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2DE identification of proteins exhibiting turnover and phosphorylation dynamics during sea urchin egg activation

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Abstract

The animal egg is a unique quiescent cell, prepackaged with maternal mRNAs and proteins that have functions in early development. Rapid, transient signaling at fertilization alters egg physiology, resulting in Ca^{2+} release from the endoplasmic reticulum (ER) and cytoplasmic alkalinization. These events trigger the zygote developmental program through initiation of DNA synthesis and entry into mitosis. Post-translational modifications of maternal proteins are responsible for the spatio-temporal regulation that orchestrates egg activation. We used functional proteomics to identify the candidate maternal proteins involved in egg activation and early development. As the first step of this analysis, we present the data on the baseline maternal proteome, in particular, on proteins exhibiting changes in abundance and in phosphorylation state upon egg activation. We identify 94 proteins that were stable, reproducibly displayed a shift in isoelectric point, or changed in relative abundance at specific times after activation. The identities of these proteins were determined by quadrupole time-of-flight tandem mass spectrometry. The set of the most dynamic proteins appear to be enriched in intermediary metabolism proteins, cytoskeletal proteins, gamete associated proteins and proteins that have Ca^{2+} mediated activities.
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Keywords: Proteomics; Phosphorylation; Sea urchin; Fertilization; Egg activation

Introduction

The cellular events occurring within seconds to minutes post fertilization are collectively termed egg activation and include changes in intracellular physiology, signaling through kinases and secondary messengers, remodeling of the cytoskeleton and a dramatic increase in the overall metabolic rate of the egg (reviewed in Epel, 1997; Runft et al., 2002; Whitaker, 2006). Reverse genetics approaches have been used to identify and characterize several maternal proteins that mediate these events. In mammals, fish, amphibians and echinoderms, the identification of egg proteins by assessing specific functional activities has revealed key signaling pathways that regulate the initiation of development (Runft et al., 2002; Whitaker, 2006). In addition, a number of forward genetic screens and genome-wide RNAi screens performed in nematodes, zebrafish and *Drosophila* have led to the identification of a broader repertoire of genes associated with the female sterile phenotype (Dosch et al., 2004; Fitch and Wakimoto, 1998; Geldziler et al., 2004; Kamath et al., 2003; Labbé et al., 2006; Ohsako et al., 2003; Perotti et al., 2001; Piano et al., 2000; Szabad et al., 1989). In most of these forward genetic model systems the fertilization event is difficult to control and quantify, thus connection between the female sterile phenotype and a specific egg activation event remains tentative. A more direct and unbiased approach to the problem of protein factors that mediate egg activation is to study a model with tractable biochemistry and *in vitro* fertilization (Sato et al., 2002). One such a model is the sea urchin *Strongylocentrotus purpuratus*. Visualization of gamete interaction and egg activation events is routine in sea urchins, and large quantities of synchronously fertilizing gametes can be obtained. While echinoderms are not yet amenable to forward genetic screens, the recent completion of the *S. purpuratus* genome sequence

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(Sodergren et al., 2006) facilitates application of many genome-scale technologies to the study of egg activation.

Proteomic approaches, which seek to identify various proteins and their modified forms in the biological sample and quantify the abundance of each variant, are especially relevant for studying cells such as eggs, where regulatory mechanisms are driven by post-transcriptional events, including mobilization and post-translational modifications (PTM) of existing, maternal proteins. Furthermore, in sea urchins, very little detectable transcription or translation occurs in the minutes following fertilization (Davidson, 1982; Epel, 1967); therefore, physical and spatial modulation of existing proteins such as phosphorylation, degradation and changes in protein solubility and availability is responsible for successful activation. Identification of these proteins also provides further validation of existing tiling array data, EST database sequencing and current genome annotation (Sodergren et al., 2006).

Recently, several large scale screens have been conducted in *C. elegans*, zebrafish and *Drosophila* to identify the global changes in the mRNA population as oocytes mature and then transition from egg to embryo (DeRenzo and Seydoux, 2004; Schier, 2007; Stitzel and Seydoux, 2007). However, only a few studies (see below) have used a proteomics based approach to visualize how maternal proteins are changing at fertilization.

Approximately 350 proteins have been detected in the mature pig oocyte (Ellenderova et al., 2004), and global proteome analysis has identified approximately 500 protein spots in the samples of mouse oocytes, including 32 spots that were less abundant at the morula stage mouse embryos than in the oocyte (Coonrod et al., 2002; Sasaki et al., 1999). A similar study in the plant *Solanum chocoense* detected 619 proteins in the unfertilized oocyte (Vyetrogon et al., 2007). Using SYPRO Ruby we recently estimated the number of proteins and their abundance in a soluble fraction of sea urchin eggs and early embryos. Approximately 600 maternal proteins were reproducibly detected in the egg and by 2 min post fertilization. In the next few minutes, this number was reduced to 464, then increased at 15 min (587), and reached a steady level (567 proteins) at 30 min (Roux et al., 2006). These data indicate rapid and significant changes in levels of total protein abundance during sea urchin egg activation and early development, at least in this subset of relatively abundant proteins.

Protein phosphorylation is an important regulatory mechanism of egg activation in all species that have been studied (Whitaker, 2006; Kinsey, 1997a; Sato et al., 1998) and the sea

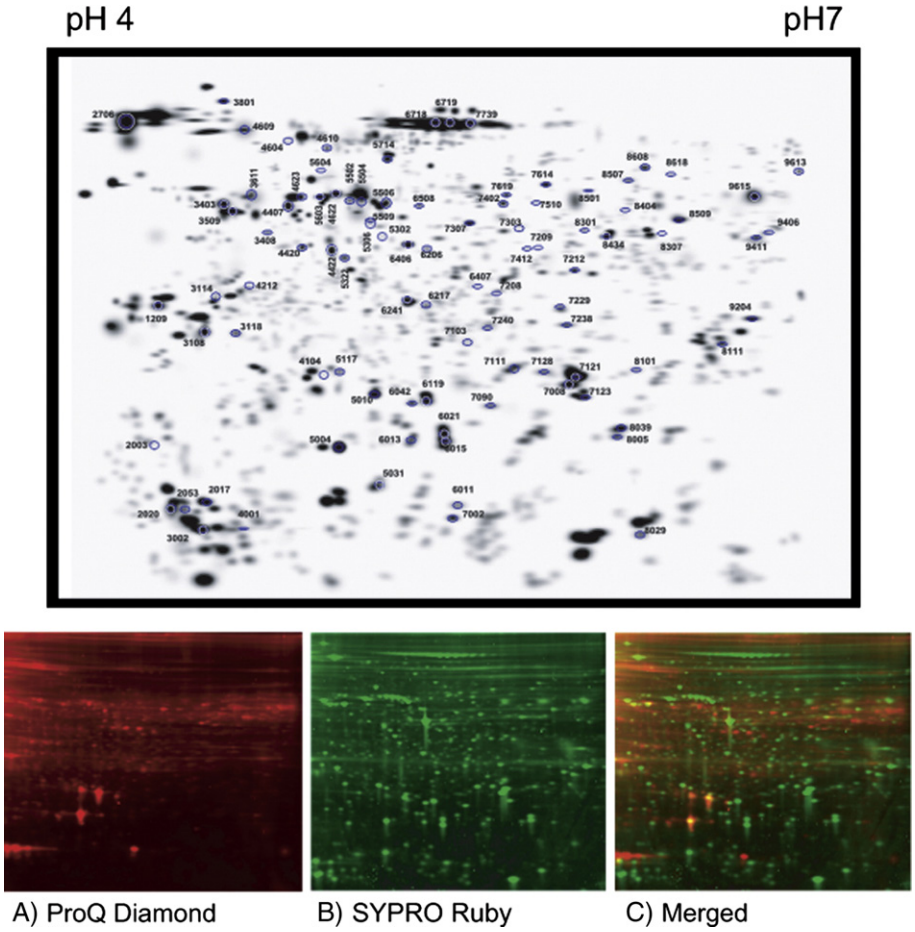


Fig. 1. Multiplexed 2DE analysis of sea urchin egg and zygote proteins. Top panel: Image of the PDQuest synthetic master gel representing all spots identified within a user-defined match set (spots detected from all time points are included, UF, F2 and F30). Circled spots were those picked for Q-ToF MS/MS identification. The numbering corresponds to the Spot ID (PDQuest spot # assignment) shown in Table 1. Lower panels: Images of a 2DE gel (UF) stained with (A) ProQ Diamond phospho-stain and (B) SYPRO Ruby total protein stain. The merged image is shown in panel C.

