RAF family kinases are RAS-activated switches that initiate signalling through the MAP kinase cascade to control cellular proliferation, differentiation and survival\(^1\)\(^-\)\(^3\). RAF activity is tightly regulated and inappropriate activation is a frequent cause of cancer\(^4\)\(^-\)\(^6\); however, the structural basis for RAF regulation is poorly understood at present. Here we use cryo-electron microscopy to determine autoinhibited and active-state structures of full-length BRAF in complexes with MEK1 and a 14-3-3 dimer. The reconstruction reveals an inactive BRAF–MEK1 complex restrained in a cradle formed by the 14-3-3 dimer, which binds the phosphorylated S365 and S729 sites that flank the BRAF kinase domain. The BRAF cysteine-rich domain occupies a central position that stabilizes this assembly, but the adjacent RAS-binding domain is poorly ordered and peripheral. The 14-3-3 cradle maintains autoinhibition by sequestering the membrane-binding cysteine-rich domain and blocking dimerization of the BRAF kinase domain. In the active state, these inhibitory interactions are released and a single 14-3-3 dimer rearranges to bridge the C-terminal pS729 binding sites of two BRAFs, which drives the formation of an active, back-to-back BRAF dimer. Our structural snapshots provide a foundation for understanding normal RAF regulation and its mutational disruption in cancer and developmental syndromes.

**Overall structure of autoinhibited BRAF**

We co-expressed full-length wild-type BRAF with full-length MEK1 in insect (SF9) cells. To eliminate potential heterogeneity due to phosphorylation of the MEK1 activation loop, we used a variant of MEK1 in which these phosphorylation sites were mutated to alanine (S218A/S222A) in our structural studies. Affinity purification of the expressed proteins yielded well-defined complexes that also contained insect-cell-derived 14-3-3\(\epsilon,\zeta\) dimers (see Methods and Extended Data Fig. 1a). Co-expression of human 14-3-3 isoforms with BRAF and MEK1 did not fully displace the insect-cell 14-3-3\(\epsilon,\zeta\) dimers (Extended Data Fig. 1b). We therefore exploited the binding of the abundant and highly conserved endogenous 14-3-3 proteins. This approach enabled us to isolate ‘monomeric’ complexes that contained a single chain of each of BRAF, MEK1 and the two 14-3-3 subunits (Extended
The BRAF kinase domain is oriented such that its active site faces away from the 14-3-3 domain, enabling it to coordinate MEK1 in a ‘face-to-face’ orientation. Both the MEK1 and the BRAF kinase domains exhibit stereotypical inactive conformations, in which their regulatory αC-helices are displaced from their active positions. The N-terminal BRAF-specific domain and the RBD of BRAF are not clearly defined in the cryo-EM map. A reconstruction that was filtered to a resolution of 5 Å and contoured at a lower level revealed density adjacent to the CRD that corresponds to the RBD domain (Extended Data Fig. 1g), but it did not provide sufficient detail to enable the positioning of an RBD model.

### The 14-3-3 dimer organizes inactive BRAF

Our BRAF complexes contain an approximately equimolar ratio of the ε and ζ isoforms (Extended Data Fig. 1a), and we expect that each side of the 14-3-3 dimer is a mixture of the two isoforms in our reconstructions. For simplicity and convenience, our model is constructed using the Spodoptera frugiperda 14-3-3ε sequence for both subunits, but with residue numbering corresponding to the human 14-3-3ζ isoform. The 14-3-3 dimer interacts with every ordered domain of BRAF, and the interacting residues are highly conserved across all 14-3-3 isoforms (Extended Data Fig. 2a). The most N-terminal portion of BRAF that is well defined in the cryo-EM maps is the CRD domain. The CRD domain fold, which is approximately 50 residues in length, contains a small β-sheet and is stabilized by two zinc coordination sites (Fig. 1c). The domain binds in the centre of the 14-3-3 cradle, with contacts to both subunits of the dimer (Extended Data Fig. 2b). Notably, two loops of the CRD domain that are expected to mediate association of the domain with the membrane (residues 239–245 and 253–260) make extensive contact with the 14-3-3 domain in the autoinhibited complex (Extended Data Table 1). Previous mutagenesis studies of the CRAF CRD have identified two residues in this region that are important for binding to 14-3-3. The corresponding residues in BRAF (R239 and T241) are indeed found at this position.

The poorly conserved linker that connects the CRD domain and the CR2 region is not visible in our map, but the phosphorylated CR2 segment is well defined in the phosphopeptide recognition groove on one side of the 14-3-3 dimer (Extended Data Fig. 2b, c). The ordered CR2 segment extends from Q359 to I371, with pS365 roughly at its centre. Beyond I371, the linker that connects CR2 to the kinase domain is not visible. The BRAF kinase C-lobe contacts both 14-3-3 subunits but interacts most extensively with the pS365-binding subunit, packing against its α9 helix and α8–α9 loop (Extended Data Fig. 3c). This portion of the 14-3-3 domain also contacts H510 and adjacent residues in the N-terminal lobe of the kinase domain. We observe continuous density connecting the C-terminus of the BRAF kinase domain with the pS729 14-3-3-binding motif, which occupies the recognition groove on the opposite side of the 14-3-3 dimer (Extended Data Fig. 3d, e). BRAF residues S732–A736 thread between the CRD and the 14-3-3 domain as they exit the recognition groove, and weak density corresponding to a few additional residues indicates that the BRAF C-terminus passes across a hydrophobic surface on the CRD domain before it becomes substantially disordered. The interactions of the 14-3-3 domain with the CR2 segment in the present structure are similar to those observed in a crystal structure of human 14-3-3ζ in complex with a CRAF peptide (Extended Data Fig. 3f).

### The autoinhibited BRAF–MEK kinase module

The BRAF and MEK1 kinase domains bind with their active-site clefts juxtaposed, and both kinases exhibit inactive conformations (Fig. 2a). Density for ATP-γ-S is visible in the BRAF active-site cleft (Extended Data Fig. 4a); in the MEK active-site we also observe density corresponding to a bound nucleotide, which is seemingly ADP (Extended Data Fig. 4b). The inactive conformation of MEK1 and the face-to-face kinase orientation seen here is similar to that previously observed for the isolated...
Inhibitory mechanisms of the 14-3-3 dimer

The crystal structure described above shows that the MEK1 and BRAF kinase domains can adopt their mutually inhibited conformations in the absence of any interactions with the 14-3-3 protein. This raises the question of the role of the 14-3-3 dimer in BRAF inhibition. Our structure suggests that, rather than inducing an inactive conformation in the kinase domain, the 14-3-3 maintains the inhibited state by sterically blocking formation of the BRAF kinase domain dimer that is required for BRAF activation. In the cryo-EM structure of the autoinhibited complex, the surface corresponding to the BRAF dimer interface is obstructed by the bound 14-3-3 dimer (Fig. 3a–c). In particular, dimer interface residues H510, D565 and Y566 are all in contact with the 14-3-3 domain. Additionally, the 14-3-3 domain sequesters the CRD domain, which is crucial for Ras-driven activation and membrane recruitment of BRAF. The surface corresponding to the membrane-binding loops of the CRD is largely occluded in the autoinhibited complex (Fig. 3d).

