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Activation of client protein kinases by the HSP90 molecular chaperone system is affected by phosphorylation at multiple sites on HSP90, the kinase-specific co-chaperone CDC37, and the kinase client itself. Removal of regulatory phosphorylation from client kinases and their release from the HSP90-CDC37 system depends on the Ser/Thr phosphatase PP5, which associates with HSP90 via its N-terminal TPR domain. Here, we present the cryoEM structure of the oncogenic protein kinase client BRAFV600E bound to HSP90-CDC37, showing how the V600E mutation favours BRAF association with HSP90-CDC37. Structures of HSP90-CDC37-BRAFV600E complexes with PP5 in autoinhibited and activated conformations, together with proteomic analysis of its phosphatase activity on BRAFV600E and CRAF, reveal how PP5 is activated by recruitment to HSP90 complexes. PP5 comprehensively dephosphorylates client proteins, removing interaction sites for regulatory partners such as 14-3-3 proteins and thus performing a ‘factory reset’ of the kinase prior to release.

Interaction with the HSP90 molecular chaperone system is a prerequisite for the stability and biological function of a large proportion of the kinome, including most of the main oncogenic protein kinases. Recruitment of kinases to the HSP90 system is mediated by CDC37, which functions as an adaptor able to interact independently with HSP90 and protein kinases and facilitate their association. CDC37 is subject to a number of phosphorylation events, one of which—phosphorylation of Ser13 by casein kinase 2 (CK2)—is critical to its function in protein kinase activation. HSP90 itself is also multiply phosphorylated, and while none are critical to its core biochemistry, several of the modified sites have nonetheless been shown to have important functions in the regulation of ATP-utilisation and/or co-chaperone and client interactions. The protein kinase clients of HSP90–CDC37 are themselves frequently phosphorylated, sometime autogenously, as part of their regulation, and can, in turn, participate in the phosphorylation of components of the chaperone complexes to which they are recruited, generating a complex network structure of post-translational regulation—the so-called Chaperone Code—the surface of which has only been scratched.

Phosphorylation is, by its nature, a reversible post-translational modification, and its role in switching the behaviour of a modified protein depends both on the kinase that ‘writes’ the modification and the phosphatase that ‘erases’ it. HSP90 is directly associated with an unusual serine/threonine protein phosphatase PP5 (Ppt1p in yeast), which has been implicated in the maturation/activation of a number of HSP90-dependent client proteins. PP5 has a tetratricopeptide (TPR) domain attached to the N-terminus of an Mn2+-dependent PP1/PP2A/PP2B family phosphatase domain. In common with several other HSP90-associated proteins, the TPR domain confers a high affinity for the MEEVD motif that forms the extreme C-terminus of HSP90. Ppt1p, the yeast homologue of PP5, has been implicated in regulating the phosphorylation of HSP90 itself, with a deficit of Ppt1p activity leading to reduced activation of a range of client proteins in vivo. Within the specific context of the HSP90–CDC37 system, activation of protein kinase clients in vivo has been shown to depend on dephosphorylation of pSer-13 in CDC37 by PP5, which only occurs when PP5 and CDC37 are bound simultaneously to the same HSP90 dimer.
To understand how PP5 operates in the context of an HSP90–CDC37 client complex, we have reconstituted an active PP5 complex with HSP90, CDC37 and the highly HSP90-dependent oncogenic V600E mutant of the protein kinase BRAF. We have determined the cryoEM structure of an HSP90–CDC37–BRAFV600E complex, and structures of HSP90–CDC37–BRAFV600E with PP5 bound in phosphate-engaged and phosphatase-autoinhibited conformations. These structures reveal how single PP5 docks with the dimeric C-terminus of HSP90, and how docked PP5 rearranges to allow the catalytic phosphatase domain to access phosphorylation sites on the chaperone, co-chaperone and client. Together with proteomic analysis of PP5 activity on HSP90–CDC37–bound BRAFV600E and HSP90–CDC37–bound CRAF, our studies reveal how the HSP90–CDC37–PP5 complex acts to comprehensively dephosphorylate the bound client.

**Results**

**PP5 dephosphorylates CDC37 within an HSP90–CDC37–BRAFV600E complex**

We previously showed that protein phosphatase 5 (PP5) was able to dephosphorylate pSer13 in CDC37 when both proteins were physically associated with HSP90. To determine whether PP5 could also do this when CDC37 was engaged in a complex with a client protein and HSP90, we co-expressed and purified an HSP90–CDC37–BRAFV600E (HCK) complex from insect cells (see Methods), and used a phosphospecific antibody to demonstrate that wild-type PP5 could dephosphorylate CDC37–pSer13 in the complex in a time-dependent manner (Supplementary Fig. 1A).

To attempt to trap a productive complex of PP5 engaged with HSP90–CDC37–BRAFV600E, we incubated the HSP90–CDC37–BRAFV600E complex with a PP5 D274N mutant which had previously been shown to catalytically inactivate PP5 with minimal disruption to substrate binding and were able to purify a stable HSP90–CDC37–BRAFV600E–PP5 complex (HCK–P) on size exclusion chromatography (Supplementary Fig. 1B).

**CryoEM structure determination**

For structural studies, the purified complex was cross-linked (Supplementary Fig. 1C) and applied to cryoEM grids, which were then plunged into a liquid ethane/propane mixture. Movies from selected regions of the grids were recorded on an FEI Titan Krios microscope equipped with a Falcon IV detector (see Methods). Motion correction, images processed, and particles picked using cryoSPARC and RELION 4.0 (Supplementary Fig. 1D, E). We determined the cryoEM structure of an HSP90–CDC37–BRAFV600E complex, which in the map, with only the C-terminal lobe of the kinase domain being well defined in the map (Fig. 1D). The polypeptide chain for this segment can be traced into clear features from Thr521 to Ile724, apart from the region corresponding to the activation segment connecting the 594-DFG-596 and 621-APE-623 motifs, which is poorly ordered. The final 42 residues at the C-terminus beyond the kinase C-lobe are also disordered.

