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PKA phosphorylation of p62/SQSTM1 regulates PB1 domain interaction partner binding

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ABSTRACT

p62, also known as SQSTM1, is a multi-domain signalling scaffold protein involved in numerous critical cellular functions such as autophagy, apoptosis and inflammation. Crucial interactions relevant to these functions are mediated by the N-terminal Phox and Bem1p (PB1) domain, which is divided into two interaction surfaces, one of predominantly acidic and one of basic character. Most known interaction partners, including atypical protein kinase C (aPKC), bind to the basic surface, and acidic–basic interactions at this interface also allow for p62 homopolymerisation. We identify here that the coupling of p62 to the cAMP signalling system is conferred by both the direct binding of cAMP degrading phosphodiesterase-4 (PDE4) to the acidic surface of the p62 PB1 domain and the phosphorylation of the basic surface of this domain by cAMP-dependent protein kinase (PKA). Such phosphorylation is a previously unknown means of regulating PB1 domain interaction partnerships by disrupting the interaction of p62 with basic surface binding partners, such as aPKCs, as well as p62 homopolymerisation. Thus, we uncover a new regulatory mechanism that connects cAMP signalling with the p62 multi-domain signalling scaffold and autophagy cargo receptor protein.

1. Introduction

The signalling adaptor p62/SQSTM1 regulates important cellular functions, such as apoptosis and inflammation, but is best known for its role in the orchestration of events involved in autophagy [1]. The vital scaffolding function of p62 is underpinned by its ability to complex with a variety of other regulatory proteins by virtue of the different interaction domains it contains. These include the atypical zinc finger (ZZ) domain, Light Chain 3 (LC3) interacting region [2] and TRAF6-binding and ubiquitin-associated (UBA) domains [1]. p62 also contains an N-terminal Phex and Bem1p (PB1) domain [3]. The PB1 motif allows p62 to interact with other PB1 domain-containing proteins and with itself *via* hetero- and homo-dimerisation (or -polymerisation). A PB1 domain may contain two different conserved motifs to allow for interaction with binding partners. One of them, the OPCA motif, consists of a conserved cluster of acidic amino acids, with the complement in the interacting partner provided by a conserved lysine residue within a predominantly basic cluster. These conserved clusters are sometimes referred to as A-type (acidic) and B-type (basic). Some proteins, such as p62, contain both conserved motifs, allowing for homo-polymerisation.

In autophagy, p62 plays the role of a cargo receptor by detecting ubiquitinated substrates using its UBA domain [4,5], aggregating them through PB1-mediated homo-polymerisation and attaching them to the autophagosome by association with LC3 [2,6]. Autophagosomes then fuse with lysosomes, thus degrading the former autophagy substrates, releasing amino acids and other nutrients as new building blocks for the cell.

Apart from the homopolymerisation of p62, many of its interaction partners bind to p62 *via* heterodimeric PB1 domain interactions, involving the conserved basic cluster in p62 that includes Lys7, Arg21 and Arg22 [7] together with the OPCA motif in the interaction partner. Examples of proteins that form a complex with p62 in this manner include the atypical PKC (aPKC) isoforms, PKCʌ/ι and PKCζ [8,9], TRAF6 [8] and MEK5 [10]. In addition to these "p62-basic cluster-binders", MEKK3 was recently identified as the first protein, apart from p62 itself, to bind in the converse fashion, *i.e.* by binding to the OPCA motif/acidic cluster of p62. It is thought that this mechanism allows MEKK3, but not MEKK2, to activate NF-κB signalling selectively *via* its association with

Abbreviations: aPKC, atypical protein kinase C; cAMP, cyclic AMP-dependent protein kinase; LC3, microtubule-associated protein 1A/B light chain 3; MEK, mitogen-activated protein kinase kinase/ERK kinase; MEKK, MEK kinase; NBR, neighbour of BRCA1 gene 1 protein; NF-κB, nuclear factor κB; OPCA, OPR/PC/AID motif; PB1, Phox and Bem1p; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; SQSTM1, sequestosome-1; TRAF6, TNF receptor associated factor 6; UBA, ubiquitin-associated domain; UCR, upstream conserved region; ZZ, atypical zinc finger

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p62 [10]. To date, it is not known if and how these diverse interactions are regulated.

We have shown recently that p62 interacts with the cyclic AMP-degrading phosphodiesterase, PDE4A4 [11,12]. This enzyme is a member of the PDE4 family of phosphodiesterases, comprising >25 isoforms encoded by 4 genes in mammals. The different isoforms of PDE4 interact with an array of signalling proteins, such as protein kinases and scaffolding proteins, and they are sequestered in this way to distinct subcellular locales, predominantly by interactions mediated through their isoform-specific N-termini [13]. This localisation allows cAMP signalling compartmentation, where the stimulus cAMP, its effectors such as cAMP-dependent protein kinase (PKA) and substrates thereof are brought closely together in one protein complex. The action of distinct PDE4 isoforms results in the production of stimulus-specific, subcellularly confined cAMP gradients. Such gradients promote the transient activation of the cAMP effector proteins, PKA, exchange proteins activated by cAMP (Epac) and cyclic nucleotide-gated (CNG) ion channels, which leads to distinct changes in cell function [14]. In the case of PKA, the enzyme recognises substrates in its vicinity through a consensus motif R–R/K–X–S/T in their amino acid sequence [15], wherein the serine or threonine is phosphorylated to bring about a change in function.

In this report we show, for the first time, that PDE4 can interact directly with the p62 PB1 domain by binding to the p62 OPCA motif. Furthermore, we identify a novel PKA phosphorylation site within the p62 PB1 domain and demonstrate that it regulates the binding interaction of certain partner proteins. Taken together, these observations are the first indication that cAMP signals can cross-talk with the well-established signalling functions of p62. We suggest that this opens up a novel regulatory mechanism for both the homopolymerisation of p62 (which is a prerequisite for autophagosome formation) and its binding to other partners involved in signalling pathways affecting nutrient homeostasis, immunological responses and cancer development.

2. Results

2.1. PDE4A4 binds directly to p62

p62 associates constitutively with the cAMP degrading PDE4A4 isoform in cells and this is required for the reversible intracellular redistribution of PDE4A4 into aggregates that is elicited by chronic exposure to certain PDE4-specific inhibitors, such as rolipram, that promote 'capping' of the active site by the regulatory UCR2 domain [11,12,16].

