CHAPTER ONE: INTRODUCTION

1.1 The DWNN gene

This study aimed to characterise the distribution patterns of the Domain With No Name (DWNN) gene and its products in colon cancer and undiseased colon tissue and ascertain the role played by the gene in apoptosis. The DWNN gene was first identified via RACE and inverse PCR at the University of the Western Cape in Chinese Hamster ovary cell lines where knockouts of the gene resulted in high resistance to cytotoxic T-lymphocyte killing and to staurosporine-induced apoptosis. This novel 36kb long gene has been mapped on chromosome 16p21 in humans and has been shown to express two transcripts of 1.1kb and 6.1kb. BLAST search analysis has shown that the DWNN protein shows no significant structural homology to any known protein. Pugh and Faro have, however, established the secondary structure of the DWNN domain and demonstrated that it is composed of α-helices and β-sheets, with a similar 3D structure as to that of ubiquitin (Pugh and Faro, unpublished). Sequence analysis has revealed that the DWNN domain is highly conserved throughout the eukaryote species, including humans, worms, flies, fungi, plants, protozoa and microsporidia (**figure 1.1**) (Rees and Pugh, unpublished).

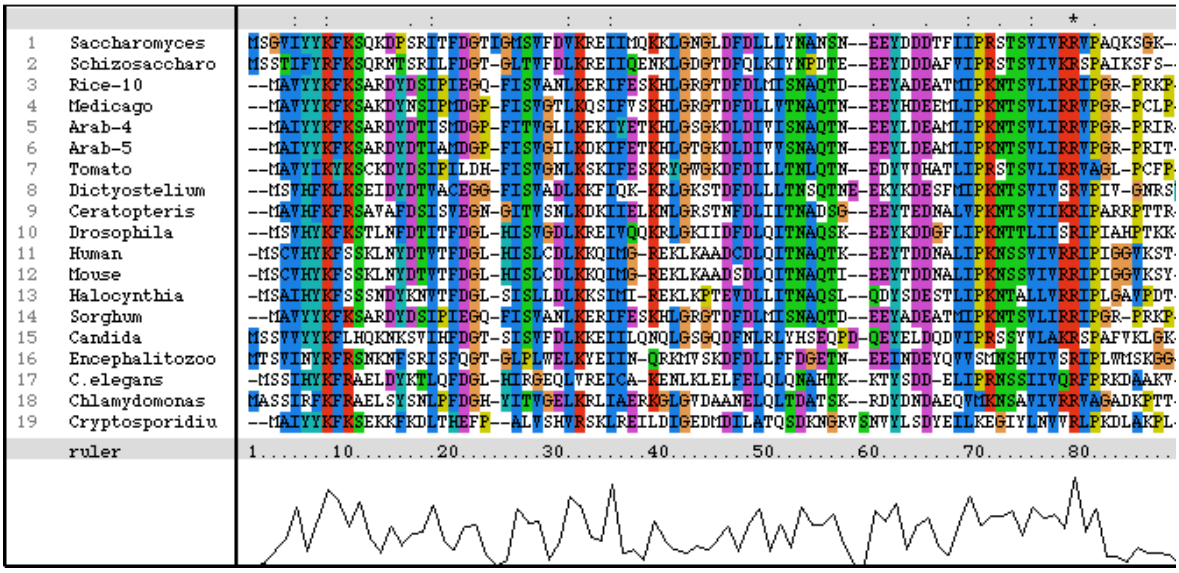

Figure 1.1: Conserved amino acids within the DWNN domain in multiple species (Rees and Pugh, unpublished).

The domain structure of the DWNN protein in humans, worms and flies are alike in that the DWNN domain is allied with other conserved domains, namely the zinc and RING finger domains and the PACT/RBBP6 domain (**figure 1.2**). The serine-rich, Rb-binding and p53-binding domains in worms and flies are still being confirmed. Plants, fungi and microsporidia all have the DWNN domain, but not the PACT/RBBP6 domain.

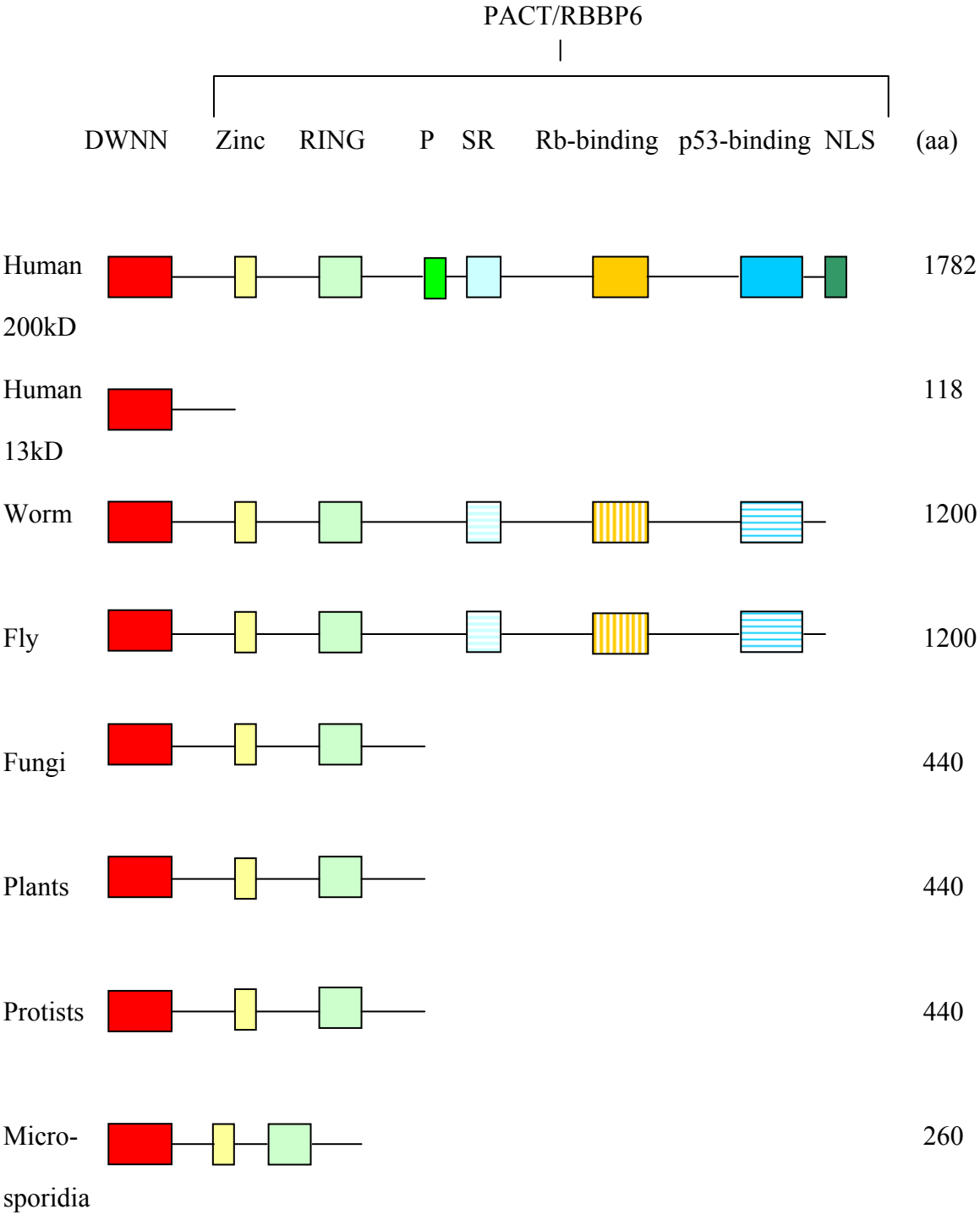

Figure 1.2: Domain structure of the DWNN protein in different species (Rees, unpublished).

P – proline-rich

SR – serine/arginine region

NLS – nuclear localisation sequence

1.1.1 The 1.1kb transcript

The 1.1kb transcript (DWNN-13) encodes for a 13kD protein, which consists of only the N-terminal DWNN domain, constituting three exons (**figure 1.3**). This transcript consists of a highly conserved region of eighty amino acids and a hydrophobic tail (**figure 1.4**) (Skepu, unpublished).

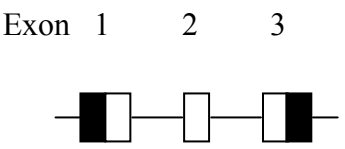

Figure 1.3: Structure of the DWNN domain making up DWNN-13

MSCVHYKFSSKLNYDTVTFDGLHISLCDLKKQIMGREKLKAAD
CDLQITNAQTKEEYTDDNALIPKNSSVIVRRIPIGGVKSTSKTYVI
SRTEPAMATTKAVCKNTISHFFYTLLLPL

Highly conserved amino acid region

Hydrophobic C-terminal tail

Figure 1.4: Protein translation of the DWNN-13 gene (Skepu, unpublished)

1.1.2 The 6.1kb transcript

The 6.1kb transcript (DWNN-200) encodes for the 200kD protein, consisting of the conserved DWNN domain and the PACT/RBBP6 region (**figure 1.5**). The PACT/RBBP6 region is composed of a CCHC zinc finger between exon 6; a C_3HC_4 RING finger domain between exons seven and ten; a proline-rich region between exons ten and fifteen; a serine/arginine domain and a Rb-binding domain in exon seventeen; a p53-binding domain in exon 18 and a nuclear localisation sequence. In total DWNN-200 is comprised of eighteen exons. The zinc finger domain associates with single stranded RNA and DNA. The RING finger domain exhibits ubiquitin-ligase activity, and this domain occurs often in E3 ubiquitin ligases (Dlamini *et al.*, 2001). DWNN-200 undergoes alternative splicing at exon 16 and has been shown via sequence data to have two promoters and the DWNN gene has therefore at least six mRNA transcripts (Dlamini *et al.*, 2001).

