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Supplementary information

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Genome-wide CRISPRi/a screens in human neurons link lysosomal failure to ferroptosis

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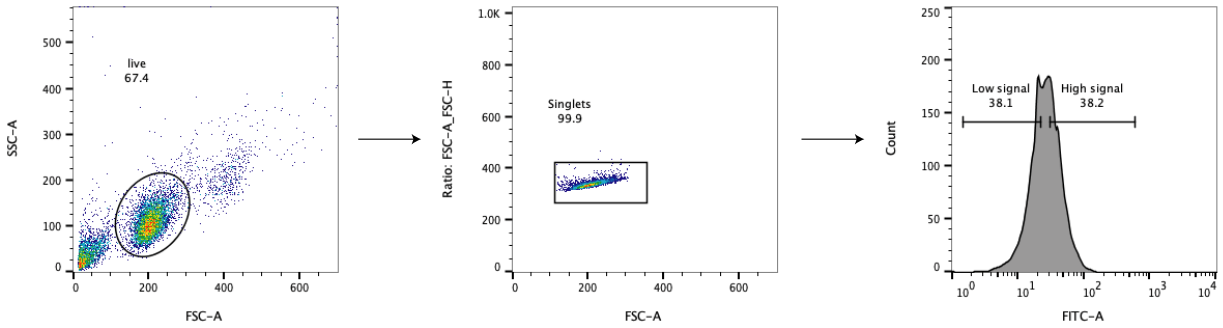

Supplementary Figure 1: Gating strategy for FACS-based screens.
Intact neurons were identified from the FSC-SSC plot and then gated for singlets. These cells were sorted into high and low signal populations corresponding to the top 40% and the bottom 40% of the staining signal distribution.

Supplementary Note

Background: Selective vulnerability of neurons to oxidative stress

Neurons, as one of the longest-living cell types in the human body, are challenged by various stresses in aging and disease. Due to their post-mitotic nature, neurons do not have the ability to 'self-renew' by cell division. Therefore, robust stress response mechanisms are required for neurons to maintain long-term health. One of the predominant types of stress in aging and neurodegenerative diseases is oxidative stress[1,2], which is induced by excessive accumulation of ROS in the cell. The brain is highly susceptible to ROS and ferroptosis, due to its high levels of oxygen consumption, abundant redox-active metals such as iron and copper, limited antioxidants and high levels of PUFAs[3]. A large body of evidence has implicated oxidative stress, iron accumulation and ferroptosis in neurodegenerative diseases[4–6], yet a comprehensive understanding of how neurons regulate redox homeostasis and maintain survival under oxidative stress is lacking. To this end, we decided to investigate neuronal redox homeostasis and survival under oxidative stress conditions using our CRISPRi/a screening platform.

Compared to other recent genetic screens focusing on oxidative stress toxicity in human cells[7,8], our screens are unique in the following aspects. First, we used post-mitotic neurons instead of

cancer cell lines. Second, we induced milder oxidative stress in cells by prolonged culture of cells in an antioxidant-free medium, compared to severe oxidative stress induced by paraquat or H_2O_2 in other studies. Third, we screened not only based on cell survival, but also on direct ROS and lipid peroxidation levels, while other studies focused on survival only. Given these major differences, it is not surprising that our screens identified many unique hits not identified in previous screens.

Insights from ROS and peroxidized lipid screens in neurons

Our screens for ROS and peroxidized lipid levels uncovered a number known ROS regulators beyond those discussed in the main text. These include positive regulators such as *PARP1*[9], *SAT1*[10] and *NOX5*[11] and negative regulators such as *PTEN*[12] and *FH*[13,14] showed the expected effects on ROS and/or peroxidized lipid levels in our screens

Numerous novel biological insights have emerged from our rich datasets. *GPX4* and genes related to *GPX4* synthesis are indispensable for neurons to survive oxidative stress. Given the major role of *GPX4* in reducing lipid peroxidation and suppressing ferroptosis, this result suggests that lipid peroxidation-induced ferroptosis, rather than other forms of cell death, may be the main cause of neuronal loss under oxidative stress conditions that are commonly found in the brains of patients with neurodegenerative diseases. This is supported by numerous studies reporting high levels of iron and lipid peroxidation in neurodegenerative disease patient brains[15,16], and by the finding that ferroptosis inhibitors such as iron chelators and the lipid-peroxidation inhibitor Ferrostatin-1 are neuroprotective in animal and cellular models of neurodegenerative diseases[17–20].

