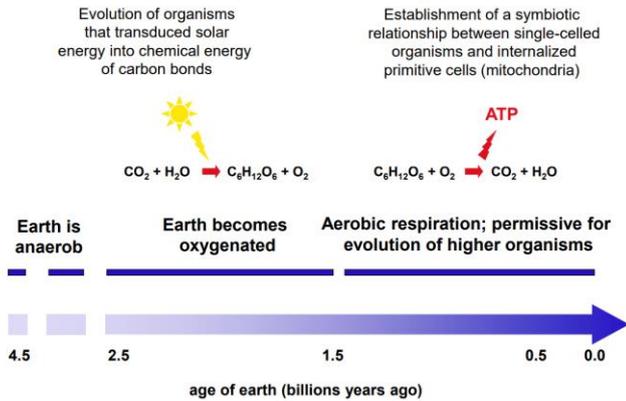


CELL BIOLOGY KOVACS

OXYGEN SENSING, SIGNALING, AND METABOLISM

LIFE WITH (AND WITHOUT) OXYGEN



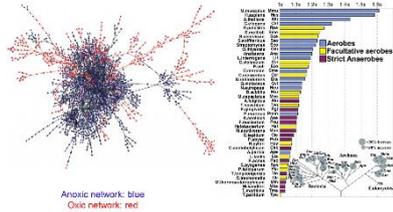
- Advantage to **generate energy** much **more efficiently** after earth got oxygenated
- Oxygenation of the earth permits the **evolution of higher organisms** by **aerobic respiration**
- Because of oxygen's **high energy potential**, mammals became addicted to it. However, now they also needed to handle the **ROS byproducts**

ORGANELLES NEEDING OXYGEN:

- Mitochondria, Peroxisomes, Chloroplasts, ER

EFFECT OF OXYGEN ON KEGG'S METABOLIC "BACKBONE"

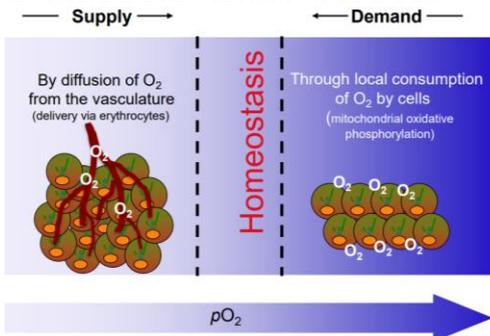
- Oxidic network: 1000 new metabolites which branch out of the dense/core metabolic network that is similar
- Anoxic network: Same core metabolites, but lacks the additional metabolites
- Oxygen needed some completely new metabolic pathways!



OXYGEN HOMEOSTASIS

= Demand of oxygen due to local consumption of oxygen by cells → If demand is fulfilled by **supply from the vasculature**, the homeostasis is reached

- Diffusion is limited → there is an oxygen gradient
- Hypoxic regions induce a demand of oxygen which **induces signalling pathways** to increase the supply and to restore the homeostasis inside the cell



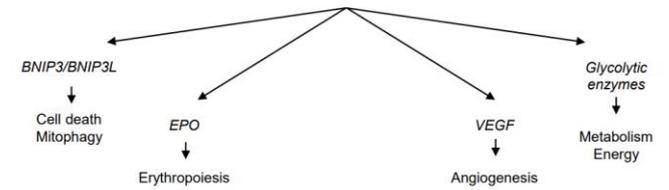
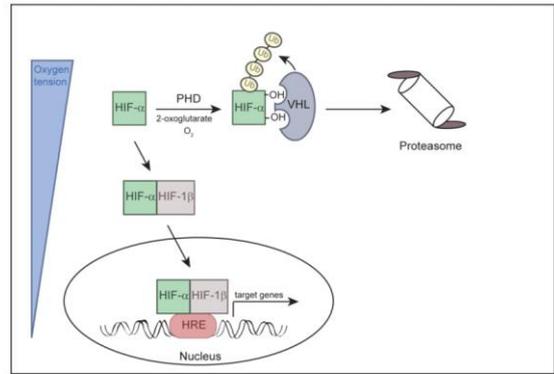
How would a cell adapt to a low oxygen level?

- **Metabolic reprogramming** → change metabolism
- Formation of **new blood vessel** (long term)

OXYGEN SENSING AND HIFs

- **HIFs = Hypoxia-inducible factors**
 - o Three isoforms exist: HIF-1, HIF-2, (HIF-3)

Family of transcription factors that **mediate adaptive responses to hypoxia**



HIF- α subunit and HIF- β subunit **dimerize** → translocate into the nucleus → bind promoter sequence **hypoxia response elements (HRE)** → induce target genes (**Glycolytic enzymes, VEGF, EPO and BNIP3/BNIP3L**)

HIF SUBUNITS AND THEIR REGULATION

- **HIF-1 α / HIF-2 α subunits:** HIF- α activity is **linked to oxygen levels** → are **O_2 -regulated** and get normally degraded (under Normoxia)
- **HIF-1 β subunit:** Constitutively expressed

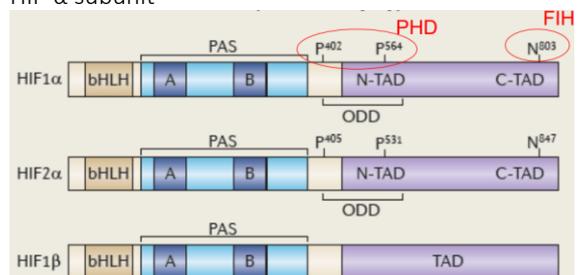
OXYGEN-DEPENDENT REGULATION OF HIF- α DEGRADATION OF HIF- α :

Normoxia: HIF- α is marked/**hydroxylated** (-OH added) by **prolyl hydroxylase domain protein (PHD)** & targeted for **proteasomal degradation** by the von **Hippel-Lindau (VHL) E3 ubiquitin ligase complex**. Further, **FIH (Factor inhibiting HIF) event (asparagine hydroxylase** hydrolyses HIF- α with oxygen as substrate) blocks the interaction between HIF- α subunit and the **transcriptional co-activators p300 and CREB** binding protein

→ **PHD-mediated hydroxylation:** Destabilizes HIF- α subunit

→ **FIH-mediated hydroxylation:** Inhibits transcriptional activity (no interaction with co-activators)

- **PHD** uses **molecular oxygen** and **2-oxoglutarate (= α -Ketoglutarat)** as substrate to hydroxylate HIF- α
- HIF- α get hydroxylated at two specific proline residues (Pro402, Pro564 in HIF-1 α) in the **O_2 -dependent degradation domain (ODD)**
- OH-groups are required for VHL to bind and degrade HIF- α subunit



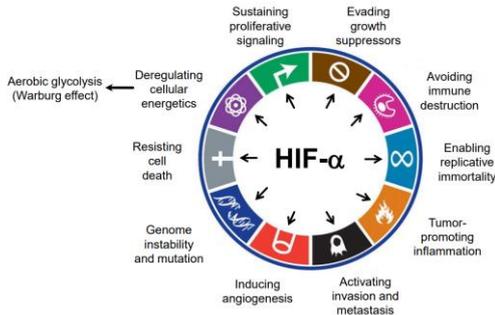
- Hypoxia:** PHD is not active → Hydroxylation is inhibited and HIF-α is **not degraded** → It **dimerises with HIF-1β** and enters the nucleus to induce target gene transcription
- Hypoxia inhibits PHD activity through various mechanisms
 - Substrate limitation → Needs oxygen for hydroxylation

PHD AS O₂ SENSORS

- **PHD is the real oxygen sensing machines**
 - o Needs oxygen to work → use O₂ as substrate for the hydroxylation reaction of HIF-α
- Treatment strategies in patients with anaemia or ischemia: **inhibit PHD to increase EPO production** with PHD inhibitor drugs

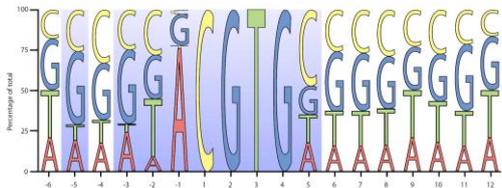
HALLMARKS OF CANCER

HIF-α signalling supports all **hallmarks of cancer**



HRE SEQUENCE AND HIF-A DNA BINDING

- Core consensus sequence of hypoxia response elements (HRE): **CGTG** → HIFα proteins bind to this sequence

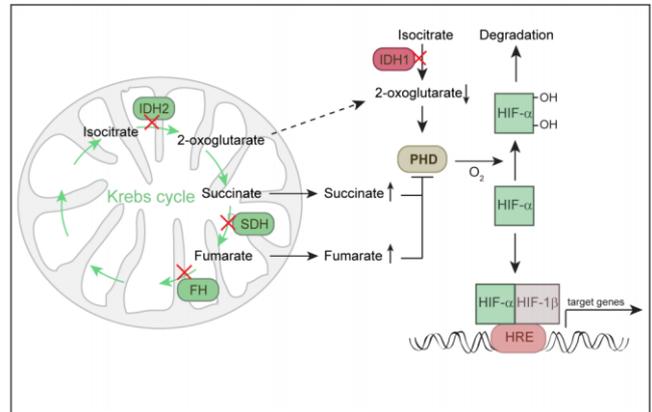


EXCURSE: METHODS TO FIND OUT IF A TRANSCRIPTION FACTOR REGULATES EXPRESSION OF A CERTAIN GENE

- CRISPR/Cas9 induced mutation in TF (HIF-1α) + hypoxic conditions → qPCR → no expression
- **Chip technique** (chromatin immune precipitation): Isolate genomic DNA, cut it, AB against HIF-1α, pull down DNA (just get the DNA fragments which are bound to HIF + ab), amplify fragments and get the promoter activity
- Luciferase assay: Clone promoter of HIF into construct with reporter gene, transfect cell → nice way to get the promoter activity
- In silico analysis: Software tools to align the promoter and look for consensus sequences of HIF

PSEUDOHYPOXIA

Pseudohypoxia: Stabilisation of HIF-1α subunits under normoxic conditions



HIF-α stabilisation under normoxia → Inhibition of PHDs

Responsible for this phenomenon are the **oncometabolites succinate, fumarate and 2oxoglutarate/R-2-hydroxyglutarate** (3 TCA cycle mutations)

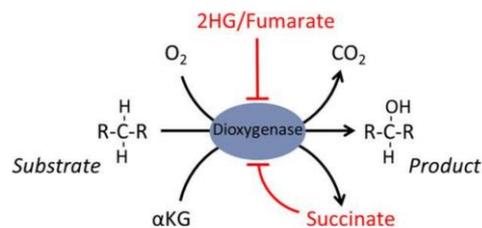
- **Increase in succinate & fumarate** → Inhibition of PHD
 - o Loss of function mutations in the enzymes **succinate dehydrogenase (SDH)** and **fumarate hydratase (FH)** lead to **accumulation of succinate and fumarate** → PHD is inhibited → HIF-α stabilised, no degradation
- **Low 2-oxoglutarate (α-KG)** → Inhibition of PHD
 - o **2-oxoglutarate** is a **substrate** of PHD and is needed for the hydroxylation reaction
 - o Mutations in **isocitrate dehydrogenases 1/2 (IDH1 (cytosolic) & IDH2)** lead to **low levels of 2-oxoglutarate** → decreasing activity of PHDs → low rate of HIF-α hydroxylation → HIF-α stabilised

Hence, **mutation in metabolic enzymes promote cancer development by promoting HIF signalling** even under normoxic conditions

CATALYTIC FUNCTION OF 2-OXOGLUTARATE-DEPENDENT DIOXYGENASES

Catalytic function of PHD

- Co-substrates:
 - o Oxygen
 - o 2-oxoglutarate (α-KG)
 - o **Fe²⁺** and **ascorbate** as co-factors also needed
- **Inhibited by 2HG, Fumarate and Succinate**



CREATION OF A PSEUDO HYPOXIC STATE:

- Fe²⁺ is important for PHD to work
- **COCC₂** and **DMOG** are chelators that bind iron → Enzymes cannot function properly → Creation of pseudo hypoxic state → Activation of hypoxic pathway under normoxic conditions

ONCOMETABOLITE 2-HYDROXYGLUTARATE

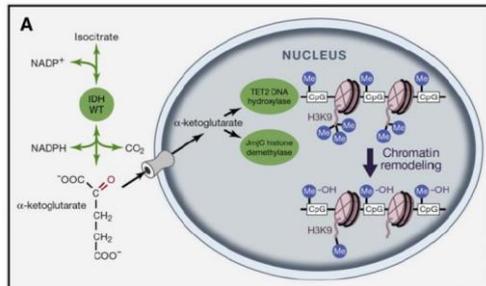
2-HYDROXYGLUTARATE AND HIF- α

- Gain of function mutation in **IDH1/IDH2** can lead to the production and accumulation of **R-2-hydroxyglutarate (2HG)** and depletion of **2-oxoglutarate**
 - o This oncometabolite is not found in a normal cell
 - o 2-hydroxyglutarate is an oncogenic metabolite in leukaemia and brain cancer

DYSREGULATES EPIGENETICS & CELL DIFFERENTIATION

Oncometabolite 2-hydroxyglutarate **dysregulates epigenetics and cell differentiation**

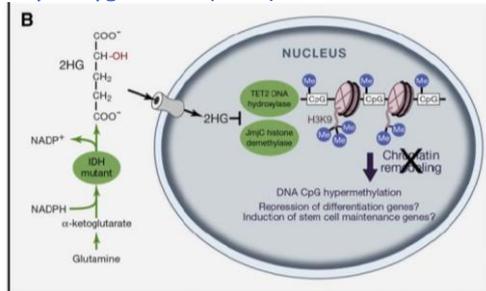
α -ketoglutarate



- Produced in part by **IDH WT**
- Can enter the nucleus and be used as a substrate for **dioxygenase enzymes** that modify epigenetic marks
 - o **TET2 DNA hydroxylase enzyme**: Involved in DNA demethylation
 - o **JmJc demethylase**: Demethylates histone tails

→ Hypomethylated state = differentiated state

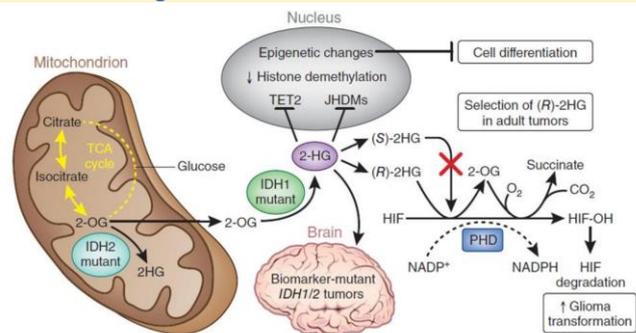
2-Hydroxyglutarate (2-HG)



- Can **competitively inhibit** α -ketoglutarate-dependent enzymes like **TET2** and **KDM** histone demethylases
 - o Impairs normal epigenetic regulation
 - o Results in **altered histone methylation marks**, DNA hypermethylation and dysregulated cellular differentiation

→ Hypermethylated state = genes are constantly accessible
→ proliferative/stem cell like state

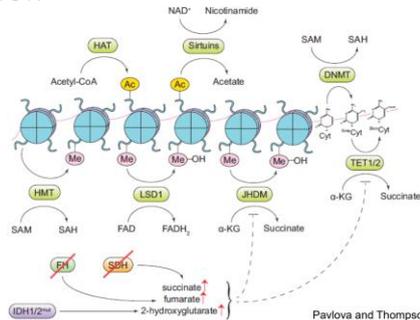
Common feature of cancer-associated **mutations in cytosolic IDH1 and mitochondrial IDH2** is the acquisition of a neomorphic enzymatic activity → **Converts glutamine-derived α -ketoglutarate to 2HG**



2-hydroxyglutarate is an oncogenic metabolite in glioma

- In adult tumors, HIF would inhibit oncogenesis
 - (R)-2-HG is selected for degradation of HIF (HIF-OH) which allows glioma transformation

ALTERATIONS IN METABOLITE-DRIVEN GENE REGULATION

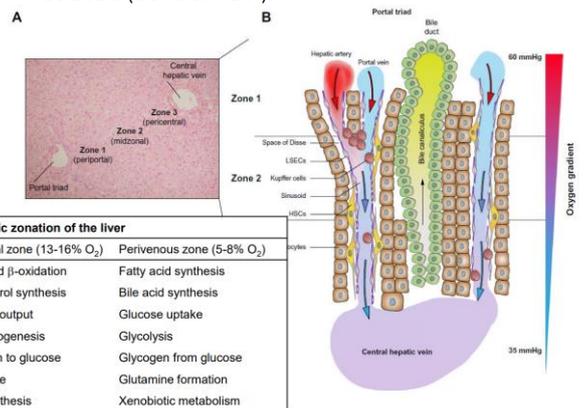


HAT, histone acetyltransferase enzymes
SAM, S-adenosylmethionine
SAH, S-adenosylhomocysteine
DNMT, DNA methyltransferase enzymes
HMT, histone methyltransferase enzymes
LSD1, lysine-specific histone demethylase
JHDM, Jumonji domain-containing histone demethylase enzymes
TET1/2, ten-eleven translocation methylcytosine dioxygenase 1/2

Link between genetic/epigenetic modification and nutrition: Metabolite driven gene regulation → increased succinate and fumarate can also inhibit TET2 and JHDM

OXYGEN GRADIENT AND METABOLIC ZONATION OF THE LIVER

- Oxygen gradient in liver **corresponds with different metabolic pathways** that need more or less oxygen
 - o High oxygen in portal triad zone (zone 1)
 - o Decreasing in direction of central hepatic vein
- Metabolic zonation
 - o **High oxygen levels (13-16%, periportal zone)**: Metabolic pathways that need oxygen → TCA cycle, FA β -oxidation, cholesterol synthesis, Gluconeogenesis, Glycogenolysis,
 - o **Low oxygen levels (5-8%, perivenous zone)**: Pathways that need less oxygen are enriched here
 - o HIF signalling is stronger in regions where oxygen is reduced (central vein)



Experiment: Nutritional stress and hypoxia in the liver:

Mice with high fat diet or high sucrose diet show hypoxic regions in the liver after 10 days → this nutritional stress leads to hypoxic regions and you can assume that here HIF signalling is up-regulated

High fat/sucrose diet induces hypoxia in the liver

Speculation: HIF might be involved in type 2 diabetes development

HYPOXIA AND METABOLIC STRESS:

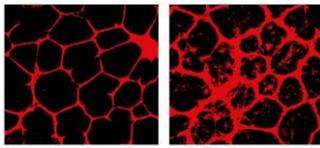
NUTRIENTS UPTAKE

- Survival requires **control systems** that **prevent aberrant cell proliferation** → Uncontrolled proliferation is prevented because cells do not normally take up nutrients unless stimulated to do so by GF
- Cancer cells overcome this GF dependence by acquiring mutations → some pathways constitutively activate the uptake and metabolism of nutrients that both promote cell survival and growth → nutrients uptake exceed the demands

NEOPLASTIC AND NON-NEOPLASTIC TISSUE EXPANSION

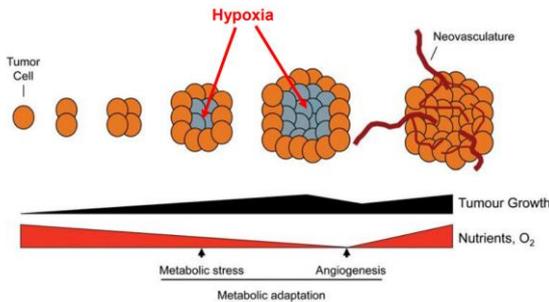
Hypoxia as a key component of neoplastic tissue expansion:

- **Metabolic stress phase:** If a tumor is growing too fast → lack **nutrients and oxygen** → supply of surrounding blood vessels is not enough
- Especially the core of the tumor is depleted of important nutrients and oxygen → hypoxia
- If the tumor is able to resolve this stress situation it can grow further → Crucial changes in this metabolic adaptation phenomenon are:
 - o Induction of **angiogenesis** (HIF → EPO, VEGF) (new blood vessels that are not perfect though)



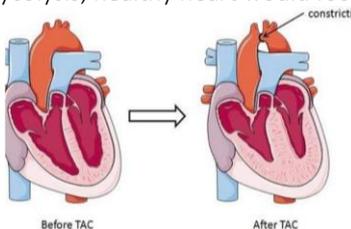
Capillary leakiness of tumor-associated vasculature

- o **Autophagy** (self-digestion of macromolecules to generate essential metabolites for energy)
- o **Metabolic reprogramming by HIF signalling:** Shifts from oxidative phosphorylation to **glycolysis**

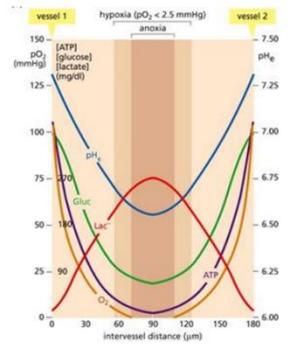


Hypoxia plays also a role in non-neoplastic tissue expansion

- **Obesity:** Nutritional overload → Adipocyte mass expansion → Hypoxia, HIF signalling → metabolic changes
- **Pathologic stressor/transaortic constriction:** Enlargement of heart (hypertrophy) → hypoxia, HIF signalling → metabolic changes (from FA oxidation to glycolysis, healthy heart would focus on FA oxidation)



Hypoxic and metabolic stress reprogramming is common in neoplastic and nonneoplastic tissue expansion



Hypoxia and necrosis of cells in poorly vascularized sections of tissues

METABOLIC STRATEGIES OF DIFFERENTIATED VS. PROLIFERATIVE CELLS

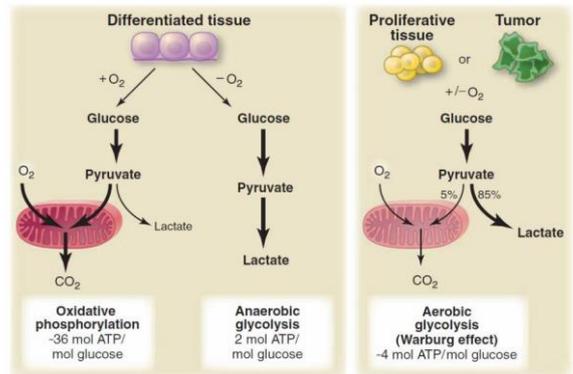
GLUCOSE METABOLISM

Differentiated tissue:

- **Normoxia/Presence of oxygen:**
 - o **Glycolysis:** Glucose is metabolized to pyruvate
 - o **Oxidative phosphorylation:** Pyruvate is nearly completely oxidized to CO₂ (in mitochondria) (Oxygen is essential for this process)
- **Hypoxia/Oxygen is limited:**
 - o **Anaerobic glycolysis:** Cells redirect pyruvate away from mitochondrial oxidative phosphorylation by generating **lactate** → Generation of lactate allows glycolysis to continue by cycling NADH back to NAD⁺, but results in **minimal ATP production (2mol)**

Proliferative tissue:

- **Independent of oxygen concentration**
- 85% (An)aerobic glycolysis
- 5% oxidative phosphorylation



- Normal differentiated cells rely primarily on **mitochondrial oxidative phosphorylation** to generate the energy needed for cellular processes
- Most **cancer** cells rely on **aerobic glycolysis** → “**Warburg effect**” → Cancer cells tend to “**ferment**” **Glucose into lactate** even in **presence of sufficient oxygen** which could support mitochondrial oxidative phosphorylation
 - o Property is shared by normal proliferative tissues
 - o Mitochondria remain functional
 - o Some oxidative phosphorylation continues in both cancer cells and normal proliferating cells

How is the metabolic strategy regulated in proliferative cells?

- Hypoxic conditions → lack of oxygen → oxidative phosphorylation cannot be driven
- HIF recognized lack of oxygen and regulates metabolism towards anaerobic glycolysis resulting in high lactate levels (Lactate is transported out of the cells to prevent strong pH changes)

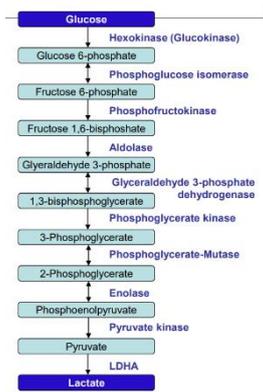
How is the metabolic strategy regulated in tumor cells?

- HIF mutated → metabolism towards aerobic glycolysis

HIF ACTIVATES GLYCOLYTIC GENES

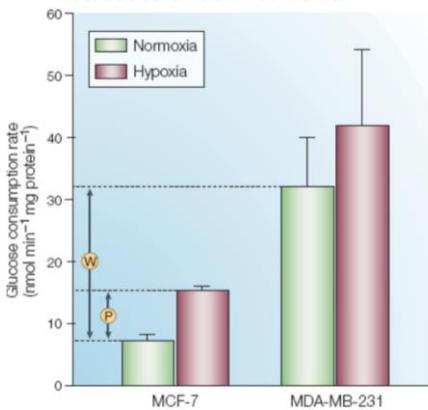
HIF signalling is a **major driver** of the **glycolytic pathway**

- HIF up-regulates all enzymes involved in the glycolysis pathway
- Constitutive HIF signalling in cancers make the transformed cells highly glycolytic



PASTEUR AND WARBURG EFFECTS IN NON-INVASIVE AND METASTATIC BREAST CANCER CELL LINES

- Glucose consumption is **increased under Hypoxia** and **decreased under Normoxia**



P: Pasteur effect, W: Warburg effect
MCF-7: non invasive cell line, MDA-MB-231: metastatic cell line

What is the difference between Pasteur and Warburg effect?

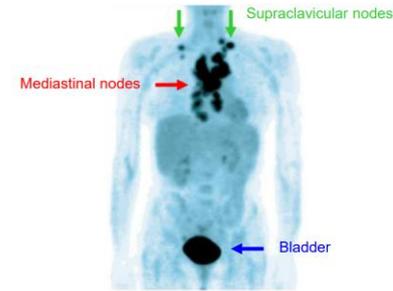
- **Pasteur effect:** Difference of Glucose consumption in cell line under **normoxia** and **hypoxia**
 - o No oxygen → Switch from oxidative phosphorylation to glycolysis = more Glucose consumption
 - o = Inhibiting effect of oxygen on the glycolysis/fermentation process

- **Warburg effect:** Higher rate of **glycolysis** even in the **presence of oxygen** (aerobic glycolysis)
 → Most **cancer** cells rely on **aerobic glycolysis**: Cells **“ferment”** Glucose into lactate even in **presence of sufficient oxygen**

- Remember: Just 2 mol of ATP are produced
- Reasons for this metabolic switch:
 - Metabolic adaptation of cancers to **promote growth, survival, proliferation, and long-term maintenance**
 - By promoting Glucose uptake and fermentation of glucose to lactate a **lot of metabolic intermediates are created** → can sustain the fast proliferation and growth (ATP not so essential)
 - Mitochondria are NOT defect

DIAGNOSTIC:

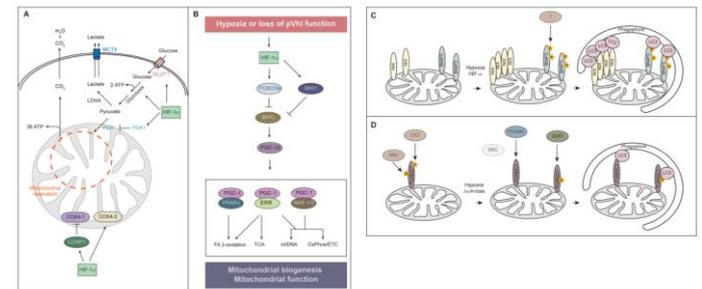
High Glucose consumption and uptake of cancers is **used for diagnostic purposes** → Marked glucose analogue is used that can be taken up but is not further metabolised → Dark spots in tomography are tissues, that takes it up quickly and accumulates it (=Cancer)



Positron-emission tomography imaging with 18 fluorodeoxyglucose of a patient with lymphoma

METABOLIC ADAPTATION: REGULATION OF MITOCH. FUNCTION AND ABUNDANCY BY HIF-1α

- HIF signalling has a crucial role in **metabolic reprogramming**
- Under hypoxia **mitochondrial metabolism is shut down** in order to reduce ROS damage
- Problem: HIF signalling in cancers → promotes cancer by inducing this metabolic reprogramming effects which involve:

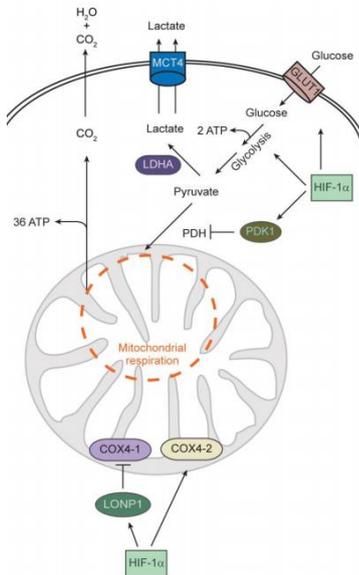


1. Mitochondrial metabolism **2. Mitochondrial bioenergetics** **3. Selective autophagy of mitochondria (Mitophagy)**

Tell me 3 ways how HIF signalling could inhibit mitochondrial function/metabolism?

