LETTERS

Identification of pathways regulating cell size and cell-cycle progression by RNAi

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Many high-throughput loss-of-function analyses of the eukaryotic cell cycle have relied on the unicellular yeast species *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In multicellular organisms, however, additional control mechanisms regulate the cell cycle to specify the size of the organism and its constituent organs[1]. To identify such genes, here we analysed the effect of the loss of function of 70% of *Drosophila* genes (including 90% of genes conserved in human) on cell-cycle progression of S2 cells using flow cytometry. To address redundancy, we also targeted genes involved in protein phosphorylation simultaneously with their homologues. We identify genes that control cell size, cytokinesis, cell death and/or apoptosis, and the G1 and G2/M phases of the cell cycle. Classification of the genes into pathways by unsupervised hierarchical clustering on the basis of these phenotypes shows that, in addition to classical regulatory mechanisms such as Myc/Max, Cyclin/Cdk and E2F, cell-cycle progression in S2 cells is controlled by vesicular and nuclear transport proteins, COP9 signalosome activity and four extracellular-signal-regulated pathways (Wnt, p38βMAPK, FRAP/TOR and JAK/STAT). In addition, by simultaneously analysing several phenotypes, we identify a translational regulator, eIF-3p66, that specifically affects the Cyclin/Cdk pathway activity.

The cell cycle can be divided into distinct phases including a synthesis (S) phase, where DNA is replicated, and a mitosis (M) phase, where cell division occurs. In animal cells, growth and the synthesis of components required for these phases are regulated by extracellular growth factors and occur mainly in two gap phases, G1 (between M and S) and G2 (between S and M)[2–4]. The ordered progression of the cell-cycle phases is orchestrated by cyclin-dependent kinases (Cdks), whose activity is controlled by phosphorylation and by association with specific regulatory subunits, the cyclins[2–4].

The cell cycle is a robust system in which compensatory mechanisms control its overall length[5]. We therefore analysed the effect of RNA-mediated interference (RNAi)-induced loss of function of *Drosophila* genes in S2 cell-cycle distribution using flow cytometry, which allows direct determination of the fraction of cells in different phases of the cell cycle (Supplementary Fig. S1).

Consistent with the high efficiency of RNAi in *Drosophila*[6–9], clear phenotypes including arrest in G1, G2/M and S, cytokinesis and DNA replication defects, and apoptosis and/or cell death were observed when targeting known regulators of these processes (Fig. 1a and Supplementary Fig. S2). We next carried out a pilot screen targeting *Drosophila* kinases and phosphatases individually (Fig. 1b and Supplementary Fig. S3), and in pools of homologues to address redundancy (Fig. 1c and Supplementary Fig. S4). Unexpectedly, the analysis of redundancy did not reveal any additional cell-cycle regulators (Fig. 1c and Supplementary Table S3). These results suggest that a considerably lower fraction of *Drosophila* kinases regulate the cell cycle (19%) than has been reported previously (35%, ref. 10; for explanation and comparison, see Supplementary Table S4 and Fig. S5). Despite this, our screen identified also kinases for which a role in the S2 cell cycle has not been appreciated (for example, AKT1, CG7177 and MEKK1/MEKK4).

We reasoned that, by screening most *Drosophila* genes, we should be able to identify the pathways regulating the S2 cell cycle. Screening of 11,971 double-stranded RNAs (dsRNAs) generated from the *Drosophila* Gene Collection (DGC) releases 1 and 2 (see Supplementary Methods and refs 7, 8) showed that loss of 270 and 169 genes resulted in significant changes in G1 and G2 populations, respectively (Supplementary Fig. S6). A strong correlation between cell size at the G1 and G2 phases was observed in individual samples, suggesting that *Drosophila* cells do not have a 'strong' cell-size checkpoint[11] that forces cells to a particular size at a defined cell-cycle phase (Supplementary Fig. S7). Components acting on the same direction in a particular process, such as ribosomal proteins or Dp, E2f and Cyclin E, appeared in discrete areas of a plot describing G1 cell size as a function of fraction of cells in G1 (Fig. 2a). The strongest phenotypes, characterized by specifically decreased cell size, were observed with dsRNAs targeting *Rbf*, a negative regulator of E2f pathway, and *cropped* (*crp*), the *Drosophila* AP4 transcription factor (Supplementary Tables S3 and S5).

