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Chapter 4

OSTEOCLAST BIOLOGY IN PAGET'S DISEASE OF BONE

Stephen McManus, Lyne Bissonnette, and Sophie Roux*
Division of Rheumatology, Faculty of Medicine, University of Sherbrooke,
Sherbrooke, Quebec, Canada

ABSTRACT

Paget's disease of bone (PDB) is characterized by focal and disorganized increases in bone turnover. Because the initial phase of PDB involves excessive bone resorption, osteoclasts have been identified as the cells primarily affected in PDB. Pagetic osteoclasts are both larger and more numerous than normal osteoclasts. They are overactive and hypersensitive to osteoclastogenic factors, and resistant to apoptosis. Although a viral etiology has been suggested for Paget's disease, several studies have revealed a marked genetic component. The discovery of mutations of the *SQSTM1* (Sequestosome1, p62) gene in numerous patients has identified the protein p62 as an important modulator of bone turnover. p62 mediates several diverse cell functions, including the control of NF-κB signaling, protein trafficking and autophagy. Since *SQSTM1* mutations do not fully explain the osteoclast phenotype of PDB, the contribution of other osteoclast-related genes, viruses or environmental factors may be involved. Here, we review the most recent advances in osteoclast biology regarding PDB with a particular attention to the impact of the p62 mutations.

PDB is the second most common human skeletal disorder, after osteoporosis, affecting up to 3% of adults over 55 years of age [1]. PDB is characterized by focal and disorganized increases in bone turnover, and osteoclasts have been identified as the cells primarily affected in PDB [2]. As the osteoclasts are abnormally overactive, this disease provides an excellent model for elucidating osteoclast behavior. Both the genetic and viral etiologies have been thoroughly studied in the last few years. The discovery of mutations of the *SQSTM1*

* Corresponding author: Dr Sophie Roux M.D. Ph.D., 3001, 12th avenue N, Sherbrooke, PQ, Canada, J1H5N4. Phone: 819-564-5261, Fax: 819-564-5265. E-mail: Sophie.Roux@USherbrooke.ca.

(Sequestosome1, p62) gene in numerous patients has identified the protein p62 as an important modulator of bone turnover, providing new insights in osteoclast biology and highlighting the major role of p62 in these cells. New loci have recently been associated to PDB implying that genes other than *SQSTM1* may contribute to the pathogenesis of PDB. Here is a review of recent advances in our understanding of the regulators of osteoclast phenotype, signaling and functions in pagetic osteoclast.

OSTEOCLAST PHENOTYPE IN PDB

Osteoclasts are multinucleated cells formed by the fusion of mononuclear cells of hematopoietic origin, a process dependent on the dendritic cell-specific transmembrane protein (DC-STAMP) [3, 4]. The osteoclast phenotype differs from that of macrophages and macrophage polykaryons in several important respects; in particular, osteoclasts and their precursors express the calcitonin receptor (CTR) and the Receptor Activator of NF-κB (RANK), and present the unique capacity to resorb bone [5, 6]. After exercising their resorption activity, osteoclasts undergo apoptosis, and they have been shown to be sensitive to apoptosis induction by a number of cytokines and factors, including Fas-ligand, TRAIL, and TGFβ [7-9]. Osteoclast differentiation and activation are supported by osteoblasts and stromal cells, and regulated by two signaling pathways, which are activated by M-CSF and RANKL (RANK Ligand) respectively, and a recently described ITAM (immunoreceptor tyrosine-based activation motif)-mediated co-stimulatory signaling [10]. The interactions between RANKL, expressed on osteoblasts, and its receptor RANK, found on mature osteoclasts as well as their progenitor cells, trigger a series of signaling events promoting and regulating differentiation, activity, and survival of the osteoclast affected. Upon stimulation, a trimeric receptor complex recruits and associates with TRAF6, resulting in signaling cascades that ultimately activate transcription factors, particularly NF-κB and NFATc1 [10] (Figure 1).

In PDB, osteoclastic bone resorption is abnormally increased in localized areas of the skeleton, and remains coupled to bone formation. This characteristic rapid and chaotic bone turnover results in an increase in bone volume that is composed of abnormal matrix with irregular and patchy arrangement of collagen fibers. Pagetic osteoclasts are both larger and more numerous, possess a higher number of nuclei per osteoclast, and cause deeper resorption lacunae [11]. They are resistant to apoptosis, and are overactive [12]. They also are hypersensitive to osteoclastogenic factors, such as RANKL, 1,25-$(OH)_2D_3$ [13, 14], and express higher levels of TAFII-17, a VDR binding protein [15].

Gene expression profile studies showed different expression pattern between osteoclast cultures from PDB patients compared to healthy controls. Pagetic osteoclasts displayed down-regulation in genes involved in apoptosis (*CASP3* and *TNFRSF10A*), in cell signaling (*TNFRSF11A*), in the osteoclast bone resorbing function (*ACP5* and *CTSK*) and in the gene coding for Tau protein (*MAPT*) [16]. Another study found an upregulation of the expression of anti-apoptotic Bcl-2 gene in pagetic osteoclasts [17].

