Bioinformatic and experimental survey of 14-3-3-binding sites

Catherine JOHNSON, Sandra CROWTHER, Margaret J. STAFFORD, David G. CAMPBELL, Rachel TOTH and Carol MACINTOSH

MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

More than 200 phosphorylated 14-3-3-binding sites in the literature were analysed to define 14-3-3 specificities, identify relevant protein kinases, and give insights into how cellular 14-3-3/phosphoprotein networks work. Mode I RXX(pS/pT)XP motifs dominate, although the +2 proline residue occurs in less than half, and LX(R/K)/SX(pS/pT)XP is prominent in plant 14-3-3-binding sites. Proline at +1 is rarely reported, and such motifs did not stand up to experimental reanalysis of human Ndel1. Instead, we discovered that 14-3-3 interacts with two residues that are phosphorylated by basophilic kinases and located in the DISC1 (disrupted-in-schizophrenia 1)-interacting region of Ndel1 that is implicated in cognitive disorders. These data conform with (disrupted-in-schizophrenia 1)-interacting region of Ndel1 that is implicated in cognitive disorders. These data conform with

INTRODUCTION

14-3-3s are dimeric proteins that dock on to phosphorylated serine and threonine residues in hundreds of intracellular target proteins, including enzymes and structural components of metabolism, vesicle and protein trafficking, cytokoskeletal regulation, DNA replication, transcription, translation, membrane receptors, reversible ubiquitination and phosphorylation, and effectors of small GTPases [1–11]. Understanding when and how 14-3-3s impact on these targets therefore offers a fantastic opportunity to gain regulatory insights into many areas of eukaryotic biology. We and others are developing differential proteomics methods to characterize the large-scale shifts in the subsets of targets that become phosphorylated and bind to 14-3-3s when specific signalling pathways are activated [12,13]. To fully interpret these global data, however, we must also have a precise understanding of the specificities with which 14-3-3s engage with individual targets.

A 14-3-3 dimer comprises two curved L-shaped monomers, arranged with diagonal symmetry to generate a boat-shaped central groove. The inner corner of each L contains a pocket for binding to phosphorylated residues on target proteins [14,15]. Inspection of the few 14-3-3-binding sites known for binding to phosphorylated residues on target proteins [14,15]. Inspection of the few 14-3-3-binding sites known

1 To whom correspondence should be addressed (email c.macintosh@dundee.ac.uk).

Abbreviations used: AANAT, serotonin acetyltransferase; AGC, protein kinase A/protein kinase G/protein kinase C family kinase; AMPK, AMP-activated protein kinase; BAD, Bcl-XL/Bcl-2-associated death promoter; CaMK, Ca2+/calmodulin-dependent protein kinase; CDK5, cyclin-dependent kinase 5; DIG, digoxigenin; DISC1, disrupted-in-schizophrenia 1; DSS, Division of Signal Transduction Therapy; EST, expressed sequence tag; FOXO, Forkhead box O; GLUT4, glucose transporter 4; GST, glutathione transferase; HA, haemagglutinin; HAP1A, Huntingtin-associated protein 1A; HDAC, histone deacetylase; HEK, human embryonic kidney; KLC, kinesin light chain; MARK, microtubule affinity-regulating kinase; PI4K, phosphoinositide 4-kinase; PKB, protein kinase B; PKC, protein kinase C; PP2A, protein phosphatase 2A; RSK, ribosomal S6 kinase; YAP1, yes-associated protein 1.
its phosphorylation is essential for phosphorylation of the real 14-3-3-interacting site elsewhere on the protein. For example, a S256A mutation of FOXO3 (forkhead box O3; also known as FKHR (forkhead in rhabdosarcoma)) inhibits phosphorylation of the Thr24 14-3-3-binding site [20]. We decided not to prejudge the collection of information, except where there were clear uncertainties, but accept that the quality of the dataset is likely to be diluted by wrongly assigned sites. Our aims were to look for patterns in the data, draw inferences about how 14-3-3 proteins work, make predictions that might help in finding novel 14-3-3-binding sites, identify research questions, and provide the foundation of a database that can be updated in future. We checked and corrected one obvious anomaly by identifying two 14-3-3-binding sites on Ndel1 (previously known as NUDEL), instead of the three phosphoSer/Thr-Pro sites that had been previously proposed to interact with 14-3-3 [21]. The deregulation of Ndel1 and associated proteins is strongly implicated in disorders of brain development and cognition, including schizophrenia, and our findings change perspectives on how 14-3-3 impacts this system. More generally, our survey highlights the special mechanical, signalling and evolutionary properties that emerge from the configuration of a 14-3-3 dimer binding to a doubly phosphorylated target.

