# DOCTOR OF PHILOSOPHY

## Functional characterisation of SPO11 in human cancer cells

Althobaiti, Fayez

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# Functional characterisation of SPO11 in human cancer cells

Ph.D. Thesis

2017

**Fayez Alhumaidi Althobaiti**

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# Abstract

Cancer is considered one of the main causes of death worldwide. A combination of various genetic or epigenetic modifications, which lead to distinct chromosomal abnormalities or mutations, cause somatic cell transformations to cancer cells. Since tumour progression is caused by multiple mechanisms and factors, estimating the risk of cancer and determining the appropriate therapy are both becoming serious challenges. Ultimately, a fundamental aim is to design targeted therapeutics to treat cancer without the limitation of potential adverse side effects on normal cells.

The identification and functional characterisation of specific genes, which are known as cancer testis antigens (CTAs) gene or cancer/germline genes provides a number of cancer-specific biomarkers. Their expression is largely limited to germline cells in normal tissues, and can play a core role in cancer diagnosis, prognosis, prevention and treatment. In this study, a novel human CTA gene, *SPO11*, is identified in terms of its expression, protein localisation and likely function in different cancer cells.

Herein, we demonstrate that human SPO11 protein is observed in testis, most cancer cells, and some tumour tissues, but it is not found in normal healthy tissues. *SPO11* knockdown in various cancer cell lines results in reduced proliferation, detached and unviable cells. We present evidence to suggest that SPO11 depletion alters the level of cell cycle regulatory proteins without inducing apoptosis or senescence, suggesting SPO11 functions in cancer cells to alter cell cycle dynamics.

Since chromosomal instability and uncontrolled cell proliferation are considered hallmarks of human cancer, the results presented herein suggest that SPO11 may play a critical role in genome stability control and be essential for cancer progression. The presence of SPO11 in cancer cells may lead to aberrant initiation of DNA double-stranded breaks and/or altered DNA replication/chromosome segregation during mitosis resulting in chromosome changes that subsequently develop towards cancer. Eventually, the SPO11 protein may have diagnostic, prognostic and therapeutic value in cancer treatment.

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# List of Abbreviations

| | |
|---|---|
| 3' | Three prime end of DNA |
| 5' | Five prime end of DNA |
| AEs | Axial elements |
| ATP | Adenosine-5’-triphosphate |
| amp | Ampicillin |
| BCA | Bicinchoninic acid |
| bp | Base pair |
| BTB | Blood-testis barrier |
| BRCA1 | Breast cancer susceptibility 1 |
| BRCA2 | Breast cancer susceptibility 2 |
| CTA | Cancer testis antigen |
| CT | Cancer-testis |
| C-terminal | Carboxy-terminal domain |
| CpG | -Cytosine-phosphate-guanine- |
| CO | Crossover |
| CE | Central element |
| cDNA | Complementary DNA |
| °C | Degrees Centigrade |
| CDK | Cyclin dependent kinase |
| CTLs | Cytotoxic T lymphocytes |
| *CTAG1B* | Cancer/testis antigen 1B |
| SCCs | Squamous cell carcinomas |
| dHJ | Double-Holliday junction |
| DMEM | Dulbecco's Modified Eagle Medium |
| DSB | Double-strand break |
| DPBS | Dulbecco’s phosphate-buffered |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| D-loop | Dissociation loop |
| DAPI | 4’-6-diamidino-2-phylindole |
| Da | Dalton |

| | |
|---|---|
| dNTPs | Four deoxynucleoside triphosphates |
| dH2O | Distilled water |
| DC | Dendritic cell |
| DMC1 | Disrupted meiotic cDNA1 |
| ECACC | European Collection of Cell Cultures |
| ELDA | Extreme limiting dilution analysis |
| *E. coli* | *Escherichia coli* |
| esiRNA | Endo-ribonuclease prepared siRNA |
| FBS | Foetal bovine serum |
| F | Forward |
| G0 | Quiescent phase of the cell cycle |
| G1 | Gap-1 phase |
| G2 | Gap-2 phase |
| g | Gram |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GTEx | Genotype-Tissue Expression |
| GBM | Glioblastoma multiforme |
| HR | Homologous recombination |
| HLA | Human leukocyte antigen |
| hrs | Hours |
| Hela | Henrietta Lacks |
| HA tag | Human influenza hemagglutinin |
| HBV | Hepatitis B |
| HCV | Hepatitis C |
| HAT | Histone acetyltransferase |
| IF | Immunofluorescent staining |
| IHC | Immunohistochemistry |
| kb | Kilobase |
| KDa | Kilodalton |
| L | Litter |
| LB | Luria bertani |
| *MAGE-A* | Melanoma antigen family A |
| MHC | Major histocompatibility complex |

| | |
|---|---|
| MRN | MRE11–RAD50–NBS1 |
| MRX | Mre11-Rad50-Xrs2 |
| M-PER | Mammalian protein extraction reagent |
| M | Mitosis phase |
| min | Minute |
| mRNA | Messenger RNA |
| mA | Milliamperes |
| μg | Microgram |
| μL | Microliter |
| ml | Milliliter |
| MCS | Multiple cloning site |
| MW | Molecular weight |
| N-terminal | Amino-terminal domain |
| NCO | Non-crossover |
| NHEJ | Non-homologous end-joining |
| ng | Nanogram |
| nM | Nanomolar |
| NCBI | National Centre for Biotechnology Information |
| *NXF2* | Nuclear RNA export factor 2 |
| NK | Natural killer |
| ORF | Open reading frame |
| *PRDM9* | PR domain zinc finger protein 9 |
| PCR | polymerase chain reaction |
| PVDF | polyvinylidene difluoride |
| PBS | phosphate buffered saline |
| pH | Power of hydrogen |
| PFA | Paraformaldehyde |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RT-PCR | Reverse transcription PCR |
| qRT-PCR | Quantitative, real time PCR |
| R | Reverse |
| RT | Room temperature |

| | |
|---|---|
| RB | Retinoblastoma-associated gene |
| SC | Synaptonemal complex |
| SIC | Synapsis initiation complex |
| ssDNA | Single strand DNA |
| siRNA | Small interfering RNA |
| S | Synthesis-phase |
| s | Seconds |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| shRNA | Short hairpin RNA |
| SW480 | Colon adenocarcinoma |
| SDS | Sodium dodecyl sulphate |
| SEREX | Serological analysis of recombinant cDNA expression libraries |
| SYCP1 | Synaptonemal complex protein 1 |
| *SAGE1* | Sarcoma antigen 1 |
| SCCs | Squamous cell carcinomas |
| TF | Transverse filament |
| TBS | Tris buffered saline |
| TAA | Tumour associated antigens |
| Tris | Tris (hydroxymethyl) aminomethane |
| TBE | Tris-borate-EDTA |
| TM | Melting temperature |
| TCGA | The Cancer Genomic Atlas |
| V | Volt |
| WB | Western blot |
| WCE | Whole cell extract |
| X-CT | X- chromosome cancer testis genes |

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# Chapter 1

## Introduction

# 1. Introduction

## 1.1 Cancer

### 1.1.1 Overview of cancer

Cancer is considered one of the main causes of death worldwide, accounting for over 8.2 million deaths annually. Although approximately 14 million annual cancer cases have been reported in recent years, this number is expected to reach 22 million cases within the next two decades. According to Cancer Treatment and Survivorship, Facts and Figures (2016-2017), the population of cancer survivors is estimated to be approximately 20.3 million; including 10 million males and 10.3 million females by January 1, 2026 in the US. Cancer affects various organs of the human body, including lung, stomach, colon, and breast. Prostate, breast, lung and colorectal cancers are the most common types of cancers in the United States, with lung, breast, prostate, colorectal and pancreatic cancers being considered the overall highest cause of death (Butterfield, 2015).

Cancer is a disease caused by uncontrolled cell division giving rise to tumours (Cavallo et al., 2011). In general, uncontrolled cell division results in the formation of benign tumours, some of which may switch to malignant tumours (Aly, 2012). The most common feature of cancer cells is proliferating quickly and aggressively with the loss of normal cell cycle regulation (Jayashree et al., 2015). Cancer is considered to arise due to a combination of various genetic or epigenetic alterations leading to distinct chromosomal abnormalities or mutations (Hatzimichael & Crook, 2013; Sharma et al., 2010; Wodarz & Zauber, 2015). These aberrations lead to altered cellular metabolism, DNA repair, DNA duplications and chromosome segregation errors. For example, errors in DNA replication may lead to abnormal genetic recombination, resulting in gene amplifications or deletions. Furthermore, changes in chromosome number may result from errors in DNA repair or chromosome segregation (Belpomme et al., 2007; Lutz, 2002; Vogelstein & Kinzler, 2004). In addition, most cancer cells present DNA replication stress, which can drive genomic instability (Macheret & Halazonetis, 2015).

The risk of cancer development can be increased by external or environmental factors, including carcinogens, age, smoking, obesity, diet, alcohol consumption, or infectious organisms (Anand et al., 2008). Human cancer can also be caused by biological agents such as hepatitis B or C viruses (HBV or HCV), which are linked to hepatocellular carcinoma (Lupinacci et al., 2013; Wodarz & Zauber, 2015).

For tumour formation, cancer cells must "escape" immune system checkpoints by overcoming immune reactivity and the "inflamed microenvironment" (Cavallo et al., 2011). Moreover, some cancers have the ability to spread (metastasise) to other tissues through the lymphatic system or bloodstream, resulting in the destruction of those tissues via the formation of secondary tumours (Figure 1.1) (Brábek et al., 2010; Fridman et al., 2014; Jemal et al., 2011).

Oncogenes, tumour suppressor genes and DNA repair genes are the most important genes associated with developing cancer (Vos et al., 2011). Despite their high regulation, mutation in these genes may be involved in the generation of tumorigenesis and can be identified as a hallmark of cancer (Hanahan & Weinberg, 2011; Negrini et al., 2010; Vogelstein & Kinzler, 2004). Table 1.1 summarises the main differences between these genes. According to the pathological features of cancerous cells, such as cancer cell origin and the stage or level of tumour progression, human cancers can be divided into four categories, namely carcinoma, sarcoma, leukaemia and lymphoma.

Malignant tumours of epithelial tissues are known as carcinoma, representing approximately 90% of human cancers and found in several organs such as skin, lung and breast. Sarcomas occur in connective tissue such as bone and muscles, whereas leukaemia (cancer of white blood cells) and lymphoma (cancer of B and T lymphocytes) initiate from haematopoietic cells (reviewed Ruddon, 2007; Weinberg, 2013).

Cancer can be classified into four types depending on the initiated tissues; including carcinoma that initiates in the epithelium, sarcoma that initiates in connective or supportive tissues, leukaemia that initiates in white blood cells, lymphoid that initiates in lymphoid tissues and myeloma that initiates in the plasma cells of the bone marrow (Siegel et al., 2012).

**Table 1-1 Examples of cancer-associated genes.**

| Gene type | Function | Example | References |
|---|---|---|---|
| Tumour-suppresser genes or anti-oncogenes | - Break cell proliferation<br>- Induce apoptosis<br>- Inhibit the formation of tumours | *TP53* and *RB* | (Thoma et al., 2011) |
| Oncogenes<br>Mutated proto-oncogenes | - Promote cell division<br>- Importance in chromatin remodelling, signal transduction and growth regulation | *RAS* subfamilies<br>Viral oncogenes<br>*Myc* | (Croce, 2008; Pylayeva et al., 2011) |
| Genomic stability genes<br>DNA repair genes<br>Caretaker genes | - DNA repair<br>- Regulation of genetic alteration | *ATM*<br>*BRCA1* and *BRCA2* | (Negrini et al., 2010) |

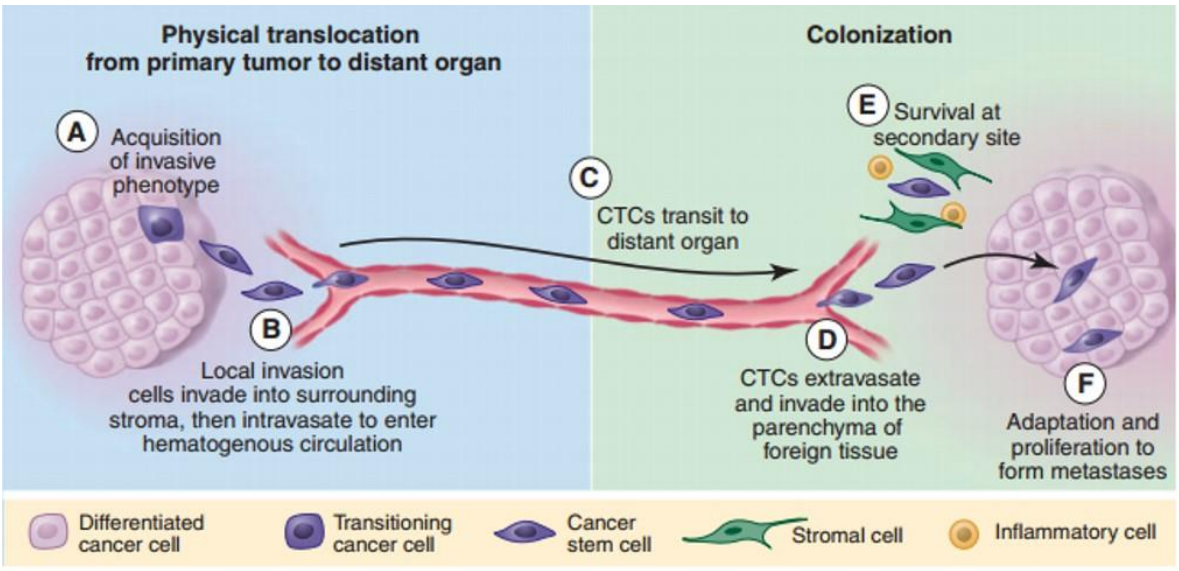



**Figure 1-1 The metastatic process.**
Metastasis consists of two phases: (1) translocation of cancer cells from a primary tumour to a distant organ, (2) followed by colonization of the distant organ and formation of a secondary tumour. (A) Invasive phenotype of cancer cells is initiated in order to commence metastasis. (B) Surrounding cells and their blood vessels are invaded by cancer cells. (C) Circulating tumour cells (CTCs) have anchorage-independent survival. (D) Foreign tissue at the distant organ is invaded by CTCs after exiting the circulation. (E) Cancer cells in the foreign site have to escape from the innate immune system and survive. (F) Cancer cells in the new location start to divide after adapting to the new microenvironment (Adapted from Chaffer & Weinberg, 2011).

### 1.1.2 Hallmarks of cancer

Cancer can be distinguished from normal tissue by the specific features of cancer cells and tissues, termed the hallmarks of cancer (Hanahan & Weinberg, 2011; Sonnenschein & Soto, 2013). According to Hanahan and Weinberg (2011), ten hallmarks of cancer are proposed, namely sustained proliferative signalling, evasion of growth suppressors, resistance to cell death, enabling of replicative immortality, induction of angiogenesis, activation of invasion and metastasis, genome instability and mutation, deregulation of cellular energetic mechanisms, tumour-promoting inflammation, and evasion of immune destruction (Figure 1.2). Table 1.2 lists the distinguishing features of all the hallmarks of cancer.

#### 1.1.2.1 Contact inhibition

Cells undergo quiescence, which is a reversible state, when there is a shortening in serum growth factor, resulting in slower proliferation. During senescence, which is an irreversible state, the cell cycle is arrested regardless of the presence of growth factors (Demidenko & Blagosklonny, 2008; Serrano et al., 1997). Senescence has an important role in tumour suppression, organism aging and embryonic development, and can be stimulated by DNA repair, the activation of oncogenes and the inactivation of tumour suppressor genes (Lapasset et al., 2011; Sun, 2014). Contact inhibition functions as another type of quiescence when cells at high density come into contact with each other, resulting in arrested growth. Contact inhibition is also reversible since the cells can re-enter the cell cycle and start dividing again when they split and re-plate at low density. The distinct features of contact-inhibited cells are low protein synthesis and metabolism and a small vertical morphology (Leontieva, 2014). It is suggested that the signalling pathways, which distinguish irreversible senescence from reversible quiescence, may promote by contact inhibition or growth factor deprivation (Leontieva, 2014). The mechanisms of contact inhibition of cell movement and proliferation are determined by nectin-like molecules (Necls), which play an essential role in the tissue regeneration of tumour phenotypes and different cellular organisms. In fact, nectins and nectin-like molecules are present in several cell types and are immunoglobulin-like transmembrane cell adhesion molecules (Takai et al., 2008). The majority of cells in living organisms are contact-inhibited and have the ability to re-proliferate under specific conditions, such as tissue damage, in order to retain their integrity. The resistance to anti-cancer drugs in tumour cells can be the result of the suppression of senescence in a densely packed tumour (Sun, 2014).

The reduction of intercellular adhesion levels leads to the loss of epithelial differentiation in carcinoma. In addition, the invasion and metastasis of carcinoma cells can be a result of the distribution and/or impairment of the integrity of intercellular junctions, including the cell-cell adhesion receptor and cell adhesion molecule E-cadherin. Therefore, the association of the adherence junction protein beta-catenin and the tumour suppressor gene as well as reducing E-cadherin expression level are the main reasons for greater invasiveness in carcinoma cells (Birchmeier., 1995; Jeanes., et al 2008). In vitro, the hydrophobic polystyrene surface will be more hydrophilic in order to obtain an acceptable level of cell attachment. Thus, cell attachment proteins such as vitronectin and fibronectin in the serum can adhere and spread on the bottom of the flask, resulting in cell attachment (Ramsey et al., 1984).

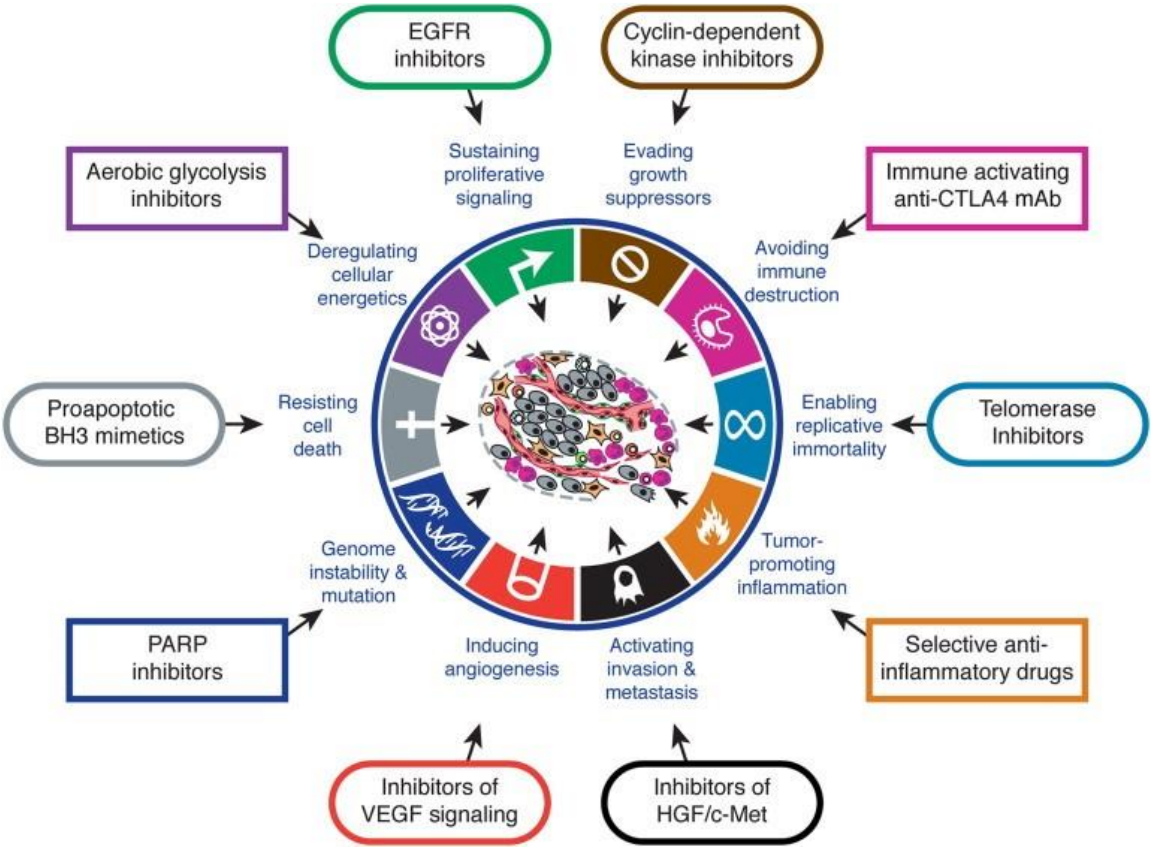



**Figure 1-2 Schematic diagram of cancer hallmarks and their therapeutic agents.**
The figure illustrates the hallmarks of cancer that lead to cancer cell immortality through a variety of mechanisms such as replication, development of resistance to cellular death or apoptosis, evasion of growth suppressors, promotion of proliferative signalling, sustaining angiogenesis, and tissue invasion and metastasis. Additionally, cancer cells have the ability to initiate inflammation, evade immune destruction and re-programme energy metabolism through mutation and genomic instability. Furthermore, promising newly developed cancer therapeutics (developed drugs), which target the specific cancer hallmarks indicated, are also shown (Adapted from Hanahan & Weinberg, 2011).

**Table 1-2 The features of the hallmarks of cancer (Hanahan & Weinberg, 2011).**

| Hallmarks of cancer | Causes | Result |
|---|---|---|
| Sustaining of proliferative signalling | Deregulation of cell cycle and cell growth proteins | Uncontrolled cell division |
| Evasion of growth suppressors | Mutation in tumour suppressor genes | Failure to regulate cell proliferation |
| Resisting cell death | Defection in the apoptotic programme | Escape from apoptosis |
| Enabling replicative immortality | Delay of cell senescence or cell elimination | unlimited cell division and DNA replication |
| Inducing angiogenesis | The requirement of metabolism materials | New blood vessels are formed |
| Activating invasion and metastasis | Spread of cancer cells via the blood and lymphatic vessels | New tumours in new organs are initiated |
| Genome instability and mutation | Alterations in the genome | Changes in genomic structure |
| Deregulation of cellular energetics | Re-programming of energy metabolism | Causing mutation in tumour suppressor genes and oncogenes |
| Tumour promotion of inflammation | Usual infecting reaction | Formation of chronic inflammation |
| Evasion of immune destruction | Poorly understood | Avoidance of the immune system |

### 1.1.3 Epigenetics and cancer

#### 1.1.3.1 DNA methylation and cancer

Cancer is associated with genetic alterations such as chromosomal abnormalities and mutations in tumour-suppressor genes and oncogenes. However, cancer is also considered an epigenetic disease since it was observed that a key feature of cancerous cells is DNA methylation deficiency at CpG dinucleotides (Feinberg & Vogelstein, 1983; Hatzimichael & Crook, 2013). In many cancerous cells, transcription silencing of DNA repair genes is connected with DNA hypomethylation (non-promoter regions) and hypermethylation (promoter regions) (Akhavan et al., 2013). Interestingly, hypermethylation can lead to increased tumorigenesis through its effects on a range of processes, including cell-cycle regulation, DNA repair and genomic instability regulation (Esteller & Herman, 2002; Wu et al., 2012). In different human cancers, DNA methyltransferase activity is high compared to normal tissues (Ibrahim et al., 2011).

One group of cancer-specific genes is known as the cancer-testis antigen genes (CTA genes) (see Section 1.2). The expression of some CTA genes can be activated by DNA hypomethylation, for example, *MAGE-A1* and the helicase antigen gene *HAGE* (De et al., 2010; Roman et al., 2007), whereas DNA hypermethylation is linked to CTA gene silencing (Yawata et al., 2010). Kim et al. (2013) demonstrated that normal testis and cancerous cells have a specific DNA hypomethylation pattern at CTA promoters (Kim, et al., 2013).

#### 1.1.3.1 Histone acetylation and cancer

The expression of human cancer genes might be affected by histone modification through changes in chromatin structure from an active to an inactive form, and vice versa (Kouzarides, 2007). In some cancer cells, the expression of different genes can be adversely regulated (downregulate/upregulate) by either histone hyperacetylation or deacetylation. It has been reported that hyperacetylation of histone H3 and H4 may stimulate the expression of CTA genes such as *MAGE-A3* (Yawata et al., 2010). In many cases of human leukaemia (Shigeno et al., 2004), as well as in some murine cancer models (Yao et al., 1998; Kung et al., 2000), histone acetyltransferase (HAT) mutations are responsible for these cancers. In addition, HAT mutations can acetylate specific proteins involved in cancer progression such as p53, p21 and myc (Taubert et al., 2004; Patel et al., 2004).

Moreover, in lung cancer cells, the expression of *NY-ESO1*, a CTA gene (see Section 1.2) can be elevated via histone deacetylation (Weiser et al., 2001). Furthermore, Timmermann et al. (2001) showed that the progression of leukaemia, colorectal and breast cancer is associated with the downregulation of HATs and histone deacetylases (Timmermann et al., 2001).

### 1.1.4 Cancer therapy

Cancer therapy is mainly focus on extending survival and/or improving the quality of life. During cancer survival, three points should be taken into the consideration; the period between cancer diagnosis and the completion of treatment, the period between treatment and extension of survival, and finally, the period after survival (Verdecchia et al., 2002). The diagnosis and treatment of cancer commonly occur late in disease progression, subsequent to metastasis. Therapeutic strategies aim to target tumour cells with a limited impact on the function of normal cells. Conventional cancer treatments, such as surgery (removal of the tumour), radiotherapy and chemotherapy (using anti-cancer drugs), can inhibit cancer progression. However, all of these methods may have a negative effect on normal cells, leading to various detrimental effects on patients (Aly, 2012; Suri, 2006; Kasibhatla & Tseng, 2003).

Surgery is an appropriate strategy for solid tumours, but recurrence of cancer after surgery is often reported. On the other hand, fatigue, anorexia, skin irritation, hair loss and nausea are common side effects of radiotherapy, and may also lead to memory loss, gait dysfunction, incontinence and endocrine dysfunction when used long-term (Aly, 2012; Eichler & Plotkin, 2008).Tumour regression can be achieved through chemotherapeutic treatment. Chemotherapeutic agents target cancer cells by targeting cell division mechanisms since cancer cells divide faster than normal cells. Nevertheless, normal cells multiplying at the same speed may also be affected and resistance as a consequence of cancer cell mutation may lead to treatment failure (Rivera & Gomez, 2010). Furthermore, regardless of its efficacy on tumour and cancer cell eradication or on reduction of tumour volume, chemotherapy affects the immune system, thus decreasing its effectiveness. Importantly, chemotherapy may lead to tumour relapse since the remaining cancer cells and cancer stem cells are thought to have the ability to escape immune system checkpoints (Nakajima et al., 2013). Additionally, chemotherapy also has severe side effects, including dizziness, loss of libido, diarrhoea, abdominal pain, weight gain, hair loss, fatigue, and loss of appetite (Carelle et al., 2002).

Although traditional cancer treatments such as surgery, radiotherapy, hormonal therapy and chemotherapy are considered mainstays of therapy, their success in curing cancer is remains limited. However, the appropriate treatment for each patients depends mostly on the size, number and types of cancers, the stage or level of tumours (metastases) and the prediction or evaluation of patients (Ahluwalia & Winkler, 2015). Recent work, demonstrated that the mechanism of the majority of cancer medications depends on causing of DNA damage and/or affecting DNA replication, but normal cells as a result may have toxic effects. However, although targeting oncogene functions directly by certain drugs seems to be promising in this field, oncogenes are often expressed at some level in somatic cells and known as proto-oncogenes; therefore, the use of this treatment approach may be limited (Bagci & Kurtgoz, 2015). On the other hand, some types of cancer showed successful treatment using this approach such as breast cancer. For example, targeting ErbB2 protein, a receptor tyrosine kinase, in breast cancer by specific monoclonal antibody (Herceptin) lead to decelerate tumour progression and improve survival (Bagci & Kurtgoz, 2015).

Because cancer promotes inflammation, the importance of poly (ADP-ribose) polymerase 1 (PARP-1) in inflammatory disease has been reported. Therefore, treating different inflammatory diseases by PARP-1 inhibitors is being tested in experimental models (BaGarg, 2011). PARP, also known as poly (ADP-ribose) synthases and poly (ADP-ribose) transferases, is considered to be a special post-translational modification (Lautier et al., 1993; Lindahl et al., 1995). Although the PARP family consists of 18 members, only PARP-1 and PARP-2 play a role in DNA damage (Otto et al., 2005). PARP-1 is a nuclear, well-characterised protein, since approximately 85% of PARP cellular activity is presented via this member (Shieh et al., 1998). In addition, PARP-1 is an enzyme that plays a critical role in the repair of damaged DNA and transcription processes. Several proteins such as histones, topoisomerases and DNA helicases can be targeted when the synthesis and attachment of highly negatively charged polymers of ADP-ribose (PARs) is catalysed via PARP-1 (BaGarg, 2011). Both PARP-1 and poly (ADP-ribose) glycohydrolase (PARG) regulate the amount of PAR formation and its attachment to other proteins, thereby controlling cell fate (Andrabi et al., 2006; Poitras et al., 2007).

Combination therapies are now thought to offer more. For example, the combination of radiotherapy and immunotherapy showed an increase in the performance of treating melanoma brain metastases since the effectiveness of immunotherapy can be stimulated by radiotherapy (Franceschini et al., 2016).