The molecular basis of PDB and the characteristic features of the pagetic osteoclasts are not fully understood, however major advances have been made over the past ten years, particularly in genetics, and have provided important new insights into the pathogenesis of this disease.

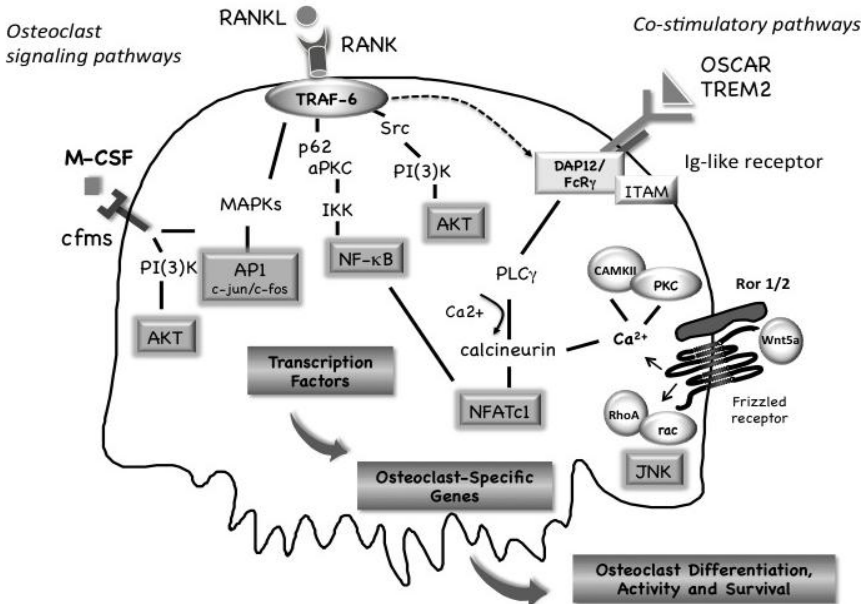

Figure 1. Osteoclast signaling pathways. In both pre-osteoclasts and mature osteoclasts, the interaction between RANKL and RANK results in transduction signals via a signaling complex containing TNFR-associated factors, mainly TRAF6. Several signaling cascades are activated, leading to the expression of specific genes involved in osteoclast differentiation, activation and survival, and include the NF-κB, both classical and alternative [106], as well as MAP kinases (JNK, p38, ERK) and Src pathways [5]. NFATc1, a transcription factor, is also strongly induced and activated after RANKL stimulation in osteoclasts via c-Fos, NF-κB and calcium signaling [107]. RANKL may activate the calcium signals that lead to the induction of NFATc1 through a co-stimulatory pathway that involves Ig-like receptors, such as OSCAR or TREM2, and adaptor molecules, such as FcRγ and DAP12, that contain an ITA motif, which is critical for activating calcium signaling. The phosphorylation of this motif is stimulated by the RANKL–RANK interaction, and by Ig-like receptors, and results in the activation of PLC and calcium signaling [10]. Non-canonical Wnt proteins (Wnt5a, Wnt11) bind another receptor complex including a frizzled receptor and receptor tyrosine kinase-like orphan receptors (Ror 1/2). This pathway activates the planar cell polarity via JNK signals, as well as Ca^{2+} signaling via PKC- and calcineurin-dependent signals [108, 109]. The non-canonical β-catenin independent Wnt pathway directly affects osteoclast precursors and stimulates their differentiation.

VIRAL AND GENETIC FACTORS IN PAGET'S DISEASE OF BONE

The involvement of a virus infection has been proposed in the pathogeny of PDB since the discovery of inclusion bodies in the nuclei and cytoplasm of osteoclasts which resemble the nucleocapsids of paramyxovirus [18, 19]. This hypothesis has since been supported by studies focusing mainly on Measles virus that have detected mRNA or protein expression of paramyxovirus nucleocapsids in osteoclasts, bone marrow cells or peripheral blood cells from PDB patients. The role of a slow infection in the pathogeny of PDB is still a matter of debate, and other studies failed to detect such an expression [20]. Studies have highlighted the role of paramyxovirus infection in some of the characteristic of pagetic osteoclasts. Transfection of Measles virus nucleocapsid (MVNP) gene in human osteoclast precursors resulted in an increase in osteoclast formation and activity, and increase the sensitivity to $1,25(OH)_2D_3$ [15,

21]. Transgenic mice expressing MVNP targeted in the osteoclast lineage also had an increase in osteoclast formation from bone marrow cultures with increases in osteoclast formation, activity, as well as increased nuclei number per osteoclast, and *in vivo* develop lesions similar to those observed in PDB [22].

