Functional interaction between Sequestosome-1/p62 and Autophagy-Linked FYVE-containing protein WDFY3 in human osteoclasts

Running title: SQSTM1 and ALFY in human osteoclasts

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ABSTRACT

Paget's disease of bone (PDB) is a late-onset disorder characterised by focal areas of increased bone resorption, with osteoclasts that are increased in size, multinuclearity, number and activity. PDB-causing missense and nonsense variants in the gene encoding Sequestosome-1/p62 (SQSTM1) have been identified, all of which cluster in and around the ubiquitin-associated (UBA) domain of the protein. SQSTM1 is ubiquitously expressed and there is, as yet, no clear reason why these mutations only appear to cause an osteoclast-related phenotype.

Using co-immunoprecipitation and tandem mass spectrometry, we identified a novel interaction in human osteoclast-like cells between SQSTM1 and Autophagy-Linked FYVE domain-containing protein (ALFY/ WDFY3). Endogenous ALFY and SQSTM1 both localised within the nuclei of osteoclasts and their mononuclear precursors. When osteoclasts were starved to induce autophagy, SQSTM1 and ALFY relocated to the cytoplasm where they formed large aggregates, with cytoplasmic relocalisation appearing more rapid in mature osteoclasts than in precursors in the same culture. Overexpression of wild-type SQSTM1 in HEK293 cells also resulted in the formation of cytoplasmic aggregates containing SQSTM1 and endogenous ALFY, as did overexpression of a PDB-causing missense mutant form of SQSTM1, indicating that this mutation does not impair the formation of SQSTM1- and ALFY-containing aggregates.

Expression of ALFY in bone cells has not previously been reported, and the process of autophagy has not been studied with respect to osteoclast activity. We have identified a functional interaction between SQSTM1 and ALFY in osteoclasts under conditions of cell

stress. The difference in response to starvation between mature osteoclasts and their precursors may begin to explain the cell-specific functional effects of SQSTM1 mutations in PDB.

KEYWORDS

Sequestosome-1

Autophagy-linked FYVE domain protein

WD repeat and FYVE domain containing 3

Paget’s disease of bone

Osteoclast

Autophagy

ABBREVIATIONS

ALFY	Autophagy-linked FYVE domain-containing protein
hOCL	Human osteoclast-like cells
PDB	Paget’s disease of bone
SQSTM1	Sequestosome-1
WDFY3	WD repeat and FYVE domain containing 3

INTRODUCTION

Inherited mutations in the gene encoding Sequestosome-1 (SQSTM1, also known as p62) have been described among individuals affected with late-onset Paget's disease of bone (PDB) (reviewed in [1]). PDB is a common metabolic bone disorder that affects around 3% Caucasians, typically diagnosed in the fifth or sixth decade, and is characterised by focal lesions of increased bone turnover [2]. Within these lesions, bone-resorbing osteoclasts are increased in size, number, multinuclearity and activity. Bone-forming osteoblasts are subsequently over-activated, and the bone deposited is disorganised, weak and prone to fracture.

SQSTM1 is ubiquitously expressed and functions as a scaffold protein to integrate and diversify signals from multiple receptors, including tumour necrosis factor receptor family members, interleukin receptors, and nerve growth factor receptors (reviewed in [3]). A role for SQSTM1 in sequestration of protein aggregates prior to their degradation by autophagy has also been described [4]. Various PDB-causing mutations in SQSTM1 have been identified, all of which cluster in and around the C-terminal ubiquitin-associated (UBA) domain. The most common mutation identified thus far results in heterozygous substitution of a proline residue at codon 392 with a leucine (P392L), and is found in around one-quarter of PDB cases examined [5].

PDB is caused by germline mutations, yet disease onset and severity are regulated both temporally (usually late-onset) and spatially (causing specific lesions within the skeleton). It has been proposed that these may be regulated by environmental factors such as viral infection (reviewed in [6]), although this remains controversial [7]. The aim of this study

therefore was to identify proteins within osteoclasts that interact with SQSTM1 and that may account for the apparent cell-specific effects of PDB-causing mutations in *SQSTM1*.

