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Clinical Investigations

SQSTM1 and Paget's Disease of Bone

R. Layfield,[1] L. J. Hocking[2]

[1]School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK
[2]University of Aberdeen Medical School, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK

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Abstract. Mutations in the Sequestosome 1 gene (*SQSTM1*; also known as *p62*) have recently been identified as the cause of 5q35-linked Paget's disease of bone (PDB). All of the mutations identified to date affect the ubiquitin-associated (UBA) domain of SQSTM1, a region of the protein that binds noncovalently to ubiquitin. In this review we consider the possible functional significance of the SQSTM1-ubiquitin interaction, and consequences of the SQSTM1 UBA domain mutations. Clarification of the *in vivo* roles of SQSTM1 in bone-cell function will be central to improving our understanding of the molecular pathogenesis of PDB and related conditions.

Key words: SQSTM1 — p62 — Paget's disease of bone — Ubiquitin — UBA domain

Paget's Disease of Bone

Paget's disease of bone (PDB [MIM 167250, 602080]) is a common skeletal disorder, affecting ~3.1% of individuals > 55 years of age in the U.K. [1]. The clinical presentation of PDB varies widely. Although many patients are asymptomatic, ~30% experience bone pain, skeletal deformity, deafness, neurological symptoms, and pathological fractures [2]. These complications can result in loss of mobility and independence. The most serious complication of PDB is osteosarcoma; although this is rare, affecting < 1% of patients, the majority of osteosarcomas that occur in adulthood do so in patients with PDB [3].

Role of Genetic Factors in the Pathogenesis of PDB

The cause of PDB is not completely understood, but genetic factors play an important role. Given that the disease is focal (rather than systemic) additional local factors may in some cases act as a disease trigger. Familial clustering of PDB has long been recognized [4] and current estimates indicate that between 15% and 40% of affected individuals have at least one affected first-degree relative [5–7]. For numerous extended families the disease has been described as being inherited in an autosomal dominant manner [7–9]. Ethnic differences in incidence of the disease have also been observed [10] and these differences persist after emigration, which is consistent with a key role for genetic rather than environmental factors in disease causation. There are also suggestions that PDB might be caused by a slow virus infection of bone cells with one of the paramyxoviruses [11], although evidence in support of this hypothesis is conflicting and the role of viruses in the pathogenesis of PDB currently remains uncertain [12]. Proponents of the hypothesis that PDB might have an environmental trigger, point, to the observation that the prevalence of PDB has decreased significantly in the United Kingdom over the past 35 years [13]. Although these apparent changes in incidence could be caused by changes in exposure to an environmental agent, another possibility is that the demographic changes in ethnic background of the population that has occurred in the United Kingdom over the past 25 years. In this regard, it is of interest that epidemiological studies over a 40-year period have shown no major changes in PDB incidence in Rochester, Minnesota [14]. In addition, archaeological studies of skeletal remains in the United Kingdom have shown that the prevalence of PDB between the fifteenth and nineteenth centuries is quite similar to the prevalence today, excluding major changes in disease incidence over the past 500 years [15].

Molecular Genetic Determinants of PDB

Over the past 5 years, tremendous advances have been made in understanding the molecular genetic basis of PDB and related conditions. The rare PDB-like bone

Correspondence to: R. Layfield; E-mail: robert.layfield@nottingham.ac.uk

dysplasia, familial expansile osteolysis (FEO [MIM 174810]) was mapped by Hughes et al. to chromosome 18q21–22 [16], and subsequently some cases of familial PDB were mapped to the same region (*PDB2*) [9, 17]. Positional cloning studies showed that the gene for receptor activator of NFκB (*RANK*) lay within the *PDB2* critical region, and mutation screening identified a heterozygous 18-bp tandem in-frame repeat within exon one of *RANK* in individuals from three families affected with FEO [18] and a 27-bp repeat in individuals from one family with a clinical syndrome suggestive of early onset PDB [19]. These mutations encode six and nine amino acid insertions, respectively, in the signal peptide region of RANK, and both activate NFκB signaling [18]. Later, a 15-bp insertion was described in patients with expansile skeletal hyperphosphatasia (ESH), which introduces a five–amino acid insertion into the RANK signal peptide [20]. Accordingly, it is currently considered that FEO, early onset familial PDB, and the related syndrome of ESH are allelic disorders caused by different activating mutations affecting the signal peptide region of RANK [20]. Although *RANK* mutations are responsible for these severe, early onset PDB-like syndromes, mutations in the *RANK* gene have been excluded as a cause of late-onset familial and sporadic PDB. Fine mapping of the 18q21–22 locus excluded linkage across the entire *PDB2* region in late-onset familial PDB [8, 21, 22], and mutation screening of *RANK* in U.K. pagetic families and in a series of individuals with sporadic PDB failed to identify any disease-causing mutations [23]. Recently, homozygous deletions of the gene for osteoprotegerin (OPG), the soluble RANK ligand (RANKL) decoy receptor, have been identified as a cause of the rare syndrome juvenile hyperphosphatasia (also known as juvenile Paget's disease) [24, 25]. Although this illustrates the importance of mutations in the RANK signaling pathway as a cause of PDB-like phenotypes, mutations in *RANKL* and *OPG* have been excluded as a common cause of late-onset sporadic and familial PDB [26].