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**Structure of HSP90–CDC37–BRAFV600E**

The structure of the HSP90–CDC37–BRAFV600E complex consists of two molecules of human HSP90β arranged in the ATP-bound closed conformation originally observed in a complex of yeast HSP90 and the co-chaperone P23/Sba1 (Fig. 1A). The polypeptide chain for both HSP90β molecules can be traced through more or less continuous, ordered density from Glu10 to Glu692, with the exception of the low-complexity linker segment from approximately 220–275 which connects the N-terminal and central domains. Consistent with the closed conformation, bound ATP (or ADP-molybdate) is present in the N-terminal domains of both HSP90 molecules (Fig. 1B).

CDC37 in the complex presents in a very similar conformation as seen in the cryoEM structure of the HSP90–CDC37–CDK4 complex, with the N-terminus (residues 1–120), which consists predominantly of a long coiled-coil α-hairpin protruding from one side of the core HSP90 dimer, while the globular helical domain that forms the bulk of the C-terminal dimer (136–378) is packed against the opposite face of the dimer (Fig. 1A). The two-halves of CDC37 are connected by an extended β-strand (121–135) which hydrogen bonds onto the edge of the central β-sheet of the middle domain of one of the HSP90 monomers. The polypeptide chain in the N-terminus can be traced from the N-terminus methionine to Cys54 and from Leu91–Glu34; however, the tip of the coiled-coil α-hairpin (residues 55–90) is not visible in the map. The C-terminal part of CDC37 is far less well defined than the N-terminus, with the structure only discernible at the level of secondary structural elements from residue 140 to residue 266, suggesting a high degree of disorder and/or multiple conformational states for this loosely bound domain.

Serine 13, whose phosphorylation and targeted dephosphorylation are critical for client kinase activation by HSP90, is clearly phosphorylated within the complex and engaged with the side chains of CDC37 residues His33 and Arg36, and Lys406 of HSP90 as previously seen in the cryoEM structure of the HSP90–CDC37–CDK4 complex (Fig. 1C).

Although the complex was formed by co-expression of the full-length proteins, relatively little of the 84 kDa BRAFV600E is visible in the cryoEM volume, with only the C-terminal lobe of the kinase domain being well defined in the map (Fig. 1D). The polypeptide chain for this segment can be traced into clear features from Thr521 to Ile724, apart from the region corresponding to the activation segment connecting the 594-DFG-596 and 621-APE-623 motifs, which is poorly ordered. The final 42 residues at the C-terminus beyond the kinase C-lobe are also disordered.

The face of the BRAFV600E kinase C-lobe that forms one wall of the ATP-binding cleft in the fully folded kinase structure interacts with a contiguous segment of CDC37 from Thr19 to Ala35, incorporating the beginning of the first helix in the coiled-coil segment (Fig. 1E). The core of the interface is provided by the side chains of His20, Ile23, Asp24, Ser27 and Trp31 of CDC37, which sit together in a channel in BRAFV600E lined by Arg562, Gly563, Tyr566, Leu567, Ile572, His574, Thr590, Lys591, Ile592, Gly593 and Asp594. One consequence of the interaction of CDC37–Trp31 with BRAFV600E is to force the catalytically important DFG motif, into a quite different conformation to that found in the folded active kinase, with the following activation segment containing the oncogenic V600E mutated residue, being disordered. V600E and other common oncogenic Val600 mutations have been shown to confer a strong dependence on association with HSP90–CDC37 for cellular stability and activation, whereas wild-type BRAF is a relatively weak ‘client’ of Val600 in wild-type BRAF forms part of a hydrophobic cluster that holds the activation segment in an ordered inhibitory conformation, which is destabilised by oncogenic mutations such as V600E, contributing to unregulated kinase activity. Such destabilisation would also facilitate the conformational switch of the DFG and attached activation segment required by the interaction with CDC37 seen here, more readily than the hydrophobic and more rigid wild-type sequence, providing a satisfactory explanation for the substantially stronger HSP90-dependence of the oncogenic BRAF mutants.

HSP90 makes only a few direct contacts with the BRAFV600E kinase C-lobe, restricted to peripheral interactions with surface-exposed side chains of Arg338, Phe341 and Trp312 from the central region of HSP90 (Fig. 1F)–the latter two previously implicated in client interactions in an earlier mutagenesis study, and a polar interaction between HSP90–Arg196 and BRAF–Asp565. The major interactions between HSP90 and the kinase client involve residues 521–533 of BRAFV600E, which would be part of the N-terminal lobe in the fully folded kinase structure, which in the complex threads between the central segment of the two HSP90 monomers
adjacent to the extended loops from Asn351 to Phe344 that come close together at the heart of the HSP90 dimer (Fig. 1G). Upstream of BRAFV600E residue 521, the chain emerges on the opposite face of the dimer, to run adjacent to the loosely bound globular domain in the C-terminal half of CDC37. However, the map in this region lacks detail due to conformational flexibility, and/or the presence of multiple conformations.

Structure of HSP90–CDC37–BRAFV600E–PP5 complexes

Two different sets of particles were obtained in which additional volume corresponding to PP5 was evident bound to the C-terminus of the HSP90 dimer within the HSP90–CDC37–BRAFV600E complex. The two structures are distinguished by whether the C-terminal phosphatase domain of PP5 is packed against the N-terminal TPR domain in a ‘closed’ conformation or is substantially separated from it in an ‘open’
sequence in the cryoEM map. E Within the complex, Trp31 displaces Phe595 of the key regulatory DFG motif into a different conformation than in the intact kinase, stabilised by the interaction of DFG Asp594 with CDC37 Arg30. This conformational switch is facilitated by the oncogenic V600E mutation in the ‘activation segment’ immediately following the DFG motif, and explains why the oncogenic BRAFV600E mutant is a strong client of the HSP90–CDC37 chaperone system, whereas the wild-type is not. F HSP90 itself only makes peripheral contact with the kinase C-lobe, but mutation of the HSP90 residues involved impairs kinase activation in vivo8. G As previously seen for CDK4 in complex with HSP90 and CDC37, the strand from the kinase N-lobe immediately upstream of the well-ordered C-lobe, becomes linearised and stretches between the two HSP90 monomers to emerge on the other face of the complex adjacent to the globular part of CDC37. No ordered structure upstream of this is visible in the cryoEM maps.