The overall architecture of autoinhibited BRAF is probably shared with both ARAF and CRAF, as key interdomain contacts are highly conserved among these proteins (Extended Data Fig. 6). Consistent with our structural findings, early structure–function studies established a key role for the CRD in maintaining RAF in an autoinhibited state. An alanine scanning mutagenesis study of the CRAF CRD domain identified mutations in II surface-exposed residues that increased the RAS(G12V)-dependent activation of CRAF, including two that fully activated CRAF in the absence of mutant RAS. All II of the corresponding residues in BRAF are located at interdomain contacts in the present structure (Extended Data Fig. 2b). Perhaps most compellingly, the BRAF CRD domain is a hot spot for germline mutations that cause Noonan syndrome and related RASopathies. Altered residues map to sites of contact with 14-3-3 or the BRAF kinase domain in the present structure, providing a structural rationale for their activating effects (Extended Data Fig. 3g).

Structures of active BRAF complexes

The autoinhibited structure described above reveals a clear role for phosphorylation of both S365 and S729 in RAF autoinhibition. To further explore the role of these modifications in RAF regulation, we...
prepared the following BRAF variants: BRAF(S365A), with a serine-to-alanine mutation at residue 365; BRAF(S729A); and the double mutant BRAF(S365A/S729A). We then expressed these variants with or without the co-expression of MEK1 in insect cells. Although we obtained soluble, stable BRAF–MEK1–14-3-3 complexes in experiments with BRAF(S365A) (Extended Data Fig. 7a), experiments with the S729A and S365A/S729A variants yielded little BRAF, and it was largely aggregated and did not co-purify with 14-3-3 proteins (data not shown). Size-exclusion chromatography of the BRAF(S365A) sample revealed a broad peak containing BRAF(S365A), MEK1 and the 14-3-3 dimer. Examination of the phosphorylation state of the BRAF(S365A) in this peak revealed near-stoichiometric phosphorylation of S729, but little phosphorylation of activation-segment sites T599 and S602 (Extended Data Fig. 1c). Nevertheless, the purified complex was highly active in MEK phosphorylation assays (Fig. 4a, Extended Data Fig. 7a), and cryo-EM imaging of the complex revealed 2D class averages that were consistent with larger, dimeric complexes (Extended Data Fig. 8a).

A three-dimensional (3D) reconstruction, at approximately 5 Å resolution, of the predominant species in this BRAF(S365A)–MEK1–14-3-3 sample revealed an active, back-to-back BRAF kinase dimer, with MEK1 bound to each BRAF kinase domain (Fig. 4b, Extended Data Fig. 8b). A single 14-3-3 dimer bridges the phosphorylated pS729 sites at the C termini of the two BRAF kinase domains. We do not observe interpretable density for BRAF regions preceding the kinase domain, nor for the C terminus beyond S734. We built a model into this cryo-EM map by domain-wise rigid-body fitting of the previously reported active MEK–BRAF kinase domain complex (PDB ID: 4MNE) and the 14-3-3 dimer from the autoinhibited complex described here. The BRAF–MEK kinase domain portion of the structure exhibits the same overall organization as the previous structure, and inspection of the cryo-EM map confirms that the BRAF C-helix is in its inward position, as expected for the active dimer.

Three-dimensional classification of particles from the same set of images enabled the reconstruction of a second particle similar to the one described above, but with only a single MEK1 bound to the BRAF dimer (Fig. 4c). In this ‘MEK-lite’ complex, the 14-3-3 dimer canters to the side of the missing MEK, assuming a more asymmetric position with respect to the back-to-back BRAF kinase domain dimer.

We also expressed wild-type BRAF alone (without co-expression of MEK1) in both insect and mammalian (HEK293) cells, and obtained soluble BRAF in complex with endogenous 14-3-3 proteins using both expression systems (Extended Data Fig. 7b–f). Quantification of phosphorylation in the elution fractions by mass spectrometry revealed near-stoichiometric phosphorylation of S729 and a high level of S365 phosphorylation in peak fractions, but negligible phosphorylation of both T599 and S602 in BRAF produced by both mammalian and insect cells (Extended Data Fig. 8c–f). The BRAF–14-3-3 complex was highly active in a MEK phosphorylation assay (Fig. 4a, Extended Data Fig. 7b), as determined by western blotting for pS218/222 MEK for the autoinhibited wild-type monomer complex (left), the BRAF(S365A) complex (centre), and a wild-type BRAF–14-3-3 complex prepared without co-expression of MEK1 in insect cells (right). Blots for BRAF (anti-strepII) and MEK are provided as loading controls for enzyme and substrate, respectively. For gel source data, see Supplementary Fig. 1.

The comparison of the 14-3-3 contact and the BRAF dimer interface (centre) and a wild-type BRAF–14-3-3 complex prepared without co-expression of MEK1 in insect cells (right). Blots for BRAF (anti-strepII) and MEK are provided as loading controls for enzyme and substrate, respectively. For gel source data, see Supplementary Fig. 1. A single 14-3-3 dimer bridges the phosphorylated pS729 sites at the C terminus of the two BRAF kinase domains. We do not observe interpretable density for BRAF regions preceding the kinase domain, nor for the C terminus beyond S734. We built a model into this cryo-EM map by domain-wise rigid-body fitting of the previously reported active MEK–BRAF kinase domain complex (PDB ID: 4MNE) and the 14-3-3 domain-wise rigid-body fitting of the previously reported active MEK–BRAF kinase domain complex (PDB ID: 4MNE).
Cryo-EM imaging of the mammalian-expressed complex revealed predominant 2D class averages that were consistent with a 14-3-3-bound BRAF dimer, as did imaging of the same sample supplemented with RAF inhibitor GDC-0879 (Extended Data Fig. 8g). We obtained a 3D reconstruction of the inhibitor-bound BRAF–14-3-3 complex at a nominal resolution of 7 Å, which confirmed the dimeric state of the complex (Extended Data Fig. Sh, i). As with the MEK-bound dimer, the BRAF kinase domain forms the expected symmetrical, back-to-back dimer in this structure and we do not observe the N-terminal domains of BRAF. Despite the fact that the 14-3-3 dimer bridges the pS729 sites of the two kinase domains, it adopts a highly asymmetric position with respect to the kinase dimer (Extended Data Fig. Si). In this skewed position, the 14-3-3 dimer approaches the active-site cleft and intrudes into the MEK-binding region of one BRAF kinase domain, but not the other.

**Discussion**

In the quiescent state, BRAF, MEK1, and a 14-3-3 dimer form a tightly integrated signalling device. In light of their extensive interactions, we propose that the RAF–MEK–14-3-3 complex—rather than RAF itself—serves as the RAS-activated switch that initiates signalling through the MAP kinase cascade. Phosphorylation of both of the 14-3-3-binding sites and engagement by a 14-3-3 dimer is required for maturation of RAS into its regulated, inactive state. Our structural and biochemical findings suggest that MEK also contributes to the stability of the inactive state of BRAF, but we do not exclude the possibility that RAFs can assemble into an autoinhibited 14-3-3 complex without MEK. The essential role of pS729 in both the autoinhibited and active states of the kinase, and its stoichiometric phosphorylation in our purified complexes, leads us to suggest that this is a structural phosphorylation, rather than a regulatory one. It is noteworthy that, although phosphorylation on T599 and S602 is widely thought to play a crucial role in BRAF activation, we find little to no phosphorylation on these sites in active BRAF–14-3-3 dimers. The potential role of activation-loop phosphorylation in RAF regulation merits further study.

The structures described here provide views of RAF in its quiescent and active states and, in light of previous functional dissection of RAF regulation, they outline a model for RAF activation (Extended Data Fig. 9). In the autoinhibited state the RBD is exposed, enabling recruitment of the quiescent complex to the membrane by activated RAS. By contrast, the CRD and its membrane-binding surface is largely buried by interactions with the 14-3-3 dimer and other segments of RAF, suggesting that its ‘extraction’ upon RAS binding and membrane localization is the key event that promotes the release of the inhibitory position of the 14-3-3 domain. When released from its inhibitory position, the 14-3-3 dimer can rearrange to bridge the pS729 sites in the C-terminal tails of two BRAFs, driving formation of the active BRAF dimer. Once activated, BRAF can phosphorylate MEK, which promotes its release. Steric effects of the 14-3-3 domain could also modulate the affinity for MEK, as evidenced by the asymmetric position assumed by 14-3-3 upon MEK release.

