LETTERS

Somatic misexpression of germline P granules and enhanced RNA interference in retinoblastoma pathway mutants

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***Caenorhabditis elegans* homologues of the retinoblastoma (Rb) tumour suppressor complex specify cell lineage during development[1,2]. Here we show that mutations in Rb pathway components enhance RNA interference (RNAi) and cause somatic cells to express genes and elaborate perinuclear structures normally limited to germline-specific P granules. Furthermore, particular gene inactivations that disrupt RNAi reverse the cell lineage transformations of Rb pathway mutants. These findings suggest that mutations in Rb pathway components cause cells to revert to patterns of gene expression normally restricted to germ cells. Rb may act by a similar mechanism to transform mammalian cells.**

A mutation in the *C. elegans* gene *lin-15* causes enhanced sensitivity to RNAi (Fig. 1a). We tested four double-stranded (ds)RNAs that induce weaker RNAi phenotypes in wild-type animals than the corresponding loss-of-function mutant phenotype. All these dsRNA induce stronger loss-of-function phenotypes in *lin-15(n765)* animals (Fig. 1a and Table 1). The *lin-15* locus comprises two genes, *lin-15A* and *lin-15B*, that are inactivated by the *n765* mutation (ref. 3). RNAi is enhanced in *lin-15B(n744)* but not in the strong loss-of-function *lin-15A(n767)* or *lin-15A(n433)* alleles (Fig. 1a). *lin-15B* is a class B synthetic multivulva (synMuv B) gene[3]. This class of genes encodes homologues of the mammalian tumour suppressor Rb[1] and Rb complex components LIN-53 (mammalian RbAp48) (ref. 1) and DPL-1 (mammalian DP)[2], as well as nuclear proteins that function in the Rb pathway, such as LIN-13, LIN-9, LIN-36 and TAM-1 (ref. 4). SynMuv B genes function redundantly with synthetic multivulva A (synMuv A) genes, such as *lin-15A*, to antagonize Ras signalling in the specification of vulval cell lineages[1–3]. Animals defective in both the synMuv A and synMuv B pathways display multiple vulva structures due to cell lineage changes, whereas animals defective in only one of the synMuv pathways have normal cell lineages in these tissues[1–3].

Other members of the *C. elegans* Rb pathway also negatively regulate RNAi. Loss-of-function mutations or RNAi inactivation of six other Rb pathway genes—*lin-35* (*Rb*), *dpl-1* (*DP*), *lin-53*, *lin-9*, *lin-13* and *hpl-2* (*HP1*); where homologous mammalian genes are denoted in parentheses—markedly enhance RNAi (Fig. 1a; see also Supplementary Fig. S1). Null or strong alleles of three other synMuv B genes, *lin-36*, *tam-1* and *lin-37*, do not enhance RNAi (Fig. 1a), suggesting that some synMuv B genes can affect vulval cell lineages without enhancing RNAi of these tester genes. Strong or null alleles of the four known synMuv A genes, *lin-15A*, *lin-8*, *lin-56*, and *lin-38* do not enhance RNAi (Fig. 1a). The enhanced RNAi of synMuv B