Although a viral etiology has been suggested for Paget's disease, numerous studies have also revealed a marked genetic component [23]. Some years ago, a germline mutation in the *SQSTM1* gene on 5q35 was identified in a high proportion of PDB patients [24-27]. This gene encodes the ubiquitin-binding protein sequestosome 1, also known as p62. Since this initial discovery, 27 other mutations have also been reported, although the P392L substitution is the most frequent [1, 23]. Mutations of this gene have been detected in about 30% of familial Paget's cases, with a high penetrance of about 80% in patients over 60 years of age, and in about 9% of sporadic cases [1, 23, 27]. A role for somatic acquired SQSTM1 mutations in affected tissues has also been suggested in PDB, but is still controversial [28, 29]. Other strong loci of susceptibility for PDB have also been investigated (5q31, 10p13), although no gene has been identified so far in these loci. In a candidate gene approach, the valosin containing protein (VCP) gene was studied in PDB. The VCP gene is involved in a rare disease in which PDB can occur as part of a disease associated with inclusion body myopathy and frontotemporal dementia. Genetic variants of this gene were not associated with PDB in a British population [30], but such an association was found in a Belgian population [31]. Two recently published genome-wide association studies (GWAS) in PDB patients identified seven significant genetic variants for susceptibility to PDB located at the 1p13 *(CSF1)*, 7q33 (*CNOT4*, *NUP205*, *SLC13A4)*, 8q22 (*TM7SF4)*, *10p13 (OPTN)*, 14q32 (*RIN3)*, 15q24 (*PML*, *GOLGA6A)*, and 18q21 *(TNFRSF11A)* loci [32, 33]. The genetic risk for PDB close to the *CSF1*, *OPTN*, *TM7SF4*, and *TNFRSF11A* genes was confirmed in other populations [34]. The data obtained from GWAS and the replications studies strongly suggest that these 7 genetic variants may predispose to PDB. Interestingly, some candidate genes have been involved in osteoclast formation, activity and survival (*CSF1* encoding MCSF; *TNFRSF11A* encoding RANK), and in osteoclast fusion (*TM7SF4* encoding DC-STAMP). This implies that genes other than *SQSTM1* may also contribute to the pathogenesis of PDB, although so far p62 is the only gene in which mutations have been identified.

ROLE OF P62 IN OSTEOCLAST SIGNALING

The discovery of mutations of the *SQSTM1* (Sequestosome1, p62) gene in PDB has led to the identification of protein p62 as an important modulator of bone turnover. Genetic inactivation of p62 in mice leads to impaired RANKL-induced osteoclastogenesis, due to defective activation of NF-κB [35], identifying p62 as a key player in osteoclast biology and in RANKL signaling pathway.

p62, an adaptor protein with multiple binding domains - p62 or sequestosome 1 (SQSTM1) is a highly conserved protein with multiple and varied functional domains. Involved in cell signaling, receptor internalization, and protein turnover, the protein contains a ubiquitin (Ub)-associated (UBA) domain at its C-terminus, two PEST sequences, between which an LC3-interaction region (LIR) stands, a binding site for the RING finger protein TRAF6, a domain binding p38 as well as LIM-containing proteins, a ZZ finger interacting

with RIP, a PB1 domain for binding atypical PKCs (aPKCs), but also ERK, NBR1, MAPKK5 (MEK5) and MEKK3, and p62 itself as well, and finally an N-terminus capable of direct interaction with the proteasome subunit component [23, 36, 37]. With such numerous protein-protein interaction motifs, p62 is considered a scaffold, playing an important role in the osteoclast, and serving as the switchboard from which the RANKL activation signal is propagated (Figure 2).

TRAF6, p62 and RANKL-induced osteoclast signaling - The p62 scaffolding protein is one of the functional links reported between RANKL and TRAF6-mediated NF-κB activation [38]. The most clearly established function of p62 is its role as a scaffold protein for the RANKL-induced activation of NF-κB, requiring interactions between TRAF6, p62, the PB1-interacting atypical Protein Kinase Cζ (PKCζ), and phosphoinositide-dependent kinase 1 (PDK1), which result in the formation of a multimeric protein complex [12, 35, 39]. The sequence of RANKL-induced activation requires the recruitment of TRAF6, which is responsible for most of the downstream events that lead to osteoclast differentiation and activation [40], and is necessary for RANKL-induced NF-κB activation [41]. RANKL-induced TRAF6 recruitment also leads to the activation of the MAP kinases p38, ERK (extracellular-signal regulated kinase), and JNK (c-jun terminal kinase), as well as activating the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.

