Identification of Candidate Regulators of Embryonic Stem Cell Differentiation by Comparative Phosphoprotein Affinity Profiling*[S]

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 phosphoprotein regulators of 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. ***Molecular & Cellular Proteomics 5:57–67, 2006.***

Embryonic stem cells (ESCs)[1] were originally identified in mice and subsequently characterized from human tissue sources (1–3). The development of living mice wholly derived from such cells has demonstrated the pluripotency of murine embryonic stem cells (4). ESCs are capable of self-propagation by symmetrical division while retaining pluripotency. This phenotype is maintained by a combination of extrinsic and intrinsic factors (5). The known extrinsic factors are leukemia-inhibitory factor (LIF) and bone morphogenetic proteins. LIF signaling through gp130 results in phosphorylation, dimerization, and nuclear translocation of the signal transducer and activator of transcription STAT3 (6). Bone morphogenetic protein treatment induces expression of Id family transcriptional modulators (5). The principle known intrinsic factors that maintain the ESC phenotype are the transcription factor OCT-4 and the novel homeodomain protein Nanog.

When ESCs are cultured in suspension in the absence of extrinsic factors, spherical multicellular structures termed embryoid bodies (EBs) are formed, and the individual cells comprising the sphere begin to differentiate along various lineages in a disorganized fashion (7). Differentiation into multicellular spheres is also a property of other stem cells, such as the formation of neurospheres from neuronal stem cells. Control of the differentiation process is a topic of intense interest because of the potential application of stem cells to regenerate diseased or injured tissues.

Apart from the small number of known intrinsic and extrinsic factors that maintain the embryonic stem cell phenotype, the molecular basis for the pluripotency and self-renewal of stem cells is not well understood. A number of studies have used RNA expression profiling techniques in an attempt to identify genes that specify stem cell properties in mice (8–10) and humans (11–13). One group of studies revealed a set of 332 genes whose expression appears to be enriched in murine ESCs (8, 9, 14). However, when data from hematopoietic and neuronal stem cells were included, cross-study compar-

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

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[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.

ison yielded almost no consensus, suggesting that the stem cell phenotype cannot be explained by transcriptional profile alone (14, 15). Clearly mechanisms operating at the post-transcriptional level may also be relevant.

To date, proteomic analysis of embryonic stem cells has been limited. Guo *et al.* (16) used comparative two-dimensional gel electrophoresis (2DGE) to examine the differentiation of ESCs into neural cells under retinoic acid treatment and found 24 differentially expressed spots of which 12 were identified. Elliott *et al.* (17) produced a two-dimension gel electrophoresis map of proteins in the mouse ESC line R1 and identified 218 proteins. Recently Nagano *et al.* (18) used 2D LC-MS/MS to identify ~1800 proteins from the ESC line E14-1. 35 of these proteins yielded mass spectra that were consistent with phosphorylation, and one phosphorylation site was mapped. To specifically examine phosphorylation, Prudhomme *et al.* (19) used quantitative Western blots to analyze how the phosphorylation status of 31 selected proteins correlated, in a combinatorial fashion, with mouse ESC proliferation or differentiation and found that the phosphorylation status of the kinases RAF1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, extracellular signal-regulated kinase, protein kinase B-α, SRC, and protein kinase C-ϵ were especially significant. As such, these data suggest that kinase activity and the phosphorylation status of target substrates may act as critical regulators of stem cell function. Here we sought to characterize the stem cell state by identifying the phosphoproteome associated with mouse ESCs and their derived EBs. Phosphoprotein enrichment and comparative 2DGE were used to profile phosphoprotein expression. Proteins detected by silver stain were identified by MALDI-MS/MS or by LC-MS/MS. The set of proteins that exhibited altered post-translational modification during differentiation included several proteins identified previously in gene expression arrays as conserved features of the stem cell phenotype. Proteins related to protein catabolism, protein folding, chromatin remodeling, and other functions were identified and found to exhibit evidence of altered phosphorylation between the ESC and EB states.

EXPERIMENTAL PROCEDURES

Cells—The murine embryonic stem cell line J1 (20) was maintained on a feeder layer of murine embryonic fibroblasts. Prior to each experiment feeder cells were removed by briefly passaging on a gelatin-coated plate. A portion of the cells were differentiated into EBs by culturing for 24 h in bacteriological grade Petri dishes, which do not support ESC adherence. The remaining cells were plated on tissue culture dishes and maintained as ESC colonies in the presence of LIF for 24 h. The successful removal of feeder cells and formation of ESC colonies and EBs were verified by direct observation of cell morphology under light microscopy.

