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Cell phenotypes can be predicted from propensities of protein conformations

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Abstract

Proteins exist as dynamic conformational ensembles. Here we suggest that the propensities of the conformations can be predictors of cell function. The conformational states that the molecules preferentially visit can be viewed as phenotypic determinants, and their mutations work by altering the relative propensities, thus the cell phenotype. Our examples include (i) inactive state variants harboring cancer driver mutations that present active state-like conformational features, as in K-Ras4B^{G12V} compared to other K-Ras4B^{G12X} mutations; (ii) mutants of the same protein presenting vastly different phenotypic and clinical profiles: cancer and neurodevelopmental disorders; and (iii), alterations in the occupancies of the conformational (sub)states influencing enzyme reactivity. Thus, protein conformational propensities can determine cell fate. They can also suggest the allosteric drugs efficiency.

Keywords

Conformational ensembles; cell fate; occupancy; cancer; RASopathies; neurodevelopmental disorders

Introduction

Recently, we called for a revision of the decades-old *sequence-structure-function* paradigm and replacing it by a modern *sequence-conformational ensemble-function* paradigm. We argued that such a revised outlook more accurately encapsulates the linkage between

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Declaration of interests
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sequence and function, and especially, is required by the updated dynamic energy landscape [1].

Proteins are not rigid, neither *in vitro* nor in the cell. They fluctuate. They sample an ensemble of states, triggering conformational heterogeneity. The states are visited with different frequencies. High energy states are visited rarely, low energy states frequently. The smaller conformational fluctuations of stable folded proteins sample states around the average structure yielding more homogeneous ensembles. Unstable, or intrinsically disordered proteins that interconvert between a broad range of conformations with relatively low barriers between them, can present high conformational heterogeneity [2]. Clustering of the conformations [3], or describing the ensembles by their associated thermodynamic weights [4], can describe the proteins' structural heterogeneity. The input can come from biophysical experiments, such as crystallography, single-molecule fluorescence (or Förster) resonance energy transfer (smFRET) experiments that measure distances at the 1-10 nM range, single-particle cryo-electron microscopy (cryo-EM), which can capture conformational transitions by observing single conformer in each condition, cryo-X-ray structural snapshots from multiple related structures [5], quantitative backbone structural dynamics by solution nuclear magnetic resonance (NMR) spectroscopy [6], computational models obtained from explicit solvent long time scales molecular dynamics (MD) simulations [7], and especially, MD/NMR combination [8,9]. As we and others have shown, this combination is powerful in clarifying the mechanism of autoinhibition [10] and in showing how oncogenic mutations can alter the dynamic nature of the K-Ras/calmodulin complex [8]. The frequencies of visits of close conformations can provide the propensities of the conformational states. Here we suggest that propensities of different states can express distinct protein and cell phenotypes (Figure 1 depicts the concept). High propensity of active state-like conformations of a mutational variant obtained from an inactive structure can point to a strong mutation with a consequent high chance of a transformed cell phenotype [7]. In a protein that can act in two vastly different phenotypes, cancer and neurodevelopmental disorders (NDDs), a stronger mutation with an active state profile may point to cancer. Our premise is that a strong mutation elicits strong signaling ([7] and references therein). However, on their own, single proteins, and single mutations do not determine cell phenotype. Homeostatic mechanisms, the types and locations of additional mutations, expression levels, cell type, timing, and chromatin structure, all play key roles [11,12].

The heterogeneity of conformational ensembles is important since distinct conformations may define functional specificities. Nuclear receptors that act as transcription factors through ligand-linked conformational changes provide one example [13]. Substitutions at a key position resulted in altered ligand specificities for multiple ligands through distinct favored conformations. The conformers' populations are specifically allosterically shifted by the different mutations, altering their propensities. Atomistic MD simulations with enhanced sampling captured the resulting allosteric population shifts in the ensembles and correlated them with ligand-specific transcriptional activation. Mechanistically, pairwise cooperativities, where binding at the 'receiving end' is modulated solely by binding at the 'initiating end' is not an accurate representation [14]. Multiple steps are involved along the favored allosteric propagation pathways [15]. These binding events

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reflect multiple conformational selection steps including collectively modulated additional binding events involving concurrent regulation in the cell. Resolving the local energetic conflicts experienced during binding induces allosteric effects [16,17]. The dynamically interchanging conformations transform the propensities of the conformers in the ensemble. Allosteric population shifts in the ensembles promoted by covalent events are not limited to mutations, but also include posttranslational modifications, which can also alter function [18]. Finally, chaperones, the nanoscale molecular machines that recognize incompletely or incorrectly folded protein clients, arrest and assist in refolding them are a remarkable example of the link between changes in conformations and phenotypes [19,20].

