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F-box proteins FBXO31 and FBX4 in regulation of cyclin D1 degradation upon DNA damage

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Cell cycle progression is precisely regulated -- positively by cyclins and cyclin dependent kinases (CDKs) and negatively by CDK inhibitors, consisting of INK (e.g. p16) and KIP (e.g. p27) family members. During a normal cell cycle, cyclin D1, an essential mediator of G1 phase progression, accumulates in mid-G1 phase as a result of transcriptional activation by mitogenic signals, whereas it decreases in S phase due to targeted degradation by SCF E3 ubiquitin ligases.

SCF ligases, consisting of Skp-1, Cullins, F-box proteins and the RING domain containing protein RBX1/ROC1 or RBX2/ROC2/SAG, are the largest E3 ubiquitin ligases that regulate a variety of biological processes by timely promoting degradation of diverse substrates. Structurally, Cullin-1 acts as a scaffold protein, where its N-terminus binds to Skp1-F-box complex, and the C-terminus binds to RBX1. The core SCF E3 ubiquitin is the complex of cullins and RBX1; the latter binds to E2 and catalyzes the transfer of ubiquitin from E2 to substrates. The substrate specificity of SCF complex is, on the other hand, determined by F-box proteins that recognize phosphorylated substrates for binding and subsequent degradation. Up to the present, three F-box proteins, FBXO31, FBX4, and FBXW8 have been identified to bind to phospho-cyclin D1^{Thr286} for targeted degradation in response to DNA damage and MAPK signals (Lin et al, 2006; Okabe et al, 2006; Pontano et al, 2008; Santra et al, 2009).

The most recent work by Michael Green's group, published in the June 4 issue of *Nature*, unveiled an essential role of F-box protein FBXO31 in targeted degradation of cyclin D1 in response to DNA damage in SK-MEL-28 melanoma cells (Santra et al, 2009). FBXO31 was initially identified in a genome-wide siRNA screen by the same group as a candidate protein required for oncogenic BRAF to induce senescence in primary fibroblasts and melanocytes (Wajapeyee et al, 2008). Functional follow-up studies revealed that ectopic expression of FBXO31 induced G1 arrest in melanoma cells with associated reduction of cyclin D1, but not other cyclins or CDKs, suggesting that cyclin D1 is a putative substrate of FBXO31. Elegant biochemical characterization showed that FBXO31 binds to and directs ubiquitination of wild type cyclin D1, but not its phospho-refractory mutant, cyclinD1-T286A, firmly establishing that cyclin D1 is a *bona fide* substrate of SCF^{FBXO31} E3 ubiquitin ligase. More importantly, FBXO31-mediated cyclin D1 degradation is a cellular response to DNA damage. Upon exposure to ionizing radiation as well as many other DNA damaging agents, FBXO31 is induced and stabilized by ATM-mediated phosphorylation. Induced FBXO31 then binds to

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Santra, M.K., Wajapeyee, N., Green, M.R. (2009). F-box protein FBXO31 mediates cyclin D1 degradation to induce G1 arrest after DNA damage. *Nature* 459, 722-5.

Pontano, L.L., Aggarwal, P., Barbash, O., Brown, E.J., Bassing, C.H., Diehl, J.A. (2008). Genotoxic stress-induced cyclin D1 phosphorylation and proteolysis are required for genomic stability. *Mol Cell Biol.* 28, 7245-58

phospho-cyclin D1^{Thr286} (phosphorylated by an undefined MAP kinase) for targeted degradation, leading to G1 arrest (Figure 1). Furthermore, FBXO31 siRNA silencing abrogates the G1 arrest after DNA damage and sensitizes melanoma cells to radiation (Santra et al, 2009). The significance of this work is multiple-fold. First, it demonstrated that FBXO31 is a novel and physiological relevant F-box protein for cyclin D1 degradation by SCF E3 ubiquitin ligase. Second, FBXO31 induction and subsequent cyclin D1 degradation appear responsible for a rapid initiation phase of G1 arrest, which differs from the p53/p21-mediated slow maintenance phase of G1 arrest after genotoxic stress. This resolves a long-standing question with regard to what else also causes cyclin D1 degradation, leading to rapid G1 arrest upon DNA damage, in addition to anaphase-promoting complex/cyclosome (APC/C) (Agami & Bernards, 2000). Third, the study suggests that FBXO31 could serve as a radiosensitizing target. A small molecule that either inhibits cyclin D1 phosphorylation at Thr286 or disrupts FBXO31-cyclin D1 binding could act as a radiosensitizer.

