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Rapid generation of deletion alleles using CRISPR/Cas9

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To understand gene function it is frequently desirable to obtain a complete molecular null allele of a gene of interest. We previously used the CRISPR/Cas9 system to generate small insertions or deletions as a result of non-homologous end joining (Waaijers et al., 2013). However, alleles generated in this fashion do not necessarily result in complete loss of function. In-frame mutations may not affect protein function, and even early stop codons can be circumvented by alternative splicing or translation initiation at a downstream ATG. For this reason, we developed a method to efficiently generate deletion alleles using CRISPR/Cas9. In experiments targeting a locus with two sgRNAs located closely together, several of the alleles obtained corresponded to a deletion of the intervening sequences (Cho et al., 2013). We tested whether the simultaneous targeting of two sites could be used to generate large deletion alleles through non-homologous end-joining (Figure 1).

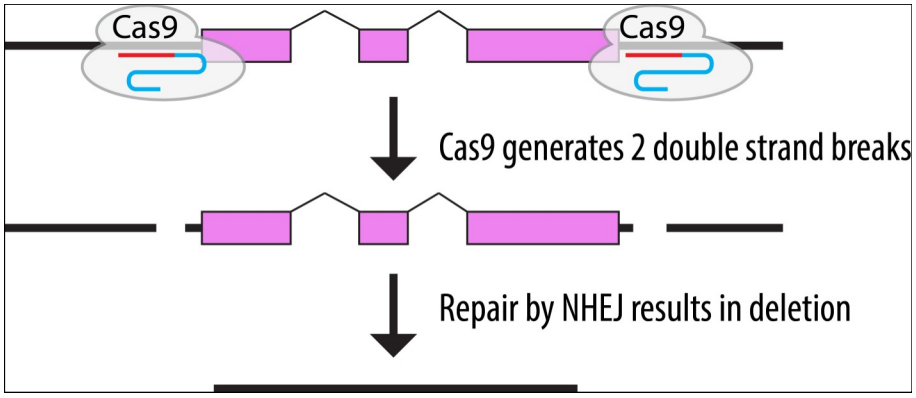

Figure 1: Generation of deletion alleles using CRISPR/Cas9. Simultaneous targeting of two sites using two distinct sgRNA sequences can result in the deletion of the intervening sequence after non-homologous end joining (NHEJ).

We selected three genes of different lengths (1 kb, 5.4 kb, and 11 kb from predicted start to stop). For each gene we designed two sgRNAs, one targeting a sequence before the start codon and the other targeting a sequence after the stop codon, and cloned these into the U6::sgRNA vector pMB70. Next, we injected 30 N2 animals with a mixture containing 5 ng/µl *Pmyo-3::mCherry* (pCFJ104), 50 ng/µl of each of the two sgRNAs and 50 ng/µl *Phsp-16.48::Cas9* (pMB67). To induce expression from the *hsp-16.48* promoter, injected animals were heat shocked for 1 h at 34°C on agar plates floating in a water bath, 30 min to 1 hr after injection. From transgenic F1 animals expressing mCherry, we PCR amplified a region surrounding the targeted region to detect the presence of a deletion.

For each of the 3 genes we successfully obtained deletion alleles with the following efficiencies: 3/84 F1 animals for the 1 kb deletion, 13/32 F1 animals for the 5.4 kb deletion, and 1/89 F1 animals for the 11 kb deletion. For 5 of the deletions, we determined the break points by sequencing. For all 5 deletions, the break points were within 11 bp of the predicted Cas9 cut site. One deletion exactly matched the predicted cut sites, and one deletion was accompanied by a small (11 bp) insertion. Thus, by targeting two sites simultaneously the CRISPR/Cas9 system can be used to generate accurately targeted deletions over a wide size range. This approach has two important benefits. First, as it makes use of non-homologous end joining the procedure may be more efficient than homologous recombination-based approaches. Second, the presence of deletions is easily detected by PCR in the F1 generation.

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A fast, marker-free method to mutate, tag, or delete any gene using linear DNAs with short homology arms

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Homology-directed repair (HDR) of double-strand breaks can be used to introduce precise edits in the genome, but is thought to be an inefficient process requiring long stretches of homology between donor and target sequences. We have found that HDR of cuts made by CRISPR/Cas9 is surprisingly efficient and requires only short homology arms, even for long, gene-sized edits (i.e., whole-gene deletions and GFP insertions).

Zhao et al. (2014) reported earlier this year that single-strand DNA oligos (ssODNs) can be used to introduce base-size edits near Cas9 sites without selection. Inspired by that report, we tested whether ssODNs could also be used to introduce small insertions, as shown for TALEN sites by Lo et al., (2013). We designed ssODNs to contain a restriction site (7 bases) or protein tags (18 to 66 bases: Tetra-Cys,V5, Myc, OLLAS, and FLAG) flanked by short homology arms (50-60 bases) and screened for edits in the first generation after injection (F1), using PCR and restriction digestion as in Zhao et al., (2014). We identified edits at frequencies ranging from 0.9 to 7% of F1s. Edit frequency was affected more by sgRNA efficiency than by edit size. We also found that ssODNs that bridge two sgRNA cut sites spaced far apart can be used to generate a precise, whole-gene deletion (1.7 kb, 3.8% of F1s). (Imprecise gene-size deletions can also be generated using two spaced sgRNAs and no repair template).

Emboldened by these results, we tried the same approach to insert GFP. We used PCR fragments made by amplifying GFP (864 bases) with primers containing ~60 bases flanking the Cas9/sgRNA site, inserting the GFP right in the sgRNA site or 27 bases away. We obtained 4% and 0.9% GFP-positive F1s overall, with 15% of injected mothers giving 20-48% edited F1s on the second day after injection ("jackpot broods"). The edits can be identified directly among the F1s by PCR or, most efficiently, by direct inspection for GFP expression. In this way, it is possible to go from injection to beautiful GFP pattern in just four days!

Based on these results, we developed a systematic protocol to mutate, delete, or tag any gene using ssODNs or PCR fragments. Using this protocol, the lab has already modified 13 genes. We are currently testing whether our approach could be streamlined even further using Co-CRISPR schemes to identify jackpot broods (Kim et al., 2014; Zhang and Glotzer, 2014; Arribere et al., 2014).