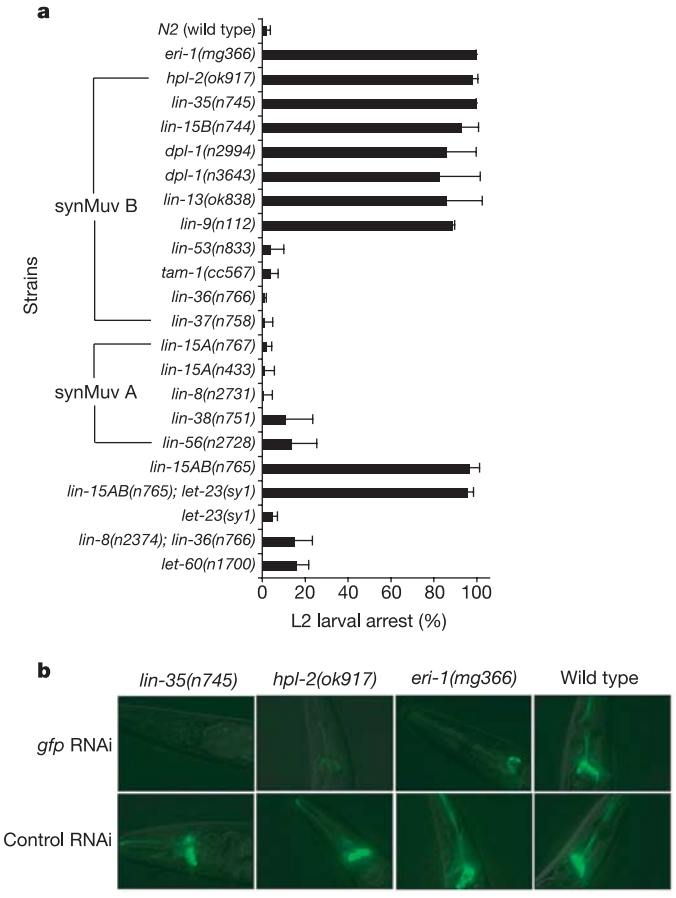

Figure 1 | Rb pathway mutants are more sensitive to RNAi than wild-type animals. a, Animals of the indicated genotype were grown on bacteria expressing *cel-1* dsRNA and the percentage of their progeny arresting at the L2 larval stage was determined. Wild-type N2 animals and *eri-1(mg366)*[5] mutants were used as negative and positive controls. Error bars represent standard deviation among the data collected from at least three independent experiments. **b**, Neuronally expressed *tub-1::gfp* is susceptible to *gfp* RNAi in the *lin-35* and *hpl-2* mutants. *lin-35(n745)*, *hpl-2(ok917)*, *eri-1(mg366)* and wild-type animals, all carrying the integrated *tub-1::gfp* transgene, were grown on bacteria expressing *gfp* dsRNA (*gfp* RNAi) or an empty vector L4440 (control RNAi). Fluorescence microscopy was performed on representative adult progeny.

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pathway mutants is not dependent on whether the animals have ectopic pseudovulvae: for example, the *lin-15AB(n765); let-23(sy1)* double mutant has enhanced RNAi but is vulvaless due to a defect in the receptor tyrosine kinase *let-23* (ref. 3) (Fig. 1a). Conversely, mutants with ectopic pseudovulvae, such as *lin-8(n2374); lin-36(n766)* or the Ras gain-of-function mutant *let-60(n1700)* have wild-type RNAi response (Fig. 1a).

Consistent with the enhanced RNAi phenotype of Rb pathway mutants, target messenger RNA abundance is markedly decreased in these mutants. The induction of movement defects by feeding nematodes bacteria that express dsRNA targeting the muscle gene *unc-22* is enhanced in Rb pathway mutants. In three independent experiments, *unc-22* RNAi in wild-type animals caused a 50% reduction in the level of *unc-22* mRNA, whereas in the *lin-35* mutant, *unc-22* mRNA was reduced by 70–80% (Supplementary Fig. S2).

RNAi is particularly enhanced in the nervous system of Rb pathway mutants. Neurons are generally refractory to RNAi[5]. In *lin-35* (*Rb*), *dpl-1* (*DP*), *hpl-2* (*HP1*), *lin-15B*, *lin-9* and *lin-13* mutants, but not in wild-type animals, dsRNA targeting *gfp* silences neuronally expressed *tub-1::gfp* (Fig. 1b). In the *lin-35* mutant, but not wild type, feeding RNAi targeting endogenous neuronal genes, such as *egl-8*, *egl-30*, *unc-17* and *unc-2*, causes resistance to the acetylcholinesterase inhibitor aldicarb, similar to mutations in these loci (Table 1; see also Supplementary Fig. S3). However, RNAi of these genes does not induce significant movement defects observed in the corresponding mutants, suggesting partial gene inactivation or inactivation only in a particular subset of neurons.

