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Review

Hepatocellular carcinoma and the ubiquitin–proteasome system

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ABSTRACT

Hepatocellular carcinoma is one of the largest causes of cancer-related deaths worldwide for which there are very limited treatment options that are currently effective. The ubiquitin–proteasome system has rapidly become acknowledged as both critical for normal cellular function and a frequent target of de-regulation leading to disease. This review appraises the evidence linking the ubiquitin–proteasome system with this devastatingly intractable cancer and asks whether it may prove to be fertile ground for the development of novel therapeutic interventions against hepatocellular carcinoma.

1. Hepatocellular carcinoma: the problem

Hepatocellular carcinoma (HCC) is generally acknowledged as the sixth most prevalent cancer in the world and is currently the third most likely cause of cancer-related death worldwide [1,2]. Current predictions suggest that the number of new cases diagnosed annually is set to rise; in 1990, it was estimated that there were 437,000 new cases [3] while by the year 2000, this had risen to an estimated 564,000 [4]. Globally, HCCs comprise the vast majority (>80%) of primary, malignant liver tumours [4,5].

The incidence of primary liver cancer varies with both gender and geographical location [4]. Rates of HCC are, on average, up to four times higher in the male population. Geographically, age-standardised incidence rates (ASRs; per 100,000) for the male population in Asia vary from ~10 in Brunei, to almost 100 in Mongolia while in Africa rates vary from ~1 in Malawi to nearly 80 in Mozambique. In Europe, America and Australasia, incidence rates are consistently observed at fewer than 10 per 100,000 (Fig. 1; [6]). Similar trends are observed, in general, within the global female population.

Mortality from liver cancer is extremely high with many reports quoting it as the being the third most-common cause of cancer-related death worldwide [1,2]. This high lethality is reflected in the observation that it exhibits a yearly fatality ratio of approximately 1 (i.e. most cases do not survive beyond one year, post-diagnosis [4]) and only approximately 12% of cases survive to 5 years, post-diagnosis, or beyond [7,8].

A number of established risk factors for primary liver cancer are now known. The most significant of these are attributable to long term, persistent infection with either Hepatitis B (HBV) or Hepatitis C (HCV) viruses, which account for approximately the 75% to 80% of the total cases [3]. In contrast to the incidence of HBV-associated HCC, which has remained relatively stable in recent times, increases in the occurrence of HCC have been observed in many so-called developed nations including Australia, America, Europe and the U.K [9–12]. Such increases have been ascribed to the increasing effects of long-term infection by HCV. The causes of the observed increased HCV infection rates can be partly ascribed to cohort effects such as those seen in Japan since the 1970s where they were ascribed to increased HCV exposure through contaminated blood transfusion and needles [13] or those seen in the Nile delta region of Egypt as a result of large scale campaigns to treat outbreaks of schistosomal infestation [14].

Other risk factors for primary liver cancer include chronic alcohol abuse (although it is as yet unclear whether the alcohol per se is the cause as chronic alcoholics show a significantly higher occurrence of HBV or HCV antigens [15]) and exposure to key environmental toxins such as aflatoxin B1 which cause specific hepatic DNA mutations [16].

In addition to primary drivers such as the Hepatitis viruses it is increasingly apparent that chronic inflammation is a major contributory factor towards the development of many cancers [17] including HCC, albeit that the precise complexities have yet to be clarified. This feature has recently been illustrated quite elegantly in a number of transgenic animal models. One study, in which an inducible IκB "super-repressor" was expressed, showed that functional inhibition of NFκB activity prevented the progression of liver cancer [18] while in contrast, a model of chemically-induced liver damage in the context of partial NFκB inhibition led to an increased incidence of cancer [19] and ablation of the IKKγ/NEMO subunit of the IKK complex in liver parenchymal cells causes both steatohepatitis and liver cancer [20]. In

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NFκB function, its role in the inflammatory responses to diverse stimuli and its contribution to the pathogenesis of HCC are complex (for good recent reviews, see [21–24]). Hepatocytes lacking NFκB activity appear to be exquisitely sensitive to apoptosis induced by cytokines such as TNFα and the activity of NFκB is also known to antagonise the pro-apoptotic and proliferative functions of the stress-induced kinase, JNK. However, the liver is the one organ that stands out by virtue of its capacity to regenerate extensively in response to damage with surviving hepatocytes stimulated to undergo rapid proliferation by the release of growth stimulatory cytokines (IL-6 and HGF amongst others) from surrounding Küppfer and Stellate cells. It is proposed that this microenvironment where hepatocytes (or their stem-cell progenitors) are stimulated to undergo continual cell death (in response to chronic viral infection, carcinogen exposure or other liver injury) followed by proliferation of the surviving cells is one where mutation is both more likely and can be passed on more efficiently to daughter cells thus potentiating the development of HCC.

The lethality of HCC is linked directly to our lack of an effective treatment for the disease, even if diagnosis is achieved at an early juncture. The gold standard for treatment is that of a complete liver transplant for an early HCC exhibiting no extra-hepatic spread [25] although the success of this procedure is restricted because of the limiting availability of appropriate donor livers. Local surgical ablation therapies can also prolong survival rates but frequent recurrence of new HCC ultimately renders such treatments of limited use. There are very limited effective chemotherapy approaches for the treatment of HCC and even the 2007 FDA approval [26] given to the Ras/MEK/ERK inhibitor [27], sorafenib, only increased the median survival time from approximately eight months to ten months [28]. Other treatments with some apparent future potential include the use of ERBB1 inhibitors such as gefitinib [29] and erlotinib [30] or approaches to target the vascular endothelial growth factor system such as the use of bevacizumab monoclonal antibody therapy [31].

As current treatments are so limited in their effectiveness, methods for prevention of HCC are increasingly being explored. To this end, some success has been achieved by using vaccination programmes against HBV [32] and also by the use of interferon (IFN) to prevent the progress towards chronic hepatitis [33,34].

The relative paucity of efficacious interventions for HCC suggests the need for new targets that may be amenable to pharmaceutical intervention.

2. The ubiquitin–proteasome system

A detailed exposition of the ubiquitin–proteasome system (UPS) is beyond the scope of this review but for good, recent reviews of the system, the reader is directed to those by Kersher et al. [35], Pickart and Cohen [36], Pickart and Eddins [37], Roos-Mattjus and Sistonen [38] and Nandi et al. [39].

