Transcriptomic analysis of loss of Gli1 in neural stem cells responding to demyelination in the mouse brain

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ABSTRACT

In the adult mammalian brain, Gli1 expressing neural stem cells reside in the subventricular zone and their progeny are recruited to sites of demyelination in the white matter where they regenerate oligodendrocytes, the myelin forming cells. Remarkably, genetic loss or pharmacologic inhibition of Gli1 enhances the efficacy of remyelination by these neural stem cells. To understand the molecular mechanisms involved, we performed a transcriptomic analysis of this Gli1-pool of neural stem cells. We compared murine NSCs with either intact or deficient Gli1 expression from adult mice on a control diet or on a cuprizone diet to induce widespread demyelination. These data will be a valuable resource for identifying therapeutic targets for enhancing remyelination in demyelinating diseases like multiple sclerosis.

BACKGROUND & SUMMARY

Multiple Sclerosis (MS) is the most common cause of neurological disability in young adults[1] and is characterized by inflammatory demyelination leading to axonal injury. In addition to preventing immune-mediated demyelination, a major therapeutic goal in MS is to enhance new myelin sheath formation by remyelination which prevents the loss of axons and restores function[2-4]. The nervous system is capable of remyelination as observed in autopsy specimens from MS patients characterized by short, thin myelin sheaths[5-8]. However, remyelination ultimately fails, particularly in MS patients with progressive disease[8,9] and the factors that limit remyelination remain poorly understood[9,10].

There are three sources of remyelinating cells in the adult brain - neural stem cells (NSCs)[11-13], oligodendrocyte progenitor cells (OPCs)[14-16] and mature oligodendrocytes[17,18]. NSCs reside in the subventricular zone (SVZ) while the OPCs and oligodendrocytes are present throughout the parenchyma in the adult brain and are recruited locally to regenerate myelin in demyelinated lesions[15,19]. Furthermore, NSCs can promote remyelination either by replenishing the adult OPCs recruited into lesions, or as a direct source of oligodendrocytes themselves. Consistently, prior studies have shown that NSCs in the SVZ are activated by local demyelination, migrate out and differentiate into OPCs that generate remyelinating oligodendrocytes[11,13,14,20]. Indirect marker studies of postmortem brains further suggest that NSCs proliferate during acute attacks of MS and are a source of remyelinating cells in the human brain[12,21,22]. More importantly, ablating NSCs results in axonal loss highlighting their significant functional contribution to remyelination[23]. Taken together, these studies suggest remyelination by

NSCs protects axons. Thus, enhancing remyelination by NSCs is expected to provide a neuroprotective strategy in MS.

The adult SVZ is comprised of a functional, quiescent neural stem cell population, which are multipotent, divide slowly, self-renew, and give rise to proliferating, transit-amplifying cells[24]. There is considerable heterogeneity among these NSCs, indicated by their expression of specific transcription factors and generation of distinct cells during homeostasis and in response to injury[25]. One NSC subset, which is enriched in the ventral SVZ, expresses the transcription factor Gli1 and generates interneurons in the olfactory bulb and astrocytes in the healthy mouse brain[26,27]. This population of NSCs is also present in the human SVZ[27] as well as in NSCs derived from human induced pluripotent stem cells and human embryonic stem cells[28]. Remarkably, and in contrast to its fate in the healthy adult brain, this Gli1 pool of NSCs is specifically recruited to the white matter in response to demyelination and go on to differentiate into remyelinating oligodendrocytes[20,27,29]. Inhibition of Gli1 in these cells further enhances their remyelination potential and is neuroprotective resulting in functional improvement in mouse models of MS[27,28]. Importantly, Gli1 expressing stem-like cells respond to injury in other organs like the liver, lung, bone marrow, kidney and heart with loss of Gli1 resulting in attenuated injury in these organs[30-37]. Thus, inhibition of Gli1 is potentially an important therapeutic strategy for regenerative medicine and the molecular mechanisms by which Gli1 affects stem cell repair may be shared in many different types of tissues.