To identify genes involved in cell death and cytokinesis, we analysed changes in the populations of cells with less than 2*N* DNA or more than 4*N* DNA, respectively (Fig. 2 and Supplementary Fig. S8). The increase in the $<2N$ population in all cases consisted of apoptotic or dead cells; the genes whose loss caused this phenotype included previously unknown effectors, the known inhibitor of apoptosis *Thread* and several mitosis regulators, consistent with induction of apoptosis by mitotic catastrophe[12] (Fig. 2b, and Supplementary Figs S8 and S9 and Table S6). The increase in the $>4N$ population was due to cells that had undergone two rounds of DNA replication without cytokinesis (8*N* DNA); dsRNAs causing this phenotype targeted several previously undescribed genes and known cytokinesis regulators[9] (Fig. 2c, and Supplementary Fig. S8 and Table S6).

There have been several large-scale RNAi screens, and in many there is very little overlap between the gene sets identified even when the same phenotypes are analysed. This is apparently due to a very large number of false positives (see Supplementary Tables S7 and S8, and Fig. S10). To reduce the number of false positives, we analysed all phenotypes by using criteria that excluded all 564 control samples ($>99.99994\%$ confidence). Roughly 4% of genes (488; Supplementary Table S3) had a cell-size, cell-cycle or cell-death phenotype. From an analysis of 19 different pathways and/or protein complexes, we estimate that our screen identified ~80% of strong non-redundant

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cell-cycle regulators (Fig. 3a and Supplementary Table S9). Despite the general effectiveness of the RNAi (Fig. 3a and Supplementary Fig. S11), however, we did not identify some genes with clearly defined functions in the cell cycle (Fig. 3a and Supplementary Fig. S12). In different cases, the weak RNAi phenotype could be explained by high protein levels or stability, redundant function and/or the fact that defects in mitotic or DNA replication fidelity often do not appreciably alter overall cell-cycle phase distribution[13] (Supplementary Table S9 and Fig. S12).

Of the 78 known interactions between the genes identified, 70 were consistent with the observed phenotypes (Supplementary Table S10). A high proportion of the diseases linked to the human orthologues of the identified genes are related to cancer (8 of 25; Supplementary Table S5), consistent with the known relationship between cell-cycle regulation and cancer. As expected, the *Drosophila* protein–protein interaction map[14] indicated a much larger number of interactions between proteins than we identified than between a random set of proteins (Fig. 3b), and most interactions were consistent with the observed phenotypes (107 of 143; Supplementary Table S11).

Of the 488 genes that we identify, 319 (65%; Supplementary Tables S1 and S3) have not been identified in previous *Drosophila* and mammalian RNAi screens. A principal difference between our screen and previous studies[6,9] is that, by using flow cytometry, we can simultaneously identify six distinct phenotypes, allowing unbiased classification of the identified genes into pathways using hierarchical clustering (Fig. 3c and Supplementary Fig. S13). Positive components of a given pathway should segregate into the same cluster, because their loss is expected to cause similar phenotypes. This was indeed observed, for example 43 of 56 dsRNAs in one 'translation' cluster targeted ribosomal subunits. Another 'G1 cell-cycle' cluster contained the classical positive G1 regulators of the Cdk/E2f pathway, including E2f, Dp, Cyclin E, Cdk2 and Cdk4 (Fig. 3c). Because of an opposite phenotype, Rbf, a known negative regulator of this pathway, was not included in this cluster.

