Identification of Candidate Regulators of Embryonic Stem Cell Differentiation by Comparative Phosphoprotein Affinity Profiling*§

Lawrence G. Puente‡§¶, Douglas J. Borris§, Jean-François Carrière§‖, John F. Kelly**; and Lynn A. Megeney‡§‡‡§§

Embryonic stem cells are a unique cell population capable both of self-renewal and of differentiation into all tissues in the adult organism. Despite the central importance of these cells, little information is available regarding the intracellular signaling pathways that govern self-renewal or early steps in the differentiation program. Embryonic stem cell growth and differentiation correlates with kinase activities, but with the exception of the JAK/STAT3 pathway, the relevant substrates are unknown. To identify candidate phosphoproteins with potential relevance to embryonic stem cell differentiation, a systems biology approach was used. Proteins were purified using phosphoprotein affinity columns, then separated by two-dimensional gel electrophoresis, and detected by silver stain before being identified by tandem mass spectrometry. By comparing preparations from undifferentiated and differentiating mouse embryonic stem cells, a set of proteins was identified that exhibited altered post-translational modifications that correlated with differentiation state. Evidence for altered post-translational modification included altered gel mobility, altered recovery after affinity purification, and direct mass spectra evidence. Affymetrix microarray analysis indicated that gene expression levels of these same proteins had minimal variability over the same differentiation period. Bioinformatic annotations indicated that this set of proteins is enriched with chromatin remodeling, catabolic, and chaperone functions. This set of candidate phosphoproteins with potential relevance to embryonic stem cell differentiation includes products of genes previously noted to be enriched in embryonic stem cells at the mRNA expression level as well as proteins not associated previously with stem cell differentiation status.

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* From the ‡Ottawa Health Research Institute, Molecular Medicine Program and the §Ontario Genomics Innovation Centre, Ottawa Hospital, 501 Smyth Road, Ottawa, Ontario K1H 8L6, Canada, the **Institute for Biological Sciences, National Research Council, Ottawa, Ontario K1A 0R6, Canada, and the ††Department of Cellular and Molecular Medicine and Centre for Neuromuscular Disease, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada. Received, June 1, 2005, and in revised form, September 21, 2005. Published, MCP Papers in Press, September 26, 2005, DOI 10.1074/mcp.M500166-MCP200

1 The abbreviations used are: ESC, embryonic stem cell; 2D, two-dimensional; 2DGE, two-dimensional gel electrophoresis; ES, embryonic stem; EB, embryoid body; LIF, leukemia-inhibitory factor; TRIM28, tripartite motif protein 28; TEBP, telomerase-binding protein; HRE, hormone response element; cPGES, cytosolic prostaglandin E synthase.
Evidence of Protein Phosphorylation—Phosphopeptide enrichment and comparative two-dimensional gel electrophoresis were performed on ESC and EB samples before identification of proteins by MS/MS. Cell lysates were passed over phosphopeptide affinity columns, and eluting proteins were detected by 2DGE and silver stain (Fig. 1). As expected for phosphopeptides (21), most spots were present at low isoelectric points in the pH 4–6 range (Fig. 1). Including both ESC and EB samples, a total of 1367 protein spots were detected over 10 gels. Within each pair of gels, the majority of spots (~80%) were present at equal apparent abundance and gel mobility when ESC and EB were compared. In total 283 spots exhibited obvious changes in intensity (estimated change of at least 30%) or mobility when pairs of ESC and EB samples were compared. From the gels, 362 spots were excised, giving preference to spots that appeared to be differentially expressed between ESC and EB. 332 identifications were made (Table I) that represented 108 different proteins (many proteins were independently identified multiple times). 30 proteins gave rise to multiple mobility species, and a total of 183 protein species were observed (Table I).

