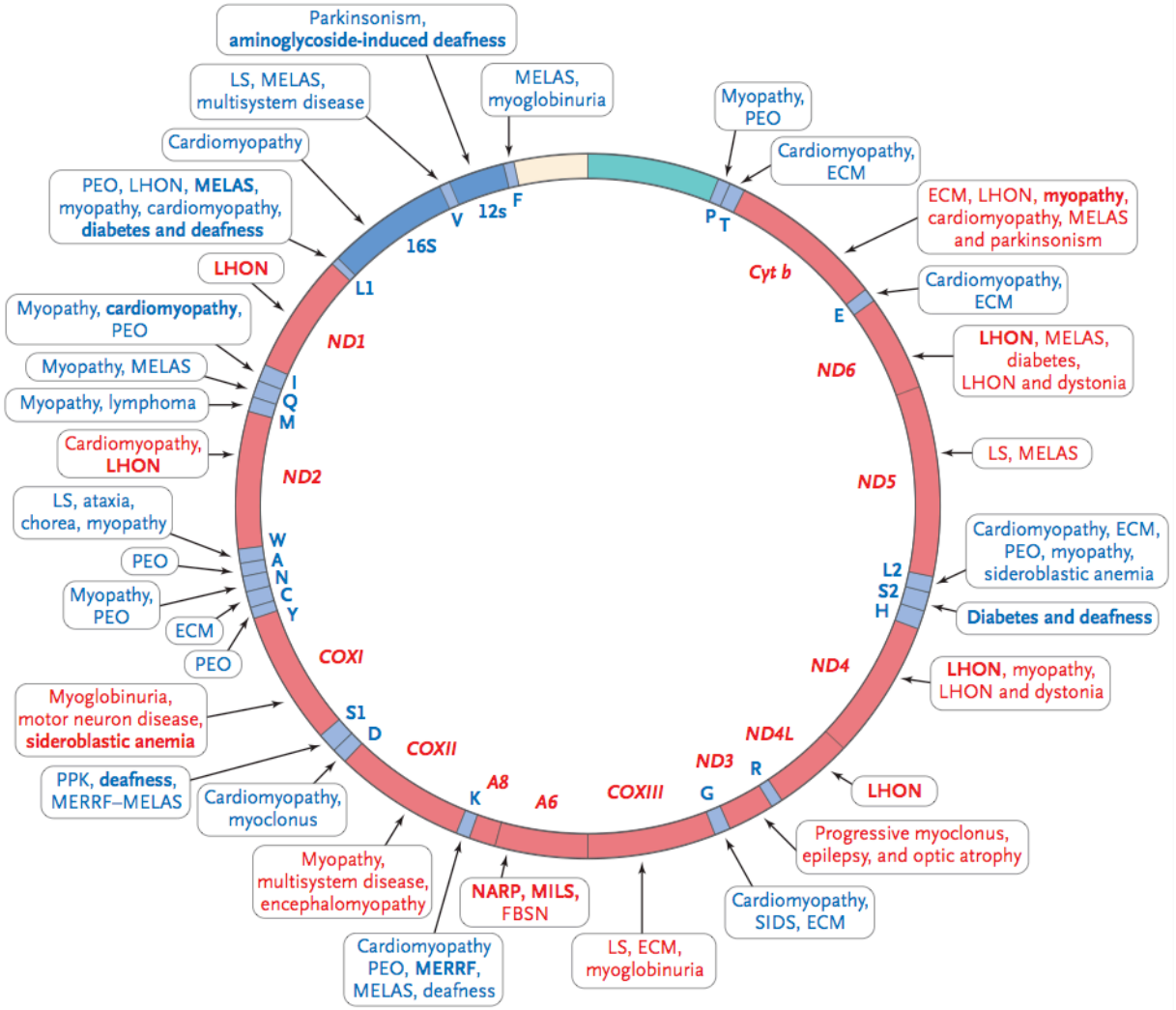
mtDNA mutations cause maternally inherited mitochondrial diseases



16.6 kb genome encodes 37 genes:

13 polypeptides, 2 rRNA, 22 tRNA

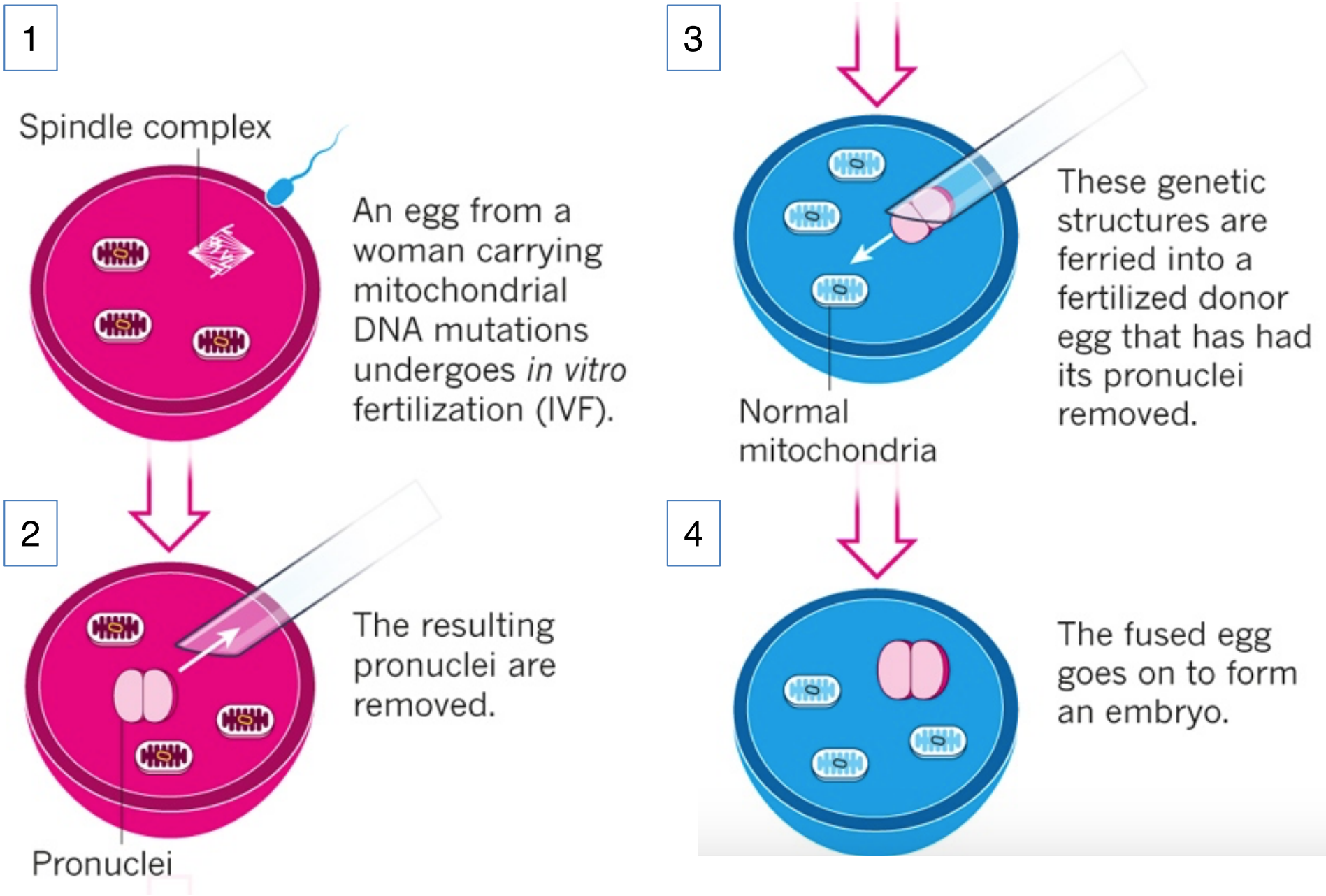
mtDNA diseases are diverse, but have common features:

- maternal inheritance
- respiratory chain defects
- neuro-muscular symptoms

Animal models have been difficult to generate.

DiMauro and Schon (2004) N Engl J Med

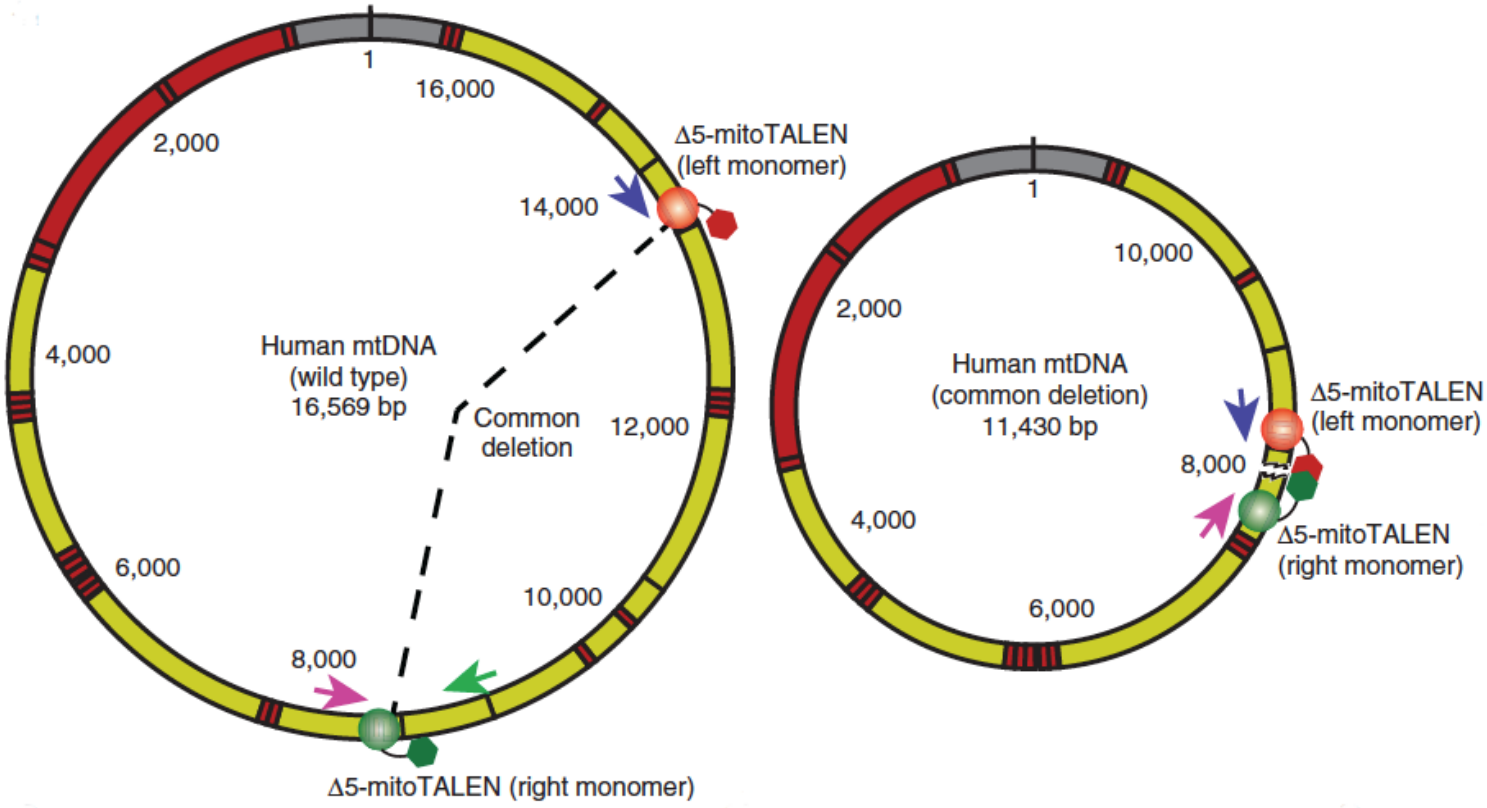
Pronuclear transfer



Related: Maternal spindle transfer; polar body transfer

Callaway (2014). Nature

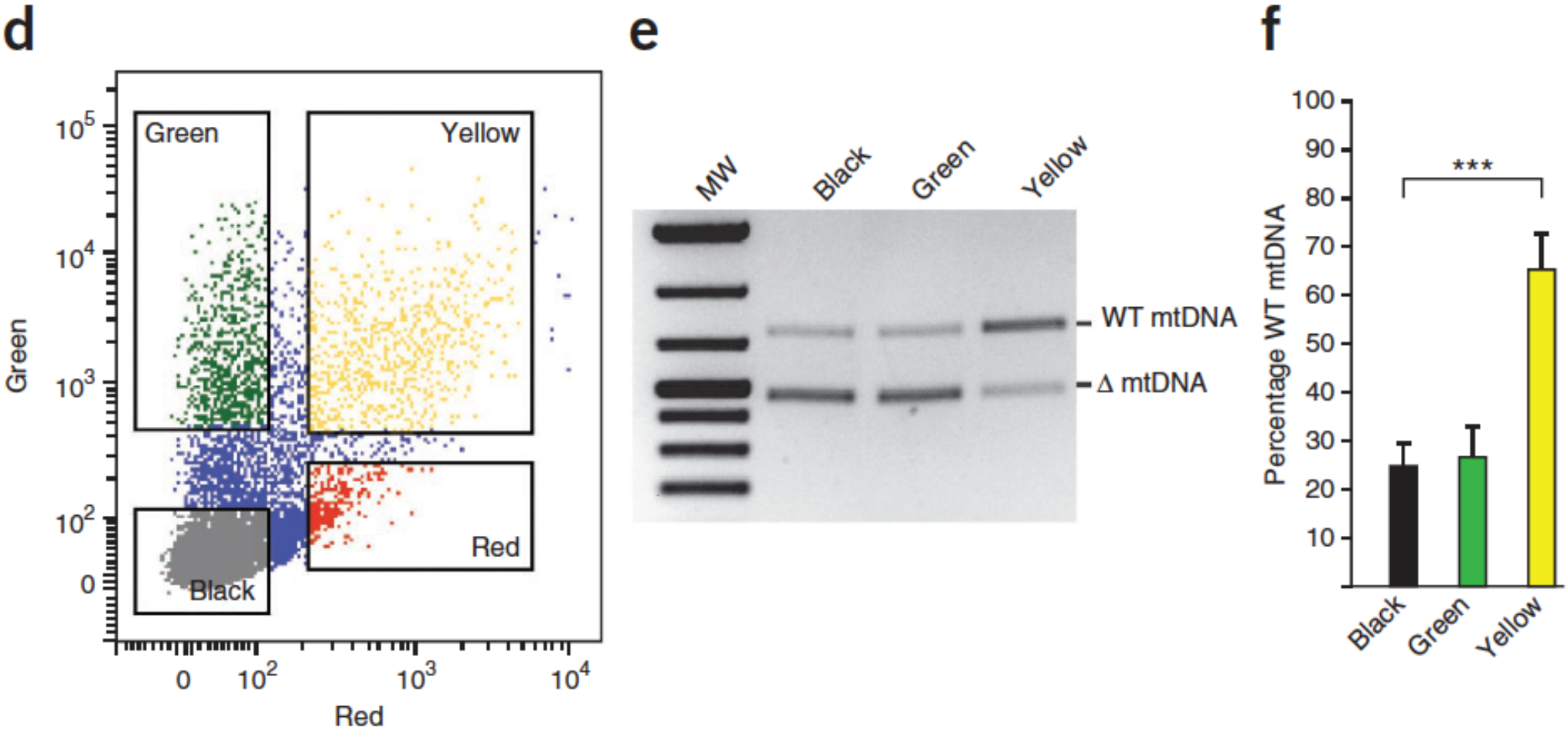
Engineering mitochondrial nucleases against specific mtDNA mutants



- Fok1 must dimerize to be functional endonuclease.
- Designing mito-TALENs against the “common” deletion.
- A similar strategy can be used to target mutant mtDNA containing a point mutation.
- Relies on the concepts: (1) threshold effect, and (2) mtDNA can be re-populated in a heteroplasmic cell

Bacman et al (2013) Nat Med

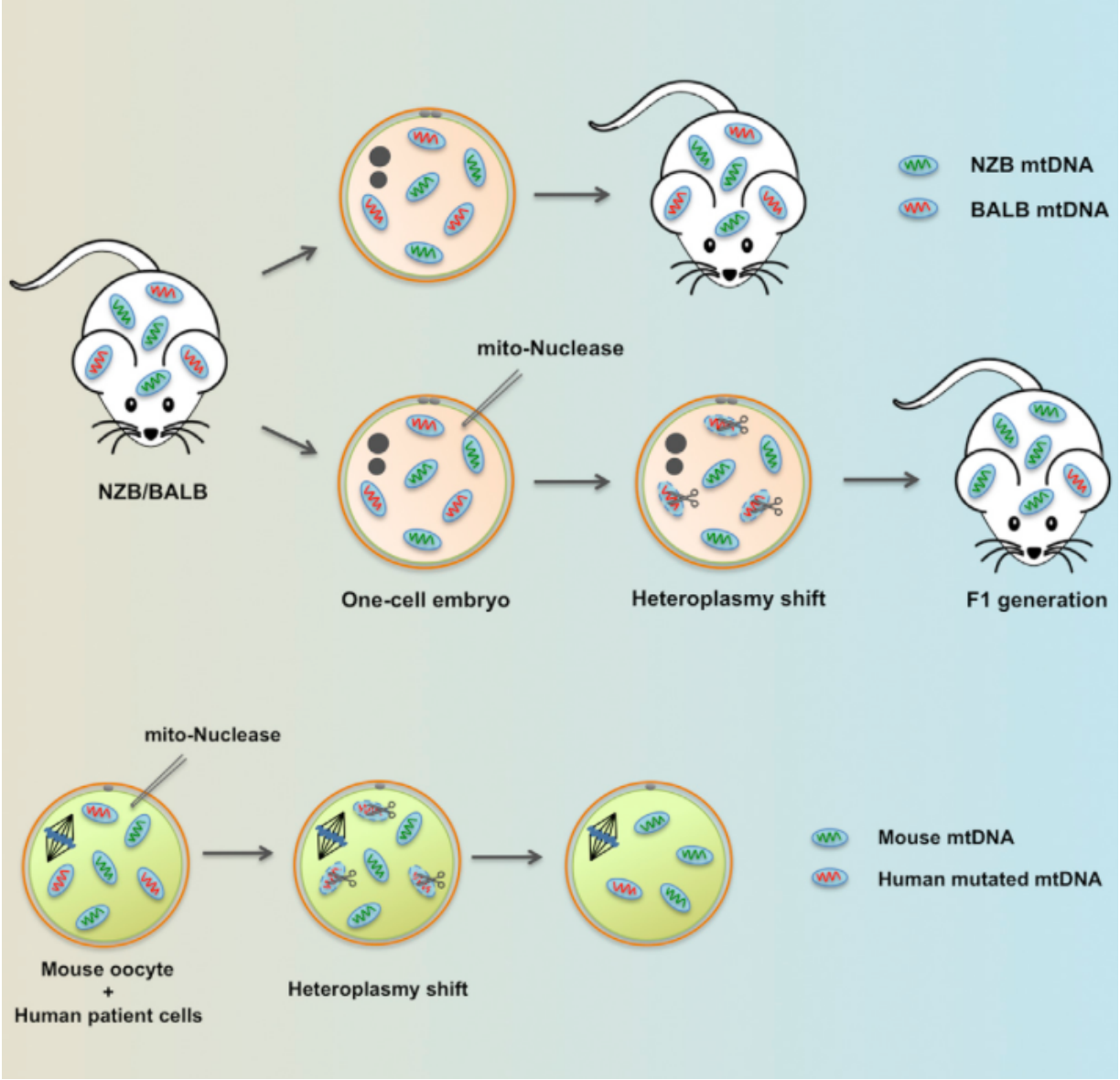
Engineering mitochondrial nucleases against specific mtDNA mutants



- One mito-TALEN fuse to EGFP; the other to mCherry. Both plasmids transfected.
- Cell sorting (d), followed by PCR analysis (e, f).

Bacman et al (2013) Nat Med

Engineering mitochondrial nucleases against specific mtDNA mutants



Redy et al (2015) Cell

mtDNA mutations in aging

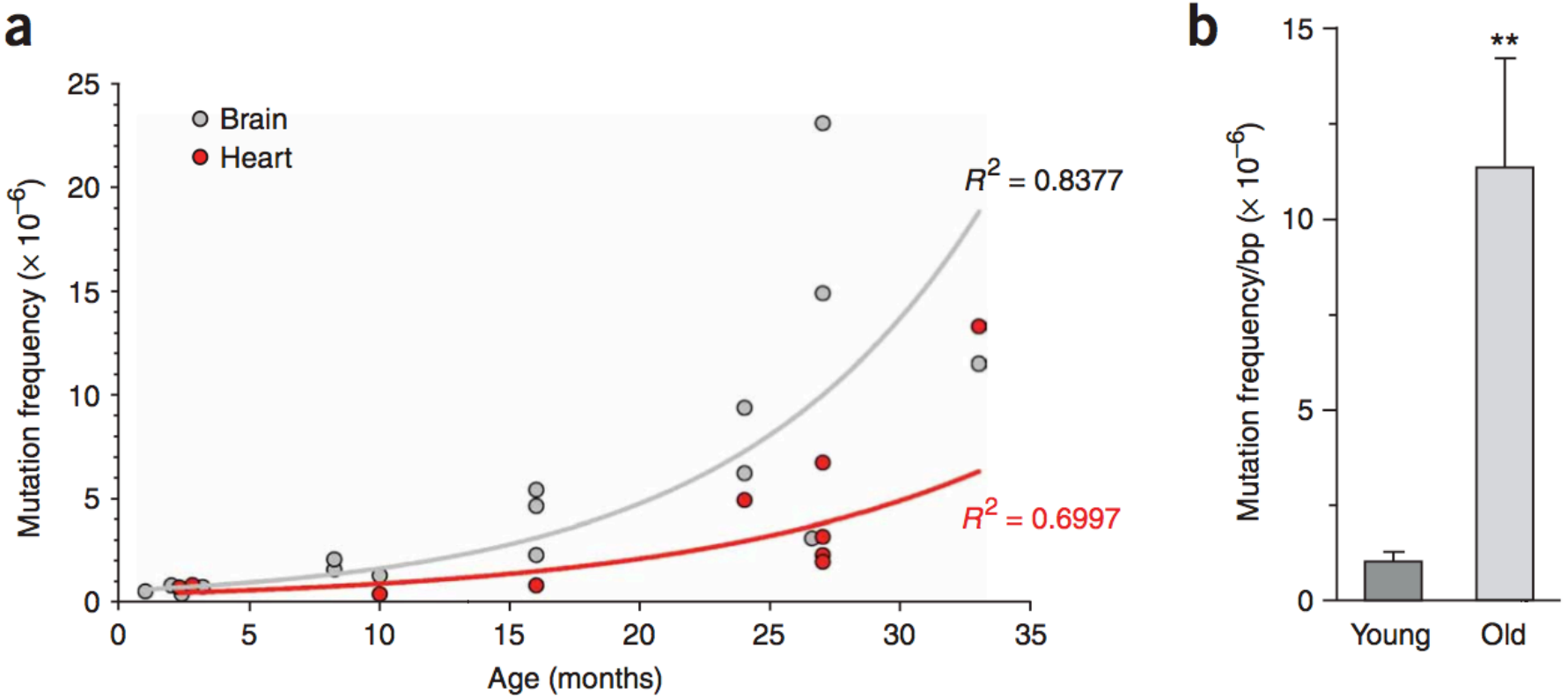
- mtDNA mutations accumulate with age
  - 2-3 mutations in every genome in older patient groups

- Mice that have been engineered with a mutation in *POLG*, the sole mtDNA polymerase, makes the mice prone to replication and repair errors in the mtDNA genome.
  - PREMATURE AGING phenotype occurs: weak, low energy, gray hair
  - Can significantly reverse these phenotypes by regular exercise.