### 1.1.5 Cancer immunotherapy

The development of new immunotherapeutic approaches to the identification, diagnosis and treatment of cancers at the early stages are based on the failure of the immune system to regulate or prevent the progression of tumours. Therefore, cancer immunotherapy, as a potential and alternative cancer treatment, has been investigated (Pardoll, 2003). The fundamental function of the immune system is to recognise and distinguish self and non-self antigens. Accordingly, the main aim of immunotherapy in treating cancer is the targeting tumour cells without damaging their normal and healthy counterparts (Aly, 2012; Iclozan & Gabrilovich, 2012; Pardoll, 2003). These goals can be achieved through two strategies: targeting the tumour directly by monoclonal antibodies or inducing the immune system, which will in turn target the tumour, by promoting T cell tumour-specific activity (Figure 1.3) (Fu et al., 2016).

In the first approach, targeting the tumour includes developing monoclonal antibodies (Ab) against tumours (naked monoclonal antibodies), while radio-immunotherapy agents may lead to the killing of tumours in unspecific cells. Immunotoxins are another means of targeting the tumour; in this method, cytokines, for example, are linked to toxins and then bound to tumour cells through their receptors to deliver the toxin into them (Olsen et al., 2001). Importantly, delayed tumour growth and inactivation of their functions can be achieved via anti-tumour antibodies (Munkley, 2016).
The second route, stimulation of T cell activity, can be achieved through various approaches, including cancer vaccines, immune checkpoint antagonists, stimulatory agonists, oncolytic viruses and cellular therapies (see Figure 1.3) (Fu et al., 2016).

In therapeutic cancer vaccines, anti-tumour T cells are tempted to work against tumour-associated or tumour-specific antigens by immunising patients, while the function of pre-existing anti-tumour T cells is induced by immune checkpoint antagonists (Gros et al., 2014; Cohen et al., 2015; Tran et al., 2014). Stimulatory agonists constitute another strategy, based on the activation of costimulatory receptors, which leads to the promotion of T cell function (Mellman et al., 2011).

In addition, tumour cells can be infected by genetically altered viruses, known as oncolytic viruses, resulting in tumour cell death (Bartlett et al., 2013). In cellular therapies, cell surface molecules in several cancers are targeted (June et al., 2015). For example, use of naked monoclonal antibodies, alone or with other conventional chemotherapy agents, yielded a significant improvement in complete remission, overall survival and tumour cell death rates in many cancers, such as colon, breast and lymphomas (Cheson & Leonard, 2008; Van et al., 2009; Vogel et al., 2002; Weiner et al., 2010).

Immunotherapy primes the immune system to attack and destroy developing cancer cells through various mechanisms, including natural killer (NK) cells, cytotoxic T lymphocytes (CTLs), NK T cells and antibodies (Iclozan & Gabrilovich, 2012). Meanwhile, dendritic cells (DCs) play a central role in the anti-tumour immunity of solid tumours by coordinating the activities of NK cells, CTLs and NK T cells. Further, dendritic cells (DCs) play a central role in the antitumor immunity of solid tumours by coordinating the activities of the NK cells, CTLs and NK T cells (Iclozan & Gabrilovich, 2012; Harris & Drake, 2013; Aly, 2012; Mellman et al., 2011; Pardoll, 2003).

Immunotherapy agents may have side effects, which are different from those caused by traditional cancer treatment since they work differently. Pneumonitis, colitis, hepatitis, pancreatitis, skin rashes and endocrine disorders are examples of the possible adverse effects of immunotherapy (Mosbech, Müller, On Behalf of the Study Group, 2000). Furthermore, immune checkpoint proteins, which are responsible for distinguishing normal cells from abnormal cells, may serve as cancer immunotherapy. One example of this is anti-programmed death 1 (PD-1). Blocking the pathway of proteins such as the PD-1 receptor on activated T-cells targets cytotoxic T-lymphocyte antigen 4 (CTLA-4), the PD-1 receptor and programmed death ligand 1 (PD-L1), resulting in the possibility of damaging healthy cells as well as cancerous cells (DolanGupta, 2014).

Although immunotherapy seems to reduce the risk of cancer, it may have side effects that cause other diseases. For instance, inflammatory bowel disease can be the result of over stimulated T-cells (PercivalMilner, 2005).

Additionally, an overactive or hyper-responsive immune system is an adverse effect of this type of therapy, leading to autoimmune disease, inflammatory disease and allergies. Therefore, suppressing the overactive immune response may benefit patients with such diseases. In autoimmune disease, self-cells (or normal cells) are attacked by the immune system because they are recognised as foreign cells. In terms of allergies, B-cells are induced to produce more antibody via stimulated T-helper 2 (TH2) cells as a result of an overactive immune system that considers pollen to be an attacking parasite (Yazdanbakhsh, Kremsner & van Ree, 2002). Interestingly, reducing the number of white blood cells and affecting the immune system may result from treating chronic myeloid leukaemia (CML) via immunotherapy (Van Driessche et al., 2005).

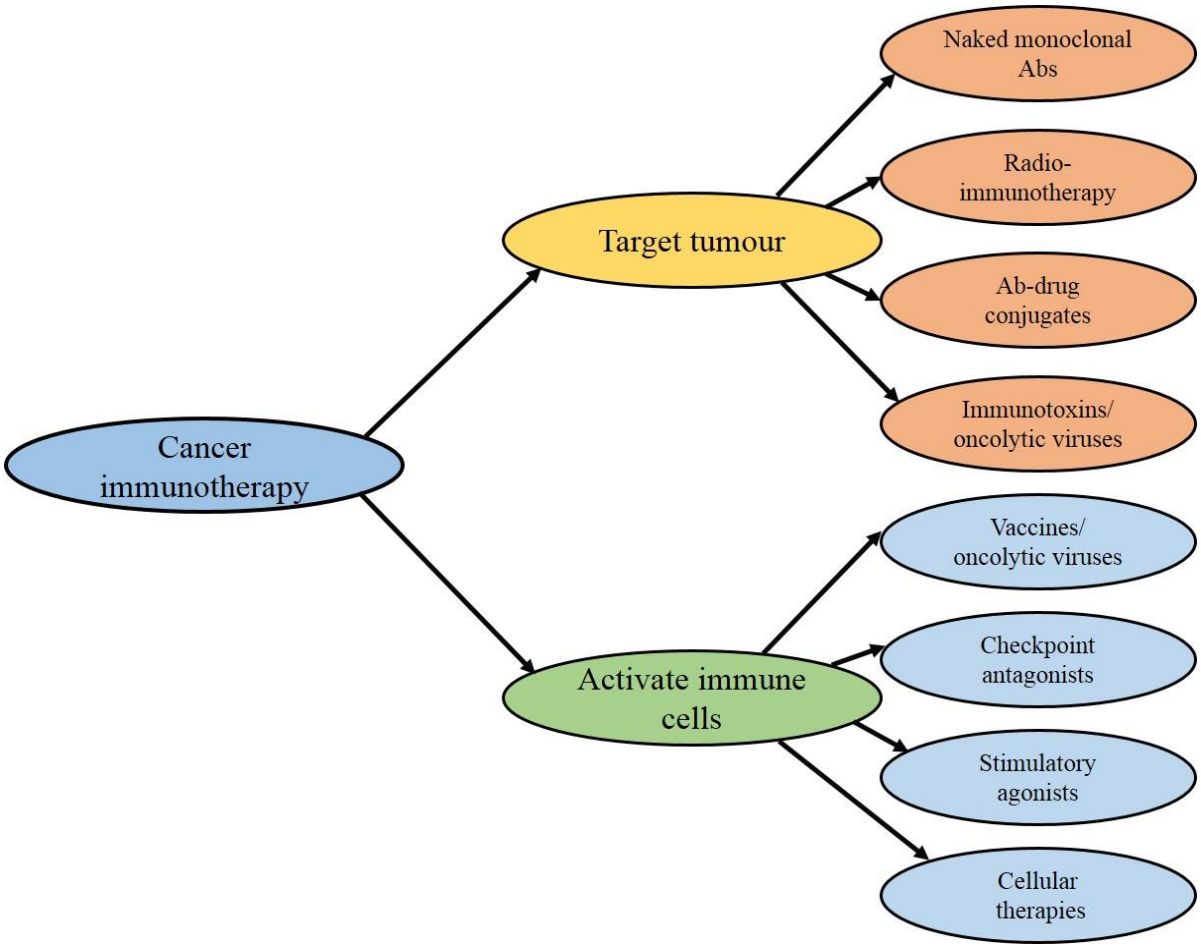



**Figure 1-3 The classification of cancer immunotherapy agents.**
Cancer immunotherapy agents are divided into two categories, depending on their goals or targets, which include targeting the tumour and activating the immune system (Adapted from Sathyanarayanan & Neelapu, 2015).

### 1.1.6 Tumour-associated antigens (TAAs)

The identification of cancer-specific antigens that can serve as biomarkers/targets, known as TAAs, is considered an essential part of cancer immunotherapy and other targeted therapies. In addition, they can be useful for cancer diagnosis and prognostics (Luborsky, 2001). TAAs are specific proteins produced after any changes in cellular gene expression and DNA sequence. Alterations in gene expression can be caused by the mutation of various cancer-related genes, such as proto-oncogenes, tumour suppressor genes and instability genes (Krishnadas et al., 2013). The immune system usually targets particular antigens present on the cancer cell surface; however, many antigens may also be present on somatic cells in small amounts, and are therefore not cancer-specific (Criscitiello, 2012). TAAs play a crucial role in triggering the immune system through the generation of a single epitope that is recognised by the immune system, leading to cancer cell destruction (Krishnadas et al., 2013). According to TAA expression patterns, there are four types of human TAAs (see Table 1.3).

While TAAs can be used for cancer immunotherapy, they must possess essential features, including exclusive expression in cancer cells, consistent presence in most tumours and status as a target for CTLs. Therefore, the targeting of TAAs in cancer immunotherapy has been widely studied in recent years (Krishnadas et al., 2013). Importantly, in tumours, these antigens are expressed at high levels and the potential of monoclonal antibodies (mAbs) to recognise tumour antigens is much higher (Luborsky, 2001). For example, in breast cancer immunotherapy, TAAs are expressed in somatic normal tissues, whereas in cancer cells, they are overexpressed or mutated (Cheever et al., 2009). Conversely, according to Tygart (2014) PSMAs (Prostate-Specific Membrane Antigen) as TAAs are not expressed in normal tissues, whereas their expression level is high in cancer cells (Tykvart et al., 2014). Additionally, the mAb designed to work against the TAAs EGFR and HER2, is identified as a target drug for solid cancers because of its substantial signals in tumour growth (Sattler et al., 2011; Sliwkowski & Mellman, 2013).

TAAs also generate a complex with HLA-class 1 molecules on the surface of tumour cells (peptide), which can be recognised by $CD8^+$ cytotoxic T cells (CTLs), for instance, leading to the destruction of these tumours (Kiessling et al., 2012; Rosenberg, 1997).

**Table 1-3 Human tumour associated antigen (TAA) types.**

| Human TAA types | Features | Examples | | References |
|---|---|---|---|---|
| | | Antigen | Associated cancer | |
| Viral antigens | Caused by viral infections | Human papillomavirus | Cervical cancer | (Vogt, 2012) |
| Differentiation antigens | Expressed in malignant and somatic cells | Tyrosine and MART-1/MelanA | Melanoma cancer | (Barrio et al., 2012; Kozlowska et al., 2013) |
| Overexpressed antigens | Overexpressed proteins generate specific peptides associated with T cell responses | Transmembrane mucin MUC1 | Many carcinomas | (Kaur et al., 2014; Singh et al., 2006) |
| Cancer testis antigens | -Expressed only in human germ lines (testis and ovary) and cancer cells<br>-Silent in normal cells<br>-Valuable targets for cancer vaccines, biomarkers and immunotherapy | REC8 | Melanoma cell lines | (Loriot et al., 2003; Whitehurst, 2014; Rosa et al., 2012; Krishnadas et al., 2013) |

## 1.2 Cancer testis antigen (CTA)

The CTAs (cancer testis antigens) are a heterogeneous group of proteins produced solely in adult normal male testis tissues including germ cells in healthy individuals, but not in other normal somatic tissues. Nevertheless, CTAs are produced in various cancer cells at high levels, such as in bladder cancer, lung cancer, melanomas and ovarian cancer, or at low levels such as in renal cancer, leukaemia and colorectal cancer (Chen et al., 1997; Fratta et al., 2011; Simpson et al., 2005; Whitehurst, 2014). The expression of cancer testis (CT) genes in cancer cells has been investigated mostly by analysing mRNA via RT-PCR, while immunohistochemistry analyses has been utilised to investigate the protein level of a number of CTAs. Various approaches have been used to discover novel CTAs, including serological analysis of recombinant cDNA expression libraries (SEREX) (Chen et al., 1997), bioinformatics, data-mining strategies (Feichtinger et al., 2012) and, more recently, a transcriptomics-multiplatform approach using genotype-tissue expression (GTEx) or The Cancer Genomic Atlas (TCGA) (Wang et al., 2016).

According to quantitative RT-PCR analysis, the expression of mRNA from distinct CTA genes in some somatic tissues, such as liver, pancreas and spleen, was less than 1% compared to their expression in testicular germ cells (Caballero & Chen, 2009). In 1991, the melanoma antigen family (*MAGE*) was the first human CT gene to be identified. Interestingly, *MAGEA-1* gene expresses in adult normal testis and placental cells as well as in many human tumours (DE et al., 1991; Fratta et al., 2011; Zendman et al., 2003). MAGE-1 was recognised by autologous typing with T cell clones isolated from a melanoma patient (DE et al., 1991). Subsequently, MAGE-A3 and NY-ESO-1 have been placed in the top 10 category of the Project for Prioritization of Cancer according to the National Cancer Institute (Cheever et al., 2009).

The surface of cancer cells has specific protein-based antigenic peptide regions such as the major histocompatibility complex (MHC) molecule or human leukocyte antigen (HLA), which contain the most tumour specific antigens. Cytotoxic T lymphocytes recognise the MHC peptide complex, leading to the eradication of tumours (Adair & Hogan, 2009). The lack of interaction between the human immune system and CTA proteins in testis may be due to the presence of the blood–testis barrier and the absence of the expression of class I HLAs on the surface of testis germ cells (Ghafouri et al., 2012; Kalejs & Erenpreisa, 2005; Li et al., 2012).

Thus, such CTAs are not recognised as self-structures and are therefore likely candidates for therapeutic targets (Ghafouri et al., 2012; Kalejs & Erenpreisa, 2005; Li et al., 2012). The production of CTAs in germlines and cancer cells and their absence in somatic cells provide them with a unique and important potential in cancer diagnosis and drug targeting (McFarlane et al., 2015).

### 1.2.1 Classification of CT genes

More than 70 gene families of potential CT genes are available on the CTA database (http://www.cta.lncc.br), with approximately 1000 of these members being known CTA genes (Fratta et al., 2011). The localisation of multiple CT genes on the X chromosome has been noted (Kalejs & Erenpreisa, 2005). CT genes can be classified into two types according to the localisation of their chromosomes. The X-CT group is located on the X chromosome and these are generally expressed in the spermatogonial stage of spermatogenesis in normal testis as well as in the placenta. *MAGE-A3*, *MAGE-8*, *MAGE-A10*, *XAGE-2* and *XAGE-3* are considered as examples of X-CT genes, which are members of large paralogue families. X-CT genes represent 52% of identified CT genes (Rajagopalan et al., 2011). The non-X-CT group are encoded on the autosomes and generally expressed in spermatocytes and throughout meiosis (Almeida et al., 2009; Caballero & Chen, 2009). *BAGE*, *BORIS*, *CT9/3BRDT*, *SCP-1* and *SPO11* are examples of non-X-CT genes, which are mostly found as single-copy (Fratta et al., 2011; Ross et al., 2005).

Although the expression of CT genes in the testis is largely restricted to spermatogenic germ cells, several CT genes are expressed at different stages of sperm development, including spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. CT antigens can be further divided, depending on their gene expression profiles, into four categories: (1) Testis-restricted, where expression can be found only in the adult testis, placenta and at least one type of cancer. (2) Testis-brain-restricted, expresses in the adult testis, central nervous system (CNS) and at least one type of cancer. (3) Testis-selective, where expression can be found in the adult testis and in no more than two normal tissues with expression lower than in the testis, and at least one type of cancer. (4) testis-brain-selective, expresses in the adult testis, CNS tissues, and no more than two normal tissues with expression lower than in the testis, and at least one type of cancer (Feichtinger et al., 2012; Hofmann et al., 2008).

### 1.2.2 The function of CTA in normal and malignant tumour tissues

CTAs have gained importance in cancer immunology due to their high specificity to testis tissues and cancers. Nevertheless, in both germline tissues and tumour cells, the biological functions of CT genes and the regulation of their expression remain poorly understood, although evidence of their role in tumorigenesis has increased. The function of some CT genes can be identified in normal tissues, including their role in cell division (Jungbluth et al., 2005) and the function of germ cells (Cronwright et al., 2005; Gedye et al., 2009). For example, SPO11 plays a key role in the formation of DNA double-strand breaks in order to initiate meiotic homologous recombination (Yamada & Ohta, 2013). Synaptonemal complex protein 1 (SYCP1) initiates chromosome synapsis during meiosis 1 (Schramm et al., 2011; Tureci et al., 1998). BORIS has an essential role in the regulation of the promoter methylation process via spermatogenesis during meiosis II of male germline cells (Klenova et al., 2002).

During oncogenesis, a large group of germline genes plays an important role in tumour development. For example, germ line genes have been shown to be essential for brain tumour development in *Drosophila melanogaster* (Janic et al., 2010; Sumiyoshi et al., 2016). The activation of the same pattern of germline genes occurs in human tumour, suggesting human germ line genes are oncogenic (Feichtinger et al., 2014). CT genes are considered a major cohort of this germ line group. Although the functions of CT proteins are poorly understood in the testis, some roles in the oncogenic processes have been demonstrated (Gjerstorff et al., 2015; Whitehurst, 2014).

Nevertheless, evidence of the function of CT genes in cancer cells remains limited, although it has been reported that they may play a role in cell growth, transcriptional regulation, putative proto-oncogenes and genetic instability (Figure 1.4) (Cheng et al., 2011; Scanlan et al., 2002). CTAs may play a significant role in the control of complex genes and the activation of abnormal genes during cancer progression (Mirandola et al., 2011; Scanlan et al., 2004; Whitehurst, 2014).

It has been proposed that one feature of cancer progression may be due to a soma–to–germline transformation (Feichtinger et al., 2014; McFarlane et al., 2014). Lindsey et al. (2013) addressed, to some extent, the relationship between genomic instability as a hallmark of human cancer and the aberrant expression of germline genes in cancers such as melanoma. This relationship may arise due to a conflict between meiotic germ cell pathways and the normal mitotic cell cycle.

In human somatic cells, the shortening of the ends of linear chromosomes (telomeres), after each round of DNA replication (Harley et al., 1990), regulates and limits cell growth through multiple mechanisms, such as induction of P53 and response of the DNA-damage checkpoint (di et al., 2003; Karlseder et al., 1999). Therefore, the alteration of normal human cells to cancerous ones requires maintenance of their telomeres through a fundamental mechanism known as Alternative Lengthening of Telomeres (ALT) (Henson et al., 2002). The activity of ALT requires several meiotic genes, such as *HOP2-MND1*, which are thought to be involved in the formation of inter-telomere homologous recombination (HR). Interestingly, due to the importance of ALT in cancer cells, these genes may be considered a potential tool in cancer diagnosis and therapy (Cho et al., 2014; Cesare & Reddel, 2010; di et al., 2003).

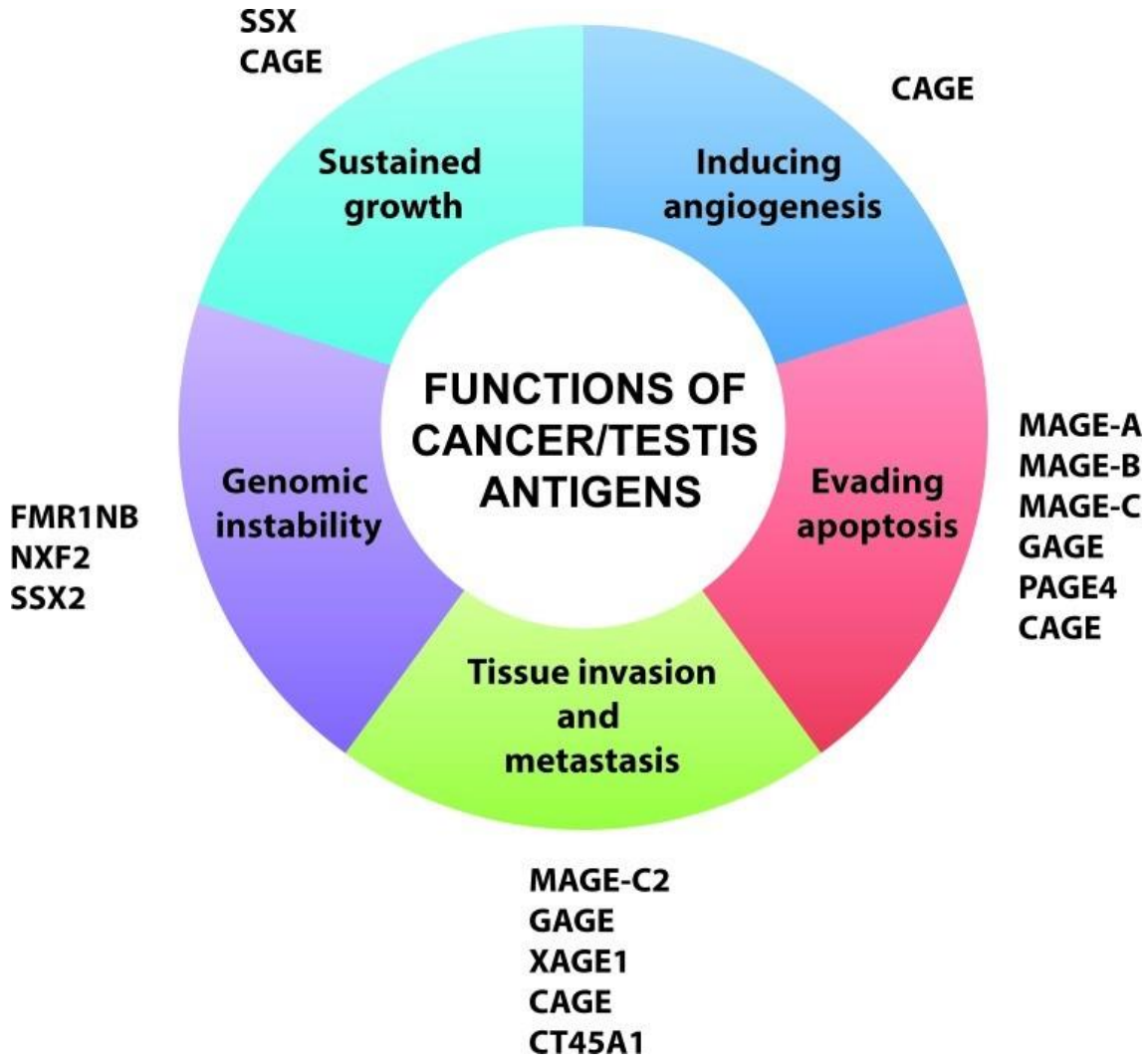



**Figure 1-4 Examples of potential functions of cancer testis antigens (CTAs) in cancer cells (Adapted from Gjerstorff et al., 2015).**

### 1.2.3 CTA and the diagnosis, prediction and prevention of cancer

CTAs are considered a family of proteins that may have a role in the diagnosis and prognosis of cancer diseases (Fratta et al., 2011; Chomez et al., 2001). The most CT-restricted and most immunogenic genes in cancer patients appear to be X-CT genes. For example, MAGEA3 and cancer/testis antigen 1B (CTAG1B) have been considered attractive targets for immunotherapy (Caballero & Chen, 2009). Since the production of X-CT antigens in malignant tumour is exclusive, it is suggested that X-CT antigens can be used as an effective diagnostic pathological tool. CTAs could have potential diagnostic applications as biomarkers through their immunohistochemical detection in order to distinguish between benign and malignant tumours and to characterise the morphology of similar tumours (Chen, 2014; Piotti et al., 2013).

In general, tumours of high-grade and late clinical stage are expected to produce CTAs at higher frequencies in some types of cancers. For example, *MAGE-A1* expresses in approximately 48% of metastatic melanoma comparing with 16% of primary melanoma (Caballero & Chen, 2009). Piotti et al. (2013) indicated that X-CT genes may be a valuable diagnostic tool, especially for squamous cell carcinoma (SCC) and its precursor lesions. Interestingly, testing SCCs for expression of eight X-CT genes (*MAGEA3*, *NY-ESO-1*, *GAGE*, *MAGEC1*, *MAGEC2*, *CT45A1*, *SAGE1*, and nuclear RNA *NXF2*) resulted in high expression of those genes and at least one was expressed in 62% of the samples. Furthermore, when examining histologically dysplastic oesophageal lesions for X-CT antigens, six were identified, confirming the frequent presence of X-CT antigens in pre-invasive early squamous malignancy. Additionally, 66% of head and neck cancer were CTA-positive (Chen, 2014). A further study on synovial sarcoma and liposarcoma confirmed that *NY-ESO-1* was highly expressed in approximately 80% of synovial sarcomas. Thus, the immunohistochemical detection of NY-ESO-1 can be used as a biomarker to diagnose synovial sarcoma (Pollack et al., 2012; Lai et al., 2012; Jungbluth et al., 2001).

While CTA genes are often highly expressed in aggressive tumours (late clinical stage), they could also serve as potential clinical biomarkers for cancer prognosis. For example, the expression of *CTCFL* was positive in glioblastoma multiforme (GBM) specimens, whereas it was negatively expressed in the early stage of cancer (Chen et al., 2010; Cheng et al., 2011). The CT gene *MAGE-A1* was expressed at 48% in metastatic melanoma and 16% in primary melanoma (Brasseur et al., 1995).

The expression of *NY-ESO-1* differed depending on the tumour grade, and was seen to be 40% in grade 3 bladder tumours, 23% in grade 2 tumours and no expression in grade 1 tumours (Kurashige et al., 2001). Sharma et al. (2006) showed that the expression of *CT10* is positively linked to the survival of urothelial carcinoma patients. However, a study published in 2013 characterised four CT genes, including *ACTL8*, *CTCFL*, *OIP5* and *XAGE3*, on GBM patients, resulting in co-expression of 3–4 of these CTAs, which leads to a significantly better survival. Therefore, although those CTAs can be a prognostic marker for GBM, they may also function as potential targets for immunotherapeutic approaches (Freitas et al., 2013).

Rousseaux et al. (2013) studied the expression of 26 testis-specific/placenta-specific (TS/PS) genes with 293 patients samples with lung cancer. After that, patients were divided into three groups depending on the number of 26 genes that expressed in their tumour; P1, P2, and P3. P1 refers to tumours expressed none of the 26 CT genes, P2 refers to tumours expressing one or two, and P3 refers to tumours expressed three or more of these genes. Comparing the clinical outcomes of P1 and P3 of patients, with the consideration of clinical stage and histological subtype, show that P3 tumours had aggressive phenotype, leading to the development of metastases and short-term fatal outcome in a large number of patients. Remarkably, this finding indicates the possibility of using these genes as attractive diagnostic and predictive cancer tools as well as cancer drug targets (Rousseaux et al., 2013).

CTAs can be used as a possible preventative vaccine for cancer, either through the reduction of the high risk of cancer in healthy individuals or by inhibition of tumour development at the early stage of cancer formation. For example, in breast cancer, women with deleterious *BRCA1* and *BRCA2* genes have a 50% likelihood of developing breast cancer (Adams et al., 2011). In addition, ovarian carcinoma and melanoma with *BRCA* mutations express CTAs frequently and therefore these CTAs could function as targets in immunoprevention (Odunsi et al., 2003; Velazquez et al., 2007). Of note, a high production of CT antigens (80%) in prostate carcinomas and their presence early in situ tumours demonstrate their importance in cancer prevention (Theurillat et al., 2007; Hudolin et al., 2006).

### 1.2.4 CTAs and the therapeutics of cancer

Due to the unique CTA gene expression profiles, these genes may serve as valuable candidates for anti-cancer vaccines and as potential tumour immunotherapeutic targets (Simpson et al., 2005; van et al., 2011; Valmori et al., 2007). Many types of cancer, such as 70% of SCCs of the head and neck and 91% of carcinomas of the breast, produce MAGE proteins, which are associated with aggressive melanoma, for instance. Therefore, a wide range of human tumours could be treated through targeting of this protein (Bhatia et al., 2011). Indeed, Bhatia et al. (2011) suggested that, when the MAGE and KAP-1 interaction is inhibited, tumour growth is also inhibited, thus indicating a promising cancer drug therapy. In one case, targeting of NY-ESO-1 in a patient diagnosed with metastatic melanoma led to a reduction in the size and number of metastasised tumours and, following adoptive T cell therapy, the patient was cancer-free for over two years (Hunder et al., 2008). During T cell therapy, the HLA-peptide complex of a tumour-associated antigen is specifically recognised by a T-cell receptor (TCR), which is transduced with T cells (Karpanen & Olweus, 2015; Morgan et al., 2006; Robbins et al., 2011). Additionally, chimeric antigen receptor (CAR) T cells can be used in T cell therapy. The transduction of the CAR's structure leads to the manufacture of T cells; the CAR is composed of an extracellular domain (antibody), which recognises tumour cells via its receptor, and an intracellular domain, which activates T cells. For example, the remission rate of patients with chronic lymphocytic leukaemia or acute lymphoblastic leukaemia is increased under CD19 CAR T cell therapies (Kalos et al., 2011; Kochenderfer et al., 2012; Porter et al., 2011; Whilding & Maher, 2015).

