THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 9, Issue of February 27, pp. 8181–8189, 2004

Printed in U.S.A.

HLS5, a Novel RBCC (Ring Finger, B Box, Coiled-coil) Family Member Isolated from a Hemopoietic Lineage Switch, Is a Candidate Tumor Suppressor*

Received for publication, June 25, 2003, and in revised form, December 5, 2003
Published, JBC Papers in Press, December 8, 2003, DOI 10.1074/jbc.M306751200

Jean-Philippe Lalonde‡§, Raelene Lim‡¶‖, Evan Ingley‡, Peta A. Tilbrook‡, Martin J. Thompson‡, Ross McCulloch‡, Jennifer G Beaumont‡, Carol Wicking, Helen J. Eyre‡‡, Grant R. Sutherland‡‡, Kathy Howe§§, Ellen Solomon¶¶, James H. Williams‡, and S. Peter Klinken‡‖‖**

*From the ‡Laboratory for Cancer Medicine, Western Australian Institute for Medical Research, Royal Perth Hospital and the Center for Medical Research, The University of Western Australia, Perth, Western Australia 6000, Australia, ¶School of Biomedical Sciences, Curtin University of Technology, Bentley, Western Australia 6102, Australia, **Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia, ‡‡Center for Medical Genetics, Department of Cytogenetics and Molecular Genetics, Women's Hospital, Adelaide, Southern Australia 5006, Australia, §§Lymphocyte Activation Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom, and ¶¶Division of Medical and Molecular Genetics, Guy's School of Medicine, King's School of Medicine, and St. Thomas' School of Medicine, London SE1 9RT, United Kingdom*

Hemopoietic cells, apparently committed to one lineage, can be reprogrammed to display the phenotype of another lineage. The J2E erythroleukemic cell line has on rare occasions developed the features of monocytic cells. Subtractive hybridization was used in an attempt to identify genes that were up-regulated during this erythroid to myeloid transition. We report here on the isolation of hemopoietic lineage switch 5 (*Hls5*), a gene expressed by the monocytoid variant cells, but not the parental J2E cells. *Hls5* is a novel member of the RBCC (Ring finger, B box, coiled-coil) family of genes, which includes *Pml*, *Herf1*, *Tif-1α*, and *Rfp*. Hls5 was expressed in a wide range of adult tissues; however, at different stages during embryogenesis, *Hls5* was detected in the branchial arches, spinal cord, dorsal root ganglia, limb buds, and brain. The protein was present in cytoplasmic granules and punctate nuclear bodies. Isolation of the human cDNA and genomic DNA revealed that the gene was located on chromosome 8p21, a region implicated in numerous leukemias and solid tumors. Enforced expression of *Hls5* in HeLa cells inhibited cell growth, clonogenicity, and tumorigenicity. It is conceivable that *HLS5* is one of the tumor suppressor genes thought to reside at the 8p21 locus.

Differentiated cells for each of the hemopoietic lineages develop from pluripotent stem cells (1), a process orchestrated via specific combinations of transcription factors (2, 3). Lineage-restricted structural/functional genes are gradually expressed as cells display the phenotype of mature cells. Although the process of lineage commitment was once considered irrevocable, data over the past decade indicate that the hemopoietic system may be more plastic than previously thought (4). Leukemic cells apparently committed to one lineage can be "reprogrammed" or undergo "lineage switching" and develop the appearance of another cell type (5, 6).

Early descriptions of lineage switching involved the conversion of B lymphoma cells overexpressing the *raf* and *myc* oncogenes into macrophages (5, 7). Other B cell to macrophage transitions involved a number of different oncogenes (8–11), whereas mast cells transformed with *Bcr-abl* have produced erythroid and megakaryocytic cells (12). A B cell to granulocytic switch has also been observed previously (13). The recent data with *Pax5*−/− mice are particularly noteworthy as committed B cells lacking the *Pax5* gene could develop into macrophages, osteoclasts, dendritic cells, granulocytes, natural killer cells, and T cells upon appropriate stimulation (14, 15).

The combination and concentration of transcription factors within hemopoietic cells play key roles in dictating lineage fate (3, 16). Indeed, by manipulating the levels of transcription factors, the plasticity of the hemopoietic system has been demonstrated, *e.g.* increasing the concentration of GATA-1 in myelomonocytic cells produced thrombocytes, eosinophils, and erythroblasts (6). Similarly, overexpression of GATA-1 or NF-E2 in M1 monoblastoid cells generated erythroid and megakaryocytic cells (17, 18). In addition, overexpression of PU.1 in murine erythroleukemia cells induced an erythroid to myeloid lineage switch (19).

One example of a spontaneous lineage switch was the conversion of J2E erythroleukemic cells into monocytoid cells (20). The parental J2E cells differentiated morphologically and synthesized hemoglobin in response to erythropoietin (21, 22), whereas the monocytic variants (J2E-m)[1] lacked all of the

* This work was supported by the National Health and Medical Research Council of Australia (Grant 212070), the National Breast Cancer Foundation, the Cancer Foundation of Western Australia, the Medical Research Foundation of Royal Perth Hospital, and Cancer Research UK. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF009514, AF009513, AAC17945, AF145374, AF494189, and AF492463.

§ Recipient of an M.D. and Ph.D. Ad Hoc scholarship awarded by the Stalker McEwan fund through the Medical Research Foundation of Royal Perth Hospital.

‖ Recipient of an Australian Postgraduate Award through Curtin University of Technology.

‖‖ To whom correspondence should be addressed: Laboratory for Cancer Medicine, Level 6, Medical Research Foundation Bldg., Rear, 50 Murray St., Perth, Western Australia 6000, Australia. Tel.: 61-8-92240334; Fax: 61-8-92240322; E-mail: pklinken@waimr.uwa.edu.au.

[1] The abbreviations used are: J2E-m, monocytic variant of J2E cell; dpc, days post-coitum; HLS or *Hls*, hemopoietic lineage switch; MLF1, myeloid leukemia factor 1; RACE, rapid analysis of the cDNA ends;

erythroid characteristics and displayed myeloid surface markers (20). To search for genes involved in this breach of lineage commitment, we have utilized cDNA representational difference analysis (RDA) (23). This PCR-based form of subtractive hybridization identified hemopoietic lineage switch 7 (*Hls7*), the murine homologue of human myeloid leukemia factor 1 (*MLF1*) (24, 25), as one molecule expressed exclusively in the myeloid variant (23, 26). Ectopic expression of *Mlf1* in parental J2E cells recapitulated the phenotypic changes associated with the erythroid to myeloid switch. Moreover, exogenous *Mlf1* impeded the differentiation of normal and immortalized erythroid cells and promoted myeloid maturation (23). These observations implicated *Mlf1* as an important hemopoietic lineage-determining gene.

In this study, we describe the isolation of *Hls5* from the RDA between J2E cells and a myeloid counterpart, J2E-m2. *Hls5* is a novel member of the Ring finger, B box, coiled-coil (RBCC) family of genes, which includes the well characterized tumor suppressor gene *Pml* (27) as well as *Tif1-α* (28), *Herf1* (29), and *Rfp* (30). The human *HLS5* gene mapped to chromosome 8p21, a locus involved in numerous leukemias and cancers (31). Significantly, expression of exogenous *Hls5* suppressed the tumorigenic phenotype of HeLa cells. Therefore, it is plausible that *Hls5* is one of the tumor suppressor genes postulated to reside at 8p21.

