Apoptin : oncogenic transformation & tumor-selective apoptosis

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Chapter 2

Introduction

Cellular proliferation and oncogenic transformation: uncovering the fundamental principles for specific killing of cancer cells

Abstract

The *Book of Genesis* gives a detailed account of how God created our planet in 7 days - or, rather, 6 - through a set of specific, sequential actions. In his *On the Origin of Species*, Charles Darwin postulated that all species of life emerged from a limited number of common ancestors, evolving over time through natural selection (Darwin, 1859). However large the contradiction, both books served an identical purpose: to explain the origin of life. So too in medicine, it was believed that illnesses were the result of supernatural or divine forces, until Hippocrates first argued that disease was the product of environmental factors, diet, and living habits (Jones, 1868). Although many of his assumptions turned out to be erroneous, the so-called 'father of medicine' did launch the idea of pathogenesis, a concept fundamental to modern life science research. Combining the insights of Hippocrates and Darwin, and of many of their colleagues in-between and since, intense scientific effort has been directed at understanding the pathogenesis of one of the world's largest contemporary health problems: cancer (WHO, 2008). While the elaborate molecular mechanisms behind tumorigenesis are being elucidated more and more clearly, therapy is still lacking in safety and effectiveness. Here, I will review the current knowledge on carcinogenic cell transformation, as well as therapeutic approaches stemming from these findings. Next, I will describe exciting new prospects in both research and therapy, where, finally, I will highlight the anti-cancer potential of the Chicken Anemia Virus-derived protein apoptin.

2.1 In the beginning, there was chaos – on the origin of cancer

Cancer is the general term for a class of diseases, characterized by uncontrolled cellular proliferation. Research has indicated that cancer development (tumorigenesis) originates with the stepwise accumulation of genetic changes, driving the progressive transformation of normal cells into highly malignant progeny (Hahn and Weinberg, 2002). These genetic changes include mutations, deletions and amplifications, producing oncogenes with dominant gain of function, and tumor suppressor genes with recessive loss of function. The vast majority of all known tumor suppressor genes are involved in DNA repair and genomic regulation (Lengauer, et al., 1998), so that tumor cells almost invariably display a large degree of genomic instability, resulting in further accumulation of malignant genetic changes.

Random mutations in the approximately six billion basepairs comprising the human genome could theoretically give rise to a huge number of different combinations of genetic alterations. However, research indicates that the process of carcinogenesis is not a random one, and it has been suggested that the more than 100 different types of human cancer share at least six crucial characteristics, the so-called core 'hallmarks' of cancer (Hanahan and Weinberg, 2000, 2011; Stratton, et al., 2009):

1. self-sufficiency in growth signals
2. insensitivity to growth-inhibitory signals
3. evasion of programmed cell death
4. limitless replicative potential
5. sustained angiogenesis
6. tissue invasion and metastasis

Researchers now also propose two additional alterations, namely a change in cellular metabolism (Weinberg and Chandel, 2009), and evasion of immune destruction (Hanahan and Weinberg, 2011). As will

be discussed in following sections, each of these acquired capabilities represents the breach of regulatory mechanisms tightly controlling the cell cycle and hence normal proliferation and homeostasis, upsetting the balance between cell survival and proliferation, and cell death. The genomic instability discussed above is regarded as an enabling characteristic, as is the tumor micro-environment, which can secrete growth and inflammatory factors to promote neoplastic progression (see below).

2.2 Normal proliferation and homeostasis: the cell cycle

At the basis of cellular proliferation and homeostasis lies the cell cycle. This set of strictly organized processes dictates if, when and under which conditions a cell reproduces itself, and provides safe-guarding mechanisms to dispose of aberrant cells.

The most fundamental function of the cell cycle is to accurately duplicate the cell's chromosomal DNA and then segregate the copies precisely into two genetically identical daughter cells. These processes define the two major phases of the cell cycle (Figure 2.1) (Heichman and Roberts, 1994). DNA duplication occurs during S phase (S for synthesis), and chromosome segregation and cell division occur in M phase (M for mitosis). Before each of these phases, eukaryotic cells go through a so-called 'gap' phase – G1 between M and S phase, and G2 between S and M phase. This is partly to allow time for growth, but also importantly to provide time for the cell to monitor the internal and external environment, ensuring that conditions are suitable and all preparations have been completed. The G1 phase is especially important in this respect. Its length can vary greatly depending on external conditions and extracellular signals from other cells. If extracellular conditions are unfavorable, for example, cells delay progress through G1 and may even enter a specialized resting state known as quiescence, or G0, in which they can remain for days, weeks, or even years before resuming proliferation (Pardee, 1989). In

fact, many cells remain permanently in G0 until they or the organism dies. Such cells have either differentiated into specialized states, or have become senescent, and do not have the ability to return to G1. Typically, cells in G2 that do not meet the requirements for completion of the cell cycle, e.g. because of extensive DNA damage, are killed. This is achieved through various modes of cell death (see section 2.5.1).

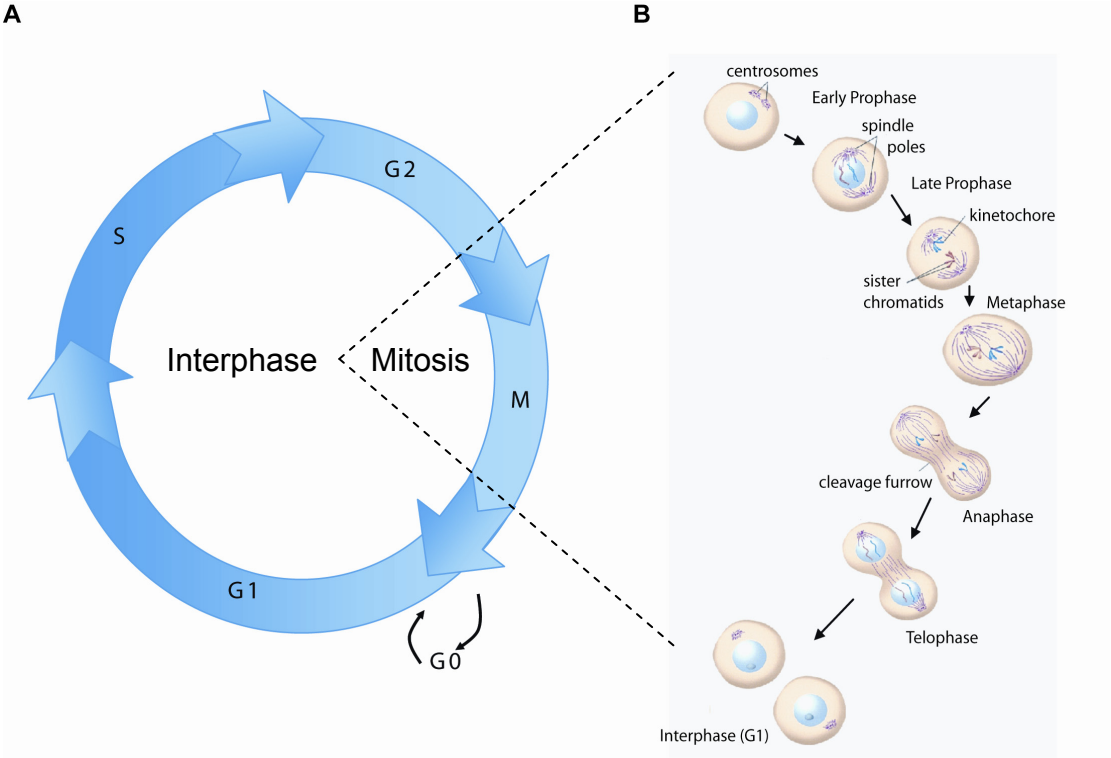

Figure 2.1. A. The eukaryotic cell cycle is traditionally divided into four sequential phases: G1, S, G2, and M. G1, S, and G2 together are called interphase. **B.** During interphase, the centrioles are also replicated, forming small daughter centrioles. Early prophase: the centrosomes, each with a daughter centriole, begin moving toward opposite poles of the cell. Chromosome condensation and nuclear membrane disintegration are initiated. Late prophase: chromosome condensation is completed; each visible chromosome structure is composed of two chromatids held together at their centromeres. The microtubular spindle fibers begin to radiate from the regions just adjacent to the centrosomes, which are moving closer to their poles. Some spindle fibers reach from pole to pole; most go to chromatids and attach at kinetochores. Metaphase: the chromosomes move toward the equator of the cell, where they become aligned in the equatorial plane. Anaphase: the two sister chromatids separate into independent chromosomes and move to one spindle pole each. Simultaneously, the cell elongates, and cytokinesis begins as the cleavage furrow starts to form. Telophase: new nuclear membranes form around the daughter nuclei; the chromosomes uncoil and become decondensed; and the nucleolus becomes visible again. Cytokinesis is nearly complete, and the spindle disappears as the microtubules and other fibers depolymerize. Upon the completion of cytokinesis, each daughter cell enters the G1 phase of the cell cycle and is ready to proceed again around the cycle. Adapted from Lodish et al. (1999)

Box 1. *Cyclins and CDKs control the cell cycle*
At the heart of the cell-cycle control system is a family of protein kinases known as cyclin-dependent kinases (Cdks), which are sequentially activated to trigger the various steps of the cell cycle (Norbury and Nurse, 1991, 1992). Cdks are activated by the binding of cyclins – as indicated by their name – as well as by phosphorylation and dephosphorylation of the kinase. They are inactivated by various Cdk inhibitory proteins (CKIs), such as p16Ink4a, p27Kip1, and p21Cip1, and by degradation of the cyclin subunits at specific stages of the cell cycle (Elledge and Harper, 1994). Each cyclin is specific for a given phase of the cell cycle, and the levels of the various cyclins rise and fall as the cell progresses through the cycle. This results directly in cyclical changes in the phosphorylation and (in)activation of intracellular proteins that initiate or regulate the major events of the cell cycle: DNA replication, mitosis, and cytokinesis. The major cell-cycle regulatory proteins are summarized in Table 2-1.

Table 2-1. Specific Cyclin-Cdk complexes act to promote each phase of the cell cycle.

Cell cycle phase	**Cyclin**	**Cdk**
G1	Cyclin D	Cdk4/6
G1/S	Cyclin E	Cdk2
S	Cyclin A	Cdk2
M	Cyclin B	Cdk1

Below, the four phases of the cell cycle are discussed in further detail.

2.2.1 G1

During the G1 phase of the cell cycle, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cycle into G0 (Sherr, 1996). G1 progression normally relies on stimulation by mitogens, e.g. Ras, and can be blocked by anti-proliferative cytokines, e.g. TNFβ.

Early in G1, D-type cyclins (see Box 1) assemble into holoenzyme complexes with one of two catalytic subunits, Cdk4 or Cdk6 (Sherr, 1994). Transcription of the cyclin D1 gene and assembly with Cdk4 depend strongly on receptor-mediated Ras and PI3-K signaling (Figure

2.2A) (Marshall, 1999). Persistent mitogenic stimulation leads to progressive accumulation of cyclin D-dependent kinases within the cell nucleus; here they collaborate with cyclin E-Cdk2 to phosphorylate pRb and pRb family members p107 and p130, canceling their growth inhibitory functions by disrupting the interaction with E2F, resulting in activation of G1/S and S-phase cyclins, thereby activating the DNA replication machinery and facilitating S phase entry (Reed, 1992).

