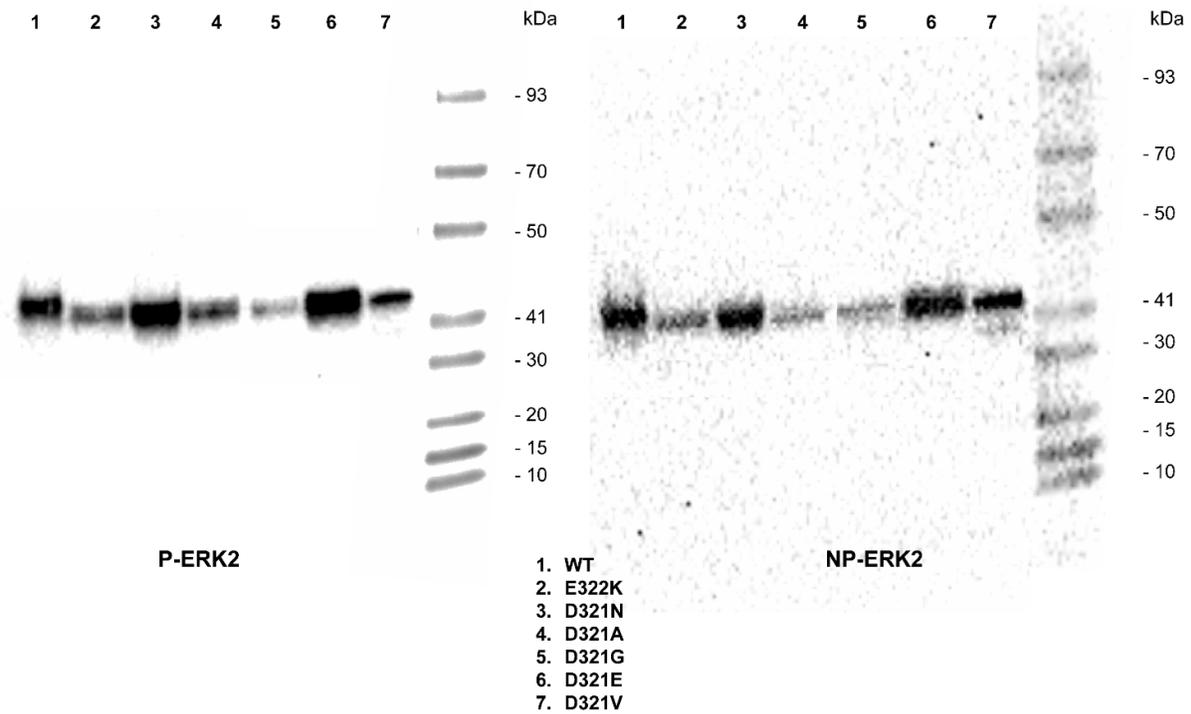


*Supplementary Material*

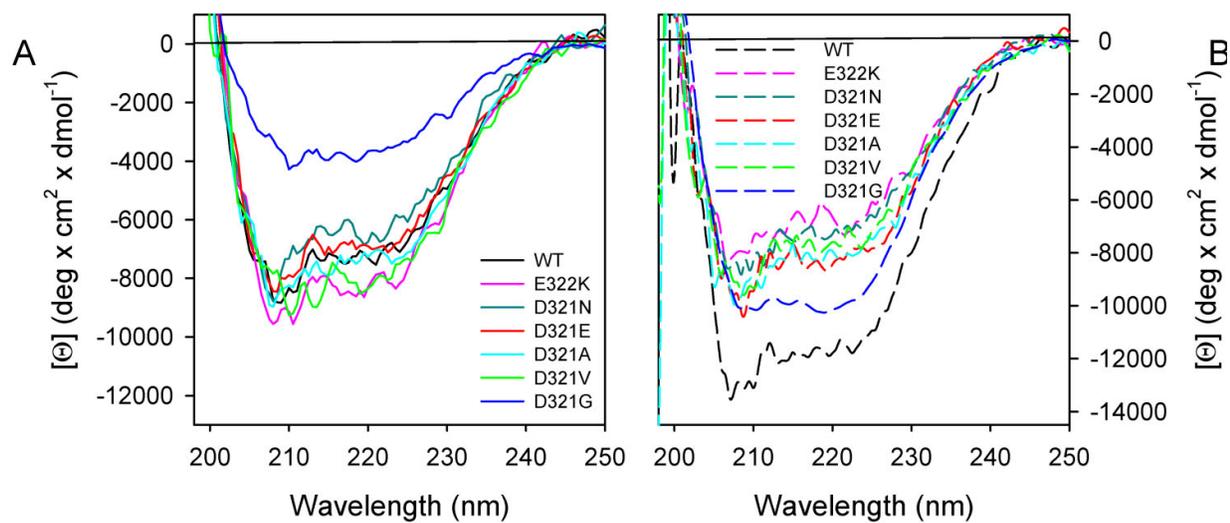
**Table S1. List and details of the analyzed ERK2 variants selected from COSMIC database**

