The *Caenorhabditis elegans ekl* (Enhancer of *ksr-1* Lethality) Genes Include Putative Components of a Germline Small RNA Pathway

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ABSTRACT

A canonical Ras–ERK signaling pathway specifies the fate of the excretory duct cell during *Caenorhabditis elegans* embryogenesis. The paralogs *ksr-1* and *ksr-2* encode scaffolding proteins that facilitate signaling through this pathway and that act redundantly to promote the excretory duct fate. In a genomewide RNAi screen for genes that, like *ksr-2*, are required in combination with *ksr-1* for the excretory duct cell fate, we identified 16 “*ekl*” (enhancer of *ksr-1* lethality) genes that are largely maternally required and that have molecular identities suggesting roles in transcriptional or post-transcriptional gene regulation. These include the Argonaute gene *csr-1* and a specific subset of other genes implicated in endogenous small RNA processes, orthologs of multiple components of the NuA4/Tip60 histone acetyltransferase and CCR4/NOT deadenylase complexes, and conserved enzymes involved in ubiquitination and deubiquitination. The identification of four small RNA regulators (*csr-1*, *drh-3*, *ego-1*, and *ekl-1*) that share the *Ekl* phenotype suggests that these genes define a functional pathway required for the production and/or function of particular germline small RNA(s). These small RNAs and the other *ekl* genes likely control the expression of one or more regulators of Ras–ERK signaling that function at or near the level of kinase suppressor of Ras (KSR).

THE Ras–Raf–MEK–ERK signaling pathway is used repeatedly during metazoan development to control many different cellular events (for example, see SIMON *et al.* 1991; MELNICK *et al.* 1993; JOHNSON *et al.* 1997; WOJNOWSKI *et al.* 1997; SUNDARAM 2006; SCHOLL *et al.* 2007). The kinase suppressor of Ras (KSR) scaffolding protein facilitates signaling through this pathway, at least in part by binding the kinase MEK and promoting its colocalization with the kinases Raf and ERK (CLAPERON and THERRIEN 2007). KSR also binds other partners that may contribute to Raf kinase activation (DOUZIECH *et al.* 2006; RITT *et al.* 2007). Although KSR is clearly important for signaling *in vivo*, its precise role and mode of regulation are not well understood. KSR was originally discovered in *Caenorhabditis elegans* and *Drosophila* (KORNFELD *et al.* 1995; SUNDARAM and HAN 1995; THERRIEN *et al.* 1995), and genetic screens in these systems are one approach for identifying additional factors important for KSR function.

In *C. elegans*, as in mammals, KSR is encoded by two paralogous genes, *ksr-1* and *ksr-2*, which have both overlapping and unique functions (OHMACHI *et al.* 2002; CHANNAVAJHALA *et al.* 2003). For example, *ksr-1* and *ksr-2* are redundantly required for Ras–ERK-dependent

specification of vulval and excretory duct cell fates, while *ksr-1* alone is required for Ras–ERK-dependent sex myoblast migration, and *ksr-2* alone is required for Ras–ERK-dependent germline meiotic progression. At least some of the unique functions of these paralogs probably can be explained by differences in soma *vs.* germline expression, with *ksr-1* (like most X-linked genes in *C. elegans*) being more highly expressed in the soma (REINKE *et al.* 2000; KELLY *et al.* 2002; MACIEJOWSKI *et al.* 2005), leaving *ksr-2* responsible for most germline functions.

One of the earliest developmental events that requires *ksr-1* and *ksr-2* is specification of the excretory duct cell fate (OHMACHI *et al.* 2002). The excretory duct is a component of the worm’s primitive renal system that functions in osmoregulation and is essential for viability (NELSON and RIDDLE 1984). It is located in the head, just ventral to the posterior pharynx, and it can be easily visualized with the reporter transgene *lin-48::gfp* (Figure 1) (JOHNSON *et al.* 2001). The duct forms during embryogenesis from the left member of the excretory duct/G1 neuroblast equivalence group (SULSTON *et al.* 1983). In *let-60 ras* loss-of-function mutants, both members of this equivalence group adopt a G1 fate, whereas in *let-60(gf)* mutants, both adopt a duct fate (YOICHEM *et al.* 1997). *let-60 ras* functions cell autonomously within the duct precursor and appears to mediate an inductive signaling event in the embryo that promotes the duct (*vs.* G1) fate (YOICHEM *et al.* 1997). LIN-3/EGF is likely to

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FIGURE 1.—Absence of the excretory duct cell in *ekl; ksr-1* animals. Differential interference contrast (DIC) (A, C, and E) and GFP fluorescence (B, D, and F) images of L1 larvae carrying the *plin-48::gfp* transgene *sals14* (JOHNSON *et al.* 2001; SEWELL *et al.* 2003) are shown. *plin-48::GFP* is expressed in the excretory duct cell (arrow), as well as a few other cells (arrowheads) in the head and the tail of L1-stage *sals14; ksr-1(n2526)* larvae (A and B). In contrast, no excretory duct cell is visible in dying larvae from *ksr-2(RNAi); sals14; ksr-1(n2526)* (C and D) and *csr-1(RNAi); sals14; ksr-1(n2526)* (E and F) strains, although *plin-48::GFP* expression is still apparent elsewhere. Similar results were obtained with the other *ekl* genes (data not shown).

be the relevant signaling ligand, as *lin-3* mutants lack an excretory duct cell, and *lin-3* is expressed in nearby embryonic cells at the relevant time (M. SUNDARAM, unpublished data). Loss of the excretory duct cell can explain the zygotic “rod-like lethality” of *let-60 ras* and other Ras pathway mutants, since without this cell larvae rapidly fill with fluid, become turgid, and die (NELSON and RIDDLE 1984; YOCHAM *et al.* 1997). Although animals lacking either *ksr-1* or *ksr-2* usually have an excretory duct cell and are viable, animals lacking both *ksr-1* and *ksr-2* lack a duct cell and are rod-like lethal (Figure 1), thus demonstrating that *ksr-1* and *ksr-2* are redundantly required for Ras-dependent excretory duct cell fate specification (OHMACHI *et al.* 2002).

To identify genes required for KSR function, we conducted a genomewide RNAi screen for “*ekl*” (enhancer of *ksr-1* lethality) genes whose depletion mimics loss of *ksr-2* and causes excretory duct loss in a *ksr-1* null mutant background. The majority of genes identified in this screen appear to be required in the maternal germline to regulate gene expression at the transcriptional or the post-transcriptional level. These include the Argonaute gene *csr-1* and a specific subset of other genes implicated in endogenous small RNA processes, orthologs of multiple components of the NuA4/Tip60 histone acetyltransferase complex and the CCR4/NOT deadenylase

complex, and conserved enzymes involved in ubiquitination and deubiquitination. We hypothesize that these genes cooperate to affect the expression and/or function of targets critical for KSR scaffold activity.

