Tools for the study of lipid pathology

Technical Journal Club

Manfredi Carta

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Contents

Lipid-droplet-accumulating microglia represent a dysfunctional and proinflammatory state in the aging brain

Julia Marschallinger, Tal Iram, Macy Zardeneta, Song E. Lee, Benoit Lehallier, Michael S. Haney, John V. Pluvinage, Vidhu Mathur, Oliver Hahn, David W. Morgens, Justin Kim, Julia Tevini, Thomas K. Felder, Heimo Wolinski, Carolyn R. Bertozzi, Michael C. Bassik, Ludwig Aigner & Tony Wyss-Coray

Nature Neuroscience **23**, 194–208(2020) | Cite this article

Sphingolipid Control of Fibroblast Heterogeneity Revealed by Single-Cell Lipidomics

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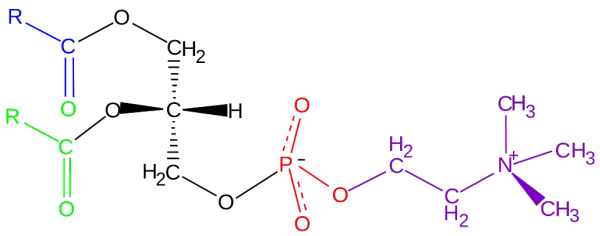
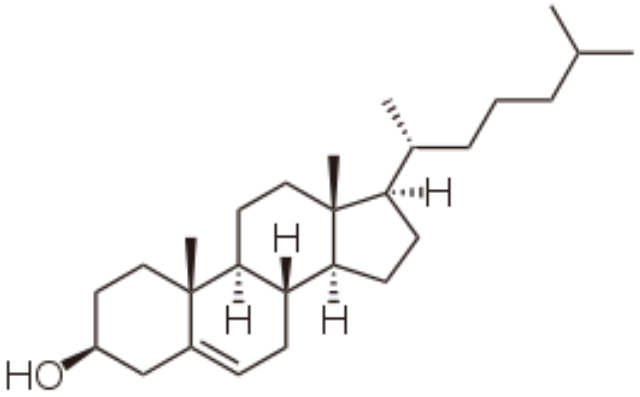
Introduction

- Human cells produce thousands of lipids
- Functions of lipids: building blocks of membranes, energy source, signalling molecules (eicosanoids, steroid hormones etc), emulsifiers (bile acids)
- Classes of lipids: phospholipids, sterols, sphingolipids



Phosphatidylcholine



Cholesterol

Images: Wikimedia Commons

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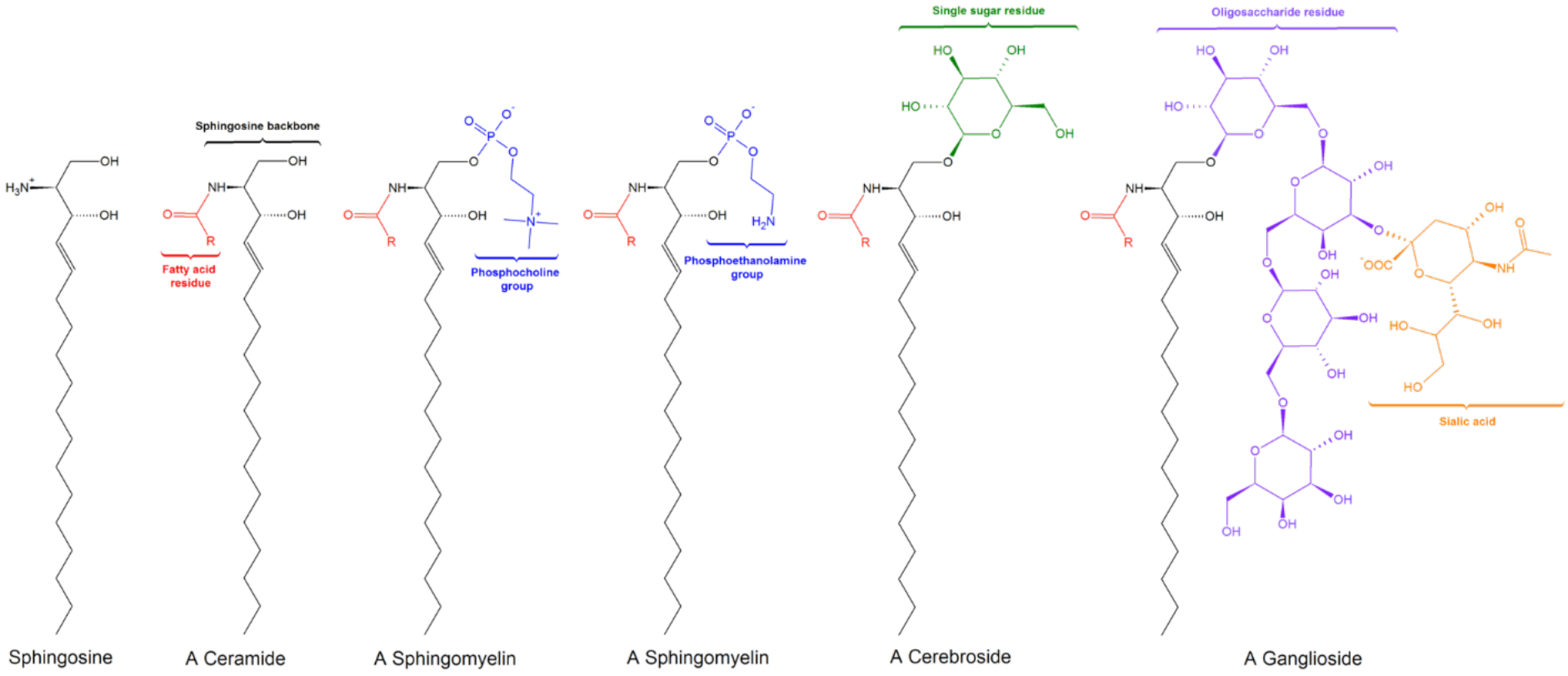


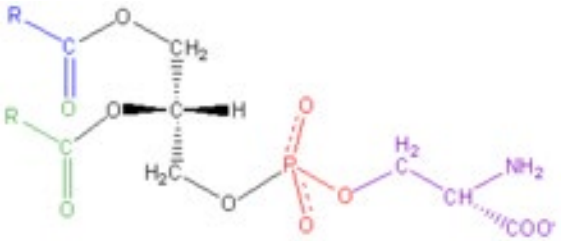
Images: Wikimedia Commons

Introduction

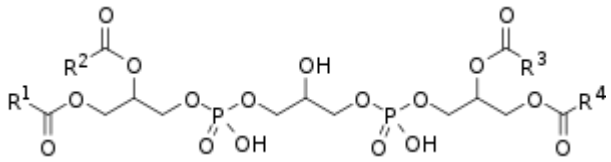
- Cellular lipid composition changes during differentiation events and also varies across individual cells of the same type
- Lipid geometry influences shape of membranes
- Lipid composition can be influenced by cell cycle and microenvironmental cues.

Some examples:

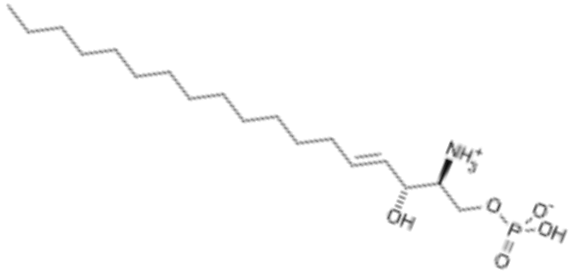
- During apoptosis, phosphatidylserine (usually in inner leaflet of plasma membrane) is flipped to outer leaflet
- Phosphatidylserine is also flipped to the outer membrane leaflet of activated blood platelets → promotes coagulation and platelet aggregation
- Cardiolipin is translocated from inner to outer mitochondrial membrane during apoptosis → activates pro-apoptotic signalling molecules (Bcl-2, Bax, Bad etc)
- Sphingosine-1-phosphate regulates chemotaxis of immune cells



Phosphatidylserine



Cardiolipin



Sphingosine-1-phosphate

Chaurio *et al*, 2009

Lipid-droplet-accumulating microglia represent a dysfunctional and proinflammatory state in the aging brain

Julia Marschallinger, Tal Iram, Macy Zardeneta, Song E. Lee, Benoit Lehallier, Michael S. Haney, John V. Pluvinage, Vidhu Mathur, Oliver Hahn, David W. Morgens, Justin Kim, Julia Tevini, Thomas K. Felder, Heimo Wolinski, Carolyn R. Bertozzi, Michael C. Bassik, Ludwig Aigner & Tony Wyss-Coray ✉

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Introduction

- Genetic studies have linked microglia to the pathogenesis of neurodegenerative diseases
  - E.g. *APOE* and *TREM2* are AD risk genes and code for proteins that are expressed in microglia, with important roles in neurodegeneration and ageing

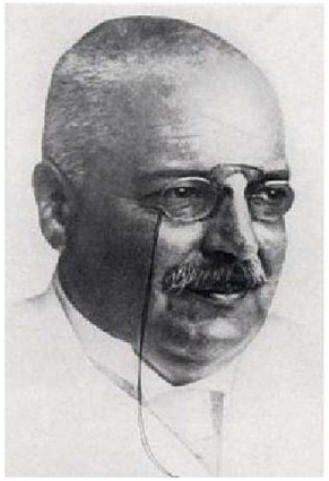
- Microglia become progressively activated and seemingly dysfunctional with age
  - Lose their homeostatic molecular signature
  - Increased production of proinflammatory cytokines
  - Elevated generation of reactive oxygen species (ROS)
  - Buildup of dysfunctional lysosomal deposits, indicative of impaired phagocytosis

- Several microglia/macrophage states related to disease and ageing have been described:
  - Disease associated microglia (DAM): presumably protective microglial population in AD models
  - Neurodegenerative microglia (MGnD): induced by apoptotic neurons in AD, ALS, MS
  - Lipid-associated macrophages: Found in adipose tissue, regulate lipid homeostasis (Jaitin et al, Cell 2014)

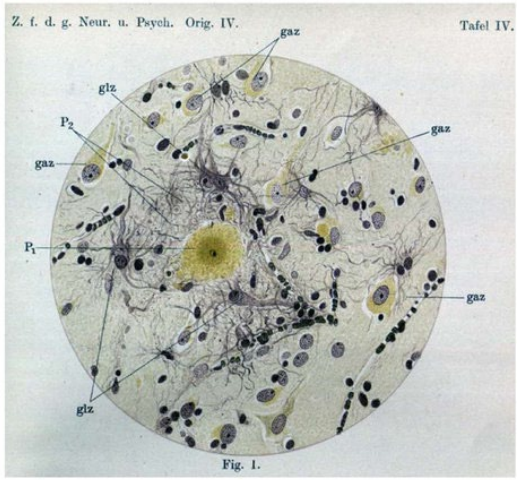
Keren-Shaul *et al.*, Cell 2017
Krasemann *et al.*, Immunity 2017
Jaitin *et al.*, Cell 2014

Lipid-droplet associated microglia

A



B

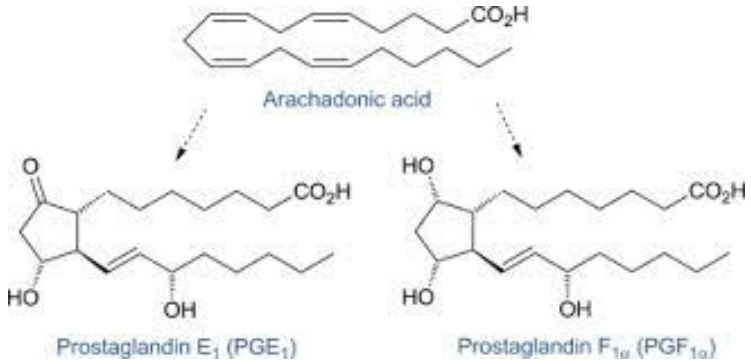


C-1

Die Glia hat reichlich Fasern gebildet, daneben zeigen viele Gliazellen große Fettsäcke.