- LONGEVITY is associated with specific mtDNA haplogroups
  - Haplogroups refer to sets of common mtDNA variants that have become evolutionarily fixed and can be used to define human populations

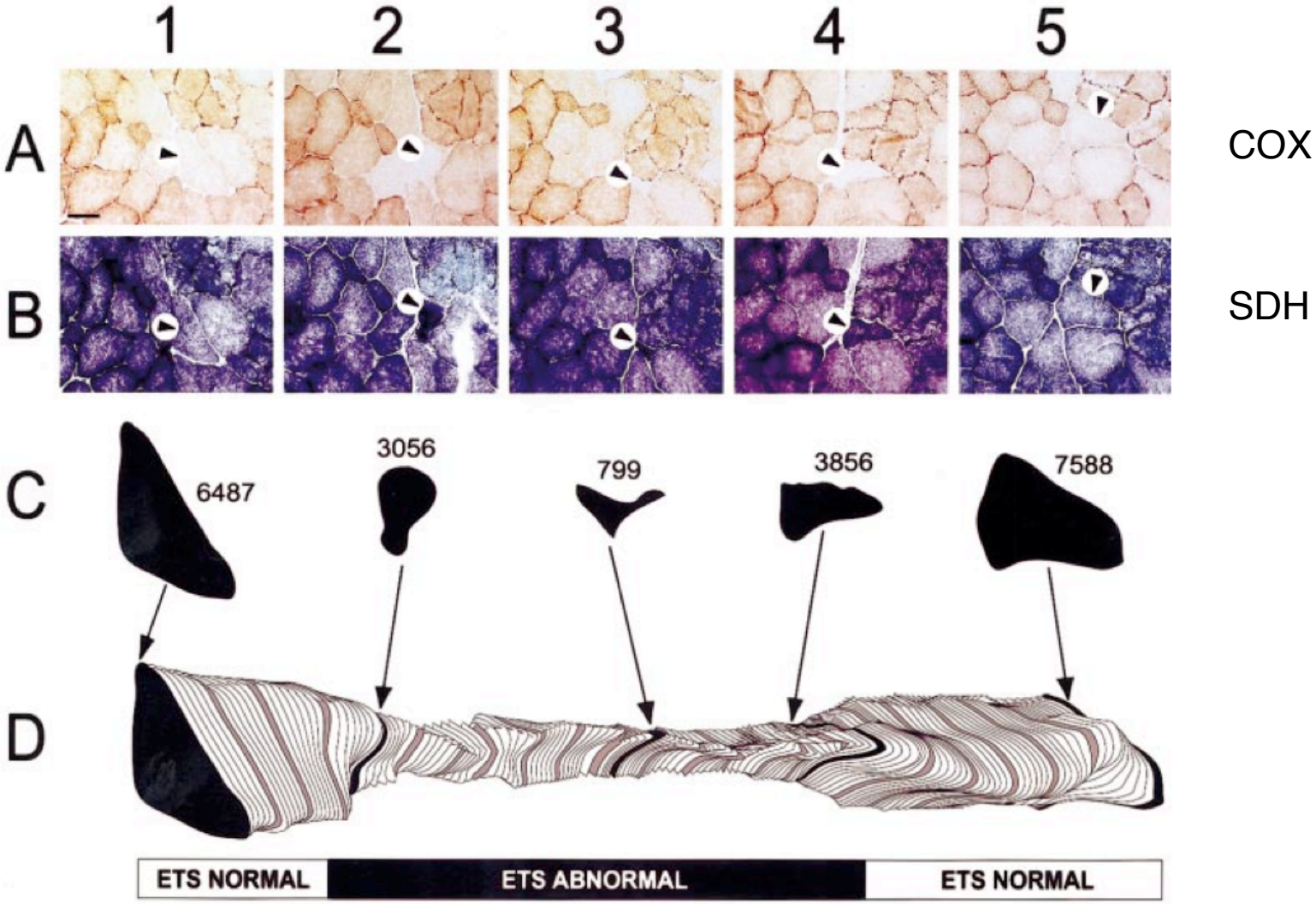
- “Sporadic” neurodegenerative disorders, such as Parkinson’s disease, often are associated with an increase in mtDNA mutations and deletions

Aging is associated with accumulation of mtDNA mutations



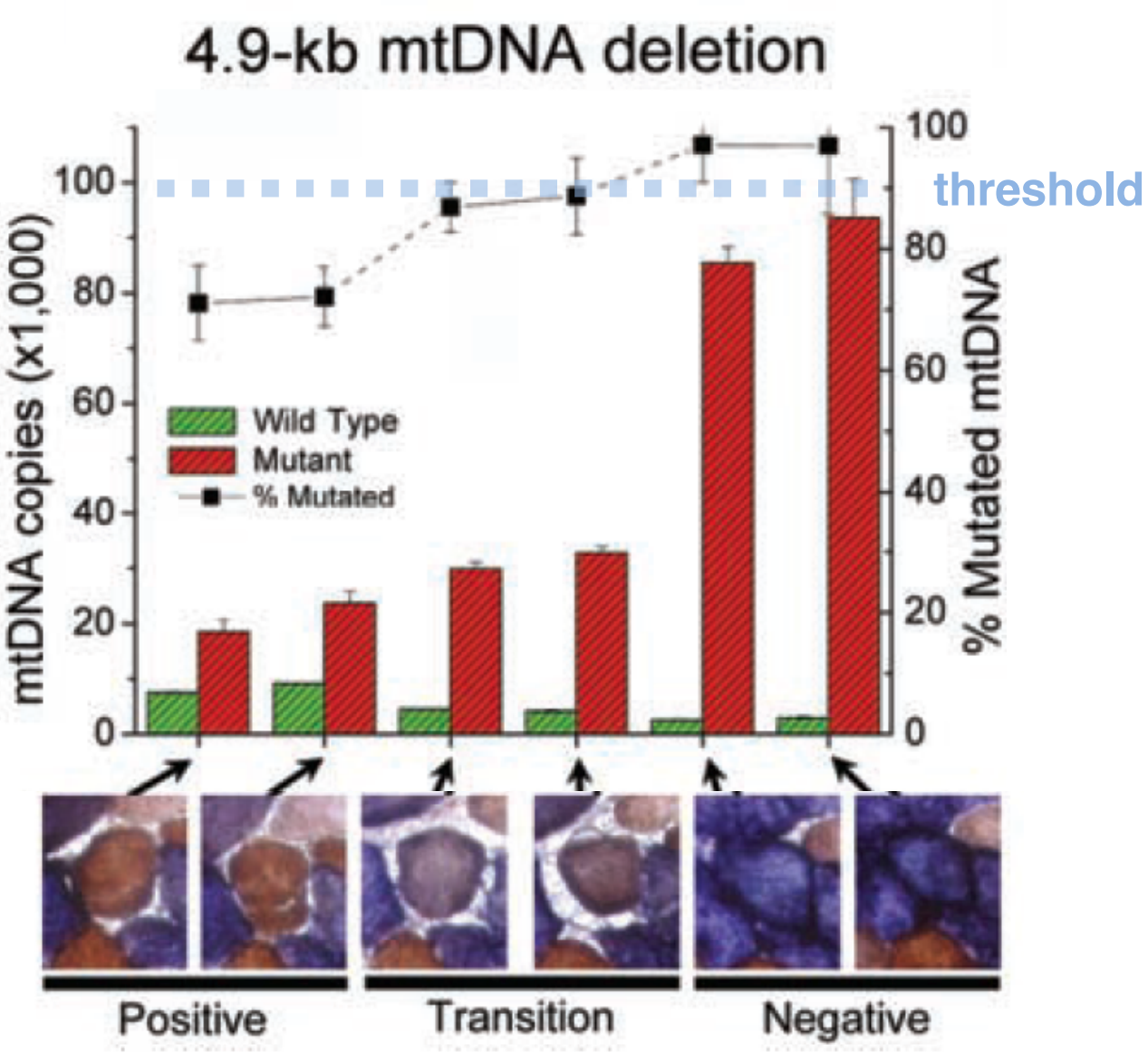
Vermulst et al. (2007) Nature Genetics

Expansion of mtDNA deletions in aged skeletal muscle



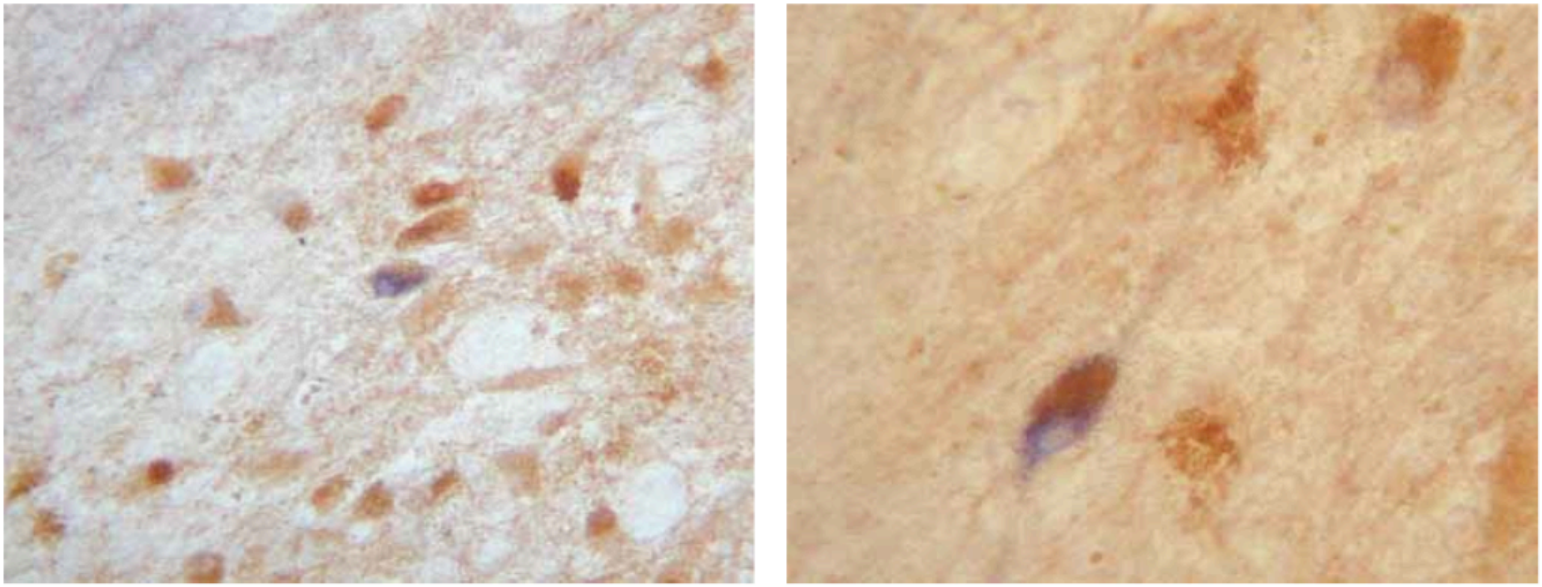
Wanagat et al. (2001) FASEB Journal

A high threshold for mtDNA accumulation before OSPHOS defects



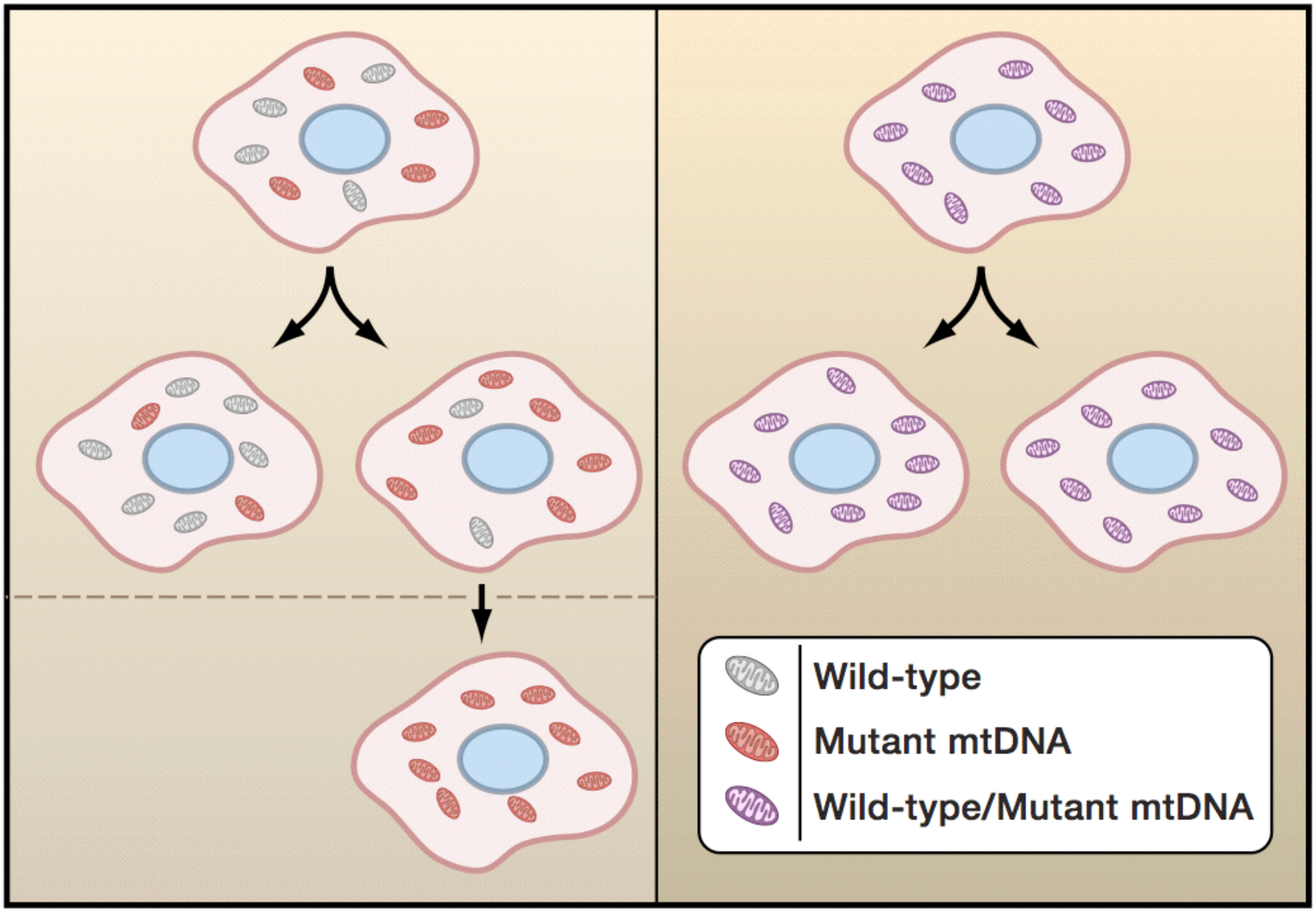
Durham et al. (2007) Am J Hum Genetics

Expansion of mtDNA deletions in substantia nigra neurons



Bender et al. (2006) Nature Genetics

Mitochondrial fusion may affect the natural history of mtDNA mutations

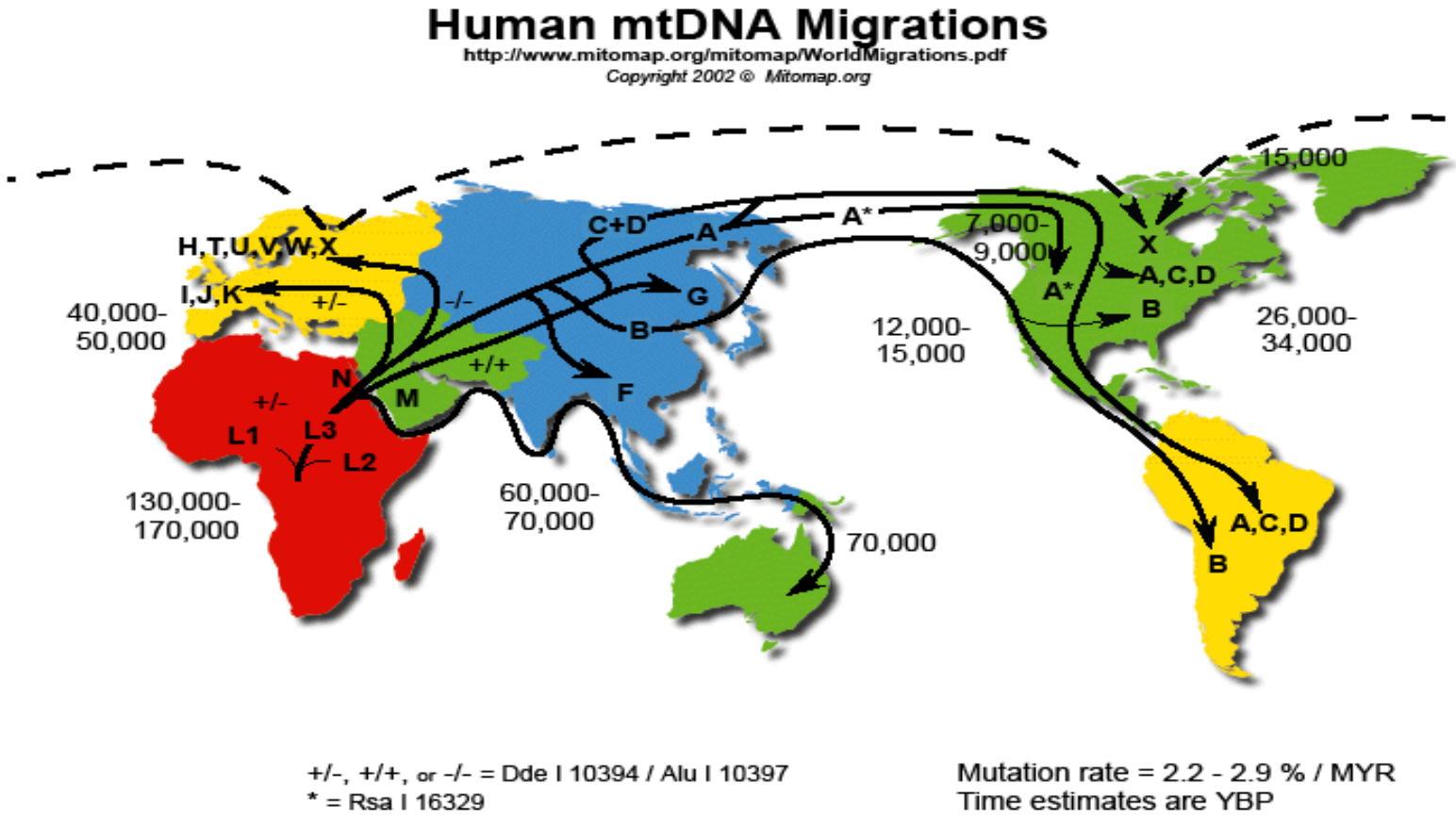


Chan (2006) Cell

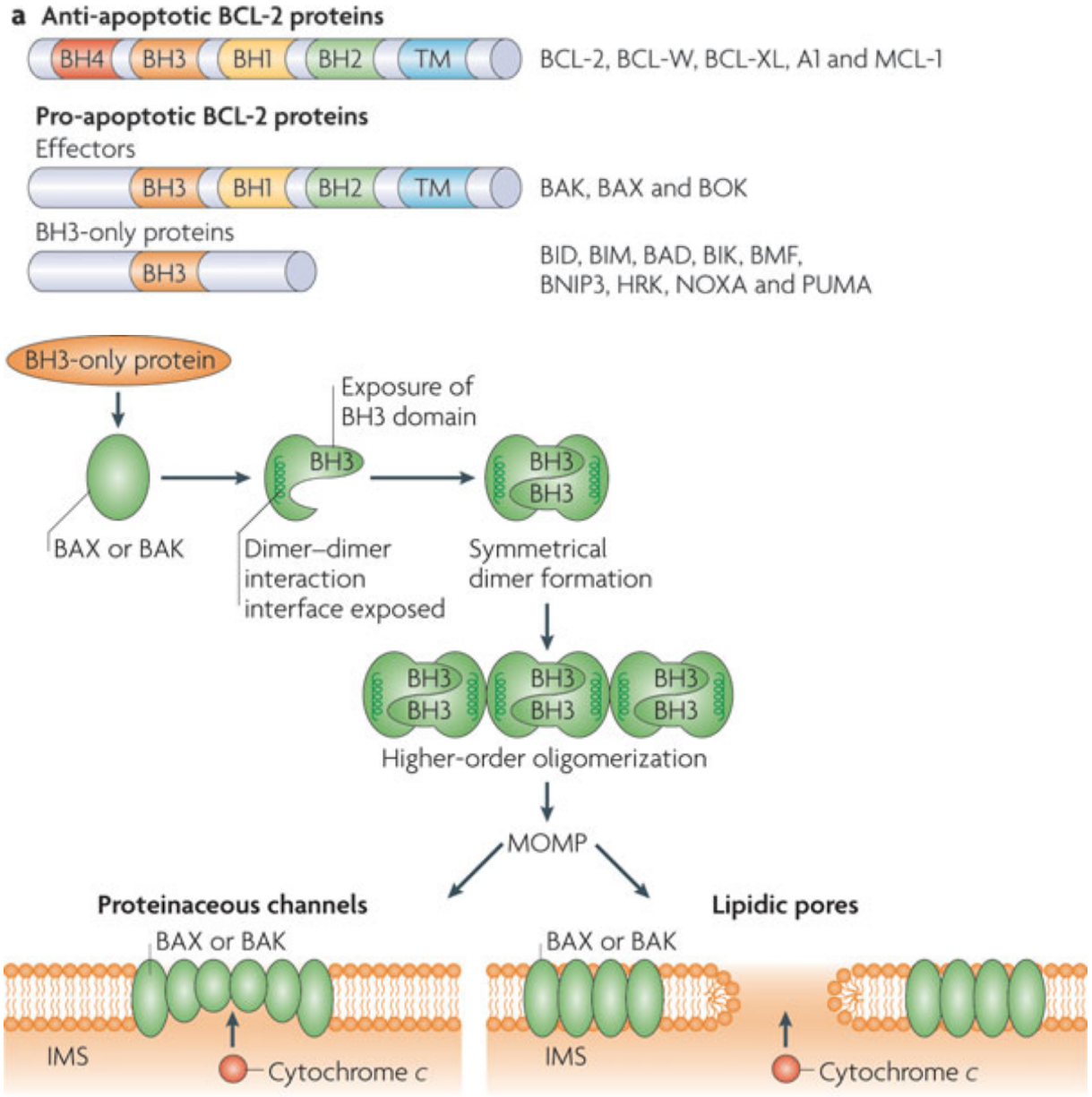
Mitochondrial Eve

- Different mtDNA lineages diverged from an ancestral woman who originated in Africa
- Sub-Saharan African individuals present the most variable mtDNA sequences
- Most haplogroups are continent specific
  - L1, L2, L3 groups are sub-Saharan African lineages
  - H, I, J, K, T, U, V, W, X encompass almost all mtDNA from European, North African, & Western Asian Caucasian
  - A, B, C, E, F, G, M represent majority of lineages described for Asia, Oceania and Native Americans

