

# **Histone Acetylation and Chemoresistance in Colorectal Cancer: an Opportunity for Effective Personalized Treatment**

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Doctor of Philosophy**

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**By**

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## Declaration of Originality

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# Acknowledgements

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Finally, I would like to thank everyone offered me advice or support.

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# Abstract

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Colorectal cancer (CRC) is the most common cause of deaths in the West. Despite many therapeutic opportunities, drug resistance or recurrence has significant rates among patients. Nearly 50% of CRC patients develop metastases. Therefore, sensitive biomarker and effective treatments with minimal toxicity are needed. Genetic and epigenetic alterations play major roles in initiation, development, and chemoresistance of CRC. Histone deacetylase2 (HDAC2) over-expression is well-known in CRC. Many studies have associated HDAC2 over-expression and *TP53* mutations with late stages of metastatic CRC (mCRC). However, the relationship between HDAC2 expression level and *TP53* status and mCRC drug resistance is unclear. Here, I have investigated HDAC2 role in drug resistance and assessed the synergistic effects of DNA chemotherapeutics agents and HDAC inhibitors (HDACIs) on *TP53* status in mCRC cell lines. I have shown for the first time that in mutated p53 mCRC cells (Sw480 and HT-29) the steady-state level of HDAC2 is low compared to wild-type p53 cells (HCT116 p53+/+). I have also found that increase in HDAC2 expression level in the highly resistant cell line HT-29 enhances drug resistance and its depletion by shRNA sensitises HT-29 to 5Fluorouracil (5FU) or Oxaliplatin (Oxa). The combined treatment of suberoylanilide hydroxamic acid (SAHA)/5FU and SAHA/Oxa was able to reduced HDAC2 expression level and induced mitotic cell death. However, SAHA/Doxorubicin combined treatment induced cell death in wild-type p53 (HCT116 p53+/+), null p53 (HCT116 p53-/-), and SW480 cell lines. This cell death associated with decrease in HDAC2 level. I have shown the association between sensitivity to treatment and reduction of HDAC2 level via bioluminescence imaging in combination with liposomal-encapsulated

SAHA/Doxorubicin delivery to monitor tumour growth. I have observed a significant decrease in tumour growth and HDAC2 level. Therefore, I suggest that unlike mutated p53, HDAC2 could be an epigenetic prognostic biomarker to predict therapeutic response in mCRC.

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# Publication

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The following paper has been submitted to Cancer Research Journal.

**Histone deacetylase2 regulation controls metastatic colorectal cancer response to HDAC inhibitor combined with DNA damaging agents**

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# List of Abbreviations

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|                |                                         |
|----------------|-----------------------------------------|
| <b>5FU</b>     | : 5Fluorouracil                         |
| <b>APC</b>     | : Adenomatous polyposis coli            |
| <b>BLI</b>     | : Bioluminescence imaging               |
| <b>CI</b>      | : Combination index                     |
| <b>CIMP</b>    | : CpG Island methylator phenotype       |
| <b>CIN</b>     | : Chromosomal instability               |
| <b>Cisp</b>    | : Cisplatin                             |
| <b>COX-2</b>   | : Cyclooxygenase-2                      |
| <b>CPT-11</b>  | : Camptothecin-11                       |
| <b>CRC</b>     | : Colorectal cancer                     |
| <b>CT</b>      | : Computed tomography                   |
| <b>DNMTIs</b>  | : DNA methyltransferase inhibitors      |
| <b>Dox</b>     | : Doxorubicin                           |
| <b>DSB</b>     | : Double-strand breaks                  |
| <b>FAP</b>     | : Familial adenomatous polyposis        |
| <b>FCM</b>     | : Flow cytometry                        |
| <b>FOBT</b>    | : Faecal occult blood testing           |
| <b>H3K9ME3</b> | : Trimethylation of histone H3 lysine 9 |
| <b>HATs</b>    | : Histone acetyltransferase enzymes     |
| <b>HDACIs</b>  | : Histone deacetylase inhibitors        |
| <b>HDACs</b>   | : Histone deacetylase enzymes           |
| <b>HCC</b>     | : Hepatocellular carcinoma              |
| <b>IBD</b>     | : Inflammatory bowel disease            |
| <b>IBS</b>     | : Irritable bowel syndrome              |
| <b>IGF2</b>    | : Insulin-like growth factor II         |
| <b>IHC</b>     | : Immunohistochemistry                  |

|                                |                                                              |
|--------------------------------|--------------------------------------------------------------|
| <b>LOH</b>                     | : Loss of heterozygosity                                     |
| <b>MAPK</b>                    | : Mitogen-activated protein kinase                           |
| <b>mCRC</b>                    | : Metastatic colorectal cancer                               |
| <b>MCD</b>                     | : Mitotic cell death                                         |
| <b>MLH1</b>                    | : MutL homolog1                                              |
| <b>MMR</b>                     | : Mismatch repair                                            |
| <b>MRI</b>                     | : Magnetic resonance imaging                                 |
| <b>MSI</b>                     | : Microsatellite instability                                 |
| <b>MSI-H</b>                   | : High-frequency MSI                                         |
| <b>MSI-L</b>                   | : Low-frequency MSI                                          |
| <b>MSS</b>                     | : Microsatellite stable                                      |
| <b>NaB</b>                     | : Sodium butyrate                                            |
| <b>NAD<sup>+</sup></b>         | : Nicotinamide adenine dinucleotide                          |
| <b>NF-<math>\kappa</math>B</b> | : Nuclear factor $\kappa$ B                                  |
| <b>NSAIDs</b>                  | : Nonsteroidal anti-inflammatory drugs                       |
| <b>Oxa</b>                     | : Oxaliplatin                                                |
| <b>PARP</b>                    | : Poly (ADP-ribose) polymerase                               |
| <b>PARPc</b>                   | : PARP cleavage                                              |
| <b>PBS</b>                     | : Phosphate buffer saline                                    |
| <b>PEI</b>                     | : Polyethylenimine                                           |
| <b>PET</b>                     | : Positive emission tomography                               |
| <b>PI</b>                      | : Propidium iodide                                           |
| <b>PK</b>                      | : Pyruvate kinase                                            |
| <b>ROI</b>                     | : Region of interest                                         |
| <b>ROS</b>                     | : Reactive oxygen species                                    |
| <b>RPM</b>                     | : Revolutions per minute                                     |
| <b>SAHA</b>                    | : Suberoylanilide hydroxamic acid                            |
| <b>SDS-PAGE</b>                | : Sodium dodecyl sulphate polyacrylamide gel electrophoresis |

|                |                                                            |
|----------------|------------------------------------------------------------|
| <b>Ser10H3</b> | : Serine 10 residue in histone 3                           |
| <b>SSA</b>     | : Sessile serrated adenomas                                |
| <b>TCF</b>     | : T-cell factor                                            |
| <b>TNM</b>     | : Tumour, node, metastasis                                 |
| <b>TRAIL</b>   | : Tumour-necrosis factor-related apoptosis inducing ligand |
| <b>TSA</b>     | : Traditional serrated adenomas                            |
| <b>TSA</b>     | : Trichostatin A                                           |
| <b>VPA</b>     | : Valproic acid                                            |

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# **Chapter 1**

## **Introduction**

## **Chapter at Glance:**

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# 1. Colorectal Cancer (CRC)

## 1.1. Definition and Epidemiology

The word colorectal is derived from the colon and the rectum which form the large bowel in the gastrointestinal tract of the human body, figure 1.1. Colorectal cancer (CRC) is a tumour which occurs in colon or rectum. CRC or cancer of the large bowel has a high incidence in the developed countries in comparison with the other parts of the world. This cancer is ranked third in prevalence in the UK after breast and lung cancer and about 110 new patients with colorectal cancer are diagnosed each day (Cancer Research UK, <http://info.cancerresearch-uk.org/cancerstats/types/bowel/incidence/>). The worldwide incidence of CRC is high, especially in the western countries (WHO website, [http://www.who.int/gho/ncd/mortality\\_morbidity/cancer\\_text/en/](http://www.who.int/gho/ncd/mortality_morbidity/cancer_text/en/)), and the highest incidence rate is the United States (Stallmach et al., 2011). In most parts of the world, CRC in women comes second in incidence after breast cancer and in men, CRC is the third most common cancer in incidence after lung and prostate cancer (Ballinger and Anggiansah, 2007); hence CRC is a major health problem globally (Siegel et al., 2011). Also, the mortality from CRC is high across the world (Cidon, 2010), and it differs among countries, depending on how early diagnosis is performed and the treatment offered, both of which can reduce deaths from CRC (Cai et al., 2011). It is estimated that approximately 16000 deaths a year are caused by CRC and the survival rate at 5 years is only 50% in the UK (Logan et al., 2011).

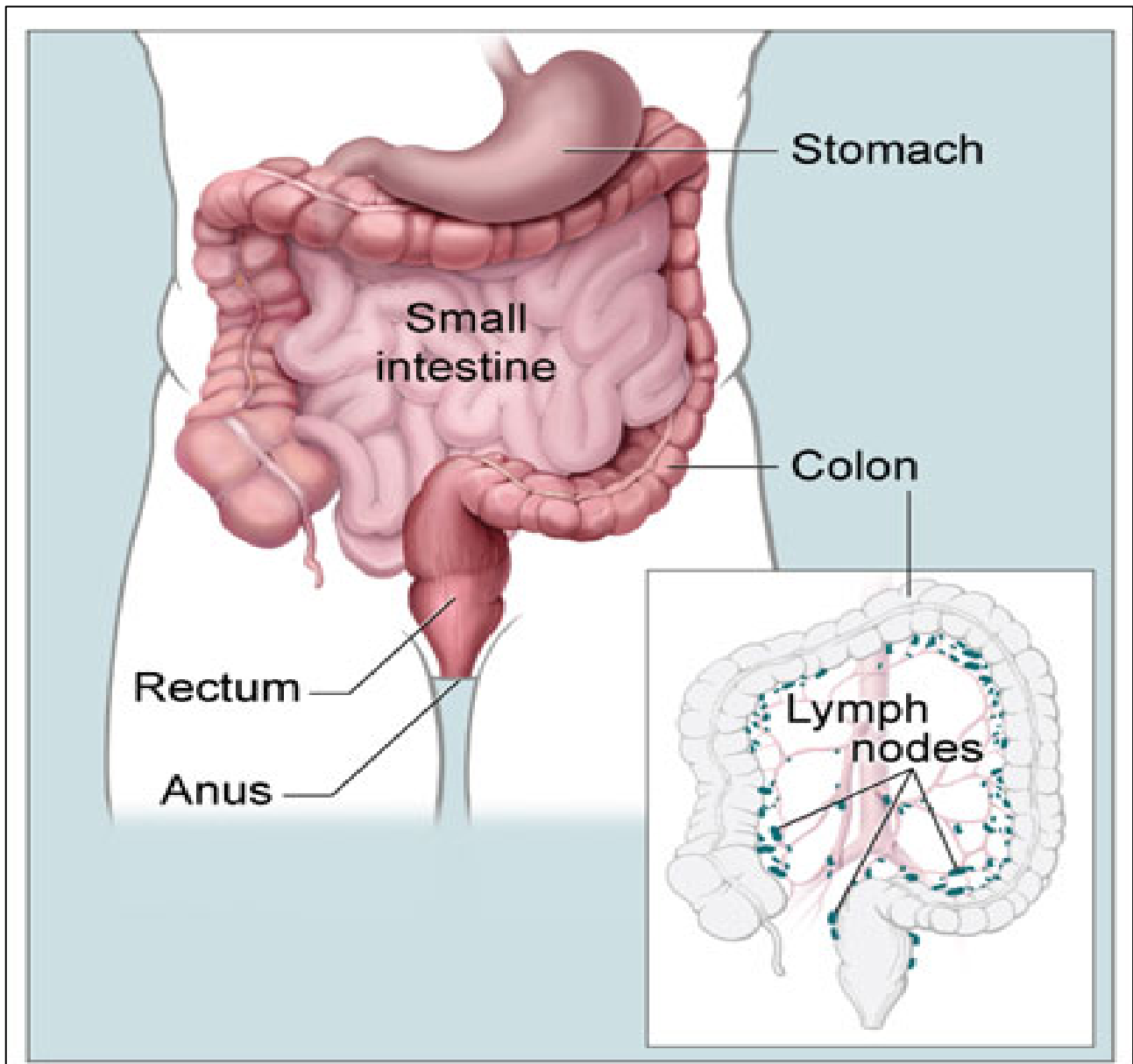


**Figure 1.1: The large bowel location inside the human abdomen.**

*The gastrointestinal tract in our bodies starts with the mouth and continues to the oesophagus which connects to the stomach. The latter, in turn, connects to the small intestine that at its end joins the colon (which has three distinct parts: the ascending colon, the transverse colon, and the descending colon). The descending colon leads to the rectum which opens into the outside of the body through the anus. Both the colon and the rectum form the large bowel which is highly surrounded by lymph nodes. The figure was taken from:*

*<http://www.cancer.gov/cancertopics/wyntk/colon-and-rectal/page2>.*

## **1.2. The aetiology of colorectal cancer**

The aetiology of CRC is multifactorial (Ponz de Leon and Roncucci, 2000). This means that genetic and non-genetic factors are involved. The risk factors which increase the predisposition to CRC include autosomal dominant inheritance, sex, age, family history, current and previous clinical history, and nutrition (Cunningham et al., 2010). Diet alone is considered to be the cause of about 70-90% of all CRC cases (Araújo et al., 2011). The high consumption of red meat, uncontrolled alcohol consumption, the presence of inflammatory bowel disease, and smoking increase the risk of developing CRC.

In addition, obesity and low levels of physical activity due to sedentary lifestyle has a noticeable role in CRC incidence (Dennis et al., 2011). However, a number of protective factors may lower the incidence of CRC. A high-fibre food such as fruit, cereals, and vegetables (van den Brandt and Goldbohm, 2006) increases faecal bulk and decreases transit time (de Kok and van Maanen, 2000). Also, it has been shown that using aspirin on a frequent basis reduces the risk of CRC to 60%. The aspirin effect is similar to the effect of the nonsteroidal anti-inflammatory drugs (NSAIDs) which decrease prostaglandin production by inhibition the over-expression of cyclooxygenase-2 (COX-2) enzyme in CRC. COX-2 expression is inducible upon an inflammatory stimulus, and it is not usually expressed in normal colorectal epithelium but it is highly expressed in many colorectal tumours in absence of the inflammatory stimulus (Boland et al., 2000). Another protective factor that has been highlighted in CRC is physical activity which reduces the risk for

CRC (Song et al., 2011). Etiologically, CRC is conventionally classified into two types (Cheah, 2009):

1) Familial (hereditary) CRC:

This type comprises about 15-20% of the CRC cases, and it arises as a result of mutations in one or more genes involved in cell cycle control such as tumour suppressor genes and mismatch repair genes (Dietrich et al., 2011). The most common syndromes representing this type of CRC are:

- Familial Adenomatous Polyposis (FAP): is an autosomal dominant syndrome caused by a germ-line mutation in the tumour suppressor gene the adenomatous polyposis coli (*APC*) gene located on the long arm of chromosome 5 (5q). Carrier patients develop hundreds to thousands of adenomatous polyps in their colon or rectum depending on the degree of penetrance. Some of these polyps transform to CRC later in life if they are not treated in early stages of their development (de Campos et al., 2010).
- Hereditary nonpolyposis colorectal cancer (HNPCC): is another autosomal dominant syndrome which involves germ-line mutations in four genes (*MLH1*, *MSH2*, *PMS2*, and *MSH6*) of the DNA mismatch repair system (MMR system). The defects in these genes lead to microsatellite instability (MSI). Microsatellites are short DNA tandem repeats found within the coding (exons) or non-coding (introns) regions of the human genome. They play crucial

roles in DNA repair function and when they have mutations (Li et al., 2004), they result in DNA instability which leads to cancer development. Patients who have HNPCC develop CRC in an early age of life (Vasen, 2005).

## 2) Sporadic CRC:

Forms around 70 to 80% of all CRC cases, and it usually takes place without any existence for a familial history as an isolated tumour in the colon or rectum (Moran et al., 2010). However, complex interactions between genetic and environmental factors are implicated in development of sporadic CRC (Naccarati et al., 2007). In contrast to the hereditary CRC, sporadic CRC usually appears in the late age of life (about 60 years old) as a result of the accumulation of multiple mutations in a single or a few epithelial cells of the colon- rectum tract (Patel et al., 2012). Studies for sporadic CRC show that most patients exhibit epigenetic changes like hypermethylation in multiple CpG islands of very important genes like the MMR genes (Park et al., 2003), hence about 15-20% of sporadic CRC cases show MSI (Imai and Yamamoto, 2008a). This PhD thesis has dealt with sporadic CRC, and cell lines used in this study were derived from sporadic CRC patients.

### **1.3. The emergence and progress of CRC**

CRC like other cancer diseases occurs as result of successive accumulation of genetic mutations and epigenetic changes in a normal cell (Pierce, 2011). The rate of mutations and the mutated genes are different among cancer types (Pritchard and Grady, 2011). In CRC, these genetic and epigenetic changes transform the cells lining the large bowel into benign neoplasia (adenoma) in the form of microscopic polyps (Pierce, 2011). These polyps grow to form invasive carcinoma which eventually becomes metastatic cancer invading other tissues, as shown in figure 1.2. The model, adenoma/carcinoma progression sequence, was proposed by Fearon and Vogelstein in 1990 (Fearon and Vogelstein, 1990).

### **1.4. Signs and Symptoms of CRC**

Signs and symptoms include loose and/or frequent stools, bleeding that may be visible (dark) or not in the stool, iron-deficiency anaemia (due to the bleeding); obstruction and palpable mass (usually with right colon cancer) which are associated with perforation or abscess formation. Secondary signs and symptoms are intermittent abdominal pain, nausea or vomiting, weight loss, and mucus in the stool (Hall, 2011, Labianca et al., 2010, de Campos et al., 2010).

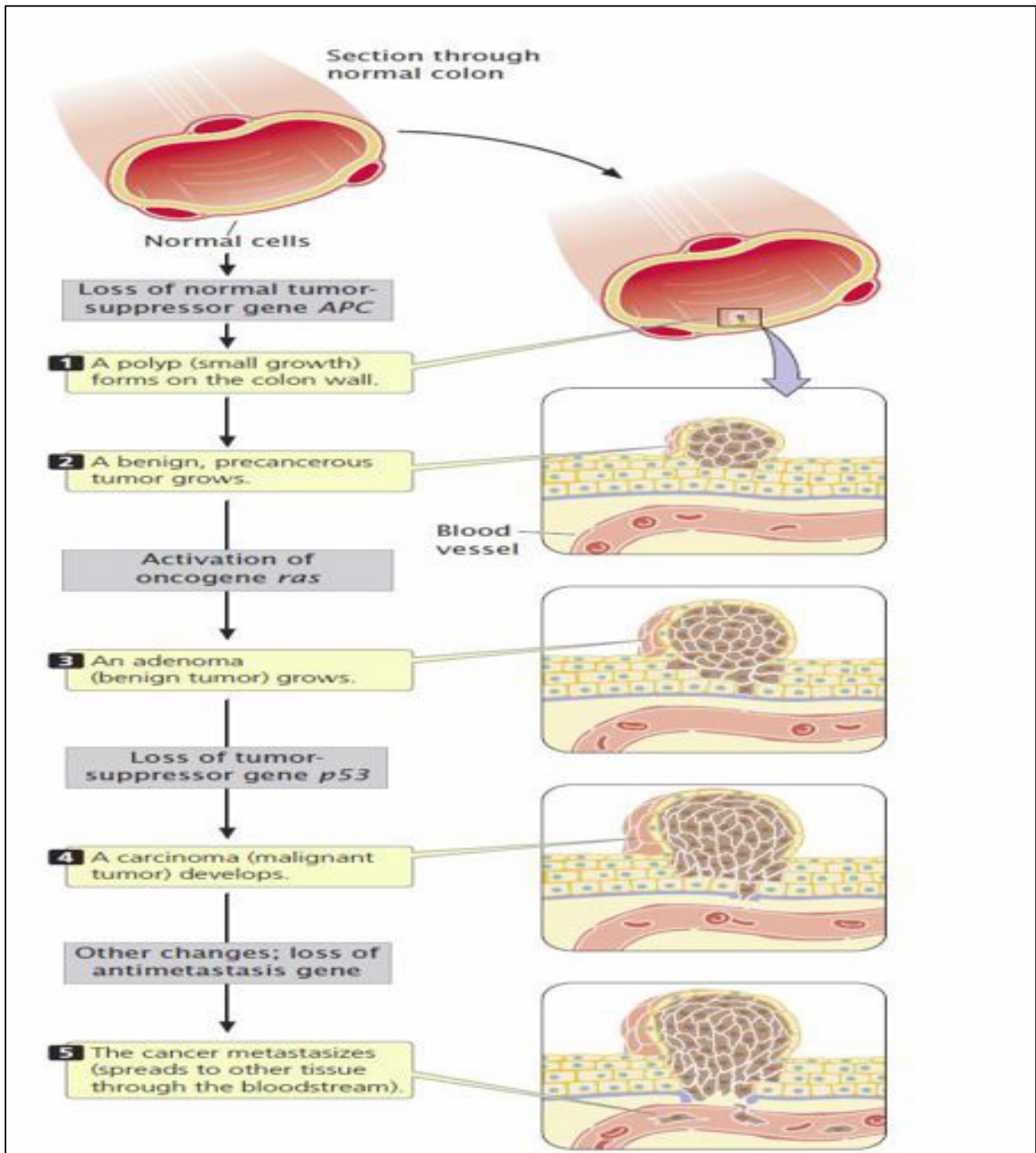


**Figure 1.2: The origin and progression of colorectal cancer.**

*CRC starts in the epithelial cells of the colon as small polyps which grow to form adenoma. The progression of genetic and epigenetic abnormality in the adenoma cells leads to formation of a carcinoma that invades other tissues. This figure was taken from: Pierce, B.A., Genetics: A Conceptual Approach 2011: W H Freeman & Company.*

## 1.5. Pathological staging of CRC

Determining the size and the spread of the tumour within the large bowel is called staging which is very important for CRC treatment plan (adjuvant treatment). The staging of CRC is performed histologically post surgery. The most commonly used classification of CRC stages in the UK is Duke's staging system (Bull et al., 1997). However tumour, node, metastasis staging system (TNM) is now being used besides Dukes' staging system. TNM staging system is more accurate than Dukes' staging system, but it has many subgroups which make it less useful clinically. Table 1.1 summarizes the Duke's stages and the equivalent TNM stages used in the UK (Hall, 2011). Figure 1.3 shows a cartoon of Dukes' stages and the corresponding TNM stages for CRC.

| <b>Table 1.1: Dukes' and TNM staging for colorectal cancer</b>                                           |                                                                                  |
|----------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| <b><i>Duke's stage<br/>(equivalent TNM stage)</i></b>                                                    | <b><i>Description</i></b>                                                        |
| <b>A (T1 and T2)</b>                                                                                     | <b>Localised to mucosa and sub mucosa</b>                                        |
| <b>B (T3 and T4)</b>                                                                                     | <b>Extending into or through muscle layer<br/>without lymph node involvement</b> |
| <b>C (N0-N2)</b>                                                                                         | <b>Lymph node involvement</b>                                                    |
| <b>D (M0-M1)</b>                                                                                         | <b>Distant metastases</b>                                                        |
| Adapted from: Hall, N., Colorectal cancer: features and investigation. Medicine, 2011. 39(5): p. 250-253 |                                                                                  |

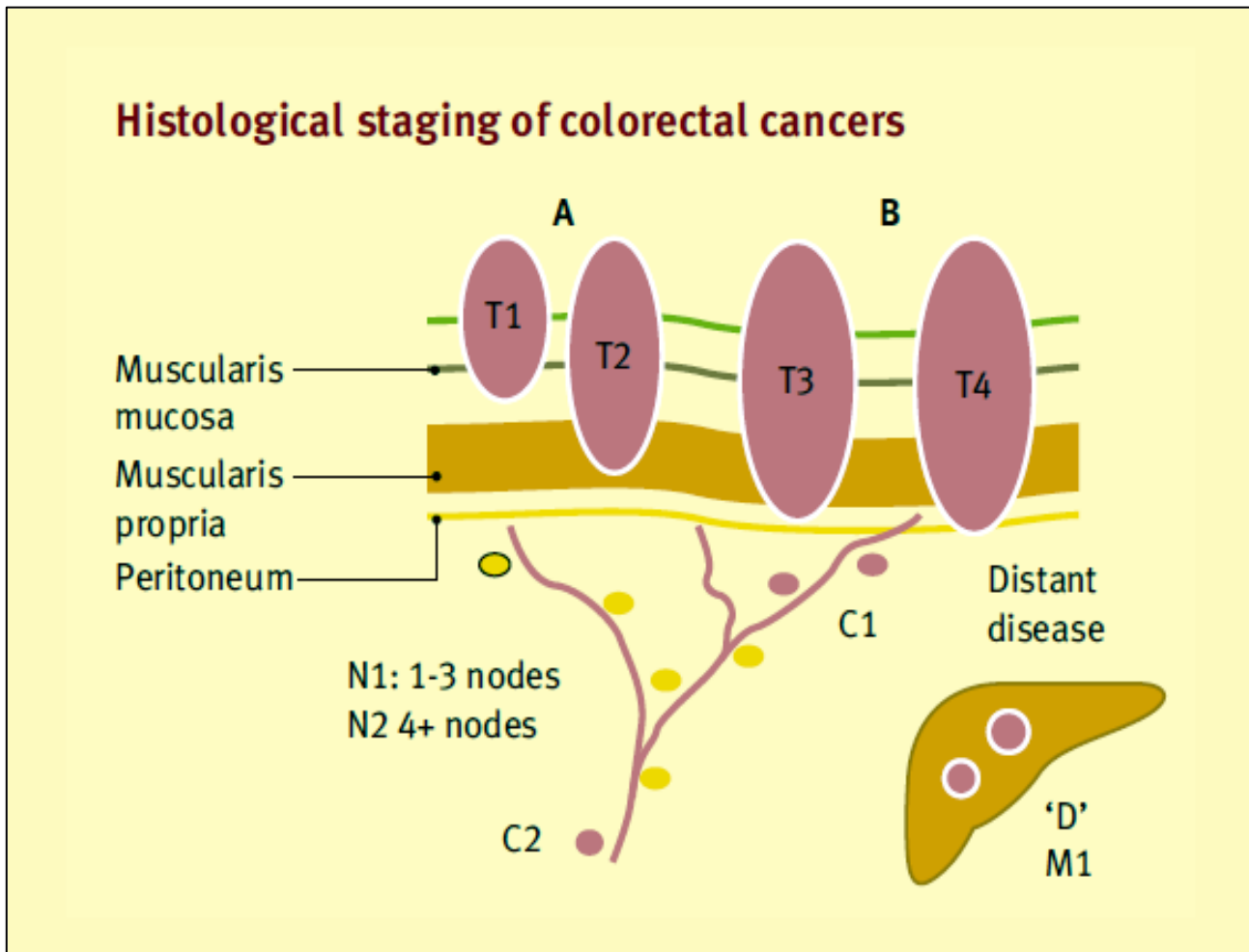


**Figure 1.3: A cartoon illustrates Duke's and TNM staging systems.**

*Duke's staging system has four stages from A to D, whereas TNM staging system has three main stages (T, N, and M), in which each stage has subgroups (T1-T4, N0-N2, M0-M1). Duke's A stage equals T1 and T2, the tumour does not pass beyond muscularis propria. Duke's B tumour matches T3 and T4 wherein the tumour penetrates the muscle layer and it could reach the peritoneum or adjacent organs as in T4 of TNM system. Duke's C stage parallels N stage of TNM system in which the tumour involves the lymph nodes. N1 refers to 1 to 3 lymph nodes having the tumour, whereas N2 denotes that 4 or more lymph nodes invaded by the tumour. Finally, Duke's D stage is M stage in TNM system. The tumour in this stage transfers to the distant organs such as the liver, ovaries, and the lungs. The figure was taken from the reference (Hall, 2011).*

## 1.6. Pathogenesis of CRC

Recent research has shown that CRC is a heterogeneous disease, and there are mainly four molecular pathways consistent with the adenoma /carcinoma progression sequence. Genetic and epigenetic alterations are implicated in these pathways which are (Harrison and Benziger, 2011):

1. Chromosomal Instability (CIN) pathway.
2. CpG Island Methylator Phenotype (CIMP) pathway.
3. Microsatellite Instability (MSI) pathway.
4. Serrated pathway.

### 1- Chromosomal Instability (CIN) pathway

It is the most common pathway in sporadic CRC as it exists in 70-85% of sporadic CRC cases. It is, therefore, known as the canonical pathway of CRC. Its initiation involves mutations in tumour suppressor genes, hence it is also called the suppressor pathway (Worthley et al., 2007). All CRC cases which fall under this pathway have loss of heterozygosity (LOH) in adenomatous polyposis coli (*APC*) gene in the early stage. *APC* is a tumour suppressor gene operating in the Wnt signalling pathway, and it plays a crucial role in  $\beta$ -catenin degradation (Kennell and Cadigan, 2009). When *APC* is mutated, increased levels of  $\beta$ -catenin accumulate in the cytoplasm and translocate to the nucleus (Sena et al., 2006) to promote transcription of genes involved in cell proliferation and survival which starts the development of adenomas in the colon or rectum (Phelps et al., 2009). Alongside

the progression of the tumour according to adenoma/carcinoma model, LOH in other genes such as *DCC*, *KRAS*, *SMAD2*, *SMAD4*, and *TP53* occurs in a sequential progression consistent with the transformation to the metastasis state (Rowan et al., 2005) as shown in figure 1.4. These changes lead to aneuploidy and structural alterations to chromosomes, for example a deletion in the long arm of chromosome 5 (5q), and a deletion in chromosome 18 or 17 (18q or 17q). These alterations together result in chromosomal instability (CIN) which characterizes this pathway (Grady, 2004).

## **2- CpG Island Methylator Phenotype (CIMP) pathway**

CIMP pathway is ranked the second most common pathway after CIN pathway as it is found in 15% of sporadic CRC cases (Worthley and Leggett, 2010). It represents the most epigenetic alterations in sporadic CRC through hypermethylation of promoters of tumour suppressor and DNA repair genes accompanied by hypomethylation of global DNA (Jass, 2007). These epigenetic alterations of methylation status in colon epithelial cells lead to the silencing of tumour suppressor genes. It can also promote the transformation of these cells to adenoma which, in turn, becomes carcinoma according to Fearon and Vogelstein model (Kim et al., 2010). Sporadic CRC cases having these alterations are called CIMP positive (CIMP+), and they are classified into: CIMP low (or CIMP-2) and CIMP high (or CIMP-1) (Shen et al., 2007). CIMP-1 tumours often associate with microsatellite instability and *BRAF* mutations, whereas CIMP-2 tumours often are characterised by a high rate of *K-RAS* mutations, rare micro-satellite instability, and

less *BRAF* and *TP53* mutations (Coppede, 2011). The difference between CIMP-1 and CIMP-2 reflects on the tumour's localization, prognosis, and therapy (Issa, 2008), figure 1.5.

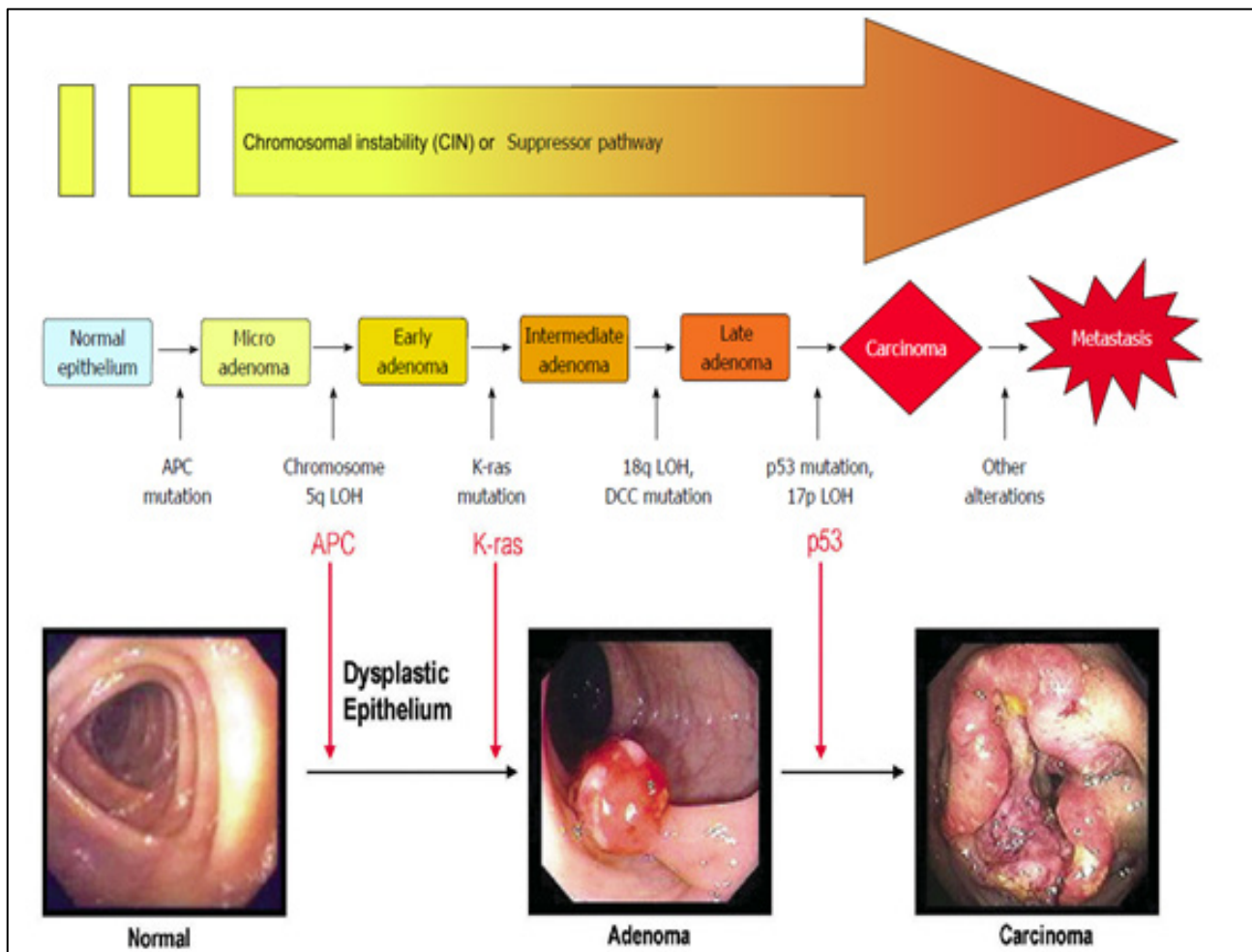


**Figure 1.4: The suppressor pathway in CRC.**

*The sequential progression of CRC pathological stages is accompanied by molecular alterations of crucial genes involved in the cell cycle control, DNA repair, cells adhesion, and chromosome segregation. Mutations and loss of heterozygosity (LOH) form the most common genetic alteration in this pathway. APC and K-RAS mutations occur in the early stage of this pathway; however P53 mutations take place in the late stage. Adapted from the references (Moran et al., 2010, Steele, 2006).*

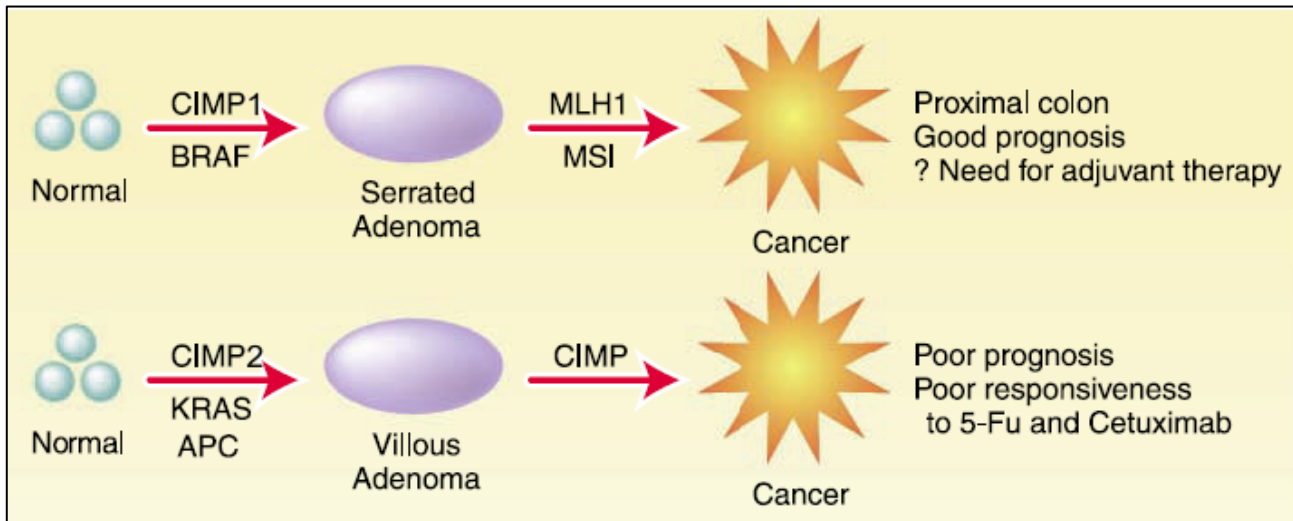


**Figure 1.5: CIMP sub-pathways of sporadic CRC.**

*CpG Island Methylator Phenotype (CIMP) pathway arises in two different ways: CIMP-1 (or CIMP high) and CIMP-2 (or CIMP low), each of them has its own clinical implications on the prognosis and the treatment of the tumour. CIMP-1 is characterised by serrated adenomas located in the proximal colon, has mostly BRAF mutations, and associates with good prognosis. However, CIMP-2 is characterised by villous adenomas, has K-RAS, APC and TP53 mutations, and shows the worse prognosis (it has poor response to chemotherapy). The figure is adapted from reference (Issa, 2008).*

### **3- Microsatellite Instability (MSI) pathway**

Microsatellites are one type of DNA repetitive sequences. They consist of 1-5 nucleotides in length occurring in tandem repeats, and hence are also called short tandem repeats. These repeats spread throughout the human genome, including the introns and

coding exons, in a unique pattern characterising each individual from another (Booth, 2007). Microsatellite instability (MSI) occurs when the Mismatch Repair (MMR) system is impaired.

MMR genes are responsible for checking and correcting the errors made by DNA polymerases during the DNA replication step. When MMR is not functional, a loss or gain happens in DNA sequence including microsatellite repeats, and it leads to MSI (2012). MMR system consists of at least seven different genes (*MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, *PMS1* and *PMS2*) that encode proteins acting together in heterodimeric form which detects and repairs mismatch errors during DNA replication (Poulogiannis et al., 2010). In familial (hereditary) CRC, the inactivation of MMR genes by germ-line mutations in one or more of the MMR genes results in microsatellite instability. However, the micro-satellite instability in sporadic CRC results from promoter hypermethylation of MMR genes (Armaghany et al., 2012), although some CRCs with intact MMR function develop MSI due to frameshift mutations at microsatellites in other genes (2012). Based on a standardized marker panel of the five mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and three dinucleotides (D2S123, D5S346, D17S250), which were chosen by a National Cancer Institute consensus conference, MSI were divided into three categories depending on the degree of the presence of unstable loci among the markers which have a high sensitivity and specificity (Bacher JW et al., 2004). These categories are (Imai and Yamamoto, 2008b):

- a) High-frequency MSI (MSI-H): the tumour has  $\geq 30$ -40% of the marker panel mutated.

- b) Low- frequency MSI (MSI-L): the tumour has < 30-40% of at least 1 of the marker panel mutated.
- c) Microsatellite stable (MSS): the tumour does not show any instability in the marker panel.

Clinically and in contrast to CIN pathway which has abnormal chromosome number, MSI pathway has a normal karyotype with unique molecular genetic changes (Walther et al., 2009). In addition, MSI CRC tumours show a better prognosis than the negative MSI tumours (Boland and Goel, 2010). Figure 1.6 shows MSI pathway development.

#### **4- The Serrated pathway**

This pathway has been recently considered as a distinct pathway in sporadic CRC carcinogenesis (Leggett and Whitehall, 2010). In the past, it was thought there are two types of polyps in CRC-adenomas and hyperplastic polyps. The latter were accounted as benign without malignant potential. However, it is now proved that these hyperplastic polyps actually are heterogeneous polyps classified now as traditional serrated adenomas (TSA), sessile serrated adenomas (SSA) and true hyperplastic polyps (Farris et al., 2008). True hyperplastic polyps form about 80–90% of all serrated polyps and mostly are innocuous lesions (Higuchi et al., 2005). In contrast, TSA and SSA together represent around 0.5 to 1.3% of CRC polyps, and have a great risk to become malignant (Patel et al., 2012).