**PP5 phosphatase targets**

While dephosphorylation of CDC37–pSer13 is the best studied HSP90-associated activity of PP5 (see Supplementary Fig. 1A), under the conditions in which HSP90–CDC37–BRAFV600E is expressed and purified to be amenable to structural studies, CDC37–pSer13 is fully buried in the core of the ATP-bound closed HSP90 complex and remains so in the presence of the catalytically dead PP5. Even though the phosphatase domain of PP5 can detach from the C-terminus of the HSP90 dimer and move substantially towards CDC37, pSer13 would only become accessible when the N-terminal domains of HSP90 separate following ATP hydrolysis, so trapping a structurally tractable complex in which PP5 is engaged with CDC37–pSer13 remains to be achieved. However, CDC37 is not the only component of the complex that is susceptible to phosphorylation, and, therefore, a potential substrate for PP5. To gain some insight into other potential substrates, we mapped the phosphorylation sites on the purified HSP90–CDC37–BRAFV600E complex with and without treatment with PP5, by mass spectrometry (see Methods, Fig. 4A, C). We identified two sites in HSP90 (Ser226, Ser255), which were significantly diminished by PP5 treatment. Both of these CK2 sites are within the charged linker segment connecting the N and middle domains of HSP90 and have been implicated in the regulation of HSP90β secretion42. We identified multiple sites in BRAFV600E whose phosphorylation was significantly (p < 0.03) decreased by PP5 treatment (Fig. 4A). One (Ser151) occurs just before the RAS binding domain (RBD), while six (Ser339, Ser365, Thr401, Ser429, Ser432, Ser446) occur within the disordered segment between the RBD and kinase domains. Ser365 plays a critical role in 14–3–3 binding43 and, along with Ser429, has been shown to have differential regulatory effects on different BRAF isoforms44, while Ser446, which maps just upstream of the kinase N-lobe, is the topological equivalent of Ser338 in CRAF whose dephosphorylation by PP5 was previously shown to deactivate kinase signalling activity19.

Within the kinase domain itself, which is the focus of interaction of the HSP90–CDC37 system, we identified no phosphorylation sites in the N-lobe, but two sites within the C-lobe, which is the only part of BRAFV600E in complex with HSP90–CDC37 that is resolved in the cryoEM structure. Ser64, identified as inhibitory phosphorylation specifically enriched in the V600E mutant19, maps to the C-terminal end of the activation segment 594–623, which is disordered in the structure, while Ser675 is involved in the regulation of BRAF ubiquitylation by the E3 ligase ITCH19 is in the middle of an extended coil that
connects two helices. Both of these residues map to parts of the surface of BRAFV600E that are not involved in interaction with HSP90 or CDC37, and would therefore be accessible to the PP5 phosphatase domain given the flexibility of its connection to its TPR anchor. We found no phosphorylation of the activation segment residues Thr599 and Ser602, whose phosphorylation by MLK343 is required for full BRAF (wt or V600E) kinase activity44.

Three further sites (Ser729, Ser750 and Thr753) all occur in the unstructured segment that follows the end of the C-lobe at residue 720. Ser729 has recently been shown to have a key role in 14-3-3 binding in concert with Ser36534,45. The proximity of the C-terminal end of the C-lobe to the substrate binding cleft of the phosphatase domain strongly suggests that one or more of these sites are engaged with the catalytically inactivated PP5.

We performed the same analysis for an HSP90–CDC37–CRAF complex, and as with the BRAF complex, observed a set of phosphorylation sites present on the kinase in the absence of PP5 treatment, that were significantly dephosphorylated when PP5 was added (Fig. 4B, C). These included Ser233, Ser259 and Ser621, which, similarly to Ser365 and Ser729 in BRAF, mediate CRAF interaction with 14-3-3 protein46. As with the BRAF complex, no activation segment phosphorylation (Ser491, Ser494) was detected in the HSP90-CDC37-associated CRAF.

As PP5-dependent dephosphorylation of 14-3-3 binding sites was observed in both BRAF and CRAF, we considered the possibility that PP5 might play a role in modulating the regulatory interaction of these kinases with 14-3-3 proteins. We have previously shown that downstream ‘partner’ proteins of HSP90-CDC37-bound kinases, such as CDK4 and CDK6, are able to extract the chaperone-bound kinase, presumably due to equilibrium exchange between the closed and open forms of the chaperone complex47. We, therefore, considered the possibility that 14-3-3 may be able to do this with HSP90-CDC37-bound BRAFV600E so long as the key 14-3-3-interacting sites on BRAFV600E were phosphorylated. Using an affinity ‘pull-down’ of the kinase in the purified HSP90–CDC37–BRAFV600E complex in the presence of 14-3-3 protein in vitro, we observed co-precipitation of 14-3-3 protein.
Western blotting confirmed that, consistent with the mass spectrometry observations, BRAF^{V600E} molecules within the purified HSP90–CDC37–BRAF^{V600E} sample retained phosphorylation on at least Ser729 which is required for high-affinity interaction with 14-3-3 (Fig. 5B). However, when PP5 was added to the HSP90–CDC37–BRAF^{V600E} complex, the amount of 14-3-3 protein co-precipitating with the BRAF-V600E was substantially diminished consistently with the dephosphorylation of BRAF-V600E-Ser729 observed when PP5 was added. The isolated TPR domain of PP5 had no effect on the phosphorylation state of BRAF^{V600E} nor the level of 14-3-3 co-precipitated. We performed the same analysis with the HSP90–CDC37–CRAF complex, and again saw a substantial decrease in the amount of 14-3-3 that co-precipitated with CRAF following dephosphorylation of CRAF-Ser621 (Fig. 5C, D).