Briefly, ubiquitin is a highly conserved 76 amino acid protein found in all eukaryotes so far examined where it acts as a post-translational modification tag for other proteins (Fig. 2). Addition of ubiquitin to target proteins is an ATP-dependent process requiring the sequential action of three essential enzymes — E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin-protein ligase enzyme). The E3 enzyme (there are many hundreds of these in the human genome) acts as the major substrate determining part of the system. Ubiquitin is conjugated, mainly, to the ε-amino groups of lysine residues within target proteins via its C-terminal carboxyl group forming an iso-peptide bond. As ubiquitin itself contains seven internal lysine residues, it follows that consecutive rounds of ubiquitin addition can occur by addition of the incoming ubiquitin to one of the lysine residues within the preceding ubiquitin moiety: in this way, poly-ubiquitin chains can be built up on a target protein. If a poly-ubiquitin chain on a target protein features linkages which are composed via K48 of each preceding ubiquitin (a so-called K48 chain) and the number of ubiquitin moieties is four or more, this acts as a recognition signal for the 26S proteasome which then acts to degrade the target protein in an ATP-dependent fashion. Ubiquitin itself is the prototypical member of the so-called ubiquitin-like proteins (UBLs): small post-translational modifier proteins which share the β-grasp 'ubiquitin superfold' structure. Members of this group of proteins share similarities in structure and many characteristics of their activation and attachment to target proteins are analogous to that of ubiquitin itself. The attachment to target proteins of UBLs other than ubiquitin does not, however, usually result in target protein degradation [40].

The UPS plays a key role in many processes important for cellular homeostasis such as regulation of the cell cycle [41], apoptosis [42–45], receptor signalling and endocytosis [46,47] and many more. The natural corollary of the above is the prediction that defects in one or more of the many UPS components will be major contributory factors to human disease; this is now widely accepted to be the case. As a function of this, the UPS is increasingly being viewed as a novel target

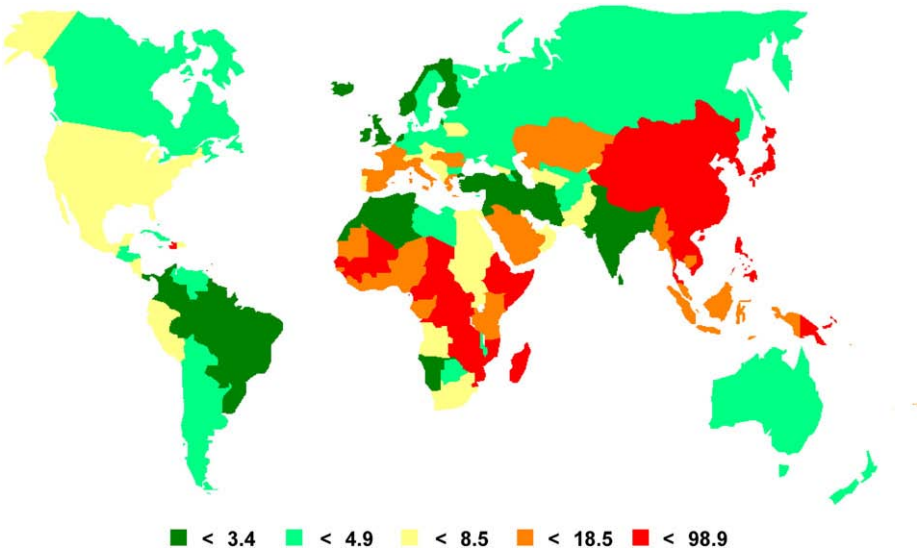

Fig. 1. Age-standardised incidence rates (ASRs) for the occurrence of HCC in the global male population expressed per 100,000. Image generated from global data accumulated for GLOBOCAN 2002 at the International Agency for Research on Cancer (IARC; http://www-dep.iarc.fr/).

for drug development and bortezomib, a proteasome inhibitor, is already in clinical use particularly in the United States as an intervention for late-stage multiple myeloma [48].

3. A role for UPS malfunction in HCC pathogenesis?

The molecular analyses of human liver cancer have highlighted many genetic and epigenetic changes including alterations to both oncogenes and tumour suppressor genes. A number of these genetic modifications impinge directly upon the UPS and its function.

3.1. Gankyrin

Gankyrin is a small (25 kDa, 226 amino acids; also known as PSMD10, 26S proteasome regulatory subunit p28 or p28^{GANK}), highly conserved protein containing seven ankyrin repeats [49] which was discovered simultaneously as a subunit of the 26S proteasome [50] which interacts specifically with the S6b ATPase [51] of the 19S regulatory cap and via the use of subtractive cDNA cloning as a protein which is routinely over-expressed at both the mRNA and protein levels in human HCC [52].

Available evidence suggests that gankyrin may have an early function in HCC pathogenesis. Increased gankyrin expression in hepatocytes occurs as one of the earliest observable events in a chemical model of liver cancer [53] and an analysis of gankyrin expression levels in normal, cirrhotic and HCC human livers showed a marked up-regulation in both hepatoma cell lines and in HCC samples where expression levels were 3.6-fold higher than in cirrhotic samples and 5.2-fold higher than para-carcinoma samples [54]. The significance of these studies linking gankyrin up-regulation to HCC pathogenesis was elegantly demonstrated by the observation that the use of RNAi to facilitate gankyrin knockdown in HCC resulted in reduced cell growth, reduction in observed levels of hyper-phosphorylated retinoblastoma protein (pRb) and caspase 8/9-dependent apoptosis [55,56]; the increased sensitivity of gankyrin knockdown cells to caspase 8/9-mediated apoptosis is likely due to increased stabilisation of the p53 tumour suppressor protein [56]. Gankyrin expression in a rat model system of hepatic regeneration is also up-regulated within oval cells (liver stem-cell progeny which mediate hepatocyte proliferation in response to injury) in reaction to a combination of chemical and partial heptectomy [57].