Anti-tumor cell vaccines targeting CTAs may play a key role in cancer treatment. In addition, human cancer cells express those antigens, named cancer vaccines, and the human CD8+ and CD4+ T-cells recognised them (Blanchard, et al., 2013; Renkvist et al., 2001). For instance, the CTAs MAGE-A1 and NY-ESO-1 are considered attractive targeted antigen-specific vaccines against several types of human tumours (Campos et al., 2013). Stimulation of the T-lymphocyte response against malignancy has been tested using those two antigens (Caballero & Chen, 2009). Furthermore, the effectiveness of immunotherapeutic vaccines was seen to improve when CT SPAG9 was used in combination with other CTAs (Suri et al., 2015).

The development of the application of CTAs as potential therapeutics for cancer may depend on the choice and targeting of the correct proteins within a particular cancer type. Further, the identification of specific partners for individual CTA proteins should be considered. Interestingly, it has been reported that, in cancerous patients, promoting the immune system through a combination of CTAs may elevate and maintain immune response, advance the efficiency of the treatment of haematological malignancies, and improve the survival rate (Figure 1.5) (Meek & Marcar, 2012; Suri et al., 2015). A study carried out with acute myeloid leukaemia or myelodysplasia showed that the production of CTA MAGE antigens can be increased by using DNA methyltransferase and histone deacetylase inhibitors; as a result, the level of circulating MAGE cytotoxic T lymphocytes was raised significantly and patient clinical responses were amended (reviewed in Meek & Marcar, 2012).

In the near future, studies improving the knowledge of the molecular biology of CTA proteins, advancing different approaches to trigger immunotherapeutic response and further targeting protein-protein correlations should shed light on the usage of these proteins as interesting and effective techniques in the field of tumour biomarkers and treatments (Meek & Marcar, 2012).

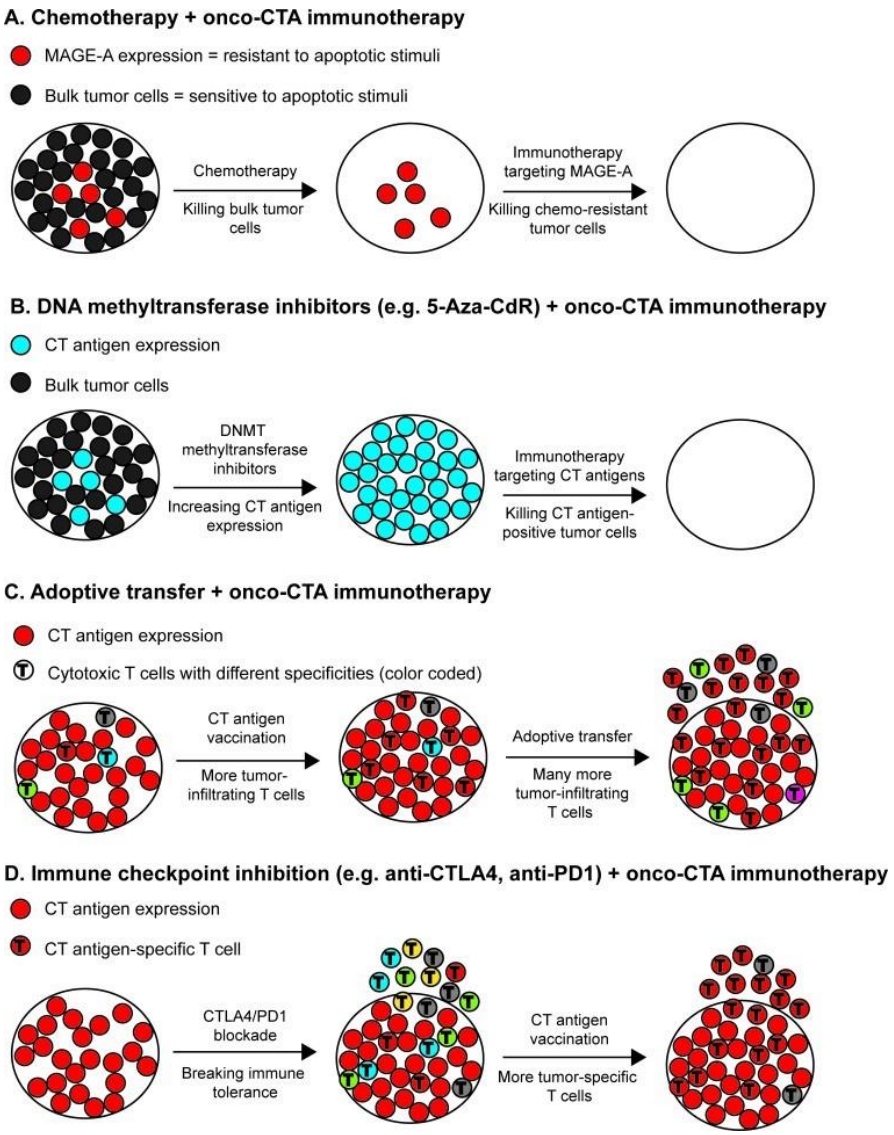



**Figure 1-5 Treating cancer via the combination of oncogenic cancer testis antigens (CTAs) and other therapies (Adapted from Gjerstorff et al., 2015).**

## 1.3 Cell cycle

### 1.3.1 Overview of the mitotic cell cycle

All living organisms undergo cell division. The cell division process involves DNA replication, cell growth and then the production of new daughter cells. In eukaryotic cells, two types of cell division exist: mitosis and meiosis. During cell division, an accurate distribution of chromosomes is needed to maintain cellular and tissue homeostasis (reviewed in Marston & Amon, 2004). Following mitosis, two genetically identical daughter cells are produced (Figure 1.6). The aim of mitotic cell division is to grow and repair tissues. On the other hand, meiosis is a germ line cell division to produce gametes.

The mitotic cell cycle mainly consists of two core phases: the interphase, including Gap-1 (G1), S (DNA synthesis), Gap-2 (G2), and mitosis (M phase), the latter consists of four sub-phases: prophase, metaphase, anaphase and telophase. Cell division preparation, including growth in size, DNA replication, RNA and protein production, take place during the interphase. Mitosis (M phase) is followed by cytokinesis to generate two daughter cells. Furthermore, G0 or the resting phase (quiescent state), is an additional phase in which cells exit the cell cycle due to external triggers (Figure 1.6) (Singh & Dalton, 2014; Kronjaet al., 2011).

Strictly regulating the progression of the cell cycle in eukaryotic cells is important to maintain genome integrity. Controlling cell division can be achieved by a series of checkpoints and cyclin-dependent kinase (CDK) and their specific activators or cyclins. For example, any error in cell division can activate a checkpoint, which leads to arrest and/or speeding up or slowing down the cell cycle (Nurse, 1990; Sorensen & Syljuasen, 2012; Williams & Stoeber, 2012).

Importantly, defective cell cycle regulators and checkpoint mechanisms may be considered as a cancer hallmark and may affect cancer detection and treatment (Aarts et al., 2013; Nojima, 1997). Importantly, inhibiting CDK activities via the creation of multiple cell cycle arrests that lead to inducing apoptosis can deliver a novel target for cancer treatment (Peyressatre et al., 2015; Suryadinata et al., 2010).

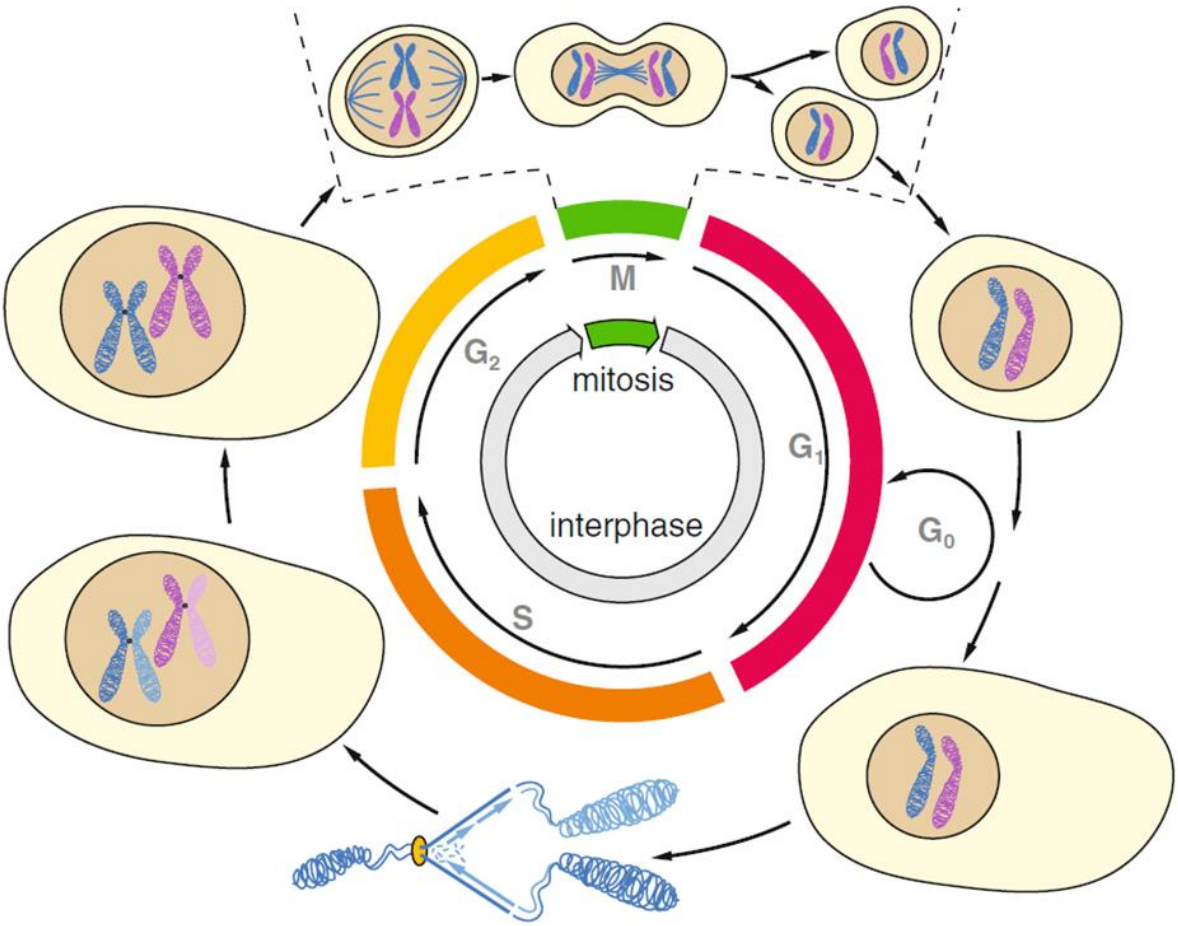



**Figure 1-6 Schematic diagram of the eukaryotic cell cycle and the stages of mitotic cell division.** The cell cycle consists of two main phases: interphase and mitosis (M). Interphase comprises three stages: Gap 1 (G1), DNA synthesis (S) and Gap 2 (G2). During G0 phase, quiescent state, cells do not progress through the cell cycle. Chromosomes are doubled and are enclosed by a nuclear envelope. Each chromosome is also comprised of two identical sister chromatids, linked together by centromeres. In the mitosis phase, two daughter cells are produced through nuclear division (mitosis) and cytoplasmic division (cytokinesis) (Adapted from Behl & Ziegler, 2014b).

## 1.4 Overview of meiotic cell division

Meiosis is a unique cell division that generates gametes (sperm cells and egg cells in advanced eukaryotes) in sexually reproducing eukaryotes. Meiosis takes place in the male testis and female ovary in mammals (reviewed in Lindsey et al., 2013). Although meiosis is critical for creating genetic diversity, it also maintains chromosome number accuracy (Longhese et al., 2008; Gerton & Hawley, 2005). One round of meiotic DNA replication is followed by two successive rounds of accurate chromosome segregation to create haploid gametes (Chicheportiche et al., 2007; Prieler et al., 2005). The first round of chromosome segregation (meiosis I) reduces the number of chromosomes by the segregation of homologous chromosomes. However, the second phase (meiosis II) equates the chromosomes when the sister chromatids are separated (Clift & Marston, 2011; Marston & Amon, 2004). Additionally, one of the distinguish features of meiosis is programmed recombination, which has an important role in genetic diversity (Zickler & Kleckner, 1998, 2015).

During an extended pre-meiotic S-phase, the replication of chromosomes occurs. This occurs prior to inter-homologous interactions, which play a significant part in meiotic recombination and in the separation of the homologous chromosomes (Lee & Amon, 2001). Four phases or stages are involved in both meiotic cell divisions, including prophase, metaphase, anaphase and telophase (Figure 1.7) (Page & Hawley, 2004; Zickler & Kleckner, 1998).

Since the reduction of the chromosome numbers is a fundamental event during meiosis I, three essential events must occur. Firstly, the connection between the homologous chromosomes has to be presented via meiotic recombination (Clift & Marston, 2011; Gerton & Hawley, 2005). Secondly, attachment to the microtubules, derived from the same spindle pole, must be established between the sister kinetochores of each homologue. Subsequently, attachment between homologous pairs via chiasmata is established. Finally, the removal of chromosome arm cohesion, which is a result of the dispersal of sister chromatid cohesion occurs (Clift & Marston, 2011; Marston & Amon, 2004). Most of the key meiosis I events occur in prophase I, which is sub-divided into five cytological division phases (leptotene, zygotene, pachytene, diplotene and diakinesis) (Table 1.4) (Zickler & Kleckner, 1999).

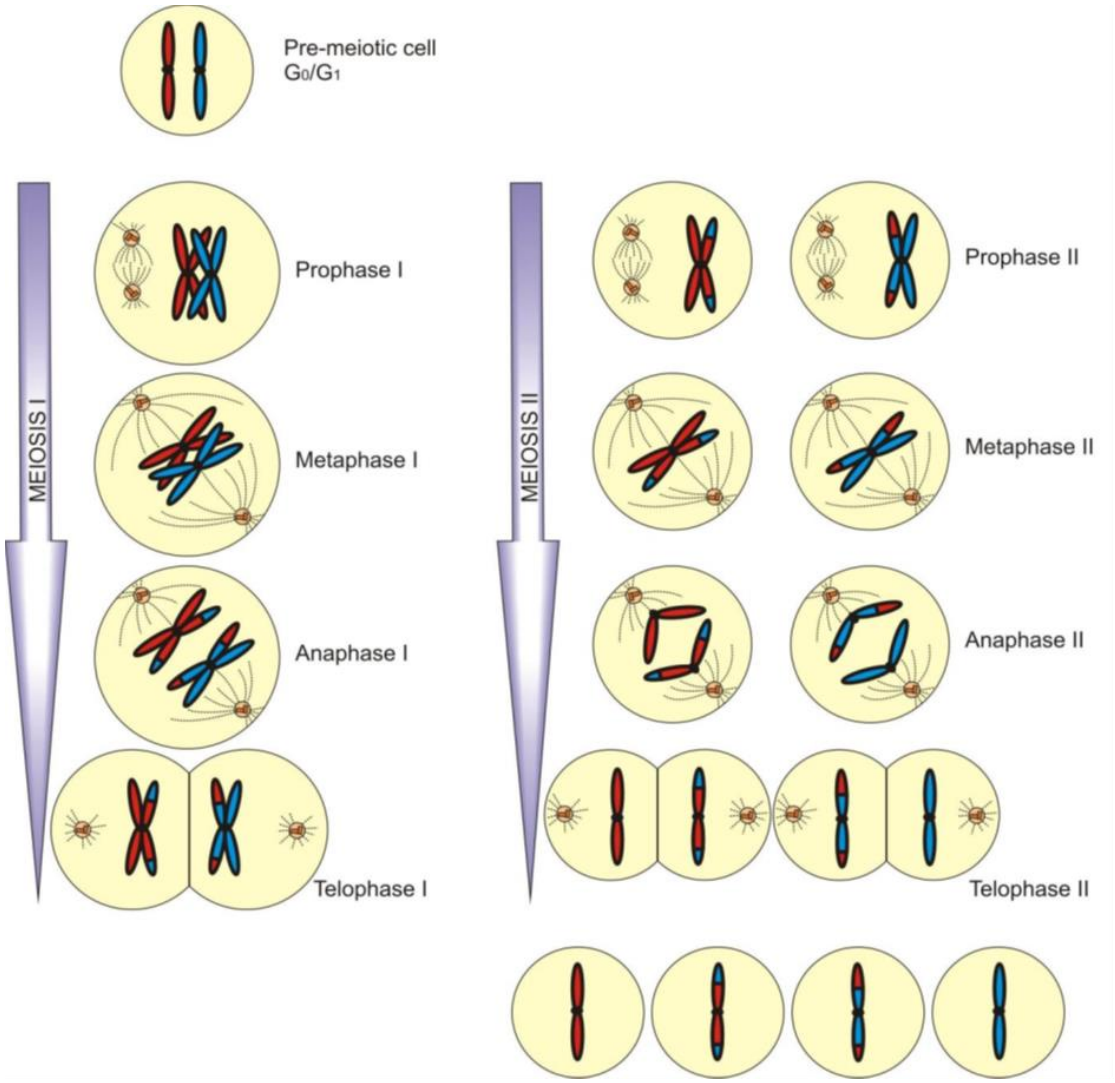



**Figure 1-7 The stages of meiotic cell division.**
Two rounds of chromosomal segregation occur in meiosis: meiosis I, which is reductional segregation, and meiosis II, which is equational segregation. In this figure, the maternal chromosome is indicated by the colour red, whilst the paternal chromosome is indicated via the colour blue. Firstly, meiosis I is divided into four phases: prophase I, metaphase I, anaphase I and telophase I. The chromosomes become thick and visible, and a spindle is generated during the prophase stage. Moreover, during this phase, the homologous chromosomes pair up, resulting in crossing over, which forms a unique combination of alleles on each chromatid. During the metaphase I, the homologous pairs align on the equator of the cell. During anaphase I, the homologous chromosomes are segregated and move to opposite ends of the cell. During telophase I, two new haploid nuclei are produced. Meiosis I is followed by meiosis II, which includes four stages: prophase II, metaphase II, anaphase II and telophase II. During prophase II, the spindle is created after breaking down the nuclear envelope. During metaphase II, the chromosomes are pulled to the cell centre and then they align randomly at the metaphase plate. During anaphase II, the centromere of each chromosome splits, leading to the separation of the sister chromatids. Finally, four haploid daughter cells are produced by the end of meiosis II (Page & Hawley, 2004; Zickler & Kleckner, 1998).

The main features of prophase I is the pairing of homologous chromosomes and establishing stable connections. Importantly, just prior the pairing, homologous telomeres are clustered and attached to the nuclear envelope, which is termed the bouquet. Chromosomes are compacted and the axial elements proteinacious structure between sister chromatids are formed. The bouquet stage occurs in the leptotene-zygotene transition, in which the telomeres in the nuclear membrane travel to a polarized configuration and attach to the centrosome. The formation of the bouquet enables telomere regions to be attached to the envelope of nucleus from leptotene until late pachytene (Figure 1.8) (Harper et al., 2004; Siderakis & Tarsounas, 2007). The ends of the chromosomes are clustered within a limited area during this stage, in which the movement of the chromosomes in and out of the bouquet is observed. Interestingly, double-strand DNA breaks (DSBs) or some downstream recombination process may be required for the exit from the bouquet (Zickler, 2006).

During prophase I phase, the homologous pairs of chromosomes are tangled together and move towards the equatorial plate. This is also the phase where crossing over can occur (Page & Hawley, 2004; Zickler & Kleckner, 1998, 2015). Prophase I is considered the distinguishing phase since it differentiates meiosis from mitosis according to the specific events that occur such as crossover.

**Table 1-4 The sub-stages of prophase I (Zickler & Kleckner, 1999).**

| Sub-stages | The Features |
|---|---|
| Leptotene | Chromosomes are obviously individualized (thin and thread-like) as they condense. The pairing of homologous chromosomes is also initiated programmed (recombination). |
| Zygotene | The homologous chromosomes come closer together, resulting in the generation of the synaptonemal complex (SC), which assembles between homologues (Qiao et al., 2012). Bivalent indicates each pair of synapsed homologous chromosome (Blanco-Rodriguez, 2012). The telomeres cluster at the nuclear envelope (bouquet stage) (Zickler, 2006). |
| Pachytene | The chromosome are short and thick (Page et al., 2003). The formation of SC is completed. Crossing over occurs (Kleckner, 1996). Pachytene checkpoints are active at this stage in order to arrest meiosis in case of any error in chromosome synapsis and/or recombination, leading to repair process or to promote apoptosis (Pellestor et al., 2011). |
| Diplotene | The release of SC starts, although the physical linkage of the homologous remains via cohesion between sister chromatins (Buonomo et al., 2000). |
| Diakinesis | The SC is released completely and the condensation of the homologous chromosomes occurs before the start of metaphase I. The formation spindle is started and the membrane of the nuclease breaks down (Ollinger et al., 2010). |

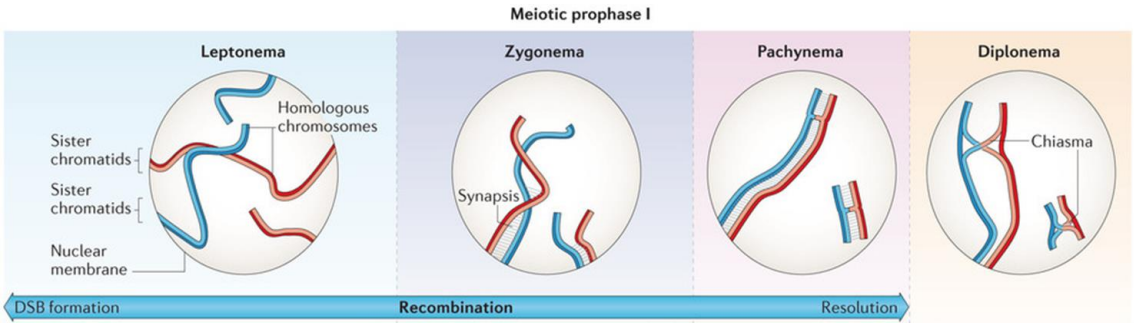



**Figure 1-8 Chromosome organisation during meiotic prophase I.**
During meiotic prophase I, chromosomes are paired and consist of homologous chromosomes. Each chromosome contains two sister chromatids. At leptonema, chromosomes are identified and associated with their partners as soon as the recombination appears through the initiation of DNA double-strand breaks (DNA-DSBs), which are introduced by SPO11. Additionally, meiotic recombination is completed before the end of pachynema. Paired chromosomes are linked to each other forming homologs by a highly structured protein known as synaptonemal complex (SC) at zygonema. During crossover repair, the homologs are used as a template rather than the sister chromatin, leading the initiation of chiasmata linkages between homologs at diplonema. Clustering telomeres from one pole of the nuclear envelope is known as bouquet stage, which indicates the transition state from leptonema to zygonema (Baudat, et al. 2013).

### 1.4.1 Meiotic homologous recombination

#### 1.4.1.1 Homologous recombination (HR) function

During meiosis, at the onset of prophase I, most sexually reproducing organisms engage in meiotic inter homologue recombination by forming DSBs, which are generated by the topoisomerase type II-like protein SPO11 (Hunter, 2015; Keeney, 2001; Prieler et al., 2005). The repair of these chromosome breaks in meiosis requires homologous recombination (HR) which takes place when genetic material is replaced or exchanged with its homologous chromosome (Oum et al., 2011). Inter homologue HR plays key roles in chromosome segregation, chiasmata formation and the preventing of chromosomal non-disjunction (Romanienko et al., 2000; Storlazzi et al., 2003; Henderson & Keeney, 2004). Meiotic recombination stages include DSB formation, exonucleolytic resection of 5′ends at the breaks, 3′ end strand invasion into a chromatid of a homologous chromosome, formation of Holliday junction and subsequent resolution (Handel & Schimenti, 2010).

#### 1.4.1.2 Homologous recombination (HR) repair

DSBs must be repaired in order to maintain genome integrity. It is worth noting that unrepaired DSBs may result in the loss of genetic information and chromosome rearrangements (Longhese et al., 2008). The repair of DSBs occurs before entering pachynema, either via COs, or NCOs (Cromie & Smith, 2007). Chromosome translocations may result from abnormal repair of DSBs (Richardson et al., 1998).

During meiotic DSB formation, the phosphodiester backbone of the DNA is attacked by the catalytic tyrosine of SPO11, resulting in the generation of a covalent SPO11-DNA complex (Figure 1.9) (Keeney et al., 1997). A protein complex, MRN, is recruited to the DSB (Prieler et al., 2005; Lisby et al., 2004). The endonucleolytic activity of the MRN (MRE11-RAD50-NBS1) complex and CtIP in mammals is responsible for the removal of SPO11 from meiotic DSB ends (Inagaki et al., 2010; Sartori et al., 2007).

The removal of SPO11 from DSB ends is achieved by single-stranded endonucleolytic cleavage, which may release SPO11 and 5′strand fully or partly (Keeney et al., 1997; Neale et al., 2005). During this processing 3′ single-stranded tails are generated; thus, 5′ ends are required to be resected. RecA family members, RAD51 and DMC1 in mammals, (Rad51 and Dmc1 in *S. cerevisiae*) are bound to the single-stranded DNA (ssDNA) in order to form nucleoprotein filaments of the homologous duplex (Chen et al., 2008; San et al., 2008). From the 3′ end, repair synthesis occurs and the dissociation (D-loop) captures the other DNA end. Consequently, a double Holliday junction (dHj) is generated at around mid-pachytene after fully formation of synaptonemal complex (SC) (see Section 1.5) (Zickler, 2006). Crossovers (COs) and an exchange of flanking markers occur via the directional resolution of the dHj at middle-late pachytene in budding yeast and mouse (Guillon et al., 2005). Synthesis-dependent strand annealing (SDSA) may occur in non-crossover (NCOs) without the exchange of flanking markers. During SDSA, the extended 3′ is excluded from the D-loop when annealing with different ends of the DSBs (Figure 1.10) (Paques & Haber, 1999; Mc et al., 2007).

During meiosis I, the process of HR is critical for the generation of CO events. In humans and mice, at least one obligate CO is required for each chromosome to obtain perfect segregation in meiosis I (Cole et al., 2010). However, gene conversions or non-crossovers are the result of the larger part of meiotic DSBs (Inagaki et al., 2010).

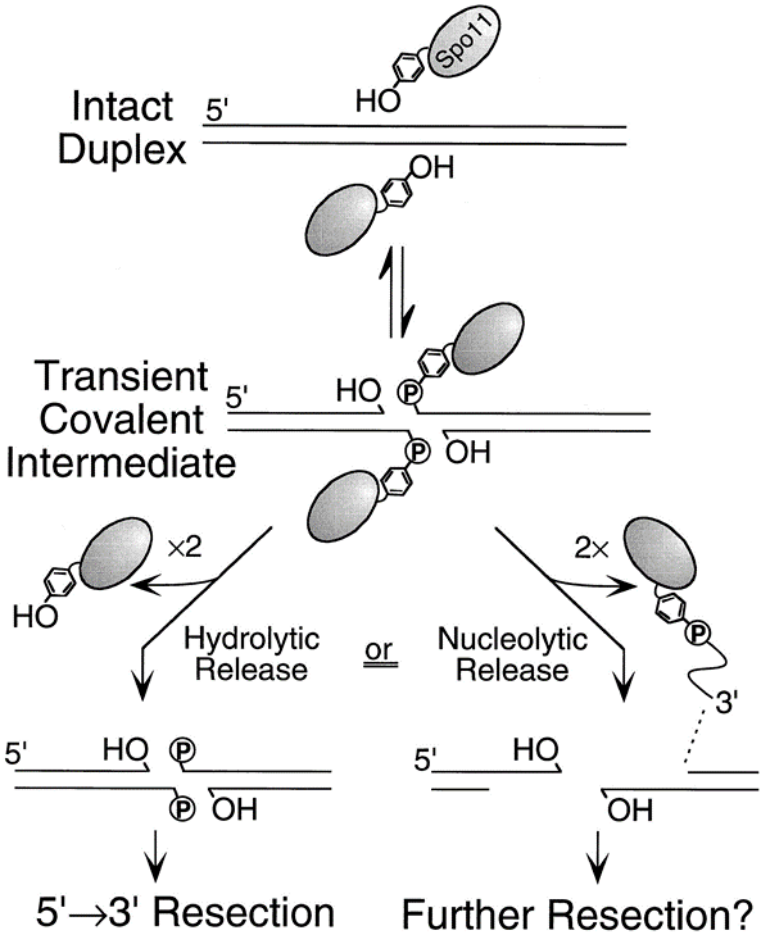



**Figure 1-9 The meiotic DSB mechanism.**
DNA is potentially cleaved by Spo11. The phosphodiester backbone is attacked by a tyrosine side chain, leading to generate the covalent bond between Spo11 and the 5′ end then a free 3′ OH was released. Two Spo11 are required to cut both DNA strands. The release of Spo11 from the cleavage reaction can be achieved via a downstream single-strand nucleolytic cleavage, although it has been shown the latter occurs (Neale et al., 2005; keeney et al., 1997).

