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Protein phosphorylation changes reveal new candidates in the regulation of egg activation and early embryogenesis in D. melanogaster

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Abstract

Egg activation is the series of events that must occur for a mature oocyte to become capable of supporting embryogenesis. These events include changes to the egg's outer coverings, the resumption and completion of meiosis, the translation of new proteins, and the degradation of specific maternal mRNAs. While we know some of the molecules that direct the initial events of egg activation, it remains unclear how multiple pathways are coordinated to change the cellular state from mature oocyte to activated egg. Using a proteomic approach we have identified new candidates for the regulation and progression of egg activation. Reasoning that phosphorylation can simultaneously and rapidly modulate the activity of many proteins, we identified proteins that are post-translationally modified during the transition from oocyte to activated egg in *Drosophila melanogaster*. We find that at least 311 proteins change in phosphorylation state between mature oocytes and activated eggs. These proteins fall into various functional classes related to the events of egg activation including calcium binding, proteolysis, and protein translation. Our set of candidates includes genes already associated with egg activation, as well as many genes not previously studied during this developmental period. RNAi knockdown of a subset of these genes revealed a new gene, *mrityu*, necessary for embryonic development past the first mitosis. Thus, by identifying phospho-modulated proteins we have produced a focused candidate set for future genetic studies to test their roles in egg activation and the initiation of embryogenesis.

Keywords

Egg activation; phosphorylation; proteomics

Introduction

For development to initiate, a number of events must take place to convert a mature oocyte to an egg capable of supporting embryogenesis. These include resumption and completion of the meiotic cell cycle, changes to the egg's outer coverings, degradation of certain maternal mRNAs, and the poly-adenylation and translation of others. These events are collectively termed "egg activation" [reviewed in (Ducibella and Fissore, 2008; Horner and Wolfner,

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2008)]. In many organisms, egg activation is triggered by fertilization and a subsequent rise in intracellular Ca^{2+} (Eisen et al., 1984; Gilkey et al., 1978; Swann and Yu, 2008). However, in *Drosophila* and other insects examined, egg activation occurs independent of fertilization (Doane, 1960). In these organisms, egg activation is triggered instead by passage of the oocyte through the reproductive tract (Heifetz et al., 2001; Went and Krause, 1974), though the importance of calcium remains conserved (Horner and Wolfner, 2008a). Thus, *Drosophila*, with its ease of genetic manipulation, is a valuable model for studying the conserved features of egg activation without interference from otherwise concurrent post-fertilization events.

However, traditional screening methods for recessive maternal-effect mutations have identified only a small number of factors necessary for egg activation in model systems such as *Drosophila* and *C. elegans* [reviewed in (Horner and Wolfner, 2008; Singson et al., 2008)]. One explanation for this modest success to date is that factors important for the oocyte-to-embryo transition may play additional essential roles at later stages of development, causing lethality and preventing their detection in a maternal-effect screen. Additionally, large-scale genetic screens to identify mutations affecting egg activation are difficult to carry out and sterile mutants can be difficult to maintain in stock. We take a different approach by identifying proteins that are post-translationally regulated during egg activation. We hypothesize that these regulated proteins will include new factors important for egg activation and early embryogenesis.

As little to no transcription occurs during egg activation, maternally provided mRNAs and proteins must be sufficient to regulate all of the events that take place during and immediately after this transition. Indeed, considerable evidence suggests that maternal proteins direct the earliest events in egg activation (McKnight and Miller, 1976; Newport and Kirschner, 1982; Zalokar, 1976). In *Drosophila*, meiosis can complete without the synthesis of any new proteins (Page and Orr-Weaver, 1997). In addition, proteomic studies of sea urchin eggs showed that in the first 2 minutes after fertilization the number of protein spots detectable by 2D gel electrophoresis decreases by 23%, suggesting that translation of new proteins is a relatively “late” event of egg activation (Horner and Wolfner, 2008; Roux et al., 2006). Characterization of the *C. elegans* oocyte proteome and transcriptome shows that the oocyte proteome appears to be biased towards factors likely to act immediately upon fertilization while the oocyte transcriptome is biased towards factors that are likely to act later in embryogenesis (Chik et al., 2011). These findings point to maternal proteins playing key roles in the initial events of egg activation. As these proteins are present in the oocyte prior to egg activation, they must also be highly regulated so that the correct subsets of proteins are active before, during, and after egg activation is triggered.

One possible mechanism for rapid regulation of many proteins is modulation of their phosphorylation state. Protein phosphorylation/dephosphorylation occurs quickly, consistent with the time frame of early activation events. Phosphorylation can also cause a large array of regulatory effects, such as altering a protein’s activity, localization, and/or association with other proteins [for examples see (Cargnello and Roux, 2011; Poon and Jans, 2005)]. Additionally, a single kinase or phosphatase can act on many substrates [for example, MAPK (Cargnello and Roux, 2011)], allowing the rapid transmission of an upstream signal to multiple downstream targets. Thus, regulation through phosphorylation can coordinate multiple events that are responsive to a single trigger. At the same time, the substrate specificity of these enzymes also allows for individual pathways to be regulated independently.

The importance of protein phosphorylation changes during egg activation is underlined by the findings that one critical regulator of this process is a kinase (CaMKII), while another is

a phosphatase (calcineurin). Ca^{2+}/calmodulin-protein kinase II (CaMKII) is required for meiotic cell cycle progression at egg activation in both mammals and amphibians (Backs et al., 2010; Chang et al., 2009; Hansen et al., 2006; Liu and Maller, 2005; Markoulaki et al., 2004; Rauh et al., 2005; Tatone et al., 1999). In *Xenopus*, the phosphatase calcineurin must be both activated and subsequently inactivated for exit from metaphase II and proper migration of male and female pronuclei (Mochida and Hunt, 2007; Nishiyama et al., 2007). In *Drosophila*, calcineurin and its regulator *sra* are required for multiple aspects of activation, including completion of meiosis and translation of new proteins (Horner et al., 2006; Takeo et al., 2010; Takeo et al., 2006).

While these protein phosphorylation regulators are clearly important, few of the proteins being phospho-modified during egg activation have been identified. In *Drosophila*, two proteins necessary for early embryogenesis are known to be dephosphorylated during egg activation – Giant Nuclei [GNU; (Lee et al., 2003; Renault et al., 2003; Tadros et al., 2007)] and Young Arrest [YA; (Lin and Wolfner, 1991; Sackton et al., 2009; Yu et al., 2002; Yu et al., 1999)]. In vertebrates, phosphorylation of the Emi2 protein at egg activation triggers its degradation, which is necessary for the resumption of meiosis (Liu and Maller, 2005; Rauh et al., 2005). It is also known that MAPKs are dephosphorylated upon egg activation in vertebrates, as well as in *Drosophila*, leading to a decrease in MAPK activity in the egg (Fan and Sun, 2004; Sackton et al., 2007). We hypothesize that, in addition to these examples, many more maternal proteins change in phosphorylation state at this time to rapidly permit the large change in cellular state that occurs at egg activation. Consistent with this hypothesis, proteomic methods have detected dynamic changes in phosphorylation at the time of fertilization/egg activation in sea urchins (Roux et al., 2006; Roux et al., 2008).