1. HIF-DEPENDENT REGULATION OF GLYCOLYSIS AND MITOCHONDRIAL METABOLISM

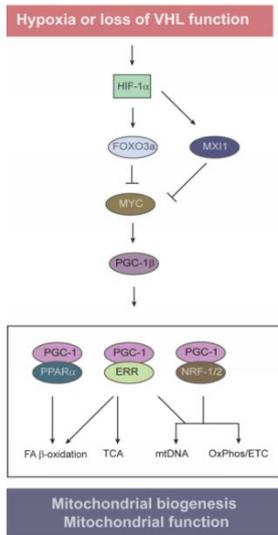
- Low oxygen: Cells undergo two **HIF-1α-mediated** alterations of cellular metabolism:
 - o O₂-independent ATP production (aerobic glycolysis)
 - o Reduction of mitochondrial O₂ consumption (avoiding ROS)
- HIF-1α signaling contributes to **aerobic glycolysis** by stimulating the expression of **GLUT1** and **glycolytic enzymes**
 - o HIF-1α induces: GLUT1, glycolysis enzymes, PDK1, LDHA, MCT4, COX4-2, LONP1
- Increased glycolysis generates increased levels of pyruvate, which is largely converted to lactate by **HIF-inducible lactate dehydrogenase A (LDHA)** and removed from the cell by the MCT4 transporter
- HIF-1α induces **pyruvate dehydrogenase kinase 1 (PDK1)**, which **inhibits PDH** and **blocks conversion of pyruvate to acetyl-CoA** → resulting in decreased flux through the TCA
 - o Decreased TCA cycle activity prevents oxidative phosphorylation and excessive mitochondrial ROS production



- Under normoxia, **COX4-1** is the predominant isoform of COX4 present in the electron transport chain, which transfers electrons to O₂
- Under hypoxia, HIF-1α upregulates the expression of **COX4-2** (more efficient, less ROS) and the mitochondria protease **LONP1**, which in turn degrades **COX4-1**

2. REGULATION OF MITOCHONDRIAL BIOGENESIS AND ABUNDANCE BY HIF-1α

- HIF-1α induces the **expression of MXI1** (= repressor of **MYC**) → Represses a subset of MYC target genes such as **PGC-1β**
- HIF-1α activates **FOXO3a** → inhibits **MYC** activity
- Impaired interaction between **PGC-1** and TF such as PPARα, ERR, and NRF-1/2 orchestrates the major functions of mitochondria

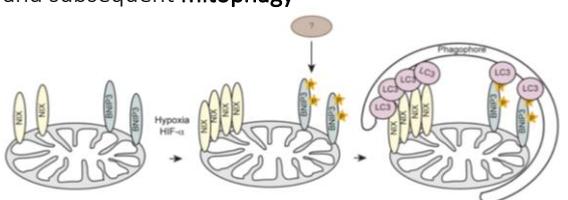


- HIF-1α mediated **inhibition of MYC and PGC-1** results in reduced mitochondrial biogenesis

3. MITOPHAGY IN MAMMALIAN CELLS:

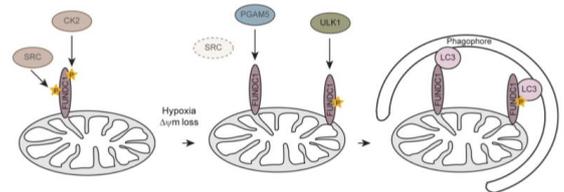
NIX/BNIP3-MEDIATED MITOPHAGY IN MAMMALIAN CELLS:

- **NIX and BNIP3**: Outer mitochondrial membrane proteins that interact with **LC3**
- Under hypoxia: HIF-1α induces NIX and BNIP3 → Phosphorylation of BNIP3 **promotes** its binding to **LC3** and subsequent **mitophagy**



FUNDC1-MEDIATED MITOPHAGY IN MAMMALIAN CELLS

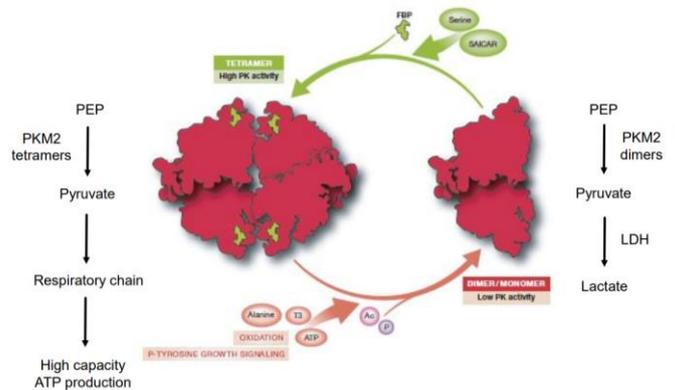
- **FUNDC1**: Outer mitochondrial membrane protein that interacts with **LC3**
- Under normal physiological conditions: FUNDC1 is phosphorylated → prevents LC3 binding
- Under hypoxia or loss of mitochondrial membrane potential: Expression of **SRC** is strongly suppressed & **PGAM5** (phosphatase) is induced by HIF signalling → dephosphorylation of FUNDC1 → Enhanced interaction between FUNDC1 and LC3 → promotes mitophagy
- Phosphorylation of FUNDC1 by **ULK1** enhances its binding to LC3



PKM2

CELLULAR SIGNALING EVENTS THAT MODULATE PKM2 ENZYMATIC ACTIVITY

- **PK**: **Pyruvate kinase** → Glycolytic enzyme → Catalyses last step in glycolysis (dephosphorylation PEP to pyruvate)
 - o Specific pyruvate kinase **isoforms** support **different energetic and biosynthetic requirements** of cells in tumors and normal tissues
 - Expression of PKM1 isoform has been associated with **differentiated tissue**
 - **PKM2 isoform** is closely linked to **embryogenesis, tissue repair, and cancer**



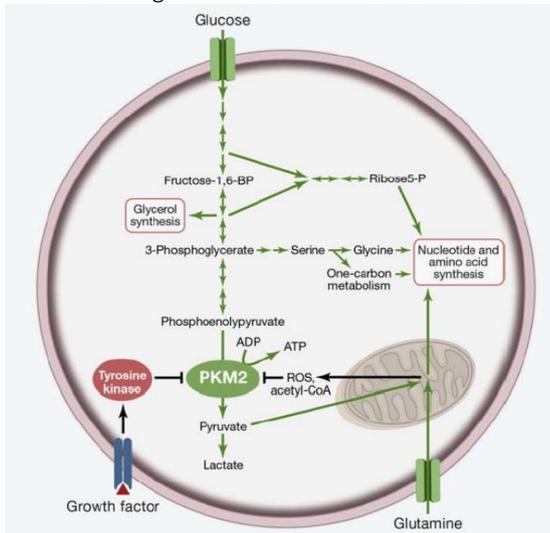
- **PKM2 tetramer**: Highly active, high PEP to pyruvate conversion → respiratory chain → high ATP
- **PKM2 dimer**: Low rate → accumulation of intermediates of glycolysis → supports anabolic growth
- There is constant switching between dimeric and tetrameric state
- Allosteric regulation is achieved through stabilization or destabilization of the enzyme tetramer
 - o **Stabilization**: Upstream glycolytic intermediate FBP, serine and SAICAR
 - o **Destabilization**: Alanine, ATP, thyroid hormone T3 and acetylation & phosphorylation (GF signalling)

PKM2 EXPRESSION IN PROLIFERATING CELLS FACILITATES MACROMOLECULAR SYNTHESIS

- Decreased enzymatic activity of PKM2 in the cytoplasm **promotes accumulation of upstream glycolytic intermediates** and their shunting into **anabolic pathways** that include:

- o Pentose phosphate pathway
- o Glycerol 3 phosphate pathway (Lipids)
- o Serine synthetic pathway (Nucleotides & AA)
- o One carbon metabolism (Epigenetic changes)

→ Metabolic growth



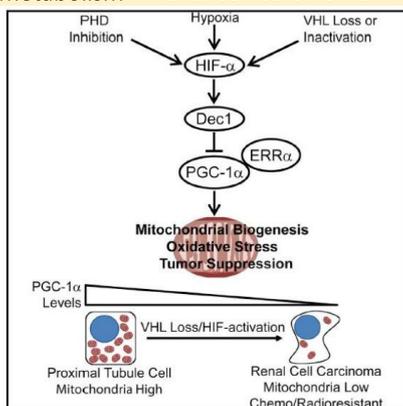
- **GF** signalling via **Tyrosine kinase** inhibits **PKM2** → link to cancer
- **Glutamine** addition of cancer: Used to refill TCA cycle in order to synthesise **AA, FA** and **lipid** under **hypoxic conditions** → allows formation of extramitochondrial acetyl CoA without the need of **PDH**

Would you inhibit or activate PKM2 for cancer treatment?

- **Activate PKM2** in order to **maintain glycolytic flux** and to **avoid accumulation of intermediates** that are crucial for fast proliferation and growth of transformed cells
- Additionally, **restore NAD+** (essential for glycolysis) by pyruvate that can be metabolised to lactate by LDH
- Further treatment options are **MCT4 inhibitors** → prevent lactate secretion → **acidification** of tumor cell

SUPPRESSION OF PGC-1α

Suppression of PGC-1α is critical for reprogramming oxidative metabolism in renal cell carcinoma



- PGC-1α expression induces oxidative stress and suppresses ccRCC tumor growth
- Low PGC-1 α expression: Marker of poor prognosis in ccRCC

SUMMARY:

How could the metabolism be modulated back towards oxidative phosphorylation?

PKM:

- **Lower activity of PKM2** leads to accumulation of upstream intermediates (needed for proliferation)
- Treatment: **Activate PKM2** in order to **maintain glycolytic flux** and to **avoid accumulation of intermediates** that are crucial for fast proliferation and growth of cancer cells

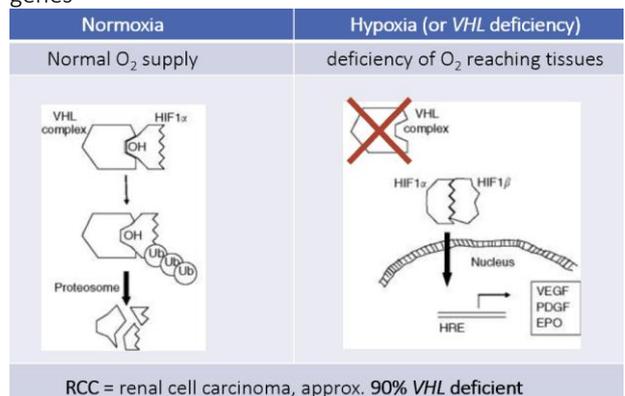
PGC-1

- HIF-α inhibits Myc → no PGC activation → no mitochondrial biogenesis
- Treatment: **Promotion of PGC-1** could bring back some of the mitochondrial functions & reduce cancer growth

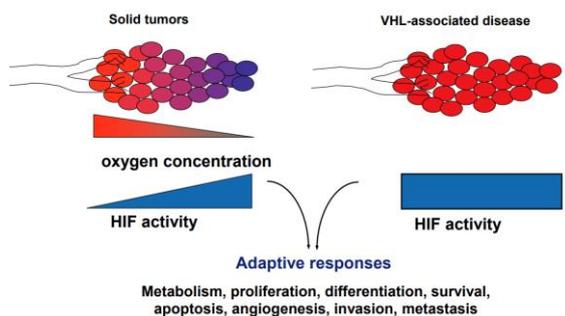
HIPPEL-LINDAU (VHL) DISEASE

- **VHL mutations** lead to the von Hippel-Lindau disease → HIF is stabilized and constantly active

- o VHL is **tumor suppressor**
- o VHL is responsible for **HIF-α degradation**
- **Hypoxia OR VHL deficiency**: HIF is not hydrolysed and therefore not degraded by VHL → leads to HIF-α and HIF-β dimerization and thus activation of several target genes



- **Multiple tumor formation** in central nervous system and visceral organs
- Often associated with **strong renal cell carcinoma (RCC)**
- Lipid accumulation in clear cell renal cell carcinoma:



LINKS BETWEEN HIFs, TYPE 2 DIABETES, AND METABOLIC SYNDROME

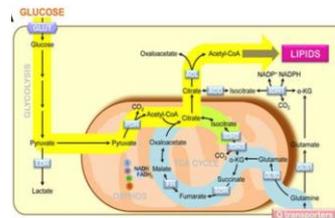
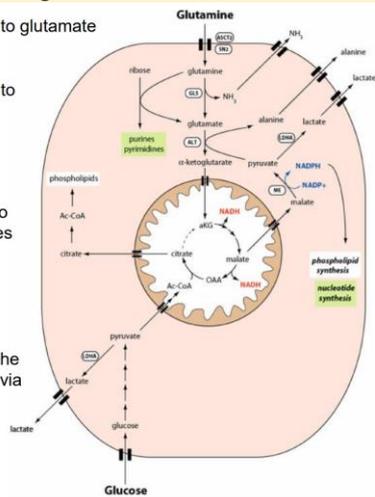
Lipid accumulation links HIFs to metabolic syndrome and type 2 diabetes

TCA CYCLE: GLUCOSE AND GLUTAMINE

Glucose and glutamine are sources for **carbon and nitrogen**
 → Needed for amino acid synthesis

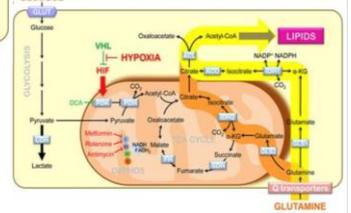
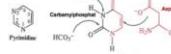
Glutaminolysis leads to the generation of NADPH

1. Deamidation of glutamine to glutamate
2. Transamidation of glutamine to glutamate through enzymes of nucleotide biosynthesis
3. Transamination of glutamate to α -ketoglutarate via transaminases (alanine aminotransferase)
4. Mitochondrial metabolism of α -ketoglutarate to malate, and the oxidation of malate to pyruvate via malic enzyme (ME)



1. Glucose oxidation
2. Glutaminolysis (Glutamine oxidation)
3. Reductive carboxylation (RC) (mitochondria and cytoplasm)

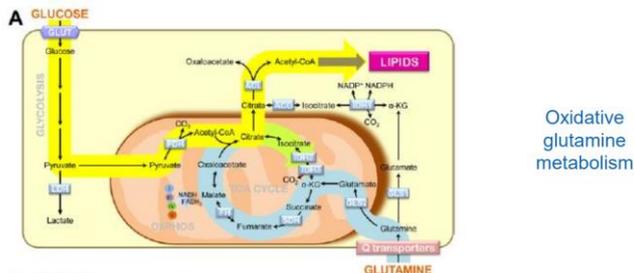
All 3 ways are used for
 - Lipid synthesis
 - Pyrimidine synthesis through aspartate



- A cell can run the TCA cycle in three different ways:
 - o **Glucose oxidation**: Glucose is used as a substrate
 - o **Glutaminolysis**: Glutamine is used as a substrate and the TCA cycle is run clockwise around
 - pathways are normally used in **normoxic conditions**
 - o **Reductive carboxylation**: Glutamine is used as a substrate and the TCA cycle is run the opposite direction
 - Only used in hypoxic conditions because the **glucose oxidation pathway is inhibited by HIF**

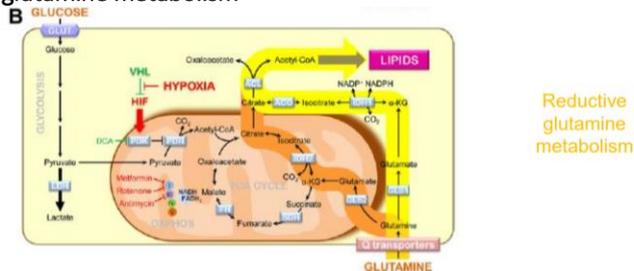
A METABOLIC SWITCH IN CARBON SOURCE FOR LIPID SYNTHESIS

Normoxia/differentiated cell: Glucose is taken up and metabolised to pyruvate, which is introduced in the TCA cycle → Formation of acetyl CoA (main substrate for lipid synthesis). Glutamine is taken up by transporter converted to glutamate and introduced into the TCA cycle as well. Here it is used to replenish the TCA cycle intermediate α -KG → Glucose is the main source of acetyl CoA for lipid synthesis → oxidative glutamine metabolism



Hypoxia/cells that have a VHL mutation: Uptake of glutamine → converted to glutamate in mitochondria or in cytoplasm → Further metabolization to α -KG → isocitrate (cytoplasm) = NADPH restored

Formation of acetyl-CoA → lipid formation → glutamine main source of acetyl CoA for lipid synthesis → **reductive glutamine metabolism**



- Regeneration of NADPH is important for **phospholipid synthesis and nucleotide synthesis** under hypoxia
- Glutamine is also used for the **synthesis of non essential AA** (carbon and nitrogen source)
- Both processes support the **metabolic adaptation of cancer cells**, which are almost **glutamine addicted**

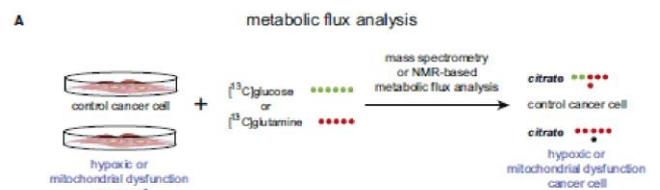
Under hypoxic conditions, there is a **reprogramming of carbon metabolism triggered by HIF alpha** → switch to a **glutamine-maintained TCA cycle** which leads to an **increased glutamine utilization for biomass and reducing equivalent production**

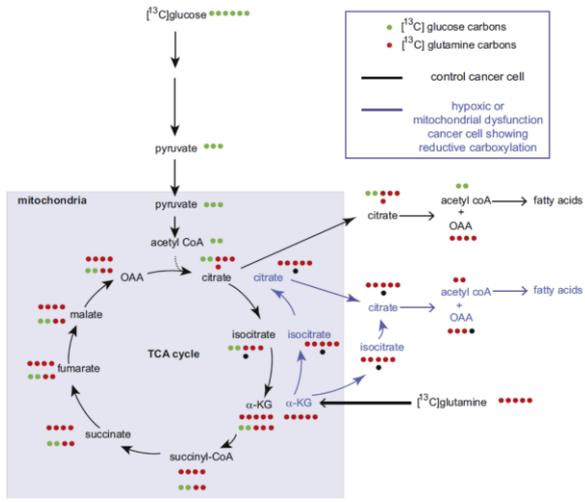
SUMMARY

- In normoxic conditions, **citrate** serves as substrate for Acetyl-CoA **for lipid formation**
- Under hypoxia, the TCA cycle does not run → no citrate → to overcome this lack of citrate, 3 steps of TCA are reserved
 - o Glutamine uptake → glutamate → ketoglutarate → isocitrate → citrate → Acetyl-CoA → lipids (= "anaplerosis")
 - o Tumor cells therefore get addicted to glutamine (also increased activity of glutamine synthetase)

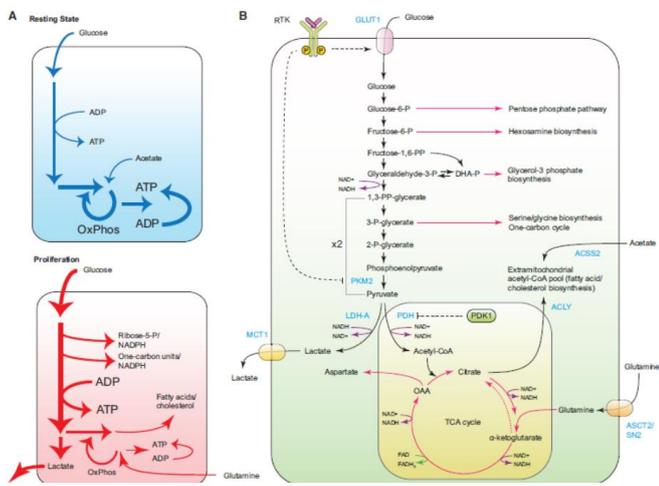
METABOLIC FLUX ANALYSIS

- Metabolic flux analysis reveal **dysregulated cancer cell metabolism** under hypoxia or mitochondrial dysfunction
- Labelled Glucose and glutamine (different carbon isotopes), analysing the metabolite citrate with mass spectrometry technique: If all carbons in citrate are from glutamine → hypoxic/mitochondrial dysfunction/cancer cell → sign that cell has a reductive glutamine metabolism





EXCURSE: USE OF GLYCOLYSIS/TCA CYCLE INTERMEDIATES FOR BIOSYNTHESIS AND NADH PRODUCTION



LIPID METABOLISM

PRINCIPLE ROLES OF LIPIDS

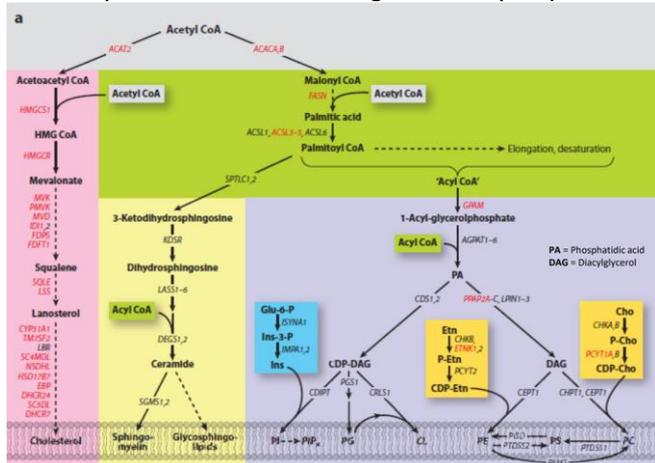
- Whole body energy homeostasis
- Transcription and signal transduction
- Environment sensing
- Protein modifications
- Generation of permeability barriers
- Protection against reactive metabolites
- Membrane integrity

DISTURBANCES IN LIPID METABOLISM

- Changes in cellular and circulating lipid composition are associated with numerous human pathologies
- It is therefore widely acknowledged that lipid homeostasis is critical for health
- Disturbances in lipid metabolism underlay several diseases:
 - o Obesity
 - o Atherosclerosis and heart attack
 - o Stroke
 - o Smith-Lemli-Opitz syndrome
 - o Zellweger syndrome
 - o Cancer clear cell phenotype
 - o (Type 2 diabetes, Liver steatosis, Coronary heart diseases)

PRINCIPAL PATHWAYS FOR MEMBRANE LIPID SYNTHESIS

- Acetyl CoA is the main building block for lipid synthesis



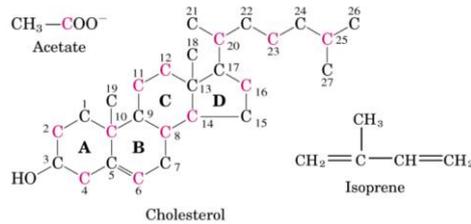
CHOLESTEROL

How can the liver maintain the homeostatic balance of cholesterol?

- De novo synthesis (needs quite a lot of energy and substrates)
 - Uptake from diet
 - Secretion (cholesterol efflux)
 - Catabolism to bile acids (secretion)
 - Storage in lipid droplets
- Different pathways work together and guarantee cholesterol homeostasis
- Dysregulation leads to diseases

CHOLESTEROL FACTS

- Cholesterol as central lipid of mammalian cells
- Transported with lipoproteins (core hydrophobic, surface hydrophilic part of phospholipids, proteins etc.)
- Structure: 27 carbon molecule, 4 rings
- Precursors: Acetate (acetyl-CoA) and isoprene



- Cholesterol biosynthesis is among the most intensely regulated processes in biology
- Transport: via LDL/HDL
 - o Cells have various pathways for the uptake of cholesterol from LDL and export to HDL
 - o Intracellular cholesterol transport/distribution: Vesicular and non-vesicular mechanisms
- Cholesterol provides membranes with special physical properties (stability and fluidity)
- Metabolites of cholesterol are important
 - o Steroids, oxysterols and bile acids
 - o Important biological roles as signal transducers and solubilizers of other lipids
- Cholesterol is critical for embryonic development
 - o Early stage of pregnancy: Cholesterol is transported from mother to embryo
 - o Late stage of pregnancy: Embryo relies on de novo synthesis of cholesterol
- Aberrant cholesterol homeostasis is involved in pathogenesis of cardiac and brain vascular diseases, dementias, diabetes, and cancer
 - o Diseases caused by a defect in cholesterol trafficking (e.g., Niemann-Pick type C, Tangier disease)

HISTORY OF CHOLESTEROL

- Isolated from gallstones in 1789

First Half—The Era of Cholesterol

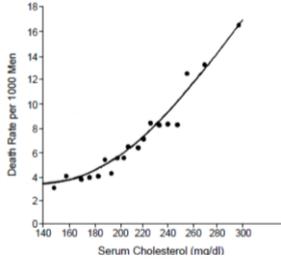
1910	Human atherosclerotic plaques contain cholesterol
1913	High cholesterol diet causes atherosclerosis in rabbits
1919	Heart attacks recognized in humans
1933	Feedback inhibition of cholesterol synthesis demonstrated
1938	Familial hypercholesterolemia described
1950	Cholesterol biosynthetic pathway elucidated
1951	High-fat diets raise plasma cholesterol in humans
1953	Risk factor concept advanced

Second Half—The Era of LDL

1955	LDL identified as risk factor for CHD
1973	LDL receptor discovered
1976	HMG CoA reductase inhibitors (statins) discovered
1981	Statins increase LDL receptors in vivo
1987	First statin (Mevacor) approved for human use
1994	Statins decrease heart attacks and prolong life
1997	SREBP pathway elucidated
2006	PCSK9: Destroyer of LDL receptors

CHOLESTEROL IN DISEASE

CHOLESTEROL IN HEART DISEASE:



Heart Disease

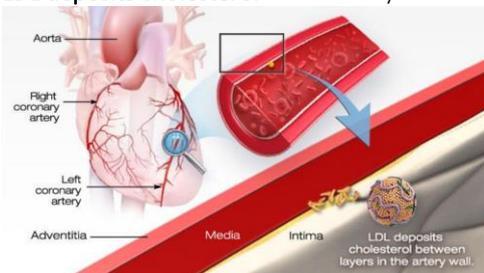
- #1 killer in USA
- Largely preventable
- Strongly linked to cholesterol
- Overall deaths linked as well (see figure)

- Link between deregulated cholesterol levels and CVD
- Link between high fat diet and high cholesterol levels which in turn correlate with heart diseases

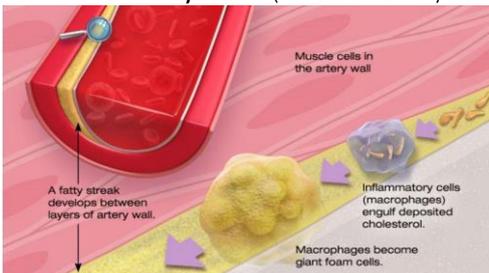
CHOLESTEROL IN ATHEROSCLEROTIC PLAQUES AND CORONARY ARTERY DISEASE

- Endothelium in blood vessel can be damaged by high blood pressure, smoking and increased levels of LDL

1. LDL deposits cholesterol between layers in artery wall



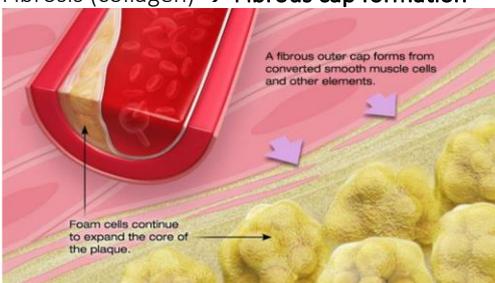
2. Formation of fatty streak (between artery muscle layers)



3. Macrophages start entering and phagocyte LDL

4. Macrophages become giant foam cells → expands plaque core

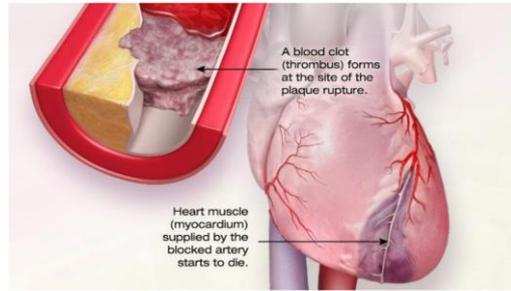
- 5. Macrophages produce pro inflammatory cytokines → Inflammation + infiltration of smooth muscle cells → Fibrosis (collagen) → **Fibrous cap formation**



6. Plaque rupture (eg. by increased streaming velocity) → blood clot formation



- 7. Blockage of blood stream → lack of muscle support → cell/muscle death
- 8. If heart muscle affected → cardiac arrest



→ Plaques are formed, vessel diameter gets smaller (higher blood pressure) (cardiac hypertrophy) → Plaques can rupture (thrombosis → ischemia/stroke)

DYSLIPIDEMIA

= Abnormal lipid amount in the blood

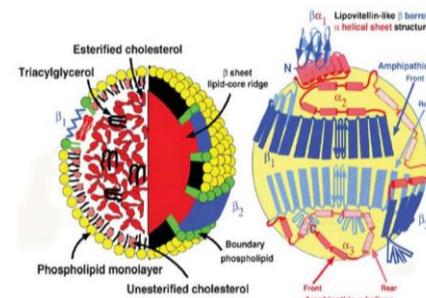
- Packaging into lipoprotein is defective
- Too high LDL-C levels, too low HDL-C levels
- Major risk factor for **atherosclerosis**

4 MAJOR CLASSES OF LIPOPROTEINS

Intercellular transport of unesterified („free“) cholesterol, cholesteryl esters and other lipids through the aqueous circulatory system is dependent on the **packaging of these hydrophobic molecules into water-soluble carriers = lipoproteins**

- Lipoproteins are defined by density and size
 - o **VLDL**: Very low-density lipoprotein: Triglyceride (TAGs) carrier from the liver
 - o **LDL**: Low-density lipoprotein (“bad” cholesterol)
 - o **IDL**: Intermediate density lipoprotein
 - o **HDL**: High-density lipoprotein (“good” cholesterol) (smallest particle)
 - o **Chylomicrons**: Dietary lipid carrier synthesized in the intestines (biggest particle)

LDL



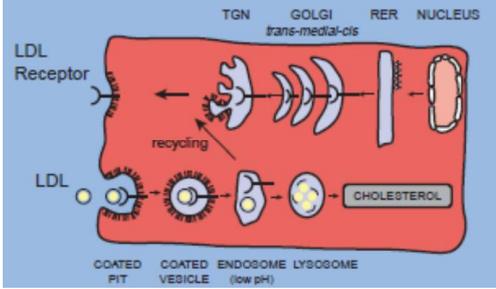
- Inside filled with triacylglycerol (TAG) and **esterified cholesterol (CE)** (LDL particle consists mainly of CE)
- Outer surface:
 - o Phospholipid monolayer
 - o Contains unesterified cholesterol
 - o Contains apoB-100 lipoprotein → important for the receptor mediated uptake in the liver via the LDLR (mediates interaction with receptor)

LDL RECEPTOR:

- Determines LDL level in blood circulation
- Binds LDL → internalisation → disassembly
- Is **regulated by cellular cholesterol concentration**
- Can be induced by “Statin” drugs
- Defective in familial hypercholesterolemia (FH)

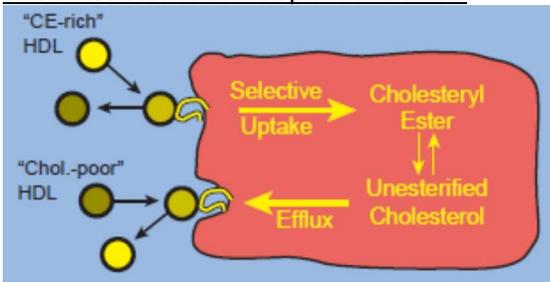
CHOLESTEROL UPTAKE OFO CELLS

a) LDLR mediated endocytosis: (receptor mediated)



