kep1 interacts genetically with *dredd*/*Caspase-8*, and *kep1* mutants alter the balance of *dredd* isoforms

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The *Drosophila kep1* gene encodes an RNA binding protein related to the murine QUAKING apoptotic inducer. We have previously shown that *kep1* can induce apoptosis when transfected into different cell lines. To better define the role of Kep1 in apoptosis, we generated *kep1* null flies. These flies were viable, but females displayed reduced fertility, with approximately half of the eggs laid from *kep1*− homozygotes failing to hatch. In addition, loss of *kep1* suppressed *GMR-rpr*-mediated apoptosis in the *Drosophila* eye, and *kep1* mutant flies had increased susceptibility to *Escherichia coli* infection. We found that Kep1 bound *dredd* RNA *in vitro*, and that extracts prepared from *kep1* mutant ovaries had markedly reduced proteolytic cleavage activity toward the caspase-8 target substrate IETD-7-amino-4-trifluoromethyl coumarin. We observed increased levels of the β isoform of *dredd* mRNA in *kep1* mutants as compared with wild-type. Taken together, our results suggest that Kep1 regulates apoptosis by influencing the processing of *dredd* RNA.

KH domain | apoptosis | *Drosophila* | caspases

Programmed cell death, or apoptosis, regulates the destruction of unnecessary cells in metazoan development, and alterations in the apoptotic program have been implicated in pathogenesis (for review, see ref. 1). KH domain-containing RNA binding proteins have been proposed as a family of apoptotic inducers (2). For example, the mouse quaking (QKI) protein and the *Drosophila* Kep1 and Sam50 proteins are potent inducers of cell death in the absence of any other signal (2–4). Expression of QKI causes apoptosis in transfected cells (4), and the C-terminal 14 aa of the QKI-7 isoform are necessary and sufficient to induce apoptosis (2). Kep1 (also referred to as Qkr58E-3; ref. 5), Sam50, and QKI all belong to the STAR protein family that contains a GSG domain (3, 6, 7). Like other single KH domain-containing proteins (3, 4, 8, 9), Kep1 can form homodimers and can bind homopolymeric RNA *in vitro*. In addition, lysates obtained from S2 cells transfected with Kep1 can induce isolated HeLa nuclei to show morphological characteristics indicative of apoptosis (3). Other KH domain-containing proteins also play a role in apoptosis. Induction of MCG10, an RNA binding protein containing two KH domains, can suppress proliferation of cells by inducing apoptosis and cell cycle arrest in G_2/M (10). Similarly, overexpression of the *Drosophila* homologue of the fragile X protein (Dfmr1) leads to apoptotic cell loss in all adult tissues examined (11). Finally, a proteomic analysis of Jurkat cells identified 21 proteins, of which 15 contained RNA binding motifs, that are modified during Fas-induced apoptosis, suggesting an important role for RNA binding proteins in the apoptotic process (12).

Many cells undergo apoptosis during embryogenesis and metamorphosis of *Drosophila* (13–15). Central to the apoptotic program are enzymes collectively referred to as caspases, which are synthesized as inactive proenzymes. These zymogens are cleaved to generate the catalytically active molecules that initiate a proteolytic cascade. Loss of the caspase CED-3 in the nematode *Caenorhabditis elegans* leads to a loss of most developmental programmed cell death (16). The functions of three other caspases encoded in the *C. elegans* genome have not yet been established. Several genes encoding caspases are present in the *Drosophila* genome: *Dcp1* (17), *Damm* (18), *decay* (19), and *Ice* (20), which encode effector caspases with short prodomains, and *dream* (21), *dredd* (22), and *Dronc* (23), which encode initiator caspases with long prodomains. The initiator caspases are cleaved to their active forms, which in turn cleave effector caspases and other molecules.

In *Drosophila*, all embryonic cell death is under the control of three closely linked genes, *reaper* (*rpr*), *grim*, and *head involution defect* (*hid*), that all fall within a chromosomal deletion *Df*(*3R*)*H99* (*H99*; refs. 24–26). These genes induce expression of genes encoding caspases, including *dredd*. In oogenesis, *dredd* mRNA is expressed beginning at stage 10 in nurse cells and the developing oocyte. *dredd* mRNA accumulates at high levels in stage-12 to -13 egg chambers, coincident with nurse cell apoptosis. *dredd* is also expressed in developing embryos, with its expression preceding the death of the cell in which it is found. *dredd* mRNA is essentially absent in *H99* embryos, and mutations that remove *dredd* are capable of suppressing *GMR-rpr*-induced apoptosis in the *Drosophila* eye (22).

dredd produces at least four different mRNAs (22). The δ and γ isoforms encode full-length Dredd proteins that differ by the presence of an additional 6 aa in the δ isoform. The protein encoded by the α isoform has most of its prodomain deleted. The β isoform of *dredd* mRNA differs from the others because of the inclusion of intron II, which can result in either the translation of a protein that contains all of the prodomain but lacks most of the catalytic domain, or possibly a protein that contains only the caspase domain. Evidence from cDNA sequencing projects suggests that the β isoform normally exists in several tissues (27).