We define propensities as the number of *active molecules* and suggest that determination of this number is significant for several reasons. Not only does it help in dissecting the relationships between the ensemble and protein (or RNA [21]) function. As we discuss below, it can help in determining, and predicting, the phenotype associated with the higher (lower) propensities. Within this framework, we recall the community effort of predicting protein function from its sequence [22] or structural features [23,24]. The relationship, and significance of heterogeneous conformational ensembles for function has increasingly been considered (e.g., [25–32]).

Here we underscore the significance and feasibility of predicting function from conformational propensities. In line with this, the effectiveness of allosteric drugs can be measured, and thus predicted, by the changes in propensities of the relevant conformations that they elicit.

Definitions and model overview

First, what is a “protein state” and what is a “protein conformation”? Both terms relate to protein ensembles with structures that are interconverting along time and conditions. Protein disorder is commonly referred to as the “disordered state”. Yet, when relating to protein shapes within an ensemble of “ordered” proteins, “state” and “conformation” are often used interchangeably. Here our definitions are based on the free energy landscape, and in line with the multiple interconverting molecules in the disordered state. In our definition, multiple shapes [(sub)states] located around the bottom of a minimum with minor differences in energy, which are separated by low kinetic barriers, constitute a conformational state (Figure 2), whereas conformational states are separated by higher barriers. An allosteric mutation can alter the relative stabilities of the states, and of the substates that they embody. Since a conformational state is described by bonded and nonbonded intramolecular interactions, different conformational states of the molecules would be impacted in different ways.

The propensities of the conformational states relate to their relative energies. Apart from repressors, under physiological conditions, the number of molecules populating the active conformational state is lower than that in the inactive states. The enhanced sampling of a variant harboring a driver mutation temporally increases the number of molecules with active conformational state features; in enzymes, making it predisposed for catalysis, in small GTPases, making it predisposed for effector activation. The number of active

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molecules decides signaling strength, thus cell function. Effective allosteric drugs reduce the number active molecules. Cellular processes depend on interactions, which are mediated by intermolecular contacts. In turn, these depend on binding affinity and the number of available, binding-competent conformations, that is, the propensities of the relevant conformational states [33–35]. Mapped occupancies of active conformational states can help foretell cell fate by harnessing the free energy landscapes and conformational dynamics.

Prediction of functional cell states and cell fates

The functions of a large number of proteins remain unknown, lacking experimental and manual annotation [36]. Computational methods have been exploiting the data, developing, and applying functional prediction algorithms most commonly from sequences, using global and local (multiple) sequence alignments searching for, and interrogating homologs. 'Function association matrices' have also been used to annotate even remote homologs, and recently, sequence-based protein language models have been developed as well. Experimentally determined structural information was not used as much for protein function prediction largely due to its paucity as compared to known sequences. AlphaFold2 [37] and other recent machine language-based structural prediction methods, such as the fast Meta AI ESMFold [38], which are filling in the 'dark matter' of the protein universe, will likely alter this landscape. AlphaFold2 has been used in large-scale prediction of protein functions through heterogeneous feature fusion [36]. Protein 3D structural data are advantageous since structure is better conserved than sequence. Amino acid contact maps have been successfully exploited as well [24]. However, none of these directly capture conformational mechanisms like allostery, which are rooted in ensembles, and controlled by their dynamic distributions [39]. Allostery and signaling are properties of populations, whereas these methods predict structural snapshots ranked by a scoring function. To obtain conformational data they can be applied multiple times with different parameters; however, they will still not provide propensities which are influenced by kinetic barrier heights that separate the states.

The quintessential nature of predictions derived from propensities of conformational states differ from predictions of function from sequences in a number of ways. First, they provide a conformational profile of a protein, whose function is already known. Second, that conformational profile, which epitomizes conformations that have been preferentially visited, and their associated propensities, can be viewed as key phenotypic determinants of the cell. Conformational profiles provide a phenotypic resolution which neither sequences nor 3D structures are able to attain. Beyond the known function of the protein, through their propensities, they may describe protein action across time. Since they point to the number of molecules that exist in the active conformational state, *this may allow us to consider questions such as, what is the extent of the protein activity.* Further, if the protein can contribute to multiple diseases, as in the case of phosphoinositide 3-kinase (PI3K) lipid kinase and Src homology 2 (SH2) domain-containing tyrosine phosphatase-2 (SHP2) phosphatase acting in cancer and NDDs, the propensities may suggest which of these diseases is the one that is more likely to emerge from certain mutations or their combinations. The extent of protein activity and the related disease can provide a signature of the cell phenotype. *Taken together, this posits the methodological challenge of how to*

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obtain propensities, which could integrate with algorithms for prediction of function, for a more complete and relevant functional description.