1. LDLR precursor is shuffled into the ER
→ LDLR synthesis in the ER
 2. Post-translational modifications in the Golgi
 3. Transport to the surface (remains membrane-bound)
 - o Plasma membrane
 - o Mature LDLR sits in clathrin coated pits
 4. LDL bind to LDLR with apoB100
 5. Vesicular internalization of LDL-LDLR-complex
 - o Coated vesicle
 6. Formation of the endosome → pH is lowered
 7. Lower pH induces dissociation of LDL/LDLR
 - o LDLR gets recycled (back to membrane, if no PCSK9)
 - o LDL gets disassembled by hydrolytic enzymes in lysosome
 - o Cholesterol is released and distributed in the cell
- Niemann-Pick type C: No cholesterol distribution from lysosomes = accumulation, stuck cholesterol

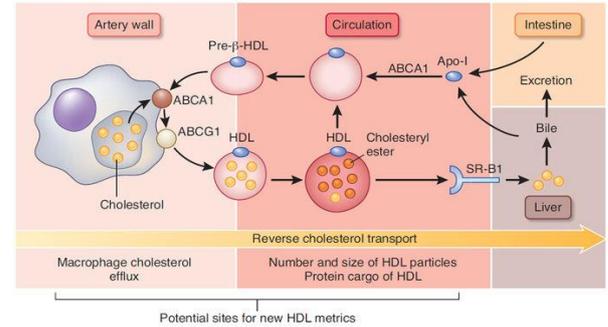
b) SR-B1 mediated selective uptake and efflux:



- Scavenger receptor B1 multifunctional receptor for cholesterol influx and efflux (No endocytosis!)
- **Uptake:** CE-rich HDL binds to SRB1 on cell surface → Selective uptake of CE (CE can be converted into unesterified cholesterol)
- **Efflux:** Reverse cholesterol transport → Unesterified cholesterol goes out of the cell into an HDL (SR-B1 bound) (HDL gets from cholesterol poor state to a cholesterol rich state)

REVERSE CHOLESTEROL TRANSPORT PATHWAY:

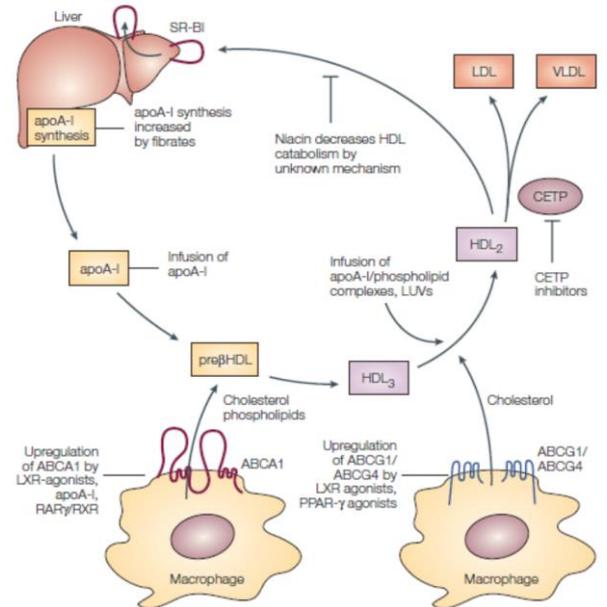
= Transport of cholesterol (ester) from periphery back to the liver → SRB1 mediated selective uptake of CE in liver



1. ApoA-I synthesis in the liver → Secretion
2. ABCA1 in macrophages transfers cholesterol phospholipids to apo1-a → nascent HDL
3. LCAT esterifies cholesterol → HDL3
4. ABCG1 in macrophages transfers cholesterol to HDL3 → HDL2
5. HDL2 goes into periphery
 - Plays a major role in avoiding atherosclerosis/plaque formation in sub-endothelial tissue of arteries
 - CE can be loaded into HDL particles via ABCA1 and ABCG1 → This CE rich HDL can then be transported to the liver and CE can be selectively taken up by hepatocytes via SRB1 → The now CE poor HDL particle can recirculate and repeat the reverse cholesterol transport from periphery to liver

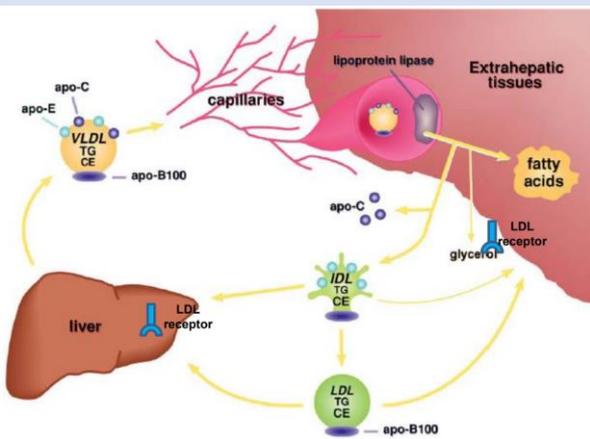
TREATMENT TARGETS OF ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

- Increase apo HDL, decrease LDL



- Fibrates: Ligands to the TF PPARα → Increase apoA1 synthesis → more cholesterol uptake into HDL
- Infusion of apoA1-q complexes → More HDL formation
- Upregulation of ABCA1 or ABCG1/4 → Secretion of cholesterol and phospholipids from macrophages to avoid formation of foam cells and atherosclerotic plaques is promoted
- CETP inhibition → Prevents transformation of HDL to LDL or VLDL
- Decrease HDL degradation
- Niacin → decreases HDL catabolism

VLDL, IDL, AND LDL PATHWAY



- VLDL particles are assembled in the liver and secreted in order to supply muscle, adipose tissue with FA
 - o VLDL consists of 3 lipoproteins (apo E, C and B100), mainly TAGs and cholesteryl ester (CE)
- Lipoprotein lipase hydrolyses TAGs in capillaries near target tissues → FA can be taken up
- Simultaneously: apoC dissociates from particle → This leads to the generation of IDL
- IDL can be taken up by the liver or also by tissues (via LDLR) or it can be transformed to the LDL

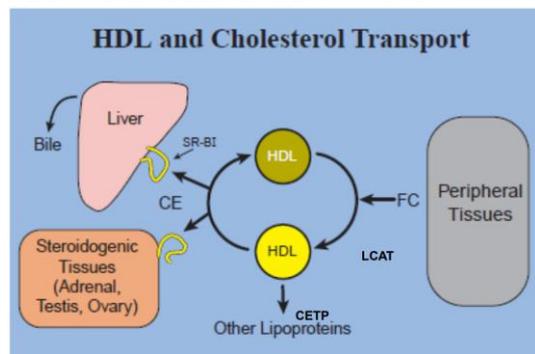
A WAY OF CHOLESTEROL THROUGH THE WHOLE BODY

1. Intestine → Liver by **chylomicrons**
2. Liver → Peripheral tissues by **HDL**
3. Peripheral tissues → HDL as free cholesterol by **LCAT** (lecithin cholesterol acyltransferase)
4. HDL can be transformed into other lipoproteins by **CETP** (Cholesteryl Ester Transfer Protein)
5. HDL → steroidogenic tissues/ liver as cholesterol esters (uptake via SR-B1)

- Cholesterol is transported from peripheral tissue mainly as cholesteryl ester
- Transformation is catalysed by LCAT enzymes
- SRB1 in hepatocytes can take up CE selectively (reverse cholesterol transport)
- CETP transfers CE from HDL to the LDL pathway
 - o CETP is therefore also potential drug target

HIGH-DENSITY LIPOPROTEIN (HDL)

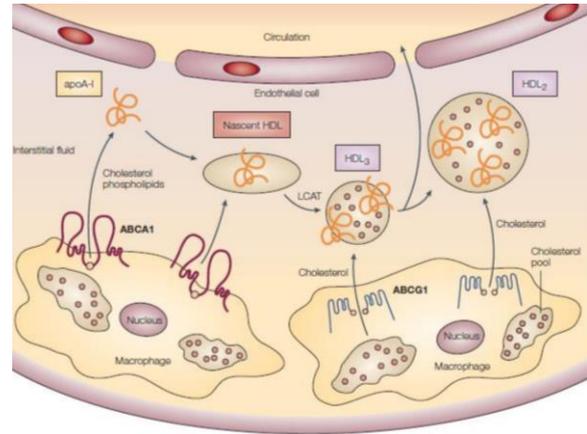
HDL AND CHOLESTEROL TRANSPORT



SR-B1: scavenger receptor class B type I
 LCAT: lecithin:cholesterol acyltransferase
 CETP: cholesteryl ester transfer protein
 CE: cholesteryl ester

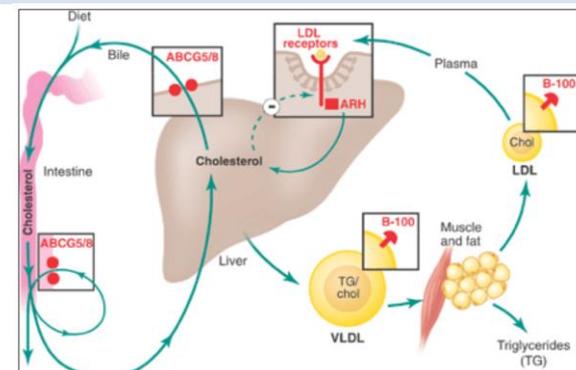
Krieger M, Annu. Rev. Biochem. (1999)

HDL PARTICLE GENERATION



- apoA1 lipoprotein is synthesised in the liver & secreted
 - o It can take up cholesterol and phospholipids which are secreted from macrophages via ABCA1 → Formation of the pre/nascent HDL
- Further cholesterol uptake and esterification through the LCAT enzymes leads to the formation of mature HDL(3/2) particles
- HDL goes into circulation and can be freed from CE via SRB1 in the liver
- Tangier disease (mutation in ABCA1): Low HDL levels since formation of the particle is impaired

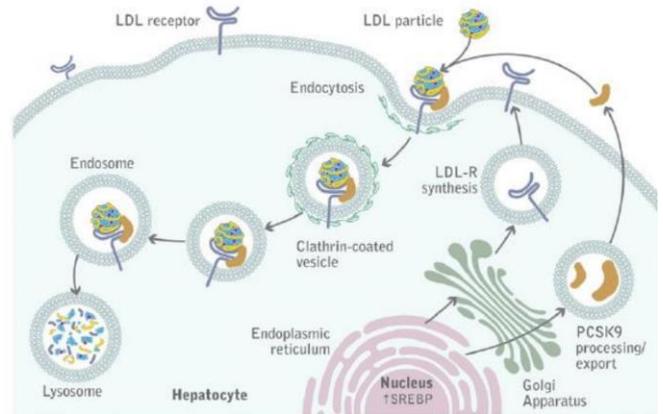
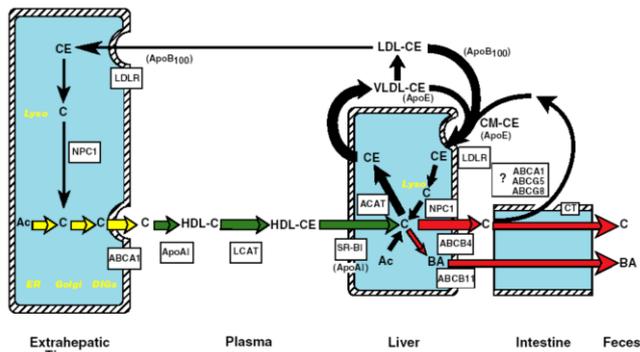
MONOGENIC DISORDERS CAUSING HYPERCHOLESTEROLEMIA



Besides high fat diet and a sedentary life-style some genetic diseases are also known to increase the levels of cholesterol in the organism and lead to early heart attacks:

- **Familial hypercholesterolemia (FH)** → LDLR mutation, loss of function
 - o Bumps on skin: Deposits of cholesterol derived from LDL
 - o Heterozygous: 2 fold increase of cholesterol levels
 - o Monozygous: Several heart attacks at the age of 6 and 8
- **Familial ligand-defective apoB-100 (FDB)** → Mutation in apoB100
 - o Impaired interaction with LDLR → Increase of cholesterol
- **Autosomal recessive hypercholesterolemia** → ARH, adaptor protein of LDLR
 - o Functional LDLR but still decreased uptake of LDL since adapter protein is needed
- **Sitosterolemia** → Mutation in ABCG5/ABCG8 transporters, bind to each-others
 - o Increase uptake of plant sterols (would be secreted if functional transporters) and cholesterol
 - o Increase cholesterol level in the liver (also because efflux of cholesterol into the bile is impaired)
 - o Accumulation of cholesterol in the liver inhibits LDLR receptor transcription (SREBP pathway) → Even more increasing LDL/cholesterol levels

FLOW OF CHOLESTEROL THROUGH THE MAJOR TISSUE COMPARTMENTS OF THE MOUSE



THE DESTROYER OF LDL-RECEPTOR: PCSK9

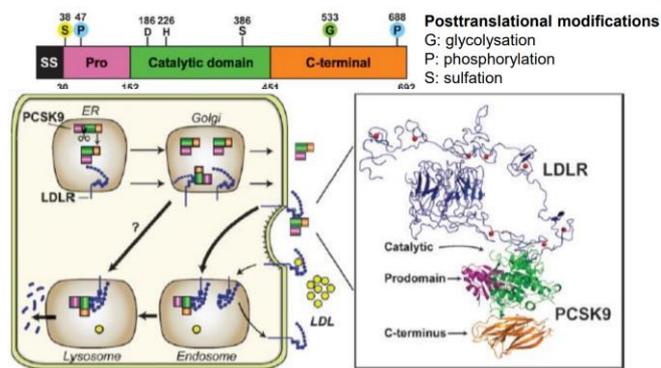
PCSK9 = Proprotein convertase subtilisin-like/kexin type 9
 - Inhibitor of LDLR → Degradation of LDLR

DISCOVERY:

- 2003: Gain of function mutations in the gene PCSK9 in two french families with autosomal dominant form of familial hypercholesterolemia (high LDL levels) were identified
- 2005: Loss of function mutation in PCSK9 was associated with low plasma LDL levels (88% lower risk of developing heart disease)
- 2007: Total PCSK9 deficiency in two women: Very low LDL levels and no health problems

STRUCTURE AND SYNTHESIS

- PCSK9 is a **secreted protein** which is synthesised and simultaneously secreted into the ER as a pro-protein
- Autocatalytic cleavage leads to the formation of two parts which attach to each other and are ready for the PTM (glycosylation, phosphorylation, sulfation) in the Golgi → **Activation upon cleavage of pro-domain**
- After this processing mature form is secreted
- Structure:
 - o SS domain
 - o Pro-domain
 - o Catalytic domain
 - o C-terminal domain



MECHANISM OF ACTION

- Usually when LDLR binds LDL it is taken up by endocytosis and switches from an open to a closed form in the endosome leading to the dissociation of LDL particle from the receptor → Receptor recycling
- PCSK9 binds to LDLR at plasma membrane (→ stays in the open conformation → no more recycling) → **lysosomal degradation**
- PCSK9 can also bind to LDLR in Golgi → Direct lysosomal degradation without presentation of LDLR on plasma membrane

PCSK9 AS NOVEL TARGET FOR LDL-C LOWERING THERAPY

- Inhibition of PCSK9 is a therapeutic approach to lower LDL levels

Therapeutic approaches for PCSK9 inhibition:

- Antibodies against PCSK9 (Inhibition of autocatalytic site)
- Small-molecule inhibitors
- Molecular scaffolds: Adnectins
- Gene silencing (siRNA lipidoid nanoparticles)
 - PCSK9 anti-sense oligonucleotide (ASO): reduction in circulating total cholesterol levels by 53% in mice.
 - Locked nucleic acid (LNA) ASO: *Pcsk9* mRNA levels reduced by ~60% in mice; 2.5-3-fold increase in hepatic LDLR levels; circulating PCSK9 reduced by ~50% and plasma LDL-C and apoB levels decreased by 35% in nonhuman primates
 - siRNA in lipidoid nanoparticles: reduced plasma PCSK9 levels by ~70% and plasma LDL-C levels by ~56% in cynomolgous monkeys
 - Induction of loss-of-function mutations in mice, using clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) genome editing, results in reduced plasma PCSK9 and LDL-C concentrations.

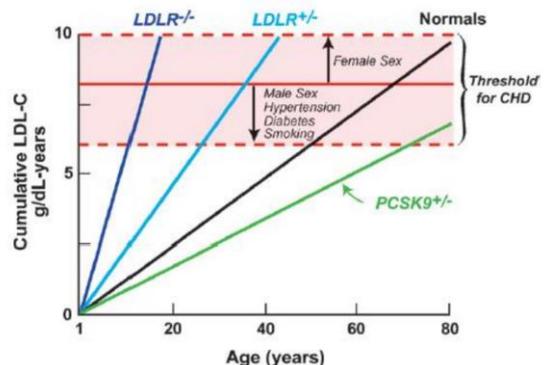
Inhibition of PCSK9 **reduces the degradation of LDLR** → more LDLR are present on the cell surface → allows **more uptake of LDL** and **reduces the amount of circulating LDL** in the blood → **less cholesterol deposition** in blood vessels

Would an inhibition of PCSK9 heal FH patients?

FH= familial hypercholesterolemia

- Homozygous FH patients (LDLR^{-/-}) do not have any functional LDLR → drug will not reduce the disease burden → Should not be treated with such drugs!
- Heterozygous patients have 50% functional LDLR → might partially reduce the LDL levels in blood → Can be treated

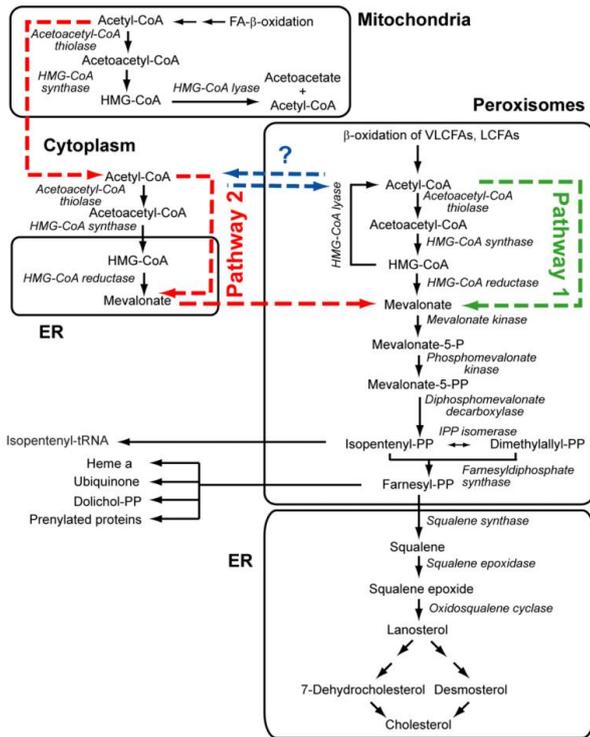
For successful treatment via PCSK9 inhibition, patients need to have functional LDLR!



Relationship between cumulative LDL-C exposure and age

REGULATION OF CHOLESTEROL SYNTHESIS IN EUKARYOTES

Regulation via HMG-CoA reductase and Statins



- **Acetyl CoA** is the **crucial building block** → Can come from FA β-oxidation in mitochondria or peroxisomes
 - o Continuous polymerisation of acetyl CoA subunits leads to the formation of cholesterol
- Synthesis needs a **lot of energy and precursors**
- More than 20 enzymes are involved in the synthesis pathways
 - o Enzymes are distributed in different organelles: Mitochondria, cytoplasm, ER, peroxisomes
 - o Signal sequences at N/C terminus direct enzymes to proper place
 - o An important enzyme is the **HMG-CoA reductase** (mainly in ER and peroxisome) → It is the rate-controlling enzyme of the **mevalonate pathway** (metabolic pathway that produces cholesterol and other isoprenoids)

Which techniques would you use to determine the subcellular localization of a protein?

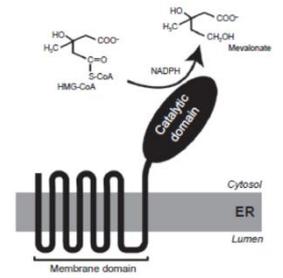
- Bioinformatics and in silico tools (consensus targeting sequence)
- Fluorescent tags/markers (need to know where signal sequence is located in protein of interest) (e.g. peroxisome proteins have C-terminal sequence, do not put it after that)
- Immunofluorescence (IF) and immunohistochemistry (IHC)
- Deletion constructs/mutations affecting target sequence → look if localisation is impaired
- Immunoelectron microscopy (differ if protein is in IMM or OMM or in space between)
- Subcellular centrifugation of lysate (separate organelles on density gradient + mass spectrometry)
- Proximity labeling (BIOID or APEX2)

HMG-COA REDUCTASE

Reduces HMG-CoA into mevalonate

DOMAIN STRUCTURE OF HMG-COA REDUCTASE

- 8 transmembrane loops
- 2 major domains:
 - o Catalytic domain: Facing cytoplasm (HMG-CoA → Mevalonate using NADPH)
 - o Membrane domain with sterol sensing domain (SSD)

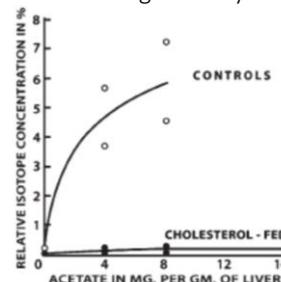


STATIN INHIBITORS OF HMG-COA REDUCTASE

- **Statins:** Competitive inhibitors of enzymes
 - o Have structural similarities with HMG-CoA and bind to the catalytic domain
 - o Induce expression of LDLR → Increase LDL uptake
 - o Higher LDL uptake → less cholesterol required → synthesis inhibited by **negative feedback mechanism** (HMG-Coa reductase inhibited)
 - o Side effects are myopathy, muscle pain to severe muscle loss (very rare)

FIRST DESCRIPTION OF CHOLESTEROL FEEDBACK

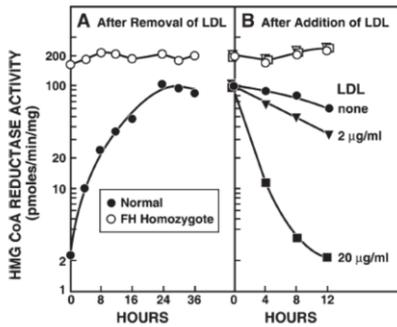
- Animal synthesises large amounts of cholesterol only when this substance **is not furnished in the diet**
- With the administration of moderate amounts of cholesterol, synthesis decreases → Mice/cell culture with no cholesterol = animal/cells induce endogenous synthesis of cholesterol



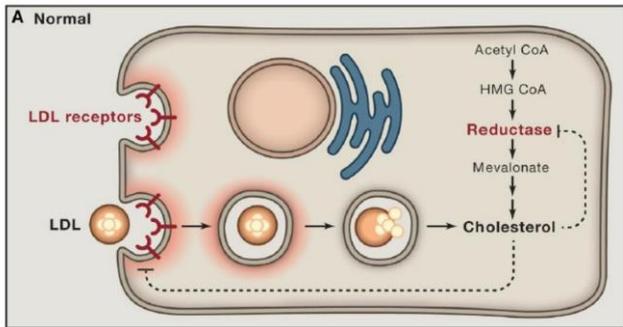
FEEDBACK REGULATION OF HMG-COA REDUCTASE

Experiment: Fibroblast cell culture of **healthy & FH patient**, medium with **removal or addition of LDL**

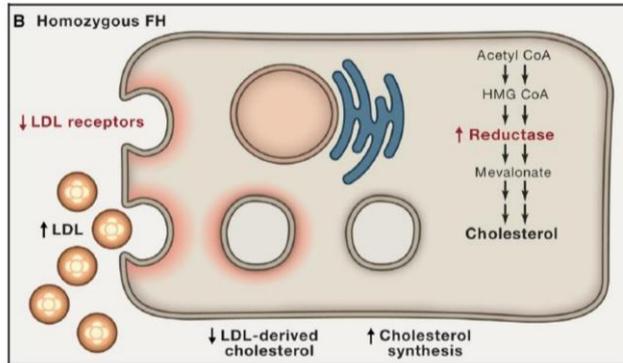
- HMG-CoA reductase active → Endogenous cholesterol synthesis → Inhibition by increasing cholesterol levels (feedback loop)
- Removal of LDL:
 - o WT: LDL endocytosis ↓ = Cholesterol ↓ = HMG-CoA activity increases
 - o FH: Impaired LDLR = No LDL uptake = HMG-CoA constitutive active
- Addition of LDL:
 - o WT: LDL endocytosis ↑ = cholesterol ↑ = HMG-CoA activity ↓
 - o FH: impaired LDLR = no LDL uptake = no cholesterol inhibition
- FH homozygote has constantly high activity of HMG-coa reductase (cause no functional LDLR at all)



- If cholesterol can be taken up via LDLR → Endogenous synthesis is suppressed



- If cell is not able to take up cholesterol → Endogenous synthesis is constitutively active



- Administration of statins to reduce blood LDL: **Inhibition of HMG-CoA + up-regulation of LDLR**

- o Blocking HMG-CoA reductase leads low cholesterol level in cells
- o If cell has low cholesterol levels inside it can induce the synthesis and also promote the uptake pathway by **inducing the transcription of LDLR**

- FH patients have no functional LDLR and can't take up cholesterol. Should they be treated with statins?

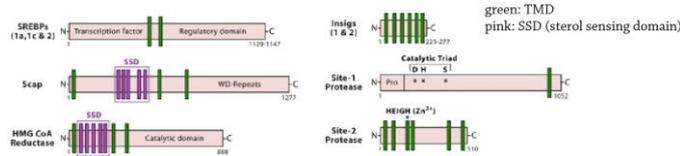
- No, since LDLR receptor are impaired!
- o Yes for heterozygous patients (still have 50% functional LDLR) → In this case statins can push the functional LDLR expression & increase the uptake

STEROL REGULATORY ELEMENT-BINDING PROTEINS

SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis

- Regulate lipid homeostasis in vertebrate cells
 - o **Membrane-bound transcription factors**
 - o Directly activate gene expression for **cholesterol & fatty acid synthesis**
 - o Synthesis and uptake of cholesterol, fatty acids, triglycerides, phospholipids and NADPH cofactor required to synthesize these molecules
- Mammalian genome encodes **three SREBP isoforms**
 - o SREBP-1a, SREBP-1c, SREBP-2
 - o SREBP-1a and -1c: Derived from a single gene → Use of alternative transcription start sites that produce alternate forms of exon 1, designated 1a and 1c
- At normal levels of expression:
 - o SREBP-1c favors the **fatty acid biosynthetic pathway**
 - o SREBP-2 favors **cholesterogenesis**
- SREBPs comprise a subfamily of **bHLH leucine zipper (bHLH-LZ) proteins**
- SREBPs bind both the **canonical inverted-repeat E-box site**, characteristic of **most bHLH proteins**, and the **SREBP-specific direct-repeat-binding element or SRE** (sterol regulatory element)

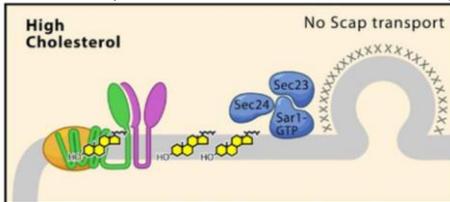
INSIG-MEDIATED REGULATION OF SCAP/SREBP TRANSPORT TO THE GOLGI



- Scap = SREBP cleavage activating protein
 - o Senses cholesterol (ER)
- **Insigs: ER anchor proteins of SREBP**
- Site-1 protease: Serine protease (Golgi)
- Site-2 protease: Metalloprotease (Golgi)

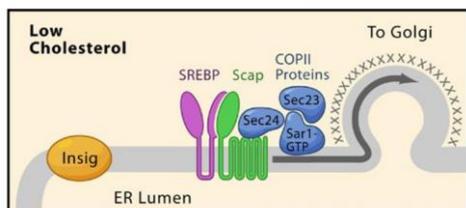
High cholesterol levels: More stable membrane SREBPs sequestered in the ER in a complex with SCAP (WD-C domain interaction) and **ER-anchored Insigs**

- SCAP binds to cholesterol → Conformational change
- **SCAP binds to Insig anchor proteins** → Inactive SREBP-SCAP(+cholesterol)-Insig complex in ER
- **COP2 protein cannot bind** → **no transport to golgi**



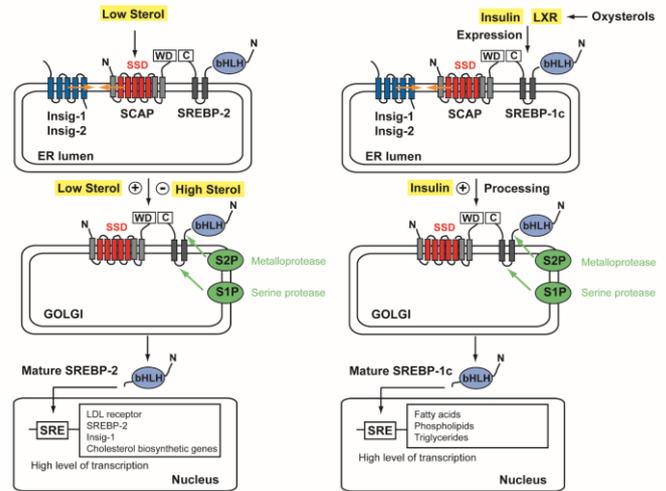
Low cholesterol levels: More floating within membranes SCAP SSD not bound to cholesterol → new conformation

- SCAP detaches from Insig anchor which get degraded
- SCAP exposes a cytosolic binding site for COPII proteins → COP2 mediated transport of SCAP-SREBP to Golgi



SREBP2 PATHWAY (LEFT)

1. Low sterol levels sensed by SSD at the ER
2. SCAP detaches from **Insig** and binds to **COP2**
3. COPII mediated transport of SCAP-SREBP to Golgi
4. S2P and S1P proteases cleave SREBP
 - o SCAP recycled to the ER
 - o Transcriptionally active N terminus of SREBP released
5. SREBP goes into the **nucleus** and binds **SRE**
6. Induction of transcription (Aim: **Resupply of cholesterol**)
 - o LDLR
 - o SREBP-2
 - o Insig (reestablishes inactive SREBP-SCAP-Insig complex)
 - o Cholesterol synthesis genes (like HMG-CoA)



SREBP-1 processing is not induced by cholesterol but by **insulin** (right picture)

- After a meal insulin induces **FA, TAGs** and **phospholipid synthesis** via **SREBP1** in liver
- Insulin → SCAP → detachment from Insig
- LXR induces transcription of SREBP1-C, not the activation/processing

OVERVIEW OF SCAP FUNCTIONS:

- o Senses cholesterol with SSD
- o Interacts with SREBP independently of cholesterol binding
- o Interacts with Insig if also bound to cholesterol
- o Mediates transport of SREBP by interaction with COPII proteins
- Overview with key points: Cholesterol is negatively regulating the transport ER → Golgi No cholesterol → SCAP and SREBP are in Golgi (Insig degraded) Cholesterol → SREBP with SCAP bound to Insig in ER

OVEREXPRESSION

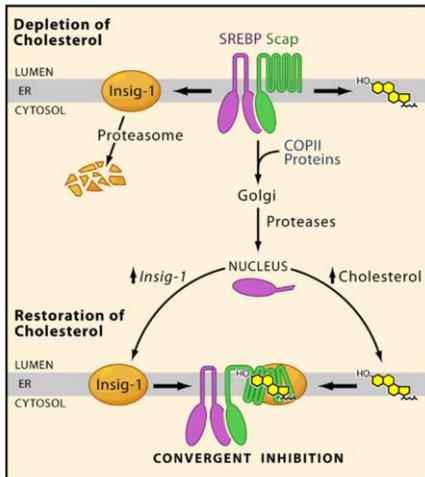
What happens if SCAP is overexpressed?