**EXPERIMENTAL**

**Plasmid construction**

The coding region for human Ndel1 (NCBI accession number AY004871.1; GI:12043568) was amplified from IMAGE consortium EST (expressed sequence tag) clone 4820855 using the primers 5′-GAGGATCCCTAGGATGTTGAGATATACCA-GATTTCCTTCAG-3′ and 5′-GTGCGGCCGCTCACACACATGGAGGGCCAGCATACC-3′. The PCR product was digested with BamHI and NotI and ligated into the pCMV5.HA expression vector [pCMV5 containing an HA (haemagglutinin) tag sequence] for expression of the tagged fusion protein HA–Ndel1. A P322T mutation originating from the EST was mutated back to proline using the mutagenic primers 5′-CAGTAAAC-GGTCTTGACCGTCTCTTCCTCCTCC-3′ and 5′-GGAGGAGGAGGGGCTTCAAGACCCCGTTACTG-3′ so that the sequence exactly matches the NCBI accession number AY004871.1; GI:12043568, and the resulting protein matches the Swiss-Prot entry Q9GZM8. S251A and S336A mutants, doublemutant S251A/S336A, triple-mutant S198A/T219A/S231A and the 5× alanine mutant (S198A/T219A/S231A/S251A/S336A) of Ndel1 were generated by PCR mutagenesis using KOD Hot Start DNA polymerase (Novagen). Bacterial expression plasmids expressing GST (glutathione transferase)-tagged wild-type and mutant versions of Ndel1 were generated by subcloning the BamHI/NotI insert from pCMV5.HA plasmids into pGEX6P-1 (Amersham).

**Cell culture, lysis and immunoprecipitations**

Human HEK (human embryonic kidney)-293 cells were cultured on 10-cm diameter dishes in medium containing 10% (v/v) FBS (foetal bovine serum), and, at 24 h after transfection with the indicated plasmids, cells were rinsed with ice-cold PBS and lysed in 0.3 ml of ice-cold lysis buffer [50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcin-LR, 0.1% 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM PMSF and one ‘Complete’ protease inhibitor cocktail tablet (Roche) per 50 ml]. For immunoprecipitations, sheep anti-HA antibody (raised against the synthetic peptide PYDYDPYDA) at 3 μg of antibody/mg of lysis was first coupled to Protein G–Sepharose (30 μl of 50% slurry in lysis buffer) by mixing at room temperature (20°C) for 1 h. Lysate (3 mg) was then added and mixed for a further 2 h at 4°C. Cells were washed twice with 50 mM Tris/HCl (pH 7.5) and 500 mM NaCl, and twice with 50 mM Tris/HCl (pH 7.5), 1 mM EDTA and 0.1% 2-mercaptoethanol, pelleting Sepharose in between washes at 12000 g. Immunoprecipitates were extracted into SDS-sample buffer, and separated by SDS/PAGE (10% gels).

**Antibodies and kinases**

Phospho-specific antibodies were raised against the following peptide sequences derived from Nde1: TPSARIpSALN [residues 245–254 where pS represents phosphorylated Ser251 (pSer)]; and CGSSRPSpAPGMLP (cysteine plus residues 330–342 where pS represents pSer336). Peptides were coupled separately via the N-terminus and added cysteine residue respectively to BSA and keyhole-limpet haemocyanin, mixed and injected into sheep at Diagnostics Scotland. The antibodies were affinity purified by the DSTT (Division of Signal Transduction Therapy, University of Dundee, Dundee, Scotland, U.K.) on phosphopeptide–Sepharose columns and the flow-throughs collected. In Western blot analysis, the phospho-specific antibodies were used at 1 μg/ml containing 10 μg/ml of the unphosphorylated peptide. Mouse anti-HA and sheep anti-HA antibodies were raised against the peptide PYDYDPYDA. Sheep anti-GST antibody was raised against GST expressed from pGEX-4T and used at 0.1 μg/ml. The kinases CDK5 (cyclin-dependent kinase 5)/p35 and Aurora A were from Upstate, while human Aurora B was produced in the DSTT.

**14-3-3 overlays and Western blot analysis**

Membranes were incubated in 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.2% Tween 20 containing 5% (w/v) dried skimmed milk powder (Marvel) and were immunoblotted at 4°C for 16 h using the antibodies indicated. Detection was performed using HRP (horseradish peroxidase)-conjugated secondary antibodies (Promega) and ECL® (enhanced chemiluminescence reagent; Amersham BioSciences) for all Western blots of recombinant proteins and DIG (digoxigenin)–14-3-3 overlays, which use DIG-labelled 14-3-3 (a mix of the Saccharomyces cerevisiae BMH1 and BMH2 14-3-3 isoforms) in place of primary antibody, as described in [12].

**In vitro phosphorylation**

GST–Ndel1 proteins were phosphorylated in vitro with the kinases indicated for 30 min at 30°C. The reaction buffer contained 25 mM Tris/HCl (pH 7.5) and 1 mM EDTA, and reactions were started by adding 0.1 mM cold ATP/10 mM magnesium acetate. SDS sample buffer was added to terminate the reactions.

**RESULTS AND DISCUSSION**

**Most reported 14-3-3-binding sites conform to mode I motifs, whereas plants reveal a mode I variant characterized by leucine at −5 and serine at −2**

We collated details of sites on target proteins that have been reported to bind directly to 14-3-3 proteins (Supplementary Table 1 at http://www.BiochemJ.org/bj/427/bj4270069add.htm).
Two is the key to 14-3-3

Figure 1  WebLogo analyses of 14-3-3-binding sites of the proteins listed in Supplementary Table 1