**Discussion**

The structures presented here provide a view of a protein kinase other than CDK4 in a complex with CDC37 and HSP90 in the ATP-bound closed state. This confirms a common mechanism of partial denaturation of the kinase domain, with the first strand of the N-lobe linearised in the molecular clamp of the closed HSP90. While the remainder of the N-lobe of CDK4 was partially visible in some subsets of particles, the smaller and less structured N-lobe of BRAF is completely disordered in the HSP90–CDC37–BRAF^{V600E} complex. The molecular details of the interaction of the DFG motif of BRAF^{V600E} with CDC37 in the complex provide a satisfactory explanation for how the oncogenic mutation of Val600 within the early part of the activation loop, converts BRAF from a weakly dependent HSP90 client with only moderate affinity for CDC37, into a highly dependent client which is rapidly degraded when cells are treated with HSP90 inhibitors. The potential importance of activation segment conformation in the client recognition process mediated by CDC37 is further underlined by the failure to detect any activation segment phosphorylation in either BRAF^{V600E} or CRAF in their purified complexes with HSP90–CDC37. This may indicate that activation segment phosphorylation only occurs after the release of the dephosphorylated kinase from the chaperone complex, or that its presence is inhibitory to recruitment by HSP90–CDC37—further work will be required to distinguish these possibilities.
PP5 docks onto HSP90–CDC37–BRAF\textsuperscript{V600E} through a bipartite interaction mediated by the TPR domain, which binds the C-terminal MEEVD motif of HSP90 in its concave channel and plugs the tip of its terminal \( \alpha \)-helix into one of two hydrophobic pockets formed at the interface of non-equivalent \( \alpha \)-helices from each of the two HSP90 monomers. This mode of interaction is markedly different from that of FKBP51 with HSP90, which uses an N-terminal extension to its TPR to bind perpendicularly between the last helices of the HSP90 dimer\textsuperscript{49}. We observe PP5 binding alternatively to both symmetry-related C-terminal pockets on HSP90, but with markedly different conformations depending on which side of the overall complex the phosphatase domain is positioned (Fig. 6A). When on the same face as the globular region of CDC37, which presents no phosphorylated substrate residues, the phosphatase domain remains associated with the TPR domain at the C-terminus of the HSP90 dimer in an auto-inhibited conformation\textsuperscript{37}. However, when bound with the phosphatase on the same face as the ordered C-lobe of the kinase, the phosphatase detaches from the TPR and docks against the middle domain of HSP90, with its substrate binding cleft in contact with the face of the kinase C-lobe from which the C-terminal segment extends, most likely held there by its interaction with one of the substrate phosphorylation sites that map to the early part of that segment (Fig. 6B, Supplementary movie 1). Considerable flexibility of the unstructured linker that connects the phosphatase to its TPR anchor would allow the phosphatase access to other substrate phosphorylation sites on the exposed surface of the C-lobe, and, indeed to parts of the kinase that are disordered in the complex, but nonetheless brought into general proximity to the phosphatase domain by their mutual binding to the HSP90–CDC37 ‘scaffold’.

**Fig. 4 | PP5 is a comprehensive remover of client phosphorylations.**

A Comparison of phosphopeptide abundances from mass spectrometry analysis of BRAF\textsuperscript{V600E} in the context of a complex with HSP90–CDC37, with or without incubation with PP5, each with two technical repeats utilising different labelling. Peptides are clustered on scaled abundance. Statistical analysis was performed with a two-sided \( t \)-test and multiple hypothesis testing corrections by permutation-based FDR. Significant hits were filtered for \( p\text{-value}<0.05, \log_{2}\text{fold-change}<1 \) and had an FDR < 0.1. B As A, but for a CRAF–HSP90–CDC37 complex. C Schematic of phosphorylation sites identified in HSP90–CDC37-associated BRAF\textsuperscript{V600E} and HSP90–CDC37-associated CRAF that are removed by the addition of PP5 (see Methods). Of the characterised globular regions, only the kinase C-lobe is ordered in complexes with HSP90–CDC37. Regulatory roles have been assigned in the literature for many of the sites identified in both proteins. Of particular interest are BRAF Ser365 and Ser729 and CRAF Ser233, Ser259 and Ser 621 (open circles) as they mediate the interaction of regulatory complexes with 14-3-3 proteins with the respective kinases\textsuperscript{34,46}. Based on the relative position of the C-lobe and PP5 phosphatase, pSer729 is most likely to be the residue bound at the active site of the catalytically inactivated PP5 in the structure of the complex with HSP90–CDC37–BRAF\textsuperscript{V600E}.

**References**

37. Nature Communications | (2022) 13:7343 https://doi.org/10.1038/s41467-022-35143-2
HSP90 sits at the heart of signal transduction within the eukaryotic cell, a substantial proportion of which is mediated by reversible phosphorylation. HSP90 in concert with its kinase-specific targeting partner CDC37 plays a critical role in the activation of the protein kinases that mediate this signalling, but the precise nature of that role remains obscure. Biochemical and structural studies (and see above) have clearly shown that interaction with the HSP90–CDC37 system results in catalytic silencing of a client kinase, through the partial unfolding of the kinase domain. However, association with HSP90–CDC37 also brings client kinases into the proximity of HSP90 co-chaperones that interact with the C-terminal MEEVD motif of the chaperone via their TPR domains. This exposes the client to modifications delivered by the catalytic domains of the TPR-cochaperones, which range from prolyl isomerases to E3-ubiquitin ligases, and of most significance for protein kinase clients, a protein phosphatase – PP5.

Our data show that the overwhelming majority of Ser/Thr phosphorylations present on BRAFV600E or CRAF bound to HSP90–CDC37, are removed by PP5. Thus, PP5 effectively provides a ‘factory reset’ of the client kinase by removing whatever regulatory modifications may have been applied to it before it bound to HSP90–CDC37, on both N- and C-terminal sides of the kinase domain that drive chaperone recruitment (Fig. 6C). This exposes the client to modifications delivered by the catalytic domains of the TPR-cochaperones, which range from prolyl isomerases to E3-ubiquitin ligases, and of most significance for protein kinase clients, a protein phosphatase – PP5.

Activity of the client post-release. We have here demonstrated chaperone-targeted kinase dephosphorylation by PP5 for two different but related kinases—BRAF and CRAF. However, given the similarity of the way kinases as different as BRAF and CDK4 have been shown to interact with HSP90–CDC37, it is reasonable to suppose that dephosphorylation by PP5 may be a common experience for all HSP90 client kinases. Together with its ability to also remove the phosphorylation of CDC37 and thereby destabilise the association of the kinase client with the chaperone complex, PP5 could then provide directionality to the interaction of the kinase with the chaperone complex, ensuring the release of the client from the HSP90–CDC37 platform as a tabula rasa, ready for whatever new phosphorylation events are required for its regulated function in the cell.