EXPERIMENTAL PROCEDURES

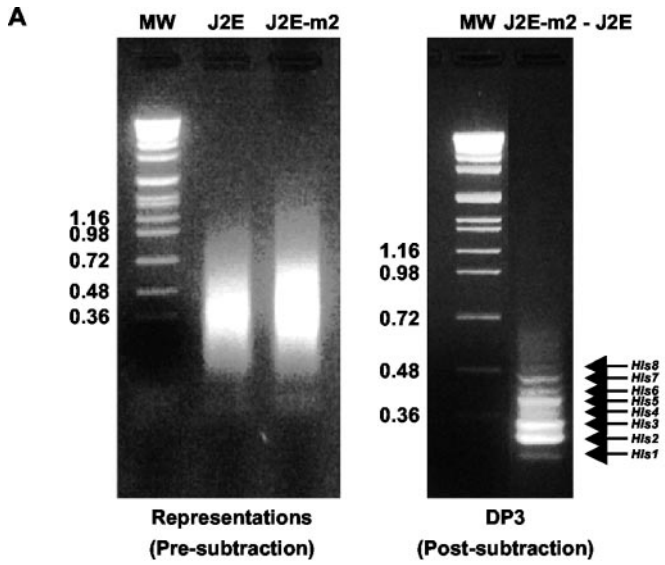
Cell Culture—Cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal calf serum, and cell viability was determined by eosin exclusion (22). Total RNA and protein isolated from various tissues and cell lines were analyzed by Northern blotting and immunoblotting as described previously (23, 32).

cDNA RDA—The RDA used was based on the method of Hubank and Schatz (33) and modified as described previously (23). Poly(A)$^+$ RNA isolated from J2E driver and J2E-m2 tester cell lines was converted to double-stranded DNA, digested with Sau3AI, and subjected to three cycles of subtraction. Fragments enriched by this procedure were subcloned into pGEM-T (Promega, Madison, WI) for further analysis.

cDNA and Genomic Library Screening—A cDNA library from the murine lymphohematopoietic progenitor cell line EML C.115 (34) was screened for full-length *Hls5* using the RDA fragment as a probe. To confirm that the 5′ end of the cDNA had been isolated, a rapid analysis of the cDNA ends (RACE) strategy was adopted using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA). A 129 SvJ murine genomic library in Lambda DASH™ II (Stratagene, La Jolla, CA) was screened for clones containing the *Hls5* gene. The complete genomic sequence was obtained from two independent clones. The human *HLS5* cDNA was isolated from a human fetal liver cDNA library (Clontech BD Biosciences), and two independent clones were sequenced.

Hls5 Constructs and Infection of HeLa Cells—A Myc epitope tag was introduced at the C terminus of *Hls5* and *HLS5* and then subcloned into pcDNA3 (Invitrogen) and pBabe-puromycin (35) to generate the pcDNA3-*Hls5*-Myc, pcDNA3-*HLS5*-Myc, and pBabe-*Hls5*-Myc, respectively. The PA317-packaging cell line (36) was transfected with linearized pBabe-*Hls5*-Myc, and viral supernatants were used to infect HeLa cells as described previously (32). Individual clones were isolated, and unique viral integration sites were determined by Southern blotting. Myc-tagged Hls5 was detected by immunoblotting using anti-Myc (9E10) antibodies followed by horseradish peroxidase-conjugated antibodies as described elsewhere (23, 32). Visualization was by enhanced chemiluminescence (Amersham Biosciences).

Immunofluorescent Microscopy—COS-7 cells were transiently transfected with pcDNA3-*Hls5*-Myc using LipofectAMINE (Invitrogen). After 24 h, cells were incubated with anti-Myc antibodies (9E10) before application of Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR). After washing, coverslips were mounted in 2.5% 1,4-diazobicyclo[2.2.2]-octane (DABCO, Fluka, New South Wales, Australia) containing 0.00005% Hoechst 33258 (Calbiochem). Fluorescence was visualized on a Bio-Rad MRC 1024 UV-laser scanning confocal microscope.



B

	Homology	Expression (mRNA) J2E	J2E-m2
Hls1	Cyclooxygenase 2	-	+
Hls2	Glutamate carboxypeptidase	-	+
Hls3	Mitochondrial DNA	nd	
Hls4	Reg. subunit PKC	nd	
Hls5	Novel RBCC	-	+
Hls6	GDP dissociation inhibitor D4	-	+
Hls7	Myeloid Leukemia Factor-1	-	+
Hls8	*Hls1,2* concatamer	nd	

FIG. 1. **RDA conducted between the erythroid (J2E) and a myeloid (J2E-m2) cell types.** *A*, the *left panel* shows the cDNA representations generated prior to the analysis. The *right panel* shows the eight individual clones obtained following three rounds of subtraction. *B*, homologies of *Hls1–8* and expression patterns determined by Northern blotting. *nd*, not determined; *MW*, molecular weight.

Clonogenicity and Tumorigenicity Assays—For clonogenicity assays, a layer of 0.8% agar was placed in 6-cm dishes and then 10^4 HeLa cells were seeded in a 0.3% agar layer as described above (37). Colonies were counted 3 weeks after initial plating. Tumorigenicity was determined by injecting 10^6 cells subcutaneously into 6–8-week-old female BALB/c nude mice. Tumor volume was determined twice a week using calipers.

Cell Cycle and Apoptosis Analyses—HeLa cells transfected with vector alone, pcDNA3-*Hls5*-Myc, or pcDNA3-*HLS5*-Myc using LipofectAMINE 2000 were cultured for 24–72 h and then stained with propidium iodide before analysis on a FACScanII instrument (BD Biosciences) as described in detail elsewhere (22, 38). TUNEL (transferase dUTP nick and label) assays were performed according to the manufacturer's instructions (Roche Applied Science). Transfected HeLa cells or those treated with nocodazole (100 ng/ml) were incubated overnight before Cdk1 immunoprecipitation (anti-Cdc2 p34; sc-54, Santa Cruz Biotechnology, Santa Cruz, CA) and assayed for kinase activity as we have described previously (32) using [γ-^{32}P]ATP and histone H1. Phosphorylated protein was separated by SDS-PAGE, transferred, and analyzed by phosphorimaging device.

In Situ Hybridization and Fluorescent in Situ Hybridization—Whole mount *in situ* hybridization on mouse embryos was performed as described previously (39). A digoxigenin-labeled riboprobe was prepared by transcription of a 903-bp fragment from the 3′-untranslated region of *Hls5*. For fluorescent *in situ* hybridization, a 2.4-kb fragment of the human *HLS5* gene was generated by PCR, labeled with biotin-14-dATP and hybridized to metaphase spreads from two normal males using a modification of the previously described technique (40). Images of met-

RDA, representational difference analysis; RBCC, Ring finger, B box, coiled-coil; ERK, extracellular signal-regulated kinase; Pml, promyelocytic leukemia; Rfp, ret finger protein; MAIR, macrophage-derived apoptosis-inducing RBCC protein.