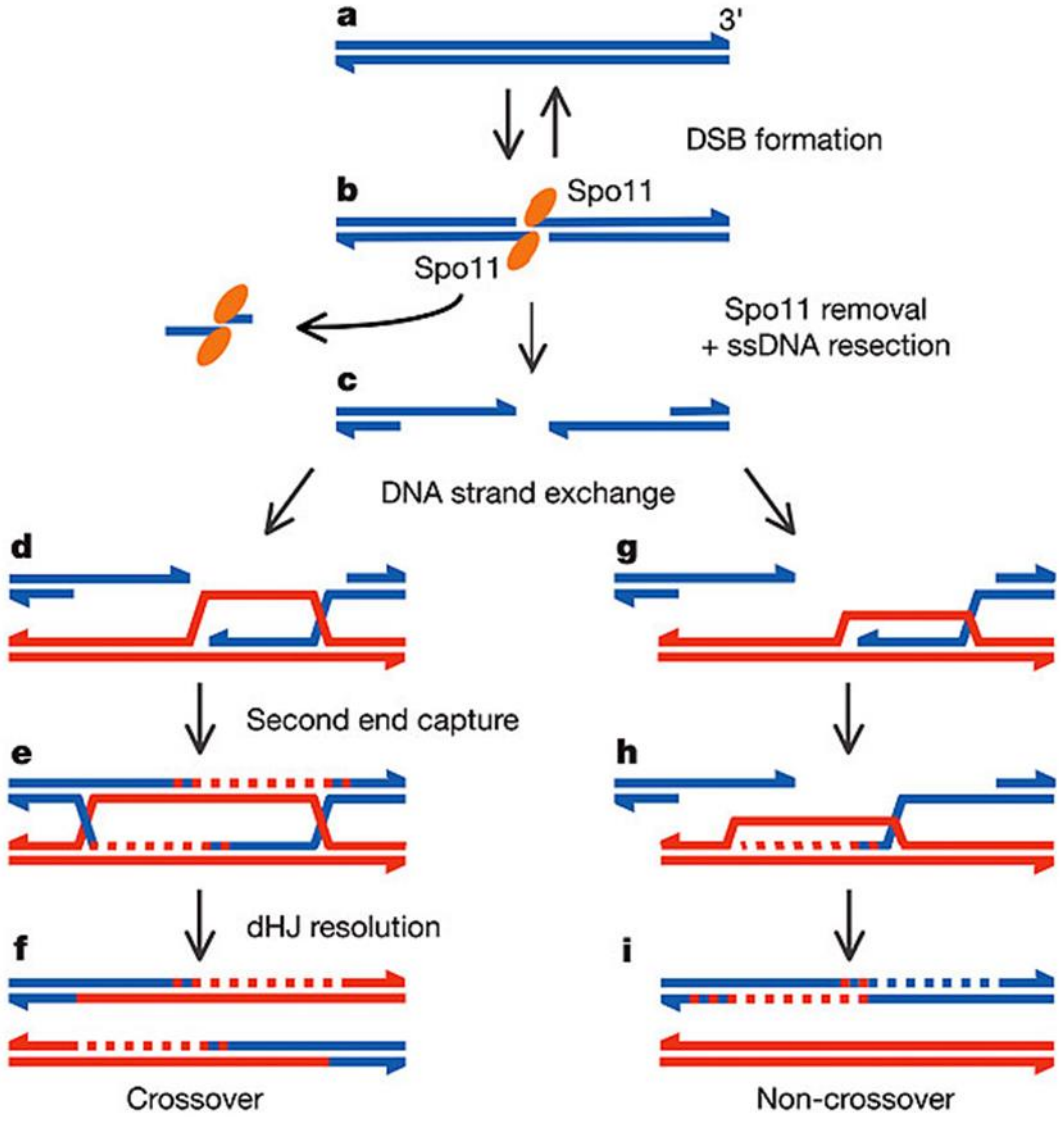



**Figure 1-10 A schematic model for the formation and repairing of meiotic DSB.**
On both strands, the duplex has been broken via Spo11. The DSBs are generated then repaired via homologous recombination (HR). (a-c) Presynapsis. A covalent Spo11-DNA complex is formed. (d-f) The formation of crossover. (d) ssDNA migration in order to repair the breaks through strand exchanging. (e) DNA synthesis and Holliday junction formation (dHJ). (f) HJ resolution and exchanged flanking DNA is yielded. (g-i) Non-crossover mechanism. Strand invasion (g) and DNA synthesis (h) are existed. (i) DNA helicase may dissociate a transient strand invasion, leading to DNA synthesis on the other stand. Finally, a mature non-crossover output is gained through additional DNA synthesis (Neale & Keeney, 2006).

## 1.5 The synaptonemal complex (SC)

The synaptonemal complex (SC) is a protein structure located between homologous chromosomes during early meiotic prophase. The SC contains two structures: the lateral elements (LEs) and the central element (CE). Axial elements (AEs) are generated when sister chromatids become organised and ultimately form LEs of SC. During zygotene, the LEs are completed and linked to the CE by the transverse filaments (TFs). SC structure is matured by the end of zygotene; thereby, the paired homologous chromosomes are synapsed (Figure 1.11).

In humans, mice and *S. cerevisiae*, the completion of meiotic recombination and the performance of the CO are thought to be the main functions of the mature full-length SC (Hunter, 2015). However, incomplete assembly of the SC may lead to failure of synapsis between impaired meiotic recombination and homologous chromosomes, resulting in cell death (Page & Hawley, 2004). Since the AEs are aligned with homologous pairing, the SC is formed, thus supporting HR (Handel & Schimenti, 2010). In addition, homologous pairing and synapsis are stimulated by recombination; therefore, synapsis steadies homologous pairing (Henderson & Keeney, 2005).

Several proteins of the SC structure have been identified in mammalian such as SYCP1, SYCE1, SYCE2, and testis-expressed protein 12 (TEX12). SYCP1 generates TF and it has two terminal domains: the first one is associated with the lateral elements and is known as carboxy-terminal domain (C-terminal) and the second domain interacts with the central elements, and is known as amino-terminal domain (N-terminal) (Liu et al., 1996), while the second interacts with SYCE1 and SYCE2, which are located in the central element of the SC (Costa et al., 2005). The central element contains TEX12 protein, which plays an important role in the formation of the CE and the association of SYCE2 (Hamer et al., 2008).

During the leptotene to zygotene stages, the AEs are initiated, which may lead to the formation of assembly by SC. Interestingly, the lack of synapsis may lead to infertility or aneuploidy in mammals (Fraune et al., 2012), whereas miscarriages may result from oocyte aneuploidy in humans (Garcia et al., 2009).

In cancer cells, SYCP3 and BRCA2 together form a complex, which deactivates the mitotic recombination DNA repair pathway resulting in genome instability (Hosoya et al., 2011). Accordingly, proteins that involve in SC formation may be abnormally expressed in cancers and functionally contribute to oncogenic evolution; for example, the SYCP1 protein expressed in brain, gastric, lung and pancreatic carcinoma and melanoma (Tureci et al., 1998; Nishikawa et al., 2012; Meuwissen et al., 1992).

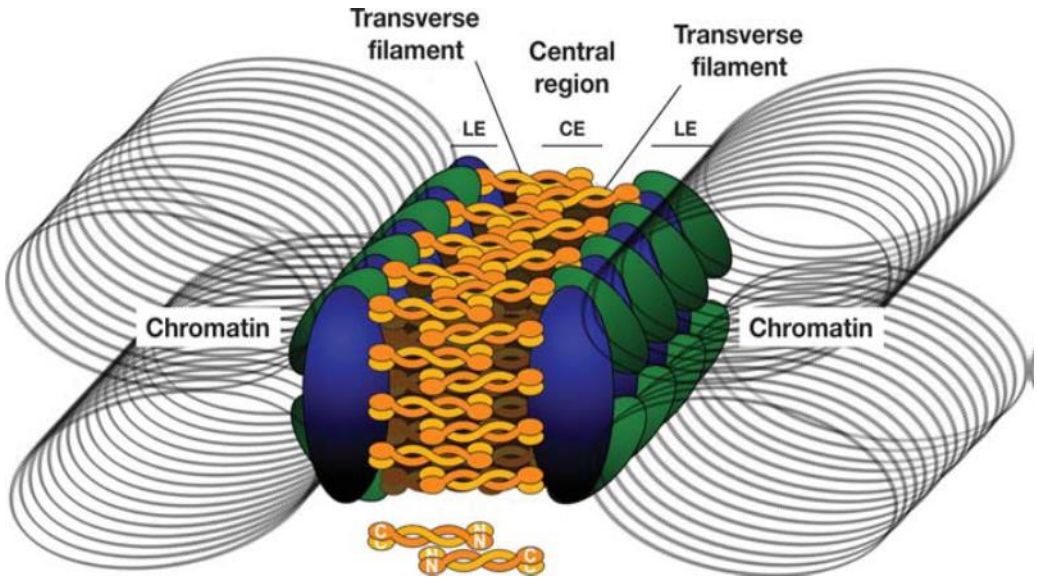



**Figure 1-11 Model of SC structure.**
This figure illustrates the components of SC; lateral element (LE), transverse filaments (TF), central element (CE), and central region. At the bottom, the arrangement of TF proteins can be seen. In this figure, hypothetical arrangement of cohesins is indicated via the blue colour, whereas other LE proteins are indicated by the green colour (Adapted from Page & Hawley, 2004).

## 1.6 The *SPO11* gene

The *SPO11* gene is conserved in a wide variety of organisms, including Yeast, *Drosophila melanogaster*, *Caenorhabditis elegans*, humans and mice. However, some plants, such as *Arabidopsis thaliana*, have three *SPO11* orthologous (Mc et al., 1998; Romanienko et al., 1999). *SPO11* is specifically expressed in the gonads in mammals and is thought to be meiosis-specific. However, a weak expression has been reported in *Drosophila*, mouse and human in somatic tissue (Baudat et al., 2000; Keeney et al., 1999; Romanienko et al., 1999).

Interestingly, plant such as *Arabidopsis*, there are three SPO11 paralogs including *AtSPO11-1*, *AtSPO11-2* and *AtSPO11-3* (An., et al 2011). In fact, meiotic homologous recombination is initiated by both *AtSPO11-1* and *AtSPO11-2*, while *AtSPO11-3* plays an important role in DNA endoreduplication. However, type 1 and 2 may work co-ordinately and seem to generate wild-type levels of DSBs since they are involving in the formation of a heterodimeric complex and it has been suggested that the cleavage of each DNA strand can be created via different AtSPO11 proteins. In addition, this functional coloration between these genes could be demonstrated by the double mutant of *Atspo11-1 Atspo11-2* since there is no difference between them in terms of phenotype (Pradillo et al., 2014).

*SPO11* homologs in humans and mice are more similar to each other than in other species. Mouse *Spo11* is on chromosome 2H4 near a telomere, whereas in humans, *SPO11* localises to 20q13.2–q13.3 (Romanienko et al., 1999). The mouse *Spo11* cDNAs encodes two proteins either 358 or 371 amino acids, while human *SPO11* encodes 396 amino acids, which are roughly 82% identical to the mouse protein (Shannon et al., 1999).

In the mouse genomes, the *Spo11* gene encodes two isoforms; the longest mRNA isoform has 13 exons and is known as *Spo11* β (44.5 kDa), while the smaller transcript skips exon 2 and has 12 exons and known as *Spo11* α (40.3 kDa). Both mRNA isoforms contain exon 5, which is critical for DSB initiation and for coding the catalytic tyrosine (Figure 1.12) (Bellani et al., 2010). Furthermore, the full length of topoisomerase VI suggests the interaction of Spo11 dimers with other proteins and is influenced by the differences in the N termini of Spo11α and –β (Corbett et al., 2007).

*SPO11* is a meiosis-specific gene not expressed during mitosis, in both male and female mammals (Baudat et al., 2000; Keeney et al., 1999; Klein et al., 2002; Romanienko et al., 1999). The SPO11 protein is considered to be a type II topoisomerases-like protein and belongs to the topoisomerase IIB (TOP6A) and topoisomerase VIB (TOP6B) protein family (Bergerat et al., 1997).According to Koslowski et al. (2002) *SPO11* is believed to be a cancer testis antigen (CTA) gene since its expression in germ lines and cancer tissues was detected by RT-PCR techniques. Shannon et al. (1999) reported that when northern blot analysis was used, the expression of *SPO11* was determined only in adult testis at a high level, but not in other somatic adult tissues. However, SPO11 is produced in some cancer cells; consequently, by considering this data, it may lead to shed new light on the proposed function of SPO11 in cancer cells (Collins et al., 1998; Shannon et al., 1999). Additionally, western blot and immunofluorescence techniques have demonstrated that SPO11 is observed in melanoma, which is considered a highly unstable genomic tumour (Chen et al., 2005; Kalejs & Erenpreisa, 2005; Lindsey et al., 2013).

The primary function of the SPO11 protein takes place in meiotic cell division (Malik et al., 2007). In fact, SPO11is responsible, with other genes such as MEI4 and MER2, for the initiation of meiotic recombination through the introduction of DSBs in early meiotic prophase (Bergerat et al., 1997; Atcheson et al., 1987; Keeney, 2001). *SPO11* gene is considered as one of the first meiotic recombination genes (Esposito & Esposito, 1969). Bellani et al. (2010) proposed a topoisomerase activity for SPO11 α, but no evidence has been found to support this idea (Bellani et al., 2010). In *S. cerevisiae*, the formation of the SC, the AE and the spindle require *Spo11* (Boateng et al., 2013; Celerin et al., 2000; Loidl, 2013; Malik et al., 2007). Moreover, budding yeast requires *Spo11* for regulating meiotic DNA replication; the functional role for this remains unclear, although it is DSB-independent (Cha et al., 2000).

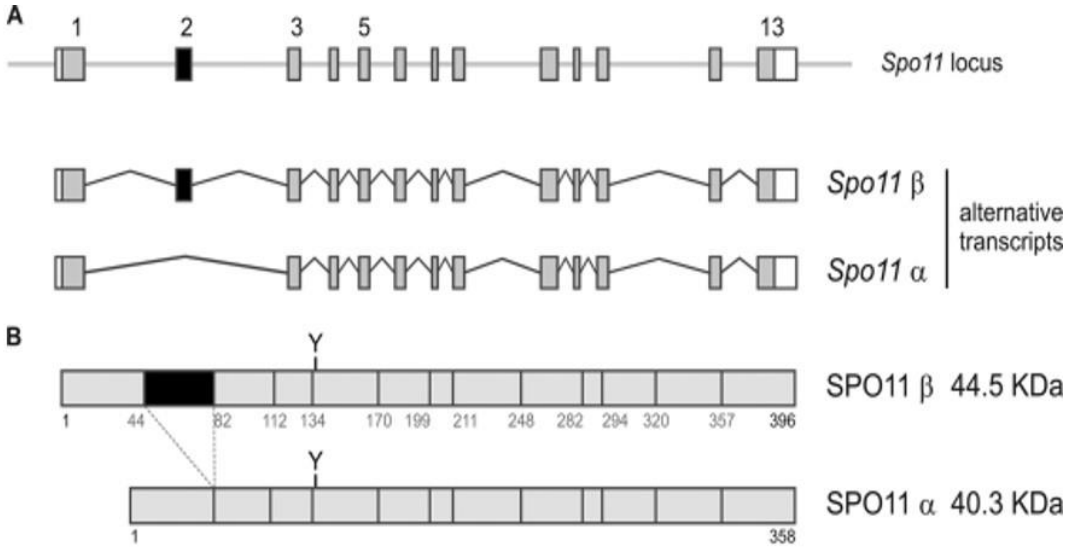



**Figure 1-12 Genomic organisation, splicing pattern and polypeptides of mouse *Spo11*.**
(A) The Spo11α transcript does not contain exon 2, while the spo11 β transcript includes exon 2. (B) The polypeptides of SPO11 α and β. The black square indicates the part encoded by exon 2. Y refers to the catalytic tyrosine encoded within exon 5 (Adapted from Bellani et al., 2010).

The absence of *SPO11* in *S. cerevisiae* has an adverse effect on the formation of mature recombination products, meiotic DBSs and Holliday junction formation (Cao et al., 1990; Keeney, 2001; Schwacha & Kleckner, 1994). In mice, male and female infertility can be the result of disruption of *Spo11* (Baudat et al., 2000; Romanienko et al., 2000). Interestingly, in mice, the homozygous null mutation of *Spo11* may lead to arrest in early prophase or spermatocyte apoptosis in mid-prophase I (Romanienko et al., 2000). In addition, the lack of *Spo11*-α in male mice results in a decrease in DSBs in the sex chromosomes, which suggests that *Spo11*-α has a role in sex chromosome recombination (Kauppi et al., 2011). The Disruption of *Spo11* leads to homologous chromosomes not synapsing or synapses with multiple partners (non-homologous) (Baudat et al., 2000; Romanienko et al., 2000). Moreover, it has been reported in *S. pombe* (Cervantes et al., 2000), *Drosophila* (Mc et al., 1998), *C. elegans* (Dernburg et al., 1998) and *Coprinus cinereus* (Celerin et al., 2000) that the interruption of *spo11* homologs may reduce the level of meiotic recombination and defect gametes, which means that meiosis fails (Malik et al., 2007). The formation and repair of DSB require the co-operation of many other genes, including *MEI4* and *MREII* in *S. cerevisiae* . Therefore, the alteration or mutation of any of these genes may affect the initiation of DSB and meiotic recombination, which leads to the notion that the products of some of these genes are associated with one another to some extent (Hunter, 2007; Keeney et al., 1997; Keeney, 2007).

ATM kinase is a protein that associated with SPO11; ATM regulates the formation of DSBs through controlling SPO11 (Loidl, 2013; Lange et al., 2011). Lange et al. (2011) suggested that in budding yeast and in mammals, DSBs might activate ATM, which leads to the promotion of a negative feedback loop. This loop results in phosphorylation of SPO11 or its accessory proteins and the control of the number of DSBs that may be generated by SPO11. Furthermore, Ski8 protein was identified as a direct partner of Spo11 since they both are associated with meiotic chromosomes. It has been reported that Ski8 plays a critical role with Spo11 in the formation of DSBs. In addition, localisation of Ski8 in the cytoplasm or in the nucleus depends on its interaction with Spo11 (Keeney & Neale, 2006). In the absence of Spo11 or Ski8, SCs are not formed and the recognition of homologous are decreased (Storlazzi et al., 2003).

The PRDM9 protein has an important part in transcriptional regulators of cellular differentiation and maturation (Hohenauer & Moore, 2012). Additionally, the PRDM9 protein is responsible for activating recombination hot spots and for exposing DNA for DSB formation. Moreover, PRDM9, is an example of a chromatin modifier and it activates the chromatin, allowing the SPO11 to bind to DNA to initiate DSB (Grey et al., 2011). *SPO11* and *PRDM9* are expressed in different cancer cell lines, while their expression in normal tissues are limited (Lindsey et al., 2013; Feichtinger et al., 2012). Therefore, these genes, as CTA genes, may be considered as good examples for cancer biomarker and drug targets.

Recently, Robert et al. (2016) and Vrielynck et al. (2016) reported that the SPO11 protein has a partner known as the TopoVIB-like protein. Moreover, the TopoVIB-like protein is presents in plants and animals, and plays a role in generating meiotic DNA DSBs. In mice, TopoVIB associates with the TopoVIA region, which in turn, corresponds to the SPO11 splice variant β (SPO11β). This variant is composed of 38 amino acids in the N terminus (Corbett et al., 2007; Graille et al., 2008). A study suggested that SPO11β is responsible for the initiation of DSBs, due to the formation of the complex with TopoVIB (Kauppi et al., 2011).

### 1.6.1 SPO11 and cancer

The expression of CTA genes is restricted to germ cells and can be detected in a variety of histological tumour types. Importantly, these genes may have a postulated function in cancer cells, which might enable CTAs to be used as valuable biomarkers and drug targets. Interestingly, SPO11 has been identified as a CTA (Koslowski et al., 2002; Shannon et al., 1999). In cancer cells, some proteins such as SPO11, SYCP3, PRDM9 and DMC1 may interrupt the mechanism that maintains genomic stability. Therefore, affecting the number of chromosomes in cells or enhancing chromosomal rearrangement can be the result of germ cell activation in cancer cells (Atanackovic et al., 2011; Costa et al., 2007; Nielsen & Gjerstorff, 2016).

Since *SPO11* is expressed in melanomas, it exhibits chromosomal instability (CIN) with other CTAs, such as *SCP1*, *REC8* and *HORMAD1*, and which suggests that it has a role in cancer initiation and chromosomal instability has been suggested. Both *SPO11* and *HORMAD1* have been confirmed to be overexpressed in melanoma using western blot analysis and immunofluorescence (Lindsey et al., 2013). Nielsen and Gjerstorff (2016) suggested that SPO11 might have a proposed oncogenic function unrelated to DSB generation since its expression is not limited to male germ cells (Nielsen & Gjerstorff, 2016).

Atanackovic et al. (2011) published their study that demonstrated that the *SPO11* gene was expressed in at least one cell line of 10 tested samples with acute myeloid leukaemia (AML); however, its expression was absent from samples of healthy bone marrow (BM) (Atanackovic et al., 2011). Other studies have suggested that enhancing genomic instability and aneuploidy by creating abnormal chromosomal recombination may be an additional function of SPO11 in cancer cells (Kalejs et al., 2006; Litvinov et al., 2014). Interestingly, some effective anti-cancer drugs, such as etoposide and doxorubicin, target DNA topoisomerase II (Top2) (Liu, 1989). These types of drugs lead to the formation of a specific enzyme that causes DNA damage. SPO11 may become important in the cancer treatment since it is considered a type IIB topoisomerase (Pommier et al., 1985; Nitiss, 2009).

As discussed, SPO11 seems to have a proposed role in cancer cells, making it a potentially valuable biomarker in terms of both cancer diagnosis and prognosis. Furthermore, *SPO11* as a CTA gene may have important applications in cancer drugs and therapeutic targets.

## 1.7 Aims and objectives

The aim of this project was to identify the potential function of the human meiosis-specific gene *SPO11* in various types of cancer cells. The main reason for choosing this gene was that it has a restricted expression in cancer cells compared with normal tissues. This resulted in the hypothesis that SPO11 might have a role in cancer cells. Additionally, it was hypothesised that this gene might potentially be used as the spcific target, and could serve as a human cancer-specific marker in terms of targeting uncontrolled cell division (cancer).

### 1.7.1 The specific aims of this study were:

1- To assess the level of SPO11 protein in different normal and cancerous tissues.

2- To knockdown the *SPO11* gene by using several techniques such as siRNA and shRNA to determine the effect of the reduction of its level on cancer cells.

3- To address how knocking down the *SPO11* gene influences various cell cycle proteins, such as cyclin A and B, apoptosis and RecA genes (Rad 51 and DMC1).

4- To validate the SPO11 antibody using two types of tags (HA and C-myc) to determine the specificity of SPO11 antibody.

# Chapter 2

## Materials and Methods

# 2. Materials and methods

## 2.1 Human cell culture

### 2.1.1 Culturing the cell lines

All cell lines that have been used in this project, were grown in a humidified atmosphere containing 5% $CO_2$ at 37°C. Those cell lines were purchased from the American Type Culture Collection (ATCC) therefore, their passage were based on recommended dilutions and confluences from this provider. The cell lines were cultured in a medium supplemented with 10% foetal bovine serum (FBS) (Invitrogen; Lot 41Q6208K). Cultures were checked regularly for mycoplasma contamination using the Mycoplasma PCR Detection kit (Sigma Aldrich; MP0035). Cell lines and culture conditions are summarised in Table 2.1.

### 2.1.2 Thawing frozen cancer cell lines

After removing a vial of cells from the liquid nitrogen tank, it was immediately thawed in a water bath at 37ºC for approximately 2 minutes. The cells were transferred into a sterile 15 ml conical tube with 5 ml of complete and warmed growth medium. Following that, the cells were pelleted by centrifuging at 1000 x g for 5 minutes. The supernatant was aspirated and the pellet was resuspended into 10 ml fresh growth medium, which was then transferred to an appropriate flask. The cells were grown for 24 hours at 37°C in a humidified incubator with the required $CO_2$ concentration.

### 2.1.3 Dissociation of adherent cells from culture

The medium was aspirated from the plates and the attached cells were washed once with 1 x Dulbecco's phosphate-buffered saline (DPBS), (Gibco®; 14190-250). After that, trypsin (Gibco®; 25300-054) was added to cover the cell layer surface of the plates and returned back to the incubator for 2-5 minutes (depending on the cell line) to allow the cells to dissociate into suspension. Next, warm fresh medium with serum was added to inhibit the trypsin activity and then the cells could be used as required.

### 2.1.4 Cancer cell line stocks preparation

When cells become 80–90 % Confluent, they were washed twice with 1x DPBS and trypsinised with 1x trypsin – EDTA (Invitrogen, GIBCO 1370163). The cells were collected into 10 ml fresh medium, and then centrifuged at 1000 x g for 5 minutes. Cell pellets were resuspended gently into 1 ml freezing containing 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich; D8418) and 90% FBS and transferred into a labelled cryotube. The vials should then be placed in a Nalgene 'Mr Frosty' freezing container filled with isopropanol at -80°C for at least 24 hours in order to obtain successful cell recovery since a continuous cooling rate of 1°C per minute from room temperature is highly recommended. The cells could be placed at -80°C for 24 hours for short-term storage or stored in liquid nitrogen tank for long-term storage.

**Table 2-1 Cell lines employed in this study and culture conditions.**

| Cell line names | Origin | $CO_2$ | Culture Media conditions |
|---|---|---|---|
| SW480 | Human colon adenocarcinoma | 5% | Dubeco's modified Eagle's medium (DMEM) + GLATAMAXTM (Invitrogen; 61965) + 10% FBS |
| HEP-G2 | Hepatocellular carcinoma | | |
| A2780 | Human ovarian carcinoma | | |
| MCF7 | Human Caucasian breast adenocarcinoma | | |
| NTERA-2 | Human Caucasian pluripotent embryonal carcinoma | | |
| 1321N1 | Human brain astrocytoma | | |
| Hela | Cervical cancer | | |
| K562 | Leukaemia | | |
| Jurkat | Leukaemia | | |
| HCT116 | Human colon carcinoma | 5% | McCoy's 5A medium + GLUTAMAXTM (Invitrogen; 36600) + 10% FBS |
| Lovo | Human colon adenocarcinoma | 5% | Ham's F12 + 2 mM Glutamine + 10% Foetal Bovine Serum (FBS) |

## 2.2 Cell counting

Firstly, when a hemocytometer slide (Sigma-Aldrich; Z169021-1EA) was used, the slide, together with its cover, was gently wiped with 70% ethanol, followed by deionized water. The cell suspension was mixed to disrupt any clumps of cells in order to obtain a reasonable estimation of the actual cell concentration. In a clean Eppendorf tube, 10 µl of cell culture was mixed with the amount of 0.4% Trypan blue (Invitrogen; 15250-061). After placing the cover slip above the grid of the hemocytometer, 10 µl volume of this mixture was loaded into the chamber through V-shaped wells using a 20P micropipette. The haemocytometer was read under a Carl Zeiss Axiostar microscope 10 × Objectives and live cells were unstained, whereas blue cells indicated dead cells (stained with Trypan Blue). The average number of cells was counted on 5 squares for both the girds, and the total number of live cells was determined by using the following calculation:

Cells/ ml = Average count of live cells × 2 (dilution by Trypan blue) × dilution factor × 104.

Secondly, Viable-cell counts as measured by the trypan blue dye exclusion method on Biorad's TC10 cell counter.

## 2.3 Western blot protocol

### 2.3.1 Protein extraction

M-PER® Mammalian Protein Extraction Reagent (Thermo; 78503) was used to extract the protein from whole cells lysates from adherent and suspension. A flask containing cells was washed with warm 1x DPBS. Following that, the attached cells were detached by using trypsin, and the resulting cell suspension was washed with warm 1x DPBS and pelleted for 10 minutes by centrifugation at 2500 × g. The supernatant was discarded and the wet pellet was weighed. A total of 10 µl of M-PER lysis buffer was added for each mg of cell pellet. Halt Protease Inhibitor Cocktail (Thermo Scientific; 87785) and Halt Phosphatase Inhibitor Cocktail (Thermo Scientific; 78420) was added (1 µl of Inhibitor Cocktail / 100 µl of lysis buffer). This mixture was mixed gently by shaking and incubated at room temperature for 10 minutes. Cell debris was removed by centrifuging the tube for 15 minutes at 14000 × g. The supernatant was transferred to a newly-labelled tube and kept at -20°C until required.

### 2.3.2 Protein concentration assay using BCA

The total extracted protein concentration was estimated by using BCA Protein Assay Kit (Thermo Scientific; 23227) in order to load equal amounts of protein into each well in the gel. According to the manufacturer's protocol, a set of standards (A- I) was prepared from the bovine serum albumin provided with the kit. Following that, working reagent was prepared by adding 50 parts of Reagent A to one part of Reagent B. Standards and tested samples were then added to the working reagent and incubated for 30 minutes at 37°C in darkness. Standard curve and protein concentration were carried out with 1 µl of each sample using a NanoDrop ND 2000c Spectrophotometer (Thermo; WZ-83061-12).