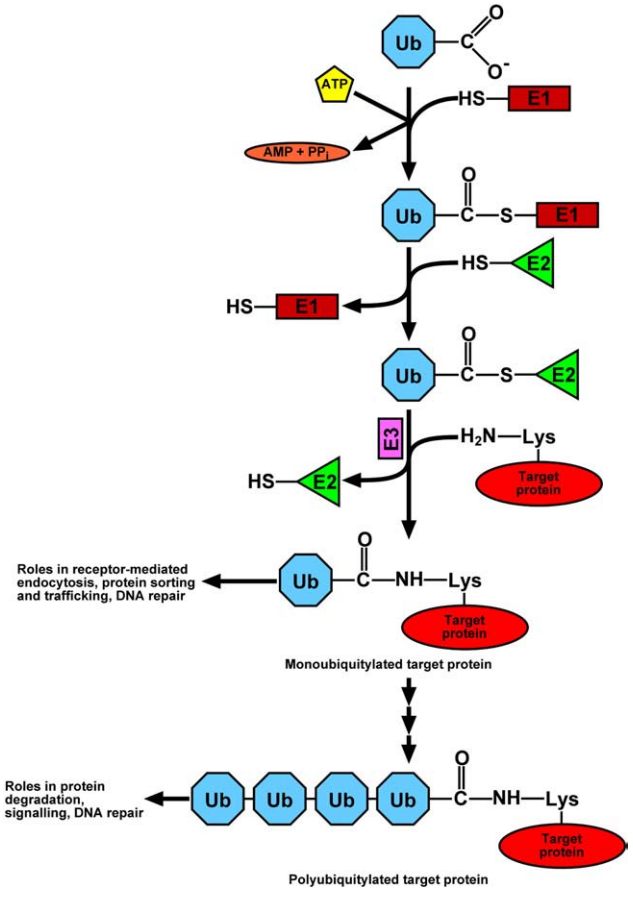

Fig. 2. The protein ubiquitylation cascade. Ubiquitin (Ub) is activated in an ATP-dependent reaction by ubiquitin-activating enzyme (E1) to form a thiolester intermediate. Activated ubiquitin is transferred, preserving the thiolester linkage, to an ubiquitin-conjugating enzyme (E2) and is finally transferred in the presence of an ubiquitin-protein ligase (E3) to the ε-amino group of a lysine residue within a target protein via an iso-peptide bond. Multiple rounds of ubiquitin addition can subsequently occur, each incoming ubiquitin being attached to an internal lysine of the preceding ubiquitin moiety.

Initial hypotheses concerning the mechanisms of gankyrin function in HCC formation were based around the identification of a LxCxE (LACDE) motif at positions 178 to 182 [52]: such LxCxE motifs are known to function as pRb-binding motifs in both viral proteins (i.e. HPV E7 and adenovirus E1a) and endogenous cellular proteins (i.e. cyclin D1 or HDAC1) [58]. The LACDE motif of gankyrin was shown to be essential for the mediating the interaction of gankyrin with pRb in vitro and was essential for conferring anchorage-independent growth on NIH 3T3 fibroblasts [52]. The crystal structure of free gankyrin [49] shows that the LACDE motif adopts a helical structure in contrast to the β-strand conformation of other known pRb-interacting LxCxE peptides and suggests that, except for Glu182, the motif is buried within the α-helix of the fifth ankyrin repeat and not accessible for pRb-binding. Nevertheless, that the LACDE motif is essential for pRb-binding was confirmed in studies examining the interaction of gankyrin with CDK4 [59] and the S6b ATPase [60] suggesting that the mode of interaction must be different to that of the classical HPV E7 LxCxE peptide.

In addition to its interaction with pRb, gankyrin has also been shown to bind the D cyclin-dependent kinase, CDK4 [51] and in doing so compete with p16^{INK4a}, removing its inhibitory influence on CDK4 kinase activity [59,61]. This effect of gankyrin on CDK4 activity is consistent with the observation that gankyrin over-expression leads to pRb hyper-phosphorylation and release of active E2F transcription factors [52] thereby presumably acting as a forward impetus to drive increased cell division.

It is interesting to note greater than 80% of human HCC show a functional disruption of the pRb/p16^{INK4a}/cyclin D1 pathway. Although promoter hyper-methylation and silencing of the p16^{INK4a} locus appears to be the most frequent alteration affecting this pathway [62] the structural studies of Nakamura et al. [60], observed over-expression of gankyrin in the majority of human HCC and the interaction of gankyrin with CDK4 are all consistent with a model in which gankyrin contributes to HCC pathogenesis by acting to ferry hyper-phosphorylated pRb to the 26S proteasome where binding of gankyrin to the S6b ATPase facilitates pRb release for subsequent degradation. The effect of gankyrin binding to the S6b ATPase has yet to be elucidated although one attractive option is that it acts to stimulate the ATP hydrolysing activity of the ATPase in a similar fashion to that described for the S7 ATPase on binding of the oncoprotein, HEC [63] (Highly Expressed in Cancer cells). In so doing, this may stimulate gating of the pore in the 20S core particle of the 26S proteasome and facilitate the degradation of pRb.

In addition to its role affecting the pRb axis, gankyrin also impinges upon the p53 tumour suppressor. In this context it is interesting to note that, although it is accepted that functional p53 deficiency contributes to the generation of HCC, the importance of p53 mutation to the initial or the latter stages of HCC pathogenesis is unclear. Evidence supporting a role for p53 mutation as a relatively late event is apparent from experiments in transgenic mice [64].

p53 is a relatively unstable protein under normal circumstances and it is inactivated through the action of the mdm2 (mouse double minute-2) protein. Mdm2 is an E3 enzyme belonging to the RING-finger family of proteins [65] that acts as a negative regulator of p53 stability through the promotion of p53 ubiquitylation and its subsequent degradation via the 26S proteasome [66,67]. Disruption of the p53-mdm2 interaction is a primary pathway towards the stabilisation of p53 upon cellular stress.

Gankyrin has been shown to provide protection against chemical agents that induce DNA damage and subsequent p53-dependent apoptosis. In addition gankyrin interacts with mdm2 both in vitro and in vivo, an interaction that appears to increase both the association and activity of mdm2 for p53. This increased, gankyrin-mediated interaction of mdm2 and p53 drives increased ubiquitylation and subsequent proteasomal degradation of p53 [56].