RESULTS

Phosphoprotein Enrichment Profiling—To profile the phosphoproteome of ESCs and to identify proteins with potential relevance to ESC differentiation, phosphoprotein enrichment and comparative two-dimensional gel electrophoresis were performed on ESC and EB samples before identification of proteins by MS/MS. Cell lysates were passed over phosphopeptide affinity columns, and eluting proteins were detected by 2DGE and silver stain (Fig. 1). As expected for phosphopeptides (21), most spots were present at low isoelectric points in the pH 4–6 range (Fig. 1). Including both ESC and EB samples, a total of 1367 protein spots were detected over 10 gels. Within each pair of gels, the majority of spots (~80%) were present at equal apparent abundance and gel mobility when ESC and EB were compared. In total 283 spots exhibited obvious changes in intensity (estimated change of at least 30%) or mobility when pairs of ESC and EB samples were compared. From the gels, 362 spots were excised, giving preference to spots that appeared to be differentially expressed between ESC and EB. 332 identifications were made (Table I) that represented 108 different proteins (many proteins were independently identified multiple times). 30 proteins gave rise to multiple mobility species, and a total of 183 protein species were observed (Table I).

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Identifying Candidate Differentiation-associated Proteins—A key goal of our study was to identify candidate regulators of stem cell differentiation by identifying proteins whose phosphorylation status is altered in a manner that correlates with ESC differentiation status. Following phosphoprotein affinity column treatment and 2DGE, protein identities and silver stain patterns were carefully correlated across the gels. For each identified spot on each pair of gels, silver staining was assessed as either greater in ESC, greater in EB, or unchanged (marked as symbols in Table I). Only strong alterations (~30% or greater increase or decrease) in silver stain intensity were scored as changes so that across all samples only 20% of spots were considered to exhibit change. 20 protein species were identified that were repeatedly detected more strongly in EB, whereas 15 species were repeatedly observed preferentially or exclusively in ES samples (Table I). 11 cases were identified in which a protein was present at different electrophoretic mobilities when ESC and EB were compared (Table II).

Correlation between Phosphoprotein Detection and Gene Expression—An important question in the interpretation of the phosphoprotein enrichment screen is whether lack of detection of a protein under a given condition reflects a lack of phosphorylation or a lack of expression. Therefore, we examined gene expression profiles for our proteins of interest. The J1 ES cell line has been extensively characterized with respect to gene expression using Affymetrix™ gene arrays (10). Gene array data for 0, 6, 12, 18, and 24 h of J1 ES cell differentiation was extracted from the StemBase (10) database (the 0- and 24-h samples in the database correspond to our ESC and EB samples, respectively). With the exception of ASCL1, every protein listed in Table II matched to one or more Affymetrix probe sets that were classed as “Present” in J1 ES cells. Gene expression data were also examined quantitatively. For each probe that matched to a protein of interest, gene chip average signal (proportional to gene expression) at 24 h of EB formation was plotted against average signal at 0 h (i.e. non-differentiated ESC) (Fig. 3). In most cases deviations from the diagonal were minimal (Fig. 3). All 24-h average signal values fell between 0.5 and 1.5 times the time 0 value. These observations support the interpretation that the proteins listed in Table II are similarly expressed in both ESC and EB but are differentially phosphorylated between the two conditions.

Bioinformatic Annotation of Protein Functions—To gain insight into the putative biological functions of the identified proteins, Gene Ontology annotations were extracted from the Mouse Genome Database (27). For the set of proteins we identified as being expressed in J1 ES cells (a subset of Table I), the most common Biological Process annotations were heat shock/chaperone, protein catabolism, protein biosynthesis, and cytoskeleton organization (Fig. 4, inner pie chart). For the set of proteins that exhibited changes in post-translational modification when undifferentiated and differentiated cells were compared (Table II), the corresponding biological process annotations were even further enriched in heat shock and protein catabolism functions as well as transcription and chromatin modulating functions (Fig. 4, outer pie chart), whereas annotations related to protein biosynthesis, cy-
TABLE I

Proteins identified from embryonic stem cells and embryoid bodies after phosphoprotein enrichment

Proteins were extracted from undifferentiated ES cells and from 24-h differentiated EBs of the murine embryonic stem cell line J1. Samples were loaded onto phosphoprotein affinity columns, and eluted proteins were separated by 2DGE and visualized by silver stain. Five experiments were performed with each experiment as a comparative pair (ES versus EB). For each protein species that was identified on each pair of gels, any strong change in the intensity of silver stain between the paired gels was noted, and such observations are symbolized as follows:

- **▲**, higher detection in EB than ESC;
- **▼**, higher detection in ESC than EB;
- **=**, comparable staining in ESC and EB.

