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Survival from Hypoxia by Inactivation of Aminoacyl-tRNA-Synthetases

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Abstract

The sensitivity of an organism and its cells to hypoxic injury varies widely; yet, few genetic determinants of metazoan hypoxic sensitivity have been identified. We report here the isolation of a profoundly hypoxia resistant mutant and its identification as a reduction-of-function allele of *rrt-1*, which encodes an arginyl-tRNA synthetase. *rrt-1* knockdown before or after the hypoxic injury rescues animals from death. RNAi knockdown of most other aminoacyl-tRNA synthetases also confers hypoxia resistance, the level of which inversely correlates with translation rate. *rrt-1*(*RNAi*) blocked hypoxic induction of the unfolded protein response and tunicamycin toxicity. Disruption of the unfolded protein response partially suppressed the hypoxia resistance of *rrt-1*(lf). The data support a model where translational suppression induces hypoxia resistance, in part by reducing unfolded protein toxicity.

Forward genetic screens offer the possibility of discovering genes not previously known to control hypoxic sensitivity. Such genes are likely to play an important role in emergent organismal traits such as habitat range and ability to hibernate. Additionally, these genes may lead to the development of novel therapies for conditions where cellular hypoxic sensitivity is a pathological determinant such as stroke, myocardial infarction, and cancer. We have previously shown that wild type *C. elegans* when placed in a severe hypoxic environment ($[O_2] < 0.3$ vol%) become immobile but fully recover when returned to normoxia within 4 hours (1). If the hypoxic incubation is prolonged past 4 hours, permanent behavioral deficits, cellular death, and eventually organismal death ensue. After a 24-hour hypoxic incubation, greater than 99% of wild type animals are dead. We used this easily scored organismal hypoxic death endpoint as the basis for a mutant screen to identify genes that control hypoxic sensitivity of the whole animal and its cells. Specifically, we screened for EMS-derived mutants that survived a 22 hour hypoxic incubation. In a screen of 3884 F1 mutant genomes, we recovered 14 mutants that had a significant hypoxia resistant phenotype (Table S1). These mutants fell into 13 complementation groups. *gc47*, one of the strongest hypoxia resistant mutants, was chosen for further characterization and mapping.

After outcrossing to the wild type strain N2, the hypoxia resistance of *gc47* was quantified and found to be substantial. Immediately after removal from a 20 hour hypoxic incubation both N2 and *gc47* were paralyzed, but *gc47* recovered the ability to move completely over the next 1 to 2 hours. After a 24 hour recovery, essentially all of the *gc47* animals were alive whereas almost all wild type failed to survive (Fig. 1); *gc47* prolonged the hypoxic incubation time required for complete killing by more than three fold (Fig. 1). The hypoxia

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resistant phenotype was fully recessive and segregated as a single locus in a Mendelian fashion (Fig. 1D and Materials and Methods). *gc47* was mapped based on its hypoxia resistant phenotype to the left arm of chromosome III (Fig. 2A). A combination of two factor and three factor mapping with visible mutations and single nucleotide polymorphisms placed the mutation in a 106 kb interval containing 32 known or predicted genes. RNAi of 29 of the 32 genes in the interval identified only one gene, *rrt-1*, whose knockdown resulted in high level hypoxia resistance (Fig. 2B). Simultaneously, five fosmids that together spanned the entire interval were individually injected along with a transformation marker to attempt transformation rescue of *gc47*; only one fosmid restored normal hypoxia sensitivity to *gc47* (Fig. 2C). The rescuing fosmid contained the *rrt-1* gene that was implicated by RNAi. Sequencing of the *rrt-1* gene in *gc47* found a single mutation, a G to A transition at nucleotide 811, resulting in a change of amino acid residue 271 from an aspartate in wild type to an asparagine in *gc47* (Fig. 2A). Based on mapping, transformation rescue, phenocopy with RNAi, and the identification of a missense mutation, we assign *gc47* as an allele of *rrt-1*. Given that the hypoxia-resistant phenotype of *gc47* is phenocopied by *rrt-1*(RNAi) and that the mutation is fully recessive, *gc47* behaves like a reduction-of-function allele of *rrt-1*.

