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REVIEW

New knowledge on critical osteoclast formation and activation pathways from study of rare genetic diseases of osteoclasts: focus on the RANK/RANKL axis

J. C. Crockett · D. J. Mellis · D. I. Scott · M. H. Helfrich

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Abstract Functional, biochemical and genetic studies have over the past decade identified many causative genes in the osteoclast diseases osteopetrosis and Paget's disease of bone. Here, we outline all osteoclast diseases and their genetic associations and then focus specifically on those diseases caused by mutations in the critical osteoclast molecule Receptor Activator of Nuclear factor Kappa B (RANK). Both loss and gain-of-function mutations have been found in humans leading to osteopetrosis and high bone turnover phenotypes, respectively. Osteopetrosis-associated RANK mutations are widely distributed over the RANK molecule. It is likely that some negatively affect ligand binding, whereas others preclude appropriate association of RANK with downstream signalling molecules. In the Paget-like disorders, familial expansile osteolysis, early onset Paget's disease and expansile skeletal hyperphosphatasia, heterozygous insertion mutations are found in the RANK signal peptide. These prevent signal peptide cleavage, trapping the protein translated from the mutated allele in the endoplasmic reticulum. Whole animal studies replicate the hyperactive osteoclast phenotype associated with these disorders and present only with heterozygous expression of the mutation, suggesting an as yet unexplained effect of the mutant allele on normal RANK function. We discuss the cell biological studies and animal models that help us to understand the nature of these different RANK defects and describe how careful dissection of these conditions can help understand critical pathways in osteoclast development and function. We highlight areas that require further study, particularly in light of the pharmacological interest in targeting the RANK signalling pathway to treat diseases caused by excessive bone resorption.

Keywords ePDB · ESH · FEO · Osteopetrosis · Paget's disease · RANK/RANKL

While this review was going to press, a study was published by Albagha et al (Nature Genetics, published online May 2010) demonstrating the association of SNPs on chromosome 18q21, close to the *TNFRSF11a* locus, with late onset Paget's Disease in patients without *SQSTM1* mutations. This highlights *RANK* as an additional susceptibility gene for development of late-onset Paget's disease.

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Introduction

A number of genetic diseases have been identified that are caused by defects in the formation or function of osteoclasts, the key cell type specialised in degradation of bone matrix. The logical expectation is that osteoclast malfunction might lead to either reduced or increased activity and indeed both types of osteoclast defects are found in humans. Here, we briefly discuss all osteoclast diseases, grouped under those that are sclerotic/osteopetrotic because of osteoclast under-activity, and those that show increased bone turnover because of osteoclast hyperactivity. We then focus specifically on those disorders caused by mutations in proteins in the Receptor Activator of Nuclear factor Kappa Beta (RANK) pathway, a signalling pathway critical for osteoclast development and activity and one in which much new information has come to light in the past few years. It has become clear that naturally occurring RANK mutations have wide ranging effects in osteoclasts leading to vastly different bone phenotypes in their hosts. The precise molecular

mechanisms by which this occurs are not fully known and deserve further study.

Osteoclasts

Osteoclasts are large multinucleated cells which are unique in their ability to resorb mineralised bone matrix. They form as a result of the fusion of mononuclear precursors derived from the monocyte/macrophage lineage, and this differentiation is controlled by interactions between osteoblasts and/or stromal cells and pre-osteoclasts [1]. Macrophage-colony stimulating factor (M-CSF) and RANK ligand (RANKL), expressed by osteoblasts and stromal cells, are essential factors for inducing osteoclast formation. M-CSF is required for both the proliferative and differentiation phase of osteoclast development [2] and RANKL is critical for osteoclastogenesis and bone resorption [3–5]. RANKL interacts with its receptor RANK, a transmembrane receptor that is a member of the tumour necrosis factor (TNF) receptor superfamily and is expressed on the surface of pre-osteoclasts and mature osteoclasts [6]. Osteoprotegerin (OPG), a soluble decoy receptor produced by osteoblasts and stromal cells within the bone environment can block osteoclast formation in vitro and bone resorption in vivo by binding to RANKL and reducing its ability to interact with RANK [7]. The RANK/RANKL axis is important in regulation of bone, but also has important roles in immunology and arterial calcification [8] and, most recently, in the control of thermoregulation [9].

When osteoclasts are activated to initiate resorption, they form a specific attachment to the bone surface via a membrane domain called the "sealing zone" (SZ). This attachment involves rearrangement of the cytoskeleton, especially the actin cytoskeleton, to form a ring of F-actin perpendicular to the bone surface. Transmission electron micrographs of osteoclasts in bone in situ clearly demonstrate the high actin content and its orientation in the SZ (Fig. 1a), while osteoclasts cultured on the surface of a cut piece of bone or dentine most easily demonstrate the ring formed by the F-actin (Fig. 1b). The plasma membrane surrounded by the actin ring then forms the "ruffled border" (RB), a highly convoluted membrane domain that provides a large surface area for release of the protons and proteolytic enzymes required to dissolve the bone matrix (Fig. 1a, c). Endocytosis and subsequent transcytosis of degradation products from the resorption area beneath the osteoclast results in release of the degradation products to nearby capillaries via the membrane domain at the cell surface opposite the RB called the "functional secretory domain" (FSD, Fig. 1a) [10, 11].

During the resorption process, acidic intracellular vesicles fuse with the plasma membrane in the RB to

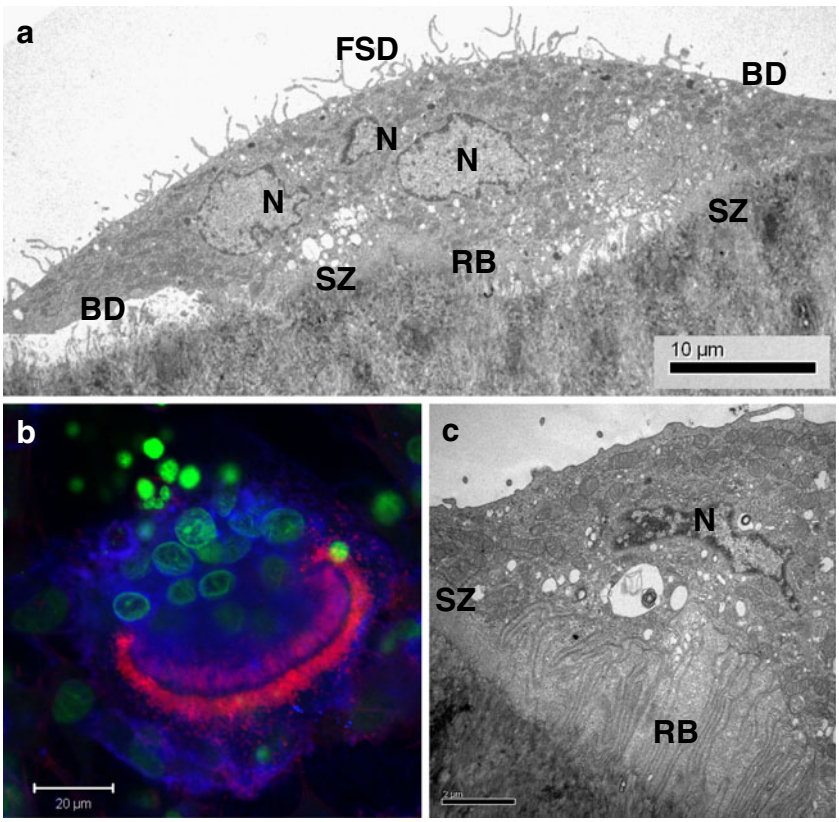

Fig. 1 Illustrations of the membrane domains of osteoclasts cultured on a mineralised surface. **a** Transmission electron micrograph: the ruffled border (RB) has a large surface area for the release of protons and proteolytic enzymes into the resorption lacunae underneath the cell. The RB is surrounded by a sealing zone (SZ) forming a tight attachment to the bone surface. The basolateral domain (BD) is the area of plasma membrane between the SZ and the functional secretory domain (FSD). *N* = nucleus. **b** Confocal micrograph of a resorbing osteoclast. The cell is stained with wheat germ agglutinin to indicate the plasma membrane (*blue*), for F-actin to indicate the SZ (*red*) and the nuclei are stained with a DNA-binding dye (*green*). **c** Higher magnification transmission electron micrograph of the RB to illustrate the extensive folding of this membrane domain (scale bar=2•m)

release acid into the space between the osteoclast and the bone matrix. Although as yet not fully understood, it is possible that this initial acidification provides the trigger for the full-scale formation of the RB. When acidic vesicles fuse with the forming RB, they insert a vacuolar-type proton ATPase (V-ATPase) into the RB membrane to transport protons to the bone matrix. The action of the V-ATPase is coupled with that of chloride channels that pump a negatively charged ion for each proton to maintain electroneutrality. In osteoclasts, it is currently thought that ClC-7, a Cl^-/H^+ antiporter [12] is coupled to the V-ATPase [13] in the RB. Ion equilibrium within the cytoplasm of the osteoclast is achieved through the production of protons and bicarbonate ions by the actions of carbonic anhydrase II (CAII) and by the exchange of bicarbonate for chloride via the HCO_3^-/Cl^- exchanger, found in the fourth main membrane region of osteoclasts: the basolateral membrane. Proteolytic enzymes secreted into the resorption area underneath the RB include the protease cathepsin K, the main collagenase produced by osteoclasts [14]. Other enzymes involved in matrix degradation and expressed at high levels in osteoclasts are tartrate-resistant acid phosphatase and matrix metalloproteinase 9 [1].

In a healthy skeleton, bone resorption is tightly coupled to bone formation, and several diseases are known where abnormal osteoclast formation and/or activity leads to an unbalanced bone homeostasis. Osteopetrosis is caused by a lack of osteoclast activity, either due to lack of osteoclasts or to defective osteoclast function (Fig. 2a, b). By contrast, Pagetic diseases feature osteoclast hyperactivity (Fig. 2c). We will first discuss these two classes of osteoclast diseases that result in diametrically opposite bone phenotypes in more detail before returning to the topic of RANK/RANKL mutations.

Osteopetrotic conditions, lack of osteoclast activity

Osteopetrosis is characterised by the presence of a high bone mass caused by osteoclast dysfunction. Despite the high bone mass, patients with osteopetrosis suffer repeated fractures due to the brittle nature of their bone and the persistence of mineralised cartilage which is not remodelled adequately. This distinguishes the condition from other high bone mass disorders, such as sclerosteosis or van Buchem disease, which are caused by osteoblast overactivity and are characterised by the absence of fractures, even after trauma [15–17]. The distinction is clinically important, as for disorders caused by intrinsic defects in osteoclasts, a descendant of the haemopoietic lineage, bone marrow transplantation (BMT) may present a realistic treatment option [18], whereas this is not the case for bone dysplasias originating from deregulated osteoblast activity. Osteopetrosis was first recorded in 1904 by radiologist Heinrich Albers-Schönberg, when he examined an adult patient with generalised bone sclerosis and multiple fractures [19]. Since then, a variety of osteopetroses have been described in adults and children. Genetic and cell biological studies in rodents with spontaneous mutations leading to high bone mass have been instrumental in identifying the osteopetrotic mutations in human patients. In particular, the groundbreaking work from Donald Walker and Sandy Marks showed why studies in rodents with inherited bone phenotypes have high relevance to understanding genetic bone disease in humans and helped to identify BMT as the best treatment option in the majority of patients with the disease, (Table 1) [20–25].

It is now clear that osteopetrosis can be grouped into three different types depending on the severity and mode of inheritance of the condition: malignant autosomal recessive osteopetrosis (ARO), intermediate autosomal recessive osteopetrosis (IARO) and autosomal dominant osteopetrosis (ADO).

ARO is a common denominator for the severest forms of the disease. ARO is usually diagnosed soon after birth because of the associated severe haematological symptoms, caused by bone marrow space occlusion, and by neurological symptoms through nerve compression or in some cases (below) through primary neurological defects. Radiological examination shows flaring of the long bones and general sclerosis. While the bone is dense, it is at the same time brittle and hence prone to fracture. IARO shows essentially similar features, but with a less severe bone phenotype. With the exception of some cases caused by CAII deficiency in which intracranial calcification has been reported [26], absence of neurological defects make the disease generally more compatible with prolonged survival. Fractures may still occur and in fact may be the first feature that alerts clinicians to the disease.