Genome-wide scans in families affected with PDB have identified potential susceptibility loci for the disease on chromosomes 5q35 (*PDB3*) [27, 28] 5q31 (*PDB4*) [28], 2q36 (*PDB5*) [27], 10p13 (*PDB6*), and 18q23 (*PDB7*) [29]. Nine different mutations in the gene encoding sequestosome 1 (*SQSTM1*; also known as p62) have now been identified as the cause of 5q35-linked PDB, all of which affect the *C*-terminal ubiquitin-associated (UBA) domain of the SQSTM1 protein (Fig. 1B). The name sequestosome 1 derives from the ability of the protein to form cellular aggregates know as "sequestosomes," which may be sites of intracellular protein degradation (see later).

A recurrent C > T transition at position +1215 on the cDNA sequence of *SQSTM1* (NM_003900), which results in a proline to leucine amino acid substitution at codon 392 (P392L), was the first to be identified [30]. This mutation was found to affect some 50% of French-Canadian PDB families and 20% of sporadic French-Canadian PDB patients.

In a study of UK-derived familial and sporadic PDB cases, the P392L mutation was found in 13/62 (19%) of families and 9% of sporadic PDB patients [31]. In the same study, a T insertion mutation was identified at position +1224 that introduces a stop codon in place of glutamine at codon 396 (E396X) in four families (6%), and a novel G > A transition identified in one family (2%) that abolishes the splice donor consensus recognition site at the start of intron seven of *SQSTM1* [31]. The latter is predicted to cause failure of splicing and generate a truncated protein of 390 amino acids owing to an in-frame stop codon 8-bp downstream from the intron seven splice junction, which like the E396X mutation effectively removes a large majority of the UBA domain in the gene product.

Deletion of 1215C has been reported in a large Australian family, causing a frameshift and premature termination of the protein at residue 394 (394X-1215delC) in the UBA domain [32].

Recently, four further mutations affecting the UBA domain of SQSTM1 have been identified in UK-derived familial and sporadic PDB cases [33]. The first of these is a T deletion mutation at position +1210 in 1/70 (1.4%) families that also results in premature termination of the protein at codon 394 (394X-1210delT). The other three are missense mutations affecting the UBA domain: an A > G transition at position +1250 resulting in a methionine to valine substitution at codon 404 (M404 V), present in 4/70 (5.7%) families and 1/288 (0.3%) individuals with sporadic PDB; (2) a G > A transition at position +1271 causing a glycine to serine substitution at codon 411 (G411S) found in 3/70 (4.3%) families; (3) and a G > A transition at position +1313 causing a glycine to arginine substitution at codon 425 (G425R) in 1/70 (1.4%) families and 1/299 (0.3%) individuals affected with sporadic PDB [33].

In addition, the 394X-1210delT and 394X-1215delC mutations were identified in a study of US PDB pedigrees, as was a novel C > T transition at position +1200 resulting in a proline to leucine substitution at codon 387 (P387L; [34]), and the P392L, M404V and G425R mutations were identified in an Italian series of patients affected with PDB [99].

Functions of the SQSTM1 Protein

The protein encoded by the human *SQSTM1* gene has been implicated in diverse cellular functions [35] including modulation of potassium channel function, control of transcriptional activation, protein recruitment to endosomes, and, probably most relevant to PDB, control of NFκB signaling. In this latter context,

