KEL-8 Is a Substrate Receptor for CUL3-dependent Ubiquitin Ligase That Regulates Synaptic Glutamate Receptor Turnover

Henry Schaefer and Christopher Rongo

INTRODUCTION

Glutamate is the most abundant excitatory neurotransmitter in the brain, and glutamatergic synapses play a critical role in learning, memory, and developmental plasticity of the central nervous system (Meldrum, 2000). Ionotropic glutamate receptors (GluRs) receive and transduce glutamatergic signals on the postsynaptic face of synapses, where these multitransmembrane spanning proteins assemble into tetrameric glutamate-gated channels of differing subunit composition (Hollmann and Heinemann, 1994; Dingledine et al., 1999). The regulation of these receptors, the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors (AMPARs) to synapses serves as an important component of synaptic signaling and plasticity. Regulated ubiquitination and endocytosis determine the synaptic levels of AMPARs, but it is unclear which factors conduct these processes. To identify genes that regulate AMPAR synaptic abundance, we screened for mutants that accumulate high synaptic levels of the AMPAR subunit GLR-1 in Caenorhabditis elegans. GLR-1 is localized to postsynaptic clusters, and mutants for the BTB-Kelch protein KEL-8 have increased GLR-1 levels at clusters, whereas the levels and localization of other synaptic proteins seem normal. KEL-8 is a neuronal protein and is localized to sites adjacent to GLR-1 postsynaptic clusters along the ventral cord neurites. KEL-8 is required for the ubiquitin-mediated turnover of GLR-1 subunits, and kel-8 mutants show an increased frequency of spontaneous reversals in locomotion, suggesting increased levels of GLR-1 are present at synapses. KEL-8 binds to CUL-3, a Cullin 3 ubiquitin ligase subunit that we also find mediates GLR-1 turnover. Our findings indicate that KEL-8 is a substrate receptor for Cullin 3 ubiquitin ligases that is required for the proteolysis of GLR-1 receptors and suggest a novel postmitotic role in neurons for Kelch/CUL3 ubiquitin ligases.

The regulated localization of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors (AMPARs) to synapses is an important component of synaptic signaling and plasticity. Regulated ubiquitination and endocytosis determine the synaptic levels of AMPARs, but it is unclear which factors conduct these processes. To identify genes that regulate AMPAR synaptic abundance, we screened for mutants that accumulate high synaptic levels of the AMPAR subunit GLR-1 in Caenorhabditis elegans. GLR-1 is localized to postsynaptic clusters, and mutants for the BTB-Kelch protein KEL-8 have increased GLR-1 levels at clusters, whereas the levels and localization of other synaptic proteins seem normal. KEL-8 is a neuronal protein and is localized to sites adjacent to GLR-1 postsynaptic clusters along the ventral cord neurites. KEL-8 is required for the ubiquitin-mediated turnover of GLR-1 subunits, and kel-8 mutants show an increased frequency of spontaneous reversals in locomotion, suggesting increased levels of GLR-1 are present at synapses. KEL-8 binds to CUL-3, a Cullin 3 ubiquitin ligase subunit that we also find mediates GLR-1 turnover. Our findings indicate that KEL-8 is a substrate receptor for Cullin 3 ubiquitin ligases that is required for the proteolysis of GLR-1 receptors and suggest a novel postmitotic role in neurons for Kelch/CUL3 ubiquitin ligases.

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Address correspondence to: Christopher Rongo (rongo@waksman.rutgers.edu).
tion is conducted in a stepwise manner by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases, which recognize target proteins and catalyze the covalent attachment of ubiquitin to these target substrates. Ubiquitinated membrane proteins are substrates for endocytosis, although the exact mechanism by which such endocytosed membrane proteins are eventually degraded is unclear (Haglund et al., 2003; Hicke and Dunn, 2003). Improper ubiquitination in the nervous system has been implicated in Parkinson’s, Huntington’s, and Alzheimer’s diseases as well as in other neurological disorders, further supporting the importance of ubiquitination and protein turnover in proper nervous system function (Ehlers, 2004).

Ubiquitination specificity is conferred by E3 ligases, including the diverse superfamily of Cullin-RING ligases (reviewed in Willems et al., 2004; Petroski and Deshaies, 2005). Cullin-RING ligases are complexes containing a RING protein, which recruits E2-conjugating enzymes, a Cullin scaffold protein, a substrate receptor, and sometimes an adaptor between the Cullin and substrate receptor. There are seven different subfamilies of Cullin-RING ligases (named CDL1–7 for Cullin-dependent ligase), and each subfamily can assemble with numerous substrate receptors. Each substrate receptor contains a domain for interacting with a specific substrate and a subfamily-specific domain for interacting with a specific Cullin subfamily member. For example, CDL1 (or SCP) is comprised of CUL1 bound to an F-box-containing substrate receptor, CDL2 is comprised of CUL2 bound to a SOCS/BC-box-containing substrate receptor, and CDL3 is comprised of CUL3 bound to a BTB-domain-containing substrate receptor. Cullin-RING ligases have been primarily studied for their role in mitosis and cell division, particularly for CDL1. Recently, CDL3s were found to regulate the meiosis/mitosis transition in C. elegans (Pintard et al., 2003; Xu et al., 2003). CDL3s assemble with BTB proteins, which are substrate receptors that directly bind to CUL3 without the aid of an adaptor (Deshaies, 1999; Joazeiro and Weissman, 2000; Geyer et al., Pintard et al., 2003; Xu et al., 2003). There are many known BTB proteins, suggesting the assembly of a diverse array of CDL3s, each with unique substrate specificity (Willems et al., 2004; Petroski and Deshaies, 2005). The biological function of these proteins is largely unknown, particularly for postmitotic Cullin-RING ligases.