	<b>Solvent Accessibility</b>	<b>Nucleotide substitution</b>	<b>Pathogenicity</b>	<b>Cancer Tissue</b>
<b>D321A</b>	<b>Exposed</b>	<b>A &gt; C</b>	<b>0.98</b>	<b>Liver</b>
<b>D321E</b>	<b>Exposed</b>	<b>C &gt; G</b>	<b>0.85</b>	<b>Urinary tract</b>
<b>D321G</b>	<b>Exposed</b>	<b>A &gt; G</b>	<b>0.97</b>	<b>Lung</b>
<b>D321V</b>	<b>Exposed</b>	<b>A &gt; T</b>	<b>0.98</b>	<b>Skin</b>
<b>D321N</b>	<b>Exposed</b>	<b>G &gt; A</b>	<b>0.98</b>	<b>Upper aerodigestive tract</b>
<b>E322K</b>	<b>Exposed</b>	<b>G &gt; A</b>	<b>0.98</b>	<b>Liver</b>

The table reports the amino acid substitution (in red), the solvent accessibility, the information about nucleotide substitution, pathogenicity and cancer tissues that are reported in the COSMIC database [24]. The pathogenicity scores for individual mutations from FATHMM-MKL are in the form of a single p-value, ranging from 0 to 1. Scores above 0.5 are deleterious, but to highlight the most significant data in COSMIC, only scores  $\geq 0.7$  are classified as 'Pathogenic'. Mutations are classed as 'Neutral' if the score is  $\leq 0.5$ .



**Figure S1.** Western blot analysis of ERK2 wild-type and variants. The phosphorylation of ERK2 wild-type and variants were analyzed by Western blotting using the monoclonal antibody raised against the doubly phosphorylated ERK2 (anti-Phospho-ERK1/ERK2-Thr185, Tyr187) and the antibody anti-ERK1/ERK2, as described in Materials and Methods. NP-ERK2: non-phosphorylated ERK2; P-ERK2: phosphorylated ERK2.