- Insig gets oversaturated and can't bind all SCAP → Increased transport of SREBP to the golgi → Proteolytic activation of TF → **increased cholesterol synthesis**

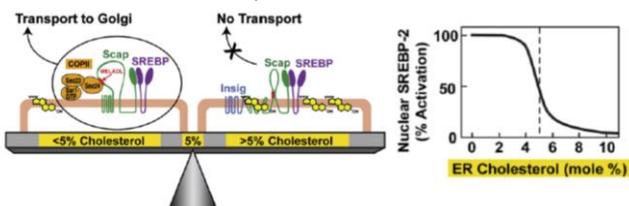
What happens if Insig-1 or Insig-2 are overexpressed?

- Insig overexpression → all Scap bound → decreased transport of SREBP into the Golgi → no pathway activation

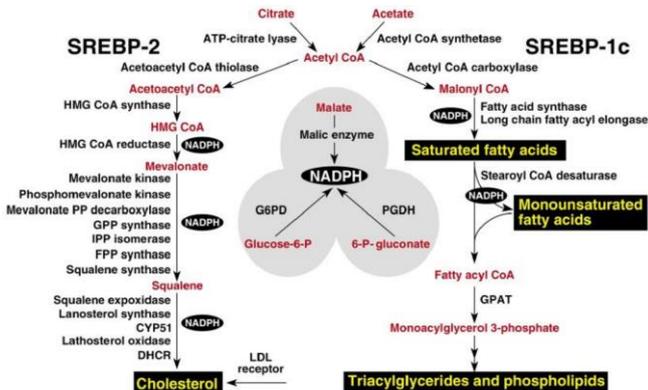
CONVERGENT FEEDBACK INHIBITION OF CHOLESTEROL SYNTHESIS AND UPTAKE AND SENSIBILITY OF PATHWAY:



- Feedback inhibition converge on function of **SCAP** and needs 2 compounds:
 - o Cholesterol & Insig
 - o Needed to keep SCAP in ER
 - o Only cholesterol is not enough → Pathway not shut down to early!
- Depletion of cholesterol → SCAP detaches from Insig → Golgi → Insig gets degraded
- Processed mature TF induces endogenous cholesterol synthesis and also the expression of Insig
- Cholesterol & Insig anchor → binds to SSD of SCAP → inhibit SCAP + SREBP transport
- ER has very low cholesterol levels. Slight fluctuations can be sensed by SSD of proteins that sit in ER membrane efficiently

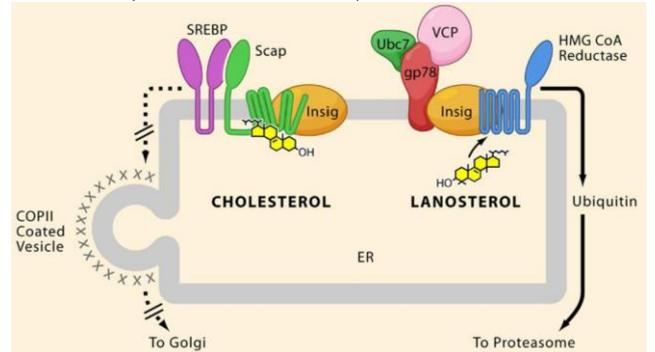


GENES REGULATED BY SREBPS



2 ACTION OF INSIGS IN CHOLESTEROL HOMEOSTASIS

- Prevents ER-Golgi transport → SREBP signalling in the presence of cholesterol (de novo synthesis is blocked)
- Regulates **ubiquitin-mediated proteasomal degradation of HMG CoA reductase** in the presence of lanosterol (de novo synthesis is turned off)

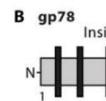


Low cholesterol → de novo synthesis induced → lanosterol/cholesterol homeostasis restored → HMG CoA degradation

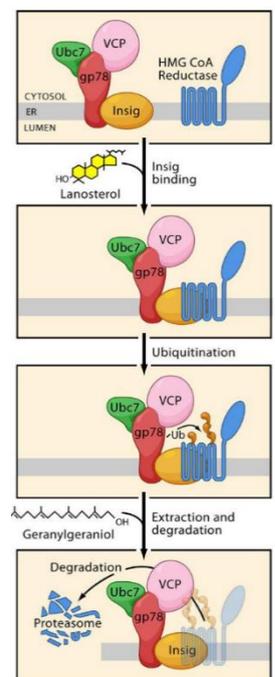
REGULATED DEGRADATION OF HMG-COA REDUCTASE

Membrane domain with SSD of HMG CoA is crucial for its regulation:

- Insig is in a complex composed of:
 - o gp78: Membrane-embedded E3 ubiquitin ligase which in turn is bound to:
 - Ubc7: ubiquitin-conjugating enzyme
 - VCP: ATPase that plays a role in extracting ubiquitinated proteins



1. Lanosterol (metabolite of biosynthetic pathway of cholesterol, much better inducer as cholesterol) binds to Insig and mediates binding to HMG CoA
2. gp78 ubiquitin ligase ubiquitinates the membrane domain of HMG CoA
3. Ubiquitinated HMG CoA reductase is extracted from the membrane by VCP and delivered to the proteasome for degradation (This post ubiquitination step is stimulated by geranylgeraniol)



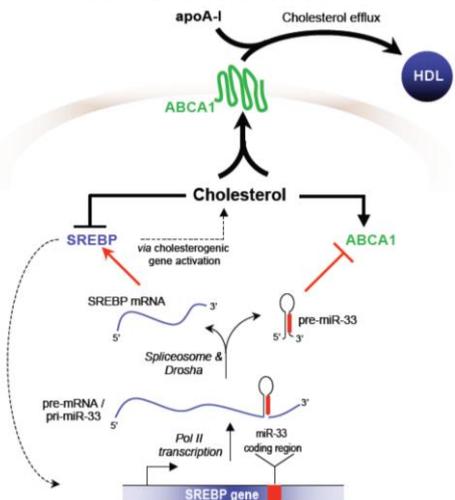
Side note: Over-expressing just the catalytic domain of HMG CoA will lead to an increased endogenous cholesterol synthesis which can't be controlled by cholesterol. Hence, cell has also to make sure that catalytic domain is not cleaved during the transport of HMG CoA to the proteasome, to avoid further cholesterol synthesis. Degradation of the whole enzyme is key!

ROLE OF miRNAS IN CONTROL OF CHOLESTEROL METABOLISM

- miRNAs are **21-24 nucleotide, non-coding RNAs**
- **Regulators of mRNA stability and translation**
 - o Reduce translation of transcripts and/or lead to their degradation by binding to partially complementary sites in 3' untranslated region (3'UTR) of mRNA transcripts
- Are often located within **introns** of protein-coding genes rather than in their own unique transcription units
- Intronic miRNAs are typically expressed and processed with the precursor mRNA in which they reside
- The human genome codes for **more than 900 miRNAs**
- miRNA families are predicted to bind and repress largely **overlapping sets of target transcripts**, with functionality influenced by tissue- and stimulus-specific expression of individual members

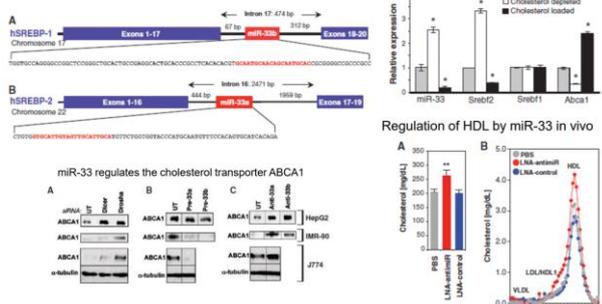
COORDINATED CHOLESTEROL HOMEOSTASIS REGULATION BY MIR33A AND SREBP2

- miRNA33a/b is located on a **SREBP intron**
- **miR33 is co-expressed** with SREBP2
- miR33 **regulates HDL** via cholesterol transporter **ABCA1 inhibition** (target the mRNA of ABCA1)
- Usually ABCA1 would allow cholesterol efflux for HDL formation → this is inhibited by miR33
- If cellular cholesterol levels are low: SREBP2 activation and thus miRNA33a expression induced → Inhibits the transcription of ABCA1 transporter = no cholesterol efflux (no retrograde transport)
- Overall result: **Cellular cholesterol homeostasis restored**
 - o SERBP2 → Induces de novo synthesis
 - o miRNA33a → Decreases efflux



- Possible treatment: Decrease miRNA33 in macrophages → More ABCA1 → More efflux of cholesterol after they take up LDL in artery walls (avoid foam cell formation)

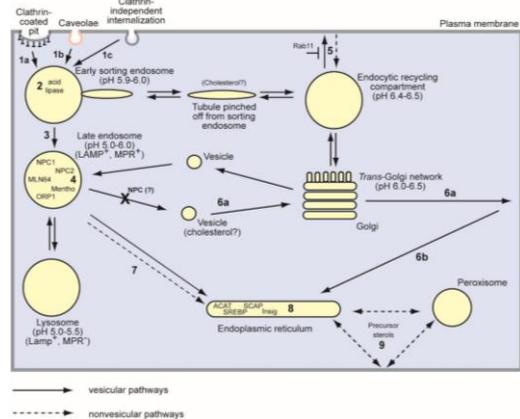
SREBPs are host genes to conserved intronic miRNAs, miR-33a/b, which are coexpressed with SREBPs



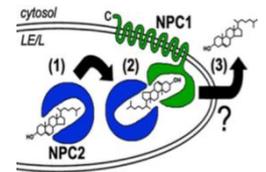
CELLULAR CHOLESTEROL TRAFFICKING

In the cell cholesterol is transported by **a vesicular and a non vesicular transport**

- Receptor mediated uptake of LDL → Endosome → Late endosome/lysosome → Cholesterol is distributed to plasma membrane and other organelles



- **NPC1 and NPC2** (lysosomal proteins) are two essential proteins for cholesterol to leave the lysosomes
- LDL particle is degraded in the lysosome → free cholesterol NPC2 & NPC1 work together and secrete cholesterol → cytoplasm
- NPC2: Binds cholesterol and hands it to its partner
- NPC1 (transmembrane protein): Distribution in cell



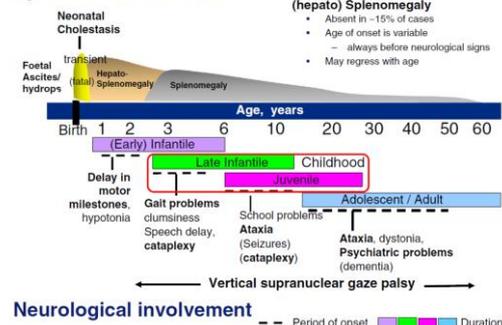
NPC -/-

- Cholesterol accumulates in late endosomes/lysosomes of cell bodies of Npc1^{-/-} mouse
 - o Have higher cholesterol tissue concentrations
 - o Have higher tissue cholesterol synthesis rate
- *NPC^{-/-}: Huge levels of cholesterol are measured in organs but they still have an increased de novo synthesis rate, why?* Cholesterol is stuck in lysosomes and can't inhibit the de novo synthesis by binding to the SSD of SCAP in the ER and in turn inducing the binding of SCAP-SERBP complex to the ER anchor protein Insig → Post-translational activation (proteolytic cleavage) of SREBP2 can't be deactivated since cholesterol is stuck in the lysosomes and can't function as regulator of this pathway because ER levels are not increasing. Additionally: Esterification of cholesterol takes place in the ER (LDL → lysosome → free cholesterol gets in ER → esterified); hence, NPC^{-/-} have decreased esterification of cholesterol and a lot of free cholesterol

NIEMANN-PICK DISEASE TYPE C

- Niemann Pick disease type C is a neurovisceral disorder
- Mutation in NPC proteins → Cholesterol accumulation in lysosomes, impaired cellular distribution

Systemic involvement



CHOLESTEROL IN CENTRAL NERVOUS SYSTEM

CHOLESTEROL IN THE CENTRAL NERVOUS SYSTEM

- **Plasma lipoproteins do not cross the blood-brain barrier**
→ Brain depends on intracerebral de novo synthesis of cholesterol within brain tissue
- Brain is the **most cholesterol-rich organ in the body** (25% of unesterified cholesterol in the whole body)
- Cholesterol concentration in the brain is 15-20 mg/g tissue, average in other tissues is ~2 mg/g tissue
- **2 major cholesterol pools in the CNS:**
 - o Myelin sheaths (i.e., oligodendroglia)
 - o Plasma membranes of astrocytes and neurons
- Cholesterol is found predominantly in white matter
- Up to 70% of the brain cholesterol is associated with myelin → Myelin consists of 70% lipids & 30% proteins
- Cholesterol is an essential signal for synaptogenesis and the formation, function and stability of synapses and are sensitive to disturbances in cholesterol metabolism
- Cholesterol has a **half-life of 4-6 months** in rat brain

CNS DISORDERS: THE CHOLESTEROL CONNECTION

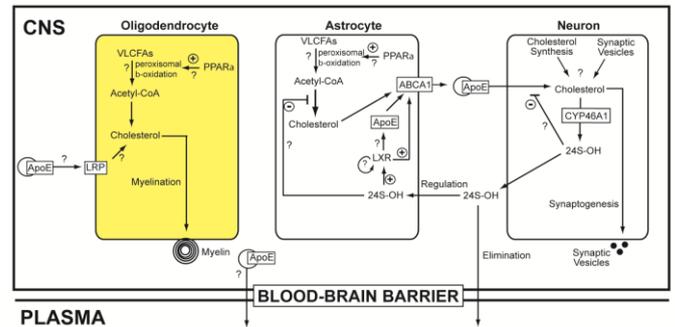
- Cholesterol is an essential signal for synaptogenesis and the formation, function and stability of synapses are sensitive to disturbances in cholesterol metabolism
- **Increased cholesterol turnover (Alzheimer's disease (AD) and Niemann-Pick type C disease)**
- **Elevated plasma cholesterol** → increased Alzheimer susceptibility
- **Hypercholesterolemia** → associated with increased brain amyloid β
- Apolipoprotein E (ApoE) (major cholesterol transporter in the brain) increased expression → Increased risk of Alzheimer's disease development at a younger age
- Determinants of cardiovascular health, especially midlife **dyslipidemia**, are associated with an increased **risk of dementia**
- Statins might have beneficial effects in several neurologic diseases (e.g., multiple sclerosis, AD, ischaemic stroke)

Brain is the only place where cholesterol levels are decreased in circulation compared to other organs; Why?

Regulation of cholesterol synthesis in the brain is different compared to other organs. One reason for this is that lipoproteins do not pass the adult bbb → organ depends on cholesterol de novo synthesis. E.g. using statins during pregnancy → Inhibit de novo synthesis of developing brain etc. → impaired myelin formation and neuron formation

MODEL OF CHOLESTEROL HOMEOSTASIS IN BRAIN

- Intracerebral transport of cholesterol via **ApoE** (no crossing of lipoproteins from blood because of bbb)
- LRP (LDLR homologs, from same receptor family) on oligodendrocytes, astrocytes and neurons mediates uptake of cholesterol from the cerebrospinal fluid
- Cholesterol efflux/turnover: Oxidation of cholesterol to 24S-OH (hydroxysterols) via CYP46A1 → can pass the bbb → Taken up by the liver → Further metabolised to bile acid



2 MAJOR CHOLESTEROL POOLS:

1. **Oligodendrocyte cholesterol in/for myelin sheets**
Olig. cholesterol pool important for neonatal development: VLCFAs (very long chain FAs) metabolised to Acetyl-CoA → Cholesterol synthesis; Strong expression of the TF PPAR α → Peroxisomal β -oxidation inducing enzymes etc. → Cholesterol is used for myelin formation
2. **Crosstalk between astrocytes & neurons:**
Both synthesize cholesterol de novo; however, only during early development neurons depend on own synthesis, afterwards they predominantly use the cholesterol pool from astrocytes (maintenance and formation of synapses etc.). Astrocytes synthesize cholesterol → Secretion with ABCA1 transporter → Transported via ApoE and taken up from neurons with LRP. 24-OH can also bind to the LXR in astrocytes → Induce ABCA1 expression → Increased secretion of cholesterol. This is the way how neurons signal to astrocytes an increased need of new cholesterol. 24-OH from neurons induces cholesterol efflux from astrocytes

CHOLESTEROL METABOLISM & EMBRYOGENESIS

ROLE OF CHOLESTEROL IN THE EMBRYO AND FETUS

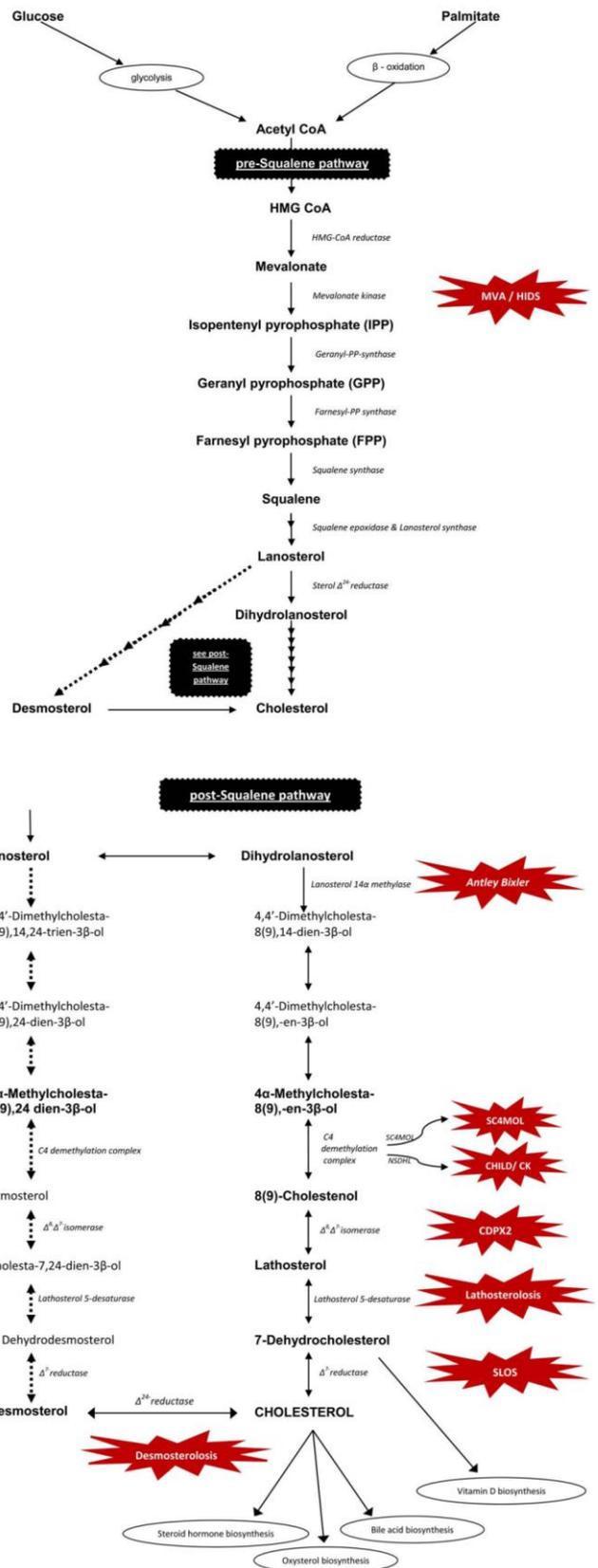
- **Membrane formation**
- **Maintains membrane integrity** and consequently the structure and function of membrane-bound proteins
- Cholesterol is **part of lipid rafts and caveolae**
 - o Membrane microdomains in which signalling processes start
 - o Critical for directing the location and thereby activity of proteins into lipid-rich or-poor membrane microdomains
- **Hedgehog processing** (limb development)
- **Precursor** for bile acids, steroid hormones, and oxysterols
 - o Bile acids are key integrators of metabolism in addition to being involved in lipid absorption
 - o Some steroid hormones are essential for normal development of the fetus (i.e., lack of estrogen affects morphology of the gonads)

THE ROLE OF MATERNAL CHOLESTEROL

- **Maternal transfer of cholesterol to the fetus occurs**
- Overall significance of transferred cholesterol debatable
 - o Early development depends on maternal cholesterol
 - o Later, endogenous cholesterol synthesis is essential
- Effect of less exogenous cholesterol
 - o Less cholesterol could be presented to the fetus due to lower maternal cholesterol concentrations
 - o Less uptake of lipoproteins
 - o Lower sterol synthesis rates in placenta or yolk sac
 - o Less transport of sterol to the basolateral side
 - o Less efflux or secretion to the fetal-facing side of trophoblasts or placental epithelial cells
- Women with lower plasma cholesterol concentrations had smaller newborns → Correlation between low plasma cholesterol and microcephaly
 - o Newborns with abnormal inutero growth rates, which lead to intrauterine growth-restricted infants and macrosomic infants (newborn with excessive birth weight), have an increased risk of developing age-related diseases

GENETIC DISORDERS OF CHOLESTEROL BIOSYNTHESIS

- Blocking synthesis: Not only impaired functions, also accumulation of intermediates; could be toxic
- **Mutations in early biosynthesis enzymes:**
 - o Rare because fatal
 - o No viability of the organism
 - o E.g. mevalonate kinase mutation (partial loss of function) → Mevalonate accumulation → acidification, inflammation
- **Mutations in late biosynthesis enzymes (post squalene):**
 - o Found in population and can lead to severe or minor disabilities
 - o E.g. Smith Lemli Opitz syndrome (SLOS); dehydrocholesterol-reductase (DHCR) involved in last step of synthesis is mutated



Explanation

Intermediates of cholesterol biosynthesis pathway get the ring structure after reaction catalysed by lanosterol synthase. Farnesyl pyrophosphate (FPP) is still a branch point and can be used as intermediate for other pathways. In contrast, lanosterol has the typical sterol ring structure → able to partially take over functions of cholesterol if further steps in synthesis impaired

INBORN ERRORS OF CHOLESTEROL SYNTHESIS

- The sooner a gene acts in cholesterol synthesis, the earlier the phenotype occurs
- Dependence of fetal development on endogenous cholesterol synthesis
 - o Inhibitors of cholesterol biosynthetic enzymes are potentially teratogenic (causing developmental malformation) → Avoid drugs inhibiting human cholesterol synthesis in early pregnancy
- Consequences of cholesterol deficiency and the potential consequence of accumulation of bioactive or toxic precursor sterols
- Disorders of sterol metabolism frequently → structural abnormalities of the brain, skeleton, and skin
- Neuro-developmental/ behavioral abnormalities are frequent and variable
- Defects in cholesterol synthesis are generally lethal in mice, while humans with impaired later steps of the pathway can survive with severe malformations

BIOLOGICAL BASIS OF CHOLESTEROL PHENOTYPES

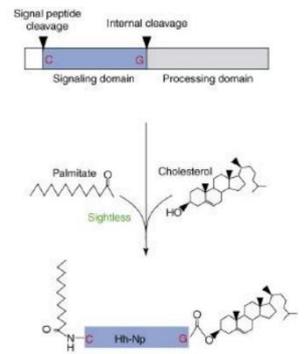
1. Deficiency of the final product cholesterol
2. Excess or deficiency of sterol intermediates, which are precursors in cholesterol synthesis
3. Modification in hedgehog signaling
 - Disorders:
 - o SLOS (Smith-Lemli-Opitz-syndrome)
 - o Lathosterolosis phenotype
 - o CHILD syndrome

PERTURBATION OF HEDGEHOG SIGNALING

- Hedgehog family is comprised of 3 different proteins:
 - o Sonic hedgehog (SHH)
 - o Indian hedgehog (IHH)
 - o Desert hedgehog
- Cholesterol is a covalent **ligand of the hedgehog family** of developmental patterning proteins
- Shh-deficient mice:
 - o Cyclopic, defects in the ventral neural tube, somite, and foregut patterning
 - o Later defects include distal limb malformation, absence of vertebrae and ribs, and failure of lung branching
- These phenotypes are consequences of **dysfunction in patterning during early embryogenesis**
- Defects in cholesterol synthesis/ lack of cholesterol might **disrupt autocatalytic hedgehog processing**
 - o Binding of cholesterol and palmitate to signaling domain after its cleavage

BIOGENESIS OF HEDGEHOG PROCESSING AND LIPID MODIFICATIONS

- Precursor proteins of hedgehog signalling are processed by autocatalytic cleavage to the mature form → for this processing cholesterol is needed
 - o Cholesterol is essential for the proper maturation of hedgehog proteins!
- No proper autocatalytic processing because of impaired cholesterol biosynthesis → no hedgehog gradient → impaired development and potential malformations of embryo.
- Disease phenotypes resulting from mutations of hedgehog signalling proteins are similar to the phenotypes of cholesterol synthesis mutations
- Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function



TOXICITIES ARISING FROM STEROL INTERMEDIATES

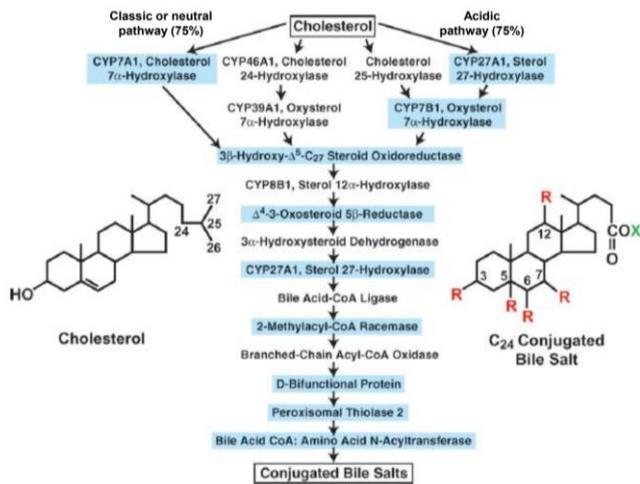
- Inhibition of enzymes early in the sterol biosynthetic pathway leads to **early embryonic lethality** (e.g., HMGCR, MVK, SREBP-2, squalene synthase)
- Most fetuses with defects in sterol biosynthesis late in the pathway **are viable until late in gestation or until just after birth**
- Early inhibition: Results in **lack of isoprenoids and cholesterol**
 - o Inhibition prior farnesol synthesis
 - o Isoprenoids are essential for basic cellular processes (e.g., cell proliferation)
 - o Isoprenoids modify proteins of the the ras, rab, and rho families; GTP-binding proteins; and G proteins
 - o Farnesyl pyrophosphate is a precursor for dolichol, which is essential for survival of blastocysts past implantation
- Late inhibition: results in a lack of cholesterol and a buildup of different intermediates

CHOLESTEROL BREAKDOWN: SYNTHESIS OF BILE ACIDS

FUNCTIONS OF BILE ACIDS

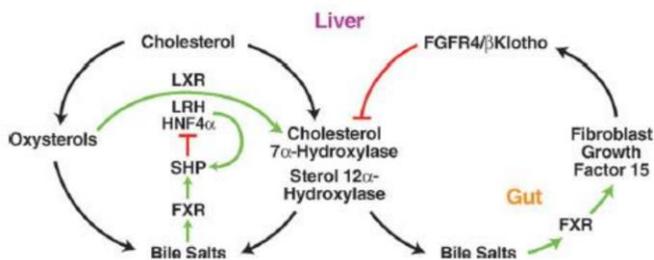
- Dietary lipid absorption
- Cholesterol homeostasis
- Inherited mutations that impair bile acid synthesis cause a spectrum of human disease; this ranges from liver failure in early childhood to progressive neuropathy in adults
- Bile acids (BAs) as signaling molecules:
 - o BAs activate mitogen-activated protein kinase (MAPK) pathways
 - o BAs are ligands for the G-protein-coupled receptor (GPCR) TGR5
 - o BAs activate nuclear hormone receptors such as FXR

ENZYMES OF BILE ACID SYNTHESIS

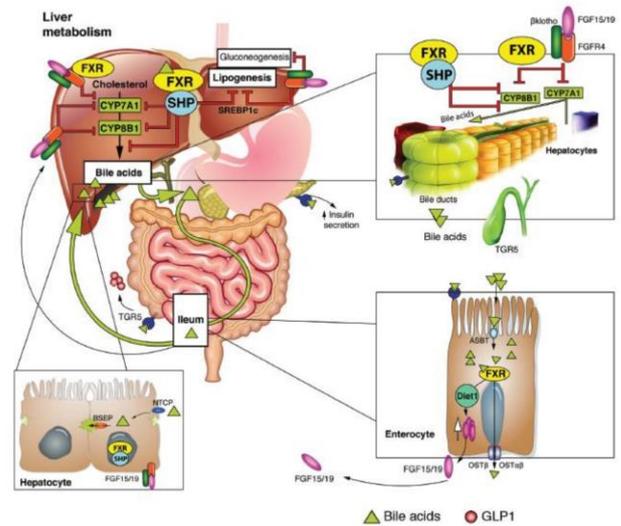


- Classic or neutral pathway
- Acidic pathway
- Initial pathways differ, but downstream they are shared
- Bile salt is conjugated at carbon 24 (to glycine or taurine)
- Initial regulation mostly via feedback loops
 - o Oxysterols act activatory
 - o FGFR4/βKlotho act inhibitory

REGULATION OF BILE ACID SYNTHESIS



BILE FLOW



METABOLIC SYNDROME

- At least 3 of:
 - o Obesity
 - o High blood pressure
 - o High blood sugar
 - o High serum TAGs and low HDL level
 → Deregulated lipid and glucose metabolism
- Disease affects several organs:
 - o Non alcoholic fatty liver
 - o Heart dysfunction/hypertrophy
 - o Atherosclerosis
 - o Defective pancreatic tissue
 - o Expanding adipose tissue ...