**Methods**

**Protein expression and purification**

Full-length human HSP90β, CDC37 and BRAFV600E were subcloned into the baculovirus vector pBIG1α with an N-terminal His6 tag on HSP90β, a C-terminal His6 on CDC37 and N-terminal His6×2×Strep tag on the BRAFV600E. Human rhinovirus 3C protease recognition sites were introduced between the proteins and the fusion tags. An identical approach was used for the expression of HSP90–CDC37–BRAFV600E.

Sf9 cells (Invitrogen) were transfected with 1 µg of pBIG1α HSP90β, CDC37 and BRAFV600E for viral production. For protein expression,
Sf9 cells were infected with HSP90β, CDC37 and BRAFV600E baculovirus at an MOI of 2 and incubated for 72 h at 26 °C.

Sf9 cells were lysed and sonicated in 40 mM Hepes pH 7.4, 150 mM NaCl, 10 mM KCl, 20 mM Na2MoO4, 20 mM imidazole, 0.5 mM TCEP, 2U/ml Turbo DNAase (Invitrogen), EDTA-free protease inhibitor cocktail tablets and phosphatase inhibitor tablets (Roche). The NaCl concentration was increased to 750 mM before incubating the lysate with talon resin (Takara Bio) for 2 hours at 4 °C. The resin was washed sequentially with lysis buffer containing 750-600-450-300 and 150 mM NaCl. The protein complex was eluted from the resin in 40 mM Hepes pH 7.5, 100 mM NaCl, 10 mM KCl, 20 mM Na2MoO4, 500 mM imidazole, 0.5 mM TCEP, 10% glycerol. The eluate from the Talon resin was applied to a 2 ml streptactin column (IBA) in Streptactin binding buffer consisting of 40 mM Hepes pH 7.4, 150 mM NaCl, 10 mM KCl, 10 mM MgCl2, 20 mM Na2MoO4, 0.5 mM TCEP, 10% glycerol and eluted with 75 mM Biotin. Elutions from the Streptactin column were applied to a Superdex S200 26/60 size exclusion column (GE Healthcare) and

**Fig. 6 | Conformational rearrangements of PP5 facilitate client dephosphorylation.**

A The HSP90 dimer provides two symmetry equivalent alternative and mutually exclusive binding sites for the TPR domain of PP5, which are both seen in the complexes described here, and directs the associated phosphatase domain to different faces of the complex. B Following binding to either of the two symmetry equivalent binding sites at the C-terminus of HSP90, the phosphatase domain of PP5 can release from its autoinhibited interaction with its own TPR domain (left), and engage with substrate phosphorylation sites on the chaperone components or the bound client (right). The model shown on the right is based on the experimental 'closed' structure (see above), but with the PP5 component rotated to the same side as the phosphatase in the experimental 'open' structure by the superimposition of the TPR domains. A movie illustrating the conformational switch between the 'closed' catalytically autoinhibited conformation and the 'open' substrate-engaged conformation is provided in Supplementary Movie 1. C By switching PP5 binding between the two TPR-binding sites on the HSP90 dimer, the flexibly attached phosphatase domain can access phosphorylation sites upstream and downstream of the core interacting domain of the kinase client, taking advantage of the partly unfolded and 'linearised' state that binding to HSP90–CDC37 facilitates.
eluted in Streptactin binding buffer. An identical approach was used for the expression of HSP90–CDC37–CRAF.

Human PP5 residues 16–499 with or without a D274N mutation were cloned into pGEX6P1 with an N-terminal GST tag and C-terminal His6 tag. PP5 was expressed in *Escherichia coli* and purified as previously described[9]. Human HSP90 TRP residues 19–147 subcloned into the pET28a vector with an N-terminal His6 tag was expressed in *E. coli*. The cells were lysed and sonicated in 40 mM Hepes pH 8.0, 300 mM NaCl, 5% glycerol, 0.5 mM TCEP and EDTA-free protease inhibitor cocktail tablets. The lysate was incubated with talon resin (Takara Bio) for 1 hour at 4 °C. The resin was washed sequentially with lysis buffer, and the protein was eluted in 40 mM Hepes pH 8.0, 300 mM NaCl, 250 mM imidazole, 0.5 mM TCEP, and 5% glycerol. The elute from the talon resin was applied to a Superdex S75 26/60 size exclusion column (GE Healthcare) in 25 mM Hepes pH 8, 150 mM NaCl, 0.5 mM TCEP and 5% glycerol.

The ξ-isofrom of 14–3–3 with an N-terminal 6×His-FLAG tag was cloned into pRSET-A and expressed in *E. coli* BL21 DE3 cells. Cells were lysed in 40 mM Tris pH 8, 0.5 mM TCEP, 500 mM NaCl, 5 mM imidazole and EDTA-free protease inhibitor cocktail tablets (Roche). The extract was purified with a Hitrap Talon Crude column and eluted in lysis buffer with 500 mM imidazole. The elution was applied to a HiPrep Desalting column, followed by ion exchange chromatography on a Hitrap Q and size exclusion on a HiLoad Superdex 75 in 20 mM Hepes pH 7.4, 0.5 mM TCEP, 50 mM NaCl 10% glycerol (all chromatography media from Cytiva/GE).