The inactive-state structures described here reveal the bona fide inactive conformation of BRAF, and thereby provide a structural foundation for understanding its activation by mutations in cancer. Oncogenic mutations of V600 and K601 in the inhibitory turn are not compatible with the structural context of these residues, providing a rationale for their activating effect via destabilization of the inhibitory turn (Fig. 2c). Other less common oncogenic BRAF mutations occur in residues that participate directly in the coordination of ATP and its associated divalent cation (Fig. 2d), and they may destabilize the autoinhibited state by weakening interactions with ATP and/or by disrupting interactions of the glycine-rich loop with the inhibitory turn in the activation segment. Many of the same BRAF residues are also altered in RASopathies (Extended Data Fig. Sf). Outside of the kinase domain, CRAF and ARAF contain somatic point mutations in or near the CR2 phosphorylation site in diverse cancers, including lung adenocarcinoma. These mutations eradicate the CR2 14-3-3-binding site, promoting formation of the active RAF dimer—as we observe here with the BRAF(S365A) mutant. The KIAA1549: BRAF truncation/fusion oncprotein that is found in paediatric low-grade gliomas lacks the entire CR1 and CR2 regions of BRAF, and is therefore constitutively active.

The integral nature of the RAF–MEK–14-3-3 switch has important pharmacologic implications. It is well established that certain MEK and RAF inhibitors can stabilize or destabilize their interaction. However, the notion that the RAF–MEK–14-3-3 complex—which is distinct from the isolated RAF and MEK kinases—may represent a relevant pharmacologic receptor for a broader range of inhibitors has not, to our knowledge, been systematically explored. Perhaps the most perplexing aspect of RAF-inhibitor pharmacology is the paradoxical activation of the MAP kinase pathway by certain RAF kinase inhibitors. Diverse RAF inhibitors disrupt autoinhibitory interactions of the BRAF kinase with its N-terminal region, and some promote dimerization of the isolated BRAF kinase domain. Considering the extensive interactions of BRAF with ATP in the autoinhibited state, we speculate that RAF inhibitors may promote conformational activation by displacing ATP from quiescent RAF. Whether this leads to observed paradoxical pathway activation will in turn depend upon ensuing cellular events—potentially including changes in RAF phosphorylation state, RAS-binding, membrane localization and 14-3-3 rearrangements—and on the potency of a particular agent as an inhibitor of activated RAF dimers.

Many questions regarding RAF regulation remain. The structures described here and the ability to prepare full-length autoactivated and active BRAF will inform and enable detailed mechanistic studies of RAF activation and RAF-inhibitor pharmacology. In the long term, a deeper understanding of RAF regulation should aid in the development of more effective and better-tolerated therapeutics for RAF-driven cancers.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-019-1660-y.

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Methods

Data reporting

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Cell lines

The insect (SF9 and Hi5) and mammalian (Exp293F) cell lines used for protein production were obtained from Thermo Fisher Scientific, and tested negative for mycoplasma contamination.

Preparation of BRAF–14-3-3 complexes from SF9 insect cells

Recombinant baculovirus expressing full-length human BRAF with an N-terminal His$_{6}$-tag and C-terminal StrepII tag was prepared using baculoviral transfer vector pAc8. Recombinant baculovirus expressing the variant BRAF(S365A) was produced in the same manner. For protein production using the baculovirus/insect cell expression system, litre-scale cultures of SF9 cells (4 l total for a typical preparation) were infected with high-titre viral stocks expressing wild-type or mutant BRAF (1% of final culture volume). Cells were collected 65–72 h post-infection, lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl$_2$, 0.5 mM TCEP, 50 μM ATP-γ-S and protease inhibitor cocktail (Thermo Fisher Scientific), and applied to Ni-NTA agarose beads (Qiagen). After washing with Buffer A supplemented with 20 mM imidazole (Buffer A contains 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl$_2$, 0.5 mM TCEP, 10 μM ATP-γ-S), bound proteins were eluted with Buffer A supplemented with 500 mM imidazole and adjusted to pH 8.0. After washing with Buffer A supplemented with 10 mM desthiobiotin. The eluted complex was concentrated to approximately 2 mg ml$^{-1}$ using an Amicon Ultra concentrator (500 MWCO, Millipore) and further purified by size-exclusion chromatography (SEC) on a Superdex 200 10/300 GL column (GE Healthcare Life Sciences) in Buffer A. Analysis of the purified samples by SDS–PAGE revealed the baculovirus-expressed BRAF co-purified at a high stoichiometry with intact BRAF (1:1 stoichiometry).

Preparation of BRAF–MEK1–14-3-3 complexes from SF9 insect cells

Recombinant baculovirus expressing full-length human BRAF with an N-terminal His$_{6}$-tag and C-terminal StrepII tag was prepared using baculoviral transfer vector pAc8. Recombinant baculovirus expressing the variant BRAF(S365A) was produced in the same manner. For protein production using the baculovirus/insect cell expression system, litre-scale cultures of SF9 cells (4 l total for a typical preparation) were infected with high-titre viral stocks expressing wild-type or mutant BRAF (1% of final culture volume). Cells were collected 65–72 h post-infection, lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl$_2$, 0.5 mM TCEP, 50 μM ATP-γ-S and protease inhibitor cocktail (Thermo Fisher Scientific), and applied to Ni-NTA agarose beads (Qiagen). After washing with Buffer A supplemented with 20 mM imidazole (Buffer A contains 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM MgCl$_2$, 0.5 mM TCEP, 10 μM ATP-γ-S), bound proteins were eluted with Buffer A supplemented with 500 mM imidazole and adjusted to pH 8.0. After washing with Buffer A supplemented with 10 mM desthiobiotin. The eluted complex was concentrated to approximately 2 mg ml$^{-1}$ using an Amicon Ultra concentrator (500 MWCO, Millipore) and further purified by size-exclusion chromatography (SEC) on a Superdex 200 10/300 GL column (GE Healthcare Life Sciences) in Buffer A. Analysis of the purified samples by SDS–PAGE revealed the baculovirus-expressed BRAF co-purified at a high stoichiometry with intact BRAF (1:1 stoichiometry).

Preparation of BRAF–MEK1–14-3-3 complexes from HEK293 mammalian cells

Full-length human BRAF bearing an N-terminal His$_{6}$-tag and C-terminal StrepII tag was cloned into pcDNA 5/FRT/TO vector. For protein production, litre-scale suspension cultures (2 l total for a typical preparation) of HEK293 cells (Exp293F) were transfected using the Exp293F expression system according to the manufacturer’s protocol (Thermo Fisher Scientific). Cells were collected by centrifugation 48–60 h post-transfection. BRAF–14-3-3 complexes were purified from mammalian cell pellets as described above for isolation BRAF–14-3-3 from insect cells. Analysis of the purified sample by SDS–PAGE revealed that BRAF co-purified with mammalian-cell-derived 14-3-3 isoforms.