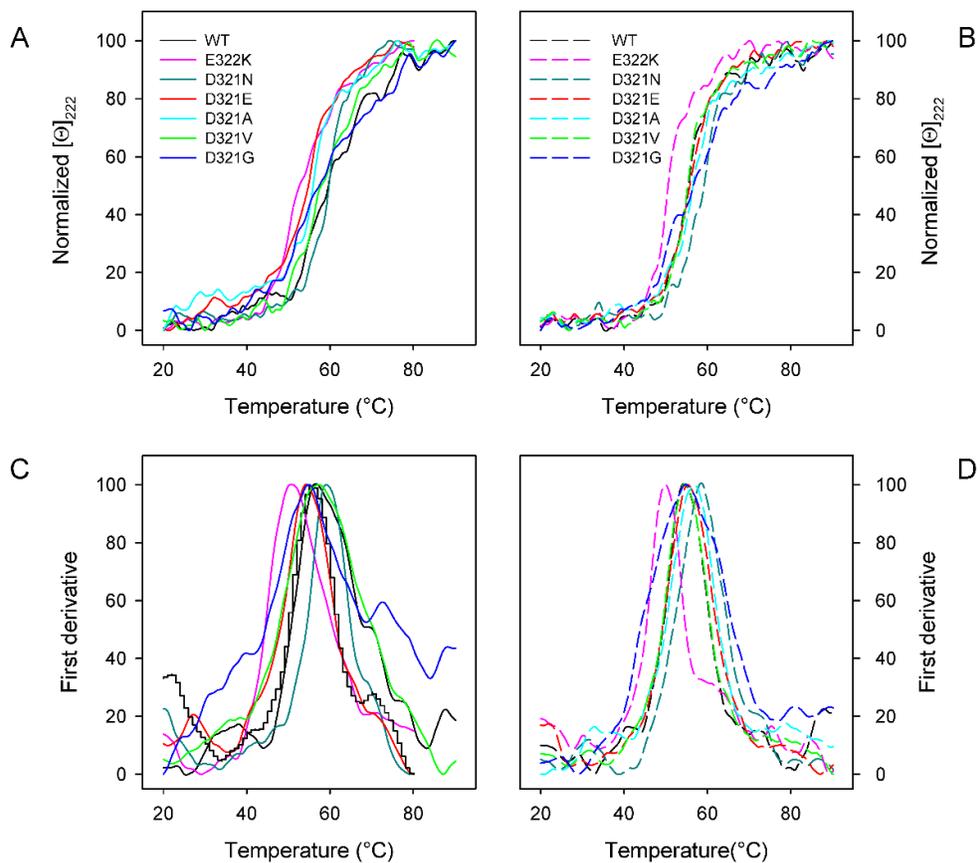


**Figure S2. FAR-UV CD spectra of ERK2 wild-type (WT) and variants. (A)** nonphosphorylated-ERK2 (NP-ERK2) WT and variants in the CD-site. **(B)** phosphorylated ERK2 (P-ERK2) WT and variants in the CD-site. All spectra were recorded at 20°C in a 0.1-cm quartz cuvette at 130-170  $\mu\text{g}/\text{mL}$  in 20 mM Tris-HCl, pH 7.5 containing 0.2 M NaCl and 0.2 mM DTT.

**Table S2. Molar ellipticity ratio of nonphosphorylated-ERK2 (NP-ERK2) and phosphorylated ERK2 (P-ERK2) wild type and variants.**

	<b>NP-ERK2</b>	<b>P-ERK2</b>
<b>ERK2</b>	<b><math>[\Theta]_{222/208}</math></b>	<b><math>[\Theta]_{222/208}</math></b>
<b>wild-type</b>	0.80	0.90
<b>D321A</b>	0.80	0.82
<b>D321E</b>	0.81	0.83
<b>D321G</b>	1.11	0.98
<b>D321N</b>	0.76	0.83
<b>D321V</b>	1.01	0.77
<b>E322K</b>	0.81	0.90

The  $[\Theta]_{222/208}$  ratio between 222 and at 208 nm is indicative of non-interacting helices (0.8-0.9) and coiled coil helices (>1.0).



**Figure S3. Thermal unfolding of nonphosphorylated-ERK2 (NP-ERK2) and phosphorylated ERK2 (P-ERK2) wild type and variants in the CD-site.** ERK2 wild-type and variants (103  $\mu\text{g/ml}$  - 130  $\mu\text{g/ml}$ ) were heated from 20°C to 95°C in 20 mM Tris-HCl, pH 8.0 containing 0.2 M NaCl and 0.2 mM DTT. The molar ellipticity at 222 nm ( $[\Theta]_{222}$ ) was monitored continuously every 0.5 °C. (A) Thermal unfolding of NP-ERK2 wild type and variants. (B) Thermal unfolding of P-ERK2 wild-type and variants. First derivative of NP-ERK2 (C) and P-ERK2 (D) wild-type and variants data reported in A and B, respectively.