Methods for observing and predicting propensities

Acquiring temporal, high resolution atomic-level ensemble models that accurately represent conformational heterogeneity is vital to understanding of how proteins and cells work [40]. Several experimental techniques, including time-resolved X-ray crystallography and cryo-EM [41–45], and spectroscopic methods [46,47], can trap conformations. However, their usefulness may fall short. The conformations need to be generated which is technically complex [47]. Modeling conformational variability at ambient temperatures directly from X-ray diffraction data has been challenging [40]. Recent advances in refinement of multiconformer ensemble models from multitemperature X-ray diffraction data have made the collection of high-quality heterogeneous diffraction data possible. Integrating automated sampling with manual refinement of diffraction datasets at different temperatures (313 to 363 K) resulted in multiconformer models, including their relative occupancies, and interconnections.

High resolution atomistic, long time scales conformational sampling by MD simulations, or Monte Carlo, can usefully predict and capture temporal propensities, as well as the kinetic barriers for conformational switching [41,48–50]. However, high barriers require lengthy time scales for exhaustive sampling of the conformational space, unless *a priori* constrained by experimental measurements [51,52]. Emerging integrated machine learning–MD methods may help in large scale systems, as well as enhanced sampling techniques [31,53–56]. MD simulations can describe molecular mechanisms by exploiting empirical potentials, which can be improved by machine learning approaches. As we discuss below, in our hands, conformational behavior of protein variants observed in explicit solvent MD simulations could distinguish between disease outcomes even though the differences may be moderate, pointing to the challenges in sampling and the requirements for longer time scales, or emerging superior sampling approaches, as the above methods aim to accomplish.

The K-Ras4B mutational variants, cancer and NDDs, and enzyme examples

Sampling of conformational space *in vivo* is influenced by the cellular environment, which is challenging to capture. However, conformational studies in solution may still unravel puzzling *in vivo* consequences. We surmised that the differences in the propensities of conformational states of K-Ras4B variants could be a key factor in the differences in the rates of GTP hydrolysis, nucleotide exchange rates, and selectivity for plasma membrane phospholipids, thus in oncogenic aggressiveness [57]. *KRAS* is the most highly mutated *RAS* gene in human cancer, causing various cancer phenotypes in different organs (Figure 3a). Among the oncogenic mutations at the active site, G12 is the most highly populated, followed by G13 and Q61. To test our conjecture, we considered two strong K-Ras4B mutations, K-Ras4B^{G12V} and K-Ras4B^{G12D} (Figure 3b). The first is the strongest K-Ras4B mutation. It is the most aggressive and chemotherapy resistant. The second is the most frequent and the key mutation in pancreatic adenocarcinoma. Both single residue substitutions decelerate intrinsic and catalyzed GTP hydrolysis, retaining the protein in a

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constitutively active state, promoting strong cell proliferation. The crystal structures of both mutants are similar, providing no clue to their differential oncogenic behavior. To discern why the difference in the aggressiveness of the mutations, we collected NMR data and performed explicit solvent MD simulations of the active and inactive conformations, aiming to explore its conformational sampling, thus occupancies [7]. We observed that the two mutational variants exhibit distinct conformational dynamics in their GDP-bound states, which in the wild-type protein is the inactive state. As expected from constitutive activating mutations, even in this state, they often visit active-like conformations, resembling that of the active GTP-bound state. However, their conformational profiles differed. K-Ras4B^{G12V} visited active-like conformations much more frequently than the K-Ras4B^{G12D} did. Inspection of the conformational details explained the reason: The fluctuating interactions of the aliphatic sidechain of the Val12 with the Switch II region of K-Ras4B^{G12V}-GDP differ from those of Asp12 in GDP-bound state, which is not observed in the crystal structure, where the contacts in the crystalline state constrain the dynamics by stabilizing the Switch I region of the protein. Thus, the differences in the G12 mutants' conformations can be explained by the contacts with the Switch II region which stabilize the active-like state, resolving the differential oncogenic aggressiveness conundrum.

