BIOLOGY CENTER AS CR

Institute of Entomology

Branišovská 31, 370 05 České Budějovice, Czech Republic

Dr. Marek Jindra, PhD
Department of Genetics
tel./fax: 420 387 775 232
e-mail: jindra@entu.cas.cz
http://www.entu.cas.cz/jindra

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Evaluation of MS thesis:

Jana Matulova

"Study of the Role of Casein Kinase I epsilon in Breast Cancer Using *Drosophila* as a Model"

The project of Jana Matulova had an extremely ambitious goal: to introduce point mutations in the CKIε gene, known from human cancer patients, into the homologous *Drosophila* gene, and test their causal relationship to neoplastic growth or cancer-like situations in the living fly system. This project alone, if it indeed included the phenotypic analyses of the obtained *Drosophila* CKIε point mutants, would normally be a subject of a five-year grant, requiring effort of the entire laboratory. Just preparation of the technical prerequisite for this work, i.e. generation of the specific fly mutants, was an extremely demanding task for a masters student.

Moreover, the two strategies employed for generating the mutants relied on recently developed techniques that are by no means routine, even in the top notch labs of the world. The first is homologous recombination, facilitated by transgenic tools in *Drosophila*, a method for which the supervisor of the thesis, Tomas Dolezal, became an expert. It was probably his positive previous experience that encouraged this approach. The second strategy, use of site-specific zinc-finger nucleases to facilitate recombination with the mutant DNA, was entirely novel, still rarely used technique, and therefore even less likely to produce the desired result. I really admire the courage of Jana to undertake this work and her ability to understand and actually conduct all of the experimental steps.

Yes, both strategies have failed, but not because the particular steps would be inproperly executed. Jana managed to prepare transgenic fly lines carrying the correct mutant constructs. The numbers of transgenic lines were clearly sufficient and the high numbers of flies screened for the recombination events were quite impressive. Most importantly, Jana rigorously found where the problem occured by doing the right experiment (a genomic Southern blot). She showed that while the inserted constructs mobilized, they failed to induce duplication of the targeted gene. Without this necessary step, further mutagenesis was impossible.

The zinc-finger nuclease approach was even more novel and more ambitious, less guaranteed to work. Again, Jana did everything to properly document that at least all the preparatory steps were done correctly. Just the cloning steps such as 7-primer PCR strategy to generate zinc-finger encoding sequence for targeting the CKIε gene surely required exquisite molecular biology skills. Instead of attempting the mutagenesis directly, Jana first used this technique in a pilot experiment – targeted insertion of the mini-white marker gene. Clearly the approach failed in its first step, causing high lethality of mRNA-injected larvae and no marker-positive flies.

Very importantly, Jana thoroughly and critically discussed the possible causes of the failure. A common cause in both approaches could be the proximity of heterochromatin near the CKIε gene, making the targeted DNA poorly accessible to the enzymes. Obviously this could not have been predicted. The problem with zinc-finger nucleases is that their specificity is still not entirely known, making it difficult to target a given DNA in an absolutely unique manner. This simply reflects that the methods is still in its development.

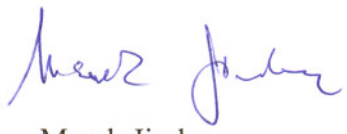
The thesis itself is very nicely written, well organized, easy to read. There are only minor (and repetitive) mistakes in English. The introductory chapters span a wide field of biology and are written at a level normally seen in PhD disertation theses. As I already mentioned, the Discussion is extremely important and useful in addressing the possible experimental pitfalls.

I have only one question. Throughout reading of the text, I kept thinking why on earth the these difficult methods were chosen, instead of putting mutant constructs randomly into the fly genome (simple germline transformation that Jana has mastered), and then crossing them into the available CKIε/dco null mutant background. This would have been the old-fashioned but feasible approach. Only in the last paragraph of the Discussion Jana suggests this approach, which is now underway. I would like to ask:

1) What is the likely effect of introducing the mutations into the CKIε/dco null mutant background?
2) Would it be better to express the mutant version under the natural CKIε/dco promoter, or would it be good to use the UAS-Gal4 system. What would be the advantage of the latter?
3) Based on what is known about he individual mutations within CKIε, would Jana expect some dominant effects of the mutant proteins upon their expression in otherwise wild-type flies? Would such dominant effects answer some of the questions about CKIε role in tumorigenesis ??

In summary, I think that this thesis represents and enormous amount of properly executed, documented and interpreted experimental work. It clearly shows Jana's ability to undertake demanding projects, even such that clearly exceed the normal scope of master projects. Despite the ultimate failure, I would like to suggest that Jana defends her MS degree with the best score.

