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Drosophila Genome-Scale Screen for PAN GU Kinase Substrates Identifies Mat89Bb as a Cell Cycle Regulator

Short Article

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Summary

Although traditional organism-based mutational analysis is powerful in identifying genes involved in specific biological processes, limitations include incomplete coverage and time required for gene identification. Biochemical screens using cell transfection or yeast two-hybrid methods are rapid, but they are limited by cDNA library quality. The recent establishment of "uni-gene sets" has made it feasible to biochemically screen an organism's entire genome. Radiolabeled protein pools prepared from the *Drosophila* Gene Collection were used in a *Drosophila* in vitro expression cloning ("DIVEC") screen for substrates of PAN GU kinase, which is crucial for S-M embryonic cell cycles. Ablation of one identified substrate, Mat89Bb, by RNAi produces a polyploid phenotype similar to that of *pan gu* mutants. *Xenopus* embryos injected with *Mat89Bb* morpholinos arrest with polyploid nuclei, and *Mat89Bb* RNAi in HeLa cells gives rise to multinucleated cells. Thus, Mat89Bb plays an evolutionarily conserved role as a crucial regulator of both cell cycle and development.

Introduction

Early embryonic cell cycles in *Drosophila* consist of rapidly alternating rounds of DNA replication (S phase) and mitosis (M phase) without intervening gaps (G1 and G2) (reviewed in Lee and Orr-Weaver, 2003). Driven by large maternal pools of RNA and protein, these streamlined cell cycles are regulated posttranscriptionally. Three *Drosophila* genes, *pan gu* (*png*), *plutonium* (*plu*), and *giant nuclei* (*gnu*), play crucial roles in coordinating these rapid S-M cycles (Freeman and Glover, 1987; Freeman et al., 1986; Shamanski and Orr-Weaver, 1991). Mutation of any of these genes disrupts coupling of S and M phases, resulting in DNA replication without mitosis ("giant nuclei" phenotype) in embryos from homozygous female mutants. A similar phenotype is observed in unfertilized eggs from *png*, *plu*, or *gnu* mutants: the female meiotic products fail to condense to form normal polar bodies, and unscheduled DNA replication results in polyploidy. *png* encodes a serine/threonine kinase (Fenger et al., 2000), and *plu* and *gnu* encode novel proteins (Axton et al., 1994; Renault et al., 2003). Recently, we have shown that all three proteins are required to form an active kinase complex, and that PNG activation requires GNU-dependent dimerization of PNG/PLU heterodimers (Lee et al., 2003).

Several lines of evidence show that Cyclin B is a key target of the giant nuclei class of genes. A weak *png* phenotype is dominantly enhanced by a 2-fold decrease in *cyclin B* gene dosage, whereas increasing the *cyclin B* copy number suppresses the giant nuclei phenotype (Lee et al., 2001). Furthermore, Cyclin B protein levels are decreased in embryos from *png*, *plu*, or *gnu* mutants (Fenger et al., 2000). Thus, one function of the PNG complex is to maintain adequate levels of Cyclin B required for chromosome condensation and mitotic entry and to prevent rereplication of DNA after S phase is completed. To gain insight into the mechanism by which the PNG kinase complex regulates embryonic cell cycles, a large maternal-effect lethal collection was recently screened to identify new giant nuclei mutants, and a yeast two-hybrid screen for PNG-interacting proteins was performed (Lee et al., 2003). Unfortunately, neither approach led to the identification of new genes that might elucidate the mechanism of action of the PNG kinase complex.

In vitro expression cloning (IVEC) is a powerful method that combines the ability to make radiolabeled proteins for use in a variety of biochemical screens with the ability to quickly isolate corresponding cDNAs (King et al., 1997; Lustig et al., 1997). In its original incarnation, pools of ~100 random clones from a cDNA expression library are added to coupled transcription-translation reactions in the presence of [^{35}S]methionine. An appropriate phage RNA polymerase (T7, T3, or SP6) drives expression of cDNAs in vectors with upstream promoters. Pools of radiolabeled proteins that are generated can then be used in a variety of biochemical screens. As opposed to other biochemical screening approaches (e.g., yeast two-hybrid), IVEC screening does not require fusion libraries, which are often of poor quality because the cDNA must be cloned in-frame with the bait, and full-length clones often have stop codons in their 5′-UTRs.

The IVEC screening approach has been used successfully to identify proteins that undergo mitosis-specific degradation (McGarry and Kirschner, 1998), apoptotic protease substrates (Cryns et al., 1997), protein kinase substrates (Gao et al., 2000; Stukenberg et al., 1997), DNA binding proteins (Mead et al., 1998), phosphoinositide binding proteins (Rao et al., 1999), and proteins with uracil-glycosylase activity (Haushalter et

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al., 1999). As with any screening approach that uses conventional cDNA libraries, a major limitation is the fact that genes are often over- or underrepresented in such libraries. Furthermore, while pools are relatively small, identification of individual clones often takes longer than the initial screen, and clones occasionally are not recovered. The use of a normalized, annotated library from an organism would represent an ideal way to perform biochemical screens using the IVEC approach. Clones would be equally represented within pools, the responsible gene within positive pools could be quickly isolated because the identity of each clone in every pool would be known a priori, and the entire genome of an organism could be sampled in a short time. Here, we describe the development of an IVEC screening approach using *Drosophila* Gene Collection and the isolation of a PNG kinase substrate, Mat89Bb, essential for S-M cycles of early *Drosophila* embryogenesis, *Xenopus* embryonic cell cycles and morphogenesis, and cell division in cultured mammalian cells.