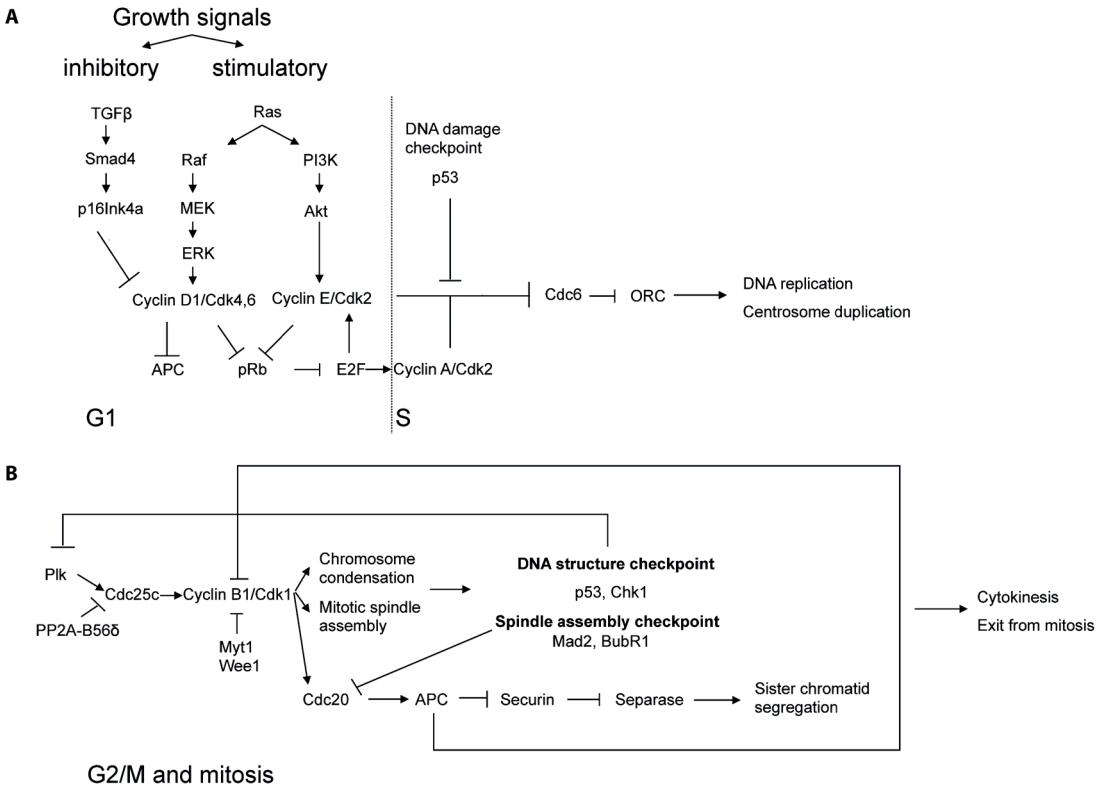

Figure 2.2. Molecular pathways comprising the four phases of the cell cycle. **A.** In G1, growth stimulatory such as Ras, and growth inhibitory signals such as TGFβ, converge on the cyclinD1/Cdk4 complex. A net balance of positive signals lead to activation of cyclinD1/Cdk4, which cooperates with cyclinE/Cdk2 to phosphorylate pRb, thus liberating E2F and initiating DNA replication. ORC, origin recognition complex. **B.** Following DNA replication, CyclinB1/Cdk1 is activated through the actions of Polo like kinase. This activity is however subject to two G2/M control checkpoints, namely the DNA structure checkpoint, which ensures the absence of unreplicated or damaged DNA, and the spindle assembly checkpoint, which ensures the attachment of all sister chromatids to microtubules connecting them to opposite poles of the spindle. Successful clearance of these checkpoints results in activation of the APC, which results in sister chromatid separation and completion of cell division. See text for further details.

The phosphorylation and thus inactivation of pRb constitutes a so-called restriction point (Blomen and Boonstra, 2007; Pardee, 1974); after this, the cells become refractory to extracellular growth regulatory signals, and are committed to enter S phase and complete the cell cycle. Beyond this point, the cell cycle can only be halted by activation of the cell cycle checkpoints (see Box 2).

Box 2. *The G1/S cell cycle checkpoint*
Although cell cycle transitions depend on the underlying CDK cycle, superimposed checkpoint controls help ensure that certain processes are completed before others begin. Components of checkpoint control need not be essential to the workings of the cycle; instead, their role is to brake the cycle in the face of stress or damage. By allowing repair to take place, they become crucial in maintaining genomic stability (Sancar, et al., 2004).

At the transition from G1 to S, there is an important such checkpoint: if the cell's DNA is damaged, p53 (along with its family members p63 and p73) is activated (Bartek, 2001). One of its roles is to ensure that, in response to genotoxic damage, cells arrest in G1 and attempt to repair their DNA before it is replicated. If the damage is too severe to be repaired, continued activation of p53 leads to programmed cell death (see section 2.5.1). If however, the damage is repaired, p53 is again inactivated, and the cell continues through to S phase.

2.2.2 S phase

S phase begins with the activation of the pre-replication complexes by cyclin A/E-Cdk2 (Wuarin and Nurse, 1996). The DNA pre-replication complexes are assembled on replication origins during G1, and are kept inactive by the binding of Cdc6. Phosphorylation of Cdc6 by S-phase Cdk complexes not only activates initiation of DNA replication but also prevents re-assembly of new pre-replication complexes. Because of this inhibition, each chromosome is replicated just once during passage through the cell cycle, ensuring that the proper chromosome number is maintained in the daughter cells.

2.2.3 G2

At the end of S-phase, before progression to M-phase, there are two checkpoints (Sancar, et al., 2004): one in early G2, to ensure all DNA has been replicated, and one in late G2, ensuring that the replicated DNA is error-free. If both checkpoints are cleared successfully, Polo-like kinase activates Cdc25c, which itself activates cyclinB/Cdk1 by removing the inhibitory phosphorylations catalyzed by the Myt1 and Wee1 kinases.

2.2.4 Mitosis

Following its activation by Cdc25c, the cyclinB/Cdk1 complex triggers chromosome condensation, assembly of the mitotic spindle, nuclear envelope breakdown, and rearrangement of the actin cytoskeleton, Golgi apparatus, and ER (Figure 2.2B) (Colanzi and Corda, 2007; Güttinger, et al., 2009). At the metaphase-to-anaphase transition, there is a final, major checkpoint: the spindle-attachment checkpoint (Musacchio and Salmon, 2007). At this point, the cell contains 4n DNA, with each replicated chromosome consisting of two identical sister chromatids glued together along their length by the action of protein complexes called cohesins. The two sister chromatids are attached to opposite poles of the mitotic spindle, with cohesion being enforced by the action of securin. Upon the initiation of anaphase, Cdc20 activates the anaphase promoting complex (APC), which then targets securin for proteolysis, freeing separase, which itself cleaves the cohesin complexes, allowing segregation of the sister chromatids (Sullivan and Morgan, 2007).

The spindle-assembly checkpoint (SAC) operates to ensure that all chromosomes are properly attached to the spindle before sister-chromatid segregation occurs. The SAC depends on a sensor mechanism that monitors the state of the kinetochore, the specialized region of the chromosome that attaches to microtubules of the spindle. The kinetochore comprises the chromosome centromere,

which is defined by the incorporation of specific histone variants, including CENP-A (Cleveland, et al., 2003), and achievement of proper kinetochore tension is dependent on proper formation of pericentric heterochromatin, which is characterized by trimethylation of histone H3 lysine 9 and H4 lysine 20 (Heit, et al., 2009). The generation of stable kinetochore-microtubule attachments depends on the B56 regulatory subunit-containing protein phosphatase PP2A, which is enriched at centromeres/kinetochores of unattached chromosomes (Foley, et al., 2011).

Any kinetochore that is not properly attached to the spindle sends out a negative signal to the cell-cycle control system, blocking Cdc20-APC activation and sister-chromatid segregation. The nature of the signal generated by an unattached kinetochore is not clear, although several proteins, including Mad2, are recruited to unattached kinetochores and are required for the SAC to function. Even a single unattached kinetochore in the cell results in Mad2 binding and the inhibition of Cdc20-APC activity and securin destruction. Furthermore, proteins such as BubR1 sense kinetochore tension, activating the SAC upon lack of proper, amphitelic (bi-oriented) attachment of sister chromatids. Thus, sister-chromatid segregation cannot occur until the final kinetochore has been attached, and sister chromatids are attached to opposite poles of the spindle.

After the chromosomes have segregated to the spindle poles, the cell must reverse the complex changes of early mitosis. The spindle must be disassembled, the chromosomes decondensed, and the nuclear envelope reformed. Cytokinesis then ensues, the cytoplasm is pinched off, and two identical daughter cells are produced, completing the cell cycle. The exit from mitosis is triggered by the inactivation of cyclinB/Cdk1 (Wolf, et al., 2007). This inactivation occurs mainly by ubiquitin-dependent proteolysis of cyclin B, triggered by the same Cdc20-APC complex that promotes the destruction of securin at the

metaphase-to-anaphase transition. Thus, the activation of the Cdc20-APC complex leads not only to anaphase, but also to inactivation of the cyclin B/Cdk1 complex — which in turn leads to all of the other events that take the cell out of mitosis.

Recent studies have shown that the cyclin B/Cdk1 complex can also be inactivated by phosphorylation and inactivation of Cdk1, providing an important contribution to the exit from mitosis. Phosphorylation of Cdk1 is achieved by inactivation of Cdc25c, which again is achieved through the activities of PP2A, specifically PP2A complexes containing the B56δ subunit (Forester, et al., 2007).

2.3 Mechanisms underlying uncontrolled proliferation in cancer: hallmarks and enabling characteristics

As indicated before, human cancer cells have acquired certain capabilities, which allow them to breach the regulatory mechanisms of the normal cell cycle, conferring upon themselves the aforementioned trademark characteristics. Each trait is described below, with a few examples illustrating the strategies by which they are acquired in human cancers.

Self-sufficiency in proliferative signaling

Oncogenic processes exert their greatest effect by targeting particular regulators of G1 phase progression. Cancer cells commonly achieve autonomy from normal growth signaling through three molecular strategies, involving alteration of:

- Extracellular growth signals: many cancer cells acquire the ability to synthesize the growth factors to which they are responsive, e.g. PDGF (Ostman and Heldin, 2007; Wang, et al., 2010), EGF and TGFα (Kalyankrishna and Grandis, 2006). Alternatively, cancer cells may send signals to stimulate the release of growth factors by surrounding (normal) stromal cells (Bhowmick, et al., 2004; Cheng, et al., 2008).

- Transcellular transducers of those signals: growth factor receptors are often overexpressed or structurally altered in many cancers, e.g. Her2/neu in breast cancer (Freudenberg, et al., 2009), either allowing cells to become hyperresponsive to ambient levels of growth factors that normally would not trigger proliferation, or eliciting ligand-independent signaling, respectively.
- Intracellular circuits that translate those signals into action: e.g. the B-Raf protein is activated in about 40% of human melanomas, continuously stimulating proliferation. Similarly, activating mutations in the catalytic subunit of PI3K are being detected in an array of tumor types (Jiang and Liu, 2009; Yuan and Cantley, 2008).

Recent results have also highlighted the importance of the disruption of negative-feedback loops in cancer cells. In approximately 20% of human tumors, the Ras oncogene is activated (Davies, 2002; Downward, 2003; Karnoub and Weinberg, 2008). However, its oncogenic effects do not result from a concomitant hyperactivation of its downstream signaling pathways. Instead, Ras GTPase activity, which normally operates as an intrinsic negative-feedback mechanism to ensure that active signaling is transitory, is compromised.

Circumventing growth-inhibitory signaling

As discussed in paragraph 2.2.1, up to the restriction point, progression through the cell cycle is controlled by the effects of extracellular signals on pRb; beyond this point, control is executed via the cell cycle checkpoints. Hence, to achieve insensitivity to inhibitory signaling, cells must disable the TGFβ-pRb pathway, as well as the cell cycle checkpoints.

Disruption of the TGFβ-pRb signaling circuit, thereby acquiring insensitivity to anti-growth signals (Massagué, 2004), can be achieved in a number of ways:

- downregulation or mutation of the TGF-β receptors (Levy and Hill, 2006);
- elimination of intracellular signal transducers, e.g. by mutation of the gene encoding for Smad4 (Levy and Hill, 2006);
- loss of functional pRb; in fact, the pRb gene was the first tumor suppressor gene to be identified (Knudson, 1971; Sherr and McCormick, 2002).

The first and most important cell-cycle checkpoint (Box 2) involves the activation of another major tumor suppressor protein, p53. Whereas pRb acts in response to signals from the outside, p53 responds to signals from within the cell. If there is significant damage to the cell's genome, or if the levels of growth-promoting signals, nucleotide pools, glucose, or oxygenation are suboptimal, p53 can halt further cell-cycle progression until these conditions have normalized, or, in the face of overwhelming or irreparable damage to such cellular subsystems, p53 may trigger apoptosis. Accordingly, p53 function is lost in over 50% of human tumors, either directly as a result of mutations in the p53 gene, or indirectly through binding to (viral) proteins, or as a result of alterations in genes whose products interact with p53 or transmit information to or from p53 (Vogelstein, et al., 2000).

Evasion of cell death

The normal cell possesses the ability to detect cellular stress, including abnormal mitogenic stimulation, and responds by preventing further division through either cell cycle arrest or programmed cell death (see section 2.5.1), preventing the survival and proliferation of cells with various disease-promoting mutations. Though the exact mechanisms underlying this 'sensing' ability remain to be fully elucidated, several key players have been identified.

