

# Biosynthesis of ionotropic acetylcholine receptors requires the evolutionarily conserved ER membrane complex

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The number of nicotinic acetylcholine receptors (AChRs) present in the plasma membrane of muscle and neuronal cells is limited by the assembly of individual subunits into mature pentameric receptors. This process is usually inefficient, and a large number of the synthesized subunits are degraded by endoplasmic reticulum (ER)-associated degradation. To identify cellular factors required for the synthesis of AChRs, we performed a genetic screen in the nematode *Caenorhabditis elegans* for mutants with decreased sensitivity to the cholinergic agonist levamisole. We isolated a partial loss-of-function allele of ER membrane protein complex-6 (*emc-6*), a previously uncharacterized gene in *C. elegans*. *emc-6* encodes an evolutionarily conserved 111-aa protein with two predicted transmembrane domains. EMC-6 is ubiquitously expressed and localizes to the ER. Partial inhibition of EMC-6 caused decreased expression of heteromeric levamisole-sensitive AChRs by destabilizing unassembled subunits in the ER. Inhibition of *emc-6* also reduced the expression of homomeric nicotine-sensitive AChRs and GABA<sub>A</sub> receptors in *C. elegans* muscle cells. *emc-6* is orthologous to the yeast and human EMC6 genes that code for a component of the recently identified ER membrane complex (EMC). Our data suggest this complex is required for protein folding and is connected to ER-associated degradation. We demonstrated that inactivation of additional EMC members in *C. elegans* also impaired AChR synthesis and induced the unfolded protein response. These results suggest that the EMC is a component of the ER folding machinery. AChRs might provide a valuable proxy to decipher the function of the EMC further.

Genes coding for nicotinic acetylcholine receptor (AChR) subunits form an evolutionarily conserved family, which includes a large repertoire of ionotropic receptors gated by acetylcholine (ACh). In mammals, there are at least 16 different genes encoding AChR subunits. Each subunit is made up of a large extracellular domain, four transmembrane segments (M1–M4), and a variably long intracellular loop between M3 and M4. Individual subunits assemble into homo- or heteropentameric receptors with distinct pharmacological and biophysical properties (reviewed in ref. 1). Interestingly, the assembly of AChRs seems rather slow and inefficient, which is a feature common to several multimeric transmembrane complexes, such as GABA<sub>A</sub> receptors (2) or cystic fibrosis transmembrane conductance regulator (CFTR) receptors (3). In vertebrate muscle cells, only 30% of the synthesized  $\alpha$  subunits finally assemble into a mature pentamer (4). In neurons, a significant number of AChRs are also found in intracellular compartments (5). Although the physiological significance of AChR intracellular retention is not completely understood, it potentially has diverse pathological implications. For example, a rare mutation in the human  $\beta 4$  AChR subunit has been linked to amyotrophic lateral sclerosis (6), and this mutation was shown to impair the export of  $\alpha 4\beta 4$  AChRs from the endoplasmic reticulum (ER) (7). ER retention of AChRs was also proposed to influence ER stress and the unfolded protein response (UPR) in dopaminergic neurons (8). In the smoker's brain, an enhancement

of AChR expression likely contributes to tobacco addiction. This up-regulation involves a chaperone activity of nicotine, which enhances the assembly of specific receptor species and their export from the ER, together with a reduced degradation of AChR subunits by the proteasome system (9–11).

The overall efficiency of AChR biosynthesis is dependent on multiple processes in the ER and in early secretory compartments (reviewed in ref. 12). AChR subunits enter the ER cotranslationally in an unfolded state via the Sec61 translocon. Transient interactions with ER resident components of the biosynthetic machinery, such as BiP (13, 14), calnexin (15, 16), and ERp57 (17), then influence the folding process and protect immature subunits from degradation. Folding subunits are exposed to a variety of enzymes that catalyze posttranslational modifications (including glycosylation and palmitoylation) and regulate protein expression (18–20). Assembly of the subunits is sequential, and intermediates of certain receptors, such as muscle AChRs, have sufficient  $t_{1/2}$ s to be isolated and characterized (reviewed in ref. 21). ER retention or retrieval signals prevent the exit of unassembled or partially assembled AChR subunits. For example, an ER retention signal present in the M1 transmembrane segment of the subunits is masked by receptor assembly to allow ER exit (22). Unassembled subunits are eventually degraded through the ER-associated degradation (ERAD) machinery (23). ER export signals have also been demonstrated to enhance the recruitment of receptors into ER exit sites (24). In addition, degradation signals present in the cytoplasmic M3–M4 subunit loop (25, 26) must be masked for ER exit licensing. The cytoplasmic M3–M4 loop is also involved in interactions with adaptor proteins, such as the small cytoplasmic chaperone 14-3-3 (27) or the UBXD4 protein (28).

Despite its physiological and pathological importance, relatively few cellular factors are known to control AChR biosynthesis at early steps. This might be due to the difficulty in identifying labile interactions with partially assembled receptors or because receptors interact with large cellular complexes. Genetic screens in model organisms provide a means to identify relevant components in biological networks involved in this specific process. Specifically, the nematode *Caenorhabditis elegans* is a powerful model organism with which to dissect AChR biosynthesis genetically. ACh is the

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main excitatory neurotransmitter in *C. elegans*, and at least 29 genes encode AChR subunits (29). Two types of ionotropic AChRs, heteromeric levamisole-sensitive AChRs (L-AChRs) and homomeric nicotine-sensitive AChRs (N-AChRs), are present at the neuromuscular junction (NMJ), the best-characterized cholinergic synapse in the worm (30). L-AChRs are heteropentameric receptors that can be activated by the nematode-specific cholinergic agonist levamisole. Levamisole causes hypercontraction of *C. elegans* body-wall muscles (BWMs) and death of WT worms at high concentrations (31, 32). Genetic screens for resistance to levamisole have identified the structural subunits of the receptor, including three  $\alpha$  subunits (LEV-8, UNC-38, and UNC-63) and two non- $\alpha$  subunits (LEV-1 and UNC-29). The second type of receptor, N-AChR, is activated by nicotine and is insensitive to levamisole. It is most likely homomeric, composed of five ACR-16 subunits (33, 34).

Apart from receptor subunits, genetic screens identified three auxiliary proteins (RIC-3, UNC-50, and UNC-74) required for receptor biosynthesis. RIC-3 was identified in a suppressor screen of neuronal degeneration caused by a gain-of-function mutation in the *C. elegans* AChR composed of DEG-3 and DES-2 subunits (35). RIC-3 is a small integral membrane protein located in the ER that is required for the assembly of at least five distinct AChR receptors in *C. elegans*, including L-AChRs and N-AChRs (35–37). RIC-3 is conserved in flies and mammals, and it can either promote or inhibit the expression of AChRs and 5-HT3 receptors in heterologous systems (reviewed in ref. 12). Recent analysis suggests that RIC-3 activity depends on its expression level; at low levels, RIC-3 behaves as an ER chaperone promoting AChR assembly, whereas high expression levels cause ER retention through long-lived interactions with AChRs (38). UNC-50 was identified in a screen for resistance to levamisole. UNC-50 is the ortholog of GMH1, a transmembrane protein conserved from yeast to humans, which interacts with a guanine nucleotide exchange factor of the small G protein Arf and potentially regulates COPI-dependent trafficking events. UNC-50 localizes to the Golgi apparatus and promotes the targeting of L-AChRs to the plasma membrane by preventing routing and degradation in lysosomes (39). UNC-74 is a predicted thioredoxin necessary for L-AChR expression, but its role has not been fully characterized. Interestingly, expression of L-AChRs in *Xenopus* oocytes not only requires the five receptor subunits but the three auxiliary proteins RIC-3, UNC-50, and UNC-74 as well (36). Recently, the ER resident transmembrane proteins NRA-2/nicalin (nicastrin-like protein) and NRA-4/nodal modulator (NOMO), were proposed to regulate L-AChR subunit composition and stoichiometry (40).