To identify the factors that regulate the turnover of AMPARs, we screened for mutants with increased synaptic abundance of GLR-1. Here, we describe mutants for the gene kel-8 (kelch-repeat containing protein 8), which have increased GLR-1 levels in neurites. Mutants for kel-8 also show an increased frequency of spontaneous reversals of locomotion, consistent with increased GLR-1 synaptic levels. By contrast, we found that the levels and localization of other synaptic proteins seem normal in kel-8 mutants. KEL-8 is a member of the BTB-Kelch superfamily of proteins and contains six Kelch repeats and a BTB domain. Kelch repeats are protein–protein interaction domains, and Kelch proteins interact with a variety of other proteins, including actin (Adams et al., 2000). We show that KEL-8 is expressed and required in GLR-1-expressing neurons and is localized to sites adjacent to GLR-1 postsynaptic clusters along the ventral cord neurites. We also show that KEL-8 is required for efficient ubiquitin-mediated turnover of GLR-1 subunits. Moreover, KEL-8 binds to CUL-3, a Cullin3-type scaffold for E3 ubiquitin ligases, and expression of a dominant negative CUL-3 results in GLR-1 accumulation. Our findings suggest that KEL-8 is a substrate receptor for CDL3 that regulates GLR-1 turnover and indicate a novel biological role for Cullin-RING ligases: the regulation of glutamate receptor localization and signaling in postmitotic neurons.

**Materials and Methods**

**Strains**

Standard methods were used to maintain *C. elegans* (Wood, 1988). Animals were grown at 20°C on standard NGM plates seeded with OP50 *Escherichia coli*. Some strains were provided by the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). Strains used in this study include *nuIs25[lgl-1::gfp], osd1[slub-1::gfp], osd1s16[lgl-1::rph], osd2[lin-10::gfp], nai68[unc-43::gfp], nai89[Mub, ttx-3::gfp], unc-11(e47), dpy-11(e224), unc-54(e153ts), rhl-119(e185), and CB4856 (Hawaiian strain).

**Isolation and Mapping of the kel-8 Mutant**

P0 *nuIs25* nematodes were ethyl methanesulfonate mutagenized using standard procedures (Brenner, 1974). F2 animals from individual plates were sampled (n = 30–50) by mounting on 2% agarose pads containing levamisole. Animals were scored by fluorescence microscopy for defects in GLR-1-::GFP localization. Mutants were recovered either directly from the slide or by isolating siblings from the parental F1 plate. The mutant containing the *kel-8(delta8)* mutation also contained a second linked mutation with an uncoordinated behavioral phenotype and its own GLR-1 localization phenotype. This second mutation was crossed away from the *kel-8(delta8)* after five rounds of backcrossing and will be described elsewhere.

The *kel-8(delta8)* mutation was mapped between dpy-11 and unc-34 on the left arm of LGV. A dpy-11 kel-8 unc-34 recombinant chromosome was constructed and used to introgress into the CB4856 LGV chromosome to allow three-factor single-nucleotide polymorphism (SNP) mapping. Multiple recombinants placed *kel-8* between SNP pkP103 (map position –12.41) and SNP C29G2.1 (map position –13.37), a region of ~20 kb. This region is spanned by six cosmids, which were independently injected into kel-8 mutants. Cosmid W0290 (coincident with rol-6dm) rescued the *kel-8* mutant phenotype with respect to GLR-1 in five of six Rod extrachromosomal lines. By contrast, we observed no rescue from five other cosmid (C2489, 0 of 2 lines; R0589, 0 of 2 lines; C0202, 0 of 2 lines; C0202, 0 of 5 lines) that were adjacent to W0290 in the genome but lacking the *kel-8* locus. We sequenced the most promising candidate on W0290 and identified a premature stop codon mutation in W0290.2 from multiple independent PCR reactions using *kel-8* genomic DNA as template. Sequences from full-length cDNAs yk109610 and yk136011 (a gift from Y. Kohara, National Institute of Genetics, Mishima, Japan), which include an SL1 splice leader marking the 5' end of transcription, were used to determine the gene structure.

**Transgenes and Germline Transformation**

To observe the localization of different synaptic proteins, we used several previously published integrated transgenes: *nuIs25[lgl-1::gfp], osd1[slub-1::gfp], osd1s16[lgl-1::rph], osd2[lin-10::gfp], nai68[unc-43::gfp], and nai89[Mub, ttx-3::gfp] (Rongo et al., 1998; Rongo and Kaplan, 1999; Burbea et al., 2000; Xu et al., 2003). These transgenic strains were isolated by microinjecting various plasmids (typically at 50 ng/ml) using rol-6dm (a gift from C. Mello, University of Massachusetts Medical School, Worcester, MA) as a marker. The following transgenes were introduced into the germline and followed as extrachromosomal arrays. The *kel-8::gfp* transgene was generated by subcloning 5 kb of upstream genomic sequence and the entire *kel-8* open reading frame from genomic cosmids W0290 into the GFP vector pPD95.75 (a gift from A. Fire, Stanford University School of Medicine, Palo Alto, CA) so that the GFP sequences were fused in frame at the carboxy terminus of *kel-8*. The *P_{unc-16}::kel-8::gfp* transgene was generated by subcloning the *kel-8* cDNA from yk362h1 (a gift from Y. Kohara) into pV6 (a gift from V. Maricq, University of Utah, Salt Lake, UT), which contains the *gfp* promoter. Yellow fluorescent protein (YFP) sequences were then inserted in frame at the *kel-8* carboxy terminus. The *P_{unc-3}::kel-8::gfp* transgene was generated by subcloning 5 kb of upstream genomic sequence and the first five exons through amino acid 219 into pPD95.75 so that GFP was fused in frame at the carboxy terminal end of the *kel-8* 3'UTR domain. The *P_{unc-3}::kel-8::gfp* transgene was generated by subcloning 5 kb of upstream genomic sequence from yk362h1 into pV6. *P_{unc-16}::kel-3*, *P_{unc-3}::kel-3(31–500)*, and *P_{unc-3}::catalyst* (777) transgenes were generated by subcloning *catalyst* cDNA sequences from pGST-CUL-3 (a gift from L. Xu and W. Harper, Harvard Medical School, Boston, MA) into pV6.