Phosphoprotein Enrichment—Phosphoprotein enrichment was performed using Phosphoprotein Purification Kit 37101 (Qiagen, Mississauga, Ontario) as described previously (21). Briefly 2.5 mg of protein was loaded onto phosphoprotein binding columns and washed extensively before eluting bound proteins that were then concentrated using the supplied ultrafiltration columns (10-kDa cut-off).

Two-dimensional Gel Electrophoresis—2DGE was performed as described previously (21) using 8–16% gradient precast gels (Bio-Rad) or 10% hand-cast gels in a Protean II electrophoresis cell (Bio-Rad). For isoelectric focusing (first separation dimension), three different pH ranges were used: 3–10 (done twice), 5–8 (done once), and 4–7 (done twice). Each experiment was done as an ES *versus* EB comparison pair for a total of 10 two-dimensional gels.

Silver Stain and In-gel Digest—Silver staining was performed by a standard method (22). For LC-MS/MS, samples were prepared as described previously (21). For MALDI-MS/MS samples, the in-gel digest was performed manually and extracted in 5% formic acid. Prior to spotting for MALDI analysis, samples were cleaned using ZipTips (Millipore, Billerica, MA) following the manufacturer's recommended procedure.

Mass Spectrometry and Protein Identification—Nano-LC-MS/MS was performed as described previously (21) using an Ultima Q-TOF hybrid tandem mass spectrometer (Waters). MALDI-MS/MS spectra were acquired using a QSTAR XL tandem mass spectrometer (ABI/Sciex) with an oMALDI-2 source and Analyst QS version 1.1, build 9865. α-Cyano-4-hydroxycinnamic acid (Agilent, Palo Alto, CA) matrix was used. Spectra were searched against the National Center for Biotechnology Information non-redundant 20050606 database using Mascot daemon version 2.0.5 on an in-house Mascot server version 2.0.04. Parameters used for queries were trypsin cleavage, two missed cleavages, $\pm$1.2-Da peptide tolerance, $\pm$0.6-Da MS/MS tolerance, and the following variable modifications: acetyl (N-terminal), carbamidomethyl (Cys), deamidation (Asn, Glu), oxidation (Met), phosphorylation (Thr, Ser, Tyr), and pyro-Glu (N-terminal Glu). The results were then evaluated manually, and search parameters were narrowed as warranted to eliminate potential false-positive identifications.

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 phosphoprotein affinity columns, and eluting proteins were detected by 2DGE and silver stain (Fig. 1). As expected for phosphoproteins (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).

Evidence of Protein Phosphorylation—Phosphopeptide

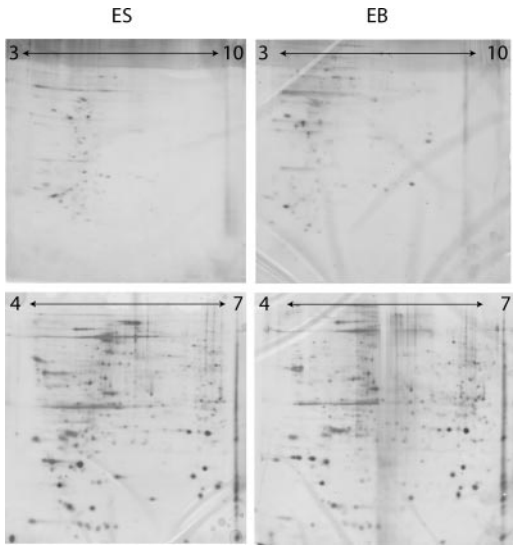

FIG. 1. **Phosphoproteome gel profiles of ESC and EB.** J1 murine embryonic stem cells were cultured and, after removal of feeder cells, were maintained as undifferentiated ES cells or were differentiated into EBs over 24 h. Proteins were extracted and loaded onto phosphoprotein affinity columns. Eluting proteins were separated by two-dimensional gel electrophoresis and detected by silver stain. The first separation dimension (isoelectric focusing) was performed over a pH range of 3–10 (*top panels*) or 4–7 (*lower panels*).

