Report on the PhD thesis of M.Eng. Sara Mikac

Thesis Title: "Identification of a novel, transcription-independent role of Nrf2 in lung cells"

This is a well-developed, well-written and logically constructed thesis addressing the effect of transcription factor Nrf2 on the expression of major histocompatibility complex class I (MHC-1) proteins. The introduction first describes the structure and function of Nrf2, its regulation by Keap1 as well as other Keap1-independent means. It then discusses the various alterations in Keap1 and Nrf2 that occur in cancer cells. This is followed by a description of the role of Nrf2 is regulating the cellular stress response to oxidants and electrophiles, its role in detoxification reactions, as well as cellular proteostasis, autophagy, intermediary metabolism and bioenergetics. The dual role of Nrf2 in cancer is also described. There is an interesting section on the role of Nrf2 in immunity, which is particularly relevant to the focus of the research described in the thesis. Finally, examples are given for some of the most widely studies Nrf2 activators and inhibitors.

The Materials and Methods chapter is well described, but could include a bit more detail on the conditions of the fluorescence imaging (such as excitation and emission wavelength rather that "UV fluorescence") and analysis of the microscopic images, and needs a separate section on Statistical Analysis.

The Results chapter consist of two parts. The first part described the investigation of the role of Nrf2 on the expression of genes encoding proteins involved in antigen presentation, such as the MHC class I proteins (HLA-A) using non-small cell lung cancer (NSCLC) cell lines that differ in Nrf2 activity, i.e. A549 cells with high Nrf2 activity and RERF-LC-AI cells that have normal Nrf2 activity. Primary NSCLC cell lines derived from patients' tumours are also used in some experiments. It was found that the dowregulation of Nrf2 led to an increase in the mRNA levels for HLA-A, but a decrease in its protein levels. Curiously however, increasing the levels of Nrf2 (by depletion of its main negative regulator, Keap1) did not have the expected (opposite to Nrf2 depletion) effect. It was then hypothesized that Nrf2 may regulate the synthesis of degradation of HLA-A. This possibility was tested using a translation elongation inhibitor (emetine), Click-iT labelling, and pulse-chase analysis, but the results were inconclusive. Subsequent co-IP, immunofluorescence-based co-localization, and proximity ligation assay experiments showed that Nrf2 interacts with HLA-I, and suggested that this interaction takes place primarily in the cytoplasm. This finding is supported by molecular modelling and molecular dynamics simulations, which further suggested that binding to Nrf2 stabilizes HLA-A.

The second part of the Results chapter is focused on the faster migrating band that is detected with the anti-Nrf2 antibody from Abcam. It is shown that this protein is not a product of post-translational modifications, such as glycation or phosphorylation. Instead, it represents a shorter isoform of Nrf2 that lacks the first N-terminal 16 amino acids (called ΔN-Nrf2 isoform 2) resulting from alternative translation initiation. Molecular modelling and molecular dynamics simulations suggest that the lack of the first 16 amino acids partly impairs binding to Keap1 and subsequent ubiquitination; this is supported by experiments using Keap1 depletion or a neddylation inhibitor. Unlike full-length Nrf2, ΔN-Nrf2 is localized

in the cytoplasm both under homeostatic conditions and upon exposure to tBHQ, and does not accumulate upon exposure to tBHQ.

The thesis contains publishable data and brings new knowledge to the field of Nrf2 biology.

General recommendations:

- It will be good to have a section describing the aims and hypotheses which are being addressed in the thesis immediately after the introduction.
- The Methods need a section on statistical analysis to explain how the statistical analysis of the data has been done.
- The abbreviations have to consistent throughout the thesis. For example, NQO1 is defined as "NAD(P)H quinone oxidoreductase 1" on page 11 and 18, but as "NAD(P)H quinone dehydrogenase 1" on page 21 and 45. Best to define once, when it is first mentioned, and use just the abbreviation from then on.
- "RNA expression" is often used throughout the thesis. It will be more precise to use "mRNA" instead.
- The positions of the molecular weight markers should be indicated on all western blots. Also, how representative are the western blots, i.e. how many times was each experiment that was analyzed by western blot performed?