A recent study by Qiu et al. [68] has highlighted both the complexity of gankyrin interactions with p53 and pRb and the importance of cross-talk between these two critical tumour suppressors. The pRb protein can prevent the gankyrin-mediated interaction of mdm2 and p53 thereby preventing the ubiquitylation and subsequent degradation of p53; a function illustrated by the fact that RNAi knockdown of pRb enhances the mdm2-p53 interaction and de-sensitises cells to DNA damage-induced apoptosis. These data are consistent with a model in which pRb can arbitrate stabilisation of p53 by disrupting the gankyrin-mediated mdm2-p53 interaction but under conditions of increased mdm2 or gankyrin expression (such as is often reported in cancer), pRb is degraded thus effecting an increased degradation of p53 (Fig. 3; [68]). A detailed mechanistic explanation of how gankyrin causes an increase in the activity of mdm2 towards p53 must, however, await data from a co-crystallographic, or similar, study.

Gankyrin appears to be a somewhat promiscuous protein; it is known to interact with the proteasomal S6b ATPase, pRb and mdm2. Two recent studies [69,70] have, however, implicated gankyrin in an interaction with RelA (p65); one half of the NFκB isoform that, together with its heterodimer partner p50, is activated via the canonical TNFα pathway [22]. Both studies produced data that suggests that gankyrin expression negatively regulates NFκB activity induced by the canonical (i.e. TNFα-induced) pathway although the mechanism(s) by which this is brought about is unknown. Chen et al.

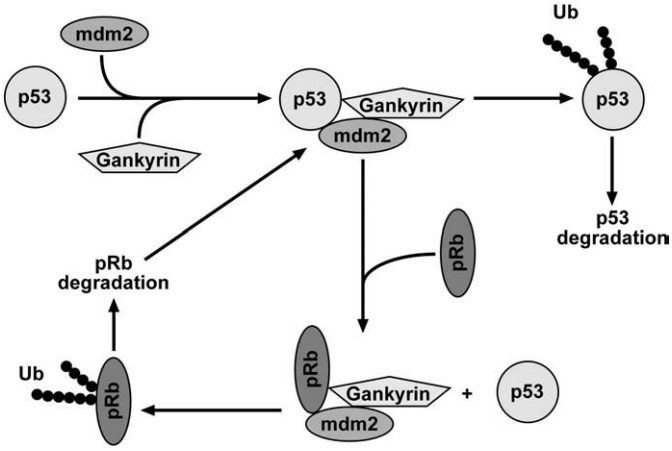

Fig. 3. A model for pRb regulation of gankyrin-mediated mdm2-p53 interaction and p53 degradation (adapted from Qiu et al., 2008 [68]). Gankyrin interacts with mdm2 facilitating increased interaction of mdm2 for p53, amplifying mdm2-mediated p53 poly-ubiquitylation leading to 26S proteasome-mediated degradation of p53. pRb binds to the central domain of mdm2 inhibiting the gankyrin–mdm2-p53 interaction thereby stabilising p53. Conditions that lead to increased pRb degradation, such as gankyrin over-expression or mdm2 over-expression due to copy number amplifications, or pRb inactivation via somatic mutation can potentiate the formation of a gankyrin–mdm2-p53 complex and p53 destabilisation.

[69] suggest that gankyrin can act as a nucleo-cytoplasmic shuttling protein which acts to shuttle NFκB from the nucleus and retain it in the cytoplasm whereas Higashitsuji et al. [70] argue for a role of gankyrin as an NFκB inhibitor via the action of the HDAC III enzyme, SIRT1 and exclude a role for gankyrin as a NFκB exporting protein as gankyrin-mediated repression of NFκB activity was unaffected by leptomycin B, an inhibitor of CRM1-mediated nuclear export.

Although it is unclear how to reconcile the mechanistic details presented in these studies, both point to a role for gankyrin as an inhibitor of TNFα-induced NFκB activity. This is of interest in the context of HCC pathogenesis as it is consistent with the recent transgenic models [19,20] that suggest NFκB inhibition is a major driver towards the development of primary liver cancer as NFκB inhibition in hepatocytes causes apoptosis in many cells and leads to compensatory proliferation favouring the accumulation of somatic mutation. Although it remains to be elucidated, it is interesting to speculate that gankyrin, a protein containing seven ankyrin repeats, may function in a similar way to the classical inhibitory molecules of NFκB signalling, the IκB proteins, of which IκBα is perhaps the most well understood. IκBα is a target gene for NFκB and one of its functions is to mediate the shuttling of NFκB from the nucleus to the cytoplasm thereby decreasing NFκB activity via a negative feedback loop [21].

3.2. HBV, HCV and the UPS

Although not part of the UPS themselves, both HBV and HCV express a number of proteins with the potential to affect the UPS, perhaps the best studied of which is the HBx protein of HBV. The fourth open reading frame of the HBV genome, giving rise to a highly conserved 16.5 kDa polypeptide, encodes the HBx protein that is known to act as a transcriptional transactivator [71]. Amongst the genes activated by HBx are oncogenes such as c-myc and c-*fos*, components of the Ras signalling pathway and the EGF receptor. The HBx protein is not essential for the HBV life cycle in vitro but is required for full productive infection in vivo [72].

Amongst the cellular targets for HBx is the 26S proteasome [73,74]. HBx interacts with both the PSMC1 (S4 ATPase) and PSMA7 (α1/C2) subunits of the proteasome by two-hybrid and immunoprecipitation assays and co-sediments with the proteasome upon sucrose gradient centrifugation. HBx interaction with the proteasome is required for efficient HBV replication [75]. Studies using wild-type and HBx-negative viral strains also illustrate that HBx-negative strains replicate at only 10% of the efficiency that is observed for the wild-type virus and that inhibition of the proteasome in cells infected with HBx deficient virus restored viral replication to wild-type levels; wild-type viral replication rates were left unaffected by proteasomal inhibition. HBx expression in the HCC cell line, HepG2 causes a decrease in the chymotrypsin- and trypsin-like activities of the proteasome that suggests that HBx can act as a proteasomal inhibitor; an interesting observation given the role of the proteasome in generating Class I antigens for the immune system [76]. The role of HBx in immune system evasion may also be seen in the observation that HBx can compete with the PA28 proteasomal activator complex for binding to the α4 subunit of the proteasome [77]. A decreased ability of PA28 to interact with the catalytic 20S core of the proteasome would be predicted to result in less efficient Class I antigen generation [78].

HBx can also affect the stability of several oncogenes and tumour suppressors. Co-expression of HBx with c-myc results in an increased stability of the c-myc protein; this is effected through an interaction between HBx and the SCF-type E3 enzyme, SCF^{Skp2} which causes destabilisation of the SCF^{Skp2} complex [79].

