

## Supplementary Discussion

### 4E-BP2 Phosphorylation

Control of *in vitro* phosphorylation of IDPs is often very challenging due to the accessibility of non-specific sites leading to excess phosphorylation. However, in the case of 4E-BP2, it is surprisingly very difficult even to obtain the phosphorylation at the five *bona fide* proline-directed phosphorylation sites (T37, T46, S65, T70 and S83). When phosphorylation was performed by mixing all components in a 50 mL Falcon<sup>TM</sup> tube (Fisher Scientific), the reaction was inefficient, resulting in a mixture of predominantly 3- and 4-fold phosphorylated 4E-BP2, in a manner that was not sensitive to large amounts of added kinase and ATP. However, phosphorylation using a dialysis technique (described in the Methods) enabled efficient phosphorylation with the expected 5 proline-directed phosphorylations using stoichiometric amounts of kinase to substrate and ~10,000-fold ATP.

To definitively rule out phosphorylation at residues other than the proline-directed sites, we compared chemical shifts between the wild type and mutants of 4E-BP2. Analysis of the <sup>1</sup>HN chemical shifts for serines/threonines in phosphorylated 4E-BP2 (Fig. 1) suggests that S44 and S25 may also be phosphorylated. For example, the <sup>1</sup>HN chemical shift of S44 (which is located on the second hairpin, one residue from the phosphorylated T46) is downfield shifted to 9.62 ppm, similar to the shift observed for the five expected sites. However, it was observed that the chemical shift for C35, which occupies the structurally equivalent position in the first hairpin and cannot be phosphorylated, is similarly downfield shifted (9.25 ppm). The similarities of the chemical shifts of <sup>36</sup>TTPGGT<sup>41</sup> and <sup>45</sup>TTPGGT<sup>40</sup> provide additional support that the two hairpins are in very similar chemical environment and that the downfield shifts of S44 are due to structural effects. Furthermore, when each of the five proline-directed kinase phosphorylation sites (serine or threonine) is substituted to alanine individually or in combination, the mass spectra and the <sup>1</sup>H-<sup>15</sup>N HSQC spectra change accordingly. For example, phosphorylation of S83A to form pT37pT46pS65pT70 results in a <sup>1</sup>H-<sup>15</sup>N HSQC spectrum with a missing pS83 peak and local chemical shift changes compared to the phosphorylated wild type protein (Extended Data Fig. 8). Similar observations were made for the T37A/T46A, (i.e. pS65/pT70/pS83, see Extended Data Fig. 2a), showing no downfield shifts for S25 or S44.

Finally, we also verified the phosphorylation of each site independently through chemical shift analysis. Results show that, for wild type phosphorylated 4E-BP2, all serine <sup>13</sup>Cα/β chemical shifts are similar outside the folded region except (i) S65 and S83, which show upfield and downfield shifts for the Cα and Cβ, respectively, as a result of the phosphorylation (Extended Data Fig. 8), and (ii) S25 and S44, which show upfield and downfield shifts, respectively, as a result of tertiary contacts within the folded region (see chemical shift values in Extended Data Fig. 8). This interpretation is supported by data on phosphorylation of mutants that disrupt the structure formation and do not show such upfield/downfield chemical shifts for S25/S44 (see Fig. 3a,b). Surprisingly,

the secondary chemical shift changes for the C $\alpha$  was higher than that of C $\beta$  for S44, pS65 and pS83. This effect probably originates from the tertiary contacts that differentially affect the backbone more than the side chain.

*Proline isomerization, disorder-to-folded state equilibrium and conformational exchange within the folded state of phosphorylated 4E-BP2.*

Upon phosphorylation, the major state of P18-R62 of 4E-BP2 becomes folded while the rest of the protein remains disordered. Interestingly, there are multiple minor peaks that remained unassigned (Extended Data Fig. 1c) and these cannot be accounted for by the cis-trans isomerization of the disordered regions of phosphorylated 4E-BP2 alone. This observation as well as the high affinity of interaction with eIF4E of the folded pT37/pT46 suggest that the folded state is in dynamic equilibrium with a disordered unfolded state. While 4E-BP2 contains eleven proline residues, three are within the phosphorylation-induced fold. P38 and P47, which form tight  $\beta$ -turns resulting in hairpin formation, are both in the trans-conformation with their cis-conformations being incompatible with the folded state. However, P31, located on the long flexible loop connecting strands  $\beta$ 1 and  $\beta$ 2, can exist in multiple conformations compatible with cis-trans isomerization (Extended Data Fig. 3b).

The significant number of NOE violations to the folded structure of the phosphorylated 4E-BP2 is evidence for conformational exchange in the folded region, including for this long loop. This ensemble has an unusually high number of violations for a folded protein at an average of 8% per model, with 5% being completely unsatisfied across all models; these violations are consistent with the contribution of minor state populations, as the vast majority occur between residues that are already in close spatial proximity to one another and which could be satisfied by minor rearrangement of the fold, such as an elongation of the strand pairing between strands 2 and 3. The overall fold of the protein is apparent in the majority of the data, with two clear hairpin contacts showing up as contacts extending perpendicular to the diagonal (the x=y line), and the one long-range parallel strand pairing demonstrated with NOEs between strands 1 and 4 (Extended Data Fig. 6). The violated NOEs cluster primarily at the open edges of the two hairpins furthest away from the turn, such that they remain in close sequence proximity to satisfied restraints, reflecting fraying hairpins.

Extended Data Fig. 6b-d show examples of NOE violations consistent with dynamic conformational exchange. A detailed look at individual NOE pairs supports the conclusion that minor conformations contribute to the high number of violations, as consistently violated restraints conflict with the majority of the data that define the major conformation. Consistently violated restraints are shown in red and selections of satisfied restraints that represent the major state are shown in yellow. In (b) the position of Y54 in the ensemble is determined by HE-HN NOE restraints holding it in a fixed orientation to the surface of the sheet. Of these restraints, the majority can be satisfied by the orientation observed in the model, at least one of which would have to be broken in order to satisfy the consistently violated restraint observed between Y54HE and L42NH. While the

major state is inconsistent with this NOE, it could derive from a minor state population containing the same sheet with Y54 flipped in the opposite direction, a simple dynamic event. (c) The violated restraint between C35NH and T37NH shows similar behavior. The pairing between strands 2 and 3 satisfies the majority of the data, including both NOEs and the secondary structure restraints derived from the chemical shifts. The unsatisfied restraint disagrees with the majority of the data but a minor state in which strand 2 breaks its beta-sheet hydrogen bonds allowing a turn conformation could enable it to be satisfied. (d) Finally, the largest cluster of medium range violated NOEs comes from the sequence leading up to strand 2, where the violated restraints would pull those residues down towards the adjacent portion of strand 3. Some of these NOEs could be consistent with cis-trans proline isomerization at P31 in this long loop N-terminal to strand 2. Many could be satisfied by an alternate conformation in which the pairing between strands 2 and 3 is elongated. However, this would require breaking the long-range pairing between strands 1 and 4. The stability of the isolated hairpins for the singly pT37 or pT46 phosphorylated proteins or for the Y54A/L59A mutant, as well as the heteronuclear NOE values of less than 0.8 and NMR resonances reflecting minor conformers, provides support for the lack of structural cooperativity, enabling dynamic exchange with alternate conformations.