Update: Paper (including protocol) is now in press at GENETICS (http://www.genetics.org/content/early/2014/09/30/genetics.114.170423).

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From injection to mutant in four days: a hybrid PCR-phenotypic CRISPR-Cas screen

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In our generation of a mutant in the gene *T07C12.15* through CRISPR-Cas, we struggled at first with a PCR-only based approach. We then switched to using a phenotypic screen as described by Dan Dickinson and colleagues (Dickinson et al., 2013; website: http://wormcas9hr.weebly.com/). Through this screen, we were able to isolate mutants using either hygromycin- or the *unc-119*-based protocols. However, we made three interesting observations: 1) similar to other reports (Waaijers et al., 2013), we have noticed considerable embryonic and larval lethality in the F1, presumably owing to high level expression of the CRISPR-Cas9 endonuclease; 2) contrary to what has been suggested, we were able to relatively easily establish lines for the CRISPR-Cas9 transgenics; and 3) we noticed the few mutants that we have obtained (at a seemingly lower frequency than has been reported by others) might have arisen late in the growth/development of the injection plates.

For these reasons, we wondered if it was possible that the mutant might arise through dsDNA breakage and transgene-based gene conversion later in the growth of the worms, as can occur in either endogenous transposon (Barrett et al., 2004)- or Mos1-based (Frokjaer-Jensen et al., 2010) mutagenesis. We set up experiments comparable to those done using the Dickinson protocol, except starting each plate with three transgenic animals derived from established transgenic lines. The results of these experiments are shown in Figure 1.

Screen type	# Injected Po	# Plates	Avg. # injected Po/Plate	# GFP(+) Plates	Percentage GFP(+) of total plates	Mutant obtained	Transgenic line(s) obtained
Hygromycin-based mutant screen	61	27	2.259259259	18	66.66666667	XU172 (xp4)	XU165
unc-119–based mutant screen	66	38	1.736842105	18	47.36842105	XU174 (xp5)	XU166 XU167 XU168
Injection of *unc-119*–based construct to obtain transgenic lines	13	8	1.625	1	12.5	none	none
Injection of hygromycin–based construct to obtain transgenic lines	36	8	4.5	7	87.5	none	XU169 XU170
Injection of hygromycin-based construct + genomic DNA (high concentration) to obtain transgenic lines	52	21	2.476190476	11	52.38095238	none	XU171
Injection of hygromycin-based construct + genomic DNA (low concentration) to obtain transgenic lines	49	23	2.130434783	3	13.04347826	none	none
Hygromycin-based mutant screen (repeat experiment)	51	23	2.217391304	12	52.17391304	none	XU176
Transgenic line-based screen (XU165)	75	25	3	25	100	none	N/A
Transgenic line-based screen (XU166)	75	25	3	25	100	none	N/A
Transgenic line-based screen (XU167)	75	25	3	25	100	none	N/A
Transgenic line-based screen (XU169)	75	25	3	25	100	none	N/A
Hybrid Hygromycin/PCR-based mutant screen	76	28	2.714285714	19	67.85714286	XU177 (xp6)	N/D

Figure 1: Results of injections and mutant screens. N/A, not applicable; N/D, not determined.

Unfortunately, we were not able to identify mutants through these lines, though our numbers (both of the number of animals and the rate with which we are obtaining the mutants) are low enough that it is worth repeating these experiments to be certain. The results with our lines are not unexpected and presumably due to silencing of the arrays. We have also tried coinjection of genomic DNA to overcome this obstacle (Figure 1), but such lines have proved to be nearly inviable (again presumably due to germline expression of the Cas9 endonuclease), and therefore seemingly unusable for this purpose.

We reasoned: if a mutant is obtained through processes occurring in the injected parent, then it should be possible to detect this mutant in the F1 generation, as has been observed by others (Friedland et al., 2013; Katic and Grosshans, 2013). We then realized that by combining the efficacy of a phenotypic hygromycin- or *unc-119*-based screen with a PCR-based approach, the whole process could be simplified, streamlined, and considerably accelerated.

Our modified workflow is shown in Figure 2, and the initial results of such an approach in the last row of Figure 1. By combining the hygromycin-based phenotypic approach with a PCR screen, we find that we are able to obtain a mutant in only four days from injection, and that the subsequent heat shock step to remove the transgenics may be unnecessary. With a hygromycin-based screen, after the second day on hygromycin, only the transgenics and putative mutants are completely healthy in appearance-and these latter two categories can be distinguished by the presence or absence of GFP or other markers. Candidate mutant animals can then be directly singled and tested by PCR, rather than waiting the additional 5-7 days to allow them to populate the plates and the additional time of subsequent heat shock followed by putative mutant selection. This hybrid PCR-phenotypic approach thus combines the "best of both worlds" and cuts the total time of mutant isolation from about 7-10 days, down to about four.

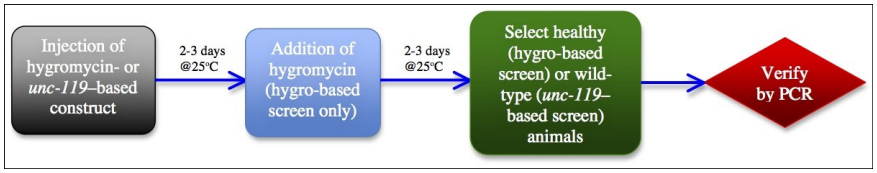

Figure 2: Modified workflow for isolation of mutants through hybrid PCR-phenotypic–based screen.

We thank Dan Dickinson for very helpful discussions over the course of these experiments.

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CRISPER'ed twitchers

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Inspired by work from the Calarco lab (Friedland et al., 2013) concerning CRISPR/CAS9, I have been investigating if *unc-22* could be utilized as a convenient marker for CRISPR/CAS9 system activity. A single sgRNA guide (GGAGAAGGAGGCGGTGCTGG) was designed to target the *unc-22* locus (termed *unc-22*-sgRNA-1000, since it was the 1000th sgRNA of those predicted to lay within the *unc-22* gene). Since Friedland and colleagues demonstrated simultaneous CRISPR activity of two independent sgRNAs on two different loci, I assumed that it would be useful to evaluate/discriminate between transformed animals by selecting only those that engage the proper CAS9 activity. Below, I describe three experiments that show that the above *unc-22*-sgRNA-1000 worked consistently and efficiently in my hands.