mass spectra are rarely observed in protein identification experiments because of the rarity of phosphopeptides among total peptides and because of the very low ionization efficiency of phosphorylated peptides in positive ion mode (23). However in our study, after phosphoprotein affinity treatment, we observed mass spectra that were consistent with phosphorylation for several proteins: HSPB1, p23/TEBP, CKAP1/TBCB, stathmin, hepatoma-derived growth factor, and cofilin. In four cases, specific sites of phosphorylation were mapped (Fig. 2). In stathmin, the phosphopeptides ESVPDFPLpSPPK (where pS is phosphoserine) (Ser-37) and ASGQAFELILpSPR (Ser-24) were observed (Fig. 2, *A* and *B*) and represent previously known sites of phosphorylation (24). Two gel mobility species of stathmin were observed of which only the lower pI form showed Ser-24 phosphorylation. Both phosphorylated and unphosphorylated forms of the HSPB1 tryptic peptide SPSWEPFR were observed with a +80 difference in mass/charge characteristic of phosphorylation (Fig. 2*C*). The MS/MS spectrum of the HSPB1 phosphopeptide was consistent with phosphorylation at Ser-13 (not shown). In other cell types, this peptide can be phosphorylated at multiple sites including Ser-15 (25). For cofilin, the peptide *N*-acetyl-ApSGVAVSDGVIK was observed (Fig. 2*D*) that matches the known biochemistry of this protein (26).

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 II). 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; Ptma, prothymosin α; VCP, valosin-containing protein.

		Silver Stain Trends					Number of Peptides Matched (Score)									
	Accession	Experiments					Exp #1		Exp #2		Exp #3		Exp #4		Exp #5	
Protein	Number	#1	#2	#3	#4	#5	ES	EB	ES	EB	ES	EB	ES	EB	ES	EB
Anp32A	gi|1763275	▼					8(304)									
Anp32A	gi|1763275	▲	▲	▲	▲			6(232)				3(107)		3(94)		
Anp32A	gi|1763275	▲	−	−	▲				1(24)	2(98)						
Anp32B	gi|18700032	▲						3(89)								
Anp32B	gi|18700032	▼					5(114)									
Anp32B	gi|18700032	▲						5(174)								
Anp32B	gi|18700032	▲		▲				2(102)				4(120)				
Anp32B	gi|18700032	−	−				5(116)	4(179)		3(84)						
Anp32E	gi|30580338	▼					2(39)									
Anp32E	gi|30580338	▲						1(35)								
Anp32E	gi|30580338	▲						1(47)								
Arfgap1	gi|4063616			▼							1(49)					
Arhgdia/RhoGDI-1	gi|12597249					▼									5(206)	2(110)
Ascl1	gi|15131817	▲	▲							1(48)						