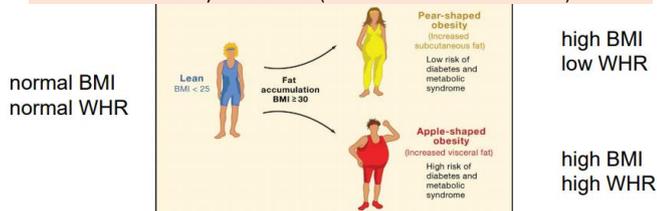
OBESITY

Obesity = Having a very **high amount of body fat** in relation to lean body mass, or **Body Mass Index (BMI) of 30 or higher**

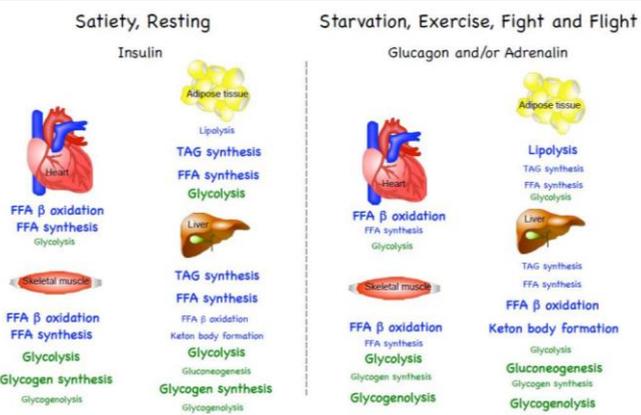
- **Body Mass Index (BMI):** Weight [kg] /square height [m²]
 - o BMI is used to assess the extent of general obesity
- **Waist-to-hip-ratio (WHR):** Waist to hip ratio
 - o Parameter for central obesity (apple-shaped or pearshaped obesity)
- Obesity is a complex trait, driven by the **interaction between genetic and environmental factors**
- Trend: Obesity is still on the rise (USA: up to 25-30% of the population)

RISKS

- **Fat distribution** influences risks associated with obesity
 - o High BMI & low WHR: Safe
 - o High BMI & high WHR: Higher risk of diabetes & metabolic syndromes (Increase in visceral fat)



LIPID AND GLUCOSE HOMEOSTASIS



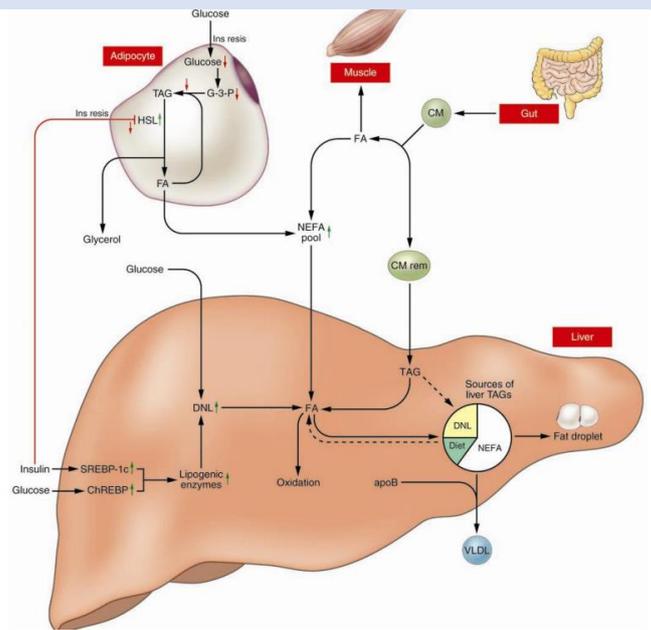
Satiety (insulin signalling):

- **Liver:**
 - o Active TAG & FA synthesis
 - o Increased glycolysis and glycogen synthesis (build up storage)
 - o Decreased FFA β-oxidation, Keton body formation, gluconeogenesis and glycogenolysis
- **Heart:** Gets its energy from β-oxidation (diseased heart: relies more on glu for energy)
- **Muscles:** Use β-oxidation to produce energy and also glycolysis

Fasting/starvation (glucagon/adrenalin signalling):

- Liver: opposite pathways to produce glucose for brain (either ketons or glucose)

MODEL OF LIPID FLUX THROUGH THE LIVER



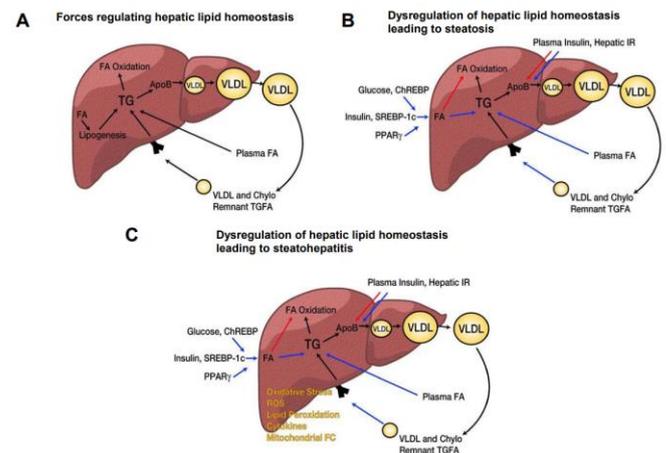
- **Uptake, de novo synthesis, oxidation, secretion and storage** contribute to lipid homeostasis in the liver
 - o Intake of FA from the gut → CM → deliver TAGs to peripheral tissues → CM rem → taken up by liver
 - o Glucose induces de novo lipogenesis in liver with collaboration of insulin using 2 Transcription factors: **SREBP1c** and **ChREBP**
- Under starvation **adipose tissue mobilises FA**
 - o **Lipolysis of TAGs** by HSL (hormone sensitive lipase) (HSL is inhibited by insulin) leads to increased non esterified FA (NEFA) bound to albumin in circulation
 - o NEFA can be taken up by the liver → cause of transient fatty liver because of starvation

NONALCOHOLIC FATTY LIVER DISEASE

DE NOVO LIPID (DNL) SYNTHESIS RATE

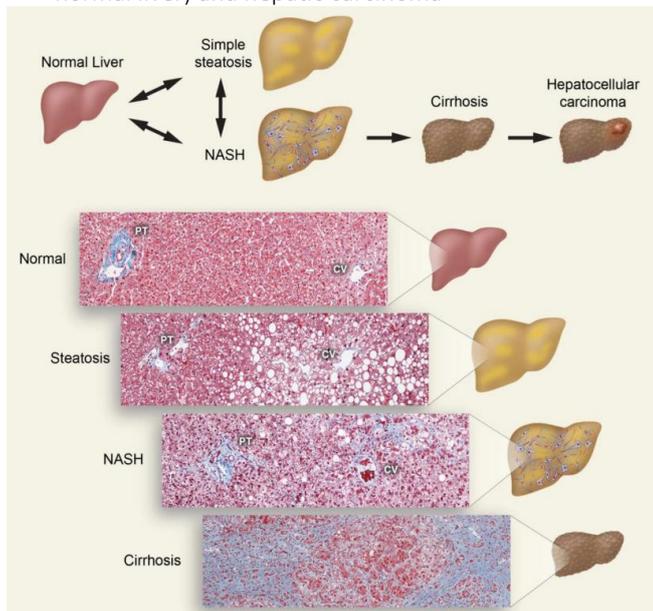
- DNL contributes to the **development of a fatty liver** (Impaired β-oxidation has less impact)
 - o Starvation: Low DNL rate (5%)
 - o After a meal: DNL rate increases to 25%
- Diabetic patients: **No difference** between fed and fasted state, DNL rate is constitutively increased
- **Increased glucose/fructose** intake also promote the development of a non-alcoholic fatty liver disease

HEPATIC LIPID METABOLISM IN STEATOSIS



DISEASE SPECTRUM

- Non-alcoholic fatty liver disease (NAFLD) can further exacerbate to NASH, nonalcoholic steatohepatitis, (fat accumulation + inflammation, ROS and start of fibrosis) and further to cirrhosis (strong fibrosis, can't go back to normal liver) and hepatic carcinoma



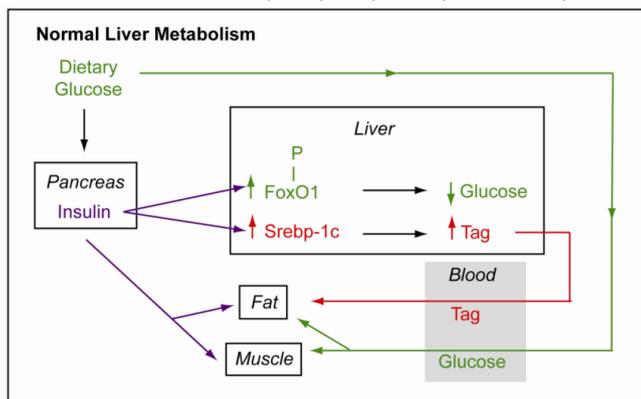
SELECTIVE INSULIN RESISTANCE

Selective insulin resistance in the liver produces a more severe metabolic defect than total insulin resistance

Normal response

- Insulin leads to:
 - o Processing of SREBP1c → leads to increased synthesis of FA and TAGs in liver
 - o TAGs are transported via VLDL to peripheral tissues (muscle (E), adipose tissue (storage))
 - o Decreased gluconeogenesis, since insulin signalling leads to the phosphorylation of the TF FoxO1
 - o Fat and muscle: Insulin leads to increased glucose uptake
- FoxO1 is regulated by phosphorylation
 - o Active: Not phosphorylated (in nucleus binding genes that are responsible for gluconeogenesis) (starvation)
 - o Inactive: phosphorylated (gets out of the nucleus) (meal)

→ Induces SERBP1c and phosphorylates (inactivates) FoxO1



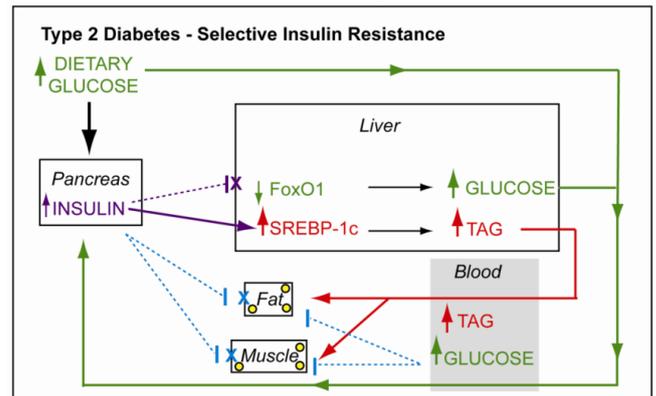
Insulin regulates glucose/TAG production in the liver:

- Insulin → pFOXO1↑ → glucose production↓
- Insulin → SREBP1c↑ → TAG production↑

Selective hepatic insulin resistance (type 2 diabetes)

(Increased glucose → pancreas compensation → hyperinsulinaemia → insulin resistance in peripheral tissues (muscle/fat), leading to impaired glucose uptake → hyperglycaemia)

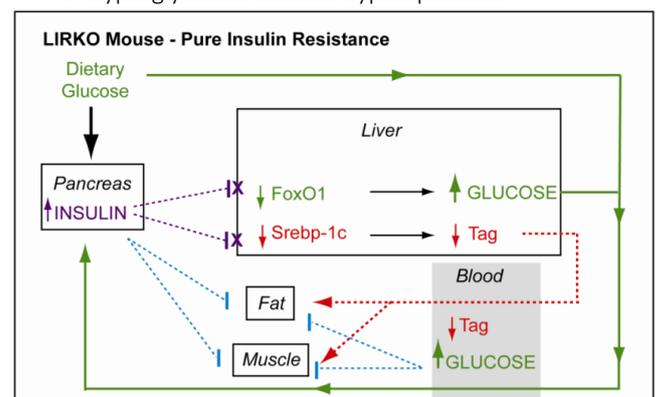
- Insulin cannot induce the uptake of glucose into tissues (resistance!) → blood glucose levels stay ↑↑↑
 - Selectivity in liver:
 - o FoxO1 regulating pathway is not responding
 - o FoxO1 is **constitutively activated** (gets not phosphorylated)
 - o Insulin can't to decrease gluconeogenesis, but it continues to stimulate synthesis of FA & TAGs via the SERBP1c pathway
- deadly combo of **hyperglycemia & hyperlipidimia**



- Insulin → no insulin signalling on pFOXO1 → glucose production↑
- Insulin → SREBP1c↑↑ → TAG production↑↑
- TAG are secreted from the liver and are stored in fat and muscle tissue

Total hepatic insulin resistance (no pathway responding)

- Mice knockout model with **no insulin receptor in liver**
 - Insulin cannot induce the uptake of glucose into tissues (resistance!) → blood glucose levels stay ↑↑↑
 - Insulin fails to decrease gluconeogenesis, and it also fails to stimulate synthesis of FA and TAGs
- Hyperglycemia without hyperlipidemia



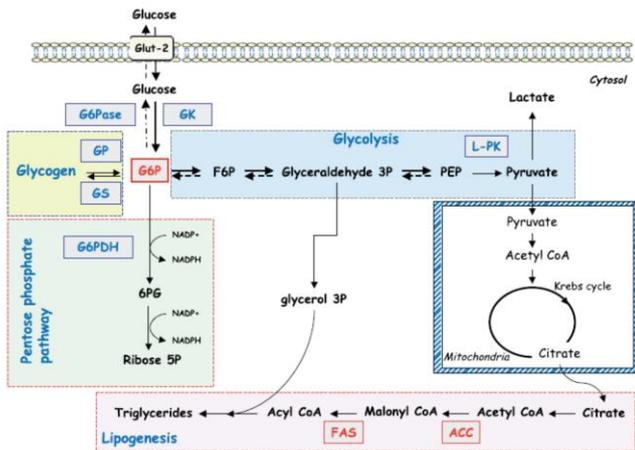
- Insulin → no pFOXO1 signalling → glucose production↑
- Insulin → no SREBP1c signalling → TAG production↓
- Only TAG get secreted from the liver

Total hepatic insulin resistance state has consequences **less severe** than those observed with the combined elevation because **there is no additional hypertriglyceridemia** (less atherosclerosis risk) → Selective hepatic insulin resistance is causing a much more severe disease phenotype (NAFLD, CVD, ...)

SYNTHESIS OF TRIGLYCERIDES IN THE LIVER

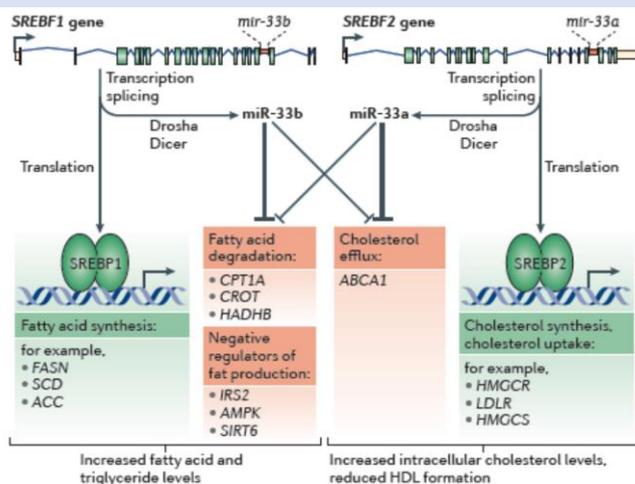
De novo lipogenesis induced with SREBP1c signalling:

- FA are generated (mainly palmitic acid) which are further modified (elongated, double bonds) and synthesised to TAGs
- Glycolysis is also important for the synthesis of TAGs → to generate glycerol 3P



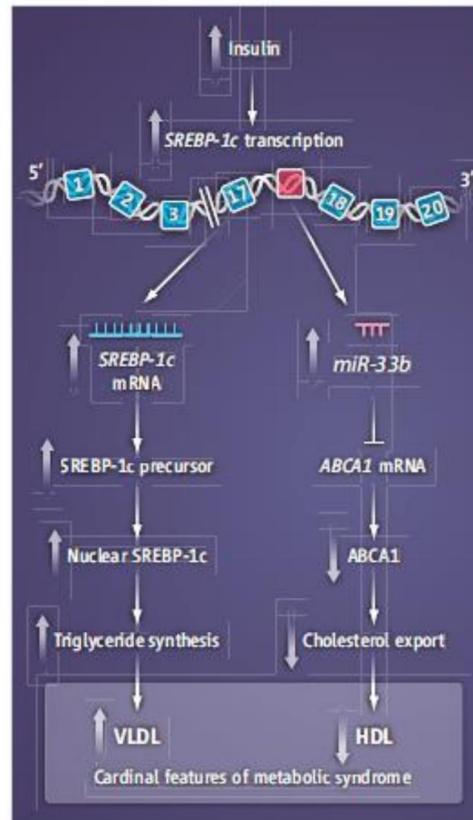
- TAGs can be incorporated in VLDL and transported to the periphery for energy (heart, muscle)
- If there are a lot of free FA → can be toxic in the cell (affect signalling pathways etc.) → storing FA in the inert form as TAGs (accumulation in lipid droplets)

MODEL OF THE SREBP AND MIR-33 CIRCUIT



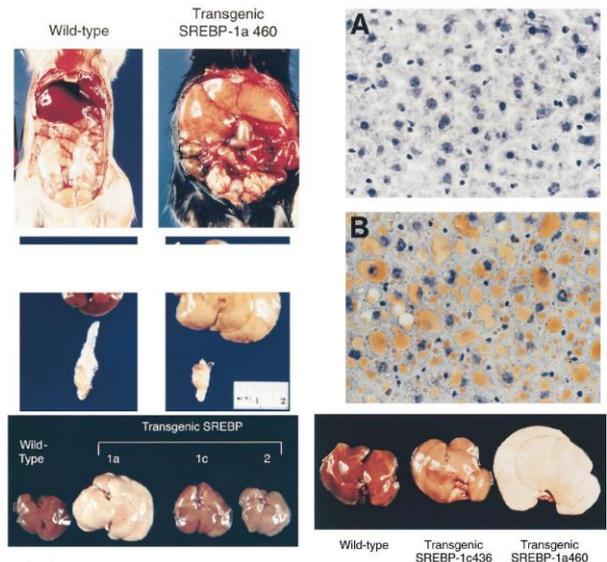
- **miRNA33b is co-expressed with SREBP1c** (located in intron of the gene)
- After meal → increased insulin in circulation → SREBP1c processing & expression SREBP1c induced = miRNA33b expression induced → inhibits the expression of enzymes that are involved in the degradation of FA
- **Result** → De novo lipogenesis (FA, TAGs and phospholipids) promoted
 - o SREBP1c → induces DNL
 - o miRNA33a → inhibits degradation of FA → increased FA and TAGs
- miR33b is also targeting ABCA1 mRNA → impaired cholesterol efflux (low HDL) → increased insulin → SREBP1c transcription → increased FA/TAGs synthesis & export (increased VLDL) and decreased cholesterol export (decreased HDL)
- No miR-33b in the SREBP1 gene of rat/mouse = obese

- insulin-resistant mice manifest all of cardinal features of metabolic syndrome except reduction in HDL



GENERATING A MOUSE MODEL WITH A CONSTITUTIVE ACTIVE SREBP1C PATHWAY

Active/mature transcription factor domain of SREBP1c (N-terminal part of SREBP1c) is introduced without its **regulatory domain** that interacts with SCAP and the transmembrane domain = Mature form (60kDa) that would be obtained after cleavage from the whole 120 kDa protein → Insulin independent **constitutive active SREBP1c**



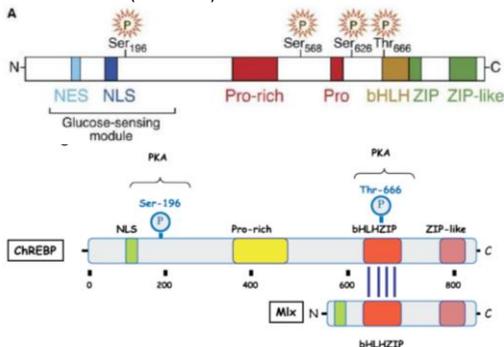
Livers from wild-type and transgenic mice expressing truncated dominant-positive SREBP-1a, -1c, and -2

- 1a shows the most severe phenotype, type 2 the least
- SREBP1c↑ → miR33↑ → ABCA1↓ → HDL↓
- SREBP1c↑ → triglyceride synthesis↑ → VLDL↑ → Metabolic syndrome: HDL↓ & VLDL↑

CARBOHYDRATE RESPONSE ELEMENT BINDING PROTEIN (CHREBP)

PROTEIN STRUCTURES

- **ChREBP** contains **several functional domains** including:
 - o N-terminal glucose sensing domain
 - (LID: inhibitory at low glucose)
 - o **Nuclear export signal (NES)**
 - o **Nuclear localization signal (NLS)**
 - o Proline-rich domains (protein-protein interactions)
 - o Basic helix-loop-helix/Zipper DNA binding domain (bHLH/ZIP)
 - o C-terminal glucose-responsive activation conserved element (GRACE)



Mlx = Max-like protein X

LOCALIZATION

- Low glucose → is cytoplasmic
- High glucose levels → translocates into the nucleus
- **ChREBP promotes TAG production**

CHREBP ACTIVITY REGULATION

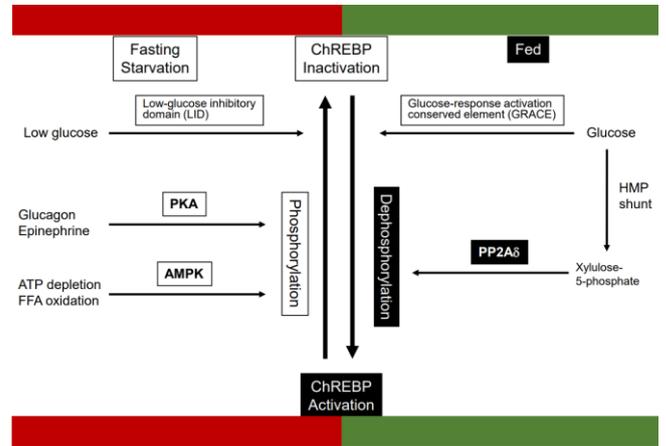
- ChREBP is regulated by **phosphorylation & dephosphorylation of serine and threonine residues** by
 - o **PKA** (cAMP-dependent protein kinase) → **Inactivate**
 - o **AMPK** (AMP-activated protein kinase) → **Inactivate**
 - o **PP2A delta** (phosphatase 2A delta) → **Activate**
- **Nutrients & hormone mediated changes in protein phosphorylation determine the activity of ChREBP**

During starvation (glucagon & epinephrine):

- o Hepatic glycolysis and DNL are suppressed
- o Gluconeogenesis, beta-oxidation and ketogenesis are upregulated
- Glucagon and epinephrine **increase the intracellular cAMP concentration and activate PKA**
- Intracellular AMP accumulation (low energy, low ATP) **activates AMPK**
- PKA and AMPK phosphorylate ChREBP → **NOT active** (cytoplasm)
 - o at (Ser196) inactivates nuclear import
 - o at Thr666 prevents DNA binding

Fed - high glucose:

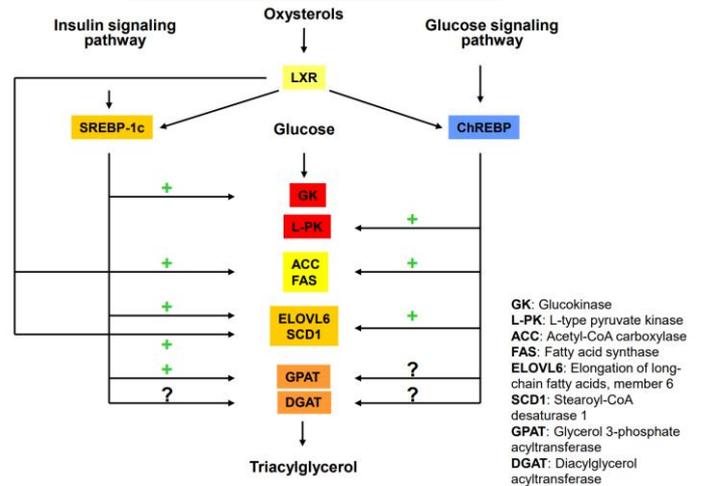
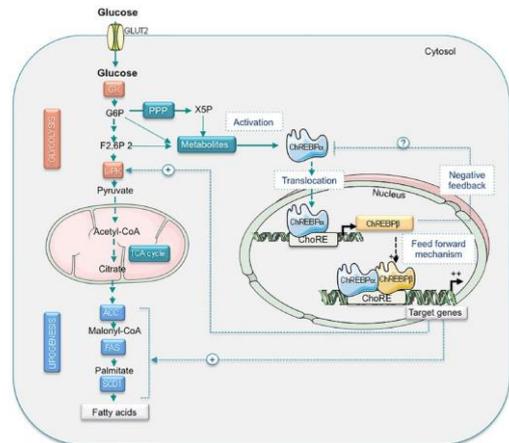
- Glucose **activates ChREBP transactivity**
- Glucose → xylulose-5-phosphate (in hexose monophosphate (HMP) shunt)
 - Xu-5-P activates **PP2A delta** → **dephosphorylates & thus activates ChREBP**
 - binds to carbohydrate response element (ChoRE) motifs in promoters of **TAG synthesis genes** (DNL genes) and some **glycolytic pathway genes** (e.g. pyruvate kinase)



REGULATION OF HEPATIC LIPOGENESIS BY INSULIN & GLUCOSE VIA CHREBP, SREBP-1C AND LXR

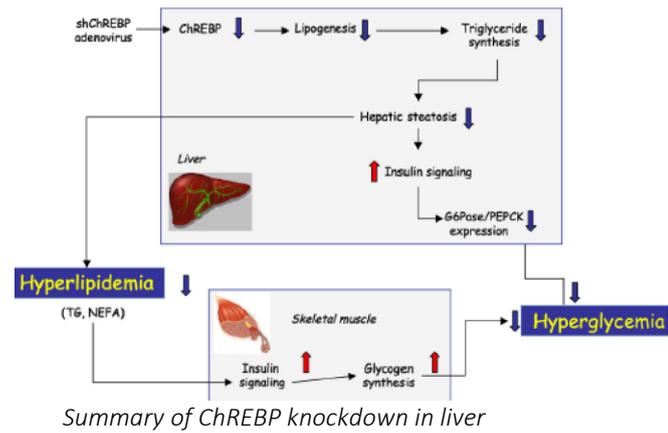
Overview of the 3 TF that regulate glycolysis and DNL

- **Insulin** → **SREBP1c** → DNL & first genes of glycolysis
- **Glucose** → **ChREBP** → DNL & later steps genes of glycolysis
- **LXR** (regulated by oxysterols) induces **the transcription of SREBP1c and ChREBP**; NOT the activation!
 - Insulin and glucose are needed for their activation



CHREBP KNOCKDOWN MICE

- Decreased levels of glycogenesis and lower TAG synthesis (decreased lipogenesis)
 - decreased hepatic steatosis
 - decreased hyperlipidemia & hyperglycemia
 - improve insulin signalling in peripheral tissue



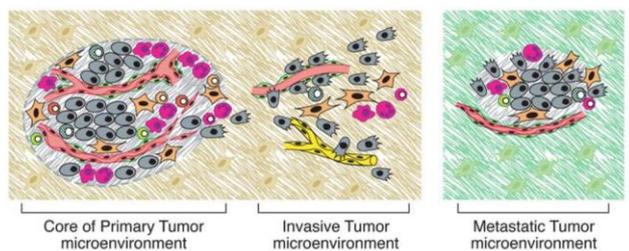
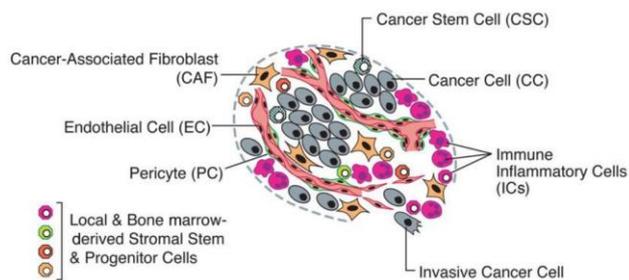
LIPID METABOLISM IN CANCER

A limited set of phenotypes exists in virtually all aggressive cancers:

- **Metabolic reprogramming** in tumors occurs as a consequence of **mutations in cancer genes** and **alterations in cellular signaling**
- **Aerobic glycolysis (Warburg effect)**: Cancer cells consume high amounts of glucose and produce lactic acid → provides cancer cells growth advantages in the tumor microenvironment
- **Increased glutamine metabolism**: Glutamine-derived α -ketoglutarate contributes to the production of citrate
- High rate of energy-consuming processes driving **increased protein synthesis** (e.g., mTOR pathway) and more **active DNA synthesis**
- **Increased de novo fatty acid synthesis**, which is functionally related to the glycolytic pathway (glycolysis provides energy and precursors for FA synthesis)

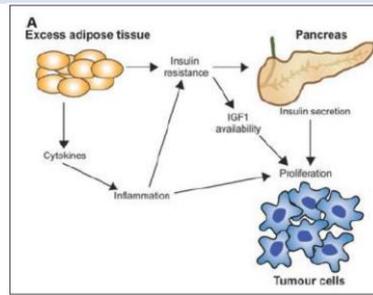
THE TUMOR MICROENVIRONMENT

- Tumors as complex tissues!



