# Investigation of pathogenic mechanisms in osteoporosis and neurodegenerative diseases through modeling of human diseases in mice

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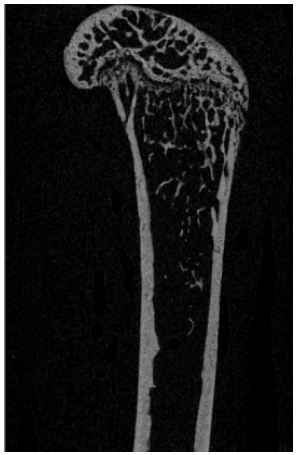
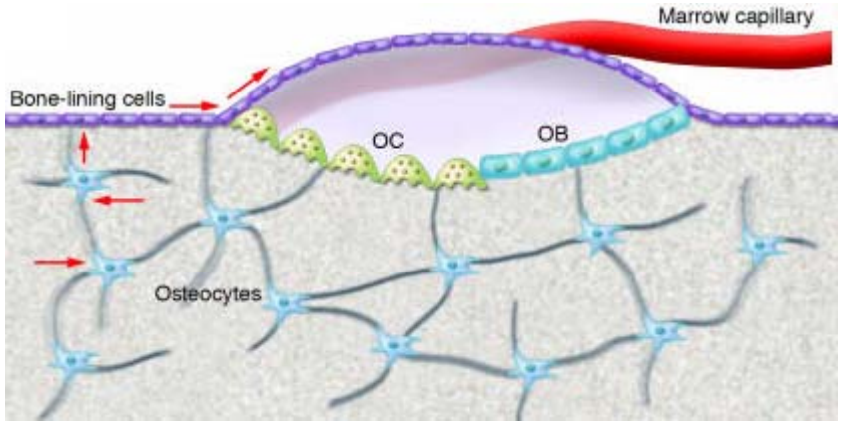
# Skeletal structure and function

- The skeleton is a dynamic organ with mechanical (movement, stability), protective (absorbs traumatic forces), and metabolic (mineral homeostasis) functions.
- Composed of two types of bone:
  - **Trabecular bone**: Network of connecting plates inside the cortical shell (~ 20% of total skeletal mass)
  - **Cortical bone**: Outer dense shell (~ 80% of total skeletal mass)