**HSP90–CDC37–BRAF**<sub>V600E</sub>–PP5 complex assembly

To assemble the PP5 complex for CryoEM, the HSP90–CDC37–BRAF<sup>V600E</sup> complex was purified as described above, but after the complex was eluted from the Strep-tactin column, the Na2MoO4 and biotin were removed from the buffer by buffer exchanging using a 100 kDa Mwt cut-off concentrator. This sample was then incubated with a 2× molar excess of PP5 for 2 h at 4 °C. The sample was loaded onto a Superdex 200 10/300 size exclusion column (GE Healthcare) and eluted in 100 mM NaCl, 25 mM Hepes, 10 mM KCl and 1 mM MnCl2, 0.2 mM MTCEP and 2% glycerol. Samples prepared for CryoEM were further crosslinked with 1 mM 25 mM Hepes, 10 mM KCl and 1 mM MnCl2, 0.2 mM MTCEP and 2% glycerol. Prior to grid preparation, the crosslinked HSP90–CDC37–BRAF<sup>V600E</sup> complex was purified by 3D re-refinement and CTF refinement were performed on these particles, followed by 3D refinement. A round of 3D classification was performed to retrieve back the three classes, which now contain polished particles. The overall resolution for all three classes improved after 3D refinement.

To improve the resolution of the PP5 domains, the particles from the two classes which contained PP5 were further classified using signal subtraction and focused 3D classification without alignments, with masks around the PP5 domains. After reverting to the original particles and applying a soft mask around the whole complex, the best-focused class for the HSP90, CDC37, BRAF and PP5 (in a more ‘closed’ conformation) refined to a resolution of 3.9 Å and the best class for the HSP90, CDC37, BRAF and PP5 (in an open conformation) refined to 4.2 Å, with improved density observed for the PP5 domains in both classes. Particles from all three classes were re-extracted at 0.86 Å/pix, and a final round of particle polishing was performed on all three classes individually, followed by postprocessing in RELION in which the nominal resolution was determined by the gold standard Fourier shell correlation (FSC) method[59]. Maps were subsequently post-processed using DeepEMhancer[60].

The data processing flow is shown in Supplementary Fig. 2, local resolution variations, and FSC curves for refinement and model fitting are shown in Supplementary Fig. 3, and 3D plots of particle orientations are shown in Supplementary Fig. 4.

**Model building**

Atomic models were derived from the cryoEM structure of an HSP90–CDC37–CDK4 complex (PDB code: 5FWK), and crystal structures of PP5 protein and domains (PDB codes: 1WAO, S1PE and 1AI7) and BRAF kinase domain (PDB code: 1UWH) docked as rigid bodies into experimental volumes using ChimeraX[55]. The local fit of the models was adjusted manually in Coot[50], and the global fit was optimised using Phenix.refine[51]. Parameters defining the data collection and the quality of the final atomic models (PDB codes: 7ZRO, 7ZR6, 7ZRS) are given in Table 1.

**Dephosphorylation assays**

To monitor dephosphorylation by PP5, 0.15 μM of HSP90–CDC37–BRAF<sup>V600E</sup> complex or HSP90–CDC37–CRAF complex was mixed with 0.3 μM of PP5s in a buffer containing 100 mM NaCl, 25 mM Hepes pH 8,

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10 mM KCl, 1 mM MnCl₂, 0.2 mM TCEP, 2% glycerol and 2.5 mM MgCl₂. The reaction was started by incubating the samples at 30 °C. Samples were taken over 45 min for SDS-PAGE analysis. Phosphorylation-site specific antibodies were used in western blots to probe the phosphorylation states of phospho-Ser13 Cdc37 (MA533209 Invitrogen) diluted 1/2500, phospho-Ser729 BRAF (ab124794 Abcam) diluted 1/2500, phospho-Ser621 CRAF (ab157201 Abcam) diluted 1/1000 and phospho-Ser621 CRAF antibodies as above, and an antibody to 14-3-3 (#8312 Cell Signalling) diluted 1/1000.

### Mass spectrometry phosphorylation analysis
Samples were split into two equal parts and diluted up to 100 μL with 100 mM triethylammonium bicarbonate (TEAB) followed by one-step reduction/alkylation with 5 mM TCEP and 10 mM iodoacetamide for 45 min at room temperature. Proteins were then digested overnight with 50 ng/μL trypsin (Pierce). Peptides were labelled with the TMT-10plex reagents (four labels used) according to the manufacturer’s instructions (Thermo), followed by C18 clean-up using the Pierce Peptide Desalting Spin Columns. Phosphopeptides were enriched with the High-Select™ Fe-NTA Phosphopeptide Enrichment Kit (Thermo). Both the enrichment eluent and flowthrough (FT) were further subjected to mass spectrometry analysis.

### LC-MS analysis
LC-MS analysis was performed on the Dionex UltiMate 3000 UHPLC system coupled with the Orbitrap Lumos Mass Spectrometer (Thermo Scientific). Each sample was reconstituted in 30 μL of 0.1% formic acid, and 15 μL were loaded to the Acclaim PepMap 100, 100 μm × 2 cm C18, 5 μm trapping column at 10 μL/min flow rate of 0.1% formic acid loading buffer. Peptides were analysed with an Acclaim PepMap (75 μm × 50 cm, 2 μm, 100 Å) C18 capillary column connected to a stainless-steel emitter with integrated liquid junction (cat# PSSEl, MSWIL) fitted on a PSS2 adaptor (MSWIL) on the EASY-Spray source at 45 °C. Mobile phase A was 0.1% formic acid, and mobile phase B was 80% acetonitrile, 0.1% formic acid. The gradient separation method at a flow rate of 300 nL/min was the following: for 65 min (or 95 min for FT) gradient from 5%–38% B, for 5 min up to 95% B, for 5 min isocratic at 95% B, re-equilibration to 5% B in 5 min, for 10 min isocratic at 5% B. Each sample was injected twice. Precursors between 375 and 1500 m/z were selected at 120,000 resolution in the top speed mode in 3 s and were isolated for HCD fragmentation (collision energy 38%) with quadrupole isolation width 0.7 Th, Orbitrap detection at 50,000 resolution (or 30,000 for FT sample), max IT 100 ms (or 50 ms for FT sample) and AGC 1 × 10⁶. Targeted MS precursors were dynamically excluded for further isolation and activation for 30 or 45 s with 7 ppm mass tolerance.