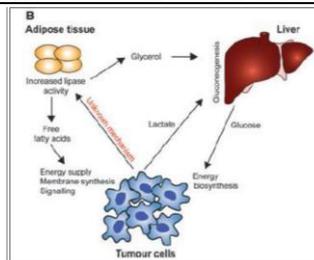
- Different metabolic pathways are active depending on the tumor microenvironment (cell types etc.)
- E.g. myeloma cells are glycolytic but if migrating/metastasis → highly mitochondrial metabolism

WHOLE-BODY LIPID METABOLISM AND CANCER



- **Obesity & insulin resistance** contribute to **cancer development** by increasing the secretion of insulin by pancreatic β -cells and by enhancing IGF1
- Secretion of inflammatory cytokines can also promote insulin resistance as well as the transformation and proliferation of tumor cells

Crosstalk between tumors and adipose tissue

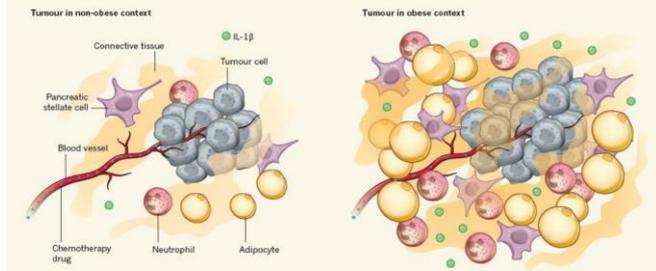


- Tumor cells can promote the breakdown of lipids in the adipose tissue → promote lipolysis (TAG breakdown), increased free FA in circulation → cause cachexia in patients with cancer
- Tumor cells can **use circulating free fatty-acids** as an **energy supply** for membrane biosynthesis or for signalling processes
- **Glycerol** produced by the breakdown of TAGs can be used for **gluconeogenesis** in the liver → glucose is thus for the cancer available
- Many cancer cells have a **lipogenic phenotype** because of their **accumulation of lipids**
 - o One example is the **clear cell renal cell carcinoma (ccRCC)**
- Additionally, **fat cells remodel the microenvironment around cancers** (e.g. the pancreatic tumor (PDAC)):

PANCREATIC DUCTAL ADENOCARCINOMA (PDAC)

- Obesity is a major risk factor for PDAC
- Is very common: Fourth most-common cause of cancer-associated death, is associated with poor response to chemotherapy and poor prognosis
- **Adipocytes, immune cells and pancreatic stellate cells** signal through IL-1 β and the AT1 angiotensin receptor to drive **migration of neutrophils** to the tumor microenvironment → increases inflammatory and fibrotic response in the tumor microenvironment
- Denser cellular microenvironment puts extra mechanical tension on the tissue → may **restrict bloodvessel perfusion**
- Therapy: Depletion of neutrophils or blocking activity of IL-1 β reduce cancer progression

Fat cell remodel microenvironment in pancreatic tumors

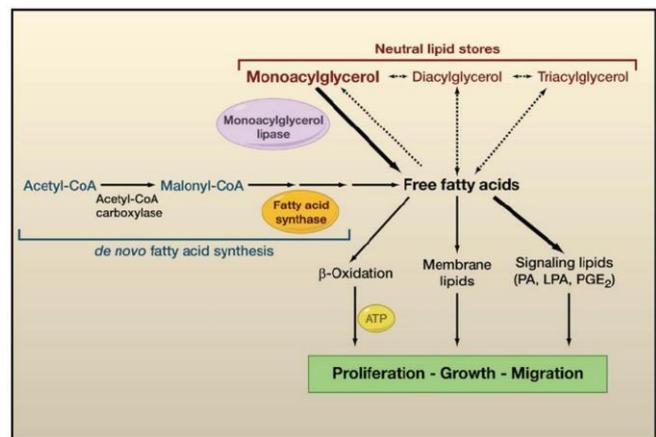
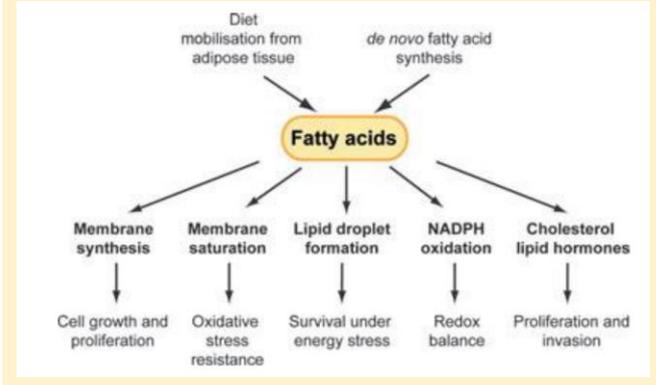


Tumor adipocyte number is significantly higher in obesity

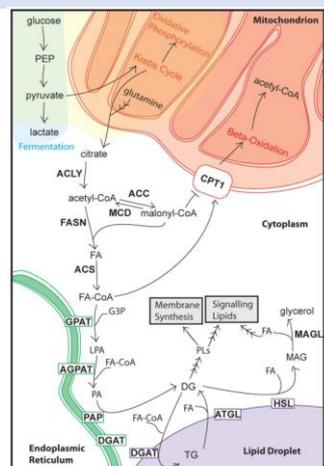
Adipocyte → migration of neutrophils → inflammatory cytokines → activate cells in microenvironment → increased fibrosis (more pressure on blood vessel, hypoxia), affects immune cell functions etc.

LIPIDS/FA IN CANCER DEVELOPEMENT

Lipids can promote different aspects of cancer development



CELLULAR FATTY ACID METABOLISM



FA, fatty acid
 LPA, lysophosphatidic acid
 PA, phosphatidic acid
 MAG, monoacylglycerol
 DG, diacylglycerol
 TG, triacylglycerol

ACLY: ATP citrate lyase
 ACC: acetyl-CoA carboxylase
 FASN: fatty acid synthase
 ACS: fatty acid-CoA ligase
 MCD: malonyl-CoA decarboxylase
 CIC, citrate carrier

CPT1: carnitine palmitoyl transferase

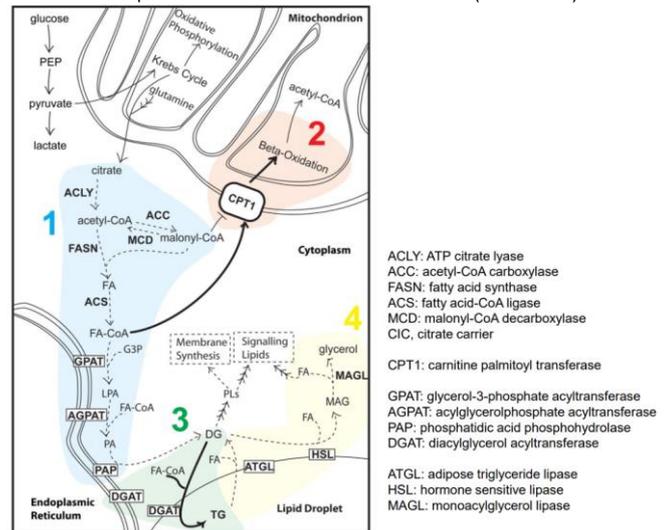
GPAT: glycerol-3-phosphate acyltransferase
 AGPAT: acylglycerolphosphate acyltransferase
 PAP: phosphatidic acid phosphohydrolase
 DGAT: diacylglycerol acyltransferase

ATGL: adipose triglyceride lipase
 HSL: hormone sensitive lipase
 MAGL: monoacylglycerol lipase

- Cancer cells often have **perturbed metabolism** allowing them to **accumulate intermediates** as sources of building blocks
 - o Most understood perturbation: **Warburg effect** (use of fermentation even in presence of oxygen; characterized by increase in glucose uptake & consumption, a decrease in oxidative phosphorylation and the production of lactate)
 - o Another commonly observed alteration: **increased glutamine metabolism**
 - o **Lipid metabolism**: Is also altered → Use of FAs for energy storage, membrane proliferation and the generation of signalling molecules

THERAPEUTIC STRATEGY:

- Since **FAs are essential for cancer proliferation** → limit availability of FA in the cell!
 - o Blocking FA synthesis
 - o Blocking expression of FA synthesis genes
 - o Increase FA degradation → promote β-oxidation
 - o Decreasing FA release from storage
 - o Diverting FAs to storage
 - Transformation of FA-CoA + diglyceride into TAG
 - Transport FA-CoA into mitochondria (via CPT1)



Limiting fatty acids in the cell might limit cancer cell proliferation

Blocking FA synthesis

- **Glucose metabolism** feeds into FA metabolism at the point of **citrate**, an intermediate in the Krebs cycle
 - o Several steps are required to convert citrate to bioactive FA
 - o Steps involve the following enzymes: ACLY, ACC1/2, FASN, ACS → inhibiting these enzymes would limit cancer cell growth
- **Localization of citrate** determines its metabolic fate
 - o Mitochondrial citrate feeds into the Krebs cycle
 - o Cytoplasmic citrate feeds into FA synthesis
- Citrate is transported by the **transporter CIC** which is over-expressed in various cancer cell lines → inhibition of CIC = reduced FA synthesis
- **ACLY**: Bridges glucose metabolism and FA metabolism by converting 6-carbon citrate to oxaloacetate and acetyl-CoA, the precursor for FA synthesis and cholesterol synthesis
 - o Acetyl-CoA is an important intermediate
 - o Inhibiting its production may have consequences for other metabolic pathways as well

- **ACC**: ACC1 is enriched in lipogenic tissues and ACC2 occurs in oxidative tissues
 - o They have different metabolic roles
 - o Malonyl-CoA made by ACC1 is thought to serve as a substrate for FA synthesis
 - o Malonyl-CoA made by ACC2 serves to inhibit CPT1, thus preventing FA degradation
- **FASN**: Successive condensation reactions to form a FA from malonyl-CoA and acetyl-CoA substrates, producing mainly 16-carbon palmitate
- **ACS**: To enter bioactive pools, they must be activated by ACS enzymes, which generate FA-CoA

Block the expression of FA synthesis genes (siRNA)

- In addition to directly targeting enzymes of FA synthesis, their activities could be reduced by reducing transcription levels
- **Inhibiting SREBP-1** expression in cancer cells could decrease FA synthesis gene expression
- **Inhibit liver X-activated receptor (LXR)** → LXR activates fatty acid synthesis by inducing SREBP-1c mRNA expression

Inhibit or activate FA degradation depending on cancer type

- Cancers that are highly lipogenic → activate FA degradation via β -oxidation (Problem: Energy produced)
- FA-CoA are transported from cytosol into mitochondrion after they are converted to FA carnitines by **CPT1** (rate-limiting step of fatty acid transport into mitochondria, inhibited by malonyl-CoA)
- Overexpression of CPT1 → Increased FA degradation

Divert FA to storage

- Diacylglycerols (DAGs) can be either transformed to phospholipids for signalling functions and membrane synthesis or they can be transformed to TAGs by DGAT
- **DGAT** catalyses the only dedicated step in TAGs formation and thus provides a key target for decreasing available lipids by increasing lipid storage
- Increased storage of FAs in neutral lipids, such as TGs or sterol esters leads to a reduction in FAs available for use as membrane building blocks or signalling lipids and inhibit cellular proliferation

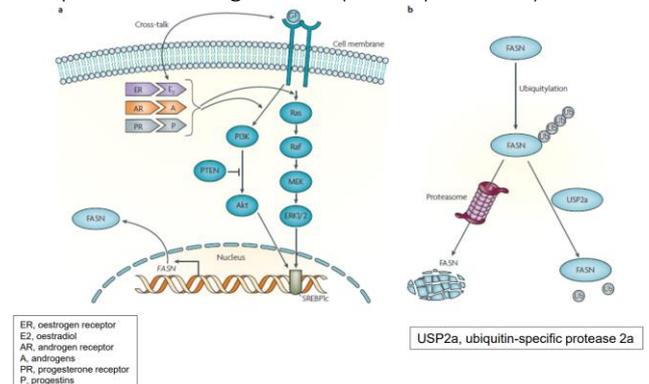
Decrease FA release from storage → prevent lypolysis

- Once stored, FAs can be released for use by specific lipases → By preventing lipolysis, the active FA pool available for cancer cell proliferation can decrease
- Mobilisation of TAGs from the storage can be achieved by different lipases (ATGL, HSL, MAGL) leading to free FA in the cytosol
- These lipases are often over-expressed in tumors and cause cachexia

Aim: **Activate DGAT** and simultaneously **inhibit the release of FA from storage**

PATHWAYS THAT REGULATE THE EXPRESSION OF TUMOR-ASSOCIATED FASN → INCREASED DNL

- **Mutated GF receptors** → constitutive active Ras/Raf or PI3K/Akt pathway → SREBP1c → FASN
- **Mutated PTEN** (tumor suppressor) → active Akt signalling → SREBP1c → FASN
- GF → Ras → Raf → MEK → Erk1/2 → SREBP1c → FASN
- **Over-expression of USP2a** → protects FASN from proteasomal degradation (no ubiquitination)



DE NOVO FATTY ACID SYNTHESIS

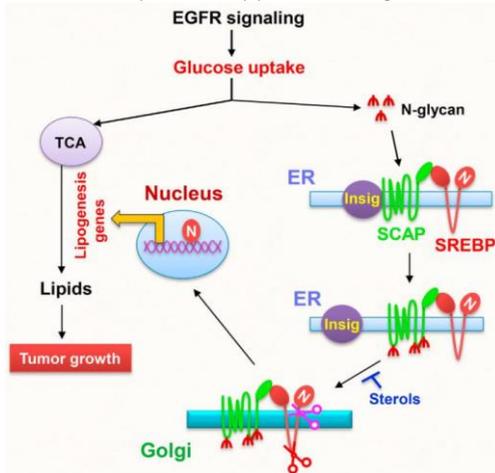
- **Two sources**:
 - o Exogenously derived FAs (*dietary*)
 - o **Endogenously synthesized FAs** (*De novo synthesis*)
- Biosynthesis is catalysed by the multifunctional, homodimeric **fatty acid synthase (FASN)**
 - o Predominant product of FASN is **palmitate** (C16:0)
 - o In well-nourished individuals the role of FASN is of minor importance → Sufficient levels of dietary fat
- Normal cells/tissues (even those with high cellular turnover) preferentially use **circulating lipids for the synthesis of new structural lipids**
- In normal conditions: **Excess carbohydrate** → FAs → **esterified to storage TAGs** (via FASN)
- De novo FA synthesis is very active during **embryogenesis** and in **fetal lungs**

DE NOVO FATTY ACID SYNTHESIS IN CANCER

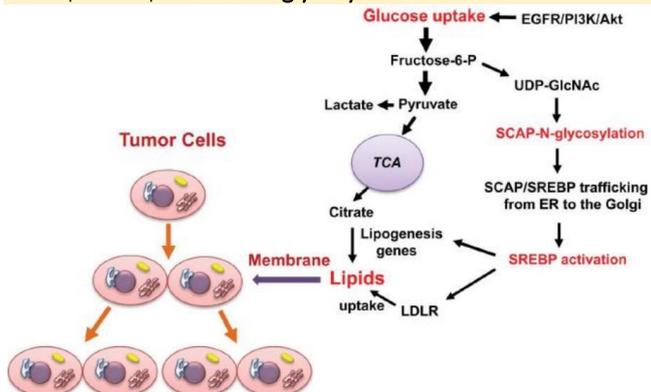
- **Increased de novo biosynthesis of FAs in a wide variety of tumors** (independent of circulating lipid/FA levels)
- Neoplastic lipogenesis: **Significantly increased activity of several lipogenic enzymes in tumor cells** [FASN, ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACACA)]
 - o Modulation of multiple lipogenic enzymes at various levels (increased transcription, enhanced protein stabilization,...)
 - o **Upregulation of FASN** represents a nearly universal phenotypic alteration in most human **malignancies**
- FAs synthesized in cancer cells are **mainly used for membrane lipid formation** by proliferating cells (esterified to phospholipids and incorporated)
- Many of the genes that encode the enzymes of the FA biosynthetic pathway, including ACLY, ACACA, FASN, reside on **human chromosome 17q**. This is a common site for gene rearrangement and is the location of many oncogene amplifications. However, only one study evaluating the correlation of the expression levels of lipogenic enzymes with gene copy number alterations has detected a significant increase in FASN copy number in prostate cancer

SCAP LINKS GLUCOSE TO LIPID METABOLISM IN CANCER CELLS

- **Increased glucose uptake** (glycolytic flux) in cancers results in the **accumulation of intermediates** like citrate (used for lipogenesis) or glucose-mediated **N-glycosylated SCAP**
- SCAP N-glycosylation promotes **SCAP-SREBP trafficking from ER to the Golgi**
 - Increased processing of active SREBP
 - Expression of lipogenesis genes and LDLR
 - Increased DNL & lipid uptake; increasing levels of cellular lipids to support tumor growth

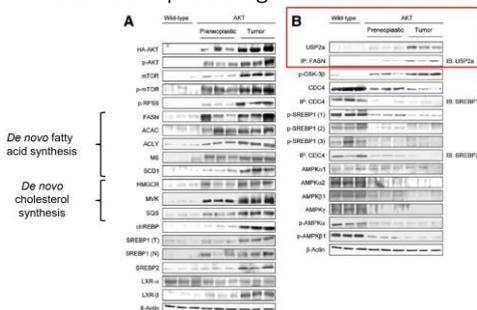


Glucose promotes tumor growth by activating lipid synthesis and uptake upon SCAP N-glycosylation



Increased lipogenesis (FA & cholesterol synthesis) promotes development of tumors

- o Cancers with poor outcomes show over-expression of de novo FA & cholesterol genes (FASN, ACC, ACLY, SREBP1/2) as well as **USP2a**
- o Also constitutive active Ras/Raf, PI3K/Akt, MAPK pathways promote development & DNL → link between lipid deregulation and cancer development



AKT-overexpressing mice → Hepatocellular carcinoma

- Upregulation of de novo cholesterol/FA synthesis
- Liver enlargement, Lipid accumulation, Activation of FASN and USP2a

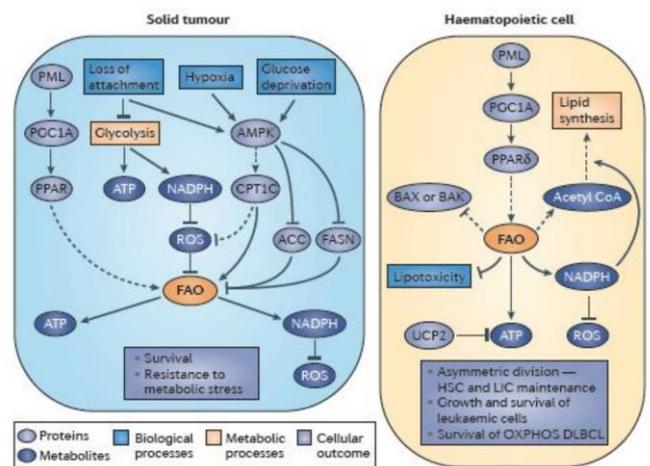
ADIPOSE TAG LIPASE CONTRIBUTES TO CANCER ASSOCIATED CACHEXIA

- Cachexia is a multifactorial wasting syndrome
 - o most common in patients with cancer that is characterized by the uncontrolled loss of adipose and muscle mass
- Functional lipolysis is essential in pathogenesis of CAC
- Pharmacological inhibition of metabolic lipases may help prevent cachexia
- **Inhibition of lipolysis** through genetic ablation of adipose triglyceride lipase (ATGL) or hormone-sensitive lipase (HSL) ameliorates certain features of cancer-associated cachexia (CAC)

TRANSCRIPTIONAL SIGNATURE AND COMMON GENE NETWORKS LINK CANCER WITH LIPID METABOLISM

- Transcriptional profiling of 2 isogenic transformation models identified a gene signature **linking cancer with inflammatory and metabolic diseases**
 - o Transformed cells up-regulate genes that are also over-expressed in metabolic disorders (obesity, diabetes, ...)
- Many drugs used for treatment of diabetes and CVD **inhibit transformation and tumor growth**
- **Lipid metabolism genes** are important for transformation and are **up regulated in cancer tissues**
- As in atherosclerosis, oxidized LDL and its receptor OLR1 activate the inflammatory pathway through NF-κB, leading to transformation
- OLR1 is important for maintaining the transformed state in diverse cancer cell lines and for tumor growth, suggesting a molecular connection between cancer and atherosclerosis

CONTRIBUTION OF FATTY ACID OXIDATION TO CANCER CELL FUNCTION



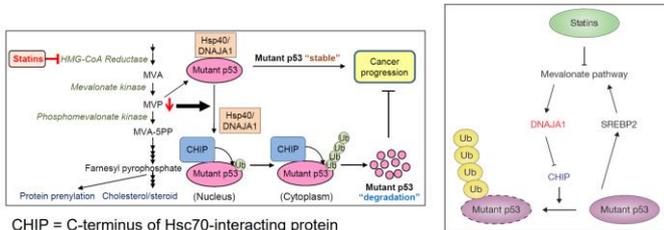
CHOLESTEROL/ISOPRENOID BIOSYNTHESIS AND CANCER

- Cancer cells have a **deficient feedback control of HMGCR** or **increased HMGCR expression** → dysregulation of the mevalonate pathway might drive malignant transformation
- **Mevalonate** is a precursor of many cell cycle regulators (e.g. dolichol, geranylgeranyldiphosphate (GGPP) and farnesyl diphosphate (FPP))
 - o **Dolichol** stimulates DNA synthesis
 - o **GGPP and FPP** regulate **RAS and RHO**, which regulate cell **proliferation, differentiation, apoptosis**
- GGPP and FPP cause isoprenylation of the intracellular G proteins Ras and Rho, which in turn regulate the signal transduction of several membrane receptors crucial for the transcription of genes involved in cell proliferation, differentiation, and apoptosis
- Statins have anticarcinogenic activity but mechanisms do not necessarily involve cholesterol lowering

MEVALONATE METABOLISM AND CANCER MALIGNANCY: REGULATION BY P53

- Deregulated mevalonate pathway is linked to cancer
- p53 can be activated by a number of cellular stressors, including DNA damage, radiation, chemical agents, hypoxia and oncogene deregulation
- In a stress response, p53 functions as a TF to suppress cell cycle progression, promote senescence, or to induce apoptosis
- The majority of cancer-associated mutations in p53 are missense mutations that result in the translation of a different full-length protein
- Mutant p53 can abrogate the protective effect of the remaining WT allele → interfere with still functional WT allele
- Mutation of p53 is not equivalent to simply losing WT p53 function, which normally prevents tumorigenesis → Instead, certain p53 mutants may acquire a **tumor-promoting function** → mutant p53 transcriptionally enhances the expression of many mevalonate pathway genes via SERBP2

MUTANT P53 AND THE MEVALONATE PATHWAY FEED-FORWARD LOOP IN ONCOGENESIS



CHIP = C-terminus of Hsc70-interacting protein
 MVP = mevalonate-5-phosphate
 MVA = mevalonate

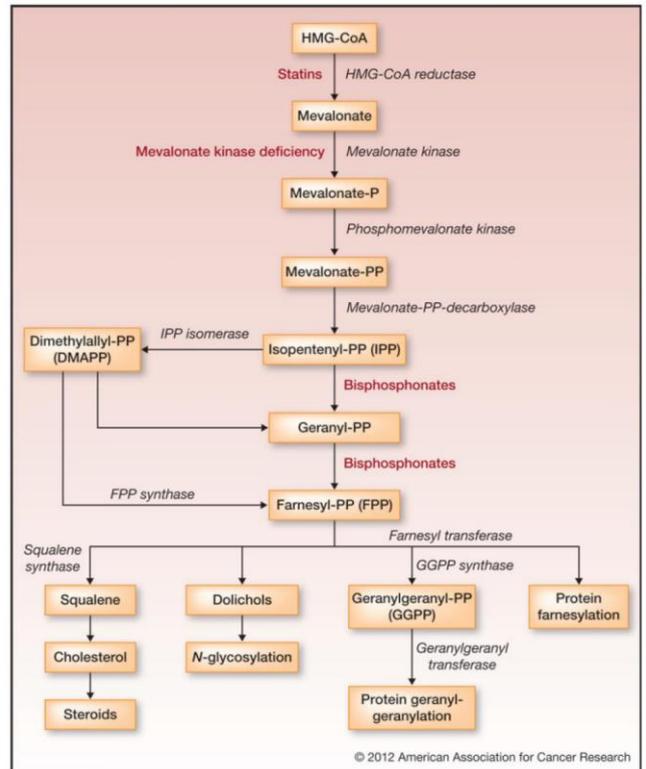
Feed forward mechanism:

- **Mutated p53 induces SREBP2** which in turn **drives the mevalonate pathway**
- Mevalonate pathway results in stabilisation of the mutated p53 by the heat-shock protein DNAJA1, which interacts with p53 and prevents the ubiquitin ligase CHIP to ubiquitinate and degrade the mutant p53

- **Stabilised mutated p53** can further induce the **mevalonate pathway** → promotes cancer development

Treatments:

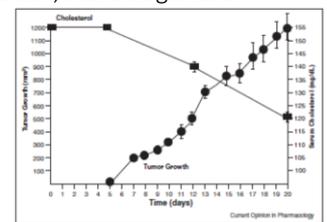
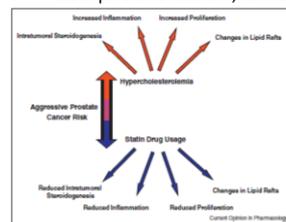
- **Statins** inhibit **HMG-CoA reductase** → decreased levels of mevalonate → inhibit cancer promoting function
- Other possibilities: Inhibit later steps of the pathway (**shown in picture**) e.g. **Biphosphonates** → accumulate isopentenyl-PP → phosphoantigen on cell surface can be recognised by NK cells → kill cancer cells



2 EXAPLES HOW CHOLESTEROL CAN PROMOTE CANCER

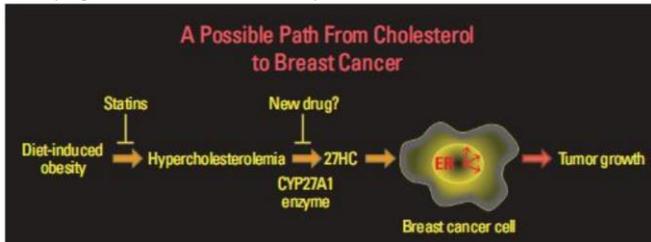
CHOLESTEROL AND PROSTATE CANCER

- Prostate cancer has a **genetic component** (but is not well defined)
- **Environmental factors play a large role in prostate cancer risk** (e.g., Western diet)
- Epidemiological studies suggest that men with **hypercholesterolemia** are at **increased risk**
- Cholesterol-sensitive cancer progression mechanisms: cell proliferation, inflammation, steroidogenesis

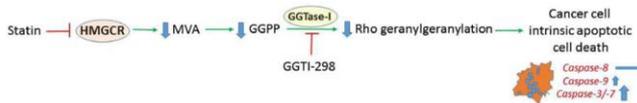


CHOLESTEROL AND BREAST CANCER

- **Hypercholesterolemia and metabolic syndrome are risk factors for breast cancer**
- Cholesterol metabolite **27-hydroxycholesterol (27-HC)** **mimics estrogen** in certain tissues → replays estrogen function (anti estrogen treatment is not effective)
- Estrogen-driven breast tumors may **rely on 27-HC to grow when estrogen isn't available**
- Aggressive breast tumors have higher **levels of CYP27A1**, which **converts cholesterol into 27-HC**
- Breast cancer patients with low levels of **CYP7B1** (Enzyme that breaks down 27-HC) live shorter
- 27-HC may play a role in other hormone-driven cancers (e.g. Endometrial cancer)

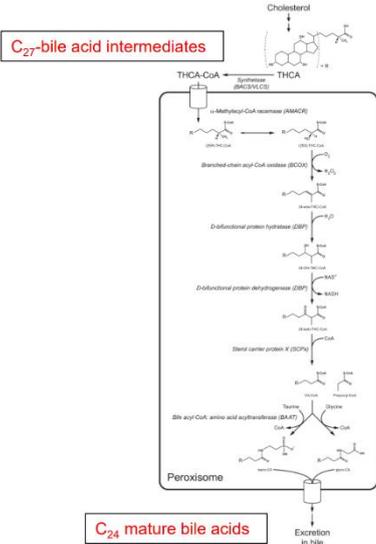


STATIN-INDUCED CELL DEATH IN CANCER CELLS



PEROXISOMAL STEPS INVOLVED IN BILE ACID BIOSYNTHESIS

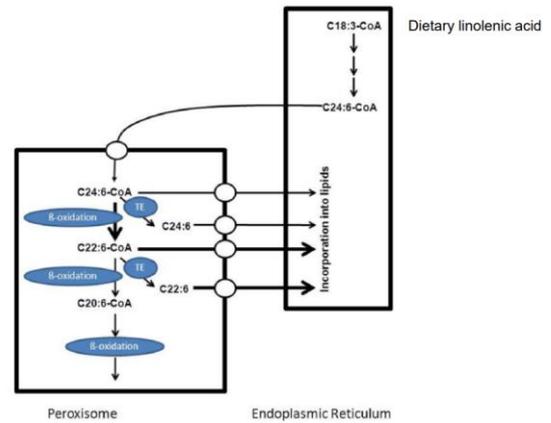
C27- bile acid intermediate (=THCA-CoA) enters peroxisome → formation of C24 mature bile acids → Excretion in bile



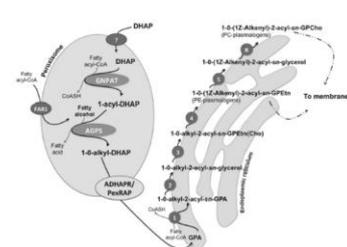
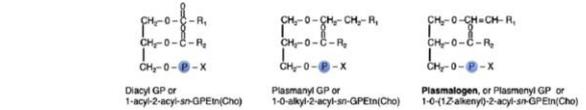
No functional peroxisomes or single enzyme deficiencies of peroxisomal steps in bile acid biosynthesis:

Decreased levels of mature C24 bile acids

Increased levels of C27-bile acid intermediates in the blood, urine, and tissues



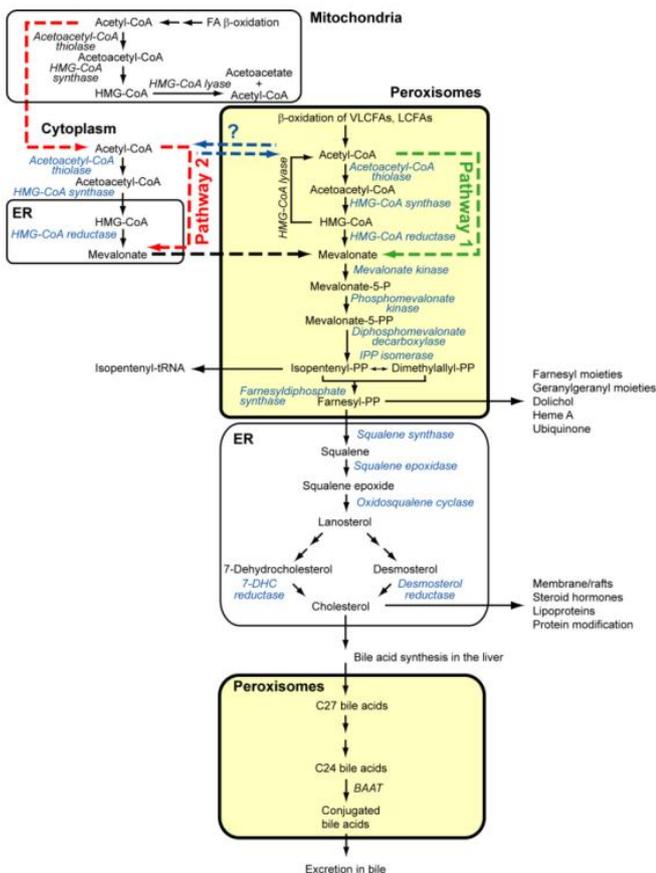
METABOLIC PATHWAY FOR PLASMALOGEN SYNTHESIS



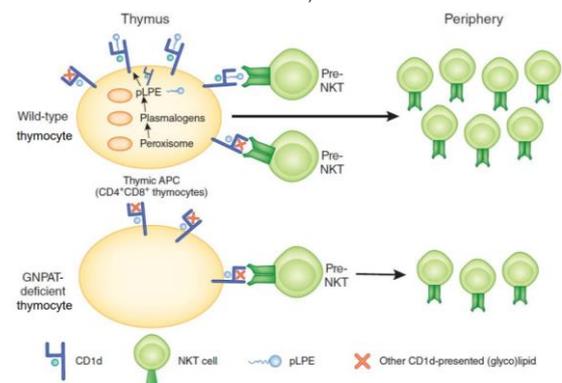
FAR1: fatty alcohol reductase
GNPAT: glycerone phosphate O-acyltransferase
AGPS: alkylglycerone phosphate synthase
ADHAPR/PexRAP/DHRST7b: alkyl/acyl DHAP reductase

- There are 2 types of ether phospholipids
 - o Plasmalmyl-phospholipids (ether bond position sn-1 to alkyl group)
 - o Plasmenyl-phospholipids (ether bond position sn-1 to alkenyl group = plasmalogens)
- Biosynthesis of plasmalogens (PLs) begins with **association of peroxisomal matrix enzymes**

SUBCELLULAR LOCALIZATION OF CHOLESTEROL BIOSYNTHESIS IN MAMMALIAN CELLS



PEROXISOME GENERATION OF INKT (INVARIANT NATURAL KILLER T CELL) LIGANDS



Under normal conditions, iNKT cells are selected by thymocytes that present a variety of glycolipids and phospholipids to preiNKT cells. Those cells then develop into mature iNKT cells that are exported to the periphery. In a GNPAT-deficient environment, the lack of ether-bonded phospholipids generated in the peroxisome, results in considerably fewer iNKT cells. Such observations indicate the importance of peroxisome-generated lipids in the development of a large subpopulation of iNKT cells.

