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DOCTORAL THESIS REVIEW REPORT

Review of the doctoral dissertation by **Hossein Khodadadi**, M.Sc., entitled "*Analysis of the Molecular Mechanism of Ubiquitinated Protein Accumulation Caused by Nfe2l1 Knockdown in Neuronal Differentiated P19 Cells*"

Performed at the Institute of Genetics and Animal Biotechnology, Polish Academy of Sciences

Degree sought: Doctor in Agricultural Science | Format: Cumulative dissertation (3 publications)

under the supervision of **dr hab. Hiroaki Taniguchi**, PhD, Associate Professor – main supervisor

and

dr. hab Dawid Winiarczyk PhD, Assistant Professor – *Auxiliary Supervisor*

1. Formal basis for the review (resolution of the Disciplinary Council): The dissertation of Hossein Khadadadi submitted for evaluation concerns research described in cumulative format, addresses the role of the transcription factor Nfe2l1 (Nrf1) in neuronal proteostasis, using a retinoic acid-induced neurogenesis model based on P19 embryonal carcinoma cells. The work is presented through three linked publications:

(1) a methodological paper describing an siRNA-mediated knockdown protocol during P19 neurogenesis (MethodsX, 2025); (2) a primary research article demonstrating that Nfe2l1 knockdown impairs neuronal differentiation, increases ubiquitinated protein accumulation, and alters the expression of Parkinson's-disease-related genes under proteasome inhibition (Scientific Reports, 2025); and (3) a literature review positioning NFE2L1 as a central regulator of proteostasis through its interplay with the proteasome, autophagy, and ferroptosis (Frontiers in Molecular Neuroscience, 2025).

The three components are logically sequenced — method development, experimental validation, and conceptual synthesis — and together form a coherent research programme. The candidate combines classical molecular biology (qPCR, western blotting, immunocytochemistry) with transcriptome-wide profiling (RNA-seq, KEGG pathway enrichment) to build a multi-layered picture of Nfe2l1 function during neurogenesis and under pharmacologically induced proteotoxic stress (MG132). The topic is timely and relevant: proteostasis collapse is a recognised feature of Parkinson's disease and related neurodegenerative disorders, and NFE2L1/NRF1 remains comparatively understudied compared with its paralogue, NFE2L2/NRF2.

Table 1. Structure of the cumulative dissertation.

Publication 1	Methods paper (siRNA/RNA-differentiation protocol)	MethodsX, 2025 (published)	Establishes a reusable experimental platform
Publication 2	Original research (qPCR, WB, ICC, RNA-seq)	Scientific Reports, 2025 (published)	Core experimental evidence for Nfe2l1 function
Publication 3	Narrative literature review	Front. Mol. Neurosci., 2025 (published)	Conceptual synthesis and disease context

The total Impact Factor (IF) of publications according to the Journal Citation Reports (JCR) database is **9.6, and 350 KBN points**. The dissertation is presented as a single-topic cycle of three publications, in which the PhD student is the first author. The dissertation also includes the PhD student's commentary in English (55 pages) with an introduction, hypothesis, and objectives (6 pages), materials and methods (4 pages), a description of the most important results obtained during the research (15 pages), a discussion and conclusions (8 pages), a bibliography (4 pages), and a summary of the results in Polish and English is missing. In total, the dissertation is 93 pages long. I declare that the series of publications constituting the scientific achievement of Hossein Khodadadi, in formal and substantive terms, meets the requirements set out in the **Act on Academic Degrees and Academic Title and on Degrees and Title in the Field of Art of 14 March 2003 (as amended) and the Regulation of the Minister of Science and Higher Education of 1 September 2011,** which are set for persons applying for the degree of Doctor of Veterinary Sciences.