Results and Discussion

DIVEC Screen for PNG Kinase Substrates

The *Drosophila* Gene Collection (Stapleton et al., 2002) comprises full-length, annotated cDNAs representing the majority of known genes in *Drosophila melanogaster*. Release 1 consists of 5849 cDNAs corresponding to ~43% of the *Drosophila* genome. Clones are arrayed in 17 384-well plates, with the first 4 plates consisting of clones in a pBlueScript vector, and the remainder consisting of clones in the vector pOT2a. Taking advantage of the fact that we can generate active recombinant PNG kinase complex and that phosphorylation of a given protein can alter its mobility on SDS-PAGE, we have applied the IVEC screening methodology to Release 1 of the *Drosophila* Gene Collection in an approach that we have termed *Drosophila* IVEC ("DIVEC") to identify PNG kinase substrates.

Clones from the *Drosophila* Gene Collection Release 1 were individually grown, and bacterial cultures were pooled in groups of 24 (each group representing a row from the original 384-well plates) for plasmid DNA isolation. These DNA pools were used to generate radiolabeled protein pools in a coupled transcription-translation reticulocyte lysate system. Initial tests indicated that, although strongly radiolabeled protein pools could be generated from pOT2a-based plasmid pools, only weakly radiolabeled protein pools were generated from pBlueScript-based clones. It is likely that poor labeling of pBlueScript-based clones is due to the GC-rich content of the polylinker site that decreases transcription/translation efficiency. Given that transcription is the limiting step in coupled transcription-translation reactions, we bypassed the requirement for transcription of pBlueScript-based clones. Consequently, pBlueScript-based clones (Release 1 plates 1–4) were individually amplified by using primers containing vector sequences flanking the polylinker. PCR products were pooled in groups of 24 for mRNA synthesis by using a T7 promoter introduced into the upstream PCR primer, and mRNA pools were added to reticulocyte lysates for direct translation. Radiolabeled protein pools made in this way had a signal several-fold higher than that obtained from coupled transcription-translation reactions of pBlueScript-based plasmid DNA.

Translated products generated from four representative pools of *Drosophila* Gene Collection clones and assessed by SDS-PAGE/autoradiography are shown in Figure 1A. A broad range of radiolabeled proteins of varying sizes was detected. In general, approximately 80%–85% of predicted proteins of the appropriate size can be readily detected within pools after an overnight exposure. To identify substrates of the cell cycle kinase PNG, pools of labeled proteins made from Release 1 were incubated in a kinase reaction with active PNG complex consisting of recombinant PNG/GST-PLU/GNU expressed and purified by using the *Sf9*/baculovirus system. Potential contribution of other kinases within reticulocyte lysates was minimized by substantially diluting (20-fold) lysate into the reaction. As a negative control, labeled protein pools were also incubated with an inactive form of the complex (GST-PLU/GNU) lacking the PNG subunit. Reaction products were analyzed by SDS-PAGE and autoradiography. Candidate substrates were identified as bands within pools that migrated with slower mobility following treatment with active PNG kinase complex compared to the control reaction (Figure 1B). Positive pools were confirmed by retesting, and the apparent molecular weight of each protein that exhibited a PNG-dependent shift was estimated by comparison to standard markers. Candidate clones within each pool were selected based on predicted sizes of the encoded proteins and tested individually as PNG substrates.

A summary of results from our DIVEC screen for PNG substrates with the *Drosophila* Gene Collection Release 1 is shown in Table 1. Seven genes were identified whose protein products had altered mobility (shifted upward) after incubation with active PNG kinase (Figure 1C). One of these seven genes is *gnu*, which we previously showed encodes both an activator and substrate of PNG kinase (Lee et al., 2003). Other than *gnu* and *CG5739*, a gene of unknown function, the other five genes are evolutionarily conserved with vertebrate homologs. Of particular interest are the genes encoding Mat89Bb and the small ribosomal protein subunit mRpS22. Mat89Bb shares a maternal expression pattern with PNG, PLU, and GNU (Stebbings et al., 1998), and the PNG kinase complex regulates Cyclin B levels post-transcriptionally, possibly via regulation of its translation (Fenger et al., 2000). Given that Mmp2, which shifts weakly after PNG treatment, is a membrane bound matrix metalloproteinase that localizes to the cell surface (Llano et al., 2002), it is unlikely to be a bona fide PNG substrate. Except for the guanyl-nucleotide exchange factor *CG9139*, the other genes identified as PNG substrates have not been previously characterized.

