Cyclin K-containing Kinase Complexes Maintain Self-renewal in Murine Embryonic Stem Cells

Received for publication, November 8, 2011, and in revised form, March 31, 2012. Published, JBC Papers in Press, April 30, 2012, DOI 10.1074/jbc.M111.21760

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Background: The physiological function of cyclin K is poorly defined.

Results: Cyclin K interacts with CDK12 and CDK13, and knockdown of cyclin K, CDK12, or CDK13 causes embryonic stem cell differentiation.

Conclusion: Cyclin K, CDK12, and CDK13 are required for embryonic stem cell self-renewal.

Significance: Novel kinase complexes are identified to maintain embryonic stem cell pluripotency.

Protein phosphorylation plays an important role in the regulation of self-renewal and differentiation of embryonic stem cells. However, the responsible intracellular kinases are not well characterized. Here, we discovered that cyclin K protein was highly expressed in pluripotent embryonic stem cells but low in their differentiated derivatives or tissue-specific stem cells. Upon cell differentiation, the level of cyclin K protein was decreased. Furthermore, knockdown of cyclin K led to cell differentiation, which could be rescued by an expression construct resistant to RNA interference. Surprisingly, cyclin K did not interact with CDK9 protein in cells as thought previously. Instead, it associated with CrkRS (also known as CDK12) and CDC2L5 (also known as CDK13). Similar to cyclin K, both CDK12 and CDK13 proteins were highly expressed in murine embryonic stem cells and were decreased upon cell differentiation. Importantly, knockdown of either kinase resulted in differentiation. Thus, our studies have uncovered two novel protein kinase complexes that maintain self-renewal in embryonic stem cells.

Pluripotent ES cells are capable of differentiating to essentially all cell lineages, whereas under appropriate tissue culture conditions, they can self-renew indefinitely in vitro (1, 2). Much progress has been made to elucidate the molecular control at the level of transcription (3). The core transcription factors Oct4, Sox2, and Nanog collaborate to activate the expression of genes that promote self-renewal and repress that of lineage-specific genes (4). Yet, our current understanding of other regulatory mechanisms remains incomplete. Protein phosphorylation has emerged recently as an important control of ES cell self-renewal and differentiation. The activity of core transcription factors Oct4 (5), Sox2 (6), and Nanog (7) are controlled tightly by phosphorylation. In addition, dynamic changes in global protein phosphorylation occur during early differentiation of ES cells (8, 9). However, key intracellular kinases that regulate self-renewal as well as differentiation are not well characterized.

The physiological function of cyclin K protein (encoded by CCNK) is defined poorly. This is reflected by the fact that the physiological form of cyclin K protein (CycK) has not been demonstrated convincingly so far. Human CCNK was cloned initially to encode a putative protein of 357 amino acid residues (calculated molecular mass, ~41 kDa) (10). However, Expressed Sequence Tag profiling studies in genome databases favor a putative alternatively spliced transcript encoding a protein of 580 amino acid residues to be the predominant form (calculated molecular mass, ~65 kDa). In addition, murine CCNK is predicted to encode only one putative transcript homologous to the longer transcript in humans. Therefore, the physiological form of CycK remains to be determined. Perhaps the most accepted function of CycK is to participate in RNA polymerase II transcription. This is because CycK has long been thought to interact with CDK9 protein, a well established elongation factor in RNA polymerase II transcription (11, 12). This interaction was initially identified in a yeast two-hybrid screen (13) but has never been demonstrated in mammalian cells. In addition, unlike cyclin T1 and T2 (11, 12), two well characterized regulatory subunits of CDK9, CycK does not stimulate transcription when artificially tethered to promoters (14). Nevertheless, CycK-containing protein complex immunoprecipitated from human cells does contain kinase activities in vitro (10, 15).