Table 1
Urchin proteins identified by LC Q-ToF tandem mass spectrometry

	Spot ID	NCBI accession	Peptide matches	Sea urchin genome ID	ProQ diamond	Role in egg activation
Calcium mediated						
	8509	GI:72110134	10	Sp-Irbit-like	Y	Y
	1209	GI:115744199	10	Sp-Annexin	–	UN
	5117	GI:115744199	3	Sp-Annexin	Y	UN
	3002	GI:50979313	5	Sp-Spec2a	Y	Y
	5504	GI:115928284	6	Sp-Spec2d	Y	Y
	3114	GI:72013987	7	Sp-Tropomyosin 1	–	Y
	5506	GI:115928284	5	Sp-Pdia4	Y	Y
	7002	GI:72155016	1	Sp-Pdia4	–	Y
Cell maintenance						
	4604	GI:115891388	3	Sp-HSP902A1	Y	UN
	4610	GI:72014565	12	SP-HSP701H	Y	Y
	6407	GI:72168826	2	Sp-RPA1	Y	UN
	7008	GI:72110987	7	Sp-GST-11	Y	UN
	7111	GI:72006659	3	Sp-Gsto1	Y	UN
	7510	GI:72115978	2	Sp-NCL-like	Y	Y
	6206	GI:115623835	1	Sp-SMARCC2	Y	UN
Gamete associated						
	2003	GI:72159079	3	Sp-Speract	Y	Y
	2017	GI:72022217	3	Sp-Speract	–	Y
	6015	GI:72022217	4	Sp-Speract	–	Y
	8005	GI:72022217	4	Sp-Speract	–	Y
	2020	GI:47550935	4	Sp-Cvp18	–	Y
	3801	GI:115784461	12	Sp-Rendezvin	–	Y
	4609	GI:47550943	8	Sp-Rendezvin	Y	Y
Ion exchange						
	9204	GI:72005582	12	Sp-Vdac2	–	UN
	9406	GI:72005582	5	Sp-Vdac2	Y	UN
	9615	GI:72005582	3	Sp-Vdac2	Y	UN
Cellular signaling						
	3118	GI:72179591	2	Sp-14-3-3_two	–	UN
	3611	GI:72065387	8	Sp-Rbbp-4	Y	UN
	4212	GI:72111397	4	Sp-PRKCSH-like	–	UN
	5604	GI:115640031	3	Sp-PR65 alpha	–	UN
Metabolism						
	2706	GI:47551123	8	Sp-MYP	Y	UN
	6718	GI:72006169	10	Sp-Vitellogenin3	–	Y
	6719	GI:72006169	12	Sp-Vitellogenin3	Y	Y
	7103	GI:72111362	3	Sp-Vitellogenin1	Y	Y
	7739	GI:72051578	9	Sp-Vitellogenin3	–	Y
	3403	GI:72008757	3	Sp-Atp5b	–	UN
	3509	GI:72008757	6	Sp-Atp5b	–	UN
	4407	GI:72008757	17	Sp-Atp5b	Y	UN
	3408	GI:72015065	12	Sp-Suclg2	Y	UN
	4001	GI:72139667	1	Sp-5-L0	Y	UN
	4420	GI:115947577	8	Sp-Potfn	Y	UN
	4422	GI:72014688	4	Sp-As3mt	–	UN
	5603	GI:115899354	15	Sp-Aldh2	–	UN
	4622	GI:115899354	11	Sp-Aldh2	–	UN
	6508	GI:115638483	10	Sp-Aldh6a1	Y	UN
	5010	GI:72065271	4	Sp-Fth1	Y	UN
	5031	GI:72065271	3	Sp-Fth1	Y	UN
	5302	GI:115733016	8	Sp-Acadl	Y	UN
	5714	GI:72077315	11	Sp-Sdha	–	UN
	6042	GI:115644435	5	Sp-Peroxiredoxin	Y	UN
	7090	GI:115644435	5	Sp-Peroxiredoxin	–	UN
	7121	GI:115637218	10	Sp-Peroxiredoxin	Y	UN
	6241	GI:115651961	12	Sp-Mdh2	Y	Y
	7123	GI:72085421	5	Sp-Sod2	–	UN

Table 1 (*continued*)

	Spot ID	NCBI accession	Peptide matches	Sea urchin genome ID	ProQ diamond	Role in egg activation
Metabolism						
	7208	GI:72108535	3	Sp-Esterase D	Y	UN
	7209	GI:72012729	4	Sp-Acads	Y	UN
	7212	GI:115752657	7	Sp-Taldo1	–	UN
	7303	GI:115715574	9	Sp-Fah	Y	UN
	7402	GI:72144184	7	Sp-Fah	Y	UN
	7412	GI:115626002	5	Sp-Mthfd1	–	UN
	7619	GI:115676711	6	Sp-Gluld1	Y	UN
	8206	GI:72124124	6	Sp-Mdh1	Y	Y
	8404	GI:72004519	10	Sp-Eno1	Y	UN
	8434	GI:115744264	14	Sp-Idh2	Y	UN
	8501	GI:72013762	8	Sp-Ugdh	Y	UN
	9411	GI:115746768	3	Sp-Acat1	Y	UN
	9613	GI:72092734	7	Sp-Tkt	–	UN
Protein biosynthesis						
	2053	GI:72112467	1	Sp-Acidic ribosomal protein	–	Y
	3108	GI:72066070	5	Sp-EF1B alpha	Y	Y
	7307	GI:68534982	16	Sp-EF1B gamma	Y	Y
	5322	GI:72010866	3	Sp-EIF3S3-like	Y	Y
	6011	GI:72025937	4	Sp-RPS12	–	UN
	7614	GI:72185290	3	Sp-CCT2	Y	UN
	8507	GI:72007611	9	Sp-CCT5	Y	UN
	8608	GI:72006577	16	Sp-CCT7	Y	UN
	8618	GI:115628085	8	Sp-CCT6A	Y	UN
Protein turnover						
	7128	GI:115903612	3	Sp-Psma2	Y	Y
	8101	GI:115905724	4	Sp-Psma2	–	Y
Structural						
	6119	GI:72165711	1	Sp-TAGLN2	–	UN
	4104	GI:72165711	1	Sp-TAGLN2	Y	UN
	4623	GI:47551017	4	Sp-Beta-tubulin-3/1	Y	Y
	6217	GI:115705411	3	Sp-Beta-tubulin-3/1	Y	Y
	5306	GI:72072990	4	Sp-Alpha-tubulin-5	–	Y
	5502	GI:72049732	5	Sp-Alpha-tubulin-10	Y	Y
	5509	GI:115736220	2	Sp-Alpha tubulin 2	Y	Y
	5004	GI:115624091	6	Sp-Cofilin	Y	Y
	6013	GI:115624091	8	Sp-Cofilin	–	Y
	8301	GI:115644616	5	Sp-Gelsolin	Y	Y
	8307	GI:115644616	5	Sp-Gelsolin	Y	Y
	6406	GI:47551035	9	Sp-Cytoplasmic actin IIIb	Y	Y
	7229	GI:115740945	5	Sp-CAPZB	–	UN
	7240	GI:72016744	9	Sp-YP30/fasciclin	–	Y
	8039	GI:72016744	6	Sp-YP30/fasciclin	–	Y
NA	7238		-	Unknown	–	NA
	8111		-	Unknown	Y	NA

Y = yes, UN = un-described. Peptide matches represent individual non-redundant and non-overlapping peptide spectra generated for each protein.

urchin has been used as a model for identification and characterization of phosphorylated proteins and kinase activity at fertilization (Ciapa and Chiri, 2000; Jaffe et al., 2001; Runft et al., 2002). Although examples of kinases, phosphatases and their substrates that play a role in early embryogenesis are accumulating, the set of the participants in regulation by phosphorylation is far from complete (Sato et al., 1998, 2002). Recently, we have applied a sensitive and quantitative method of detecting protein phosphorylation, coupled to two dimensional electrophoresis (2DE), to the characterization of global phosphoproteome dynamics in the sea urchin egg and zygote and estimated that about 30% of the proteins found in the unfertilized egg are phosphorylated (Roux et al., 2006). This number of phosphorylated proteins increases more than two-fold by 2 min after fertilization and returns to the initial levels by 30 min.

Here, we have carried out a proteomic screen on the egg and zygote to identify proteins that exhibit reproducible, dynamic changes in both abundance and phosphorylation at fertilization. The unbiased (with respect to function) identification and functional classification of maternal proteins generate a list of candidate proteins for further investigation regarding their potential role in orchestrating egg activation events and the egg to embryo transition. This study, along with the recent characterization of sperm proteomes in the sea urchin (Nomura

and Vacquier, 2006; Su et al., 2005), will provide important clues about species specific gamete recognition, sperm–egg binding/fusion and activation of the developmental program.