rrt-1 encodes an arginyl-tRNA synthetase, one of the aminoacyl-tRNA synthetases (aaRS). aaRS catalyze the ATP-dependent acylation of their cognate tRNA(s) with a specific amino acid (2). aaRS fall into two distinct structural classes. RRT-1 is a class I enzyme, which is characterized by HIGH and MRSK domains (3, 4). aaRS can also be grouped according to whether they can form a multisubunit complex; in higher eukaryotes, the RRT-1 ortholog has been isolated in a cytoplasmic complex with six other aaRS and three accessory subunits (5, 6). Finally, some aaRS are specific for mitochondrial tRNAs. Besides their role in translation, a subset of the aaRS has been implicated in non-translation-related functions (7, 8). Thus, a critical question to answer is whether *rrt-1* is unique among aaRS in controlling hypoxic sensitivity or if additional aaRS also have this property and if so, whether these fall into a particular structural or functional class. To test the other aaRS, we used feeding RNAi constructs against 23 of the 33 predicted aaRS in the *C. elegans* genome. All but one of the 23 RNAi constructs conferred significant hypoxia resistance (Table S2). Thus, most, if not all, aaRS control hypoxic sensitivity. However, knockdown of some aaRS produced a strong hypoxia-resistant phenotype and some did not. This variable strength of phenotype was not explained by the class of aaRS or whether the tRNA substrate was cytoplasmic or mitochondrial. One trivial explanation for this range of phenotypes is that the RNAi constructs are variably effective at knocking-down the target gene. Indeed, quantitative real-time PCR to compare transcript abundance before and after RNAi knockdown found that some of the non-hypoxia resistant RNAis produced a small or even undetectable reduction in transcript abundance (Table S2). However, RNAi efficacy did not explain all of the phenotypic variance. For example, the levels of the methionine-tRNA synthetase transcript were knocked down seven-fold, yet the level of hypoxia resistance was modest and much less than that seen with histidine-, tyrosine, or isoleucine-tRNA synthetases with a smaller degree of knockdown observed. To examine whether the variable levels of hypoxia resistance can be explained by differences in the degree of translational suppression, we measured ^{35}S-methionine incorporation in animals treated with aaRS RNAis spanning the range of hypoxia resistant phenotypes as well as in *rrt-1(gc47)* (Figure S1). The level of hypoxia resistance had a strong inverse correlation with the relative translation rate. However, the absolute amount of translational suppression was relatively modest even in the strongest hypoxia resistant animals where the ^{35}S-methionine incorporation was about half that of vector controls. Thus, hypoxic sensitivity appears to be exquisitely sensitive to even small changes in translation rate. Consistent with translational suppression as the proximate mechanism of hypoxia resistance, treatment with cycloheximide also conferred hypoxia resistance (Fig. S2).

A reduction in translation rate might be expected to produce a variety of phenotypes besides hypoxia resistance. In particular, reduced translation has been shown to lengthen lifespan in *C. elegans* and other organisms (9-11). Additionally, we have previously reported that a known, very long-lived mutant was also highly hypoxia resistant (1). Thus, we hypothesized that *rrt-1*(*gc47*) would be long lived. Indeed, *rrt-1*(*gc47*) had a small but significantly increased lifespan (Figure S3). While this further links hypoxia resistance and long lifespan, the striking difference in the strength of the two phenotypes suggests that their mechanisms downstream of RRT-1 are distinct or that hypoxic sensitivity is much more responsive than lifespan to alterations in the translation machinery. Other phenotypes seen in *gc47* were less remarkable. It had a modest decrease in fecundity, a small but significant level of embryonic lethality, moved about half as fast as wild type animals, and developed to adults about 15% slower than wild type (Table S3). Thus, as expected for mutation of a gene with such an essential function, *rrt-1*(*gc47*) has a pleiotropic phenotype but the only strong phenotype was hypoxia resistance.

