

Mapping of dynamic allostery within p38 alpha kinase via network analyses and NMR spectroscopy

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Abstract

Kinases are major drug targets especially in cancer therapy. However, the high degree of conservation of their active sites hinders the development of selective inhibitors, motivating a deeper understanding of kinase conformational ensembles and allosteric communication pathways. Here, we use dynamical network analysis to identify key residues involved in a dynamic allostery between the N- and C-lobes that connects the major functional units of the MAP kinase p38 α . By combining NMR spectroscopy, activity assays, and in silico analysis of wildtype protein and mutants in the presence or absence of an active-site inhibitor, we experimentally validate the obtained architecture with respect to global protein motion and long-range allosteric modulation. Notably, the identified network highlights communication pathways across several functional sites, prominently involving the allosteric site, the activation loop, and the lipid-binding domain with its embedded cryptic pocket in the C-lobe. These findings provide mechanistic insight into p38 α allostery and suggest new opportunities for the rational design of allosteric modulators of MAP kinases.

Introduction

The family of p38 mitogen-activated protein kinases (MAPK) represents one of the principal elements within the MAPK signalling cascade, critically involved in mediating cellular responses to stress and inflammatory stimuli. A robust cluster of experimental evidence suggests that p38 can exert pro-oncogenic functions in various types of cancer¹, which has made p38 one of the most important drug targets across a wide range of cancers. p38 kinases are prototypical kinases comprising a bi-lobal structural core that is divided into a smaller N-terminal and a larger C-terminal lobe. The catalytic active site lies between the two domains, forming a hinge bearing high flexibility, which is considered to be an important parameter for nucleotide exchange². The regulation of p38 α activity involves several highly dynamic structural elements, including the catalytic loop, the N-terminal segment of the activation loop containing the conserved “DFG-motif”, the α C helix, and the hinge region³. Drug design efforts for this kinase have been focused predominantly on small-molecule inhibitors of the ATP binding site, prohibiting the activating phosphorylation of residues T180 and Y182. Addressing this critical functional element, however, a component widely conserved across the entire kinome, entails dose-limiting adverse effects owing to non-selective inhibition.

Consequently, the identification of other potential locales that bind substrates, inhibitors, or allosteric effectors is of great interest¹. Modulators that bind in the “DFG-out” conformation, exploiting an additional site opening up close to the active site, have also been designed⁴. Sorafenib (Nexavar®) is such a (“type II”) inhibitor, binding to this site but potently inhibiting nine other kinases⁵ (**Fig. 1a**). Its strategic use in combination with the drug SB202190 at the ATP-binding site, e.g., entails a synergistic effect that increases the apoptotic response in colorectal cancer cells⁵. However, the resemblance of this site to the conserved phosphate-binding site has resulted in little or no increase in the specificity of drug binding, toxicity, and dosage constraints. A rather distant, cryptic pocket is associated to the lipid-

binding domain, hosting the “MAP kinase insert”⁶, which – in conjunction with a significant conformational change – has been shown to accommodate a range of lipophilic molecules like *n*-octyl- β -glucopyranoside (β -OG) as well as suitable covalent binders addressing a conserved Cys closeby⁷. This hidden pocket in the C-terminal region of the protein, the “lipid pocket”⁶ (**Fig. 1a**), has been shown to bear a primary sequence conserved over the p38 family and potentially allows for binding of more drug-like small molecules specifically generated in the quest for new allosteric p38 inhibitors⁸.

Allosteric communication refers to an intramolecular mechanistic crosstalk between spatially distant sites that participates in modulating functionality as a function of external events⁹. Allostery, in a wider sense, is the event in which one site somehow “feels” changes in a different site^{10,11}. As such, it can either be based on significant structural changes or rather – even in the complete absence of the latter – hinge on dynamic features that are mutually dependent across the protein¹². This latter mechanism, commonly referred to as “dynamic allostery”⁹, involves the redistribution of correlated motions across the protein, which can be modulated or induced by regulatory events such as ligand binding, protein–protein interactions, or phosphorylation.

In p38 α complexes, evidence for long-range dynamic coupling is provided by both structural and dynamical observations: Structural superimposition of the “DFG-out” p38 α complexes bound to Sorafenib alone (PDB 3HEG¹³) and to both Sorafenib and β -OG (PDB 3GCS¹⁴) reveals a flip of the pyridyl nitrogen and methyl substituent of Sorafenib, despite a separation of more than 30 Å from β -OG bound in the lipid pocket (**Fig. 1b**). This slight but noteworthy difference in ground-state structures suggests that ligand binding at the distal lipid pocket alters the conformational ensemble of the kinase in solution. In addition, activation of p38 α incurs dynamics differentially modulated across timescales, with activation-loop phosphorylation having been observed to quench ps–ns motions without altering the average conformation. Instead, uniform μ s–ms backbone dynamics are induced by substrate binding, flattening the energy landscape and rendering key allosteric sites accessible¹⁵. A cross-lobe interdependency in the realm of dynamic allostery also seems to involve the lipid pocket, occupation of which was observed to lead to widespread chemical-shift perturbations and changes in dynamics¹⁶. Similarly, for p38 γ kinase, another one of the four MAPK isoforms and a close relative to p38 α with ~60 % sequence identity, motional changes in the kinase due to distant effectors have been assessed from various angles^{17, 18, 19}. These observations align with the identification of long-range community networks in protein kinase A (PKA), fundamentally orchestrating overall dynamics and hence functionality²⁰, as well as findings in the Src kinases²¹, epidermal growth factor receptor (EGFR)²², and Abl kinase²³, where catalytic activity was seen to be modulated by very distant sites.¹⁹ Corroborating Cooper and Dryden’s original theory, activation of PKA has recently been ascribed to a redistribution of fast and intermediate-timescale thermal fluctuations (the “violin model”), which explains the lack of apparent structural changes. Thereby, the formation of “hydrophobic spines”, specifically the C-spine assembled upon ATP binding, and the R-spine, linked to positioning of the α C-helix, an assembly regulated by upstream processes (also shown in **Fig. 1a**), is thought to aid in orchestrating suitable dynamic networks.²⁴ In MAP kinases,

insights into such motional dependencies are of general interest from a biological perspective. However, they are of particular importance in the light of a motional connectivity between the different regulatory sites, the active site, or the lipid binding domain. A residue-specific mapping of such a dynamic network and identification of its key participants may aid understanding the molecular underpinnings of p38 regulation and even launch future pharmacological avenues based on the underlying pathways (**Fig. 1c**).

An in-depth interrogation of dynamic networks has become possible using molecular-dynamics simulations in combination with dynamic network analyses, where the correlations of motions assessed as generalised correlation coefficients based on mutual information are exploited to determine the strength of dynamic connectivity between different residues of the system.²⁵ Whereas these in-silico findings are extremely rich in information, they do require verification by experimental means. Providing site-specific access to a large range of chemical and motional parameters, NMR spectroscopy is particularly well suited as a complement to such simulations²⁶. Here, we use state-of-the-art computational approaches based on a dynamical network analysis software (Dynetan)²⁵ to characterize p38 dynamic networks from an NAMD²⁷-based molecular-dynamics simulation. Perturbing those residues that turn out to be pivotal for the motional network through site-directed mutagenesis, we then use NMR in solution to monitor the consequences for the various mutants experimentally. Together, these data reveal an extended dynamic network spanning both kinase lobes and comprising several distinct allosteric “hotspots” in p38 α . In addition to features common to other kinases, our analysis identifies the MAP kinase-specific lipid-binding domain and its associated cryptic lipid pocket as integral components of the dynamic network, highlighting their potential relevance for allosteric therapeutic intervention.

Results

Following the workflow summarized in Fig. S1, and the system setup summarized in Table. S1, we performed molecular dynamics (MD) simulations of apo p38 α , sorafenib-bound p38 α , and p38 α bound to both sorafenib and β -OG using NAMD 3²⁷ with the CHARMM36 force field²⁹. (See methodological details in the SI text. For the apo protein, Figs. S2 and S3 show root-mean-square deviations as a function of time as well as root-mean-square fluctuations as a function of residue.) For each system, five independent replica simulations of 1 μ s each were carried out. For subsequent network and correlation analyses, the final 200 ns of each replica were concatenated and analyzed using Dynetan²⁵ (described in more detail in the Methods and graphically summarized in Fig. S4). This analysis included community detection and quantification of betweenness centrality, which identifies residues that are central to allosteric communication.

