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Jian Liu
Wayne State University

Elizabeth E. Puscheck
Wayne State University

Fangfei Wang
Wayne State University

Anna Trostinskaia
Wayne State University

Dusan Barisic
Wayne State University

See next page for additional authors

Authors
Jian Liu, Elizabeth E. Puscheck, Fangfei Wang, Anna Trostinskaia, Dusan Barisic, Gordon Maniere, Dana Wygle, W Zhong, Edmond HHM Rings, and Daniel A. Rappolee

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Jian Liu[1], Elizabeth E Puscheck[1], Fangfei Wang[1], Anna Trostinskaia[1], Dusan Barisic[1], Gordon Maniere[1], Dana Wygle[1], W Zhong[2], Edmond H H M Rings[5] and Daniel A Rappolee[1,2–4]

[1]C S Mott Center for Human Growth and Development, Department of Obstetrics and Gynecology, Hutzel Hospital, Wayne State University School of Medicine, [2]Department of Anatomy and Cell Biology, Wayne State University School of Medicine, [3]Karmanos Cancer Institute, Wayne State University School of Medicine and [4]Insitute for Environmental Health Science, Wayne State University School of Medicine, Detroit, Michigan 48201, USA and [5]Department of Pediatrics, Center for Liver, Digestive and Metabolic Diseases, Academic Hospital Groningen, Groningen, The Netherlands

Correspondence should be addressed to Daniel A Rappolee, C S Mott Center for Human Growth and Development, Wayne State University School of Medicine, 275 East Hancock, Detroit, Michigan 48201, USA; Email: drappole@med.wayne.edu

Abstract

Serine-threonine kinases and transcription factors play important roles in the G1-S phase progression of the cell cycle. Assays that use quantitative fluorescence by immunocytochemical means, or that measure band strength during Western blot analysis, may have confused interpretations if the intention is to measure G1-S phase commitment of a small subpopulation of phosphorylated proteins, when a larger conversion of the same population of proteins can occur during late G2 and M phases. In mouse trophoblast stem cells (TSC), a human placental cell line (HTR), and/or mouse preimplantation embryos, 8/19 serine-threonine and tyrosine kinases, 3/8 transcription factors, and 8/14 phospho substrate and miscellaneous proteins were phosphorylated at higher levels in M phase than in interphase. Most phosphoproteins appeared to associate with the spindle complex during M phase, but one (p38MAPK) associated with the spindle pole and five (Cdx2, MEK1, 2, p27, and RSK1) associated with the DNA. Phosphorylation was detected throughout apparent metaphase, anaphase and telophase for some proteins, or for only one of these segments for others. The phosphorylation was from 2.1- to 6.2-fold higher during M phase compared with interphase. These data suggest that, when planning and interpreting quantitative data and perturbation experiments, consideration must be given to the role of serine-threonine kinases and transcription factors during decision making in M phase as well as in G1-S phase.

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Introduction

About 70% of fertilized human embryos are lost before birth and the majority of these are lost at the time of implantation in the uterus (Cross *et al.* 1994, Rappolee 1999). Since insufficient trophoblast proliferation is one of the causes for loss of embryos, studies on trophoblast proliferation will improve our understanding of the mechanisms underlying trophoblast growth and will help prevent loss of embryos. Cell cycle progression is an important part of cell proliferation. Serine-threonine kinases and transcription factors play important roles in the progression of the cell cycle (Roovers & Assoian 2000, Wilkinson & Millar 2000, Rappolee 2003).

Many investigations on the roles of serine-threonine kinases and transcription factors in mitosis have been carried out in mammalian somatic cells, but little has been done in preimplantation embryos. G1-S phase mitogenic signal transduction is largely mediated by protein kinases; examples include the Raf family, the mitogen activated protein kinase (MAPK) family, the MAPK activating kinase (MEK) family (Roovers & Assoian 2000, Wilkinson & Millar 2000) and transcription factors (e.g. MycC, fos; Sears &

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Nevins 2002). These transcription factors are downstream of receptor tyrosine kinases (RTK) such as the fibroblast growth factor (FGF) receptor family that mediate a necessary mitogenic input into placental trophoblasts in the preimplantation mouse embryo (Chai *et al.* 1998). During this important period of decision-making, only small subpopulations of protein kinases may be phosphorylated and perform downstream functions. However, elevated (putatively quantitative) conversion of populations of protein kinases and transcription factors has been observed during late G2, and throughout M phase in somatic cells (Willard & Crouch 2001). Western blotting was used to determine that some serine-threonine kinases were hyper-phosphorylated at M phase in the first two cell divisions of the mouse embryo, but later preimplantation cell divisions showed no hyper-phosphorylation (Iwamori *et al.* 2000).

The unique role of kinases during M phase is intriguing, but not totally understood. It is likely that (1) there is a change in the substrate range of kinases during M phase, (2) the change must happen quickly due to the short duration of mitosis, and (3) a great number of M phase structural molecules (such as cytoskeletal tubulin) must be phosphorylated. It is likely that nearly 100% of kinases of one type may be converted for about 1 h of mitosis, but less than 10% are converted for a few hours of G1 preceding S phase (Whitmarsh & Davis 1999, 2000, Willard & Crouch 2001). It is therefore important to know about the kinases and transcription factors that are used by the preimplantation embryo for M phase, if correct interpretations about function at the G1-S phase are to be made.

The purpose of this study was to investigate the expression of activated phosphorylated serine-threonine kinases and transcription factors during mitosis in mouse preimplantation embryos and mouse and human trophoblast cells.

Materials and Methods

Reagents

The antibodies used were: 14-3-3 phospho substrate (CS9601), Akt phospho SER473 (CS9271), Akt all forms (SC5298), Akt1 phospho substrate (CS9611), ATF2 phospho THR71 (CS9221), ATF2 all forms (CS9222), ATM phospho ser1981 (CS4526), ATM/ATR phospho substrate (CS2851), CDK2-phospho THR160 (CS2561), Elk-1 phospho SER383 (CS9186), Elk-1 all forms (SC355, CS9182), Ets2 (SC351), FGFR1 phospho TYR766 (SC12935), FGFR1 all forms (SC121), FRS2alpha phospho (CS3861), FRS2α all forms (SC7131), GSK3α-phospho SER21/9 (CS9331), Jun-C phospho SER63 (CS9261, SC7980-R), jun-C all forms (CS9262), MAPK/ERK phospho THR202/TYR204 (CS9101), MAPK-ERK all forms (CS9102), MAPK/ERK phospho THR202/TYR204 (CS9106), MAPK-phospho THR202/TYR204 (CS9106), MAPK-phospho THR183/TYR185 (M8159, Sigma Chemical Co., St Louis, MO, USA), MAPK phospho (UBI 06-64206, Upstate Biotechnology Inc, Lake Placid, NY, USA), MAPK5/ERK5 all forms (SC1284, SC1285), ERK5 phospho Thr215 Tyr220 (CS3371), MEK1 all forms (SC219), MEK1 all forms (SC436), MEK1,2 all forms (CS9122), MEK1,2 phospho SER217/SER221 (CS9121), MEK5 all forms (SC10795), MEK5 all forms (SC9320), MSK-1 phospho SER360 (CS9594), MycB phospho SER68 (SC16303R), myc C all forms rabbit (SC788), MycC phospho THR58/SER62 (CS9401, SC8000R), p27 (SC528), p27KIP1 all forms (MS-256-P0, clone 16P07 Ab1), p27 phospho SER10 (SC12939R), p38MAPK all forms (CS9212), p38MAPK phospho THR180/TYR182 (CS9211), p53 phospho SER15 (SC11764-R), p57 (SC8298), p57KIP2 all forms (MS-1062-P0, Ab6, clone 16P07), 3-phosphoinositide-dependent protein kinase (PDK) phospho SER241 (CS3061), PDK phospho substrate (CS2291, protein kinase (PK) A (PKA) phospho substrate (CS9621), PKC phospho substrate (CS2261), Raf1 all forms (SC7198), retinoblastoma (Rb) protein phospho SER795 (CS9301, SC7986R), ribosomal S6 kinase (RSK) 1 phospho THR573 (RSK1) (CS9346), RSK1 all forms (SC231), RSK1 phospho SER380 (CS9341), 90RSK3 all forms (SC1431), 90RSK2 all forms (SC1430), RSK1 all forms (RSK1) (CS9342), RSK1 phospho THR359/SER363 linker (RSK1) (CS9344), RSK2 all forms (SC1430), RSK3 all forms (SC1431), RSK2,1 phospho SER380 (SC11756), RSK3 phospho (CS9345), stress activated protein kinase (SAPK)/JNK all forms (CS9252), SAPK/JNK phospho THR183/TYR185 (CS9251), SOS1 all forms (SC10803, SC256), STAT1 phospho TYR701 (CS9171), THR phospho (SC9381), TYR phospho (PY350)(SC18182). All antibodies designated CS are from Cell Signaling (Beverly, MA, USA), all antibodies designated SC are from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and all antibodies designated MS are from Lab Vision (Fremont, CA, USA).

