Table 2. The kinomes of the nematodes *B. malayi*, *C. elegans*, and *C. briggsae* in comparison with that of *Homo sapiens*. Eukaryotic protein kinases are mainly defined on the basis of sequence similarity of their catalytic domains, plus knowledge of accessory domains and any known modes of regulation. Conventional protein kinases (EPKs) include AGC [adenosine 3′,5′-monophosphate–dependent protein kinase/protein kinase C (PKC)] kinases regulated by cyclic-nucleotide and calcium-phospholipid binding; casein kinase 1 and close relatives (CK1); calmodulin-dependent kinases (CaMK), cyclin-dependent kinases (CDK)/mitogen-activated protein kinases/glycogen synthase kinases/CDK-like kinases (CMGC); receptor guanylate cyclase (RGC); a group including many kinases functioning in MAP kinase cascades (STE); tyrosine kinases (TK); tyrosine kinase-like kinases (TKL). Atypical protein kinases (APKs) include Alpha (exemplified by myosin heavy chain kinase of *Dictyostelium discoideum*); phosphatidylinositol 3-kinase–related kinases (PIKK); pyruvate dehydrogenase kinases (PHDK); “right open reading frame” (RIO) [named as such as it was one of two adjacent genes that were found to be transcribed divergently from the same intergenic region].

<table>
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<tr>
<th>Protein kinases</th>
<th>Organism</th>
<th>Kinas shared by all 3 nematodes</th>
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5. Materials and methods are available as supporting material on Science Online.
30. Funding for this project was provided by a grant from the National Institute for Allergy and Infectious Diseases, NIH (NIAID/NIH U01-AI50903) awarded to E.G. and A.L.S. We would like to acknowledge our colleagues in the Filarial Genome Consortium and the filarial research community for their continued support and encouragement. The Filarial Genome Consortium was initiated by grants from the United Nations Special Programme for Research and Training in Tropical Diseases (TDR), which is cosponsored by the U.N. Children’s Fund (UNICEF), U.N. Development Programme (UNDP), World Bank, and World Health Organization (WHO) (T23/99/12 to A.L.S., T23/99/15 to B.S. and T23/99/157 to S.A.W.). This whole-genome shotgun project has been deposited at the DNA Databank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and GenBank under the project accession AAQA00000000. The version described in this paper is the first version AAQA01000000. The data are also available in WormBase release W5175.

UHRF1 Plays a Role in Maintaining DNA Methylation in Mammalian Cells

Magnolia Bostick1,2, Jong Kyong Kim2, Pierre-Olivier Estève, Amander Clark, Sriharsa Pradhan, Steven E. Jacobsen1,3

Epigenetic inheritance in mammals relies in part on robust propagation of DNA methylation patterns throughout development. We show that the protein UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1), also known as NP95 in mouse and ICBP90 in human, is required for maintaining DNA methylation. UHRF1 colocalizes with the maintenance DNA methyltransferase protein DNMT1 throughout S phase. UHRF1 appears to tether DNMT1 to chromatin through its direct interaction with DNMT1. Furthermore UHRF1 contains a methyl DNA binding domain, the SRA (SET and RING associated) domain, that shows strong preferential binding to hemimethylated CG sites, the physiological substrate for DNMT1. These data suggest that UHRF1 may help recruit DNMT1 to hemimethylated DNA to facilitate faithful maintenance of DNA methylation.

Cytosine methylation is an epigenetic mark used for the silencing of transposable elements and for the regulation of development (1, 2). Once established, DNA methylation is often stable through mitosis, in part because CG methylation is faithfully main-
tained after DNA replication by DNMT1 (3). The SRA domain present in the Arabidopsis KRYPTONITE histone methyltransferase, and also in UHRF1 family proteins found in plants and animals, was found to bind methylated DNA in either a CG, CHG (where H indicates A, T, or C), or asymmetrical sequence context (4–7). KRYPTONITE is required for maintenance of CHG DNA methylation, and the Arabidopsis KRYPTONITE homolog VIM1/ORTH2 is required for maintenance of CG methylation at centromeric repeat sequences (6, 7). Mammalian UHRF1, formerly known as NP95 (nuclear protein 95) in mouse and ICBP90 (inverted CCAAT box binding protein 90) in human, contains several domains, including UBL (ubiquitin-like), RING (really interesting new gene), and PHD (plant homeo domain) domains and an SRA domain that can bind methylated DNA (4, 5, 8–10). Prior analysis has implicated UHRF1 in DNA damage control, regulation of S phase, and transformation to malignancy (5, 11–14), although the mechanism behind these effects is unknown. In this study, we explored the interaction between UHRF1 and DNMT1 in maintaining mammalian CG DNA methylation.