2. General characteristics and rationale of the dissertation, including scientific problem, its formulation, and relevance: The Introduction builds its case in three logical steps that are worth summarising here because they frame how the rest of the thesis should be judged. First, post-mitotic neurons are argued to be uniquely dependent on proteostasis because they cannot dilute damaged proteins through division, a point well supported by citation to Hetz and Saxena (2017) and Hipp et al. (2019). Second, the ubiquitin-proteasome system (UPS), autophagy, and — more recently — ferroptosis-preventing mechanisms are presented as an interlocking, multilayered defence network, with failure in one system placing additional burden on the others. Third, NFE2L1/NRF1 is introduced as a stress-responsive transcription factor sitting at the intersection of these systems, activated by proteasome inhibition and translocating to the nucleus to drive a "proteasome recovery pathway". This framing is logical and well-referenced, and it motivates the central experimental question addressed by Publication 2: what happens to this recovery pathway and to neuronal differentiation itself when Nfe2l1 is depleted specifically during neurogenesis under proteotoxic challenge? The stated hypotheses and objectives (Sections 2–3 of the thesis) are appropriately scoped to what is actually tested: they do not overreach into claims about in vivo relevance or therapeutic efficacy, and each hypothesis maps onto a specific, identifiable results subsection. This hypothesis-to-result mapping is a genuine strength of the thesis's organisation and makes the document easier to audit than many cumulative dissertations, where the connection between stated aims and delivered results is often looser.

3. Summary of Scientific Content

3.1 Publication 1 — Methodological protocol (MethodsX): Establishes and validates a two-stage RA-induced P19 differentiation protocol combined with a two-hit siRNA transfection schedule (days 5 and 8) for Nfe2l1 knockdown. Differentiation is confirmed morphologically (embryoid body and neurite formation) and molecularly (Map2/Pax6 up, Oct4 down); knockdown is confirmed by western blot under MG132-stabilised conditions. This is a solid, reusable protocol paper of modest but genuine value to the field.

3.2 Publication 2 — Primary research article (Scientific Reports): The core experimental study and key findings of PhD thesis were: (i) Nfe2l1 and Nfe2l2 mRNA rise during neuronal differentiation; (ii) NFE2L1 protein and mRNA are both induced by proteasome inhibition (MG132) in undifferentiated and differentiated cells; (iii) RNA-seq shows that MG132 treatment of P19 neurons enriches neurodegeneration-associated KEGG pathways, with Parkinson's disease the top hit; (iv) a panel of 12 non-proteasomal PD-associated genes upregulated under proteasome inhibition (Atf6, Camk2d, Casp3, Daxx, Eif2ak3, Keap1, Sod1, Slc39a9, Tuba4a, Tuba8, Tubb2a, Ubb) was tested after Nfe2l1 knockdown, with Atf6, Camk2d and Sod1 showing Nfe2l1-dependent induction; (v) Nfe2l1 knockdown reduces Map2/Pax6 expression and neurite formation, consistent with impaired neuronal maturation; (vi) Nfe2l1 knockdown increases polyubiquitinated protein accumulation, especially under MG132; and (vii) the lncRNA Neat1 (but not Malat1) is regulated by Nfe2l1 during proteasome inhibition.

It is worth noting the internal logical structure of this publication in more detail, since it is the evidentiary backbone of the whole thesis. The RNA-seq comparisons are staged hierarchically: first, neuronal differentiation itself is shown to reshape the transcriptome extensively (1,549 genes uniquely altered under DMSO-only differentiation, 1,655 uniquely altered under MG132-treated differentiation, with 8,810 genes commonly affected by both); second, proteasome inhibition is compared between undifferentiated stem cells and differentiated neurons (1,397 versus 3,952 uniquely altered genes, with 2,237 genes shared); and third, KEGG enrichment on the Mg132-treated-neuron-versus-DMSO-treated-neuron contrast specifically identifies Parkinson's disease as the top-ranked pathway, ahead of general "pathways of neurodegeneration", Alzheimer's disease, and amyotrophic lateral sclerosis. This staged design is a reasonable way to isolate a differentiation-specific, disease-relevant signature, and the candidate is correct to focus subsequent validation on the neuron-specific MG132 contrast rather than the stem-cell contrast. The subsequent narrowing from the full DEG list, to the KEGG-defined PD pathway genes, to the 27 upregulated PD-associated genes, to the final 12-gene non-proteasomal validation panel is transparent and traceable, which is commendable. The Nfe2l1-dependence results themselves are appropriately three-way factorial in design (siRNA: siNC vs. siNfe2l1; treatment: DMSO vs. MG132), which allows the candidate to distinguish genes whose MG132 induction is Nfe2l1-independent (Keap1, Ubb, Casp3, Eif2ak3, Tuba4a, Tuba8 — all still induced by MG132 in the Nfe2l1-knockdown background) from the smaller set whose induction requires Nfe2l1 (Atf6, Camk2d, Sod1). This factorial logic is the most rigorous element of the analysis and should be highlighted as such in the thesis's own summary, since it is easy to overlook amid the volume of individual gene panels reported.