The raw files were processed in Proteome Discoverer 2.4 (Thermo Scientific) with the SequestHT search engine for peptide identification and quantification. The precursor and fragment ion mass tolerances were 20 ppm and 0.02 Da, respectively. Spectra were searched for fully tryptic peptides with a maximum of 2 missed cleavages and a minimum length of 6 amino acids. TMTplex at N-terminus/K and Carbamidomethyl at C were selected as static modifications. Oxidation of M, Deamidation of N/Q and Phosphorylation of S/T/Y were selected as dynamic modifications. Spectra were searched against reviewed UniProt Homo sapiens protein entries (downloaded 18/01/2022), peptide confidence was estimated with the Percolator node, and peptides were filtered at q-value < 0.01 based on decoy database search. Results were reported at the peptide level, and at least two unique peptides were required for the identification of the proteins of interest. The reporter ion quantifier node included a TMT quantification method with an integration window tolerance of 15 ppm. Only peptides with average reporter signal-to-noise >3 were used, and phosphorylation localisation probabilities were estimated with the IMP-ptmRS node with an initial minimum probability of 25%. Selected phosphosites had a minimum localisation probability of 98%. Statistical analysis was performed in Perseus software using a two-sided t-test and multiple hypothesis testing corrections by permutation-based FDR. Significant hits were filtered for p-value < 0.05, log2fold-change <-1 and had an FDR < 0.1.
Neckers, L. & Workman, P. Hsp90 molecular chaperone inhibitors.

Mollapour, M. et al. Swe1Wee1-dependent tyrosine phosphorylation.

Xu, W. et al. Hsp90 middle domain phosphorylation initiates a

Woodford, M. R. et al. Mps1 mediated phosphorylation of Hsp90

References

1. Taipale, M. et al. Quantitative analysis of hsp90-client interactions reveals principles of substrate recognition. Cell 150, 987–1001 (2012).

2. Pearl, L. H. Hsp90 and Cdc37.

3. Stepanova, L., Leng, X. H., Parker, S. B. & Harper, J. W. Mammalian

4. Mollapour, M. et al. Threonine 22 phosphorylation attenuates

5. Xu, W. et al. Dynamic tyrosine phosphorylation modulates cycling of the HSP90-P50(CDC37)-AHA1 chaperone system. Nat. Chem. Biol. 9, 307–312 (2013).

6. Miyata, Y. & Nishida, E. CK2 controls multiple protein kinases by phosphorylating a kinase-targeting molecular chaperone, Cdc37. Mol. Cell Biol. 24, 4065–4074 (2004).

7. Shao, J., Prince, T., Hartson, S. D. & Matts, R. L. Phosphorylation of serine 13 is required for the proper function of the Hsp90 co-chaperone, Cdc37. J. Biol. Chem. 278, 38117–38120 (2003).

8. Neckers, L. & Workman, P. Hsp90 molecular chaperone inhibitors: are we there yet? Clin. Cancer Res. 18, 64–76 (2012).

9. Mollapour, M. et al. Swe1Wee1-dependent tyrosine phosphorylation of Hsp90 regulates distinct facets of chaperone function. Mol. Cell 37, 333–343 (2010).

10. Xu, W. et al. Hsp90 middle domain phosphorylation initiates a complex conformational program to recruit the ATPase-stimulating cochaperone Aha1. Nat. Commun. 10, 2574 (2019).

11. Woodford, M. R. et al. Mps1 mediated phosphorylation of Hsp90 confers renal cell carcinoma sensitivity and selectivity to Hsp90 inhibitors. Cell Rep. 14, 872–884 (2016).

12. Mollapour, M. et al. Threonine 22 phosphorylation attenuates Hsp90 interaction with cochaperones and affects its chaperone activity. Mol. Cell 41, 672–681 (2011).

13. Nguyen, M. T. N. et al. Isoform-specific phosphorylation in human Hsp90beta affects interaction with clients and the cochaperone Cdc37. J. Mol. Biol. 429, 732–752 (2017).

14. Backe, S. J., Sager, R. A., Woodford, M. R., Makedon, A. M. & Mollapour, M. Post-translational modifications of Hsp90 and translating the chaperone code. J. Biol. Chem. 295, 11099–11117 (2020).

15. Biswas, S. & Rao, C. M. Epigenetic tools (The Writers, The Readers and The Erasers) and their implications in cancer therapy. Eur. J. Pharm. 837, 8–24 (2018).

16. von Kriegsheim, A., Pitt, A., Grindlay, G. J., Kolch, W. & Dhillon, A. S. Regulation of the Raf-MEK-ERK pathway by protein phosphatase 5. Nat. Cell Biol. 8, 1011–1016 (2006).

17. Conde, R., Xavier, J., McLoughlin, C., Chinkers, M. & Ovsenek, N. Protein phosphatase 5 is a negative modulator of heat shock factor 1. J. Biol. Chem. 280, 28989–28996 (2005).

18. Shao, J., Hartson, S. D. & Matts, R. L. Evidence that protein phosphatase 5 functions to negatively modulate the maturation of the Hsp90-dependent heme-regulated elf2 alpha kinase. Biochemistry 41, 6770–6779 (2002).

19. Silverstein, A. M. et al. Protein phosphatase 5 is a major component of glucocorticoid receptor-hsp90 complexes with properties of an FK506-binding immunophilin. J. Biol. Chem. 272, 16224–16230 (1997).

20. Chen, M. X. et al. A novel human protein serine/threonine phosphatase, which possesses four tetratricopeptide repeat motifs and localizes to the nucleus. EMBO J. 13, 4278–4290 (1994).

21. Cliff, M. J., Harris, R., Barford, D., Ladbury, J. E. & Williams, M. A. Conformational diversity in the TPR domain-mediated interaction of protein phosphatase 5 with Hsp90. Structure 14, 415–426 (2006).

22. Wawinger, C., Suhre, M. H., Wegele, H. & Buchner, J. The phosphatase Ppt1 is a dedicated regulator of the molecular chaperone Hsp90. EMBO J. 25, 367–376 (2006).

23. Vaughan, C. K. et al. Hsp90-dependent activation of protein kinases is regulated by chaperone-targeted dephosphorylation of Cdc37. Mol. Cell 31, 886–895 (2008).

24. da Rocha Dias, S. et al. Activated B-RAF is an Hsp90 client protein that is targeted by the anticancer drug 17-allylamino-17-demethoxygeldanamycin. Cancer Res. 65, 10686–10691 (2005).