MATERIALS AND METHODS

Cell Culture

With approval from the North of Scotland Research Ethics Committee (UK), human osteoclast-like (hOCL) cells were generated from peripheral blood mononuclear cells donated by health volunteers, as described previously [8]. Briefly, venous blood samples were collected into EDTA-coated Vacutainer tubes by venepuncture, and mononuclear cells isolated by density gradient centrifugation using Lymphoprep (Axis-Shield Diagnostics Ltd, UK) according to manufacturer's instructions. Cells were resuspended in αMEM (Sigma-Aldrich) containing 10% foetal calf serum, 2mM L-glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin and 20ng/ml recombinant human macrophage-colony stimulating factor (rhMCSF; R&D Systems) at $1x10^6$ cells/ml, seeded into $75cm^2$ flasks and incubated at 37°C, 5% CO_2 until cells reached ~80% confluence.

For co-immunoprecipitation analysis, culture medium in $75cm^2$ flasks was additionally supplemented with 100ng/ml recombinant human receptor activator of NFκB ligand (rhRANKL; Peprotech) and the culture continued until multinucleated osteoclast-like cells had formed. For immunohistochemical analysis, the M-CSF-dependent mononuclear cells in $75cm^2$ flasks were trypsinised and seeded onto serum-coated glass coverslips in 48-well plates at 40,000 cells per well in medium containing 20ng/ml rhMCSF and 100ng/ml rhRANKL, and incubated at 37°C, 5% CO_2. Once cells had fused to form multinucleated hOCLs, they were fixed in 4% paraformaldehyde.

Human embryonic kidney (HEK) 293 cell lines were obtained from the European Collection of Cell Cultures and maintained in αMEM containing 10% foetal calf serum, 2mM L-

glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin. For immunohistochemical analysis, cells in 75cm^2 flasks were trypsinised and seeded onto serum-coated glass coverslips in 48-well plates at 40,000 cells per well and incubated at 37°C, 5% CO_2 until they reached ~80% confluence.

Immunoprecipitation

Lysates were prepared from hOCLs in 75cm^2 flasks when the majority of cells had fused to form large, multinucleated cells. Cells were scraped on ice into cold lysis buffer consisting of 25mM HEPES pH7.5, 150mM sodium chloride, 1% v/v Triton, 25mM sodium fluoride, 1mM EDTA, 1mM sodium orthovanadate, 1% v/v mammalian protein inhibitor cocktail (Sigma) and 0.4% v/v phosphatase inhibitor cocktail (Sigma). The lysate was incubated with Protein-G Agarose beads (Calbiochem) to remove proteins that bound non-specifically to the beads. The cleared lysate was then incubated for one hour with guinea-pig N-terminal specific anti-SQSTM1 antibody (GP62N, Progen) and for a further hour with Protein-G Agarose beads. The beads were washed three times with lysis buffer then the bound proteins were eluted from the beads by incubation at 95°C for five minutes in reducing sample buffer.

Protein Identification

Eluted proteins, as well as proteins that bound non-specifically to Protein-G Agarose beads, unbound proteins (first wash) and total lysate were subjected to SDS-PAGE on 12% Bis-Tris HCl gel (Biorad) and visualised using GelCode Blue Stain Reagent (Pierce). Bands that appeared specifically among the eluted proteins and not in the non-specific binding or wash fractions were excised from the gel and subjected to in-gel trypsin digestion. Briefly, proteins were reduced with DTT (60°C, 20 min), S-alkylated with iodoacetamide (25°C, 10 min) then digested with trypsin (37°C, 8 h). The resulting tryptic peptide extract was dried by

rotary evaporation (SC110 Speedvac; Savant Instruments, USA) and dissolved in 0.1% formic acid for liquid chromatography-tandem mass spectrometry analysis.

Peptide solutions were analysed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd, UK) coupled to an UltiMate 3000 LC System (Dionex (UK) Ltd, UK). Peptides were separated on a Monolithic Capillary Column (200 μm i.d. x 5 cm; Dionex part no. 161409). Eluent A was 3% acetonitrile in water containing 0.05% formic acid; Eluent B was 80% acetonitrile in water containing 0.04% formic acid with a gradient of 3% - 45% Eluent B in 12 minutes at a flow rate of 2.5μL/min. Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode with a scan range of 300-1500 m/z, 3 averages, and up to 3 precursor ions selected from the MS scan (100-2200 m/z). Precursors were actively excluded within a 1.0 min window, and all singly charged ions were excluded.

