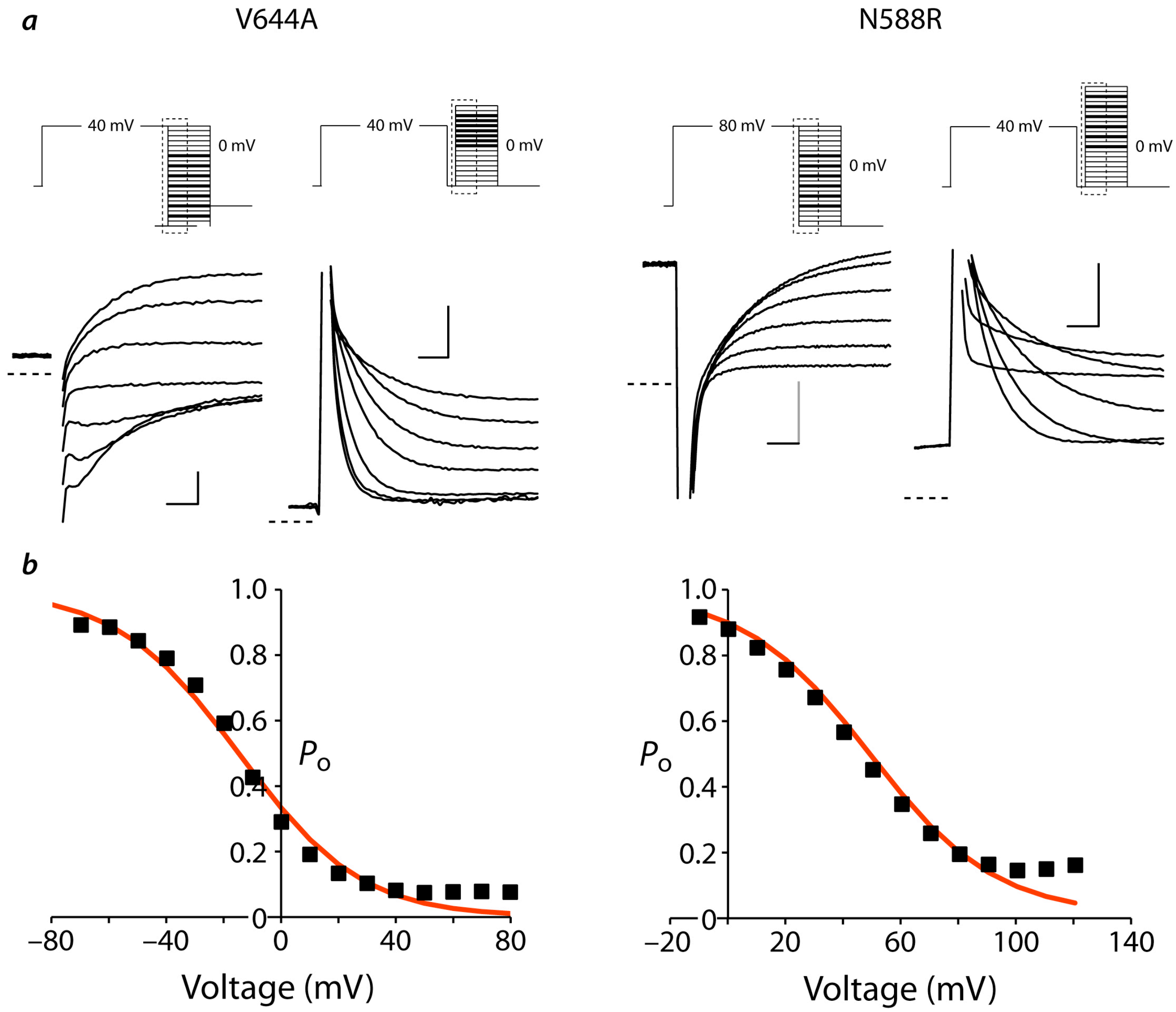
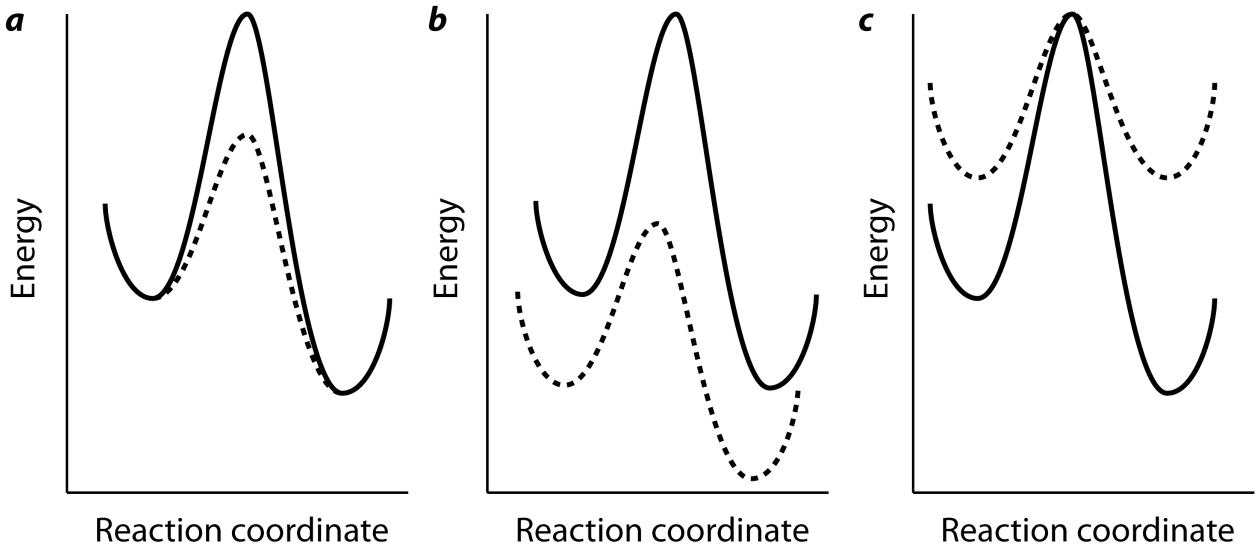
Supplementary Figure 1: Comparison of two independent methods for calculation of equilibrium for open – inactivated state transition