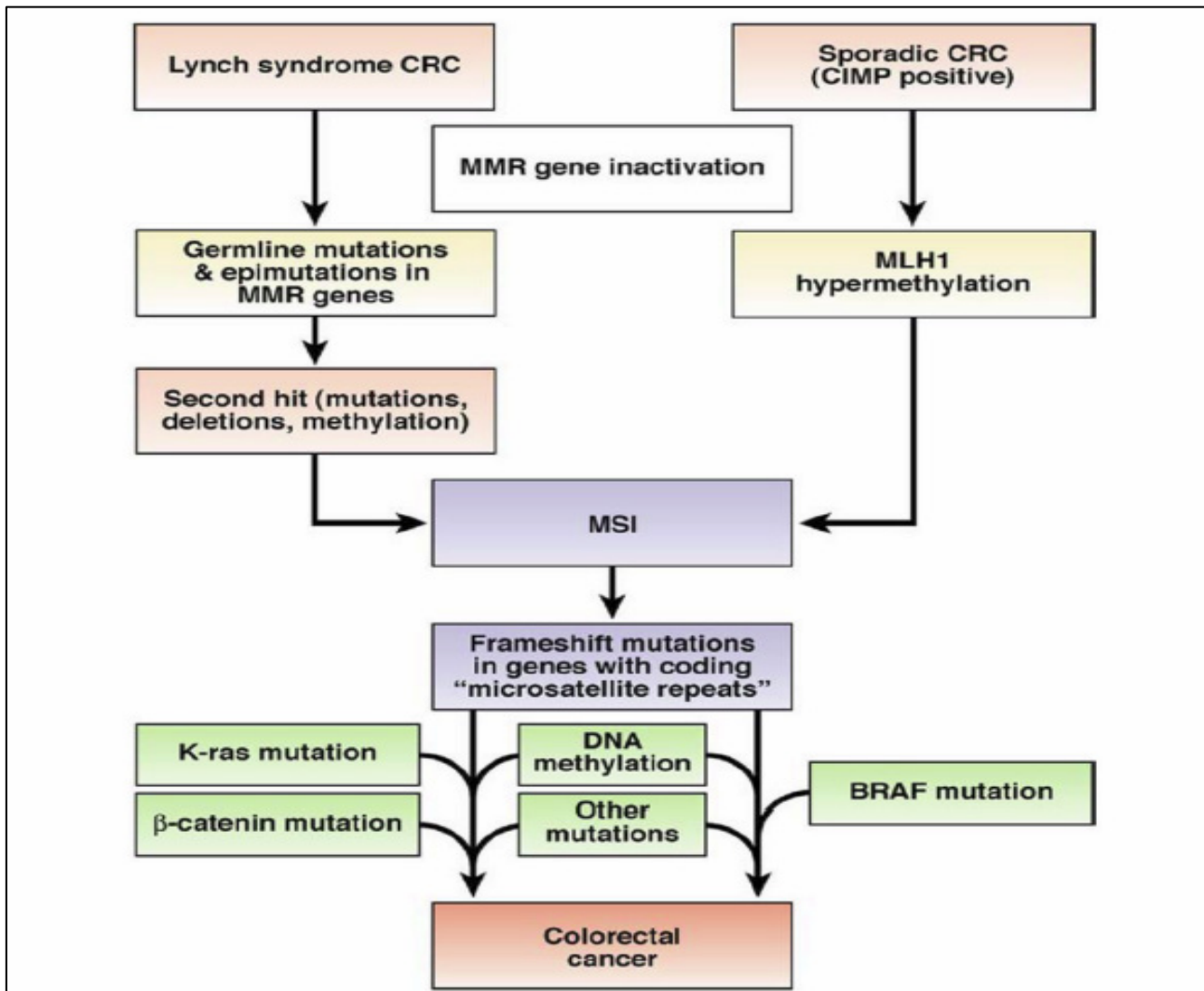


**Figure 1.6: MSI pathway development.**

*MSI arises as a result of the inactivation of MMR system, which happens in two different ways. 1) Lynch syndrome: individuals have a germ-line mutation in one of the MMR genes. When a second mutation hits the wild-type copy (functional) via LOH, methylation, or point mutation, MMR system becomes defective. This results in MSI and rapid accumulation of somatic mutations, for example KRAS and β-catenin. 2) Sporadic methylation-induced silencing of MLH1 which is one gene of MMR system, and its inactivation in this situation is not inherited, but it involves the presence of CIMP. Methylated promoter of MLH1 leads to the MMR activity failure and MSI development. BRAF mutations are observed in most CIMP and MSI tumours, but do not exist in tumours of patients having Lynch syndrome. The figure is taken from reference (Boland and Goel, 2010).*

Morphologically, TSA has a homogeneous population of abnormal cells, whereas SSA does not (Torlakovic et al., 2008). This difference also reflects on the molecular level as most serrated adenocarcinomas have microsatellite stable (MSS) or low-level microsatellite instability (MSI-L), and they arise from TSA. On the other hand, 15-20% of serrated adenocarcinomas are MSI-H and they arise from SSA polyps (O'Brien et al., 2006). In addition, *KRAS* mutations are present in 80% of TSA lesions, but they are rare in SSA (Harvey and Ruszkiewicz, 2007). In contrast, SSA polyps show a high ratio of *BRAF* mutations which are rare in TSA (Sandmeier et al., 2009). Both oncogenes (*BRAF* and *KRAS*) are key components of the ERK/MAPK pathway, and their activation through the normal pathway sequence or through a point mutation in each of them leads to inhibition of apoptosis, cell proliferation, survival (Leggett and Whitehall, 2010), and trigger malignant change in intact colorectal tissue (Young et al., 2007), as shown in figure 1.7.

Furthermore, TSA and SSA differ in the location of incidence. TSA usually occurs in the left colon. However, SSA is found in the right colon like enlarged folds (Noffsinger, 2009). Figure 1.8 summaries the difference between TSA and SSA pathways.

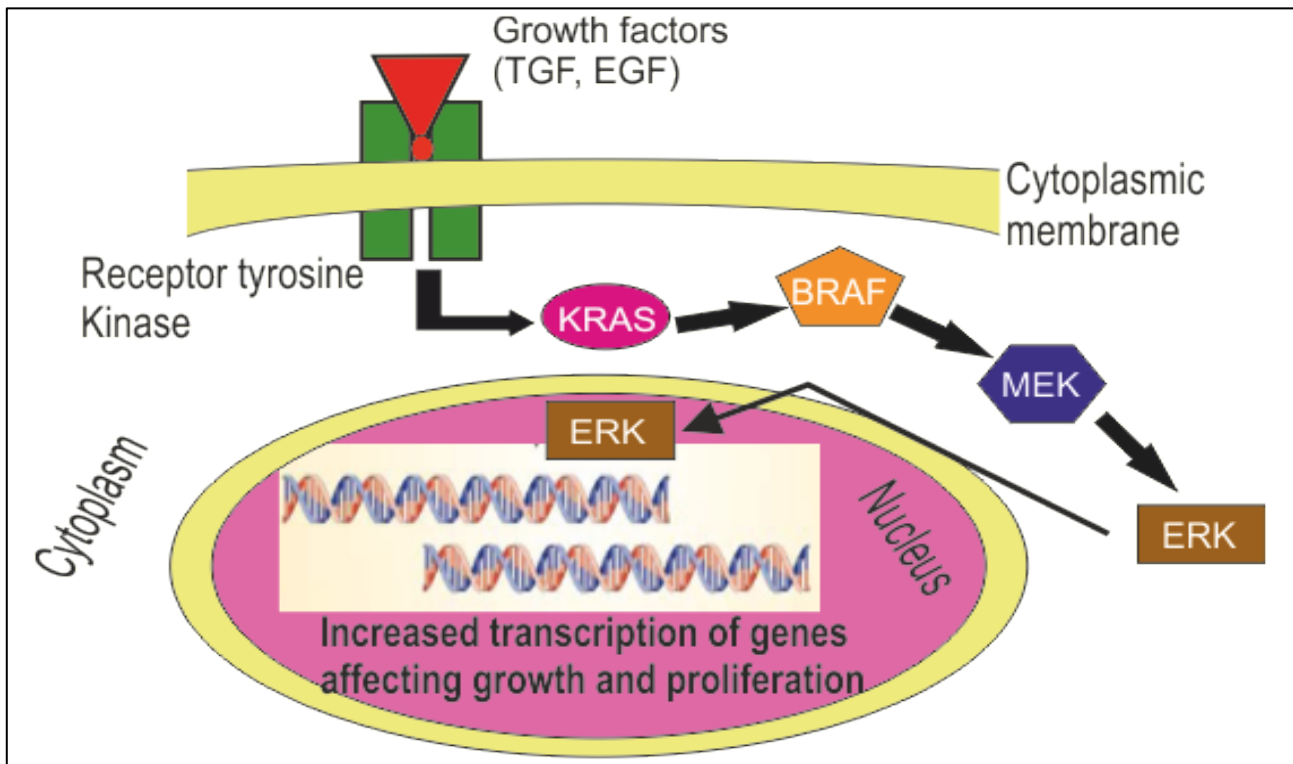


**Figure 1.7: KRAS/BRAF pathway and CRC.**

*Both KRAS and BRAF proteins participate in the transduction of intracellular signals via the mitogen-activated protein kinase (MAPK) signalling. The final activated kinase in this pathway, extracellular signal-regulated kinase (ERK), translocates to the nucleus and induces transcription genes that regulate cell growth and proliferation. KRAS and BRAF genes are preferentially mutated in most CRC pathways. The figure was modified from reference (Leggett and Whitehall, 2010).*

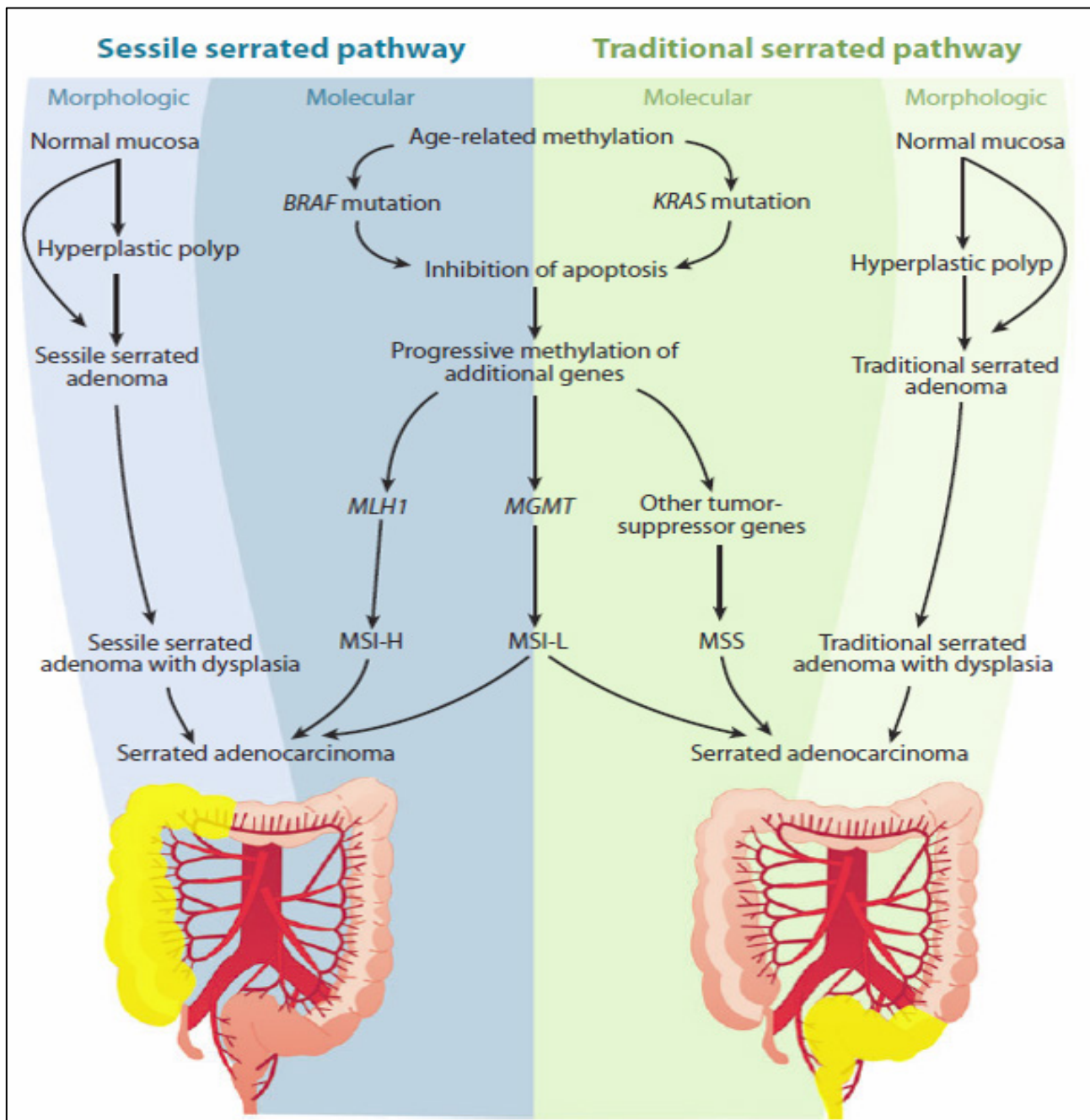


**Figure 1.8: SSA versus TSA pathway in sporadic CRC.**

*This illustrative figure shows the differences between the two pathways (SSA and TSA) of serrated adenocarcinoma. The green area represents the features of the traditional serrated pathway: morphologic (light shade) and molecular (dark shade), whereas the blue area represents the features of the sessile serrated pathway. The yellow colour depicts the most common colon area affected in each pathway. The figure was taken from reference (Noffsinger, 2009).*

## 1.7. Human p53 and CRC

As mentioned above, the chromosomal instability pathway is characterized by mutations in tumour suppressor genes in which *TP53* is mutated in more than 50% of CRC cases. Mutations in *TP53* gene occur in the late stages of CRC (Einspahr JG et al., 2006). Here, I am giving a brief background about p53, and then I will talk about its mutations in CRC.

### • General background

Human p53 protein was discovered in 1979 by Crawford and his colleagues while they were working on Simian Virus 40-transformed cells (the p53 web site, [http://p53.free.fr/p53\\_info/p53\\_disco.html](http://p53.free.fr/p53_info/p53_disco.html)). First, p53 was considered a tumour antigen with transforming properties because it interacts with the viral SV40 T-antigen (Lane and Crawford, 1979). However, it has been revealed later that p53 is actually a tumour suppressor and mutant p53 induce cancer (Weisz et al., 2007). The discovery of p53 has opened the door to many ongoing studies about the structure and functionality of p53 in the cell (Braithwaite et al., 2005, Momand et al., 1992, Feng Z et al., 2007). Even more than 30 years after p53 discovery, there is much to learn about it. The *TP53* website (<http://p53.free.fr/index.html>) database contains useful and huge amount of scientific work on p53.

p53 is a 393 amino acids protein encoded by *TP53* gene which is located in 17p13.1 (OMIM, <http://omim.org/entry/191170>). This protein structurally consists of 5 main domains (Joerger and Fersht, 2010), as shown in figure 1.9.

- 1- The transactivation domain starts from the amino terminal. This region plays a role in activation and stabilization of p53. It also contains the mdm2 protein binding site (the p53 web site, [http://p53.free.fr/p53\\_info/p53\\_Protein.html](http://p53.free.fr/p53_info/p53_Protein.html)).
- 2- Prolin rich cassette region which is conserved in the majority of p53 and is essential for p53 apoptotic function (the p53 web site, [http://p53.free.fr/p53\\_info/p53\\_Protein.html](http://p53.free.fr/p53_info/p53_Protein.html)).
- 3- DNA binding domain in the central region of p53. This domain is the target of 90% of p53 mutations (the p53 web site, [http://p53.free.fr/p53\\_info/p53\\_Protein.html](http://p53.free.fr/p53_info/p53_Protein.html)).
- 4- The oligomerization or tetramerization domain which is necessary for dimerization as p53 works as a dimer of two dimers which each of them consists of two p53 molecule (the p53 web site, [http://p53.free.fr/p53\\_info/p53\\_Protein.html](http://p53.free.fr/p53_info/p53_Protein.html)).
- 5- The carboxy-terminus of p53 contains three nuclear localization signals (NLS) and a non-specific DNA binding domain (it bind to damaged DNA). This region plays a role in downregulation of DNA binding of the central domain (the p53 web site, [http://p53.free.fr/p53\\_info/p53\\_Protein.html](http://p53.free.fr/p53_info/p53_Protein.html)).

p53 is active biologically as a homotetramer, especially to perform its function as a transcription factor (Weinberg et al., 2004). p53 binds to its double- stranded DNA target in a sequence-specific manner which consists of two motifs separated by 0–13 base pairs (Wei CL et al., 2006). Each motif (half-site) consists of 10 base pairs of the general form RRRCWWGYYY whereas (R = A, G; W = A, T; Y = C, T) (Ho et al., 2006).

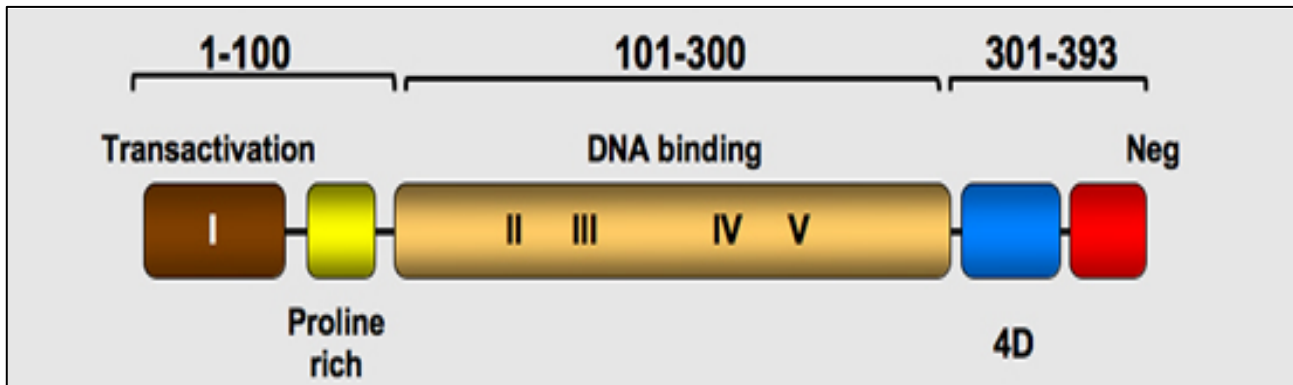


**Figure 1.9: The domains of p53.**

*Human p53 protein consists of five domains, each has specific functions.*

- 1) The amino-terminus region begins from 1-42 amino acid. This region contains the acidic transactivation domain and the mdm2 protein binding site. It also includes the Highly Conserved Domain I (HCD I)*
- 2) Proline rich domain starts from 40-92 amino acids. The proline residues are conserved in the majority of p53.*
- 3) The DNA binding domain expands from 101-306. It contains HCD II to V. Almost 90% of p53 mutations occur in this region.*
- 4) The oligomerization domain starts from 307-355 (4D in the figure). It is necessary for dimerization of p53, and also contains a nuclear export signal (NES).*
- 5) The carboxy-terminus of p53 from 356-393. It contains 3 nuclear localization signals (NLS) and a non-specific DNA binding domain. This region is also involved in down regulation of DNA binding of the central domain. The figure was taken from reference (the p53 web site, [http://p53.free.fr/p53\\_info/p53\\_Protein.html](http://p53.free.fr/p53_info/p53_Protein.html)).*

p53 is a member of a proteins family that includes p63 and p73 (Wei et al., 2012). However, p53 differs from p63 and p73 in structure and function (Dötsch V et al., 2010). p53 lacks a sterile alpha motif (SAM) domain which forms an additional extension for the tetramerization domain in p63 and p73 (Lu et al., 2009), as shown in figure 1.10. In addition, specific amino-acid substitutions are found different within the canonical tetramerization domain motif (Coutandin et al., 2009). This may explain why p53 forms a homotetramer and does not interact with tetramerization domain of p63 and p73 which form mixed tetramers in vitro (Joerger et al., 2009).

p63 and p73 are a tissue-specific and play essential roles in normal development (Moll and Slade, 2004). It has been shown that mice deficient for either of p63 or p73 genes are embryonic lethal (Yang A et al., 1999, Yang A et al., 2000), whereas mice deficient for p53 are viable but have a tendency to develop tumours (Berrigan et al., 2002). However, in some circumstances p63 and p73 can function as tumour suppressors (Finlan and Hupp, 2007, Rosenbluth and Pieterpol, 2008). This means that in response to DNA damage, both proteins p63 and p73 can bind DNA, transactivate p53-responsive genes, and mediate cell cycle arrest cellular senescence, and apoptosis (Keyes et al., 2005, Melino G et al., 2004).

In normal tissues, wild type p53 is often maintained at low levels by its negative regulators mdm2 (also known as hDM2) and mdm4 (also known as mdmx) (Iwakuma and Lozano, 2003). While Mdm2 is E3 ubiquitin ligase which tags p53 with ubiquitin promoting its degradation through the ubiquitin-dependent proteasome pathway

(Marine JC et al., 2006, Toledo and Wahl, 2006), mdmx per se is not E3 ligase (Finch et al., 2002). Nonetheless, Mdmx binds to p53 DNA binding domain and inhibits the role of p53 in inducing cell cycle arrest and apoptosis (Jin et al., 2006).

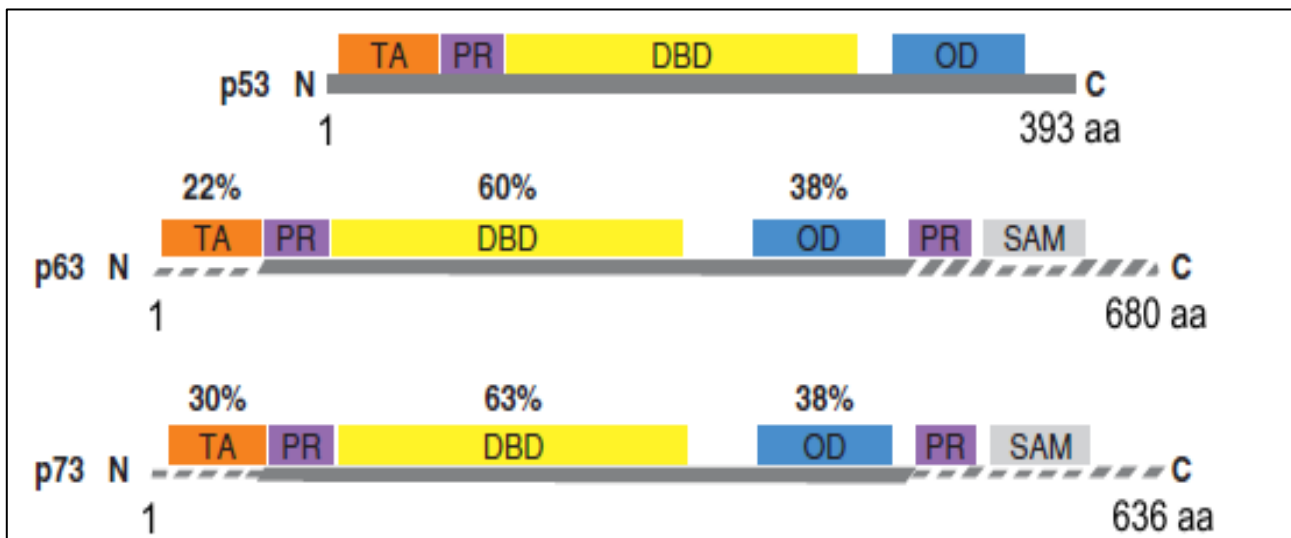


**Figure 1.10: Schematic representation of the protein structure of the p53 family members.**

*p63 and p73 contain a sterile alpha motif (SAM), a putative protein–protein interaction domain found in many signalling proteins and transcription factors. Identity shared by p73 and p63 with p53 is indicated by percentage (%). The three proteins consist of an amino-terminal transactivation domain (TAD), a central DNA binding domain (DBD) and a carboxy-terminal oligomerization domain (OD). PR denotes Proline rich sequence, aa denotes amino acids. The figure was reshaped from reference (Dötsch V et al., 2010).*

Upon various cellular stresses, including oncogenes activation and DNA damage, naturally low level of p53 in the cell markedly increase and translocate nucleus to regulate (activate or repress) the transcription of many genes involved in growth arrest, apoptosis, DNA repair and differentiation in damaged cells (Oren, 2003).

p53 function and regulation are controlled by post-translational modifications which are a part of signalling processes to direct p53 to perform a specific task according to the inducing stimulus (Hoeller et al., 2006). More than 50 individual post-translational modifications modify p53 function and regulation, and these modifications vary in type, position, and function (Meek and Anderson, 2009). The known post-translational modifications in p53 are phosphorylation, poly-ribosylation, glycosylation, acetylation, methylation, ubiquitination, sumoylation, and neddylation (Gu and Zhu, 2012). Figure 1.11 summarises these modifications with their functions and sites of action. The paradigm for these modifications is phosphorylation of p53 at amino-terminal in specific residues including Ser15, Thr18, and Ser20 blocks MDM2–p53 binding and leads to decrease of p53 degradation (p53 stabilization) (Shieh SY et al., 1997, Schon O et al., 2002, Dumaz et al., 2001). Moreover, it has been shown that the phosphorylation of p53 at these residues induces p53 association with p300/CBP and stimulates p53 transactivation function (Lambert et al., 1998, Dornan et al., 2003, Finlan and Hupp, 2004). Such these proteins which modulate p53 transactivation at specific promoters are called p53 co-factors. They are divided to co-activators (which enhance p53 transactivation function) and co-repressors (which repress p53 transactivation function) (Vousden and Prives, 2009,

Espinosa, 2008). Most of these co-factors are histone-modifying enzymes, for example, histone acetyltransferases (p300/CBP and PCAF), histone deacetylases (HDAC1), histone methyltransferase (PRMT1 and CARM1) (An et al., 2004), and other co-activator complexes such as NuA4–Tip60 and SAGA (Ard et al., 2002, Berger, 2002). However, there are a number of proteins that selectively alter the function of p53. These proteins are called p53- binding partners (Naumovski and Cleary, 1996, Iwabuchi et al., 1994). p53-binding partners work either by changing the p53 affinity to a specific subset of response elements in p53 target genes, or by affecting the ability of p53 to recruit transcriptional coactivators at specific loci (Beckerman and Prives, 2010). Figure 1.12 shows p53-binding partners, their interaction sites on p53, and their functions. One well-known example of p53-binding partner is the three members of the apoptosis-stimulating of p53 protein (ASPP) family of proteins, ASPP1, ASPP2 and iASPP (inhibitory member of the ASPP family) (Samuels-Lev Y et al., 2001, Bergamaschi D et al., 2003, Notari M et al., 2011). While ASPP1 and ASPP2 bind to p53 DNA binding domain to direct the cell toward an apoptosis by inducing transactivation of bax and puma but not p21 (Patel S et al., 2008), iASPP protein binds to p53 and repress the transactivation of pro-apoptotic genes (Bergamaschi et al., 2006). Interaction of p53 with its binding partners is affected by p53 post-translational modifications. For example, phosphorylation p53 at S46 triggers the binding of prolyl-isomerase Pin1 (p53-binding partner) with the p53 transactivation domain. This important interaction promotes the release of iASPP from p53 allowing the apoptosis induction upon DNA damage (Mantovani F et al., 2007).

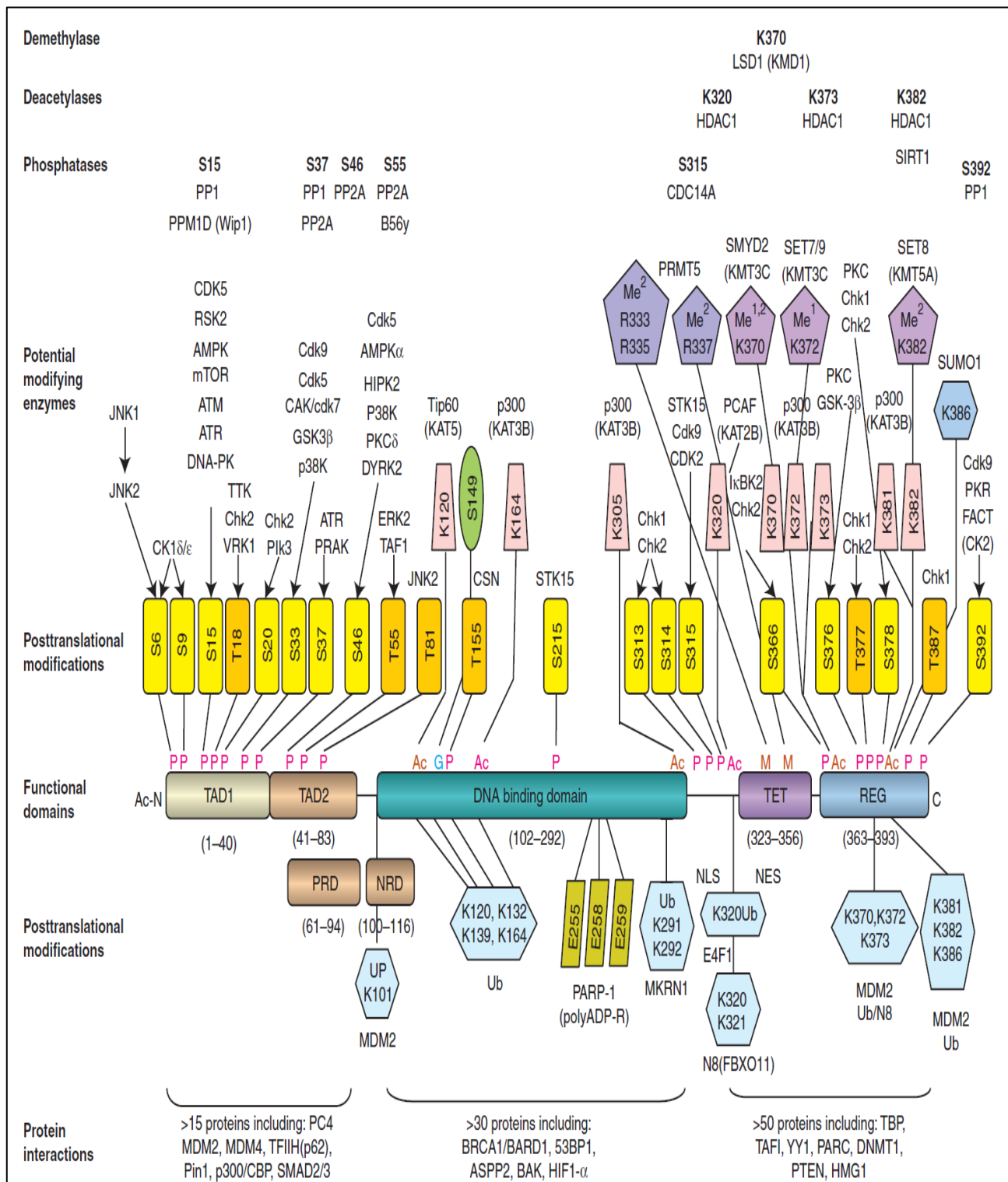


**Figure 1.11: Human p53 post-translational modifications.**

The figure shows the functional domains of p53 protein with the post-translational modifications in each domain and their effects. Also, the responsible modifying and demodifying enzymes for these modifications are shown. The figure was taken from reference (Meek and Anderson, 2009).

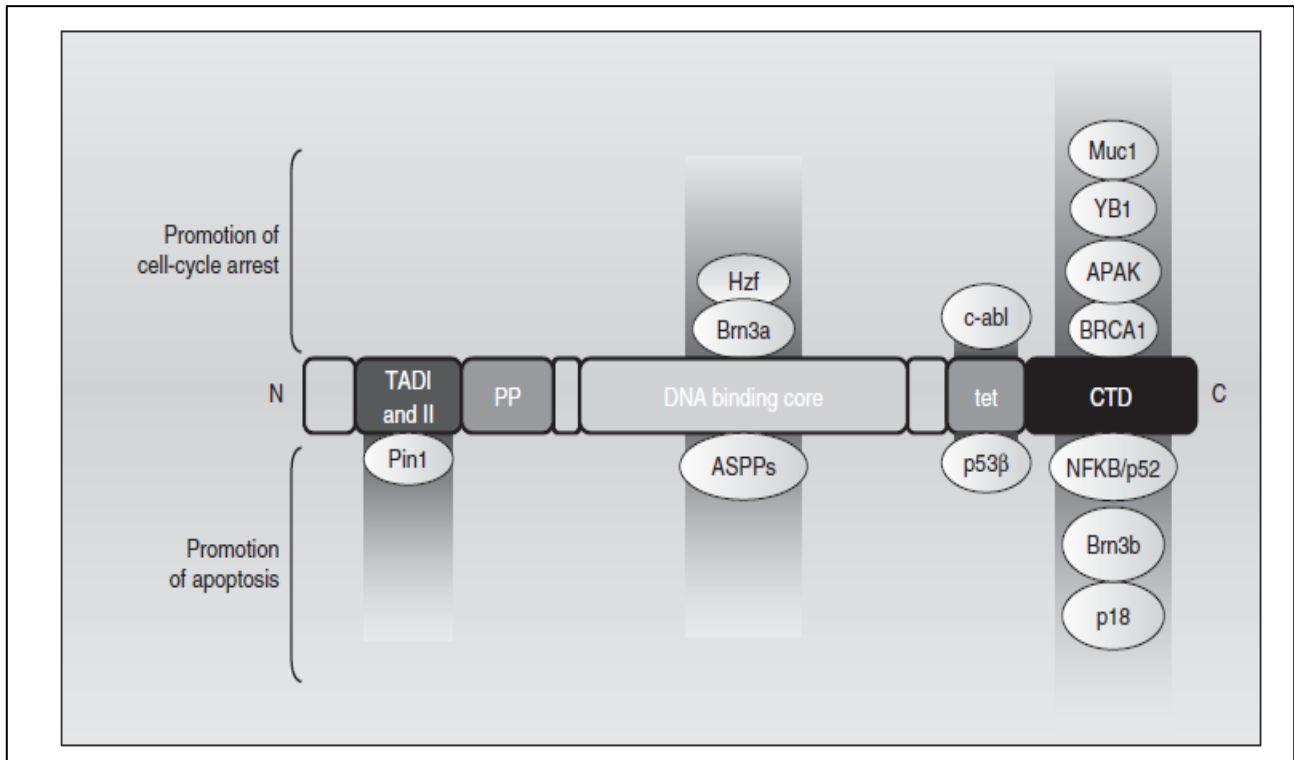


**Figure 1.12: p53-binding partners.**

*p53 function is affected by its binding to different proteins called p53-binding partners. These proteins have a specificity binding to p53 domains to induce differential transactivation of target genes and outcome as shown in the figure. For example, Muc1, YB1, APAK, BRCA, Hzf, Brn3a, and c-abl induce transcription of genes involved in cell cycle arrest, whereas ASPP1, ASPP2, JMY, Pin1, NFκB/p52, Brn3b, and p53b induce transcription of apoptotic genes. The figure was taken from reference (Beckerman and Prives, 2010).*

## • p53 in CRC

The function of p53 is almost compromised in many human cancers, usually because of somatic mutations which take place during tumorigenesis (Vogelstein et al., 2000). The rates of reported *TP53* mutations vary between cancer types. For example, *TP53* mutations range around 10% in haematopoietic cancer (Peller and Rotter, 2003) to 50 - 70% in colorectal (Iacopetta, 2003), ovarian (Schuijjer and Berns, 2003) and head and neck (Blons and Laurent-Puig, 2003). Usually, germline *TP53* mutations are rare (Ginsburg et al., 2009) and lead to predisposition to cancer type known as Li - Fraumeni Syndrome (LFS) (Malkin D et al., 1990, Dickson, 2012). However, somatic *TP53* mutations are found in the vast majority of sporadic cancers (Olivier M et al., 2010). All *TP53* mutations can be classified as DNA contact (the mutations occur in the DNA binding domain and do not affect p53 structure) and structural mutations (the mutations usually occur in the p53 tetramerization domain and lead to change in p53 conformation) (Bullock and Fersht, 2001). According to their effects on function, *TP53* mutations fall in two categories, which are (Sigal and Rotter, 2000):

1. Loss of function: p53 loses its tumour suppressive function and acquire dominant-negative activities (Lubin et al., 2010).
2. Gain of function: p53 gains new oncogenic properties (Oren and Rotter, 2010).

Half of all colorectal cancer cases have *TP53* mutations (Berg et al., 2010), and this occurs mostly in the late stages of the sequential progression from adenoma to carcinoma (Rivlin et al., 2011). These mutations lead to loss of heterozygosity (LOH) in the late stages after the early deletion of chromosome 17p region containing the p53 gene which occurs frequently in CRC (Iacopetta, 2003). The mutant p53 associates with its nuclear accumulation in CRC tissues, and p53 over-expression is linked with poor prognosis in human CRC patients (Liu and Bodmer, 2006, Iacopetta, 2003, Vogtmann E et al., 2013, Nasif WA et al., 2006). According to the data published on IARC TP53 Database (<http://p53.iarc.fr/>), the majority of *TP53* mutations in CRC are point mutations in which missense mutations comprising more than 89% , as shown in figure 1.13. These missense mutations result from transitions GC to AT at cytosine phosphate guanine dinucleotides in seven hotspot codons (175, 213, 244, 245, 248, 273, and 282) (Russo A et al., 2005), figure 1.14. In addition, most of *TP53* mutations occur in exons 5 to 8, as appeared in figure 1.15. These mutations result in non-functional p53 protein as it cannot bind to DNA at responsive elements (Vogelstein et al., 2000, Russo A et al., 2005) <http://p53.iarc.fr/TP53SomaticMutations.aspx>

**Point mutation (N = 1527)**  
© IARC TP53 Database, R16, November 2012

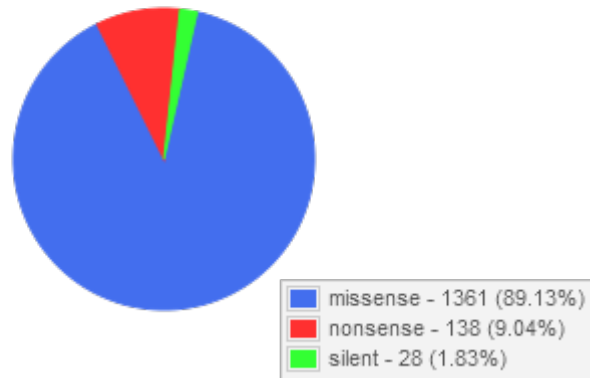


**Figure 1.13: Types of TP53 mutation in CRC.**

*The majority of mutations in TP53 gene are point mutation in CRC. More than 89% of TP53 mutations are missense and about 9% are nonsense. These mutations result in non-functional p53. Less than 2% of TP53 mutations are silent and do not mostly affect p53 functionality. The figure was taken from IARC TP53 Database, <http://p53.iarc.fr/TP53SomaticMutations.aspx>.*

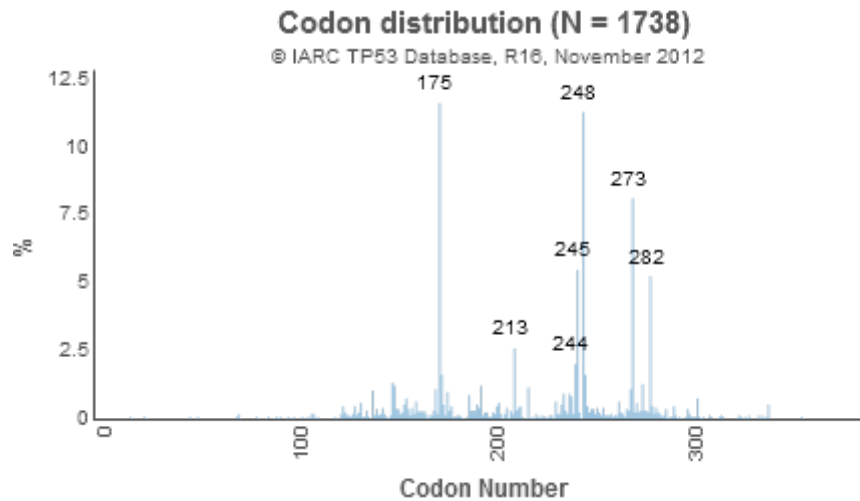


**Figure 1.14: Hotspot positions of TP53 point mutations in CRC.**

*Seven codons in DNA binding domain of TP53 gene show high rate of substitution mutations from GC to AT. These hotspot positions are 175, 213, 244, 245, 248, 273, and 282. The figure was taken from IARC TP53 Database, <http://p53.iarc.fr/TP53SomaticMutations.aspx>.*

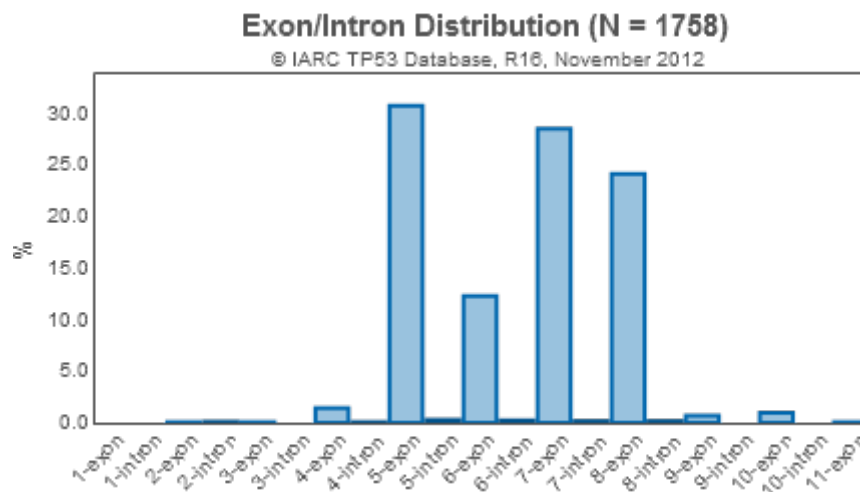


**Figure 1.15: Distribution of the point mutations within TP53 exon/intron in CRC.**

*Most TP53 point mutations fall within exon 5 to 8 region which encode DNA binding domain. These mutations result in non-functional p53 protein. The figure was taken from IARC TP53 Database, <http://p53.iarc.fr/TP53SomaticMutations.aspx>.*

## 1.8. Diagnosis of CRC

The symptoms involved in CRC do not necessarily indicate the real existence of cancer as these symptoms also occur with other colorectal problems such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), haemorrhoids, and diverticulosis and its complications (Labianca et al., 2010). Therefore, the need for accurate diagnosis is essential for confirming CRC. Generally, the time of any cancer diagnosis (including CRC) significantly impacts on the survival time for patients. The earlier the diagnosis time of CRC is, the longer survival time and the better treatment management for patients is (Steele, 2006). The available diagnosis methods are:

### ❖ Biochemical tests:

- 1- Faecal occult blood testing (FOBT): blood existence in the stool does not confirm colorectal cancer as the bleeding could be due to several reasons; also the blood test should be accompanied with a full blood count to detect anaemia. However, this test is a good, non-invasive indicator for further investigations and is widely used in CRC screening. The test used for detecting the blood in the faeces is called faecal occult blood testing (FOBT) (Lindholm et al., 2008).
- 2- Genetic stool testing: another non-invasive test involves genetic screening of the colonic cancer cells shed in the stool samples for tumour-specific mutations, particularly for the mutations in genes implicated in CRC (*KRAS*, *BRAF*, *P53*, *APC*, and *BAT26*).

Compared to analyses of DNA extracted from colon tumours, genetic stool testing gives consistent results (Calistri et al., 2003).

- 3- M2-PK Testing: this test also depends on the faecal samples to detect M2-PK enzyme which is an isomer of the pyruvate kinase (PK) enzyme. M2-PK is involved in the glycolysis pathway as it catalyzes the final step in this pathway in which phosphoenolpyruvate is converted to pyruvate. It has been demonstrated that M2-PK is found in elevated levels in proliferating gastrointestinal cancer cells and is detectable in the stool (Haug et al., 2008). Recently, this test has reached overall sensitivity of 79% and specificity of 81% for CRC detection (Li et al., 2012).

#### ❖ **Imaging tests:**

- 1- Colonoscopy: This has superseded the methods depending on barium enema (Spinzi and Minoli, 2001). Colonoscopy is considered the gold-standard investigation for polyps in colorectal track, and is the only technique that enables the identification, biopsy, and removal of premalignant polyps (in their early stages) throughout the entire colon and rectum. Moreover, Colonoscopy has the advantage of placing a tattoo at the sites of the biopsies to recognize them at subsequent laparoscopic resection (Zauber et al., 2012), however colonoscopy has risks of bleeding and perforation which are

associated with the invasive techniques (Rabeneck L et al., 2008).

2- Computed tomography (CT) scans: these are non-invasive, preoperative clinical staging techniques. Particularly, they are used for elderly patients to scan the abdomen and pelvis, and chest in order to detect distant spread after CRC has been confirmed. The most widely used CT scans are conventional computed tomography, positive emission tomography (PET), and CT Colonography (Lucidarme et al., 2012, Flanagan et al., 1998, Chaparro et al., 2009). In addition, magnetic resonance imaging (MRI) has recently come to the fore as a preferred evaluation for CRC metastases in the liver among patients who had not previously undergone therapy (Niekel et al., 2010).