MATERIALS AND METHODS

General methods and alleles: General methods for the handling, culturing, and EMS mutagenesis of nematodes were as previously described (BRENNER 1974). *C. elegans* var Bristol strain N2 is the wild-type parent for all strains used in this work. Specific genes and alleles are described on Wormbase (<http://www.wormbase.org>) unless otherwise noted here. Deletion alleles *unc-25(tm1531) I*, *ccr-4(tm1312) IV*, and *ekl-5(tm2531) X* were kindly provided by S. Mitani (National Bioresource Project, Japan). Deletion allele *unc-25(cs77) I* was isolated by PCR screening of an EMS-mutagenized worm library made in our own laboratory. All except *ekl-5* were outcrossed at least six times prior to analysis. The *ego-1(om84)* allele was used to test for genetic interactions with *ksr-1(n2526)* (QIAO *et al.* 1995). Transgene *sals14 [lin-48::gfp] II* was obtained from Helen Chamberlin (JOHNSON *et al.* 2001; SEWELL *et al.* 2003). Transgene *czIs10 [unc-119(+); pie-1::GFP::KSR-2a]* was obtained from Monica Gotta (ETH Zürich) and is described below.

RNAi-feeding screen: RNAi feeding was performed essentially as described in KAMATH *et al.* (2001). RNA production was induced using 1 mM IPTG. For chromosome I, the screen was performed in duplicate using both *ksr-1(n2526)* single mutants (strain MT8677) and the RNAi-hypersensitive strain *rrf-3(pk1426); ksr-1(n2526)* (strain UP1017); however, all chromosome I *ekl* genes were identified in both strain backgrounds. Subsequent chromosomes were screened with only the *ksr-1(n2526)* strain. Multiple L2–L3 animals were plated on each bacterial strain expressing a different RNAi clone and the progeny were screened 3 and 4 days later for the presence of rod-like lethality. All clones in which the rod-like lethality was perceived to be higher than background were retested in triplicate. For clones that passed this retest, the rod-like lethal phenotype was quantified by collecting eggs laid by multiple RNAi-treated mothers over an 8- to 16-hr period and then counting the numbers of rod-like larvae and live progeny (dead embryos and other dying larvae were often present but not counted). Clones that consistently resulted in a rod/(rod + live) ratio of at least 20% were considered positive. The identities of these positive clones were verified by PCR amplification and restriction digestion, sequencing, and/or RNAi with an independent clone. This verification step was important as we found several positive hits whose identities differed from the expected clone in that library well.

After screening the entire RNAi library and identifying 15 positive hits, we performed selective retesting of genes related to our initial hits—*i.e.*, known components of the core Ras pathway and genes implicated in small RNA processes, the NuA4/Tip60 complex, the CCR4/NOT complex, and protein ubiquitination. These retests of initially negative clones (supplemental Table S1 at <http://www.genetics.org/supplemental/>) identified another three positive hits (*trr-1*, *gfl-1*, and *let-711/ntl-1*). Notably, other than *cnk-1*, we did not identify known Ras pathway genes in our screen, and when deliberately retested, 0/6 such genes showed a strong *ekl* phenotype. This high false negative rate indicates that our screen has identified only a small proportion of the genes that could potentially interact with *ksr-1*.

Phenotype analysis: Newly hatched L1 larvae from RNAi-treated mothers were scored for the presence or the absence of

the excretory duct cell using the *plin-48::gfp* transgene in strain UP1252 [*saIs14; ksr-1(n2526)*]. We specifically scored larvae that were already beginning to accumulate pockets of fluid and thus were destined to manifest the rod-like lethal phenotype. All *ekl* dsRNAs caused a high proportion of duct loss in this assay, whereas unrelated rod-like lethal mutants such as *ceh-10* and *vha-5* did not, demonstrating that fluid accumulation itself does not obscure *lin-48::gfp* expression.

All *ekl* dsRNAs were tested in additional strain backgrounds to probe the nature of their effect on excretory duct development. With the exception of *rab-7* and *cnk-1*, the *ekl* dsRNAs did not significantly increase rod-like lethality in any of the following strain backgrounds tested: N2 [*wild-type*], UP189 [*lin-45(ku112)*], MH731 [*mek-2(ku114)*], UP1199 [*cnk-1(ok836)*], and MT1175 [*lin-25(n545)*]. For zygotic RNAi experiments, mothers from strain UP916 [*rde-1(ne300) unc-42(e270); lon-2(e678) ksr-1(n2526)*] were exposed to dsRNA and then mated to *ksr-1(n2526)/0* males.

For the *ksr-2* rescue experiments, full-length *ksr-2a* cDNA was amplified from clone yk343d6 using the oligos (forward: atgagcgatgaaagaagaaaaagc) and (reverse: ctaaacagtggtattctcgtg) and cloned into the modified Gateway vector pID3.01 to create an amino-terminal GFP fusion, pMG290 (*pie-1::GFP::KSR-2a*). The pID3.01 vectors utilize the *pie-1* 5'- and 3'-UTR sequences to drive expression in the maternal germline (PELLETTIERI *et al.* 2003; HAO *et al.* 2006). Integrated transgenic strains were made by microparticle bombardment as described in PRAITIS *et al.* (2001) to create the strain ZU32 {*unc-119(ed3) III; czIs10[unc-119(+); pie-1::GFP::KSR-2a]*}. Germline expression of *ksr-2(+)* is sufficient to rescue the sterility, rod-like lethality, and Vulvaless phenotypes of *ksr-2(dx27); ksr-1(n2526)* animals (Figure 2D and data not shown). The *ekl* RNAi feeding was performed, as described above, using *unc-119(ed3); lon-2(e678) ksr-1(n2526); czIs10* and either *ksr-1(n2526)* or *lon-2(e678) ksr-1(n2526)* as a control.

Tests for the Rde phenotype were performed by feeding the viable deletion mutants *ccr-4(tm1312)*, *ekl-5(tm2531)*, *ubc-25(cs77)*, and *ubc-25(tm1531)* dsRNA corresponding to *pos-1*, which is required for embryonic viability (TABARA *et al.* 1999). L4-stage animals for each genotype were plated on *pos-1(RNAi)* feeding plates and eggs laid between 25 and 48 hr postplating were scored for viability (hatching). For all four deletion strains, 100% ($n > 100$) of eggs from *pos-1(RNAi)* animals failed to hatch after 24 hr. These data plus the RNAi data from the synergy tests (Figure 4) suggest that *ccr-4*, *ekl-5*, and *ubc-25* are not required for RNAi.

The remaining *ekl* genes were tested for the ability to suppress *gfp(RNAi)*. L4-stage animals of the strain AZ212 that expresses GFP::H2B in the germline were plated on *ekl(RNAi)* for 24 hr and transferred to *ekl(RNAi) + gfp(RNAi)* plates for an additional 24 hr, after which animals were analyzed for germline GFP expression. Untreated animals show strong GFP expression in the germline, while animals treated with only *gfp(RNAi)* had no GFP expression. As expected, animals pretreated with *ekl-1*, *ego-1*, and *drh-3* dsRNA displayed strong GFP expression. *csr-1*, *let-711/ntl-1*, and *math-33* displayed reduced GFP expression, and little or no expression was seen with *mys-1*, *epc-1*, *trr-1*, *gfl-1*, *ekl-4*, *ntl-2*, *ekl-6*, and *rab-7*.