Initially, we set out here to determine whether the interaction between p62 and PDE4A4 is direct. To do this we developed an ELISA-based assay using purified recombinant full length MBP-p62 and GST-PDE4A4 proteins (Fig. 1A). We detected increases in chemiluminescence only when increasing amounts of MBP-p62 were combined with GST-PDE4A4. This was not the case for any combination including GST or MBP alone, suggesting that the MBP and GST tags were not involved in the interaction between p62 and PDE4A4 and that the two proteins interact directly. To support the notion of a direct interaction between p62 and PDE4A4 and to gain insight into the nature of the interaction sites between the proteins, we utilised peptide array technology [17]. Peptide array technology has been used successfully by us to map the interfaces between interacting proteins (see *e.g.* [18,19]). In order to map the sites of interaction between p62 and PDE4A4, we synthesised a peptide array representing

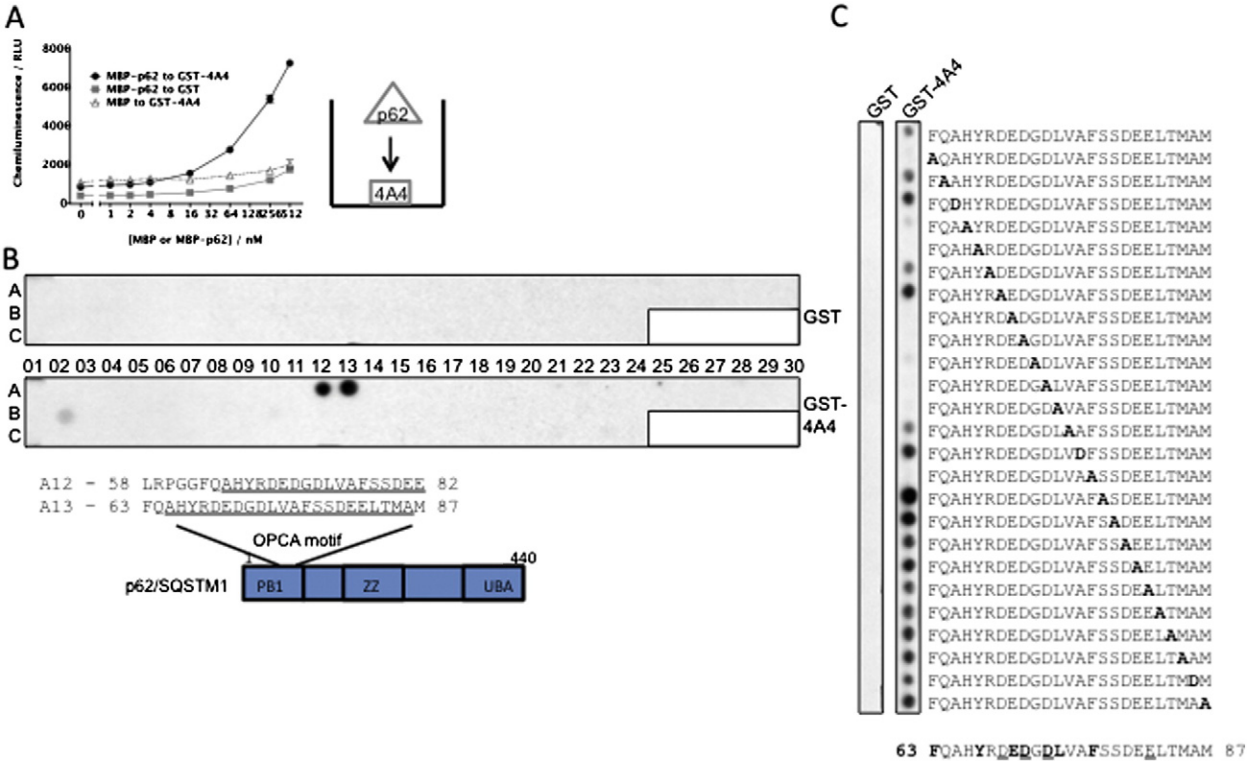

Fig. 1. PDE4A4 and p62 interact directly. (A) Full-length purified MBP-p62 and GST-PDE4A4 interact directly in an ELISA. 384 well microplates were coated with 256 nM GST or GST-PDE4A4. Free binding sites were blocked, and increasing concentrations (1–512 nM) of MBP or MBP-p62 were added to the wells. After washing, the bound protein was detected using an anti-MBP-HRP conjugate and a chemiluminescence substrate. (B) A peptide array representing the full length sequence of p62 screened with purified GST-PDE4A4 (5 μg/ml) reveals interaction of the PDE with the OPCA motif (underlined) located within the PB1 domain of p62, spanning amino acids 58 to 87. Bound protein was detected using an anti-GST-HRP conjugate. (C) A peptide array alanine scan across the p62 OPCA motif (spot A13 from panel B) overlaid with GST-PDE4A4 (5 μg/ml) reveals essential amino acids of p62 required for PDE4A4 binding. The bottom line summarises amino acids essential for PDE4A4 binding (in bold) *versus* those essential for the interactions of the OPCA motif with the basic motif in other PB1 domains (underlined).

p62 consisting of 25mer peptides overlapping by 5 amino acids that encompassed the entire p62 sequence (Fig. 1B). We then probed this array with either GST or GST-PDE4A4 and detected areas of interaction by blotting for the GST tag. A robust interaction of GST-PDE4A4 (but not GST alone) was identified on peptide spots A12 and A13, which correspond to amino acids 58–87 within the p62 sequence (Fig. 1B). This part of the sequence is located within the Phox and Bem1p (PB1) domain, an evolutionarily well-conserved protein interaction domain [3] located at the N-terminus of p62. The sequence contained within the interacting peptides (A12, A13) contains the OPCA motif of the PB1 domain, which is known to be essential for interaction with other PB1 domain-containing proteins *via* its conserved cluster of acidic amino acids. A detailed alanine scan of this region, where successive residues were substituted with alanine residues (with endogenous alanine residues replaced by aspartate), shows that the major relevant residues involved in the interaction are Phe63, Tyr67, Glu70, Asp71, Asp73, Leu74 and Tyr77 (Fig. 1C). Hence, two of the aspartates conserved in OPCA motifs, Asp71 and Asp73, appear to be crucial for the interaction, while the conserved Asp69 and Glu82 are not.

Consequently, p62 is a new example for a signalling scaffold protein binding a PDE4 isoform directly.

2.2. p62 is phosphorylated at Ser24 by PKA

Intrigued by this finding, and by previous studies where we found scaffolding proteins to bind PDE4 isoforms as well as PKA substrates, allowing for regulation of substrate phosphorylation [20,21], we were curious whether p62 or one of its interaction partners could be a PKA substrate. Indeed, upon examining the amino acid sequence of p62 we noticed an optimal PKA consensus phosphorylation sequence 21-RRFS-24 [15] within the PB1 domain. In order to see if PKA can phosphorylate the serine within this motif (Ser24), a peptide array covering the full-length sequence of p62 was incubated with purified, recombinant PKA catalytic subunit and γ-^{32}P-ATP, followed by autoradiography (Fig. 2A). The first five peptides on the array contained the PKA phosphorylation motif and were phosphorylated by PKA. Phosphorylation was not observed with the adjacent peptides (from peptide 6), suggesting that Ser24 can be phosphorylated by recombinant PKA *in vitro*. This

