INTRODUCTION

Cell-cycle progression is controlled by a combination of transcriptional and posttranslational regulatory events (Morgan, 2006). Transcriptionally, the retinoblastoma protein/E2F pathway directly regulates expression of cyclin E and other target genes to drive the G1/S transition (Duronio and O’Farrell, 1995; Duronio and Xiong, 2013; Dyson, 1998; Geng et al., 1996; Ohtani et al., 1995). In quiescent mammalian cells, overexpression of E2F can induce S phase entry (Johnson et al., 1993), and in Drosophila, ectopic E2F can accelerate cell-cycle progression (Neufeld et al., 1998). Similarly, overexpression of G1 cyclins results in truncated G1 phases (Johnson et al., 1993; Ohtsubo and Roberts, 1993; Resnitzky et al., 1994) and is reported to induce mammary gland tumors in mice (Smith et al., 2006; Wang et al., 1994). These studies collectively demonstrate the importance of proper cell-cycle-associated transcription and thus raise a critical question: How much of the genome is periodically transcribed in a cell-cycle-associated manner?

To address this issue, a paradigm for understanding global cell-cycle-associated transcription has emerged from studies of synchronized cells in humans (Cho et al., 2001; Whitfield et al., 2002), budding yeast (Cho et al., 1998; Spellman et al., 1998), fission yeast (Oliva et al., 2005; Rustici et al., 2004), bacteria (Laub et al., 2000), and plant cell culture (Menges et al., 2003). Together these studies have identified hundreds of periodic genes, a large number of which are involved in cell-cycle-specific processes and expressed at peak levels when their functions are required. However, to date, global analyses of periodic transcription have focused on single-cell systems, and the potential intricacies of the periodic transcriptome in complex multicellular tissues remain poorly understood.

Unlike single-cell cultures, cell division in a developing tissue has to be coordinated with the developmental control of growth, patterning, and morphogenesis. In the vertebrate neural tube, for example, nuclei migrate during cell-cycle progression such that mitotic events are confined to the apical epithelial surface (Sauer, 1935). This process, interkinetic nuclear migration (IKNM), is proposed to be essential for the maintenance of tissue architecture in complex epithelia (Nakajima et al., 2013), and it is also important for determining the cell fate of neural progenitors in vertebrates (Cappello et al., 2006; Del Bene et al., 2008; Murciano et al., 2002; Xie et al., 2007). Nevertheless, despite the ubiquity of this conserved mitotic cell behavior (Meyer et al., 2011), the mechanisms linking nuclear cell-cycle progression to IKNM remain unclear, and the potential contributions of periodically expressed genes to the regulation of IKNM and other tissue-specific processes have received little direct attention.

In Drosophila, the adult wings and other appendage structures are derived from imaginal discs, monolayer epithelial sacs that undergo rapid and continuous proliferation during larval development (Cohen, 1993). Although wing growth is directed by patterning signals, cell division occurs ubiquitously without an obvious spatial pattern until late in development (Garcia-Bellido and Merriam, 1971; Johnston and Edgar, 1998). In the present study, in order to gain insight into global aspects of cell-cycle-associated transcription and the role of periodic genes in wing development, we profiled gene expression in G1 and G2/M phase wing disc cells isolated using a dissociation-fluorescence-activated cell sorting (FACS) protocol. By directly comparing the cell-cycle-associated transcriptome of wing disc cells with that of cultured S2 cells, we identified both common and context-dependent periodic genes. These genes were further tested for their function in tissue development, cell proliferation, cell-cycle phasing, and mitosis in the developing wing. The vast majority of genes identified using this approach were not
revealed in a previous S2 RNAi screen in vitro (Björklund et al., 2006). Notably, we also implicate two periodic genes in the control of mitotic nuclear position during IKNM, highlighting the importance of understanding the regulation of cell-cycle progression in a context-dependent manner.

RESULTS

Global Analysis of Cell-Cycle-Associated Transcription

To define the global cell-cycle-associated transcriptional profile in the developing wing, we first developed a physical and enzymatic disruption protocol to rapidly dissociate whole discs into a suspension of single cells. Compared with the conventional 2–4-hr-long enzymatic protocol for imaginal disc dissociation, our method recovered approximately three times more live cells (20 min compared with 120 min). Using this approach, dissociated wing disc cells were stained live for DNA content and then sorted into G1 and G2/M populations by FACS (Figures S1A–S1C available online). To compare cell-cycle-associated transcription in the wing epithelium with that observed in cell culture, we performed a parallel series of FACS experiments in cultured Drosophila S2 cells (Figure 1A; Figures S1D–S1F).

From the sorted G1 and G2/M populations, RNA samples were subjected to microarray analysis. Three biological replicates were examined for each condition and evaluated using a moderated t statistic (Smyth, 2004) to define the most significantly periodic genes in both wing discs and S2 cells (Figure 1B; adjusted p value < 0.05).

Based on the statistical analysis described above, we identified more than 700 cell-cycle-associated genes in wing discs and more than 600 in S2 cells (Figure 1B). The intersection of these sets included 150 genes with similar patterns of periodic expression in both cell types (defined as common genes in Figure 1C and Table S1A). Intriguingly, 200 genes were periodic exclusively in wing disc cells, and 91 were periodic only in S2 cells (defined as wing disc specific and S2 specific in Figures 1D and 1E; Table S1A). Furthermore, 16 genes displayed inverse periodic behavior; these genes were periodic in both cell types but peaked in different phases (and were thus defined as
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opposite; Figure 1B; Table S1A). The observed variability between periodic transcription in wing discs and S2 cells is not likely to represent experimental noise, because we found a strong correlation in global periodic gene expression between control wing disc samples from two different laboratory strains, OreR and w1118 (Figure S1G; Pearson correlation = 0.909). In this case, only 19 probe sets (among 18,952 analyzed) exhibited a significant difference in periodic expression (Table S1B).