### 2.3.3 Detection of target protein

Approximately 20-30 µg of total protein extract was combined with 4x Bolt LDS Sample Buffer (Life Technologies; B0007) and 10x Bolt Sample Reducing Agent (Life Technologies; B0009). Prior to electrophoresis, samples were placed in a heating block for 10 minutes at 70°C, in order to denature those samples. Then, proteins were separated alongside the protein ladder (Precision Plus Protein Dual Color Standards, Biorad; 161-0374) on commercially different concentrations of polyacrylamide, Bolt 4-12% Bis-Tris Plus Gel, 15 well (Life Technologies; BG04125BOX) and NuPAGE® Novex® 4-12% Bis-Tris Plus Gel, 12 well (Life Technologies; NP0322BOX) using Bolt MES SDS Running Buffer (Life Technologies; B0002). A running buffer was prepared as concentrated reagents (20×) and was diluted with distilled water before use.

The gels were run at 120 V for 1 hour. Using the electrotransfer technique, the protein was transferred electrophoretically onto a methanol-soaked PVDF membrane (Immobilon-P, Millipore, IPVH00010) at 500 mA for 2-4 hours using transfer buffer [30.3 g of Trizma® base (SIGMA; T1503), 144 g of Glycine (SIGMA; G8898) and distilled water to 1 litre]. After transferring the protein, the membrane was washed once with distilled water for 5 minutes, and followed by blocking in 5% skimmed milk powder in PBS/0.5% Tween 20 (milk solution) for at least an hour at room temperature. The blotted membrane was probed with required concentration of primary antibody (diluted in blocking solution) (Table 2.2) and incubated overnight at 4°C on a rocker plate.

The membrane was then washed three times for 5 minutes with PBS/0.1% Tween 20. After washing, the membrane was incubated with corresponding secondary antibody in proper dilution (Table 2.3) for an hour at room temperature, followed by washing three times for 5 minutes with in PBS/0.1% Tween 20. The protein of interest was detected by incubating the membrane for 5 minutes with Signal-generating solution, for example Chemiluminescent Peroxidase Substrate-3 (Sigma, #CPS3100-1KT), Super Signal West Pico Chemiluminescent Substrate (Thermo, #34080), in order to amplify light that can be detected by X-ray film. The membrane then was placed in X-Ray Cassettes and exposed to CL-X Posure film (Thermo Scientific, 34091) using optimal exposure times. In the dark room, the film was developed in an X-Ray Film Processor according to the manufacturer's (MI-5) guide.

**Table 2-2 Primary antibodies used in western blot (WB) and immunoflourescence (IF).**

| Primary antibody | Cat. No. | Source | Host | Clonality | W.B. dilution | IF dilution |
|---|---|---|---|---|---|---|
| Anti-SPO11 | Ab81695 | Abcam | Rabbit | Monoclonal | 1/1500 | 1/3000 |
| Anti-MAGEC1 | ab61404 | Abcam | Mouse | Monoclonal | 1/500 | |
| Anti-GAPDH | Sc-365062 | Santa Cruz | Mouse | Monoclonal | 1/5000 | - |
| Anti-Cleaved Caspase-3 | 9664 | Cell Signaling | Rabbit | Monoclonal | 1/1000 | - |
| Anti-c-Myc | ab32072 | Abcam | Rabbit | Monoclonal | 1/5000 | - |
| Anti-Cyclin A2 | 4656 | Cell Signaling | Mouse | Monoclonal | 1/1000 | - |
| Anti-Cyclin B1 | 4138 | Cell Signaling | Rabbit | Polyclonal | 1/1000 | - |
| Anti-Cyclin E1 | 4129 | Cell Signaling | Mouse | Monoclonal | 1/1000 | - |
| Anti-p21 | 2947 | Cell Signaling | Rabbit | Monoclonal | 1/1000 | - |
| Anti-p27 | 3686 | Cell Signaling | Rabbit | Monoclonal | 1/1000 | - |
| Anti-Phospho-Rb | 8516 | Cell Signaling | Rabbit | Monoclonal | 1/1000 | - |
| Anti-Rb | 9309 | Cell Signaling | Mouse | Monoclonal | 1/1000 | - |
| Anti-HA-Tag | 3724 | Cell Signaling | Rabbit | Monoclonal | 1/1000 | 1/1600 |
| Anti-HA-Tag | 2367 | Cell Signaling | Mouse | Monoclonal | 1/1000 | 1/100 |
| Anti-c-Myc-Tag | 2276 | Cell Signaling | Mouse | Monoclonal | 1/1000 | - |
| Anti-Rad51 | 8875 | Cell Signaling | Rabbit | Monoclonal | 1/1000 | - |

**Table 2-3 Secondary antibodies used in western blot (WB) and immunofluorescence (IF).**

| Secondary antibodies | Cat. No. | Source | Host | Application | Dilution |
|---|---|---|---|---|---|
| Anti-rabbit IgG, HRP-linked Antibody | 7074 | Cell Signalling | Rabbit | WB | 1/3000 |
| Anti-mouse IgG, HRP-linked antibody | 7076 | Cell Signalling | Mouse | WB | 1/3000 |
| Peroxidase- Donkey Anti-Rabbit IgG (H+L) | 711-035-152 | Jackson immuno | Rabbit | WB | 1/25000 |
| Peroxidase- Donkey Anti Mouse IgG (H+L) | 715-035-150 | Jackson immuno | Mouse | WB | 1/25000 |
| Goat anti-Mouse IgG (H + L), Alexa Fluor 488 conjugate | A-11029 | Life technologies | Mouse | IF | 1/250 |
| Goat anti-Rabbit IgG (H + L), Alexa Fluor 568 conjugate | A-11011 | Life technologies | Rabbit | IF | 1/250 |
| Donkey anti-Goat IgG (H + L), Alexa Fluor 568 conjugate | A-11057 | Life technologies | Goat | IF | 1/400 |
| Goat anti-Rabbit IgG (H + L), Alexa Fluor 488 conjugate | A-11034 | Life technologies | Rabbit | IF | 1/250 |

### 2.3.4 Source of human normal tissue lysates

Human normal lysates were provided from a different company as described in Table 2.4.

**Table 2-4 Source of normal lysates used in western blot.**

| Normal tissues | Source | Cat. No. | Protein amount |
|---|---|---|---|
| Testis | Abcam | Ab30257 | 20 µg |
| Thymus | Abcam | Ab30146 | 20 µg |
| Skeletal muscle | Abcam | Ab29331 | 20 µg |
| Small intestine | Abcam | Ab29276 | 20 µg |
| Ovary | Abcam | Ab30222 | 20 µg |
| Colon | Abcam | Ab30051 | 20 µg |
| Breast | Abcam | Ab30090 | 20 µg |
| Testis | Novus Biological | NB820-59266 | 20 µg |
| Lung | Novus Biological | NBP2-27734 | 20 µg |
| Ovary | Novus Biological | NBP2-28454 | 20 µg |
| Liver | Novus Biological | NBP2-29220 | 20 µg |
| Colon | Novus Biological | NBP2-28208 | 20 µg |

## 2.4 Extreme limiting dilution analysis (ELDA)

Extreme limiting dilution analysis (ELDA) was performed as Hu and Smyth, 2009 described. Cells were seeded in 96 well plates at concentrations of 1000, 100, 10 and 1 cell in 100 µl of medium together with serum. A further 12 well repeats were used for untreated cells, negative control siRNA, Hiperfect treated cells and SPO11 siRNA 2 and siRNA 4 treated cells. The transfection complex was made by adding 10 nM siRNA (Qiagen) containing 0.3 µl HiPerfect reagent (Qiagen; 301705) to 4.7 µl medium serum free and incubating for 20 minutes at room temperature. A negative control non-interference siRNA (Qiagen; 1022076) was prepared using the same procedure. After that, the SPO11 siRNA transfection mixture, negative control siRNA and Hiperfect only were added drop wise to the medium. The cells were incubated in a humidified incubator at 37°C with 5% $CO_2$ for 10 days. Cells were supplemented with 50 µl of medium with serum and the transfection complexes re-applied after 2 and 6 days of incubation. At the end of 10 days of culture, the numbers of wells showing positive cells growth were counted using light microscopy. The frequency of cell proliferation was determined by the ELDA web tool (http://bioinf.wehi.edu.au/software/elda/).

## 2.5 Immunostaining protocol

Cells were seeded into 24 well plates at a density of 50,000 cells per well, cultured on glass cover slips. After 24 hours, they should be ready for staining when they are sub-confluent. The cells were washed with DPBS after removing the medium. The cells were then fixed with 4% paraformaldehyde (PFA) (Thermo; RA21476410) in DPBS for 10 minutes at room temperature in order to preserves the morphology and antigenicity of the cells. The cells were Incubated with 0.2% Triton X-100 (Sigma; 019K01512) for 10 minutes at room temperature was the next step, followed by blocking the cells with 10% FBS/DPBS at 37°C for at least one hour at room temperature to block unspecific binding and permeabilize membranes. Next, the cells were stained with the primary antibodies, diluted in 10% FBS/DPBS (Table 2.2) overnight at 4°C on a rocker plate. The cells were then washed three washes using DPBS for 10 minutes at room temperature, and then the cells were incubated with appropriate Alexa Fluor secondary antibody, diluted in 10% FBS/DPBS (Table 2.3) for two hours at room temperature in the dark. This was followed by washing the cells three times with DPBS for 10 minutes each at room temperature in the dark.

After washing, the cover slips were mounted with Prolong® Gold Antifade Reagent with DAPI (Cell Signalling; 8961), and then the cover glass was sealed to the slide with nail polish and stored at 4°C for at least 24 hours. A Zeiss LSM 710 confocal microscope and ZEN software (Zeiss) was used to detect immunofluorescence. Negative control samples were required by only using secondary antibody to test the specificity of the immunostaining reactions.

## 2.6 Transfection methods

### 2.6.1 siRNA transfection protocols

Fresh complete medium cells were seeded in 6-well plates and then incubated under normal growth conditions for 24 hours. Before starting the experiments, the cell confluency should be calculated for every cell line, since they are different in their seeding number, for instance for Hela cells $3\times10^5$ cells per well should be used. Prior to the transfection, the siRNA was diluted in 100 µl serum free-media and mixed gently with 10 µl of Hiperfect Transfection Reagent (Qiagen; 301705) and 1.5 µl of a 10/20 µM siRNA of the gene of interest or 1.5 µl of Negative Control siRNA. The names and sequences of the siRNAs used in the study are listed in Table 2.5. Transfection was also carried out with 10 µl Lipofectamine® RNAiMAX (Invitrogen; 13778-150). Additionally, Green Viromer (Lipocalyx; VG-01LB-03) was used as a transfection reagent, and manufacturer's directions were followed. The mixture was then incubated for 20 minutes at room temperature, in order to form the transfection complexes and then added drop-wise onto the cells whilst gently swirling the plates. After 24 hours, the transfection was carried out twice or three times, depending on the cell line without changing the complete medium. After a period of either 48 or 72 hours, post-transfection, the cells were harvested and the efficiency of knockdown of the targeted protein was assessed by western blot (see Section 2.3). In addition, untreated cells were used as a positive control, whereas AllStars Negative Control siRNA (Qiagen; 97315239) was used as a negative control to measure the expression level of the gene of interest.

**Table 2-5 siRNA used for gene knockdown.**

| Gene target | Product name | Qiagen cat no. | Target sequence |
|---|---|---|---|
| Negative control | Negative Control siRNA | 1022076 | 5'-AATTCTCCGAACGTGTCACGT-3' |
| SPO11 | Hs_SPO11_2 FlexiTube siRNA | SI00100373 | 5'-ACAACTAATGTTAACGCATAA-3' |
| | Hs_SPO11_4 FlexiTube siRNA | SI00100387 | 5'-TACCTTCTACGATACAACTAA-3' |
| Rad 51 | | | 5'-CTGGCTACATAGTAAATCAAA-3' |

### 2.6.2 esiRNA (endo-ribonuclease prepared siRNA) transfection protocols

In fresh complete medium cells were seeded in 6-well plates then incubated under normal growth conditions for 24 hours. Before starting the experiments, the cell confluency was calculated for every cell lines since they are different in their seeding number, for instance for Hela cells $3\times10^5$ cells per well were used. Before transfection, different reagents were prepared; firstly, transfection reagent, 150 µl Opti-MEM medium (no FBS) was added 9 µl of Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen, # 13778-150). Secondly, negative control reagent, 150 µl Opti-MEM medium (no FBS) was added 6 µl of BLOK-iT™ Fluorescent Oligo. Thirdly, positive control reagent, 150 µl Opti-MEM medium (no FBS) was added 6 µl of EHU063791Lamin-A/C. Finally, gene knockdown reagent, 150 µl Opti-MEM medium (no FBS) was added 6 µl of esiRNA gene-specific knockdown (SPO11-open; XX-90204-1-20UG; HMT; 8021602). After that, 150 µl of diluted Lipofectamine was added to 150 µl of each diluted esiRNA (negative, positive and gene specific knockdown). The mixture was incubated for 10 minutes at room temperature. After incubation, 250 µl of the complexes was added drop-wise onto the cells with gently swirled the plates. After 24 hours, the transfection was carried out twice without changing the complete medium. 48 or 72 hours post-transfection the cells were harvested and the efficiency of knockdown of the targeted protein was assessed by western blot.

## 2.7 Senescence staining

Cells were plated onto 6 well plates at a concentration of $1x10^5$ cells in 10 ml of serum with media. After 24 hours, transfection took place using SPO11 siRNA and negative control siRNA at a final concentration of 10 nM and HiPerFect Reagent was used as described in Section 2.6.1. After three hits. Senescence staining was performed using a β-galactosidase staining kit (Cell Signalling; 9860) following the manufacturer's protocol.

## 2.8 Cell viability

### 2.8.1 Cell viability counting

After SPO11 siRNA knockdown, cell viability counts were performed. Cell viability was determined by either using a hemocytometer or Biorad's TC10 automated cell counter. Cell viability was calculated as the number of live and dead cells.

### 2.8.2 RealTime-Glo™ MT Cell Viability Assay

Cells were plated in 96 well plates at an appropriate concentration depending on the cell line; for instance, in the case of SW480 cells, $1 \times 10^3$ cells/ml per well was used. The cells were incubated for 24 hours prior to the transfection. Following that, cells were transfected with siRNA using Hiperfect Transfection Reagent and non-interference as a siRNA negative Control (see Section 2.6.1). Three hits with siRNA were performed, and after a period of 24 hours from the last hit, RealTime-Glo™ MT cell viability assay (Promega; G9711/2/3) was performed according to the manufacturer's instructions.

### 2.8.3 CellTiter 96 AQ ueous One Solution Cell Proliferation Assay

Cells were plated in 96 well plates at an appropriate concentration depending on the cell line; for instance, in the case of SW480 cells, $1 \times 10^3$ cells/ml per well was used. The cell were incubated for 24 hours prior to the transfection. Following that, cells were transfected with siRNA using Hiperfect Transfection Reagent and non-interference as a siRNA negative Control (see Section 2.6.1). Three hits with siRNA were performed and after period of 24 hours from the last hit, CellTiter 96 AQ ueous One Solution Cell Proliferation Assay (Promega; G3582) was used according to the manufacturer's instructions.

## 2.9 Growth curve

Cells were seeded at equivalent densities ($1 \times 10^5$ cells/ml) in 6 well plates and incubated for 24 hours, in order to attach and recover. Following that, transfection with SPO11 siRNA was performed every day for 8 days. Media was changed after 3 and 5 days. At daily intervals, cells were trypsinzed and counted every day using Biorad's TC 20 automated cell counter with the trypan blue. Western blot then was carried out after harvesting the cells to determine any change in the protein level.

## 2.10 Primer design for RT-PCR

The National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) was used to obtain the specific sequence of all genes. The Primer3 software (available from: http://www.genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi) was used to design the primers each gene codon sequence. All primers were designed to span at least one intron where possible between the forward and reverse. Eurofins MWG operon (http://www.eurofinsgenomics.eu/) was used to synthesis all primers in this study, and sterile distilled water was used to dilute the primers to final concentration of 10 pmol. Gene-specific primers used for RT-PCR screening and their expected size are listed in Table 2.6. The PCR reaction took place in 25/50 µl final volume, using BioMix Red (Bioline BIO-25005) or 1x MyTaq Red Mix (Bioline, BIO-25043). The target sequence was amplified with initial heating at 96°C for 5 minutes, followed by 40 cycles of denaturing at 96°C for 30 seconds, annealing temperature is shown in Table 2-6 for 30 seconds extension at 72°C for 40 seconds, followed by a final elongation step 72°C for 5 minutes.

**Table 2-6 Primer sequences and their expected product size in base pairs.**

| Gene | Primer direction | Primer sequence (5` to 3`) | Tm (°C) | Product size (bp) |
|---|---|---|---|---|
| B-Actin | F | 5'-TGCTATCCCTGTACGCCTCT-3' | 58 | 675 |
| | R | 5'-CGTCATACTCCTGCTTGCTG-3' | | |
| PTRE-3G | F | 5'- ATTCCACAACACTTTTGTCT- 3' | 55 | 3431 |
| | R | 5'- GGTTCCTTCACAAAGATCCTC- 3' | | |
| *SPO11* | F | 5'- GACTGGATCCATGGCCTTTGC- 3' | 55 | 1191 |
| | R | 5'- GACTGGATCCTTATATCCATC- 3' | | |
| *SPO11* | F | 5'- AAACGTCGAAGAACGAGGCC- 3' | 55 | 600 |
| | R | 5'- CGACCACAGGTACAATTCAC- 3' | | |

## 2.11 The cloning of N-HA::SPO11 and C-Myc::SPO11 into PTRE-3G vector

### 2.11.1 Primers design for cloning

Human SPO11 open reading frame was cloned into PCMV MCS N-HA and pCMV MCS C-Myc by Dr. Ellen Vernon, in our lab. Ellen cloned SPO11 cDNA into pCMV MCS N-HA vector and pCMV MCS C-Myc vector. What we did after that is cutting SPO11cDNA from these vectors with both tags N-HA and C-Myc then we cloned them into pTRE-3G vector with the Tet-On 3G System. The forward and reverse primers were both designed with restriction enzyme sites for N-HA and C-Myc tags (Table 2.7). With regards to SPO11::N-HA, *Pst*I and *NedI* restriction, enzymes were added to the primers. However, *Pst*l and *Bam*Hl were used with SPO11::C-Myc. Restriction enzymes and their recognition site are shown in Table 2.8. A couple of random bases were added to the 5' end of the primers to preserve the restriction site during PCR. Eurofins MWG operon was used to synthesise all primers for cloning; therefore, primers were used following the manufacturer's instructions, to dilute to a concentration of 100 pmol, and then diluted, from 100 pmol to 10 pmol.

**Table 2-7 Primers sequences used for cloning into PTRE-3G vector and their expected size.**

| Primer name | Primer sequence | Tm (°C) | Product size (bp) |
|---|---|---|---|
| NHA-*Nde*I F<br>NHA-*Pst*I R | 5'-GACCATATGACTATAGGGCGAATTAATACGA-3'<br>5'-CTACTGCAGGATATCGGATCCCTCGAG-3' | 53 | 1300 |
| C-Myc -*Pst*I F<br>C-Myc -*Bam*HI R | 5'-GTACTGCAGATGGCCTTTGCACCTATGGG -3'<br>5'-CTCGGATCCTCATAAGTTAACCAGGTCC-3' | 55 | 1341 |

### 2.11.2 Purification and DNA digestion

The PCR reactions were carried out in a total volume of 50 µl, by using MyTaqTM HS Red Max (Bioline; MTHRX-41408). Afterwards, the PCR products were run on 1% agarose gel after mixing it with blue loading dye (6x) (NEB; B7021S), and specific bands were extracted. The purification took place using Nucleospin Gel and PCR clean-up (MACHEREY-NAGEL;740609-250) following the manufacturer's protocol. The purified PCR product (≈25 µl) was digested with 1 µl of restriction enzymes, 5 µl of cutsmart TM Buffer (Bioline; B7204S) buffer, and 18 µl of $ddH_2O$ in a total volume of 50 µl. Incubating the samples at 37°C for a period of 2 hours was taken into account, and then run on a 1% agarose gel. After digestion,

specific bands were selected and purified with Nucleospin Gel and PCR clean-up. In terms of PTRE-3G vector, dephosphorylating step took place, after the cleaning-up step, by adding 2 µl of shrimp Alkaline phosphatase (SAP) (Bioline; M0371L), 5 µl of cut smart buffer, and 17 µl of $ddH_2O$ in a total volume of 50 µl. The mixture was incubated at 37°C for 30 minutes, and then heat inactivation step at 56°C for 5 minutes took place.

**Table 2-8 Restriction enzymes used for cloning.**

| Restriction enzyme | Enzymes buffer | Recognition site | Cat. No. | Source |
|---|---|---|---|---|
| *Nde*I | Cutsmart buffer | 5'…CA▼TATG…3'<br>3'…GTAT▲AC…5' | R0111S | Bioline |
| *Pst*I | Cutsmart buffer | 5'…CTGCA▼G…3'<br>3'…G▲ACGTC…5' | R0410S | Bioline |
| *Bam*Hl | Buffer 3 | 5'…G▼GATCC…3'<br>3'…CCTAG▲G…5' | R602A | Promega |

### 2.11.3 Ligation and transformation

The H-HA: SPO11 and C-Myc: SPO11 were ligated into the PTRE-3G plasmid. The insert DNA and plasmid concentration needed to be measured by using NanoDrop (ND_1000). Therefore, the molar ratio of vector to insert to optimise the ligation was determined using in-silico calculator (http://www.insilico.uni-duesseldorf.de/Lig_Input.html). A 50 ng of vector was mixed with determined molar ratio of the insert, 1 µl of 10x ligase buffer (Promega; C126B), 1 µl of T4 DNA ligase (Promega; M180A), and the volume of the DNA ligation mix was adjusted to 20 µl with sterile water (Sigma; W4502). The ligation mixtures were gently mixed and briefly centrifuged and then incubated overnight at 4°C. In terms of transformation, a tube of NEB 10-beta competent *E. coli* (BioLabs; C3019H) was placed on ice for 10 minutes to thaw. After that, the procedures according to manufacturer's instructions were followed. Several 10-fold serial dilutions of transformation reaction were performed and then spread out onto LB agar Petri dishes containing 100 mg/ml ampicillin (Sigma; A9518) and then incubated at 37°C overnight. The LB (Luria Bertani) and LB agar media components were listed in Table 2.9.

**Table 2-9 Preparation of LB and LB agar media for *E. coli* culture.**

| | LB medium (1 L) | | | LB agar medium (1L) | | | |
|---|---|---|---|---|---|---|---|
| **Reagent** | Bacto®-Tryptone 211705 | Bacto®-Yeast Extract; 212750 | NaCl | Bacto®-Tryptone | Bacto®-Yeast Extract | NaCl | Bacto®-Agar; 214030 |
| **Volume** | 10 g/L | 5 g/L | 10 g/L | 10 g/L | 5 g/L | 10 g/L | 15 g/L |

### 2.11.4 Colony screening

A few colonies were randomly picked from the overnight plates after transformation by using a micropipette tip and rinsing it into 20 µl of sterile water. The PCR reaction was performed using internal primers as seen in Table 3.6, for SPO11 in order to determine the presence of SPO11 in the selected colonies. PCR reaction containing 5 µl of sterile water/colony mixture. 12.5 µl of 2x MyTaq redMax, 0.5 µl of each primer and 6 µl of distilled water. The SPO11 primers that were used detailed in Table 3.6. In case of the positive PCR colonies, the remaining water/colony mixtures were plated into 5 ml of LB liquid medium with 100 mg/ml ampicillin, and incubated overnight at 37°C.

### 2.11.5 Plasmid isolation from *E. coli*

5 ml of *E. coli* overnight culture was used to extract the plasmids via a QIAprep Spin Miniprep Kit (250) (Qiagen; 27106), according to the manufacturer's protocol. A 3 µg of each purified plasmid was digested with appropriate restriction enzymes to confirm the cloning. Afterwards, the correct cloned genes were sequenced to confirm the correct orientation as well as to detect any gene mutations.

### 2.11.6 Sequencing PCR products

1500 ng/µl of DNA samples were used for sequencing and mixed in a ready labelled tube with 2 µl of 10 pmol of forward or reverse primer and then adjusted to 17 µl with sterile water. This plasmid was sequenced with PTRE-3G vector forward and reverse primers (Table 2.5) to confirm that the SPO11 with tags was cloned in the correct orientation, with no mutations. These tubes were sent at room temperature to Eurofins MWG for DNA sequencing. The sequencing results were blasted and aligned against corresponding genes using the Basic Local Alignment Search Tool (BLAST) website https://blast.ncbi.nlm.nih.gov/Blast.cgi.

## 2.12 Establishment of a double Tet-On 3G stable cell line

### 2.12.1 Puromycin selection (Kill curve)

HeLa Tet-On 3G cell line and HCT116 cell line were seeded into 6 well plates using the appropriate medium (Table 2.1) with 100 µg/ml G418. When the cells become confluent, seven doses of puromycin were added to the plate in order to optimise the minimum dose of puromycin required to kill all cells after 3-5 days. As a result, in the case of the Hela cells, 0.8 µg/ml was the lowest optimum dose chosen for single colony selection whereas 2.0 µg/ml was chosen for HCT116.

### 2.12.2 Generation of double stable HeLa / HCT116 Tet-On 3G stable cell lines

Double stable HeLa/HCT116 Tet-On 3G cell lines with *N-HA::SPO11* and *C-Myc::SPO11* were created by growing a HeLa / HCT116 Tet-On 3G cell lines in 6 well until they become almost confluent. Following that, the transfection procedure, using Xfect transfection reagent (Clontech; PT5003-2) according to the manufacturer's protocols, took place. Consequently, 2.5 µg and 5 µg of recombinant vector pTRE-3G containing N-HA::SPO11 and C-Myc::SPO11 were added to each well, along with 100 ng of a linear puromycin selection marker (20:1 ratio) was added to each well. Transfection with only the pTRE3G vector without any cloning was taken into account. After a period of a 48 hours, cells were split into 4 x 10 cm dishes and grown for a further 48 hours with fresh media with appropriate concentration of puromycin (Hela = 0.8 µg/ml, HCT116 = 2 µg/ml) and 100 µg/ml Geneticin (G418) and the media. Puromycin and G418 were refreshed every 4 days. 6-8 days later, single healthy colony was transferred into 24 well plates. The cells were refreshed with a suitable concentration of selective antibiotic (puromycin and G418). When the cells became confluent, they were split into 3 wells of 6 well plates, and then into T75 flasks. Some cells were used for screening, while the rest of them were frozen in liquid nitrogen for long-term storage.

### 2.12.3 Screening of a double stable HeLa / HCT116 Tet-On 3G stable cell lines

Doxycycline (Sigma; D9891-5G) was used to promote gene induction for each colony. Each colony was seeded into 10 cm dishes and 1 μg/ml doxycycline was added to positive plates whereas the negative plates were cultured in the absence of doxycycline. All plates were incubated for 48 hours at 37°C in 5% $CO_2$. The proteins were then extracted to perform western blot, in order to determine the induction and N-HA and C-Myc tags cloning.

### 2.12.4 Sequencing PCR products

Samples of 1500 ng/μl of DNA were used for sequencing and mixed in ready labelled tubes with 2 μl of 10 pmol of forward or reverse primer and then adjusted to 17 μl with sterile water. This plasmid was sequenced with pTRE-3G vector forward and reverse primers (Table 2.6) to confirm that the *SPO11* cDNA with tags was cloned in the correct orientation, with no mutations. These tubes were sent at room temperature to Eurofins MWG for DNA sequencing. The sequencing results were blasted and aligned against corresponding genes using the Basic Local Alignment Search Tool (BLAST) website https://blast.ncbi.nlm.nih.gov/Blast.cgi.

# Chapter 3

## Influence of SPO11 knockdown on cell proliferation

# 3. Influence of SPO11 knockdown on cell proliferation

## 3.1 Introduction

A fundamental property of CTA genes is their restricted expression in the human germ line and in malignant cells, whereas they may not be expressed or may be expressed at an extremely low levels in healthy somatic cells, except when they are subject to carcinogenesis (Cheng et al., 2011; Hofmann et al., 2008; Simpson et al., 2005). In testes, different stages of spermatogenesis can be identified using CTAs. For example, SPO11 can be found in the early meiotic phase, MAGE-A or NY-ESO-1 is detected in spermatogonia with mitotic clonal prevalence and ADAM2 is active at the end of meiosis in haploid cells (Simpson et al., 2005).

Although the available data suggests that CT genes expression might play a role in tumourigenesis, the biological function of many of these proteins has yet to be fully understood. The role of the MAGE protein family has been given more attention than that of other CTA proteins. The involvement of MAGE proteins in cell survival, in increasing tumourigenic properties and in malignancy progression have all been reported (Bai et al., 2005; Doyle et al., 2010). Furthermore, in the melanoma cell line, the depletion of some CTAs (for instance, SSX4, XAGE1 and GAGE) causes tumour cell invasion and a cell viability decline following siRNA depletion (Caballero et al., 2013). The effectiveness of XAGE1 knockdown was found to be consistent in different tumours, including lung adenocarcinoma, prostate cancer and melanoma cell lines (Caballero et al., 2013). *TSP50* is a CT gene that is thought to be an oncogene and is highly expressed in breast cancer (Xu et al., 2007; Zhou et al., 2010). Zhou et al. (2010) demonstrated that reducing the TSP50 level by 70% in murine embryonal carcinoma stem cells using a shRNA technique had a negative effect on cell growth and migration, resulting in apoptosis.

