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Functional Genomic Analysis of RNA Interference in *C. elegans*

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RNA interference (RNAi) of target genes is triggered by double-stranded RNAs (dsRNAs) processed by conserved nucleases and accessory factors. To identify the genetic components required for RNAi, we performed a genome-wide screen using an engineered RNAi sensor strain of *Caenorhabditis elegans*. The RNAi screen identified 90 genes. These included Piwi/PAZ proteins, DEAH helicases, RNA binding/processing factors, chromatin-associated factors, DNA recombination proteins, nuclear import/export factors, and 11 known components of the RNAi machinery. We demonstrate that some of these genes are also required for germline and somatic transgene silencing. Moreover, the physical interactions among these potential RNAi factors suggest links to other RNA-dependent gene regulatory pathways.

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 rearrangements, 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 initiation 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 penetrance 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 non-specific; 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 a 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

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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 Y38A10A.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 (CF I_m/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 (guanosine 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*, *pqn-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 RuvB and the mitogen-activated protein (MAP) kinase path-

way 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 germline silencing (fig. S2) (19), demonstrating that germline 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

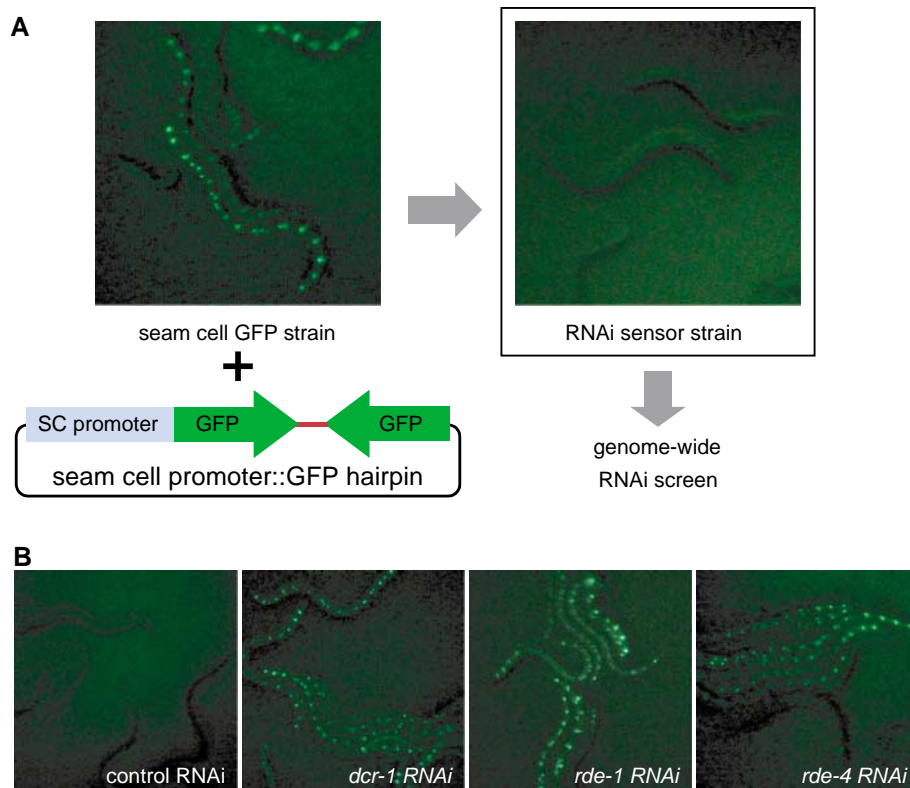


Fig. 1. A scheme for the genome-wide screen to identify factors required for RNAi. (A) The RNAi sensor strain (GR1401) is composed of a dsRNA GFP hairpin that silences the GFP reporter expression in the seam cells by RNAi. (B) Inhibiting the RNAi pathway by feeding the RNAi sensor strain *Escherichia coli* that expresses dsRNA corresponding to the core RNAi genes *dcr-1*, *rde-1*, and *rde-4* (but not control vector) restores the reporter GFP expression in the seam cells.