Proteins were recovered by in-gel digestion and identified using tandem mass spectrometry as described under “Experimental Procedures.” The confidence of protein identification in each case is given by two measures: the number of non-redundant peptides sequenced by MS/MS followed by the probability-based MOWSE score shown in parentheses. For 2DGE separation, a variety of isoelectric focusing ranges were used. Experiments 1 and 2 used pH range 3–10, experiments 3 and 4 used pH range 4–7, and experiment 5 used pH range 3–6. Hdgf, hepatoma-derived growth factor; Calr, calreticulin; HnRNP, heterogeneous nuclear ribonucleoprotein; NAC, nascent polypeptide-associated complex; Npm, nucleophosmin; Pdhb, pyruvate dehydrogenase β; Ptna, prothymosin α; VCP, valosin-containing protein.

| Protein        | Accession Number | Silver Stain Trends | Number of Peptides Matched (Score) |
|----------------|------------------|---------------------|------------------------------------|
|                |                  | Expt #1 | Expt #2 | Expt #3 | Expt #4 | Expt #5 |
| Anp32A         | gi|1763275         | ▼        |         |         |         |         |
| Anp32A         | gi|1763275         | ▼ ▲ ▲ ▲   |         |         |         |         |
| Anp32B         | gi|1670537         | ▼         |         |         |         |         |
| Anp32B         | gi|1670537         | ▼         |         |         |         |         |
| Anp32B         | gi|1670537         | ▼         |         |         |         |         |
| Anp32B         | gi|1670537         | ▼         |         |         |         |         |
| Anp32B         | gi|1670537         | ▼         |         |         |         |         |
| Anp32E         | gi|35808339        | ▼         |         |         |         |         |
| Anp32E         | gi|35808339        | ▼         |         |         |         |         |
| Anp32E         | gi|35808339        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
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| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |
| Arfap1         | gi|30463616        | ▼         |         |         |         |         |

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toskeleton organization, and other functions were less abundant.

Tripartite Motif Protein 28 (TRIM28)/TIF1β—After 2DGE, peptides related to TRIM28 (TIF1β) were detected in two distinct gel mobility species. One species was a high molecular mass spot that contained multiple peptides that were matched to various regions of the 90-kDa TRIM28 sequence by MS/MS data (Fig. 5). A second species of ~17-kDa was identified in two independent experiments that contained the peptides ADVQSIIGLQR and LSPPYSSPQEFAQDVGR. These peptide sequences are known to occur in only two cases: TRIM28 and unnamed protein product gi|26354228 whose
sequence is identical to the C-terminal 240 amino acids of TRIM28 (predicted mass, 26 kDa). These data demonstrate that a protein product identical to or smaller than the predicted protein gi126354228 and containing a sequence identical to the TRIM28 BROMO domain is present in mouse ESCs.