(A) The sequences containing each of the phosphorylated 14-3-3-binding sites listed in Supplementary Table 1 were aligned relative to one another, centred on the phosphorylated residue with 20 residues either side (Supplementary Table 2). The sequence logo was created using WebLogo Version 2.8.2 (http://weblogo.berkeley.edu/; [82,83]), with 'frequency plot' selected. The height of symbols for each position in the sequence indicates the relative frequency of each amino acid at that position. Also shown are separate sequence logos for 14-3-3-binding sites from mammalian and plant species respectively. (B) WebLogo plots of 14-3-3-binding sites with and without leucine, arginine and histidine residues in the −5 position. (C) WebLogo plots of 14-3-3-binding sites for proteins where pairs of sites have been identified.

and searched these data for notable features (Figure 1 and Supplementary Table 2 at http://www.BiochemJ.org/bj/427/bj4270069add.htm). A WebLogo frequency plot of the combined >200 reported sites (Figure 1A) shows that mode I RXX(pS/pT)XP motifs dominate, and leucine and arginine are the most common residues in the −5 position. Serine and proline are the most common residues in the extended sequences flanking the 14-3-3-binding sites. However, the proline residue at +2 is found in only ∼50 % of target sites. Structural data show that the proline residue at +2 in the canonical motifs twists the peptide back out of the docking site [19,22]. Other residues may also allow such a twist, but it is not clear whether this is always essential for 14-3-3 binding.

Few reported 14-3-3-binding sites have a +1 proline residue, which contrasts with phosphoproteomic studies of cell lysates and subcellular fractions, where phosphoSer-Pro is the most commonly reported phosphorylation motif overall [23]. Proline-directed kinases therefore do not phosphorylate 14-3-3-binding sites, although they can play a regulatory role by phosphorylating the 14-3-3 themselves [24]. Similarly, no reported 14-3-3-binding sites conform to the canonical consensus for phosphorylation by casein kinase II, [namely (pS/pT)(X1)(X2)(D/S/pS) where X1 is not proline], which is probably the second most common type of motif in the mammalian phosphoproteome as a whole [25]. Generally, it has been reported that glutamate and aspartate do not provide good phosphomimetic residues with respect to 14-3-3 binding to target proteins, although there are exceptions such as synaptophysin 2/myopodin [26].

While the optimal mode I and II 14-3-3-binding motifs were defined using phosphopeptides [18,19], target sites in proteins must satisfy the specificity requirements for both 14-3-3s and the protein kinases that create the sites in the first place. Theoretically, kinases and 14-3-3s could each use distinct specificity determinants to home-in on the same site. The data indicate congruence, however, in that basic residues in positions −3 to −5 relative to the phosphorylated site are found in most 14-3-3-binding sites, and basophilic kinases in the AGC [protein kinase A/protein kinase G/PKC (protein kinase C) family

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kinase] and CaMK (Ca\(^{2+}\)/calmodulin-dependent protein kinase) subfamilies of the kinase are most commonly implicated in the phosphorylation of 14-3-3 binding sites [27–29].

Linking specific kinases firmly to target sites is challenging. However, it should be possible to sort 14-3-3-binding sites into motif subsets to aid the task of defining relevant protein kinases. One striking subset comprises the plant 14-3-3-binding sites, for which the distinct motif LX(R/K)SX (pS/pT)XP is most prevalent (Figures 1A and 1B, and Supplementary Table 2). This motif resembles a mode I site, except that that either an arginine or lysine residue can be in the −3 position and there is an additional leucine residue at −5 and a serine residue at −2. Three plant proteins with a leucine at −5 in their 14-3-3-binding proteins, namely nitrate reductase (NIA1 and NIA2), TPS5 (trehalose-phosphate synthase 5) and F2KP (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), have been proposed to be co-ordinately regulated in leaves in the dark [30], raising the question of whether the brassinosteroid receptor (BZR1) and vacuolar potassium channel (KCO1) with similar motifs might also be targets of the same kinase(s). A similar motif was also identified in 30 mammalian 14-3-3-binding sites, except that a leucine residue at −5 was accompanied by either a serine or threonine residue at −2 (Figure 1B). Several CaMK enzymes exhibit a preference for a leucine residue at −5, including mammalian PKD (protein kinase D), Chk (checkpoint kinase) 1, Chk2, AMPK (AMP-activated protein kinase). AMPK-related kinases such as C-TAK1/MARK3 (microtubule affinity-regulating kinase 3), and the plant AMPK-related SrKks [31]. Further experiments are needed to clarify any reason for the apparent coupling between a leucine residue at −5 and a serine residue at −2 in the plant 14-3-3-binding proteins. Because several of the plant proteins have been studied in the context of the same dark-activated signalling pathway [30–32], it seems likely that the −5 leucine and −2 serine guide phosphorylation by the relevant kinase(s), rather than subsequent selection of 14-3-3 isoforms. Defining the points of contact of the −5 leucine with the kinases and 14-3-3s should be informative.