RRT-1 presumably functions in all cells to mediate translation. However because of their high metabolic activity, germ cells might be particularly vulnerable to hypoxic injury and thereby determine the hypoxic sensitivity of the whole organism. To determine if RRT-1 acts exclusively in the germline to control whole organismal hypoxia sensitivity or if it acts in somatic cells such as neurons and myocytes, we made use of a mutation in the *rrf-1* gene. *rrf-1* encodes an RNA-directed RNA polymerase required for the somatic but not germline actions of RNAi (12). Thus the hypoxia resistant phenotype of *rrt-1*(RNAi) in an *rrf-1* mutant background would be diminished if RRT-1 acts in somatic cells to control organismal hypoxic sensitivity whereas no effect of the *rrf-1* mutant would be seen if RRT-1 acted exclusively in germ cells. Indeed, the hypoxia resistance of *rrt-1*(RNAi) was greatly reduced in an *rrf-1* mutant versus wild type background (Figure S4). We next directly examined neurons and myocytes to determine if *rrt-1*(*gc47*) prevents hypoxic injury of these somatic cell types. We have previously observed that a sublethal hypoxic insult produces characteristic pathological changes including axonal beading and myocyte nuclear fragmentation (1, 13). Both axonal beading and myocyte nuclear fragmentation were abated in *rrt-1*(*gc47*) (Fig. S5).

When does RRT-1 function to regulate hypoxic sensitivity? In particular, we wanted to know whether *rrt-1* acts around the time of the hypoxic insult, either during or after hypoxia. Alternatively, given the fundamental role of RRT-1 in translation, reduction of *rrt-1* function might produce developmental defects that indirectly confer hypoxia resistance. In order to answer this question, we applied RNAi to wild type animals early in development, early in adulthood prior to the hypoxic insult, or in adulthood after the hypoxic insult (Fig. 3). RNAi either during early development or during early adulthood protected equally well from subsequent hypoxic death (Fig. 3B). Thus, the hypoxia resistant phenotype of *rrt-1* reduction-of-function is not dependent on developmental stage and can be induced after development is complete. Importantly, *rrt-1*(RNAi) after the hypoxic insult increased survival from delayed hypoxic death (Fig. 3C). This result has significant implications for the mechanism whereby RRT-1 controls hypoxic sensitivity. *rrt-1* reduction-of-function almost certainly reduces global translation rate, and as a consequence, should reduce oxygen consumption. Indeed, the rate of paralysis by hypoxia, which should correlate with oxygen consumption, is decreased in *rrt-1(gc47)* (Figure S6). Reduced oxygen consumption by translational arrest is a logical and established mechanism for reducing cellular injury during hypoxia but not after (14-16). Thus, the mechanism of protection by *rrt-1* knockdown, at least that functioning after the hypoxic insult, appears to be more complex than a global reduction in oxygen consumption by translational arrest.

Hypoxia has been shown to produce intracellular misfolded proteins and thereby induce the unfolded protein response (UPR)(17). One effect of UPR induction is phosphorylation of the translation initiation factor eIF2-α, thereby suppressing translation (18). Translational suppression by the UPR has been proposed as an adaptive mechanism to reduce the load of newly synthesized and unfolded proteins, particularly in the context of cancer cell biology. Thus, we considered the hypothesis that the hypoxia resistant phenotype of *rrt-1*(rf) may be, at least in part, due to a reduction in unfolded proteins. To examine this question, we utilized strains carrying a transgene consisting of a fusion between the hsp-4 promoter and GFP, P*hsp-4*::GFP. P*hsp-4*::GFP expression has been shown to be a reliable indicator of the level of unfolded proteins and of activation of the UPR(19-21). Hypoxia induced a significant increase in expression of P*hsp-4*::GFP that peaked four hours after recovery from hypoxia and was dependent on the length of hypoxic incubation (Fig. 4A,B,C). As would be expected, the glycosylation-inhbitor tunicamycin, which increases the level of unfolded proteins, also induced P*hsp-4*::GFP expression. Induction of P*hsp-4*::GFP expression by either hypoxia or tunicamycin was blocked virtually completely by a loss-of-function mutation in *ire-1*, which encodes an ER transluminal kinase essential for the UPR (21, 22) (Fig. 4A,B). Consistent with a reduction in unfolded protein load, *rrt-1*(*RNAi*) completely blocked hypoxic induction of P*hsp-4*::GFP (Fig. 4A,B); however, it did not diminish induction by tunicamycin. Thus importantly, the level of translational suppression by *rrt-1*(*RNAi*) does not preclude synthesis of the GFP marker under strong inducing conditions. Further supporting the hypothesis that *rrt-1*(rf) reduces the load of unfolded proteins, *rrt-1*(*gc47*) was highly resistant to tunicamycin-induced developmental arrest (Fig. 4D). Finally, we found that *ire-1*(lf) and *xbp-1*(lf), a downstream target of *ire-1*(21), significantly suppressed the hypoxia resistance produced by *rrt-1*(*RNA*i), but neither *ire-1*(lf) nor *xbp-1*(lf) induced hypersensitivity in *rrt-1*(+) animals (Fig. 4E). Rather, the *ire-1*(lf) and *xbp-1*(lf) animals were weakly resistant. These data indicate that inhibition of translation by *rrt-1*(lf) and the UPR interact synergistically to reduce hypoxic sensitivity, but that in the absence of translational suppression by *rrt-1*(lf), an intact UPR weakly promotes death after a severe hypoxic insult.