- In 1907, Alois Alzheimer observed “glial cells showing large adipose sacks” (probably microglia), but this phenomenon remained largely unstudied
- Lipid accumulation in immune cells also takes place in other pathologies:
  - “Foamy macrophages” in atherosclerosis
  - Foamy macrophages show hallmarks of senescence
  - Myeloid cells form lipid droplets in response to inflammation, leukocytes in arthritis, eosinophils in allergic inflammation

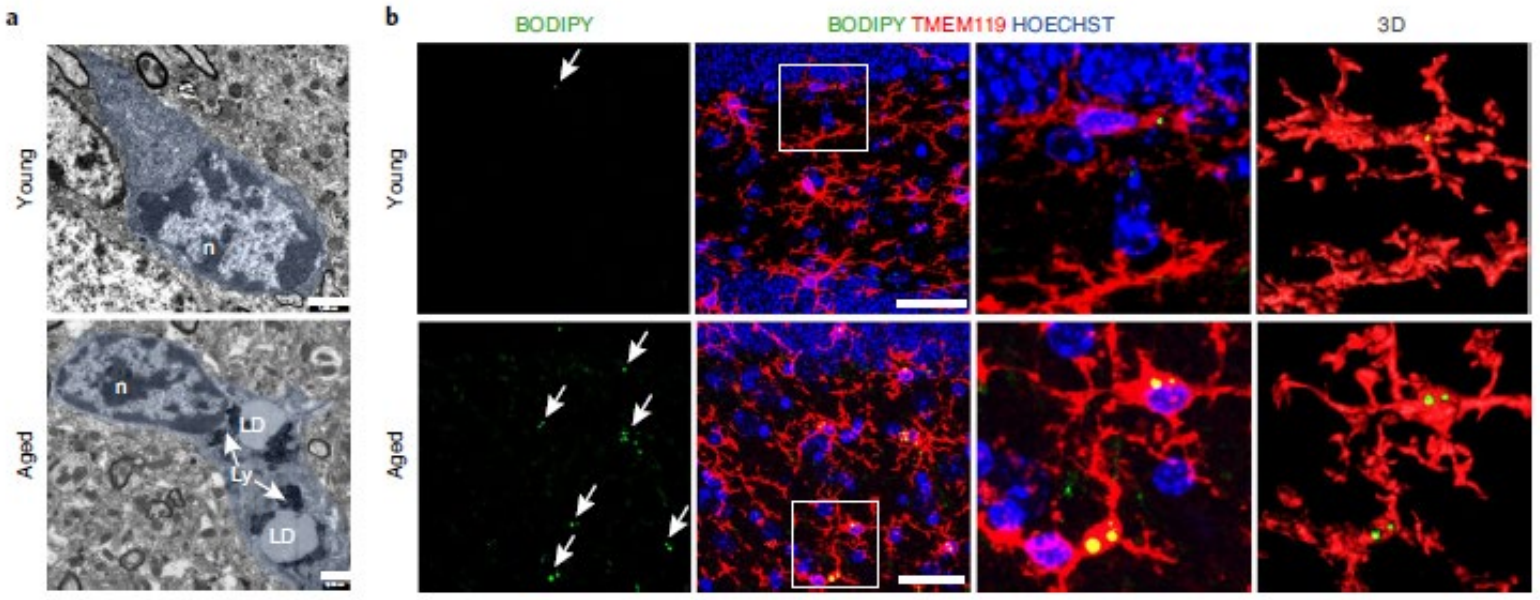
Lipid droplets



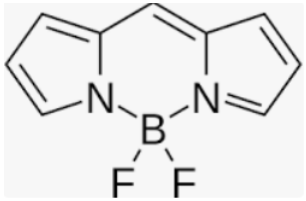
- Lipid droplets contain glycerolipids (e.g. mono-, di- and triacylglycerols) and cholesteryl esters
- Lipid droplets are sites of production and storage for eicosanoids and cytokines, are involved in antigen presentation and pathogen clearance
- In mice, Oil-red-O (lipid stain) positive “lipid-laden” cells increase with age: neurons, astrocytes, ependymal cells and microglia
- Lipid droplets are also induced in hippocampal slices cultures by treatment with lipopolysaccharide (molecule found in bacterial membranes, recognised by innate immune system)

What is the role of lipid droplets in neurodegeneration?

Microscopy of lipid droplets



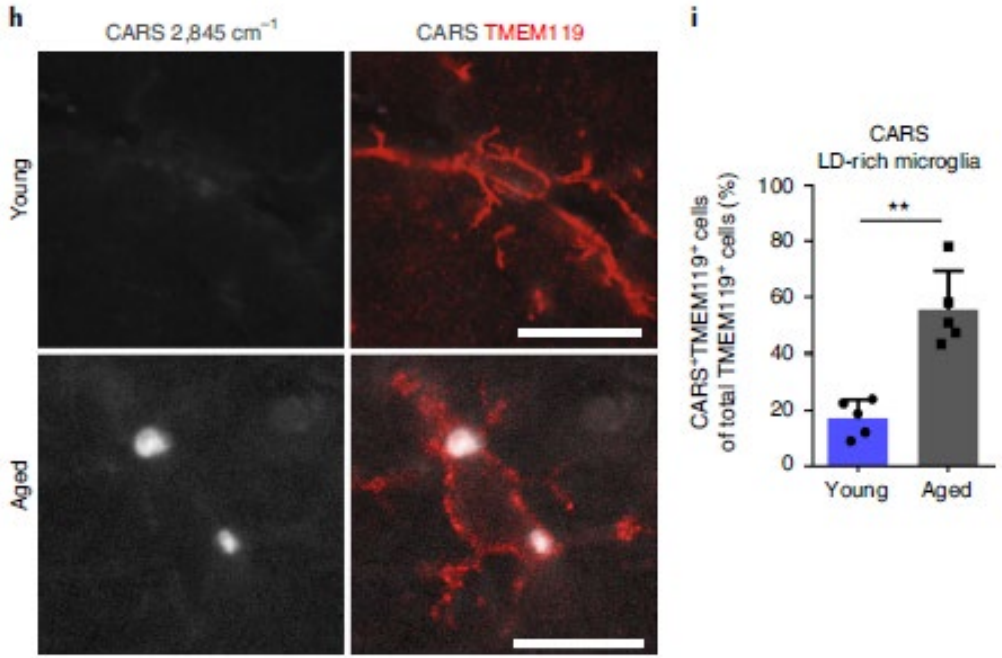
- Transmission EM: lipid droplets were more abundant im aged (20 months) mice compared to young animals (3 month), frequently located next to dense lysosomal material
- BODIPY stain (labels neutral lipids) : percentage of BODIPY+ TMEM119+ microglia was 4x higher in aged mice, droplets were larger



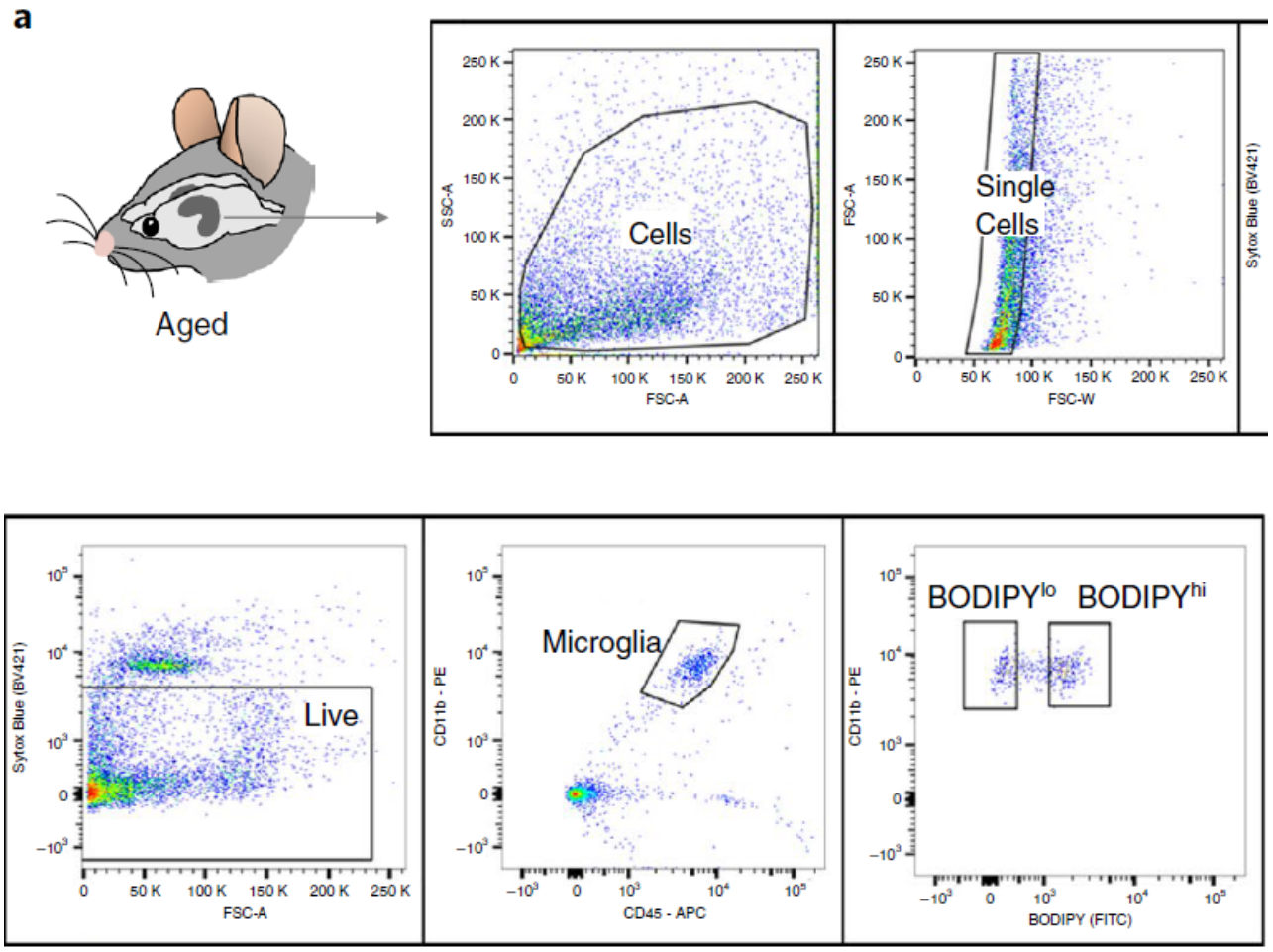
BODIPY

Coherent anti-Stokes Raman scattering

- Different types of molecules show characteristic vibrational energy states
- Electromagnetic energy at appropriate (infrared) wavelengths will excite the molecules into these states
- Two laser beams with frequencies adjusted to the vibrational energy excite the molecule of interest
- Molecules relax back, releasing photons, which are used for imaging
- Here: CARS was adjusted to vibration of neutral lipids (CH_2) → lipid-laden microglia were more frequent in aged mice



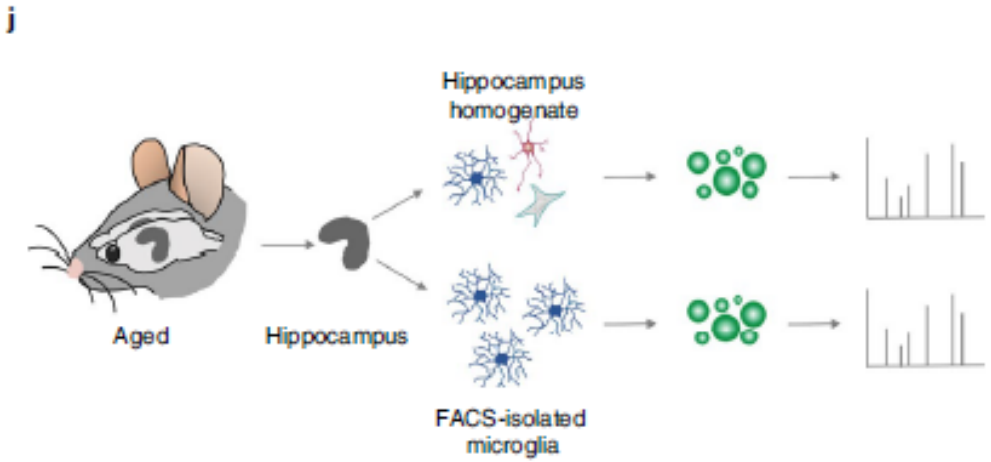
Isolation of lipid-laden microglia

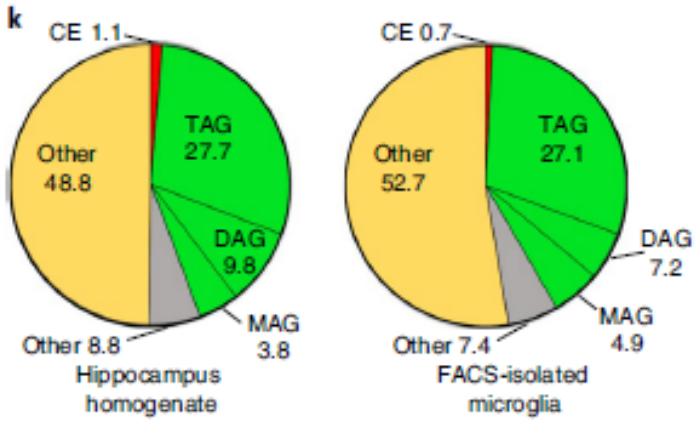


Lipidomics

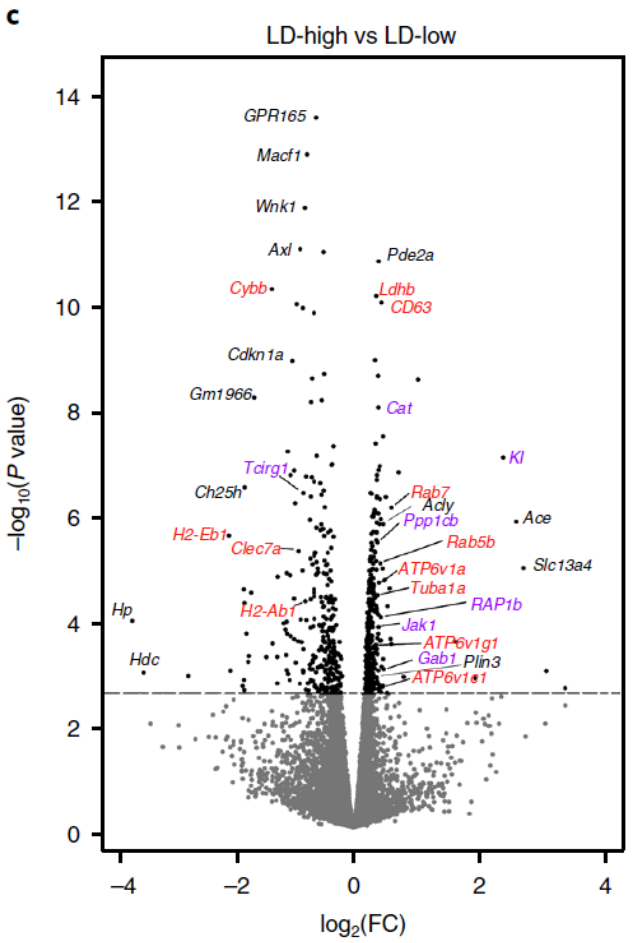
- TMEM119+ microglia were sorted by FACS
- Lipid droplets were extracted from bulk hippocampus and sorted microglia by homogenising tissue/cells and centrifugation (commercial kit) → lipids float to top