To identify Gli1-regulated genes that affect NSC recruitment and generation of remyelinating oligodendrocytes, we performed a transcriptomic analysis. We isolated and compared RNAs from NSCs with genetic loss of Gli1 vs. those with intact Gli1

expression from both healthy and demyelinated adult mouse brains (Fig. 1). These data will be valuable not only for future remyelination studies but may also provide mechanistic insights into the shared principles of regeneration across organs.

METHODS

Fate-mapping of neural stem cells and demyelination: All the genetically modified mice on C57bl/6 background were allowed access to diet and water *ad libitum* and maintained according to protocols approved by New York University Medical Center's IACUC. The *Gli1*$^{CreERT2/+}$ mice (Jax # 007913) which have CreERT2 knocked into the Gli1 locus were bred with the reporter Rosa-CAG-EGFP-LoxP (RCE) (Jax# 032037) mice to generate *Gli1*^{Het} (*Gli1*$^{CreERT2/+}$*;RCE*) with intact *Gli1* expression and *Gli1*^{Null} (*Gli1*$^{CreERT2/CreERT2}$*;RCE*) mice with global knockout of *Gli1*. To permanently label the Gli1 pool of NSCs with GFP, we administered 5 mg tamoxifen (Sigma) in corn oil on alternate days for a total of four intraperitoneal injections to 10 week old mice. We did not observe any labeling in the absence of tamoxifen administration. Mice used for GFP negative controls received intraperitoneal injections of corn oil without Tamoxifen. A week after the last dose of tamoxifen/corn oil, the demyelinated group was fed 0.2% cuprizone in the chow for 3 weeks while the healthy control group remained on regular chow. All mice had access to food and water *ad libitum* and were housed in a room with 12 hour dark/light cycle. Cuprizone diet induces apoptosis of mature oligodendrocytes leading to maximum demyelination of the white matter corpus callosum after 5 weeks of continuous feeding[38]. Here, we used 3 weeks of cuprizone diet for our analysis since we previously found that *Gli1*^{Null} NSCs in the SVZ have significantly higher proliferation at

this timepoint compared to *Gli1*^{Het} SVZ, suggesting maximal activation coincides with this early stage of demyelination[27,39].

Dissection of SVZ: Brain tissues were harvested from 6 (3 Males and 3 Females) mice per genotype (*Gli1*^{Het} and *Gli1*^{Null}) per diet (cuprizone and control) after euthanizing them with CO_2. The brain was removed gently out of the skull and immediately transferred to a Petri dish chilled on ice and placed with the dorsal surface facing up on a chilled acrylic mouse brain matrix. The brains of mice were dissected according to previously published protocols[40]. Coronal sections of 1mm thickness were made from the rostral end of the brain up to the dorsal hippocampus posteriorly, using a sharp and chilled razor blade. The SVZ was identified as a thin layer of tissue lining the medial and lateral walls of the lateral ventricles and carefully dissected under a microscope from each coronal brain section.

Dissociation and FACsorting of neural stem cells: For each RNA sample, the SVZ from one female and one male mice was pooled together. Overall, we had 3 RNA samples/genotype from control diet group and 3 RNA samples/genotype from cuprizone diet group in this study. The SVZ were then dissociated into single cell suspension following the manufacturer's protocol in the papain dissociation kit (Worthington Biochemicals # LK003150). Briefly, the dissected SVZ tissue was minced using iris scissors and digested by incubating in a solution containing 20 Units/ml papain and 0.005% DNase for 30minutes at 37˚C. Subsequently, the papain was inactivated with albumin-ovomucoid inhibitor and the digested tissue was needle triturated to obtain a cell suspension.