We also found that that disruption of lipid metabolism, in particular glycosphingolipid degradation in the lysosome, by loss of prosaposin (*PSAP*), drives the formation of lipofuscin in neurons, which leads to iron accumulation and strongly induces ROS production, oxidizing lipids and leading to neuronal ferroptosis under oxidative stress. While lipofuscin has traditionally been considered a byproduct of aging and as a consequence of defective cellular homeostasis, its physiological and pathological functions have not been well characterized. This is largely due to the lack of robust systems to model lipofuscin given the fact that lipofuscin is normally only formed in aged post-mitotic cells. Our *PSAP* KO neurons provide a reliable genetic system to model and study the biology of lipofuscin in young, live human neurons. Our result suggests a direct pathogenic role of lipofuscin in inducing neuronal ferroptosis. The accumulation of lipofuscin is a pathological hallmark of many degenerative diseases, such as neuronal ceroid lipofuscinosis (NCL)[21] and inherited age-related macular degenerations[22,23]. It has also been characterized in Alzheimer's disease[24,25], Parkinson's disease[26], Hungtinton's disease[27,28] and GRN-associated frontotemporal dementia (FTD)[29]. Our findings suggest that inhibiting lipofuscin formation or subsequent ferroptosis may serve as new therapeutic strategies for these diseases.

Our results highlight the importance of lipid homeostasis, in particular balanced levels of glycosphingolipids, for neuronal health. Among all tissues in the human body, the brain is one of the richest in lipid content and lipid diversity. Lipids are not only an essential structural component of membranes, but also important signaling molecules in the brain[30]. Therefore,

maintaining lipid homeostasis is of vital importance for brain cells, especially neurons with long neurites and dynamic synaptic vesicles release and recycling. Abnormal lipid metabolism has been observed in neurodegenerative diseases[31–33]. Accumulation of some glycosphingolipids, especially simple gangliosides, caused by inhibition of lysosome membrane recycling, contributes to neurodegeneration both in cultured neurons and in animal models[34].

Insights from CROP-Seq screens

Knockdown of *VPS54*, *PAXIP1*, and *PON2* caused highly correlated transcriptomic changes. *VPS54* and PON2 have been implicated in Amyotrophic Lateral Sclerosis (ALS)[35,36] whereas PAXIP1 is associated with Alzheimer's disease (AD)[37]. Shared transcriptomic changes included: (i) increased expression of EBF3, an apoptosis inducer[38] that is also upregulated in the hippocampus of AD model mice[39], (ii) decreased expression of components of neurofilaments, namely *NEFL*, *NEFM*, and *NEFH*. Importantly, significantly decreased expression of *NEFL* and *NEFM* was also found in neurons of early-stage AD patients in a recent single-cell transcriptomic study[40] and (iii) decreased expression of other genes important for neuronal function, including *PCDH11X* and *PCDH11Y*, encoding protocadherin proteins, and *SYT2*, encoding synaptotagmin 2.

We found that overexpression of NQO1 was toxic to neurons, which was unexpected since elevated levels and activity of NQO1 have been found in the brains of patients with different neurodegenerative diseases[41–46], as well as in patient iPSC-derived neurons[46].

Open questions

Many important questions around neuronal lipid and redox homeostasis and ferroptosis remain to be investigated. For example, since neurons have very long neurites, how do neurons sense and respond to a local lipid peroxidation event on the cell membrane? Are GPX4 or other enzymes recruited to sites of lipid peroxidation, or are peroxidized lipids internalized and delivered to GPX4 or other enzymes for detoxification? What are the sensors and mediators in these processes? How do peroxidized lipids cause death? Is it a passive physical process or a regulated biological program, and are there neuron-specific aspects of ferroptosis? If the latter is true, what are the players mediating cell death downstream of lipid peroxidation? Many of these questions can be readily investigated using our functional genomics platform.