**Fluorescent Microscopy**

GFP-, cyan fluorescent protein (CFP)-, and YFP-tagged fluorescent proteins were visualized in nematodes by mounting L4 and young adults on 2% agarose pads with 10 mM levamisole at room temperature. Fluorescent images were observed using a Zeiss Axiopt 2.0 or a luminescence FLUOIII or FL400 (1.4 numerical aperture PlanApo for both) objective and imaged with an ORCA charge-coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ) using Image-Pro version 4.1 (Media Cybernetics, Silver Spring, MD) and VayTek...
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KEL-8 protein was produced in COS-7 cells by subcloning

sequences of the first 100 amino acids into pGEX-KG (Guan and

Dixon, 1991). GST-KEL-8(1-100) protein was expressed and purified using

glutathione-Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire,

United Kingdom) and used to immunize rabbits using Freund’s (Pocono

Rabbit Farms, Canadensis, PA). The resulting antiserum recognized both glu-
tathione S-transferase (GST) and KEL-8 proteins.

Behavioral Assays

Nose-touch sensory responses were assayed as described previously (Hart et al., 1995). Each animal was tested on food for reversal of locomotion after a

forward collision with a hair. Each animal was tested 10 times, and 20 or more animals were tested for each genotype. The reversal frequency for fluorescent signals that were 2 SDs above the

unlocalized baseline using a macro written for Image-Pro. We found this

algorithm agreed with puncta assessed by eye. Cluster size was measured as

the maximum radius for each outlined cluster. Cluster number was calculated

by counting the average number of clusters per 10 μm of dendrite length.

Anti-KEL-8 Antibody

GST-KEL-8(1-100) protein was produced in BL21 E. coli by subcloning kel-8
cDNA sequences encoding the first 100 amino acids into pGEX-KG (Guan and

Dixon, 1991). GST-KEL-8(1-100) protein was expressed and purified using

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RESULTS

GLR-1 Accumulates in kel-8 Mutants

Chimeric GLR-1 receptors tagged with the green fluorescent protein (GLR-1::GFP) are localized to synaptic clusters at neuron–neuron synapses within the C. elegans neuropil in

living animals (Figure 1A; Rongo et al., 1998; Rongo and

Kaplan, 1999; Burbea et al., 2002). From a direct visual screen for mutants with defects in GLR-1::GFP localization, we

identified an allele, d38, of a gene that we have named kel-8

based on its sequence (Figure 2; see below). Mutants for

kel-8(d38) lack small synaptic clusters of GLR-1::GFP and

instead accumulate large accretions of GLR-1::GFP (Figure

1B). The size of GLR-1::GFP accretions in kel-8 mutants

Figure 1. KEL-8 regulates GLR-1 abundance in dendrites. GLR-1::GFP (A and B), SNB-1::GFP (C and D), UNC-43::GFP (E and F), and LIN-10::GFP (G and H) fluorescence was observed along the ventral cord dendrites of wild-type nematodes (A, C, E, and G) or kel-8 mutants (B, D, F, and H). Whereas wild-type animals have small clusters of GLR-1::GFP (100%; n = 20), most kel-8 mutants have large (>2-μm) clusters of GLR-1::GFP (95%; n = 20). Bar, 5 μm.

The mean cluster area (I) and the mean number of clusters per 10 μm of dendrite length is plotted for adult nematodes of the

given genotype and expressing the transgene indicated beneath the

diagram. White bars indicate wild-type animals, whereas black bars

indicate kel-8 mutants. AU, arbitrary units. Error bars are SEM for all

graphs. **p < 0.001 and ***p < 0.0001 compared with wild type

expressing the corresponding transgene by Student’s t test. n = 15–20 animals for each genotype–transgene combination.

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version 6.2 software (VayTek, Fairfield, IA). Exposure times were chosen to

fill the 12-bit dynamic range without saturation, and out-of-focus light was

removal with a constrained iterative deconvolution algorithm (VayTek).

To quantify the fluorescently tagged proteins, images of nematodes were

captured by CCD as described above using a constant gain and exposure time

(filling the 12 bit dynamic range) for all samples. Background fluorescence

coverslip and from nonspecific tissue autofluorescence was removed by

subtracting an image filtered with a low pass Gaussian filter. Cluster

outlines were calculated for fluorescent signals that were 2 SDs above the

unlocalized baseline using a macro written for Image-Pro. We found this

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Figure 2. KEL-8 encodes a member of the Kelch Superfamily. (A) The predicted intron/exon gene structure of kel-8 based on cDNA sequence is shown at top. Black boxes indicate coding sequences, whereas white boxes indicate untranslated regions. At bottom is the predicted protein domain structure, including the BTB, BACK, and six Kelch repeats. Amino acid identities and similarities to human Kelch-like 8 (KHL8) for each domain are shown. The molecular nature of the od38 mutation is indicated. (B) Phylogenetic tree for KEL-8 and various Kelch proteins in the human and C. elegans genomes. KEL-8 is most similar to human Kelch-like 8. (C) Amino acid alignment of KEL-8, human KHL8, and Drosophila KELCH. Black highlighting indicates identities, and gray highlighting indicates similarities. Overlines indicate specific protein domains. (D) Fluorescence from GLR-1::GFP in the ventral cord bundle of kel-8 mutants. (E) kel-8 mutants rescued with genomic cosmid W02G9 containing the kel-8 locus. (F) kel-8 mutants rescued cell autonomously with a $\text{P}_{\text{glr-1}}::\text{kel-8}$ transgene containing wild-type kel-8 cDNA fused to the glr-1 promoter. Bar, 5 μm.
ranges from 2 to 10 times larger compared with GLR-1::GFP clusters in wild-type animals (Figure 1, B and I). There is a 40% decrease in the number of accretions per length of neurite in kel-8 mutants compared with the number of clusters in wild-type animals (Figure 1), although the drop in number observed in kel-8 mutants is a likely consequence of the large accretion size. These results suggest that KEL-8 regulates the abundance of GLR-1 receptors in ventral cord neurites.