In addition to its role in apoptosis, *dredd* is involved in the *Drosophila* antibacterial response (28, 29). In *dredd* mutants, the expression of all genes that code for peptides with antibacterial activity following Gram-negative bacterial infection is repressed (29). Activation of the *Drosophila* immune response is preceded by the proteolytic cleavage of the NF-κB factor Relish. The cleavage of Relish is independent of the proteosome but has an absolute requirement for Dredd (30). These data suggest either a direct role for Dredd in the cleavage of Relish or in the participation in a proteolytic cascade that results in Relish activation.

Although *kep1* is an inducer of apoptosis, how it regulates cell death and its broader cellular role are unknown. To investigate the function of *kep1* in *Drosophila* development, we generated *kep1*− null flies. We found that *kep1*− null flies are viable but display reduced fertility. Furthermore, we show that the Kep1 protein can bind *dredd* mRNA *in vitro*. Mutations in *dredd* and

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Abbreviations: AFC, 7-amino-4-trifluoromethyl coumarin; OreR, Oregon-R.

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kep1 behave in a similar fashion when infected with *Escherichia coli*. Loss of either *dredd* or *kep1* could also suppress the *GMR-rpr*-induced apoptosis in the *Drosophila* eye. We conclude that Kep1 is a positive regulator of apoptosis in *Drosophila* that exerts its effect in part through *dredd*.

Methods

P-Element Mobilization. To create a null allele of *kep1*, excision lines were generated by the introduction of a transposase source into *EP2493/CyO* (obtained from Todd Laverty, Berkeley *Drosophila* Genome Project, Berkeley, CA). *EP2493/CyO* males were crossed to *w*;*Bic-D*^{PA66} *Su*(*Bic-D*^{PA66}) *cn/CyO*;Δ*2-3 Sb/TM3 Ser* virgin females (31). *EP2493/CyO*;Δ *2–3 Sb/+* males were individually crossed with *w*;*P*[*w*+]/*CyO* virgin females. Individual white-eyed males that have lost the P element, *EP2493*/CyO*, were crossed to *P*[*w*+]/*CyO* virgin females, and white-eyed balanced progeny were crossed to obtain individual balanced lines. Excision lines were screened by Southern analysis using the complete *kep1* cDNA as a probe.

Fertility Testing. *Drosophila* lines were tested for fertility by crossing to Oregon-R (OreR) flies. Flies of the appropriate genotype were introduced into cages and were subjected to a 24-h prelay on apple juice plates. Eggs were subsequently collected for a 24-h period and were scored 30 h later according to the number and phenotype of unhatched progeny.

Characterization of the KEX15 Excision. Genomic DNA was isolated from homozygous *kep1* flies and used as a template for PCR. Oligonucleotides 5′-CTCAGTAAACTCGCTTGG-3′ and 5′-CCTAATACTAGTTAAAGCC-3′ were designed to amplify between positions 89907 and 91364 of the BAC clone BACR48M13 (coordinates are those of GenBank accession no. AC005714). The product isolated from the PCR was cloned into the *Eco*RV site of pBlueScript II and sequenced using T7 and T3 sequencing primers (Sheldon Biotechnology Centre, McGill University; ref. 3).

Generation of Transgenic Flies. *Myc-kep1* was excised from the pBlueScript II vector (3) by using a *Kpn*I/*Xba*I digest and cloned into the *Kpn*I/*Xba*I sites of pCasper4. A 1.45-kb genomic DNA fragment containing all of the sequence upstream of *kep1* was amplified from a *yw* background by using oligonucleotides 5′-AAATCTAGACCTAATACTAGTTAAAGCC-3′ and 5′-AAATCTAGATTTTATCATAACAATCTTTAC-3′. The PCR fragment was digested with *Xba*I and cloned upstream of *kep1* in the myc-*kep1* pCasper4 construct. Transgenic flies were generated using standard microinjection techniques (32) in a *yw* recipient, using the pTurbo plasmid as a transposase source.