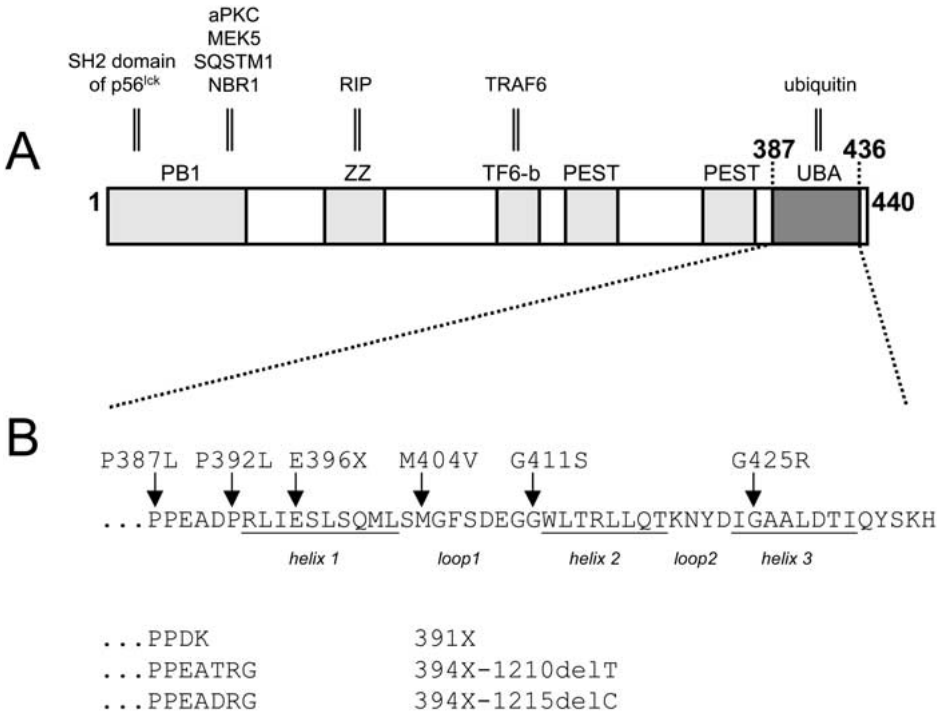

Fig. 1. (**A**) Schematic representation of the SQSTM1 protein, PBI, ZZ, and TF6-b refer to binding sites for the indicated proteins (top). PEST indicates the presence of PEST domains within SQSTM1, and UBA refers to the ubiquitin-binding ubiquitin-associated domain, between residues 387 and 436 of the 440 amino-acid residue protein. (**B**) Schematic representation of the 5q35-linked PDB SQSTM1 UBA domain mutations. Five of the PDB mutations cause amino acid substitutions (P387L, P392L, M404V, G411S, and G425R) in the SQSTM1 UBA domain. An insertion mutation generates a 395-residue protein lacking most of the UBA domain (E396X), and three other mutations lead to frameshifts, which generate proteins with truncated UBA domains (390 and 393 residues; two different mutations generate the latter). "Helix" and "loop" indicate the positions of alpha-helix and connecting loop regions, respectively, of the three-helix bundle UBA domain, as determined by protein NMR [78].

SQSTM1 acts as a scaffold in a number of signaling pathways, which led to activation of NFκB. Through stimulation-dependent interaction with TRAF6, SQSTM1 is able to recruit atypical protein kinase C (aPKC) proteins, which regulate NFκB activation via phosphorylation of IκB kinase ß (IKKß) [36], to interleukin-1 (IL-1) and nerve growth factor (NGF)–signaling complexes [37, 38]. Similarly, SQSTM1 also binds the scaffold protein RIP (receptor interacting protein), recruiting aPKCs to tumor necrosis factor-alpha (TNFα)–signaling complexes [39]. According, in these cases depletion of SQSTM1 severely inhibits NFκB signaling [37–39]. NFκB activity is central to osteoclastogenesis, and may be required for mature osteoclast homeostasis (reviewed in [40]). Significantly, RANK is a member of the TNF receptor family [41] that directly interacts with and requires TRAF6 for activation of NFκB signaling [42], and TRAF6-deficient mice lack osteoclasts or show defective osteoclast function [43, 44]. SQSTM1 and RANK are, therefore, likely to be common components of a TRAF6-dependent signaling pathway that regulates bone cell function via NFκB activity [45, 46] (Fig. 2). Because the *RANK* mutations activate NFκB signaling [18] and phenotypic similarities with PDB are evident, it is feasible that *SQSTM1* mutations will be found to have similar functional effects [30, 31]. Indeed, recent observations that genetic inactivation of *SQSTM1* in mice leads to impaired osteoclastogenesis and inhibition of IKK activation, and that ectopic expression of a P392L-SQSTM1 construct in cultured cells activates NFκB more efficiently than the wild type construct [46], are supportive of this proposal.

Structure of the SQSTM1 Protein

SQSTM1 is a 440-amino-acid-residue protein consisting of several well-characterized motifs and sequences that mediate protein–protein interactions (Fig. 1A).

