Pharmacology of bisphosphonates

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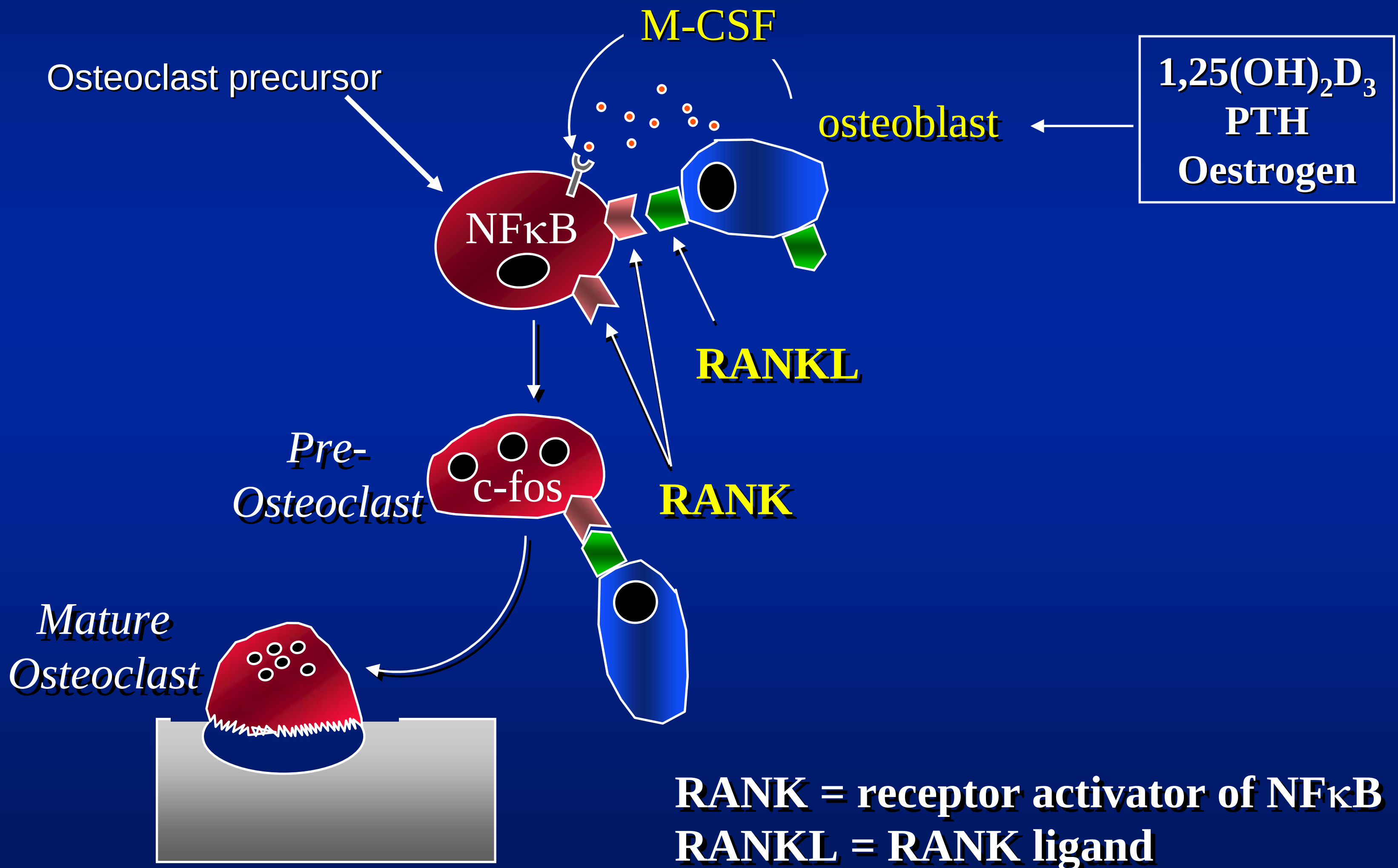
Outline

- Introduction to osteoclasts
- Culture and analysis of osteoclasts *in vitro*
- Mechanism of action of bisphosphonates
- Mechanism of action of phosphonocarboxylate analogues of bisphosphonates
- Localisation of these compounds in bone

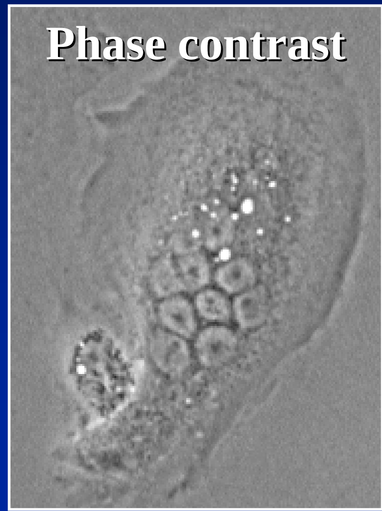
Formation of osteoclasts

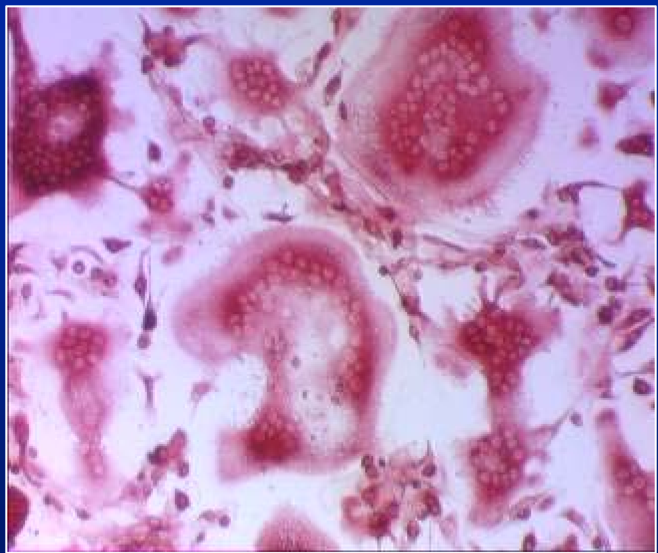


RANK = receptor activator of NFκB
RANKL = RANK ligand

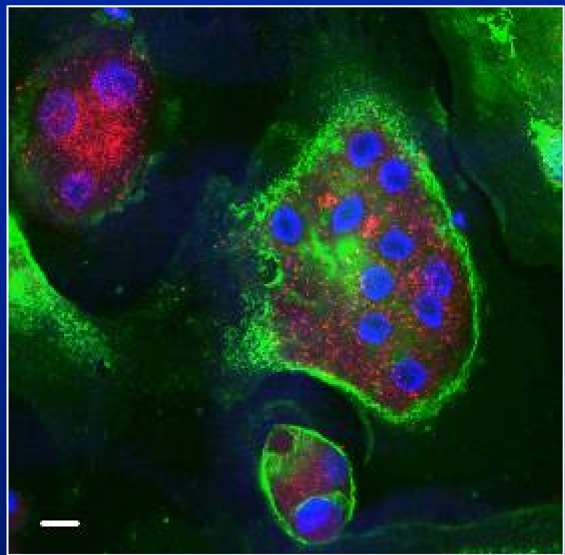
Identifying features of osteoclasts

- Multinucleated
- Have high levels of TRAP (tartrate-resistant acid phosphatase)
- Abundance of acidic vesicles
- $\alpha_v\beta_3$ integrin (vitronectin receptor) on the cell surface
- Expression of cathepsin K

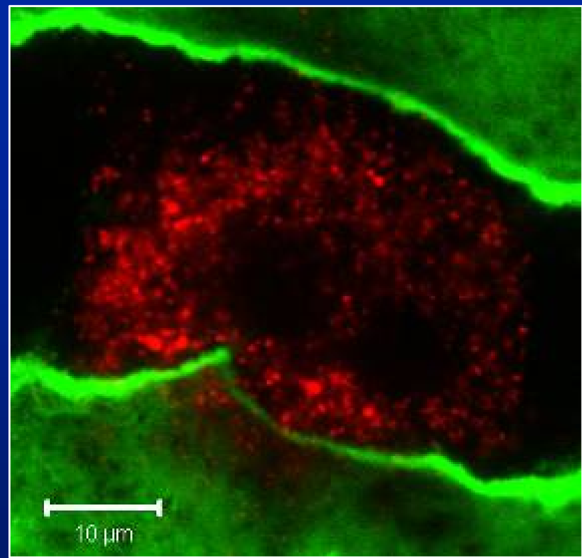




Staining for TRAP activity



vitronectin receptor (green) acidic vesicles (red)

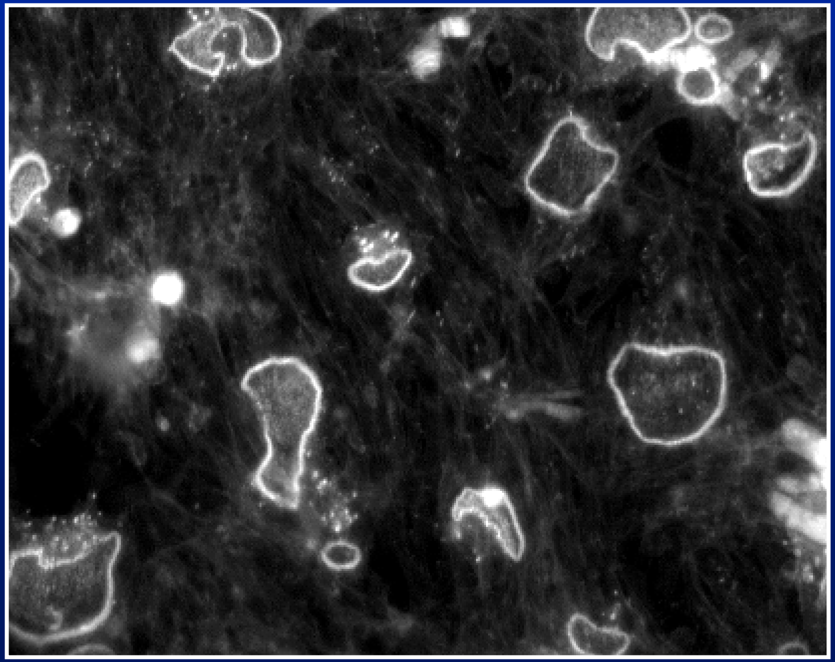


Cath K activity (red) (live osteoclast)

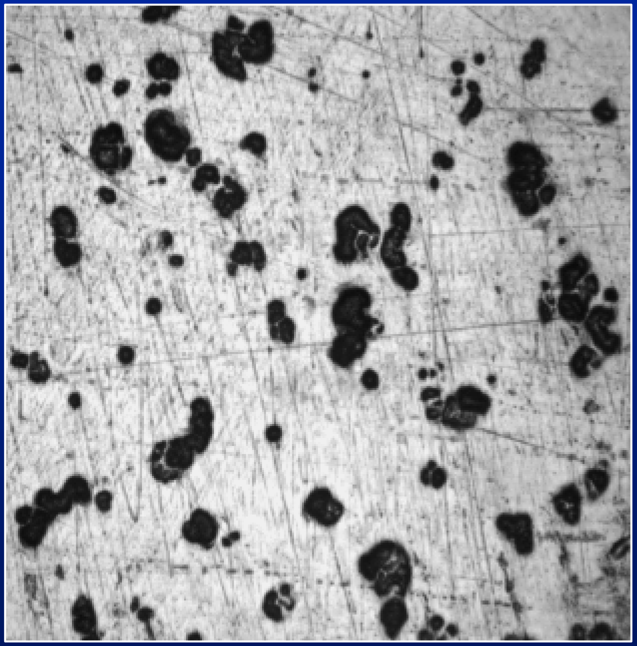
Identifying features of resorbing osteoclasts

- Formation of distinct membrane domains (e.g. ruffled border)
- Polarisation of cytoskeleton into an F-actin ring
- Resorption of bone!

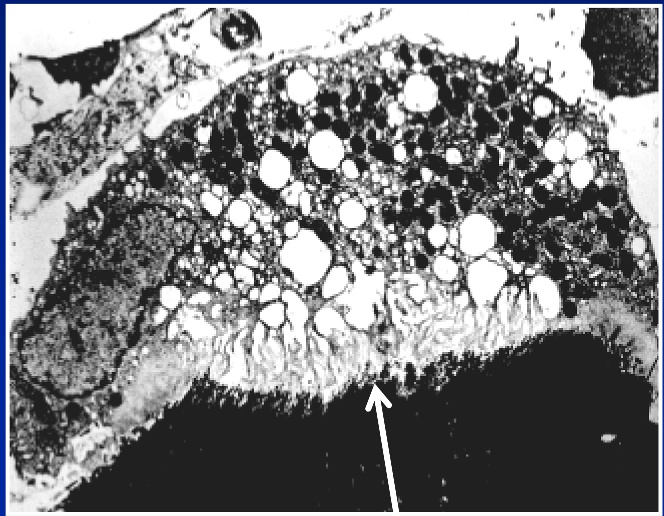
Actin rings visualised by staining with fluorescent phalloidin conjugates

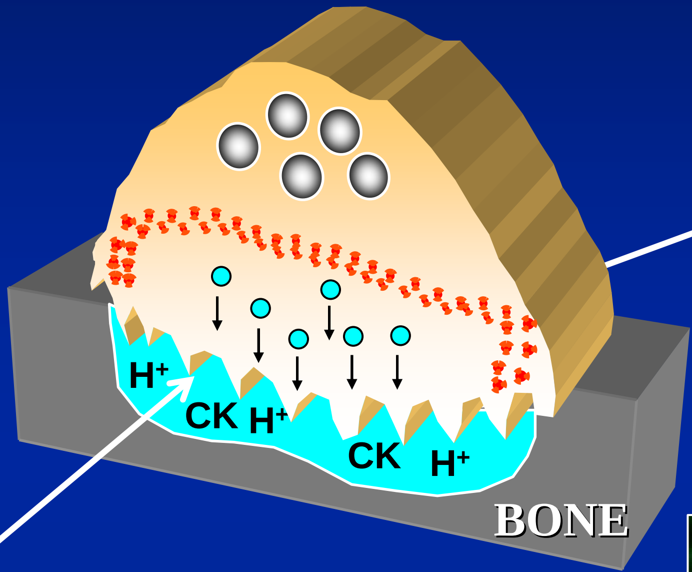


Resorption pits on bone surface visualised reflected light microscopy

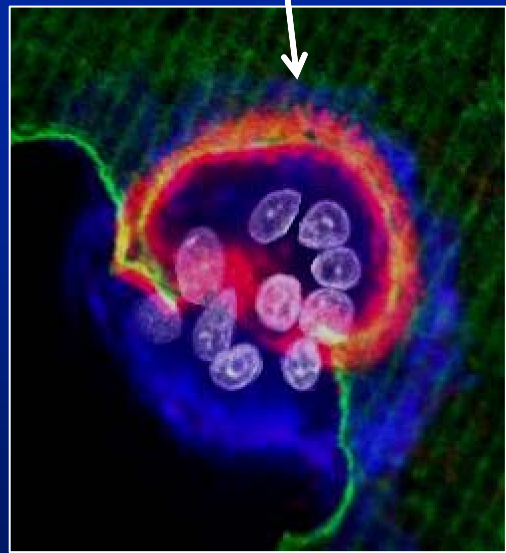


Osteoclasts are specialised bone-resorbing cells





F-actin 'ring'
sealing zone



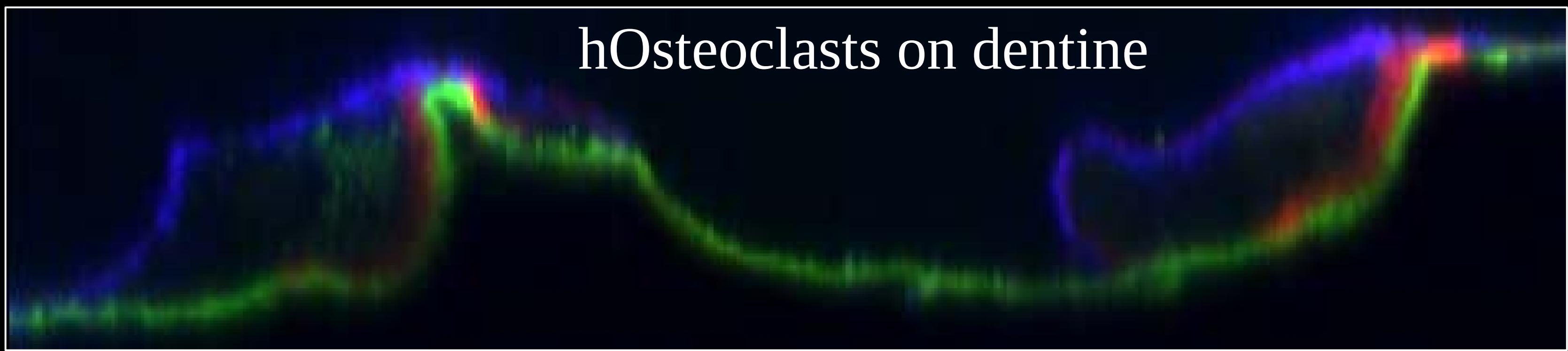
ruffled border

Bone is resorbed by the secretion of acid and cathepsin K (CK) at the ruffled border of the osteoclast

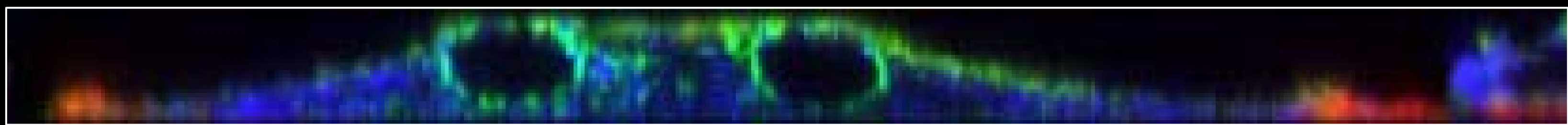
Analysis of *resorbing* osteoclasts

- Osteoclasts need to be cultured on a mineralised substrate
  - Dentine
  - Cortical bone
  - Hydroxyapatite discs

axial views:



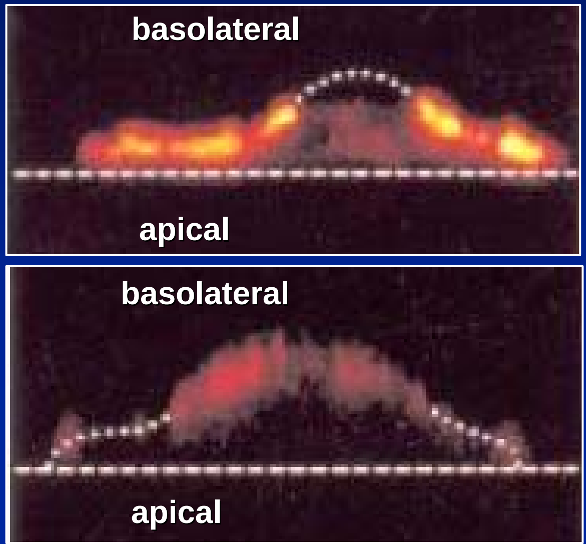
30µm



10µm

hOsteoclast on glass

Unusual membrane domains in osteoclasts

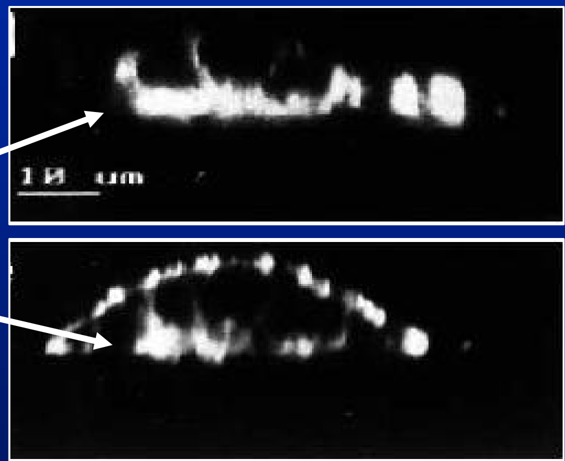


Vesicular stomatitis virus — *- basolateral domain in epithelial cells*

Influenza virus — *- apical domain in epithelial cells*

*Salo et al. 1996 J Cell Sci **109**, 391*

Ruffled border:
Properties of an endosomal/lysosomal membrane

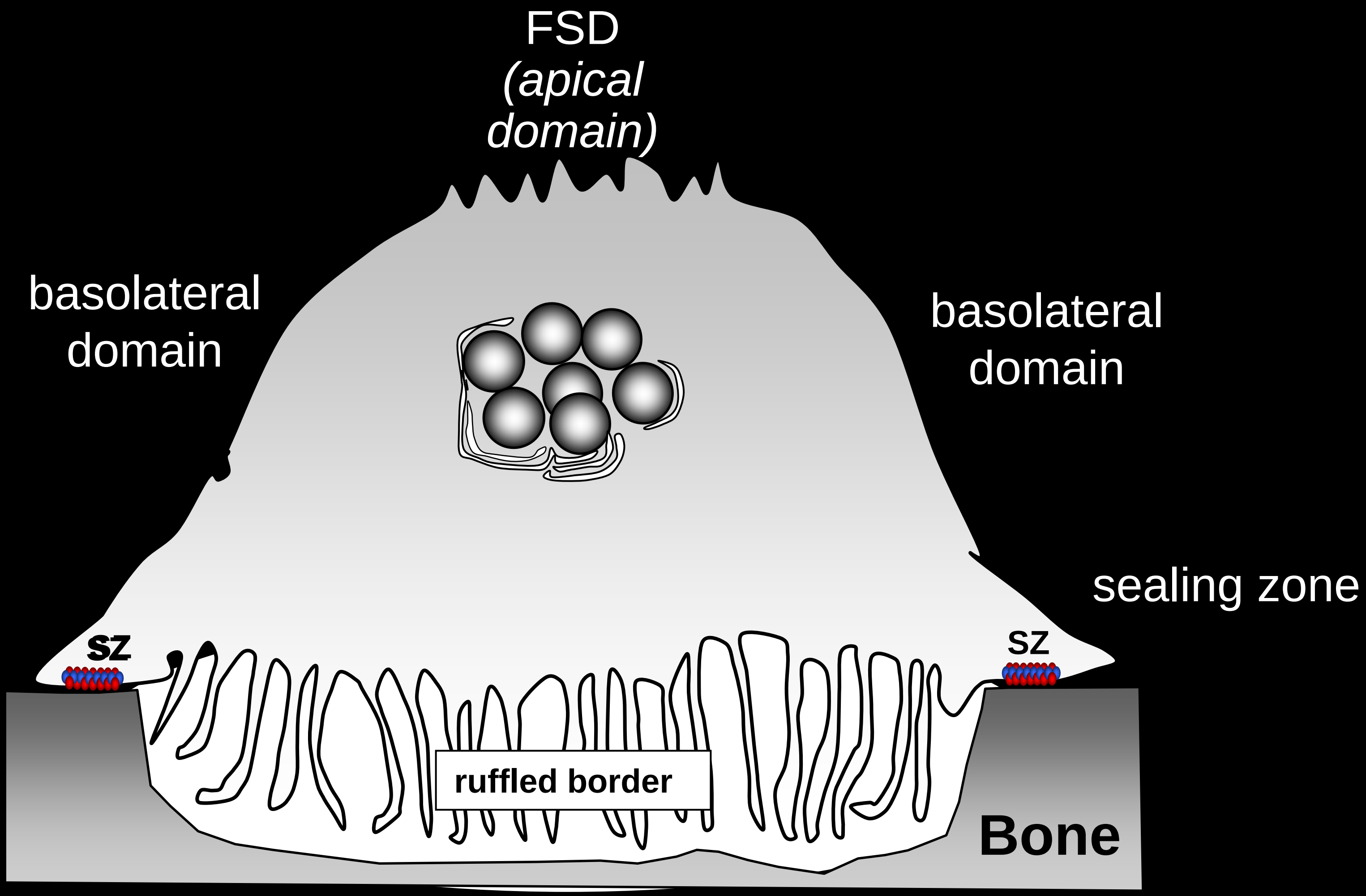


LAMP-2

Vitronectin receptor

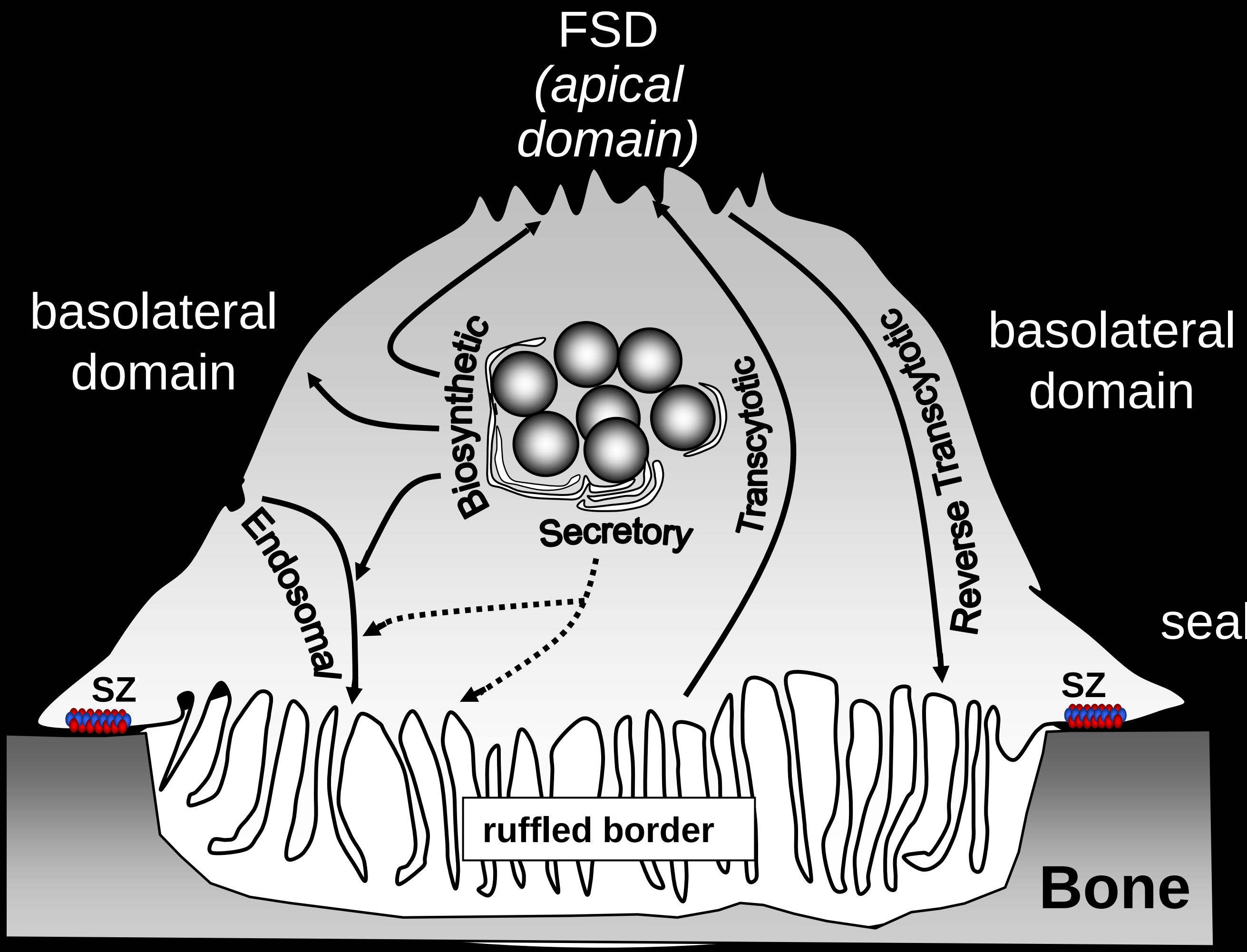
*Palokangas et al 1997 J Cell Sci **110**, 1767*

Unique membrane domains in osteoclasts



FSD- Functional secretory domain
SZ- sealing zone (F-actin ring)

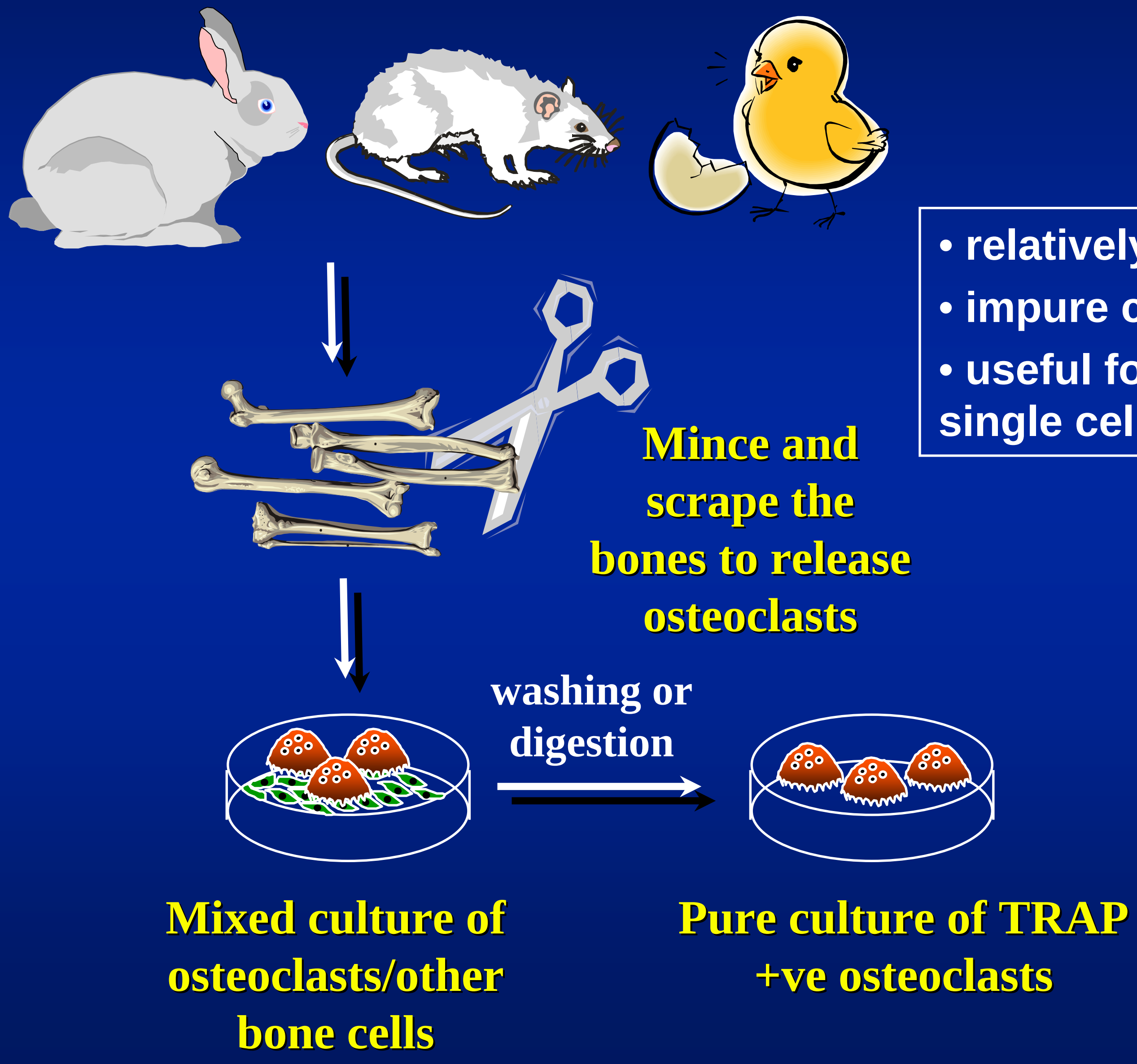
Vesicular trafficking routes in osteoclasts



FSD- Functional secretory domain
SZ- sealing zone (F-actin ring)

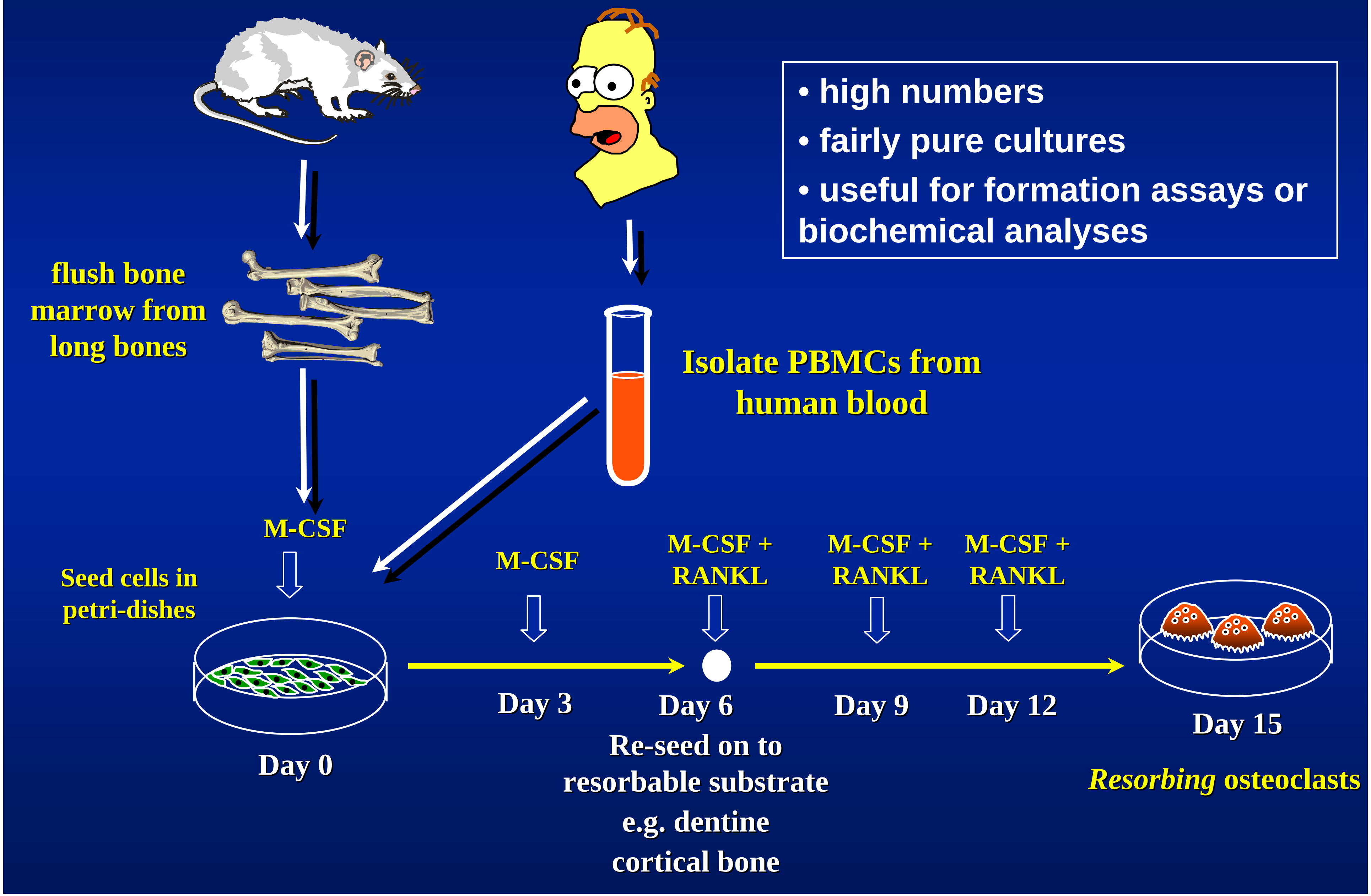
Sources of osteoclast cultures

Isolation of mature osteoclasts

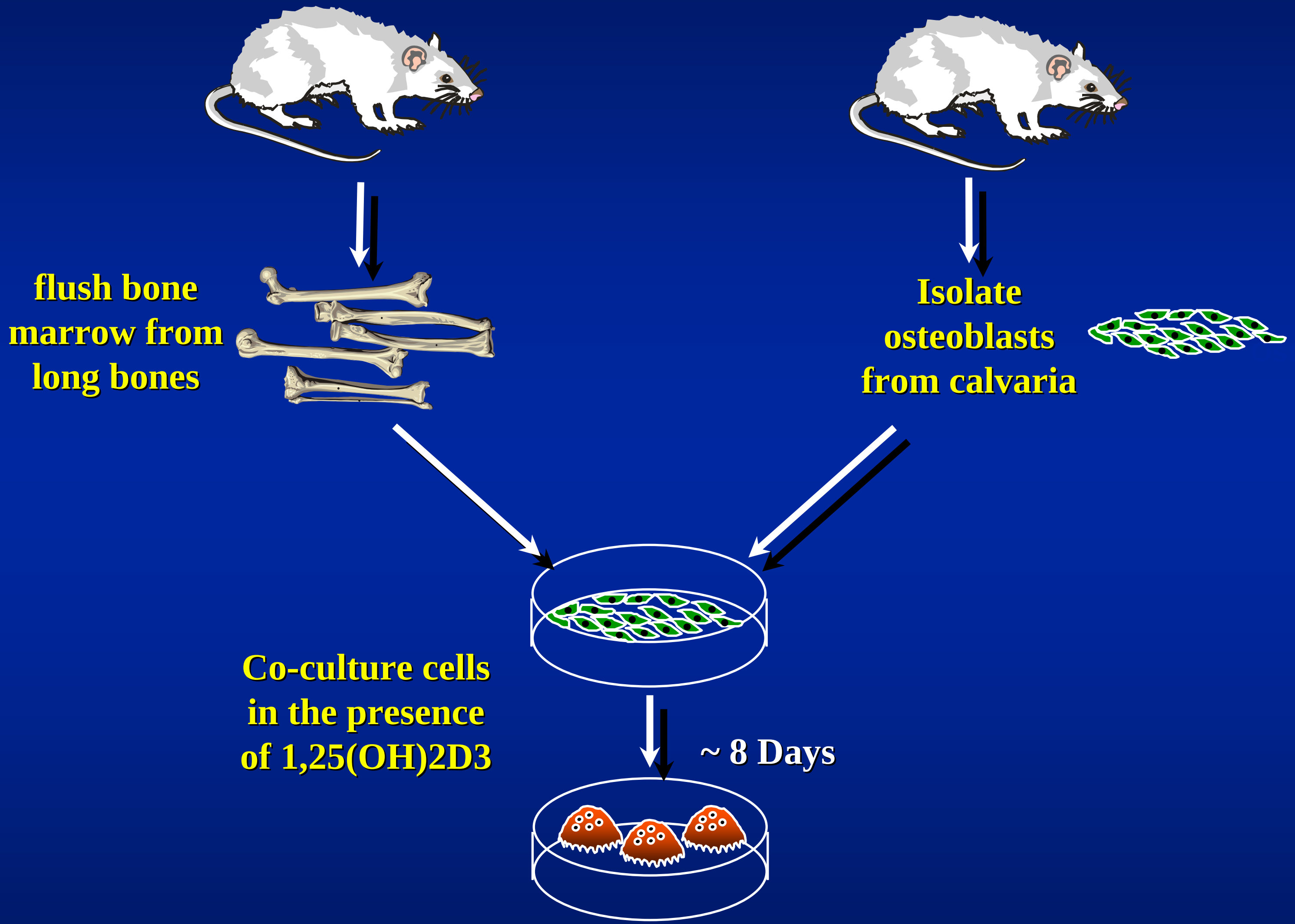


- relatively low numbers
- impure cultures
- useful for resorption assays or single cell studies

Generation of osteoclasts *in vitro*

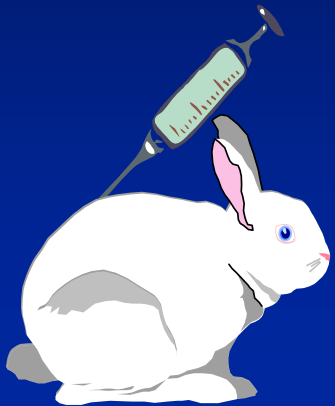


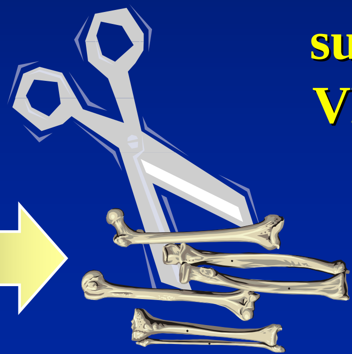
Generation of osteoclasts *in vitro*- before RANKL!



