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Cyclin Degradation for Cancer Therapy and Chemoprevention

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Abstract Cancer is characterized by uncontrolled cell division resulting from multiple mutagenic events. Cancer chemoprevention strategies aim to inhibit or reverse these events using natural or synthetic pharmacologic agents. Ideally, this restores normal growth control mechanisms. Diverse classes of compounds have been identified with chemopreventive activity. What unites many of them is an ability to inhibit the cell cycle by specifically modulating key components. This delays division long enough for cells to respond to mutagenic damage. In some cases, damage is repaired and in others cellular damage is sufficient to trigger apoptosis. It is now known that pathways responsible for targeting G1 cyclins for proteasomal degradation can be engaged pharmacologically. Emergence of induced cyclin degradation as a target for cancer therapy and chemoprevention in pre-clinical models is discussed in this article. Evidence for cyclin D1 as a molecular pharmacologic target and biological marker for clinical response is based on experience of proof of principle trials. J. Cell. Biochem. 102: 869–877, 2007.

Key words: cyclins; proteolysis; ubiquitin; proteasome; ISG15-UBE1L; cancer therapy; cancer chemoprevention

THE CARCINOGENESIS CONTINUUM

Cancer formation and the carcinogenesis process involve multiple steps and may occur over many years. Major steps are summarized in Figure 1 and include initiation, promotion, invasion, and metastatic events. These are part of the carcinogenesis continuum and are potential targets for life-style, dietary or nutritional interventions to prevent cancers or for pharmacologic strategies to reduce, reverse or chemoprevent cancers, as reviewed [Sporn et al., 1976; Talalay, 1989; Stoner et al., 1997; Conney, 2003; Freemantle et al., 2003]. Figure 1A depicts accumulated alterations during carcinogenesis. Many alterations are recognized as surrogate end points or biological markers (biomarkers) of carcinogenesis; some are also molecular pharmacologic targets, as reviewed [Dragnev et al., 2003]. What needs to be revealed in each tumor cell context is which alterations are rate-limiting in the maintenance or progression of carcinogenesis (Fig. 1B). This article highlights deregulation of cyclin expression as a key step in lung carcinogenesis. Indeed, pharmacologic triggering of cyclin proteolysis has been uncovered as a novel therapeutic and candidate chemopreventive target in the lung [Dragnev et al., 2007a].

Several chemopreventive mechanisms target the initiation step of carcinogenesis, as reviewed [De Flora and Ferguson, 2005]. These include scavenging of reactive oxygen species, which inhibit DNA oxidative damage, and regulation of phase I enzymes that affect reactive carcinogenic metabolites and phase II detoxification enzymes that conjugate carcinogens, both

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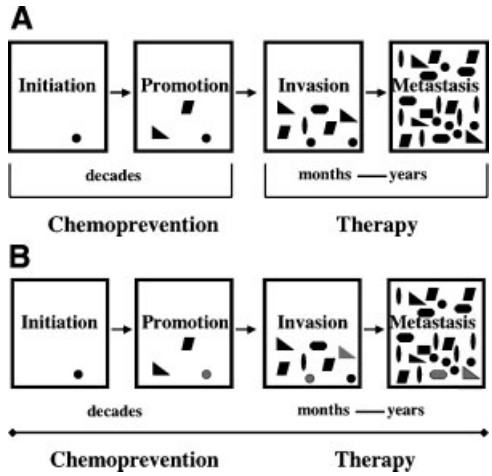

Fig. 1. The carcinogenesis continuum. **A**: Distinct steps have been identified in cancer formation, as displayed in this figure. Coincident with these steps is the appearance of diverse changes in cells and tissues that accumulate in the later stages of carcinogenesis. Changes are depicted as black symbols in this figure. Somewhat arbitrarily, molecular targets for chemoprevention have been conceptualized as affecting only early steps of carcinogenesis while therapeutic targets were viewed as those evident in later stages. Given the continuum of the carcinogenesis process, clinically beneficial chemopreventive effects could be engaged throughout the multi-steps of carcinogenesis. **B**: Ideally, rate-limiting steps in carcinogenesis need to be targeted. These play critical roles in the maintenance and/or progression of this process. These steps are displayed by the gray symbols in this figure and include aberrant cyclin expression, as discussed in the text. The gray symbols are meant to convey presence of aberrant expression of either cyclin D1 or cyclin E, which together represent surrogate markers of carcinogenesis and also anti-neoplastic targets. The solid line is meant to convey the continuum of the carcinogenesis process.