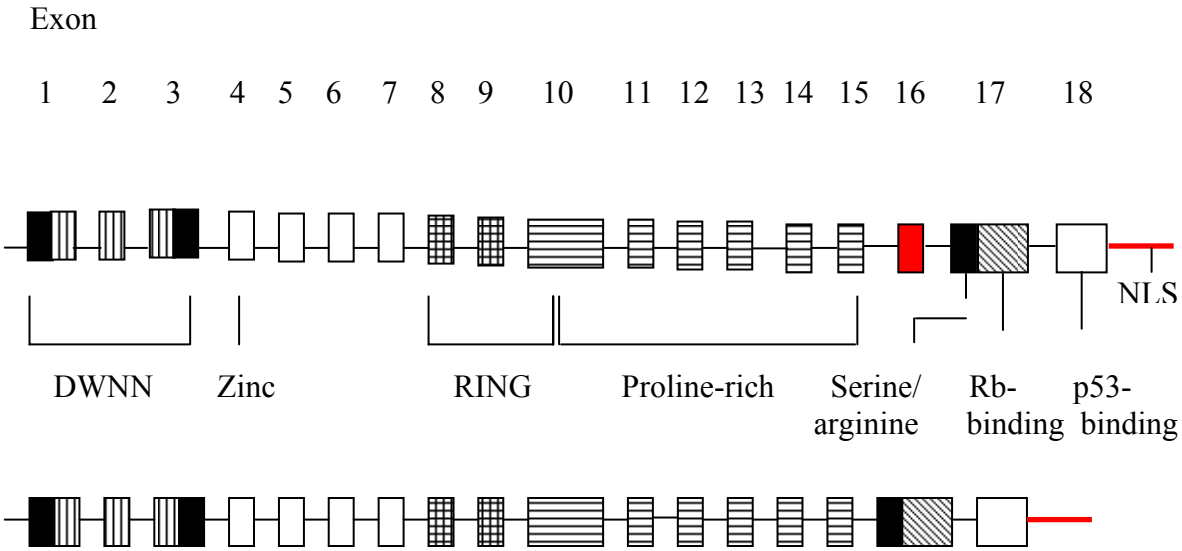

Figure 1.5: DWNN-200 domain structure showing the protein without and with alternative splicing (Dlamini *et al.*, 2001)

1.1.3 The ubiquitin-proteasome system

The highly conserved ubiquitin-proteasome system (UP-S), which was identified in the early 1980's, is critical in the homeostasis of proteins and is essential for the regulation of normal and cancerous cellular functioning. This system displays great potential for the development of new anticancer therapies because it presents an abundant array of molecular targets for precise interference (Burger and Seth, 2004). Degradation via this system constitutes specific steps involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Once a protein has been tagged with ubiquitin (Ub), the proteasome identifies and targets it for digestion and

fragmentation. This process of degradation provides a mode of eradicating damaged or mutant proteins, whose buildup poses a possible risk to the cell (Rock and Goldberg, 1999).

The two main pathways for cellular protein turnover are lysosomal and proteasomal degradation. The lysosome is responsible for eliminating cell surface proteins by endocytosis, accountable for 10-20% of normal protein turnover (Glickman and Ciechanover, 2002; Rock and Goldberg, 1999). 80% of cellular proteins are tagged with Ub and subsequently degraded in the cytoplasm by the proteasome. In rare circumstances, such as for membrane-anchored proteins, tagging with Ub can result in degradation in the lysosome (Glickman and Ciechanover, 2002; Rock and Goldberg, 1999). The Ub-activating enzyme, E1, is responsible for an ATP-dependent transfer of Ub to a Ub-conjugating enzyme, E2. E2 functions by either directly transferring an activated Ub molecule to the substrate or to an Ub-protein isopeptide ligase, E3, which also tags substrates with Ub (Hershko *et al.*, 1983). The mechanism of ubiquitination is as follows: using ATP, the E1 enzyme catalyses the development of a reactive thiolester bond with Ub, which is conveyed to the active cysteine site of E2. E2-ubiquitin thiolester specifically ligates to E3, resulting in the formation of an isopeptide bond between Ub and the targeted protein (Badciong and Haas, 2002). Poly-ubiquitinated proteins are usually degraded by the 26S proteasome (Adams, 2003). Proteasomes occur in the nucleus and cytosol and make up 1% of the eukaryotic cellular protein matter (Gerards *et al.*, 1998).

Proteins implicated in cancer and that are regulated by the UP-S include p53 and p27 tumour suppressors, epidermal growth factor receptor (EGFR), the transforming growth factor-β receptor (TGF-βR) complex, as well as cell cycle and oncogenic transcription regulators such as NF-κB and *c-myc* (Adams, 2003; Glickman and Ciechanover, 2002; Sakamoto, 2002; Seth *et al.*, 2003; Zhang *et al.*, 2004). Therefore because of this regulatory system, the UP-S is closely linked with the incidence and progression of cancers (Zhang *et al.*, 2004). Despite the abundance of cancer-related proteins which are degraded in this way, only a few of the ubiquitin-conjugating enzymes and ligases have been studied in terms of expression patterns in human tumours (Nalepa and Harper, 2003; Okomoto *et al.*, 2003; Sakamoto, 2002). Loss of the cell-cycle inhibitory protein p27 is correlated with aggressive tumour growth and an unfavourable prognosis in colon cancer. Decreased levels are a direct result of increased proteasome-dependent degradation, and this rate of degradation is controlled by the specific Ub ligase subunits, Skp2 and Cks1, with overexpression of these subunits being associated with low levels of p27 and poor tumour differentiation. Expression levels of these subunits are now used as novel prognostic markers in colon cancer as they directly correlate with survival (Hershko *et al.*, 2004).

The deletion of the E1 enzyme responsible for the primary activation of Ub in mammalian cells is lethal, yet no association with cancer has been identified (Ciechanover *et al.*, 2000). The E2 enzyme, UbcH10, and certain E3 ligases have been identified as being expressed at high levels in cancerous cells (Nalepa and Harper, 2003; Okomoto *et al.*, 2003). The UP-S has been shown to be inhibited by peptide aldehyde,

borofax peptide, 3,4-dichloro isocoumarin and MG-132 (Zhang *et al.*, 2004). Inhibition of proteasome activity results in the death of proliferating cells, therefore presenting the therapeutic potential of this in certain diseases, such as cancer. With the general inhibition of the UP-S, key proteins are regulated non-specifically. However, with the targeting of specific E3s, these proteins can be specifically stabilized, thereby reducing unwanted side effects, such as stabilising undesirable proteins (Fang *et al.*, 2003). The activation of NF-κB promotes diseased cell proliferation and minimizes the efficiency of chemotherapy and radiation. With proteasome inhibition using PS-341, the inhibitor of NF-κB activation, IκB, is stabilized, and cell proliferation therefore decreases (Adams, 2001).

The tumour suppressor p53, which has a highly significant influence on cell cycle control, DNA repair and apoptosis, plays a critical role in a number of cancers and is regulated by substrate-specific E3 ubiquitin ligases, namely the RING-type Mdm2 and the HECT-type E6-AP (Burger and Seth, 2004; Glickman and Ciechanover, 2002; Nalepa and Harper, 2003; Sakamoto, 2002). The human papilloma virus E6 protein takes control over the HECT domain E3 E6-AP, resulting in proteasomal degradation of p53 and the subsequent promotion of cervical uterine carcinomas (Scheffner *et al.*, 1994).

The oncoprotein Mdm2 is the main Ub ligase for p53 and is overexpressed in 5-10% of human tumours (Fang *et al.*, 2000). It inhibits p53 transcriptional functioning or targets it for ubiquitination through an atypical C-terminal RING finger, with consequent degradation by proteasomes. Mdm2 therefore has the same effect as p53 mutation or

deletion, which results in cancer progression (Yang *et al.*, 2004). Like a number of other RING finger ligases, Mdm2 is capable of catalysing its own ubiquitination, thereby is able to autoregulate itself (Fang *et al.*, 2000). Mdm2 and its homologue MdmX both possess a p53 binding domain, a zinc finger motif and a C-terminal RING finger domain (Lorick *et al.*, 1999). The observed interaction between Mdm2 and MdmX has been shown to increase Mdm2 ubiquitination. It has been demonstrated that both proteins are necessary for normal development and that they have non-overlapping functions (Badciong and Haas, 2002). MdmX expression is also increased in a number of tumour cell lines (Ramos *et al.*, 2001). The potential to specifically inhibiting Mdm2 E3 activity is very appealing with regards to promoting apoptosis mediated by p53 in cancer (Fang *et al.*, 2000).

A number of the E3 HECT and RING proteins have interestingly showed an association with the induction and regulation of immune self-tolerance. These proteins, which include c-Cbl, Cbl-b, GRAIL, Itch and Nedd4, inhibit T cell growth factor production and proliferation (Mueller, 2004).

1.1.4 DWNN homologues

Proteins similar to the 3' end of the DWNN-200 protein have been identified and include PACT, P2P-R, RBBP6 and Mpe1 proteins (**figure 1.6**). These are partial cDNAs in which the N-terminal conserved DWNN domain is absent, however they have been shown to bind the tumour suppressors p53 and Rb.

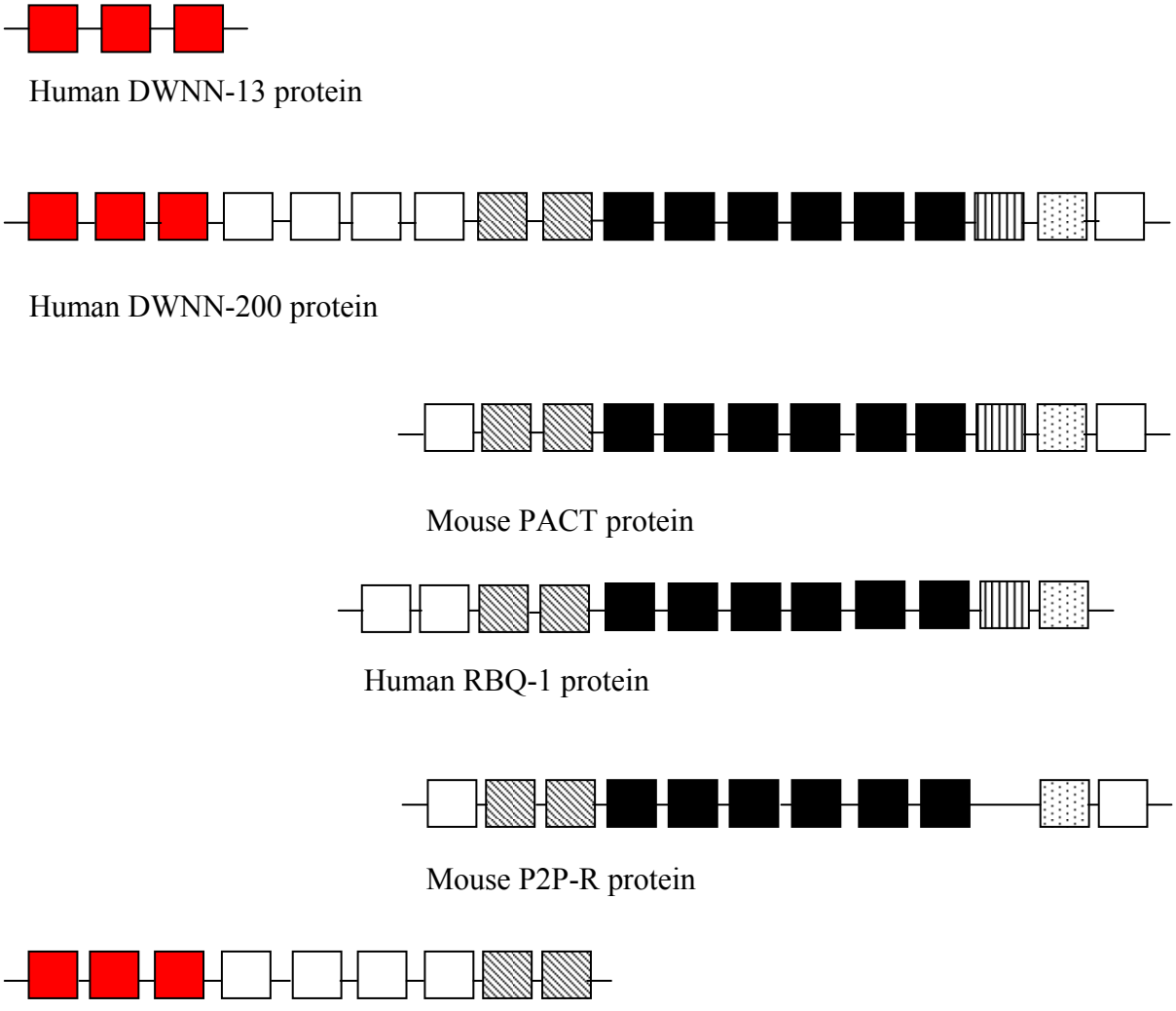

Figure 1.6: The DWNN proteins and the DWNN partial cDNAs (Skepu, unpublished)

1.1.4.1 PACT

p53 associated cellular protein-testes derived (PACT) is 5177bp long and codes for 1583 amino acids. First isolated from the mouse testes expression library by employing p53 as a probe (Simons *et al.*, 1997), it translates into a 250kD protein. The 1583 amino acid sequence of this highly charged protein has a 34 amino acid alternatively spliced region, a serine/arginine region and a 54 amino acid lysine-rich region. Studies have shown that PACT localises at the same areas where a number of pre-mRNA splicing constituents are situated (Simons *et al.*, 1997; Spector, 1993), suggesting that PACT plays a role in cellular pre-mRNA splicing.