Community detection, the first output of network analyses, partitions the network into clusters that share common motion. This enables probing of consistency between the rich, atomic-resolution MD data here with coarser experimental studies on this or similar systems in the past. For the wild type, apo protein, this analysis yields 14 clusters, shown by different colors in **Fig. 2a**. Here, each community denotes a

group of residues that exhibit strongly correlated motions with each other but weaker correlations with residues outside the group, reflecting coherent dynamical behavior within the protein. Despite the ~60 % sequence identity between the isoforms only as well the stark differences in methodology, the clusters obtained from the in-silico assessment here have a remarkable congruency to those (15) clusters found via purely experimental analyses (methyl scanning and chemical-shift perturbations) for p38g¹⁷: Like in this previous study, our clusters divide the N-lobe into different active-site (magenta) and allosteric-pocket (brown) regions and take accountability of the R-spine (gray) and C-spine regions (pink). The C-lobe is divided into three clusters, where the MAP kinase insert (lipid binding domain) is the cluster shown in green in Fig. 2a and deviates from the cluster containing the activation loop (shown in purple). Notably, several of these dynamic features also align with community structures identified previously for a different kinase (protein kinase A, PKA) using Girvan–Newman-based network analysis²⁰. Here, communities were segregated into 13 regions: In the N-lobe, the ATP binding pocket (magenta) is reminiscent of the PKA “Com A1”, the C-helix; the extended allosteric pocket of the p38α (brown) corresponds to “Com B”, and the R-spine of PKA (“Com C”) matches the gray cluster in p38α here²⁰. In the C-lobe of PKA, “Com D” is a cluster congruent to the activation loop in p38α (purple), “Com E”, the C-spine cluster in PKA, matches the pink cluster in p38α, and “Com F” in the PKA case is the substrate binding region, which bears at least a slight similarity to the lipid pocket in p38α (green), which is part of the lipid binding domain only applicable for MAP kinases²⁰.

More interestingly, we interrogated betweenness-centrality, which property identifies allosteric-network hotspots on the basis of their participation extent within different communication pathways. These results are shown in **Fig. 2b** and more specifically in Table S2. Whereas naturally, a one-to-one comparison with other kinases is compromised due to the different primary structures and differences in the techniques applied, these pathways again seem qualitatively consistent with the experiment-based pathways connecting different regions of p38γ, where methyl mutations of several conserved sites were explored.¹⁷ For example, the memory node L170 in p38γ that takes additional input from the DFG-motif, appears as a motional hotspot in our study as well. Likewise, the hinge cluster of Abl kinase, constituting the D400 of the DFG-motif, had similar dominance in the long-range communication, suggesting the aspartate of the kinase to be a significant player in dynamic allostery³⁰. In case of PKA, the F185 of the DFG motif coordinated together was found to be part of the allosteric communication instead of the vicinal aspartate, evocating the hypothesis whether the DFG motif itself is a necessary allosteric modulator³¹. D150, another node identified by our betweenness centrality study, belongs to the catalytic HRD-motif. In PKA, the homologous aspartate D166 was found to be the most crucial node that preorganizes the substrate’s phospho-acceptor site for efficient phosphotransfer³². (For Abl kinase and EGFR, the residues of this central catalytic machinery are the homologous D363 and D813, respectively.^{33, 34, 35}) R49, another mediator that we identified as a residue with a high betweenness centrality, is a part of the extended allosteric pocket in p38α. While it reinstates its allosteric role in p38α in our study, a homologous residue in similar kinases that pinpoints its governance in their allosteric mechanism has not been found³⁶. Most interestingly, however, W207, identified here as another key

residue of the network, is positioned near the substrate binding groove. In PKA, the homologous W222 has been found to be an allosteric hub in the α F/FC region and a structural keystone anchoring the C-lobe.^{37, 38} (Abl and EGFR kinases have similar hydrophobes but there is no exact analogue to pinpoint in this region³⁹.) In p38 α , this residue plays a particular role as it directly faces the lipid pocket, for which the possibility of designing allosteric effectors has been speculated.⁷ Its high betweenness centrality hence directly rationalizes a participation of the lipid pocket as part of the dynamic network.

Beyond the overall inter-lobe correlations (shown in **Fig. 2c** and Table S3), which confirm both short-range connectivities dictated by the three-dimensional fold of the protein (e.g., R70/F169) and longer-range cross-talk such as between residues L104 and L167 or N102 and K165, we specifically examined the communication pathways linking the allosteric pocket, the activation loop, and the lipid pocket. For this reason, we picked one representative residue of the network for each of these sites/clusters and computed the cumulative pairwise correlation (the sum of degrees of correlation between any two neighbors) within the shortest path of communication. For the allosteric site, represented by residue K66, towards the activation loop (Y188), the information flux traverses the D168 and G170 of the DFG motif, similar to p38 γ (**Fig. 3b**, compare Table S5)^{17, 18, 19}. Accordingly, the shortest path between the activation loop and the lipid pocket, with residues such as K233 and I235, is shown in **Fig. 3c**. Pathways between the two pockets (between K66 in the allosteric pocket and K233, and I235 in the lipid pocket) were also calculated without specific consideration of the activation loop. This path involves the DFG motif, but bypasses the activation loop to channelise the communication to the lipid binding domain (**Fig. 3e**). Importantly, all of the above analyses identify a network of allosteric communication that interconnects not only the N- and the C-lobe generally but also involves the lipid-binding-domain and specifically the lipid pocket. These *in silico* results rationalizes the existence of functional communication pathways between the active site and the lipid-binding domain, supporting potential avenues for targeting MAP kinases through allosteric modulation.

To assess whether those networks are inherently apparent already from crystallography, the nodes identified were further examined in terms of B-factors derived from the crystal structures²⁹. Crystallographic B-factors provide an experimental proxy for local rigidity and structural order. Fig. S5 shows a depiction of B-factors. However, the B-factors of the hotspots of the dynamic network are mostly in the intermediate range (see a list of B-factors for all major nodes of the network in Table S4), denoting that these residues are not particularly disordered in the crystal. (For example, residues R49, D150, and D168 have values of 0.509, 0.342, and 0.497, respectively. W207 shows an even lower value of 0.056, making it a very well-defined residue.) Even though a flexible character/flexibility of specific residues at physiological temperatures can sometimes be forecasted from the cryogenic crystallographic viewpoint, it seems that the allosteric hotspots are not recognizable through a specific degree of disorder. For further elucidation of changes to the networks, we hence turned to room temperature dynamics data, assessed by NMR spectroscopy (see below).

To validate the existence and characteristics of the dynamic allosteric network identified by dynamical network analysis, we pursued a two-level approach involving additional *in silico* analyses and experimental validation. In both cases, the network was probed by “mutating” residues identified as key nodes based on high betweenness centrality. For this downstream analysis, in particular correlation analyses, ligand (sorafenib) binding studies, solution NMR chemical-shift perturbations and NMR relaxation, as well as biochemical assays (see below), four (R49, D150, D168, and W207) were selected and replaced by alanine. W207 is of particular interest as it forms part of the lipid pocket in the C-lobe. MD data (five 1 μ s trajectories for each of the four mutants) were obtained using similar strategies as for the wild type (Fig. S6) and re-analyzed with the mentioned network framework. These analyses confirmed both, differences in betweenness-derived pathways and altered community structure (Fig. S7) compared to the wild type. The effects of mutation on long-range communication were also quantified by evaluating inter-lobe (N–C) correlations and correlations along the wild-type-defined shortest communication paths (Tables S2, S3, and S5). Total correlation maps for all ensembles are provided in Fig. S8 and S9. Whereas the tertiary structure and hence many correlations are necessarily maintained with respect to **Fig. 2c**, changes with respect to long-range correlations can be witnessed in each of the cases. (Difference maps (mutant – wild type) are shown in Fig. S10.) To focus on pathway-level effects, we again selected one central residue per structural element (K66 for the allosteric pocket, Y188 for the activation loop, and I235 for the lipid pocket as before) and computed the shortest paths between them also for the mutants. The wild-type path served as the reference, and the cumulative amounts of pairwise correlations between any two consecutive residues along this path were evaluated for wild type and each mutant. Uncertainty was estimated from five replicas, each partitioned into five windows (Table S6). Across all paths (allosteric-to-activation loop, **Fig. 3b**; lipid-pocket-to-activation loop, **Fig. 3d**; and allosteric-to-lipid pocket paths, **Fig. 3f**), mutant correlations were consistently lower than in the wild type protein. Interestingly, W207A, despite its residence in the C-lobe, has a very strong effect on the allosteric site-to-activation loop path (~ 20 %, **Fig. 3b**). Conversely, for the lipid-pocket-to-activation loop, D168A, the DFG-mutant lying in the hinge of the N-lobe has a strong impact. (~ 10 %, **Fig. 3d**); both residues lie distal to the respective pathways, consistent with a dense allosteric network. (**Fig. 3d**). Intriguingly, with respect to the direct pathway between the allosteric pocket and the lipid pocket, all the nodes identified by the network mutated out (R49A with ~ 10 %, D150A, D170A, both with ~ 20 %, and W207A, ~ 25 %) show a substantial, statistically significant drop (**Fig. 3f**).