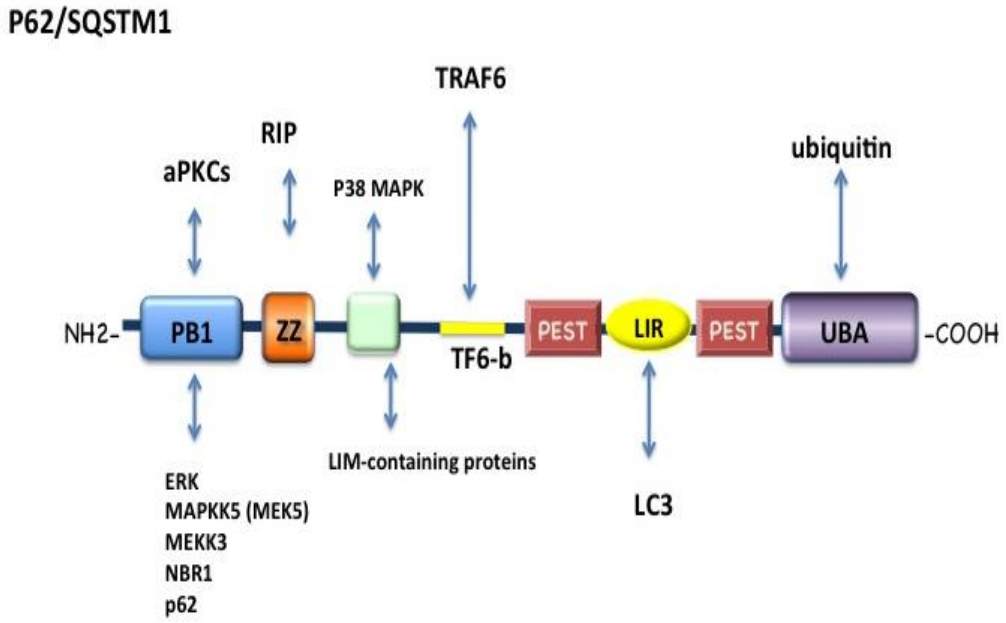

Figure 2. p62/SQSTM1 linear representation. Multiple interaction motifs located within p62 enable recruitment of specific proteins and regulation of downstream signaling pathways. Thus, the aPKCs and ERK interact with the PB1 domain whereas RIP1 binds to the ZZ domain, and TRAF6 interacts with the TF6-b sequence. The UBA domain binds to polyubiquitin chains. PB1: Phox and Bem1p ; ZZ ZNF: ZZ-type zinc finger; TF6-b: TRAF6 binding sequence; PEST: (P, Proline; E, Glutamate; S, Serine; T, Threonine) rich sequence; LIR: LC3 interacting region; UBA, ubiquitin associated.