ADO presents later in life than the recessive forms and is usually diagnosed by coincidental radiological examination or by fractures. Despite the association with “benign” in the older literature, more recent systematic reviews have highlighted the significant morbidity associated with this condition [27, 28].

Rather than classification by mode of inheritance, osteopetrosis can also be classified as either an osteoclast-rich or osteoclast-poor disease, dependent on whether the disease is caused by a defect in osteoclast activity or by a problem in osteoclastogenesis [18]. We, however, prefer to classify the different types of disease by the name of the gene mutated, since this is now possible in over 75% of osteopetrosis cases. This type of classification is important as the treatment options depend on knowing whether the defect is osteoclast autonomous (i.e., an osteoclast-expressed gene is defective) or lies in an environmental

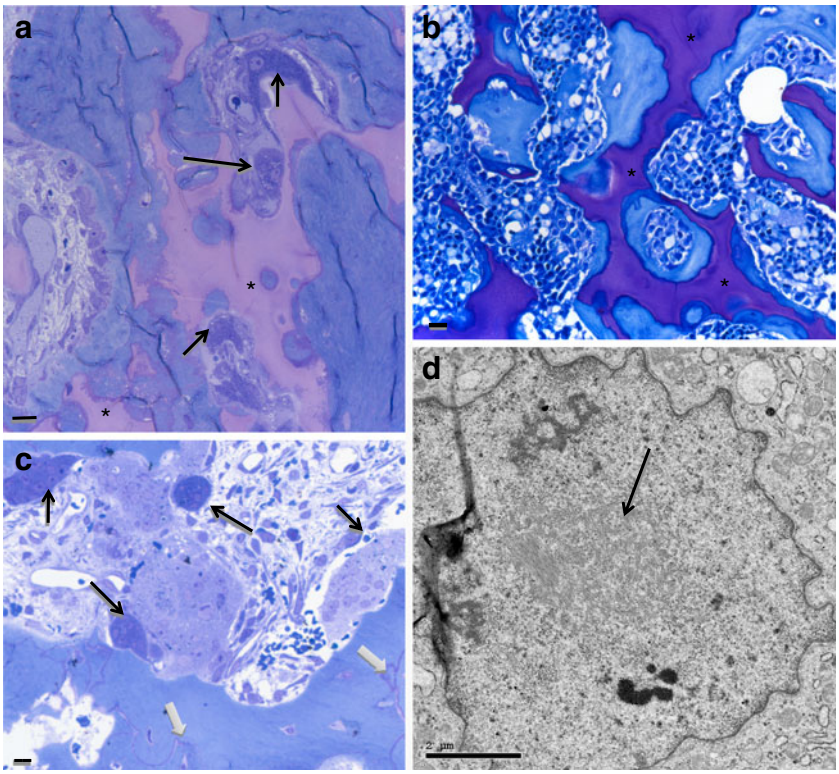

Fig. 2 Light micrographs of bone biopsies from patients with osteoclast-rich osteopetrosis (**a**) osteoclastpoor osteopetrosis (**b**) and Paget's disease of bone (**c**). Note the retention of cartilage (*pink/purple*) in the cases of osteopetrosis (**a**, **b**) and the relative smooth surface of the matrix, even when osteoclasts are abundant (*arrows* in **a**). Bone is *blue*. By contrast, in the biopsy from a patient with PDB, osteoclasts are abundant (*closed arrows*), but bone resorption is clearly evident from the scalloped bone surface. Within the bone matrix cement lines are visible (*open arrows*) indicating the high rate of bone turnover. **d** Transmission electron micrograph of a large inclusion in the nucleus of an osteoclast in PDB (*arrow*). Scale bars in **a**–**c** are 20 μm, scale bar in **d** is 2 μm. **a**, **c** are semithin (1 μm) sections of demineralised epon-embedded bone stained with toluidine blue; **b** is a 5-μm section of a demineralised wax section stained with toluidine blue

factor controlling osteoclast function (in which case BMT will not be indicated) [29]. In Table 1, we summarise the available information and group the diseases by gene defect and by the ability of BMT to provide a clinical cure. We also list the genes involved, describe the osteoclast phenotype in vivo and in vitro and list relevant animal mutations that have helped understand the nature of the biological defects with the seminal references to the genetic defects and animal studies.

In short, the osteopetrosis-like disease pycnodysostosis is caused by recessive loss-of-function mutations in the enzyme cathepsin K. This results in inefficient collagen degradation and hence ineffective bone resorption, coupled with dysmorphic features, such as missing terminal phalanges. Mutations in the a3 subunit of V-ATPase, in the Cl^-/H^+ antiporter ClC-7 and its associated molecule OSTM1 (together responsible for chloride transport coupled to proton transport), in the protein PLEKHM1 (with a role in vesicular trafficking) and in the enzyme CAII (responsible for the first enzymatic step in proton synthesis) are all associated with recessive osteopetrosis and mutations within the signalling molecule NEMO (with a role in NFκB activation) cause a X-linked form of osteoclast-rich osteopetrosis. All these mutations result in the presence of normal or excessive numbers of non-functional osteoclasts with an inability to traffic vesicles containing protons and proteolytic enzymes to the bone surface (Fig. 2a). Intriguingly, as well as an inability to resorb the organic and the inorganic matrix of bone, this lack of vesicular trafficking in itself leads to a defect in RB formation. Although in principle, all these genetic causes of osteoclast malfunction might be successfully treated by BMT, it is clear that some mutations do not act solely on osteoclast function, i.e. are not entirely osteoclast-specific.

Most patients with recessive mutations in ClC-7 and all those with mutations in OSTM1 suffer from severe primary neurological defects, especially cerebral/cerebellar atrophy, retinal degeneration and neuronal lysosomal storage disease. These problems are not corrected by replacement of the bone marrow so that BMT is contraindicated in OSTM1 related disease and must be performed with careful patient selection and parental counselling in CLC-7-associated disease [30].

Patients with mutations in RANKL and in RANK form a distinct subgroup of recessive osteopetrosis. Bone biopsies from these patients do not show osteoclasts (Fig. 2b). Skeletal pathology in patients with RANK mutations may benefit from BMT [31], but no data on longer-term follow-up are available as yet. Patients with RANKL mutations do not respond to BMT and surprisingly display a less severe phenotype with several cases now alive and relatively well in their teens. A relatively simple functional test on osteoclast formation in vitro will easily distinguish between the two types of disease if the bone biopsy has identified osteoclast-poor osteopetrosis (Fig. 3) [29]. The clinical and genetic assessments that should be made before deciding on BMT in osteopetrosis have recently been reviewed elsewhere [32].

Immunological investigations in patients with RANKL defects showed few immunological abnormalities, but importantly and in keeping with the RANKL null mouse that lacks lymph nodes, lymph nodes were not palpable. B and T cell numbers in the human patients, however, appeared normal and the overall effect of RANKL absence on immunological markers appeared minimal [33]. Most patients with RANK mutations showed more severe immunological defects with a defect in memory B cell differentiation and in most, but not all reduced immunoglobulin levels [31]. Lymph node examinations were not carried out in the cases reported. Although at first glance, there appears to be a difference in the severity of the immunological defects between mice and humans lacking RANK/RANKL activity, it is becoming clear that, in the mouse, as probably in humans, other molecules can compensate for the lack of RANK signalling in immune cells, as discussed in more detail elsewhere [8]. As new osteopetrosis patients with RANK and RANKL deficiencies are diagnosed, it will be important to perform full immunological investigations to help understand the difference between these two types of defects and the relative roles of RANK and RANKL in normal immunology and physiology. New roles for RANK and RANKL are still being elucidated; most recently, RANK has been shown to have a critical role in the control of fever with RANK expression demonstrated in neuronal cells, including astrocytes in the brain [9]. This may explain why two of the osteoclast-poor ARO patients with RANK mutations did not develop a fever during episodes of pneumonia [9].

It is also clear that a number of other TNF superfamily members, for example LIGHT and APRIL, substitute for RANKL in vitro and allow differentiation of osteoclasts [34]. The levels of these molecules in vivo and especially in patients with RANKL deficiencies are not known, but it is conceivable that low-level osteoclast formation is sustained in this way, offering an explanation for the fact that some bone modelling is clearly taking place in these patients with very low or undetectable levels of osteoclasts in bone biopsies.

Paget's disease and related disorders, hyperactive osteoclasts

Common, late-onset Paget's disease

Paget's disease of bone (PDB) is a common late-onset metabolic bone disease characterised by focal areas of increased bone remodelling affecting 1–2% of white Caucasian population over the age of 55 years and 8% of men and 5% women over the age of 80 years (http://www.paget.org.uk/). The disease is most prevalent in the UK and Northern Europe, but is also common in Australia, New Zealand and USA [35, 36]. Patients suffer from a range of symptoms including bone pain, bone deformity, deafness, osteoarthritis and may develop osteosarcoma [37, 38]. Paget's disease is the result of deregulated bone turnover. The disease is driven primarily by increased activity of osteoclasts, but it is possible that intrinsic defects in other cell types in the bone environment contribute to disease onset and severity [39]. Pagetic lesions occur either within a single bone (monostotic) or, more commonly, at multiple sites throughout the skeleton (polyostotic) with affected bones most likely to be the femur, tibia, pelvis and skull. Pagetic bone lesions show increased numbers of enlarged, highly active osteoclasts with more nuclei than osteoclasts in non-Pagetic bone [40, 41], suggesting either increased fusion or increased lifespan of osteoclasts (Fig. 2c). In response to an increase in osteoclast activity, osteoblast activity is also increased resulting in high levels of alkaline phosphatase in serum. The combined uncontrolled activity of osteoblasts and osteoclasts also results in the persistence of woven bone which is not remodelled into lamellar bone (Fig. 2c). This results in a skeleton that is, at least in affected sites, structurally weaker than normal and that fractures more easily.

PDB has a strong genetic component, although it is also clear that there is a substantial influence from environmental and possibly other factors, including other genes. Discussion of this complex topic is outwith the remit of this review and we refer the reader to recent discussions elsewhere [38, 40]. The only mutated gene thus far reported and confirmed in several cohorts of PDB patients, is *SQSTM1* encoding the protein sequestosome-1/p62 (from now on referred to as p62). A range of mutations has been reported, all of which are located in, or near the C-terminal ubiquitin-associated (UBA) domain of the protein and prevent p62 binding to ubiquitin [42]. p62 is a multi-domain protein which acts as a dimer and has an important role in the activation of NFκB pathway downstream of

Table 1 Functional effects of genes mutated in osteopetrosis and Paget-like diseases