In addition to the apo proteins, we also examined how the presence of an active/allosteric-site binder would influence the network. For this purpose, we employed the *in-silico* interrogation of wild type and mutants outlined above, however, starting from the sorafenib-bound X-ray structure 3HEG. Fig. S11 and Table S8 show the embedding of this ligand as interrogated using Discovery Studio⁴⁰. MD simulations again afforded trajectories of 5 replicas each (Fig. S12). At first, we assessed motional correlations between the protein and the drug molecules. Fig. S13 shows these correlations, where for simplicity the ligand sites were coarse-grained into a Western and an Eastern part analysed individually. Interestingly, we observed that the total amount of cumulated correlation drops slightly upon mutation, most prominently for mutant R49A (Fig. S13). More importantly, however, we then pursued the above

examination of interdomain communication within the ligand-bound protein. For the wild-type complex, shortest communication paths connecting the allosteric pocket, activation loop, and lipid pocket were identified using the same representative residues as in the apo analyses. Structural representations (Fig. S14A, C, E) reveal that inhibitor binding now reroutes the preferred communication pathways linking the allosteric pocket, the activation loop, and the lipid pocket with each other. For each complex, we quantified the cumulative pairwise correlations along these paths, using the ligand-bound WT as the reference. Now, taking the new, ligand-bound routes of the wild type protein as the reference and mutating the above-mentioned network hotspots, no significant reduction in correlation is observed anymore (Fig. S14B, D, F). This indicates that ligand binding rigidifies the protein and largely suppresses its intrinsic dynamic network. Consistently, no systematic gains or losses in cumulative correlations are detected along the ligand-bound paths.

Together, these results demonstrate that (i) mutations at network sites attenuate communication in a pathway- and residue-dependent manner, (ii) sorafenib reshapes the communication routes relative to the apo protein, and (iii) ligand binding to the active site globally rigidifies the protein, effectively quenching dynamic network propagation. Assessing a single shortest communication path neglects the fact that allosteric signaling typically arises from the collective contribution of multiple pathways. Moreover, the *in silico* identification of long-range communication paths spanning many network edges is intrinsically less sensitive than the analysis of shorter network fragments, such as those shown in **Fig. 2b**. Even with the extensive sampling afforded by five independent microsecond-long MD replicas, these pathway-level correlations retain a certain statistical error, underscoring the need for experimental validation based on vastly larger ensembles of molecules. To experimentally (with a much bigger ensemble) probe the functional consequences of hijacking the dynamic network, we therefore first assessed the enzymatic integrity of the individual mutants relative to the wild-type protein using Homogeneous Time-Resolved Fluorescence (HTRF) assays⁴¹ (**Fig. 3g**, see details in the Methods).

HTRF is a robust, high-throughput, low-background FRET-based technique that combines the sensitivity of time-resolved fluorescence with a homogeneous assay format. In this study, HTRF was employed to quantitatively compare protein substrate binding efficacy (using GST-tagged activating transcription factor 2 as a substrate) as well as the integrity of binding of ATP between wild-type and mutant constructs⁴¹. Adding to the wild-type protein, whose expression and purification has been described in the literature⁴², all of the above mentioned mutants were generated via site-directed mutagenesis and the protein expressed recombinantly in *E. coli* and purified according to the existing protocols (see the Methods and Fig. S15, for biochemistry details). The dependence of reaction speed on either ATP or substrate concentration serves as a quantitative proxy for binding of either interaction partner (see details in the Methods). Kinetic characterisation was pursued via Michaelis-Menten fits (**Fig. 3h** and **Fig. 3i**), confirming highest binding competencies, with the lowest Michaelis-Menten constants (K_M) for the wild type enzyme (Table S7). Strongly impaired affinity occurs in mutants D168A, R49A, with a twofold decrease in ATP binding each. Similarly, for the mutant D150A, the binding to the substrate is diminished, and ATP binding competency is slightly lower. Also for W207A, even though the mutation is deep in the

C-lobe, the ATP-driven regulation and catalysis appear to be lost. The most severe phenotypes were observed in **D150** and **W207**, even though neither of these sites is part of the actual enzymatic process, consistent with the possibility of disrupted enzymatic potency due to allosteric modulation from distant sites of mutation. Even though the consistent decrease for both interaction partners even for mutation sites far away from binding interfaces and in particular for W207 as a lipid pocket residue is noteworthy, more specific motional analyses were sought to corroborate the findings.

If protein motions were purely local and independent, a point mutation would be expected to affect primarily the substituted residue and its immediate environment. By contrast, NMR studies have shown that perturbations within dynamic allosteric networks can induce long-range changes in both, site-specific chemical shifts and relaxation properties across multiple timescales^{17 43}. To assess such putative long-range changes in protein dynamics, we used solution-state NMR on ¹³C/¹⁵N/²H labelled protein samples expressed in triple labelled media and purified using successive rounds of Ni-affinity, anion exchange, and size exclusion chromatography according to published procedures⁴². In spite of multiple attempts and in contrast to all other proteins mentioned here, the plasmid of mutant W207A could not be successfully expressed as a stable triple-labeled construct in deuterated minimal media, precluding its structural and dynamic interrogation by NMR. Peak assignments were achieved via 3D HNCA spectra for both wild-type and mutant constructs, complemented by TROSY-HSQC used as fingerprint spectra (Fig. S16), upon which existing assignments (BMRB entry: 17471) could be transferred in a stepwise manner. To complement the missing fourth mutant, we newly introduced V38A, in this case, however, specifically as an experimental negative control. V38 lies in a folded element of the tertiary structure, but outside the predicted dynamic network, allowing us to compare any “regular” alterations induced locally to global changes entailed by a tight dynamic network.

We first employed chemical-shift perturbation (CSP) analysis to probe changes in the average chemical environment of backbone amides across the protein. In the absence of allosteric communication, the chemical shifts of very distant residues would not be expected to change even in the case of slight structural changes around the mutation site. Instead, the analysis revealed widespread deviations across the backbone for all functional mutants involved in the allosteric coupling. Notably, D168A, the “DFG-loop mutant”, exhibited the most dramatic global extent of CSPs across the structure (**Fig. 4a, c, and d**), including long-range effects such as the complete disappearance of the W207 peak in the C-lobe. As mentioned above, apart from its identification as a spatially distant key residue involved in the interdomain communication, W207 also represents a marker for the lipid pocket. This corroborates both, D168A’s sensitive influence on allosteric interactions and domain coupling as well as the participation of W207 in the allosteric network. The distributed nature of dynamic perturbations throughout the protein can be recognized from **Fig. 4b**, exemplarily depicting chemical-shift perturbations for this variant compared to the wildtype. (CSPs and spectral overlays for the other mutants are shown in Fig. S17.) In contrast to the mutants focused on hotspots found for the dynamic network, the negative control V38A showed indeed very limited chemical-shift changes (see **Fig. 4d** and Fig. S17). The stark differences in chemical-shift perturbations between mutations within the dynamic network and the negative control is

also seen from the violin plots shown in **Fig. 4c**, supporting V38A's role as a mutation benign to the conformational ensemble and underscoring the global conformational remodeling induced by the actual network-disruptive mutations on the contrary. These findings provide experimental support for mutations within hotspots identified by dynamical network analysis to indeed induce substantial allosteric rewiring of the kinase conformational ensemble.