25. Garnett, M. J. & Marais, R. Guilty as charged: B-RAF is a human oncogene. Cancer Cell 6, 313–319 (2004).

26. Oberoi, J. et al. Structural and functional basis of protein phosphatase 5 substrate specificity. Proc. Natl Acad. Sci. USA 113, 9009–9014 (2016).

27. Dilorio, M. C. & Kulczyk, A. W. A robust single-particle cryo-electron microscopy (cryo-EM) processing workflow with cryoSPARC, RELION, and Scipion. J. Vis. Exp. 179, e63387 (2022).

28. Kimanius, D., Dong, L., Sharov, G., Nakane, T. & Scheres, S. H. W. New tools for automated cryo-EM single-particle analysis in RELION-4.0. Biochem. J. 478, 4169–4185 (2021).

29. Ali, M. M. et al. Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. Nature 440, 1013–1017 (2006).

30. Verba, K. A. et al. Atomic structure of Hsp90-Cdc37-Cdk4 reveals that Hsp90 traps and stabilizes an unfolded kinase. Science 352, 1542–1547 (2016).

31. Roe, S. M. et al. The Mechanism of Hsp90 regulation by the protein kinase-specific cochaperone p50(cdc37). Cell 116, 87–98 (2004).

32. Johnson, L. N., Noble, M. E. M. & Owen, D. J. Active and inactive protein kinases: structural basis for regulation. Cell 85, 149–158 (1996).

33. Wang, X. T. et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 116, 855–867 (2004).

34. Park, E. et al. Architecture of autoinhibited and active BRAF-MEK1-14-3-3 complexes. Nature 575, 545–550 (2019).

35. Davies, H. et al. Mutations of the BRAF gene in human cancer. Nature 417, 949–954 (2002).

36. Meyer, P. et al. Structural and functional analysis of the middle segment of hsp90: implications for ATP hydrolysis and client protein and cochaperone interactions. Mol. Cell 11, 647–658 (2003).

37. Yang, J. et al. Molecular basis for TPR domain-mediated regulation of protein phosphatase 5. EMBO J. 24, 1–10 (2005).

38. Das, A. K., Cohen, P. W. & Barford, D. The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. EMBO J. 17, 1192–1199 (1998).
39. Weidenauer, L. & Quadrani, M. Phosphorylation in the charged linker modules interactions and secretion of Hsp90beta. Cells 10, 1701 (2021).

40. Hmitou, I., Drullilennec, S., Valluet, A., Peyssonnaux, C. & Eychene, A. Differential regulation of B-raf isoforms by phosphorylation and autoinhibitory mechanisms. Mol. Cell. Biol. 27, 31–43 (2007).

41. Demey, L. et al. Autophosphorylation on S614 inhibits the activity and the transforming potential of B-Raf. Cell Signal 28, 1432–1439 (2016).

42. Yin, Q. et al. K27-linked ubiquitination of BRAF by ITCH engages cytosolic response to maintain MEK-ERK signaling. Nat. Commun. 10, 1870 (2019).

43. Chadee, D. N. & Kyriakis, J. M. MLK3 is required for mitogen activation of B-Raf, ERK and cell proliferation. Nat. Cell Biol. 7, 770–776 (2004).

44. Kohler, M. et al. Activation loop phosphorylation regulates B-Raf in vivo and transformation by B-Raf mutants. EMBO J. 35, 143–161 (2016).

45. Martinez-Fiesco, J. A., Durrant, D. E., Morrison, D. K. & Zhang, P. Structural insights into the BRAF monomer-to-dimer transition mediated by Ras binding. Nat. Commun. 13, 486 (2022).

46. Dumaz, N. & Marais, R. Protein kinase A blocks Raf-1 activity by stimulating 14–3-3 binding and blocking Raf-1 interaction with Ras. J. Biol. Chem. 278, 29819–29823 (2003).

47. Hallet, S. T. et al. Differential regulation of G1 CDK complexes by the Hsp90-Cdc37 chaperone system. Cell Rep. 21, 1386–1398 (2017).

48. Acquaviva, J. et al. Overcoming acquired BRAF inhibitor resistance in melanoma via targeted inhibition of Hsp90 with ganetespib. Mol. Cancer Ther. 13, 353–363 (2014).

49. Lee, K. et al. The structure of an Hsp90-immunophilin complex reveals co-chaperone recognition of the client maturation state. Mol. Cell 91, 3496–3508.e5 (2021).

50. Taipale, M., Jarosz, D. F. & Lindquist, S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat. Rev. Mol. Cell Biol. 11, 515–528 (2010).

51. Blundell, K. L., Pal, M., Roe, S. M., Pearl, L. H. & Prodromou, C. The structure of FKBP38 in complex with the MEEVD tetra-ricopeptide binding-motif of Hsp90. PLoS ONE 12, e0173543 (2017).

52. Zhang, M. et al. Chaperoned ubiquitylation-crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex. Mol. Cell 20, 525–538 (2005).

53. Ballinger, C. A. et al. Identification of CHIP, a novel tetracopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. Mol. Cell Biol. 19, 4535–4545 (1999).

54. Weissmann, F. & Peters, J. M. Expressing multi-subunit complexes using biGbac. Methods Mol. Biol. 1764, 329–343 (2018).

55. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. Nat. Methods 14, 331–332 (2017).

56. Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoS-PARC: algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods 14, 290–296 (2017).

57. Bepler, T. et al. Positive-unlabeled convolutional neural networks for particle picking in cryo-electron micrographs. Nat. Methods 16, 1153–1160 (2019).

58. Zivanov, J. et al. New tools for automated high-resolution cryo-EM structure determination in RELION-3. Elife 7, e42166 (2018).

59. Henderson, R. et al. Outcome of the first electron microscopy validation task force meeting. Structure 20, 205–214 (2012).

60. Sanchez-Garcia, R. et al. DeepEMhancer: a deep learning solution for cryo-EM volume post-processing. Commun. Biol. 4, 874 (2021).

61. Pettersen, E. F. et al. UCSF ChimeraX: structure visualization for researchers, educators, and developers. Protein Sci. 30, 70–82 (2021).