Final Narrative Report

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"Modulation of Necrosis: *In vivo* Analysis of a Novel Gene Implicated in Injury-induced Neuronal Death"

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BODY OF REPORT

The report should cover the following information in 2 - 5 pages, in addition to photographs, figures, charts, etc. Use language suitable for lay readers.

1. Original aims of the project.
2. Project successes.
3. Project challenges.
4. Implications for future research and/or clinical treatment.
5. Plans to continue this research, including applications submitted to other sources for ongoing support.
6. List and include a copy of all publications emerging from this research, including those in preparation.

Background and Overview

Necrotic neuronal death initiated by ion channel hyper-activation plays a major role in both the initial and prolonged death of neurons consequent to SCI. Necrosis induced by injury occurs in two phases--the first due to the physical injury, and the second induced in neighboring cells by the necrosis of the neurons directly injured. Blocking or delaying such secondary necrotic cell death would significantly limit incapacitation. Regrettably, our understanding of molecular necrosis mechanisms is poor, and no truly effective therapeutics are available to block injury-induced necrosis.

A major goal of the Driscoll lab is to identify genes critical for the progression through ion channel-induced neuronal necrosis using a powerful experimental model--the nematode *Caenorhabditis elegans*. In this simple animal, we identified mutant ion channels (for example, the *mec-4(d)* channel) that are genetically hyper-activated and induce necrosis of the neurons in which they are expressed. Interestingly, hyper-activation of mammalian MEC-4 homolog ASIC1a makes a huge contribution to brain neuronal loss in mammalian ischemia.

Dissection of death mechanisms associated with ion channel hyper-activation in *C. elegans* has revealed that nematode and mammalian models of channel-mediated toxicity share a common mechanism requiring a rise in intracellular Ca^{2+} dependent on ER calcium release and activation of calpain and cathepsin proteases. Overall the lab has been exploiting the powerful genetics of *C. elegans* to identify physiological modulators of necrosis, which are logical targets for intervention.

1. Original Aims of the project:

My project rationale and goals. One of the key gaps in our current understanding of necrosis mechanisms involves an early step in the process—we do not understand how ion channel hyper-activation leads to catastrophic ER calcium release. I am interested in determining how this calcium overload is initiated and what gene activities might be knocked down or out to prevent death as calcium homeostasis is disrupted. Previously, we had found that RNAi knockdown of *C. elegans* calmyrin, a 311 amino acid protein with 3 copies of the EF hand calcium-binding domain that has mammalian homologs implicated in death regulation and expressed in the nervous system, was partially protective against *mec-4(d)*-induced necrosis. I hypothesized that calmyrin may act an executor factor in necrosis activated by the intracellular calcium rise. I proposed to gain evidence in support of this hypothesis using the powerful tools of *C. elegans* genetics to determine how *Ce-calmyrin* fits into the necrosis pathway and by initiating analysis of a possible parallel process in mammalian necrosis. My original aims were:

Aim 1. To generate and perform basic characterization of a true *Ce-calmyrin* knockout mutant.

Aim 2. To characterize the role of *Ce-calmyrin* in nematode cell death.

Aim 3. To test whether the human counterpart of *Ce-calmyrin* plays a conserved role in necrotic cell death.

I expanded the project to include a full genome survey of possible actions of EF-hand calcium-binding proteins on necrosis.

2. Project Successes

Aim 1. Molecular characterization of a calmyrin deletion allele.