To determine whether the observed chemical-shift perturbations really reflect changes in global protein dynamics, we employed solution-state ^{15}N relaxation, more specifically, [^{15}N , ^1H] heteronuclear NOE (hetNOE) and ^{15}N transverse relaxation rates (R_2). These partly complementary relaxation parameters probe site-specific motion on the ps-ns timescale, with R_2 being additionally influenced by motion on the μs timescale. Among all mutants tested, D150A exhibited the most pronounced deviations from wild-type behavior across both, hetNOE and R_2 datasets (**Figs. 5** and S18). The further reduction in hetNOE values compared to the wildtype at several positions throughout the protein suggests a further increase in fast-timescale local flexibility. In parallel, increased R_2 values, especially in the C-lobe (residues around 200 – 250), indicate an additional increase of conformational-exchange contributions, stemming from increased μs timescale fluctuations and suggesting a widely altered energy landscape introduced by the mutation. These observations also align with significant exchange broadening, which is usually incurred by enhanced μs – ms conformational dynamics. The differences to the wildtype are also visualized through structural mapping of per-residue deviations (**Fig. 5a-b**), witnessing dynamic perturbations extending well beyond the respective mutation sites. Intriguingly, D168A and R49A also show vivid alterations in the C-lobe regarding the R_2 rates, and more slightly for hetNOE values, again supporting that these sites represent key nodes in long-range allosteric coupling. Also the extent of dynamic reorganization in D150A appears to be globally distributed. In disparity, the control variant V38A, with its mutation site outside the predicted dynamic network, exhibited again only minimal, local changes. Both its hetNOE and R_2 profiles remain largely aligned with the wild-type except for subtle shifts near the site of mutation (Fig. S18), confirming its neutrality regarding the kinase's conformational ensemble. Boxplot comparisons across the variants (**Fig. 5c-d**) support a general enhancement in both, fast-timescale flexibility (decreased hetNOE) and a concurrent rise in μs timescale dynamics (higher R_2 values), suggesting that mutations in dynamic network positions generally increase protein plasticity and conformational exchange. These shifts correlate with a loosening of the dynamic network upon mutation, upon which motion of the individual residues becomes less coherent and less restricted overall.

To probe conformational-exchange processes on the microsecond-to-millisecond timescale, we finally performed ^{15}N CPMG relaxation dispersion (RD) measurements on wildtype protein and the mutants (Fig. S19). Data quality was insufficient to perform a more comprehensive analysis, but exchange contributions R_{ex} to the effective transverse relaxation values could be obtained with reasonable accuracy, offering residue-specific identification of transient conformational substates. In RD experiments, generally, the exchange contribution R_{ex} is derived from the difference in $R_{2\text{eff}}$ measured at varying CPMG refocusing efficiency. High R_{ex} values are indicative of residues undergoing chemical

exchange (allosteric transitions, functional loop dynamics, or domain breathing motions) between conformers in particular on the fast ms timescale, being stronger both, for elevated excited-state populations and increased chemical-shift differences for those transient states. Globally, wild-type samples exhibited the largest number of residues with significant R_{ex} contributions throughout the N-lobe and the C-lobe, indicating widespread conformational plasticity (Fig. S19A-B). As expected, a highly similar pattern as for the wild type is observed for V38A, both with respect to the residue level (Fig. S19A) and regarding the distribution of R_{ex} contributions depicted in the form of a histogram (Fig. S19B), consistent with its position outside the dynamic network and confirming its dynamically silent nature in terms of allosteric communication. The mutant construct R49A shows a slight loss of ms timescale exchange in the N-lobe and a slight increase in the C-lobe, confirming the above reorganization of motional behavior (Fig. S19C). For mutants D150A and D168A, by contrast, a slight decrease of R_{ex} in the C-lobe and a slight increase in the N-lobe was observed (Fig. S18C). All of these changes are, however, rather sporadic and any trends are difficult to discern. The apparent lack of strong differences between the samples results on the one hand from a rather noticeable error associated with the individual $R_{2\text{eff}}$ rates. Fig. S18D exemplifies this for A172, a residue lying in between the DFG motif and the activation loop, via comparison of dispersion curves for the different samples. A number of other dispersion curves are shown in Fig. S19. However, the higher overall similarities across the R_{ex} histograms in Fig. S18B suggests that the differences induced by the perturbations to the dynamic networks, as consistently seen upon CSPs, hetNOEs, and R_2 analyses, are rather limited to motions faster than the ms regime.

Discussion

The results presented here demonstrate that p38 α kinase harbors a dynamic allosteric network that connects the N- and C-lobes and explicitly incorporates the lipid-binding domain and its associated lipid pocket. Mutations at residues identified as central nodes within this network induce substantial perturbations in both local and global dynamics, as evidenced by a consistent set of computational and experimental observations, including molecular dynamics simulations, dynamical network analysis, pathway-level correlation changes, chemical-shift perturbations, heteronuclear NOE measurements, and transverse relaxation rates. While ligand binding partially modulates these effects, the dominant impact of network disruption is observed in the apo ensembles. Collectively, these findings provide experimental validation for the simulation-derived prediction that the p38 α scaffold supports long-range dynamic communication between distinct functional regions of the protein. Whereas the firm connectivity between dynamic-network residues tethers the plastic kinase architecture tightly together, thereby conducting concerted dynamics across long distances, mutations at key network sites abrogate long-range communication, entailing increased fast-timescale flexibility and conformational exchange. This conclusion can rationalize prior observations in a more biological context in a residue-specific manner. For example, local perturbations, such as activation-loop phosphorylation or binding of upstream regulators, can induce dynamical changes at distal sites within the kinase, albeit on slower timescales than those primarily interrogated here¹⁵. With the revelation of a widespread dynamic network prominently involving the activation loop and encompassing in particular the active site, the top part of

the C-lobe, and the lipidic pocket, the various prior observations hinting to dynamic allostery across the kinase architecture and regulation come to no surprise.

Several residues identified as key network nodes coincide with sites previously implicated in pathological or regulatory phenotypes, despite not being directly involved in catalysis. D150, located in proximity to the catalytic site⁴⁴ of the protein, forms an intramolecular H-bond with T185 that is required for TAB1-induced autoactivation⁴⁵. R49K/A is used as a convenient tool mutation for studying PRMT1-mediated control of p38 α ⁴⁶. Also D168 mutation is known to render the enzyme dysfunctional and has been used as a valuable mechanistic probe^{47, 48, 49}. Its prominent functional role, being key for the in/out conformational switch of the DFG motif, required for exchanging ATP/ADP at the active site, clearly exceeds the role as a mere dynamics-transducing element of the network. It is still important to realize that this site can also be ascribed a prominent role in transducing information across the protein scaffold, which would mean an impact on the motional properties and allosteric sensing associated with the conformational changes at the DFG motif. Importantly, our data establish the lipid-binding domain as an integral component of the p38 α dynamic network, which provides further prospects for pharmacological avenues based on lipid-pocket-binding allosteric modulators.⁷ Taken together, these observations demonstrate how wide-spread dynamic networks, rather than the local mobility associated with the individual structural elements, underly the integration of regulatory signals in p38 α and likely in other kinases as well.

Beyond the sheer identification/mapping of communication pathways between the p38 α N- and C-lobes, in particular including the so-far largely unexploited lipid pocket, in the presence and absence of the inhibitor, the above results may also offer a conceptual framework for the in-silico identification of possible allosteric sites in a kinase drug discovery context. The knowledge about, in particular, the existence and whereabouts of surface sites associated to dynamic networks could facilitate the design of next-generation allosteric modulators that do not target the known pockets, such as the ATP binding site or the “allosteric pocket”. It is so far unclear whether the lipid pocket will ever be addressed pharmacologically. The confirmation that a dynamic network extends to this site and in particular the identification of W207 at the rim of the lipid pocket suggests the site may serve as a useful starting point for new allosteric p38 α inhibitors. With first-generation, low-affinity binders (lipid pocket ligands), lipid pocket occupation (at the low populations obtained) has not been found to bear a strong impact on kinase activity.⁷ However, given that for a desired systemic effect even a weak molecular impact can suffice, the above data encourage further work, involving bulkier or sterically optimized ligands, which might result in new perspectives to overcome the off-site effects usually associated with targeting kinases. More generally, awareness and spatial mapping of dynamic networks in enzymes may guide the identification of regulatory sites amenable to therapeutic intervention or rational engineering. It remains to be seen whether dynamic allostery will prove valuable not only for pharmacological inhibition but also for fine-tuning of enzymatic activity in biotechnological applications through targeted allosteric mutations^{50, 51, 52}.

In summary, we identify and map a dynamic allosteric network within p38 α MAP kinase that spans both lobes, prominently involves inter-lobe segments of the architecture and the activation loop, and explicitly includes the lipid-binding domain and its associated lipid pocket. By integrating computational and experimental structural biology approaches, we reveal specific residues that act as key information relays within a coordinated motional framework, transmitting dynamics across spatially distant regions of the protein. Mutations at these hotspots largely corrupt the integrity of the network, thereby significantly reducing inter-lobe communication and inducing global changes in residue-specific dynamics, underscoring their central role in long-range allostery. Together, these findings provide new insights into the dynamic basis of kinase regulation and pathology. With the lipid pocket surface, in particular residue W207, found to form an integral part of the dynamic network, the study also highlights opportunities for identifying and targeting allosteric sites in future pharmacological strategies.