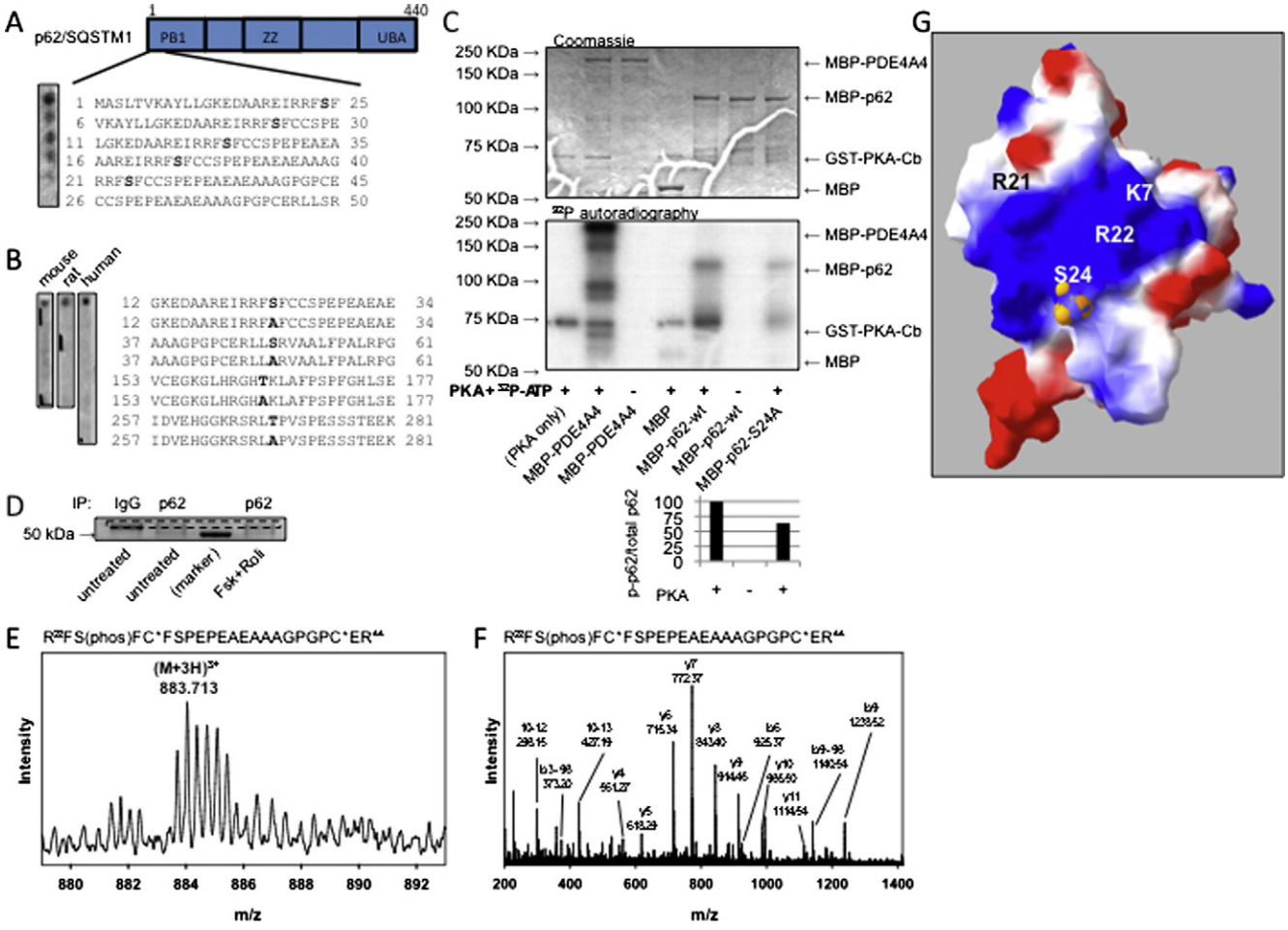

Fig. 2. PKA phosphorylates p62 at Ser24. (A–C) *In vitro* phosphorylation of p62 by PKA. (A) A peptide array covering the sequence of the N-terminal 50 amino acids of human p62 by overlapping 25mers was incubated with recombinant PKA catalytic subunit and γ-^{32}P-ATP. Phosphorylation was detected by autoradiography. (B) The Ser24 phosphorylation site as well as all other sites resembling the PKA consensus site RXXS/T were synthesised as wildtype 25mer peptides, or with the phospho-target amino acid replaced by alanine. The peptide array was then PKA-phosphorylated and detected as in (A). (C) Full length MBP fusion proteins of p62, p62-S24A and PDE4A4 were expressed in *E. coli* and purified. 2 mg of each protein were incubated with γ-^{32}P-ATP and with (+) or without (−) recombinant purified PKA catalytic subunit, and then run by SDS-PAGE. A Coomassie staining and autoradiography were carried out. (D–F) *In vivo* phosphorylation of p62 by PKA. (D) Rat PC12 cells were stimulated for 10 min with forskolin and rolipram or left untreated (control and IgG). Cell lysates were prepared, and immunoprecipitated with IgG or a p62-specific antibody. After cysteines were reduced and modified by carbamidomethylation, IP eluates were separated on a gel and stained with Coomassie blue. A band of approx. 62 kDa present in the p62-IPs, but not in the IgG control, was excised (dashed box) and subjected to mass spectrometric analysis. The protein was digested with trypsin and analysed by nanoLC–MS/MS. The peak with m/z 883.713 in the MS spectrum (E) corresponds to the triply charged ion of the phosphorylated sequence 22–44 (m_{calc} 2648.083) of p62. C-terminal fragment ions (y4–y11) of the CID spectrum (F) confirm the phosphorylated amino acid sequence, whereas the b ions (b3–98, b6, and b9) indicate phosphorylation *in vivo* at serine 24 after forskolin + rolipram treatment. C*, cysteines are alkylated by iodoacetamide. (G) Structure of the p62 PB1 domain showing the electrostatic surface potential and position of Ser24 (orange) within the conserved basic motif (based on the PDB-deposited structure, 2KKC [25], and modified using Swiss-PdbViewer [50]).

notion is reinforced by a control peptide which has Ser24 replaced by an alanine, and which cannot be phosphorylated in this way (Fig. 2B). The same holds true for homologous sequences from mouse and rat p62. As an additional control, all other sequences from human, mouse and rat p62 containing other putative (though non-ideal) PKA phosphorylation consensus sequences were synthesised and probed in the same way (Fig. 2B). Only peptides containing the PKA phosphorylation motif that incorporates Ser24 were found to be PKA substrates, suggesting that Ser24 is the sole PKA site within p62.

As short peptide substrates might be subject to non-specific kinase action, and the target Ser24 might be less accessible in a full-length protein due to conformational hindrance, we purified recombinant full length MBP-p62 and a S24A mutant thereof. Both MBP-p62 wild type and the S24A mutant, as well as MBP-PDE4A4, which is an established PKA substrate [22] and therefore serves as a positive control, were incubated with the PKA catalytic subunit and γ-^{32}P-ATP, followed by autoradiography (Fig. 2C). Phosphorylation of MBP-PDE4A4 and MBP-p62 at the corresponding molecular weights was observed. Phosphorylation of the S24A mutant of MBP-p62 was reduced strongly, with the residual phosphorylation likely to be caused by non-specific phosphorylation of the fused MBP moiety that, on its own, appeared as a weak intensity band (Fig. 2C lower panel).

Though recombinant PKA catalytic subunit is representative of both the activity and specificity of PKA *in vivo* [23,24], to determine whether the observed p62-Ser24 phosphorylation also takes place in a cellular context, and to further validate Ser24 as a bona fide PKA substrate, we subjected p62 immunopurifications to analysis by mass spectrometry. Immunopurification of p62 from rat pheochromocytoma (PC12) cells was undertaken following either no treatment or treatment with the adenylyl cyclase activator, forskolin (100 μM), and the PDE4 inhibitor, rolipram (10 μM). Gel bands corresponding to the molecular weight of p62 were cut from a Coomassie-stained gel (Fig. 2D) and proteins were in-gel digested by trypsin. Nano-liquid chromatography–tandem mass spectrometry (nanoLC–MS/MS; Fig. 2E and F) unambigously identified a tryptic peptide corresponding to Ser24-phosphorylated p62 in the forskolin/rolipram-treated immunoprecipitate but not in the untreated cells. Such data indicates that the PDE4A4 activity associated with p62 may regulate phosphorylation at this site on p62 within cells.