Here, we report the identification of proteins whose phosphorylation state changes upon egg activation in the genetic model system *Drosophila melanogaster*. Using multiple proteomic methods we identify 311 proteins that are phospho-regulated at the time of egg activation. These proteins fall into a number of functional classes that are biologically relevant to this developmental time, including calcium binding and regulation, proteolysis, and protein translation. Through a pilot RNAi screen we show that one of these proteins, mrityu, is important for early embryogenesis. We also find that the majority of these phospho-regulated proteins are conserved from *Drosophila* to vertebrates. These methods should be broadly applicable to other systems as an efficient way to identify important molecules that act in egg activation and early embryogenesis. As these proteins are not only present, but also regulated, during this critical transition, they provide a targeted set of candidates to test in future studies for roles in the regulatory cascades and downstream events of egg activation.

Materials and Methods

Fly culture

Drosophila melanogaster stocks were raised on yeast-glucose-agar medium at 23 ± 2°C in a 12-h L:12-h D photoperiod. All experiments were carried out with females from the P2 strain of Oregon R (ORP2) (Allis et al., 1977).

Collection of mature oocytes and laid eggs

Stage 14 mature oocytes were obtained from 3–5 day old wild-type virgin females that had been reared on heavily-yeasted food. Dissection was performed in Isolation Buffer, a hypertonic solution that does not activate eggs (Page and Orr-Weaver, 1997). Oocytes were dissected in one hour blocks of time, then flash-frozen in liquid nitrogen and stored at −80°C until use.

Newly eclosed virgin females were aged on yeasted vials for 3–5 days and then mated to spermless males (Boswell and Mahowald, 1985) (produced by crossing *tud*^{1} *bw sp* ♀ ×Canton-S ♂) to obtain unfertilized, but activated eggs. For all experiments with activated laid eggs, mated females were allowed to deposit eggs on petri plates containing grape juice-agar for 0–30 minute periods. Eggs were washed off the plates in Egg Wash (Karr and Alberts, 1986), frozen in liquid nitrogen and stored at −80°C until use. Eggs collected for Western blotting were dechorionated in a 50% bleach solution for 2 minutes before being flash frozen.

Immunoblotting

Samples of 50–100 mature oocytes or activated eggs were homogenized in Extraction Buffer (10 mM Tris pH 7.5; 20 mM NaF, 2 mM EGTA, 10 mM DTT, 400 nM okadaic acid, and 2% SDS), followed by the addition of an equal amount of SDS sample buffer. Extracts were electrophoresed on polyacrylamide SDS gels containing 0– 25 μM Phos-tag (Wako Pure Chemical Industries, Ltd., Richmond, VA) and subjected to Western blotting analysis as previously described (Kinoshita et al., 2009; Kinoshita et al., 2006; Sackton et al., 2007). Primary antibodies, kind gifts of H. Bellen, R. Vale, J. Raff, F.R. Jackson, and T. Hays were used at the following dilutions in 1% milk: guinea pig anti-Vap-33-1, 1:10,000 (Pennetta et al., 2002); rabbit anti-Spindly, 1:1000 (Griffis et al., 2007); rabbit anti-Spd-2, 1:500 (Dix and Raff, 2007); rabbit anti-lark, 1:5000 (Newby and Jackson, 1996); mouse anti-DLIC, 1:3000 (Mische et al., 2008). HRP-conjugated secondary antibodies were used at a 1:2000 dilution and visualized with the ECL Plus Western Blotting Detection system (GE Healthcare, Piscataway, NJ).

Immunostaining and microscopy

Embryos and ovaries were fixed in methanol/heptane and stained with DAPI, or as described in (Horner et al., 2006). For DAPI staining, fixed embryos were incubated in PBS containing 1 μg/ml DAPI for 5 minutes, followed by five 15 minute washes in PBST. Rat anti-sperm tail antibody (kind gift of T. Karr) was used at a dilution of 1:800 (Karr, 1991; T. Karr, ASU, personal communication). Mouse anti-tubulin antibody was used at 1:400 (Sigma, St. Louis, MO, catalog#T5168) and Alexa secondary antibodies were used at 1:200 (Invitrogen, Grand Island, NY). RNaseA (Roche, Indianapolis, IN) was added at a final concentration of 5 μg/ml and propidium iodide (Molecular Probes, Eugene, OR) was used at a final concentration of 10 μg/ml. Images were collected using a Zeiss Axioskop compound microscope or a Leica TCS SP2 confocal microscope as described in (Horner et al., 2006).

mrityu RNAi flies

A fly line with an RNAi construct to the gene CG1216 (*mrityu*) was obtained from the Transgenic RNAi Resource Project at Harvard Medical School (line GL00033). Females from this line were crossed to nanos-GAL4 males (Bloomington stock 4937) to specifically knockdown *mrityu* in the female germline (referred to as RNAi females). Control females were nanos-GAL4/TM3 siblings.

2D-gel electrophoresis

Oocytes and embryos, stored at −80°C until use, were consolidated into batches of approximately 1500 mature oocytes or unfertilized laid eggs and homogenized in 50 μl protease inhibiting buffer (Monsma and Wolfner, 1988) containing 1 mM Na-orthovanadate, 10 mM β-glycerophosphate, and 20 mM NaF as phosphatase inhibitors (PPIHB). Buffer exchange was performed three times for extracted proteins against 500 μl lysis buffer (7 M urea/2 M thio urea, 4% CHAPS) using a 10 kDa cut-off membrane filter (Millipore, Billerica, MA). Protein concentrations were determined by the Bradford assay using BSA as

a standard, and approximately 100 μg of oocyte or egg protein was used for each of the 2-D gel analyses. The first-dimensional separation was performed by immobilized pH gradient isoelectric focusing (13cm IPG, nonlinear pH 3–10 strips; GE Healthcare). Isoelectric focusing was conducted using Multiphor II (GE Healthcare) according to the manufacturer's instructions after 12 h in-gel rehydration. For electrophoresis in the second dimension, the strips were then transferred and apposed to 12.5% SDS-polyacryalmide vertical gels that were cast in-house at 14 × 16 cm using a Hoefer SE600 gel cast apparatus and run using a Hoefer vertical gel running system (Hoefer, Inc., Holliston, MA). Peppermint Stick molecular weight markers (Molecular Probes) were applied to each gel at a concentration of 0.25 μg/protein. Gels were first run to observe the extent of phosphorylation changes occurring during egg activation. A second set of gels was run for protein identification from specific spots that changed in phosphorylation. Both oocyte and egg samples were run either in triplicate (first set) or duplicate (second set).

Two-dimensional electrophoresis was performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Gel staining and imaging

Gels were fixed in 40% methanol/10% acetic acid and stored overnight in 10% methanol/7% acetic acid. Each gel was then incubated in Pro-Q Diamond Phosphorylation Stain (Molecular Probes) for the detection of phosphoproteins. Following de-staining, the gels were scanned using a Typhoon 9400 laser scanner (GE Healthcare) with 532 nm excitation and 560 nm long pass emission filters. The gels were further post-stained with SYPRO-Ruby (Molecular Probes) or Colloidal Coomassie Blue (Invitrogen) for detection of all protein spots and once again scanned with the Typhoon 9400 with 488 nm excitation and 610 nm band pass 30 emission filters.