Gene	Protein name	Key references	Animal model and key references	Protein function	In vitro osteoclast phenotype	In vivo osteoclast phenotype	Clinical phenotype	Additional information/symptoms
TCIRG1	T cell, immune regulator 1, ATPase, H^+ transporting, lysosomal V0 subunit A3	[106–108] (OMIM 604952)	Mouse *oc/oc* [109]	Acidification of the resorption lacunae	No ruffled border, no resorption	Defective proton pump, high number of osteoclasts present, no ruffled border	ARO	Classic osteoclast-rich osteopetrosis with neurological defects due to nerve compression, anaemia and infections. BMT is indicated
CLCN7	Chloride channel 7	[13] (OMIM 602727)	Mouse *Clcn7*$^{-/-}$ [13]	Cl^-/H^+ antiporter	No ruffled border, no resorption	Defective Cl^-/H^+ antiporter, high number of osteoclasts present, no ruffled border	ARO/IARO/ADOII	Severe osteoclast-rich osteopetrosis with lysosomal storage disease in the brain and primary retinal degeneration. BMT requires careful consideration
OSTM1	Osteopetrosis associated transmembrane protein 1	[110] (OMIM 607649)	Mouse *gl/gl* [111]	β subunit for ClC-7	Increased osteoclast numbers, poorly developed ruffled border, disrupted cytoskeleton	No bone histology available	ARO	Rare, very severe osteoclast-rich osteopetrosis with lysosomal storage disease in the brain. Often results in perinatal death. BMT not indicated
PLEKHM1	Pleckstrin homology domain containing, family M (with RUN domain) member 1	[112, 113] (OMIM 611466)	Rat *ia/ia* [114]	Vesicular trafficking	Poorly developed ruffed border, lack of resorption, increased intracellular TRAP levels	No bone histology available	IARO	Mild type of osteopetrosis with no other clinical symptoms. BMT not indicated.
NEMO	Nuclear factor-κB essential modulator	[58, 115] (OMIM 300248)	Mouse *Nemo*$^{-/-}$ [116]	NFκB activation	Not performed	Normal osteoclast numbers, enlarged trabeculae with cartilage core	Mild X-linked osteopetrosis	Immunodeficiency, multiple infections, lymphoedema, malabsorption. Indication for BMT not clear–hepatotoxicity associated with pre-conditioning
CAII	Carbonic anhydrase II	[117] (OMIM 611492)	Mouse *Car2*$^{-/-}$ [118]	Intracellular acidification	Lack of proton secretion	No bone histology available	IARO	Osteopetrosis with renal tubular acidosis and cerebral calcifications. Range of clinical severity. BMT not indicated.
TNFSF11 (RANKL)	Tumour necrosis factor superfamily, member 11	[33] (OMIM 602642)	Mouse *Tnfsf11*$^{-/-}$ [3]	Osteoclast formation function and survival	Normal osteoclast formation, polarisation and resorption in presence of recombinant wild-type RANKL	No osteoclasts present (lack of formation)	ARO	Osteoclast-poor osteopetrosis. No obvious defects in immunological parameters. BMT not indicated.
TNFRSF11A (RANK)	Tumour necrosis factor receptor superfamily, member 11A	[31] (OMIM 603499)	Mouse *Tnfrsf11a*$^{-/-}$ [6]	Osteoclast formation function and survival	No osteoclast formation	No osteoclasts present (lack of formation)	ARO	Osteoclast-poor osteopetrosis. Visual impairment, hypogammaglobulinemia, nystagmus. BMT indicated
VCP	Valosin containing protein	[90] (OMIM 167320)	Mouse overexpressing *Vcp* mutant R155H [119]	Proteasomal degradation of phosphorylated IκB-α	Data not available	Increased bone turnover, presence of inclusions bodies in osteoclast nuclei and in cytoplasm	Inclusion body myopathy, Paget's disease and frontotemporal dementia (IBMPFD)	Syndrome combining muscle weakness, dementia, and Paget-like bone lesions. Not all three organs systems are affected in all patients. Management is tailored to suit individual patients,

								in most cases bisphosphonates are used to treat the bone disease
TNFRSF11B (OPG)	Tumour necrosis factor (ligand) superfamily, member 11B	[44, 45] (OMIM 239000)	Mouse *Tnfrsf11b*$^{-/-}$ [65]	Decoy receptor for RANKL	Data not available	Increased bone turnover, disorganised trabecular bone	Juvenile Paget's disease (JPD)	Plate-like formation of the trabecular bone in the iliac crest. Treatment is generally with bisphosphonates and/or recombinant OPG
SQSTM1 (p62)	Sequestosome-1 (p62)	[120] (OMIM 602080)	Global *Sqstm1*$^{-/-}$[55]	Scaffold protein	Osteoclasts are hypersensitive to vitamin D3 and RANKL	Focal areas of increased bone turnover, presence of inclusion bodies in osteoclast nuclei and in cytoplasm, incr[121] ease in osteoclast size and number	Paget's disease of bone (PDB)	Deafness and neurological defects
			Oc-specific *Sqstm1*^{P392L} [121]					Treatment is usually with bisphosphonates but analgesics such as paracetamol and non-steroidal anti-inflammatory drugs (NSAIDS) are often used to treat pain [122]
			Global *Sqstm1*^{P392L} [102]					
TNFRSF11A (RANK)	Tumour necrosis factor receptor superfamily, member 11A	[48, 101] (OMIM 174810)	n/a	Osteoclast formation function and survival	Data not available	Focal areas of increased bone turnover, presence of inclusion bodies in osteoclast nuclei and in cytoplasm, presence of woven bone	Familial expansile osteolysis (FEO)	Focal areas of expansile osteolytic bone lesions, early tooth loss and deafness
								Bisphosphonates (and sometimes calcitonin) are used to treat bone pain and reduce disease activity [46]
		[49] (OMIM – n/a)	n/a	Osteoclast formation function and survival	Data not available	Focal areas of increased bone turnover, presence of inclusions bodies in osteoclast nuclei and in cytoplasm, disorganised collagen bundles, presence of woven bone	Expansile skeletal hyperphosphatasia (ESH)	Hyperostotic long bones, early tooth loss and deafness.
								Bisphosphonates and NSAIDs are used to treat hypercalcaemia and bone pain [46]
		[50] (OMIM – n/a)	*ePDB-Rank* knock-in mouse [52]	Osteoclast formation function and survival	Data not available	Focal areas of increased bone turnover, presence of inclusions bodies in osteoclast nuclei and in cytoplasm, presence of woven bone	Early onset Paget's disease of bone (ePDB)	Osteolytic and sclerotic bone lesions, early tooth loss and deafness.
								Bisphosphonates are used to reduce bone resorption, surgery can be effective in some cases to correct bone deformities [46]

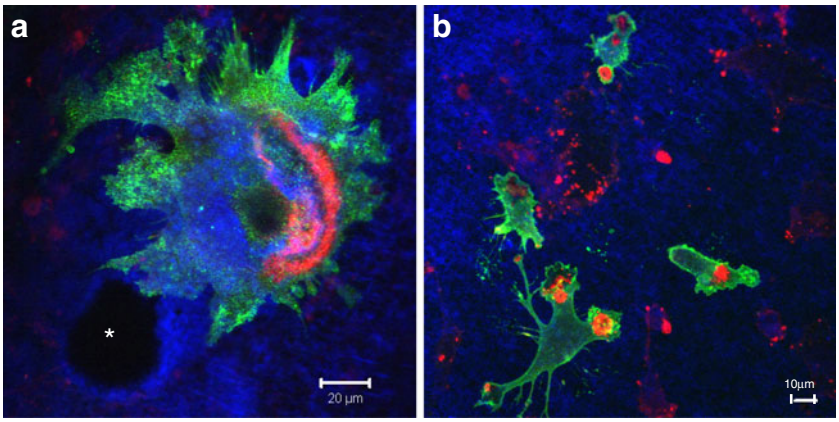

Fig. 3 Osteoclast formation in vitro distinguishes between the two groups of patients with osteoclast-poor osteopetrosis. Osteoclasts are stained for vitronectin receptor (*green*), F-actin (*red*) and the dentine surface is stained with a fluorescent bisphosphonate (*blue*). Resorption lacunae are visible by absence of blue staining and identified by an *asterisk*. **a** Normal resorbing osteoclasts are seen when mononuclear cells from patients with RANKL mutations are cultured with MCSF and wild-type recombinant RANKL. **b** No multinuclear, resorbing osteoclasts are seen when mononuclear cells from patients with RANK mutations are cultured with MCSF and wild-type recombinant RANKL (reproduced with permission from [31])

RANK [43]. We will return to the possible functional consequences of the p62 mutations further below when discussing more fully the functional consequences of mutations in the RANK signalling pathway.

Early-onset high turnover diseases caused by OPG mutations

Juvenile Paget's disease (JPD) is a rare, early-onset disease of high bone turnover presenting in early childhood. Both an increase in osteoclast and in osteoblast activity is seen leading to the production of weak and disorganised bone that is prone to fracture. Autosomal recessive mutations within the gene encoding OPG (*TNFRSF11B*) are the cause of JPD [44, 45]. Depending on the nature of the mutation patients either produce less efficient forms of OPG with reduced affinity for RANKL or fail to produce OPG entirely.

Early-onset high bone turnover diseases caused by RANK mutations

Familial expansile osteolysis (FEO), early-onset Paget's disease of bone (ePDB) and expansile skeletal hyperphosphatasia (ESH) are extremely rare diseases that share features with late-onset PDB but each have their own characteristic symptom profiles [46, 47]. The early age of onset, within the first two decades, is the main distinguishing feature between these conditions and the more common, late-onset PDB described above. FEO predominantly affects the major bones of the appendicular skeleton which can lead to gross deformities, but also affects the jaw and the bones of the middle ear resulting in deafness and premature tooth loss. ESH patients also suffer from the premature loss of hearing and tooth loss but in addition show involvement of the long bones of the fingers causing bone pain in the hands. e-PDB shares all the above clinical features with involvement of both the appendicular and axial skeleton. In all disorders, bone pain and bone fractures are the main clinical symptoms and lead to the diagnosis. Since the patients generally have suffered from these progressive diseases for some time before a formal diagnosis is made, little is known so far about the early stages of the disease pathology.

Heterozygous insertion duplication mutations located within the signal peptide region of the RANK gene have been identified as the cause of these diseases. FEO is associated with an 18-base pair (84dup18) tandem duplication leading to an additional six amino acids in the RANK protein, ePDB with an insertion of 27 bp (75dup27 or 78dup27), leading to an additional nine amino acids and ESH with a 15 bp duplication (84dup15), adding five amino acids to RANK [48–51].

These similar mutations in the same region of the gene interestingly lead to phenotypes that were clinically distinguished before molecular information was available and even more remarkably, the in vitro studies also find subtle differences between the different forms of RANK when expressed in model cell systems. Elucidation of the molecular mechanisms leading to the hyperactive osteoclast phenotype that is the key feature of the early onset Pagetic disorders has been ongoing in a number of laboratories, but has proved difficult. The first animal model engineered to express the ePDB gene has been reported in abstract form and is showing a bone phenotype similar to that seen in human patients [52].

To try to understand how insertion mutations in RANK lead to hyperactive osteoclasts and how mutations in other

regions of RANK lead to absence of osteoclasts and osteopetrosis, we will now first discuss the normal process by which RANK interacts with RANKL and activates signalling pathways resulting in the activation of the transcription factor NFκB.

The RANK signalling pathway

The RANK signal transduction pathway is composed of several key elements (Fig. 4). In addition to the mutations in RANK, RANKL and OPG mentioned above, mutations within signalling intermediates in the signalling pathway downstream of RANK also lead to abnormal bone phenotypes in mice [53–56] or bone disease in humans [57, 58]. RANKL is expressed on the surface of osteoblasts and stromal cells but also exists in a soluble form, generated by proteolytic cleavage of the ectodomain from the surface of cells [59], a process that may be important in the development of tumour-induced osteolytic lesions [60, 61]. Based on homology to ligand/receptor interactions between other members of the TNF/TNFR superfamilies, trimeric RANKL (with three identical subunits) is predicted to bind to trimeric RANK [62, 63] to initiate the signalling cascade leading to osteoclast formation. The activity of RANKL is modulated by OPG by preventing RANKL binding to RANK. Disruption in the production or activities of any of these molecules results, as predicted, in a change in osteoclast formation or activity. In mice, deletion of the genes for RANK or RANKL leads to profound osteopetrosis [3, 6, 64], whereas deletion of the gene for OPG leads to a high bone turnover osteoporosis [65].