Bioinformatic analysis was then carried out to provide insight into the molecular location of this novel PKA phosphorylation site in p62 and the possible consequences due to such phosphorylation. The NMR solution structure of the p62 PB1 domain [25] was used to locate the position of Ser24 on the surface of the protein domain. It was found

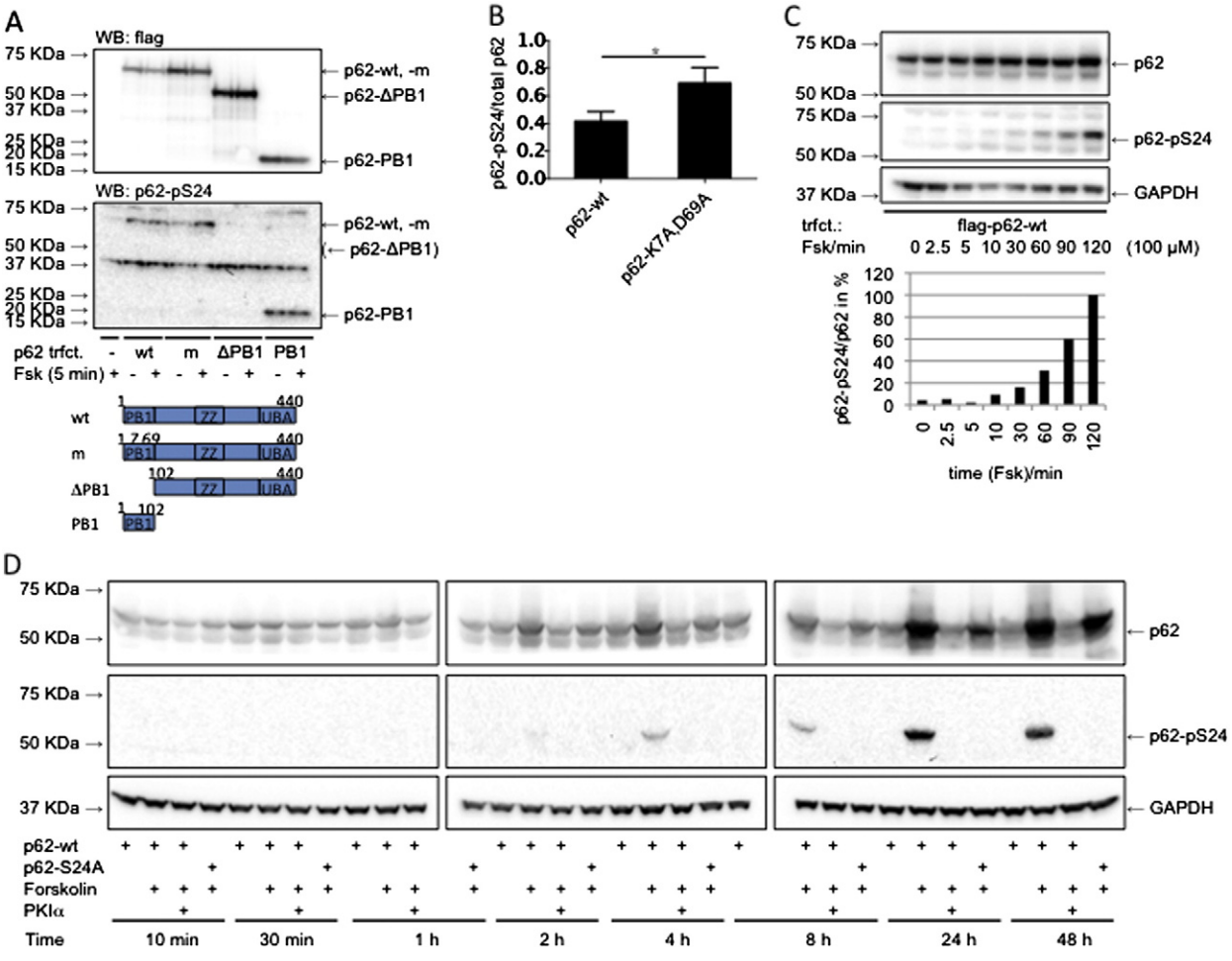

Fig. 3. Phosphorylation of p62 at Ser24 in cells is PKA-dependent. (A) The p62 PB1 domain is both necessary and sufficient for phosphorylation at Ser24. HEK293 cells were left untransfected (−) or transiently transfected with equal amounts of flag-p62-wt (wt), -K7A/D69A (m), -ΔPB1 or the p62 PB1 domain alone (PB1). After 24 h, cells were treated with 100 μM forskolin for 5 min as indicated. Lysates were prepared and analysed by Western blot using specific flag- or p62-pS24 antibodies as indicated in the figure. (B) Under basal conditions, p62-K7A/D69A is stronger phosphorylated at Ser24 than p62-wt. Quantification for m (p62-K7A/D69A) from panel A. Statistical analysis by paired *t*-test n = 3, P = 0.03. (C) Forskolin evokes a slow phosphorylation of p62 at Ser24. HEK293 cells were transiently transfected with equal amounts of flag-p62-wt. After 24 h, cells were treated with 100 μM forskolin for the indicated times. Lysates were prepared and analysed by Western blot using specific p62-, p62-pS24 or GAPDH antibodies as indicated in the figure. The quantification shows phospho-p62 normalised to total p62 band intensity. (D) p62 phosphorylation at Ser24 peaks after forskolin stimulation for 24 h and is blocked by PKI or a S24A mutant. HEK293 cells were transiently transfected with equal amounts of flag-p62-wt or -S24A in combination with either pcDNA3 or myc-PKIα. After 24 h, cells were treated with 100 μM forskolin for the indicated times. Lysates were prepared and analysed by Western blot using specific p62-, p62-pS24 or GAPDH antibodies as indicated in the figure.

to be within the largely basic (positively charged) region around the conserved Lys7 (Fig. 2G). Hence, as phosphorylation of Ser24 would introduce a negatively charged phosphate group at the site, it is likely that this would change its electrostatic potential and that this might serve to affect the binding of partner proteins through electrostatic interactions. This has been shown to be the case for PB1–PB1 interactions involving the conserved basic and acidic (OPCA) motifs [7,26,27]. Indeed, when the electrostatic surface potential was calculated for the PB1 domain, it was found that the phosphorylated Ser24 can be expected to change the electrostatic appearance of the basic motif surface (Fig. 2G).

Intrigued by this finding, and in order to monitor the dynamic phosphorylation of p62 at Ser24 directly, we raised a phospho-specific antibody against the Ser24 site (Fig. S2). To validate the antibody, and to prove further that the Ser24 site is the sole point of PKA phosphorylation on p62, we transfected HEK293 cells with plasmids encoding different flag-tagged p62 constructs: a full length wild type p62 (wt); a mutant containing the double substitution K7A and D69A within the PB1 domain which has been shown to disable polymerisation of p62 (m) [7]; a truncated p62 with the PB1 domain (amino acids 1–101) deleted (ΔPB1); or a construct corresponding to only the PB1 domain of p62 (PB1) (Fig. 3A). Cells were then either treated with forskolin (50 μM) alone for 5 min or left untreated. Cell lysates were subsequently probed with the p62-pS24 antibody (Fig. 3A, lower panel) and a FLAG antibody (Fig. 3A, upper panel). Although expression of all constructs was confirmed using the FLAG antibody, phospho-bands were only detected for the wild type (wt), the non-polymerising mutant (m) and the PB1 domain alone (PB1). Phosphorylation at S24 was not apparent for the p62 construct lacking the PB1 domain (ΔPB1). These data suggest that the p62 PB1 domain is both necessary and sufficient to promote p62 phosphorylation by PKA. Surprisingly, forskolin treatment alone failed to trigger an increase in Ser24 phosphorylation in either the wild type or PB1 only constructs. The K7A/D69A non-polymerising mutant (Fig. 3B), however, showed significantly increased Ser24 phosphorylation under basal conditions. We hypothesise that polymerisation of the PB1 domain physically restricts access of PKA to the S24 phosphorylation site, resulting in a dramatic reduction of this post-translational modification.

