FHA: A New Phosphoprotein Binding Domain in Signal Transduction (1999-present). FHA is a newly discovered domain in signal transduction. Our lab was again the first group to determine the solution structure of an FHA domain. Subsequently the crystal structure of a complex has been determined by X-ray, and we have also determined the solution structures of many more structures of free and complexed FHA domains. Most importantly, we have used combinatorial libraries to demonstrate that FHA domains can bind both phosphotyrosine peptides and phosphothreonine peptides, and that FHA domains from different proteins confer different ligand specificity. For example, the FHA1 domain of yeast Rad53 is specific to pTXXD motif, while the FHA2 domain from Rad53 is specific to pTXXL as well as pYXL motifs. The structural basis of different ligand specificity has been elucidated by solution structures of several FHA-phosphopeptide complexes. Subsequently, the project has ventured into a few uncharted terrories as summarized below:

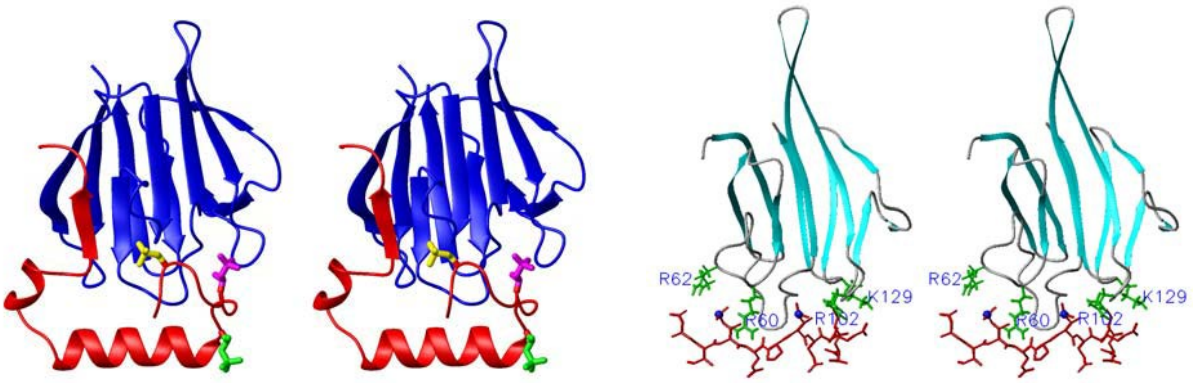
Human Ki67 FHA. Ki-67 antigen protein, with 3256 amino acid residues including a FHA domain near its N-terminus, is widely used as a cancer marker because of its absence in resting cells and its appearance in the nucleus and on the chromosomal surface in interphase and mitotis respectively. However its biological function is relatively unknown. After unsuccessful attempts to identify short phosphopeptides that can bind to Ki67 FHA, we showed that the synthetic fragment 226-269 of its binding partner protein hNIFK binds Ki67 tightly if Thr234 is phosphorylated. In vitro kinase assays showed that Thr234 can be phosphorylated by GSK3, but only if Thr238 is first phosphorylated by CDC2/cyclin B. The structure of the Ki67 FHA complex with NIFK(226-269) phosphorylated at S230, T234 and T238 was then solved by NMR. The structure shows an extended binding surface for the protein-protein interactions (Figure 4). This structure represents the most extensive structural information of an FHA domain-phosphoprotein complex. It clearly shows that the interaction goes beyond the short stretch of the pThr site. It also provides a basis for quantitative evaluation of specific interactions. For example, changing pThr to pSer led to a decrease of binding affinity by a factor of 70, and deleting the β-strand led to a loss of a factor of 180.

Yeast Rad53 and Dun1 Kinases. Recently we have discovered a new mechanism of signaling, the "phospho-counting" mechanism involving by FHA domains. FHA domains and SQ/TQ cluster domains (SCDs) play important roles in DNA damage signalling. The Rad53-SCD1 has dual, genetically separable functions in regulating the activation of the Rad53 kinase, and in the subsequent phosphorylation-dependent and FHA domain-dependent activation of Dun1 kinase by Rad53 in *Saccharomyces cerevisiae*. However, the molecular mechanism how a single phosphorylation site cluster can regulate the ordered, sequential activation of a kinase cascade has remained a conundrum. Our results show that the SCD1 of Rad53 serves as an FHA domain-dependent phospho-counting switch for sequential activation of the Rad53-Dun1 checkpoint kinase cascade. We found that the Dun1-FHA domain has ~100-fold increased affinity for di-phosphorylated as compared to mono-phosphorylated Rad53-SCD1. NMR structures of the complexes demonstrate that the specificity for di-phosphorylated SCD1 results from a second phospho-threonine binding site not reported in other FHA domains. *In vivo*, any single threonine of Rad53-SCD1 is sufficient for *RAD53*-dependent survival of replication fork stalling, but two adjacent threonines in the Rad53-SCD1 are necessary for the *DUN1*-dependent transcriptional induction of ribonuclease-reductase (*RNR*) genes following replication blocks. The results indicate an SCD1 phosphorylation state counting mechanism that tunes the activation of the Rad53-Dun1 kinase cascade to the strength of the checkpoint signal, with a lower

threshold for Rad53 activation and urgent replication fork stabilisation, and a higher threshold for Dun1 activation to increase nucleotide supply for restart of multiple stalled forks. This study thus provides the biochemical, structural and biological basis for a novel phospho-counting switch mechanism in signal transduction.

Current and Future Directions. The two systems described above, Ki67 and Rad53/Dun1, will both be further continued. The goals are to uncover molecular mechanisms of signaling, by combining biochemical, structural, and biological approaches. In addition, we are also characterizing the functions and structures of a newly identified human cancer-related protein, TIFA, particularly in its interaction with TRAF2. A major emphasis of our work will be to identify phosphorylation sites in vivo, and then characterize FHA-phosphoprotein interactions both in vitro and in vivo. This is a new frontier of structural biology and we are already at the forefront of this field.

Figure 4. Structures of the Ki67FHA-NIFK(226-269, triphosphorylated) complex (left), and the Dun1FHA-Rad53SCD1(diphosphorylated) complex determined by NMR.



Recent Results (from paper 18 below):

Mammalian MDC1 interacts with CHK2 in the regulation of DNA damage-induced S-phase checkpoint and apoptosis, which is directed by the association of MDC1-FHA and CHK2-pThr68. However, different ligand specificities of MDC1-FHA have been reported, and no structure is available. Here we report the crystal structures of MDC1-FHA and its complex with a CHK2 peptide containing pThr68. Unlike other FHA domains, MDC1-FHA exists as an intrinsic dimer in solution and in crystals. Structural and binding analyses support the pThr+3 ligand specificity, and provide structural insight for MDC1-CHK2 interaction.

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