

Autophagic receptors Nbr1 and p62 coregulate skeletal remodeling

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Skeletal remodeling is an ongoing process requiring the coordinated action of different cell types to maintain homeostatic control of bone synthesis and degradation. Mutations in p62/SQSTM1 are associated with sporadic and 5q35-linked Paget Disease of Bone (PDB), characterized by focal increased bone turnover. These mutations cluster in the ubiquitin associated (UBA) domain and are thought to lead to enhancement of NFκB pathway activation involved in osteoclastogenesis and hyper-responsiveness to receptor activator of nuclear factor-κB ligand (RANKL). The structurally similar selective autophagic receptor, Nbr1, binds to LC3 and p62 and is sequestered into autophagosomes, whereas it accumulates in autophagic-deficient tissues. We have shown that truncation of Nbr1 in a murine model, where it can still interact with p62 but not LC3, leads to increased osteoblast differentiation and activity in vivo. This results in an age-dependent increase in bone mass and bone mineral density. This is a molecular consequence of loss of autophagy receptor function via deletion of its C-terminal UBA domain, and/or modulation of the p38 MAPK cellular signaling pathway.

modulation and bone remodeling through NFκB during osteoclastogenesis. p62 also has far-reaching roles in regulation of apoptosis, innate immunity and cancer. Less is known about the function of the structurally similar protein Nbr1, which interacts with the N-terminus of p62 via a common oligomerization motif (PB1). Nbr1 also shares a ubiquitin-associated domain (UBA) and interacts with the key autophagic membrane component LC3.

Bone remodeling involves a complex interplay between the activity of osteoblasts, which produce extracellular matrix and are responsible for its mineralization, multinucleated osteoclasts, which resorb mineralized bone and osteocytes, terminally differentiated long-lived master regulators of bone homeostasis. PDB is caused by aberrant osteoclastogenesis, and it is associated with rapid bone remodeling, leading to focal areas of increased bone formation that are deformed, weak and liable to fracture. The molecular mechanism whereby p62 mutations are proposed to lead to PDB involves regulation of signal-dependent ubiquitination/deubiquitination of TRAF6 via binding to the p62 UBA domain and downstream deregulation of NFκB signaling leading to increased osteoclast formation.

We identified Nbr1 as a regulator of osteoblastic bone formation through creation of a knock-in mouse model expressing a truncated form of Nbr1. The truncated polypeptide encoding the first 135 amino acids of Nbr1 (trNbr1), including the p62-Nbr1 PB1 interface, but lacking the Nbr1 homodimerization (CC), LC3 interaction (LIR1/2) and the C-terminal (UBA) ubiquitin binding domains, was expressed at equivalent levels to wild-type

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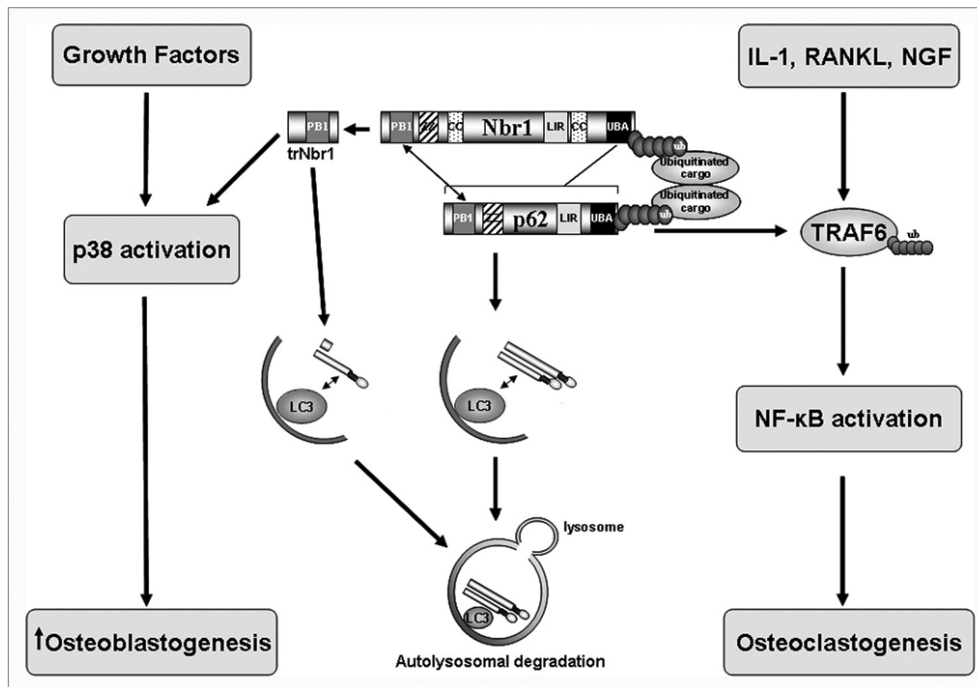