Areas for technology development

There are several areas for future technology development. First, a robust inducible CRISPRi system that allows temporal control of gene knockdown in mature neurons will help avoiding false-positive screening phenotypes due to interference with the differentiation process. For example, in the CRISPRi survival screen, we unexpectedly identified genes in the N6-methyltransferase writer complex, including *ZC3H13*, *METTL3*, and *METTL4*, as strong protective hits when knocked down (Fig. 1e). The N6-methyltransferase writer complex is responsible for m6A RNA modification, which regulates a variety of biological processes including neuronal differentiation[47,48]. The inducible CRISPRi system will help to determine whether the strong protective phenotypes of these genes are false positive due to defects in

neuronal differentiation. Second, we have previously used our platform for arrayed high-content imaging screens for complex neuronal phenotypes, such as neurite outgrowth[49]. Such imaging-based screens could be applied to study other neuronal phenotypes, such as electrophysiological signals (by voltage imaging or calcium imaging), synaptic vesicle dynamics and axonal transport, and cell non-autonomous phenotypes such as glia-neuron interaction. Imaging phenotyping can also be coupled with simultaneous transcriptomic profiling, thus allowing the association of gene expression with imaging phenotypes for a given genetic perturbation. Lastly, by combining our platform with base editor or prime editing technology[50], we will be able to directly assess the effect of disease-associated mutations in human neurons on various cellular phenotypes in a scalable, massively parallel format. While base editor screens to assess human variants have been recently conducted in cancer cell lines[51,52], it is of great interest to investigate how variants associated with different human diseases could affect various cell types differently given the fact that a lot of human diseases are tissue-specific.

References

1. Barnham, K. J., Masters, C. L. & Bush, A. I. Neurodegenerative diseases and oxidative stress. *Nat. Rev. Drug Discov.* **3,** 205–214 (2004).
2. Finkel, T. & Holbrook, N. J. Oxidants, oxidative stress and the biology of ageing. *Nature* **408,** 239–247 (2000).
3. Patel, M. Targeting oxidative stress in central nervous system disorders. *Trends Pharmacol. Sci.* **37,** 768–778 (2016).
4. Lin, M. T. & Beal, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* **443,** 787–795 (2006).
5. Rouault, T. A. Iron metabolism in the CNS: implications for neurodegenerative diseases. *Nat. Rev. Neurosci.* **14,** 551–564 (2013).
6. Stockwell, B. R. *et al.* Ferroptosis: A regulated cell death nexus linking metabolism, redox biology, and disease. *Cell* **171,** 273–285 (2017).
7. Dubreuil, M. M. *et al.* Systematic Identification of Regulators of Oxidative Stress Reveals Non-canonical Roles for Peroxisomal Import and the Pentose Phosphate Pathway. *Cell Rep.* **30,** 1417–1433.e7 (2020).
8. Reczek, C. R. *et al.* A CRISPR screen identifies a pathway required for paraquat-induced cell death. *Nat. Chem. Biol.* **13,** 1274–1279 (2017).
9. Hocsak, E. *et al.* PARP inhibition protects mitochondria and reduces ROS production via PARP-1-ATF4-MKP-1-MAPK retrograde pathway. *Free Radic. Biol. Med.* **108,** 770–784 (2017).
10. Ou, Y., Wang, S.-J., Li, D., Chu, B. & Gu, W. Activation of SAT1 engages polyamine metabolism with p53-mediated ferroptotic responses. *Proc. Natl. Acad. Sci. USA* **113,** E6806–E6812 (2016).