a. Typical examples of current records from V644A and N588R channels using voltage protocols shown in insets. Dashed lines indicate zero current level and scale bars are 5 ms and 500 nA. Rates of recovery from inactivation and rates of inactivation were calculated by fitting exponential functions to the current traces as shown in Fig. 2 (in the main text). ***b.*** Channel open probability ([O] / ([O] + [I]) determined from k_{inact} and k_{rec} (red line) and ratio of I_{ss} / I_{peak} (black symbols) (as described for Fig. 2 in the main text).

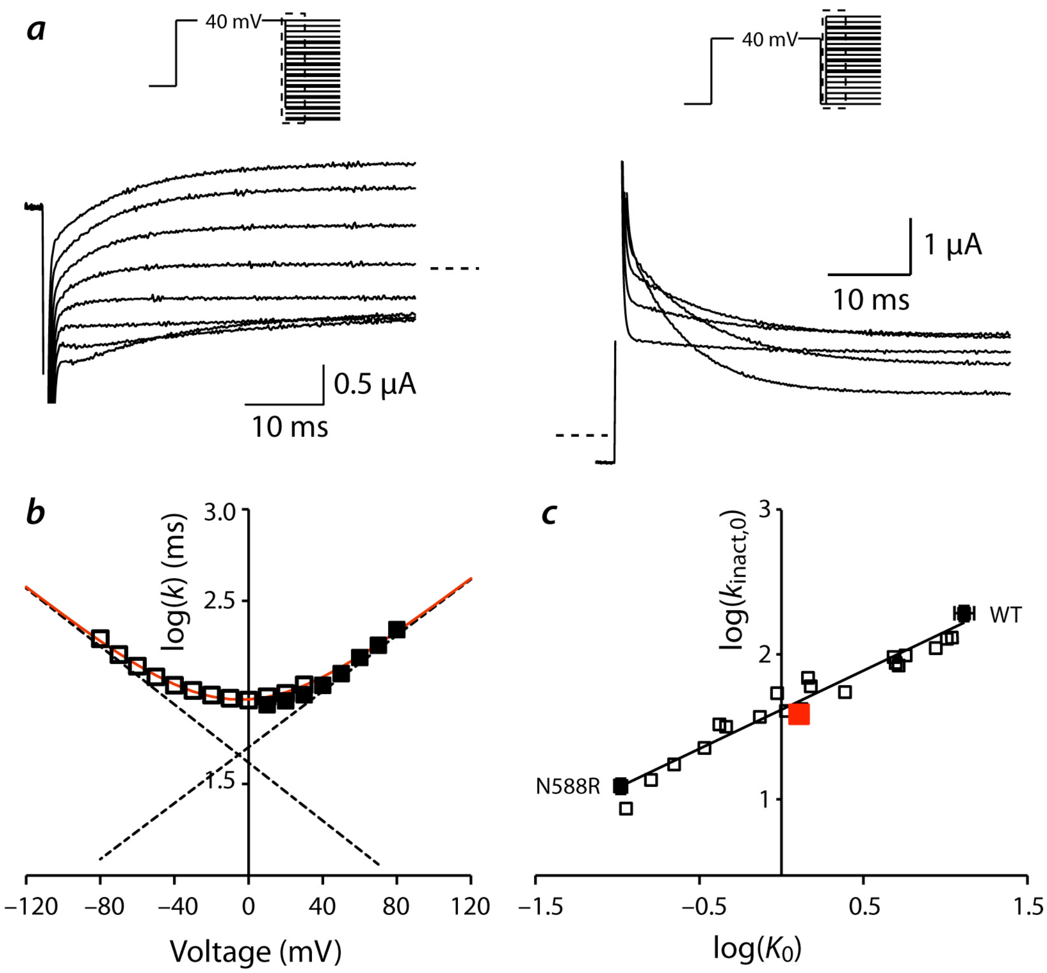
Supplementary Figure 2: Mutations that disrupt the ground states and/or transition state are usually not amenable to Φ–value analysis**.**



The effect of mutations that significantly alter the ground states and/or transition state can be considered as analogous to climbing over a mountain.
a. mutations that affect the transition state but do not affect the ground states, i.e. catalytic mutations, would be analogous to bypassing the summit and taking an easier route to get from one side of the mountain to the other. As such these mutations do not tell you anything about the transition pathway for the WT protein.
b mutations that significantly affect the ground states – would be analogous to traversing a different mountain with a different summit and so would not tell you anything about the transition pathway for the WT protein.
c. mutations that significantly alter the ground states, but not the transition state, may or may not reveal information about the transition pathway for the WT protein.

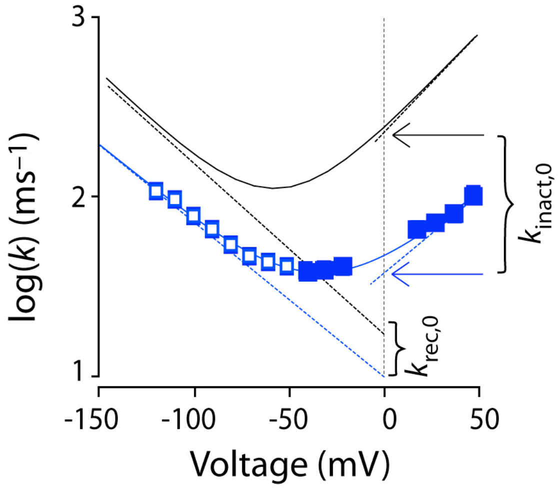
It is possible for some mutations to cause significant perturbations as outlined above, whilst other mutations at the same residue may not. In our study mutations to Ser631 provide such an example, where S631C and S631H appear to be examples of catalytic mutations (type a), S631Q, S631N, S631E appear to be examples of type b and S631A and S631Y appear to be informative mutations. It is possible for individual mutations of type b or type c to give a Φ-value in the range 0-1. However, it would be very unlikely that multiple type b or type c mutations at the same residue would give you identical Φ-values. It is therefore standard practice to require more than one mutant at any given residue to have the same Φ-value to be confident that it is an informative residue.