reducing the carcinogenic insult [Talalay, 1989; Stoner et al., 1997]. Pharmacologic and nutritional agents, including isothiocyanates, resveratrol, sulforaphane, and selenium affect initiation, promotion and progression steps of carcinogenesis. These agents act through multiple mechanisms that can include the effects described above plus inhibition of inflammation, proliferation, and angiogenesis as well as induction of differentiation and apoptosis [Jang et al., 1997; Rose and Connolly, 1999; Birt et al., 2001; Murillo and Mehta, 2001; Conaway et al., 2002; Conney, 2003; Sun et al., 2004; Juge et al., 2007].

Despite extensive pre-clinical evidence for chemopreventive activity of structurally diverse agents, translation of these discoveries into the clinic has met with limited success. Yet, clinical evidence for chemoprevention was reported with selective estrogen receptor modulation (SERM) to reduce breast cancer risk [Fisher et al., 1998], selective cyclooxygenase-2 inhibition to reduce polyp formation in familial adenomatous polyposis and with use of non-steroidal anti-inflammatory drugs (NSAIDs) to reduce colon carcinogenesis, as reviewed [Rao and Reddy, 2004]. A novel pathway will be discussed here that triggers cyclin proteolysis and anti-neoplastic effects in relevant pre-clinical models and also within proof of principle clinical trials. Emphasis is placed on studies of lung carcinogenesis where this mechanism was first uncovered.

G1 CYCLINS AND S-PHASE ENTRY

G1 cyclins are activating regulators of cyclin dependent kinases (CDK) 2, 4, and 6 [see reviews Malumbres and Barbacid, 2001, 2005 and Fig. 2]. CDK4 and CDK6 bind to D-type cyclins for activity in early G1; CDK2 binds sequentially to E-type cyclins later in G1 and to A-type cyclins during S-phase, as reviewed [Malumbres and Barbacid, 2005]. The expression of G1 cyclins is tightly controlled at transcriptional and post-transcriptional levels. Cyclin D1 is regulated by multiple extracellular signals and is central to the decision to enter S-phase.

CDK activity is negatively regulated by CDK inhibitors (CKIs). CKIs p15, p16, p18, and p19 bind to CDK4 and CDK6 and prevent binding to D-type cyclins. CKIs p21, p27 and p57 bind to the G1 cyclin–CDK complexes, although only cyclin E-CDK2 activity is stoichiometrically inhibited [Malumbres and Barbacid, 2001; Ortega et al., 2002]. While cyclin D1 is upregulated in response to mitogenic signals, CKIs are induced in response to cellular stress. A classic target gene of p53 is p21, which is activated in response to DNA damage and diverse stimuli [el-Deiry et al., 1993]. Like most biological systems, it is the relative amounts of activators versus inhibitors and not the absolute levels, which determine the cellular response to exogenous signals, see Figure 2. This accounts for small changes in cyclins or CKIs exerting profound consequences on the cell cycle.

CYCLIN D-CDK4/6: AN ONCOGENE AND PHARMACOLOGICAL TARGET

Cyclin D1 and cyclin E1 expression is deregulated in many cancers [Malumbres and Barba-

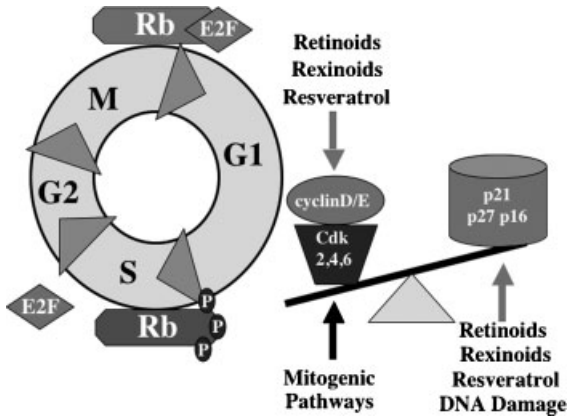

Fig. 2. A schema depicting a simplified model of the cell cycle that emphasizes how chemopreventive compounds delay S-phase entry. Cell cycle progression into S-phase depends on the formation of holoenzymes consisting of cyclin dependent kinases (CDKs) and their regulatory components, the cyclins. Cyclin–CDK complex activity is inhibited by cyclin dependent kinase inhibitors (CKIs), such as p27 and p21. The precise balance between these complex components determines whether cells progress through the cell cycle or not. Representative chemopreventive agents are shown in this figure that delay S-phase entry either by repressing G1 Cyclin-CDK components through transcriptional or post-transcriptional mechanisms or by increasing the levels of specific CKIs. Growth promoting mitogenic signals are shown that enhance cell cycle progression by increasing G1 cyclin expression.