The main challenge in CRC diagnosis is not related very much to the primary tumour detection, but rather to the accurate identification of the presence of lymph node invasion and/ or micro-metastatic disease which have an essential impact on patient management (Kim et al., 2006).

## **1.9.Treatment for CRC**

Treatment management for CRC is associated with the stage of the tumour and the patient's general health. The following choices are available depending on the situation.

**Surgery:** involves local removal of the tumour, and is performed for invasive carcinoma. Surgery of CRC has a risk of recurrence with or without metastasized state (Burt et al., 2010).

**Radiation:** is the local application of high energy X-rays to kill cancer cells. It is effective in specific stages of CRC development (particularly primary tumours). This therapy may be used alone or in combination with other treatments. The side effects of radiation treatment are fatigue, diarrhoea, hair loss, dental problems, and skin irritation in some patients (Cunningham et al., 2010).

**Chemotherapy:** is the use of anti-cancer drugs to kill or stop the proliferation of cancer cells. It is used before or after the surgery, hence it is called adjuvant chemotherapy. Many drugs have been approved for use in CRC chemotherapy, for example: doxorubicin, fluorouracil, irinotecan, oxaliplatin, and others (Meyerhardt and Mayer, 2005). They can be given intravenously, orally, intramuscularly, and applied directly to skin. The risk of chemotherapy drugs is drug resistance, toxicity, loss of specificity, and other side effects such as nausea, fatigue, weight loss, and hair loss (2000).

## 2. Epigenetics and Colorectal Cancer

Epigenetic changes are defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence. As discussed above in the pathways of CRC, epigenetics play a key role in CRC development (van Engeland et al., 2011). The most important epigenetic alterations in CRC are summarised below.

### •DNA Hypomethylation

This is the sharp reduction in the overall amount of 5-methylcytosine at CpG dinucleotides in repetitive sequences (Ehrlich, 2002). DNA hypo-methylation is one of the first noticeable epigenetic changes in cancer cells. It is linked to oncogene activation and chromosomal instability (CIN) in CRC (Rodriguez et al., 2006).

### •DNA Hypermethylation

This takes place in the 5' region of a gene promoter, specifically in CpG islands which are normally unmethylated and transcriptionally active (Baylin and Herman, 2000). In most cancer types, including CRC, CpG islands in the promoters of crucial genes such as tumour suppressor and DNA repair genes become hypermethylated as a result of the epigenetic alterations associated with genetic changes. Eventually, these genes suffer down regulation transcriptionally (Baylin et al., 2001). The biallelic promoter CpG island hypermethylation of the mismatch repair gene, mutL homolog1 gene, *MLH1* is one of the best known examples of CRC epigenetic alterations.

### •Loss of imprinting

When DNA hypomethylation in CRC occurs in imprinted loci, it abnormally activates them. One confirmed loss of imprinting example in CRC is the insulin-like growth factor II (*IGF2*) gene. This gene is normally silent by imprinting of the maternally inherited allele in normal cells. However, hypomethylation of the proximal promoter of *IGF2* of the imprinted allele in CRC leads to activation of this gene, and this activation (over-expression of IGF2 protein) activates the IGF1 receptor (IGFR), which autophosphorylates and activates the IRS-1/PI3K/AKT and GRB2/RAS/ERK pathways which increase cell proliferation and tumour risk (Kaneda et al., 2007).

### •Post-Translational Histone Modifications

Post-translational histone modifications play an important role in regulation of transcription within the cell (Kouzarides, 2007). They act within and between nucleosomes or recruit nonhistone proteins to change the higher-order chromatin structure. The change of the higher-order chromatin structure may be either relaxing the chromatin (transcription is active) or condensing the chromatin (transcription is deactivated) (Kouzarides, 2007). The results of post-translational modifications are determined by the amino acid type, the position in the histone tail, and the type of modification (Imhof, 2006), see table 1.2. Post-translational modifications activate or repress crucial processes in the cell such as transcription, DNA replication, and DNA repair (Sawan and Herceg, 2010). Figure 2.1 represents the nucleosome with the histones tail post-translational modifications.

**Table 1.2: different histone modifications and their effects**

| Chromatin Modifications | Residues Modified      | Functions Regulated                              |
|-------------------------|------------------------|--------------------------------------------------|
| Acetylation             | K-ac                   | Transcription, Repair, Replication, Condensation |
| Methylation (lysines)   | K-me1 K-me2<br>K-me3   | Transcription, Repair                            |
| Methylation (arginines) | R-me1 R-me2a<br>R-me2s | Transcription                                    |
| Phosphorylation         | S-ph T-ph              | Transcription, Repair, Condensation              |
| Ubiquitylation          | K-ub                   | Transcription, Repair                            |
| Sumoylation             | K-su                   | Transcription                                    |
| ADP ribosylation        | E-ar                   | Transcription                                    |
| Deimination             | R > Cit                | Transcription                                    |
| Proline Isomerization   | P-cis > P-trans        | Transcription                                    |

**The table lists histone modifications, their occurrence sites, and the associated function.**

*K: denotes lysine, ac: denotes acetylation, me: denotes methylation, me 1: denotes adding one methyl group, me 2a: denotes adding two methyl groups in an asymmetric position, me 2s: denotes adding two methyl groups in a symmetric position, Cit: denotes citrulline, S: denotes serine, T: denotes threonine, R: denotes arginine, E: denotes glutamic acid, P: donates proline, Ub: denotes ubiquitylation, Su: denotes sumoylation, and Ar: denotes ADP ribosylation. Adapted from: Kouzarides, T., Chromatin Modifications and Their Function. Cell, 2007. 128(4): p. 693-705.*

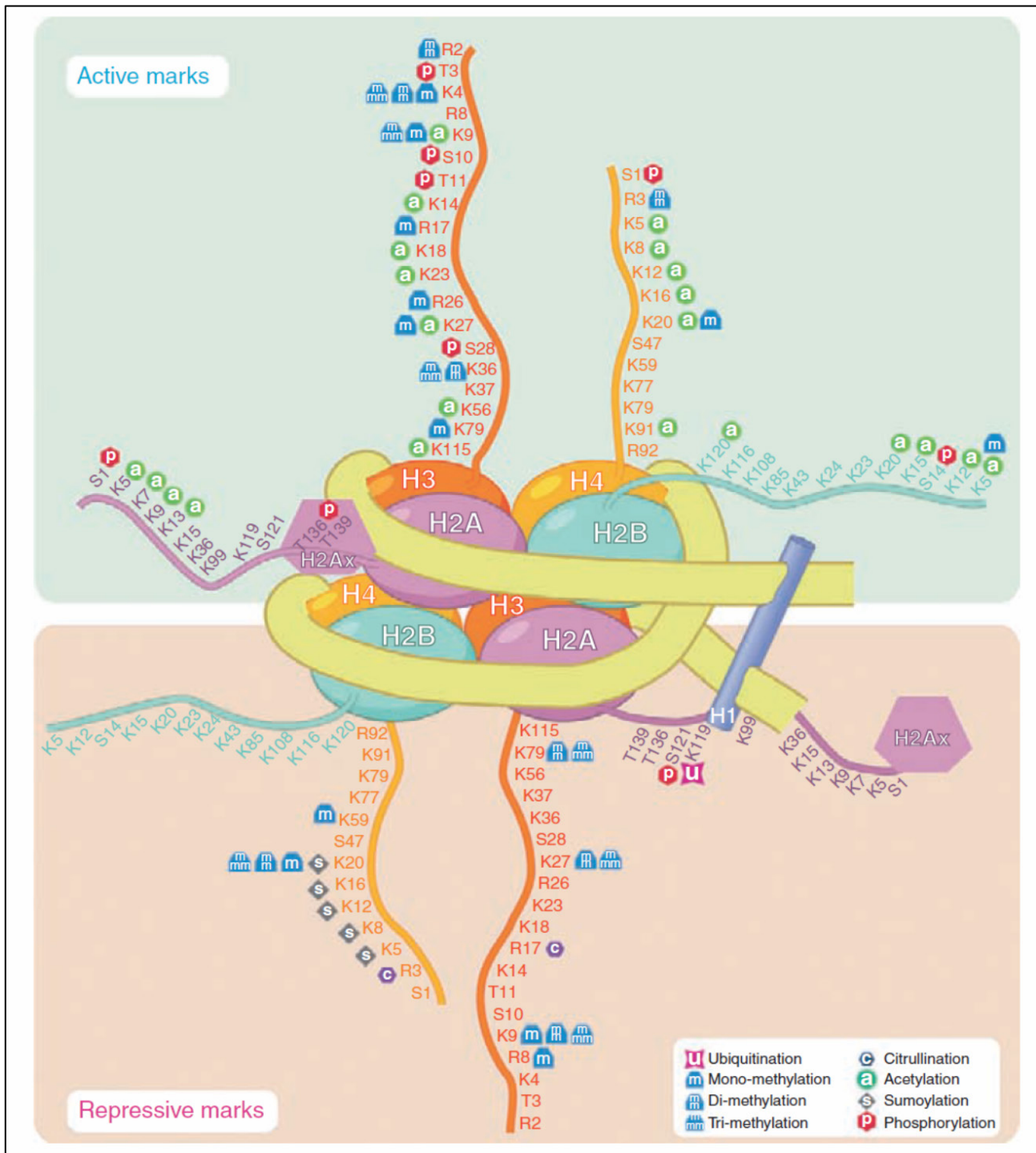


**Figure 2.1: Post-translational histone modifications.**

*A schematic illustration of the nucleosome showing different covalent modifications in histone tails residues. Active modifications which promote gene expression are represented in the upper part of the figure and the repressive modifications which deactivate gene expression are represented in the lower part of the figure. Lysine (K), arginine (R), serine (S), and threonine (T). Adapted from reference (Sawan and Herceg, 2010).*

## **•Post-translational histone modifications in CRC**

### **1. CRC histone methylation:**

This mainly occurs on the lysine (K) residues of histone 3 and 4. In CRC, it has been shown that di- and trimethylation of histone H3 lysine 4 (H3K4me2/me3) is abundant near the transcription start site of transcriptionally active genes, while trimethylation of histone H3 lysines 9 and 27 (H3K9me3 and H3K27me3) characterizes transcriptionally inactive genes (van Engeland et al., 2011).

### **2. CRC histone phosphorylation:**

Phosphorylation of Serine 10 in histone 3 (Ser10H3) is crucial in chromatin remodelling and cell cycle regulation during mitosis and meiosis (Nowak and Corces, 2004). The phosphorylation modification in Ser10H3 plays a dual action as it is associated with chromatin relaxation and transcriptionally active genes in interphase, but also it is associated with chromosome condensation in mitosis (Nowak and Corces, 2004). In CRC, it has been found that a mammalian Ipl1/ aurora kinase (AIM-1) which is involved in phosphorylation of Ser10H3, is over-expressed, and this is associated with Chromosome Number Instability (CIN) (Ota et al., 2002).

### **3. CRC histone acetylation:**

Acetylation is the most important post-translational histone modification because it contributes substantially to unfolding of

chromatin through neutralizing the basic charge of the lysine residue (K) (Kouzarides, 2007), hence acetylation is associated with active transcription process of genes (Sterner and Berger, 2000). The acetylation is controlled by the balance between two types of enzymes: histone acetyl-transferase enzymes (HATs) and histone deacetylase enzymes (HDACs) (Yang and Seto, 2007), as shown in figure 2.2. Human HATs and HDACs are classified in super-families and classes respectively, as summarized in table 1.3. It has been shown in many human cancers that the balance between HATs and HDACs is lost (Peserico and Simone, 2011). A loss or decrease of acetylation in important residues of H3 and H4 such as lys9-H3 and lys16-H4 is a common characteristic of human cancer, including CRC. This leads to gene transcription-deregulation of crucial tumour suppressor genes, and induces cells transformation and tumour development (Pruitt K et al., 2006).

Moreover, it has been demonstrated that histone acetylation decrease is not only involved in gastrointestinal tumour initiation, but also in tumour invasion and metastasis (Yasui et al., 2003). The change in acetylation status in cancer cells has been linked to increased expression of individual HDACs in indefinite patterns. For example, number of studies showed an increase in HDAC1 expression in gastric (Choi et al., 2001), prostate (Halkidou et al., 2004), and colon (Wilson et al., 2006) cancers. Over expression of HDAC2 is well confirmed in all colorectal carcinoma having *APC* mutation (Zhu P et al., 2004).

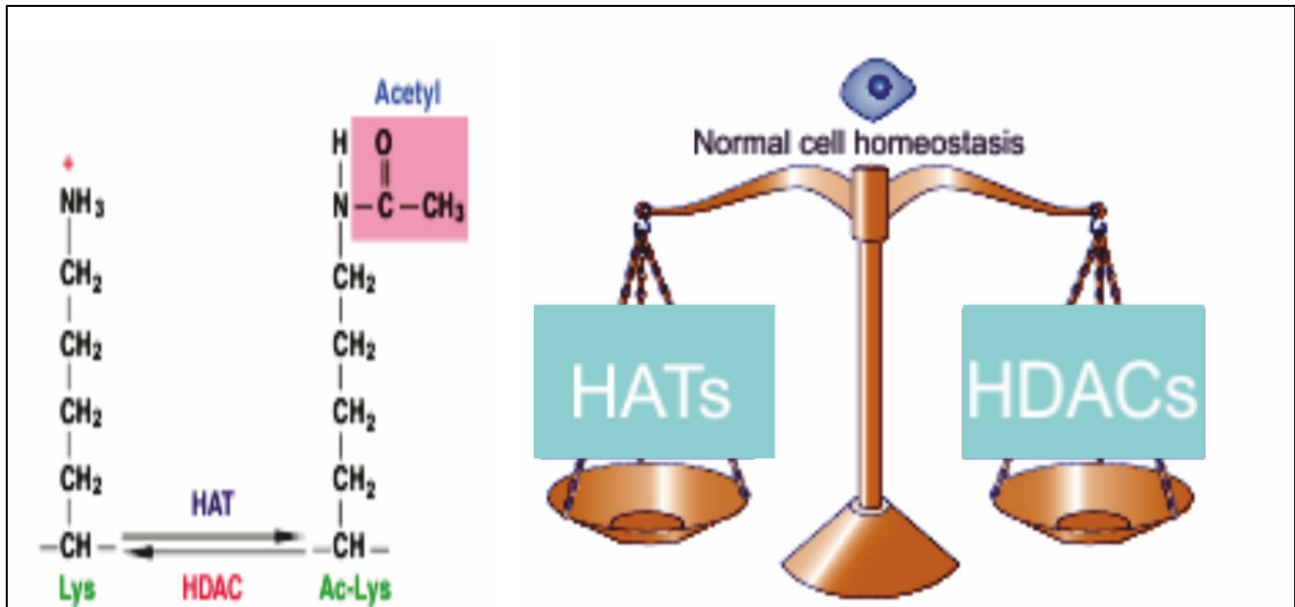


**Figure 2.1: balance between HATs and HDACs in a normal cell**

*Addition of acetyl group to lysine residue in histone tail is catalysed by HATs enzymes activity, whereas the opposite process is catalysed by HDACs enzymes activity. Specific gene expression and cell homeostasis as a result are maintained by the normal balance of HATs and HDACs activity in the cell. The figure is adapted from reference (Yang and Seto, 2007).*

| <b>Table 1.3: human histone acetyltransferases (HATs) and histone deacetylases (HDACs)</b>                                                                                                                                                        |                                                                                                                       |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------|
| <b>HATs super-families</b>                                                                                                                                                                                                                        |                                                                                                                       |
| GNATs super- family                                                                                                                                                                                                                               | General control non-derepressible 5 (Gcn5)-related N-acetyltransferases family (GNATs) includes Hat1, Gcn5, and PCAF. |
| MYST super- family                                                                                                                                                                                                                                | Includes Tip60, MOZ, MORF, and HBO1.                                                                                  |
| P300/CBP proteins                                                                                                                                                                                                                                 | Two proteins: p300 and CBP work as a single entity.                                                                   |
| <b>HDACs classes</b>                                                                                                                                                                                                                              |                                                                                                                       |
| Class I                                                                                                                                                                                                                                           | Includes HDAC (1, 2, 3, and 8)                                                                                        |
| Class IIa                                                                                                                                                                                                                                         | Includes HDAC (4, 5, 7, and 9)                                                                                        |
| Class IIb                                                                                                                                                                                                                                         | Includes HDAC (6 and 10)                                                                                              |
| Class III                                                                                                                                                                                                                                         | Includes (Sirt1-7)                                                                                                    |
| Class IV                                                                                                                                                                                                                                          | HDAC 11                                                                                                               |
| <p><b>The table shows brief list of human HATs and HDACs.</b></p> <p>Human GANTs and MYST are super-families. They contain subfamilies listed in the table. Adapted from the references (Sterner and Berger, 2000) and (Yang and Seto, 2007).</p> |                                                                                                                       |

### **3. Histone deacetylase inhibitors (HDACIs)**

#### **1- History and discovery**

The story of HDAC inhibitors (HDACIs) development as therapeutic drugs is the opposite of the conventional principle of a targeted therapy development, in which a drug is usually designed to target an already studied cancer cell specific gene or pathway. However, in HDAC inhibitors case, the drugs came first and their target was defined later (Witt and Lindemann, 2009). In the late 1970s, it was noticed that the short chain fatty acid butyric acid induces accumulation of acetylated histone proteins, which lead to erythroid differentiation of Friend leukemic cells when applied to cell culture in millimolar concentrations (Mai A et al., 2005, Miller et al., 2003). Subsequently, other chemical compounds were discovered having the effects of induction of differentiation, cell cycle inhibition and apoptosis of transformed cells in culture accompanied by accumulation of acetylated histone proteins. Eventually, it was discovered that targets of these drugs were histone deacetylase enzymes (HDACs) (Witt and Lindemann, 2009).

#### **2- Effect and structure**

Nowadays, it is well known that HDACIs have the potency to induce growth arrest, differentiation, and/or apoptotic cell death of transformed cells in vitro and in vivo (Marks et al., 2000), whereas normal cells show relative resistance to the HDACIs in the same concentrations applied to transformed cells (Ungerstedt et al., 2005), as pictured in figure 2.3.

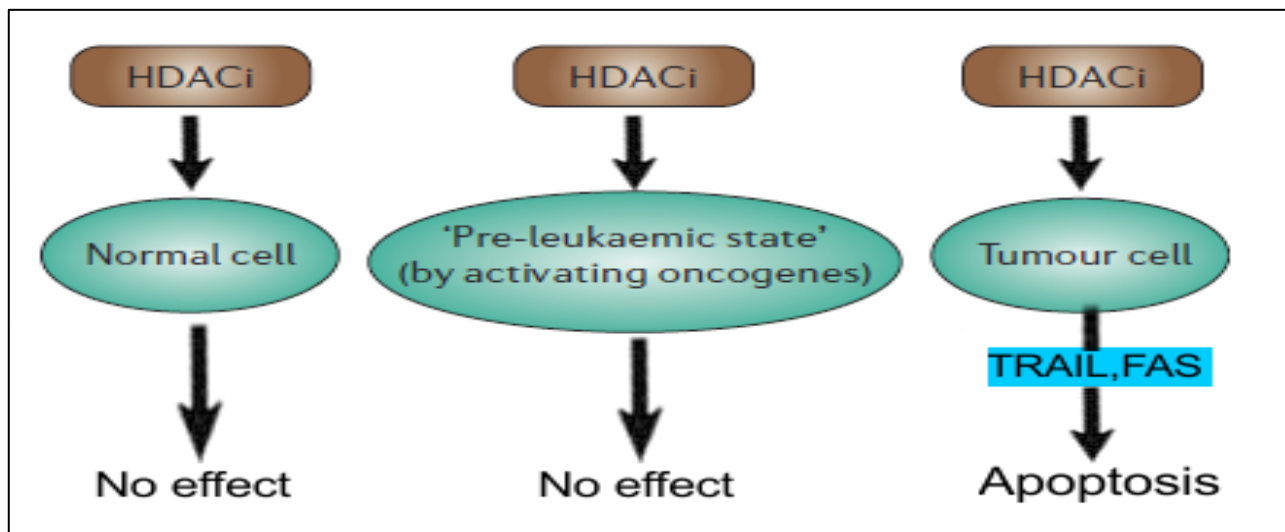


**Figure 2.3: tumour-specific effects of HDACIs in acute promyelocytic leukaemia.**

*Histone deacetylase inhibitors (HDACIs) have little or no effect when they are applied to normal cells. For example, in acute promyelocytic leukaemia, HDACIs are able to induce apoptotic cell death through tumour-necrosis factor-related apoptosis inducing ligand (TRAIL) or FAS pathways, whereas there are no effects of HDACIs on cultures of normal haematopoietic progenitors in vitro or on haematopoiesis in normal mice. In addition, no effects of HDACIs have been noticed on preleukaemic stage cells which have an activation of oncogenes, but they have not reached the full transformation state. The figure is adapted from reference (Balasubramanian et al., 2008).*

Many kinds of HDACIs are available for their potential therapeutic effects in cancer; some of them in clinical trials. Some HDACIs were derived from biological sources, for example Trichostatin A (TSA) which comes from a bacterial origin as an antifungal antibiotic, and many were designed and synthesized to target their HDAC (Zhang et al., 2010). Depending on the chemical structure, HDACIs were classified in classes summarised in tables 2.3.

| <b>Table 1.4: Classification of Histone Deacetylase Inhibitors</b>                                                                                                                                                                                                                                                      |                                                    |                                |                                        |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------|--------------------------------|----------------------------------------|
| <b><u>Class</u></b>                                                                                                                                                                                                                                                                                                     | <b><u>Inhibitor</u></b>                            | <b><u>HDAC specificity</u></b> | <b><u>Clinical Trial Status</u></b>    |
| Short-chain fatty acid                                                                                                                                                                                                                                                                                                  | Butyrate                                           | Class I, IIa                   | Phase II                               |
|                                                                                                                                                                                                                                                                                                                         | Valproic acid (VPA)                                | Class I, IIa                   | Phase II                               |
| Hydroxamate                                                                                                                                                                                                                                                                                                             | Trichostatin A (TSA)                               | Class I, II                    | No clinical trial                      |
|                                                                                                                                                                                                                                                                                                                         | Suberoylanilide hydroxamic acid (SAHA, Vorinostat) | Class I, II                    | Approved for cutaneous T-cell lymphoma |
|                                                                                                                                                                                                                                                                                                                         | Tubacin                                            | Class IIb                      | No clinical trial                      |
| Benzamide                                                                                                                                                                                                                                                                                                               | MS-275                                             | Class I                        | Phase I, II                            |
|                                                                                                                                                                                                                                                                                                                         | CI-994 (tacedinaline)                              | Unknown                        | Phase I, II,III                        |
| Cyclic tetrapeptide                                                                                                                                                                                                                                                                                                     | Apicidin                                           | HDACs 1 and 3                  | No clinical trial                      |
|                                                                                                                                                                                                                                                                                                                         | Depsipeptide                                       | Class I                        | Phase I, II                            |
| Electrophilic-ketone                                                                                                                                                                                                                                                                                                    | Trifluoro-methylketone                             | Unknown                        | No clinical trial                      |
| <p><b>The table shows HDACIs classes accompanied by brief examples of each class, specificity, and the corresponding clinical trial.</b></p> <p>There are five classes of HDACIs classified according to the chemical structure. This table is adapted from the references (Bolden et al., 2006, New et al., 2012).</p> |                                                    |                                |                                        |

### 3- Efficacy and functionality

From activity perspective, some HDACIs have a broad inhibition effect such as Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA, Vorinostat), and LBH589. Therefore they are called pan HDACIs as they inhibit all HDAC members of class 1, and 2. Other HDACIs inhibit one class or some members of HDACs, for example valproic acid (VPA) inhibits Class I, IIa, whereas MS-275 inhibits just Class I (Bolden et al., 2006, New et al., 2012). Very few HDACIs work specifically to inhibit one HDAC per se, for example PCI-34051 is a HDAC8-specific inhibitor (Balasubramanian et al., 2008). It is worth taking into consideration that the above mentioned HDACIs do not have any effects on class III of HDACs, (Sirt1-7) because this class depends on Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) for its activity. Therefore HDACIs classes in table 1.4 do not inhibit Sirt 1-7 (Dokmanovic and Marks, 2005a). All HDACs members of class I, class II, and class IV are Zn<sup>+</sup> dependent (Balasubramanian et al., 2009) as their active sites contain the zinc atom occupying the bottom of a channel delimited by a rim, which corresponds to the substrate pocket (acetyl-lysine). HDACIs exploit the zinc atom to inhibit HDACs activity, hence all HDACIs (either from natural or synthetic sources) pharmacophores primarily have a metal-binding domain (ZnC), which chelates zinc and blocks the enzymatic activity of HDACs, and two other domains known as a linker domain and a surface domain (Minucci and Pelicci, 2006), figure 2.4.

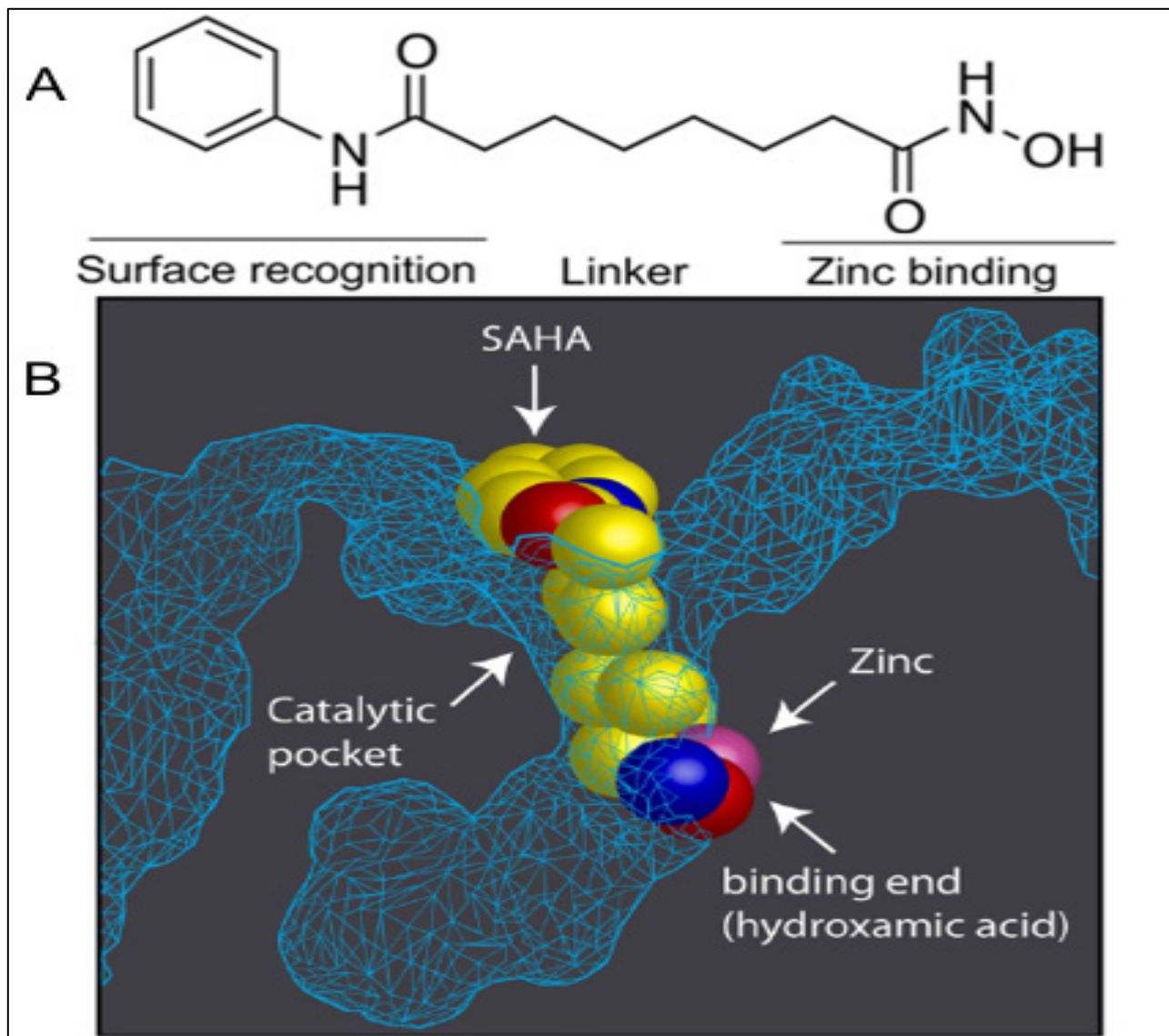


**Figure 2.4: a representation of the crystal structure of the binding between HDACs and HDACIs.**

*A: general structure of a HDACI: a surface recognition, a linker, and a zinc binding domain. B: a representation of the binding between a HDAC (the blue net) and SAHA (coloured beads excluding the pink bead). SAHA inserts into the pocket-like catalytic site of a HDAC enzyme, and it binds by its hydroxamic moiety to the zinc atom (the pink bead). This chelates the zinc and blocks the enzymatic activity of the HDAC enzyme. The figure is taken from reference (Marks, 2010).*

#### **4- Combination therapies with HDACIs**

HDACIs are promising new anti-cancer agents especially for the therapy of hematologic and solid neoplasms. Research is focused on developing more specific and effective HDACIs (Marks, 2010). Their use in combination with other types of cancer therapy (radiation, DNA damaging agents, and DNA demethylating agents) shows synergistic effects depending on the type of the combination and the sequence of drug administration (Bots and Johnstone, 2009). Moreover, many studies show that combination therapies with HDACIs may be the best therapeutic strategy in cancer treatment (Nolan L et al., 2008, Gore et al., 2006, Bishton et al., 2007). The role of HDACIs in combination treatments mainly falls in two concurrent ways (Miller et al., 2011). The first way is the mechanistic effect of HDACIs in combination treatments with DNA damaging drugs, in which HDACIs increase the acetylation of the histones leading to relaxed chromatin which facilitates the way for DNA damaging drugs to act (Hajji et al., 2010), figure 2.5. The second way is the transcriptional effect of HDACIs in combination treatments. For example, the combination of HDACIs with DNA methyltransferase inhibitors (DNMTIs) restores the expression of the epigenetically silenced tumour suppressor genes, and induces biological responses resulting in tumour regression (Seidel et al., 2012), figure 2.5. Also, it has been shown that HDACIs treatment increase the expression of several proapoptotic proteins, such as Bim and Bmf (Zhang Y et al., 2006).

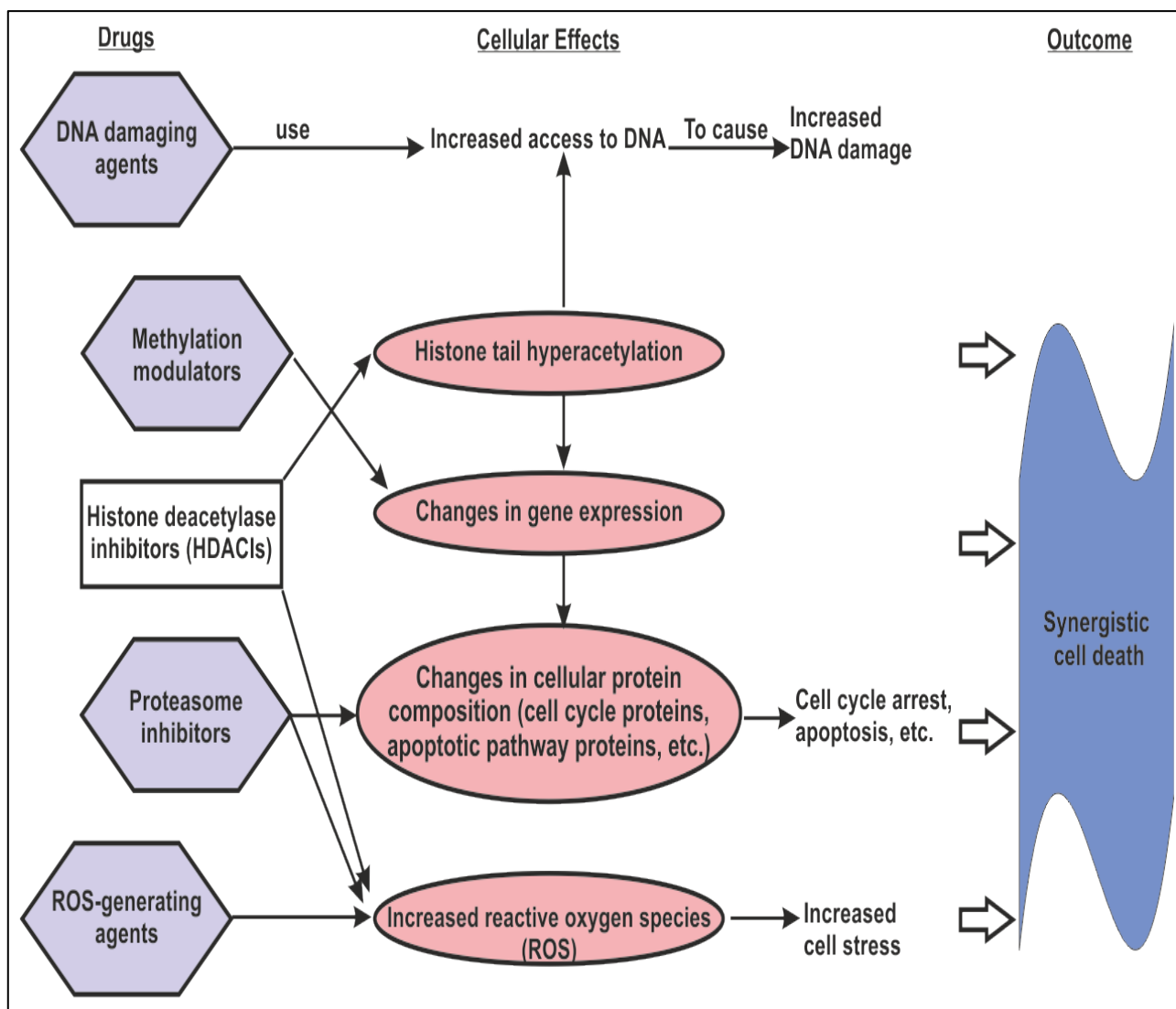


**Figure 2.5: types of therapeutic combinations with HDACIs.**

*Combinatorial HDACIs treatments showed synergistic cell death in different ways depending on the combined drug. For example, HDACIs with DNA-damaging agents, such as radiation and chemotherapies caused dramatic DNA damage in comparison with the singular treatment with each drug. This synergistic effect happens because of the increased accessibility to DNA which HDACIs cause. Also, histone demethylase inhibitors and DNA methyltransferase inhibitors acted synergistically with the actions of HDACIs. In addition, the combination of HDACIs and proteasome inhibitors increased the cell death in a synergistic way through cell cycle arrest and apoptosis owing to the increase (toxic levels) of reactive oxygen species (ROS) production which also occurred when HDACIs were combined with ROS-generating agents. The figure is adapted from reference (Miller et al., 2011).*

## 5- Resistance to HDACIs

Similar to any cancer drugs, HDACIs encounter a resistance in the cancerous cells, and this limits the HDACIs actions (Wagner et al., 2010). A number of studies demonstrated several mechanisms involved in the biology of resistance to HDACIs. However, this resistance to HDACIs is not completely understood and more investigation is needed (Xu et al., 2007). The main mechanisms of resistance to HDACIs are:

### 1. Drug efflux

This mechanism is commonly associated with the multidrug resistance in cancer cells. It operates through overexpression of efflux pumps in the ATP-binding cassette transporter family, and it is associated with high levels of P-glycoprotein, the protein of the multidrug resistance-1 gene (*MDR1*), which activates effluxing chemotoxins from cells (Ruefli et al., 2002). The drug efflux mechanism does not affect all HDACIs. However, romidepsin (depsipeptide) (Tabe Y et al., 2006) and sodium butyrate (Morrow CS et al., 1994) are to date the only HDACIs shown to undergo efflux from the cancer cells including CRC cells (Jain and Zain, 2011).

### 2. HDACs overexpression and desensitization

Alterations in the level of HDAC enzymes in the treated cells render the cells resistant to HDACIs (Yoshida et al., 1990). For instance, overexpression of HDAC1 in melanoma cells

confers resistance to sodium butyrate (Bandyopadhyay et al., 2004). In addition, inactivation mutations in HDACs play a role in building resistance to specific HDACIs. Recent studies showed that a frameshift mutation in *HDAC2* are found in various CRC cell lines, and the deactivation of HDAC2 induced a failure in triggering histone acetylation and inhibiting proliferation after trichostatin A treatment. However, the cells remained sensitive to valproate and butyrate derivatives (Ropero et al., 2006).

### 3. Epigenetic and chromatin alterations

Silencing of tumour suppressor genes and induction of malignancy in many cells happens as results of epigenetic mechanisms such as hypermethylation at the promoter regions of these genes and hypoacetylation of histones H3 and H4 (Jones and Baylin, 2007). It has been shown that methylation of DNA hinders the effects of HDACIs in fully restoring the expression of epigenetically silenced tumour suppressor genes, and this represents a mechanism of resistance to HDACIs. To prove the effect of DNA methylation in provoking resistance to HDACIs, a combination treatment of a demethylation agent (for example, 5-aza-2'-deoxycytidine) and a HDACI restores the effects of the HDACI (Zhang C et al., 2007). Besides the methylation of DNA, cellular polyamines (such as spermidine and spermine) that regulate gene expression through their effects on the chromatin microenvironment play a key a role in increasing

sensitization to HDACIs. Moreover, polyamines' depletion increases resistance to HDACIs treatment (Saunders and Verdin, 2006).

#### 4. Stress response mechanisms

Oxidative damage by producing reactive oxygen species is crucial in cell death induced by HDACIs treatment (Rosato RR et al., 2006, Rosato et al., 2003). According to this mechanism of resistance, cells showing resistance to HDACIs circumvent the oxidative stress by overexpressing proteins that work as reactive oxygen species scavengers, for example thioredoxins (Powis and Kirkpatrick, 2007). In contrast, down regulation of thioredoxin proteins through thioredoxin-binding protein 2 augments the response to SAHA (vorinostat) (Butler et al., 2002).

#### 5. Antiapoptotic/prosurvival mechanisms

The apoptotic cell death induced by HDACIs in various malignant cells proceeds via the intrinsic pathway which involves mitochondrial damage, cytochrome c release, and production of reactive oxygen species (Peart MJ et al., 2003). Therefore, overexpression of antiapoptotic proteins such as Bcl-2 or Bcl-XL is a key means by which transformed cells avoid the apoptotic effects of HDACIs (Maiso P et al., 2006). Moreover, it has been identified that the antiapoptotic transcription nuclear factor  $\kappa$ B (NF- $\kappa$ B) is a mediator of HDACIs resistance (Mayo MW et al., 2003). HDACIs

treatment induces transcriptional activation of NF- $\kappa$ B which hinders HDACIs ability to trigger cell death in non-small cell lung cancer lines and leukaemia cell lines (Dai et al., 2005, Rundall BK et al., 2004). Therefore, using BAY-11-7085 (an inhibitor of NF- $\kappa$ B activation) restores the response to the effects of HDACIs in the malignant cells (Fantin and Richon, 2007). Besides the above mentioned antiapoptotic mechanisms, there are prosurvival mechanisms playing a role in HDACIs resistance. For example, the cyclin-dependent kinase inhibitor p21<sup>(CIP1/WAF1)</sup> which is involved in cell cycle arrest, has also a role in the regulation of programmed cell death (Gartel, 2005). It has been shown through several studies that HDACIs treatments in cancer cells up-regulate the expression of p21<sup>(CIP1/WAF1)</sup> which, in turn, either mediates cell cycle arrest, differentiation, and apoptosis (Ocker and Schneider-Stock, 2007, Ju and Muller, 2003) or confers protection to colon cancer cells against the apoptotic effects of HDACIs (Wagner and Roemer, 2005). This dual effect of p21<sup>(CIP1/WAF1)</sup> is HDACIs dose dependent, as low concentration of Entinostat (MS-275, a potent HDAC inhibitor from Benzamide class, see table 1.4) caused cell cycle arrest in leukaemia cells, whereas that effect disappeared at high concentrations of Entinostat (Rosato et al., 2003, Frew et al., 2009). Finally, it has been demonstrated that autophagy mimics the dual effect of p21<sup>(CIP1/WAF1)</sup> in the response of cancer cells to HDACIs, and this depends on the cellular context. While activation of autophagy results in cell death and enhances therapeutic efficacy in some cancer cells,

it induces a protective effect through coping with therapy induced oxidative stress and cellular damage in other cells (Amaravadi et al., 2007, Kondo and Kondo, 2006). In the latter scenario, combining autophagy inhibitors with HDACIs treatment enhances the therapeutic effects (Shao et al., 2004). Figure 2.6 summaries the above mentioned mechanisms of resistance to HDACIs. Another antiapoptotic protein which plays a role in CRC resistance to treatment and reduces apoptosis is survivin (Yang et al., 2009). The next paragraph will talk about survivin and its role in mCRC.

## **6- Survivin and CRC**

The inhibitor-of-apoptosis (IAP) family of proteins were originally discovered in baculoviruses . 8 proteins of IAP family were identified in human: c-IAP1, c-IAP2, NAIP, Survivin, XIAP, Bruce, ILP-2, and Livin (Liston et al., 2003). IAP proteins share the ability to bind and inhibit specific caspases, hence they block apoptosis and participate in controlling the delicate balance between life and death in the cell (Deveraux and Reed, 1999). Survivin is a 16.5 Kd protein encoded by *BIRC5* gene which is located in 17q25.3 . The 142 amino acids protein is the only member in IAP family that plays an important role as a chromosome passenger during cell division (Altieri, 2006). Thus survivin has two roles: 1) inhibition of apoptosis, 2) regulation of mitosis (Knauer et al., 2007).