For example, excessive mitogenic stimulation leads to the production of a cell-cycle inhibitor protein called p14ARF, which binds and inhibits the p53-inhibitor Mdm2, therefore causing p53 levels to increase, inducing either cell-cycle arrest or, if prolonged, apoptotic cell death (Sherr, 2001). As discussed before, p53 is also activated in response to DNA damage. Furthermore, insufficient survival factor signaling can also trigger apoptosis (section 2.5.1).

Cancer cells acquire resistance to apoptosis through various mechanisms:

- the p53 tumor suppressor gene is inactivated by mutation in approximately half of all human cancers (Brosh and Rotter, 2009; Sherr and McCormick, 2002);
- the anti-apoptotic Bcl-2 oncogene is often up-regulated (Reed, 2008);
- the Fas death-inducing signal has been shown to be titrated away from the Fas death receptor by upregulation of a non-functional (decoy) Fas ligand in cancer cell lines (Pitti, et al., 1998).

Besides apoptosis, emerging evidence suggests that still other devices are in place to prevent abnormal cellular proliferation. These include autophagy, necrosis and senescence. However, it also seems that tumor cells might actively engage in these processes in order to achieve survival. Each pathway is discussed in detail in paragraph 2.5.1, though senescence will also be discussed in the next section.

Acquiring limitless replicative potential

In principle, the combination of growth signal autonomy, insensitivity to anti-growth signals and resistance to apoptosis should suffice to enable the generation of the vast cell mass constituting a tumor. However, Hayflick showed that cells in culture have a finite replication potential and stop growing after a certain number of doublings (60-70

for normal human cells) – a process termed senescence (Hayflick, 1965; Hayflick and Moorhead, 1961). Others showed that senescence could be circumvented by disabling the p53 and pRb tumor suppressor proteins, after which cells continue to multiply until they enter a second state, labeled crisis, which is characterized by massive cell death and end-to-end fusion of chromosomes (Hara, et al., 1991; Shay, et al., 1991).

It is this latter trait that provided the clue to cellular immortalization. The ends of chromosomes, telomeres, are progressively shortened with each cycle of cell division, due to the inability of DNA polymerases to completely replicate the 3' ends of the linear chromosomal DNA during S phase (Harley, et al., 1990; Zhao, et al., 2009). Once telomeres are shortened beyond a critical length, the protein complexes capping the ends are lost, and they are no longer able to protect the ends of chromosomal DNA. The unprotected chromosomal ends trigger a widespread DNA damage response, resulting in end-to-end fusions and death of the cell (Blackburn, 2000; d'Adda di Fagagna, et al., 2003).

In order to prevent telomere shortening and achieve immortalization, malignant cells must therefore activate a system for telomere maintenance (Samassekou, et al., 2010). The large majority (85-90%) does so by upregulating the expression of the telomerase enzyme (Counter, et al., 1994; Kim, et al., 1994; Shay and Bacchetti, 1997), which elongates telomeric DNA, while the remainder uses a mechanism termed "alternative lengthening of telomeres" (ALT), which appears to maintain telomeres through recombination-based interchromosomal exchanges (Bryan, et al., 1997, 1998; Morrish and Greider, 2009).

Angiogenesis

In order to attain and sustain their rapid proliferation rate, tumor cells need to generate an ample amount of ATP for energy and *de novo* synthesis of nucleotides, lipids and proteins. This results on the one hand in an increased demand for oxygen cq vasculature, and on the other hand a fundamental switch in cellular metabolism (the 'seventh' hallmark, see below). The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 μm of a capillary blood vessel. In order to progress to a larger size, tumors must therefore develop angiogenic ability (Bergers and Benjamin, 2003). This "angiogenic switch" is activated by changing the balance of angiogenesis inducers and countervailing inhibitors. One common strategy involves increased expression of vascular endothelial growth factor (VEGF) (Cook and Figg, 2010); VEGF gene expression can be up-regulated by both hypoxia and oncogene signaling (Carmeliet, 2005; Ferrara, 2009; Mac Gabhann and Popel, 2008). Surprisingly, in both animal and human models, angiogenesis was found to be induced relatively early during the development of invasive cancers. It is therefore likely that the angiogenesis switch also contributes to the premalignant phase of neoplastic progression.

Tissue invasion and metastasis

In reality, the vast majority of human cancer deaths are not caused by the primary tumor, but rather by the metastases arising from it. Successful invasion and metastasis depend on the other hallmark acquired capabilities, as well as on the loss of adherence with the surrounding tissue. The most widely observed alteration in cell-cell adhesion in cancer involves E-cadherin (Berx and van Roy, 2009). Normally, coupling of adjacent cells by E-cadherin bridges results in the transmission of anti-growth and other signals via cytoplasmic contacts with beta-catenin to intracellular signaling circuits. Such "contact inhibition" is further enhanced by the actions of e.g. Merlin,

and LKB1. However, in the majority of epithelial cancers E-cadherin function is lost (e.g. by promoter hypermethylation), freeing the path to metastasis (Lombaerts, et al., 2006). Though it remains to be seen how frequently Merlin is compromised in human cancers, it is already known that the loss of the *NF2* gene, which encodes Merlin, triggers a form of human neurofibromatosis. Similarly *LKB1* has been identified as a tumor suppressor gene that is lost in certain human malignancies (Shaw, 2009), and suppression of LKB1 expression destabilizes epithelial integrity and renders epithelial cells susceptible to Myc-induced transformation (Hezel and Bardeesy, 2008; Partanen, et al., 2009).

The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the invasion-metastasis cascade (Talmadge and Fidler, 2010). This depiction envisions a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of these vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed colonization. The epithelial-mesenchymal transition (EMT), a program normally occurring during embryonic development and wound healing, has become prominently implicated in this cascade. Several of the transcription factors responsible for EMT (e.g. Snail, and Slug) can directly repress E-cadherin gene expression, and have been shown in experimental models of carcinoma formation to be causally important for programming invasion; ectopic over-expression of some of these factors has even been found to elicit metastasis (Micalizzi, et al., 2010; Schmalhofer, et al., 2009). It remains to be determined whether EMT also contributes to invasion of non-epithelial tumor types,

although expression of EMT-inducing transcription factors has been observed in some cases.

Two additional, distinct modes of cancer cell invasion have been identified (Friedl and Wolf, 2008). In one, termed "collective invasion", nodules of cancer cells advance *en masse* into adjacent tissues. This is characteristic of e.g. squamous cell carcinomas; coincidentally, these cancers are rarely metastatic, suggesting that collective invasion lacks certain functional attributes to facilitate metastasis. The second mode of invasion, in which individual cancer cells gain morphological plasticity, enabling them to slither through existing interstices in the extracellular matrix, is termed "amoeboid" (Madsen and Sahai, 2010). It is not yet clear whether either of these modes of invasion employs any components of the EMT program, or whether there are still other cell-biologic pathways contributing to invasion and metastasis.

The physical dissemination of cancer cells from the primary tumor to distant tissues is only one aspect of metastasis; the other major phase of metastasis relates to the adaptation of these cells to foreign tissue micro-environments, resulting in successful colonization. Little is known about the precise steps involved in colonization. Carcinoma cells that have undergone EMT during initial invasion and metastasis, might - when no longer under the influence of EMT-inducing signals from the original tumor micro-environment, - undergo a reversal process (termed the mesenchymal-epithelial transition, or MET), resulting in the formation of new tumor colonies. The explosive metastatic growth observed in the clinic for certain cancers, soon after resection of the primary tumor, suggests that the primary tumor might release factors that initially render micrometastases dormant. On the other hand, metastases that erupt decades after treatment of the primary tumor reflect the heterogeneity of the primary tumor (see below): the disseminated cells might lack certain hallmark capabilities, such as sustained proliferative signaling in the absence of

growth factors in the new micro-environment, insensitivity to growth signals present in this new micro-environment, or induction of angiogenesis. Nutrient starvation might induce intense autophagy (see 2.5.1), causing cells to adopt a state of dormancy, which is reversed upon favorable changes in the new micro-environment.

Alternatively, metastatic dissemination may also lead to "re-seeding" of cancer cells at the site of the primary lesion. It is likely that the micro-environment at the primary tumor site is intrinsically hospitable to malignant cells that 'return home', resulting in successful recolonization. Finally, while metastatic dissemination is generally regarded as the final step in neoplastic progression, there are reports indicating that cells can disseminate remarkably early, dispersing from noninvasive premalignant lesions in both mice and humans (Coghlin and Murray, 2010; Klein, 2009). The clinical significance of this phenomenon is however yet to be established, as the ability of such premalignant cells to successfully colonize distant sites remains unproven.

Alteration of cellular metabolism

As briefly alluded to before, the onset of proliferation introduces important problems in not only the cell cycle, but in cellular metabolism as well, for each passage through the cycle requires a doubling of total biomass. Consequently, if cells are to proliferate rapidly and uncontrollably, as is the case in cancer, a profound metabolic reprogramming is required (DeBerardinis, et al., 2008).

At rest, basal levels of growth-factor signaling allow cells to take up sufficient nutrients to provide for the low levels of ATP production and macromolecular synthesis needed to maintain cellular homeostasis. In the absence of any extrinsic signals, mammalian cells lose surface expression of nutrient transporters. To survive in the absence of the ability to take up extracellular nutrients, growth-factor-deprived cells

engage in autophagic degradation of macromolecules and organelles. This is afinite survival strategy, which can ultimately result in cell death. In contrast, mitogenic signaling instructs cells to begin taking up nutrients at a high rate and to allocate them into metabolic pathways that support production of ATP and macromolecules including proteins, lipids, and nucleic acids. The resulting increase in aerobic glycolysis, de novo lipid biosynthesis, and glutamine-dependent anaplerosis, culminating in a net increase in cellular biomass (growth) and, ultimately, the formation of daughter cells, is now regarded as the seventh hallmark of tumorigenicity (Hanahan and Weinberg, 2011; Weinberg and Chandel, 2009).

These features were first observed by Otto Warburg over 80 years ago, who noted that rapidly proliferating tumor cells consume glucose at a higher rate than normal cells, secreting most of the glucose-derived carbon as lactate rather than oxidizing it completely (a phenomenon known as the 'Warburg effect') (Warburg, 1925, 1956). Many reports have since corroborated that an increase in (aerobic) glycolysis is indeed a hallmark of tumorigenicity (Gatenby and Gillies, 2004), though aerobic glycolysis itself is not unique to tumor cells, as it also occurs in rapidly proliferating primary cells. The high glycolitic rate provides several advantages for proliferating cells. It allows cells to use the most abundant extracellular nutrient, glucose, to produce abundant ATP. Notably, the glucose transporter GLUT1 is up-regulated in many human tumors (DeBerardinis, et al., 2008). Although the yield of ATP per glucose consumed is lower compared to oxidative phosphorylation, the rate of ATP production during glycolysis is higher (Pfeiffer, et al., 2001). Also, further compensating for the lower efficiency of aerobic glycolysis compared to oxidative phosphorylation, is the fact that glucose degradation provides cells with intermediates needed for biosynthetic pathways (van der Heiden, et al., 2009). There is even advantage in the clinic, where positron emission tomography (PET) exploits the increased uptake and

utilization of glucose in cancer cells by using a radio-labeled analog of glucose (^{18}F-fluorodeoxyglucose, FDG) to visualize metastatic lesions.

The molecular mechanism behind the metabolic switch observed in tumor cells is regulated by the PI3K/AKT/mTOR pathway. PI3K activation can increase glucose uptake and utilization through AKT (Elstrom, et al., 2004; Rathmell, et al., 2003); mTOR stimulation activates the transcription factor HIF-1 (Majumder, et al., 2004), which enhances glycolysis by increasing the expression of genes that encode glycolytic enzymes and glucose transporters (Semenza, 2000, 2007). Oncogenes such as Ras and Myc also stimulate glycolysis through induction of glycolytic enzymes and glucose transporters (Dang and Semenza, 1999), and activating mutations have been reported for the isocitrate dehydrogenase 1/2 (IDH) enzymes in certain types of cancer (Yen, et al., 2010). Furthermore, the PI3K/AKT/mTOR pathway also stimulates ribosome biogenesis, which is fundamental to achieve rapid cell growth and proliferation (Dufner and Thomas, 1999; Gingras, et al., 2004).