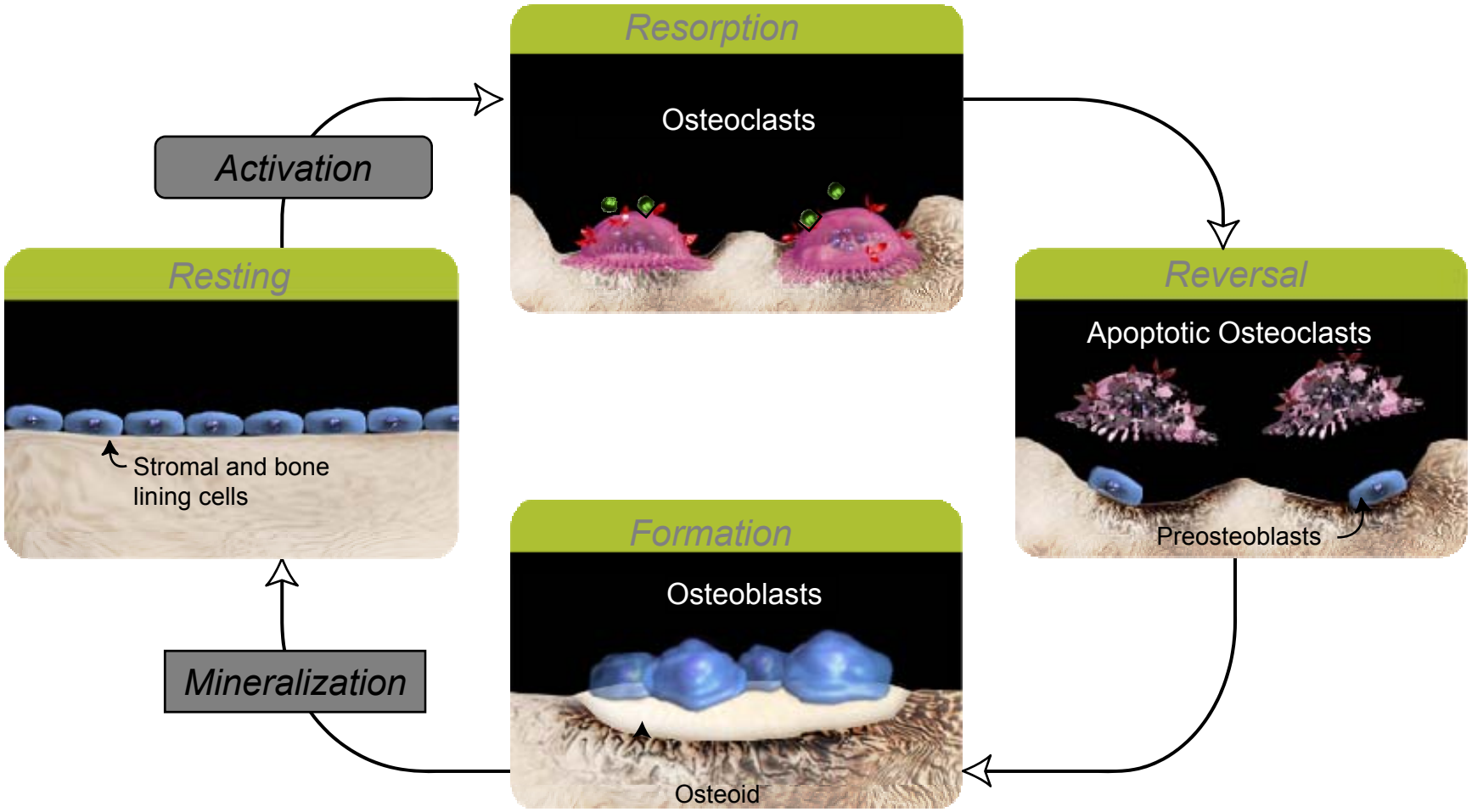


Bone types

- Osteoclasts
- Osteoblasts
- Osteocytes



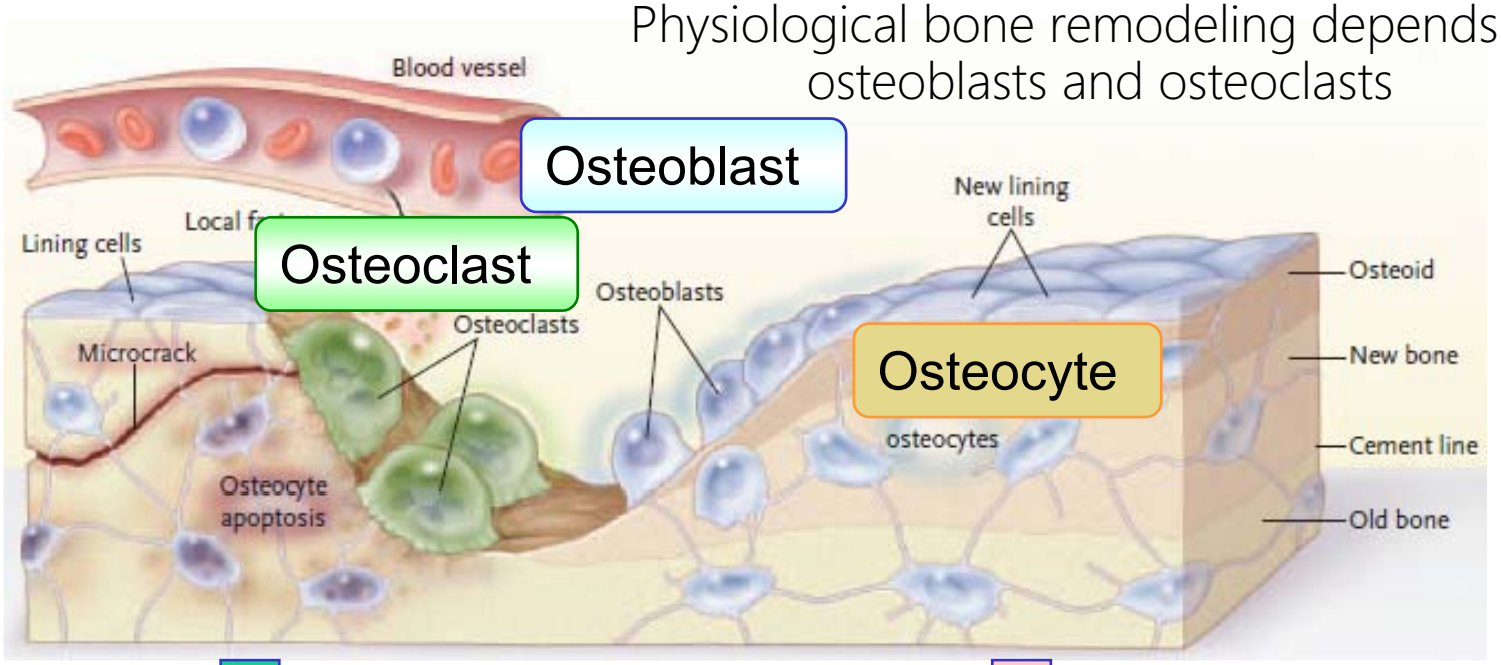
# Bone remodeling steps




When bone turnover is increased, bone loss dominates

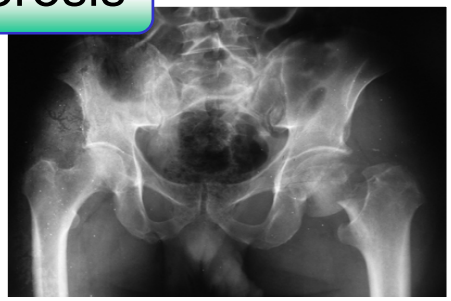
# Bone remodeling & diseases

Physiological bone remodeling depends on osteoblasts and osteoclasts

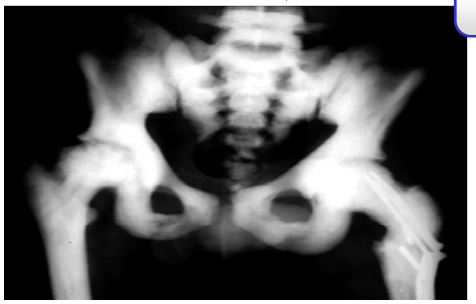



Imbalance

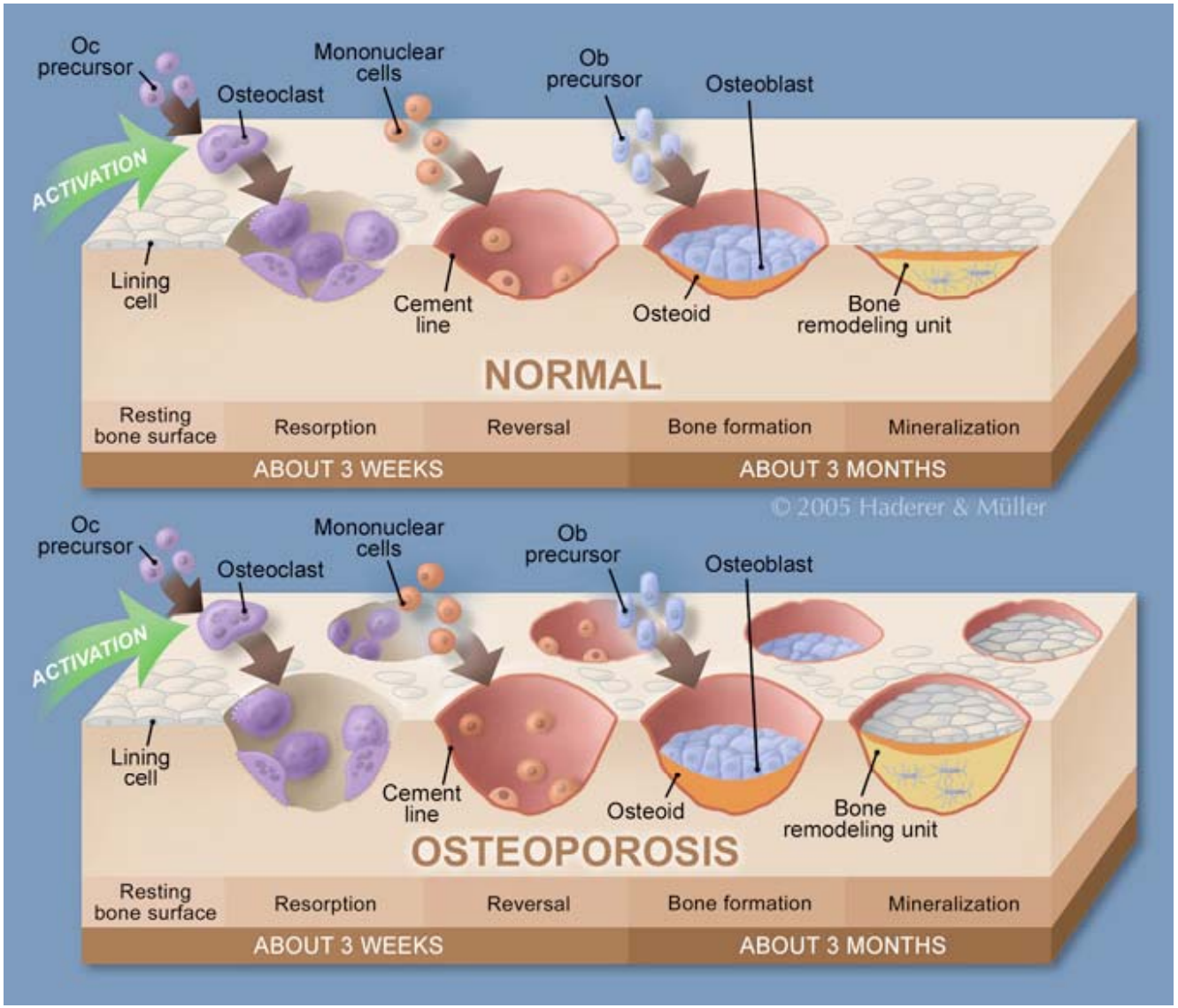
Osteoporosis



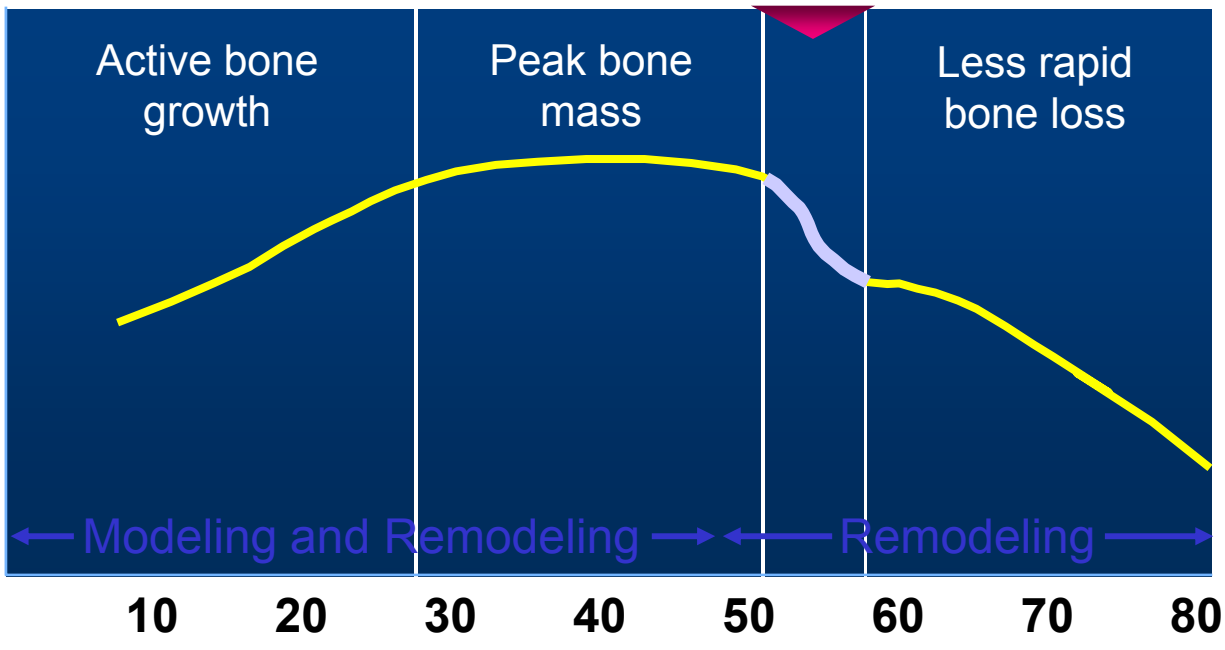
Osteopetrosis



# Bone remodeling and osteoporosis

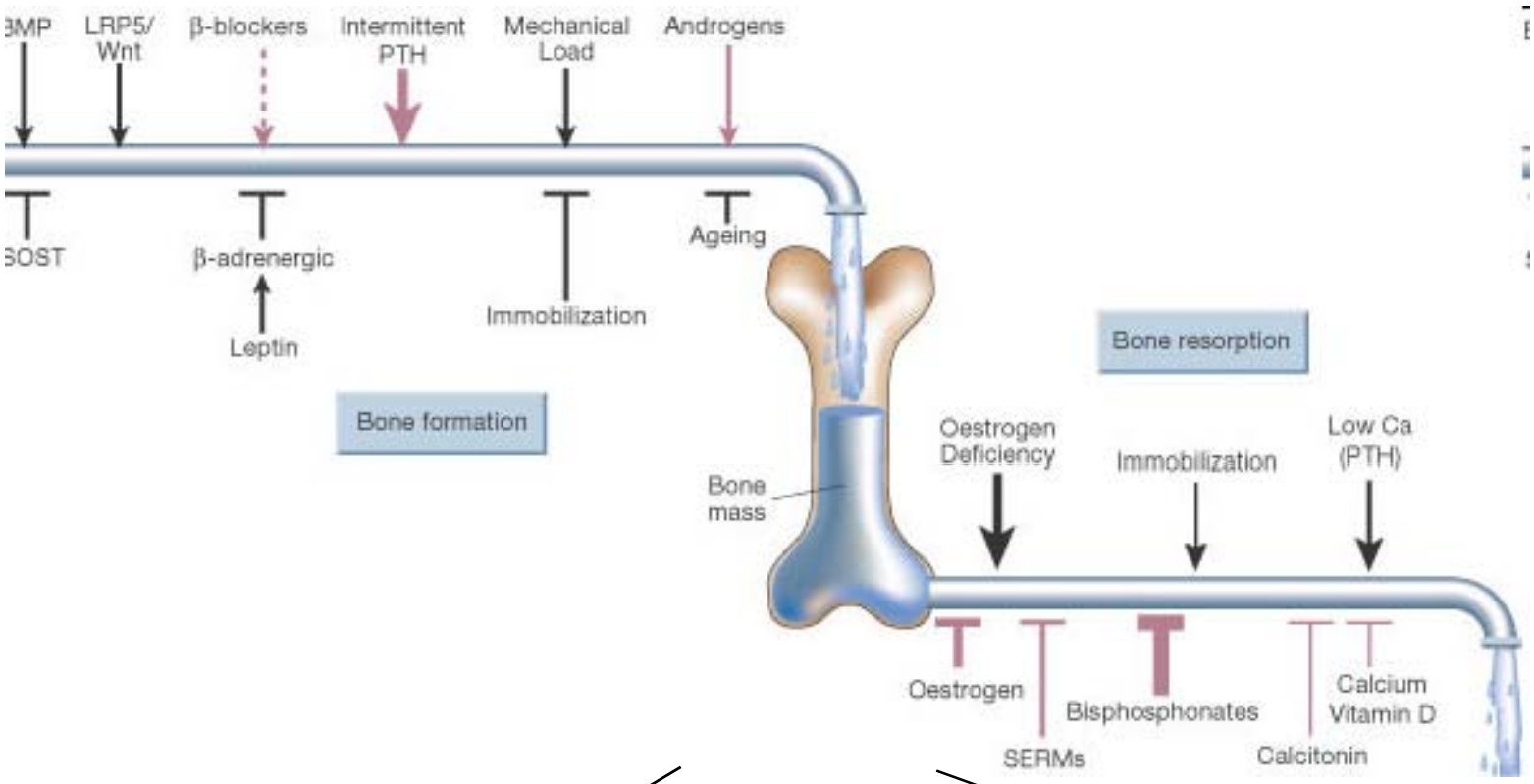


# Bone loss after menopause




*Adapted from: Finkelstein JS. Cecil Textbook of Medicine. 21st ed. 1999:1366-1373.*

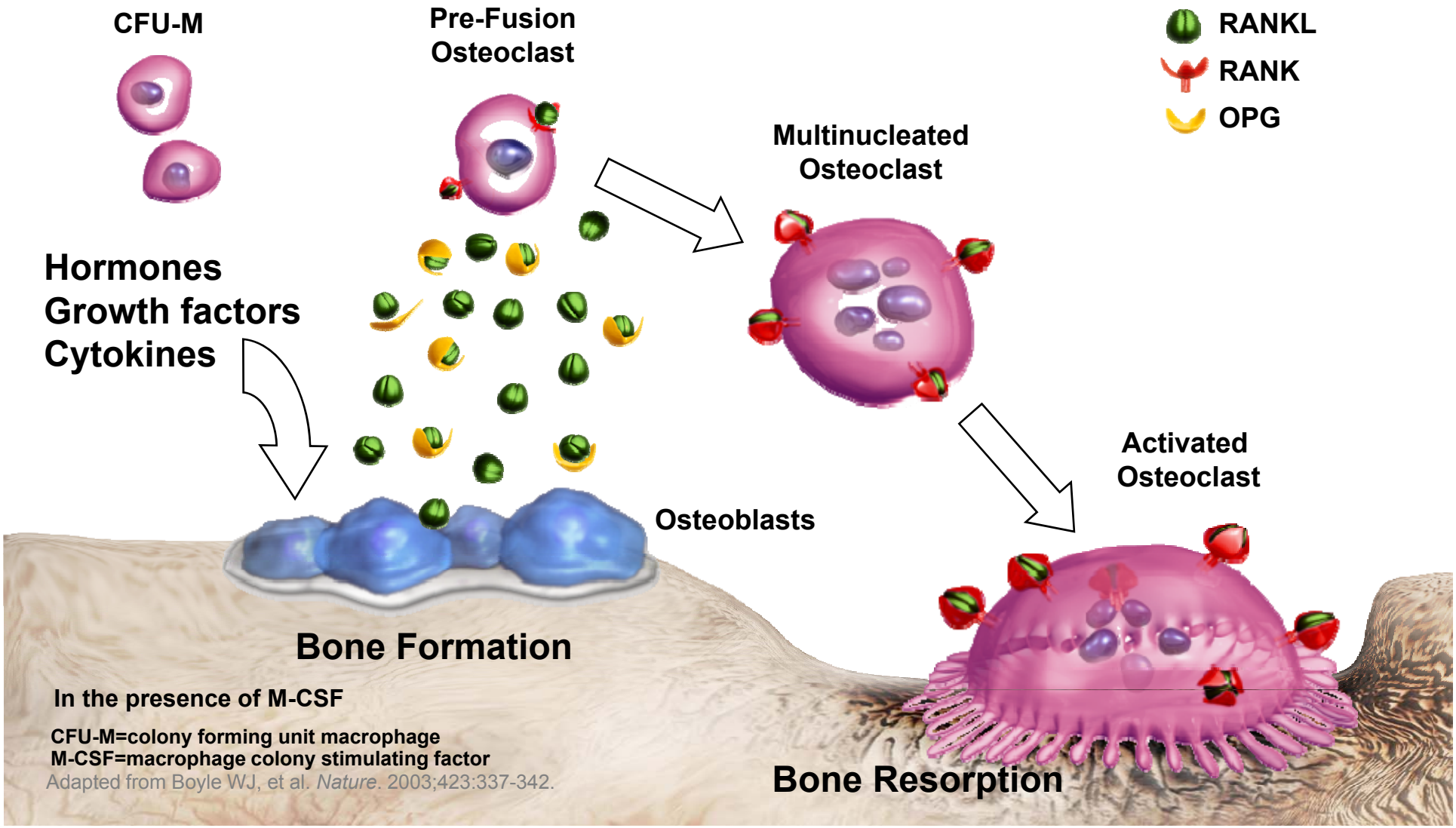
# Treatments in Osteoporosis




PTH

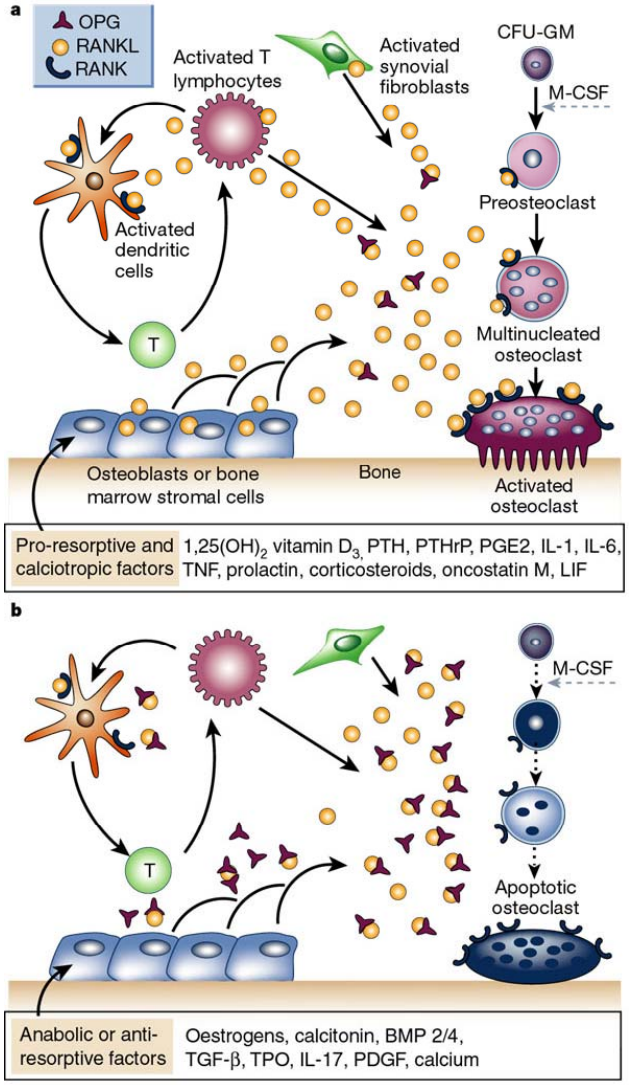
Bisphosphonates
Oestrogen replacement therapy, ERT
SERMS (raloxifene)
Calcitonin
Anti-RANKL

# RANKL is the master regulator of osteoclast formation, function and survival




Adapted from Boyle WJ, et al. *Nature*. 2003;423:337-342.