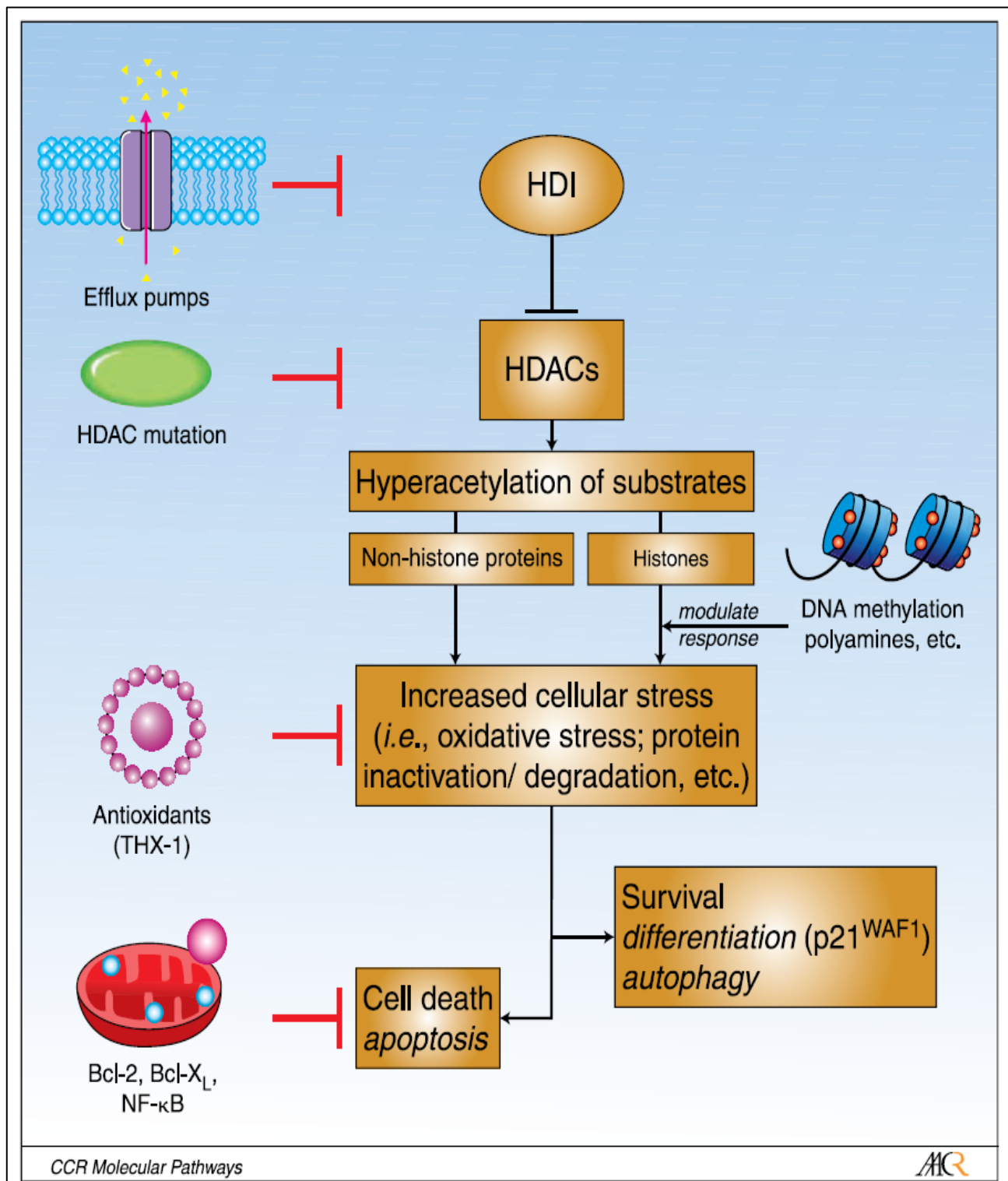


**Figure 2.6: Mechanisms of resistance to HDACIs apoptotic effects.**

*The cascade of events and outcomes following use of HDACIs in vitro are shown in the figure. Also the figure shows the factors that block HDACIs activity and confer resistance along the pathway of their action. The figure is taken from reference (Fantin and Richon, 2007).*

The cytoplasmic survivin expression associates with its role in inhibiting apoptosis by binding and deactivating caspases 3, 7, and 9 (Nachmias B et al., 2004). The role of survivin in cell division is associated with its nuclear expression which increases dramatically the G2/M phase (Li et al., 1998 , Liston et al., 2003). Survivin performs its role in cell division through interacting with Aurora kinase B (AURKB), inner centromere protein antigens (INCENP), and borealin (CDCA8) to form Chromosomal passenger complex. This complex then localizes to kinetochores at metaphase and facilitates chromosomes segregation on spindle microtubules (Kanwar et al., 2010). This explains why survivin is over-expressed in many cancer including CRC (Altieri, 2003, Kim et al., 2003). Moreover, it has been shown that survivin is involved in the early stage of CRC tumorigenesis (Kim et al., 2003, Svec J et al., 2010), and its expression is stimulated by up-regulated (TCF)/ $\beta$ -catenin pathway as a result of the deactivation of *APC* gene which happens in the majority of sporadic CRC (Zhang T et al., 2001), see chromosomal instability (CIN) pathway of CRC. Over-expression of survivin is linked with poor survival and resistance to apoptosis in CRC (Sarela AI et al., 2000, Wen et al., 2013, Di Stefano et al., 2010). In addition, survivin participates in CRC chemo-resistance as it inhibits drug-induced apoptosis (Prabhudesai et al., 2007). The anti-apoptotic effect of survivin can be strengthened by mutant p53 which takes place in the late stage of CRC. Wild-type p53 transcriptionally represses survivin (Hoffman et al., 2002b, Mirza et al., 2002, Hoffman et al., 2002a). As above mentioned, p53 becomes mutant the late stage of metastatic CRC with the most mutation in DNA binding domain of *TP53*, and

this will lead to increase in survivin expression levels which are already elevated with initiation of chromosomal instability (CIN) pathway for CRC (Watson, 2006), as shown in figure 2.7.

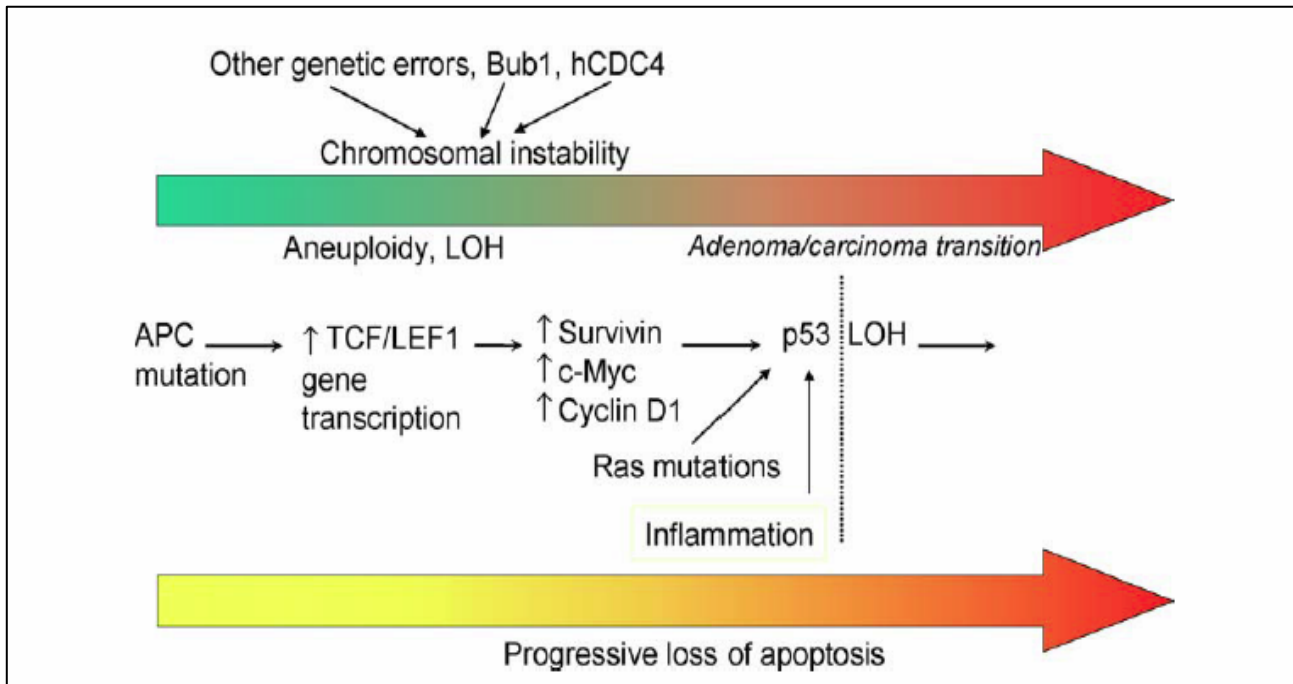


**Figure 2.7: Up-regulation of survivin levels in the chromosomal instability pathway of colorectal cancer.**

*Mutation in APC gene which occurs early in the chromosomal instability pathway leads to up-regulation of many genes transcribed by TCF/LEF1. Among these genes is survivin which happens in the early stage. TP53 becomes mutated in the late stage of CRC. All these alterations correlate with loss of apoptosis. Bub1 encodes a mitotic checkpoint serine/threonine-protein kinase Bub1; hCDC4 encodes F-box protein which targets cyclin E for ubiquitin-mediated proteolysis. The figure was taken from reference (Watson, 2006).*

## 4. Objectives and Hypothesis

In the beginning of this project, I aimed to check the effect of exogenous wild-type p53 and mutated p53 transfection on the response of isogenic null p53 colorectal cancer cell line (HCT116 p53<sup>-/-</sup>) to DNA damaging agent (Doxorubicin). The Ser15A, K373R, and K381R *TP53* mutations were used. Therefore, HCT116 p53 were transfected with wild-type p53 and above mentioned mutations for p53. Stable clone for wild-type and the mutations were generated. The exogenous wild-type p53 and mutant p53 cells were treated with 0.75 $\mu$ M and 1 $\mu$ M Doxorubicin for 24 hours. I checked the cell death by flow cytometry using Propidium iodide (PI) dye. Unfortunately, the results have not shown any difference in cell death between the exogenous wild-type p53 and mutant p53 cell lines, figure 2.8. This drew my attention to involvement of other factors that may play role in resistance of isogenic null p53 colorectal cancer cell line (HCT116 p53<sup>-/-</sup>) to doxorubicin in comparison to its endogenous wild-type cells, HCT116 p53<sup>+/+</sup>. This could be on the epigenetic level as HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup> theoretically have the same genetic background. It is well-known that histone deacetylase2 enzyme (HDAC2) is over-expressed in mCRC. However, its role in the resistance to combined treatment of HDACIs and DNA damaging agents have not been well-investigated.

**The hypothesis of this project is:** HDAC2 may play a role in response of metastatic colorectal cancer to combined treatment of HDACIs and DNA damaging agent.

### **The aims of this study are:**

- 1- To characterize the response of early stage mCRC cell lines (p53 is intact) and late stage mCRC cell lines (p53 is mutated) to combined treatment of HDACIs and DNA damaging agent.
  - 2- To investigate the HDAC2 role in mCRC established cell lines upon specific combined treatments of HDACIs and DNA damaging agents.
  - 3- To select synergistic combined treatment of HDACIs/DNA damaging agents which induces cell death with minimal toxicity in mCRC established cell lines.
  - 4- To test the effect of the selected synergistic combined treatment of HDACIs/DNA damaging agents in vivo using xenograft mice.
-

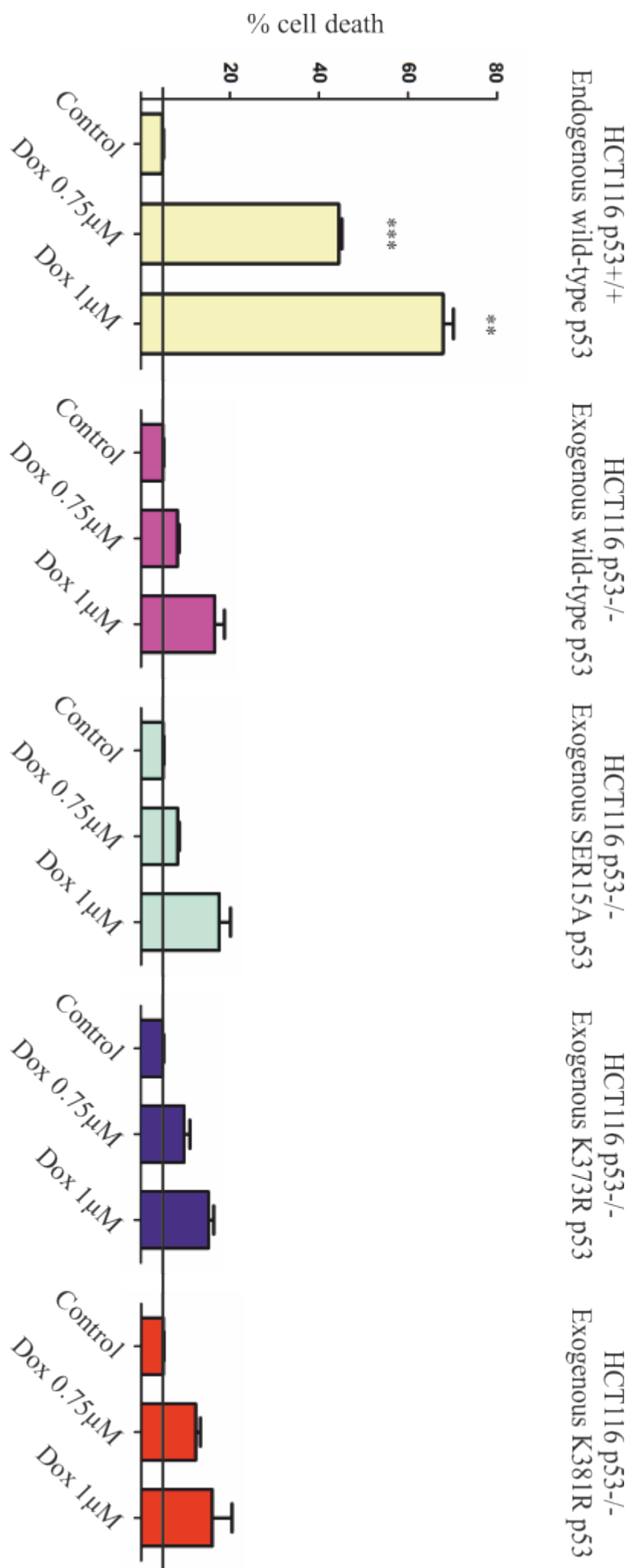


**Figure 2.8: Wild-type p53 or mutant p53 transfection has not improved the response to doxorubicin in isogenic HCT116 P53-/- cells.**

Cell death was investigated by flow cytometry using propidium iodide dye staining. Isogenic null p53 cells were transfected with either wild-type p53 or mutant p53 (Ser15A, K373R, K381R). The results showed that neither the wild-type p53 nor any of the used mutants induced any significant change in response to doxorubicin treatment. All the measurements were normalized to HCT116 p53+/+ control sample. Data was presented as mean  $\pm$  S.E.M.;  $n=3$ . \* $P$  between 0.001-0.01, \*\* $P$ <0.001.

# **Chapter 2**

## **Materials and methods**

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# 1-In vitro study

## • Cell lines used in this study

All the cell lines used in this study are established human metastatic colorectal cancer lines. I used four cell lines which are:

1- HCT116 p53<sup>+/+</sup> cells: have wild type P53.

2- HCT116 p53<sup>-/-</sup> cells: do not express P53 protein as both *TP53* gene copies were knocked down.

3- SW480 cells: have two substitution mutations in *TP53* gene. The first mutation is a G→A substitution in codon 273 of the DNA binding domain of *P53* gene resulting in an Arg→His change and the second mutation is C→T in codon 309 of the oligomerization domain of *TP53* gene resulting in a Pro→Ser substitution .

4- HT-29 cells: have one mutation in the DNA binding domain of *TP53* gene. This mutation is similar to the first mutation in SW480 (a G→A mutation in codon 273 resulting in Arg→His) .

## • Reagents used in this study

| Name of reagent           | The source       |
|---------------------------|------------------|
| Acetone                   | (VWR, UK)        |
| Alexa Fluor® antibodies   | (Invitrogen, UK) |
| Ammonium persulfate (APS) | (Sigma, UK)      |

|                                                                 |                                      |
|-----------------------------------------------------------------|--------------------------------------|
| Bovine serum albumin (BSA)                                      | (Sigma, UK)                          |
| Chemiluminescence film                                          | (GE Healthcare, UK)                  |
| Chemi-luminescent reagent                                       | (Thermo, UK)                         |
| Cisplatin (Cisp)                                                | (Sigma, UK)                          |
| Control shRNA Plasmid (sc-108060)                               | (Santa Cruz Biotechnology, Inc, USA) |
| Coomassie® Brilliant Blue G-250                                 | (Bio-Rad, UK)                        |
| D-luciferin                                                     | (Gold Biotechnology, USA)            |
| Doxorubicin                                                     | (Sigma, UK)                          |
| Dulbecco's modified Eagle's medium                              | (Sigma-Aldrich, St. Louis, MO, USA)  |
| EDTA                                                            | (Sigma, UK)                          |
| Fluorouracil or 5 Fluorouracil (5FU)                            | (Sigma, UK)                          |
| Foetal bovine serum (FBS)                                       | (Sigma, UK)                          |
| Glycerol (99%)                                                  | (Sigma, UK)                          |
| HEPES                                                           | (Sigma, UK)                          |
| Irinotecan or Camptothecin-11                                   | (Sigma, UK)                          |
| MgCl <sub>2</sub>                                               | (Sigma, UK)                          |
| NaCl                                                            | (VWR Prolab, UK)                     |
| Nitrocellulose membranes                                        | (GE Healthcare, UK)                  |
| Nonidet P40                                                     | (Fluka, UK)                          |
| Nupage lds sample buffer 4X                                     | (Invitrogen, UK)                     |
| Opti-MEM media                                                  | (Invitrogen, UK)                     |
| Platinum <sup>®</sup> SYBR <sup>®</sup> Green qPCR SuperMix UDG | (Invitrogen, UK)                     |
| ThermoScript <sup>™</sup> RT PCR System                         | (Invitrogen, UK)                     |
| Oxaliplatin (Oxa)                                               | (Sigma, UK)                          |
| PageRuler <sup>™</sup> Prestained Protein Ladder                | (Fermentas, UK)                      |
| Paraformaldehyde                                                | (Thermo, UK)                         |
| Penicillin- streptomycin                                        | (Sigma, UK)                          |
| pGL4.20 [luc2/Puro] Vector                                      | (Promega, UK)                        |
| Phosphate buffer saline (PBS) 10X                               | (GIBCO, CA)                          |
| Polyacrylamide 40%                                              | (Bio-Rad, UK)                        |
| Polyethylenimine (PEI)                                          | (Polysciences Inc, US)               |
| Protease inhibitors                                             | (Roche, Welwyn, UK)                  |
| SDS (10%)                                                       | (Bio-Rad, UK)                        |

## • Cell culture

All the cells were maintained at 37°C in Dulbecco's modified Eagle medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS; Sigma, UK) and penicillin (100U/ml) – streptomycin (100µg/ml) (Sigma, UK) in the presence of 5% CO<sub>2</sub>.

## • Cell collection

Cell collection either before or after treatment was done as follows:

- 1- Medium was transferred into a collection tube.
- 2- Cells were washed with pre-warm trypsin 1X (Sigma, UK).  
The amount of washing trypsin depended on the cells container (1ml of trypsin was used for washing the cells seeded in 10 cm dish or T25 flask, whereas 3 ml of trypsin was used for T75 flask).
- 3- The washing trypsin was then transferred into the collection tube which has the medium from the first step. Then another amount of pre-warm trypsin was put into the cells container to detach them (1ml of trypsin was used for the cells seeded in 10 cm dish, 3ml for T25 flask, and 5 ml of trypsin was used for T75 flask).
- 4- The cells container was incubated in 37°C incubator for 5 minutes.
- 5- The cells were homogenised with a pipette controller, then 1 ml of complete growth medium was added over the cells to homogenise them very well.

- 6- Then the cells suspension was transferred into the corresponding collection tube from the first step.
- 7- The cells container was washed with 1ml of pre-warm phosphate buffer saline (PBS) 1X (GIBCO, CA) to remove any remaining cells from the previous step. Then the washing PBS was transferred to the corresponding collection tube from the first step and the cells container (flask or dish) was discarded.
- 8- The collection tube was centrifuged at 2600 RPM (revolutions per minute) for 5 minutes, and the supernatant was removed with a tissue culture pump.
- 9- The cells pellet was suspended in 1 ml of pre-warm PBS buffer 1X and transferred to an eppendorf tube.
- 10- The eppendorf tube was centrifuged at 2600 RPM for 5 minutes.
- 11- The supernatant was removed and either the cells pellets were transferred to a specific tubes for flow cytometry analyses, or the eppendorf containing the cells pellet immediately was dropped in liquid nitrogen flask to kill the cells.
- 12- The eppendorf was stored in -20°C freezer for further processing, proteins and histones extraction.

## • Cells Subculturing

Cancer cells grow very fast and they need to be split and transferred to a new container when they become confluent in their container. The process is called subculturing or passaging and it should be done under the safety cabinet class II to prevent any contamination for the cells.

1. Culture medium was removed and discarded with a vacuum pump.
2. Cells were rinsed with pre-warm trypsin 1X to remove any trace of cells medium. The amount of washing trypsin depends on the cells container (1ml of trypsin was used for washing the cells seeded in T25 flask, whereas 3 ml of trypsin was used for T75 flask).
3. Then another amount of pre-warm trypsin was put into the cells container to detach them (3ml for T25 flask, and 5 ml of trypsin was used for T75 flask).
4. The container was incubated in 37°C incubator for 5 minutes.
5. The cells were homogenised with a pipette controller, then 1 ml of medium was added over the cells and homogenised very well.
6. 6 ml of complete growth medium was added for T25 flask or 10 ml for T75 flask to neutralize the effect of trypsin, and very well homogenisation was applied.
7. 1 ml was taken from the cells suspension in the previous step and transferred to a new flask and enough amount of complete growth medium was added according to the size of the flask (10 ml for T25 and 20ml for T75).
8. The flask was incubated at 37°C, in CO<sub>2</sub> 5% incubator. Replacing the medium is necessary in the next day to remove dead cells.

- **Luciferase-stable HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup>**

Xenograft mouse models become instrumental tools for increasing the understanding of human cancer (Aguirre et al., 2003). In addition, they provide an essential means to evaluate the efficacy of therapeutic intervention (Shah NP et al., 2004). Through the application of recent available imaging techniques and reagents, it is possible to measure the effects of a drug on xenograft tumour proliferation or apoptosis rate. One of these techniques is bioluminescence imaging (BLI) using luciferase and its substrate. The advantages of luciferase bioluminescence imaging are: non-invasive, sensitive, and provides relative measure of cell viability or cell function and metabolism (Lyons, 2005).

For generating luciferase-stable clones from HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup> for in vivo study, each cell line was cultured in a 10 cm dish until the cells reach 60-70% confluency. The transfection was carried out with Polyethylenimine (PEI) (Polysciences Inc, US) polymer mixed with the pGL4.20 [luc2/Puro] Vector (Promega, UK) (a gift from Dr. Amin Hajitou, Department of Medicine, Imperial College London) in ratio 4:1 (V/W), and the mixture was completed to 200  $\mu$ L with Opti-MEM media (Invitrogen, UK). The mixture was then dropped over the cells covered with the minimum amount of Dulbecco's modified Eagle's medium. Two days after the transfection, the stable clones from each cell line (HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup>) were selected in Puromycin for two weeks. To confirm the success of the stable clones and to check the luciferase

expression levels, Steady-Glo<sup>®</sup> Luciferase Assay System (Promega, UK) was used.

- **HDAC2-Knocked down HCT116 p53<sup>-/-</sup> and HT-29 stable clones**

Each cell line was cultured in a 10 cm dish until the cells reached 60-70% confluency. The transfection was carried out with Polyethylenimine (PEI) (Polysciences Inc, US) polymer mixed with HDAC2 shRNA Plasmid (sc-29345-SH, Santa Cruz Biotechnology, Inc.) in ratio 4:1, and the mixture was completed to 200 µL with Opti-MEM media (Invitrogen, UK). The mixture was then dropped over the cells covered with the minimum amount of Dulbecco's modified Eagle's medium. Two days after the transfection, the stable clones from each cell line (HCT116 p53<sup>-/-</sup> and HT-29) were selected in Puromycin for two weeks. The efficiency of HDAC2-knocking down compared to scramble shRNA Plasmid (sc-108060, Santa Cruz Biotechnology, Inc.) was assessed by immuno-blotting.

- **Transient HDAC2-overexpression in SW480 cells**

SW480 cells were cultured in a 10 cm dish until the cells reach 60-70% confluency. The transfection was carried out with Polyethylenimine (PEI) (Polysciences Inc, US) polymer mixed with pcDNA3.1-HDAC2 plasmid in ratio 4:1 (the plasmid was obtained from collaboration with Dr. Nicolas Mercado, Airway Disease Section, National Heart and Lung Institute, Imperial College

London). The mixture was then dropped over the cells covered with the minimum amount of Dulbecco's modified Eagle's medium. 12 hours after the transfection, the cells were treated with Dox, VPA, SAHA and a combination of them for 24 hours. Afterwards flow cytometry and immunoblotting analysis were performed.

## • Flow cytometry (FCM)

This method could be applied to fixed or live cells. In this study, the flow cytometry (FCM) analyses were applied on live cells to measure their viability upon treatment using Propidium iodide (PI) dye. After cells were collected as stated above in the cells collection protocol (step 1 to 11), they were then incubated with PI dye as in the table 2.6 and analysed using the FCM machine (BD FACSCalibur<sup>TM</sup>, BD Biosciences). The data were analyzed using the Flowing Software (free software downloaded from <http://www.flowingsoftware.com/>). Raw data was analysed using dot plots in which X-axis forward scatter (FSC) Y-axis (side scatter SSC). Cellular debris was excluded by gating. Percentages of live and dead cells were assessed by a histogram using X-axis FL3-H as shown in figure 2.9.

| Table 2.6: FCM dye used in this study |                            |                 |
|---------------------------------------|----------------------------|-----------------|
| Dye                                   | Incubation period          | Analysis filter |
| PI                                    | 30 minutes at 37°C in dark | FL3             |

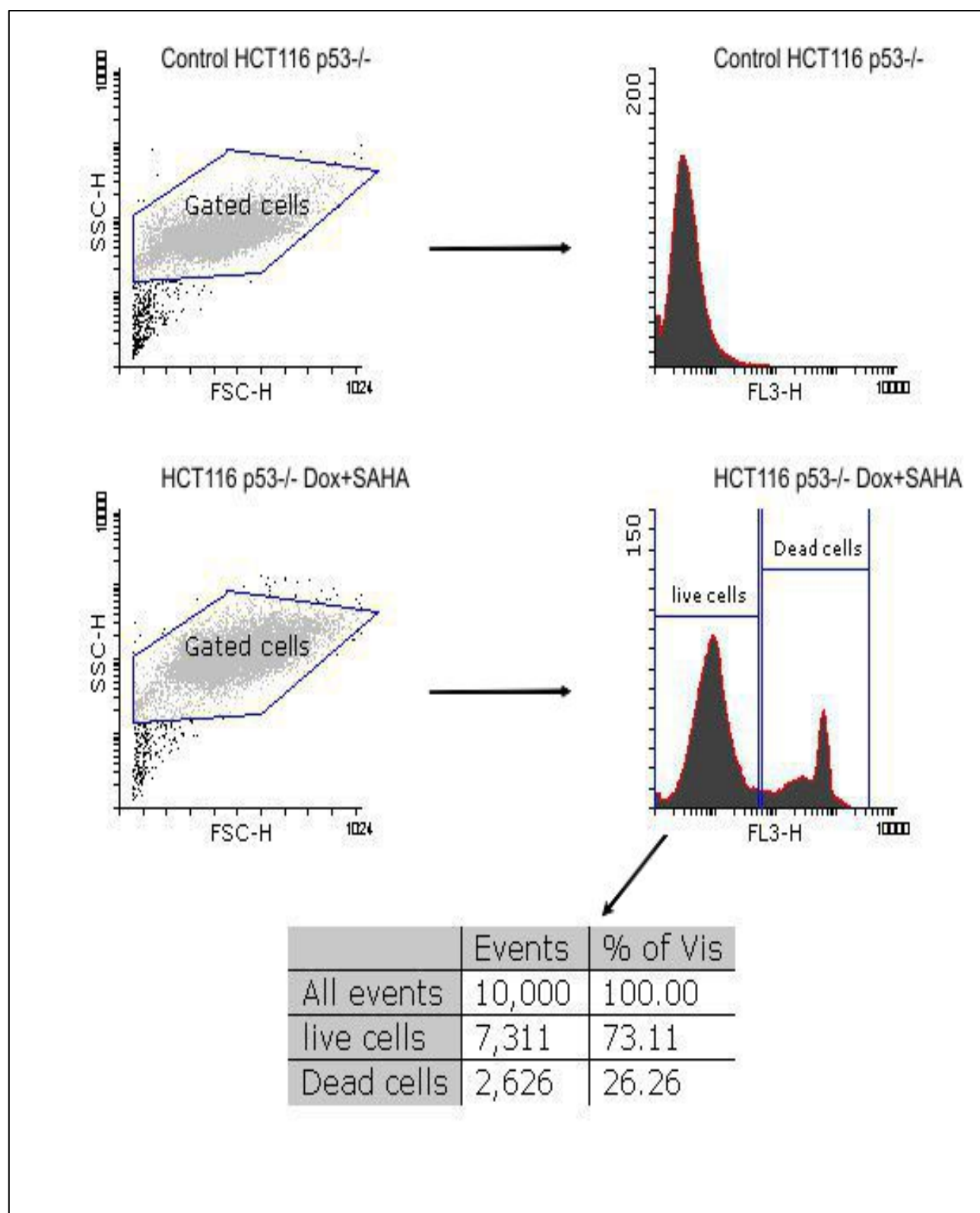


**Figure 2.9: Acquiring and gating the cells for FACS after PI staining.**

*Cells were gated in order to exclude cellular debris and aggregates. The percentage of dead cells were acquired from 10000 cells after plotting the gated cells in histogram using X-axis FL3-H.*

- **Immunofluorescence**

It is a technique used for identifying simultaneously the localization, distribution, and quantifications of proteins within cells using labelled antibodies which transmit fluorescence when they are excited by laser. Confocal microscope TCS SP5 II (Leica) was used for this purpose. The protocol was as follows:

Day 1: under the safety cabinet class II, three autoclaved glass cover-slips were placed in each well of a 6 wells plate. The cells were then seeded over the cover-slips in a density of 150,000 - 200,000 cells/well and the plate was incubated at 37°C.

Day 2: the medium was replaced with new one and the required treatment was applied. The plate was re-incubated at 37°C.

Day 3: the medium was removed and the cells were rinsed twice with pre-warm PBS 1X at room temperature. The cells were fixed with 1 ml/well of 4% (w/v) paraformaldehyde in PBS 1X for 10 min at 4°C. After the fixation, the cover-slips were rinsed three times with PBS 1X followed by adding 1 ml/well of blocking/permeabilization buffer (10mM HEPES+ 0.3% TX-100+3% BSA, PH=7.4) for 1 hour at room temperature. The cover-slips were then incubated with the primary antibodies (the concentration is applied according to the manufacturer's instruction) in a humid chamber over night at 4°C.

Day 4: the cover-slips were placed in the 6 wells plate and washed three times with PBS 1X. Afterwards the cover-slips were incubated with the corresponding secondary antibodies, Alexa Fluor® (Invitrogen, UK) for 1 hour in a humid chamber at 37°C. After washing three times with PBS 1X, the cover-slips were mounted on immuno-staining slides over a drop (10µl) of Vectashield mounting

medium (Vector Laboratories, UK) and sealed with nail polish. The slide were examined directly under the confocal microscope or stored in the dark at 4°C for later use.

## • Total protein extraction

This step starts from the frozen cells pellets which were prepared as in the cells collection protocol. The extraction was done as follows:

1. The eppendorfs containing the cells pellets were put on ice and between 80-200µl (depending on the size of the pellet) of total protein extraction buffer (TGN) was added. The composition of TGN buffer is shown in table 2.1. The stock can be distributed in 10 ml aliquot and stored in the freezer. 2 tablets of protease inhibitor (Roche, UK) were added to 10ml TGN before use.

| Table 2.1: TGN buffer stock (50 ml)          |        |
|----------------------------------------------|--------|
| Substances                                   | Volume |
| 1 M Tris-HCL PH=6.5 (Sigma, UK)              | 2,5 ml |
| 2.5 M NaCl (VWR Prolab, UK)                  | 3 ml   |
| Glyserol (99%) (Sigma, UK)                   | 5 ml   |
| 0.5 M β glycerophosphate (Sigma, UK)         | 5 ml   |
| Tween 20 (Sigma, UK)                         | 0.5 ml |
| Nonidet P40 (Fluka, UK)                      | 100µl  |
| Fill up with miliQ dH2O (total volume 50 ml) |        |

2. The eppendorfs were mixed and kept on ice for 10 minutes.
3. The following cycle was repeated 3 times (mixing by vortexing, 10 minutes incubation).

4. Then the eppendorfs were centrifuged at maximum speed (13000-14000 RPM), at 4° C for 10 minutes.
5. The supernatant containing total proteins was transferred to a new eppendorf and the pellet was discarded.
6. The total proteins eppendorfs were stored in the -20°C freezer for further processing.

## ● **Histones extraction**

This protocol was used to extract pure histones from cells to investigate post-translational histone modifications. The procedure starts from the frozen cells pellets which were prepared in the cells collection protocol.

1. The eppendorfs containing the cells pellets were put on ice (it is necessary for histones extraction to be done on ice), and 1 ml of lysis buffer (table 2.2) was added to the cells and the mixture was homogenized very well. Then the eppendorfs were incubated on ice for 15 minutes. The stock of lysis buffer should be stored at 4°C.
2. The eppendorfs were centrifuged at 3500 RPM for 10 minutes at 4°C and the supernatant was removed.
3. The pellets were washed again with 1 ml of lysis and incubated on ice for 15 minutes.
4. The eppendorfs were centrifuged at 3500 RPM for 10 minutes at 4°C and the supernatant was removed.
5. Then the pellets were washed with 1 ml of ice-cold (1ml) Tris-EDTA prepared as shown in table 2.3.

| <b>Table 2.2: Histones lysis buffer stock (500 ml)</b>     |                       |                            |
|------------------------------------------------------------|-----------------------|----------------------------|
| <b>Substances</b>                                          | <b>Volume/ amount</b> | <b>Final concentration</b> |
| Tris-HCL PH=6.5 (Sigma, UK)                                | 0.605 g               | 10 mM                      |
| Sodium bisulphate (Sigma, UK)                              | 3.452 g               | 50 mM                      |
| MgCl <sub>2</sub> (Sigma, UK)                              | 0.47605 g             | 10 mM                      |
| Sucrose (VWR Prolab, UK)                                   | 43 g                  | 8.6%                       |
| Triton X-100 (Sigma, UK)                                   | 5 ml                  | 1%                         |
| Fill up with miliQ dH <sub>2</sub> O (total volume 500 ml) |                       |                            |

- The eppendorfs were centrifuged at 3500 RPM for 10 minutes at 4°C and the supernatant was removed, the pellets (the nuclei) were then suspended in either 50 or 100 µl of ice-cold miliQ dH<sub>2</sub>O depending on the size of the pellet.

| <b>Table 2.3: Tris-EDTA buffer stock (500 ml)</b>          |                       |                            |
|------------------------------------------------------------|-----------------------|----------------------------|
| <b>Substances</b>                                          | <b>Volume/ amount</b> | <b>Final concentration</b> |
| Tris-HCL PH=7.4 (Sigma, UK)                                | 0.605 g               | 10 mM                      |
| EDTA (Sigma, UK)                                           | 2.4 g                 | 13 mM                      |
| Fill up with miliQ dH <sub>2</sub> O (total volume 500 ml) |                       |                            |

- The eppendorfs were centrifuged at 3500 RPM for 10 minutes at 4°C and the supernatant was removed, the pellets (the nuclei) were then suspended in either 50 or 100 µl of ice-cold miliQ dH<sub>2</sub>O depending on the size of the pellet.
- Then cold sulphuric acid, H<sub>2</sub>SO<sub>4</sub> (VWR, UK) 0.4 M, was added in amounts similar to the water amounts (50 µl of H<sub>2</sub>SO<sub>4</sub> if 50 µl of water is added).
- The eppendorfs were vortexed and incubated on ice for 1 hour.

10. The eppendorfs were centrifuged at 15000 RPM for 60 minutes at 4°C and the supernatant was transferred to the new tube.
11. Acetone (VWR, UK) (1 ml) was added to each new eppendorf, and brief centrifugation at high speed (15 seconds) was applied before overnight incubation at -20°C freezer.
12. The next day, the eppendorfs were centrifuged at 15000 RPM for 10 minutes at 4°C and the supernatant was removed.
13. The white pellet in each eppendorf was left to air-dry.
14. Finally, the pellet was dissolved in 50-100 µl miliQ dH<sub>2</sub>O depending on the size of the pellet, and the eppendorfs were stored for further processing.

- **Bradford protein assay**

This assay is used to measure the concentration of proteins (including histones) depending on generating a standard line for serial dilutions (from 1 to 5 µg/µl) of a known concentration solution of Bovine serum albumin (BSA) (Sigma, UK). The reagent for this assay is called Coomassie® Brilliant Blue G-250 (Bio-Rad, UK) or commonly known Bradford solution. It was diluted 1/5 in miliQ dH<sub>2</sub>O before use, then it was distributed in amount of 200 µl in each well of a 96 wells plate. Each unknown sample (1 µL) was added to one well containing Bradford solution and the absorbance was measured at 600 nm wave-length using a GloMax®-Multi Detection System (Promega, UK). The measured absorbance values were dealt according to the standard line equation ( $Y = a.X + b$ ) to obtain the protein concentration. Y=the protein's measured absorbance, X= the protein's required concentration, (a, b) are constants obtained from

plotting the known concentrations of BSA serial dilutions against their corresponding absorbances.

- **Immunoblotting (western blot)**

An analytical technique allows measuring the level of a specific protein in cells using an antibody against that protein. The sequential steps of this technique were as follows:

1. Sample preparation

Total proteins or histones samples were mixed with Nupage lds sample buffer 4X (Invitrogen, UK) and miliQ dH<sub>2</sub>O according to table 2.4:

| <b>Table 2.4: Samples preparation (1X) for immunoblotting</b> |               |                   |            |
|---------------------------------------------------------------|---------------|-------------------|------------|
|                                                               | Sample volume | Nupage lds buffer | water      |
| Total proteins                                                | Contains 80µg | 5 µl              | Up to 25µl |
| Histones                                                      | Contains 10µg | 5 µl              | Up to 25µl |

The mixtures were boiled at 95°C heating block for 3 minutes to denature and reduce the protein disulfide bonds, and then the samples were centrifuged briefly and used either directly or stored in -20°C freezer. In the latter case, the boiling step must be applied to the samples before using them again.

## 2. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, UK) was used for carrying out this step. Sodium dodecyl sulphate polyacrylamide gels were cast in percentage of 12% for total proteins and 15% for histones separation, table 2.5. PageRuler™ Prestained Protein Ladder (Fermentas, UK) and TGS 1X running buffer (Bio-Rad, UK) were used in all SDS-PAGE. The electrophoresis was performed at 80V until separation of the protein ladder marker was visualized and then continued at 120V. At the end of the procedure, the gels were removed from the glass plates and prepared for the next step by removing the stacking gels. Then gels were transferred to a solid support for immunodetection.

| Table 2.5: One gel's composition |                       |                 |
|----------------------------------|-----------------------|-----------------|
| Materials                        | Total proteins<br>12% | Histones<br>15% |
| Resolving gel                    |                       |                 |
| MiliQ dH <sub>2</sub> O          | 3.2 ml                | 2.45 ml         |
| 40% polyacrylamide               | 3 ml                  | 3.75 ml         |
| 1,5M tris 8.8                    | 3.75 ml               | 3.75 ml         |
| 10% SDS                          | 0.1 ml                | 0.1 ml          |
| TEMED                            | 0.006 ml              | 0.006 ml        |
| 10% APS                          | 0.1 ml                | 0.1 ml          |
| Stacking gel                     |                       |                 |
| MiliQ dH <sub>2</sub> O          | 1.9 ml                |                 |
| 40% polyacrylamide               | 0.34 ml               |                 |
| 1,5M tris 6.8                    | 0.75 ml               |                 |
| 10% SDS                          | 0.03 ml               |                 |
| TEMED                            | 0.005 ml              |                 |
| 10% APS                          | 0.009 ml              |                 |

### 3. Transfer and immunoblotting

The proteins on gels were transferred to nitrocellulose membranes (GE Healthcare, UK) using Mini Trans-Blot Cell (Bio-Rad, UK) filled with 1X transfer buffer (20% methanol in 1X TGS buffer). The transfer was performed at 400mA for 2 hours. The nitrocellulose membranes were then blocked with 5% powdered milk in PBS-Tween buffer for 30 minutes at room temperature. After the blocking step, the membranes were washed three times for 5 minutes in 1X PBS-0.1% Tween buffer on a rocker shaker machine. After preparing the required primary antibodies according to their manufacturer's directions, the nitrocellulose membranes were incubated with them at 4°C overnight on a rocker shaker machine. The next day, the membranes were washed three times for 5 minutes in 1X PBS-0.1% Tween buffer and the corresponding species-specific horseradish peroxidase-conjugated secondary antibody was applied. The secondary antibodies, either anti-mouse (GE Healthcare, UK) or anti-rabbit (Sigma, UK), were prepared in 5% powdered milk in PBS-Tween buffer in dilution of 1/1000 (vol/vol). After 1 hour incubation with a secondary antibody at room temperature, the membranes were washed three times for 5 minutes in 1X PBS-0.1% Tween buffer, and they were then treated with an enhanced chemi-luminescent reagent (Thermo, UK) followed by an incubation in high performance chemiluminescence film (GE Healthcare, UK). A Kodak machine or a manual developing was used. The densitometry analysis of scanned films was done via ImageJ programme.

- **Real time PCR**

ThermoScript<sup>™</sup> RT-PCR System kit (Invitrogen, UK) was used in generating cDNA as follows: Total RNA (1µg) and the primer oligo (dT)<sub>20</sub> (50 pmoles), 2µl of 10 mM dNTP mix, mixed with DEPC-H<sub>2</sub>O (up to 12µl), were pre-heated to 65° C for 5 min and then place on ice. Then 8µl of the following mastermix (4µl of 5x cDNA synthesis buffer, 1µl of 0.1 M DTT, 1µl of RNaseOUT<sup>™</sup> (40 U/Yl), 1µl of DEPC-treated water, and 1µl ThermoScript<sup>™</sup> RT) was added. The final mix was transferred to a thermal cycler preheated to the appropriate cDNA synthesis temperature and incubated as 30-60 min at 50° C. Then the cDNA synthesis reaction was terminated by incubating at 85° C for 5 min.

mRNA expression of *HDAC2*, *α-TUBLIN*, and *PPIA* was quantified by Platinum<sup>®</sup> SYBR<sup>®</sup> Green qPCR SuperMix UDG (Invitrogen, UK) with a GeneAmp 7500 Fast System (Applied Biosystems, Carlsbad, CA, USA). 2µl of cDNA from each sample was mixed with 5µl SYBR green mix, 0.5µl Primer Fwd, 10 µM, 0.5µl Primer REV, 10 µM, 0.2 µl ROX, and finally the reaction was completed up to 10µl with autoclaved distilled water. Real-time PCR reactions were set following the manufacture instructions: one initial step at 95° C for 2 minutes hold followed by a 40-cycle step, which was 15 seconds at 95° C for denaturation and 30 seconds at 56° C for annealing and 30 seconds at 72° C. The dissociation stage was 15 seconds at 95° C, one minute at 56° C, and 15 seconds at 95° C. Threshold cycle (Ct) values obtained for each gene were normalized to *α-TUBLIN* and *PPIA* which are housekeeping genes.