The Rb pathway regulates RNAi synergistically with other negative regulators of RNAi, such as the 3′ exonuclease ERI-1 (ref. 5) and the RNA-dependent RNA polymerase RRF-3 (ref. 6) that both act in the same pathway[5]. Rb pathway null mutants and *eri-1* (or *rrf-3*) null mutants are hypersensitive to RNAi of distinct sets of target genes (Table 1). The Rb pathway and *eri-1* have distinct responses to injected short interfering RNAs (siRNAs): whereas an *eri-1* mutant is more responsive to injection of *unc-22* siRNA (23 base pairs long) than wild type, *lin-35* and *lin-15B* mutants are not more responsive (Table 1), suggesting that the Rb pathway acts at a step upstream of siRNA production. Strains carrying mutations in both *eri-1* and *lin-15b* were synergistically sensitive to RNAi. The release from negative regulation of RNAi in strains carrying mutations in both the Rb and *eri-1*/*rrf-3* pathways increases the intensity of RNAi in the *C. elegans* nervous system. These double mutants allow RNAi to be used for the functional genomic dissection of neuronal functions (see the accompanying paper (ref. 7)) as well as for the identification of neural components in physiological circuits, such as the regulation of metabolism, feeding and ageing.

The enhanced RNAi of Rb pathway mutants depends on many of the canonical RNAi pathway genes, with one exception that gave an indication of how the Rb pathway may enhance RNAi. Double mutants between *lin-35* and the RNAi-defective genes *rde-1*, *rde-4* and *mut-7*, and between *lin-15b* and *mut-16*, are unresponsive to *nsf-1* dsRNA; they are RNAi defective, like the single *rde-1*, *rde-4, mut-7,* or *mut-16* mutants (Supplementary Table S1). However, although animals with a mutation in the RNA-dependent RNA polymerase (RdRP) gene *rrf-1* are unresponsive to *unc-22* and *nsf-1* dsRNAs, *lin-15b; rrf-1* double mutant animals are responsive to both dsRNAs (Supplementary Table S2). RdRPs enhance RNAi by allowing siRNAs from the primary response to dsRNAs to prime further replication on target mRNAs, which in turn amplify the response[8]. The RRF-1 RdRP is essential for RNAi of somatic genes[8], whereas the EGO-1 RdRP is enriched in the germ line and is required for RNAi of genes expressed in the germ line[8,9]. One model for the relaxed requirement for *rrf-1* in the Rb pathway mutants is ectopic expression in somatic tissues of the germline-specific EGO-1 RdRP, making *rrf-1* and *ego-1* redundant genes.

The model of misexpression of RNAi components in Rb pathway mutants is supported by the somatic expression of normally germline-specific P-granule-like structures in the *Rb* pathway mutants (Fig. 2a). *pgl-1* encodes an essential RNA-binding component of P granules that mark the *C. elegans* germ plasm in wild type[10]. In the *lin-35* (*Rb*) mutant, PGL-1 is ectopically expressed in intestine, hypoderm and particular cells near the nerve ring, where it accumulates into perinuclear structures that closely resemble the normally germline-restricted P granules (Fig. 2a). Similar PGL-1-containing structures are also expressed in the somatic cells of *lin-13*, *dpl-1*, *hpl-2*, *lin-15B* and *lin-9* mutants (data not shown). In contrast, ectopic PGL-1 is not detected or is present at much lower levels in the soma of the synMuv B mutants that do not enhance RNAi—*lin-53(n833)* or *lin-37(n758)*—or the synMuv A mutants (data not shown). *pgl-1* is also misexpressed in the somatic cells of the synMuv B NuRD component mutants *let-418* (*Mi2*) and *mep-1* (ref. 11). The fact that the somatic PGL-1 in Rb pathway mutants accumulates in perinuclear structures as in germline cells suggests that additional P granule components and germline-specific factors are also ectopically expressed to allow the assembly of P granules[11].