Sincerely,



Marek Jindra

The University of Utah
Department of Biochemistry

Review of Master Thesis:
Study of the Role of Casein Kinase I epsilon in Breast Cancer Using Drosophila as a Model; Jana Matulova; 2009

Kelly Beumer
5/19/2009

A particular hyomorphic allele of the gene *discs overgrown, dco*^{3}, leads to hyperplastic overgrowth of imaginal discs. The human homolog of *dco*, CKIε, is a candidate for implication in cancer, as a high rate of nonsynonymous amino acid changes have been found repeatedly in breast cancer tissue samples. Thus, the ability to explore the effect of AA changes in Drosophila would be advantageous. However, various alleles of *dco* show a wide variety of phenotypes, indicating that the best way of exploring these mutations would be to introduce the changes into the native locus, thus preserving normal regulation of the gene.

Currently two methods exist for introducing site directed sequence changes into Drosophila genes via homologous recombination. The first is the targeted mutagenesis protocol developed by Rong and Golic in 2000. In this protocol, a mutated version of the sequence, the donor, is introduced into the genome flanked by FLP recombinase recognition sites and I-SceI recognition sites. The homologous sequences are oriented "ends in" so that when the donor is liberated by expression of FLP and I-SceI, it can recombine into the native site, resulting in the introduction of a *white* gene, flanked by two copies of the sequence of interest, one of which carries the desired sequence changes. An additional round of recombination is stimulated, and animals are chosen who carry the desired mutation.

The second method is the protocol developed by the Carroll lab, in which nucleases are designed to target the desired sequence, introducing a DSB into the native sequence, thus stimulating recombination with a donor carrying the desired mutations. The nucleases are introduced as RNA by embryo injection, and in the best cases, produce mutations at a frequency high enough to identify them solely by molecular means without a known phenotype.

Both protocols have their advantages and drawbacks, and a variable success rate in different hands, and at different loci. The two protocols have not been compared head to head at many loci, leaving open the question of whether some loci may be more amenable to one method than the other. Thus, Jana chose to pursue both protocols, in hopes of success with one.

In both cases, an important aspect of the protocol is to identify the desired mutants. The Rong and Golic protocol relies first on the localization of the *w* gene to discern desirable homologous recombinants, as opposed to nonhomologous integrants, followed by molecular characterization to determine that the new DNA is integrated

Department of Biochemistry
15 N Medical Dr. East, Rm 4100
Salt Lake City, Utah 84112-5650
Phone (801) 581-2117
Fax (801) 581-7959

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appropriately. The PCR primers Jana designed could not be tested independently to provide a positive control, so when she did not recover a positive signal, she devised two methods to independently show that that her failure to recover mutants was a not the result of ineffective testing. The first was to use control primers that allowed her to test each experimental primer independently. This reduced the probability of PCR failure, but did not eliminate it, so she then used southern analysis to confirm that each independent candidate was in reality a nonhomologous integrant.

In using the Carroll lab protocol Jana designed her donor to be marked with a *w* gene, hoping to use eye color to easily identify targeted mutants. Although there is no good reason to suspect that it should not be possible to recover mutants in this way when using ZFNs, there is no report of the technique having succeeded, and her success would have been of significant interest. However, faced with an inability to recover red-eyed mutations, she appropriately stepped back a stage and tested the ability of the nucleases to created targeted breaks in the *dco* gene by testing for a loss of viability found in *dco* null flies. Her results indicated that the ZFNs were not cutting at the target. Jana correctly proposes that failure of ZFN specificity is the most likely cause of failure.

There are a couple of questions that arise in this experimental description. First, the concentrations of nucleic acids Jana reports using are much higher than are published as ideal in our papers, and I suspect much of the lethality she reports may be due to this. The difficulty she experienced in injecting such high nucleic acid concentrations definitely reflects our experience. Her recovery of fertile adults in her reported injections were respectively 4% and 6%, when we would more likely expect 10%-30%. This type of result is behind our recommendation of lower concentrations. Were lower nucleic acid concentrations tested, and what was the reasoning behind the chosen concentrations?

A second question is whether all injections were done using the same RNA prep. I don't think this is published, but we definitely see differences in results from one RNA prep to another, though we see no observable difference in the RNA. Having used multiple preps would eliminate this variable.

Jana designed her experiments carefully, addressing variables and choosing controls that allowed her to distinguish potential failure of the protocol from experimental error. Although she was not successful in producing the desired mutants, and may have focused on experimental detail more than necessary, she has carefully analyzed her results and presents plausible, testable hypotheses for her failure, as well as an alternate plan. She appears to be an excellent candidate for a Masters degree, and should succeed nicely if she chooses to continue her education.

Kelly Beaver

Department of Biochemistry
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