Experiment 1

In a preliminary attempt, I injected unpurified PCR product (synthesized by PCR stitching, resulting in pU6:*unc-22*-sg1000) diluted with an injection mix containing ~200ng/µl of *peft-3*::CAS9 encoding plasmid and fluorescent markers, into 12 *him-6(e1104)* animals (Hodgkin et al., 1979). 9 P0 plates segregated some number of marker positive F1's. Among 60 singled F1's, I recovered one plate with apparent F2 twitchers. Upon sequencing, I confirmed 2 lesions in the guide-specified region [a (-9bp) deletion resulting in weak twitcher phenotype and a (-42;+2 indel) resulting in a strong twitcher phenotype] transmitted to F3.

Experiment 2

In the second experiment, the above pU6:*unc-22*-sg1000 was injected as a plasmid (~25 ng/µl in the same injection mix as in Experiment 1) into 24 *him-6(e1104)* animals. Among the first 60 marker positive F1's, I isolated *F1 Twitcher*. This phenotypicaly affected F1 *unc-22(-)* animal enthusiastically segregated a strong twitching phenotype in the F2. Sequencing confirmed 2 lesions in the *unc-22*-sgRNA-1000 specified region [(-61bp) and (-105bp) deletions] transmitted to F3.

Experiment 3

In this attempt I injected the mix used in Experiment 2 into animals of a more complex genotype [*smg-1(cc546ts); dvIs27*] (Link et al., 2003). A temperature sensitive *smg-1(cc546ts)* mutation was developed by the Fire lab as a means to engineer conditional transgene expression (see WBG 14 (5) February, 1997, Getz et al., Fire lab vector kit 97, and source vectors www.addgene.org/static/cms/files/Vec97.pdf for detailed description). Surprisingly, among the first 60 marker positive F1's, three animals (of two independently injected P0) segregated spectacularly spastic F2 twitchers. Sequencing of the progeny of the above F2 twitchers confirmed at least 8 different lesions in the *unc-22*-sgRNA-1000 specified region [(-61bp), (-42bp), (-32bp), (-15bp), (-12bp), (-9bp) deletions and two indels (-51;+7bp) and (-26;+7bp)]. At least three other F1's (all siblings of the above strong F1) segregated weaker twitchers that contained two additional small frame restituting deletions [(-9bp) and (-15bp)].

Interestingly, amongst the singled F2 progeny, we recovered two sibling twitcher lines with a confirmed (-61bp) deletion growing considerably slower than the other lines. Both lines contained homozygous insertions of the extrachromosomal array, presumably containing *peft-3*::CAS9 and pU6:*unc-22* -sgRNA-1000 plasmids.

From the above experiments I conclude that both *him-6(e1104)* and *smg-1(cc546ts)*; *dvIs27* appear permissive for the *unc-22*--sgRNA-1000 dependent CAS9 effects on *unc-22*. Due to some *smg-1 (ts)* properties or the presence of *dvIs27* (possibly aggravating the twitches), *smg-1*(*cc546ts*); *dvIs27* might be somehow favorable in recovering heritable *unc-22* phenotypes (however both, above explanations are not mutually exclusive and both remain speculative). On the other hand, *him-6(e1104)* might perhaps sometimes lead to early identification of heritable *unc-22* phenotypes in the F1.

The most surprising observation was that some identified deletions (-61bp), (-15bp), and (-9bp) were confirmed in the F2's from independently injected parental animals. Strikingly, an identical (-61bp) deletion was identified in the experiments conducted in *him(-)* and *smg(-)* backgrounds. Of the above observation, I conclude that in the independent experiments the most prevalent lesion [i.e., the (-61bp) deletion] apparently reoccurs. This may imply that the initial CAS9 cleavage specified by *unc-22* -sgRNA-1000 operates under some genomic constraint (e.g., due to the local chromatin structure or other intrinsic properties of the specified region), and/or perhaps the repair mechanism is biased.

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CRISPR/Cas *C. elegans* web sites

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http://web.science.uu.nl/developmentalbiology/CRISPR.html
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http://www.genome-engineering.org/crispr/
CRISPR resources, Feng Zhang, MIT

http://crispr.mit.edu/
CRISPR design tool

https://www.addgene.org/CRISPR/worm/
CRISPR/Cas plasmids for use in *C. elegans*

https://groups.google.com/forum/#!forum/crispr
Genome engineering using CRISPR/Cas Systems discussion group

https://groups.google.com/forum/#!forum/worm-crispr
Worm CRISPR discussion group

C. elegans CRISPR/Cas papers

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Sharing is caring, helping is developing

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Help Us Help You

Have you ever had a burning technical question that no one in your lab could answer, or found yourself sobbing uncontrollably in the microscope room because your results were unexplainable and nobody was there to help you?

Have you ever wanted to discuss your project or talk through potential experiments and wanted feedback from people outside your immediate scientific circle?

Community is important. We're here for you. Unless you're Sydney Brenner, you had to learn the worm tools of the trade from someone. We're writing this note in the Gazette in the hope that it sparks interest in the worm community to use the unparalleled online resources that are freely available to us all.

All right, but apart from the sanitation, the medicine, education, wine, public order, irrigation, roads, the fresh-water system, and public health, what have the people at 'Wormbase' ever done for us?
Well they did give us the:

Worm Community Forum

The Worm Community Forum (forums.wormbase.org) is a great resource, filled with invaluable information and answers to common troubleshooting questions regarding worm work. Tweaked a protocol and want to share or tried a protocol and it hasn't worked? Want feedback on your scientific fails (firm but fair advice always given) and wins? Looking for a new position or want to advertise a position in your lab/institution? This is the place to do it!

OK, they didn't give us the next thing….but they would have:

Worm Internet Relay Chat (IRC) Channel

Want to interact with other researchers in real time, worldwide and not just someone sat in a call centre? Come visit the Worm IRC Channel (##worms) on the freenode IRC Network (freenode.net). If you don't use an IRC client, you can use a browser version to chat with us. It's a casual environment to talk freely about worms, or to brainstorm ideas on how to wow your journal reviewers, or maybe if you want to get some technical help and can't wait for someone to reply on the forum. So what are you waiting for, come on down! We also are fun people too, we promise.