C. elegans P granules are normally assembled in the germline

Table 1 | Rb pathway mutants display enhanced RNAi distinct from the *eri-1* and *rrf-3* mutants

Strains	Feeding RNAi							Injection of *unc-22* siRNA (% twitchers)#
	hmr-1 (% Emb)*	*unc-15* (Unc/Prz)†	*unc-17* (resistant to aldicarb)‡	*egl-8* (resistant to aldicarb)‡	*myo-2* (% L1 let.)§	*dpy-13* (Dpy) ‖	*daf-2* (% dauer)¶	
Wild type (N2)	11 ± 12	+	−	−	0	+	0	19 ± 2
lin-15AB(n765)	99 ± 1	+++	+	++	0	+	0	NT
lin-15A(n767)	15 ± 6	+	−	−	0	+	0	NT
lin-15B(n744)	71 ± 10	+++	+	++	0	+	0	21 ± 6
lin-35(n745)	82 ± 9	+++	+	++	0	+	0	0
eri-1(mg366)	98 ± 2	+++	−	+	89 ± 8	+++	0	73 ± 7
rrf-3(pk1426)	97 ± 4	+++	−	+	92 ± 5	+++	0	NT
lin-35(n745); eri-1(mg366)	100 ± 0	+++	++	+++	90 ± 4	+++	95 ± 7	NT
rrf-3(pk1426); eri-1(mg366)	100 ± 0	+++	−	+	87 ± 11	+++	0	NT

Nematodes of the indicated genotype were grown on bacteria expressing the indicated dsRNAs (Feeding RNAi) or microinjected with synthetic *unc-22* siRNA (23 bp with two-nucleotide overhangs)[5] at 20 °C. All data were collected from at least three independent experiments.

*Progeny were scored for the percentage of dead embryos (Emb).

†Progeny were scored for uncoordinated (Unc) (+) or paralysed (Prz) (+++) phenotype.

‡Progeny were scored for no (−), weak (+) or strong resistance (+++) to 1.0 mM of the acetylcholinesterase inhibitor aldicarb.

§Progeny were scored for L1 larval lethality (% L1 let.).

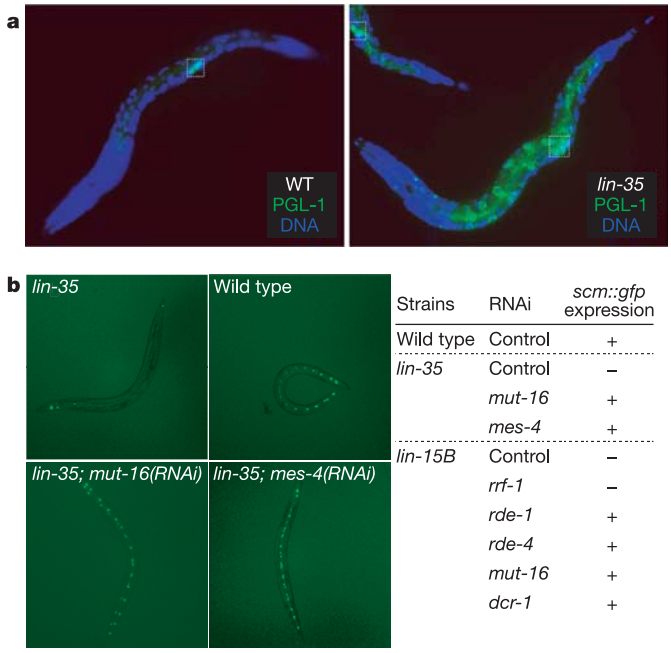
‖Nematodes were scored as having a slightly (+) or severe (+++) dumpy body shape (Dpy).

¶Nematodes were scored for the percentage of animals arrested as dauer larvae (% dauer).

#Young adult nematodes were incubated in 1.0 mM of the nicotinic agonist levamisole, and the percentage of twitching animals was counted 3 min later (% twitchers). NT indicates that certain strains were not tested for the experiment.

blastomeres from maternally expressed gene products, and are segregated exclusively to the germline lineage during early development. P granule components continue to be expressed only in the developing germline where they are assembled into complex structures at the nuclear pores[12]. Their perinuclear localization may reflect a nuclear transport function for the RNAs that accumulate in the P granules. The P granules are homologous to polar granules in *Drosophila*; both are composed of homologous helicases and RNA-binding proteins, as well as a number of mRNAs that specify germline fates and somatic patterning[10,12].