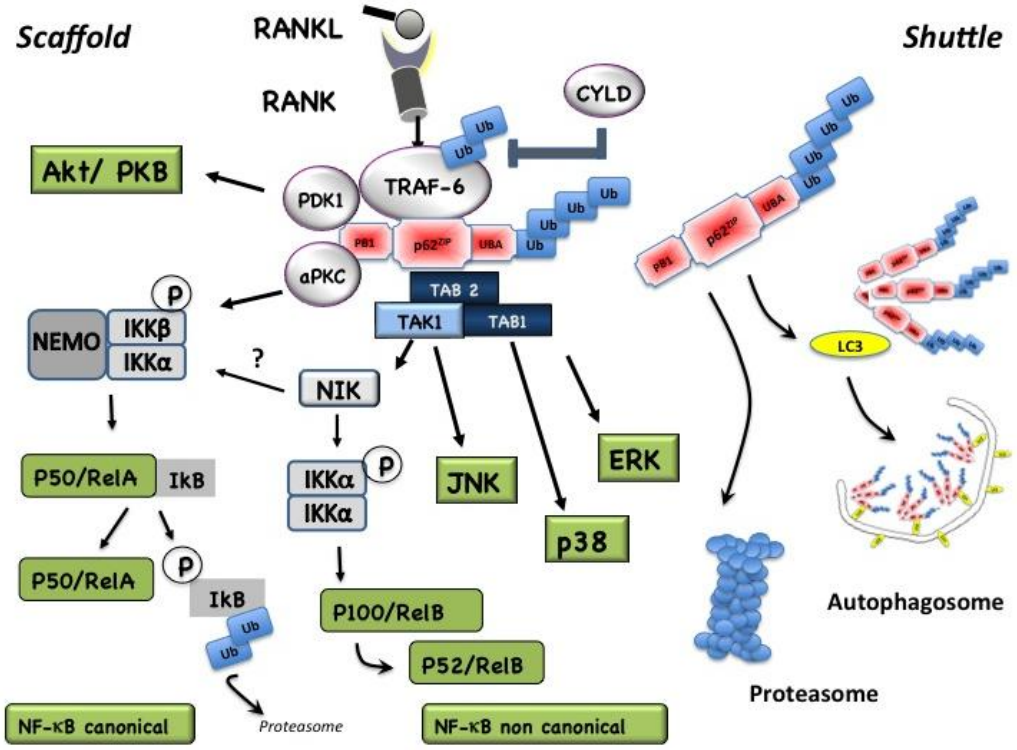

Figure 3. p62 in osteoclast signaling. In both precursors and mature OCLs, the interaction between RANKL and RANK results in signaling cascades that require the recruitment of TNFR-associated factor 6 (TRAF6), and ultimately activate transcription factors, particularly NF-κB and NFATc1. The cytosolic p62 protein, encoded by the SQSTM1 gene, is a scaffolding protein that interacts with the RANK signaling complex, and is one of the functional links reported between RANKL and TRAF6-mediated NF-κB activation. p62 may be involved in the formation of mutimeric protein complexes, because of the interactions between its PB1 domain with other proteins. Due to its ability to bind polyubiquitinated substrates at the C-terminal UBA domain, it may also play a role in protein degradation through its association with the proteasome at its N-terminal ubiquitin-like (UBL) domain, and in autophagy as an LC3-interacting protein.

Another function of TRAF6 in RANK signal propagation is the formation of protein complexes with TGFβ-activated kinase 1 (TAK1) and adaptor proteins TAB1 and TAB2 [42]. When TAK1 is activated, it in turn phosphorylates NF-κB-inducing kinase (NIK), which activates the IκB kinase (IKK) complex, leading to NF-κB pathway activation. As for the MAP kinases, TAK1 will also activate the JNK pathway, while TAB1 recruits and binds p38 to the TRAF6 complex, leading to activation of its pathway [43, 44]. RANKL also activates the Akt/PKB pathway with the aid of TRAF6 and associated signaling proteins [45]. The key to these pathways is the scaffolding protein p62, which is recruited and bound to the TRAF6/RANK complex, and acting as a scaffold regulates these signaling pathways (Figure 3).

Once bound to TRAF6, p62 permits the recruitment of atypical protein kinase C (aPKC) proteins [46]. After stimulation by interleukin-1 (IL-1) or nerve growth factor (NGF), these kinases regulate NF-κB activation via the phosphorylation of IκB kinase β (IKKβ) [39, 47].

The p62 complex also binds the scaffolding receptor interacting protein (RIP) and kinase PDK1, recruiting aPKCs to TNF-α signaling complexes [48, 49].

p62, TRAF6 and CYLD- p62 interactions with TRAF6 stimulate TRAF6 K63-linked autoubiquitination and E3 ligase activity, as well as regulate the synthesis of K63 chains on target substrates. This ubiquitination of TRAF6 is an important mechanism mediating its signaling functions [50, 51]. Activated TRAF6 may then stimulate NF-κB activity by activation of the IKK complex, either through aPKC or TAK1-dependent phosphorylation, and requiring NEMO (IKKγ) ubiquitination for optimal activation [52]. In addition to its well established role in the activation of TRAF6, the role of p62 in regulating a deubiquitinating enzyme (DUB) is a current subject of research. p62 appears to be a molecular adaptor associating deubiquitinase CYLD and TRAF6 [53]. In addition to interacting with TRAF6, CYLD has specificity for Lys^{63} (K63) chains, and reverses the processing of protein ubiquitination. The decrease in the activity of CYLD leads to the accumulation of Lys^{63} (K63)-ubiquitinated substrates [53]. By interacting with CYLD at its C-terminal domain, p62 promotes the binding of CYLD to TRAF6 [54]. CYLD thus negatively regulates NF-κB activity by reducing the autoubiquitination of TRAF6 [55, 56]. It therefore negatively regulates the activation of the IKK and JNK pathways, and is markedly upregulated under conditions of RANKL-induced osteoclastogenesis, acting as a natural feedback inhibition regulator [54]. CYLD is a crucial down-regulator of RANK signaling in osteoclasts, and accordingly, it has been shown that CYLD-deficient mice are severely osteoporotic, originating from aberrant osteoclast differentiation, having larger and more numerous osteoclasts which are hypersensitive to RANKL [54].

P62 AS A SHUTTLING FACTOR

UBA and PB1 domain function - It has been suggested that p62 is a shuttling factor in the delivery of poly-ubiquinated substrates to the proteasome for degradation [57]. p62 has been proven to sequester polyubiquitin into aggresomes or sequestosomes, common pathological features of many diseases [58, 59]. However, outside of a few examples, evidence suggests that p62 functions primarily as a scaffolding protein for ubiquitinated proteins in processes not directly related to proteasomal degradation, like NF-κB signaling and macro-autophagy [35, 60]. p62 may be involved in the formation of multimeric protein complexes, due to interactions between its PB1 (Phox and Bem1p) domain with other proteins [61]. Given the ability of p62 to bind polyubiquitinated substrates the C-terminal UBA domain, and its association with the proteasome at its N-terminal ubiquitin-like domain, it likely plays a role in protein turnover as well [62]. The aforementioned N-terminal PB1 permits p62 to act as a shuttling factor, directing for turnover polyubiquitinated proteins that interact with the UBA domain [57]. Proteins containing the PB1 domain include PDK1, Par6, the aPKCs, and many more, and can form specific heterodimers between family members [63]. Therefore, in addition to potentially playing a role in protein turnover via proteasome association, p62 may also participate in the formation of multimeric protein complexes through interactions between PB1 domains. This domain allows flexibility, either favoring this oligomeric function or the formation of self-associating p62 molecules into sequestosomes [62].