Methods

Dynamical Network Analysis

All atomic molecular dynamics (MD) simulations were performed using NAMD 3.0²⁷ in conjunction with the QwikMD plugin⁵³. To quantify ensemble-level differences associated with the reported ligand-dependent conformational changes, simulations were initiated from two crystallographic structures of p38 α in which the inhibitor ring flip was observed (PDB IDs: 3HEG¹³ and 3GCS¹⁴). For apo p38 α (1WFC²⁹), missing segments were rebuilt by comparative modeling. NOESY-derived restraints were applied during energy minimization (20,000 steps), slow heating to 300 K, and a $\sim$ 1 μ s restrained equilibration; restraints were then released and five independent 1 μ s production replicas were generated. Dynamical network analysis identified betweenness-centrality hotspots, which were alanine-scanned using QwikMD; each mutant underwent the same minimize–anneal–equilibrate protocol, followed by five 1 μ s replicas for correlation and network metrics. Sorafenib was docked into the allosteric pocket (Table S6), prepared identically, and simulated in five 1 μ s replicas. Correlations and communication pathways were compared across apo, mutant, and ligand-bound ensembles. A total of $\sim$ 50 μ s simulations are used for this study.

All simulations employed the CHARMM36²⁹ force field for proteins and ligands. Covalent bonds involving hydrogen atoms were constrained using SHAKE, and equations of motion were integrated using a 1 fs time step. Long-range electrostatics were treated using the particle-mesh Ewald method under periodic boundary conditions, with a real-space cutoff of 12 Å and a pair-list distance of 14 Å. Systems were equilibrated under NVT conditions for thermalization and NPT conditions for pressure coupling prior to production. Temperature was maintained at 300 K using Langevin dynamics, and pressure was controlled at 1 atm using the Langevin piston method under periodic boundary conditions.

The topology and trajectory files from the MD simulations were incorporated into the Python notebooks of Dynetan²⁵. The software uses the incorporation of MDAnalysis to analyze the trajectory simulation files⁵⁴. Contact detection optimization of the code was already performed via Numba⁵⁵ and Cython⁵⁶.

Network statistics and the determination of optimal paths were carried out using the Floyd–Warshall algorithm, provided by the NetworkX package. For Floyd–Warshall calculations, the “distance” between nodes was defined as

$$d = -\log(r_{MI}),$$

which uses mutual information as a method for contact detection consistent with previous applications of this method which can be seen as:

$$r_{MI}[i,j] = (1 - e^{-2/3 I[i,j]})^{1/2}$$

where i and j are the position of the alpha carbon of each residue and $I[i,j]$ are the mutual information between them which is computed by the density estimator described as:

$$I[i,j] = \psi(k) - 1/k - \langle \psi(n_i) + \psi(n_j) \rangle + \psi(N)$$

where here N is the total number of simulation frames, $\psi(x)$ is the digamma function, n_i is the number of frames in which residue i is close to the one in the reference, and $\langle \rangle$ stands for the average of the trajectory, using a neighboring parameter k of 6.

Each amino acid residue is regarded as a node. The network is structured by these nodes and nodes lie within a cutoff distance (4.5 Å) for at least 75% of an MD trajectory. The residues which lie farther than the cutoff value are excluded which mainly includes some solvent and ion molecules. The nodes are connected via links known as “edges”.

Betweenness centrality was computed as :

$$b_i = 1/c \sum_{s,t \in V} \sigma(s,t|i) / \sigma(s,t)$$

where $\sigma(s,t)$ is the number of shortest paths between nodes s and t , V is the ensemble of graph nodes, and C is a normalization factor.

The simulations were analyzed using in-house Python and TCL scripts along with VMD⁵⁷. Contact maps for residue–residue interactions were generated with VMD and PyContact. For the statistical testing, we normalized all measurements by the cumulative of the WT (or LWT) row. For each variant we report the per-variant mean and plot error bars as the sample standard deviation across replicates. Group comparisons to WT used a non-parametric permutation bootstrap: we pooled the two groups, permuted labels, recomputed the mean difference, and repeated this 10,000 times (two-sided). The p-value is the proportion of permuted differences whose absolute value exceeded the observed absolute difference. No parametric assumptions (normality or equal variances) were required and plotted using python notebooks. All the structural figures for simulations are rendered through VMD. The figures showing residue-wise properties are extracted through Chimera attributes.

Sample Preparation

The plasmid map of the kinase was elucidated by nanopore sequencing to entail the specific design of mutagenesis primers. The primers for the mutants were designed such that the mutation residue lies at the center for the forward and reverse primers. *Phusion™ High-Fidelity DNA Polymerase*, a fusion polymerase containing a Mastermix of nucleotides, enzymes, and buffers were used to carry out the polymerase chain reaction. The PCR product was digested using the DpnI enzyme at 37 °C for 1 hr and was immediately transformed overnight at 37 °C to *E.coli* XL 10 gold cells. The transformed colonies were grown in an overnight culture at 37 °C and the plasmid was extracted using a PEG gold miniprep kit and sent to Eurofins for Sanger Sequencing. The wildtype as well as the mutants was expressed into BL21(DE3) *E. coli* cells using ^{13}C and ^{15}N labelling for assignment and relaxation experiments. D_2O adaptation was carried out and the cultures were grown in 1 L at 37 °C to reach the OD_{600} of 0.6-0.7. They were then cooled for 30 min to room temperature, and induced with 1 mM IPTG overnight (~20 h) at 18 °C while shaking at 180 rpm. For the mutant W207A, expression in deuterated minimal media could not be achieved. The cells were lysed using a 50 mM Tris Buffer, benzonase, and lysozyme in the presence of a protease inhibitor, centrifuged, and the supernatant was exposed to affinity chromatography (Binding Buffer: 50 mM Tris, 500 mM NaCl, 25 mM Imidazole and 5 % Glycerol; Elution Buffer: 50 mM Tris, 500 mM NaCl, 500 mM Imidazole and 5 % Glycerol) using a nickel column. The eluted fractions were dialyzed to a pH of 7 and were subjected to an overnight cleavage in the presence of thrombin. Anion exchange (Binding Buffer: 25 mM Hepes and 5 % Glycerol; Elution Buffer: 25 mM Hepes, 1 M NaCl, 5 % Glycerol) and Size Exclusion Chromatography (SEC Buffer: 20 mM Hepes, 50 mM NaCl, 100 mg/L methionine and 5 % Glycerol) was then used to obtain pure protein.

HTRF Analysis

Wild-type and mutant p38 α constructs (unlabelled) were activated with constitutively active MKK6^{S207E/T211E} (Thermo Scientific, Lot 877061F) in activation buffer (50 mM Tris, 10 mM MgCl_2 , 1 mM ATP, 1 mM DTT, 0.001% Tween-20, pH 7.4) at 37 °C for 90 min with shaking at 400 rpm. Reactions were dialyzed overnight at 4 °C into storage buffer (20 mM HEPES, 50 mM NaCl, 5% glycerol, pH 7.1), concentrated to ~0.2 mg/mL, and stored at -80 °C. ATP K_M was determined using the HTRF® KinEASE™ kit (Cisbio) following the manufacturer's instructions. Activated p38 α was added per well at 0.04 ng (WT), 3.5 ng (mutant/unlabelled), or 20 ng (mutant/labelled) and incubated for 10/10/20 min, respectively, with 1 μM GST-ATF2 substrate across 0.4–900 μM ATP in reaction buffer (50 mM HEPES, 0.1 mM Na_3VO_4 , 0.02% NaN_3 , 0.01% w/v BSA, 10 mM MgCl_2 , 1 mM MnCl_2 , 1 mM DTT, 0.01% Triton X-100, pH 7.0) in 384-well black flat-bottom plates (Greiner Bio-One). Reactions were stopped with detection solution (50 mM HEPES, 0.1% w/v BSA, 800 mM KF, 20 mM EDTA, 0.666 nM anti-phospho-ATF2-Eu(K) antibody, 100 nM anti-GST-d2 antibody, pH 7.0) and incubated 60 min at room temperature. Time-resolved fluorescence was read on an EnVision 2104 (PerkinElmer) at 620 nm and 665 nm, 60 μs after excitation at 317 nm. The 665/620 signal ratio was plotted versus ATP concentration and fitted to the Michaelis–Menten equation in Origin (OriginLab).