Evasion of immune destruction

Yet another particular feature of cancer cells concerns their relationship to the immune system. Ordinarily, cells of the innate and adaptive immune response cooperate to protect the body against harmful agents, including bacteria, viruses and parasites. Evidence suggests, however, that these cells also function in "tumor surveillance", in which cells and tissues are constantly monitored for nascent tumors, recognizing and eliminating incipient cancer cells. While this is obviously plausible for virus-induced cancers, it seems less so for the >80% of tumors of non-viral etiology. Still, human tumors frequently have defects in MHC class I antigen presentation (Seliger, 2008), and deficiencies in the development or function of cytotoxic T lymphocytes (CTLs), helper T cells or natural killer (NK) cells each led to demonstrable increases in cancer incidence in mouse

models (Kim, et al., 2007; Teng, et al., 2008). Clinical epidemiology also increasingly supports the existence of anti-tumoral immune responses in human cancer; for example, patients with colon and ovarian tumors that are heavily infiltrated with CTLs and NK cells have a better prognosis than those lacking this abundant immune response (Bindea, et al., 2010). Furthermore, cancer cells may paralyze infiltrating CTLs and NK cells by secreting e.g. TGFβ (Yang, et al., 2010), or suppress their actions by recruiting inflammatory cells that are actively immunosuppressive, such as regulatory T cells and myeloid-derived suppressor cells (MDSC) (Mougiakakos, et al., 2010; Ostrand-Rosenberg and Sinha, 2009).

Another class of cells pertaining to the immune system comprises the dendritic cells (DCs). As antigen-presenting cells, DCs play a central role in both innate and adaptive immunity. DCs can be found in tumors in both humans and mice; however, cancer cells have been shown to suppress DCs through the expression of cytokines such as IL-6 and -10, and VEGF (which, coincidentally, also stimulates angiogenesis). Alternatively, tumors may condition DCs to form suppressive T cells, and studies have shown that in multiple myeloma, DCs even support clonogenic growth (Steinman and Banchereau, 2007, and references therein). Thus, much like certain infectious agents (e.g. HIV), cancer cells have developed strategies to evade, and in some instances even exploit, DCs.

Taken altogether, the data imply that anti-tumor immunity might be a significant barrier to tumor formation and progression, imposing upon tumor cells the need to acquire the ability to either evade immune suppression, or adapt it to promote proliferation.

Genomic instability

Acquisition of the features discussed above depends in large part on a succession of alterations in the genomes of neoplastic cells. This

entails mutations, but also epigenetic modifications. Ordinarily, genome maintenance systems (often referred to as the caretakers of the genome) ensure that the rates of spontaneous mutations per cell cycle are very low. Additionally, as discussed above, p53, the "guardian of the genome", plays a central role in the surveillance systems that normally monitor genomic integrity and inhibit proliferation of genetically damaged cells. Analysis of cancer cell genomes has shown that many tumor cells appear to specifically target the caretakers and guardians of the genome for deletions and inactivating mutations, further accelerating the accumulation of tumor-promoting genomic alterations. Conversely, other genomic regions, harboring genes whose expression favors neoplastic progression, are often amplified in cancer cells. Genomic imbalance is thus an enabling characteristic, exploited by cancer cells to acquire the hallmark capabilities required for malignant transformation.

Telomerase has ambiguous roles in this regard: in the absence of telomerase expression, sustained proliferation results in loss of telomeric DNA, leading to end-to-end fusions and general karyotypic instability. While the resulting genetic alterations could be advantageous to the cancer cell, they may also induce cellular senescence. Increased expression of telomerase, while bypassing senescence, may reduce genomic instability and delay neoplastic progression; prolonged expression of telomerase may again lead to genomic imbalance due to fusion and breakage of excessively elongated telomeres.

The immune system and other cells of the tumor micro-environment

As discussed before, some tumors are densely infiltrated by cells of both the innate and adaptive arms of the immune system. What's more, it's becoming increasingly clear that practically every neoplastic lesion contains immune cells – ranging from subtle infiltrations to gross inflammations. This is largely though to reflect an attempt by

the immune system to eradicate cancerous cells. However, the tumor-associated inflammatory response has been shown to have a paradoxical effect, enhancing tumorigenesis and progression, in fact helping incipient neoplasias to acquire hallmark capabilities.

Inflammatory cells supply growth factors to sustain proliferative signaling, survival factors limiting cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes facilitating angiogenesis, invasion and metastasis, and EMT-inducing signals (DeNardo, 2010; Grivennikov, 2010; Karnoub and Weinberg, 2006, 2007; Kessenbrock, et al., 2010; Qian and Pollard 2010), and have even been shown to release mutagenic factors, promoting genomic imbalance (Grivennikov, 2010). Concurrently, inflammation is in some cases evident at the earliest stages of neoplastic progression, and is demonstrably capable of fostering the development of incipient neoplasias into full-blown cancers (Qian and Pollard, 2010: de Visser, 2006). The tumor-stroma interaction is not one-sided: not only do cancer cells secrete factors to suppress elimination by the cells of the immune system, but they have also been shown to stimulate these cells. In an experimental model of metastatic breast cancer, the cancer cells secreted CSF-1, stimulating tumor-associated macrophages, while the latter reciprocated by supplying epidermal growth factor (EGF) to the breast cancer cells (Qian and Pollard, 2010).

Evidently, these interactions also extend to the other cells in the tumor micro-environment. For contrary to earlier views, tumors are now regarded as complex, organized networks of heterogeneous, specialized cells – comparable to organs. Besides the cells of the immune system, these include endothelial cells and pericytes, which form the tumor-associated vasculature, as well as fibroblasts and other stromal cells.

Another important constituent of the tumor micro-environment concerns the so-called "cancer stem cells" (CSCs). Traditionally, tumors have been portrayed as reasonably homogeneous cell populations – principally arising from a single cell that managed to acquire the hallmark capabilities - until relatively late in the course of tumor progression, when hyperproliferation combined with increased genetic instability would spawn distinct clonal subpopulations. However, there is increasing evidence that certain cancer cells assume a stem cell-like character. CSCs, like their normal counterparts, may self-renew as well as spawn more differentiated derivatives. The origins of these CSCs is not entirely clear, though it is proposed that they arise either through de-differentiation, or through oncogenic transformation of normal tissue stem cells (Cho and Clarke, 2008; Lobo, et al., 2007). Additionally, CSCs have been shown to express markers of their corresponding normal tissue stem cells (Al-Hajj, et al., 2003). They were originally implicated in the pathogenesis of hematopoietic malignancies, but have now also been identified in e.g. breast carcinomas and neuroectodermal tumors. In fact, induction of the EMT program in certain model systems has been shown to induce many of the defining features of stem cells (Mani, et al., 2008).

One important implication of the above-discussed, recently acquired knowledge on the tumor micro-environment, is that all the core hallmark capabilities might not need to reside within a single cell. For instance, the ability to negotiate the invasion-metastasis cascade may be acquired in certain cancers via inflammatory cells in their micro-environment, without the requirement that the cancer cells themselves undergo additional mutations beyond those that were needed for primary tumor formation. Another is that the dynamic interactions between cancer cells and their micro-environment, and the development of CSCs, complicates not only the elucidation of the mechanisms of cancer pathogenesis, but also the development of novel therapies to successfully target primary and metastatic tumors.

2.4 Oncogenic transformation: the making of a human tumor cell

Regardless of the many remaining uncertainties, the set of cancer-typical traits discussed above does allow for a tentative model of oncogenic transformation (Figure 2.3). Experiments using the viral oncoproteins Simian Virus 40 (SV40) large and small T antigens have elegantly demonstrated that full malignant transformation of human cells can be achieved in a limited number of steps, requiring (Hahn and Weinberg, 2002b):

- Oncogenic activation of Ras, e.g. through activating mutations, conferring growth signal autonomy;
- Bypassing replicative senescence and evasion of apoptosis by the introduction of SV40 LT, which binds to and inhibits the functions of pRb and p53, respectively (Ali and DeCaprio, 2001);
- Activation of telomerase to achieve immortalization;
- Co-expression of SV40 ST, which associates with PP2A and alters its cellular function (Yu, et al., 2001). Though PP2A has many cellular functions and has been shown to be an important tumor suppressor, exactly how inhibition of PP2A contributes to malignant transformation remains unclear (Mumby, 2007).

Intriguingly, while the fifth and sixth hallmarks are not required for malignant transformation, but rather promote continued proliferation, invasion and metastasis once the tumor has been formed, the seventh hallmark is indeed activated by Ras. Similarly, the eighth proposed hallmark appears not to be required for initial malignant transformation, though one might speculate that the SV40 antigens could perhaps either trigger the activation of the immune system, eliciting tumor-promoting inflammation, or actively suppress antigen presentation, aiding in immune escape of infected cells. Furthermore, owing to the inhibition of pRb and p53, cells are predisposed to genomic instability, facilitating the acquisition of the remaining hallmarks and thus further neoplastic progression.

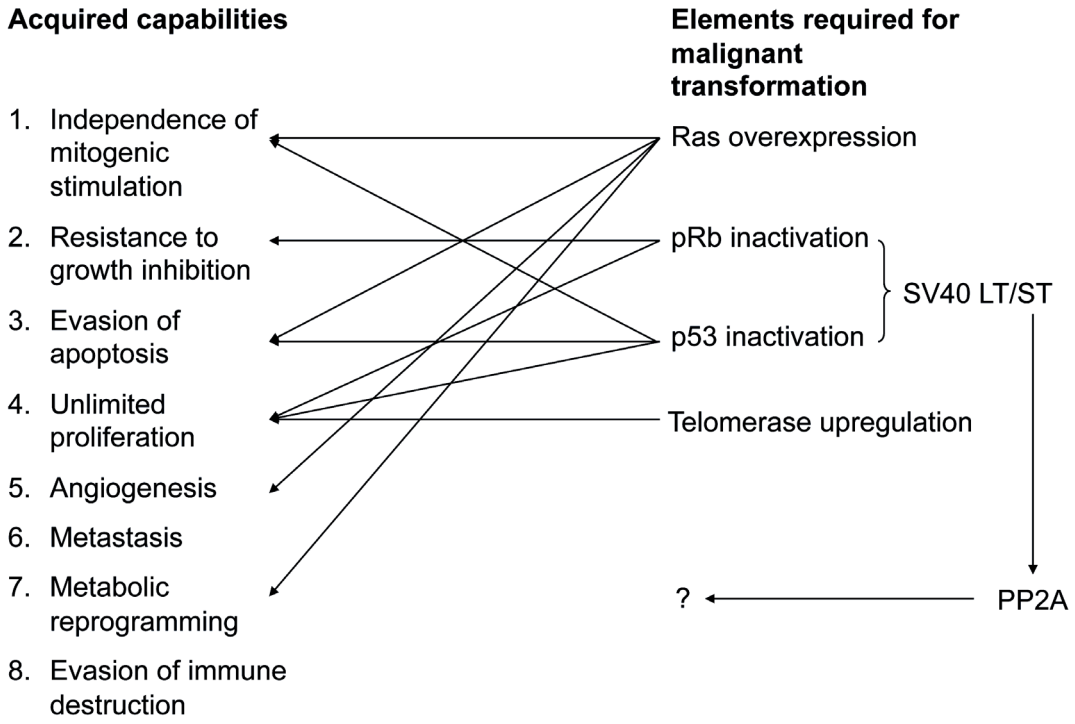

Figure 2.3 Experimental findings demonstrate that only a few steps are necessary for malignant transformation of human cells. Over-expression of Ras confers independence from mitogenic signaling, while inactivation of the tumor suppressors pRb and p53 confer immortalization, which is sustained by upregulation of telomerase. Ras over-expression also induces angiogenesis and the seventh proposed hallmark, namely the metabolic switch, which is postulated to be required to provide the energy and nutrients necessary for rapid cellular proliferation. PP2A inactivation by SV40 ST has been demonstrated to be required for full malignant transformation, though how this contributes to tumorigenesis has yet to be elucidated. Adapted from Hahn and Weinberg, 2002b.

2.5 Killing tumor cells in the 21st century

Cancer is traditionally treated by debulking through surgery, and killing any remaining cells by a combination of radio- and chemotherapy. As the conventional therapies have been designed to target rapidly proliferating cells in general, and do not target the tumor cells specifically, they are also toxic to normally rapidly proliferating cells, causing serious side-effects, such as anemia, and suppression of the immune system. Furthermore, they rely heavily on the induction of apoptosis, whereas, as discussed previously, cancer cells typically accumulate alterations to the apoptotic machinery, conferring on them the ability to evade apoptosis. Recent

understanding of the molecular pathogenesis of cancer has led to the development of targeted therapies, and increasing attention is being directed towards other types of cell death, including autophagy, mitotic catastrophe, necrosis and senescence. The various pathways leading to cell death are discussed in section 2.5.1, and the novel anticancer strategies designed to effectuate cancer cell death are presented in section 2.5.2.