## Primer pairs used in real time PCR analysis

| Gene                              | Forward primer<br>5'->3' | Reverse prime<br>5'->3' |
|-----------------------------------|--------------------------|-------------------------|
| <i>HDAC2</i>                      | GACAGTGGAGATGAAGATGGA    | TTCTGATTTGGTTCCTTTGG    |
| <i>PPIA</i>                       | CTGCACTGCCAAGACTGA       | GCCATTCCTGGACCCAAA      |
| <i><math>\alpha</math>-TUBLIN</i> | GCCAAGCGTGCTTTGTTC       | CACACCAACCTCCTCATAATCC  |

### • Statistical analysis for in vitro study

Results were expressed as mean  $\pm$  SEM of three independent experiments measured in triplicate. Statistical significance was assessed by one-way ANOVA with Tukey's post-hoc test, setting statistical significance at  $p < 0.05$  using GraphPad Prism version 5.3 (GraphPad Software, CA, USA).

### • List of the antibodies used for immunoblotting

Table 2.6 lists antibodies used in this study for immunoblotting, their manufacturers, sources, and dilutions.

| Antigen                         | Source | Dilution   | Company                    |
|---------------------------------|--------|------------|----------------------------|
| PARP/ cleaved PARP              | Rabbit | 1/1000     | Cell Signaling Technology® |
| Phospho-p53 (Ser15)             | Mouse  | 1/1000     | Cell Signaling Technology® |
| Anit-HDAC1                      | Mouse  | 1/1000     | Cell Signaling Technology® |
| Anit-HDAC2                      | Mouse  | 1/1000     | Cell Signaling Technology® |
| Phospho-p53 (Ser20)             | Rabbit | 1/1000     | Cell Signaling Technology® |
| Phospho-p53 (Ser37)             | Rabbit | 1/1000     | Cell Signaling Technology® |
| Acetyl-p53 (Lys382)             | Rabbit | 1/1000     | Cell Signaling Technology® |
| Anti-total p53 [DO1]            | Mouse  | 1/5000     | GeneTex                    |
| Anti-MDM2                       | Mouse  | 1 µg/ml    | Merck Millipore            |
| Anti-Phospho-Histone H3 (Ser10) | Mouse  | 1 µg/ml    | Millipore                  |
| Acetyl-Histone H3 (Lys9)        | Rabbit | 1/500      | Millipore                  |
| Anti-Histone H3, CT, pan        | Rabbit | 1/25000    | Millipore                  |
| Anti-acetyl-Histone H4 (Lys16)  | Rabbit | 0.1 µg/ml  | Millipore                  |
| Anti-acetyl-Histone H4 (Lys12)  | Rabbit | 1/20000    | Millipore                  |
| Anti-Histone H4, pan            | Rabbit | 1/30000    | Millipore                  |
| Anti-dimethyl-Histone H3 (Lys9) | Rabbit | 1/2000     | Millipore                  |
| Anti-acetyl-Histone H3          | Rabbit | 0.05 µg/ml | Millipore                  |
| Survivin                        | Rabbit | 1 µg/ml    | Novus                      |
| β-Actin                         | mouse  | 1 µg/ml    | Sigma                      |
| Secondary antibody: anti-rabbit | mouse  | 1/1000     | Sigma                      |
| Secondary antibody: anti-mouse  | Rabbit | 1/1000     | GE Healthcare              |

- **List of chemotherapeutic drugs used in this study**

1. Doxorubicin (Dox): is a powerful anti-neoplastic and antibiotic drug produced by *Streptomyces peucetius* bacterium species. Dox belongs to the anthracycline class, and it is used to treat wide spectrum of cancer types. Its mechanism of action depends on forming complexes with DNA by intercalation, and inhibiting topoisomerase II enzyme activity. Therefore, Dox induces DNA damage presented by DNA double-strand breaks (DSB) leading to cell death. The major Dox's side-effect is cardiotoxicity (*Drugbank*, <http://www.drugbank.ca/drugs/DB00997>). This drug was purchased from (Sigma, UK).
2. Camptothecin-11 (CPT-11): is an effective drug mainly used in CRC treatment. CPT-11 is one drug from the camptothecin class. The mechanism of action for CPT-11 and its active metabolite SN-38 relies on topoisomerase I enzyme inhibition. This leads to DNA single-strand breaks (SSB) which accumulate, resulting in DSB and consequently apoptosis occurs. Toxicity of CPT-11 manifests in gastrointestinal complications, such as diarrhoea, vomiting, nausea, abdominal cramping, and infection (*Drugbank*, <http://www.drugbank.ca/drugs/DB00762>). This drug was obtained from (Sigma, UK).
3. Valproic Acid (VPA): is a short fatty acid used for epilepsy treatment. However, its mechanism of action as an anticonvulsant and mood-stabilizing drug is not completely understood. It is

thought that VPA may increase gamma-aminobutyric acid levels in the brain or alter the properties of voltage dependent sodium channels (Rosenberg, 2007). Also, it has been noticed that VPA has the ability to work as a histone deacetylase inhibitor (HDACI) for Class I, IIa of HDACs and is recently under investigation for treatment of HIV and other types of cancer (*Drugbank*, <http://www.drugbank.ca/drugs/DB00313>). This drug was obtained from (Sigma, UK).

4. Vorinostat or Suberoylanilide Hydroxamic Acid (SAHA): is a well known HDACs inhibitor drug from hydroxamate class. SAHA inhibits Class I and Class II of HDACs at nanomolar concentrations. As a result, SAHA induces cell cycle arrest and/or apoptosis of some transformed cells. SAHA is under pre-registration stage for the treatment of cutaneous T cell lymphoma (CTCL) (*Drugbank*, <http://www.drugbank.ca/drugs/DB02546>). This drug was purchased from (Sigma, UK).
5. Sodium Butyrate (NaB): is a short fatty acid like VPA. The effects of NaB on cultured CRC mammalian cells are well known for a long time (Davie, 2003). NaB at millimolar concentrations inhibits proliferation, induces differentiation and induces or represses gene expression (<http://www.hdacis.com/Sodium-Butyrate.html>). This is attributed to its role as HDACI for Class I, IIa of HDACs (Smith and Workman, 2009). This drug was purchased from (Sigma, UK).

6. Cisplatin (Cisp): is a chemotherapeutic drug used to treat diverse types of metastatic cancers such as sarcomas, small cell lung cancer, ovarian cancer, and lymphomas. Cisp was the first member of Platinum-based antineoplastic drugs class which includes Oxaliplatin and Carboplatin. Cisp like all alkylating agents has three mechanisms of action: 1- Cisp attaches alkyl groups to DNA bases. This action is followed by DNA fragmentation by repair enzymes which attempt to replace the alkylated bases. As a result, DNA synthesis and RNA transcription from the affected DNA stop. 2- Cisp forms cross-link bonds between atoms in the DNA and this blocks the DNA separation for synthesis or transcription, resulting in DNA damage. 3- Cisp provokes DNA mutations due to mispairing of the nucleotides (*Drugbank*, <http://www.drugbank.ca/drugs/DB00515>). This drug was purchased from (Sigma, UK).
7. Oxaliplatin (Oxa): is in the same family of Cisp. Chemically, Oxa differs from Cisp in having a cyclohexyldiamine group replacing the two amine groups of Cisp to improve the antitumour activity. Oxa is used for the treatment of advanced carcinoma of the colon or rectum. Also it is administrated as adjuvant treatment for colon cancer patients who have had complete resection of the primary tumour. Its mechanism of action is similar to Cisp, but Oxa binds preferentially to the guanine and cytosine nucleotides of DNA (*Drugbank*, <http://www.drugbank.ca/drugs/DB00526>). This drug was purchased from (Sigma, UK).

8. 5 Fluorouracil (5FU): is a pyrimidine analog which belongs to antimetabolites family. 5FU is used as anticancer drug to treat different cancers including colon, oesophageal, gastric, rectum, and breast. Also, 5FU is used in a cream form for actinic (solar) keratoses and superficial basal cell carcinomas of the skin. The main 5FU mechanism of action is that it inhibits DNA and RNA synthesis by blocking the thymidylate synthetase conversion of deoxyuridylic acid to thymidylic acid. This inhibition leads to cell suicide and cell death (*Drugbank*, <http://www.drugbank.ca/drugs/-DB00544>). This drug was purchased from (Sigma, UK).

- **The quantification of synergistic and antagonistic effects of combined drugs used in this study**

The synergistic effect of the combination of two drugs means that the effect of using them together is higher than the sum of the effect of each drug alone. On the other hand, the antagonistic effect of the combination of two drugs together means that they produce less effect than the sum of the effect of each drug alone (Chou, 2010). The evaluation of effects of combined treatment was done using Chou and Talalay method (Chou, 2006). For each drug of a combination treatment, dose-effect curves were generated and they were used to analyze the results obtained from the combination treatment in the same experiment. The data were analyzed using CalcuSyn software (Biosoft, Cambridge, UK) to calculate the combination index (CI), when  $CI=1$  indicates an additive effect,  $CI<1$  indicates a synergistic effect, and when  $CI>1$  indicates an antagonistic effect.

## **2-In vivo study**

The in vivo work has been done by Prof Jimmy Bell's group (Ms Leigh Brody, PhD student) in collaboration. I prepared and provided luciferase-stable cell lines.

- **Cells preparation**

The above mentioned luciferase-stable clones from HCT116 P53<sup>+/+</sup> and HCT116 P53<sup>-/-</sup> were seeded at  $2.5 \times 10^5$  cells per T-25 flask (Nunc, USA) and grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK), supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich, UK) and penicillin (100U/ml)-streptomycin (100 $\mu$ g/ml) (Sigma, UK) in the presence of 5% CO<sub>2</sub> at 37°C incubator. Cells were passaged every 3-5 days while never exceeding 15 passages. Once the cells were approximately 80% confluent, they were harvested by removal of the medium, washing with PBS 1X, and followed by the addition of trypsin (Sigma-Aldrich, UK) for approximately 5 minutes. The cells were transferred to a tube, washed with PBS three times and re-suspended in serum-free DMEM and centrifuged at 1,000 RPM for 5 minutes. After the cells were counted on a haemocytometer, the medium was removed and the cells were finally re-suspended at  $1 \times 10^7$  cells per ml of serum-free DMEM for injections into the mice.

- **Liposomes' preparation**

Liposomes were prepared from stock solutions in organic solvent of N1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN),

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (polyethylene glycol)-2000 (DSPE-PEG2000) using a spontaneous vesicle formation procedure. The solvent was then removed in vacuo to ensure production of an even lipid. The film was re-hydrated and remotely loaded with a defined volume of either 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (4mM, NaCl 135mM, pH 6.5) or with the combination of Doxorubicin and SAHA, giving lipid suspensions in either case with predetermined total lipid concentration of 2.88mg/mL. The concentration of doxorubicin and SAHA was selected to approximate a 20g mouse receiving liposomal doxorubicin at a dosage of 10 mg/kg and a liposomal dosage of SAHA to be 50mg/kg. After the thin-film rehydration, the nanoparticle solution was sonicated for 1hr at 30°C, in order to form the required uniform PEGylated liposome, and buffered to a pH of 7.0. The liposomal solution was dialyzed for 18 h using the Float-A-Lyzer G2 device to remove any unencapsulated materials. The particles had a component molar ratio of 32/24/8/30 (CDAN/DOPC/DSPE-PEG PEG2000/cholesterol). Particle size and zeta potential were determined using a Malvern Zetasizer. The size of the vesicles was typically 100nm.

- **In vivo tumour model development**

All experiments were carried out in compliance with Animals (Scientific Procedure) Act 1986 under the project licence 70/6656. Cells from the previous step were injected into male Balb-c

nude/nude mice subcutaneously into the right flank in concentration of  $1 \times 10^6$  cells in 100  $\mu$ l of serum-free media, using a 25 gauge needle. Mice were divided into groups bearing tumours from two cell lines of the colon cancer origin which contain p53 in varying states of activity: Group 1 (n=8) consisted of mice bearing tumours from HCT116 p53 +/+; Group 2 (n=8) consisted of mice bearing tumours from HCT116 p53 -/-. Identification of subcutaneous nude mouse xenograft tumours took 14 to 20 days. Once tumours were palpable, they were measured by a pair of callipers and the volume estimated using the modified ellipsoidal shape equation (Jensen et al., 2008):

$$\text{Tumor volume} = 1/2(\text{length} \times \text{width}^2)$$

- **Tumour regression assessment by size and by bioluminescent imaging luciferase**

For an assessment of tumour growth, a bioluminescence imaging approach was employed using IVIS Spectrum pre-clinical optical in vivo imaging system (PerkinElmer, Massachusetts, USA). At the initial identification of the xenograft tumour, a baseline measurement was obtained using callipers and IVIS Spectrum. After baseline measurements, mice were divided into 2 groups (n=4 per group). In the first group, mice were treated intraperitoneally with a dose of 40mmol/day of Doxorubicin/SAHA combination liposome (200  $\mu$ L). In the second group, mice were treated intraperitoneally with the control HEPES liposome (200  $\mu$ L) in parallel. For the duration of the study, intraperitoneally injections were administered

three times a week with parallel callipers and IVIS measurements taken weekly. For visualization by luminescence, the Xeogen IVIS-200 (PerkinElmer, Massachusetts, USA) optical imaging system was used. Briefly, an IVIS 200 cooled CCD camera system was used for emitted light acquisition and Living Image software (PerkinElmer, Massachusetts, USA) for data analysis as an overlay on IGOR software (Wavemetrics Corporation, USA). Animals were given a 100  $\mu$ l subcutaneous injections in the scruff of the neck of D-luciferin (Gold Biotechnology, USA) at a concentration of 15 mg/ml under 1-2% inhaled Isoflurane anaesthesia. An average of 10 kinetic bioluminescent acquisitions were collected between 0 and 30 minutes after substrate injection to confirm a peak photon emission recorded as maximum photon efflux per second. Data analyses and background correction were carried out by using total photon flux emission (photons/s) in a region of interest (ROI) covering the entire xenograft tumour region.

- **Tumour histological study**

The total immunohistochemistry (IHC) work was done by Dr Mona A El-Bahrawy's group in collaboration. Animals were killed and the tumours were carefully excised to make sure that the samples did not include any surrounding normal tissue. Tumours were weighed and portioned to formalin solution (Sigma-Aldrich, UK) for histological evaluation by immunohistochemistry (IHC) studies as follows: After mounting the tumours' sections on slides, they were de-waxed and rehydrated by passing them through xylene and descending grades

of alcohol then rinsed in water. Then the slides were incubated for 15 minutes with 0.6% hydrogen peroxide solution. The slides were rinsed and immersed in 0.1M citrate buffer pH 6.0 and microwaved for 15 minutes in a microwave oven (750 watts) for antigen retrieval. They were then immediately cooled under running water and rinsed in phosphate buffered solution (PBS). 100µl of Protein block was added to each slide for 5 minutes. After rinsing with 0.05% PBS/Tween 20 solution for 5 minutes, the slides were incubated with 100µl of the primary antibody at 4°C overnight. The survivin (Novus biological, Cambridge), P53 (Dako, CA), and HDAC2 (Cell Signaling Technology, MA) antibodies were used at the following dilutions 1/400, 1/200, and 1/200 respectively. Following overnight incubation, slides were washed with 0.05% PBS/ Tween 20 solution. The slides were then incubated with secondary antibody (rabbit anti-mouse, Vector laboratories) for 30 minutes at room temperature. The slides were stained using the ABC kit (Vector laboratories), and counterstained by haematoxylin for 2 minutes then rinsed in tap water for 5 minutes. Slides were then dehydrated in ascending grades of alcohol and cleared in 3 changes of xylene. Finally, the slides were mounted using Di-N-Butyl Phthalate in Xylene (DPX) mountant and covered with a glass coverslip. For negative controls, duplicate slides from each case were used. These slides were incubated with 100µl antibody diluent instead of primary antibody.

- **Human samples used in this study**

In collaboration with Dr Mona El-Bahrawy (Department of Histopathology, Imperial College London), 10 human normal liver samples and 10 human CRC hepatic metastases were obtained from the archived material of the Department of Histopathology, Hammersmith Hospital, London, United Kingdom. Ethics approval for the use of human tissue for research was obtained from the Institutional Board of the Tissue Bank of Imperial College Healthcare NHS Trust.

- **Statistical analysis for in vivo study**

Results were expressed as mean  $\pm$  SEM of three independent experiments. Statistical significance was assessed by one-way ANOVA with Tukey's post-hoc test, setting statistical significance at  $p < 0.05$  using GraphPad Prism version 5.3 (GraphPad Software, CA, USA).



# **Chapter 3**

## **Results**

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# **1-Characterization of response of wild-type, null, and mutated p53 mCRC cell lines to DNA damaging agents.**

## **Summary:**

Mutations in *TP53* suppressor gene are a notable event which takes place in the late stages of the complex tumourigenesis of colorectal cancer (Iacopetta et al., 2006), whereas the early stages maintain a wild-type *TP53* gene but acquire other genetic (*APC* and *RAS*) or epigenetic alterations (e.g. DNA methylation promoter silencing) that compromise the p53 response (Worthley et al., 2007). However, the alteration of p53 protein function followed by increased aggressiveness of tumour growth is controversial (Kern et al., 2002). In this section, I investigated the importance of p53 protein in the induction of metastatic colorectal cancer (mCRC) cell death by DNA damaging agents. I found that in the four mCRC cell lines used in this study, wild-type p53 protein is a key element in the sensitivity to DNA damaging agents. However, p53 mutations alone do not play an important role in resistance to DNA damaging agents. SW480 cells have two p53 mutations (one of them is similar to the only p53 mutation in HT-29 cells) yet these cells are more sensitive than HCT116 p53<sup>-/-</sup> (lack *TP53* gene) and HT-29 cells (have one p53 mutation). Moreover, HCT116 p53<sup>-/-</sup> cells lack p53 proteins but they showed better response than HT-29 cells to DNA damaging agents.

## **Laboratory work and results:**

Wild-type p53 HCT116 cell line (HCT116 p53<sup>+/+</sup>) was treated with an increasing concentration (0.1-3  $\mu$ M) of Doxorubicin (Dox). In p53 positive cells the use of 0.5 $\mu$ M of Dox was sufficient to phosphorylate the p53's serine residues (Ser15, Ser37, and Ser20) in sequence, and to stabilize p53

as a result. Also 0.5μM of Dox induced PARP cleavage (a hallmark of apoptotic cell death). Acetylated K382 residue in p53 and substantial PARP cleavage (PARPc) appear with 1 μM Dox, figure 3.1A. To check the role of p53 in controlling the sensitivity to doxorubicin, p53 wild-type (HCT116 p53+/+) and null isogenic *TP53* (HCT116 p53-/-) were treated with 1μM Dox and PARP cleavage was checked by immunoblotting. As expected, HCT116 p53-/- cells were less sensitive to 1μM Dox treatment and showed less cell death in comparison with HCT116 p53+/+, figure 3.1B.

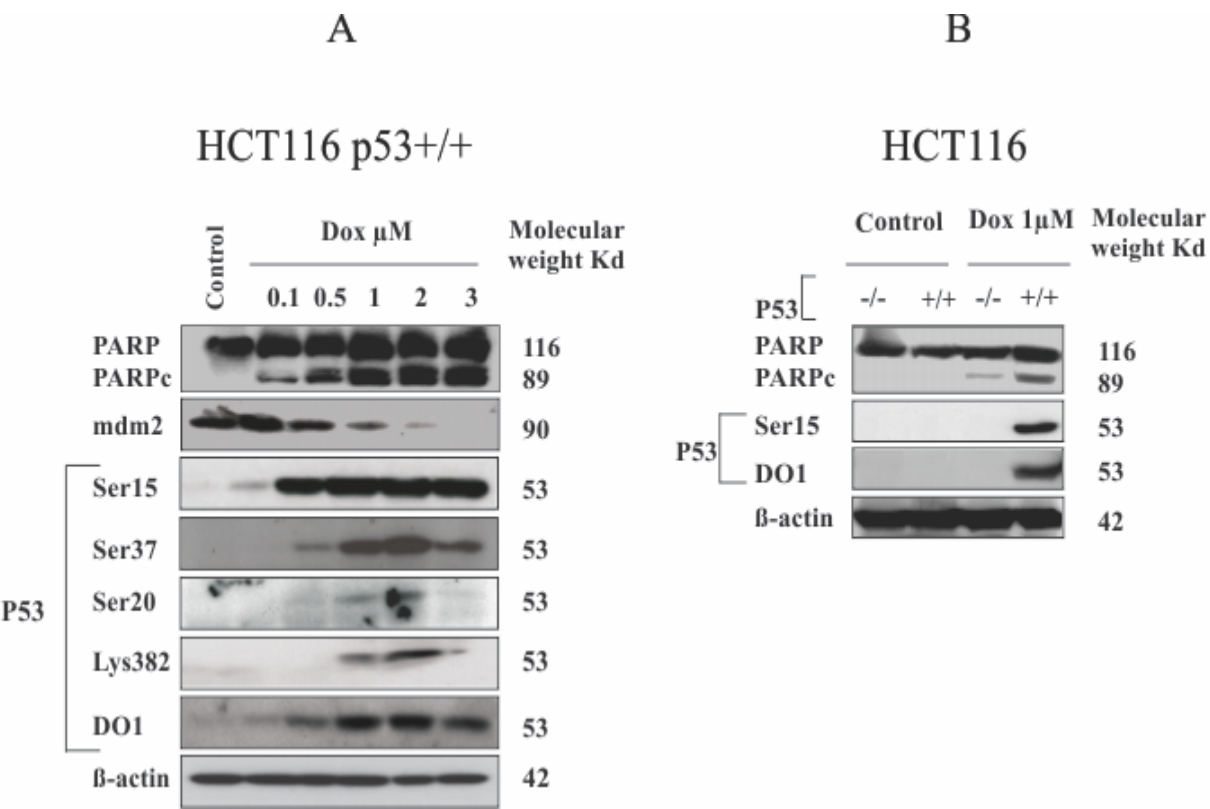


**Figure 3.1: Characterization of HCT116 p53+/+ and HCT116 p53-/- response to Dox.**

A) HCT116 p53+/+ cells were treated with dose-increase of Dox for 24 hours, cell death was confirmed by PARP cleavage (PARPc) expression, also activation and stabilization of p53 were assessed. B) Comparison of cell death upon Dox 1μM between HCT116 p53+/+ and HCT116 p53-/. β-actin was used as a loading control.

In order to substantiate once more the importance of intact *TP53* gene in regulating DNA damaging response, HT-29 and SW480 cells (both have a mutated *TP53* gene, see cell lines used in this study paragraph for more details) were also treated with a dose-increase of Dox for 24 hours followed by cell death measured by Poly (ADP-ribose) polymerase (PARP) and its cleaved form (PARP cleavage which is a hallmark of apoptosis) expression. I found that HT-29 cells (which have one mutation in the DNA binding domain of *TP53* gene) are the most resistant to Dox among the tested cell lines as this treatment failed to induce PARPc in HT-29 even at high concentration (2  $\mu$ M), whereas SW480 cells (which have two mutations in *TP53* gene, one of them similar to HT-29 P53 mutation and the other mutation is in oligomerization domain) responded to Dox, and they were even more sensitive than HCT116 p53<sup>-/-</sup> cells, figure 3.2.

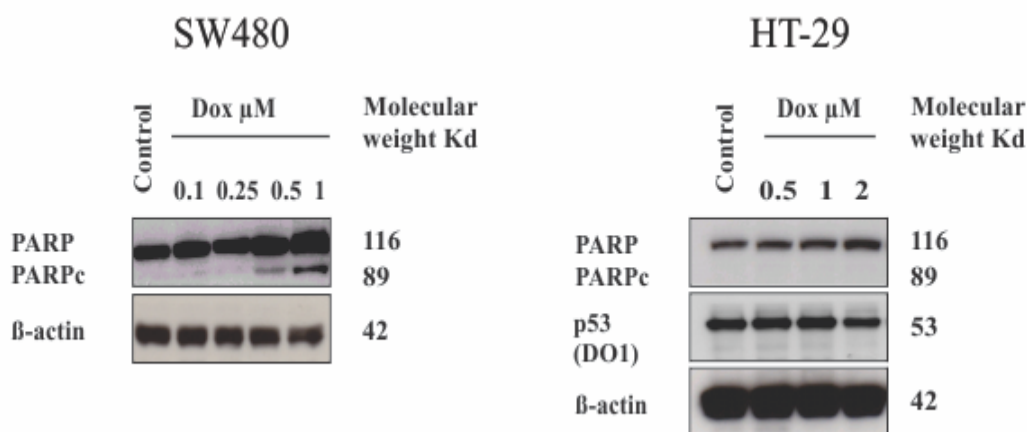


**Figure 3.2: Characterization of SW480 and HT-29 response to Dox.**

A) Cell death in SW480 upon an increasing dose of Dox. B) Checking cell death by measuring PARPc expression and p53 level in HT-29 cells after 24 hour treatment for Dox 0.5, 1 $\mu$ M, and 2 $\mu$ M.  $\beta$ -actin was used as a loading control.

To show the treatment response of Dox among the four tested cell lines in a clear and unified way, quantification of PARPc bands upon Dox 1 $\mu$ M in each cell line was presented in figure 3.3. The graph shows that wild type p53 cells (HCT116 p53+/+) are the most sensitive cells followed by SW480 cells (which have two p53 mutations). In addition, null p53 cells (HCT116 p53-/-) is less sensitive than SW480, and HT-29 cells (which have one p53 mutation) is the most resistant cells in this study.

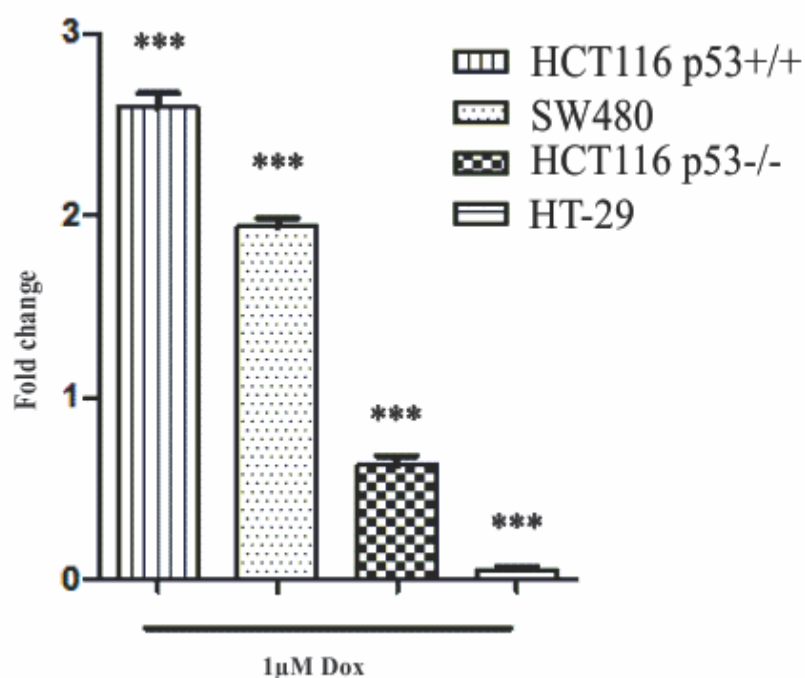


**Figure 3.3: Quantification of PARPc upon 1 $\mu$ M Dox treatment in HCT116 P53+/+, HCT116 P53-/-, SW480, and HT-29.**

*The band of PARPc corresponding to Dox 1 $\mu$ M for each cell line was quantified relative to the control cells of each cell line using ImageJ program. Data was presented as mean  $\pm$  S.E.M.; n=3. One-way ANOVA, Tukey's post-hoc test, statistical level 95%. \*\*\* denotes statistical significance ( $p < 0.0001$ ).*

To investigate whether or not the difference in sensitivity of analysed cell lines is Dox-specific, several more drugs were tested. Therefore, the first-line chemotherapy to treat mCRC: 5-fluorouracil (5FU) and Oxaliplatin (Oxa), and the most widely used chemotherapeutic agents in cancer treatment, Cisplatin (Cisp) and Irinotecan or Camptothecin-11 (CPT-11) were applied to HCT116 p53<sup>+/+</sup> cells (as the most sensitive among the tested cells) and HT-29 cells (as the most resistant cells among the tested cells). Selected low and high concentrations were used of each drug for HCT116 p53<sup>+/+</sup> cells, whereas the high concentrations of selected drugs were used for HT-29 cells, figure 3.4A. The cell death checked by PARPc showed that HCT116 p53<sup>+/+</sup> cells responded in a dose-dependent manner with applied treatment. However, HT-29 cells were resistant to all drugs as they did not show any cell death measured by PARPc and flow cytometry, figure 3.4B. These results revealed that difference in response to treatment is not Dox-specific, and it is general characterizations of each cell type.

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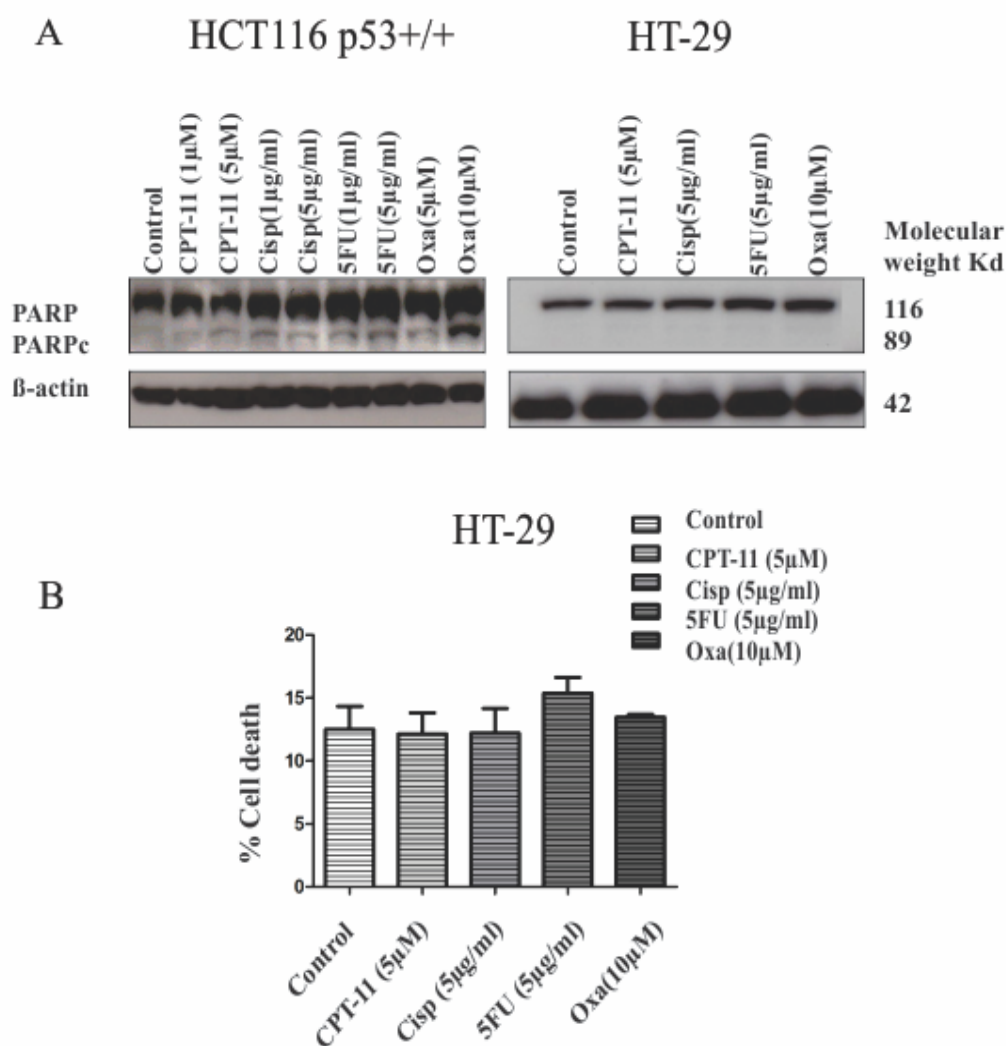


**Figure 3.4: Characterization of HCT116 P53+/+ and HT-29 response to CPT-11, Cisp, 5FU, and Oxa.**

A) Cell death investigated by measuring PARPc expression upon low and high dose treatment of Irinotecan or Camptothecin-11 (CPT-11), Cisplatin (Cisp), 5-fluorouracil (5FU) and Oxaliplatin (Oxa) in HCT116 P53 and HT-29 cells. As HT-29 showed a strong resistance to Dox, just high concentrations of CPT-11, Cisp, 5FU, and Oxa were applied to these cells. B) HT-29 cell death investigated by flow cytometry using Propidium iodide (PI) staining. The flow cytometry analyses were represented by mean  $\pm$  S.E.M.;  $n=3$ . The cell death upon the treatments (CPT-11, Cisp, 5FU, and Oxa) were not significant in comparison with the control ( $P=0.55$ ), and this supports the immunoblotting for these treatments in HT-29 as no PARPc bands were detected.

## **2-Selective combined treatment with HDAC inhibitors and DNA damaging agents induces different levels of sensitivity in mCRC cells.**

### **Summary:**

HDACIs modulate cellular responses to DNA damaging agents including chemotherapeutic drugs (Chen et al., 2007). Although many combinatorial strategies showed both effective and synergistic effects (Frew et al., 2009, Bruzzese et al., 2009), the exact mechanisms for this synergy are not clearly understood and they seem different according to the employed combination regimen (Dokmanovic and Marks, 2005b). Despite the fact that several HDACs inhibitors are potential drugs for CRC treatment, including Vorinostat (SAHA), Valproic acid (VPA), and sodium butyrate (NaB), few have shown promising preclinical results (Sakajiri S et al., 2005). In this section, I investigated the effects of combinations of low doses of HDACIs (VPA, SAHA, and NaB) and low doses of DNA damaging agents (Dox, CPT-11, 5FU, and Oxa) in HCT116 p53<sup>+/+</sup>, HCT116 p53<sup>-/-</sup>, and HT-29 (the combinations of 5FU and Oxa were just used for HT-29 cells as these cells showed high resistance to Dox in the previous section). The concentrations of the 5FU and Oxa were chosen from the literature, whereas the concentrations of the other drugs were from dose-increasing experiments. I found that the synergistic effects of combinations of HDACIs and DNA damaging agents are obtained in the early stages of colorectal cancer represented by HCT116 p53<sup>+/+</sup> cells, regardless of the identity of the HDACIs and DNA damaging agents. However, in the late stages of colorectal cancer (where P53 is mutated) represented by HT-29 cells; Just SAHA (as a HDAC inhibitor) combined

with specific DNA damaging agents (5FU and Oxa) can induce the synergistic effects.

### **Laboratory work and results:**

HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup> were treated with a low concentration of either a DNA damaging agent alone, Dox (0.5 $\mu$ M) and Camptothecin-11(CPT-11) (5 $\mu$ M) or combined with a HDAC inhibitor, VPA (1mM), SAHA (0.5 $\mu$ M), and sodium butyrate (1mM) for 24h. Cells were incubated with propidium iodide (PI) and then analysed for cell death by flow cytometry. Significant cell death was observed upon SAHA and NaB as a single treatment in HCT116 P53<sup>+/+</sup> but only a slight increase in HCT116 p53<sup>-/-</sup>, figure 3.5 A. In addition, all combined treatments in HCT116 p53<sup>+/+</sup> induced a synergistic effect on cell death in comparison with the single treatment, whereas HCT116 p53<sup>-/-</sup> showed a synergistic effect only with Dox/SAHA. Moreover, an antagonistic effect on cell death was observed with the combinations Dox/VPA, Dox/NaB, CPT/SAHA, and CPT/NaB in the HCT116 p53<sup>-/-</sup>, figure 3.5 A. As HT-29 cells were more resistant to Dox treatment than the other two cell lines (HCT p53<sup>+/+</sup> and HCT p53<sup>-/-</sup>), more drugs were tested with this cell line. Therefore, in addition to Dox (0.5 and 2  $\mu$ M) and CPT-11(5  $\mu$ M), 5-fluorouracil (5FU) and Oxaliplatin (Oxa) were used as a single treatment or combined with SAHA (0.5  $\mu$ M). The combined treatments, SAHA/5-FU (5  $\mu$ g/ml) and SAHA/Oxa (10  $\mu$ M) analysed by flow cytometry showed synergistic effects on cell death in comparison with the single treatment. However, Dox 0.5  $\mu$ M or 2  $\mu$ M combined with SAHA and CPT/SAHA exhibit antagonistic effects, figure 3.5 B.

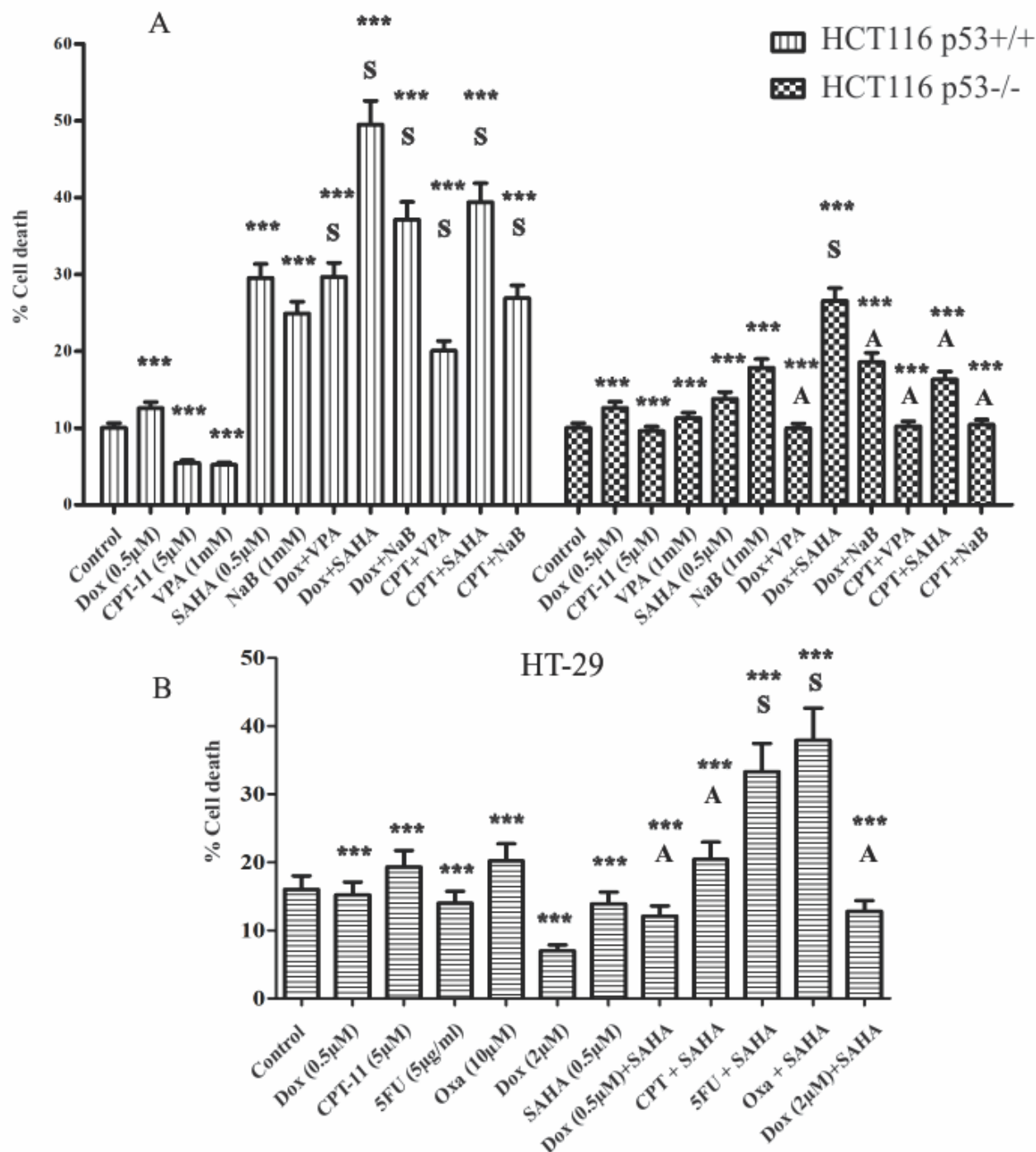


**Figure 3.5: Selective combined treatment of HDAC inhibitors and DNA damaging agents induces different levels of sensitivity to cell death in HCT116 p53+/+, HCT116 p53-/-, and HT-29.**

Cell death was analysed by flow cytometry using Propidium iodide (PI) staining after different treatment combinations of Dox, CPT, 5FU and SAHA for 24 hours. A) HCT116 p53+/+, HCT116 p53-/-. B) HT-29 cells. S denotes a synergistic effect and A denotes an antagonistic effect. Error bars were represented by the mean  $\pm$  S.E.M.;  $n=3$ . \*\*\* $P<0.001$ .

### **3-Doxorubicin combined with SAHA or VPA triggers decrease in histone acetylation correlating with the degree of response to applied treatments.**

#### **Summary:**

Modifications of histones taking place in several histone lysine residues play a role in gene activity (Jenuwein, 2006). For example, the modifications (methylation and acetylation) of specific lysines in histone H3 or H4 is associated with gene silencing and cancer development (Kondo et al., 2008, Fraga et al., 2005). In addition, histone lysine residues modifications are considered to be informative of tumour stage in different types of cancer (Stirzaker et al., 2004). Moreover, it has been found that elevated acetylation level in specific lysine 9 residues in H3 participates in the development of multidrug resistance in breast cancer cells (Toth et al., 2012). In this section, I investigated the acetylation levels in lysine 9 in H3 (H3K9ac) and lysine 12 (H4K12ac) and 16 in H4 (H4K16ac) upon a combinatorial treatment with Dox (DNA damaging agent) and VPA or SAHA (HDACIs) in HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29 cell lines. I found that in HT-29 cells which were very resistant to the applied treatment, both combination treatments (Dox/VPA or Dox/SAHA) induced elevated levels of acetylation in the three tested lysine residues. In contrast a sharp decrease in acetylation levels in those residues occurred in HCT116 p53+/+ cells which were highly sensitive to the applied treatment. However, moderate decrease in acetylation levels in lysine 9 in H3 and lysine 12 in H4 and no change in lysine 16 in H4 took place in HCT116 p53-/- and SW480 cells which were moderately sensitive upon both combined treatments. It seems that the effect of combined treatment

on histone acetylation is p53-independent. This correlation between response to treatment and acetylation levels in the tested lysine residues was an indication for HDAC investigation which I did in the next section.

### **Laboratory work and results:**

HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29 cell lines were treated with low concentrations of Dox (0.5 $\mu$ M), VPA (1mM), and SAHA (0.5 $\mu$ M) and their combinations Dox/VPA and Dox/SAHA for 24 hours. Cells were collected and total proteins and histones were then extracted and exposed to SDS-PAGE on 12% for total proteins and 15% gels for histones. After the transfer and blocking steps, antibodies against PARPc,  $\beta$ -actin, H3K9ac, total histone 3 (H3), H4K12ac, H4K16ac, and total histone 4 (H4) were used. HCT116 p53+/+ cells which responded positively to the applied treatment (figure 3.6A) showed noticeable decrease in acetylation levels in H3K9ac, H4K12ac, and H4K16ac upon combined treatments Dox/VPA and Dox/SAHA, as appeared in immunoblotting images and their quantifications (figure 3.6 A and B). However, HT-29 cells which were very resistant to high concentrations of Dox or its combinations (figure 3.2, 3.6A) exhibited very high levels of acetylation in the three lysine residues upon all single or combined treatments Dox/VPA and Dox/SAHA, figure 3.6 A and B. HCT116 P53-/- and SW480 cell lines which were between HT-29 and HCT116 p53+/+ in their sensitivity to Dox and its combinations (figure 3.2 and 3.6A) displayed moderate decrease in acetylation levels in H3K9ac and H4K12ac with no change in H4K16ac residues, figure 3.6 A and B. These results reveal a correlation between the acetylation levels in the tested lysine residues and sensitivity to chemotherapeutic drugs, and they

indicate the involvement of HDAC members in this relationship. This indication was investigated and established in the next section.