UV Crosslinking Assay. Radiolabeled RNA was generated from expressed sequence tag GH10971 by using the T7 RNA polymerase from PCR products generated using T7 sequencing primer and 5′-CATATTATCCCTGCTAAGATTGGC-3′ (primer 01–58) or 5′-CCACATTGTATCCCATCGAGG-3′ (primer 01–59) or using oligonucleotides designed to amplify intron II containing a T7 RNA polymerase site at the 5′ end oligonucleotide 5′ T7-CGGAATGTTAGCAGGGATAATATG-3′ (primer 01–165) and 5′-GTGGAGAAGGGCAATGTTGTACTTC-3′ (primer 01–166). RNAs were incubated on ice for 30 min with HeLa cell extracts obtained after transfection of *myc-kep1* or *myc-Sam50*. Samples were UV-crosslinked in a Stratalinker (Stratagene) for 20 min on ice, followed by RNase A treatment before Myc immunoprecipitation. Samples were run on a 10% PAGE.

Caspase-8 Assays. Dredd activity was assessed using the Apo-alert kit (CLONTECH) following the manufacturer's protocol. The equivalent of two pairs of ovaries from well fed flies were used per assay. We followed the conversion of the caspase-8 7-amino-4-trifluoromethyl coumarin (AFC) substrate (IETD-AFC) to AFC over a time course of 1 h at 25°C. All assays were performed in duplicates on three separate days by using independent ovary extracts. Experiments were designed to maintain the rate of conversion of the substrate throughout the time course of the experiment within the linear range of the instrument (Fluoromark PM microplate reader, Bio-Rad).

RT-PCR and the Generation and Screening of Ovary cDNA Libraries. Libraries were generated using the Stratagene cDNA synthesis kit according to the manufacturer's protocol. cDNAs were cloned into the *Eco*RI/*Xho*I site of the Uni Zap vector by using poly(A)+ RNA obtained from 2- to 3-day-old, well fed females. Approximately 750,000 plaque-forming units were screened for each library by using a 500-bp fragment obtained from the 5′ end of the *dredd* EST LP05556. We isolated eight cDNA clones (four from the *yw* library, and four from the *kep1*− library), and all were completely sequenced. All eight cDNA clones obtained had different 5′ ends and were therefore independent clones. For RT-PCR experiments, 2 pmol of oligonucleotide 5′-GTCGATGTGCTGGTCCGGCG-3′ (primer 01–60) was used to prime the reverse transcription reaction by using 1 μg of poly(A)$^+$ mRNA isolated from *yw* or *kep1*− ovaries. One-tenth of each reverse transcription reaction was used in each of the subsequent PCR by using the cycling conditions 94°C for 30 s, 60°C for 30 s, and 74°C for 45 s over 30 cycles employing Vent polymerase (New England Biolabs). Oligonucleotide pairs were either 5′-ATGGCCGGATCAAACCTGTTG-3′ (primer 01–55), located in exon 1, and 5′-GGAGAAGGGCAATGTTGTACTTC-3′ (primer 01–57), located in intron II, or primers 01–55 and 01–59 located in exon 2. One-tenth of each PCR was loaded onto a 1.5% agarose gel.

***E. coli* Infections of *dredd* and *kep1* Mutant Flies.** Two- to 4-day-old female flies were pricked with a 30-gauge needle dipped in a concentrated solution of *E. coli* (5 ml of bacteria grown to OD 0.6 were recovered and suspended in 500 μl of media). Flies were allowed to recover for 8 h at room temperature and all subsequent values were relative to the number of flies alive at this time point. Flies were incubated at 29°C and, after daily examination for survivors, were transferred to fresh vials. Counts were taken every day for 7 days.

Electron Microscopy. Flies were dissected and placed onto a platform. Scans were performed on unfixed tissues by using the S-3000N (Hitachi, Tokyo) scanning electron microscope at the Institute of Materials and Intelligent Systems, University of Sherbrooke. Scans were at ×200 magnification, using a 3-kV beam.

Results

Generation of *kep1* Mutants. The line *EP2493* (33) carries an EP element inserted 67 bp 5′ from the predicted transcriptional start site of *kep1*. We used this line to generate *kep1* knockout flies by using imprecise P-element excision. Seventy-seven lines were initially generated, of which seven lines contained a rearrangement in their genome as assessed by Southern blot analysis and genomic PCR (data not shown). Sequence analysis confirmed that one of the above lines, which we termed *kep1*−, contained a 1.4-kb deletion spanning exons 1–5 of the *kep1* gene (Fig. 1*A*).