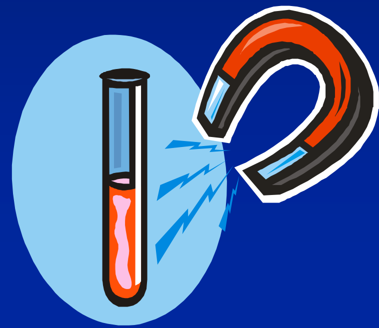
Useful for assessing osteoblast-osteoclast interactions in osteoclast formation e.g. whether a defect is in the osteoblast or osteoclast lineage

Isolation of osteoclasts ex vivo

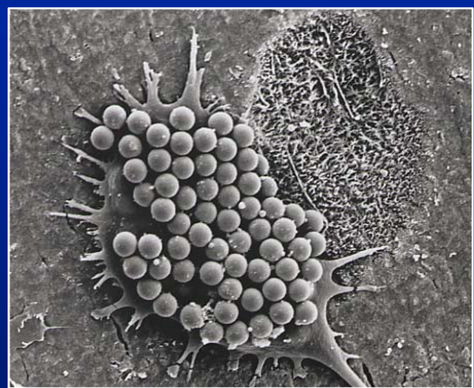




Isolate osteoclasts using antibody to surface marker (e.g. VNR) and magnetic bead separation



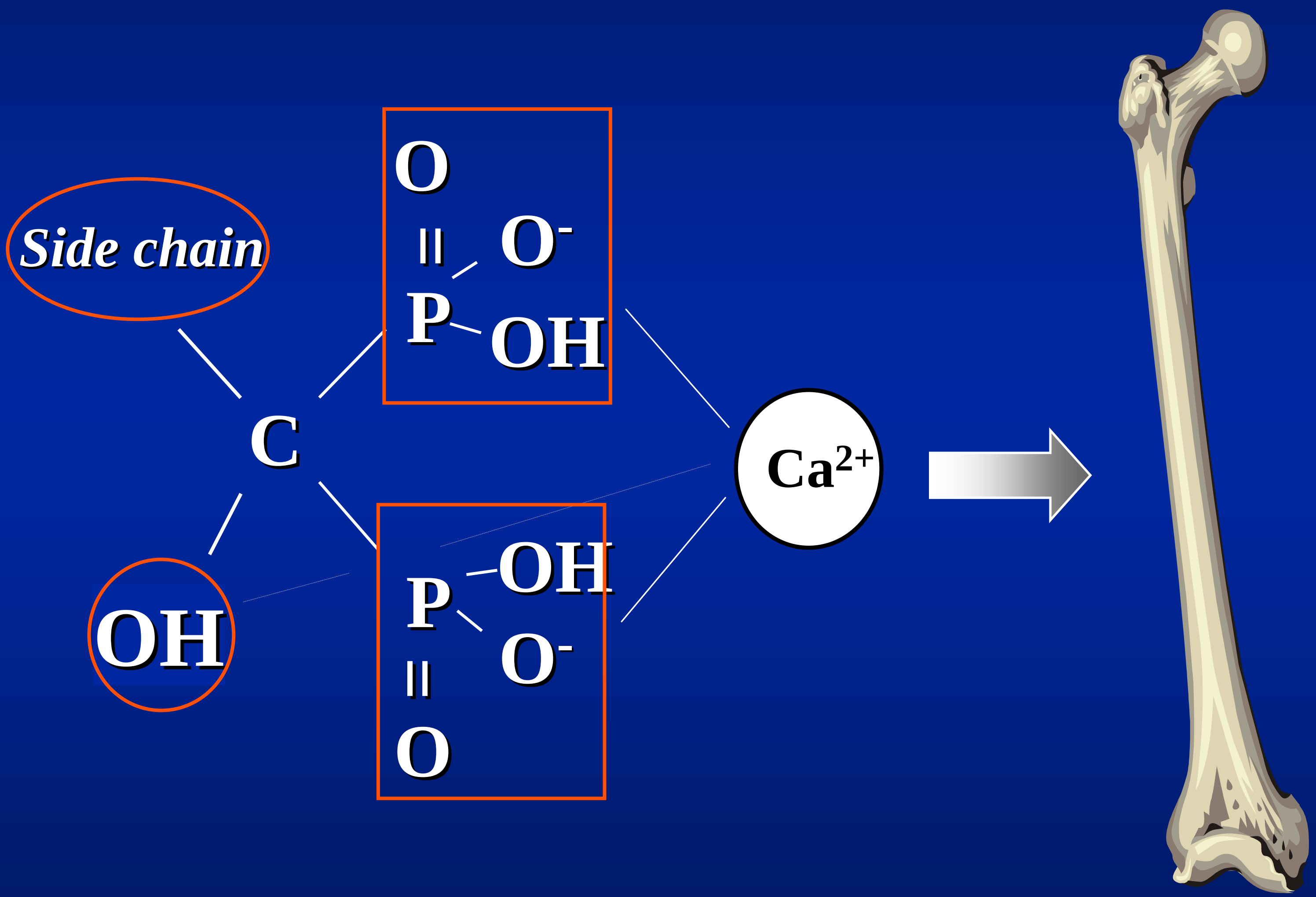
Pure culture of VNR^{+ve} osteoclasts



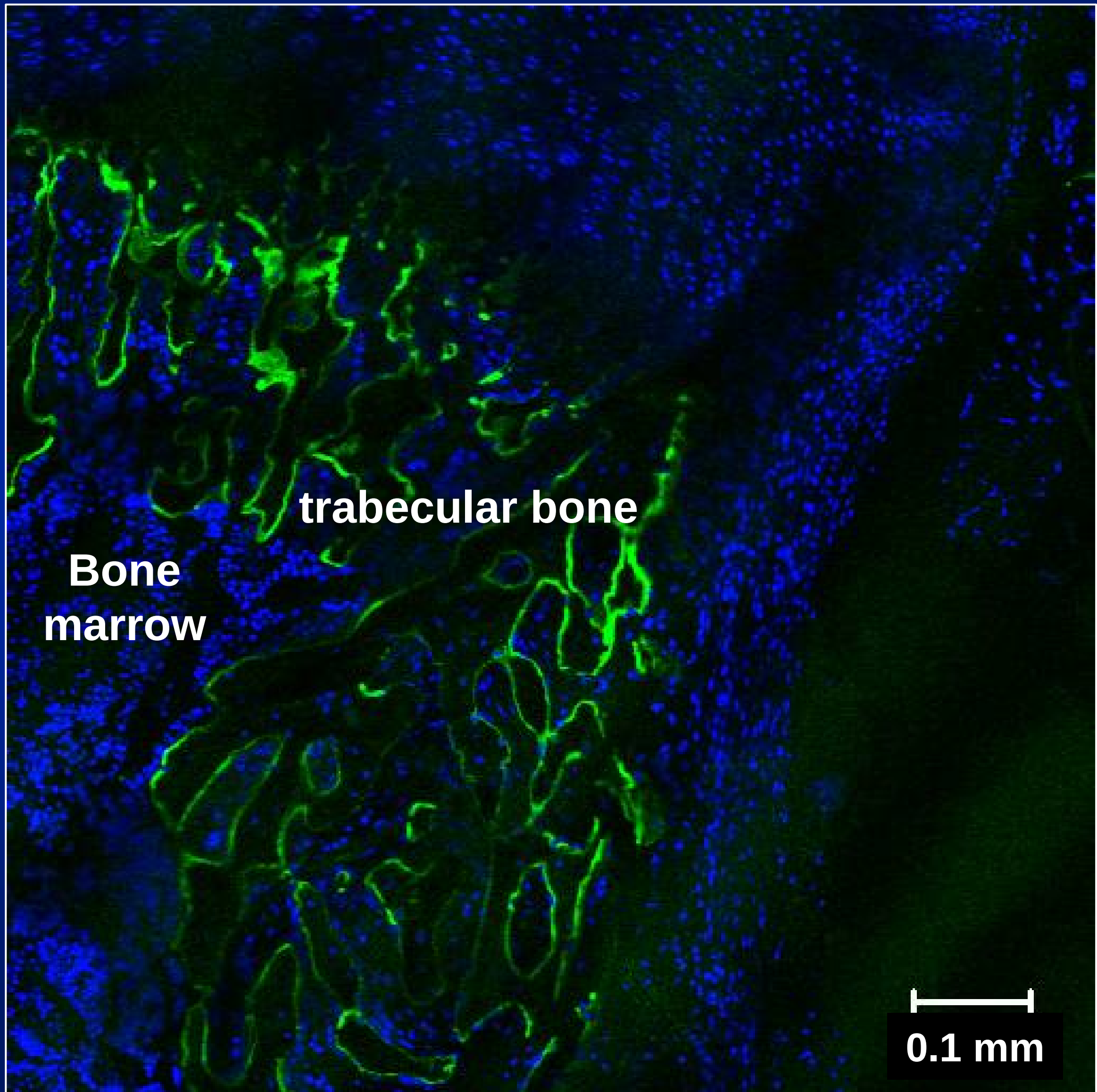
Useful for assessing the effect of a drug on osteoclasts *in vivo*

Bisphosphonates

Bisphosphonates bind calcium ions and target bone mineral



Localisation of a fluorescent BP (RIS) in mouse vertebra



cartilage

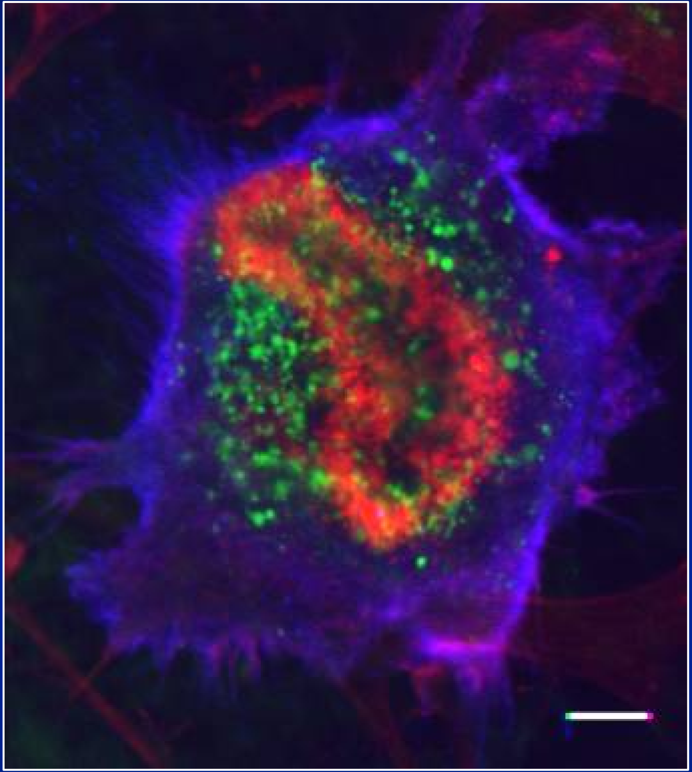
Bone

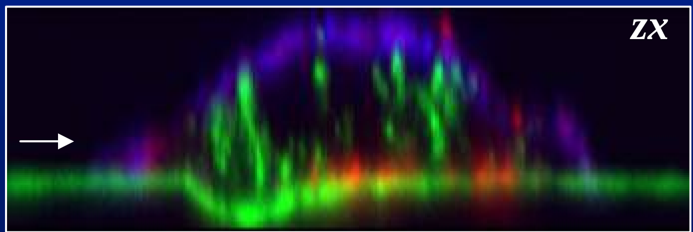
Green = FAM-RIS

Blue = nuclei

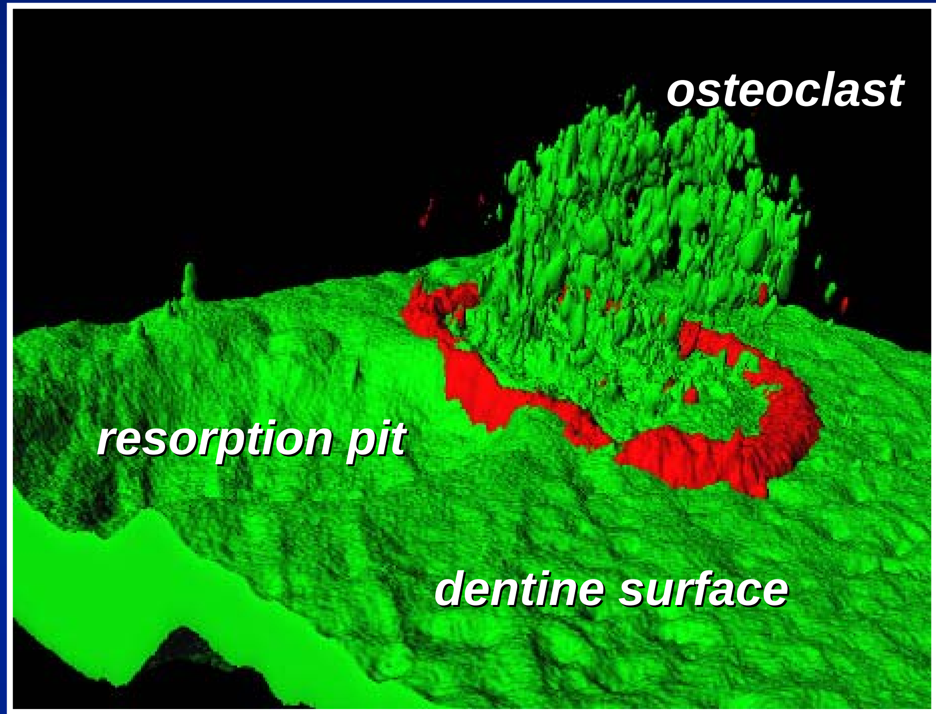
Osteoclasts engulf bisphosphonate into intracellular vesicles

Coxon *et al* 2008, *Bone* 42:848-60





Green- F-ALN
Red- F-actin
Blue-VNR

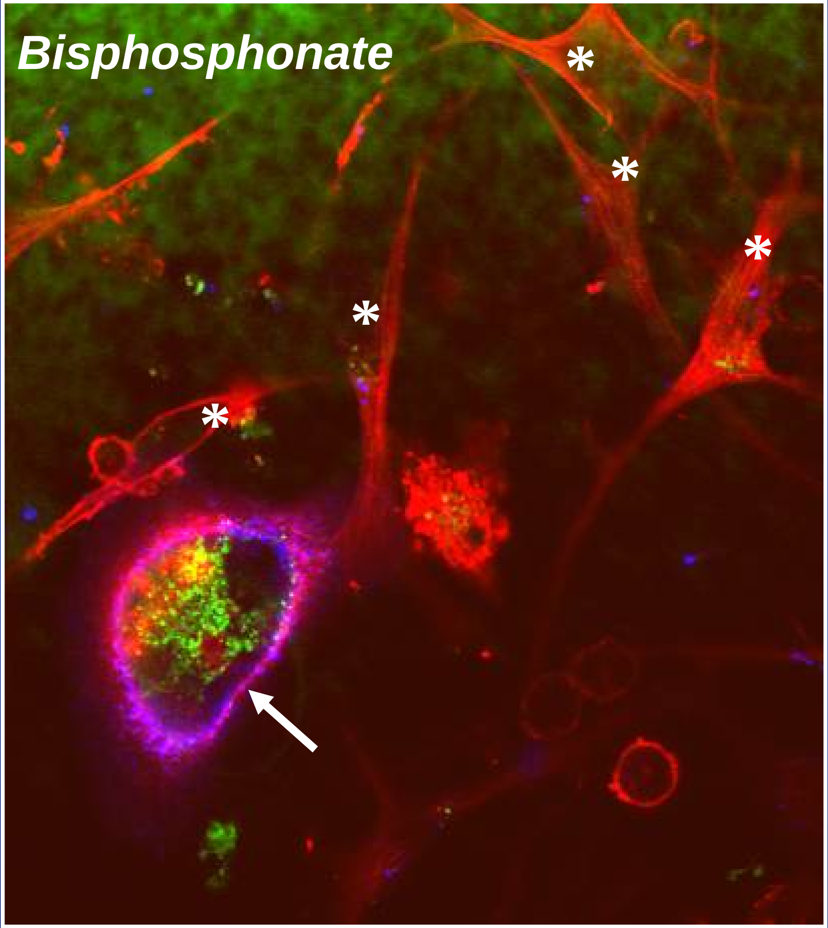


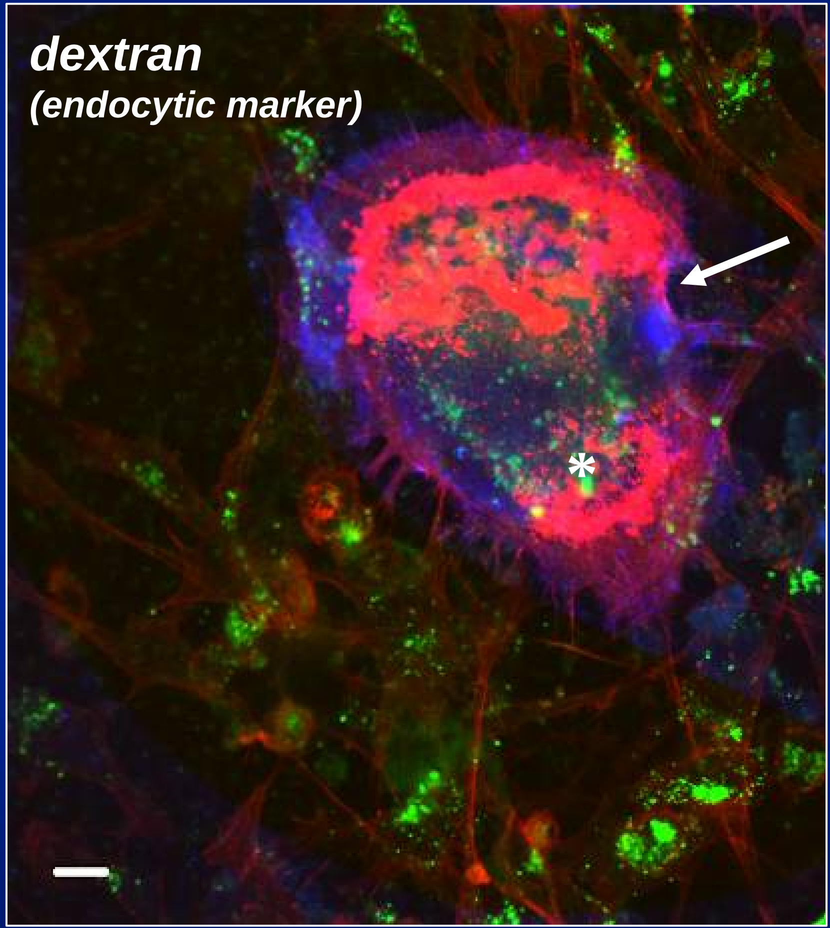
green = bisphosphonate

red = actin cytoskeleton

Bisphosphonates are selectively internalised from mineralised surfaces by osteoclasts

Rabbit osteoclasts cultured on dentine labelled with fluorescent alendronate





Green- F-ALN
Red- F-actin
Blue-VNR

Green- dextran
Red- F-actin
Blue-VNR

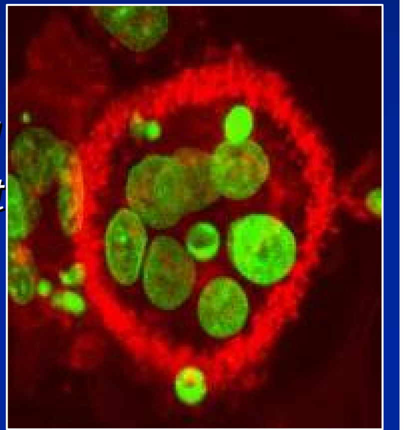
Effects of BPs on osteoclast morphology

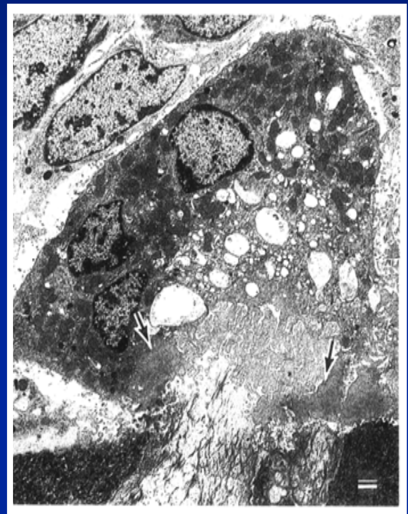
cytoskeleton
(F-actin ring)

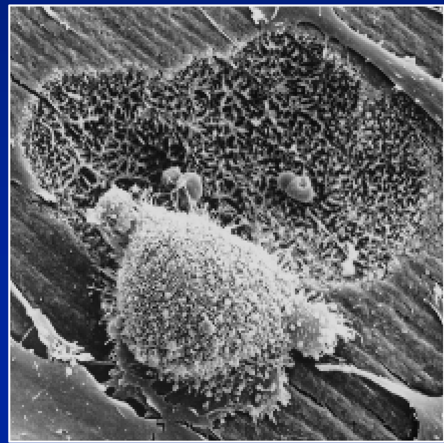
Ruffled border
in vivo

cell morphology
in vitro

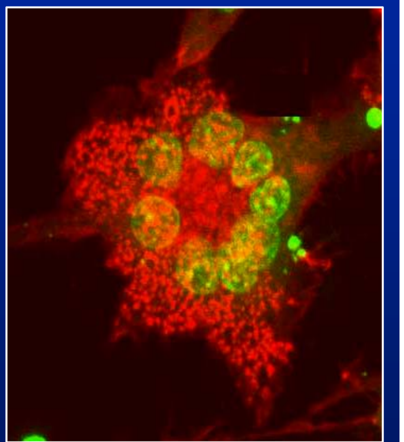
resorbing osteoclast

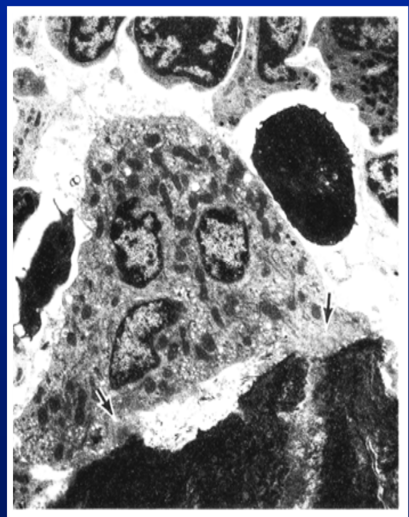






+ RIS





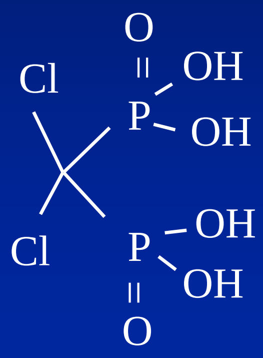
retraction

apoptosis

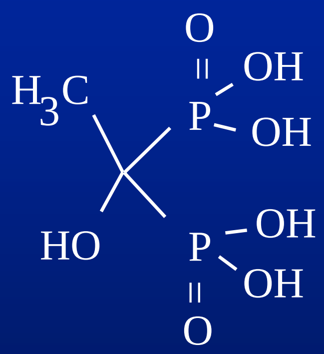
Sato *et al* 1991, *J Clin Invest* 88, 2095