Abnormal accumulation of β-catenin is a hallmark of many HCCs and a strong positive driving force during HCC pathogenesis although the mechanism by which β-catenin accumulation occurs is unclear. A recent study that may shed some light on the mechanistic details of β-

catenin accumulation during HCC development suggests HBx is able to differentially regulate the levels of β-catenin via proteasomal degradation depending on the status of cellular p53 [80]. In the presence of p53, HBx down-regulates β-catenin via p53-dependent transcriptional activation of the SIAH1 gene (the product of which is the RING-finger E3 enzyme, siah1) whereas in cells lacking p53, HBx mediates the stabilisation of β-catenin via inhibition of the glycogen synthase-3β-dependent pathway. Many tumours with high β-catenin expression levels exhibit high frequencies of p53 mutation that suggests there may be selective pressure for p53 loss in these tumours.

An intriguing recent discovery concerns the interaction of HBx with the WD40-like repeat-containing Damaged DNA-Binding protein (DDB1) [81,82]. DDB1 hetero-dimerises with another WD40 repeat-containing protein DDB2 as part a CUL4-based SCF E3 enzyme complex important for the recognition and repair of UV- and chemical mutagen-induced DNA lesions [83,84]. Interaction of HBx with DDB1 interferes with cell viability and growth in culture, a function that has been implicated in the establishment of infection [85] and interestingly, other viral proteins also interact with the SCF^{DDB1} complex during their life cycle to influence its activity and substrate range [86–88]. Whether HBx subverts the activity of the SCF^{DDB1} complex to facilitate efficient HBV replication via increased or decreased ubiquitylation activity or altered substrate specificity remains to be determined but is intriguing nonetheless.

A characteristic of all SCF E3 complexes so far studied is that they are regulated by the NEDD8-CAND1 cycle [89]. The cullin subunit of SCF-type E3s is modified by the ubiquitin-like protein NEDD8, a modification that is generally thought to enhance the ubiquitylation activity of the ligase by preventing the inhibitory binding to the complex of the CAND1 protein. Removal of the NEDD8 modification is achieved by the action of the COP9/signalosome (CSN) complex via its integral CSN5/Jab1 subunit, something that would be expected to decrease the activity of the SCF ligases. However, a number of studies have illustrated that an active CSN complex is required for optimal SCF ligase activity, an apparent paradox that has been explained by the need for a CSN-mediated decrease in SCF ligase activity to counter the effects of SCF ligase autocatalytic adapter instability [90–92]. The link between HBx and SCF^{DDB1} ligase complexes is further enhanced by the observation that the CSN5/Jab1 gene in chromosomal region 8q is often amplified in HCCs [93] and its over-expression in Hep3B cells increases their proliferation while CSN5-specific siRNA knockdown decreases their growth rate.

While the role of HCV gene products and their direct influence on the pathogenesis of HCC is much less well understood than for the HBx protein of HBV, some pertinent information has been forthcoming. Recently, the non-structural (NS) NS5b protein of HCV that fulfils the role of the viral RNA-dependent RNA polymerase has been implicated in the degradation of the pRb tumour suppressor protein [94,95]. The mechanism by which NS5b decreases cellular pRb levels appears to via the restriction of pRb to the cytoplasm followed by the recruitment of the HECT-family member E3, E6AP, leading to the ubiquitylation and proteasomal degradation of pRb with concomitant E2F transcription factor release and cellular proliferation. The subversion of the E6AP ubiquitin-ligase by the NS5b protein is reminiscent of a similar function performed by the E6 protein of oncogenic transforming strains of the human papilloma virus in the degradation of p53 [96].

3.3. SIAH1

Siah1 is a member of the RING-finger family of ubiquitin-protein ligases which was originally described as a p53-induced gene up-regulated during apoptosis [97] and recent studies have highlighted the interaction of siah1 with the C-terminus of the Adenomatous Polyposis Coli (APC) protein and its mediation of the β-catenin degradation [98].

The SIAH1 gene is located in chromosomal region 16q12.1, a region that frequently undergoes loss-of-heterozygosity (LOH) during HCC pathogenesis [99] and expression of the SIAH1 gene is markedly down-regulated in advanced HCCs including those that are larger and/or poorly differentiated [100]. However, mutational analysis of the SIAH1 gene in the same study revealed that there were no observable somatic mutations found in 35 HCCs studied. Presumably, therefore, other mechanisms such as promoter hyper-methylation must be invoked to explain the observed down-regulation of SIAH1 gene expression.

As discussed previously, siah1 is a negative regulator of β-catenin accumulation in response to DNA damage, a function that is mediated via activation of p53 and elevated β-catenin levels are commonly observed in many HCCs. In HCCs that have maintained wild-type p53 function down-regulation of SIAH1 gene expression, possibly via promoter hyper-methylation, is one possible route towards maintaining or elevating β-catenin levels.

3.4. Parkin

The product of the PARK2 gene, parkin, is, like siah1, a member of the RING-finger family of E3 enzymes. Parkin differs from siah1 in its domain organisation and contains two RING domains separated by an IBR (in-between-RING) domain [101]; siah1 contains a single RING domain.

Parkin was originally characterised as the product of the PARK2 gene implicated in Autosomal Recessive Juvenile Parkinsonism (AR-JP), the most frequent form of hereditary Parkinson's disease [102]. The PARK2 gene is located on chromosome 6q26 in the highly unstable FRA6E common fragile site, a region often altered in various solid tumours including HCCs [103] and also subject to frequent LOH in malignant breast and ovarian tumours [104].

Evidence to support a tumour suppressive role for parkin has come from a number of recent studies. A systematic analysis of 50 cancer-derived cell lines including 11 from HCCs revealed one HCC line which contained a homozygous exon 3 deletion of the PARK2 gene, 4 of 11 HCCs containing heterozygous deletions of PARK2 exons and one with an exon duplication [105]. Furthermore, the same study identified that more than 50% of HCC primary tumours showed a significant decrease in PARK2 gene expression and parkin over-expression in HCC cell lines slowed cell growth and rendered transfected lines sensitive to apoptosis induced by inhibitors of the cell cycle.

A recent study using a parkin −/− transgenic mouse model in which homozygous deletion of exon 3 of the PARK2 gene was created [106] generated mice in which hepatocyte proliferation was enhanced and which developed hepatomegaly with increasing frequency of macroscopic liver tumour development at 72 and 96 weeks of age (33% and 45%, respectively). Heterozygous parkin +/− siblings showed no such tumour development and hepatocytes from parkin −/− mice showed a reduced susceptibility to apoptotic stimuli and a deficiency in caspase activation.