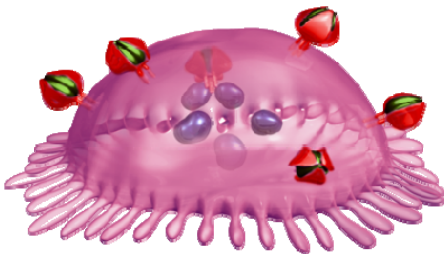
# Diseases associated with RANKL




Ratio RANKL/OPG ⟶ Imbalances in bone remodeling

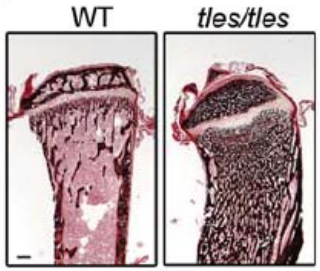
**Increased RANKL:**

Osteoporosis
Inflammatory arthritis
Multiple myeloma
Bone metastasis



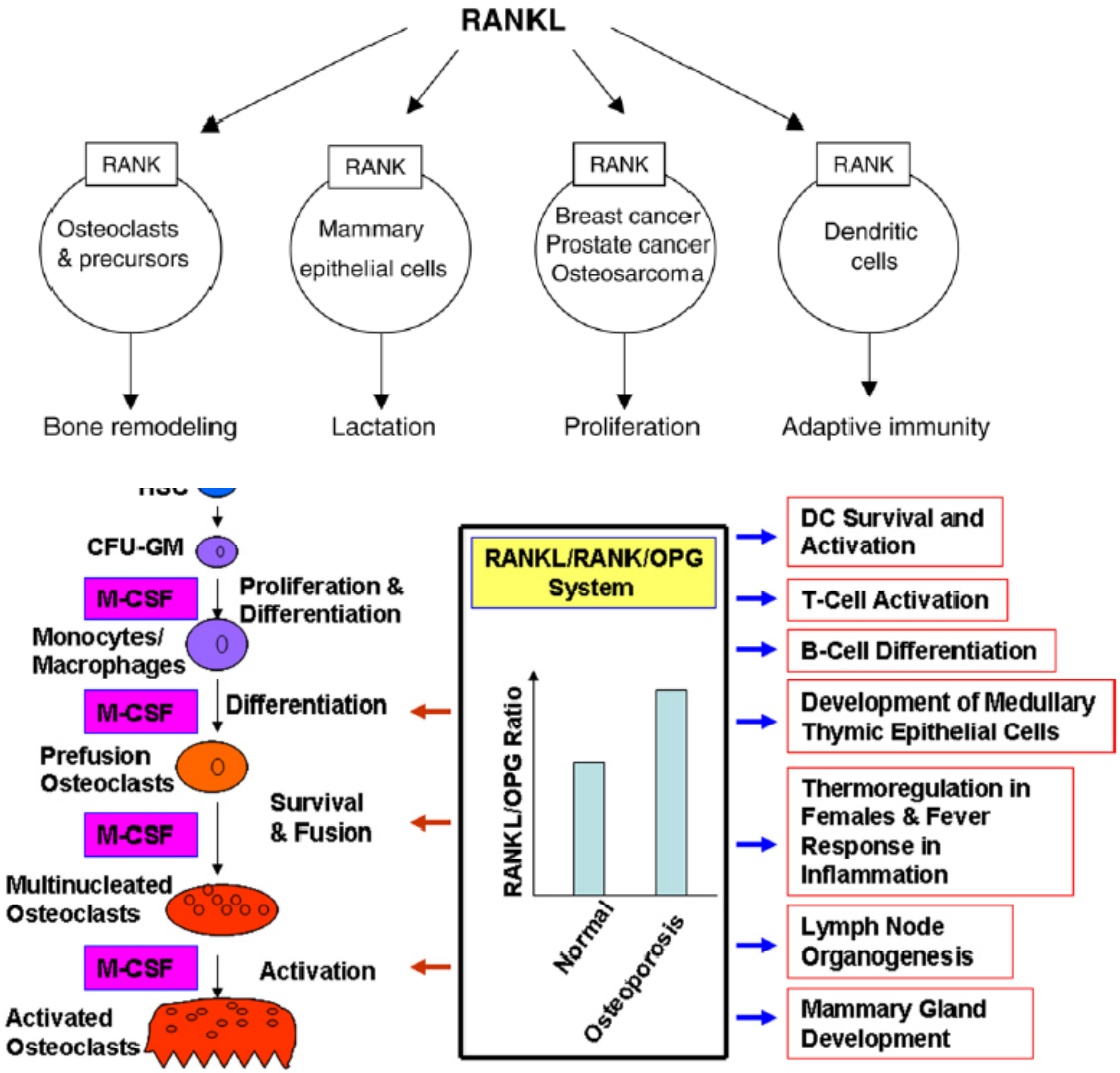
**Decreased, lack or defective RANKL:**

Osteopetrosis
(children with autosomal recessive osteopetrosis, ARO)
Osteopetrosis (RANKL KO or RANK KO mice)



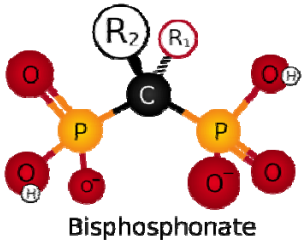

*Douni et al, Hum Mol Genet. 2012*

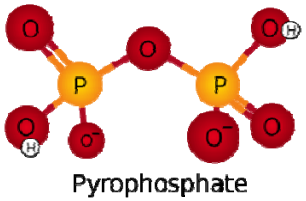
# Pleiotropic effects of RANKL

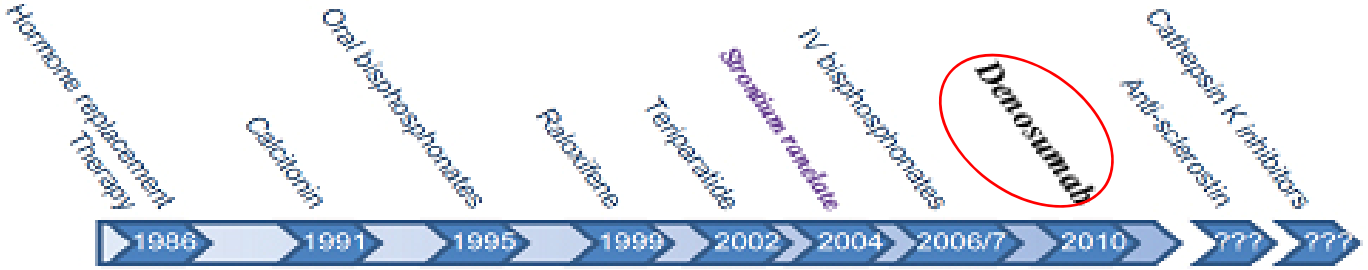


# DENOSUMAB (anti-RANKL): new therapy against osteoporosis

Post-Menopausal Osteoporosis




Specificity!

- FDA approved for post-menopausal osteoporosis and prostate cancer patients.
- Phase 2 and 3 clinical trails in patients with a variety of bone disorders has resulted in significant inhibition of bone resorption without any obvious significant adverse effects.

New inhibitors? New animal models?

# Our RANKL animal models

- Identification of a functional mutation in RANKL
- Screen and identification of novel RANKL inhibitors
- Generation of human RANKL transgenic mice for preclinical studies

# Functional Genetics

**REVERSE GENETICS** (gene → phenotype)

- Expression of exogenous genes (transgenic mice)
- Modification of endogenous genes
  - Gene disruption (knockout mice)
  - Subtle mutations
  - Conditional gene expression (conditional mice)

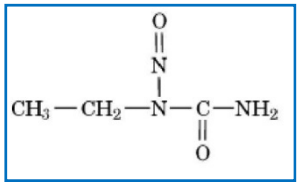
HYPOTHESIS

**FORWARD GENETICS** (phenotype → gene)

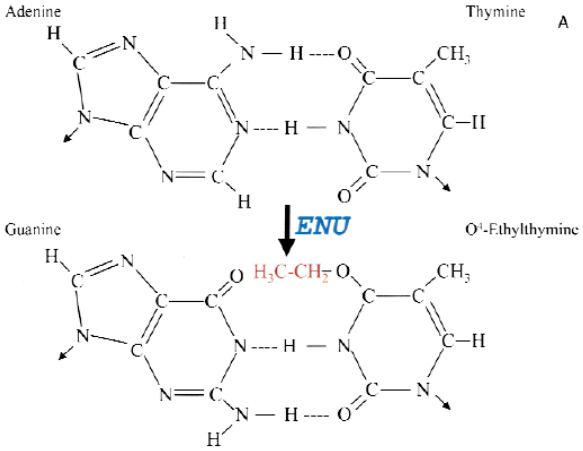
- Spontaneous mutants
- Random mutagenesis

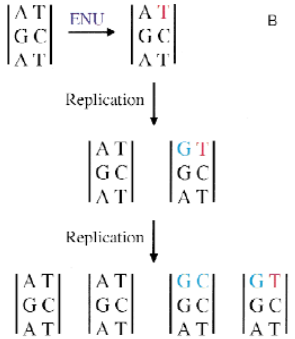
NO HYPOTHESIS

# N-ethyl- N-nitrosourea (ENU)




ENU is the most potent mutagen in mice → random point mutations




Spermatogonia stem cells

A series of mutant alleles at single loci

- 64% missense mutations (substitution of an aminoacid)
- 10% nonsense mutations (introduction of a stop codon)
- 26% splicing errors

- Loss-of-function
- Gain-of-function
- Hypomorphic (partial loss of function)
- Antimorphic (antagonizing WT)
- Hypermorphic (exaggerated function)

Various phenotypes (Rare diseases)
- Skeletal system
- Neuromuscular system

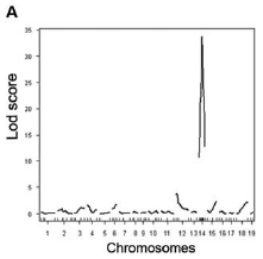
Identification of novel pathogenic genes

# Identification of a missense mutation in RANKL causing osteopetrosis (missense mutation)

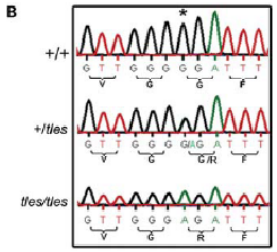
## ENU-derived toothless mice

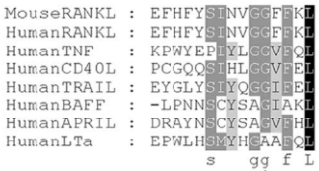
- Failure of tooth eruption
- Growth retardation
- Thymic hypoplasia
- Absence of lymph nodes