Materials and methods

Gamete collection, handling and fertilization

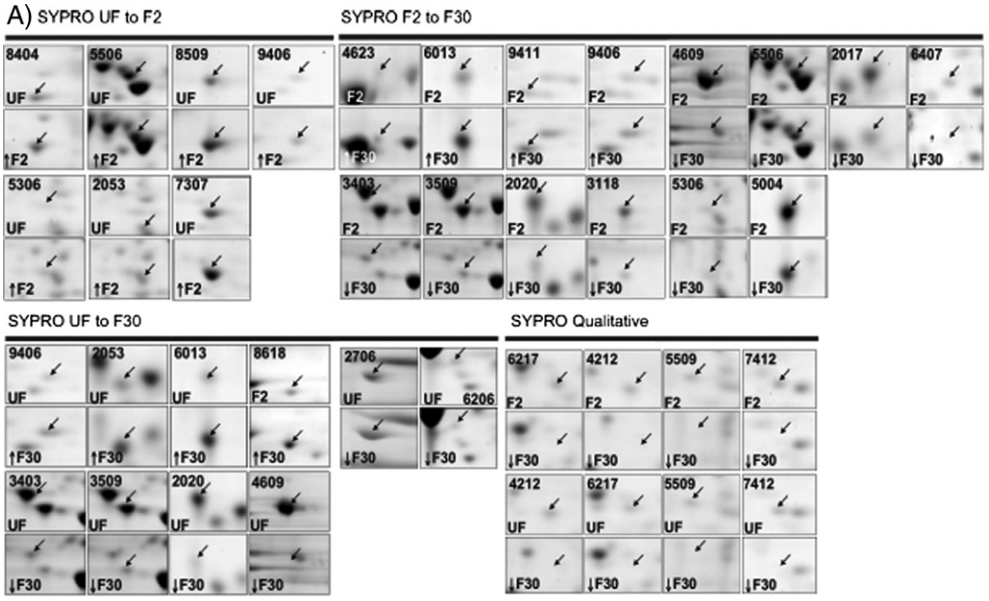
California purple sea urchins, *S. pupuratus*, were collected from the Santa Barbara Channel (Goleta Pier, Goleta, CA) and kept in 10–12 °C temperature-controlled aquaria. Fertilization was performed using dry sperm diluted 1:25,000 in filtered sea water (FSW):jelly water (1:1 v/v). All collections times post fertilization (F) refer to minutes following sperm addition.

Preparation of NP-40-soluble protein lysate

Total NP-40-soluble protein from unfertilized eggs or embryos collected 2 and 30 min after sperm addition was prepared as described (Kumano et al., 2001) and the protein concentration was determined by the BCA method (Pierce Biotechnology, Inc.; Rockford, IL). Freshly prepared lysate was immediately separated by isoelectric focusing (IEF) and SDS PAGE.

Two-dimensional gel electrophoresis (2DE), imaging and analysis

NP-40-soluble protein lysates were treated as described (Roux et al., 2006) with the following exceptions; 100 μg soluble protein was used for 2DE on a pH 4–7 gradient (GE Healthcare; Piscataway, NJ) as follows—300 volts (V) 0:01 h,



B)

SYPRO UF to F2

Spot ID	Sea urchin genome ID	Relative difference
8404	Sp-Eno1	3.77
5506	Sp-Pdia4	2.96
8509	Sp-Irbit-like	1.82
9406	Sp-Vdac2	1.82
5306	Sp-Alpha-tubulin-5	1.82
2053	Sp-Acidic ribosomal protein	1.64
7307	Sp-EF1B gamma	1.60

SYPRO F2 to F30

Spot ID	Sea urchin genome ID	Relative difference
4623	Sp-Beta-tubulin-3/1	8.70
6013	Sp-Cofilin	2.04
9411	Sp-Acat1	1.54
9406	Sp-Vdac2	1.59
3403	Sp-Atp5b	-7.21
3509	Sp-Atp5b	-7.18
2020	Sp-Cvp18	-5.85
3118	Sp-14-3-3_two	-5.78
4609	Sp-Rendezvin theta	-5.32
5506	Sp-Pdia4	-3.15
2017	Sp-Speract	-2.60
6407	Sp-RPA1	-2.17
5306	Sp-Alpha-tubulin-5	-1.91
5004	Sp-Cofilin	-1.67

SYPRO UF to F30

Spot ID	Sea urchin genome ID	Relative difference
9406	Sp-Vdac2	2.88
2053	Sp-Acidic ribosomal protein	2.07
6013	Sp-Cofilin	2.03
8618	Sp-CCT6A	1.66
3403	Sp-Atp5b	-9.11
3509	Sp-Atp5b	-8.68
2020	Sp-Cvp18	-6.42
4609	Sp-Rendezvin theta	-6.34
2706	Sp-Vitellogenin	-1.59
6206	Sp-SWI/SNF	-1.58

C)

SYPRO Qualitative UF to F30

Spot ID	Sea urchin genome ID	Relative difference
4212	Sp-PKC substrate 80K-H	-699.43
6217	Sp-Beta-tubulin 1/3	-603.15
5509	Sp-Alpha-tubulin 2	-423.98
7412	Sp-Mthfd1	-298.83

SYPRO Qualitative F2 to F30

Spot ID	Sea urchin genome ID	Relative difference
6217	Sp-Beta-tubulin-3/1	-595.48
4212	Sp-PRKCSH-like	-503.20
5509	Sp-Alpha tubulin 2	-472.90
7412	C-1-tetrahydrofolate synthase	-339.25

Fig. 2. Analysis of proteins exhibiting changes in relative abundance during egg activation. Each panel in panel A represents an example of single, identified spots (arrows) that exhibited a difference in abundance ≥1.5-fold between the time points indicated (UF, F2 and F30). Spot ID numbers are indicated and correspond to those in panel B, listing the relative increase or decrease in total protein abundance. Individual spots were compared over time post fertilization and fold differences were calculated. Protein abundance is shown in units relative to the ratio of normalized pixel density for each individual spot measured in parts per million. Data points are grouped according to time points and levels of expression. Positive values indicate a fold increase in protein abundance, negative values indicate a decrease. (C) Subset of protein spots present in eggs that were undetectable by F30. Relative difference is indicated as the net intensity (PPM) of the spot. For both panels B and C, only samples having a maximum coefficient of variance <30% were included in the analysis.

3500 V 1:30 h, and 3500 V 3:00 h. All samples were run simultaneously and in quadruplicate.

2D gels were fixed and stained with ProQ Diamond and SYPRO Ruby stains and imaged as described (Roux et al., 2006) (Molecular Probes Inc, Eugene, OR). Images were imported into PDQuest (version 7.4) for spot detection, analysis and gel excision using the ProteomeWorks Spot Cutter (Bio-Rad, Hercules, CA). Images were then organized within PDQuest as matched sets. Replicate treatments (UF=unfertilized, F2 and F30, $n=4$) were grouped and ambiguous matches were manually reconciled. Analysis was then performed to identify spots with qualitative (presence/absence) and quantitative $\geq$1.5-fold increase/decrease. In order to be considered for further analysis, a spot had to fulfill these criteria on at least three of four gels. For ProQ Diamond stained gels all spots also were required to be detected and matched in SYPRO Ruby image replicate groups in order to be successfully excised. Based on these criteria, 94 spots were selected for protein identification.

Quantitative analysis of these 94 spots was performed as a confirmation of analysis set detection and visual determination of protein dynamics. Relative spot intensity was measured in PPM and the coefficient of variance (CV) within each replicate group was calculated. Only spots whose replicate group CV was <30% were subject to quantitative differential detection analysis. The CV threshold was determined based on the combined amount of error induced by both experimental variance and PDQuest software (Wheelock and Buckpitt, 2005). Quantitative differences between replicate groups were calculated using Excel for both SYPRO Ruby and ProQ Diamond images and a threshold was set at $\geq$1.5-fold. Proteins reported as having differential phosphorylation did not show a significant change in total protein detection using SYPRO Ruby, ensuring selection based on level of phosphorylation, not protein abundance.

Quadrupole time of flight tandem mass spectrometry (Q-ToF MS/MS)

Selected proteins were picked using a ProteomeWorks Spot Cutter (Bio-Rad, Hercules, CA) followed by in-gel digestion with trypsin using mass spectrometry grade trypsin (Trypsin Gold, Promega, Inc.; Madison, WI). Peptides were fractionated by nanoflow reverse phase chromatography and their MS/MS spectra were collected using electrospray ionization, Q-ToF MS/MS (Q-ToF II, Waters Millford, MA). The MS/MS spectra were analyzed using ProteinLynx Global Server (PLGS) software (Waters, Milford, MA) and the proteins were identified by spectral matching using the sea urchin protein database (NCBI). Putative protein identities were assigned based on best BLAST match assessed by E-value and then were cross checked by tBLASTn analysis (http://www.ncbi.nlm.nih.gov/BLAST/) and by BLASTp against, respectively, the Baylor *S. purpuratus* GLEAN3 gene predictions and corresponding protein translations (http://www.hgsc.bcm.tmc.edu/blast/blast.cgi%3Forganism%3DSpurpuratus). The expression of each identified protein was evaluated by manual inspection of the embryonic tiling array data (Samanta et al., 2006). Proteins selected for quantitative analysis reported at least 2 non-overlapping peptide matches. Peptide sequences are available upon request. Functional assignment was performed using the NCBI KOG framework as described (Goel and Mushegian, 2006).

Immunoblotting

NP-40-soluble lysates used in the same 2DE experiment and from two additional sea urchin cultures were separated by SDS PAGE and transferred to nitrocellulose membrane. Blots were probed with rendezvin theta polyclonal rabbit antisera (a generous gift from Dr. Gary Wessel and Dr. Julian Wong; Wong and Wessel, 2006b) diluted 1:5000 in 5% milk in Tris buffered saline with 0.1% tween 20 overnight. Antibody detection was by HRP conjugated secondary antibodies and chemiluminescent detection (Pierce Endogen, Inc.; Rockford, IL). Imaging and quantitation were performed using a Versa-doc platform and Quantity One software (Bio-Rad, Hercules, CA). Blots were stripped and re-probed with anti-alpha tubulin monoclonal antibody (Sigma, St. Louis, MO; clone B-5-1-2T/5168) diluted 1:10,000 and signal was detected in the manner stated above. Images were normalized to alpha tubulin and Student's *t*-tests were performed to determine significance between the time points indicated.