The data from the above studies is consistent with parkin acting as a tumour suppressor for the development of HCC.

Finally, the PARK2 gene shares a bidirectional promoter with an adjacent gene, parkin co-regulated gene (PACRG). A recent study has shown that abnormal hyper-methylation of the common PARK/PACRG promoter is integral to decreases in parkin expression in human leukemias [107]; such a mechanism may also function in some HCCs where decreases in PARK2 gene expression are observed in the absence of PARK2 somatic mutation.

3.5. NFκB signalling, the UPS and HCC

The role of chronic inflammation is now accepted as critical in the pathogenesis of HCC and other cancers [17] and chronic hepatic inflammation as a result of long-term infection by HBV and HCV is a major contributory factor in cases where Hepatitis virus infection is implicated.

The watchful cellular regulation of the transcriptional response to diverse immunological stimuli is mediated mainly through the "master" regulatory transcription factor, NFκB [21,108] and much work over the last ten to fifteen years has established that the UPS plays many and varied roles within the signalling cascades which result in an activation of NFκB [109]. A detailed exposition of NFκB signalling is beyond the scope of this review but the role of ubiquitylation in the control of NFκB activation is typified by the action of TNFα, the prototypical cytokine activator of the so-called "classical" NFκB pathway (Fig. 4).

A number of recent studies have described defects within the NFκB signalling system manifesting from genetic abnormalities of UPS components correlating with HCC pathogenesis [110,111] or have provided insight into how selective inhibition of UPS components of NFκB signalling may provide novel and fertile targets for drug design [112–115].

The product of the human CYLD gene is a deubiquitylating enzyme (DUB) that was initially characterised as the gene commonly mutated in the disease cylindromatosis/turban tumour syndrome where it acts to deubiquitylate the TRAF2 RING-finger E3 [116,117] in the classical NFκB pathway (Fig. 4). It was also subsequently shown to deubiquitylate BCL-3 [118], a member of the IκB family of NFκB inhibitory proteins. Although an IκB member, BCL-3 appears to form transcriptionally active heterodimers with both p50 and p52 [119–121] and transgenic BCL-3 knockout mice are unable to generate an appropriate humoral immune response [122,123]. CYLD has subsequently also been shown to target other proteins important in the NFκB pathway including RIP, Lck, TAK1 and NEMO proteins [124,125].

A comparative systematic study of CYLD expression in HCC and colon carcinoma cell lines revealed that CYLD mRNA expression is significantly reduced in all tumour cell lines examined [110]. In addition, evaluation of CYLD mRNA expression levels in tumour tissue isolated from patients in comparison with surrounding non-neoplastic tissue revealed that seven (of nine) HCC and ten (of ten) colon carcinoma samples exhibited either reduced or absent CYLD expression. Hyper-methylation as a possible cause of CYLD down-regulation