Gel staining and imaging were performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Gel image analysis

Images from Pro-Q Diamond and Colloidal Coomassie Blue (CCB) or SYPRO-Ruby stains were analyzed using the Image Master 2D Platinum software (GE Healthcare).

For the first set of gels, the replicate Pro-Q Diamond and CCB images for each sample type (mature oocyte or activated egg) were combined into a single Pro-Q or CCB synthetic gel image, containing the average volume of each spot. Once values were obtained for each spot, Pro-Q spot volumes were normalized by dividing each spot by its corresponding CCB spot volume. Two known phosphorylated proteins, ovalbumin and beta-casein, present in the PeppermintStick protein marker were used to set a Pro-Q/CCB ratio minimum which we used to determine which spots were phosphorylated. The lowest Pro-Q/CCB ratio obtained for these control proteins was 0.91. Therefore, a protein spot was considered phosphorylated if the Pro-Q/CCB ratio was greater than or equal to 0.91. Due to this normalization process, only the Pro-Q spots with a matching CCB spot could be analyzed for potential phosphorylation. The final normalized values were used in comparison between samples, with a 2-fold or higher difference in volume considered significant.

For the second set of gels, stringent criteria were applied to select spots for quantification and expression profiling. Only spots that were present in both biological duplicate gels of this second set were analyzed by Image Master software. The intensity of matching spots on the gels was compared between the Pro-Q Diamond and SYPRO-Ruby stained gels to detect potential phosphoprotein candidates. If the intensity of the Sypro Ruby stained spot was greater than 50% of the corresponding ProQ Diamond stained spot intensity, the spot was

selected as a phosphoprotein. ProQ Diamond nonspecific staining of the nonphosphorylated proteins in the Peppermint Stick MW Standard was nearly undetectable. For spots corresponding to potential phosphoproteins, we compared Pro-Q Diamond intensities between oocytes and eggs in order to determine changes in phosphorylation levels. A similar comparison was made in the SYPRO-Ruby gels to determine quantitative changes in protein abundance between oocytes and eggs. The average normalized volumes for each spot (% of total spot volume) from each sample group were compared, and spots with at least a 1.5-fold differential expression between two samples were considered to be up or down-regulated. With a single exception, spots with changes only in Pro-Q Diamond staining, and not Sypro Ruby staining, were chosen for subsequent protein identification analysis. (The one exception was spot number 1068, which showed a decrease in Sypro Ruby staining from oocyte to egg in addition to staining with Pro-Q Diamond only on the oocyte gels.)

Computer analysis of the gels was performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Relative abundance of proteins within a single spot was estimated by the exponentially modified protein abundance index (emPAI) as described by Yang et al. (2007).

Immobilized Metal Affinity Chromatography (IMAC)

500–600 mature oocytes or unfertilized, activated laid eggs were combined and homogenized in PPIHB (containing 0.1% SDS) as described above. After homogenization, additional PPIHB was added to a final volume of 250 μl. Samples were centrifuged at 20,000 × g for 30 minutes at 4°C and the supernatant was collected. 10 mM DTT (60°C for 30–60 min) was used as a reducing agent and 60 mM iodoacetamide was used for alkylation of cysteine residues. Trypsin was added at a ratio of 1:15 trypsin:total protein and samples were incubated overnight at 37°C. Reactions were stopped with 5% formic acid and samples were acidified to pH 2.5–3.0 with 1 M HCl. Samples were added to equilibrated PHOS-Select Iron Affinity Gel (Sigma) and incubated for a minimum of one hour before the gel was washed to remove unphosphorylated peptide fractions. Phosphopeptides were eluted by adding 200–400 μL Elution Solution (400 mM ammonium hydroxide) for subsequent mass spectrometry analysis.

IMAC enrichment was performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Protein identification

Spots of interest from the 2D gels were picked robotically from a master gel containing both mature oocyte and activated egg samples (Investigator ProPic; Genomic Solutions, Ann Arbor, MI) for in-gel digestion with trypsin and extraction by robotic ProPrep (Genomic Solutions) using standard protocols (Shevchenko et al., 1996). Spots of interest and IMAC enriched peptides were subjected to nanoLC-ESI-MS/MS analysis on a hybrid triple quadrupole linear ion trap mass spectrometer, the 4000 Q Trap (ABI/MDS Sciex, Framingham, MA). A second mass spectrometry analysis of the IMAC peptides was also performed on a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA). The MS/MS data generated from the nanoLCMS/ MS IDA analysis on 2D gel spots and IMAC samples were submitted to Mascot 2.2 for database searching using an in-house licensed Mascot local server. The search was performed to query the NCBInr database (Taxonomy: Drosophila) downloaded from NCBI (July 2008), allowing one missed cleavage site by trypsin. Carbamidomethyl modification of cysteine, methionine oxidation and phosphorylation of serine/threonine/tyrosine were set as variable modifications. All matches that occurred above a 95% confidence interval (CI), with significant scores for the

peptides defined by Mascot probability analysis greater than "identity" were considered for the protein identifications. Resulting spectra were inspected manually to verify phosphorylation site identifications. Only peptides with an identified phosphorylation site were included in further analyses. Peptide sequences from the Orbitrap results were manually run through a BLAST analysis (http://blast.ncbi.nlm.nih.gov) to determine the proteins from which they were derived.

Mass spectrometry was performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Characterization of candidate proteins

Function was chosen from the GO terms listed by AmiGO (amigo.geneontology.org). Tissue expression was mined from FlyAtlas (www.flyatlas.org). Unless otherwise indicated, independent evidence for phosphorylation was found at the Uniprot database (www.uniprot.org). In cases where the only evidence for phosphorylation was from Zhai et al. (2008), the protein was considered a known Drosophila phosphoprotein only if the same phosphopeptide was found in our IMAC experiment. If there was no evidence of phosphorylation in Drosophila, homologs were searched in the Uniprot database for reports of phosphorylation, beginning with human and followed by mouse, rat, and Xenopus. Both Uniprot and Pubmed were searched for previously known evidence of phosphorylation. InParanoid (http://inparanoid.sbc.su.se/cgi-bin/index.cgi) gene search was used to identify orthologs in any vertebrate species.

Results

2D gel electrophoresis detects dynamic phosphorylation changes

To visualize proteomic changes that occur upon egg activation in *Drosophila*, we performed two-dimensional (2D) gel analysis of samples containing protein from mature, stage 14 oocytes or 0–30 minute activated, unfertilized laid eggs. We detected 830 protein spots in samples from mature oocytes and 822 protein spots in samples from activated eggs. A subset of the spots detected in mature oocytes were not detected in activated eggs (231/830), suggesting that these proteins are degraded or undergo posttranslational modifications upon egg activation. We also detected protein spots unique to activated eggs (223/822), indicative of translation or post-translational modifications upon activation.

In samples from both stages, approximately 30 percent of the total detected proteins were phosphorylated. This result is similar to the percentage of phosphorylated proteins found in sea urchin unfertilized eggs, and to the percentage of the proteome that has been observed to be phosphorylated in mammalian cells (Ahn and Resing, 2001; Roux et al., 2006; Roux et al., 2008). Of the phosphoproteins we analyzed, 40% were more highly phosphorylated (≥ 2 fold) in mature oocytes and 38% were more highly phosphorylated in the activated egg, indicating that there is a high level of phospho-modulation during egg activation in *Drosophila* (Figure 1 and Supplementary Table S1).