The other genes in the G1 cell-cycle cluster included two cullins (Cul-1/lin19 and Cul-4), involved in ubiquitin-mediated protein degradation, and five components of the COP9 signalosome (Fig. 3c), a protein complex linked to regulation of cullin activity[15]. In

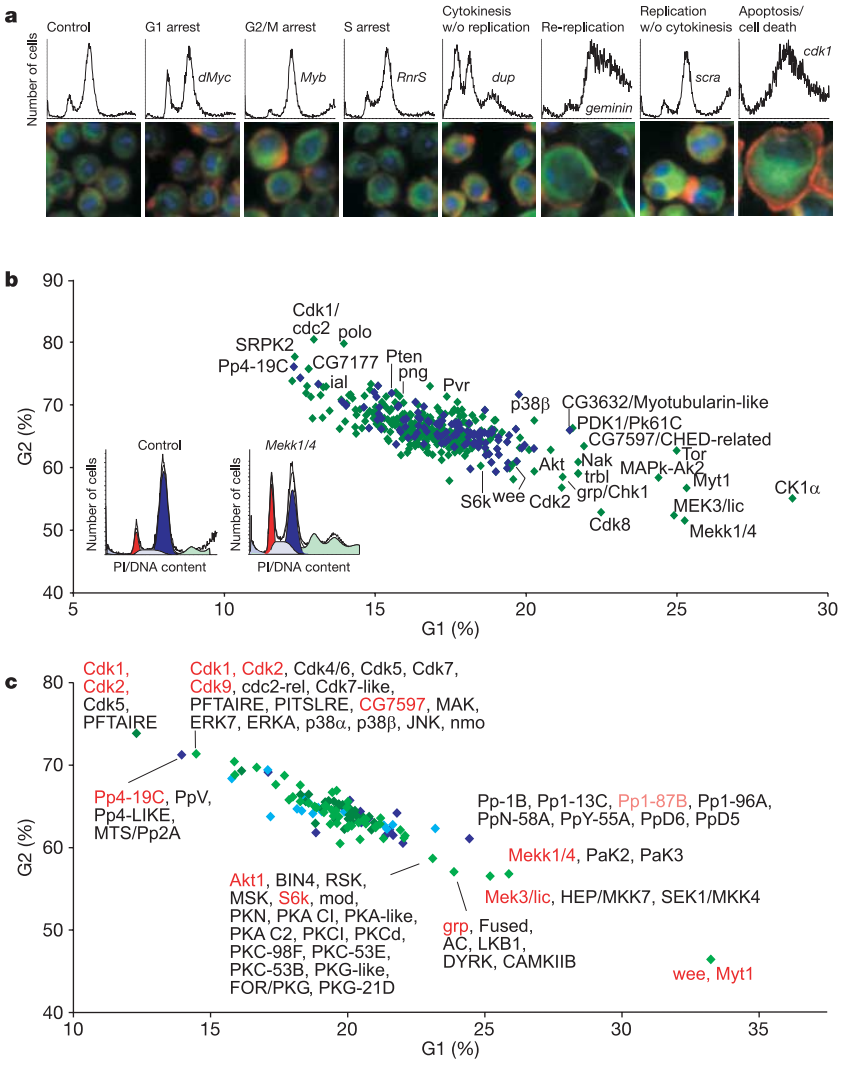

Figure 1 | Cell-cycle regulation by protein phosphorylation in S2 cells. **a**, Flow cytometry can detect multiple RNAi phenotypes. Shown are histograms (top; DNA content) and micrographs (bottom; blue, DNA; red, F-actin; green, α-tubulin) of dsRNA-treated cells. Note that, similar to *geminin* dsRNA, *Cdk1* dsRNA causes cell death and also a giant cell and re-replication phenotype. *scra* dsRNA induces binucleate cells with 8*N* DNA, whereas *Myb* dsRNA induces a small increase in cells having single nuclei with 8*N* DNA. **b**, **c**, Redundancy of protein kinase (green) and phosphatase (blue) function. Fraction of G1 (*x* axis) and G2 (*y* axis) phase cells after 4 d of treatment with individual dsRNAs (**b**) or pooled dsRNAs (**c**) against related genes (dark colour, close homologues; light colour, more distant homologues; red text, genes identified previously in individual screen). Note that a pool containing *wee* and *Myt1* has a much stronger phenotype than *wee* or *Myt1* alone. *Pp1-87B* (pink) was identified only in the DGC screen. Inset, phenotypic analysis of the control and MEKK1/MEKK4 samples.