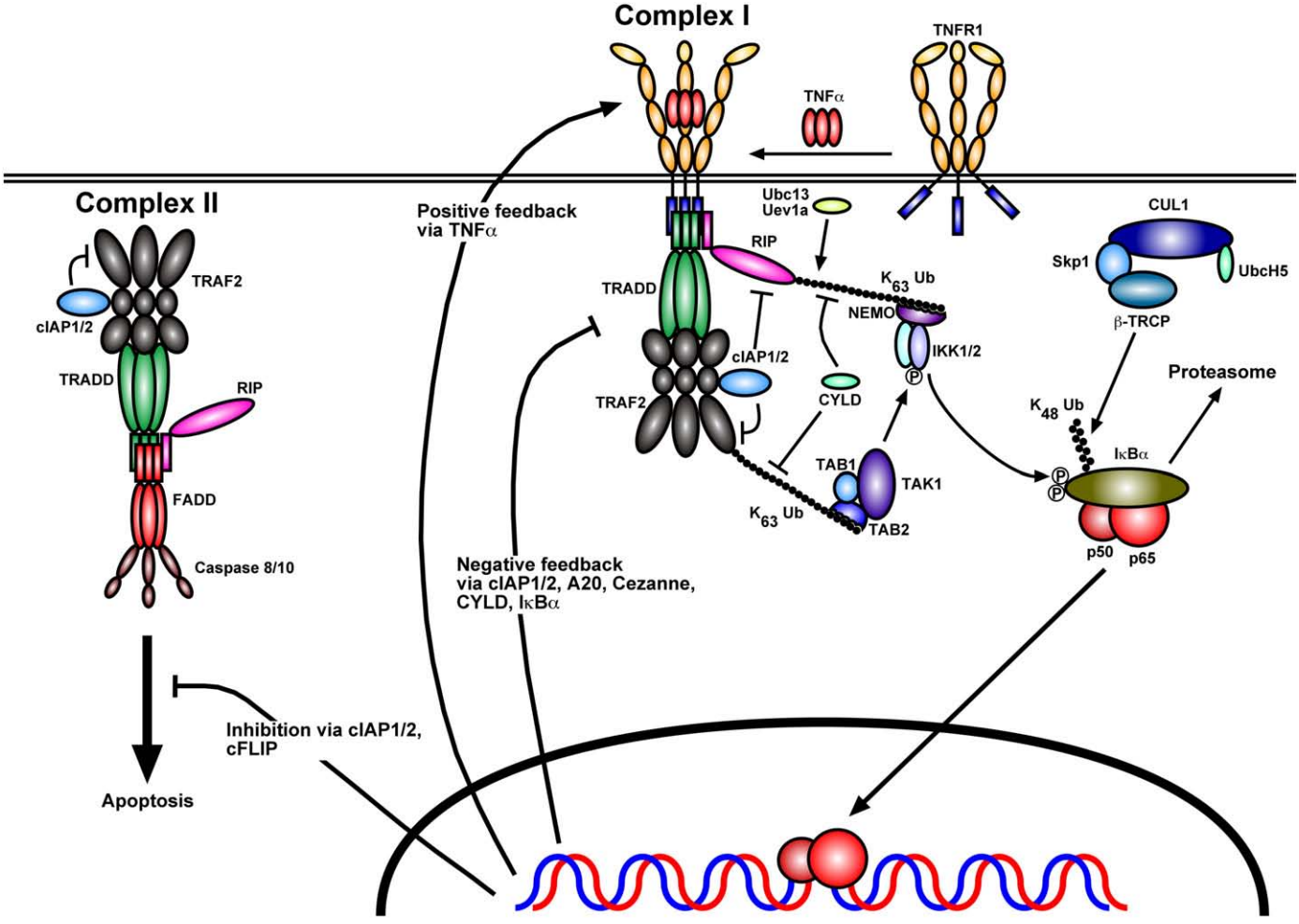

Fig. 4. The "classical" NFκB signalling pathway. Trimeric TNFα binds to the TNFR1 receptor leading to intracellular recruitment of TRADD (TNF receptor-associated death domain) via interaction of their respective death domains (DDs). Subsequent recruitment of RIP (receptor-interacting protein) via its DD and TRAF2 (TNF receptor-associated factor 2) leads to formation of Complex I. TRAF2 (in combination with the Ubc13/Uev1a E2 enzyme) catalyses poly-ubiquitylation, via K63 linkages of itself, RIP and NEMO. Poly-ubiquitin chains attached to TRAF2 mediate the recruitment of the TAK1 (TGFβ-activated kinase)/TAB1 (TAK1-binding protein 1)/TAB2 (TAK1-binding protein 2) complex via the direct binding of TAB2 to the K63-linked ubiquitin chain. TRAF2 also catalyses the formation of K63-linked poly-ubiquitin chains on RIP leading to recruitment of the inactive IκB kinase (IKK) complex via binding of NEMO to the K63-linked poly-ubiquitin chain. Active TAK1 subsequently catalyses the direct phosphorylation of the IKK2 subunit of IKK leading to its consequent activation. Active IKK catalyses the phosphorylation of the inhibitory IκBα protein leading to its K48-linked poly-ubiquitylation by the SCF E3 enzyme, $SCF^{\beta\text{-}TRCP}$ and subsequent degradation by the proteasome. This allows transport of the p50/p65 NFκB into the nucleus where it drives the expression of many genes including those of feedback inhibitory proteins such as A20, cIAP1/2, the DUB enzyme CYLD and cFLIP (cellular FLICE-inhibitory protein; a catalytically inactive caspase-8 homologue). cFLIP competes with caspase-8 for binding to Complex II (a TNFR1-related complex postulated to contain FADD [Fas-associated death domain] and caspase 8; TNFR1 appears to be absent from this complex). A20 acts as a DUB (via its OTU domain) removing K63-linked chains from RIP (a similar DUB activity is ascribed to Cezanne) replacing them with K48-linked poly-ubiquitin chains (via its zinc-finger domains) leading to RIP degradation and repression of NFκB signalling. cIAP1/2 act via their RING-finger domains to K48 poly-ubiquitylate TRAF2 and RIP leading to suppression of NFκB signalling. In addition cIAP1/2-mediated ubiquitylation of TRAF2 can suppress apoptotic signalling via Complex II.

as determined by exposure of cells to 5-azacytidine was ruled out. Additionally, there is a significant inverse correlation between reduced CYLD expression levels and NFκB activities measured via luciferase reporter assays.

Interestingly, the location of the CYLD gene (chromosome 16q12.1) falls on a region that is frequently subject to LOH during HCC pathogenesis [99] and that also contains the SIAH1 gene that has also been implicated in the generation of HCC.

An exciting recent discovery using cross-species comparative oncogenomics [111] has implicated the cIAP1 protein as oncogenic in the context of HCC pathogenesis. cIAP1 is a member of a group of eight proteins in humans characterised by possession of one or more baculoviral IAP (inhibitor of apoptosis) repeat (BIR) domains [126]. Interestingly, five of the eight contain a RING-finger domain characteristic of E3s and one, BRUCE/Apollon, an enormous protein of 4856 residues contains an ubiquitin-conjugation (UBC) domain characteristic of E2 enzymes in addition to its single BIR domain. cIAP1 (and its close relative cIAP2) contain, in addition to multiple BIR and RING domains, a CARD domain which is involved in apoptotic signalling: CARD domains mediate the association of adaptor proteins and procaspases through hetero-dimerisation of the respective CARDs, recruiting procaspases to upstream signalling complexes and allowing procaspase activation. The expression of at least three IAP family members (XIAP, cIAP1 and cIAP2) occurs in response to stimuli that activate NFκB signalling.

The study by Zender et al. [111] using a mouse model of HCC induced by over-expression of c-myc revealed an amplified segment in a murine chromosomal region syntenic with human chromosome 11q22. This region of human chromosome 11q harbours two genes (cIAP and YAP1) which were subsequently confirmed as oncogenic in the context of c-myc over-expression: no oncogenic effect was seen in the context of either H-ras or Akt over-expression highlighting that cIAP1 oncogenicity is context-dependent. Interestingly, the authors noticed that simultaneous over-expression of both cIAP1 and YAP1 gave a synergistic rather than additive amplification of oncogenicity compared with either oncogene alone.

The IAP domain-containing proteins were initially characterised as inhibitors of the pro-apoptotic caspase enzymes [127–129] although the exact mechanistic details, particularly for cIAP1 and cIAP2, of how this effect is mediated is still unclear. cIAP1 can also act as an E3 enzyme for both TRAF2 and RIP leading to UPS-mediated degradation of these proteins and down-regulation of NFκB signalling [113,130–132] (Fig. 4) and sensitisation of cells to TNFα-induced cell killing. The complexities surrounding the roles of the cIAPs in signalling and cancer in also supported by recent studies of multiple myeloma (MM) using comparative genomic hybridisation and microarray analyses [133,134] which revealed that significant numbers of MM patient samples and cell lines harboured inactivating mutations in the cIAP1 and cIAP2 genes (in addition to other mutations) leading to increased, constitutive NFκB activation. While the above data appear to suggest contradictory roles for the cIAPs, there is much yet to be discovered about how these proteins regulate both NFκB and apoptotic signalling and a fine balance may exist between pro- and anti-apoptotic signalling: cIAP1 over-expression may favour anti-apoptotic inhibition of the caspase-mediated cell death pathway and cell survival at the expense of cell death.