11. Bánfi, B. *et al.* A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J. Biol. Chem.* **276,** 37594–37601 (2001).
12. Bankoglu, E. E. *et al.* Role of PTEN in Oxidative Stress and DNA Damage in the Liver of Whole-Body Pten Haplodeficient Mice. *PLoS One* **11,** e0166956 (2016).
13. Sudarshan, S. *et al.* Fumarate hydratase deficiency in renal cancer induces glycolytic addiction and hypoxia-inducible transcription factor 1alpha stabilization by glucose-dependent generation of reactive oxygen species. *Mol. Cell. Biol.* **29,** 4080–4090 (2009).
14. Bardella, C. *et al.* Cells lacking the fumarase tumor suppressor are protected from apoptosis through a hypoxia-inducible factor-independent, AMPK-dependent mechanism. *Mol. Cell. Biol.* **32,** 3081–3094 (2012).
15. Ndayisaba, A., Kaindlstorfer, C. & Wenning, G. K. Iron in Neurodegeneration - Cause or Consequence? *Front. Neurosci.* **13,** 180 (2019).
16. Han, C. *et al.* Ferroptosis and its potential role in human diseases. *Front. Pharmacol.* **11,** 239 (2020).
17. Kupershmidt, L., Amit, T., Bar-Am, O., Weinreb, O. & Youdim, M. B. H. Multi-target, neuroprotective and neurorestorative M30 improves cognitive impairment and reduces Alzheimer's-like neuropathology and age-related alterations in mice. *Mol. Neurobiol.* **46,** 217–220 (2012).
18. Kaur, D. *et al.* Genetic or pharmacological iron chelation prevents MPTP-induced neurotoxicity in vivo: a novel therapy for Parkinson's disease. *Neuron* **37,** 899–909 (2003).
19. Chen, J. *et al.* Iron accumulates in Huntington's disease neurons: protection by deferoxamine. *PLoS One* **8,** e77023 (2013).
20. Skouta, R. *et al.* Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. *J. Am. Chem. Soc.* **136,** 4551–4556 (2014).
21. Anderson, G. W., Goebel, H. H. & Simonati, A. Human pathology in NCL. *Biochim. Biophys. Acta* **1832,** 1807–1826 (2013).
22. Delori, F. C. *et al.* In vivo measurement of lipofuscin in Stargardt's disease--Fundus flavimaculatus. *Invest. Ophthalmol. Vis. Sci.* **36,** 2327–2331 (1995).
23. Dorey, C. K., Wu, G., Ebenstein, D., Garsd, A. & Weiter, J. J. Cell loss in the aging retina. Relationship to lipofuscin accumulation and macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **30,** 1691–1699 (1989).
24. Dowson, J. H., Mountjoy, C. Q., Cairns, M. R. & Wilton-Cox, H. Changes in intraneuronal lipopigment in Alzheimer's disease. *Neurobiol. Aging* **13,** 493–500 (1992).
25. Dowson, J. H., Mountjoy, C. Q., Cairns, M. R. & Wilton-Cox, H. Alzheimer's disease: distribution of changes in intraneuronal lipopigment in the frontal cortex. *Dementia* **6,** 334–342 (1995).
26. Braak, E. *et al.* alpha-synuclein immunopositive Parkinson's disease-related inclusion bodies in lower brain stem nuclei. *Acta Neuropathol.* **101,** 195–201 (2001).
27. Braak, H. & Braak, E. Allocortical involvement in Huntington's disease. *Neuropathol Appl Neurobiol* **18,** 539–547 (1992).
28. Goebel, H. H., Heipertz, R., Scholz, W., Iqbal, K. & Tellez-Nagel, I. Juvenile Huntington chorea: clinical, ultrastructural, and biochemical studies. *Neurology* **28,** 23–31 (1978).
29. Ward, M. E. *et al.* Individuals with progranulin haploinsufficiency exhibit features of neuronal ceroid lipofuscinosis. *Sci. Transl. Med.* **9,** (2017).
30. Bazan, N. G. Lipid signaling in neural plasticity, brain repair, and neuroprotection. *Mol. Neurobiol.* **32,** 89–103 (2005).