RNAi of the nine germline expressed genes that are known targets of the *drh-3*-dependent and *err*-independent endo-siRNAs (DUCHAINÉ *et al.* 2006) were tested for the ability to enhance *ksr-1* lethality or to suppress the *drh-3(RNAi); ksr-1(n2526)* Ekl phenotype. RNAi clones for *T01A4.3*, *Y56A3A.14*, *F16D3.5*, *B0250.8*, *C04F12.9*, *B0047.1*, *T12G3.1*, and *F54F2.2* were obtained from the Ahinger library, and the *F39F10.2* feeding clone was generated by cloning the corresponding genomic DNA into the L4440 feeding vector. All clones failed to enhance the rod-like lethal phenotype of *ksr-1*, producing 0–2%

rod-like lethality ($n > 176$) as compared to <1% for *gfp(RNAi)* ($n = 1095$). To test for suppression of the *drh-3* Ekl phenotype, bacteria expressing each clone were mixed with bacteria expressing dsRNA corresponding to *drh-3*. No significant reduction in rod-like lethality was observed [30–48% ($n > 195$) as compared to 36% for *gfp + drh-3* ($n = 951$)].

cDNA and deletion mutant analysis: *ubc-25* gene structure was confirmed by sequencing the coding region of EST clone yk1102b10—this clone sequence differed at two coding base positions from a previously reported cDNA (SCHULZE *et al.* 2003), but matched the reported genomic sequence at those positions (www.wormbase.org). *ekl-5* gene structure was confirmed by sequencing EST clones yk458f3 and yk497g4—these clone sequences perfectly matched prior predictions (www.wormbase.org). We could not detect evidence for *trans*-splicing of *ekl-5*, and no stop codons are present upstream of the predicted start codon in the EST clones, leaving open the possibility of additional 5' exons.

The molecular breakpoints of deletion alleles *ccr-4(tm1312)*, *ubc-25(tm1531)*, and *ekl-5(tm2531)* have been characterized (<http://www.grs.nig.ac.jp/c.elegans/index.jsp>). We further verified these deletions by PCR analysis using primers both within and outside the deletion. We sequenced *ubc-25(cs77)* to show that it is a 381-bp deletion plus 12-bp insertion that removes intron 2 and most of exon 3. With respect to the wild-type *ubc-25* genomic sequence from ATG to stop (1–1364), the *cs77* sequence is 1–458/CACATCTGAGGC/840–1364. There is also a C → T point mutation at nucleotide 396, which would result in a Pro → Leu substitution. Analysis of RT-PCR products shows that *cs77* leads to a frameshift and truncation prior to the UBC catalytic domain. *cs77* and the other deletion alleles all would be predicted to severely reduce and most likely eliminate gene function.

Mutant lethality was quantified by counting the total number of eggs laid by multiple mothers over an ~8-hr period and then counting the numbers of rod-like larvae and live L4 progeny observed over the subsequent 2–5 days. Total lethality was calculated as (total – live)/total.

Real-time PCR: *ksr-2* mRNA levels in *csr-1(RNAi)*, *mys-1(RNAi)*, *ccr-4(tm1312)*, *ubc-25(cs77)*, and *ekl-5(tm2531)* mutants were compared to those in wild-type or *GFP(RNAi)* strains using Taqman (Applied Biosystems, Foster City, CA) real-time PCR. Total RNA was prepared from embryos using Trizol reagent (Invitrogen, Carlsbad, CA) and RNeasy (QIAGEN, Valencia, CA) and reverse transcribed using the Superscript first-strand synthesis kit (Invitrogen). Two independent biological samples were prepared for each of the RNAi experiments. Taqman primers for *ksr-2* and the reference gene *smr-1* were obtained from Applied Biosystems; the *ksr-2* primers amplify regions of exons 6 and 7 and thus recognize both known splice isoforms (OHMACHI *et al.* 2002). All reactions were performed in triplicate on an ABI 7900 HT machine. *ksr-2* levels were either equivalent or very slightly (less than twofold) higher in the experimental samples compared to controls. We also used this assay to verify that *ksr-1(n2526)* mutants do not have elevated levels of *ksr-2* expression.

RESULTS

An RNAi screen for enhancers of *ksr-1* lethality: Specification of the excretory duct fate occurs during mid- to late embryogenesis (SULSTON *et al.* 1983) and relies on a combination of maternally provided and zygotically expressed Ras pathway gene products (SUNDARAM 2006). Maternally provided *ksr-2* is sufficient to allow the duct fate, as *ksr-2*; *ksr-1* double mutants, which lack

FIGURE 2.—RNA-mediated interference of *ekl* genes enhances the rod-like lethality of *ksr-1* larvae. (A) For each *ekl* gene, the progeny of dsRNA-fed MT8677 [*ksr-1(n2526)*] mothers were collected at least 24 and not more than 48 hr after the initial dsRNA exposure (except for *let-711/ntl-1*, where progeny were collected as early as 5 hr after the initial dsRNA exposure to minimize embryonic lethality). The percentage of rod-like lethality was calculated as no. rods/(no. rods + no. live), thus excluding embryonic and other pleiotropic lethals. Shown is average rod-like lethality across all experiments. Error bars indicate actual range of percentages obtained across different experiments. In general, longer maternal preexposure resulted in higher rod-like lethality and increased pleiotropy. All $P < 0.001$ by Fisher's exact test compared to GFP control. (B) For each *ekl* gene, the progeny of two dsRNA-fed MH734 [*ksr-1(ku68)*] mothers were collected and scored as above. All $P < 0.001$ by Fisher's exact test compared to GFP control, except *let-711/ntl-1* and *ekl-6*, $P < 0.01$. *ku68* is a missense allele with slight dominant-negative properties (SUNDARAM and HAN 1995; ROCHELEAU *et al.* 2002). (C) For each *ekl* gene, the progeny of two dsRNA-treated UP1260 [*rrf-1; ksr-1(n2526)*] mothers were collected and scored as above (solid bars); the broods of similarly treated MT8677 mothers were scored in parallel (shaded bars). All $P < 0.001$ by Fisher's exact test compared to GFP control, except *trr-1* ($P = 0.002$), *gfl-1* ($P = 0.02$), *let-711/ntl-1* ($P = 0.14$, not significant), and *ekl-6* ($P = 0.012$) in the UP1260 background. There is a trend for the UP1260 values to be lower than the MT8677 values, suggesting some somatic contribution; this difference is significant at $P < 0.001$ for *csr-1*, *trr-1*, *let-711/ntl-1*, *ntl-2*, *math-33*, *ekl-5*, *ekl-6*, and *cnk-1* and at $P < 0.01$ for *ccr-4* and *rab-7*. (D) *ksr-2(dx27); ksr-1(n2526)* animals are maternally rescued for viability but sterile. The germline-expressed transgene *pie-1::GFP::KSR-2* (*czIs10*) rescues the sterility and rod-like lethality of *ksr-2*; *ksr-1*, allowing the homozygous strain to be propagated. *pie-1::GFP::KSR-2* only partially rescues other *ekl* gene phenotypes. For each *ekl* gene, the progeny of two or four dsRNA-treated *unc-119(ed3); lon-2(e678) ksr-1(n2526); czIs10* mothers were collected and scored as above (solid bars); the broods of similarly treated MT8677 and/or UP166 [*lon-2(e678) ksr-1(n2526)*] mothers were scored in parallel (shaded bars). All $P < 0.001$ by Fisher's exact test compared to *ksr-2(dx27)* control, except *drh-3* ($P = 0.0012$), *trr-1* ($P = 0.028$), *gfl-1* ($P = 0.2$, not significant), and *rab-7* ($P = 0.074$, not significant) in the *ksr-1; czIs10* background. There is a trend for the *ksr-1; czIs10* values to be lower than the *ksr-1* values, suggesting that increasing germline *ksr-2(+)* can partially rescue *ekl(RNAi)*, *ksr-1(n2526)*, or both; this difference is significant at $P < 0.01$ for all except *ekl-1*, *ntl-2*, and *math-33*. Data for *let-711/ntl-1* were not included due to high embryonic lethality.