In this study, we sought to determine the physiological function of CycK protein. Our data are the first to show that the predominant form of CycK protein consists of 554 and 580 amino acid residues in murine and human cells, respectively. We further discovered that cyclin K protein is highly expressed...
in murine ES cells, and its knockdown results in cell differentiation. Surprisingly, cyclin K does not interact with CDK9 in mammalian cells. Instead, it associates with CDK12 and CDK13 proteins. Similar to cyclin K, both CDK12 and CDK13 are highly expressed in murine ES cells, and their knockdown leads to differentiation. Thus, our studies have uncovered two novel protein kinase complexes that maintain self-renewal in embryonic stem cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Feeder-free R1 murine ES cells were cultured in DMEM containing 15% ES cell-grade fetal bovine serum (Gemini Bio-Products), supplemented with 105 units/ml LIF (Strategene), 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 0.1 mM non-essential amino acids. The pluri potency of R1 ES cell culture was monitored routinely by teratoma formation assay. Briefly, 105 ES cells in PBS were injected subcutaneously into the dorsal flank of nude mice. After six to eight weeks, tumors were surgically dissected from the mice, fixed in PBS containing 4% formaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) and characterized by trained medical pathologists (supplemental Fig. 3). Alkaline phosphatase (AP)3 staining of cells was performed following manufacturer’s instructions (Sigma). Derivation and differentiation of dermal stem cells were carried out as described previously (16). Other cell lines were cultured according to ATCC’s guidelines. All cell lines were cultured at 37 °C in a 5% CO2 incubator.

Antibodies—Anti-cyclin K and anti-FLAG M2 antibodies were purchased from Sigma; anti-CDK12, CDK9, CycT1, Oct4, Sox2, and HA antibodies were from Santa Cruz; and anti-actin antibody was from Millipore. Anti-CDK13 antibody was a gift from Dr. Geneviere (Universite Pierre et Marie Curie).

Generation of Anti-CycK Antibody—Synthetic peptide GLDPATEARYRREGAF was used to immunize rabbits (Zen Bioscience, Chengdu, China). Crude antiserum was incubated at 54 °C for 20 min and passaged over recombinant CycK(1–150) covalently attached to Actigel ADL resin (Sterogene) following the manufacturer’s instructions. After washing with PBS, affinity-purified anti-CycK antibody was eluted with 100 mM glycine (pH 2.5), and neutralized with 1 M Tris base. The antibody was aliquoted and stored at −80 °C.

Plasmid Construction—The coding region of murine CycK was cloned from ES cells and verified by sequencing. For rescue experiment in ES cells, three silent mutations (G651A, C654T, and A657G) were introduced by site-directed mutagenesis (150 mM NaCl, 1.5 mM MgCl2, 10 mM KCl, 20 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.1% PMSF, EDTA-free complete protease inhibitor mixture (Roche) and 0.5% Nonidet P-40). Total cell lysates were cleared by centrifugation for 10 min at 17,000 × g/4 °C. Supernatant was then mixed with indicated antibodies immobilized on protein A/G beads (Santa Cruz) and rotated for at least 90 min at 4 °C. Protein A/G beads were then washed by buffer C, supplemented with 5 mM NaCl to final concentration of 500 mM. Protein complexes were eluted by 2 × protein loading buffer for subsequent Western blot analyses.

Immunoprecipitation—Cells were lysed in ice-cold buffer C (150 mM NaCl, 1.5 mM MgCl2, 10 mM KCl, 20 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 0.1% PMSF, EDTA-free complete protease inhibitor mixture (Roche) and 0.5% Nonidet P-40). Total cell lysates were cleared by centrifugation for 10 min at 17,000 × g/4 °C. Supernatant was then mixed with indicated antibodies immobilized on protein A/G beads (Santa Cruz) and rotated for at least 90 min at 4 °C. Protein A/G beads were then washed by buffer C, supplemented with 5 mM NaCl to final concentration of 500 mM. Protein complexes were eluted by 2 × protein loading buffer for subsequent Western blot analyses.