Natural mtDNA variation defines haplogroups and human evolution



Bax and Bak cause mitochondrial outer membrane permeabilization (MOMP)



BH domains= Bcl2 homology domains

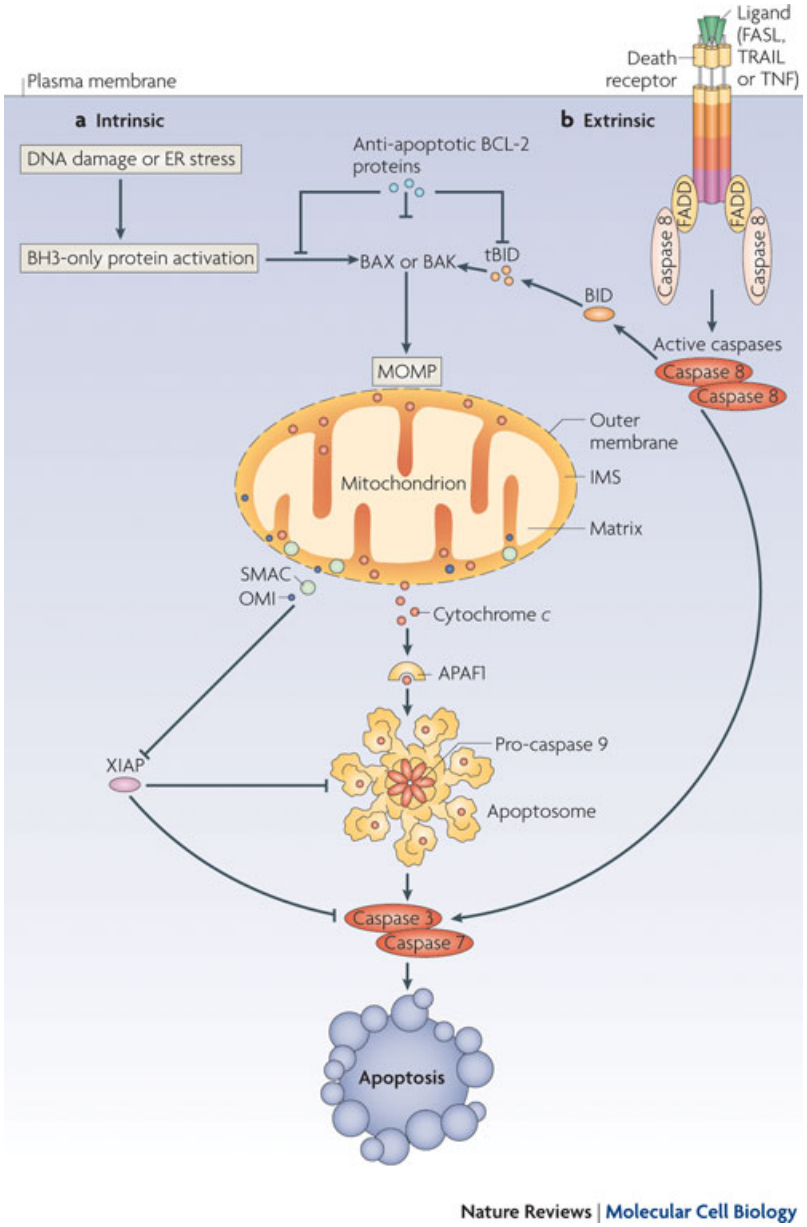
Bid=BH3 interacting domain protein death agonist
tBid=truncated Bid (activated)
Bax=Bcl2 associated X protein
Bak=Bcl2 antagonist or killer

BH3-only proteins (e.g., Bid) activate Bax or Bak, leading to their oligomerization.

These oligomers appears to permeabilize the mitochondrial outer membrane, perhaps by forming pores.

Tait & Green. (2010) Nat Rev MCB

Intrinsic and extrinsic cell death pathways involve MOMP



Intermembrane space proteins implicated in cell death:

Cytochrome *c*: required for caspase activation

SMAC/Diablo: second mitochondria-derived activator of caspase; binds and antagonizes XIAP (X-linked inhibitor of apoptosis protein)

OMI/HtrA2: mitochondrial protease; binds and cleaves XIAP

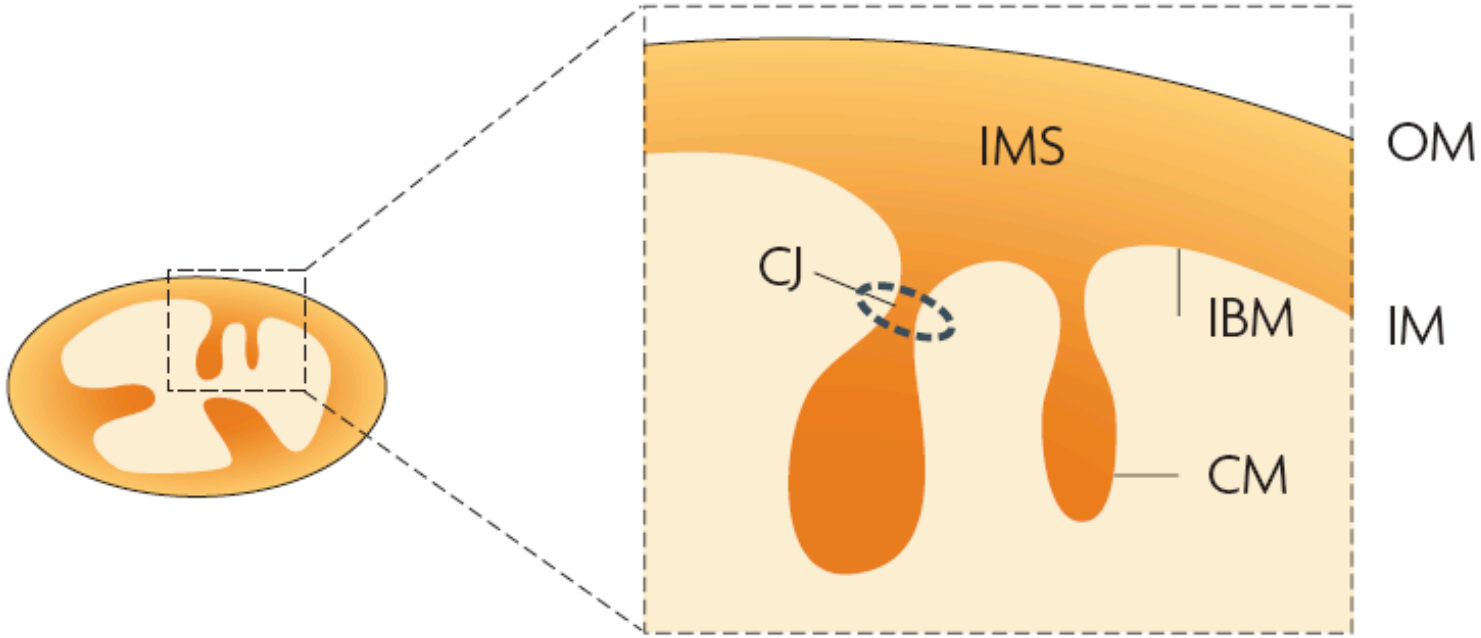
AIF: Apoptosis-inducing factor

Endonuclease G: Nuclease in mitochondria.

However, knockout mice studies indicate that apoptosis can occur in the absence of the latter 4.

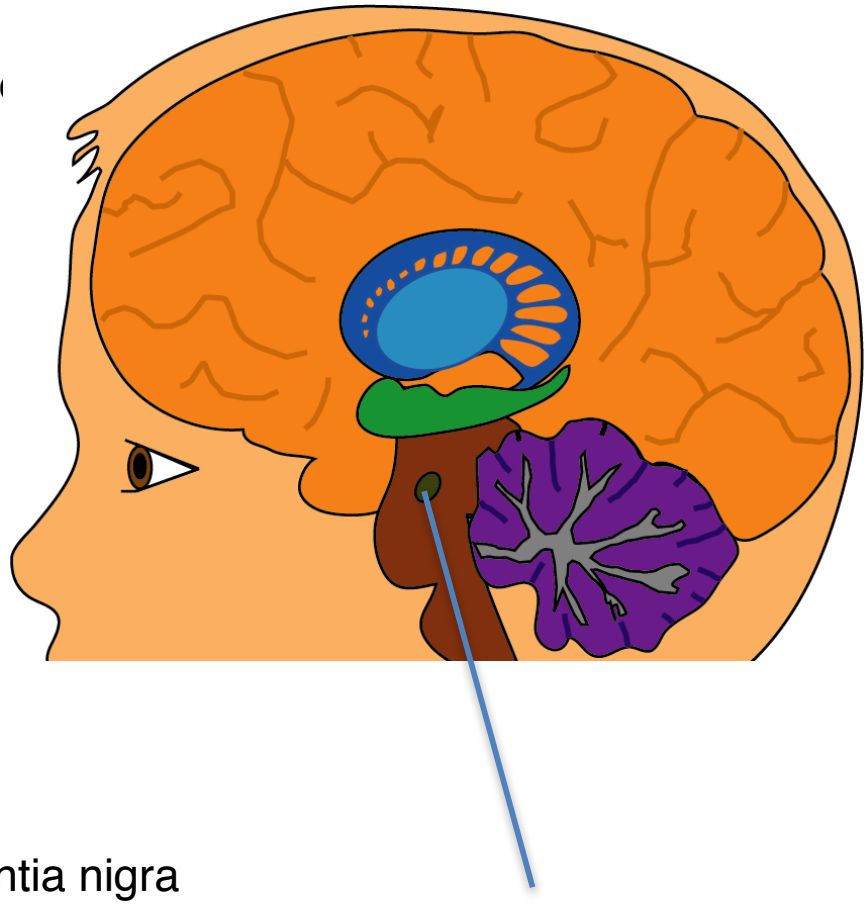
Tait & Green. (2010) Nat Rev MCB

Structural changes in mitochondria during apoptosis



Parkinson's disease

Second most common neurodegenerative dis
movement disorder



substantia nigra of midbrain

4 cardinal clinical symptoms

- movement disorder with muscle rigidity
- slow movements (bradykinesia)
- tremor
- postural instability.

Pathology

- Loss of dopaminergic neurons in the substantia nigra
- Decreased stimulation of the motor cortex
- Intraneuronal (cytoplasmic) protein aggregates= Lewy bodies
    - Inclusions contain α-synuclein and ubiquitin

Parkinson's disease and mitochondrial dysfunction

Most PD cases are sporadic, but 5-10% are familial.

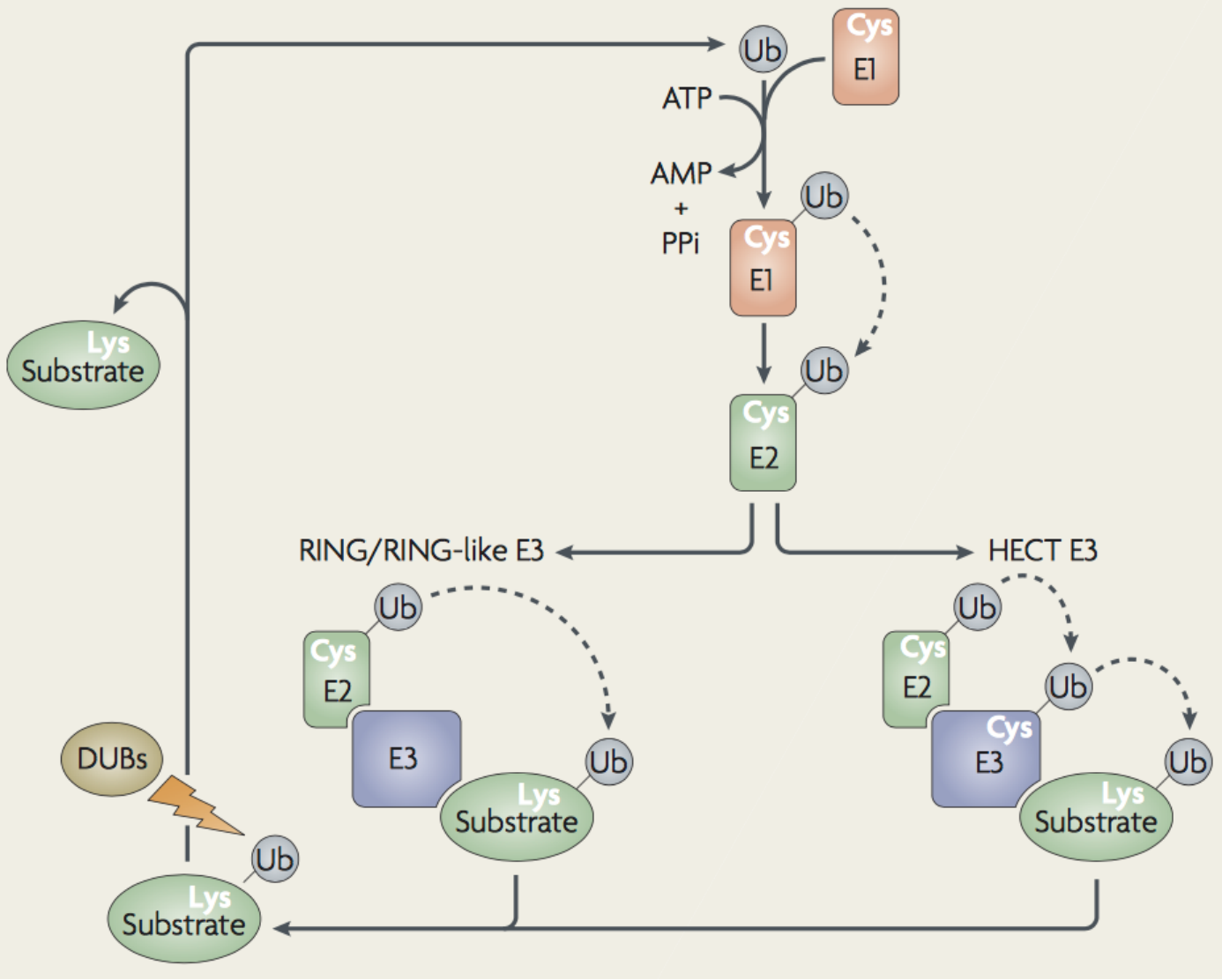
Genes involved include:

- α-synuclein
- leucine-rich repeat kinase 2
- DJ-1 (may be chaperone)
- PINK1 and Parkin

Mitochondrial connection

- The mitochondrial toxins MPTP (contaminant of synthetic opiate) and rotenone block Complex I function and cause Parkinson's symptoms in humans and rodents.
- Evidence of mitochondrial dysfunction in sporadic PD.
- Several genes causing familial Parkinson's are involved in mitochondrial function
    - Parkin= E3 ubiquitin ligase with 2 RING domains
    - PINK1=PTEN-induced kinase (serine/threonine)

Two types of E3 Ubiquitin ligases



- HECT (**h**omologous to the **E**6-AP **c**arboxyl **t**erminus)
    - Accepts ubiquitin transiently from E2, before it transfers ubiquitin to the substrate
- RING finger (**R**eally **i**nteresting **n**ew **g**ene)
    - Zinc finger that contains a Cys3HisCys4 motif that binds 2 Zn ions.
    - Binds to E2 and substrate, causing the Ubiquitin to transfer from the E2 to substrate.

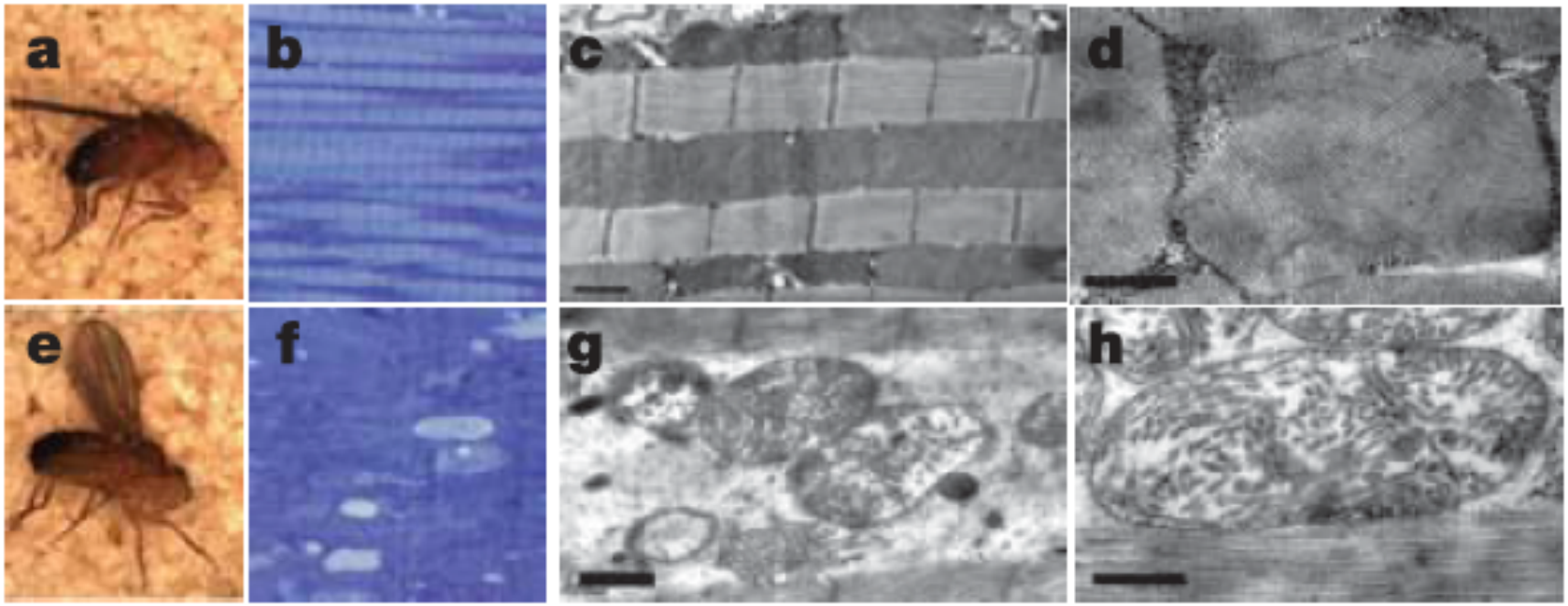
Ravid & Hochstrasser (2008) Nat Rev MCB

Muscle degeneration in *pink1* flies

Background: Mouse knockouts of Pink1 and Parkin have little phenotype. However, previous studies showed that Parkin mutant flies have reduced lifespan and locomotor defects. Apoptotic death found in muscles. Here, *pink1* flies are found to have a similar phenotype.

Wildtype

pink1 mutant



b) and f): toluidine blue staining of indirect flight muscles; mutant has vacuolations and muscle fibers disorganized.

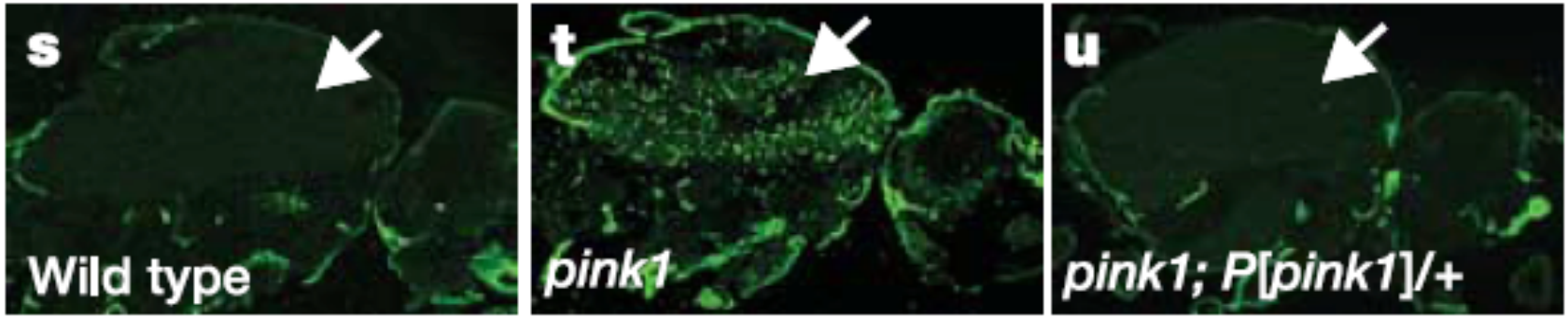
c), d): wt mitochondria

g), and h): mutant mitochondria have changes in cristae

e): raised wings

Clark et al. (2006) Nature

Apoptotic muscle degeneration in *pink1* flies



t) In indirect flight muscles, TUNEL staining show positive nuclei in mutant.

Clark et al. (2006) Nature

pink1 flies are sensitive to mitochondrial stress

Mutant flies more sensitive to paraquat (herbicide that induces ROS) and rotenone (Complex I inhibitor). Both compounds can cause PD-like symptoms in animal models.

