Perspective

Fbxo7 Gets Proactive with Cyclin D/ckd6

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ABSTRACT

Although all three D cyclins bind and activate cdk2, 4 and 6, Fbxo7 has been characterised as a selective enhancer of cdk6 activity. It increases activation by directly facilitating cdk6 interaction with viral and cellular D cyclins. Fbxo7 overexpression has transforming activity in murine fibroblasts, and it is also highly expressed in human cancer, suggesting it is a potential oncogene. Fbxo7 has the ability to activate cell cycle regulators, and is part of an E3 ubiquitin ligase. We postulate Fbxo7 coordinates the ubiquitination of its substrates with cell cycle entry. It may therefore represent a means to integrate cell signals and control disparate biological processes during the early part of the cell cycle.

INTRODUCTION

Normal cell growth is characterised by regulated waves of synthesis and degradation of cell cycle regulators. Many diverse signalling pathways, variously affecting transcription, translation, assembly, localisation, activation and degradation converge on the activity of a class of serine/threonine kinases known as cyclin-dependent kinases (cdk) which promote cell cycle progression. Two classes of multi-subunitted E3 ubiquitin ligases, SCF (Skp1-Cdc53/Cul1-F box protein) and APC (anaphase promoting complex), are directly involved in the proteolysis of cell cycle proteins, including cyclins, the activators of cdk5. The APC functions during anaphase until the following S phase and is responsible for the degradation of mitotic cyclins and proteins involved in sister chromatid separation. Its activity enables the completion of mitosis and imparts irreversibility and thus directionality to the cell cycle.1 SCF E3 ligases, on the other hand, are active throughout the cell cycle, and engage with their target proteins through an F box protein (Fbp). Fbps are a diverse family of proteins with a defining motif, the F box motif, which binds to Skp1, linking it to the rest of the E3 scaffold. There are over 70 Fbps, and they contain a collage of protein-protein interaction motifs, like WD40 repeats and leucine zippers, which recognise substrates via phosphorylated motifs known as ‘degrons.’ In screens for Skp1 binding proteins using yeast two hybrid methodology and by genome wide sequence analysis for F box motifs within proteins, this large family of human F box motif-containing proteins was identified.2-4 In simple terms, the juxtaposition of a protein-interacting domain with an F box should create a ‘ubiquitinator’ for any interacting proteins.

F box proteins contact the substrate and thus provide the specificity for subsequent ubiquitination. In actual fact, it is not an exclusive relationship in that several substrates have been implicated as being targeted by a single F box protein, e.g. Skp2 has been reported as ubiquitinating p27,5-7 p21,8,9 free cyclin E,10 Orc1,11 c-Myc,12,13 FOXO1;14 Fbw7 targets cyclin E,15,16 Notch1,17-20 c-Jun21,22 and C-Myc23,24 while β-TrCP ubiquitinates IKBα/β and β-catenin.21-24 Moreover, another E3 ubiquitin ligase, KPC, ubiquitinates p27 in G1 phase,25 indicating several different ubiquitinating activities can converge on a single protein. The cell therefore has a large repertoire of ubiquitinating enzymes, perhaps with varying affinities for their substrates or capacities for ubiquitination. To date, the majority of SCF-type E3 ligases remain orphan ligases as their targets are unknown.

The canonical Skp2 and Fbw7 are well characterised as interacting with p27 and cyclin E, respectively catalyzing their ubiquitination, precipitating their degradation by the 26S proteasome.26 As p27 and cyclin E influence passage through the G1 restriction point, so in effect do Skp2 and Fbw7 as their regulators. It has been more recently demonstrated
that the functional consequences of ubiquitination for the target protein can vary from degradation to differential trafficking to altered functionality.\textsuperscript{27-30} This indicates that ubiquitination is as pleiotropic a signal as phosphorylation, and therefore that ubiquitin ligases have the potential to bring about different functional consequences to their interacting partners.