We also evaluated the time-dependency of p62 phosphorylation at the Ser24 site in forskolin-challenged HEK293 cells expressing flag-tagged p62 (Fig. 3C), showing that this reached a maximum after a 2 h period. To see whether phosphorylation increases further after 2 h forskolin stimulation, we increased the timecourse to 48 h. This revealed a maximum at 24 h (Fig. 3D). At the same time, in order to demonstrate that this action was PKA-specific we co-transfected cells with PKI, a potent and highly selective inhibitor of PKA [28]. Consistent with this action being mediated by PKA, we found that PKI ablated the forskolin-induced phosphorylation of p62 at Ser24 across the whole timecourse (Fig. 3D). The specificity of the Ser24 phosphorylation site was further confirmed by a S24A mutant p62 that under the same conditions was not phosphorylated at all.

Other AGC kinases have similar substrate consensus sequences to that of PKA. For example, Akt/PKB kinase has the consensus motif RXRXXSφ, with φ denoting a bulky hydrophobic residue [29]. However, stimulation of HEK293 cells with insulin, the prototypical Akt activator [30], did not lead to any detectable phosphorylation of p62 at Ser24 (Fig. S1), even though Akt itself was clearly activated under these conditions, as measured by its phosphorylation at Ser473 [31].

2.3. Phosphorylation at Ser24 regulates p62 polymerisation and PB1 interaction partner binding

We propose that the functional relevance of Ser24 phosphorylation of p62 by PKA is the introduction of a negatively charged phosphate group to the basic surface of the OPCA motif leading to a disruption of the PB1 domain interaction partner binding through electrostatic interference (Fig. 4A). To address this, we introduced S24A (phospho-null) and S24D (phospho-mimic) mutations into a cherry-tagged p62 wild-type construct.

Work from other groups has shown that p62 forms cytoplasmic bodies both endogenously and when overexpressed in cells and that this process requires both the PB1 and the UBA domains [2,32]. Consistent with this we observed extensive cytoplasmic body formation in HEK293 cells expressing either recombinant wild-type or mutant S24A cherry-tagged p62 (Fig. 4B, upper and middle panel). Strikingly, however, this was not evident when cells were transfected with the PKA phospho-mimicking cherry-tagged p62-S24D, which exhibited a dispersed distribution in the cytoplasm (Fig. 4B, lower panel). This suggests that the single PKA phospho-mimicking mutation suffices to prevent p62 polymerisation.

We then set out to determine whether the binding of other interaction partners of the p62 PB1 domain was affected by the phospho-mimicking S24D mutation. We used such a mutation as it reflects a homogeneous population of fully phosphorylated p62, which would be difficult to realise using forskolin-challenged cells where p62 is slowly phosphorylated and there will be a mixture of phosphorylated and non-phosphorylated species. Thus we carried out a series of co-immunoprecipitation (IP) experiments using either cherry-p62-S24A, which mimics the fully de-phosphorylated state at Ser24, or S24D, which mimics the fully phosphorylated state at Ser24. Such immunoprecipitates were then subject to Western blotting using specific antisera to established endogenous interaction partners of p62 ([7, 33]; Fig. 4C).

Doing this we found that various proteins that are known to bind to the p62 PB1 domain basic motif, namely NBR1, PKCι, PKCζ and MEK5, while co-immunoprecipitating with the S24A p62 mutant, failed to bind to the S24D p62 mutant (Fig. 4C). This suggests that PKA phosphorylation of S24 regulates the binding of those partner proteins to p62 that require the PB1 interface.

In order to test this hypothesis we evaluated the binding of LC3, which is known to bind to a distinct region on p62, which has been termed the LC3-interacting region (LIR; [32]). Investigating LC3 interaction, we found that LC3 co-immunoprecipitated with both the S24A and S24D mutant p62 constructs, although binding was somewhat less with the S24D mutant. PDE4A4 also binds to a different surface patch on p62, namely the acidic, rather than the basic domain of the PB1 interface (Fig. 1B). As endogenous PDE4A5 (the rodent orthologue of human PDE4A4) is only expressed at vanishingly low levels in PC12 cells we used the HEK293-PDE4A4 cell line, which stably overexpresses PDE4A4 (Fig. 4D). When transfecting these cells with tagged p62 mutants, we found that both the S24A and the S24D mutant p62 constructs exhibited similar abilities to bind PDE4A4 (Fig. 4D). This observation was reinforced using an *in vitro* ELISA assay that evaluated the association between MBP, MBP-p62-wt and -p62-S24D to GST-PDE4A4 (Fig. 4E). Using this approach we found that the p62-wt and -S24D proteins exhibit very similar binding characteristics to PDE4A4, while MBP alone does not bind at all.

3. Discussion

We report here the discovery of cAMP degrading PDE4A4 as a new interaction partner for the signalling adaptor p62/SQSTM1. We propose that this protein complex represents a previously undiscovered point of cross-talk between the cAMP signalling system and p62-regulated processes. In this, sequestered PDE4A4 may influence access of cAMP to PKA able to phosphorylate p62 and, thereby, selectively regulate its interaction with certain partner proteins.

Peptide mapping of the interaction sites between p62 and PDE4A4 allowed us to identify a well defined binding site on p62 encompassing the conserved acidic OPCA motif (amino acids 58–87), which is known to be crucial for PB1 domain interactions. So far, only two interaction partners have been shown to bind that part of the p62