IMAC enrichment of phosphopeptides identifies numerous proteins that change in phosphorylation state during egg activation

After observing the extent of the phosphorylation changes that take place during egg activation by 2D gel, we set out to determine the identities of the proteins that undergo these changes. We applied a peptide-based approach with enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC). IMAC allowed us to identify a large number of phosphorylated proteins in both mature oocytes and activated eggs; including proteins that may not have been detectable, or appeared differently, on the 2D gels. IMAC

also provides the additional advantage that each protein is associated with an identified phosphorylation site.

Comparisons were made between the phosphopeptides that we found in either mature oocyte or activated egg samples, resulting in a total of 273 phosphopeptides that were identified in only one sample or the other; 205 in the mature oocyte and 68 in the activated egg (Supplementary Table 2). In most cases the phosphorylation(s) of a given protein was found within a single peptide sequence, although we also identified a few proteins phosphorylated within multiple phosphopeptide sequences. Thus, these 273 unique phosphopeptides define 238 proteins: 174 with mature oocyte specific phosphorylations (Supplementary Table 2A), 58 with activated egg specific phosphorylations (Supplementary Table 2B), and 6 proteins that were phosphorylated in both samples, but at different sites. We consider these candidates for proteins that change in phosphorylation state during egg activation. Our finding of more phosphorylated peptides in mature oocytes than in activated eggs also suggests that egg activation is accompanied by more dephosphorylation events than phosphorylation events.

Since the IMAC experiment does not control for changes in protein abundance, some of the changes observed could be due to protein translation or degradation instead of a change in phosphorylation state. However, we believe that majority of the changes we identified represent true changes in phosphorylation state. Among the phosphoproteins identified only in the mature oocyte samples are GNU and ERK (encoded by the *rolled* gene), two proteins previously known to be dephosphorylated during egg activation (Renault et al., 2003; Sackton et al., 2007), demonstrating our ability to detect relevant phosphorylation changes by this method. In addition, data from previous mass spectrometry experiments informed us that for approximately 21% of the proteins identified by IMAC the protein is present in both oocytes and activated eggs (unpublished results).

Additional proteins that change in phosphorylation state are identified from 2D gels

Recognizing that any single proteomic method is not saturating for the proteome, we ran a second set of 2D gels in an effort to identify additional proteins undergoing phosphorylation changes. While we were unable to control for abundance changes in the IMAC experiment, we chose to control for it here. We applied strict criteria for selecting spots for identification and selected spots that only showed differences between oocytes and activated eggs in phosphorylation (ProQ Diamond) but not protein abundance (SYPRO-Ruby). Even though these criteria reduced the number of proteins that we identified from the 2D gels, they ensured that the spot changes we focused on were specifically due to phosphorylation differences between the samples. Thirty spots met these criteria; eighteen of these represented proteins that were more highly phosphorylated in the mature oocyte, while 12 were more highly phosphorylated in activated eggs (Figure 2). Mass spectrometry of the selected spots resulted in a total of 83 protein IDs, as most spots contained more than one protein (Supplementary Table 3). We hypothesize that the phosphorylation change of a given spot is most likely due to the most highly abundant protein present within the spot and/or the proteins for which we identified a phosphorylated peptide. However, it is possible that multiple co-migrating proteins contributed to the observed ProQ Diamond staining. We therefore considered all proteins identified as candidate proteins that may be changing in phosphorylation state during egg activation.

Only ten proteins were identified by both the 2D gels and IMAC. Thus, neither method was saturating for the proteome. For six of these proteins (vig2, Dlic, HSP26, Smt3 activating enzyme 2, CG8209, and CG14309) the results were consistent between the two methods. The other four proteins (Nucleosome assembly protein 1, yolk protein 1, A kinase anchor protein 200, and CG18190) were either identified in multiple spots on the 2D gels or were

identified from spots more highly phosphorylated in the opposite sample from which we identified a phosphopeptide by IMAC. It is possible that these proteins have additional phosphorylation sites that we failed to identify by IMAC and the overall change in phosphorylation state is in the direction indicated by the 2D gels. Additional studies will be required to fully define the phosphorylation state of these proteins in oocytes and activated eggs.

Western blots confirm phosphorylation state change for select proteins

The phosphorylation of a protein can affect its electrophoretic mobility through a gel: a more highly phosphorylated protein will often have a slower mobility than a less phosphorylated form of the same protein. This difference in mobility can be enhanced by the addition of Phos-tag, which binds the phosphate groups on phosphorylated proteins, slowing their movement through a polyacrylamide gel (Kinoshita et al., 2006). Thus, if a protein is present in different phosphorylation states between samples, this may appear as a band shift on a Western blot. We tested a subset of seven of our IMAC candidates with available antibodies by Western blot to see if we could observe a shift for any of these proteins when comparing samples from mature oocytes and activated eggs. We observed a shift for five candidates – lark, Dynein light intermediate chain (DLIC), Spindly, Spd-2, and Vap-33-1 (Figure 3). Treatment of the samples with a phosphatase (CIAP) confirms that the shifts seen on the Western blot are due to phosphorylation differences (Supplementary Figure S1).

The ability to detect a phosphorylation shift depends on the size and nature of the protein, as well as the number of phosphorylation sites that it contains. Because phosphorylation does not guarantee a change in electrophoretic mobility, our failure to observe a shift for the other two candidates (Cup and Slender lobes) does not rule out that they are phosphoregulated. Since Cup and Slender lobes are large proteins (125 kD and 160 kD, respectively), a phosphorylation change may be unlikely to result in a detectable change in protein mobility. In addition, we found Cup to be phosphorylated in both oocytes and activated eggs, but on different residues, again reducing the likelihood that the phosphorylation states would migrate differently on a SDS Page gel. As such, these were not ideal candidates for confirmation by Western blotting.

Spindly, DLIC, Spd-2, and lark show shifts indicative of dephosphorylation during egg activation and Vap-33-1 appears to exist in an intermediate phosphorylation state that is oocyte specific. Spindly protein migrates more slowly in mature oocytes than in activated eggs, indicating that a more highly phosphorylated form of the protein is present in the oocyte (Figure 3A). DLIC protein runs as a doublet, the upper band of which was shown by Mische et al. (2008) to represent a phosphorylated form of the protein. We observe this doublet in mature oocytes, however only the lower band is detectable in unfertilized activated eggs (Figure 3B). Spd-2 protein appears as a smear in the oocyte lane when run on a Phos-tag gel (5% acrylamide, 3.5 μM Phos-tag), indicating that the protein is phosphorylated. This smear is no longer present in the activated egg lane, consistent with dephosphorylation of Spd-2 during egg activation (Figure 3C). Phos-tag gels (10.6% acrylamide, 25 μM Phos-tag) also allow us to visualize slower mobility fractions of lark and Vap-33-1 proteins which correspond to phosphorylated forms of the proteins. For lark, the phosphorylated state is specific to the mature oocyte, again consistent with dephosphorylation of the protein during egg activation (Figure 3D). Vap-33-1 protein appears to exist in several distinct phosphorylation states, one of which is oocyte specific (Figure 3E). These results are consistent with our identification of mature oocyte specific phosphopeptides for these five proteins, supporting the validity of the candidates we have identified.