Peptide peaks were detected and deconvoluted automatically using data analysis software (Bruker). Mass lists in the form of Mascot Generic Files were created automatically and used as the input for Mascot MS/MS Ions searches of the NCBInr database using the Matrix Science web server. The default search parameters used were: Enzyme = Trypsin, Max. Missed cleavages = 1; Fixed modifications = Carbamidomethyl (C); Variable modifications = Oxidation (M); Peptide tolerance ± 1.5 Da; MS/MS tolerance ± 0.5 Da; Peptide charge = 2+ and 3+; Instrument = ESI-TRAP. Any protein with an overall score above zero is listed; scores are a composite from individual peptide scores and are used to rank hits. Data from this study are deposited in PRIDE [9], accession number 13762, and were converted from Mascot Generic File format using PRIDE Converter [10] version 2.4.2.

Generation and transfection of FLAG-tagged SQSTM1 Plasmids

Plasmids containing wild-type or 392L SQSTM1 in pGEX4-T1 were kindly donated by Dr Rob Layfield, University of Nottingham and have been described previously [11]. N-terminal FLAG-tagged SQSTM1 variants were prepared by excising full-length SQSTM1 from pGEX4-T1 vectors using EcoRI and XhoI restriction sites then subcloning the insert into pCMV-Tag2B (Stratagene). Plasmids containing FLAG-tagged SQSTM1 were transformed into XL1-Blue Supercompetent Cells (Stratagene), grown overnight in 100ml LB-broth containing 50µg/ml kanamycin, and purified using Endotoxin-free Plasmid Midiprep kits (Qiagen) according to manufacturer's instructions. Inserts in plasmids were sequenced at the University of Dundee Sequencing Service using gene-specific primers (N-terminus: 5'- ACCAAGTCCCCGTCCTCATCGC-3' and C-terminus: 5'-CCCGTCTACAGGTGAACTCCAG-3') to ensure correct orientation and confirm presence of the expected SQSTM1 mutation.

HEK293 cells on glass coverslips were transfected with FLAG-SQSTM1 when they reached ~80% confluence. Cells were transfected with 250ng plasmid DNA (wild-type or P392L) using 3:1 FuGENE6 (Roche): plasmid. Following transfection, cells were incubated at 37°C, 5% CO_2 for 24 hours, then fixed in 4% paraformaldehyde.

Immunofluorescence Detection of SQSTM1 and ALFY

Fixed hOCLs or HEK293 cells on glass coverslips were permeabilised with 0.5% Triton X-100 and blocked with 10% FCS in PBS. Endogenous SQSTM1 was detected using 1:100 guinea-pig anti-p62, N-terminal-specific (Progen) in 5% FCS followed by 1:100 AlexaFluor® 594 goat anti-guinea pig IgG (Molecular Probes) in 5% FCS. Endogenous ALFY was detected with 1:100 rabbit anti-ALFY [12] in 5% FCS and 1:150 AlexaFluor® 488 goat anti-rabbit IgG (Molecular Probes) in 5% FCS. FLAG-tagged SQSTM1 was detected using 1:200

mouse anti-FLAG (Sigma) in 5% FCS followed by 1:150 AlexaFluor® 488 goat anti-mouse IgG (Molecular Probes) in 5% FCS. Nuclei were counterstained using TO-PRO®-3 iodide (Molecular Probes). Subcellular localisation was visualised by laser scanning confocal microscopy (LSM510 Meta, Zeiss). Images were captured using multi-track image capture for far red (633nm), red (543nm), and green (488nm) channels using the same acquisition parameters for all images. No image processing was performed.

Cell Starvation

hOCLs were starved of serum, growth factors and amino acids for various durations by culturing cells in Hanks Buffered Saline Solution (HBSS) as described previously [12]. Briefly, medium was removed and cells were washed three times with HBSS pre-warmed to 37°C before incubation in HBSS at 37°C for the specified duration. Following starvation, cells were fixed in 4% paraformaldehyde for immunohistochemical detection of SQSTM1 and ALFY as described above.

RESULTS

SQSTM1 functionally interacts with ALFY

Immunoprecipitation of p62 from human osteoclast lysates produced five bands of co-precipitated proteins that appeared to be bound specifically to p62 and which were not apparent in the non-specific binding fraction or in the unbound (first wash) fraction (Figure 1); these ranged in mass from ~30kDa to ~180kDa. Each of the five bands was excised and analysed separately to identify proteins present. For all bands, immunoglobulin heavy and light chains were identified, with protein scores ranging from 29-99. Bands 1 & 2 also contained Autophagy-linked FYVE domain protein (ALFY, also known as WD40- and FYVE-domain containing protein 3, WDFY3). The overall protein score for ALFY was 31 for both bands; protein coverage was 2%, and the predicted peptide fragments were unique to ALFY. No other proteins were identified for bands 1-5 with overall scores above zero.