A couple of new tools for the disorganized or pedantic researcher

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We previously published methods to allow worm researchers to
(i) identify potentially useful Mos1 insertions from the NEMAGENETAG collection (MosLocator)
(ii) convert and/or update lists of genes (**WormBase Converter**; see Griffon and Ewbank, WBG 18 #4)

We've now added 2 more tools specifically designed for worm labs, with the aim of making life easier:

- **ICeE**, a worm-centered electronic notebook that facilitates storage of data and metadata. It comes pre-loaded with the CGC list of strains and the RNAi clones from the main collections.
- **Clone Mapper**, which does three things:

1. **Map your clones**: Automatically determines whether a clone taken from the Ahringer or Vidal RNAi library is what it is supposed to be, by mapping your clone sequences to an in-silico RNAi clone library.
2. **Find Targets**: Predicts gene targets for RNAi clones, on the basis of matching n-mer sequences, overcoming some of the limitations of current Wormbase predictions.
3. **Retrieve Sequences**: Allows single or multiple clone and transcript sequences to be retrieved.

Many people contributed to the elaboration of these tools, and we benefited from invaluable input from numerous members of the WormBase staff.

All these tools are freely available via our website. Feedback is welcome.

If you use any of them, please do cite the relevant publications:
MosLocator: Vallin et al., 2012 PMID: 22347378
Wormbase Converter: Engelmann et al., 2011 PMID: 21602919
ICeE: Montanana et al., 2014 http://dx.doi.org/10.4161/worm.32160
Clone Mapper: Thakur et al., 2014 PMID: 25187039

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Batch_Fusion_Primer: a tool to generate the necessary primers for GFP fusion experiments from files containing any number of sequences

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Here, I present a simple script written in Perl for generating the necessary primers for use in GFP fusion experiments (Hobert, 2002; Reporter gene fusions) in *C. elegans* using multi sequence files. The program returns forward and reverse GFP fusion primers based on parameters determined by the user. These parameters include: length of search areas at the 5' and 3' ends of the input sequence; forward primer length, and reverse primer length (to be appended to the GFP specific oligo); and 3' end GC clamp. The main feature of this script is that it works with files containing any number of FASTA formatted DNA sequences. The script uses the BioPerl SeqIO module (Stajich et al., 2002) to parse FASTA formatted sequences, and can easily be adapted to return primers from a large number of formats, including GenBank, EMBL, ABI, FASTQ, and KEGG (Stajich et al., 2002). The program returns primers with GC% between 40 and 60, and primer Tm values between 52°C and 68°C. The program selects against highly repetitive sequences composed of multiple tandem identical base pairs or multiple di-nucleotide repeats. The program also examines self-complementarity within each primer and enumerates a 'selfie_score' by generating reverse complement substrings of each primer and checking for pattern matches. Two 'selfie_score' values are returned for the fusion primer: the first one examines self-complementarity to the designed reverse primer only, and the second score checks complementarity between the designed portion and also the GFP specific portion.

The script is organized such that parameters for each primer (forward or reverse) can be easily modified (and improved) by the user independently, and contains comments describing each step. The program executes from the command line and the output provides tab separated features that prints to the screen and also writes to an output file: tab1: start position of primer; tab2: primer Tm; tab3: primer sequence; tab4: selfie_score {two scores for the fusion primer}; tab5: percent GC content.

This simple script might be of use to researchers working on multi-gene families, alternatively spliced transcripts, or really any type of large gene data set where GFP expression is desired. If only forward primers are desired the user can enter 'zero' for 3' space to be sampled, and using such modifications to the code may provide further utility in traditional PCR experiments for large gene data sets. Hosted at SourceForge and freely available for download here - http://batchfusionprimerfetch.sourceforge.net/

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Carnivorous *elegans*

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Well, *C. elegans* is not really a meat eater, but if you read the recipe for Nematode Growth Medium (NGM) , you would be forgiven for thinking so. Bacto-peptone, contrary to what its name implies, is not a bacterial extract but is made from animal tissue. It is an enzymatic digest of beef and/or pork meat. Peptones were introduced into microbiology around 1880 by Naegeli and have become a standard ingredient for bacteriological culture media ever since.

It is not hard to imagine that bacto-peptone is not made out of prime steak or sirloin, but from meat that doesn't sell. Because of the possible danger of bovine spongiform encephalopathy (BSE) coming from these tissues, BD Biosciences (a major bacto-peptone producer) began tracking the origin of their meat sources and requiring all bovine meat to come from BSE-free countries. Based on catalogue and lot numbers, a certificate of analysis can be requested that states the animal source and the different tissues that went into the production of your specific lot of bacto-peptone. One batch my lab used a couple of years ago had the lot-number 8052930 and was made out of bovine and porcine tissue from animals farmed in the US. It contained tissue categories I, II and IV which include: bone, bone marrow, mucosa, pancreas, brain, retina, pituitary gland, pineal gland, optic nerve, peripheral nerves, albumin, adipose tissue, cartilage, milk, prostate, ovary and plasma.

Incidentally, many of the tissues out of which this particular lot was made from, and probably most batches are made from, are tissues known to be involved in endocrine signaling. Not surprisingly, significant amounts of estrogen and estradiol have been found in peptone (Feldman and Krishnan, 1995) as well as at least 9 different bile acids (Kamekura et al., 1988), a class of acids to which dafachronic acids belongs (Motola et al., 2006).

How does this affect day-to-day *C. elegans* experiments? Using solvents in combination with NGM may have unintended consequences. The most widely used solvents, besides water, are ethanol and dimethyl sulfoxide (DMSO). DMSO is used in drug discovery and pharmacology because of its ability to pass through biological membranes and to carry other molecules with it. It is to be expected that DMSO will carry some of the constituents of bacto-peptone into *C. elegans* and potentially have some unintended consequences. If you use solvents like DMSO in your experiments in combination with NGM you should be at least aware of this possibility. Ideally, use a culture method that does not contain bacto-peptone. Otherwise, you may make your worms "eat some meat", carnivorous indeed.

