Review of the master thesis written by Alexandr Pospěch and named "Attempts on chromatin immunoprecipitation with *C.elegans* nuclear receptor NHR-25.

Summary:

Alexandr Pospech's thesis focuses on optimization of the chromatin immunoprecipitation method (ChIP). Since it is very difficult to perform ChIP from *C. elegans* lysates he tries to optimize this method using human HEK 293 cells. He uses well established system, in which NHR-25 protein binds to sf-1 DNA motif. As a negative control the mutated sf-1 DNA motif is used. Conventional and qPCR are used to analyze ChIP-ed samples. AP shows higher level of the NHR-25 binding to the wt version of sf-1 DNA sequence comparing to the mutated sf-1 DNA. Furthermore he attempts verify the binding of NHR-25 to Cyclin E promoter region. These ChIP-ed samples are analyzed by qPCR, but unfortunately due to high background these results are inconclusive. Finally AP attempts to immunoprecipitate GFP-tagged MBF-1 protein from *C. elegans*. In summary all the results showed here might be promising, however further optimization is needed before this method can be widely used.

Questions regarding the Introduction section:

1. Is the expression of *NHR-25* developmentally regulated? If yes, how.
2. Is the expression of the *NHR-25* gene tissue specific? If yes, in which cells or tissues *Nhr-25* is expressed?
3. Would be possible to screen the *C. elegans* genome to look for TCA motif in the promoter regions of all genes?
4. Chromatin immunoprecipitation is not the only method to study DNA-protein interaction. Would AP know other methods? If yes, can he briefly mention their advantages and disadvantages and can he explain why did he choose ChIP?

Questions regarding the Results section:

1. Chapter 4.3.1., page 38, line
   What are the specific control primers? Are they serving as negative control for ChIP experiment or positive control for sheared DNA? Please explain.
   Since you detected signal in lanes 3,7,11 and 15, does it mean that NHR-25 binds to its own sequence?
   Why did you detect a band in lane 24?
   Note: Moreover, I suppose that the column 24 was supposed to be in green colour and thus the first sentence of the second paragraph on the page 38 is incorrect.
2. Chapter 4.3.2.

Why did you decrease amount of the DNA? What did you expect to happen?
Why did you use different set of primers for your control (primers to amplify EGFP region comparing to primers specific for NHR-25 used in the first ChIP experiment)?
Why do you suddenly call these primers "a negative control sequence"?
I am sorry but this part was very confusing to me. Please explain the differences.
Is there any way how to avoid the fixation of the transfected plasmids after transfection?

3. Figure 4.8.a
   Honestly, this figure required much more attention and could have been explained in much better way. Please explain differences between lane 1 and 3 regarding to ctrl primers (btw. these primers are suddenly called a "non-specific primers" in the figure legend)
4. Chapter 4.3.3. and 4.3.4.
   Unfortunately, due to the high background (signals are detected in ChIP-ed samples using GAPDH primers) all the results are inconclusive. What can be improved next? How do you explain the high background level in this case? GAPDH primers are directed against genomic DNA and thus the high background is not due to "fixation of the transfected plasmids". If I misunderstood the fundamentals, please explain.
5. Chapter 4.4
   Author claims that he successfully pulled-down MBF-1 protein tagged to GFP. I am afraid that this conclusion is not right for two obvious reasons: 1. there are no differences between the immunoprecipates using anti-GFP and anti-MET antibodies. I guess the negative control was not selected properly. 2. Positive control anti-MBF-1 antibody does not recognize its target. I understand that this antibody was created against drosophila protein and now it is used against *C. elegans* protein. However if you were not sure if this antibody recognizes the *C.elegans* MBF-1 protein then you were not supposed to use this antibody as a positive control (last sentence of the first paragraph, page 45). Can author comment on this?

In summary, Alexandr Pospěch had a great opportunity to work in the excellent lab and he learned and performed a lot of general molecular biology methods. Since his tasks were pretty difficult, I can't wonder that the project has not been finished and a lot of steps need to be still optimized.

This master thesis fulfils all the criteria for master theses given by the Faculty of Natural Sciences and I recommend it for the defense.

V Ceskych Budejovicich, 24.1.2010

Alena Zikova

Opponent's Review of Alexandr Pospěch's Master Thesis:
Attempts on chromatin immunoprecipitation with C. elegans nuclear receptor NHR-25

The goal of the thesis was to establish chromatin immunoprecipitation (ChIP) protocol in the laboratory and apply this technique to analyze NHR-25 receptor interaction with its target sequence.

The introduction chapter on C. elegans biology and on nuclear receptors is limited only to 3 pages of the text and that I found really not sufficient for the Master Thesis where the student should demonstrate much broader and insightful review on the topic.
Not much attention was paid to Materials and Methods section as well since it rather looks like cut'n paste from the laboratory protocol book. Order is not logic, e.g. 1st strand synthesis is placed in C. elegans culture chapter (3.3) and not in Molecular biology methods chapter (3.4.) as it probably came from single protocol source. More importantly, the protocols are very often too trivial for state-of the-art of the current biology and often not really related to the thesis subject (Buerker cell description?, worm microinjections – was it really used? I did not find it in the thesis since the only experiment worms were used was a pilot immunoprecipitation from established transgenic mbf-1::gfp line).
The major problem I see is the misunderstanding what ChIP technology is about. It is a technique that was developed to validate data coming from other experiments in vitro, namely mobility shift assays (EMSA for DNA-binding) and transient transfections (for measuring the transcriptional activity) in more "physiological" context of chromatin. For pilot experiments one might use transient transfections of the respective transcription factor (e.g. NHR-25) in HEK293 cells but never use the target sequence on the co-transfected plasmid where no real chromatin context exists. This will make the obtained results less meaningful than transient transactivation assays. Also the number of target sequence plasmid copies transfected into a single cell is several orders of magnitude higher than the endogenous two copies in the promoter of two gene alleles and this will make any quantifications by PCR or qPCR impossible or very difficult to interpret. Therefore I will not comment on the experiments dealing with ChIP.
I have, however, two specific questions:

1. Is EGFP::NHR25 protein able to bind DNA and transactivate e.g. SF-1 reporter similar to bona fide NHR-25 protein?
2. Why there were not tried the natural SF-1 sites found in e.g. cyp21 gene for pilot ChIP experiments if these sites are recognized by NHR-25 in HEK293 cells in transient mode?

In summary, Alexandr Pospěch demonstrated basic molecular and cell biology skills and the ability to gather his work in the written form. However, his work lacks high standards of thesis usually defended from both tutor's laboratories and has multiple formal and scientific deficiencies described above.

Taken together, I recommend this Master thesis to be defended with reservations and I suggest the grade Good.

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