To identify additional components required for AChR assembly and folding, we designed a genetic screen for mutants with only partially decreased sensitivity to the cholinergic agonist levamisole, because screens for complete resistance to levamisole are likely saturated. Such mutants completely paralyze on high levamisole concentration within a few hours but subsequently adapt within 12–16 h and recover motility in contrast to WT worms (41, 42). Here, we identify and characterize *in vivo* the ER membrane protein complex-6 (*emc-6*) gene. We demonstrate that EMC-6 is required for the proper assembly of AChRs through the function of an evolutionarily conserved complex involved in ER homeostasis.

## Results

**Disruption of the Evolutionarily Conserved Gene *emc-6* Confers Partial Resistance to the Cholinergic Agonist Levamisole.** To identify new components involved in AChR metabolism, we used *Mos1*-mediated insertional mutagenesis to generate mutants resistant to the cholinergic agonist levamisole (42). A strain carrying the mutant allele *kr150* was isolated based on its ability to adapt to overnight exposure to 0.6 mM levamisole. A *Mos1* insertion was identified in *kr150* by inverse PCR in the ORF F33D4.7 (Fig. 1A).

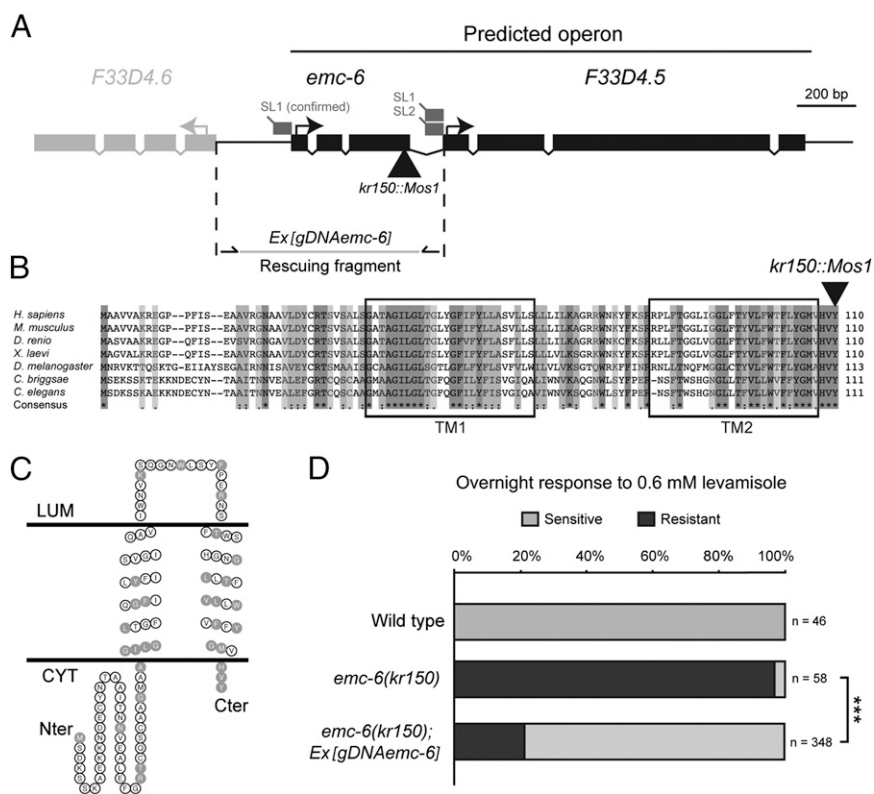
This gene is conserved from yeast to humans (Fig. 1B and Table S1), and it was tentatively named *emc-6* because of its homology with the yeast gene *EMC6* (43). Based on cDNA sequencing, the *C. elegans emc-6* messenger is transspliced to the splice leader SL1 and encodes a predicted 111-aa protein with two transmembrane domains (Fig. 1C). Topology prediction programs suggest that the N-terminal region of the protein is cytosolic. The decreased sensitivity to levamisole of *kr150* mutants was rescued by providing a 900-bp genomic fragment containing the *emc-6* coding region (Fig. 1A and D), thus demonstrating that mutating *emc-6* causes the partial resistance to levamisole in this mutant.

Nonquantitative RT-PCR analysis demonstrated that *emc-6* transcript remains in *kr150* mutants. Insertion of the *Mos1* transposon causes the replacement of the conserved ultimate amino acid by four unrelated residues followed by a STOP codon, a cryptic polyadenylation site being used in the *Mos1* sequence (Fig. S1A). Quantitative RT-PCR analysis indicated, however, that the level of this *emc-6* mRNA was severely reduced compared with the WT [ $21 \pm 5\%$  at L2 stage and  $8 \pm 1\%$  at L4 stage (mean  $\pm$  SEM);  $n = 4$ ] (Fig. S1B). These data suggested that *emc-6(kr150)* is a strong loss-of-function mutant but may not be a null allele (details are provided in Fig. S2). Our attempts to generate a second allele of *emc-6* by noncomplementation screening after EMS mutagenesis (30,155 F1 animals screened) was unsuccessful, suggesting that *kr150* is a particular hypomorphic allele of *emc-6* and that *emc-6* might be an essential gene.

Apart from resistance to levamisole, the *kr150* mutation is associated with general phenotypic defects, including embryonic lethality, growth retardation, and reduced brood size (Fig. S2). Unfortunately, the rescue of these phenotypes could not be quantitatively scored because the transgene expressing *emc-6* from a genomic fragment was, by itself, causing partial embryonic lethality and significant developmental defects. Because adaptive resistance to levamisole is usually not associated with developmental phenotypes (e.g., *lev-10*, *lev-9*, *oig-4*, *molo-1* mutant), these data suggest that *emc-6* supports general functions apart from its role in cholinergic neurotransmission.