mammals Cul-4 has been linked to DNA damage response[16], but our results indicate that Cul-4 is also involved in normal cell-cycle regulation in animals. Consistent with the similar phenotypes of the cullins, COP9 subunits (CSNs) and several Cdk/E2f pathway components (Fig. 3c), the increase in G1 induced by dsRNAs targeting CSNs could be reversed by simultaneously targeting the Cdk inhibitor dacapo (Supplementary Fig. S15). Our results are thus consistent with a loss of COP9 leading to G1 arrest owing to failure of cullin-mediated degradation of dacapo. The positive role of COP9 in the cell cycle is in agreement with mouse knockout studies[15,17]. In *Drosophila* ovaries, however, COP9 negatively regulates the cell cycle[18], suggesting that its effects may be tissue-specific.

Notably, a translational initiation factor, eIF-3p66, clustered with the G1 cell-cycle regulators. Expression of *eIF-3p66* also correlates well with that of Rbf, E2f and Cyclin E during *Drosophila* development (Supplementary Fig. S14). dsRNAs targeting *eIF-3p66* resulted in an increased cell size, indicating that, unlike loss of other translational regulators, loss of eIF-3p66 does not generally inhibit translation. Furthermore, the phenotype caused by dsRNA targeting *eIF-3p66* was reversed by dsRNA targeting *dacapo* (Supplementary Fig. S15 and data not shown), indicating that eIF-3p66 specifically affects the Cyclin/Cdk pathway.

The cluster of genes whose loss resulted in an increase in G1 cell size with no G1 DNA content phenotype contained several genes involved in transcription and messenger RNA processing (Fig. 3c). The lack of G1 phenotype is probably due to a uniform effect of transcriptional inhibition on all cell-cycle phases, and continued growth of the cells is possible owing to translation of stable mRNAs. The distinct phenotype of transcriptional and translational regulators suggests that cell size may be controlled at the RNA level, either directly by regulatory RNAs or through differential stability of mRNAs of proteins controlling cell division and growth.

A number of genes whose loss resulted in increased G1 content were classified into a defined 'signalling' cluster, which contained two regulators of translation (eIF-4G and Ef1α48D), a ubiquitin E3-ligase (URE-B1/CG8184), and components of several extracellular ligand-regulated signalling pathways (Fig. 3c). These genes differed from those in the G1 cell-cycle cluster in that the dsRNAs targeting them resulted in decreased cell size. Thus, the signalling pathways either affect cell division through regulation of growth, or affect both cell growth and cell division in parallel (see ref. 19).

By manually comparing the genes identified (Supplementary Table S3) with literature, we identified four signalling pathways that regulate the cell cycle: the FRAP/TOR, JAK/STAT, Wnt and p38βMAPK pathways. The FRAP/TOR[20] pathway is known to regulate cell proliferation and, consistently, we identified eight known components of this pathway as G1 regulators (Supplementary Fig. S15). In S2 cells, another known cell-cycle regulatory pathway, the JAK/STAT pathway[21], had a relatively weak effect, as indicated by the mild phenotypes observed by dsRNAs targeting several of its components (Supplementary Fig. S15). The identified genes known to regulate the Wnt pathway (Supplementary Fig. S15) indicated that this pathway is not active in S2 cells, but that treatments that activate it inhibit S2 cell proliferation.