The cell suspension was passed through a 50 µm celltrix filter (Sysmex-Partec # 04-0042-2317) immediately before FACsorting the GFP+ NSCs using a MoFlo XDP cell sorter (Beckman Coulter) in the NYU Cytometry core facility. NSCs harvested from *Gli1*^{Het} or *Gli1*^{Null} mice treated with corn oil served as negative controls for FACsorting. A forward scatter vs. side scatter dot plot was used to gate the primary cell population which was then sorted for fluorescence using a FITC filter. The histograms were set as follows: (R1) FSC-area vs. SSC-area, (R2) SSC-width vs. SSC-area, (R3) FITC-log vs. PE-log. NSCs in (R1) were gated to (R2) which were then gated to (R3) (Fig.2). On average, the cell sorting yielded 17,666.7 ± 4163.3 GFP+ cells in the *Gli1*^{Het} healthy group on control diet, 7,833.3 ± 2,112.7 GFP+ cells in the *Gli1*^{Het} demyelination group on cuprizone diet, 41,333.3 ± 5033.2 GFP+ cells in the *Gli1*^{Null} healthy group on control diet and 34,000 ± 10,816.7 GFP+ cells in the *Gli1*^{Null} demyelination group on curpizone diet.

Total RNA isolation: The GFP positive neural stem cells were collected in RNA extraction buffer (Arcturus PicoPure RNA isolation kit, Applied Biosystems # 12204-01) after FACsorting and total RNA was isolated following the manufacturer's instructions. The isolated RNA was eluted in 15 µl of elution buffer following on column DNAse1 treatment using RNase-free DNase set (Qiagen # 79254). The total RNA was assessed for RNA integrity with a bioanalyzer (Agilent) in the NYU Genome Technology Center. RNA samples with a RIN of more than 8 were submitted to NYU Genome Technology Center for Next Generation Sequencing (NGS).

Library preparation and RNA sequencing: mRNA was purified and the cDNA was synthesized with the mRNA fragments as templates using SMART-Seq V4 RNA kit

(Clontech # 634889). The sequencing libraries were generated using Nextera XT DNA Library Preparation Kit (Illumina #FC-131-1024), which uses an engineered transposome to simultaneously fragment and barcode the cDNA, adding unique short adapter sequences. The barcoded cDNAs were then amplified by 6 cycles of PCR and their quality was checked for size, quantity and integrity by TapeStation system (Agilent) as well as qPCR for precise quantification. The generated libraries were then mixed for multiplexing on 3 lanes and sequenced on Illumina HiSeq 2500 platform with 150bp paired end modules, which yielded mapped reads in the range of 16 - 39 million per sample.

Data transformation and downstream analysis: The data were transformed and analyzed by a bioinformatician in the NYU Genome Technology Center. Briefly, the raw sequencing data files were initially processed using FASTQC v.0.11.5 in order to perform quality control checks. Subsequently the Fastq files were aligned to the mm10 mouse reference genome utilizing TopHat v2.1.1. About 93% to 96% of the RNAseq reads, including R1 reads in the 5` to 3` forward direction and R2 reads in the reverse direction, mapped to the mouse genome. The aligned bam files were then quantified for gene expression values utilizing HTSeq-count 0.11.1. Finally DESeq2 v1.12 was used for analysis of the quantified count matrix[41]. Genes that had an adjusted p-value (False Discovery Rate) of less than 0.05 were considered significantly different for respective pairwise comparisons.

DATA RECORDS

The sequencing data were deposited at the NCBI Gene Expression Omnibus (GEO) under the accession number GSE162683 (Table 2). Tables with data including RNA

quality, barcode lane statistics, RNA read counts, alignment statistics, DEseq2 expression counts and the data comparison between the groups can be accessed in Figshare.

TECHNICAL VALIDATION

FACsorting: As our negative control for FACsorting, we used cells isolated from the brains of mice with the same genotype but injected with corn oil instead of tamoxifen such that GFP is not expressed. These GFP negative cells were used to set up the gating parameters before GFP positive cells were sorted which ensured that only GFP positive neural stem cells were collected after sorting (Fig. 2).

RNA quality: The quality of total RNA was assessed by RNA Integrity Number (RIN) using the Agilent Bioanalyzer. Following the standard practice in NGS, we used RNA with RIN>8 for our sequencing analysis.