**EMC-6 Is Required in Muscle for L-AChR Expression.** To characterize the *emc-6* expression pattern, we built a transcriptional reporter expressing GFP under the control of *emc-6* regulatory sequences. Surprisingly, we observed that transgenes driving GFP expression under the control of the 263-bp promoter sequence used for *emc-6* rescue experiments induced lethality and did not transmit GFP expression after one generation. Despite significant efforts, we failed to establish stable expression of this transcriptional reporter and could only acquire pictures of mosaic F1 animals (Fig. S3A). In these mosaic animals, GFP was detected in almost all tissues, including neurons and muscle cells. The very unusual toxicity of a small promoter fragment suggested that it might trigger sequence-specific epigenetic mechanisms. To bypass this technical issue, we characterized the expression pattern of the *Caenorhabditis briggsae* ortholog of *emc-6* (*Cb-emc-6*) in *C. elegans*. *Cb-EMC-6* and *C. elegans* Ce-EMC-6 protein sequences are 98% identical. Expression of the genomic locus of *Cb-emc-6* in *emc-6(kr150)* was sufficient to rescue the levamisole resistance of *emc-6(kr150)* mutants (Fig. S3B). Interestingly, transgenic expression of *Cb-emc-6* was nontoxic in *C. elegans*. We therefore expressed a nuclear GFP under the control of the *C. briggsae*-promoting sequence of *emc-6* in *C. elegans* (Fig. S3C). We were able to detect GFP expression in every tissue from the threefold stage to adulthood. Altogether, these results suggest that *emc-6* is ubiquitously expressed.

To test in which tissue EMC-6 was required for L-AChR expression, we used the muscle-specific *Pmyo-3* promoter or the neuron-specific *Prab-3* promoter to express the *emc-6* cDNA in *emc-6(kr150)* mutants (Fig. 2A). Unlike the *Prab-3::emc-6* construct, *Pmyo-3::emc-6* fully rescued levamisole resistance of *emc-6*



**Fig. 1.** *emc-6* encodes a conserved transmembrane protein. (A) Structure of the *emc-6* genomic locus. *emc-6* is predicted to be the upstream gene of an operon ([www.wormbase.org](http://www.wormbase.org)). Black boxes represent the coding regions, and gray boxes represent the 5' untranslated region. The black triangle illustrates the site of *Mos1* insertion in *kr150*. SL1, SL1 transspliced leader; SL2, SL2 transspliced leader. (B) ClustalX alignment of *C. elegans* EMC-6 with orthologs from nematodes, fly, and vertebrates. Predicted transmembrane regions (TM1 and TM2) are boxed. Residues conserved in all species are highlighted in dark gray, residues with strongly similar properties (scoring >0.5) are highlighted in medium gray, and residues with weakly similar properties (scoring <0.5) are highlighted in light gray. The position of the *kr150* mutation is indicated by a triangle. (C) EMC-6 topology model based on TMHMM and Topo2 predictions. Residues conserved in all species are labeled in gray. Cter, C-terminus; CYT, cytoplasm; LUM, lumen; Nter, N-terminus. (D) Expression of an *emc-6* genomic fragment rescues levamisole resistance in *kr150* mutants. Gray bars illustrate the percentage of dead animals after overnight exposure to 0.6 mM levamisole, and black bars illustrate the percentage of surviving animals. Three independent transgenic lines were tested. n, number of animals tested. \*\*\* $P < 0.001$ , Fisher exact probability test.

(*kr150*) mutants. This indicates that *emc-6* functions in BWMs to promote L-AChR surface expression.

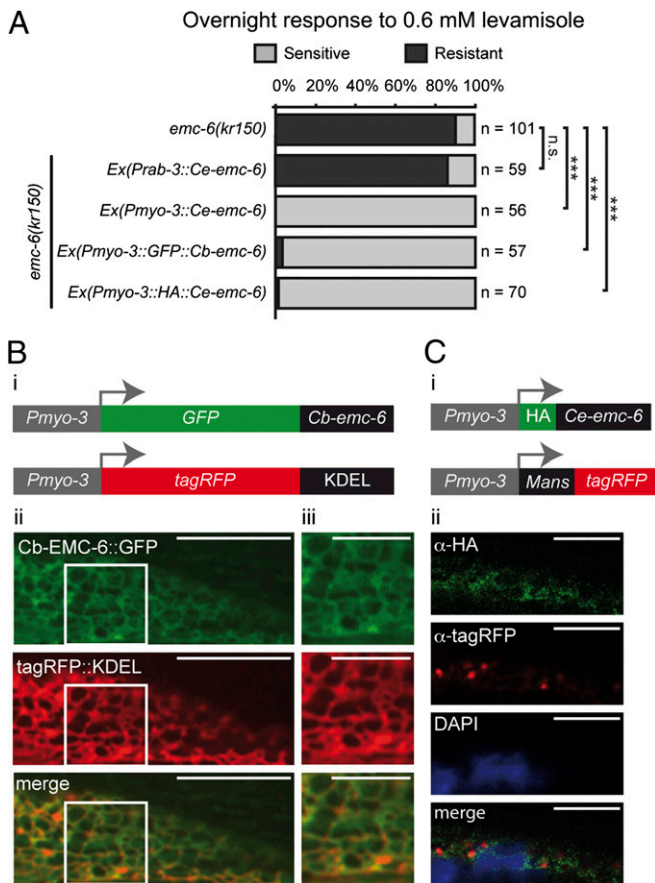
**EMC-6 Localizes to the ER.** To assay the subcellular localization of the EMC-6 protein, we expressed an N-terminally tagged EMC-6 construct in the BWMs. As mentioned above, transgenes driving *Ce-emc-6* expression were extremely toxic, and we could only generate a single transgenic line, prompting us to express a tagged version of *C. briggsae* EMC-6 also. BWM expression of either HA::Ce-EMC-6 or GFP::Cb-EMC-6 rescued the *emc-6(kr150)* levamisole resistance phenotype (Fig. 2A). With both constructs, tagged EMC-6 localized to a perinuclear intracellular network reminiscent of ER. Consistently, GFP::Cb-EMC-6 colocalized with tagRFP fused to the ER retention signal KDEL (44) (Fig. 2B). Conversely, the Golgi marker  $\alpha$ -MannosidaseII-tagRFP (45) did not colocalize with HA::Ce-EMC-6 (Fig. 2C). These results demonstrate that both *C. briggsae* and *C. elegans* EMC-6 primarily localize to the ER.

***emc-6* Is Required for Surface Expression of Ligand-Gated Ion Channels at NMJs.** The resistance of *emc-6(kr150)* mutants to levamisole suggested that disrupting *emc-6* impairs L-AChR function. To analyze this defect, we first recorded the electrophysiological response of BWM cells to pressure-applied levamisole and observed a 73% decrease in the response of *emc-6(kr150)* mutants compared with the WT (Fig. 3A). To evaluate the number of L-AChRs present at the synapse, we quantified the L-AChR-

dependent evoked response after stimulation of cholinergic motor neurons and found a 66% reduction in *emc-6(kr150)*. These data suggest that the amount of functional L-AChRs is severely reduced in *emc-6(kr150)* but that the remaining receptors are still clustered at NMJs (Fig. 3B).

To test if these electrophysiological phenotypes might reflect a synaptogenesis defect, we stained cholinergic boutons by immunofluorescence. The staining pattern of the vesicular acetylcholine transporter UNC-17 was indistinguishable between *emc-6(kr150)* and WT animals. By contrast, the staining of L-AChRs was dramatically decreased in *emc-6(kr150)* mutants (Fig. 3C). To quantify the synaptic decrease of the L-AChRs in *emc-6(kr150)*, we generated a knock-in strain carrying the *unc-29* L-AChR subunit endogenously tagged with tagRFP using the *MosTIC* technique (46). Consistent with immunostaining of L-AChRs, we observed an 82% decrease of the amount of L-AChR at the NMJ (Fig. 3D and E). The remaining L-AChRs still formed synaptic clusters in *emc-6(kr150)* mutants. Altogether, these electrophysiological and histological results indicate that *emc-6* is required for the surface expression of L-AChRs.