Several genes linked to the p38βMAPK pathway[22,23] (Fig. 1b and Supplementary Table S1) were also identified, and we verified the function of MEKK1/MEKK4 and MEK3 in this pathway by analysing p38βMAPK phosphorylation in response to dsRNA treatments (Supplementary Fig. S15). Although regulation of the cell cycle by the p38βMAPK pathway in the absence of stress or DNA damage has

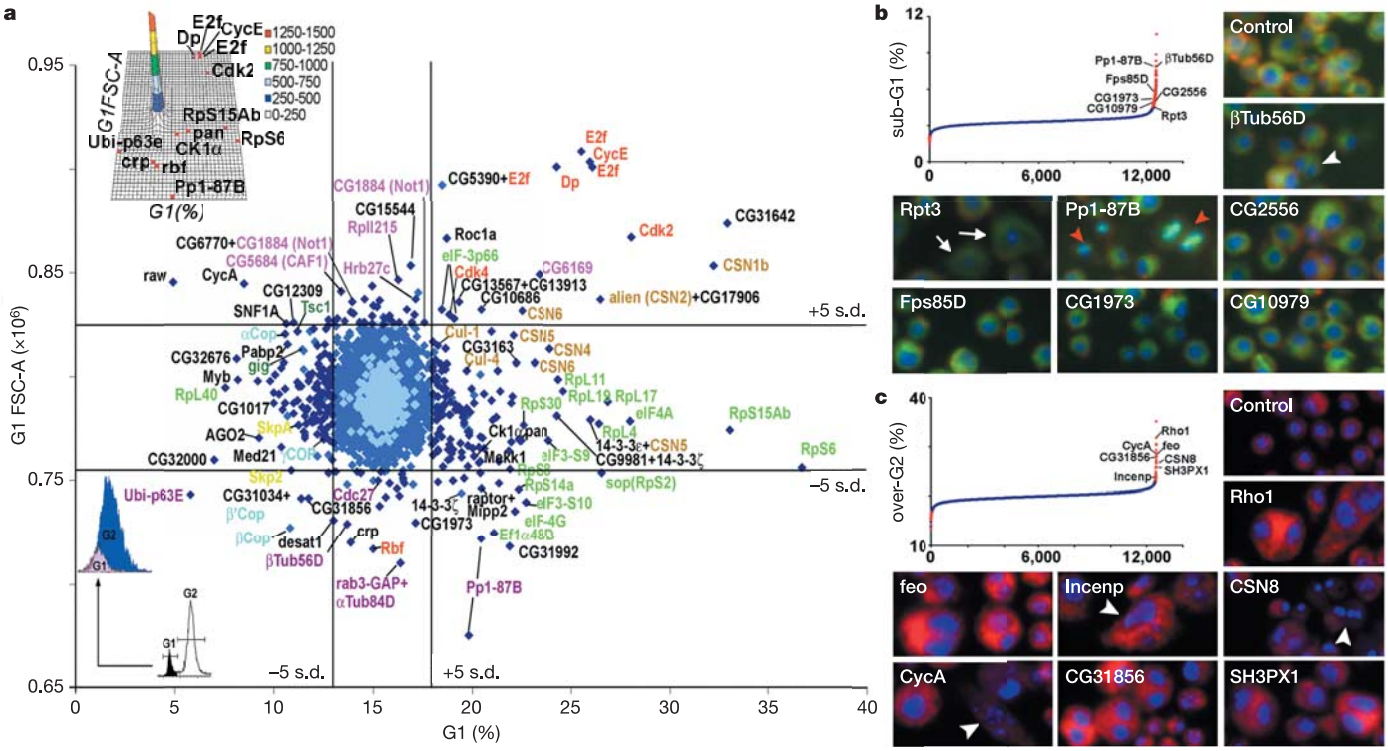

Figure 2 | The DGC RNAi library screen. a, Cell size (FSC-A) as a function of fraction of cells in G1. Control samples are indicated in light blue, and samples that do not and do differ significantly from the mean (Supplementary Table S3) in blue and dark blue, respectively. Genes involved in the Cdk/E2f pathway (red), COP9 signalosome activity (brown), translation (light green), apoptosis and/or cell death (purple; see Supplementary Fig. S6a), vesicular transport (cyan), SCF^{Skp2} (yellow-green), RNA transcription or splicing (light purple), and tuberous sclerosis (dark green) are indicated. Lines indicate 5 s.d. of control samples. Inset, sample density (*z* axis) as a function of G1 DNA content (*x* axis) and G1 size (*y* axis). **b**, dsRNAs inducing cell death (blue, DNA; red, F-actin; green, α-tubulin). Note that some dsRNAs also induce a mitotic (red arrowhead), flat cell (arrow) or multinucleate (white arrowhead) phenotype. **c**, dsRNAs inducing over-4*N* DNA content (blue, DNA; red, protein). Arrowheads indicate abnormal nuclei. Graphs in **b** and **c** show ranking of phenotypic scores; genes outside the 5 s.d. limits are in red.