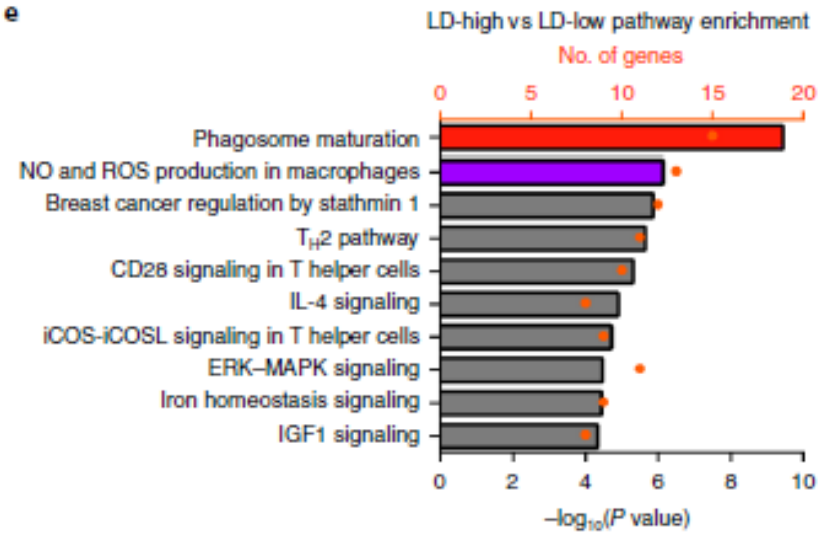
- Lipids were analysed by mass spectrometry





RNA-seq of sorted microglia

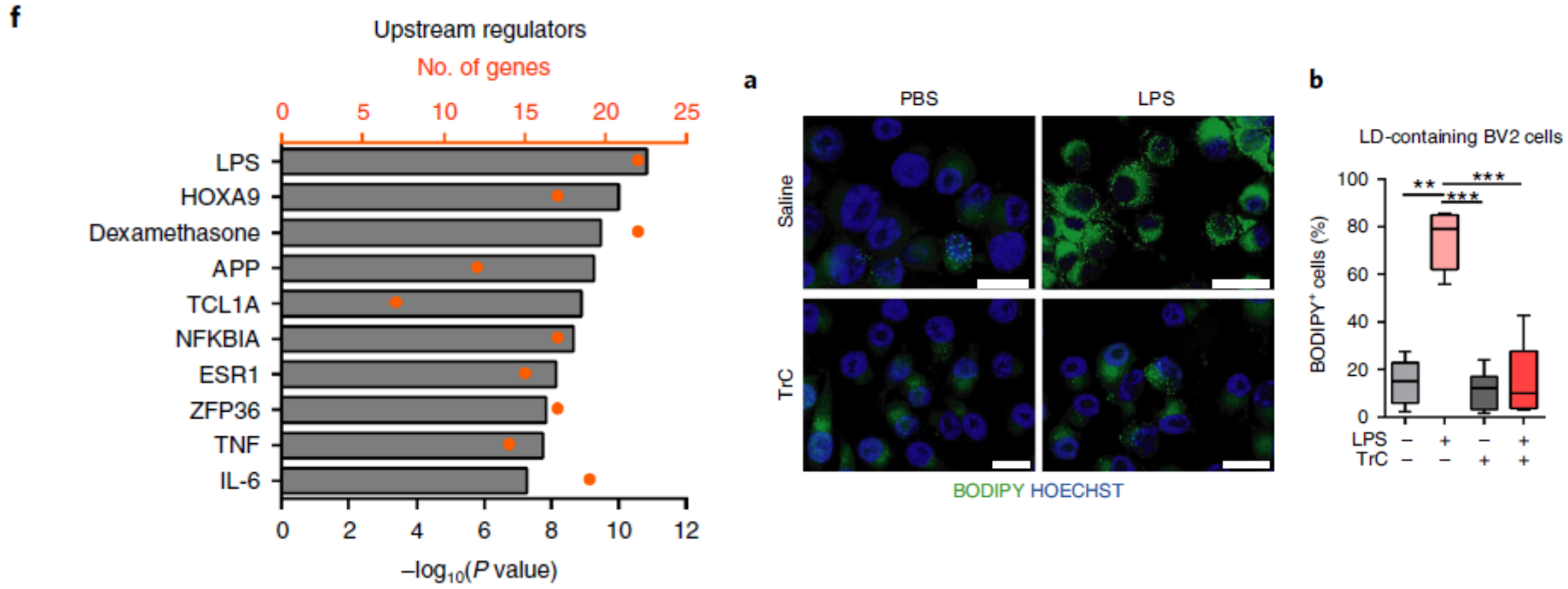




Lipid-laden microglia: upregulation of lysosomal, nitric oxide and ROS synthesis genes

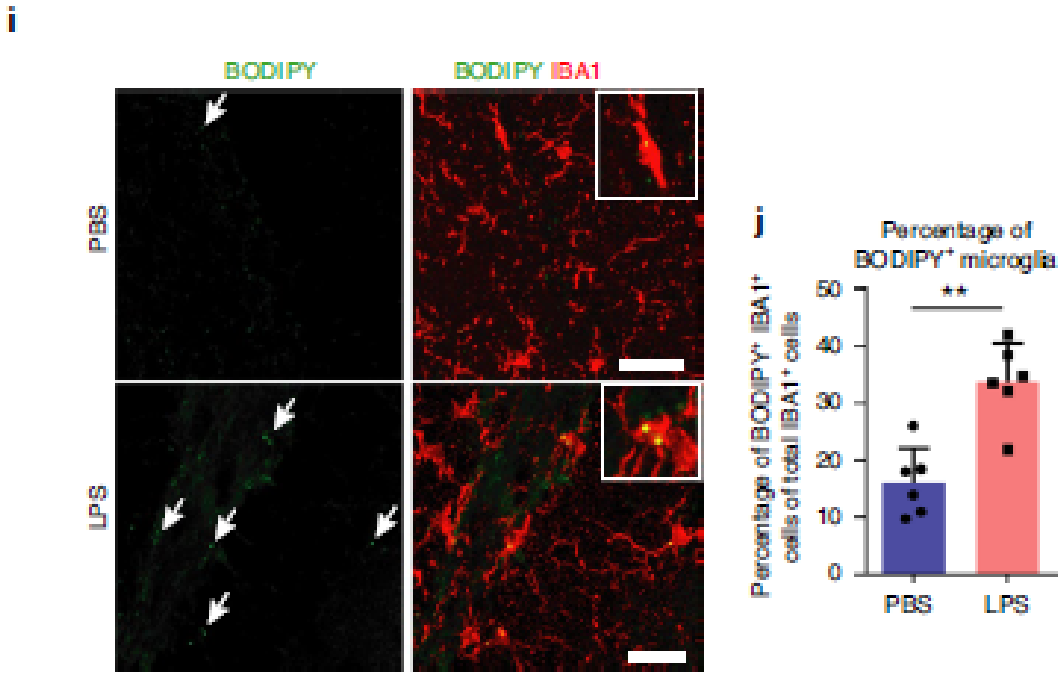
Lipid-high microglia: enriched in lipid-related genes, e.g. ATP citrate synthase (*ACLY*, involved in lipid synthesis)

Lipopolysaccharide as a regulator of lipid synthesis?



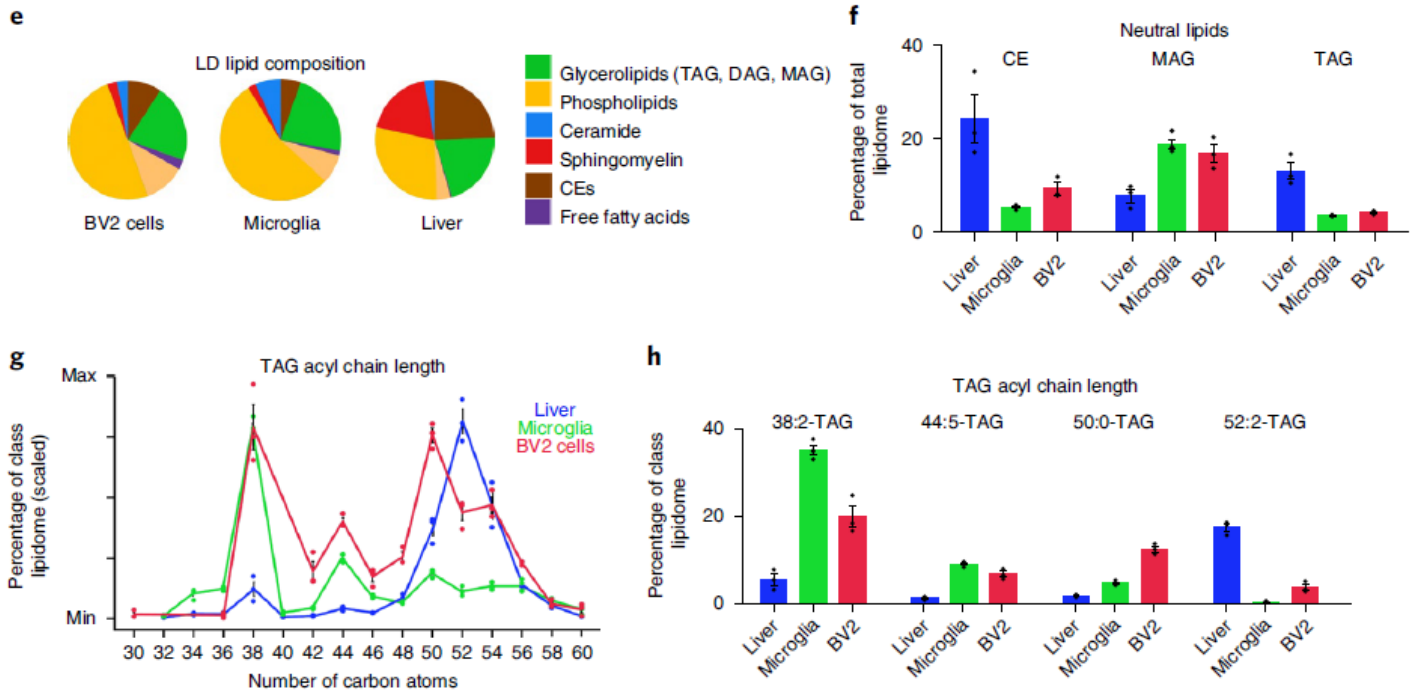
- LPS was predicted to be one of the main regulators of the genes enriched in lipid-laden microglia
- Indeed, LPS induced formation of lipid droplets in the microglial cell line BV2
- Lipid droplet formation could be inhibited by triacsin C (TrC, inhibitor of fatty acyl CoA synthetase)

LPS also induced lipid droplets with similar compositions in vivo



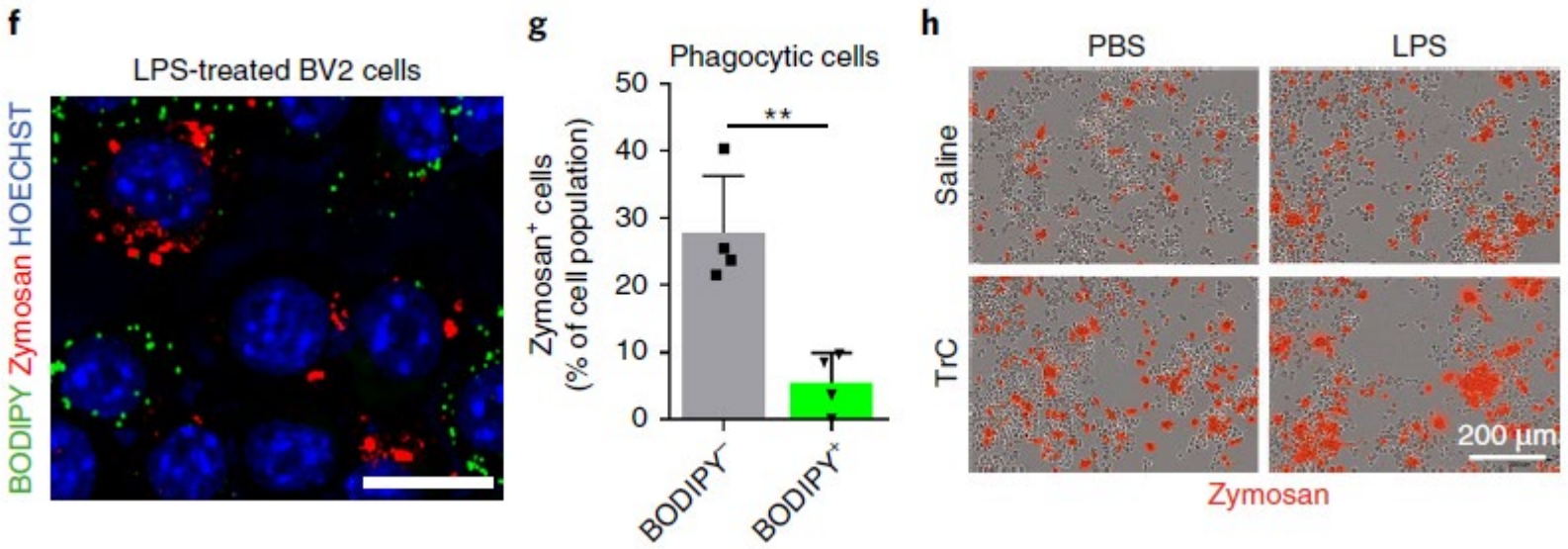
Mice injected with LPS for 4 d showed increased lipid-laden microglia