Results

Identification of sea urchin egg and zygote proteins

About 600 protein spots were reproducibly detected by the SYPRO RUBY stain and ~200 spots were stained with phosphoprotein-specific stain in the unfertilized egg, consistent with our previous report (Roux et al., 2006). At the next step, we identified proteins that exhibited changes in abundance or phosphorylation during early zygotic development using the same quantitative multiplexed technique. For each time point (UF, F2 and F30), sets of 4 gels were stained with ProQ Diamond followed by SYPRO Ruby, imaged and analyzed as described in the Materials and methods section. A master gel image representing all of the protein spots detected and an example of one of the raw gel images are shown in Fig. 1. Of the ~600 reproducibly detectable SYPRO Ruby spots, 94 were chosen for identification by LC MS/MS (Table 1, Fig. 1 top panel). These were selected from user-defined analysis sets which included hundreds of spots whose abundance and phosphorylation remained unchanged or displayed $\geq$1.5-fold increase or decrease between time points. Each analysis set was visually inspected in order to reconcile computer-mediated spot detection. Spots selected for MS/MS met the following criteria: (1) reproducible detection among replicates using both SYPRO Ruby and ProQ Diamond; (2) adequate abundance for successful generation of MS/MS spectra; and (3) representation of the entire biochemical range of the proteome with respect to both Mr and p*I*. Selected spots make up roughly 1/6 of the detectable, NP-40-soluble egg proteome. Of the 94 spots chosen for analysis by mass spectrometry, all but two were identified using the predicted sea urchin proteome as a reference. The

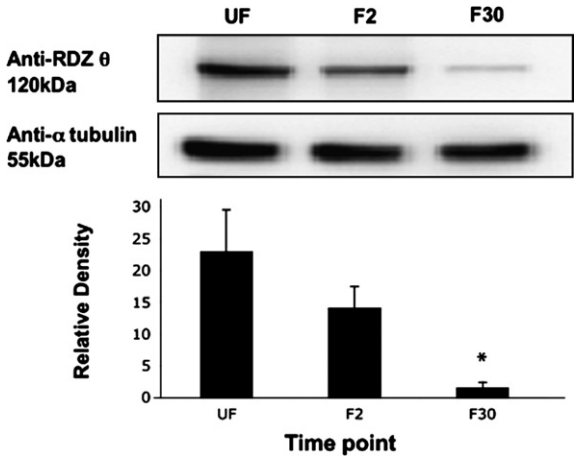


Fig. 3. Immunoblot analysis of Sp-Rendezvin theta. NP-40 lysates from a 2DE experiment and two additional sea urchin embryo cultures ($n=3$) were analyzed for the presence of Sp-Rendezvin theta. Top panel depicts a representative blot of 50 μg of soluble lysate from unfertilized (UF) eggs and zygotes at 2 and 30 min post sperm addition (F2 and F30). Blots were re-probed with alpha tubulin antibody for normalization (lower panel). Quantitation is reported in relative density based on normalized values generated using Quantity One software and is shown graphically in the bottom panel. Asterisk (*) indicates statistically significant difference in detected protein abundance (Student's *t*-test $p=0.03$). Compare with the 2DE results shown in Fig. 2, Spot ID #4609.

Table 2
Phosphorylated proteins identified from the sea urchin egg and embryo proteome

Functional group	Sea urchin genome ID	Spot ID	Biological function	In urchin		Role in egg activation[a]	Refs
				Eggs	Embryo		
Calcium mediated							
	Sp-Irbit-like	8509	IP3 receptor/Ca^{2+} homeostasis	UN	UN	Y	(Ando et al., 2006, 2003; Mikoshiba, 2007)
	Sp-Annexin	5117	Bind Ca^{2+} and phospholipids and mediates membrane exocytosis/ trafficking, cytoskeletal rearrangement and mitotic signaling Ca^{2+} binding through EF hand motifs regulates cellular processes.	UN	UN	UN	(Gerke and Moss, 2002; Okamoto et al., 2004)
	Sp-Spec2a[b]	3002	Ectoderm specification marker	UN	Y	UN	(Brandhorst and Klein, 1992; Carpenter et al., 1984; Hardin et al., 1988; Mao et al., 1994; Villinski et al., 2005; Xiang et al., 1988; Yuh et al., 2001)
	Sp-Spec2d[b]	7002	See above	UN	Y	UN	(Brandhorst and Klein, 1992; Carpenter et al., 1984; Hardin et al., 1988; Mao et al., 1994; Villinski et al., 2005; Xiang et al., 1988; Yuh et al., 2001)
	Sp-Pdia4	5506	Protein disulfide isomerase-calcium storage	Y	Y	Y	(Lucero et al., 1994, 1998)
Cell maintenance							
	Sp-HSP902A1	4604	Molecular chaperone	Y	Y	UN	(Bedard and Brandhorst, 1986; Bishop et al., 2002; Bishop and Brandhorst, 2001; Giovanni et al., 1999)
	SP-HSP701H	4610	Molecular chaperone	Y	Y	Y	(Agueli et al., 2001; Fabiana et al., 2003, 2004; Geraci et al., 2003, 2004; Giovanni et al., 1999; Sconzo et al., 1995; Sconzo et al., 1999)
	Sp-RPA1	6407	DNA replication/repair	UN	Y	UN	(Fanning et al., 2006; Fernandez-Guerra et al., 2006)
	Sp-GST-11[b]	7008	Compound detoxification	UN	UN	UN	
	Sp-Gsto1[b]	7111	Compound detoxification	Y	Y	UN	(Cunha et al., 2005; Goldstone et al., 2006)
	Sp-NCL-like	7510	Nucleolin-transcription, histone chaperone, ribosomal processing	UN	UN	Y	(Fair et al., 2001; Schwab and Dreyer, 1997)
	Sp-SMARCC2[b]	6206	SWI/SNF related-chromotin reorganization, repair and replication	UN	UN	UN	
Gamete associated							
	Sp-Speract[b]	2003	Sperm chemotaxis	Y	UN	Y	(Hansbrough and Garbers, 1981; Bentley et al., 1987; Cardullo et al., 1994; Ramarao et al., 1990; Shiba et al., 2005; Wood et al., 2003)
	Sp-Rendezvin	4609	Cortical granule exocytosis	Y	UN	Y	
Ion exchange							
	Sp-Vdac2	9615	Fluid transport, acidification, volume control, polarization	UN	UN	UN	(Steinacker et al., 2000; Yin et al., 2007)
	Sp-Vdac2	9204	See above	UN	UN	UN	(Steinacker et al., 2000; Yin et al., 2007)
	Sp-Rendezvin[b]	4609	Cortical granule exocytosis	Y	UN	Y	(Wong and Wessel, 2006b)
Cellular signaling							
	Sp-Rbbp-4	3611	Retinoblastoma binding proteins-cell cycle regulation	UN	UN	UN	(Binne et al., 2007; Nevins, 2001)

Table 2 (*continued*)