PB1 Domain

The N-terminus (~80 residues) of the SQSTM1 protein contains a PB1 (Phox and Bem1) domain through which the protein is able to bind corresponding PB1 domains in aPKCs [47, 48]. In addition, the PB1 domain of SQSTM1 mediates interactions with other PB1 domain–containing proteins including the mitogen-activated protein kinase MEK5 (an upstream activator of ERK5)

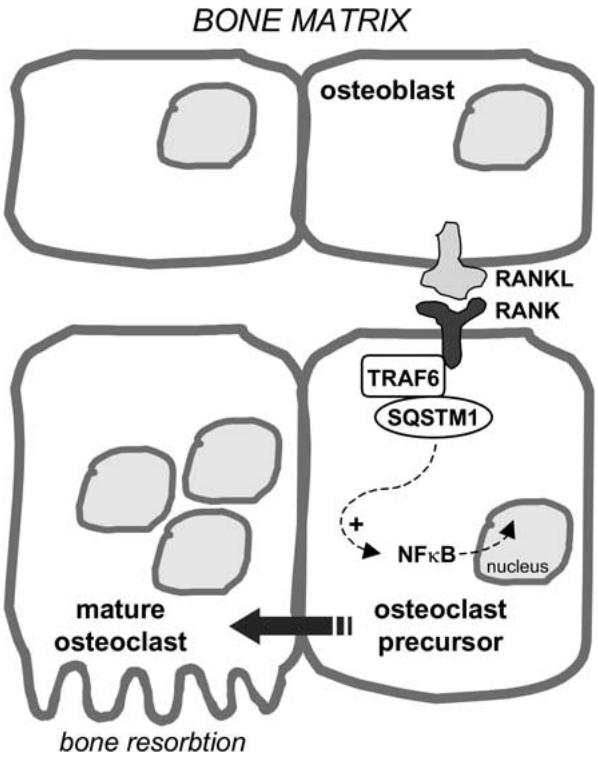

Fig. 2. RANK and SQSTM1 are proposed to be common components of a TRAF6-dependent NFκB signaling pathway that is central to osteoclastogenesis, and may be required for mature osteoclast homeostasis.

as well as NBR1 (next to breast cancer 1) [49, 50], the latter of which shows a domain organization similar to that of SQSTM1. Through its PB1 domain, SQSTM1 is able to assemble into homotypic complexes in so called "front-to-back" arrays [48–50], which may be related to the ability of the protein to form "sequestosomes" (hence, the name sequestosome 1 or SQSTM1) [51]. These are punctate cellular aggregates that are probably functionally related to the SQSTM1- and ubiquitin-positive inclusions that characterize certain human degenerative conditions [52, 53]. The extreme N-terminus of the SQSTM1 protein also binds the Src homology 2 (SH2) domain of $p56^{lck}$ [54], although in this case the interaction is not based on PB1–PB1 domain interactions.

ZZ Domain

The ZZ domain of SQSTM1 is a novel zinc finger motif [55] between residues 128 and 163 of the protein, which is critical for the interaction of SQSTM1 with RIP [39], as it links aPKCs to TNFα signaling complexes.

TRAF6-Binding Motif

The binding of SQSTM1 to TRAF6 is stimulation dependent [37, 46] and is mediated by a region of SQSTM1 C-terminal of the RIP binding site (the interaction is abrogated by deletion of residues 225–251) allowing SQSTM1 to bind TRAF6 and RIP simultaneously, thereby consistent with SQSTM1's role as a scaffold protein in NFκB signaling.

PEST Sequences

SQSTM1 contains two PEST sequences, regions of the protein that are rich in the amino acids proline (P), glutamic acid (E), serine (S), and threonine (T). PEST sequences occur in proteins that are targets of ubiquitin-dependent proteolysis (see subsequent text) [56], and cell stress associated with arsenite or arsenate is reported to lead to the accumulation of ubiquitylated forms of SQSTM1 in cultured osteoblasts [57].

UBA Domain

The C-terminus of the SQSTM1 protein (residues 387–436) contains a UBA domain, and, as noted previously, all nine of the SQSTM1 5q35-linked PDB mutations identified to date affect this region of the protein (Fig. 1B). This observation, combined with the fact that the mutations show phenotypic similarities, is consistent with the proposal that *SQSTM1* mutations may cause a common loss or gain of function that is predisposing to PDB. In order to understand how *SQSTM1* mutations relate to disease progression, it is, therefore, vitally important to understand the functional significance of the presence of the UBA domain within the protein, and, further, the functional consequences of the mutations.