Ageing and LPS induced lipid droplets with similar compositions



Mass spectrometry: LPS-treated BV2 cells and aged microglia contained similar lipids, with similar acyl chain lengths

Impaired phagocytosis in lapid-laden microglia



- Lipid droplets were induced in BV2 cells with LPS
- Microglia were exposed to fluorescent zymosan (polysaccharide found in fungi) particles
- LPS increases phagocytosis, but zymosan particles were mainly found in BODIPY- cells and less in BODIPY+
- Triacsin C treatment increased zymosan phagocytosis in LPS-treated cells

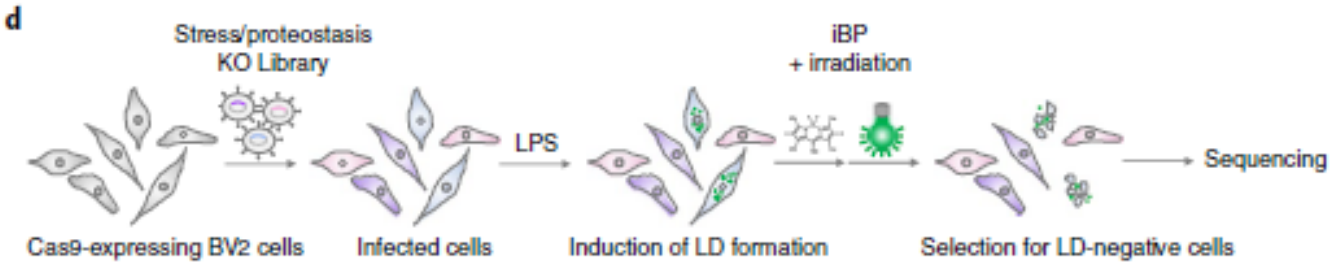
- Aged plasma also induced lipid droplet formation
- Similar results in slice cultures (not shown)

CRISPR screen for regulators of lipid droplet formation

- A sgRNA library based on RNA seq of lipid-laden microglia targeting ~2000 genes was constructed (lysosomal genes, protein degradation, celullar stress)
- Every gene was targeted by 10 sgRNAs
- 1000 negative control sgRNAs
- Pooled library transfected into BV2 cells with lentiviruses → LPS treatment

Selection strategy for enrichment of lipid droplet negative cells (in which gene knockdown might have inhibited droplet formation):

- Addition of iodine atoms to BODIPY
- Upon photoexcitation of iodo-BODIPY, singlet oxygen (a ROS) is formed → kills cells
- 3 rounds of photoexcitation selection
- sgRNA composition was sequenced → enriched / depleted genes?



Genes regulating lipid droplet formation

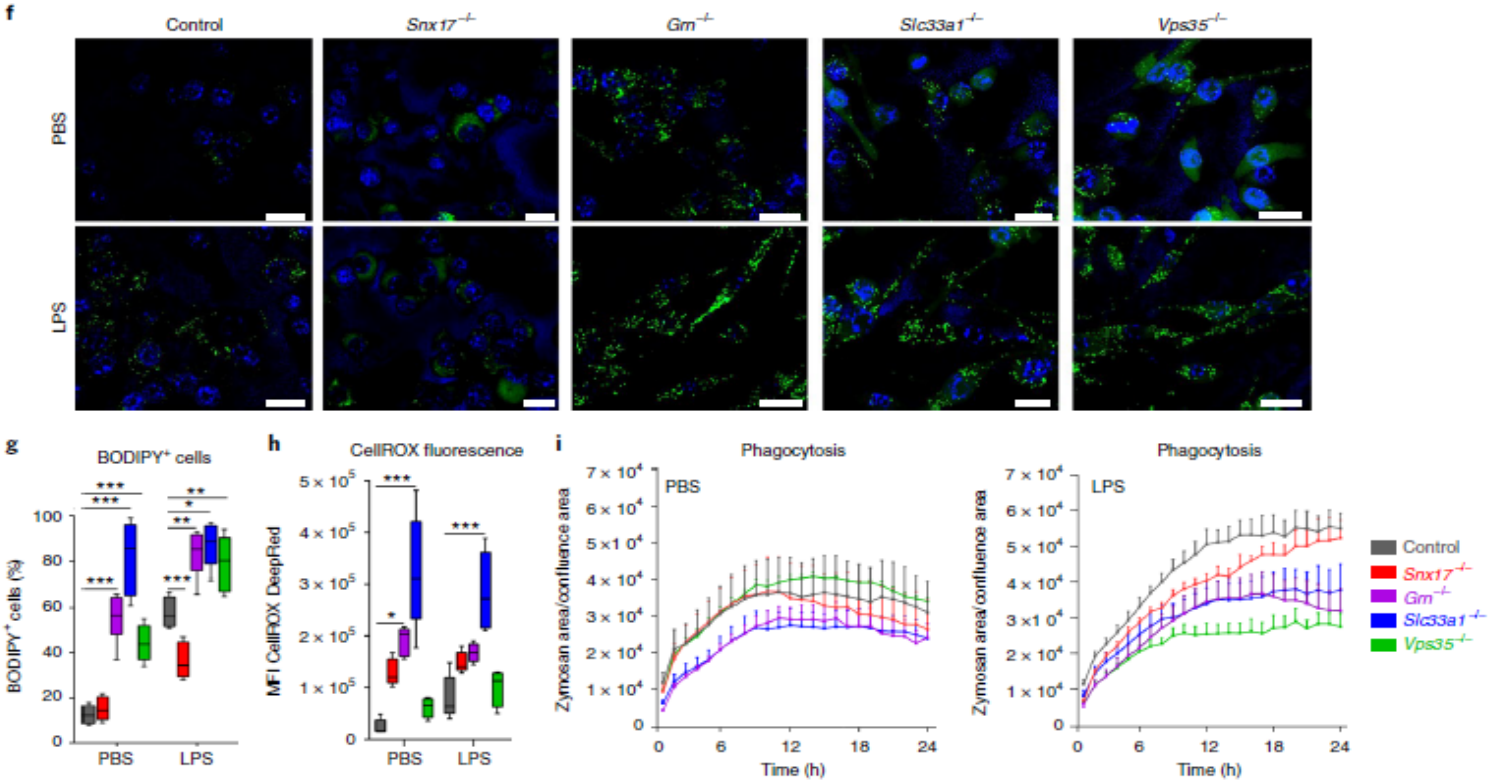
- 112 enriched/depleted genes were identified
- Included known neurodegeneration risk genes, hinting at a possible relationship between lipid storage in microglia and neurodegeneration
  - *Slc33a1* (acetyl-coenzyme A transporter 1): sgRNA depleted; required for ganglioside synth.
  - *Vps35* (vacuolar protein sorting 35): sgRNA depleted; vesicle formation, autophagy
  - *Grn* (granulin precursor): sgRNA depleted; inflammation, protein homeost., lysosomes
  - *Snx17* (sorting nexin): sgRNA enriched; interacts with phospholipids

- Individual BV2 knockout lines with CRISPR deletions of the genes of interest were treated with LPS

- sgRNAs targeting *Grn*, *Slc33a1*, *Vps35*: significantly more lipid droplets
- sgRNAs targeting *Snx17*: significantly fewer lipid droplets
- All in line with CRISPR screen results

- Also significantly increased ROS generation and decreased zymosan phagocytosis if *Grn* and *Slc33a1* were targeted

Genes regulating lipid droplet formation



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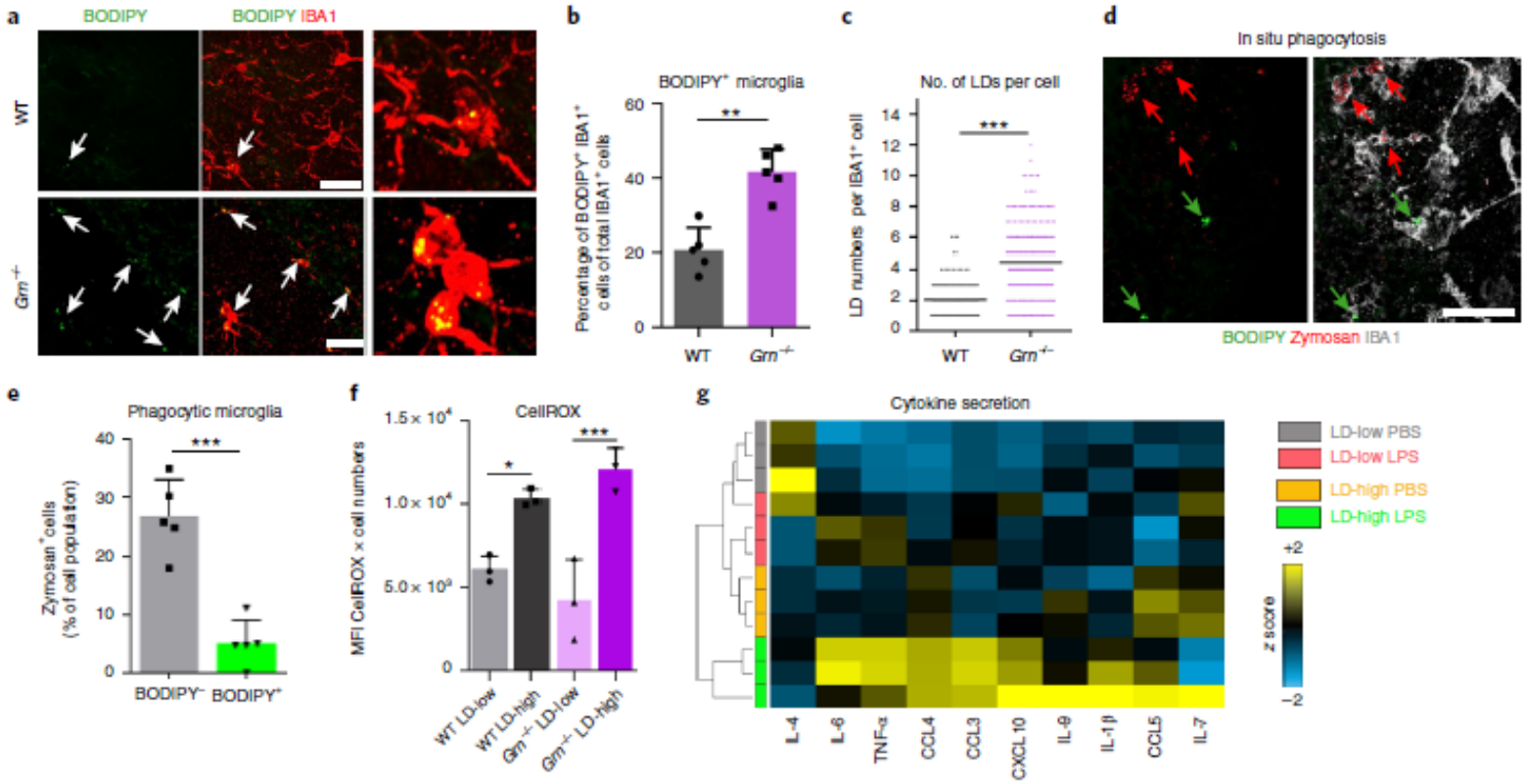
Grn-KO mice



- *GRN* mutations are linked to the development of frontotemporal dementia (FTD)
- *Grn*-KO mice are used as a model for FTD and are characterized by microglial changes, neuroinflammation and cognitive deficits
- As expected, hippocampus of *Grn*-KO mice contained high numbers of BODIPY+ microglia, twice as many lipid droplets per cell
- Decreased phagocytosis of zymosan
- Increased ROS production (CellROX)
- Elevated secretion of proinflammatory cytokines

Summary

- Novel microglia state in the ageing brain: LDAM (lipid droplet-associated microglia) with unique transcriptional profile
- Comprised >50% of microglia in the aged hippocampus
- Showed typical age-related functional impairments: production of proinflammatory cytokines, decreased phagocytosis, elevated ROS production
- Formed in response to LPS treatment, similarly to peripheral immune cells that form LDs in the context of inflammation
- Are they formed in response to age-related neuroinflammation?
- Probably aren't related to demyelination (would be expected to contain more cholesterol, in line with lipid droplets observed in experimental autoimmune encephalomyelitis)
- LDs were often located next to lysosomes: perhaps they accumulate as a consequence of impaired lysosomal degradation
- Also formed in *Grn*-KO mice. Microglia showed similar RNA-seq profiles as LDAM (not shown)

- Targeting LDAM might represent a promising therapeutic strategy

Introduction

Sphingolipid Control of Fibroblast Heterogeneity Revealed by Single-Cell Lipidomics

Laura Capolupo, Irina Khven, Luigi Mazzeo, Galina Glousker, Francesco Russo, Jonathan Paz Montoya, Sylvia Ho, Dhaka R. Bhandari, Andrew P. Bowman, Shane R. Ellis, Romain Guiet, Johannes Muthing, Bernhard Spengler, Ron M.A. Heeren, Gian Paolo Dotto, Gioele La Manno, Giovanni D'Angelo

doi: https://doi.org/10.1101/2021.02.23.432420

Overview: Differential role of fibroblasts

Fibroblasts synthesise and remodel the extracellular matrix (ECM) and maintain the structural integrity of tissues

Fibroblasts exhibit differences in gene expression, depending on their activity:

- Fibrogenic (e.g. formation of scar tissue)
- ECM remodelling (e.g. tumour infiltration)
- Inflammatory (e.g. cartilage degeneration in arthritis)

Following tissue damage, fibroblasts experience phenotypic interconversion by adopting matrix secreting and inflammatory states

Changes in fibroblast subpopulations are associated with fibrosis, cancer growth

How fibroblast heterogeneity and plasticity is regulated is unclear

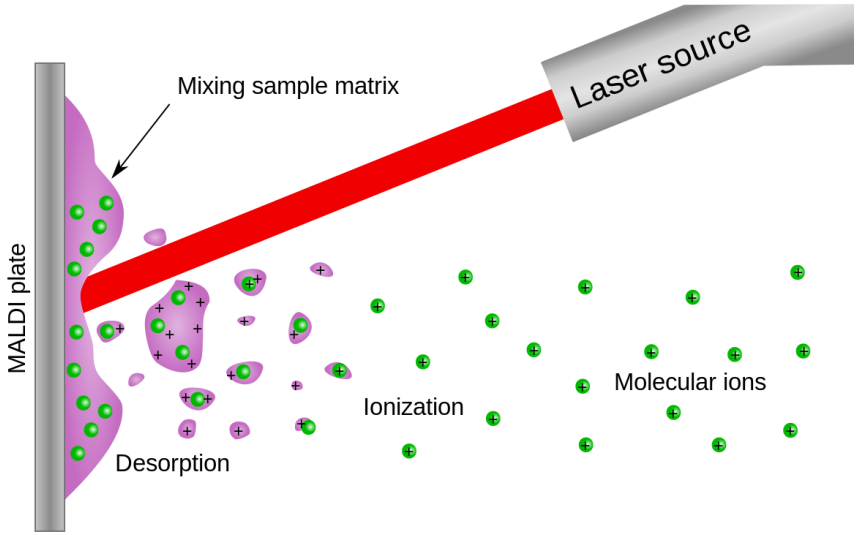
Fibroblasts within single cultures and biopsies contain different lipids, but the significance of this is unknown → maybe lipids are components of regulatory pathways?