CTAs can be detected in almost all types of cancers, including melanoma and lung cancer cell lines. Therefore, CTAs may serve as a potential cancer biomarker for use in cancer immunotherapy, vaccination and drug targeting (Simpson et al., 2005; van et al., 2011). For example, in breast cancer cells, metastatic events can be promoted through particular members of CTA45 and CTA45A1 families. Therefore, tumours developed to the metastatic stage can be predicted by detecting CTAs (Shang et al., 2014).

The transcripts of human *SPO11* and mouse *Spo11* genes can be detected at high levels only in normal testes by northern blotting but not in other somatic tissues (Shannon et al., 1999). Koslowski et al. (2002) identified the *SPO11* gene as a CTA gene using a combination of northern blotting and RT-PCR with NCBI GenBank mining in somatic and cancer tissues. The *SPO11* gene was not detected in a panel of multiple normal non-testicular tissues, including liver, lung and ovary tissues, whereas the expression of *SPO11* was found in testes and in five different tumour samples, including 1 melanoma cell line, 2 cervical cancer cell lines and 2 lung cancer cell lines, of the total 119 tumours and cancer samples tested (Koslowski et al., 2002). Furthermore, Romanienko et al. (1999) showed that, human *SPO11* and mouse *Spo11* genes are not expressed in normal tissues, except thymus tissues in mice, while, in human carcinoma cell lines, *SPO11* is expressed in prostate, colon and ovary cells (Romanienko et al., 1999). In addition, the production of the human SPO11 protein in 17 different cancer cell lines was differentiated, but none found in 14 different normal tissues using anti-SPO11 antibody (Abnova) (I. Aldeailej, PhD thesis, Bangor University).

In mitotic cells, the potential function of CTA genes in cancer progression can be determined by several methods, such as a proliferation dynamics, an extreme limiting dilution assay (ELDA) or by measuring cell viability (Molina et al., 2004; Assanga & Lujan, 2013). Proliferation curves allow for the evaluation of the effectiveness of disturbing CTA genes, drugs or other agents on cancer cells, stimulating, inhibiting and/or affecting cell growth and viability (Assanga & Lujan, 2013). In addition, ELDA assays detect the responses of cellular self-renewal to any change in certain conditions, such as a reduction in the gene expression level, compared with positive or negative controls (Hu & Smyth, 2009; Taswell, 1987).

We hypothesis that the *SPO11* gene may be necessary to drive or to maintain the oncogenic processes. In this chapter, the SPO11 protein level was investigated in different normal, cancer and tumour tissues in order to confirm SPO11 as CTA. Furthermore, since *SPO11* was identified as a CTA gene, this chapter will also address the question of whether *SPO11* is required for cancer cell proliferation using different cancer cell lines.

## 3.2 Results

### 3.2.1 SPO11 presence in normal human tissues, human cancer cells and human tumour tissues

To detect the presence of the SPO11 protein in normal tissues, cancer cells and tumour tissues, western blotting was used. The normal tissue protein extract were provided by two companies: Abcam and Novus.

The anti-SPO11 antibody (ab81695) was used in this study and has been validated by SPO11 depletion (see Chapter 4), production of SPO11 in *E. coli* and immunoprecipitations / mass spectrometry, which has been performed by one of our group members. The SPO11 protein is only detected in the testes, not in other normal tissues, except two very faint bands in the thymus, one at the exacted size and the other has lower size (Figure 3.1). The SPO11 protein level was assessed using seven human cancer cell lines; testis tissues were used as a positive control. All the cancer cells used in this study showed a band of the SPO11 protein at relatively high levels (Figure 3.2 A). In addition, a SPO11 band was detected in the tumour tissues (liver, ovary and lung) used in the western blot (Figure 3.2 B).

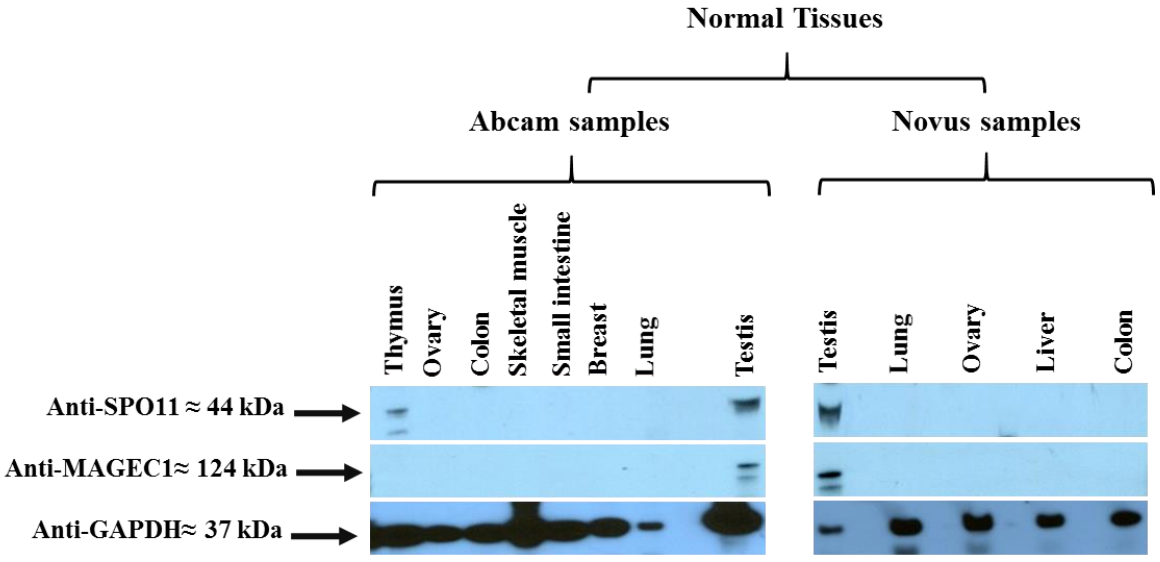



**Figure 3-1 Western blot analysis to detect SPO11 protein levels in diiferent normal tissue lysates.**
SPO11 is produced in normal testis tissues and in a very faint band in thymus tissues and was detected by the rabbit polyclonal anti-SPO11 antibody (Abcam, #ab81695). Anti-GAPDH was used as a control, whereas anti-MAGE-C1 was used as a CTA control. The samples were provided by Abcam and Novus companies.

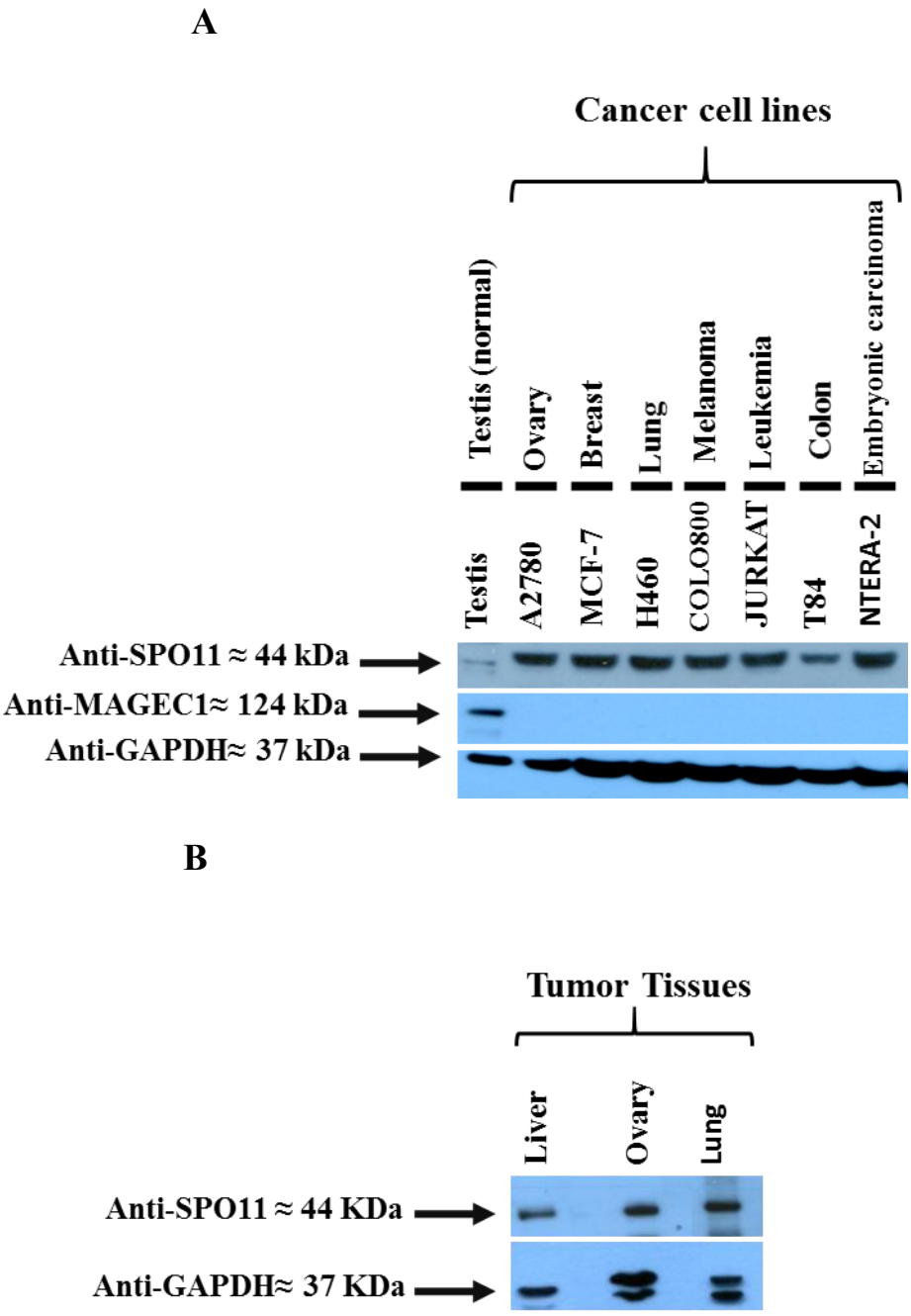



**Figure 3-2 Western blot analysis to detect SPO11 protein levels in different cancer cell line lysates and tumour tissues.**
GAPDH was used as a loading control, whereas MAGE-C1 was used as a CTA control. (A) SPO11 was present in all the tested cancer cell lines and was detected by the rabbit polyclonal SPO11 antibody (Abcam, #ab81695). (B) Positive signals were seen in all the tumour tissues included in the study.

### 3.2.2 SPO11 controls the mitotic proliferative potential of cancer cells

To address whether SPO11 might function in a non-meiotic context in cancer cells, we employed siRNA to deplete *SPO11* mRNA. Two independent *SPO11* siRNAs were used: siRNA 2 and siRNA 4. Four different cancer cell lines SW480, Hela, A2780 and HCT116 were cultured to measure cell viability. The SW480 and Hela cells were treated with both siRNAs, whereas the A2780 and HCT116 cells were only treated with siRNA 2. The SW480 and Hela cells were grown for 8 days with the treatment of siRNA 2 and 4, as shown in Figure 3.3 A and Figure 3.4 A, respectively. However, the A2780 and HCT116 cells were seeded for 8 and 4 days as shown in Figure 3.5 A and Figure 3.6 A, respectively. The cells were seeded in 6-well plates with different cell concentrations depending on the cell lines. After 24 hours, the cells were transfected every day for 4–8 days with non-interfering siRNA as negative controls as well as *SPO11* siRNA 2 and 4. The cells were then harvested every day and counted using trypan blue, which stains the membranes of dead cells with a blue dye. The cells were counted using either a haemocytometer or an automatic cell counter under an optical microscope. As a result, the cell count curves represent the total number of cells and show that the number of surviving cells transfected with siRNA 2 and 4 was much lower than the number of surviving cells in the untreated and non-interfering conditions, especially in the last two days of treatment.

The images were taken for all cell lines just before harvesting the cells. Images of the SW480 and Hela cells (Figure 3.3 B and Figure 3.4 B, respectively) were taken after day 8, as the number of cells decreased significantly. Images of the A2780 cells were taken on day 8 (Figure 3.5 B), whereas images of the HCT116 cells were taken on day 4 (Figure 3.6 B). The cell numbers for all the cells was decreased due to SPO11 siRNA 2 and 4 knockdown compared with the negative control.

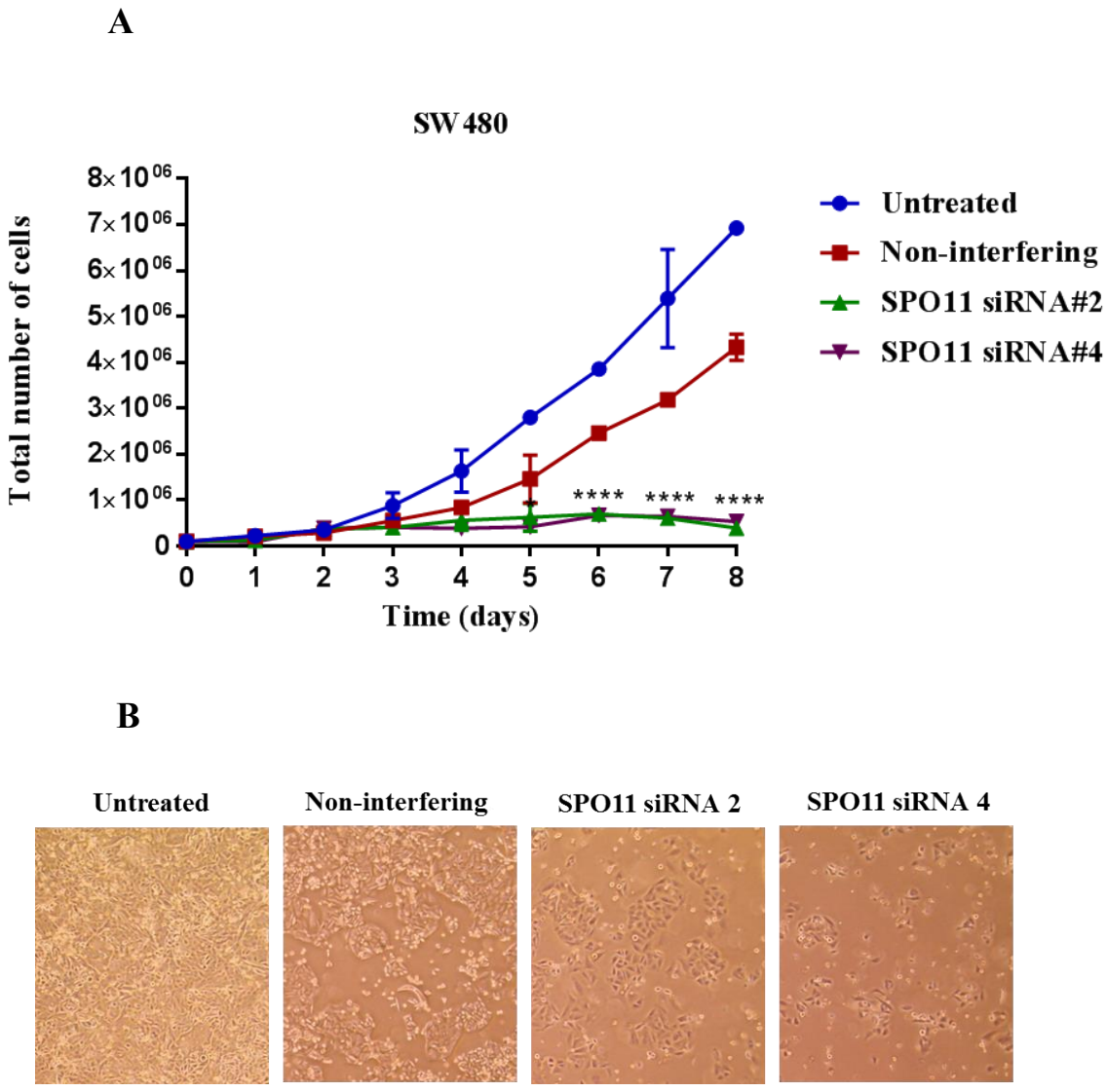



**Figure 3-3 SPO11 is required for the proliferation of SW480.**
(A) siRNA depletion of *SPO11* mRNA inhibits SW480 cells proliferation. Untreated SW480 cells were utilised as positive controls and cells treated with non-interfering siRNA were used as negative controls for SPO11 knockdown. The error bars represent the standard error for the total number of cells, as calculated for three repeats (****P < 0.0001). (B) Cell images taken before trypsinisation to assess the cell density on day 8 using an ECLIPSE-inverted microscope (5 X lens).

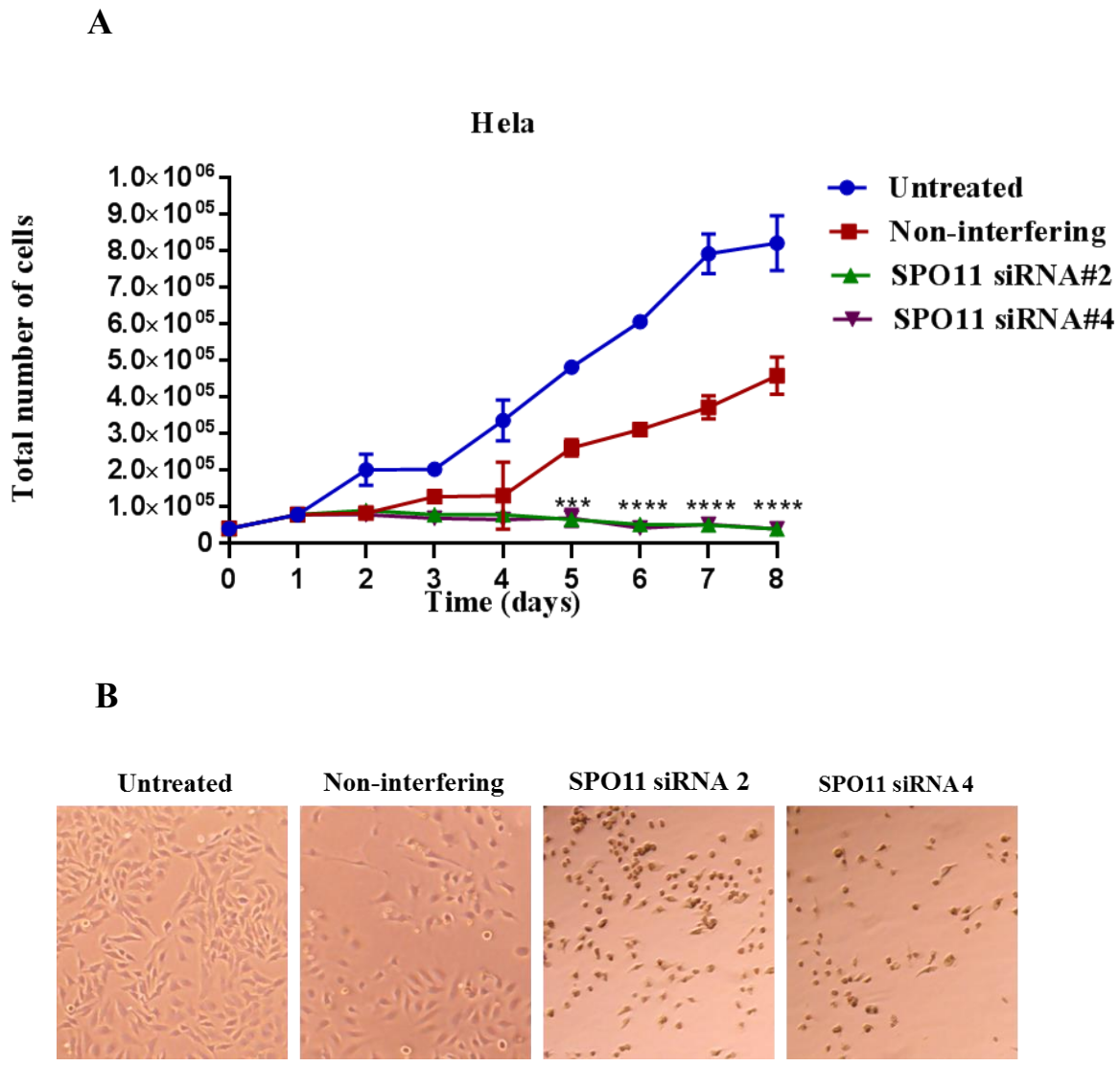



**Figure 3-4 SPO11 is required for the proliferation of Hela cells.**
(A) siRNA depletion of *SPO11* mRNA inhibits Hela cell proliferation. Untreated Hela cells were utilised as a positive control, whereas cells treated with non-interfering siRNA was used as a negative control, for SPO11 knockdown. The error bars represent the standard error for the total number of cells, as calculated for three repeats (***P < 0.001, ****P < 0.0001). (B) Cell images taken before trypsinisation to assess the cell density on day 8 using an ECLIPSE-inverted microscope (5 X lens).

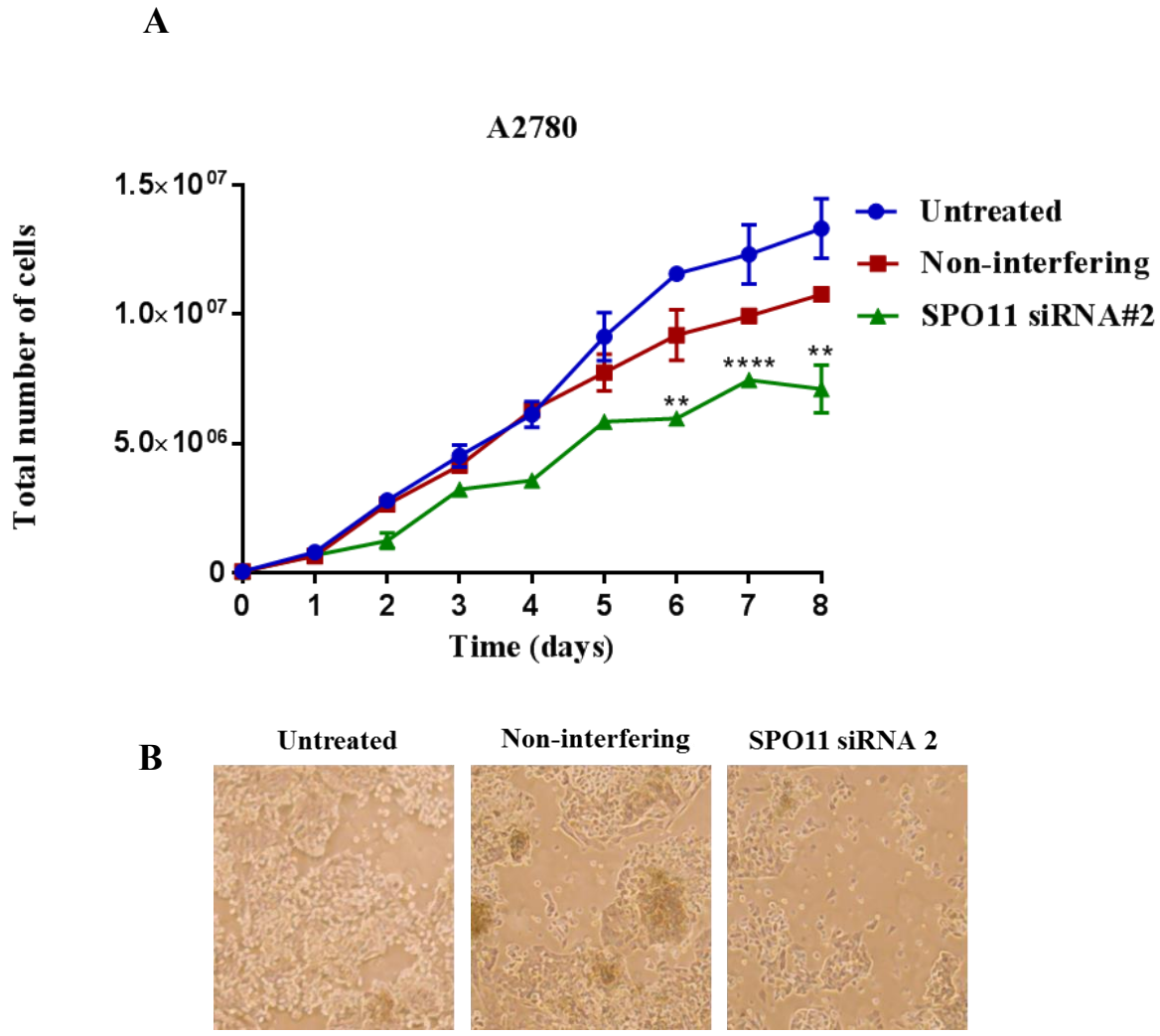



**Figure 3-5 SPO11 required for the proliferation of A2780.**
(A) siRNA depletion of SPO11 mRNA inhibits A2780 cell proliferation. Untreated A2780 cells were utilised as a positive control, whereas cells treated with non-interfering siRNA were used as a negative control, for SPO11 knockdown. The error bars represent the standard error for the total number of cells, as calculated for three repeats (**P < 0.01, ****P < 0.0001). (B) Cell images taken before trypsinisation to assess the cell density on day 8 using an ECLIPSE-inverted microscope (5 X lens).

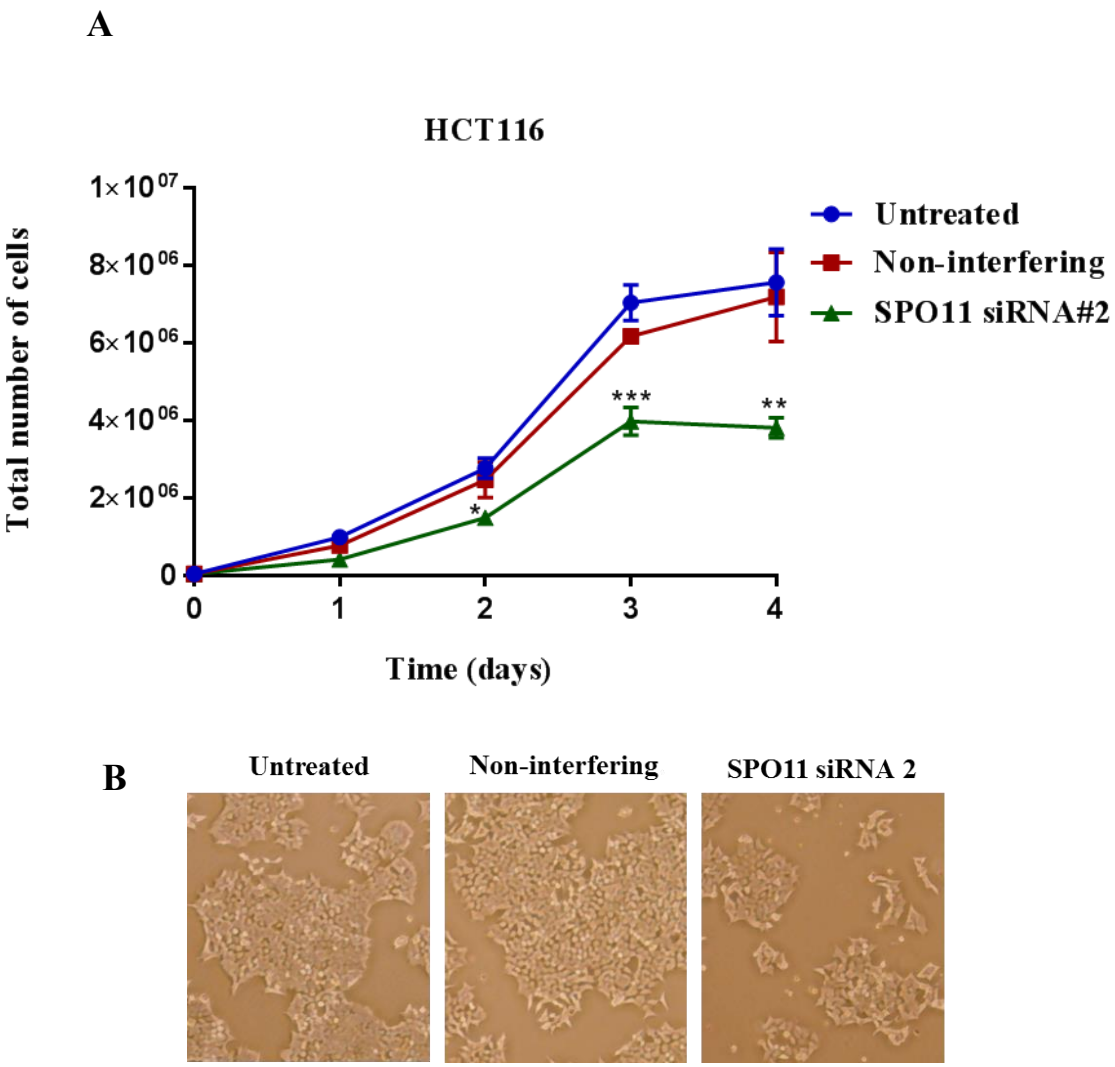



**Figure 3-6 SPO11 is required for the proliferation of HCT116 cells.**
(A) siRNA depletion of SPO11 mRNA inhibits HCT116 cell proliferation. Untreated HCT116 cells were utilised as a positive control, whereas cells treated with non-interfering siRNA were used as a negative control, for SPO11 knockdown. The error bars represent the standard error for the total number of cells, as calculated for three repeats (*P < 0.05, **P < 0.0079, ***P < 0.0005). (B) Cell images taken before trypsinisation to assess the cell density on day 4 using an ECLIPSE-inverted microscope (5 X lens).