Table S3 Thermodynamic parameters for GdmCl-induced unfolding equilibrium of nonphosphorylated-ERK2 (NP-ERK2) and phosphorylated ERK2 (P-ERK2) wild-type and variants measured by far-UV CD and fluorescence spectroscopy.

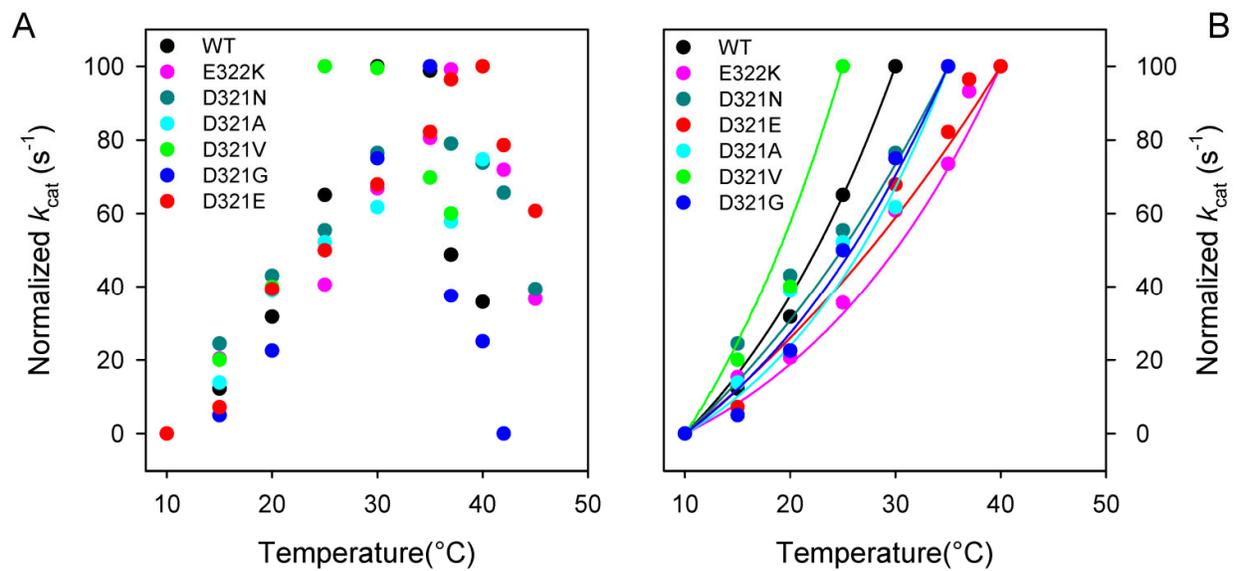
ERK2	$\Delta G^{H_2O}$ (kcal/mol)		$m$ (kcal/mol/M)		[GdmCl] <sub>0.5</sub> (M)	
	CD ([ $\Theta$ ] <sub>222</sub> )	Fluorescence ( $\bar{\lambda}$ )	CD ([ $\Theta$ ] <sub>222</sub> )	Fluorescence ( $\bar{\lambda}$ )	CD ([ $\Theta$ ] <sub>222</sub> )	Fluorescence ( $\bar{\lambda}$ )
NP-wild-type	2.51 + 0.22	2.79 + 0.16	0.91 + 0.09	1.34 + 0.08	2.76	2.08
P- wild-type	2.30 + 0.16	2.88 + 0.20	0.97 + 0.07	1.19 + 0.09	2.37	2.42
NP-D321A	2.16 + 0.19	2.91 + 0.20	1.08 + 0.10	1.39 + 0.10	2.00	2.09
P-D321A	1.83 + 0.14	2.89 + 0.28	0.90 + 0.07	1.51 + 0.15	2.03	1.92
NP- D321E <sup>b</sup>	2.43 + 0.20	$\Delta G1 = 2.93$ $\Delta G2 = 4.76$	1.13 + 0.10	$m1 = 2.15 + 0.15$ $m2 = 1.41 + 0.38$	2.16	[GdmCl] <sub>0.5</sub> = 1.36 + 0.02 [GdmCl] <sub>0.5</sub> = 3.36 + 0.13
P-D321E <sup>a</sup>	$\Delta G1 = 3.39$ $\Delta G2 = 6.99$	2.09 + 0.13	$m1 = 2.50 + 0.39$ $m2 = 1.96 + 0.57$	1.13 + 0.10	[Gdm] <sub>0.5</sub> = 1.59 + 0.05 [Gdm] <sub>0.5</sub> = 3.57 + 0.08	2.02
NP-D321G <sup>a</sup>	$\Delta G1 = 2.95$ $\Delta G2 = 4.41$	2.91 + 0.23	$m1 = 2.45 + 0.43$ $m2 = 1.27 + 0.47$	1.37 + 0.11	[Gdm] <sub>0.5</sub> = 1.20 + 0.04 [Gdm] <sub>0.5</sub> = 3.48 + 0.21	2.12
P-D321G	2.31 + 0.13	2.97 + 0.26	1.01 + 0.07	1.26 + 0.12	2.28	2.35
NP-D321N	2.07 + 0.16	2.57 + 0.20	1.00 + 0.08	1.17 + 0.10	2.06	2.20