Germ lines are more protected than somatic cells from foreign genetic elements, such as transposons and viruses[13–15]. An increased level of RNAi in the *C. elegans* germ line is suggested by the silencing of transgenes and transposons specifically in the germ line and by the activation of transgenes and transposition by mutations that attenuate germline RNAi[14,15]. In Rb pathway mutants, germline-specific RNAi components may be de-repressed in somatic cells, which in turn enhances RNAi. Consistent with this model, Rb pathway mutations cause silencing of a repetitive *gfp* transgene in somatic cells (Fig. 2b) similar to germline-specific transgene silencing normally observed in the wild type[4]. Inactivation of *mut-16* by RNAi restores somatic GFP expression in the *lin-35* or *lin-15B* mutants (Fig. 2b), and RNAi in Rb pathway mutants requires the germline RNAi factor MUT-7 (Supplementary Table S1). PGL-1 is required for germline co-suppression[15], suggesting that the somatic PGL-1 expression in Rb pathway mutants could also be necessary for the enhanced RNAi.



Strains	RNAi	*scm::gfp* expression
Wild type	Control	+
lin-35	Control	–
	mut-16	+
	mes-4	+
lin-15B	Control	–
	rrf-1	–
	rde-1	+
	rde-4	+
	mut-16	+
	dcr-1	+

Figure 2 | Mutations in the Rb pathway cause soma to germline transformation and transgene silencing. a, The *lin-35* null mutant *lin-35(n745)* exhibits somatic expression of the germline-specific gene *pgl-1*. Wild-type and *lin-35(n745)* L1 larvae were fixed and immunostained with antibodies against the germline-specific P granule protein PGL-1 (green). The germ cells Z2/Z3 are marked in dashed boxes. DNA was counterstained with DAPI (blue). **b**, Transgene silencing in the soma of Rb pathway mutants. A seam cell-specific *gfp* transgene (*scm::gfp*) is silenced in *lin-35(n745)* and *lin-15B(n744)*, but not in wild type; inactivation of *mut-16* or *mes-4* by feeding RNAi restores expression of *scm::gfp* in *lin-35(n745)* or *lin-15B(n744)* animals. Fluorescence microscopy was performed on representative animals at the L4 larval stage. + and − represent the expression and silencing of the *scm::gfp* transgene, respectively. Bacteria expressing an empty vector L4440 are used as the control feeding RNAi.

Because mutations in the Rb pathway affect the cell divisions of vulval cells as well as enhance RNAi, we asked whether genes implicated as possible components in the RNAi pathway mediate the cell lineage defects of Rb pathway mutants. *C. elegans* RNAi gene inactivations that in turn attenuate RNAi have been identified[13]. We tested whether any of 36 viable RNAi-suppressing gene inactivations could also suppress the multivulvae phenotype of Rb pathway and other multivulvae mutants. Six gene inactivations suppress the multivulvae phenotype of three Rb pathway synMuv A and synMuv B double mutants: *lin-15AB(n765)*, *lin-35(n745); lin-8(n2731)*, and *lin-36(n766); lin-8(n2374)*. These six gene inactivations are *mes-4* (SET domain, trithorax class gene), *pqn-28* (SIN3 component), *gfl-1* (chromatin protein, orthologue of mammalian glioma-amplified sequence 41 (GAS41)), *zfp-1* (PHD domain chromatin protein, orthologue of mammalian AF10), M03C11.3 (weakly homologous to a chromatin-associated protein) and ZK1127.3 (novel protein) (Fig. 3a and data not shown). None of these 36 gene inactivations suppressed the multivulvae phenotype of the Ras gain-of-function mutant *let-60(n1700)* or the ETS transcription factor loss-of-function mutant *lin-1(e1777)* (Fig. 3a and data not shown), showing that these clones are not general multivulvae suppressors or Ras pathway genes. In a control RNAi screen of a hand-picked library of 434 clones encoding a variety of known RNAi, RNA-binding and RNA-processing factors, only *pqn-28* (*SIN3*)—the only one out of the six multivulvae-suppressing clones in this library—suppressed the multivulvae phenotype of *lin-35(n745); lin-8(n2731)* animals. Thus, a high hit rate was specific to the RNAi-attenuating clones. Neither RNAi of *dcr-1* nor null mutations in other genes necessary for RNAi, such as *rde-1* and *rde-4*, restore the wild-type vulval cell lineage in the Rb pathway mutants (Fig. 3a; see also Supplementary Table S1), suggesting that excessive RNAi is