Asna1	gi|17391018					−										2(81)
Banf1	gi|6753178	−	▲				1(51)	1(42)		3(148)						
Btf3	gi|2851417	▲	−					5(208)		1(53)						
Calm1	gi|50274	▲	▲				2(112)	5(260)	1(51)	5(213)						
Calr	gi|6680836				▲									2(50)		
Calr	gi|6680836		▲	▲						1(47)		9(445)				
Calr	gi|6680836	▲	−	−	−			13(364)	8(450)	8(425)	1(52)	5(136)	6(272)	6(208)		
Cbx1	gi|7767191	−			−		1(60)	1(72)						4(152)		
Cbx3	gi|6680860	−	▼	▼	−	▼		2(42)	1(43)		2(90)	2(147)			1(45)	
Cdc37	gi|7949018	▲		▲			2(131)					2(42)				
Cfl1	gi|6680924	▲					3(137)	10(293)								
Chmp4b	gi|28077049	▲						2(53)								
Ckap1/Tbcb	gi|14715044			▼	▼						3(71)	1(47)				
Clns1a/pICLn	gi|544031	▼	▼				2(121)	1(79)								
Clns1a/pICLn	gi|544031			▼		▼					1(52)	2(80)			2(81)	
Cnn3	gi|55391513				▼								2(64)			
CUTA	gi|21702737	▲						1(60)								
Destrin	gi|9790219	▲					4(181)	8(296)								
DN38/Nap1l1	gi|109866			▲		▲						2(72)				2(101)
Eef1b2	gi|31980922	▼					4(167)									
Eef1b2	gi|31980922	−					3(104)	4(174)								
Eef1b2	gi|31980922	−	−	−	−		5(299)	5(224)	3(232)	3(138)	2(134)	3(199)	3(169)			
Eef1b2	gi|31980922	▼		▲								1(46)				
eEF1g	gi|27754099		−		▲				6(268)					3(57)		
eIF2b2	gi|14149756	▼					2(37)									
eIF2b2	gi|14149756	▲						3(131)								
eIF2b2	gi|14149756	−		−			6(242)	10(434)			3(88)	5(172)				
eIF3 s1	gi|26349519	▼					2(99)									
eIF3 s2	gi|9055370				▲	▲								3(85)		5(134)
eIF3 s3	gi|18079341			▲								1(35)				
eIF3 s5	gi|21313620			▼	▲						3(44)			4(142)		
eIF-4e	gi|110568			▲	▼	▲						1(37)				2(100)
Enolase			▲							1(36)						
G3bp	gi|7305075	▲						1(33)								
Habp1/C1qbp	gi|6680816			▼							1(60)					
Habp1/C1qbp	gi|6680816			▲								2(97)				
Habp1/C1qbp	gi|6680816			▲	▲							2(201)	2(125)	2(159)		
Habp1/C1qbp	gi|6680816	▼	▲	−			3(69)	2(55)	1(29)	1(107)						
Habp1/C1qbp	gi|6680816	▲	▼					3(160)	2(120)	2(121)						
Hdgf	gi|22261803	▼	▼				10(212)									
Hmga1	gi|7710034	▲						2(43)								
Hmgb1	gi|40352857		▼						3(78)							
Hmgb2	gi|55777186	▲	▲					6(240)	5(240)	6(369)						
Hmgb2l1	gi|30794254	▼					1(38)									
Hmgb2l1	gi|30794254	▼					1(27)									
HnRNP-C	gi|4139188	▲						1(27)								
HnRNP-K	gi|13384620					▼									2(48)	
HnRNP-K	gi|13384620			▼	▼	▲					3(152)		4(115)			6(260)
HnRNP-K	gi|13384620				▼	▲					2(91)		5(207)	4(117)		5(221)
HnRNP-K	gi|13384620					−									4(164)	3(98)