In WT animals, BWMs sense excitatory cholinergic inputs through heteromeric L-AChRs and homomeric ACR-16-containing N-AChRs, as well as through inhibitory GABAergic input through the UNC-49 GABA<sub>A</sub> receptors. To decipher the specificity of the *emc-6(kr150)* phenotype at NMJs, we recorded the electrophysiological responses of BWM to pressure-ejected nicotine, which stimulates only ACR-16-containing



**Fig. 2.** *emc-6* is required in muscle cells for L-AChR function and localizes to the ER. (A) Expression of an *emc-6* cDNA and GFP::Cb-EMC-6 or HA::Ce-EMC-6 translational fusion in muscle (*Pmyo-3* promoter) but not in neurons (*Prab-3* promoter) rescues levamisole resistance of *emc-6(kr150)* mutants. n, number of tested animals. n.s. (nonsignificant),  $P > 0.05$ ; \*\*\* $P < 0.001$ , Fisher exact probability test. (B) GFP::Cb-EMC-6 and tagRFP::KDEL translational fusions used for expression in BWM (*Pmyo-3* promoter). (ii) Live images of GFP::Cb-EMC-6 and tagRFP::KDEL displaying a reticular staining pattern throughout the cytoplasm surrounding the nucleus. (Scale bars, 5  $\mu$ m.) (iii) Magnified view of the reticulum network. (Scale bars, 2.5  $\mu$ m.) (C) HA::Ce-EMC-6 fusion does not colocalize with a Golgi-resident tagRFP-tagged Mannosidase II protein (MANS::tagRFP). (i) HA::Ce-EMC-6 and MANS::tagRFP used for expression in BWMs (*Pmyo-3* promoter). (ii) Immunostaining with anti-HA and anti-tagRFP antibodies. DAPI staining of cell nuclei. (Scale bars, 5  $\mu$ m.)

N-AChRs, (Fig. 3F) or to GABA (Fig. 3G). We found a 36% decreased response to nicotine and a 57% decreased response to GABA in *emc-6(kr150)* mutants compared with WT animals. These results demonstrate that *emc-6* is required for the surface expression of at least three different types of multimeric ion channels.

***emc-6* Stabilizes L-AChR Subunits Before or During Receptor Assembly.** To test if the reduction of L-AChR at the NMJ of *emc-6(kr150)* mutants reflected a decrease in total receptor expression, we quantified the amount of the UNC-29 L-AChR subunit by Western blot analysis (Fig. 4A). UNC-29 expression was decreased by 72% in *emc-6(kr150)* compared with the WT. Decreased L-AChR expression could be caused at transcriptional or translational steps. To test if *emc-6* was required for gene expression, we measured the mRNA levels of *unc-29*, *unc-38*, and *unc-63*, the three essential L-AChR subunits, and the mRNA levels of *acr-16* and *unc-49*, the constitutive subunits of homomeric N-AChRs

and GABA<sub>A</sub> receptors, respectively (Fig. S4A and B). Compared with the WT, we did observe a slight but not significant decrease of these receptors' mRNA levels in *emc-6(kr150)* mutants.

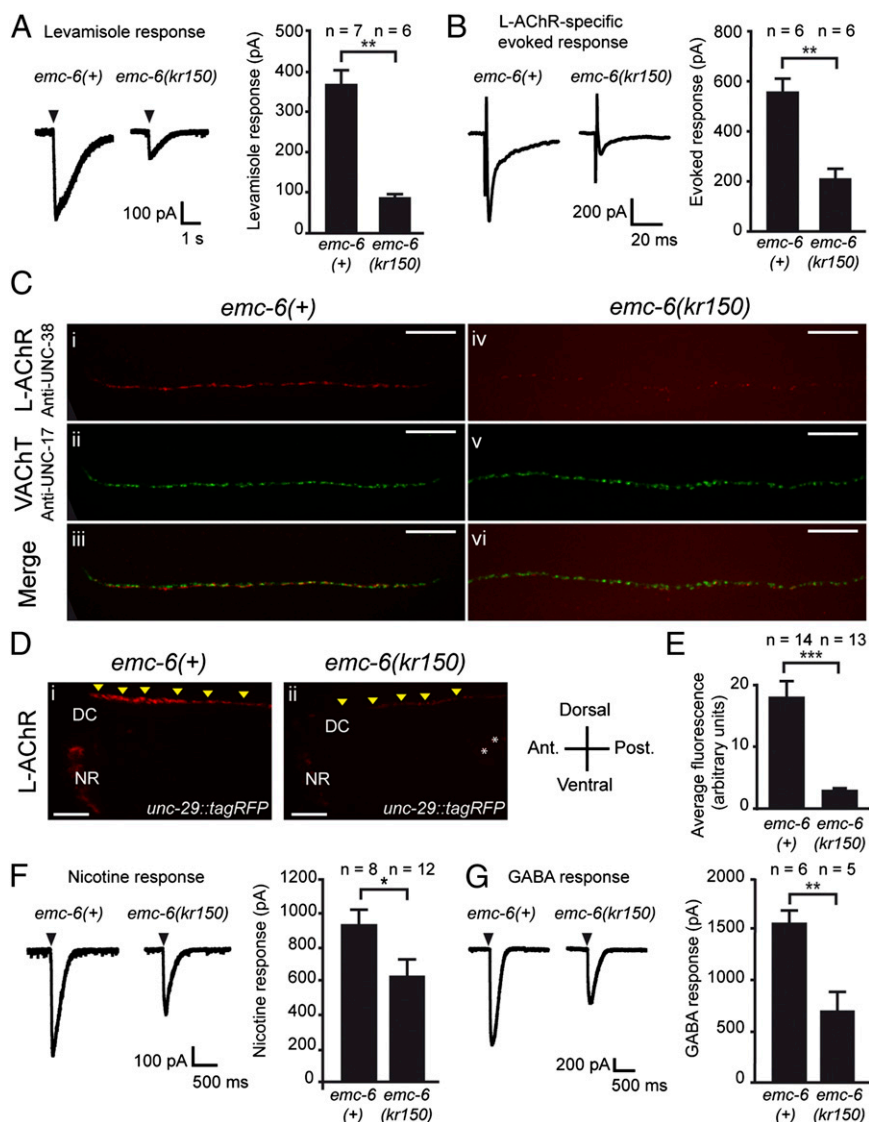
To test if EMC-6 was required for L-AChR biosynthesis within the ER, we analyzed the effect of *emc-6* inactivation on unassembled L-AChR subunits retained in the ER. If EMC-6 is required for the folding or stability of unassembled L-AChR subunits, impairing *emc-6* function should reduce the level of remaining unassembled subunits. Conversely, if EMC-6 is required after the assembly of the receptor, we predict that ER-retained unassembled subunits would not be affected by *emc-6(kr150)* mutation. We have previously demonstrated that L-AChR subunits do not traffic to the plasma membrane when the essential UNC-63 subunit is absent (38). In *unc-63(0)* mutants, unassembled UNC-29 subunits accumulate in the ER and are readily detected by Western blot (Fig. 4A). In *unc-63(0); emc-6(kr150)* double mutants, the amount of UNC-29 was decreased [compare with *unc-63(0)*] to a similar extent as in *emc-6(kr150)* single mutants (compared with WT) (Fig. 4A), suggesting that *emc-6* is indeed required for the stability of ER-retained unassembled subunits.