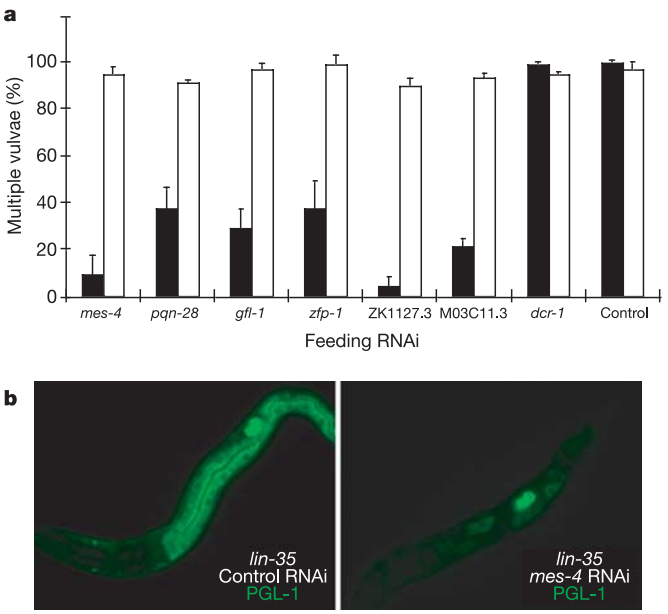


Figure 3 | Inactivation of six RNAi factors suppresses the multiple vulvae phenotype of the Rb pathway mutants. a, *lin-35(n745); lin-8(n2731)* (filled columns) and the Ras gain-of-function mutant *let-60(n1700)* (open columns) were grown on bacteria expressing the indicated dsRNA (feeding RNAi) or an empty vector L4440 (control) at 22 °C. The percentage of progeny with multiple vulvae was scored. The error bars represent standard deviation among the data collected from at least three independent experiments. **b**, RNAi of *mes-4* suppresses the somatic expression of *pgl-1* in *lin-35(n745)* animals. The *lin-35(n745)* animals were fed bacteria expressing *mes-4* dsRNA (*mes-4* RNAi) or an empty vector L4440 (control RNAi) at 22 °C. Their progeny at larval stage L1 were fixed and immunostained with antibodies against PGL-1 (green).

not the cause of the cell lineage transformations of Rb pathway mutants.

Five of the six multivulvae-suppressing clones are annotated as chromatin factors that directly interact with each other and with synMuv B pathway proteins in other species. *Drosophila* homologues of MES-4 interact with SWI/SNF and NuRD complexes and antagonize Polycomb complexes[16]. MES-4 is homologous to the human MLL protein that is the fusion partner to AF10, the orthologue of ZFP-1, in leukaemic translocations[17]. The *Drosophila* homologue of ZFP-1, dAF10, binds to HP1, an orthologue of *C. elegans* HPL-2 (ref. 18). Mammalian AF10 binds the GFL-1 orthologue GAS41. GAS41 interacts with the SWI/SNF complex[17] that contains mSIN3 histone deacetylase, the orthologue of PQN-28, and is a direct target of the oncogene *Myb*[19]. Mammalian SIN3 interacts directly with Rb chromatin complexes[20]. Myb co-purifies with Rb and E2F in the *Drosophila* dREAM complex, and seven out of eight dREAM subunits are orthologues of *C. elegans* synMuv B genes[21]. The dREAM complex binds to particular chromosomal domains on the *Drosophila* polytene chromosomes, suggesting that, similar to the Polycomb complex, an antagonism between Rb-repressing and MES-4-activating complexes may take place at particular chromatin loci[21]. MES-4 coats the autosomes in the *C. elegans* germ line; the MES-2, MES-3 and MES-6 proteins antagonize the binding of MES-4 to the X chromosome to mediate the silencing of the X chromosome in the *C. elegans* germ line[22].