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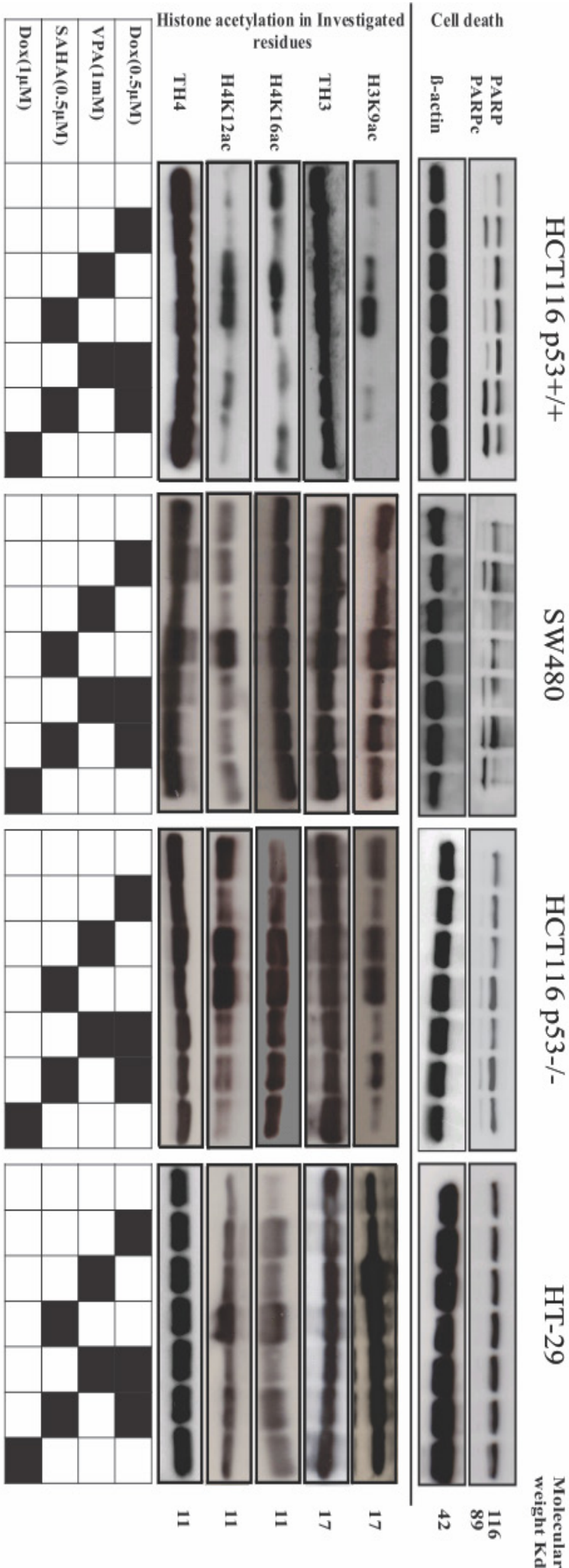
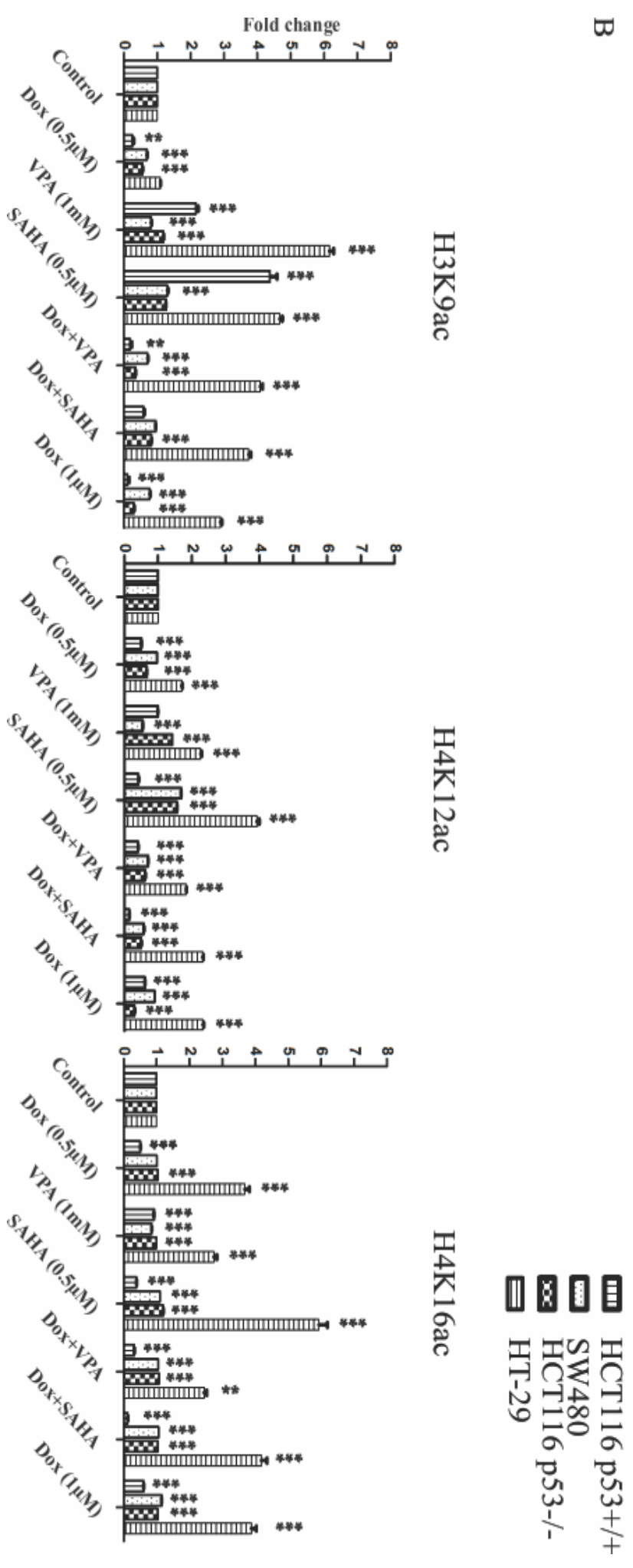


**Figure 3.6 A: Doxorubicin combined with SAHA or VPA triggers decrease in histone acetylation correlating with the degree of cell death upon applied treatments.**

*HCT116 p53<sup>+/+</sup>, HCT116 p53<sup>-/-</sup>, SW480, and HT-29 cell lines were treated with Dox (0.5μM and 1μM), VPA (1mM), SAHA (0.5μM) and their combinations (Dox/VPA and Dox/SAHA) for 24 hours. Cell death was investigated by measuring PARPc expression in parallel with the acetylation status in the following histone lysine residues: H3K9, H4K12, and H4K16. TH3, TH4=total histone 3 and 4, respectively.*



#### **4-Increased HDAC2 expression linked with p53 status represents a key factor in drug resistance when Dox is combined with VPA or SAHA in the mCRC cell lines used in this study.**

##### **Summary:**

HDAC2 over-expression and p53 mutations are significantly associated with advanced stages and poor prognosis in CRC patients (Hanigan et al., 2008). However, the relationship between HDAC2 and p53 status is not completely understood (Ito et al., 2002) nor the role and regulation of HDAC2 expression level upon CRC drug treatments. In this section, I checked the levels of HDAC1 and 2 and their relationship with p53 status in untreated mCRC cell lines. In addition, I examined the HDAC2 alterations in response to Dox and its combined treatment with HDACIs, VPA and SAHA. I found that there is no correlation between HDAC1 and the response to the applied treatment. However, I found that the relationship between HDAC2 levels and p53 status depends on the functionality of p53 per se in normal conditions (without treatment) as the untreated HCT116 p53<sup>+/+</sup> cell line (which has intact and functional p53, wild-type p53) expresses high level of HDAC2, whereas the level of HDAC2 is very low in the cell line which either lacks p53 totally (HCT116 p53<sup>-/-</sup>) or has mutated p53 (SW480 and HT-29). Moreover, the high level of HDAC2 in HCT116 p53<sup>+/+</sup> decreases along-side the response to Dox combined with VPA or SAHA treatment while the low level of HDAC2 in HCT116 p53<sup>-/-</sup>, SW480, and HT-29 increases alongside the resistance to the applied treatment, and the highest HDAC2 increase is in HT-29. These findings are consistent with the results found

in the previous section. In addition, these results show that HDAC2 is a crucial player in chemotherapy resistance in the tested cell lines.

### **Laboratory work and results:**

In the previous section, I found that elevated levels of acetylation developed alongside the resistance to Dox as single treatment or combined with VPA and SAHA in HT-29 cell line, whereas the acetylation levels decreased alongside the response to the same treatment in HCT116 p53<sup>+/+</sup>, HCT116 p53<sup>-/-</sup>, and SW480 cell lines, figure 3.6 A and B. This was an indicator to check the main HDAC enzymes, HDAC2 which is over-expressed in mCRC (Song J et al., 2005) and HDAC1 which usually forms a complex with HDAC2 (Hayakawa and Nakayama, 2011). Therefore, I applied Dox and its combination with VPA and SAHA treatment which was used for checking the acetylation status in the previous section. HCT116 p53<sup>+/+</sup>, HCT116 p53<sup>-/-</sup>, SW480, and HT-29 cell lines were treated for 6 and 24 hours to check respectively for the early and late effects of the treatment. Expression of HDAC1 and 2 were investigated alongside PARPc (cell death indicator) for each treatment. At 6 hours treatment the HCT116 p53<sup>+/+</sup> cell line exhibited noticeable sensitivity to both combination VPA/Dox and SAHA/Dox but not to the single treatment as measured by PARPc, figure 3.7 A. This sensitivity correlated with marked decrease in HDAC2 expression figure 3.7 A and B. However, HCT116 p53<sup>-/-</sup>, SW480 and HT-29 showed pronounced increase of HDAC2 upon single or combined treatments which correlated with resistance to the treatment, figure 3.7 A and B. To check whether the protein changes of HDAC2 levels are transcriptional or not, a preliminary real time PCR analysis was done in HCT116 p53<sup>+/+</sup> and HT-29 upon 6

hours treatment of Dox, VPA and VPA/Dox. Interestingly, I found that changes in HDAC2 levels are on transcriptional levels, figure 3.8. At 24 hours treatments all cell lines showed different cell death responses to the applied treatment and they can be classified in their sensitivity as follows: HCT116 p53<sup>+/+</sup> being the most sensitive, then SW480, HCT116 p53<sup>-/-</sup>, and finally HT-29 as shown in figure 3.9 A. The sensitivity response was in parallel with the decrease in HDAC2 in HCT116 p53<sup>+/+</sup> and SW480 cell lines, but the resistance response was in parallel with increase in HDAC2 in HCT116 p53<sup>-/-</sup> and HT-29 cell lines, figure 3.9 A and B. No response to the applied treatment has been shown in HT-29 cell line and this correlated with increasing levels of HDAC2. While HDAC2 expression level increased significantly with treatment and this correlated with drug resistance, HDAC 1 showed no clear correlation in all cell lines at 6 and 24 hours, figure 3.7 and 3.9.

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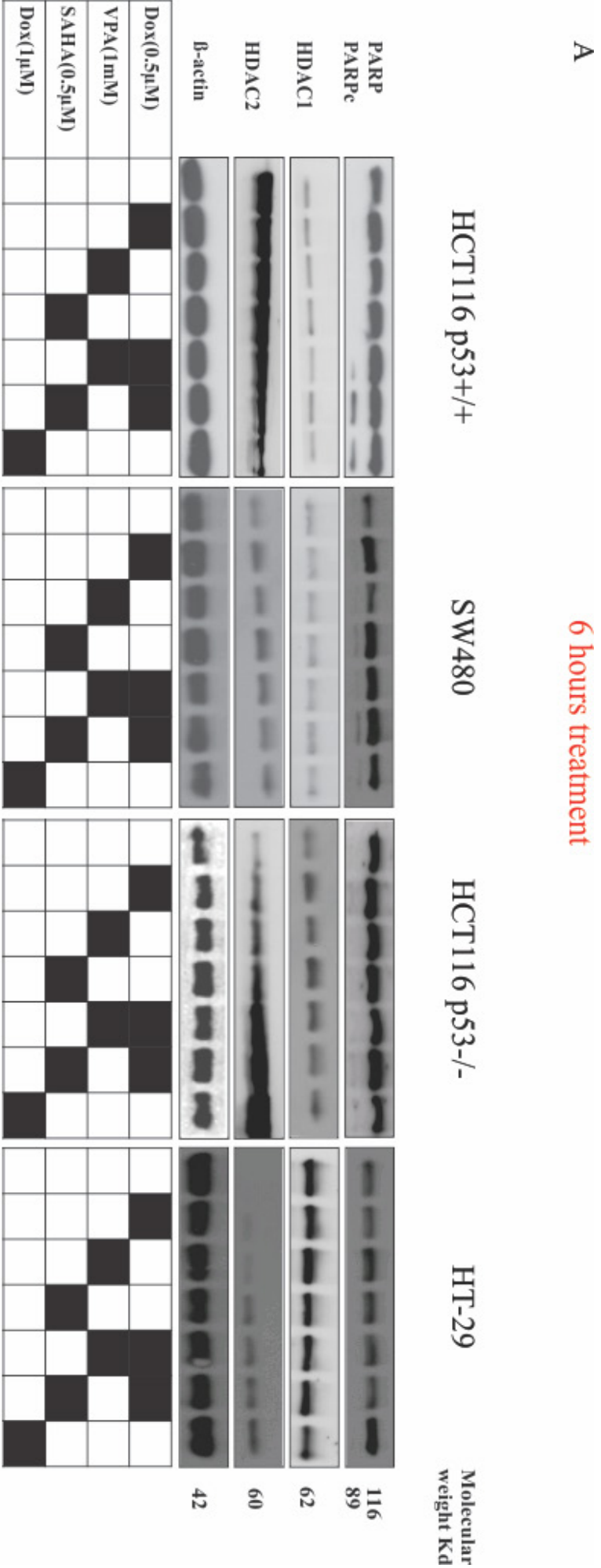


**Figure 3.7 A: HDAC1 and 2 levels upon 6 hours treatment with Dox, VPA, SAHA and their combination in HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29 cells.**

*A. PARP cleavage (PARPc), HDAC1, and HDAC 2 levels after 6 hours treatment with Dox (0.5 $\mu$ M and 1 $\mu$ M), VPA (1mM), SAHA (0.5 $\mu$ M) and their combinations (Dox/VPA and Dox/SAHA).  $\beta$ -actin was used as a loading control.*

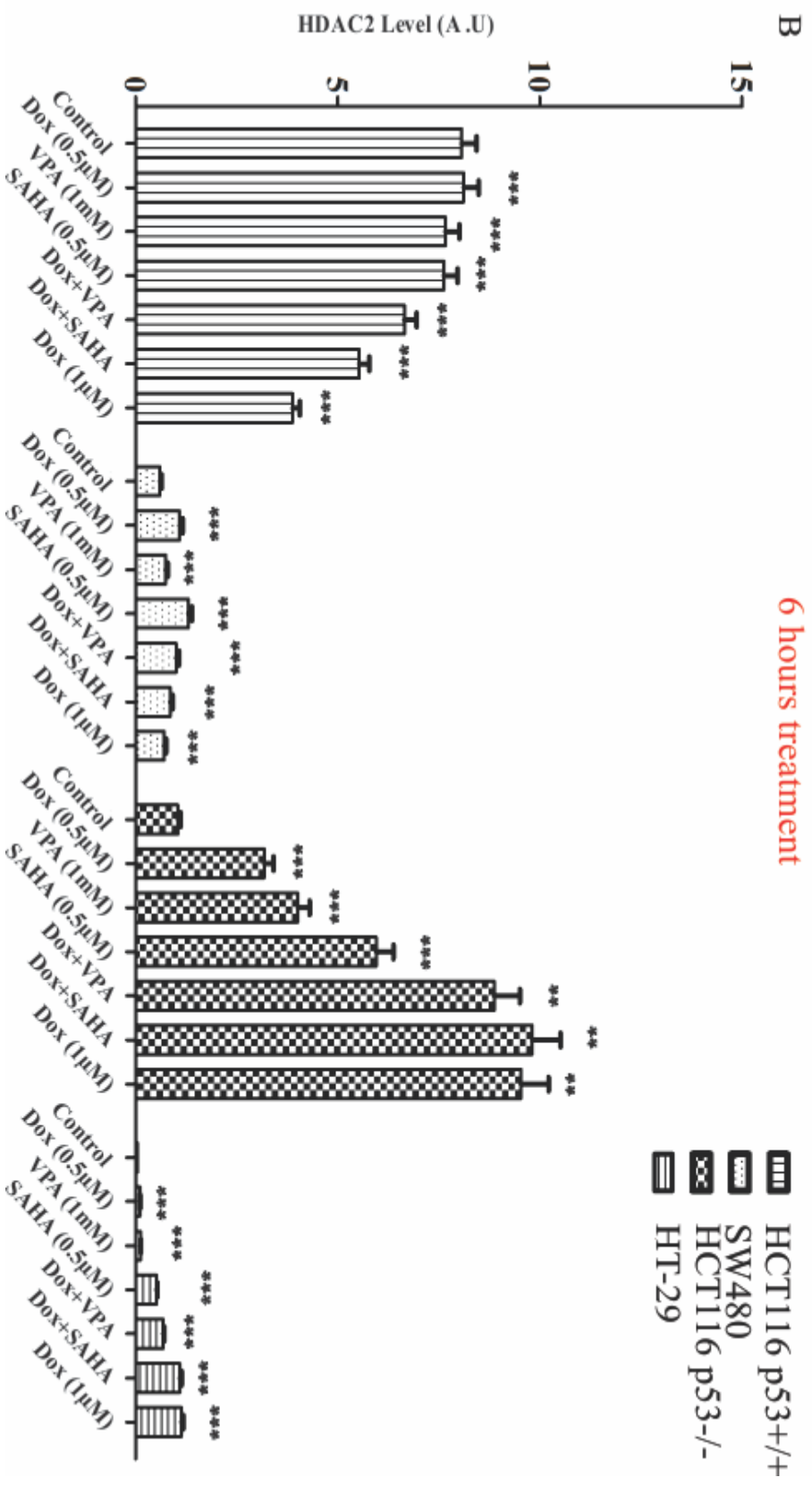


**Figure 3.7 B: Quantification of alterations in HDAC2 levels upon 6 hours treatment with Dox, VPA, SAHA and their combination in HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29 cells.**

*The bands of HDAC2 levels in figure 3.7 A upon the applied treatment for each cell line were quantified following scanning densitometry using ImageJ program. Data was presented as mean  $\pm$  S.E.M.; n=3. \*\*P between 0.001-0.01, \*\*\*P<0.001.*

6 hours treatment

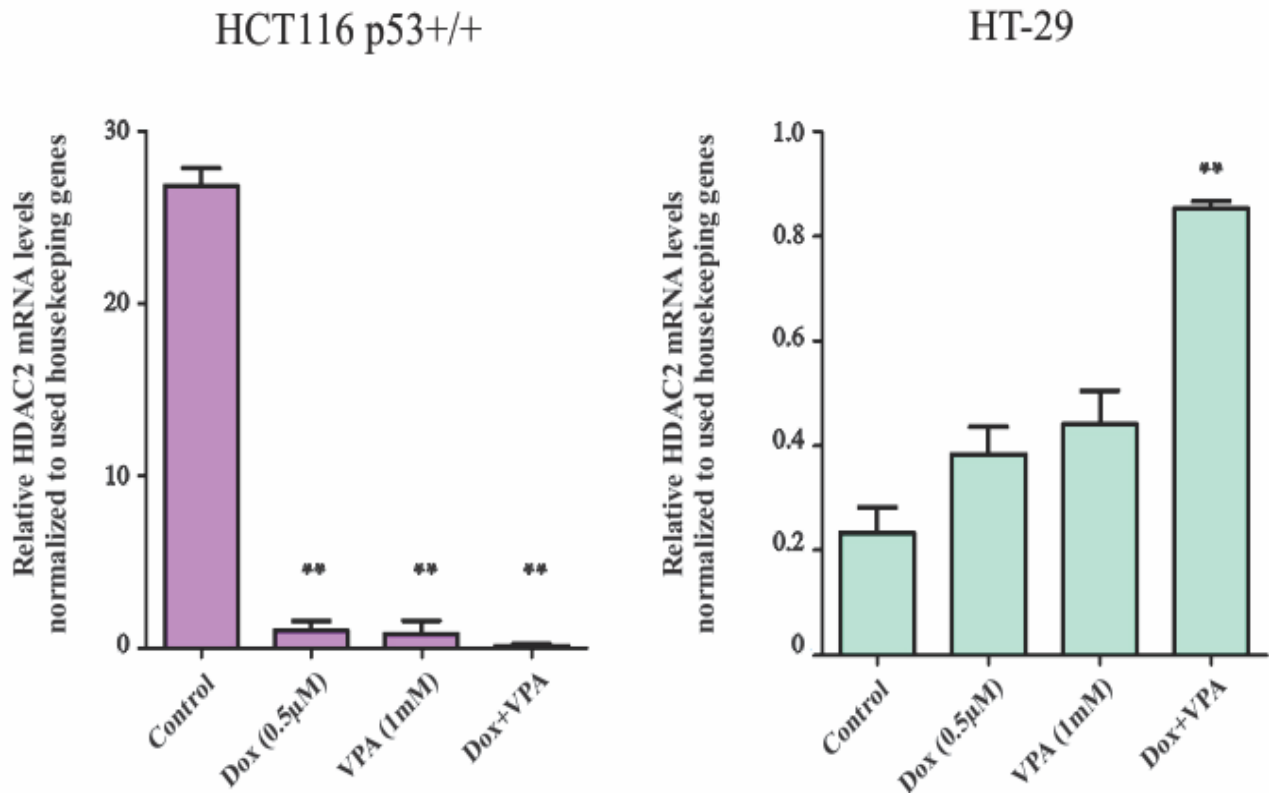


**Figure 3.8: Real time PCR analysis for HDAC2 mRNA levels in HCT116 p53+/+ and HT-29 upon 6 hours treatment of Dox, VPA, and VPA/Dox.**

*Both cell lines were treated for 6 hours treatment with Dox (0.5μM), VPA (1mM), and their combinations (Dox/VPA). mRNA was extracted and quantified by real time PCR for HDAC2, α-TUBLIN, and PPIA genes. mRNA of HDAC2 levels were normalized against α-TUBLIN and PPIA mRNA levels. Data was presented as mean ± S.E.M.; n=3. \*\*P between 0.001-0.01.*

A 24 hours treatment

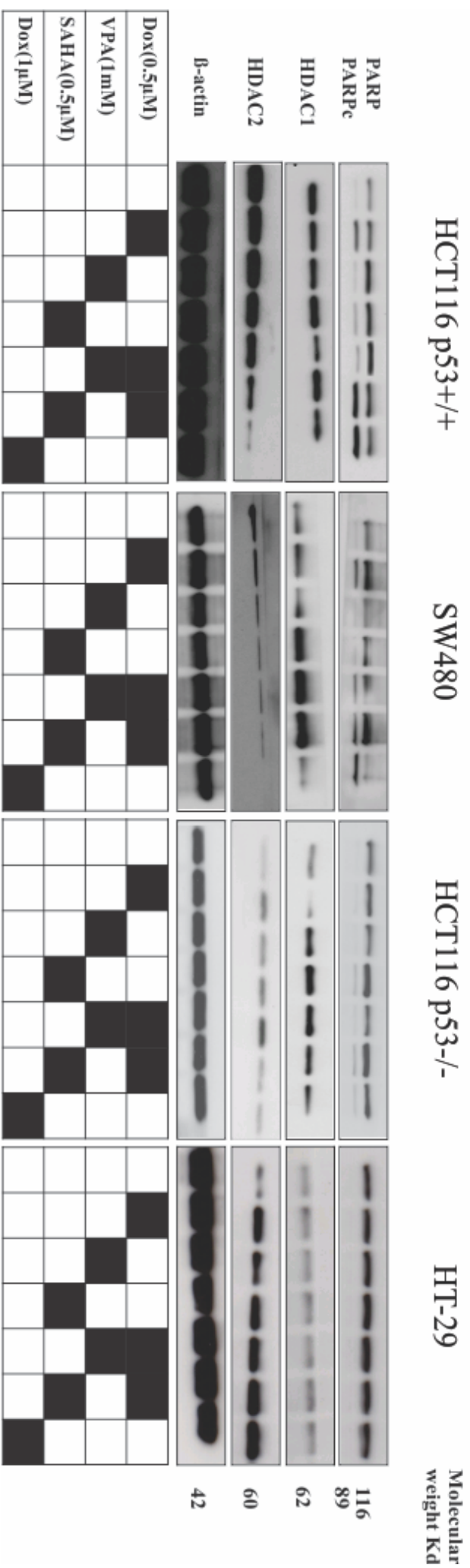


**Figure 3.9 A: HDAC1 and 2 levels upon 24 hours treatment with Dox, VPA, SAHA and their combination in HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29 cells.**

*A. PARP cleavage (PARPc), HDAC1, and HDAC 2 levels after 24 hours treatment with Dox (0.5μM and 1μM), VPA (1mM), SAHA (0.5μM) and their combinations (Dox/VPA and Dox/SAHA). β-actin was used as a loading control.*

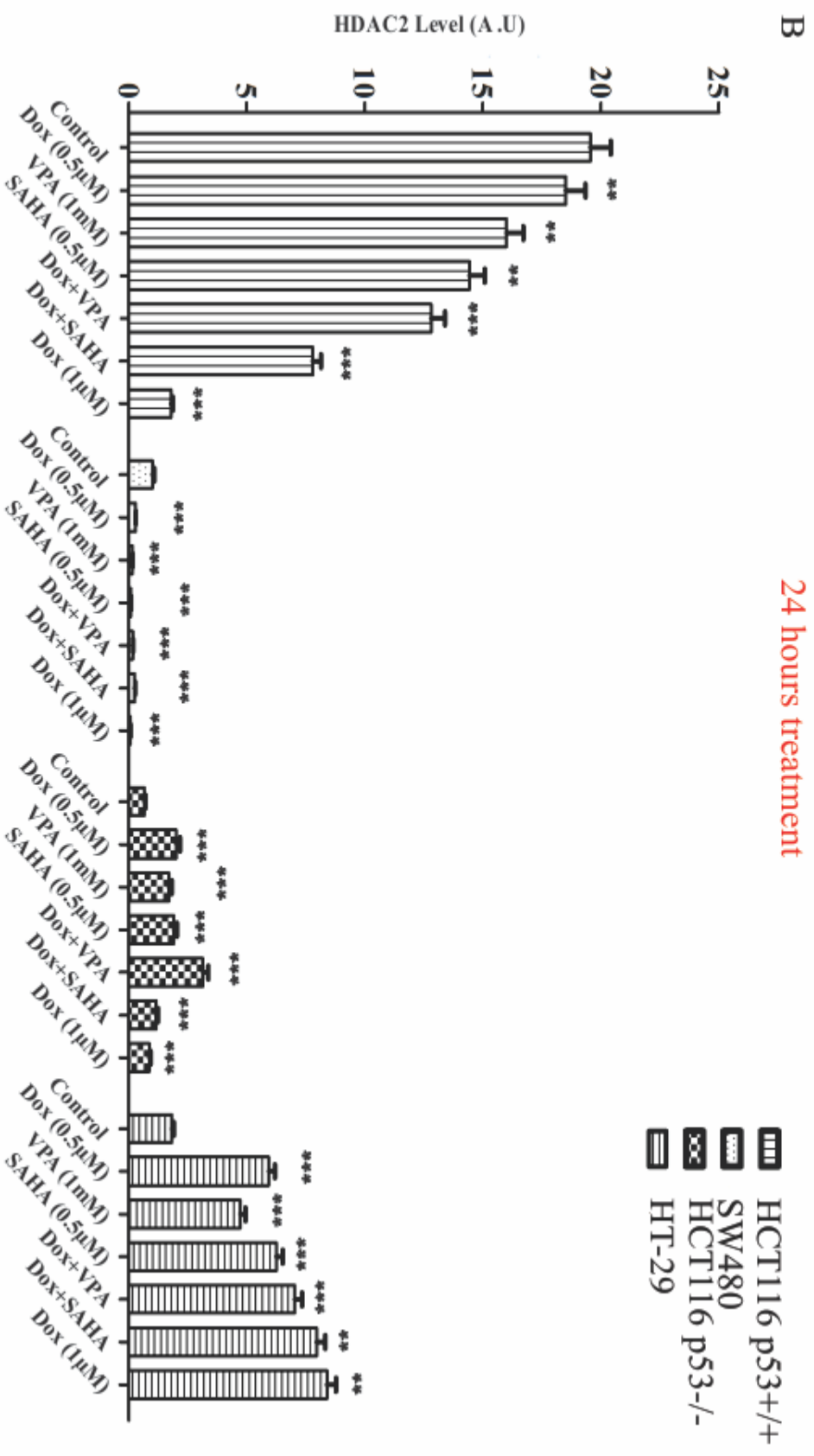


**Figure 3.9 B: Quantification of HDAC2 levels alterations upon 24 hours treatment with Dox, VPA, SAHA and their combination treatment in HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29 cells.**

*The bands of HDAC2 levels in figure 3.9 A upon the applied treatment for each cell line were quantified following scanning densitometry using ImageJ program. Data was presented as mean  $\pm$  S.E.M.; n=3. \*\*P between 0.001-0.01, \*\*\*P<0.001.*

## **5-Correlation between survivin and HDAC2 levels determines cell death on treatment with Dox combined with VPA or SAHA in the mCRC cell lines used in this study.**

### **Summary:**

Survivin is a bifunctional protein encoded by *BIRC5* gene. It prevents apoptosis by inhibiting caspase 3 and 7 activation. Also survivin regulates mitosis (Knauer et al., 2007), hence survivin is over-expressed in the majority of cancer types (Kennedy et al., 2003). However, the relationship between its expression and either status of P53 or HDAC2 in mCRC is unclear. I found in the previous section that HDCA2 levels increased alongside the resistance to applied treatment. Therefore, in this section, I checked the survivin levels (an inhibitor of apoptosis) in untreated and Dox/VPA and Dox/SAHA treated mCRC cell lines displaying different p53 status: wild-type p53 (HCT116 p53+/+), mutated p53 (SW480 and HT-29), and null p53 (HCT116 p53-/-) mCRC cell lines. I found that survivin expression levels correlate with their HDAC2 counterparts. In untreated wild-type p53 cell line (HCT116 p53+/+), Survivin is over-expressed, whereas in the untreated and mutated or null p53 (SW480, HT-29, and HCT116 p53-/-) survivin is expressed at low levels. (Also HDAC2 has the same status, see the previous section). In addition, I found that the high level of survivin in HCT116 p53+/+ decreases alongside the response to Dox combined with VPA or SAHA treatment while the low level of survivin in SW480, HT-29 and HCT116 p53-/- increases alongside the resistance to the applied treatment. The changes in survivin protein level following the applied treatment are similar to the alterations of HDAC2 expression level upon the same treatments (see the previous section). This

correlation between HDAC2 and survivin explains the relationship between HDAC2 levels and the response to Dox as a single treatment or combined with VPA and SAHA in mCRC.

### **Laboratory work and results:**

In the previous section, I found that HDAC2 levels increase alongside the absence or reduction of apoptosis response upon Dox and its combination with VPA and SAHA treatment in HCT116 p53<sup>+/+</sup>, SW480, HCT116 p53<sup>-/-</sup>, and HT-29. As survivin is an inhibitor of apoptosis and is over-expressed in mCRC, I checked, therefore, the status of its level and the occurred change upon Dox and its combination with VPA and SAHA treatment. The four cell lines were treated with Dox (0.5 $\mu$ M), VPA (1mM), and SAHA (0.5 $\mu$ M) and their combinations Dox/VPA and Dox/SAHA for 24 hours. Total proteins were extracted and immunoblotted in nitrocellulose membranes (see materials and methods for more details). p53, HDAC2, and survivin were investigated, figure 3.10 A. Untreated (control) wild-type P53 cells (HCT116 p53<sup>+/+</sup>) showed high level of survivin and HDAC2, whereas untreated and mutated p53 cells (SW480 and HT-29) and untreated null p53 cells (HCT116 p53<sup>-/-</sup>) displayed low level of survivin and HDAC2 compared to their counterparts in HCT116 p53<sup>+/+</sup>, figure 3.10 A. Moreover, the alterations of survivin levels towards the sensitivity or resistance to the applied treatment were the same as HDAC2 levels alterations. In HCT116 p53<sup>+/+</sup>, SW480, and HCT116 p53<sup>-/-</sup>, the survivin level reduced with sensitivity and response to the treatment, figure 3.10 B. However, in HT-29 cells, the survivin levels significantly increased with the strength of resistance to the applied drug, figure 3.10 B. The correlation between the increase of HDAC2 level and the resistance to the treatment was reflected by corresponding increase of survivin level.

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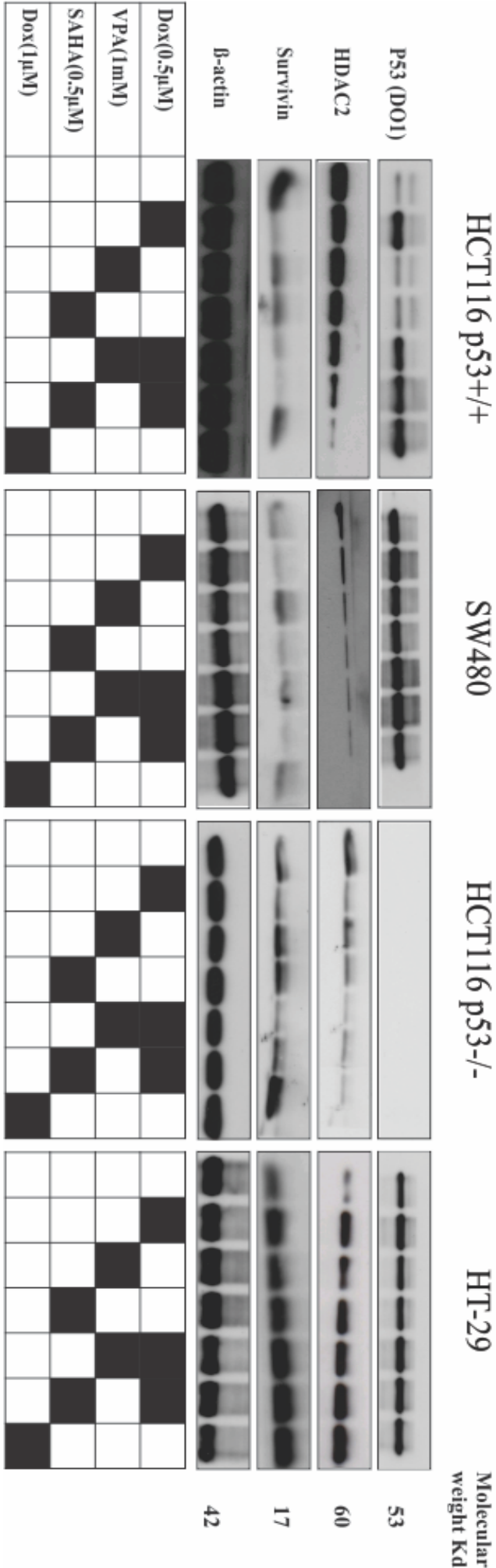


**Figure 3.10 A:** Correlation between HDAC2 and survivin levels upon 24 hours treatment with Dox, VPA, SAHA and their combination in HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29 cells.

*The four cell lines (HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29) were treated for 24 hours with Dox, VPA and SAHA as single or combined, then total proteins extracted and immunoblotted with antibodies against p53, HDAC2, Survivin, and  $\beta$ -actin.*

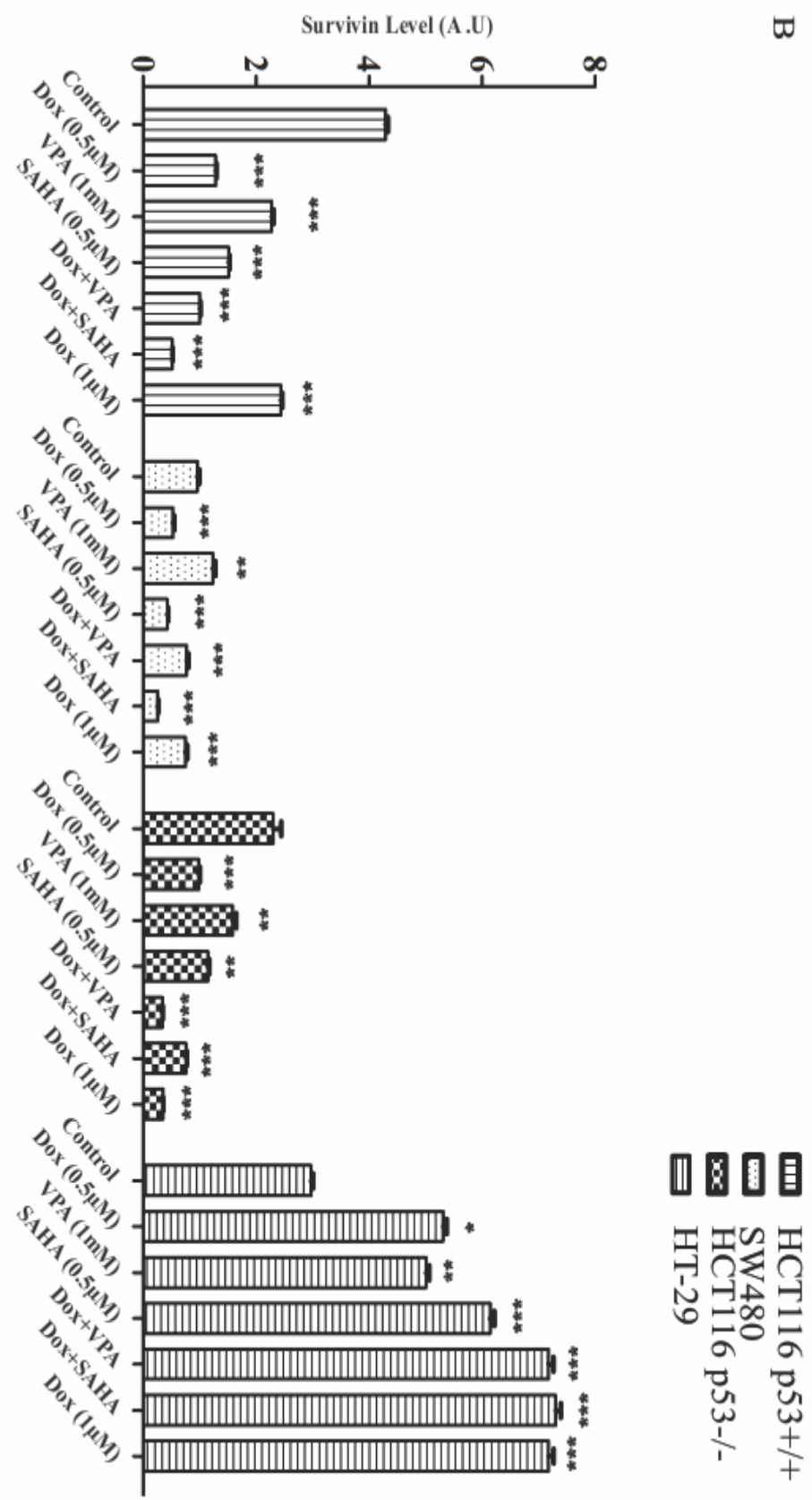


**Figure 3.10 B: Quantification of survivin level alterations upon 24 hours treatment with Dox, VPA, SAHA and their combination treatment in HCT116 P53+/, HCT116 P53-/-, SW480, and HT-29 cells.**

*The bands of survivin levels in figure 3.10 A upon the applied treatment for each cell line were quantified following densitometric scanning using ImageJ program. Data was presented as mean ± S.E.M.; n=3. \*P between 0.01-0.05, \*\*P between 0.001-0.01, \*\*\*P<0.001.*

## **6-HDAC2 depletion sensitizes HCT 116 p53<sup>-/-</sup> to Dox/VPA treatment, whereas its over-expression erases SW480 sensitivity to the same treatment.**

### **Summary:**

Throughout the previous sections, I showed the correlation between HDAC2 level and the response to the applied treatment. While the sensitivity to treatment is accompanied by a reduction in HDAC2 level, the resistance to treatment is accompanied by an increase of HDAC2 level in the tested mCRC. However, until this point there is still an urgent question to be addressed which is: ‘does HDAC2 play a direct role in conferring resistance or sensitivity to treatment’ or in other words, ‘If HDAC2 has been knocked down in the resistant cells, would this sensitize these cells to the treatment?’ and vice versa, ‘if HDAC2 has been over-expressed in the sensitive cells, would this confer resistance to these cells’. In this section, I answered these questions and I proved that HDAC2 play a direct role in conferring resistance or sensitivity to treatment. HCT116 p53<sup>-/-</sup> cells showed resistance against Dox (0.5 $\mu$ M), VPA (1mM), and their combination Dox/VPA treatment. This resistance is accompanied by HDAC2 level increase, section 4- figure 3.9A. However, shRNA- HDAC2 HCT116 p53<sup>-/-</sup> stable clone cells (HDAC2 knocked-down by shRNA) showed clear response and cell death measured by PARP cleavage expression. On the other hand, SW480 cells are sensitive to Dox (0.5 $\mu$ M), VPA (1mM), and their combination Dox/VPA treatment. This sensitivity is accompanied by HDAC2 level decrease, section 4- figure 3.9A. However, over-expressed HDAC2 SW480 cells become resistant to the same treatment and PARP cleavage bands disappeared. These results

prove that HDAC2 expression levels play a direct and crucial role in conferring resistance or sensitivity to treatment.

### **Laboratory work and results:**

ShRNA-HDAC2 HCT116 p53<sup>-/-</sup> stable clone and transient over-expressed HDAC2 SW480 were generated as described in materials and methods. The efficiency of Knock-down HDAC2 in HCT116 p53<sup>-/-</sup> was about 85% measured by the quantification of HDAC2 levels in untreated parental HCT116 p53<sup>-/-</sup> and shRNA-HDAC2 HCT116 p53<sup>-/-</sup> stable clone, figure 3.11 A and B. To compare the sensitivity between parental HCT116 p53<sup>-/-</sup> (which showed resistance against the treatment used, section 4- figure 3.9A) and shRNA-HDAC2 HCT116 p53<sup>-/-</sup>, Dox (0.5 $\mu$ M), VPA (1mM), and their combination Dox/VPA treatment was used for 24 hours. Cell death was measured by PARPc expression. Figure 3.11 C shows that HDAC2 depletion renders HCT116 p53<sup>-/-</sup> responsive and sensitive to the treatment to which the parental cells HCT116 p53<sup>-/-</sup> cells were resistant. This increase in sensitivity to the treatment upon knocking down HDAC2 was also confirmed by flow cytometry analysis using PI dye, figure 3.11 D. On the other hand, over-expression of HDAC2 (about 1.5 fold, figure 3.12 A and B) in SW480 cells renders these cells completely resistant to 24 hours treatment of Dox (0.5 $\mu$ M), VPA (1mM), and SAHA (0.5 $\mu$ M) and their combinations Dox/VPA, Dox/SAHA. Moreover, over-expression of HDAC2 in SW480 cells erased the PARPc cleavage bands which were developed upon this treatment in the parental SW480 cells, and made SW480+HDAC2 cells resistant to the applied treatment comparing with their parental SW480 cells, figure 3.12 C. This resistance was also confirmed by flow cytometry analysis using PI dye, figure 3.12 D.