***kep1* Mutant Females Have Reduced Fertility and Produce Defective Eggs.** *kep1*− homozygous flies were viable and displayed no obvious morphological phenotype. We compared the fertility of *kep1*− females to heterozygous and wild-type controls, and found that the percentage of eggs hatching from *kep1*−/*CyO*

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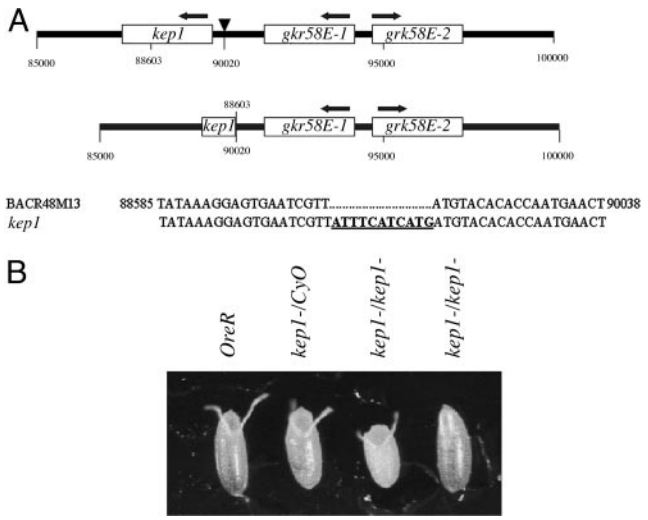

Fig. 1. Characterization of *kep1*− mutants. (*A*) Schematic diagram of the arrangement of *kep1* and two neighboring genes in region 58E of chromosome 2 in wild-type (*Top*) and in the *kep1*− deletion (*Middle*). Note that much of the *kep1* gene is deleted in the *kep1*− deletion. The wild-type and *kep1*− genomic sequences are given at the *Bottom*. PCR was used to amplify the region surrounding the P-element excision site in *kep1*− homozygotes, the amplified fragment was sequenced, and its sequence was compared with that of a BAC clone that did not contain the original P element (BACR48M13). The start of the *kep1* cDNA in BACR48M13 is at position 89990. The sequence of the remaining fragment of the P element, after the imprecise excision event, is underlined. (*B*) Phenotypes of eggs laid by *kep1*− mutant females; details and frequencies of the different phenotypes are described in Table 1.

females was indistinguishable from that of wild-type controls (Table 1). In contrast, homozygous *kep1*− females displayed reduced fertility, with only 46% of the *kep1*− eggs hatching. This reduced fertility was rescued with the introduction of a *kep1* transgene. Female *kep1*− homozygotes carrying two copies of the *kep1* transgene had fertility similar to the *OreR* controls (Table 1). The unhatched eggs produced by homozygous *kep1*− females fell into three major phenotypic classes (Fig. 1*B*). Eggs belonging to the first class (17.7%) were short and fat in appearance but had the proper amount of dorsal appendage material (Table 2). The second class (7%) included eggs that were normal in size but had little or no dorsal appendage material. The remaining 75.3% had a normal appearance (Table 2).

Kep1 Interacts *in Vitro* with *dredd* mRNA. To identify potential RNA targets for Kep1, we used an *in vitro* RNA crosslinking assay to test whether Kep1 could associate with several mRNAs that encode proteins involved in the apoptotic process. We found that *dredd* mRNA bound myc-tagged Kep1, whereas the mRNAs encoding the effector caspase *Dcp1* and the *Drosophila inhibitor of apoptosis 2* (*diap2*; ref. 34) could not be crosslinked to the Kep1 protein (Fig. 2*A* and data not shown). The interaction observed between Kep1 and *dredd* mRNA was specific for Kep1,

Table 1. Fertility of *kep1*− flies

Females	Males	Total eggs	Hatched	% Hatching
OreR	OreR	164	151	92
kep1−/*CyO*	OreR	128	120	94
kep1−/*kep1*−	OreR	261	121	46
P[*kep1*+]; −/−	OreR	1,102	941	85

Female and male flies of the genotypes listed above were introduced into cages and were allowed to lay eggs for a 24-h period. Eggs were scored 30 h later for their ability to hatch.

Table 2. Phenotypes of eggs produced by *kep1*− flies

Egg phenotype	OreR		*kep1*−/*CyO*		*kep1*−/*kep1*−	
	No. of eggs	%	No. of eggs	%	No. of eggs	%
Normal	803	100	771	96.2	310	75.3
Short eggs	0	0	18	2.2	73	17.7
DA defects	0	0	13	1.6	29	7.0

Flies of the genotypes listed above were introduced into cages and allowed to lay eggs on apple juice plates supplemented with live yeast (two changes, 1 h each). Eggs were then collected for 1 h and scored for their phenotypes 8 h later. The data above are the cumulative numbers obtained from egg collections carried out on three consecutive days. DA, dorsal appendage.

because a closely related *Drosophila* KH domain-containing protein Sam50 failed to bind *dredd* mRNA (Fig. 2 *A* and *B*).

