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Review

Wrenches in the works: drug discovery targeting the SCF ubiquitin ligase and APC/C complexes

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Abstract

Recently, the ubiquitin proteasome system (UPS) has matured as a drug discovery arena, largely on the strength of the proven clinical activity of the proteasome inhibitor Velcade in multiple myeloma. Ubiquitin ligases tag cellular proteins, such as oncogenes and tumor suppressors, with ubiquitin. Once tagged, these proteins are degraded by the proteasome. The specificity of this degradation system for particular substrates lies with the E3 component of the ubiquitin ligase system (ubiquitin is transferred from an E1 enzyme to an E2 enzyme and finally, thanks to an E3 enzyme, directly to a specific substrate). The clinical effectiveness of Velcade (as it theoretically should inhibit the output of all ubiquitin ligases active in the cell simultaneously) suggests that modulating specific ubiquitin ligases could result in an even better therapeutic ratio. At present, the only ubiquitin ligase leads that have been reported inhibit the degradation of p53 by Mdm2, but these have not yet been developed into clinical therapeutics. In this review, we discuss the biological rationale, assays, genomics, proteomics and three-dimensional structures pertaining to key targets within the UPS (SCF^{Skp2} and APC/C) in order to assess their drug development potential.

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Function

The known roles of two RING E3 ubiquitin ligase complexes, the SCF ligases and the anaphase-promoting complex or cyclosome (APC/C), exemplify the cellular utility of regulated protein degradation. Both these complexes execute a precisely timed degradation of key proteins in the cell cycle [1-3]. Degradation ensures that the consequences are unidirectional and temporarily irreversible, intrinsic requirements of passage through a cell division cycle checkpoint [4]. SCF complexes rely on their F-box protein component as a substrate-specific adaptor, and it is therefore the F-box protein (and the cellular activity of its targeted substrate) that determines the genetic and cellular effect of any particular SCF complex [5]. A summary of F-box proteins and their known substrates and functions has recently been published [6]. The function of the APC/C is more closely tied to the cell cycle machinery: this E3 ligase mediates progression through and exit from mitosis, and the maintenance of the G0/G1 state [7].

SCF^{Skp2} ubiquitylates the Cdk inhibitor p27 in the presence of the small accessory factor Cks1 [8]. The ubiquitylation is dependent on a specific Cdk phosphorylation site on p27, and was demonstrated *in vitro* using purified components, as well as in cell cycle arrested cellular extracts and in mammalian cells using RNAi. This phos-

phorylation site and Cks1 form a complex, the binding of which to Skp2 has now been crystallographically visualized [9]. The three-dimensional structure of the complex perfectly recapitulates the biochemistry of the tripartite interaction.

The APC/C has a similarly convincing mechanism, at least for part of its function. The APC/C comes in two forms depending on whether it incorporates the subunit Cdh1 or the subunit Cdc20, both from the Fizzy family of proteins [10]. The two different forms play distinct, high impact roles in the cell cycle. Timely degradation of securins and mitotic cyclins via the APC/C^{Cdc20} (demonstrated in fission yeast studies) accomplishes the metaphase to anaphase transition and the exit from mitosis, respectively, thus providing a dramatic illustration of how the cell can biochemically accomplish a precise, rapid ultrastructural event like chromatid separation [11]. Clearly, transcription and translation are not sufficiently rapid or compartment-specific to accomplish such a large cellular movement at precisely the right time. Instead, the immense, activated machinery needed for the movement of sister chromatids is assembled with a key brake, the securins, in place [12]. When assembly is complete and the appropriate signals converge, the brake is degraded and the machinery seeks its equilibrium, which results in separated chromatids [11]. Concomitant degradation of the mitotic cyclins via the APC/C^{Cdc20} resets the driving force of the cell cycle (Cdk activity) to zero for the G1 phase of the next cell cycle [12].