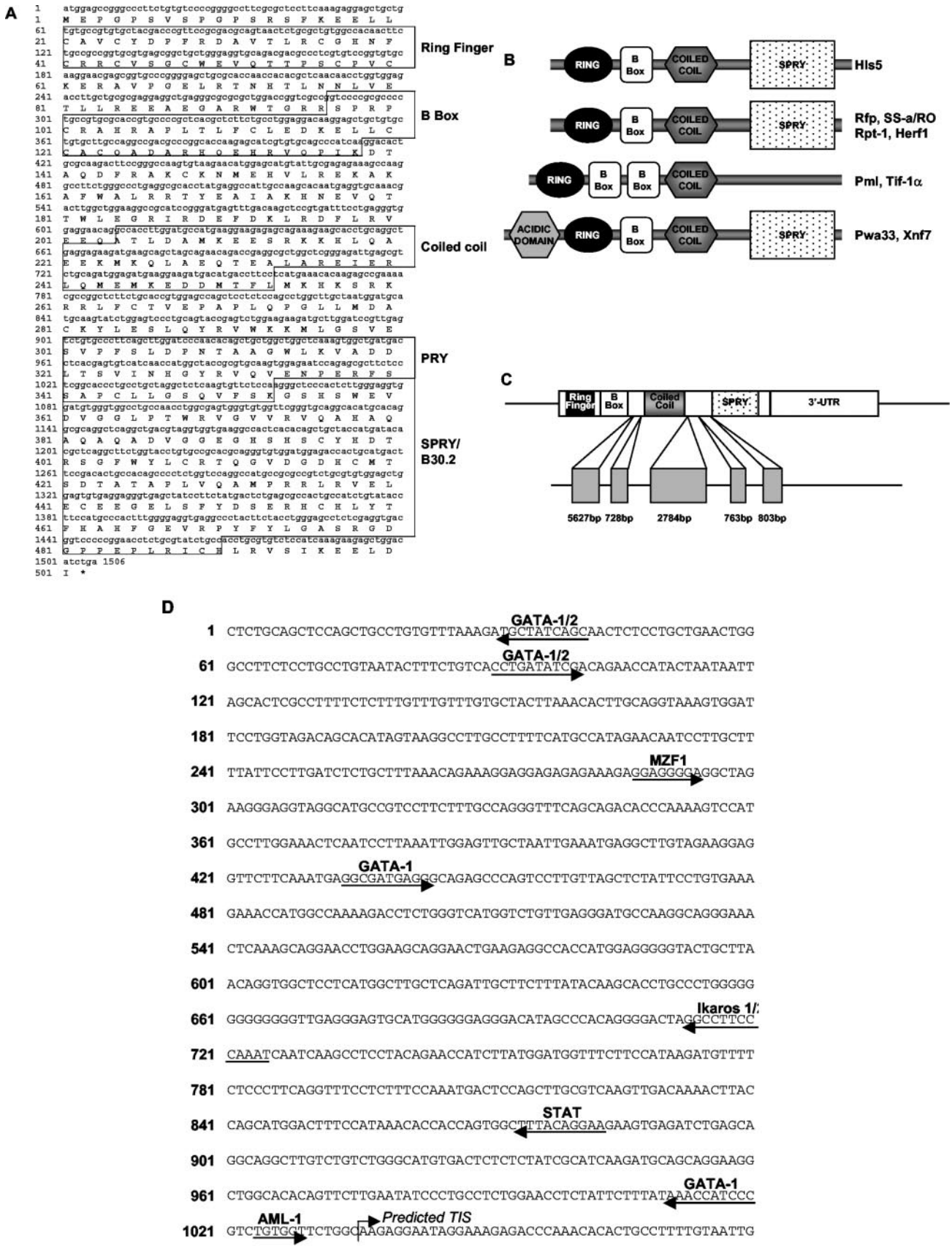


FIG. 2. **Hls5 is a novel member of the RBCC family.** *A*, cDNA and amino acid sequence of murine Hls5. Sequence analysis of the cDNA (*nucleotides numbered above*) identified a 501 amino acid open reading frame (*amino acids numbered below*). The various domains are *boxed* and displayed at the *right*. *B*, structural organization of Hls5 and other members of the RBCC family of proteins. *C*, genomic structure of murine *Hls5* with introns *boxed*. *D*, approximately 1 kb of *Hls5* promoter region with potential transcription factor binding sites and predicted transcription initiation site (*TIS*) identified. *UTR*, untranslated region.

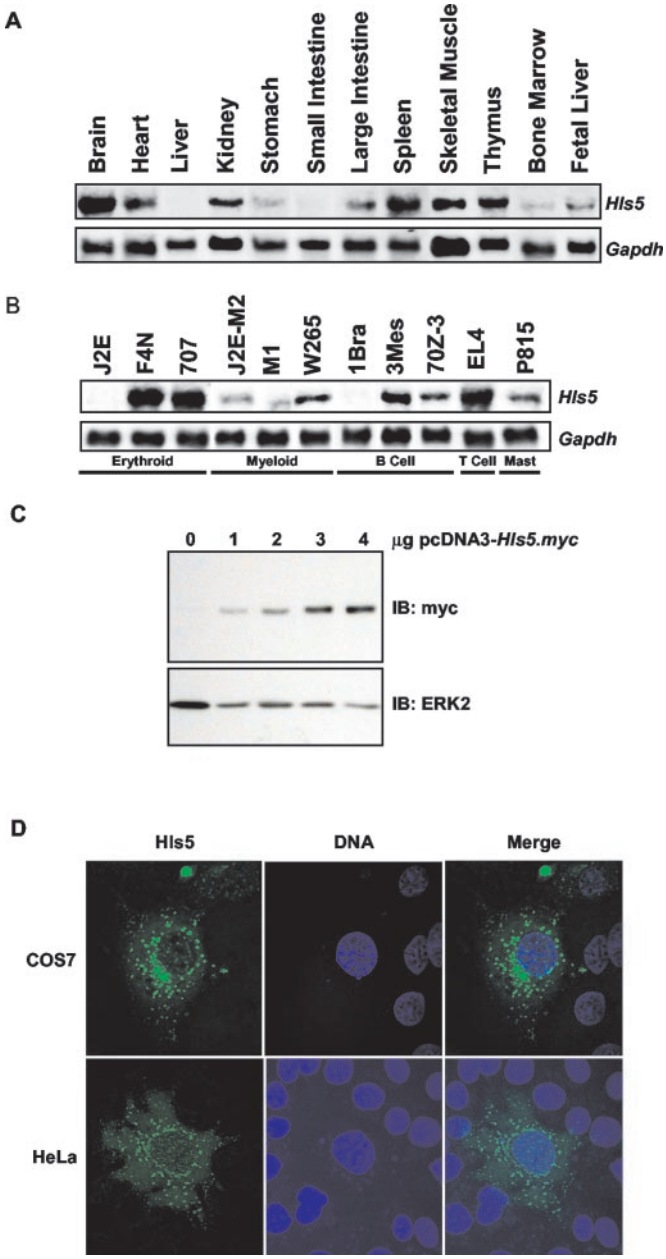


FIG. 3. **Expression of *Hls5* and subcellular localization.** *A*, Northern blot of total RNA from different organs in adult mice. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as a loading control. *B*, Northern blot of total RNA extracted from murine hemopoietic cell lines. *C*, COS cells were transfected with pcDNA-*Hls5*-Myc (1–4 μg) and the protein identified by immunoblotting (*IB*) with anti-myc antibodies. The blot was reprobed with anti-ERK2 antibodies, which served as a loading control. *D*, COS and HeLa cells were transfected with pcDNA-*Hls5*-Myc, and 24 h later, the protein was localized by confocal microscopy using an anti-Myc antibody. DNA was identified by Hoescht staining.

aphase preparations were captured by a cooled CCD camera using the ChromoScan image collection and enhancement system (Applied Imaging International Ltd.).

RESULTS

Isolation of Hls5, a Novel RBCC Family Member—In an attempt to identify genes that were activated when J2E cells became monocytoid, an RDA was conducted between the erythroid and myeloid cell types. Fig. 1*A* shows the representations from J2E and the myeloid variant J2E-m2. Following three rounds of PCR-based subtraction, eight clones were isolated, which were termed *Hls1–8* (Fig. 1*B*). *Hls1* and *Hls6* represented previously characterized myeloid genes, whereas *Hls7* is the murine homologue of *MLF1* (23–25). *Hls3*, *Hls4*, and *Hls8* were not studied further because they included a mitochondrial contaminant, a known signaling molecule and a concatamer.

When originally isolated, *Hls2* was a novel molecule. Full-length *Hls2* was represented by a 1,716-bp cDNA and contained an open reading frame encoding a protein of 433 amino acids (GenBank™ accession numbers AF009513 and AAC17945). However, overexpression and underexpression studies of *Hls2* in a variety of hemopoietic cell lines failed to alter their phenotype. A yeast two-hybrid screen did not identify any binding partners for *Hls2*. This gene was subsequently shown to have 88% identity and 92% homology with the secreted protein, human plasma glutamate carboxypeptidase (41), and was not analyzed further.

The 361-bp *Hls5* fragment (GenBank™ accession number AF009514) was used to screen a murine cDNA library, and then RACE was employed to delineate the 5′ end of the cDNA. These approaches generated a 3,687-bp cDNA (GenBank™ accession number AF145374) with a 501 amino acid open reading frame (Fig. 2*A*). The putative translation initiation site contained a consensus Kozak (42, 43) sequence.