Methylation of genomic DNA from wild-type and mUhrf1−/− (mouse Uhrf1) embryonic stem (ES) cells (15) was analyzed by Southern blots and genomic bisulfite sequencing (16). We observed massive global losses of DNA methylation in mUhrf1−/− cells, as evidenced by a decrease in the size of genomic DNA after digestion with the CG methylation–sensitive restriction enzyme HpaII (Fig. 1A). We also observed decreased methylation of two well-characterized, heavily methylated, high copy number loci, the minor satellite and IAP (intracisternal A particle) elements (Fig. 1, B and C). We quantified the change in DNA methylation by using bisulfite sequencing of wild-type and mUhrf1−/− genomic DNA. We observed a decrease of IAP element CG DNA methylation from 91% in the

Fig. 1. Decreased DNA methylation in mUhrf1−/− ESC. (A) Ethidium bromide–stained genomic DNA from two wild-type (E14) and three mUhrf1−/− independent sets of ES cells digested with a CG methylation–sensitive enzyme (HpaII) or –insensitive enzyme (MspI). (B and C) Southern blots probed with a minor satellite (B) or IAP (C) probe. (D) Bisulfite sequencing methylation patterns at IAP (left) or Line-1 (right) sequences. CG dinucleotides are represented by circles, solid if methylated and open if unmethylated. Percentages of methylated CG dinucleotides are below each pattern.
wild type to 19% in mUhrf1−/− and a decrease in Line-1 (another abundant element) methylation from 49% to 14% (Fig. 1D). This loss of methylation phenotype was not a secondary consequence of decreases in DNA methyltransferases, because we observed that DNMT1, DNMT3a, and DNMT3b amounts were not reduced in the mUhrf1−/− cells (fig. S1).

Loss of DNMT1 activity also led to decreased genomic DNA methylation at Line-1 (17), IAP (18), and minor satellite (19) sequences. Furthermore, both UHRF1- and DNMT1-depleted ES cells failed to properly differentiate and showed embryonic lethality, but both remained proliferative as ES cells (13, 20). Lastly, the subnuclear localization of DNMT1 (21, 22) and UHRF1 (14, 23) have been studied as cultured cells progress through the cell cycle, and both were found at multiple foci during S phase. During mid-S phase, both UHRF1 and DNMT1 were shown to be at replication foci, as judged by colocalization with proliferating cell nuclear antigen (PCNA) and 5-bromo-2′-deoxyuridine (BrdU), and in late S phase both proteins were also found associated with 4′,6′-diamidino-2-phenylindole (DAPI)-staining heterochromatin (14, 23–25). Consistent with these earlier findings, we found that, in COS-7 cells, mUHRF1 and DNMT1 colocalized as synchronized cells were allowed to progress from G1 through S phase (Fig. 2). As the cells entered S phase, both proteins moved from a diffuse pattern throughout the nucleus (excluded from the nucleolus) to primarily being localized to multiple foci (Fig. 2). Merged images of DNMT1 and mUHRF1 confirmed that these foci contained both proteins. The colocalization was especially evident in late S phase, when highly methylated heterochromatin is replicated (Fig. 2). This colocalization was repeated with hUHRF1 (human UHRF1) and DNMT1, giving very similar results (fig. S2).

Consistent with the immunolocalization data, we found that, in COS-7 cells, mUHRF1 and DNMT1 colocalized as synchronized cells were allowed to progress from G1 through S phase (Fig. 2). As the cells entered S phase, both proteins moved from a diffuse pattern throughout the nucleus (excluded from the nucleolus) to primarily being localized to multiple foci (Fig. 2). Merged images of DNMT1 and mUHRF1 confirmed that these foci contained both proteins. The colocalization was especially evident in late S phase, when highly methylated heterochromatin is replicated (Fig. 2). This colocalization was repeated with hUHRF1 (human UHRF1) and DNMT1, giving very similar results (fig. S2). Consistent with the immunolocalization data, we found that hUHRF1 and DNMT1 were chromatin-associated proteins whose abundance in HeLa cell chromatin increased as cells progressed from G1 through S phase (fig. S3).

By using a coprecipitation assay, we found that UHRF1 and DNMT1 were physically associated as ES cells (

![Fig. 2. Colocalization of mUHRF1 and DNMT1. Detection of DsRed-DNMT1 and green fluorescent protein (GFP)–mUHRF1 transiently expressed in synchronized COS-7 cells. Cells were released from G1 arrest and followed for the given number of hours (h) through S phase. DNA was visualized with Hoechst stain.](image)