Two groups of bisphosphonates with different modes of action

Simple Bisphosphonates

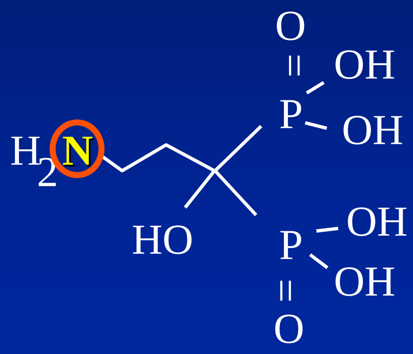


clodronate

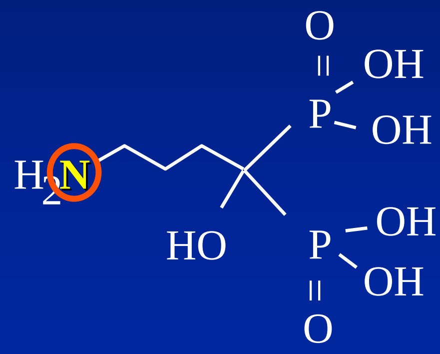


etidronate

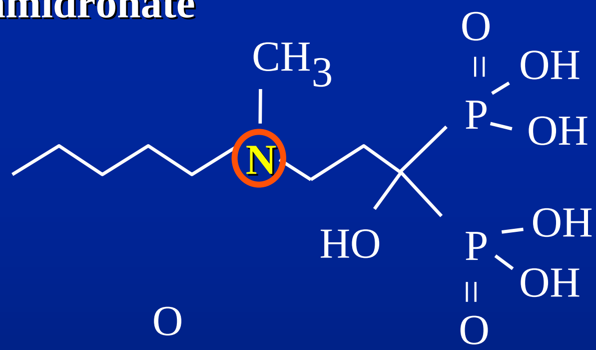
Nitrogen-containing Bisphosphonates



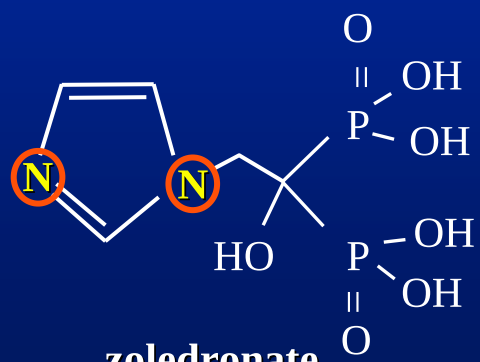
pamidronate



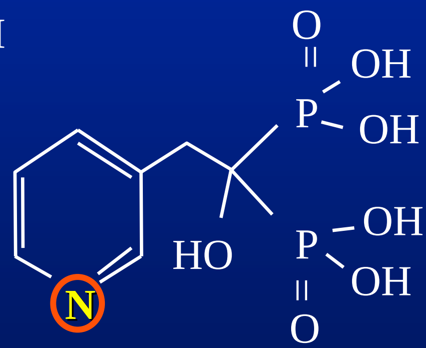
alendronate



ibandronate



zoledronate



risedronate

Bisphosphonates inhibit farnesyl diphosphate synthase in the mevalonate pathway

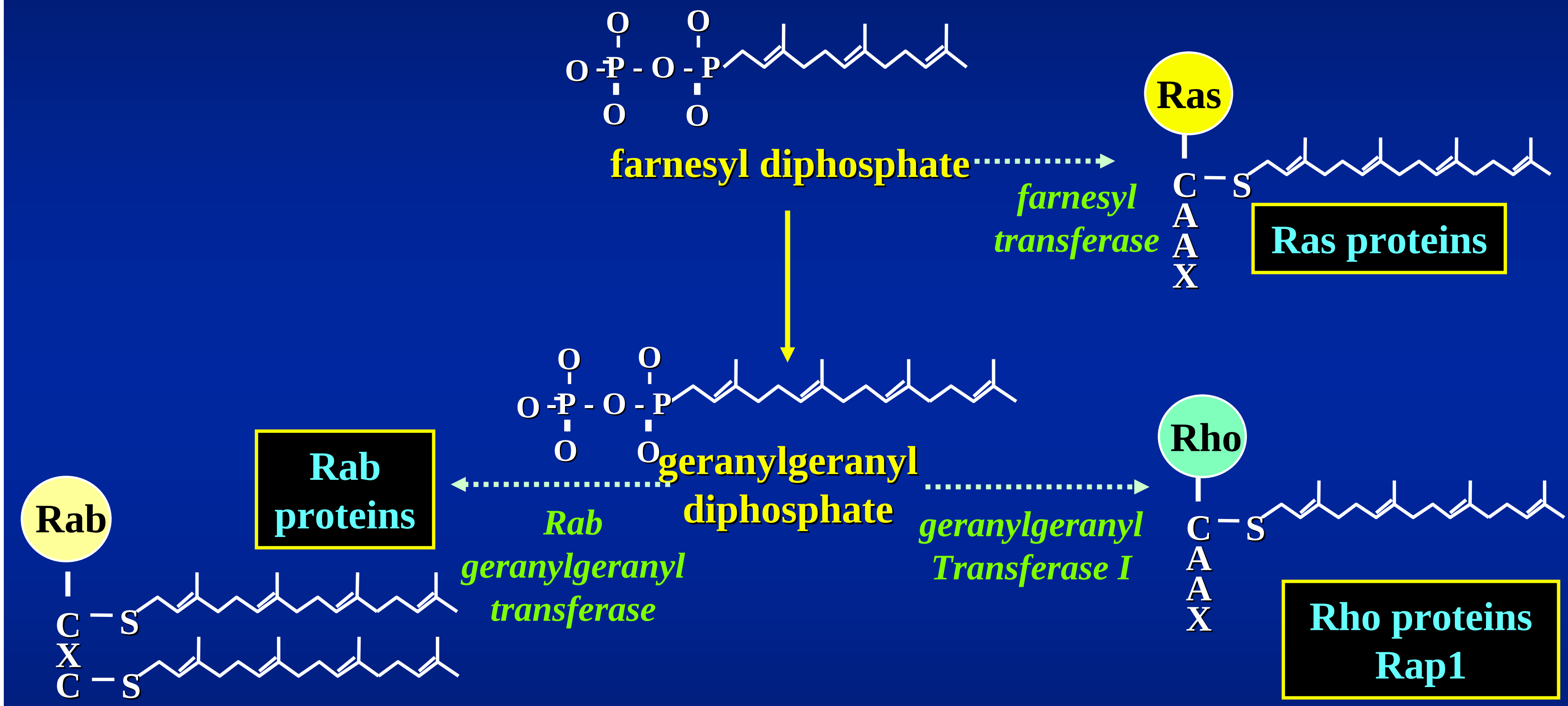
Luckman *et al*, 1998
Fisher *et al*, 1999
Van Beek *et al*, 1999
Keller *et al*, 1999
Bergstrom *et al*, 2000
Grove *et al*, 2000
Dunford *et al*, 2001

HMG-CoA
↓
mevalonate
↓
phosphomevalonate
↓
mevalonate diphosphate
↓
dimethylallyl diphosphate ←→ **isopentenyl diphosphate**
↓

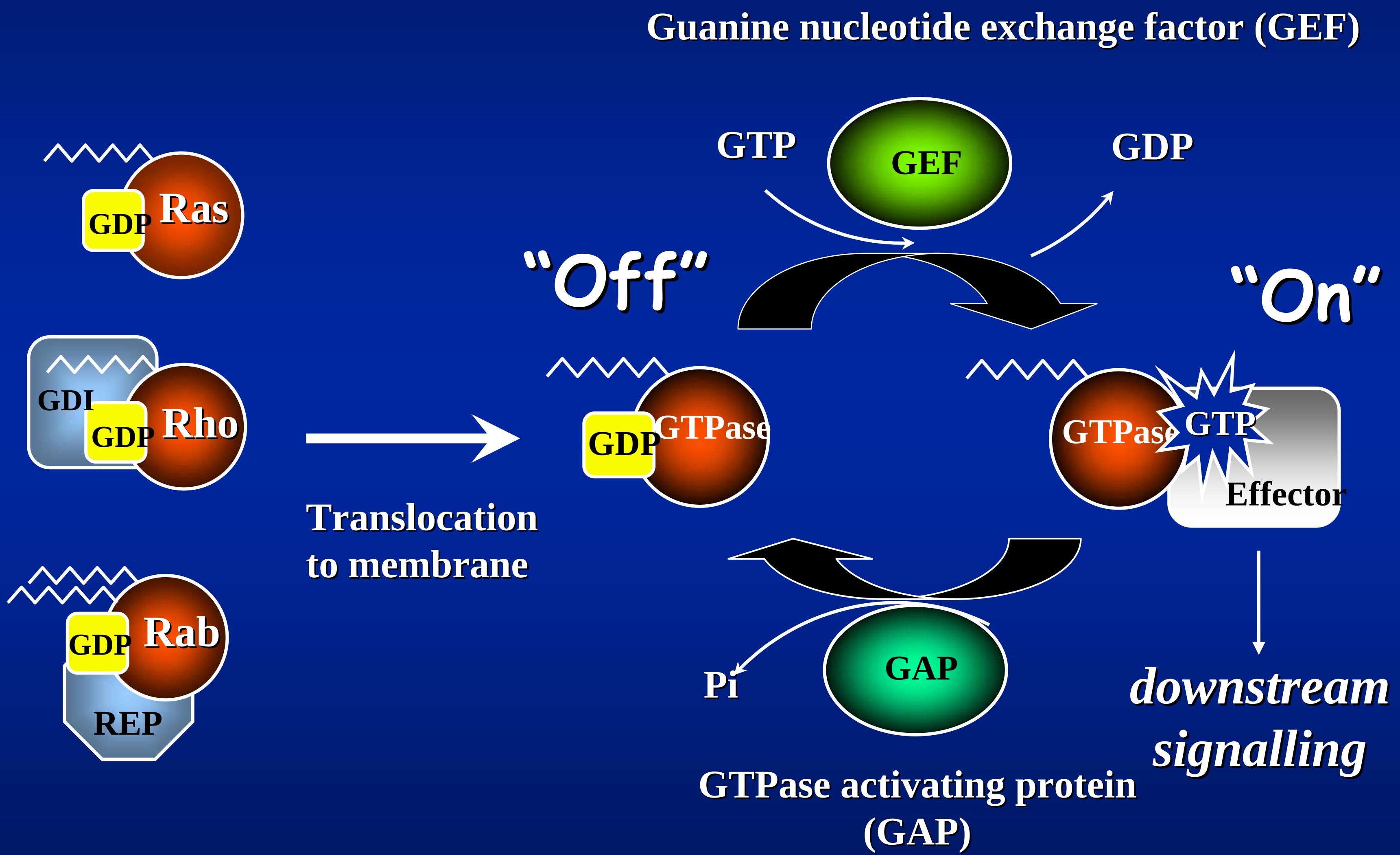

geranyl diphosphate ✕ ***FPP synthase***
↓
farnesyl diphosphate → **squalene** ⇢ **cholesterol**
↓
geranylgeranyl diphosphate

N-BPs e.g.
pamidronate
alendronate
Ibandronate
risedronate
zoledronate

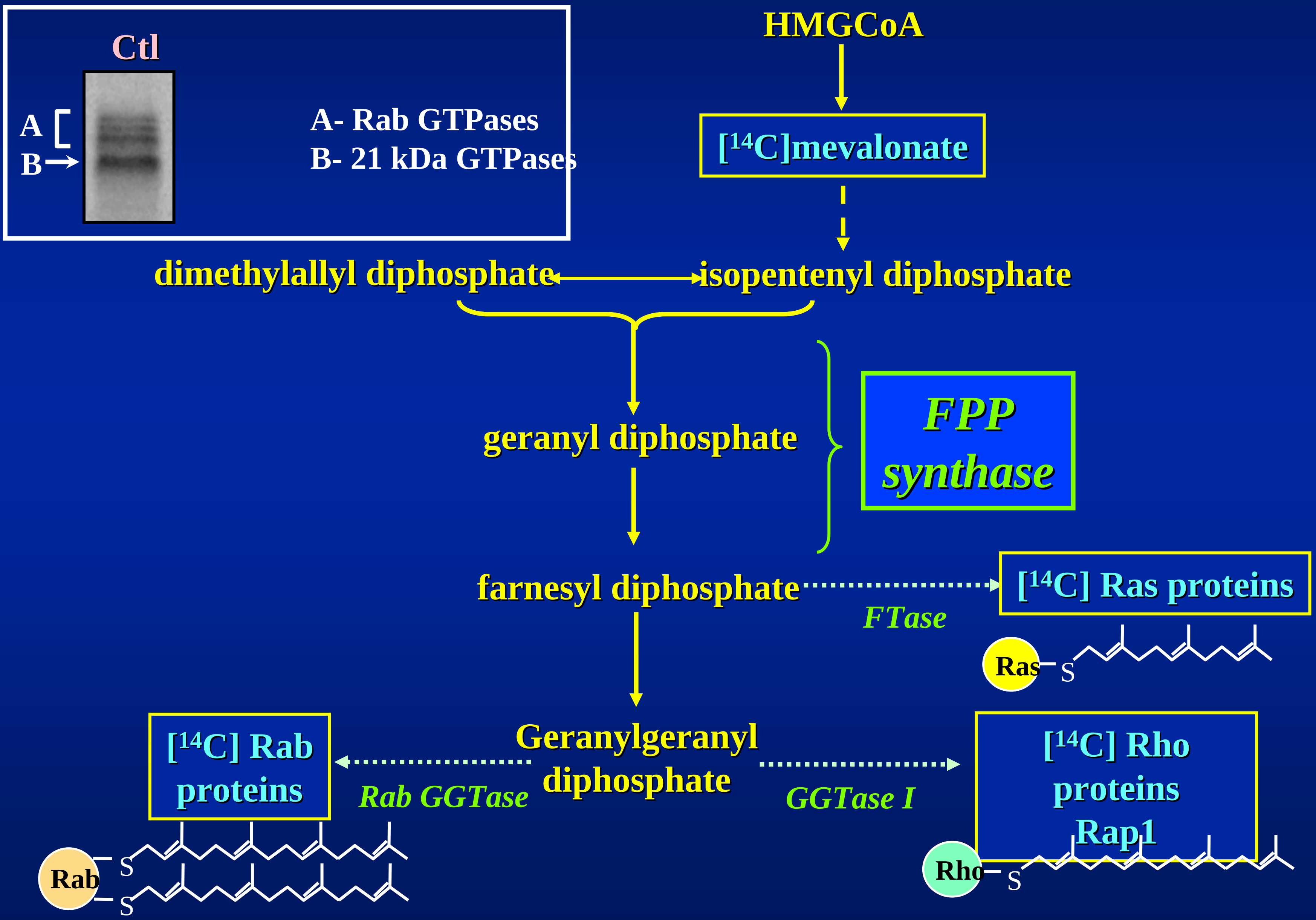
Most small GTPases are modified by prenylation



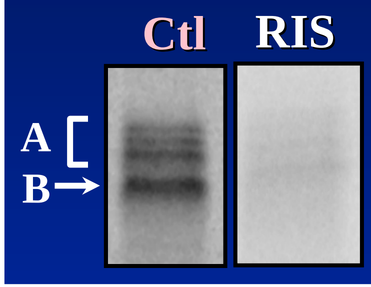
Small GTPases act as molecular switches



Bisphosphonates inhibit protein prenylation



Bisphosphonates inhibit protein prenylation



A- Rab GTPases
B- 21 kDa GTPases

HMGCoA

[^{14}C]mevalonate

dimethylallyl diphosphate ↔ isopentenyl diphosphate

BPs
e.g. RIS

FPP synthase

FTase

Ras

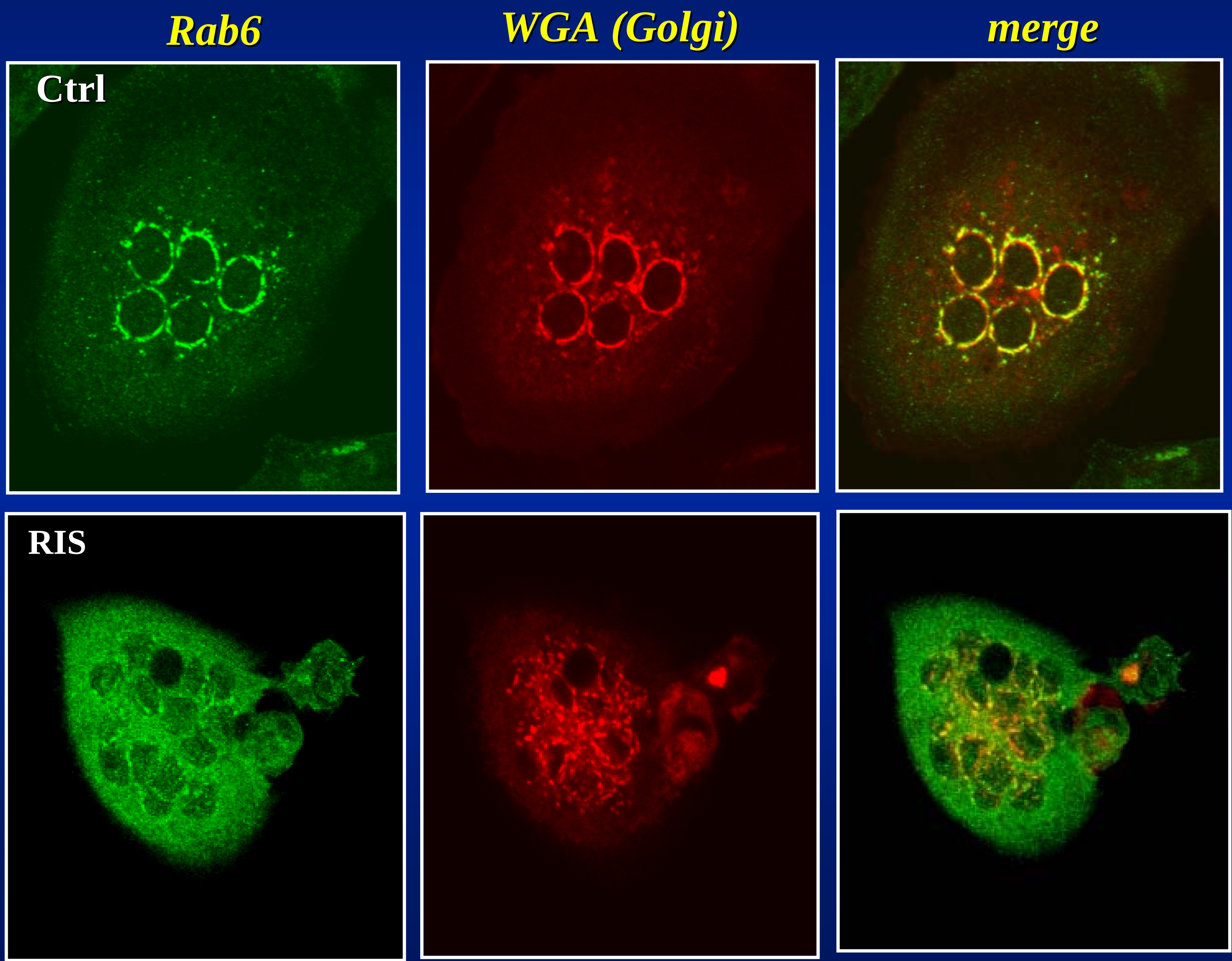
Rab GGTase

GGTase I

Rab

Rho

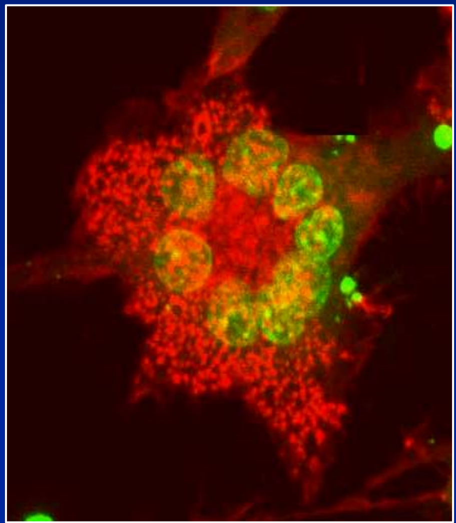
RIS disrupts the localisation of Rab6 in rabbit osteoclasts



Loss of prenylated small GTPases disrupts osteoclast function

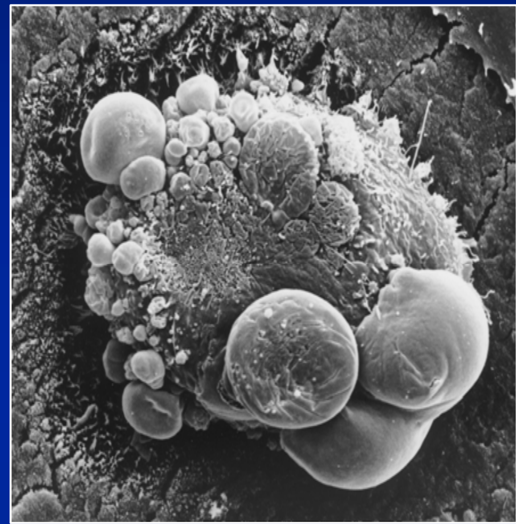
loss of prenylated small GTPases

cytoskeleton disruption



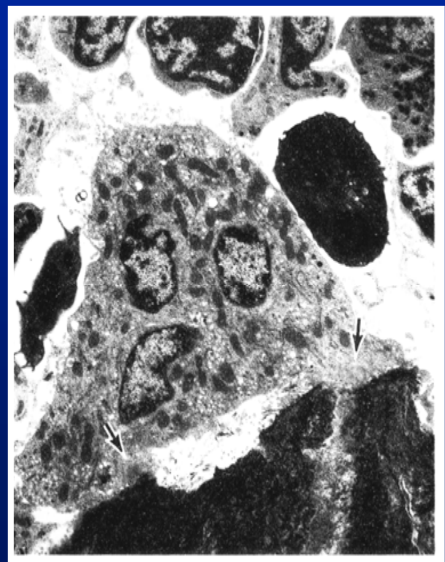
Rho, Rac, Cdc42
(regulate the actin cytoskeleton)