**DISCUSSION**

Proteomic Profiling of Stem Cells—By examining the phosphoprotein-enriched proteome of mouse ESC and EB, 108 proteins and 183 protein species were identified (Table I). Importantly 39 proteins (46 protein species) were identified that reproducibly exhibited distinct patterns of post-translational modification (as indicated by affinity for phosphoprotein binding columns and/or altered electrophoretic gel mobility) between the undifferentiated ESC and differentiating EB states (Table II). Such observations are correlative but nevertheless suggest these proteins may be functionally linked to ESC differentiation. Current phosphoprotein profiling methods are by no means comprehensive, and the candidates identified here undoubtedly represent only a subset of the complete phosphoproteome. An additional method to address sample complexity is by using HPLC separation technology in concert with mass spectrometry (18). Indeed in one experiment analyzed by LC-MS/MS we noted 51 additional protein species that were not seen in the MALDI-MS/MS experiments (this accounts for a substantial portion of the apparent experiment-to-experiment variability seen in Table I). In practical terms, however, MALDI-MS/MS was found to offer a significant savings in terms of time required for processing large numbers of samples. Phosphoprotein profiling techniques that dispense with 2D gels and use only HPLC separation can achieve high proteome coverage (18). However, in our study 108 different proteins produced 183 gel mobility species indicating that a large amount of protein modification data may be overlooked in gel-free experiments.

**Consensus for Embryonic Stem Cell Protein Profiles**—Two previous studies have examined the general proteome of ES cells (17, 18). 70 of the proteins identified in our study were

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**Table I—continued**

| Protein Name | GI/Accession | Affinity | Mobility | Affinity | Mobility |
|--------------|--------------|----------|----------|----------|----------|
| RIKEN cDNA 2010003.03 | gi22137813 | \(\downarrow\) | 1| 1\(\uparrow\) |
| RIKEN cDNA 2410005.409 | gi2222719 | \(\downarrow\) | 2| 1\(\uparrow\) |
| RIKEN cDNA 2900001.123 | gi30424800 | \(\downarrow\) | 2\(\uparrow\) | 3\(\downarrow\) |
| RIKEN cDNA 2570343103 | gi28174932 | \(\downarrow\) | 2| 1\(\uparrow\) |
| Rpl1 | gi51762880 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Rpl1 | gi51762889 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Rpl1 | gi51762908 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Rpl2 | gi24273435 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
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| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
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| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
| Tdol | gi30856940 | \(\downarrow\) | 2\(\uparrow\) | 1\(\downarrow\) |
Fig. 2. Representative phosphoprotein and phosphopeptide mass spectra. A, MS/MS spectrum assigned to the phosphopeptide ESVDFPLpSPPK of stathmin. B, MS/MS spectrum assigned to the phosphopeptide ASGQAFELILpSPR of stathmin. C, MS spectrum assigned to the phosphoprotein Hspb1 showing the position of peptides and phosphopeptides assigned by MS/MS. D, MS/MS spectrum of the N-terminal acetylated phosphopeptide ApSGAVAVS-DGVIK of coflin. A and C are from MALDI-MS/MS experiments; B and D are from LC-MS/MS experiments.
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Fig. 3. Gene expression for selected proteins. The database StemBase (www.scgo.ca:8080/StemBase/) contains gene expression data for a large number of cell types, including J1 cells, that was obtained using the Affymetrix Mouse Expression Set 430 GeneChip Arrays (10). Protein identities from Table II were mapped to corresponding GeneChip probe identification numbers using either StemBase functions or the NetAffx service (Affymetrix, www.affymetrix.com). For each matched probe set, gene chip average signal values, which are proportional to mRNA level, were extracted from StemBase. Values obtained from 24-h differentiated J1 embryoid bodies were plotted against the corresponding 0-h (undifferentiated) values.

not identified by Elliott et al. (17), consistent with the interpretation that the phosphoprotein enrichment method selectively captures a less abundant subset of the proteome. Nagano et al. (18) identified over 1700 proteins from E14-1 cells of which 35 had features that suggested potential phosphorylation. A subset of these 35 proteins, G3BP, PSMA2, TRIM28, nudix, nucleophosmin, and heat shock protein 1, were also detected in our study. From a meta-analysis of gene expression studies, Fortunel et al. (14) proposed a list of 332 genes to be specifically enriched in mouse ESC. Interestingly nine of these proteins were independently identified in our study, and except for RUVB-L1 and HNRNP-K, all exhibited evidence of altered phosphorylation between ESC and EB, suggesting a functional relationship to ESC differentiation (Table III). Conversely the majority of proteins whose phosphorylation state correlated with the ESC differentiation state in our study were not classified as ESC-relevant by mRNA transcript profiling, reinforcing the complementary nature of proteomic and genomic analyses.