Relatively unusual among ubiquitinating proteins of its type, p62's UBA is rendered biologically inactive by stabilization of the classical canonical UBA conformation via dimer formation [64, 65]. And by making the active and less stable monomer unavailable, p62 further regulates its own function [66]. Once free in monomeric form, p62 undergoes a conformational change favoring K63-linked polyubiquitination, granting the protein additional functions as a multifunctional scaffold protein, affecting processes of NF-κB, autophagy, and more [62, 66].

Autophagy and LC3 interaction – Whereas proteosomal degradation targets soluble proteins, autophagy (macro-autophagy) is a regulated process that can also eliminate large structures such as aggregates, misfolded macromolecules, organelles or pathogens through the formation of autophagosomes. These structures are large vesicles that wrap up damaged material and fuse with lysosomes to deliver their content for subsequent degradation. Autophagy takes place in all cells, in order to maintain cell homeostasis or in response to stress or starvation, by eliminating damaged components, and providing cells with energy and nutrient resourcing [67, 68].

p62 has been identified as an LC3-interacting protein implicated in autophagy [60, 69, 70]. Studies have shown that p62, along with ubiquitinated proteins, is transported into autophagosomes, suggesting that p62 is a receptor for these proteins that directs them to lysosomes [60, 70]. This is made possible by its LRS (LC3 recognition sequence) located between the zinc finger and UBA domains of p62, where residues interact with the N-terminus and ubiquitin domain of LC3 [71, 72]. LC3 covalently conjugates with phosphatidylethanolamine (PE) through an enzymatic cascade, and is found on the inner and outer membranes of the isolation membrane of the autophagosomes, and is vital to membrane biogenesis as well as closure of the isolation membrane [73-76]. This interaction leads to relocation of p62 to the autophagosome, submitting it to degradation by the autophagy-lysosome system. However, when autophagy is impaired, p62 is allowed to accumulate, which can result in failure to terminate some of its signaling processes, as well as the formation of p62-positive inclusions [59, 69].

Findings support the hypothesis that ubiquitinated proteins interact with p62, followed by aggregation of the protein complex in a p62 dependent manner, and terminating with degradation of these aggregates by autophagy [77, 78]. The exact mechanisms and impact of this pathway are still being developed, as some details are still unknown; like the quantities of cellular ubiquitinated proteins being transported into autophagosomes by p62, or the signals that are responsible for modulating selective degradation of ubiquitinated protein aggregates by autophagy. Interestingly, knockout of p62 does not appear to markedly affect levels of ubiquitinated proteins in the cell, possibly due to compensatory action by Nbr1 (neighbor of BRCA1 gene 1), which interacts with LC3 in a similar manner [69, 79]. Loss of function of Nbr1 leads to perturbation of p62 levels and hyperactivation of p38 MAPK that favors osteoblastogenesis [80].

As previously mentioned, the interaction of p62 with TRAF6 promotes the oligomerization and subsequent K63 polyubiquitination and activation of TRAF6, resulting in NF-κB activation [81]. In the case of cell death, cell-surface death receptors like those of TRAIL trigger apoptosis through signalization after initiator caspase-8 activation by polyubiquitination, via interaction of the death-inducing signaling complex (DISC) with cullin3-based ubiquitin ligase (CUL3) [82]. p62 can promote aggregation of the CUL3-modified caspase-8, leading to its full activation and processing, favoring commitment to cell

death [82]. It has thus been suggested that p62-positive aggregates are signaling hubs that can determine whether cells die by caspase activation or survive through TRAF6-NF-κB pathways [83]. p62 also interacts with Keap1, a ubiquitin ligase for Nrf2 [84]. Nrf2 regulates gene expression of a variety of antioxidant proteins and detoxification enzymes [85]. So, a surplus of p62, either by faulty autophagic processes or overproduction, can lead to competitive binding of Keap1, stabilizing Nrf2 and the activation of its target genes. Therefore, because the level of p62 protein is controlled by autophagy, p62-associated autophagy can regulate not just the NF-κB and other TRAF6-associated activation pathways, but apoptosis and environmental stress response as well [84].

Autophagy has been involved in hypoxia-induced osteoclast differentiation [86], in MCP-1 (Monocyte chemotactic protein-1)-induced osteoclast differentiation through MCPIP-mediated induction of oxidative stress [87], and p62 may play a role in the starvation-induced autophagy in human osteoclasts [88]. Autophagy proteins have been shown to participate in the polarized secretion of lysosomal contents into the extracellular space by directing lysosomes to fuse with the osteoclast ruffled membrane [89].