UBA domains are short (~50 residue) sequences that are present in enzymes of the ubiquitin conjugation and deconjugation pathway [58], as well as in proteins that are regulators of ubiquitin-dependent proteolysis or other ubiquitin-mediated processes (reviewed in [59]). Ubiquitin is a small (~8.5 kDa) protein that controls a variety of cellular processes by the posttranslational covalent modification (ubiquitylation) of other proteins (Fig. 3). Ubiquitin forms isopeptide linkages via its C-terminal glycine76 (G76) residue to lysine (K) side chains in its targets as a result of sequential actions of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligase (E3) enzymes (reviewed in [60]), with the E3s conferring substrate recognition. In addition, family of deubiquitylating enzymes exists that reverse the actions of ubiquitylation [61]. The attachment of multi-ubiquitin chains, with individual ubiquitins in the chain linked via K48 (or in rare cases K29), signals ATP-dependent proteolysis of the target protein by the 26S proteasome complex [62–64]. In contrast, conjugation of multi-ubiquitin chains linked via K63 does not signal protein degradation but instead regulating processes including endocytosis [65], DNA repair [66], and

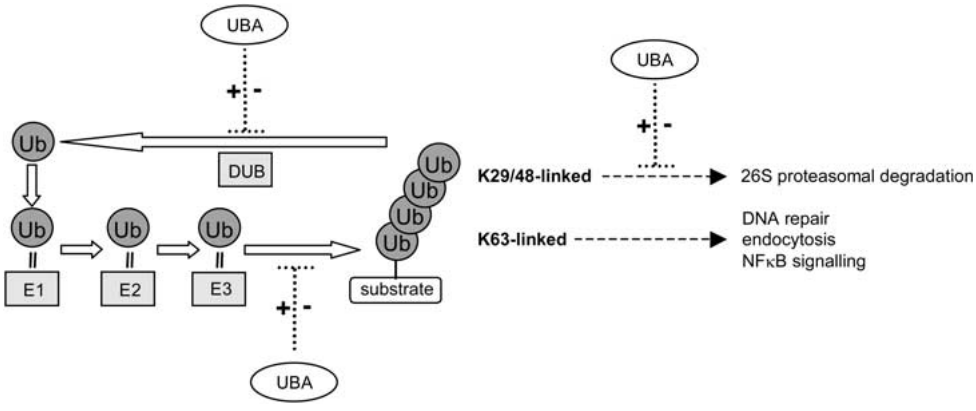

Fig. 3. Functions of protein ubiquitylation. Substrate proteins are ubiquitylated (covalently conjugated with ubiquitin [Ub]) by the sequential actions of ubiquitin-activating (E1), -conjugating (E2) and -ligase (E3) enzymes, a modification reversed by deubiquitylating (DUB) enzymes. K29- and K48-linked multi-ubiquitin chains signal substrate degradation by the 26S proteasome. Conjugation of K63-linked multi-ubiquitin chains signals nondegradative processes. Some E2 and DUB enzymes contain UBA domains. In addition, other UBA domain proteins may act as positive (+) or negative (−) regulators of ubiquitin chain assembly/disassembly, or of delivery of ubiquitylated substrates to the 26S proteasome complex.

activation of NFκB signaling [67, 68]. The latter is mediated by the multiple auto-ubiquitylation of TRAF6, which itself is a so-called RING (really interesting new gene) domain protein, and like other RING domain proteins has intrinsic E3 ubiquitin-ligase activity. In this case, assembly of a K63-linked multi-ubiquitin chain on TRAF6 is required for TAK1 (a mitogen-activated protein kinase kinase kinase)–mediated phosphorylation and activation of IKKß by IL-1ß and other proinflammatory cytokines [67, 68]. TAK1 is also a component of a complex that includes RANK and TRAF6 [69], suggesting that K63-linked ubiquitylation of TRAF6 may be a mechanism that regulates RANK signaling (Fig. 4). Interestingly, in addition to the ubiquitylation of TRAF6 that positively regulates NFκB signaling, deubiquitylation of TRAF6 may also in some cases be used as a mechanism to negatively regulate NFκB signaling [70, 71].

All of the UBA domain proteins characterized to date appear to act as regulators of the turnover of multi-ubiquitylated proteins, which is achieved through direct noncovalent high-affinity binding of the UBA domains with multi-ubiquitin chains [72–75] although some UBA domains also apparently interact with monomeric ubiquitin [73], the physiological relevance of which is unclear. Consistent with these observations, the UBA domain of SQSTM1 [76, 77] is reported to mediate interaction with both multi-ubiquitin chains [51, 78] and monomeric ubiquitin [79]. Proposed mechanisms of regulation of ubiquitin-dependent proteolysis by UBA domain proteins include delivery of multi-ubiquitylated targets to the 26S proteasome [80], sequestration of multi-ubiquitylated targets precluding proteasomal proteolysis [81], and regulation of ubiquitin chain assembly [74] or disassembly [82].

Functional Significance of the SQSTM1 UBA Domain

The precise functional significance of the SQSTM1 UBA domain is currently unclear, however possible *in vivo* roles are considered below.