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 abrogates 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

Viable Clones										Lethal Clones										
Gene Targeted	Locus	Description	Homology			GFP Score	Co-injection	Germline Silencing	Transgene Silencing		Gene Targeted	Locus	Description	Homology			GFP Score	Transgene Silencing		
			Hs	Mm	Dm				Sp	scm				sur-5	Hs	Mm			Dm	Sp
Known RNAi										Chromatin factors										
B0379.3	<i>mut-16</i>	Novel nematode protein				4.0	+	+	+	+	C26D10.1	<i>ran-3</i>	GTPase exchange factor RCC1				3.1	+		
K12H4.8	<i>dcr-1</i>	DICER RNase III				4.0	+	+	+	+	K07A1.11	<i>rba-1</i>	Rb-binding protein; RbAp48, CAF-1-like				2.7	+		
T20G5.11	<i>rde-4</i>	dsRNA binding protein				4.0	+	-	+	+	D2096.8		Nucleosome assembly protein				3.3	+		
F15B10.2	<i>drh-1</i>	DEAD/DEAH box helicase				2.4	+	+	+	+	T08G11.4		Methyltransferase domain				2.9	+		
M04B2.3	<i>gfl-1</i>	Chromatin associated protein				2.3	+	+	+	+	ZK1127.7		DNA topoisomerase type II				2.3	+		
K08H10.7	<i>rde-1</i>	Piwi and PAZ domain protein				4.0	+	-	+	+	DNA damage repair									
Y2H9A.1	<i>mes-4</i>	SET domain nuclear protein				4.0	+	+	+	+	C27H6.2		RuvB-like 1				2.4	+		
F52G2.2	<i>rsd-2</i>	Required for siRNA spreading				4.0	+	+	+	+	T22D1.10		RuvB-like 2				2.7	+		
Y48G8AL.6	<i>smg-2</i>	NMD protein; RNAi persistence				2.4	+	-	+	-	ZK1127.4		BRCA2 interacting protein				3.2	+		
ZK1098.8	<i>mut-7</i>	3'-5' exonuclease domain				2.5	+	+	+	+	Nuclear import/export									
F54F2.2	<i>zfp-1</i>	Chromatin-associated protein				3.2	+	-	+	+	F32E10.4	<i>ima-3</i>	Importin- α family				4.0	+		
Chromatin factors										R06A4.4	<i>imb-2</i>	Importin- β family				1.9	+			
F02E9.4	<i>pqn-28</i>	SIN3 component				2.8	-	-	+	+	Y48G1A.5	<i>imb-5</i>	Importin- β family				3.8	+		
R06C1.1	<i>hda-3</i>	Histone deacetylase				2.5	+	+	+	+	K07F5.13	<i>npp-1</i>	Nuclear pore protein				2.6	+		
M03C11.3		Chromatin associated protein				3.6	-	+	+	+	F59A2.1	<i>npp-9</i>	Ran GTPase-binding protein				3.0	+		
T23B12.1		Polycarb-like PHD Zn-finger				2.3	-	-	-	-	C07E3.2		Nuclear export of pre-ribosomes				3.7	+		
Nuclear import/export										Other										
Y56A3A.17	<i>npp-16</i>	Nuclear pore complex component				2.2	-	-	+	+	F54H12.1	<i>aco-2</i>	Mitochondrial aconitase homolog				2.5	+		
Other										T09A5.10	<i>lin-5</i>	Spindle apparatus component				2.3	+			
F37B12.4		Ubiquitin carboxyl-terminal hydrolase				2.3	-	-	+	-	F46A9.5	<i>skr-1</i>	SCF ubiquitin ligase				3.0	+		
Piwi/Paz, Tudor, or DEAD/DEAH helicase domains										F56A3.4	<i>spd-5</i>	Mitotic spindle assembly				2.8	+			
C04F12.1		Piwi domain protein				4.0	+	-	+	+	Y38F2AL.3	<i>vha-11</i>	Vacuolar H ⁺ -ATPase V1 sector				2.3	+		
K12B6.1		Piwi and PAZ domain protein				2.5	-	-	+	-	F43G9.1		Mitochondrial isocitrate dehydrogenase				2.6	+		
F22D6.6		Tudor domain				2.3	-	+	+	+	F43G9.10		Microfibrillar-associated protein MFAP1				2.3	+		
Y38A10A.6		DEAD/DEAH-box RNA helicase				2.9	+	-	+	+	W04C9.1	<i>haf-4</i>	ABC transporter				3.0	+		
RNA binding & processing										DEAD/DEAH helicase domains										
F43G9.5		Pre-mRNA cleavage factor subunit				3.5	-	+	+	-	C06E1.10		DEAD/DEAH-box RNA helicase				3.0	+		
ZK112.2	<i>ncl-1</i>	B-box Zn-finger protein				2.7	+	-	+	+	F56D2.6		DEAD/DEAH-box RNA helicase				2.9	+		
K08D10.4	<i>mip-2</i>	Spliceosomal protein				2.9	-	+	+	+	Protein synthesis									
W05H7.4		Zn-finger protein				2.3	+	-	+	-	C15F1.4	<i>ppp-1</i>	Translation initiation factor eIF2B subunit				2.2	+		
T19B10.4	<i>pqn-70</i>	mRNP component				2.3	-	-	+	+	F52B5.6	<i>rpl-25.2</i>	60S ribosomal subunit L23a protein				3.7	+		
Y71G10AL.1		S.c H/ACA snoRNP homolog				2.4	+	-	+	+	F59A3.3		KOW motif found in ribosomal proteins				2.8	+		