Collection of mouse embryos

Standard techniques were used for obtaining mouse embryos (Hogan *et al.* 2002). Female MF-1 mice (4–5 weeks old, Harlan Sprague Dawley, Indianapolis, IN, USA) were injected intraperitoneally with 10 IU pregnant mares' serum gonadotropin (Sigma Chemical Co.), followed by an injection of 7.5 IU human chorionic gonadotropin (Sigma Chemical Co.) 44–48 h later. After the second injection, females were housed overnight with C57BL/6J × SJL/J F1 hybrid males (Jackson Laboratories, Bar Harbor, ME, USA). Noon of the day following coitus was considered day E0.5. For immunocytochemical (ICC) analysis, embryos were obtained at the morula/early cavitation blastocyst (E3.5), or at the 8-cell/compaction (E2.5) stage; for reverse transcriptase-polymerase chain reaction (RT-PCR), embryos were collected at the following stages: unfertilized egg, 2-cell stage (E1.5), 8-cell/compaction stage (E2.5) and morula-early blastocyst (E3.5) stage. The animal use protocols were approved by the Wayne State University Animal Investigation Committee.

Placenta cell culture

Mouse TSC (Tanaka *et al.* 1998) and SV40 large T transformed human trophoblast cells (HTR) (Graham *et al.* 1993) were cultured as described. In a few experiments, TSC were cultured overnight with 0.5 μg/ml of a commercial colchicine analog, Karyomax Colcemid (Gibco/BRL Gaithersburg, MD, USA), which blocked most cells in late G2-prophase, not in metaphase.

Indirect immunocytochemistry and nuclear staining

For immunocytochemical analysis, TSC, HTR and E3.5 mouse embryos were fixed for 30 min in 2% fresh paraformaldehyde (pH 7.4) in phosphate-buffered saline (PBS), quenched with 0.1 M glycine, and permeabilized for 10 min with 0.25% Triton X-100. The embryos were stained with primary antibodies (diluted at 1:100 in PBS-Tween with 10% fetal calf serum). The primary antibody was followed by staining with biotinylated IgG (Vector Labs, Burlingame, CA, USA). Proteins were visualized with fluorescein isothiocyanate (FITC) coupled to streptavidin (Vector Labs). Nuclear counterstaining was carried out with Hoechst 33258 (10 μg/ml). Photomicrography was carried out with a Leica DM IRE2 epifluorescence microscope with a Retiga 1350 Ex cooled charge coupled device controlled electronically by SimplePCI AI module software. Nearest or no neighbor deconvolution was performed using the SimplePCI DNN module. Photographs were analyzed using Photodex CPIC and C-Imaging Simple PCI intensity analysis software (Compix Inc., Imaging Systems, Cranberry Township, PA, USA) and formatted for presentation using Adobe Photoshop 6.0 (San Jose, CA, USA). All fluorescence photos were handled and analyzed in the same way. All experiments were repeated at least twice with similar results.

Statistical analysis

The data in this study are representative of 2–3 independent studies and are given as means ± S.D. Statistical significance of differences between different samples was calculated by Student's *t*-test (SPSS 10.0 and SISA website, Uitenbroek 1997). *P*-values of less than 0.05 were considered significant.

Results

We investigated the high levels of phosphorylation of 19 serine-threonine and tyrosine kinases, 8 transcription factors, and 14 other proteins or substrate groups during mitosis (Fig. 1) in embryos, and in TSC and HTR cells. In general, all threonine substrates are elevated at mitosis (Fig. 2; note that figures including all controls for published Figs 2–8 are provided as supplementary figures to the online version of *Reproduction* (see http://reproduction-online.org/content/vol128/issue5/index.shtml); supplemental Fig. 6), suggesting that serine-threonine kinase plays a role in the regulation of mitosis. In embryos, TSC and HTR it was found that among the individual serine-threonine and tyrosine kinases studied, the following had a high level of phosphorylation during mitosis: ATM (ataxia telangiectasia mutants, note that 5 additional figures not published are available at an on-line database; supplemental Fig. 1), MEK1, 2 and p27 (Fig. 3, supplemental Fig. 7), RSK1, 3 (Figs 4 and 5, supplemental Figs 8 and 9), and p38MAPK (Fig. 6, supplemental Fig. 10) (Table 1). Among the transcription factors, we found a high mitotic phosphorylation of MycB (supplemental Fig. 2), MycC (Fig. 7, supplemental Fig. 11), and Cdx (Fig. 8, supplemental Fig. 12), in embryos, HTR, and TSC (3/8 phosphoprotein transcription factors studied had elevated M phase fluorescence) (Table 2).

The signaling enzymes and transcription factors studied that are (or are not) highly phosphorylated at the M phases are listed in Tables 1 and 2. The duration of their elevation during mitosis is shown in Fig. 9. Mitosis typically lasts for approximately one hour in somatic cells, and only a few of the proteins studied had elevated phosphorylation during all of mitosis. The cell cycles in both HTR and TSC cells are close to 24 h (data not shown) and the preimplantation mouse embryo has a cell cycle at E3.5 of about 12 h (Pedersen 1987, Hogan *et al.* 2002). Confidence in our ability to detect low frequency events such as metaphase, or other segments of the M phase, was based on a survey of over 60 000 micrographs of embryos and placental cell lines. The morphology of mitotic cells was clear in HTR and TSC. In embryos, it was more difficult to detect classic metaphase plates although they were visible

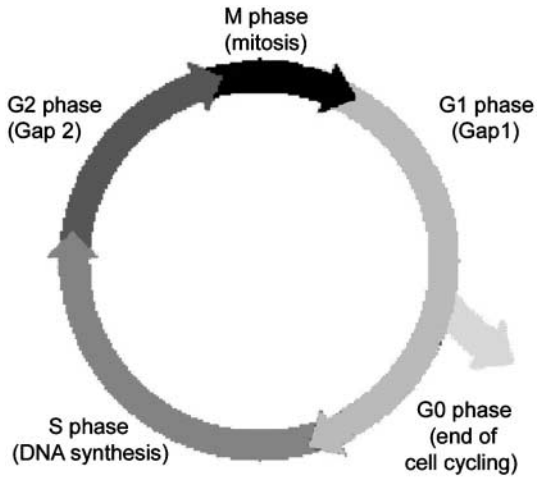

Figure 1 Cell cycle diagram showing the short mitosis phase that averages about 1 h and the longer S phase (DNA synthesis) that averages about 8 h in somatic cells. Other cell cycle phases include G1 (Gap1) when the decision to commit to S phase is made, G2 (Gap2) when DNA repair is made after S phase and before mitosis, M phase (mitosis), when cell and nuclear division occurs, and GO phase, when a cell leaves the cell cycle and requires longer (than G1 phase), or may completely loose the ability, to restart *S* phase.