To confirm that the decreased SDS-PAGE mobility of candidate PNG substrates is actually due to phosphorylation, samples were treated with Lambda Phosphatase after PNG kinase incubation. Treatment with Lambda Phosphatase completely reversed the mobility shifts of Mat89Bb (Figure 1D, upper panel) and four other candidate PNG substrates (data not shown), thereby con-

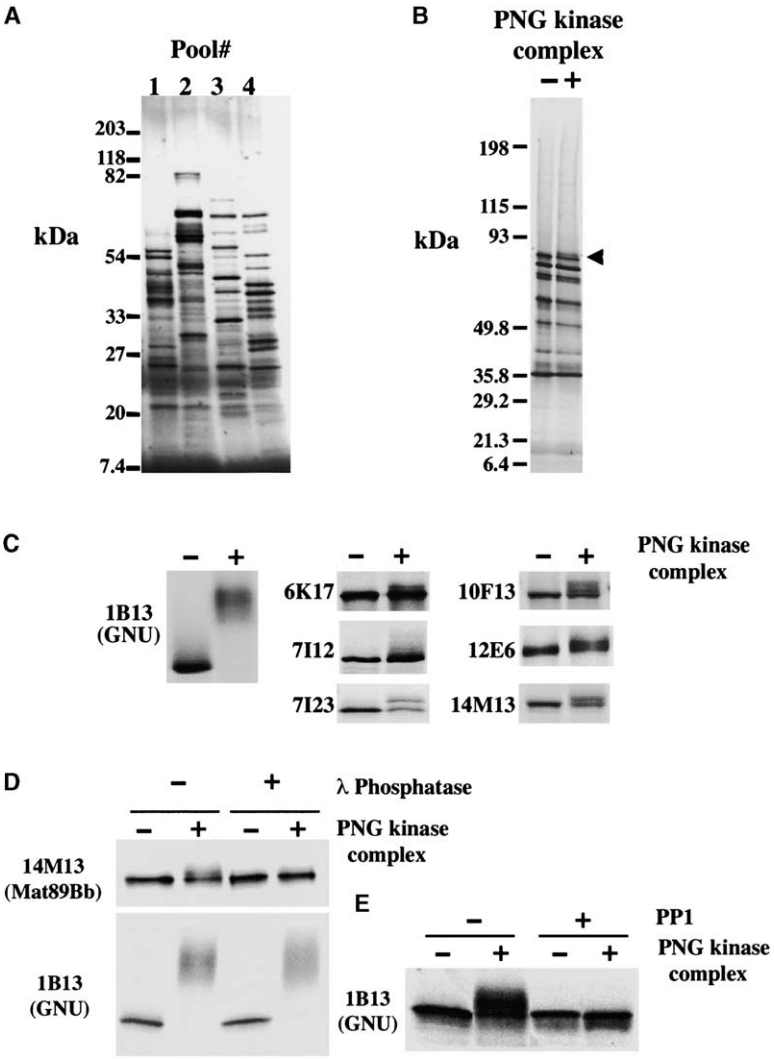

Figure 1. DIVEC Screen for PNG Kinase Substrates Identifies Seven Candidates

(A) Four representative pools (0.5 μl each) of in vitro-translated radiolabeled proteins made from the *Drosophila* Gene Collection. Samples were analyzed by SDS-PAGE/autoradiography and were exposed for 16 hr.

(B) Example of a positive pool from the DIVEC screen for PNG substrates. PNG kinase treatment (+) results in decreased mobility of a specific band (arrowhead) relative to control (–). This particular pool was generated from a DNA pool with only 12 clones (most pools had 24 clones).

(C) Mobility shifts of the seven individual proteins identified as PNG substrates.

(D) Incubation with Lambda Phosphatase after PNG kinase treatment reverses the mobility shift of Mat89Bb, but not GNU.

(E) Incubation with Protein Phosphatase 1 (PP1) after PNG treatment reverses the mobility shift of GNU.

firming that these shifts are a consequence of phosphorylation. In contrast, the mobility shifts of GNU and mRpS22 observed after incubation with PNG kinase were not altered by subsequent treatment with Lambda Phosphatase (Figure 1D, lower panel). This result, however, did not rule out the possibility that these mobility shifts are due to phosphorylation by PNG (as opposed to another modification) because not all phosphoproteins are susceptible to Lambda Phosphatase.