How are proteins directed to their subcellular localization in the cell?

Which techniques would you use to determine the subcellular localization of a protein?

KEY ROLE OF PEROXISOMES IN THE FORMATION OF DOCOSAHEXAENOIC ACID (DHA; C22:6 N-3)

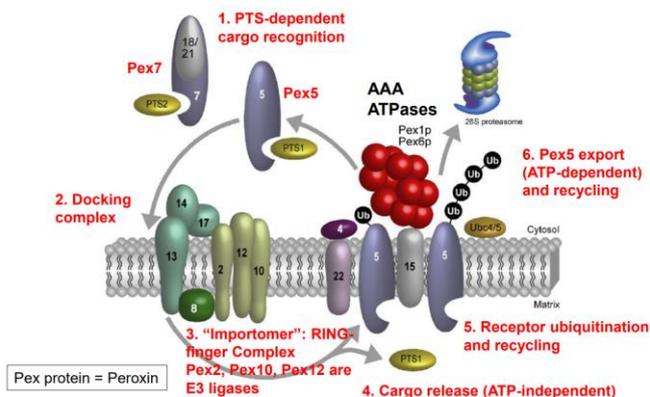
- Docosaheptaenoic acid is synthesized from C18:3 n-3 which first undergoes a number of elongation and desaturation steps in the ER to produce C24:6-CoA which is then transported to the peroxisome
- Within peroxisomes **C24:6-CoA** undergoes one cycle of beta-oxidation to produce the corresponding C22:6-CoA which can then be exported out of the peroxisome for subsequent incorporation into lipids in the ER
- longer C24 precursor to C22 by single β-oxidation cycle

PEROXISOMAL IMPORT

- Targeting proteins for **peroxisomal import** relies on 2 conserved **peroxisomal targeting signals (PTS)**:
 - o **PTS1** at the very C-terminus (majority of peroxisomal proteins) recognized by import receptor **Pex5**
 - o **PTS2** near the N-terminus (few peroxisomal proteins) recognized by import receptor **Pex7**
- Completely folded proteins can be imported
- Peroxisomal import can be divided in four steps:
 1. PTS dependent cargo recognition
 2. Docking & translocation of the receptor cargo complex to the luminal site
 3. Cargo release
 4. Receptor ubiquitination and recycling

PEROXISOMAL MATRIX PROTEIN IMPORT CASCADE

Consensus Peroxisomal targeting signal 1 (PTS1): (S/C/A)(K/R/H)(L/M)
 Consensus Peroxisomal targeting signal 2 (PTS2): (R/K)(L/V/I)X5(H/Q)(L/A)



1. Proteins harboring a peroxisomal targeting signal (PTS) are **recognized** and bound by the import receptors **Pex5** and **Pex7** in the cytosol
2. Cargo-loaded receptor is directed to the peroxisomal membrane and binds to the **docking complex (Pex13/Pex14/Pex17)**
3. The **import** receptor assembles with **Pex14** to form a transient pore and cargo proteins are transported into the peroxisomal matrix in an unknown manner
4. **Cargo release** might involve functions of Pex8 or Pex14
5. The import **receptor** is **monoubiquitinated** at a conserved cysteine by the **E2-enzyme complex Pex4/Pex22** in tandem with **E3-ligases** of the RING-complex (Pex2/Pex10/Pex12)
6. The ubiquitinated receptor is **exported** released from the peroxisomal membrane in an ATPdependent manner by the AAA-peroxins Pex1 and Pex6, which are anchored to the peroxisomal membrane via Pex15. As the last step of the cycle, the ubiquitin moiety is removed and the receptor enters a new round of import

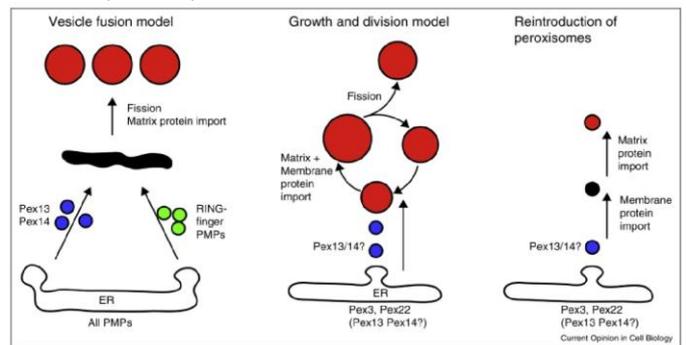
- **Pex8** connects the docking complex to the RING-finger complex (Pex2/10/12; E3 ubiquitin ligases)
 - o The multiprotein complex; docking & RING-complex is called the **“importomer”** → mediates cargo translocation/release
- Ubiquitination of Pex5 by E2-enzyme complex Pex4/22 in tandem with E3-ligases of RING-complex can have 2 outcomes:

- o Monoubiquitination → Membrane extraction by AAA ATPases (Pex1/6) anchored to peroxisomal membrane via Pex15: ATP dependent export & recycling of Pex5
- o Polyubiquitination: proteasomal degradation of Pex5
- Generation of Pex5^{-/-} mice model: impaired import → peroxisome biogenesis defect

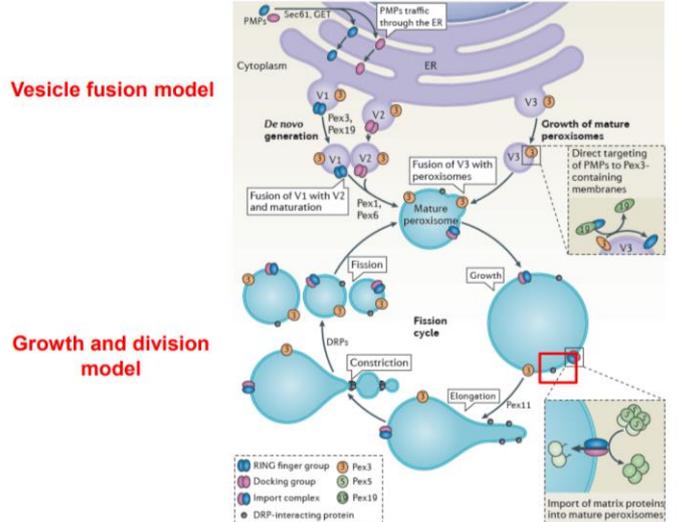
PEROXISOME BIOGENESIS

MODELS FOR PEROXISOME MULTIPLICATION:

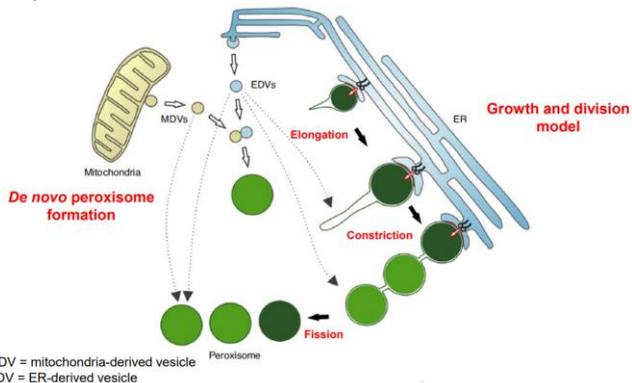
- **Vesicle fusion model:** Vesicles containing different peroxins (Pex proteins) fuse and form a preperoxisomal membrane structure → This contains a complete importomer → matrix protein import starts Fission (extension with constriction; same machinery as mitochondria) results in peroxisomes of final size and membrane protein composition
- **Growth division mode:** Peroxisomes are derived from existing peroxisomes by fission (faster compared to vesicle fusion model)
- **Reintroduction of peroxisomes:** Absence of peroxisomes, ER-derived Pex containing vesicle matures slowly into a peroxisome



- **Vesicle fusion model**
 1. ER contains all peroxisomal membrane proteins (PMPs)
 2. Membrane segregation, exit by different vesicles
 3. Vesicles containing Pex13/14 or RING-finger complex
 4. These vesicles fuse → preperoxisomal membrane structure
 5. Complete importomers on surface → matrix protein import
 6. Subsequent fission: right size and membrane protein composition
- **Growth and division model**
 1. Peroxisomes derive from existing peroxisomes by fission
 2. Fission from existing peroxisome (containing Pex19) + vesicle deriving from ER (packed with some class 2 PMPs) → Fusion
 3. Growth of new peroxisome
- **Reintroduction of peroxisomes**
 1. In absence of peroxisomes: ER-derived Pex3 on vesicle (Pex19 docking site)
 2. Vesicle gains all PMPs
 3. Import of matrix proteins → growth and division



- Usually, peroxisomes are **formed de novo from the ER** through budding & heterotypic fusion of 2 vesicle types
 - o This mechanism separates RING finger and docking components of the import complex into different vesicles (not import competent) until after fusion and assembly of a complete and functional import complex
- Mature peroxisomes can **multiply by growth**, with proteins and membranes from the ER and fission



PEROXISOMES VS MITOCHONDRIA DIVISION

- Peroxisomes and mitochondria are divided by similar machineries (share fission machinery)
 - o Mitochondria divide by fission
- Shared components of the peroxisomal and mitochondrial division/fission machinery are:
 - o Fission 1 (Fis1)
 - o Dynamin-like protein 1 (Dlp1)
 - o Mitochondrial fission factor (Mff)
- Peroxisomal only: Pex11β

Shared components of the peroxisomal and mitochondrial division machinery in mammals:
 Fis1: Fission 1
 Dlp1, Drp1: Dynamin-like protein 1
 Mff: Mitochondrial fission factor

Dynamin-like protein 1 (DLP1) mutation!

- Fission machinery forms a ring around the fission tube
- Dlp1 mutation leads to elongation of both mitochondria and peroxisomes

PEROXISOMES: FLEXIBLE & DYNAMIC ORGANELLES

- Keeping yeast in glu medium → few peroxisomes
- Transfer yeast in oleic acid medium or methylotrophic medium → increased number of peroxisomes → peroxisomal biogenesis allows the metabolisation of oleic acid/methanol = yeast can proliferate
- Transfer yeast back in glu medium → selective degradation of peroxisomes (selective autophagy) → flexible induction of biogenesis and degradation of peroxisomes

PEROXISOMAL DISORDERS

- Peroxisomes play a role in pathogenesis of nonalcoholic fatty liver disease and steatohepatitis, aging, diabetes, and neurodegenerative disorders
 - o A decrease in peroxisome abundance has been observed in various tumor cells
- The importance of peroxisomal metabolism is illustrated by the marked abnormalities in brain and systemic organs in peroxisome biogenesis disorders of the Zellweger spectrum in which functional peroxisomes are absent or disorders caused by single peroxisomal enzyme deficiencies

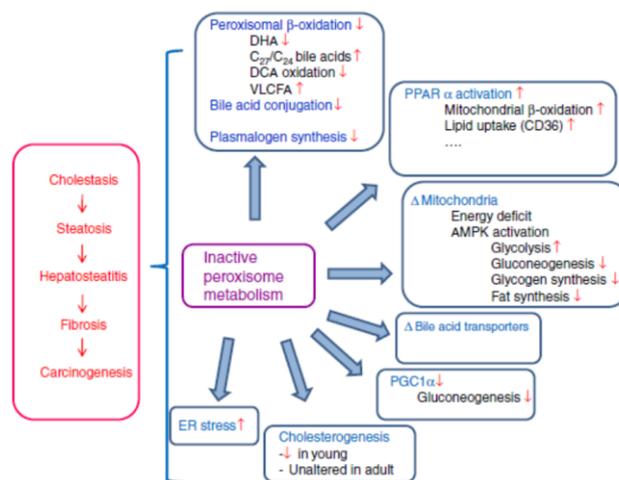
Peroxisome biogenesis disorders	Single peroxisomal enzyme deficiencies
Zellweger spectrum disorders*	X-linked Adrenoleukodystrophy*/
Zellweger syndrome*	Adrenomyeloneuropathy*
Neonatal adrenoleukodystrophy*	3-Ketoacyl-CoA thiolase deficiency*
Infantile Refsum disease*	D-Bifunctional enzyme deficiency*
Rhizomelic chondrodysplasia punctata*	Classical Refsum disease*
Hyperpipecolic acidemia*	Dihydroxyacetonephosphate (DHAP) Acyltransferase deficiency*
	Alkyl-DHAP synthase deficiency*
	Mevalonate kinase deficiency*
	α-Methylacyl-CoA racemase deficiency*
	Acatalasiaemia
	Glutaric aciduria type III*
	Hyperoxaluria type I

*Nervous system affected

X-LINKED ADRENOLEUKODYSTROPHY AND LORENZO'S OIL

- Most frequent peroxisomal disorder
- Incidence of 1:17,000
- Disorder of peroxisomal fatty acid β-oxidation
- Progressive behavioral, cognitive, and neurological deterioration leading to death within a few years after onset

METABOLIC ABNORMALITIES IN LIVER OF MICE WITH PEROXISOME BIOGENESIS DEFECTS



Overall disease progression

PEX GENES OF PEROXISOME DEFICIENCIES

- **Pex protein mutations** result in severe **peroxisomal biogenesis** disorders that are often fatal → Liver, kidney and brain are most affected
 - o Cerebrohepato renal syndroms
 - o Hypomyelination
 - o Hearing loss
 - o Catharates (loss of sight)
 - o Accumulation of metabolites
 - o Lipid accumulation (steatosis)

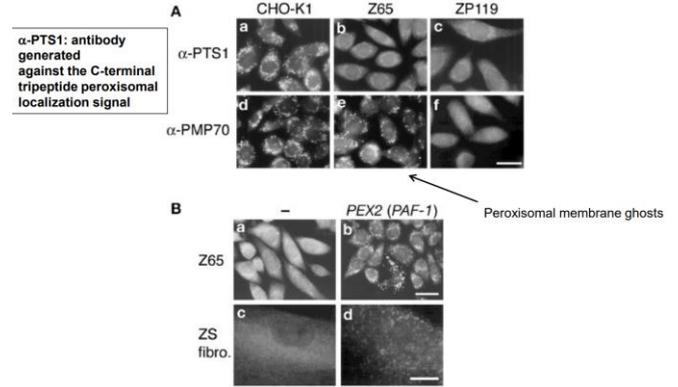
o ER and mitochondrial stress

Gene	CG		Phenotype	Peroxisome ghosts	Peroxin Characteristics
	US/EU	Japan			
PEX1	1	E	ZS, NALD*, IRD*	+	AAA family
PEX2	10	F	ZS, IRD*	+	PMP RING
PEX3	12	G	ZS	-	PMP PMP-DP
PEX5	2		ZS, NALD	+	PTS1 receptor, TPR family
PEX6	4(6)	C	ZS, NALD*	+	AAA family
PEX7	11	R	RCDP	+	PTS2 receptor, WD motif
PEX10	7(5)	B	ZS, NALD	+	PMP RING
PEX11 β	16		ZS	+	PMP
PEX12	3		ZS, NALD, IRD	+	PMP RING
PEX13	13		ZS, NALD*	+	PMP PTS1-DP SH3
PEX14	15	K	ZS	+	PMP PTS1-DP PTS2-DP
PEX16	9	D	ZS	-	PMP PMP-DP
PEX19	14	J	ZS	-	CAAX motif, PMP receptor
PEX26	8	A	ZS, NALD*, IRD*	+	PMP, Pex1p-Pex5p recruiter

ZS, Zellweger syndrome; NALD, neonatal adrenoleukodystrophy; IRD, infantile Refsum disease; RCDP, rhizomelic chondrodysplasia punctata.

PEROXISOMAL GHOSTS

- Chinese hamster ovary (CHO) cell mutants are **defective in peroxisome assembly (biogenesis)**



- Peroxisomes are apparently missing in Zellweger syndrome; nevertheless, some of the integral membrane proteins of the organelle are present
 - o Peroxisomal membrane proteins are located in unusual empty membrane structures of larger size
- Peroxisomal ghosts are **intracellular structures** distinct from lysosomal compartments in Zellweger syndrome
 - o Are aberrant peroxisomal structures found in cultured skin fibroblasts from patients affected by Zellweger Syndrome (ZS), a genetic disorder of peroxisomal assembly
 - o Contain peroxisomal integral membrane proteins (PxIMPs) and they lack most of the matrix enzymes that should be inside the organelle
- May be a result from genetic defects in the cellular machinery for importing newly-synthesized peroxisomal proteins into the organelle
- Peroxisomal ghosts are distinct subcellular structures, occupying separate subcellular locations

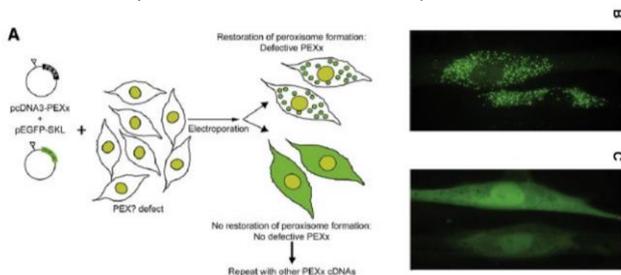
PEROXISOME-DEFICIENT PEX2^{-/-} ZELLWEGER MOUSE

- Disruption of the Pex2 locus
- 2 genetic backgrounds
 - o Congenic 129S6/SvEv background, Mice die usually <12 h after birth
 - o Hybrid Swiss Webster-129 (SW/129) background
- Approx. 20-30% of mice survive for 7-10 days, lower number survives 12-13 days, rare mice survive up to P36
- Bile acid feeding significantly prolonged survival of
- SW/129 PEX2^{-/-} mice
- Retarded somatic growth of Pex2^{-/-} mice

SCREENING FOR PEX MUTATIONS: PEX CDNA TRANSFECTION COMPLEMENTATION ASSAY

Principle of **PEX cDNA transfection complementation assay** used to **identify the defective PEX gene** in cells from patients

- Cells are **co-transfected** with 2 different expression plasmids:
 - o **Functional Pex-containing plasmid** (the one that you suspect is mutated in patient)
 - o **GFP-SKL(PTS1) plasmid** (GFP with peroxisomal targeting sequence)
- If **genetic complementation occurs** (GFP signal in peroxisomes; functional import) → patient has a **defect in the Pex protein** that was added via transfection
- **No genetic complementation occurs** when the reporter protein is localized in the cytosol (still impaired import machinery = another Pex is affected)

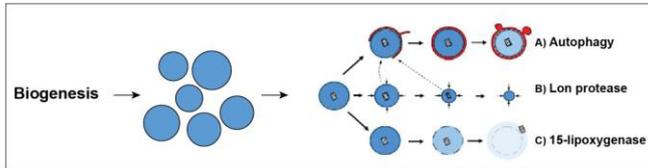


- **Nowadays:** Whole genome studies are performed and analysed if there are mutations

DEGRADATION PATHWAYS OF PEROXISOMES

- Peroxisomes are highly dynamic organelles
- Peroxisome homeostasis is achieved via a **tightly regulated interplay between peroxisome biogenesis and degradation** via selective autophagy, which is commonly known as “**pexophagy**”
- Several stress conditions have been shown to stimulate pexophagy and decrease peroxisome abundance
- **Pexophagy (70-80%)** is critical for the maintenance of cellular homeostasis by maintaining both organelle integrity and number in the context of varying environments and stresses

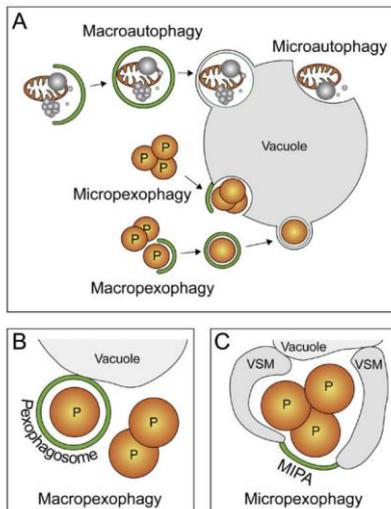
AUTOPHAGY



- The major degradation pathway of peroxisomes is **autophagy** (self eating)
 - o This is also a survival mechanism to obtain energy
- We distinguish between **2 pathways**:
 - o **Cvt** (cytoplasm to vacuole targeting) = **Selective Autophagy**:
 - o **PAS** (phagophore assembly site) pathway = **Macroautophagy**

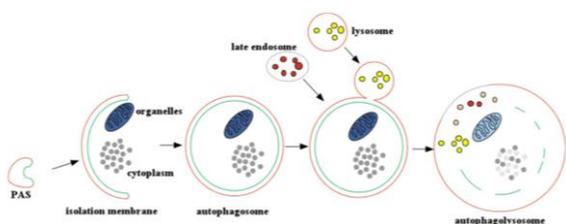
MODES OF GENERAL AUTOPHAGY AND PEXOPHAGY

- There are two main modes of pexophagy in yeast
- Macropexophagy and Micropexophagy



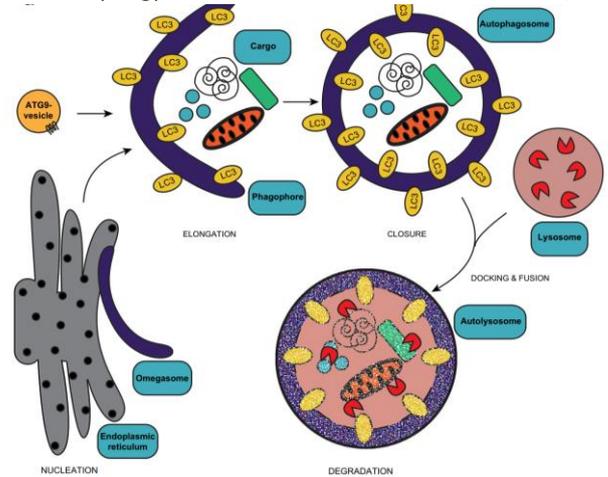
MACROAUTOPHAGY

- Not selective
- Phagophore is formation → enfold organelles or part of the cytoplasm → Enclosed autophagosome → Fuses with lysosome → Further degradation of engulfed (*verschlingte*) components and release of broken-down nutrients



- Autophagosome formation and maturation is a **highly regulated process**
 - o Key components in this process are **autophagy-related (ATG) proteins**

Macroautophagy can be divided into six sequential steps:



- 1. Initiation**
 - o Initiation of autophagosome formation is regulated by the UNC-51-like kinase 1/2 (ULK1/2) and class III phosphatidylinositol 3-kinase (PI3K) complexes
- 2. Membrane nucleation and phagophore formation**
 - o ULK complex activates PI3K complex 1 leading to its translocation to the ER, where it produces phosphatidylinositol 3-phosphate to drive omegasome formation
 - o ULK complex translocates subdomains of the endoplasmic reticulum (ER) (=omegasomes) to drive the nucleation of autophagosomes
- 3. Phagophore elongation with concomitant cargo sequestration**
 - o Phagophore elongation is facilitated by transient interactions with the ATG9 compartment, which delivers lipids for membrane formation from various sources including the ER, recycling endosomes, plasma membrane, mitochondria, and Golgi
 - o Elongation and closure of phagophore membrane is controlled by two ubiquitin-like conjugation pathways that conjugate ATG12 to ATG5 and ATG8 (= LC3 proteins) to the lipid phosphatidylethanolamine (PE)
- 4. Phagophore closure forming an autophagosome, a double-membrane vesicle**
- 5. Fusion of the autophagosome with the lysosome forming an autolysosome**
- 6. Degradation of sequestered cargo in the autolysosome**
 - o The inner membrane of the autolysosome is lysed and the cargos are degraded through the activity of lysosomal hydrolases
 - o Degradation products are transported out of the autolysosome into the cytosol for reuse

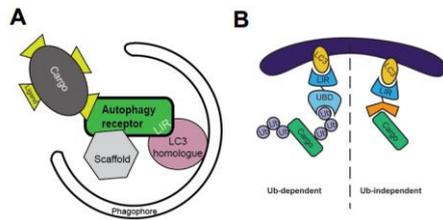
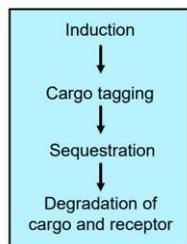
SELECTIVE AUTOPHAGY:

- In selective autophagy, specific cargo-recognizing receptors connect the cargo to the core autophagic machinery
- Selective parts/organelles in the cytoplasm are degraded (ER, peroxisomes, lipids, protein aggregates...)
- The 4 key steps are:

- o Induction
- o Cargo tagging
- o Sequestration
- o Degradation of cargo and receptor

- Enclosure of the phagophore leads to the cargo sequestration and the selective degradation of cargo as well as the cargo/autophagy receptor
- Selectivity is mediated by autophagy/cargo receptors which form the bridge between the cargo and the autophagic machinery:
 - o Bind to the cargo (cargo tagging)
 - o Bind to LC3 protein (connected to phagophore) with their LIR domain (LC3 interacting domain)
 - o Bind to scaffold proteins

The 4 key steps of selective autophagy



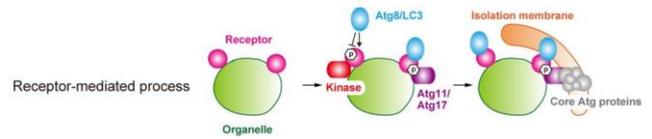
- **LC3-interacting region (LIR)** motif ensures the targeting of autophagy receptors to LC3 (or other ATG8 family proteins) anchored in the phagophore membrane
- The selective autophagy receptors are often synthesized even under conditions wherein the cargoes are not degraded, but receptor activation often relies on protein modifications, like phosphorylation or ubiquitination. E.g. transfer yeast back glu medium → phosphorylation of already bound autophagy receptor interacts also with autophagy machinery → degradation of peroxisomes

MOLECULAR MECHANISMS OF SELECTIVE AUTOPHAGY

- Selective autophagy can be mediated by 2 different molecular mechanisms for cargo recognition:
 - o **Receptor-mediated processes**
 - o **Ubiquitin-mediated processes**
- Both involve protein phosphorylation that activates or inactivates their downstream events

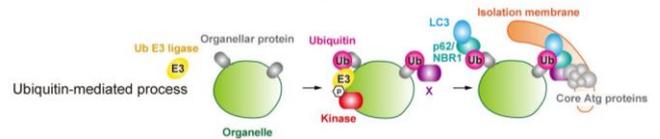
Receptor-mediated process:

- Autophagy/cargo receptors associated on the cargo surface interact with LC3 conjugated to the phospholipid phosphatidyl-ethanolamine and localized to phagophores
- Protein kinases phosphorylate receptors and regulate receptor interactions with LC3

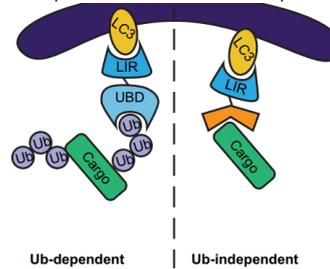


Ubiquitin-mediated process:

- E3 ubiquitin ligases target the organelle and ubiquitinate proteins on organelle surface. The ubiquitin chains then interact with proteins that recognise ubiquitination and have the LIR domain → interaction with LC3
- Protein kinases phosphorylate the ubiquitin ligases and promote targeting and activation of the E3 enzymes → common way of pexophagy



Ub-dependent and Ub-independent selective autophagy:



- **Ub-dependent selective autophagy:** Prototypical selective autophagy receptor with an **UBD** recognizes **Ub** chains attached to **intracellular cargo** and physically links the targeted material to the autophagosomal membrane via a **LIR** motif that binds to lipidated and autophagosome membrane associated **LC3**
- **Ub-independent selective autophagy:** **Autophagy receptors** directly bind to **intracellular cargo** and connect it to the autophagosomal membrane through their **LIR** motif

GENERAL THEMES OF SELECTIVE AUTOPHAGY PATHWAYS

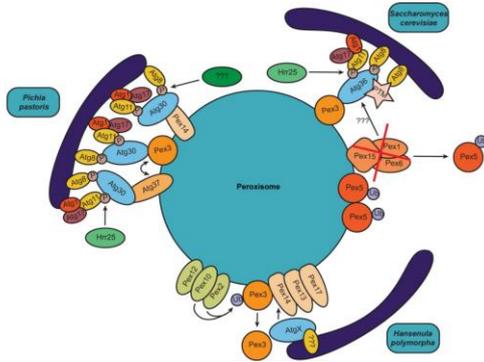
The key decision point in any selective autophagy pathway is the mechanism by which the **core autophagy machinery** is redirected to degrade primarily selective cargo

1. Every selective autophagy pathway studied to date requires a specific cargo receptor
2. These cargo receptors typically have a tripartite role in (a) cargo binding, (b) interaction with Atg11, and (c) interaction with Atg8 (LC3 in mammals) via an Atg8-interaction motif (LIR motif)
3. The selective autophagy receptors are often synthesized even under conditions wherein the cargoes are not degraded, but receptor activation often relies on protein modifications, such as phosphorylation or ubiquitination.
4. Specialized membrane structures, such as the MIPA, are needed for micropexophagy, and not for macropexophagy
5. Generally the receptors are degraded in the vacuole (lysosome) along with the cargo

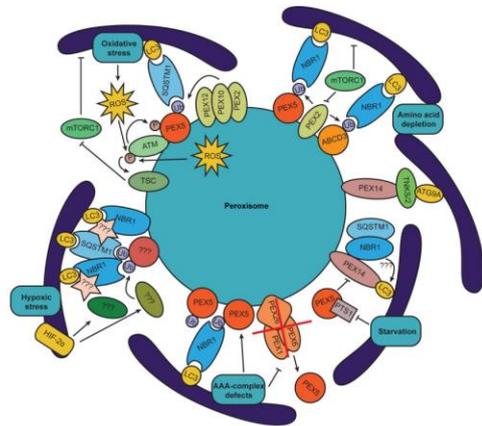
AUTOPHAGY IN YEAST VS. MAMMALS

See paper at the end!