Another subset of 36 14-3-3-binding sites has an arginine residue in the −5 position, which is always accompanied by an arginine residue at −3 (Figure 1B). Arginine residues at −5 and −3 are determinants for phosphorylation by enzymes including PKB (protein kinase B)/Akt, PIM kinases and RSKs (ribosomal S6 kinases), although the presence of these residues does not guarantee phosphorylation by these kinases, and each kinase has further selection criteria [23,32]. None of the plant 14-3-3-binding sites has an arginine at −5. This could reflect the small sample size for the plant proteins, or might be biologically meaningful, given that plants do not have direct counterparts of PKB/Akt, PIM kinases or RSKs [33,34], although they have many other CaMK and AGC protein kinases [35]. We note that PKB/Akt has been assigned responsibility for phosphorylating a number of 14-3-3-binding sites that do not conform to the canonical consensus for this kinase. In the case of the mammalian WW-domain transcriptional regulator YAP1 (yes-associated protein 1), the 14-3-3-binding sites have a histidine residue at −5 and an arginine residue at −3, and phosphorylation of YAP1 was originally attributed to PKB/Akt [36], but later discovered to be catalysed by the Warts/Lats kinases in the NDR branch of the AGC kinases [37,38], as is the related protein TAZ (WWTR1). FAM82A2 and SRPK2 also have reported 14-3-3-binding sites with a histidine residue at −5 (Figure 1B and Supplementary Table 1).

As the dataset of 14-3-3 target sites grows, the sorting into different motif subsets should become more obvious, facilitating the task of matching sites with potential protein kinases.

### Mode III 14-3-3-binding sites

Unlike most targets, 14-3-3s activate the plant plasma membrane H\(^+\)-ATPase by binding to a so-called C-terminal mode III binding motif SW(pT)X-COOH in which the phosphorylated residue is the penultimate threonine residue in the C-terminal autoinhibitory domain [15]. It has been suggested that mode III 14-3-3-binding sites are more widespread in nature [39,40], with the mammalian IL (interleukin)-9 receptor α-chain [41], two-pore-domain potassium channels TASK1, 3 and 5 [42], the nicotinic acetylcholine α4β2 receptor [43] and HAP1A (Huntingtin-associated protein 1A) [44] being proposed as mode III interactors. However, to our knowledge, phosphorylation of the penultimate residue has not been demonstrated directly for the channels and receptors, whereas the threonine residue of the mode III site on HAP1A is restricted to the rat version of this protein. Some proteins, such as AANAT (serotonin acetyltransferase) have one mode I and one mode III site [45]. While the mode III sites are at the C-termini, we note that several proteins have 14-3-3-binding sites that are close to their N-termini (Supplementary Tables 1 and 2).

### 14-3-3 binding to unphosphorylated proteins

Generally, 14-3-3s do not bind appreciably to unphosphorylated proteins, with the exception of the ExoS, which is injected into host mammalian cells by the pathogenic bacterium *Pseudomonas aeruginosa* and contains a novel binding motif G\(^{32}\)LLDALDLAS\(^{130}\), in which the hydrophobic residues (Leu\(^{32}\), Leu\(^{42}\) and Leu\(^{59}\)) are essential for binding to cellular 14-3-3s and pathogenesis [46,47]. Similarly, the rolB protein from the hairy root bacterium *Agrobacterium rhizogenes* interacts with 14-3-3 inside Arabidopsis cells, possibly in a phosphorylation-independent interaction [48]. Phosphorylation-independent interactions between physiological targets and 14-3-3s have also been reported [49,50], although these studies do not exclude the possibility that the reported targets interact with an unknown intermediary protein that is phosphorylated and binds to 14-3-3s.

### Two tandem 14-3-3-binding sites within disordered regions and straddling functional domains

The 14-3-3s are dimeric proteins with diagonal symmetry, such that the two phosphate-binding sites are diagonally opposite each other and ~34.4 Å (1 Å = 0.1 nm) apart across the floor of the binding groove for the human 14-3-3ζ dimer (Figure 2), although the spacing may be greater due to some flexibility between the 14-3-3 monomers [51]. In theory, 34 Å could be bridged by a minimum of 15 residues from one phosphorylated residue to the other (inclusive) in fully extended conformation, although more would be needed for a dimer that adopts a more open conformation [51]. For two tandem phosphorylated sites to dock into a single 14-3-3 dimer, the target protein must thread out and in, in anti-parallel orientation, as has been shown in the structure of the 14-3-3 bound to a doubly phosphorylated peptide derived from PKCζ [14].

Thus far, approx. 50 proteins have been reported to contain two phosphorylated 14-3-3-binding sites (Figure 1C and Supplementary Tables 1 and 2). For a few targets, there is sufficient structural and kinetic binding data to support the hypothesis that the two phosphorylated sites on the same target bind to either side of a 14-3-3 dimer [14]. It seems reasonable to propose that the configuration of a 14-3-3 dimer engaging with two tandem phosphorylated sites on the same target might be a more general
phenomenon. If so, defining the locations of the two sites should give clues about how the 14-3-3 dimer acts mechanically to change the function of the target.

The paired sites can be considered in two broad groups: one set has a short stretch of 40 amino acid residues or less between the phosphorylated sites, which are in regions of predicted disorder in N- and C-terminal tails or between functional domains on the protein, and it may be that the binding of a 14-3-3 dimer imposes order [52,53]. This class includes enzymes whose activities are altered by 14-3-3 binding. For tyrosine hydroxylase, for example, biophysical measurements indicate that the bound 14-3-3 dimer acts as a lever that forces a conformational change which activates the enzyme [54]. In other proteins, the dual 14-3-3-binding sites are further apart and straddle either side of one or more folded domains. Structural analyses show how the two 14-3-3-binding sites at phosphoThr and phosphoSer of FOXO4 dock its forkhead domain into the central channel of the 14-3-3 dimer, occluding the DNA-binding interface of the transcription factor without causing any dramatic conformational change [55]. There are sometimes much longer distances between two phosphorylated sites on the listed targets, 360 residues for TBC1D1, for example [56], which is too much for the intervening sequences to be enclosed within the central groove of 14-3-3. In the absence of structural data, one can speculate that the 14-3-3 dimer provides a partial mask, or perhaps the 14-3-3 dimer pins back the flanking regions to better present the intervening domain for other interactions.