Single cell lipidomics with mass spectrometry

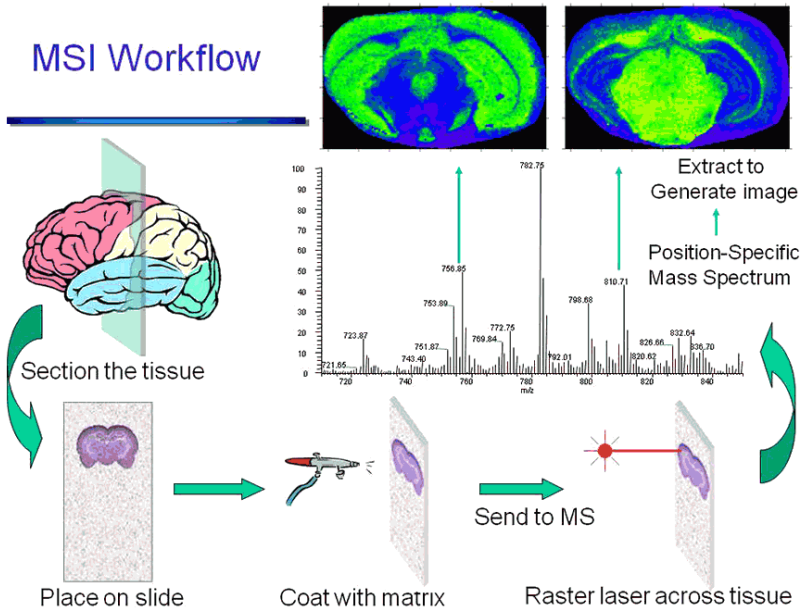
MS, particularly MALDI-MSI, is now sensitive enough to study the lipid composition of single cells

MALDI-MSI: matrix-assisted laser desorption/ionization mass spectrometry imaging

Mass spectrometry imaging (MSI): visualises spatial distribution of molecules by collecting mass spectra from individual spots



MALDI



Images: Wikimedia Commons, Univ. of Florida (ufl.edu)

MALDI-MSI workflow

Set-up

1. Dermal human fibroblasts (dHFs) were cultured and fixated (0.25% glutaraldehyde)
2. Stained and imaged (confocal microscopy)
3. Prepared for MALDI (addition of dihydroxybenzoic acid = matrix component)

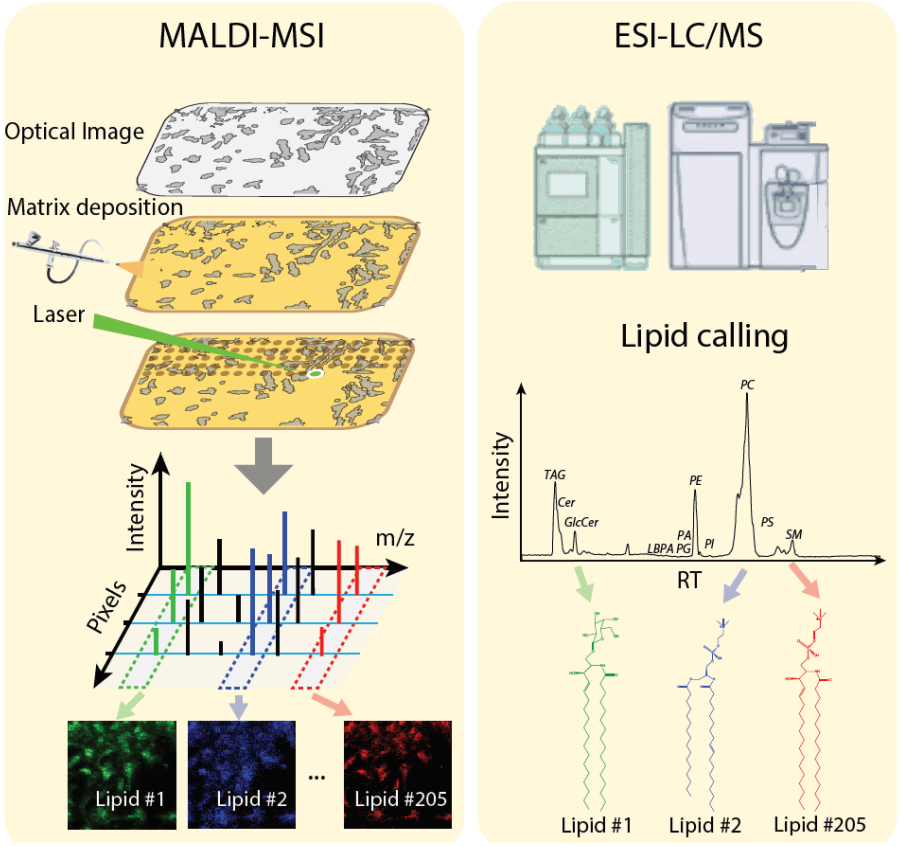
Analysis

1. Mass spectra acquired by MALDI
2. Total lipid composition also analysed by ESI (electrospray ionization-liquid chromatography MS)

Metabolite annotation: spectra are compared to public databases and to libraries of lipid ionization data

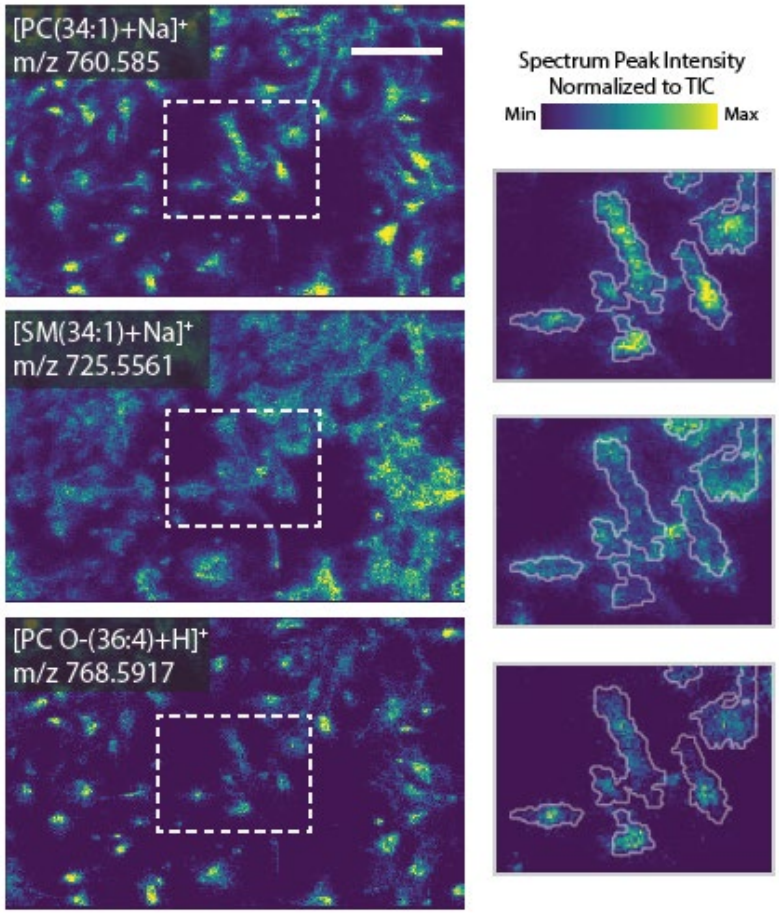
Inferred lipid composition was assigned to segmented images of single dHFs

MALDI-MSI workflow: MALDI and ESI-LC MS



Spatial mass spectra acquired by MALDI, then validated by ESI-LC/MS

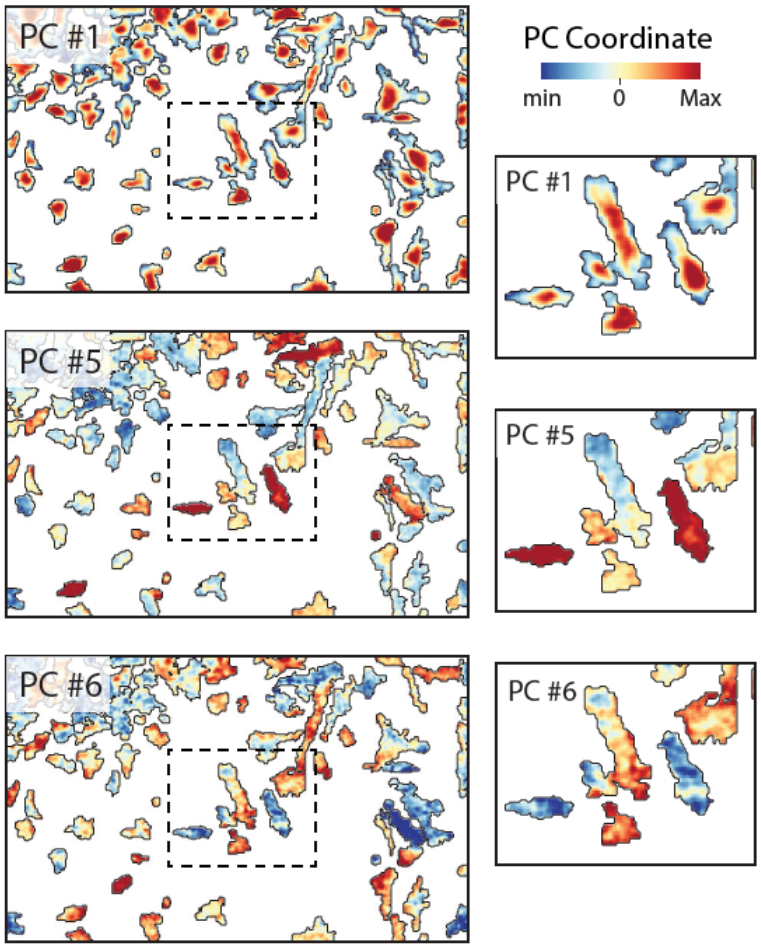
MALDI-MSI workflow: Ion images

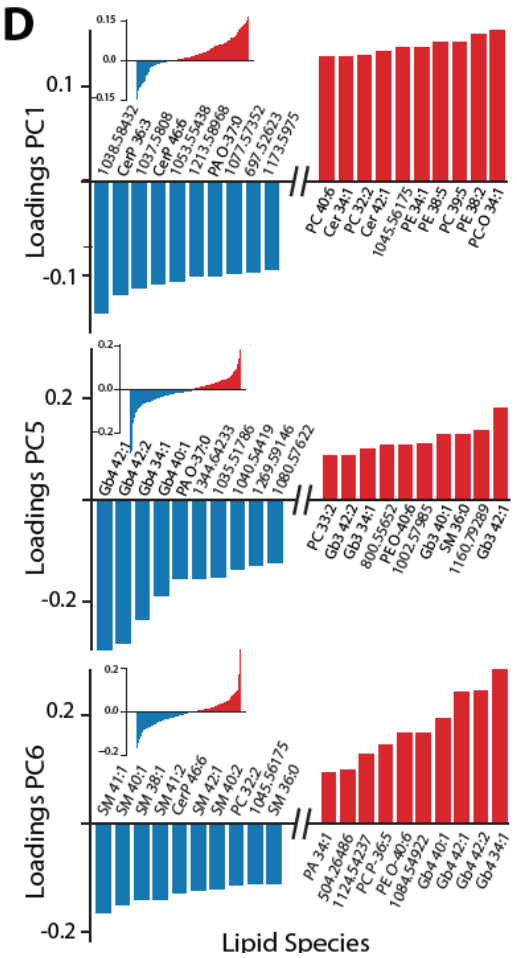


Abundance of peaks, defined by mass to charge ratio (m/z) is mapped to laser spots

As there were many different peaks, a principal component (PC) analysis was performed to summarise ions into PCs