![Fig. 3. hUHRF1 interacts with DNMT1 and is required for its association with chromatin. (A) Wild-type HEK293T cells (wt) or those stably expressing DNMT1 fused to CBD (D1) were used for coprecipitation experiments. Chitin sepharose was used to precipitate DNMT1. hUHRF1 and DNMT1 were detected by immunoblot analysis from total protein fractions (Input) or precipitation fractions (Bound) using antibodies against DNMT1 or hUHRF1. (B) Total proteins were extracted from mock-treated control HeLa cells (Control) or cells treated with siRNAs for DNMT1 (DNMT1 kd) or hUHRF1 (hUHRF1 kd). Decreased protein quantities were verified by immunoblot analysis, with antibodies against actin used as a loading control. (C) Chromatin was purified from wild-type HeLa cells (Control), DNMT1 kd cells, or hUHRF1 kd cells, and DNMT1 and hUHRF1 were detected by immunoblot analysis. Anti-histone H3 (H3) immunoblot analysis was used to confirm visualization of the chromatin fraction and as a loading control. (D) COS-7 cells transiently expressing GFP-DNMT1 were treated with mock (top) or hUHRF1 (bottom) siRNAs. PCNA was visualized with a monoclonal antibody, and DNA was visualized with Hoechst stain.](image)
We studied the methyl DNA binding properties of the UHRF1 SRA domain by using electrophoretic mobility shift assays and a bacterially expressed and purified glutathione-S-transferase (GST) fusion with the mUHRF1 SRA domain. We used double-stranded oligonucleotides that contained either unmethylated, fully methylated, or hemimethylated cytosines found in three different sequence contexts (CG, CHG, and asymmetric CHH; fig. S7). The mUHRF1 SRA only bound to DNA methylated in a CG context (fig. 4A), consistent with the prevalence of this type of methylation in mammalian genomes. In addition, the mUHRF1 SRA domain bound better to hemimethylated CG DNA, whether it was methylated on the sense strand (hs) or the antisense strand (ha), than it did to fully methylated DNA (fig. 4, A and B). We quantified this difference and found a sevenfold higher affinity for hemimethylated DNA (K\text{d} = 1.8 ± 0.4 nM for hs and K\text{d} = 1.7 ± 0.1 nM for ha) than for fully methylated DNA (K\text{d} = 12.1 ± 1.7 nM) (mean ± SD, Fig. 4B and fig. S8). The mUHRF1 SRA domain did not bind to hemimethylated CNG-containing DNA or to asymmetrically methylated DNA (which is also methylated on only one strand), confirming its preference for CG methylation (fig. 4A). In addition, negative control binding assays with purified GST alone showed no detectable binding even at greatly increased GST concentrations. We confirmed the specificity of the mUHRF1 SRA domain for hemimethylated DNA by performing competition studies where GST-mUHRF1SRA bound to the hemimethylated CG DNA (hs) was competed with unmethylated, fully methylated, or hemimethylated DNA (Fig. 4, C and D). Lastly, we found that hUHRF1 also shows a preference for hemimethylated CG DNA (fig. S9).

We found that the affinity for binding to hemimethylated DNA is not a general property of SRA domains, because this specificity was not observed for the Arabidopsis SRA-SET protein, SUVH6 (fig. S10) (6). This confirms our earlier finding that SRA domains from different proteins show a wide variation in binding properties for differentially methylated DNA (6).

In summary, we found that UHRF1 is required for maintenance of CG DNA methylation, physically interacts and colocalizes with DNMT1, and is required for the stable association of DNMT1 with chromatin. The affinity of the UHRF1 SRA domain for hemimethylated DNA suggests a model in which UHRF1 can bind to hemimethylated DNA and recruit DNMT1 to facilitate efficient maintenance CG methylation. Two other important factors are known to contribute to maintenance methylation. The first is the inherent preference of DNMT1's catalytic activity for hemimethylated DNA (26, 27). The second is the recruitment of DNMT1 to replication foci through its interaction with PCNA.

Fig. 4. SRA domain of mUHRF1 preferentially binds hemimethylated CG dinucleotides. (A) Electrophoretic mobility shift assays with GST-mUHRF1SRA binding to double-stranded oligonucleotides containing unmethylated (U), fully methylated (M), or hemimethylated DNA with the methyl cytosine on sense (Hs) or antisense (Ha) strands, in either a CG, CHG, or CHH sequence context. Antibody against GST (GST) added to the assay before electrophoresis supershifted the GST-mUHRF1SRA/DNA complex, whereas the nonspecific control antibody GFP (GFP) did not. (B) Quantification of binding of GST-mUHRF1SRA to unmethylated (CGu, circles), fully methylated (CGm, diamonds), or hemimethylated (CGhs, squares; CGha, solid circles) oligonucleotides. Points represent mean ± SD. (C and D) Competition assays with GST-mUHRF1 bound to CGhs and competed with 10×, 100×, 500×, and 1000× of unmethylated, fully methylated, or hemimethylated cold DNA. fp is free probe.
MicroRNA Inhibition of Translation Initiation in Vitro by Targeting the Cap-Binding Complex elf4F