I obtained and molecularly characterized a deletion allele of the *C. elegans* gene calmyrin, here referred to as *clm-1(tm1353)*. I out-crossed the *clm-1(tm1353)* mutant strain with wild type 5X in order to rid the genome of other unwanted mutations that potentially occurred during the deletion mutagenesis. I sequenced the calmyrin deletion allele *clm-1(tm1353)* and found the following (Fig. 1):

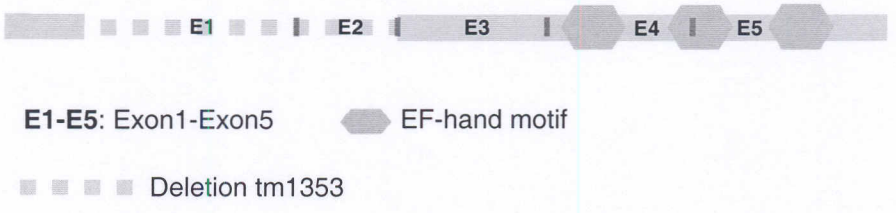
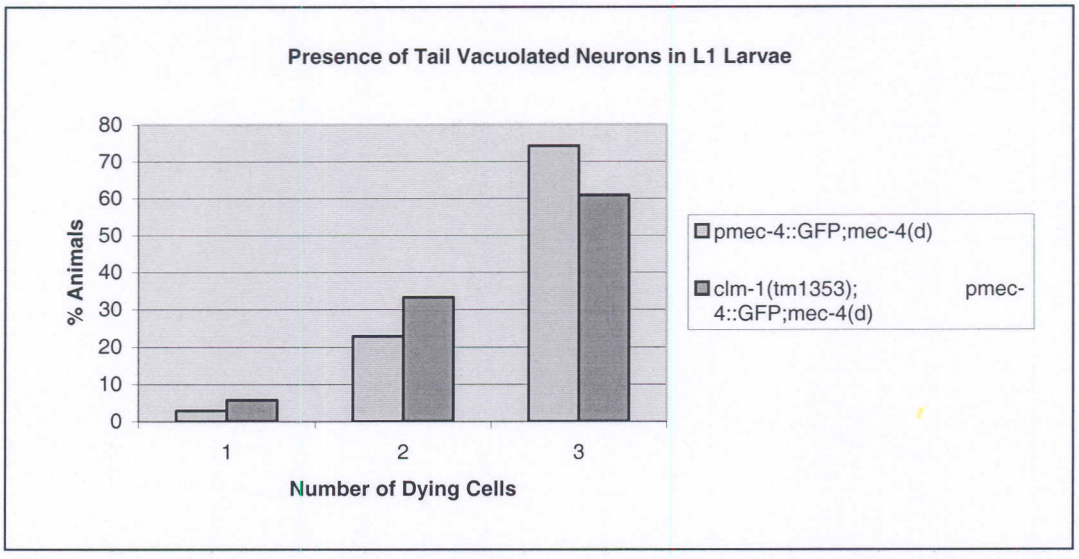


Figure 1: Diagram of *Ce-calmyrin* coding sequence showing the deletion tm1353 and the 3 EF-hand motifs.

Deletion *clm-1(tm1353)* eliminates most of Exon1 and all of Exon2 of the coding sequence, most likely producing a null mutation. *clm-1(tm1353)* mutants do not show gross morphological or behavioral defects indicating that the calmyrin gene is not essential for *C. elegans* development.

Aim 2. Characterization of the role of Ce-calmyrin in nematode cell death

***clm-1(tm1353)* confers partial neuroprotection against necrotic cell death.** I tested the effects of calmyrin deletion on necrotic cell death in our worm model of neuronal necrosis ($p_{mec\text{-}4}$GFP; *mec-4(d)*). *mec-4(d)* kills and thus obliterates the fluorescent signal of $p_{mec\text{-}4}$GFP-labeled neurons. I generated animals *clm-1(tm1353)*; $p_{mec\text{-}4}$*GFP; mec-4(d)* so I could measure *clm-1(tm1353)* protection to neuronal cell death triggered by *mec-4(d)* based on reduced number of dying *mec-4(d)* neurons (vacuolated neurons) (Fig. 2A) and increased number of fluorescent $p_{mec\text{-}4}$GFP neurons (Fig. 2B). I conclude that calmyrin provides mild neuroprotection. RNAi knockdown of *clm-1* in the *clm-1(tm1353)* background did not significantly enhance death suppression, indicating that general RNAi effects do not have a major impact on necrosis suppression and suggesting that *clm-1(tm1353)* is a null allele.