We previously identified the type I phosphatase *PP1 87B* as a dominant suppressor of *png* in a sensitized screen for genes that suppress or enhance *png* (Lee et al., 2001). One explanation for this genetic interaction is that PP1 may dephosphorylate substrates of PNG, thereby counteracting its activity. We therefore tested whether PP1 could dephosphorylate GNU after incubation with the PNG complex. Treatment with PP1 completely reversed the effects of PNG kinase on GNU mo-

Table 1. PNG Kinase Substrates Identified by *Drosophila* Genome-Scale IVEC Screen

DGC	Clone ID	Gene	Description	Size (kDa)	Vertebrate Homologs
1B13	LD12084	*CG5272, gnu*	Cell cycle regulator, maternal	27	No
6K17	GH07821	*CG12261*, *mRpS22*	Mitochondrial small ribosomal protein subunit	46	Yes
7I12	SD03462	*CG1794, Mmp2*	Matrix metalloproteinase	89	Yes
7I23	LD45253	*CG5739*	Uncharacterized	12	No
10F13	LD28817	*CG6962*	Uncharacterized	87	Yes
12E6	SD03358	*CG9139*	Guanyl-nucleotide exchange factor	77	Yes
14M13	LD33046	*CG6814, Mat89Bb*	Uncharacterized, maternal	76	Yes

bility (Figure 1E); a similar result was obtained for mRpS22 (data not shown). Thus, the altered mobilities of all candidate PNG substrates identified in our DIVEC screen represent PNG-phosphorylated forms of the proteins.

The PNG Substrate Mat89Bb Is Essential for S-M Cycles of Early *Drosophila* Embryogenesis

Of the seven in vitro substrates for the PNG kinase identified by DIVEC screening, loss-of-function mutations have been described for only *gnu* (Freeman et al., 1986) and *Mmp2* (Page-McCaw et al., 2003). Because mutation of any of the members of the PNG kinase complex (*png*, *plu*, or *gnu*) results in a giant nuclei phenotype (DNA replication uncoupled from mitosis, leading to polyploidy in embryos from mutant females) (Freeman and Glover, 1987; Freeman et al., 1986; Shamanski and Orr-Weaver, 1991), the PNG substrates identified by DIVEC screening might similarly be required for normal embryonic cell cycles. Given the small number of PNG substrates identified in our DIVEC screen and the lack of pre-existing mutations in the genes encoding these proteins, we used RNA interference to rapidly assess the consequences of "knocking down" the gene function of each identified PNG substrate. RNA interference was performed by injection of double-stranded RNA corresponding to each candidate substrate into syncytial embryos and unfertilized eggs from wild-type *Drosophila* females, followed by DAPI staining to visualize the DNA.

RNA interference of *Mat89Bb* gave rise to a phenotype similar to that of the giant nuclei mutants, and this phenotype is consistent with excessive DNA replication in both fertilized and unfertilized eggs (Figure 2). In unfertilized eggs from wild-type females, the four meiotic products are maintained in a condensed state to form distinctive rosette-like structures known as polar bodies. In unfertilized eggs from giant nuclei mutants (*png*, *plu*, or *gnu*), the meiotic products are decondensed and undergo inappropriate DNA replication to become polyploid (Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991). Remarkably, RNA interference of *Mat89Bb* in unfertilized *Drosophila* eggs (Figure 2C) yields a giant nuclei phenotype in roughly one-third of injected eggs that is essentially identical to that of *png*-derived eggs (Figure 2E). Injection of unfertilized eggs with either buffer (Figure 2A) or unrelated dsRNA (data not shown) as a negative control had no effect on polar body structure. Considering the lapse of time between the deposition of unfertilized eggs and injection of double-stranded RNA in these experiments (at least 38 min), these results suggest that the continued presence of Mat89Bb is required to maintain the characteristic rosette structure of polar bodies in unfertilized eggs.

Aberrant embryonic cell cycles also occur as a consequence of mutations in *png*, *plu*, or *gnu*, and uncoupling of S and M phases leads to cell cycle arrest with highly polyploid nuclei (Figure 2F) (Freeman et al., 1986; Shamanski and Orr-Weaver, 1991). RNA interference of *Mat89Bb* in *Drosophila* syncytial embryos similarly results in intensely DAPI-stained nuclei (Figure 2D) in approximately one-fourth of injected embryos relative to embryos injected with either buffer (Figure 2B) or unrelated dsRNA (data not shown) as a negative control. These effects of decreasing *Mat89Bb* gene function on DNA content in both unfertilized eggs and embryos are consistent with a role for Mat89Bb in the PNG/PLU/GNU pathway and suggest that it is a true in vivo substrate of the PNG kinase complex.

Although decreasing *Mat89Bb* gene function by RNA interference in *Drosophila* syncytial embryos resulted in increased DNA content relative to buffer-injected control embryos (compare Figures 2B and 2D), the degree of polyploidy was less than that observed in embryos from weak *png* mutants (compare Figures 2D and 2F). One possible explanation for this difference is that Mat89Bb is only one of several in vivo substrates of the PNG kinase complex that mediates its effects on the cell cycle. Another consideration is the experimental constraints that limit how soon after egg collection that injections of double-stranded RNA can be performed (at least 38 min in our experience). The giant nuclei genes (and presumably substrates of the PNG kinase complex) are required as early as the first embryonic mitotic division following fertilization (Freeman et al., 1986; Shamanski and Orr-Weaver, 1991). In order for RNA interference to have an effect on a cellular process over a narrow window of time, such as during the early syncytial divisions, the targeted protein must be turned over rapidly. In our attempts to knock down the function of the giant nuclei genes *png* or *gnu* by RNA interference in syncytial embryos or unfertilized eggs as a positive control, we observed no effect on cell cycles or polar body structure, as assessed by DAPI staining (data not shown). RNA interference of the remaining five PNG kinase substrates identified by DIVEC screening in syncytial embryos or unfertilized eggs similarly resulted in no obvious defects (data not shown). Thus, negative RNA interference results do not rule out the possibility that these other in vitro substrates of PNG kinase may be bona fide substrates required for normal embryonic cell cycles and maintenance of polar bodies.