Regulation of Ubiquitin-Dependent Proteolysis

The SQSTM1 protein, *via* its UBA domain, is able to bind K48-linked multi-ubiquitin chains [51, 78] which, as noted previously, are proteasomal degradation signals when attached to substrate proteins [64]. SQSTM1 is reported to bind multi-ubiquitylated proteins that accumulate when the proteasome degradative function is impaired [51, 83], and in cultured cells treated with proteasome inhibitors SQSTM1 is able to sequester ubiquitylated substrates into "sequestosomes" [51]. In this regard, SQSTM1 may function like some other UBA domain proteins as a negative-regulator of ubiquitin-dependent proteolysis, by preventing the delivery of ubiquitylated substrates to the 26S proteasome [81]. Such substrates could include other components of the osteoclast NFκB signaling pathway, including TRAF6, which under certain conditions, e.g., following exposure of cells to interferon-gamma [84], is subject to proteasomal degradation (presumably in this case mediated by K48-linked multi-ubiquitylation).

Regulation of TRAF6 K63-Linked Ubiquitylation

Although the temporal relationship between TRAF6 ubiquitylation and SQSTM1 binding is not clearly defined, the combined observations that the TRAF6-SQSTM1 interaction is stimulation dependent and that the K63-linked multi-ubiquitylation of TRAF6 occurs

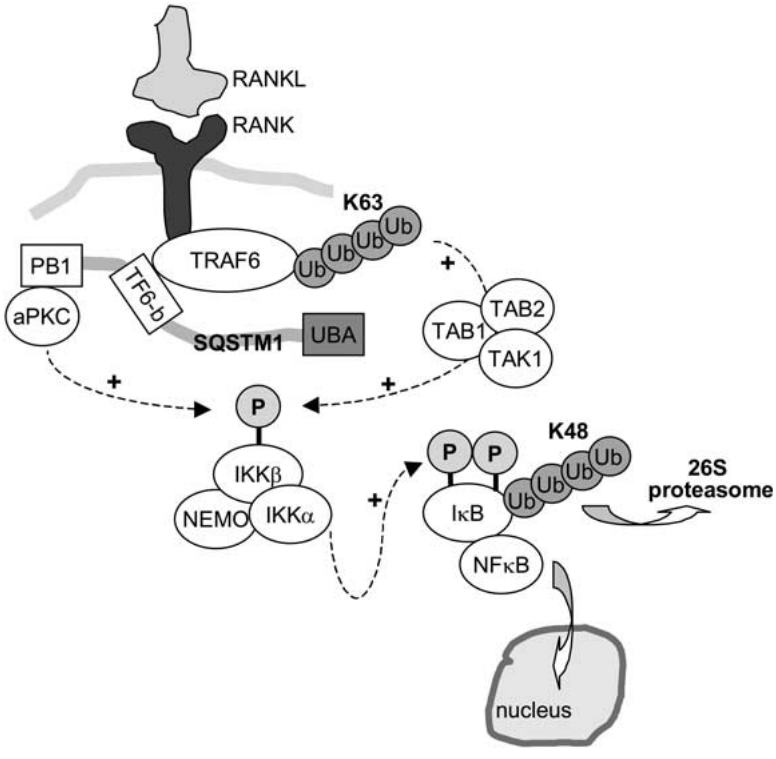

Fig. 4. Proposed RANK-TRAF6-SQSTM1 signaling cascade. TRAF6 directly interacts with RANK and both proteins can form a complex which includes TAK1 and TAB2. Receptor stimulation by RANKL leads to association of TRAF6 with RANK, and (we propose) to the subsequent K63-linked polyubiquitylation of TRAF6. An alternative degradative pathway mediated by K48-linked polyubiquitylation of TRAF6 also exists (not shown). Stimulation-dependent binding of SQSTM1 to TRAF6 is mediated by a binding site (TF6-b) distinct from the SQSTM1 UBA domain and may precede TRAF6 ubiquitylation Through its PB1 domain, SQSTM1 is able to recruit aPKCs to the signaling complex, which can regulate NFκB activation *via* phosphorylation of IκB kinase ß (IKKß). K63-linked ubiquitylation of TRAF6 promotes TAK1 kinase–mediated phosphorylation and activation of IKKß (as is the case of IL-1ß signaling). The activated IKK complex phosphorylates IκB leading to K48-linked polyubiquitylation of IκB and its subsequent 26S proteasomal degradation, allowing NFκB to enter the nucleus and activate target genes.