TABLE I—*continued*

HnRNP-K	gi|13384620					—									3(180)	10(636)
Hspa4	gi|2495342		▼						3(148)							
Hspa8	gi|1661134	▲		▼		▲		3(68)			2(68)					15(677)
Hspb1/HSP27	gi|17390597			▲	▲							7(193)				
Hspb1/HSP27	gi|17390597			▲	—						5(151)	3(68)				
Hspca/Hsp90a	gi|6754254		▼						3(128)							
Hspca/Hsp90a	gi|6754254	▲						9(337)								
Hspca/Hsp90a	gi|6754254	▲						11(405)								
Hspca/Hsp90a	gi|6754254				▼	▼							5(95)		4(166)	
Hspca/Hsp90a	gi|6754254	▼	▼				4(102)			1(74)						
Hspca/Hsp90a	gi|6754254	—	—				3(77)	3(108)		3(113)						
Hspca/Hsp90a	gi|6754254	▲			▲			6(225)						3(90)		
Hspca/Hsp90a	gi|6754254		—						3(70)	6(215)						
Hspca/Hsp90a	gi|6754254		—							2(91)						
Hspca/Hsp90a	gi|6754254	▲	▼					8(196)	4(183)							
Hspca/Hsp90a	gi|6754254	▼					2(39)									
Hspcb/HSP84	gi|123681	▼					2(52)									
Hspcb/HSP84	gi|123681		▼						1(36)							
Hspcb/HSP84	gi|123681				▼								2(67)			
Hspcb/HSP84	gi|123681				▲									4(93)		
Hspcb/HSP84	gi|123681	▼	▼						1(49)	1(62)						
Hspcb/HSP84	gi|123681			—							3(77)	3(74)				
Hspcb/HSP84	gi|123681		▼	▲					3(147)	2(75)		2(188)				
Hspcb/HSP84	gi|123681		—		▲				2(159)							
Hspcb/HSP84	gi|123681	—					1(53)									
Lamr1	gi|31560560					▲										7(378)
Mak	gi|29477134			—								1(40)				
Mapre1	gi|7106301					▼									4(142)	2(75)
Mrgpra5	gi|50401114					▲										1(47)
Myl6	gi|33620739		▲							2(58)						
NAC alpha	gi|20867687					▼									4(166)	
NAC alpha	gi|20867687	—	—	—		—	3(235)	3(218)	3(100)		4(157)	3(212)			4(192)	4(177)
Npm 1	gi|6679108		▼						1(58)							
Npm 1	gi|6679108				▲	▲								3(111)		2(87)
Npm 1	gi|6679108	▲						1(44)								
Nudt5	gi|29436910					▼									3(158)	2(124)
Pa2g4/p38-2G4	gi|790470		—						3(104)	1(30)						
Pdap1/PDGF-AP	gi|20848839		▼						4(137)							
Pdcd5	gi|9790259	▼					5(249)									
Pdha1	gi|6679261		▲							4(109)						
Pdhb	gi|18152793					▲										2(87)
Pdia4/Erp-72	gi|119531	▲						1(35)								
Pdia4/Erp-72	gi|119531	▲						4(86)								
PPP1R7	gi|15488779				▼								3(53)			
Psma1	gi|33563282		—	—	—				5(211)	3(64)						
Psma2	gi|6679497		▼						4(135)							
Psma2	gi|6679497				▲									2(72)		
Psma2	gi|6679497	▲	▲					4(171)		2(99)						
Psma3	gi|31981534				▲									3(62)		
Psma3	gi|31981534			▼	▼						1(26)		7(244)	2(81)		
Psma3	gi|31981534	▲						1(33)								
Psma4	gi|6755196	▲	▼					5(189)								
Psma5	gi|7106387	▲	—	—	▲				3(130)	1(32)						
Psma7	gi|7106389	▲						4(139)								
Psmb2	gi|14198410		—						4(168)	3(125)						
Psmb3	gi|6755202			—	—						3(98)					
Psmb4	gi|14198355			▲	—						1(46)	3(60)				
Psmb6	gi|31982099	▲	▲	▼	—	▼		3(182)		2(57)			2(47)		3(126)	1(32)
Psmb6	gi|31982099	▼					1(32)									
Psmc2	gi|33859604			▲	▲							3(111)				
Psmc3	gi|6679503	▲						1(33)								