3.3 Publication 3 — Review (Frontiers in Molecular Neuroscience): A well-referenced narrative review integrating NFE2L1's role across the ubiquitin-proteasome system, autophagy/aggrephagy, mitochondrial homeostasis, and ferroptosis, with a dedicated section on the pathological evidence linking NFE2L1 deficiency to Parkinson's and Alzheimer's disease (including in vivo knockout phenotypes and postmortem substantia nigra data). The review is comprehensive and appropriately hedged, and the authors transparently disclose the use of a generative-AI tool for language proofreading, which is good practice.

4. Assessment of Significance and Originality

Assessment of knowledge of the literature relevant to the research topic and correctness and originality of the method and scope of the scientific problem: The thesis makes a credible, incremental contribution. Its principal novel finding is the demonstration, in a neuronal-differentiation context, that Nfe2l1 knockdown selectively blunts the MG132-induced upregulation of a defined subset of Parkinson's-disease-associated genes

(Atf6, Camk2d, Sod1) and of the lncRNA Neat1, while other MG132-responsive genes (Keap1, Ubb, Casp3, Eif2ak3, Tuba4a, Tuba8) remain inducible independently of Nfe2l1. This selectivity is scientifically interesting because it suggests Nfe2l1 sits within a specific sub-circuit of the proteotoxic stress response rather than being a generic amplifier of the MG132 response, and it is consistent with (and extends) the candidate's own prior published work on Nfe2l1 in proteostasis. The P19 model and the accompanying siRNA protocol, published separately as a methods paper, add practical value for other groups working with this system.

The originality is best described as confirmatory-and-extending rather than paradigm-shifting: the core biological premise (Nfe2l1 as a proteostasis regulator relevant to neurodegeneration) was already established by prior work, including the candidate's own supervisory group (Taniguchi et al., 2017; Łuczyńska et al., 2023/2024), and by other groups (e.g., Ofoghi et al., 2024 on Nfe2l1–ferroptosis; Hatanaka et al., 2023 on Nfe2l1–aggrephagy). The thesis's distinct contribution is testing this axis specifically in a neuronal differentiation paradigm and linking it mechanistically, if only correlatively, to Neat1 and to PD-associated transcripts. This is a legitimate and useful, if modest, incremental advance.

Positioned against the wider field, the thesis is comparable in scope and ambition to other recent cumulative dissertations combining a methods paper, a primary research article and a review — a format increasingly common in European doctoral programmes, including in Poland. The decision to build the entire experimental programme around a single, well-characterised in vitro model rather than spreading effort across multiple systems is defensible for a doctoral timeline and allowed the candidate to generate a genuinely multi-layered dataset (targeted validation plus unbiased transcriptomics) rather than a single superficial survey. The trade-off, discussed further in Section 7.3, is that conclusions about in vivo or human disease relevance necessarily remain inferential rather than demonstrated.

5. Evaluation of Methodological Rigor by advanced laboratory techniques

5.1 Cell culture and differentiation model: The RA-induced P19 differentiation protocol is standard in the field and is described with sufficient granularity (media composition, RA concentration, embryoid-body formation time, plating schedule) to be reproducible; this is corroborated by its independent publication as a stand-alone MethodsX protocol. Differentiation is validated by three independent readouts (morphology, Map2/Pax6 induction, Oct4 repression), which is good practice and reduces the risk that a single noisy assay drives the reported "successful differentiation" claim.

5.2 qPCR: Primer sequences and amplicon sizes are tabulated (Table 1 of the main thesis and the equivalent table in Publication 2), GAPDH is used consistently as the reference gene, and the 2^(-ΔΔCt) method is applied appropriately. One area for improvement: reference-gene stability itself (e.g., whether Gapdh expression is stable across the differentiation time-course and across MG132 treatment) is assumed rather than empirically verified against a second housekeeping gene, which would have strengthened confidence in the fold-change values, particularly given that differentiation itself is known to alter cellular metabolism and could plausibly shift housekeeping gene expression.

