings: Anti-human Klf6 was used to probe d.m. Luna and human KLF6 over-expression in Drosophila eye discs tissue and on Western blots thereof. The equivalent of 40 eye discs of each genotype was loaded on a gel. Eye disc staining of the same genotypes revealed only a Klf6
specific pattern. Human tissue culture cells: Blot detection was performed using standard HRP coupled secondary antibodies and ECL detection according to protocol.

Supporting Information

Figure S1 luna mutant tissue in developing eyes and wings and in adult eyes, do not show defects. Anterior is to the left and dorsal is up in all panels. 3rd larval instar eye (A) and wing (B) imaginal disc tissue mutant for lunaA#565 or #c7, respectively (marked by GFP in green), stained for DE-cadherin (red and monochrome in A’, B’) and metaphase (phospho-histone H3 in blue, monochrome in A”, B”). (C) Adult eye section of lunaA#410 loss-of-function clone marked by the loss of pigment granules next to rhabdomeres. (C’) Schematic representation of mutant eye tissue in grey.

Figure S2 Luna and KLF6 overexpression affect eye and wing development. A–C: Lateral and dorsal views of adult wings and in adult eyes, do not show defects. H3 in blue, monochrome in A(B)

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Author Contributions

Conceived and designed the experiments: UW ER JM MM. Performed the experiments: UW ER. Analyzed the data: UW ER JM MM. Contributed reagents/materials/analysis tools: UW ER JM MM. Wrote the paper: UW ER MM.
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