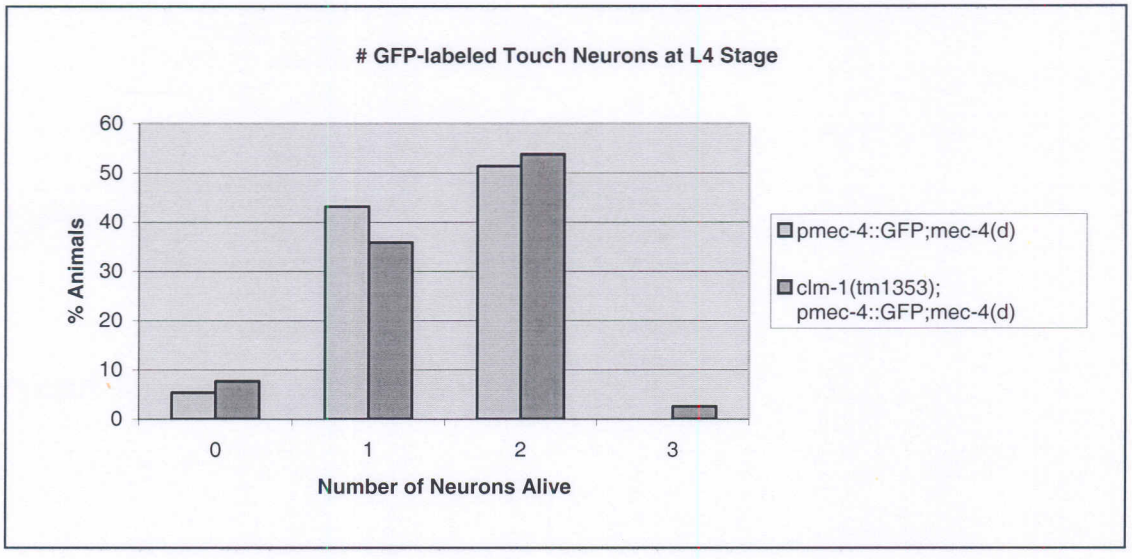


Figure 2: Neuron survival is increased in *clm-1(tm1353)*; p_{mec-4}*GFP*; *mec-4(d)* animals.
A: Lack of calmyrin slightly decreases the number of dying tail neurons visualized as swollen, vacuolated cells in L1 larvae using inverted Nomarski microscopy. There are 3 cell neurons in the mid-tail section of the animal; most of these cells succumb to *mec-4(d)*-induced necrosis in the p_{mec-4}*::GFP;mec-4(d)* control strain. *clm-1(tm1353);*p_{mec-4}*::GFP;mec-4(d)* show reduced incidence of *mec-4(d)* cell death as identified by a decreased percentage of animals with 3 dying cells and an increased percentage of worms displaying 1-2 dying cells.
B: Absence of calmyrin slightly increases neuronal survival as determined by scoring the number of GFP-expressing neurons. There is lower number of animals with 0 and 1 cell surviving and an increased number of animals with 2 or more neurons surviving. Note that 1-2 neuronal cells tend to survive *mec-4(d)*-induced necrosis in the *clm-1(+)* strain p_{mec-4}*::GFP*; *mec-4(d),* which is the negative control in this assay.

3. Experimental Challenges

I had identified a calcium-sensitive protein with impact on progression through necrosis, but the weak suppressor activity of this single gene disruption suggested that additional calcium regulated genes might contribute to the progression through necrosis. After some discussion we agreed that the most prudent experimental course to take could be to address more thoroughly the potential contributions of other EF hand proteins before we invested too heavily in *clm*-1 follow-up. We felt that this experimental course might reveal multiple processes and calcium sensors that work in concert; combination knockouts might be needed for their potential application in therapy. Alternatively, a more potent modulator might emerge.