The crystal structure of RANK remains to be determined, hence the precise interaction of RANK with RANKL is not known. The binding of RANKL to RANK is highly specific since RANKL does not bind to any of the

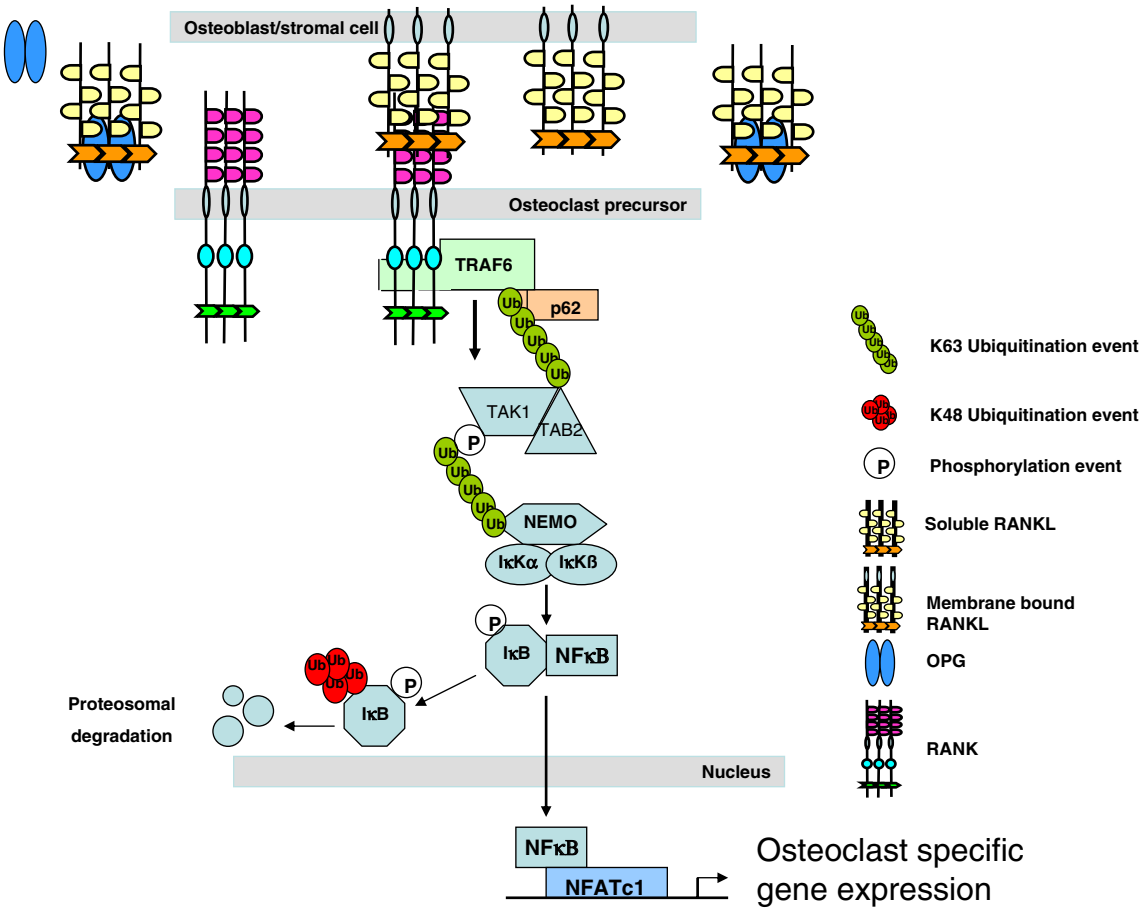

Fig. 4 A summary of the signalling pathway downstream of RANK/RANKL interaction that results in NFκB activation. Trimeric RANKL binds to trimeric RANK receptor resulting in recruitment of TRAF6 to the cytoplasmic domain of RANK. p62 facilitates the formation of K63 polyubiquitin chains on TRAF6 that form a platform for the assembly of the TAB2/TAK1 complex. Phosphorylation of TAK1 results in the K63 polyubiquitination of NEMO and the subsequent phosphorylation and activation of IKKα and β. These kinases phosphorylate IkBα which targets this inhibitory protein for degradation within the proteasome. NFκB is released upon degradation of IkBα and enters the nucleus to regulate expression of genes required for osteoclastogenesis

other TNFR superfamily members except for the decoy receptor OPG [66]. Equally, RANK interacts exclusively with RANKL and does not bind any other member of the TNF family [66]. Despite this, many groups have used structural information from other members of the TNFR superfamily to infer information about RANK. TNFR1 and TNFR2 have a unique site within their *extracellular* domain known as the pre-ligand assembly domain (PLAD) that is critical for TNFR trimerisation and receptor function [67]. This suggests that receptor trimerisation may be important for other members of this receptor superfamily [63]. By contrast to TNFR1 and TNFR2, RANK does not have a PLAD domain, but instead, a six-amino acid motif (534 IIVVYV 539) within the *cytoplasmic domain* that acts as the ligand-independent oligomerisation site [68]. When overexpressed, RANK self-associates in the absence of RANKL and induces osteoclast formation. This is likely to be as a result of trimer formation since artificial induction of RANK *dimer* formation is not sufficient to induce expression of critical osteoclast differentiation genes or to support osteoclast formation and function [68]. Although a role for this RANKL-independent RANK activation has still to be formally demonstrated in vivo, it has been suggested that it may explain the osteoclast phenotype observed in patients carrying the FEORANK mutations [68].

The pathway downstream of RANK, and activated upon binding of RANKL, comprises a series of steps in which signalling intermediates are either activated or degraded. This ultimately results in activation of NFκB and expression of genes required for osteoclastogenesis (Fig. 4). Activation and degradation of signalling intermediates is generally achieved by ubiquitination and phosphorylation. Ubiquitination is the term used to describe the addition of chains of ubiquitin molecules to substrates. The exact chemical linkage between ubiquitin molecules within the chains determines the fate of the target protein. Linkage via lysine residue 48 (K48) marks the targeted protein for degradation by the proteasome, whereas, by contrast, linkage via lysine residue 63 (K63) facilitates the next step in the signalling pathway [69].

The cytoplasmic domain of RANK contains a binding site for TNFR-associated factor 6 (TRAF6) [70]. There are seven members of the TRAF family, but since TRAF6-deficient mice exhibit reduced numbers of osteoclasts due to impairment in RANK signalling [54, 56], TRAF6 is considered the key modulator of RANK signalling. The exact mechanism by which RANK trimerisation facilitates the recruitment of TRAF6 remains to be confirmed. We have recently studied a truncating mutation in RANK that retains the TRAF6 binding site but lacks the oligomerisation motif. This mutant RANK protein does not bind to wild-type RANK confirming the inability of the protein to trimerise, but it does associate with TRAF6. Interestingly, NFκB is activated downstream of this mutant receptor, suggesting that trimerisation is not required for TRAF6 interaction with the cytoplasmic domain of RANK or for its ability to transduce the signal (Crockett and Mellis, unpublished).

When associated with RANK, TRAF6 ubiquitinates itself via interaction with the p62 UBA domain leading to the formation of a K63-linked ubiquitin chain [71]. TRAF6 ubiquitination is essential for recruitment of signalling intermediates [72] including TAB2 and TAK1, leading to formation of a complex that is absolutely required for RANK signalling [73, 74]. Phosphorylation of TAK 1 (by TAB2) initiates the formation of a K63-linked ubiquitin chain on NFκB essential modulator (NEMO; IKKγ), critical for NFκB activation [75]. This in turn triggers the phosphorylation and activation of IKKα/β which phosphorylates IKBα. The resulting conformational change in IKBα triggers K48-linked ubiquitination and subsequent proteasomal degradation of IKBα releases NFκB to translocate to the nucleus to initiate transcription of genes essential for osteoclastogenesis (Fig. 5) [71, 76].

In any signal transduction pathway, the balance between activation and inactivation is critical in determining the duration of the signal. To turn off RANK signalling, a number of mechanisms exist or have been postulated. Firstly, NFκB increases expression of IKBα. This in turn stabilises the IKB/NFκB complex in the cytoplasm, preventing NFκB translocation and reducing expression of its target genes [76]. Secondly, de-ubiquitinases, enzymes involved in de-ubiquitinating substrates, are involved in regulating RANK signalling. Two de-ubiquitinases are known that reduce NFκB translocation by suppressing signalling at a stage upstream of the IKK complex. The first, cylindromastosis tumour suppressor protein (CYLD), cleaves the K63–linked ubiquitin chain from TRAF6, thereby destabilising the TRAF6/p62 and the TAB2/TAK1 complexes and resulting in the termination of the RANK signal. The second, A20, is an NFκB-dependent inhibitor of NFκB and another negative feedback loop in the pathway [71]. Thirdly, signalling pathways are generally also regulated by the degradation of the receptor and by the process of receptor recycling to the cell surface. In the case of RANK, there is as yet little knowledge about receptor degradation and recycling, but there is some evidence that the Cbl family of proteins are involved. The Cbl proteins (c-Cbl and Cbl-b) are ubiquitin ligases that down-regulate receptors for M-CSF and EGF [77]. Cbl proteins are recruited to the RANK complex upon ligand binding in an Src-dependent manner [78]. Activation of Cbl-b has been shown to decrease the levels of the RANK protein via ubiquitin-mediated proteosomal degradation, suggesting that Cbl-b interaction with RANK is a major player in

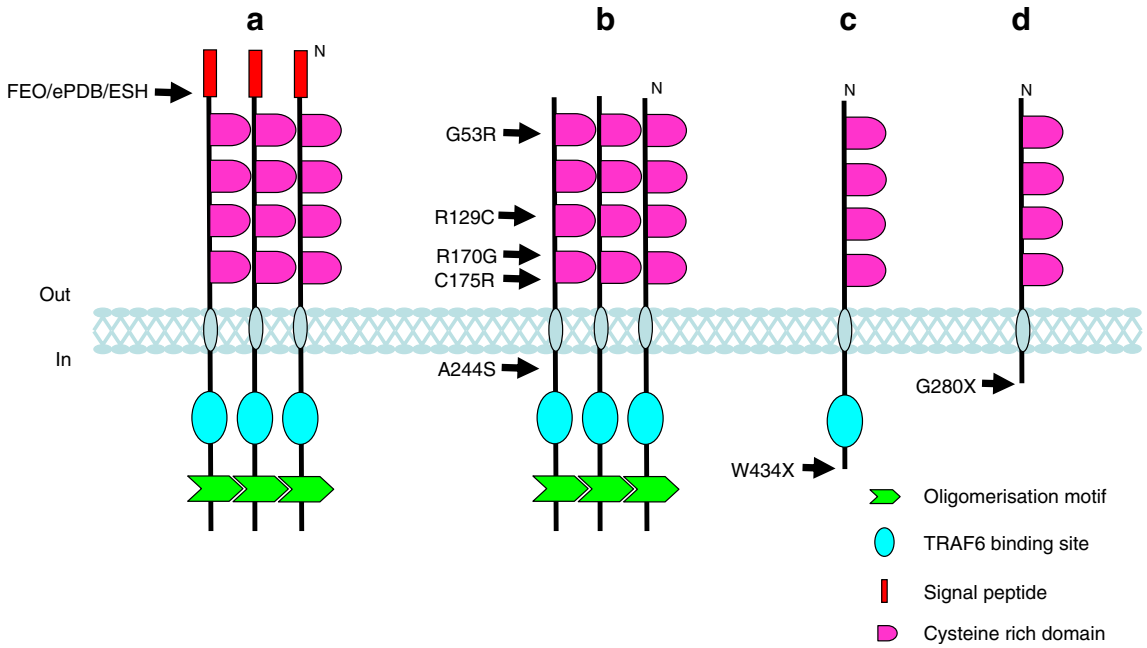

Fig. 5 Domains in RANK associated with disease-associated mutations in RANK (derived from data presented in [31]). **a** RANK contains a signal peptide that is normally cleaved during the post-translational modification process. In RANK containing the FEO, ePDB and ESH mutations, the signal peptide is not cleaved, preventing translocation of RANK to the plasma membrane. **b** The extracellular domain of RANK contains four cysteine-rich domains that are important in RANKL binding. Four of the point mutations associated with osteoclast-poor osteopetrosis–G53R, R129C, R170G and C175R–are within this region of the protein and are predicted to interfere with RANKL/RANK interaction. The A244S mutation occurs just within the cytoplasmic domain in a region that has not been associated with receptor function. **c** The oligomerisation motif mediates ligand-independent oligomerisation of the RANK receptor. The W434X mutation associated with osteoclast-poor osteopetrosis truncates the protein resulting in lack of this critical domain and prevents receptor oligomerisation with as yet undetermined effects on RANKL-dependent signalling. **d** The TRAF6 binding site in the cytoplasmic domain of RANK is critical for downstream signalling. The G280X protein associated with osteoclast-poor osteopetrosis lacks both the TRAF6 domain and the oligomerisation motif and would therefore be predicted to prevent downstream signalling activation in addition to the effects of lack of trimer formation

RANK degradation [79]. c-Cbl may have an additional effect by promoting RANK recycling [79]. Finally, surface expression of receptors is often regulated by proteolytic enzymes that cleave the extracellular ligand-binding domain, preventing activation of signalling pathways. TNFα converting enzyme (TACE; ADAM-17) is one such enzyme and generates the soluble forms of TNFα as well as RANKL [59, 80]. Inhibition of TACE activity upregulates surface RANK expression on monocytes [81], and, most recently, RANKL was shown to upregulate TACE-mediated shedding of the RANK ectodomain in a TRAF6-dependent manner indicating a negative feedback mechanism to regulate RANK surface expression [82]. Therefore, these observations strongly suggest that activation or inhibition of TACE could indeed play a significant role in the regulation of RANK signalling. Taken together, RANK signalling is dependent upon the combined actions of the complex activating and inactivating mechanisms described above, and it is not surprising that a number of conditions have now been identified where RANK signalling is deregulated as a result of mutations in either the receptor itself or in steps in the signal transduction pathway.