2.5.1 Cell death pathways and response to antitumor therapy

The various modes of cell death have long been classified according to their morphological features (Kroemer, et al., 2009). Recent breakthroughs in cell death research have, however, allowed for the tentative introduction of a novel characterization based on measurable biochemical features (Galluzzi, et al., 2011). Both the morphological and biochemical features of the various cell death types are summed up in Table 2-2 and schematically depicted in Figure 2.4. Even though the various modes of cell death are discussed as separate entities, one must keep in mind that many interconnections exist: e.g., the apoptosis and autophagy pathways share a number of components (Maiuri, et al., 2007), while autophagy is required to mediate the senescence transition (Young, et al., 2009).

Apoptosis

Apoptosis is the term for programmed cell death, in which the cell membrane is disrupted, the cytoplasmic and nuclear skeletons are broken down, the nucleus is fragmented, chromosomes are degraded, and the shriveled cell corpse, neatly packaged, is engulfed by nearby cells and disappears, without eliciting an inflammatory response (Kroemer, et al., 2009).

The apoptotic machinery, depicted in Figure 2.4A, consists of sensor proteins and a family of effector proteins called caspases (Kurokawa

Table 2-2. The morphological features of the different modes of cell death. Adapted from Wlodkowic, et al., 2010 and Galluzzi, et al., 2011. MAP1LC3, micro-tubule-associated protein 1 light chain 3; SQSTM1, sequestosome 1

Type of cell death	**Morphological features**	**Distinctive biochemical features**
Apoptosis	Rounding-up of the cell Reduction of cellular and nuclear volume Nuclear fragmentation Plasma membrane blebbing Minor modification of cytoplasmic organelles Engulfment by resident phagocytes *in vivo*	Internucleosomal DNA fragmentation Phosphatidylserine exposure Intrinsic apoptosis - caspase-dependent, cytochrome c release - caspase-independent Extrinsic apoptosis - death receptor signaling, caspase-8/-10 activation - dependence receptor signaling, caspase-9 activation
Autophagy	Lack of DNA fragmentation Accumulation of (double-membraned) autophagic vacuoles Little or no uptake by phagocytic cells *in vivo*	Increased lysosomal activity Initially perceived as caspase-independent although recent reports indicate cross-talk with apoptosis MAP1LC3 lipidation SQSTM1 degradation
Necrosis	Dissolution of chromatin Swelling of cytoplasm and cytoplasmic organelles Rupture of plasma membrane	Lack of caspase cascade activation RIP1/3 activation
Mitotic catastrophe	During mitosis: multiple micronuclei, aberrant mitotic spindles Following mitotic failure: formation of giant polykaryons	Mitotic arrest Caspase-2 activation (in some cases) p53/p73 activation (in some cases)
Senescence	Appearance of characteristic heterochromatic foci Flattened cytoplasm Increased cellular granularity	Initiated by telomere shortening Activation of SA-β-gal Caspase-independent

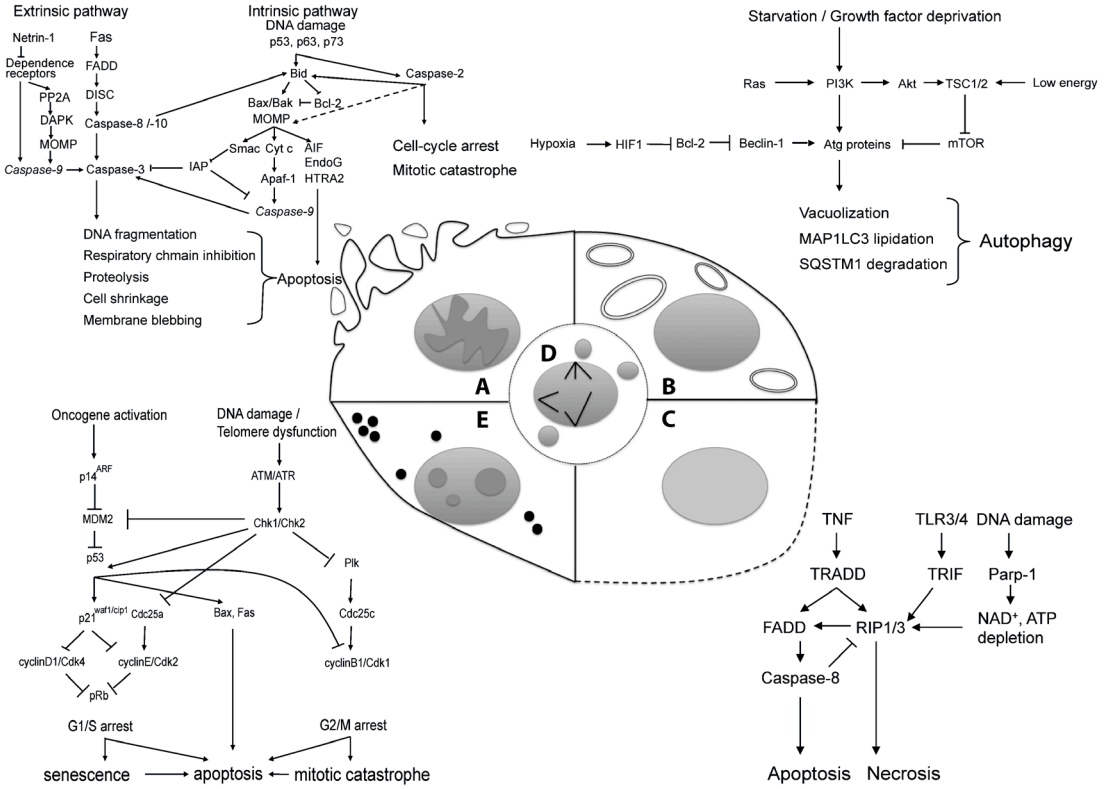

Figure 2.4. Schematic depiction of the various modes of cell death, and a general overview of the most important molecular players involved, also indicating the cross-talks existing between the different pathways. **A.** Apoptosis is characterized by nuclear chromatin condensation and fragmentation, cell shrinkage and blebbing of the cytoplasmic membrane. It can be induced extrinsically by stimulation of death receptors, e.g. FADD and insufficient survival signaling, or intrinsically, by e.g. DNA damage. Both pathways converge on the activation of the executioner caspase, caspase-3; however, DNA damage-induced activation of caspase-2 can also result in cell cycle arrest and mitotic catastrophe. Release of AIF, EndoG, and HTRA2 proteins from the mitochondria can also induce caspase-independent apoptosis. MOMP, mitochondrial outer membrane permeabilization. **B.** Autophagic cell death is characterized by the appearance of double-membraned autophagic vacuoles and the lack of chromatin condensation. Autophagy is induced by starvation and/or growth factor deprivation, which stimulates PI3K to induce the formation of autophagosomes comprising Beclin-1 and various Atg proteins. Other cellular stress signals, such as hypoxia and low energy also stimulate autophagy, respectively by removing Bcl-2 sequestration of Beclin-1, and mTOR suppression of autophagosome assembly. **C.** Necrotic cell death is characterized by chromatin dissolution, cytoplasmic swelling and rupture of the cell membrane. The kinase RIP1 and its homolog RIP3 are central players in this process, and induce necrosis in the case of caspase inhibition. **D.** Mitotic catastrophe is the result of damaged DNA and aberrant mitotic spindle formation. Abrupt interruption of mitosis at metaphase/anaphase results in the formation of multiple micronuclei. Otherwise, in the case of mitotic failure, the spindle is disassembled and cells enter G1 without having undergone cytokinesis, forming giant polykaryons (not depicted). **E.** DNA-damage- and oncogene-induced senescence is characterized by the appearance of characteristic heterochromatic foci, cytoplasmic granules, and flattening of the cytoplasmic membrane. See text for further details.

and Kornbluth, 2009). Apoptosis can be initiated by two distinct pathways, respectively conveying intra- and extracellular stress signals. Intracellular stress signals, such as growth factor withdrawal, DNA damage, oxidative stress or oncogene activation, lead to release of cytochrome c from the intermembrane space of the mitochondria to the cytoplasm. This process is tightly regulated by the Bcl-2 family of both pro- and anti-apoptotic proteins, and results in the activation of caspase-9. The extrinsic pathway is activated in one of two ways: either by the binding of death-inducing ligands, such as Fas and TNFα, inducing formation of the death-inducing signaling complex (DISC), and activation of caspase-8 and -10, or alternatively, through the actions of "dependence receptors", when the concentration of their specific ligands fall below a certain threshold (Mehlen and Bredesen, 2011). Both apoptotic pathways lead to activation of the executioner caspases, caspase-3, -6 and -7, which are the main proteases responsible for cellular degradation.

In addition, experiments with caspase inhibitors, wherein cell death could be delayed but not inhibited, led to the proposal of a caspase-independent mode of intrinsic apoptosis. This would entail the release of AIF, EndoG and HTRA2 from the mitochondria in response to intrinsic stress signals, leading to large-scale DNA fragmentation and cleaving of a wide array of proteins, including cytoskeletal proteins.

The last stage of apoptosis involves the uptake of apoptotic cells by phagocytosis. This process is initiated by externalization of phosphatidylserine on the surface of apoptotic cells, facilitating recognition, uptake and removal of apoptotic cell debris by phagocytes.

Autophagy

Autophagy is characterized by the sequestration of cytoplasmic material (proteins and organelles) within autophagosomes for bulk

degradation by lysosomes (Kroemer, et al., 2009). Typically, autophagic cell death occurs in the absence of chromatin condensation, but is accompanied by massive autophagic vacuolization of the cytoplasm. These so-called "autophagosomes" originate from two conjugation systems, involving the autophagy-associated Atg proteins (de Bruin and Medema, 2008) (Figure 2.4B). In fact, lipidation of Atg8 (MAP1LC3) is a defining biochemical feature of autophagy, as is degradation of the autophagic substrate sequestosome 1 (SQSTM1) (Table 2-2). The autophagic pathway is regulated by the PI3K/AKT/mTOR pathway (Petiot, et al., 2000; Wang and Klionsky, 2003), which, coincidentally, is also responsible for the metabolic switch observed in rapidly proliferating cells (the seventh hallmark of cancer).

Rather than being simply a cell death pathway, autophagy is actually quite important for cell survival, providing an alternative source of nutrients (Klionsky and Emr, 2000). In yeast, autophagy is induced under nutrient-limiting conditions as a mechanism to survive; however, in *Drosophila melanogaster*, autophagic structures are formed during morphogenesis, corroborating its role in cell death (Baehrecke, 2003). It has therefore been considered that, under conditions of cellular stress, autophagy might start as an adaptive response in order to enhance cell survival, but that, beyond a certain threshold, it can result in cell death. Importantly, some reports indicate that cells displaying features of autophagic cell death can still recover upon withdrawal of the death-inducing stimulus (Boya, et al., 2005).

During cellular transformation, autophagy may prevent a normal cell from becoming a malignant one by degrading damaged organelles and thereby reducing cellular stress, or by degrading specific proteins that enhance tumor formation (Jin and White, 2007; Mathew, et al., 2007). It may also limit chromosome instability and thereby tumor

progression (Mathew, et al., 2007). Alternatively, autophagy may prevent tumorigenesis by killing premalignant cells (Karantza-Wadsworth, et al., 2007). Besides its potential tumor-suppressive roles in the early stages of tumorigenesis, autophagy has also been proposed to play a tumor-promoting role during the later stages of tumor growth (Amaravadi, et al., 2007; Lum, et al., 2005). In this case, autophagy protects cells against stressful conditions. Notably radio- and chemotherapy treatment can induce autophagy, leading to a state of reversible dormancy, enabling the resistance, persistence and regrowth of tumors (Apel, et al., 2009; White and DiPaola, 2009).

Necrosis

Necrotic cell death is characterized by cellular swelling, rupture of the plasma membrane and subsequent loss of intracellular contents, often provoking an inflammatory response (Kroemer, et al., 2009). As opposed to apoptosis, necrosis has long been considered to be an uncontrolled form of cell death. However, evidence is accumulating that the execution of necrotic cell death may be finely regulated by death domain receptors and Toll-like receptors, and is dependent on the activity of the kinase RIP1 and its homolog RIP3 (Festjens, et al., 2007) (Figure 2.4C).