upon receptor stimulation [67, 68], is consistent with a model in which the UBA domain of SQSTM1 mediates recruitment of the protein along with aPKCs to signaling complexes *via* binding to the TRAF6 ubiquitin chain. However, the TRAF6-binding region of SQSTM1 (residues 225–251) is some distance from the UBA domain (residues 387–436), suggesting that the latter is not directly involved in the interaction. Although there is no evidence that SQSTM1 is directly ubiquitylated to mediate TRAF6-NFκB signaling, it is feasible that SQSTM1 could interact noncovalently, via its UBA domain, to regulate ubiquitylation of other signaling proteins. For example, binding of SQSTM1 to nonubiquitylated TRAF6 could allow SQSTM1 to subsequently use its UBA domain to regulate K63-linked ubiquitin chain assembly or disassembly [74, 82] on TRAF6. Subtle alterations in the length of the K63-linked chain length could be used to regulate TAK1 activity, in the same way that K48-linked chain length determines the affinity of substrate binding (and degradation) by the 26S proteasome [64]. Such a model is supported by the recent observations that the deubiquitylating enzyme CYLD (a tumor suppressor that is mutated in familial cylindromatosis) is able to deubiquitylate K63-linked ubiquitylated TRAF6 leading to negative regulation of TNF-dependent NFκB signaling [70, 71], although the role of CYLD in RANK signaling has not been investigated. It is also noteworthy that the TAB2 (TAK1-binding protein 2) protein, which links TAK1 to TRAF6 [85], contains a putative ubiquitin-binding CUE domain (coupling of ubiquitin conjugation to ER degradation domains are closely related to UBA domains), but like SQSTM1, the region of TAB2 that binds TRAF6 does not include this ubiquitin-binding element.

Cellular Inclusion Formation

As already discussed, under certain experimental paradigms SQSTM1 is able to sequester ubiquitylated substrates into cellular inclusions, termed 'sequestosomes' [51], which may be equivalent to "aggresomes" and functionally related to SQSTM1-positive neuronal inclusions in human neurodegenerative [52] and liver disorders [53]. In Alzheimer's disease, SQSTM1 is an early component of neurofibrillary tangles, suggesting that the protein may play a role in the maturation and recruitment of ubiquitylated proteins to these lesions [52]. It may be significant that osteoclasts from pagetic tissue show characteristic nuclear and cytoplasmic inclusions [86], although it is not know if these lesions also contain the SQSTM1 protein and/or ubiquitin. Interestingly in some models of neurological disorders (polyglutamine diseases), ubiquitin-positive nuclear inclusions appear to be neuroprotective [87, 88]. It is therefore, possible that SQSTM1-mediated inclusion formation is part of a normal osteoclastic response to cell stress. Indeed recent studies have shown that a functional UBA domain is required for SQSTM1-dependent nuclear inclusion formation in a cell-based

model, although whether the presence of inclusions in this case correlated with cell survival was not addressed [89].

Interactions with Non ubiquitylated Proteins

Non ubiquitylated binding partners have to date not been identified for the SQSTM1 UBA domain; however, a number of such protein interactions have been described for other UBA domains. These include interactions between the UBA domains of hHR23A and the human immunodeficiency virus Vpr protein [90] and 3-methyladenine-DNA glycosylase [91]; the PLIC-1 UBA domain and the α1 subunit of the $GABA_A$ receptor [92]; the hPLIC-1 UBA domain and presenilins 1 and 2 [93]; and the hPLIC-2 UBA domain and the 26S protesome [94]. In addition, there are also reports of UBA domain hetero- and homodimerization [95]. It seems likely therefore that SQSTM1 UBA domain–binding proteins could also exist, which may be related to osteoclast activity. Such interactions might, for example, serve to recruit other proteins to SQSTM1 signaling complexes.

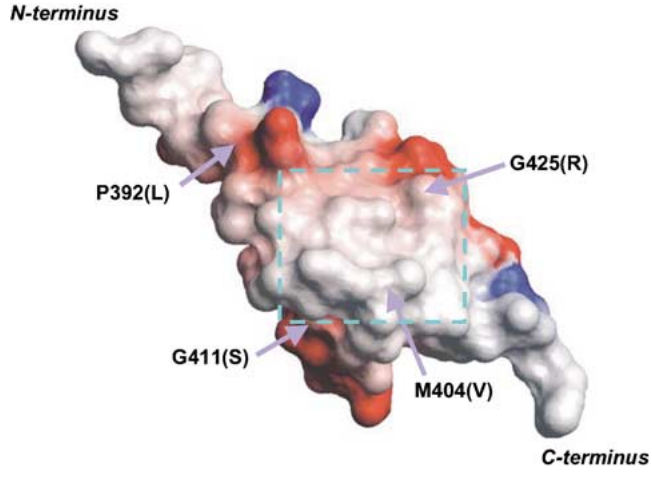

Fig. 5. Surface representation of the SQSTM1 UBA domain (residues 387–436), derived from the three-dimensional structure of the isolated SQSTM1 UBA domain that was recently determined by protein NMR [78]. The boxed area indicates the hydrophobic patch (white) that is implicated in ubiquitin-binding. The M404 and G425 residues, which when mutated to V and R, respectively, ablate ubiquitin-chain binding and are involved in this surface patch. P392 and G411 are located outside of the ubiquitin-binding surface, and mutations (to L and S, respectively) do not affect ubiquitin-chain binding.