zygotic gene function but retain maternal *ksr-2*, have a duct and are viable (though sterile) (OHMACHI *et al.* 2002), and germline-specific expression of *ksr-2* rescues the lethality of *ksr-2; ksr-1* double mutants (Figure 2D and MATERIALS AND METHODS). In the absence of *ksr-1*, maternally provided *ksr-2* may also be necessary for the duct fate, since when we specifically RNAi depleted maternal *ksr-2* in *rrf-1; ksr-1* animals, which are RNAi-resistant somatically (SJTEN *et al.* 2001), those animals arrested as rod-like larvae (Figure 2C).

To identify genes important for KSR function, we screened the Ahringer RNAi feeding library (FRASER *et al.* 2000; KAMATH *et al.* 2003) for clones that could

mimic the effect of *ksr-2* and consistently cause strong rod-like lethality in the background of *ksr-1(n2526)*, a nonsense mutation and putative null allele (KORNFELD *et al.* 1995) (see MATERIALS AND METHODS). After screening a total of 16,748 RNAi clones, we identified 18 clones that consistently caused strong rod-like larval lethality in multiple *ksr-1* mutant backgrounds, but not in a wild-type background (Figure 2 and data not shown). We call this phenotype and the corresponding class of genes *ekl* for enhancer of *ksr-1* lethality. In all cases, a significant proportion of *ekl; ksr-1* animals lacked a *plin-48::gfp*-positive excretory duct cell, suggesting that these genes cooperate with *ksr-1* and the Ras pathway to specify the

excretory duct cell fate (Figure 1 and data not shown). Surviving animals had normal vulval development and normal P11/P12 ectoblast fates, suggesting that other Ras-dependent signaling events were unperturbed. The specific nature of this phenotype and the fact that only 0.1% of clones tested showed an Ekl phenotype indicate that *ksr-1* enhancement does not result from a general reduction in health or vigor. Furthermore, many of the *ekl* genes point to specific processes or protein complexes whose perturbation leads to this phenotype (Table 1, see below).

The *ekl* genes interact specifically with *ksr-1* and not with other mutants in the Ras pathway: Although the original goal of our RNAi screen was to identify genes that specifically regulate KSR function and/or EGFR/Ras/ERK signaling, the molecular identities of many of the *ekl* genes (Table 1) suggest a broader role in gene regulation. Furthermore, when we tested our *ekl* RNAi clones against a panel of other Ras pathway mutants (MATERIALS AND METHODS), including hypomorphic alleles of *lin-45* *raf* and *mek-2*, we found that only 2 RNAi clones caused rod-like lethality in those backgrounds as would be expected if Ras signaling or the excretory duct fate were perturbed directly (data not shown); these corresponded to the known Ras pathway scaffold *cnk-1* (ROCHELEAU *et al.* 2005) and the GTPase *rab-7* (L. CHOTARD and C. E. ROCHELEAU, unpublished results). The remaining 16 *ekl* RNAi clones showed rod-like lethality exclusively in the background of *ksr-1* alleles, suggesting that these *ekl* genes influence a process to which *ksr-1* mutants are particularly sensitive.

We considered the hypothesis that the *ekl* genes affect *ksr-2* expression or function, because as discussed above, *ksr-1* mutants are particularly sensitive to loss of *ksr-2*. In contrast, other Ras pathway mutants do not show genetic interactions with *ksr-2* (OHMACHI *et al.* 2002 and data not shown). Consistent with (though not proving) a defect at the level of KSR-2 scaffold function, the *ekl* genes appear to function in the germline (see below) and show similar patterns of epistasis to *ksr-2*: the Ekl phenotype was reduced but not eliminated by a constitutive allele of *let-60* *ras* and was eliminated by mutation of the downstream MPK-1/ERK target *lin-1* (Figure 3). However, using real-time PCR, we have not detected any reduction in endogenous *ksr-2* mRNA expression in embryos from *ekl* RNAi or *ekl* mutant strains (see MATERIALS AND METHODS). Furthermore, a *pie-1::GFP::KSR-2* transgene (which lacks endogenous *ksr-2* regulatory elements) only partially rescued the *ekl*(RNAi); *ksr-1* phenotype whereas it fully rescued the *ksr-2*; *ksr-1* phenotype (Figure 2D). Thus, the *ekl* genes are unlikely to affect *ksr-2* expression directly, but may instead alter the expression or function of an unknown interacting factor(s) (see DISCUSSION).

The *ekl* genes act in the maternal germline: Under strong RNAi conditions, many of the *ekl* genes have pleiotropic RNAi phenotypes that include significant

embryonic lethality irrespective of the *ksr-1* mutant background (Table 1), suggesting that these genes regulate the expression of multiple targets. Both the embryonic lethality and the Ekl phenotype reflect (at least in part) a maternal requirement for these genes, as germline-specific RNAi (in an *rrf-1*; *ksr-1* background) continued to elicit both phenotypes (Figure 2C and data not shown), whereas zygotic-specific RNAi for *ekl-1*, *drh-3*, or *ekl-4* (in the RNAi-competent *rde-1/+*; *ksr-1* progeny of RNAi-defective *rde-1*; *ksr-1* mothers) did not elicit these phenotypes (data not shown) (see HERMAN 2001 for a discussion of zygotic RNAi). Furthermore, for all *ekl* deletion mutants tested (*ego-1*, *ccr-4*, *ubc-25*, and *ekl-5*; see below), *ekl*; *ksr-1* animals obtained from *ekl/+*; *ksr-1* mothers were viable (data not shown). The maternal-effect phenotype of the *ekl* genes suggests that these genes must be expressed in the germline to influence excretory duct cell fate specification in the embryo. Available *in situ* hybridization data (<http://nematode.lab.nig.ac.jp/db2/>), microarray expression data (KIM *et al.* 2001; REINKE *et al.* 2004), and the sterile phenotypes of several existing deletion mutants (QIAO *et al.* 1995; SMARDON *et al.* 2000; CEOL and HORVITZ 2004; DUCHAINE *et al.* 2006; YIGIT *et al.* 2006) are also consistent with a germline function for the *ekl* genes.