The β Isoform of *dredd* mRNA Is More Abundant in *kep1* Ovaries. To test the possibility that Kep1 has an effect on *dredd* RNA metabolism, we probed Northern blots with the 3′-untranslated

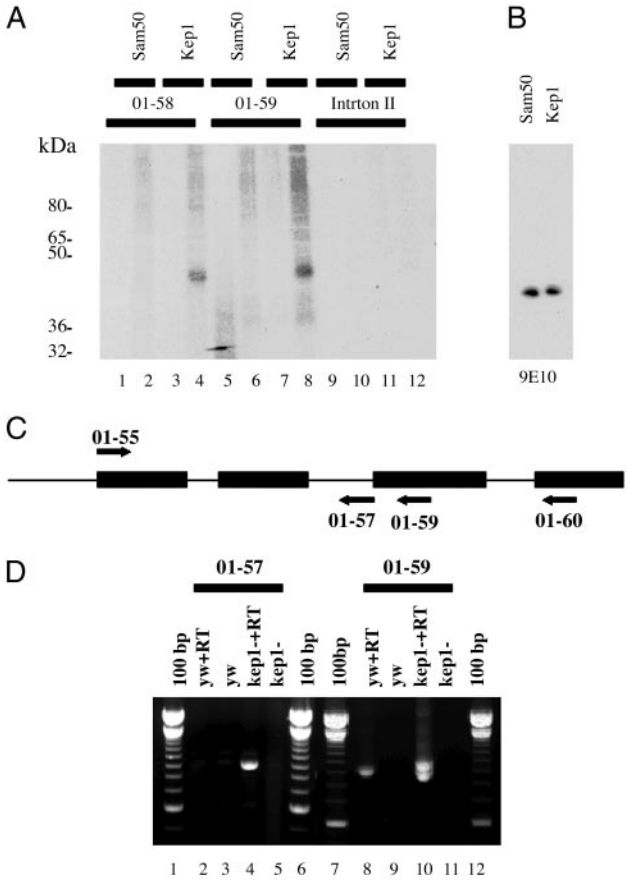

Fig. 2. Kep1 protein can be UV crosslinked to *dredd* mRNA. (*A*) Radiolabeled RNAs (refer to *Methods*) were incubated in the presence of HeLa cell extracts transfected with either *myc-Sam50* (lanes 1, 2, 5, 6, 9, and 10) or *myc-kep1* (lanes 3, 4, 7, 8, 11, and 12). These extracts were immunoprecipitated with control serum (odd-numbered lanes) or 9E10 anti-Myc antiserum (even-numbered lanes). (*B*) Myc immunoblot using 9E10 anti-Myc antiserum showing the presence of the transfected proteins in both extracts. (*C*) Organization of part of the *dredd* gene. Exons are shown as dark boxes; arrows indicate the relative position of the oligonucleotides used in the RT-PCR experiments to investigate the nature of the different *dredd* mRNA isoforms in various mutants. (*D*) RT-PCR were fractionated on a 1.5% agarose gel. Lanes indicate the source of the mRNA used in the reverse transcription reactions in the presence (+RT) or absence of reverse transcriptase.

