Posttranscriptional gene silencing by RNAi is a conserved process by which dsRNA triggers the destruction of homologous target mRNAs (1). RNAi-related mechanisms also mediate heterochromatin formation, silencing of transposable elements, antiviral defense, genome reorganization, cell proliferation, cell differentiation, cell death, and developmental timing and patterning (2–5).

Although genetic and biochemical studies have identified components of RNAi, including the dsRNA processing enzyme Dicer (DCR-1) and the effector complexes RISC (RNA induced silencing complex) and RITS (RNA-induced trans-activation of transcriptional gene silencing (TGS)) (1, 2), a comprehensive genomic analysis by RNAi should in principle identify the complete pathway. Using RNAi to identify RNAi factors has been demonstrated previously (6). In addition, the production of non-null phenotypes by RNAi enables the study by RNAi of essential genes, such as dcr-1, the only C. elegans Dicer. In this study, we have used a genome-wide approach to identify an extensive set of genes required for RNAi in C. elegans.

To monitor RNAi in vivo, we designed an “RNAi sensor” strain (GR1401) that expresses both a gfp (green fluorescence protein) dsRNA hairpin and a gfp reporter gene in C. elegans epithelial seam cells (Fig. 1A). In wild-type animals, gfp dsRNA targets the gfp mRNA for degradation, abrogating GFP expression. Feeding the C. elegans RNAi sensor strain bacteria that express dsRNA corresponding to genes previously implicated in RNAi robustly restored GFP expression, whereas control dsRNA did not (Fig. 1B).

We screened a library of bacterial clones expressing dsRNAs designed to individually inactivate 94% of the ~19,000 predicted genes in the worm genome (7, 8) [table S1 (9)]. L1-stage larvae of the RNAi sensor strain were fed each bacterial clone, and GFP fluorescence was monitored in their progeny. For the 945 genes annotated as embryonic lethal (7), L1-stage animals were fed the bacterial clone and GFP fluorescence was monitored in the later larval or adult stage of the same generation. All experiments were scored on a GFP intensity and penetration scale of 0 (no GFP expression) to 4 (highly penetrant, strong GFP expression), and those that scored an average of ~2 or greater were designated candidate RNAi genes (Table 1). All candidate clones were retested no fewer than five independent times in triplicate.

Screening of the genome-wide RNAi library identified 90 clones (0.5%) that reproducibly disrupt RNAi. Eleven of these correspond to loci known to be required for RNAi, including the core RNAi machinery such as dcr-1, rde-1, and rde-4 (Table 1 and table S2). Fifty-four of the new genes are essential for viability, and one-third of the viable 25 new genes exhibit reduced brood sizes (P < 0.01, Student’s t test) (table S3); 85% of the new genes have human homologs, suggesting conserved functions (Table 1 and table S4). It is possible that some of the identified factors could be nonspecific; for example, inactivation of a factor (e.g., dpy-20) could inhibit the expression from one epidermal promoter of the RNAi sensor strain but not the other. However, because this may be observed in large majority of the RNAi clones tested also affect transgene silencing in a variety of other tissues (see below), most are likely to act in the RNAi pathway.

To verify that genes uncovered in our screen are required for RNAi of endogenous
genes, we coinjected animals with dsRNA of each candidate RNAi gene together with dsRNA of mom-2, a gene essential for viability (fig. S1) (6). The survival of progeny indicates that inactivation of the candidate gene renders animals resistant to the lethality of mom-2 RNAi. Because the coinjection assay relies on detecting a phenotype in the progeny of injected worms, only the 36 RNAi genes that are not essential for viability were examined (Table 1 and fig. S1). Inactivation of 10 newly identified and 11 known RNAi genes rescued the lethality associated with injection of mom-2 dsRNA (>45% viability, P < 0.05), compared to coinjection with dsRNAs targeting genes dispensable for RNAi [fig. S1 (9)] or injection of mom-2 dsRNA alone.

Among the new RNAi candidates, we identified six proteins with domains found in known RNAi factors: Two proteins contain either a PIWI domain (C04F12.1) or both PIWI and PAZ (K12B6.1) domains found in Argonaute and the RNAi factor RDE-1; F22D6.6 possesses a Tudor RNA binding domain identified in the TSN micrococcal nuclease of RISC; and Y38A10.6, F56D2.6, and C06E1.10 have DEAD/DEAH-box motifs found in Dicer, MUT-14, DRH-1, and DRH-2 (1).

RNA binding and processing factors constitute the largest class of new RNAi factors identified and suggest new steps in the RNAi pathway as well as overlap with other RNA-mediated gene regulatory pathways (Table 1). We identified components of the pre-mRNA cleavage and polyadenylation complex that functions in the formation of mRNA 3’ ends (10), including F09G2.4, cpf-2, and F43G9.5, key components of the cleavage and polyadenylation specificity factor (CPSF/F09G2.4), the cleavage stimulation factor (CstF/CPF-2), and the cleavage factor I (CF1/F43G9.5), respectively. A mutation in a predicted polyadenylate [poly(A)] polymerase component exhibits an Rde phenotype in C. elegans (11), whereas poly(A) polymerase (Cid12) associates with the RITS complex in Schizosaccharomyces pombe (12).