Neither the precise role of the kinase activity of RIP1 nor its downstream targets are known. Previously, it was shown that mitochondria-produced reactive oxygen species (ROS) are important players in the execution of necrotic cell death (Festjens, et al., 2006). Therefore, it is conceivable that RIP1 directly or indirectly targets mitochondria. Indeed, in tumor necrosis factor (TNF)-stimulated cells, RIP1 translocates to the mitochondria. In addition, RIP1 has also been shown to be essential for TNF-induced production of ceramide, the latter mediating TNF-induced caspase-independent cell death. As the phospholipase cPLA2 contributes to TNF-induced necrosis (Thon, et al., 2005), it is conceivable that a RIP1-cPLA2-acid sphingomyelinase

pathway may lead to necrotic cell death. Because inhibition of ceramide accumulation clearly diminished caspase-independent cell death but not as completely as inhibition of RIP1, ceramide obviously may represent a central factor, but most likely not the only one, transmitting the death signals generated by RIP1 in response to TNF.

Notably in some studies, RIP1-dependent autophagic cell death instead of necrosis was observed (Yu, et al., 2006). However, the induction of autophagic cell death was much slower than the induction of death receptor-induced necrotic cell death. Thus, whether necrosis or autophagy ensues when apoptosis is inhibited, will surely depend on cells and circumstances.

Necrosis can also be induced through DNA damage (Festjens, et al., 2006). This type of cell death is mediated by PARP-1, a protein involved in DNA damage repair. Activation of PARP-1 catalyzes the hydrolysis of NAD+ into nicotinamide and poly-ADP ribose, causing depletion of NAD+. This results in cellular energy failure and caspase-independent death of different cell types.

Unfortunately, the inflammatory response which accompanies necrotic cell death (in contrast to apoptosis and autophagy), can in fact promote neoplastic progression, given that the inflammatory cells can foster proliferation, angiogenesis and tissue invasion and metastasis (see paragraph 2.3). Additionally, necrotic cells can release factors like IL-1α, which can directly stimulate viable neighboring cells to proliferate, again facilitating neoplastic progression.

Mitotic catastrophe

Mitotic catastrophe is a type of cell death that follows aberrant mitosis, occurring either during or shortly thereafter. In mammalian cells, and particularly in tumor cells, mitotic catastrophe is mainly associated with activation of the G2/M cell cycle checkpoints for DNA

damage/structure and spindle assembly, and involves numerous players involved in these checkpoints, including Chk2, cyclinB/Cdk1, and members of the p53 family, including p53 and the p73 variant TAp73 (Figure 2.4D) (de Bruin and Medema, 2008). Following mitotic catastrophe, cells are ultimately killed by engaging the apoptotic or necrotic pathways, or by induction of cellular senescence (Figure 2.4E) (Galluzzi, et al., 2011).

At least two subtypes of mitotic catastrophe can be distinguished (Castedo, et al., 2004). First, mitotic catastrophe can kill the cell during or close to metaphase, in a p53-independent manner involving the activation of caspase-2. Second, mitotic catastrophe can occur after failed mitosis, in a partially p53-dependent manner involving the activation of the polyploidy checkpoint in G1. Even though mitotic catastrophe is accompanied by chromatin condensation and mitochondrial release of apoptosis-inducing factor and cytochrome c, which are key features of apoptosis, there are a number of fundamental differences. Importantly, it has been shown that over-expression of Bcl-2 does not block and might actually enhance mitotic catastrophe (Lock and Stribinskiene, 1996). Cell death occurring during the metaphase-to-anaphase transition is characterized by the activation of caspase-2, which is activated in the nucleus in response to DNA damage (Lassus, et al., 2002; Paroni, et al., 2002), and is a process that cannot be inhibited by Bcl-2 (Peart, et al., 2003; Read, et al., 2002; Robertson, et al., 2002).

Senescence

Analogously to the replicative senescence induced in primary cells as a result of shortened telomeres, treatment of malignancies may result in a permanently growth-arrested state (Gewirtz, et al., 2008). This is termed 'accelerated senescence', and is sensed as a permanent state of DNA damage, while the cell remains viable and metabolically active (Figure 2.4E). DNA damage signaling activates the p53 and pRb

proteins (or their respective family members, as p53 and pRb function are often lost during tumorigenesis), which respectively results in first a temporary, then a prolonged arrest in G1. Senescent cells characteristically display senescent associated DNA damage foci (SDF) and senescent associated heterochromatin foci (SAHF) (Campisi and d'Adda di Fagagna, 2007); SAHF are often found at the promoters of E2F target genes where they are thought to inhibit transcription, thereby enforcing growth arrest (Narita, et al., 2003).

2.5.2 Novel approaches to the treatment of cancer

The success of anticancer therapies depends on their ability to distinguish between normal and cancer cells and specifically exert their toxic effect on the malignant cells. Novel anticancer strategies therefore involve a targeted approach, utilizing knowledge of the cancer hallmarks and enabling characteristics discussed above, and the tumor suppressor and oncogenic pathways involved. Accordingly, the following strategies will be discussed:

- inhibition of growth signaling pathways
- induction of programmed cell death
- disruption of telomere maintenance and, hence, cellular immortalization
- targeting the tumor and its micro-environment to prevent angiogenesis and metastasis
- attenuation of tumor cell metabolism
- targeting cancer cells for immune destruction
- exploiting genomic instability
- proteins selectively killing tumor cells

Clinical experience with therapies selectively targeting only one each of these characteristics has shown that the effect is often transitory. This suggests the existence of at least some (partial) redundancy, in the form of multiple pathways governing each capability, and/or an adaptive shift from one capability to another, facilitated by genomic

instability and the tumor micro-environment. Hence, successful cancer therapies must comprise a combination of modalities.

Targeting growth signaling pathways in cancer

The first two acquired capabilities discussed in section 2.3 concerned self-sufficiency in growth signaling and insensitivity to growth-inhibitory signaling. Thus, growth-signaling pathways, and especially the receptor proteins, are interesting targets for anticancer strategies (Christoffersen, et al., 2009). Therapeutic approaches involve both hormone therapy and monoclonal antibodies. For example, targeting the EGF dependent signaling pathway has been successfully applied in the clinic. One of the receptors in this pathway is Her2 (Her2/neu, ErbB2), and it has been shown to be overexpressed in 20-25% of breast tumors. Targeting this receptor via the antibody Herceptin (trastuzumab) has proven to be very effective in the treatment of this type of cancer (Chang, 2010).

Another approach involves the development of Cdk inhibitors to halt the cell cycle, with several compounds already being evaluated in clinical trials (De Falco and De Luca, 2010).

Inducing programmed cell death

Overturning the cancer cell's apoptosis blockade has been an appealing approach in the design of anti-cancer therapies. For example, in cells where p53 function has been lost, this might be substituted for by activation of the p53 family member p73, either alone or in combination with other anti-cancer therapies (El-Rifai and Zaika, 2008; Vilgelm, 2008). The same might also be achieved by inhibition of the negative apoptosis regulator Bcl-2 (Kang and Reynolds, 2009), e.g. through so-called BH3-mimicking compounds (Chonghaile and Letai, 2008). Another promising approach is the development of small therapeutic compounds, referred to as Smac mimetics, which are designed to block the function of members of the

inhibitor of apoptosis (IAP) protein family (Gyrd-Hansen and Meier, 2010). Alterations in IAPs are found in many types of human cancer and are associated with chemoresistance, disease progression and poor prognosis (Hunter, et al., 2007; LaCasse, et al., 2008). Consistent with the idea that different types of cancer cells are dependent on ("addicted to") IAPs for their survival, the inactivation of IAPs, particularly when combined with other treatments, results in the death of most tumour cells *in vitro*. Though inactivation of IAPs does not seem to be detrimental to normal cells, loss of IAPs is also associated with the development of certain types of cancer. Several compounds are therefore currently being assessed for safety in phase I clinical trials.

As induction of the other modes of cell death may cause undesirable tumor-promoting effects, studies to define optimal strategies to modulate and exploit these other forms of cell death for cancer therapy are still ongoing. For instance, several autophagy modulators are currently being investigated (Chen and Karantza, 2011), and mechanisms being considered for the induction of senescence include restoration and/or promotion of p53 function, modulation of the cell cycle through Cdk inhibitors, and inhibition of telomerase action (see below) (Nardella, et al., 2011).

Attacking cellular immortalization via disruption of telomere maintenance

While normal human somatic cells do not or only transiently express telomerase and therefore shorten their telomeres with each cell division, most human cancer cells typically express high levels of telomerase and show unlimited cell proliferation (acquired capability 4). Telomerase is thus an attractive therapeutic cancer target, and novel anti-cancer strategies include the direct targeting of components of telomerase: the protein component hTERT or RNA component hTR (Phatak and Burger, 2007). Examples of such agents include the small

molecule hTERT inhibitor BIBR1532 and Imelstat (GRN163L), a thio-phosphoramidate oligonucleotide targeting the template region of hTR as a "template antagonist" (Kelland, 2007). Anti-tumor effects of both compounds have been observed in cell lines and, particularly for Imelstat, also in xenografted human tumors in mice. Imelstat treatment of human glioblastoma tumor-initiating cells *in vitro* led to progressive telomere shortening, reduced rates of proliferation, and eventually cell death (Marian, et al., 2010). In combination with radiation and the DNA alkylating agent temozolomide, Imelstat had a dramatic effect on cell survival and activated the DNA damage response pathway. *In vivo*, chronic systemic treatment produced a marked decrease in the rate of xenograft subcutaneous tumor growth.

Effects of anti-telomerase treatment are largely dependent upon initial telomere length, which can result in a substantial lag before antitumor activity is observed in tumors possessing relatively long telomeres. An alternative approach is therefore to target the telomere itself (Telomere Targeting Agents, TTAs) (Kelland, 2005). Several classes of small molecules have been described that induce the G-rich single-stranded overhang of telomeric DNA to fold into 4-stranded G-quadruplex structures. Such folding is incompatible with telomerase function and may induce rapid telomere uncapping. These molecules have shown potent telomerase inhibition in nanomolar concentrations *in vitro* and the rapid induction of senescence in cancer cells. The TTA BRACO19 has demonstrated single agent activity against human tumour xenografts with anti-tumour effects apparent from only 7 days of treatment.

So far, Imelstat is the only drug of its class in clinical trials. In the near future, it is expected that other direct telomerase targeted agents as well as those targeting telomeres (e.g., AS1410 based on BRACO19) will enter Phase I clinical trials (Parkinson and Minty, 2007).

Targeting angiogenesis and metastasis

As discussed above, tumor growth requires the malignant cell to be able to e.g. form new vasculature, as well as produce autologous growth factors. These characteristics need not be united in a single cell, as cells in the tumor micro-environment can contribute to neoplastic progression by providing the necessary factors, aiding not only in e.g. the angiogenic switch, but also in fostering tissue invasion and metastasis.

Furthermore, the cancer stem cells (CSCs) found in many tumors have important properties that must be considered in the development of effective cancer therapies. Their relative quiescence allows them to escape cell death by therapies that attack only rapidly proliferating cells. Also, their ability for self-renewal allows them to recover from the anti-cancer attack, repair their DNA and again initiate the formation of a new tumor. Thus, even if the cancer appears to be cured, the CSCs survive, and in time the tumor reappears. Furthermore, as CSCs are able to modify the tumor microenvironment, providing trophic factors to support tumor growth, they harbor a considerable potential for recurrence, as well as successful colonization of distant metastases.

Therapeutic strategies that could be employed to target angiogenesis and metastasis in general (acquired capabilities 5 and 6), and cancer stem cells in particular, are:

- anti-angiogenic therapy, e.g. through inhibition of VEGF using the monoclonal antibody bevacizumab, to reduce the vasculature, thereby depleting the tumor of oxygen and essential nutrients (Cook and Figg, 2010)
- blocking adhesion between cells and to the extracellular matrix to prevent successful colonization of metastases, e.g. by targeting tumor-specific cadherins and integrins (Blaschuk and Devemy, 2009; Desgrosellier and Cheresh, 2010)

- another way to prevent metastasis might be through blocking the EMT chemotaxis pathways that are active in the tumor and its micro-environment (Nieto, 2011; Roussos, et al., 2011)
- inducing differentiation, e.g. through BMP signaling to revert the capacity of CSCs to form tumors and increase their sensitivity to therapy (Ghotra, et al., 2009)
- targeting self-renewal and quiescence, e.g. through PTEN or Wnt signaling pathways, to decrease the CSC population (Ghotra, et al., 2009)

Targeting cancer cell metabolism

In addition to acquiring a complex array of genetic changes, tumor cells develop an alteration in the metabolism of glucose and oxygen (acquired capability 7). As this altered metabolism does not appear to be subject to the high genetic variability of tumors, it may represent a more reliable target for cancer therapy.