Functional group	Sea urchin genome ID	Spot ID	Biological function	In urchin		Role in egg activation [a]	Refs
				Eggs	Embryo		
Metabolism							
	Sp-MYP	2706	Yolk platelet protein, iron binding	Y	Y	UN	(Brooks and Wessel, 2002, 2003, 2004; Mallya et al., 1992; Yokota et al., 2003)
	Sp-Vitellogenin3	6719	Egg yolk biosynthesis	Y	Y	Y	(Shenless and Sellers, 2001; Wang and Williams, 1982; Brooks and Wessel, 2002; Brooks and Wessel, 2003; Brooks and Wessel, 2004; Mallya et al., 1992; Yokota et al., 2003)
	Sp-Vitellogenin1	7103	Egg yolk biosynthesis	Y	Y	Y	(Shenless and Sellers, 2001; Wang and Williams, 1982; Brooks and Wessel, 2002, 2003, 2004; Mallya et al., 1992; Yokota et al., 2003)
	Sp-Atp5b	4407	ATP production	UN	UN	UN	(Satoh et al., 1994)
	Sp-Suclg2	3408	Succinate Co-enzyme A ligase-cellular respiration	UN	UN	UN	
	Sp-5-L0	4001	Fatty acid metabolism	Y	UN	UN	(Brash et al., 1991; Hawkins and Brash, 1987; Perry and Epel, 1985)
	Sp-Potfu[b]	4420	Protein *O*-fucosyltransferase-NTCH glycosylation	UN	UN	UN	(Okajima et al., 2003)
	Sp-Aldh6a1[b]	6508	Aldehyde dehydrogenase	UN	UN	UN	
	Sp-Fth1	5010	Ferritin heavy chain-intracellular iron storage	UN	UN	UN	(Huang et al., 2003)
	Sp-Fth1	5031	See above	UN	UN	UN	(Huang et al., 2003)
	Sp-Acadl	5302	Fatty acid metabolism	UN	UN	UN	(Macheroux et al., 1997)
	Sp-Peroxiredoxin	6042	Antioxidant	UN	UN	UN	(Leyens et al., 2004)
	Sp-Peroxiredoxin	7121	Antioxidant	UN	UN	UN	(Leyens et al., 2004)
	Sp-Mdh2	6241	Gluconeogenesis	Y	Y	Y	(Biliar et al., 1966; Okabayashi and Nakano, 1984; Ozaki and Whiteley, 1970)
	Sp-EsteraseD[b]	7208	Hydrolysis of ester bonds	Y	Y	UN	(Westin, 1970)
	Sp-Acads	7209	Fatty acid metabolism	UN	UN	UN	
	Sp-Fah[b]	7303	Fumarylacetoacetase-tyrosine catabolism	UN	UN	UN	
	Sp-Fah[b]	7402	Fumarylacetoacetase-tyrosine catabolism	UN	UN	UN	
	Sp-Gluld1[b]	7619	Glutamine synthetase	UN	UN	UN	
	Sp-Mdh1	8206	Gluconeogenesis	Y	Y	Y	(Biliar et al., 1966; Okabayashi and Nakano, 1984; Ozaki and Whiteley, 1970)
	Sp-Sod2	7123	Oxidative stress	UN	Y	UN	(Pagano et al., 2001; Pangano et al., 2001)
	Sp-Eno1	8404	Enolase-glycolosis	UN	UN	UN	
	Sp-Idh2	8434	Citric acid cycle/Redox	UN	UN	UN	(Pollak et al., 2007)
	Sp-Ugdh	8501	UDP glucose dehydrogenase-proteoglycan synthesis	UN	UN	UN	(Vigetti et al., 2006)
	Sp-Acat1	9411	Ketone catabolism	UN	UN	UN	
Protein biosynthesis							
	Sp-EF1B alpha	3108	Protein translation	Y	Y	UN	(Boulben et al., 2003; Le Sourd et al., 2006)
	Sp-EF1B gamma	7307	Protein translation	Y	Y	UN	(Boulben et al., 2003; Le Sourd et al., 2006)
	Sp-EIF3S3-like	5322	Protein translation	UN	UN	UN	
	Sp-CCT2[b]	7614	Cytosolic protein chaperone	UN	UN	UN	
	Sp-CCT5[b]	8507	Cytosolic protein chaperone	UN	UN	UN	
	Sp-CCT7[b]	8608	Cytosolic protein chaperone	UN	UN	UN	
	Sp-CCT6A[b]	8618	Cytosolic protein chaperone	UN	UN	UN	

(continued on next page)

Table 2 (*continued*)

Functional group	Sea urchin genome ID	Spot ID	Biological function	In urchin		Role in egg activation[a]	Refs
				Eggs	Embryo		
Protein turnover							
	Sp-Psma2	7128	Proteasome subunit alpha 2-protein degradation	Y	Y	Y	(Chiba et al., 1999; Iwafune et al., 2002; Kawahara et al., 2000)
Structural							
	Sp-TAGLN2[b]	4104	Transgelin-actin binding	UN	UN	UN	
	Sp-Beta-tubulin-3/1	4623	Cytoskeletal structure/function	Y	Y	Y	(Alexandraki and Ruderman, 1985a,b; Meng et al., 2004)
	Sp-Beta-tubulin-3/1	6217	Cytoskeletal structure/function	Y	Y	Y	(Alexandraki and Ruderman, 1985a,b; Meng et al., 2004)
	Sp-Alpha-tubulin-10	5502	Cytoskeletal structure/function	Y	Y	Y	(Alexandraki and Ruderman, 1985a,b; Meng et al., 2004)
	Sp-Alpha tubulin-2	5509	Cytoskeletal structure/function	Y	Y	Y	(Alexandraki and Ruderman, 1985a,b; Meng et al., 2004)
	Sp-Cofilin	5004	Actin disassembly	Y	Y	Y	(Abe et al., 1996; Alexandraki and Ruderman, 1985b; Golsteyn and Waisman, 1989; Hoyosa et al., 1982; Nusco et al., 2006)
	Sp-Gelsolin	8301	Actin severing and capping	Y	Y	Y	(Golsteyn and Waisman, 1989; Mabuchi et al., 1985)
	Sp-Gelsolin	8307	Actin severing and capping	Y	Y	Y	(Golsteyn and Waisman, 1989; Mabuchi, 1990)
	Sp-Cytoplasmic actin IIIb	6406	Cytoskeletal structure/function	Y	Y	Y	(Morris et al., 2006; Sun and Schatten, 2006; Wong et al., 1997)
NA	Unknown	8111	Not determined	NA	NA	NA	NA

In urchin refer to reports in literature that provide evidence for the presence of the specified protein as phosphorylated in either the egg or embryo.

[a] UN = un-described.

[b] No phosphorylation shown in literature.

identities of these proteins and their functional classification are provided in Table 1 and provide the first step toward a more comprehensive analysis of proteins involved in egg activation.

Dynamic changes in abundance of egg and zygote proteins

Quantitative detection of changes in total soluble protein abundance was set at the threshold of ≥1.5-fold with <30% sample to sample variance. Using these criteria, 23 of 94 proteins changed in total abundance (SYPRO Ruby) over the experimental time course (0–30 min post insemination) (Fig. 2, Supplemental Table 1). By this method, changes cannot necessarily be assigned to protein degradation or synthesis as PTMs may cause shifts on the 2D gels; rather, this method allows for tracking of dynamic changes in overall abundance of a specific protein species.

Several examples of specific areas of the 2D gels exhibiting these dynamic changes are presented in Fig. 2. Seven spots become more intense 2 min after fertilization. These proteins were detected as having ≥1.5-fold increase in abundance relative to levels in unfertilized eggs (UF to F2; Fig. 2). Comparisons of changes in total protein abundance between 2 and 30 min post fertilization (F2 to F30) and unfertilized to F30 min (UF to F30) showed a significant population of proteins that decrease in abundance (Fig. 2). This group includes proteins with diverse functions, including loss of proteins that are known to be specifically released during cortical granule exocytosis (CGE), such as 18 kDa Cvp and Rendezvin theta (Wong and Wessel, 2006b).

To confirm the 2DE results, western blotting of 1D gels using specific antibodies was conducted. For example, an antibody specific for the endogenous theta isoform of Sp-Rendezvin detected the protein in the UF egg and at F2; however, it decreased 15.7-fold by F30 (Fig. 3). Antibody detection was ~3-fold more sensitive than protein staining alone with SYPRO, but consistent with the loss of Sp-Rendezvin protein over time observed by 2DE (Fig. 2; Spot ID #4609, Supplemental Table 1). It is likely that cross-linking of Sp-Rendezvin theta during hardening of the fertilization envelope (FE) creates an insoluble protein complex that is subsequently excluded from total NP-40 lysates, as opposed to degradation of the protein (Wong and Wessel, 2006a). Thus, although we were not able to comprehensively test each of the proteins identified by the 2DE screen (due to a lack of specific antibodies), internal replicate consistency and the confirmation by antibody analyses when feasible indicate that this 2DE method provides a reliable first pass to identify proteins that exhibit dynamic changes after fertilization.

Phosphorylated egg and zygote proteins

Sixty-two of the ninety-four identified proteins were detected with the ProQ Diamond stain in the unfertilized egg and thus scored as phosphorylated (Table 2). The ProQ Diamond stain appears to be quite specific (Stasyk et al., 2005; Steinberg et al., 2003), and ~70% of the ProQ Diamond stained proteins in our dataset have been previously identified as phosphoproteins (Table 2). Thus, it is likely that we have identified about 20 new phosphorylation targets in the unfertilized eggs of sea urchin.

Nineteen of the ProQ Diamond-positive proteins showed dynamic phosphorylation patterns following fertilization (Supplemental Table 1, Fig. 4). Many exhibited an altered phosphorylation state or ≥1.5-fold change in abundance of the phosphorylated form during fertilization (Fig. 4). The largest number of detectable increases in individual protein phosphorylation occurred during the first 2 min after fertilization. This was followed by a decrease in the number of phosphorylated proteins by 30 min (Fig. 4), consistent with our previous results (Roux et al., 2006) and the reported changes in bulk kinase activity after fertilization (Kinsey, 1995; Kinsey, 1997b). A substantial number of these proteins (66.1%; $n=41$) of the phosphoproteins identified are un-described with respect to their role in egg activation (Table 2, UN) and represent targets of future studies.

Predicted function of egg and zygote proteins

Functional classification of proteins was conducted using gene ontology and eukaryotic subset of NCBI Clusters of Orthologous Groups (KOGs; Tatusov et al., 2003) is presented in Supplemental Table 1 and graphically in Fig. 5. The majority of proteins identified are involved in intermediary metabolism ($n=37$), protein biosynthesis ($n=9$) or function as structural components of the oocyte ($n=15$) (Fig. 5). Proteins involved in calcium-mediated interactions ($n=8$), cell maintenance ($n=7$) and signaling ($n=4$) were also identified in the egg and zygote. KOG classification for proteins involved in metabolism is shown in Fig. 5 and for all identified proteins in Supplemental Table 1. The significance of the functional groups is discussed below.

