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Binding of disordered proteins to a protein hub

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A small number of proteins, called hubs, have high connectivity and are essential for interactome functionality and integrity. Keap1 is a crucial hub in the oxidative stress response and apoptosis. The Kelch domain of Keap1 preferentially binds to disordered regions of its partners, which share similar binding motifs, but have a wide range of binding affinities. Isothermal titration calorimetry (ITC) and multi-microsecond molecular dynamics (MD) simulations were used to determine the factors that govern the affinity of all currently known disordered binding partners to Kelch. Our results show that the affinities to this hub are largely determined by the extent of preformed bound state-like conformation in the free state structures of these disordered targets. Based on our findings, we have designed a high-affinity peptide that can specifically disrupt the Keap1-NRF2 interaction and has the potential for therapeutic applications.

Characterization of protein interaction networks is essential for understanding the functions of individual proteins, signaling pathways and their interconnections^{1,2}. Some proteins have higher connectivity relative to others and they are commonly referred to as hubs^{3,4}. Hub proteins are essential for interactome functionality and stability; therefore, it is not surprising that their disruptions in the human interactome are frequently associated with diseases^{4,5}.

The structural plasticity of intrinsically disordered proteins (IDPs) allows them to interact with numerous different targets. It is also common for well-structured hub proteins to preferentially interact with disordered partners⁶. In contrast to protein-protein interactions between well-folded proteins, which usually involve larger interaction surfaces that are discontinuous in sequence, IDPs typically bind to targets using short (~6 residues) consecutive stretches of amino acid residues called linear motifs (LMs)^{7,8}. These motifs often have distinct sequence characteristics with the primary difference being an increased hydrophobic content⁹, which may promote local structure formation^{10–13}. Accurate detection of LMs and detailed characterization of their structures or conformational propensities would provide unique insight into the relationships between sequence and structure.

Here, we used a combined experimental and computational strategy to examine the interactions between the well-folded Kelch domain hub, from Keap1 (Kelch-like ECH-associated protein 1), and all of its identified (to date) disordered partners (Fig. 1a). NRF2 is arguably the most well known target of Keap1. The interaction of these proteins is critical for regulating the cellular anti-inflammatory oxidative stress responses¹⁴. The C-terminal Kelch domain of Keap1, which adopts a ~32 kDa β -propeller structure, is responsible for mediating the interaction with the Neh2 domain of NRF2. Structural studies have shown that Neh2 is intrinsically disordered¹⁵. It has high and low affinity 'ETGE' and 'DLG' containing Kelch domain binding regions, respectively, which will be referred to as sites 1 and 2, respectively hereafter^{15,16}. These sites are located at separate ends of the ~100 residue Neh2 domain, connected by a segment with high helical propensity¹⁵. When both sites are bound to two separate Kelch domains of a Keap1 dimer, NRF2 is ubiquitinated, which targets it for proteasomal degradation. When only site 1 is bound, NRF2 avoids the degradation pathway and can promote expression of its target genes¹⁶. In addition to NRF2, at least 10 other proteins have been shown to interact with the Kelch domain of Keap1 to date. These include WTX¹⁷, p62¹⁸, PGAM5¹⁹, PALB2²⁰, FAC1²¹, PTMA²², IKK β ²³ and BCL2²⁴. Several of these partners have been identified to disrupt the low affinity site 2-Kelch domain interaction, allowing NRF2 to promote cytoprotective gene expression. Most of the Kelch domain interacting proteins contain sequences resembling the site 1 motif of the Neh2 domain (Fig. 1a) and will be referred to as site 1-type proteins hereafter. The one exception is BCL2, which contains a site 2-type sequence and will be referred to as a site 2-type protein (Fig. 1a). Although not included in this study, as it does not appear to be directly linked to the oxidative stress response or apoptosis, myosin-VIIa has also been shown to interact with Kelch²⁵. The region of human myosin-VIIa capable of binding Kelch includes a site 1-type sequence¹⁶³⁵LDHDTGE¹⁶⁴¹.

SUBJECT AREAS:
SOLUTION-STATE NMR
COMPUTATIONAL BIOPHYSICS
INTRINSICALLY DISORDERED
PROTEINS
PROTEIN FOLDINGReceived
10 May 2013Accepted
12 July 2013Published
29 July 2013Correspondence and
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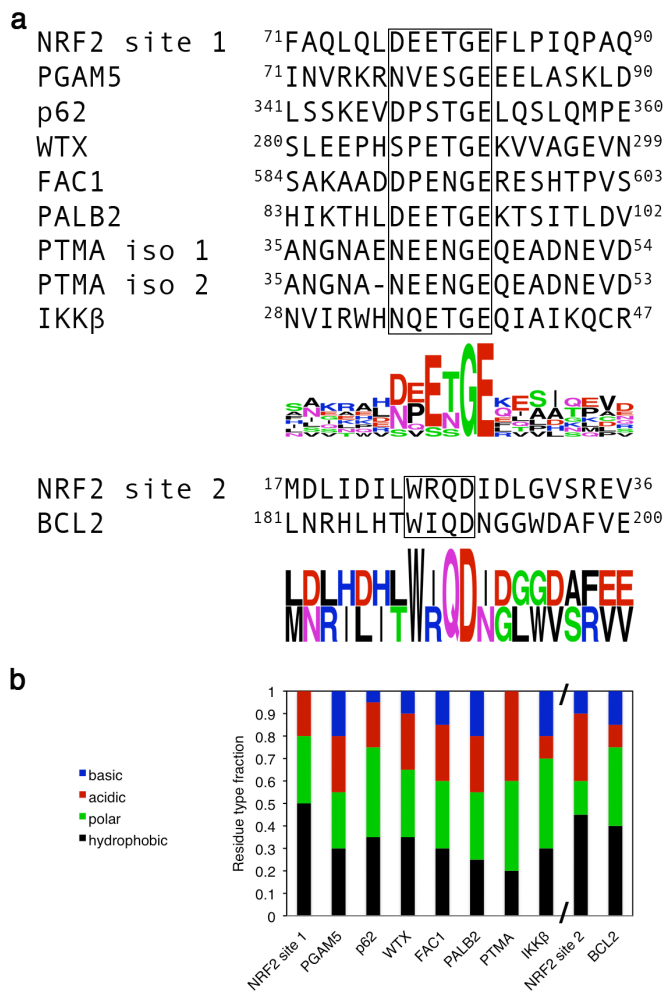