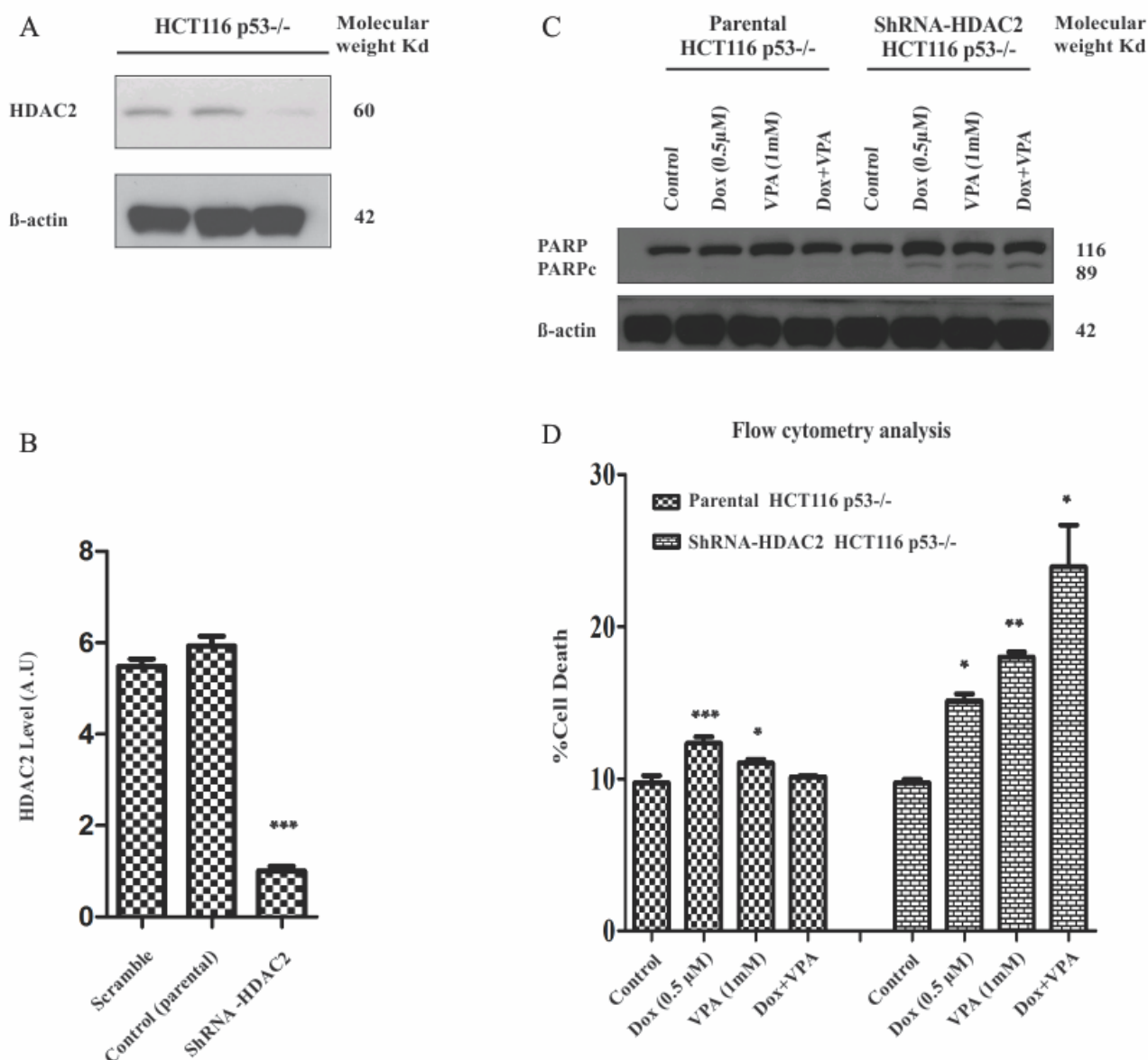


**Figure 3.11: HDAC2 depletion sensitizes HCT 116 p53<sup>-/-</sup> cells to Dox/VPA treatment alone and in combination.**

*A: Immunoblotting for HDAC2 in HDAC2-knocked down HCT 116 p53<sup>-/-</sup> cells. Parental HCT 116 p53<sup>-/-</sup> cells were transfected with scrambled shRNA or HDAC2-specific shRNA. B: Quantification of HDAC2 bands in panel A by densitometric scanning shows that the efficiency of HDAC2 knock-down is about 85%. C: Parental HCT116 p53<sup>-/-</sup> and shRNA-HDAC2 HCT116 p53<sup>-/-</sup> cells were treated with Dox (0.5μM), VPA (1mM), and Dox/VPA for 24 hours. Cell death was investigated by PARPc expression (a hallmark of apoptosis). D: Sensitivity to the treatment upon knocking down HDAC2 was confirmed by flow cytometry analysis using PI dye to measure cell death. Data was presented as mean ± S.E.M.; n=3. \*P between 0.01-0.05, \*\*P between 0.001-0.01, \*\*\*P<0.0001.*

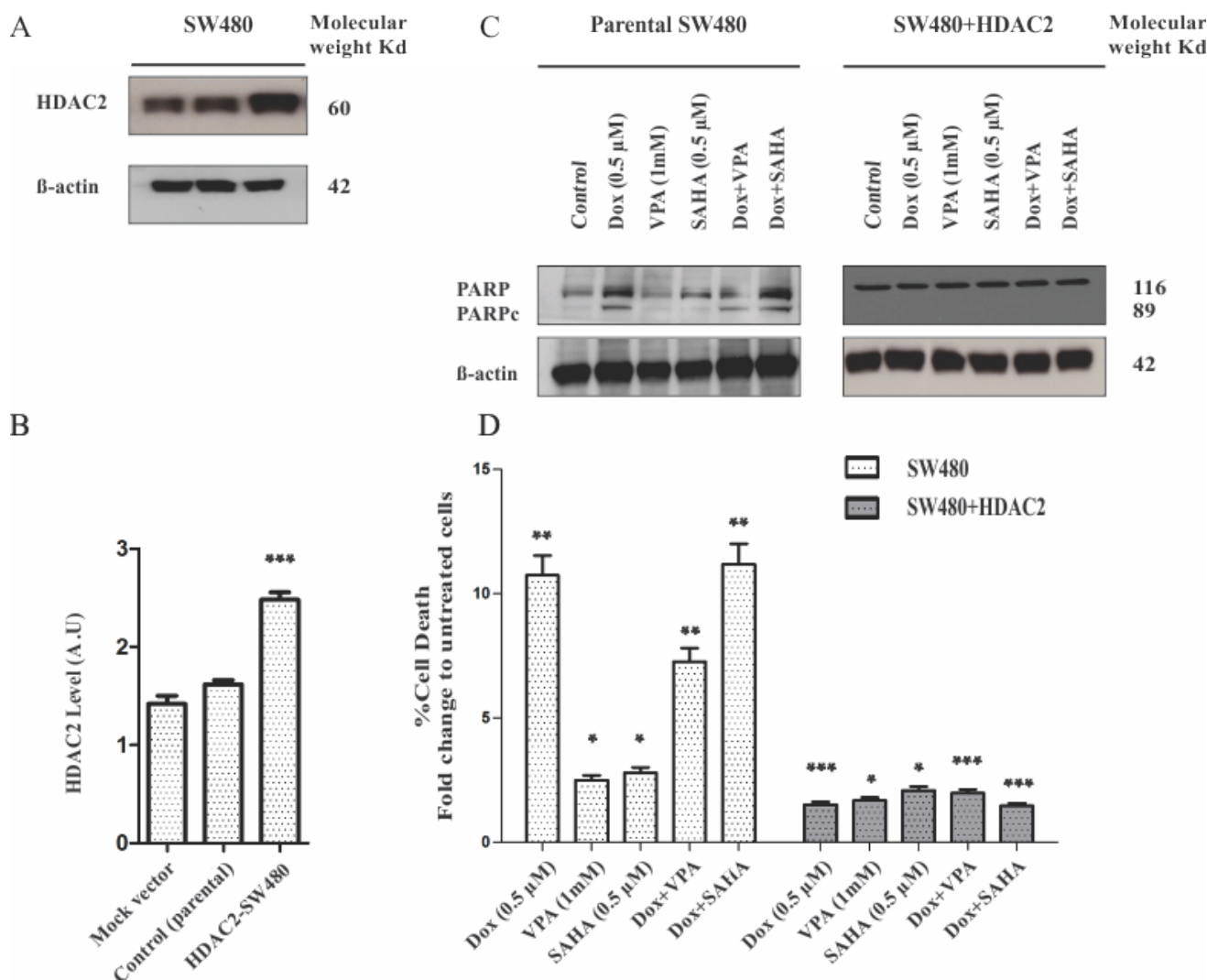


**Figure 3.12: HDAC2 over-expression renders SW480 cells resistant to Dox/SAHA treatment.**

*A: Immunoblotting for HDAC2 in HDAC2-overexpressed SW480 cells. Parental SW480 cells were transiently transfected with mock vector or HDAC2-expression vector. B: Quantification of HDAC2 bands in panel A by densitometric scanning shows that the efficiency of HDAC2 overexpression is about 1.5 fold. C: Parental SW480 and SW480+HDAC2 cells were treated with Dox (0.5μM), VPA (1mM), SAHA (0.5μM) and their combination for 24 hours. Apoptosis was investigated by PARPc expression levels. D: Resistance to the treatment was confirmed by flow cytometry analysis using PI dye to measure cell death. Data was presented as mean ± S.E.M.; n=3. \*P between 0.01-0.05, \*\*P between 0.001-0.01, \*\*\*P<0.0001.*

## **7-HDAC2 controls chromatin plasticity and its depletion enhances mitotic cell death in drug resistant HT-29 cells upon 5FU and Oxa treatments.**

### **Summary:**

HT-29 cells were the most resistant cells in this study and they expressed high levels of HDAC2 alongside their resistance to treatment. In this section, I experimentally answered the question “If HDAC2 was knocked down in these resistant cells, would this sensitize the cells to treatment”. I found that HT-29 cells undergo caspase-independent cell death, specifically mitotic cell death (MCD) by chromosome fragmentation (Stevens JB et al., 2007), upon 5FU/SAHA and Oxa/SAHA combined treatment. This MCD explains the absence of PARPc after 5FU/SAHA and Oxa/SAHA combined treatment, although there was cell death as investigated by flow cytometry. In addition, I found that HDAC2 depletion via shRNA was sufficient to sensitize HT-29 cells to 5FU or Oxa as a single treatment. Moreover, HDAC2 depletion significantly increased MCD in shRNA-HT-29 cells compared to parental HT-29 cells after exposing both cell lines to the same treatment with 5FU or Oxa. These results again confirm the crucial role of HDAC2 in determining response to chemotherapy.

### **Laboratory work and results:**

HT-29 cells have shown resistance to 5FU (5 $\mu$ g/ml) or Oxa (10 $\mu$ M) as a single treatment, figure 3.13 A. However, 5FU/SAHA (0.5 $\mu$ M) or Oxa/SAHA (0.5 $\mu$ M) combined treatment sensitized HT-29 cells and

induced significant cell death as investigated by flow cytometry and microscopy analysis, figure 3.13 B and C. Nevertheless, this significant cell death was not accompanied by PARPc induction, figure 3.13 D. This indicates that the cell death induced by both combinations in HT-29 cells is caspase-independent and does not involve PARPc. To investigate further, HT-29 were exposed to the same treatment above and cell death was investigated by confocal microscopy, with immunofluorescence using phosphorylated serine 10 histone H3 antibody (Mitotic Marker, Abcam, UK) and 4',6-diamidino-2-phenylindole (DAPI) dye (a nuclear and chromosome counterstain, Invitrogen, UK). Interestingly, the confocal microscope images showed that the cell death induced upon 5FU/SAHA and Oxa/SAHA combined treatment is mainly mitotic cell death (MCD), figure 3.14 A, B. This type of cell death occurs during metaphase and involves the degradation of condensed chromosomes (Stevens JB et al., 2007). However, MCD does not lead to PARPc because it is caspase-independent form of cell death. This explains why there is no PARPc in HT-29 cells upon 5FU/SAHA and Oxa/SAHA combined treatment even though there is cell death shown by flow cytometry and microscopy analysis. To check if HDAC2 depletion in HT-29 can sensitize the cells to treatment with 5FU or Oxa as a single treatment, to which HT-29 cells were resistant, shRNA-HDAC2 HT-29 stable clone was generated as described in materials and methods, and shRNA HT-29 and parental HT-29 cells were treated with SAHA (0.5  $\mu$ M), 5FU (5  $\mu$ g/ml), and Oxa (10  $\mu$ M) for 24 hours. Mitotic cell death was investigated by immunofluorescence as there was no PARPc induced after treatment (as mentioned above these cells undergo caspase-independent cell death). Interestingly, shRNA-HDAC2 HT-29 cells showed significant increase in MCD compared to parental HT-29 cells, figure 3.15 A, B. Therefore,

HDAC2 depletion was sufficient to sensitize HT-29 cells to 5FU or Oxa as a single treatment. Moreover, HDAC2 depletion disturbs the higher order chromatin structure in a similar manner to SAHA combined with 5FU or Oxa, figure 3.15 C and figure 3.16.

## HT-29

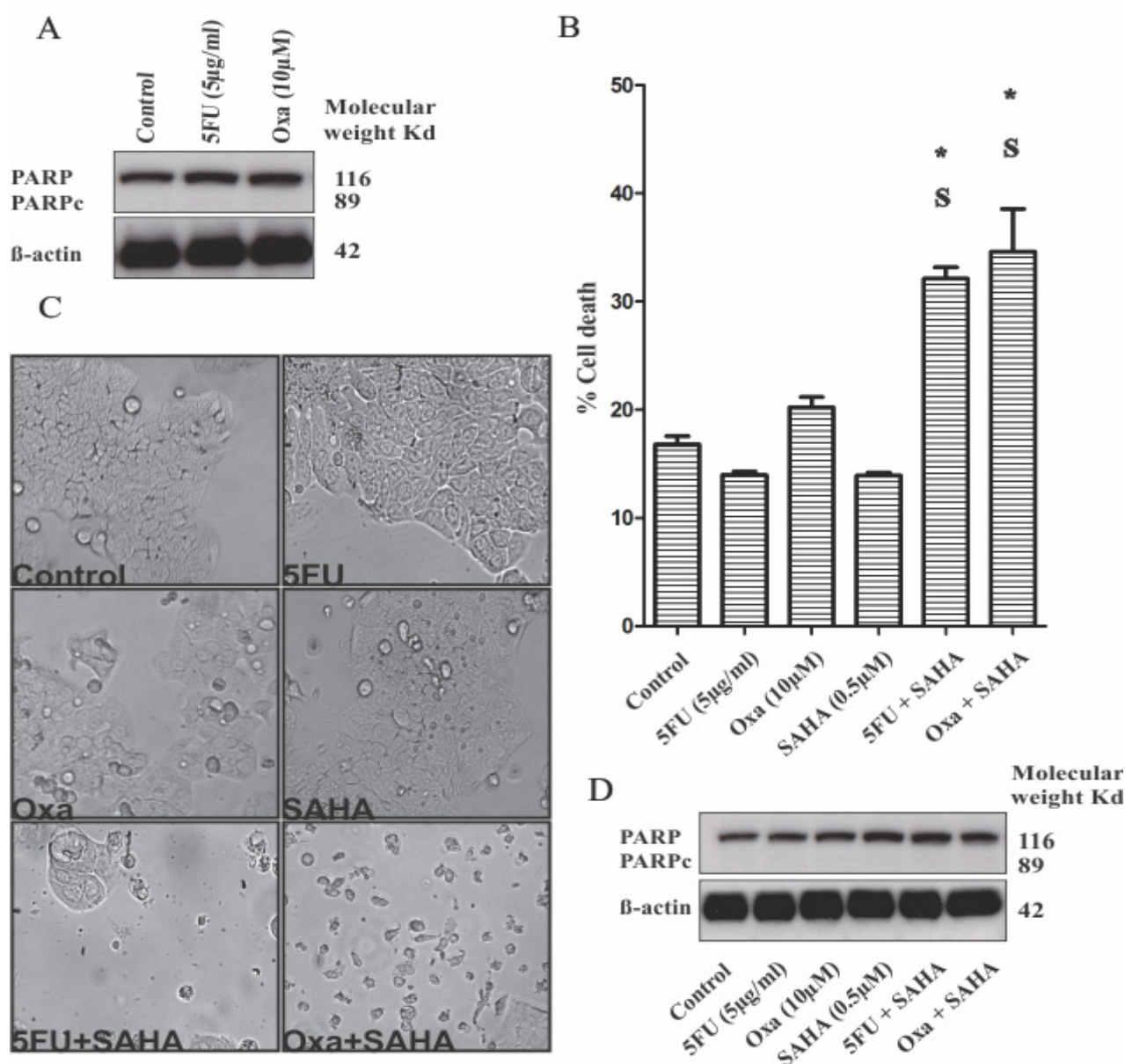


**Figure 3.13: HT-29 cells undergo a caspase-independent cell death upon SAHA/5FU and SAHA/Oxa combined treatments.**

Checking cell death by PARP cleavage (PARPc) in HT-29 upon treatment by 5FU (5 $\mu$ g/ml) and Oxa (10 $\mu$ M) as a single treatment for 24 hours (A). HT-29 cells were treated with SAHA (0.5 $\mu$ M), 5FU (5 $\mu$ g/ml), Oxa (10 $\mu$ M), and their combined treatment SAHA+5FU and SAHA+SAHA for 24 hours, then cell death was investigated by: flow cytometry (B) using Propidium iodide (PI) staining, by microscopic observations (C), and by PARPc (D). S denotes a synergistic effect. Error bars represent the mean  $\pm$  S.E.M.; n=3. \*P between 0.01-0.05.

## HT-29 Mitotic Cell Death (MCD)

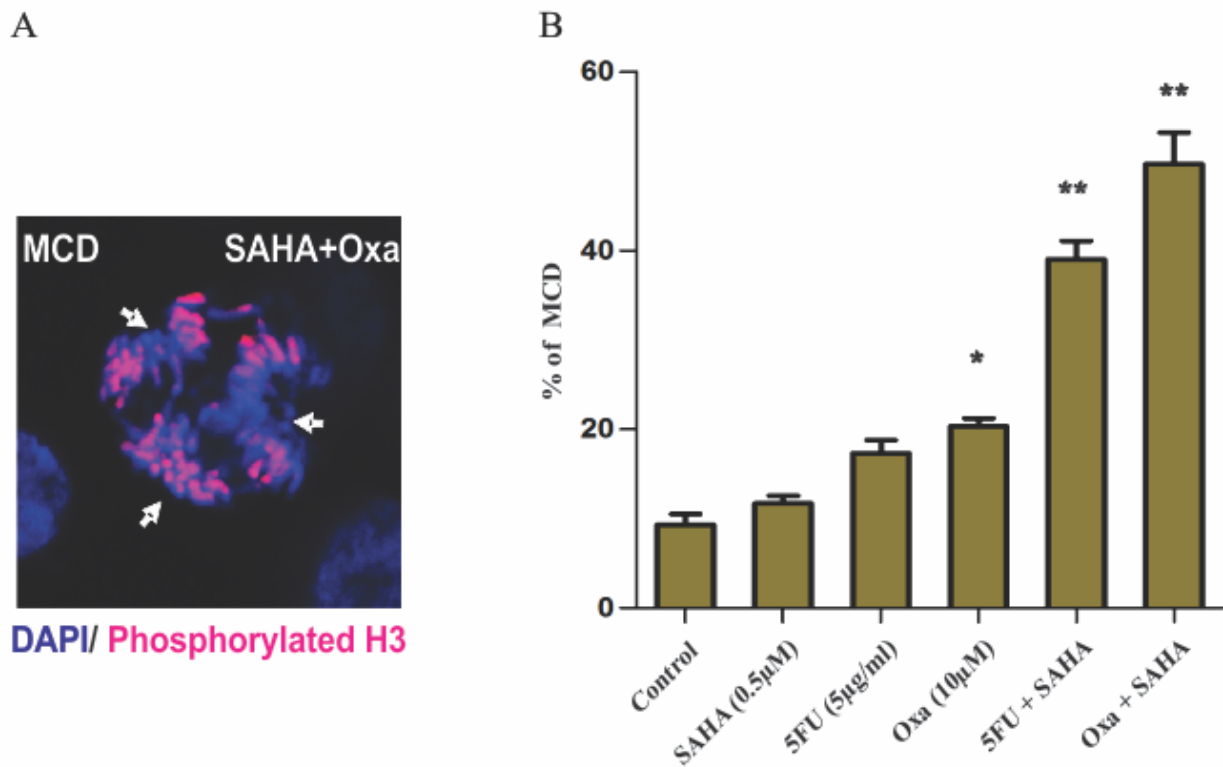


**Figure 3.14: HT-29 mitotic cell death (MCD) by chromosome fragmentation upon SAHA/5FU and SAHA/Oxa combined treatments.**

*A: Mitotic cell death (MCD) in HT-29 upon SAHA+Oxa combined treatment observed under a confocal microscope after immunostaining with DAPI and phosphorylated H3. B: Quantifications of mitotic cell death (MCD) in HT-29 upon 5FU, Oxa combined with SAHA. Error bars represent the mean  $\pm$  S.E.M.;  $n=3$ . \* $P$  between 0.01-0.05, \*\* $P$  between 0.001-0.01.*

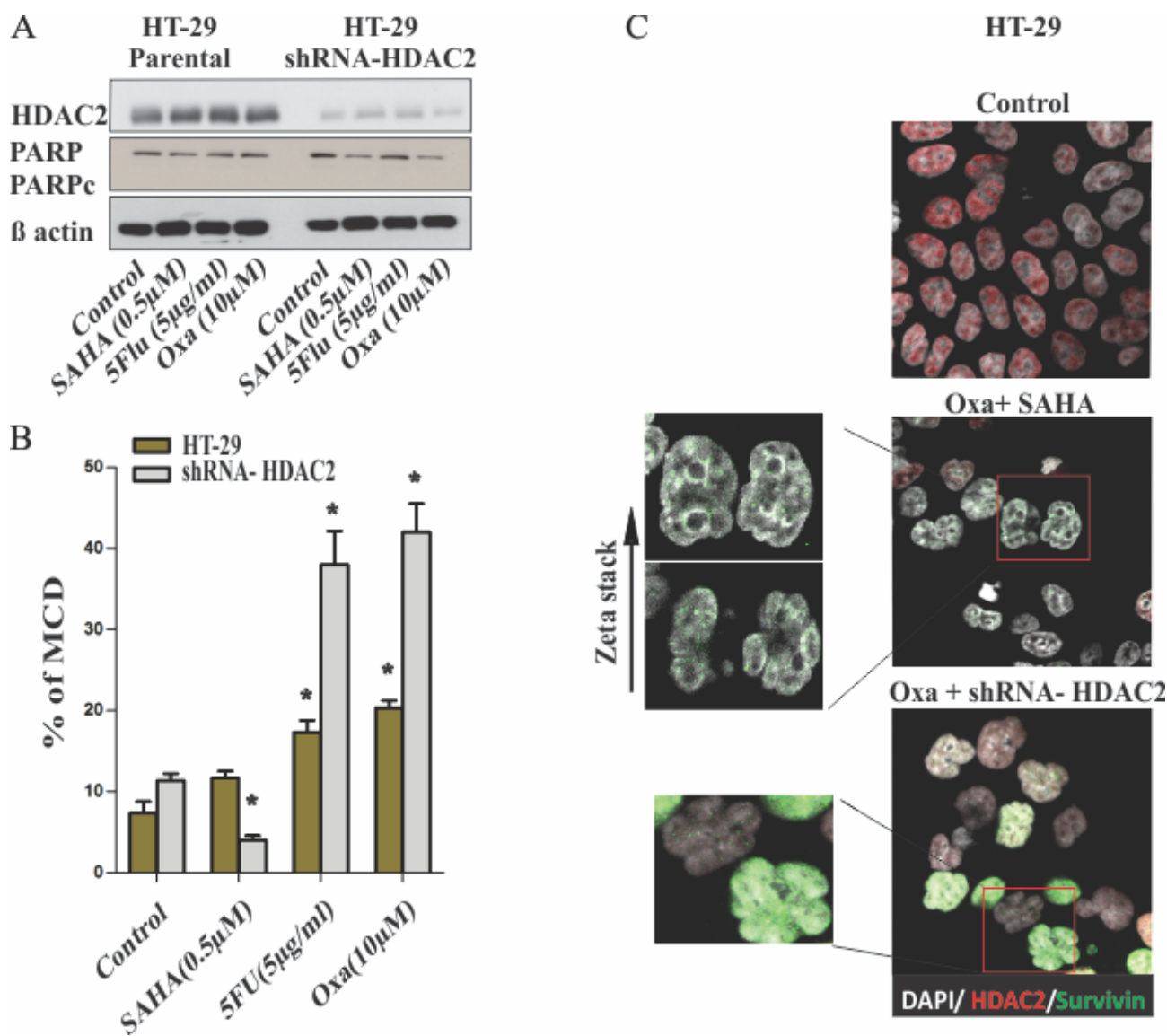


**Figure 3.15: HDAC2 controls the chromatin plasticity and its depletion enhances mitotic cell death in drug resistant HT-29 cells upon 5FU and Oxa treatments.**

*A: Checking cell death by PARP cleavage (PARPc) in parental and shRNA-HDAC2 HT-29 cells and upon 5FU, Oxa combined with SAHA. B: Quantifications of MCD in parental and shRNA-HDAC2 HT-29 cells upon treatment by 5FU, Oxa combined with SAHA. The observations of cell death were done under a confocal microscope after immunostaining. C: Zeta stack images for parental and shRNA-HDAC2 HT-29 cells upon Oxa+SAHA (in parental HT-29 cells) and Oxa (in shRNA-HDAC2 HT-29, which mimics the effect of SAHA). Both cell lines were stained with DAPI and incubated with HDAC2 and Survivin primary antibodies. Error bars represent the mean  $\pm$  S.E.M.; n=3. \*P between 0.01-0.05.*

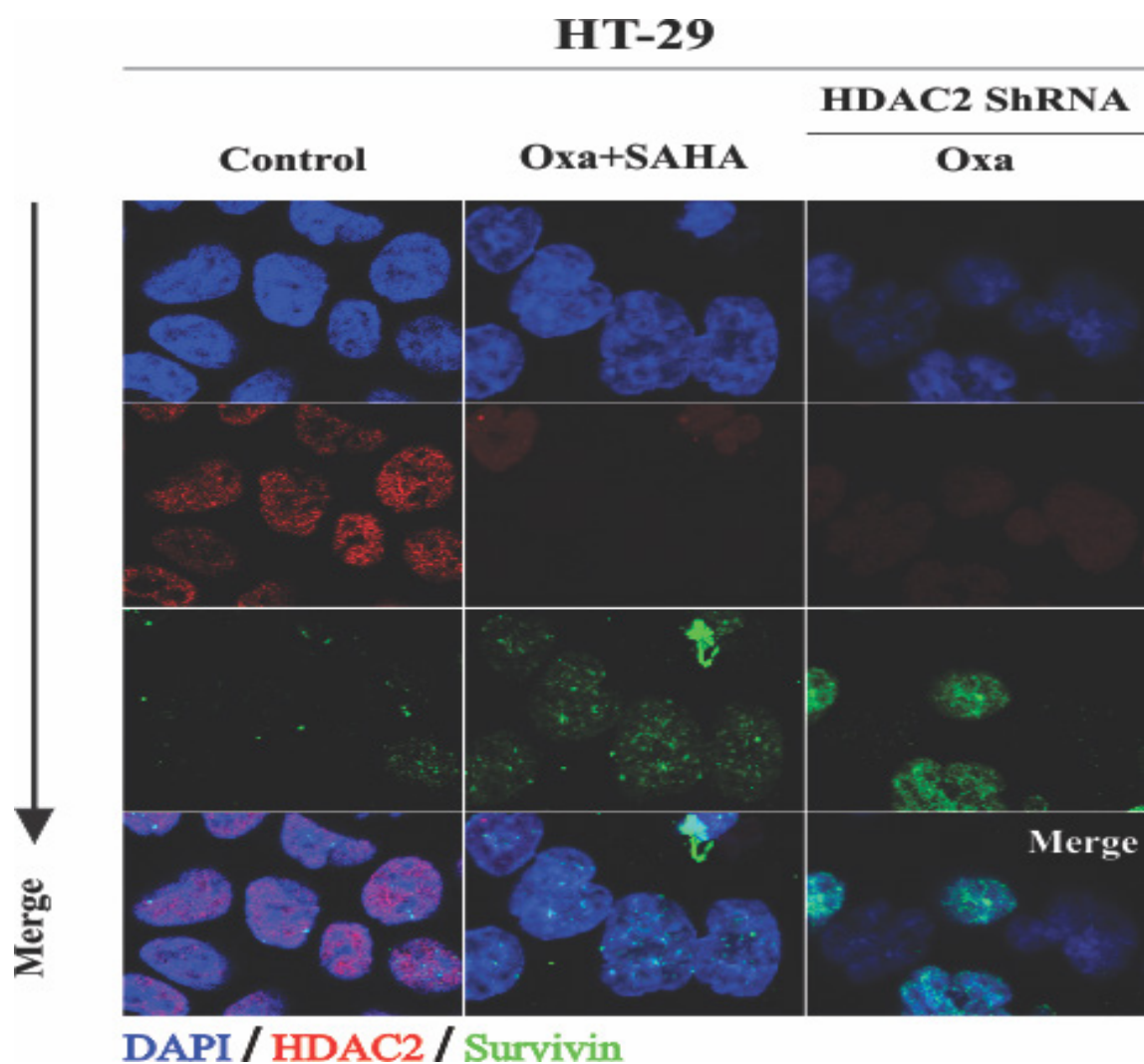


**Figure 3.16: Oxa combined with SAHA or ShRNA-HDAC2 has similar effects on HT-29 mitotic cell death.**

*Confocal microscope images after immunostaining for parental and shRNA-HDAC2 HT-29 cells upon Oxa+SAHA (in parental HT-29 cells) and Oxa (in shRNA-HDAC2 HT-29). HDAC2-knock down mimics the effect of SAHA in combined treatment with Oxa. Both cell lines were stained with DAPI and incubated with HDAC2 and Survivin primary antibodies.*

## **8-In vivo imaging and immunohistochemical confirmation of Dox/SAHA combined treatment effects and the related role of HDAC2 in obtained response.**

### **Summary:**

In vivo studies are complementary to their counterparts in vitro, and both are crucial for modern cancer research and clinical care. A key advantage of in vivo imaging (as a part of in vivo studies) is that it provides information about treatment efficacy in the real or similar milieu or microenvironment of a tumour (Condeelis and Weissleder, 2010). In this section, I managed to confirm the synergistic effect of the combined treatment Dox/SAHA in HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup> in a murine xenograft model. Dox/SAHA combined treatment induced cell death in both HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>-/-</sup>, also decreased HDAC2 and survivin expression levels in vitro (see result section 2). In this preliminary in vivo study I showed by immunohistochemistry (IHC) analyses as proof of concept that Dox/SAHA treated tumours isolated from HCT116 p53<sup>+/+</sup> xenograft mice dramatically decreased their cellular HDAC2 and survivin levels (tumours isolated from HCT116 p53<sup>-/-</sup> xenograft mice showed necrosis, so IHC for them was not successful). The decrease in HDAC2 and survivin levels is consistent with the in vitro results (result section 4 and 5). In this section, I also had the chance to compare the HDAC2, survivin, and p53 levels between normal human liver tissues and human CRC metastases in liver tissues using IHC analyses. All CRC metastases in liver tissues showed high increase of HDAC2, survivin, and p53 levels. This increase is consistent with the metastatic status.

## **Laboratory work and results:**

In order to substantiate the association between HDAC2 expression level and cell death in vivo, non-invasive spatiotemporal visualization was used based on bioluminescent molecular imaging system of a murine xenograft model. Luciferase stable cell lines HCT116 p53  $+/+$  and HCT116 p53  $-/-$  were generated and subcutaneously transplanted into nude mice. Since the combined treatment Dox/SAHA produces significant cell death in both HCT116 p53  $+/+$  and HCT116 p53  $-/-$  cell lines in vitro, the in vivo validation of this effect was explored. Therefore, liposomal-encapsulated SAHA/Dox was prepared and intraperitoneally delivered to the mice bearing human mCRC xenografts. This innovative delivery strategy allowed for easier delivery and increased toxicity reduction. Eight nude mice were transplanted subcutaneously with each luciferase stable clone cell line. After the tumour growth arose in the mice (2-3 weeks), the control group (n=4 mice per cell line) was separated from the treated group. The size of the tumour was measured using callipers and recorded before treatment in parallel with luciferase activity measurement to ensure the presence and viability of the transplanted cells, figure 3.17. The results showed noticeable decrease in the size of tumour together with luciferase activity upon the combined treatment in HCT116 p53  $+/+$  (figure 3.18 A and B), and in HCT116 p53  $-/-$  (figure 3.19 A and B). Moreover, the IHC analyses for HDAC2 level on the tumour isolated from HCT116 p53  $+/+$  xenograft mice showed that HDAC2 level decreased by more than 50% after Dox/SAHA combined treatment, figure 3.18 C (tumours isolated from HCT116 p53  $-/-$  xenograft mice showed necrosis, so IHC for them was not successful). This clearly supports the relationship between HDAC2 expression level and sensitivity to the combined treatment. In

addition, the IHC analyses for survivin showed a decrease in survivin level in tumours isolated from Dox/SAHA treated HCT116 p53<sup>+/+</sup> xenograft mice, figure 3.18 C. The decrease in HDAC2 and survivin levels in Dox/SAHA treated HCT116 p53<sup>+/+</sup> xenograft mice was in parallel with cell death and tumour shrinkage. These results confirm again the relationship between HDAC2 levels and resistance or response to Dox/SAHA treatment. Also, IHC analyses for 10 samples from normal human liver tissues and 10 samples from human CRC metastases in liver showed high levels of HDAC2, p53, and survivin compared with the normal tissues. Figure 3.20 shows the IHC analyses for HDAC2, survivin, and p53 in representative samples. Table 3.1 (below the figure 3.20) summarizes the immunohistochemistry observations for the 10 samples of human CRC metastases in liver.

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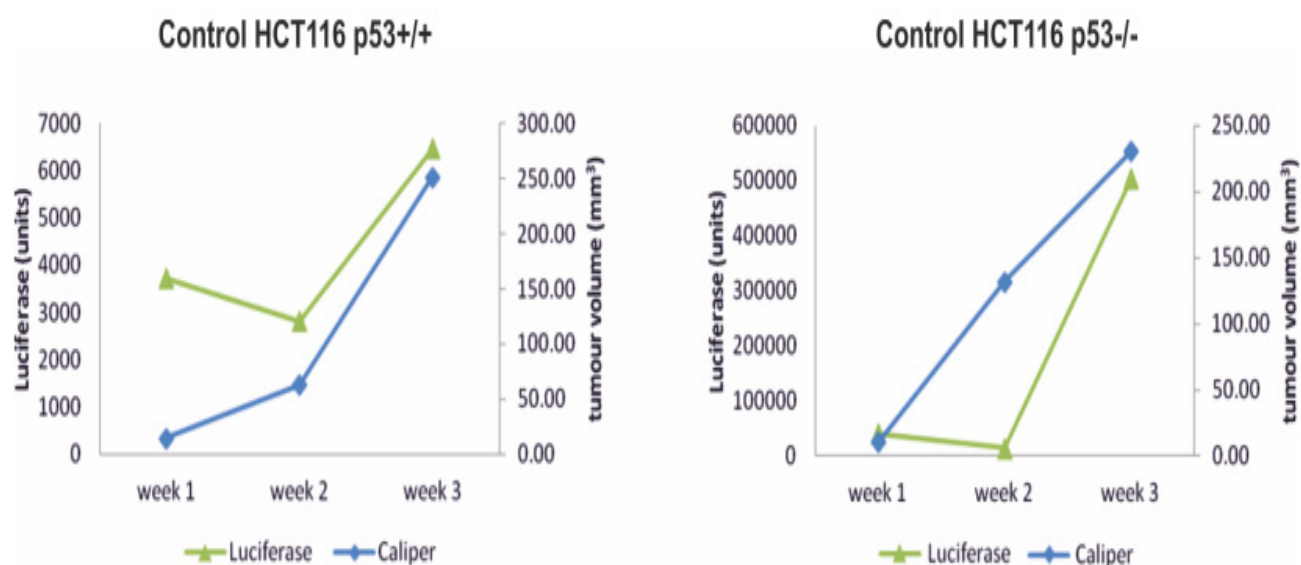
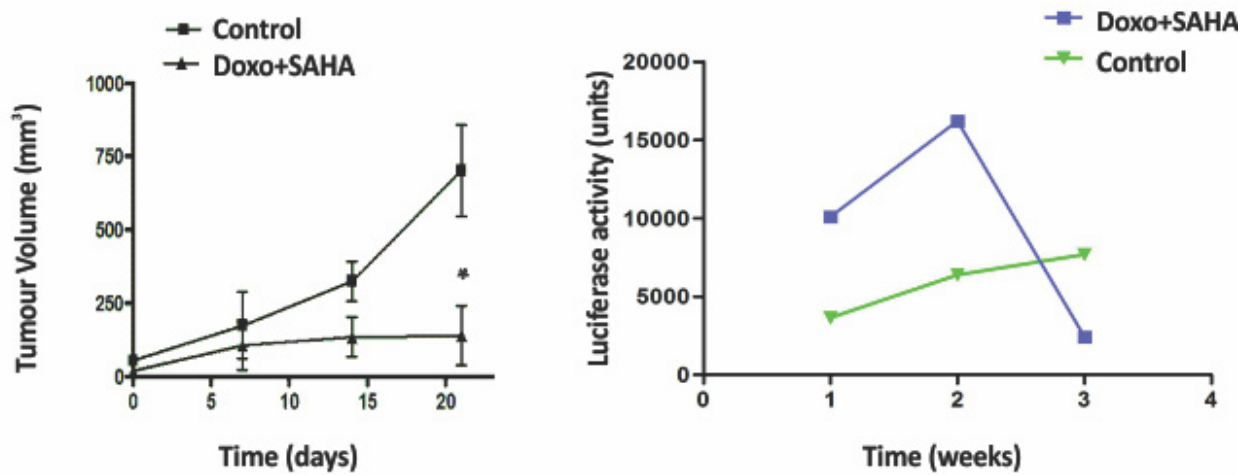


**Figure 3.17: Monitoring the establishment of HCT116 p53+/+ and HCT116 p53-/- in mice by size and luciferase activity.**

*The correlation between calipers and luciferase activity measurements confirms the establishment and viability of HCT116 p53+/+ and HCT116 p53-/- xenograft in mice.*

## HCT116 p53+/+

A



B

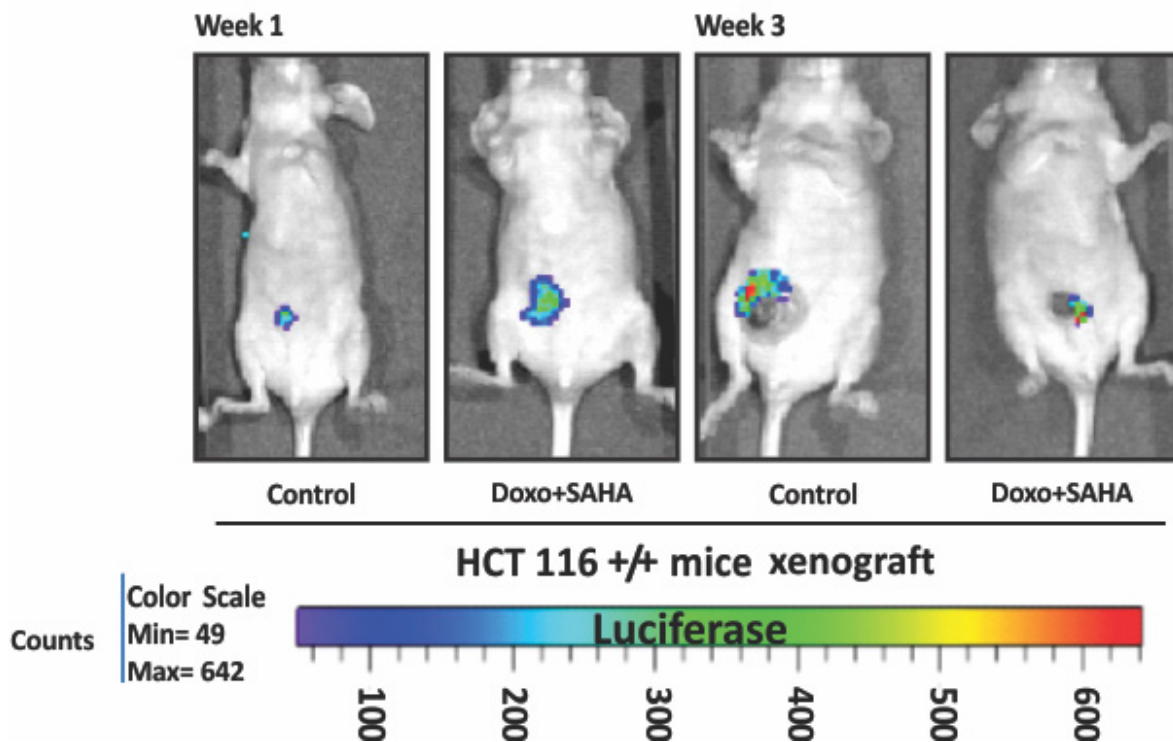


**Figure 3.18 A-B: In vivo imaging of liposome-encapsulated Dox/SAHA treatment effects in HCT116 p53+/+ xenograft mice.**

*A: The tumour size and luciferase activity in control and liposomal Dox/SAHA treated HCT116 p53+/+ xenograft groups. Error bars represent by the mean  $\pm$  S.E.M.;  $n=3$ . Statistical significance at  $p < 0.05$ . B: Bioluminescence imaging for control and Dox/SAHA treated HCT116 p53+/+ xenograft mice throughout three weeks shows sharp decrease in tumour size.*