To validate periodic expression in vivo, we selected a set of 24 genes that included both known cell-cycle genes (e.g., pcna, Cdc6, and Bora) and newly identified periodic genes (e.g., CG1218 and CG10200), and then we examined their expression in the eye imaginal disc. Twenty-two of 24 genes exhibited cell-cycle-associated expression (Figures S2A and S2B) relative to the morphogenetic furrow, a stripe of eye disc cells arrested in G1. Most of the periodic genes we analyzed were expressed broadly in the wing disc (Figures S2A and S2B), except for one G1 gene with elevated expression in myoblasts (Sox100B) and one G2 gene (CG3168) with enriched expression in neural lineages (Figure S2C). Together these results identify a core set of periodic genes shared between wing disc and S2 cells, demonstrate the robustness of periodic transcription in the wing disc, and reveal substantial differences in periodic gene expression between different cellular contexts.

The Context Dependence of Cell-Cycle-Associated Gene Expression

To gain a global perspective on functional implications of cell-cycle-entrained transcription, we applied gene ontology (GO) analysis to the periodic genes from six main categories (Figures 1C–1E; Table S1A). GO analysis showed each of these categories to be enriched for distinct biological processes (Figure 2A). As expected, G1 genes with similar expression profiles in wing discs and S2 cells (G1-common [Com]) were enriched for DNA replication. Likewise, G2 genes exhibiting similar regulation in both cell types (G2-Com) were enriched for functions in mitosis. Unexpectedly, several core elements of the DNA replication machinery exhibited elevated G1 expression only in wing disc cells (G1-WD). These included Orc1-Orc3, Mcm2-Mcm5, and dup (Cdt1) of the prereplication complex (pre-RC), and Sld5 of the GINS complex (Figures 2B and 2C). Furthermore, CDC6, another component of the pre-RC (Cocker et al., 1996), was one of the 16 genes exhibiting opposing patterns of periodic transcription between wing disc and S2 cells (Figures 2B and 2C; Table S1A). This result is particularly interesting because CDC6 plays an essential role in the initiation of DNA synthesis by loading minichromosome maintenance (MCM) proteins onto chromatin (Coleman et al., 1996). In budding yeast, this process requires de novo synthesis of CDC6, consistent with its peak expression near the G1/S transition (Donovan et al., 1997; Zhou and Jong, 1990). In keeping with the importance of its temporal regulation, ectopic CDC6 causes an M phase delay in fission and budding yeast (Boronat and Campbell, 2007; Bueno and Russell, 1993), and it induces epithelial-mesenchymal transition-like changes in mouse and human epithelial cell lines (Sideridou et al., 2011). On a more general level, it is unclear why key elements of the DNA replication machinery would exhibit different periodic expression profiles in wing disc versus S2 cells, because most of these genes are known E2F targets (Dimova et al., 2003). Indeed, computational analysis of regulatory regions from both common and wing disc-specific G1 genes revealed significant enrichment for the consensus motifs recognized by E2F (Figure S2D). These results suggest that there may be context-dependent transcriptional regulation of cell-cycle factors targeted by E2F (e.g., DNA replication factors). Whether this context-dependent activity is controlled through distinctive E2F binding sites (e.g., see Figure S2D) remains unclear.

The differences between the periodic transcriptomes of wing disc and S2 cells were not limited to G1 genes. Many known mitotic genes exhibited elevated G2 expression only in S2 cells (Figures 2D and 2E). These include pim (encoding Drosophila Securin), wee (encoding Drosophila Wee1 kinase), and CycB3, as well as the checkpoint-protein-encoding genes iok (homolog with human CHEK2) and mad2. In addition, a few genes with known functions in mitosis exhibited higher G1 expression only in the wing disc, such as string (Drosophila CDC25), cid (CENP-A), and Borr (borealin-related) (Figures 2D and 2E; Figure S2A). This suggests that some genes are transcribed before their protein function is required, or that they may have unexplored functions outside of G2/M in wing disc cells.

Taken in sum, our transcriptional profiling experiments revealed an unexpected degree of context dependence in cell-cycle-associated transcription, even for genes encoding core components of the DNA replication and mitotic machineries. Additionally, these experiments allowed us to define the core periodic transcriptome of proliferating epithelial cells in vivo. Many of the periodic genes we identified were uncharacterized or not thought to play a role in cell proliferation. We therefore systematically disrupted these genes to determine their functions in vivo. Detailed results of the transcriptional profiling and phenotypic experiments can be accessed through a searchable online database at http://odr.stowers.org/FlyCycle.

Functional Identification of Periodic Genes Required for Wing Development

To circumvent the limitations of whole-animal mutant analysis, we used tissue-specific RNAi to interrogate requirements for genes from the common, wing disc-specific, and opposite classes during wing development. We obtained transgenic RNAi lines from the Vienna Drosophila RNAi Center and screened 461 RNAi lines targeting 311 genes (multiple lines from the VDRC P-Element RNAi Library were tested whenever possible; Table S2A). To identify the subset of periodic genes that functionally contribute to growth of the wing, male flies carrying each UAS-RNAi construct were crossed to females of the genotype: Bx-GAL4; UAS-dicer2 (Figure S3A). For each cross, we scored for adult viability and wing phenotypes using specific terms described in Table S2A. When there were multiple RNAi constructs available for a single gene, the relevant phenotypes were quantified and averaged (Table S2B; Figure S3B). In sum, almost 80% of all genes tested were required for normal wing development (244/311; Figure 3A; Table S2B), suggesting a significant enrichment of developmental function among wing disc periodic genes (compared with 32/66 S2-specific periodic genes [Table S2C] and 35/67 random genes required for wing development [Table S2D]; p < 0.005, Fisher’s exact test). Among these, 107 gene knockdowns led to a small wing phenotype, representing a strong growth defect. In addition, 137
Figure 2. Context-Specific Periodic Transcription of Core Cell-Cycle Genes

(A) GO category enrichment for the periodic gene classes. The gray boxes on the right indicate the development, mitosis, and DNA replication-related GO categories.