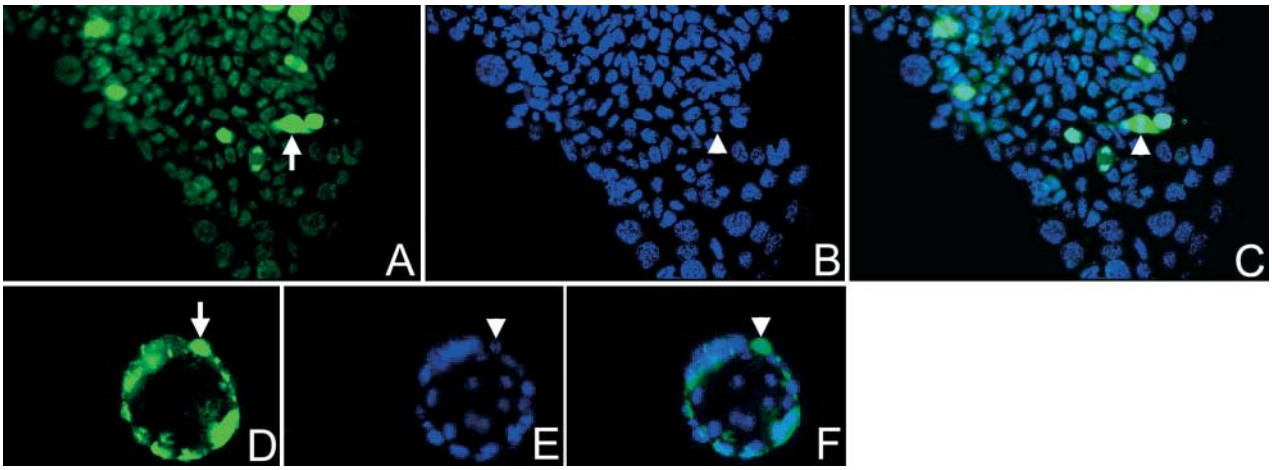

Figure 2 Threonine (Thr) phospho is detected at elevated levels in TSC at mitosis. TSC were cultured, fixed, and developed by immunocytochemical means for Thr phospho. (A, B, C) TSC stained with Thr phospho stain, Hoechst stain, and after merging respectively. TSC in apparent metaphase with elevated expression localized in the apparent mitotic spindle are shown by an arrow in A and arrowheads in B and C. (D, E, F) E3.5 embryos stained with Thr phospho stain, Hoechst stain, and after merging respectively. Arrow (in D) shows position of nucleus and arrowheads (in E, F) show nucleus.

(RSK1, Wang *et al.* 2004). However, the elevation in fluorescence intensity in a fraction of the cells in embryos occurred only for those antibodies that also showed an elevation of fluorescence intensity in obviously mitotic HTR and TSC. In addition, the fraction of nuclei of TSC, HTR, or embryos that had an elevated fluorescence intensity was in the range of 1–4% (data not shown), corresponding to the predicted fraction of unperturbed cells that would be in M phase (or some segment of M-phase) for 1 h or less/24 h cell cycle.

The majority of proteins studied had elevated phosphorylation only at M phase as indicated by obvious morphologic criteria. It is interesting to note that a few proteins with elevated fluorescence had additional non-M phase fluorescence (Table 2; p53 phospho SER15, supplemental Fig. 3) or the fluorescence was elevated during cell cycle phases besides the M phase (Table 2; p57, Ets2, supplemental Figs 4 and 5 respectively). This is in agreement with previous studies that showed that p53 phospho SER15 is highest in cyclic somatic cells in early G1 phase (Buschmann *et al.* 2000), and that p57 is activated during endocycle S phase and during the terminal G1 phase of mitotic trophoblasts (Zhang *et al.* 1998, Hattori *et al.* 2000).

The location of phosphoproteins elevated during mitosis is tabulated in Table 3. The majority of phosphoproteins were detected in the spindle complex, but p38MAPK phospho was detected in the spindle pole, and Cdx2 was localized with the DNA. This suggests, since many of the phosphoproteins are in the MAPK pathway, that MAPK may be involved with the regulation of the embryonic spindle complex, consistent with a role for MEK-MAPK in mitosis in Swiss 3T3 cells (Willard & Crouch 2001). Note that the implied mitotic function of a given phosphoprotein may change quickly over developmental time, as we could find no elevated MAPK phospho during mitosis in preimplantation embryos using four anti-MAPK phospho antibodies, although this has been reported in post-implantation embryos (Corson *et al.* 2003). Although the great majority of this study was undertaken in reviewing large numbers of micrographs of unperturbed cultured placental cells and embryos, we did perform one study with a commercial colcemid analog, Karyomax. We found that, as in some other cell types (manufacturer's notes), overnight incubation of TSC with Karyomax elevated the fraction of cells with condensed, smaller, circular nuclei that were putative late G2/prophase. Normally, MEK1, 2 phospho was elevated and detected in cells in metaphase or anaphase where it appeared to be in the spindle complex, or in a very small fraction of cells with condensed nuclei where phosphoprotein was detected as spots on the DNA (Fig. 3A, B, C, supplemental Fig. 7). The Karyomax protocol also elevated the fraction of cells with elevated fluorescence for MEK1, 2 phospho (Fig. 3G, H, I, supplemental Fig. 7) and p27 phospho (Fig. 3J, K, L, supplemental Fig. 7). Interestingly, this treatment also created similar 1-, 2-, and 4-spotted circular nuclei that were correlated with TSC sheet dispersion and nuclear size increase, suggesting that the differentiation state of trophoblast cells might correlate with MEK1,2 and p27 expression around M phase. This brief study produced the desired result of increasing the fraction of cells with elevated phosphoprotein expression at or around M phase. However, the lack of metaphase block in this cell type, and the inability to associate localization of nuclear spots with function in unperturbed cells, led us to discontinue this avenue of M phase testing. It is interesting to note that (1) possible co-localization of p27 and MEK1,2 might suggest an enzyme substrate interaction, and (2) at a very low frequency MEK1,2 nuclear spots were seen in unperturbed TSC. Point number (2) suggests that the localization in the DNA may not be an artifact of tubulin