In many papers that define one 14-3-3-interaction motif, there are also data suggestive of a second unidentified motif on the same target. For example, Demmel et al. [57] pinpoint phosphoSer as essential for 14-3-3 binding to yeast PI4K (PIk1p; phosphoinositide 4-kinase), and their data indicate that a second unknown binding site exists in the catalytic region of this enzyme. In cases where one phosphate dominates for high-affinity binding, it is possible that a second lower-affinity-binding site on the same target may be needed for the target to slot into place properly so that the 14-3-3 dimer can impose a functional change in the target, as proposed in the ‘gatekeeper’ hypothesis of Yaffe [58]. These secondary low-affinity phosphorylation sites can be difficult to spot by mutagenesis analysis if they do not contribute much to the affinity of binding to 14-3-3.

14-3-3 dimers as logic gates and coincidence detectors that integrate incoming signals from two different protein kinases

As well as enabling a 14-3-3 dimer to act mechanically as a lever, clamp or mask, docking on to two phosphorylated residues confers a 14-3-3 with the special signalling properties of a digital logic gate that converts two inputs into a single output. If either phosphorylated site is sufficient to generate an output, the system would be an ‘OR’ gate, whereas if two phosphorylated sites must be engaged before the 14-3-3 can generate a mechanical action on the target, the 14-3-3 would be an ‘AND’ gate. An ‘AND’ gate would suppress noise because no action would occur when only one 14-3-3-binding site is phosphorylated, and be a coincidence detector if each site is phosphorylated by a different protein kinase and/or dephosphorylated by a different protein phosphatase. Figure 3 summarizes the variety of configurations in which a 14-3-3 dimer can engage with one or two phosphorylated sites on target proteins.

Several authors have highlighted cases where two tandem phosphorylated sites can be phosphorylated by distinct protein kinases, namely TBC1D1, MARK2/Par-1b, BAD (Bcl-XL/Bcl-2-associated death promotor) and myopodin [25,56,59,60]. For example, the two 14-3-3-binding sites on the apoptosis regulator BAD can be phosphorylated variously by ERK1/2 (extracellular-signal-regulated kinase 1/2)-activated kinases, PKB/Akt and PKA. Phosphorylation of either 14-3-3-binding site is sufficient to trigger dissociation of BAD from the anti-apoptotic protein Bcl-XL, inhibiting the pro-apoptotic activity of BAD. For BAD, therefore, the 14-3-3 appears to act as an ‘OR’ gate because phosphorylation of either 14-3-3-binding site induces the interaction and thereby inhibits apoptosis [59].

As a start to breaking down the task of assigning each 14-3-3-target interaction into ‘AND’ gate, ‘OR’ gate and/or ‘coincidence detector’ categories, we examined the 50 targets with paired 14-3-3-binding sites to determine whether they were pairs of similar or different motifs. Several proteins contained RXRXXSpS at both sites, including mammalian ADAM2 (a disintegrin and metalloproteinase 2), Mdm2 (murine double minute 2), TBC1D4, FOXO1, FOXO2, FOXO3, BAD, therefore, the 14-3-3 appears to act as an ‘OR’ gate because phosphorylation of either 14-3-3-binding site induces the interaction and thereby inhibits apoptosis [59].

Figure 2 Structure of a 14-3-3 dimer, based on that within the AANAT/14-3-3 structure [22] in which the two phosphate-binding sites are 34.4 Å apart

We consider that the two binding sites are arranged in an antiparallel orientation, it is also useful to consider the distance of ~30 Å between the C-atom of the proline of a six-amino-acid phosphopeptide [RRH(pT)LP derived from AANAT] bound to one side of the binding groove and the C-atom of the N-terminal residue of the second peptide (arginine). This distance could be covered with a peptide containing approx. 8 amino acids in fully extended conformation, which would make 8 + 3 + 4 = 15 residues from one phosphorylated residue to the other (inclusive of the phosphorylated residues). Allowing (say) 4 amino acid residues for turns, would give a minimum of ~19 amino acid residues. This Figure is provided courtesy of Thomas Obsil (Charles University in Prague, Prague, Czech Republic).

Figure 3 The variety of configurations in which a 14-3-3 dimer can engage with one or two phosphorylated sites on target proteins.
proteins with the plant plasma membrane H+-ATPase is inhibited by tyrosine phosphorylation [61], whereas dephosphorylation of Ser376 in response to IR radiation enables 14-3-3s to bind to the nearby phosphoSer378 on p53 [62], and acetylation at Lys9 or Lys10 enhances the phosphoserine-dependent binding of 14-3-3s, although the plant and animal enzymes differ considerably in their overall architectures and biological contexts [65,66].

As summarized in Supplementary Table 1, the class IIa HDACs (histone deacetylases; comprising HDACs 4, 5 6 and 9) share identical 14-3-3-binding sites that regulate their nuclear–cytoplasmic shuttling and are phosphorylated by various protein kinases, including AMPK [67]. Similarly, the 14-3-3-binding sites are conserved within FOXO transcription factors (1, 3 and 4) [55], and these families presumably evolved with differences in other structural and functional aspects of the proteins.