Differentiation of ES Cells—ES cells grown on six-well tissue-culture plates were washed in PBS, trypsinized, and resuspended in ES cell growth medium without LIF (ES/−LIF). Cells

3 The abbreviations used are: AP, alkaline phosphatase; qPCR, quantitative PCR; MEF, murine embryonic fibroblast; LIF, leukemia inhibitory factor.
were then seeded onto a 6-cm bacterial Petri dish and grew in suspension to form embryoid bodies. ES/LIF medium was refreshed every other day. After 7 days, embryoid bodies were transferred onto gelatin-coated tissue-culture plates and cultured for another seven to 14 days in ES/LIF medium.

**Immunofluorescence**—R1 mouse ES cells were grown on six-well tissue-culture plates, fixed for 20 min in 4% paraformaldehyde/PBS, and washed in PBS twice. Cells were permeabilized with 0.3% Triton-X 100 in PBS for 10 min, and then washed in PBS. After being blocked in 3% BSA in PBS for 1 h, cells were incubated with primary antibodies (1:200 dilution) overnight at 4 °C. After washing in PBS for 20 min, cells were incubated in 1:400 dilution of Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 568 goat anti-mouse IgG secondary antibodies (Molecular Probes) for 1 h, followed by washing in PBS for 20 min. To visualize DNA, cells were stained with 0.5 mg/ml of DAPI after secondary antibody incubation.

**Tissue Extracts**—Tissues (~0.3 cm³) were surgically dissected from male BALB/c mice and homogenized in radioimmune precipitation assay buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% PMSF, 1 mM Na₃VO₄, EDTA-free complete protease inhibitor mixture (Roche), and 50 mM NaF). Lysates were cleared by centrifugation for 5 min at 17,000 × g/4 °C. Supernatant was then collected for subsequent Western blot analyses.

**RESULTS**

Determine Physiological Form of CycK Protein—To determine the physiological form of CycK protein, Western blot analysis was carried out using a commercial antibody against the N-terminal region identical in two putative human isoforms. Only one band with a molecular mass of ~70 kDa was detected in various human and murine cell lines (Fig. 1A), indicating that predominant form of CycK protein contains 580 and 554 amino acid residues in human and murine cells, respectively. To verify this result, an anti-CycK antibody was generated against an epitope identical in putative murine and human isoforms (Fig. 1B). Although the antiserum detected three bands above 50 kDa, the affinity-purified antibody only recognized one band with molecular mass ~70 kDa in HeLa cell lysate (Fig. 1C). In addition, preincubation of affinity-purified antibody with the epitope peptide abolished this signal (Fig. 1D), confirming specificity of the antibody. Importantly, two short hairpin RNA (shRNA) constructs (hK-1 and hK-2), targeting different regions identical in putative human CCNK isoforms, but not scramble shRNA efficiently reduced the signal detected (Fig. 1E). The specificity of shRNA was further verified by reduction of CCNK mRNA, revealed by a qPCR experiment (Fig. 1F). The corresponding murine cDNA, encoding a protein of 554 amino acid residues, was subsequently cloned and utilized in the rest of experiments. Consistently, transient expression of this cDNA generated one band that overlapped with endogenous CycK in F9 cells (data not shown). Taken together, we concluded that the predominant form of CycK protein contains 580 and 554 amino acid residues in human and murine cells, respectively. In the following experiments, our customer-made, affinity-purified antibody was used to detect the expression of CycK unless otherwise specified.

CycK Is Highly Expressed in ES Cells—To further characterize the physiological function of CycK, its expression profile was investigated in various murine tissue and cell types. Consistent with the previous report (19), CDK9 was expressed ubiquitously in tissues with a higher ratio of CDK9/55 in testis and CDK9/22 in brain and liver (Fig. 2A). Surprisingly, the expression of CycK was only detectable in testis (Fig. 2A). Because tissues contain mostly fully differentiated cells, we reasoned that CycK might be expressed in less differentiated cell types. Indeed, the protein level of CycK was high in murine ES cells but hardly detectable in highly differentiated murine embryonic fibroblasts (MEFs) under the same condition (Fig. 2B). A weak signal could be detected in MEFs only after a much longer exposure time. In addition, various signals including proliferation (Fig. 2C), serum stimulation (Fig. 2D), and UV irradiation (Fig. 2E) failed to increase the level of CycK in MEFs. Furthermore, when ES cells were induced to differentiate in vitro using the standard embryoid body formation procedure, the protein level of CycK was gradually decreased, concomitant