Genome-wide RNAi screen for suppression of cell death by EF-hand proteins

The progression of injured neurons to death occurs through Ca^{2+} signaling. Our previous RNAi screen for necrosis suppressors included all genes on Chr I but missed genes in other chromosomes that could potentially be involved in cell death, such as Ca^{2+}-sensitive EF-hand genes. I therefore examined the annotated *C. elegans* genome sequence for EF-hand genes and identified 107 predicted open reading frames with this motif. 65 of these are in the RNAi library we have in the lab. I tested these for RNAi effects on necrosis and identified 5 genes that exert effects 2 or more fold above the empty vector negative control (L4440); about half as potent as the positive control calreticulin(RNAi) (Fig. 3). These genes--corresponding to open reading frames K10B3.10; F54C1.7; C47C12.4, B0511.1, C56C10.9--are strong candidates for carrying out calcium-dependent processes in necrosis. I have requested deletion alleles of these genes from large-scale efforts to knockout all *C. elegans* genes. In the meantime, I have identified deletions for several other genes on our original test list: M04F3.4 (a moderately strong candidate), F54G8.2, F10G8.5, Y116A8C.36, F25H2.8, and C44C1.3 (the last two are very weak and could help set the baseline).

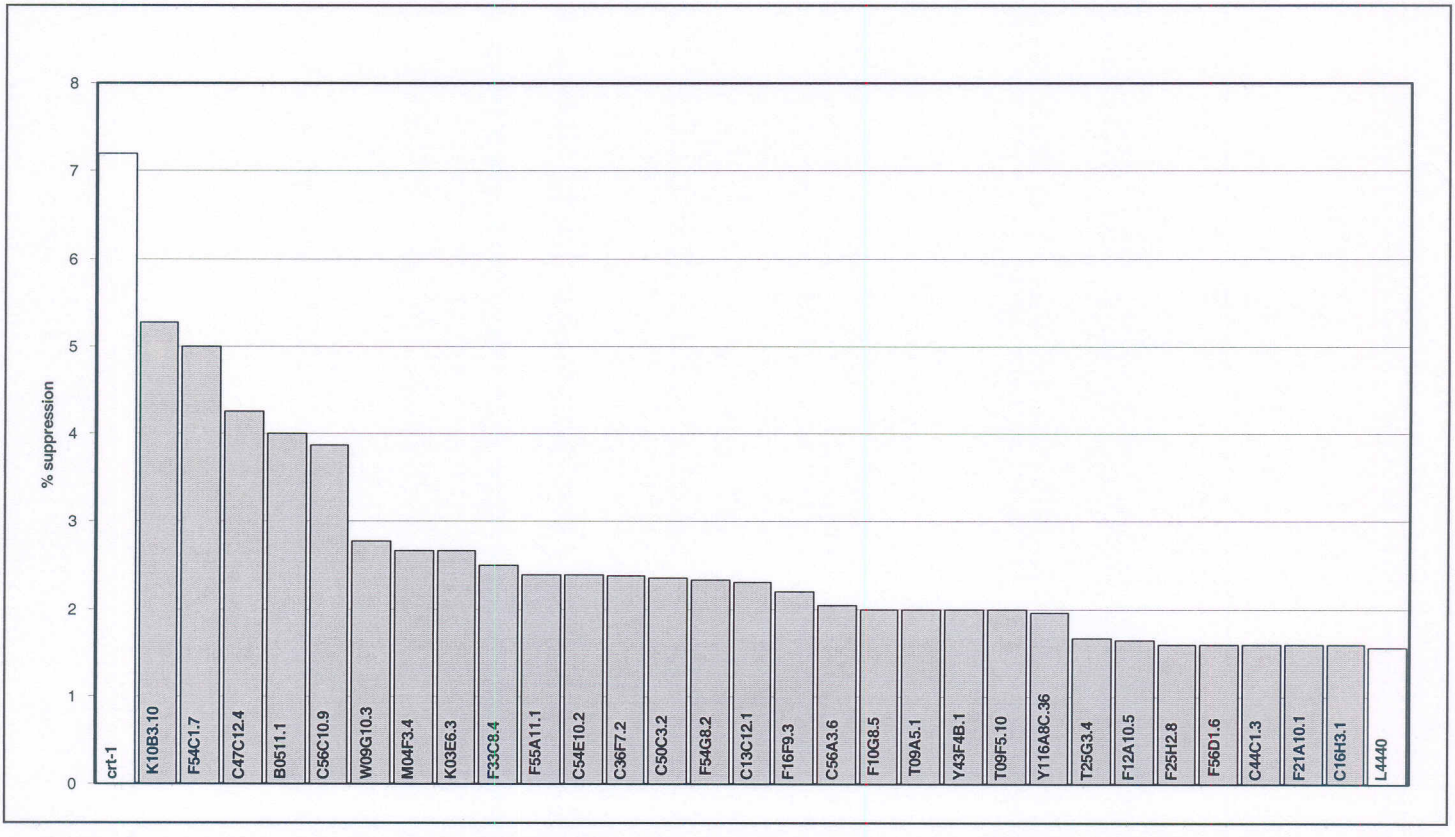


Figure 3: Suppression effects of EF-hand genes on mec-4(d)-induced cell death. Of the 67 EF-hand genes tested by RNAi, 29 (this graph) showed suppression of mec-4(d) necrosis to some extent. L4440 is an RNAi empty vector used as negative control in this analysis.

Summary. Overall, I report the isolation and analysis of a deletion in the EF-hand calcium-binding protein, calmyrin, testing for a possible role in necrosis. I report a weak effect on necrosis but my data suggest that other calcium sensitive proteins may act in parallel to promote toxicity. I report an initial test of this hypothesis that identifies 5 potential new suppressors among the EF hand family, and that supports more extensive study of these genes, which is ongoing. The support on this project has provided an excellent training period for me, and has built up a new research focus in the Driscoll lab.

4. Implications for future research and/or clinical treatment.