**Fbxo7, a selective enhancer of cdk6.** We have recently reported on the characterisation of another Fbp which interacts with proteins that control the G1 restriction point.\textsuperscript{31} Fbxo7 was cloned in a yeast two hybrid screen using the cyclin from *Herpesvirus saimiri* as bait. Viral cyclins from oncogenic herpesviruses preferentially bind and robustly activate cdk6, transforming it into an inhibitor-resistant kinase with an enhanced substrate range.\textsuperscript{32} Viral cyclins have sequence homology with cellular D-type cyclins but when complexed to cdk6, function like three cyclin/cdk complexes which control the G1 to S phase transition: D/cdk6, cyclin E/cdk2, and cyclin A/cdk2. We identified Fbxo7, as interacting with viral and cellular cyclin D/cdk6 complexes. Fbxo7 had been identified in screens and characterised as interacting with the components of SCF1 ligases and as having ubiquitin ligase activity, although its substrates for ubiquitination were not known.\textsuperscript{2,4} The interaction of Fbxo7 with cyclins suggested that it may be part of an SCF-type E3 ubiquitin ligase responsible for the ubiquitin-mediated degradation of cyclins. Cyclins are ubiquitinylated proteins which are very short lived proteins, having a half-life of less than 15 minutes when located in the cytoplasm.\textsuperscript{35} However, neither the over-expression of Fbxo7 nor the reduction of its levels impacted on the abundance of cyclins (D-type, E or A) or of cdk6. Instead of potentiating their degradation, Fbxo7 specifically increased the abundance of D-type cyclin/cdk6 complexes.\textsuperscript{31} Fbxo7 achieved this through selective and direct interaction with cdk6 but not cdk4 or cdk2, the other cdk's which are bound and activated by D-type cyclins. This preference for cdk6 was seen both in vitro and in vivo, although the molecular basis for the selectivity is not known.

Fbxo7 behaved as a facilitator of cyclin D/cdk6 complexes which can potentially occur via a variety of mechanisms in vivo. Firstly, an enhancer may act as an assembly factor, facilitating the interaction of one subunit with the other, or it could also stabilise the complex after its formation, preventing its dissociation. Alternatively, it could shield the complex from degradation by enhancing its import into the nucleus, where cyclin D has a much longer half-life,\textsuperscript{35} preventing its export from the nucleus, or preventing its interaction with other proteins that promote its degradation (other ubiquitin ligases or the proteasome). All of these activities, alone or in combination, would result in an overall increase in cyclin/cdk6 complexes and activity.

When the ability of Fbxo7 to facilitate cyclin D association with cdk6 was tested by in vitro binding assays, it was compared to a known assembly factor for cyclin D/cdk4 complexes, p27.\textsuperscript{31} Surprisingly cyclin D1 and cyclin D3 showed different affinities for cdk6 binding. Cyclin D3 interacted with cdk6, but its binding to cdk6 was enhanced by the presence of p27 and Fbxo7 in concert. Cyclin D1 bound cdk6, but only in the presence of p27, and the abundance of complexes was not greatly affected by the presence of Fbxo7. These different affinities and sensitivities of the D cyclins for cdk6 to the presence of p27 and Fbxo7 might temporally order or sharpen a cell's responsiveness to mitogenic stimuli. Moreover, these in vitro sensitivities also suggest that Fbxo7 could have a greater influence as an assembly factor in cells where cdk6 and its activation by cyclin D3 is important, for example in the hematopoietic compartment. In vivo, Fbxo7 increased cdk6 activity in the absence of p21 and p27, suggesting its activity acts in parallel to them. Thus Fbxo7 is capable of fielding signals and translating outcomes channelled specifically through the cyclin D/cdk6 pathway, its activity being distinct from the known assembly factors.

While investigating motifs required for binding to cdk6, (neither an N-terminal Ubl motif nor the F box motif were required), we identified two independent domains within Fbxo7 that were independently sufficient to mediate an in vivo interaction with cdk6; one is a discrete motif, approximately a third of the way along the length of the protein and the other is within the C terminal proline rich domain (our unpublished results). This is indicative of a bipartite interaction of Fbxo7 with cdk6, potentially when it is in complex with a D-type cyclin. This could indicate that Fbxo7 physically holds cyclin D together with cdk6, like molecular forceps, and explain an assembly factor function of Fbxo7.

Fbxo7 also localises in and out of the nucleus, utilising a Crm1-dependent pathway, suggesting that it could chaperone cyclin D/cdk6 complexes into the nucleus.\textsuperscript{31} In addition, by virtue of its direct interaction with cyclin D/cdk6 complexes in vivo, Fbxo7 could prevent a different E3 ubiquitin ligase, one involved in the ubiquitin-mediated degradation of D-cyclins, from targeting these complexes. Thus the stabilising activity of Fbxo7 could occur at any point in the life cycle of cyclin D (1, 2, or 3)/cdk6 complexes.

**Fbxo7 as an integrator of cell signalling: linking ubiquitination with assembly.** Given the dominant hypotheses about the function and mechanisms of SCFs, it was perplexing that Fbxo7 did not increase the degradation of those proteins with which it interacted but rather increased their assembly and activity. However, a more 'traditional' outcome for the ubiquitination of proteins by Fbxo7 has been reported for a protein, HURP (hepatoma upregulated protein), whose phosphorylation by cyclin B/cdk1 results in its recognition by the proline-rich domain of Fbxo7 and its subsequent degradation.\textsuperscript{34} Although the function of HURP protein has not been elucidated, an increase in its abundance may be important in liver cancers. We reported Fbxo7 to be upregulated in its expression in human lung and colon cancers, and have transforming activity through cdk6. However, we did not address its expression in hepatocytes or whether the deregulation of Fbxo7 expression might be important in the development of hepatomas.