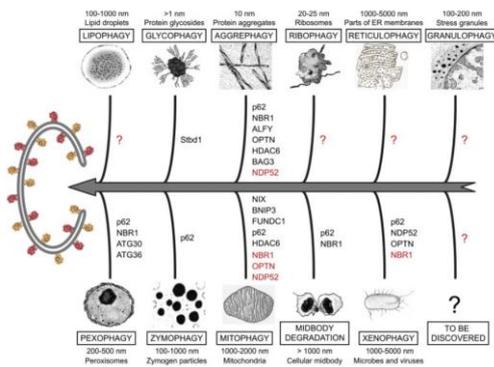
YEAST PEXOPHAGY



MAMMALIAN PEXOPHAGY

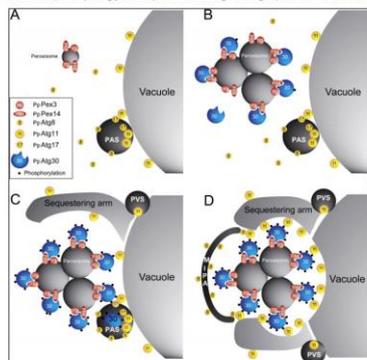


TYPES OF SELECTIVE AUTOPHAGY IN MAMMALS



PEROXISOME TURNOVER BY SELECTIVE AUTOPHAGY (PEXOPHAGY) IN YEAST

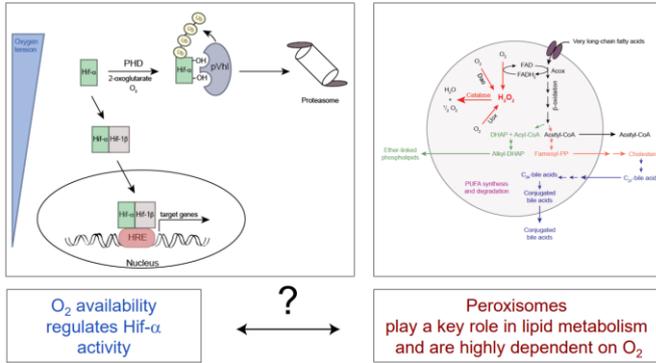
PpAtg30 tags peroxisomes for pexophagy in *P. pastoris*, Atg36 tags peroxisomes for pexophagy in *S. cer.*!



Experimental setup to induce pexophagy in yeast?

HYPOXIA SIGNALING & PEROXISOME HOMEOSTASIS

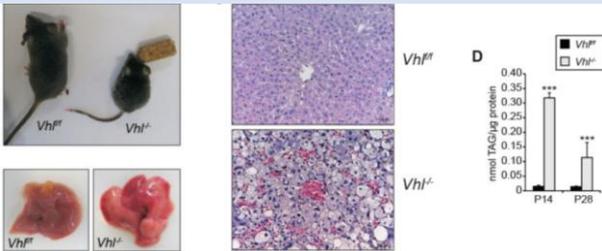
Are peroxisomes interconnected with Hif- α signaling?



Hypothesis

- To minimize O₂ consumption under hypoxic conditions, Hif- α signaling either inhibits O₂-dependent peroxisomal metabolic pathways or decreases peroxisome abundance
- Reduced peroxisomal activity would be part of metabolic reprogramming causing changes in lipid metabolism
- We tested this hypothesis in the liver because peroxisomes are highly abundant in the liver and liver-specific loss of VHL causes severe lipid accumulation

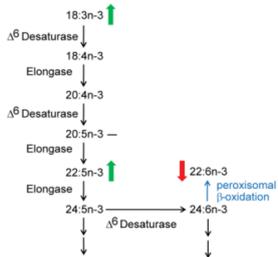
LIVER-SPECIFIC LOSS OF VHL



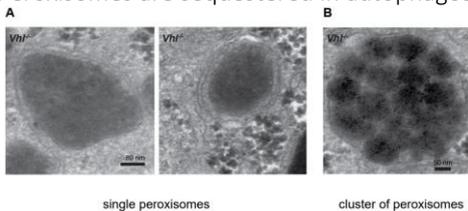
Vhl^{-/-} livers or Vhl^{-/-}/Hif1 α ^{-/-} livers:

Loss of tumor suppressor Vhl and thus constitutive active HIF

- **Reduced number of peroxisomes**
- Contain intact peroxisomes
- Causes **hepatic steatosis** → Accumulation of FA with many double bonds (complex, long chain FA) → Accumulation of bile acid intermediates → decreased DHA



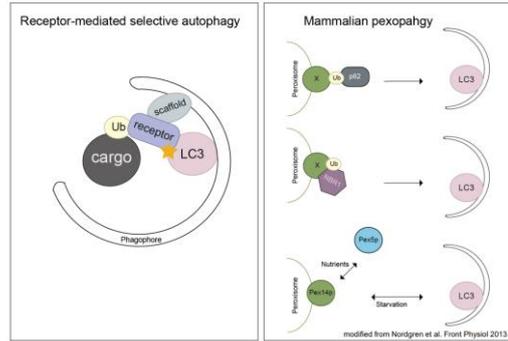
- Normal peroxisome biogenesis machinery (functional)
- Peroxisomes colocalize with autophagosomes
- Peroxisomes are sequestered in autophagosomes



Electron microscopy demonstrates the autophagic sequestration of peroxisomes in Vhl^{-/-} livers

- Therapy: Inhibition of autophagy increases peroxisome abundance in Vhl^{-/-} livers

- Protein levels of Nbr1 and p62 are decreased
 - o Nbr1 colocalizes with peroxisomes in livers
 - o p62/Sqstm1 colocalizes with peroxisomes



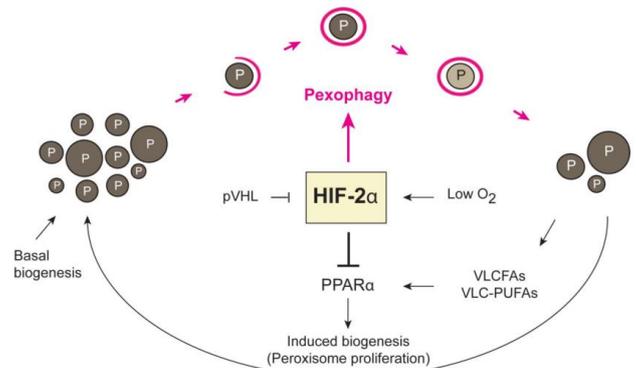
Selective degradation of peroxisomes via autophagy

- Hif-2 α (low oxygen) but not Hif-1 α activation mediates the decrease of peroxisome abundance in Vhl^{-/-} livers → Hif-2 α promotes pexophagy (Hif-1 α has no effect)
- Hif-2 α (low oxygen) mediates changes in lipid composition reminiscent of peroxisomal disorders
- Human ccRCCs with high HIF-2 α (low oxygen) levels have decreased peroxisome abundance → correlation between high HIF-2 α levels and loss of peroxisomes
- **HIF-2 α gain-of-function** leads to similar lipid changes as in Vhl^{-/-} and Vhl^{-/-}/Hif1 α ^{-/-} livers

How does Hif-2 α decrease peroxisome abundance?

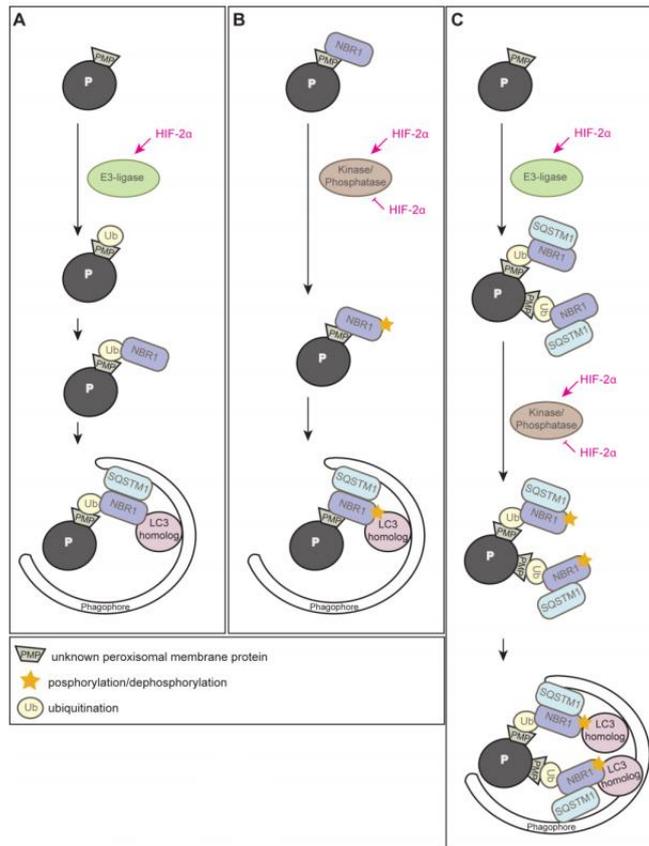
HIF-2A AND PEROXISOME DEGRADATION

- Hif-2 α promotes degradation of mammalian peroxisomes by selective autophagy
- Peroxisomes play a central role in lipid metabolism, and their function depends on molecular oxygen
- Low oxygen tension or von Hippel-Lindau (Vhl) tumor suppressor loss is known to **stabilize hypoxia-inducible factors alpha (Hif-1 α and Hif-2 α)**
- Are thus peroxisome homeostasis and metabolism interconnected with Hif- α signaling? → YES → Hif signalling induces mitophagy and pexophagy
- Hif-2 α activation raise peroxisome turnover by selective autophagy (pexophagy) and thereby changes lipid composition reminiscent of peroxisomal disorders
- Peroxisome abundance is reduced in VHL-deficient human clear cell renal cell carcinomas with high HIF-2 α levels → **Hif-2 α is as a negative regulator of peroxisome abundance and metabolism**



- Hif-2 α promotes mammalian pexophagy and is thus a negative regulator of peroxisome abundance and metabolism

MODELS ILLUSTRATING HOW HIF-2A MIGHT TRIGGER PEXOPHAGY



OPEN QUESTIONS

- How does Hif-2α induce pexophagy at the molecular level?
- Which posttranslational modifications of peroxisomal membrane proteins and/or autophagy receptors regulate pexophagy?
- Is loss of peroxisomes an early event in tumorigenesis and promoting or slowing down tumor growth?
- How does induction of pexophagy in cancer cells affect the tumor microenvironment and tumor growth?
- Does decreased peroxisome abundance affect the metastatic potential of cancer cells?
- What are the consequences of decreased peroxisome abundance on cellular metabolism in cancer cells?

PEROXISOME ABUNDANCE IN TUMORS

- Peroxisome number is reduced in clear cell renal cell carcinoma (ccRCC) with high HIF-2α levels
- HIF-2α stabilization is observed in the vast majority of solid tumors and might lead to reduced peroxisome abundance in other cancer types
- Excessive peroxisome proliferation leads to hepatocellular carcinomas in rodents
- Peroxisomal branched-chain fatty acid β-oxidation enzymes are induced in prostate cancer. Is peroxisome abundance also increased?
- Peroxisomes are essential for ether lipid synthesis – aggressive cancers have high levels of ether lipids, and inhibition of ether lipid synthesis reduces tumor growth → if tumor has increased Hif-2α signalling and still a lot of peroxysomes it is highly aggressive
- Decrease in peroxisome abundance has also been observed in other tumors such as hepatocellular carcinoma, colon carcinoma, breast cancer

PAPER

HIF-2α PROMOTES DEGRADATION OF MAMMALIAN PEROXISOMES BY SELECTIVE AUTOPHAGY

Hypotheses: HIF-α signalling may inhibit O₂-dependent peroxisomal metabolism and/or decrease the number of peroxisomes. In addition, HIF-mediated reduction in peroxisomal activity could promote metabolic reprogramming causing changes in lipid metabolism

HIF-1

- Low oxygen tension or von Hippel-Lindau (Vhl) tumor suppressor loss is known to stabilize hypoxia-inducible factors alpha (Hif-1α & Hif-2α) to mediate adaptive responses
- HIF-1α and HIF-2 α have both common and distinct target genes and are differentially regulated in various physiological and pathophysiological conditions
- HIF-induced genes promote angiogenesis, erythropoiesis, metastasis, and metabolic reprogramming, such as shifting metabolism from oxidative phosphorylation to glycolysis
- Activation of HIFs also reprograms lipid metabolism leading to lipid accumulation

Peroxisomes

- Are extremely versatile and dynamic organelles whose number, size, and function are dependent on cell type and metabolic needs
- Peroxisomes adapt rapidly to cellular demands, and their homeostasis is achieved by the counterbalance between organelle biogenesis and degradation
- Peroxisomes play a central role in lipid metabolism: Degradation of fatty acids (i.e., very long-chain fatty acids and polyunsaturated fatty acids), ether lipid synthesis, cholesterol and bile acid synthesis, and metabolism of reactive oxygen species
- Peroxisomal function depends on O₂
- Lack of peroxisomal metabolism leads to extensive cellular metabolic disarray → patients with peroxisomal disorders display severe hepatic dysfunction with fatty liver, cholestasis, and eventually cirrhosis

Autophagy

- Autophagy is an evolutionary conserved catabolic process for degradation of macromolecules and organelles
- Three mechanisms for peroxisome degradation have been described in mammalian cells: Selective autophagy (pexophagy), proteolysis by Lon protease 2 (Lonp2), 15-lipoxygenase-1 (Alox15)-mediated autolysis
- Key factor in selective autophagy is specific cargo-recognizing receptors that connect the cargo to the core autophagic machinery
- Two pexophagy receptors, Atg30 and Atg36, have been identified in yeast and their overexpression stimulates pexophagy even under peroxisome-inducing conditions. However, there are no orthologous genes in mammals, and no pexophagy receptors have been identified

MOUSE MODELS:Liver-specific Vhl, Vhl/Hif1 α , and Vhl/Hif2 α knockout mice

- Peroxisomes are highly abundant in the liver
- Liver-specific loss of pVhl causes severe lipid accumulation

RESULTS

- **Peroxisome abundance is reduced** in Vhl^{-/-} livers (but they are intact)
- Reduced number of peroxisomes has consequences on lipid composition → **Changes in lipid composition** in Vhl^{-/-} livers are similar to peroxisomal disorders
 - o Most TAG species showed accumulation of 10-fold
 - o TAGs with high number of double bonds increased 30- to 100-fold
 - o Accumulation of VLCFAs and VLC-PUFAs
 - o Fatty acids: DHA and AA deficiencies
 - o Alterations are due to defective peroxisomal β -oxidation
- **Hif-2 α but not Hif-1 α activation** mediates the decrease of peroxisome abundance in Vhl^{-/-} livers
 - o Peroxisomal phenotype rescue in Vhl^{-/-}/Hif-2 α ^{-/-}
- Expression of peroxisome biogenesis machinery was not impaired → **peroxisome biogenesis and proliferation are functional**
- Pex11a was significant transcriptional upregulated
 - o Pex genes encode peroxins essential for peroxisome biogenesis
 - o Pex11a was the only peroxin that showed significant transcriptional upregulation in Vhl^{-/-}
 - o Overexpression of Pex11a **promotes peroxisome division** and **induces peroxisome abundance** (positive regulator) → There might be a feedback loop in which Vhl^{-/-} hepatocytes sense reduction of peroxisomes and attempt to counterbalance their lack by inducing a positive regulator of peroxisome biogenesis
- Peroxisome proliferation can be induced in Vhl^{-/-} livers
 - o WY and PBA induce peroxisome proliferation
- Hif-2 α signaling has a **repressive effect on ligand-dependent Ppara** transcriptional activity
 - o Ppara is a promoter of peroxisome proliferation
 - o Hif-2 α -mediated pexophagy is associated with decreased peroxisomal metabolism → Results in accumulation of VLCFAs and VLC-PUFAs → these lipids **are activating ligands for the transcription factor Ppara** → However, Hif-2 α restrains ligand-induced Ppara-mediated peroxisome proliferation → HIF2 α induces pexophagy and counteracts Ppara → ensures efficient depletion of the peroxisome pool
- **Autophagy is induced** in Vhl^{-/-} mice → increased degradation of peroxisomes
 - o Autophagy machinery is required for **HIF-2 α -mediated pexophagy**
- **HIF-2 α gain-of-function** leads to similar lipid changes as Vhl^{-/-} livers
- Pharmacological inhibition of autophagy increases peroxisome abundance in Vhl^{-/-} livers

- o Treatment with 3-methyladenine (3-MA) (inhibits autophagy) increases peroxisome abundance and Nbr1 and p62 protein levels (receptors for selective autophagy of ubiquitinated targets)
- o Overexpression of Nbr1 and p62 has been shown to induce degradation of peroxisomes
- o 3-MA treatment induced significant clustering of Nbr1- and p62-positive peroxisomes → Hif-2 α signaling might increase the rate of Nbr1 interaction with peroxisomes
- Autophagy receptor **Nbr1** localizes to peroxisomes and **is likewise degraded by Hif-2 α -mediated pexophagy**
 - o Nbr1 and p62 are recruited to peroxisomes and participate in Hif2 α -mediated pexophagy where they are degraded together with peroxisomes in autolysosomes
- Peroxisome abundance is reduced in clear cell renal cell carcinomas with **high HIF-2 α levels**
 - o Negative regulatory effect of HIF-2 α signaling on peroxisome abundance is not restricted to hepatocytes and may extend to other tissues
- HIF-2 α is considered to be a core oncogenic driver in ccRCC and is upregulated in the vast majority of solid tumors → Alterations in lipid metabolism as a result of deregulated peroxisome homeostasis → Accumulation of neutral lipids and glycogen → Fatty acids support cancer growth by providing substrates for energy production or by generating building blocks for membranes and signaling lipids in proliferating cells

CONCLUSION

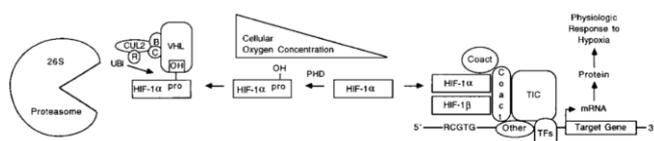
Are peroxisome homeostasis & metabolism interconnected with Hif- α signaling?

- **Hif-2 α** signaling has a regulatory function on peroxisomes → Hif-2 α activation **raises peroxisome turnover by selective autophagy (pexophagy)** and thereby changes lipid composition
- Peroxisome abundance is reduced in VHL-deficient human clear cell renal cell carcinomas with high HIF-2 α levels
- Hif-2 α is a **negative regulator of peroxisome abundance and metabolism and promotes pexophagy**

ADDITIONAL FROM THE BLOCKCOURSE:

In a multicellular organism, cells are exposed to a wide range of oxygen concentrations, typically ranging from 1 to 15% O₂, in contrast to 20.9% O₂ in the air. Whereas O₂ is actively transported in the blood, it diffuses passively into target tissues. Direct measurements of oxygen concentrations have shown that O₂ can diffuse for about 100µm into a tissue, before it is used up. Thus any cell more than 100µm away from a blood supply will have very low oxygen concentrations (termed hypoxia).

The maintenance of oxygen homeostasis is an essential cellular and systemic function and thus the questions how oxygen is sensed by cells, and how adaptive responses to hypoxia are implemented, have fascinated biologists for more than 100 years. During the past decade there has been enormous progress in understanding adaptation to hypoxia at the molecular level and its connections to cell growth, development, physiology and the pathophysiology of diseases such as heart disease and cancer. Cells undergo a variety of biological responses in response to hypoxic conditions. The earliest recognized pathway was that hypoxic cells undergo a shift from aerobic to anaerobic metabolism. The transcriptional activator hypoxia-inducible factor 1 (HIF1) functions as a global regulator of O₂ homeostasis that facilitates both O₂ delivery and adaptation to hypoxia (below normal levels of oxygen) via the activation of over 70 target genes. Among the critical physiological processes regulated by HIF-1 target genes are erythropoiesis [erythropoietin (EPO) production], angiogenesis [vascular endothelial growth factor (VEGF) production], and glycolysis, which are examples of systemic, local tissue, and intracellular adaptive responses to hypoxia, respectively. HIF is a heterodimeric protein that is composed of HIF-1α and HIF-1β subunits. In contrast to the β-subunit, which is constitutively expressed, the expression and activity of the HIF-1α subunit are precisely regulated by the cellular O₂ concentration. Under normoxic conditions, HIF-1α subunits have an exceptionally short half-life and are targeted for ubiquitin-mediated proteolysis, whereas they are stabilized under hypoxia. The molecular basis for this regulation is the O₂-dependent hydroxylation of proline residues in HIF-1α by a novel class of enzymes, the prolyl hydroxylases (PHDs). This specific modification enables specific binding of HIF-1α to VHL (von HippelLindau) tumor suppressor protein. pVHL functions as a substrate recognition component of an E3 ubiquitin-protein ligase complex that targets HIF-1α subunits for ubiquitin-mediated proteolysis. This in turn prevents the activation of HIF target genes. As PHDs require molecular oxygen for their function, their enzymatic activity is inhibited by hypoxia. Consequently, HIF-1α is not recognized by pVHL and thus not targeted for degradation. Subsequently, HIF-1α dimerizes with HIF-1β and activates its target genes. This mechanism ensures that HIF-dependent transcriptional programs are only engaged when oxygen levels drop



HYPOXIA SIGNALING AND CANCER

Background Hypoxia and cancer. Our understanding of the molecular basis of cancer has been illuminated greatly by the study of familial cancer syndromes. Investigations of the specific mutations responsible for these syndromes and the cellular signaling circuits disrupted by the mutant tumor suppressor proteins have provided unprecedented insight into the molecular origin and pathogenesis of inherited and sporadic forms of cancer. These investigations have also invariably helped to shed light on fundamental biological processes such as cell growth and division, apoptosis as well as angiogenesis and metastasis. Studies on the molecular basis of the von Hippel-Lindau hereditary cancer syndrome illustrate these principles very well. In particular, functional studies of the VHL gene product, pVHL, have helped to decipher one of the most fundamental biological processes namely how cells sense hypoxia (low oxygen levels) and what signaling pathways mediate the adaptive cellular responses. It is well known that for incipient neoplasias to progress to a larger size, they must undergo adaptive changes that allow them to survive and even proliferate in a hypoxic environment. This is because the oxygen supplied by the vasculature obligates virtually all cells in a tissue to reside within 100 µm of a capillary blood vessel. Oxygen limitation is central in controlling neovascularization, glucose metabolism, survival and tumor spread. This pleiotropic action is orchestrated by hypoxia-inducible factor (HIF), which is a master transcriptional factor in nutrient stress signaling and has become recognized as a strong promoter of tumor growth. Angiogenesis induced by hypoxia. One of the most well-studied hypoxia responses is production of growth factors such as vascular endothelial growth factor (VEGF) that induces new blood vessel formation (angiogenesis) and thus enhances oxygen supply to the growing tumor. In hypoxia the binding of HIF to the vegf promoter is a key determinant in its expression. Renal cell carcinomas have a particularly high level of VEGF expression, consistent with the notion that inactivating VHL mutations occur in about 50% of such tumors. VEGF attracts and guides sprouting neovessels into oxygen-depleted regions of the tumor mass. Endothelial cells situated at the tip of the sprouts sense and navigate through the environment using long filopodia that are rich in VEGF receptor-2 (VEGFR-2). Thus, migration of the tip cells is guided by a graded distribution of VEGF. The inhibition of angiogenesis, in particular through the blockade of VEGF, is a promising strategy for treatment of cancer, and the first antiangiogenic agents have been recently approved for use. Hypoxia and the glycolytic phenotype in tumors. A near-universal property of primary and metastatic cancers is upregulation of glycolysis, resulting in increased glucose consumption. Otto Warburg discovered in the 1920s that tumors, unlike normal cells, converted glucose to pyruvate and then to lactate, even in the presence of abundant amounts of oxygen (known as aerobic glycolysis or the “Warburg effect”). Normal cells also convert glucose to pyruvate but in contrast transport it to the mitochondria where it enters the tricarboxylic acid (TCA) cycle and undergoes oxidative phosphorylation. The inhibition of glycolysis by the presence of oxygen, which allows mitochondria to oxidize pyruvate to CO₂ and H₂O is termed the “Pasteur effect”. Since the anaerobic metabolism of glucose is inefficient – it produces only 2 ATP per glucose,

whereas complete oxidation produces 38 ATP per glucose – hypoxic tumor cells in order to obtain enough ATP increase their rate of glycolysis through active HIF that induces the expression of glucose transporters such as Glut-1 and the enzymes involved in glycolysis. HIF also drives the conversion of pyruvate to lactate by the HIF-target gene lactate dehydrogenase A that is upregulated in transformed cells. The lactate is excreted from cells through a H⁺/lactate cotransporter, the monocarboxylate transporter, leading to an acidification of the extracellular space of tumors. Hypoxic tumor cells counteract local acidosis by HIF-dependent induction of the membrane-bound ectoenzyme carbonic anhydrase IX, which converts metabolically generated CO₂ into bicarbonate. The bicarbonate formed enters cells through the action of the ubiquitous bicarbonate/Cl⁻-exchanger family, leading to cellular alkalization and subsequent acidification of the extracellular milieu, to the benefit of the cell. Cell populations with upregulated glycolysis and acid resistance have a powerful growth advantage, which promotes unconstrained proliferation and invasion. Manipulation of tumor acidosis and/or glycolysis is a promising strategy to reduce tumor progression.

Also the chemokine receptor CXCR4, which is involved in organ-specific metastasis, is regulated by the VHL-HIF circuit. In fact, clear cell renal cell carcinoma that manifest mutation of the VHL gene in the majority of cases, revealed an association of strong CXCR4 expression with poor tumor-specific survival. Thus, the propensity of evolving tumor cells to invade and metastasize and thus kill patients, appears to be intimately linked to the disruption of the VHL-HIF circuitry.

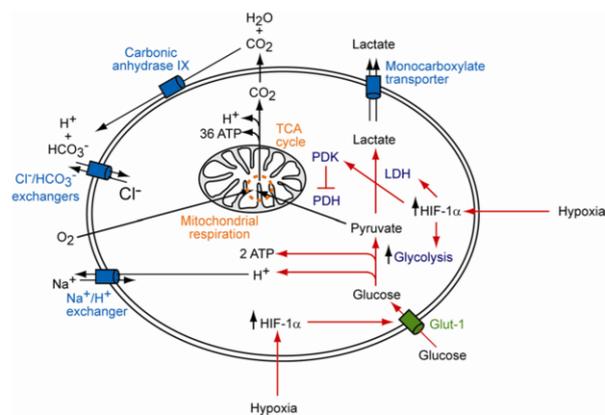


Figure 3. Glucose metabolism in mammalian cells. Glucose is taken up by specific transporters (e.g., Glut-1) and is converted to pyruvate, generating 2 ATP per glucose. In normal cells the pyruvate generated by glycolysis is metabolized through the TCA cycle and oxidative phosphorylation, which is efficient in energy production and generating 36 additional ATP per glucose. In hypoxic tumor cells pyruvate is reduced to lactate because oxidative phosphorylation that requires oxygen is limiting. Since this option is less efficient in producing energy the tumor cells increase their uptake and consumption of glucose through an increase in the production of glucose transporters and enzymes of the glycolytic pathway. The overproduction of lactic and carbonic acids contributes to the acidosis characteristic of tumors. To survive and proliferate, cells must extrude these acids and maintain a balance between the extracellular and intracellular pH through the activity of several pumps, exchangers, and transporters. LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase.