ASPP proteins discriminate between PP1 catalytic subunits through their SH3 domain and the PP1 C-tail

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Serine/threonine phosphatases such as PP1 lack substrate specificity and associate with a large array of targeting subunits to achieve the requisite selectivity. The tumour suppressor ASPP (apoptosis-stimulating protein of p53) proteins associate with PP1 catalytic subunits and are implicated in multiple functions from transcriptional regulation to cell junction remodelling. Here we show that Drosophila ASPP is part of a multiprotein PP1 complex and that PP1 association is necessary for several in vivo functions of Drosophila ASPP. We solve the crystal structure of the human ASPP2/PP1 complex and show that ASPP2 recruits PP1 using both its canonical RVxF motif, which binds the PP1 catalytic domain, and its SH3 domain, which engages the PP1 C-terminal tail. The ASPP2 SH3 domain can discriminate between PP1 isoforms using an acidic specificity pocket in the n-Src domain, providing an exquisite mechanism where multiple motifs are used combinatorially to tune binding affinity to PP1.
The large number of serine/threonine kinases (over 500 in humans) dwarfs the ~40 serine/threonine phosphatase catalytic subunits, posing a major challenge for the specific dephosphorylation of kinase substrates. For the most abundant of these phosphatase catalytic subunits, PP1 and PP2A (phosphoprotein phosphatase 1/2A), this challenge is met by a large collection of accessory subunits, which recruit specific substrates, prevent promiscuous dephosphorylation events, and/or tether phosphatases to discrete subcellular locations. Over 200 PP1-interacting proteins (PIPs) have been identified to date, ~70% of which contain the small linear motif (SLiM) RVxF, which binds a hydrophobic groove in the PP1 catalytic subunit [8-6]. In addition to the RVxF motif, many other PP1-binding SLiMs have been identified, such as SILK [8-9], ΦΦ [8-10], and KIR (Ki67–RepoMan) motifs [8-11]. Several SLiMs are often combined within an intrinsically disordered domain to form a high affinity PIP/PP1 complex. This is the case for several PIPs where the structural basis for PP1-binding has been elucidated [8-12], including the targeting subunits MYPT1 (Myosin phosphatase targeting subunit 1), which uses an RVxF and a MYPT-Bc motif to contact PP1 [8-13] and Spinophilin (also known as Neurephin, RVxF and ΦΦ [8-14]) [8-15]. A subset of these PIPs occludes (e.g. Spinophilin, PNUTS) or extends (e.g. MYPT1) some of the three PP1 substrate-binding grooves, thereby increasing catalytic subunit specificity. Others promote specificity by recruiting PP1 catalytic subunits to their substrates or a particular subcellular localisation [8-4].

In mammals, four PP1 catalytic subunits encoded by three genes exist: the broadly expressed PP1α, PP1β and PP1γ, and the testis-specific PP1y2. Genetic analysis in model organisms has shown that different PP1 isoforms perform overlapping but distinct cellular functions. Complementation tests substituting the PP1 catalytic subunit [8-2] for loss of PP1 [8-3] display distinct functional consequences. For example, PP1α and PP1β [8-4,8-5] have non-redundant functions in the Drosophila ASPP/PP1 complex. Our data show that ASPP proteins use their SH3 binding SLiMs have been identified, such as SILK [8-9], ΦΦ [8-10], and KIR (Ki67–RepoMan) motifs [8-11]. Several SLiMs are often combined within an intrinsically disordered domain to form a high affinity PIP/PP1 complex. This is the case for several PIPs where the structural basis for PP1-binding has been elucidated [8-12], including the targeting subunits MYPT1 (Myosin phosphatase targeting subunit 1), which uses an RVxF and a MYPT-Bc motif to contact PP1 [8-13] and Spinophilin (also known as Neurephin, RVxF and ΦΦ [8-14]) [8-15]. A subset of these PIPs occludes (e.g. Spinophilin, PNUTS) or extends (e.g. MYPT1) some of the three PP1 substrate-binding grooves, thereby increasing catalytic subunit specificity. Others promote specificity by recruiting PP1 catalytic subunits to their substrates or a particular subcellular localisation [8-4].

Thus, isoform selectivity is thought to be a key feature of regulatory PIPs, several of which display isoform preferences, such as MYPT1 [8-13]. Spinophilin [8-14,15], RepoMan [8-16,17] and Ki67 [8-18,19], though limited mechanistic information exists on how this is achieved [8-4,8-12,22]. In the case of Ki67 and RepoMan, two PIPs involved in mitotic exit, the Ki67–SLiM motif determines preference toward PP1γ through a single amino acid change in a binding pocket in the catalytic domain of PP1β. Human PP1s differ mainly in their N- and, most notably, C-termini (see Fig. 4a22), suggesting that in many cases these could provide the basis for subunit selectivity. The PP1 termini are unstructured [8-23], therefore their involvement in PP1 recruitment by PIPs has been difficult to dissect molecularly. An exception is MYPT1, whose ankyrin repeats associate with amino acids 301–309 in the PP1 C-tail, and are proposed to drive selectivity towards PP1γ [8-13], though the MYPT-Bc-binding region of PP1γ may also participate in selectivity [8-24]. However, the extreme C-terminus (PP1α [8-29–30], PP1β [8-31], and PP1γ [8-32]), which contains a type 2 SH3-binding motif (PII – PxxPxR) and a variable C-tail, has never been crystallised and its contribution to PIP recruitment remains under-studied.

The ASPP (apoptosis-stimulating protein of p53) protein family, which in mammals is composed of ASPP1, ASPP2 and iASPP (inhibitor of ASPP), are RVxF-containing PIPs (RARL in the case of iASPP [8-25]). p53BP2, a fragment of ASPP2, was one of the first RVxF-containing PP1 interactors to be identified [8-26], while Drosophila ASPP was recovered in a two-hybrid screen for PIPs [8-27]. ASPP proteins have well characterized functions as modulators of gene transcription through the p53 family [8-28] and also regulate cell–cell contact remodelling in mammals and flies [8-29–31]. Indeed, mammalian ASPP2 and Drosophila ASPP localise at tight junctions and adherens junctions (AJs), respectively [8-29–31], and are required for junctional stability, at least in part by recruiting the polarity protein Par-3 (Bazooka in flies) [8-29–32], although the role of the ASPP/PP1 association has not been examined in this context.

The function of ASPP proteins in recruiting PP1 catalytic subunits has been examined in two contexts. First, during the cell cycle, ASPP1/2 and PP1α dephosphorylate the kinetochore component Hecl to promote microtubule/kinetochore attachment [8-33], and c-Nap1 to induce centrosomal linker reassembly at the end of mitosis [8-34]. Second, the growth-promoting transcriptional coactivator targets of the Hippo pathway, YAP (yes-activated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) are dephosphorylated on an inhibitory site by ASPP2/PP1, promoting their transcriptional activity [8-35,36]. As well as their PP1-binding RVxF/RARL motifs, ASPP proteins have C-terminal ankyrin repeats (like MYPT1) followed by an SH3 domain (Supplementary Fig. 1a). Mutation of the PP1 C-terminal PPII compromises binding to ASPP proteins [8-25], while an SH3 domain mutation in iASPP impairs PP1 association [8-37], suggesting that ASPP family members can engage the PP1 C-tail. Here, we characterize the ASPP/PP1 complex structurally and functionally. Our data show that ASPP proteins use their SH3 domain to discriminate between different PP1 isoforms based on their C-tail and provide structural insights into the function of the PP1 C-tail.

### Results

**Characterisation of the Drosophila ASPP/PP1 complex**

Our previous affinity purification–mass spectrometry (AP-MS) data [8-38] suggested that ASPP1/2 are part of a tightly interlinked protein network comprising PP1 catalytic subunits, as well as the PP1 inhibitor IPP2 (Fig. 1a). In addition, this putative complex contains N-terminal RASSF (ras-association domain family) (RASSF7-10) scaffold proteins [8-39] and different isoforms of the small coiled-coil protein CCDC85. Prior to performing a genetic analysis of this complex, we first sought to biochemically characterise the Drosophila ASPP/PP1 complex. First, we verified the binary interaction between ASPP and the four Drosophila PP1 catalytic subunits by co-immunoprecipitation (co-IP) from Drosophila S2 cell lysates (Fig. 1b). As expected, ASPP associated with PP1 catalytic subunits, though it displayed a marked preference for PP1e96A and PP1p96C, with PP1a87B and PP1e13C weakly associating with ASPP. Like mammalian ASPP1 and ASPP2, Drosophila ASPP possesses a PP1-binding RVxF motif at its C-terminus, preceding the ankyrin repeats. We mutated the ASPP RVxF motifs (RVSF to RASA; ASPPFA, see Supplementary Fig. 1a). Association of ASPPFA to the PP1 catalytic subunits was substantially reduced in co-IP experiments compared with ASPP wildtype (Fig. 1b), suggesting the RVxF is required for the Drosophila ASPP/PP1 interaction, as is the case for mammalian ASPP1/2 [8-33,35].

We previously showed that the Drosophila ASPP and RASSF8 mutants share many phenotypic similarities, suggesting common biological functions [8-41], and our Mass Spectrometry analysis suggested that mammalian RASSF7 and RASSF8 (the Drosophila RASSF orthologues) are associated with ASPP/PP1 complexes in HEK293 cells [8-38]. Interestingly, in S2 cells, which do not express endogenous ASPP or RASSF8 [8-31], PP1e96A associated with RASSF8 only in the presence of exogenous ASPP (Fig. 1c), suggesting that RASSF8 indirectly associates with PP1 via ASPP.
and 796 Drosophila complex in recruitment of both RASSF8 and Ccdc85 to the ASPP/PP1 revealed that the ASPP coiled-coil domain is required for the and SH3 domain) (Supplementary Fig. 1a). These experiments had no effect on the ASPP interaction with RASSF8 or Ccdc85. Mutating the PP1-binding RVxF motif at the ASPP C-terminus within the RVxF motif of ASPP (ASPPFA) decreases the binding between PP1 isoforms and ASPP.

Next, we examined Ccdc85, which was also associated with PP1, ASPP1/2 and RASSF7-10 in HEK293 cells. In the absence of ASPP, FLAG-Ccdc85 IPs recovered a small amount of Ccdc85 only in the presence of ASPP (Fig. 1d). Thus, Ccdc85 can interact with PP1 through ASPP, though a low affinity direct interaction is possible.

Since RASSF8 and Ccdc85 could form a complex with ASPP/PP1, we tested whether these might represent separate complexes or if a tetrameric complex was possible. First, we mapped the domain of ASPP involved in the RASSF8 and Ccdc85 interactions by performing co-IPs with three truncations: 1–234 (comprising the coiled-coiled region), 234–795 (the divergent middle region) and 796–1020 (comprising the RVxF motif, the ankyrin repeats and SH3 domain) (Supplementary Fig. 1a). These experiments revealed that the ASPP coiled-coil domain is required for the recruitment of both RASSF8 and Ccdc85 to the ASPP/PP1 complex in Drosophila S2 cells (Supplementary Fig. 1b, c). Mutating the PP1-binding RVxF motif at the ASPP C-terminus had no effect on the ASPP interaction with RASSF8 or Ccdc85 (Supplementary Fig. 1d, e). Reciprocal co-IP of RASSF8 with Ccdc85 was only possible in the presence of ASPP (Fig. 1e). Thus, our biochemical data indicate that an ASPP/PP1 complex also comprising Ccdc85 and RASSF8 can exist in cultured S2 cells. In Human Embryonic Kidney (HEK) 293T cells, we were able to IP RASSF8 and endogenous PP1a with ASPP2, confirming the Mass Spectrometry and S2 cell results (Supplementary Fig. 1f).

ccdc85 mutants phenocopy the ASPP eye phenotypes. We had previously shown that RASSF8 and RASSF8 mutants share many phenotypic similarities. In particular, these mutants display a rough eye phenotype due to an excess number and aberrant organisation of the inter-ommatidial cells (IOCs), which separate each unit of the Drosophila compound eye (see Fig. 2a for a schematic of wild type ommatidial organization). Since Ccdc85 is associated with the ASPP/PP1 complex in cell culture, we wished to analyse the effects of its genetic inactivation. We generated a ccdc85 mutant (ccdc85C1.1) by transposon mobilisation (Supplementary Fig. 2a–c, see the Methods section). ccdc85C1.1 animals were viable but displayed a rough eye phenotype similar to that of
ASPP and RASSF8 mutants, either as homozygotes or in trans to a deficiency (Df(2L)Exel7014) or the original P(XP)d06579 transposon insertion.

We quantified IOC numbers and bristle placement in ccdc85^{C1.1} pupal retinas at 40 h APF (after puparium formation) stained with an anti-E-cadherin (E-cad) antibody to visualise cell boundaries (Fig. 2). Like ASPP and RASSF8 mutants (Fig. 2i, l and ref. 31), ccdc85^{C1.1} mutants displayed increased IOC numbers and a substantial number of bristle displacements (Fig. 2b, c, f, g) though the IOC increase was milder than ASPP mutants (ccdc85: 13.37 vs ASPP: 14.15, compared with 12.37 for control retinas). Conversely, Ccdc85 overexpression under the control of the GAL/UAS system using the eye-specific GMR-GAL4 driver reduced IOC numbers and also induced bristle displacements (Fig. 2d–g).
Thus, cdc85 disruption elicits a similar eye phenotype to ASPP and RASSF8 loss, while its overexpression has the opposite effect, suggesting that these three binding partners share common functions in vivo.

**Loss of the RVxF motif disrupts ASPP function in vivo.** Although the ASPP and cdc85 mutants share phenotypic similarities, this does not prove that their shared developmental function pertains to PP1 regulation. To address this question, we tested the effect of disrupting the ASPP/PP1 interaction in vivo. We generated an ASPP rescue construct where the ASPP cDNA is expressed at low levels under the control of the ubiquitin 63E promoter with a N-terminal GFP tag (ubi > ASPP, see experimental procedures). Expression of this construct in ASPP mutant animals fully rescued both the increased IOC number (Fig. 2h–j and 1) and bristle displacement defects (Fig. 2h–j and m). In contrast, expression of ASPPFA inserted at the same locus only partially rescued both defects (Fig. 2h, k–m). Mosaic expression of the ubi > ASPPwt and ubi > ASPPFA constructs using the Flp/FRT system indicated that both constructs are normally localized at the AJ’s of epithelial cells, and expressed at similar levels (Supplementary Fig. 2d–i).

Next, we examined the effect of the ASPPFA mutation on other ASPP phenotypes8. While ubi > ASPPwt fully rescued the wing size increase (Fig. 3a, b, d and e) and wing notching upon single copy loss of csk (C-terminal src kinase, Fig. 3f, h), ubi > ASPPwt only partially rescued both phenotypes (Fig. 3c, d, e, g and h). ASPP mutant animals frequently display a duplication of the anterior scutellar macrochaete of the adult thorax (Supplementary Fig. 3a, b). This defect is partially rescued by ubi > ASPPwt and not at all by ubi > ASPPFA. Finally, opposite to the loss-of-function (Fig. 3e), overexpression of ASPP using the GAL4/UAS system under the control of the wing-specific MS1096-GAL4 driver reduced wing size by ~10% (Fig. 3l, j, l and m), while ASPPFA expression had little effect (Fig. 3k, l and m). Thus, association with PP1 is required for several in vivo functions of ASPP.

**ASPP association with PP1 subunits requires multiple motifs.** An interesting characteristic of the different PP1 catalytic subunits in Drosophila and mammals is the diversity of their C-terminal tails (hereafter referred to as C-tail) (Fig. 4a). In particular, two of the Drosophila PP1s (PP1α87B and PP1α13C) have truncated C-tails (Fig. 4a) and show a markedly reduced association with ASPP in co-IP experiments (Fig. 1b) compared with PP1α96A and PP1β9C, which have an extended C-tail, like all the mammalian PP1c isoforms (Fig. 4a). We generated C-terminally truncated versions of PP1α96A and PP1β9C lacking the C-tail and observed that these displayed a strongly reduced ability to associate with ASPP in co-IP experiments (Fig. 4b). This suggests that ASPP can discriminate between different PP1 isoforms based on the PP1 C-tail.

Like mammalian ASPP1/2, Drosophila ASPP has an SH3 domain at its C-terminus (Supplementary Fig. 1a), while the PP1 C-tail of PP1α96A and PP1β9C and all the mammalian PP1c isoforms contain a highly conserved PPII (ref. 23 and 4a). Indeed, recent work showed that deletion or mutation of the PP1 C-tail compromises its ability to bind ASPP1, ASPP2 and iASP25. We generated a mutant ASPP construct with a cryptophan to lysine mutation in the hydrophobic patch of the SH3 domains (W897K - ASPPWK), which abolishes ligand binding while preserving SH3 domain structure40. ASPPWK had reduced binding to PP1α96A and PP1β9C in co-IP experiments (Fig. 4c). In contrast, ASPPWK binding to PP1α87B and PP1α13C was not affected, since these lack the C-terminal SH3-binding motif (Fig. 4c). Mutation of both the RVxF and the SH3 domains (ASPPFA-WK) further reduced the binding between ASPP and PP1α96A to background levels (Fig. 4d). Thus, both the RVxF and SH3 domains of ASPP participate in PP1 docking. The ASPP/PP1 interaction is an example of a PIP that engages the PP1 motif of the PP1 C-tail. In order to structurally characterize this interaction modality, we co-crystallized human ASPP2 with PP1α.

**Crystal structure of ASPP2:PP1α.** We solved the crystal structure of human PP1α77-330 bound to ASPP2R292-1120 (hereafter referred to as PP1α:ASPP2 complex) at a resolution of 2.15 Å (Table 1). The overall structure of the PP1 catalytic domain is very similar to other reported PP1 structures12. ASPP2 binds to PP1α through three surfaces of interaction, covering ~1200 Å² of solvent-accessible area: the RVxF motif, the first ankyrin motif and the SH3 domain, respectively, burying 530, 120 and 500 Å² of solvent-accessible area (Fig. 5a, Supplementary Fig. 4a). ASPP2 residues R291RVKF294, constitute the canonical RVxF motif, which binds PP1 hydrophobic pocket in an extended conformation similar to what has been observed previously for other PP1-binding proteins (Fig. 5b)8,10,11,13,14,41–43. The second patch of interactions is formed by four acidic residues from the ASPP2 first ankyrin motif facing PP1αK260 and PP1αR261 (Fig. 5c). PP1αR261 forms a network of hydrogen bonds with ASPP2D932 and ASPP2E938. This observation is in agreement with previous work showing that PP1αR261 mutation reduced ASPP2 binding to PP1α25. A similar interaction has been observed in the structure of PP1β bound to MYPT113.

The electron density observed for the PP1α:ASPP2 structure allowed the modelling of the seven conserved residues from its PP1 motif (PP1α317RPTTPPP325) bound to ASPP2 SH3 domain and forming the third surface of interaction (Fig. 5d, e and Supplementary Fig. 4b). Native mass spectrometry analysis
indicated that the PP1:ASPP2 complex shows no evidence of degradation after 7 days at 20 °C, therefore the C-tail residues not observed in the electron density are most likely flexible rather than proteolytically removed (Figure Supplementary Fig. 4c).

PP1\(^\alpha\)\(_{317-323}\) adopts the characteristic right handed type II polyproline helix containing three residues per turn\(^{44}\). The two conserved prolines PP1\(^\alpha\)P318 and PP1\(^\alpha\)P321 sit in two xP binding grooves formed by ASPP2 L113/W1066 and ASPP2 P1110/W1097, respectively (Fig. 5a, d, e). PP1\(^\alpha\)R323 is tightly coordinated by three acidic residues from the ASPP2 SH3 domain: ASPP2 D1074 and ASPP2 E1094 from the RT loop and ASPP2 E1075 from the n-Src loop.

**ASPP2’s SH3 acidic specificity pocket promotes PP1\(^\alpha\) binding.**

The affinity of PP1\(^\alpha\)\(_{7-330}\)-ASPP2\(_{220-1120}\) was measured by Isothermal Calorimetry (ITC) and by Bio Layer Interferometry (BLI). PP1\(^\alpha\) was found to bind tightly to ASPP2 with an affinity of 6.1 and 13.6 nM by ITC and BLI methods, respectively (Fig. 6a, b). Truncation of the PP1 C-tail (PP1\(^\alpha\)\(_{7-300}\)) has a dramatic effect, with no binding detected by ITC (Fig. 6a). To gain a better
understanding of the role of the PP1 C-tail in PP1α:ASPP2 complex formation, we measured the binding affinity of various peptides corresponding to PP1 C-tails to ASPP2\textsuperscript{920-1120} by ITC (Fig. 6c, Supplementary Fig. 5). The PP1α\textsuperscript{301-330} C-tail binds ASPP2 with a surprisingly high affinity of 143 nM (Fig. 6c, Supplementary Fig. 5a). Mutation of PP1α\textsuperscript{R323} to alanine completely abolished ASPP2 binding, highlighting the critical role of PP1α\textsuperscript{R323} for ASPP2 binding (Fig. 6c, Supplementary Fig. 5b). The expected binding affinity of a PP1 motif for its SH3 domain is in the 5–20 μM range\textsuperscript{45,46}. The fact that ASPP2\textsuperscript{920-1120} binds to the PP1α\textsuperscript{301-330} C-tail almost two orders of magnitude above this value prompted us to consider the possibility that additional contacts not observed in the crystal structure may explain this high affinity.

The core PPII motif sequence PP1α\textsuperscript{317–323} found in all PP1 C-tails binds ASPP2 with a much lower affinity of 5650 nM, whereas the same sequence extended by seven residues at its C-terminal extremity (PP1α\textsuperscript{317–330}) binds with a much stronger affinity of 270 nM, comparable with PP1α\textsuperscript{301-330} (Fig. 6c, Supplementary Fig. 5c–d). Thus, the C-terminal residues following the PPII motif are critical for ASPP2 binding. A similar observation has previously been reported for a few other PP motifs displaying unusually strong affinities for their respective SH3 domain\textsuperscript{47–51}. In those complexes, the increased affinity arises from additional interactions between residues flanking the core PP sequence and the SH3 domain specificity pocket constituted by the variable region of the RT and n-Src loops\textsuperscript{52}. A careful inspection of the electrostatic potential surface of ASPP2:PP1α structure reveals that ASPP2 SH3 domain contains a very acidic specificity pocket, formed by \textsuperscript{1090}DEDE\textsuperscript{1094} from the n-Src loop and \textsuperscript{1073}DDE\textsuperscript{1075} from the RT loop (Fig. 5e, Supplementary Fig. 4d). We therefore hypothesized that this specificity pocket could accommodate the basic PP1α C-terminal residues \textsuperscript{324}NSAKAKK\textsuperscript{330} through a number of electrostatic interactions tethering the PP1α C-tail on

### Figure 4
The SH3 domain of ASPP binds to the PP1 C-tail. a Alignment of the C-termini of human and Drosophila PP1s. PP1α3C and PP1α78B have shorter C-termini that lack the PxxPxr motif (class II SH3 domain binding motif). All human PP1 isoforms possess this motif. The PxxPxr motif is highlighted in grey. Positively charged residues after the PPII are highlighted in blue.

b Western blots of co-IP experiments from lysates of transfected S2 cells, probed with indicated antibodies. c Western blots of co-IP experiments from lysates of transfected S2 cells, probed with indicated antibodies. d Western blots of co-IP experiments from lysates of transfected S2 cells, probed with indicated antibodies.
Table 1 Data collection and refinement statistics (molecular replacement)

|                         | PP1:ASPP2 |
|-------------------------|-----------|
| **Data collection**     | P1        |
| **Space group**         | P1        |
| **Cell dimensions**     | a, b, c (Å) | 46.8, 81.6, 87.9 |
|                         | α, β, γ (°) | 91.0, 91.8, 103.9 |
| **Resolution (Å)**      | 59–2.15 (2.20–2.15) |
| **R_sym or R_free**     | 0.28 (1.0)  |
| **Completeness (%)**    | 99.8 (98.1) |
| **Redundancy**          | 6.9 (5.7)  |
| **Refinement**          | Resolution (Å) | 50–2.15 (2.18–2.15) |
|                         | No. reflections | 68,468 |
|                         | R_work/R_free | 17.7/21.4 |
| **Protein**             | 7860      |
| **Ligand/ion**          | 154       |
| **Water**               | 245       |
| **B-factors (Å²)**      | PP1       | 41.7   |
|                         | ASPP      | 59.7   |
|                         | Ligand/ion| 60.5   |
|                         | Water     | 50.3   |
| **R.m.s. deviations**   | Bond lengths (Å) | 0.06   |
|                         | Bond angles (°) | 0.74   |

*One crystal was used for data collection. Values in parentheses are for highest-resolution shell.

the ASPP2 SH3 domain specificity pocket (Fig. 5e). Alanine substitution of the three C-terminal lysine residues PP1αK327, PP1αK329 and PP1αK330 (hereafter referred to as PP1αK301–330 3KA mutation) reduces the affinity for ASPP2 by 13-fold, down to 1900 nM (Fig. 6c, Supplementary Fig. 5e). These data strongly support a critical role of PP1αK327, PP1αK329 and PP1αK330 in providing additional affinity and specificity to PP1α C-tail towards ASPP2 SH3 domain. Individual alanine mutations of these lysine residues show that they all contribute to ASPP2 binding (Fig. 6c, Supplementary Fig. 5e–h). However, mutation of PP1αK327 to alanine showed the strongest effect with a fourfold decrease in affinity (Fig. 6c, Supplementary Fig. 5e).

PP1 C-tails determine ASPP2 isoform preference. All human PP1 isoforms share a high sequence similarity (92.5–100%) within their catalytic domains and differ mainly in their N-terminal and C-terminal extremities. They all contain a highly variable C-tail except for their conserved PPII motif (Fig. 4a). To evaluate the PP1 isoform preference of ASPP family members, we measured the affinity of human iASPP, ASPP1 and ASPP2 for each full-length PP1 isoform by BLI. ASPP1 did not display any PP1 isoform selectivity in vitro (Fig. 6d, Supplementary Fig. 6). iASPP showed a modest (twofold) preference for PP1α and PP1β versus PP1γ (Fig. 6d, Supplementary Fig. 6). In contrast, ASPP2 showed a strong preference for PP1α with an affinity of 13.6 nM which is 13- and 14-fold higher than that observed for PP1β and PP1γ, respectively (Fig. 6d, Supplementary Fig. 6).

To test whether ASPP2 PP1 isoform preference is a consequence of the high sequence variability observed within the PP1 C-tail, we measured the affinity of each PP1 C-tail for ASPP2 by ITC. PP1αK301–330 binds ASPP2 with an affinity of 143 nM, which is, respectively, 7, 5 and 15 times greater than the affinity measured for PP1β[297–327], PP1γ[298–323] and PP1γ[297–324] C-tails (Fig. 6c, Supplementary Fig. 5i–k). The differential affinities between ASPP2 and the different PP1 C-tails measured by ITC reflect those observed between ASPP2 and the full-length PP1 isoforms by BLI with PP1α binding over 13-fold stronger compared to PP1β and PP1γ (Fig. 6c, d). The chimeras generated by substituting the PP1β[297–327], PP1γ[298–323] and PP1γ[297–324] sequences C-terminal to the PP1I motif by the seven C-terminal residues of PP1α (NSAKAKK) show elevated affinity to ASPP2, comparable to PP1α[301–330] (Fig. 6c, Supplementary Fig. 5l–m).

Therefore, the reduced affinity observed for PP1β[297–327], PP1γ[298–323] and PP1γ[297–324] is the result of the sequence variability observed in their C-tails (Fig. 4a). All PP1 isoforms C-tails contain three basic residues within their last five C-terminal residues (except for PP1γ which has only one). However, the distance of the linker between the PP1I motifs and those basic residues vary from 3 to 10 amino acid with the shorter one observed in PP1α. In addition, the longer PP1β, PP1γ1 and PP1γ2 linkers contain some glycine and proline residues that might influence the geometry of the C-tail (Fig. 4a). Together, these data show that ASPP2 discriminates between PP1 isoforms based on the PP1 C-tail, and more specifically on the basic residues following the PPII motif.

ASPP2 specificity for PP1α resides in its n-Src loop. Although ASPP1, ASPP2 and iASPP share high sequence homology in their C-terminal region, only ASPP2 displays selectivity for PP1α (Fig. 6d). Interestingly, we noticed some sequence differences within the variable n-Src loop (Supplementary Fig. 7a). For clarity, we numbered those n-Src loop residues from 1 to 5 (Supplementary Fig. 7a). The most acidic n-Src loop is found in ASPP2, while the other ASPPs lack some of the acidic residues. The n-Src loop of human ASPP1 and Drosophila ASPP are both missing acidic residues in positions 1 and 4, whereas human and C. elegans iASPP are both missing two acidic residues in positions 2 and 3, with the latter also lacking one in position 5. ASPP1 does not show specificity for PP1α (Fig. 6d), but mutation of the n-Src loop residues 1 and 4 in ASPP1 to the equivalent acidic ASPP2 residues (ASPP1K1052E/D1093S and ASPP2D1091G/E1092P) imparts specificiity towards PP1α (Fig. 6d). Conversely, the single and double ASPP2 mutants mimicking the ASPP1 n-Src loop (ASPP2E1090K/D1093S and ASPP2D1093S) display a drop in specificity for PP1α (Fig. 6d). These data highlight the critical role of the n-Src loop acidic specificity pocket in modulating ASPP1/2 selectivity towards PP1α.

The binding kinetics of human iASPP to all PP1 isoforms are very different compared to those observed for ASPP1/2 (Supplementary Fig. 6). iASPP binds PP1α with a lower apparent $K_D$ (2.6 nM instead of 13.6 nM for ASPP2), however, it shows a sevenfold lower $K_{on}$ and a 110-fold lower $K_{off}$ for PP1α compared to ASPP2. Therefore, once assembled, the PP1α:iASPP complex is more stable, with a half-life of 58 min versus 31 s for PP1α:ASPP2 complex. In order to understand how iASPP interacts with PP1α C-tails, we performed ITC measurement, showing that, though human iASPP binds the PP1α C-tail with relatively high affinity (395 nM), the binding is only modestly affected by the PP1α 3KA mutation (690 nM) (Fig. 6e, Supplementary Fig. 5n–o), showing that the iASPP:PP1 interaction is less reliant than ASPP2 on the PP1α C-terminal lysines and therefore likely involves other interactions. In support of this view, the iASPP SH3 domain specificity pocket is less acidic than that of ASPP2 (Supplementary Fig. 7a).

Interestingly, the double mutation ASPP2D1091G/E1092P in positions 2 and 3 of the ASPP2 n-Src loop mimicking the human
iASPP had little effect on binding to the PP1α C-tail by ITC or binding of the PP1α full-length by BLI (Fig. 6d–e, Supplementary Fig. 5p, Supplementary Fig. 6). However, the triple mutant ASPP2D1091K/E1092V/E1094K mimicking the C. elegans iASPP n-Src loop with two charges inversion in positions 2 and 5 had a dramatic effect and abolished ASPP2 binding to the PP1α C-tail by ITC and reduced the affinity for full-length PP1α by almost 600-fold from 13.6 nM to 8.2 μM by BLI (Fig. 6d, e, Supplementary Fig. 5q, Supplementary Fig. 6). Thus, the PP1α C-tail lysines are recognised by the ASPP2 SH3 domain, while the ASPP1 and iASPP are less able to discriminate between PP1 isoforms because of their less acidic SH3 domain specificity pockets.
The extreme PPIα C-tail interacts directly with ASP2P. Although our ITC data support the importance of the PPIα C-tail lysines in ASP2P binding, these residues were not detectable in our crystal structure. To identify the PPIα C-tail residues implicated in ASP2P binding, we isotopically labelled (15N) the PPIα C-tail for NMR spectroscopy. First, we acquired the 2D [1H,15N]-HSQC spectrum in its unbound form. The spectrum displayed all the hallmarks for a peptide including no chemical shift dispersion in the 1H dimension due to lack of secondary structure elements (Fig. 7a). Next, we performed the sequence-specific backbone assignment of the PPIα C-tail (97% complete) using a 13C, 15N-labelled peptide. The secondary chemical shift (CSI) plot confirmed the lack of preformed secondary structure elements in the free PPIα C-tail (Supplementary Fig. 8).

We then titrated unlabelled ASP2P into 15N-labelled PPIα C-tail in order to observe either chemical shift perturbations or peak intensity changes that are indicative of binding (Fig. 7b). Indeed, PPIα C-tail cross peaks either shifted or had dramatically reduced peak intensities (e.g. PPIα310-325) upon ASP2P titration. This indicates that these residues are the core binding residues, correlating well with those observed in the electron density of the PPIα-ASP2P complex (PPIα317-323). Interestingly, the PPIα C-tail residues that flank the core binding domain also showed chemical shift perturbations, indicating that they also contribute to binding. Thus, to comprehensively identify all interacting residues, we recorded a 15N-[1H] heteronuclear NOE and a 15N transverse relaxation (R2) experiment of the PPIα C-tail in the free and ASP2P bound states. These experiments showed that all PPIα C-tail residues, including the three C-terminal lysines, have reduced flexibility in the presence of ASP2P (Fig. 7c, d). Thus, these lysines clearly contribute to the overall binding between these two proteins, as also confirmed by ITC (Fig. 6c), and do so via a fuzzy charge–charge interaction (lysines can flexibly move about the negatively charged pocket provided by the n-Src loop and thus contribute to binding without the need of locking into a single conformation, which would be visible in the crystal structure).

Function of the ASP2P SH3 domain/PPIα C-tail interaction. To test the role of the SH3 acidic n-Src loop in the context of the full ASPP/PP1 complex, we used an AP-MS approach. We stably expressed Strep-HA tagged ASP2P or ASP2PΔKK (ASP2PΔ1091K/E1092V/E1094K) in HEK 293T cells using the Flp-In system (see Methods). After pulldown of ASP2P or ASP2PΔKK from cell lysates, we used Mass Spectrometry to quantify associated peptides (Fig. 8a). In agreement with the biophysical data, ASP2P was associated with all the PPI1 isoforms, and the ASP2PΔKK mutation significantly decreased the amount of PPI1 binding to ASP2P, while binding to PPIβ and PPIγ1 was not affected (Fig. 8a). Indeed, PPIα was the only interactor of ASP2P reduced upon mutation of the n-Src loop (Supplementary Fig. 9).

To address PPI isoform specific interaction in a cellular context, we stably expressed Strep-HA tagged PPIα, PPIαAC (a truncation lacking amino acids 301–330, including the PPII and C-tail lysines), PPIβ and PPIγ1 in HEK293T and performed AP-MS experiments (Fig. 8b). As observed using ITC and BLI, ASP2P showed a marked preference for PPIα, compared to PPIβ and PPIγ1, while this selectivity was not observed for ASP1 (Fig. 8b). iASPP does not discriminate between PPIα and PPIβ, but shows low association to PPIγ1 (Fig. 8b). As expected, deletion of the C-tail of PPIα (comprising both the PPI and C-tail lysines) severely impaired binding between PPIα and all ASPP isoforms (Fig. 8b, c). In addition, binding of the other members of the ASPP/PP1 complex, RASSF7/8 and CCDC85 are severely reduced by the PPIαAC truncation, clearly indicating the importance of the PPI-C tail in the assembly of the ASPP/PP1 tetrameric complex (Fig. 8c).

As the ASP2PΔKK mutation specifically disrupted PPIα recruitment to the ASP2P/PP1 complex (Fig. 8a), we tested the effect of this mutation on the dephosphorylation of TAZ, a known substrate of the complex36. As previously reported, overexpression of wild type ASP2P in HEK293T cells promotes TAZ dephosphorylation on Serine 89, which is prevented by mutation of the RVxF SLiM (Fig. 8d, e and ref. 36). Interestingly, ASP2PΔKK was also compromised in its ability to dephosphorylate TAZ (Fig. 8d and e), suggesting that the interaction between the ASP2P SH3 domain specific pocket and PPIα is required for ASP2P/PP1 substrate dephosphorylation.

The SH3 domain is required for ASP2P in vivo function. To test the in vivo effect of disrupting the interaction between the ASP2P and SH3, and PPI1, we generated ASP2P rescue constructs with mutations in the RVxF motif (ASPPFA), the SH3 domain (ASPPWK) and a combination of mutations in both domains (ASPPFA-WK). Expression of wild type ASP2P in ASP2P mutant flies fully rescues the increased number of IOCs and bristle misplacement (Supplementary Fig. 10a–c, g, h). The RVxF mutant, (ASPPFA) did not fully rescue the IOC and bristle phenotypes, and mutation of both the RVxF and SH3 (ASPPFA-WK) enhanced this effect (Supplementary Fig. 10a–d, f–h). In contrast, the SH3 specificity pocket mutant ASPPFK (ASPPD981K/D982V/E984K) was able to rescue the increase in IOC number phenotype, though bristle misplacements were still apparent (Supplementary Fig. 10e, g and h). The relatively mild effect of the ASPPFA-KV mutation was surprising, since the equivalent mutation in ASP2P strongly reduced affinity towards PPIα (Figs. 6d and 8a).

Since Drosophila Ccd85 can associate with PPI1 in cell culture (Fig. 1d), we hypothesized that it might provide an extra contact point between the ASP complex and PPI1, thereby partially rescuing the effect of ASP2P/PP1-binding surface mutations. Consistent with this hypothesis, mutation of ASP2P and ccd85 together dramatically increased the IOCs number and the bristle misplacement phenotypes (Fig. 9a–c and i–j). These defects could be rescued back to ccd85 single mutant levels by the expression of the wild type form of ASP in an ASP2P, ccd85 mutant background (Fig. 9d and i–j). In contrast, all the ASPP mutant forms (ASPPFA, ASPPWK, ASPPFK, ASPPFA-WK) failed to rescue...
the IOC number and bristle misplacement phenotypes (Fig. 9e–h and i–j). Thus, consistent with our binding data, both the RVxF and the SH3 domain are important for ASPP function in vivo.

The ASPP complex promotes PP1β9C and PP1α96A AJ localisation. Drosophila ASPP preferentially binds to two PP1 isoforms, PP1α96A and PP1β9C (Fig. 1b). Analysis of GFP tagged forms of these two isoforms expressed under their endogenous promoter revealed an adherens junction pool localised with E-cadherin in retinas at 26 h APF (Supplementary Fig. 10k–n), where we have previously shown ASPP and RASSF8 to be localised31. In contrast, GFP tagged PP1α87B, which has low affinity

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for ASPP (Fig. 1b), localises in the cytoplasm (Supplementary Fig. 10i–j). To test whether the junctional pool of PP1α96A and PP1β9C is dependent on the ASPP complex, we examined their localisation in ASPP, ccdc85 double mutant animals (Fig. 9k–t and Supplementary Fig. 10o–u). Interestingly, junctional localisation of PP1α96A and PP1β9C was disrupted in ASPP, ccdc85 double mutant (Fig. 9k–m, q–t and Supplementary Fig. 10o–u), while ASPP single mutants showed unaltered PP1β9C localisation (Fig. 9n–p and t). Thus, the ASPP complex is required to recruit a junctional pool of PP1β9C and PP1α96A.

Discussion
How highly specific phosphatase complexes are assembled to induce precise spatially and temporally controlled dephosphorylation of kinase substrates remains a key open question with important therapeutic ramifications. Here, we show that ASPP proteins form a PP1-containing complex with Ccdc85 and members of the N-terminal RASSF family (Figs. 1, 8 and S1). Depletion of these proteins in Drosophila leads to similar phenotypes and mutations in the ASPP PP1-binding motifs compromise its in vivo functions (Figs. 2, 3, 9, Supplementary Fig. 2, Supplementary Fig. 3, Supplementary Fig. 10). Our structural and biophysical data indicate that human ASPP2 can interact with PP1 catalytic subunits both through its RVxF SLiM and its SH3 domain, which binds an extended PPII motif in the PP1 C-tail (Figs. 5–7). Interestingly, at least in Drosophila, Ccdc85 appears to provide an additional PP1-binding surface to the ASPP/PP1 complex. Indeed, the phenotypes elicited by mutations in the ASPP RVxF motif or SH3 domains are markedly enhanced by loss of ccdc85 (Fig. 9). Our structure indicates that ASPP proteins do not select appropriate substrates for dephosphorylation by restricting access to the PP1 catalytic cleft (Fig. 5), as reported for some PIPs such as Spinophilin. Instead, in vivo analysis suggests that the ASPP complex is required for the recruitment of a
Fig. 8 Function of the ASPP2 SH3 domain/PP1α C-tail interaction. **a** Quantitative AP-MS from HEK293T cells expressing Strep-HA tagged ASPP2 or ASPP2^KVK. Chart shows protein abundance normalised to the bait (%) relative to PP1α, PP1β, PP1γ. Protein abundance is measured on the basis of the average intensity of the three most intense and unique peptide precursors. Error bars indicate the standard deviation. A two-way ANOVA test was done to determine if the differences among means were significantly different from each other. Three pairwise comparisons were carried out (ASPP2 PP1α vs ASPP2^KVK PP1α, ASPP2 PP1β vs ASPP2^KVK PP1β and ASPP2 PP1γ vs ASPP2^KVK PP1γ) and p-values were adjusted using Bonferroni correction. Significant differences are marked. ** indicates p < 0.0001. **b** Quantitative AP-MS from HEK 293T cells expressing Strep-SH tagged PP1α, PP1αΔC (PP1α1-300), PP1β and PP1γ. Protein abundance of ASPP1, ASPP2 and iASPP is measured on the basis of the three most intense and unique peptide precursors. Each intensity value is normalised to the respective bait and to PP1α. Error bars indicate standard deviation. A two-way ANOVA was done to determine if differences among means were significantly different from each other. Three pairwise comparison among each group were carried out (PP1α vs PP1αΔC, PP1α vs PP1β and PP1α vs PP1γ) and p-values were adjusted using a Bonferroni correction. Significant differences are marked. * indicates p < 0.05, ** indicates p < 0.005.

c) Comparison of PP1α and PP1αΔC protein interactions. Heatmap of ASPP2-PP1 complex proteins intensity after purification with PP1α or PP1αΔC.
d) Western blot of transfected HEK293T cell lysates probed with the indicated antibodies.
e) Quantification of the ratio between P-TAZ^{589} and total HA-TAZ protein levels normalised to control phosphorylation. Error bars represent standard deviation. A one-way ANOVA test was carried out to determine if the differences among means were significantly different from each other. Two pairwise comparisons were carried out (ASPP2wt vs ASPPFA and ASPPwt vs ASPP^KVK) and p-values were adjusted using a Bonferroni correction. Significant differences are marked. * indicates p < 0.05 (n = 3 independent experiments)
junctional pool of PP1α96A and PP1β9C (Fig. 9, Supplementary Fig. 10).

The structure of the ASPP2:PP1α complex represents observation of the PP1 C-tail interacting with a PIP and providing isoform specificity. Since most PP1-binding motifs are SLiMs present within intrinsically disordered regions, this constitutes a rare occurrence of a folded protein domain involved in PP1 recruitment. The ASPP2 SH3 domain accommodates the highly conserved PP1α PPII motif while its acidic specificity pocket enhances affinity and specificity for PP1α C-tail basic
residues PP1αK327, PP1αK329 and PP1αK330 (Figs. 5e, 6c, d), through fuzzy electrostatic interactions (Fig. 7). The variability observed in this region for the other PP1 isoforms results in reduced affinity. The first lysine (PP1αK327) appears the most critical for ASPP2 binding (Fig. 6c), suggesting that the spacing between the PP1II and C-terminal lysines is important for ASPP2 selectivity towards PP1α, which has the shortest spacer of all PP1 isoforms (Fig. 4a). In addition, the presence of residues such as glycines or prolines in the PP1β and PP1γ spacers, which likely alter or constrain C-tail geometry also reduce affinity for PP1α, as suggested by chimera experiments (Fig. 6c). Indeed, our AP-MS data indicate that the ASPP2 SH3 domain constitutes an attractive affinity are sufﬁcient for achieving a common isoform selectivity in vivo, as illustrated for RepoMan/Ki-67, which selectively bind to PP1γ based on a single amino acid change in the PP1 catalytic domain11. Indeed, our AP-MS data show that mutating the ASPP2 SH3 domain specificity pocket (ASPP2KKV) dramatically reduces binding to PP1α but not other isoforms (Fig. 8a). Furthermore, ASPP2 shows a marked preference for PP1α in HEK293T cells, while ASPP1 does not (Fig. 8b). It is interesting to note, however, that ASPP1 has a phosphatase activity for cancer therapy58, inhibitors of the ASPP2/PP1 complex, which would increase YAP/TAZ inhibitory phosphorylation, might prove an interesting therapeutic avenue. Our work indicates that the ASPP2 SH3 domain constitutes an attractive target for the specific inactivation of the ASPP2/PP1 holoenzyme. Indeed, mutation of the ASPP2 SH3 domain specificity pocket (ASPP2KKV) compromises the dephosphorylation of TAZ at its inhibitory site, serine 89 (Fig. 8). The ability to target speciﬁc SLiM interaction pockets in Ser/Thr phosphatases is supported by the discovery that the widely used immunosuppressants cyclosporin A and FK506 bind calcineurin in the LxVP SLiM binding pocket59. Thus, a better understanding of the PP1 regulatory code will impact future drug discovery efforts34,60.

Methods

Cell culture. Drosophila S2 cells (Drosophila Genome Resource Center; RRID: CVCL_2232) were maintained at 25 °C in Schneider’s Drosophila Medium (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 100 unit/ml penicillin and 100 mg/ml streptomycin (Gibco) in 75 cm² culture flasks (Corning). For transfections, 30 min prior to transfection, 3 × 10⁵ S2 cells were seeded per well of a 6-well plate (Corning) in 2 ml of serum-containing Schneider’s Drosophila Medium (Gibco). Two hundred to four hundred nanograms of DNA per plasmid were transfected per well using Effectene Transfection Reagent (Qia- gen) according to the manufacturer’s protocol. Cells were lysed 48 h after transient transfection. Human embryonic kidney cells (HEK293T) (Crick Cell Services) were cultured in 5% CO₂ atmosphere and 37 °C in DMEM (Dulbecco’s modified Eagle’s medium, SIGMA) supplemented with 10% FBS (fetal bovine serum, Gibco) and penicillin/streptomycin (100 µg/ml, Gibco). Cells were transfected with Lipofecta- mine * 2000 (Thermo Fisher) according to the manufacturer instructions. Cells were lysed 48 h after transfection. Cell lines were probed by STR and tested negative for mycoplasma by the Cell Services Technology Platform at the Francis Crick Institute.
Co-IPs and western blotting. S2 and HEK293T cells were lysed with HEPEs lysis buffer (150 mM NaCl, 50 mM HEPEs pH 7.5, 0.5% (v/v) Triton X-100 supplemented with protease inhibitor cocktail with EDTA (Roche) and the soluble fraction was obtained by centrifugation (16,000 x g, 20 min). For co-IP experiments cleared lysates were added to 20 μl of ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich) or 15 μl of GFP-Trap agarose (Chromotek) in 100 μl HEPEs lysis buffer. The lysate and the beads were incubated for 1 h at 4 °C and washed four times with HEPEs lysis buffer. Input and co-IP samples were analysed by SDS-PAGE and western blot. Primary antibodies were: mouse anti-FLAG (M2), 1/5000 (Sigma-Aldrich F1804); mouse anti-GFP (3E11), 1/1000 (Cancer Research UK); rat anti-HA (3F10), 1/2000 (Roche); mouse anti-Myc (9E10), 1/40 (Invitrogen); mouse anti-α-tubulin (Developmental Hybridoma Bank); mouse anti-V5 (R860-25), 1/5000 (Thermo Fisher), rabbit anti-P-TAZSer89 1/1000 (E1X9C) (Cell Signalling Technologies 9979), rabbit anti-HA 1/1000 (C294) (Cell Signalling Technologies 3724). Secondary antibodies were from GE Healthcare (1:5000).

Molecular biology. Fly genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen). For RT-PCR, total RNA was isolated using the RNeasy Mini Kit (Qiagen) and treated with DNase (Promega) for 37 °C for 30 min before adding DNase Stop Solution (Promega). cDNA was synthesised from 1 µg of isolated RNA with oligo(dT) primers using the First Strand cDNA Synthesis Kit for RT-PCR (Roche) according to the manufacturer’s instructions.

All Drosophila S2 and human cell expression vectors were obtained using Gateway cloning technology (Invitrogen). Entry vectors into Gateway pDONR/Zeo Vector (Invitrogen) or pENTRTM-TOPO (Invitrogen) Gateway BP clonase II Enzyme mix (Invitrogen) or TOPO cloning (Invitrogen) according to the manufacturer’s protocol. Site-directed mutagenesis was performed using PfuTurbo DNA Polymerase (Stratagene) or were previously described13. Expression vectors were obtained using Gateway system (Invitrogen) according to the manufacturer’s protocol into Drosophila Gateway Vector Collection plasmids (Drosophila Genomics Resource Center), pDEST-DNAx-VA5, pDEST-3xFLAG-CMV-7/1 or pCDNAs/FRT/T0/Sh/GW15. To generate the ASP2 rescue constructs, full-length ASPP cDNAs were cloned into the Gateway system into pBAD-30 expression plasmid and the resulting plasmids were then transformed in E. coli DH5α and the verified sequences were then used for the expression in human cell lines. In HEK293 cells were co-transfected with the corresponding expression plasmids (ASPP1, ASPP2, ASPP2KVK, PP1α, PP1β, PP1γ) and the pOG4 vector (Thermo Fisher) for co-expression of the Flp-recombinase using FuGENE 6 transfection reagent according to manufacturer’s specifications (Promega). Two days after transfection, cells were collected and lysed using lysis buffer (100 µg/ml). Stable isogenic cell pools were grown in four 14 cm Nunc dishes, followed by 1 µM/mL doxycycline for 24 h for the expression of SH-tagged proteins, harvested with PBS and frozen in liquid nitrogen. Each cell line was processed for three biological replicates. The frozen cell pellets were resuspended in 50 µl Triton X-100 buffer (50 mM NaCl, 50 mM NaF, 0.5% Igepal CA-630, 200 µM NaVO3, 1 mM PMSF, 20 µg/ml Avidin and 1 mM Protease Inhibitor mix (Sigma-Aldrich)) and incubated on ice for 10 min. Cleared lysates were incubated with 100 µl slurry Strep-Tactin sepharose beads (IBA GmbH) in spin column (Bio-Rad). The beads were washed with 2 ml HNN lysis buffer and 2 ml 200 mM HEPES buffer (50 mM NaF, 0.5% Igepal CA-630, 200 µM NaVO3) and HNN buffer and processed following the FASP protocol88. Briefly, the eluate was loaded on a 10 kDa molecular weight cut-off spin column (Vivaspin 5000, 50,000) and centrifuged at 14,000 × g for 30 min until dryness. Samples were denatured, reduced (8 M Urea and 5 mM TCEP in 50 mM ammonium bicarbonate, 30 min) and alkylated (10 mM iodoacetamide, 30 min). Each sample was subsequently washed three times by flushing the column with 25 mM ammonium bicarbonate and proteolyzed with 0.5 µg of Trypsin (Promega, sequencing grade) for 16 h at 37 °C. Proteolyis was quenched by 0.1% TFA and peptides were purified with a C18 micropipen column (Next Generation). The dried peptides were reconstituted in 0.1% formic acid and 2% acetonitrile. IRT peptides (Biognosy) were spiked to each sample before LC-MS/MS analysis for quality control. LC-MS/MS was performed on Orbitrap Fusion Trividib mass spectrometer (Thermo Fisher) coupled with a Thermo easyLC-1000 liquid chromatography system (Thermo Fisher). Peptides were separated using reverse phase column (NanoeX C18 100 Å, 1.8 µm, 75 µm x 250 mm) across 60 min linear gradient from 5 to 35% (buffer A: 0.1% v/v formic acid, 2% (v/v) acetonitrile; buffer B: 0.1% (v/v) formic acid, 98% (v/v) acetonitrile). The data acquisition mode (data-dependent acquisition) was set to perform a cycle of 3 s with high resolution MS scan in the Orbitrap (120,000 at 400 m/z) to acquire MS/MS spectra in the top 5 MS/MS ions in the mass range 60-1000 m/z. Charge state (2+, 3+) ions were selected for fragmentation. The dynamic exclusion window was set to 25 s. Precursors with MS signal that exceeded a threshold of 5000 were fragmented (HCD, Collision Energy 20%). The ion accumulation time was set to 50 ms (MS) and 80 ms (MS/MS).

Acquired spectra were searched using the MaxQuant software package version 1.5.2.8 embedded with the Andromeda search engine89 against humanproteome reference database (http://www.uniprot.org/) extended with reverse decoy sequences. The search parameter were set to include only full tryptic peptides, carbamidomethyl as static peptide modification, oxidation (M) and phosphorylation (S,T,Y) as variable modifications. The MS and MS/MS mass tolerance was set, respectively, to 20 ppm and 0.5 Da. False discovery rate of <1% was used at the protein level to infer the protein presence. The protein abundance was determined from the intensity of top two unique peptides for each protein. Raw data are provided in Tables 2 and 3.

Image analysis. The cell outlines of 40 APF retinas were visualised using anti-E- cadherin antibodies. IOCs were counted within a hexagonal region of interest using ImageJ. Each vertex of the hexagon was placed at the centre of a retinal amastigada surrounding one central ommatidium. Cells that were bisected by the lines of the hexagon were counted as half a cell. Bristle clusters were not counted as IOCs. Bristle displacement was defined as any hexagon containing either two bristle clusters directly next to each other or when two bristle clusters were not separated by two proximal pigment cells. Missing bristle clusters were not counted as bristle misplacement.

Adults fly wings were mounted in Euparal (Agar Scientific) and imaged with a LeicaDFC420C camera mounted on a Zeiss Axioplan2 microscope. Image was used to trace the outline of wing blade. The identification of junctions of PP1–FP–FP198 and GFP–FP1–AAP66A was performed using ImageJ. Statistical analysis were performed using the Prism Graphpad 7 software.
Protein preparation. An overnight pre-culture of PP1α-330, PP1α-700, PP1β-327, PP1γ1-11 with a plasmid encoding PP1α-330, PP1α-700, PP1β-327, PP1γ1-11 was grown at 37 °C in LB medium supplemented with Kan, Cam and 1 mM MnCl2. PP1 protein production was initiated by inoculating a 1001 fermenter with 400 ml of pre-culture. The cells were grown in LB medium supplemented with 1 mM MnCl2 at 30 °C to an OD600 of ~0.5 when 2 g/l of arabinose was added to induce the expression of the GroEL/GroES chaperone. When OD600 reach ~1, the temperature was lowered to 10 °C and PP1:ASPP2 expression was induced with 0.1 mM IPTG for 24 h. Harvested cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM imidazole, 700 mM NaCl, 1 mM MnCl2, 1% TX-100, 0.5 mM TCEP, 0.5 mM AEBSF, 15 μg/ml benzamidine and complete EDTA-free protease inhibitor tablets) and lysed by French press. The supernatants is clarified by centrifugation and stored at ~80 °C.

Crystallization and data collection and refinement. For PP1α:ASPP2 complex expression the plasmids encoding PP1α-330 and ASPP2-1120 were co-transformed with a pGEX-6P2 plasmid (GE Healthcare) containing a cleavable GST tag. ASPP2-1120 expression was at 30 °C in E. coli Rosetta (DE3) pLysS (Merck). Bacteria were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1% TX-100, 0.5 mM TCEP, 0.5 mM AEBSF, 15 μg/ml benzamidine and complete EDTA-free protease inhibitor tablets) and lysed by French press. The supernatants is clarified by centrifugation and stored at ~80 °C. ASPP2-1120 expression was induced with 0.1 mM IPTG for ~18 h. The cells were harvested by centrifugation, resuspended in fresh LB medium (again supplemented with 1 mM MnCl2 and 200 μg/ml chloramphenicol to eliminate all ribosome activity) and agitated for 2 h at 10 °C. Harvested cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.5 mM TCEP, 0.5 mM AEBSF, 15 μg/ml benzamidine and complete EDTA-free protease inhibitor tablets) and lysed by French press. The supernatants is clarified by centrifugation and stored at ~80 °C. PP1α:ASPP2 complex is then purified by size exclusion chromatography using a Superdex 200 column equilibrated and run in 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl. Peptides for ITC measurements were synthesized by the LRI peptide synthesis core facility.

Expression and purification of PP1α-330 for NMR. Human PP1α-330, was cloned into the pET-M8-MBP plasmid that encodes an N-terminal Histag tag followed by maltose binding protein (MBP) and a TEV (tobacco etch virus) protease cleavage site and expressed in E. coli BL21 (DE3) CodonPlus-RIL cells (Agilent Technologies). Cells were grown in Lysogeny Broth in the presence of selective antibiotics at 37 °C up to OD600 of 0.6–0.8, and expression was induced by addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Proteins were expressed for ~14 h at 18 °C prior to harvesting by centrifugation at 6,000 x g. Cell pellets were stored at ~80 °C.

Cell pellets were suspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM Imidazole, 0.1% Triton X-100; EDTA-free protease inhibitor, (Roche), and lysed using high pressure homogenization (Avestin C3 Emuliflex)). The cell lysate was centrifuged at 45,500 x g and the soluble fraction was loaded onto 10 ml of pre-equilibrated Ni-NTA resin (GE Healthcare). After 1 h at 4 °C, the resin was washed with five column volumes of wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole) and eluted with 20 ml of elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 500 mM imidazole). The purified protein was cleaved overnight at 4 °C with tobacco etch viral (TEV) protease and then subjected to a Ni-NTA purification step to remove the cleaved His-tagged MBP, any uncleaved protein and TEV protease. Purified PP1α-330 was pooled and heat purified (95 °C, 30 min; centrifuged at 12,000 x g) and the supernatant concentrated and immediately frozen and stored at ~20 °C.

For NMR measurements, expression of uniformly 15N- and/or 13C-labeled PP1α-330 was facilitated by growing cells in M9 minimal media containing 1 g/l 15NH4Cl and/or 4 g/l 13C-D-glucose (CIL) as the sole nitrogen and carbon sources, respectively.

NMR spectroscopy. The sequence-specific backbone assignment of 15N- and 13C-labeled PP1α-330 was achieved by series of 3D NMR spectra including 3D HNCA, 3D CBCA(CO)NH, 3D HNCACB and 3D HN(CO)CA. All the spectra for PP1α-330 were obtained at 283 K using a Bruker Neo 600 MHz spectrometer equipped with a TCI HCN z-gradient cryoprobe. NMR spectra were processed with NMRPipe and Topspin (Bruker) and analysed using either CARA (http://www.cara.nmr.ch) or Sparky (http://www.cgl.ucsf.edu/home/sparky). 

1H, 15N and 13C chemical shifts were indirectly referenced to 3-trimethyl-silyl-1-propanesulphonic acid, sodium salt (DSS). Chemical shift index (CSI) and secondary structure propensity (SSP) were calculated using the RefDB database. CSI (deviations of chemical shifts from their expected random core values, also known as secondary chemical shifts determined using PCCS – ΔCS = ΔObserved – ΔExpected, and ΔCS = ΔObserved – ΔExpected) and SSP scores were calculated using 13C and 15O chemical shifts, five weight averaged and default parameters, using the RefDB programme. Cysteine and residues immediately preceding proline were ignored in secondary chemical shift calculations.

PP1α-330 and PP1γ1-11 co-crystallized with ASPP2 (twofold excess) stable state heteronuclear 13N/H NOE spectra (128 scans) were obtained using a 5 s recycle delay (with and without pre-stimulation). Additionally, 15N transverse relaxation (CPMG) measurements (64 scans) were recorded using relaxation delays (16.96, 33.92, 67.84, 115.68, 203.52, 271.36, 339.2, 407.04 and 542.72 ms for PP1α-330 and PP1γ1-11). Data were analysed using SPICE and SEDPHAT and GuASS.

Bio-layer interferometry assay. Bio-layer interferometry (BLI) is an optical analytical technique for measuring kinetics of interactions in real-time. The biosensor tip surface immobilized with a ligand is incubated with an analyte in solution, resulting in an increase in optical thickness of the biosensor tip and a wavelength shift, which is a direct measure of the change in thickness. Bio-layer interferometry analysis of ASPP bindind to PP1 were studied using Octet Red 96 (Fortebio). In total, 50 μg/ml HIS tagged PpiA, PpiB or PpiY1 were immobilized on Nickel coated biosensor (Ni-NTA, Fortebio) and the typical immobilization levels were above 3 nm. Ligands-loaded Ni-NTA biosensors were then incubated with different concentrations of ASPP in the kinetics buffer. Global fitting of the binding curves generated a best fit with the 1:1 model and the kinetic association and dissociation constants were calculated. All binding experiments were performed in solid-black 96-well plates containing 200 μl of solution in each well at 25 °C with an angular velocity of 1000 rpm. Curve fitting, steady state analysis, and calculation of kinetic parameters (Kd, kcat, and kcat/km) were done using Octet software version 7.0 (Fortebio).
13. Terrak, M., Kerff, F., Langsetmo, K., Tao, T. & Dominguez, R. Structural basis for regulation of protein phosphatase 1.

14. Ragusa, M. J. et al. Spinophilin directs protein phosphatase 1 specificity toward interacting proteins. Transl. Res. 164, 366–391 (2014).

15. Goldberg, J. et al. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase 1. Nature 376, 745–753 (1995).

16. Scotti-Lavinio, E., Garcia-Diaz, M., Du, G. & Frohman, M. A. Basis for the isoform-specific interaction of myosin phosphatase subunits with protein phosphatase 1c beta and myosin phosphatase targeting subunit 1. J. Biol. Chem. 285, 6419–6424 (2010).

17. Skene-Arnold, T. D. et al. Molecular mechanisms underlying the interaction of protein phosphatase 1c with ASPP proteins. Biochem. J. 449, 649–659 (2013).

18. Helps, N. R., Barker, H. M., Elledge, S. J. & Cohen, P. T. Protein phosphatase 1 interacts with p53BP2, a protein which binds to the tumour suppressor p53. FEBS Lett. 377, 295–300 (1995).

19. Bennett, D., Lyučeva, L., Alphey, L. & Hawcroft, G. Towards a comprehensive analysis of the protein phosphatase 1 interactorome in Drosophila. J. Mol. Biol. 364, 196–212 (2006).

20. Sullivan, A. & Lu, X. ASPP: a new family of oncogenes and tumour suppressor genes. Br. J. Cancer 96, 196–200 (2007).

21. Virshup, D. M. & Shenolikar, S. From promiscuity to precision: protein phosphatases get a makeover. Mol. Cell 33, 537–545 (2009).

22. Verheyen, T. et al. Genome-wide promoter binding profiles of the p53 family (ASSP) interacts with protein phosphatase 1 via a noncanonical binding motif. J. Biol. Chem. 286, 34039–34044 (2011).

23. Huang, H. B. et al. Characterization of the inhibition of protein phosphatase 1 by DARPP-32 and inhibitor-2. J. Biol. Chem. 274, 7870–7878 (1999).

24. O’Connell, N. et al. The molecular basis for substrate specificity of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. J. Biol. Chem. 278, 18817–18823 (2003).

25. Skene-Arnold, T. D. et al. Molecular mechanisms underlying the interaction of protein phosphatase 1c with ASPP proteins. Biochem. J. 449, 649–659 (2013).

26. Sullivan, A. & Lu, X. ASPP: a new family of oncogenes and tumour suppressor genes. Br. J. Cancer 96, 196–200 (2007).

27. Bennett, D., Lyučeva, L., Alphey, L. & Hawcroft, G. Towards a comprehensive analysis of the protein phosphatase 1 interactorome in Drosophila. J. Mol. Biol. 364, 196–212 (2006).

28. Sullivan, A. & Lu, X. ASPP: a new family of oncogenes and tumour suppressor genes. Br. J. Cancer 96, 196–200 (2007).

29. Connell, N. et al. The molecular basis for substrate specificity of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. J. Biol. Chem. 278, 18817–18823 (2003).

30. Cong, W. et al. ASPP2 regulates epithelial cell polarity through the PAR complex. Curr. Biol. 20, 1408–1414 (2010).

31. Langton, P. F. et al. The DSAPP-DRASSF8 complex regulates cell-cell adhesion during Drosophila retinal morphogenesis. Curr. Biol. 19, 1969–1978 (2009).

32. Zausinger, S., Zhou, Y., Bray, S. J., Tapon, N. & Dijane, A. Drosophila MAGI interacts with RASSF8 to regulate E-Cadherin-based adherens junctions in the developing eye. Development 142, 1102–1112 (2015).

33. Zhang, Z. P. et al. ASPP1/2/PP1 complexes are required for chromosome segregation and kinetochore-microtubule attachments. Oncotarget 6, 41550–41565 (2015).

34. Zhang, Y. et al. The tumor suppressor proteins ASPP1 and ASPP2 interact with C-Nap1 and regulate centrosome linker reassembly. Biochem. Biophys. Res. Commun. 458, 494–500 (2015).

35. Royer, C. et al. ASPP2 links the apical lateral polarity complex to the regulation of YAP activity in epithelial cells. PLoS One 9, e113384 (2014).

36. Liu, C. Y. et al. PPI cooperates with ASPP2 to dephosphorylate and activate TAZ. J. Biol. Chem. 286, 5558–5566 (2011).

37. Yoon, S. et al. Essential, overlapping and redundant roles of the Drosophila protein phosphatase 1 alpha and beta subunits. J. Cell. Biol. 6829, 2012 (1995).
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Author contributions

S.M. and R.L. solved the crystal structure and measured ITC affinities, S.M. performed the BLI measurements. Y.Z., M.T.B. and J.B. performed all cell culture and Drosophila experiments and performed molecular cloning for the structural and biophysical studies. N.O.R. synthesized peptides. R.B. performed the NMR studies; G.S.K. measured ITC affinities of full-length PP1. F.U., A.v.D., S.H. and M.G. provided Mass Spectrometry data. N.T., S.M., M.G., R.P. and W.P. supervised the study. All authors contributed to manuscript writing.

Additional information

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