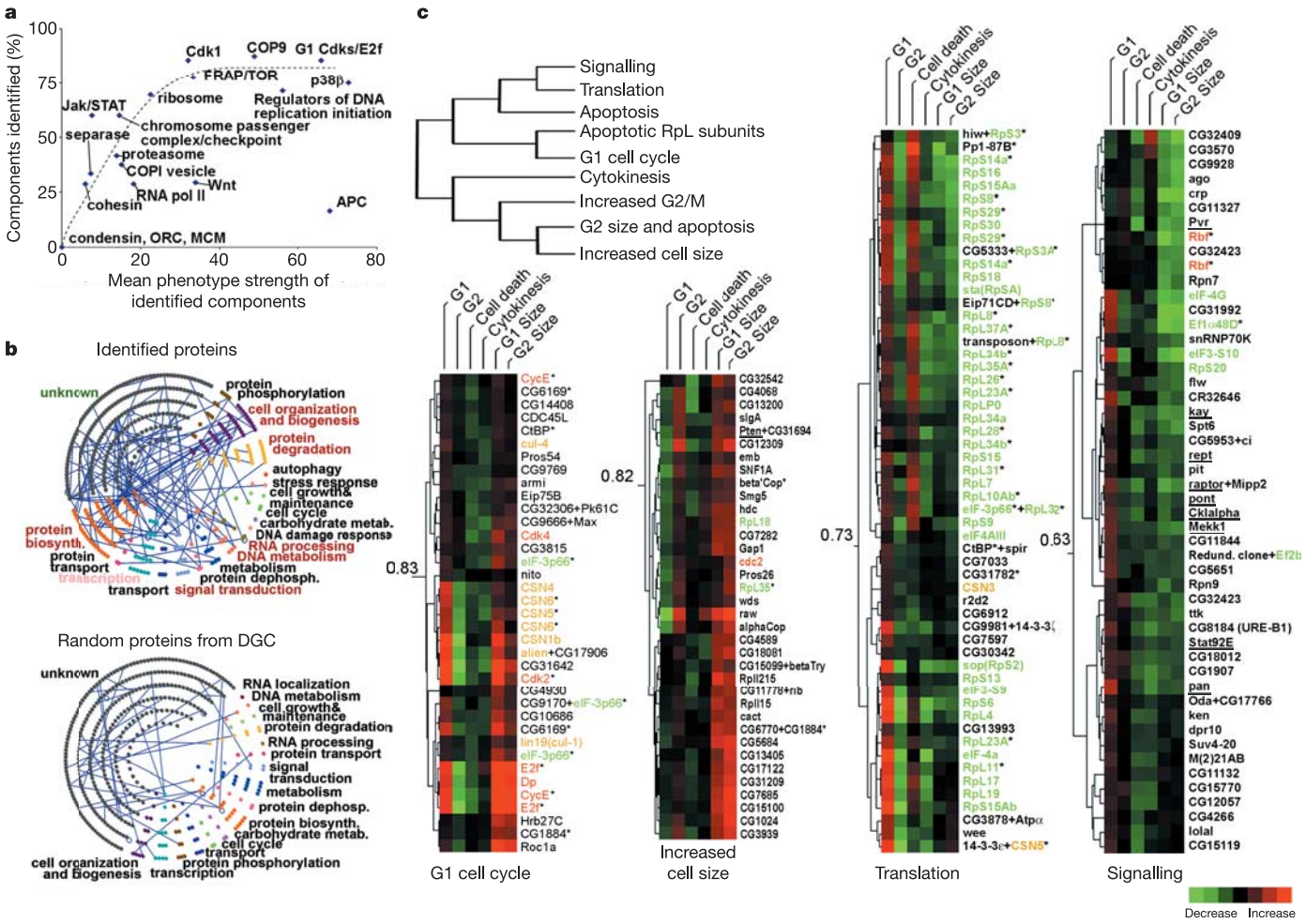

Figure 3 | Classification of genes identified in the DGC screen. **a**, Percentage of genes identified in the 19 indicated pathways or protein complexes increases as a function of the mean phenotype strength of the complex (approximated by the dotted line). **b**, Identified proteins (top) have a much larger number of interactions[14] (coloured lines) than does a random set of proteins (bottom; the median example of five random sets is shown). Overrepresented (red, >2 ×; light red, >1.5 ×) and underrepresented (green) gene ontology classes are indicated. **c**, Hierarchical clustering of genes based on their phenotypic scores. Top left, major sub-branches, named according to phenotype and/or known activity of the included genes. Four sub-branches are shown in detail (for complete analysis, see Supplementary Fig. S13). Note that genes that are present more than once (asterisks) typically cluster tightly and that genes involved in the Cdk/E2f pathway (red), COP9 signalosome activity (yellow), translation (light green) and signal transduction (underlined) are predominantly found in specific clusters. Pearson correlation coefficients of the clusters are also indicated.

not previously been appreciated, our data are consistent with the identification of MAPk-Ak2 as a checkpoint kinase[24].

Loss of several kinases that negatively regulate Cdk1 activity led to an increase in G1 content (Supplementary Fig. S15), probably owing to an accelerated G2/M phase[5]. We also observed that Chk1 and MAPk-Ak2, two kinases that have been linked to DNA damage checkpoints[24–26], operate in normal cell-cycle regulation of cultured S2 cells. Together with the finding that in human cells Chk1 shields Cdk1 from premature activation[27], our results suggest that during normal cell cycle these kinases have basal activity that slows down G2 progression. The basal activity could represent partial activation that occurs locally in response to damage during normal replication, possibly effecting localized changes in DNA replication and repair.