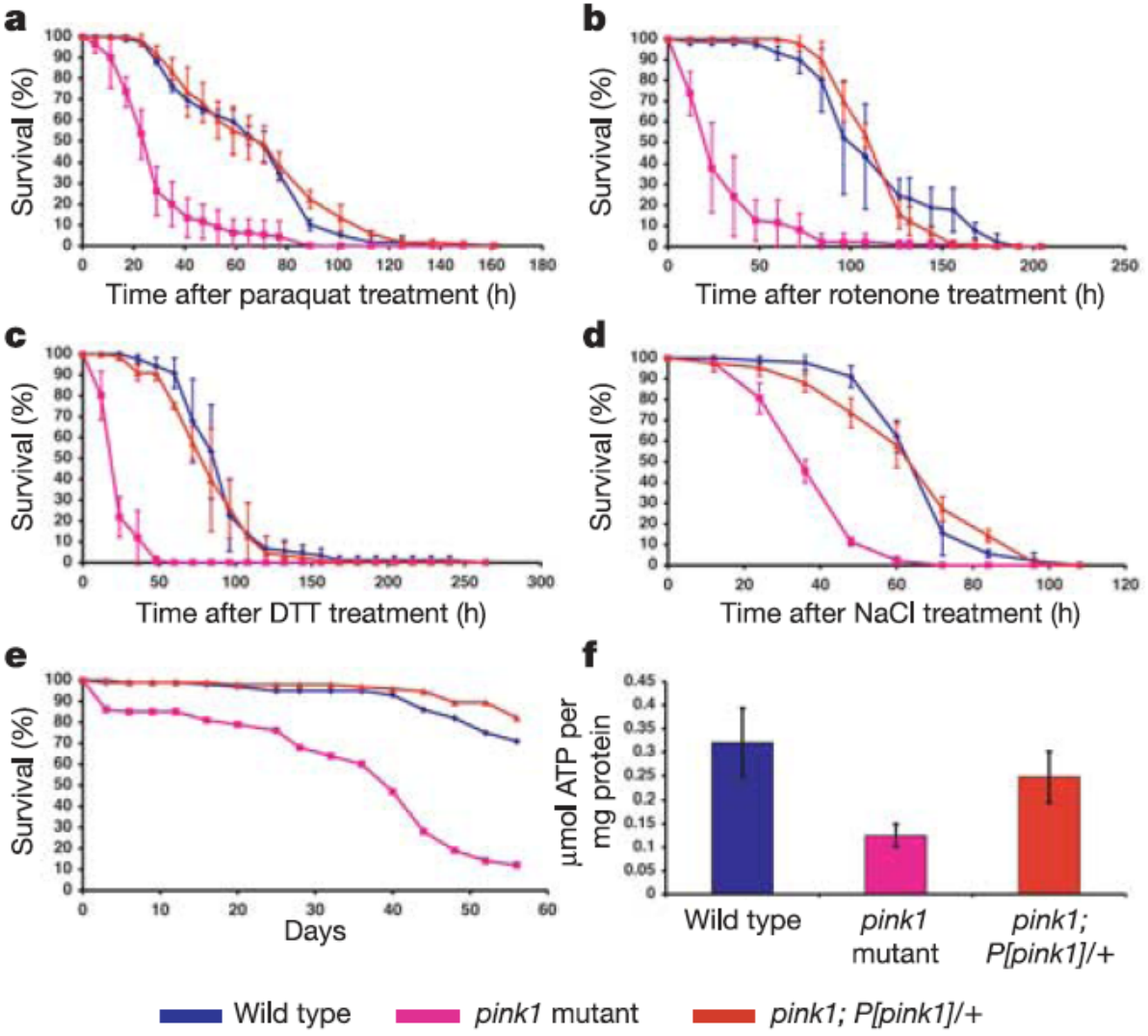
(a) paraquat (b) rotenone (c) DTT (c) NaCl.

(e) lifespan f) mean ATP levels.

Mutant flies also sensitive to other stresses.

Conclusion: Pink1 mutants resemble Parkin mutants.

How are Pink1 and Parkin related?

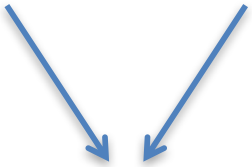


Clark et al. (2006) Nature

Models of Pink1/Parkin function

Parallel pathways

Pink1 Parkin



Normal mito

What is the phenotype of a Pink1/Parkin double mutant?

same as single mutant

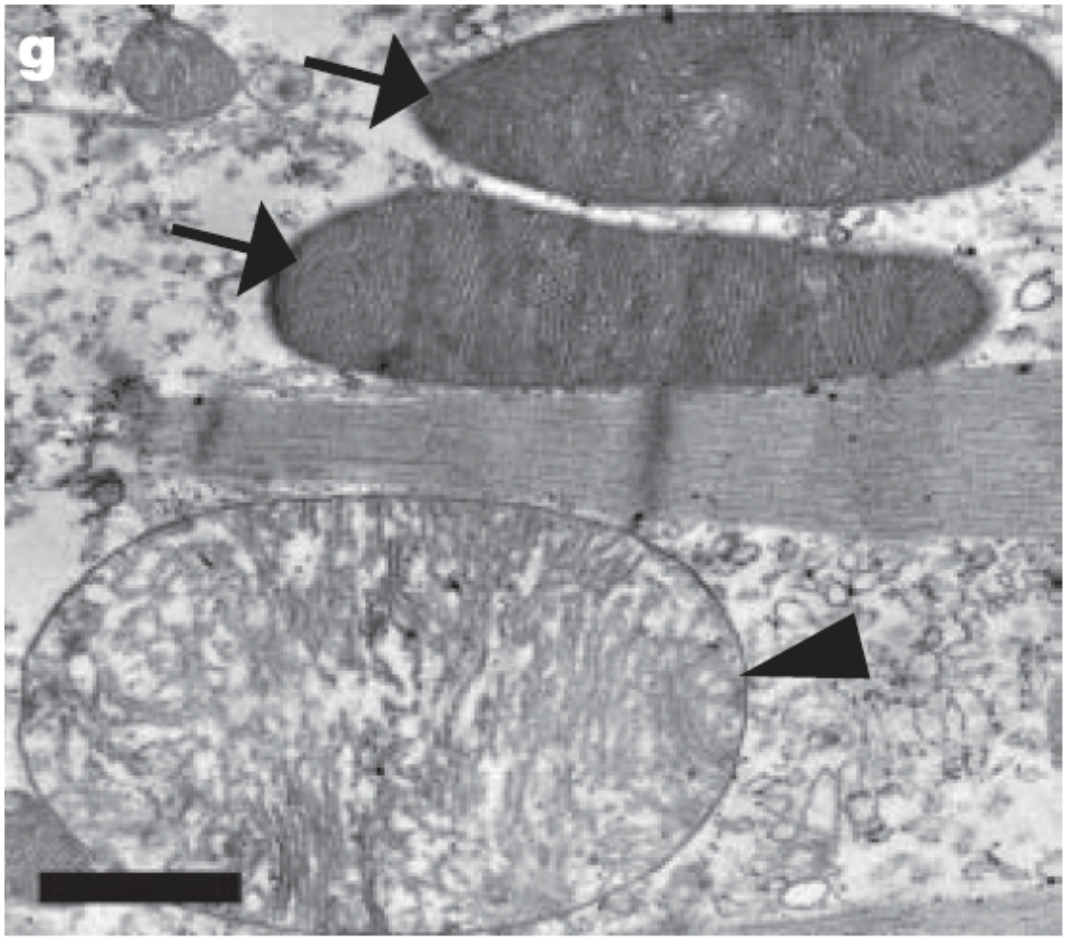
Same/linear pathway

Pink1/Parkin

Normal mito

What is the order of Pink1 versus Parkin?

Overexpression of Parkin rescues *pink1* flies



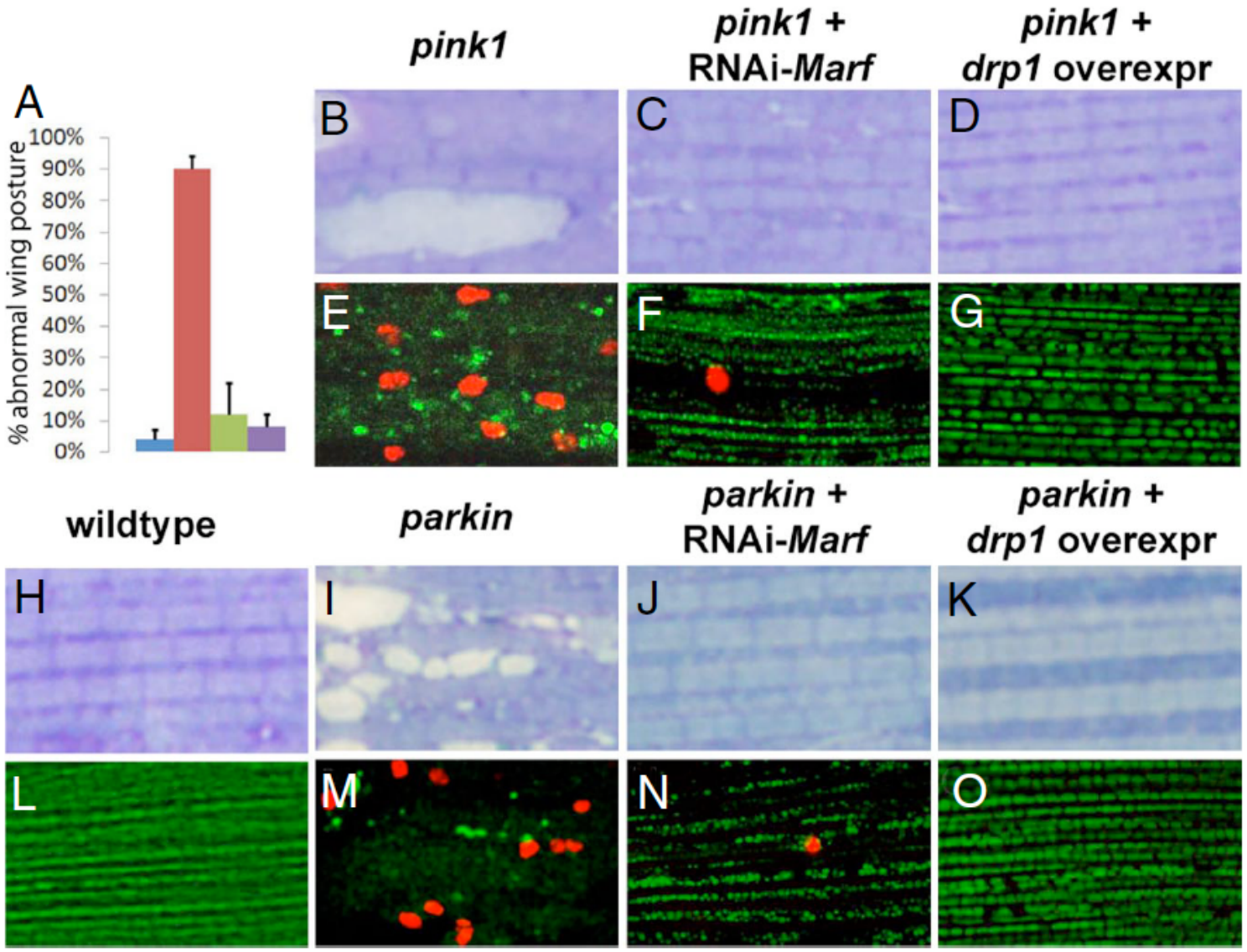
EM show rescue of cristae in pink1 mutant overexpressing parkin. Two mitochondria have normal cristae.

Clark et al. (2006) Nature

Genetic interactions of pink1 and parkin mutants with mitochondrial fusion and fission

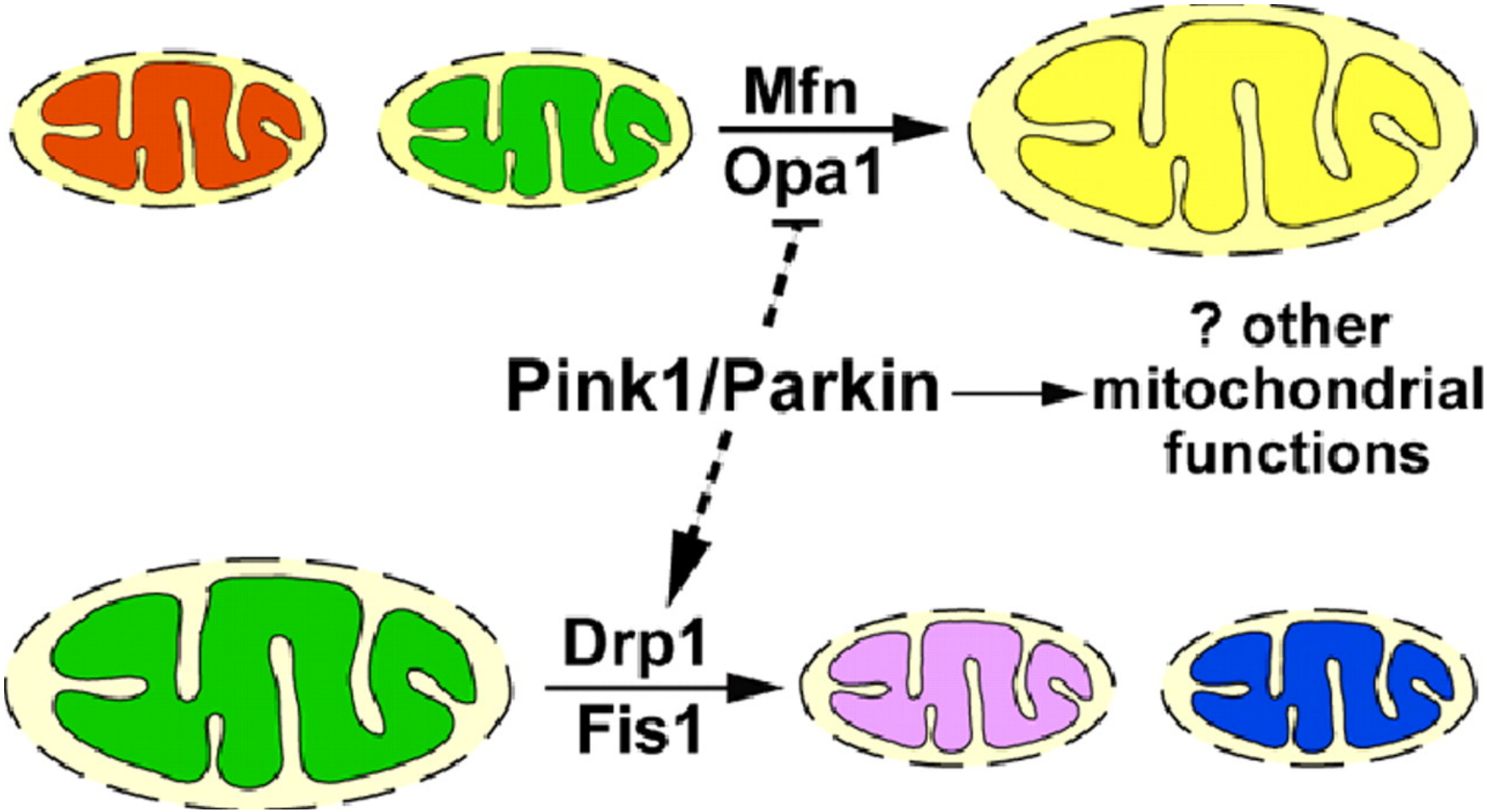
wt
pink1 mutant
***pink1* mutant *+ Marf* RNAi**
***pink1* mutant + Drp1 overexpression**

e-g) and m-0): TUNEL (red)/mitoGFP in flight muscles



Deng et al. (2008) PNAS

Model of PINK1/Parkin link to dynamics



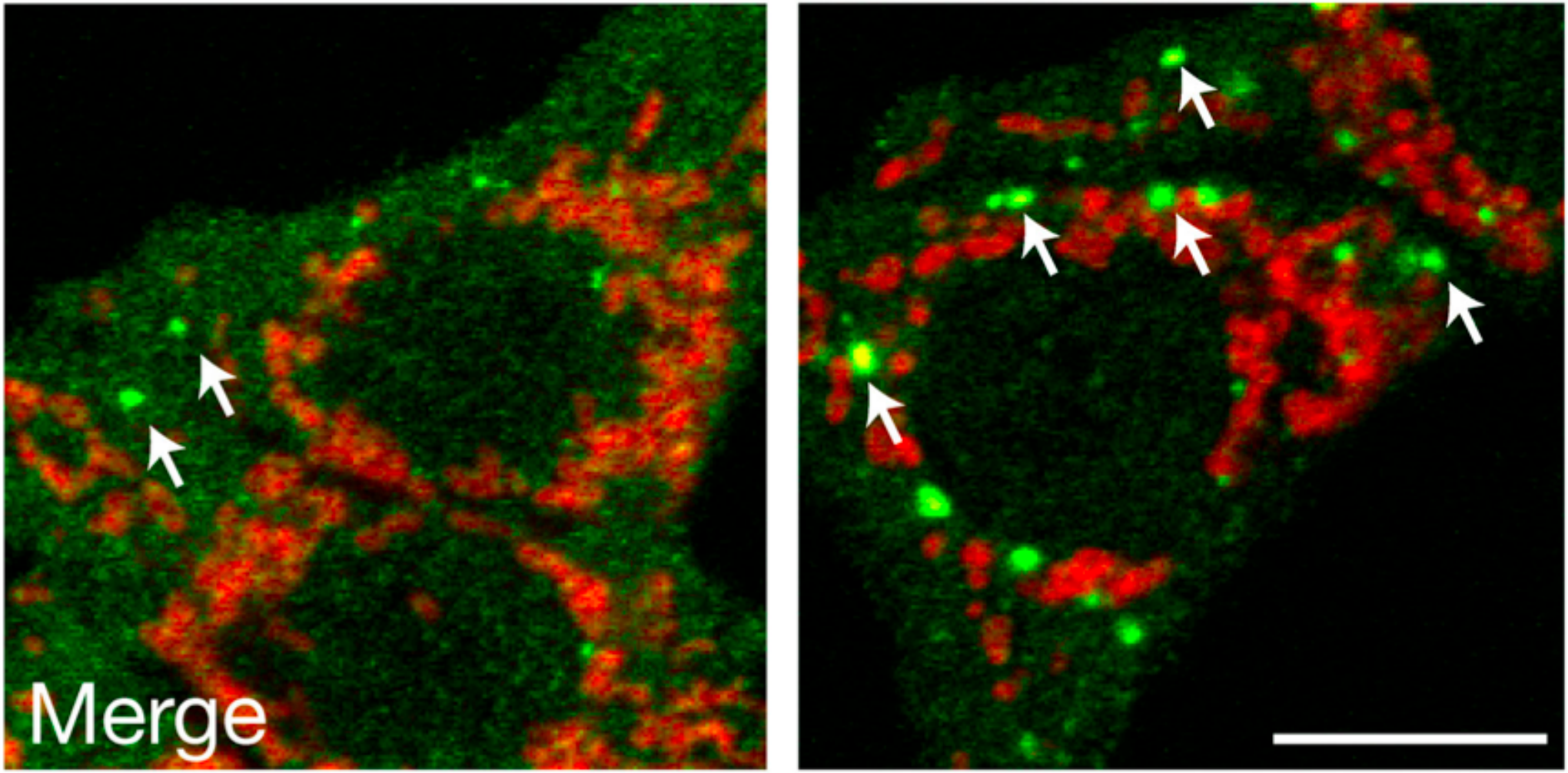
Hypothesis: Pink1 and Parkin promote fission and/or inhibit fusion, either directly or indirectly.

Deng et al. (2008) PNAS

Translocation of Parkin to mitochondria

control (DMSO)

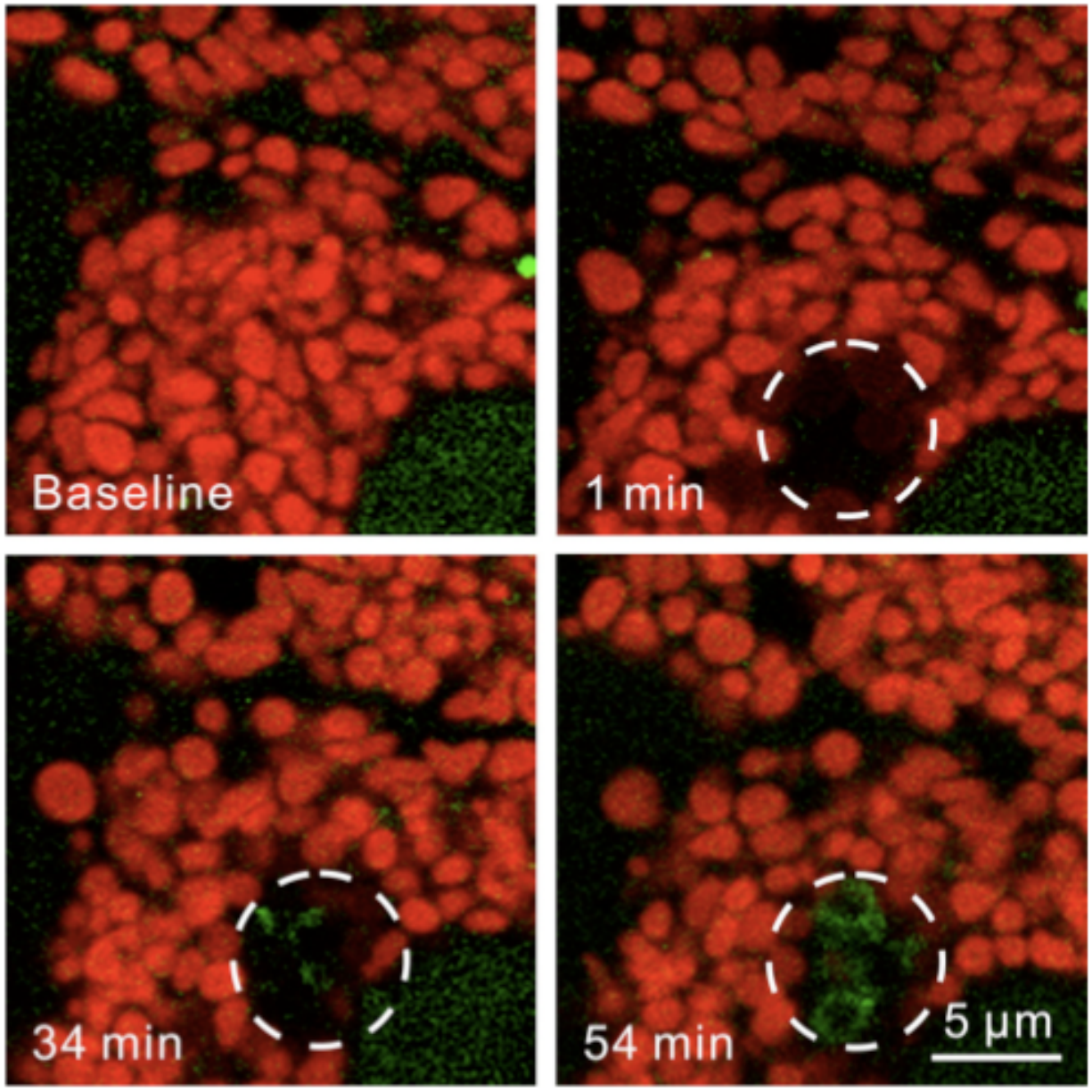
10 μM CCCP



HEK293 cells immunostained for endogenous Parkin (green) and Tom20 (red)

Narendra et al. (2008) JCB

Mitophagy may be selective



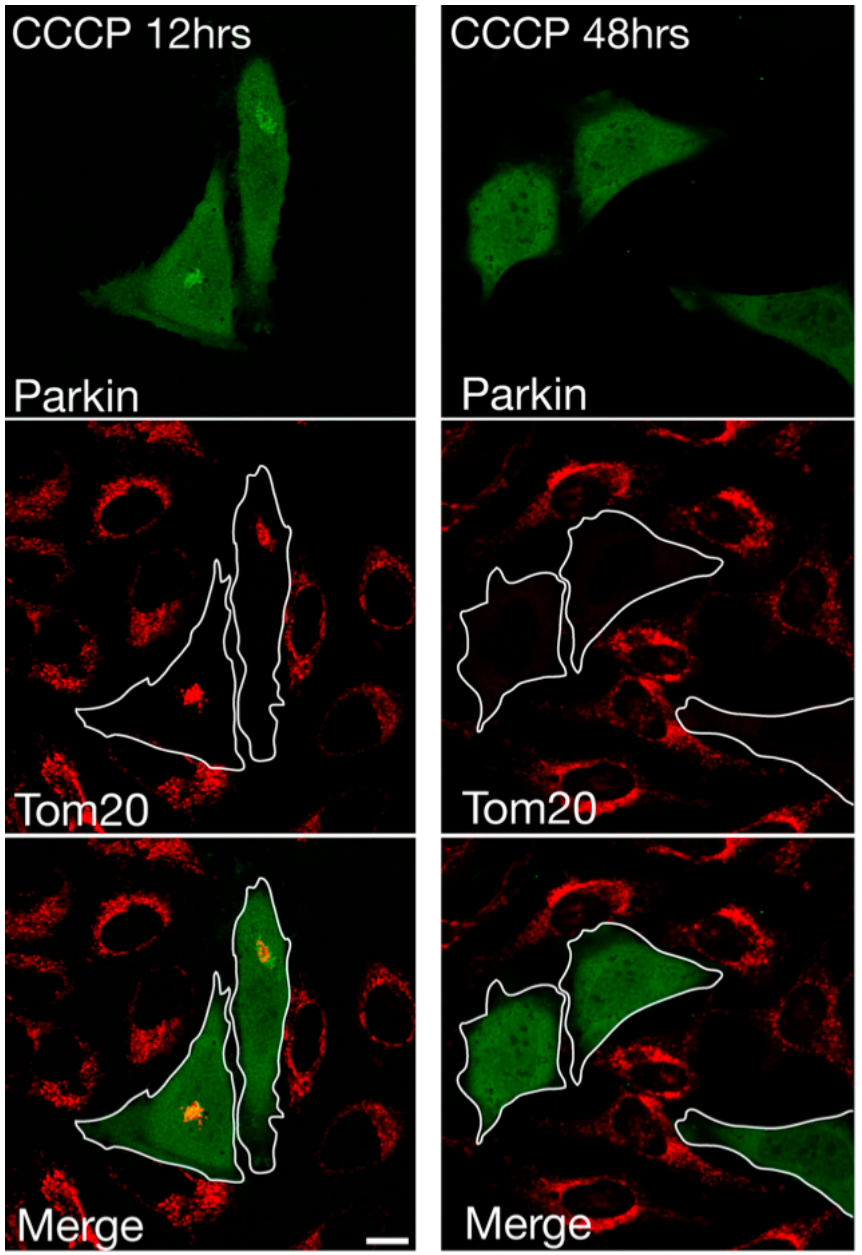
GFP-LC3 transgenic hepatocytes loaded with TMRM; 488 nm laser used to damage mitochondria within circle; GFP-LC3 appears within an hour.