As demonstrated in elegant yeast studies, APC/C^{Cdh1} contributes to the degradation of mitotic cyclin at the end of mitosis, and stays active during the next G1, thereby inducing the degradation of a variety of pro-S phase and pro-mitotic substrates [10]. APC/C^{Cdh1} is now known to mediate the degradation of both Cdc20 and, surprisingly, its G1 counterpart Skp2/Cks1 [1,13].

Only four other SCF ligases, $SCF^{\beta TrCP}$, SCF^{Fbw7}, SCF^{Fbxl3} and SCF^{Fbx4}, have been definitely matched to their substrates in purified biochemistry and cellular studies [5]. For two of these targets, atomic resolution three-dimensional structural data and established *in vitro* and *in vivo* assays are available [14,15]. However, the biological rationale for both targets is not as consistent across the spectrum of genetics, biochemistry, cell biology, histopathology and animal studies as it is for Skp2 [16]. Interestingly, although these SCF ligases operate on key substrates within the cell division cycle (such as PDCD4 [17], Cdc25A [18,19], Claspin [20], Wee1 [21], Emi1 [22], cyclin E [23] and cyclin D1 [24]), they also influence substrates that are active in prominent cellular signaling pathways upstream of the cell division cycle, such as the NFκB pathway, the Notch pathway, the translational machinery and pathways converging on c-MYC [25] and c-Jun [16]. Fbw7 may also play a significant role in angiogenesis, adding to its interest as a target [4]. Given the multitude of cellular pathways involved, the effects of inhibitors of $SCF^{\beta TrCP}$, SCF^{Fbw7}, SCF^{Fbxl3} and SCF^{Fbx4} appear difficult to predict at present.

Disease, mutation, expression

Several lines of evidence directly support a role for Skp2 as an oncogene in cancer tissues. A multitude of human cancer mutations map to the cell cycle arena in which Skp2 operates [4] and, more importantly, several specific findings demonstrate that the biochemical and cellular growth-promoting effects of Skp2 translate to tumorigenesis [26,27]. Histopathologically, Skp2 overexpression correlates with a higher tumor grade and inversely correlates with prognosis in both epithelial cancer and lymphomas [28,29]. In human tumors, low levels of p27 often correlate with Skp2 overexpression, suggesting that the latter contributes to suppression of p27 abundance in cancer cells [30,31]. Interestingly, Cks1 is also highly expressed in certain epithelial cancers [32-36]. The introduction of Skp2 appears to lead to extracellular matrix- and cell contact-independent growth [37,38] and, significantly, Skp2 contributes to the formation of tumors in animal models [28,39].

Skp2-/- mice, developed by the Nakayama group at AIST in Japan, are hypoplastic, while p27-/- mice are hyperplastic. Interestingly, a genetically induced double-deficiency of Skp2 *and* p27 in this mouse line reversed the Skp2 hypoplastic phenotype and corrected ploidy and mitotic defects observed in some Skp2-/- mouse tissues [40].

In addition to Skp2, there are at least 67 other F-box proteins available in the human genome to form SCF ligases [6], a few of which have intriguing phenotypes related to disease. The most prominent of these is Atrogin, which cellular extract studies demonstrated can target the muscle differentiation factor MyoD for degradation [41]. Atrogin (also known as MAFbx) is upregulated in muscle atrophy and could play a role in the skeletal wasting seen in neurological disorders such as amyotrophic lateral sclerosis and cardiac muscle abnormalities in certain cardiomyopathies [42-44]. An F-box protein, Dactylin/Fbw4, is the cause of a specific genetic skeletal malformation [45] and another, Fbw8, which associates with Cullin7, is implicated in the developmental genetic abnormality known as 3-M syndrome [46].

Only a few mutations in the APC/C have been described in human cancers [47], but as expected from the biochemical understanding of Cdh1, they are inactivating mutations. Interestingly, an inhibitor of the APC/C, Emi1, is also found at high levels in certain epithelial cancers [48].