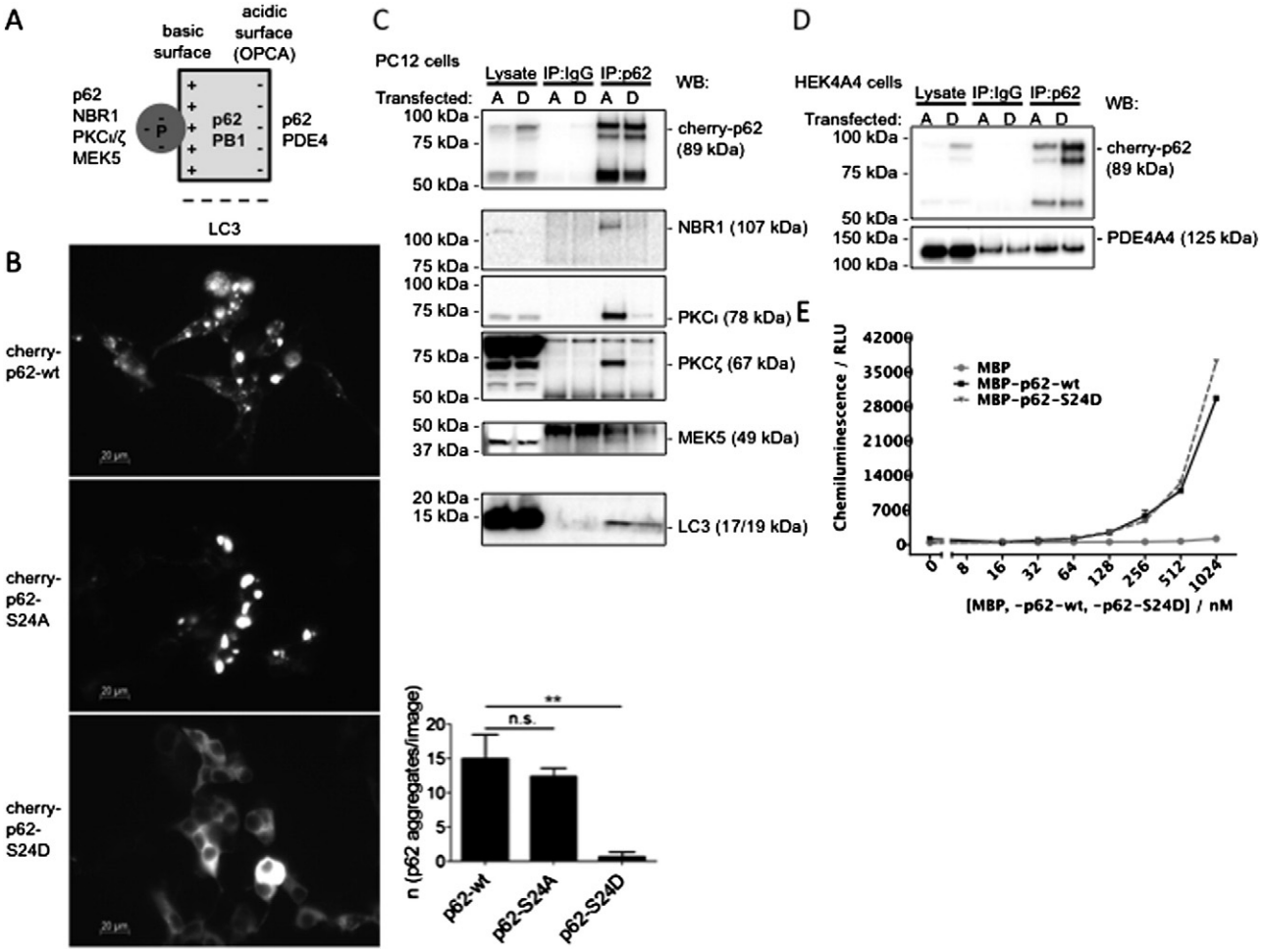

Fig. 4. Mimicking phosphorylation at p62-Ser24 determines polymerisation and interaction partner binding. (A) Schematic of the p62 PB1 domain and known interaction partners of its basic and acidic surfaces. (B) S24D mutation of cherry-p62 causes a dispersed phenotype inside cells. HEK293 cells were transfected with cherry-p62-wt, -S24A or -S24D and analysed using a fluorescence microscope 24 h later. While p62-wt and -S24A are present in large aggregates, p62-S24D is dispersed throughout the cytosol. Images are representative for experiments done independently at least 3 times. Statistical analysis by one-way ANOVA (all means): $n = 3$, $P = 0.0072$, and by unpaired *t*-test (p62-wt *vs.* -S24A): $n = 3$, $P = 0.5073$. (C) S24D mutation of p62 specifically disrupts binding to established interaction partners of the PB1 domain. PC12 cells were transfected with either cherry-p62-S24A or -S24D. After 24 h, lysates were prepared and immunoprecipitated using IgG or a p62-specific antibody. After washing, lysates and eluates from the IPs were separated by SDS-PAGE and blotted onto PVDF membranes. The membranes were detected using specific antibodies as indicated in the figure. LC3 is a known p62 interaction partner, but it binds outside the PB1 domain. Blots are representative for experiments done independently at least 3 times. (D) p62-S24A and -S24D both co-immunoprecipitate PDE4A4. HEK293 cells stably transfected to express PDE4A4 (lower panel) were transfected and analysed as in (C). Blots were detected using a PDE4A4-specific antibody. (E) MBP-p62-wt and -S24A display similar binding affinities in an ELISA using microplates coated with GST-PDE4A4. Experimental procedure as in Fig. 1A.

PB1 domain, namely p62 itself when homo-polymerising, and the mitogen-activated protein kinase kinase kinase, MEKK3 [10]. Comparison of binding motifs for the established p62 partners against those utilised by PDE4A4 suggests that PDE4A4 binds in a novel fashion to the OPCA motif of the p62 PB1 domain. The scanning alanine replacement peptide array (Fig. 1C) showed that PDE4A4 is utilising some (Asp71 and Asp73), but not all, of the conserved acidic amino acid residues of the PB1 domain. Furthermore, the array suggests that the binding of PDE4A4 to p62 utilises additional non-acidic amino acids previously unknown to be involved in other PB1 domain interactions (Phe63, Tyr67, Glu70, Leu74 and Tyr77). There is no sequence homology between PDE4 proteins and any other PB1 domain protein sequence, supporting the notion of a unique binding mode. The uniqueness of the interaction, along with a well defined short binding region, could be exploited to develop highly specific peptide disruptors for use as a therapeutic or research tool to further probe the functional relevance of the p62–PDE4A4 interaction. We have recently developed such agents for other PDE containing protein complexes that have potential as therapeutics in both the oncology and cardiovascular disease fields [18,19,34].

The PKA substrate consensus sequence identified in p62 PB1 domain conforms to the most common physiological motif, RRXS [35] and the PKA specific inhibitor PKI ablates phosphorylation at this site (Fig. 3D). Indeed, our mass spectrometric analysis (Fig. 2D–F) identified the phosphorylated p62 sequence 22–44 (RFS(phos)FCFSPEPEAEAAAGPGPCER), containing such motif, suggesting p62 is a physiological substrate of PKA. Furthermore, we ruled out Akt/Protein kinase B as a representative for other AGC kinases, some of which have similar substrate consensus sequences, as the responsible kinase for p62-Ser24 phosphorylation, as insulin stimulation did not induce measurable p62 phosphorylation over up to 48 h (Fig. S1). Taken together, the evidence presented here suggests, for the first time, that p62 is a substrate for PKA.

There are two clear primary functional outcomes of the p62 phosphorylation at Ser24 by PKA that go along with each other: one is the effect on homopolymerisation, and the other is the selective action on

proteins binding to the basic part of the PB1 domain. In the following, we will detail these primary outcomes, along with possible secondary consequences.

We were intrigued by the fact that the Ser24 PKA site is positioned at the centre of the basic cluster of the OPCA region, close to K7, R21 and R22 (Fig. 2G). K7 is highly conserved across B-type PB1 domains, and it is crucial for interaction with A-type domains, akin to the ones that underpin homopolymerisation [26]. R21 and R22, which are also part of the PKA substrate consensus sequence, were found to be crucial for the interaction of p62 with PKCλ [7] and it is likely that PKA phosphorylation of p62 would negate this interaction. As Ser24 sits within the p62 polymerisation interface, phosphorylation represents a dynamic and controllable means to regulate polymerisation, with possible consequences for autophagy initiation (see below). Conversely, the degree of p62 phosphorylation at Ser24 should be affected by the scaffold's homopolymerisation state. Our data fits with this model, as a K7A/D69A mutant blocks polymerisation, results in better PKA access to Ser24 and leads to increased phosphorylation (Fig. 3B). Furthermore, our assumptions based on the model (Fig. 2G) with regards to interaction partner binding appear to be correct as p62 homopolymerisation is blocked in a S24D phosphomimic p62 (Fig. 4B). Likewise, NBR1, PKCι, PKCζ and MEK5, all binding to the basic B-type part of the p62 PB1 domain, do not bind to p62-S24D (Fig. 4C).