## Immunohistochemistry analyses

C

### HCT 116 p53<sup>+/+</sup> Xenograft mice

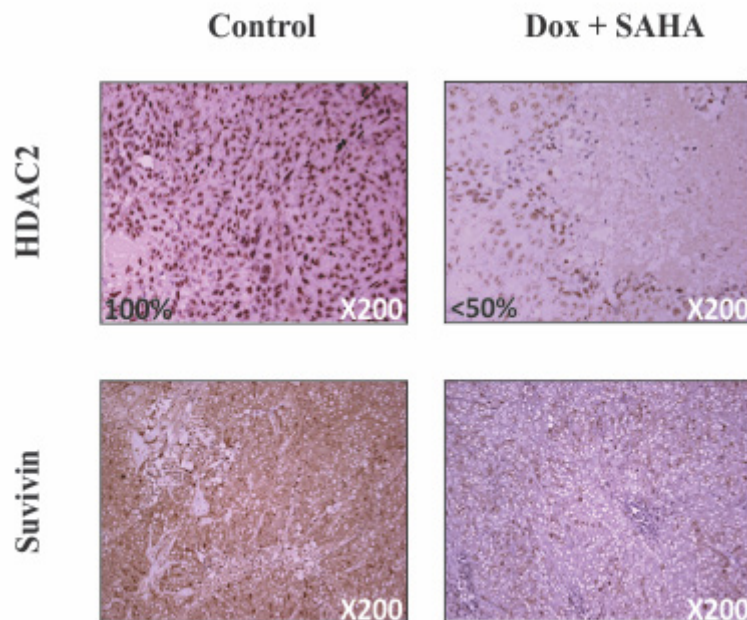
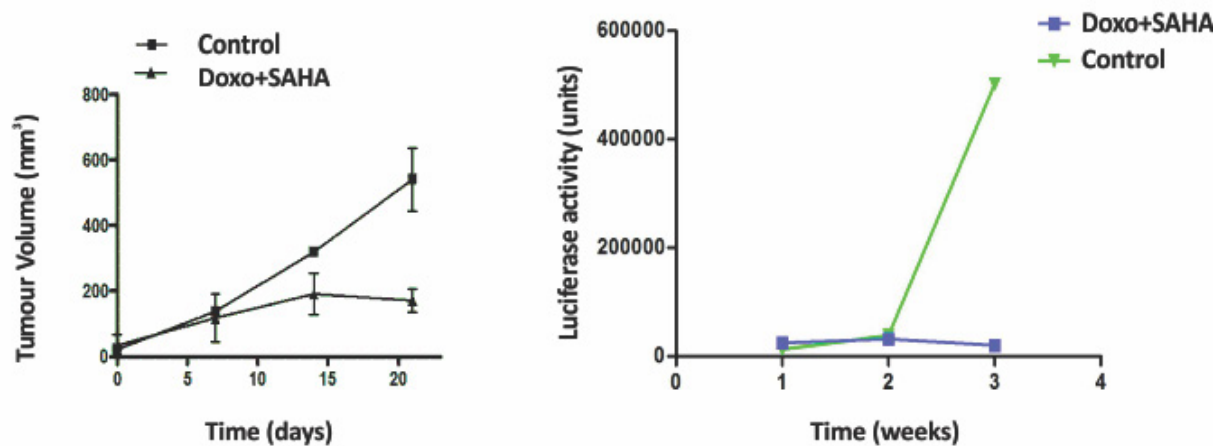


**Figure 3.18 C: Immunohistochemistry analyses for HCT116 p53<sup>+/+</sup> xenograft in mice.**

*Immunohistochemistry analyses for HDAC2 and survivin on the control and the treated tumour extracted from HCT116 P53<sup>+/+</sup> xenograft mice. HDAC2 and survivin levels have decreased by more than 50% after Dox/SAHA combined treatment.*

A



B

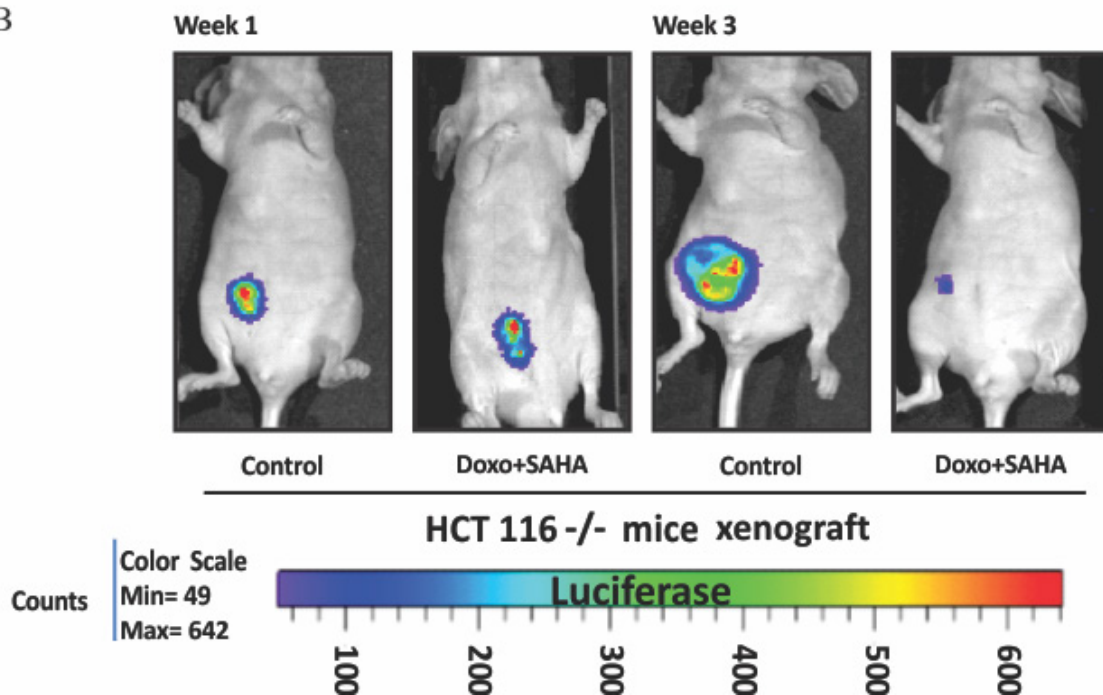


**Figure 3.19: In vivo imaging of liposome-encapsulated Dox/SAHA treatment effects in HCT116 p53-/- xenograft mice.**

*A: The tumour size and luciferase activity in control and liposomal Dox/SAHA treated HCT116 p53-/- xenograft groups. Error bars represent by the mean  $\pm$  S.E.M.;  $n=3$ . Statistical significance at  $p < 0.05$ . B: Bioluminescence imaging for control and Dox/SAHA treated HCT116 p53-/- xenograft mice throughout three weeks shows a decrease in tumour size.*

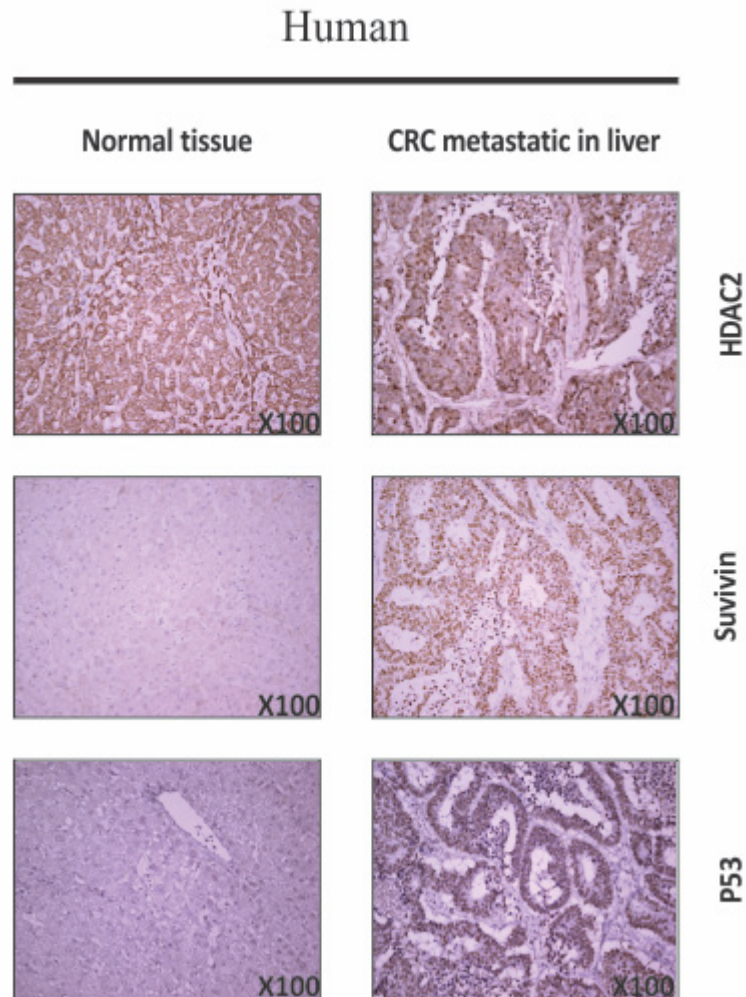


**Figure 3.20: Immunohistochemical analyses for HDAC2, survivin, and p53 expression levels in 3 samples of normal human liver and CRC hepatic metastases.**

*Metastatic CRC tissues have shown high levels of HDAC2, survivin, and p53 in comparison with their human normal tissues. This confirms in vitro results found in this project.*

Table 3.1: Immunohistochemistry observations for 10 samples of human CRC metastases in liver.

| Sample ID            | P53 levels                                          | HDAC2 levels                                | Survivin levels                                                                |
|----------------------|-----------------------------------------------------|---------------------------------------------|--------------------------------------------------------------------------------|
| (intact and mutated) |                                                     |                                             |                                                                                |
| HS08-11131           | negative                                            | moderate nuclear in 100% of cells           | weak cytoplasmic in all cells and moderate nuclear in < 50% of cells           |
| HS09-5038            | moderate to strong nuclear staining in 90% of cells | moderate nuclear in 100% of cells           | weak cytoplasmic in all cells and moderate to strong nuclear in > 50% of cells |
| HS09-8283            | weak nuclear staining in 1% of cells                | moderate to strong nuclear in 100% of cells | weak cytoplasmic in all cells and moderate nuclear in < 50% of cells           |
| HS10-313             | negative                                            | moderate to strong nuclear in 100% of cells | weak cytoplasmic in all cells and moderate to strong nuclear in > 50% of cells |
| HS08-12482           | strong nuclear in 75% of cells                      | moderate to strong nuclear in 100% of cells | weak cytoplasmic in all cells and moderate nuclear in < 50% of cells           |
| HS08-2468            | strong nuclear in 95% of cells                      | weak to moderate nuclear in 100% of cells   | weak cytoplasmic in all cells and strong nuclear in < 50%                      |
| HS09-8935            | negative                                            | moderate to strong nuclear in 100% of cells | weak cytoplasmic in all cells and moderate to strong nuclear in > 50% of cells |
| HS10-8435            | moderate nuclear in 45% of cells                    | moderate to strong nuclear in 100% of cells | weak cytoplasmic in all cells and moderate to strong nuclear in < 50% of cells |
| HS09-4289            | moderate nuclear in 15% of cells                    | strong nuclear in 100% of cells             | weak cytoplasmic in all cells and moderate to strong nuclear in > 50% of cells |
| HS08-11129           | moderate nuclear in 95% of cells                    | moderate nuclear in 100% of cells           | weak cytoplasmic in all cells and moderate to strong nuclear in > 50% of cells |
| General comment      | No nuclear staining in normal tissue                | No nuclear staining in normal tissue        | No nuclear staining in normal tissue                                           |

# **Chapter 4**

## **Discussion**

## **Chapter at Glance:**

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# 1. Discussion

The ultimate role of p53 protein is to maintain the integrity of the genome in the cell; hence it is dubbed “the guardian of the genome” (Efeyan and Serrano, 2007). To accomplish this role, p53 is involved in several pathways forming a functional circuit called the p53 functional circuit (Jin and Levine, 2001) which opt for DNA repair, cell cycle arrest, or apoptosis when the cell has stress signals, as shown in figure 4.1. Giving this importance of p53, more than 50% of human cancers have mutated p53 (Miller et al., 2005). The great bulk of the studies in cancer research field have shown that mutated p53 induces resistance to chemotherapy treatment in cancer cells (Mogi and Kuwano, 2011, Crea et al., 2011). Consistent with these studies, however, in this research project, I have shown that reduction in HDAC2 expression level plays an essential role in mCRC response to DNA damaging agents as single treatment or combined with HDACIs, whereas *TP53* mutation status was found to be a less significant drug resistance factor in mCRC. While, mutated *TP53* HT-29 cells exhibited extreme resistance to drugs, mutated *TP53* SW480 cells showed drug sensitivity comparable to that of wild-type p53 HCT116 cells (HCT116 p53<sup>+/+</sup>). Although, all tested combined-treatments were found to exert a synergistic effect on the induction of cell death in mCRC HCT116 p53<sup>+/+</sup> cells, cell death induced in HCT116 p53<sup>-/-</sup> (null p53

cells) was shifted from synergistic with Dox/SAHA combinations to antagonistic with Dox/VPA, Dox/NaB, and all CPT-11 combinations.

These results point to the nonessential role of *TP53* in the effects of Dox/SAHA combinations since this treatment induces a synergistic effect on cell death in wild type and null p53 cells. Also, this is supported by previous finding where in the absence of functional p53, SIRT1 (HDAC class III) expression level is a critical parameter in trichostatin A or VPA-mediated sensitization of several multidrug-resistant cancer cells to topoisomerase II inhibitor etoposide (Hajji et al., 2010). Additionally, the introduction of wild-type p53 alone is not sufficient to substantially alter the sensitivity of cancer cells to a given chemotherapeutic agent (Breen et al., 2007). Mutated p53 expression in different cell backgrounds causes distinct patterns of drug resistance, indicating that cell context can influence this phenotype (Sampath J et al., 2001). These differences in drug resistance might be explained by epigenetic regulation of certain genes which are influenced by mutated p53 (Sampath J et al., 2001). In fact, reconstitution of p53 protein in HT-29 was not able to increase sensitivity to drug treatment in these cells (work was done by my colleague who continues this research).

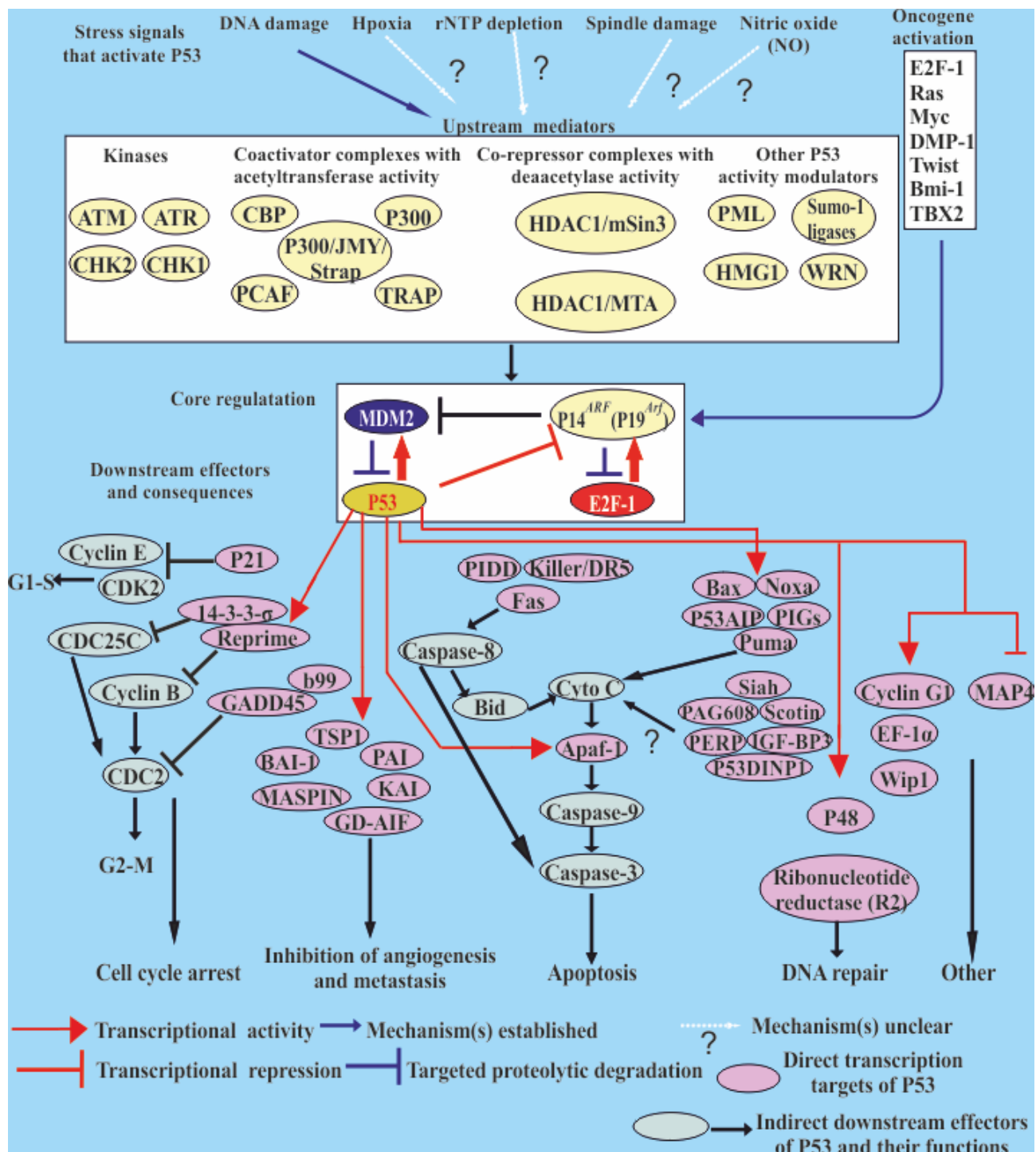


**Figure 4.1: The p53 functional circuit.**

*P53 is maintained at a low level in cells under normal conditions. However, upon many stress signals p53 is activated and stabilized to transcriptionally regulate or directly activate proteins involved in DNA repair process, Apoptosis, inhibition of transformation, and cell cycle arrest. Through its pathways, p53 retains intact genome in cells and suppresses tumour formation. The figure was re-shaped from (Jin and Levine, 2001).*

In wild type p53, null p53 and mutated p53 (SW480) cell lines which were treated with SAHA or VPA combined with Dox, significant decrease in the expression level of HDAC2 is observed when apoptosis is induced, as shown by PARP cleavage as a hallmark of apoptosis. In contrast upon the same combinations, mutated p53 (HT-29) cell line shows an increase in HDAC2 expression with resistance to cell death. Remarkably HDAC2 overexpression renders mutated p53 SW480 cells resistant to the combined treatment. These results suggest that p53 mutation in mCRC does not confer protection against the effect of HDACIs (SAHA or VPA) combined with Dox, and provide evidence for the importance of HDAC2 level in drug combination response. Like HDAC2 expression, HDAC1 also seems to be upregulated in colorectal cancer (Lucio-Eterovic et al., 2008). Here I have shown, while HDAC2 expression level increased significantly with resistance to the treatment, HDAC 1 had no clear correlation in all tested cell lines. The change in HDAC2 expression levels does not associate with change in HDAC2 activity upon SAHA/Dox treatment in the cell lines used in this study. This was investigated by HDAC2 activity assay which was kindly done by Dr Nicolas Mercado as he has the facility in his laboratory (Airway Disease Section, National Heart and Lung Institute, Imperial College). After I treated the four cell lines (HCT116 p53+/+, SW480, HCT116 p53-/-, and HT-29) with SAHA/Dox combination. The

cells were collected and sent out for measuring HDAC2 activity. Interestingly, the four cell lines have shown no significant change in HDAC2 activity upon the treatment, as shown in figure 4.2. However, measuring HDAC2 activity supported that wild-type p53 cells have high expression levels and more activity of HDAC2 than mutated p53 cell lines.

## HDAC2 activity (2hours, immunoprecipitation)

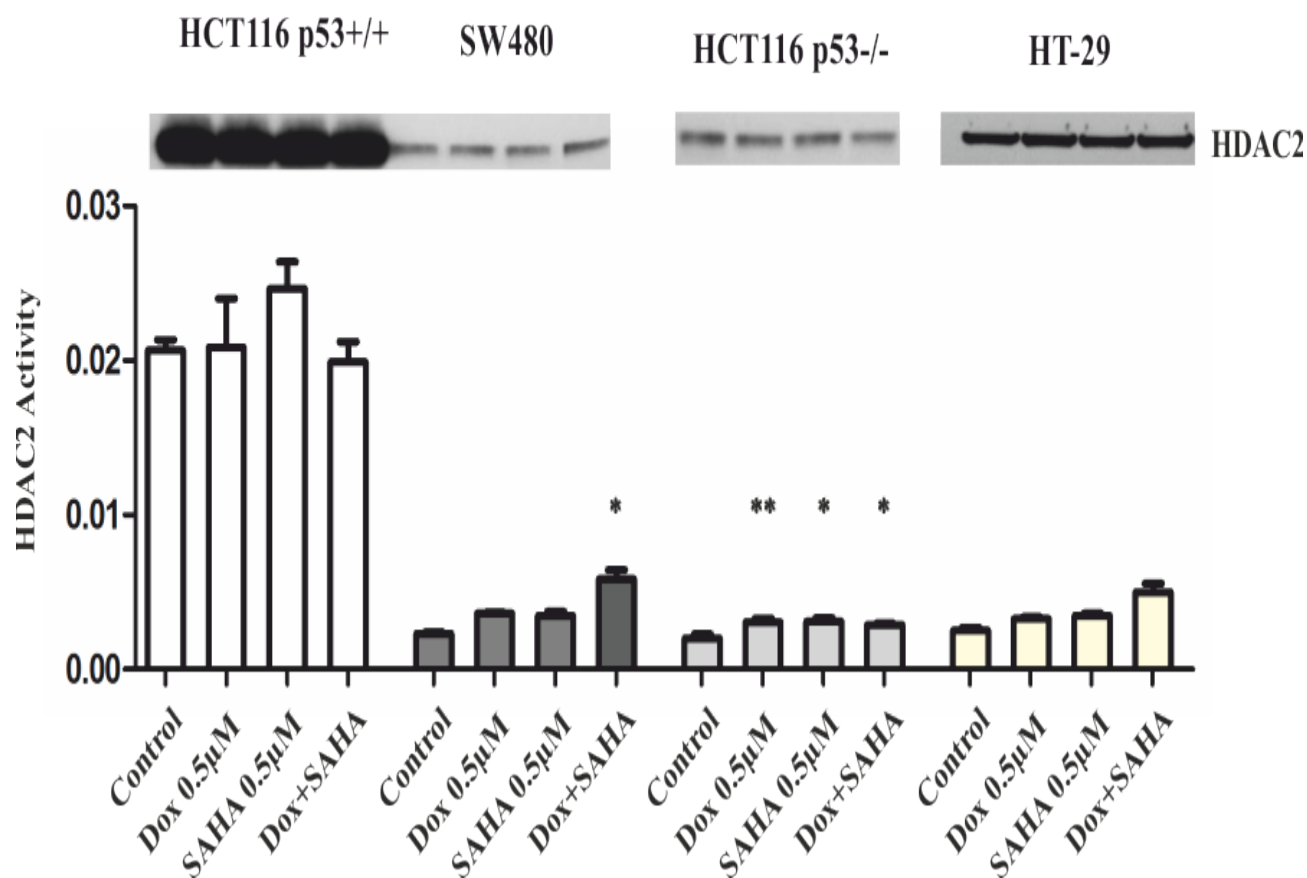


**Figure 4.2: HDAC2 activity measurement upon SAHA/Dox treatment.**

The four cell lines (HCT116 p53+/+, HCT116 p53-/-, SW480, and HT-29) were treated for 24 hours with Dox and SAHA as single or combined. The cells were then collected and sent out for measuring HDAC2 activity. Interestingly, each cell line has shown no clear change in HDAC2 activity. However, this measurement supports what I found about high expression level of HDAC2 in wild-type p53, whereas low expression level in mutated p53 cell line. Data was presented as mean ± S.E.M.; n=3. \*P between 0.01-0.05, \*\*P between 0.001-0.01.

Also, I have established an in vivo model for verification of HDAC2 level and the response to combined treatment, where liposomal-encapsulated SAHA/Dox was used to treat human mCRC xenografts mice as an innovative delivery strategy for toxicity reduction. According to data available on the internet web, this is the first time that a nanoparticle (liposomal) approach has been applied to deliver a HDACI/DNA damaging agent (SAHA/Dox in this project) combined therapy directed to the site of action for mCRC treatment. I have found that the combined treatment induces a proportional and significant decrease in HDAC2 expression level associated with tumour shrinkage as measured by tumour size and luciferase activity. The combined treatment induces similar reduction in both wild type p53 and null p53 tumour. Therefore, this verifies the nonessential role of p53 against the level of HDAC2 as potential marker for positive response to this combined treatment. Interestingly, I have found that mutated p53 (SW480 and HT-29) and null p53 cell lines express a low level of HDAC2 in comparison with wild-type p53 cell line which expresses a high level of HDAC2 in untreated cells. The mechanism of HDAC2 inactivation and drug treatment inducing cell death remains largely unknown. In HT-29 cell line, I found that HDAC2 depletion disturbs the higher order chromatin structure in a similar manner as HDACIs and increases HT-29 sensitivity to 5FU or Oxa treatment

resulting in mitotic cell death (MCD). Recently, whole genome expression microarray analyses performed on HDAC2 knockdown human hepatocellular carcinoma (HCC) cells has identified up-regulation of many of cell cycle inhibitors genes and down-regulation of cyclins genes (Noh et al., 2011). Therefore, I investigated the mechanism of HDAC2 depletion on cell death in mCRC. The results in my research support what has been found in the above mentioned studies, and they further elucidate the underlying mechanism of the HDAC inhibitor SAHA combined with 5FU or Oxa and the role of HDAC2 in inducing mitotic cell death in HT-29 cell line. The increased HDAC2 expression has been found in colorectal cancer patients at mRNA and protein level indicating that HDAC2 overexpression is due to transcriptional activation (Zhu P et al., 2004). This overexpression of HDAC2 appears to be implicated in cancer through its aberrant recruitment and consequent silencing of tumour suppressor genes. For example,  $\beta$ -catenin/T-cell factor (TCF) signalling pathway which is deregulated in colon cancer regulates HDAC2 transcription. Also, histone acetyltransferase p300/CREB-binding protein (CBP) is found to be a critical coactivator for  $\beta$ -catenin-TCF-mediated survivin transcription (Ma, 2005, Ma et al., 2005) which is enhanced by  $\beta$ -catenin and repressed by p53 [32]. However, the relationship among survivin, mutated *TP53*, and HDAC2 on cell death has not been explored upon the combined treatment

in mCRC. Here I have shown that combined treatment VPA/Dox or SAHA/Dox induced dramatic reduction in survivin and HDAC2 expression level in wild-type p53 and SW480 but not in HT-29 cells. The overexpression of survivin in HT-29 appears to correlate with increased HDAC2 and the resistance to the combined treatments. Therefore, p53 appears not to be involved in survivin and HDAC2 regulation of cell death upon these combined treatments. Moreover, the results in this research show that SAHA/Oxa or shRNA-HDAC2/Oxa triggered clear increase in survivin level with dramatic decrease in the HDAC2 expression in HT-29 cells. This unexpected increase in survivin level upon cell death supports the special cell death (mitotic cell death) induced after SAHA/Oxa or shRNA-HDAC2/Oxa in HT-29. A body of evidence indicates that compact chromatin is crucial for the protection against agents causing DNA breaks and oxidative DNA damage. This protection disappears via chromatin relaxation (Ljungman and Hanawalt, 1992). HDACIs increase histone acetylation and the subsequent chromatin relaxation renders DNA more susceptible to a number of DNA-damaging agents (Rajendran P et al., 2011). It may also allow for the increased binding of transcription factors that regulate genes involved in cell death. Interestingly, it has been suggested that p53 has the biochemical potential for inducing chromatin relaxation, as it can employ the histone acetyltransferase (HAT) p300 to

chromatin and thus initiate histone acetylation (Espinosa and Emerson, 2001) and subsequently enhance chromatin accessibility to enable efficient lesion detection (Green and Almouzni, 2002, Friedberg, 2001, Rubbi and Milner, 2003). On the contrary, recent studies showed that the elevated acetylation levels in H3 correlate with cancer proliferation and drug resistance (Toth et al., 2012, Simpson NE et al., 2010). However, it is not clear whether such differences in acetylation steady state levels form a marker for resistance to drugs as single therapy or combined with HDACIs in cancer cells. With this perspective in mind, I have demonstrated that doxorubicin combined with SAHA or VPA triggers a decrease in histone acetylation in sensitive mCRC cells (wild-type p53 and SW480 cells) but not in highly resistant HT-29 cells. Therefore, in HT-29 the H3K9ac, H4K12ac and H4K16ac levels remain elevated after the combined treatments compared to the other cells. These results are in agreement with the elevated levels of lysine 9-acetylated histone H3 that occur at the multidrug-resistance protein 1 (MDR1) promoter in multidrug-resistant cells (To KK et al., 2008). Hence, the association between histone acetylation levels and mCRC sensitivity could have particular importance, since the level of acetylation may predict the combination therapy outcomes. However, the level of histone acetylation appears to be independent on HDAC2 regulation in drug resistance, and possibly

HDAC2 expression represses activation of transcription factors which control cancer cell death. For example, it has been found that overexpression of HDAC2, but not HDAC1, represses NOXA gene expression and induces resistance towards VPA and etoposide combined treatment in pancreatic cancer cells, while HDAC2 depletion upregulates NOXA protein level and sensitizes these cells to the VPA and etoposide combined treatment (Fritsche et al., 2009). In summary, HDAC2, in a given tumour entity, might not only be of prognostic value, but may also predict the response to combined (HDACIs/DNA damaging drug) therapy. I think that identification of HDAC2 expression as a possible sensitive “epigenetic biomarker” associated with HDACIs/ DNA damaging drug resistance may lead to a new molecular target for mCRC therapy, help to improve treatment of CRC and provide a more robust mechanistic rationale for the use of HDACIs. It is, therefore, highly probable that the overall response to conventional treatment may be less effective in patients that strongly express HDAC2 in their cancer cells. Also, it may be rational to recommend that translational protocols for future clinical trials should include HDACIs as adjuvant therapeutic agents with conventional mCRC drugs.

## **2. Study limitation**

I would like to mention that the time was a major limitation. Also, lack of specific HDAC2 inhibitors until now is a potential limitation in my research. All HDAC inhibitors (SAHA, VPA, and NaB) used in this project are not specific for HDAC2.

## **3. Future works emerging from this study**

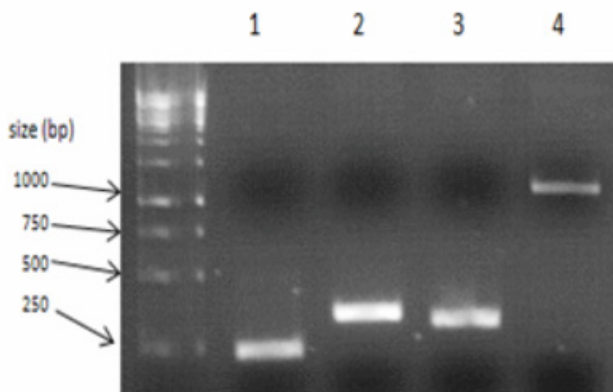
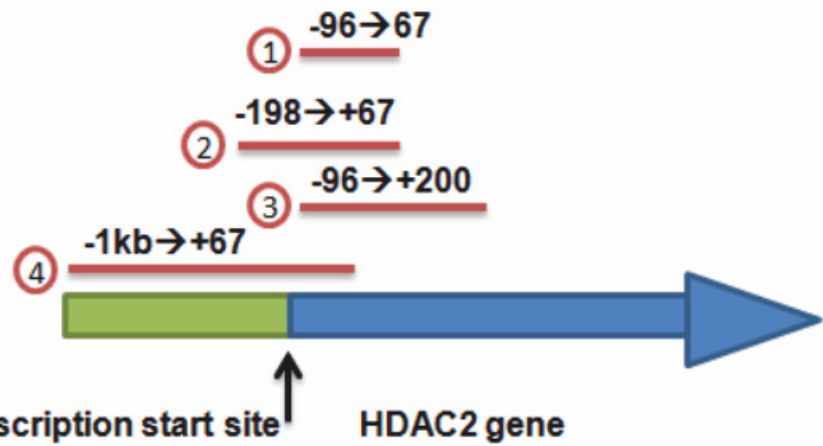
- The in vivo experiment done in this project was a preliminary and proof of concept for Dox/SAHA combined treatment effect. In the future, therefore, the in vivo work will be expanded to include Dox and SAHA as single treatment.
- Also, xenograft mice bearing ShRNA-HDAC2 HCT116 p53<sup>-/-</sup> or ShRNA-HDAC2 HT-29 tumours will be established.
- Testing more mCRC cell lines for HDAC2 role found in this project.
- Introduction of wild-type p53 into the mutated p53 cells line (SW480 and HT-29) and investigation into effects on cell death would be encouraging (My colleague who continues this project did some preliminary work on HT-29 as mentioned in the discussion).
- In addition, examination of the lysine residues in histones tails and other proteins controlled via HDAC2 expression level and

identification of histone or non-histone cell death code will be investigated in future work. Also, exploration of HATS enzymes which counteract HDAC2 action and their balance in mCRC cell lines will support this study.

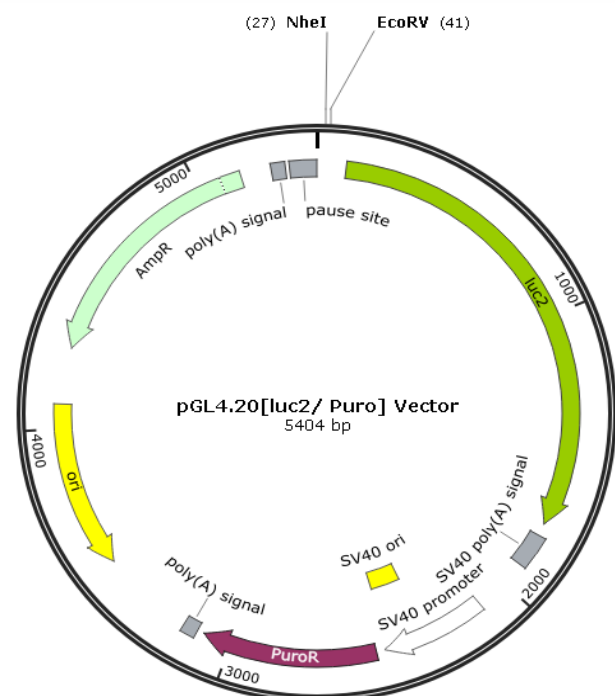
- Using Micrococcal Nuclease enzyme (MNase) digestion for chromatin extracted from mCRC cell lines established in this project will be done. MNase digestion is a good and simple assay to map nucleosome positions in chromatin. Applying quantitative real-time PCR (qPCR) technique after extensive MNase digestion up to mononucleosome-sized fragments of DNA will give an idea about DNA sequence that was protected from digestion (i.e. transcriptionally inactive DNA sequence).
- Finally, assessment of HDAC2 recruitment into DNA and its control of the pro-survival genes in mCRC will be in the future work. Therefore, my colleague started generating a reporter gene using luciferase gene within pGL4.20 plasmid and different parts of HDAC2 promoter as shown below. The idea is to check which part of HDAC2 promoter plays a role in transcription process, and then transcription factors and proteins which bind to the effective part of HDAC2 promoter will be investigated.

## Generating HDAC2 promoter luciferase reporter constructs

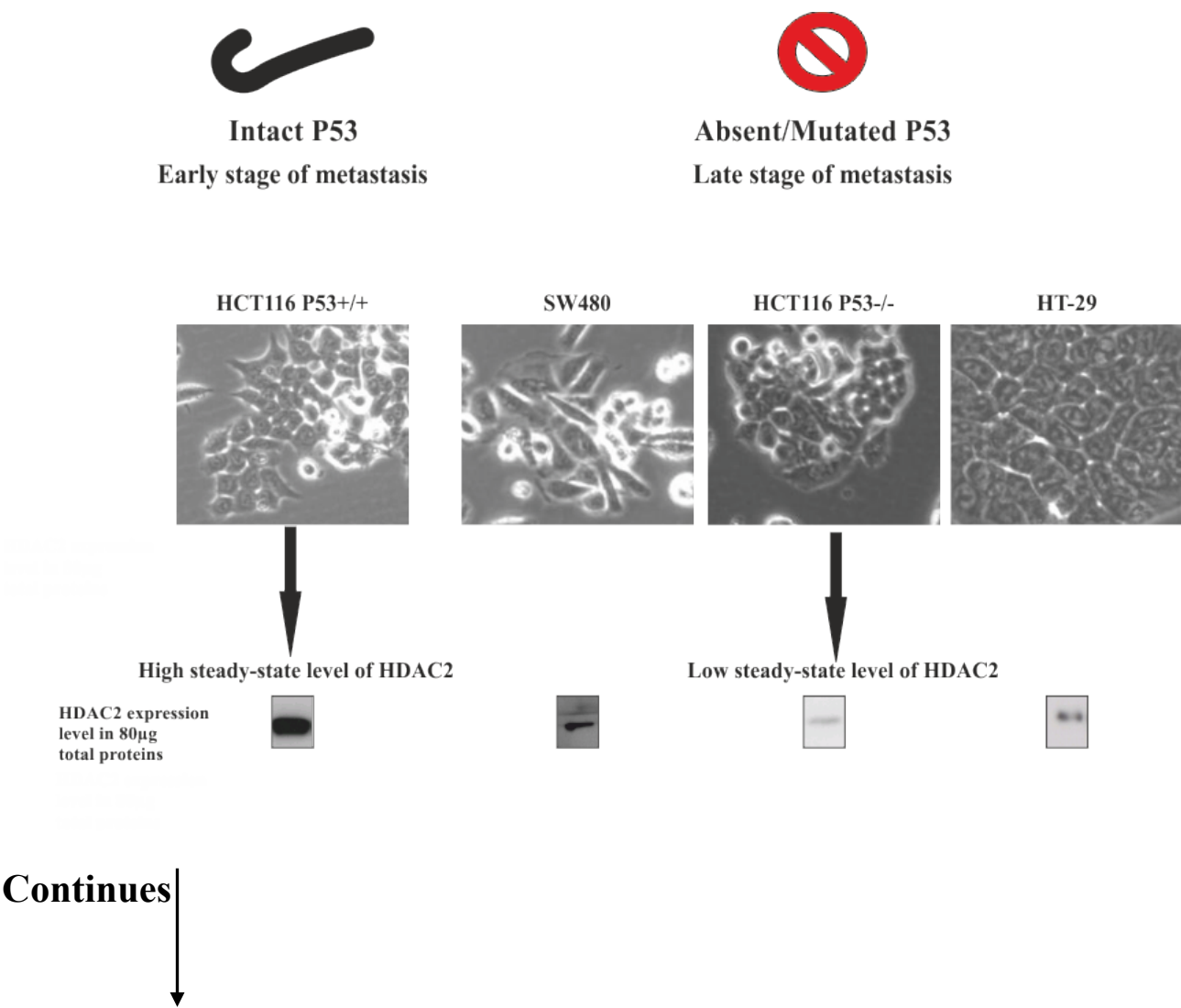
*Cloning different parts of HDAC2 promoter*



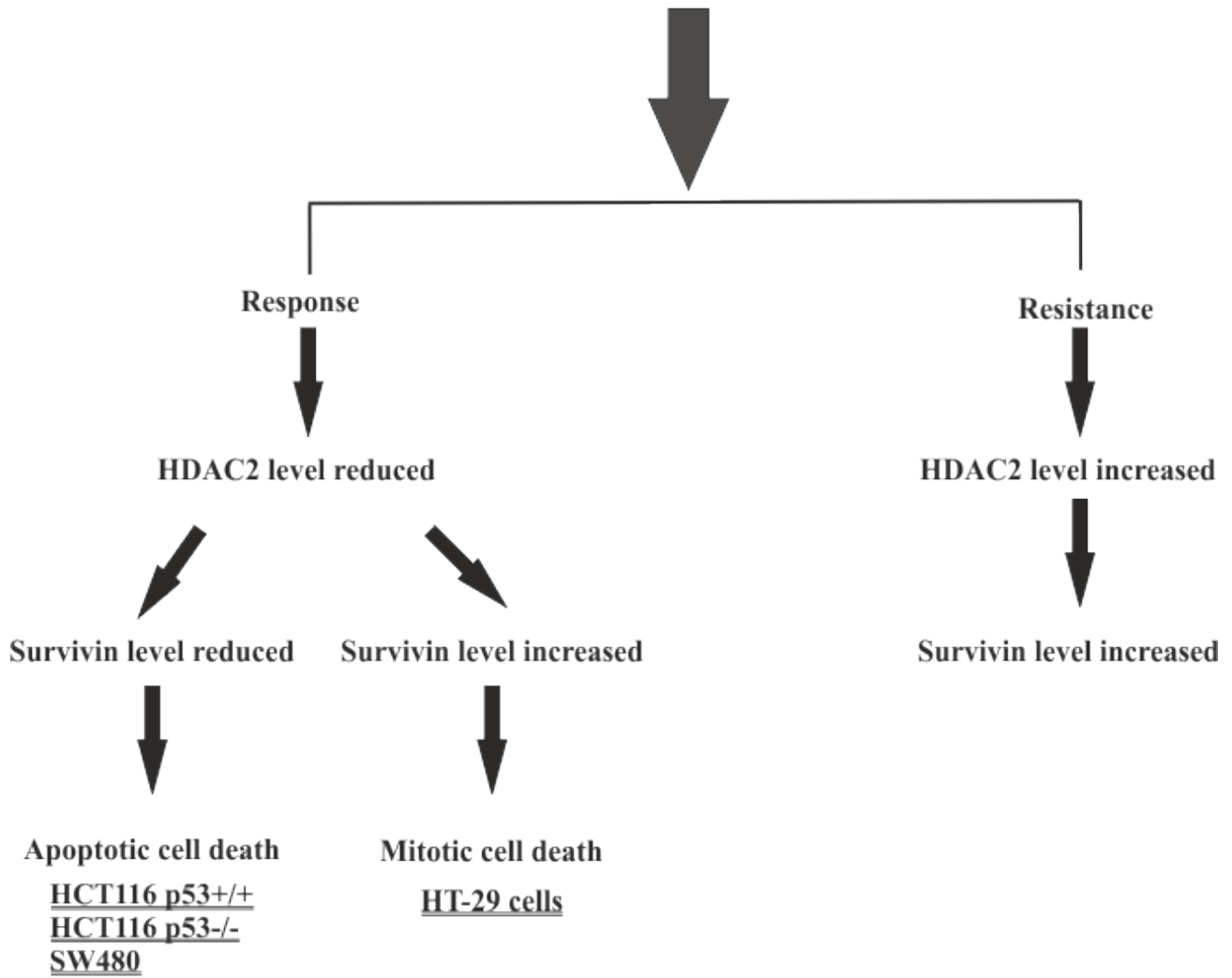
*pGL 4.2 Luciferase plasmid*



4. A schematic summary of the results obtained in this project



## HDACIs/DNA damaging agents treatment



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