Genotypic and phenotypic assessment: Before harvesting the neural stem cells, we collected the tail tissue from all the mice following euthanasia. These tissue were used for PCR analysis to reconfirm the genotypes of the mice used to isolate neural stem cells. We also obtained a higher number of GFP positive cells from *Gli1*^{Null} mice compared to *Gli1*^{Het} mice, consistent with higher proliferation rates of NSCs in the *Gli1*^{Null} mice[27], further validating their phenotype. An analysis of differential gene expression in healthy *Gli1*^{Het} NSCs vs. healthy *Gli1*^{Null} NSCs showed a significant difference in the expression on only one gene i.e. *Gli1*, which not only confirmed the genotype but also confirmed the well-established lack of phenotype in the healthy *Gli1*^{Null} mice[42] (Fig. 3 and Table 1). In contrast, there were substantial differences (i.e. 1507 genes) in the transcriptomes of het vs. null NSCs on cuprizone for 3 weeks (Fig. 3,

Table 2), consistent with their very distinct responses to demyelination. Many of these genes were downregulated in the nulls in agreement with the role of Gli1 as a transcriptional activator [43,44].

Clustering of RNAseq data: A 3D Principle Component Analysis (PCA) plot visualized by Plotly (Fig.4) showed clustering of the samples in the control diet groups from both *Gli1*^{Het} and *Gli1*^{Null} neural stem cells; similarly the cuprizone diet RNA samples from both genotypes clustered together but away from the control diet groups.

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AUTHOR CONTRIBUTIONS

JS designed and performed the experiments, and wrote the manuscript. JLS oversaw the project and edited the manuscript.

COMPETING INTERESTS

A patent on the method of targeting GLI1 as a strategy to promote remyelination has been awarded, with J. L. Salzer, J. Samanta and G. Fishell listed as co-inventors. JLS is a consultant for and has ownership interests in Glixogen Therapeutics.

ADDITIONAL INFORMATION

Correspondence and request for materials should be addressed to JS and JLS.

FIGURE LEGEND

Figure 1: Schematic overview of the study design.

A flowchart demonstrates the experimental design and data analysis.

Figure 2: Fluorescence-activated cell sorting of fate-mapped neural stem cells.

Top panel shows the gating parameters used to delineate the R3 region with GFP negative cells which were then used to isolate GFP-positive cells as shown in the bottom panel. SSC- Side scatter, FSC- Forward scatter.

Figure 3: Visualization of differential gene expression in Gli1 het and null NSCs

MA plots of the RNAseq data show the differences in expression of genes between the two genotypes on control and cuprizone diets. The blue box highlights the only gene differentially expressed between $Gli1^{Het}$ and $Gli1^{Null}$ NSCs on the control diet groups, corresponding to Gli1 itself. Ctrl.- Control diet group, Cup.-Cuprizone diet group.

Figure 4: Principle component analysis (PCA) of the sequencing data.

A 3D PCA plot shows segregation of the control diet samples from the cuprizone diet samples. The variance is higher in the $Gli1^{Het}$ samples compared to the $Gli1^{Null}$ samples.

Table 1: Number of differentially expressed genes.

Genes that had an adjusted p-value (False Discovery Rate) of less than 0.05 and fold change of more than 2 or less than 0.5 in the compared groups are quantified.

Table 2: NCBI GEO accession numbers of samples

Accession number of the samples included in the the NCBI GEO submission GSE162683.