## Genetic mapping

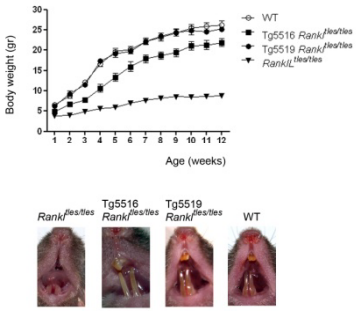



## Identification of RANKL mutation

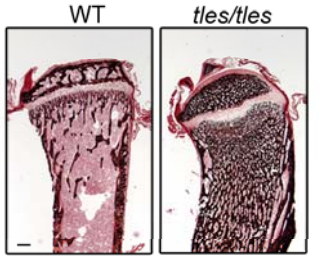



## Genetic confirmation

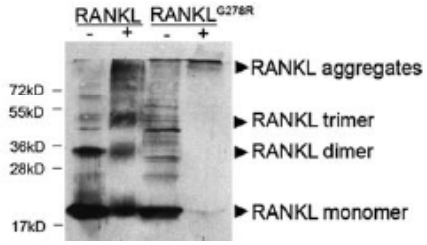


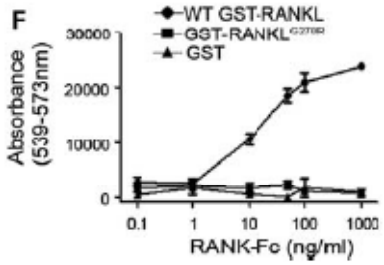

## Histological analysis





## Biochemical analysis



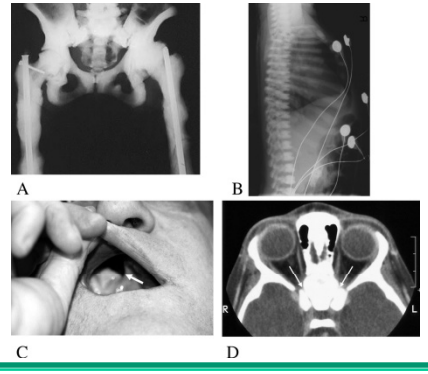




## Hypothesis

*Douni et al., Hum Mol Genetics, 2012*

Osteoclast-poor human osteopetrosis due to mutations in the gene encoding RANKL


Cristina Sobacchi[1,2], Annalisa Frattini[1], Matteo M Guerrini[1,2], Mario Abinun[3], Alessandra Pangrazio[1], Lucia Susani[1], Robbert Bredius[4], Grazia Mancini[5], Andrew Cant[3], Nick Bishop[6], Peter Grabowski[6], Andrea Del Fattore[7], Chiara Messina[8], Gabriella Errigo[8], Fraser P Coxon[9], Debbie I Scott[9], Anna Teti[7], Michael J Rogers[9], Paolo Vezzoni[1], Anna Villa[1,10] & Miep H Helfrich[9]

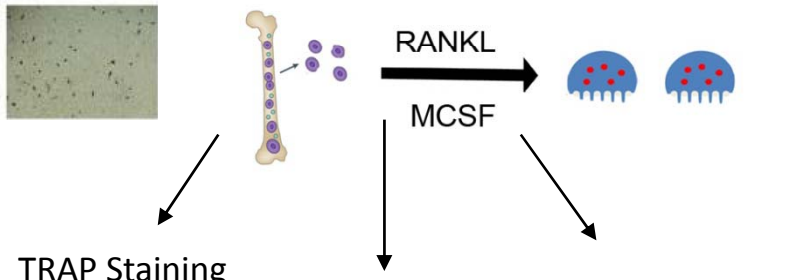
# Pipeline for the evaluation of novel RANKL inhibitors

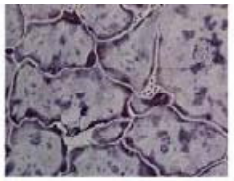
## Biochemical assays

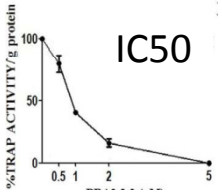
Elisa

Crosslinking /western blot

## Osteoclastogenesis

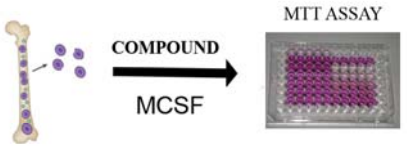


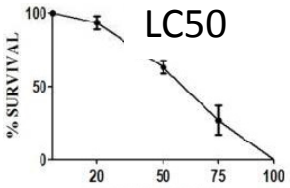






IC50

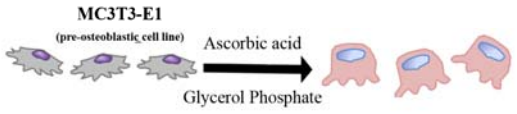
## Cytotoxicity




LC50

## Osteoblastogenesis




Alizarin Red Staining

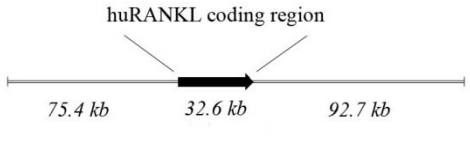
ALP Activity

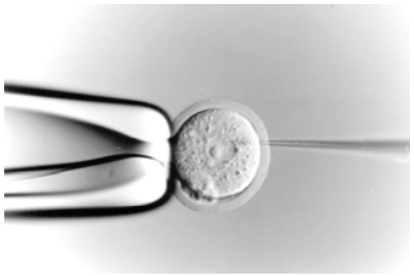
## Treatment of osteoporotic mouse models

WT - Model - Model +

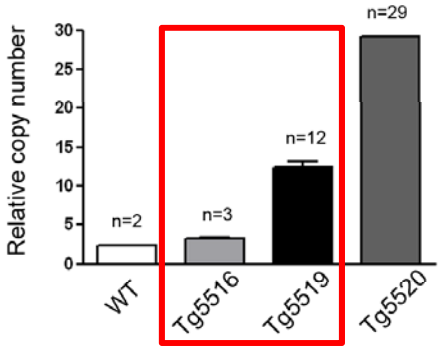
# Generation of human RANKL transgenic mice

200kb genomic fragment containing:
human RANKL



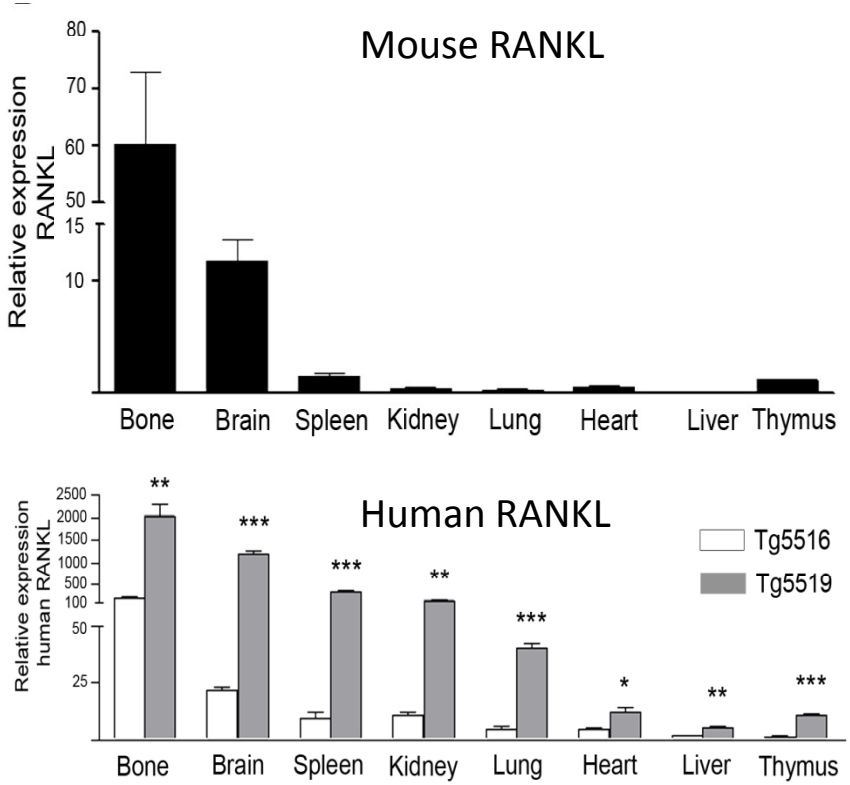



Transgene copy number




*Rinotas et.al., J Bone Miner Res 2014*

Physiological expression pattern



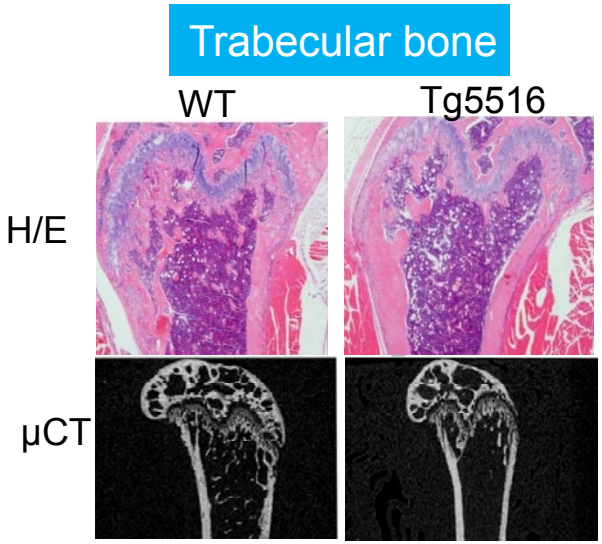

**Model I: Low copy and low expressing Tg5516 line**

**Model II: High copy and high expressing Tg5519 line**

# Generation of human RANKL transgenic mice: Novel genetic models of osteoporosis

**Tg5516 mice**

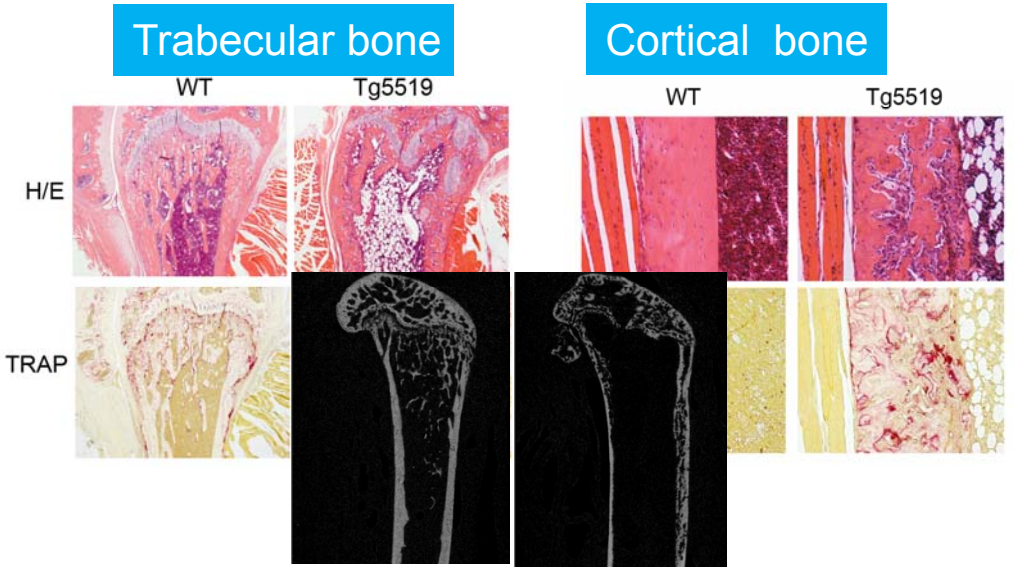
- Low expression of human RANKL
- Trabecular bone loss
- Increased bone fragility




Tg5516 mice represent a model of mild osteoporosis

**Tg5519 mice**

- High expression of human RANKL
- Trabecular bone loss
- Cortical porosity
- Increased bone fragility