Kim et al. (2007) Arch Biochem Biophy

Parkin + CCCP lead to loss of mitochondria

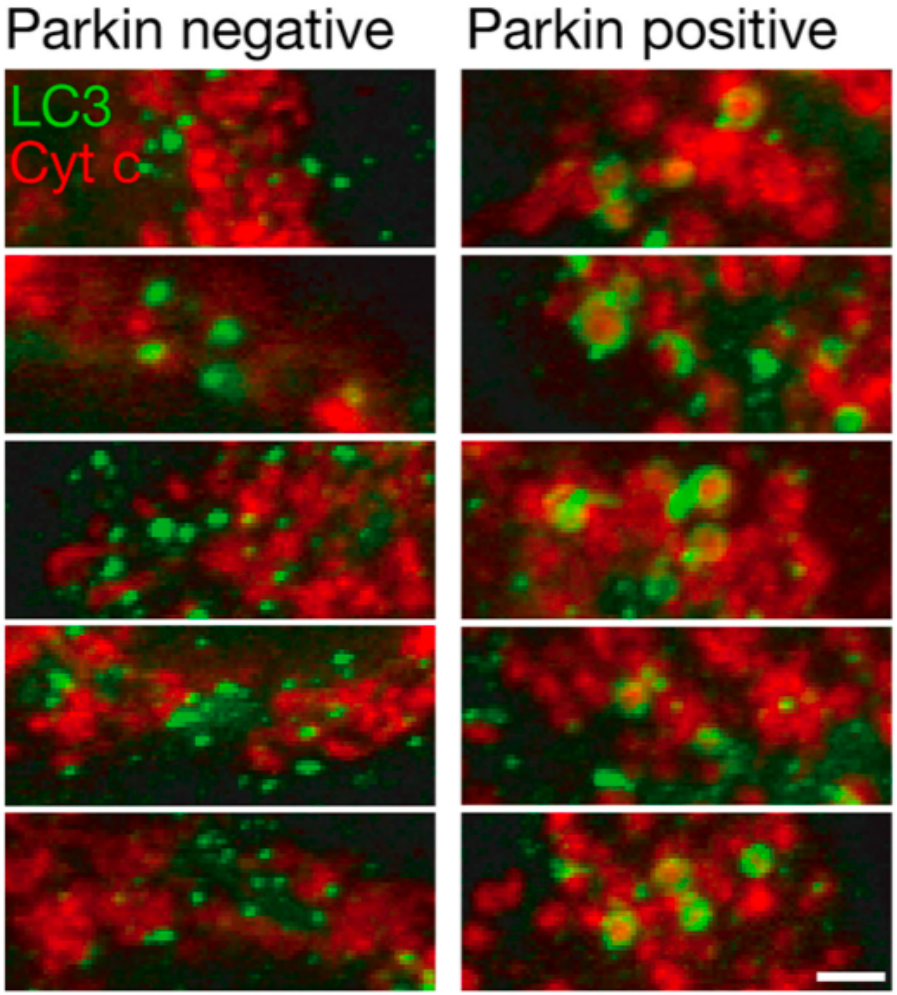
HeLa cells do not express Parkin.
Expression of YFP-Parkin and incubated with 10 μM CCCP for 12 and 48 hours.

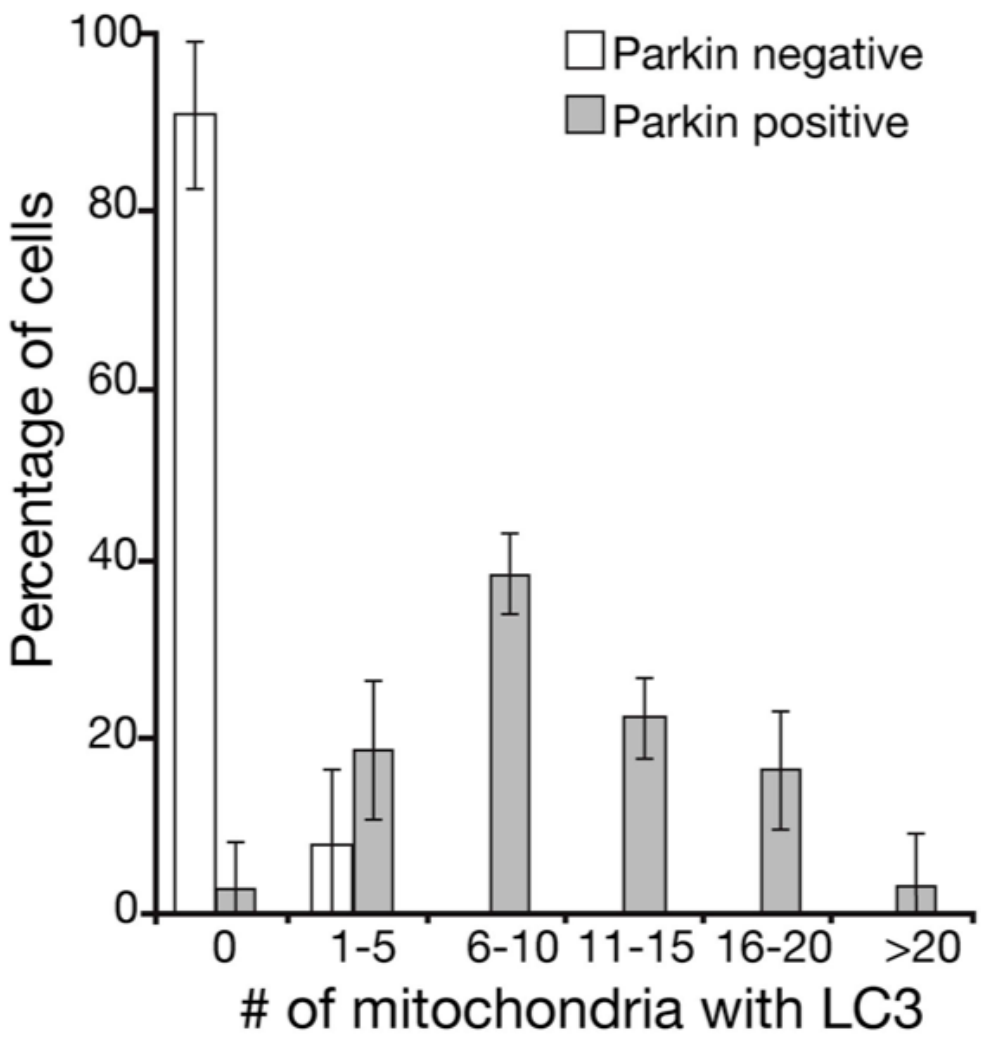
- Aggregation of mitochondria at 12 hrs
- Disappearance of mitochondria at 48 hrs



Narendra et al. (2008) JCB

Parkin-stimulated mitophagy

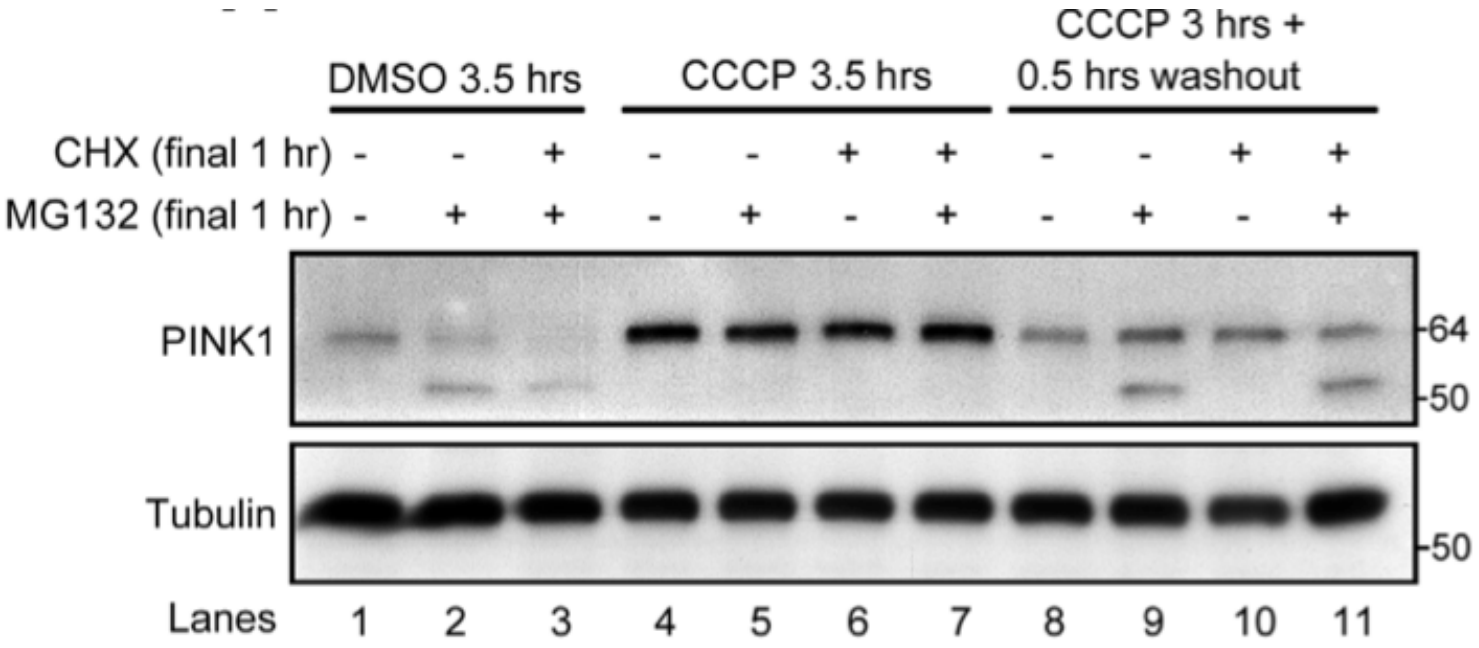


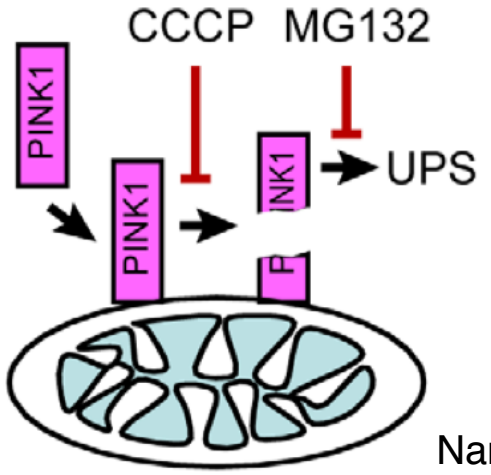


right panel: HeLa cells expressing GFP-LC3 and Parkin, treated with 10 μM CCCP

Narendra et al. (2008) JCB

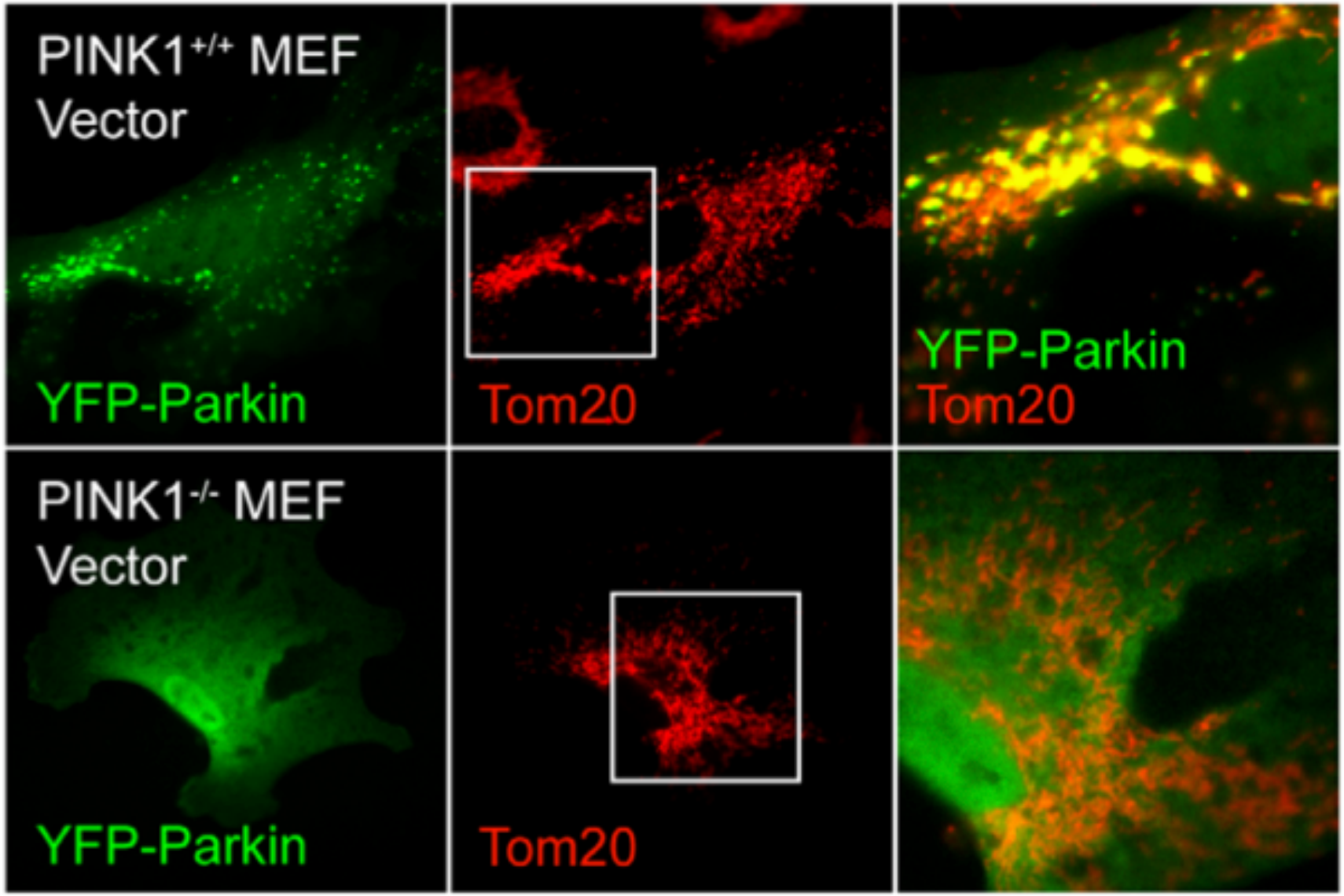
Stabilization of PINK1 on depolarized mitochondria





Narendra et al. (2010) PLOS Biol.

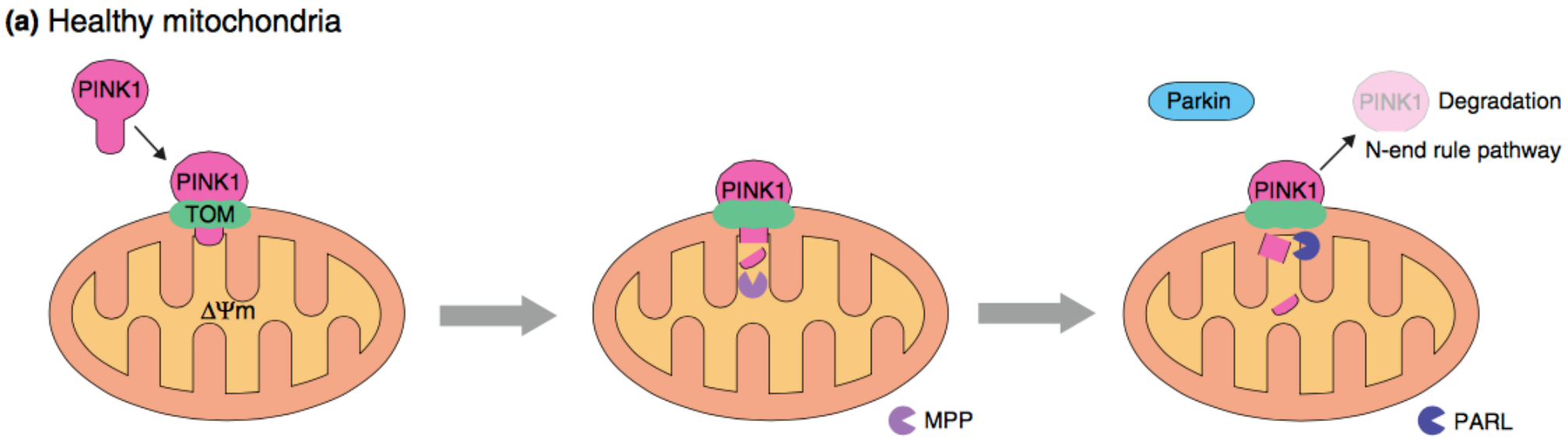
Parkin translocation is PINK1-dependent



Primary MEFs expressing YFP-Parkin, treated with 20 μM CCCP for 3 hours. Immunostained for Tom20.

Narendra et al. (2010) PLOS Biol.

Pink1 degradation on healthy mitochondria

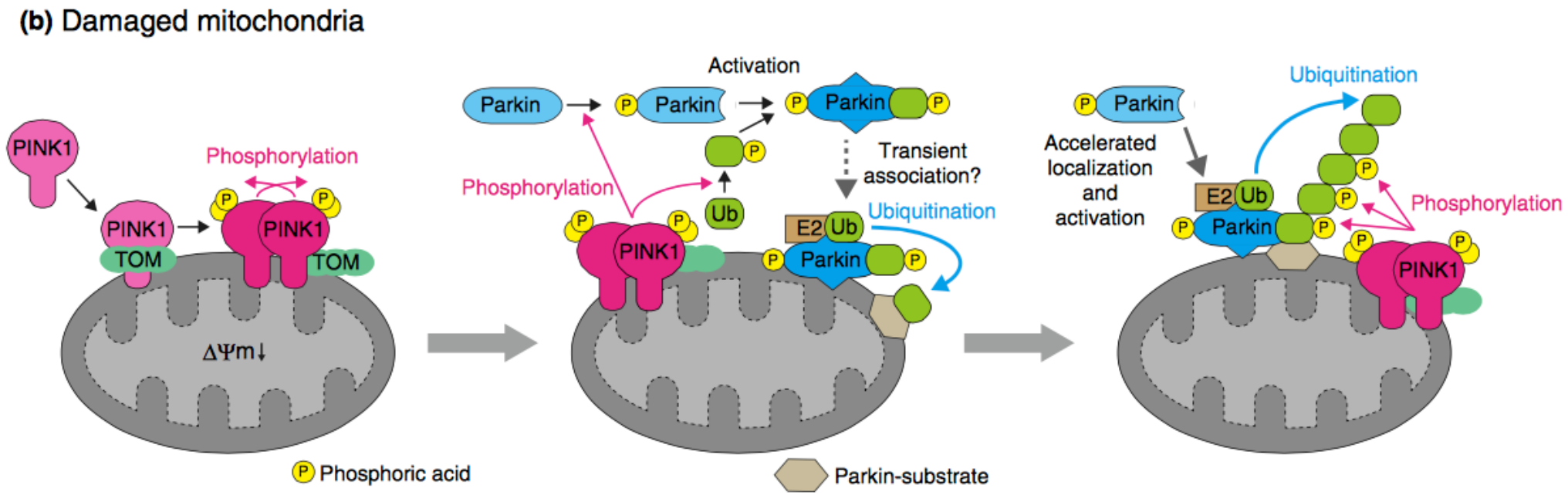


Pink1 levels are kept low on healthy mitochondria:

- Pink1 is partially translocated (membrane potential dependent), the MTS is removed by the mitochondria processing protease, TM segment is stalled in IM, processed by PARL protease.
- The cleaved Pink1 is released to cytosol, degraded by N-end rule and the UPS.