5.3 Western blotting and densitometry: Antibody catalogue numbers, dilutions, and detection reagents are consistently reported, which is good practice for reproducibility. Densitometric quantification is presented relative to β-actin loading controls throughout. As already noted in Section 6.1, the n=2 replication for the siRNA-titration experiment (Fig. 4a, three different siRNA sequences) is a genuine limitation; for a dose- or sequence-optimisation experiment intended to justify selection of a lead siRNA (siNFE2L1-1) for all downstream work, a somewhat larger n, or at least a clear statement that this particular panel was for selection purposes only and not intended to support a formal statistical claim, would be preferable.

5.4 Immunocytochemistry and image quantification: Confocal imaging parameters (objectives, laser lines, acquisition software) are described in useful detail. Quantification of corrected MAP2 fluorescence intensity and of ubiquitin aggregate counts per cell (based on 10 images from 2 biological replicates, 100 cells per

group) is a reasonable, if not extremely deep, sampling strategy; reporting whether image analysis was performed blind to experimental condition would strengthen confidence in these counts, particularly for the more subjective aggregate-counting metric.

5.5 RNA sequencing and bioinformatics: The pipeline (BGI sequencing, DESeq2 for differential expression, DAVID/KEGG for enrichment, the Dr. Tom platform for visualisation) is a conventional and appropriate choice. As discussed in Section 6.1, the principal gap is the absence of a clearly stated number of biological replicates per RNA-seq condition; this single omission is the most consequential methodological point raised in this report, because so much of the thesis's downstream candidate-gene selection rests on the RNA-seq DEG calls.

6. Strengths of the Thesis and the practical applicability of the obtained results

- Coherent narrative arc across the three publications, each building logically on the last (method → mechanism → synthesis).
- Appropriate and complementary combination of techniques: qPCR, western blot, immunocytochemistry with quantified signal intensity, and unbiased RNA-seq/KEGG enrichment, rather than reliance on a single readout.
- The RNA-seq-to-candidate-gene pipeline (differential expression → KEGG enrichment → PD-gene cross-referencing → targeted validation) is methodologically sound and transparently described.
- Data availability is properly handled: RNA-seq deposited in NCBI SRA under a public BioProject accession (PRJNA1196185), and reagent/antibody catalogue numbers are consistently reported, supporting reproducibility.
- The candidate has already achieved peer-reviewed publication of all three components in respectable, indexed journals, which is itself independent quality assurance of the work.
- The General Discussion and Conclusion sections honestly acknowledge the principal limitation of the work (in vitro, P19-based, not primary neurons or an in vivo model) and propose sensible, concrete future directions.
- The thesis's hypothesis-to-objective-to-result mapping (Sections 2–3 and 6 of the main text) is unusually clean for a cumulative dissertation, making it straightforward for the committee to verify that each stated aim was actually addressed by a corresponding experiment.
- The candidate demonstrates competence across a genuinely broad technical range — cell culture, RNAi, qPCR, western blotting, confocal immunocytochemistry with quantitative image analysis, and next-generation sequencing analytics — which is appropriate breadth for a doctoral training portfolio and suggests good preparation for independent research.

Taken together, these strengths indicate a candidate who has been trained to a good standard in molecular neuroscience methodology and who has already demonstrated the ability to bring a research programme through to peer-reviewed publication three times over. This is a meaningful marker of readiness for independent scientific work, independent of the specific critiques raised in the following sections.

7. Major Comments and Concerns

7.1 Statistical presentation and internal consistency: Throughout Section 6.2 and the accompanying figures, statistical significance is repeatedly reported with the wrong inequality direction — e.g., "Atf6 (**p>0.01)", "Camk2d (***p>0.001)", "Sod1 (**p>0.01)" — where the intended meaning, based on the reported asterisk convention and the underlying publication, is clearly $p<0.01$ / $p<0.001$. This is very likely a systematic transcription artefact (the ">" and "<" symbols appear transposed at multiple points in the thesis text), but it recurs consistently enough that it should be corrected throughout the final version before defense, since a reader relying on the thesis text alone (rather than the published PDF) would otherwise be misled about the direction of the reported effects.