Chromatin-regulatory Proteins—Epigenetic factors, namely histone and DNA modification, are thought to play an important role in regulating transcription in early embryos and stem cells (28). Moreover gene array studies have detailed the extensive differences in transcription that exist between stem cells and their differentiated progeny (10), but only a small number of transcription factors have been specifically associated with the ESC transcriptional program. In our study, a number of proteins that recognize or modify chromatin were identified. Remarkably a set of these proteins consistently exhibited evidence of differential phosphorylation when ESC and EB were compared. CBX3/HP1γ, DN38/NAP1L-1, HMGB2, TRIM28/KRIP-1/TIF1β, ANP32A (pp32), and SET are associated with chromatin modification and related processes including gene silencing (29–32). These observations are consistent with the concept that maintenance of the transcriptional program of stem cells may be explained in part by epigenetic regulation. Indeed several recent studies support this concept. For example, Oct-4 gene expression appears to be influenced by epigenetic mechanisms and chromatin remodeling during both normal development and during the “nuclear reprogramming” that accompanies somatic cell nuclear transfer (33, 34). Conversion of oligodendrocyte precursor cells to multipotent neural stemlike cells was shown to be associated with chromatin remodeling (35), and histone methylation was found to exhibit specific patterns during mouse embryo development (36). Our data demonstrate that the phosphorylation status of chromatin-remodeling proteins is modulated at the earliest stages of ESC differentiation and as such may provide significant regulatory control over the stem cell genome.

**TRIM28/TIF1β**—One of the chromatin phosphoproteins was of particular interest. Specifically a small (<20-kDa) protein containing amino acid sequences identical to the C-terminal region of TRIM28 was detected in ESC but not EB after phosphoprotein enrichment. Whether this protein results from cleavage of full-length TRIM28 or is the product of a distinct transcript (potentially predicted protein gi|26354228) is unknown at this time. Given that only the 100–200 most abundant products after phosphoprotein enrichment are evident on our 2D gels, it is unlikely that this protein is simply the result of general protein degradation. Because the sequenced peptides correspond to the BROMO domain (Fig. 5), this smaller protein likely retains the ability to bind acetylated lysine. Full-length TRIM28 and HP1 are known to interact, resulting in phosphorylation of HP1 and gene silencing (29). In the small TRIM28-related protein, we predict that the HP1 interaction domain would be absent (Fig. 5). Mutational analysis of TRIM28 showed that abrogating the HP1-TRIM28 interaction prevented differentiation in an embryonic carcinoma cell model (37). As such, the small TRIM28-related protein detected in our experiments might repress ESC differentiation by acting as a dominant negative form of TRIM28.

**ESC-enriched Chaperone Phosphoproteins**—Protein p23 (TEBP; telomerase-binding protein/cPGES; cytoplasmic pro-
taglandin E synthase) was detected more strongly in ESC than EB in the phosphoprotein screen (Tables I and II) while showing no change at the mRNA level (Fig. 2). One function of p23 is regulation of HSP90/HSPCB (38), which was also detected as an ESC-enriched putatively phosphorylated protein (Tables I and II). The chaperone activity of HSP90 is limited to a specific set of “client” proteins including telomerase, prostaglandin receptor, and certain kinases (39). Both p23 and HSP90 are required for efficient telomerase complex assembly, and high telomerase activity is present in ESC (40). Casein kinase II can phosphorylate p23 and potentiate cPGES activity (41). These observations suggest that kinase signaling could be linked to the coordinated assembly of specific protein complexes via regulated chaperone activity. In addition, p23 can negatively regulate transcription by disassembling transcriptional regulatory complexes at hormone response elements (HREs) (42). Interestingly expression of the stem cell-specific factor Oct-4 is negatively regulated by retinoic acid via HREs (43). Furthermore p23 gene expression is enriched in ESC relative to non-stem cell populations (8, 9, 14). Collectively these observations suggest a model in which high p23 expression may simultaneously promote telomerase activity and prevent Oct-4 repression. The proteins PSMC5/TRIP1 (Tables I and II) and PSMC3/TBP1 (Table I) have also been functionally linked to HREs (44, 45).