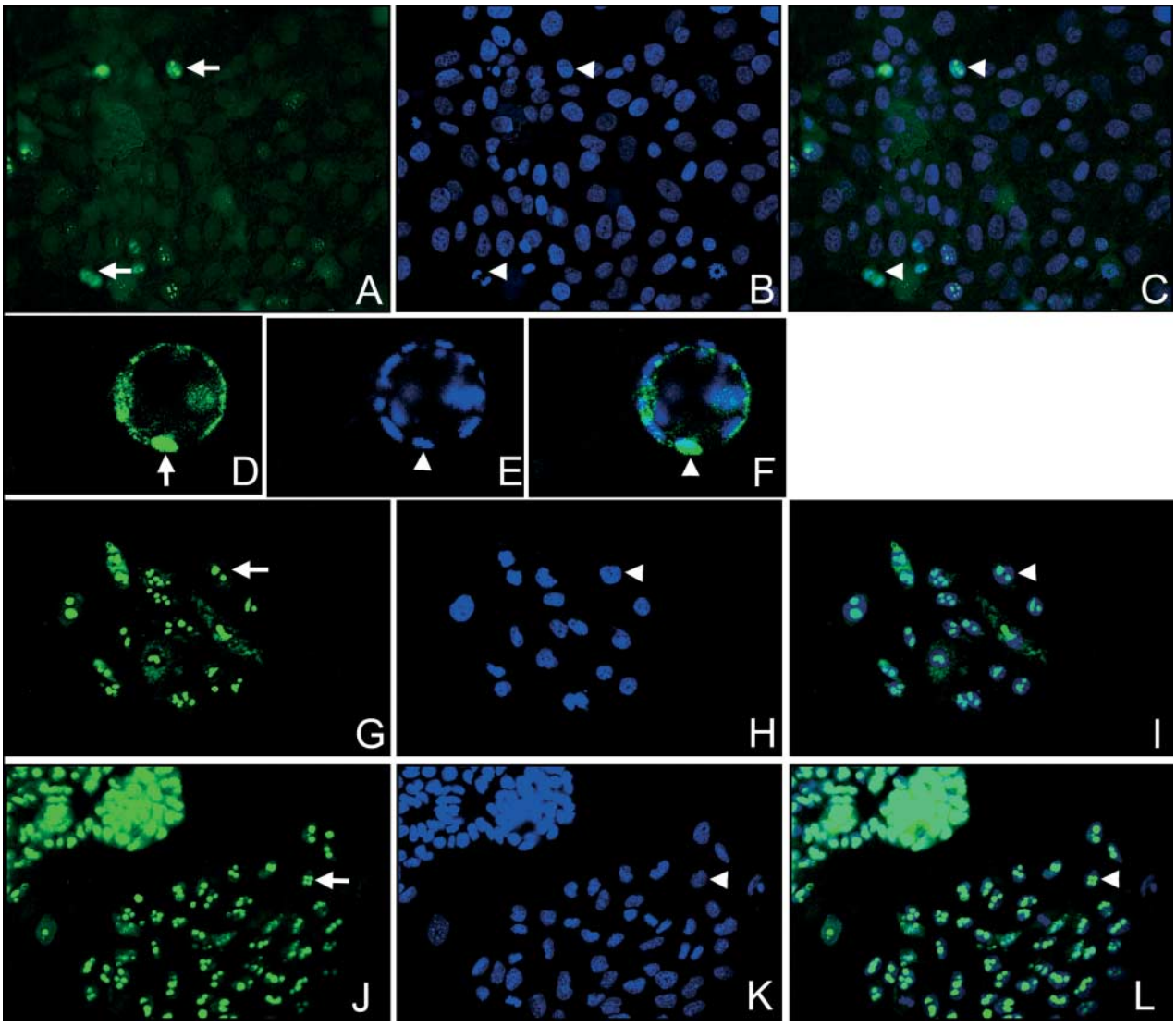

Figure 3 MEK1, 2 phospho and p27 phospho proteins are detected at elevated levels in TSC and E3.5 embryos at mitosis. MEK1, 2 phospho proteins are detected at elevated levels in TSC and E3.5 embryos at apparent prophase and anaphase mitosis. TSC were cultured, fixed, and developed by immunocytochemical means for MEK1, 2 phospho. (A, B, C) TSC stained with MEK1, 2 phospho stain, Hoechst stain, and after merging respectively. TSC in apparent anaphase with elevated expression localized in the mitotic spindle are shown by the bottom arrow in A while the top arrow shows a putative prophase cell MEK1, 2 phospho spots on the DNA. Corresponding arrowheads show nuclear position in B and C. (D, E, F) E3.5 embryos stained with MEK1, 2 phospho stain, Hoechst stain, and after merging respectively. (G, H, I) Uniform nuclear spotting of MEK1, 2 phospho in Karyomax-treated TSC corresponding to Hoechst stain, and after merging respectively. (J, K, L) Karyomax-treated TSC stained with p27 phospho stain, Hoechst stain, and after merging respectively. Arrows and arrowheads in D–L show position of nuclei.

depolymerization caused by Karyomax, but may reflect a low frequency event in unperturbed cells. This suggests that the proper colchicine derivative might be used in studies of M phase function of phosphoproteins in TSC and embryos.

To quantitate the elevation in fluorescence intensity at M phase, we performed a comparison of the levels of intensity of mitotic cells compared with interphase cells (Table 4). All phosphoproteins had a higher intensity at M phase than in interphase cells, in the range of 1.5–5.6 (mean = 2.8) for ratios of raw quantitated immunofluorescence to 2.0–6.2 (mean = 3.8) for the same ratios after a no-antibody background was subtracted from both the means for the M phase (numerator) and interphase (denominator). In baseline studies for each antibody, it was shown that the fluorescence for each antibody was hgiher than with no-antibody, non-immune serum, or for antibody with co-addition of excess immunizing antigen (data not shown). One weakness of the study is that the pictures were taken by different individuals and they had a tendency to shoot micrographs on the high or low intensity range of the 0–255 units/pixel intensity range of the Simple PCI software. The micrographs were taken to emphasize the differences between interphase and M-phase fluorescence. In baseline studies for each antibody, it was shown that the fluorescence for each antibody was

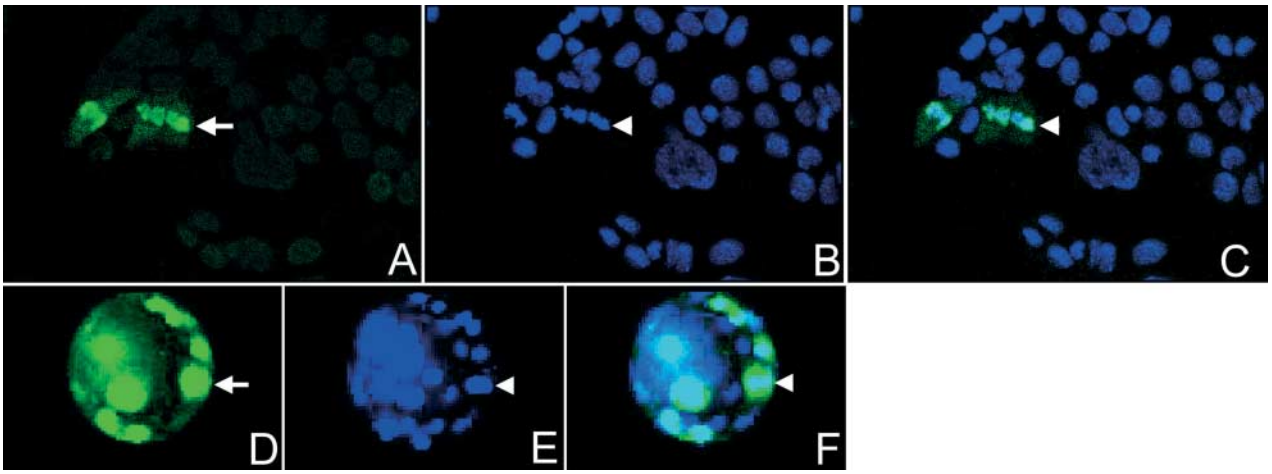

Figure 4 RSK1 phospho is detected at elevated levels in TSC and E3.5 embryos at mitosis. TSC were cultured and E3.5 embryos were isolated and fixed and developed by immunocytochemical means for RSK1 phospho. (A, B, C) TSC in apparent anaphase with elevated expression localized in the mitotic spindle. (D, E, F) Elevated expression of RSK1 phospho is detected in E3.5 embryos in mitotic cells, with condensed chromosomes and in apparent anaphase. Arrowheads (B, C, E, F) show nuclei and arrows (A, D) show position of nucleus.