1.1.4.2 RBQ-1 (RBBP6)

Also known as RBBP6, the RBQ-1 protein was isolated from a human small cell lung carcinoma library. This novel 140kD protein binds the Rb gene and the underphosphorylated pRb (Sakai *et al.*, 1995). Sequence analysis demonstrates a 94% amino acid homology between mouse PACT and human RBQ-1 (Simons *et al.*, 1997). The 3011bp cDNA has an open reading frame encoding for 948 amino acids, with an alternatively spliced 34 amino acid region. The 140kD protein is composed of 12 exons.

1.1.4.3 P2P-R

Proliferation potential protein related (P2P-R) is a nuclear protein, which was identified by cloning from a mouse cDNA library, and is expressed in a number of murine tissues and cells (Witte and Scott, 1997). The 5173bp cDNA has a 4214bp open reading frame, which encodes a 156.9kD protein. This protein, which is a result of alternative splicing omitting an exon of 34 amino acids, binds the tumour suppressor proteins, p53 and Rb (Scott *et al.* 2003). The cDNA contains an N-terminal RING type zinc finger, a heterogenous nuclear ribonucleoprotein (hnRNP)- associated domain, an Rb-1 and p53 binding domain, a proline rich domain, a serine/arginine region, a single stranded nucleotide binding domain and a C-terminal lysine-rich domain. The cDNA of RBQ-1 shows significant homology to the 5' region of the cDNA of P2P-R (Sakai *et al.*, 1995). P2P-R is involved in the regulation of RNA metabolism, apoptosis and p53-dependent transcription (Simons *et al.*, 1997). It has been shown to bind to the proline-rich and C-terminal regulatory domains of p53, and these domains regulate the expression of pro- and anti-apoptotic genes (Gao *et al.*, 2002).

1.1.4.4 Mpe-1 protein

This novel gene was isolated from *Saccharomyces cerevisiae* and encodes a 49.5kD protein of 441 amino acids, which contains the conserved DWNN domain, zinc finger and RING finger. Required for the specific cleavage and polyadenylation of pre-mRNA, it is a constituent of the cleavage and polyadenylation factor complex (CPF). This conserved protein has homologies in *S. pombe*, *A. thaliana*, *D. melanogaster* and humans (Vo *et al.*, 1995).

1.2 Cancer

Cancer has affected mankind for thousands of years and has been found in the bones of Egyptian and Peruvian mummies dating back to 3000 BC. The term carcinoma, from the Greek karkinoma, meaning crab, was first used around 400 BC by Hippocrates, who compared the spreading effect of the disease to the claw-like extensions of a crab. The German pathologist, Johannes Müller, first identified malignant tissue by microscopy in 1836 and noted the disorganised, abnormal inconsistency of these cells compared to normal cells. Today many forms of cancer can be effectively treated by surgery, radiation therapy, chemotherapy, phototherapy, immunotherapy and biological modifiers (McAllister *et al.*, 1993).

1.2.1 Effects of cancer cells

All forms of cancer are typified by uncontrolled cell proliferation and metastasis, the movement of tumour cells from their site of origin to new locations where they attach to proliferate further. These tumour cells begin to interfere with the activities of normal cells, and result in the loss of important functions as a consequence of vital cells being rendered inoperative. Solid tumours compress the adjacent normal tissues and interfere with nerve function and blood supply. Tumours are also able to cause damage by rupturing barriers such as internal membranes, the outer skin and gut wall, resulting in bleeding, infection, and loss of compartmentalisation. As the malignant tumour mass increases, the nutritional demand from the cancerous cells may cause normal cells to be deprived, resulting in weakened body functions, tiredness, muscular weakness and weight loss (McAllister *et al.*, 1993).

1.2.2 Carcinogenesis

Carcinogenesis is typically regarded as the sequence of initiation (mutation), followed by promotion (clone expansion). This can be compared to the neo-Darwinian theory of evolution, with the first stage of genetic change (including recombination) and the second stage of selection. Selection is based mainly on the elimination of the less fit. The mutated cells would be selected as a result of their resistance to apoptosis. There is a wide variation in cancer rates across different countries, a difference that can only adequately be explained by genetic differences. The risk of cancer in migrants gradually changes to that of the population into which they immigrate, an adaptation that cannot entirely be due to environmental mutagens. There is a definite gene-environment interaction, which encompasses the history of the cell's genetic changes and the type of environment it lives in. It may be that mutated cells are more efficient at adapting to environmental niches than are normal cells (Vineis, 2003).

The transformation of normal cells to malignant cells is estimated to be reliant on between four to seven rate-limiting genetic alterations. Critical events in this progression include the loss of proliferative control, failure to undergo apoptosis, the onset of neoangiogenesis, remodelling of tissues, the invasion of tumour cells into adjacent tissue, and ultimately, the metastatic dissemination of tumour cells to remote organs (Herzig and Christofori, 2002).

1.2.3 Colon cancer

1.2.3.1 Prevalence

In South Africa, Asian men have the highest incidence of colorectal cancer compared to all other cancers (1 in 43); in Caucasian women, it is the second most prevalent (1 in 44) and the third most prevalent cancer in Caucasian men (1 in 34) (based on statistics from 1993-1995; Sitas *et al.*, 1998). Between 1993 and 1995, an average of 939 men and 890 women were reported to have colorectal cancer. In 1994, the Central Statistics Service (CSS) reported 614 male and 660 female deaths as a consequence of colorectal cancer. The rates for South African Blacks are two in 100 000, in both men and women, similar to other African countries.

Table 1.1: Lifetime risks of colorectal cancers per population group in South Africa (1993-1995) (Sitas *et al.*, 1998)

	Male	**Female**
Asian	1 in 43	1 in 79
White	1 in 34	1 in 44
Black	1 in 400	1 in 500
Coloured	1 in 200	1 in 294
All	1 in 94	1 in 130

Typically the rates of colon cancer incidence tend to be higher in westernised populations that are economically privileged. Studies have shown that the risk of colorectal cancer changes in migrants after a period of 20-30 years in a high-risk country, and that the risk of developing colorectal cancer may decrease after migrating from a high to a low risk country (Schottenfeld, 1995). Studies show that 35% of all colonic cancers occur in the sigmoid colon; 22% in the caecum; 12% in the ascending colon; 10% in the transverse colon and 7% in the descending colon. Interestingly, in countries of high rates with colon cancer incidence, there is a larger number of sigmoid cancers, and in countries with low rates, a larger number of cancers of the cecum and ascending colon (Schottenfeld, 1995).

1.2.3.2 Anatomy of the colon

The colon is a section of the large intestine, which is also comprised of the appendix, cecum, rectum and anal canal. The main functions of the colon are the collection, dehydration, transit and temporary storage of stool. The colon is differentiated into the ascending, transverse, descending and sigmoid sections. It is composed of four layers of tissue: the serosa, muscularis propria, submucosal layer and the innermost mucosa. The outermost layer, the serosa, is comparable to a membrane, while the deeper layer of muscularis propria is a muscular layer. The submucosal layer contains the nervous tissue, connective tissue and blood and lymph vessels. The innermost mucosa has three constituents: the epithelium, lamina propria and muscularis mucosae. Most cancers of the colon are initiated in the epithelial layer, from where it infiltrates the outer tissue layers, eventually spreading to other organs through the blood and lymph system (Owen and Kelly, 1994).

1.2.3.3 Pathology of Colon Cancer

In normal colon tissue, stem cells at the bottom of crypts divide continuously in an asymmetric fashion, adding cells to the crypt's proliferative area (Bykorez and Ivashchenko, 1984; Bach *et al.* 2000). These intermediary cells will either differentiate further into Goblet cells or migrate towards the lumen (Luebeck and Moolgavkar, 2002). In malignant tissue, however, genomic alterations disrupt normal division, resulting in symmetric division, with the possible result that both daughter cells may preserve the clonogenic traits of the stem cell. If apoptotic activity is not adequate, this leads to the build up of altered stem cells and the risk of clonal expansion (Luebeck and Moolgavkar, 2002).

One of the foremost premalignant lesions detected in colorectal cancer are aberrant crypt foci (ACF) (Luebeck and Moolgavkar, 2002). Dysplastic ACF, also termed adenomatous crypts or microadenoma, are precursors of adenomatous polyps, and show loss of heterozygosity (LOH) on 5q, the locus of adenomatous polyposis coli (APC) (Roncucci *et al.,* 2000). Adenomatous polyps are in turn the precursors of colon carcinoma (Luebeck and Moolgavkar, 2002). The progression from adenoma to high-grade dysplasia (HGD) implicates the p53 gene (Hanahan and Weinberg, 2000). HGD is the catalyst for malignant transformation (Boland *et al.,* 1995).

A hypothesis by Luebeck and Moolgavkar suggests that after mutations at the two APC loci occur in a stem cell, normal asymmetrical division continues to produce daughter cells that move to the proliferative zone of the crypt. Here they escape the constraints of

the microenvironment at the stem cell zone at the bottom of the crypt and after symmetric cell division, are able to expand clonally. This theory proposes that the nature of the microenvironment is an important factor in sustaining normal cell division (Luebeck and Moolgavkar, 2002). Evidence supporting this hypothesis is provided by genetic and immunohistological studies of dysplastic crypts in small adenomas. Two distinct areas could be deciphered: near the bottom of the crypts, cells appeared morphologically normal and near the colonic lumen, cells were dysplastic and had APC inactivating mutations (Shih *et al.* 2001).