Several *ekl* genes, including *csr-1*, may act together in small RNA biogenesis or function: Four *ekl* genes have been previously implicated in RNAi-related processes and may define a novel small RNA pathway that regulates germline gene expression (Table 1). *csr-1* is one of a large number of genes coding for Argonaute-like PAZ-PIWI domain proteins in *C. elegans* (GRISHOK *et al.* 2001) and is required for transgene-mediated cosuppression (ROBERT *et al.* 2005) and efficient RNAi in the germline (YIGIT *et al.* 2006). *drh-3* encodes a Dicer-related helicase that is associated with the Dicer protein, DCR-1, and is required for the production of endogenous small RNAs as well as for RNAi in the germline (DUCHAINE *et al.* 2006). *ego-1* encodes an RNA-dependent RNA polymerase (RdRP) required for multiple aspects of germline development and for RNAi in the germline (QIAO *et al.* 1995; SMARDON *et al.* 2000; MAINE *et al.* 2005; VOUGHT *et al.* 2005). *ekl-1* (F22D6.6) encodes a Tudor-domain protein required for RNAi, transgene silencing, and transgene-mediated cosuppression in the germline (GRISHOK *et al.* 2005; KIM *et al.* 2005; ROBERT *et al.* 2005). *ekl-1* also has a paralog, *eri-5*, that is an enhancer of RNAi (Eri phenotype) and whose protein product is associated with DCR-1 (DUCHAINE *et al.* 2006). Although we directly tested numerous other genes implicated in RNAi (supplemental Table S1), only one other (*gfl-1*, see below) was found to share the Ekl phenotype, suggesting some unique property of *csr-1*, *drh-3*, *ego-1*, and *ekl-1*. In addition to sharing the Ekl and RNAi-defective phenotypes, these four genes all share a similar chromosome segregation-defective and embryonic-lethal phenotype (DUCHAINE *et al.* 2006; YIGIT *et al.* 2006; K. M. PANG and C. C. MELLO, personal communication and our unpublished observa-

TABLE 1
Molecular identities of *ekl* gene products

Ekl gene product		Protein motifs	Homologous proteins	Phenotypes
Group I: RISC-like Piwi-AGO family				
CSR-1	F20D12.1	PAZ, PIWI		Emb, Lvl, Ste, Rde (KAMATH <i>et al.</i> 2003; SIMMER <i>et al.</i> 2003; RUAL <i>et al.</i> 2004; SONNICHSEN <i>et al.</i> 2005; YIGIT <i>et al.</i> 2006)
DRH-3/ EKL-3	D2005.5	DEAD-box helicase	DRH-1, -2	Emb, Rde (FRASER <i>et al.</i> 2000; FERNANDEZ <i>et al.</i> 2005; SONNICHSEN <i>et al.</i> 2005; DUCHAINE <i>et al.</i> 2006)
EGO-1	F26A3.3	RdRP	RRF-1–3	Emb, Lvl, Ste, Rde (QIAO <i>et al.</i> 1995; FRASER <i>et al.</i> 2000; SMARDON <i>et al.</i> 2000; SIMMER <i>et al.</i> 2003; SONNICHSEN <i>et al.</i> 2005; LEHNER <i>et al.</i> 2006)
EKL-1	F22D6.6	2 Tudor domains	ERI-5	Emb, Abs, Rde (FRASER <i>et al.</i> 2000; MAEDA <i>et al.</i> 2001; ZIPPERLEN <i>et al.</i> 2001; SIMMER <i>et al.</i> 2003; RUAL <i>et al.</i> 2004; SONNICHSEN <i>et al.</i> 2005)
Group II: NuA4/Tip60-like				
MYS-1	VC5.4	Chromo, Myst-family HAT	ESA1, Tip60	Emb, Lvl, Ste, SynMuvC (CEOL and HORVITZ 2004; FERNANDEZ <i>et al.</i> 2005; SONNICHSEN <i>et al.</i> 2005; LEHNER <i>et al.</i> 2006; UPDIKE and MANGO 2006)
TRR-1	C47D12.1	PI3K	TRRAP, Nipped-A	Emb, Lvl, Ste, SynMuvC (MAEDA <i>et al.</i> 2001; SIMMER <i>et al.</i> 2003; CEOL and HORVITZ 2004; RUAL <i>et al.</i> 2004; SONNICHSEN <i>et al.</i> 2005; LEHNER <i>et al.</i> 2006)
EPC-1	Y111B2A.11		EPL1, E(Pc)	Emb, Lvl, Ste, SynMuvC (MAEDA <i>et al.</i> 2001; KAMATH <i>et al.</i> 2003; SIMMER <i>et al.</i> 2003; CEOL and HORVITZ 2004; RUAL <i>et al.</i> 2004; SONNICHSEN <i>et al.</i> 2005)
EKL-4	Y105E8A.17	SANT domain	SWC4, DMAP1	Emb, Lvl, Sup of SynMuv (FRASER <i>et al.</i> 2000; MAEDA <i>et al.</i> 2001; SIMMER <i>et al.</i> 2003; SONNICHSEN <i>et al.</i> 2005; CUI <i>et al.</i> 2006b)
GFL-1	M04B2.3	YEATS domain	YAF9, GAS41	Lvl, Sup of SynMuv, Rde (DUDLEY <i>et al.</i> 2002; KIM <i>et al.</i> 2005; CUI <i>et al.</i> 2006b)
Group III: CCR4/NOT-like				
CCR-4	ZC518.3	LRR, nuclease	CCR4, twin	Lvl, semiviable, Emb (KAMATH <i>et al.</i> 2003; SIMMER <i>et al.</i> 2003; SONNICHSEN <i>et al.</i> 2005). Allele <i>tm1312</i> .
LET-711/ NTL-1	F57B9.2		NOT1	Emb, Lvl, Ste (MAEDA <i>et al.</i> 2001; KAMATH <i>et al.</i> 2003; SIMMER <i>et al.</i> 2003; SONNICHSEN <i>et al.</i> 2005; DEBELLA <i>et al.</i> 2006)
NTL-2	B0286.4		NOT2	Emb, Lvl, Ste (KAMATH <i>et al.</i> 2003; SIMMER <i>et al.</i> 2003; RUAL <i>et al.</i> 2004)
Others				
UBC-25	F25H2.8	RWD-like, UBC	Ube2q	Viable, alleles <i>cs77</i> , <i>tm1531</i>
MATH-33	H19N07.2	MATH and ubiq protease	Usp7	Gro (KAMATH <i>et al.</i> 2003; SIMMER <i>et al.</i> 2003)
EKL-5	Y26E6A.1	CCHC Zn finger		Viable, allele <i>tm2531</i>
EKL-6	T16G12.5	Armadillo-like helical	TMCO7	Lvl, Ste (KAMATH <i>et al.</i> 2003; SIMMER <i>et al.</i> 2003)
RAB-7	W03C9.3	Rab GTPase	Rab7	Emb (KAMATH <i>et al.</i> 2003; SIMMER <i>et al.</i> 2003; RUAL <i>et al.</i> 2004; SONNICHSEN <i>et al.</i> 2005)
CNK-1	R01H10.8	SAM, CRIC, PDZ, PH	CNK family	Viable (ROCHELEAU <i>et al.</i> 2005)

Protein motifs were identified on the basis of protein homology, Wormbase annotations, and SMART analysis (SCHULTZ *et al.* 1998; LETUNIC *et al.* 2006). Emb, embryonic lethality; Lvl, larval lethality; Ste, sterility; Rde, RNAi defective; SynMuvC, class “C” synthetic multivulva; Sup of SynMuv, suppressor of synthetic multivulva; Gro, slow growth; Abs, abnormal spindle.

tions). These similarities suggest that CSR-1, DRH-3, EGO-1, and EKL-1 all function together to mediate the effects of small endogenous RNAs in the germline.