Recently, Rer1, a Golgi-ER retrieval receptor, was demonstrated to control the expression of vertebrate muscle AChRs by ensuring the retention of unassembled subunits in the ER (47). To test the possibility that EMC-6 might also be involved in the control of the ER exit of receptor subunits, we analyzed the UNC-29 glycosylation profile in *unc-63(0)* and *emc-6(kr150)* (Fig. 4B). Mature N-glycosylations on membrane proteins are resistant to Endoglycosidase H (EndoH) cleavage after trafficking through the secretory pathway, whereas the nascent sugar side chains synthesized in the ER are cleaved by EndoH. By contrast, peptide-N-glycosidase F (PNGase) eliminates all carbohydrates from N-linked glycosylation. To distinguish between mature and immature L-AChRs, we analyzed the digestion profiles of the UNC-29 subunit after treatments by EndoH or PNGase. In the WT, UNC-29 is resistant to EndoH, whereas it is fully sensitive in *unc-63(0)* in agreement with ER retention of the remaining unassembled L-AChR subunits (the doublet that was observed in the WT likely corresponds to partially glycosylated subunits generated during L-AChR maturation processes, in addition to fully resistant mature species). In *emc-6(kr150)*, the remaining UNC-29 subunits were also resistant to EndoH, similar to the WT, indicating that these subunits were properly matured in the absence of EMC-6. In contrast, UNC-29 was fully sensitive to EndoH in the double mutant *unc-63(0); emc-6(kr150)*, indicating that even in the absence of EMC-6, unassembled subunits are still retained in the ER.

Altogether, these results suggest that EMC-6 is not involved in the regulation of the ER exit of AChRs and point to a stabilizing role of EMC-6 during receptor subunit maturation and assembly within the ER.

***emc-6* Is a Component of the EMC, a Conserved Complex Required for L-AChR Maturation in *C. elegans*.** In yeast, the EMC-6 ortholog (EMC6p) forms a physical complex with five additional proteins (43). This complex localizes to the ER and was accordingly named the "ER membrane protein complex." Inactivation of this complex triggers ER stress and activation of the UPR pathway. It was also recently linked to the ERAD machinery in human cell lines (48). The EMC members EMC1 to EMC6 are conserved from yeast to humans (Table S1). We tentatively named the *C. elegans* orthologs *emc-1* through *emc-6* (corresponding ORFs are shown in Table S1).

The L-AChR biosynthesis defects in *emc-6(kr150)* might be caused by a global dysfunction of the EMC. Alternatively, the very selective *kr150* mutation might have unmasked a specific contribution of the EMC-6 protein for multimeric transmembrane receptor biosynthesis. To test these hypotheses, we inactivated the *emc-1* through *emc-6* genes by RNAi because no mutations exist