(B) Context-specific periodic transcription of genes involved in DNA replication. Note that Orc1-Orc3 and Mcm2-Mcm5 were significantly periodic only in wing disc cells (dark blue).

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produced other morphological defects such as curly, canoeshaped, blistered, or notched wings (Table S2B; Figure S3C). The curly and canoeshaped wing phenotypes may reflect imbalanced growth of dorsal and ventral sides of the wing blade due to the stronger dorsal expression of Bx-GAL4 (Figures S3A and S3C). This initial screen defined putative loss-of-function phenotypes for 244 periodically expressed genes, many of which were growth defects. Hypothetically, these genes could directly control cell-cycle progression, but they might also indirectly affect growth by regulating related processes. Thus, we next tested the cellular basis for the observed wing defects using both cytometric analysis of DNA content as well as direct confocal imaging.

Identification of Periodic Genes Required for Cell-Cycle Progression In Vivo

To determine which periodic genes were required for wing development through effects on cell-cycle progression, we analyzed the phenotypes of RNAi knockdown cells by flow cytometry. For these experiments, we knocked down each gene in GFP-labeled cells of the posterior compartment of the wing disc using flies of the genotype: UAS-dicer2; engrailed-GAL4, UAS-EGFP. For each analysis, the corresponding GFP-negative internal compartment cells were used as an internal control (Figure 3B). In total, 156 RNAi lines were tested, representing 133 genes that produced wing defects in the primary RNAi screen (Figure 3C). Among these, RNAi knockdown for 13 genes could not be analyzed owing to early lethality or severely reduced wing disc sizes (Figure 3C). Flow cytometry data for the other 120 genes (138 lines) was analyzed for defects in cell proliferation and cell-cycle phasing (Figure 3C; Table S2E; Table S2F; http://odr.stowers.org/FlyCycle).

Using quantitative analysis of DNA content and cell numbers from individual RNAi knockdowns, we next clustered the lines according to phenotypic categories (Figure 3D). Knockdown of 36 genes produced a highly significant reduction in GFP\(^+\) cell numbers relative to GFP\(^-\) controls (proliferation defects \([>3 \text{ SD}]; \text{Figure 3D}; \text{Figure S3D}; \text{Table S2E}). Compared with cell-cycle phasing in the cognate GFP\(^-\) internal controls, 27 of these knockdowns also caused highly significant changes in the distribution of cells in G1, S, and G2/M phases (increased G1, increased S, increased G2/M \([>3 \text{ SD}]; \text{Figures 3D and S3E}; \text{Figures S3E–S3G}; \text{Table 1})). In addition, nine gene knockdowns led to a decrease in cell number and no significant effect on cell-cycle phasing (Figures 3D and 3E). These genes may therefore function in the control of other developmental processes, such as apoptosis (e.g., CG5491; data not shown). Conversely, knockdown of 12 genes did not significantly affect cell numbers but did produce defects in cell-cycle phasing (Figure 3D; Table 1).

As described above, we identified a total of 39 periodically expressed genes that were required for normal cell-cycle phasing (Figures 3D; Figures S3E–S3G; Table 1). These included many known genes, including cell-cycle regulators (e.g., Cyclin E [CycE]) and DNA replication factors (e.g., mus209 [pnc1], CDC45L, Sld5, dpa [Mcm4], and lat [Orc3]) for G1/S phase, and the anaphase cytokine protease-encoding gene Separa se (Sse) for G2/M (Figure 3D; Figures S3E–S3G; Table 1). Besides known cell-cycle regulators, we also identified several periodic genes with unknown cell-cycle functions, including NTF2-related export protein 1 (Nxt1), trus, Nop60B, ovo, CG14781, CG10200, CG31344, and CG16734 (Figures 3D; Figures S3E–S3G; Table 1). Strikingly, of the 39 genes required for cell-cycle phasing in the wing, only four genes were previously identified in genome-wide Drosophila RNAi screens in S2 cells (Table 1; Björklund et al., 2006). Only two of these genes (CycE and CDC45L) showed similar phenotypes after knockdown in wing discs and S2 cells. In contrast, trus knockdown increased the G2/M population in discs but decreased the G2/M population in S2 cells (Table 1; Björklund et al., 2006). Knockdown of CG9772 (Skp2; Shibutani et al., 2008) increased the G2/M population in both discs and S2 cells, but it also caused an accumulation of aneu poloid or polyploid cells not reported in cell culture (with >4C DNA content; Figure 3E). In general, the divergence in genes identified by the two screens may reflect technical differences but may also suggest a critical difference in cell-cycle regulation and periodic gene activity between different cell types or between the in vivo and in vitro contexts.

