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In vitro FANCD2 monoubiquitination by HHR6 and hRad18

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Fanconi anemia is a rare hereditary disorder characterized by short stature, progressive bone marrow failure, and a high susceptibility to several different forms of cancer. It is caused by a deficiency in one of several proteins that comprise the FA pathway (FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N, O, and P), which is involved in the regulation of various forms of DNA damage repair. Cells with an impaired FA pathway are particularly sensitive to DNA cross-linking agents, such as MMC, cisplatin, and UV radiation. Several of the proteins (FANCA, B, C, E, F, G, L, and M) form the FA core complex, which acts as an E3 ubiquitin ligase to monoubiquitinate FANCD2 and FANCI following DNA damage. Upon ubiquitination, FANCD2 and FANCI form a heterodimer which aggregates in nuclear foci with several downstream proteins, such as FANCD1/BRCA2, FANCN/PALB2, and FANCJ/BRIP1, which function in interstrand crosslink or homologous recombination repair mechanisms.[1,2] The monoubiquitination of FANCD2 is considered to be the focal point of the pathway, as the phenotypic result, including human cancer, of most FA mutations stems from the inability of FANCD2 to be ubiquitinated in response to DNA damage. In response to DNA lesions resulting from stalled replication forks, the human homolog of yeast rad 6 (HHR6)/hRad18 pathway is responsible for the ubiquitination of a number of proteins involved in homologous recombination repair and translesion synthesis by the E2 and E3 enzymes HHR6 and hRad18, respectively.[3] As a deficiency of the HHR6 ortholog in yeast cells, Rad6, causes a sensitivity to DNA crosslinking agents similar to that found in FA-deficient cells, a link between the 2 pathways was hypothesized. Indeed, our studies show that HHR6 and hRAD18 are closely involved in the regulation of FANCD2 ubiquitination;[3-5] however, whether this regulation is direct or indirect remains unknown. Currently 8 proteins are known to be included in the FA core complex; however, it is possible that the core may contain other proteins yet unidentified. For that reason a system to study the monoubiquitination of FANCD2 by the FA core complex in vitro is not yet a feasible method. It is known, though, that HHR6 and hRAD18 are fully capable of acting as E2/E3 partners in ubiquitination reactions,[6] and therefore could be used in simple, effective ubiquitination reactions in vitro. If HHR6 and hRAD18 are indeed able to directly ubiquitinate FANCD2, they would be vital in creating a system for studying FANCD2 monoubiquitination in vitro, which would be a valuable tool for Fanconi anemia research and for the investigation into the convergence of the FA and HHR6/hRad18 pathways. To test the ability of HHR6 and hRad18 to directly ubiquitinate FANCD2, GST-HHR6, and MBP-hRad18 fusion proteins were created, as well as His-wtFANCD2 and His-mtFANCD2, a form of FANCD2 containing a K561R mutation eliminating the lysine residue onto which ubiquitin is covalently bonded. These proteins were purified from either baculovirus or bacteria expression systems. Based upon the working system established from Dr Wang's laboratory (NIH-NIA), an in vitro ubiquitination assay was modified by using biotinated ubiquitin to replace the isotope-labeled one. Surprisingly, Avidin-HRP detects wtFANCD2 but not k561R mtFANCD2 at the size, similar to FANCD2 protein, detected by FANCD2 antibody on the same blot after stripping. These results demonstrate that HHR6 and hRad18 can monoubiquitinate FANCD2 at K561 in vitro. At the moment, it remains unknown of whether only HHR6 pairing with hRad18 can deliver the ubiquitination reaction, which needs to further dissect E2-E3 partnership with many other known E2s or E3s. However, this in vitro monoubiquitination of FANCD2 can serve as a research tool in studying the FA tumor-suppressor pathway, which has been found to play a significant role in the suppression of the development of non-FA cancers.[5,7] In addition, the importance of the observations made goes beyond the development of a new assay. Given that FANCD2 is relatively well conserved across a variety of species,[1] compared with the other FA proteins (those in the FA core complex are generally only found in mammalian cells), the monoubiquitination of FANCD2 by HHR6 and hRad18 may represent a novel stress response pathway equipped at the beginning of evolution. This is because that orthologs of HHR6 and hRad18 are present in most species.[8] The FA proteins, however, join this hypothetical primordial stress-response pathway for fine-tuning to meet the signaling plasticity essential to mammalian cells. Nonetheless, it is certain that the successful demonstration of FANCD2 monoubiquitination by HHR6 and hRad18 will have a greater impact on FA cancer research than what we can imagine for now.

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