RNAi knockdown of candidates identifies a new gene important for the first embryonic mitosis

We next used RNAi to test our hypothesis that our candidates include new genes that act during egg activation or embryogenesis. We attempted to knock down 18 of our candidates by female germline-specific RNAi and found that 6 are essential for female fertility and that knockdown of a seventh severely reduced fertility (only 33% of laid eggs produced adult progeny)(Table S4). The lack of a phenotype for the remaining 11 candidates indicates that maternal expression of these genes is either not essential for early development, perhaps due to redundant molecules, or that we failed to successfully knock down the genes by RNAi. Of the 6 genes whose knockdown caused sterility, knockdown of 4 prevented the production of eggs, indicating roles for these genes in oogenesis. Knockdown of the other 2 resulted in eggs that were defective in embryogenesis. Knockdown of one of these genes, *PyK*, leads to embryonic arrest, but the arrested embryos have been able to complete the early embryonic cell cycles (data not shown), though it is possible that there was insufficient knockdown to reveal earlier functions of the protein. In contrast, we find the early arrest caused by knockdown of the other gene, *mrityu* (*mri*), to be of particular interest.

Mri, which our IMAC experiments detected as a protein that is phosphorylated during egg activation, is a BTB/POZ domain-containing protein with no previously known function (flybase.org). *mri* RNAi females lay eggs, but those eggs fail to hatch (Supplementary Table S5). RT-PCR confirms that *mri* is knocked down in mature oocytes of RNAi females, indicating that *mri* expression in the germline is necessary for female fertility (Supplementary Figure S2). To determine if the embryos laid by *mri* RNAi mothers arrested early or late in embryogenesis we stained 1–3 hr old embryos with DAPI to observe the number of nuclei present. We failed to observe more than 5 nuclei in any *mri* arrested embryos (n = 30), indicating an early arrest point. To confirm that embryos from *mri* RNAi females are fertilized prior to their developmental arrest, we stained 0–90 minute old embryos with an anti-sperm tail antibody. A sperm tail was clearly visible within both control and RNAi embryos (7/8 control; 11/12 RNAi), indicating that the sterility of *mri* RNAi females is not due to a lack of fertilization (Figure 4A).

To determine the specific arrest point of the RNAi embryos, we then stained them with propidium iodide and anti-tubulin antibody, to visualize DNA and microtubules, respectively. While 1–3 hour old control embryos have undergone multiple rounds of syncytial mitosis (Figure 4B), we never observe such divisions in *mri* RNAi embryos. Instead, these embryos arrest during metaphase of the first 1–3 mitotic divisions (Figure 4C and Supplementary Figure S3). In approximately half of the embryos observed (14/30) only one nucleus is present within the egg, while another 30% of the embryos (9/30) contain only 2 nuclei. Our ability to find a polar body in one-third of these embryos suggest that meiosis able to resume and complete (Figure 4D), leading to our hypothesis that the arrest is during the first or second embryonic mitosis. However, we can not currently rule out the possibility that we are observing either the single male pronucleus, or the male and female pronuclei. Additionally, we observed two embryos that appear to still be arrested in meiosis I. We attribute the slight variability in the arrest point to the variable efficiency of RNAi and the fact that we are able to reduce the levels of, but not eliminate, maternal *mri* transcript in the oocyte. Further studies of *mrityu* will benefit from the creation of a mutant line that eliminates all mrityu protein. This will allow us to pinpoint the arrest point and more fully characterize the mechanism by which *mrityu* contributes to the regulation of early embryonic development. Additional targeted mutations will be necessary to determine the role of phosphorylation in *mrityu*'s function.

By screening a small subset of the phosphoproteins we identified in this study, we have found a new gene, *mrityu*, that is required for early embryonic development. These findings

indicate that the phosphoregulated proteins we identified can be a rich source of molecules needed for the oocyte-to-embryo transition and that further RNAi screening will reveal additional genes that act during this early developmental period.

Discussion

Egg activation is a rapid and highly regulated set of events that allow a relatively quiescent oocyte to transition to the cellular state necessary for embryo development. Much work has been done to understand the initial calcium signal that triggers egg activation in most organisms, and a few of the immediate downstream molecules that transduce this calcium signal have been identified, primarily the kinase CaMKII and the phosphatase calcineurin (Backs et al., 2010; Chang et al., 2009; Takeo et al., 2010)]. In addition, we know the ultimate cellular events that occur when egg activation is successful – modifications of the egg coverings, resumption and completion of meiosis, translation of new proteins, and degradation of specific maternal macromolecules [reviewed in (Horner and Wolfner, 2008)]. However, the pathways and proteins that connect the early triggers of activation to the final downstream events remain largely unknown.

We have identified a set of proteins that will help us to better understand the connections between the early triggers and final events of egg activation. As this transition occurs before zygotic transcription and too early for major effects of any new protein synthesis, we explored the possibility that post-translational modifications might activate or inactivate critical molecules during egg activation. Identifying the molecules that are regulated during this transition will provide new molecular insights into this important developmental period, as well as new candidates to test for roles in egg activation. Here we report that large-scale phosphoproteome changes occur during *Drosophila* egg activation. We identified a total of 311 proteins that change in phosphorylation state during this transition; these include proteins that we found to be phosphorylated in only mature oocytes or activated eggs, as well as proteins that changed in the specific phosphorylation site between samples. Our results show the importance of combining proteomic approaches and the complementary, rather than redundant, nature of these methods; consistent with studies that compared protein-based and peptide-based proteomic methods (Wu et al., 2006). The 2D gels provide a global view of the protein differences found between the two states and do not require the specific phosphorylated peptide of a protein to be detectable by mass spectrometry. In contrast, IMAC does require the identification of the phosphorylated peptide, but does not depend on the protein being detectable, or appearing differently, on a gel. Thus, by combining methods with different strengths we were able to expand the number, and type, of proteins within our data set. Even so, we do not expect that the 311 proteins we identified represent the full list of proteins that are phospho-regulated during egg activation. However, they provide an important start in providing new candidates to test for roles in egg activation and improving our understanding of the importance of phosphorylation during this period of development. Through a pilot genetic screen, which identified *mrityu* as a new gene with an essential role in early *Drosophila* development, we verified that the phosphomodified proteins we detected will be a source of new regulators of this process.

Large scale phosphorylation changes occur during egg activation

We showed here that a relatively large fraction of the *Drosophila* oocyte proteome (~30%) is phospho-modified during egg activation. Of the 311 phospho-modulated proteins that we identified by proteomic comparison of mature oocytes (prior to egg activation) and activated eggs, we found 84 *Drosophila* proteins, and the *Drosophila* homologs of another 123 proteins from other organisms, that were known to be phospho-modified in at least one cell type. The proteins that we identified include two (ERK and GNU) that had previously been shown to be phospho-modified during this time-window (Sackton et al.,2007; Renault et al.,

2003) and five more (Spd2, Spindly, lark, DLIC, and Vap33) whose phosphorylation changes during egg activation we verified independently by Western blotting.