The change in GLR-1 ventral cord accumulation in kel-8 mutants could reflect a general defect in protein trafficking, or in synapse formation per se. To test this possibility, we examined the localization of three other synaptic proteins: SNB-1 (synaptobrevin), UNC-43 (CaMKII), and LIN-10 (Mint2). We previously generated transgenes that express GFP-tagged versions of these proteins using the glr-1 promoter (Rongo et al., 1998; Rongo and Kaplan, 1999; Shim et al., 2004). We introduced these transgenes into wild-type and kel-8 mutant animals to observe the subcellular localization of their protein products in the ventral cord neurites. SNB-1::GFP is localized to presynaptic terminals (Rongo et al., 1998; Nonet, 1999), and we found no significant change in the size and number of SNB-1::GFP-labeled terminals in kel-8 mutants compared with wild type (Figure 1, C, D, I, and J). UNC-43::GFP and LIN-10::GFP colocalize with GLR-1 at postsynaptic elements in the ventral cord (Rongo and Kaplan, 1999; Glodowski et al., 2005; Umemura et al., 2005), and we found no significant difference in the size or number of either UNC-43::GFP (Figure 1, E, F, I, and J) or LIN-10::GFP (Figure 1, G–J) clusters in kel-8 mutants compared with wild type. These results indicate that the accumulation of GLR-1 in kel-8 mutants is not because of gross defects in trafficking or synapse formation in the GLR-1-expressing neurons.

**KEL-8 Encodes a BTB-Kelch-like Protein**

We identified the kel-8 gene as predicted coding region W02G9.2 by genetic mapping and transformation rescue (Figure 2, A, D, and E; see Materials and Methods for details). The kel-8 gene encodes a 690-amino acid protein in the BTB-Kelch superfamily that is predicted to contain a BTB domain, a BACK domain, and six Kelch repeats (Bork and Doolittle, 1994; Ahmad et al., 1998; Adams et al., 2000; Stogios and Privé, 2004). Multiple complete cDNA clones of kel-8 were obtained from the C. elegans expressed sequence tag project. Multiple subtypes of BTB-Kelch-like proteins have been identified in vertebrates based on their sequence similarity to Drosophila KELCH. Of the vertebrate subtypes, we found that the KEL-8 gene product shows the highest similarity to Kelch-like 8 (Figure 2B). Two other BTB-Kelch-like proteins, KEL-1 and SPE-26, have been identified in the C. elegans genome, and a BLAST search of the C. elegans genome revealed five additional genes encoding BTB-Kelch-related proteins. Based on the similarity to Kelch-like 8, we named the W02G9.2 gene kel-8 for kelch-repeat containing protein 8 (Figure 2C).

To determine the molecular nature of the kel-8(od38) allele, we sequenced genomic DNA from kel-8 mutants. The mutation alters the conceptual translation of KEL-8 protein from arginine to an Opal stop codon at amino acid 102, resulting in a protein lacking all functional domains (Figure 2A). Thus, kel-8(od38) is a likely null allele in the kel-8 gene.

**KEL-8 Negatively Regulates GLR-1 Function**

KEL-8 negatively regulates GLR-1 abundance, which could result in increased levels of GLR-1 on the membrane surface of kel-8 mutants. The levels of GLR-1 on the postsynaptic membrane can be monitored through changes in behavior (Burbea et al., 2002; Juo and Kaplan, 2004). C. elegans spend the majority of their time moving forward; however, this forward locomotion is occasionally halted by spontaneous reversals in the direction of movement, and GLR-1 signaling positively regulates these spontaneous reversals (Zheng et al., 1999; Mellem et al., 2002). C. elegans backward locomotion can also be induced by stimulating the mechanosensory neuron ASH, which makes glutamatergic connections to the GLR-1-expressing interneurons (White et al., 1986; Kaplan and Horvitz, 1993). Mutants with reduced GLR-1 signaling have a lower frequency of spontaneous reversal and are nose-touch insensitive, whereas mutants with increased GLR-1 signaling or higher levels of cell surface GLR-1 have a higher frequency of spontaneous reversal (Hart et al., 1995; Maricq et al., 1995). Wild-type animals reverse direction in response to nose-touch with a frequency of ~83% (20 animals, 10 trials per animal), whereas glr-1 mutants only reverse direction in response to nose-touch with a frequency of ~7% (Figure 3A). We found that kel-8 mutants responded to nose-touch with a frequency of ~82%. We also examined spontaneous reversal frequency. Wild-type animals spontaneously reversed ~2.6 times per minute (20 animals, 5 min trial per animal), whereas glr-1 mutants only spontaneously reverse direction ~1.4 times per minute (Figure 3B). We found that kel-8 mutants spontaneously reversed direction ~4.1 times per minute, a frequency that was statistically greater than that for wild-type animals (Figure 3B). To determine whether the increased reversal frequency is because of increased GLR-1 in kel-8 mutants, we examined glr-1; kel-8 double mutants. The double mutants behave similarly to glr-1 single mutants, demonstrating that glr-1 suppresses the behavioral defects of kel-8 and that the increased reversal frequency in kel-8 mutants requires GLR-1 function. Our results suggest that the increase in GLR-1::GFP abundance in kel-8 mutants correlates with an increase in GLR-1-mediated locomotion behavior and is consistent with increased synaptic strength.
KEL-8 Is Expressed in Neurons