drh-3 together with *dcr-1*, *rff-3*, *eri-1*, *eri-3*, and *eri-5* is required for the production of several classes of endogenous small RNAs (DUCHAINE *et al.* 2006). Interestingly,

FIGURE 3.—*ekl* epistasis. For each *ekl* gene, the progeny of dsRNA-fed MT8677 [*ksr-1(n2526)*] (shaded bars), UP754 [*lon-2 ksr-1(n2526) lin-15(n309)*] (open bars), UP28 [*let-60(n1046gf); ksr-1(n2526)*] (solid bars), or UP764 [*lin-1(n304); lon-2 ksr-1(n2526)*] (bars with light shading; not visible for most *ekl* genes as *lin-1* completely suppressed the rod-like lethality) mothers were collected in parallel 24–40 hr after the initial dsRNA exposure. Shown is average rod-like lethality across all broods. All *ekl* dsRNAs except *trr-1* and *gfl-1* enhanced UP28 with a significance of $P < 0.01$ (Fisher's exact test). *lin-15* is a synthetic multivulva mutant believed to elevate *lin-3*/EGF expression (FERGUSON and HORVITZ 1989; CUI *et al.* 2006a). *let-60(n1046gf)* is a hypermorphic allele of the *ras* gene (BEITEL *et al.* 1990; HAN and STERNBERG 1990; HAN *et al.* 1990). *lin-1(n304)* is a null mutation of a Ras/ERK target gene encoding an Ets domain transcription factor (BEITEL *et al.* 1995). ^, no data. In the UP754 mutant background, these dsRNAs caused extensive embryonic and/or other larval lethality, which prevented meaningful quantification of rod-like lethality (which was also present). This may be due to the reported RNAi hypersensitivity of *lin-15* mutants (WANG *et al.* 2005) or to a specific genetic interaction with *lin-15*.

only *drh-3* is required for the production of a specific class of small RNAs corresponding to germline-expressed genes. Similarly, only *drh-3* showed an Ekl phenotype, whereas *dcr-1*, *rff-3*, *eri-1*, *eri-3*, and *eri-5* did not (supplemental Table S1). We therefore hypothesized that *drh-3*, along with *csr-1*, *ego-1*, and *ekl-1*, might affect Ras signaling via a mechanism involving this unique class of small RNAs. However, RNAi against targets of the nine reported siRNAs in this class did not cause an Ekl phenotype and did not significantly suppress the *drh-3* Ekl phenotype (see MATERIALS AND METHODS). Therefore, the group I genes may act through distinct siRNAs of this class or through an as yet uncharacterized class of *drh-3*-dependent but *rff-3*- and Eri-independent small RNAs (see DISCUSSION).

ego-1 is required for histone H3K9 methylation of unpaired DNA in the meiotic germline (MAINE *et al.* 2005). As H3K9 methylation is usually associated with repressed chromatin (RICE and ALLIS 2001), the loss of this mark in *ego-1* mutants implies that many X chromosome loci may be erroneously expressed; it is possible that such misexpression leads to the Ekl phenotype. However, *csr-1*, *drh-3*, and *ekl-1* mutants do not lose H3K9 methylation (X. SHE and E. M. MAINE, personal communication) and we did not identify any putative histone methyltransferases (HMTases) in our RNAi screen. Thus

the relationship between H3K9 methylation and the Ekl phenotype is still unclear.

We do note, however, that one shared feature of the group I *ekl* genes is that the Ekl phenotype is strongly reduced in a *lin-15* mutant background (Figure 3). *lin-15* is a “synthetic multivulva” (SynMuv) mutant believed to cause elevated *lin-3*/EGF transcription due to changes in chromatin structure (FERGUSON and HORVITZ 1989; CUI *et al.* 2006a; FAY and YOCHEM 2007). *lin-15* mutants are also RNAi hypersensitive (Eri), another defect thought to be caused by changes in chromatin structure (WANG *et al.* 2005). Suppression of group I Ekl defects by *lin-15* therefore could be a consequence of increased *lin-3*-dependent signaling or could be a consequence of other chromatin or RNAi-related changes.

Several *ekl* genes encode orthologs of the NuA4/Tip60 histone acetylase complex: Five *ekl* genes encode orthologs of proteins found in the NuA4/Tip60 chromatin-remodeling complex. MYS-1 is related to yeast Esa1p and mammalian TIP60, the histone acetyltransferases (HATs) of the yeast NuA4 and mammalian Tip60 complexes (CEOL and HORVITZ 2004). TRR-1, EPC-1, GFL-1, and EKL-4 (Y105E8A.17) are related to other well-characterized components of these complexes (DUDLEY *et al.* 2002; CEOL and HORVITZ 2004; CUI *et al.* 2006b) (Table 1). The NuA4 and Tip60 complexes are thought to

influence the expression of large numbers of genes via acetylation of histones (DOYON and COTE 2004; SQUATRITO *et al.* 2006). The molecular identities and shared Ekl phenotypes suggest that the *C. elegans* proteins are likely to form a related complex with similar properties.

Interestingly, *mys-1*, *trr-1*, and *epc-1* were previously found to share a “SynMuv C” phenotype, suggesting that they antagonize Ras signaling during vulval development (CEOL and HORVITZ 2004). Furthermore, *ekl-4* and *gfl-1* function antagonistically to the SynMuv C genes to promote Ras-mediated vulval development (WANG *et al.* 2005; CUI *et al.* 2006b), whereas we have seen that *ekl-4* and *gfl-1* function cooperatively with *mys-1*, *trr-1*, and *epc-1* to promote Ras-mediated excretory duct cell development. It is likely that multiple versions of a NuA4/Tip60-like chromatin remodeling complex exist in *C. elegans* and that these regulate different sets of genes. Recently, *mys-1* and *trr-1* were identified as having genetic interactions with several signal transduction pathways, including the Wnt and Notch pathways, consistent with their regulating the expression of a large number of genes (LEHNER *et al.* 2006). The SynMuv C and Ekl phenotypes probably result from misregulation of different specific targets that impact Ras signaling in opposite ways.

A potential link between the NuA4/Tip60-like (group II) and RISC-like (group I) *ekl* genes is suggested by the fact that *gfl-1* (group II) is also required for RNAi (DUDLEY *et al.* 2002; KIM *et al.* 2005). However, other group II genes such as *ekl-4* are not required for RNAi (CUI *et al.* 2006b and MATERIALS AND METHODS). Thus it is unlikely that the NuA4/Tip60 complex is required merely for the transcription of one or more group I *ekl* genes. Instead, we favor the model that group I and group II *ekl* genes cooperate, perhaps through chromatin modifications, to modulate some common target(s).