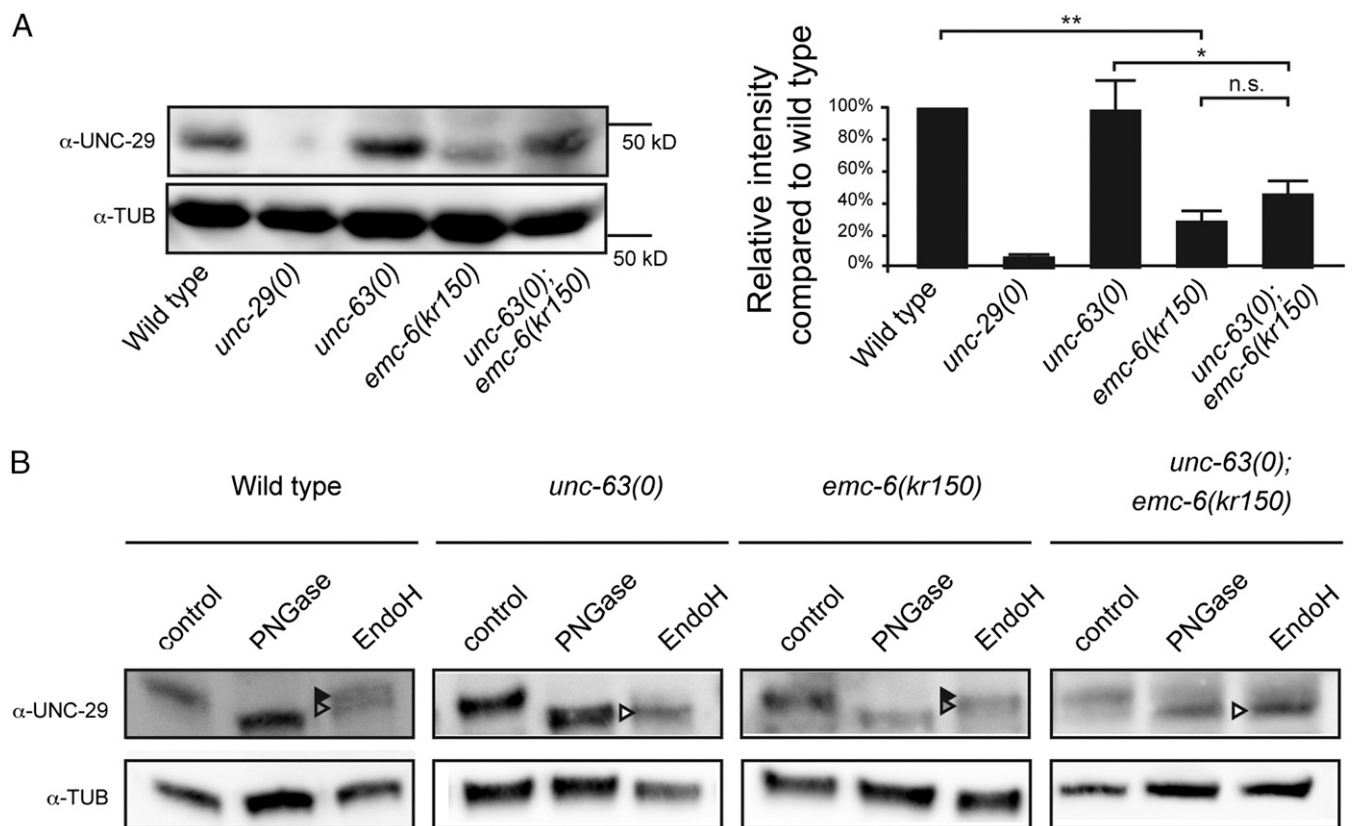


**Fig. 3.** EMC-6 is required for surface expression of ionotropic receptors. (A) Response to pressure ejection of levamisole in voltage-clamped ventral BWMs is reduced in *emc-6(kr150)* [mean  $\pm$  SEM (WT):  $367 \pm 37$  pA,  $n = 7$ ; *emc-6(kr150)*:  $87 \pm 8$  pA,  $n = 6$ ;  $P = 0.0012$ ]. Arrowheads mark application onset. (B) L-AChR-dependent evoked currents recorded from BWMs after ventral nerve cord stimulation are decreased in *emc-6(kr150)*. Electrically evoked responses were obtained in a *unc-49(e407); acr-16(ok789)* genetic background to eliminate GABA<sub>A</sub> receptor and N-AChR contributions (WT:  $553 \pm 50$  pA,  $n = 6$ ; *emc-6(kr150)*:  $210 \pm 31$  pA,  $n = 6$ ;  $P = 0.0022$ ). (C) L-AChR expression is decreased at NMJs of *emc-6(kr150)* mutants (*i*, *iii*, *iv*, and *vi*), whereas presynaptic differentiation is unaffected (*ii*, *iii*, *v*, and *vi*). L-AChRs are labeled using anti-UNC-38. Cholinergic boutons are labeled using an anti-vesicular acetylcholine transporter UNC-17 (VAcHT) antibody. (Scale bars, 10  $\mu$ m.) (D) Visualization of L-AChRs in living worms using a knock-in UNC-29::tagRFP L-AChR subunit engineered by *MosTIC* homologous recombination. DC, dorsal cord; NR, nerve ring. Arrowheads indicate L-AChR clusters at the dorsal nerve cord. Intestinal autofluorescence is indicated with an asterisk. (Scale bars, 10  $\mu$ m.) (E) UNC-29::tagRFP fluorescence is reduced in *emc-6(kr150)* mutants (WT:  $18.1 \pm 2.5$  arbitrary units,  $n = 14$ ; *emc-6(kr150)*:  $2.9 \pm 0.3$  arbitrary units,  $n = 13$ ;  $***P < 0.001$ , Mann-Whitney test). (F) EMC-6 is required for N-AChR function. Response to pressure ejection of nicotine (WT:  $931 \pm 78$  pA,  $n = 8$ ; *emc-6(kr150)*:  $633 \pm 94$  pA,  $n = 12$ ;  $P = 0.023$ ). Arrowheads mark application onset. (G) EMC-6 is required for GABA<sub>A</sub> receptor function. Response to pressure ejection of GABA (WT:  $1552 \pm 118$  pA,  $n = 6$ ; *emc-6(kr150)*:  $710 \pm 172$  pA,  $n = 5$ ;  $P = 0.0043$ ). Arrowheads mark application onset.

in any of the *C. elegans* EMC member orthologs except for *emc-6(kr150)*. Inactivation of EMC members led to various phenotypic defects, including embryonic lethality and developmental arrest (Table S2). Strikingly, inactivation of *emc-1*, *emc-2*, *emc-3*, *emc-4*, and *emc-6* caused significant resistance to levamisole compared with worms fed with an empty RNAi vector (Fig. 5A). The lack of effect of *emc-5* RNAi might reflect a specific function of EMC-5 in the EMC or heterogeneity of EMC composition according to its substrate.

These data provide indirect support for the existence of an EMC in *C. elegans* and suggest that the entire complex is required to ensure proper maturation of L-AChRs.

**C. elegans EMC Is Involved in ER Homeostasis.** The EMC was identified as a complex involved in ER homeostasis in yeast and in ERAD in human cells. To test if EMC inactivation leads to ER stress in *C. elegans*, we used the UPR reporter *Phsp-4::gfp*, whose expression is strongly induced in the gut and hypodermis by UPR activation (49). Inactivation of *emc-1*, *emc-2*, *emc-3*, *emc-4*, and *emc-6* by RNAi was associated with significant ER stress (Fig. 5B). Inactivation of *emc-5* seemed to have no significant effect on UPR activation. To test whether UPR activation was a side effect of decreased L-AChR function, we inactivated *unc-50* by RNAi and saw no ER stress induction despite strong resistance to levamisole (Fig. 5A and B).



**Fig. 4.** *emc-6* is required before or during L-AChR assembly. (A) L-AChR expression is reduced in *emc-6(kr150)* mutants. Levels of unassembled UNC-29 L-AChR subunits detected in *unc-63(kr13)* are further decreased in *unc-63(0); emc-6(kr150)* double mutants. UNC-29 levels are quantified by Western blot using anti-UNC-29 antibodies and normalized to tubulin levels. The migration profile of UNC-29 subunit appears to be slightly different in *emc-6(kr150)* compared with other genotypes, which might reflect slight changes of UNC-29 glycosylation. Seven independent experiments were quantified (mean  $\pm$  SEM). n.s. (not significant),  $P > 0.05$ ;  $*P < 0.05$ ;  $**P < 0.01$  after Holm correction, Mann-Whitney–Wilcoxon test. (B) Remaining L-AChRs exit the ER in *emc-6(kr150)*. Treatments with EndoH or N-Glycosidase F (PNGase) were performed on protein extracts of mixed-stage animals before SDS/PAGE analysis. Black arrowheads indicate glycosylated forms resistant to EndoH, gray arrowheads indicate partially glycosylated forms partially resistant to EndoH, and white arrowheads indicate unglycosylated forms sensitive to EndoH.

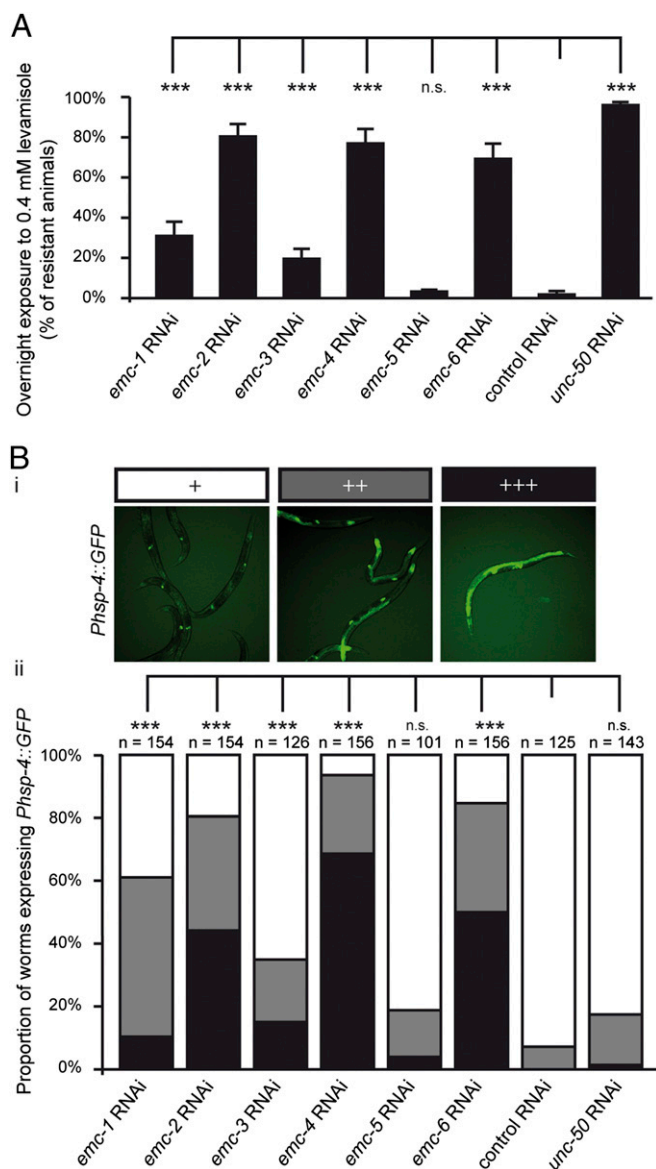
Because EMC inactivation caused ER stress, we asked whether the L-AChR biosynthesis defects of *kr150* mutants could be an indirect consequence of ER stress. We therefore tested whether down-regulation or overactivation of the UPR pathway would decrease L-AChR expression. Mutants of the two major components of the UPR pathway in *C. elegans*, *ire-1* and *xbp-1* (49), were fully sensitive to levamisole and had normal levels of L-AChRs at the NMJ based on immunostaining (Fig. S5A and B). Similarly, the *upr-1(zc6)* mutation, which leads to constitutive activation of the UPR pathway (49), did not cause resistance to levamisole. These results demonstrate that *C. elegans* EMC inactivation triggers ER stress and activation of the UPR independent of its role in L-AChR biosynthesis.