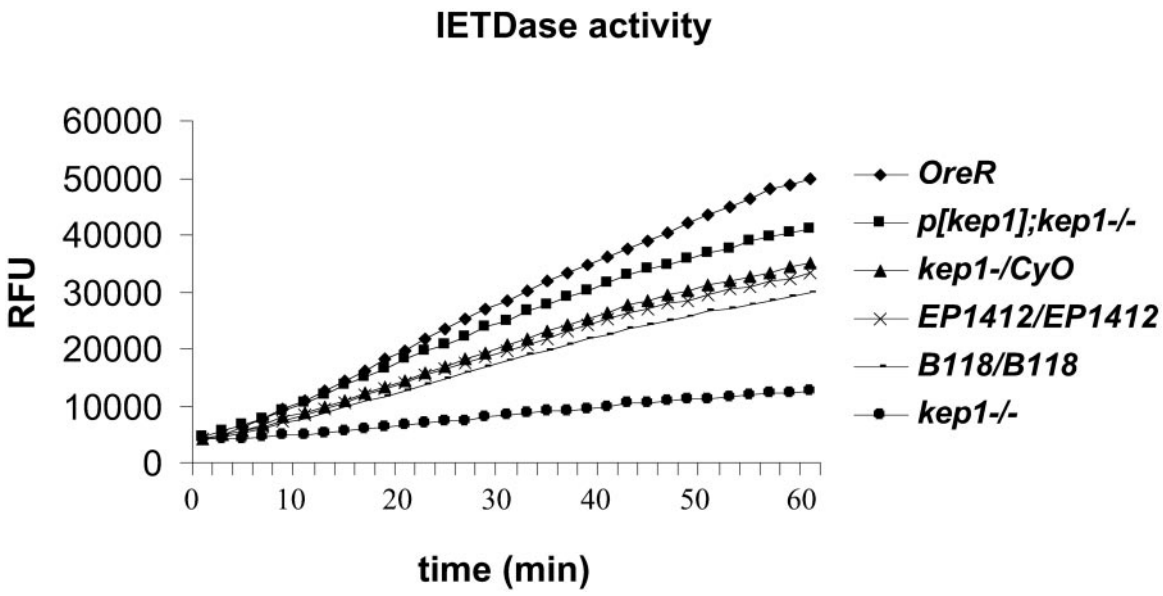

Fig. 3. IETDase activity in ovary extracts. Extracts were prepared and IETDase activity was measured as described in *Methods*. Results are the average readings from duplicate samples from one representative experiment. RFU, relative fluorescent units. OreR was used as the wild-type control, and other extracts were analyzed from *kep1*− heterozygotes (*kep1*−/*CyO*) and homozygotes (*kep1*−/−), homozygotes for two alleles of *dredd* (*EP1412*/*EP1412*, *B118*/*B118*), and ovaries obtained from flies carrying two copies of the *kep1* transgene in a *kep1* mutant homozygous background (*P*[*kep1*];*kep1*−/−).

region of *dredd* that detects all known *dredd* isoforms. We initially focused on RNA isolated from ovaries because *dredd* is expressed in this tissue (22). A single band of ≈1.6 kb was observed in extracts from wild-type and *kep1*− ovaries consistent with the size of mRNAs encoding the β, δ, or γ, but not the α, isoforms of *dredd* (data not shown). To determine whether the ratio of mRNAs encoding these *dredd* isoforms is altered in *kep1*− ovaries, we generated cDNA libraries from *kep1*− homozygous ovaries and wild-type controls. Four independent *dredd* clones were isolated from the *kep1*− library: two encoded the δ isoform of *dredd* and two encoded the truncated β isoform. In contrast, all four independent clones recovered from a wild-type library encoded the δ isoform of *dredd*. These data suggest that *kep1* activity influences the ratio between the β and δ isoforms. To extend this result, the isoforms of *dredd* mRNA were examined in wild-type and *kep1*− mutant populations by using an RT-PCR assay. The relative positions of the oligonucleotides used in the RT-PCR experiments are illustrated in Fig. 2*C*. RT-PCR products corresponding to mRNAs encoding the β and δ isoforms were present in RNA isolated from *kep1*− ovaries, whereas only the mRNA that encodes the δ isoform was observed in wild-type samples (Fig. 2*D*). These data indicate that *kep1* activity negatively influences the accumulation of the β isoform of *dredd*.

Reduced Dredd/Caspase-8 Activity in *kep1*−/− Ovaries. Dredd contains a long prodomain typical of a group III apical caspase (35). Because Dredd is most closely related to caspase-8 (36), we measured caspase-8 activity in *Drosophila* ovaries obtained from different genotypes by using a fluorometric-based assay that relies on the ability to cleave the nonfluorometric substrate IETD-AFC to free the fluorescent AFC moiety. As expected, mutations in *dredd* reduced caspase-8 activity relative to the wild-type control (Fig. 3, *B118*/*B118* vs. *OreR*). However, this activity was still above background levels (≈3,000 RFU), indicating that our assay is not strictly specific for Dredd, but also detects additional group III caspase activities that can cleave the IETD sequence (35). We observed a dramatic decrease of IETDase activity in *kep1*− homozygous ovary extracts (*kep1*−/−), even more pronounced than for the *dredd* allele tested. Introduction of two copies of the *kep1* transgene into the *kep1*− homozygous background restored the IETDase activity to near wild-type control levels (*P*[*kep1*];*kep1*−). Ovary extracts obtained from *kep1*− heterozygotes had an intermediate level of IETDase activity. These data indicate that IETDase activity in ovaries depends on the level of Kep1.