Figure 1 | Sequence analysis of the Kelch domain interacting proteins. a) Manual sequence alignment and sequence WebLogos of the site 1- and site 2-type regions of the Kelch domain binding proteins^{60,61}. B) Residue type fractions of the sequences. A, F, I, L, M, P, V, W and Y are hydrophobic; C, G, N, Q, S and T are polar; D and E are acidic; K, H and R are basic.

Through sequence analysis, disorder predictions, binding parameter measurements and MD simulations, the factors that govern the binding affinities and specificities of the different IDP-Kelch interactions were determined. Our findings were also used to design a new higher affinity Kelch-binding peptide.

Results

Kelch domain interacting proteins are predominantly disordered.

Even though a number of targets of the Kelch domain of Keap1 have been identified, structural information of only a few of them is currently available. NMR studies show that the sites 1 and 2 Kelch binding regions of Neh2 are located in disordered regions¹⁵. Similarly, the site 1-type binding motif of PTMA have also been found to be largely unstructured in its free state²⁶. Based on the amino acids sequences of the various proteins (Fig. 1a), intrinsic disorder in the Kelch domain binding regions of the site 1-type binders may be a common attribute. Predictions by PONDR-FIT²⁷, a meta-predictor of intrinsically disordered amino acids, show that for NRF2, p62, WTX, FAC1, PALB2, and PTMA, their site 1-type Kelch binding motifs are all located in long stretches of disordered regions (Supplementary Fig. 1). PGAM5 and IKKβ were the only site 1-type proteins with disorder scores < 0.5. Based on the prediction, the Kelch binding region of PGAM5 is located on the border of the

N-terminal disordered region and C-terminal structured domain. Meanwhile, the binding site of IKKβ is predicted to be in a structured part of the protein (Supplementary Fig. 1), which is consistent with the homology model²⁸ that illustrates that IKKβ is a well-folded protein (Supplementary Fig. 2). For the site 2-type binders, NRF2 and BCL2 were predicted to have disorder tendencies of 0.40 and 0.07, respectively (Supplementary Fig. 1). The predicted low disorder tendency of BCL2 was not surprising because its binding region is found in a well-folded part of the protein²⁹. Overall, the results suggest that while both intrinsically disordered and well-folded protein segments are able to bind to the Kelch domain, the majority of the partners (identified to date) are disordered, particularly around the site 1-type binding regions.

Sequence and structure comparison of the Kelch domain interacting proteins. Sequence comparison shows that the site 1-type proteins share high sequence similarity in a 6-residue stretch corresponding to the ‘DEETGE’ of NRF2 site 1 (Fig. 1a). These residues comprise the Kelch domain binding interface³⁰ and will be referred to as positions $i - i + 5$. Notably, G and E always occupy positions $i + 4$ and $i + 5$, respectively. E is found at $i + 2$ in all of the proteins, except p62, which contains an S at this position. The other positions are more variable. Outside of this 6-residue stretch, there are no clear sequence similarities between the different site 1-type proteins (Fig. 1a). On the other hand, the two site 2-type proteins have a short, 4-residue, ‘WXQD’ consensus region. Like the site 1-type proteins, these two proteins do not share apparent sequence consensus outside of this short motif (Fig. 1a).

The hydrophobic content amongst the site 1-type binders varied considerably between 20–50%, with the NRF2 site 1 having the highest fraction of hydrophobic residues, followed by p62/WTX, PGAM5/FAC1/IKKβ, PALB2 and PTMA. Meanwhile, acidic content fell into a narrower range of 10–25% for all proteins, except PTMA, which had a considerably higher fraction of 40% (Fig. 1b). For the site 2-type sequences, NRF2 and BCL2 have similar amounts of hydrophobic content between 40–45%, whereas the latter has considerably higher polar and less acidic content (Fig. 1b).

Crystal structures of p62, PTMA and Neh2 peptides in complex with the Kelch domain are currently available. The structures show that the site 1-type regions of these three proteins all bind to the same site on Kelch^{18,22,31}. Further, in their bound states, the PTMA and p62 peptides both adopt β-hairpin structures with low backbone rmsd (<0.3 Å Cα rmsd for 8 atom pairs) and similar sidechain conformations to the NRF2 site 1 peptide bound to the Kelch domain^{18,22,31} (Supplementary Fig. 2 and Supplementary Table 1). Notably, even though the structure of Kelch-IKKβ complex is currently not available, the free-state structure of IKKβ reveals that the Kelch domain binding region of this protein²⁸ also forms a β-hairpin that has considerable resemblance (~0.5 Å Cα rmsd for 8 atom pairs) to the bound state structure of the NRF2 site 1 peptide (Supplementary Fig. 2). When comparing the site 2-type binders, it is clear that in its free state, the ‘WIQD’ sequence of BCL2²⁹ has considerable structural resemblance (<0.5 Å Cα rmsd for 4 atom pairs) to the ‘WRQD’ sequence of the NRF2 site 2 peptide bound to Kelch³². These residues appear to adopt a ‘turn’ conformation and share similar backbone, but not χ^1 , dihedral angles (Supplementary Fig. 2 and Supplementary Table 1). Intriguingly, although the site 1- and 2-type Kelch domain interacting proteins do not have obvious sequence similarities, the residues that are largely buried in the Kelch domain binding interface (EE in NRF2 site 1 and PTMA, PS in p62, QE in IKKβ and QD in NRF2 site 2 and BCL2) all have similar ϕ and ψ angles (Supplementary Table 1).