Induction of apoptosis



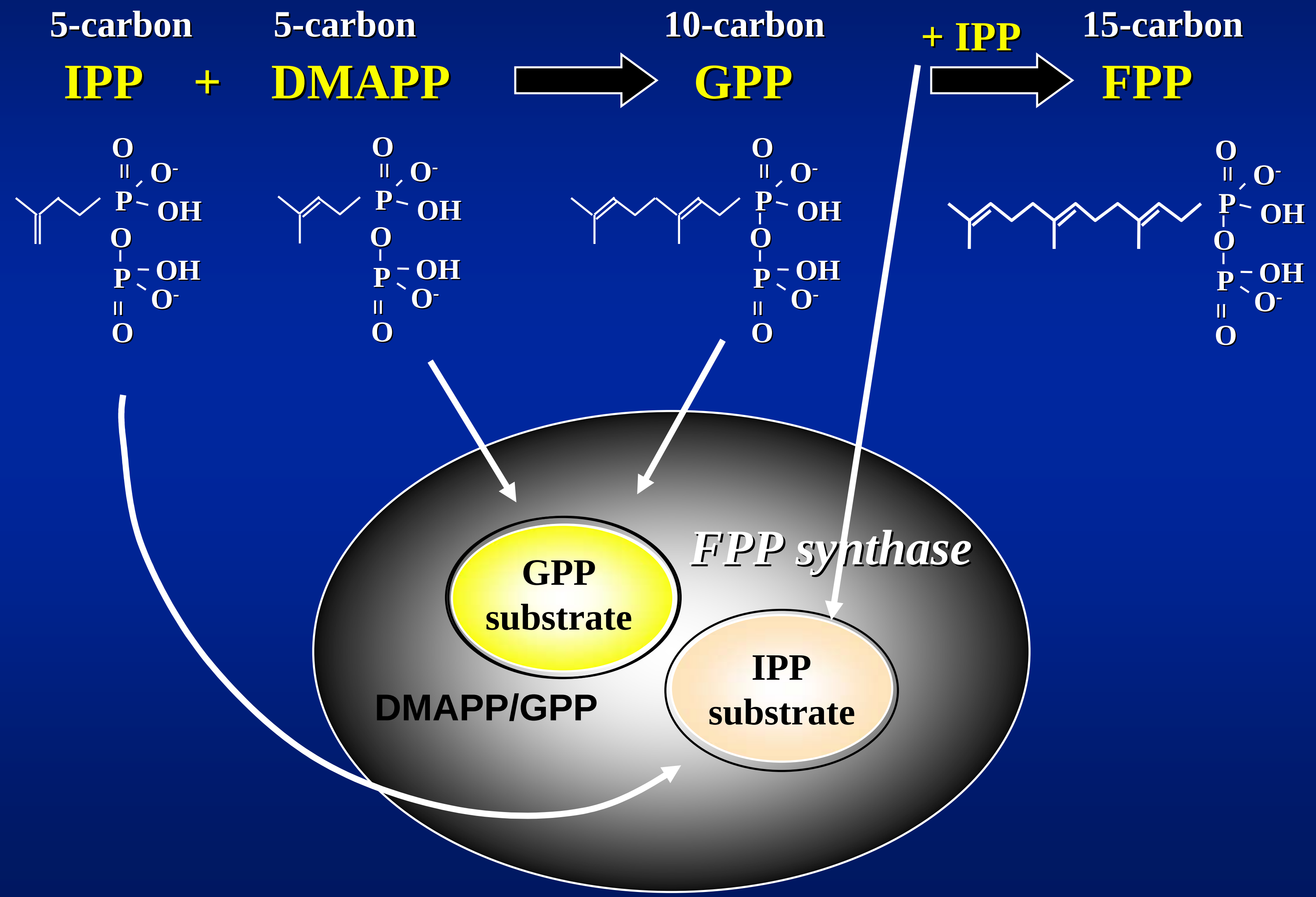
Rho, Rac
(regulate cell survival)

Loss of the ruffled border



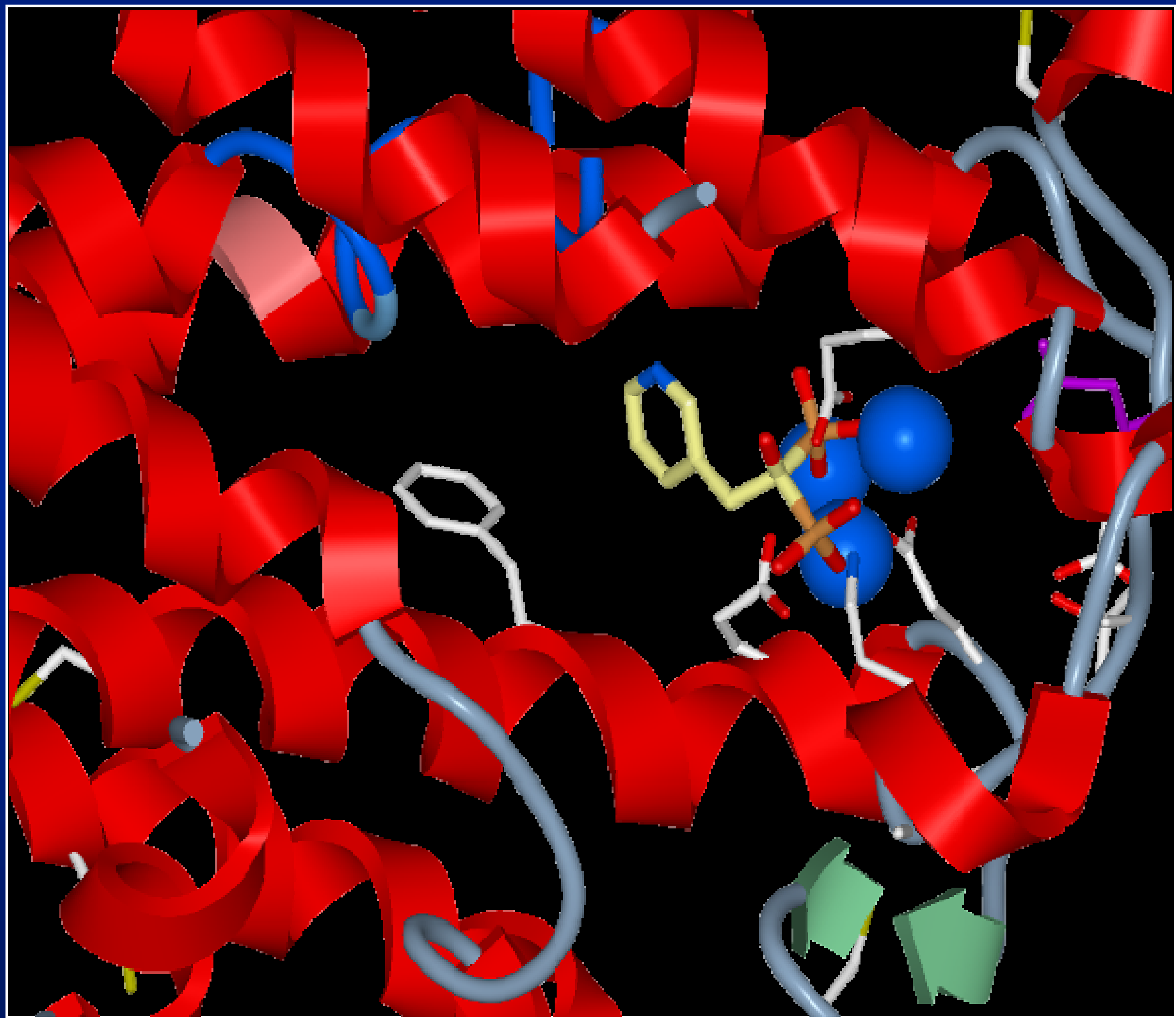
Rabs
(regulate vesicular Transport)

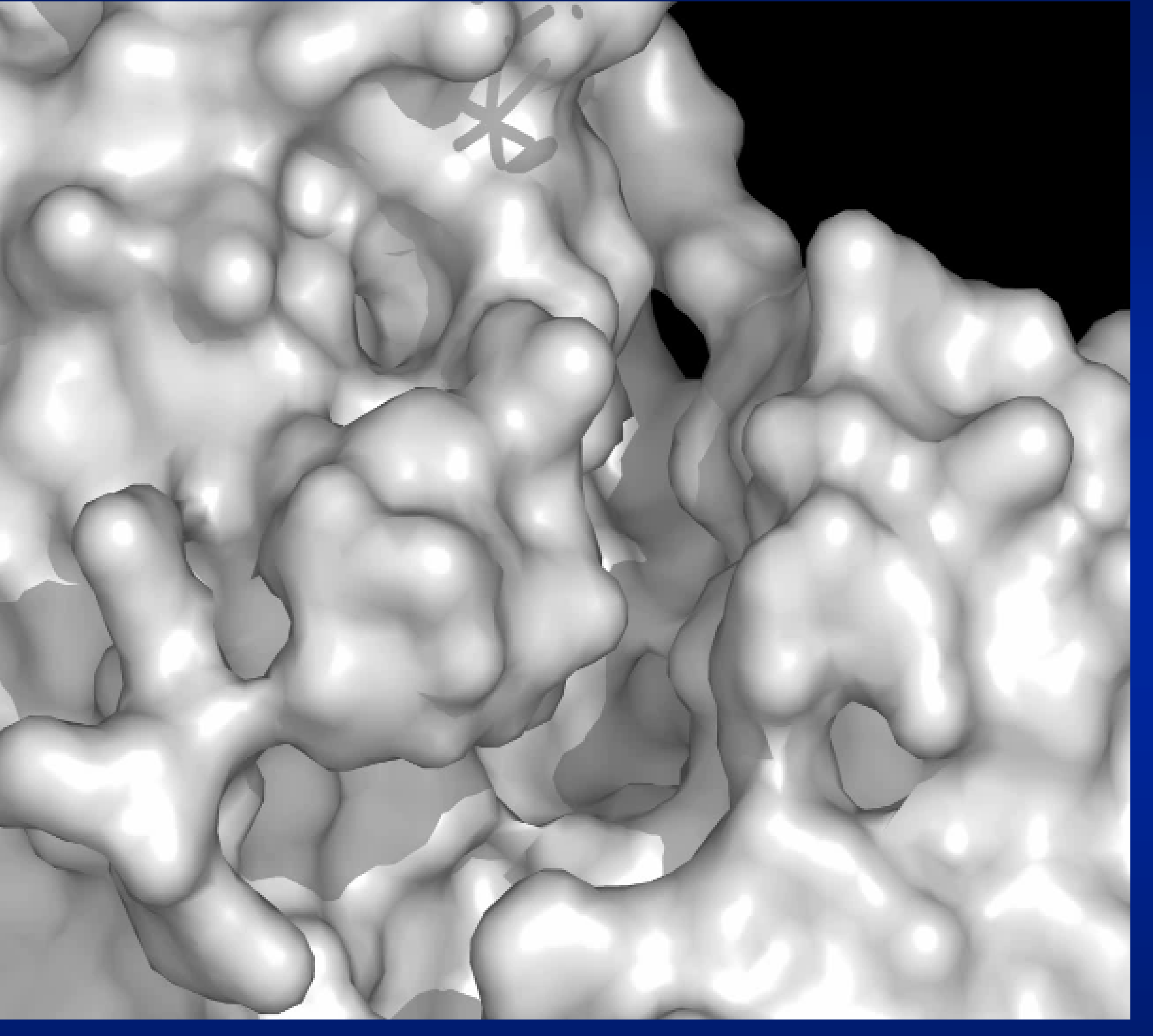
FPP synthase catalyses the condensation of isoprenoid lipid chains



Risedronate co-ordinates with 3 magnesium ions in the GGP binding site of FPPS

Kavanagh *et al* 2006, *Proc. Natl. Acad. Sci* 103, 7829-7834

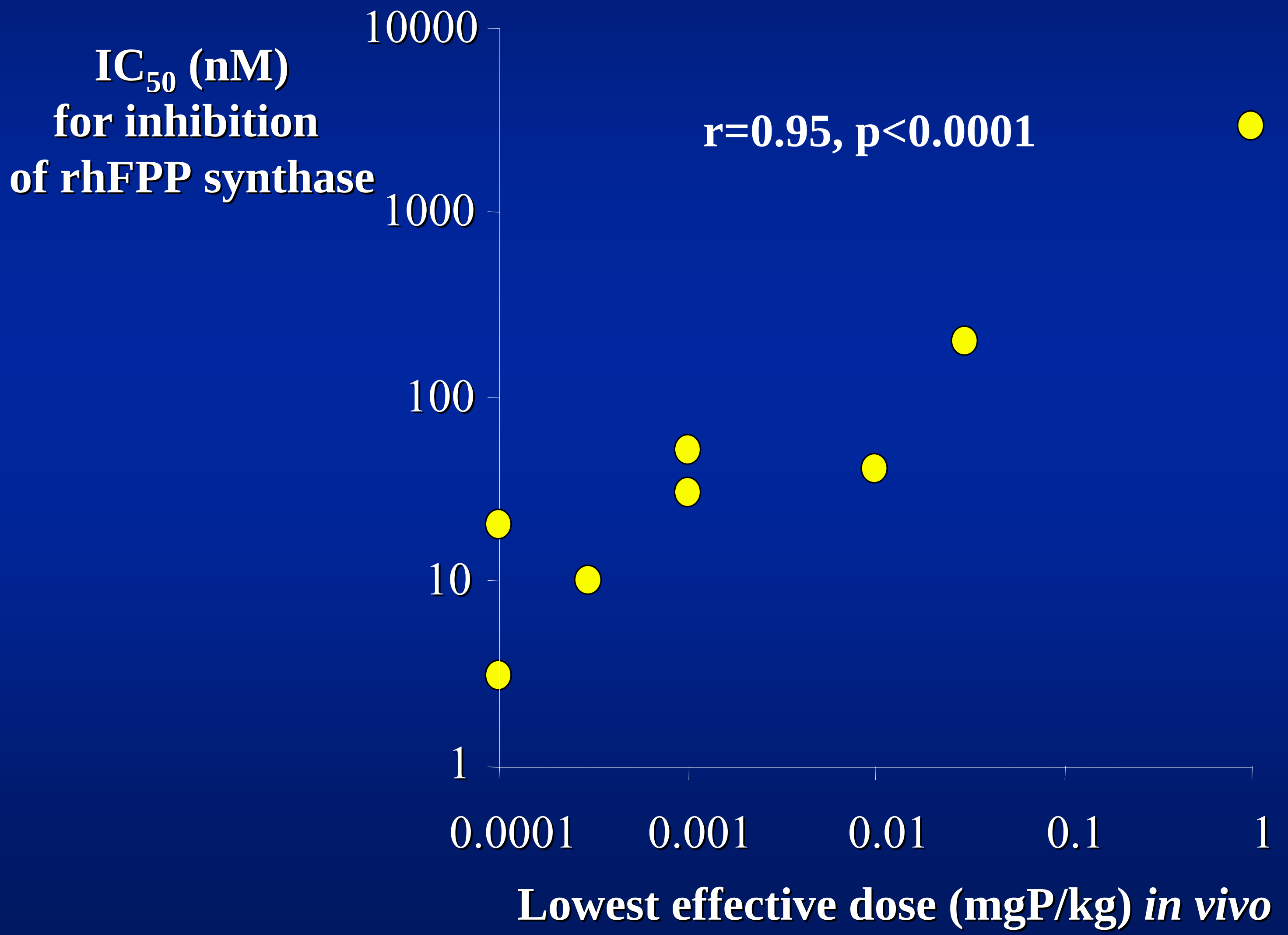




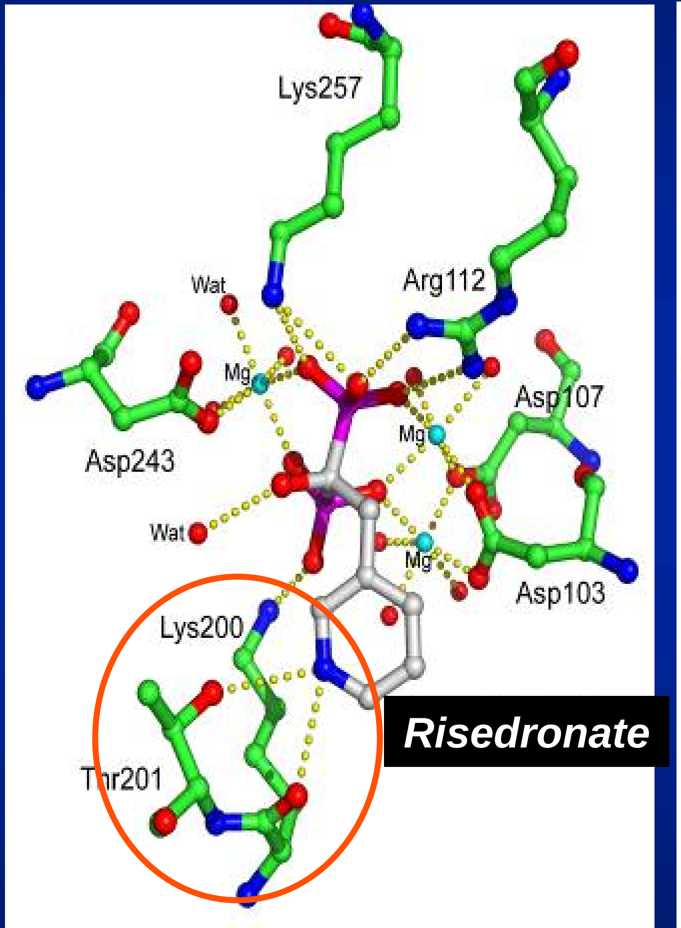
Courtesy of Jean-Michel Rondeau, Novartis

Correlation between inhibition of FPP synthase *in vitro* and anti-resorptive potency *in vivo*

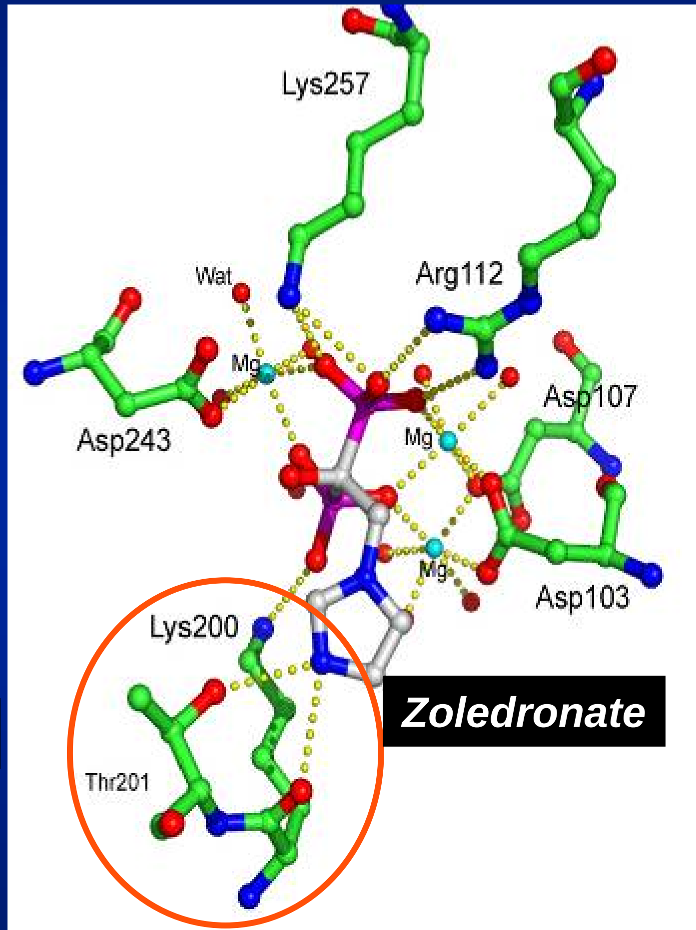
Dunford *et al, J Pharm Exp Ther* 2001



The nitrogen in the side chain of risedronate or zoledronate interacts with a conserved threonine and lysine residue in FPP synthase