The importance of IAPs in preventing cell death and as a potential drug target of significant value to HCC treatment has recently been strikingly illustrated [112,114]. The anti-apoptotic activity of the IAP proteins can be inhibited by natural IAP antagonists that include *Drosophila* Grim, mammalian SMAC/Diablo and Omi/HtrA2 [135–137]. Mature SMAC and Omi are both mitochondrial proteins released into the cytosol on mitochondrial outer membrane disruption during apoptosis and interact with the BIR domain(s) of IAPs thereby inhibiting the negative effect of IAPs on caspase activation and allowing apoptosis to proceed [138–140]. Interaction of these inhibitory proteins with the IAPs is mediated via conserved tetrapeptide motifs at the N-termini of the mature proteins that bind within a surface groove in the IAP BIR domain(s) [140,141]. The studies by Vince et al. [114] and Varfolomeev et al. [112] reveal that small molecule inhibitors of IAP function manifest their ability by binding the BIR domains of cIAP1 at the same site as the natural tetrapeptide ligands of Grim, SMAC/Diablo and Omi/HtrA2 leading to rapid auto-ubiquitylation and proteasome-mediated degradation of cIAP1, a function that requires both the BIR and RING domains of cIAP1. The loss of cIAP1 leads to activation of the classical NFκB signalling pathway, TNFα-dependent cell death and also activates the non-canonical NFκB signalling pathway via stabilisation of NIK, the kinase required to activate this pathway that is normally almost undetectable in cells.

The X-linked inhibitor of apoptosis (XIAP) protein is another member of the IAP family, induced like cIAP1/2 in response to NFκB activation, that has recently been identified as a target of potential therapeutic importance to the pathogenesis of HCC [115]. Many signals that activate NFκB also activate the JNK pathway [142,143], the consequence of which is frequently the activation of pro-apoptotic signals and cell death. NFκB activation circumvents these pro-apoptotic drivers during, for example, TGFβ signalling by up-regulating the expression of anti-apoptotic molecules such as XIAP and cIAP1/2. XIAP is able to form a TGFβ-inducible complex with the protein kinase TAK1 that is critical for the phosphorylation and activation of the IKK complex (leading to degradation of the IκBα inhibitor of NFκB and activation of classical NFκB signalling; Fig. 4) and MKK7, the upstream kinase essential for activation of the JNK signalling pathway leading to apoptosis [109]. Once bound, the RING domain of XIAP mediates the poly-ubiquitylation of TAK1 and its subsequent degradation by the proteasome thus ablating the activation of JNK signalling and inhibiting apoptosis. The role of XIAP in mediating the degradation of TAK1 is intriguing given the fact that many HCCs acquire resistance to TGFβ-mediated cell killing, a property of normal hepatocytes. Small molecule inhibitors of the interaction between XIAP and TAK1 may therefore provide another potentially fertile avenue for exploration of novel, efficacious treatments for HCC.

3.6. FAT10

FAT10 is a recently characterised member of the UBL family with a molecular weight of ~18 kDa comprising 165 amino acids. It was originally identified as a novel gene within the genomic HLA-F locus [144]. Based on the location of the FAT10 gene and its ability to undergo induction of expression in response to IFNγ and TNFα [145], it was proposed that FAT10 may play a role which might be important for the correct functioning of the immune system. FAT10 (along with UCRP/ISG15) is unusual amongst UBL proteins in that it is composed of two tandem, head-to-tail β-grasp ubiquitin superfolds [35,40]. At the level of primary sequence the N-terminal UBL domain is ~30% identical while the C-terminal UBL domain is ~35% identical to ubiquitin itself. In common with ubiquitin and most of the other UBL proteins, FAT10 contains the conserved Gly-Gly di-peptide at its C-terminus, a feature that is essential for the formation of iso-peptide bonds with ε-amino groups of lysine side chains within target proteins [146] and FAT10 has been observed to form conjugates with target proteins that may play a role in promoting apoptosis [147]. Interestingly, there is also a conserved lysine residue within the FAT10 sequence analogous to the K48 of ubiquitin raising the possibility of FAT10 chain formation or possibly modification by ubiquitin or other UBLs.

Recently, several reports have linked FAT10 over-expression with the development of HCC in humans [148–150]. Northern blot analyses for FAT10 mRNA expression in 23 patient HCC samples revealed that there was a significant up-regulation of FAT10 expression in 90% of patients. This data was substantiated by the use of in situ hybridisation and immunohistochemistry using anti-FAT10 antibodies revealed the

highest levels of FAT10 protein localised to the nucleus of HCC hepatocytes and not the surrounding non-HCC or immune cells [148]. A more recent study of FAT10 expression in HCC and colon cancer cells [150] revealed that FAT10 expression was induced up to 100-fold by the synergistic action of both IFNγ and TNFα. Experiments investigating whether FAT10 itself is an oncogene revealed however that it is incapable of transforming NIH3T3 cells, a property of many genuine oncogenes such as H-ras.

A study by Oliva et al. [149] also revealed an almost 200-fold increase in FAT10 expression through the use of the drug 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), a compound which induces the formation of Mallory–Denk Bodies (MDBs) in the liver: chronic liver disease which includes the presence of MDBs is associated with the later formation of HCC [151–153]. The increase in FAT10 expression in this model appears to be due to epigenetic alterations as animals re-fed S-adenosylmethionine showed no FAT10 expression increases. The results of these studies suggest that FAT10 may be important for HCC pathogenesis itself and may also provide a good marker for the identification of liver pre-neoplasia.

4. Conclusions

The global problem of hepatocellular carcinoma is significant with frequently poor prognoses and short life expectancies post-diagnosis combined with current treatment options that are often ineffective. New avenues are therefore required to develop more effective treatments and drug regimens.

Twenty-five years ago, the UPS was unheard of outside a few dedicated enthusiasts. In the intervening years we have discovered that the UPS is critical for normal homeostasis in every cell and the importance of its de-regulation is becoming increasingly apparent to a myriad of disease processes from neurodegeneration to cancer. The first specifically designed drug (bortezomib) that affects the UPS is showing promise in the clinical setting even though it is effectively a fairly crude broadsword with which to tackle disease. Second and third generation molecules that selectively target specific E3 or DUB enzymes are already in development and should provide more of a rapier to specifically target only the key enzyme critical for effective therapeutic intervention.

The increasing evidence of multiple roles for the UPS within the pathogenesis of HCC suggests that it may prove to be fertile ground on which to develop novel therapies that will prove effective in the treatment of this most devastating disease.

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