Binding parameters of the Kelch domain interacting proteins.

With the aim to identify the mechanisms by which the Kelch domain binds to different disordered targets, we have determined the thermodynamic parameters of binding (Table 1) for peptides

Table 1 | Thermodynamic parameters for the binding of the peptides to the human Kelch domain^a

Protein	n ^b	K _d ^c (10 ⁻⁶ M)	ΔH ^c (kcal/mol)	TΔS ^c (kcal/mol)	ΔG ^c (kcal/mol)
NRF2 site 1	1.08	0.023 ± 0.002	-16.96 ± 0.05	-6.56	-10.40 ± 0.03
NRF2 site 1 E78P	0.99	0.007 ± 0.001	-16.76 ± 0.05	-5.64	-11.12 ± 0.03
PALB2	1.01	0.087 ± 0.007	-19.29 ± 0.11	-9.66	-9.63 ± 0.05
PGAM5	1.07	0.23 ± 0.01	-19.12 ± 0.06	-10.05	-9.07 ± 0.03
WTX	1.04	0.25 ± 0.02	-18.04 ± 0.09	-9.03	-9.01 ± 0.04
FAC1	0.99	1.1 ± 0.05	-15.11 ± 0.08	-6.98	-8.13 ± 0.04
p62	0.97	1.3 ± 0.02	-18.38 ± 0.07	-10.34	-8.04 ± 0.03
WTX pS286	0.98	1.5 ± 0.14	-10.83 ± 0.14	-2.88	-7.95 ± 0.10
PTMA iso 2	1.05	2.62 ± 0.05	-17.29 ± 0.06	-9.69	-7.60 ± 0.03
PTMA iso 1	1.07	11.6 ± 0.23	-14.74 ± 0.12	-8.02	-6.72 ± 0.05

^aThe peptide sequences are shown in Fig. 1a.

^bBinding stoichiometry.

^cK_d is the dissociation constant. ΔH, ΔS and ΔG are the change in enthalpy, entropy and Gibbs free energy upon binding at T = 298.15 K, respectively.

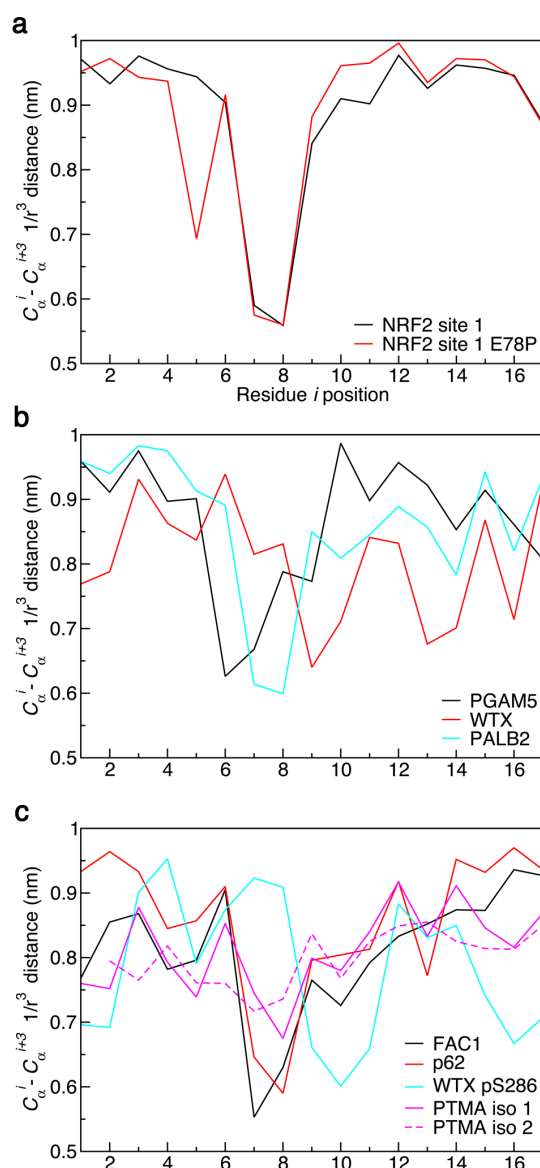


Figure 2 | $C_{\alpha}^i - C_{\alpha}^{i+3} / r^3$ averaged distances from the MD simulations. The $1/r^3$ averaged distances were calculated using the `g_rmsdist` tool in GROMACS 4.5 over the last 0.5 μ s of the trajectories.

encoding the binding regions of site 1-type Kelch domain interacting proteins with high disorder tendencies. For systematic comparisons, peptides of equal length (20 amino acids) were used and the experiments were performed using the same buffer conditions.