In contrast, 14-3-3s display distinct modes of binding to two related Rab GTPase-activating proteins, namely TBC1D4 (AS160) and TBC1D1, involved in trafficking of the GLUT4 (glucose transporter 4) transporter to the cell surface for uptake of glucose into cells. Both AS160 and TBC1D1 contain two 14-3-3-binding sites straddling either side of the PTB2 (phosphotyrosine-binding domain 2) domain on these proteins. The phosphoThr642 14-3-3-binding site on AS160 is similar to phosphoThr596 on TBC1D1, and these residues appear to be phosphorylated by PKB/Akt in response to insulin and IGF1 (insulin-like growth factor 1). In contrast, the phosphoSer341 site on AS160 is distinct from the phosphoSer237 14-3-3-binding site on TBC1D1, with the latter being phosphorylated by AMPK and/or enzyme with similar specificity [56]. We hypothesize, therefore, that differential expression and regulation of AS160 and TBC1D1 may help explain why GLUT4 trafficking is more or less sensitive to insulin and AMPK activators in different tissue types.

TBC1D1 is the more ancient protein [56], raising the question of how the phosphoSer341 region of TBC1D1 transmuted into phosphoSer341 of AS160. In one possible scenario, we propose that the dimeric 14-3-3 may have facilitated this evolutionary event (Figure 4). Suppose that a copy of TBC1D1 arose by gene duplication. In the copy, if one 14-3-3-binding site (Thr642/Thr596) remained unchanged, this might provide ‘good enough’ regulation by 14-3-3 to avoid serious loss of control, thereby allowing the second site to vary. In time, a new and useful consensus for a different protein kinase arose at the second site, and the resulting AS160 became a paralogue with distinct regulatory properties from the original TBC1D1.

**Figure 3 14-3-3 dimers read digital codes: a model exploring the signalling implications of 14-3-3 binding to two phosphorylated sites on the same target**

The term ‘digital code’ usually refers to a binary system of zeros and ones. Because 14-3-3s are dimeric, they can be considered as a coupled binary device that can distinguish patterns of zero, one or two phosphorylated target sites. Generally, zero phosphates means no binding, with the exception of ExoS from *P. aeruginosa* [47]. One phosphate is sometimes enough to create a sufficiently high-affinity 14-3-3-binding site, and sometimes not. But even when one phosphate dominates for high-affinity binding, sometimes a second lower-affinity-binding site is needed for the target to slot in properly. These second low-affinity phosphorylation sites can be difficult to spot by mutagenesis analysis if they do not contribute much to the affinity of binding to 14-3-3s, but secondary sites may have to be in place for the 14-3-3 to impose a functional change in the target (the ‘gatekeeper’ hypothesis; [58]). The two phosphorylated sites can be inserted by the same kinase, or in one or two cases, different kinases have been found to phosphorylate each site on the same target. The configuration with two kinases turns a 14-3-3 dimer into a coincidence-detecting device where two different signalling inputs have to be received before 14-3-3s can bind to the target and a functional change is induced. In addition, multisite phosphorylation at other sites can enhance or inhibit 14-3-3 interactions, either by influencing the phosphorylation status of the 14-3-3-binding sites, by direct steric modulation of the binding to 14-3-3s, and/or by phosphorylation of the 14-3-3s themselves.

Multi-protein families that bind to 14-3-3s

14-3-3s, particularly residues within the central groove, are highly conserved in all eukaryotes. Thus far, however, only one pair of protein orthologues share a conserved 14-3-3-binding site across the animal and fungal Kingdoms, namely the Golgi trafficking regulator PI4K (mammalian IIIβ isoform and *Saccharomyces cerevisiae* Pik1) (Supplementary Table 1). While the Cdc25 (cell division cycle 25) cell-cycle-regulating protein phosphatases in mammalian cells and the fission yeast bind directly to 14-3-3s, the precise site has not been defined in the yeast protein [64] (Supplementary Table 1). Mammalian and plant forms of fructose 2,6-bisphosphate kinase/phosphatase interact with 14-3-3s, although the plant and animal enzymes differ considerably in their overall architectures and biological contexts [65,66].

As summarized in Supplementary Table 1, the class IIa HDACs (histone deacetylases; comprising HDACs 4, 5 6 and 9) share identical 14-3-3-binding sites that regulate their nuclear–cytoplasmic shuttling and are phosphorylated by various protein kinases, including AMPK [67]. Similarly, the 14-3-3-binding sites are conserved within FOXO transcription factors (1, 3 and 4) [55], and these families presumably evolved with differences in other structural and functional aspects of the proteins.
Figure 4 Hypothesis for the role of 14-3-3 dimers in driving evolutionary change of 14-3-3-binding sites in paralogous proteins

If a copy of a 14-3-3-binding protein were to arise by gene duplication, we hypothesize that because the 14-3-3s are dimers, the function of the copy could be kept under sufficient control if one 14-3-3-binding site remained unchanged ‘the lynchpin site’, while allowing sequence divergence to occur at the second 14-3-3-binding site. If a useful consensus for phosphorylation by a different kinase were to arise at the evolving site, the result would be the generation of a parologue with distinct regulatory inputs from the original. Whether or not 14-3-3s have actually driven evolution in this way, this theory should stimulate experimentation to compare the regulatory details of 14-3-3 binding to paralogous members of multi-protein families.