Recommended minor corrections:

Introduction:

- Page 15, second paragraph: "In response to oxidative stress, Nrf2 is liberated from Keap1-mediated degradation..." gives the impression the Nrf2 is released from Keap1, which is not correct. It would be better to say: "In response to oxidative stress, the ability of Keap1 to target Nrf2 for degradation is impaired..."
- End of page 15 and start of page 16: It is important to briefly mention here that these motifs are actually phosphodegrons, i.e. phosphorylation of these motifs is required for binding to beta-TrCP, as described later in detail on page 19.
- Page 17, top: The statement: "The IVR region is rich with highly reactive Cys residues, as a consequence of the positively charged environment of basic amino acids K131, R135, K150 and H154 near to the Cys-rich region" is not correct. All of tehse amino acids are in the BTB domain of Keap1, not in the IVR. It will be good to include the amino acid numbers when defining the domains of Keap1 (as well as Nrf2 in the previous section). In the next sentence: "...human Keap1 has up to 27 Cys...", "..up to..." has to be removed. Human Keap1 has 27 Cys.
- Section 1.2.1. and Figure 3 decribe the "hinge and latch model", but this is only applicable to Keap1-Nrf2 protein-protein interaction (PPI) inhibitors, not oxidants or electrophiles, which modify Cys in Keap1 (see Horie et al. Commun Biol. 2021 May 14;4(1):576). This needs to be correctly reflected in the text and figure 3.
- Page 20, end of section 1.2.3. "...while keeping the gene functionality intact" It would be better to say "...while keeping the protein functionality intact".
- Section 1.2.4. describes some of the known post-translational modifications of Nrf2 and their roles, focusing on phosphorylation and acetylation. It will be also good to add glycation to this section (see Sanghvi et al. Cell. 2019 Aug 8;178(4):807-819). This is relevant to the experiment shown in Figure 34 on page 78.

- Page 21, section 1.3.1.: It says "...and heme oxygenase 1 (HO-1) are catalyzing the conjugation reactions". This is not correct. HO-1 is not catalyzing a conjugation reaction, but the oxidative cleavage of heme.
- Page 21, section 1.3.1.: The last paragraph discusses the enzymes involved in the biosynthesis and regeneration of GSH, and it will be good to also add glutathione reductase.
- Page 28: "It would also explain a significant reduction of Th1 and Th17 cytokines, including IL-6, upon Nrf2 activation with derivatives of the triterpene oleanolic acid" Considering that oleanolic acid itself or its non-electrophilic derivatives do not activate Nrf2, it would be good to add "electrophilic" before "derivatives of the triterpene oleanolic acid".
- Page 29, Figure 7: The A) and B) labels of the two panels are missing.
- Page 30, section 1.5.3.: Because a large part of the thesis is focused on the regulation of MHC molecules by Nrf2, it will be good to add a figure or a Table illustrating the concepts described in this section.
- Page 31, section 1.6.1.1. Reference 130 focuses much more on 1,2-dithiole-3-thiones rather than isothiocyanates, such as sulforaphane, and could be replaced. There are better references to illustrate the effect of sulforaphane on the Nrf2-Keap1 pathway (e.g. see Yagishita et al. Molecules. 2019 Oct 6;24(19):3593).
- Page 33, Figure 8: "target sides" should be "target sites".

Results

- Page 44, section 3.1.1.: It is important here to also mention that a third reason for the high Nrf2 activity in A549 cells is promoter hypermethylation of the *KEAP1* gene, which leads to its lower expression (Guo et al. Biochem Biophys Res Commun. 2012 Nov 9;428(1):80-5).
- Page 45, section 3.1.2.1. and Figure 9 on page 46: As pointed out, the downreguation of Nrf2 by siNrf2 lowers the protein levels of HLA-A, but the upregulation of Nrf2 by siKeap1 does not lead to an increase in the protein levels of HLA-A. It is concluded that "The reason could be the involvement of Keap1 in different pathways and therefore, its influence on HLA-I expression can be independent from Nrf2 (186)." This is certainly one possible explanation, but a western blot showing the protein levels of Keap1 is necessary to confirm the extent of Keap1 knockdown, even though the increases in the Nrf2 and NQO1 levels strongly suggest that the knockdown of Keap1 was indeed successful.
- Page 50, line 6 from bottom: "...fluorescent microscope..." should be "...fluorescence microscopy..."
- Page 67, Figure 28: It is not clear how many times this experiment has been performed. How robust and reproducible is it? The finding is interesting and is worth of further investigation using this quantitative approach. This is particularly important considering the opposite result for the effect of Nrf2 on the cytoplasmic HLA-A, i.e. the co-localization experiments suggest binding between Nrf2 and HLA-A in the cytoplasm, which should increase the stability of HLA-A. This conclusion is in conflict with the results from the flow cytometry experiments, which suggest increased stability of the intracellular HLA-A in the absence of Nrf2. What could be the explanation?

- Page 74-75 and Figure 31: The statement "However, the 105 kDa Nrf2 was not completely knocked-down in the case of A549 and NSCLC 1, indicating its high stability, that was previously observed (Fig. 31A and B)." At this point of the study, an alternative explanation could be that this is a non-specific band, and this could be mentioned.

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