cid, 2001]. The increased CDK activity driven by these cyclins overcomes checkpoint inhibition from CKIs and permits unsanctioned entry into the cell cycle. DNA damage remains unrepaired and additional damage can occur leading to accumulation of neoplastic changes. In this regard, mice lacking cyclin D1 are reported as resistant to breast cancers induced by MMTV-driven H-*ras* and c-*neu*/*erbB-2* oncogenes [Yu et al., 2001]. These experiments highlight a central role for cyclin D1 in breast cancers caused by oncogenic *ras* and HER-2 receptor overexpression.

Subsequent studies confirmed that the CDK-dependent activity of cyclin D1 was responsible for HER-2 mediated breast carcinogenesis, highlighting the therapeutic potential of targeting CDKs [Landis et al., 2006; Yu et al., 2006]. Several small molecule inhibitors of CDKs are undergoing clinical evaluation, as reviewed [Collins and Garrett, 2005; Depinto et al., 2006; de Carcer et al., 2007]. Flavopiridol is a pan-CDK inhibitor preferentially targeting CDK2/cyclin E activity and has shown clinical activity in phase I and II trials. A long-standing goal has been to obtain specific CDK4/6 inhibitors and that objective is being addressed by several small molecule and natural product inhibitors, for example, PD 0332991, fascaplysin and fascaplysin analogues [Toogood et al., 2005; Mahale et al., 2006; Lin et al., 2007].

PROTEASOMAL DEGRADATION AND CYCLIN D1

Ubiquitin is added to proteins through a series of specific enzymatic reactions. E1 activates ubiquitin in an ATP-dependent reaction that allows passage to the E2 ubiquitin-conjugating enzyme. The ubiquitin-charged E2 is then the optimal substrate for binding to target specific E3 ubiquitin ligases. Recently, E4 polyubiquitin chain conjugation factors were identified that control the extent of polyubiquitination or in some cases polyubiquitin-recognition adding another layer of regulation to this system, as reviewed [Kuhlbrodt et al., 2005]. There are seven lysines in ubiquitin; each has been shown to form ubiquitin chains in vitro, as reviewed [Herrmann et al., 2007]. The proteasome selectively degrades proteins modified by multiple ubiquitin molecules linked through the ubiquitin lysine K48 residue of ubiquitin. Conversely, polyubiquitin linked through K63 primarily alters protein function, but does not lead to degradation. Monoubiquitinated proteins are not targeted to the proteasome, but may impact activities such as transcriptional potential, protein processing and subcellular distribution.

The complexity of the ubiquitin and ubiquitin-like systems has been compared to another protein modification, phosphorylation [Herrmann et al., 2007]. Much like phosphorylation, ubiquitination is reversible. Many ubiquitin deconjugation enzymes exist, several of which are involved in recycling ubiquitin from degraded proteins in the proteasome. However, the large and diverse families of deubiquitinases found in cells indicate they likely have additional context specific functions, as reviewed [Wilkinson, 2000].

Cyclin D1 is regulated transcriptionally by a broad range of mitogenic stimuli, as reviewed [Wang et al., 2004]. Cyclin D1 protein stability is regulated through targeted ubiquitin-mediated proteolysis [Alao, 2007]. During the cell cycle, cyclin D1 levels are required to decrease at the G1-S phase boundary. Phosphorylation of the T286 cyclin D1 residue allows it to be recognized

by the nuclear exporter CRM1, which transports it to the cytoplasm where it is rapidly degraded. Glycogen synthase kinase-3β (GSK-3β) phosphorylates T286 of cyclin D1, and activation of GSK-3β has been shown to result in cyclin D1 nuclear export and degradation [Diehl et al., 1998]. Other kinases also phosphorylate cyclin D1 at the T286 position, including $p38^{SAPK2}$ and ERK2, as reviewed [Alao, 2007]. Varying forms of cellular stress can trigger cyclin D1 degradation, including ionizing radiation and a variety of genotoxic, osmotic, viral, and oxidative stimuli, as reviewed [Alao, 2007].