Supplementary Figure 3: Co-expression of WT and N588R channels results in an intermediate inactivation phenotype.



a. Families of currents recorded from an oocyte with ~50:50 WT:N588R K_v11.1 channels. Left panel: during double pulse voltage protocol to measure recovery from inactivation. Right panel: triple pulse voltage protocol to measure rates of inactivation.
b. Chevron plot for rates of inactivation and recovery from inactivation obtained from the currents shown in panel a.
c. rate-equilibrium free energy plot for N588R (solid square to left), WT (solid square to right) and mixtures of WT and N588R (open symbols). The red symbol highlights the example shown in panels a and b.

Supplementary Figure 4: Chevron plot for WT channels in presence of 98 mM external K^+.



Plot of logarithm of rates of inactivation (solid symbols) and recovery from inactivation (open symbols) in presence of 98 mM external K^+. Solid lines are fits of the equation: $k_{obs,V} = k_{inact,V} + k_{rec,V}$ and dashed lines show the plots for $k_{rec,V}$ and $k_{inact,V}$ for 98 mM external K^+ (blue) and 2mM external K^+ (black, reproduced from Fig. 6, main text).

Supplementary Table 1: log($k_{inact,0}$) and log(K_0) values for all mutants

mutant	n	log(K_0)	log($k_{inact,0}$)	Δlog (K_0) [‡]
S4				
K525C	3	0.84 ± 0.08	2.14 ± 0.08	–0.27
K525Q	7	1.13 ± 0.04	2.32 ± 0.02	0.20
K525R	5	1.17 ± 0.09	2.29 ± 0.01	0.06
K525D	6	1.34 ± 0.04	2.38 ± 0.01	0.23
R528A	5	1.04 ± 0.07	2.32 ± 0.04	–0.08
R531A	4	0.86 ± 0.14	2.10 ± 0.07	–0.26
R531T	4	0.94 ± 0.09	2.27 ± 0.05	–0.17
R531N	8	1.01 ± 0.05	2.22 ± 0.02	–0.11
R531Q	5	0.80 ± 0.11	2.20 ± 0.07	–0.31
R534A	2	0.80 ± 0.07	1.92 ± 0.04	–0.31
R534D	4	0.65 ± 0.03	1.98 ± 0.04	–0.47
R534Q	8	0.91 ± 0.09	2.09 ± 0.05	–0.20
R537A	3	0.85 ± 0.07	2.14 ± 0.03	–0.26
R537S	5	0.80 ± 0.05	2.10 ± 0.03	–0.32
R537Q	5	0.63 ± 0.09	1.97 ± 0.07	–0.49
R537D	6	0.82 ± 0.04	2.07 ± 0.03	–0.29
R537T	6	0.89 ± 0.06	2.13 ± 0.03	–0.23
K538R	4	1.01 ± 0.06	2.18 ± 0.03	–0.11
K538Q	3	1.11 ± 0.07	2.26 ± 0.02	0.00
K538D	7	1.02 ± 0.07	2.22 ± 0.04	–0.10
K538A	14	0.97 ± 0.06	2.14 ± 0.03	–0.14
K538S	8	1.04 ± 0.08	2.32 ± 0.04	–0.08
K538T	4	1.14 ± 0.11	2.27 ± 0.06	0.02
K538N	11	1.02 ± 0.06	2.11 ± 0.02	–0.09
S4S5				
L539A	9	1.04 ± 0.03	2.17 ± 0.01	–0.07
L539V	10	1.31 ± 0.08	2.43 ± 0.04	0.2
L539K	6	1.23 ± 0.07	2.33 ± 0.02	0.12
L539D	8	0.73 ± 0.05	1.95 ± 0.03	–0.39
D540A	9	0.50 ± 0.10	2.05 ± 0.06	–0.61
D540K	8	0.46 ± 0.06	2.01 ± 0.02	–0.65
D540S	10	0.48 ± 0.04	1.99 ± 0.02	–0.63
D540H	4	1.76 ± 0.13	2.56 ± 0.06	0.65
R541A	9	0.71 ± 0.03	2.01 ± 0.04	–0.4
R541D	8	0.62 ± 0.07	1.92 ± 0.04	–0.49
R541E	9	0.80 ± 0.04	2.00 ± 0.03	–0.31
R541H	8	0.92 ± 0.07	2.21 ± 0.06	–0.19
R541M	8	0.84 ± 0.05	2.14 ± 0.05	–0.27
R541Q	13	0.96 ± 0.04	2.08 ± 0.02	–0.15
R541S	8	0.66 ± 0.04	1.94 ± 0.03	–0.45
Y542A	8	0.22 ± 0.04	2.00 ± 0.03	–0.89