**Summary**—Phosphoprotein enrichment coupled to 2DGE and MS/MS led to the identification of 108 different proteins from undifferentiated and early differentiated mouse embryonic stem cells. 39 of these proteins exhibited differential recovery from phosphoprotein affinity columns and/or altered 2DGE mobility when ESC and EB were compared (Table II). We propose that these proteins be considered as having potential relevance to ESC differentiation. Of these proteins, p23, HNRP-K, NAP1L1, pICln, PSMC5, SET, and TRIM28 have been proposed previously to be potential determinants of the pluripotent stem cell state on the basis of enriched gene expression (14). Our observations support this hypothesis and provide evidence that these proteins are phosphorylated in stem cells in a differentiation-specific manner. Two forms of TRIM28 were detected in ESC including a truncated form whose expression may have functional consequences. Altered post-translational modification was detected in a number of proteins related to HSP90 chaperone function, to protein catabolism, and to chromatin remodeling suggesting that these processes may be highly relevant to stem cell fate and...
TABLE II
Proteins exhibiting differential post-translational modifications between ESC and EB

Expressed proteins were identified from J1 ESC and EB after phosphoprotein enrichment and 2DGE as described under "Experimental Procedures." Protein identities and silver stain patterns were carefully correlated across five pairs of gels to detect proteins in which recovery through phosphoprotein affinity columns or mobility under 2DGE was altered between the ESC and EB samples. Asterisks indicate cases where multiple gel mobility species of the protein exhibited the same behavior.

A. Protein species with increased detection in ESCs
   Cbx3, Ckap/Tbcb, Cln1a/plCln*, Hdgfl, Hspca*, Hspcb, Psma3, Ptna, Ranbp1, Tebp/cPGES, Tpm2, Trim28a, Trim28-related fragment

B. Protein species with increased detection in EBs
   Anp32a, Anp32b, Ascf1, Caln1, Calr, Cdc37, DN35/Nap111, elf3 s2, Hapb1/C1qbp, Hmgb2, Hspb1, Hspca, Npm1, Psma2, Psmc2, Psmc5/Trip1, Psmc6, Stip1*, Tubulin

C. Evidence of altered gel mobility in ESC vs. EB
   Anp32a, Anp32b, Anp32e, elf2b2, Hapb1/C1qbp, Hspca, Hspcb, Psma3, Psmb6, SET, Tra1

TABLE III
Detection of gene products previously proposed to be embryonic stem cell-associated

A list of 332 cDNAs proposed to be embryonic stem cell-enriched (14) was compared against the proteins detected here in ESC and EB at the protein level after phosphoprotein enrichment. Proteins that were found in both studies are listed along with our observations of protein detection after phosphoprotein affinity column treatment.

| ES-enriched gene | Phosphoprotein detection |
|------------------|--------------------------|
|                  | ES          | EB          |
| Clns1a/plCln    | Yes         | Weakly      |
| G3bp             | No          | Weakly      |
| Hn rpk           | Yes         | Yes         |
| Nap111           | No          | Yes         |
| Psmc5            | No          | Yes         |
| Ruvb1l           | Yes         | Yes         |
| SET*             | Yes         | Yes         |
| Tebp/cPGES/p23   | Yes         | Weakly      |
| Trim28           | Yes         | No          |

* Protein was detected in both ESC and EB but at different electrophoretic mobilities under 2DGE in each case.

Proteomic characterization of early-stage differentiation of mouse embryonic stem cells into neural cells induced by all-trans retinoic acid in

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