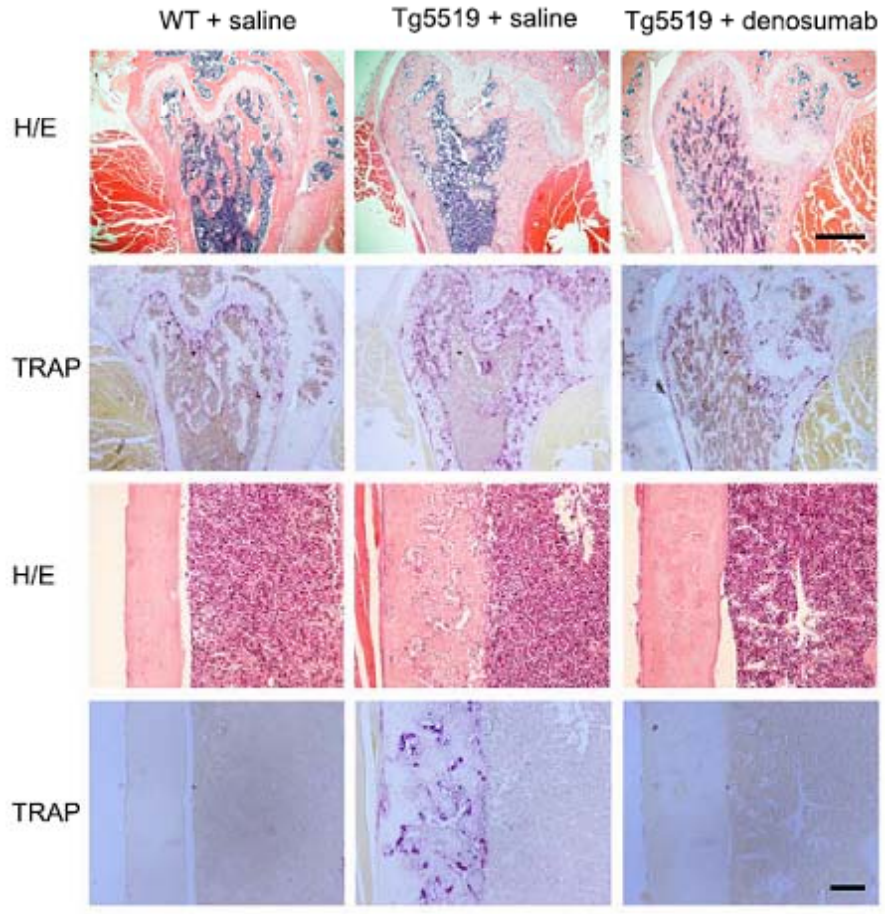


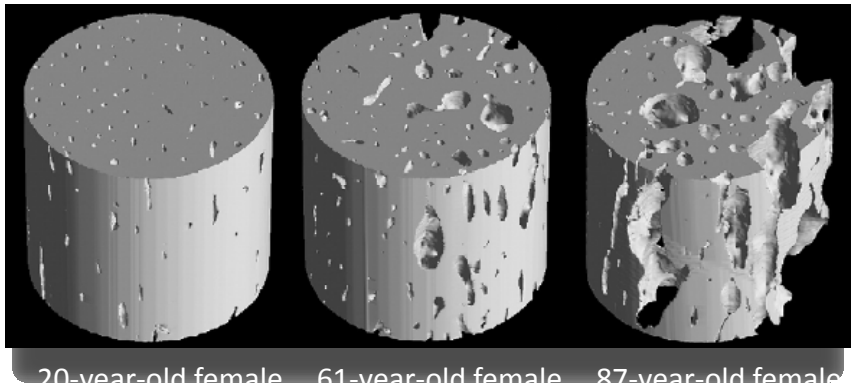

Tg5519 mice represent a model of severe osteoporosis

*Rinotas et.al., J Bone Miner Res 2014*

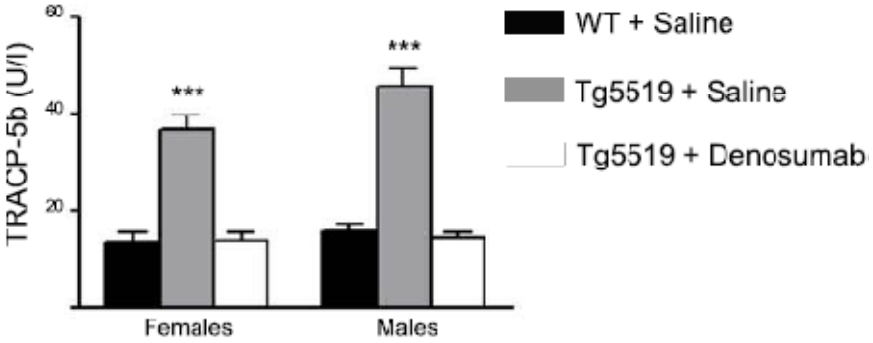
# TgRANKL mice as models for preclinical trials

- Subcutaneously administration of DENOSUMAB twice per week (wk4-wk10)
- Dosage:10mg/kg



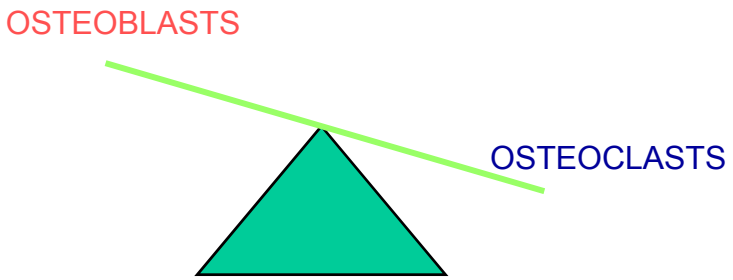




*Cooper et al. Bone (2007)*

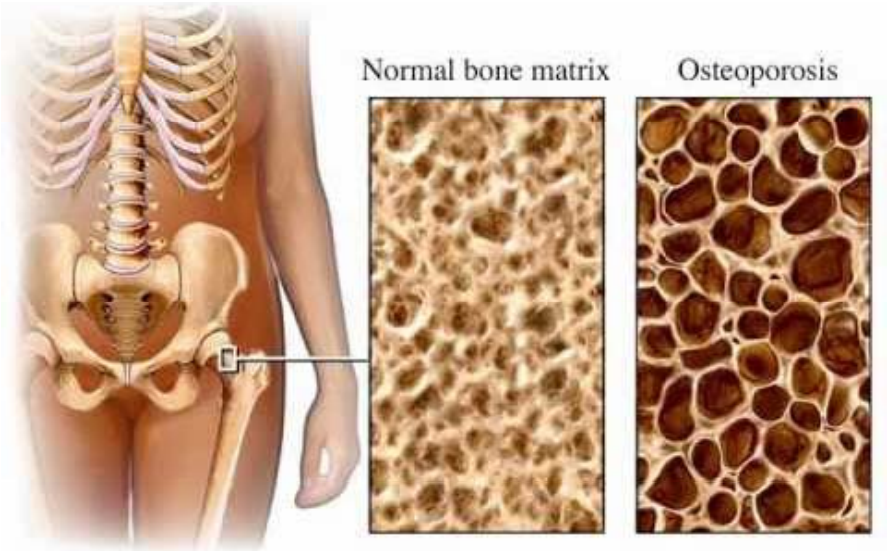


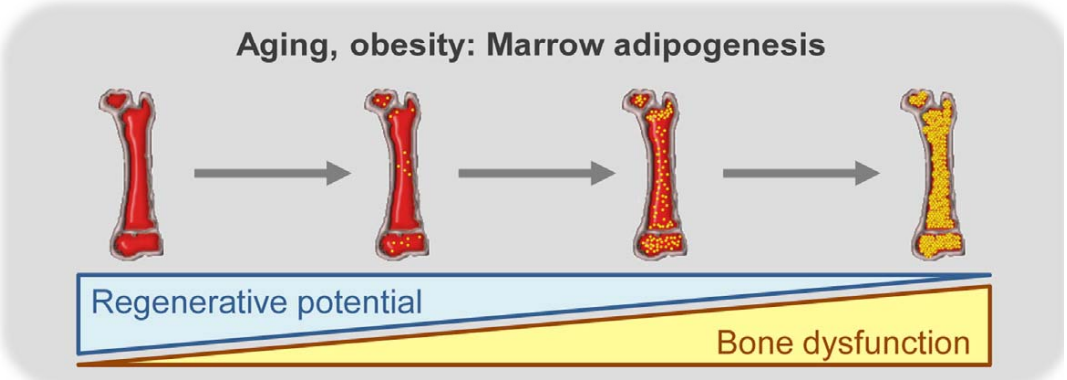

*Rinotas et.al., J Bone Miner Res, 2014*

# Aging, Osteoporosis and Bone Marrow Adiposity




- Low Bone Mineral Density
- Low bone mass (bone loss)
- Increased Osteoclastogenesis
- Cortical Porosity
- Increased fracture incidents
- Estrogen Deficiency
- Increased Bone Marrow Adiposity in women with Osteoporosis



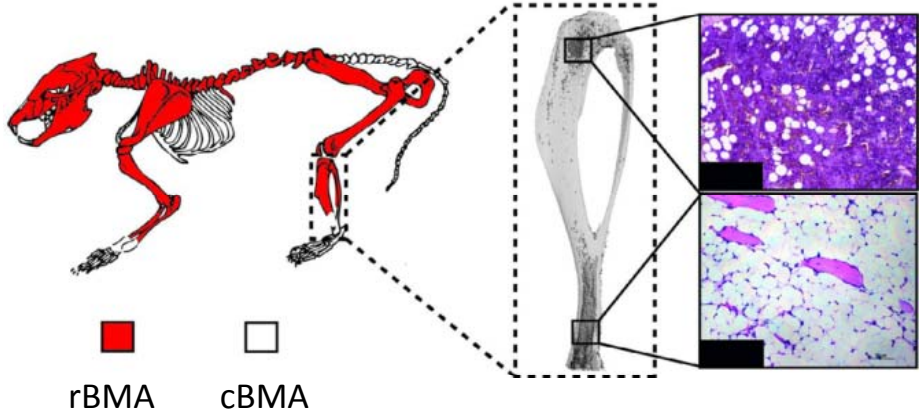



# Adipocyte types

- **White Adipocytes**→specialized to store energy as triacylglycerol
- **Brown Adipocytes**→store less lipids and allows energy to be converted at heat-located at intercapular region
- **Bone Marrow Adipocytes (yellow fat)**→incomplete known-no specific marker distinguishing from other types of adipocytes

**Bone Marrow Adipocytes**

Constitutive BMA  Regulatory BMA



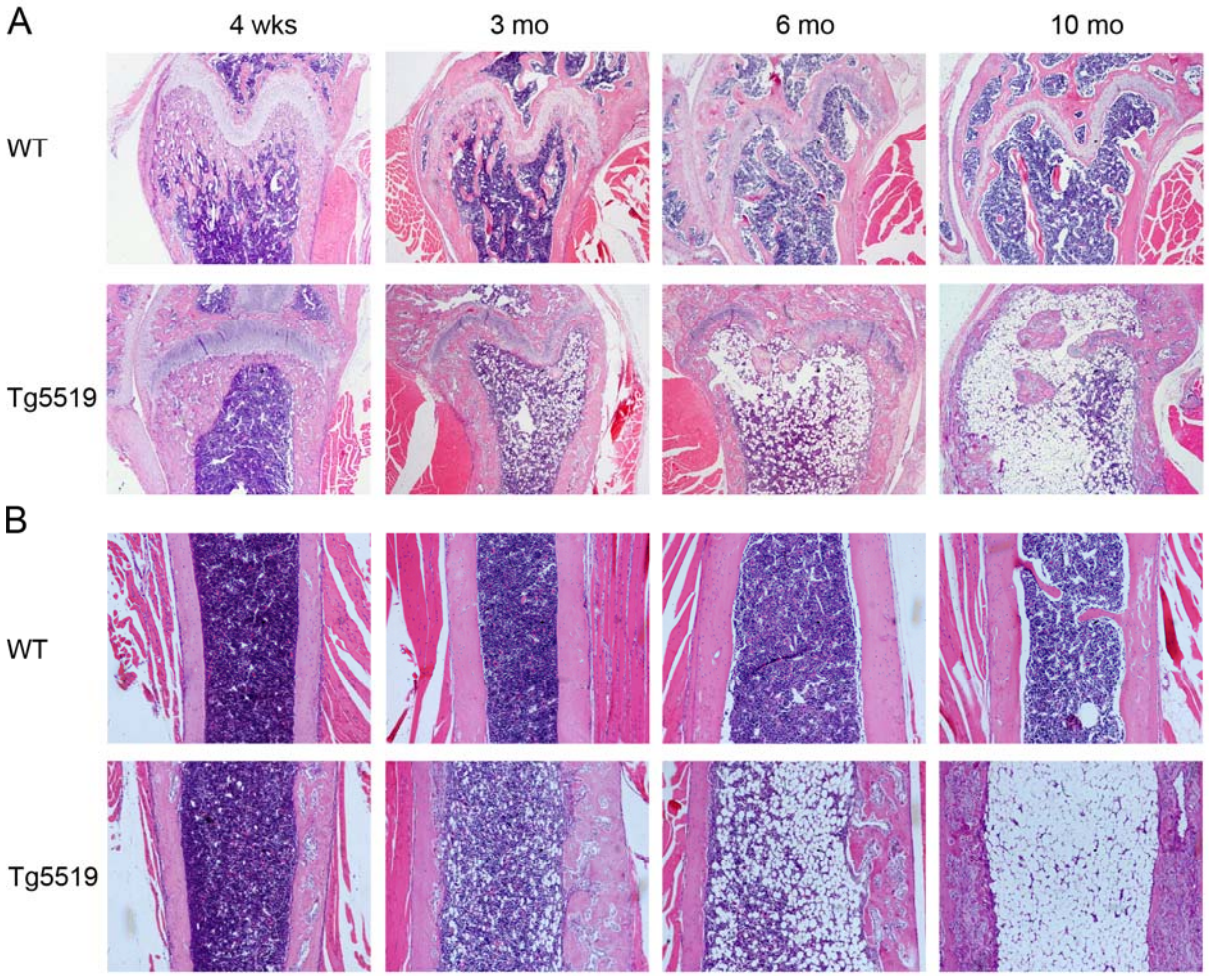

*Scheller et.al., Trends Endocrinol Metab 2016*

Marrow Adipocytes express adipokines such as adiponectin, leptin, IL-6, TNF, RANKL