MALDI-MSI workflow: 8 Principal components (PCs) summarise 205 lipids

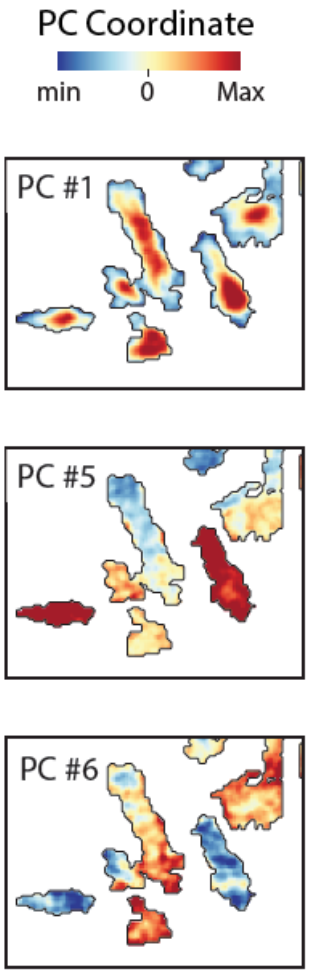




Left: Principal component analysis → PCs are mapped
Right: Contribution of top and bottom 10 lipids in every PC

PC coordinates were distributed in different ways

PC coordinates were distributed in different ways



PC1:

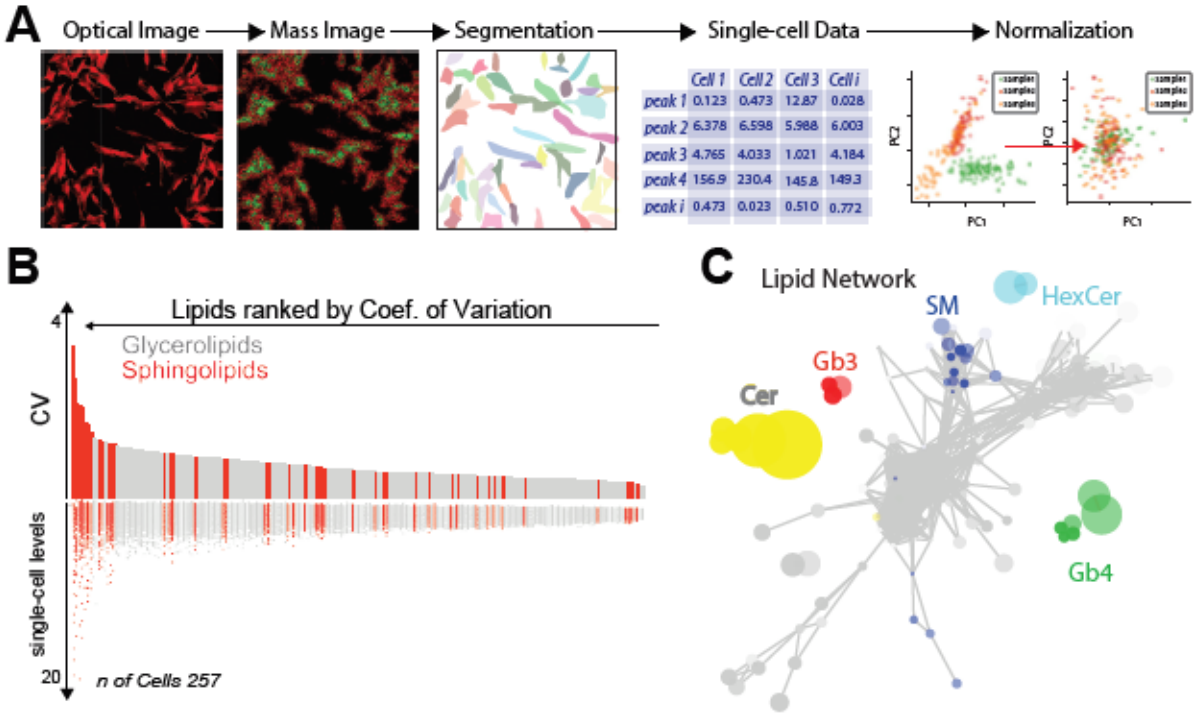
- changed moving from inner part of cell to periphery
- determined by glycerolipids and sphingolipids, reflects composition of perinuclear and peripheral cell membranes

Other PCs:

- distributed differently among cells
- depended on certain sphingolipids (ceramides, spingomyelins, globosides)
- confirmed what was already known: selected sphingolipids vary across cells

Two axes of lipid variation in dHF: intracellular organisation, intercellular differences

Specific lipid classes varied across cells: biological significance?



A. Using MALDI-MSI, a total of 257 single-cell lipidomes were obtained and segmented to images

B. Coefficient of variation was calculated for each individual lipid → Sphingolipids showed the highest variability

C. Lipid correlation network: Lipids that co-vary (= show similar variation patterns) are connected and cluster together

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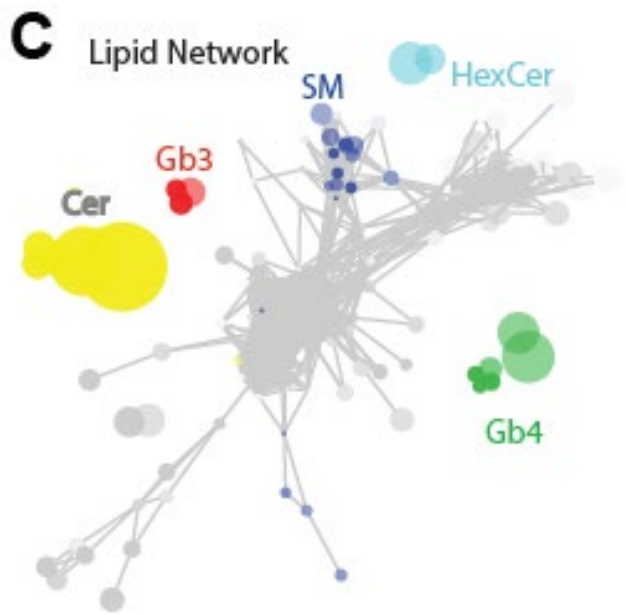
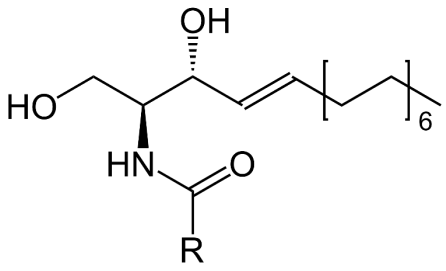
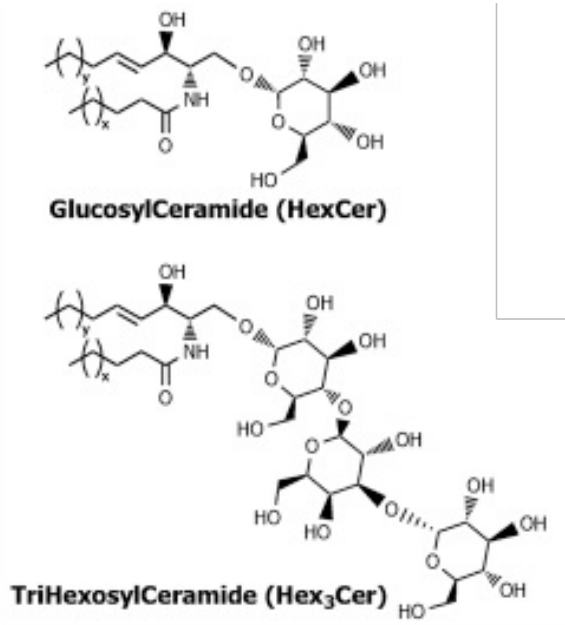




ceramide



C. Lipid correlation network: Lipids that co-vary (= show similar variation patterns) are connected and cluster together

Lipids were clustered in groups consisting of compounds bearing the same headgroup

- OH: ceramides (Cer)
- Hexose: hexosylceramides (HexCer)
- Trihexose: trihexosylceramides (Gb3)
- N-acetyl-hexose-trihexose (Gb4)

Ceramide processing appears to be cell-cell variable (rather than production of different ceramide backbones)

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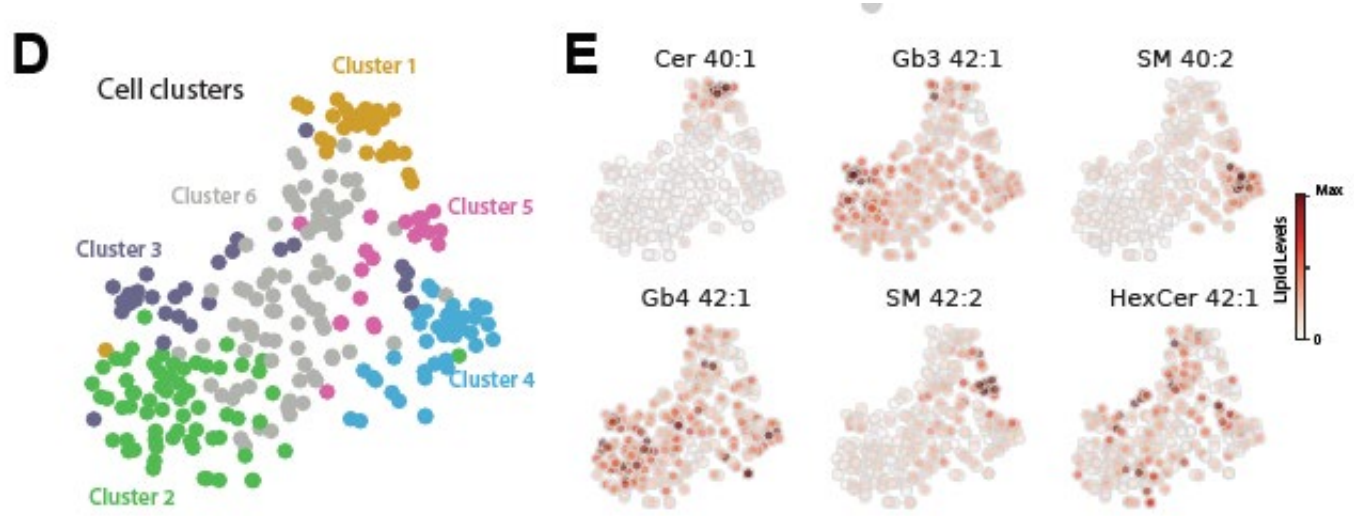
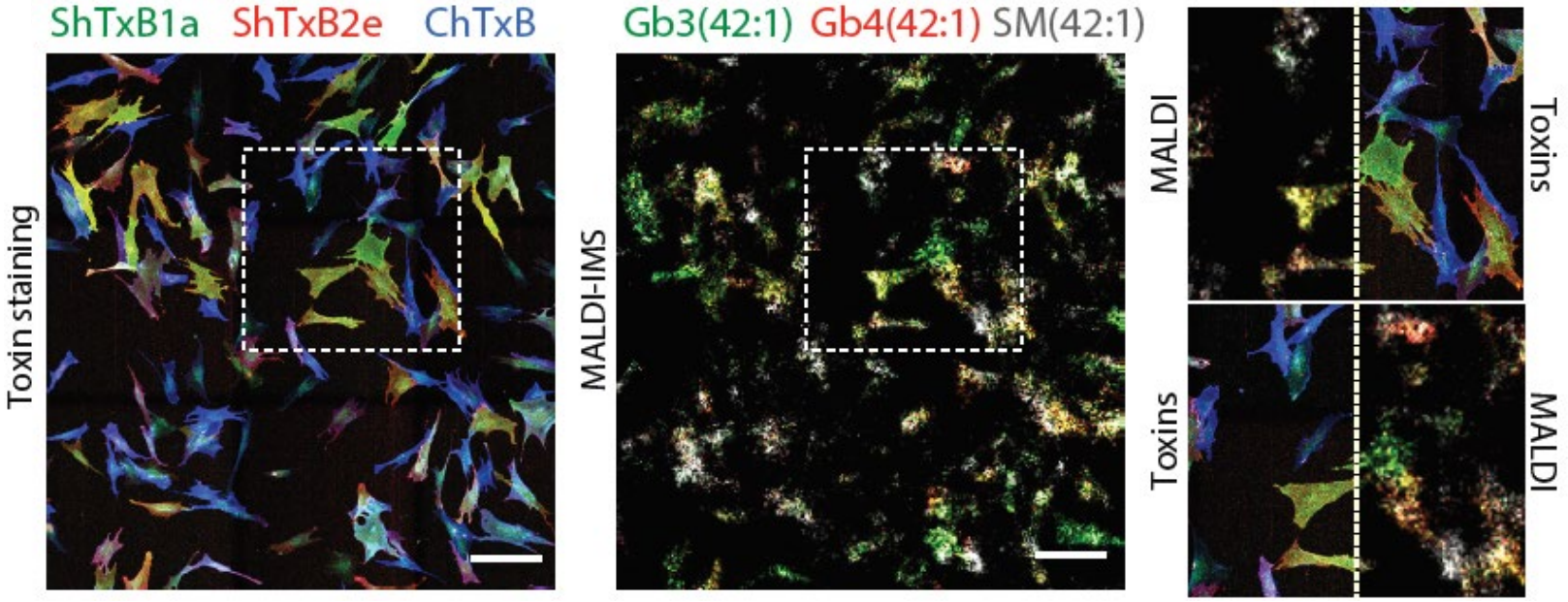


Left: Cells could be grouped according to lipid composition (t-SNE)

Right: certain lipids were enriched in certain clusters, suggesting that dHFs exist in distinct sphingolipid metabolic states

Similar patterns were found in 4 distinct cell lines and in fibroblast biopsies

Lipid labelling with bacterial toxins



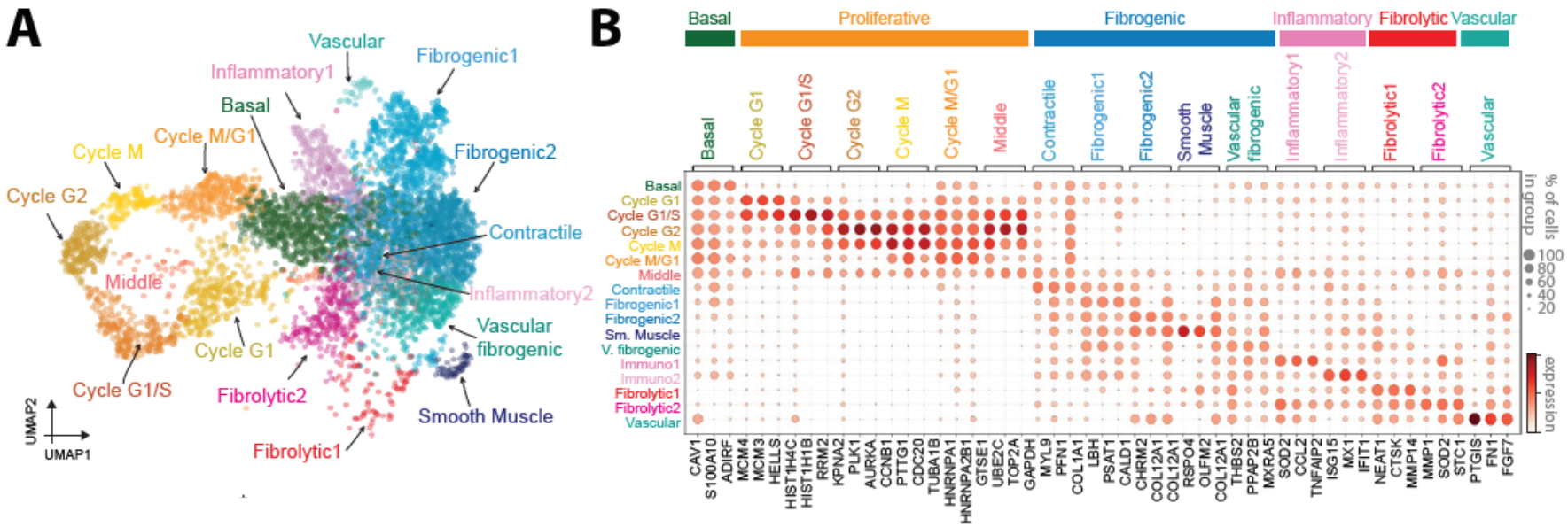
Bacterial toxins conjugated to fluorescent molecules were used to label lipids

- E.g. Shiga toxin 1 binds trihexosylceramides, Cholera toxin binds ganglioside GM1 etc.

Toxin stain matched lipid composition detected in MALDI

Toxin stain intensity decreased after treatment with inhibitors of sphingolipid metabolism (not shown)

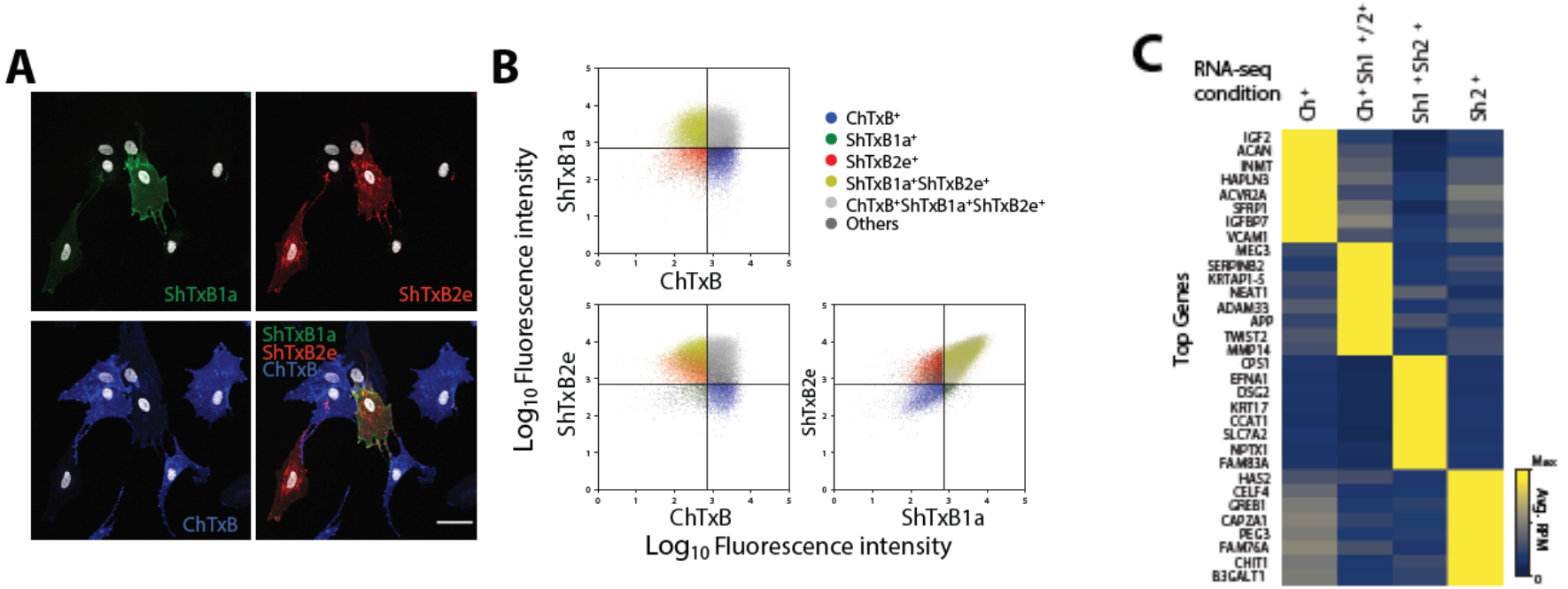
scRNA-seq of 5652 fibroblasts



Fibroblasts clustered according to biological processes (UMAP):

- proliferation (cell cycle genes), pro-inflammatory cytokine secretion (inflammatory), pro-fibrotic secretion (fibrogenic), extracellular matrix remodeling (fibrolytic) and pro-angiogenic factor secretion (vascular)

FACS was used to separate dHFs according to their lipotype (toxin stain)



A. dHFs stained by bacterial toxins

B. FACS gating scheme

C. RNA sequencing of sorted cells yielded distinct transcriptional profiles

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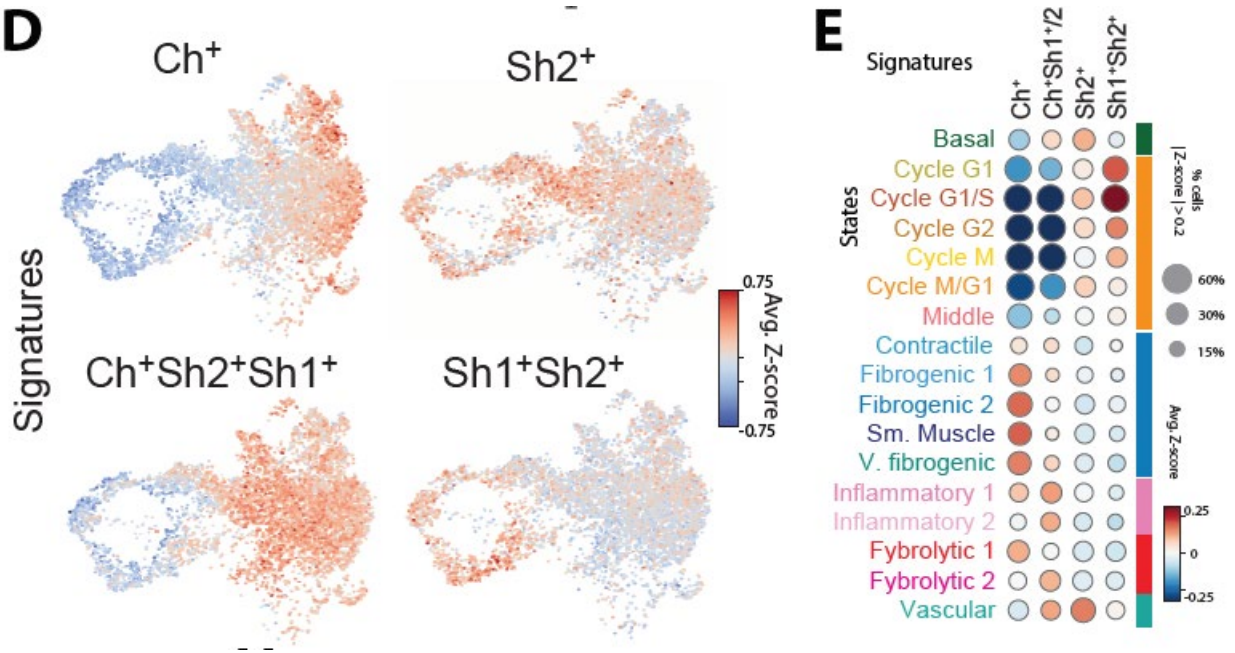


UMAP embedding coloured according to matching lipotype

Ch+: fibrogenic fibroblasts

Sh2+: basal state fibroblasts

Sh2Sh1+: proliferating cells

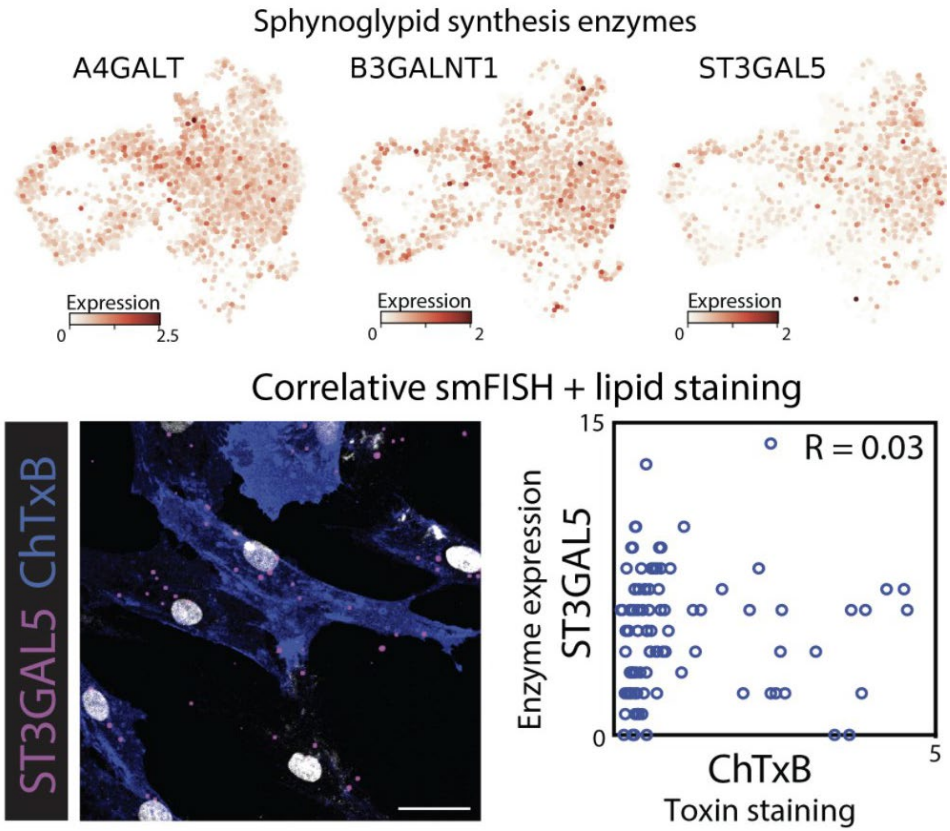
Triple positives: inflammatory, fibrolytic (remodelling) and vascular fibroblasts

Cells are not endowed with transcript. programmes that clearly account for cell lipotype

Surprisingly, genes encoding sphingolipid metab. enzymes were expressed homogenously across cells and did not correlate with cell-state marker transcripts

Are lipotypes causally upstream of cell states, meaning that lipid composition influences cell-cell transcriptional heterogeneity?

Correspondence between lipotypes and cell states could depend on post-transcriptional regulation



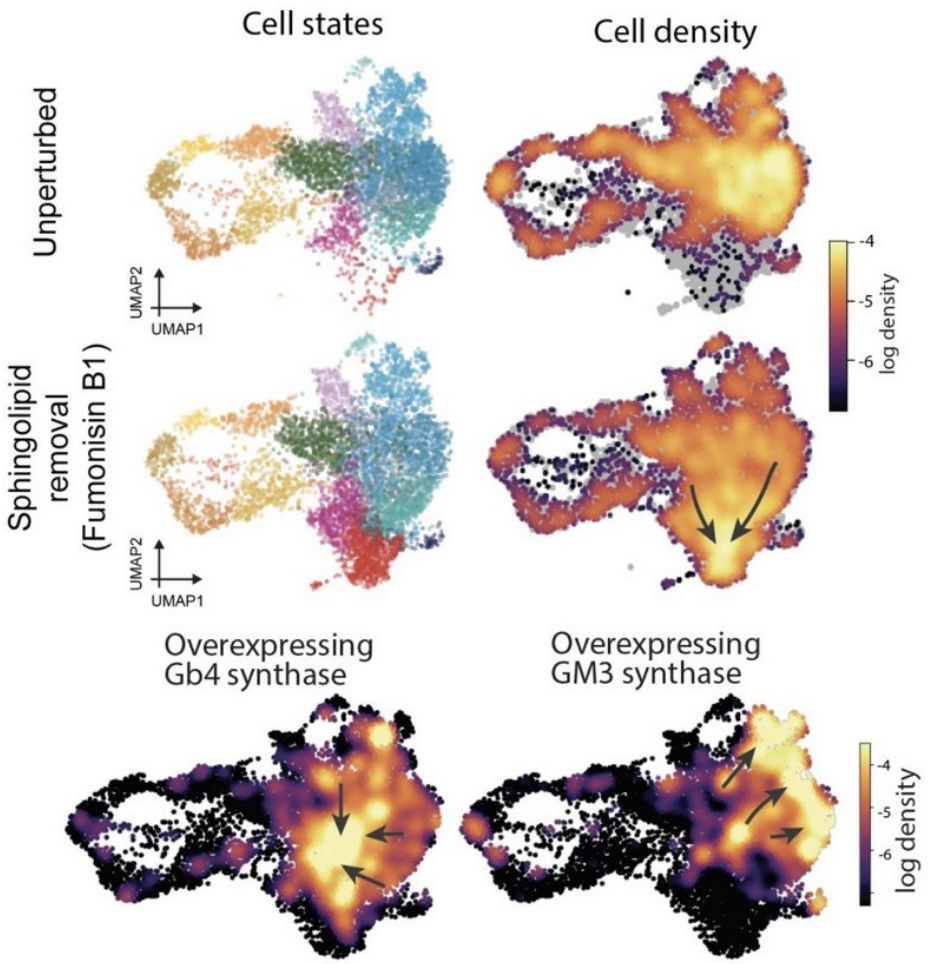
Sphingolipid composition influences cell states

Treating dHF with ceramide synthase inhibitor (FB1) alters cell state distribution

- More frequently fibrolytic and vascular, less often fibrogenic and inflammatory

Alterations in cell state could also be induced by overexpressing sphingolipid metabolic enzymes critical for synthesis of lipotype-defining molecules

→ Overproduction of lipids lead to induction of respective cell state



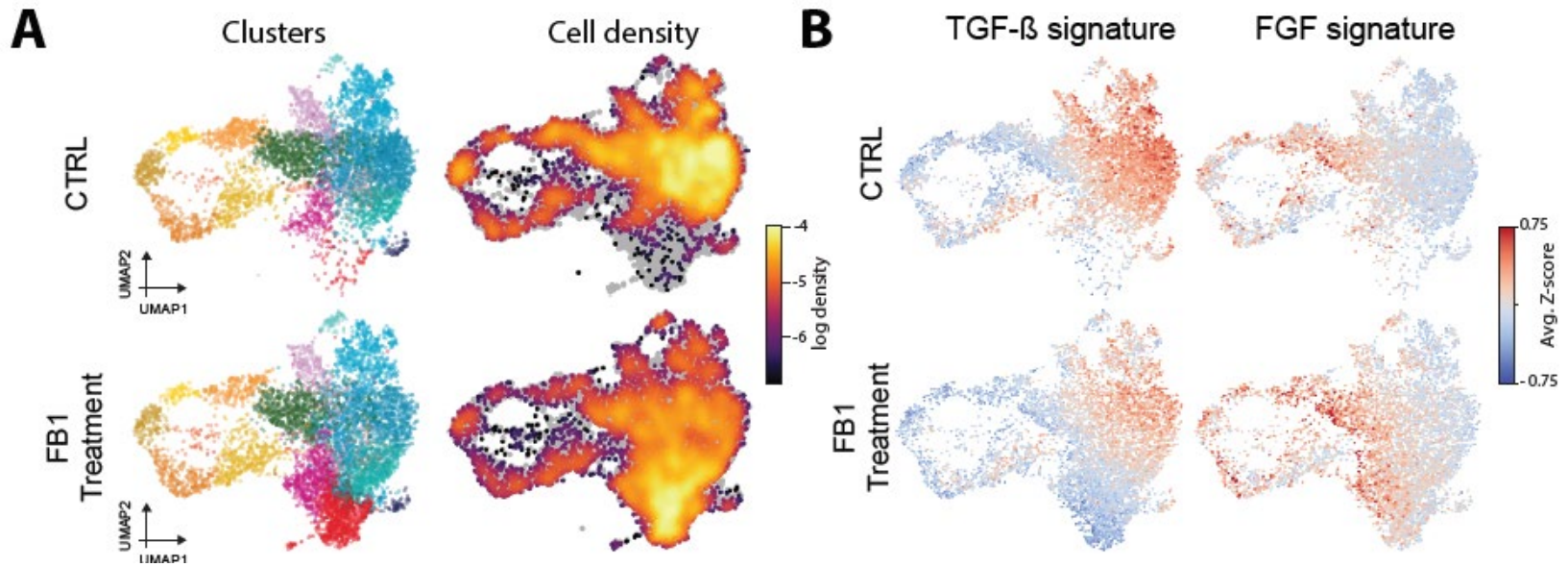
MMP1: matrix metallopeptidase 1
CCL2: C-C chemokine ligand 2
ACTA2: Actin Alpha 2

COL12A1: collagen 12 A1 chain
VCAN: versican

Inflammatory & Fibrolytic
MMP1 ↑, CCL2 ↑, ACTA2 ↑

Fibrogenic
COL12A1 ↑, VCAN ↑

Sphingolipid composition influences cell states



Treating dHF with ceramide synthase inhibitor (FB1) alters cell state distribution

- More frequently fibrolytic and vascular, less often fibrogenic and inflammatory
- **Genes repressed by TGF-beta or activated by FGF2 were particularly affected**

→ Altering lipid metabolism alters activity of regulatory pathways. Is the reverse also true?

TGF: transforming growth factor. FGF: fibroblast growth factor

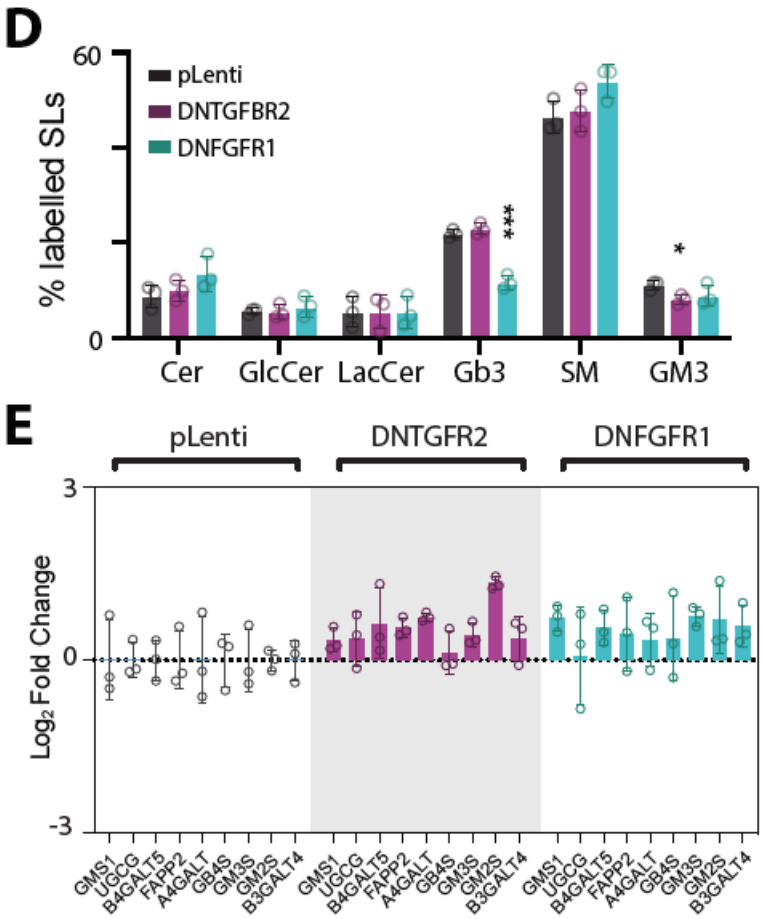
Regulation of fibroblast activity by sphingolipids

Fibroblasts were transfected with lentiviruses:

- Control (pLenti)
- DNTGFBR2: dominant negative TGF-beta receptor 2
  → a mutation that **inactivates TGBR2**
- DNFGFR1: dominant negative FGF-beta receptor 1
  → a mutation that **inactivates FGFR1**

D. Pulse labelling of cultured cells with [H^3]-D-Sphingosine (radioactive), analysis of lipid composition with chromatography → only Gb3 production is lowered in TGFBR mutant

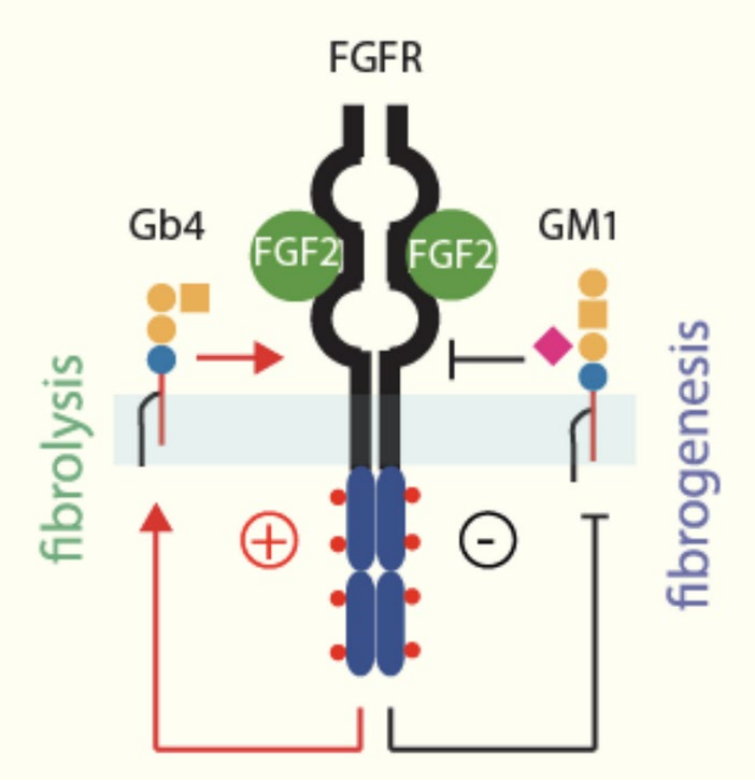
E. qPCR of sphingol. synthesis enzymes → no significant changes – regulation of Gb3 may be post-transcriptional?



GM3: monosialodihexosylganglioside
Gb4: globotetrahexosylceramide

Summary: single cell lipidomics reveals regulation of fibroblast activity by sphingolipids

- Fibroblasts exist in distinct states that are highly relevant to physiology and disease
- MALDI-MSI methodology for spatial analysis of lipid composition was established
- Lipid composition is heterogeneous in cultured fibroblasts
- Bacterial toxins were used to stain lipids
- Toxin stains allowed correlation of lipidomic and FACS / transcriptomic data



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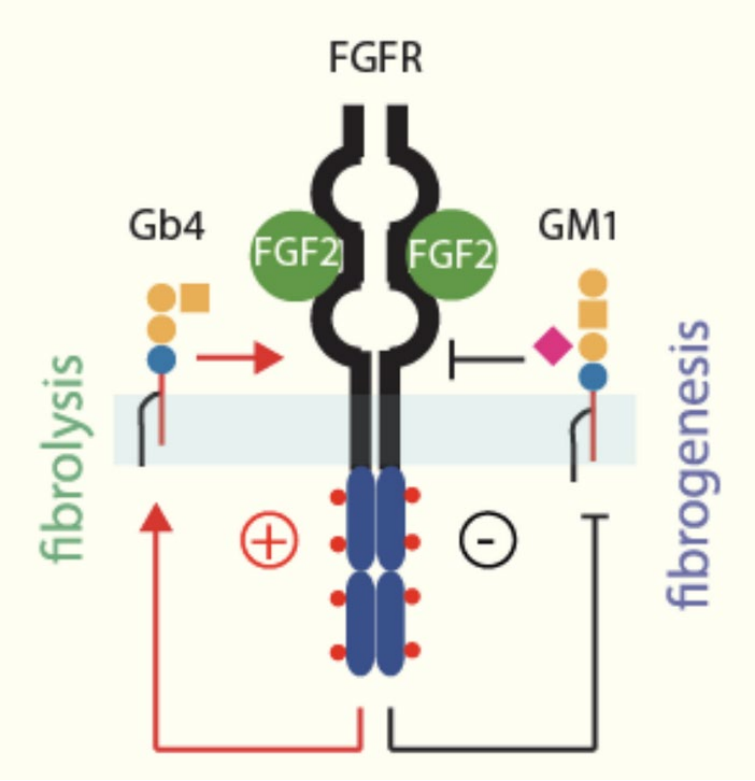
Summary: single cell lipidomics reveals regulation of fibroblast activity by sphingolipids

- Production of GM3 fostered the TGF-beta transcriptional programme → fibrolytic (remodelling) or proinflammatory state with production of MMPs and chemokines
- GM1 and Gb4 had the opposite effect: fibrogenic with production of ECM proteins, downregulation of MMPs

- Lipid remodelling could be an early driver in the establishment of cell identity
- Differences in lipid synthesis cause cells to adopt different cell states

- **Possible mechanism**: Lipids influence cell signalling via regulation of receptors (e.g. FGF receptor)



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Summary: regulation of fibroblast cell states by lipids

The precise differences in lipid composition that directly affect cell phenotypes remain unknown.

Dermal fibroblasts can adopt several transcriptional states that are of relevance to many diseases

- The lipidomes and transcriptomes of individual human dermal fibroblasts were measured, revealing differential lipid compositions
- high-resolution mass spectrometry was coupled to single-cell transcriptomics.
- cell-to-cell variation of specific lipid metabolic pathways contributes to the establishment of cell states involved in wound repair and in skin cancer growth
- Sphingolipid composition defined fibroblast subpopulations while sphingolipid metabolism influenced cell transitions
- Lipid composition: a new regulatory component to the homeostasis and self-organization of multicellular systems.

Thank you for your attention