Eiyama & Okamoto (2015) COCB

Phosphorylated ubiquitin acts as a Parkin receptor



Pink1 activates Parkin in positive feedback loop:

- Loss of membrane potential prevents Pink1 translocation. Dimerization and cross-phosphorylation activates Pink1. Also phosphorylates Parkin and Ub.

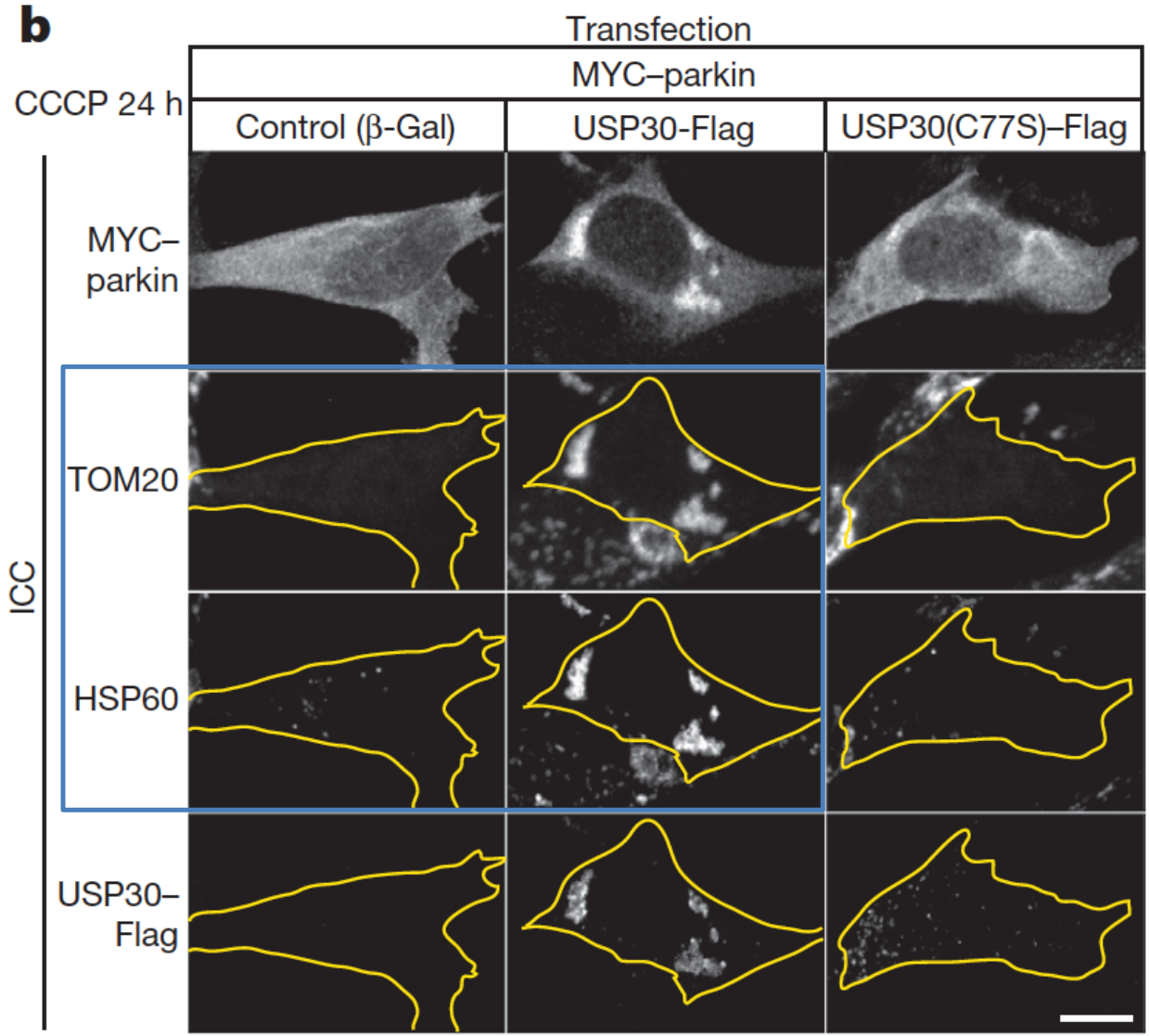
- Phosphorylation of Parkin's UBL domain removes auto-inhibition; increased ligase activity

- Phosphorylation of polyubiquitin on mitochondria causes Parkin recruitment

Eiyama & Okamoto (2015) COCB

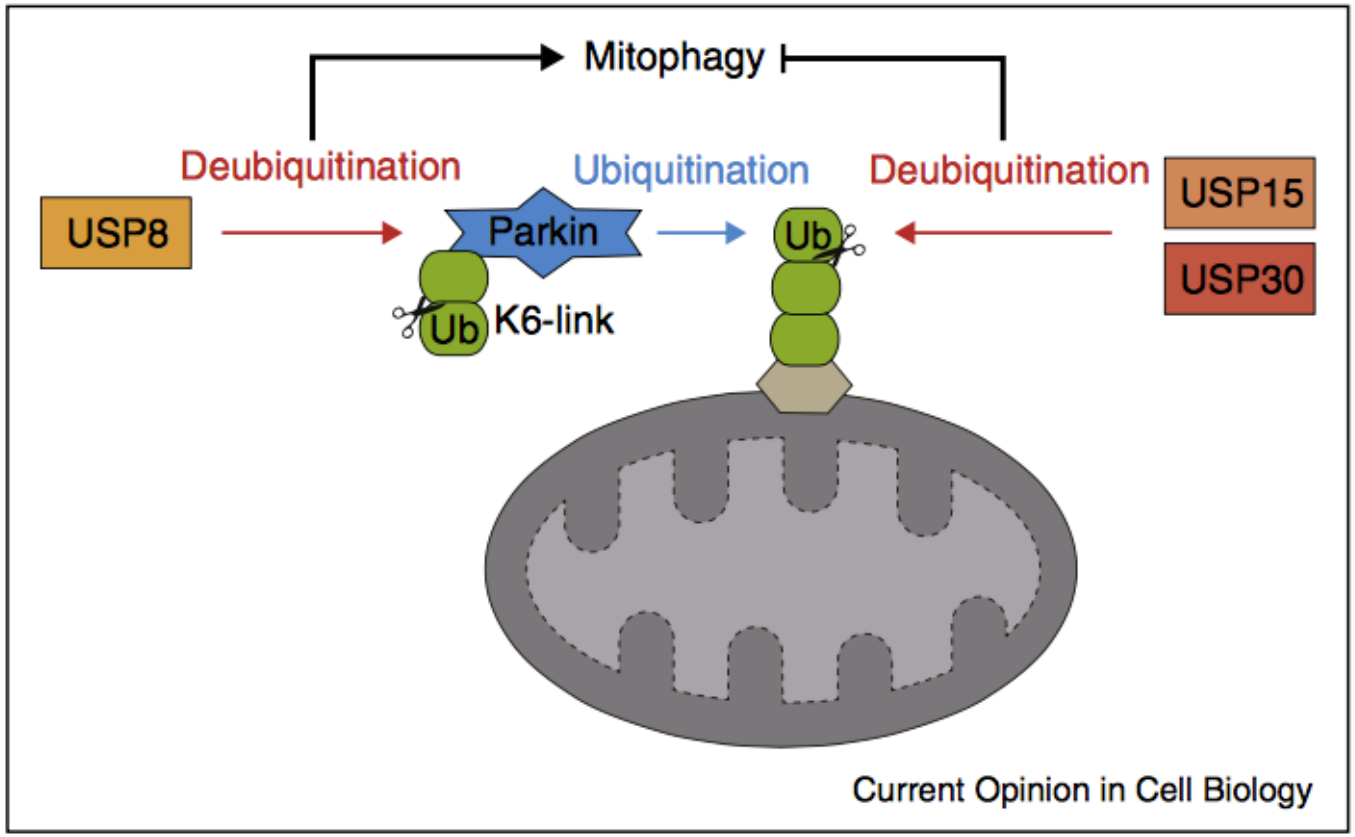
The level of ubiquitination regulates mitophagy

- From library screening, the deubiquitinase USP30 (ubiquitin specific peptidase 30) antagonizes Parkin-mediated mitophagy
- Figure shows dopaminergic SH-SY5Y neuronal cells. Cells transfected with Parkin and treated with CCCP.



Bingol et al. (2014) Nature

Ubiquitination/deubiquitination regulates mitophagy

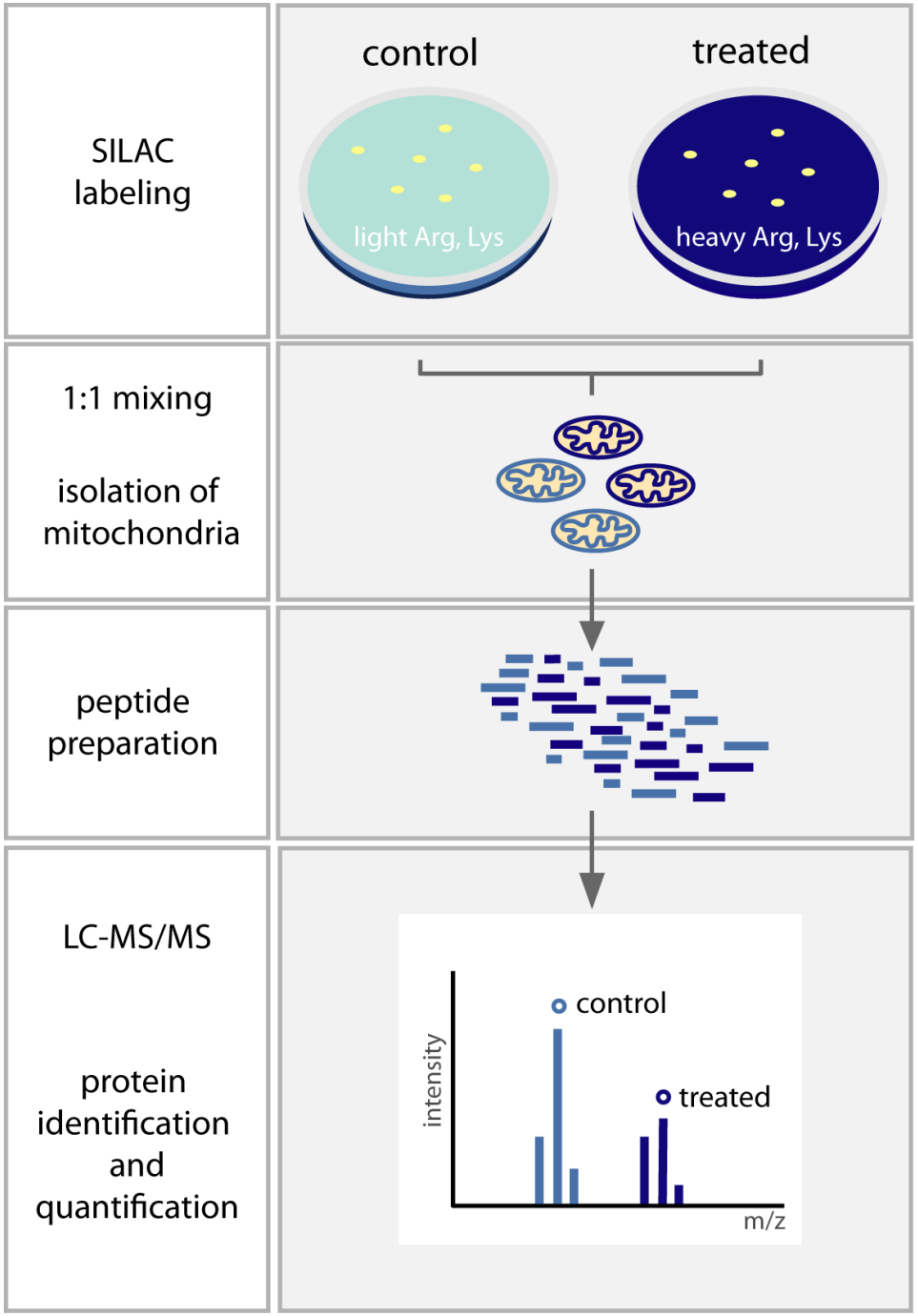


The balance of ubiquitination/deubiquitination is critical:

- Overexpression of USP15 or USP30 can block Parkin-mediated mitophagy

Eiyama & Okamoto (2015) COCB

Quantitative proteomics of depolarized mitochondria by SILAC



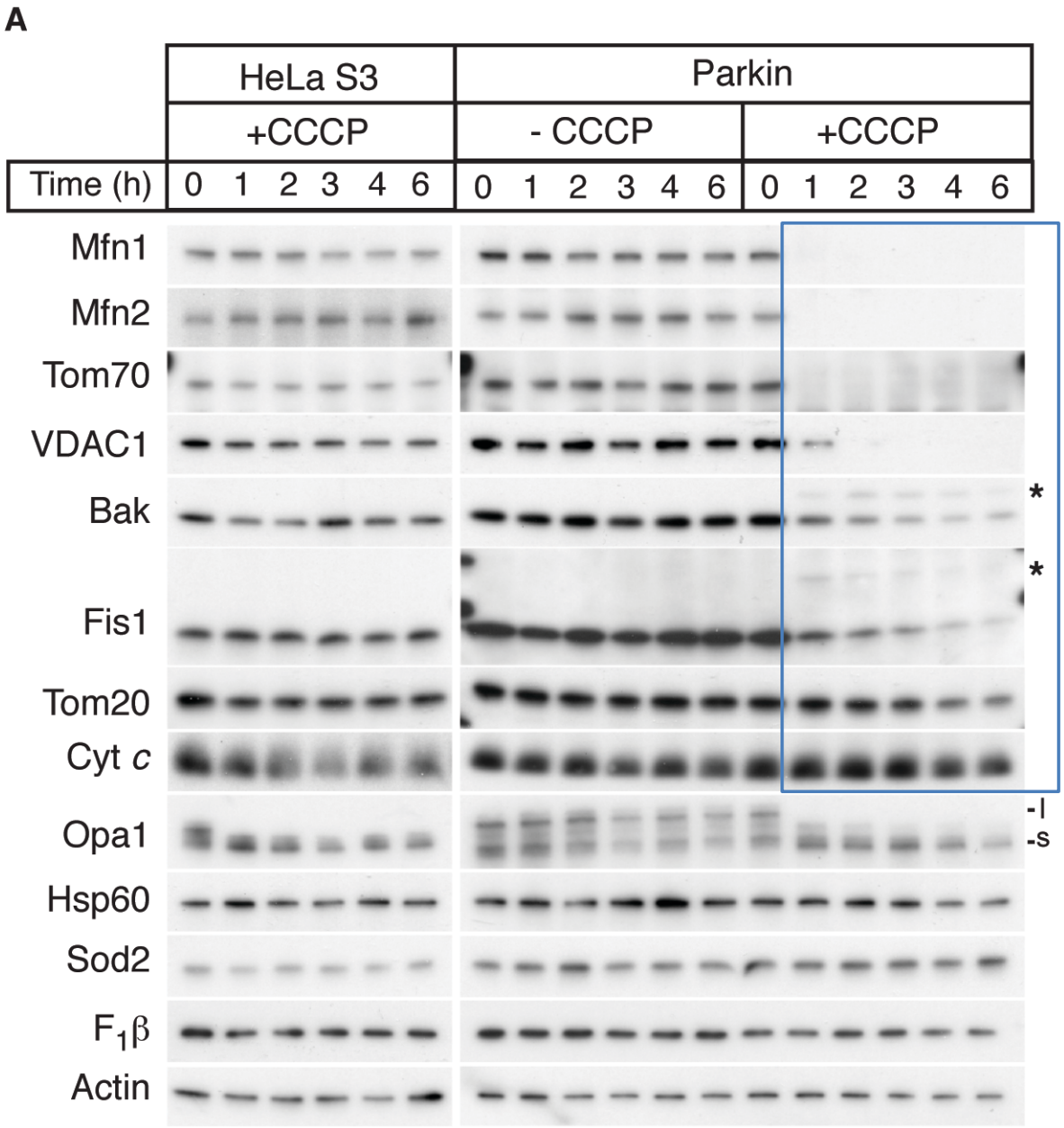
human MitoCarta proteins:	1013
MitoCarta proteins identified:	777
MitoCarta proteins quantified:	766

Protein	Biological function	SILAC ratio	Significance
PARKIN	E3 ubiquitin ligase	13	4.42E-20
DRP1	Mitochondrial fission	6.3	4.78E-07
Autophagy-related			
NBR1	Autophagy adaptor	8.3	2.90E-06
p62/SQSTM1	Autophagy adaptor	5.8	1.75E-06
MAP1LC3B2;MAP1LC3B	Autophagosome component	5.4	3.70E-06
GABARAPL2	Autophagosome component	3.4	7.87E-06
ATP6V1B2	V-type proton ATPase subunit	3.3	1.64E-05
ATP6V1E1	V-type proton ATPase subunit	3.1	4.77E-03
ATP6V1C1	V-type proton ATPase subunit	2.9	7.23E-03
ATP6V1A	V-type proton ATPase subunit	2.9	2.45E-03

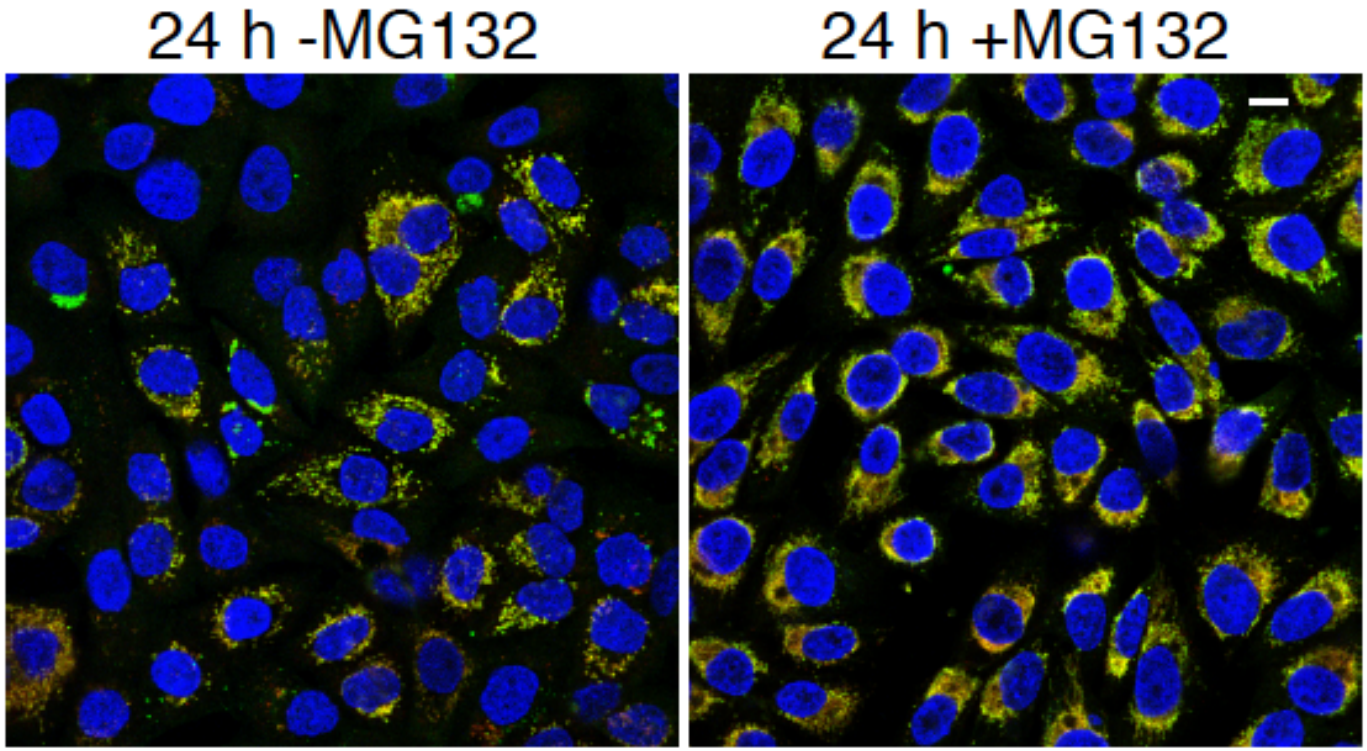
Protein	Biological function	SILAC ratio	Significance
Ubiquitin	protein modification	8.9	9.58E-15
PSMA2	20S Proteasome subunit	4.2	8.08E-05
PSMB5	20S Proteasome subunit	4.2	4.21E-04
PSMA1	20S Proteasome subunit	4.0	5.38E-04
PSMB3	20S Proteasome subunit	4.0	6.20E-04
PSMB6	20S Proteasome subunit	3.9	2.30E-04
PSMA6	20S Proteasome subunit	3.7	1.18E-03
PSMB4	20S Proteasome subunit	3.7	1.96E-03
PSMA7	20S Proteasome subunit	3.7	1.29E-03
PSMA4	20S Proteasome subunit	3.6	4.70E-04
PSMA3	20S Proteasome subunit	3.6	4.85E-04
PSMB1	20S Proteasome subunit	3.3	2.66E-03

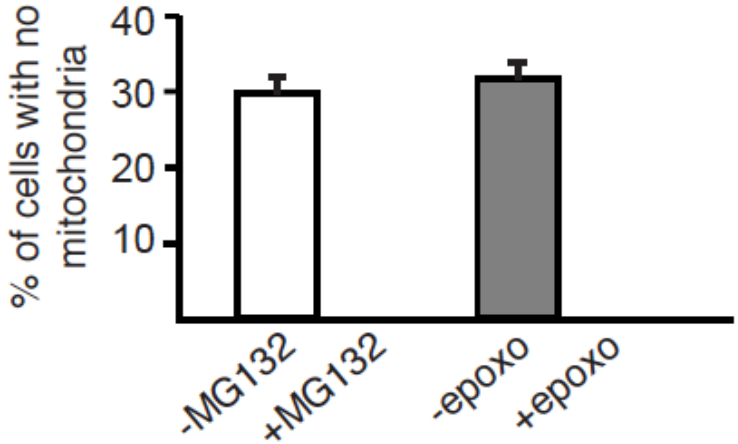
Protein	Biological function	SILAC ratio	Significance
MFN1	Mitochondrial fusion	0.09	6.94E-18
MFN2	Mitochondrial fusion	0.10	2.51E-35
TOM70	Mitochondrial import	0.13	3.78E-40
MIRO1/RHOT1	Mitochondrial transport	0.16	4.29E-15
CPT1A	fatty acid metabolism	0.23	1.62E-21
MOSC2	oxidoreductase	0.26	6.67E-10
MITONEET/CISD 1	regulation of respiration	0.26	8.74E-18
GPAM	glycerolipid synthesis	0.42	3.39E-04
MIRO2/RHOT2	Mitochondrial transport	0.44	4.94E-06
FIS1	Mitochondrial fission	0.55	1.33E-03