Psmc5	gi|7110703	▲	▲					3(131)								
Psmc6	gi|28175479	▲	▲					5(186)								
Psme3	gi|6755214			▲	—						2(52)	1(60)				
Ptma	gi|7110705	▼		▼			1(47)				1(62)	1(45)				
Rad23a	gi|34447211	▲						3(84)								
RanBP1	gi|6755284	—	▼		▼	▼			3(126)	3(121)			3(41)		4(139)	2(110)
Rbbp4	gi|47059484				▲									3(73)		
Rbm8a	gi|37231642		▲							6(234)						
RIKEN cDNA 2010003J03	gi|22137613	▲						1(47)								

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

TABLE I—*continued*

RIKEN cDNA 2010003J03	gi|22137613	▲						1(40)								
RIKEN cDNA 2410005H09	gi|22122719	▲						1(40)								
RIKEN cDNA 2900010J23	gi|30424800	▼					3(128)									
RIKEN cDNA 2900010J23	gi|30424800	▼					3(116)									
RIKEN cDNA 5730434I03	gi|28174932	▼					2(41)									
Rplp1	gi|51762888					▼									1(32)	
Rplp1	gi|51762888					▼									1(32)	
Rplp1	gi|51762888					—									1(38)	1(54)
Rplp2	gi|42476345	—	—			—	5(384)	7(458)	2(90)	4(296)					3(191)	4(310)
Rrm2	gi|54887418			▼							4(88)					
Ruvbl1	gi|9790083					▼									1(51)	
Ruvbl1	gi|9790083	▲	—					6(250)	1(42)							
Septin-11	gi|39963577	▲						4(97)								
SET	gi|3953617	▲			▲			1(78)						3(71)		
SET	gi|3953617	▼					1(69)									
SET	gi|3953617	▼					1(36)									
SET	gi|3953617	▲						1(41)								
Skp1a/Tceb1l	gi|25777713	—					1(56)	1(30)								
St13	gi|19526912	▲		—	—			6(255)			6(246)	7(235)				
Stathmin	gi|14625464	—	—		—		7(250)	7(283)	1(42)	5(179)			2(89)	6(187)		
Stathmin	gi|14625464	▼	▲			▲	6(240)	6(251)	1(44)	7(306)						3(185)
Stip1	gi|13277819	▲	▲	▲				5(176)		4(144)		2(41)				
Stip1	gi|13277819	▲	▲					3(77)	1(24)	5(118)						
Taldo1	gi|33859640	▲						3(59)								
Taldo1	gi|33859640	▲	—	—	—			6(260)			2(46)	8(260)				
Taldo1	gi|33859640		—	—	—				7(214)	6(209)			9(270)			
Tebp/cPGEs	gi|30721855	—	▼	▼	▼	▼			2(49)	4(260)	6(251)	2(74)	4(158)		3(160)	3(185)
Thumpd1	gi|21704176	▲						4(139)								
Tpm2	gi|16507925	▼				▼	1(45)								2(87)	
Tpm3	gi|111212	—	—	—			9(396)	17(763)			5(282)	15(828)				
Tpm3	gi|40254525	▼					1(53)									
Tpm4	gi|47894398			▼							1(49)					
Tra1	gi|6755863		▼						4(99)							
Tra1	gi|6755863		▲							1(36)						
Trim28a	gi|12275870				▼								4(179)			
Trim28a	gi|12275870			▼	▼						8(388)		3(118)			
tubulin		▲	▲					8(384)		6(281)						
tubulin, alpha		▼	▲				3(102)									
tubulin, alpha						▼									3(134)	
tubulin, alpha				▲								4(113)				
tubulin, alpha						▲										6(331)
tubulin, beta		▲						2(66)								
unnamed (similar to PSMA5)	gi|51765976	▼					1(52)									
unnamed (similar to PTX1)	gi|12846043	▲						1(43)								
unnamed (TRIM-related)	gi|26354228		▼	▼					1(53)		2(122)					
VCP	gi|55217		▼						2(85)							
Vil2	gi|6678571			▲								2(82)				
Vil2	gi|6678571			—	—						6(251)	8(257)	3(64)			
Wnt16	gi|6249635			▼							1(34)					