To assess potential functional relationships between periodic genes required for normal cell-cycle phasing, we next constructed a protein-protein interaction (PPI) map using known physical interactions in Drosophila and predicted interactions based on orthology (Warde-Farley et al., 2010). The resulting network (Figure 4A; Figure S4A) exhibited tight interactions between genes whose knockdown led to an increase in the G1/S population, but no direct interactions between genes whose knockdown increased the G2/M population. This indicates that G2/M accumulation may be triggered by the disruption of diverse and unrelated pathways, whereas G1/S accumulation may be primarily attributable to defects in DNA replication. Among the genes with G1/S defects, 21/27 were expressed at elevated levels in G1, and 19 of these formed a tight PPI network (Figures 4A and 4B; Figures S4A and S4B). Within the network, protein complexes involved in DNA replication emerged, including ORC, MCM, GINS, and DNA polymerases (Figure 4B; Figure S4B). Additionally, a few other genes were connected to the DNA replication protein network, including Nop60B (encoding a pseudouridine synthase) and CG31344 (a putative target of E2F in Drosophila [Roy et al., 2010]; Figure 4B; Figure S4B). Their periodic expression, requirements for cell-cycle progression, and interactions with known DNA replication factors indicate a likely function in DNA replication-related processes. There were also six genes highly expressed in G2 whose RNAi led to

(C) Normalized expression levels of individual DNA replication genes by microarray. Values in (C) and (E) are the mean ± SD of three biological replicates. Asterisks indicate significance of periodic expression by t statistics with a Benjamini-Hochberg adjusted p value < 0.05. The colors under the gene names at the bottom correlate with the colors in (B).
(D) Model of the context-specific periodic transcription of genes involved in mitosis.
(E) Normalized gene expression level of individual genes involved in mitosis, plotted as in (C). The colors under the gene names at the bottom correlate with the colors in (D).
See also Figure S2.
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A  Bx-GAL4 > 311 gene RNAi

- Lethal 1.0% (n=3)
- 20.6% (n=64) Small wing
- 34.4% (n=107) Other defects
- 44.1% (n=137) No defects

B engrailed-GAL4, UAS-GFP > UAS-RNAi

- GFP+ DAPI
- GFP

C en-GAL4 > 133 gene RNAi

- Lethal 4.5% (n=6)
- Tiny or no wing disc 5.3% (n=37)

D

- Increased G2/M
- Increased G1/S
- Decreased G2/M
- Decreased G1/S
- Not significant

- Sox100B
- yellow-d
- CG9080
- CG31133
- CG14781
- CG9772
- CG3168
- scrib
- Sae
- trus-a
- Nn11-a
- CG31344-a
- Nn1-b
- trus-b
- Fen1
- CG5491
- Orc2-a
- CG12018
- cdc
- Determin
- CG1218
- Flo-2
- Orc2-b
- sll
- CG8569
- Mcm5
- Polo2
- CDC45L-a
- Cdt1-180
- DNApol-epsilon
- mxc
- lat
- DNApol-delta
- dpa
- CG16734
- Bci
- MCM6
- Orc1
- yin
- mus209
- ovo
- Sld5
- Nop60B
- Ts-a
- Ts-b
- CDC45L-b
- APC4-a
- Caf1-105
- APC4-b
- CG31344-b
- Rbl
- CG10209
- CG16883
- CycE
- bfr

E

- Adult wing
- Larval wing disc

- Control
- CycE
- mxc
- scrib
- CG14781
- Sld5
- trus
- CG9772
- CG1218
- CR32027

(legend on next page)
Periodic Genes Required for Mitotic Chromosome Segregation and Cell Size

The cytometry screen described above provided us with a rough profile of cell proliferation and cell-cycle phasing defects for a large number of periodic gene knockdowns. To better understand the wing growth defects and identify periodic genes directly involved in mitosis or other cellular processes, we also analyzed a subset of phenotypes at the cellular level. To achieve a relatively late wing pouch-specific knockdown, 71 RNAi lines that produced strong growth defects in the primary screen were crossed with nubbin-GAL4 (Figure S5A). Sixty-three of 71 of these RNAi lines caused a similar growth reduction as in the primary screen; among these we identified defects in chromosome segregation and mitotic cell size. The knockdown of eight genes resulted in significant chromosome alignment and

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segregation defects (Figures 5A–5I; Figures S5B and S5C). Among these, only cid (Figure 5G) is known to directly function in kinetochore assembly and chromosome segregation (Blower and Karpen, 2001). Six other genes have direct or indirect functions in the process of DNA replication, including dpa (Mcm4; Figure 5B), CDC45L (Figure 5C; Figure S5B), pcna (Figure 5D), RPA2 (Figure 5E), Rnrs (Figure 5F), and DNA-ligI (Figure 5I). Insufficient levels of DNA replication factors and incomplete DNA replication could lead to an accumulation of cells in S phase. Under these conditions, abnormal cells that are able to pass through the DNA replication checkpoint may have incompletely replicated DNA and broken chromosomes, resulting in mitotic arrest or chromosome segregation defects (Loupart et al., 2000; Pflumm and Botchan, 2001). One final gene associated with defects in chromosome segregation was HipHop, which encodes a protein localized to telomeric regions (Gao et al., 2010). After HipHop knockdown, we observed tightly bound anaphase chromosomes (97.6%, n = 41 anaphase figures; Figure 5H; Figure S5C). This could reflect telomere fusion, which has been reported in HipHop RNAi S2 cells (Gao et al., 2010). In HipHop RNAi wing discs, aberrant chromosome bridges were largely resolved by telophase, although a thread of DNA often persisted between daughter nuclei (Figure S5C).

Defects in chromosome segregation were occasionally associated with additional cellular phenotypes. In addition to lagging chromosomes, Deterin RNAi wing disc cells exhibited a high frequency of multipolar spindles (31/49 mitotic figures; Figure 5J). Mitotic figures with multipolar spindles typically either segregated the chromosomes into two units or failed at segregation entirely (Figures S5D and S5E; Movie S2). It is possible that these multipolar spindles formed as a consequence of a previous incomplete cytokinesis. However, by live imaging of Deterin RNAi wing discs, we did not observe an obvious delay or arrest in mitotic progression as was observed after knockdowns of HipHop and the DNA replication factor DNA-ligI (Figures S5D and S5E; Movies S1 and S2). Deterin’s human homolog has been shown to function in the chromosomal passenger complex and is required for normal mitotic division (Lens et al., 2006; Li et al., 1999). Here, our microarray expression data (Figures 2D and 2E) are consistent with the known cell-cycle-dependent expression of Deterin during G2/M phase in human cells (Li et al., 1998). Furthermore, our results indicate a function for Deterin in chromosome segregation and perhaps mitotic checkpoint activation in Drosophila.