We have also identified additional proteins which interact with Fbxo7 (R. Kirk, H. Laman, and N. McDonald, unpublished results), which are being investigated as candidates for Fbxo7-mediated ubiquitination, resulting in possible degradation, altered function, assembly or localisation. We hypothesize that Fbxo7 provides a means to link entry into the cell cycle with the ubiquitination of other Fbxo7-interacting proteins, thus coordinating cell cycle progression with other biological processes. It remains to be tested whether other F box proteins have an assembly or other functions in addition to their capacities to promote ubiquitin-mediated degradation.

**SCF deregulation and cancer.** The proteins which directly impact on the G1 restriction point, cyclin D/cdk4/6/INK4a/pRb, are commonly mutated during tumor formation.\textsuperscript{35,36} Consequently it was hypothesized that mutation of Fbps might also contribute to tumorigenesis as they regulate the abundance of the key proteins directing the G1 to S phase transition. Subsequently the increased expression of Skp2 and the mutation of Fbw7 were found indicating that these proteins are indeed deregulated in human cancers.\textsuperscript{16,37-46} Because Fbxo7 stimulates cdk6 activity, we investigated the transforming capacity of Fbxo7 overexpression in immortalised murine
fibroblasts. Fbxo7 expressing cells had increased cdk6 activity, selectively assembled more cyclin D/cdk6 complexes, and had higher levels E2F transactivation and expression of E2F target genes. Significantly, these cells were transformed, being invasive, capable of anchorage-independent growth, and forming tumors in athymic nude mice. These transformed properties were reversed when cdk6 expression was ablated arguing strongly for the involvement of this pathway. However, the proliferative capacity of these cells was unchanged, presumably due to uncompromised cdk4 activity. These data suggest that increasing cdk6 specifically can affect transformed properties like invasiveness and anchorage independent growth.

We and others have shown that Fbxo7 binds to the other components of SCF E3 ubiquitin ligases and is an active ligase.2,31 However, we were unable to identify which protein Fbxo7 ubiquitinates to affect cyclin D/cdk6 activity, and our attempts to demonstrate in vitro ubiquitination of D cyclins or cdk6 alone or in complex were unsuccessful. However, it is possible that additional co-factors, like p27 or Cks1, may be required in these in vitro assays. While the F-box motif was not necessary for binding to cdk6, its presence did significantly enhance the transforming activity of Fbxo7, indicating that enzymatic activity played a role in transforming cells. By comparison, the ubiquitin like motif (Ubl) at the N-terminus was not required for either Fbxo7 binding to cdk6 or for its transforming activity. The presence of a Ubl motif suggests that any Fbxo7-interacting proteins could potentially be directly ferried to the proteasome as such motifs have been reported to directly interact with subunits of the 20S core of the proteasome.47 However, the function of the Ubl motif in Fbxo7 function has not yet been determined.

Cdk activities (cdk2, cdk4 and cdk6) have recently been shown to be highly overlapping and nonessential.48,49 Nevertheless, Fbxo7 is responsible for selectively activating one of these redundant activities. This discovery shows the cdk6 pathway is independently regulated, and indicates while the cell utilizes signalling pathways which can converge to promote growth, the machinery regulating their activity remains distinct and separate. The specialisation of signalling pathways converging on the cell cycle regulators to effect cell proliferation has been previously described.50 We postulate that in the early mitogen-sensitive part of the cell cycle, multiple pathways feed signals into the regulators of cell cycle entry, and Fbxo7 represents a mechanism to relay and integrate these signals. Under physiological conditions it is more likely that cells receive intermittent, disconnected or weak signals. Therefore, kinase activity may be more subtly primed or differentially responsive to allow for variation in cellular inputs and responses. This may be relevant in development or in specialised tissue compartments, like the hematopoietic compartments, where cells differentiate, quiesce, and reactivate in response to transient cell-cell contact, antigen binding, or cytokine signalling. Thus in different tumors, it may be more relevant to identify and develop treatments to inhibit the signalling pathways that are selectively activated, thereby potentially reducing toxicity to adjacent cells that take a different route to mitogenic activation. The identification of proteins that specifically activate oncopgenic signalling pathways can lead to tailored pharmacogenetic therapeutics for subsets of susceptible cancers.

References


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