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 gi|26354228 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

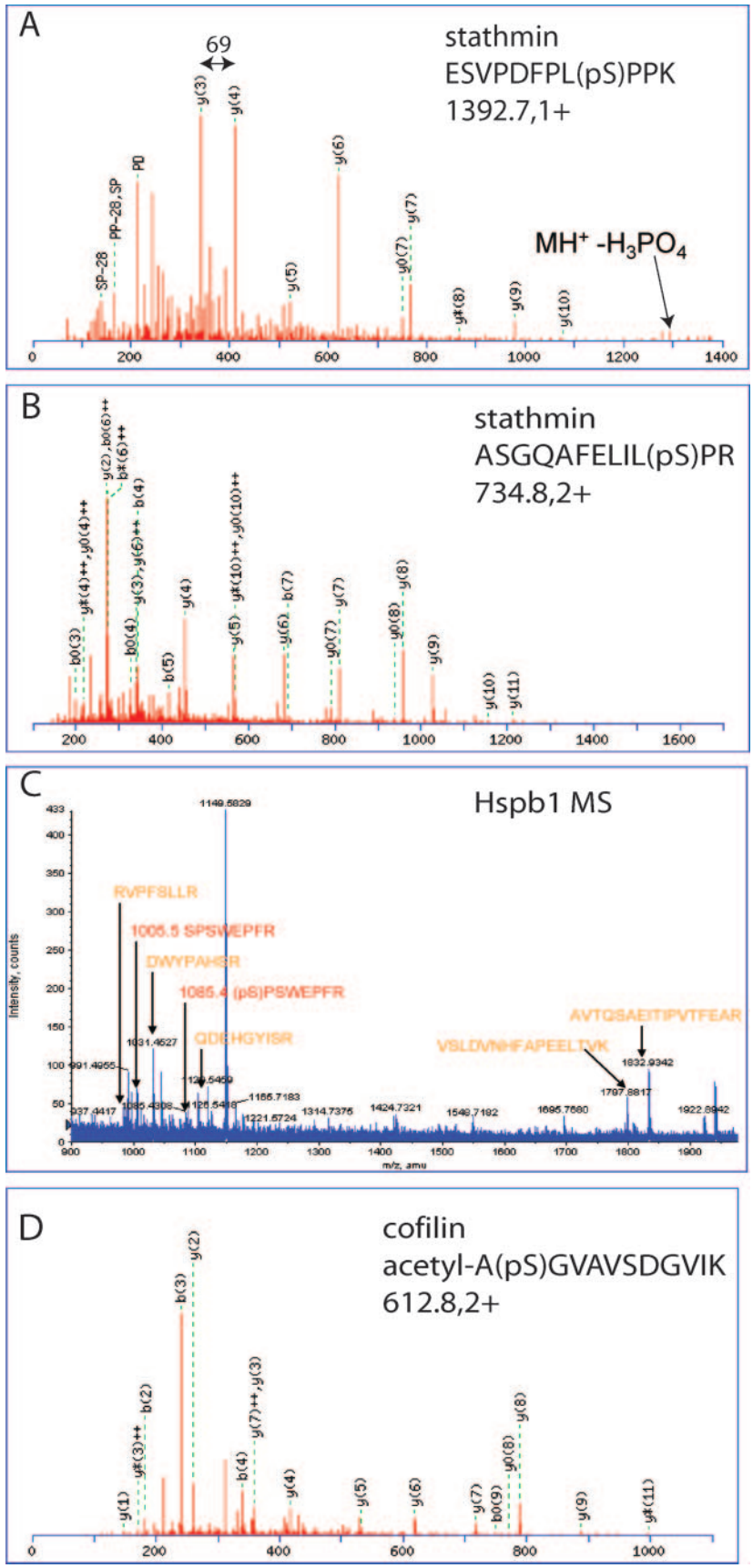

FIG. 2. **Representative phosphoprotein and phosphopeptide mass spectra.** *A*, MS/MS spectrum assigned to the phosphopeptide ESVDFPLpSPPK of stathmin. *B*, MS/MS spectrum assigned to the phosphopeptide ASGQAFELILpSPR of stathmin. *C*, MS spectrum assigned to the phosphoprotein Hspb1 showing the position of peptides and phosphopeptides assigned by MS/MS. *D*, MS/MS spectrum of the N-terminal acetylated phosphopeptide ApSGVAVSDGVIK of cofilin. *A* and *C* are from MALDI-MS/MS experiments; *B* and *D* are from LC-MS/MS experiments.

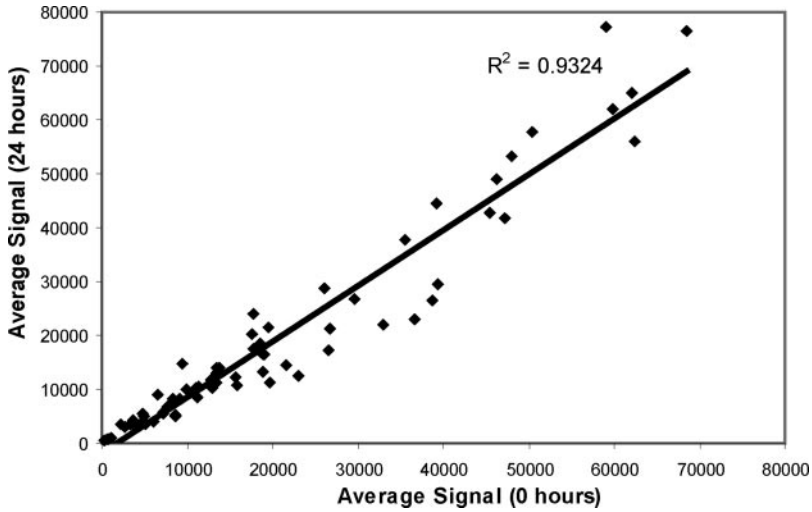

FIG. 3. **Gene expression for selected proteins.** The database StemBase (www.scgp.ca:8080/StemBase/) contains gene expression data for a large number of cell types, including J1 cells, that was obtained using the Affymetrix Mouse Expression Set 430 GeneChip Arrays (10). Protein identities from Table 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 HNRP-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 pros-

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).