The proteins identified in this study fall into a number of functional classes (based on known and/or predicted functions) (Supplementary Table S6). These include calcium binding/regulation, cell cycle, metabolism/glycolysis, proteolysis, and protein translation; all classes which are anticipated to act during egg activation. Our finding of phosphomodulated proteins in these classes also agrees with a previous report that found proteins phospho-modulated at the time of fertilization in sea urchins in the classes of calcium binding/regulation, metabolism, and protein translation (Roux et al., 2008) and supports the hypothesis that phosphorylation is a key regulator of many of the events of egg activation. Our results also identify proteins whose functions suggest new pathways to examine for roles in or immediately after egg activation. For example, our finding of phospho-regulation of both the SUMO activating enzyme Uba2 and the SUMO-specific protease Ulp1 suggests that sumoylation may be important during this developmental transition. Consistent with this idea, SUMO germline clones are reported to have defects in both patterning of the embryo and in the syncytial mitotic cell cycles (Nie et al., 2009). Additionally, our identification of multiple zinc-binding proteins suggests that zinc-based processes may be important during this developmental transition in *Drosophila*, as was recently proposed for mammals (Kim et al., 2010; Suzuki et al., 2010).

Phospho-regulated proteins include new regulators of egg activation and embryogenesis

The proteins identified in this study represent a focused set of candidates that we can now test genetically for roles in egg activation and early embryogenesis. This includes a number of proteins that would not have been identified through a traditional genetic screen for female steriles, as approximately one-third of these candidates are essential for viability (Supplementary Table S7)(flybase.org). We can now take advantage of recent advances in RNAi tools that allow successful knockdown of genes (and their protein products) in the female germline (Ni et al., 2011) and test these candidates for functional roles during or immediately after egg activation.

Verifying that our approach can uncover players in the oocyte-to-embryo transition, approximately 15% of the proteins we identified are known to be important for female fertility, playing roles in oogenesis and embryogenesis (Supplementary Table S7) (flybase.org) and others are implicated in the meiotic cell cycle. For example, our finding of phospho-modulation of Spd-2 and Spindly suggested roles for these proteins during or immediately after egg activation. Consistent with this, fertilized embryos from *spd-2* mutant mothers fail to enter the first mitosis due to problems in pronuclear migration and fusion (Dix and Raff, 2007). Likewise, Spindly (a mitotic phosphoprotein in humans) is necessary for the metaphase-anaphase transition in both mitosis and meiosis (Barisic et al., 2010; Chan et al., 2009; Griffis et al., 2007; Zhang et al., 2010). As *Drosophila* oocytes are arrested in metaphase prior to egg activation, we hypothesize that the dephosphorylation of Spindly is involved in regulating its association with dynein and the transition to anaphase.

To test our hypothesis that the proteins identified in this study include novel factors required for egg activation or early embryogenesis we performed a pilot study testing 18 of our candidates. In addition to four proteins required for oogenesis, we found that activity of the protein encoded by the *mrityu* gene is required for initiation of embryo development. We expect future extension of this RNAi screen to the remaining proteins we detected as phospho-modulated will reveal additional new essential players in the egg-to-embryo transition. Once we understand the functional role of these proteins, we will then be able to progress to understanding the importance of their phospho-regulation by creating transgenic

lines that mutate the phosphorylation sites (to residues that can not be phosphorylated and/or phospho-mimetic residues).

Since 82.9% of our candidates have homologs in vertebrates, the pathways thus uncovered are likely to be conserved. Indeed, the importance of calcium, and the early signaling through calcineurin in egg activation, are conserved between *Drosophila*, sea urchins, and vertebrates [reviewed in Ducibella and Fissore, 2008 and Horner and Wolfner, 2008; Takeo et al., 2010]. Our results suggest that in addition to these early events, many other pathways controlling egg activation may be conserved across species. Consistent with this idea, two proteins that we find to be dephosphorylated during *Drosophila* egg activation (Cup and eIF-2alpha), are also dephosphorylated in mouse oocytes at the time of fertilization (Alves et al., 2009; Villaescusa et al., 2006). Thus, in addition to identifying the phospho-regulated proteins during *Drosophila* egg activation, the results we reported here will likely help to uncover the molecular mechanisms that regulate the egg-to-embryo transition even in animals such as mammals where such screens would be prohibitive.

Conclusions

This study took an alternative proteomic approach to find new genes important for the oocyte-to-embryo transition. By identifying proteins that are not only present, but phospho-modulated during this transition, we have produced an enriched source of new candidate genes important for egg activation and early embryogenesis. Comparison of the proteomes of *Drosophila* mature oocytes and unfertilized activated eggs identified 311 proteins that change in phosphorylation state during this transition. Many of these proteins fall into functional categories such as calcium binding and regulation of translation, which are related to the known events of egg activation. As the 82.9% of these genes are conserved from *Drosophila* to vertebrates, they also represent candidates that may be tested for roles in egg activation and embryogenesis in other species of interest. Using the genetic tools available for *Drosophila*, including new RNAi lines that successfully knockdown genes in the female germline, we have begun testing these genes for their roles in egg activation and the initiation of development. Within the set of genes we tested, we identified one new gene, *mrityu*, which is necessary for embryos to progress past the first mitosis. We expect that continued testing of these candidates will reveal additional genes previously not known to act during egg activation or embryogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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This is the first report of proteome changes during Drosophila egg activation.

We identified 311 proteins whose phosphorylation changes during egg activation.

These proteins likely include factors necessary for the egg-to-embryo transition.

RNAi of a subset of them identified *mrityu* as essential to initiate embryogenesis.

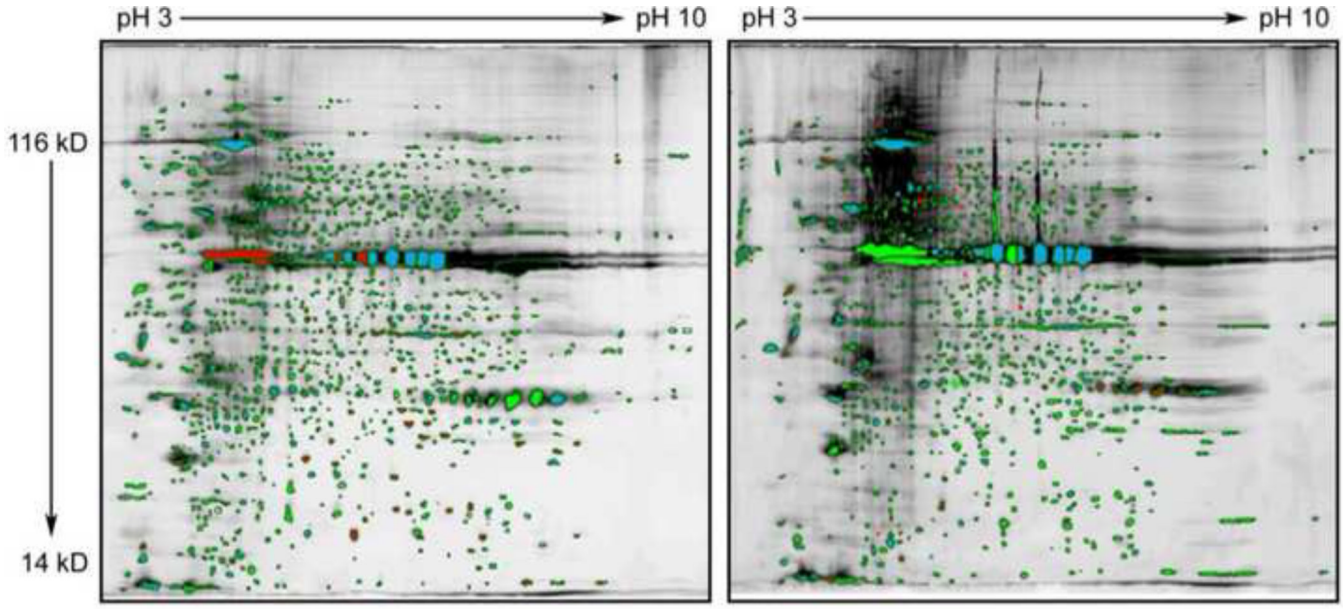

Figure 1.