1.2.3.4 Bacterial Carcinogenesis

1.2.3.4.1 *Escherichia coli* infection

The geographical distribution of colon cancer implies that environmental factors contribute to resistance of endemic populations to colon cancer. There is an inverse relationship between the occurrence of colon cancer and enterotoxic *Escherichia coli* (ETEC) infections. Colon cancer cell proliferation is restricted by a novel intracellular signalling pathway initiated by specific peptides that are elaborated from ETEC, and diarrhoea mediated by these peptides occurs repeatedly throughout a person's life-span in developing countries and contributes to the low prevalence of colon cancer (Pitari *et al.*, 2003).

1.2.3.4.2 *Helicobacter pylori* infection

Helicobacter pylori is a microaerophillic Gram-negative bacterium that has been classified as a Group 1 carcinogen by the International Agency for Research on Cancer (Han *et al.*, 2003; IARC, 1994; Mahady *et al.*, 2002), and in particular those strains that are positive for CagA protein are thought to cause intestinal adenomas and hyperplastic polyps (Nakano *et al.*, 1990; Shmuely *et al.*, 2001; Tabata *et al.*, 1999). Bacterial infections contribute to the induction of cancer via two modes: the initiation of chronic inflammation, and the assembly of carcinogenic bacterial metabolites (Parsonnet, 1995). *H. pylori* infection causes cancer via an inflammatory mechanism. Inflammation induces cancer by initiating cell proliferation and the generation of mutagenic free radicals and N-nitroso compounds. Colonic cell proliferation is promoted by resident colonic flora exogenous compounds, such as rutin metabolised into mutagens, which contributes to colon carcinogenesis. Bacteria are able to make high concentrations of fecapentaenes, which are potent *in vitro* mutagens (Parsonnet, 1995).

A correlation between *H. pylori* infection and the high incidence of gastric cancer has been found in Japanese patients, compared to the low occurrence of the disease in Western countries (Huang *et al.*, 1998; Tabata *et al.*, 1999; Yamagata *et al.*, 2000). A study showed the presence of *H. pylori* antibodies in 49% of control subjects, 71.4% of patients with colonic polyps, 69.2% of colon cancer patients evaluated at time of diagnosis and 48% of colon cancer patients evaluated 1-9 years after surgery (Meucci *et al.*, 1997). Another study showed the presence of *H. pylori* IgG antibodies in 79% of patients with colonic polyps compared to 62% of control patients (Breuer-Katschinski *et*

al., 1999). Curcumin, a polyphenol chemical component drawn from turmeric (*Curcuma longa*), has been shown to have chemopreventative properties, with the ability to impede colon cancer in rats (Mahady *et al.*, 2002). It has also been shown to restrict the growth of CagA+ strains of *H. pylori in vitro*, which may explain the mechanism of its chemopreventative action (Mahady *et al.*, 2002). Alterations in expression levels of p53, c-erbB-2, c-met, APC and DCC were observed in gastric cancer studies, however this could not be correlated with infections of *H. pylori* (Wu *et al.*, 1997).

1.2.3.5 Aetiology

1.2.3.5.1 Age

A number of risk factors increase the chances of colon cancer. Age is one of them, with 90% of colon cancer patients being over the age of fifty. However, it can occur in younger individuals. In an individual who has had a previous adenomatous polyp, or a history of chronic bowel inflammation, the risk is also increased. Reproductive factors associated with increased risk of colorectal cancer include: family history of colorectal cancer among first-degree relatives; late age at first pregnancy; regular menstrual cycles and late age at menopause (Yoo *et al.*, 1999).

1.2.3.5.2 Body weight

Some studies show that there is an increasing amount of evidence that excess body weight may also be associated with a greater risk of colon cancer (Caan *et al.*, 1998). Other studies look closer and have found that obesity is associated with an increased risk of colon cancer in premenopausal women, but this correlation does not extend to postmenopausal women (Terry *et al.*, 2002).

1.2.3.5.3 Diet

The extent of colonic cell differentiation is regulated by the colonic microfloral environment and diet (Bry *et al.*, 1996; Yang *et al.*, 1996). Studies have shown that a substantial consumption of alcohol, in conjunction with an inadequate intake of folate and methionine, may increase the chances of developing colon cancer (Giovannucci *et al.*, 1995). Further studies have shown that the risk of colorectal cancer is increased by a diet that is high in refined grain and red meat (Levi *et al.*, 1999). On the other hand, dietary fat (e.g. fish oil) and dietary fibre (e.g. cellulose) have been shown to have a protective role against the development of colon tumours. They are believed to act on the balance between cell division, maturation and death (Heitman and Cameron, 1990; Lindner, 1991); for example, wheat bran protects against colon cancer at early and late stages of tumour development (Alabaster *et al.*, 1995). This protective effect is a consequence of wheat bran's high dietary fibre and phytic acid content (Jenab and Thompson, 1998). Studies have also shown that a diet rich in fish, starch and β-carotene results in lower colorectal mucosa proliferation and a normal cell proliferation pattern along the crypt (Caderni *et al.*, 1999).

Yoghurt has been shown to influence the balance between mitosis and apoptosis by increasing cellular apoptosis in colon cancer cells, and is associated with a decreased risk of colon cancer (Rachid *et al.*, 2002). Epidemiological studies, dietary history, *in vitro* studies and human trials have shown that an increase in dietary calcium intake reduces colonic carcinogenesis (Buset *et al.*, 1986; Slattery *et al.*, 1988; Thomas *et al.*, 1993). Studies suggest that the consumption of excess dietary energy leads to the development of insulin resistance and therefore an increase in the circulating levels of insulin, triglycerides and non-esterified fatty acids. These serve as a proliferative stimulus to the colonic epithelial cells and the accumulation of oxygen intermediates. Such long-term exposure promotes colon cancer (Bruce *et al.*, 2000).

1.2.3.6 Induction of colon cancer

In a normal cell cycle, the action of growth-promoting oncogenes is balanced by the action of growth-restraining tumour suppressor genes. Genetic aberrations of proto-oncogenes result in oncogenes, which produce tumour cells. Mutations also render suppressor genes dysfunctional, thereby giving tumour growth free reign (Weinberg, 1991). For a tumour to reach full malignancy, both types of mutations are usually necessary.

Malignant cells form as a result of a series of events in which genetically altered growth factors and their receptors are involved (Aaronson, 1991). Two genes affect the process of colonic neoplasia: gatekeeper genes, which regulate cell birth and cell death, and caretaker genes, which direct the rate of gene mutations. A malignant tumour is formed

when gatekeeper genes, such as adenomatous polyposis coli (APC) mutate, resulting in cell birth exceeding cell death. Tumorigenesis is accelerated when caretaker genes mutate as well. Mutations in the APC or β-catenin gene result in the production of a small, benign tumour. This tumour progresses in growth through other pathways, such as ones involving transforming growth factor B or the p53 gene.

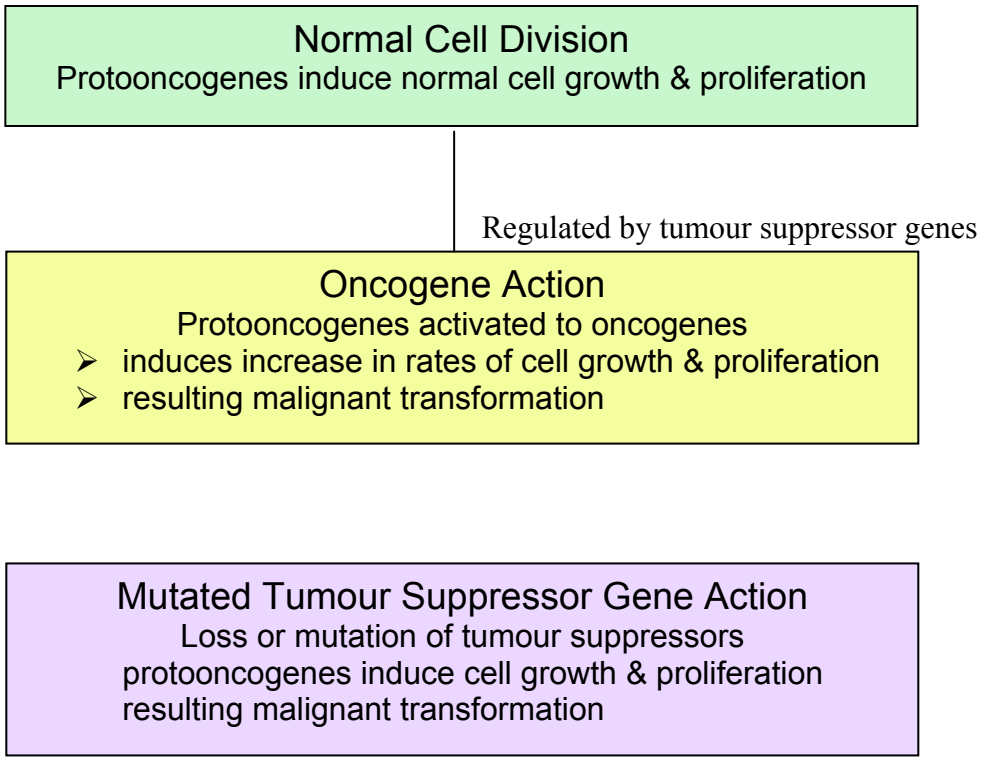

Figure 1.7: The initiation of cancer (adapted from www.intouchlive.com)

1.2.3.7. Genetic basis of colon cancer

Complex genetic and epigenetic alterations of the intestinal mucosa are involved in the development of colon cancer. Genetic alterations involved in colorectal cancers include: allelic losses on chromosome arms; mutations of oncogenes, mismatch repair genes and tumour suppressor genes; methylation defects in gene promoters and instability in the coding of repeat sequences of target genes (Gayet *et al.*, 2001). Chromosome instability (CIN) causes cancer cells to lose or gain large sections of chromosomes at a much higher rate than normal cells. CIN develops in the very early stages of colon tumorigenesis. Tumours are formed as a result of a number of genetic alterations and attain a range of new phenotypic characteristics, one of which is the loss of normal cell cycle control (Asker *et al.*, 1999).

Specific chromosomal regions that have been reported as being deleted in colorectal tumours include 5q, 17q, 18q and 22q (Vogelstein *et al.*, 1989). Deletions of alleles in the short arm of chromosome 17 are found in over 75% of colorectal tumours. These 17p deletions usually occur late in colorectal tumour development, and are associated with the progression from the benign (adenomatous) to the malignant (carcinomatous) state. Genetic alterations that occur relatively early in colorectal tumour development include *ras* mutations (Baker *et al.*, 1989; Fearon and Vogelstein, 1990). The molecular events arbitrating the transition from uniformly curable adenomas to often lethal carcinomas are very important. Understanding these events allows for more effective therapies to be developed. The allelic deletion of chromosome 17p occurs in many cancers besides the colon, such as the breast and lungs.

Germ-line mutations are present in all cells. However, those that occur in genes that are fundamental to the cell cycle cause cancer in specific cell lines only, such as mismatch repair genes in the colon (Mitchell *et al.*, 2002). The rate of gene mutation, besides that of mismatch repair genes or the p53 gene, is the same in colon cancer cells as in normal cells. This suggests that a mutator phenotype, which increases the mutation rate in many genes, is not a vital element of sporadic cancers (Vineis, 2003). The mismatch repair system functions to limit colorectal neoplasia. The system operates by repairing subtle errors made during copying of the genome by DNA polymerases. Inherited mutations of the mismatch repair genes can result in hereditary nonpolyposis colorectal cancer (HNPCC), which is the most common hereditary cancer.