The altered metabolism of cancer cells is associated with increased glycocolytic activity and repression of oxidative phosphorylation. The harmful effects of the concurrent increase in H_2O_2 production is counterbalanced by the increase in glycolytic activity, creating a self-reinforced loop. This loop can be interrupted by increasing the cellular levels of H_2O_2 (using e.g. pro-oxidant agents), or by attenuating glycolysis (using glycolysis inhibitors), or a combination of both (López-Lázaro, 2010).

In fact, many anticancer agents, such as paclitaxel, doxorubicin and arsenic trioxide, produce H_2O_2 (Alexandre, et al., 2006; Jing, et al., 1999; Ubezio and Civoli, 1994). Also, using several cancer and normal cell lines, Chen, et al. (2005) observed that high concentrations of ascorbic acid selectively killed cancer cells and that this effect was mediated by H_2O_2. Several glycolysis inhibitors have shown anticancer effects (e.g. 2-deoxy-D-glucose, lonidamine, 3-bromopyruvate and

dichloroacetate) and some of them have already entered clinical trials (Chen, et al., 2007; Gatenby and Gillies, 2007; Lopez-Lazaro, 2008; Martin, 2006; Pelicano, 2006; Xu and Huang, 2006). For example, it has been shown that dichloroacetate, a known glycolysis inhibitor that has been used in humans for decades in the treatment of lactic acidosis and inherited mitochondrial diseases, induced marked anticancer effects in mice (Bonnet, et al., 2007). Other strategies that might also be used to exploit the increased glycolytic activity of cancer cells and selectively kill these cells, are inhibition of the Na+/K+-ATPase pump (e.g. by cardiac glycosides), or of cellular systems involved in the extrusion of (acid-death inducing) protons from the cytosol.

Because of the dual role of autophagy in cancer, it is difficult to predict whether inhibition or stimulation of autophagy may result in tumor cell death (Apel, et al., 2009). Preclinical studies with chloroquine, which inhibits lysosome acidification and thereby autophagy, in conjunction with alkylating agents, showed remarkable efficacy inhibiting tumour growth in mice (Amaravadi, et al., 2007). Alternatively, promoting autophagy might also be expected to limit tumor progression. Hence, before autophagy can be targeted for the treatment of cancer, further studies investigating the dichotomy of its roles in tumor prevention and promotion are warranted (Rosenfeldt and Ryan, 2009).

Another aspect of the metabolic switch that takes place during neoplastic progression concerns the concurrent increase in protein production, and hence, ribosome biogenesis. Though this has long remained a largely unexploited target in cancer therapy, increasing attention is being paid to inhibitors of rRNA synthesis for the development of novel therapeutic strategies (Drygin, et al., 2010). For example, CX-3543, a small molecule nucleolus-targeting agent that selectively disrupts nucleolin/rDNA complexes in the nucleolus,

thereby inhibiting Pol I transcription and inducing apoptosis in cancer cells, is currently being evaluated for treatment of carcinoid/neuroendocrine tumors in a phase II clinical trial (Drygin, et al., 2009). Some classic anticancer therapeutics, including cisplatin and 5-fluorouracil, have even been shown to exert their activity, at least partially, through disruption of ribosome biogenesis (Ghoshal and Jacob, 1997; Jordan and Carmo-Fonseca, 1998).

In addition, studies indicate that many common and specialized mRNA export factors, including CRM1 and eukaryotic translation initiation factor 4E (eIF4E), are disregulated in cancer, making them also attractive therapeutic targets (Siddiqui and Borden, 2011). Indeed, specific targeting of the eIF4E-dependent mRNA export pathway in a phase II proof-of-principle trial with ribavirin led to impaired eIF4E-dependent mRNA export, correlating with clinical responses including remissions in leukemia patients (Assouline, et al., 2009).

Immune destruction of cancer cells

The ability of cells of the immune response system to infiltrate tumors presents a unique opportunity for combating cancer cells. Tumors are replete with potential antigens, which can become immunogenic when presented by DCs, activating the different arms of cell-mediated resistance (Steinman and Banchereau, 2007). This means that the resulting immune attack can encompass multiple targets, diminishing the cancer's chances of immune escape. Following recognition of tumor-specific antigens, T lymphocytes exert their cytotoxic effects on tumor cells via the extrinsic apoptosis pathway, involving Fas, and via the secretion of perforin and serine proteases granzyme A and B (Pardo, et al., 2004). Perforin is a transmembrane pore-forming molecule, which allows granzyme A and B to enter the target cell and induce apoptosis. Granzyme A activates caspase-independent pathways by inducing single-stranded DNA damage, while granzyme B

directly activates caspase-3, and can also cleave Bid to induce the release of cytochrome c (Rousalova and Krepela, 2010). There is also evidence that DCs themselves can acquire killer activity and express granzyme and perforin.

Several strategies are being employed in the field of tumor immunology, including the use of monoclonal antibodies against specific tumor-associated antigens to achieve steric inhibition and neutralization, complement activation, and activation of cell-mediated cytotoxicity, and so-called "cancer vaccines" (Dougan and Dranoff, 2009).

Vaccination against infectious diseases has proven to be one of the great successes of modern medicine, inducing efficient, specific activation of cytotoxic T lymphocytes, as well as the generation of memory cells, protecting against future infection. Translation of this knowledge for the prevention and treatment of cancer has not been straightforward, with, among other things, the selection of appropriate target antigens proving a difficult task, as well as the design and interpretation of clinical trials for this novel class of cancer therapeutics (Lesterhuis, et al., 2011; Palucka, et al., 2011). One example showing great promise with regard to cancer prevention is the development of two human papilloma virus-derived vaccines for the prevention of cervical cancer (Lowy and Schiller, 2006). As for therapeutic vaccination, a series of clinical trials have recently yielded encouraging results. First, treatment of metastatic prostate cancer with sipuleucel-T, a cellular vaccine based on enriched blood DCs briefly cultured with a fusion protein of prostatic acid phosphatase with GM-CSF, resulted in an approximately 4-month–prolonged median survival in phase III trials (Kantoff, et al., 2010). Sipuleucel-T has been approved by the FDA for treatment of metastatic prostate cancer, thereby paving the clinical development and regulatory path for the next generation of cellular immunotherapy products. Second, a

phase III trial in metastatic melanoma testing peptide vaccine in combination with high dose IL-2 versus IL-2 alone showed significant improvement in overall response rate and progression-free survival in patients who received the vaccine (Schwartzentruber, et al., 2011). Third, a phase III trial in patients with follicular lymphoma showed that idiotype vaccine therapy (BiovaxID) significantly prolongs the duration of chemotherapy-induced remission (Morse and Whelan, 2010). Furthermore, a randomized phase II trial of a poxviral-based vaccine targeting a prostate-specific antigen (PROSTVAC) in men with metastatic castration-resistant prostate cancer showed an improved overall survival in patients when compared with patients receiving control vectors (an observed difference in median survival of 8.5 months) (Kantoff, et al., 2010b).

In the sipuleucel-T example described above, DCs are generated *ex vivo*, loaded with tumor antigens, and re-injected to induce strong T-cell and perhaps also natural killer immunity. Another, novel, approach to cancer vaccines is based on the delivery of antigens directly to dendritic cells (DCs) *in vivo*, using chimeric proteins made of anti-DC receptor antibody fused to a selected antigen (DC targeting). Studies in mice demonstrate that DC targeting results in considerable potentiation of antigen-specific T cell immunity. The induction of immunity is observed only when the DC maturation signal is provided, as, otherwise, tolerance ensues (Bonifaz, et al., 2002; Hawiger, et al., 2001). A major challenge of this approach will be to elicit T cell responses that are sufficiently robust and long lasting so as to be clinically active. Indeed, the efficacy of DC targeting *in vivo* needs to be established in clinical trials in patients, and early studies are ongoing.

Besides the molecular make-up of the tumor itself, immunotherapeutic approaches also need to consider that of the tumor micro-environment, and aim to trigger a multi-facetted immune

response involving humoral, cellular, and innate immunity (Poschke, et al., 2011). Recent studies have attempted to relieve the suppression of immune activity in the tumor micro-environment (imposed by the cancer cells) by blocking inhibitory signals using monoclonal antibodies, showing promising results (such as blocking cytotoxic T-lymphocyte antigen-4, CTLA-4, with the monoclonal antibody ipilimumab) (Hoos, et al., 2010). Additional efforts to either directly target MDSC or their suppressive mechanisms should aid the development of successful combination-immunotherapies. Due to their immature nature, a potential way to remove MDSC is to force them to differentiate, for example, by using all-trans retinoic acid (ATRA) or vitamin D3, which promotes myeloid differentiation and has been clinically applied (Lathers, et al., 2004; Mirza, et al., 2006).

Exploiting genomic instability

The genetic instability that is characteristic of cancer cells can be both good and bad for anticancer therapy. Although it seems to provide an Achilles' heel that many conventional therapies exploit, genetic instability can also make eradicating cancer more difficult. Because of the abnormally high mutability of many cancer cells, most malignant tumor cell populations are heterogeneous in many respects, which may make them difficult to target with a single type of treatment. Moreover, this mutability allows many cancers to evolve resistance to therapeutic drugs at an alarming rate (Rajagopalan and Lengauer, 2004).

The novel “synthetic lethal” approach aims to exploit defects in DNA repair pathways using a new theory (Yap, et al., 2011). This theory proposes that targeting tumor cells, genetically defective in one given pathway, with a specific molecular therapy, designed to inhibit a “synthetic lethal” gene partner involved in a complementary pathway, results in selective tumor cell killing. Studies have shown that breast cancer cells, defective in homologous recombination due to BRCA1/2

defects are highly sensitive to blockade of the base excision repair (BER) pathway via inhibition of the poly (ADP-ribose) polymerase (PARP) enzyme, providing strong evidence for the clinical application of this approach (Bryant, et al., 2005; Farmer, et al., 2005). PARP inhibitors might also be used in combination with standard radio- and chemotherapy to sensitize cells to cytotoxic DNA damage; additionally, some PARP inhibitors seem to possess anti-angiogenic activity, making their application in the clinic even more attractive (Mangerich and Bürkle, 2011).

The synthetic lethal concept has also been applied for the treatment of p53-deficient cancers, which, as discussed before, occur rather frequently. Loss of p53 function renders cells dependent on the checkpoint kinase Chk1 for activation of cell cycle checkpoints; hence, inhibition of Chk1 in the presence of DNA damage or replicative stress should lead to mitotic catastrophe and cell death in p53-deficient tumors while sparing normal cells. Several Chk1 inhibitors have now been developed; after showing promising results in preclinical models, they are currently being evaluated in phase I clinical trials (Ma, et al., 2011).

Proteins selectively killing tumor cells

In recent years, a unique set of molecules possessing a remarkable ability have been identified: these proteins are able to detect the malfunctioning of a presumably shared set of cancer-critical genes, and selectively kill the corresponding tumor cells, leaving the normal cells unharmed. Among these proteins killing tumor cells (PKTC) are the human cytokines TRAIL and MDA7/IL24, the frog-derived Brevinin-2R, the human pro-apoptotic Par-4 and Noxa proteins and the organic cation transporter-like 3 (ORCTL3), the alpha-lactalbumin and oleic acid complex HAMLET, and the viral proteins adenovirus E4ORF4, parvovirus NS1, and the Chicken Anemia Virus-encoded protein apoptin (Bruno, et al., 2009; Grimm and Noteborn, 2010;

Argiris, et al., 2011). As these PKTC appear to function independently of the type of cancer, their therapeutic potential seems unprecedented. Apoptin, the first of these proteins to be discovered, and the subject of the present thesis, will be discussed in the next section.

2.6 Apoptin

Apoptin is the protein product of the VP3 gene of the Chicken Anemia Virus (CAV; Noteborn, et al., 1994). The pathogenesis of CAV infection and discovery of apoptin will be discussed in section 2.6.1, while the characteristics of apoptin and perspectives for application as an anti-cancer agent are discussed in sections 2.6.2 and 2.6.3, respectively.

2.6.1 Chicken Anemia Virus

In young chicks, CAV infection results in severe anemia and immunodeficiency (among other symptoms), owing to apoptosis of the cells in the thymus, bone marrow and spleen (Noteborn and Koch, 1995). Early after infection, a 2.3 kb polycistronic mRNA is transcribed, encoding three genes: the capsid protein VP1, the scaffold protein and phosphatase VP2, and VP3 (apoptin) (Noteborn, 2004). Later on in the infection, splicing of the CAV mRNA produces additional RNA products; it is however not known whether these result in functional proteins (Kamada, et al., 2006).