Figure 1: A schematic overview of the study design

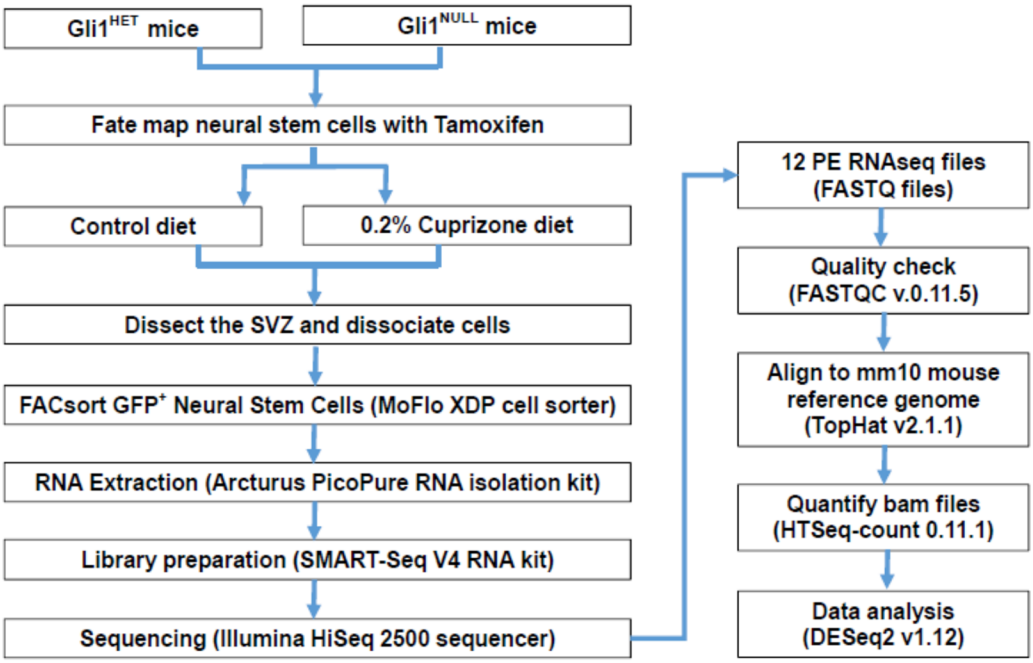

Figure 2: FACsorting neural stem cells

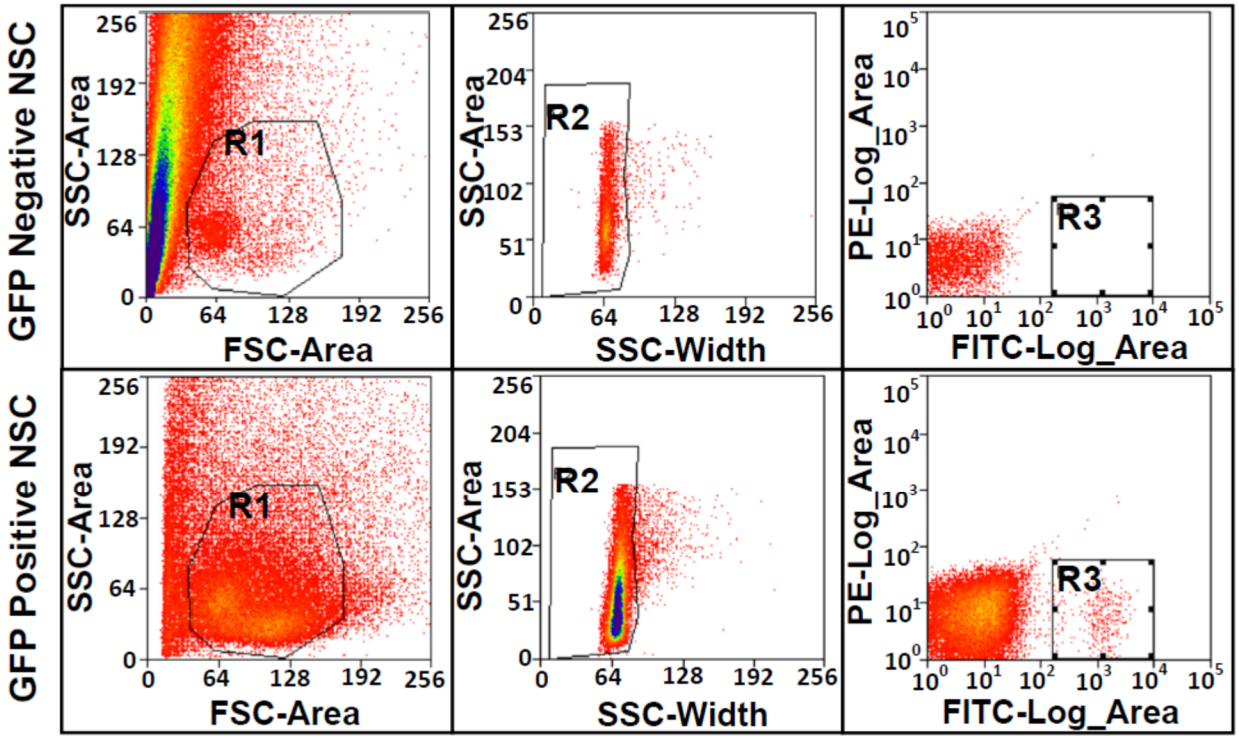

Figure 3: MA plots of the RNAseq data

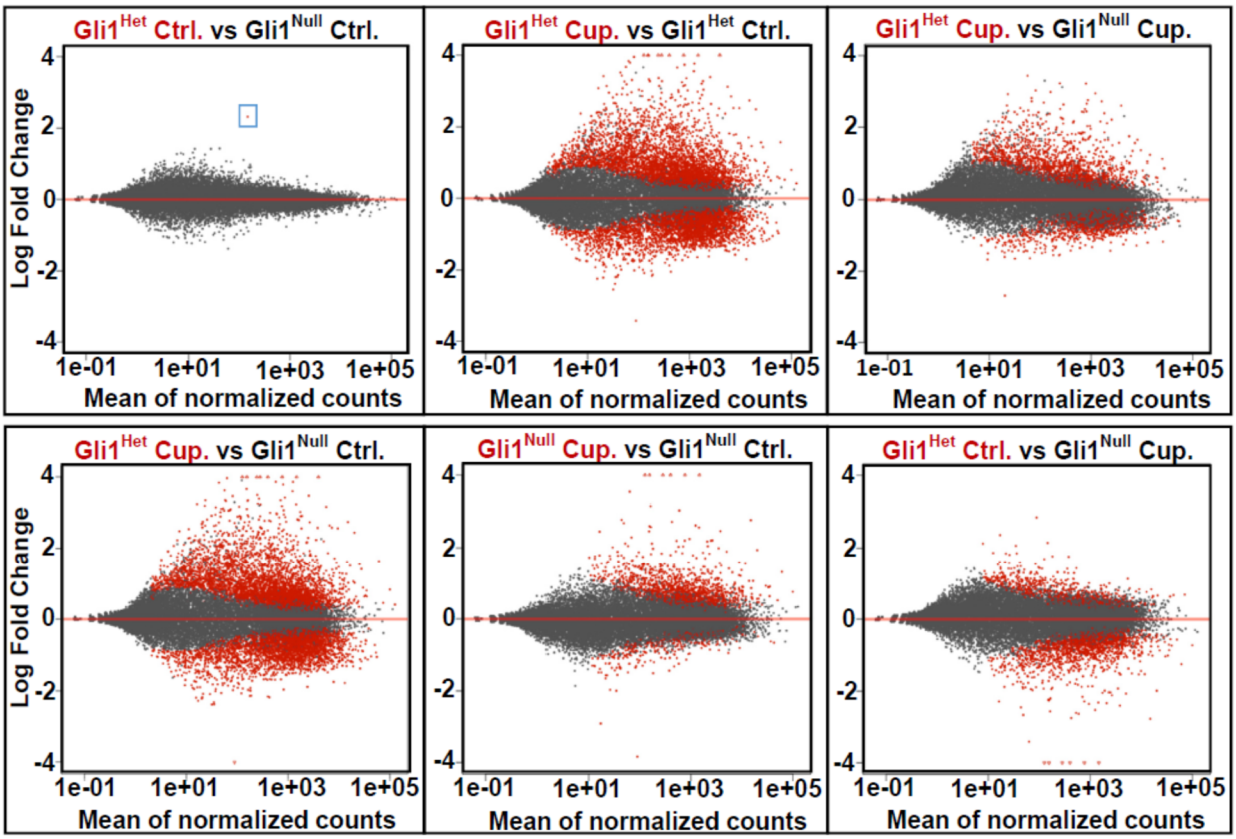

Figure 4: Principle component analysis of the sequencing data

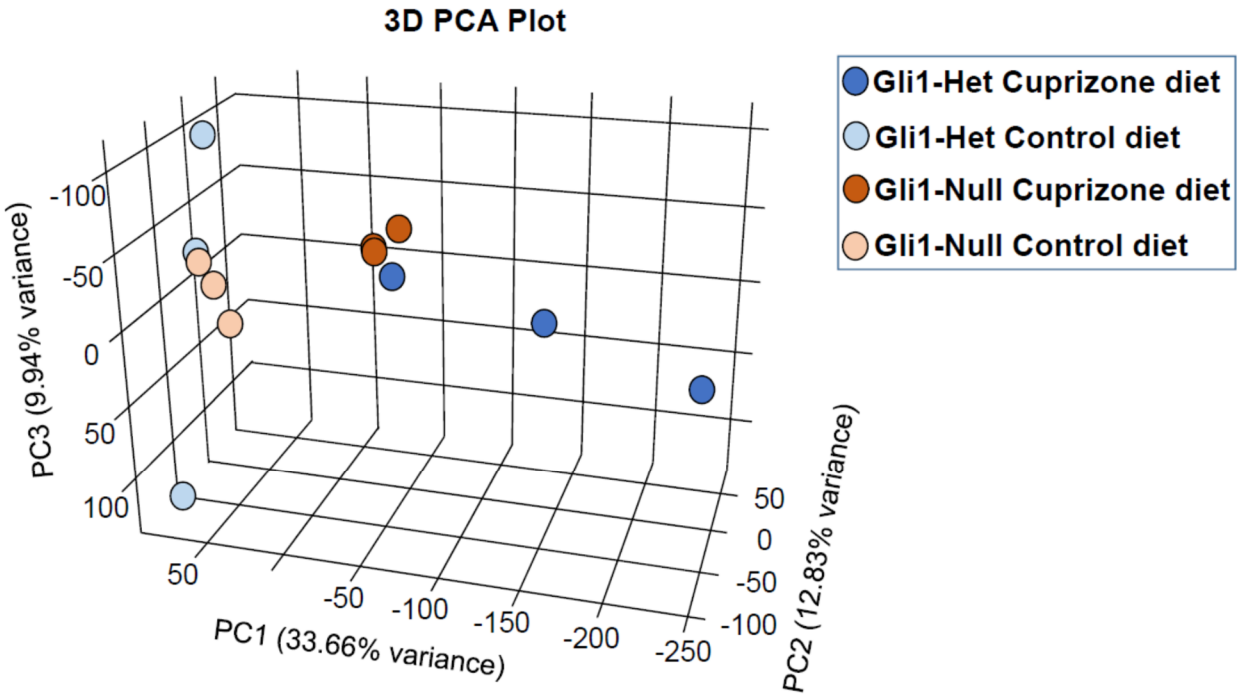

Table 1: NCBI Geo accession numbers of samples

Samples	**Accession**
Gli1-Het control diet-1	GSM4956878
Gli1-Het control diet-2	GSM4956879