The model of colon carcinogenesis set up by Fearon and Vogelstein explains the genetic changes occurring in the adenoma-carcinoma sequence (Fearon and Vogelstein, 1990). Carcinogenic factors result in the hypomethylation of DNA allowing for chromosomal irregularities (Counts and Goodman, 1995). With the mutation of APC and K-*ras* genes, proliferation is initiated in normal epithelial cells, resulting in the formation of an adenoma. Mutation of the DCC gene allows for an early adenoma to progress to an intermediate and late adenoma. For the malignancy to reach its full potential, in terms of invasive and metastatic capabilities, mutation of the p53 tumour suppressor gene is necessary. During this process, discrepancies occur in at least four DNA mismatch repair genes including DNA replication errors at microsatellite areas, resulting in the initiation of chromosomal irregularities (Fearon and Jones, 1992).

1.2.3.8 Tumour progression

Colon tumours are very good research materials for studying the genetic changes involved in the development of a common neoplasm, as tumours of differing stages of development can be accessed (Fearon and Vogelstein, 1990). Initially, the hyperplastic epithelium appears normal, advancing to adenomatous polyps at different stages of dysplasia, to a non-invasive and finally, invasive carcinomatous stage (Weinberg, 1991). Colorectal adenomas are a group of mucosal neoplasms, sharing vital acquired genetic and phenotypic traits. Histologically, adenomas are classified as tubular, villous and tubulovillous, on the basis of the percentage of villous content. Adenomas have dysplastic cells, namely cells that exhibit low or high-grade atypia. Low-grade dysplastic adenomas exhibit mild to moderate architectural irregularities. High-grade dysplastic adenomas, on the other hand, show heightened changes in glandular patterns and/or extreme cytologic irregularities. These include high nuclear:cytoplasm ratios, loss of nuclear basal polarity, irregular nuclear membranes, exaggerated nucleoli and aggregated chromatin (O'Brien, 1995).

Precursor adenomas are uniformly curable, while the more advanced carcinomas are usually lethal. The pathway of molecular events negotiating this transition is therefore crucial (Baker *et al.*, 1989). It is understood that there are a number of factors leading to the development of cancer; however, cancer is primarily a genetic disease resulting from genetic alterations. It is caused by the overexpression of oncogenes and the inactivation of tumour suppressor genes. Oncogene overexpression is a consequence of mutation in regulatory genes and/or gene amplification (Kovvali *et al.*, 2003). Both environmental

and hereditary factors contribute to the development of colorectal tumours, enabling one to examine both inherited and somatic genetic alterations (Fearon and Vogelstein, 1990).

It has been established that 60% of the large adenomas in the colon show the presence of a mutated, activated K-*ras* oncogene. The development of polyps into carcinomas is generally correlated with the inactivation-mutation of the chromosome 18-associated deleted-in-colorectal-cancer (DCC) tumour suppressor gene, followed by the inactivation-mutation of the chromosome 17 p53 suppressor gene. The order is not always followed precisely, but is the favoured sequence of events. It is estimated that approximately seven distinct genetic changes occur in a cell's progression from adenoma to carcinoma (**Figure 1.8**). Therefore, carcinogenesis can be regarded as a sequence of definable genetic alterations, which successively accumulate in the genome of an evolving malignant cell. In most colon cancers these alterations are effected through somatic mutations (Vogelstein *et al.*, 1989). It is evident that there is a clear relationship between LOH of the DCC gene (i.e. impaired function) and the development of colon cancer, yet there is inconsistency concerning the chronology of these alterations in carcinogenesis (Kataoka *et al.*, 2000). Gastric and colon cancer probably share a genetic basis for carcinogenesis (Uchino *et al.*, 1993). Uchino *et al.* observed that LOH occurred at all stages of gastric cancer; Fang *et al.* observed it late in cancer progression; Wu *et al.* identified LOH in advanced gastric cancer, and as infrequently in the early stages (Fang *et al.*, 1998; Uchino *et al.*, 1992; Wu *et al.*, 1997).

Colon carcinomas develop from adenomas that may be identified as polypoidal, flat or depressed (Kudo, 1993; Morson, 1984; Muto *et al.*, 1985). Flat or depressed tumours have been identified as having a greater malignant potential than polypoidal tumours (Wolber and Owen, 1991). The APC-Ras-p53 pathway is the induction pathway resulting in colon cancer (**Figure 1.8**). Two components of this pathway have been proposed: the chromosomal instability pathway (CIN) and the microsatellite instability pathway (MIN). The CIN pathway is characterized by loss of heterozygosity (LOH) and the MIN pathway by microsatellite instability (MSI) (Lengauer *et al.*, 1998). The MIN pathway involves the extensive accumulation of mutations of DNA mismatch repair genes, resulting in clonal selection. The MSI+ phenotype is characterized by allelic alterations of repetitive regions in the genome (Aaltonen *et al.*, 1993). Genes containing repeated domains in their coding sequences are commonly targeted by MSI, such as the bax gene, whose loss of function aids in colon tumorigenesis (Rampino *et al.*, 1997).

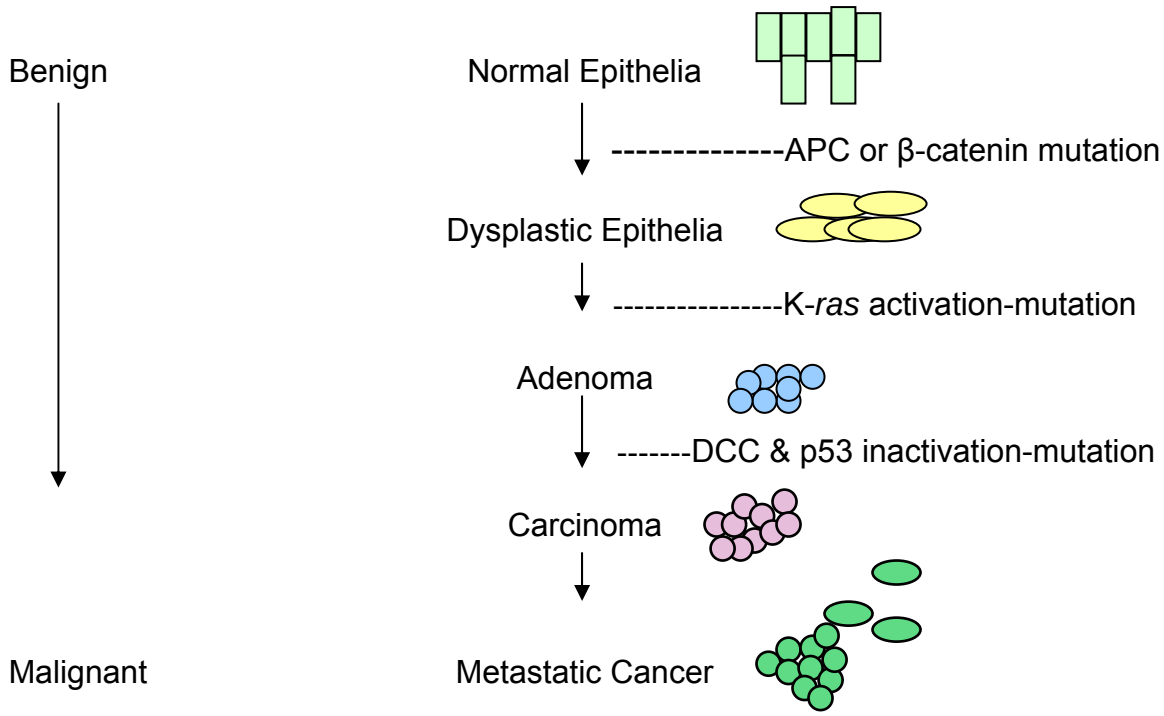

Figure 1.8: The genetic changes involved in colon cancer development

(adapted from Smith *et al.*, 2002)

1.2.3.9 Key genes

1.2.3.9.1 APC gene

An inherited mutation in the pro-apoptotic adenomatous polyposis coli (APC) gene is the initiating stage for colorectal polyp induction (Ichii *et al.,* 1992). 90% of sporadic colorectal adenomas, resulting in familial adenomatous polyposis (FAP), have stop codon mutations in, or deletions of, APC and show irregularities in APC-dependent signalling (Groden *et al.,* 1991; Iwamoto *et al.,* 2000; Nishisho *et al.,* 1991). A comparable APC mutation occurs in 60% of spontaneous colorectal cancers, 63% of adenomas and in 4.6% of aberrant crypt foci, implying that defects in the APC gene are an early factor in sporadic colon cancer (Powell *et al.,* 1992; Smith *et al.,* 1994). It was found that cases of FAP originating from an inherited APC mutation, showed morphologically normal flat colonic mucosa with cells displaying increased resistance to stress-induced apoptosis (Bedi *et al.,* 1995).

APC affects the expression of many genes, including the c-*myc* oncogene and its antagonist *Mad-1* (He *et al.,* 1998). The family of proteins that includes myc regulates transcription by binding to E-boxes (i.e. specific myc-binding elements: CACGTG) of genes influencing both proliferation and apoptosis (Jamerson *et al.,* 2000). C-*myc* regulates the transcription of ornithine decarboxylase (ODC), which is the first enzyme in polyamine synthesis (Meyskens and Gerner, 1999). Wild-type APC has been shown to suppress c-*myc* and inactivate *Mad1*. The expression of ODC is then reduced, correlating with reduced colon tumorigenesis. On the other hand, mutant APC was shown to up-

regulate ODC RNA and polyamine contents, and this correlated with heightened colon tumorigenesis (Martinez *et al.,* 2003).

1.2.3.9.2 p53 Tumour suppressor gene

Mutations within the p53 tumour suppressor gene, resulting in loss of p53 activity, occur in approximately half of all human cancers. Many of the tumours with wild type p53 bear mutations in the pathway, which still allow for full p53 activation (Vousden, 2002). It has been observed that many p53 mutations in colorectal cancer do not promote carcinoma progression and that some mutants, especially those within the conserved regions, can act against the negative functions of other p53 mutations (Forslund *et al.*, 2001). Studies show that the wild-type p53 gene in human cancers correlates well with successful anti-cancer treatments and that restoring wild-type p53 function to cancer cells devoid of the wild-type gene may improve the outcome of cancer therapies (Brachmann *et al.*, 1998).

Mutations of the p53 tumour suppressor gene are common in colon cancer, occurring late in colon tumorigenesis (Fazeli *et al.*, 1997). Why does the loss of p53 function occur so late in colon tumorigenesis? If p53 functioned in colon cancer cells by restricting the proliferation of DNA damaged cells, this activity would be cancelled in tumour cells early on in their progression. Therefore, the role of p53 in colon carcinogenesis may be the induction of apoptosis in response to mutations of APC, *ras* and other genes, and hence p53 is inactivated at a late stage of tumour development (Fazeli *et al.*, 1997).