Kavanagh *et al* 2006
Proc. Natl. Acad. Sci



Rondeau JM *et al 2006*
Chem Med Chem

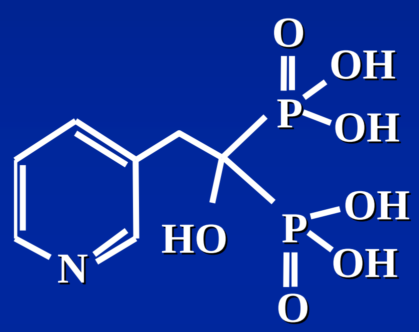
ideal distance is ~3Å

Zoledronate	2.96
Risedronate	2.97
Alendronate	3.84
Ibandronate	4.03

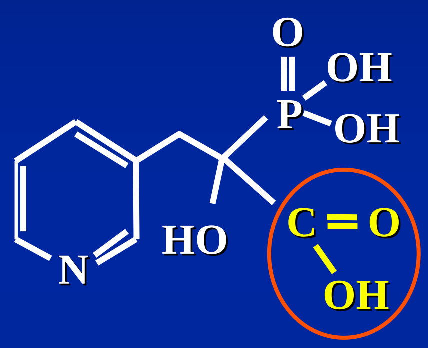
This interaction enables 'slow tight binding' of the enzyme

Phosphonocarboxylate analogue of RIS

Bisphosphonate	Phosphonocarboxylate



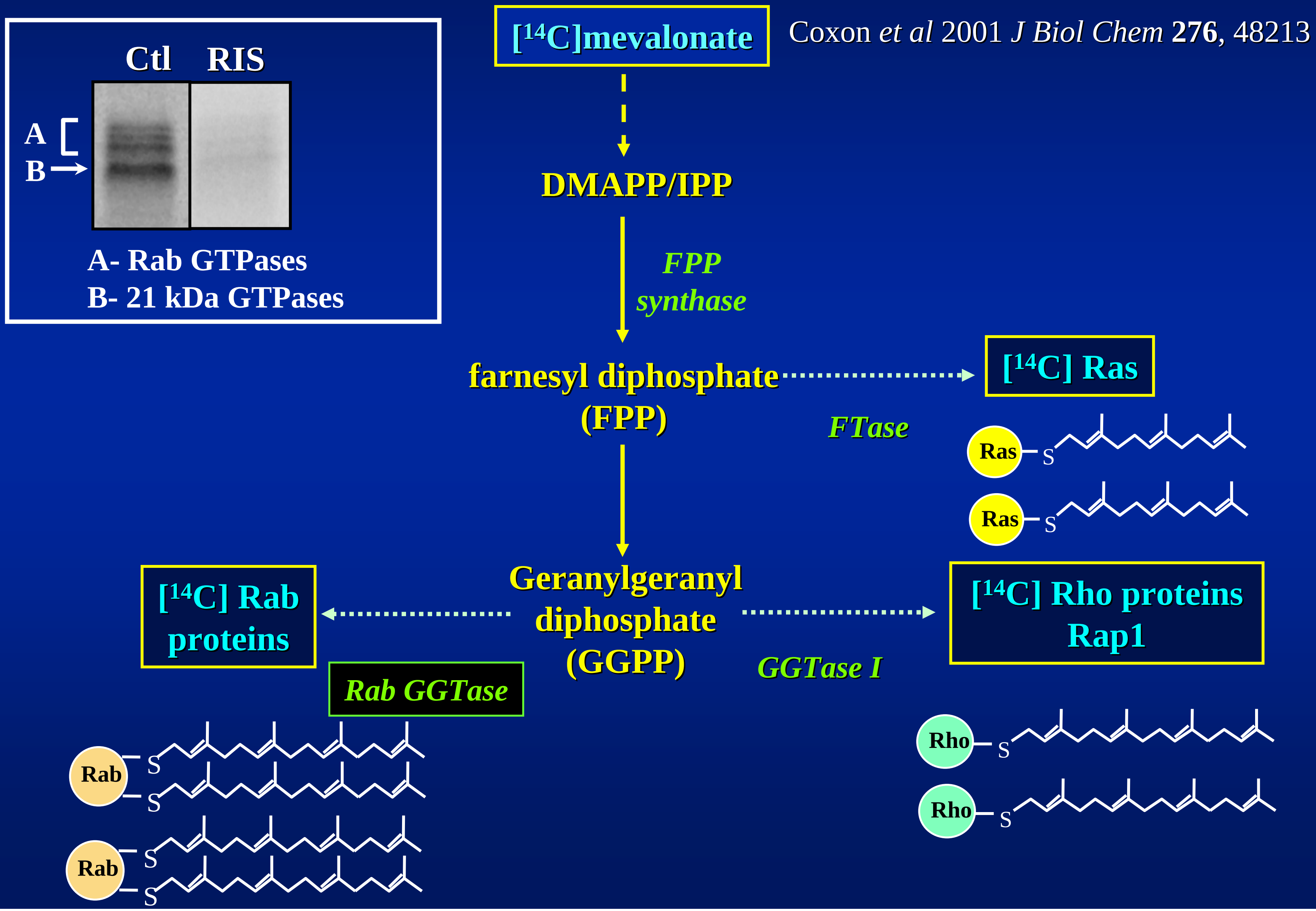
Risedronate (RIS)



NE10790
(3-PEHPC)

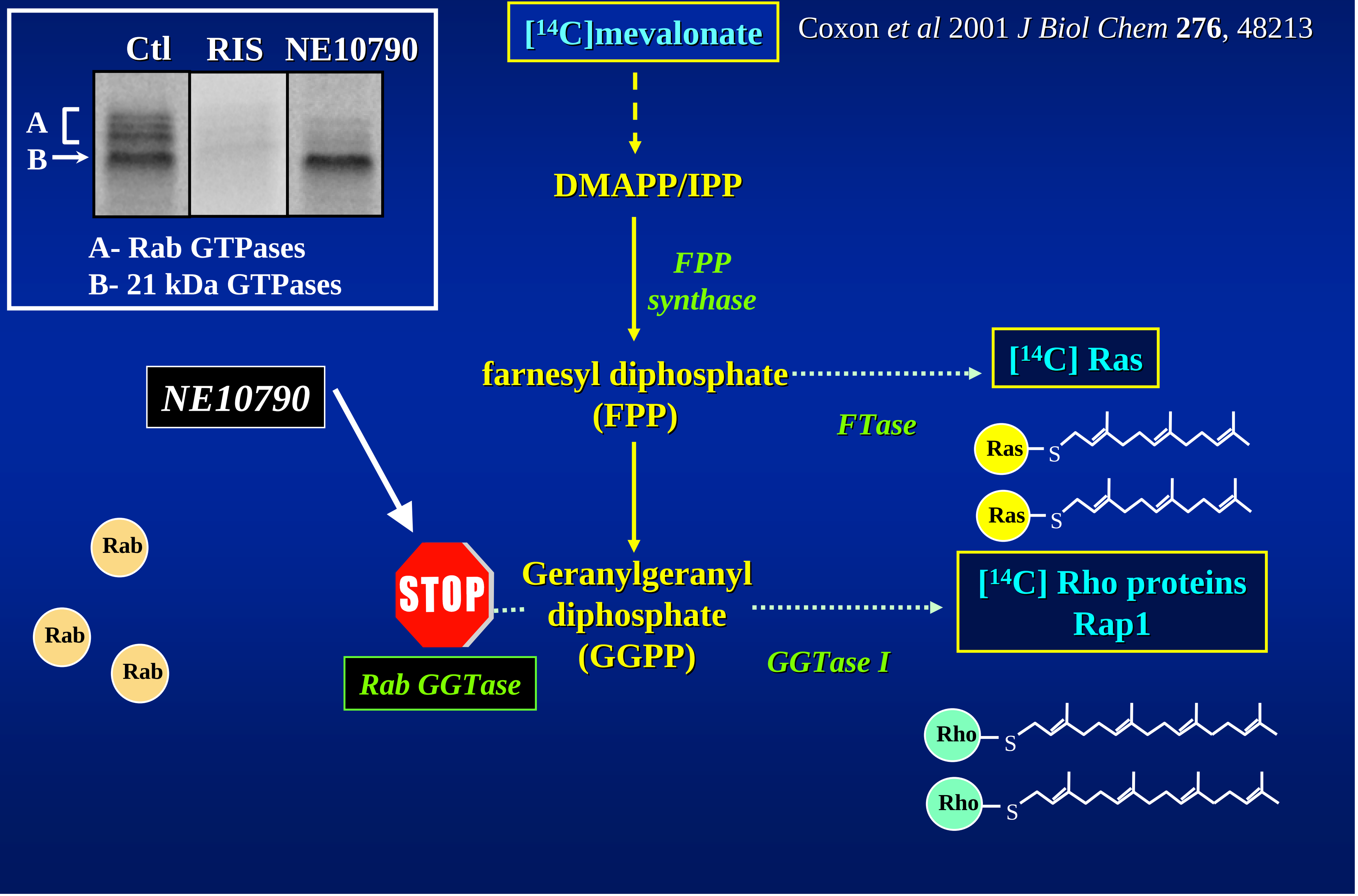
50x lower affinity for bone
5,000x lower anti-resorptive potency

NE10790 is a novel inhibitor of Rab GGTase

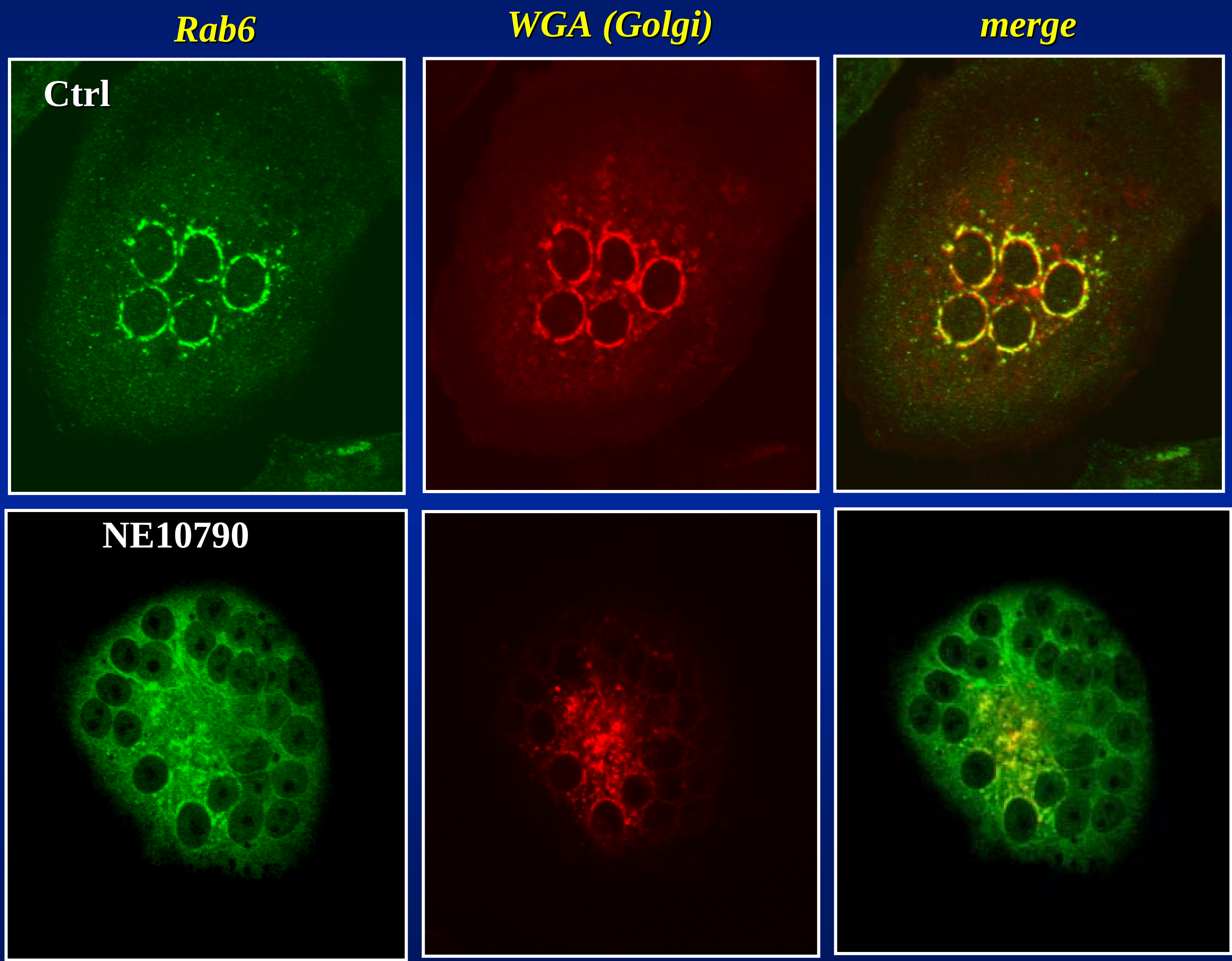


NE10790 is a novel inhibitor of Rab GGTase

Coxon *et al* 2001 *J Biol Chem* **276**, 48213



NE10790 disrupts localisation of Rab6 in rabbit osteoclasts

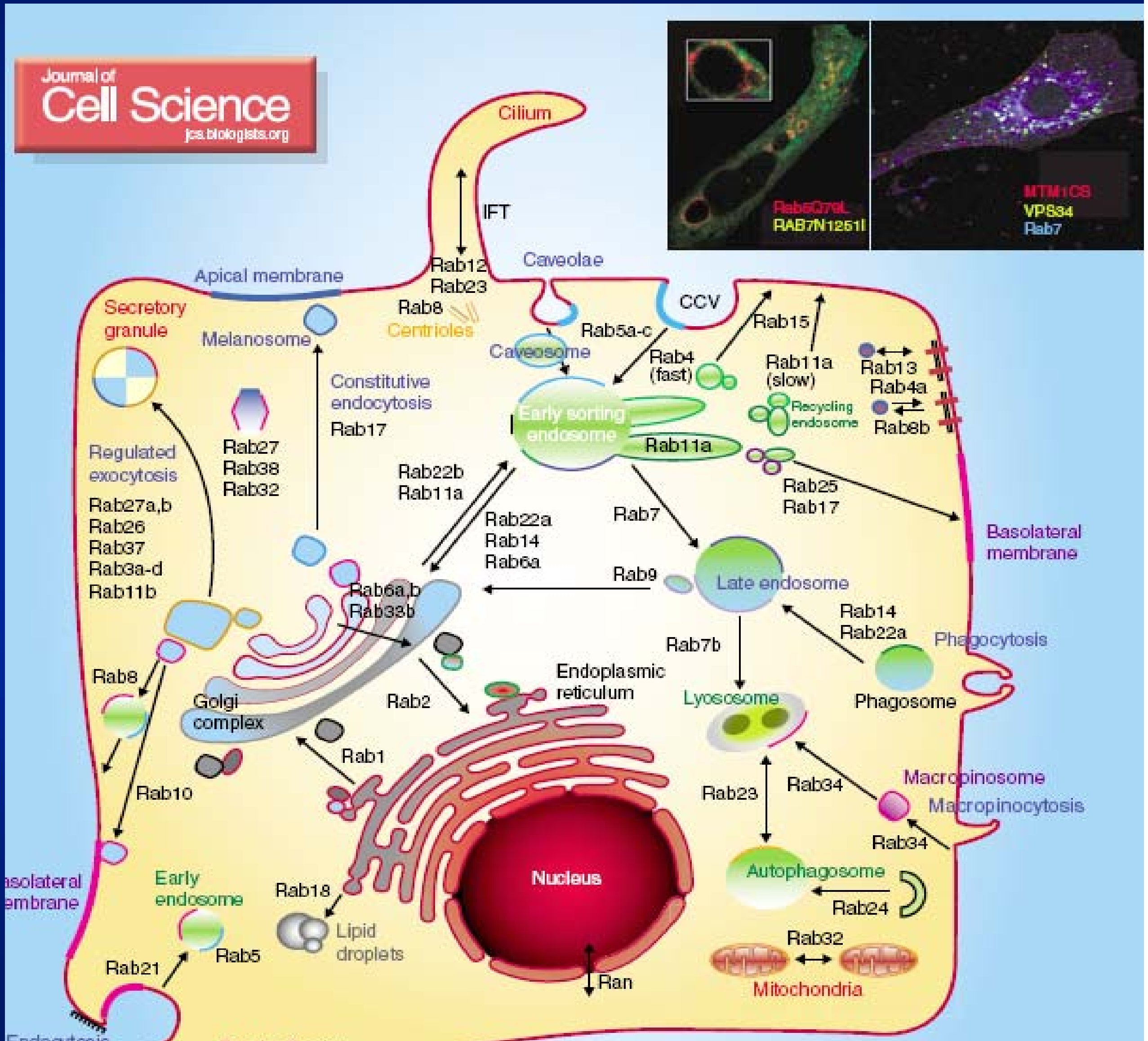


The Rab family of small GTPases

Family of small GTPases (21-28kDa) with more than 70 human isoforms

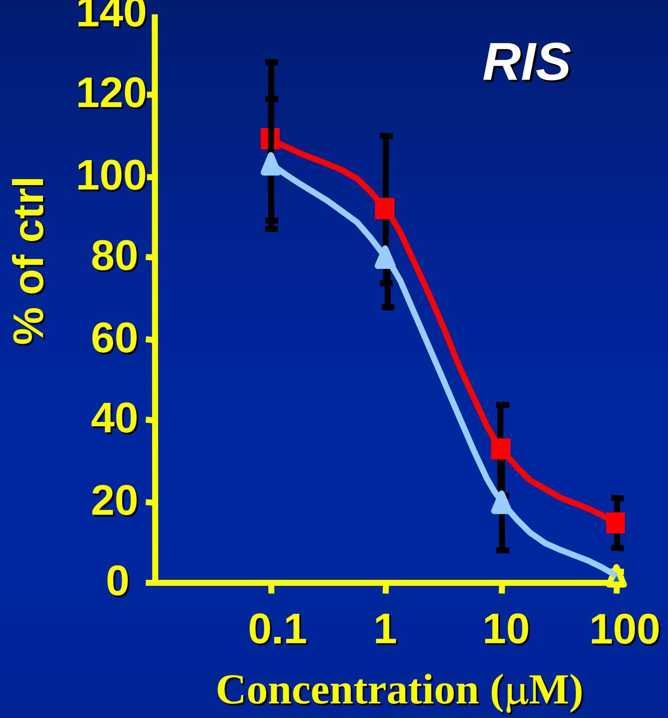
Rab GTPases are crucial regulators of vesicle trafficking:

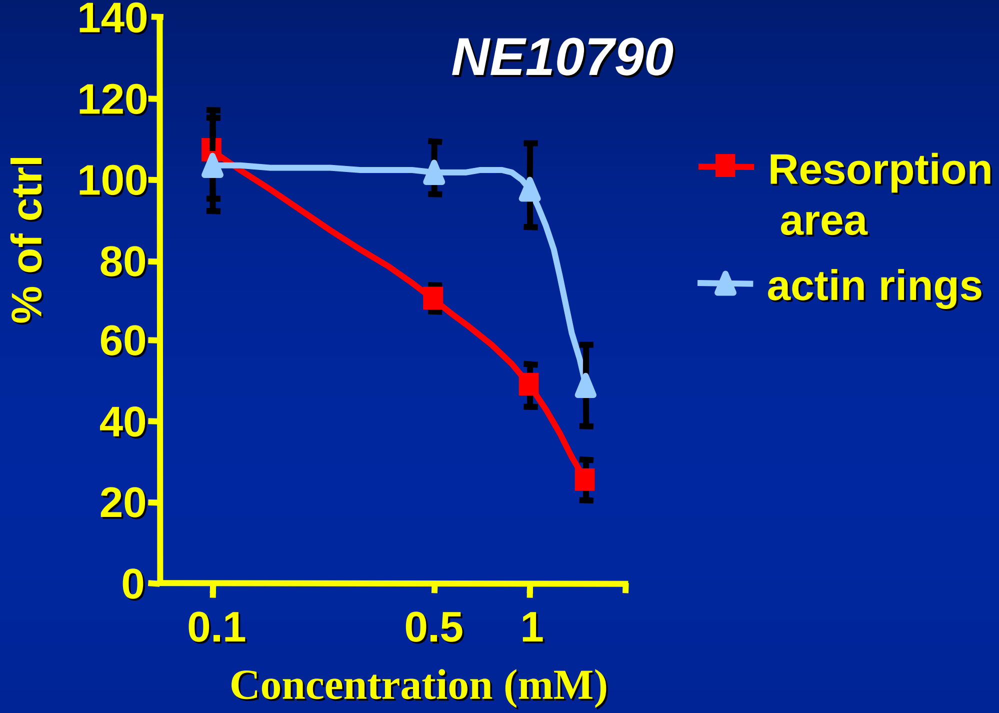
- Vesicle formation
- Vesicle motility
- Vesicle docking
- Membrane fusion



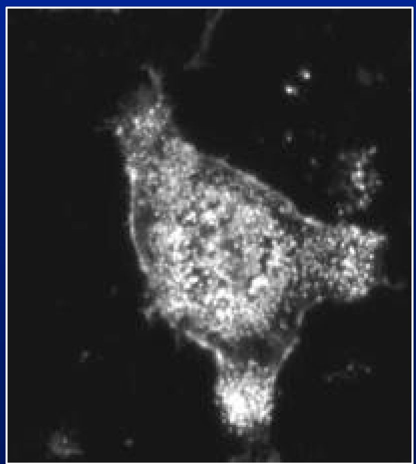
Schwartz et al (2007) J Cell Sci 120: 3905-3910