The *Drosophila Mat89Bb* gene encodes a 76 kDa protein with a maternal expression pattern (Stebbings et al., 1998). A comparison of the sequences of *Drosophila* and human Mat89Bb revealed that they are 36% identical and 61% similar at the amino acid level (Supplemental Figure S1; see the Supplemental Data available with this article online). While homologs of the PNG kinase complex have not yet been identified in organisms other than dipteran flies (Renault and Axton, 2003), our identification of the evolutionarily conserved protein Mat89Bb as a substrate of PNG kinase combined with our *Drosophila* RNA interference data suggested that this complex impinges on molecules that have a conserved cell cycle function in diverse organisms. Therefore, we tested whether decreasing Mat89Bb function in *Xenopus* embryos or in cultured mammalian cells would similarly disrupt their cell cycles.

Mat89Bb Plays an Essential Cell Cycle Role in Early *Xenopus* Development

As in *Drosophila*, *Xenopus* embryos undergo rapid S-M cycles prior to the midblastula transition. Based on our

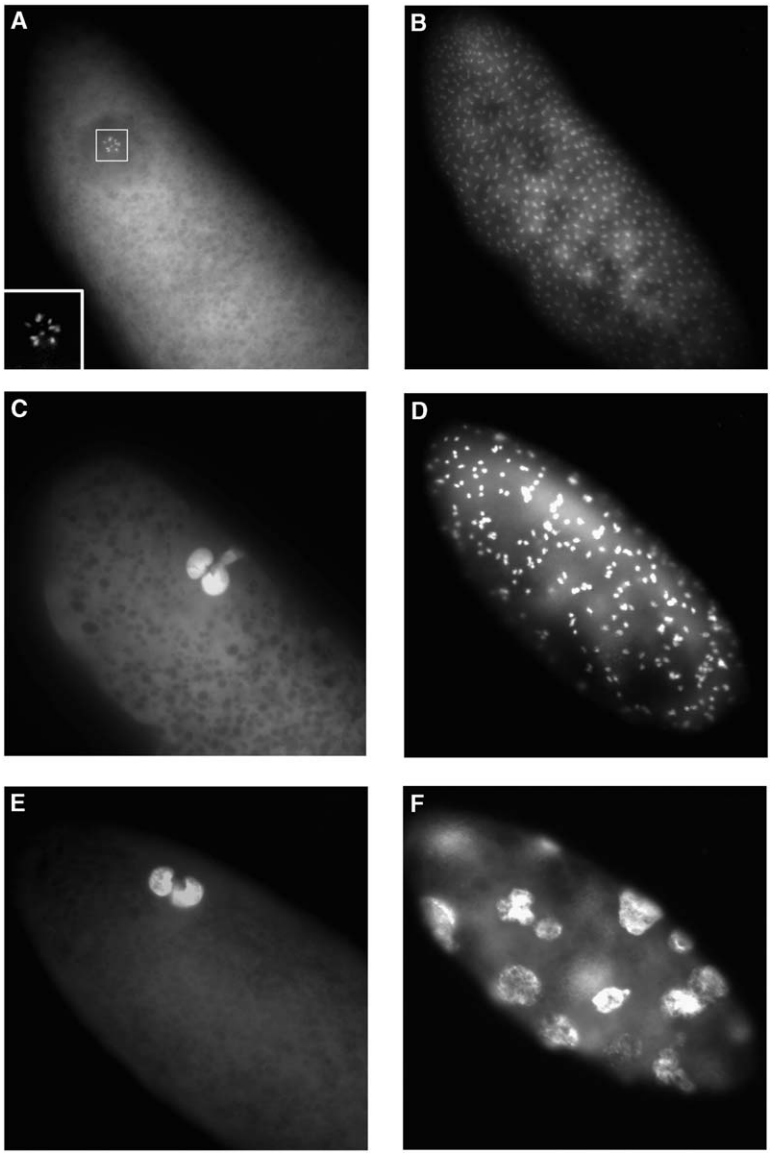

Figure 2. RNA Interference of *Mat89Bb* Results in a *png*-like Phenotype in *Drosophila* Unfertilized Eggs and Embryos

(A–D) DAPI staining of unfertilized eggs and embryos from wild-type females following injection. Buffer-injected (A) unfertilized eggs and (B) embryos have a normal DNA staining pattern. Injection of *Mat89Bb* dsRNA into (C) unfertilized eggs and (D) embryos results in intensely DAPI-stained nuclei.

(E and F) DAPI staining of (E) unfertilized eggs and (F) embryos from weak *png* mutants (png^{3318}) is shown for comparison.