The initial stages of p53-induced apoptosis may be reversible, following the removal of the apoptotic stimulus (Geske *et al.*, 2000). In the early stages of p53-induced apoptosis, it has been shown that general DNA repair activity begins to increase. The p53 gene assists in affirming that mitosis does not progress until the completion of DNA synthesis and oversees the quality of newly replicated DNA (Taylor *et al.*, 1999). The p53 gene is located on region D1 of chromosome 17p, which is within the region often deleted in colorectal tumours (Baker *et al.*, 1989). p53-mediated apoptosis may be enhanced by several chemotherapeutic agents. The status of the endogenous p53 gene in cancerous cells may therefore be a vital component in the end result of cancer therapy (Zhu *et al.*, 2001).

1.2.3.9.3 Mdm2

The murine double minute 2 (Mdm2) protein is the main cellular ubiquitin ligase accountable for p53 downregulation (Pray *et al.,* 2002). By forming a compact complex with p53 it inhibits transactivation, cell cycle arrest and apoptosis mediated by p53 (Momand *et al.*, 1992; Oliner *et al.*, 1993). This is accomplished by inhibition of the transcriptional activity of p53, or the targeting of p53 for ubiquitination and degradation by proteasomes (Haupt *et al.*, 1997; Momand *et al.*, 1992). Transcription is prevented by the binding of the Mdm2 N-terminal domain to the activation domain of p53 (Thut *et al.*, 1995). The C-terminal domain of Mdm2 degrades itself and the p53 protein as it contains a RING-finger domain exhibiting ubiquitin ligase activity (Honda and Yasuda, 1999). p53 turnover by Mdm2 may also occur independently of ubiquitination by a mechanism that is regulated by phosphorylation (Blattner *et al.*, 2000). Mdmx is a

homologue of Mdm2, and formation of Mdmx/Mdm2 oligomers protects both p53 and Mdm2 from degradation (Jackson and Berberich, 2000; Sharp *et al.*, 1999).

1.2.3.9.4 Retinoblastoma gene

The retinoblastoma (Rb) susceptibility gene was the first and therefore prototype tumour suppressor gene identified, with its mutation resulting in childhood retinal cancer (Bookstein and Lee, 1991). The 180kb long gene encodes a 105kD nuclear phosphoprotein (pRb) (Weinberg, 1991). Mutations of the Rb gene aid in the formation of tumours, with its inactivation resulting in multiple genetic alterations, which lead to tumorigenesis. Rb is responsible for the movement of cells through the G1 phase-restriction checkpoint, and this point of control is often found lacking in cancerous cells. Furthermore, the gene promotes terminal differentiation, prevents cell cycle re-entry, and by guarding the passage through mitosis, ensuring accurate chromosomal segregation and chromatin remodelling, is a key factor in maintaining genetic integrity (Zheng and Lee, 2001). Like other tumour suppressor genes, reintroduction of a wild-type Rb allele into Rb-deficient cells is able to suppress cancer cells and the neoplastic state, and depletion of reconstituted Rb reduces cells back to their cancerous status (Chen *et al.*, 1992; Riley *et al.*, 1994).

1.2.3.9.5 The deleted-in-colon-cancer gene

The deleted-in-colon-cancer (DCC) gene, which is made up of more than one million base pairs on chromosome 18, encodes a 190kD transmembrane phosphoprotein. It has structures similar to cell adhesion molecules in its extracellular domain, which indicates binding to an extracellular matrix or basement membrane component. However, DCC does not only mechanically hold the cell to the extracellular matrix; it is probably a signal-transducing receptor, whose loss of function gives evolving tumour cells a growth advantage (Weinberg, 1991).

This tumour suppressor gene encodes for the netrin-1 receptor. Netrin-1 is a molecule that participates in axon guidance. When DCC is engaged by netrin-1, apoptosis is blocked and in the absence of ligand binding, apoptosis is induced by DCC. DCC is a caspase substrate and mutation at the site where DCC is cleaved by caspase-3 completely suppresses the pro-apoptotic effect of DCC (Mehlen *et al.*, 1998).

1.3 The cell cycle and tumorigenesis

The cell cycle is essential for the development and differentiation of the individual. Mitotic cell division takes place in a series of stages making up the cell cycle, which is regulated by a range of genetic and biochemical factors. The function of abnormal genes, oncogenes, which cause cells to enter rapid, uncontrolled cell cycles of division, result directly or indirectly in the formation of tumours. Oncogenes have a normal counterpart, the proto-oncogene.

The initial stage of the cell cycle is interphase, at which point a cell from a previous division enters the G1 stage. During this stage, growth is promoted with the synthesis of proteins, carbohydrates, lipids and other molecules, but DNA replication is inhibited. As DNA replication is initiated, the G1 stage ends and the S stage of interphase begins, during which period the entire cellular DNA, along with the histone and nonhistone chromosomal proteins associated with DNA, are replicated. With the completion of DNA replication, the S stage ends with the commencement of the brief G2 stage, which signifies the termination of interphase. During this stage, proteins needed for mitosis progression are synthesized. During the G2 and S stages, assembly of the main classes of molecules continues. The cell then enters the mitosis phase of the cell cycle to produce two genetically identical daughter cells, which then re-enter the G1 stage (Wolfe, 1995).

The main cell cycle modifiers are those that operate through plasma membrane receptors. They present the main channels for the coordination of development and maintenance of a range of organ systems. In combination with an external protein, such as a growth factor, the receptor transmits a signal into the cytoplasm that induces a reaction cascade, culminating directly or indirectly in cell division. Many oncogenes encode for dysfunctional cell receptors or proteins participating in the reactions, thereby disenabling regulation of cell proliferation (Wolfe, 1995).

Since DNA is so vital in genetic inheritance, cells must be able to respond to DNA damage. They do this either by undergoing cell cycle arrest, to allow for easier repair of DNA, or by undergoing cell death. In germ cells, genome alterations are limited for

faithful propagation of the species, while in somatic cells, genome alterations must be dealt with to prevent abnormal cell proliferation. DNA damaging agents can be used in cancer therapies to activate tumour suppressors and therefore take advantage of the cell suicide and growth arrest systems, which they regulate (Coultas and Strasser, 2000).

1.3.1 p53 and Rb genes in the cell cycle

The functions of the p53 and Rb genes are required for the critical period of G1 to S-phase transition. Overexpression of Rb at the early G1 stage of the cell cycle instigates reversible G1 arrest. However, injection of Rb at the late G1 stage or early S stage of the cell cycle does not influence DNA synthesis. The inhibition of transforming growth factor β (TGFβ) correlates with the phosphorylation of pRB (the product of the Rb gene), which inactivates its function. A correlation has been identified between the lack of TGFβ responsiveness in tumours and the inactivation of the RB gene. This suggests that some tumour suppressor genes may encode proteins that are involved in the biochemical cascade that is stimulated by the inhibition of cytokines (Aaronson, 1991).

The nuclear localization of pRb and its DNA binding ability implies that it has a role in transcriptional regulation, while its tissue distribution suggests that it is involved in the growth regulation of a range of cells and tumour types. Its inactivation, however, only occurs in a small group of tumours, in which gene activation is exclusively derived from somatic events. pRb moves between a hyper-phosphorylated and relatively unphosphorylated condition in a cell cycle-specific approach. In G1 it is under-phosphorylated; in the transition to S phase it becomes heavily phosphorylated; it remains

phosphorylated through the S, G2 and most of the M phase; and reverts to an under-phosphorylated state at or before the transition from M to G1. This pattern suggests that pRb may be involved in the regulation of the cell's progression through the cell cycle (Weinberg, 1991).

1.3.2 Anticancer therapies applied to the cell cycle

It is possible that exogenous growth factors may be applied to synchronize tumour cell proliferation and thereby increase the effectiveness of chemotherapy at a particular point of the cell cycle, e.g. the cytokines interferon and interleukin-2 are already being used in cancer treatment. Work is being done to specifically and effectively target tumour cells by intervening at the critical points in mitogenic signalling where oncogenes are usually activated. A more promising target for precise therapy would be the growth factor receptor itself. A possible strategy for this involves using monoclonal antibodies that cause down-regulation of the receptors. This has been shown to restrict tumour proliferation both *in vitro* and *in vivo*. Overexpression of receptors in tumours may be targeted with radioisotopes or toxins linked to monoclonal antibodies to the receptor or the particular ligand (Aaronson, 1991).

The tumour-suppressor gene p16 INK4/CDKN2 (p16) is an important cell cycle regulator and a cyclin-dependent kinase (Cdk) inhibitor. It has been shown that the adenovirus-mediated gene transfer of p16 (AdCMV.p16) into colon cancer cells initiates a halting of the S phase, mitosis and apoptosis (Tamm *et al*., 2002). After treatment of mice colorectal cancer cells with AdCMV.p16, tumour volume was significantly reduced and

cell survival prolonged. This result was Bax-independent since identical results were obtained when using cells that were wild type or mutant for p53 or Bax. This treatment may be particularly effective in Bax-negative colon cancer patients for whom the overexpression of p53 does not appear to be of therapeutic benefit (Tamm *et al*., 2002).

1.4 Apoptosis

Apoptosis is a programmed cell-suicide mechanism. Irregularities in this system are directly or indirectly the cause of a number of medical illnesses. These irregularities result in either cell accumulation, when cell eradication is non-functional, or cell loss, when apoptosis is inappropriately triggered (Reed, 2002). The balance between death signals and a cell's counteracting survival factors is an important factor in the decision as to whether a cell will live or die. If this balance can be shifted in favour of one or the other, injured cells can be saved on one hand, and undesirable cells killed on the other (Ray *et al.*, 2000). Apoptosis can be indirectly affected by altering inputs into apoptotic pathways through protein phosphatases, protein kinases, transcription factors and cell surface receptors for cytokines, cardiotrophins, neurotrophins and growth factors. There are a small number of candidate drug discovery targets that directly alter the expression or function of core genes and proteins (Reed, 2002).

Cell proliferation and cell death must be controlled to sustain tissue homeostasis in multicellular organisms. This can be achieved by regulating a shared set of factors between the processes of cell cycle progression and apoptosis. Evidence of a link between these two processes is that when the cell cycle is manipulated, an apoptotic

response is either induced or inhibited. This has been identified for tumour suppressor genes such as p53, Rb, c-*myc* and a number of cyclin-dependent kinases (Cdks) and their regulators. These proteins are involved in proliferative pathways but are also able to sensitise cells to apoptosis (Pucci *et al.*, 2000).

1.4.1 Apoptosis and cancer

During tumour development, cells encounter apoptosis-inducing stimuli. Genetic mutations that result in the inhibition of apoptosis give these cells a selective advantage. The process and regulation of apoptosis is therefore crucial with regards to tumour initiation, progression and metastasis (Ng, 2002). Apoptosis is essential in preventing the proliferation of cells with a higher-than-normal mutation rate, thereby decreasing the levels of malignant transformation. The majority of cancer treatments aim to activate apoptosis. Resistance to apoptosis induction may be the consequence of specific inhibition of apoptotic signalling. In these cancerous cells, chemotherapy or radiation may actually encourage tumour progression by increasing the mutation rate (Konstantinidou *et al.*, 2002).