Unexpectedly, several genes required for chromosome segregation also disrupted mitotic cell size (Figures 5K–5P). Cell size increased significantly after knockdown of Rnrs, Rpa2, DNA-ligI, and HipHop in wing discs, possibly due to cell-cycle delay. Metaphase spindle length increased proportionally with the cell size.
diameter for RnrS and RPA2 RNAi (Figures 5L, 5M, and 5P), consistent with the scaling of spindle length with cell size (Brown et al., 2007; Goshima and Scholey, 2010; Haras and Kimura, 2009). In contrast, the spindle did not scale to the increased cell size after HipHop knockdown, and it was actually slightly smaller than controls in enlarged DNA-ligI RNAi cells (Figures 5N–5P). Both HipHop and DNA-ligI are involved in the regulation of chromosome integrity and structure. Our data are consistent with the conjecture that spindle size scales with cell size and that abnormalities in chromosome structure may disrupt the scaling mechanism.

**Two Wing Disc-Specific Periodic Genes Implicated in IKNM**

Interkinetic nuclear migration is a fundamental cellular process by which mitotic nuclei translocate to the apical epithelial surface during prophase (Sauer, 1935; Meyer et al., 2011). These events permit the subsequent alignment of the mitotic spindle to the plane of the epithelium as defined by apically polarized cell junctions (Nakajima et al., 2013). Despite its likely conservation in pseudostratified epithelia throughout animals, the molecular mechanisms that link apically directed nuclear movements with cell-cycle progression remain poorly understood. Our imaging analysis revealed genes whose knockdown led to significant mitotic defects (Figures 5B–5J), some of which were also coupled with significant increases in mitotic cell size (Figures 5K–5P) or to a dramatic increase of mitotic figures (e.g., APC4A; data not shown). Nevertheless, even in the most severe cases, mitotic nuclei remained restricted to the apical epithelial surface.

To search for genes specifically involved in IKNM, we analyzed mitotic nuclear position after knockdown of the 71 periodic genes strongly required for wing development. Among these, IKNM was specifically disrupted by RNAi lines targeting CR32027 (predicted to be a long noncoding RNA) and CG10479 (predicted to encode an Src homology 2 domain-containing protein). In both cases, the total number of mitotic nuclei localized below the septate junction-delimited mitotic zone (MZ) was significantly increased (Figures 6A and 6B). Also in both cases, the normal polarized architecture of the epithelium was largely intact, confirmed by localization of the septate junction-associated protein Discs large (Dig) (Figure 6A).

It is proposed that Rho-kinase controls IKNM, at least in part, through phosphorylation of myosin activity (Meyer et al., 2011). Consistent with this, we observed strong cortical anti-phospho-myosin regulatory light chain (anti-p-MRLC) staining in mitotic cells of control discs (Figure 6C). After CG10479 RNAi, however, anti-p-MRLC staining was severely reduced in basally mislocalized mitotic cells (Figure 6C). This indicates that CG10479 is not only periodically expressed but also may function in the regulation of IKNM at or above the level of Rho-kinase activity. In contrast with this result, normal anti-p-MRLC signal levels were observed after disruption of CR32027, even in basally mislocalized mitotic cells (Figure 6C). Consistently, these mitotic cells exhibited mislocalized anti-phospho-Histone H3 (anti-PH3+) nuclei, but they had normal cortical F-actin accumulation and underwent normal mitotic rounding (Figures 6A, 6C, and 6D). These experiments indicate that CR32027 likely functions in the regulation of IKNM independently from both Rho-kinase activity and actomyosin contractility. Indeed, we observed a strong additive effect on nuclear position when we inhibited actin dynamics with cytochalasin D in CR32027 RNAi wing discs (Figures 6E and 6F). Under these conditions, anti-PH3+ mitotic nuclei showed a roughly uniform distribution throughout the epithelium (Figure 6G), consistent with a maximal defect in IKNM.

To extend these results, we validated the function of CR32027 using a second RNAi construct (CR32027-IR2; Figures 7A and 7B; Figures S6A and S6B) and also confirmed the reduction of CR32027 transcript levels in experimental wing discs (Figure S6C). Phenotypically, in addition to mislocalized mitotic nuclei, expression of both CR32027-IR1 and CR32027-IR2 resulted in a significant increase in basally mislocalized microtubule-organizing centers (MTOCs, marked by the pericentriolar matrix marker Centrosomin; Figures 7A and 7C). In CR32027-IR1, we observed abnormally basal cells in every mitotic phase, including telophase (7.7% ± 2.3%; n = 128), indicating that these cells can still complete division. Furthermore, using live single plane illumination microscopy (SPIM), we observed aberrant basal mitoses in cultured wing discs (Figures 7D and 7E; Movie S3). In some cases, aberrant mitotic figures first appeared basally and then moved apically to complete mitosis (Figure S6D; Movie S3). This may explain how a small but relatively normal adult wing phenotype was observed in these lines (Figure 3E).

Importantly, mitotic timing in CR32027 RNAi cells was normal compared with controls (Figures 7D and 7E; Figure S6D; Movie S3). Because CR32027 RNAi did not disrupt cell-cycle timing or phasing (Figure 3E), our results suggest that IKNM is not required for mitosis and that CR32027 may link mitotic division with nuclear migration without affecting other mitotic processes (i.e., cell rounding, cell-cycle phasing, and mitotic progression). One possible interpretation is that CR32027 directly or indirectly regulates centrosome positioning and function, and misregulation of this process may allow aberrant cell division to happen at basal positions in the epithelium. Consistent with this, centrioles in mitotic CR32027 RNAI wing disc cells were not consistently associated with the spindle poles or microtubules (MTs) (Figures 7F and 7G; Figures S6E and S6F). Further suggesting a link between CR32027 expression and centrosome function, CR32027 transcript abundance was reported to be sensitive to centrosomal manipulations in Drosophila (Baumbach et al., 2012).