Disease targets and ligands

Of the ubiquitin ligases executing the cell cycle phases and checkpoints, SCF^{Skp2} has arguably the strongest biological rationale as a drug discovery target. Along with established ubiquitylation and binding assays, the crystal structure of SCF^{Skp2} and previous crystal structures of Skp1-Skp2 and Cks1 [49-52] are exceptional assets for drug discovery targeting the ubiquitylation of p27 by Skp2. The established biochemical assays offer the possibility of high-throughput screening (HTS), and inhibitory compounds may theoretically be designed directly *in silico* based on the structure. Though the caveats of protein interface drug discovery, a historically unproductive approach, are applicable, two features of the interface between p27 and Skp2-Cks1 essentially confirm that drug discovery targeting this interface can succeed [5,53]. Firstly, a single point mutation in Glu185 of p27 abolishes the interaction [9]. This means that a molecule as small as the amino acid side chain of Glu is likely capable of disrupting this interface by competition. Secondly, the pocket in which Glu185 of p27 sits at the interface is sufficiently large to support a drug (Figure 1). Previous surveys have demonstrated that there could be a threshold pocket size required for an adequate pharmacophore space, and this one is above the threshold [53,54].

More importantly, the configuration of the SCF and APC/C as multi-subunit complexes without clear active sites means that compounds discovered through non-rational means (e.g. HTS) should have unknown binding sites on these proteins and unknown sites of interaction in general. Indeed, non-F-box binding sites (located, for example, at Cullin or Roc1/Rbx1 interfaces) could be promising therapeutic targets, but only if they are distinguishable from F-box binding sites. This is a significant obstacle to lead optimization and to target specificity. Nevertheless, compounds promising to inhibit APC/C and to inhibit the interaction of Skp2 with Cks1 have been identified [55,56]. Reported by the Vassilev group (Hoffman-La Roche, Nutley, NJ, USA) that identified the p53-Mdm2 inhibitor, chemical details of these compounds have not been revealed, although their IC_{50} is reported as < 20 uM. The availability of the three-dimensional structure of the key interface allows the possibility of identifying (*in silico* or by subsequent crystallographic experiments) the binding mode of a promising inhibitory compound. The binding mode, or receptor pharmacophore space, is then an invaluable asset to lead optimization and clinical deployment.

Therefore, in the case of SCF^{Skp2}, the resources appear to be in place for discovery of small molecules inhibiting the p27-Skp2 interface, and thus the biochemically pure ubiquitylation of p27. Is this ubiquitylation relevant in the complex environment of the mammalian cell? It appears yes, since p27 is clearly degraded during the C phase (a recently proposed term referring to the cell cycle window of Cdk activity and amplification encompassing S, G2 and M phases) [8]; since this degradation is crucial for the progression through the cell cycle and since the growth inhibiting effects of stabilizing p27 are easily observed [26,57]. SCF^{Skp2} also appears to degrade in a similarly precise manner the additional tumor suppressor substrates p21, p57, p130, FoxO1, Tob1 and perhaps BRCA2. The mitogenic effects of degradation of each of these additional substrates are reinforcing: multiple parallel investigations on different substrates converge to the same mitogenic effect of Skp2 activity.

The biological rationale for targeting Skp2 in human cancer is therefore exceptionally strong and consistent, from atomic structure to pure biochemistry to human cancer tissues and whole animal mouse homeostasis. The data suggests, in addition, that Skp2 could be an important target for inhibition in carcinomas (human epithelial cancers), which have seen notoriously few broadly effective new cancer drugs when compared with hematologic cancers. The presence of widely used *in vitro* and *in vivo* interaction and activity assays further suggests that the tools to execute a drug discovery program are in place. The requirement for protein interface targeting appears to be the only remaining obstacle, perhaps requiring a rational or structure-based discovery approach. However, the recent discovery of p53-Mdm2 inhibitors [58-60], as well as the deep library of crystallographic structural information informing the key SCF protein interfaces, suggests that this obstacle is not insurmountable. Finally, the existing mouse models set the stage for tissue-specific models of Skp2 and p27: an important asset in the validation of Skp2 as a drug target in specific cancers such as skin, breast, lung or colon.