Sample sizes for some western blot quantifications are very small: Figure 4 (siRNA knockdown validation) is explicitly reported as n=2 per group, yet is analysed with one-way ANOVA and asterisked as significant (*, **). With only two biological replicates, ANOVA assumptions (e.g., normally distributed variance estimates) cannot really be assessed, and the reported p-values should be interpreted and presented with more caution — for example by showing individual replicate values rather than only mean ± SEM with significance stars. The same caveat applies less severely to several n=3 comparisons, which is a common and largely accepted minimum in this field but leaves limited power to detect anything but large effects. No biological replicate number is stated for the RNA-seq experiment itself (Sections 4.7 and 6.2) — the Methods describe submission of RNA "from neurons treated with MG132, DMSO, and untreated wild-type controls" to BGI, and DESeq2 was used for differential expression, but DESeq2 is designed around replicated designs, and the number of biological replicates per condition is not stated anywhere in the thesis or Methods. This should be explicitly clarified, as it materially affects how much confidence can be placed in the reported DEG counts (1,549/1,655/8,810 genes, etc.) and the KEGG enrichment results that follow from them.

7.2 Table and cross-reference inconsistencies: There is a numbering inconsistency between the in-text citation and the table caption for the PD-related gene list: the running text states "…27 upregulated and 27 downregulated genes… Notably, 15 of the upregulated genes were found to be proteasome genes, while the remaining 12 belonged to other functional categories (Table 1)", yet the table that follows on the next page is captioned "Table 2: PD-Related genes Upregulated During Proteasome Inhibition in Neuronal Differentiated P19". This should be reconciled (either the in-text citation or the table numbering is off by one) before the final version is bound. Figure numbering restarts within each publication section (each of the three publications has its own "Fig. 1", "Fig. 2", etc.), which is acceptable convention for a cumulative/sandwich thesis but can be disorienting in the combined document, particularly in the General Discussion, where figures from different publications are discussed together without a unifying reference scheme. A global figure/table numbering scheme (or at minimum a per-publication prefix, e.g., "Fig. P2-3") would improve navigability for the committee.

7.3 Model and causal-inference limitations: The P19 embryonal-carcinoma-derived cells, while a well-established and convenient model for RA-induced neurogenesis, are repeatedly referred to in the text as "neurons" or "P19 neurons". They remain an immortalised, embryonal-carcinoma-derived line differentiated toward a neuronal-like phenotype, and are not primary post-mitotic neurons. The thesis's own Discussion and Conclusion appropriately flag this as a limitation, which is commendable, but the terminology used in the Results sections ("P19 neurons", "neuronal differentiated P19 cells") could more consistently signal this distinction, particularly given that a central argument of the Introduction is the special vulnerability of post-mitotic neurons to proteostasis failure.

The regulatory relationship between Nfe2l1 and Neat1 is described throughout as Nfe2l1 "regulating" or having a "specific regulatory role in Neat1 transcription", but the evidence presented is correlative (co-varying expression under knockdown and MG132 treatment); no direct mechanistic evidence (e.g., ChIP for Nfe2l1 binding at the Neat1 locus, or a rescue experiment) is provided to establish that this is a direct transcriptional effect rather than an indirect consequence of the broader disruption to differentiation and proteostasis caused by Nfe2l1 loss. The same caveat applies to the proposed links to Atf6, Camk2d and Sod1: these are consistent, plausible, and well-discussed, but should be described as associative rather than mechanistically demonstrated until a binding or epistasis experiment is performed. The Discussion is reasonably careful about this in places ("a potential regulatory role", "warrants further investigation"), and this careful hedging should be applied uniformly throughout. Several of the PD-gene mechanistic narratives in the General Discussion (e.g., on ATF6/UPR, CAMK2D/calcium signalling, SOD1/oxidative stress) draw heavily on studies in other cell types,

tissues, or disease models (fibroblasts, dopaminergic neuron cultures, other transgenic mice) to contextualise the P19 findings. This is reasonable scholarly practice, but the writing sometimes moves too quickly from "this has been observed elsewhere" to language implying that the same mechanism operates in the P19 system, without an explicit caveat each time. A brief, single unifying paragraph noting which of the proposed mechanisms are directly tested in this thesis versus inferred from the wider literature would sharpen the argument.