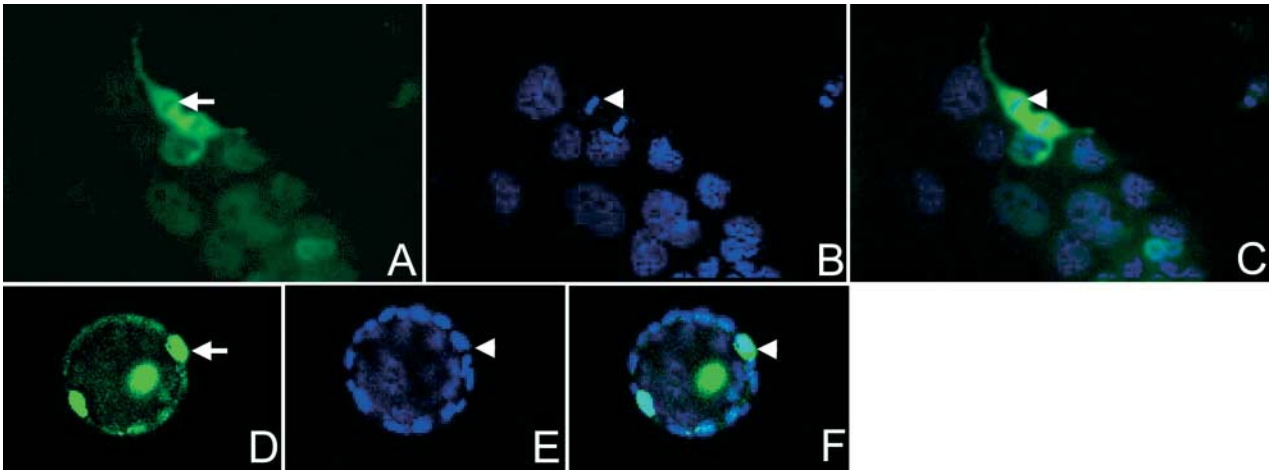

Figure 5 RSK3 phospho is detected at elevated levels in TSC and E3.5 embryos at mitosis. TSC were cultured and E3.5 embryos were isolated and fixed and developed by immunocytochemical means for RSK3 phospho. (A, B, C) TSC stained by RSK3 phospho stain, Hoechst stain, and after merging respectively, showing TSC in telephase with elevated expression colocalized with the reforming cytoplasm. (D, E, F) E3.5 embryos stained with RSK3 phospho stain, Hoechst stain, and after merging respectively, showing elevated expression in mitotic cells, with condensed chromosomes, Arrows in A and D show positions of nuclei, and arrowheads in B, C, E, F show nuclei.

higher than with no-antibody, non-immune serum, or for antibody with co-addition of excess immunizing antigen (data not shown). The high end of the fluorescence range is not linear due to pixel saturation and so the ratios may be an underestimate of the intensity of M-phase fluorescence and the corresponding fractions of protein populations that are phosphorylated.

Discussion

A little under half of serine-threonine kinases and transcription factors have a high level of phosphorylation at M phase. Although this is a small sample size, it seems likely that these fractions may extend to yet-to-be tested transcription factors and kinases.

Mitosis requires a large reorganization of the cellular cytoskeleton. Microtubules are reorganized to form a bipolar spindle; the chromosomes become condensed, attach to the spindles at their kinetochores and are segregated into two daughter cells. Reorganization of the mitotic cytoskeleton requires a large, rapid surge of serine-threonine protein phosphorylation, controlling signaling events that coordinate mitotic processes. Our data (Fig. 2) are consistent with the increase in all threonine phosphorylation substrates at M phase. An important trigger of the phosphorylation surge is the cyclin-dependent kinase (CDK), Cdc2. After CDK is activated, mitotic

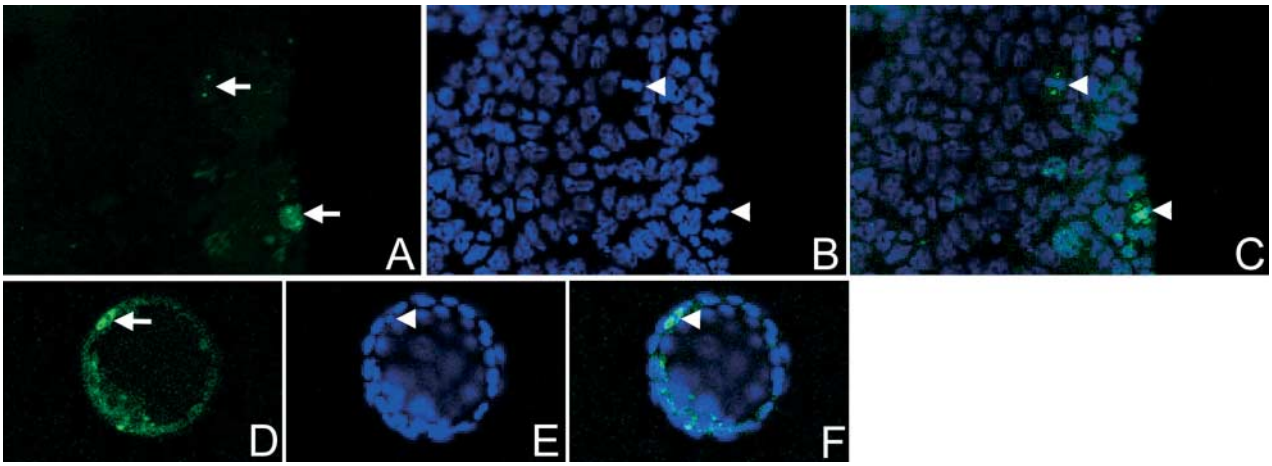

Figure 6 p38MAPK phospho proteins are detected at elevated levels in TSC and E3.5 embryos at mitosis. (A, B, C) TSC in metaphase with elevated expression of p38MAPK phospho in spindle poles. (D, E, F) Elevated expression of p38MAPK phospho is detected in E3.5 embryos in a mitotic cell, with condensed chromosomes. Arrowheads (B, C, E, F) show nuclei and arrows (A, D) show the position of the nuclei.

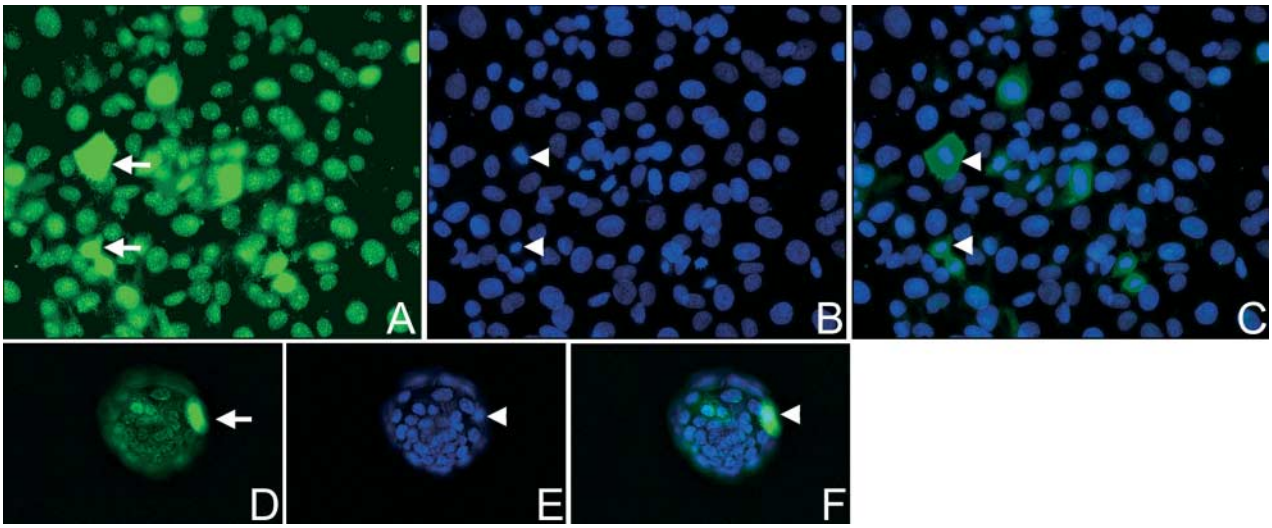

Figure 7 MycC phospho proteins are detected at elevated levels in TSC and E3.5 embryos at mitosis. (A, B, C) TSC in metaphase, anaphase, and telophase with elevated expression of MycC phospho localized in the mitotic spinctle. (D, E, F) Elevated expression of MycC phospho is detected in E3.5 embryos in mitotic cells, with condensed chromosomes. Arrowheads (B, C, E, F) show nuclei and arrows (A, D) show position of the nuclei.