# Tg5519 develop BMA

Trabecular

Cortical




*Rinotas et.al., J Bone Miner Res 2014*

# Mitochondrial dysfunction in neurodegenerative diseases

## STRUCTURE
- Outer membrane
- Intermembrane space
- Inner membrane
- Inner boundary membrane
- Cristae
- Cristae junction
- Matrix
- DNA

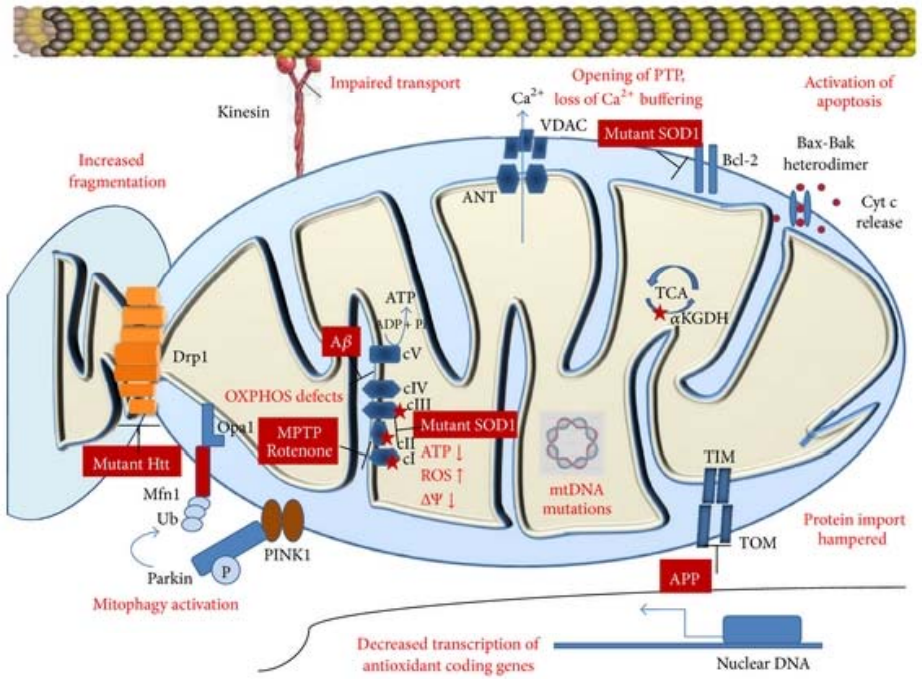
## FUNCTION
- Oxidative phosphorylation
- ROS production
- Metabolism of lipids, aminoacids
- Ca2+ buffering
- Apoptosis

## DISEASES
- Neurodegenerative
- Rare diseases
- Optic atrophies
- Ataxias
- Huntington's disease
- Alzheimer's disease
- Parkinson's disease

## DYNAMICS (repair)
- Fussion

(Opa1, MFN1/2)
- Fission

Drp1




## Pathogenic mechanisms
- Impaired dynamics
- Impaired transport
- Oxidative stress
- Apoptosis

↓

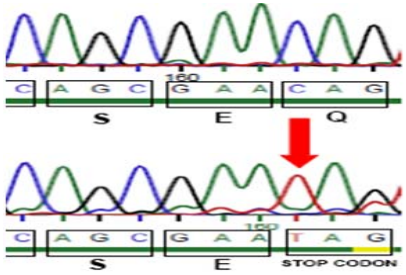
Neuronal cell death

# Identification of a novel gene causing ataxia/epilepsy : SLC25A46

**ENU-derived neurological phenotype**

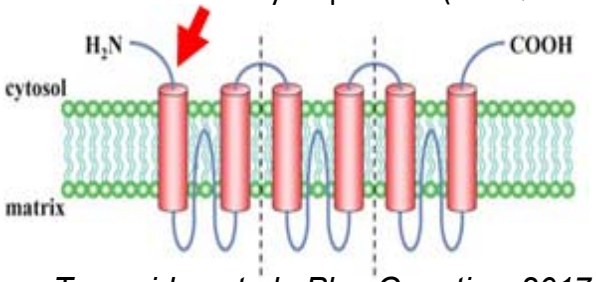
- Ataxia
- Epileptic fits
- Reduced muscle strength
- Thymus/spleen hypoplasia
- Growth retardation
- Premature death

**Genetic mapping**




**Identification of causal mutation in *Slc25a46***

Early stop codon (418 → 95 aa)



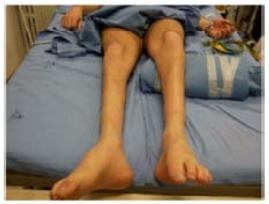

*Terzenidou et al., Plos Genetics, 2017*

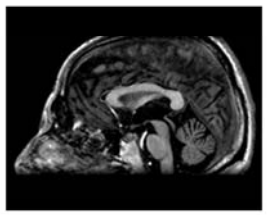
**Mutations in patients**

- Dominant Optic Atrophy and Charcot-Marie-Tooth type 2, CMT2 (Abrams et al., 2015)
- Optic atrophy spectrum disorder (Nguyen et al., 2016)
- Leigh syndrome (Janer et al., 2016)
- Progressive Myoclonic Ataxia with optic atrophy and Neuropathy (Charlesworth et al., 2016)
- Lethal congenital pontocerebellar hypoplasia (Wan et al., 2016)

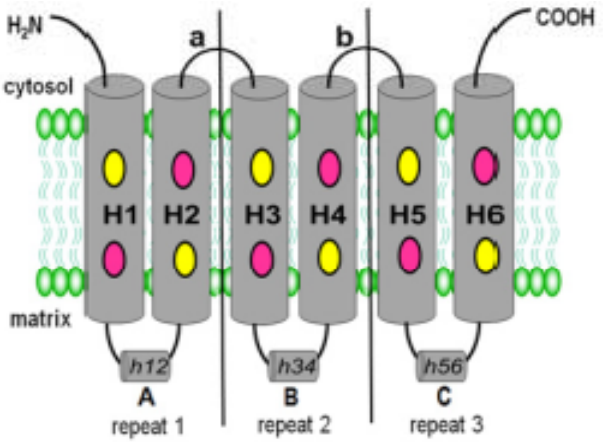
**Symptoms**

- Optic atrophy
- Ataxia / Epileptic seizures
- Cerebellum atrophy
- Neuropathy / Muscle atrophy
- Early lethality

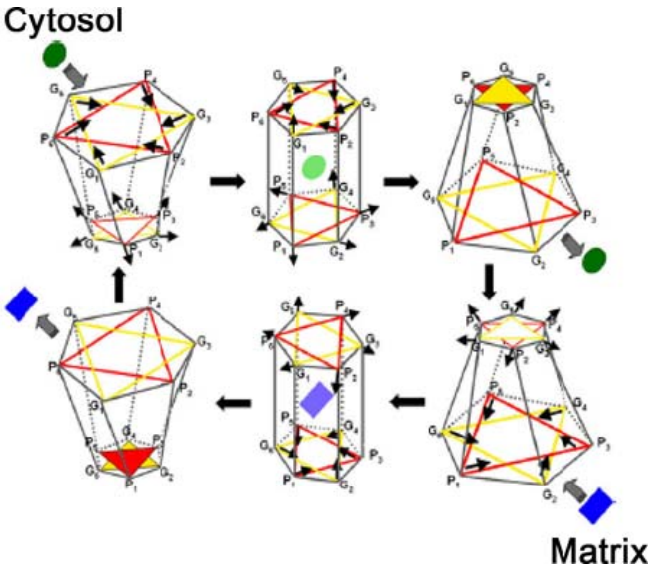





# Family of mitochondrial transporters SLC25A46




*Palmieri, 2013*



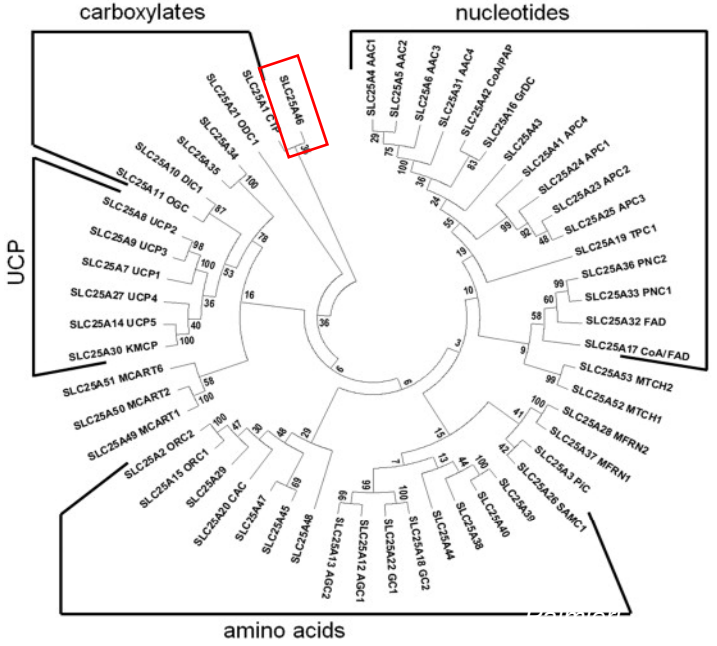

*Palmieri, 2010*

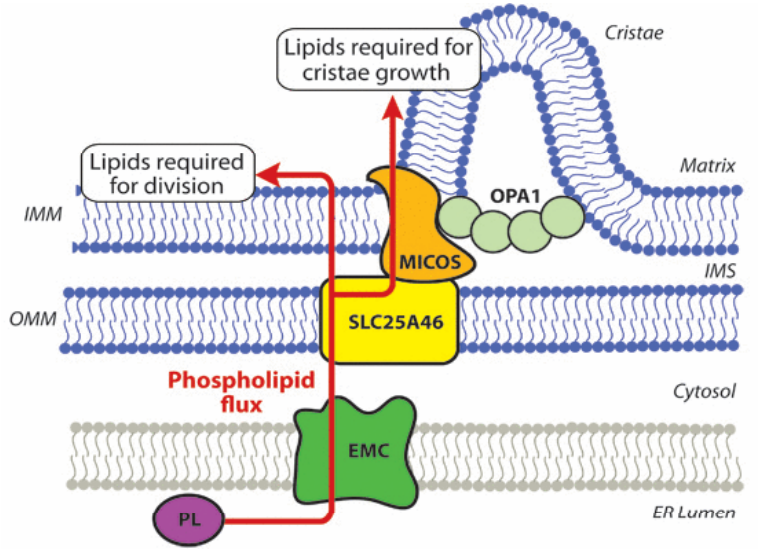
| Ασθένεια | Γονίδιο | Μεταφορέας | Υπόστρωμα |
|---|---|---|---|
| Ανεπάρκεια PiC | SLC25A3 | PiC | Φωσφορικό |
| Ανεπάρκεια AAC1 | SLC25A4 | AAC1 | ADP/ATP |
| Αυτοσωμική επικρατής προοδευτική εξωτερική οφθαλμοπληγία | SLC25A4 | AAC1 | ADP/ATP |
| Ανεπάρκεια AGC1 | SLC25A12 | AGC1 | Ασπαρτικό/ Γλουταμινικό |
| Ανεπάρκεια AGC2 | SLC25A13 | AGC2 | Ασπαρτικό/ Γλουταμινικό |
| Σύνδρομο HHH | SLC25A15 | ORC1 | Ορνιθίνη/ Κιτρουλίνη |
| Συγγενής μικροκεφαλία Amish | SLC25A19 | TPC | Πυροφωσφορική θειαμίνη |
| Νευροπάθεια με αμφίπλευρη νέκρωση του ραβδωτού σώματος | SLC25A19 | TPC | Πυροφωσφορική θειαμίνη |
| Ανεπάρκεια CAC | SLC25A20 | CAC | Καρνιτίνη/ Ακυλοκαρνιτίνη |
| Πρόωρη επιληπτική εγκεφαλοπάθεια | SLC25A22 | GC1 | Γλουταμινικό |
| Συγγενής σιδηροβλαστική αναιμία | SLC25A38 | - | ? |
| DOA, CMT2, σύνδρομο Leigh, προοδευτική μυοκλονική αταξία, γεφυροπαρεγκεφαλιδική υποπλασία | SLC25A46 | - | ? |