Data base searches revealed that the predicted protein had significant homology with several members of the RBCC family of proteins (Fig. 2*B*). The highest similarity was found with the Rfp (30, 44), SS-a/Ro-52 (45), and Herf1 proteins (29). In addition, these molecules contained an SPRY/B30.2/NHL domain (46). Hls5 also displayed structural similarities to Pml (27) and Tif1-α (28) with the exception that these molecules have two B-box domains and lack the SPRY region. KIAA1098 and TRIM35 represent *Hls5*-related expressed sequence tags (47, 48). While this paper was in preparation, Kimura *et al.* (49) reported the cloning of a cDNA induced by macrophage colony-stimulating factor they named MAIR, which is identical to *Hls5*.

Isolation of genomic clones revealed that the gene contained six exons and five introns ranging in size from 728 bp to 5.6 kb (Fig. 2*C*). Potential binding sites for numerous transcription factors were identified within the putative promoter region (Fig. 2*D*).

Expression of Hls5 and Intracellular Localization—Northern blotting was used to determine the organs in which *Hls5* was expressed. Fig. 3*A* shows that *Hls5* mRNA, ~4 kb in length, was detected in a wide variety of adult organs. Abundant mRNA was present in brain, heart, kidney, spleen, skeletal muscle, and thymus with lower levels found in stomach, large intestine, and bone marrow. Interestingly, *Hls5* was not detectable in adult liver, whereas it was present in fetal liver.

As *Hls5* was isolated from an erythroid to myeloid lineage switch, the expression pattern of *Hls5* was examined in a panel of hemopoietic cell lines (Fig. 3*B*). Although the levels varied considerably, *Hls5* mRNA was present in most erythroid, myeloid, and lymphoid lines tested; however, the transcript was not detected in J2E cells or the pre-B cell line 1-Bra. The difference in *Hls5* expression between J2E cells and other erythroleukemic lines (F4N, 707) could be attributed to immortalization of these cells at different stages of red cell maturation in which J2E cells displayed a more mature phenotype than the other erythroid lines (23).

The predicted molecular mass of the Hls5 protein was 59 kDa; however, immunoblotting of transfected COS cells identified a 66-kDa protein (Fig. 3*C*). The endogenous protein could not be detected in a variety of cell lines, presumably because of low levels of expression.

Indirect immunofluorescence and confocal microscopy were then employed to determine the intracellular location of Hls5

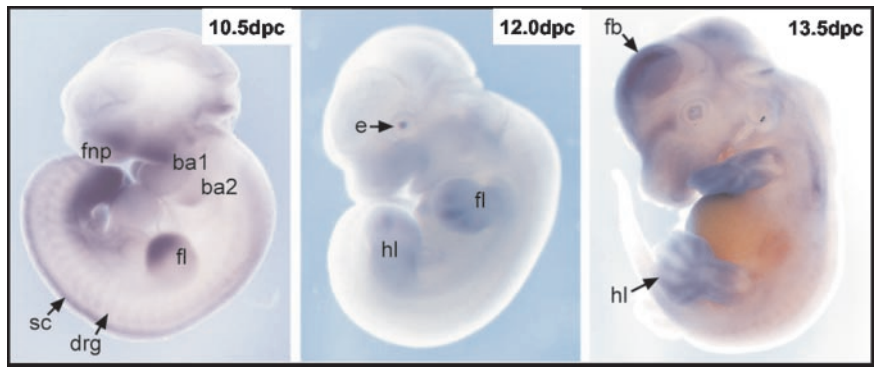


FIG. 4. **Expression of *Hls5* in murine embryos.** Whole mount *in situ* hybridization analysis of embryos at various dpc is shown. *ba1*, branchial arch 1; *ba2*, branchial arch 2; *fnp*, frontonasal process; *fl*, forelimb; *hl*, hind limb; *drg*, dorsal root ganglia; *sc*, spinal cord; *e*, eye; *fb*, forebrain.

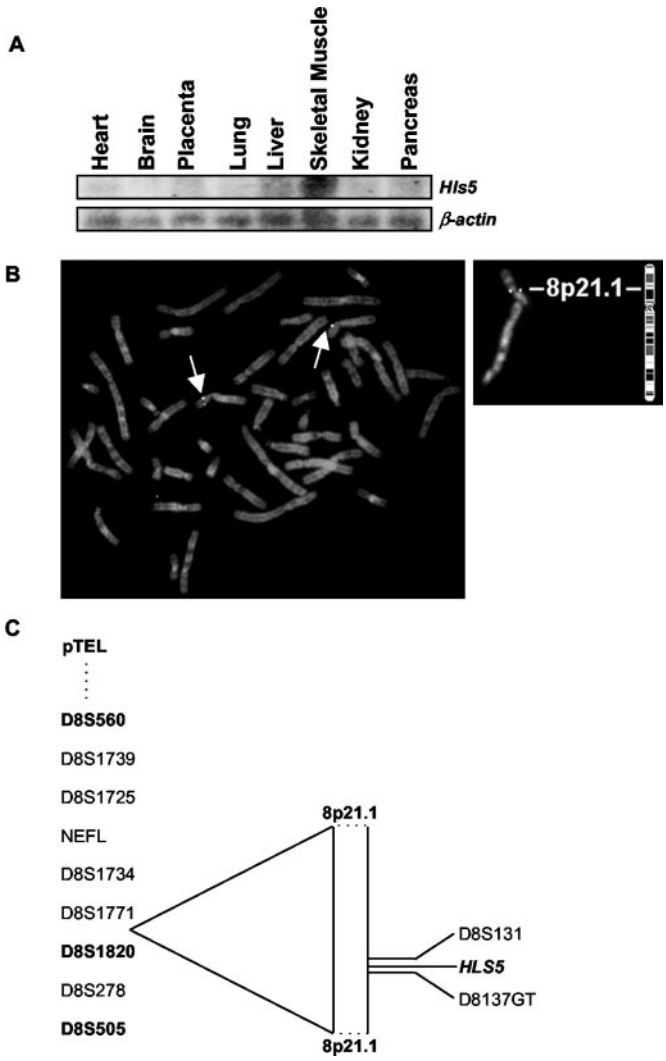


FIG. 5. ***HLS5* localizes to chromosome 8p21.1.** *A*, Northern blot of RNA extracted from different human organs. β-actin was used as a loading control. *B*, chromosomal localization of *HLS5* by fluorescent *in situ* hybridization of human metaphase spreads. *C*, DNA data base and chromosome map analysis identified *HLS5* between markers D8S131 and D8137GT.

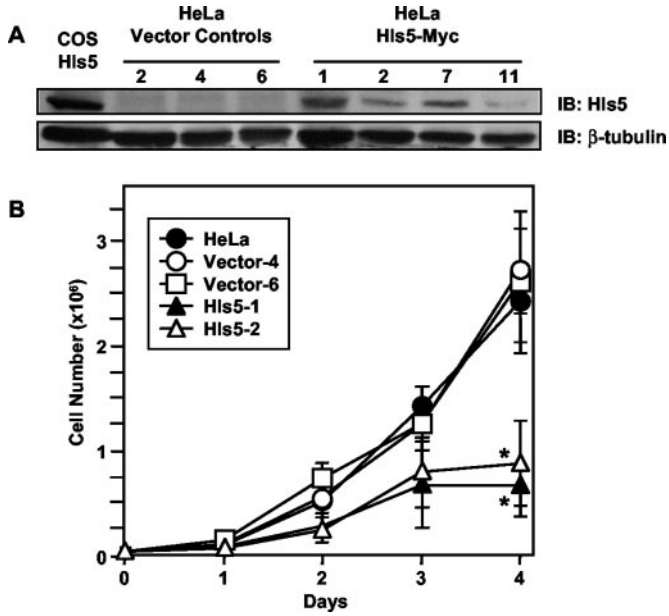


FIG. 6. ***Hls5* suppresses HeLa cell growth.** *A*, immunoblot (*IB*) analysis of parental HeLa cells and individual HeLa cell clones infected with retroviral Myc-tagged Hls5 or vector alone. Transiently transfected COS cells were used as a positive control. *B*, growth rate of representative HeLa clones over 4 days. Each value shown is the mean ± S.D. ($n = 3$). *, $p < 0.05$.

protein. As the antisera produced to Hls5 were unable to detect the subcellular localization of the endogenous protein, COS and HeLa cells were transiently transfected with a Myc-tagged *Hls5* construct. Fig. 3*D* shows that Hls5 protein was detected in discrete cytoplasmic granules as well as punctate nuclear bodies. The cytoplasmic localization did not coincide with markers for Golgi, mitochondria, or lysosomes, and the nuclear spots were not PML bodies (data not shown). In cells expressing higher levels of Hls5, the protein also concentrated in the perinuclear region. Similar subcellular compartmentalization of Hls5 was observed in NIH3T3 and 293T cells (data not shown).

Expression of Hls5 during Embryogenesis—To pursue the expression profile of *Hls5* during embryogenesis, whole mount *in situ* hybridization analysis was performed over a range of embryonic stages. At 10.5 days post-coitum (dpc), *Hls5* expression was detected in branchial arches 1 and 2 and the frontonasal process, limb buds, spinal cord, and dorsal root ganglia (Fig. 4). There was also evidence of relatively weak somite-associated staining. Hemi-sectioning of the embryo confirmed staining in the dorsal root ganglia and revealed that neural expression was predominantly in the dorsal half of the spinal cord, although weaker expression was also detected in the ventral region (data not shown). At 12.0 dpc, *Hls5* was expressed primarily in the limbs and transiently in the developing eye. By 13.5 dpc, expression in the limb was restricted to the interdigital regions and expression was evident in the telencephalic region of forebrain. This analysis suggests that expression of *Hls5* in the developing embryo is far more restricted than that seen in adult organs.

Human HLS5 Localizes to Chromosome 8p21—A full-length human cDNA was isolated (GenBank™ accession number AF492463), and low levels of mRNA were detected in a number

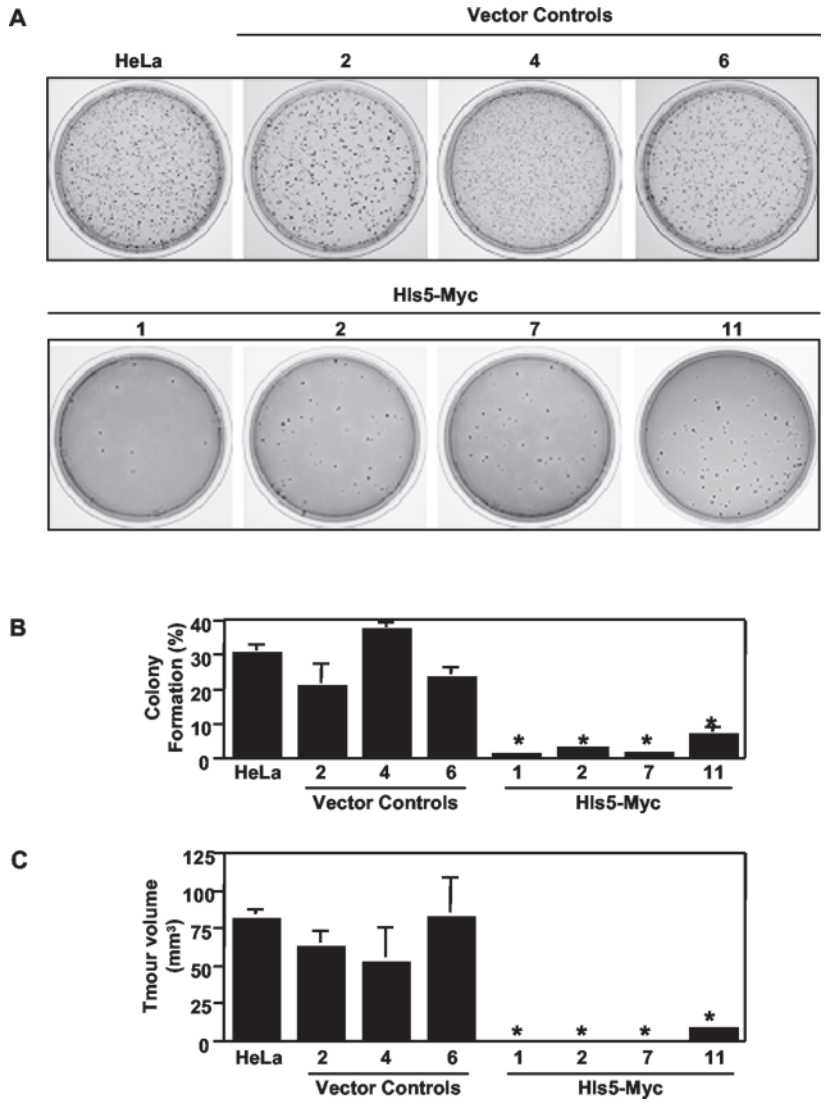


FIG. 7. ***Hls5* suppresses HeLa clonogenicity and tumorigenicity.** *A*, the clonogenicity of various HeLa cell transfectants was determined using soft agar. *B*, colony formation of HeLa cell clones as a percentage of cells plated. *C*, tumor volume of HeLa cell clones injected into nude mice. Each value shown in *panels B* and *C* is the mean ± S.D. ($n = 3$). *, $p < 0.01$.

of human organs and tissues (Fig. 5*A*). A 2.4-kb human genomic fragment (GenBank™ accession number AF494189) was used for fluorescent *in situ* hybridization, which showed that the gene was located on chromosome 8p21.1 (Fig. 5*B*). An analysis of the DNA databases and chromosome maps showed that expressed sequence tag KIAA1098 (47), which is identical to human *HLS5,* resided between markers D8S137GT and D8S131 (Fig. 5*C*). These data confirm that *HLS5* is located at 8p21.1, a region that has been implicated in numerous leukemias and solid tumors, and is thought to contain at least one tumor suppressor gene (31).

Hls5 Suppresses HeLa Cell Growth and Tumorigenicity—As *HLS5* resides within a tumor suppressor locus and is related to the known tumor suppressor gene *Pml*, which restricts cell growth (27, 37), the effects of ectopic *Hls5* expression were investigated in HeLa cells. Using a retroviral vector, Hls5 protein expression was increased in several independent HeLa transfectants (Fig. 6*A*). Each clone expressing Hls5 grew slower (Fig. 6*B*) and generated fewer colonies in soft agar (Fig. 7, *A* and *B*). Significantly, the development of tumors in nude mice was reduced substantially in cells transfected with *Hls5* (Fig. 7*C*). Together, these data indicate that *Hls5* is capable of inhibiting the growth and tumorigenic phenotype of HeLa cells and is therefore a candidate tumor suppressor gene within the 8p21 locus.

It is noteworthy that within a few weeks of culture, Hls5 protein could no longer be detected in each of the *Hls5* transfectants (data not shown). To circumvent the problem of reduced Hls5 expression following stable transfection, HeLa cells were also transiently transfected with *Hls5*. Fig. 8*A* shows that cells accumulated in G_2/M phase of the cell cycle, indicating that one mechanism by which Hls5 may inhibit cell growth is by impeding passage through the cell cycle. Similar to nocodazole, a G_2/M inhibitor, Hls5 increased the activity of cdk1 (Fig. 8*B*); however, no significant changes were observed in the levels of p53, cyclins A, B_1, or E, Cdk1, Cdk2, $p21^{waf1}$, $p27^{kip1}$, and $p57^{kip2}$ or the tyrosine phosphorylation status of Cdk1 (data not shown).

Human HLS5 induced a similar build up of HeLa cells in G_2/M 30 h post-transfection (Fig. 8*C*). Significantly, overexpression of both the murine and human genes ultimately led to increased cell death. Fig. 8*C* shows a marked accumulation of sub-G_1 DNA 70 h after transfection, indicative of DNA breakdown and apoptosis. This effect was dependent on the level of HLS5, increasing the amount transfected correlated well with the degree of DNA degradation until saturation was reached (Fig. 8*D*). The increase in sub-G_1 DNA coincided with reduced cell viability and increased TUNEL-positive cells (Fig. 8*E*). These data demonstrate that HLS5 overexpression induces cell death. Thus, the growth inhibitory effects of *Hls5* may result from a combination of delayed transit through the cell cycle and elevated cell death.