![FIGURE 1. Identification of the physiological form of CycK protein.](image-url)
with the reduction of Oct4, a master regulator of self-renewal (Fig. 2F). qPCR analyses further revealed a reduction in CycK mRNA level (data not shown), indicative of transcription regulation. The expression of CycK in pluripotent ES cells was also confirmed by immunofluorescence staining, which occurred only in Oct4-positive cells (Fig. 2G).

CycK Is Not Expressed in Dermal Stem Cells—The expression of CycK was further explored in other stem cell types. Dermal stem cells (also known as Skin-derived precursors) (20) were chosen because of its availability in the lab (Fig. 3A). Consistent with literature (20, 21), our dermal stem cells were multipotent in that they could be induced to generate osteoblasts as well as adipocytes (Fig. 3, B and C). However, unlike in ES cells, CycK was hardly detectable in dermal stem cells (Fig. 3D).

**Knockdown of CycK Leads to Differentiation of ES Cells**—Based on the above observations, we hypothesized that CycK is required for ES cell self-renewal. To test this, we first asked whether knockdown of CycK by shRNA induces differentia-
tion. The level of CycK in murine ES cells was efficiently reduced by transient transfection with shRNA specific for murine CycK (mK-1), but not with scramble or two different shRNA constructs specific for human CycK (hK-1 and hK-2) (Fig. 4A). Notably, mK-1 and hK-1 differs in only one nucleotide in the seed region, which is important for shRNA to recognize its cognate mRNA, demonstrating that knockdown by mK-1 was highly specific. Undifferentiated ES cells grow in compact colonies and show strong staining for AP. Stable knockdown by scramble shRNA did not change the compact morphology or positive AP staining of ES cells. In sharp contrast, stable knockdown of CycK by mK-1 induced flat cell morphology and negative AP staining, indicating differentiation of ES cells (Fig. 4B). Differentiation was further demonstrated by a great reduction in Sox2, a master regulator of self-renewal (Fig. 4C, lane 3). We noticed that after stable knockdown, a few ES cell-like colonies survived and kept growing. This could be because CycK was not knocked down efficiently in those cells. Alternatively, this might indicate that a subpopulation of ES cells does not require CycK for self-renewal. To distinguish these two possibilities, cells stably transfected with mK-1 were trypsinized, reseeded, and grown for 7 days before harvest (mK-1-split). Most cells did not attach upon passage. The remaining cells grew in an ES cell-like morphology.

results suggest that expression of CycK is strongly correlated with self-renewal of ES cells.

To verify the specificity of knockdown, a rescue experiment was carried out. GFP was fused to the C terminus of CycK cDNA (CycK-R) containing silent mutations that would disrupt the recognition by mK-1 shRNA. This fusion protein had a

FIGURE 3. CycK protein is not expressed in dermal stem cells. A, the morphology of dermal stem cells observed under microscope. B, dermal stem cells were induced in vitro to generate osteoblasts (indicated by arrows), revealed by Alizarin Red S staining for calcium. C, dermal stem cells were induced in vitro to generate adipocytes (indicated by arrows), revealed by Oil Red O staining for lipid deposits. D, expression of CycK was analyzed in ES and dermal stem cell extracts by Western blotting.