Although it remains unclear how a putative noncoding RNA might directly regulate centrosome localization, several long noncoding RNAs have been proposed to function by directly controlling transcription of specific targets or by regulating the basal transcriptional machinery (Rinn and Chang, 2012). We therefore used transcriptional profiling to identify gene expression changes in the wing pouch after CR32027 RNAi. For both CR32027-IR1 and CR32027-IR2 knockdown, the putative target genes Cyp6A17 and the kinesin-like protein Klp54D were highly downregulated (≥500-fold). We used RNAi to test the function of both genes in IKNM, and we observed a high frequency of basally mislocalized mitotic nuclei in Klp54D RNAi wing discs. Similar results were obtained with two independent lines targeting Klp54D, whereas no defects were observed after Cyp6A17 knockdown (Figures 7H and 7J). Notably, Klp54D knockdown did not produce a small wing phenotype, presumably because aberrant basal mitotic figures ultimately moved apically to
Figure 5. Mitotic Abnormalities Associated with Periodic Gene Knockdown In Vivo

(A) Normal anaphase chromosome segregation in a control wing disc. Cells are labeled for α-tubulin (MT, green), DNA (blue), and F-actin (red). Scale bar, 5 μm.

(B–E) Abnormal anaphase segregation with lagging chromosomes after dpa (B), CDC45L (C), pcna (D), and RPA2 (E) knockdown. Scale bar, 5 μm.

(F) Abnormal anaphase segregation with lagging chromosomes after RnrS knockdown. Scale bar, 5 μm.

(G) Abnormal anaphase segregation with lagging chromosomes after cid knockdown. Scale bar, 5 μm.

(H) Abnormal anaphase segregation with lagging chromosomes after HipHop knockdown. Scale bar, 5 μm.

(I) Abnormal anaphase segregation with lagging chromosomes after DNA-ligI knockdown. Scale bar, 5 μm.

(J) Abnormal anaphase segregation with lagging chromosomes after Deterin knockdown. Scale bar, 5 μm.

(K) Abnormal anaphase segregation with lagging chromosomes after control knockdown. Scale bar, 5 μm.

(L) Abnormal anaphase segregation with lagging chromosomes after RnrS knockdown. Scale bar, 5 μm.

(M) Abnormal anaphase segregation with lagging chromosomes after RPA2 knockdown. Scale bar, 5 μm.

(N) Abnormal anaphase segregation with lagging chromosomes after HipHop knockdown. Scale bar, 5 μm.

(O) Abnormal anaphase segregation with lagging chromosomes after DNA-ligI knockdown. Scale bar, 5 μm.

(P) Graph showing the relationship between cell diameter and spindle length for different conditions. The graph includes bars for control, RnrS, RPA2, HipHop, and DNA-ligI, with statistical data points indicating n values.
complete mitosis (data not shown). Together these results suggest that CR32027 may function directly or indirectly through transcriptional control of Klp54D, which could in turn regulate centrosome positioning or other mitotic processes. Looking forward, the regulation of centrosome positioning and dynamics may represent an important avenue for future studies of epithelial cell proliferation in vivo.

**DISCUSSION**

In this report we provide a global functional perspective on cell-cycle-dependent periodic genes in the *Drosophila* wing disc. On a genomic level, our results reveal an unexpected degree of plasticity in global periodic transcription between different cell types from the same organism. Experimentally, using a phenotypic screen validated by both flow cytometry and direct confocal analysis, we identify a large number of periodically expressed genes required for the regulation of cell proliferation in vivo. Looking forward, similar approaches in vertebrate systems could uncover new regulators of mitotic processes that may be difficult or impossible to study in vitro.

**Context Dependence of the Global Periodic Transcriptome**

One key functional implication of periodic gene expression is to ensure “just-in-time” assembly, a conserved process in eukaryotes (de Lichtenberg et al., 2005; Jensen et al., 2006). It is therefore surprising that many well-known DNA replication genes exhibited different patterns of periodic transcription in wing discs and cultured S2 cells (Figures 2B and 2C). Notably, third-instar wing disc cells have a doubling time of ~12 hr (Neufeld et al., 1998), and S2 cells have a cell cycle of roughly 24 hr with a relatively shorter duration of G1 and a longer G2/M phase (Figures S1A and S1D). This timing difference may be one of the factors underlying periodic differential transcription in the two contexts. Conversely, the differential periodic expression of certain genes could contribute to the temporal regulation of cell division. For example, peak expression of CDC6 during G2/M correlates with a longer G2/M duration in S2 cells. Because CDC6 stabilizes anaphase-promoting complex (APC) and delays mitosis (Borona and Campbell, 2007; Bueno and Russell, 1992), we reason that the periodic expression of CDC6 could contribute to the control of cell-cycle length in *Drosophila*.

Differential periodic transcription is only one aspect of context-dependent cell-cycle regulation. Periodically expressed subunits of protein complexes involved in core cell-cycle processes are proposed to be three times as likely to be phosphorylated as constitutively expressed components (Jensen et al., 2006). This raises the intriguing possibility of context-dependent regulation at the posttranslational level. These findings have some potential implications for human health. For example, Meier-Gorlin syndrome is a disease caused by mutations in pre-RC complex components but is associated with tissue-specific effects, such as reduced ear size (Bicknell et al., 2011a, 2011b; Guemsey et al., 2011). A deeper understanding of context-specific cell-cycle regulation could shed light on the tissue-specific effects in such conditions. It may also provide further insight into context-dependent features of cell-cycle progression during proliferative disease.