Molecular and functional consequences of mutations in the RANK/RANKL axis

Loss-of-function mutations in RANK

Seven different mutations in RANK have so far been identified in patients with osteoclast-poor osteopetrosis, with mutations occurring in all domains of the protein (Fig. 5) [31]. G53R, R129C, R170G and C175R are single base pair substitutions within the extracellular domain and are likely to result in changes to the ligand binding domain of RANK affecting the binding to RANKL and preventing downstream signalling. Indeed, the R170G mutation causes a complete lack of downstream activation of p38 and ERK1/2 through RANK [31]. The G280X and W434X mutations truncate the intracellular domain of RANK and cause deletion of the region which is essential for the

commitment of macrophages to the osteoclast lineage and for oligomerisation of RANK monomers [68, 83]. Whilst W434X leaves one of the TRAF6-binding domains intact, the G280X mutation results in deletion of the entire TRAF6 binding region. By investigating these mutations in vitro new data on the absolute requirement for receptor trimerisation and signal activation can be obtained, increasing our understanding of RANK signalling. It is however likely that such truncated mutant proteins are not translated to the same extent as wild-type protein and hence do not act in the same way in vivo as in the cellular expression models. In fact, the cellular quality-control process of "nonsense–mediated mRNA decay" is likely to lead to the destruction of a significant proportion of the mRNA molecules transcribed from these mutant genes [84]. Therefore, the patient phenotype seen as a result of harbouring such truncating mutations is likely the combined functional effect of the expression of the truncated protein together with that of reduced levels of RANK expression. The effect of the mutation A244S in the cytoplasmic domain of RANK is not clear as deletion of this region does not affect osteoclast formation [82], and the patient carrying this mutation was a compound heterozygote carrying C175R on the other allele complicating the functional analysis.

Gain-of-function mutations in RANK

The initial description and early in vitro characterisation of the FEO and ePDB associated mutations suggested that the mutations resulted in ligand-independent overactivation of NFκB which would help to explain the clinical phenotype [48]. However, in more recent studies, we found that there was no ligand-independent overactivation of NFκB expression when wild-type, and mutant proteins were expressed at physiological levels, rather than overexpressed as in the earlier studies. Furthermore, in these studies, only cells expressing the wild-type protein responded to RANKL [85] (Fig. 6a). The interaction between RANKL and RANK at the plasma membrane is the key initiating event in the RANK signalling process. Our in vitro overexpression studies [85] have shown, by confocal microscopy and immunoEM, that the mutant forms of the RANK receptor that cause FEO, ePDB and ESH do not reach the plasma membrane and are retained within an extended form of the endoplasmic reticulum known as "organised smooth endoplasmic reticulum" (OSER) [86]. Although each of these mutant proteins accumulated within OSER when overexpressed, there were nevertheless distinct differences: the FEORANK accumulated in OSER structures in a perinuclear region, whereas the ePDB and ESH forms of RANK were found in OSER throughout the cytosol. Overall though, the change in protein localisation compared to wild-type RANK suggests that mutant RANK is trapped within the protein synthesis pathway (Fig. 6b) and has thereby lost the ability to interact with RANKL on the cell surface, mimicking the situation in RANK loss-of-function mutants. Indeed, in a genetically modified mouse expressing ePDBRANK, homozygous expression of the transgene leads to severe osteopetrosis, whereas heterozygous expression leads to the hyperactive osteoclast phenotype as seen in patients with early onset Pagetic disorders [52].

With this dramatic difference between homozygous and heterozygous expression and the knowledge that patients are heterozygous, we have started to study the subcellular localisation of the mutant receptors in vitro when co-expressed with wild-type RANK to mimic the heterozygous nature of the patients. When co-expressed, FEORANK co-localised with wild-type RANK on the plasma membrane, suggesting that wild-type RANK can rescue the membrane localisation and thereby allow interaction with RANKL (Fig. 6c). Below, we discuss three hypotheses that may explain how such heterozygous expression may result in the osteoclast phenotype seen in patients.

Firstly, using immunoprecipitation, we determined that wild-type RANK can directly interact with FEORANK (Crockett and Mellis, unpublished). Together with the colocalisation of both proteins seen by immunostaining, this suggests that a heterotrimeric RANK could exist in cells carrying a wild-type and mutant allele. Such heterotrimeric RANK could potentially alter the kinetics of RANKL binding as signal peptide retention may alter the structural conformation of the ligand binding pocket, lead to tighter binding of RANKL. In addition, since these signal peptide mutations alter the N-terminal extracellular domain it is possible this may affect the susceptibility of the protein to cleavage by TACE, perhaps increasing the amount of surface expression of RANK. Secondly, hyperactivation of RANK signalling could be achieved by interference with the recycling and degradation of RANK, thought to be regulated by the balanced activities of the E3 ubiquitin ligases c-Cbl and Cbl-b as discussed above. Our preliminary analysis of proteasomal degradation of wild-type and mutant RANK has not demonstrated any differences, but this requires further investigation, especially in the context of heterozygous expression. Thirdly, accumulation of misfolded proteins within cells upregulates the unfolded protein response and other ER stress pathways such as the ER overload response [87, 88], pathways that by themselves can lead to increased NFκB signalling. Inclusion bodies containing proteins found in diseases associated with defective protein degradation and similar to inclusions regularly seen in osteoclasts from patients with late-onset PDB have also been described in patients with FEO (discussed in [40] and illustrated in Fig. 1c). The interaction of wild-type with mutant protein leads to

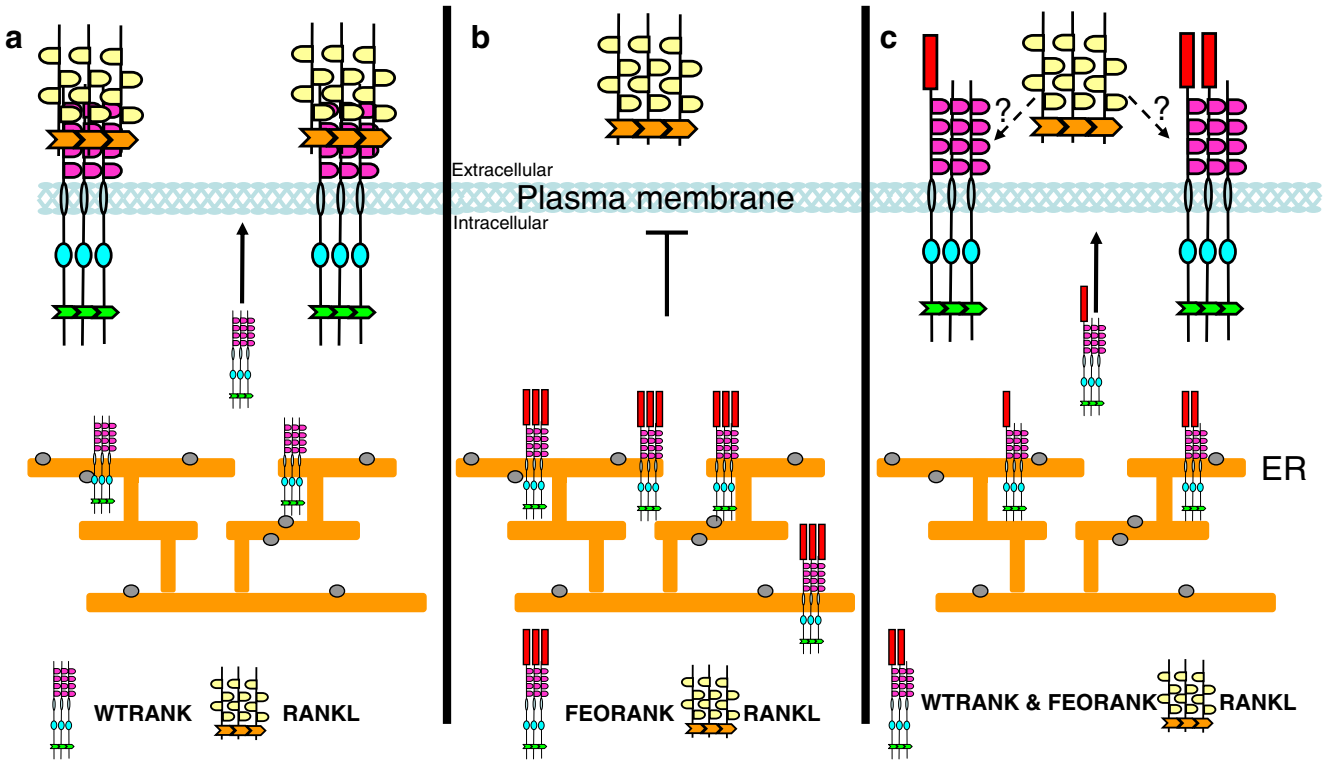

Fig. 6 A hypothetical mechanism by which heterozygous expression of mutant RANK can lead to an activated osteoclast phenotype in early onset PDB-like diseases. **a** The signal peptide is cleaved from wild-type RANK allowing it to be processed through the endoplasmic reticulum and expressed at the plasma membrane where it interacts with RANKL. **b** Mutations preclude cleavage of the signal peptide from RANK in the early-onset Pagetic diseases, trapping the mutant proteins within the endoplasmic reticulum and preventing RANK expression at the plasma membrane resulting in lack of RANKL-mediated activation of RANK signalling. **c** Our own observations support the hypothesis that, in the heterozygous situation found in the patients, wild-type RANK can interact with the mutant RANK receptors. If this occurs in the endoplasmic reticulum, the mutant receptor may "piggy-back" to the plasma membrane, allowing interaction with RANKL. However, since the signal peptide is retained on the mutant receptor, this could change the conformation of the protein and result in altered ligand binding, recycling or degradation kinetics. Such mechanisms may potentially lead to the increased osteoclast activity seen in these disorders

expression of RANK on the plasma membrane as discussed above, but at the same time it is possible that some of the mutant protein is retained within the ER and can activate ER overload pathways. Together with the RANKL-dependent signalling at the plasma membrane, such ER overload pathways through additional increases in NFκB activation, might lead to hyperactivation of NFκB and to increased osteoclast function.

Taken together, the mutations associated with FEO, ePDB and ESH are not in themselves classic gain-of-function mutations. Their effect, however, is likely to be an activation of the key pathway that can lead to osteoclast activation. Further detailed biochemical analyses are required to understand whether any of the above hypotheses are correct.

Loss-of function mutations in RANKL

The mechanistic basis of the osteopetroses resulting from the known mutations in RANKL in humans are most likely complete loss-of-function. The three mutations found so far are illustrated in Fig. 7 [33]. They comprise a single amino acid substitution in a highly conserved region of the protein, but with an as yet unknown consequence on biological activity; partial loss of a region (probably involved in RANK binding) known to be important for biological function of the protein [62] and deletion of the trimerisation domain. It has proved difficult to establish the precise loss-of-function of any of these mutant proteins. In expression studies, we were unable to obtain definitive data on any possible retention of biological activity, especially of the M199K mutant, where this might be the case. Although we were unable to generate osteoclasts with any of the mutant proteins, technical issues such as lower levels of protein obtained from the mutants and variable biological activity of the wild-type protein, precluded firm conclusions. Further studies are needed to deduce the extent to which these mutations lead to loss-of-function of RANKL in vivo.