Preparation of SpyTag–MEK1 from Hi5 insect cells

We prepared kinase-dead MEK1 (fused to SpyTag to alter its electrophoretic mobility) for use as a substrate in in vitro BRAF-activity assays. Recombinant baculovirus encoding full-length human MEK1(D190N) bearing an N-terminal His$_{6}$-tag for purification and a C-terminal Spy-tag was prepared using baculoviral transfer vector pAc8. For protein production, litre-scale cultures (2 l total for a typical preparation) were infected with high-titre viral stocks expressing Spy-tagged MEK1(D190N) (1% of final culture volume). Cells were collected 55–65 h post-infection, lysed in MEK lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM MgCl$_2$, 0.5 mM TCEP) and applied to Ni-NTA agarose beads (Qiagen). After washing with MEK lysis buffer supplemented with 20 mM imidazole, bound proteins were eluted with lysis buffer supplemented with 500 mM imidazole and adjusted to pH 7.4. To ensure that MEK1 was not phosphorylated, eluted protein was treated with lambda phosphatase overnight at 4°C before further purification by SEC on a Superdex 75 Increase 10/300 column in SEC Buffer (50 mM Tris pH 8.0, 150 mM NaCl, 2 mM MgCl$_2$, 1 mM TCEP). Pooled SEC fractions containing Spy-tagged MEK1(D190N) were incubated with SpyTag protein for covalent linkage, as described previously. Analysis of the purified SpyTag–MEK1(D190N) protein by SDS–PAGE confirmed that it migrated as expected for a protein of approximately 55 kDa. Mass spectrometry and western blotting with pMEK1/2 antibody confirmed little or no phosphorylation on the MEK1 activation loop (S218 and S222).

Size-exclusion chromatography with multilangle light scattering

The BRAF–MEK1–14-3-3 complex (prepared with MEK1(S218A/S222A)) was applied to a Superdex 10/100 GL column (GE Healthcare) in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MgCl$_2$, 0.5 mM TCEP, 10 μM ATP-γ-S, 2 μM GDC-0623. In-line multi-angle light scattering analysis was performed with an OptiLab rEX refractive index detector followed by a miniDAWN TREOS light scattering detector, and data were analysed with ASTRA (Wyatt Technology).

Kinase-activity assay

BRAF activity in SEC elution fractions was measured by diluting an aliquot of each fraction fivefold, and adding 1 μl of the diluted sample to 14 μl of a reaction mixture containing assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM MgCl$_2$, 0.5 mM TCEP) and 1 μM sodium vanadate) supplemented with 2.67 μM SpyTag–MEK1(D190N) as a substrate. Kinase reactions were started by addition of 5 μM of 4 mM ATP in assay buffer. After incubation for 20 min at 25°C, reactions were stopped by addition of SDS–PAGE sample buffer and heating to 95°C. Reaction products were resolved on a Novex 12% Tris-Glycine Midi gel (Invitrogen), and subsequently western-blotted with anti-phosphoMEK1/2(S218/222) antibody (Cell Signaling Technology) to detect phosphorylation of the SpyTag–MEK1 substrate (approximately 55 kDa).

Time-course kinase assays of BRAF–MEK1–14-3-3, BRAF(S365A)–MEK1–14-3-3 and BRAF–14-3-3 samples were performed using the same reaction buffer and substrate concentrations described above, but each sample was diluted to 100 nM, and 20 μl was used in a final reaction volume of 200 μl (final enzyme concentration was 10 nM in the reaction mixture). After initiating the assay by addition of ATP, 20 μl aliquots were removed from the reaction at the designated time points and stopped by mixing with an equal volume of 5X SDS–PAGE sample buffer and heating to 95°C. Reaction products were analysed by SDS–PAGE and western blotting for phosphoMEK1/2 as described above. The BRAF–MEK1–14-3-3 complexes were prepared with MEK1(S218A/S222A).
Pull-down assay for 14-3-3 association

Recombinant baculoviruses expressing full-length human 14-3-3β/α, 14-3-3γ, 14-3-3ζ/χ or 14-3-3ε and bearing an N-terminal Flag-tag were prepared using baculoviral transfer vector pAc8. BRAF and MEK1 were co-expressed with each of the four different human 14-3-3 isoforms in insect cells by co-infection. Co-infected SF9 cells (100 ml) were collected 55–65 h post-infection, lysed in lysis buffer, and then parallel aliquots of clarified lysate were applied to Strept-TactinXT magnetic beads (IBA GmbH) or Anti-DYKDDDDK magnetic Agarose (anti-Flag, Pierce). After washing beads with lysis buffer, bound proteins were eluted with SDS–PAGE sample buffer and resolved on 8% Bis-Tris PAGE gels. Parallel gels were western-blotted using anti-14-3-3 (pan) and anti-Flag antibodies (Cell Signaling Technology).

Expression and purification of the BRAF–MEK kinase domain complex

For insect-cell expression of the BRAF kinase domain in complex with MEK1, two recombinant baculovirus species were used. The first was prepared using baculoviral transfer vector pFastBac Dual and encoded the BRAF kinase domain (BRAF residues 445–723, fused to an N-terminal His₆-tag and a C-terminal chitin-binding domain) and human chaperone CDC37. The second baculovirus encoded full-length human MEK1 (S218A/S222A), as described above. For protein production, litre-scale suspension cultures of SF9 cells were co-infected with both viruses. Cells were collected 60–66 h post-infection and resuspended in lysis buffer (50 mM Tris pH 8.0, 250 mM NaCl, 5% glycerol and 20 mM imidazole, 1 mM TCEP) with protease inhibitor cocktail (Roche). Resuspended cells were disrupted by sonication on wet ice, and the lysate was clarified by ultracentrifugation at 40,000 r.p.m. for two hours. Clarified lysate was batch-bound to Ni-NTA beads and washed extensively with binding buffer before elution with elution buffer (50 mM Tris pH 8.0, 250 mM NaCl, 250 mM imidazole, 1 mM TCEP). The elution fractions were pooled and treated with 1.000 molar ratio of TEV protease and 100 mM β-mercaptoethanesulfonic acid (MESNA) overnight to cleave N-terminal and C-terminal tags, and further purified by SEC (Superdex 200 10/300, GE Healthcare) in storage buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM TCEP). The fractions were analysed by SDS–PAGE, and fractions corresponding to the BRAF–MEK1 kinase domain complex were pooled, concentrated to 8 mg ml⁻¹, and flash-frozen.
BRAF–MEK kinase domain crystallization and structure determination

For crystallization, an aliquot of the BRAF–MEK1(521A/S222A) kinase complex was incubated with 5 mM MgCl₂, 2 mM adenosine 5’-(β,γ-imido)triphosphate (AMP-PNP), and 0.2 mM GDC-0623 in storage buffer at 4 °C overnight. Rod-shaped crystals suitable for structure determination were obtained by vapour diffusion in hanging drops using a reservoir solution consisting of 100 mM Bis-Tris pH 6.5, 200 mM ammonium sulfate, and 22% PEG 3350 at room temperature. Crystals were collected and flash-frozen in liquid nitrogen using additional 20% glycerol as a cryoprotectant. X-ray diffraction data were collected at 100 K using NE-CAT beamline ID-24-C at the Advance Photon Source, Argonne National Laboratory, at a wavelength of 0.979 Å. Data were integrated and merged using XDS²⁹ and scaled using Aimless in the CCP4 suite⁴⁰. The structure was phased by molecular replacement in PHASER²⁹ using the relevant domains of the autoinhibited cryo-EM structure and PDB entry 4MNE as initial search models. GDC-0623 was placed into positive density in an initial Fo–Fc map and included in subsequent rounds of refinement using PHENIX.REFINE⁴⁰. Successive manual refinement was performed using Coot⁴¹. The structure was refined to Rwork/Rfree values of 0.22/0.25 at a resolution of 2.38 Å. Data collection and refinement statistics are presented in Extended Data Fig. 5a.