1.4.2 Pathways of apoptosis

Apoptosis can be induced by a range of external stimuli, such as antibiotics, UV and hormones, and by internal stimuli, such as DNA damage and transcriptional regulators. Apoptosis is carried out via two pathways: the intrinsic pathway is initiated by mitochondria; whereas the extrinsic pathway is initiated by death receptors on the cell surface belonging to the tumour necrosis factor receptor family. Both pathways are induced by the activation of apical caspases.

1.4.2.1 Caspases

The central modulators of apoptosis are enzymes that implement cell death by cleaving a variety of intracellular substrates that trigger cell dissolution. The caspases are a large family of cysteine proteases that cause the morphological changes observed in cells undergoing apoptosis (**figure 1.9**) (Nicholson and Thornberry, 1997). Most apoptosis-based drugs aim to target caspases to block cell death. These death proteases are homologous to one another and are highly conserved through evolution. Caspases are subdivided, based on their substrate preference, extent of sequence identity and structural similarities. Inactivation of caspase activity slows down or inhibits apoptosis (Earnshaw *et al*., 1999).

Caspases are synthesized as enzymatically inert zymogens. The zymogens are composed of three domains; an N-terminal prodomain, and the p20 and p10 domains that are found in the mature enzyme (Hengartner, 2000). All known caspases cleave their substrates at bonds immediately after Asp residues. Of the 12 known human caspases, six (caspase-3, -

6, -7, -8, -9 and -10) are definitely involved in apoptosis in various model systems. A classification scheme divides these apoptotic caspases into two classes, based on their prodomains and roles in cell death, namely effector (or "downstream'') caspases, which are responsible for most of the cleavages that disassemble the cell, and initiator (or "upstream") caspases, which initiate the proteolytic cascade. The upstream or initiator caspases have longer prodomains that mediate the transduction of death signals, and the assembly of activating complexes (Bangs *et al*., 2000). The most characterized caspases, caspase-3, -6 and –7, are the major effector caspases (**figure 1.9**). Once activated, these caspases cleave polypetides that subsequently undergo proteolysis in apoptotic cells (Earnshaw *et al*., 1999).

Caspases-8 and -9 are the major initiator caspases that, once activated, acquire the ability to cleave and activate effector caspases. Induction of apoptosis via death receptors results in the activation of an initiator caspase such as caspase 8 or caspase 10. These caspases can then activate other caspases in a cascade that eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6, responsible for the cleavage of the key cellular proteins that cause the typical morphological changes observed in cells undergoing apoptosis.

Apart from the ligation of death receptors there are a number of other mechanisms through which the caspase cascade can be activated. Granzyme B can be delivered into cells by cytotoxic T lymphocytes, and is able to directly activate caspases 3, 7, 8 and 10. The mitochondria are also key regulators of the caspase cascade and apoptosis. Release

of cytochrome c from mitochondria can lead to the activation of caspase 9, and then of caspase 3. Current understanding suggests that the protein-protein interactions leading to activation of caspases-8 and-9 are the end result of two very distinct signaling processes (Earnshaw *et al*., 1999) Events leading to caspase-8 activation start with binding of one of the death receptors namely Fas, by Fas L or cross-linking antibody that results in receptor trimerisation followed by attachment of the adaptor molecule FADD to the cytoplasmic domain of the receptor. FADD in turn recruits procaspase 8. The end result is the rapid liberation of cleaved, fully active caspase 8 (Medema *et al.*, 1997).

Upon activation, p53 increases the expression of Bax and the Fas receptor. While Bax promotes apoptosis, Bcl-2, Bcl-$_{XL}$ and Mcl-l inhibit apoptosis. FasL is activated by the death receptor Fas, with the consequent activation of caspase 8 and caspase 3, and the onset of apoptotic cell death (**figure 1.10**) (Nagata and Goldstein, 1995; Nagata, 1997). If these factors fail to be regulated, there is loss of control of either cell cycle arrest or apoptosis, resulting in unrestrained cell growth (Backus *et al.*, 2002).

1.4.2.2 The intrinsic/mitochondrial pathway

Mitochondria have been implicated as playing an essential role in the execution phase of apoptosis. In mitochondrion-dependent apoptosis, the release of cytochrome-*c* from the mitochondrial intermembrane space into the cytosol leads to the formation of the apoptosome, which is made up of cytochrome-*c*, APAF-1 and procaspase-9 (**figure 1.9**). This triggers the activation of caspase-9, which consecutively activates the effector caspases 7, 6 and 3, resulting in cell death by cleavage. The mechanisms responsible for

this release may include mitochondrial permeability transition and Bcl-2 regulated swelling of the mitochondrial matrix (Wilson, 1998). In most cells, apoptosis progresses with the translocation of apoptogenic factors such as cytochrome-*c* and apoptosis inducing factor (AIF) from the mitochondria to the cytosol.

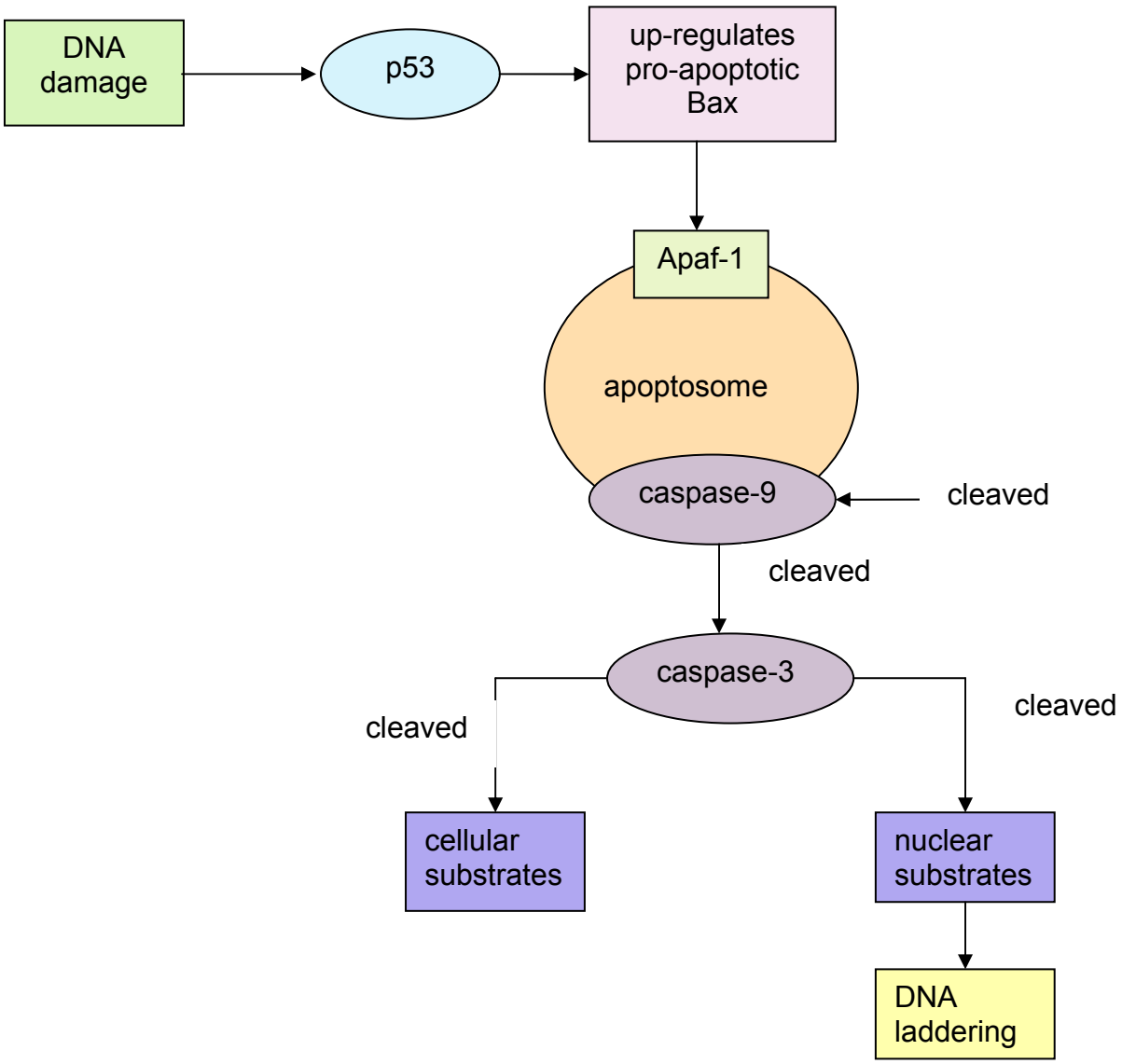

Figure 1.9: The intrinsic apoptotic pathway

1.4.2.3 The extrinsic/death-receptor pathway

In death-receptor-mediated apoptosis, activation of the death receptors results in the formation of the death-inducing signalling complex (DISC) which contains the death receptors, adaptor proteins, caspase-8 and caspase-10 (**figure 1.10**). Caspases 8, 9 and 10 are regarded as the initiator caspases at the head of the caspase-signalling cascade. Once part of the DISC (and the apoptosome in the intrinsic pathway) the caspases are activated by dimer formation (Chen and Wang, 2002). Caspase-8 is activated by the engagement of Fas (CD95) or tumour necrosis factor 1 (TNF-1). Caspase-8 then induces a cascade of caspase activations, namely caspases 3 and 7. Apoptosis inducing factor (AIF) moves into the nucleus where it triggers DNA destruction and therefore apoptosis. This pathway is regulated at a number of levels by death receptor expression and fluctuates in various cell types.