Mutants for kel-8 have no obvious morphological or developmental defects and have apparently regular, coordinated locomotion. We reasoned that the relatively specific phenotype of kel-8 mutants might be because of limited expression of the gene. We generated a transcriptional reporter by fusing 5.0 kb of upstream sequences and the entire genomic kel-8 coding sequences in frame to GFP sequences. The resulting kel-8::gfp transgene was introduced into nematodes, where it expressed KEL-8::GFP protein in a subset of neurons (Figure 4A). We also introduced the kel-8::gfp transgene into nematodes expressing GLR-1::RFP (monomeric red fluorescent protein [RFP]; Campbell et al., 2002) from a previously described transgene (Figure 4, B and C; Glodowski et al., 2005). We found that KEL-8::GFP and GLR-1::RFP were expressed in the same subset of interneurons, including AVA, AVB, AVD, and AVE (Figure 4, G–I) as well as PVC (Figure 4, J–L), suggesting that KEL-8 functions in the same cells as GLR-1. To confirm this possibility, we made a transgene, Pglr-1::kel-8, containing kel-8 cDNA sequences under the control of the glr-1 promoter. We introduced Pglr-1::kel-8 into kel-8 mutants and found that mutant nematodes carrying the Pglr-1::kel-8 extrachromosomal array (4 independent lines, 15–25 animals examined per line) were rescued for the GLR-1 localization phenotype (Figure 2F), whereas their untransformed siblings were not (Figure 2D; our unpublished data), indicating that KEL-8 functions cell autonomously.

We observed that KEL-8::GFP was localized to clusters along the ventral cord neurites (Figure 4D). Coexpression of KEL-8::GFP and GLR-1::RFP resulted in KEL-8::GFP clusters that were localized adjacent to clusters of GLR-1::RFP along the ventral cord (merged image in F). KEL-8::GFP and GLR-1::RFP are expressed in the same lateral and lumbar cells bodies (merged images in 1 and L, respectively; cell identities are indicated). (M and N) GLR-1::CFP fluorescence was observed in kel-8 mutants (M) or kel-8 mutants (N) rescued cell autonomously with a Pglr-1::kel-8::gfp transgene containing wild-type kel-8 cDNA fused in frame to YFP. The KEL-8::YFP chimeric protein functions to rescue the kel-8 mutant phenotype in these neurons. Bars, 20 μm (A–C), 5 μm (D–L), and 10 μm (M and N).

Figure 4. KEL-8 is expressed in neurons and localized to clusters in the ventral cord dendrites. KEL-8::GFP (A, D, G, and J) and GLR-1::RFP (B, E, H, and K) fluorescence were observed throughout the entire animal (A–C), along ventral cord neurites (D–F), along lateral head ganglia (G–I), and in the lumbar ganglia of the tail (J–L). KEL-8::GFP is observed in the nerve ring (NR), lateral and ventral ganglia (LV&VG), the ventral nerve cord (VNC), and the lumbar ganglia (LG) of the tail (merged image in C). Intestinal autofluorescence (IA), which is nonspecific and does not indicate expression from either transgene, is observed throughout the mid-body. Along the ventral cord, KEL-8::GFP is localized to clusters adjacent to clusters of GLR-1::RFP along the ventral cord (merged image in F). KEL-8::GFP and GLR-1::RFP are expressed in the same lateral and lumbar cells bodies (merged images in 1 and L, respectively; cell identities are indicated). (M and N) GLR-1::CFP fluorescence was observed in kel-8 mutants (M) or kel-8 mutants (N) rescued cell autonomously with a Pglr-1::kel-8::gfp transgene containing wild-type kel-8 cDNA fused in frame to YFP. The KEL-8::YFP chimeric protein functions to rescue the kel-8 mutant phenotype in these neurons. Bars, 20 μm (A–C), 5 μm (D–L), and 10 μm (M and N).
transgene have properly-localized GLR-1::CFP (4 independent lines, 15-20 animals examined per line; Figure 4N). These results indicate that the KEL-8 fusion protein is functional.

*Drosophila* KELCH homodimerizes in vivo via the BTB domain, and BTB dimerization is sufficient for endogenous KELCH protein to localize an exogenous KELCH BTB domain to the actin-rich ring canals (Robinson and Cooley, 1997). To determine whether the BTB domain is playing a similar role in KEL-8, we made a transgene, *kel-8*(1-219)::gfp, that expresses the amino terminus of KEL-8 (amino acids 1–219, which contains the BTB domain) in frame with GFP under the *kel-8* promoter. We introduced *kel-8*(1-219)::gfp into *kel-8* mutants but could not detect KEL-8(1-219)::GFP protein (our unpublished data). We then introduced wild-type endogenous *kel-8*(+) and observed the same expression and punctate localization pattern for KEL-8(1-219)::GFP protein in ventral cord neurites (our unpublished data) that we observed for full-length KEL-8::GFP (4 of 4 transgenic lines). These results indicate that full-length endogenous KEL-8 stabilizes and localizes the exogenous BTB domain protein and suggest that the KEL-8 BTB domain dimerizes in vivo.

**KEL-8 Is Required for Ubiquitin-mediated Degradation of GLR-1**

KEL-8 negatively regulates GLR-1 abundance, and one mechanism by which this could occur is ubiquitination. Because of a limiting cellular concentration of monoubiquitin, overexpression of Myc epitope-tagged ubiquitin (MUb) by a *nuIs89* transgene has been shown to negatively regulate GLR-1 abundance in neurites (Papa and Hochstrasser, 1993; Hegde et al., 1997; Swaminathan et al., 1999; Burbea et al., 2002). Because *kel-8* mutants accumulate GLR-1 (Figure 5E), KEL-8 could be needed for ubiquitin-mediated turnover of GLR-1. To test this idea, we introduced *nuIs89* into *kel-8* mutants and found that mutations in *kel-8* partially block the turnover of GLR-1 because of overexpressed ubiquitin (Figure 5F). Our quantification of GLR-1 cluster size (area) and number per length of ventral cord support these conclusions (Figure 5I). Overexpression of ubiquitin in wild-type animals results in fewer GLR-1 clusters, although cluster size remains unchanged. By contrast, overexpression of ubiquitin in *kel-8* mutants does not result in fewer GLR-1 clusters. However, whereas the size of GLR-1 clusters in *nuIs89*MUb; *kel-8* nematodes is larger than in *nuIs89*[MUb] nematodes, it is not as large as in *kel-8* single mutants. These results demonstrate that KEL-8 is required for part of the ubiquitin-mediated degradation of GLR-1. They also suggest that overexpression of ubiquitin can partially decrease GLR-1 levels by a second mechanism that is independent of KEL-8.