ProQ Diamond Phosphorylation stained images of mature oocytes (left) and 0– 30 minute activated eggs (right) show global phosphorylation changes take place upon egg activation. The gels were stained with ProQ Diamond Phosphorylation Stain to detect phosphoproteins. Spots are colored to indicate a difference in spot volume between the two samples; a spot with a 2-fold or higher volume in one sample is green, a 2-fold or lower volume in one sample is red, and spots that are approximately the same volume in both samples are blue.

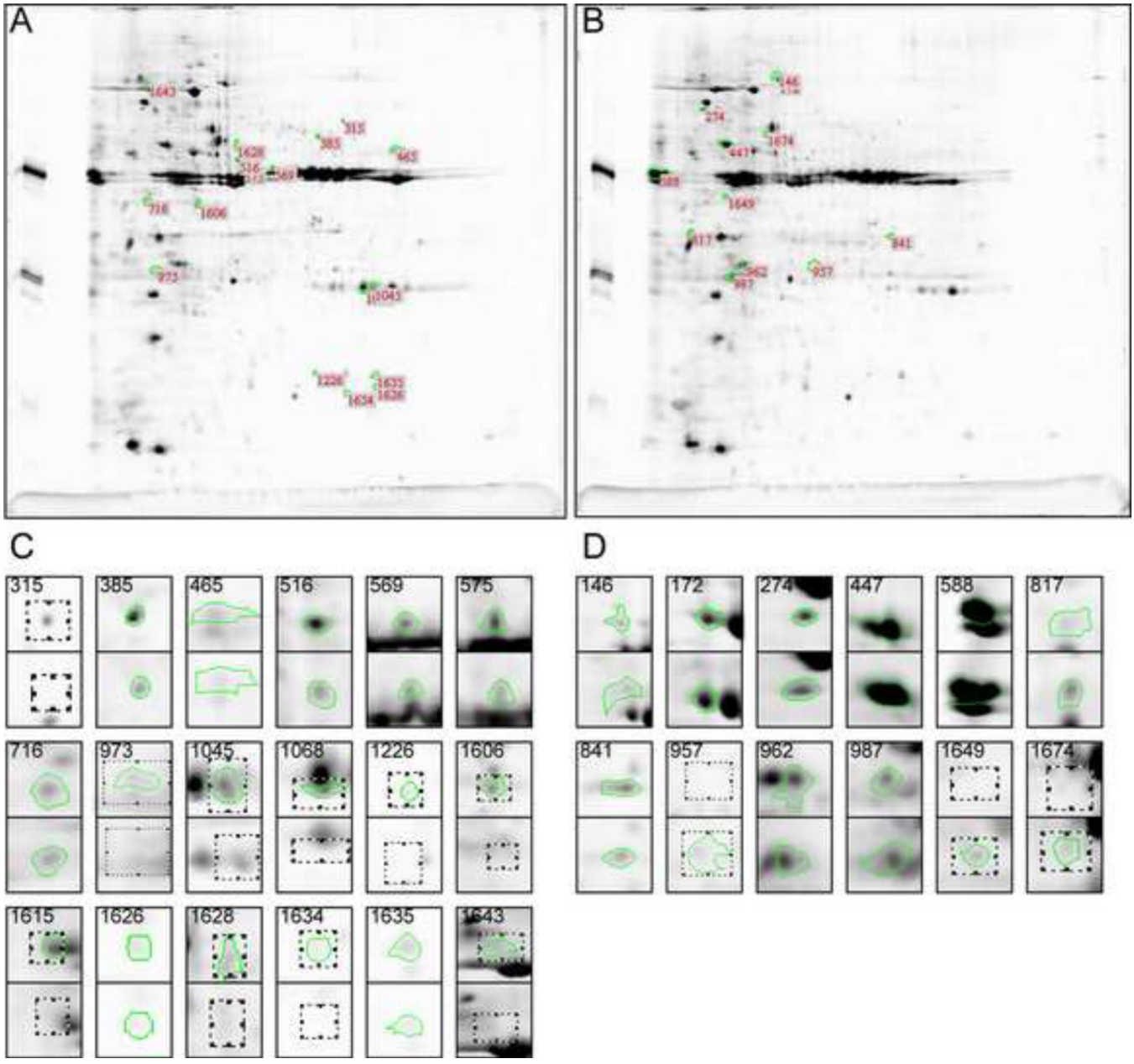

Figure 2.

2D gels stained with ProQ Diamond phosphorylation stain are shown for A) mature oocyte proteome and B) unfertilized activated egg proteome. Spots chosen for identification by mass spectrometry are indicated with their corresponding spot ID#. Images of each individual spot chosen for identification are shown below for C) spots that were upregulated or unmatched in the mature oocyte and D) spots that were upregulated or unmatched in the activated egg.