Degradation of outer membrane proteins by Parkin

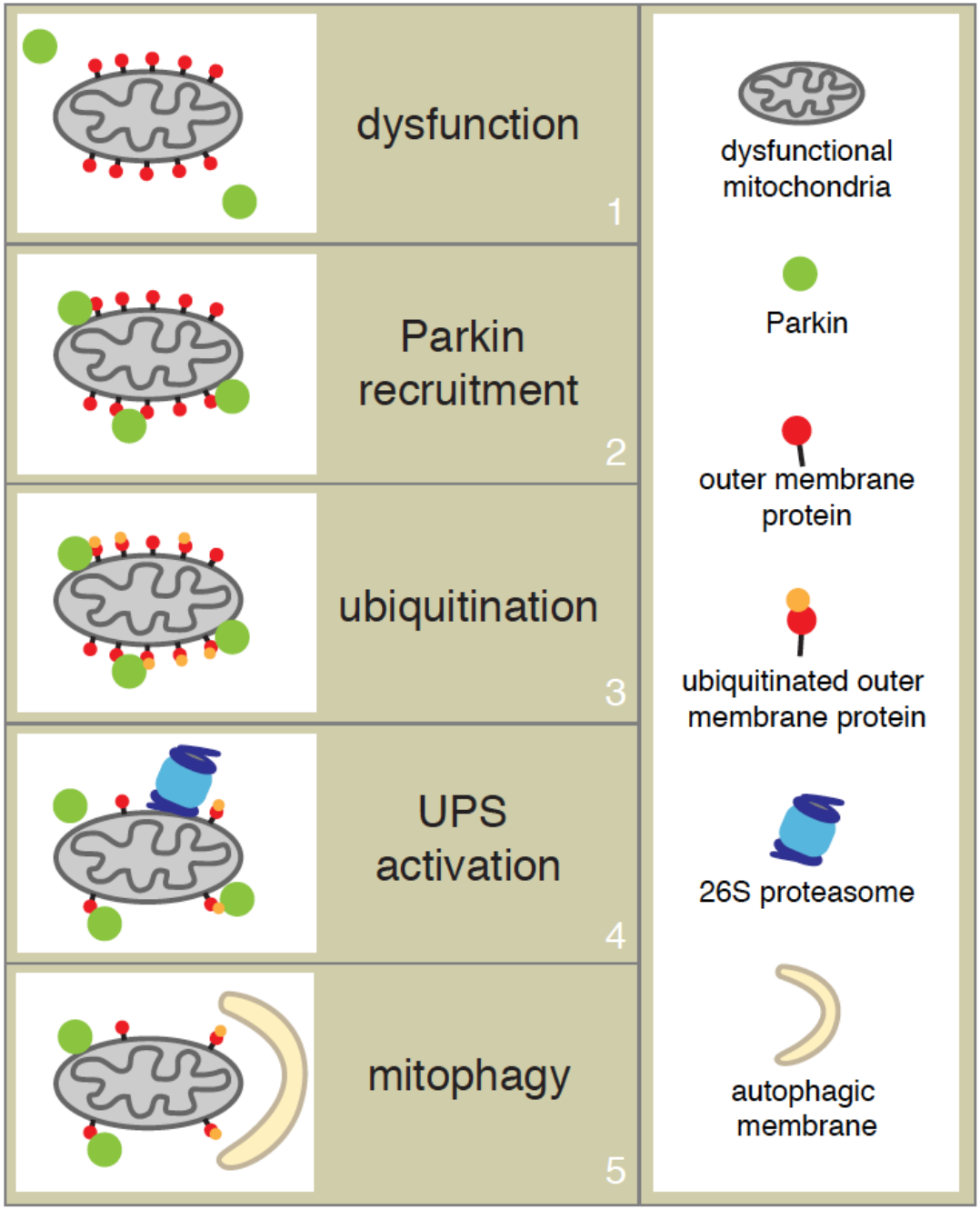


Inhibition of the UPS blocks mitophagy





Parkin functions via the UPS to mediate mitophagy



Cholesterol is a major risk factor for heart disease

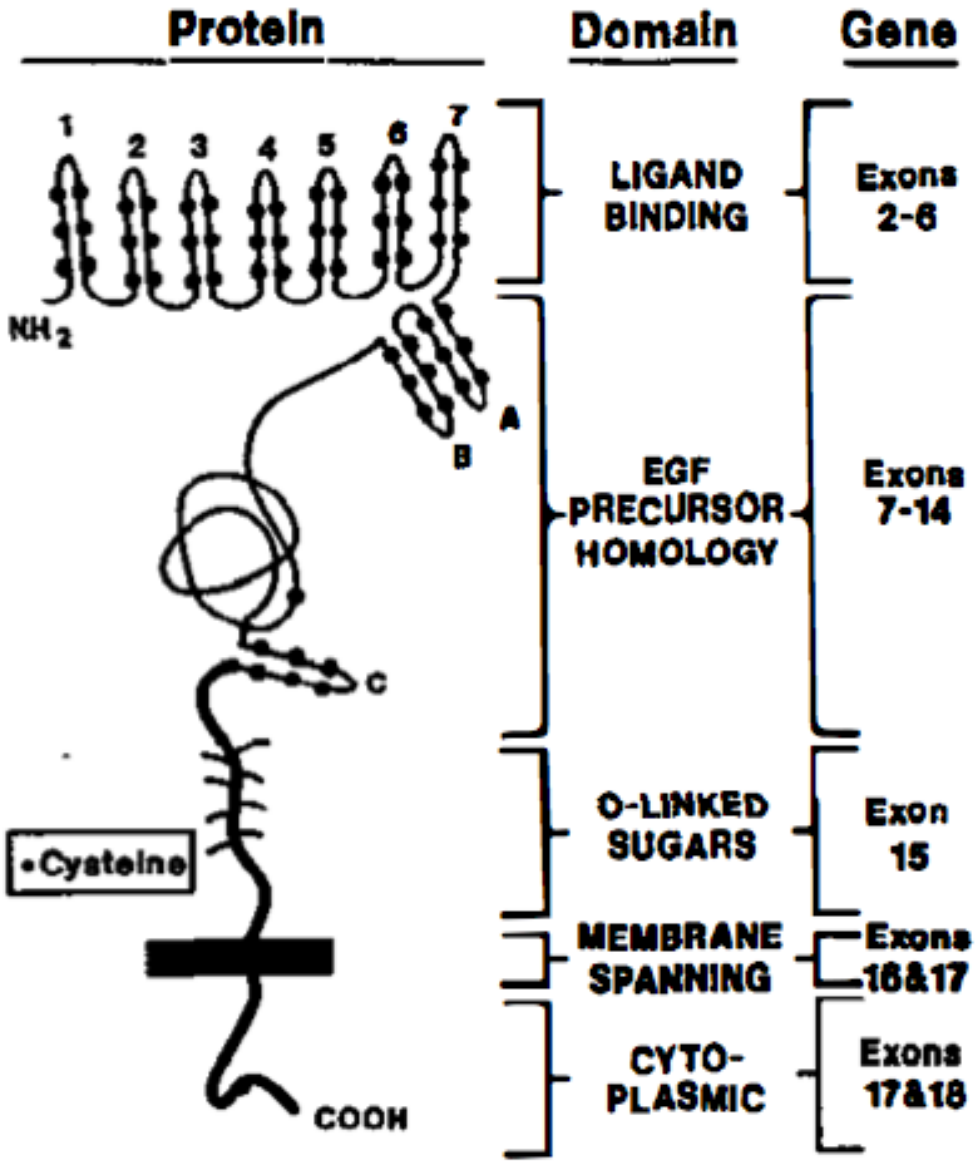
• The Framingham Heart Study: a several decade, longitudinal study of factors that contribute to cardiovascular disease, in the town of Framingham, MA.

• Studied thousands of participants who had no overt heart disease, to identify risk factors or lifestyle factors.

• Among many findings, showed that high serum cholesterol is associated with cardiovascular disease.

• http://www.framinghamheartstudy.org/about/milestones.html

The LDL receptor



- LDL receptor=839 aa

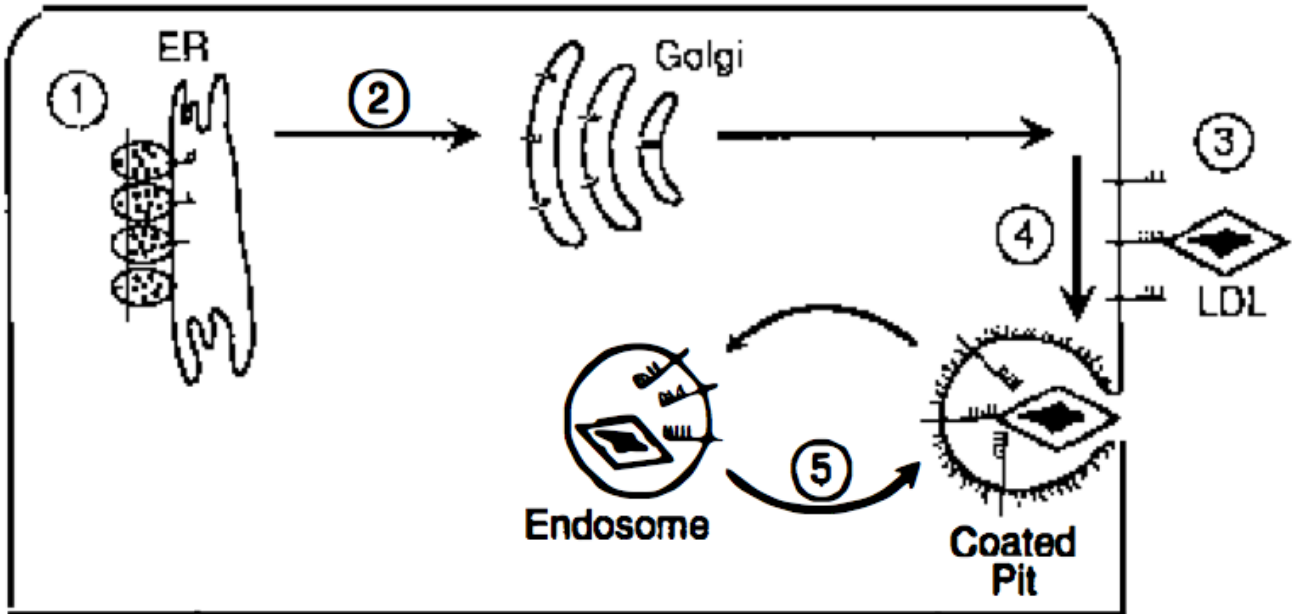
- Ligand binding domain: 7 repeats of LDL module, each ~40 aa, cysteine-rich; each stabilized by disulfide bond formation and calcium binding.

- Each repeat makes an independent contribution to ligand binding.

- EGF Precursor region involved in low pH release and recycling of receptor to the cell surface.

Hobbs et al (1990) Annu. Rev. Genet

Several class of disease mutations reveal LDLR biology



Mutation Class	Synthesis	Transport	Binding	Clustering	Recycling
1	X				
2	→	X			
3	→	→	X		
4	→	→	→	X	
5	→	→	→	→	X

Over 800 mutant alleles are known.

•**Class 1**: null alleles- no detectable protein; relatively rare

• **Class 2**: defective transport from ER to Golgi in pulse-chase experiments; protein is trapped in ER and does not contain complex sugars; common

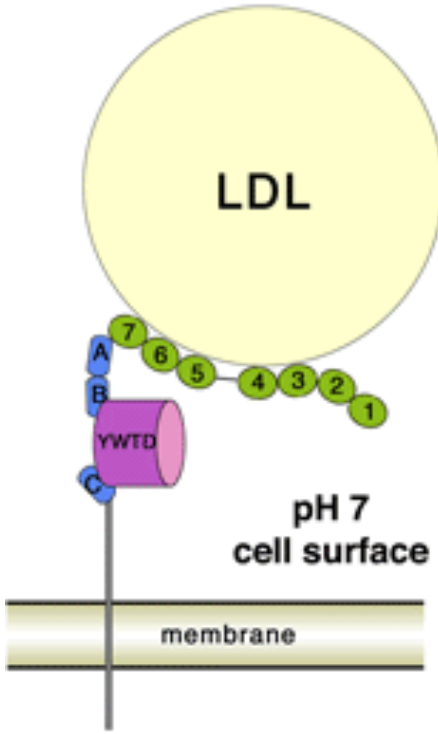
• **Class 3**: protein is on cell surface, but does not bind LDL; mutations in the LDL repeat region or EGF precursor region.

• **Class 4**: internalization-defective; mutations located in the 50 aa cytoplasmic domain; led to identification of PNxY signal in clathrin-mediated endocytosis.

• **Class 5**: recycling defective; mutations in the EGF precursor region; receptor fails to dissociate from ligand in low-pH endosome; receptor degraded.

Hobbs et al (1990) Annu. Rev. Genet

Recycling mutants of LDL receptor



Blacklow lab (HMS)

Several class of lipoproteins transport triacylglycerols and cholesterol in the blood

Lipoproteins are complexes of lipids and proteins. Five major types are:

• Chylomicrons: transport dietary triacylglycerols and cholesterol from the intestines to the rest of the body.

• Very low density lipoprotein (VLDL)
• Intermediate density lipoprotein (IDL)
• Low density liporotein (LDL)
    - transport internally produced triacylglycerols and cholesterol from the liver to other tissues

• High density lipoprotein (HDL): transport internally produced cholesterol from tissues to liver.

Mutations in ApoB found in FH

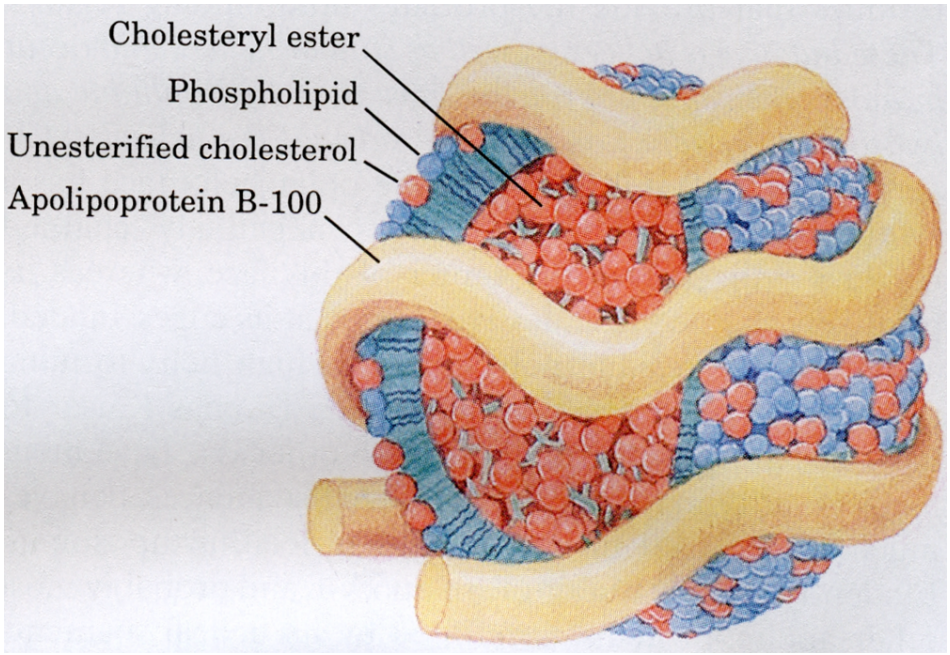
• Apolipoprotein B is the main protein component of LDL particles. An LDL particle contains a single molecule of Apo B-100.

• High Apo B levels are associated with higher rates of cardiovascular disease.

• In some FH patients, the LDL does not bind to normal LDL receptor.

• Binding of LDL to LDL receptor occurs via the apolipoprotein B component of LDL

• Point mutations in residue 3500 cause defective LDL receptor binding.



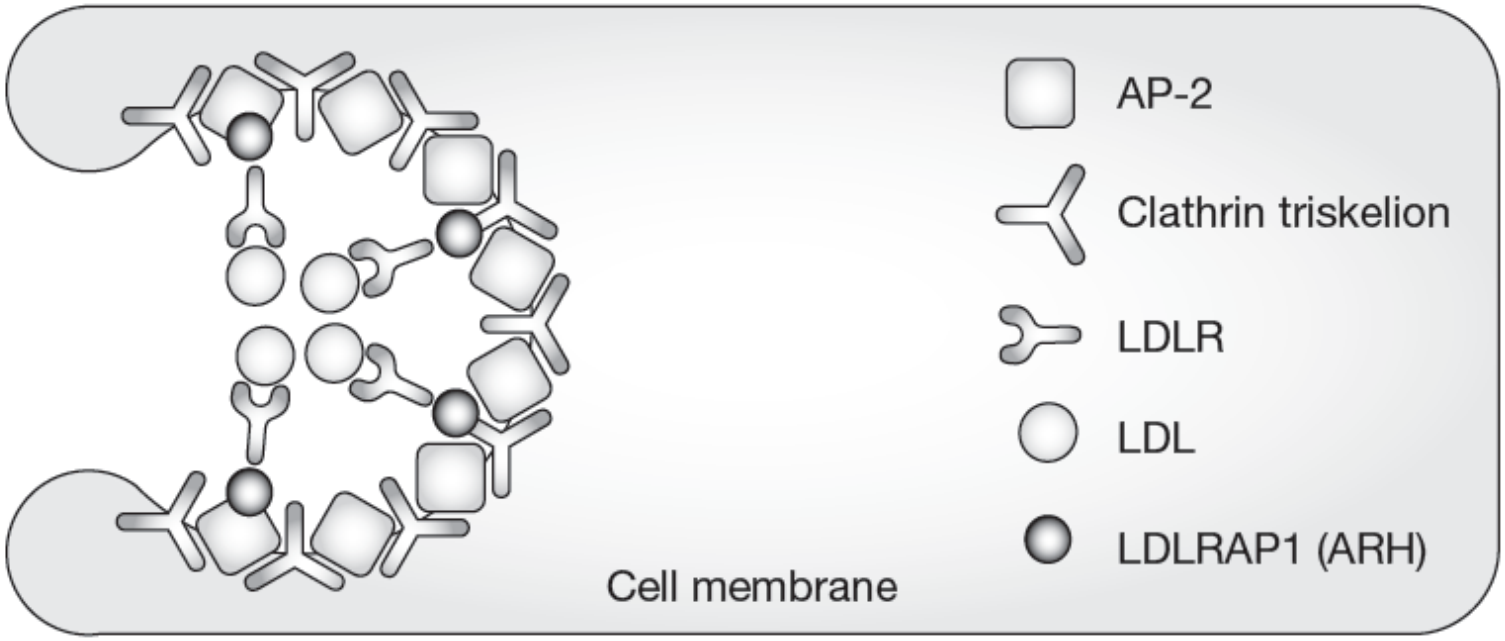
Autosomal recessive form of FH caused by mutations in LDLRAP1

• Most FH has an autosomal dominant pattern of inheritance.

• There is an autosomal recessive form in which homozygous individuals have normal production of LDL receptor, but they fail to be internalized. (this defect found in patient lymphocytes, but not fibroblasts)

• Loss of function mutations in LDLRAP1 (LDL receptor adapter protein 1).

• Thought to be an adaptor protein that interacts with the cytosolic domain of the LDL receptor to recruit it to clathrin-coated pits.



Soutar & Naoumova (2007) Nat Clinical Practice

The correlation between serum cholesterol and heart disease

- High serum LDL ("bad cholesterol") associated with higher rates of heart disease.

- High serum HDL ("good cholesterol") associated with lower rates of heart disease. However, it is unclear whether increasing HDL can reduce incidence of heart disease. In addition, some populations with naturally high HDL do not show protection against heart disease.

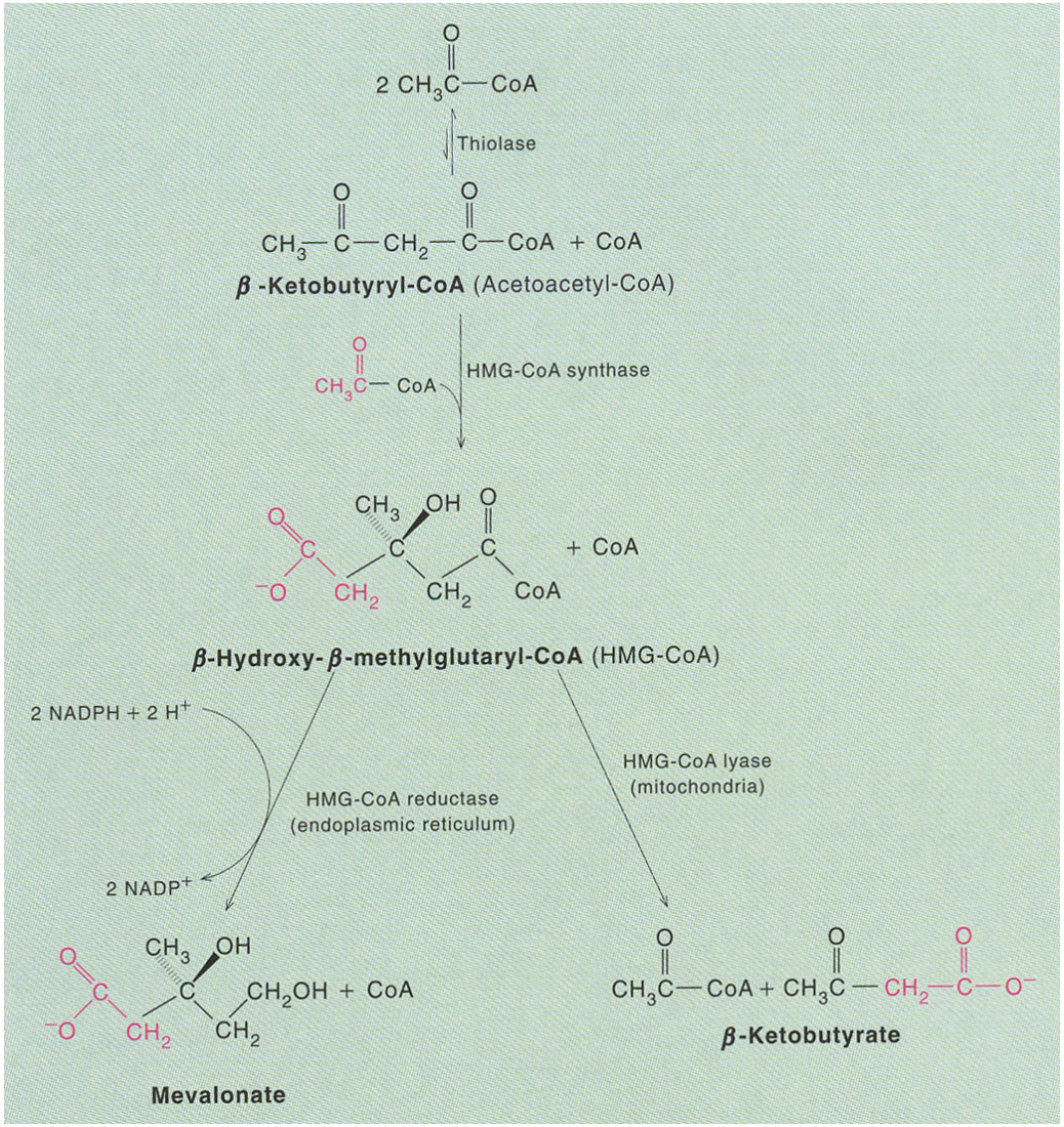
- Commonly argued that lowering total cholesterol and LDL would improve cardiovascular health.