ROLE OF P62 MUTATIONS IN PAGET'S DISEASE OF BONE

Impact of $p62^{P392L}$ in osteoclast phenotype - The significance of the most common p62 mutation, P392L, has been studied in a series of *in-vitro* experiments, using osteoclast precursors derived from the peripheral blood of PDB patients carrying the $p62^{P392L}$ gene or not, and from bone marrow cells or cord blood monocytes of normal subjects transfected with the $p62^{wt}$ or $p62^{P392L}$ gene. These studies strongly suggest that the $p62^{P392L}$ mutation affects the osteoclast phenotype by inducing overactive osteoclasts that are resistant to apoptosis, have an increased ability to resorb bone, and display some of the characteristics of the Paget osteoclast phenotype including an hyper-responsiveness to osteoclastogenetic factors, such as RANKL and TNFα [12, 90, 91].

Impact of $p62^{P392L}$ on osteoclast signaling - Previous findings revealed that in human osteoclasts, RANKL stimulation induced the formation of a multiprotein complex containing not only PKCζ and p62, but activated PDK1 as well [12]. Interestingly, even prior to RANKL stimulation p62 was associated with both activated PDK1 and PKCζ/λ in PDB osteoclasts, in osteoclasts from healthy donors harboring the $p62^{P392L}$ gene, and in $p62^{P392L}$ transfected osteoclasts [12]. Moreover, the signaling complex formed in response to RANKL stimulation normally results in NF-κB activation, which was observed in the non-transfected osteoclasts of CBM cultures. Although both wild-type and mutated p62 over-expression favored the formation of activated protein kinases/p62 complexes, only that of the mutated gene led to an increased basal level of NF-κB activation [12]. These findings strongly suggest that $p62^{P392L}$ contributes at least in part to the induction of an activated stage in osteoclasts by stimulating signaling pathways involving PDK1-PKCζ/λ that could lead to NF-κB activation. Similarly, overexpression of the $p62^{P392L}$, $p62^{K378X}$ or $p62^{E396X}$ mutants in HEK293 or Cos-1 cells increased basal NF-κB activation, and overexpression of the $p62^{K378X}$ or $p62^{E396X}$ mutants also increased RANKL-induced NF-κB activation more than overexpression of the p62 wild type mutant [91].

Mutant p62 transfects have been shown to upregulate NFATc1 expression in pre-osteoclasts, favoring increased osteoclastogenesis and osteoclast activity [92]. Naturally, TRAF6 activity is affected by p62 interaction, affording p62 some measure of indirect control over NFATc1 signaling. However, kinases may also contribute to the nuclear shuttling of this factor, as PKCζ has been shown to interact with NFATc1, and may modulate NFAT-mediated transcription by increasing the activity of its N-terminal transactivation domain [93]. Therefore RANKL stimulation may further contribute to osteoclast activation via formation of the p62-aPKC complex, leading to increased NFATc1 activity.

As noted earlier, CYLD is a crucial down-regulator of RANK signaling in osteoclasts. Recent studies have further confirmed that CYLD knockdown significantly increased c-Fos expression in cells transduced to express both wild-type and mutant $p62^{P392L}$, without necessitating RANKL stimulation [92]. Likewise, mutations to the UBA domain of p62 lead to a reduction in CYLD activity (and thus an increase in osteoclast development and resorption) [92], given that binding between the two takes place at the C-terminal end of the p62 protein.

Although the presence of the P392L substitution cannot account for all aspects of the PDB osteoclast phenotype, it clearly affects osteoclast behavior, although the mechanisms involved in such p62-driven misregulation are not fully understood. All these studies demonstrate that the $p62^{P392L}$ mutation affects the osteoclast phenotype, inducing overactive osteoclasts that display several characteristics of the Paget osteoclast phenotype.

Experimental models of PDB - In vivo, transgenic mice with targeted expression of the human $p62^{P392L}$ gene in the osteoclast lineage developed osteopenia, with an elevated osteoclast perimeter, although no PDB-related bone lesions were observed. Once again, osteoclast precursors were hyperresponsive to RANKL and TNFα, and they demonstrated increased proliferation rates, but no change in either nucleus numbers or apoptosis rates [90]. As p62 is ubiquitously expressed, p62 mutations may also affect cells other than osteoclasts, particularly stromal cells or osteoblasts. This hypothesis has been investigated in a study where $p62^{P394L}$ (the murine equivalent of human $p62^{P392L}$) knock-in mice were generated. In bone marrow cultures, the expression of $p62^{P394L}$ in stromal cells was associated with increased RANKL expression in response to $1,25(OH)_2D_3$. However, the $p62^{P394L}$ knock-in mice had histologically normal bones up to 18 months of age [94]. This implies that the presence of the p62 mutation in bone cells other than osteoclasts may increase RANKL production in the bone microenvironment, thus indirectly enhancing osteoclast formation and activity, and that additional factors are necessary for the development of PDB *in vivo*. Transgenic mice coexpressing the measles virus nucleocapsid （MVNP) under the TRAP promoter and knocked-in $p62^{P394L}$ develop PDB-like bone lesions, present in 40% of the mice over 18 months [95]. Finally, in another study, transgenic mice expressing the $p62^{P394L}$ gene develop focal osteolytic lesions, which were present in 95% of homozygous mice by 12 months of age, with some of them resembling PDB lesions [96].