serine/threonine kinases in three families - the polo kinases, aurora kinases and the NIMA-related kinases (Nrk) - govern mitosis (Piwnica-Worms 1996, O'Connell *et al.* 2003). These mitosis-specific enzymes may also control enzymes recruited from other pathways that function during interphase. For example, phosphatidylinositol-3 kinase (PI3Kinase), 3-phosphoinositide-dependent protein kinase (PDK), Akt1 (Dangi *et al.* 2003), CDK2 (Doree & Galas 1994), MycC (Niklinski *et al.* 2000), Raf1, MEK1,2, MAPK1,2 and RSK1, 2, 3 (Willard & Crouch 2001) are involved with M phase in somatic cells, and in G1-S phase signaling (Sears *et al.* 2000, Wilkinson & Millar 2000, Rappolee 2003). The entire MAPK pathway is detected in preimplantation mouse embryos (Wang *et al.* 2004, Xie *et al.* 2004). Little work has been done on the interaction of the MAPK pathway with the three mitosis-specific enzyme families during mitosis. But, during meiosis, MAPK can be upstream of polo-like kinase in starfish oocytes (Okano-Uchida *et al.* 2003), MAPK can regulate NIMA-related kinases in mouse spermatogenesis (Di Agostino *et al.* 2002), but MAPK pathway is apparently independent of aurora kinases in frog oocytes (Maton *et al.* 2003). This is the first report of high levels of expression for these MAPK pathway phosphoproteins in late preimplantation embryos and placental cells at M phase. These data are consistent with a putative M phase function of MAPK pathway enzymes in early placental lineage cells.

It is important to keep these findings in mind when planning experiments and interpreting data regarding

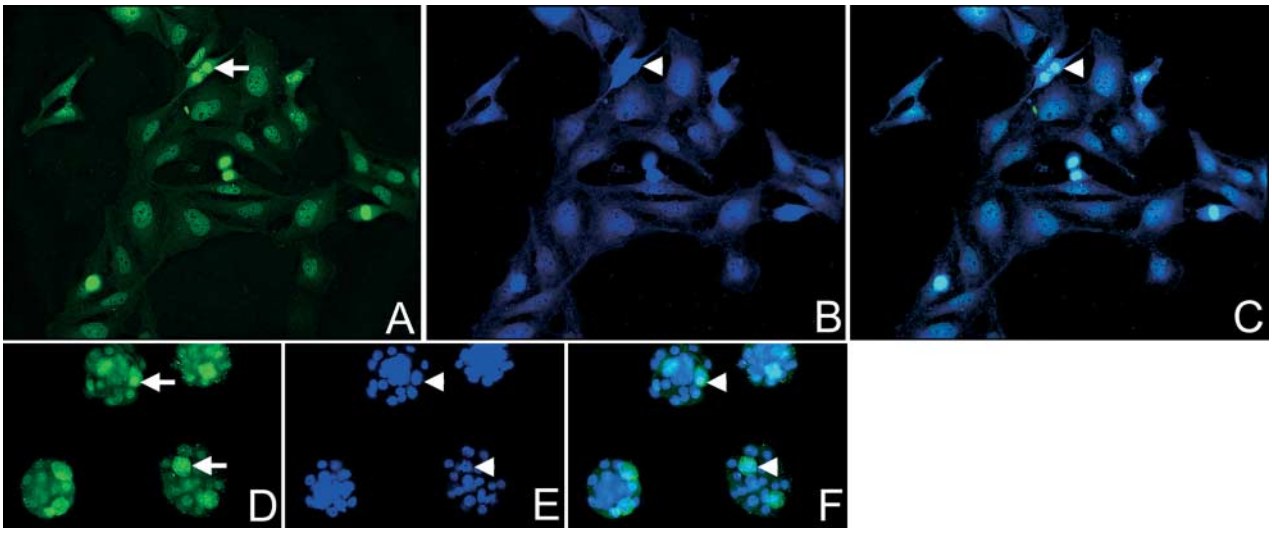

Figure 8 Cdx phospho proteins are detected at elevated levels in HTR and E3.5 embryos at mitosis. (A, B, C) HTR in metaphase and anaphase with elevated expression of Cdx phospho localized with DNA. (D, E. F) Elevated expression of Cdx phospho is detected in E3.5 embryos in mitotic cells, with condensed chromosomes. Note that these embryos are not at the same scale as in Figs 2–7. The arrow on top of A shows a cell at anaphase, the arrow at the botton of A shows a cell at metaphase. The arrows at D show positions of nuclei, arrowheads in B, C, E, F show nuclei.

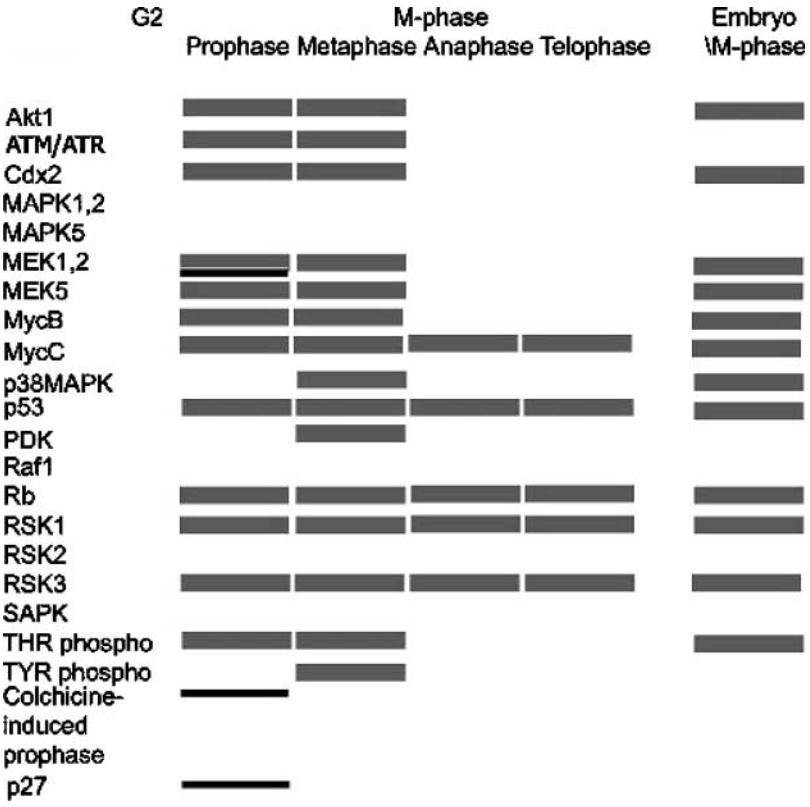

Figure 9 Summary of temporal expression of threonine-serine kinases and transcription factors that are phosphorylated at high levels during mitosis. Large grey boxes represent three M-phase segments when fluorescence intensity is elevated in normal cells, and smaller black rectangles show additional effects in colchicine/Karyomax-treated cells.

G1-S phase decision making in the early embryo. If measuring induction of interphase responses to growth factors by kinases or transcription factors using quantitative immunofluorescence, elevated fluorescence in mitotic cells ('mitotic hotspots') should be avoided. Alternatively, attempts can be made to synchronize cell divisions in order to avoid M phase. Similarly, if loss-of-function perturbations are performed, investigators should be alert for M phase phenotypes. An additional consideration is that alternative functions of the enzyme or transcription factor besides those controlling G1-S phase progression may also occur during G1-S phase.

The mitotic function of kinases changes during development. It is suggested, for example, that MEK1, 2 function is required for M phase completion as the MEK1, 2, 5 inhibitor, U0126, can block mitosis (Willard & Crouch

Table 1 Signaling enzymes that have elevated phosphorylation during mitosis: intermediary serine- threonine kinases signal transduction proteins.