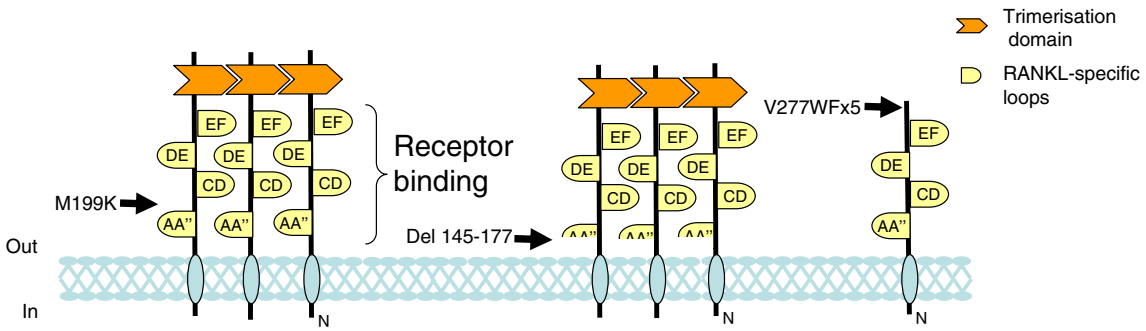

Fig. 7 RANKL mutations as found in patients with osteoclast-poor osteopetrosis (derived from data presented in [33]). The single amino acid substitution M199K is in a highly conserved domain, the deletion 145-177 removes part of a region known to be essential for biological activity and the deletion V277WFx5 removes the trimerisation domain

p62 mutations and gain-of-function in osteoclasts

Having discussed the various ways in which mutations in the RANK/RANKL pathway can lead to osteoclast dysfunction, we will briefly return to the mutations in p62 which lead to a phenotype so similar to that seen in patients with gain-of-function RANK mutations. How is it possible that mutations in these different genes lead to a phenotype of activated osteoclasts? What are the mechanistic connections between the pathways? Initially, it was considered that UBA mutations in p62 would lead to upregulation of NFκB signalling in osteoclasts because of a diminished ability of the protein to act in protein degradation pathways, especially by directing proteins to the proteasome. It seemed logical that impaired degradation of signalling intermediaries, which might not be degraded because of p62 mutations, could lead to prolonged signal transduction leading to osteoclast activation. Cellular studies, however, did not support this hypothesis, and in fact it appeared that overexpression of wild-type p62 reduced, rather than increased NFκB signalling (discussed in [40]). It is now considered that the main role of the scaffold protein p62 in NFκB signalling is by facilitating K63-linked TRAF6 ubiquitination (see Fig. 4), rather than K48-linked ubiquitination to mark proteins for proteasomal degradation. Intriguingly though, a major role of p62, which has only more recently come to light, is in autophagy, since p62 can bind to light chain 3 (LC3), a key molecule in this process [89]. Autophagy is the mechanism by which cells enclose organelles or part of their cytoplasm by a membrane to degrade its contents after fusion with lysosomes and recycle its contents. Impairment of autophagy is known to lead to accumulation of inclusion bodies in diseases such as neuropathies and myopathies. Presence of nuclear inclusion bodies is a hallmark of PDB and of the syndrome IBMPFD, which combines PDB(P) with inclusion body myositis (IBM) and/or frontotemporal dementia (FD), a condition in which inclusion bodies are seen in all affected organs. IBMPFD is caused by mutations in valosin-containing protein (VCP; Table 1; [90]). VCP has a known role in protein degradation via the proteasomal pathway and has recently also been implicated in autophagy [91]. Autophagy is known to decrease in efficiency with increasing age [92] and age is a known risk factor for PDB. Given the involvement of both p62 and VCP in autophagy, it seems plausible that mutations in these genes lead to deregulation of autophagy and that this plays a role in the aetiology of PDB. p62 levels appear to be increased in osteoclasts in patients with PDB, and the protein appears to localise in inclusions or aggregates [93], structures that are normally degraded by autophagy. Since in the early-onset Pagetic diseases, mutant RANK proteins are likely to accumulate within the ER, it is possible that autophagy (so-called ER-phagy) could play a role in maintaining ER integrity in these conditions [94]. Although at present, the way in which p62 has such profound effects specifically in osteoclasts is unknown, further knowledge of the other genes mutated in patients with PDB (in up to 75% of patients no mutation in p62 is found) should help the mechanistic studies and should elucidate how similar activated osteoclast phenotypes can result from mutations in genes such as RANK, p62 and VCP.

In addition, such studies may help determine the underlying cause of the extreme focal nature of increased bone remodelling in both early and late-onset Paget's disease. A range of local genetic, infectious, age-related, mechanical, hormonal and lifestyle factors have been suggested to hold the key to the focal nature of the lesions. So, what could these factors be? A plausible hypothesis for the presence of a local pathology, be it a tumour or a local lesion such as in PDB, is the occurrence of a somatic mutation. Two studies have recently tested this hypothesis by analysing bone cells from affected regions in patients with sporadic PDB for somatic mutations in p62. While in one study the authors observed P392L (*C1215T*) mutations

within p62 in bone cells while none were observed in peripheral blood cells from the same patients [95]; no somatic P392L mutations were identified in the second study [96]. While this indicates that somatic mutations in p62 may occur, but are not essential to develop PDB, it does not exclude the possibility that somatic mutations in other, as yet unidentified, PDB-associated genes may trigger the focal increase in remodelling. We would expect that this would most commonly occur in combination with a germline mutation in p62, or another, yet to be identified PDB gene, as a pre-disposing factor. So far this "double hit" hypothesis has not been investigated, but next-generation sequencing makes it possible to simultaneously screen multiple genes associated with key osteoclast pathways in patients. Such studies could highlight additional PDB genes or reveal gene–gene interactions that are associated with PDB.

There has been a longstanding debate over the connection of persistent viral infection and development of PDB (discussed in [40]). Although some investigators have identified viral mRNA in bone and blood samples [97] and described Pagetic lesions in mouse bone after infection with measles virus [98], others have not been able to find any evidence of viral persistence in Paget's patients [41, 99]. While it remains possible that a viral agent is involved, it remains difficult to understand how this could lead to a focal disorder, as, by contrast to neurological diseases due to persistent viral infection, none of the cell types in bone are long-lived, and persistence of the agent in local precursors with transmission to local progeny must be assumed.

In many patients with Paget's disease (early or late-onset forms), development of lesions has been linked to previous trauma, to surgery or to pregnancy [100, 101], pointing to additional risk factors which may lead to local effects. In all cases though, we would assume that these factors would act in a predisposing genetic background. In addition, the severity of FEO and the number of bones affected has been shown to differ between kindreds, suggesting again that either environmental differences, or genetic factors in addition to the RANK mutations, influence the severity of the disease.

With these multiple unproven suggestions for the occurrence of local bone lesions in Paget-like disorders, it is anticipated that the animal models may provide key insights. So far, the bone lesions reported in mice carrying p62, VCP or RANK mutations have been local, and have, as in humans, only appeared with increasing age, with interestingly RANK-associated lesions being more severe and appearing earlier that p62 and VCP-induced lesions [52, 102, 103].

The cell biological studies we discussed above have so far not given any clearer insights into the focal nature of the bone lesions. Our preferred hypothesis at present is that an osteoclast-activating stimulus, which could be any of those suggested in the paragraphs above, precipitates that hyperactive osteoclast phenotype and that this involves protein degradation pathways. We predict that mutated p62 is less able to regulate protein degradation (either of itself or of protein aggregates and complexes) by proteasomal and autophagy pathways, and that RANK mutations induce ER-phagy and possibly ER stress responses. Since in both cases the resulting osteoclast phenotype is a hyperactive one, we must assume that upregulation of intracellular protein degradation is essential for osteoclastic resorption. We are currently actively investigating this testable hypothesis.

Conclusions

Over the past 10 years, major progress has been made in the genetic characterisation of diseases caused by osteoclast dysfunction. Many of the genes found have been in the RANK/RANKL axis. Mutations in OPG and RANKL are in essence endocrine disorders which ideally should be treated with hormone replacement therapies where these can be administered without major side effects. Although this appears possible in the case of OPG, a safe way to deliver RANKL to osteoclast precursors has yet to be devised. Mutations in RANK that lead to osteopetrosis should be considered for BMT wherever a suitable donor exists. The gain-of-function mutations in RANK and p62 that lead to the overactive osteoclast phenotypes remain at present the most enigmatic. We do not at present understand how these mutations lead to profound osteoclast dysfunction, but tantalising links to protein degradation pathways have been discovered. This is perhaps not surprising as osteoclasts are, above all, exocrine cells with huge protein synthesis, requiring robust quality control mechanisms. Furthermore, transcytosis of degraded bone matrix through osteoclasts [10, 11] suggests additional links with protein degradation pathways that are as yet unexplored. Although our overall knowledge in this area has increased substantially, the functional implication of the gene mutations have been difficult to establish. With our incomplete knowledge of the molecular interactions between RANKL and RANK and with the likely interplay between NFκB signalling and protein degradation pathways in osteoclasts, this should remain an area of intense investigation, especially since the RANK/RANKL pathway is now a prime target for anti-resorptive therapies some of which (anti RANKL antibodies, recombinant OPG) are already in clinical use. With the key role of the RANK/RANKL axis in immunology [8], the careful functional dissection of the RANK mutations that cause osteopetrosis

and immunological defects will provide insights into receptor-ligand interactions and downstream signalling.

Overall, we expect that single gene mutations found in rare genetic osteoclast diseases will help to understand key metabolic pathways in this unique cell type and thereby inform our understanding of more common skeletal disorders associated with osteoclast function such as osteoporosis, a condition clearly associated with single nucleotide polymorphisms in RANK, RANKL and OPG [104, 105].

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Conflict of interest None

References

1. Teitelbaum SL (2007) Osteoclasts: what do they do and how do they do it? Am J Pathol 170:427–435
2. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson DM, Suda T (1999) Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med 190:1741–1754
3. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira dSA, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 397:315–23
4. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176
5. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602
6. Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliott R, Scully S, Van G, Kaufman S, Juan SC, Sun Y, Tarpley J, Martin L, Christensen K, McCabe J, Kostenuik P, Hsu H, Fletcher F, Dunstan CR, Lacey DL, Boyle WJ (2000) RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci USA 97:1566–1571
7. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89:309–319
8. Leibbrandt A, Penninger JM (2008) RANK/RANKL: regulators of immune responses and bone physiology. Ann N Y Acad Sci 1143:123–150
9. Hanada R, Leibbrandt A, Hanada T, Kitaoka S, Furuyashiki T, Fujihara H, Trichereau J, Paolino M, Qadri F, Plehm R, Klaere S, Komnenovic V, Mimata H, Yoshimatsu H, Takahashi N, von HA, Bader M, Kilic SS, Ueta Y, Pifl C, Narumiya S, Penninger JM (2009) Central control of fever and female body temperature by RANKL/RANK. Nature 462:505–9
10. Salo J, Lehenkari P, Mulari M, Metsikko K, Väänänen HK (1997) Removal of osteoclast bone resorption products by transcytosis. Science 276:270–273
11. Nesbitt SA, Horton MA (1997) Trafficking of matrix collagens through bone-resorbing osteoclasts. Science 276:266–269
12. Graves AR, Curran PK, Smith CL, Mindell JA (2008) The Cl-/H+antiporter ClC-7 is the primary chloride permeation pathway in lysosomes. Nature 453:788–792
13. Kornak U, Kasper D, Bosl MR, Kaiser E, Schweizer M, Schulz A, Friedrich W, Delling G, Jentsch TJ (2001) Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. Cell 104:205–215
14. Gelb BD, Shi GP, Chapman HA, Desnick RJ (1996) Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. Science 273:1236–1238
15. Balemans W, Patel N, Ebeling M, Van HE, Wuyts W, Lacza C, Dioszegi M, Dikkers FG, Hildering P, Willems PJ, Verheij JB, Lindpaintner K, Vickery B, Foernzler D, Van HW (2002) Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. J Med Genet 39:91–97
16. Van Wesenbeeck L, Cleiren E, Gram J, Beals RK, Bénichou O, Scopelliti D, Key L, Renton T, Bartels C, Gong Y, Warman ML, de Vernejoul MC, Bollerslev J, Van HW (2003) Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with an increased bone density. Am J Hum Genet 72:763–771
17. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, Lacza C, Wuyts W, Van Den EJ, Willems P, Paes-Alves AF, Hill S, Bueno M, Ramos FJ, Tacconi P, Dikkers FG, Stratakis C, Lindpaintner K, Vickery B, Foernzler D, Van Hul W (2001) Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet 10:537–543
18. Villa A, Guerrini MM, Cassani B, Pangrazio A, Sobacchi C (2009) Infantile malignant, autosomal recessive osteopetrosis: the rich and the poor. Calcif Tissue Int 84:1–12
19. Albers-Schönberg HE (1904) Röntgenbilder einer seltener Knochenerkrankung. Münch Med Wochenschr 51:368
20. Marks SCJ, Walker DG (1976) Mammalian osteopetrosis. A model for studying cellular and humoral factors in bone resorption. 227-301
21. Marks SC Jr (1984) Congenital osteopetrotic mutations as probes of the origin, structure, and function of osteoclasts. Clin Orthop:239-263
22. Marks SC Jr (1989) Osteoclast biology: lessons from mammalian mutations. Am J Med Genet 34:43–54
23. Walker DG (1975) Spleen cells transmit osteopetrosis in mice. Science 190:785–787
24. Walker DG (1975) Bone resorption restored in osteopetrotic mice by transplants of normal bone marrow and spleen cells. Science 190:784–785
25. Walker DG (1973) Osteopetrosis cured by temporary parabiosis. Science 180:875
26. Cumming WA, Ohlsson A (1985) Intracranial calcification in children with osteopetrosis caused by carbonic anhydrase II deficiency. Radiology 157:325–327
27. Bénichou OD, Laredo JD, de Vernejoul MC (2000) Type II autosomal dominant osteopetrosis (Albers-Schönberg disease):