Gli1-Het control diet-3	GSM4956880
Gli1-Het cuprizone diet-1	GSM4956881
Gli1-Het cuprizone diet-2	GSM4956882
Gli1-Het cuprizone diet-3	GSM4956883
Gli1-Null control diet-1	GSM4956884
Gli1-Null control diet-2	GSM4956885
Gli1-Null control diet-3	GSM4956886
Gli1-Null cuprizone diet-1	GSM4956887
Gli1-Null cuprizone diet-2	GSM4956888
Gli1-Null cuprizone diet-3	GSM4956889

Table 2: Number of differentially expressed genes

Comparison	No. of genes: *P*<0.05	No. of up-regulated genes: *P*<0.05	No. of down-regulated genes: *P*<0.05	No. of genes: *P*<0.05 & FC>2	No. of genes: *P*<0.05 & FC<0.5
Gli1-Het Control diet vs Gli1-Null Control diet	1	1	0	1	0
Gli1-Het Cuprizone diet vs Gli1-Het Control diet	6442	3481	2961	1447	525
Gli1-Het Cuprizone diet vs Gli1-Null Cuprizone diet	1507	955	552	500	64
Gli1-Het Cuprizone diet vs Gli1-Null Control diet	6447	3499	2948	1482	526
Gli1-Null Cuprizone diet vs Gli1-Null Control diet	1209	1015	194	294	34
Gli1-Het Control diet vs Gli1-Null Cuprizone diet	1730	436	1294	86	242