7.4a Presentation and readability of the compiled thesis: Because this is a cumulative dissertation, the same experiments are effectively described twice: once in the candidate's own synthesis (Sections 6.1–6.3 and 7 of the main text) and again in the reprinted full-text publications (Appendix C). This is standard practice for this thesis format and is not in itself a defect. Still, the degree of near-verbatim repetition between the synthesis chapters and the reprinted articles is quite high in places (for example, the Results narrative in Section 6.2 and the Results section of the reprinted Scientific Reports article are almost identical in wording). A somewhat more differentiated synthesis — for instance, one that spends relatively more space on cross-publication integration, on results that did not make it into any single paper, or on connecting Publication 3's broader ferroptosis/autophagy framework back to the specific Publication 2 findings — would make fuller use of the opportunity that the synthesis chapters provide, beyond re-presenting the published material.

7.4 Selection and justification of candidate genes: The rationale for testing exactly 12 non-proteasomal PD-associated genes (out of 27 upregulated) is clearly explained. However, it would strengthen the thesis to state explicitly why the 15 proteasome-subunit genes (Psmb2, Psmc2, Psmd1, etc., Supplementary Table 1) were excluded from the Nfe2l1-knockdown validation experiments, given that Nfe2l1 is classically defined by its role in transcribing proteasome subunit genes and might have been expected to show its clearest, most mechanistically direct effect there. If this was a deliberate choice to focus on less-studied, non-canonical targets, this should be stated as such.

8. Publication-by-Publication Comments

Publication 1 (MethodsX): This is a compact, competently executed methods paper. Its main scientific merit lies not in novel biology but in reproducibility infrastructure: a two-step embryonic-body/adherent differentiation protocol and a two-hit siRNA transfection schedule (days 5 and 8) are both described in sufficient step-by-step detail, including exact reagent catalogue numbers, incubation times and media formulations, that another laboratory could plausibly reproduce the workflow without needing to contact the authors. Validation is appropriately layered: morphological confirmation of embryonic body and neurite formation, qPCR confirmation of Map2/Pax6 induction and Oct4 repression, and western blot confirmation of NFE2L1 knockdown under MG132-stabilised conditions (since basal NFE2L1 protein is otherwise difficult to detect due to rapid proteasome turnover).

- The stated limitation that siRNA efficiency is sequence- and reagent-dependent is appropriate; consider explicitly reporting the knockdown percentage achieved (rather than only "dramatic reduction") for readers wanting to benchmark their own experiments.
- Because this protocol underlies both of the other two publications, any revision to its reported parameters (e.g., final knockdown efficiency, siRNA sequence selection rationale) should be kept consistent across all three components of the thesis.

Publication 2 (Scientific Reports): This is the central scientific contribution of the thesis and is held to the highest standard in this review. Its major strength, discussed already in Section 3.2 above, is the staged analytical pipeline moving from genome-wide RNA-seq, through KEGG pathway enrichment, to a defined candidate gene list, and finally to hypothesis-driven, factorial-designed validation experiments. This is good practice and distinguishes the work from purely descriptive candidate-gene studies.

- The immunocytochemistry quantification of MAP2 intensity and ubiquitin aggregate counts (Figs. 5–6) is a nice complement to western blotting, though cell/replicate counting methodology (e.g., blinding of the observer to condition) is not explicitly described and should be clarified.
- The claim that "ubiquitination is a normal physiological process enhanced under proteasome inhibition" is well supported; the additional claim that Nfe2l1 knockdown "further stimulates ubiquitin accumulation" is also well supported by both western blot and ICC, and is one of the more robust findings in the thesis.
- The selective Nfe2l1-dependence of Atf6, Camk2d and Sod1 induction, contrasted against the Nfe2l1-independence of Keap1, Ubb, Casp3, Eif2ak3, Tuba4a and Tuba8, is the single most interesting and best-supported novel result in the entire thesis, and should arguably be foregrounded even more prominently in the abstract and conclusion than it currently is.
- The Neat1/Malat1 comparison (Nfe2l1-dependent vs. Nfe2l1-independent lncRNA induction, respectively) is a nice internal control demonstrating specificity of the Nfe2l1 effect, though as noted in Section 7.3, the mechanism remains to be established.