Physiologically, the availability of differentially regulated target paralogues could allow the signalling inputs into a given process to be tailored to suit the particular demands of different organs and tissues. We note that Supplementary Table 1 lists representatives of several multi-protein families. In the large 14-3-3-phosphoproteomics studies, further protein families are also being identified to display affinity for binding to 14-3-3 because the 14-3-3s are dimers, the function of the copy could be kept under sufficient control if one 14-3-3-binding site remained unchanged ‘the lynchpin site’, while allowing sequence divergence to occur at the second 14-3-3-binding site. If a useful consensus for phosphorylation by a different kinase were to arise at the evolving site, the result would be the generation of a parologue with distinct regulatory inputs from the original. Whether or not 14-3-3s have actually driven evolution in this way, this theory should stimulate experimentation to compare the regulatory details of 14-3-3 binding to paralogous members of multi-protein families.

Variants of a protein can also be generated by alternative splicing of mRNA transcripts. We note that in the 14-3-3-binding phosphoproteome there are at least two cases where a 14-3-3-binding site exists in one splice variant of a protein, but not another. Thus the KCN2 gene generates many forms of KLC1 (kinesin light chain 1) with different C-termini due to differing exon selection that may specify loading of different cargoes on to the kinesin motor complex. A residue equivalent to the 14-3-3-binding phosphoSer382 in KLC2 is found only in KLC1 variants expressing exon 17 [68]. Similarly, only variants of tumour protein D52 that express exon 6 contain the 14-3-3-binding site identified on this protein [69].

14-3-3 binds to phosphoSer251 and phosphoSer336 in the DISC1 (disrupted-in-schizophrenia 1)-interacting site of human Ndel1

One obvious anomaly in our survey was Ndel1 (formerly named NUDEL), for which mutation of three residues (Ser198, Thr219, and Ser231) each with a proline residue at +1 and phosphorylated by the proline-directed kinase CDK5/p35, has been reported to prevent Ndel1 binding to 14-3-3s (Supplementary Table 1) [70]. We therefore re-examined the binding of 14-3-3 to recombinant Ndel1 expressed in HEK-293 cells, and first confirmed that HA–Ndel1 isolated from transfected HEK-293 cells binds directly to 14-3-3, and this interaction is abolished by dephosphorylation with PP2A (protein phosphatase 2A) (Figure 5A). Ndel1 has a potential mode I 14-3-3-binding site at Ser198 [SSRPS(S336*)AP, where * indicates the residue that would have to be phosphorylated to become a potential mode I site]. In addition, Ndel1 is phosphorylated by Aurora A at Ser251 [SARI(pS251)ALN], which is consistent with Ndel1 and Aurora A co-localizing at the centrosome during prophase [71] and lies within a fragment of Ndel1 (residues 189–256) that can interact with 14-3-3c in a yeast two-hybrid assay [70]. Mutation of Ser251 to alanine markedly decreased the binding of 14-3-3, while the S251A mutation also reduced the interaction, and the double S251A/S336A mutant displayed only trace binding to 14-3-3 (Figure 5B).

HEK-293 cells do not have active CDK5 because they lack its activators p35 and p39, and we therefore tested the effect of in vitro phosphorylation of Ndel1 with the candidate kinases Aurora A, Aurora B and CDK5/p35. Aurora B was included because Ndel1 at the kinetochore has been shown to play a role in M-phase progression [72], and Aurora B is one of the major kinases known to regulate M-phase progression. While incubation with Mg-ATP and each of the three kinases induced an upwards band shift of the GST–Ndel1 substrate, indicative of phosphorylation, a 14-3-3-binding signal was only seen after phosphorylation by the Aurora kinases, either singly or in combination (Figure 5C). In contrast, while CDK5/p35 phosphorylates Ndel1, it did not induce 14-3-3 binding appreciably above background levels. Use of phospho-specific antibodies, and mutated GST–Ndel1, confirmed that 14-3-3 binding correlated with phosphorylation at Ser251 and Ser336, and was almost eliminated by mutation of Ser and Ser336 (Figure 5D). In contrast, the 14-3-3-binding signal of the Ser198/Thr219/Ser231 triple alanine mutant was comparable with the wild-type Ndel1 (Figure 5D).

Having confirmed the specificity of the anti-phosphoSer251 and anti-phosphoSer336 antibodies (Figures 5C and 5D), we checked whether phosphorylation of Ser198, Thr219 and/or Ser231 was responsible for the residual 14-3-3-binding signal seen with the S251A/S336A double mutant isolated from HEK-293 cells (Figure 5B). HA–Ndel1 was not phosphorylated on Ser251 under these cellular conditions, but binding to 14-3-3 mainly reflected the phosphorylation of Ser336. Note that Ser336 is only in isoform 1 of Ndel1 (Q9GZM8) and whether other isoforms of Ndel1 also interact with 14-3-3 is unclear.