ALFY is localised to the nuclei of human osteoclasts and their precursors

We used immunofluorescence staining to examine whether endogenous ALFY was present in hOCLs and whether it colocalised with endogenous SQSTM1 (Figure 2). ALFY was detectable as weak staining throughout the nuclei of multinucleated hOCL and in mononuclear precursors, with intense perinuclear staining. Staining of SQSTM1 was weak and diffuse in the cytoplasm of mature hOCLs and precursors, with more intense staining throughout the nuclei. Colocalisation of SQSTM1 and ALFY was not apparent under normal culture conditions.

SQSTM1 and ALFY relocalise into cytoplasmic aggregates upon starvation

hOCL cultures were starved for 0-120 minutes in HBSS and immunostained for endogenous ALFY and SQSTM1 (Figure 3). In unstarved cells, SQSTM1 and ALFY localised predominantly to the nucleus and perinuclear region in both mononuclear and multinucleated cells, as described above (Figure 2). After 60 minutes of starvation, the abundance of nuclear SQSTM1 and ALFY appeared to increase, particularly in multinucleated osteoclasts. By 90 minutes of starvation, SQSTM1 and ALFY staining was evident in the cytoplasm as well as the nucleus of osteoclasts, but was still present only in the nuclei of mononuclear precursor cells. After 120 minutes of starvation, ALFY and SQSTM1 colocalised in cytoplasmic aggregates in osteoclasts but not precursor cells; at this time, cytoplasmic ALFY staining was first evident in mononuclear precursor cells. Multinucleated cells thus appeared to respond more rapidly than mononuclear cells in the same culture to amino acid starvation, in terms of ALFY relocalisation to the cytoplasm (90' for hOCL versus 120' for precursors) and aggregate formation (present at 120' in hOCL but not precursors).

SQSTM1 and ALFY colocalisation in cytoplasmic aggregates is not affected by the PDB-causing P392L mutation in SQSTM1

Wild-type (392P) and Pagetic mutant (392L) SQSTM1 were transiently overexpressed in HEK293 cells. In untransfected cells, endogenous ALFY displayed typical perinuclear localisation. In cells transfected with wild-type FLAG-SQSTM1, endogenous ALFY was detected in the cytoplasm, where it colocalised with FLAG-SQSTM1 in cytoplasmic aggregates. This colocalisation occurred in the absence of cell starvation. Similarly, in cells transfected with 392L-FLAG-SQSTM1, SQSTM1 colocalised with endogenous ALFY in cytoplasmic aggregates (Figure 4).

DISCUSSION

ALFY is a recently-described evolutionarily conserved protein involved in autophagic degradation of aggregated proteins [12]. We have shown for the first time that this protein is expressed in human osteoclasts and their precursors. In these cells, ALFY translocates rapidly from a perinuclear localisation to form cytoplasmic aggregates with SQSTM1 upon amino acid starvation. Similar localisation of ALFY has been reported previously in HeLa cells [12].

A sequence comparison of the proteins in human osteoclasts that precipitated with SQSTM1 identified peptides matching ALFY in two separate molecular mass bands between ~100-180kDa. An antibody specific to residues 2969-2984 and 3302-3316 in the full-length ALFY protein was reported previously to detect two bands of ~400kDa and ~150kDa kDa by Western blot analysis of HeLa cells [12], and three transcript variants for ALFY are reported in Entrez Gene [13] with predicted molecular masses of 395kDa, 90kDa and 32kDa. It therefore remains to be investigated which isoforms of ALFY are present and functional in osteoclasts.

We initially identified an interaction in osteoclasts between ALFY and SQSTM1 by coimmunoprecipitation from cell lysates. However, by immunofluorescence staining, these proteins only appeared to colocalise in the cytoplasm under conditions of cell stress (starvation) and did not colocalise in the nuclei of osteoclasts under normal culture conditions. It is therefore possible that we identified this interaction between ALFY and SQSTM1 by inducing the stress response during the process of preparing the cultures for lysis and immunoprecipitation.