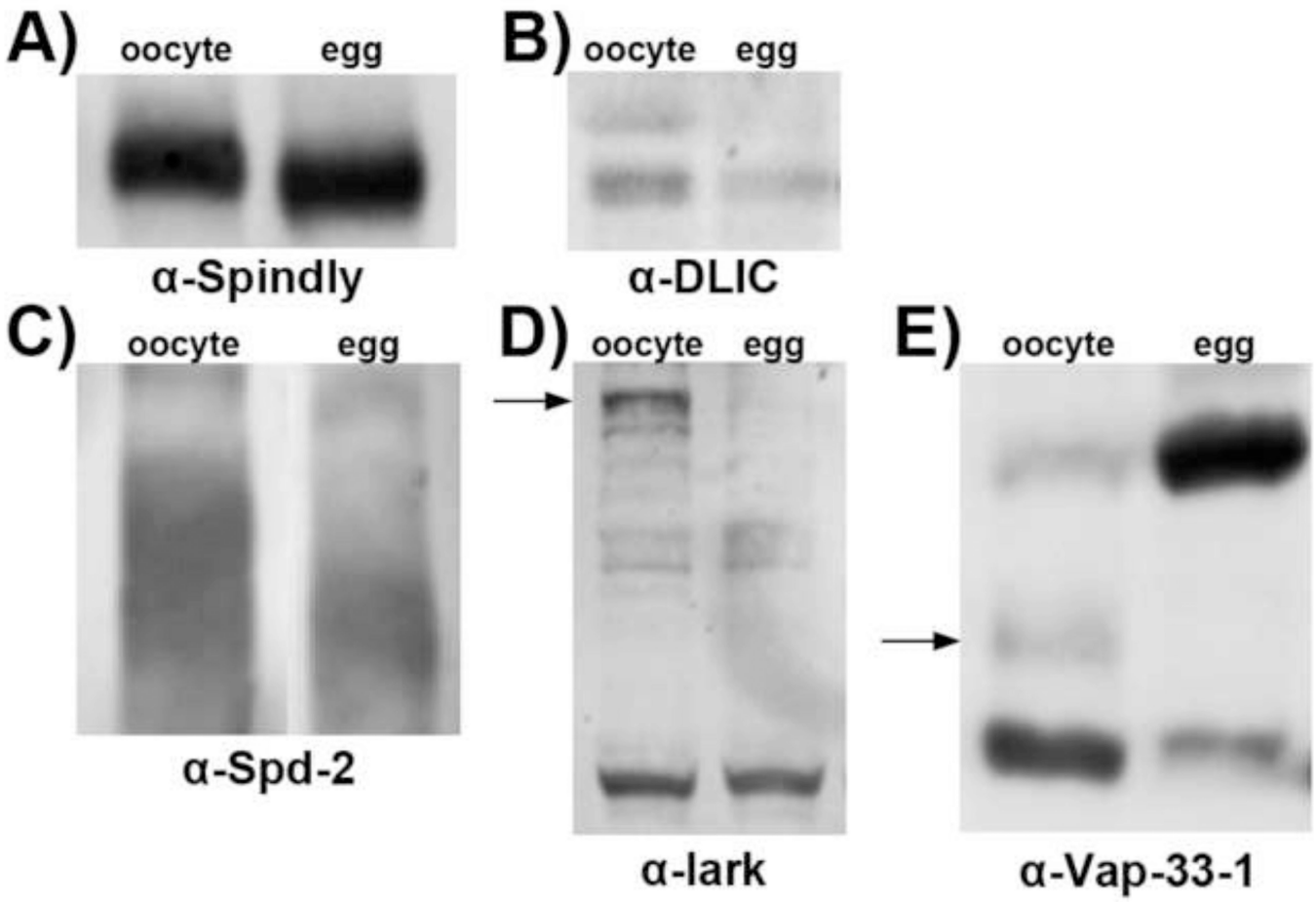

Figure 3.
Western blots confirm a change in phosphorylation state for A) Spindly B) DLIC C) Spd-2 D) lark and E) Vap-33-1 upon egg activation. Oocyte specific phosphorylation states of lark and Vap-33-1 are indicated by arrows.

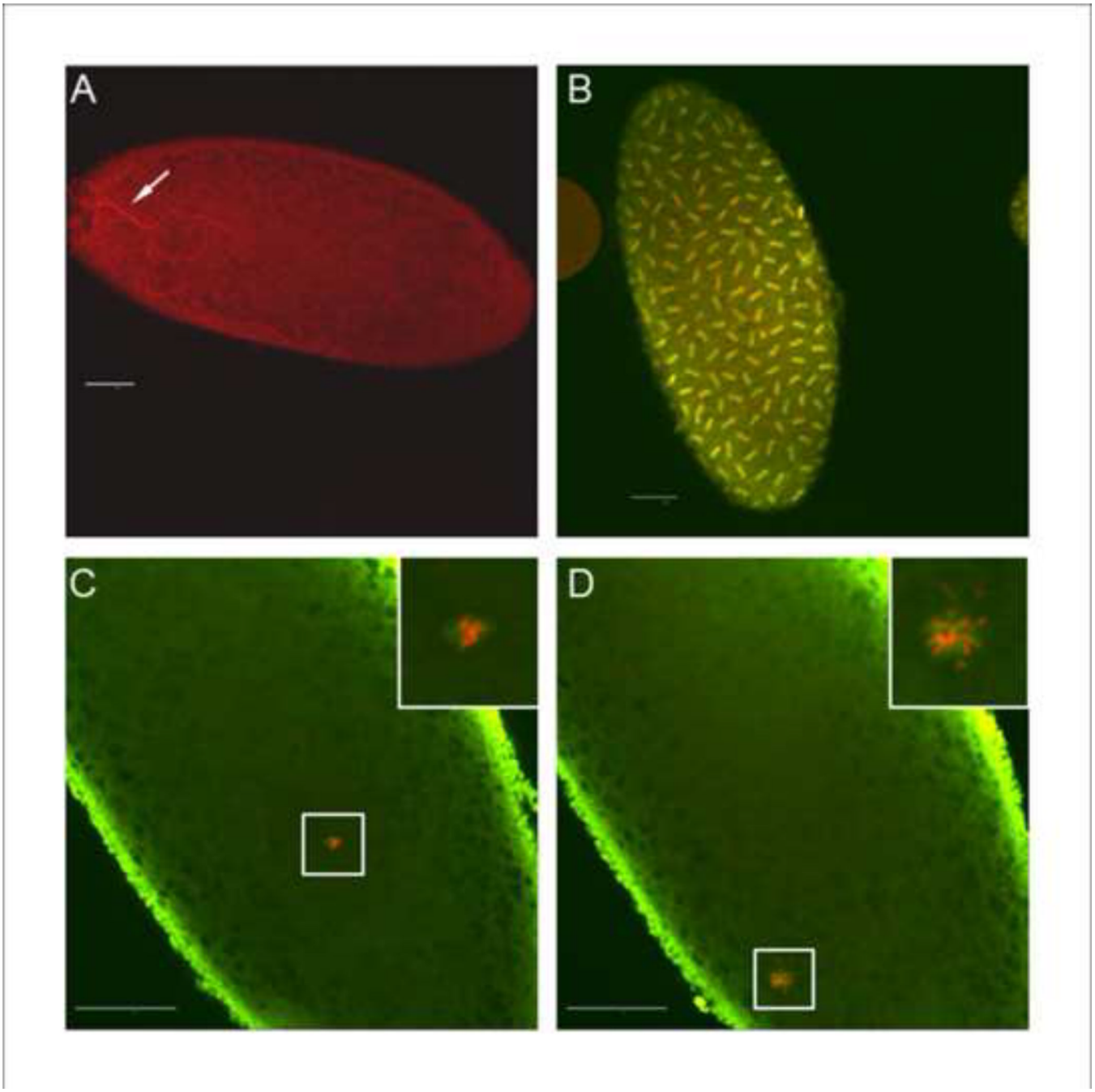

Figure 4.

Embryos are fertilized but arrest early when *mrityu* is knocked-down in the mother. A) Anti-sperm tail antibody (red) was used to visualize the sperm within 0–90 minute embryos laid by *mri* RNAi females. B-D) One to three hour old embryos laid by *mri* RNAi or control females were stained with propidium iodide to visualize DNA (red) and anti-tubulin antibody to visualize microtubules (green). Control embryos undergo mitotic divisions (B) while RNAi embryos typically arrest with a single nuclei (C). The visualization of a polar body rosette (D) suggests that meiosis is able to complete in these embryos. Insets are zoomed in images of the boxed region.