Figure 1. A model for the regulation of bone remodeling and autophagic protein degradation by Nbr1 and p62. Truncation of Nbr1 causes an increase in p38 MAPK activation and osteoblast differentiation. p62 acts to regulate bone remodeling by activating the NF- κ B pathway in response to RANKL in osteoclasts. Nbr1 and p62, via their interaction with LC3 also act as autophagic receptors for the degradation of ubiquitinated proteins. The UBA domain of Nbr1 interacts with p62 and may act to target p62 for degradation. Truncated Nbr1 (trNbr1) is also present in autophagosomes presumably via its interaction with p62. PB1, Phox and Bem1p domain; ZZ, zinc binding domain; CC, coiled-coiled domain; UBA, ubiquitin-associated domain; LIR, LC3-interacting region.

protein. The truncated Nbr1 homozygous mouse ($Nbr1^{tr/tr}$) shows a significant age-dependent increase in bone mass driven by elevated osteoblast differentiation and mineralization activity. Osteoblast and osteoclast activity are tightly linked, as osteoblasts secrete RANKL to stimulate osteoclastogenesis, which ensures control of bone turnover and formation. We see an early concomitant increase of in vivo bone surface covered by osteoclasts but this is not maintained, nor is there an increase in osteoclastogenesis. The $Nbr1^{tr/tr}$ -derived osteoclasts show no differences in differentiation potential or bone resorption capacity compared to wild-type osteoclasts ex vivo. Together, this suggests uncoupling of the osteoclast-osteoblast homeostatic regulation of bone remodeling in $Nbr1^{tr/tr}$ mice.

To determine the molecular mechanism behind this increase in bone formation, we analyzed molecular signaling pathways that were likely candidates to be misregulated by Nbr1 truncation. We show that both ERK1/2 and p38 MAPK signaling are increased in $Nbr1^{tr/tr}$ osteoclast

precursor cells in response to RANKL stimulation. p38 MAPK activity is also elevated in ex vivo osteoblast cultures from $Nbr1^{tr/tr}$ mice compared to controls. We also show, by co-immunoprecipitation in transient transfection assays, that Nbr1 and p38 MAPK are part of an interactome upon p38 MAPK activation, but that the truncated Nbr1 protein (trNbr1) does not form a complex with active or inactive p38 MAPK, suggesting the increased bone mass in $Nbr1^{tr/tr}$ mice may be due to misregulation of p38 MAPK activity. Finally we are able to rescue the ex vivo increased osteoblast differentiation and mineralization phenotype by culture in the presence of the p38 MAPK-specific inhibitor SB203580.

Despite loss of the Nbr1 LIR1 and LIR2 domains through which Nbr1 binds the autophagic marker LC3, we show by confocal microscopy that endogenous trNbr1 protein still localizes to autophagosomes with p62 and LC3. We propose that this is probably through heterooligomerization with p62 via the retained PB1 domain. We also show that, despite

loss of the LC3 interaction motifs in the trNbr1 protein, trNbr1 is still degraded by autophagy, as protein levels increase upon bafilomycin A_1 (an inhibitor of lysosomal lumen acidification) treatment. We confirm that trNbr1 interacts with p62 through in vitro binding assays and identify a novel interaction of the Nbr1 UBA domain with p62. Steady state levels of p62 protein are also doubled in $Nbr1^{tr/tr}$ cells compared to wild type, suggesting that Nbr1 may regulate p62 turnover itself, by acting as a cargo through interaction via its UBA domain.

A number of age-related degenerative disorders such as insulin resistance, myocardial hypertrophy and neurological disorders (e.g., Huntington and Alzheimer diseases) share pathogenic mechanisms, including long-term increases in oxidative stress and failure of protein aggregate clearance by autophagy. Mutations in p97/VCP (vasolin-containing protein) cause inclusion body myopathy associated with PDB and frontotemporal dementia. This is thought to be caused by accumulation of autophagic structures and

ubiquitinated protein inclusions. Similarly the p62 knockout and Nbr1^{tr/tr} mice both have a mature onset phenotype with high bone mass, but primarily due to alterations in osteoclast and osteoblast activity, respectively (Fig. 1). The molecular identification of TRAF6 as a p62 ubiquitinated target has clarified its role in bone homeostasis, but elucidation of the ubiquitinated autophagic target of Nbr1 in osteoblasts will shed further light on the role of autophagy in bone remodeling.

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