Interestingly, mutations in the AP180 clathrin adaptor protein *UNC-11* result in an increase of GLR-1 abundance (Burbea et al., 2002; Figure 5C), suggesting that clathrin-mediated endocytosis is required for the turnover of GLR-1 protein. Moreover, mutations in *unc-11*, like in *kel-8*, can partially prevent the turnover of GLR-1 because of overexpressed MUb (Burbea et al., 2002; Figure 5D). Thus, KEL-8 and UNC-11 could act together or could act independently in parallel processes to regulate GLR-1 abundance. If KEL-8 and UNC-11 work in parallel processes, then GLR-1 localization defects in *kel-8 unc-11* double mutants should be dramatically more severe than in either single mutant. Instead, *unc-11 kel-8* double mutants contained GLR-1 accumulations that were only slightly larger compared with those found in *kel-8* or *unc-11* single mutants (Figure 5, G and H), suggesting that a mutation in one of these genes can par...
Our results are most consistent with UNC-11 and KEL-8 working together in the same linear pathway to regulate GLR-1 abundance and suggest that KEL-8 is required for the ubiquitin-mediated turnover of GLR-1.

**KEL-8 Interacts with the CUL-3 Subunit of Cullin 3 Ubiquitin Ligases**

Several BTB proteins have been shown to be substrate receptors for Cullin 3 ubiquitin ligases (CDL3s) (Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003; Kobayashi et al., 2004; Zhang et al., 2004). Because KEL-8 contains a BTB domain, it might interact directly with CUL-3, the *C. elegans* Cullin 3 orthologue. To test whether these proteins interact, we used a transient expression system in COS-7 cells. We cotransfected either GST::CUL-3 protein or GST alone with FLAG-tagged KEL-8 protein (FLAG::KEL-8) in COS-7 cells. We detected GST, GST::CUL-3, and FLAG::KEL-8 on the same blot using an antibody raised against GST::KEL-8, which recognizes both GST and KEL-8 epitopes. Using glutathione agarose beads, we could pull down KEL-8 from COS-7 lysates cotransfected with GST::CUL-3 but not GST alone (Figure 6A). In addition, we immunoprecipitated GST::CUL-3 but not GST alone from cotransfected COS-7 lysates with anti-FLAG antibodies (Figure 6B). Reduced amounts of GST::CUL-3(H2) (only detectable with significantly longer chemiluminescence exposures than the blot shown) are coprecipitated compared with wild-type GST::CUL-3. For both A and B, arrowheads indicate the specific pulled down proteins. Brackets above the gels indicate the cotransfected constructs. “Load” indicates 2.5% of the original lysate. Similar results were observed in six separate transfection experiments.

![Figure 6](image-url)

**Figure 6.** KEL-8 interacts with CUL-3 Cullin. (A) GST::CUL-3 and FLAG::KEL-8 were cotransfected into COS-7 cells, which were solubilized in RIPA buffer. GST affinity chromatography was performed, and bound proteins were detected by Western blotting (WB) with an antisera that recognizes both GST and KEL-8. GST::CUL-3 but not GST alone pulls down KEL-8 protein. GST::CUL-3(H2), which reduces the affinity of CUL-3 for other BTB proteins, pulls down reduced amounts of KEL-8. (B) Immunoprecipitations were performed with anti-FLAG antisera, and commmunoprecipitated proteins were detected by Western blotting with an anti-GST antisera. FLAG::KEL-8 coprecipitates GST::CUL-3 but not GST alone. Reduced amounts of GST::CUL-3(H2) (only detectable with significantly longer chemiluminescence exposures than the blot shown) are coprecipitated compared with wild-type GST::CUL-3. For both A and B, arrowheads indicate the specific pulled down proteins. Brackets above the gels indicate the cotransfected constructs. “Load” indicates 2.5% of the original lysate. Similar results were observed in six separate transfection experiments. (C) AH109 yeast expressing CUL-3 fused to Gal4 DNA binding domain (DB CUL-3) and KEL-8 fused to an activation domain (AD KEL-8) are able to support growth on two independent yeast two-hybrid reporter genes (HIS3 and ADE2). The DB CUL-3 and AD KEL-8 constructs, present individually in AH109 and streaked out in separate quadrants, are unable to support growth. (D–H) GLR-1::GFP clusters were visualized in wild-type nematodes (D), nematodes expressing full-length CUL-3 protein (E), nematodes expressing amino-terminal residues 1–500 (F), nematodes expressing carboxy-terminal residues 501–777 (G), and rfl-1 mutants (H). Expression of either CUL-3(1-500) or CUL-3(501-777), or a mutation in the Nedd8-activating enzyme RFL-1 results in the accumulation of GLR-1. Bar, 5 μm.
ture, with an amino-terminal domain that interacts with F-box and BTB domain proteins and a carboxy-terminal domain that interacts with RING domain proteins and E2 ubiquitin-conjugating enzymes. Partial CUL-3 proteins can act as dominant negatives (Zhang et al., 2005). Thus, to interfere with endogenous CUL-3 function, we generated \textit{P}_{glr-1::cub-3(1-500)} and \textit{P}_{glr-1::cub-3(501-777)} transgenes that express the amino terminus and carboxy terminus of CUL-3, respectively, using the \textit{glr-1} promoter. We also generated a \textit{P}_{glr-1::cub-3(full length)} transgene that expresses full-length CUL-3 protein as a control. We introduced the transgenes into nematodes separately and found that expression of full-length CUL-3 had little effect on GLR-1 clusters (4 independent lines, 15–25 animals examined per line; Figure 6, D and E). By contrast, expression of either the CUL-3 amino terminus (2 independent lines, 15–25 animals per line; Figure 6F) or carboxy terminus (2 independent lines, 15–25 animals per line; Figure 6G) resulted in accumulation of GLR-1, similar to \textit{kel-8} mutants.