Discussion

Dramatic changes in cellular physiology occur during the first 30 min of egg activation. We chose to examine the proteome of unfertilized eggs and of zygotes at 2 min and 30 min post insemination in order to capture proteins which might regulate or mediate the egg to embryo transition. The first 2 min post insemination marks an increase in tyrosine kinase signaling which in turn modulates Ca^{2+} release from the egg's ER. This enables cortical granule exocytosis as the permanent block to polyspermy and initiates cytoskeletal remodeling and cell cycle reentry. By 30 min, the newly formed zygote is preparing for mitotic cell division, has nearly completed the first round of DNA synthesis and has reached a steady protein turnover state. The analysis presented here has enabled the identification of proteins whose presence and dynamic behavior occurred during these two critical time points and are discussed below in the context of the corresponding cellular events.

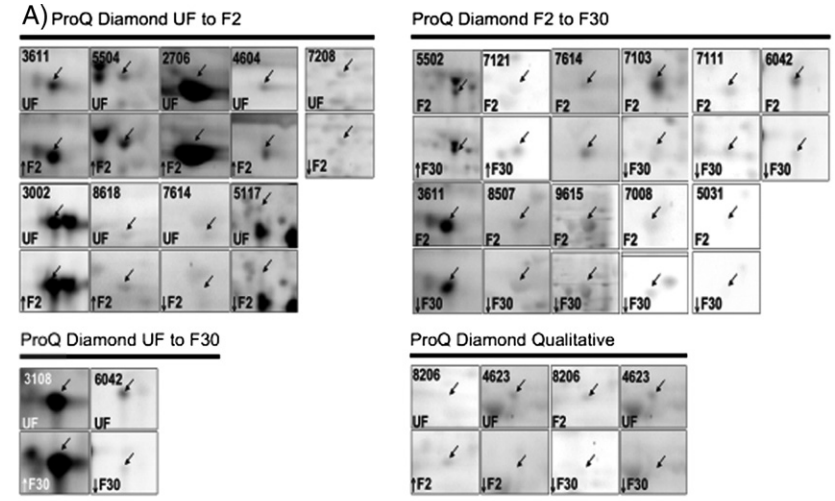
Validation of 2DE multiplexed detection and protein identification

Our previous work (Roux et al., 2006) suggested the optimal conditions for 2DE resolution of the detectable NP-40-soluble egg proteins. The proteins identified in this study represent a broad range of molecular weights and isoelectric points. As noted above, the multiplex method allows detection of proteins that change in relative abundance or phosphorylation. While it is not feasible to confirm changes in protein abundance by 1D blotting analysis in all cases, representative examples (such as Rendezvin, Fig. 3) confirmed that changes in abundance detected by the 2DE method were valid.

It is also expected that the multiplex method will detect PTMs that affect p*I*. In support of this, several spots with different p*I*s were determined to be identical proteins, indicative of PTMs (Fig. 1, Table 1). Detection of these changes is consistent with previous studies and can be used as internal controls for the 2DE multiplex analysis applied here. The 94 proteins identified have a wide variety of biological functions (Table 1, Fig. 5, Supplemental Table 1). Several proteins have been found in the egg and early zygote previously, while others appear to be novel (see Table 2).

Sp-Speract was identified in the egg proteome, decreased in abundance by F2 and was barely detected at F30 (Fig. 2). Speract is secreted from the egg into the jelly coat where it acts as a chemo-attractant for sperm (Cardullo et al., 1994; Cook et al., 1994; Kaupp et al., 2003; Shiba et al., 2005; Wood et al., 2003). After fertilization, Speract is no longer needed and may be degraded as a consequence of the "sweep" or removal of proteins no longer necessary as the egg transitions to the embryo (Stitzel and Seydoux, 2007).

Cortical granule (CG) exocytosis and formation of the fertilization envelope (FE) provide the mechanical permanent block to polyspermy (Larabell and Chandler, 1991; Vogel et al., 1991; Wong and Wessel, 2006a). In response to the Ca^{2+} rise, multiple protein components are released from the CGs as they fuse with the plasma membrane. Many CG proteins associate with the vitelline layer where they are cross-linked in the extracellular space resulting in formation of the FE. One of these proteins belongs to a class of extra-cellular matrix proteins called Rendezvin (Wong and Wessel, 2006b). The theta isoform of Sp-Rendezvin was identified in the egg proteome and 2DE analysis revealed its disappearance from the zygote proteome after cortical granule exocytosis, following the Ca^{2+} wave at 2 min post fertilization (Figs. 2, 3). The 18 kDa cortical vesicle protein (Sp-Cvp18) was also identified as a maternal protein in our analysis (Fig. 2). Sp-Cvp18 protein is expressed in sea urchin eggs, sperm, pluteus larvae and adult tissues and shows similarity to cell adhesion proteins (G. Wessel, unpublished, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&



B)

ProQ Diamond UF to F2

Spot ID	Sea urchin genome ID	Relative difference
3611	Sp-Rbbp-4	4.57
5504	Sp-Pdia4	2.37
2706	Sp-Vitellogenin	2.06
4604	Sp-HSP902A1	1.91
3002	Sp-Spec2a	1.57
8618	Sp-CCT6A	1.57
7614	Sp-CCT2	-3.23
5117	Sp-Annexin	-1.54
7208	Sp-Esterase D	-1.51

ProQ Diamond F2 to F30

Spot ID	Sea urchin genome ID	Relative difference
5502	Sp-Alpaha-tubulin-10	11.32
7121	Sp-Peroxiredoxin	6.38
7614	Sp-CCT2	3.09
7103	Sp-Vitellogenin	-9.27
7111	Sp-Gsto1	-5.30
6042	Sp-Peroxiredoxin	-4.47
3611	Sp-Rbbp-4	-3.97
8507	Sp-CCT5	-3.90
9615	Sp-Vdac	-2.36
7008	Sp-GST-11	-1.99
5031	Sp-Fth1	-1.61

ProQ Diamond UF to F30

Spot ID	Sea urchin genome ID	Relative difference
3108	Sp-EF1B alpha	1.62
6042	Sp-Peroxiredoxin	-5.53

C)

ProQ Diamond Qualitative UF to F2

Spot ID	Sea urchin genome ID	Relative difference
8206	Sp-Mdh1	298.75
4623	Sp-Beta-tubulin-3/1	-262.00

ProQ Diamond Qualitative F2 to F30

Spot ID	Sea urchin genome ID	Relative difference
8206	Sp-Mdh1	-298.75

ProQ Diamond Qualitative UF to F30

Spot ID	Sea urchin genome ID	Relative difference
4623	Sp-Beta-tubulin-3/1	-204.77

Fig. 4. Analysis of proteins detected by the phospho-stain, ProQ Diamond. Each panel in panel A represents an example of single, identified spots (arrows) that exhibited a difference in staining ≥1.5-fold between the time points indicated (UF, F2 min and F30 min). Spot ID numbers are indicated and correspond to those in panel B, listing the relative increase or decrease in phospho-staining. Individual spots were compared over time post fertilization and fold differences were calculated. Data points are grouped according to time point and levels of expression. Staining is shown in units relative to the ratio of normalized pixel density for each individual spot measured in parts per million. Positive values indicate ≥1.5-fold increase in protein abundance, negative values indicate a decrease. (C) Subset of protein spots present in eggs that were undetectable by F30. Relative difference is indicated as the net intensity (PPM) of the spot. For both panels B and C, only samples having a maximum coefficient of variance <30% were included in the analysis.

val=11934664). However, its potential role at fertilization remains to be determined. Our results show that Sp-Cvp18 is present in the UF egg and decreases between 2 min and 30 min post insemination (Fig. 2), indicating a role in oogenesis, cortical exocytosis or some early aspect of the egg to embryo transition.