The most direct and obvious outcome of p62 phosphorylation on Ser24 is its effect on p62 homopolymerisation, which has been found to be required for one of the earliest steps in autophagy, the translocation of p62 (with its cargo) to the site of autophagosome formation [36]. It is possible that the cell can adjust the level of specific p62-dependent autophagy *via* cAMP and PKA activation. NBR1 has been shown recently to act as an alternative autophagy receptor, either alone or in conjunction with p62 [6,37], so it seems feasible that phosphorylation of the PB1 domain may reduce p62- or NBR1-mediated specific autophagy. It is noteworthy that suppression of autophagy leads to p62- and ubiquitin-positive aggregates. Such aggregates are also found in pathological states such as neurodegenerative diseases (Alzheimer's, Parkinson's, amyotrophic lateral sclerosis), liver disorders (alcoholic hepatitis, steatohepatitis) and cancers (malignant glioma and hepatocellular carcinoma) [38]. Consequently, in addition to p62 and the ubiquitination machinery, cAMP signalling could be misregulated in these cases, leading to hyperphosphorylation of p62 at Ser24, suppression of homopolymerisation and hence a reduction in autophagy. Interestingly, recent data from the yeast *S. cerevisiae* shows that increased cAMP/PKA activity blocks autophagy [39], and that inactivation of the cAMP pathway induces autophagy [40]. However, yeast do not express p62 or homologues thereof, and the autophagy-regulating protein Atg13 was identified as the responsible PKA substrate in this case. It is also worth mentioning that the three PKA substrate sites of Atg13 are not conserved in the human homologue, so it is probable that a different point of signalling crosstalk exists in mammals.

A recent, comprehensive analysis of p62 phospho-substrates found that autophagy is regulated through posttranslational modification of p62, with phosphorylation at Ser403 by casein kinase 2 increasing the interaction with ubiquitinated autophagy substrate proteins [41]. The same study found Ser24 to be phosphorylated, however no kinase and no functional consequences of its phosphorylation were identified. The authors also found that Bafilomycin A1 (an autophagosome–lysosome fusion inhibitor) did not induce this phosphorylation, suggesting that phospho-Ser24 does not induce autophagy, which is in line with our results as we propose that this modification acts, instead, to inhibit homopolymerisation (Fig. 4B), a requirement for autophagy [36]. A further example of p62 regulation through phosphorylation is found in the interplay between the Keap1–Nrf2 system and autophagy. In this case, S351 is phosphorylated in a mTORC1-dependent fashion, regulating the host defence mechanism and oxidative stress response [42].

Another major cellular pathway in which p62 has a pivotal role is the NF-κB signalling system. The underlying mechanism involves PB1-mediated binding of atypical PKC isoforms to p62 [9,33]. One route leading to downstream activation of NF-κB, is the phosphorylation and inhibition of IκB kinase β (IKKβ) through the aPKCs [43]. It is thought that p62 provides an essential scaffold to organise the signalling pathway by binding either receptor interacting protein 1 (RIP1), which is connected to the tumour necrosis factor α (TNFα) receptor through tumour necrosis factor receptor type 1-associated DEATH domain protein (TRADD), or to TNF receptor associated factor 6 (TRAF6), which links through interleukin-1 receptor-associated kinase (IRAK) and myeloid differentiation primary response gene 88 (MyD88) to the interleukin-1 (IL-1) receptor. P62 is also linked to the NF-κB signalling pathway through the association that it makes with TRAF6 and MEKK3. This complex regulates NF-κB activation through ubiquitination of the IKKβ complex [10]. It is noteworthy that both PDE4A4 and MEKK3 bind to the p62 OPCA motif and it may be possible that a cAMP-dependent switch exists between NF-κB activation and other p62-organised pathways, such as autophagosome formation or MEK5-dependent signalling.

Models constructed to investigate the binding modes of p62 partner proteins have suggested that PKCλ interacts with the p62 PB1 domain, completely occupying the basic cluster, but leaving the OPCA loop free [7]. PDE4A4 could presumably bind to the A-type face of the PB1 domain at the same time as other interactors were binding to the B-type face. In this manner, the association of the PDE4 would effectively increase the chances of B-type face binding events as the PDE would maintain the B-type face in its unphosphorylated state. Thus, with regards to the aPKCs, increased local cAMP levels could lead to increased PKA activity, decreasing the bound aPKC level, which is required to activate NF-κB, providing a novel crosstalk node between the two signalling pathways. Furthermore, the possible inhibitory effect of Ser24 phosphorylation on autophagy induction could affect other p62-mediated signalling pathways as it would enhance p62–partner complexes by saving them from the fate of autophagy. The bound PDE4A4 could also cause a shift in the binding equilibrium of the p62 PB1 domain away from the polymerisation and binding of OPCA motif interactors towards monovalent interactions with the basic PB1 surface (such as aPKC, MEK5, NBR) as it should lower Ser24 phosphorylation whilst occupying the acidic OPCA motif.

In summary, we have identified the cAMP-specific PDE4A4 as a new direct binding partner of the signalling scaffold p62. Furthermore, p62 was identified as a new PKA substrate, and phosphorylation at Ser24 of its PB1 domain has been demonstrated to affect PB1 domain interaction partner binding. This new signalling axis has the potential to be harnessed in order to modulate the two main functions of p62: the induction of autophagy through PB1-dependent p62 homopolymerisation and regulation of cellular signalling such as NF-κB activation through the disruption of the PB1 domain-mediated binding of aPKCs.

4. Materials and methods

4.1. Plasmids

The plasmids used in this study were obtained through standard molecular cloning techniques. The plasmids pTH1-p62 (encoding MBP-p62) and pDest-mCherry-p62 were generously provided by Terje Johansen [2,32].

The flag-p62 plasmids were generously provided by Yu-shin Sou [44]. All p62-Ser24 mutants were generated by PCR-based site-directed mutagenesis using the QuikChange mutagenesis kit (Agilent).

4.2. Cell culture and DNA transfection

HEK293 and PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (FBS), 5 mM glutamine, and 100 units/ml penicillin/streptomycin at

37 °C in humidified air supplemented with 5% CO_2. HEK293 cells stably transfected with PDE4A4 [45] were cultured in standard HEK293 medium supplied with 1 mg/ml G418 sulphate.

4.3. DNA transfection

HEK293 and PC12 cells were transfected using PolyFect reagent (Qiagen) according to the manufacturer's guidelines and kept in culture for 24–48 h before experiments were performed.

4.4. Protein expression & purification

Proteins were expressed in the *E. coli* BL21 (DE3) strain in LB medium by induction using 1 mM IPTG at an OD_{600} of 0.6. Cells were then grown overnight at 30 °C before being harvested by a 10 min centrifugation at 5000 *g* and 4 °C. MBP fusion proteins were purified using amylose affinity resin (New England Biolabs), and GST fusion proteins using glutathione sepharose (GE Healthcare).

In brief, the cell pellets were resuspended in 25 ml (per litre of initial culture) ice cold PBS containing protease inhibitors (Complete, Roche) and 1 mM DTT. The suspension was incubated for 20 min with 30 mg lysozyme (Sigma) before being lysed by sonication for 10 min on ice. This lysate was centrifuged for 45 min at 45,000 *g* to pellet insoluble materials. 2 ml of washed amylose resin (MBP) or glutathione sepharose (GST) was added to the clear supernatant before being rotated for 2 h at 4 °C. The beads were transferred to a disposable column, washed 5 times with ice cold PBS and eluted three times with either 10 mM maltose (MBP) or 10 mM reduced glutathione solution (GST) in PBS. The eluted proteins were dialysed three times in protein buffer (50 mM Tris–HCl pH 8, 100 mM NaCl) and stored at −80 °C until used.