The involvement of cyclin D1 degradation in response to DNA damage was originally reported by Agami and Bernards in 2000 by APC/C via a destruction motif, RXXL on cyclin D1 (Agami & Bernards, 2000). A similar observation was also reported by Alan Diehl's group last year, but by SCF E3 ubiquitin ligase with associated F-box protein being Fbx4- α B crystalline complex, following cyclin D1 phosphorylation on Thr286 (Pontano et al, 2008). Previously, Diehl's group characterized cyclin D1 as a substrate of SCF^{Fbx4- α B crystalline} upon GSK3 β -mediated phosphorylation on Thr286 (Lin et al, 2006) and found *FBX4* mutations in human esophageal carcinoma that abrogate SCF^{Fbx4} ligase activity, contributing to cyclin D1 overexpression (Barbash et al, 2008). In this recent study mainly conducted in NIH3T3 cells, Diehl's group reported that upon genotoxic stress, cyclin D1 was rapidly phosphorylated at Thr286 by GSK3 β in an ATM signaling-dependent manner, followed by binding to FBX4- α B crystallin, leading to cyclin D1 degradation by SCF^{FBX4- α B crystalline} E3 ubiquitin ligase. Loss of FBX4-dependent cyclin D1 degradation via expression of cyclin D1-T286A mutant or siRNA silencing of FBX4 triggers radio-resistant DNA synthesis and compromises the intra-S-phase checkpoint response to DNA damage. These changes lead to accumulation of chromatid breaks, and sensitization of NIH3T3 cells to an S-phase-specific chemotherapeutic drug, camptothecin (Pontano et al, 2008) (Figure 1).

It is well-established that DNA damage checkpoints are required for cells to repair damaged DNA and to maintain genomic stability upon genotoxic stress. These new findings by Green' and Diehl's groups clearly demonstrated that cyclin D1 degradation via SCF E3 ubiquitin ligase upon DNA damage is required for G1 arrest in melanoma cells and for genomic stability in NIH3T3 cells. While these findings are novel and important, several mechanistic questions are worth further investigation.

First, what is the molecular determinant for recruitment of different F-box proteins (FBXO31 in melanoma cells vs. FBX4 in NIH3T3 cells) to mediate cyclin D1 degradation in response to DNA damage (Pontano et al, 2008; Santra et al, 2009)? Both FBX4 (also called FBXO4) and FBXW8, another F-box protein known to promote cyclin D1 degradation in response to MAP kinase signals (Okabe et al, 2006), are expressed in melanoma SK-MEL-28 cells. However, neither is induced by ionizing radiation, nor does siRNA silencing of either protein affect cyclin D1 degradation (Santra et al, 2009). Thus, at least in SK-MEL-28 melanoma cells, FBX4 and FBXW8 appear not involved in cyclin D1 degradation in response to ionizing radiation.

Second, it is unclear how phosphorylation of cyclin D1 at the same Thr286 site is mediated by different kinases in different cells after exposure to the same DNA damaging agent (ionizing radiation). In melanoma cells, FBXO31-mediated cyclin D1 degradation does not require GSK3 β , but an undefined MAP kinase which is sensitive to a MAPK inhibitor (Santra et al,

2009). In contrast, in NIH3T3 cells, FBX4-mediated cyclin D1 degradation does require GSK3 β , but not p38^{SAPK}, a kinase implicated in stress-induced cyclin D1 phosphorylation in another system (Pontano et al, 2008). Thus, different F-box proteins/kinases used for cyclin D1 degradation in response to the same ionizing radiation may explain a different biological consequence in tumor cells (melanoma, for G1 arrest) vs. normal cells (NIH3T3, for S phase checkpoint and genomic stability) (Figure 1).

Finally, mammalian cells arrested in the G1 phase of cell cycle are, in general, more resistant to radiation or chemotherapeutic drugs. The *Nature* study showed that abrogation of FBXO31-mediated cyclin D1 degradation results in abrogation of G1 arrest and sensitization of SK-MEL-28 melanoma cells to radiation (Santra et al, 2009), suggesting that FBXO31-cyclin D1 complex could be a potential radiosensitizing target in melanoma cells. The next obvious question is to determine if this observation can be extended to other lines of melanoma cells, but not to normal melanocytes, since therapeutic value can only be gained when radiosensitization by targeting FBXO31-cyclin D1 is melanoma-specific.

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Figure 1. FBXO31 and FBX4 regulate cyclin D1 degradation via distinct pathways

DNA damage induced by ionizing radiation activates ATM. In melanoma cells, activated ATM phosphorylates and stabilizes F-box protein, FBXO31, which binds to phospho-cyclin D1^{Thr286} (by a MAPK) and directs its ubiquitination and subsequent degradation, leading to G1 arrest (Santra et al, 2009). In NIH3T3 cells, ATM activation potentiates cyclin D1 phosphorylation at Thr286 by GSK3β. Phospho-cyclin D1^{Thr286} is then recognized by F-box FBX4/αB crystalline complex for targeted degradation by SCF^{FBX4/αB crystalline} E3 ubiquitin ligase, leading to intra S phase checkpoint control and genomic stability (Pontano et al, 2008).