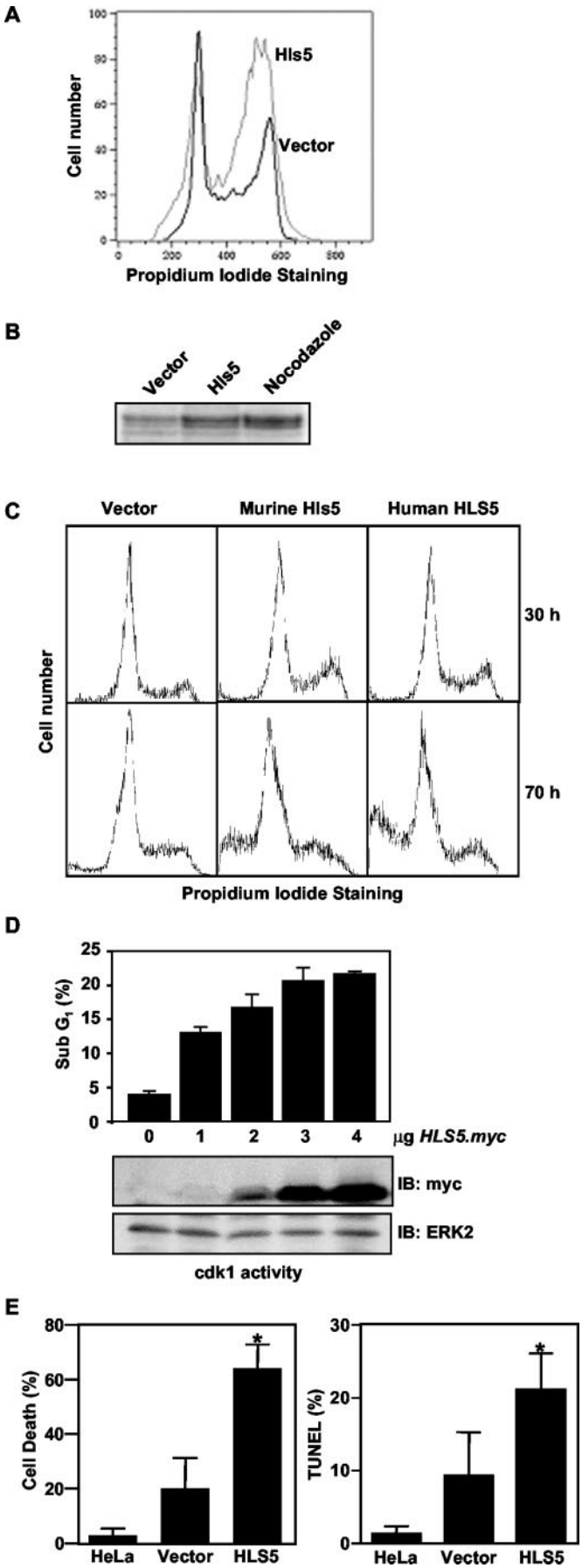


FIG. 8. ***Hls5* slows cell cycle and induces apoptosis.** *A*, cell cycle analysis of HeLa cells transfected with *Hls5* (*gray line*) or vector alone (*black line*). *B*, HeLa cells were either transfected with vector alone or *Hls5* or treated with nocodazole. Cells were lysed 24 h later, and then Cdk1 was immunoprecipitated and assayed for kinase activity using [γ-^{32}P]ATP and histone H1. *C*, cell cycle analysis of HeLa cells transfected with murine *Hls5* or human *HLS5*, 30 and 70 h post-transfection. *D*, increasing amounts (1–4 μg) of *HLS5* DNA were transfected into HeLa cells, and the percentage of cells with sub-G_1 DNA content was determined after 72 h. Immunoblotting (*IB*) of cell lysates revealed that the amount of HLS5 protein present in the cells compared with the loading control ERK2. Each value is the mean ± S.D. ($n = 3$). $p < 0.0001$ (ANOVA, analysis of variance). *E*, HeLa cell death after transfection with *Hls5* or vector alone was determined by eosin exclusion, whereas DNA degradation was demonstrated by TUNEL staining. *, $p < 0.05$. Each value is the mean ± S.D. ($n = 3$).

DISCUSSION

In this paper, we report on the isolation of *Hls5,* a novel gene that was induced when the erythroleukemic J2E cell line spontaneously developed a myeloid phenotype. Hls5 is a new member of the growing family of RBCC proteins. Other members include Pml (27), Herf1 (29), TIF1-α (28, 50), SS-A/Ro (45), and Rfp (30, 44). The tripartite organization of the RBCC domains is evolutionarily conserved, suggesting that these proteins have functional similarities (48). It has been proposed that the coiled-coil domain of the RBCC family is required for homodimerization, whereas the other motifs are involved in recruitment of additional proteins, which specify intracellular compartmentalization (48). The subcellular localization of Hls5 in cytoplasmic granules and nuclear bodies is similar to that observed with a striking number of RBCC proteins (44, 48). Whereas these cytoplasmic vesicles or speckles remain undefined, some punctate nuclear structures have been well characterized, *e.g.* PML bodies (27, 51).

Pml, Rfp, and Tif-1α are RBCC family members involved in chromosomal translocations, which induce neoplasms (50, 52–55), with PML being recognized as a *bona fide* tumor suppressor gene (27, 37, 38, 56). Efp, another RBCC family member, is associated with the development of breast cancer (57). The localization of human *HLS5* to chromosome 8p21 is significant because this locus is implicated in several leukemias and solid tumors. It has been proposed that one or more tumor suppressor genes reside on the short arm of chromosome 8 (31, 58–65). This region has been associated with numerous cancers including prostate, breast, lung, bladder, ovary, and liver (58, 59, 61, 62, 64–68). Importantly, three studies on liver, breast, and colon cancer have mapped a deleted region, which overlaps with the location of *HLS5* (59, 62, 68). Like Pml (69), Hls5 was able to reduce the growth of HeLa cells, inhibit colony formation, and suppress tumor development, indicating that *HLS5* is a potential tumor suppressor gene at this locus.

The reduced growth and tumorigenicity of HeLa cells expressing exogenous Hls5 may be explained, at least in part, by the accumulation of cells in G_2/M. Impaired transit through the cell cycle could result in Hls5-expressing cells displaying slower growth rates. Additionally, overexpression of Hls5 induced apoptosis in HeLa cells. This observation is compatible with the independent isolation of *HLS5* as MAIR, a pro-apoptotic molecule in TF-1 and NIH3T3 cells. MAIR reduced mitochondrial membrane potential and activated caspases 7, 8, and 9 but not caspase 3; however, additional studies are required to define the precise biochemical mechanisms for Hls5/MAIR-inducing cell death (49). It is possible that the effects of Hls5 are concentration-dependent, *i.e.* at lower levels, Hls5 interferes with cell cycle passage, whereas at higher concentrations the protein promotes cell death. Alternatively, restricting movement through the cell cycle may be a prelude to apoptosis. Therefore, a loss of a gene at 8p21.1, which affects the G_2/M transition and apoptosis, is potentially deleterious to cells.

It is noteworthy that Hls5 was isolated from an erythroid to myeloid lineage switch and MAIR was cloned as a gene induced by macrophage colony-stimulating factor (49). The expression of *Hls5* in bone marrow, spleen, and fetal liver is consistent with a role for this gene in hemopoiesis. Significantly, deletions, translocations, and inversions of 8p21 have resulted in acute myeloid leukemias (70) and a loss of this region has been implicated as a secondary event producing a worse prognosis in Mantle cell lymphoma (60). Intriguingly, the 8p21 locus is also involved in a t(8;17) (p21;q21), which produces rare acute promyelocytic leukemias (71, 72). Although the vast majority of acute promyelocytic leukemias are caused by the t(15;17) involving *PML-RARα* (27, 73), several variant translocations have been identified previously (73, 74). It is tempting to speculate that *HLS5* could be involved in the t(8;17) abnormality.