(A, C, and E) Unfertilized eggs were photographed at 400× magnification. (B, D, and F) Embryos were photographed at 250× magnification. The inset in (A) shows the rosette structure of polar bodies photographed at 1000× magnification.

RNA interference results in *Drosophila* embryos, we hypothesized that morpholino-mediated downregulation of Mat89Bb in *Xenopus* embryos would similarly affect both cell cycle and development. Injection of *Mat89Bb* morpholinos (200 ng) into one-cell *Xenopus* embryos resulted in a developmental delay at gastrulation in 100% (50/50) of injected embryos (Figure 3A) and subsequent defects in which the embryos were shortened along the anterior-posterior axis (Figure 3B); complete arrest occurred in all (50/50) embryos following injection of higher concentrations (>200 ng) of *Mat89Bb* morpholinos (data not shown). A similar delay in *Xenopus* embryogenesis has been reported following morpholino-mediated downregulation of Cyclin E (Audic et al., 2001), which is normally required for proper initiation of DNA replication. Unlike Cyclin E, however, *Xenopus* embryos with decreased Mat89Bb levels do not develop normally and have defects consistent with abnormal gastrulation. Both the developmental delay (data not shown) and shortened axis phenotype observed following injection of *Xenopus* embryos with *Mat89Bb* morpholinos were rescued (96%; 48/50) by coinjection of human *Mat89Bb-GFP* mRNA (Figure 3B), indicating that the morpholino effects were specific.

Given that RNA interference of *Mat89Bb* in *Drosophila* syncytial embryos results in polyploidization, we performed DNA staining of the *Mat89Bb* morpholino-injected *Xenopus* embryos to determine whether they were similarly affected. 100% of the *Mat89Bb* morpholino-injected embryos stained more intensely with propidium iodide than embryos injected with control morpholinos, suggesting that they have an increased DNA content (Figure 3C). Because DNA of *Xenopus* embryos is difficult to visualize by microscopy due to the large amount of yolk protein, we isolated animal cap explants from embryos injected with either control or *Mat89Bb*

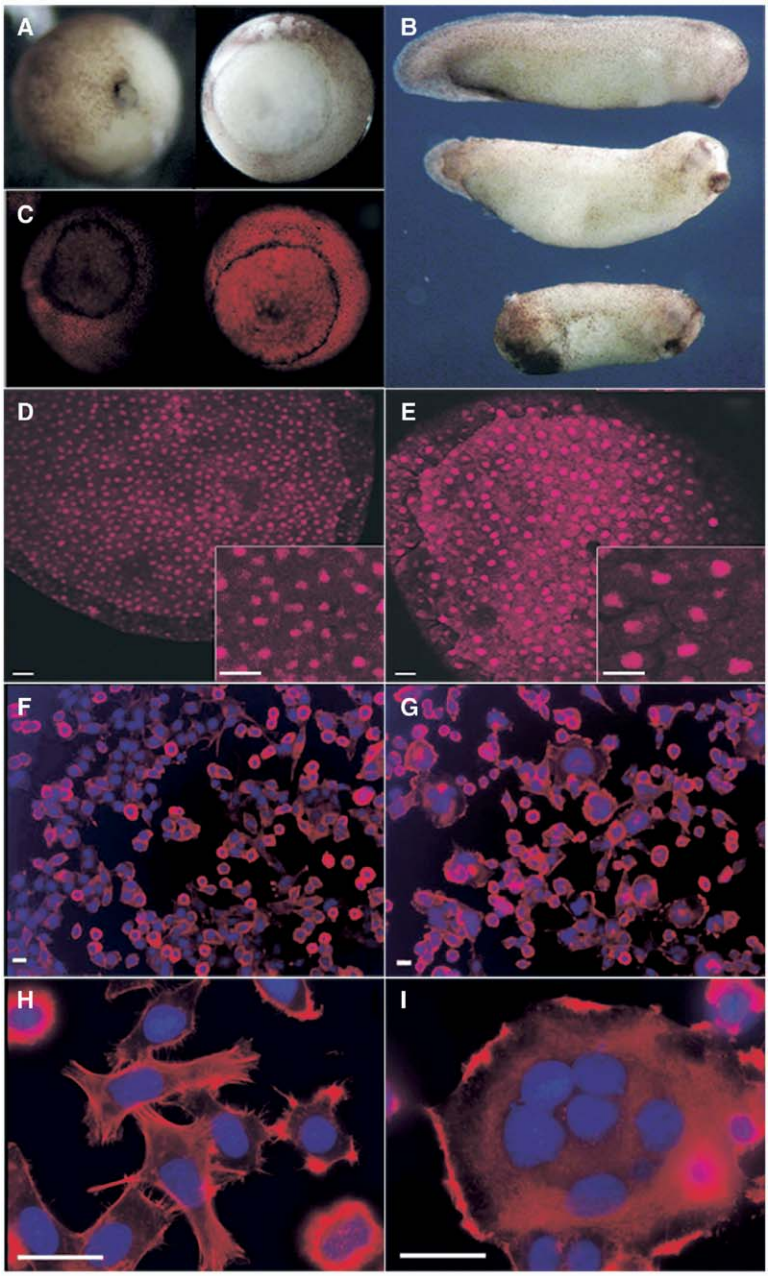

Figure 3. Downregulation of *Mat89Bb* Disrupts Development and Cell Cycles of *Xenopus* Embryos and Mitotic Divisions of Cultured Mammalian Cells