**Periodically Expressed Genes Implicated in Cell-Cycle Progression In Vivo**

In the wing disc, periodically expressed genes were enriched for factors required for normal cell-cycle phasing. A previous genome-wide screen in S2 cells using a similar flow cytometry approach found that roughly 4% of all genes screened were required for cell-cycle progression, cell size, or apoptosis (Björklund et al., 2006). Here, by functionally screening 311 periodically expressed genes in vivo, we identified more than a hundred factors involved in wing growth and 39 (among 120 genes screened by flow cytometry) required for normal cell-cycle phasing. Among the 39 periodic genes required for normal cell-cycle phasing in the wing disc, only four were previously identified by S2 cell-based screening approaches (Björklund et al., 2006; Gilsdorf et al., 2010). Furthermore, the subset identified only in our screen included some very well-studied cell-cycle genes, including *pcna, Sep15, Separase,* and *Rbf* (Table 1). The differential identification of these and other factors may simply be due to differences in RNAi efficiency or other technical discrepancies between the two systems. It is also possible that the cell-cycle machinery is more sensitive to gene expression levels in the developmental context. Nevertheless, our ability to identify both known and unknown cell-cycle-genes points to the potential of the in vivo approach. Because we only tested approximately 20% of the total wing disc periodic genes by flow cytometry and confocal imaging, additional periodic genes regulating cell proliferation may yet be identified.

**Periodically Expressed Genes Implicated in IKNM**

IKNM represents a facet of cell proliferation control that can only be fully understood in vivo. From our list of periodically expressed genes, two candidates were implicated in the regulation of IKNM in the developing wing disc. Both CR32027 and CG10479 showed ubiquitous expression in the wing disc (Figure S2A) and exhibited elevated G1 phase expression only in the wing disc and not in the S2 cells. The knockdown of both genes by RNAi caused wing growth defects (Figure 3E; Table S2A) and led to a significant increase in basal mitotic nuclei without a corresponding disruption of epithelial integrity (Figures 6A and 6B). Although we could not validate requirements for...
Figure 6. Roles of CR32027 and CG10479 in IKNM
(A) Basally mislocalized mitotic nuclei (anti-PH3+, blue; white arrowheads) in CR32027 and CG10479 RNAi (-IR) wing discs (driven by UAS-dicer2, w1118; nubbin-GAL4). Samples were also stained for F-actin (red) and septate junctions, labeled by Dlg (green).
(B) Scatter plot shows the distance of 400 anti-PH3+ nuclei from the apical surface of wing discs in controls and after CR32027 and CG10479 RNAi. Error bars show mean ± SD. The asterisks indicate statistical significance by Mann-Whitney U test with a p value < 0.0001.

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The *Drosophila* Periodic Transcriptome

CG10479 with multiple RNAi constructs, our data suggest that it controls mitotic rounding upstream of the known actomyosin contractility cassette (Meyer et al., 2011). In contrast, it appears that CR32027 functions through an independent mechanism, perhaps related to the regulation of MTOC localization and centrosome function (Figures 7A and 7C–7G; Figures S6D–S6F). Mechanistically, CR32027 may regulate this process through transcriptional regulation of the kinesin family motor protein Klp54D (Figures 7H and 7J). Interestingly, a human periodic long noncoding RNA, MALAT1, controls expression of the centromere-associated kinesin-like protein CENPE (Tripathi et al., 2013). Many kinesin-like proteins (e.g., Xklp2, kinesin-12/KIF15) localize to the spindle poles and regulate centrosome separation in *Xenopus*, mouse embryos, and human cells (Boleti et al., 1996; Courtois et al., 2012; Sturgill and Ohi, 2013). Based on our results, elevated G1 expression of the putative long noncoding RNA CR32027 could play a temporal role in controlling Klp54D abundance, thus linking cell-cycle progression with centrosome dynamics and nuclear movement. Along similar lines, it was recently reported that dynein is recruited to nuclei during G2 to drive IKNM in the vertebrate neurocortex (Hu et al., 2013). Combined, these independent results suggest that the cell-cycle phase-specific regulation of motor proteins could be a conserved feature of cell proliferation in epithelial tissues.

One of the most surprising observations in CR32027 RNAi wing discs was that mitotic progression can reach completion on the basal side of the epithelium (Figure 7E). Thus, CR32027 RNAi represents a separation of function between the control of mitotic nuclear position and the control of cell division itself. This indicates that apical rounding, mitosis, and mitotic nuclear migration, although normally tightly associated, are regulated by partially independent mechanisms. Given the importance of these issues and their likely implications for epithelial cell division in a multitude of systems, further study of CR32027 will be important to expand our understanding of the molecular mechanisms of IKNM.

**EXPERIMENTAL PROCEDURES**

FACS analysis of S2 cells, microarrays, RNA-seq experiments, bioinformatics, quantitative PCR (qPCR), in situ hybridization, fly strains, immunofluorescence, transmission electron microscopy, wing disc ex vivo culture, and drug experiments are described in Supplemental Experimental Procedures.

**Wing Disc Dissociation, FACS, and Live-Cell-Cycle Analysis of Wing Disc**

For live-cell FACS assays, *Drosophila* larvae were raised at 25°C at low density until the third-instar wandering stage, and then they were washed and dissected in PBS (pH 7.4). Forty wing discs were transferred into 300 μl of 25°C 0.25% Trypsin-EDTA (Sigma), drawn through an 18G11/2 needle ten times, and incubated for 15 min at 25°C. Then, 150 μl of heat-inactivated FBS (hi-FBS; Gibco) was added to stop the enzymatic reaction, and samples were again drawn through the 18G11/2 needle ten times. After a brief low-speed centrifugation, cells were washed and resuspended in staining solution (Schneider’s *Drosophila* Medium [Gibco], containing 2% Hi-FBS, and 1 μg/ml Hoechst 33342 [Invitrogen]) for 20 min at 25°C before sorting or cell-cycle analysis using an Influx flow cytometer (BD Biosciences). Then, 1 μg/ml 7-aminomethylcoumarin (Invitrogen) was added 5 min before cell-cycle analysis to label dying cells. Flow cytometry data were analyzed using FlowJo software (Tree Star) and Modfit software (Verity Software House).