# SLC25A46 function?

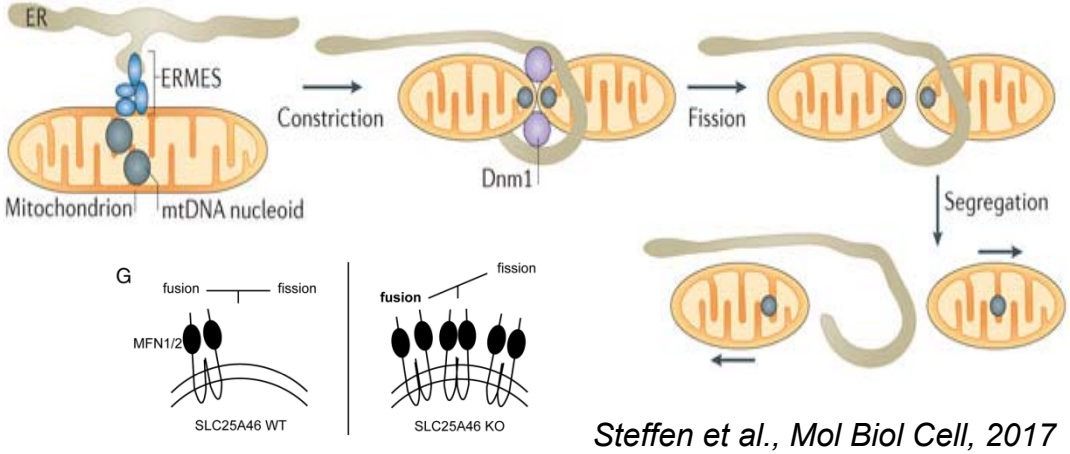
- Topology: outer mitochondrial membrane
- Interacts with fusion proteins OPA1, MFN1/2
- Interacts with members of the MICOS complex

  Mitofilin και CHCHD3
- Interacts with the EMC complex involved in lipid transport between ER and mitochondria



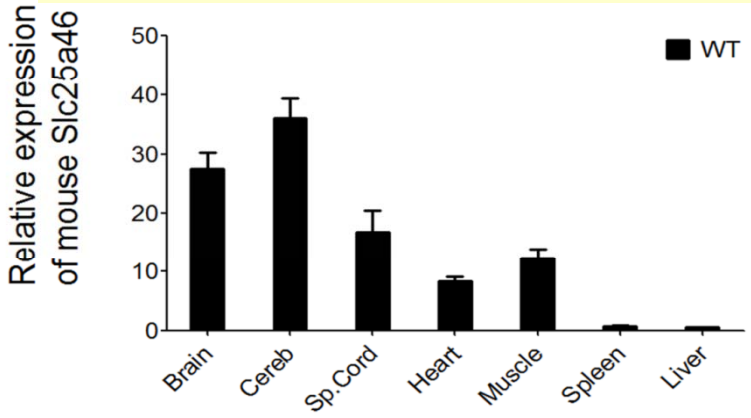

Janer et al., EMBO Molecular Medicine, 2017



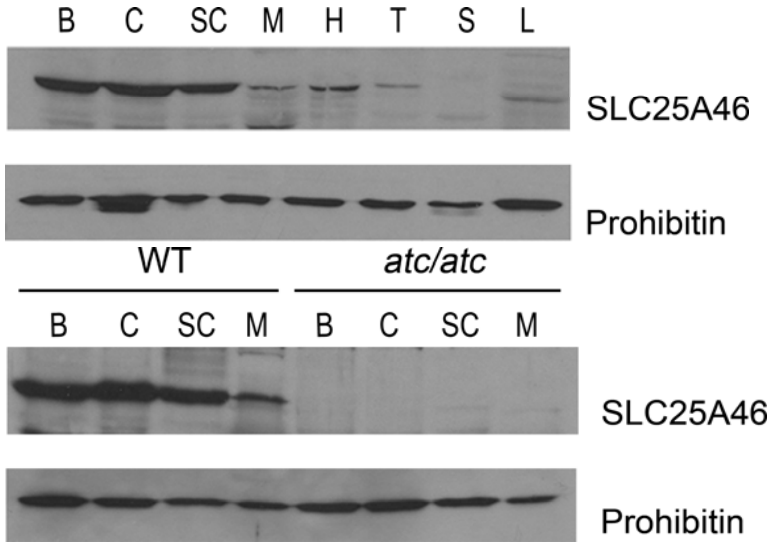

Steffen et al., Mol Biol Cell, 2017

# Expression profile of *Slc25a46*

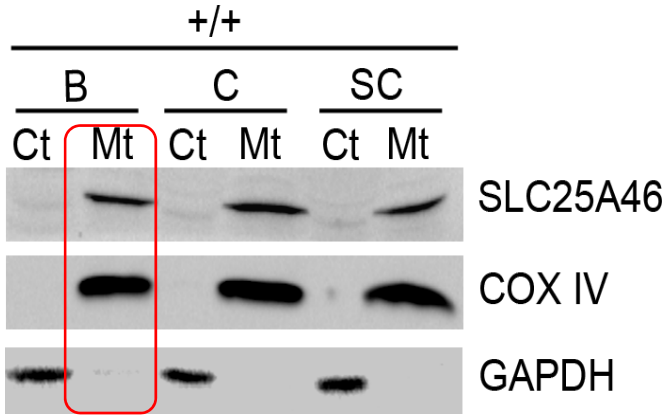
- mRNA (qPCR)

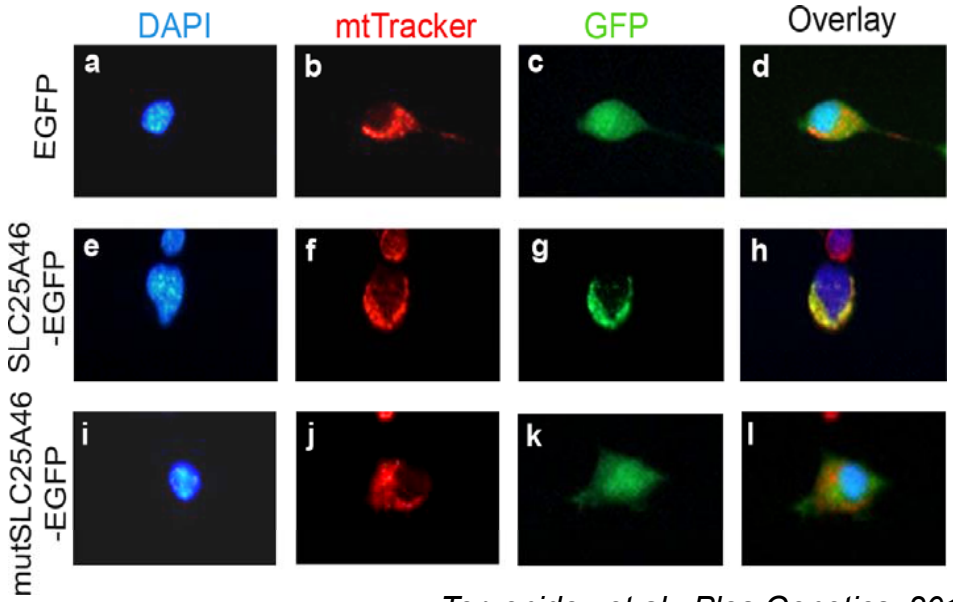



- Protein (Western)



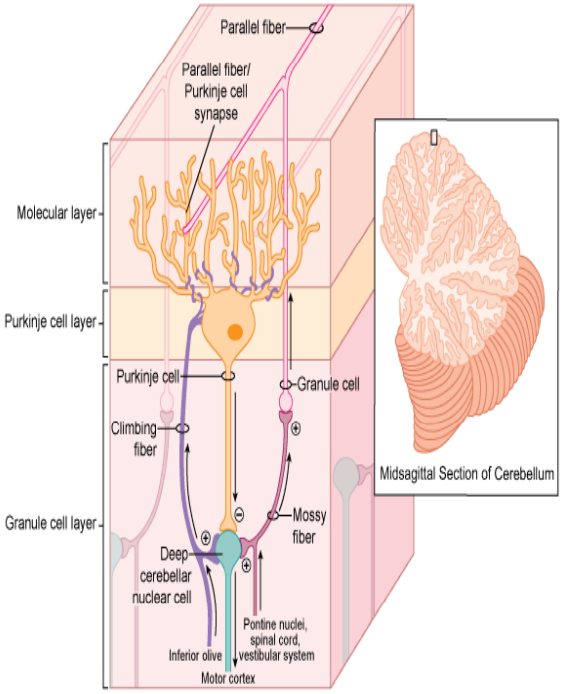

- Mitochondrial Localization

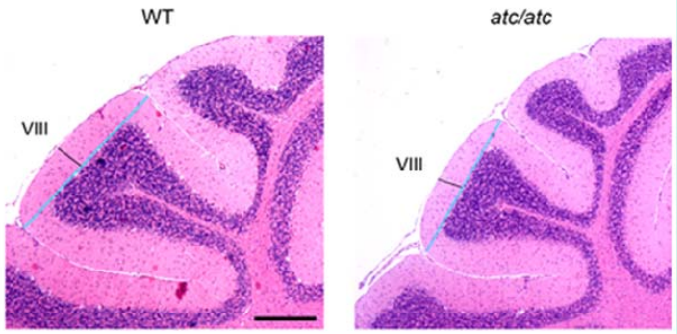


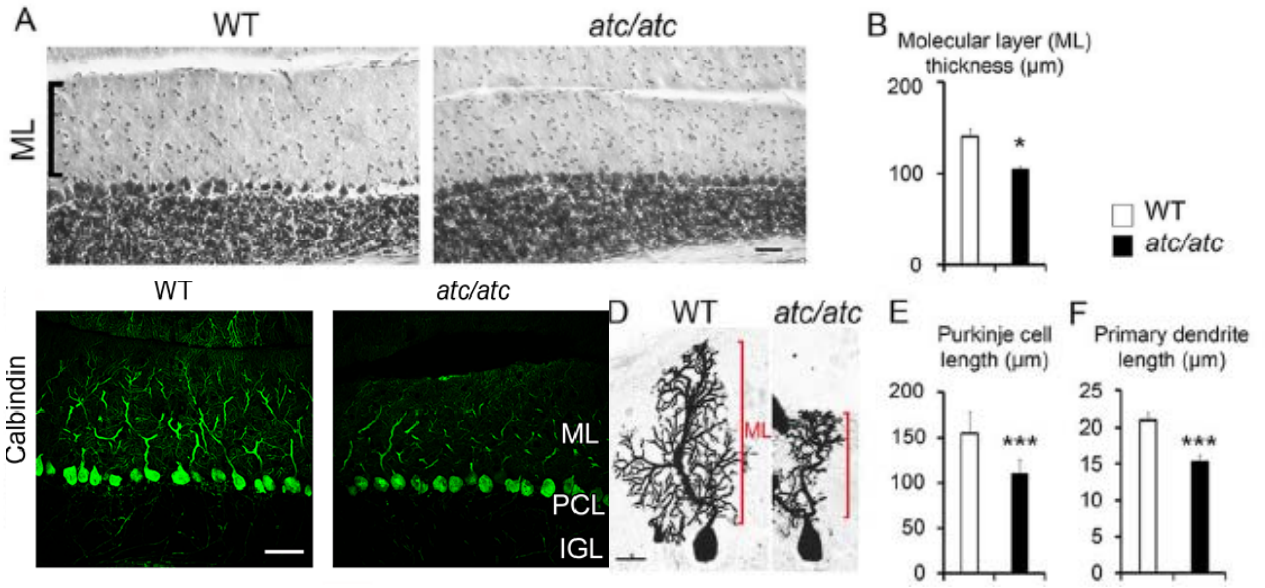

*Terzenidou et al., Plos Genetics, 2017*

# Histopathological analysis in cerebellum



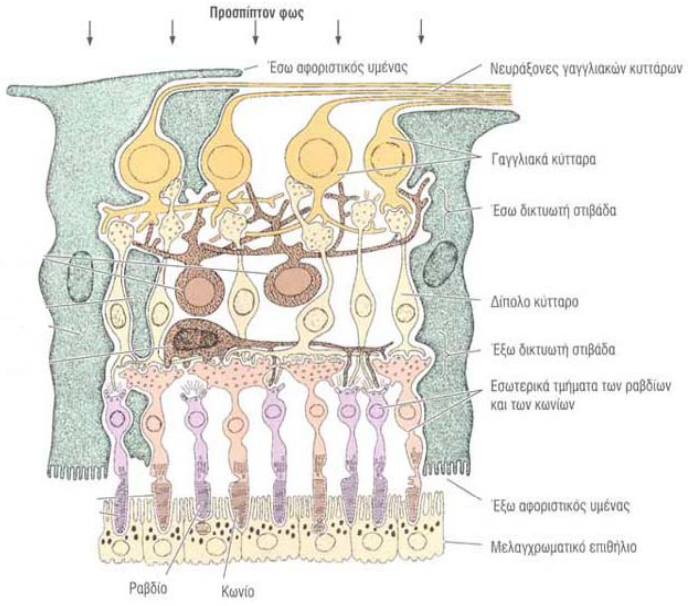




- Cerebellar hypoplasia
- Reduced thickness of the Molecular Layer
- Underdeveloped PC dendritic arborization in Molecular Layer
- Reduced glutamatergic synapses