Mass spectrometry analysis

BRAF complexes were digested separately with trypsin and Lys-C, desalted by C18, dried by vacuum centrifugation, and analysed in triplicate by capillary electrophoresis coupled to mass spectrometry (CE–MS) using a ZipChip autosampler and CE instrument (908 Devices) interfaced to a QExactive HF mass spectrometer (Thermo Fisher Scientific). Peptides were loaded for 20 s on an HR chip and electrophoresis was performed at 700 V cm⁻¹ for 10 min, with pressure assist activated at 1 min. To identify BRAF phosphopeptides, digests were analysed by data-dependent tandem mass spectrometry (MS/MS). The five most abundant ions in each MS scan (60K resolution) were subjected to MS/MS experiments using either BRAF protein digests or synthetic BRAF digests to confirm using mzStudio software⁵³. In experiments to determine the stoichiometry of phosphorylation, digests were analysed by CE–MS (MS1 scans with 15K resolution), with precursor peak areas used for quantification according to the following equations:

\[
\text{%Phosphorylation} = 1 - \frac{A_{\text{Corr. P—pep}}}{A_{\text{Tot}}} \\
A_{\text{Tot}} = A_{\text{Corr. P—pep}} + A_{\text{NonP—pep}} \\
A_{\text{Corr. P—pep}} = A_{P—pep} \times \text{Corr. factor}
\]

where \(A_{\text{Corr. P—pep}}\) is the area of the phosphopeptide corrected for differences in ionization efficiency due to phosphorylation, \(A_{\text{Tot}}\) is the total peptide peak area, \(A_{\text{NonP—pep}}\) is the peak area of the unphosphorylated peptide, \(A_{P—pep}\) is the uncorrected peak area of the phosphopeptide, and Corr. factor is the correction factor for ionization efficiency. Correction factors for ionization efficiency were determined in separate experiments using either BRAF protein digests or synthetic BRAF peptide standards. Peptides, with or without treatment with alkaline phosphatase (pptase), were analysed in triplicate by CE–MS as described above. After normalizing for loading amounts (using non-phosphorylatable BRAF peptides VFLPNK and LIDIIAR for BRAF digests or a spiked standard peptide for BRAF synthetics), correction factors were calculated according to:

\[
\text{Corr. factor} = \frac{A_{\text{NonP—pep}(+\text{pptase})} - A_{\text{NonP—pep}(−\text{pptase})}}{A_{P—pep}(−\text{pptase})}
\]

where \(A_{\text{NonP—pep}(+\text{pptase})}\) is the area of the non-phosphorylated peptide after phosphatase treatment, \(A_{\text{NonP—pep}(−\text{pptase})}\) is the area of the non-phosphorylated peptide without phosphatase treatment, and \(A_{P—pep(−\text{pptase})}\) is the area of the phosphorylated peptide without phosphatase treatment. Because discovery experiments did not detect phosphorylation of MEK1 activation loop sites 218/222 (peptide \(209_{\text{LCDFGSVLQDDLNSFVGT}_{222}}\)) we estimated an upper bound of phosphorylation of these residues by analysing digests with or without pptase treatment as above. In these experiments, we used targeted selected ion monitoring scans of \(209_{\text{LCDFGSVLQDDLNSFVGT}_{222}}\) and normalization peptides, and then calculated MEK1 activation loop phosphorylation as:

\[
\text{%Phosphorylation} = 1 - \frac{A_{\text{NonP—MEK}(−\text{pptase})}}{A_{\text{NonP—MEK}(+\text{pptase})}}
\]

where \(A_{\text{NonP—MEK}(+\text{pptase})}\) corresponds to the peak area of the unphosphorylated MEK peptide without phosphatase treatment and \(A_{\text{NonP—MEK}(−\text{pptase})}\) corresponds to the peak area of the unphosphorylated MEK peptide after phosphatase treatment. Data analysis and peak integration were performed using msStudio software⁵³. To identify 14–3–3 proteins, MS/MS spectra from data-dependent CE–MS analyses of trypsin and Lys-C digested BRAF complexes were converted to .mgf format using multiplierz software⁵¹,⁵², and searched against a forward-reverse human protein database (uniprot) with Mascot 2.6.1 (using the same search parameters as described above). Data were filtered to a false discovery rate of around 1%, and peptide sequences mapped to genes using the multiplierz pep2gene tool⁵².

Sequence alignments and software

For Extended Data Figs. 2a, 6, sequences were aligned using ClustalW and figures were prepared with ESPript 3.0⁴⁴. Structural biology applications used in this project were compiled and configured by SBGrid⁵⁵.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

Three-dimensional cryo-EM density maps have been deposited in the Electron Microscopy Data Bank (EMDB) with accession codes EMD-0541, EMD-20550, EMD-20552 and EMD-20551. Atomic coordinates of these residues by analysing digests with or without pptase treatment as above. In these experiments, we used targeted selected ion monitoring scans of \(209_{\text{LCDFGSVLQDDLNSFVGT}_{222}}\) and normalization peptides, and then calculated MEK1 activation loop phosphorylation as:

\[
\text{%Phosphorylation} = 1 - \frac{A_{\text{NonP—MEK}(−\text{pptase})}}{A_{\text{NonP—MEK}(+\text{pptase})}}
\]

where \(A_{\text{NonP—MEK}(+\text{pptase})}\) corresponds to the peak area of the unphosphorylated MEK peptide without phosphatase treatment and \(A_{\text{NonP—MEK}(−\text{pptase})}\) corresponds to the peak area of the unphosphorylated MEK peptide after phosphatase treatment. Data analysis and peak integration were performed using msStudio software⁵³. To identify 14–3–3 proteins, MS/MS spectra from data-dependent CE–MS analyses of trypsin and Lys-C digested BRAF complexes were converted to .mgf format using multiplierz software⁵¹,⁵², and searched against a forward-reverse human protein database (uniprot) with Mascot 2.6.1 (using the same search parameters as described above). Data were filtered to a false discovery rate of around 1%, and peptide sequences mapped to genes using the multiplierz pep2gene tool⁵².

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Author contributions E.P. expressed, purified and biochemically characterized all BRAF–MEK1–14-3-3 and BRAF–14-3-3 complexes. E.P. and H.J. prepared the samples and collected electron microscopy data. S.R., together with H.J. and M.J.E., processed the electron microscopy data to obtain the 3D reconstructions. E.P., S.R., B.-W.K., K.L., H.J. and M.J.E. built and analysed cryo-EM models. K.L. and G.G.-D.P. determined the BRAF–MEK1 kinase domain crystal structure. S.B.F. and J.A.M. were responsible for the mass spectrometry experiments. H.S. carried out preliminary expression and purification studies for the complex. H.J. and M.J.E. directed the project, and M.J.E. drafted the manuscript with input from all authors.