Mechanisms affecting protein stability are important to respond rapidly to exogenous signals. Two well-known examples are DNA damage-induced stabilization of p53, and stabilization of cytosolic β-catenin in response to Wnt signaling. In these cases, transcriptional mechanisms play only a minor role in these responses. In the case of cyclin D1, rapid changes in protein stability occur at the end of S-phase and in response to cellular stress. This provides a sensitive pharmacological target for cell cycle inhibition in therapeutic and chemopreventive settings. Beyond the inhibitors of CDK activity now available, several pharmacological agents were found to accelerate ubiquitin-mediated cyclin D1 degradation. These include all-*trans*-retinoic acid (RA), resveratrol, lovastatin, aspirin, and curcumin, as reviewed [Alao, 2007]. Several of these are being evaluated in cancer therapeutic or chemopreventive clinical trials [http://www.clinicaltrials.gov/]. Although structurally and biologically diverse, each has the ability to delay S-phase cell cycle entry via inhibition of CDK activity.

F box proteins are the substrate recognition components for the E3 ubiquitin ligase, as reviewed [Ho et al., 2006]. The F box protein for cyclin D1 was thought to be Skp2. However, recently F box proteins FBX4 and FBXW8 were shown to directly interact with T286 phosphorylated cyclin D1. FBX4 requires αB crystallin for activity and repression of either component increases cyclin D1 and may be responsible for cyclin D1 deregulation in some cancers [Lin et al., 2006]. FBXW8 depletion also increased cyclin D1 protein levels in colorectal carcinoma cells, but the cell proliferation rate was decreased [Okabe et al., 2006]. Future studies should precisely determine the roles these proteins play in cyclin D1 regulation.

REGULATION OF CYCLIN D1 PROTEASOMAL DEGRADATION AND LOCALIZATION

Retinoids and rexinoids (Retinoid X Receptor, RXR, agonists) induce proteasomal degradation of cyclin D1 [Langenfeld et al., 1996, 1997; Boyle et al., 1999; Spinella et al., 1999; Dragnev et al., 2004; Freemantle et al., 2007]. A study was initiated to determine which of the eighteen lysines in cyclin D1 are required for ubiquitin modification. Single, double, and multiple cyclin D1 lysine mutations were engineered before transfection into cells to evaluate stabilities before and after retinoid or cycloheximide treatments. Mutations affecting lysines surrounding the cyclin box domain and mutations of all lysines present in cyclin D1 markedly reduced polyubiquitination and increased protein stability [Feng et al., 2007]. Mutation of the N-terminus, but not the C-terminus enhanced cyclin D1 stabilization, despite RA-treatment. This indicated an additional site could influence cyclin D1 stability.

Stabilized forms of cyclin D1 were found to preferentially localize to the nucleus [Feng et al., 2007]. This finding agreed with previous observations where an exclusively cytosolic form of cyclin D1 was less stable and a predominantly nuclear form of cyclin D1 was more stable than wild-type cyclin D1 [Diehl and Sherr, 1997; Diehl et al., 1998]. A naturally occurring alternatively spliced cyclin D1 variant (cyclin D1b), which lacks the C-terminal amino acids including residue T286, is predominantly localized to the nucleus and exhibits oncogenic activity [Lu et al., 2003; Solomon et al., 2003]. A specific polymorphism of cyclin D1 (G/A870) at the splice junction of exons 4/5 is proposed to influence the relative amounts of the spliced forms. Tumors homozygous for the A allele have an increased cancer risk and poor disease outcome, which is thought to be due to relatively higher levels of cyclin D1b, as reviewed [Knudsen et al., 2006]. Additional tumor-specific mutations of cyclin D1 have been described in a small subset of patients with endometrial and esophageal carcinomas. Mutations were found in the C-terminal domain of the protein at or adjacent to the T286 residue and were shown to increase nuclear localization of cyclin D1 [Moreno-Bueno et al., 2003; Benzeno et al., 2006].

The precise mechanism of retinoid-mediated cyclin D1 repression is not yet fully delineated.

Retinoids affect expression of hundreds of gene products. Likewise, it is known that cyclin D1 transcription is also repressed in response to retinoid treatment [Freemantle et al., 2007; Huang et al., 2007; Yu et al., 2007]. Cyclin D1 levels are impacted by so many different cell-signaling pathways that it is hypothesized as a surrogate marker of cell cycle activity and even an anti-neoplastic target, as demonstrated below.