### 3.2.3 Self-renewal potential of SW480 and HCT116 cancer cell lines transfected with SPO11 siRNA 2 and 4

The cell count curves demonstrate that SPO11 knockdown affects cell proliferation. Therefore, an ELDA assay was used to evaluate cancer cell self-renewal after treatment with SPO11 siRNA 2 and 4. An ELDA assay was conducted to determine the ability of cancer cells to self-renew from single cells in the presence or absence of SPO11.

Two parental colon cancer cell lines, SW480 and HCT116 cells, were cultured to quantify the proportion of cancer cells. The cells were seeded in serial dilutions of 10, 100 and 1000 cells per well using 96-well plates. After allowing the cells to attach to the plates (24 hours), the cells treated with non-interfering and the HiPerFect Transfection Reagent were used as a negative control for SPO11 siRNA 2 and 4. Moreover, cells images were obtained after 10 days using a light microscope to determine any changes in cell proliferation self-renewal.

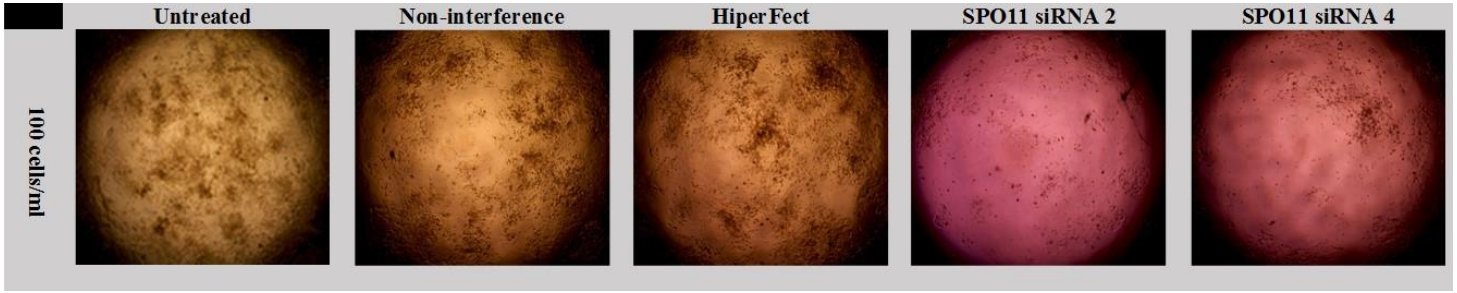
The renewal of SW480 cells treated with siRNA 2 and 4 were significantly affected compared with the negative control (Figure 3.7 A, B and C). When comparing untreated SW480 cells to non-interfering or HiPerFect treated cells, no significant difference was observed ($P > 0.05$) in the self-renewal frequency. Conversely, highly significant differences were noted between the non-interfering and the SPO11 siRNA-transfected cells ($P < 0.05$) (Figure 3.8 A and B).

The number of HCT116 cells that survived after transfection with siRNA 2 and 4 was much lower compared with those of the untreated or negative controls (Figure 3.9 A, B and C). As in the SW480 cells, there were no significant differences ($P > 0.05$) when comparing the parental cells formation frequency between the untreated cells (positive controls) and the non-interfering or HiPerFect treated cells (negative controls). However, when comparing HCT116 cells treated with siRNA 2 and 4, highly significant differences were found ($P < 0.05$) (Figure 3.10 A and B).

A

| Number of cells per well | Number of wells plated | Number of wells showing Parental cells (SW480) | | | | |
|---|---|---|---|---|---|---|
| | | Untreated cells | Negative control siRNA transfected cells | Hiperfect-only treated cells | siRNA (2) transfected cells | siRNA (4) transfected cells |
| **Number of well showing positive growth of SW480 cells after 10 days of culture** | | | | | | |
| 1000 | 12 | 12 | 12 | 12 | 12 | 12 |
| 100 | 12 | 12 | 10 | 10 | 4 | 6 |
| 10 | 12 | 12 | 9 | 9 | 1 | 5 |
| Parental cells forming frequency (95% CI) | | 1/3 (1/7-1/1) | 1/3(1/8-1/2) | 1/9 (1/19-1/4) | 1/202 (1/419-1/97) | 1/27 (1/55-1/14) |
| P value | 3.18e-05 | | | | | |

B




C

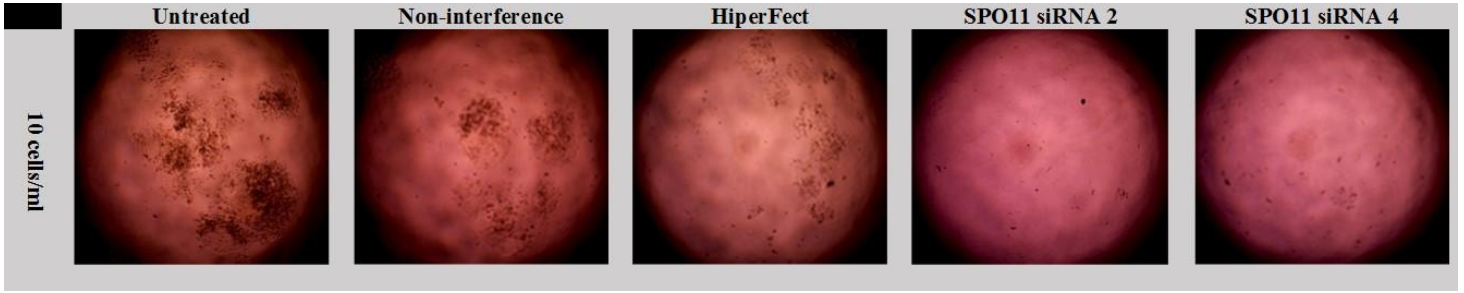
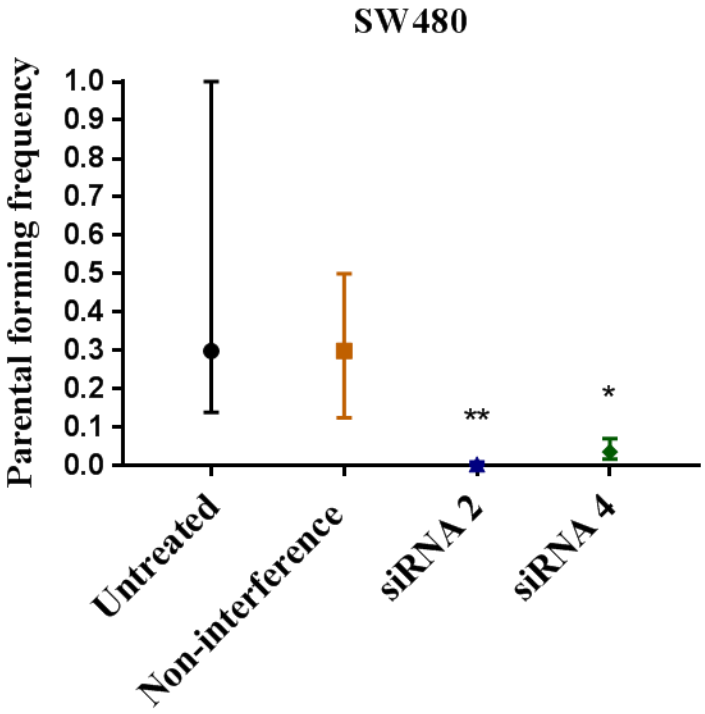



**Figure 3-7 Effect of SPO11 depletion on SW480 cell self-renewal as determined by an extreme limiting dilution analysis.**
(A) ELDA assay showing the influence of SPO11 knockdown on the self-renewal of SW480 cells. (B, C) renewal of SW480 cells transfected with siRNA 2 and 4 for 10 days with 100 and 10 cells/ml seeded, respectively. Non-interferening and HiPerFect treated cells served as negative controls, whereas untreated cells were used as a positive control. Knocking down SPO11 with siRNA 2 and 4 was found to affect SW480 cell self-renewal.

**A**




**B**

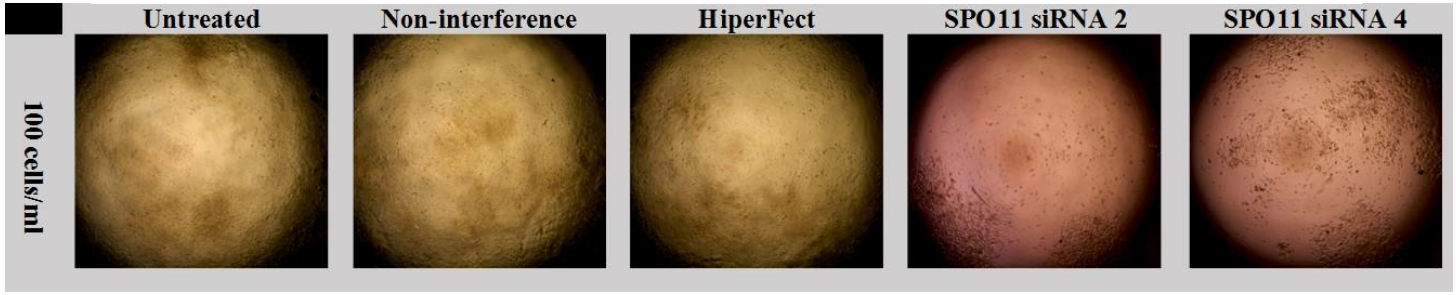
| SW480 cells / SPO11 siRNA 2 and 4 | | | | |
|---|---|---|---|---|
| **Group1** | **Group 2** | **Chisq** | **DF** | **Pr(>Chisq)** |
| Non-interference | Untreated | 0.0687 | 1 | 0.793 |
| Non-interference | SPO11 siRNA2 | 35 | 1 | 3.30-e09 |
| Non-interference | SPO11 siRNA4 | 15.5 | 1 | 8.35e-05 |
| Untreated | SPO11 siRNA2 | 62.6 | 1 | 2.52e-15 |
| Untreated | SPO11 siRNA4 | 47.3 | 1 | 6.09e-012 |
| SPO11 siRNA2 | SPO11 siRNA4 | 8.61 | 1 | 0.00333 |

**Figure 3-8 Effect of SPO11knockdown on SW480 cell self-renewal as determined by an extreme limiting dilution analysis.**
(A) Influence of siRNA 2 and 4 knockdown on SW480 cells compared to non-interfering cells, which were used as a negative control. (B) Comparison of cells transfected with SPO11 siRNA 2 and 4 to untreated and non-interfering. Chisq indicates Chi-Square. DF refers to Degrees of Freedom. Pr(>Chisq) points probability value and T test was used. Pr(>Chisq) < 0.05 means the differences are significant. The results reveal that transfected SW480 cells with siRNA 2 and 4 result in significant affect of self-renewal.

**A**

| Number of cells per well | Number of wells plated | Number of wells showing Parental cells (HCT116) | | | | |
|---|---|---|---|---|---|---|
| | | Untreated cells | Negative control siRNA transfected cells | Hiperfect-only treated cells | siRNA (2) transfected cells | siRNA (4) transfected cells |
| **Number of well showing positive growth of HCT116 cells after 10 days of culture** | | | | | | |
| 1000 | 12 | 12 | 12 | 12 | 11 | 12 |
| 100 | 12 | 11 | 11 | 11 | 7 | 8 |
| 10 | 12 | 12 | 11 | 11 | 5 | 6 |
| Parental cells forming frequency (95% CI) | | 1/13(1/38-1/6) | 1/14(1/32-1/7) | 1/14(1/32 -1/7) | 1/157(1/331-1/75) | 1/155(1/105-1/29) |
| P value | 4.15e-12 | | | | | |

**B**




**C**

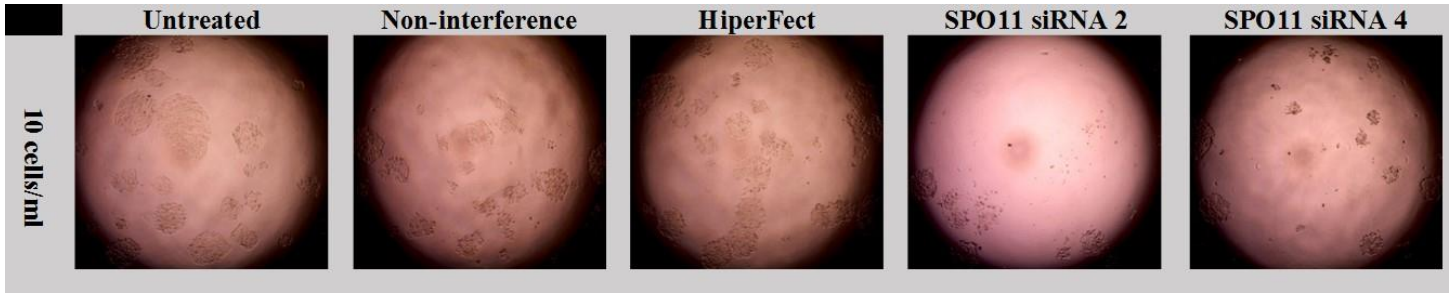
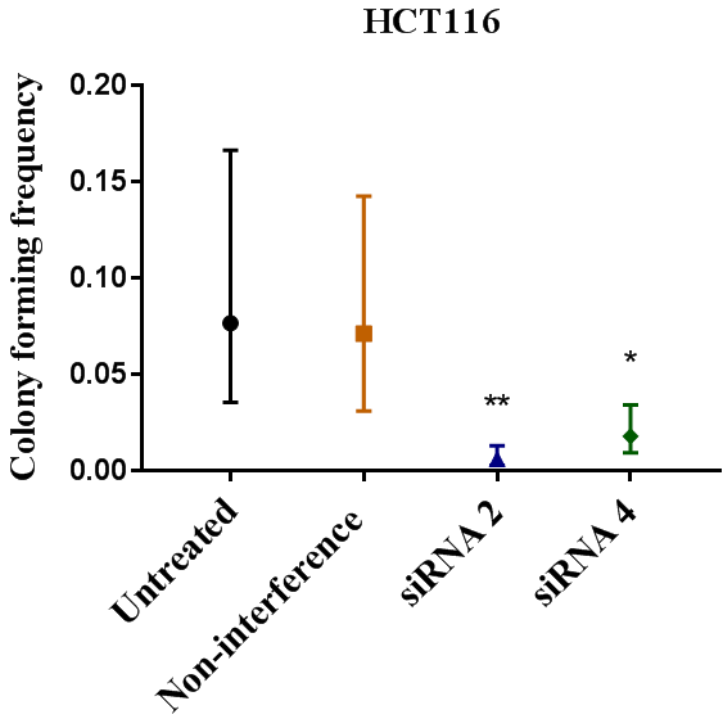



**Figure 3-9 Effect of SPO11 depletion on HCT116 cell self-renewal as determined by an extreme limiting dilution analysis.**
(A) ELDA assay showing the influence of SPO11 knockdown on the proliferation/ self-renewal (colony formaing ability) of HCT116 cells. (B, C) Growth of HCT. cells transfected with siRNA 2 and 4 for 10 days with 100, 10 cells/ml seeded respectively. Non-interfering and HiPerFect transection served as negative control whereas untreated cells used as positive control. The growth of cells transfected with SPO11 siRNA 2 and 4 is significantly affected compared with the growth of untreated and non-interfering cells.

A




B

| HCT116 cells / SPO11 siRNA 2 and 4 | | | | |
|---|---|---|---|---|
| **Group1** | **Group 2** | **Chisq** | **DF** | **Pr(>Chisq)** |
| Non-interference | Untreated | 0.0885 | 1 | 0.766 |
| Non-interference | SPO11 siRNA2 | 33.4 | 1 | 7.46e-09 |
| Non-interference | SPO11 siRNA4 | 10.4 | 1 | 0.00124 |
| Untreated | SPO11 siRNA2 | 37.4 | 1 | 9.7e-10 |
| Untreated | SPO11 siRNA4 | 12.6 | 1 | 0.000379 |
| SPO11 siRNA2 | SPO11 siRNA4 | 6.54 | 1 | 0.0105 |

**Figure 3-10 Effect of SPO11 on HCT116 cells as determined by an extreme limiting dilution analysis.**
(A) Influence of siRNA 2 and 4 knockdown on HCT116 cells compared to non-interfering cells, which were used as a negative control. (B) Comparison of cells transfected with SPO11 siRNA 2 and 4 to untreated and non-interfering. Chisq indicates Chi-Square. DF refers to Degrees of Freedom. Pr(>Chisq) points probability value and T test was used. Pr(>Chisq) < 0.05 means the differences are significant. The results reveal that transfected HCT116 cells with siRNA 2 and 4 result in significant affecte of self-renewal.

### 3.2.4 Cell viability assays

In this study, three cell viability assays were used following SPO11 depletion: the cell viability count analysis, the RealTime-Glo$^{TM}$ MT Cell Viability Assay and the CellTiter 96 AQueous One Solution Assay. First, trypan blue stain was used to assess cell viability by calculating live cells with and without SPO11 treatment. Second, absorbance luminescence was used to determine the number of viable cells in the culture by measuring the reduction potential of cells when the RealTime-GloTM MT Cell Viability Assay was used. Finally, when the CellTiter 96 AQueous One Solution Assay was used, absorbance at 490 nm was recorded to determine the number of viable cells in the proliferation assay.

Three hits of SPO11 siRNA 2 and 4 were carried out before counting or stimulating the cells for the cell viability assays; three cancer cell lines (132N1, MCF7 and SW480) were used. In addition, 6-well plates were utilised for the cell viability count technique, while 96-well plates were used for the other two cell viability assays. The 132N1 and MCF7 cell viability counts (Figure 3.11 A and Figure 3.12 A, respectively) indicated a significant reduction in the viable cells treated with SPO11 siRNA 2. In addition, the P value indicates that SPO11 siRNA knockdown produced a significant reduction in the number of cells compared to the non-interfering treated cells. The results showed no significant reduction in the number of cells between the non-interfering treated cells and the untreated cells. Further, the confluence of the cells transfected with SPO11 siRNA 2 and 4 was much lower than that of the non-interfering and untreated cells (Figure 3.11 B and Figure 3.12 B, respectively).

The survival of the SW480 cells used for the RealTime-Glo$^{TM}$ MT Cell Viability Assay and the CellTiter 96 AQueous One Solution Assay significantly decreased after SPO11 knockdown using siRNA 2 and 4. In these assays, untreated and non-interfering cells were used as controls. The cell survival of the SW480 cells after SPO11 siRNA knockdown is shown in Figure 3.13 A, B, C and D.

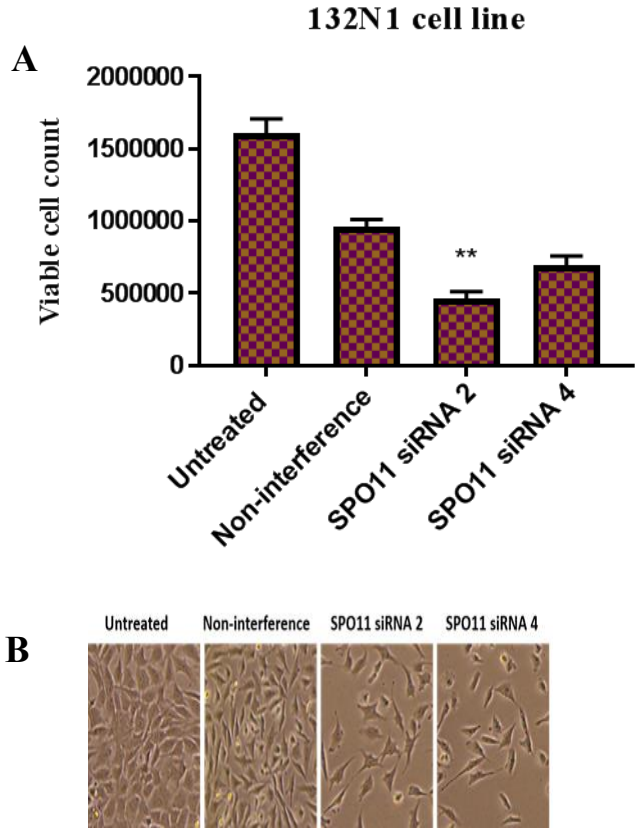



**Figure 3-11 132N1 cell viability after knocking down SPO11 with siRNA 2 and 4.**
(A) Cell viability of 132N1 cells subjected to three siRNA 2 and 4 hits. Cells treated with non-interfering siRNA were used as a negative control for SPO11 siRNA knockdown, whereas untreated cells were used as a positive control. The error bars represent the standard error for the total number of cells, as calculated for three repeats .(**P < 0.01). (B) Cell images taken before trypsinisation to assess the cell density. BioRad's TC20 Automated Cell Counter was used with trypan blue. There was a clear reduction in the number of transfected cells when compared to the negative and positive controls.

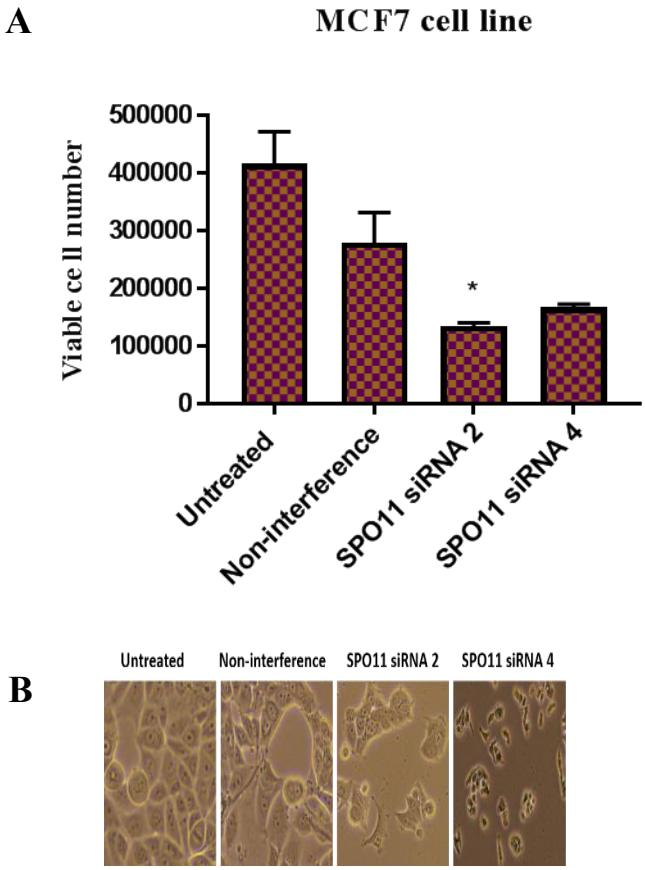



**Figure 3-12 MCF7 cell viability after knocking down SPO11 with siRNA 2 and 4.**
(A) Cell viability of MCF7 cells subjected to three siRNA 2 and 4 hits. Cells treated with non-interfering siRNA were used as a negative control for SPO11 siRNA knockdown, whereas untreated cells were used as a positive control. The error bars represent the standard error for the total number of cells, as calculated for three repeats (*$P < 0.05$). (B) Cell images taken before trypsinisation to assess the cell density. BioRad's TC20 Automated Cell Counter was used with trypan blue. There was a clear reduction in the number of transfected cells when compared to the negative and positive controls.

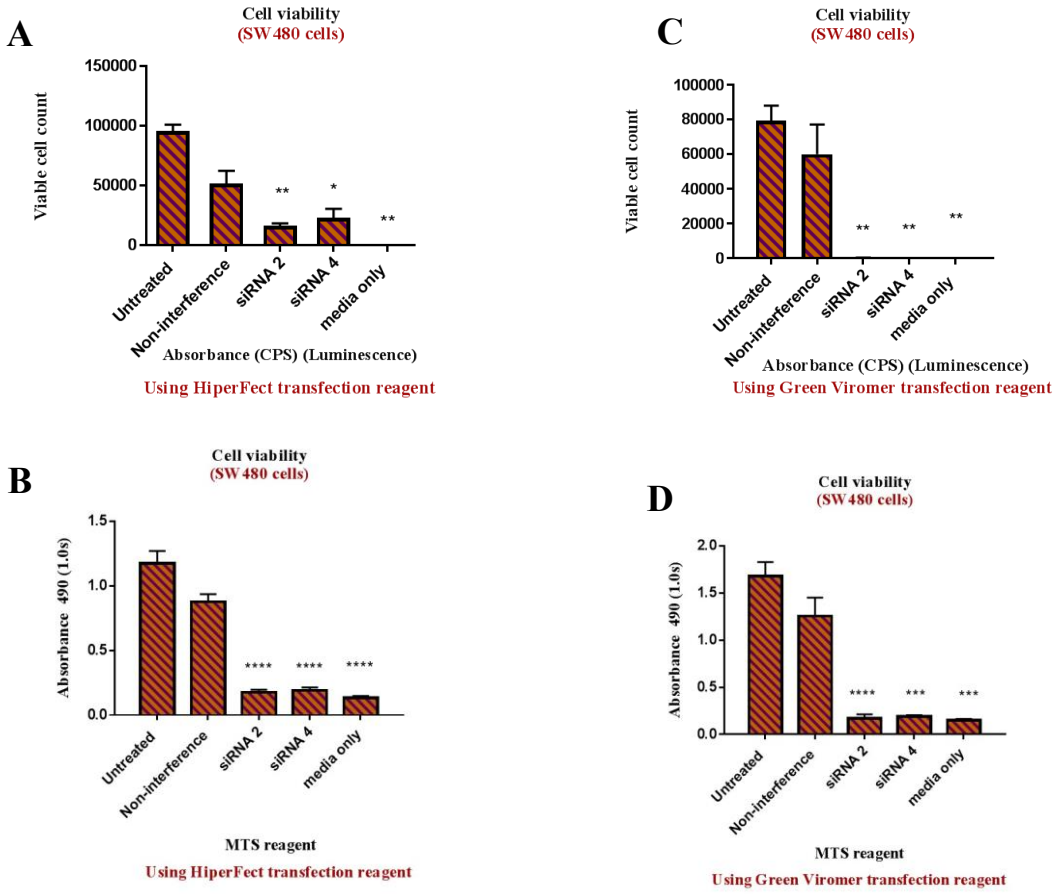



**Figure 3-13 SW480 cell viability after knocking down SPO11 with siRNA 2 and 4.**
Cells treated with non-interfering siRNA were used as a negative control for SPO11 siRNA knockdown, and untreated cells were utilised as a positive control, whereas media only (no cells) was utilised as a treatment control. The graphs show the cell viability of the SW480 cells subjected to three siRNA 2 and 4 hits using (A) HiPerFect, (B) Viromer Green or (C) a transfection reagent, and a 96-well plate was used. In panels (A) and (C), the RealTime-Glo™ MT Cell Viability Assay was used. In panels (B) and (D), the CellTiter 96 AQueous One Solution Assay was used. There was a clear reduction in the number of transfected cells when compared to the negative and positive controls.

### 3.2.5 Immunofluorescence (IF) staining of SPO11 in normal tissues

In IF staining and cell imaging techniques, antibodies are used to label a particular protein of interest using fluorescent dye. Tissue sections, cultured cells or individual cells can be used in IF to determine both the localisation and the endogenous levels of a specific protein. There are two types of IF; direct IF, which uses a single antibody to detect the target, and indirect IF, which utilises two antibodies (primary, or unlabelled, and secondary, or labelled). Indirect IF was used in this study. Fluorescence can be visualised using fluorescence or confocal microscopy (Sawant et al., 2014); confocal microscopy was used in this study.

IF was used to investigate SPO11 localisation in various normal tissues. Normal testis tissues were utilised as a positive control for SPO11. MAGE-C1 was used as a positive CTA control in normal testis tissues, while staining with only secondary antibodies was served as a negative control. All tissues were fixed and stained with DAPI, which stained DNA. Normal testis tissues was staned with only secondary antibody as shown in Figure 3-14. MAGE-C1 was present in normal adult testis in the spermatogonial layer (Figure 3.15). Furthermore, SPO11 was found in the spermatocyte layer, as shown in Figure 3.15.

The IF staining of only secondary antibodies in normal ovary tissues is shown in Figure 3.16. Figure 3.17 shows the IF staining of the SPO11 and MAGEC-1 proteins in normal ovary tissues. Additionally, Figure 3.18 shows the IF staining of the SPO11 and MAGEC-1 proteins in ovarian cancer tissues. Interestingly, SPO11 staining was weak in normal ovary tissues, while SPO11 protein was detected at strong signal in cancer ovary tissues. The IF staining of only secondary antibodies in cancer colon tissues is shown in Figure 3.19. The IF staining of normal colon tissues for the SPO11 and MAGEC-1 proteins is shown in Figure 3.20, while Figure 3.21 shows the IF staining of the SPO11 and MAGEC-1 proteins in colorectal cancer tissues. SPO11 staining shows very weak signal in normal colon tissues comparing to cancer colon tissues.