Protein	Antibody*	Cell type	M phase**
Akt1 phospho	SC1618*	HTR, TSC, embryo	+
Akt phospho	SC5298	TSC	+
ATM phospho	CS4526	HTR, embryo	+
MEK1,2 all forms	CS9122*	TSC, HTR, embryo	+
MEK1,2 phospho	CS9121*	TSC, HTR, embryo	+
MEK5 all forms	SC10795*	TSC, HTR, embryo	+
PDK phospho	CS3061*	HTR, embryo	+
RSK1 phospho	CS9344*	TSC, HTR, embryo	+
RSK3 phospho	CS9345*	TSC, HTR, embryo	+
p38MAPK all forms	CS9212*	TSC, HTR, embryo	+
p38MAPK phospho	CS9211*	TSC, HTR, embryo	+
CDK2 phospho	CS2561*	HTR, embryo	−
FGFR1 phospho	SC12935	TSC, HTR, embryo	−
GSK3alpha phospho	CS9331	HTR, embryo	−
MAPK1,2 phospho	CS9101*	TSC, HTR, embryo	−
MAPK1,2 phospho	M8159*	TSC, HTR, embryo	−
MAPK1,2 phospho	CS9106*	TSC, HTR, embryo	−
MAPK1,2 phospho	UBI 06-624*	TSC, HTR, embryo	−
ERK5, phospho	CS3371*	HTR, embryo	−
Raf1 phospho	CS9421*	TSC, HTR, embryo	−
Raf1 phospho	SC7198*	TSC, HTR, embryo	−
Raf1,2 phospho	SC12358	HTR, embryo	−
SAPK phospho	CS9251*	TSC, HTR, embryo	−
SAPK all forms	CS9251*	TSC, HTR, embryo	−

* Western blots showed a single or major band of the correct size from lysates of TSC or HTR (Wang *et al.* 2004, Xie *et al.* 2004) (not shown).
** + Signifies that elevated fluorescence intensity in apparent mitotic cells was detected, and − signifies that elevated fluorescence intensity in apparent mitotic cells was never detected.

2001). Therefore, MEK seems to be a dominant G1-S phase kinase used at M phase, but MAPK seems not to be as important in somatic cells (Harding *et al.* 2003) or in mouse embryos during M phase of the first two cleavage divisions (Iwamori *et al.* 2000). Our data are in agreement with these reports. Like the 1- to 4-cell stage embryo (Iwamori *et al.* 2000), MEK1, 2 participates in M phase later in preimplantation development as shown by immunocytochemical means. In contrast to the 1- to 4-cell stage embryos, Raf1 is not highly phosphorylated during M phase in later stage preimplantation embryos or in TSC and HTR. Dissimilar to our data and that of Iwamori and colleagues, MAPK phospho is elevated in post-implantation embryos at M phase (Corson *et al.* 2003). It seems that the embryo may use different sets of kinases at M phase during different phases of development. Therefore, an additional caution may be that the data presented here may apply to preimplantation development, but each researcher may have to re-examine the expression of these kinases and transcription factors during each period of embryonic development studied.

This study was based upon a large scan of proteins and phosphoproteins using 153 antibodies and 49 phospho-specific antibodies in 'unperturbed' embryos *ex vivo*, and placental TSC and HTR cultured under normal proliferation-promoting conditions. Approximately, 60 000 fluorescence micrographs were recorded, allowing low frequency, short-duration events such as metaphase or anaphase a good opportunity to be observed. Since the preimplantation embryo is translucent and small (having less than 100 cells through E3.5), it lends itself to immunocytochemistry more than to Western blot analysis. In this study, we focused on a mini-proteomics approach where immunocytochemistry was performed in embryos and cell lines. In other studies (Wang *et al.* 2004, Xie *et al.* 2004, data not shown), Western blots were analyzed for TSC and HTR, cell lines that represent about 75% of cells in the E3.5 embryo, and the antibodies tested in TSC or embryos are indicated in Tables 1 and 2. A weakness of this study is that Western blots were not performed in the embryos themselves. Such a study would be very prohibitive in cost. A strength of this study is that 29 of the proteins reported here had at least two antibodies that yielded similar results, and that all phosphoproteins, except p53, had similar frequencies of cells with elevated phosphoprotein fluorescence in TSC/HTR as in embryos. In the case of p53, elevated p53 occurred during obvious M phase and also during other apparent cell cycle phases. This made these results difficult to interpret as HTR is transgenic for SV40 large T antigens and this transforming may affect p53 stability and the dynamics of phosphorylation (Graham *et al.* 1993, Meek 2002). In addition, TSC may partially represent a period of placental lineage development slightly after preimplantation development. All the antibodies with no elevated phosphoprotein fluorescence in TSC/HTR also had no elevated phosphoprotein fluorescence in embryos. The congruence of negative and positive findings for elevation of phosphoproteins at M phase was complete for TSC/HTR compared with embryos except for one phosphoprotein.

Table 2 Transcription factors and other proteins that have elevated phosphorylation during mitosis.

Protein	Antibody	Cell type	M phase
Transcription factors			
ATF2 phospho	CS9221	HTR, TSC, embryo	−
Elk1 phospho	CS9186	TSC, embryo	−
JunC phospho	CS9261*	HTR, TSC, embryo	−
STAT1 phospho	CS9171	TSC, embryo	−
Ets2 all forms	SC351*	TSC, HTR,	−(only putative S phase)*
Cdx2 phospho	Rings *et al.*[a]	HTR, embryo	+
MycB phospho	SC16303*	TSC, HTR, embryo	+
MycC phospho	SC8000*	TSC, HTR, embryo	+
Phospho-protein substrates (sub) and others			
TYR phospho sub	SC18182	TSC, embryo	+
THR phospho sub	SC9381	TSC, embryo	+
Akt1 phospho sub	CS9611	HTR, embryo	+
ATM phospho sub	CS2851	HTR	+
PKA phospho sub	CS9621	HTR	+
Rb phospho	CS9301*	TSC, HTR, embryo	+
Rb phospho	SC7986R*	TSC, HTR, embryo	+
PDK phospho sub	CS2291	HTR	+
p53 phospho	SC11764*	HTR	+(+putative G1 phase)***
p57 all forms	SC8298	TSC, HTR	−(only S phase)**
p57KIP2	MS-1062-P0	TSC, HTR	− (only S phase)**
p21 phospho SER146	SC12902R	HTR, embryo	−
p21WAF1	MS-387-P0	HTR, embryo	−
p21WAF1	MS-387-P0	HTR, embryo	−
p27 phospho	SC12939R	HTR, embryo	−
p27KIP1	MS-256-P0	HTR, embryo	−
Estrogen R-α phospho	CS2514	HTR, embryo	−
Estrogen R-α phospho	CS2515	HTR, embryo	−
PKC phospho sub	CS2261	HTR, embryo	−
14-3-3 sub	CS9601	HTR, embryo	−
FRS2alpha phospho	CS3861*	HTR, embryo	−

* Western blots showed a single or major band of the correct size from lysates of TSC or HTR (not shown).
** Similar distribution in fraction of cells and morphogenetic identity of nuclei as observed previously for p57 as cells leave cytokinetic proliferation and enter endoreduplication in late G1 (Zhang *et al.* 1998, Hattori *et al.* 2000).
*** Similar distribution in fraction of cells and morphogenetic identity of nuclei as suggested for p53 phospho SER15 for largest activation during G1 phase (Buschmann *et al.* 2000).
[a] (Rings *et al.* 2001).

Table 3 Cellular location of phosphorylated threonine-serine kinases and transcription factors at mitosis.