## Discussion

A genetic screen for mutants partially resistant to the cholinergic agonist levamisole identified *emc-6*, a previously uncharacterized gene coding for a small transmembrane protein of the ER. Our data show that EMC-6 is cell-autonomously required in muscle cells to ensure the expression of L-AChRs as well as two other multimeric ligand-gated ion channels. It likely functions within the recently identified EMC to stabilize unassembled L-AChR subunits and promote their assembly into mature receptors. More generally, the EMC seems to be essential for the viability of the organism and is required for maintaining ER homeostasis in vivo.

**EMC Is an Evolutionarily Conserved Protein Complex.** *emc-6* is the ortholog of the yeast gene *EMC6*, which was identified in 2009 by Jonikas et al. (43) over the course of a genome-wide identification of genes contributing to protein folding in the ER. Comprehensive analysis of the interactions between these genes identified a functional cluster of six poorly characterized genes subsequently named *EMC1* to *EMC6*. Immunoprecipitation experiments demonstrated that these six proteins form an apparently stoichiometric complex. They are all predicted to be ER resident transmembrane proteins, except for EMC2. This complex was further identified independently by Kopito and colleagues (48) in 2012 in a high-throughput approach combining proteomics and functional genomics to define ERAD networks in human cells. Physical interactors of 25 baits involved in ERAD were identified after protein complex purification and liquid chromatography-tandem MS. Specifically, reciprocal interactions were detected among 10 proteins, including orthologs of the six yeast EMC members. The four additional proteins are not present in yeast but seem to be conserved in *C. elegans* (Table S1). Remarkably, almost nothing is known about the function of these genes, either in yeast or in mammals, and none of these genes have previously been studied in *C. elegans*.

Here, we have characterized EMC-6 and demonstrated that it is an ER protein likely expressed in all tissues. The shared phenotypes observed after inactivation of other *emc* genes suggests that EMC-6 functions together with other EMC members in *C. elegans*. For example RNAi inactivation of *emc-1*, *emc-2*, *emc-4*, or



**Fig. 5.** EMC is required for L-AChR expression and ER homeostasis. (A) EMC inactivation by RNAi leads to resistance to levamisole. Bars represent the percentage of resistant animals after overnight exposure to 0.4 mM levamisole following preembryonic RNAi in *eri-1(mg366)*. Four to 13 independent experiments were performed (20–45 animals per condition per experiment) (mean  $\pm$  SEM). n.s. (not significant),  $P > 0.05$ ; \*\*\* $P < 0.001$  after Holm correction, Mann–Whitney–Wilcoxon test. (B) EMC inactivation triggers the UPR. (i) Representative pictures of young adults expressing GFP under the control of the *hsp-4* promoter reporting the extent of the UPR (intensity: +, white; ++, gray; +++ black). (ii) Bar graph represents UPR in worms exposed to control (empty vector), *emc*, or *unc-50* RNAi. RNAi was performed preembryonically by feeding the parent WT worms with bacteria expressing dsRNA. n, number of animals tested. Four to eight independent experiments were pooled. n.s. (not significant),  $P > 0.05$ ; \*\*\* $P < 0.001$  after Bonferroni correction, Fisher exact test.

*emc-6* causes embryonic lethality or developmental arrest, and inactivation of any *emc* gene except *emc-5* decreased the expression of L-AChRs (Fig. 5A). The phenotypic differences that we observed after inactivating individual *emc* genes might arise for several nonexclusive reasons. First, they might reflect variation of RNAi efficiency for individual *emc* genes and the tissues in which they are expressed (50) because our phenotypic readout is at the organismal level. Second, the EMC might contain core components whose inactivation destabilizes the entire complex and has

more drastic effects than when targeting peripheral components. Third, the composition of the complex might vary according to its substrates. Fourth, it is very likely that individual components contribute specific functions to the whole complex. Testing these different hypotheses will require the detailed characterization of each EMC subunit and the biochemical reconstitution of the complex.

**EMC Requirement for ER Homeostasis Can Be Genetically Dissociated from Specific Functions for Cys-Loop Receptor Biosynthesis.** We showed that disruption of the EMC in *C. elegans* causes induction of the UPR (Fig. 5B). Several lines of evidence suggest that UPR induction in *emc* mutants is a direct consequence of protein maturation defects in the ER. First, EMC inactivation in yeast causes a clear UPR induction related to an accumulation of unfolded protein in the ER (51). Interestingly, Jonikas et al. (43) noticed that in yeast, “the pattern of genetic interactions of strains deleted for EMC members most closely resembled that seen in a strain overexpressing the misfolded transmembrane protein Sec61-2p (a mutated form of the Sec61 translocon) (52) and is similar to the pattern of a strain overexpressing the misfolded transmembrane protein KWS (53).” Second, induction of ER stress and UPR by tunicamycin treatment causes the transcriptional induction of 9 of the 10 EMC members in human cell lines, a typical feature of genes involved in protein folding and degradation (reviewed in ref. 54). Third, *emc* inactivation in *C. elegans* causes developmental defects that are usually not related to constitutive UPR activation (49), indicating that these defects, as well as UPR activation, are likely to be downstream consequences of impairment of ER folding capacity in *emc* mutants.