Although the current understanding positions the APC/C at the central aspects of both mitosis and the establishment/maintenance of ploidy in the cell, the biological rationale of modulating the APC/C for cancer is not as well established as for Skp2 at the tissue and organism levels. Pharmacologic inhibition of APC/C^{Cdc20} might be expected to inhibit cell growth and, possibly, cause mitotic catastrophe. This form of the APC/C could therefore be an attractive drug target in human cancers. Inhibition of APC/C^{Cdh1} could blur the boundaries of the cell cycle phases, possibly leading to genomic instability. These phenotypes would not be desirable in the context of human cancer. Agonists of APC/C^{Cdh1} are theoretically possible, however, since it is a large multi-protein complex. If available, such agonists could be useful anti-cancer therapeutics, as they would be expected to arrest the cell cycle in the G_0/G_1 phase.

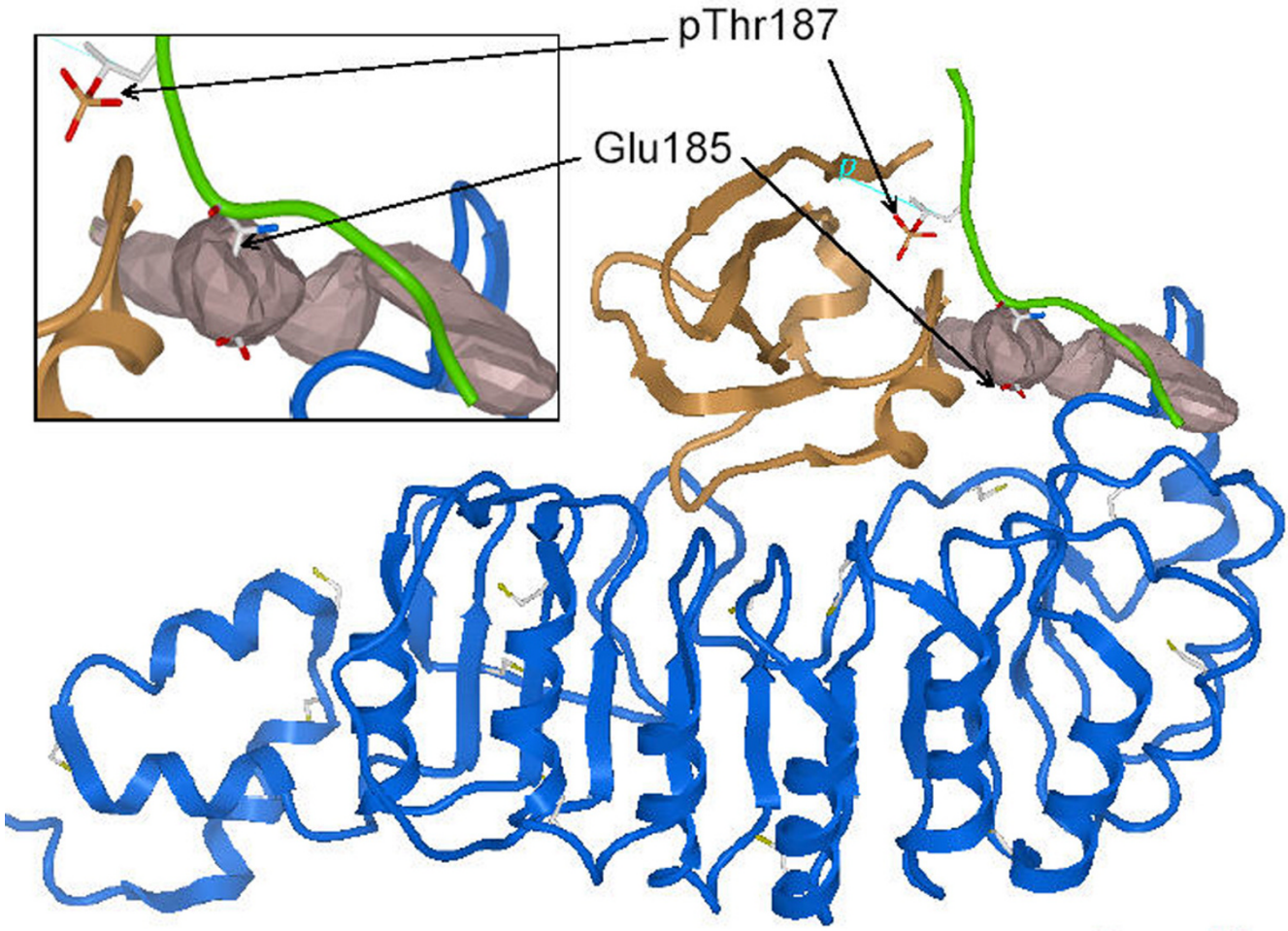

Figure 1
A drug binding pocket of sufficient size coincides with p27 Glu185 in the complex of p27, Cks1 and Skp2. Blue ribbon: Skp2. Gold ribbon: Cks1. Green ribbon: p27 peptide. Stick display: pThr187 and Glu185 of p27. Grey/pink geometric object: potential drug binding solvent pocket at Cks1-Skp2 interface seen in the absence of p27, as computed by the method of An *et al*. [54]. Inset upper left: zoomed in view of the pocket, pThr187 and Glu185 of p27.

The interplay between APC/C and SCF^{Skp2} across the cell division cycle represents a fascinating aspect of this drug discovery scenario. One would expect precise feedback between the two bookend phases of the cell cycle: the DNA synthesis (S) phase and mitosis (M). The cell would certainly not benefit from DNA synthesis occurring simultaneously with mitosis and vice versa. Indeed, the degradation of Skp2 by APC/C^{Cdh1}, the degradation of Emi1 by $SCF^{\beta TrCP}$, and the degradation of Cdc20 by Cdh1 represent some of the mechanisms by which this synchronization takes place (for review see [5]). The interdependence of these factors across the cell cycle phases suggests that specific small molecule inhibitors could be useful for both chemical genetic dissection of the details of the system and for clinical combination therapy (for example an antagonist of Skp2 and agonist of APC/C^{Cdh1} reinforcing each other).