Protein	Location at M phase
Serine-threonine protein kinases	
Akt1 phospho	Mitotic spindle colocalization
MEK1, 2 all forms	Mitotic spindle colocalization at metaphase, 2 spots on DNA with colchicine at putative prophase*
MEK1, 2 phospho	Mitotic spindle colocalization at metaphase, 2 spots on DNA with colchicine at putative prophase*
MEK5 all forms	Mitotic spindle colocalization
RSK1 phospho	Mitotic spindle colocalization, DNA
RSK3 phospho	Mitotic spindle colocalization
p38MAPK all forms	Mitotic spindle, pole
p38MAPK phospho	Mitotic spindle, pole
Transcription factors	
Cdx2 phospho	Condensed DNA colocalization
MycB phospho	Mitotic spindle colocalization
MycC phospho	Mitotic spindle colocalization
Other proteins	
TYR phospho sub	Mitotic spindle
THR phospho sub	Mitotic spindle, pole
ATM phospho sub	Mitotic spindle
Akt1 phospho sub	Mitotic spindle
p27	1–4 spots on DNA with colchicine; similar spots at very low frequency in unperturbed cells*
Rb phospho	Mitotic spindle

* Note that these double spots also occur at very low frequencies in normal TSC, untreated by colchicine/Karyomax. This suggests that the spotting is not an artifact of Karyomax, but a trapping of a low frequency event whose frequency is increased by Karyomax.
sub, substrate.

Table 4 Increase in phosphorylated threonine-serine kinases and transcription factors at mitosis.

Gene product (n = M-phase, n = interphase)	**M-phase intensity** mean ± S.D. (no antibody)	**Interphase intensity** mean ± S.D. (no antibody)	***P* value**	**Stimulation Index (SI)/SI -no antibody**
Akt phospho BWM (4, 12)	143.8 ± 12.2 (120.4)	77.4 ± 10.1 (44)	0.0001	1.9/2.7
Akt phospho TSC (6, 12)	170.3 ± 15.3 (141.5)	79.6 ± 16.6 (50.8)	<0.0001	2.1/2.8
Akt substrate HTR (6, 12)	173.5 ± 23.3 (138.1)	86 ± 9.2 (50.6)	0.0001	2.0/2.7
ATM/ATR subs. HTR (6, 12)	186.1 ± 26.4 (150.7)	82.7 ± 7.0 (47.3)	0.0001	2.3/3.2
Cdx2 phospho BWM (4, 12)	156.3 ± 12.8 (126.4)	83.1 ± 7.7 (53.2)	0.006	1.9/2.4
Cdx2 phospho HTR (12, 12)	212.5 ± 26.4 (182.5)	94.1 ± 6.4 (64)	<0.0001	2.3/2.9
Ets2 (TSC) (4, 12)	180.7 ± 40.8	32.3 ± 9.6	0.006	5.6
Ets2 (HTR) (4, 12)	169.9 ± 21.1	42.3 ± 5.9	0.001	4.0
MEK1, 2 (BWM) (4, 12)	60.2 ± 11.1 (45.1)	37.4 ± 8.2 (22.3)	0.02	1.6/2.0
MEK1, 2 (HTR) (8, 12)	238.0 ± 39.5 (211.3)	65.2 ± 12.9 (38.5)	<0.0001	3.7/5.5
MycB TSC (6, 12)	208.1 ± 17.9 (190.6)	70.5 ± 12.1 (53)	<0.0001	3.0/3.6
MycB HTR (6, 12)	200 ± 16.6 (165.9)	81.3 ± 8.8 (47.2)	<0.0001	2.5/3.5
MycB BWM (6, 12)	101.6 ± 20.3 (68.1)	67.7 ± 15.2 (32.2)	0.007	1.5/2.1
MycC TSC (8, 12)	205.2 ± 24.9 (184.4)	67.6 ± 10.0 (46.8)	<0.0001	3.0/3.9
MycC HTR (12, 12)	249 ± 13.2 (213.6)	113.7 ± 21.6 (78.3)	<0.0001	2.2/2.7
MycC BWM (10, 12)	136.7 ± 15.3 (125.6)	40.8 ± 7.4 (31.7)	<0.0001	3.4/4.0
p38MAPK TSC (5, 12)	74.3 ± 9.0 (58.8)	44.0 ± 9.9 (28.5)	0.0003	1.7/2.1
p38MAPK BWM (4, 12)	108.3 ± 24.1 (92.4)	47.1 ± 6.2 (31.2)	0.02	2.3/3.0
p53 phospho TSC (12, 12)	199.4 ± 23.8 (163.4)	94.8 ± 10.2 (58.8)	<0.0001	2.1/2.8
p53 phospho HTR (12, 12)	215.6 ± 19.5 (194.2)	66.9 ± 5.4 (45.4)	<0.0001	3.2/4.3
p57 all forms TSC (6, 12)	221.4 ± 44.7 (197.1)	56.2 ± 3.9 (31.9)	<0.0001	3.9/6.2
p57 all forms HTR (6, 12)	154.4 ± 32.3 (143.8)	55.1 ± 9.4 (44.5)	<0.0001	2.8/3.2
PDK phospho TSC (6, 12)	151.7 ± 31.2 (126.2)	60 ± 19.7 (34.5)	0.003	2.5/3.7
PDK substrate HTR (4, 12)	125.3 ± 20.5 (89.9)	63.1 ± 6.3 (27.7)	0.01	2.0/3.2
PKA phospho HTR (4, 12)	140.2 ± 33.5 (116.1)	56.1 ± 8.4 (32)	0.02	2.5/3.6
Rb phospho CS9301TSC (6, 12)	211.2 ± 18.2 (192.4)	56.0 ± 6.3 (37.2)	<0.0001	3.8/5.2
Rb phospho SC7986 TSC (6, 12)	165.0 ± 14.6 (143.0)	46.2 ± 3.5 (24.2)	<0.0001	3.6/5.9
Rb phospho SC7986 HTR (6, 12)	194.7 ± 25.6 (162.7)	60.5 ± 4.7 (28.5)	0.0001	3.2/5.7
RSK1 phospho TSC (12, 12)	225.1 ± 17.8 (201.7)	58.0 ± 3.6 (34.6)	<0.0001	3.9/5.8
RSK1 phospho BWM (10, 12)	239.8 ± 25.0 (217.1)	69.9 ± 15.1 (42.2)	<0.0001	3.4/5.1
RSK3 phospho TSC (12, 12)	154.3 ± 13.1 (123.2)	72.0 ± 10.3 (40.9)	<0.0001	2.1/3.0
RSK3 phospho HTR (12, 12)	195.7 ± 22.5 (161.3)	61.6 ± 7.2 (26.2)	<0.0001	3.2/6.2
RSK3 phospho BWM (12, 12)	173.6 ± 21.1 (150.1)	55.9 ± 4.4 (32.4)	<0.0001	3.1/4.6
THR phospho BWM (10, 12)	218.8 ± 12.2 (188.1)	75.2 ± 10.7 (44.5)	<0.0001	2.9/4.2
THR phospho TSC (12, 12)	212.1 ± 20.7 (194.3)	74.1 ± 5.8 (56.3)	<0.0001	2.9/3.5

BWM = blastocyst wholemount, HTR = human trophoblast cells, TSC = mouse trophoblast stem cells, subs = substrate.

Several proteins had a unique M phase localization. Cdx2 is, like aurora-B kinase, a cell cycle M-phase passenger (Higuchi & Uhlmann 2003) that moves from centromeres to the spindle midzone during mitosis, functioning throughout mitosis in chromosome condensation and segregation (Adams *et al.* 2001).

p38MAPK was detected in the spindle poles. p38MAPK is detected throughout preimplantation mouse development (Zhong *et al.* 2004), and mediates mitosis during early *Xenopus* cleavage divisions and is located in the spindle pole (Takenaka *et al.* 1998). The majority of phosphoproteins were observed in the putative spindle complex.

In summary, this study shows that a little under half of the signaling enzymes and transcription factors studied had elevated levels of phosphorylation at M phase in preimplantation mouse embryos and placental cell lines, suggesting that caution must be used in interpreting expression studies and cause-and-effect experiments aimed at testing the role of these phosphoproteins during G1 to S phase decision making. The results also suggest that the studies primarily aimed at testing the role of these phosphoproteins in early embryo/placental M phase function would also be of interest.

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