Géraldine Mathonnet, 1, 2 Marc R. Fabian, 1* Yuri V. Svitkin, 3 Armen Parsyan, 4 Laurent Huck, 1 Takayuki Murata, 5 Stefano Biffo, 1, 2 William C. Merrick, 4 Edward Darzynkiewicz, 5 Ramesh S. Pillai, 6 Witold Filipowicz, 6 Thomas F. Duchaine, 1† Nahum Sonenberg 1†

MicroRNAs (miRNAs) play an important role in gene regulatory networks in animals. Yet, the mechanistic details of their function in translation inhibition or messenger RNA (mRNA) destabilization remain controversial. To directly examine the earliest events in this process, we have developed an in vitro translation system using mouse Krebs-2 ascites cell–free extract that exhibits an authentic miRNA response. We show here that translation initiation, specifically the 5′ cap recognition process, is repressed by endogenous let-7 miRNAs within the first 15 minutes of mRNA exposure to the extract when no destabilization of the transcript is observed. Our results indicate that inhibition of translation initiation is the earliest molecular event effected by miRNAs. Other mechanisms, such as mRNA degradation, may subsequently consolidate mRNA silencing.

MicroRNAs (miRNAs) are short (~21 nucleotides (nt) in length) regulatory RNAs encoded within the genomes of organisms ranging from plants to animals. They are implicated in the regulation of a wide variety of biological processes (1–7). miRNAs act in association with Argonaute (Ago) proteins as components of the RNA-induced silencing complex (RISC) to repress mRNA expression (8, 9). Studies using in vivo systems reported that miRNAs either inhibit translation or lead to the degradation of the target mRNA or protein synthesis of the nascent polypeptide (10–17). An important limitation to the in vivo studies is the fact that the outcome of mRNA silencing has been examined hours or days after the initial mRNA target recognition. Thus, the development of an in vitro system is necessary to understand the biochemistry of miRNA function, especially the early steps after the recruitment of RISC to the mRNA.

We chose the mouse Krebs-2 ascites cell extract as an in vitro translation system because it supports efficient translation and exhibits many endogenous and viral translational control mechanisms (18, 19). We generated two constructs encoding the Renilla luciferase (Rluc) open reading frame fused to a 3′UTR (untranslated region) containing or lacking six target sites for let-7 miRNA (termed RL and RL-6xB, respectively), followed by a poly(A) tail of 98 nucleotides (20) (Fig. 1A). On the basis of quantitative real-time polymerase chain reaction (PCR) analyses, we estimated the total concentration of let-7a and let-7f miRNAs [which are the most abundant let-7 miRNAs in human tissues (21)] in the extract at ~150 pM. In vitro transcribed miRNAs were translated at concentrations varying from 3 pM to 3 nM. At the lowest concentration (3 pM), RL-6xB mRNA translation was only 25% of that of RL mRNA (Fig. 1B). In contrast, a decrease in translation was not observed at an mRNA concentration of 3 nM, consistent with a limiting concentration of let-7 miRNAs in the extract (Fig. 1B and fig. S1, A and B). In addition, the same degree of translation inhibition was observed when Rluc activity was normalized against firefly luciferase (Fluc) activity, expressed from Fluc mRNA used as an internal control (fig. S1, A and B). Moreover, the RL and RL-6xB miRNAs were translated similar efficiency in a wheat germ extract where let-7 is absent (fig. S1C). These results show that target mRNA repression is sensitive to the relative concentrations of the mRNA and miRNA. The degree of inhibition by let-7 miRNA for a relative concentrations of the mRNA and miRNA.

To address the specificity of the inhibition of RL-6xB mRNA translation by let-7, we supplemented the Krebs-2 ascites extract with an antisense 2′-O-methyl (2′-O-Me) oligoribo-nucleotide complementary to let-7 miRNA. This resulted in an ~2.5-fold increase in translation of RL-6xB mRNA (an increase from 28 to 71% relative to RL mRNA at 10 nM), but had no effect on translation of the control RL mRNA (Fig. 1C). Control 2′-O-Me oligonucleotides tar-

References and Notes
16. Materials and methods are available on Science Online.
31. We thank L. Johnson, A. Khattar, A. Conway, and M. Mohan for technical assistance and H. Koseki, M. Muto, G. Fan, and T. Bestor for reagents and advice. M.B. was partially funded by the American Heart Association Postdoctoral Fellowship 0625014Y. S.P. would like to thank R. Roberts, D. Comb, and New England Biolabs for support. Work in the Jacobsen lab is funded by NIH grant GM065398. S.E. is an Investigator of the Howard Hughes Medical Institute.
33. Materials and methods are available on Science Online.
34. To whom correspondence should be addressed. E-mail: nahum.sonenberg@mcgill.ca (N.S.); thomas.duchaine@mcgill.ca (T.F.D.).