Proteins with potential novel roles in egg activation: structural proteins

Sea urchin has been used for many years as a model for the study of cytoskeleton dynamics at fertilization and during cytokinesis. During fertilization, massive rearrangements of actin networks (filamentous or cortical) occur beginning around 2 min post insemination and continuing through the first 30 min, in response to intracellular Ca^{2+} increase and cellular alkalinization (Wong et al., 1997). The relative abundance and phosphorylation state of cytoplasmic actin (Sp-Cytoplasmic actin IIIb) and several beta- and alpha-tubulin isomers varied during the time points analyzed, further supporting the importance of cytoskeletal fluidity during these time points (Figs. 2, 4). Actin-binding proteins were also identified, including F-actin capping proteins Sp-CAPZB and actin-severing Sp-Gelsolin, along with the actin cross-linking protein Sp-TAGLN2. Detection of Sp-Cofilin, an actin-destabilizing protein (ADP) (Tables 1, 2, Fig. 4), was of particular interest because *Xenopus* cofilin (XAC) is necessary for the progression of normal cytokinesis during the first cleavage and is regulated by phosphorylation (Abe et al., 1996) and because a recent study in the related echinoderm, the sea star *Asterina pectinifera*, demonstrates that cofilin plays a role in Ca^{2+} homeostasis during fertilization through multiple secondary messengers, although the mechanism is not yet known (Nusco et al., 2006). We report the identification of different Sp-Cofilin

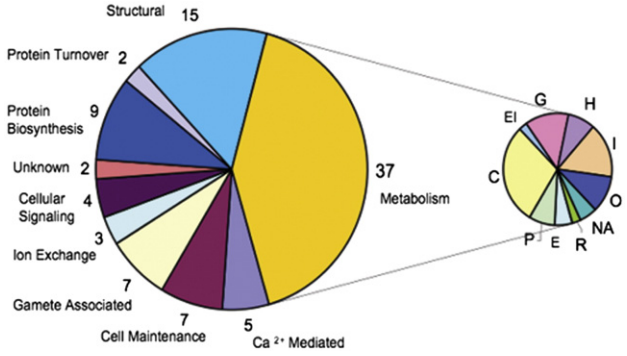


Fig. 5. Functional grouping of proteins identified in the sea urchin egg and zygote proteomes. Protein identities were parsed into functional groups based on analysis of the predicted sequence and published information (see Materials and methods). The subset of 94 identified proteins is graphically represented. Eukaryotic clusters of orthologous groups (KOG) analysis of the proteins grouped under the metabolism heading are represented to the right, with classes identified with letters as follows: (E) amino acid transport and metabolism, (P) inorganic ion transport and metabolism, (C) energy production and conversion, (EI) amino acid/lipid transport and metabolism, (G) carbohydrate transport and metabolism, (H) coenzyme transport and metabolism, (I) lipid transport and metabolism, (O) posttranslational modification, turnover, chaperones, (R) general function prediction. See text for details.

protein spots as changing in abundance during the period of Ca^{2+} mediated cytoskeletal remodeling (F2–F30, Fig. 2) and as a stably phosphorylated protein (Supplemental Table 1).

Calcium binding and regulatory proteins

In deuterostome eggs, sperm–egg interaction triggers a transient Ca^{2+} influx followed by a wave of Ca^{2+} release from the egg's endoplasmic reticulum, which originates at the site of sperm egg interaction and propagates across the egg (Jaffe et al., 2001; Townley et al., 2006; Whitaker, 2006). Many proteins are known to be involved in Ca^{2+} homeostasis during fertilization and throughout the development of the sea urchin (Roux et al., 2006; Townley et al., 2006). A recent genome-wide analysis of conserved sequence domains has led to the compilation of a comprehensive list of genes encoding the sea urchin "Calcium toolkit" (Roux et al., 2006). Many of these genes are represented by maternal mRNAs in the egg, and some of those have been identified in this proteomic study.

Ca^{2+} release at fertilization occurs via IP_3 binding to its receptor (IP_3R) on the endoplasmic reticulum (ER), but little is known about the modulation of the IP_3R in eggs. Recently, the IP_3R binding protein, IRBIT, was identified and characterized as a regulator of Ca^{2+} release from the ER in the absence of physiological levels of IP_3 in mouse and rat cerebellum extracts or when expressed in Cos cell lines (Ando et al., 2006, 2003; Devogelaere et al., 2006). IRBIT is proposed to compete with IP_3 binding to the IP_3R to inhibit or modulate Ca^{2+} release (Devogelaere et al., 2006). An ortholog of mammalian IRBIT is present in the sea urchin genome (Roux et al., 2006) and the proteomic screen described here detected the Sp-IRBIT protein in eggs and zygotes, increasing in abundance during the Ca^{2+} rise (UF-F2, Fig. 2, Table 1). The maintenance of Ca^{2+} homeostasis in Cos cells by IRBIT is dependent on levels of cytoplasmic IP_3 and phosphorylation of serine residues within its binding domain (Ando et al., 2006, 2003; Devogelaere et al., 2006). Once IRBIT is released from the ER, the phosphorylation of these residues is also important for downstream signaling by binding to the Na^+/HCO_3 co-transporter (NBC1) and increasing its activity (Shirakabe et al., 2006). IRBIT, which to date has not been studied at fertilization, is particularly intriguing with respect to egg activation since Ca^{2+} release as well as pH changes are necessary for egg activation. Furthermore, Sp-IRBIT was also recognized as a phosphorylated protein present in eggs and early zygotes of the sea urchin (Table 2). This protein has successfully been used to manipulate IP_3 mediated Ca^{2+} signaling in zebrafish embryos (Ashworth et al., 2007) and will be an important tool for further characterization of Ca^{2+} release and cellular alkalinization at fertilization using the sea urchin model.

Other calcium-binding proteins identified in this study include Sp-Spec2A, Sp-Annexin and Sp-Tropomyosin. Sp-Annexin and Sp-Spec2A were also detected in phosphorylated form (Table 2). All three proteins are present in eggs and zygotes and contain Ca^{2+} binding domains that presumably modulate their activities. Members of the annexin protein family bind Ca^{2+} and phospholipids and mediate a variety of cellular events including membrane exocytosis/trafficking, cytoskeletal rearrangement and mitotic signaling (Gerke and Moss, 2002). A recent analysis of maize egg cells suggests that annexin mediates exocytosis of the materials involved in cell wall formation following sperm–egg fusion (Okamoto et al., 2004). Sp-Annexin is present in eggs (UF) and early zygotes (F2, F30) identified in this study (Tables 1, 2, Supplemental Table 1) and could therefore be tested as a novel regulator of Ca^{2+} dependant cortical granule exocytosis. Sp-Tropomyosin is an F-actin binding protein that responds to an intracellular rise in Ca^{2+}. It has been shown to localize to the fertilization cone in sea urchin eggs, where it could be involved in mediating sperm entry (Ishimoda-Takagi, 1978; Mabuchi et al., 1985; Maekawa et al., 1989).

New candidates for involvement in sperm–egg interactions are intriguing for both fertilization biology and in the study of cell–cell fusion. Sp-Spec2A belongs to a class of Ca^{2+} binding proteins that regulate ectoderm specification during early embryogenesis (Carpenter et al., 1984). Spec protein has been detected in sea urchin eggs and embryos and is thought to bind Ca^{2+} as a means of regulating the cytoskeleton (Xiang et al., 1988). *Sp-Spec* gene regulation is well documented in sea urchin ectoderm specification (Carpenter et al., 1984; Hardin et al., 1988; Lynn et al., 1983; Tomlinson et al., 1990; Xiang et al., 1988), but little is known about its function, if any, in the egg and zygote (Table 2).

In addition to Ca^{2+}, anions also play an important role in the maintenance of cellular physiology. The voltage dependent anion channel 2 (Sp-VDAC2) was detected in UF eggs, F2 and F30 embryos (Table 1, Supplemental Table 1). VDAC like channels have been detected by immunocytochemistry in *Xenopus* oocytes and they may control a wide variety of chloride-mediated processes such as regulation of cell volume

(Steinacker et al., 2000). VDACs have not been previously characterized in sea urchin eggs and could be tested for similar functions during egg activation.

Cellular signaling

Several sea urchin egg kinases (in particular, Src family kinases, protein kinase C, CaMKII and MAPK) are known to exhibit dynamic activity in response to fertilization (Whitaker, 2006). The observation that 30% of spots detected in the egg proteome are phosphorylated and that a large majority of those exhibit dynamic changes in phosphorylation state after fertilization (Roux et al., 2006) is consistent with the model that rapid PTMs – especially phosphorylation – of maternal proteins serve to regulate and transition the egg into active development. Although signaling proteins clearly play a major role in early egg activation, the current experimental design detected only a small complement of signaling proteins (Fig. 5, Table 1). This may be explained in several ways. For example, many signaling molecules are expected to be active immediately following sperm–egg interaction and it is likely that their dynamic signaling has peaked and returned to pre-fertilization levels prior to the F2 time point analyzed here. It is also possible that due to the switch-like nature of signaling events at fertilization, the signaling proteins may be present in low (though, perhaps, locally enriched) quantities, difficult to detect using this multiplexed imaging of NP-40-soluble proteins. Instead, this screen should identify more persistent, downstream targets, especially those that remain soluble and in relatively higher abundance in the zygote.

Consistent with this rationale are the signaling proteins detected here (Table 1), including retinoblastoma binding protein (Sp-Rbbp-4), protein phosphatase 2 (PP2A, Sp-PR65 alpha), the protein kinase C substrate 80K-H (Sp-PRKCSH) and Sp-14-3-3_two (Table 1). Following fertilization in sea urchin eggs, PP2A increases in activity. PP2A activity modulates with the activity of A-kinase, CaM kinase and casein kinase until the pre-hatching blastula stage (Kawamoto et al., 2000). It is thought that PP2A activity regulates many of the rapid de-phosphorylation events occurring during this time. Interestingly, a recent report demonstrates that de-phosphorylation of the early mitotic inhibitor (Emi2) by PP2A is the mechanism which controls slow degradation of Cyclin B and maintains CSF arrest in *Xenopus* oocytes (Wu et al., 2007). It is likely that PP2A has multiple roles in both the oocyte and fertilized egg that regulate many aspects of cell cycle arrest and progression. Identification of PP2A enables its use as a tool with which to study signaling events that are modulated by phosphatase activity during egg activation. Sp-Rbbp-4 and Sp-14-3-3_two are inhibitors of division and therefore cell cycle progression in somatic cells (Aitken, 2006; Nevins, 2001); their role in egg activation and zygotic cell cycle progression is unknown.