In the second example, same protein single mutation variants can encode the vastly different phenotypic and clinical profiles of cancer and NDDs, leading us to query *how* [12,58]? To resolve this mystery, we selected key cancer mutations (E76K and D61Y/V) in SHP2 phosphatase (Figure 4) which regulates MAPK, and compared them with Noonan syndrome (NS, a RASopathy, a group of NDDs [59–62]) mutations of the same residues (E76D and D61G). We observed that the cancer drivers could induce a shift in the SHP2 ensemble toward the active state. As to the RASopathy mutations, they presented only limited conformational transitions, thus are less likely to promote proliferation. Thus, again, as the K-Ras4B variants above, conformational behavior and propensities of occupying active states-like conformations can predict not only mutation strength and clinical relevance, but are also capable in distinguishing, thus forecasting, disease outcome.

Our third example [5] involves substituting a tyrosine in the ketosteroid isomerase (KSI) enzyme with phenylalanine, changing the bound and the reactive ensemble, but with the ninefold rate decrease arising from a weakened hydrogen bond, thus conformational dynamics, which impacts protein function and enzyme catalysis. Finally, conformational variants can influence aggregation [63,64].

Conclusions

Structures captured in crystals cannot point to the cell phenotype. Despite their vast merit, crystal structures capture static structural snapshots that are populated under the conditions of the experiments. As to single cell transcriptomics, if followed and measured at different cell states and time frames, it can point to the cell's phenotype [65], including our example when differentiating between cancer cells and NDDS, where we observed different levels at different cell states [66].

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Here we suggested that propensities of conformational states obtained by detailed MD simulations can do this too. Each method has its pluses and drawbacks. Single cell transcriptomics require time, laboratory set-up, tools and are costly. Simulations are handicapped by hurdles too. Still, they are more affordable and with recent developments in machine language/AI assistance, they get faster, increasingly accomplishing their aims [67].

Here our linchpin theory is that propensities of protein conformational states may powerfully predict cell phenotypes [1], and we believe that constructions of such software tools are feasible, albeit challenging. Data of mutation outcomes are available, and MD simulations are becoming increasingly routine. We further note that since effective allosteric drugs can also bias ensembles, their action may be captured as well. We offer that eventually, prediction of cell function from conformational ensembles can extend prediction of protein function and is worth considering.

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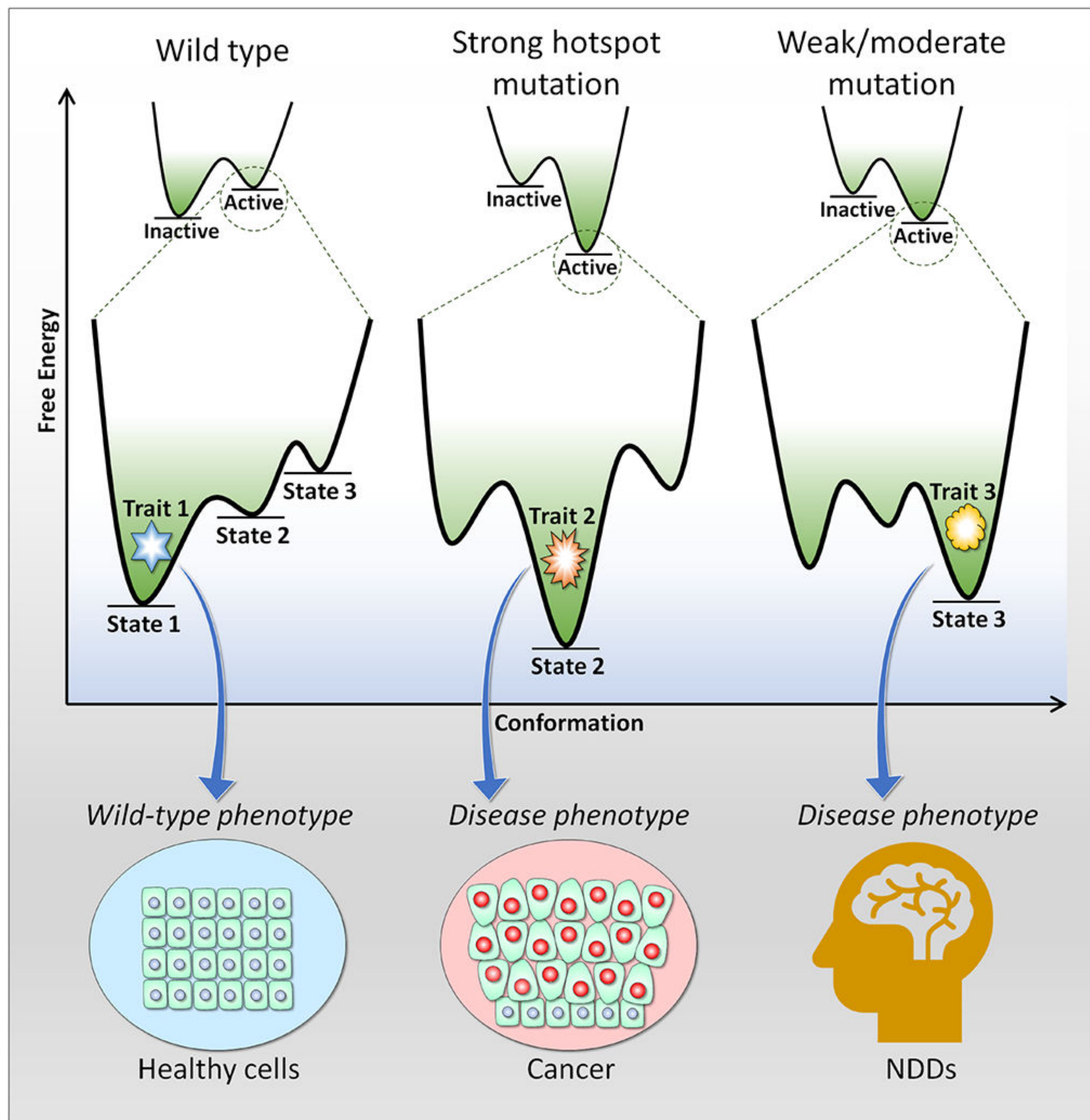