The protein from the SEC Buffer was exchanged to “NMR Buffer” containing 50 mM of HEPES and 150 mM NaCl (pH ~6.8), and the sample was concentrated to approximately 450 μ M. NMR experiments were recorded on an 800 MHz Bruker NEO spectrometer. 2D ^{15}N - ^1H TROSY HSQC and 3D TROSY HNCA were recorded to gain the assignments for the mutants via assignment transfer from the BMRB (entry: 17471). CCPN version 3.1 was used for assigning the mutant peaks⁵⁸. ^{15}N - ^1H TROSY hetNOE was performed to assess fast-timescale dynamics and was analysed using a CCPN macro for relaxation. ^{15}N TROSY-CPMG experiments were acquired in an interleaved manner as follows for the CPMG frequencies: 0, 2000, 25, 1500, 1000, 100, 750, 200, 1250, 500, 200 Hz with a CPMG delay of a total of 0.022 s and a recycling delay of 1.5 seconds. The experiments were analyzed with NESSY software⁵⁹. Graphs and analysis were produced using in house python scripts.

All the raw data has been deposited in the public repository of Technical University Dortmund (<https://data.tu-dortmund.de/previewurl.xhtml?token=ff748a60-a45c-4c14-8f09-b7e397880aca>) and can be freely accessed⁶⁰.

Declarations

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Figures

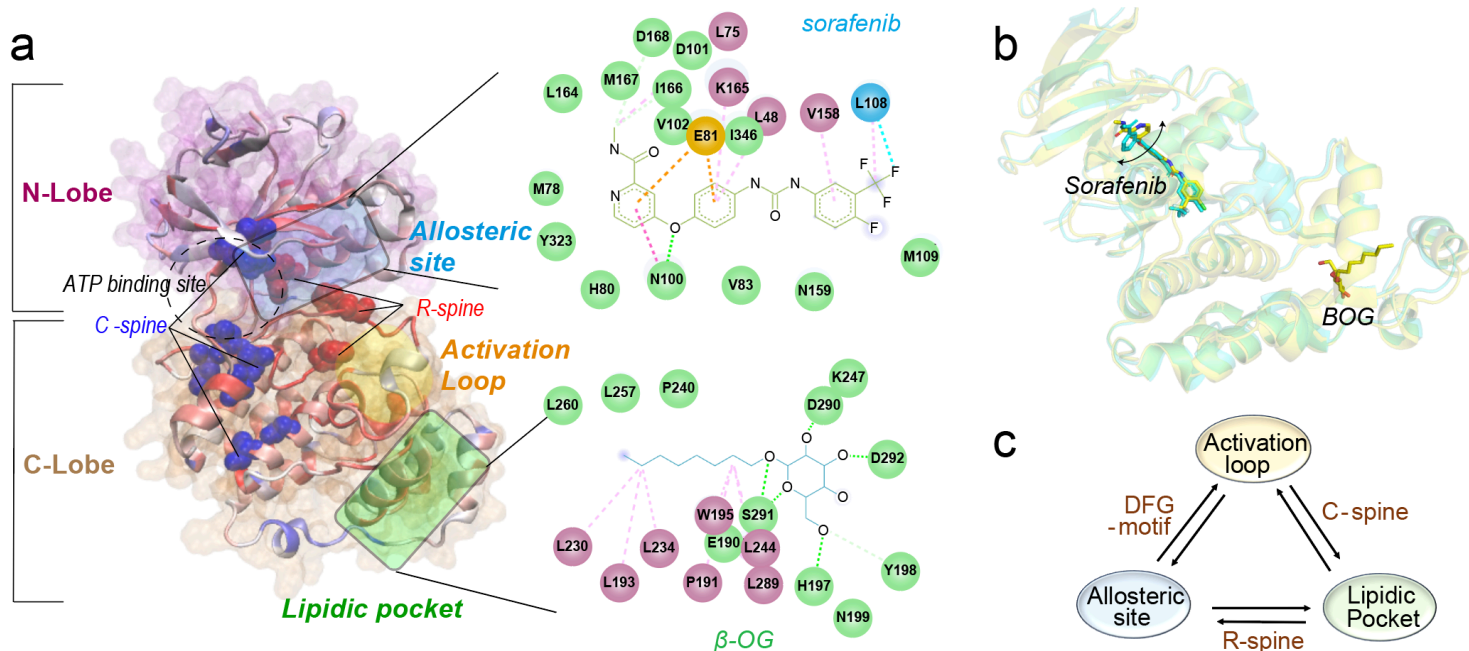


Figure 1

The interdomain communication to the lipid binding domain in p38 α in question.

a Structural representation of p38 α kinase, highlighting the interactions of ligands binding to the allosteric pocket (exemplified for sorafenib) and the lipid pocket (shown for β -OG), as produced using Discovery studio²⁸. Key structural features such as the activation loop, the catalytic C- spine, and the regulatory R-spine are highlighted. **b** Observed ring flip of the type-II inhibitor sorafenib between structures in the presence (semi-transparent yellow, PDB 3GCS) or absence (semi-transparent cyan, PDB 3HEG) of a lipid pocket binder (β -OG). **c** Sketch of the allosteric pathways between the active site, the adjacent, so-called allosteric pocket, and the lipid pocket in question.

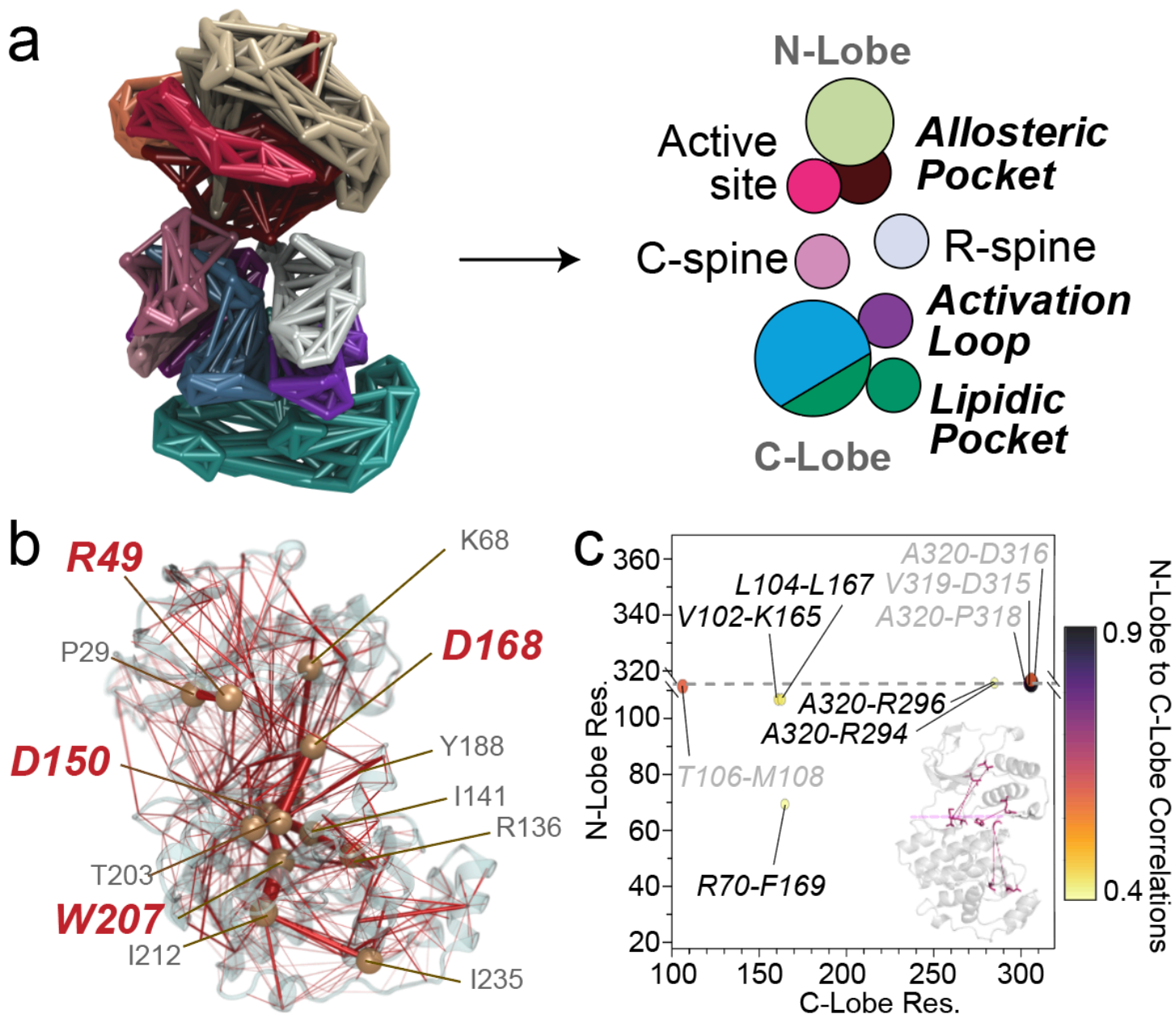


Figure 2

Dynamic network analysis of p38α kinase

a Clustering of the protein into 14 communities, largely reminiscent of the different structural elements of the kinase (right). **b** Residues with top betweenness centrality, i.e., the highest degree of information flow traversing them, also highlighting those network sites chosen for mutagenesis studies for network validation (red highlights). **c** Map of inter-residue dynamic-network correlations, specifically focusing on connections between N- and C-lobe residues. Pairs in black writing denote long-range correlations with a correlation coefficient above 0.3. Note that part of the C-terminal (320-360) lies in the N-Lobe, while the first transition from N- to C-lobe occurs at around residue 107. Correlations involving these covalent linkages are grayed out.