ERK2	$\Delta G^{H_2O}$ (kcal/mol)		$m$ (kcal/mol/M)		[GdmCl] <sub>0.5</sub> (M)	
	CD ([ $\Theta$ ] <sub>222</sub> )	Fluorescence ( $\bar{\lambda}$ )	CD ([ $\Theta$ ] <sub>222</sub> )	Fluorescence ( $\bar{\lambda}$ )	CD ([ $\Theta$ ] <sub>222</sub> )	Fluorescence ( $\bar{\lambda}$ )

<b>P-D321N<sup>a</sup></b>	$\Delta G1 = 2.39$	$3.23 + 0.29$	$m1 = 2.03 + 0.22$	$1.38 + 0.13$	$[Gdm]_{0.5} = 1.18 + 0.03$	2.35
	$\Delta G2 = 8.09$		$m2 = 2.43 + 0.71$		$[Gdm]_{0.5} = 3.34 + 0.08$	
<b>NP-D321V</b>	$1.26 + 0.10$	$2.48 + 0.18$	$0.99 + 0.07$	$1.08 + 0.08$	1.27	2.29
<b>P-D321V<sup>a</sup></b>	$\Delta G1 = 5.00$	$2.69 + 0.22$	$m1 = 5.02 + 0.22$	$1.32 + 0.12$	$[Gdm]_{0.5} = 1.00 + 0.03$	2.29
	$\Delta G2 = 4.92$		$m2 = 1.56 + 0.25$		$[Gdm]_{0.5} = 3.16 + 0.07$	
<b>NP-E322K</b>	$1.93 + 0.14$	$2.50 + 0.18$	$0.76 + 0.06$	$1.14 + 0.09$	2.53	2.19
<b>P-E322K<sup>a,b</sup></b>	$\Delta G1 = 4.02$	$\Delta G1 = 5.08$	$m1 = 4.62 + 0.49$	$m1 = 4.73 + 0.57$	$[Gdm]_{0.5} = 0.87 + 0.01$	$[Gdm]_{0.5} = 1.07 + 0.02$
	$\Delta G2 = 3.97$	$\Delta G2 = 5.78$	$m2 = 1.27 + 0.13$	$m2 = 1.53 + 0.52$	$[Gdm]_{0.5} = 3.13 + 0.06$	$[Gdm]_{0.5} = 3.77 + 0.27$

GdmCl-induced unfolding equilibrium data were measured as described in Materials and Methods by monitoring the ellipticity at 222 nm ( $[\Theta_{222}]$ ) and the fluorescence intensity averaged emission wavelength ( $\bar{\lambda}$ , Equation (2)).  $\Delta G^{H_2O}$  and  $m$  values were obtained from Equation (3);  $[GdmCl]_{0.5}$  was calculated from Equation (5). <sup>a</sup> $[\Theta_{222}]$  data were fitted to Equation (6). <sup>b</sup>  $\bar{\lambda}$  data were fitted to Equation (6). Data are reported as the mean  $\pm$  SE of the fit.