***kep1* Mutant Flies Have Increased Susceptibility to *E. coli* Infection.** To investigate whether *kep1*− and *dredd* mutants have other common phenotypes, we examined whether *kep1*− flies were also susceptible to Gram-negative bacterial infection, as are *dredd* flies (28, 29). We challenged flies by pricking them with a needle dipped in either a concentrated solution of *E. coli* or a solution of PBS as a control. Under the conditions used, control flies showed a slight decrease in viability after an *E. coli* insult (Fig. 4*A*), similar to that observed for injection with PBS alone (Fig. 4*B*). In contrast, *dredd* mutants showed a dramatic decrease in viability after *E. coli* infection, with only 10% of the flies still alive by the end of the observation period. *kep1* mutants also showed a decrease in viability, but this decrease was not as dramatic as that obtained for *dredd*. The survival rates for *kep1* mutants were 30% and 40% for homozygotes and heterozygotes, respectively. Unlike *OreR* or *kep1*− heterozygous flies, homozygous *kep1*− flies, and flies of both alleles of *dredd*, show a decrease in viability after PBS injections (Fig. 4*B*). This decrease is possibly a result of a bacterial infection being established at the wound site.

The *kep1* Mutation Suppresses *rpr*-Induced Apoptosis in the Eye. *dredd* mRNA accumulation requires the activity of the genes present at the *H99* locus, including *rpr*. Overexpression of *rpr* in photoreceptor precursor cells by using the *GMR-rpr* transgene leads to severe apoptosis that depends on *dredd* activity; so, a *dredd* mutation suppresses this phenotype (22). We assessed whether mutations in *kep1*− were also capable of suppressing *GMR-rpr*-mediated apoptosis by using the same experimental system. Scanning electron microscopic images obtained from unfixed wild-type eyes showed normal morphology (Fig. 5*A*). In

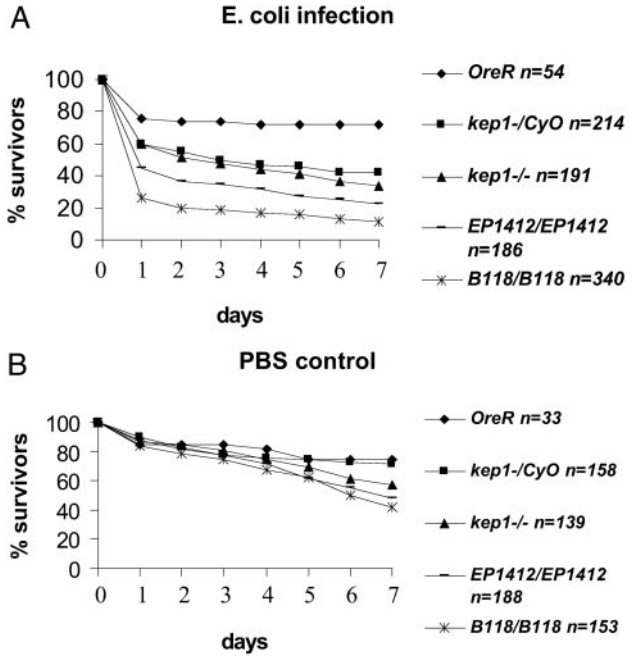

Fig. 4. Susceptibility of flies to *E. coli* infection. Flies were challenged with *E. coli* or subjected to a mock infection as described in *Methods*. OreR was used as the wild-type control, and survival was also scored for *kep1*− heterozygotes (*kep1*−/*CyO*) and homozygotes (*kep1*−/−), and homozygotes for two alleles of *dredd* (*EP1412*/*EP1412*, *B118*/*B118*).

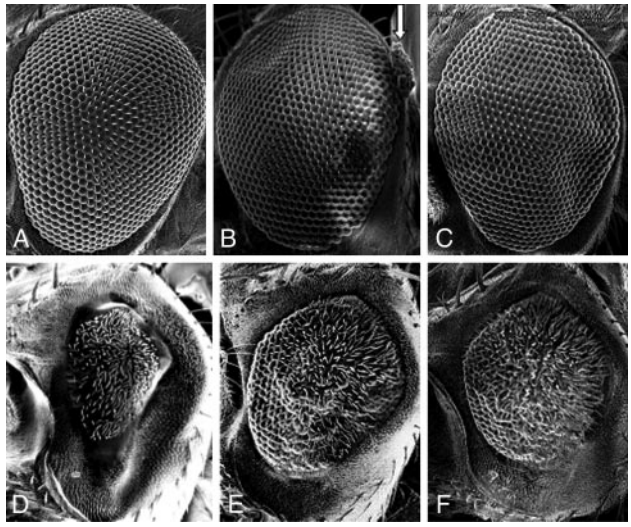

Fig. 5. Scanning electron micrographs of compound eyes. Flies of various genotypes were visualized by scanning electron micrographs as described in *Methods*. (*A*) *yw*. (*B*) *kep1*−/*kep1*−. (*C*) *B118*/*B118*. (*D*) *GMR-rpr*/+. (*E*) *kep1*−/*kep1*−;*GMR-rpr*/+. (*F*) *B118*/*B118*;*GMR-rpr*/+. The arrow illustrates an example of cell overgrowth occasionally seen in *kep1*−/*kep1*− mutant eyes.