Consequences of PDB Mutations

Considering the proposed functions of the SQSTM1 UBA domain described previously, the PDB mutations could impinge on a number of (perhaps related) cellular processes relevant to disease pathogenesis such as cell signaling, inclusion formation, or intracellular proteolysis. Certainly defective ubiquitin-dependent proteolysis can directly cause or contribute to major human diseases [96], and cellular inclusion formation is relevant to cell death/survival in diverse disease states [97]. In addition, defective NFκB signaling can clearly lead to PDB-like phenotypes [18, 20, 24, 25], and preliminary evidence suggests (at least the most common P392L) SQSTM1 PDB mutation(s) may indeed lead to activation of NFκB signaling [46].

The consequences of the PDB-associated mutations on SQSTM1 function are only just beginning to be fully addressed, and the emerging picture is already a complex one. The *SQSTM1* truncating mutations remove the majority of the UBA domain in the gene product [78] and completely ablate multi-ubiquitin chain-binding (R.L., unpublished observations). In contrast, the missense mutations analyzed to date can be resolved into those that retain (P392L, G411S) or cause loss (M404V, G425R) of K48-linked multi-ubiquitin chain-binding, at least in the isolated UBA domain [33, 78], although the P392L substitution does lead to localized modification of secondary structure [78]. The loss of ubiquitin-binding in the M404V and G425R mutants can be rationalized with reference to the three-dimensional structure of the isolated SQSTM1 UBA domain, which was recently determined by protein NMR [78]. Although neither of these substitutions affect overall folding of the UBA domain, both involve residues on the surface patch of the domain that has been implicated in ubiquitin-binding [33, 78] (Fig. 5). There is however no obvious correlation between the ability of the mutant UBA domains to bind ubiquitin chains and disease extent, although patients with truncating mutations have apparently more extensive PDB than those with missense mutations [33]. Consequently, despite the clustering of all of the PDB mutations in the UBA domain of the SQSTM1 protein, a simple model in which different mutations cause a common loss of ubiquitin chain-binding that predisposes to disease, is not apparent.

An important caveat is that functional (i.e., ubiquitin-binding) analyses of the wild type and mutant SQSTM1 UBA domains have so far been conducted using only the UBA domains in isolation (rather than in the context of the holoprotein) and with K48-linked unanchored (i.e., not attached to substrate proteins) multi-ubiquitin chains [33, 78]. Thus, in the context of the holoprotein, the ubiquitin-binding properties of the UBA domain may be different (in the same way that the ubiquitin-binding interface in the dimeric CUE domain of the Vps9p protein involves residues that are not required for ubiquitin-binding in the isolated monomer [98]) such that the P392L and G411S mutations may in fact affect ubiquitin-binding. It is also possible that the SQSTM1 UBA domain could, in addition to K48-linked ubiquitin chains, recognize multi-ubiquitin chains linked

through K63, and that all the missense mutations cause selective loss of binding of K63-linked chains or other ubiquitin oligomers. In addition, a model is feasible in which individual ubiquitylated proteins bind the SQSTM1 UBA domain at two sites, an ubiquitin-binding and a substrate-binding site, the latter of which could include P392 and G411. In this regard, PDB missense mutations in either of the sites could be sufficient to perturb protein–protein interactions required for normal osteoclast function. Alternatively, the SQSTM1 UBA domain may serve as a scaffold between ubiquitylated and nonubiquitylated proteins, with the M404V/G425R mutations peturbuing the former and P392L/G411S mutations the latter interactions. Finally, all of the missense mutations could simply cause a common loss of binding of a nonubiquitylated protein. Other formal possibilities include generalized effects of the mutations on SQSTM1 protein stability or localization. A final important consideration is that the SQSTM1 mutations are heterozygous, and the SQSTM1 protein is able to form homotypic complexes [48–50], which may be functionally relevant; entrapment of wild type subunits into unstable or inactive complexes is also, therefore, possible.

Concluding Remarks

In summary, UBA domain–specific mutations in the SQSTM1 protein are clearly important in predisposing to 5q35-linked PDB, although their mechanism of action requires clarification. Addressing the broader functional significance and consequences of SQSTM1 UBA domain mutations in cell and animal models—and in particular determining if (and how) the mutations cause defective ubiquitin-dependent proteolysis, or like *RANK* mutations, lead to activation of NFκB signaling—should direct future advances in our understanding of the molecular pathogenesis of PDB and related rheumatic conditions.

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