31. Isacson, O., Brekk, O. R. & Hallett, P. J. Novel results and concepts emerging from lipid cell biology relevant to degenerative brain aging and disease. *Front. Neurol.* **10,** 1053 (2019).
32. Hallett, P. J., Engelender, S. & Isacson, O. Lipid and immune abnormalities causing age-dependent neurodegeneration and Parkinson's disease. *J. Neuroinflammation* **16,** 153 (2019).
33. Yadav, R. S. & Tiwari, N. K. Lipid integration in neurodegeneration: an overview of Alzheimer's disease. *Mol. Neurobiol.* **50,** 168–176 (2014).
34. Boutry, M. *et al.* Inhibition of Lysosome Membrane Recycling Causes Accumulation of Gangliosides that Contribute to Neurodegeneration. *Cell Rep.* **23,** 3813–3826 (2018).
35. Slowik, A. *et al.* Paraoxonase gene polymorphisms and sporadic ALS. *Neurology* **67,** 766–770 (2006).
36. Schmitt-John, T. *et al.* Mutation of Vps54 causes motor neuron disease and defective spermiogenesis in the wobbler mouse. *Nat. Genet.* **37,** 1213–1215 (2005).
37. Chen, J. A. *et al.* A multiancestral genome-wide exome array study of Alzheimer disease, frontotemporal dementia, and progressive supranuclear palsy. *JAMA Neurol.* **72,** 414–422 (2015).
38. Zhao, L. Y. *et al.* An EBF3-mediated transcriptional program that induces cell cycle arrest and apoptosis. *Cancer Res.* **66,** 9445–9452 (2006).
39. Gu, C. *et al.* Long Noncoding RNA EBF3-AS Promotes Neuron Apoptosis in Alzheimer's Disease. *DNA Cell Biol* **37,** 220–226 (2018).
40. Mathys, H. *et al.* Single-cell transcriptomic analysis of Alzheimer's disease. *Nature* **570,** 332–337 (2019).
41. SantaCruz, K. S., Yazlovitskaya, E., Collins, J., Johnson, J. & DeCarli, C. Regional NAD(P)H:quinone oxidoreductase activity in Alzheimer's disease. *Neurobiol. Aging* **25,** 63–69 (2004).
42. Wang, Y., Santa-Cruz, K., DeCarli, C. & Johnson, J. A. NAD(P)H:quinone oxidoreductase activity is increased in hippocampal pyramidal neurons of patients with Aalzheimer's disease. *Neurobiol. Aging* **21,** 525–531 (2000).
43. Bian, J.-T., Zhao, H.-L., Zhang, Z.-X., Bi, X.-H. & Zhang, J.-W. Association of NAD(P)H:quinone oxidoreductase 1 polymorphism and Alzheimer's disease in Chinese. *J. Mol. Neurosci.* **34,** 235–240 (2008).
44. van Muiswinkel, F. L. *et al.* Expression of NAD(P)H:quinone oxidoreductase in the normal and Parkinsonian substantia nigra. *Neurobiol. Aging* **25,** 1253–1262 (2004).
45. Raina, A. K., Templeton, D. J., Deak, J. C., Perry, G. & Smith, M. A. Quinone reductase (NQO1), a sensitive redox indicator, is increased in Alzheimer's disease. *Redox Rep* **4,** 23–27 (1999).
46. Imaizumi, Y. *et al.* Mitochondrial dysfunction associated with increased oxidative stress and α-synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue. *Mol. Brain* **5,** 35 (2012).
47. Edens, B. M. *et al.* FMRP Modulates Neural Differentiation through m6A-Dependent mRNA Nuclear Export. *Cell Rep.* **28,** 845–854.e5 (2019).
48. Li, M. *et al.* Ythdf2-mediated m6A mRNA clearance modulates neural development in mice. *Genome Biol.* **19,** 69 (2018).
49. Tian, R. *et al.* CRISPR Interference-Based Platform for Multimodal Genetic Screens in Human iPSC-Derived Neurons. *Neuron* **104,** 239–255.e12 (2019).

50. Anzalone, A. V. *et al.* Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576,** 149–157 (2019).
51. Hanna, R. E. *et al.* Massively parallel assessment of human variants with base editor screens. *Cell* **184,** 1064–1080.e20 (2021).
52. Cuella-Martin, R. *et al.* Functional interrogation of DNA damage response variants with base editing screens. *Cell* **184,** 1081–1097.e19 (2021).