Despite having similar Kelch-binding motifs, a large variation in the binding affinity (K_d ranging from $\sim 12 \mu\text{M}$ to 23 nM) was observed for different disordered Kelch domain interacting proteins (Table 1 and Supplementary Table 2 and Supplementary Fig. 3). Among these binders, the NRF2 site 1 peptide displays the highest affinity to Kelch, with a K_d of 23 ± 2 nM. The value agrees well with what has been measured for a 16-mer peptide (~ 20 nM)³⁰. It is interesting that PALB2, which contains the same 'LDEETGE' sequence as NRF2 site 1, was ~ 4 -fold weaker. Our ITC data showed that while the interaction between the PALB2 peptide and Kelch domain is enthalpically more favorable than that for the NRF2 site 1 peptide, it lost considerable entropy upon binding. Compared to NRF2 site 1, PALB2 has less hydrophobic content in its binding region, which may hamper local folding and allow for more conformational freedom. This could possibly explain the more significant entropy loss of PALB2 upon binding, resulting in a weaker interaction. The unphosphorylated WTX peptide and PGAM5 bind to Kelch with similar affinity. However, upon phosphorylation of S286, the binding affinity of WTX was substantially decreased by ~ 6 -fold (Table 1). The affinity of the p62 peptide for the Kelch domain was on par with the reported value of 1851 ± 103 nM for a (mouse) fragment containing residues 168–391¹⁸. The high similarity between these measurements indicates that regions distant from the binding regions may not contribute to the interaction. The ~ 5 fold lower affinity of PTMA isoform 1 compared to isoform 2 (Table 1) was intriguing. The two isoforms have nearly identical sequences, with the only difference being a deletion of E at position $i - 1$ in isoform 2 (Fig. 1a). Factors that contribute to the difference in binding affinity are not clear, but we speculate that having amino acids with the same charge (E40 and E48) in isoform 1 somewhat close to each other in a β -turn conformation is unfavorable. Similarly, the D589 and E597 pair in FAC1 would also be in close proximity, assuming a β -turn conformation is adopted.

Free state structures of Kelch domain interacting peptides. To help identify factors that cause the large variation in binding affinity, MD simulations were performed to assess the relationships between peptide conformation in the free and bound states of the various Kelch domain interacting peptides. The results show that most peptides had free state contacts that were generally suggestive of turn or hairpin formations, with the turns occurring at the Kelch binding site (Figs. 2–4 and Supplementary Figs. 4–6).

While most of the peptides had a region of compactness at their Kelch binding regions (Fig. 2), there were some variations. For instance, it was clear that the region of compactness in the WTX

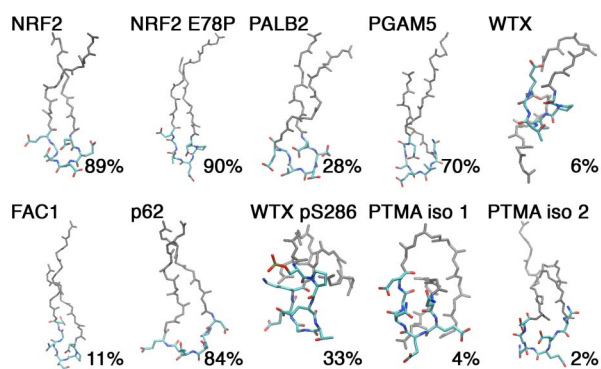


Figure 3 | Cluster analysis of the peptide structures. The center structures and percentages of structures in the highest population cluster are shown. Snapshots at 0.4 ns intervals from the 1 μ s trajectories were clustered based on the rmsd of all C_{α} atoms using a 0.15 nm cutoff^{33,34}. The Kelch binding ‘DEETGE’ like regions (Fig. 1a) are indicated in atomistic detail. The mainchain (N, C_{α} , C’, and CO) rmsd of the atoms from the ‘DEETGE’ like regions (Fig. 1a) were compared to the corresponding atoms of the bound state NRF2-Kelch structure (PDB id: 2FLU)³⁰: NRF2, 0.04 nm; NRF2 E78P 0.08 nm; PALB2, 0.10 nm; PGAM5, 0.21 nm; WTX, 0.24 nm; p62, 0.19 nm; FAC1, 0.17 nm; WTX pS286, 0.24 nm; PTMA isoform 1, 0.12 nm; PTMA isoform 2, 0.23 nm.

pS286 simulation was shifted from its expected location, towards the C-terminal end of the peptide, relative to the unphosphorylated version (Figs. 2b and c). The MD data indicates that interactions between the pS286 residue and a lysine on the opposite side of the turn could potentially be the cause of the turn distortion (Fig. 2 and Supplementary Fig. 5). In our previous MD simulations, we found that phosphorylation of T80 in an NRF2 peptide severely inhibited formation of the expected β -hairpin structure¹⁰. In the case of WTX, it appears that S286 phosphorylation may actually enhance free state structure formation. This is supported by our ITC data (Table 1), which revealed that the binding of WTX pS286 peptide to Kelch had the smallest entropy change. However, this peptide interacted with the Kelch domain with the least favorable enthalpy, which suggests that the peptide conformation induced by phosphorylation is not optimal for binding.

The conformational ensembles and bound state resemblance of the peptides was examined by cluster analysis and rmsd comparison to the NRF2 site 1 bound state^{33,34}. It was apparent that NRF2 adopted a relatively stable bound-state-like conformation with the majority of structures occupying a single cluster (Fig. 3 and 4). Bound-state-like structures formation was more infrequent in the other peptides and their largest clusters were less populated, relative to NRF2 (Fig. 3 and 4). Secondary structure analysis by DSSP further illustrates that turn or hairpin formations at the Kelch binding regions were more transient for PALB2, WTX, FAC1 and PTMA (Supplementary Fig. 6).

The MD simulations also provide insights into factors that stabilize the binding regions of the peptides. For instance, in addition to cross-strand hydrophobic contacts, electrostatic interactions between oppositely charged residues and hydrogen bonding pairs in cross-strand locations were commonly observed in the simulations (Supplementary Figs. 4 and 5).