NE10790 inhibits bone resorption *in vitro* without affecting the cytoskeleton

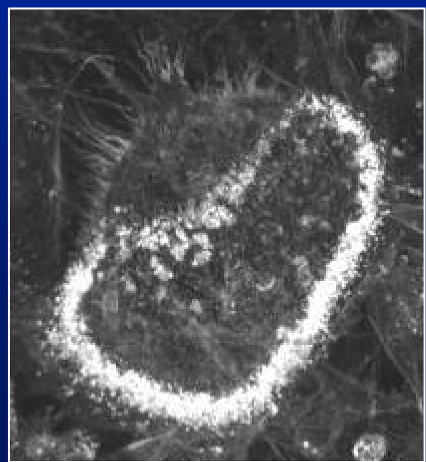




RIS

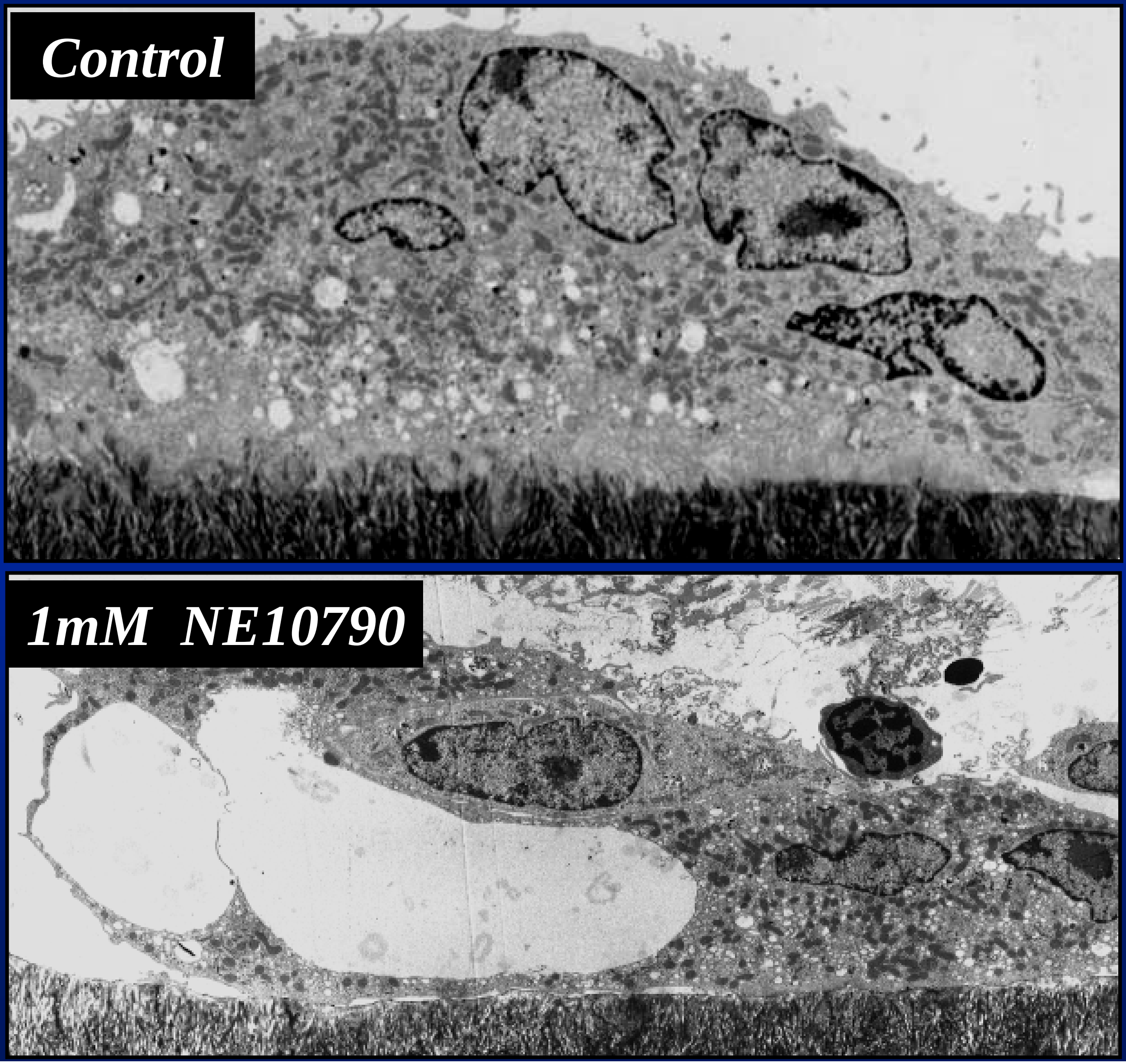


NE10790



Inhibition of Rab prenylation alters vesicular trafficking in osteoclasts *in vitro*

Coxon *et al* 2001, *J Biol Chem* 276, 48213-48222



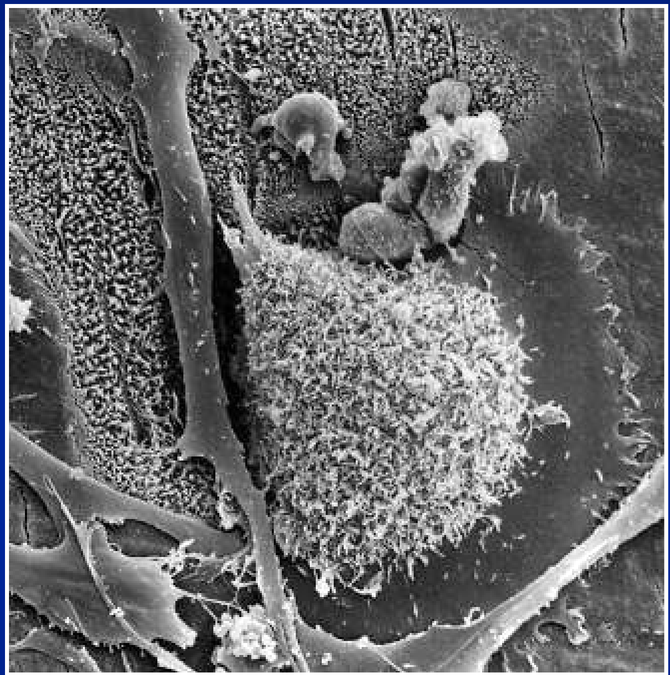
Inhibition of Rab prenylation alters vesicular trafficking in osteoclasts *in vitro*

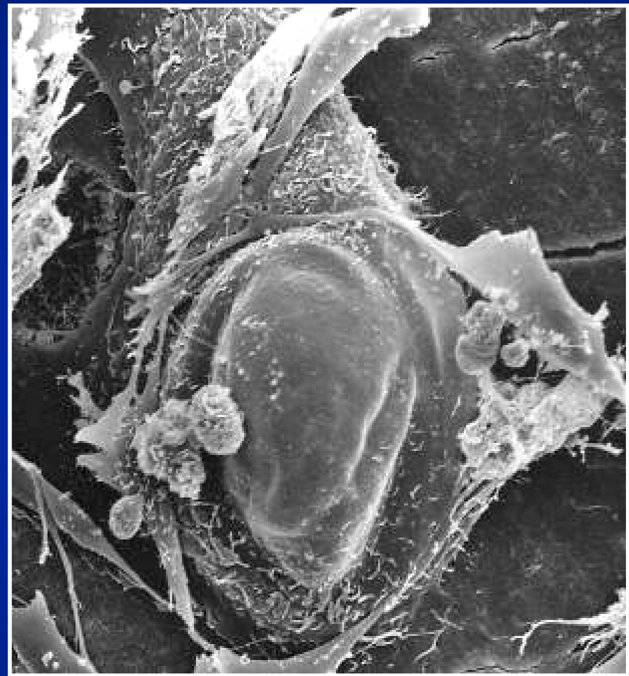
Coxon *et al* 2001, *J Biol Chem* 276, 48213-48222

Control

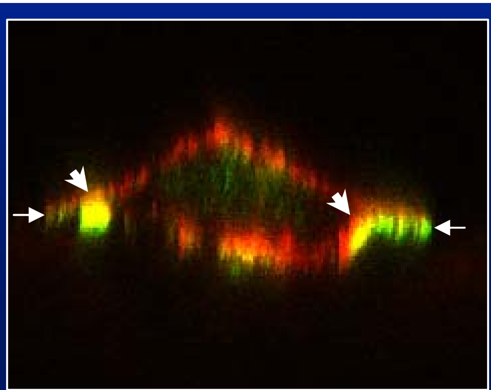
1mM NE10790

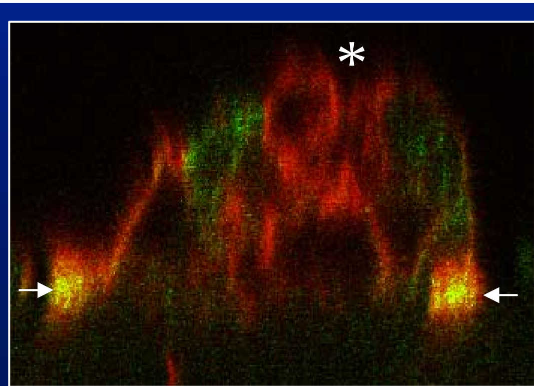
SEM





confocal
(axial views)

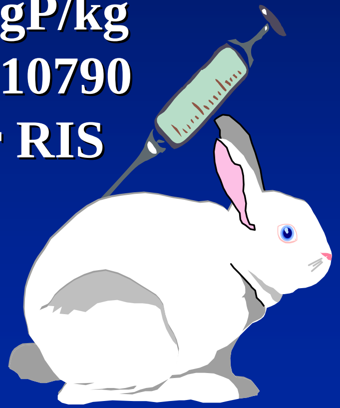




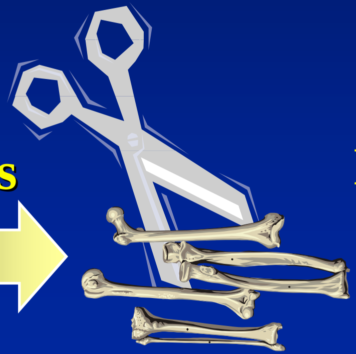
Do these drugs inhibit protein prenylation in osteoclasts *in vivo*?

Do these compounds inhibit protein prenylation *in vivo*?

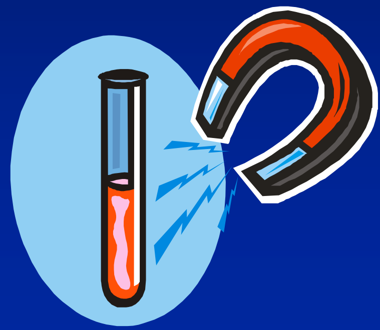
2mgP/kg NE10790 or RIS



24 hrs

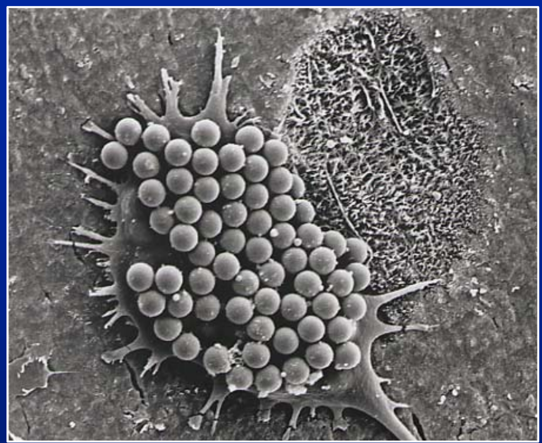


23c6 & anti-mouse IgG-coated Dynal beads



Purified osteoclast pellet

VNR^{+ve} osteoclasts

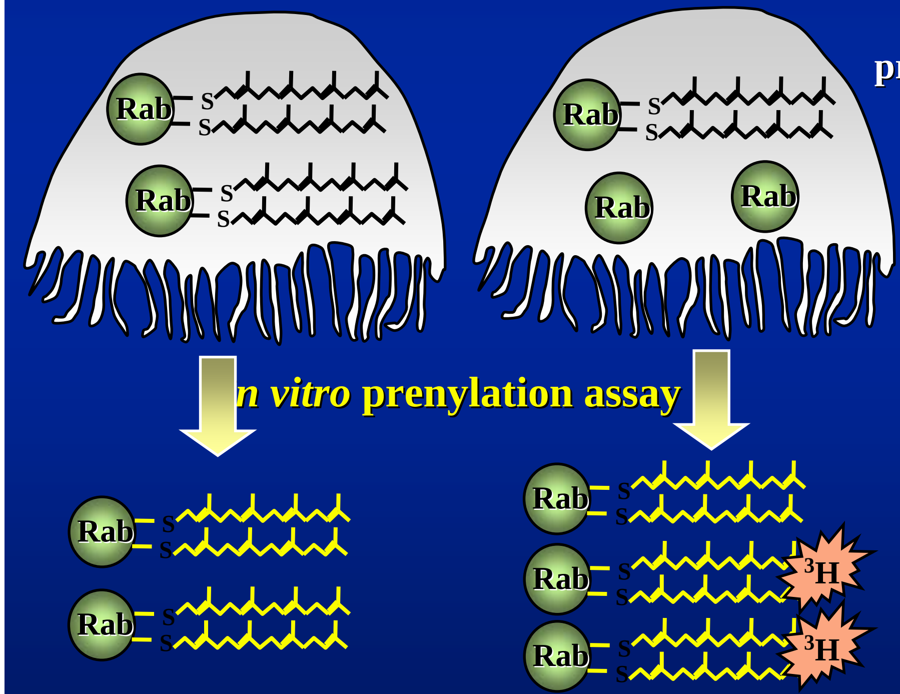


***In vitro* prenylation assay using rh Rab GGTase**

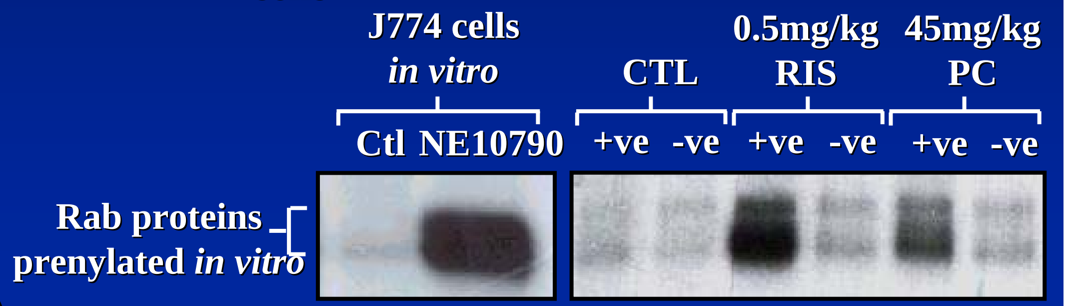
Assay for unprenylated Rap1A

BPs and PCs inhibit protein prenylation in osteoclasts *in vivo*

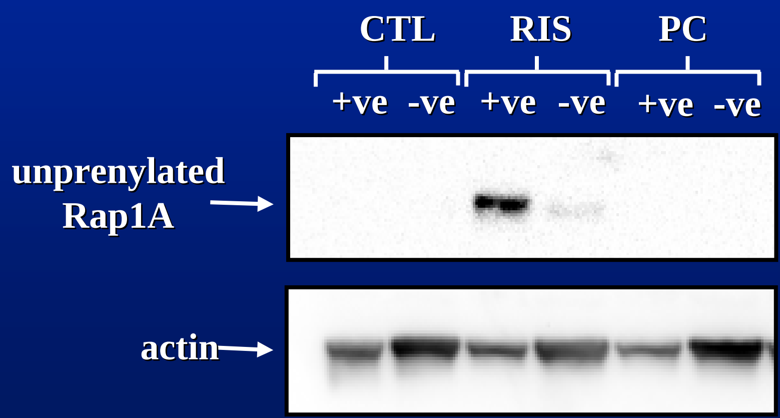
Unprenylated Rabs detected by prenylating with [^{3}H]GGPP *in vitro:*



+ve: osteoclast fraction
-ve: non-osteoclast bone marrow cells



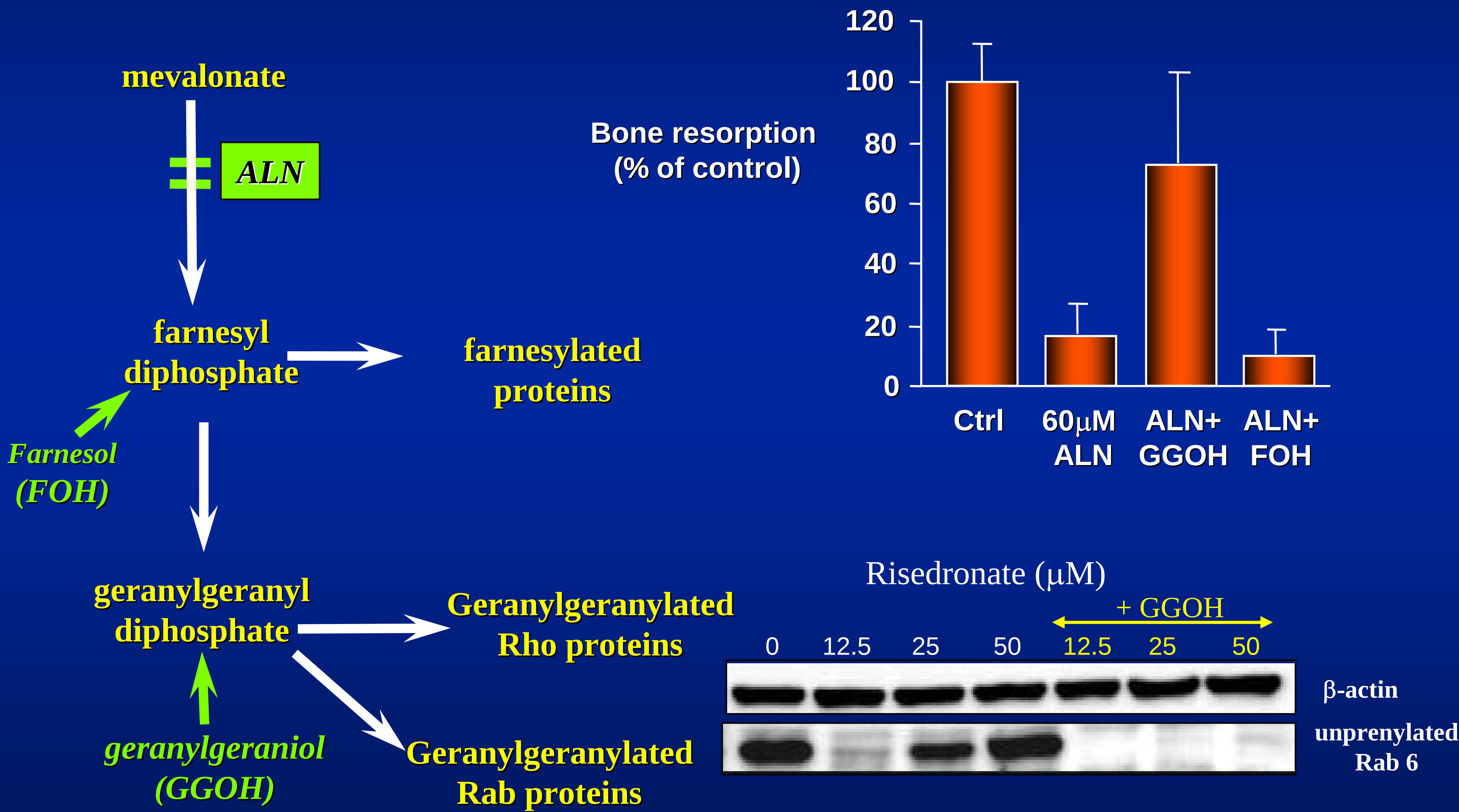
western blotting for unprenylated Rap1A:



Are the effects of BPs really due to inhibition of FPPS?