clinical and radiological manifestations in 42 patients. Bone 26:87–93
28. Del Fattore A, Peruzzi B, Rucci N, Recchia I, Cappariello A, Longo M, Fortunati D, Ballanti P, Iacobini M, Luciani M, Devito R, Pinto R, Caniglia M, Lanino E, Messina C, Cesaro S, Letizia C, Bianchini G, Fryssira H, Grabowski P, Shaw N, Bishop N, Hughes D, Kapur RP, Datta HK, Taranta A, Fornari R, Migliaccio S, Teti A (2006) Clinical, genetic, and cellular analysis of 49 osteopetrotic patients: implications for diagnosis and treatment. J Med Genet 43:315–325
29. Helfrich M, Perdu B, Coxon F (2009) Human recessive osteopetrosis. New understanding of osteoclast function through molecular and functional analysis of a rare genetic bone disease. Osteologie 18:260–267
30. Pangrazio A, Pusch M, Caldana E, Frattini A, Lanino E, Tamhankar PM, Phadke S, Lopez AG, Orchard P, Mihci E, Abinun M, Wright M, Vettenranta K, Bariae I, Melis D, Tezcan I, Baumann C, Locatelli F, Zecca M, Horwitz E, Mansour LS, Van RM, Vezzoni P, Villa A, Sobacchi C (2010) Molecular and clinical heterogeneity in CLCN7-dependent osteopetrosis: report of 20 novel mutations. Hum Mutat 31:E1071–E1080
31. Guerrini MM, Sobacchi C, Cassani B, Abinun M, Kilic SS, Pangrazio A, Moratto D, Mazzolari E, Clayton-Smith J, Orchard P, Coxon FP, Helfrich MH, Crockett JC, Mellis D, Vellodi A, Tezcan I, Notarangelo LD, Rogers MJ, Vezzoni P, Villa A, Frattini A (2008) Human osteoclast-poor osteopetrosis with hypogammaglobulinemia due to TNFRSF11A (RANK) mutations. Am J Hum Genet 83:64–76
32. Steward CG (2010) Hematopoietic Stem Cell Transplantation for Osteopetrosis. Pediatr Clin N Am 57:171–180
33. Sobacchi C, Frattini A, Guerrini MM, Abinun M, Pangrazio A, Susani L, Bredius R, Mancini G, Cant A, Bishop N, Grabowski P, Del FA, Messina C, Errigo G, Coxon FP, Scott DI, Teti A, Rogers MJ, Vezzoni P, Villa A, Helfrich MH (2007) Osteoclast-poor human osteopetrosis due to mutations in the gene encoding RANKL. Nat Genet 39:960–962
34. Knowles HJ, Athanasou NA (2009) Canonical and non-canonical pathways of osteoclast formation. Histol Histopathol 24:337–346
35. Tiegs RD, Lohse CM, Wollan PC, Melton LJ (2000) Long-term trends in the incidence of Paget's disease of bone. Bone 27:423–427
36. Bastin S, Bird H, Gamble G, Cundy T (2009) Paget's disease of bone–becoming a rarity? Rheumatology (Oxford) 48:1232–1235
37. Selby P, Davie M, Ralston S, Stone M (2002) Guidelines on the management of Paget's disease of bone. Bone 31:366–373
38. Ralston SH, Langston AL, Reid IR (2008) Pathogenesis and management of Paget's disease of bone. Lancet 372:155–163
39. Naot D, Bava U, Matthews B, Callon KE, Gamble GD, Black M, Song S, Pitto RP, Cundy T, Cornish J, Reid IR (2007) Differential gene expression in cultured osteoblasts and bone marrow stromal cells from patients with Paget's disease of bone. J Bone Miner Res 22:298–309
40. Helfrich MH, Hocking LJ (2008) Genetics and aetiology of Pagetic diseases of bone. Arch Biochem Biophys 473:172–182
41. Helfrich MH, Hobson RP, Grabowski PS, Zurbriggen A, Cosby SL, Dickson GR, Fraser WD, Ooi CG, Selby PL, Crisp AJ, Wallace RG, Kahn S, Ralston SH (2000) A negative search for a paramyxoviral etiology of Paget's disease of bone: molecular, immunological, and ultrastructural studies in UK patients. J Bone Miner Res 15:2315–2329
42. Cavey JR, Ralston SH, Hocking LJ, Sheppard PW, Ciani B, Searle MS, Layfield R (2005) Loss of ubiquitin-binding associated with Paget's disease of bone p62 (SQSTM1) mutations. J Bone Miner Res 20:619–624
43. Layfield R, Shaw B (2007) Ubiquitin-mediated signalling and Paget's disease of bone. BMC Biochem 8(Suppl 1):S5
44. Whyte MP, Obrecht SE, Finnegan PM, Jones JL, Podgornik MN, McAlister WH, Mumm S (2002) Osteoprotegerin deficiency and juvenile Paget's disease. N Engl J Med 347:175–184
45. Cundy T, Hegde M, Naot D, Chong B, King A, Wallace R, Mulley J, Love DR, Seidel J, Fawkner M, Banovic T, Callon KE, Grey AB, Reid IR, Middleton-Hardie CA, Cornish J (2002) A mutation in the gene TNFRSF11B encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype. Hum Mol Genet 11:2119–2127
46. Ralston SH (2008) Juvenile Paget's disease, familial expansile osteolysis and other genetic osteolytic disorders. Best Pract Res Clin Rheumatol 22:101–111
47. Takata S, Yasui N, Nakatsuka K, Ralston SH (2004) Evolution of understanding of genetics of Paget's disease of bone and related diseases. J Bone Miner Metab 22:519–523
48. Hughes AE, Ralston SH, Marken J, Bell C, MacPherson H, Wallace RG, Van Hul W, Whyte MP, Nakatsuka K, Hovy L, Anderson DM (2000) Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis. Nat Genet 24:45–48
49. Whyte MP, Hughes AE (2002) Expansile skeletal hyperphosphatasia is caused by a 15-base pair tandem duplication in TNFRSF11A encoding RANK and is allelic to familial expansile osteolysis. J Bone Miner Res 17:26–29
50. Nakatsuka K, Nishizawa Y, Ralston SH (2003) Phenotypic characterization of early onset Paget's disease of bone caused by a 27-bp duplication in the TNFRSF11A gene. J Bone Miner Res 18:1381–1385
51. Ke YH, Yue H, He JW, Liu YJ, Zhang ZL (2009) Early onset Paget's disease of bone caused by a novel mutation (78dup27) of the TNFRSF11A gene in a Chinese family. Acta Pharmacol Sin 30:1204–1210
52. Albagha OME, Rojas J, van 't Hof R, Dorin J, Ralston SH (2007) A mouse model of early onset Paget's disease of bone caused by an insertion mutation affecting the RANK signal peptide. Calcif Tissue Int 80(suppl 1):s34
53. Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R (1997) Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nat Med 3:1285–1289
54. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S, der HA van, Itie A, Wakeham A, Khoo W, Sasaki T, Cao Z, Penninger JM, Paige CJ, Lacey DL, Dunstan CR, Boyle WJ, Goeddel DV, Mak TW (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 13:1015–24
55. Duran A, Serrano M, Leitges M, Flores JM, Picard S, Brown JP, Moscat J, Diaz-Meco MT (2004) The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. Dev Cell 6:303–309
56. Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, Nakao K, Nakamura K, Katsuki M, Yamamoto T, Inoue J (1999) Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. Genes Cells 4:353–362
57. Hocking LJ, Lucas GJ, Daroszewska A, Mangion J, Olavesen M, Cundy T, Nicholson GC, Ward L, Bennett ST, Wuyts W, Van Hul W, Ralston SH (2002) Domain-specific mutations in sequestosome 1 (SQSTM1) cause familial and sporadic Paget's disease. Hum Mol Genet 11:2735–2739
58. Doffinger R, Smahi A, Bessia C, Geissmann F, Feinberg J, Durandy A, Bodemer C, Kenwrick S, Dupuis-Girod S, Blanche S, Wood P, Rabia SH, Headon DJ, Overbeek PA, Le DF, Holland SM, Belani K, Kumararatne DS, Fischer A, Shapiro R, Conley ME, Reimund E, Kalhoff H, Abinun M, Munnich A, Israel A,