Whether the EMC is similarly required for all transmembrane proteins or is more specifically involved in the biosynthesis of a subclass of proteins, such as ACh and GABA ionotropic receptors, is difficult to answer. However, we observed that the *emc-6* mutation isolated in our genetic screen does not impair the synthesis of all transmembrane proteins. First, we monitored the expression of the AMPA-like glutamate receptor subunit GLR-1, which assembles into a tetrameric ligand-gated ion channel (55), and we saw no decrease in the *emc-6(kr150)* background compared with the WT (Fig. S6A). Second, we analyzed vulval development of animals carrying the *emc-6(kr150)* mutation, which provides indirect access to the functionality of the EGF receptor LET-23, a cell surface glycoprotein with a single transmembrane domain. Decreased activity of *let-23* causes vulval induction defects (56). The lack of vulval defects in *emc-6(kr150)* mutants (Fig. S6B) suggests that the EGF receptor LET-23 is properly expressed. These data do not exclude the likely possibility that EMC-6 is required for the synthesis of other unidentified proteins, yet the *emc-6(kr150)* mutation unmasked some *in vivo* requirements of EMC-6 that are specific for the biosynthesis of GABA and ACh receptors and that all belong to the same superfamily of proteins called the Cys-loop receptors (reviewed in ref. 57).

**EMC Stabilizes Unassembled Subunits of L-AChR.** We have shown in this work that loss of EMC function decreases L-AChR expression, likely by impairing the stability of unassembled subunits or assembly intermediates in the ER (Fig. 4). Interestingly, our results functionally connect the EMC to ERAD because AChR subunits are established integral-membrane ERAD substrates (23). We propose that the EMC regulates the amount of subunits available to assemble into mature L-AChRs either by behaving as a chaperone for unassembled or partially assembled subunits or by modulating ERAD of AChR subunits.

The best-characterized factors that are known to influence AChR subunit stability in the ER are ER chaperones. In Cos cells and in myotubes, binding of AChR subunits with BIP was proposed to act as a protective pathway to prevent misfolded subunits from altering receptor assembly (58). This hypothesis has been indirectly con-

firmed in *C. elegans*, where the interaction between BIP and un-assembled L-AChR subunits is much stronger than with assembled L-AChRs (59). The calnexin and ERp57 ER-resident component of the biosynthetic machinery also form transient complexes with newly synthesized AChRs before full assembly occurs (17). The association of AChR subunits with ER proteins would decrease the rate of ERAD-dependent subunit degradation (17). By analogy, the EMC might provide a functional chaperone activity to stabilize or enhance the folding of partially assembled AChR subunits.

L-AChRs are the first proteins, to our knowledge, whose synthesis has been demonstrated to depend on EMC activity. The isolation of the *emc-6(kr150)* mutant may have been serendipitous. Alternatively, screening for L-AChR synthesis defects might be a very sensitive assay for EMC activity because of the very inefficient folding of these oligomeric transmembrane proteins. Specifically, AChR subunits contain an amphipathic transmembrane helix, M2, with a series of hydrophilic residues. In mature receptors, the M2 helices from each of the five subunits are organized around a pseudosymmetry axis and form a transmembrane cationic pore. It is not known how the hydrophilic face of the M2 helix of a partially assembled subunit is stabilized in the ER membrane before being buried in mature receptors. This topological issue is shared by all Cys-loop receptors, including the N-AChRs and UNC-49/GABA<sub>A</sub> receptor, which we have demonstrated also depend on the EMC for their synthesis in *C. elegans*. To gain further insight into the role of this complex in the biosynthesis of AChRs, it will be of great interest to identify other “substrates” of the EMC and test if the EMC indeed behaves as a chaperone for additional transmembrane proteins.

## Materials and Methods

**General Methods and Strains.** Unless indicated otherwise, strains were maintained at 20 °C on nematode growth medium (NGM) agar plates under standard conditions. OP50 *Escherichia coli* was generally used for feeding, except for strains exposed to RNAi, which were maintained on 1-mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) plates with HT115 for feeding. The WT reference strain was N2 Bristol. Strains, molecular constructs, transgenes, and primers are described in *SI Materials and Methods*.

**Levamisole Assay.** Assays for levamisole sensitivity after overnight exposure were performed as previously described (60). Further details are provided in *SI Materials and Methods*.

**Electrophysiology.** Electrophysiological methods were as previously described by Richmond and Jorgensen (30) and Gendrel et al. (60). L-AChR evoked recording analysis was performed in a *unc-49(e407); acr-16(ok789)* background to eliminate currents arising from activation of GABA<sub>A</sub> receptors and N-AChRs.

**Immunocytochemical Staining.** Worms were prepared by the freeze-crack method described previously (60). Further details are provided in *SI Materials and Methods*.

**Western Blotting.** Mixed-stage populations were collected from OP50-seeded NGM plates. They were rinsed three times with water and allowed to sedi-

ment on ice to wash out bacteria. Pellets were solubilized in Laemmli buffer with 2% (vol/vol)  $\beta$ -mercaptoethanol, boiled at 90 °C for 10 min, sonicated for 1 min, and centrifuged for 5 min at 15,700  $\times$  g. For glycosylation profiles, samples were treated with denaturation buffer for 10 min at 90 °C, incubated with endoglycosidase H (EndoH; New England Biolabs) or PNGaseF (New England Biolabs) for 1 h at 37 °C, and subsequently treated with Laemmli buffer as described above. Membranes were imaged with a LAS4000 (GE Healthcare Life Sciences) luminescence detector and band intensity-quantified with LAS4000 software. Further details are provided in *SI Materials and Methods*.

**Isolation of RNA and Quantitative Real-Time PCR Assay.** Synchronized first-stage larval (L1) cultures were obtained by treating young gravid adults with a bleaching solution for 5 min. Surviving eggs were allowed to reach the L1 stage during an overnight immersion in M9 solution. Total RNA was isolated from this synchronized population using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. All samples were treated with DNase (Fermentas). First-strand cDNA was synthesized from 200 ng of total RNA using an oligo(dT) primer and a Moloney murine leukemia virus reverse transcriptase (Fermentas) at 42 °C for 1 h. Quantitative PCR was performed using Light-Cycler 480 SYBR Green I Master (Roche). Primer sequences are listed in *SI Materials and Methods*. A relative quantification model, including kinetic PCR efficiency correction, was used to evaluate the relative expression ratio of the target genes (61). RNA levels were normalized to the three housekeeping genes *cdc-42*, *pmp-3*, and *Y45F10D.4* according to the method of Hoogewijs et al. (62). Further details are provided in *SI Materials and Methods*.

**RNAi.** RNAi was performed using the bacterial feeding protocol (63) with the following modifications. NGM agar plates containing 1 mM IPTG and 100  $\mu$ g/mL ampicillin were seeded with bacteria expressing dsRNA. L4-staged hermaphrodites were placed onto these plates and grown at 20 °C. After 4 d, the F1 progeny of these worms were scored for phenotypes of interest. If not available in the library of Kamath and Ahringer (63), PCR was used to amplify fragments flanked by the T7 promoter at the 5' and 3' ends. Further details are provided in *SI Materials and Methods*.

**Light Microscopy and Fluorescence Quantification.** For surface AChR quantification, animals were anesthetized with M9 buffer containing 100 mM sodium azide mounted on 2% (wt/vol) agarose in M9 pads and examined by epifluorescence using a Nikon macrozoom microscope equipped with a chilled CCD camera (stress induction) or a Leica DM5000B microscope with a spinning disk. Images were acquired using METAMORPH 7.1 software (Molecular Devices) and analyzed with IMAGEJ software (National Institutes of Health).

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