**Figure S4. Effect of temperature on the activity of phosphorylated ERK2 (P-ERK2) wild-type and variants.** (A) Temperature dependence of kinase activity of ERK2 wild-type and variants. (B) Non-linear fit of the temperature dependence of ERK2 activity to the Arrhenius equation (Equation (1)). Assays were performed under the conditions described in Materials and Methods.

**Table S4. Selected inhibitors of ERK2.** The inhibitors were analyzed by DSF for ERK2 wild-type and variants, as described in Materials and Methods.

<b>Compound</b>	<b>Supplier</b>	<b>Compound Description</b>	<b>Reference</b>
SCH772984	Cayman	ERK inhibitor	[47]
AZD0364	Cayman	ERK inhibitor	[48]
KO-947	Cayman	ERK inhibitor	[49]
LY3214996	Cayman	ERK inhibitor	[50]
FR 180204	Cayman	ERK inhibitor	[51]
GDC-0994	Cayman	ERK inhibitor	[52]
Ulixertinib	Cayman	ERK inhibitor	[53]
Magnolin	Cayman	ERK inhibitor	[54]

**Table S5. Effect of the binding with inhibitors on melting temperature of ERK2 wild-type (WT) and variants.** The values below 1 suggest that the ligand binds to the protein preferentially to the unfolded state or to non-native states and stabilizes (increases the molar fraction or the population of) the unfolded or non-native states.

Compound	$\Delta T_m$ (°C)						
	WT	D321A	D321E	D321G	D321N	D321V	E322K
<b>AZD0346</b>	12.80 $\pm$ 0.05	13.30 $\pm$ 0.51	13.70 $\pm$ 1.03	13.90 $\pm$ 0.89	14.50 $\pm$ 0.21	13.00 $\pm$ 0.03	10.20 $\pm$ 0.01
<b>KO-947</b>	11.80 $\pm$ 0.22	12.30 $\pm$ 0.37	12.80 $\pm$ 1.01	13.10 $\pm$ 0.86	13.60 $\pm$ 0.23	11.80 $\pm$ 0.02	9.20 $\pm$ 0.08
<b>SCH772984</b>	11.60 $\pm$ 0.27	11.00 $\pm$ 0.09	11.70 $\pm$ 1.15	12.20 $\pm$ 0.89	12.70 $\pm$ 0.24	11.60 $\pm$ 0.13	8.70 $\pm$ 0.15
<b>GDC-0994</b>	10.30 $\pm$ 0.30	10.70 $\pm$ 0.20	11.20 $\pm$ 0.74	11.80 $\pm$ 0.58	12.20 $\pm$ 0.15	10.30 $\pm$ 0.28	6.80 $\pm$ 0.49
<b>LY3214996</b>	9.40 $\pm$ 0.21	9.80 $\pm$ 0.02	10.30 $\pm$ 0.88	10.80 $\pm$ 0.67	11.20 $\pm$ 0.14	10.00 $\pm$ 0.02	6.60 $\pm$ 0.01
<b>Ulixertinib</b>	7.90 $\pm$ 0.39	8.30 $\pm$ 0.31	8.80 $\pm$ 0.95	9.10 $\pm$ 0.79	9.50 $\pm$ 0.24	8.30 $\pm$ 0.09	4.60 $\pm$ 0.14
<b>FR 180204</b>	2.80 $\pm$ 1.27	3.20 $\pm$ 0.20	3.70 $\pm$ 0.86	4.60 $\pm$ 0.94	5.40 $\pm$ 0.58	5.00 $\pm$ 0.44	-1.30 $\pm$ 0.08
<b>Magnolin</b>	0.20 $\pm$ 0.11	0.60 $\pm$ 0.24	1.40 $\pm$ 0.43	1.90 $\pm$ 1.13	2.60 $\pm$ 0.35	0.30 $\pm$ 0.10	-5.30 $\pm$ 0.35