In summary, we have reported that several pathways and processes are involved in cell-cycle and cell-size regulation, including basal transcription, vesicular trafficking, nuclear export, ubiquitin-mediated protein degradation, and the Cdk/E2f, JAK/STAT, FRAP/TOR, p38βMAPK and Wnt signalling pathways (Supplementary Table S1). The genes identified are likely to include previously unknown components of these pathways (Fig. 3a), ubiquitin ligase and kinase substrates (Supplementary Table S12), and targets of transcription factors (Supplementary Table S13) such as E2f and Myc/Max that are relevant for cell-cycle progression. Thus, our results will also serve as a solid foundation for a systems biology analysis of the metazoan cell cycle.

METHODS

RNAi libraries, cell culture and transfections. The RNAi libraries targeting *Drosophila* protein and lipid kinases and phosphatases, and the genes in the publicly available clone collections (BACPAC Resources Centre), consisting of about 70% of the genes annotated by the Berkeley *Drosophila* genome project[28,29], are described in the Supplementary Methods (for sequence information, see http://www.fruitfly.org/sequence/dlcDNA.shtml). Each dsRNA was screened individually. The DGC libraries occasionally contain clones in which sequences from two genes were ligated during cDNA cloning. In such clones, both genes are indicated separated by a plus sign, as the dsRNA transcribed from such a clone will target both genes. Single gene targeting could identify some genes with overlapping function (see Fig. 1). Although simultaneous targeting is required for identifying redundant genes, identification of genes when targeted alone does not rule out overlapping functions. If multiple proteins have similar activity that is rate limiting for cell-cycle progression, loss of even one protein should result in a weak phenotype. Because most pools containing known effectors were identified (Fig. 1), pooling did not substantially reduce the RNAi efficiency.