NMR experiments were also performed on the peptides to assess their free state structures. Small $^1H_{\alpha}$ chemical shifts deviations from the random coil values, and narrow range of amide 1H peak dispersions (~ 1 ppm) suggest that the peptides do not adopt stable structures in solution (Supplementary Table 3). NOESY cross-peaks between protons > 2 residues apart were evident in some of the peptides (Supplementary Fig. 4) and these contacts were consistent with turn formation at the Kelch binding motifs.

The NRF2 site 1 peptide clearly had the largest number of NOESY cross-peaks of all the peptides. Several of these cross-peaks were between residues comprising the β -turn motif that forms the binding interface with the Kelch domain. The NOEs span about the same area as documented for the full-length mouse Neh2 domain¹⁵. NOESY cross-peaks between residues in i and $i + 3$ positions were also found in the PGAM5 and p62 peptides (Supplementary Fig. 4). Even though NOESY cross-peaks between residues > 2 residues apart were not clearly observed for the WTX, FAC1, PALB2 and PTMA peptides, the presence of such contacts cannot be ruled out. Incomplete resonance assignments and overcrowding made analysis of these spectra challenging. It is also important to note that the observed NOEs > 2 residues apart were weak-moderate for all peptides. This, along with near random coil chemical shifts for most protons suggest low folded populations in general. While the NMR results are consistent with the MD findings, further experimental work is necessary for a comprehensive comparison.

Overall, the MD simulations reveal that several, if not all, of the Kelch domain binding peptides analyzed contain β -turn like LMs at their binding sites. These structures likely display resemblance to their bound state conformations to certain extents. It is anticipated that preformed structures are important features in regulating the binding affinities and other thermodynamic parameters of the different interactions.

Binding affinity correlations. Because all experiments and simulations were conducted with the same conditions for each peptide, it was possible to assess correlations between the affinities of binding and their physical properties (Fig. 5). A good correlation ($r^2 = 0.77$) was found between Kyte Doolittle hydrophathy index (higher values indicates more hydrophobic content in the sequence) and the free energy of binding (ΔG) (Fig. 5a). This analysis helps to gauge how hydrophobic residues flanking the common binding motif may stabilize free state structure and affect binding affinity. The two main outliers in this correlation are the p62 and WTX pS286 peptides. For p62, this deviation is probably due to the lack of an E in position $i + 2$. The overestimated affinity of the WTX pS286 peptide is understandable considering the Kyte Doolittle scale does not include values for phosphorylated amino acids³⁵. Although phosphoserine was assigned the maximum negative value on the scale (-4.5), a more negative value is probably appropriate.

Circular variance values, a measure of the amplitude of backbone dihedral angle fluctuations, extracted from the MD simulations, also correlate well ($r^2 = 0.75$) with ΔG (Fig. 5b). The trend clearly illustrates that the binding affinity is heavily determined by the free state dynamics. By combining hydrophathy indices with circular variances (Fig. 5c), the correlation was moderately improved ($r^2 = 0.81$). Interestingly, the binding affinities correlate to a lesser extent ($r^2 = 0.50$) to the PONDR-FIT disorder tendencies (Fig. 5d). The major outlier here is PGAM5, with an average disorder probability of 0.36 for the 20-mer region of its sequence (Fig. 5d).

A higher affinity Kelch domain interacting peptide. Based on the findings from our analysis of the site 1-type proteins, a peptide with a higher affinity for the Kelch domain than any of the natural peptides (identified to date) was generated. This peptide aided our interpretation and understanding of the thermodynamics of interaction with the Kelch domain and, even more importantly, it may be a potential therapeutic agent³⁶. Based on the results obtained here and in our previous work¹¹, we hypothesize that by restricting a peptide to sample preferentially the bound state like conformation, the entropic cost of binding may be reduced, potentially increasing binding affinity. Therefore, our attempt at designing a higher affinity peptide focused on reducing free state entropy.

MD data indicated that β -turn formation at the Kelch domain binding sites is a common feature of the various peptides, therefore,