Publication 3 (Frontiers review): This review is comprehensive, current (with an explicit and sensible decision to prioritise 2020–2024 literature), and well organised around the three proteostasis pathways (UPS, autophagy, ferroptosis) that structure the entire thesis. It is at its best when synthesising primary literature on NFE2L1 knockout phenotypes in vivo (Kobayashi et al., 2011; Lee et al., 2011) and post-mortem human tissue findings (reduced NFE2L1 in the Parkinson's disease substantia nigra), which meaningfully complement the in vitro findings of Publication 2 and help the candidate build a translational argument in the General Discussion.

- Comprehensive and up to date; disclosure of AI-assisted proofreading is transparent and appropriate, and the authors state clearly that they take responsibility for the accuracy of all statements, which is the correct way to handle such disclosures.
- The ferroptosis section, in particular, honestly notes that "there is no direct connection between NFE2L1 and ferroptosis in neurons" and that the arguments are extrapolated from non-neuronal tissue — this kind of explicit epistemic hedging is a model for how the more speculative parts of the General Discussion (Section 7 of the thesis) should also be framed.
- Table 1 within this review ("NFE2L1's impact on ferroptosis regulation in neurodegenerative diseases") is a useful, clearly organised synthesis, though it would benefit from an explicit column distinguishing findings that are directly demonstrated in neurons from those extrapolated from other cell types, consistent with the recommendation in Section 7.3.

9. Minor and Editorial Comments

- Systematic "p>" / "p<" inversion throughout Section 6.2 and Figures 7–8 legends (see Section 5.1 above) should be corrected.
- "Table 1" vs. "Table 2" cross-reference mismatch for the PD-related gene list (Section 5.2 above).
- Minor typographical issues: a duplicated word ("andand") appears in a figure legend; "Sic39a9" is used inconsistently for "Slc39a9" in some supplementary figure legends and table headers.
- The Appendix section heading skips from "10.1 Appendix A" to "10.2 Appendix C" with no Appendix B — likely a renumbering artefact that should be fixed for the final bound copy.
- Reference list formatting is generally good, but the same review (Łuczyńska et al., Redox Biology, volume 69, article 103003) is cited with publication years 2023 and 2024 in different parts of the reference list and in-text citations; this should be reconciled to a single, correct year.

- Consider adding page or figure cross-references between the thesis narrative (Sections 6.1–6.3) and the reprinted full-text publications in Appendix C, to help the committee move between the synthesis and the primary sources.

10. Questions for the Author (Suggested for the Defense)

1. How many independent biological replicates (not technical replicates) underlie the RNA-seq differential expression analysis, and how does this affect confidence in the DESeq2-derived gene counts and the downstream KEGG enrichment (Fig. 3)?
2. Given that Nfe2l1 is canonically defined by its transcriptional control of proteasome subunit genes, why were the 15 upregulated proteasome-subunit genes (Supplementary Table 1) not included in the functional Nfe2l1-knockdown validation panel, and would you predict a stronger or weaker Nfe2l1-dependence for these genes than for the 12 non-proteasomal genes that were tested?
3. What is the proposed mechanism by which Nfe2l1 knockdown selectively affects Atf6, Camk2d, Sod1 and Neat1 but not Keap1, Ubb, Casp3, Eif2ak3, Tuba4a or Tuba8 under the same MG132 challenge? Is there evidence (e.g., shared promoter elements, ARE motifs) that could distinguish direct from indirect Nfe2l1 targets among these genes?
4. Could the reduced Map2/Pax6 expression and neurite formation after Nfe2l1 knockdown reflect a general delay or toxicity effect of siRNA transfection/proteasome stress on differentiation, rather than an Nfe2l1-specific effect on neurogenesis per se? What controls (e.g., knockdown of an unrelated, non-proteostasis-related gene) would help distinguish these possibilities?
5. To what extent do you consider the P19 RA-differentiation model to recapitulate the vulnerability of terminally post-mitotic dopaminergic neurons specifically, given that Parkinson's disease pathology is highlighted throughout the thesis as the primary disease context?
6. Do you have preliminary evidence — ChIP, reporter assay, or otherwise — that would support a direct transcriptional relationship between NFE2L1 and NEAT1, as opposed to an indirect consequence of impaired proteostasis or differentiation?
7. Publication 3 discusses NFE2L1's role in mitochondrial homeostasis and mitophagy at some length, but this axis was not tested experimentally in Publication 2. Do you have a view on whether mitochondrial dysfunction could explain any of the phenotypes you observed (e.g., reduced Map2 expression, altered PD-gene expression) in the P19 model, and would this be a natural next experiment?
8. The KEGG enrichment analysis identifies Parkinson's disease as the top pathway under proteasome inhibition, but several of the other top-ranked pathways (e.g., "pathways of neurodegeneration – multiple diseases", spliceosome, ribosome biogenesis, cell cycle) are very broad, generic stress-response categories. How confident are you that the Parkinson's-disease enrichment reflects genuine disease-relevant biology specific to PD, as opposed to a general proteotoxic-stress signature that happens to overlap with the KEGG Parkinson's disease gene set?