Figure 1.
Free energy profiles of different conformational states expressing different phenotypic cell traits. For example, strong hotspot mutations alter the conformational states of the protein, generating a cancer disease phenotype, while weak/moderate mutations express a NDD disease phenotype.

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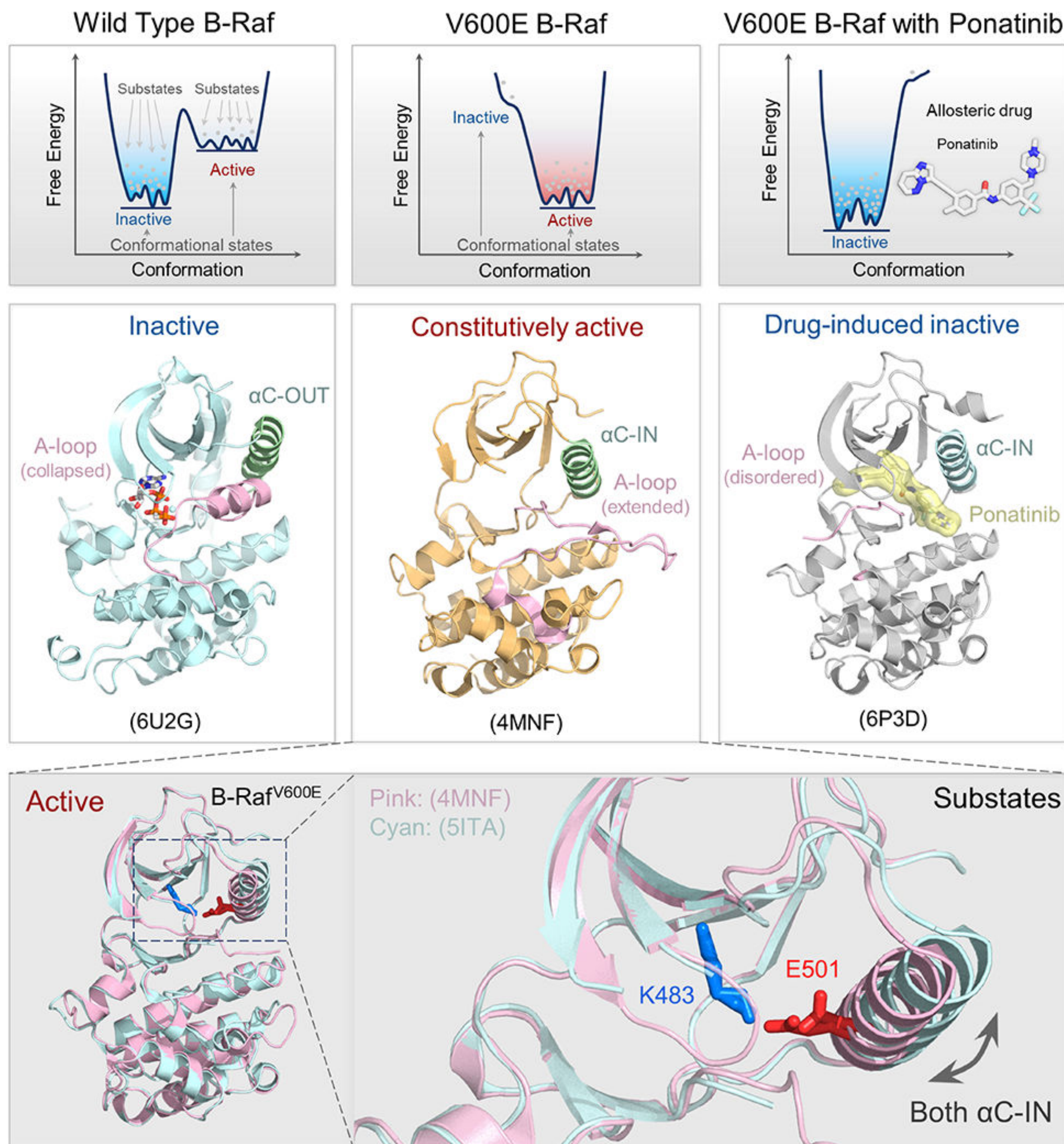