(A–E) Injection of *Mat89Bb* morpholinos in *Xenopus* embryos leads to developmental delay, with gastrulation defects and polyploidization. (A) Embryos injected with *Mat89Bb* morpholinos at the one-cell stage appear morphologically to be at (right panel) stage 10.5, whereas age-matched siblings similarly injected with control morpholinos are at (left panel) stage 26. Embryos injected with *Mat89Bb* morpholinos ([B], bottom) show defects consistent with disruption of gastrulation (e.g., shortened anterior-posterior axis) that are rescued ([B], middle) by coinjection of human *Mat89Bb-GFP* RNA. ([B], top) An uninjected embryo is shown for comparison. (C) Embryos injected with (right panel) *Mat89Bb* morpholinos have a greater intensity of DNA staining than (left panel) control morpholino-injected embryos at a similar stage of development. (D and E) Animal caps dissected from (E) *Mat89Bb* morpholino-injected embryos have larger nuclei than caps from (D) control morpholino-injected embryos; a corresponding increase in cell size is apparent in caps kept flat under a coverslip bridge and viewed at higher magnification (compare insets). Each bar represents 50 μm.

(F–I) RNA interference of human *Mat89Bb* in HeLa cells results in a multinucleated phenotype. (F and G) Low and (H and I) high magnification of stained HeLa cells fixed at day 6 postinfection. (G and I) Infection with lentivirus encoding *Mat89Bb* shRNA results in cells with multiple nuclei. (F and H) Cells infected with control virus encoding luciferase shRNA appear normal. DNA is blue, and actin is red. Each bar represents 10 μm.

morpholinos for DNA staining and confocal microscopy. Nuclei of animal cap cells from *Mat89Bb* morpholino-injected embryos had an average area ~1.6-fold larger than that of controls (compare Figures 3D and 3E; Supplemental Table S1); assuming a spherical shape, this increase in area represents a 2-fold increase in nuclear volume. The average intensity of DNA staining per unit area was identical, however, confirming that the *Mat89Bb* morpholino caused polyploidy (Supplemental Table S1). Cell size was increased, also consistent with mitotic failure and early developmental arrest. These results indicate that the biological function of Mat89Bb in cell cycle control and development during *Drosophila* embryogenesis has been conserved in vertebrates such as *Xenopus*.

Mat89Bb Is Required for Normal Mitosis in Cultured Mammalian Cells

RNA interference of *Mat89Bb* in cultured human (HeLa) cells with lentiviral-based shRNA gave rise to multinucleated cells (Figures 3F–3I). This observed increase in the DNA content of HeLa cells following downregulation of *Mat89Bb* is remarkably similar to our results with *Drosophila* embryos and eggs and *Xenopus* embryos and is a phenotype not commonly observed in cultured mammalian cells treated with shRNA (David Sabatini, personal communication). The differences in phenotypes observed between cultured mammalian cells versus *Drosophila* and *Xenopus* early embryos following downregulation of Mat89Bb function (i.e., multinucleated cells versus polyploid nuclei, respectively) may re-

flect inherent differences in the nature of their cell cycles (canonical G1-S-G2-M versus modified S-M cycles, respectively). For example, Mat89Bb appears to be required in HeLa cells for later stages of mitosis such as cytokinesis, which does not occur in the *Drosophila* syncytial embryo. Finally, these differences may also reflect the fact that downregulation of *Mat89Bb* transcripts in HeLa cells appears to be incomplete, with ~18% of control levels remaining, as determined by RT-PCR (Supplemental Figure S2); assessment of *Mat89Bb* transcript levels following RNA interference in *Drosophila* was technically difficult due to the relatively low numbers of embryos that could feasibly be injected. Despite the observed differences in phenotypes, these studies clearly demonstrate that Mat89Bb plays a fundamental role in the regulation of mitosis in mammalian cells as well as *Drosophila* and *Xenopus* embryos.

Applications of DIVEC Screening Methodology

Previous attempts to identify additional genes in the PNG/PLU/GNU pathway by screening large maternal-effect lethal collections and by yeast two-hybrid analysis were unsuccessful (Lee et al., 2003). We now demonstrate that by applying IVEC screening methodology (King et al., 1997; Lustig et al., 1997) to the *Drosophila* Gene Collection (DIVEC screening), we have successfully identified substrates of the cell cycle kinase PNG. Our current study highlights the general applicability and advantages of using such an approach. The IVEC approach allows one to utilize a wide variety of biochemical assays for screening purposes (as opposed to two-hybrid approaches in which one is solely relying on protein-protein interactions), and the *Drosophila* Gene Collection allows for rapid and complete coverage of an organism's entire genome with equal representation of all genes. Furthermore, annotation of the *Drosophila* Gene Collection makes it feasible to identify positive clones within pools rapidly because the identity of all clones in each pool is known. Finally, in vivo verification of the biological roles of candidate genes identified by DIVEC screening can be rapidly achieved in *Drosophila* by analysis of existing mutants and/or RNA interference. As demonstrated by our studies of Mat89Bb function in diverse organisms, the ability to perform rapid in vivo analysis of candidate genes is enhanced when homologs in other organisms can be readily identified.