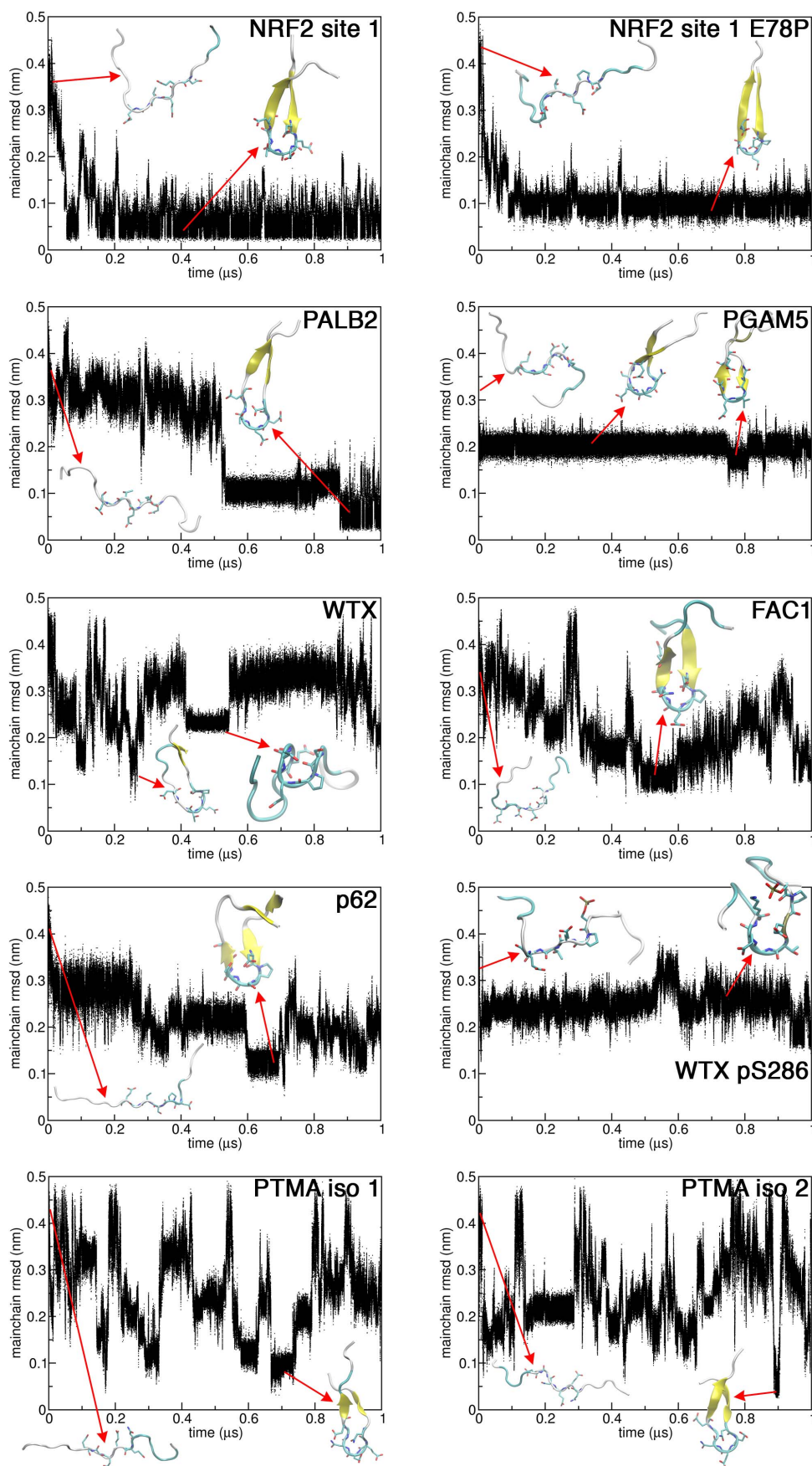


Figure 4 | Uncomplexed to complexed state comparison. For each peptide, 6 residues, including 24 mainchain (N, C $_{\alpha}$, C', and CO) atoms from the 'DEETGE' like regions (Fig. 1a) were compared to the corresponding atoms of the bound state NRF2-Kelch structure (PDB id: 2FLU)³⁰. Snapshots of the peptide conformations were rendered with VMD⁶².