Figure 2.

Illustration of the conformational states and substates of wild-type B-Raf, B-Raf^{V600E}, and allosterically inhibited B-Raf^{V600E}. Dynamic free energy landscapes and structures of B-Raf, B-Raf^{V600E}, and ponatinib bound B-Raf^{V600E} (*top panels*). The free energy shows the distribution and population of protein states. Each well represents a distinct conformational state (active or inactive), with the depth of the well indicating the stability of the state. Within each well, multiple dots indicate the population of substates that a protein can adopt. The barriers between these substates are lower than the barriers separating

different conformational states. In the left column, wide type B-Raf primarily adopts an inactive state; in the middle column, B-Raf^{V600E} primarily shows an active state; in the right column, B-Raf^{V600E} bound to an allosteric drug ponatinib which disrupts its ability to phosphorylate, resulting in population shift towards the inactive state. Their predominant structures are shown below their respective free energy landscape plots (*middle panels*). Note that the V600E mutation shifts the relative stability of the protein from favoring the inactive state to the active state. We annotate the PDB code in each panel for reference. Within each conformational state, the protein can adopt a range of substates (*bottom panels*). For example, active B-Raf^{V600E} shows structural variations in the position of the αC helix. The important salt bridge between K483-E501 represents a key feature of the active protein kinase conformation.

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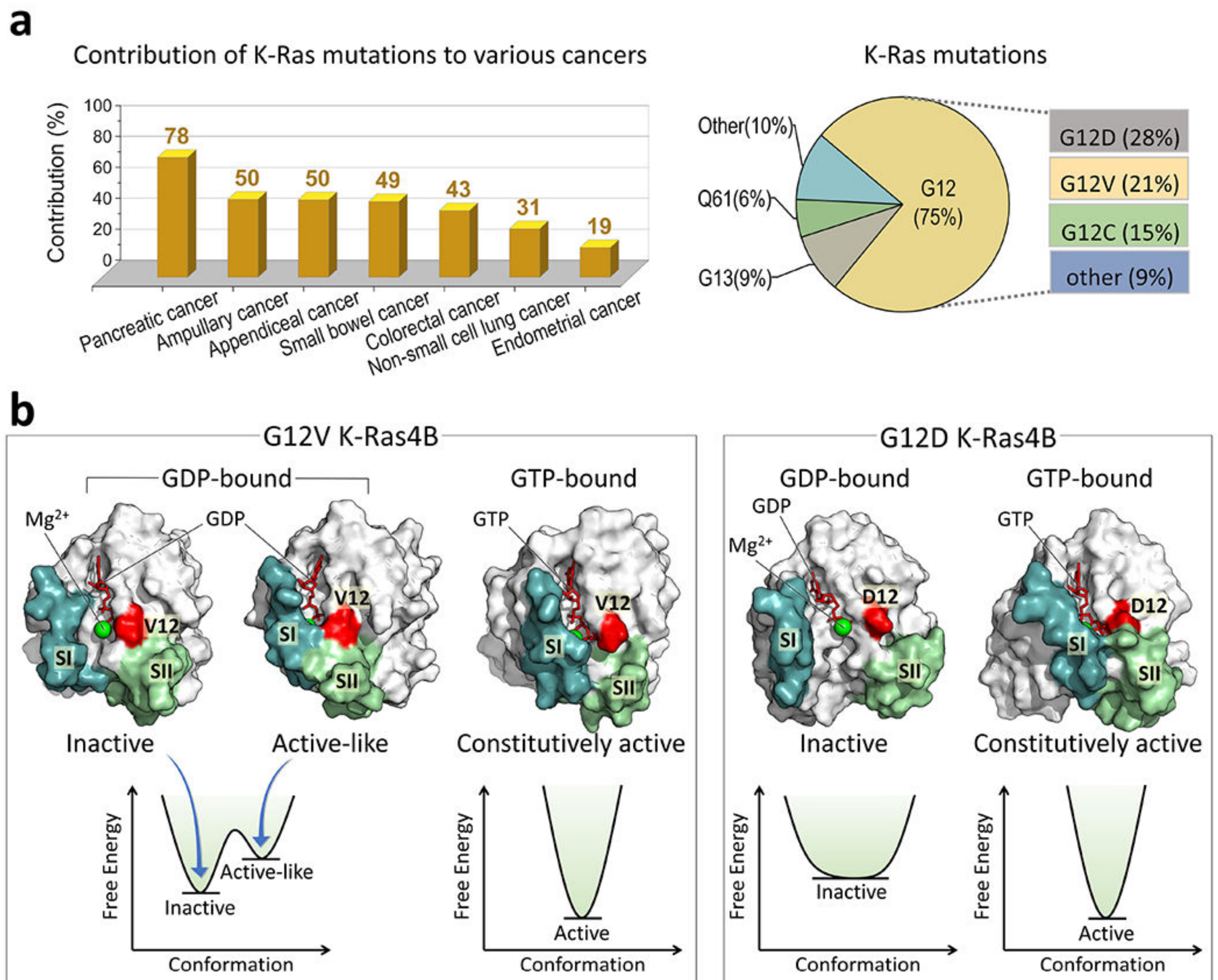