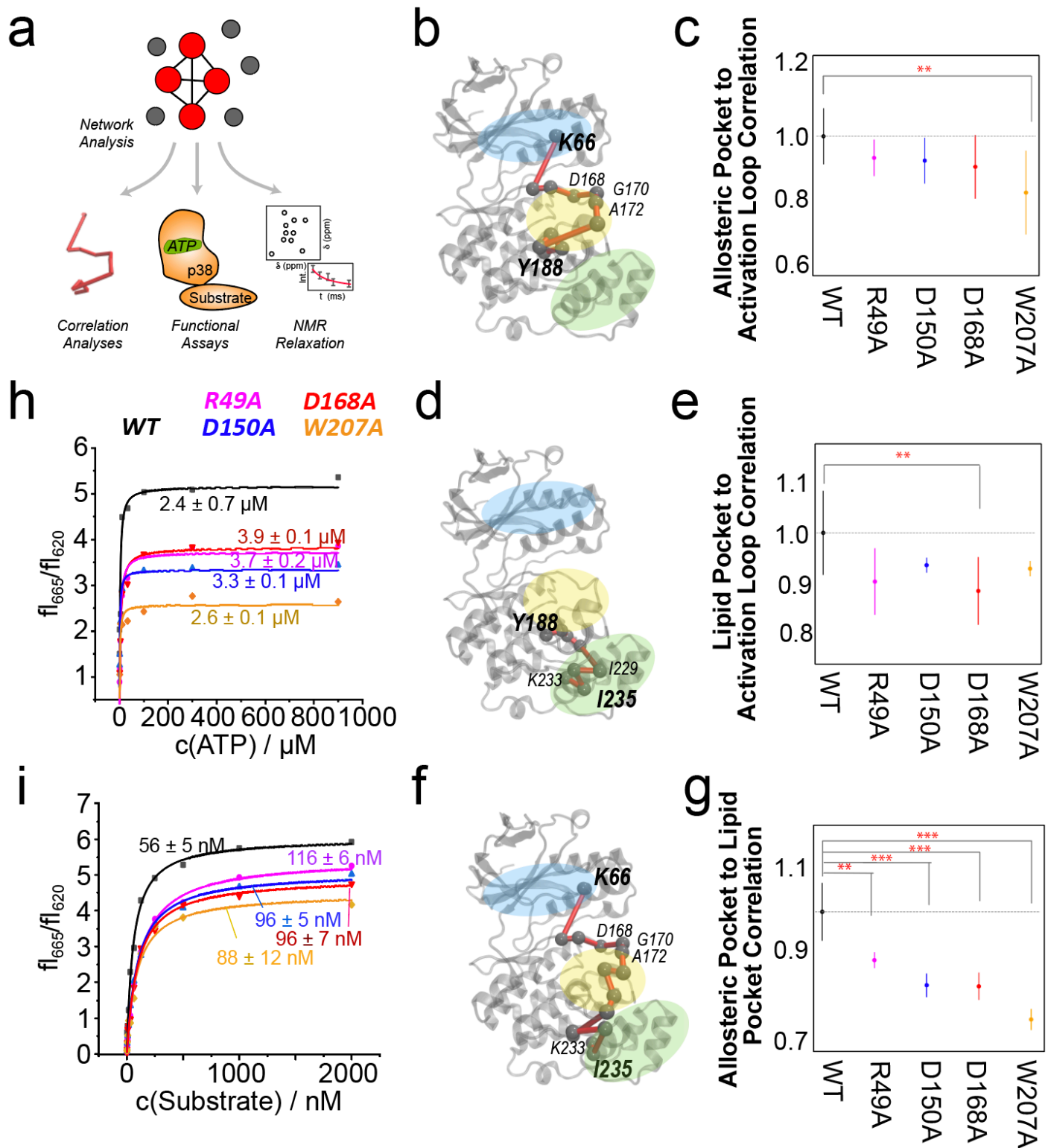


Figure 3

Comparison of shortest-path correlations and kinase activity upon dynamic-network mutation

a Strategy to verify the results of dynamic-network analyses, using in-silico mutagenesis, functional assays of mutants, and NMR chemical-shift perturbation and relaxation. **b** Residue-wise depiction of the shortest communication path from the allosteric pocket to the activation loop. **c** Total correlation of

residue pairs along the allosteric-pocket-to-activation-loop pathway, normalized to wild-type (WT = 1) and with statistical annotations ** for $p < 0.05$ and *** for $p < 0.01$. Statistical significance was assessed by non-parametric bootstrap tests, see the Methods for details. A marked reduction is observed in all mutants, with W207A exhibiting the most significant drop ($p = 0.039$). **d** Shortest path from the lipid pocket to the activation loop with key residues highlighted. **e** Correlation sums along the lipid-pocket-to-activation-loop pathway, normalized to WT, D168A highlighting a significant decrease ($p = 0.043$). **f** Residue pathway showing the shortest communication route from the allosteric pocket to the lipid pocket. **g** Total correlation for the allosteric-pocket-to-lipid-pocket pathway, normalized to WT, with all mutants showing statistically significant reduced correlations ($p = 0.017$ for R49A; $p = 0.0026$ for D150A; $p = 0.0022$ for D168A; $p = 0.005$ for W207A). **h** HTRF-based ATP titration assay comparing the integrity of ATP binding between wild-type and mutant p38 α kinase constructs across a concentration range of 0–1000 μ M ATP. The result of a Michaelis-Menten fit of the fluorescence ratio f_{665}/f_{620} obtained after a given time in the initial-rate regime serves as a quantitative proxy for ATP binding (best-fit K_M shown for each curve). **i** HTRF substrate titration assay quantifying substrate binding affinity of wild-type and mutant kinases using increasing concentrations of a GST-tagged activating transcription factor 2 (ATF2) substrate. K_M fit values are again shown for each curve. While wild-type shows high fidelity of substrate association, the data for the dynamic-network mutants consistently indicate slightly impaired ATP and substrate binding competency.