Several *ekl* genes encode orthologs of the CCR4/NOT deadenylase complex: Three *ekl* genes encode orthologs of proteins found in the CCR4/NOT deadenylase complex. CCR4 is related to the catalytic subunit of this complex, which in yeast possesses 3′–5′ exonuclease activity toward single-stranded RNA and DNA (TUCKER *et al.* 2001, 2002; CHEN *et al.* 2002; TEMME *et al.* 2004) (Table 1). LET-711/NTL-1 and NTL-2 are related to NOT1 and NOT2, two other components of the complex (LIU *et al.* 1998). The CCR4/NOT complex appears to be a major mRNA deadenylase in yeast and *Drosophila* (TUCKER *et al.* 2001; TEMME *et al.* 2004) and has been implicated in miRNA-dependent mRNA degradation (BEHM-ANSMANT *et al.* 2006). In addition, the CCR4/NOT complex may have roles in transcriptional regulation (WINKLER *et al.* 2006) and protein ubiquitination (PANASENKO *et al.* 2006). In *C. elegans*, *let-711/ntl-1*, *ntl-2*, and most other putative subunits of the CCR/NOT complex are essential, as might be expected for genes that play widespread roles in mRNA metabolism (GONCZY *et al.* 2000; MAEDA *et al.* 2001; KAMATH *et al.*

TABLE 2

Analysis of *ccr-4*, *ubc-25*, and *ekl-5* deletion mutants

Genotype	% rod ^a	% lethal ^a	n
<i>ksr-1(n2526)</i>	1	7	177
<i>ccr-4(tm1312)</i>	0	29	212
<i>ccr-4(tm1312); ksr-1(n2526)</i>	70	87	219
<i>ubc-25(cs77)</i>	0	6	177
<i>ubc-25(cs77); ksr-1(n2526)</i>	66	82	162
<i>ubc-25(tm1531)</i>	0	23	235
<i>ubc-25(tm1531); ksr-1(n2526)</i>	48	80	215
<i>ekl-5(tm2531)</i>	0	8	218
<i>ksr-1(n2526) ekl-5(tm2531)</i>	40	47	124
<i>ubc-25(cs77); ccr-4(tm1312)</i>	0	50	169
<i>ubc-25(cs77); ccr-4(tm1312); ksr-1(n2526)</i>	87*	100*	177
<i>ubc-25(cs77); ekl-5(tm2531)</i>	0	9	218
<i>ubc-25(cs77); ksr-1(n2526) ekl-5(tm2531)</i>	91*	99*	109

**P* < 0.001 (Fisher's exact test) compared to animals mutant for only one of the two *ekl* genes.

^a Rod-like larval lethality and total lethality were calculated on the basis of total embryos laid. Surviving animals had normal vulval development and normal P11/P12 fates.

2003; SIMMER *et al.* 2003; RUAL *et al.* 2004; MOLIN and PUISIEUX 2005; SONNICHSEN *et al.* 2005; DEBELLA *et al.* 2006). However, the *ccr-4* gene does not appear to be essential, suggesting either that *let-711/ntl-1* and *ntl-2* have distinct *ccr-4*-independent functions or that the paralogous gene W02G9.5 compensates for *ccr-4* loss.

We used a *ccr-4* deletion allele (*tm1312*) to confirm our RNAi result and investigate further the genetic interaction with *ksr-1* (Table 2, Figure 4A). *tm1312* removes most of the predicted CCR4 catalytic domain and thus is likely to be null (see MATERIALS AND METHODS). The majority of *ccr-4(tm1312)* homozygotes are viable and superficially normal, and while some do arrest as young larvae, none show the rod-like lethality characteristic of excretory defects. In contrast, the majority of *ccr-4; ksr-1* animals arrest with rod-like morphology. The animals that survive have normal development of other Ras-dependent cell types such as the vulva (data not shown). Thus, consistent with our previous RNAi results, *ccr-4* strongly enhances the *ksr-1* excretory duct loss phenotype, but does not enhance other postembryonic *ksr-1* phenotypes.

Additional *ekl* genes encode conserved proteins involved in ubiquitination and deubiquitination: The remaining four *ekl* genes encode a variety of proteins with possible roles in transcriptional or post-transcriptional gene regulation.

ubc-25 encodes a putative E2 ubiquitin-conjugating enzyme (UBC) orthologous to mammalian Ube2q (SCHULZE *et al.* 2003) (Table 1). This particular UBC family is absent from yeast and its biochemical properties are not well characterized, although Ube2q can be ubiquitin charged *in vitro* by the E1 Ube1 (JIN *et al.* 2007). Consistent with our RNAi results, we found that *ubc-*

FIGURE 4.—Synergistic interactions among the *ekl* genes. For each *ekl* gene, the progeny of multiple dsRNA-fed (A) UP1573 [*ccr-4*(*tm1312*); *ksr-1*(*n2526*)], (B) UP1372 [*unc-25*(*cs77*); *ksr-1*(*n2526*)], or (C) UP1576 [*lon-2* *ksr-1*(*n2526*) *ekl-5*(*tm2531*)] mothers were collected 24–40 hr after the initial dsRNA exposure. None, strain grown on standard NGM/OP50 plates as shown in Table 2. Shown is average rod-like lethality (no. rods/no. (rod + live)) across all broods. Error bars indicate the actual range of values obtained in different broods. All $P < 0.001$ by Fisher's exact test compared to GFP control, except $P > 0.05$ for *dih-3* and *unc-25* in UP1372 and *ekl-5* in UP1576. ^, these dsRNAs caused extensive embryonic and/or other larval lethality, preventing meaningful assessment of rod-like lethality.

25(cs77) or *unc-25(tm1531)* deletion mutants are mostly viable and normal, but *unc-25*; *ksr-1* double mutants have strong rod-like lethal and excretory duct loss phenotypes (Table 2, Figure 4B, and data not shown). Both deletions are likely to severely reduce or eliminate *unc-25* activity (see MATERIALS AND METHODS). As was true for *ccr-4*, *unc-25* does not enhance other postembryonic *ksr-1* phenotypes (data not shown).

math-33 encodes a putative ubiquitin-specific protease orthologous to Usp7/HAUSP (Table 1). Drosophila and mammalian Usp7 proteins have been found to deubiquitinate multiple substrates including histone H2B, the transcription factor FOXO, and the p53 regulator Mdm2 (LI *et al.* 2002; VAN DER KNAAP *et al.* 2005; TANG *et al.* 2006; VAN DER HORST *et al.* 2006). Mutants in *math-33* are not

yet available, but RNAi suggests that MATH-33 and UBC-25 act cooperatively, despite their predicted opposing molecular activities. Usp7 can process ubiquitin precursors *in vitro* to generate functional ubiquitin (LAYFIELD *et al.* 1999) and can also protect some E3 ubiquitin ligases from autoubiquitination and degradation (CANNING *et al.* 2004; TANG *et al.* 2006), suggesting two possible mechanisms by which MATH-33/Usp7 could promote a ubiquitin-dependent process. *math-33*(RNAi) causes a stronger Ekl phenotype than does *unc-25* and the two genes show additive effects (Figure 4B), indicating that *math-33* does not act completely through effects on *unc-25*.

ekl-5 encodes a novel protein with two copies of an unusual CCHC motif—it does not have obvious relatives outside of nematodes. Consistent with our RNAi results,

FIGURE 5.—Model for regulation of embryonic Ras signaling by the *ekl* genes. The *ekl* genes are required maternally and affect Ras-dependent excretory duct fate specification in the embryo; they may regulate expression of their unknown targets in the maternal germline or in the early embryo. Genes are grouped according to molecular identities. See text for details.

we found that *ekl-5(tm2531)* deletion mutants are mostly viable and normal, but *ksr-1 ekl-5* double mutants have partial rod-like lethal and excretory duct loss phenotypes (Table 2, Figure 4C). No other *ras*-like defects were observed in these double mutants (data not shown). The genetic behavior of *ekl-5* is thus very similar to that of *ccr-4* and *ubc-25*. Note, however, that the *ekl-5(tm2531)* phenotype is weaker than that seen with *ekl-5* RNAi, yet cannot be further enhanced by *ekl-5* RNAi (Figures 2A and 4C). This anomaly suggests either that partial depletion of *ekl-5* causes a stronger *ekl* phenotype than complete *ekl-5* loss or that there is some secondary modifier mutation in the *ekl-5(tm2531)* mutant strain. Note that *ekl-5* is similar to the group I *ekl* genes in that the Ekl phenotype is strongly suppressed by the *lin-15* SynMuv mutation (Figure 3).