One limitation of our DIVEC screen for PNG kinase substrates is that not all phosphorylation events result in a detectable mobility shift by SDS-PAGE. As an alternative, future DIVEC screens for kinase substrates could be designed to directly detect phosphorylation of in vitro-translated proteins by measuring ^{32}P incorporation. We have recently modified our DIVEC approach to allow high-throughput screening for proteins that physically interact with affinity-tagged "bait" protein immobilized on beads. We expect this variation of DIVEC screening to be more broadly applicable because it does not require a priori knowledge of the biological function of a given protein. Identification of Mat89Bb-interacting proteins by using this approach should provide important biological insight into its role in the cell cycle and development of metazoan organisms.

Experimental Procedures

Preparation of DNA and RNA Pools

See the Supplemental Data.

Preparation of Radiolabeled Proteins from DNA and RNA Pools

Radiolabeled proteins were prepared from DNA and RNA pools from *Drosophila* Gene Collection Release 1 according to the manufacturer's protocol (Promega Gold TNT T7 kit) and were used in a DIVEC screen for PNG substrates as described below.

DIVEC Screen for PNG Kinase Substrates

Recombinant protein complexes containing PNG-Myc/GST-PLU/GNU (active) or GST-PLU/GNU (inactive) were purified and eluted from *Sf9* cells by using glutathione beads as previously described (Lee et al., 2003). Pools of radiolabeled proteins (0.5 µl each) were incubated with 10 µl inactive or active PNG kinase complexes for 30 min at 30°C, and reactions were stopped by the addition of SDS-PAGE sample buffer. Reaction products were analyzed by SDS-PAGE/autoradiography, and films were examined for PNG-dependent mobility shifts of individual proteins. For each positive pool, individual clones encoding PNG substrates were identified by testing radiolabeled pools of increasingly smaller size. Identity of positive clones was confirmed by DNA sequencing.

Phosphatase Experiments

PNG substrates were individually ^{35}S labeled by IVT and were treated with or without active PNG kinase complex as described above. Samples were subsequently diluted into phosphatase buffer and treated without or with Lambda Phosphatase or Protein Phosphatase 1 (New England Biolabs) for 30 min at 30°C. Reaction products were analyzed by SDS-PAGE/autoradiography.

Drosophila Embryo Injections

See the Supplemental Data.

Xenopus Morpholino Injections

Xenopus laevis embryos obtained by in vitro fertilization were dejellied, cultured at 14°C–20°C in 10% Marc's Modified Ringer's (MMR) solution (Newport and Kirschner, 1982), and staged (Nieuwkpp and Faber, 1967). The anti-sense morpholino oligonucleotide based on the sequence of the sole *Xenopus laevis Mat89Bb* and the control morpholino (see Supplemental Data) were from Gene Tools. Morpholinos (200 ng) resuspended in water were injected into the animal pole of one-cell embryos (50 embryos per morpholino for each experiment unless otherwise indicated). For rescue, synthetic human *Mat89Bb* mRNA (2.7 ng) transcribed from linearized *pCS2(+)-Mat89Bb-GFP* template (SP6 Message Machine kit, Ambion) was coinjected with *Mat89Bb* morpholinos. Ectodermal explants (animal caps) dissected from stage-9 embryos after morpholino injection at the one-cell stage were cultured in 0.75× MMR until stage 11, fixed in MEMFA, and stained with propidium iodide. Stained animal caps (six per morpholino) were visualized by laser confocal scanning microscopy (LSM 510 Meta, Zeiss) as previously described for embryos (Pfleger et al., 2001). See the Supplemental Data (Supplemental Table S1) for a description of analysis of nuclear DNA content.

Lentiviral shRNA Cloning, Production, and Infection

To generate lentivirus expressing *Mat89Bb* shRNA, double-stranded oligonucleotides (see the Supplemental Data) were cloned into the *LKO.1* vector (Stewart et al., 2003). *Mat89Bb* shRNA DNA was co-transfected with *Delta VPR CMV VSVG* DNA into HeLa cells by using Fugene (Roche) as described (Stewart et al., 2003). HeLa cells were infected twice with virus-containing supernatants and were selected for puromycin resistance. At 5 days postinfection, cells were cultured overnight on fibronectin-coated coverslips and processed for staining as described (Sarbassov dos et al., 2004).

Cells stained with Texas red-conjugated phalloidin (1:500) and Hoechst (1:1000) were visualized by fluorescence microscopy.

Supplemental Data

Supplemental Data including supplemental Experimental Procedures, figures, and a table are available at http://www.developmentalcell.com/cgi/content/full/8/3/435/DC1/.

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