Figure 3.

(a) K-Ras mutations can drive cancers. Analysis of the AACR cancer cohort (GENIE Cohort v13.1-public) reveals that K-Ras mutations have high associations with pancreatic, ampullary, appendiceal, small bowel, colorectal, non-small cell lung, and endometrial cancers. The most prevalent K-Ras mutation sites are at codon 12, 13, and 61, with G12 mutations being the most frequent, accounting for ~75% of all K-Ras mutations. Among these mutations, G12D is the most common (~28%), followed by G12V (~23%) and G12C (~15%). (b) K-Ras4B encompasses two critical regions, Switch I (SI) and Switch II (SII). In the inactive GDP-bound state, the two regions are separated (referred as the open SI-SII conformation), which prevents the K-Ras interactions with its effectors. In the active GTP-bound state, these two regions come into closer proximity (referred as the closed SI-SII conformation), favorable for the effector binding. Oncogenic mutations in K-Ras4B can shift the equilibrium towards the active state. The G12V and G12D mutants have a high population in the GTP-bound state with the closed SI-SII conformation, and a low population in the GDP-bound state with the open SI-SII conformation. The G12V

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mutation induces a more potent activation of K-Ras compared to the G12D mutation. This difference in activation strength may arise from the distinct dynamic ensembles of the two mutants. In the GDP-bound state, K-Ras carrying the most aggressive G12V mutation visits frequently the active-like conformation, featuring instances of SI and SII separation. This further amplifies the likelihood of downstream effectors binding, thereby intensifying the signal transduction.

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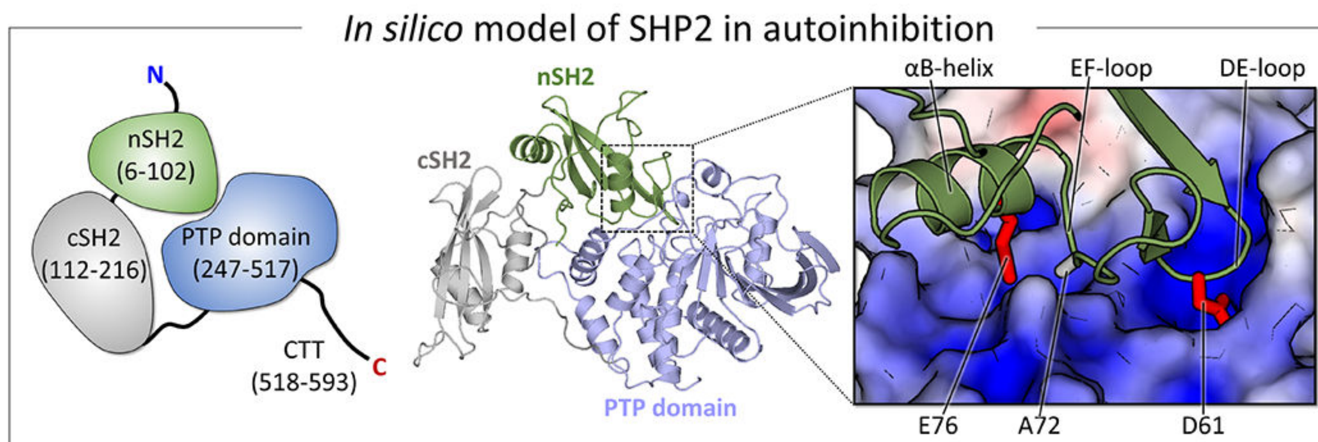