Although *Hls5* was expressed very widely in adult organs, the developmental expression profile was intriguing. In the embryo *Hls5*, mRNA was expressed predominately in the facial primordia, limbs, spinal cord, brain, and dorsal root ganglia. *Hls5* transcripts were also detected in the somites and the eye. Expression in these sites of the developing embryo suggests that *Hls5* may be involved in remodeling of tissues and/or apoptosis. These observations support the notion that Hls5/MAIR may be pro-apoptotic (49) and that this effect may be cell type-specific. The role of Hls5 in development may become clearer with the completion of *Hls5*−/− mice.

It is interesting to note that two of the genes obtained from the erythroid to myeloid lineage switch are implicated in cancer development. *Hls7* (23) was also identified as *MLF1*, a novel oncogene involved in translocations between chromosomes 3 and 5, that causes acute myeloid leukemias (24). Furthermore, elevated levels of wild type MLF1 have been observed in non-t(3;5) leukemias and are associated with poor prognosis (75). Conversely, *HLS5* bears features of a tumor suppressor gene. It would appear that the RDA performed on this hemopoietic lineage switch has isolated two important genes, which when dysregulated may induce neoplasia.

Acknowledgments—We thank Dr. Paul Rigby (Biomedical Confocal Microscopy Research Center, Department of Pharmacology, University of Western Australia) and Shane Meakins from Royal Perth Hospital for help and advice with the confocal microscopy and nude mice experiments, respectively.

REFERENCES

1. Metcalf, D. (1988) *Med. J. Aust.* **148,** 516–519
2. Orkin, S. H. (1995) *J. Biol. Chem.* **270,** 4955–4958
3. Shivdasani, R. A., and Orkin, S. H. (1996) *Blood* **87,** 4025–4039
4. Graf, T. (2002) *Blood* **99,** 3089–3101
5. Klinken, S. P., Alexander, W. S., and Adams, J. M. (1988) *Cell* **53,** 857–867
6. Kulessa, H., Frampton, J., and Graf, T. (1995) *Gene Dev.* **9,** 1250–1262
7. Principato, M., Cleveland, J. L., Rapp, U. R., Holmes, K. L., Pierce, J. H., Morse III, H. C., and Klinken, S. P. (1990) *Mol. Cell. Biol.* **10,** 3562–3568
8. Boyd, A. W., and Schrader, J. W. (1982) *Nature* **297,** 691–693
9. Holmes, K. L., Pierce, J. H., Davidson, W. F., and Morse, H. C., III. (1986) *J. Exp. Med.* **164,** 443–457
10. Davidson, W. F., Pierce, J. H., Rudikoff, S., and Morse, H. C., III. (1988) *J. Exp. Med.* **168,** 389–407
11. Borzillo, G. V., Ashmun, R. A., and Sherr, C. J. (1990) *Mol. Cell. Biol.* **10,** 2703–2714
12. Elefanty, A. G., and Cory, S. (1992) *Blood* **79,** 1271–1281
13. Lindeman, G. J., Adams, J. M., Cory, S., and Harris, A. W. (1994) *Immunity* **1,** 517–527
14. Nutt, S. L., Heavey, B., Rolink, A. G., and Busslinger, M. (1999) *Nature* **401,** 556–562
15. Rolink, A. G., Nutt, S. L., Melchers, F., and Busslinger, M. (1999) *Nature* **401,** 603–606
16. Tenen, D. G., Hromas, R., Licht, J. D., and Zhang, D. E. (1997) *Blood* **90,** 489–519
17. Yamaguchi, Y., Zon, L. I., Ackerman, S. J., Yamamoto, M., and Suda, T. (1998) *Blood* **91,** 450–457
18. Sayer, M. S., Tilbrook, P. A., Spadaccini, A., Ingley, E., Sarna, M. K., Williams, J. H., Andrews, N. C., and Klinken, S. P. (2000) *J. Biol. Chem.* **275,** 25292–25298
19. Yamada, T., Abe, M., Higashi, T., Yamamoto, H., Kihara-Negishi, F., Sakurai, T., Shirai, T., and Oikawa, T. (2001) *Blood* **97,** 2300–2307
20. Keil, U., Busfield, S. J., Farr, T. J., Papadimitriou, J., Green, A. R., Begley, C. G., and Klinken, S. P. (1995) *Cell Growth Differ.* **6,** 439–448
21. Klinken, S. P., Nicola, N. A., and Johnson, G. R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8506–8510
22. Busfield, S. J., and Klinken, S. P. (1992) *Blood* **80,** 412–419
23. Williams, J. H., Daly, L. N., Ingley, E., Beaumont, J. G., Tilbrook, P. A., Lalonde, J. P., Stillitano, J. P., and Klinken, S. P. (1999) *EMBO J.* **18,** 5559–5566
24. Yoneda-Kato, N., Look, A. T., Kirstein, M. N., Valentine, M. B., Raimondi, S. C., Cohen, K. J., Carroll, A. J., and Morris, S. W. (1996) *Oncogene* **12,** 265–275
25. Hitzler, J. K., Witte, D. P., Jenkins, N. A., Copeland, N. G., Gilbert, D. J., Naeve, C. W., Look, A. T., and Morris, S. W. (1999) *Am. J. Pathol.* **155,** 53–59
26. Lim, R., Winteringham, L. N., Williams, J. H., McCulloch, R. K., Ingley, E., Tiao, J. Y., Lalonde, J. P., Tsai, S., Tilbrook, P. A., Sun, Y., Wu, X., Morris, S. W., and Klinken, S. P. (2002) *J. Biol. Chem.* **277,** 40997–41008
27. Zhong, S., Salomoni, P., and Pandolfi, P. P. (2000) *Nat. Cell Biol.* **2,** E85–90
28. Le Douarin, B., You, J., Nielsen, A. L., Chambon, P., and Losson, R. (1998) *J. Steroid Biochem. Mol. Biol.* **65,** 43–50
29. Harada, H., Harada, Y., O'Brien, D. P., Rice, D. S., Naeve, C. W., and Downing, J. R. (1999) *Mol. Cell. Biol.* **19,** 3808–3815
30. Takahashi, M., Inaguma, Y., Hiai, H., and Hirose, F. (1988) *Mol. Cell. Biol.* **8,** 1853–1856
31. Knuutila, S., Aalto, Y., Autio, K., Bjorkqvist, A. M., El-Rifai, W., Hemmer, S., Huhta, T., Kettunen, E., Kiuru-Kuhlefelt, S., Larramendy, M. L., Lushnikova, T., Monni, O., Pere, H., Tapper, J., Tarkkanen, M., Varis, A., Wasenius, V. M., Wolf, M., and Zhu, Y. (1999) *Am. J. Pathol.* **155,** 683–694
32. Tilbrook, P. A., Ingley, E., Williams, J. H., Hibbs, M. L., and Klinken, S. P. (1997) *EMBO J.* **16,** 1610–1619
33. Hubank, M., and Schatz, D. G. (1994) *Nucleic Acids Res.* **22,** 5640–5648
34. Tsai, S., Bartelmez, S., Sitnicka, E., and Collins, S. (1994) *Genes Dev.* **8,** 2831–2841
35. Morgenstern, J. P., and Land, H. (1990) *Nucleic Acids Res.* **18,** 3587–3596
36. Mann, R., Mulligan, R. C., and Baltimore, D. (1983) *Cell* **33,** 153–159
37. Mu, Z. M., Chin, K. V., Liu, J. H., Lozano, G., and Chang, K. S. (1994) *Mol. Cell. Biol.* **14,** 6858–6867
38. Quignon, F., De Bels, F., Koken, M., Feunteun, J., Ameisen, J. C., and de The, H. (1998) *Nat. Genet.* **20,** 259–265
39. Christiansen, J. H., Dennis, C. L., Wicking, C. A., Monkley, S. J., Wilkinson, D. G., and Wainwright, B. J. (1995) *Mech. Dev.* **51,** 341–350
40. Callen, D. F., Baker, E., Eyre, H. J., Chernos, J. E., Bell, J. A., and Sutherland, G. R. (1990) *Ann. Genet.* **33,** 219–221
41. Gingras, R., Richard, C., El-Alfy, M., Morales, C. R., Potier, M., and Pshezhetsky, A. V. (1999) *J. Biol. Chem.* **274,** 11742–11750
42. Kozak, M. (1986) *Cell* **44,** 283–292
43. Kozak, M. (1987) *J. Mol. Biol.* **196,** 947–950
44. Cao, T., Borden, K. L., Freemont, P. S., and Etkin, L. D. (1997) *J. Cell Sci.* **110,** 1563–1571
45. Chan, E. K., Hamel, J. C., Buyon, J. P., and Tan, E. M. (1991) *J. Clin. Invest.* **87,** 68–76
46. Ponting, C., Schultz, J., and Bork, P. (1997) *Trends Biochem. Sci.* **22,** 193–194
47. Kikuno, R., Nagase, T., Ishikawa, K., Hirosawa, M., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N., and Ohara, O. (1999) *DNA Res.* **6,** 197–205
48. Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S., Guffanti, A., Minucci, S., Pelicci, P. G., and Ballabio, A. (2001) *EMBO J.* **20,** 2140–2151
49. Kimura, F., Suzu, S., Nakamura, Y., Nakata, Y., Yamada, M., Kuwada, N., Matsumura, T., Yamashita, T., Ikeda, T., Sato, K., and Motoyoshi, K. (2003) *J. Biol. Chem.* **278,** 25046–25054
50. Le Douarin, B., Zechel, C., Garnier, J. M., Lutz, Y., Tora, L., Pierrat, P., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. (1995) *EMBO J.* **14,** 2020–2033
51. Koken, M. H., Reid, A., Quignon, F., Chelbi-Alix, M. K., Davies, J. M., Kabarowski, J. H., Zhu, J., Dong, S., Chen, S., Chen, Z., Tan, C. C., Licht, J., Waxman, S., de The, H., and Zelent, A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 10255–10260
52. de The, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L., and Dejean, A. (1991) *Cell* **66,** 675–684
53. Goddard, A. D., Borrow, J., Freemont, P. S., and Solomon, E. (1991) *Science* **254,** 1371–1374
54. Kakizuka, A., Miller, W. H., Jr., Umesono, K., Warrell, R. P., Jr., Frankel, S. R., Murty, V. V., Dmitrovsky, E., and Evans, R. M. (1991) *Cell* **66,** 663–674
55. Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H., and Hiai, H. (1988) *Oncogene* **3,** 571–578
56. Wang, Z. G., Delva, L., Gaboli, M., Rivi, R., Giorgio, M., Cordon-Cardo, C., Grosveld, F., and Pandolfi, P. P. (1998) *Science* **279,** 1547–1551
57. Urano, T., Saito, T., Tsukui, T., Fujita, M., Hosoi, T., Muramatsu, M., Ouchi, Y., and Inoue, S. (2002) *Nature* **417,** 871–875
58. Fujiwara, Y., Emi, M., Ohata, H., Kato, Y., Nakajima, T., Mori, T., and Nakamura, Y. (1993) *Cancer Res.* **53,** 1172–1174
59. Lerebours, F., Olschwang, S., Thuille, B., Schmitz, A., Fouchet, P., Laurent-Puig, P., Boman, F., Flejou, J. F., Monges, G., Paraf, F., Bedossa, P., Sabourin, J. C., Salmon, R. J., Parc, R., and Thomas, G. (1999) *Genes Chromosomes Cancer* **25,** 147–153
60. Martinez-Climent, J. A., Vizcarra, E., Sanchez, D., Blesa, D., Marugan, I., Benet, I., Sole, F., Rubio-Moscardo, F., Terol, M. J., Climent, J., Sarsotti, E., Tormo, M., Andreu, E., Salido, M., Ruiz, M. A., Prosper, F., Siebert, R., Dyer, M. J., and Garcia-Conde, J. (2001) *Blood* **98,** 3479–3482
61. Farrington, S. M., Cunningham, C., Boyle, S. M., Wyllie, A. H., and Dunlop, M. G. (1996) *Oncogene* **12,** 1803–1808
62. Pineau, P., Nagai, H., Prigent, S., Wei, Y., Gyapay, G., Weissenbach, J., Tiollais, P., Buendia, M. A., and Dejean, A. (1999) *Oncogene* **18,** 3127–3134