Y542F	8	0.65 ± 0.08	1.97 ± 0.05	–0.46
Y542S	NE			
S543C	8	0.84 ± 0.07	2.18 ± 0.04	–0.27
S543D	8	0.51 ± 0.04	2.05 ± 0.02	–0.6
S543G	8	0.99 ± 0.09	2.19 ± 0.02	–0.12
S543H	8	0.36 ± 0.03	1.94 ± 0.02	–0.75
S543K	8	0.34 ± 0.09	1.93 ± 0.05	–0.77
S543T	8	1.11 ± 0.05	2.28 ± 0.05	0
S543V	8	0.97 ± 0.03	2.16 ± 0.02	–0.14
E544H	4	1.10 ± 0.07	2.35 ± 0.06	0.01
G546C	8	0.92 ± 0.08	2.11 ± 0.07	–0.19
G546P	10	0.91 ± 0.03	2.11 ± 0.06	–0.2
G546S	8	0.88 ± 0.06	2.18 ± 0.05	–0.23
G546V	7	0.72 ± 0.05	2.10 ± 0.03	–0.39
A547K	5	1.11 ± 0.05	2.29 ± 0.03	0
A547S	5	1.14 ± 0.02	2.23 ± 0.02	0.03
A548K	5	0.82 ± 0.04	1.98 ± 0.03	–0.29
A548S	6	1.13 ± 0.04	2.21 ± 0.02	0.02
A548D	4	0.67 ± 0.08	2.28 ± 0.02	–0.45
V549D	7	1.24 ± 0.03	2.34 ± 0.02	0.13
V549K	3	1.13 ± 0.08	2.27 ± 0.02	0.01
V549S	NE			
S5				
L550A	8	0.58 ± 0.13	1.94 ± 0.12	–0.53
L550C	7	0.88 ± 0.11	2.25 ± 0.02	–0.23
L550S	4	0.96 ± 0.12	2.25 ± 0.03	–0.15
L550D	6	1.05 ± 0.06	2.43 ± 0.04	–0.07
L552S	4	0.84 ± 0.05	2.09 ± 0.03	–0.27
F557A	11	1.43 ± 0.1	2.55 ± 0.06	0.31
F557M	6	1.00 ± 0.07	2.24 ± 0.02	–0.11
F557I	10	1.34 ± 0.04	2.56 ± 0.01	0.22
F557L	5	1.11 ± 0.07	2.29 ± 0.01	0
F557W	6	1.17 ± 0.04	2.34 ± 0.04	0.06
F557E	NE			
L564A	8	0.34 ± 0.06	1.76 ± 0.03	–0.78
L564C	13	0.45 ± 0.07	1.74 ± 0.03	–0.67
L564I	4	1.21 ± 0.09	2.36 ± 0.01	0.09
L564V	5	0.82 ± 0.05	1.96 ± 0.04	–0.29
L564R	NE			
L564W	NE			
L564M	NE			
S5P				
G584S	4	0.73 ± 0.11	1.96 ± 0.05	–0.38
H587D	4	1.10 ± 0.06	2.27 ± 0.01	–0.01
H587K	6	0.97 ± 0.04	2.31 ± 0.03	–0.14