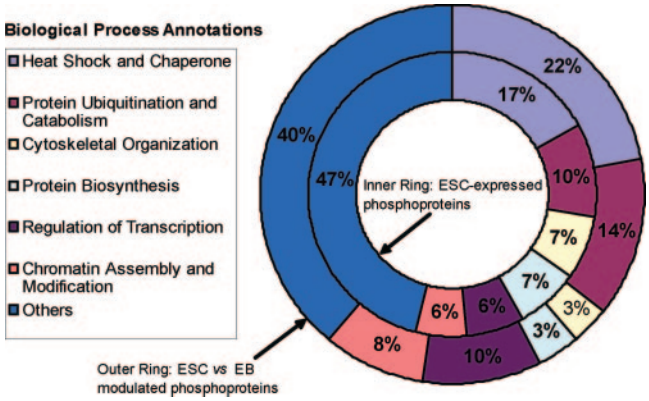

FIG. 4. **Bioinformatic annotation of phosphoprotein functions.** Gene Ontology biological process annotations were extracted from the Mouse Genome Database (27) for proteins that were detected in the phosphoprotein-enriched proteome of J1 ES cells (Table I, ES columns) and also for those proteins that exhibited altered post-translational modifications that correlated with the differentiation state (Table II). The most abundant annotation terms are shown as a percentage of total annotations. *Inner pie chart ring*, annotations for ESC-expressed putatively phosphorylated proteins. *Outer pie chart ring*, annotations for differentiation-dependent putatively phosphorylated proteins (*i.e.* proteins listed in Table II). The Heat Shock annotation includes the terms protein folding, response to unfolded protein, and response to heat. The Catabolism annotation includes the terms ubiquitin-dependent protein catabolism, protein catabolism, and protein ubiquitination. The Regulation of Transcription annotation includes the terms regulation of transcription and negative regulation of transcription. The Chromatin annotation includes the terms nucleosome assembly, chromatin assembly, and DNA packaging.

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

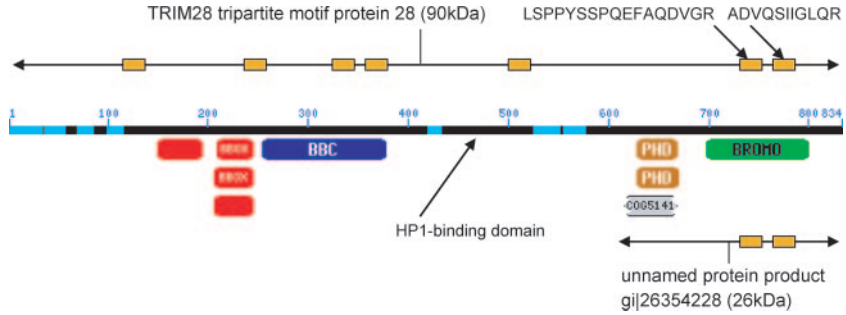

FIG. 5. **Identification of TRIM28 and a TRIM28-related product.** Unnamed protein product gi|26354228 (46) is proposed to be identical to the C-terminal region of TRIM28. Peptides with sequences corresponding to the C-terminal region of TRIM28 were detected in two distinct protein species observed by 2DGE and silver stain, one a high molecular mass spot (~90 kDa) and the other a low molecular mass spot (<20 kDa). A domain structure diagram of TRIM28 was generated using the Conserved Domain Search feature (47) at NCBI (www.ncbi.nlm.nih.gov). *Orange rectangles above* and *below* the TRIM28 domain sequence show the approximate position of peptides detected in the high mass (above) and low mass (below) species. The predicted sequence span of gi|26354228 in comparison with TRIM28 is also shown (*double headed arrow below* the TRIM28 domain diagram).

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/pICLn*, Hdgf, Hspca*, Hspcb, Psma3, Ptma, Ranbp1, Tebp/cPGEs, Tpm2, Trim28a, Trim28-related fragment

B. Protein species with increased detection in EBs
Anp32a, Anp32b, Ascl1, Calm1, Calr, Cdc37, DN38/Nap111, eIF3 s2, Habp/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, eIF2b2, Habp1/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/pICln	Yes	Weakly
G3bp	No	Weakly
Hnrpk	Yes	Yes
Nap111	No	Yes
Psmc5	No	Yes
Ruvbl1	Yes	Yes
SET[a]	Yes	Yes
Tebp/cPGES/p23	Yes	Weakly
Trim28	Yes	No

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

may be dependent on phosphorylation events. Future studies will aim to identify the kinases that mediate this phosphorylation profile. Importantly if the factors maintaining pluripotency in ESC were sufficiently understood at a mechanistic level, it might be possible to devise means to return somatic cells to a pluripotent state through targeted phosphorylation and without using nuclear transfer, a process that currently represents a significant practical and ethical limitation to potential clinical applications of stem cells.

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‖ Present address: Dionex Canada, 1540 Cornwall Rd., Oakville, Ontario L6J 7W5, Canada.

§§ Holds the Mach Gaennelsen Chair in Cardiac Research at the Ottawa Health Research Institute. To whom correspondence should be addressed. Tel.: 613-737-8618; Fax: 613-737-8803; E-mail: lmegeney@ohri.ca.

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