We also identified the nonsense-mediated decay (NMD) gene smg-2, as well as three genes predicted to function in NMD—T25G3.3, paa-1, and F26A3.2—as modulators of RNAi, consistent with previous observations implicating smg-2 and, to a lesser degree, smg-5 and smg-6, in RNAi (13). dsRNA processing and initial degradation of the target mRNA are unaffected in smg (−) mutants, suggesting that the NMD factors act downstream of siRNA production and initial target cleavage (13).

We identified factors required for nuclear import and export, including the Ran GTPase (guanine triphosphatase) exchange factor RCC1 (ran-3), the Ran GTPase binding protein 1 (npp-9), and nucleoporins (npp-1 and npp-16) (14). In addition, the identification of nuclear import receptors of the importin-α and -β families (imb-2, imb-5, and ima-3) (15) suggest a mechanism whereby siRNAs generated in the cytoplasm may subsequently be reimported into the nucleus for TGS.

dsRNAs targeting a genomic locus are known to recruit heterochromatin factors and drive heterochromatin formation and TGS in an RNAi-dependent manner (2). A number of RNAi genes encode predicted chromatin factors that may mediate TGS in response to dsRNAs. We identified two Polycomb-related components, MES-4 and T23B12.1, consistent with the finding that TGS requires Polycomb in Drosophila and germline transgene silencing requires MES-4 in C. elegans (2, 16) (fig. S2). In addition, we identified Sin3 and histone deacetylase complex (HDAC) genes hda-3, pgn-28, and rba-1. Components of Polycomb and HDAC, in addition to the RNAi machinery and RITS, are all required for heterochromatin formation (2, 6). The finding that these chromatin factors are also essential for RNAi implicates the Polycomb and HDAC complexes in an RNAi-mediated gene silencing mechanism and strongly suggests a TGS component to C. elegans RNAi.

Other classes of factors that regulate RNAi include the DNA repair factor RuB and the mitogen-activated protein (MAP) kinase pathway factors ZC449.3 and MTK-1. The DNA repair and recombination factors suggest that the RNA replication or TGS steps in RNAi may include checkpoints and control mechanisms related to those used in DNA replication and recombination. The established role of the ancient p38/MAP kinase pathway in the innate immune response to pathogens suggests that RNAi mechanisms may also be coupled to these stress and pathogen sensing pathways (17). A group of genes of unknown function includes the new rde-5 gene identified by forward genetics (18). Five members of this group have orthologs in humans, suggesting conserved functions (Table 1 and table S4).

Transgene silencing in C. elegans is mechanistically related to RNAi. A subset of genes required for RNAi are essential for transgene silencing in the germ line, including dcr-1, mut-7, and mut-16 (19, 20). However, other genes, including rde-1 and rde-4, are essential for RNAi but dispensable for germ line silencing (fig. S2) (19), demonstrating that germ line gene silencing and RNAi may require distinct, possibly paralogous, sets of genes.

We tested the roles of the new RNAi factors in germline transgene silencing using let-858p::gfp (PD7271), which is silenced in the germ line but is expressed in somatic tissues (21). Expression of let-858p::gfp in the germ
line is restored when RNAi of dcr-1, mut-7, or mut-16 is sustained for two generations (fig. S2). We assayed expression of let858p::gfp in the germ line after inactivation of the 36 RNAi candidates that are viable for multiple generations. Inactivation of 14 of these genes abolishes germline gene silencing, including 9 newly identified RNAi genes (fig. S2 and Table 1). These findings demonstrate a substantial overlap between factors required for RNAi and germline silencing.

To monitor transgene silencing in somatic cells, we developed an assay based on the observation that the seam-cell GFP transgene (in the absence of a transgene expressing GFP dsRNA) is completely silenced in the eri-1 enhanced RNAi background (22). Inactivation of 87 of the 90 genes identified in our screen restored expression of the seam-cell GFP transgene (fig. S3A and Table 1). In addition, expression of a second ubiquitously expressed reporter, sur-5::gfp (23), which is also silenced by eri-1, was restored in nonneuronal tissues in most of the gene inactivations tested (fig. S3B and Table 1). Similar results were observed using another enhanced RNAi strain, rrf-3 (24) (table S3). These findings indicate a near-complete functional overlap of factors required for RNAi and somatic transgene silencing and strongly suggest that the factors identified do not simply affect the epidermal promoters used in the RNAi sensor strain. Silencing in the germ line may use paralogs of

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**Table 1.** Best reciprocal BLASTP matches (dark gray box) or homologs (light gray box) with BLASTP e-value ≤ 10−6 in human (H), mouse (Mm), fly (Dm), and fission yeast (Sp) are indicated. Also indicated: GFP score from the screen, rescue (+) from mom-2 dsRNA lethality by coinjection of dsRNA of each candidate RNAi gene, germline transgene silencing assay, and somatic transgene silencing using the seam-cell marker (smc) GFP (for viable and lethal clones) or the sur-5::gfp (sur-5) reporters (viable clones only). See text and (9).