Translational repression is well-established as a mechanism of survival for hibernating animals in a prolonged hypoxic environment (23). Translational mechanisms are important in the tumorigenicity of cancer cells (24) and are increasingly implicated in the sensitivity of normal cell types to hypoxic and ischemic injury (14, 25). In these diverse scenarios, translational repression results in several secondary changes in the biology of the cell, including a decrease in both ATP consumption and protein aggregates, and an alteration of relative protein abundance. Our data show that a modest suppression of translation that still allows relatively normal growth and physiology can produce a profound hypoxia resistance that requires the UPR for its full phenotypic expression. An important point to reiterate in this regard is that one effect of UPR activation is translational suppression by a mechanism distinct from limiting aminoacylated-tRNA levels. A logical model derived from our data is that a reduction in translation rate by a decrement in aaRS activity reduces the unfolded protein load to a level that is manageable by the UPR and may synergize with the translational suppression produced by the UPR itself. However, without translational inhibition, the activity of the UPR is maladaptive in the context of hypoxic injury. Understanding fully this interaction between translational activity and the UPR is critical if these cellular processes are to be exploited to regulate hypoxic cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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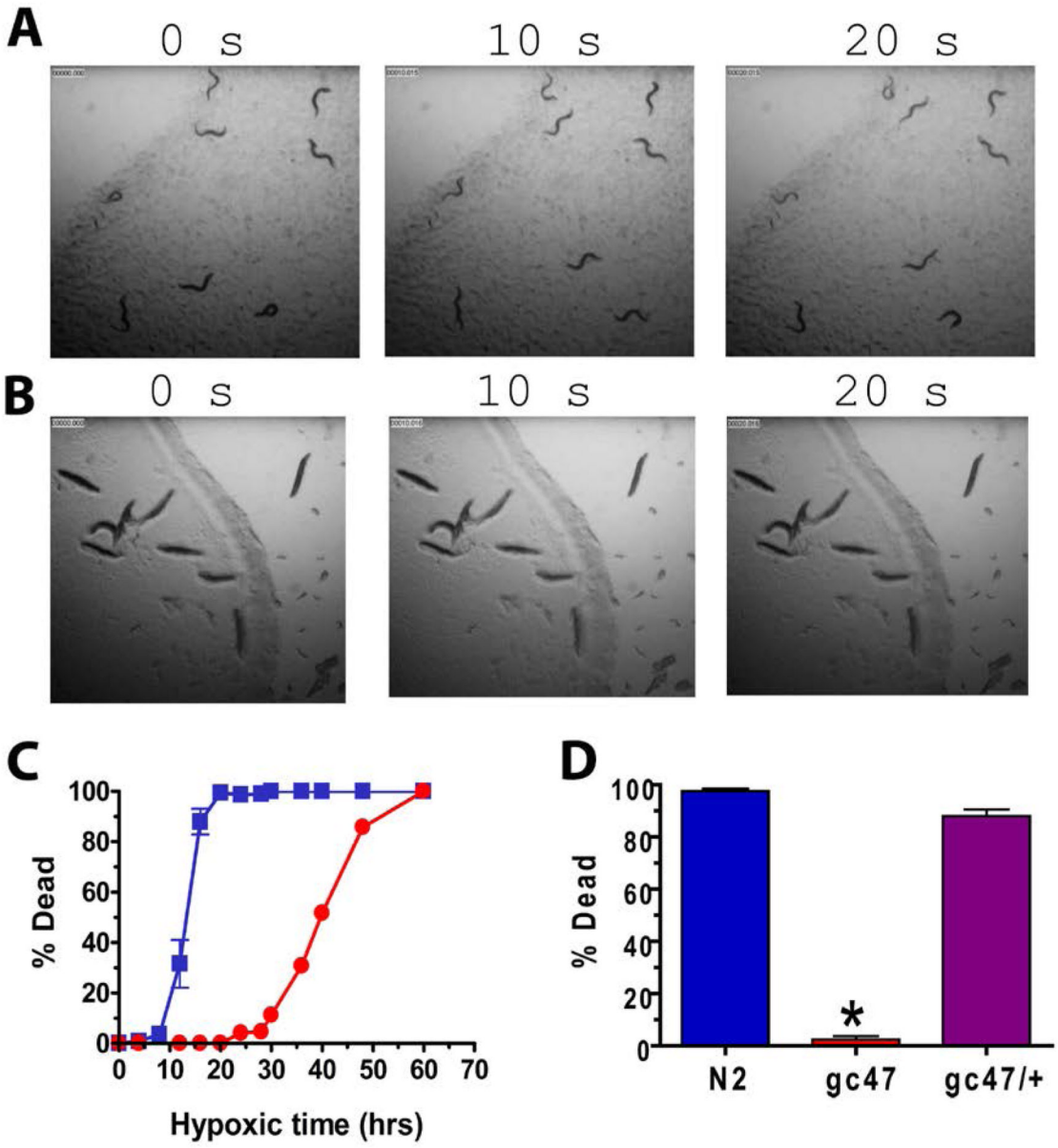