Next frontiers

Unfortunately, the technical drug discovery resources targeting the APC/C are limited, mostly due to the immense size and complexity of this complex, which has at least 12 subunits. Although the APC/C has been purified and fascinating new structural information obtained in the form of cryo-electron microscopic envelopes [61,62], these are immature drug discovery resources compared with purified *in vitro* assays and atomic resolution crystallographic structures. Lysate- or cellular-based screening assays could be employed to find lead compounds influencing the APC/C, but there is a need for three-dimensional resolution of key protein interfaces. The APC/C is therefore an intriguing complex that would benefit from a prior drug discovery yield from Skp2, but significant gaps remain both in the validation of this target and in the tools needed to ensure drug discovery success.

The current state-of-the-art clearly maps the SCF ligases and the APC/C to an emerging network of inter-relationships that drive and time the cell cycle. Small molecule inhibitors could be wrenches in the works of this key, but complex, cellular activity that underpins the growth of cancer cells. SCF^{Skp2} represents the most mature target in this context, but fortunately several of the key issues that will be resolved in targeting Skp2 for drug discovery will likely apply to the subsequent targeting of the APC/C and other SCF ubiquitin ligases. These issues include 1) whether the compounds found to inhibit Skp2 protein interfaces will have appropriate bioavailability and toxicity profiles; 2) how specific molecules designed for substrate binding interfaces will be for specific substrates (as opposed to inhibiting all the substrates for a particular ubiquitin ligase equally) and 3) the final *in vivo* or clinical effects of the compounds inhibiting these complexes, the activity of which will depend on the interplay of the various substrates affected.

Competing interests

The authors declare that they have no competing interests.

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