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Extended Data Fig. 1 | See next page for caption.
Biochemical characterization of purified BRAF complexes and cryo-EM analysis of the autoinhibited BRAF–MEK1–14-3-3 complex.

a, The full-length, autoinhibited BRAF–MEK1–14-3-3ζ complex used for cryo-EM structure determination. Left, the elution profile from SEC on a Superdex 200 column; centre, Coomassie-stained SDS–PAGE analysis of elution fractions; right, analysis by size-exclusion chromatography with multi-angle light scattering. A molar mass of 196 kDa was indicated; the calculated molecular weight of the complex is 192 kDa.

b, Analysis of co-expression of human 14-3-3 isoforms with BRAF and MEK1 in insect cells. Left, Strep-tagged BRAF, MEK1(S218A/S222A) and the indicated Flag-tagged human 14-3-3 isoforms were co-expressed in Sf9 cells and BRAF–MEK1–14-3-3 complexes were affinity-isolated from clarified lysates with either Strep-TactinXT (left four lanes) or anti-Flag (right four lanes) magnetic beads. Right, parallel gels were blotted with an anti-14-3-3 antibody that recognizes all 14-3-3 isoforms (top blot) or with an anti-Flag antibody (bottom blot). Note that even in the presence of robust overexpression of these human isoforms, BRAF preferentially associated with the endogenous insect cell 14-3-3 proteins (as seen in the Strep-TactinXT-precipitated lanes of the Coomassie-stained gel).

c, Mass-spectrometry-based quantification of selected phosphorylation sites in complexes with wild-type BRAF and with BRAF(S365A) purified for structural analysis. Note that the BRAF(S365A) complex was prepared with MEK1(S218A/S222A), whereas the wild-type BRAF complex used in this analysis contained wild-type MEK1.

d, Portion of a representative micrograph used for reconstruction of the autoinhibited BRAF–MEK1–14-3-3 complex.

e, Representative 2D class averages for reconstruction of the autoinhibited BRAF–MEK1–14-3-3 complex. Scale bar, 10 nm.

f, Fourier shell correlation (FSC) curves for the reconstruction. The horizontal line indicates a correlation of 0.143; the FSC curve for two half-maps (blue) crosses this threshold at a resolution of 4.1Å. A correlation curve for the map versus the atomic model is plotted in red.

g, The cryo-EM map of the autoinhibited BRAF–MEK1–14-3-3 complex filtered to 5 Å resolution and contoured at a lower level to reveal weaker density corresponding to the RBD domain. The map surface is coloured by domain as in Fig. 1. Unassigned densities (grey) can be ascribed to the RBD domain and other poorly structured elements as indicated. For gel source data, see Supplementary Fig. 1. Experiments in a and b were repeated at least twice with similar results. Imaging experiments in d and e were repeated four times with similar results.
Extended Data Fig. 2 | See next page for caption.
**Extended Data Fig. 2** | 14-3-3 domain sequence alignment and interactions of the CRD domain in the autoinhibited state. 

**a**, Sequence alignment of insect-cell (*S. frugiperda*) and human 14-3-3 isoforms. Secondary structure is indicated above the alignment. Identically conserved residues are shaded red. Symbols above the alignment indicate contacts with the BRAF CRD domain (purple squares), kinase domain (blue triangles), and pS365 or pS729 segments (black circles). 

**b**, Interactions of the CRD domain. Domains that contact the CRD are shown with a transparent surface and the CRD domain is shown as a purple ribbon with grey spheres representing bound zinc atoms. Sidechains are shown for CRD residues that correspond to 7 (of 11 total) residues identified in an alanine scanning mutagenesis study of the CRAF CRD domain. Alanine mutations in the corresponding residues increased RAS(G12V)-dependent activation of CRAF. Two mutations in this study fully activated CRAF in the absence of RAS(G12V); the corresponding BRAF residues are F247 and D249. F247 makes hydrophobic contacts with both the kinase C-lobe and the 14-3-3 domain, whereas D249 is positioned to form a salt bridge with R691 in the kinase C-lobe. The remaining four residues are also at sites of interdomain contacts but are not illustrated (T241, K253, Q262 and K267).
Extended Data Fig. 3 | Interactions of the 14-3-3 dimer with BRAF in the autoinhibited state.  