Drosophila S2 cells were cultured at controlled temperature (23.5 °C; Binder

KB cooling incubator) in *Drosophila* SFM (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. Under these culture conditions, most of the S2 cells were in the G2 phase of the cell cycle with 4*N* DNA content, as described previously[30]. For DGC screen transfections, 80,000 S2 cells were plated on all wells of 96-well plates. On the following day, the cells were transfected with dsRNAs in triplicate using Effectene (Qiagen) according to manufacturer's instructions, except that 50 ng dsRNA, 0.4 μl enhancer and 0.5 μl Effectene in 50 μl EC buffer were used per well (25 ng dsRNA, 25% transfection reagents and 10,000 cells were used in 384-well plates). Transfections were done similarly for the kinase/phosphatase screen, except that 450,000 cells, 125 ng dsRNA, 0.8 μl enhancer and 5 μl Effectene were used in 24-well plates (Fig. 1 and Supplementary Fig. S3).

Flow cytometry. Cells cultured for 4 days after transfection were collected by centrifugation, the culture media was removed, and the cells were fixed with ice-cold 70% ethanol overnight. The cells were then suspended in PBS containing 30 μg ml^{-1} propidium iodide (P1304MP; Molecular Probes) and 30 μg ml^{-1} RNase A (R5125; Sigma). After 30 min incubation at 37 °C, the cells were analysed with a FACSArray (DGC screen) or LSR (kinase/phosphatase screen) flow cytometer (Beckton Dickinson). Cells that were not immediately analysed were stored at 4 °C, and equilibrated to room temperature before analysis. The dsRNAs were screened individually in triplicate, and phenotypes were scored by automated analysis. A minimum confidence interval of 99.99994%, excluding all 564 'mock transfection' control samples, was used to identify hits; thus, the data set is essentially free of statistical error, as only 0.04 false positives should occur in the 72,000 hypotheses (12,000 × 6 phenotypes) tested. Data analysis is described in detail in the Supplementary Methods. Small fluctuations in G1 and G2 peak widths prevented statistically reliable automated analysis of S phase. Visual inspection was additionally used to classify sub-G1 and over-G2 phenotypes. Apart from *double parked*, *Cdk1* and *geminin*, which were all re-identified in our screen, no other dsRNAs resulted in a division without replication or (aneuploid) DNA re-replication phenotypes (see Fig. 1a). Mean phenotype strength of complexes was calculated by using the strongest of the six measured phenotypes (normalized to 0–100%; see Supplementary Table S9). Only genes that scored as hits were included in the analysis to determine the hit rate as a function of mean phenotype strength.

MTS assay and microscopy. MTS metabolic activity assay was assayed using a CellTiter96AQ kit (Promega) according to the manufacturer's instructions in triplicate 384-well plates after 4 days of dsRNA treatments. For microscopy, cells were plated on 96- or 384-well Packard ViewPlates, transfected with dsRNAs, and stained after 4 days with Hoechst33342 (Invitrogen) and Celltrace Far-Red SSAOE whole protein stain (for counting of multinucleate cells; Invitrogen) or with Hoechst33342, AlexaFluor546-conjugated phalloidin (Invitrogen) and fluorescein isothiocyanate (FITC)-conjugated anti-α-tubulin (for analysis of mitosis). The cells were imaged and analysed by a Cellomics ArrayScan high content microscope with the Morphology Explorer BioApplication. Total cell counts were obtained using automated analysis, and the mitotic and multinucleated cells were counted visually by a researcher who was blind to the samples. Antibodies are described in Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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