4.5. Immunoblotting antibodies

Samples were separated by SDS-PAGE (4–12% gradient Bis/Tris gels) and transferred to Immobilon-FL PVDF membranes (Millipore) which were immunoblotted using antibodies against GST (sc-138, Santa Cruz Biotechnology), flag (A8592, Sigma-Aldrich), p62 (ab56416, Abcam), GAPDH (MAB374, Millipore), NBR1 (5202, Cell Signaling Technology), PKCi (2998, Cell Signaling Technology), PKCz (ab51157, Abcam), MEK5 (AB3184, Millipore), LC3 (ab48394, Abcam), phospho-Akt (Ser473; 4051, Cell Signaling Technology) and Akt (9272, Cell Signaling Technology).

For specific detection of Ser24-phosphorylated p62 we commissioned the development of a rabbit polyclonal antiserum based on the epitope CREIRRFpSF (encompassing R18-F25 of the p62 sequence in human, mouse and rat; Amsbio). See Fig. S2 for characterisation details.

Quantification of Western blots was carried out using densitometry in the ImageJ software. Details regarding statistical analysis are included in the figure legends.

4.6. ELISA

The ELISA to measure protein–protein interactions was carried out as described earlier [46,47]. Briefly, wells of ELISA plates (3576, Corning), were coated with GST or GST-PDE4A4 protein (256 nM). After blocking free binding sites with 0.5% skimmed milk in TBS-T, either MBP, MBP-p62-wt or MBP-p62-S24D were added in various concentrations (see Figs. 1A, 4E for details). Plates were washed with TBS-T, and the amount of bound protein determined using an anti-MBP-HRP fusion antibody (ab49923, Abcam).

4.7. Peptide array

Peptide libraries were synthesized by automated SPOT synthesis [17]. Overlapping peptides (25 amino acids in length) were spotted on Whatman 50 cellulose membranes according to standard protocols by using Fmoc chemistry with the AutoSpot Robot ASS 222 (Intavis Bioanalytical Instruments AG). Membranes were overlaid with 10 μg/ml recombinant GST or GST-PDE4A4. Bound recombinant protein was detected using HRP-fused anti-GST (1:2000; Santa Cruz Biotechnology). Alanine scanning was performed as described [48].

4.8. ^{32}P kinase assay (array + FL)

In order to test phosphorylation of a peptide array through recombinant kinase, the array membrane was blocked overnight with 5% phosphoblocker (Cell Biolabs) in phosphorylation buffer (20 mM Tris, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM ATP, pH 7.5) before being incubated with 0.37 MBq of γ-^{32}P-ATP EasyTides (Perkin Elmer) and 25 U recombinant PKA catalytic subunit (V5161, Promega) for 30 min at 30 °C. After stringent washing with 1 M NaCl, 5% phosphoric acid and water, the membranes were dried and exposed for 3 days to film in a Kodak BioMax cassette.

For phosphorylation of purified full length proteins, 1 μg each of MBP, MBP-p62-wt, MBP-p62-S24A and MBP-PDE4A4 were diluted in phosphorylation buffer. For the phosphorylation, 0.37 MBq of γ-^{32}P-ATP and 0.2 μg of PKA catalytic subunit (60837, Abcam) were added and the mixture incubated at 30 °C for 30 min. 5× Laemmli sample buffer was added to the samples which were run on a 4–12% gradient SDS-PAGE Bis/Tris gel. This was dried and exposed for 3 days to film in a Kodak BioMax cassette.

4.9. Immunoprecipitation

For immunoprecipitations, either native cells or transfected cells were washed with ice-cold PBS before the addition of lysis buffer (25 mM Hepes, pH 7.5, 2.5 mM EDTA, 225 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, PhosStop phosphatase inhibitor tablets (Roche) and Complete EDTA-free protease inhibitor cocktail tablets (Roche)). Lysates were cleared by centrifugation at 14,000 *g* for 20 min, and protein content determined by Bradford assay. Lysates containing equal amounts of protein were then pre-cleared by incubation with Protein G sepharose (4 Fast Flow, Amersham Biosciences), before immunoprecipitation of p62 by the addition of a p62-specific antibody (ab56416, Abcam) along with Protein G sepharose overnight at 4 °C. Immunoprecipitates were then washed 5× with lysis buffer, and proteins eluted by the addition of Laemmli sample buffer. Samples were subjected to SDS-PAGE before being transferred to Immobilon-FL PVDF membrane for immunoblot.

4.10. Mass spectrometry

Excised protein gel bands were washed, shrunken, and dried in a vacuum centrifuge. Before tryptic digestion, disulphide bonds were reduced with 10 mM DTT in 25 mM ammonium bicarbonate for 45 min at 55 °C. Alkylation was performed by replacing the DTT solution with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min at 25 °C in the dark. The dried gel pieces were incubated with 50 ng of trypsin (sequencing grade; Promega, Madison, WI) in 20 μl of 50 mM ammonium bicarbonate at 37 °C overnight. To extract the peptides, 20 μl of 0.5% TFA in acetonitrile was added, and the separated liquid was taken to dryness under vacuum. For MS analysis, the peptide mixture was dissolved in 6 μl of 0.1% TFA in acetonitrile/water (5:95). NanoLC–MS/MS experiments were performed on a quadrupole time-of-flight mass spectrometer, Q-Tof Ultima (Micromass, Manchester, UK) coupled on a CapLC liquid chromatography system as previously described [49].

In brief, peptides were separated using an analytical column (Atlantis dC18, 3 μm, 100 Å, 150 mm × 75 μm i.d.; Waters GmbH, Eschborn, Germany) at an eluent flow rate of 150 nl/min using a linear gradient 4–40% of acetonitrile in 0.1% aqueous formic acid. Data-dependent acquisition was performed using one MS scan followed by MS/MS

scans of the most abundant peaks. The processed MS/MS spectra and MASCOT server version 2.0 (Matrix Science Ltd., London, UK) were used to search in house against the NCBI non-redundant protein database (version 20072010). A maximum of two missed cleavages was allowed, and the mass tolerance of the precursor and sequence ions was set to 100 ppm and 0.05 Da, respectively. The search includes carbamidomethyl modification of cysteine, methionine oxidation, and phosphorylation of serine or threonine. To identify phosphorylated peptides, probability-based scoring (MASCOT identity score, $p < 0.05$) was used. In addition, tandem mass spectra of phosphopeptides were manually verified.

4.11. Fluorescence microscopy

Cells in 6-well-plates transfected with either mCherry-p62-wt or -S24D were observed under an Axiovert 200 M fluorescence microscope (Zeiss) 24 h after transfection using a 40× LD PlanNeoFluar objective. Several photos were taken from each of several wells transfected under identical conditions using the fitted AxioCamMR camera. Quantification was carried out by counting all cytoplasmic bodies in equally sized microscopic image sections. For each condition, images from n = 3 independent experiments were used. Statistical analysis was done by one-way ANOVA to compare all means (P = 0.0072) and by unpaired *t*-test to compare p62-wt *vs.* -S24A (P = 0.5073; not significant).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.07.021.

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