**RNA Extraction for Microarray and RNA-Seq**

For the FACS/microarray experiment, wing discs and S2 cells were partitioned into G1 and G2/M populations. Gating was applied based on 2C and 4C peak positions. Cells were sorted directly into RNAProtect Cell Reagent (QIAGEN), and total RNA was extracted using an RNeasy Plus Micro Kit (QIAGEN) in triplicate for S2 and OreR wing disc cells, and in duplicate for w1118 wing disc cells. For RNA-seq, 30 third-instar wing discs of each genotype were dissected in Ringer’s solution; nota were removed to isolate the domain of Bx-GAL4. Dissected material was frozen in liquid nitrogen for total RNA extraction in triplicate (QIAGEN RNeasy Mini Kit). Microarray and RNA-seq data were deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE54928.

**Fly Crosses, RNAi Screens, and Periodic Gene Function Website**

Males from UAS-IR lines were crossed with virgin GAL4 females. All crosses were conducted at 25°C, with exceptions at 18°C and 29°C as noted. Phenotypes were scored blind. For image analysis and RNA-seq experiments, only male larvae were used. Representative RNAi phenotypes can be accessed at http://odr.stowers.org/FlyCycle.

**Confocal and Live Imaging**

Confocal images were captured using an SP5 confocal microscope (Leica Microsystems). An UltraVIEW spinning disk microscope (PerkinElmer; mounted on an Axio Observer Z1 inverted microscope [Zeiss]) was used for live imaging of mitotic cell division. Spinning disk images were taken and viewed using Velocity (Improvement, PerkinElmer), and deconvolution was performed with Huygens software (Scientific Volume Imaging). The IKNM time lapses were taken using a SPIM system built in the Stowers Institute Imaging Core. All images were analyzed using ImageJ (Schnieder et al., 2012). Live wing imaging discs were mounted between two pieces of Scotch double-sided tape (3M) in a 35 mm glass-bottomed culture dish (MatTek) and covered by a round 5 mm coverslip (Fisher Scientific; for spinning disk system) at room temperature, or in 1% agarose in Ringer’s solution (for SPIM) at 30°C. For quantification of the distance of anti-PH3+ nuclei from the apical epithelial surface, nuclei from the strong knockdown regions (wing pouch for *rub-GAL4* and dorsal wing pouch for A9-GAL4) were used.

**ACCESSION NUMBERS**

The Gene Expression Omnibus accession number for the microarray and RNA-seq data reported in this paper is GSE54928.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, two tables, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.02.018.

(C) Anti-p-MRLC (green) accumulates at the cortex of rounded mitotic cells (arrowheads; nuclei are anti-PH3+, blue; F-actin, red) in controls and after CR32027 RNAi, but not in mitotic cells after CG10479 RNAi (driven by UAS-dicer2, w1118; nubbin-GAL4). (D) Schematic summary of phenotypes for CR32027 and CG10479 RNAi. Knockdown of either gene produced basally mislocalized mitotic nuclei (blue) without disrupting the sepalite junctions (yellow). Notably, in CR32027 RNAi, mitotic rounding appears to be normal and anti-p-MRLC (green) accumulates at the cortex. (E) Increased numbers of basally mislocalized mitotic nuclei (anti-PH3+, green; white arrowheads) were observed in A9-Gal4 > UAS-CR32027 RNAI wing discs treated with 100 μM cytochalasin D (CytoD) for 30 min. Samples were again stained for F-actin (red) and DNA (blue). (F) Percentage of basal anti-PH3+ nuclei in controls and in CR32027 RNAI wing discs with and without cytoskeletal inhibitor treatments. Values are mean ± SD. The asterisks indicate statistical significance compared with control by t test with a p value < 0.005. (G) Distribution of anti-PH3+ nuclei relative to the apical epithelial surface in controls and CR32027 RNAI wing discs, with and without cytoskeletal inhibitor treatments. Mann-Whitney U test between all four groups is significant (p < 0.005).
Figure 7. CR32027 Is Required for IKNM, Potentially through Modulation of Centrosome Function and the Transcriptional Regulation of Klp54D

(A) Basally mislocalized mitotic nuclei (anti-PH3+, red; DNA, blue; arrowheads) and MTOCs (GFP-Cnn, green) in both CR32027-IR1 and CR32027-IR2 wing discs (driven by A9-GAL4).

(B) Percentage of anti-PH3+ nuclei outside the MZ in control, CR32027-IR1, and CR32027-IR2 wing discs. Values are mean ± SD. The asterisks indicate statistical significance compared with control by t test with a p value < 0.005.

(C) Percentage of basal anti-PH3+ nuclei associated with basal MTOCs in control, CR32027-IR1, and CR32027-IR2 wing discs. Values are mean ± SD. The asterisks indicate statistical significance compared with control by t test with a p value < 0.005.

Figure 7 cont. (legend continued on next page)
AUTHOR CONTRIBUTIONS

M.C.G. and L.L. designed the experiments, and L.L. performed most of the experiments. L.L. and J.S.H. performed flow cytometry experiments. C.W.S. performed data analysis of microarray and RNA-seq. L.L. and M.C.G. prepared the manuscript. All authors read and agreed with the content of the paper.

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