FIGURE 4. CycK protein maintains self-renewal in mouse ES cells. A, transient knockdown by scramble (scra), human-specific (hK-1 and hK-2), or murine-specific (mK-1) shRNA constructs in murine ES cells. Knockdown efficiency was analyzed by Western blotting. B, stable knockdown by scramble or mK-1 shRNA. Cell differentiation was indicated by negative AP staining as well as flat cell morphology. C, protein expression was analyzed by Western blotting in extracts from untransfected ES cells (—), cells stably transfected with scramble, or mK-1 shRNA. Cells stably transfected with mK-1 were also trypsinized, reseeded, and grown for 7 days before harvest (mK-1-split). Most cells did not attach upon passage. The remaining cells grew in an ES cell-like morphology. D, rescue of CycK expression by an RNAi-resistant CycK expression vector (CycK-R). Cell extracts from untransfected cells and cells transiently transfected with mK-1, CycK-R, or both were analyzed by Western blotting. E, rescue of self-renewal by CycK-R at the presence of mK-1 shRNA. Cells were stably transfected with indicated constructs and stained by AP. F, stable transfection with another CycK-specific shRNA (mK-2) led to differentiation, revealed by loss of Oct4 immunofluorescence staining. Background fluorescence was shown on the lower panel when only secondary antibody (2nd) was used. G, protein expression was examined by Western blotting in extracts from cells stably transfected with scramble or mK-2 shRNA. DNA was revealed by DAPI staining.
Cyclin K-CDK12/13 Maintain Stem Cell Self-renewal

FIGURE 5. Cyclin K interacts with CDK12 and CDK13 but not CDK9.
A, endogenous CDK9 was immunoprecipitated from ES cell extract, followed by Western blotting (WB) with indicated antibodies. B, the experimental flow-chart to identify Cyclin K-associated kinases by an unbiased proteomic approach. C, identical purification procedure was carried out using extracts from naïve NIH 3T3 cells (Mock) or cells stably expressing tagged Cyclin K (FH-Cyclin K). Purified materials were visualized by silver. Specific polypeptides in FH-Cyclin K were analyzed by LC-MS/MS. D, purified materials in CDK12-F were analyzed by LC-MS/MS. Identity of Cyclin K was revealed by Western blotting. F, domain mapping of CDK12 and CDK13. cDNAs encoding individual domains were transfected into HEK293 cells, followed by anti-FLAG immunoprecipitation, and a sequential Western blotting with anti-Cyclin K as well as FLAG antibodies. In, Input.

The identified interactions were further verified by reciprocal purifications. Two independent HEK 293-based cell lines stably expressing FLAG-tagged CDK12 (CDK12-F) were established and utilized for the purification of CDK12-containing protein complex using anti-FLAG antibodies. The purified complex was eluted by protein loading buffer, separated by SDS-PAGE, and visualized by silver (Fig. 5E). Mass spectrometric analyses revealed the presence of Cyclin K in both purifications. The identity of Cyclin K was further verified by Western blot analysis (Fig. 5E). In addition, the originally cloned Cyclin K (357 amino acids) interacted with CDK12 and CDK13 (supplemental Fig. 2). Thus, we concluded that Cyclin K interact with CDK12 and CDK13 but not CDK9.

Cyclin K Does Not Interact with CDK9 in Mammalian Cells—Although Cyclin K has long been thought to interact with CDK9, this interaction has never been demonstrated in mammalian cells. To verify this interaction, cDNA encoding FLAG-tagged Cyclin K (the originally cloned form containing 357 amino acid residues) or FLAG-tagged cyclin T1 (Cyclin T1), a well-established regulatory subunit of CDK9, was transfected into HEK 293 cells. Endogenous CDK9 efficiently pulled down FLAG-tagged Cyclin T1 but surprisingly not Cyclin K (supplemental Fig. 1).

As we have shown that the physiological form of murine Cyclin K consists of 554 amino acid residues (Fig. 1), we examined the interaction between endogenous CDK9 and this newly identified form of Cyclin K in ES cells. Endogenous CDK9 was immunoprecipitated from ES cell extract and probed for Cyclin T1 or Cyclin K. Again, Cyclin K was not detectable in anti-CDK9 immunoprecipitate, whereas Cyclin T1 was pulled down efficiently (Fig. 5A).