Fig. 1. *gc47* is a potent regulator of hypoxic cell death in *C. elegans*

(A,B) Time lapse images of **(A)** *gc47* and **(B)** N2 adult worms following a 24 hr recovery from a 20 hr hypoxic insult. **(C)** Percent dead animals for N2 (blue squares) and *gc47* (red circles) after a 24 hour recovery as a function of length of hypoxic insult. **(D)** *gc47* is recessive. Percent death of homozygous *gc47* (red; trials = 13), heterozygous *gc47*/+ (purple; trials = 9), and N2 (blue; trials = 16). mean ± s.e.m with at least 30 animals/trial.; * $p < 0.01$ (two-tailed t test).

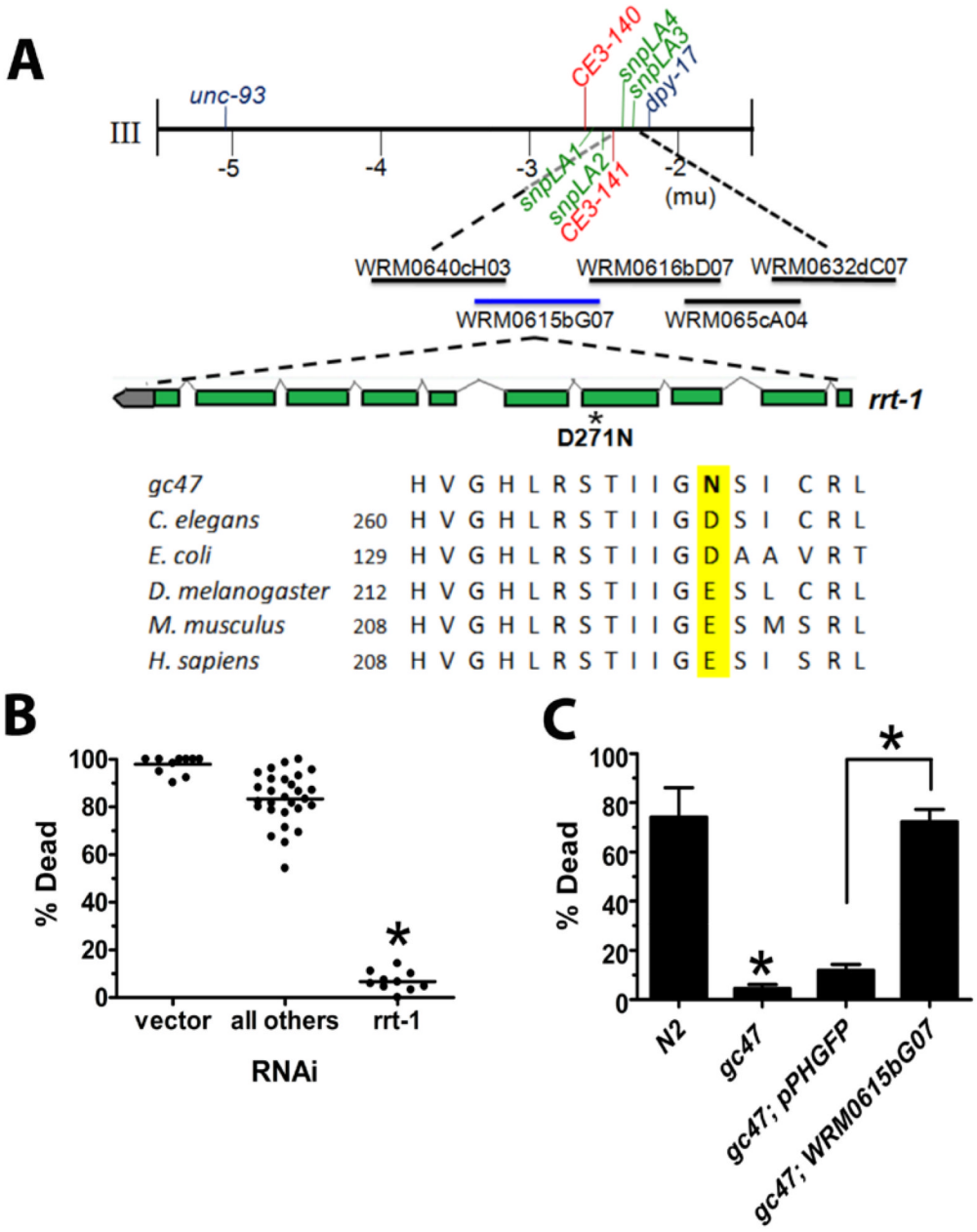

Fig. 2. *gc47* is an allele of *rrt-1*

(A) Genetic mapping of *gc47*. A portion of the genetic map of chromosome III is shown, with genes causing visible phenotypes in blue and relevant single nucleotide polymorphisms between Hawaiian CB4856 and N2 indicated in red (previously reported) or green (reported herein). Three-factor mapping with the visible markers and SNPs placed *gc47* in a 106 kb interval between CE3-141 and snpLA3. Fosmids used to attempt transformation rescue are shown below with the rescuing fosmid shown in blue. Alignment is with *rrt-1* orthologs. **(B)** RNAi of genes within the 106 kbp mapping interval. Hypoxia-induced death of animals treated with 29/32 predicted genes within the mapping interval are shown. The empty vector is shown as a non-resistant control. *rrt-1* RNAi confers highly significant hypoxia resistance ($P < 0.01$; two-tailed t test, n = 30 animals/data point). **(C)** Transformation rescue of *gc47*. Hypoxia-induced animal death was scored in N2 wild type, *gc47* (full genotype: *rrt-1(gc47)*

dpy-17(e164)), and *gc47* transformed with the transformation marker pPHGFP alone or in addition, the rescuing fosmid WRM0615bG07. mean ± s.e.m. of > 2 trials of > 20 animals/trial); * $p < 0.01$ (two-tailed t test).