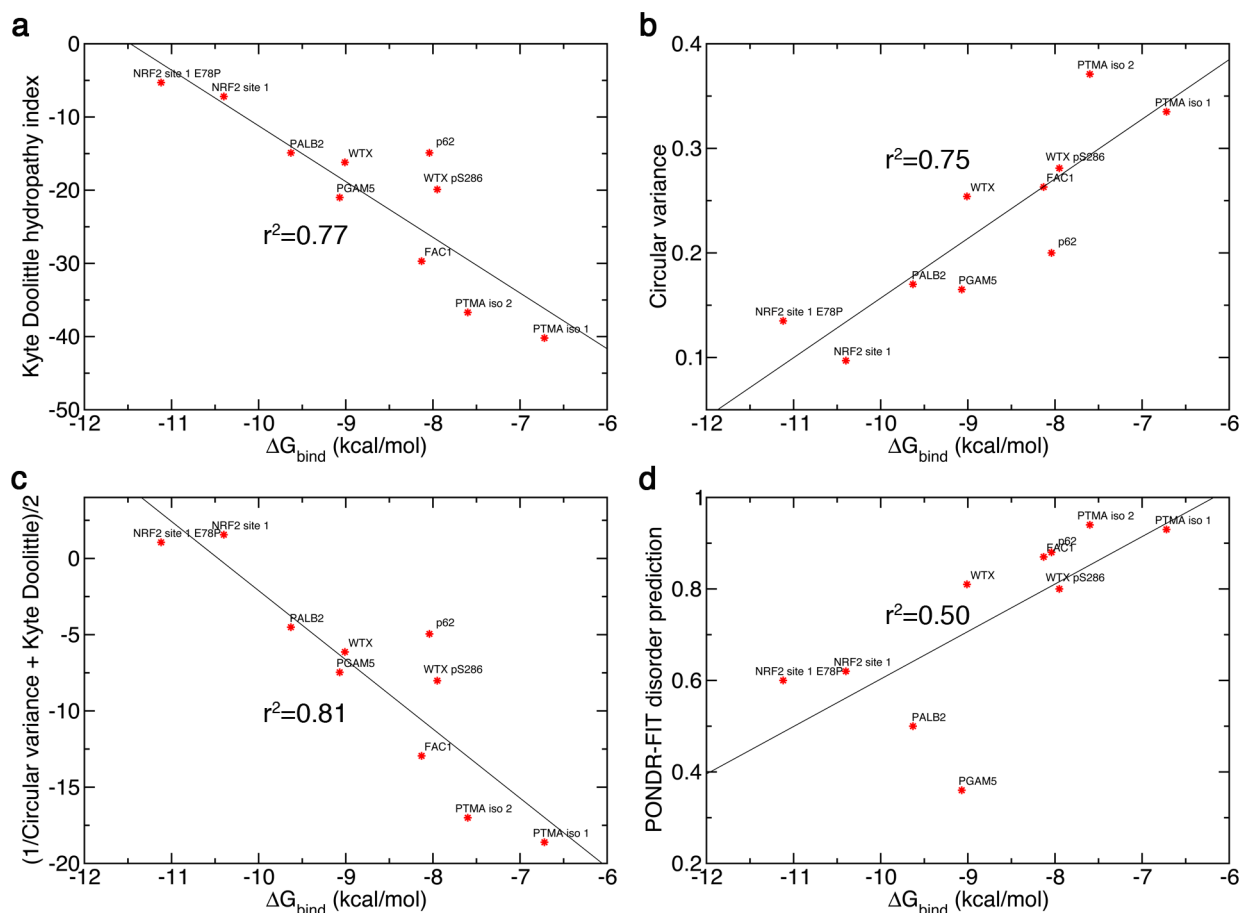


Figure 5 | Correlations between ΔG of binding and hydrophathy, disorderness, and circular variances. ΔG values were plotted against Kyte Doolittle hydrophathy indexes³⁵ (a), average circular variances (b), combined circular variances and Kyte Doolittle indexes (c), and PONDR-FIT disorder predictions (d) of the peptides shown in Fig. 1. For the WTX pS286 peptide, phosphoserine was assigned the maximum hydrophilic value on the scale (-4.5). The circular variances were calculated over the last 0.5 μ s of the MD trajectories. Disorder predictions were performed on the full-length sequences and the average values for the segments in Fig. 1a were plotted. For the WTX pS286 peptide, phosphoserine was changed to glutamic acid.

we aimed to increase the turn propensity of this region. The likely sites of turn formation in the site 1-type binders are the ‘DEET’-like regions¹¹ (Fig. 1a). The residue-specific and overall turn potentials of the ‘DEET’-like regions of the various proteins show that the high turn potentials of p62, FAC1 and WTX result primarily from their sequences containing proline at position $i + 1$ (Supplementary Table 5). Based upon this analysis, a single point mutation, E78P, was made to the natural, 20-mer NRF2 site 1 peptide (Fig. 1a). This single mutation increases the turn potential to 6.13, possibly enriching the population of molecules with defined structure in solution. ITC measurement showed that the E78P mutation indeed increases the binding affinity of the peptide (7 ± 1 nM) compared to the natural sequence by 3–4 fold (Table 1). Notably, this increase in binding affinity arises primarily due to a decreased entropic cost of binding. MD simulations were then used to examine the free state structures of the E78P peptide. The data confirms that the E78P peptide is able to adopt a hairpin structure with similar cross-strand contacts, region of compactness and bound state resemblance as the natural peptide (Fig. 2–4 and Supplementary Fig. 4–6).

Discussion

Using a combined experimental and computational approach, we have revealed factors that govern the binding affinity and specificity of different disordered partners to the Kelch hub. Our findings provide insights into the biological roles of the various protein-protein interactions and importantly, led to the design

of a high affinity Kelch-binding peptide, which may have therapeutic purposes.

We have grouped the proteins based on their binding affinities to Kelch as this may be helpful in deciphering the relationships between binding affinity and the biological functions of the various protein-protein interactions. Tier 1 consists of NRF2 only, which is the master regulator of the cellular oxidative stress response pathway^{14,37}. Tier 2 consists of PALB2, PGAM5 and WTX, which have K_d 's in the ~ 100 – 200 nM range. These proteins have been shown to promote NRF2-mediated cytoprotective gene expression, by presumably, disrupting the low affinity (K_d of ~ 1 μ M) site 2-Kelch domain interaction¹⁶. The last group of proteins, consisting of FAC1, p62 and PTMA, have dissociation constants > 1000 nM. PTMA contains a nuclear localization signal, and is thought to function as a vehicle for shuttling Keap1 into the nucleus³⁸. The transient nature of its shuttling role may explain its lower affinity. It should be noted that while the proteins discussed here interact with the Kelch domain of Keap1, the purpose of many of the interactions are not well established. The binding parameters reported here and hypothesis presented in several recent review articles may give insights into their possible roles^{39,40}.

The development of higher affinity Kelch domain ligands, which can compete with NRF2, is an area of active research. Several NRF2 inducers are currently in development or clinical trials for the treatment of chronic kidney disease, diabetes, cancer prevention, multiple sclerosis and oxidative tissue damage^{41–45}. However, many of these



compounds do not disrupt the NRF2-Kelch domain interaction directly. Compounds, such as our E78P peptide, which specifically bind to the NRF2 binding site on the Kelch domain can be alternative therapeutic agents. Head-to-tail cyclization or attachment of our E78P peptide to a cell-penetration peptide may further improve this peptide as a drug candidate⁶³.

In conclusion, our findings suggest that intrinsic disorder coupled with a preformed β -turn resembling LM located at the binding site is a common feature among many of the Kelch domain interacting proteins. The LMs are differentially stabilized by intramolecular contacts flanking and in close proximity to the Kelch binding region. The extent of motif stabilization is likely an important factor in modulating binding affinity. We found that the hydrophobic indices and free state dynamics of the peptides were well correlated with the measured free energy of binding. These parameters will be useful for predicting the affinities of other possible Kelch domain interactions. Based on this knowledge, we have selectively mutated the turn region of the NRF2 site 1 peptide to increase its binding affinity by reducing the conformational freedom of the free state. Importantly, this modified higher affinity peptide may have potential therapeutic applications. Our results provide insight into the biological roles of the various Kelch domain interactions and development of specific NRF2 inducers.