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The roles of talin splice isoforms in *C. elegans* gonad morphogenesis and muscle function

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Talin localizes to cell adhesion sites, where it binds integrins, actin and vinculin (Petit and Thiery, 2000). In *C. elegans*, *tln-1*/talin is necessary for muscle cell attachment to the basal lamina via dense bodies (Lecroisey et al., 2007) and for gonad morphogenesis, which is driven by distal tip cell (DTC) migration (Wong and Schwarzbauer, 2012). In addition to the full-length transcript (*tln-1a*), two shorter splice isoforms (*tln-1b*, *tln-1c*) of the *tln-1* transcript are predicted in *C. elegans* (Fig. 1A). Insights gained about specific *tln-1* splice isoforms have implications for the functional profile of talin and roles of its various domains, which enhances our understanding of the mechanisms behind cell attachment and migration.

We confirmed *tln-1b* and *tln-1c* transcripts in N2 *C. elegans* cDNA through RT-PCR using primers targeted to their predicted unique exon sequences, indicating that they are transcribed (Fig. 1A). Based on conserved domain searches and sequence alignments, we identified domains encoded by the predicted amino acid sequences of *tln-1b* and *tln-1c*. TLN-1b contains the integrin-binding head domain and at least one vinculin binding site in the rod, while TLN-1c contains multiple actin and vinculin binding sites in the rod (Fig. 1B).

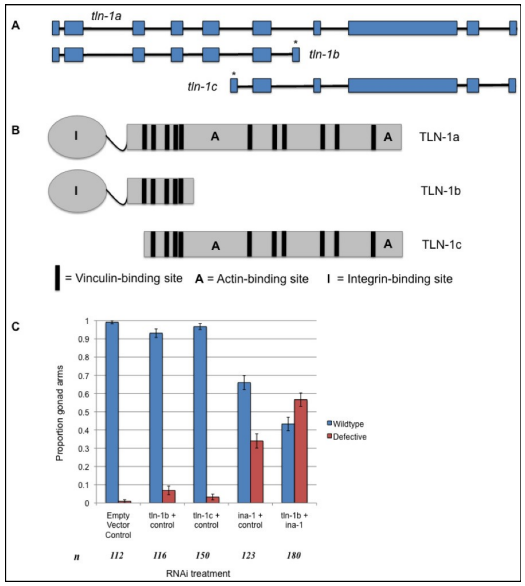

Figure 1: (A) Exons and introns of the talin isoforms, with the unique exons targeted for knockdown marked by (*). (B) Talin isoform domain schematic. (C) Gonad arm phenotypes observed upon RNAi knockdown.

Knockdown of *tln-1a* using an RNAi construct that also targets *tln-1c* causes paralysis and DTC migration defects (Cram et al., 2003). To determine the individual roles of *tln-1b* and *tln-1c*, we performed RNAi feeding experiments using novel RNAi constructs that specifically targeted either *tln-1b* or *tln-1c* (Fig. 1A). *tln-1b* RNAi caused progressive total body paralysis in *rrf-3(pk1426)* nematodes: 97% of late-L4/adult animals were paralyzed by 55 hours of RNAi treatment (n=178). We next examined the localization of PAT-2/a-integrin using the JE2222 strain that carries the PAT-2::GFP transgene (Meighan and Schwarzbauer, 2007). Following *tln-1b* RNAi and paralysis, 66% of nematodes displayed aberrant muscle cell shape and PAT-2 localization (n=44), while 86% of nematodes showed disrupted actin filamentation (n=49). This suggests that *tln-1b* is important for muscle cell shape, PAT-2 localization, and cytoskeletal attachments. RNAi against *tln-1c* did not cause paralysis or muscle morphology defects.

Although loss of either *tln-1b* or *tln-1c* did not affect gonad morphology, double RNAi knockdown of *tln-1b* and *ina-1*/a-integrin produced a significantly greater proportion of defective gonad arms ($p<0.05$), including defects in migratory direction and early cessation, when compared to *ina-1* reduction (Fig. 1C). The TLN-1b protein may interact with the INA-1 receptor during DTC migration. No synergistic effects were observed upon *tln-1c* + *ina-1* double RNAi knockdown.

Our analyses indicate that *tln-1b* is important for muscle function and DTC migration, while specific functions of *tln-1c* remain to be elucidated. These findings suggest that multiple isoforms perform the biological roles of *C. elegans* talin and that the talin head domain is especially important for these processes. We propose that the structural simplicity of TLN-1b and its potential for strong integrin binding may be necessary for integrin clustering during adhesion formation, displacement of TLN-1a during adhesion disassembly, or integrin activation during dense body protein accretion, thus making it relevant for cell migration and muscle attachment.

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Sensitization of *C. elegans* nAChRs by noxious heat

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The ability to sense and react to noxious high environmental temperature is critical for animals' survival. *C. elegans* uses distinct escape and avoidance regimes to minimize exposure to noxious heat. It has been shown that *C. elegans* heat avoidance requires several polymodal nociceptors, such as the FLP neurons, ASH neurons and others, but many of the key molecules involved in *C. elegans* thermosensation must be identified (Liu et al., 2012). Moreover the neural circuit mechanisms by which thermosensory behavior is generated remain incompletely understood especially with respect to heat avoidance (Liu et al., 2012). Therefore we proposed that *C. elegans* behavior response to noxious heat includes adaptive changes in the activity of cholinergic synapses in neural circuit regulating worm's movement (Jospin et al., 2009).

To test this hypothesis we used pharmacological analysis of noxious heat effect on steady state of cholinergic synapses of *C. elegans* in conditions of moderate rise in temperature tolerated by worms. Pharmacological analysis of steady state of *C. elegans* cholinergic synapses in vivo usually consists in measuring of locomotion sensitivity to ACh-esterase inhibitor aldicarb and nAChRs' agonists levamisole and nicotine. In both cases enormous rise in ACh content caused by aldicarb or overactivation of nAChRs by their agonists induced locomotion disturbances. In numerous genetic investigations this analysis was used to identify molecular mechanisms of neuromuscular synapses' function. In all these investigations aldicarb or levamisole sensitivity was measured by registration of worms' full inability to move on agar plate after slight touch by needle ("paralysis"). In contrast, in our experiments performed in liquid medium (NG buffer) we registered swimming disturbances, such as failure of muscle contraction in undulatory pattern or inability to forward swimming during 10 seconds, induced by aldicarb, levamisole and nicotine in concentrations which are subthreshold for nematode paralysis. Aldicarb concentration effective to induce behavior disturbances in 50% of worms after 20-minutes exposure at 23° C was 32 µM, while such for 20-minutes exposure at 33°C was 15 µM.