When Ser251 and Ser336 were mutated, there was still a residual 14-3-3-binding signal that could be eliminated by also mutating Ser198, Thr219 and Ser336 into alanine residues (Figure 5E). This background signal probably explains why Toya-oka et al. [70] pinpointed phosphoSer198, phosphoThr219 and phosphoSer336 as the 14-3-3-binding sites following their exciting discoveries linking Ndel1 and 14-3-3s to the aetiology of lissencephaly (smooth brain) [70,73]. Briefly, the N-terminal coiled-coil domain of Ndel1 binds to Lis1, a protein whose deletion or mutation causes lissencephaly due to defective migration of cortical neurons [21]. Children with deletions in Lis1 and the adjacent 14-3-3ε gene have a more severe disorder called MDS (Miller–Dieker syndrome), which is phenocopied in mice with haplodeficiency in Lis1 and 14-3-3ε [70]. Our findings place the 14-3-3 interaction at the C-terminal part of Ndel1, which has endo-oligopeptidase activity [74] and interacts with multiple protein partners including DISC1. © 2010 The Author(s)

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Figure 5 Identification of Ser251 and Ser336 as 14-3-3-binding sites in human Ndel1

(A) HEK-293 cells were transfected with a plasmid to express HA–Ndel1 and lysed after 24 h. HA–Ndel1 was immunoprecipitated from lysates (3 mg) and incubated in vitro for 30 min at 30°C in the absence or presence of 50 m-units/ml PP2A with or without its inhibitor microcystin-LR (MC-LR) at 5 μM, as indicated. The samples were separated by SDS/PAGE, transferred to nitrocellulose and probed for direct binding to DIG–14-3-3s in a far-Western overlay assay, with anti-HA immunoblot as the loading control. (B) HA–Ndel1 (WT, wild-type), HA–S251A-Ndel1, HA–S336A-Ndel1 and HA–S251A/S336A double mutant proteins were expressed in HEK-293 cells. Proteins were immunoprecipitated from lysates (3 mg) and analysed for direct binding to DIG–14-3-3s in a far-Western overlay assay with an anti-HA immunoblot as a loading control. (C) GST–Ndel1 (4 μg) expressed and purified from Escherichia coli was phosphorylated in vitro in the absence or presence of Mg-ATP with 5 units/ml Aurora A, 1 unit/ml Aurora B and 2.5 units/ml CDK5/p35 for 30 min at 30°C. Samples (25%) of the reactions were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with the indicated antibodies. The arrow indicates the signal representing phosphoSer251 Ndel1. (D) Wild-type and mutant GST–Ndel1 proteins expressed and purified from E.coli were phosphorylated in vitro with 5 units/ml Aurora A kinase in the absence or presence of Mg-ATP for 30 min at 30°C. After separation by SDS/PAGE and transfer on to nitrocellulose, membranes were immunoblotted with the antibodies indicated. (E) HEK-293 cells were transfected with plasmids encoding the wild-type and indicated mutant HA-tagged Ndel1 proteins. After 24 h, cells were lysed and HA proteins were immunoprecipitated from lysates (3 mg). The immunoprecipitates were separated by SDS/PAGE, transferred to nitrocellulose and analysed by immunoblotting with the specified antibodies. HA–PACS2 and HA–PACS2–S487A mutant proteins extracted from transfected HEK-293 cells were used as positive and negative controls respectively, for 14-3-3 binding in the overlay assay (Ser487 is equivalent to the phosphoSer437 14-3-3-binding site in [84]; Supplementary Table 1). Numbers on the right-hand side show the migration position of molecular mass standards (in kDa). 5xA represents the HA–Ndel1–S198A/T219A/S231A/S251A/S336A mutant protein.

Disruption of the DISC1 gene segregates with schizophrenia, other psychiatric disorders and cognitive variation [75–80]. 14-3-3 genes, in particular 14-3-3η, have also been implicated in schizophrenia and bipolar disorder [81]. Solving the regulation of 14-3-3 binding to Ndel1 should therefore give insights into the dynamics of processes that underlie lissencephaly and schizophrenia.

Concluding remarks

Collating all reported 14-3-3-binding sites was a valuable exercise. Even though the datasets are incomplete and flawed, tangible rules about how 14-3-3 dimers engage with their phosphoprotein targets are emerging. The running head title ‘two is the key to 14-3-3’ highlights that the collective data suggest how a 14-3-3 dimer engaging with dual phosphorylation sites has special mechanical and signalling properties, and may even have facilitated evolutionary change in target protein families. These features should have useful predictive power for dissecting the cellular regulation of the many phosphoproteins that have been isolated from cell extracts by various 14-3-3-affinity capture and release procedures [1–11]. Further sorting of 14-3-3-binding motifs into different categories should facilitate the daunting task of defining relevant protein kinases, and understanding how networks of hundreds of phosphoprotein–14-3-3 interactions control the behaviour of every eukaryotic cell.
A future plan is to create and maintain a database that shows the location of 14-3-3-binding sites in relation to functional domains on the target proteins, residue conservation across species and links to relevant protein kinases. Towards this goal, authors are invited to submit additions and corrections for Supplementary Table 1 to c.mackintosh@dundee.ac.uk.

AUTHOR CONTRIBUTION
Carol MacKintosh and Catherine Johnson conceived the study and wrote the paper. Catherine Johnson, Rachel Toth and David Campbell performed the biochemical experiments. Carol MacKintosh, Catherine Johnson, Sandra Crowther and Margaret Stafford collated and analysed sequences, and proposed hypotheses.

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