Methods

Protein purification and peptide synthesis. The Kelch domain of human Keap1 (residues 321–609), subcloned into the pET15b expression vector (from Dr. Mark Hannink, University of Missouri-Columbia), was expressed as an N-terminally His-tagged protein in *Escherichia coli* BL21 (DE3) and grown in minimal M9 medium. Protein expression was induced by addition of 0.5 mM IPTG at 18°C for 24 h. The protein was purified from the crude cell lysate by affinity chromatography using Ni Sepharose™ 6 Fast Flow beads (Amersham Biosciences). The His-tag was then cleaved by incubation with human α -thrombin (Haematologic Technologies Inc.) overnight at 4°C. The Kelch domain was purified from the cleavage mixture using a HiLoad Superdex-75 size-exclusion column (GE Healthcare) equilibrated with 50 mM sodium phosphate buffer, 100 mM NaCl, 1 mM DTT at pH 7.

Isothermal titration calorimetry (ITC) experiments. ITC experiments were carried out on a VP-ITC instrument (MicroCal) at 25°C. The protein and peptide samples were dialyzed into a buffer containing 50 mM sodium phosphate, 100 mM NaCl, 1 mM DTT at pH 7 and degassed before the experiments. ~40 μ M Kelch was added to the 1.4 mL sample cell and subjected to stepwise titration with 5 μ L aliquots of ~500 μ M peptide. Concentrations of Kelch and peptides were determined by amino acid analysis (Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, ON). The dissociation constant (K_d), molar binding stoichiometry (n) and the binding enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG) were determined by fitting the binding isotherm to a single-binding-site model with Origin7 software (MicroCal). All ITC experiments were performed in duplicate.

MD simulations. The amino acid sequences of all simulated peptides were the same as those used in the ITC experiments (Fig. 1a & Supplementary Table 4). We used the Crystallography & NMR System (CNS)⁴⁶ to generate an extended structure from each sequence. Simulated annealing was performed on each structure and resulting conformations that did not resemble the Neh2 domain site 1 region bound to the Kelch domain (PDB id: 2FLU) were used as starting structures³⁰. The N- and C-terminus of each structure was capped with acetyl (ACE) and NH₂ groups, respectively, using Chimera⁴⁷. For the WTX peptide with S286 phosphorylated (WTX pS286), a dianionic phosphate group (PO₄²⁻) was modeled onto S286 of the non-phosphorylated WTX peptide structure.

The MD simulations were performed using GROMACS (Groningen Machine for Chemical Simulations) version 4.5⁴⁸. GROMOS96 53a6 force field^{49,50} was used (Except in the WTX pS286 peptide simulation, where the GROMOS96 43a1p⁵¹ force field was used). This force field has been extensively tested and shown to perform well in simulations of IDPs^{10,11}. The starting structures were solvated in cubic boxes of linear size of 6 nm with periodic boundary conditions applied in all directions. The SPC (simple point charge) water model was used⁵². Protonation states of all ionizable residues were chosen based on their most probable state at pH 7. Histidine residues were protonated on ND1 only (a set of trajectories using the NE2 only histidine protonation state were highly similar; data for these simulations is shown in Supplementary Fig 7). Each system was overall charge-neutral and brought to an ionic strength of 0.1 M with sodium (Na⁺) and chloride (Cl⁻) ions. The simulations followed the protocols established in^{10,11,53,54} to avoid physical artifacts. Protein and non-protein atoms were coupled to their own temperature baths at 310 K using the Parrinello-Donadio-Bussi v-rescale algorithm⁵⁵. Pressure was maintained isotropically at 1 bar using the Parrinello-Rahman barostat⁵⁶. A 2 fs timestep was used. Prior to the simulations, the energy of each system was minimized using the steepest

descents algorithm. This was followed by 2 ps of position-restrained dynamics with all non-hydrogen atoms restrained with a 1000 kJ mol⁻¹ force constant. Initial atom velocities were taken from a Maxwellian distribution at 310 K. All bond lengths were constrained using the LINCS algorithm⁵⁷. A 1.0 nm cut-off was used for Lennard-Jones interactions. Dispersion corrections for energy and pressure were applied. Electrostatic interactions were calculated using the Particle-Mesh Ewald (PME) method⁵⁸ with 0.12 nm grid-spacing and a 1.0 nm real-space cut-off. Charge groups were not used (single atom charge groups)⁵⁴. Data was collected at 4 ps intervals and each simulation was run for at least 1 μ s. The total simulation time was 10 μ s.

Peptide dynamics were quantified by analyzing the circular variance ($C.V.$) of the ϕ and ψ dihedral angles over time. $C.V.$ is defined as⁵⁹:

$$C.V. = 1 - R/m \quad (1)$$

where m is the number of structures included in the analysis, and R is calculated using:

$$R = \sqrt{\frac{1}{2} \left[\left(\sum_{i=1}^m \cos \phi \right)^2 + \left(\sum_{i=1}^m \sin \phi \right)^2 + \left(\sum_{i=1}^m \cos \psi \right)^2 + \left(\sum_{i=1}^m \sin \psi \right)^2 \right]} \quad (2)$$

The value of $C.V.$ ranges between 0 and 1. Lower values represent tighter clustering about the mean and higher values are indicative of greater ϕ and ψ variability.

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Acknowledgements

We thank SharcNet (www.sharcnet.ca), the SciNet HPC Consortium and Compute Canada for providing the computational resources. Financial support was provided by the Ontario Early Researcher Award Program, Canadian Institutes of Health Research (CIHR; MOP 74679), University of Waterloo and Natural Sciences and Engineering Research Council of Canada (NSERC).

Author contributions

Conceived and designed the experiments: E.A.C., M.K., W.Y.C. Performed the experiments: E.A.C., R.C.K. Analyzed the data: E.A.C., R.C.K. Wrote the paper: E.A.C., M.K., W.Y.C.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Cino, E.A., Killoran, R.C., Karttunen, M. & Choy, W.Y. Binding of disordered proteins to a protein hub. *Sci. Rep.* **3**, 2305; DOI:10.1038/srep02305 (2013).



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