As an independent test for the role of CUL-3 in KEL-8 function, we reasoned that neddylation should be required for GLR-1 degradation. Neddylation is the attachment of the ubiquitin-like NEDD8 specifically to cullin family members and is needed for the assembly and function of Cullin-RING ligases (Willems et al., 2004; Petroshi and Deshaies, 2005). We took advantage of the temperature-sensitive \textit{rfl-1(or198ts)} mutant, which is inviable at higher temperatures because of the decreased activity of the RFL-1 Nedd8-activating enzyme (Kurz et al., 2002). We introduced GLR-1::GFP into \textit{rfl-1(or198ts)} and found that GLR-1:GFP accumulates in viable \textit{rfl-1} mutants at 15°C (Figure 6H). Together, our results suggest that KEL-8 and CUL-3 form a cullin complex, CDL3-KEL-8, that is essential for proper degradation of GLR-1 receptors.

**DISCUSSION**

In this study, we used a genetic approach to identify \textit{kel-8}, a gene required to regulate GLR-1 synaptic abundance. First, we showed that KEL-8 is a member of the Kelch superfamily of proteins that regulates the levels of GLR-1 but not other synaptic proteins. Second, we demonstrated that KEL-8 is expressed and required in neurons and is localized in clusters that are adjacent to GLR-1-containing postsynaptic elements. Finally, we found that KEL-8 binds to the CUL-3 subunit of the CDL3 ubiquitin ligase and is required for the ubiquitin-mediated turnover of GLR-1. Based upon these results, we propose that KEL-8 serves as a substrate receptor for CDL3 and that this interaction with the ubiquitin–proteasome apparatus is critical for regulating glutamate receptor abundance and synaptic signaling.

**KEL-8 Regulates GLR-1 Synaptic Levels**

Regulated turnover of GLR-1 subunits was first observed in \textit{unc-11}/clathrin adaptor AP180 mutants, where endocytosis is blocked (Burbea et al., 2002). Ubiquitination of several critical lysines in the carboxy-terminal tail of GLR-1 results in the endocytosis and degradation of GLR-1. The ubiquitination and turnover of GLR-1 can be facilitated by elevating the levels of free ubiquitin using the \textit{nuls89[MUb]} transgene, and mutations in \textit{unc-11}, components of the anaphase-promoting complex (APC), and \textit{kel-8} block some of the effects of elevated ubiquitin levels, suggesting that these proteins are needed for ubiquitin-mediated turnover of GLR-1 (Burbea et al., 2002; Juo and Kaplan, 2004). The effect of overexpressing ubiquitin from the \textit{nuls89[MUb]} transgene cannot be entirely mediated through KEL-8, because we would expect that \textit{nuls89[MUb]}; \textit{kel-8} nematodes would have a similar number and size of GLR-1 clusters to \textit{kel-8} single mutants. Whereas \textit{nuls89[MUb]}; \textit{kel-8} animals have a similar number of GLR-1 clusters relative to \textit{kel-8} single mutants, by contrast, the GLR-1 clusters of \textit{nuls89[MUb]}; \textit{kel-8} animals are of an intermediate size compared with either \textit{nuls89[MUb]} animals or \textit{kel-8} mutants alone. One explanation is that overexpression of ubiquitin might independently affect different facets (e.g., cluster size versus number) of GLR-1 synaptic localization and that KEL-8 mediates this effect on a specific facet: cluster number. Alternatively, all facets of GLR-1 localization might be determined solely by GLR-1 abundance, such that the large size of GLR-1 clusters in \textit{kel-8} mutants might be a secondary consequence resulting from the increased synaptic abundance of GLR-1. In this model, overexpression of ubiquitin results in decreased GLR-1 abundance and thus decreased cluster number. Mutations in \textit{kel-8} can only partially block this effect, allowing enough of an increase in GLR-1 abundance to raise the detectable number of GLR-1 clusters but not enough to enlarge the size of GLR-1 clusters.

We think that the latter model is more likely for several reasons. First, the shifts in both GLR-1 cluster size and number between \textit{nuls89[MUb]} animals, \textit{kel-8} mutants, and \textit{nuls89[MUb]}; \textit{kel-8} doubles are present at all points along their respective distributions, suggesting that ubiquitination and KEL-8 do not regulate subsets of clusters but rather regulate the global population of clusters (our unpublished data). Second, we see an increase in GLR-1 cluster size in transgenic lines that express GLR-1 to higher levels than the \textit{nuls25} transgene used in our study, suggesting that as GLR-1 abundance increases, the excess receptors spread beyond the postsynaptic region and appear as large clusters (our unpublished data).