Identify Cyclin K-interacting Kinases by Mass Spectrometry—An unbiased proteomic approach was utilized to identify Cyclin K-associated kinases. For the cost reason, NIH 3T3 instead of ES cells were used. NIH 3T3 cells had a much lower but nevertheless detectable level of endogenous Cyclin K than ES cells. A cell line stably expressing Cyclin K cDNA fused with a FLAG-His$_6$ tag at the N terminus (FH-Cyclin K) was established. The nuclear extract was generated and utilized for a sequential affinity purification procedure using anti-FLAG antibody followed by nickel-nitrilotriacetic acid (Fig. 5B). The purified complex was separated by SDS-PAGE and visualized by silver staining. Compared with mock purification, several specific polypeptides were detected in Cyclin K-containing protein complex (Fig. 5C).

Mass spectrometric analyses of these polypeptides identified CDC2L5 (also known as CDK13) protein. To verify this result, purification was repeated, and an independent mass spectrometric analysis was carried out. Surprisingly, CrkR5 (also known as CDK12) but not CDK13 was identified. A close examination of two mass spectrometric data sets revealed that none of ten unique peptides assigned to either CDK12 or CDK13 each time were actually shared by both kinases. Indeed, both CDK12 and CDK13 but not CDK9 protein were specifically present in purified Cyclin K-containing protein complex, revealed by corresponding antibodies (Fig. 5D).

slower mobility than endogenous Cyclin K on SDS-PAGE. When co-transfected with mK-1 shRNA, the expression of Cyclin K-R but not endogenous Cyclin K remained (Fig. 4D, lane 4). As expected, only a few AP-positive colonies remained after stable knockdown by mK-1 shRNA. In contrast, when mK-1 shRNA and Cyclin K-R cDNA in a ratio of 3:1 were co-transfected into ES cells and stably selected, a reproducible, severalfold increase in AP-positive colonies was observed repeatedly (Fig. 4E), demonstrating that self-renewal was rescued by expression of Cyclin K-R.

Knockdown of Cyclin K by a different shRNA, mK-2, targeting the 3’-UTR of Cyclin K mRNA was also carried out. Similarly to mK-1, stable transfection of mK-2 efficiently induced flat cell morphology and loss of Oct4 staining (Fig. 4F). In addition, both Oct4 and Sox2 proteins were reduced greatly (Fig. 4G). Taken together, we concluded that Cyclin K is required to maintain self-renewal in ES cells.

Acell cells were used. NIH 3T3 cells had a much lower but nevertheless detectable level of endogenous Cyclin K than ES cells. Acell cells were used. NIH 3T3 cells had a much lower but nevertheless detectable level of endogenous Cyclin K than ES cells.
Cyclin K-CDK12/13 Maintain Stem Cell Self-renewal

Knockdown of CDK12 or CDK13 Leads to ES Cell Differentiation—The expression profile of CDK12 and CDK13 was examined in ES cells. ES cells were induced to differentiate in vitro using the standard embryoid body formation procedure for 2 weeks (Fig. 6A). Cells were collected every week and analyzed for the expression of CDK12 and CDK13. Similar to CycK, both CDK12 and CDK13 were highly expressed in ES cells, and this expression was reduced rapidly during differentiation (Fig. 6B).

The role of CDK12 and CDK13 in self-renewal was further investigated. Similar to CycK, stable knockdown of either CDK12 or CDK13 by shRNA efficiently reduced the level of Oct4 and Sox2, master regulators of self-renewal (Fig. 6C). In addition, flattened cell morphology was observed, concomitant with loss of Oct4 staining (Fig. 6D), demonstrating that both CDK12 and CDK13 are required to maintain self-renewal. Expression profiles of a panel of self-renewal as well as differentiation marker genes were analyzed by qPCR and RT-PCR, respectively. Consistent with their roles in maintaining ES cells, knockdown of CDK12, CDK13, or CycK led to reduced expression of self-renewal markers (Fig. 6E) and increased expression of differentiation markers (Fig. 6F). Importantly, the expression profile of differentiation marker genes was different when CDK12 or CDK13 was knocked down, indicating that the functions of these two kinases are not redundant. Taken together, we concluded that CDK12 and CDK13, together with CycK, play an important role in maintaining self-renewal in ES cells.