Discussion and polemical remarks: I have no objections to making and regarding the merits of the dissertation and the results presented within it. Above all, I highly value the PhD student's commitment to carrying out extensive, labour-intensive research. His comprehensive approach to the subject matter presented in the dissertation deserves special emphasis.

11. Compliance, Data Availability, and Research Integrity

As an in vitro study using an established immortalised cell line, the thesis correctly states that institutional animal-ethics approval was not required, and this is documented consistently across all three publications.

RNA-seq data are deposited in the NCBI Sequence Read Archive under a public BioProject accession (PRJNA1196185), which is good practice and should be commended; the candidate is encouraged to confirm that this accession is public (rather than embargoed) at the time of the defense, since reviewers or committee members may wish to verify the deposited data directly. No conflicts of interest are declared, and authorship contribution statements (CRediT) are provided for at least one of the three publications (MethodsX), which is good practice; including equivalent CRediT statements for the other two publications in the thesis appendix, if not already present in the published versions, would further strengthen transparency around the candidate's individual contribution relative to co-authors, which is relevant for the committee's assessment of independent scholarly contribution.

The use of a generative AI tool for language proofreading in Publication 3 is disclosed appropriately, with an explicit statement that the authors reviewed and take responsibility for all AI-assisted text. This is consistent with current best practice guidance from most journals and funding bodies and should not be treated as a concern; if anything, its transparent disclosure reflects well on the candidate's research integrity practices more broadly.

12. Overall Recommendation

This is a competent, well-organised cumulative doctoral thesis built around three already peer-reviewed publications. The experimental work in Publication 2 is the scientific core and is generally sound, combining targeted molecular validation with unbiased transcriptomic profiling in a sensible and well-motivated pipeline; Publication 1 provides a useful methodological resource; and Publication 3 situates the experimental findings within a broad and current literature context. The principal weaknesses are presentational and statistical rather than conceptual: a recurring inequality-direction error in reported p-values, at least one table/text cross-reference mismatch, under-specified RNA-seq replication, and language that at times outruns the correlative nature of some of the reported gene-regulatory relationships (particularly regarding Neat1). None of these issues undermine the core contribution of the thesis, but all should be corrected in the final bound version, and several would benefit from clarification at the oral defense.

13. Summary of Results Final Conclusion

Recommendation for acceptance: In summary, the doctoral dissertation of Hossein Khodadadi deserves a very high rating. The doctoral student demonstrated well-grounded knowledge of the subject matter, as evidenced by his familiarity with the literature. The selection of the topic, the experimental approach to hypothesis testing, the use of advanced research techniques, and statistical analysis methods deserve special attention. Therefore, I believe that the doctoral dissertation of Hossein Khodadadi meets the requirements of the **Act of 14 March 2003 on Academic Degrees and Academic Titles (Journal of Laws No. 65/03 item 595, as amended)**. I hereby recommend and request the Scientific Council for the Discipline of Animal Sciences at the PAN Jastrzebiec to accept the doctoral thesis of Hossein Khodadadi to the next stages of the doctoral degree process. Finally, I recommend the PhD award with distinction (***wyróżnienie***).

22.07.2026

[Prof dr hab n wet. Chandra S Pareek]