ALTERNATIVE CYCLIN D1 DEGRADATION PATHWAYS

More than 10 ubiquitin-like modifiers have been reported in mammals [Herrmann et al., 2007], including interferon-stimulated gene-15 (ISG15). ISG15 conjugation of proteins proceeds via a similar pathway as ubiquitin conjugation. UBE1L is the E1 for ISG15, it can engage some of the same E2 and E3 components as ubiquitin, which has led to the hypothesis these systems might antagonize each other, as reviewed [Pitha-Rowe and Pitha, in press]. ISG15ylation of proteins can alter binding activity, and inhibit enzymatic activity of targeted proteins, among other effects [Takeuchi et al., 2006; Okumura et al., 2007].

Gene profiling experiments determined UBE1L and ISG15 are upregulated in response to retinoids [Pitha-Rowe et al., 2003, 2004a,b; Dao et al., 2006]. ISG15 was originally cloned as an interferon inducible gene; it is upregulated in response to microbial invasion and cell stress, as reviewed [Pitha-Rowe and Pitha, in press]. In acute promyelocytic leukemia (APL) cells, elevated UBE1L expression leads to repression of the oncogenic t(15;17) product, PML/RARα [Kitareewan et al., 2002]. The UBE1L gene is located on chromosome 3 (3p21), a region of frequent loss of heterozygosity in cancers including lung cancers [Kok et al., 1993; Pitterle et al., 1998]. UBE1L expression in histologically normal bronchial epithelium is abundant when compared to some lung cancers and lung cancer cell lines [McLaughlin et al., 2000; Pitha-Rowe et al., 2004b]. Engineered co-expression of UBE1L and cyclin D1 in bronchial epithelial cells resulted in cyclin D1 repression [Pitha-Rowe et al., 2004b]. Taken together, these findings implicate the UBE1L-ISG15 pathway in exerting a tumor suppressive effect. Likewise, this raises the possibility that pharmacological induction or activation of UBE1L-ISG15 would confer therapeutic or chemopreventive effects.

It is interesting to note that antizyme was recently shown to interact with and accelerate the degradation of cyclin D1. This activity is proteasome dependent, but T286 phosphorylation and ubiquitin independent. Therefore, this represents a novel mechanism for delivering cyclin D1 to the proteasome [Newman et al., 2004]. This is a useful tool to probe cyclin D1 as a target for cancer therapy or chemoprevention.

TARGETING G1 CYCLINS IN LUNG CANCER

The molecular pathogenesis of lung cancer and acquisition of genetic and epigenetic changes have been recently reviewed [Sato et al., 2007]. Cyclin D1 deregulation is frequently found in lung cancers along with alterations in several other pathways, which impact cyclin D1 mRNA or protein levels. These include epidermal growth factor receptor (EGFR), AKT, c-kit and *ras*. Initial studies of tobacco-carcinogen exposed immortalized human bronchial epithelial (HBE) cells showed increased expression of cyclin D1, cyclin E, and EGFR as cells became transformed [Langenfeld et al., 1996; Boyle et al., 1999]. Treatment with RA inhibited these alterations and chemoprevented carcinogen-dependent transformation [Langenfeld et al., 1996].

RA is a classical retinoid that activates nuclear retinoic acid receptors (RARs). Certain non-classical retinoids, including those activating RXRs also caused ubiquitin-proteasomal degradation of both cyclin D1 and cyclin E [Langenfeld et al., 1996, 1997; Dragnev et al., 2004]. Degradation of cyclin D1 was dependent on the presence of the T286 residue [Dragnev et al., 2004; Ma et al., 2005]. GSK-3β inhibitors reduced T286 phosphorylation and inhibited RA-mediated cyclin D1 repression [Diehl et al., 1998; Ma et al., 2005]. Cyclin D3, cyclin E, and CDK4 are also repressed with retinoid treatment through the ubiquitin-proteasome pathway [Langenfeld et al., 1996; Sueoka et al., 1999; Ma et al., 2005]. The net result of this degradation is engagement of G1 checkpoint arrest, a hallmark of retinoid response in many cell types that permits repair of genomic DNA damage, as reviewed [Freemantle et al., 2003; Niles, 2004].