C12D8.1		KH domain protein				2.1	+	-	+	-	Y61A9LA.10		GTP-binding protein AARP2				2.6	+		
Signaling										RNA binding & processing										
B0414.7	<i>mtk-1</i>	MAPKKK; ortholog of H.s. MEKK4				3.0	+	-	+	+	R06F6.1	<i>cdl-1</i>	Cell Death Lethal-1				1.9	+		
ZC4F49.3		MAPKK; homolog of H.s. MKK4				3.0	-	-	-	-	F56A8.6	<i>cpf-2</i>	mRNA cleavage stimulation factor				2.1	+		
Transcription										F48E8.5	<i>paa-1</i>	PP2A subunit A; binds SMG-5				1.9	+			
R03D7.4		RNA pol II elongation factor				2.8	+	+	+	+	W07E6.4	<i>prp-21</i>	PRP splicing factor related protein				2.8	+		
ZK1127.6, 9¶		Transcription elongation factor CA150				3.5	-	+	+	+	D2089.1	<i>rsp-7</i>	Splicing factor, arginine/serine-rich				2.5	+		
T22B3.1	<i>dpy-20</i>	BED zinc finger DNA-binding protein				2.5	-	-	+	+	Y71F9B.4	<i>snr-7</i>	Small nuclear ribonucleoprotein G				3.3	+		
Unknown										E02H1.1		Ribosomal RNA adenine dimethylase				3.0	+			
T01C3.8	<i>rde-5</i>	New <i>rde</i> gene (18)				3.9	-	+	+	-	F09G2.4		mRNA cleavage and polyA factor				2.9	+		
T19B4.5						2.5	+	-	+	+	F26A3.2		Nuclear cap-binding protein complex				2.8	+		
ZK1127.3						2.7	-	+	+	+	F49D11.1		Pre-mRNA splicing factor PRP17				2.0	+		
None/vector						0.1	-	-	-	-	T25G3.3		Upf1p-interacting protein in yeast				3.5	+		
											ZK1127.5		RNA 3'-terminal phosphate cyclase				3.3	+		
Signaling										Transcription										
T01G9.6	<i>kin-10</i>	Casein kinase II, beta subunit									T01G9.6	<i>kin-10</i>	Casein kinase II, beta subunit				3.8	+		
Transcription										W10C8.2	<i>pop-1</i>	Transcription factor TCF-4				3.8	+			
C16A3.4		C2H2-type Zn-finger domain									C16A3.4		C2H2-type Zn-finger domain				2.9	+		
C55B7.5		Prefoldin chaperone domain									C55B7.5		Prefoldin chaperone domain				2.4	+		
F43G9.12		Transcriptional regulator									F43G9.12		Transcriptional regulator				2.2	+		
T12D8.1		Transcriptional regulator									T12D8.1		Transcriptional regulator				2.3	+		
W06E11.1		RNA polymerase III subunit									W06E11.1		RNA polymerase III subunit				2.2	+		
Unknown										Unknown										
C06A5.1											C06A5.1						2.7	+		
C29E4.2											C29E4.2						2.3	+		
F26E4.4		Predicted nuclear localization									F26E4.4		Predicted nuclear localization				3.3	+		
K12H4.5											K12H4.5						2.2	-		
T23D8.3											T23D8.3						3.2	+		
W04A4.5											W04A4.5						2.3	+		
Y110A7A.19		Contains TPR-like domain									Y110A7A.19		Contains TPR-like domain				3.0	+		
None/vector											None/vector						0.1	-		

Table 1. Best reciprocal BLASTP matches (dark gray box) or homologs (light gray box) with BLASTP e-value $\leq 10^{-6}$ in human (*Hs*), 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 (*scm*) GFP (for viable and lethal clones) or the *sur-5::gfp* (*sur-5*) reporters (viable clones only). See text and (9). ¶ One clone targets two genes, *ZK1127.6* (top cell) and *ZK1127.9* (bottom cell) (BLASTP e-value 80% of protein).

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 heterochronic pathway 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 Interactome map (WI5) (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.

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Supporting Online Material

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Materials and Methods
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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 $\alpha 1$ 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. Severe cases have drastic consequences, including profound disability and death. Infants who survive 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