In this study, we showed that overexpression of wild-type SQSTM1 in HEK293 cells results in the formation of cytoplasmic aggregates of SQSTM1 that colocalise with ALFY, similar to the aggregate formation observed in hOCL upon amino acid starvation. Further, we showed that this aggregate formation and colocalisation was not impaired by the PDB-causing SQSTM1 UBA domain 392L mutation. Overexpression of wild-type and mutated forms of SQSTM1 (including 392L) in HEK293 cells has been reported previously to result in the formation of SQSTM1-containing cytoplasmic aggregates, with mutated SQSTM1 forming larger aggregates than wild-type SQSTM1 [14]. We did not observe larger aggregate formation in cells expressing 392L-SQSTM1 compared to wild-type, although this could be because we used smaller amounts of plasmid DNA and examined cells at 24 hours after transfection rather than after 48 hours as per the previous study.

During preparation of this manuscript, it was reported that SQSTM1 and ALFY interact to organise misfolded, ubiquitinated proteins into aggregates that become degraded by autophagy [15], with SQSTM1 responsible for shuttling of ALFY from the nucleus into the cytoplasm. Interestingly, deletion of the entire UBA domain of SQSTM1 retained the nucleo-cytoplasmic shuttling of ALFY but prevented formation of SQSTM- and ALFY-containing aggregates [15], suggesting that an intact UBA domain of SQSTM1 is required for recruitment of ALFY to cytoplasmic aggregates. We have now shown that this recruitment is not impaired by the presence of the PDB-causing 392L mutation in the UBA domain of SQSTM1. The interaction between SQSTM1 and ALFY has since also been shown to be important for the selective autophagic degradation of misfolded proteins rather than for bulk autophagic degradation in response to cell stress [16]. It is likely that the mutated SQSTM1 found in PDB accumulates in aggregates as the misfolded *target* of selective degradation as

well as being a facilitator of the degradation process itself, and the effect of mutated SQSTM1 on selective autophagic degradation requires further investigation. The mechanism by which this would lead to the phenotype in patients of over-activated osteoclasts (with other cells apparently unaffected) is not yet clear. The more rapid induction of (presumably autophagic) aggregate formation in response to cell stress in mature osteoclasts than their precursors observed in this study suggests that osteoclasts may have a greater dependence upon autophagy to maintain cellular function. Indeed, preliminary reports suggest that autophagy proteins regulate bone resorption by osteoclasts [17]. Together these point to a role for the process of selective autophagy in regulating osteoclast formation and function that warrants further investigation, and suggest a potential mechanism by which PDB-causing mutations in SQSTM1 exert osteoclast-specific effects.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to the following people: Mrs Denise Tosh for support with cell culture; Mrs Elizabeth Stewart (Aberdeen Proteomics Facility) for running samples through LC-MS/MS; Mr Kevin Mackenzie (Microscopy and Imaging Facility, University of Aberdeen) for assistance with confocal imaging; and Mrs Angela Duthie for technical support for cloning. Anti-ALFY antibody was kindly provided by Dr Anne Simonsen, University of Oslo, Norway.

This work was funded in part by the Paget's Association, UK. LJH was funded by the Scottish Funding Council Strategic Research Development Grant, "Generation Scotland: Genetic Health in the 21st Century". PSM was generously supported by the Association of Physicians of Great Britain and Ireland.

WEB RESOURCES

Aberdeen Proteomics - www.abdn.ac.uk/ims/proteomics

Entrez Gene (ALFY) - www.ncbi.nlm.nih.gov/gene/23001 (accessed 6th September 2010)

Matrix Science web server - www.matrixscience.com

PRIDE - www.ebi.ac.uk/pride

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FIGURE LEGENDS

Figure 1 – Immunoprecipitation gel showing bands excised

Coomassie-stained gel of SQSTM1 immunoprecipitation from hOCL showing proteins (a) in total lysate, (b) bound non-specifically to agarose beads, (c) not bound to SQSTM1 (present in first wash), and (d) bound to SQSTM1. Bands 1-5 were excised for proteomic analysis.

Figure 2 – Endogenous localisation of ALFY & SQSTM1 in mature osteoclasts and mononuclear precursors

Representative confocal image of hOCL cultures, stained for endogenous SQSTM1 (red), ALFY (green) and nuclei (blue). Merged image shows ALFY and SQSTM1 only. Mature, multinucleated osteoclasts (circled) and mononuclear precursors are present in the same culture. In all cells, ALFY is present in the nuclei only whereas SQSTM1 is both nuclear and cytoplasmic. No colocalisation between SQSTM1 and ALFY was apparent.