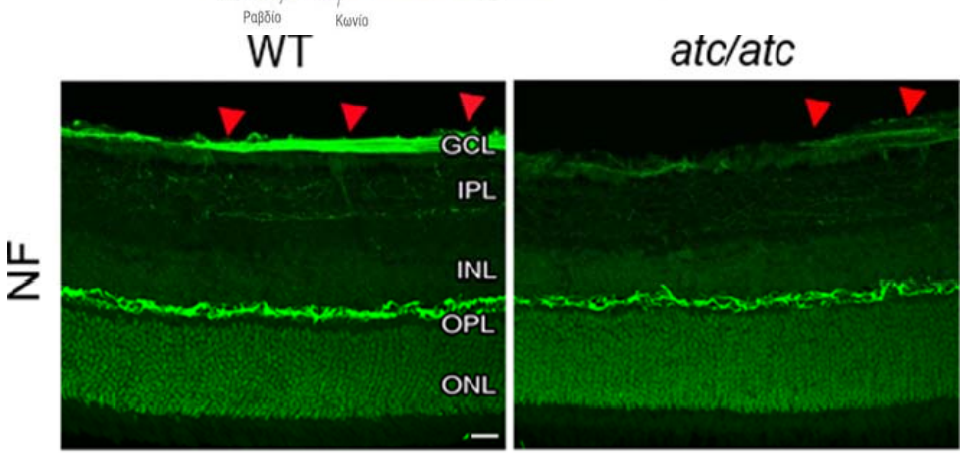


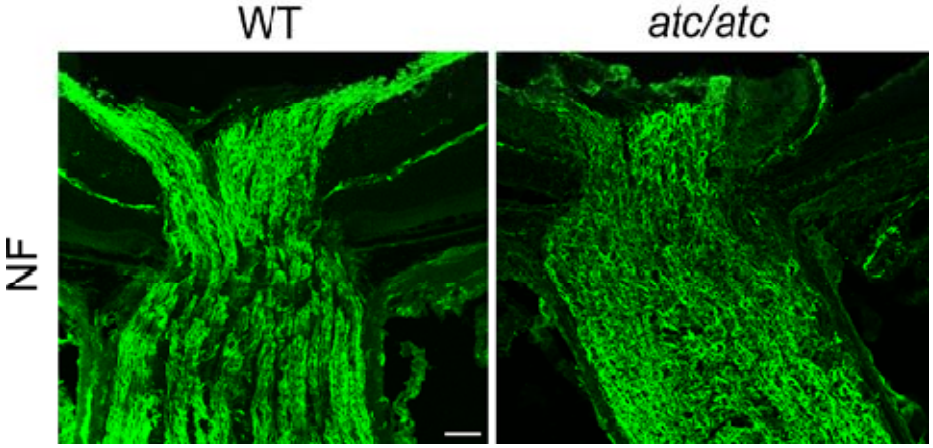

*Terzenidou et al., Plos Genetics, 2017*

# Cellular alterations in retina and optic nerve




- Reduced expression of Neurofilament (NF) in RGC axons.
- Disorganized optic nerve head of mutant mice
- Underdeveloped Retinal Ganglion cell (RGCs) dendrites
- Reduced RGC synapses



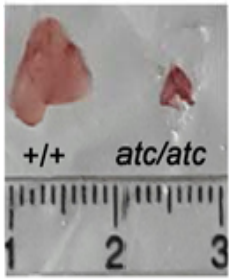



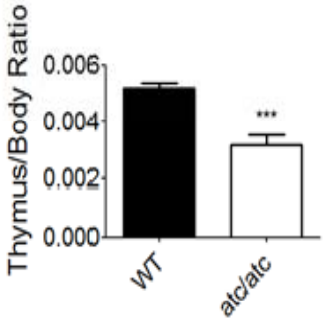

*Terzenidou et al., Plos Genetics, 2017*

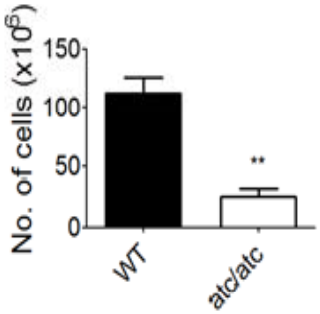
# Lymphoid abnormalities

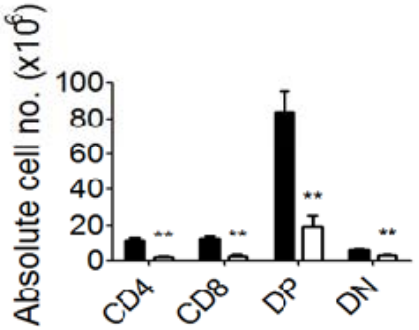
- Mutant mice develop severe hypoplasia in thymus and spleen

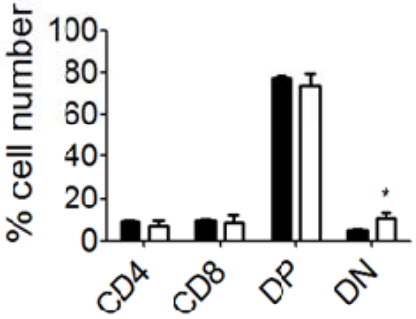
n=4-5

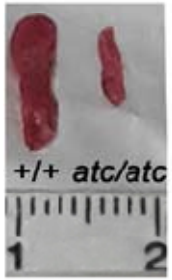


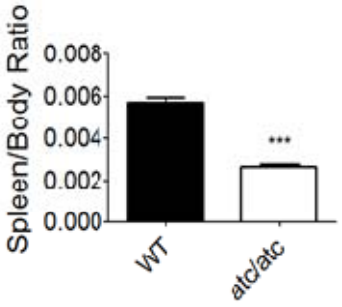



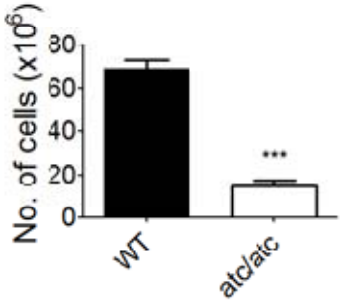



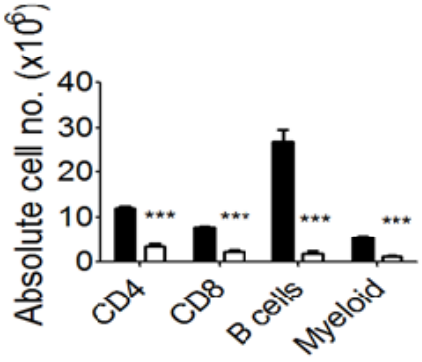



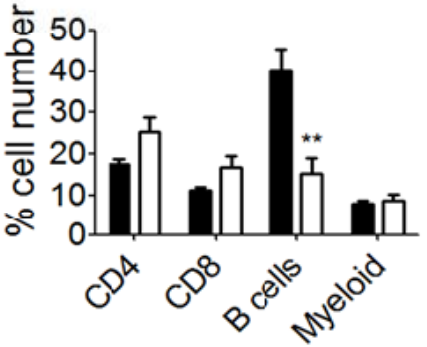



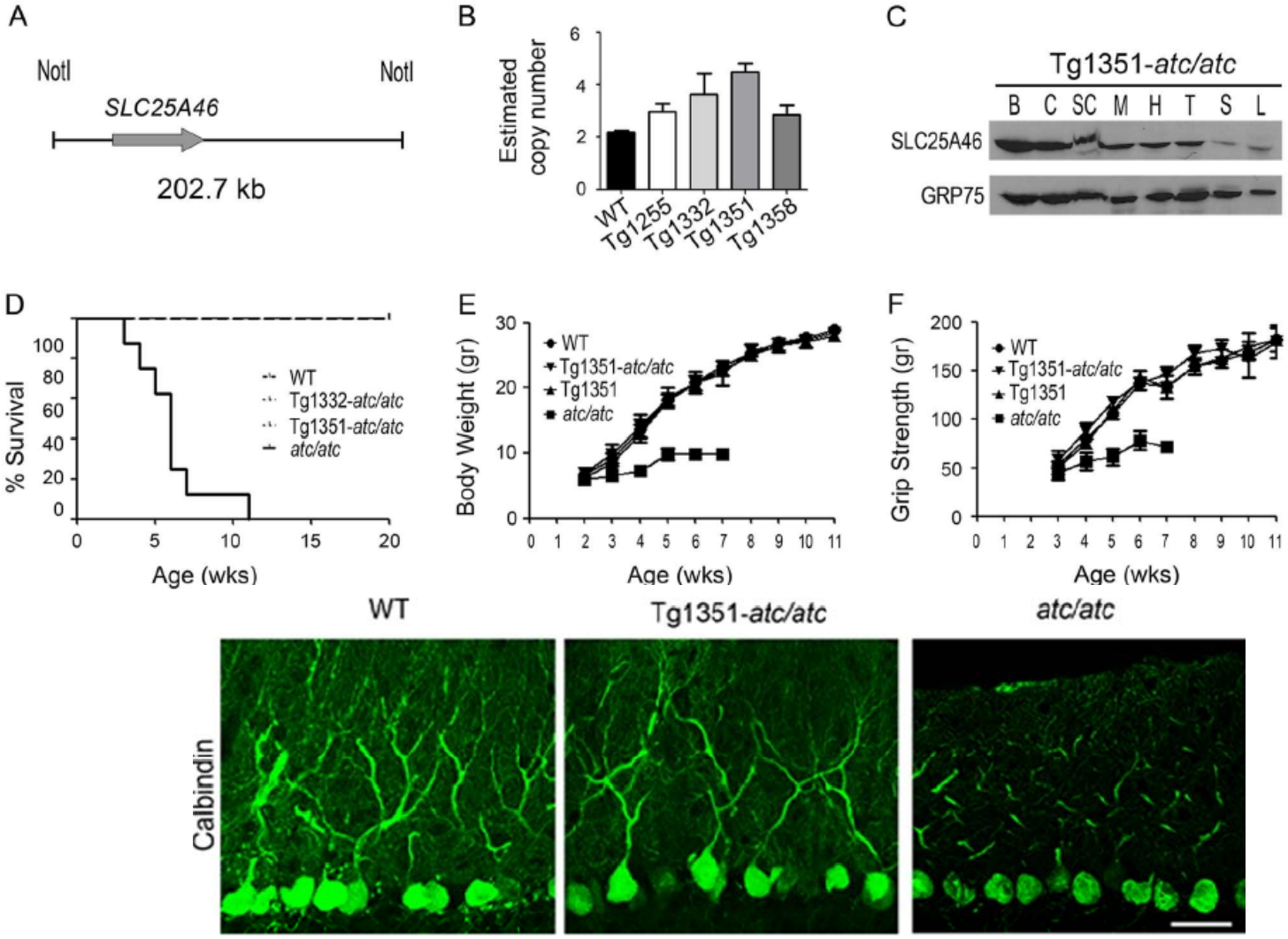




*Terzenidou et al., Plos Genetics, 2017*

# Genetic confirmation




*Terzenidou et al., Plos Genetics, 2017*

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Vassiliki Perivolidi, undergraduate student
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- Prof. Elias Couladouros, Agric Uni Athens
- Prof. George Kondopidis, Uni Thessaly
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- Dr Rebecca Matsas, Hellenic Pasteur Institute

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