- The main source of cholesterol in the body is generated internally (versus coming directly from the diet).

HMG-CoA reductase controls the rate limiting step in cholesterol synthesis

Inhibition of HMG-CoA reductase:

- decreases endogenous production of cholesterol
- increases expression of LDL receptors on hepatocytes, thereby removing LDL particles from blood
- should reduce serum cholesterol levels



Zubay, 1998

Discovery of natural statins from fungi: Akira Endo

"While living in New York, I was very surprised by the large number of elderly and overweight people, and by the rather rich dietary habits of Americans compared to those of the Japanese. In the residential area of the Bronx where I lived, there were many elderly couples living by themselves, and I often saw ambulances coming to take an elderly person who had suffered a heart attack to the hospital.

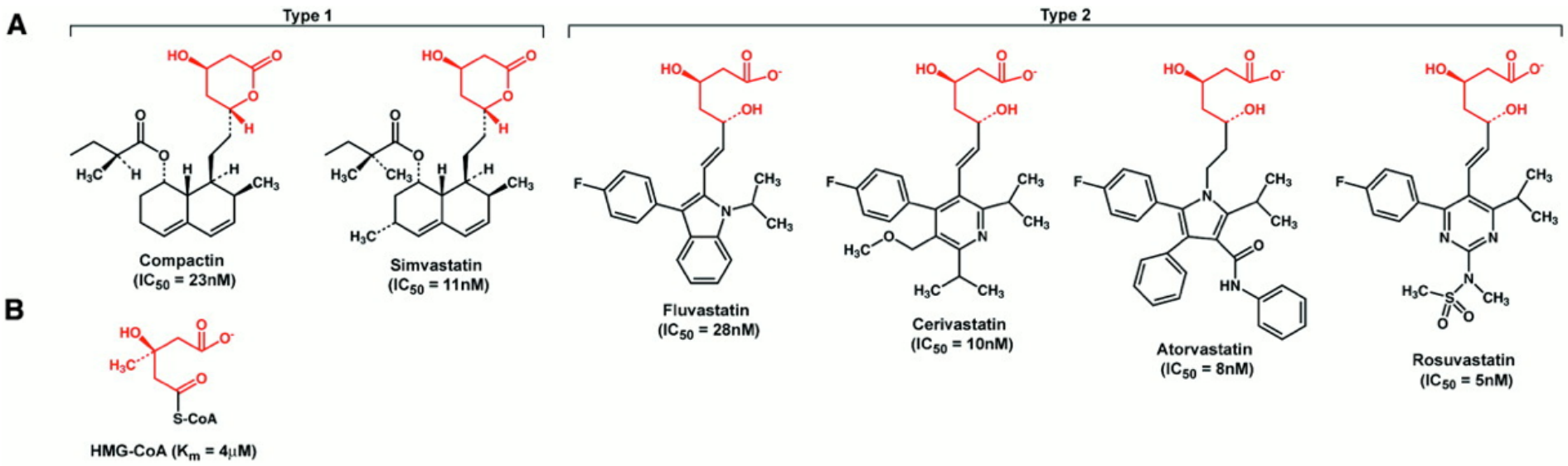
... My experience of living in New York made me realize the importance of developing a cholesterol-lowering drug.

...I speculated that a cholesterol-synthesis inhibitor, particularly a HMG-CoA reductase inhibitor, would be an effective cholesterol-lowering agent.

In 1971, I speculated that microbes would produce antibiotics that inhibited HMG-CoA reductase as a defense mechanism against other microbes that require sterols and/or other mevalonate-derived isoprenoids for their growth, and we created a research unit to isolate such products, focusing on fungi as a source of these metabolites."

Akira Endo, Nature Medicine (2008)

Statins inhibit HMG-CoA reductase



- Statin inhibit the committed step of cholesterol biosynthesis:

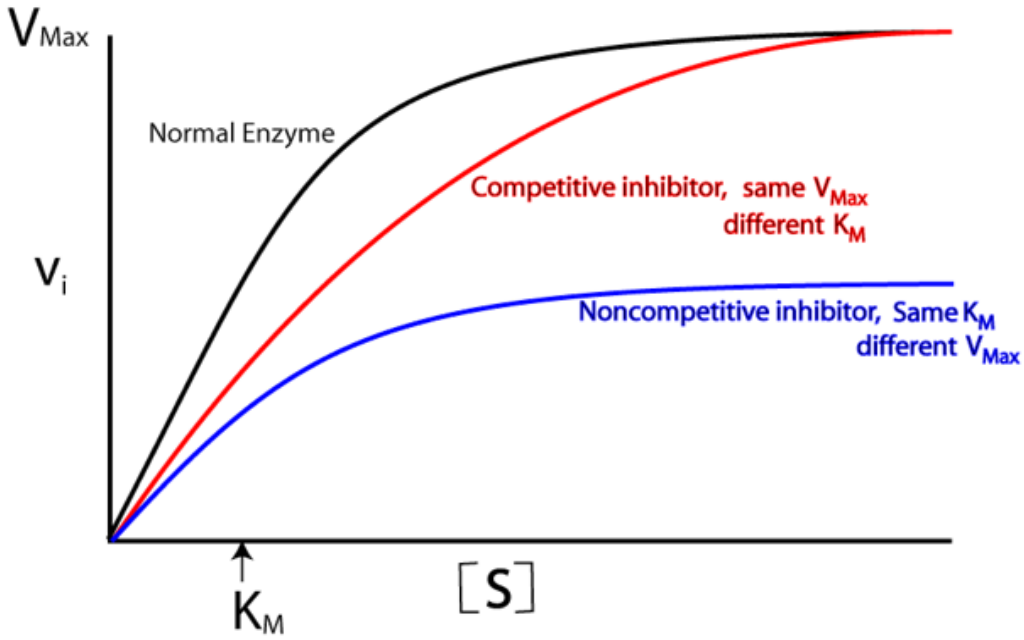
  (S)-HMG-CoA + 2 NADPH + $2H^+$ ➔ (R)-mevalonate + 2 $NADP^+$ + CoASH

- HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A

- Statins have an HMG-like moiety and additional hydrophobic rings.

- Statins are competitive inhibitors of HMG-CoA but not NADPH

Features of competitive inhibitor



http://faculty.une.edu/

• Competitive inhibitor: binding of inhibitor and substrate to enzyme is mutually exclusive.

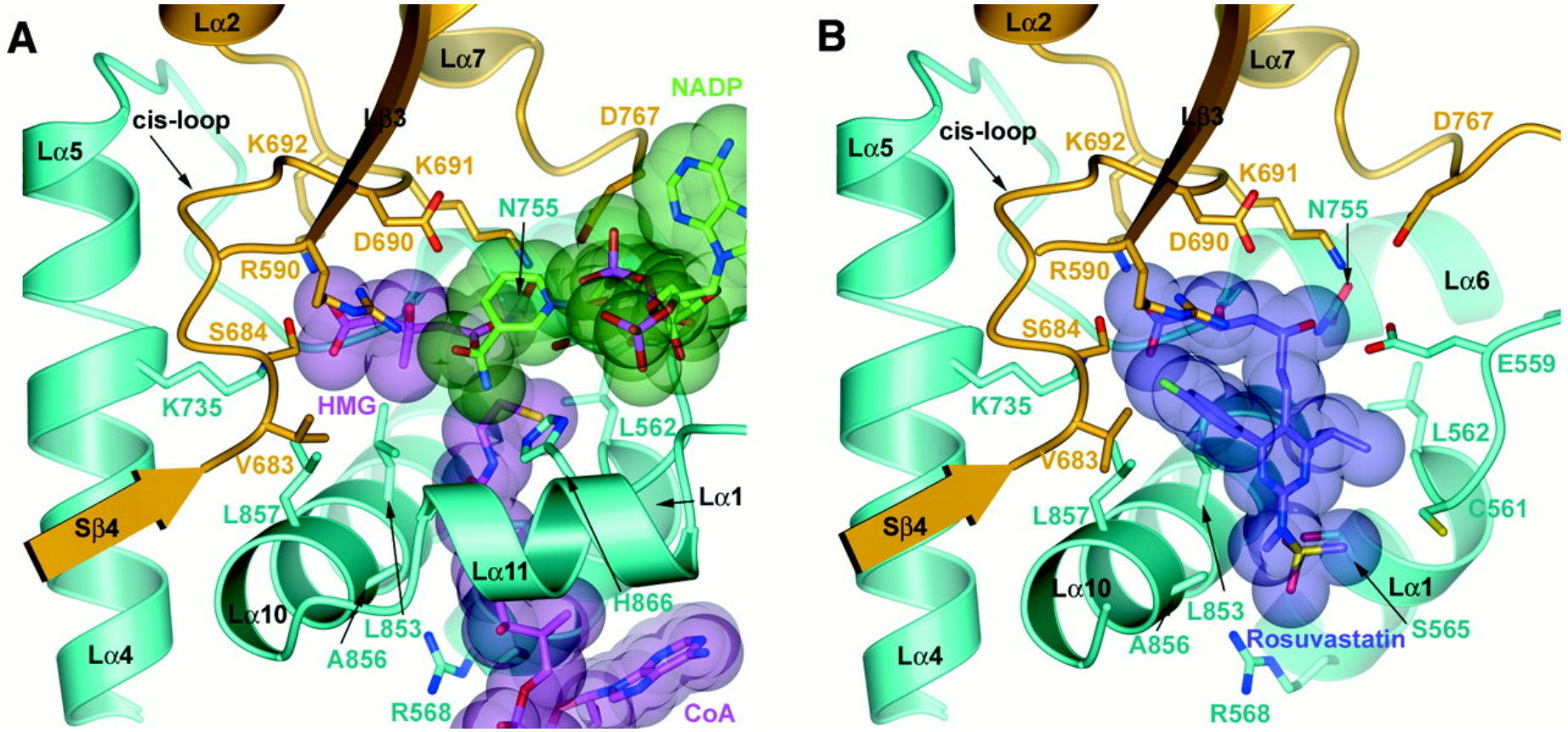
• Most commonly, this is due to binding of the inhibitor to the active site of the enzyme, but other modes possible.

• In the presence of the inhibitor, the velocity of the reaction is reduced.

• In presence of inhibitor, the apparent affinity of the enzyme and substrate is reduced.

• High concentrations of the substrate can out-compete the inhibitor.

Structure of statin bound to HMG-CoA reductase



(S)-HMG-CoA + 2 NADPH + 2H$^+$Ⓟ (R)-mevalonate + 2 NADP$^+$ + CoASH

A: Active site of HMG CoA reductase (HMGR) bound to HMG, CoA (both magenta), and NADP (green). HMGR is a dimer (one monomer yellow; other teal).
B: Rosuvastatin (Crestor) (purple) bound to HMGR

Structure explains why statins compete for HMG-CoA binding but not NADPH binding.

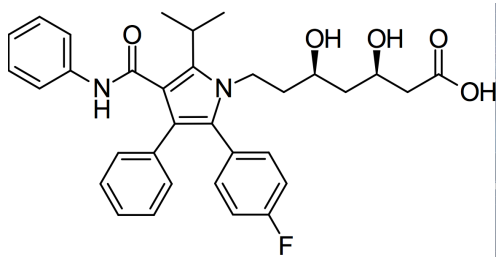
Istvan & Deisenhofer (2001) Science

Statins are among the most highly prescribed drugs

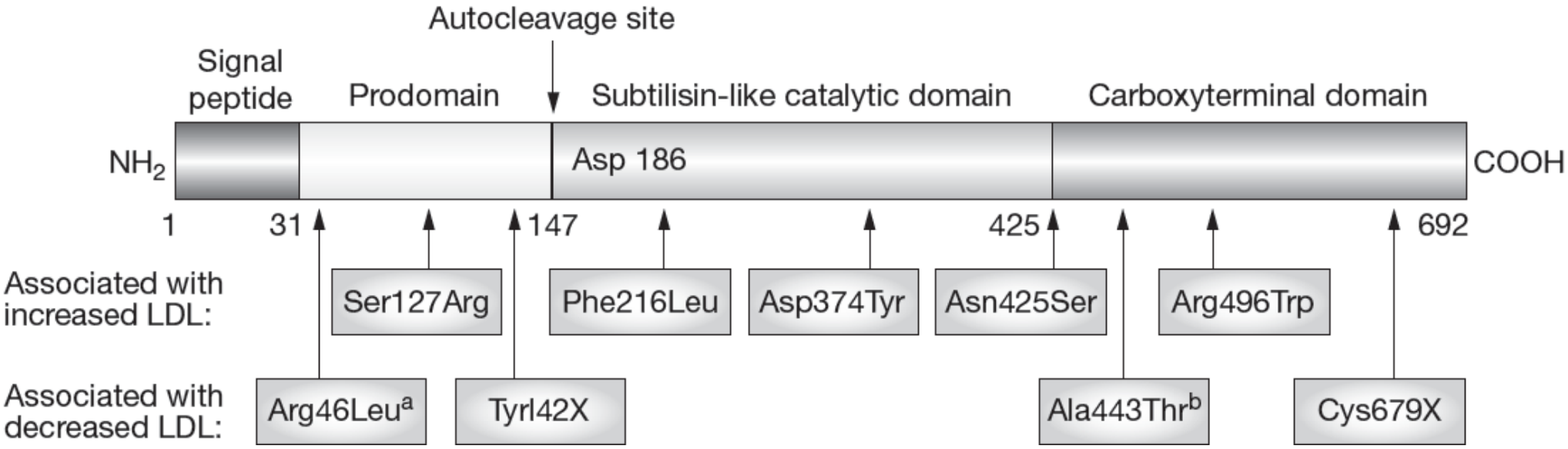
• Lipitor (Atorvastatin) is the most popular statin.

• The best-selling drug in history, but generics are now on the market.

• Some question their utility in people without a history of heart disease.



Mutations in PCSK9 associated with FH or protection against heart disease



• PCSK9: proprotein convertase subtilisin/kexin type 9, a putative protease secreted into the plasma by the hepatocytes.

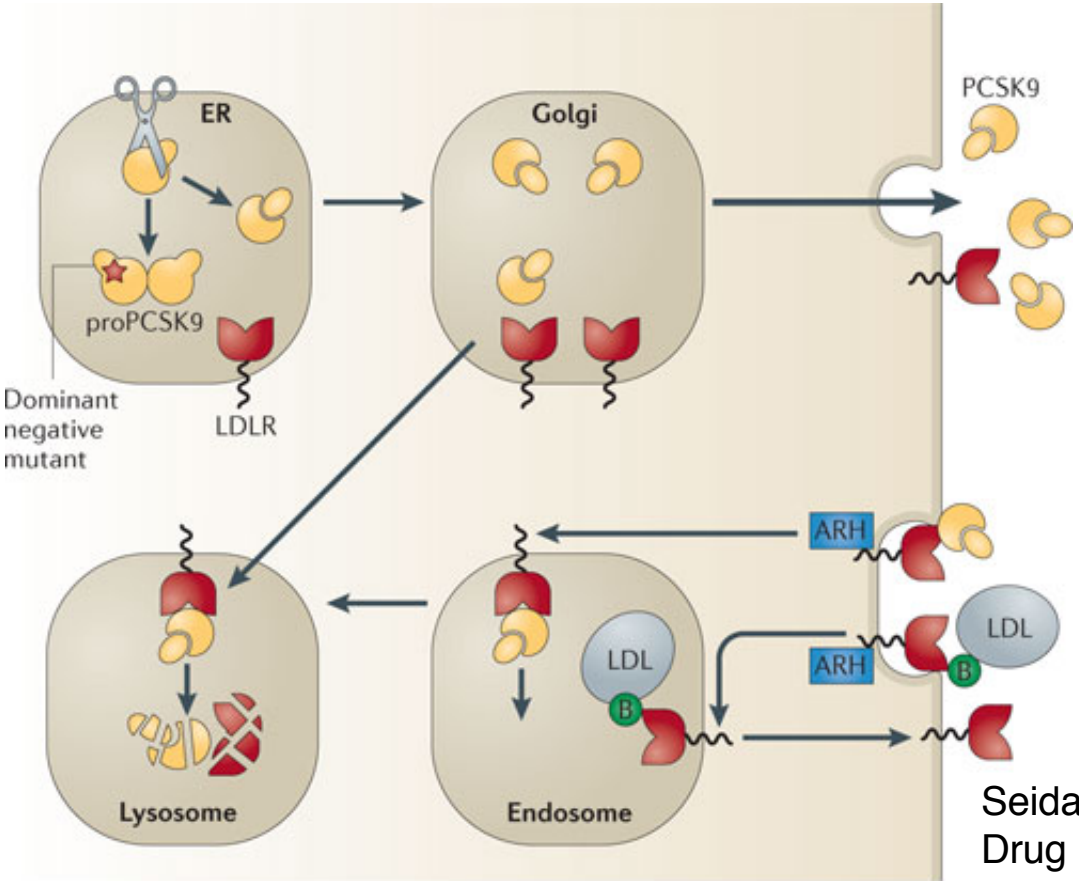
• Some gain-of-function mutations cause autosomal dominant FH.

• Overexpression of PCSK9 reduces LDL receptor levels.

• Some loss of function mutations associated with lower LDL levels and protection against cardiovascular disease, with no adverse effects

Soutar & Naoumova (2007) Nat Clinical Practice

PCSK9 promotes downregulation of LDL receptor



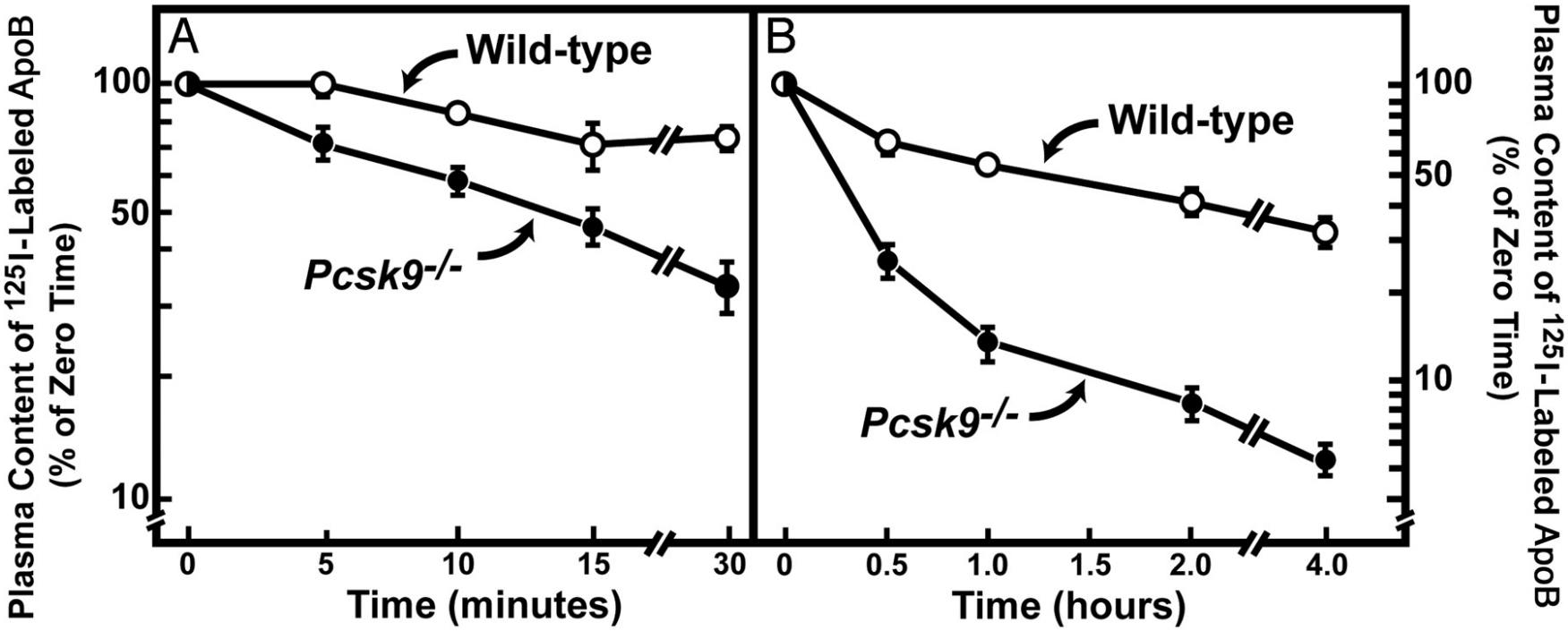
Seidah & Prat (2012) Nat Rev Drug Discovery

• Model: PCSK9 (nonenzymatically) binds to LDL receptor (acting as chaperone) and promotes its degradation via the endosome and lysosome (versus recycling LDLR back to cell surface).

• Gain of function mutations result in lower LDL receptor levels.

• Loss of function mutations result in enhanced LDL receptor levels.

Plasma clearance of 125I-labeled LDL in WT and Pcsk9–/– mice.



Liver specific knockout of PCSK9 in mice. 3-fold increase in LDL receptor levels in liver are associated with ~5-fold increase in LDL clearance from blood.

Rashid S et al. PNAS 2005;102:5374-5379

A monoclonal antibody against PCSK9 lowers serum cholesterol

Variable	Placebo	REGN727		
		50-mg Dose	100-mg Dose	150-mg Dose
Subjects with FH taking atorvastatin				
No. of subjects	6	5	5	5
LDL cholesterol				
At baseline (mg/dl)	133.2±20.7	125.0±12.1	135.8±41.1	140.2±26.2
On day 57 (mg/dl)	137.2±12.5	80.6±21.9	60.0±15.7	65.4±21.2
Difference in percent change from baseline vs. placebo (percentage points)†		−41.4	−57.6	−55.7
P value vs. placebo†		<0.001	<0.001	<0.001

• REGN727 (Regeneron) is a humanize monoclonal antibody against PCSK9. It blocks the binding of PCSK9 to the LDL receptor.

• Intravenous or subcutaneous treatment led to lowered cholesterol levels. Works in combination with statins.

• Other monoclonal antibodies have been developed by other companies.

Stein et al (2012) NEJM