**DISCUSSION**

ES cells are self-renewing, pluripotent cells, capable of differentiating into essentially all cell types. These properties make

Background fluorescence was shown on the lower panel when only secondary antibody (2nd) was used. DNA was revealed by DAPI staining. E, expression levels of pluripotency genes were analyzed by qPCR in cells stably transfected with scramble (sra), CycK, CDK12, or CDK13-specific shRNA. Expression levels were normalized to actin level and relative to the expression levels of the same transcripts in cells transfected with scramble shRNA. Expression ratios are average measurements from three independent analyses; S.D. are shown. F, expression levels of marker genes representative of differentiation were analyzed by RT-PCR in cells stably transfected with scramble, CycK, CDK12, or CDK13-specific shRNA. Fgf5 and Sox9 (ectoderm), T (mesoderm), Pdx1, Gata4 and Sox17 (endoderm), Hand1, Cdx2, Pax3 (mesoderm), Pax6 (ectoderm), and Dermo (dermal differentiation).
ES cells an ideal model system to study mammalian development and promise future medical applications in regenerative medicine (1, 2). Much of the transcriptional network that controls self-renewal has been elucidated. The core transcription factors Oct4, Sox2, and Nanog collaborate to activate the expression of genes that promote self-renewal and repress that of lineage-specific genes (3, 4). However, our understanding of molecular controls at other levels is behind. Protein phosphorylation is a major transducer of developmental as well as environmental stimuli. For this reason, perhaps it is surprising that intracellular protein kinases important for self-renewal are not well characterized in ES cells.

In this study, we identified two novel protein complexes, CycK-CDK12 and CycK-CDK13. We showed that they are highly expressed in murine ES cells. We further demonstrated that CycK, CDK12, and CDK13 are required to maintain self-renewal in ES cells. The functions of both CDK12 (also known as CrkRS) (22) and CDK13 (also known as CDC2L5) (23) are not well understood. Several lines of evidence indicate that they may regulate certain aspect of transcription. Both proteins contain serine/arginine-rich regions at their N termini, a motif frequently present in splicing factors (22, 24). Indeed, both human CDK12 and CDK13 proteins are coocolated with spliceosome components in nuclear speckles (22, 24). CDK13 can phosphorylate splicing factor ASF/SF2 in vitro (25), although it is not clear whether this happens in vivo or its functional outcomes. Drosophila CDK12 protein has been shown recently to be a transcription elongation-associated kinase (26). As the transcription of many developmental genes is controlled at the step of elongation in ES cells (27), it is tempting to speculate that CDK12 may regulate the expression of these genes. Interestingly, Oct4, Sox2, and Nanog, master regulators of self-renewal, recently have been shown to be regulated by phosphorylation in ES cells, although the responsible kinases are not known (5–7).

As there are several consensus CDK phosphorylation sites in these factors, it will be of interest to determine whether CycK-CDK12 and CycK-CDK13 are directly responsible for these phosphorylations.

During the preparation of our manuscript, Blazek et al. (28) showed that CycK interacts with CDK12 and CDK13 but not CDK9 protein, consistent with our results. In addition, in an attempt to generate CycK knock-out mice, they failed to generate any homozygous CycK−/− offspring and did not detect any CycK−/− embryos (28). In light of our findings in this manuscript, we suspect that this early embryonic lethal phenotype might be caused by failure to maintain inner cell mass, from which ES cells are derived (1). Interestingly, they also showed that CDK12 may regulate expression of a subset of genes involved in DNA damage response (28). It will be of interest to determine whether these are downstream effectors of CDK12 in ES cells as genomic stability has previously been linked to ES cell differentiation (29).

In summary, we identified two cyclin K-dependent kinase complexes that are required to maintain self-renewal in ES cells. Identification of their substrates in ES cells in future will be the key to elucidate the underlying molecular mechanisms.

Acknowledgments—We thank Jonathon Pines (University of Cambridge) for CDK12 cDNA, Anne-Marie Genevière (Université Pierre et Marie Curie) for anti-CDK13 antibody and CDK13 cDNA.

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