C. elegans exposition to temperature 33°C didn't cause disturbances in worms' swimming induced by mechanical stimulus, but induced rapid (less than 30 minutes) rise in this behavior sensitivity to partial inhibition of ACh-esterase by aldicarb. These data indicate that noxious heat caused a rise of steady state cholinergic synaptic transmission in cholinergic synapses, involved in regulation of *C. elegans* swimming.

Temperature rise up to 33°C strongly elevated swimming sensitivity not only to aldicarb, but also to nAChRs' agonists levamisole and nicotine. Concentrations of levamisole and nicotine effective to induce behavior disturbances in 50% of worms after 20-minutes exposure were respectively 62 µM and 710 µM at 23°C and respectively 15 µM and 320 µM at 33°C. Therefore it is evident that moderate heat stress activates steady state of synaptic transmission by sensitization of nAChRs, but not by stimulation of ACh release from neurons. Sensitization of nAChRs by noxious heat can be involved in rise of running away speed caused by heat stress.

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Extreme high temperature inhibits ACh release from *C. elegans* neurons

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In our previous work we have shown that partial inhibition of ACh-esterase by neostigmine protected *C. elegans* behavior against disturbances caused by extreme high temperature 36°C (Kalinnikova et al., 2013). These data show that extreme high temperature can inhibit ACh release from *C. elegans* neurons. For further analysis of this possibility we investigated the effect of extreme high temperature on toxicity of other ACh-esterase inhibitor aldicarb for organisms of two closely-related species of free-living soil nematodes *C. elegans* and *C. briggsae*. It is known that numerous mutations leading to decrease in ACh secretion cause *C. elegans* resistance to aldicarb. Therefore hyperthermia must have similar effect on aldicarb resistance if extreme high temperature inhibits ACh release.

Our experiments were carried out in liquid medium (NG buffer). In these experiments either temperature 36°C or aldicarb caused *C. elegans* paralysis (worms' inability to swim, induced by mechanical stimulus), depending on exposure time to high temperature or toxicant. The temperature rise from 23 to 33°C didn't cause worms' paralysis but accelerated time course for paralysis induced by aldicarb. In contrast, aldicarb concentrations which are toxic at 33°C (50–200 μM) were nontoxic at 36°C. Moreover, these aldicarb concentrations protected *C. elegans* against paralysis induced by hyperthermia. These data can be explained by inhibition of ACh release by hyperthermia, because partial inhibition of ACh-esterase can compensate ACh deficiency. The additional evidence for this explanation was obtained from experiments with worms preadapted for 2 hours to moderate high temperature 30°C. Such adaptation in accordance with our previous data (Kalinnikova et al., 2013) caused the rise in *C. elegans* resistance to temperature 36°C but sensitized worms to toxic aldicarb effect at 23°C. Under these conditions extreme high temperature 36°C strongly protected *C. elegans* against toxic aldicarb effect while aldicarb protected *C. elegans* against extreme high temperature.

In order to reveal the possible role of inhibition of ACh release from *C. elegans* neurons in the organism's resistance to hyperthermia we have compared the dependence of behavior sensitivity to aldicarb from rise of temperature in two closely-related species – *C. elegans* and *C. briggsae*. Since behavior thermotolerance of *C. briggsae* is significantly higher than such of *C. elegans* (Kalinnikova et al., 2011) one might propose that this difference in thermotolerance can correlate with thermostability of cholinergic synapses of these species. The dependence of aldicarb toxicity for *C. briggsae* in temperature range 23–33°C is similar with such for *C. elegans*. However at 36°C there was great difference in aldicarb action on organisms of *C. elegans* and *C. briggsae*. At this temperature aldicarb caused paralysis of *C. briggsae*, but protected *C. elegans* behavior against negative action of hyperthermia. It is known that *C. briggsae* resistance to temperature 36°C is strongly higher than such of *C. elegans* (Kalinnikova et al., 2011). These data indicate that exposure to temperature 36°C caused the inhibition of ACh release in *C. elegans*, but not in *C. briggsae* organism. Therefore it is evident that in *C. briggsae* organism the threshold both for thermal paralysis and inhibition of ACh release is higher than in *C. elegans*.

Therefore it is evident that cholinergic system is one of targets for high temperature effect on nematodes' nervous system.

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Identification of *klf-3/F54H5.3* as a novel operon and genetic circuit controlling fat deposition and FA beta oxidation in *C. elegans*

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Fatty acid biosynthesis and oxidative degradation play a key role in lipid metabolism and their homeostatic balance controls energy storage. Its deregulation and dysfunction is linked to a wide range of disease risk factors or clinical manifestations from excessive deposit (obesity) to extreme lipodystrophy (cachexia). Cancer-cachexia is a complex metabolic condition in which disease-associated metabolic changes lead to a severe loss of skeletal muscle. Alterations in mitochondrial content, morphology, and function lead to several muscle-wasting conditions. Mitochondrial dysfunction has been reported in mouse models of cancer cachexia (Constantinou et al., 2011; Fermoselle et al., 2013). We have shown that one of the three Krüppel-like factors in *C. elegans*, KLF-3, a highly conserved homologue, possessed an important function in lipid metabolism probably via its transcriptional regulation (Zhang et al., 2009; Zhang et al., 2011; Zhang et al., 2013). *klf-3* regulates beta-oxidation of long-chain fatty acids (Zhang et al., 2011) and impacts on the biosynthesis and mobilization of triglycerides (Zhang et al., 2013). We investigated if *klf-3* influenced mitochondrial activity to modulate FA beta oxidation. We analyzed mitochondrial proliferation in synchronized populations of both wild type and *klf-3* mutants using a modified Mito Tracker staining protocol (Sherwood et al., 2005). We found that mitochondrial content in the wild-type worm was notably high (Figure 1A), whereas *klf-3* elimination reduced the mitochondrial content in the mutant worm (Figure 1B). Our data suggests that KLF-3-associated lipid accumulation in the mutant affects mitochondrial contents, leading to its dysfunction and reduced catabolism.