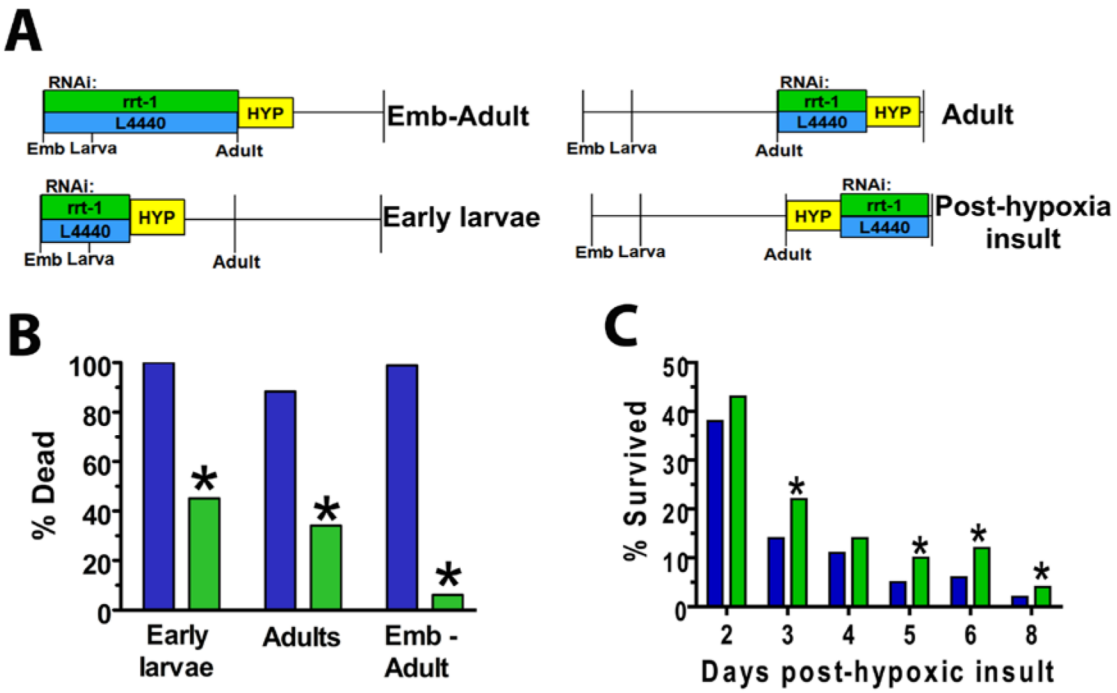

Fig. 3. *rrt-1* acutely controls hypoxic sensitivity, during and after the insult

(**A)** Schematic of the experimental protocol. Wild-type animals were exposed to *rrt-1* or L4440 empty vector RNAi at the times indicated and treated with a 20 hour **(B)** or 16 hour **(C)** hypoxic incubation (HYP, yellow boxes). "Emb" represents embryo. **(B)** Hypoxia resistance by RNAi knockdown of *rrt-1* is not developmental stage dependent. n = 40 animals/condition. *p < 0.05 versus L4440 (Fisher's exact test, two-sided). **(C)** Inhibition of *rrt-1* is effective after hypoxic insult. % Survived = 100(#animals alive at day of interest/# animals alive initially after 24 hr recovery). *p < 0.05 vs L4440 (Fisher's exact test, two-sided). n > 300 initially alive worms per RNAi over 3 independent trials.