KEL-8 cannot be the sole factor through which overexpressed ubiquitin mediates it affect on GLR-1 localization. Indeed, mutations in components of the APC result in a subtle (~20%) increase in GLR-1 abundance, although GLR-1 is not the direct ubiquitination target of the APC (Juo and Kaplan, 2004). Thus, the subtle changes in GLR-1 abundance in APC mutants might be a secondary consequence of disrupted APC function. The LIN-23 ubiquitin ligase has recently been shown to regulate GLR-1 abundance by ubiquitinating BAR-1 β-catenin (Dreier et al., 2005). BAR-1::GFP levels are elevated in \textit{lin-23} mutants, and \textit{bar-1} mutations can partially suppress the GLR-1 accumulation observed in \textit{lin-23} mutants. Mutations that stabilize BAR-1 result in a subtle (~20%) increase in GLR-1 abundance, suggesting that Wnt signaling plays a minor role in regulating GLR-1 abundance. In contrast to APC and Wnt signaling mutants, \textit{kel-8} mutants showed a dramatic enough increase (200–300% compared with wild type) to allow us to identify \textit{kel-8} in a forward genetic screen. Moreover, KEL-8 is expressed specifically in GLR-1-containing neurons, whereas APC and Wnt signaling components are broadly expressed. We currently do not know the substrate for KEL-8, and we have not been able to detect a binding interaction between KEL-8 and GLR-1 (our unpublished data), although the transient nature of the ubiquitination reaction makes this difficult to interpret. Together, our findings suggest that KEL-8 is a major, dedicated regulator of GLR-1 abundance.

What is the physiological relevance of KEL-8 acting to negatively regulate GLR-1 at the synapse? In nematode interneurons, GLR-1 signals to regulate the direction of movement by triggering a reversal in direction either in response to nose-touch stimuli or occasionally spontaneously (Hart et al., 1995; Maricq et al., 1995; Zheng et al., 1999; Mellem et al., 2002). Spontaneous reversals seem to allow the animals to
change their overall direction of travel. Elevated levels of GLR-1 protein or GLR-1 activity result in an increase in spontaneous reversal frequency, suggesting that GLR-1 abundance is regulated to control locomotion behavior (Zheng et al., 1999; Mellem et al., 2002). Similar to these observations, we found that kel-8 mutants have a high frequency of spontaneous reversal, consistent with the observed increase in GLR-1 synaptic abundance in these mutants. Because kel-8 mutants have increased GLR-1 protein levels, our results suggest that a significant fraction of GLR-1 receptors is normally degraded. We speculate that the physiological role of KEL-8 is to regulate locomotory behavior by regulating GLR-1 synaptic levels.

**KEL-8 Is Localized Adjacent to GLR-1**

We found that KEL-8::GFP is localized in clusters along the ventral cord neurites. A KEL-8::GFP chimeric protein containing the BTB domain alone is also localized in clusters; however, the localization of this chimera requires an endogenous copy of wild-type kel-8. This observation is similar to that of *Drosophila* KELCH protein, which is localized to actin-rich ring canals (Robinson and Cooley, 1997; Kelso et al., 2002). The BTB domain of KELCH is also localized to ring canals via its interaction with endogenous KELCH through BTB homodimerization (Robinson and Cooley, 1997). Because the KEL-8 BTB domain also requires endogenous kel-8 for proper localization, we speculate that KEL-8 homodimerizes through its BTB domains.

KEL-8::GFP is localized to clusters that are adjacent to the postsynaptic clusters of GLR-1::RFP. One possible explanation for the proximal but not overlapping colocalization of KEL-8 and GLR-1 is that KEL-8 is at presynaptic terminals. We think this unlikely for several reasons. KEL-8::GFP is expressed in the same neurons as GLR-1::RFP. Moreover, expression of *kel-8* cDNA by the glr-1 promoter rescues the *kel-8* mutant phenotype, indicating that KEL-8 functions in the same cells as GLR-1. The GLR-1-expressing neurons make some interneuron-to-interneuron synaptic connections, accounting for ~38% of clusters in the anterior portion of the ventral cord (White et al., 1986; Burbela et al., 2002). By contrast, >90% of GLR-1 clusters are adjacent to KEL-8 clusters, which is far more than can be explained if KEL-8 were at the presynaptic terminals of the interneuron-to-interneuron synapses.

Interestingly, recent studies of mammalian AMPARs indicate that these receptors move laterally and undergo endocytosis in membrane regions that are adjacent to the postsynaptic density (Racz et al., 2004). Thus, one possible explanation for KEL-8 localization adjacent to GLR-1 clusters is that KEL-8 is localized to tangential sites of endocytosis for GLR-1 receptors, similar to what has been observed for mammalian receptors.

**KEL-8 Is a Substrate Receptor for CDL3**

KEL-8 binds to a CUL-3 ubiquitin ligase subunit. BTB domain proteins have recently been shown to function as substrate receptors for CDL3 ubiquitin ligases (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003; Zhang et al., 2004). The BTB domain of this new class of substrate receptor (e.g., MEL-26) interacts with the Cullin repeats of Cul3, analogous to the manner by which Skp1 interacts with Cul1 in the CDL1/SCF complex. CDL-3 in turn recruits RING domain proteins and E2-conjugating enzymes into the complex, where the conjugating enzymes transfer ubiquitin to the substrate. Cullin-RING ligases are thought to assemble with numerous different substrate receptors, allowing them to recognize a large repertoire of substrates; however, the characterization of most Cullin-RING ligases has been limited to their role in cell division (Willems et al., 2004; Petroski and Deshaies, 2005). An exception is the BTB-Kelch protein Keap1, which interacts with Cullin 3 and regulates the levels of the transcription factor Nrf2 in response to oxidative stress (Zhang et al., 2004). There are 10 BTB-Kelch proteins in the *C. elegans* genome and 125 in the human genome. However, how many of these proteins assemble with Cullin-RING ligases, and the nature of their biological functions remains unclear. We speculate that KEL-8 represents a new group of BTB-Kelch superfamily members that function as Cullin substrate receptors and that many of these proteins will have critical postmitotic functions. In postmitotic neurons, rapid degradation of many synaptic proteins has been observed, and Cullin-RING ligases such as CDL3KEL-8 might provide a mechanism underlying some forms of synaptic plasticity.

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