Figure 3 – Relocalisation of SQSTM1 and ALFY in osteoclasts and precursors upon amino acid starvation

Confocal images from hOCL cultures are shown, stained for endogenous SQSTM1 (red), ALFY (green) and nuclei (blue) for unstarved cells (0') and cells starved in HBSS for 60, 90 and 120 minutes. Merged images show ALFY and SQSTM1 only. All images were taken using same acquisition settings. By 90', ALFY and SQSTM1 colocalise in the cytoplasm of a multinucleated hOCL (black arrow), but not in mononuclear precursors in the same culture. After 120', ALFY has started to relocate to the cytoplasm of mononuclear precursors (black arrow) while, in mature hOCLs, cytoplasmic aggregates have formed. The magnified image of the osteoclast at 120' is taken from a slightly different optical slice through the cell, and

illustrates the colocalisation of ALFY and SQSTM1 in cytoplasmic aggregates (white arrows).

Figure 4 – Colocalisation of overexpressed SQSTM1 with endogenous ALFY in HEK293 cells is not affected by SQSTM1 P392L mutation

Confocal images of HEK293 cells transfected with FLAG-tagged wild-type (392P) or Pagetic mutant (392L) SQSTM1, and untransfected cells. Cells were stained for FLAG (red; except untransfected), endogenous ALFY (green) and nuclei (blue). In untransfected cells, ALFY displays typical perinuclear localisation. Overexpression of SQSTM1 (wild-type or P392L) results in colocalisation of SQSTM1 with endogenous ALFY in cytoplasmic aggregates (single aggregate indicated by white arrows for wild-type and 392L).