INDUCING CYCLIN D1 REPRESSION IN THE CLINIC

Studies were undertaken to learn whether cyclin repression was a therapeutic or chemopreventive target. Aberrant cyclin D1 and cyclin E expression is often detected within premalignant and malignant tissues relative to normal lung tissues; this early onset of cyclin deregulation in carcinogenesis implied it was an attractive target for chemoprevention [Lonardo et al., 1999]. Animal models of chemoprevention validated D-type cyclins as biomarkers of chemopreventive activity [Witschi et al., 2002]. This led to the design of studies to examine cyclin expression in clinical tissues harvested during chemoprevention trials. That approach highlighted cyclin D1 as a surrogate marker of beneficial clinical response in a retrospective clinical study using 13-*cis* retinoic acid, IFN-alpha, and alpha-tocopherol for biochemoprevention [Papadimitrakopoulou et al., 2001].

Large scale randomized chemoprevention trials using classical retinoids have not resulted in significant clinical benefits, as reviewed [Freemantle et al., 2006]. One reason for this is that the nuclear receptor for classical retinoid response, RAR-β, is frequently silenced during lung carcinogenesis, as reviewed [Freemantle et al., 2003]. For this reason, proof of principle clinical trials using alternative treatment strategies were conducted to learn whether cyclins were directly targeted. In patients with early stage resectable lung cancer, a pre-treatment tumor biopsy was obtained to confirm that this expected pharmacodynamic target was basally expressed. Following a short-term treatment (typically 8–10 days) tumors were resected; plasma and intratumoral drug levels were measured. In one of these proof of principle trials, the rexinoid bexarotene was administered [Dragnev et al., 2007b] and in another the EGFR-tyrosine kinase inhibitor (TKI) erlotinib was used [Petty et al., 2004]. Expression profiles of the proliferation marker Ki-67 and cyclin D1 were immunohistochemically compared in post-treatment and pre-treatment biopsies. In both trials, repression of cyclin D1 occurred, but only when "therapeutic" intratumoral drug levels were achieved, that is, drug levels comparable to those producing cyclin D1 repression in vitro.

These clinical observations established that it is possible to affect cyclin D1 expression in human trials with candidate chemopreventive agents. Notably, this pharmacodynamic action depended on intratumoral pharmacokinetic effects. A phase I dose escalation trial combining bexarotene with erlotinib found use of these agents affected cyclin D1 expression in surrogate tissues and conferred desired therapeutic effects even in advanced stage lung cancers resistant to chemotherapy [Dragnev et al., 2005]. Notably, this oral combination therapy regimen was clinically well tolerated and demonstrated broader anti-neoplastic activity than following treatment with either erlotinib or bexarotene treatments alone [Dragnev et al., 2005].

To learn directly whether cyclin degradation plays a critical role in carcinogenesis, transgenic mouse models were engineered. Wild-type cyclin E and proteasome degradation resistant cyclin E (harboring threonine to alanine mutations at residues 62 and 380) were independently driven by the human surfactant C promoter to target expression in the mouse lung. Engineered overexpression of wild-type cyclin E and proteasome degradation resistant cyclin E caused pulmonary dysplasia and adenocarcinomas as well as metastases to form along with onset of chromosome instability, aberrant cyclin D1 expression (Xi Liu, unpublished work), hedgehog pathway activation, and other features reminiscent of human lung carcinogenesis [Ma et al., 2007]. The stabilized cyclin E, relative to wild-type cyclin E transgenic lines, exhibited a highly statistically significant increase in dysplastic lesions and multiple lung cancers, indicating the critical role for cyclin E in lung carcinogenesis [Ma et al., 2007].

These findings not only establish a direct role for cyclin E in carcinogenesis, but also indicate a need to explore the therapeutic potential of targeting G1 cyclins using mouse cancer models. This strategy, if successful, would provide a strong rationale to translate findings into the clinic as part of hypothesis-driven clinical trials. Structurally diverse anti-neoplastic agents can target cyclins for proteasomal degradation [Dragnev et al., 2004]. One approach that has already proven successful is the use of classical or non-classical retinoids and other agents to promote cyclin proteasomal degradation [Dragnev et al., 2004]. Another strategy is to use agents developed to specifically target CDKs 2, 4, and 6, as reviewed [Shapiro, 2006]. Several of these agents are entering into clinical

trials to determine safety, efficacy, as well as pharmacodynamic, and pharmacokinetic profiles [Benson et al., 2007]. How these cyclin kinase targeting agents will be used in hypothesis-driven combination trials is the subject of future work. As is the case for the use of these single agents, defined experimental or genetically engineered animal models such as the recently reported human surfactant C driven cyclin E transgenic lines [Ma et al., 2007] should prove useful to determine therapeutic or chemopreventive activity of agents in the mouse before undertaking phase I, phase II, or ultimately randomized phase III trials.

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