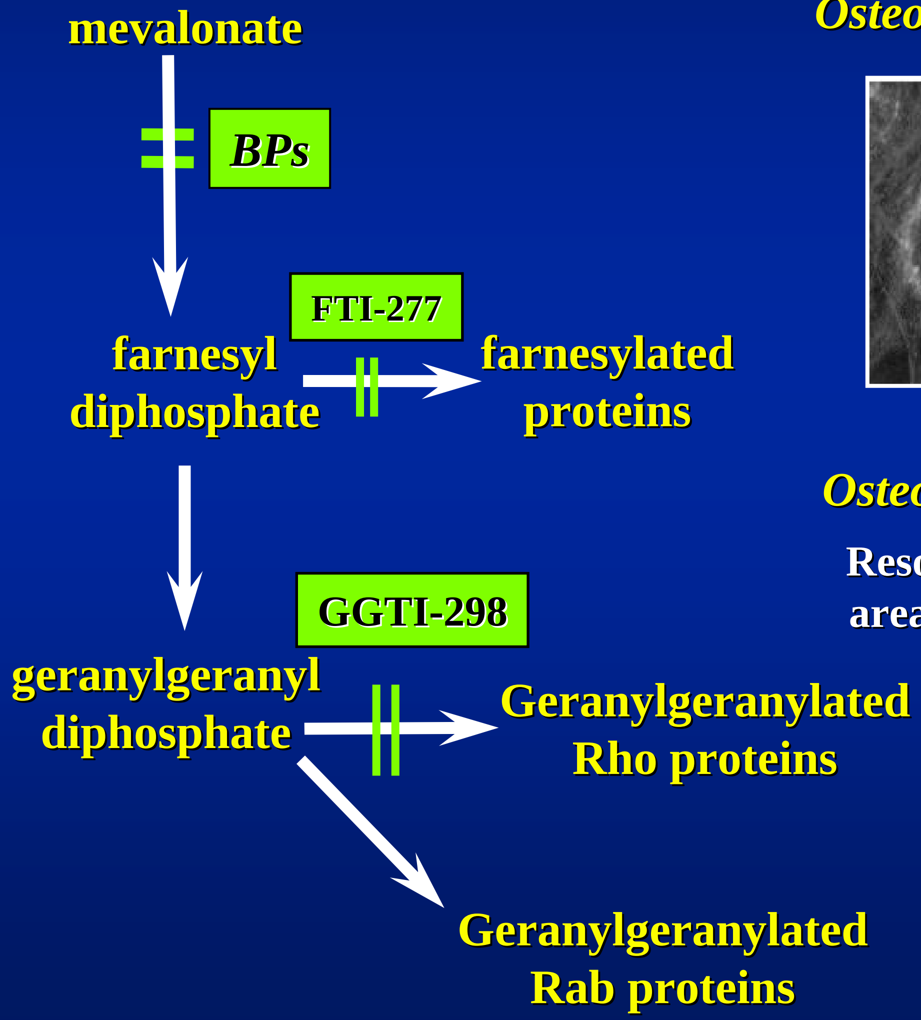
Loss of geranylgeranylated proteins accounts for inhibition of resorption by BPs

Fisher *et al* 1999, *Proc Natl Acad Sci*, 96:133-138; Coxon *et al* 2000, *J Bone Miner Res* 15:1467-1476

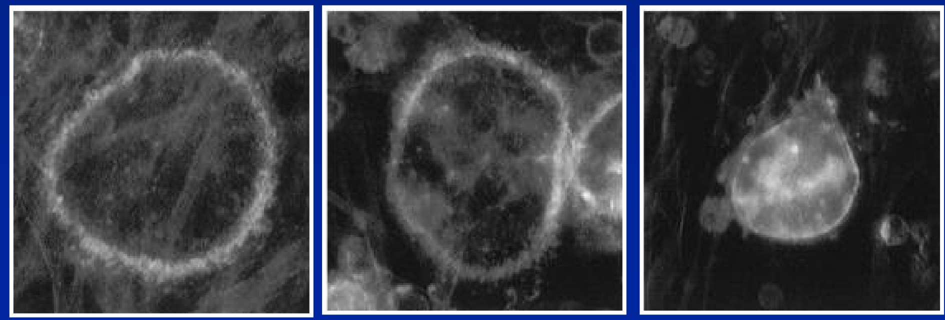


Loss of geranylgeranylated proteins accounts for inhibition of resorption by BPs

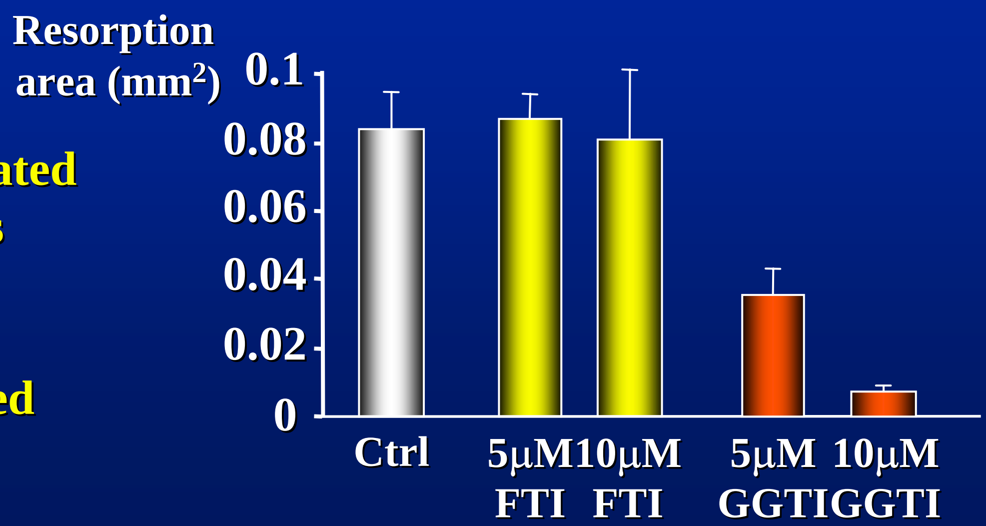
Coxon *et al* 2000, *J Bone Miner Res* 15:1467-1476
Coxon et al 2003, *Calcif Tissue Int* 72:80-84



Osteoclast polarisation:



Osteoclast resorption:



Summary

- Assays using osteoclasts in vitro and ex vivo have enabled us to determine the mechanism of action of bisphosphonates and the related phosphonocarboxylates
  - Inhibition of prenylation of small GTPases
- Morphological analysis of osteoclasts treated with these compounds has also shed light on the importance of prenylated small GTPases for osteoclast function

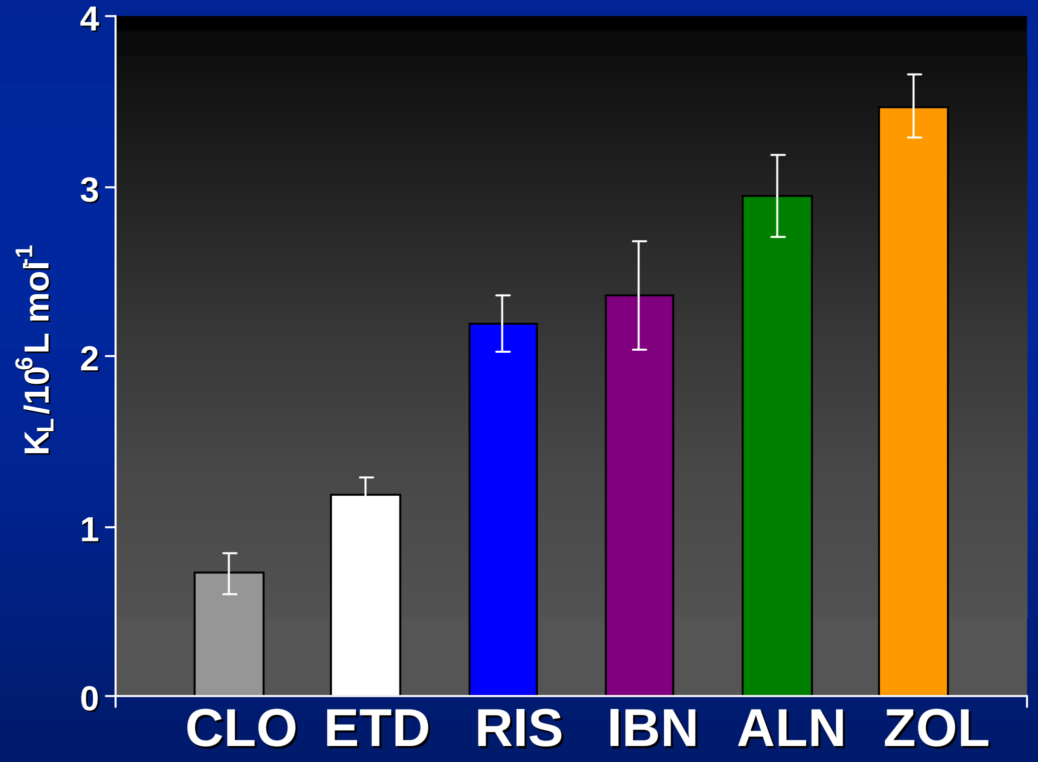
Localisation of BPs and PCs in bone

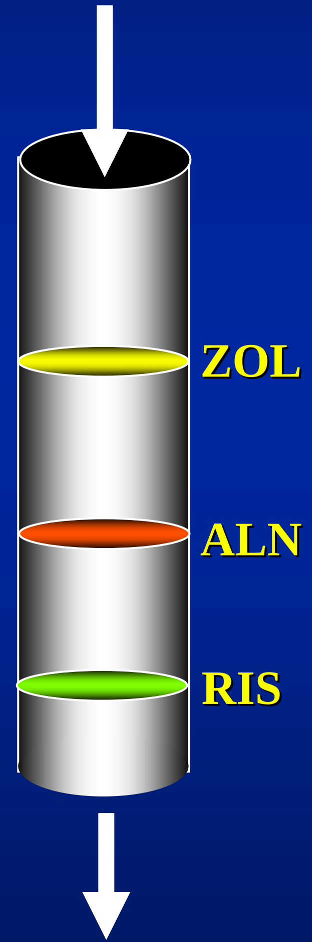
Bisphosphonates have different affinities for hydroxyapatite

Nancollas *et al* 2006, *Bone* 38: 617-627

The R_2 side-chain of BPs also contributes to bone affinity…

HAP Adsorption Affinity Constants at pH 7.4





Lawson, Triffitt, Ebetino & Russell
ASBMR & Davos 2005 & 2006

Are osteocytes exposed to bisphosphonates *in vivo*?

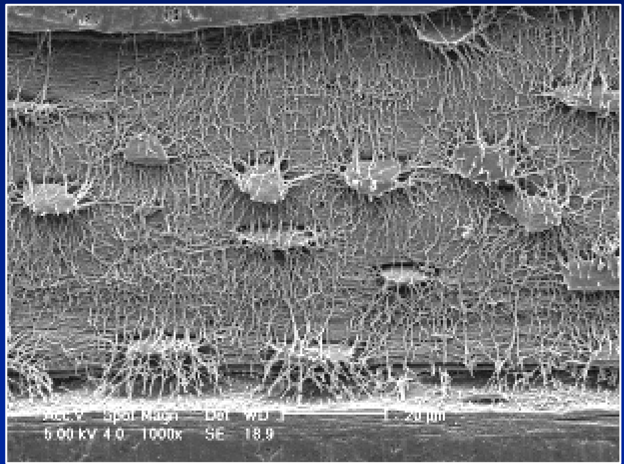
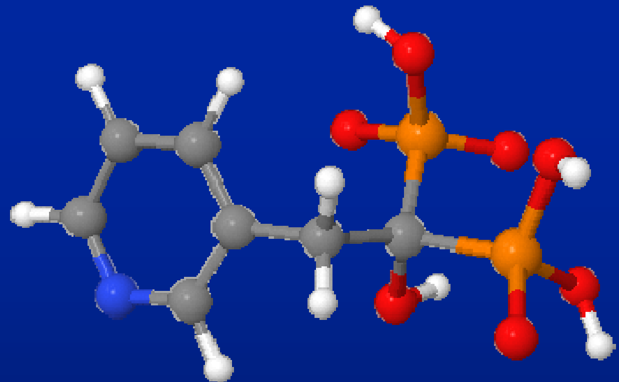
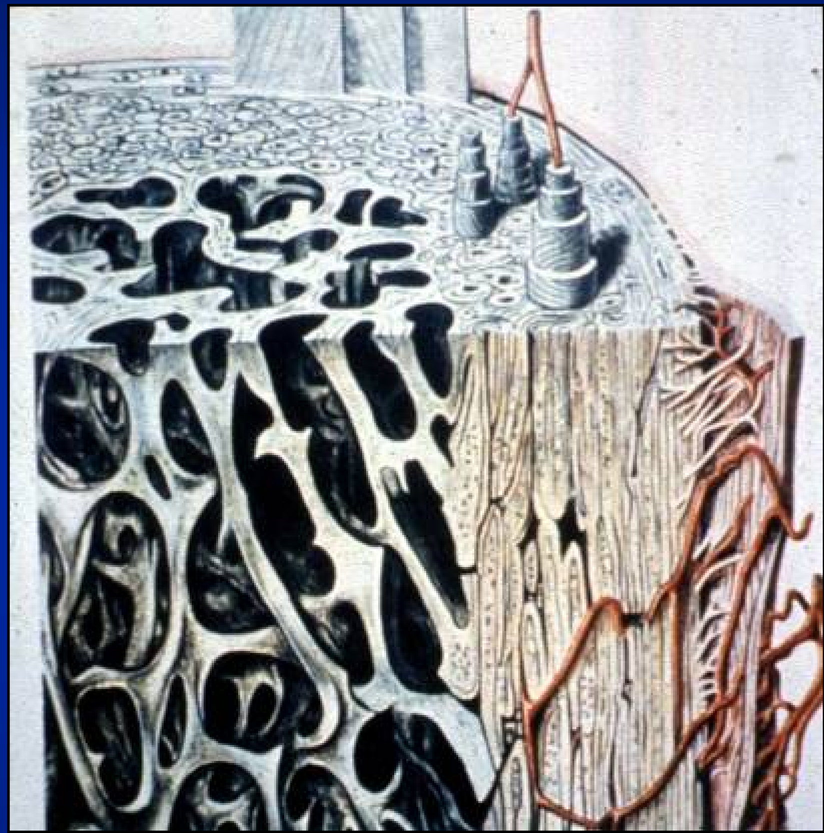


Photo by Lilian Plotkin and Lynda Bonewald

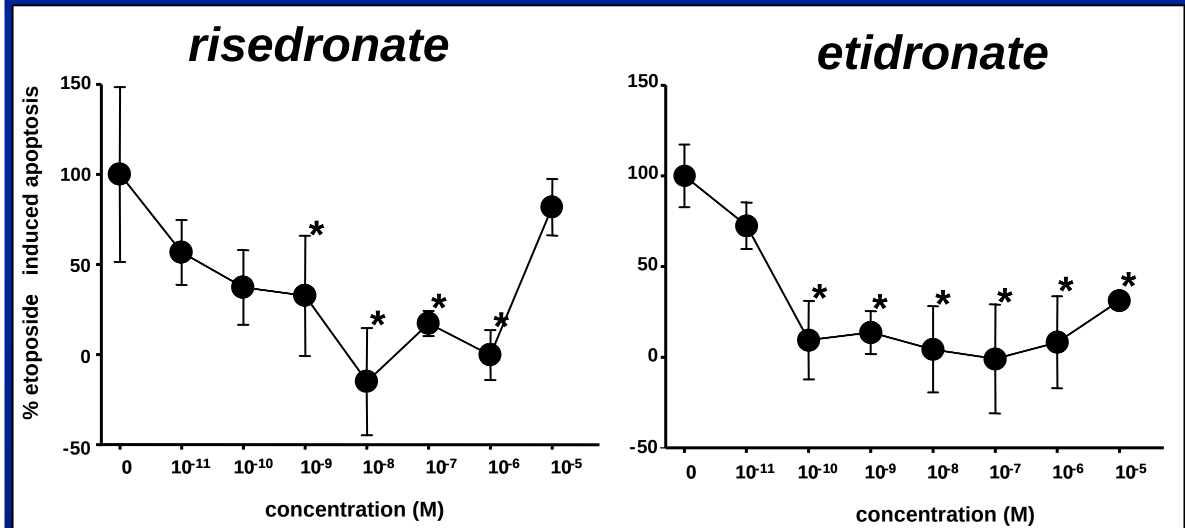


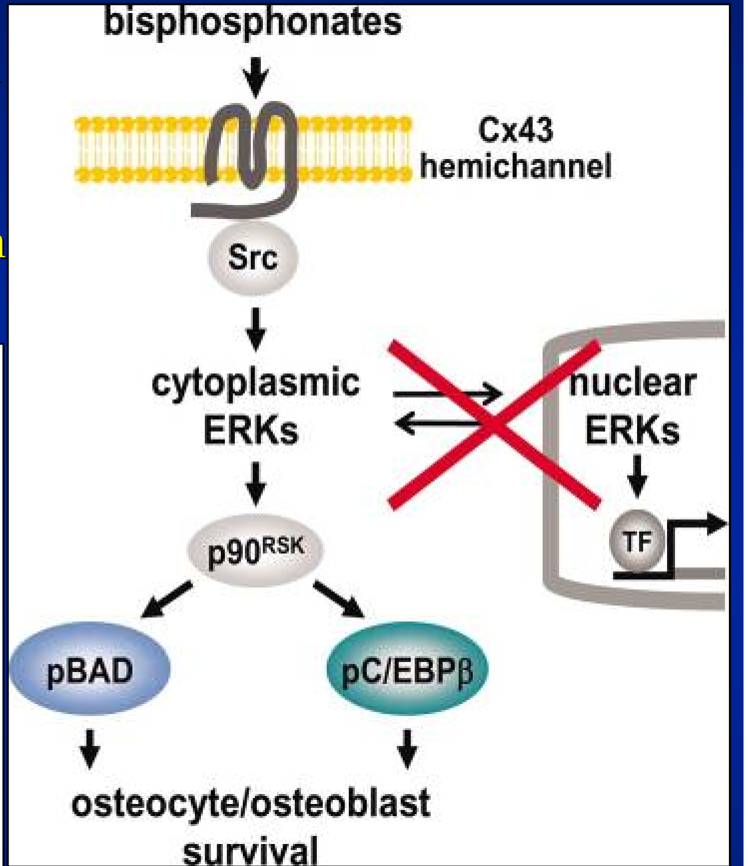


Lower affinity BPs should be able to gain access to more sites in bone than higher affinity BPs which will get ‘stuck’ at sites of first contact

Anti-apoptotic effect of BPs on osteocytes (MLO-Y4 cells)

BPs can open Connexin 43 hemichannels and activate anti-apoptotic signalling pathways via ERKS





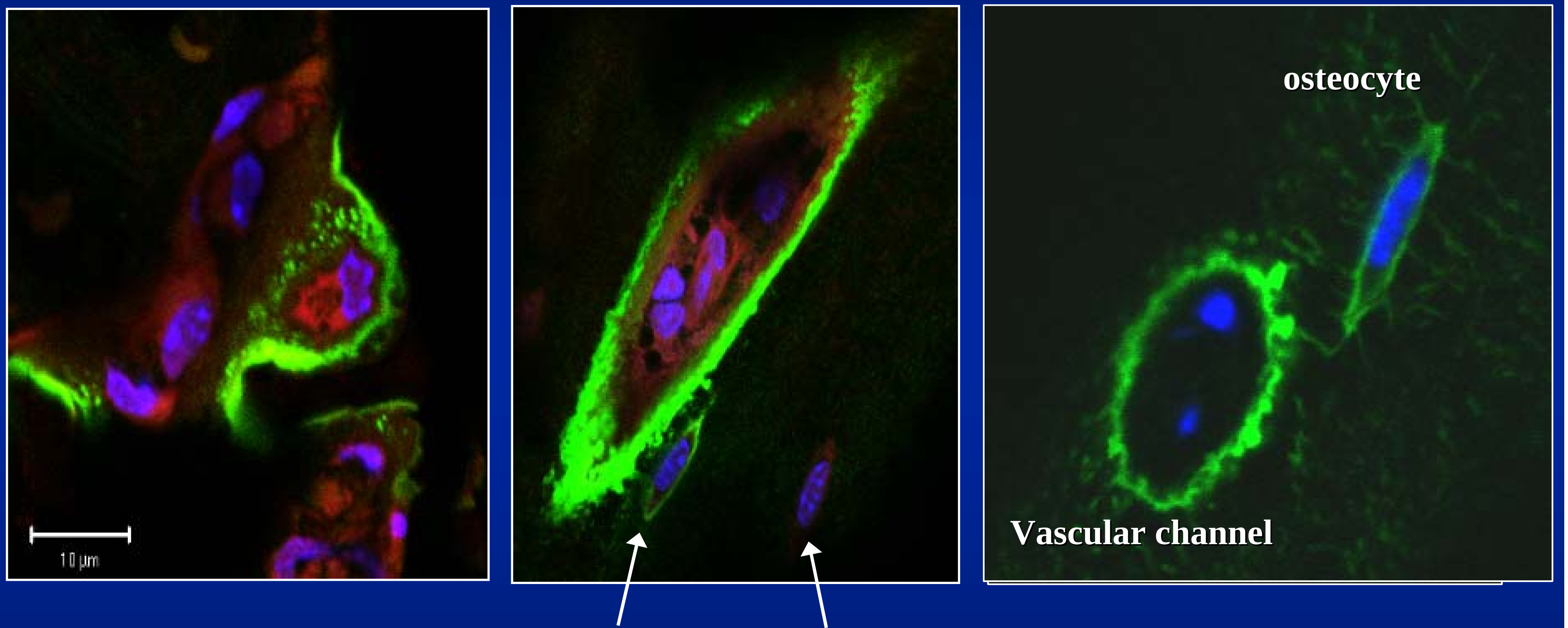
IC_{50} values in the nM range!

Plotkin, Manolagas, Bellido, Bone 39 (2006) 443-452

Methods

- Mice or rats injected subcutaneously with fluorescently labelled conjugates *(fluorescein, rhodamine or AlexaFluor 647)* of RIS and 3-PEHPC
- Animals sacrificed 24h later and tibiae fixed and embedded in MMA resin
- Resin blocks cut transversely and bone examined directly by confocal microscopy

F-RIS localises to osteocyte lacunae in mouse cortical bone



Green- F-RIS

Blue-nuclei

Red- wheat germ agglutinin

Acknowledgements

Bone Research Group, Aberdeen

Mike Rogers
Miep Helfrich
Adam Taylor
Anke Roelofs
Debbie Scott
Aysha Khalid

University of Oxford

Graham Russell
Jim Dunford

Procter & Gamble Pharmaceuticals

Hal Ebetino
Mark Lundy

University of Southern California

Charles McKenna

Imperial College, London

Miguel Seabra
Mimi Mules

Queen Mary, University of London

Alan Boyde

Funded by
Arthritis Research Campaign

P&G Pharmaceuticals

SHERT
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