Finally, *ekl-6* encodes a novel protein with a predicted armadillo-like helical motif—it has uncharacterized relatives in fish and mammals. Available RNAi and mutant data suggest that *ekl-6* may be an essential gene (Table 1).

***ekl-5* may act with the group I (RISC-like) *ekl* genes in a subset of processes:** As discussed above, the molecular identities of many of the *ekl* genes suggest three likely functional groupings (RISC-like, Tip60/NuA4-like, and CCR4/NOT-like). If true, then removing two members of a group should have consequences no more severe than removing just one member. Unfortunately, the nonnull nature of RNAi treatments and the essential requirements for the individual genes preclude such rigorous tests of interdependency. Nevertheless, additional shared phenotypes (see above) and genetic interactions (*e.g.*, Figures 3 and 4C) tend to support these functional groupings.

To test if any of the nonessential *ekl* genes could act closely with each other or with one of the three molecularly defined groups, we tested the effects of RNAi-depleting various *ekl* genes in the viable *ccr-4*, *ubc-25*, and *ekl-5* deletion backgrounds (Figure 4). We also analyzed true double mutants where possible (Table 2). In most cases, strong additive or synergistic effects were observed, suggesting that the different *ekl* genes can function independently of *ccr-4*, *ubc-25*, and *ekl-5*. This was true even of

the *ntl-2* and *ccr-4* combination (Figure 4A), consistent with the idea that there is a CCR4-independent (though potentially W02G9.5-dependent) NOT complex. It is striking, however, that RNAi depletion of the group I (RISC-like) *ekl* genes did not have more severe consequences in the *ksr-1 ekl-5* background compared to the *ksr-1* single-mutant background (compare Figures 2A and 4C). These data, together with the similar *lin-15* epistasis data (Figure 3), suggest that group I is a *bona fide* functional group and that the group I genes could function in part through *ekl-5* to promote Ras signaling. However, unlike the group I genes, *ekl-5* is not required for viability, fertility, or efficient germline RNAi (MATERIALS AND METHODS) and thus does not appear to function with the group I genes in all processes.

DISCUSSION

We have described 16 *ekl* genes that are required in combination with *ksr-1* for Ras-dependent excretory duct cell fate specification. These *ekl* genes appear to affect excretory duct development only under conditions where *ksr-1* is absent and not in any other strain backgrounds in which the Ras pathway is compromised. On the basis of the genetic properties and molecular identities of the *ekl* gene products, we hypothesize that these genes are not directly involved in signal transduction, but rather that *ekl* depletion alters the expression of one or more target genes that ultimately influence a KSR-dependent step of Ras signaling.

The role we are proposing for the *ekl* genes would be similar conceptually to that of the previously characterized synthetic multivulva (SynMuv) genes, which encode nuclear factors whose depletion mimics Ras pathway activation in the vulva and elsewhere (FERGUSON and HORVITZ 1989; FAY and YOCHER 2007). The SynMuv gene class includes the *C. elegans* Retinoblastoma (Rb) and E2F orthologs (CEOL and HORVITZ 2004) as well as other proteins found in the conserved DREAM and Myb-MuvB chromatin remodeling complexes (LIPSICK 2004). Although the mechanism by which the SynMuv genes affect Ras signaling was mysterious for many years

and is still not completely understood, the shared genetic properties of SynMuv genes allowed researchers to propose that these genes function together, a prediction that has been borne out by subsequent molecular and biochemical studies. The SynMuv genes have recently been shown to prevent ectopic *lin-3*/EGF expression (CUI *et al.* 2006a).

By analogy to the SynMuv genes, we propose that the *ekl* genes act coordinately to regulate the expression of one or more targets that ultimately impinge on the Ras-dependent excretory duct cell fate in the embryo (Figure 5). Since the *ekl* genes are largely maternally required, they may regulate target expression in the maternal germline or in the early embryo. Although the simplest model would be that the *ekl* genes affect maternal *ksr-2* expression, we find no evidence that this is the case. Instead, the *ekl* genes may normally promote the expression of other target(s) that facilitate KSR scaffold function, or they may normally inhibit the expression of target(s) that antagonize KSR scaffold function. We consider the latter possibility most likely for the group I (RISC-like) and group III (CCR4/NOT-like) *ekl* genes, given that small RNAs and the CCR4 deadenylase most often inhibit target gene expression. On the other hand, there is mounting evidence that some small RNAs can promote target gene expression (KUWABARA *et al.* 2004; LI *et al.* 2006; LIN 2007; YIN and LIN 2007). The group II (NuA4/Tip60-like) *ekl* genes also could act through either mechanism, as the Tip60 complex is implicated in both target gene activation and repression (SAPOUNTZI *et al.* 2006). Of course it is possible that the *ekl* genes act on targets that are several steps removed from the ultimate relevant effector(s) of Ras signaling.

Our study provides the first evidence that a novel small RNA pathway involving *csr-1*, *drh-3*, *ego-1*, and *ekl-1*, but not the Eri genes, acts in the *C. elegans* germline to regulate endogenous gene expression. Germline-specific small RNAs known as “piRNAs” and “rasiRNAs” have been described recently in *Drosophila* and mammals; these interact with Piwi—rather than Argonaute—subfamily proteins and are generated from single-stranded precursors in a Dicer-independent manner (VAGIN *et al.* 2006; ARAVIN *et al.* 2007; HARTIG *et al.* 2007; LIN 2007). The small RNAs relevant to the *C. elegans* Ekl phenotype could be analogous to piRNAs, as CSR-1 is equally similar to the Piwi and Argonaute subfamilies (YIGIT *et al.* 2006) and we have been unable to detect an Ekl phenotype after *dcr-1* depletion (supplemental Table S1 and data not shown), suggesting that these RNAs also may be generated in a Dicer-independent manner. However, *C. elegans* has numerous Piwi—Argonaute family proteins (YIGIT *et al.* 2006) and numerous types of small RNAs (AMBROS *et al.* 2003; RUBY *et al.* 2006; PAK and FIRE 2007), none of which (so far) closely resemble piRNAs.

Our screen identified additional genes whose functions could be relevant to those of the group I (RISC-like) *ekl* genes. In particular, genetic interaction data are

consistent with the model that a CSR-1-containing RISC modulates its relevant target via a mechanism that involves the novel protein EKL-5. As *ekl-5* mutants do not share many of the other pleiotropic defects seen in *csr-1*, *drh-3*, *ego-1*, or *ekl-1* mutants, these mutants should prove useful in identifying a relevant target responsible for the Ekl phenotype and elucidating its mechanism of regulation.

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