Courtois G, Casanova JL (2001) X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. Nat Genet 27:277–285
59. Lum L, Wong BR, Josien R, Becherer JD, Erdjument-Bromage H, Schlondorff J, Tempst P, Choi Y, Blobel CP (1999) Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. J Biol Chem 274:13613–13618
60. Nannuru KC, Futakuchi M, Sadanandam A, Wilson TJ, Varney ML, Myers KJ, Li X, Marcusson EG, Singh RK (2009) Enhanced expression and shedding of receptor activator of NF-kappaB ligand during tumor-bone interaction potentiates mammary tumor-induced osteolysis. Clin Exp Metastasis 26:797–808
61. Lynch CC, Hikosaka A, Acuff HB, Martin MD, Kawai N, Singh RK, Vargo-Gogola TC, Begtrup JL, Peterson TE, Fingleton B, Shirai T, Matrisian LM, Futakuchi M (2005) MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. Cancer Cell 7:485–496
62. Lam J, Nelson CA, Ross FP, Teitelbaum SL, Fremont DH (2001) Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. J Clin Invest 108:971–979
63. Chan FK (2007) Three is better than one: pre-ligand receptor assembly in the regulation of TNF receptor signaling. Cytokine 37:101–107
64. Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, Daro E, Smith J, Tometsko ME, Maliszewski CR, Armstrong A, Shen V, Bain S, Cosman D, Anderson D, Morrissey PJ, Peschon JJ, Schuh J (1999) RANK is essential for osteoclast and lymph node development. Genes Dev 13:2412–2424
65. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS (1998) osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12:1260–1268
66. Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, Ambrose C, Tschopp J, Schneider P (2006) Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. J Biol Chem 281:13964–13971
67. Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ (2000) A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science 288:2351–2354
68. Kanazawa K, Kudo A (2005) Self-assembled RANK induces osteoclastogenesis ligand-independently. J Bone Miner Res 20:2053–2060
69. Chen ZJ, Sun LJ (2009) Nonproteolytic functions of ubiquitin in cell signaling. Mol Cell 33:275–286
70. Darnay BG, Ni J, Moore PA, Aggarwal BB (1999) Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif. J Biol Chem 274:7724–7731
71. Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. Cell 132:344–362
72. Lamothe B, Webster WK, Gopinathan A, Besse A, Campos AD, Darnay BG (2007) TRAF6 ubiquitin ligase is essential for RANKL signaling and osteoclast differentiation. Biochem Biophys Res Commun 359:1044–1049
73. Mizukami J, Takaesu G, Akatsuka H, Sakurai H, Ninomiya-Tsuji J, Matsumoto K, Sakurai N (2002) Receptor activator of NF-kappaB ligand (RANKL) activates TAK1 mitogen-activated protein kinase kinase kinase complex through a signaling complex containing RANK, TAB2, and TRAF6. Mol Cell Biol 22:992–1000
74. Besse A, Lamothe B, Campos AD, Webster WK, Maddineni U, Lin SC, Wu H, Darnay BG (2007) TAK1-dependent signaling requires functional interaction with TAB2/TAB3. J Biol Chem 282:3918–3928
75. Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD (2006) Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat Cell Biol 8:398–406
76. Chen ZJ (2005) Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol 7:758–765
77. Horne WC, Sanjay A, Bruzzaniti A, Baron R (2005) The role(s) of Src kinase and Cbl proteins in the regulation of osteoclast differentiation and function. Immunol Rev 208:106–125
78. Arron JR, Vologodskaia M, Wong BR, Naramura M, Kim N, Gu H, Choi Y (2001) A positive regulatory role for Cbl family proteins in tumor necrosis factor-related activation-induced cytokine (trance) and CD40L-mediated Akt activation. J Biol Chem 276:30011–30017
79. Nakajima A, Sanjay A, Chiusaroli R, Adapala NS, Neff L, Itzsteink C, Horne WC, Baron R (2009) Loss of Cbl-b increases osteoclast bone-resorbing activity and induces osteopenia. J Bone Miner Res 24:1162–1172
80. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385:729–733
81. Hiasa M, Abe M, Nakano A, Oda A, Amou H, Kido S, Takeuchi K, Kagawa K, Yata K, Hashimoto T, Ozaki S, Asaoka K, Tanaka E, Moriyama K, Matsumoto T (2009) GM-CSF and IL-4 induce dendritic cell differentiation and disrupt osteoclastogenesis through M-CSF receptor shedding by up-regulation of TNF-alpha converting enzyme (TACE). Blood 114:4517–4526
82. Hakozaki A, Yoda M, Tohmonda T, Furukawa M, Hikata T, Uchikawa S, Takaishi H, Matsumoto M, Chiba K, Horiuchi K, Toyama Y (2010) Receptor Activator of NF-{kappa}B Ligand Induces Ectodomain Shedding of Receptor Activator of NF-{kappa}B in Murine RAW264.7 Macrophages. J Immunol
83. Xu D, Wang S, Liu W, Liu J, Feng X (2006) A novel receptor activator of NF-kappaB (RANK) cytoplasmic motif plays an essential role in osteoclastogenesis by committing macrophages to the osteoclast lineage. J Biol Chem 281:4678–4690
84. Kuzmiak HA, Maquat LE (2006) Applying nonsense-mediated mRNA decay research to the clinic: progress and challenges. Trends Mol Med 12:306–316
85. Crockett JC, Helfrich M, Duthie A, Scott D, Greenhorn J, Ralston S, Rogers MJ (2006) Mutations in the RANK signal peptide alter the subcellular localisation of RANK and prevent ligand-dependent activation of NFkappa B. J Bone Miner Res 21 (suppl 1):S103
86. Snapp EL, Hegde RS, Francolini M, Lombardo F, Colombo S, Pedrazzini E, Borgese N, Lippincott-Schwartz J (2003) Formation of stacked ER cisternae by low affinity protein interactions. J Cell Biol 163:257–269
87. Vembar SS, Brodsky JL (2008) One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 9:944–957
88. Pahl HL (1999) Signal transduction from the endoplasmic reticulum to the cell nucleus. Physiol Rev 79:683–701
89. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282:24131–24145
90. Watts GD, Wymer J, Kovach MJ, Mehta SG, Mumm S, Darvish D, Pestronk A, Whyte MP, Kimonis VE (2004) Inclusion body myopathy associated with Paget disease of bone and frontotemporal

dementia is caused by mutant valosin-containing protein. Nat Genet 36:377–381
91. Ju JS, Fuentealba RA, Miller SE, Jackson E, Piwnica-Worms D, Baloh RH, Weihl CC (2009) Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. J Cell Biol 187:875–888
92. Terman A, Gustafsson B, Brunk UT (2007) Autophagy, organelles and ageing. J Pathol 211:134–143
93. Azzam EA, Scott DI, Gregory JS, McKinnon AD, Helfrich MH (2009) Expresssion of inclusion body-associated proteins in Paget's disease of bone. Bone 44(Suppl):S326–S327
94. Bernales S, McDonald KL, Walter P (2006) Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS Biol 4:e423
95. Merchant A, Smielewska M, Patel N, Akunowicz JD, Saria EA, Delaney JD, Leach RJ, Seton M, Hansen MF (2009) Somatic mutations in SQSTM1 detected in affected tissues from patients with sporadic Paget's disease of bone. J Bone Miner Res 24:484–494
96. Matthews BG, Naot D, Bava U, Callon KE, Pitto RP, McCowan SA, Wattie D, Cundy T, Cornish J, Reid IR (2009) Absence of somatic SQSTM1 mutations in Paget's disease of bone. J Clin Endocrinol Metab 94:691–694
97. Roodman GD, Windle JJ (2005) Paget disease of bone. J Clin Invest 115:200–208
98. Kurihara N, Zhou H, Reddy SV, Garcia PV, Subler MA, Dempster DW, Windle JJ, Roodman GD (2006) Expression of measles virus nucleocapsid protein in osteoclasts induces Paget's disease-like bone lesions in mice. J Bone Miner Res 21:446–455
99. Matthews BG, Afzal MA, Minor PD, Bava U, Callon KE, Pitto RP, Cundy T, Cornish J, Reid IR, Naot D (2008) Failure to detect measles virus ribonucleic acid in bone cells from patients with Paget's disease. J Clin Endocrinol Metab 93:1398–1401
100. Osterberg PH, Wallace RGH, Adams DA, Crone RS, Dickson GR, Kanis JA, Mollan RAB, Nevin NC, Sloan J, Toner PG (1988) Familial expansile osteolysis:a new dysplasia. J Bone Joint Surg 70-B:255–260
101. Whyte MP, Reinus WR, Podgornik MN, Mills BG (2002) Familial expansile osteolysis (excessive RANK effect) in a 5-generation American kindred. Medicine (Baltimore) 81:101–121
102. Daroszewska A, Rojas J, Rose L, van 't Hof RJ, Ralston SH (2008) Knock-in of the P392L mutation of SQSTM1 causes a phenotype similar to Paget's disease in mice. Calcif Tissue Int 82 (suppl 1):S34–S35
103. Daroszewska A, Rose L, Rose K, Sedlmeier R, van 't Hof RJ, Ralston SH (2009) Development of a novel animal model for the syndrome of inclusions body myopathy, Paget's disease and fronto-temporal dementia. Bone 44(Suppl):S247
104. Richards JB, Kavvoura FK, Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Zillikens MC, Wilson SG, Mullin BH, Amin N, Aulchenko YS, Cupples LA, Deloukas P, Demissie S, Hofman A, Kong A, Karasik D, van Meurs JB, Oostra BA, Pols HA, Sigurdsson G, Thorsteinsdottir U, Soranzo N, Williams FM, Zhou Y, Ralston SH, Thorleifsson G, van Duijn CM, Kiel DP, Stefansson K, Uitterlinden AG, Ioannidis JP, Spector TD (2009) Collaborative meta-analysis: associations of 150 candidate genes with osteoporosis and osteoporotic fracture. Ann Intern Med 151:528–537
105. Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, Zillikens MC, Kavvoura FK, Amin N, Aulchenko YS, Cupples LA, Deloukas P, Demissie S, Grundberg E, Hofman A, Kong A, Karasik D, van Meurs JB, Oostra B, Pastinen T, Pols HA, Sigurdsson G, Soranzo N, Thorleifsson G, Thorsteinsdottir U, Williams FM, Wilson SG, Zhou Y, Ralston SH, van Duijn CM, Spector T, Kiel DP, Stefansson K, Ioannidis JP, Uitterlinden AG (2009) Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet 41:1199–1206
106. Scimeca JC, Franchi A, Trojani C, Parrinello H, Grosgeorge J, Robert C, Jaillon O, Poirier C, Gaudray P, Carle GF (2000) The gene encoding the mouse homologue of the human osteoclast-specific 116-kDa V-ATPase subunit bears a deletion in osteosclerotic (oc/oc) mutants. Bone 26:207–213
107. Frattini A, Orchard PJ, Sobacchi C, Giliani S, Abinun M, Mattsson JP, Keeling DJ, Andersson AK, Wallbrandt P, Zecca L, Notarangelo LD, Vezzoni P, Villa A (2000) Defects in TCIRG1 subunit of the vacuolar proton pump are responsible for a subset of human autosomal recessive osteopetrosis. Nat Genet 25:343–346
108. Kornak U, Schulz A, Friedrich W, Uhlhaas S, Kremens B, Voit T, Hasan C, Bode U, Jentsch TJ, Kubisch C (2000) Mutations in the a3 subunit of the vacuolar H(+)-ATPase cause infantile malignant osteopetrosis. Hum Mol Genet 9:2059–2063
109. Li YP, Chen W, Liang Y, Li E, Stashenko P (1999) Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. Nat Genet 23:447–451
110. Chalhoub N, Benachenhou N, Rajapurohitam V, Pata M, Ferron M, Frattini A, Villa A, Vacher J (2003) Grey-lethal mutation induces severe malignant autosomal recessive osteopetrosis in mouse and human. Nat Med 9:399–406
111. Rajapurohitam V, Chalhoub N, Benachenhou N, Neff L, Baron R, Vacher J (2001) The mouse osteopetrotic grey-lethal mutation induces a defect in osteoclast maturation/function. Bone 28:513–523
112. Van Wesenbeeck L, Odgren PR, Coxon FP, Frattini A, Moens P, Perdu B, MacKay CA, Van HE, Timmermans JP, Vanhoenacker F, Jacobs R, Peruzzi B, Teti A, Helfrich MH, Rogers MJ, Villa A, Van HW (2007) Involvement of PLEKHM1 in osteoclastic vesicular transport and osteopetrosis in incisors absent rats and humans. J Clin Invest 117:919–930
113. Del Fattore A, Fornari R, Van WL, de FF T, JP PB, Cappariello A, Rucci N, Spera G, Helfrich MH, Van HW, Migliaccio S, Teti A (2008) A new heterozygous mutation (R714C) of the osteopetrosis gene, pleckstrin homolog domain containing family M (with run domain) member 1 (PLEKHM1), impairs vesicular acidification and increases TRACP secretion in osteoclasts. J Bone Miner Res 23:380–391
114. Reinholt FP, Hultenby K, Heinegård D, Marks SC Jr, Norgard M, Anderson G (1999) Extensive clear zone and defective ruffled border formation in osteoclasts of osteopetrotic (ia/ia) rats: implications for secretory function. Exp Cell Res 251:477–491
115. Dupuis-Girod S, Corradini N, Hadj-Rabia S, Fournet JC, Faivre L, Le DF, Durand P, Doffinger R, Smahi A, Israel A, Courtois G, Brousse N, Blanche S, Munnich A, Fischer A, Casanova JL, Bodemer C (2002) Osteopetrosis, lymphedema, anhidrotic ectodermal dysplasia, and immunodeficiency in a boy and incontinentia pigmenti in his mother. Pediatrics 109:e97
116. Rudolph D, Yeh WC, Wakeham A, Rudolph B, Nallainathan D, Potter J, Elia AJ, Mak TW (2000) Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. Genes Dev 14:854–862
117. Sly WS, Hewett-Emmett D, Whyte MP, Yu YS, Tashian RE (1983) Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. Proc Natl Acad Sci USA 80:2752–2756
118. Margolis DS, Szivek JA, Lai LW, Lien YH (2008) Phenotypic characteristics of bone in carbonic anhydrase II-deficient mice. Calcif Tissue Int 82:66–76
119. Weihl CC, Miller SE, Hanson PI, Pestronk A (2007) Transgenic expression of inclusion body myopathy associated mutant p97/

VCP causes weakness and ubiquitinated protein inclusions in mice. Hum Mol Genet 16:919–928
120. Laurin N, Brown JP, Morissette J, Raymond V (2002) Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. Am J Hum Genet 70:1582–1588
121. Kurihara N, Hiruma Y, Zhou H, Subler MA, Dempster DW, Singer FR, Reddy SV, Gruber HE, Windle JJ, Roodman GD (2007) Mutation of the sequestosome 1 (p62) gene increases osteoclastogenesis but does not induce Paget disease. J Clin Invest 117:133–142
122. Langston AL, Campbell MK, Fraser WD, Maclennan GS, Selby PL, Ralston SH (2009) Randomised Trial of Intensive Bisphosphonate Treatment Versus Symptomatic Management in Paget's Disease of Bone. J Bone Miner Res