Cellular maintenance, proteins biosynthesis and turnover

While signaling cascades fulfill the need for a poised, rapid response to fertilization, it is also necessary to maintain control over general cellular functions such as DNA replication, protein translation and protein folding. Molecular chaperones comprised a significant proportion of the identified proteins involved in cell maintenance and protein biosynthesis (Tables 1, 2, Fig. 5). For example, heat shock proteins (HSPs) 70 and 90 were detected consistently and in abundance during the first 30 min post fertilization (Supplemental Table 1). HSP70 and 90 genes are expressed in the early embryo where they stabilize proteins against stresses such as environmental toxicants (Goldstone et al., 2006). Several chaperone-dependent T-complex proteins (TCPs) were also identified (Tables 1, 2). HSP70 associates with TCPs on the mitotic spindle and at centromeres during the first cell division in the sea urchin *Paracentrotus lividis* (Agueli et al., 2001). It is thought that HSP70 chaperones TCP-regulated tubulin folding in the zygote as well as in other cellular environments (Agueli et al., 2001; Liang and MacRae, 1997), making these interesting candidates for future studies of cytoskeletal rearrangement during fertilization.

In unfertilized sea urchin eggs, very little transcription or translation is detectable (Davidson, 1982; Epel, 1967). Between 6 and 10 min post fertilization, translation increases 5-fold (Epel, 1967), whereas transcription remains barely detectable until late blastula–early gastrula stage and is not required for successful development until late blastula stage, ~18 h post fertilization (Davidson, 1982). Therefore, it is essential that maternal proteins which make up the synthesis machinery are present when new transcription and translation are needed in the embryo, and these types of proteins were expected in our initial screen of the egg proteome. Two protein components of the ribosomal complex, Sp-Acidic ribosomal protein and a member of the small ribosomal subunit (Sp-RPS12), as well as translation initiation and elongation factors, were identified (Table 1) (the alpha and gamma subunits of the eukaryotic translation elongation factor 1 complex are discussed in more detail below). Protein degradation is also occurring and can be detected by 15–20 min post fertilization (Kawahara et al., 2000); as expected, proteasome subunits 2 and 4 are present in the egg and embryo. Interestingly all seven of the proteins in the cellular maintenance, biosynthesis and protein turnover class were also detected in phosphorylated form (Table 2). This may be indicative of a significant regulatory role and is discussed in more detail below.

Intermediary metabolism

Proteins involved in metabolism make up the largest number of proteins identified in this proteomic screen (Table 1, Fig. 5). It is estimated that 8% (2297) of the predicted *S. purpuratus* genes encode metabolism-related proteins (Goel and Mushegian, 2006), and analysis of the egg and zygotic proteomes indicates that proteins involved in intermediary metabolism make up over 40% of the total proteins identified (Table 1, Fig. 5), many of which exhibit relative changes in abundance (Fig. 2) as well as phosphorylation state (Fig. 4). These maternal metabolism proteins represent 10 different KOG categories (Fig. 5) with the majority involved in energy production and conversion.

Energy production related to redox changes at fertilization is a well-described egg activation event (Epel, 1964; Schomer and Epel, 1998), although a majority of the proteins and mechanism have yet to be identified and studied on a molecular level (Tables 1, 2). In the sea urchin, NAD kinase activity is elevated by 30–120 s post fertilization (Epel et al., 1981). NAD kinase supplies NADP and ultimately leads to increases in the NADPH:NADP redox ratio (Epel, 1964). The proteomic screen described here detected NADPH-dependent proteins in the egg and early embryo (Tables 1, 2). These proteins are involved in a wide range of bioenergetic processes. Antioxidants such as peroxiredoxin (Sp-peroxiredoxin) and superoxide dismutase (Sp-Sod2) are also present (Table 1) and likely play a role in protecting the embryo against oxidative stress (Goldstone et al., 2006). Little is known about the roles of these metabolic enzymes in early embryogenesis and this study serves to identify them as candidates for future study.

Quantitative changes in abundance of egg and zygote proteins

One goal of this study was to identify the proteins that are changing during the earliest stages following fertilization in order to better understand pathways that may be contributing to a variety of egg activation events. These changes can be attributed to protein degradation, synthesis, exocytosis, solubility or a shift in isoelectric mobility due to PTMs. Although induction of translation is fairly rapid (detectable by 6 min; Epel, 1967), the apparent increase in the abundance of a subset of proteins by 2 min post fertilization (Supplemental Table 1; Fig. 2) probably is not due to new protein synthesis. Instead, the increase is more likely due to increased solubility or enrichment. Proteins that have increased by 30 min post fertilization may, however, be the result of new synthesis or enrichment or some combination of these two factors. Of particular interest was the increase in detected levels of Sp-eF1B gamma by 2 min post fertilization (Fig. 2). Although most studies focus on the role of the sea urchin eF1B complex in regulating mitosis, it also has been suggested to also play a role in global translational regulation of mRNA (Boulben et al., 2003; Le Sourd et al., 2006). The abundance and regulation of the translation elongation factor complex during early egg activation has not been investigated in any detail and could provide clues about protein translation in the first few minutes following fertilization.

A number of maternal proteins in the egg have fulfilled their functional roles in oogenesis or cell cycle arrest and will be turned over after fertilization (Kawahara et al., 2000). Other proteins packaged into the egg are awaiting fertilization in order to facilitate rapid egg activation or the egg to embryo transition. These proteins may not need to be maintained at the same levels once a successful fertilization event has occurred and, in fact, may be necessary to remove in order for successful egg to embryo transition (Greenstein and Lee, 2006; Shirayama et al., 2006; Stitzel et al., 2006). As described in the Results section, some of the proteins, such as Sp-Rendezvin, that decrease in abundance after fertilization are exocytosed or transition to the insoluble fraction. Others may be marked for specific degradation via the proteasome, which is present and active in early sea urchin zygotes (Kawahara et al., 2000). The subset of proteins that decrease in abundance (Supplemental Table 1, Fig. 2) represents candidates for further study in this regard.

Quantitative changes in phosphorylated egg and zygote proteins

PTMs, especially protein phosphorylation, of maternal proteins are immediate regulatory devices used at egg activation. Global protein phosphorylation on tyrosine is necessary for successful fertilization (Kinsey, 1997a; Tokmakov et al., 2002; Whitaker, 2006) and several proteins that are regulated by tyrosine phosphorylation are known to be involved in egg activation and early development (Townley et al., 2006). Serine–threonine kinase pathways such as the MAP kinase cascade are also key regulators of maturation, fertilization and early zygotic cell cycles in all eggs, including those of the echinoderm (Carroll et al., 2000; Chiri et al., 1998; Kishimoto, 2004; Kumano et al., 2001; Philipova and Whitaker, 1998; Tachibana et al., 1997, 2000; Zhang et al., 2006, 2005). In the previous study using this multiplex 2DE approach, roughly 30% of the proteins present in the egg are phosphorylated. The number of phosphorylated proteins increases more than two-fold by 2 min after fertilization and returns to baseline levels by 30 min (Roux et al., 2006).

Although a subset of the phosphoproteins identified here has been described previously in the eggs and embryos of sea urchins, the majority are uncharacterized in terms of their role in fertilization and egg activation in any species (Table 2). Twenty-two of the phosphoproteins identified here have a role in intermediary metabolism. Of these, proteins Sp-Vitellogenin and Sp-MYP, Sp-Esterase D and malate dehydrogenase (Sp-Mdh1) are present in yolk and during egg activation (Table 2). Changes in redox states, oxidative stress and increase cellular metabolism are important during egg activation (Epel, 1964; Epel, 1997; Schomer and Epel, 1998) although a thorough analysis of proteins involved in metabolism has not been investigated at fertilization. The identification here of phosphorylated proteins involved in metabolism provides an extensive list of candidates that could be critical regulators of cellular homeostasis during fertilization. Proteins increasing in phosphorylation include those involved in cellular defense such as Sp-Peroxiredoxin and Sp-HSP90 (Fig. 4, Supplemental Table 1). A large increase in alpha tubulin 1 phosphorylation is seen at F2, suggesting a role for phosphorylation in remodeling of the cytoskeleton (Fig. 4, Supplemental Table 1) (Luduena, 1998).

Decreased phosphorylation of the Ca^{2+} binding protein Sp-Annexin occurs by F2, during the peak of the Ca^{2+} wave (Fig. 4). This same trend (decreased phosphorylation) also was observed for Sp-GST and Sp-Thioredoxin peroxidase (Fig. 4), which have been identified as part of the sea urchin defensome and are involved in protection from oxidative and environmental stress (Goldstone et al., 2006). The phosphoproteins identified here represent a first look at the dynamics of a PTM occurring during egg activation. However, this is only a small portion of the total phosphorylated proteins present and an eventual goal is to identify and characterize the entire sea urchin egg and early embryo phospho-proteomes.

The proteins identified in this study serve as candidates for future work that will hopefully lead to a comprehensive analysis of the collective changes that are required during the egg to embryo transition triggered by fertilization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.10.053.

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