Protein turnover and autophagy in PDB osteoclast and impact of the $p62^{P392L}$ mutation - In PDB, virtually all p62-related mutations are clustered in or around the UBA-domain, indicating that ubiquitin-binding alteration may play a role in p62-related bone diseases [97, 98], and the failure of specific polyubiquitinated substrates to interact with the p62 UBA domain may contribute to the pathophysiology of PDB. One attractive hypothesis that could explain aberrant cellular functions is the accumulation of p62-associated proteins, due to

impaired degradation through proteosomal or autophagic pathways. For instance, p62 is commonly found in protein aggregates described in degenerative diseases [59], and may be linked to increased cell survival [60]. However, ubiquitin-binding activity is not related to PDB occurrence or severity [99], and does not correlate with the UBA-mediated inhibition of proteasomal degradation [100]. Notably, even when their ubiquitin-binding activity has been severely impaired, p62 UBA domains have been shown to still be capable of inhibiting proteasomal degradation. While the mechanism by which this is accomplished is not yet definite, the results suggest that the protective effect of mutant UBA domains on p62 proteasomal degradation may primarily depend on their conformational integrity rather than their ubiquitin-binding capacity [100]. In addition, while some p62 mutants exhibit increased basal NF-κB activity relative to wild-type p62 [91, 98], it has also been observed that mutants in which p62-UBA dimerisation was impaired exhibited reduced NF-κB signaling activity [66].

As p62 is a key player in selective autophagy, and may have a role in the formation of inclusion bodies [69, 101]; because the inclusion bodies found in pagetic osteoclasts resemble those observed in diseases with defective autophagy, a dysregulation of the autophagy process may well be part of the pathogeny in PDB, although so far no direct evidence has been provided for its role in the phenotype of pagetic osteoclast [102]. In addition, the $p62^{P392L}$ mutation did not affect p62-related aggregate formation in human osteoclasts [88]. Interestingly, the $p62^{D335E}$ mutation is located in the LC3-interacting domain (position 321-342), although its impact on autophagy has not yet been studied [70, 103].

Apoptosis in PDB osteoclast and impact of the $p62^{P392L}$ mutation - In osteoclast cultures derived from PDB patients, lower rates of apoptosis were induced by the deprivation of survival factors or by death inducers, such as TRAIL, Fas activating antibody or TGF-β [12]. An interesting observation is that osteoclasts from healthy carriers of the $p62^{P392L}$ gene had an intermediate phenotype, with greater resistance to apoptosis than osteoclasts from normal controls, but less than that of osteoclasts from PDB patients. In cord blood monocytes transfected with $p62^{wt}$ or $p62^{P392L}$ genes, apoptosis rates were lower in osteoclasts overexpressing either $p62^{wt}$ or $p62^{P392L}$ than in empty-vector transfected osteoclasts [12]. An increase in p62 expression has been observed in PDB osteoclasts [12, 104], and this could contribute to their resistance to apoptosis as, according to studies in other systems, increased expression of p62 could in itself have a protective effect against cell death by preventing the build-up of potentially cell-damaging proteins [60, 105]. In addition, NF-κB is known to be involved in apoptosis regulation, and a basal activation of NF-κB was observed in osteoclasts transfected with the $p62^{P392L}$ gene, but not those transfected with $p62^{wt}$. Thus basal and RANKL-induced NF-κB activation may influence osteoclast survival in PDB. Finally, gene expression studies have suggested that genes involved in osteoclast apoptosis were significantly downregulated in pagetic osteoclasts (genes encoding caspase-3, TRAIL-R1), with a trend towards a decrease for others (TRAIL-R2, TGFβ-R1) [16], while the anti-apoptotic Bcl-2 gene's expression was upregulated [17]. So, it seems clear that apoptosis is hindered in PDB osteoclasts, and that the presence of PDB mutations in the p62 gene is involved, at least in part.

CONCLUSION

In PDB, p62/SQSTM1 mutations contribute at least in part to inducing an activated phase in osteoclast by stimulating signaling pathways that can lead to NF-κB activation, and to apoptosis resistance. In addition to its role as a scaffold protein in the control of RANKL-induced NF-κB signaling, protein p62 mediates several other cell functions including protein trafficking and autophagy, a new research area in osteoclast physiology. However, *SQSTM1* mutations do not fully account for the osteoclast phenotype of PDB, and other osteoclast-related genes may also contribute to the osteoclast phenotype. It is also likely that in addition to genetic factors, environmental factors contribute to the development of the disease.

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