Studies in transformed chicken cells in culture proved that apoptin was responsible for the CAV-induced apoptosis (Noteborn, et al., 1994). Apoptin demonstrated the same apoptosis activity in human cancer cell lines, but, surprisingly, not in normal human cells (Backendorf, et al., 2008; Danen-van Oorschot, et al., 1997; Noteborn, et al., 2005; Tavassoli, et al., 2005). This makes apoptin itself an interesting protein for therapeutic treatment of cancer cells, but also an interesting tool to investigate the process of oncogenic transformation, thereby uncovering other novel targets for anticancer

therapies. Furthermore, apoptin may also prove useful in the diagnosis of cancer, e.g. based on its ability to be specifically phosphorylated by tumor cell lysates (see below).

2.6.2 Characteristics of apoptin-induced apoptosis

Apoptin is a small (121 aa), proline-rich protein, exhibiting little homology to any other known protein – including its functional equivalent TAIP, encoded by the TT virus (Kooistra, et al., 2004). Tumor-selective apoptosis induction by apoptin is preceded by a) phosphorylation at T108 (Rohn, et al., 2002), and b) nuclear translocation (Danen-van Oorschot, et al., 2003) (Figure 2.5). These effects are characteristic for the transformed environment, as they are also elicited upon transient transformation of normal human cells by the SV40 LT and ST proteins (Zhang, et al., 2004).

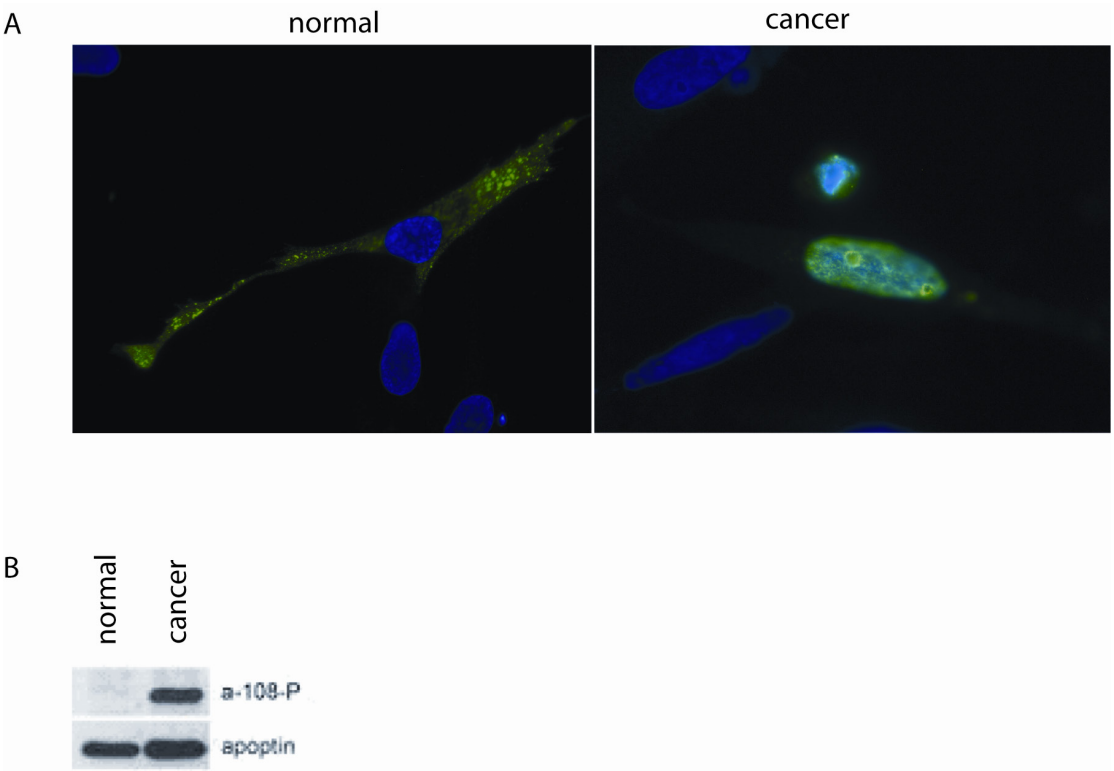

Figure 5. Characteristics of tumor-selective apoptosis induction by apoptin. **A.** In normal cells (left panel), apoptin is located in the cytoplasm, and can be found in granules extending to the cell membrane. In tumor cells (right panel), apoptin is located in the nucleus and induces apoptosis (arrow indicates dying cell). **B.** In tumor cells, but not in normal cells, apoptin is phosphorylated at position T108.

The detailed mechanisms behind both the up- and downstream pathways of apoptin activity in transformed and cancer cells are yet to be elucidated. A number of apoptin-interacting proteins have been identified, including Hippi, the protein interactor and apoptosis co-mediator of the huntingtin interacting protein 1 (Cheng, et al., 2003), and Rybp/DEDAF, a transcription factor and proapoptotic protein known to associate with the death effector domain-containing DNA binding protein DEDD (Danen-van Oorschot, et al., 2004).

Furthermore, apoptin has been shown to act through the intracellular apoptosis pathway, involving the translocation of Nur77 from the nucleus to the cytoplasm, as well as p73 and PUMA activity (Klanrit, et al., 2008; Maddika, et al., 2005). Apoptin expression enhances the level of the tumor suppressor ceramide in tumor cells, also implicating the involvement of sphingolipids in apoptin-induced cell death (Liu, et al., 2006a, b).

2.6.3 Perspectives for apoptin anti-tumor therapy

Though its pathway of action remains to be clarified, apoptin has demonstrated several features that make it well suited for cancer therapy. Besides the fact that it has been shown to selectively kill tumor and transformed cells, apoptin has also been shown to act independently of p53 (Teodoro, et al., 2004; Zhuang, et al., 1995). Furthermore, it is insensitive to BCR-ABL and Bcl-XL (Backendorf, et al., 2008; Noteborn, et al., 2005), which suggests that apoptin might induce apoptosis in cases where other (chemo)therapeutics might fail. The results of the first preclinical therapeutic studies, discussed below, are very promising.

Apoptin cancer therapy using adenoviral vectors

Pietersen and colleagues developed a strategy for the use of apoptin in cancer therapy based on adenoviral vectors (Pietersen, et al., 1999). A single injection of the apoptin-producing adenovirus in xenografted

hepatomas in nude mice resulted in a delay in tumor growth. The number of proliferating cells as detected by BrdU-labeling was dramatically decreased in the apoptin-transduced regions versus control-treated tumors (Van der Eb, et al., 2002). Importantly, the apoptin-producing adenovirus did not have appreciable toxic effects when injected intraperitoneally, intravenously, or subcutaneously into healthy rats. Further studies using these and other non-replicative viruses, including a fowlpox virus–based vector, used a regimen of multiple intratumoral injections during several days. These approaches resulted in a significant overall survival benefit for the apoptin-treated mice and, in some cases, depending on the overall transduction efficiency, complete regression of the established tumor (Noteborn, 2009).

PDT4-apoptin - topical cancer treatment

Guelen and coworkers fused apoptin to the HIV-TAT protein transduction domain, demonstrating efficient transduction of apoptin into both normal and tumor cells, while preserving its tumor-selective apoptotic properties (Guelen, et al., 2004). Sun and colleagues used a similar fusion product, employing protein transduction domain 4 (PTD4)-mediated transduction of recombinant apoptin protein, to treat tumors that had been xenografted onto nude mice (Sun, et al., 2009). In contrast to the gene therapy-based studies discussed above, PTD4-apoptin was administered by simple application onto the epidermis. Remarkably, though the protein could be detected in the epidermal tissue covering the subcutaneous tumor tissue and in several internal organs of the mice, cell death was only observed inside the tumor mass.

Recently, Jin and colleagues (Jin, et al., 2011) reported that PTD4-apoptin protein and dacarbazine acted synergistically to reduce tumor growth in a mouse melanoma model. Importantly, the combination with PTD4-apoptin allowed for a 50% reduction in the dosage of

dacarbazine, resulting in comparable reduction of tumor-growth, without any detectable hematological side-effect.

Systemic apoptin treatment and organ-specific targeting

Peng et al. showed that apoptin can be safely administered systemically, and used a specific ligand to the asialoglycoprotein receptor to target apoptin specifically to the liver (Peng, et al., 2007). Delivery of this Asor-apoptin via the tail vein into mice bearing in situ hepatocarcinomas resulted in specific and efficient distribution of apoptin in both hepatocarcinoma cells and normal liver cells. Whereas the former cells showed significant signs of regression, the normal hepatocytes were clearly not affected.

Specific targeting of apoptin to the brain is currently being developed by de Boer and coworkers. Using the nontoxic ligand CRM197 (a mutant of the diphtheria toxin), the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF), also known as the diphtheria toxin receptor (DTR), can be targeted. This receptor is constitutively present at the blood-brain barrier and is strongly up-regulated in many tumors, including human glioblastoma (Mishima, et al., 1998). Biopharmaceutical drugs have been selectively delivered to the brain via this receptor (Gaillard, et al., 2005). An apoptin-expressing plasmid coupled to CRM197 has been successfully delivered to human glioblastoma cells *in vitro* through receptor-mediated endocytosis (Rip, et al., 2009). The combination of apoptin antitumor therapy and CRM197-targeting technology thus provides a great opportunity for development of targeted therapy for brain tumors.

Combination therapy with apoptin and chemo- or radiotherapy

The combination of apoptin therapy with chemotherapeutic agents has been reported to enhance cytotoxicity to human tumor cells *in vitro* (Olijslagers, et al., 2007). Combined treatment of recombinant

adenovirus expressing apoptin with different concentrations of etoposide clearly showed an additive cytotoxic effect in human osteosarcoma U2OS cells. Paclitaxel combined with apoptin acted additively in p53-positive human osteosarcoma U2OS and nonsmall cell lung carcinoma A549 cells, p53-negative osteosarcoma Saos-2 cells, and p53-mutant prostate cancer Du145 cells. Finally, apoptin was proven to be coeffective when combined with the chemotherapeutic agent methotrexate (Zhang, et al., 2007).

Recently, apoptin has also been combined with other treatments *in vivo*. As discussed above, apoptin expression modulates the ceramide-sphingolipid pathways leading to enhanced ceramide levels (Liu, et al., 2006). The majority of prostate tumors have elevated acid ceramidase levels compared with neighboring normal prostate tissue (Liu, et al., 2006). *In vitro*, up-regulated acid-ceramidase protected cells from apoptin-induced apoptosis, whereas cotreatment with the acid-ceramidase inhibitor LCL204 sensitized cells for apoptosis. *In vivo*, combined treatment enhanced the antitumor activity of apoptin in xenografted prostate tumors in mice, resulting in significantly reduced tumor growth and increased animal survival.

Lian and colleagues combined apoptin treatment with interleukin-18 (IL-18) and reported that combined administration results in an even higher induction of an effective antitumor immune response and tumor regression (Lian, et al., 2007). IL-18 and apoptin appear to affect tumors via complementary pathways. Whereas apoptin directly targets the tumor cells, IL-18 treatment appears to act via enhancing the immune response toward tumor cells. Finally, Schoop et al. showed that treatment of a radioresistant head- and neck cancer cell line with apoptin concurrently with exposure to irradiation sensitized these cells to apoptosis (Schoop, et al., 2010).

Tumor-selective targeting using apoptin NLS

Rather than using apoptin itself, or one of its cellular targets, to design anti-cancer therapies, Wagstaff and Jans propose the use of apoptin's tumor-selective NLS (74-121) to target drugs specifically to the nucleus of tumor cells (Wagstaff and Jans, 2009). Delivery of cytotoxic agents directly to the heart of the tumor cell should result in efficient tumor cell killing without affecting healthy neighboring cells.

2.7 Synopsis

The new era of genomics and proteomics carries with it a great promise: that for every disease, the blueprint of the defective cell can be compared with that of a healthy one, thereby not only facilitating the identification of the root of the problem, but also, and more importantly, allow one to charter an efficient route to fix it. Drawing up the blueprint of a cancer cell has proven to be a difficult task. Even so, a complex map of intracellular pathways is starting to emerge, with a number of essential traits being seemingly shared by all transformed cells. The CAV-derived apoptin apparently senses these characteristics and effectively charters its own route to selectively kill malignant cells. The properties of apoptin and functionally related proteins, combined with newly developed therapeutics targeting the hallmark capabilities and enabling characteristics of cancer cells, should finally lead to the development of selective, efficient and robust therapies for cancer – instead of the long sought-after "magical bullet" cure for cancer, we will be able to build a magical cluster bomb.

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