FIGURES

Figure 1

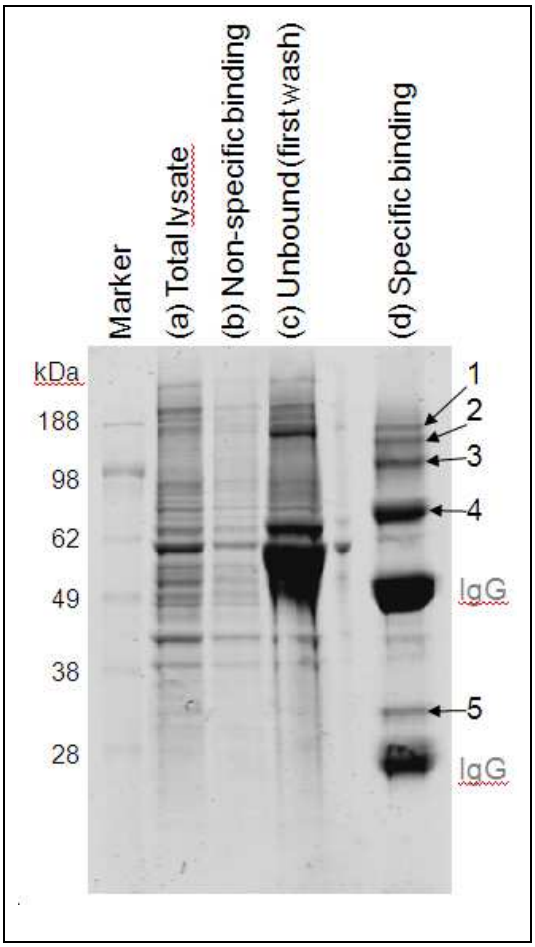


Figure 2

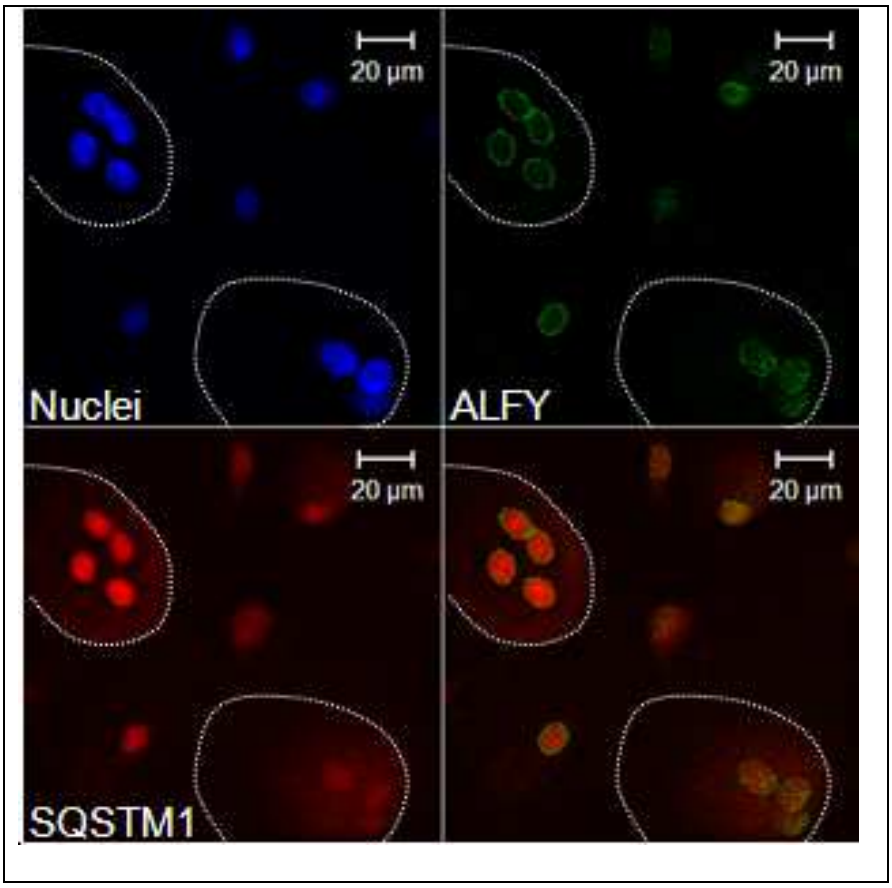


Figure 3

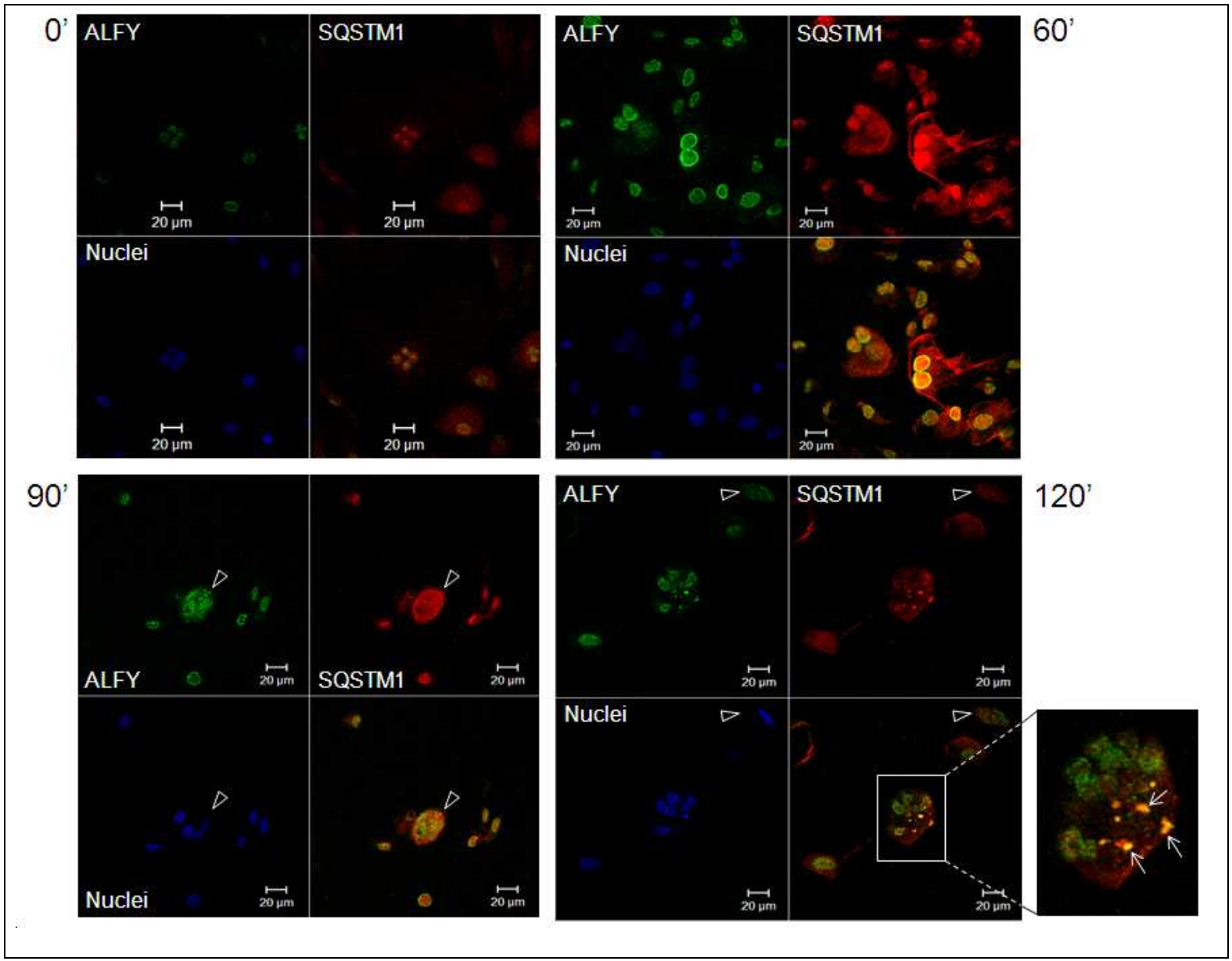