Necrotic cell death makes a devastating contribution to spinal cord injury, yet we know very little of the key genes and proteins required for necrosis. We do know that disruption of cellular calcium balance is a conserved feature of necrosis and that calcium elevation is essential for progression through necrosis. Calcium appears to be a death-promoting feature in two key cellular steps, first in amplifying the "damage" signal associated with hyper-activated calcium channels to provoke additional ER calcium release, and second, in activating cytoplasmic proteases. In *C. elegans* we have the capacity to identify candidate calcium sensors by virtue of calcium-binding motifs and then to manipulate genetically each one of these in a physiological context, to test their contributions to necrosis.

My work on EF-hand protein *Ce-calmyrin* indicates an effect on necrosis and at the same time suggests that additional calcium sensors may work in parallel during necrosis—defining more precisely how these proteins influence necrosis is significant in elaborating the little-understood death mechanism. Although this research is very basic in nature, we expect that the facility of experimental manipulation in *C. elegans*, coupled with demonstrated conservation of death mechanisms, will provide molecular information that will suggest specific therapeutic interventions.

5. Plans to continue this research, including applications submitted to other sources for ongoing support.
As we expanded our scope on EF hand proteins, much work is ongoing in the Driscoll lab on what will finally constitute the first genome-wide screen of all EF hand genes for contributions to necrosis. In particular, we will: 1) construct feeder RNAi clones to screen all 107 EF hand genes; 2) fast-test all 107 for impact on basic actions of hyperactive DEG/ENaC channel *unc-105(d)* and on stress reporter $p_{hsp\text{-}4}$GFP (these target reporters in non-neuronal tissues that are highly susceptible to RNAi and are fast, sensitive assays that can reveal candidates most worthy of focus); 3) test all EF-hand genes for roles in necrosis induced by multiple paradigms using RNAi strategies; 4) evaluate effects of true deletion mutations. At the moment the plan is to use this preliminary work in support of obtaining further NIH funding of the Driscoll lab.

6. List and include a copy of all publications emerging from this research, including those in preparation.

Ibanez-Ventoso, C., Gerstbrein, B. Xue, J. A genome-wide survey of calcium-binding EF-hand Protein Roles in neuronal necrosis. *Planned submission on continuing work.*