Recently we explored whether there is connection between fat metabolism dysfunction, reduced mitochondrial content, and muscle-wasting conditions. We characterized a joined deletion *(rh160)* of *klf-3* and its downstream *F54H5.3* gene in *C. elegans* strain NJ701. Through DNA sequencing, we determined the exact breakpoints of this deletion, which spans several kb of genomic sequence. RNA interference of *F54H5.3* in wild-type N2 worms revealed a partial embryonic lethality and defective reproduction. However, *F54H5.3* RNAi on the background of *klf-3(ok1975)*, a smaller deletion that affects only *klf-3*, alleviated the fatty phenotype of the *ok1975* mutant but recapitulated the severe muscle-wasting phenotypes associated with *klf-3(rh160)*. To-

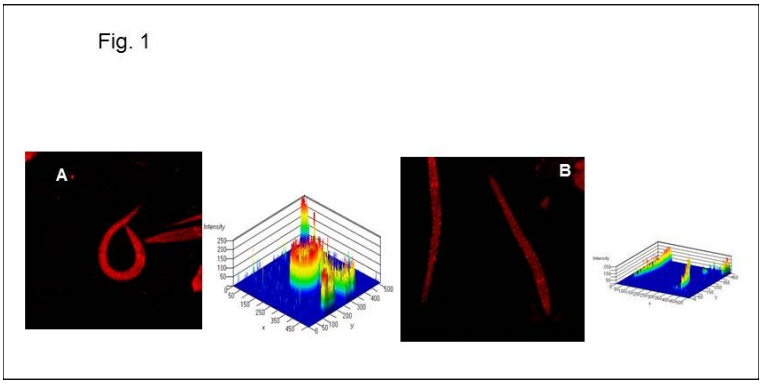

Figure 1: *klf-3* deficiency reduces mitochondrial proliferation. The changes in mitochondrial proliferation in both wild-type and *klf-3* mutants were observed after staining with MitoTracker Red in M9 buffer for two hours in dark. The worms were washed twice with M9 buffer and then transferred to NGM plate and allowed to recover for 30 min. Then worms were transferred onto 2% agarose pad, observed and imaged under LSM 510 Zeiss confocal microscope. (A) wild-type worm showing large number of mitochondria, and (B) *klf-3* worm with reduced mitochondria. Three independent experiments were performed and results were consistent between experiments.

gether, our data suggest that *klf-3* and *F54H5.3* interact in the same epistatic genetic circuit and potentially form an operon-like structure to coordinate their joint regulation in lipid metabolism. These observations bear novel implications for both obesity and type 2 diabetes and cachexia in cancer patients. Although our data is preliminary it does suggest a link between mitochondrial dysfunction, fat metabolism and cancer-cachexia. Our study may provide a simpler animal model to deepen our understanding of their underlying mechanisms.

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Worm humor: Identification of a novel mutation that enhances robust experimental reproducibility in *C. elegans*

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While slaving away hard at the bench, we have serendipitously discovered a new mutation that has increased repeatability and significance of any assay we have performed. This mutation significantly reduces the level of experimental error so intensely that we can actually report standard deviation instead of standard error of the mean. P-values are minuscule and our graphs have more stars than ever (Figure 1). In addition, putting our strains in this background has allowed us to perform extremely beautiful Western blots, so beautiful that we do not need to use Photoshop to 'robustify' our figures (Figure 2).

As typical naming of genes follows the phenotype they induce, we believe we have identified a mutation affecting a new class of genes, hereby called *robust*, or *rbst*. Mutations affecting genes belonging to this class affect experimental performance and data reproducibility. Tim Schedl has yet to reply to our request to be formally assigned this new class of genes, but we are confident that Jonathan Hodgkin would approve. We have mapped this mutation, *win1*, to either the left arm of LG III or LG IV. The graduate student currently performing the mapping studies is ironically failing to achieve a robust confirmation.

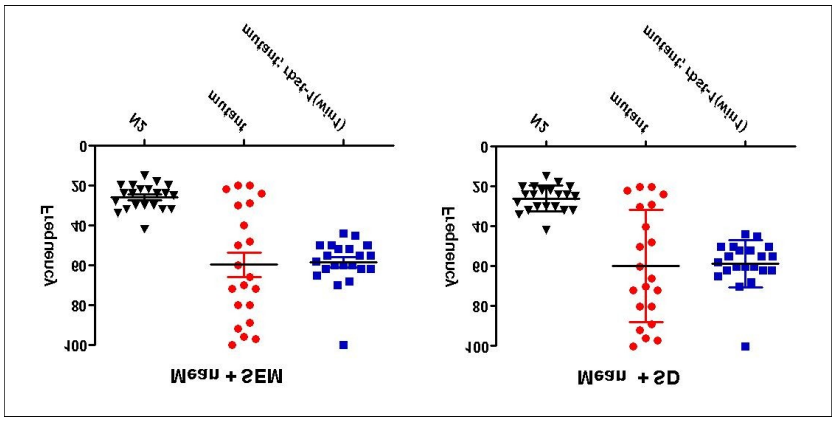

Figure 1: *rbst-1(win1)* enhances mutant phenotypes and makes data seem much more believable.

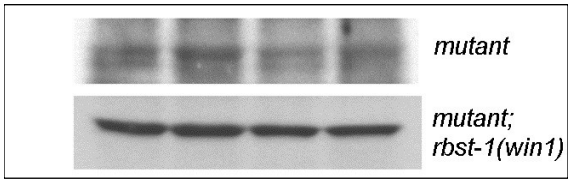

Figure 2: *rbst-1 (win1)* eliminates need for 'image processing and enhancement' by Photoshop.

In conclusion, we report identification of a new allele, *rbst-1(win1) III?; IV?* which makes all results significantly more robust and will be sure to get your papers accepted without revisions. Stay tuned for more updates on our robust progress. No, we are not sharing our magical strain just yet, but if you would like to form a robust collaboration with us to pursue further robust experiments, please feel free to contact us. We look forward to hearing your robust ideas. Thanks to Steve Hodgkinson for critical reading and verbal abuse regarding this data. Thanks to BC-V for technical advice and moral support. Thanks to Chib, raela and dunnp for robust shenanigans.