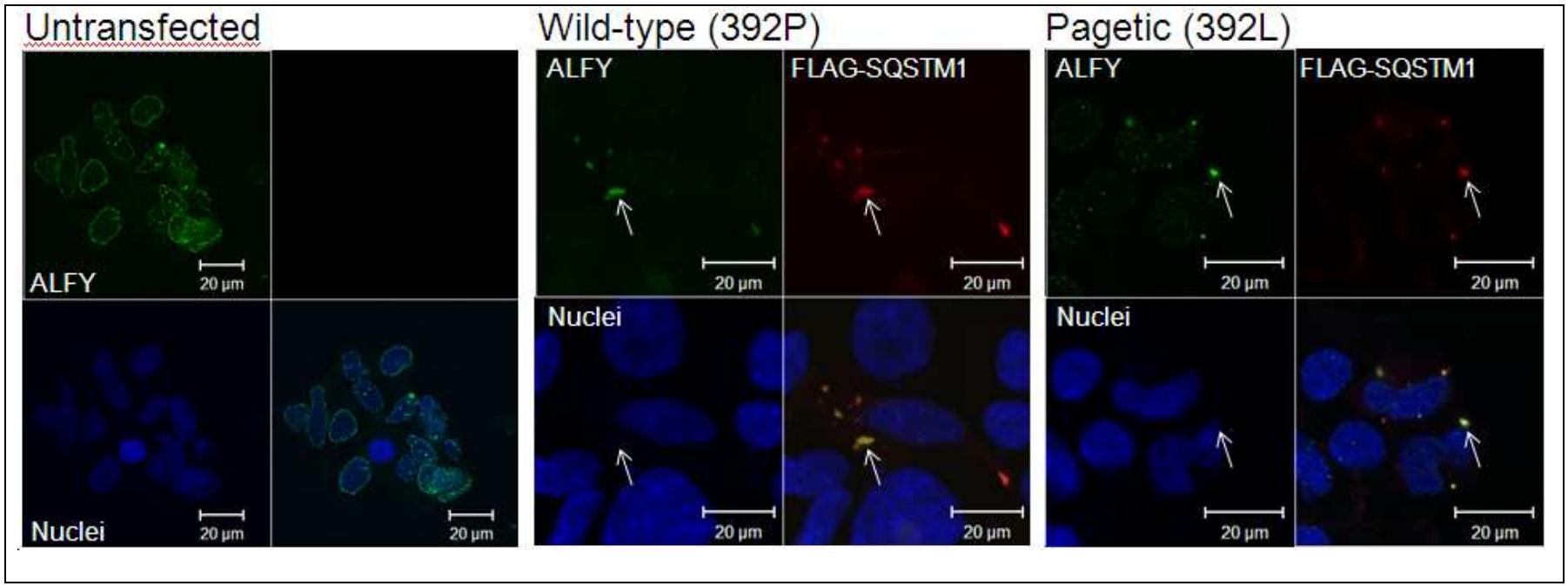


Figure 4


Untransfected
Wild-type (392P)
Pagetic (392L)
ALFY
FLAG-SQSTM1
ALFY
FLAG-SQSTM1
Nuclei
Nuclei
Nuclei
20 µm