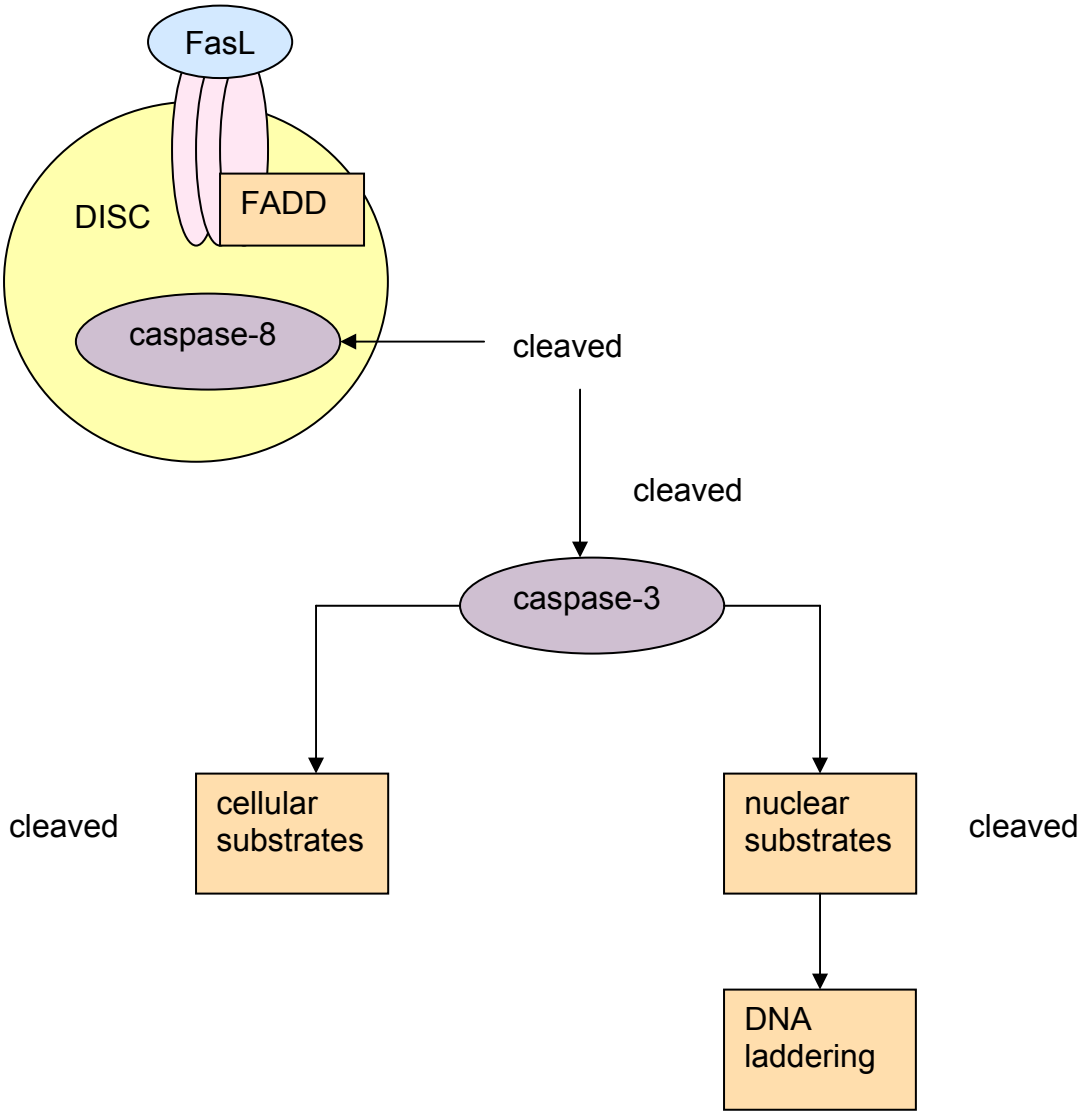

Figure 1.10: The extrinsic apoptotic pathway

1.4.3 Bcl-2 family

The Bcl-2 family is one of the most important classes of proteins regulating apoptosis. It includes the pro-apoptotic (Bax, Bad, Bak, Bid, Bcl-Xs, Bim, Bik) and the anti-apoptotic (Bcl-2, Bcl-$X_{L,}$ Bcl_W) proteins. They are found mostly at the outer membranes of mitochondria, the endoplasmic reticulum and the nucleus. Studies show that expression of the met proto-oncogene regulates apoptosis via Bcl-w (a member of the anti-apoptotic Bcl-2 family) expression in colorectal tumours (Kitamura *et al.*, 2000). The proto-oncogene Bcl-2 was first isolated from human follicular lymphomas and is mainly found at the outer-inner mitochondrial membrane.

Bcl-2 inhibits apoptosis in response to a wide range of stimuli, and inhibits the mitochondrial and nuclear manifestations of cell death. Its anti-apoptotic functions are due to the reduction of reactive oxygen species, the effect on mitochondrial proton flux and modulation of mitochondrial calcium homeostasis. Bcl-2 is expressed in organelle membranes, where it acts to protect cells from endogenous-induced apoptosis. It has been shown that Bcl-2 located at the endoplasmic reticulum (ER), impedes apoptosis induced by Bax, ionizing radiation, c-*myc* expression, serum withdrawal and ceramides. Bcl-2 obstructs caspase activation at the ER by interacting with the Bap31 protein (Rudner *et al.*, 2002). The highly conserved proapoptotic Bcl-xl/Bcl-2-associated death promoter homolog (BAD) interacts with varied Bcl-2 members to regulate apoptosis (Bae *et al.*, 2001).

A Bcl-2-independent pathway involves the apoptosis of cells triggered by Fas (CD95) and some members of the TNF family, without mitochondrial participation, indicating that there are parallel pathways which may be related but independent of Bcl-2 (Anjum and Khar, 2001).

The Bcl-2 oncoprotein is a compelling inhibitor of apoptosis. Bcl-2 overexpression results in uncontrolled cell survival and contributes to tumour formation and the development of autoimmune diseases. It is thought to avert activation of the ced3/caspase-3 subfamily, which causes the death effector machinery to be suppressed. Conventional cancer drugs most often target this pathway; therefore Bcl-2 overexpression is also correlated with drug resistance. Gene expression is inhibited by the binding of small synthetic oligodeoxyribonucleotides to a complementary base sequence of the mRNA (Packham, 1998; Zangemeister-Wittke and Ziegler, 1998). Bcl-2 inhibits apoptosis and is recurrently overexpressed in colorectal adenomas and carcinomas, with high expression in non-dysplastic epithelium adjacent to dysplastic lesions. This indicates that Bcl-2 expression is elevated before the development of morphological dysplasia (Bronner *et al*., 1995; Hague *et al*., 1994). Nondysplastic epithelium flanking a malignant or dysplastic lesion usually shows elevated expression of Bcl-2 along the entire length of the crypt villus axis. Moving away from the lesion, the expression steadily decreases to a normal pattern of expression, which involves only the crypt base (Bronner *et al*., 1995).

Bid is an abundant pro-apoptotic Bcl-2 protein, vital for death receptor-mediated apoptosis. Its mode of function involves the re-location of its truncated form, tBid, to the mitochondria, where it assists in the release of cytochrome-*c* and other apoptogenic proteins. The basic steps of Bid activation are caspase cleavage, dissociation of tBid and lipid-mediated mitochondrial transfer (Esposti, 2002).

Studies show that following the microinjection of cytochrome *c* into different cell types, apoptosis is rapidly induced. This is characterized by the externalization of phosphatidyl serine, detachment of cells from the substratum and from adjacent cells, membrane budding, chromatin condensation and cell shrinkage. Cells that were injected with Bcl-2 were noticeably less sensitive to the injected cytochrome-*c*. From this study, it is seen that extra-mitochondrial cytochrome-*c* induces apoptosis in cells and that Bcl-2 is not only able to prevent apoptosis by regulating the release of cytochrome-*c* from mitochondria, but also by interfering with caspase activation after the injection of cytochrome-*c* directly into the cytoplasm (Brustugun *et al.*, 1998).

During apoptosis, pro-apoptotic Bcl-2 proteins are activated by undergoing a conformational change which exposes the pro-apoptotic BH3 domain through dephosphorylation and proteolytic cleavage by caspases, such as Bid. Different pro-apoptotic members initiate cytochrome-c release: Bid is involved in Fas-mediated apoptosis, Bax in DNA damage-induced apoptosis and Bad in lymphokine deprivation-induced apoptosis (Tsujimoto and Shimzu, 2000).

Mitochondria are regarded as the control centres of the apoptotic cascade. The expression levels of the Bcl-2 proteins control apoptosis by regulating mitochondrial homeostasis. In the absence of a death stimulus, the ectopic expression of Bax initiates cytochrome *c* release from mitochondria. It has been shown that Bcl-2 inhibits the ion-conducting channel forming activity of Bax (Anjum and Khar, 2001). Apoptosis is induced by the p53 tumour suppressor protein, which inhibits tumour formation. p53-dependent apoptosis initiates a conformational change in Bax, which is independent of bid, as well as a Bax-Bak interaction, the release of cytochrome *c* from mitochondria, the activation of caspase-9 and caspase-3, the cleavage of caspase substrates, and apoptosis (Henry *et al.*, 2002).

1.4.3.1 PUMA

The 'BH3-only' group of proteins belongs to the Bcl-2 family and responds to distinct apoptotic stimuli by dissuading or inducing the Bcl-2 family proteins (Huang and Strasser, 2000; Letai *et al.*, 2002; Zong *et al.*, 2001). A BH3-only protein, PUMA (p53 up-regulated modulator of apoptosis), was identified as being directly regulated by p53 (Yu *et al.*, 2003). PUMA is a key factor required for apoptosis stimulated by exogenous or endogenous p53 in colon cancer cells. PUMA-induced apoptosis relies on Bax. With tumour development, hypoxia sets in as the angiogenic rate decreases (Graeber *et al.*, 1996). This induces p53 and consequently, p21 and PUMA. p21 induction causes growth arrest, while PUMA induction causes mitochondrion-dependent death (Yu *et al.*, 2003). This explains the late occurrence of p53 mutation in colon tumorigenesis, as this

is when marked hypoxia sets in. Numerous necrotic areas can be found in the cancerous tissue at this stage (Yu *et al.*, 2003).

1.4.4 Tumour necrosis factor family

The binding of tumour necrosis factor α (TNFα) at the cell surface initiates a series of signalling events which result in the caspase cascade, which is at the core of apoptosis. Caspase dysfunction, resulting in the inability to commit to apoptosis after treatment with TNF or other stimuli, may contribute to cancer development (Hedge and Williams, 2002).

1.4.4.1 TRAIL

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family and through the activation of the caspase pathway induces apoptosis in TRAIL-sensitive tumours. Studies indicate that combining TRAIL with an agent such as sodium butyrate (NaBT), which induces differentiation and apoptosis in certain colorectal cancers, has the potential of a more effective and less toxic treatment for resistant colon cancers (Hernandez *et al.*, 2001).

1.4.4.2 Fas ligand and Fas receptor

Upon activation of the Fas receptor (CD95) by Fas ligand (FasL), which is a member of the tumour necrosis factor family, apoptosis is induced in transformed and nontransformed cells (Nagata and Goldstein, 1995). FasL expression aids in creating immunologically advantaged niches where tumours are undetected by the immune system (Shiraki *et al.*, 1997). In previous work, Fas receptor expression, which is highly

expressed in normal mucosa, was not present in cancer cells (Möller *et al.*, 1994). However, FasL expression was found to be high in liver metastases of colonic adenocarcinomas, implying that it functions by eliminating activated T lymphocytes assigned to destroy tumour cells. It thereby promotes survival of cancer cells (Hahne *et al.*, 1996).

FasL is also believed to be important in the colonization of colon cancer cells in the liver (Shiraki *et al.*, 1997). Without the inhibitory effects of Fas, cancer cells can avoid the action of cytotoxic T cells. Fas-mediated apoptosis has an immunoregulatory role. Colon tumours are able to take advantage of this death program by expressing FasL, which allows the tumours to manipulate Fas into attacking antitumour lymphocytes, thereby impairing immune responses. Normal colon epithelial cells are sensitive to Fas-mediated apoptosis, whereas colon cancer cells show resistance to this cell death pathway (O'Connell *et al.*, 2000).