Worm humor: Nematode news in brief

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Slam-Dunk Grant Fails to Write Itself (Minneapolis, MN). In spite of widespread projections, R01 proposal GM088686-A1 has, "for reasons that defy all logic", failed to write itself, according to sources close to the proposal. Moreover, the competitive renewal, which is reputed to strike the perfect balance between mildly risky high-payoff studies and guaranteed publishable units, has yet to commit a single sentence to paper. "How this set of perfectly meshed yet entirely non-interdependent aims could fail to auto-compose onto a hard drive is nearly inconceivable", stated one of grant's lead investigators. That the six-point bulleted Significance section, which segues seamlessly into the Innovation component, has so completely fallen short of crafting itself, was also unanticipated. Admittedly, we thought we might have to help out with entering some Endnote references or maybe take up some slack on the budget section", stated a co-PI close to the project, "but the response to previous criticisms should have literally fallen out of the sky and onto the page given that every experiment since the initial submission has completely put reviewer's concerns to rest." Regardless of the revised proposal's failure to meet expectations, scientists associated with the grant remain optimistic that the "eight high-impact papers" generated during the previous funding period are more than likely to "submit themselves" to top journals within the next few months.

Genes within long-standing operon calling it quits (X chromosome). At a joint press conference held on the campus of Caltech, genes within the *C. elegans* *lin-15* operon recently announced their decision to split due to "creative differences". Despite co-existing for at least 50-100 million years, the individual genes known as *lin-15a* and *lin-15b* indicated their mutual desire to move on in a prepared statement. "We've done our thing together and we don't think we have anything to prove", affirmed the genes, which have been sharing regulatory elements prior to the separation of *C. elegans* and *C. briggsae*. According to sources close to the locus, having nearly identical names has been a long-standing source of irritation to the tandem genes, which share virtually no identity at the peptide level. "It's already bad enough to be part of the "*lin*" group", said *lin-15a*, "but having the same number followed by a letter is just total bullshit". "It's not like our discoverer was George Foreman or something", *lin-15b* added. Despite their intention to separate "a minimum of 5-10 map units apart or even to different chromosomes", the functionally interconnected genes plan to continue their long-standing collaboration on processes such as vulval induction while freeing their expression domains to pursue other developmental projects. Although details on the custody of 5' regulatory elements and the 3' UTR have not yet been disclosed, geneticists familiar with the process are expecting a protracted battle.

New lab announcement: Kevin Collins

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I started my independent research group from August 2014 in the Department of Biology at the University of Miami in Coral Gables, Florida. Our fundamental goal is to understand how neurons communicate in circuits to establish an appropriate level of activity that produces a robust, stable behavior. Our approach is to analyze in detail a model neural circuit that controls egg-laying behavior in *C. elegans*. We are taking advantage of the optical clarity and powerful genetics in this experimental system to literally watch the activity of every cell in the circuit in behaving animals using fluorescent Ca^{2+} reporters, and also to manipulate their activity using optogenetic tools. Using mutations and transgenes to discover and alter molecular signaling events between cells, we are determining how the complex pattern of activity in a circuit creates a coherent, regulated behavior. We expect these studies will reveal general principles of neurotransmitter signaling and neural circuit function with applications to understanding the human nervous system and its dysfunction in disease.

Website: http://www.as.miami.edu/biology/people/faculty/kevin-collins/

Ph.D. student and Postdoc positions are currently available. To apply, please send a CV and description of research interests to kcollins@bio.miami.edu

New lab announcement: Eric Guisbert laboratory at the Florida Institute of Technology

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My laboratory was established in the Department of Biological Sciences at the Florida Institute of Technology in August 2014. We are interested in the basic biological questions of how organisms sense their environment and respond to stress. Our focus is on the heat shock response (HSR) due to its important roles in normal growth and its involvement in human diseases including neurodegenerative diseases and cancer. We are identifying mechanisms of action for novel regulators of the HSR, exploring the basis for HSR tissue-specificity, and extending our findings from *C. elegans* using cultured human cell lines. We currently have openings for undergraduate, masters, and doctoral students.

New lab announcement: David Q. Matus at Stony Brook University

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The Matus lab opened its doors in January 2014 at Stony Brook University in the Department of Biochemistry and Cell Biology. We are the first official *C. elegans* lab on campus! Our laboratory is broadly interested in trying to understand how morphogenetic events are programmed during development, how these processes evolve between species and how they might go awry during pathogenic events such as cancer. We use nematode uterine-vulval attachment as a model to understand how cells acquire the ability to breach the basement membrane, an ancient form of extracellular matrix unique to the animal kingdom. Specifically, we are interested in identifying transcription factors and their targets that program cell invasive behavior in *C. elegans* and related nematode species. Please visit our website at: you.stonybrook.edu/matuslab/

Interested PhD students should contact me directly and I can provide more information about Stony Brook University graduate programs. Please check our website for potential postdoctoral opportunities as they may arise. We look forward to hearing from you and seeing you at meetings!

Worm Picking 101

By Jessie MNG Lopez 2014

VBK Lab CSUN

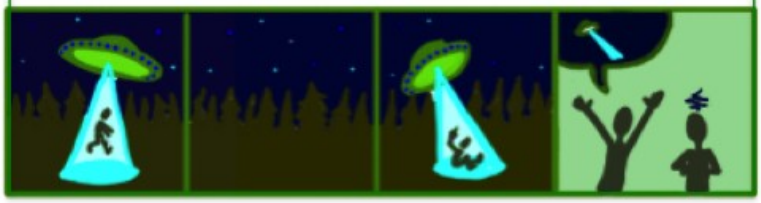
Every veteran worm researcher has their own techniques for picking worms. Here I will share some of mine. This is meant as a guide to help new nematode researchers start developing their own style. Enjoy!

- Jessie

Picking up a worm is like...

A very gentle alien abduction.

I like to imagine them trying to explain their bizarre otherworldly abduction experience to their disbelieving friends. The event is inherently traumatizing. Your job is to make it as gentle as possible.



Picking up a worm is like...

Licking your finger to pick up an eyelash.

Sometimes the *E. coli* is a thin lawn, or the plates are wet and you can't get much on the pick. That's okay!