We suggest that in the absence of the antagonistic Rb pathway complex, a MES-4 chromatin remodelling complex that includes the five other proteins identified above is inappropriately active in the soma to activate the expression of P granule genes like *pgl-1*. In the hypodermis, where ectopic P granules are manifest in the Rb mutant, inappropriate activity of the MES-4 complex may promote the expression of vulva-specific genes in the adjacent vulval precursor cells[23]. The loss of Rb pathway function may activate latent programmes of gene expression characteristic of more primitive, less differentiated cells. Consistent with this model, *mes-4* RNAi suppresses the transgene silencing (Fig. 2b), vulval cell lineage defect (Fig. 3a) and somatic misexpression of PGL-1 (Fig. 3b) in the *lin-35* (*Rb*) mutant. A mutation in *mes-4* also suppresses the lethality and ectopic P granule phenotypes of *mep-1* and *let-418* (ref. 11), and *mes-4* is required for germline co-suppression[13,15]. Two of the other five multivulvae-suppressing clones—*gfl-1* (*GAS41*) and ZK1127.3—also reverse the transgene silencing of Rb pathway mutants, but none of the other five suppresses somatic P granule formation as markedly as *mes-4* RNAi (data not shown). Consistent with the activity of these genes normally in the germ line, inactivation of these genes causes decreased brood size or sterility[13]. Because these proteins may form a biochemical complex, they probably regulate the choice between germ line and soma via the same molecular mechanism. However, *mes-4* has an additional function in X chromosome inactivation and could be more pleiotropic[22].

A related model that incorporates precedents for Rb control of cell cycle and endoreduplication[21] is that the Rb pathway chromatin factors inhibit the endoreduplication of particular target loci such as the ones that generate the abundant P granule components in the germ line. *mes-4*, *zfp-1*, *gfl-1*, *pqn-28*, ZK1123.3 and M03C11.3 may be these amplified targets in the normal germ line. In the absence of Rb, these genome regions may be amplified in soma to activate biogenesis of P granules; RNAi of the *mes-4* complex genes may interfere with these amplifications or with the assembly of P granules and other germline components from these amplified gene products. This model is favoured by the observation that mammalian homologues of *mes-4*, *zfp-1*, *gfl-1* and *pqn-28* are amplified in some tumours that may also carry Rb mutations[24,25]. In support of an endoreduplication model, five RNAi regulatory genes[13] including ZK1127.3 are clustered in one genomic region (ZK1127.3, ZK1127.7, ZK1127.4, ZK1127.9/6, ZK1127.5), and *gfl-1* (M04B2.3) clusters with its neighbour, the synMuv B gene *mep-1* (M04B2.1); these clusters may correspond to domains of amplification regulated by the Rb pathway chromatin remodelling factors.

Rb pathway mutations may also enhance RNAi by inactivating a chromatin silencing pathway that competes with the RNAi machinery for shared silencing components. The methylated histone-binding protein HP1 associates with RNAi components on yeast[26] and fly[27] heterochromatin. In *C. elegans*, mutants lacking the HP1 orthologue *hpl-2* display an enhanced RNAi phenotype as well as vulval cell lineage defects when combined with a synMuv A mutation, showing that *hpl-2* is a synMuv B gene with enhanced RNAi (ref. 28) (Fig. 1a, b). Consistent with a role in transcriptional gene silencing, the *hpl-2* mutation causes transgene de-silencing[28]. In the absence of Rb and its associated chromatin remodelling complex, including HP1, RNAi factors such as DCR-1 and Argonaute proteins may become available to mediate RNAi in the cytoplasm, leading to enhanced RNAi. However, PGL-1 is also misexpressed in the soma of the *hpl-2* mutant, suggesting that this mutant does not just release RNAi factors from chromatin to enhance RNAi. The expression of germline components in somatic cells and a release of RNAi factors from their role specifying heterochromatin regions may conspire to activate RNAi in Rb pathway mutants.