a–d, Cryo-EM density is shown at key sites of interaction that stabilize the autoinhibited complex, and domains are coloured as in Fig. 1.  
a, A portion of the interface between the CRD and 14-3-3 domain.  
b, The pS365 segment (CR2) bound in the recognition groove of the 14-3-3 domain.  
c, Contact between the α8–α9 loop of the 14-3-3 domain and the BRAF kinase domain.  
d, The C-terminal pS729 segment coordinated in the opposite recognition groove of the 14-3-3 dimer. The map is contoured at the same level in a–d.  
e, Front and back views of the reconstruction. We observe continuous density connecting the C terminus of the BRAF kinase and the pS729 14-3-3 binding site (inset).  
f, Comparison of the binding mode of the pS365 segment in the present structure with that in a previously determined crystal structure of an isolated CRAF peptide bound to 14-3-3ζ (PDB ID: 3NKX). The corresponding region of the present structure (the pS365 segment is shown with orange carbon atoms and the 14-3-3 domain is shown in tan) is superimposed on the 3NKX crystal structure (shown in blue and cyan), revealing a close correspondence in conformations of the bound peptides.  
g, The BRAF CRD is a hot spot for RASopathy mutations, which map to sites of contact between the CRD (purple), kinase (blue) and 14-3-3 domains (tan), and are expected to destabilize the autoinhibited assembly. Sites of RASopathy mutations are shown in stick form and are labelled. RASopathy mutations in the BRAF kinase domain (Q709) and CR2 region (red, S365) are also expected to destabilize these inhibitory intramolecular contacts.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Additional views and analysis of the BRAF and MEK kinase domains in the autoinhibited BRAF–MEK1-14-3-3 complex. a, Cryo-EM density map in the region of the BRAF active site showing bound ATP-γ-S. b, Cryo-EM density map in the region of the MEK1 active site indicating bound ADP, which is probably hydrolysed from ATP-γ-S. Maps in a and b are contoured at the same level. c, Superposition of the BRAF–MEK1 component of the present autoinhibited cryo-EM structure (green and dark blue) with the previously reported crystal structure of a BRAF and MEK1 kinase domain complex (yellow and light blue; PDB ID: 4MNE). The superposition is based on the MEK component of the structures, and it reveals a relative rotation of BRAF of approximately 5° about the C-lobe contact. d, Superposition of the BRAF kinase domain from the present structure with that of previously isolated BRAF–MEK kinase domain complex (PDB ID: 4MNE). Note that the present structure (dark blue, with C-helix coloured purple and the activation segment orange) exhibits key features of an autoinhibited state (C-helix out, with an inhibitory turn in the activation segment), whereas the previous structure (light blue) adopts an overall active conformation. e, Detailed view of a portion of the C-lobe contact between BRAF (blue) and MEK1 (green). f, Portions of the BRAF (blue) and MEK1 (green) activation segments interact in an anti-parallel orientation. Activating phosphorylation sites in the MEK1 activation loop are substituted with alanine in this structure (S218A/S222A), but neither residue is positioned appropriately for phosphorylation by BRAF. Note that our discussion of these interactions relies in part on the crystal structures referenced to build the atomic model, as the cryo-EM map in this region does not unambiguously define all sidechain conformations. g–j, Comparison of BRAF kinase domain conformations and relative N- and C-lobe orientations. g, Sulfonamide-containing BRAF inhibitors perturb the inactive conformation of BRAF. The BRAF kinase domain in the present structure (blue ribbon, with C-helix coloured red and the activation segment orange) is superimposed on the structure of the BRAF kinase domain crystallized as a monomer with PLX4720 (grey, PDB ID: 4WO5). The superposition is based on the C-lobes of both kinases, revealing an altered orientation of the N-lobe in the inhibitor-bound structure (a rotation of around 15°). Note also that the inhibitory turn in the activation segment helix is replaced by a short helix in the PLX4720 complex. h, Alternative view of the superposition shown in g, highlighting the axis of rotation (pink arrow) between the N-lobes. i, As in h, but with a representative inhibitor-bound dimeric BRAF structure superimposed (PDB ID: 5CSW, a dabrafenib complex). The rotation axes for N-lobe rotations of dimer structures are shown as green arrows. Note that the orientation of the rotation axis is similar for all of the dimer structures, but almost orthogonal to that of the monomer structure in h. In both h and i, the Cα atoms of K522 are shown as spheres as a point of reference. j, Relative N-lobe rotation of wild-type and BRAF(V600E) crystal structures available in the Protein Data Bank (PDB) are compared with the present nucleotide-bound, autoinhibited structure. As illustrated in h and i, C-lobes of the BRAF kinase domains were superimposed, and the rotation required to bring the kinase N-lobes into register were calculated using PyMOL. With the exception of 4MNE, all structures compared were determined in complex with inhibitors.
Extended Data Fig. 5 | Additional analysis of the crystal structure of the autoinhibited BRAF—MEK1 kinase domain complex. a, Crystallographic data collection and refinement statistics for the structure of the BRAF kinase domain (BRAFKD) in complex with MEK1(S218A/S222A), AMP-PNP and MEK inhibitor GDC-0623. Data were recorded from a single crystal. b, The crystal structure of the autoinhibited BRAF—MEK1 kinase domain complex is superimposed on the corresponding region of the autoinhibited cryo-EM structure. c, ATP-analogue AMP-PNP is extensively coordinated in the autoinhibited state. Hydrogen bonds from coordinating residues are indicated by dashed lines. d, MEK1 residue E102 in the β3–αC loop is positioned to form a hydrogen bond with a ribose hydroxyl of the nucleotide bound in the BRAF active site. e, Rare but recurrent oncogenic mutations in MEK1 map to the region of the N-terminal helix. A small, in-frame deletion of two residues (E102, I103) in the β3–αC loop maps to the region of the interface between BRAF and MEK1. f, RASopathy mutations in BRAF illustrated in the inactive conformation of the kinase domain. As with oncogenic mutations in many of the same residues, RASopathy-associated mutations will perturb nucleotide binding and/or the stability of the inhibitory turn. Notably, residues E501 and T599 form a hydrogen bond (dashed line) that appears to contribute to the stability of the inhibitory turn.
Extended Data Fig. 6 | RAF sequence alignment. Human ARAF, BRAF and CRAF sequences are aligned and identically conserved residues are shaded red. Secondary structure elements are indicated above the alignment. Symbols above the alignment indicate residues that, in the autoinhibited structure, lie at the interface with MEK1 (cyan circles), the CRD/14-3-3 interface (violet squares), the CRD/kinase domain interface (blue triangles), and the 14-3-3/kinase domain interface (yellow stars).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Purification and characterization of wild-type and BRAF(S365A) complexes. a, BRAF(S365A) was co-expressed with MEK1(S218A/S222A) in insect cells, purified by serial Ni-NTA agarose and StrepTrapHP affinity chromatography, and subjected to SEC on Superose 6 column. The SEC elution trace is shown on the left with a Coomassie-stained SDS–PAGE gel of elution fractions on the right. A parallel gel was blotted with an antibody against pS729 (bottom right). BRAF activity in each fraction was measured in a MEK phosphorylation assay (top right; see Methods for assay details.). b, c, Side-by-side comparison of wild-type BRAF complexes isolated from insect cells without (b) and with (c) co-expression of MEK1(S218A/S222A). Complexes were purified by serial Ni-NTA agarose and StrepTrapHP affinity chromatography and subjected to SEC on Superose 6. The SEC elution traces are shown on the left with Coomassie-stained SDS–PAGE gels of elution fractions on the right. BRAF activity in each fraction was measured in a MEK phosphorylation assay as described above (top right). Note that co-expression of MEK1 markedly decreases the void peak and enables the isolation of a late-eluting peak (around 15 ml) with little MEK-phosphorylation activity that corresponds to the autoinhibited BRAF–MEK1–14-3-3 monomer complex (c, fractions B8–C3). d, Wild-type BRAF was expressed in mammalian HEK293 cells, purified by serial Ni-NTA agarose and StrepTrapHP affinity chromatography, and subjected to SEC on Superdex 200. e, Elution fractions from the wild-type BRAF–14-3-3 SEC run in d are analysed by SDS–PAGE and western blotting, revealing that BRAF co-purifies with endogenous human 14-3-3 proteins. Fractions were also blotted for total BRAF (anti-StrepII), pS365 and pS729. f, Mass spectrometry analysis of trypsin and Lys-C protease digests of peak fractions of the BRAF–14-3-3 complex from HEK293 cells revealed multiple peptide sequences that mapped uniquely to six of the seven human 14-3-3 isoforms. The δ and α isoforms are phosphorylation variants of ζ and β, respectively. For gel source data, see Supplementary Fig. 1. SEC experiments were repeated at least three times (a–e), activity assays twice (a) and once (b, c), and blotting twice (e) with similar results.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Cryo-EM imaging of dimeric, active-state BRAF complexes and mass-spectrometry-based measurement of phosphorylation stoichiometry in BRAF–MEK1–14-3-3 complexes. 

**a**, Representative 2D class averages for the BRAF(S365A)–MEK1–14-3-3 complex. Scale bar, 10 nm. 

**b**, FSC curves for the BRAF(S365A)–MEK1–14-3-3 reconstructions presented in Fig. 4. 

**c**, SEC (Superose 6) traces for the indicated affinity-isolated BRAF complexes, prepared using insect or mammalian cells as described in Extended Data Fig. 7 and Methods. SEC experiments were repeated at least three times with similar results. 

**d**–**f**, Per cent phosphorylation of selected BRAF sites in successive elution fractions is plotted for each sample analysed in c. Fractional phosphorylation of these sites was measured using a mass-spectrometry-based assay (see Methods). 

**d**, Wild-type BRAF–14-3-3 complex produced by insect cells. 

**e**, Wild-type BRAF–14-3-3 complex produced by mammalian cells. 

**f**, BRAF–MEK1–14-3-3 complex produced in insect cells, prepared by co-expression of wild-type BRAF and MEK1(S218A/S222A). In d–f, note the high fractional phosphorylation of S729 in all samples, and the negligible phosphorylation of activation segment sites T599 and S602. 

**g**, Representative 2D class averages for wild-type BRAF–14-3-3 complexes prepared from mammalian cells with (top) and without (bottom) the addition of BRAF inhibitor GDC-0879 (1 μM). Both samples yielded class averages indicative of the same particle architecture, but those of the drug-treated sample revealed better-defined secondary structure. Scale bar, 10 nm. 

**h**, FSC curve for the wild-type BRAF–14-3-3 reconstruction. 

**i**, Single-particle reconstruction of the wild-type BRAF–14-3-3 complex produced in mammalian cells treated with GDC-0879. The reconstruction reveals a back-to-back BRAF kinase domain dimer with a 14-3-3 dimer bridging between its C-terminal pS729 tails. Comparison of these front and back views reveals the highly asymmetric position of the 14-3-3 dimer with respect to the dimerized kinase domains. Imaging experiments in a and g were repeated twice with independent preparations, and gave similar results.
Extended Data Fig. 9 | Structural snapshots outline a model for RAF activation. The RBD domain is exposed in the context of the autoinhibited BRAF–MEK1–14-3-3 monomer complex, enabling high-affinity binding to farnesylated, GTP-loaded RAS at the plasma membrane. We propose that ‘extraction’ of the CRD domain upon binding to prenylated RAS at the membrane is a key step in RAF activation. Without the stabilizing interactions of the CRD domain, the 14-3-3 domain can release from the BRAF kinase domain and pS365 segment to form an ‘open’ monomer. We expect the RAF–MEK kinase module of the open monomer to maintain its inactive, ATP-bound conformation as observed in the crystal structure described here. Finally, the 14-3-3 domain can rearrange to bind the C-terminal pS729 sites of two open RAF molecules, driving formation of the active, back-to-back RAF dimer. As illustrated here, the stoichiometry of 14-3-3 binding changes upon activation, but we do not exclude the possibility that a second 14-3-3 dimer remains associated with the complex, for example by bridging the pS365 segments. KD, RAF kinase domain; red circles (pSer) represent the pS365 and pS729 14-3-3 binding segments.
Extended Data Table 1 | Cryo-EM data collection, refinement and validation statistics