contrast, in *kep1*− mutants we sometimes observed an outgrowth of extra cells (Fig. 5*B*). We crossed flies carrying the *GMR-rpr* transgene with flies carrying a mutation either in *dredd* or *kep1*. As previously reported, *GMR-rpr* apoptosis was partially suppressed when both copies of *dredd* were removed (Fig. 5*F*), and we observed that the *kep1* mutation also suppressed *GMR-rpr*-mediated apoptosis (Fig. 5*E*). *dredd* has been shown to be unable to suppress *GMR-hid* apoptosis (22); consistent with this, we were unable to detect any suppression of *GMR-hid* apoptosis with either *dredd* or *kep1* mutations (data not shown).

Discussion

Our study of *kep1*− mutant flies confirms the *in vitro* data previously obtained that link *kep1* to the apoptotic process, and implicates Kep1 in *dredd* RNA processing. The β isoform of *dredd* that accumulates in *kep1*− mutants encodes a truncated protein that may have a similar function to the decoy caspase-8 molecules identified in vertebrates (28). These molecules are similar in sequence to caspase-8, except that they lack essential catalytic residues and therefore may compete with caspase-8 for sites on the receptor. Regulation of *dredd* RNA processing may be an alternative strategy that *Drosophila* has evolved to establish an additional level of control of caspase-8 activity. The protein produced by the β isoform of *dredd* may act in a dominant-negative fashion, either by binding to dFADD and down-regulating a Fas-like signaling pathway, or at the apoptosome through its ability to interact with Dark (37). A recent report (38) implicates dFADD in regulating the response to infection by Gram-negative bacteria, which, taken together with our results, suggests that a dominant-negative isoform of Dredd could operate at the level of receptor binding.

Although Dredd is the *Drosophila* caspase most similar to caspase-8, because of its long prodomain and DED sequences, our assays of caspase-8 activity indicate that other *Drosophila* caspases possess IETDase activity as well, and these caspases also appear to be regulated by Kep1. Dronc, another group III caspase with substantial IETDase activity despite its active site, which is very divergent from that of Dredd (39), may be responsible for this additional activity. Dronc is induced by ecdysone (23, 40), and ecdysone inhibits apoptosis of nurse cells (41, 42); so in developing ovaries, Dronc, although present, must be in an inactive form. In our ovary extracts, however, we may have diluted out any short-lived inhibitory proteins, for example Diap1, which can bind Dronc (43). Although there is no reported evidence for alternative splicing of *dronc*, it will be informative to determine whether Kep1 can bind *dronc* or other *Drosophila* caspase mRNAs, and to examine whether any alternative transcripts are present in *kep1*− mutants.

BLASTP analysis identifies the closest mammalian homologue of Kep1 as the SLM2 protein (44), also referred to as Étoile (45). We have observed that transfection of SLM2 into NIH 3T3 cells results in activation of apoptosis, which can be inhibited by cotransfection of the *Drosophila* inhibitor of apoptosis DIAP1 (M.D.F. and S.R., unpublished data). Recent work (46) supports the involvement of SLM2 in splice site selection, because rat SLM2 was shown to interact with various proteins involved in alternative splicing, including SRp30c and the splicing associated factor YT521-B. SLM2 can also influence the splicing pattern, in a concentration-dependent manner, of the CD44v5, human transformer-2β and τ minigenes in cotransfection experiments (46). Alternative splicing of the genes encoding the caspase Ich-1 (47), Bcl-X (48), CED-4 (49), caspase 9 (50), and interleukin-1β converting enzyme (51) have also been reported, although the RNA binding proteins involved have not been identified. In all of these cases, the spliced isoforms show the opposite apoptotic functions of their parental products. Thus, regulation of RNA processing may be a general mechanism of regulating the balance between active and repressor forms of proteins involved in the apoptotic pathway, and Kep1 may belong to a family of RNA binding proteins that influence expression of these genes at the level of RNA processing.

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