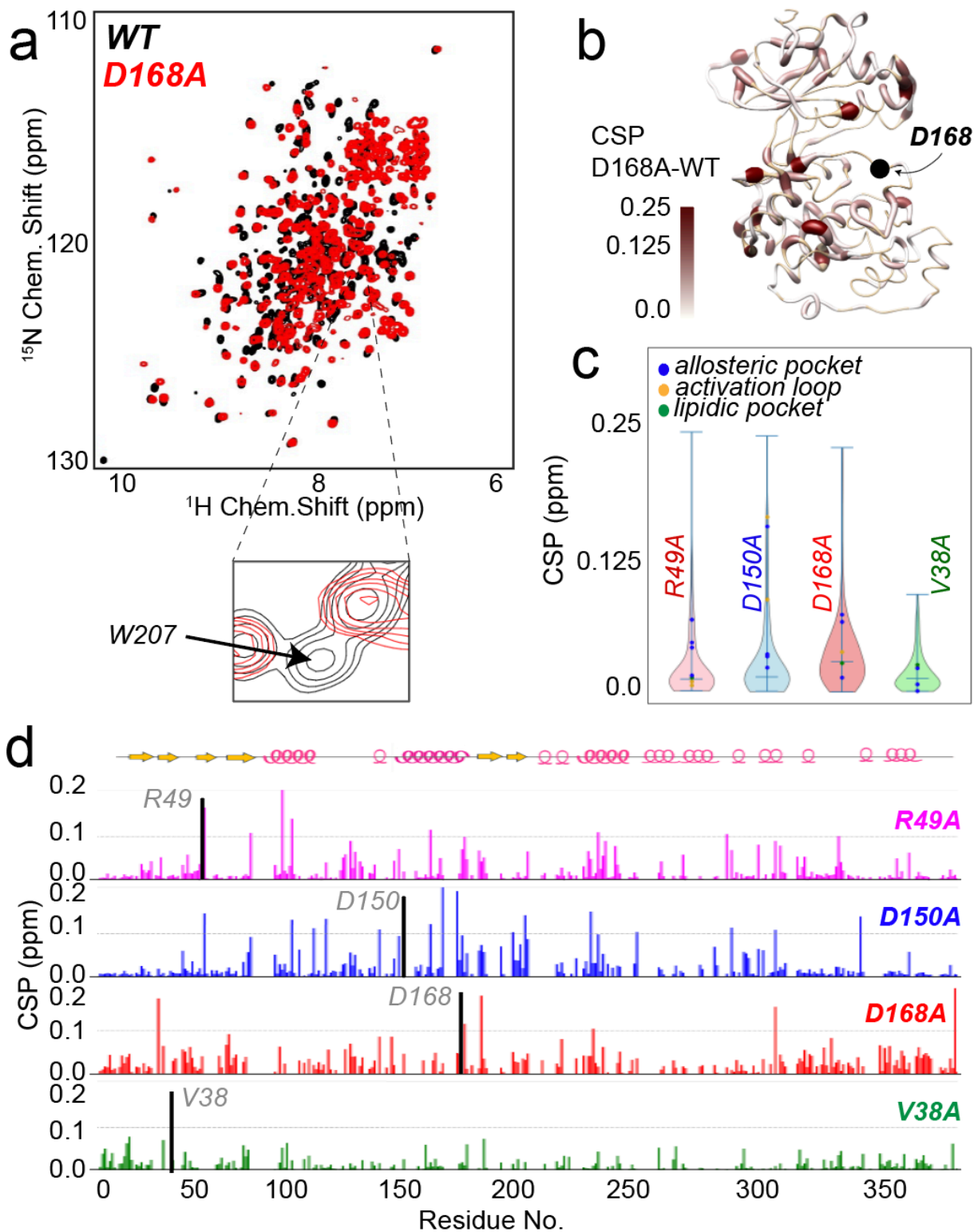


Figure 4

Chemical-shift perturbations (CSPs) induced upon mutation of key dynamic-network residues.

a Overlay of [^{15}N , ^1H] TROSY-HSQC spectra for wild-type (black) and mutant D168A (red). The mutation results in significant spectral reorganization, including disappearance of the W207 resonance in the lipid pocket. (Note that, in contrast to 2D spectrum shown here, the actual CSPs were read out from 3D

spectra.) **b** CSPs between wild-type and D168A mapped on the protein scaffold. Perturbations extend beyond the local environment of the mutation, indicating widespread conformational remodeling throughout the protein. CSPs for the other mutants are shown on the protein structure in Fig. S17. **c** Violin plots summarizing CSP distributions across network mutants (R49A, D150A, D168A) and the control variant (V38A). Blue and red dots mark residues from the lipid pocket and active site, respectively. **d** Quantitative comparison of CSPs as a function of sequence. Dynamic network mutants display various elevated perturbation even at structurally remote positions, whereas the control mutant V38A induces minimal CSPs outside its immediate vicinity.

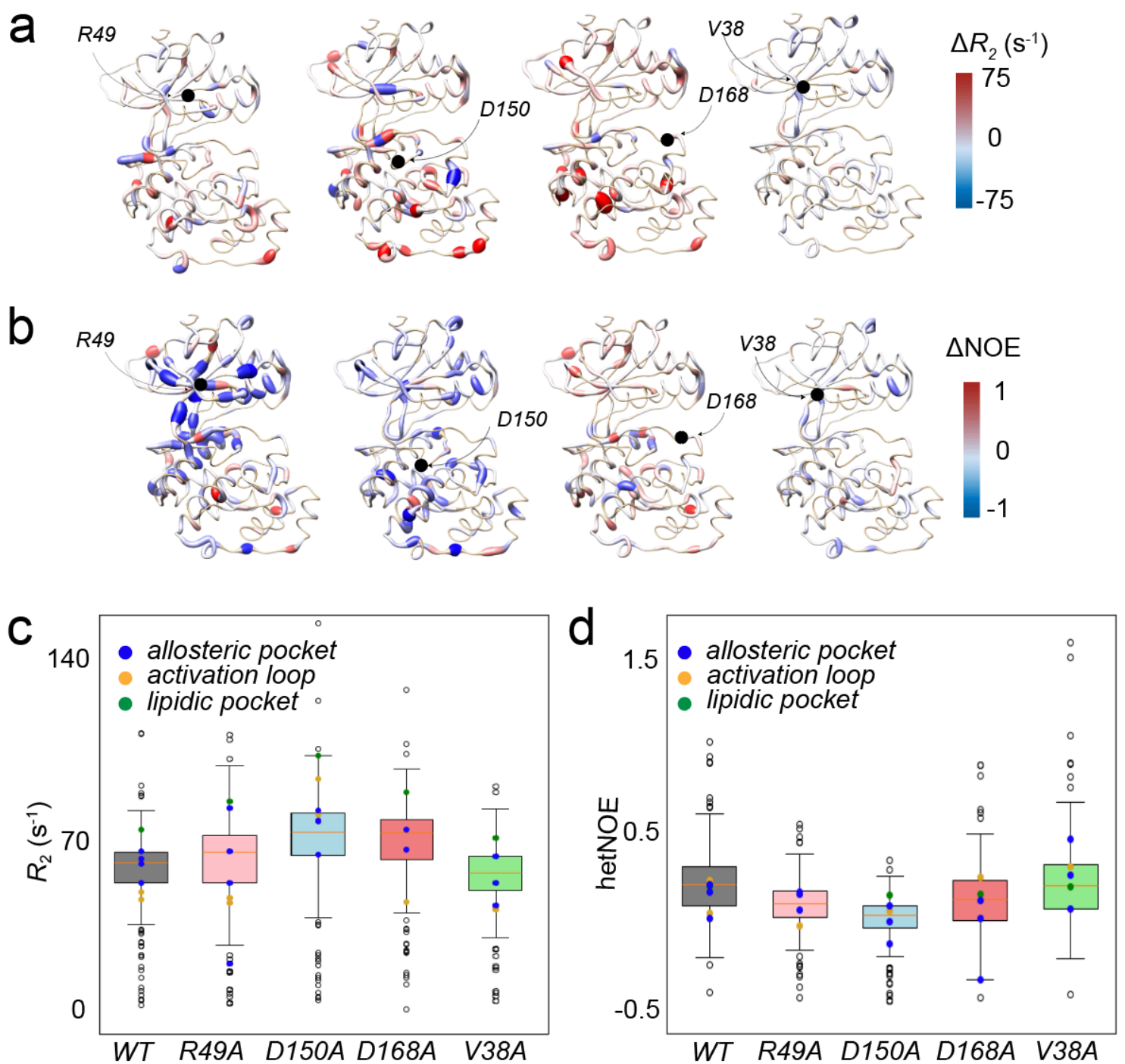


Figure 5

Changes in p38 α fast-timescale dynamics, as seen by heteronuclear NOEs and R_2 relaxation

a Structural mapping of ΔR_2 (mutant – WT) values onto the three-dimensional structure of p38 α kinase. Red regions indicate residues with R_2 higher in the mutants, predominantly localized to the catalytic core and distal C-lobe, supporting widespread conformational exchange. **b** Structural visualization of ΔhetNOE values. Blue regions highlight residues with reduced NOE values in the mutants, indicative of enhanced backbone flexibility. **c** Box plot distribution of R_2 values for wild-type, dynamic network mutants (R49A, D168A, D150A), and the negative control (V38A). Active-site residues (red) and lipid pocket residues (blue) are specifically highlighted by colored spheres. All dynamic-network mutants exhibit significantly elevated R_2 values compared to the wild-type and control, consistent with a global enhancement in slow conformational exchange. **d** Box plots of hetNOE values across the same variants. Dynamic network mutants show a marked reduction in NOE values, reflecting increased local flexibility, including at functionally critical sites. By contrast, the control mutant V38A shows limited perturbation, confirming an impact on the p38 dynamic network to be absent.

Supplementary Files

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