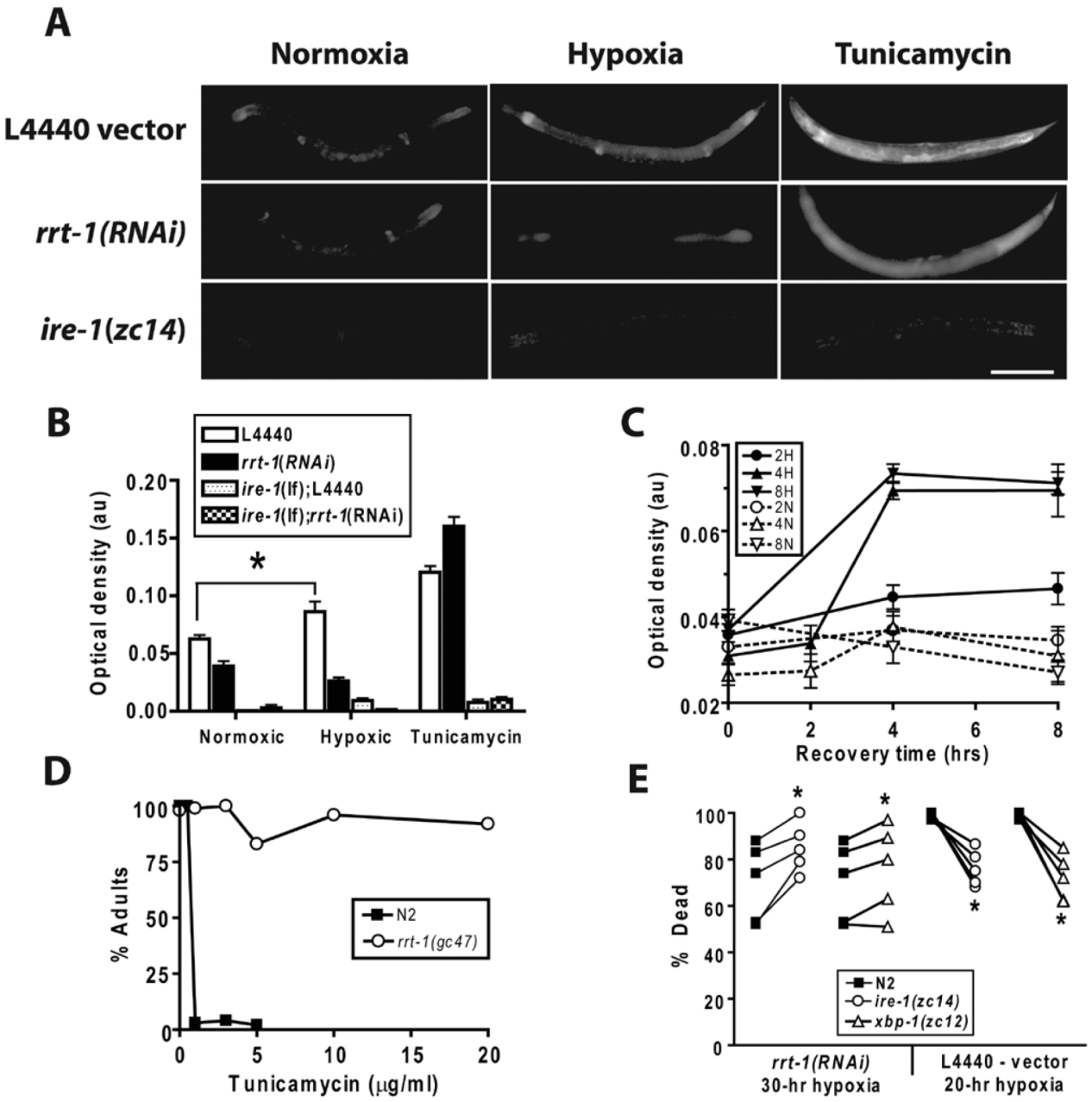

Fig. 4. The unfolded protein response is induced by hypoxia and is required for high level hypoxia resistance of *rrt-1*(*RNAi*)

(A) P*hsp-4*::GFP expression in age matched young adult *C. elegans* after incubation for six hours in M9 buffer in a normoxic or hypoxic environment or with 25 μg/ml tunicamycin. *zcIs4*[P*hsp-4*::GFP] animals were raised on empty vector or *rrt-1(RNAi)* bacteria; *ire-1(zc14);zcIs4* animals were raised on empty vector. scale bar = 200 μm **(B)** Quantification of P*hsp-4*::GFP expression under the various conditions and genetic backgrounds. * - $p < 0.01$, unpaired 2-sided t-test. **(C)** Time course of P*hsp-4::*GFP induction after hypoxic or normoxic incubations of 2, 4, or 8 hours. **(D)** Sensitivity to developmental arrest by tunicamycin in wild type and *rrt-1(gc47)* animals. Freshly laid eggs

were allowed to develop on agar plates containing the indicated concentrations of tunicamycin. The percent of animals reaching adulthood after seven days of development was scored. **(E)** Hypoxic sensitivity of wild type or UPR pathway mutant animals exposed to *rrt-1*(*RNAi*) (30 hour hypoxic incubation) or empty vector control (20 hour hypoxic incubation). * - $p < 0.05$, paired t-test.