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**Figure Caption:**

To monitor transgene silencing in somatic cells, we developed an assay based on the observation that the seam-cell GFP transgene (in the absence of a transgene expressing GFP dsRNA) is completely silenced in the eri-1 enhanced RNAi background (22). Inactivation of 87 of the 90 genes identified in our screen restored expression of the seam-cell GFP transgene (fig. S3A and Table 1). In addition, expression of a second ubiquitously expressed reporter, sur-5::gfp (23), which is also silenced by eri-1, was restored in nonneuronal tissues in most of the gene inactivations tested (fig. S3B and Table 1). Similar results were observed using another enhanced RNAi strain, rrf-3 (24) (table S3). These findings indicate a near-complete functional overlap of factors required for RNAi and somatic transgene silencing and strongly suggest that the factors identified do not simply affect the epidermal promoters used in the RNAi sensor strain. Silencing in the germ line may use paralogs of
these somatic factors or other, unrelated layers of gene regulation.

The microRNA pathway and RNAi share an overall mechanistic framework. However, to date, DCR-1 is the only identified component shared between the two related small-RNA pathways. Inactivation by RNAi of each of the 90 RNAi candidates did not affect precursor microRNA processing (25), underscoring the lack of overlap between RNAi and the microRNA pathways at this step. We also examined phenotypes associated with defects in the heterochronin path controlled by the let-7 microRNA (5). Mutations in let-7 cause supernumerary (>16) seam cells (26). Of the 90 RNAi factors, inactivation of six genes, including dcr-1, caused an increased number of seam cells and, of those, only three (dcr-1, pop-1, and kin-10) also significantly enhanced the weak let-7-protruding vulva phenotype (tables S3 and S5); furthermore, pop-1 RNAi may cause cell fate transformations rather than microRNA defects. These findings indicate little molecular overlap between the new RNAi factors and factors required for the microRNA pathway.

Protein-protein interaction maps (“interactomes”) can facilitate the identification of complex molecular networks. Interrogating the Worm Interactive map (WIS) (27) and our screening of four additional factors provided protein interaction data for 42 of the 90 RNAi factors, giving a total of 161 interactions (table S6). We then tested and found that 21 of these interactors not identified by the initial RNAi screen were nonetheless required for transgene silencing, thus supporting the validity of many of the interactions for the RNAi pathway (fig. S4). The interaction map provides a useful tool from which to postulate connections among the new RNAi factors that were not predicted a priori. For example, the interactome map links the NMD factor, SMG-2, with T25G3.3; the known RNAi factors, RSD-2 and RSD-6 (28); and a cleavage and polyadenylation component, F56A8.6 (CPF-2).

The new factors we have identified suggest new steps in dsRNA-triggered gene silencing, including nuclear import/export and downstream stages that use NMD and mRNA polyadenylation/cleavage factors. We also found a near-complete overlap among factors required for RNAi and those required for transgene silencing in somatic tissues. Further, we showed that many of these factors are required for silencing in the germ line, possibly contributing to the maintenance of germ line–soma distinctions, genome integrity, and protection from parasitic genetic elements. Overall, these findings provide a global view of how the machinery of RNAi is integrated into RNA-mediated cellular processes.

References and Notes
9. Supporting online material is available on Science Online.
18. C. C. Mello, personal communication.

Mutations in Col4a1 Cause Perinatal Cerebral Hemorrhage and Porencephaly

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Porencephaly is a rare neurological disease, typically manifest in infants, which is characterized by the existence of degenerative cavities in the brain. To investigate the molecular pathogenesis of porencephaly, we studied a mouse mutant that develops porencephaly secondary to focal disruptions of vascular basement membranes. Half of the mutant mice died with cerebral hemorrhage within a day of birth, and ~18% of survivors had porencephaly. We show that vascular defects are caused by a semidominant mutation in the procollagen type IV α1 gene (Col4a1) in mice, which inhibits the secretion of mutant and normal type IV collagen. We also show that COL4A1 mutations segregate with porencephaly in human families. Because not all mutant mice develop porencephaly, we propose that Col4a1 mutations conspire with environmental trauma in causing the disease.

Porencephaly [Online Mendelian Inheritance in Man (OMIM) record 175780] is a rare central nervous system disease usually diagnosed in infants. Type I or encephaloclastic porencephaly is characterized by cerebral white-matter lesions and degenerative cavities. Severely affected infants are often diagnosed with poor or absent speech development, epilepsy, hydrocephalus, seizures, mental retardation, and cerebral palsy. It has been suggested that porencephalic cavities in humans result from focal cerebral degeneration involving hemorrhages (1). Association studies suggest that clotting-factor genes may contribute to genetic susceptibility by predisposing to thrombophilia (2). Despite these associations, the genetic and environmental etiology of familial cases is not established (3–10), and it seems reasonable that a distinct mechanism involving primary defects of vasculature could predispose to hemorrhage and porencephaly.

To advance the understanding of porencephaly, we have identified and characterized a new mouse mutant (generated by random mutagenesis) with severe perinatal cerebral hemorrhage. In addition to cerebral hemorrhage, mutant mice are smaller than control littermates.