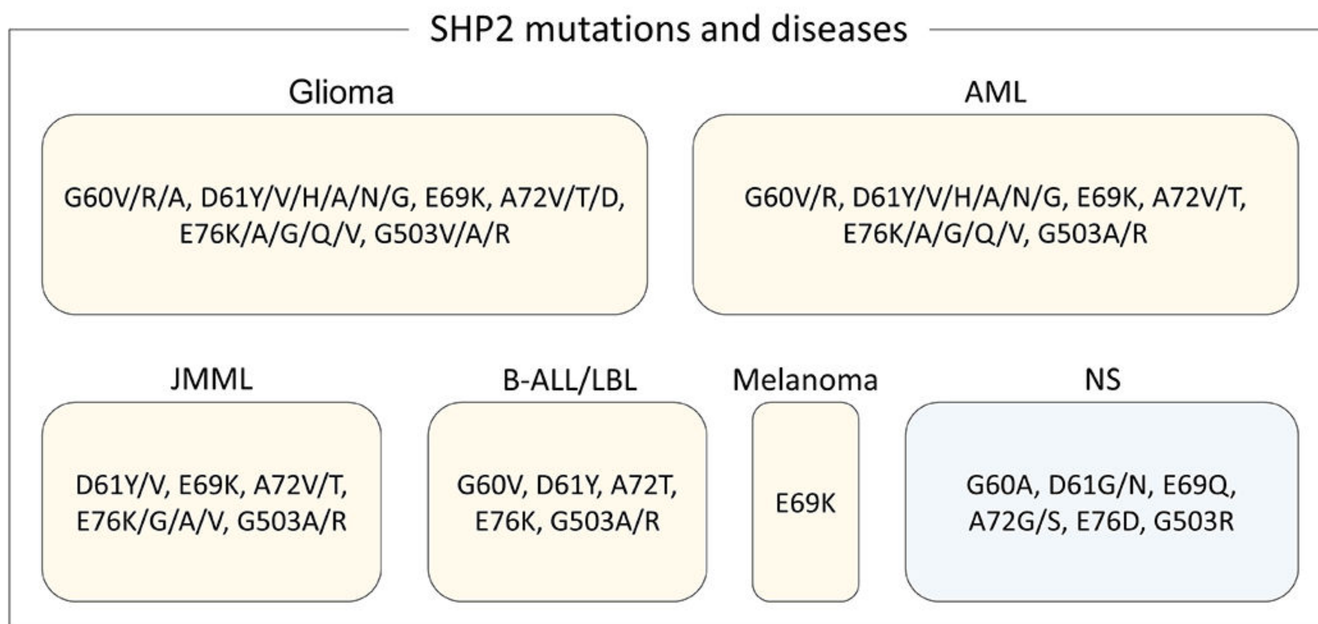

Figure 4.
SHP2, a tyrosine phosphatase, consists of the nSH2, cSH2, and PTP domains. In its autoinhibited state, the nSH2 domain tightly binds to the catalytic cleft of the PTP domain, with D61 and E76/A72 orienting towards the active and allosteric sites, respectively. Analysis of the TCGA and GENIE databases identifies hotspot mutation sites as G60, D61, E69, A72, and E76 in the nSH2 domain, and G503 in the PTP domain. Mutations at these sites can drive various cancers, including juvenile myelomonocytic leukemia (JMML), acute myeloid leukemia (AML), B lymphoblastic leukemia/lymphoma (B-ALL/LBL), glioma, and melanoma, as well as neurodevelopmental disorders (NDD) like Noonan syndrome (NS). Interestingly, some mutations are shared between cancer and NS, but they are significantly rare in cancer cases. Some mutations at the hotspot mutation sites for several representative cancers and NS are listed.