|                      | BRAF–MEK1–14-3-3 (EMD-0541) | BRAF(S365A)-MEK1–14-3-3 (EMD-20550) | BRAF–14-3-3 (EMD-20551) |
|----------------------|------------------------------|-------------------------------------|-------------------------|
|                      | (PDB 6NYB)                   | (PDB 6Q0J)                          | (PDB 6Q0K)              |
| **Data collection and processing** |                              |                                     |                         |
| Magnification        | 130,000 x                    | 36,000 x                            | 36,000 x                | 105,000 x               |
| Voltage (kV)         | 300                          | 200                                 | 200                     | 300                     |
| Electron exposure (e-/Å²) | -50                         | -50                                 | -50                     | -70                     |
| Defocus range (µm)   | -1.8 - 3.3                   | -2.0 - 3.0                          | -2.0 - 3.0              | -1.7 - 2.7              |
| Pixel size (Å)       | 1.06 (2x binned)             | 1.11                                | 1.11                    | 1.7 (2x binned)         |
| Symmetry imposed     | C1                           | C1                                  | C1                      | C1                      |
| Initial particle images (no.) | 3,531,955               | 2,008,323                           | 2,008,323               | 365,083                 |
| Final particle images (no.) | 165,298                   | 425,135                             | 595,672                 | 66,215                  |
| Map resolution (Å)   | 4.1                          | 4.9                                 | 5.7                     | 6.8                     |
| 0.143 FSC threshold  |                              |                                     |                         |                         |
| **Refinement**       |                              |                                     |                         |                         |
| Initial model used (PDB code) | 5FD2, 4MNE, 6PP9, 4FJ3, 1FAR, 3NKX | 4MNE, 6NYB                      | 4MNE, 6NYB              | 4MNE, 3NKX              |
| Map sharpening B factor (Å²) | -225                       |                                     |                         |                         |
| Model composition    |                              |                                     |                         |                         |
| Non-hydrogen atoms   | 8811                         |                                     |                         |                         |
| Protein residues     | 1097                         |                                     |                         |                         |
| Ligands              | 3                            |                                     |                         |                         |
| Metals               | 3                            |                                     |                         |                         |
| B factors (Å²)       |                              |                                     |                         |                         |
| Protein              | 125.4                        |                                     |                         |                         |
| Ligand               | 146.2                        |                                     |                         |                         |
| R.m.s. deviations    |                              |                                     |                         |                         |
| Bond lengths (Å)     | 0.014                        |                                     |                         |                         |
| Bond angles (°)      | 1.750                        |                                     |                         |                         |
| Validation           |                              |                                     |                         |                         |
| MolProbity score     | 2.18                         |                                     |                         |                         |
| Clashscore           | 6.69                         |                                     |                         |                         |
| Poor rotamers (%)    | 3.66                         |                                     |                         |                         |
| Ramachandran plot    |                              |                                     |                         |                         |
| Favored (%)          | 94.43                        |                                     |                         |                         |
| Allowed (%)          | 5.39                         |                                     |                         |                         |
| Disallowed (%)       | 0.19                         |                                     |                         |                         |
| Model vs Data        |                              |                                     |                         |                         |
| CC (mask)            | 0.74                         |                                     |                         |                         |
| CC (box)             | 0.75                         |                                     |                         |                         |
| CC (peaks)           | 0.62                         |                                     |                         |                         |
| CC (volume)          | 0.73                         |                                     |                         |                         |
| Mean CC for ligands  | 0.77                         |                                     |                         |                         |
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
SerialEM for Krios microscope and K2, K3 detector

Data analysis
Standard widely available software was used for structure determination, including RELION version 3, cryOLO, Coot and PHENIX.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The cryo-EM MAP and atomic coordinates have been deposited with the EMDB and PDB, respectively (EMD-0541, EMD-20550, EMD-20552, EMD-20551, PDB: 6NYB, 6Q0J, 6Q0T, 6Q0K, 6PP9).
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences   ☐ Behavioural & social sciences   ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Not applicable |
|-------------|----------------|
| Data exclusions | Not applicable |
| Replication | Not applicable |
| Randomization | Not applicable |
| Blinding | Not applicable |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| Antibodies | Involved in the study |
| Eukaryotic cell lines | ☑ |
| Palaeontology | ☑ |
| Animals and other organisms | ☑ |
| Human research participants | ☑ |
| Clinical data | ☑ |
| n/a | Involved in the study |
| ChIP-seq | ☑ |
| Flow cytometry | ☑ |
| MRI-based neuroimaging | ☑ |

Antibodies

Antibodies used

- Antibodies
- Vender
- Cat#/RRID
- Mouse monoclonal Penta-His Antibody
  - Qiagen
  - 34660
  - RRID:AB_2619735
- Rabbit polyclonal BRAF antibody
  - Thermofisher Scientific
  - PAS-14926
  - RRID:AB_10975898
- Rabbit monoclonal Anti-BRAF (Phospho S729)
  - abcam
  - Ab124794
- Rabbit polyclonal Anti Phospho-CRAF(S259) antibody
  - Cell Signaling Technology
  - 9421S
  - RRID:AB_330759
- Rabbit polyclonal Anti-Strep-tag II
  - abcam
  - Ab76940
  - RRID:AB_1524455
- Mouse monoclonal DYKDDDDK tag (9A3)
  - Cell Signaling Technology
  - 8146S
| Antibody Description                                      | Catalog Number | RRID       |
|-----------------------------------------------------------|----------------|------------|
| Rabbit polyclonal Anti 14-3-3 Beta/alpha                 | 9636S          | AB_10950495|
| Rabbit monoclonal Anti 14-3-3 zeta/delta(D7H5)           | 7413S          | AB_560823  |
| Rabbit monoclonal Anti 14-3-3 gamma (D15B7)              | 5522S          | AB_10950820|
| Rabbit polyclonal Anti 14-3-3 epsilon                    | 9635S          | AB_10827887|
| Rabbit monoclonal Anti 14-3-3 eta (D23B7)                | 5521S          | AB_2217758 |
| Rabbit monoclonal Anti 14-3-3 tau                        | 9638S          | AB_10829034|
| Rabbit polyclonal Anti 14-3-3 (pan)                      | CAT#8321S      | AB_10860606|
| Rabbit polyclonal, Anti-phospho –MEK1/2 (S217/221) antibody | Cat#9121S      | AB_330745  |
| Rabbit polyclonal, Anti- MEK1/2 antibody                 | Cat#9122S      | AB_823567  |
| Anti-mouse IgG, HRP-linked secondary antibody            | Cat#7076s      | AB_772191  |

**Validation**

Listed RRID for each antibody.

**Eukaryotic cell lines**

Policy information about [cell lines](#)

| Cell line source(s) | HEK293 (Expi293F) cells from Thermo-Fisher Scientific |
|---------------------|--------------------------------------------------------|
| Authentication      | Expi293F cells from were obtained directly from Thermo-Fisher Scientific, and were used only for protein production. |
| Mycoplasma contamination | negative                          |
| Commonly misidentified lines | n/a                              |
| (See ICLAC register)                        |                                        |