63. Sunwoo, J. B., Holt, M. S., Radford, D. M., Deeker, C., and Scholnick, S. B. (1996) *Genes Chromosomes Cancer* **16,** 164–169
64. Vocke, C. D., Pozzatti, R. O., Bostwick, D. G., Florence, C. D., Jennings, S. B., Strup, S. E., Duray, P. H., Liotta, L. A., Emmert-Buck, M. R., and Linehan, W. M. (1996) *Cancer Res.* **56,** 2411–2416
65. Wright, K., Wilson, P. J., Kerr, J., Do, K., Hurst, T., Khoo, S. K., Ward, B., and Chenevix-Trench, G. (1998) *Oncogene* **17,** 1185–1188
66. Gustafson, C. E., Wilson, P. J., Lukeis, R., Baker, E., Woollatt, E., Annab, L., Hawke, L., Barrett, J. C., and Chenevix-Trench, G. (1996) *Cancer Res.* **56,** 5238–5245
67. Seitz, S., Rohde, K., Bender, E., Nothnagel, A., Kolble, K., Schlag, P. M., and Scherneck, S. (1997) *Oncogene* **14,** 741–743
68. Seitz, S., Rohde, K., Bender, E., Nothnagel, A., Pidde, H., Ullrich, O. M., El-Zehairy, A., Haensch, W., Jandrig, B., Kolble, K., Schlag, P. M., and Scherneck, S. (1997) *Br. J. Cancer* **76,** 983–991
69. Mu, Z. M., Le, X. F., Vallian, S., Glassman, A. B., and Chang, K. S. (1997) *Carcinogenesis* **18,** 2063–2069
70. El-Rifai, W., Larramendy, M. L., Ruutu, T., and Knuutila, S. (1996) *Genes Chromosomes Cancer* **15,** 34–37
71. Miura, I., Nishinari, T., Hashimoto, K., Nimura, T., Miura, S., and Miura, A. B. (1994) *Cancer Genet. Cytogenet.* **72,** 75–77
72. Sonoda, Y., Misawa, S., Maekawa, T., Taniwaki, M., Abe, T., and Takino, T. (1985) *Cancer Genet. Cytogenet.* **17,** 61–67
73. Melnick, A., and Licht, J. D. (1999) *Blood* **93,** 3167–3215
74. Grimwade, D., Biondi, A., Mozziconacci, M. J., Hagemeijer, A., Berger, R., Neat, M., Howe, K., Dastugue, N., Jansen, J., Radford-Weiss, I., Coco, F. L., Lessard, M., Hernandez, J. M., Delabesse, E., Head, D., Liso, V., Sainty, D., Flandrin, G., Solomon, E., Birg, F., and Lafage-Pochitaloff, M. (2000) *Blood* **96,** 1297–1308
75. Matsumoto, N., Yoneda-Kato, N., Iguchi, T., Kishimoto, Y., Kyo, T., Sawada, H., Tatsumi, E., and Fukuhara, S. (2000) *Leukemia* **14,** 1757–1765

HLS5, a Novel RBCC (Ring Finger, B Box, Coiled-coil) Family Member Isolated from a Hemopoietic Lineage Switch, Is a Candidate Tumor Suppressor

Jean-Philippe Lalonde, Raelene Lim, Evan Ingley, Peta A. Tilbrook, Martin J. Thompson, Ross McCulloch, Jennifer G Beaumont, Carol Wicking, Helen J. Eyre, Grant R. Sutherland, Kathy Howe, Ellen Solomon, James H. Williams and S. Peter Klinken

J. Biol. Chem. 2004, 279:8181-8189.
doi: 10.1074/jbc.M306751200 originally published online December 8, 2003