N588D	4	1.00 ± 0.04	1.89 ± 0.05	–0.11
N588E	4	1.30 ± 0.13	2.43 ± 0.02	0.19
N588K	3	–1.16 ± 0.07	0.75 ± 0.06	–2.26
N588T	4	1.12 ± 0.05	1.98 ± 0.03	0.01
N588Q	5	1.27 ± 0.07	2.34 ± 0.03	0.16
N588R	4	–0.98 ± 0.04	1.09 ± 0.02	–2.09
D591Q	5	0.70 ± 0.14	1.92 ± 0.06	–0.41
D591K	5	0.22 ± 0.07	1.70 ± 0.08	–0.89
Q592K	5	–1.08 ± 0.03	0.95 ± 0.01	–2.19
Q592D	6	0.61 ± 0.06	2.01 ± 0.03	–0.50
Q592H	3	0.21 ± 0.07	1.67 ± 0.07	–0.90
Q592S	3	1.06 ± 0.09	2.25 ± 0.05	–0.05
Q592R	4	–0.93 ± 0.14	0.99 ± 0.11	–2.04
Q592A	5	0.66 ± 0.03	1.98 ± 0.01	–0.45
K595R	8	0.77 ± 0.08	2.04 ± 0.02	–0.34
K595Q	8	1.03 ± 0.07	2.22 ± 0.05	–0.07
P-helix–SF linker				
S621A	5	0.5 ± 0.03	2.11 ± 0.01	–0.61
S621T	NE			
S621C	*			
S621G	*			
S621V	NE			
S621D	NE			
T623A	*			
T623S	4	1.37 ± 0.00.03	2.30 ± 0.028	0.20
T623V	NE			
S624A	5	1.53 ± 0.07	2.03 ± 0.02	0.42
S624G	4	0.7 ± 0.09	2.46 ± 0.02	–0.41
S624V	NE			
S624C	*			
V625A	*			
V625T	8	0.39 ± 0.06	2.03 ± 0.02	–0.73
V625I	*			
F627M	†			
F627Y	†			
F627L	NE			
F627I	NE			
F627W	NE			
F627C	NE			
F627A	NE			
N629H	NE			
N629D	*			
PS6				
S631A	5	–0.26 ± 0.07	1.9 ± 0.09	–1.37

	S631H	3	1.21 ± 0.15	1.51 ± 0.03	0.10
	S631Y	7	1.62 ± 0.07	2.39 ± 0.04	0.50
	S631C	5	1.18 ± 0.05	2.85 ± 0.07	0.07
	S631Q	*			
	S631E	*			
	S631N	NE			
	N633H	2	1.20 ± 0.08	2.23 ± 0.01	0.09
	S636A	5	1.28 ± 0.03	2.18 ± 0.03	0.16
	S636H	3	0.91 ± 0.07	2.09 ± 0.03	–0.21
S6					
	V644C	5	0.58 ± 0.07	2.04 ± 0.06	–0.54
	V644L	8	1.53 ± 0.10	2.40 ± 0.02	0.42
	V644A	8	0.29 ± 0.09	2.02 ± 0.04	–0.82
	V644D	NE			
	V644I	8	1.09 ± 0.08	2.23 ± 0.03	–0.03
	M651A	5	1.40 ± 0.05	2.31 ± 0.01	0.29
	M651C	7	1.45 ± 0.03	2.45 ± 0.03	0.34
	M651F	5	1.52 ± 0.05	2.42 ± 0.01	0.4
	M651L	5	1.14 ± 0.05	2.21 ± 0.01	0.02
	Y652A	5	1.10 ± 0.04	2.39 ± 0.02	–0.02
	Y652F	5	1.23 ± 0.05	2.23 ± 0.02	0.12
	Y652I	*			
	Y652W	5	1.2 ± 0.09	2.22 ± 0.02	0.08

NE No expression detected or level too low to permit accurate determination of rate constants

* Channels have reduced selectivity for K^+ over Na^+ (defined as E_{rev} > –90 mV when perfused with ND96, typically these mutants had E_{rev} > –60 mV)

† Mutants where inactivation was too fast to permit accurate estimation of rate constants

‡ Calculated as $\log(K_{0,mut}) - \log(K_{0,WT})$