It may be significant to the action of human retinoblastoma that *C. elegans* Rb pathway mutations cause a somatic to germline cell transformation, and that some gene inactivations that disrupt RNAi can reverse the cell lineage transformations of Rb pathway mutants. The mammalian cell cycle transformations induced by Rb inactivation may also be triggered by expression of germ line or other stem-cell-like programmes in somatic cells. Gene microarrays have revealed increased expression in *Drosophila* Rb mutant cells of genes annotated to function in oocyte development, including the polar granule RNA helicase *vasa*[29]. Similarly, the RNA helicase DDX1 is amplified in Rb tumours and neuroblastomas, and is a marker for stem cell fate in the mouse[30,31]. If Rb pathway mutations cause amplification of particular chromatin regions, gene dosage studies of tumours could reveal those target genes, such as the *MLL, GAS41* and *AF10* genes. Inactivation of the mammalian homologues of the four multivulvae-suppressing genes that have good mammalian homologues—*mes-4*, *pqn-28*, *gfl-1* and *zfp-1*—for example by specific siRNAs or pharmaceuticals, might reverse the cell cycle defects and genome instability of Rb mutant tumours and cell lines. Conversely, activation of these Rb-regulated genes may confer more totipotent cell fates, which could be useful in inducing cells to recapitulate developmental pathways.

METHODS

Strains. The strains and alleles used were: N2 Bristol (wild type); *let-23(sy1)*II, *let-60(n1700)*IV, *lin-1(e1777)*IV, *lin-15(n765)*X; the doubly mutants *let-23(sy1)*II; *lin-15(n765)*X and *lin-8(n2374)*II; *lin-36(n766)*III. SynMuv A mutants include *lin-8(n2731)*II, *lin-8(n111)*II, *lin-38(n751)*II, *lin-56(n2728)*II, *lin-15A(n433)*X, *lin-15A(n767)*X. SynMuv B mutants include *lin-35(n745)*I, *lin-53(n833)*I, *dpl-1(n2994)*II, *dpl-1(n3663)*II, *hpl-2(ok917)*III, *lin-9(n112)*III, *lin-13(ok838)*III, *lin-36(n766)*III, *lin-37(n758)*III, *tam-1(cc567)*V, *lin-15B(n744)*X. RNAi-defective mutants used were *mut-16(ne322)*I, *rrf-1(pk1417)*I, *mut-7(pk204)*III, *rde-4(ne299)*III, *mut-14(pk738)*V, *rde-1(ne300)*V. RNAi-enhanced mutants used were *rrf-3(pk1426)*II, *eri-1(mg366)*III.

Immunofluorescence staining. L1 larvae were permeablized using a freeze–crack method by freezing between a polylysine-coated slide and a coverslip followed by rapid removal of the coverslip. Slides were immediately immersed in ice-cold 100% methanol for 10 min followed by ice-cold 100% acetone for 5 min. Larvae were stained using monoclonal (1:20 dilution) or affinity purified polyclonal (1:2,000 dilution) anti-PGL-1 antibodies[10] (gifts from S. Strome) followed by TRITC-conjugated donkey anti-mouse IgM (1:200 dilution; Jackson Immunoresearch) or Alexa Fluor-conjugated goat anti-rabbit IgG (1:1,000 dilution; Molecular Probes).

Behavioural test. For the aldicarb resistance test, young adult animals were transplanted to agar plates containing 1.0 mM of the acetylcholinesterase inhibitor aldicarb, and the percentage of animals paralysed over a time course of 140 min was measured. For the levamisole resistance test, young adult

nematodes were incubated in M9 solution with 1.0 mM of the nicotinic agonist levamisole, and the percentage of twitching animals was counted 3 min later.

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