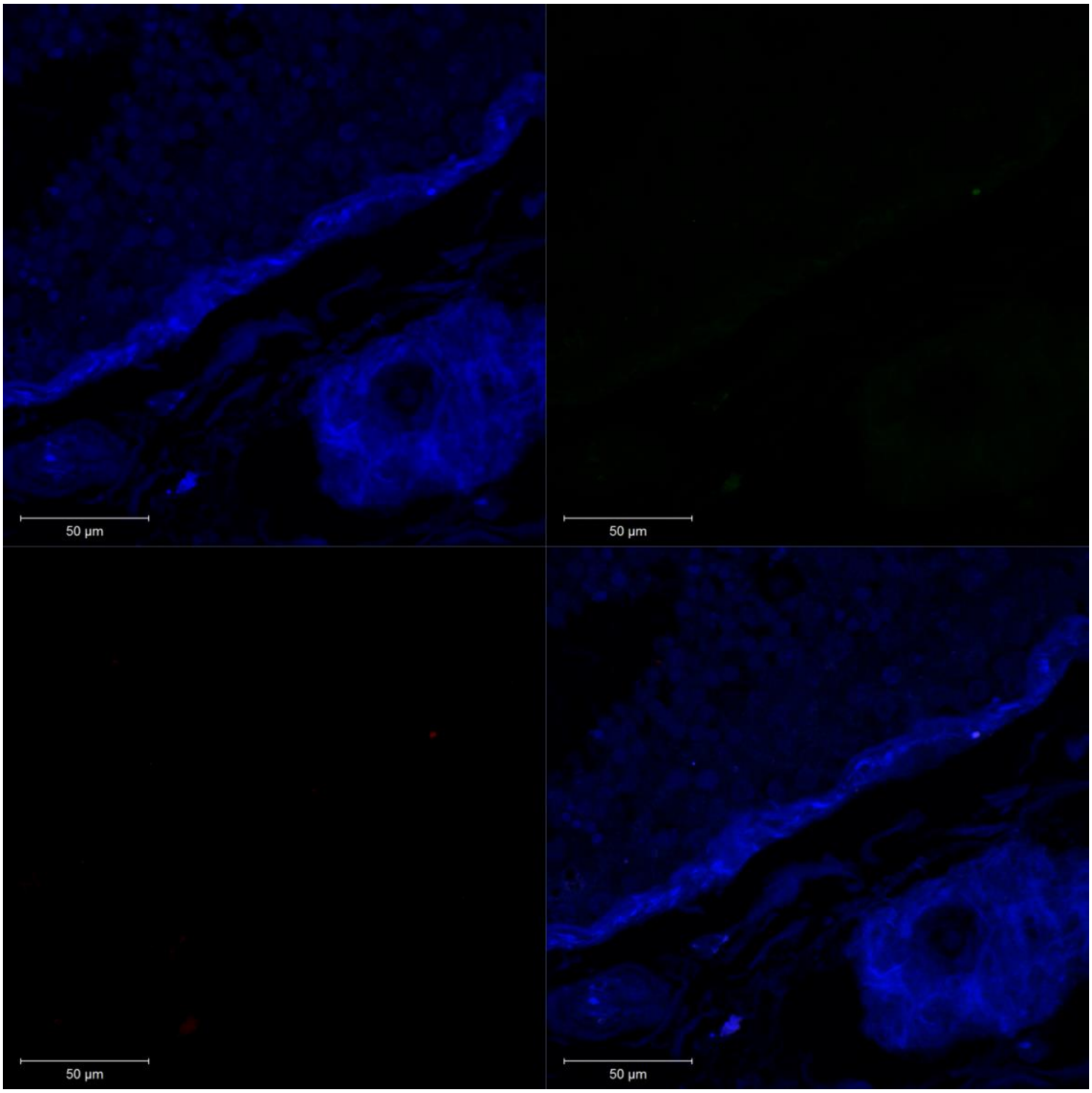



**Figure 3-14 IF analysis of the negative control in normal testis tissues (X40).**
IF image staining of only secondary antibodies, which were used as a negative control. The top left and bottom right show the blue staining (DAPI). The top right shows the staining of anti-rabbit secondary antibody (green) only, whereas the bottom left shows the staining of. anti-mouse secondary antibody (red) only. The images were taken with a ZEISS LSM 710 confocal microscope.

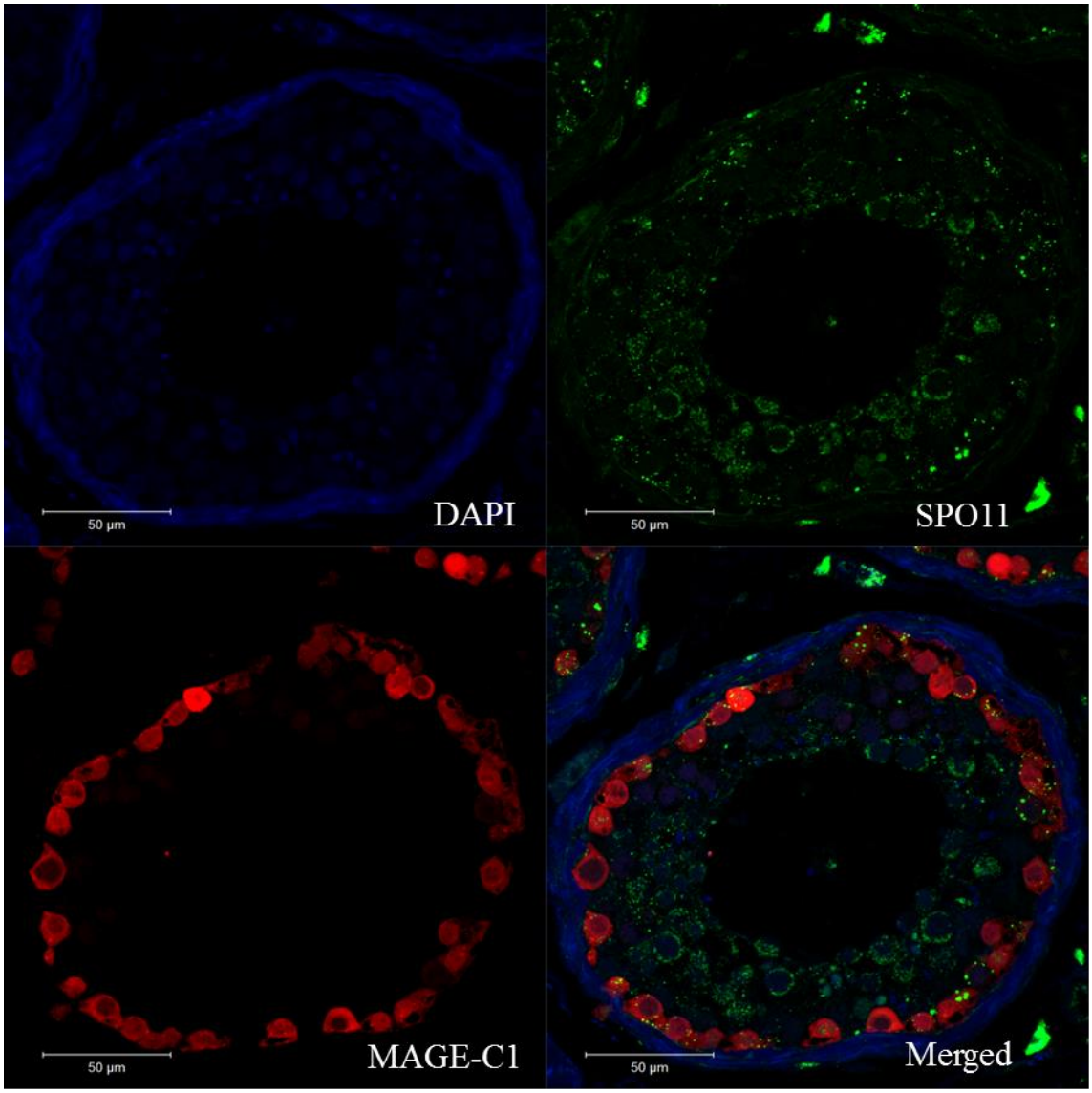



**Figure 3-15 IF staining of SPO11 and MAGEC1 proteins in normal testis tissues.**
Top left shows the blue staining (DAPI). The bottom left shows the staining of anti-MAGEC1 antibody (red), whereas the top right shows the staining of anti-SPO11 antibody (green) and was detected by the rabbit polyclonal anti-SPO11 antibody (Abcam, #ab81695). The bottom right shows the staining of DAPI, anti-MAGEC1 and anti-SPO11 antibodies. The images were taken with a ZEISS LSM 710 confocal microscope.

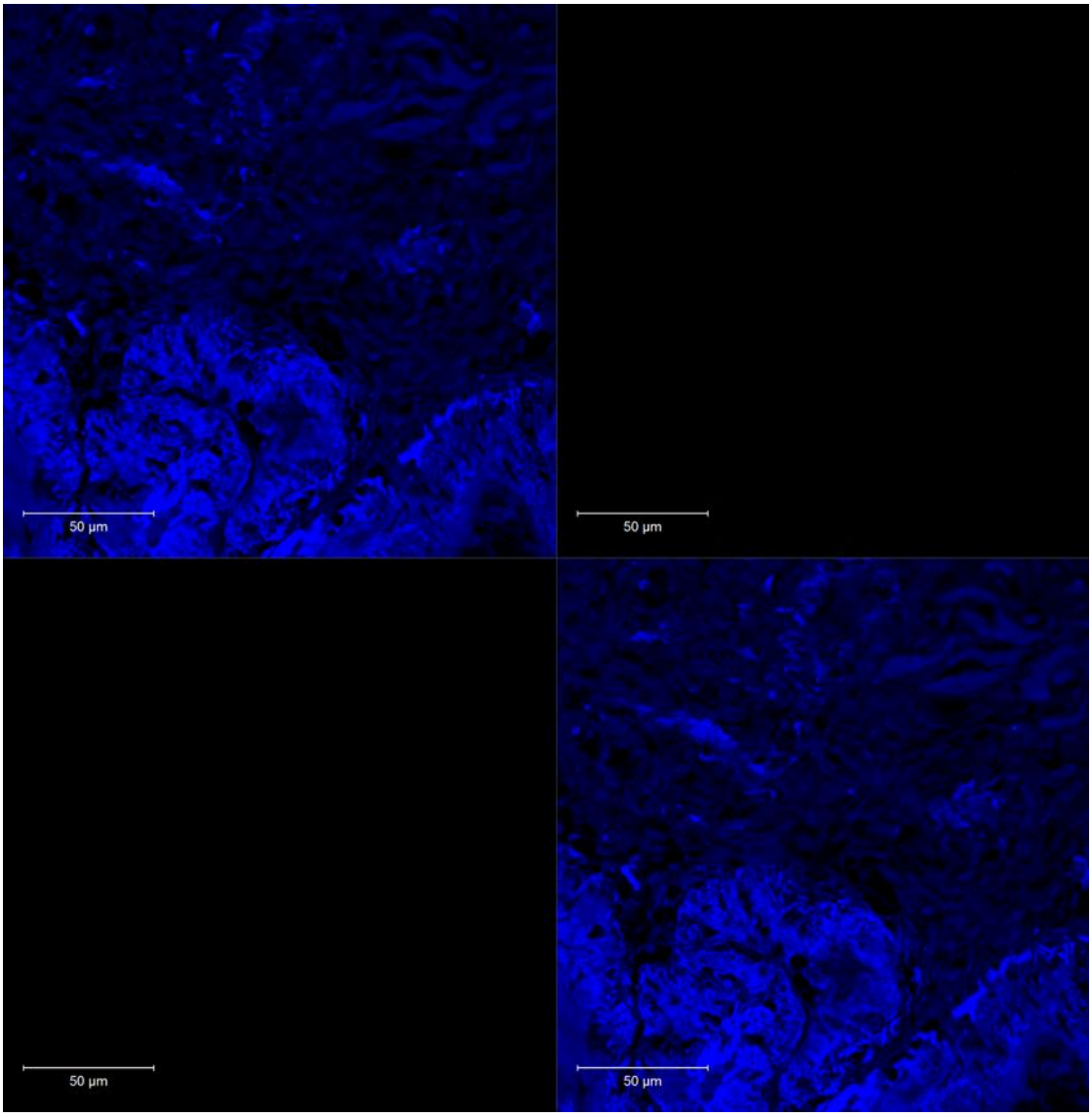



**Figure 3-16 IF staining of the negative control in normal ovary tissues (X40).**
IF image staining of only secondary antibodies, which were used as a negative control. The top left and bottom right show the blue staining (DAPI). The bottom left shows the staining of anti-mouse secondary antibody (red) only, whereas the top right shows the staining of anti-rabbit secondary antibody (green) only. The images were taken with a ZEISS LSM 710 confocal microscope.

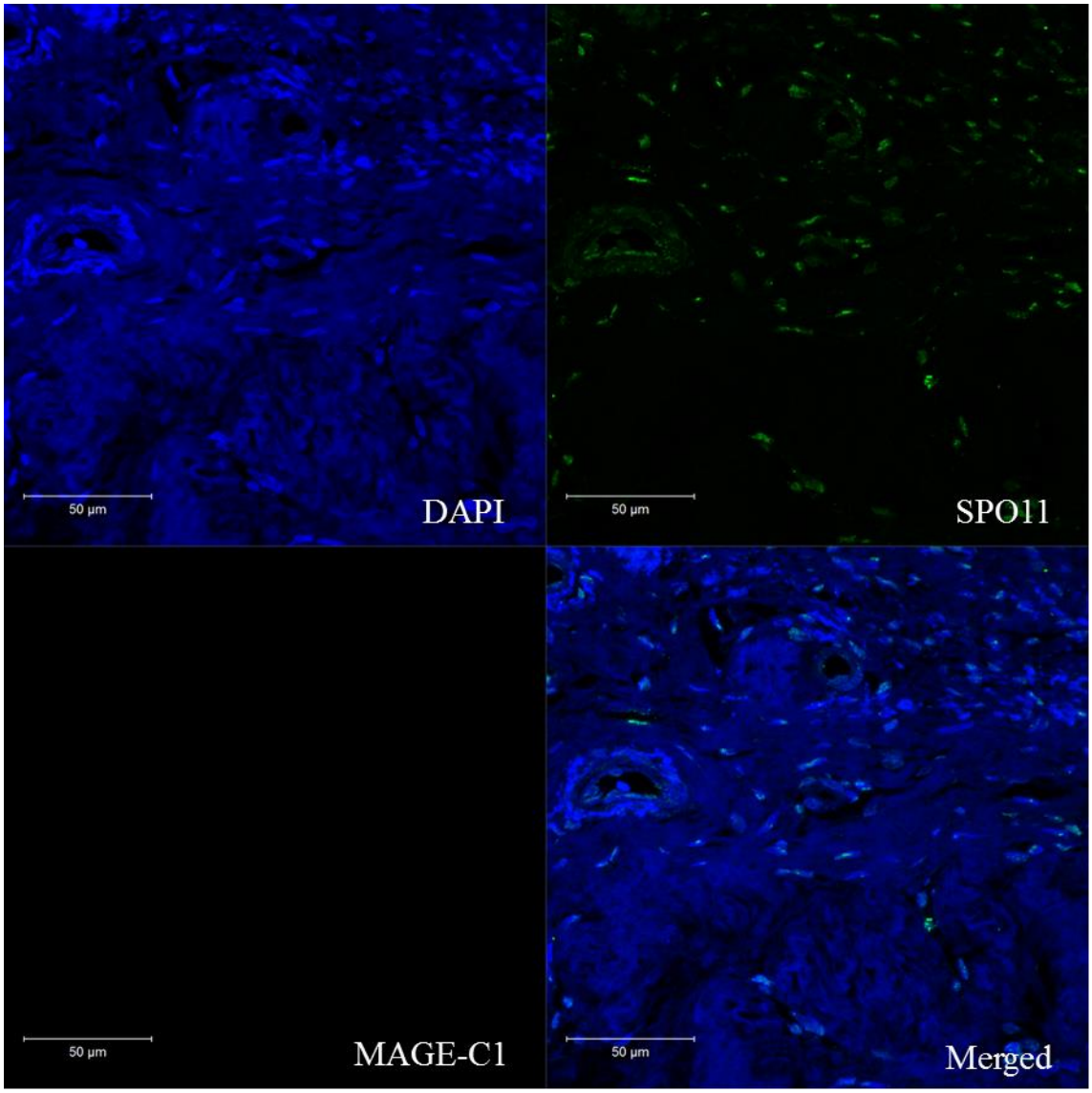



**Figure 3-17 IF staining analysis of the localisation of SPO11 in normal ovary tissues (X40).** Top left shows the blue staining (DAPI). The bottom left shows the staining of anti-MAGEC1 antibody (red), whereas the top right shows the staining of anti-SPO11 antibody (green) and was detected by the rabbit polyclonal anti-SPO11 antibody (Abcam, #ab81695). The bottom right shows the staining of DAPI, anti-MAGEC1 and anti-SPO11 antibodies. The images were taken with a ZEISS LSM 710 confocal microscope.

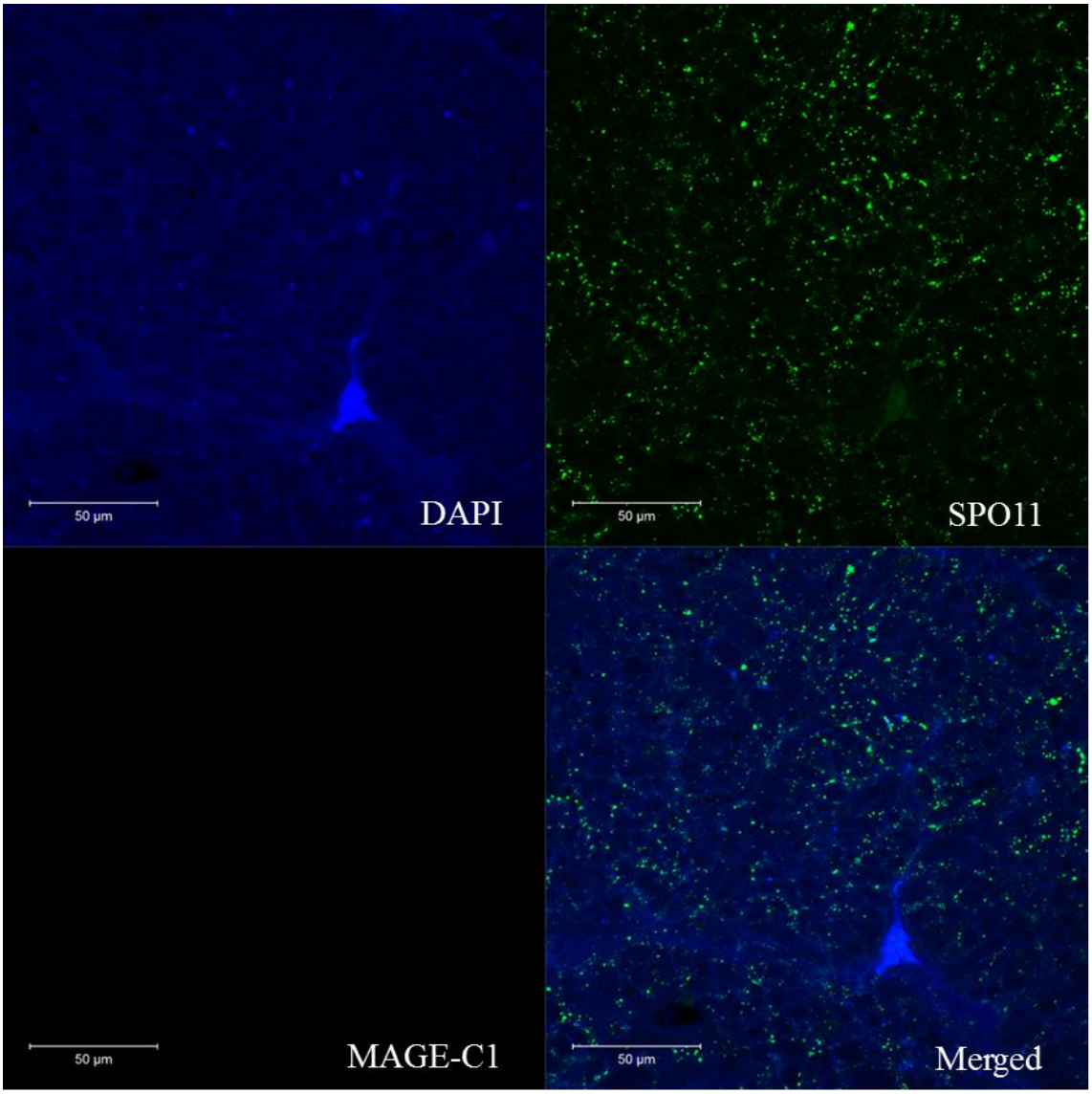



**Figure 3-18 IF staining analysis of the localisation of SPO11 in ovarian cancer tissues (X40).** The top left shows the blue staining (DAPI). The bottom left shows the staining of anti-MAGEC1 antibody (red), whereas the top right shows the staining of anti-SPO11 antibody (green) and was detected by the rabbit polyclonal anti-SPO11 antibody (Abcam, #ab81695) The bottom right shows the staining of DAPI, anti-MAGEC1 and anti-SPO11 antibodies. The images were taken with a ZEISS LSM 710 confocal microscope.

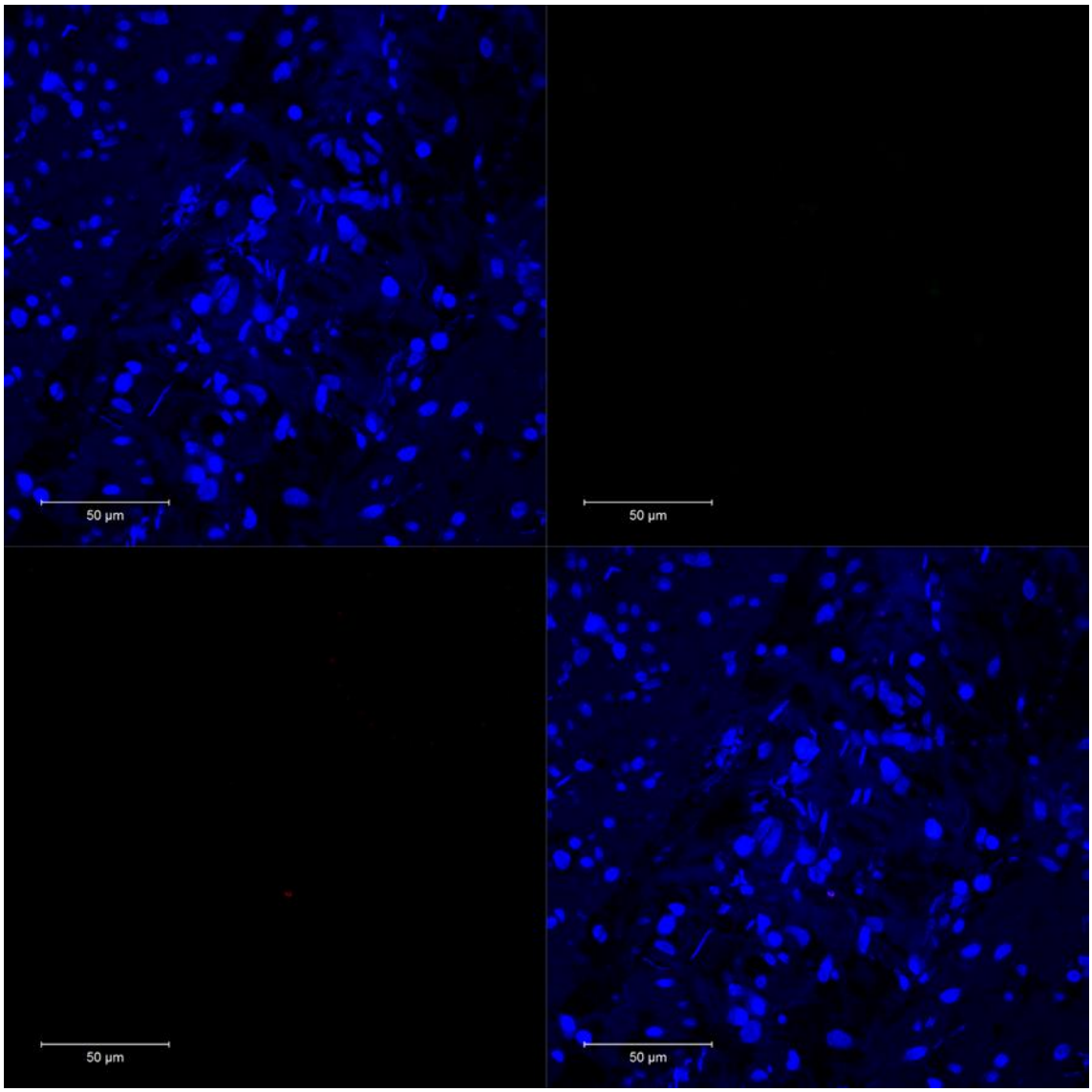



**Figure 3-19 IF staining of the negative control in colorectal cancer tissues (X40).**
IF image staining of only secondary antibodies, which were used as a negative control. The top left and bottom right show the blue staining (DAPI). The bottom left shows the staining of anti-mouse secondary antibody (red) only, whereas the top right shows the staining of anti-rabbit secondary antibody (green) only. The images were taken with a ZEISS LSM 710 confocal microscope.

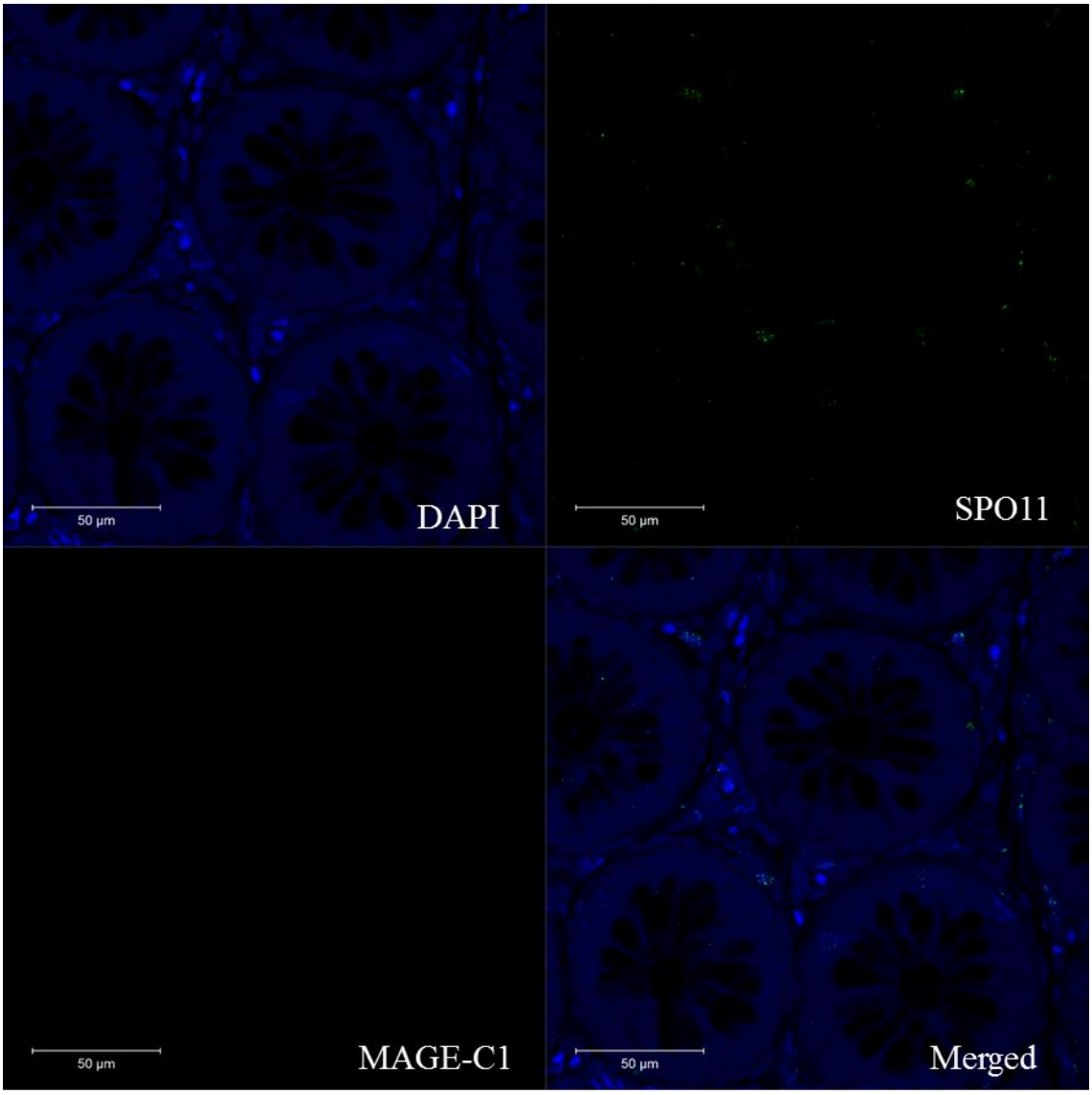



**Figure 3-20 IF staining analysis of the localisation of SPO11 in normal colon tissues (X40).**
The top left shows the blue staining (DAPI). The bottom left shows the staining of anti-MAGEC1 secondary antibody (red), whereas the top right shows the staining of anti-SPO11 antibody (green) and was detected by the rabbit polyclonal anti-SPO11 antibody (Abcam, #ab81695). The bottom right shows the staining of DAPI, anti-MAGEC1 and anti-SPO11 antibodies. The images were taken with a ZEISS LSM 710 confocal microscope.

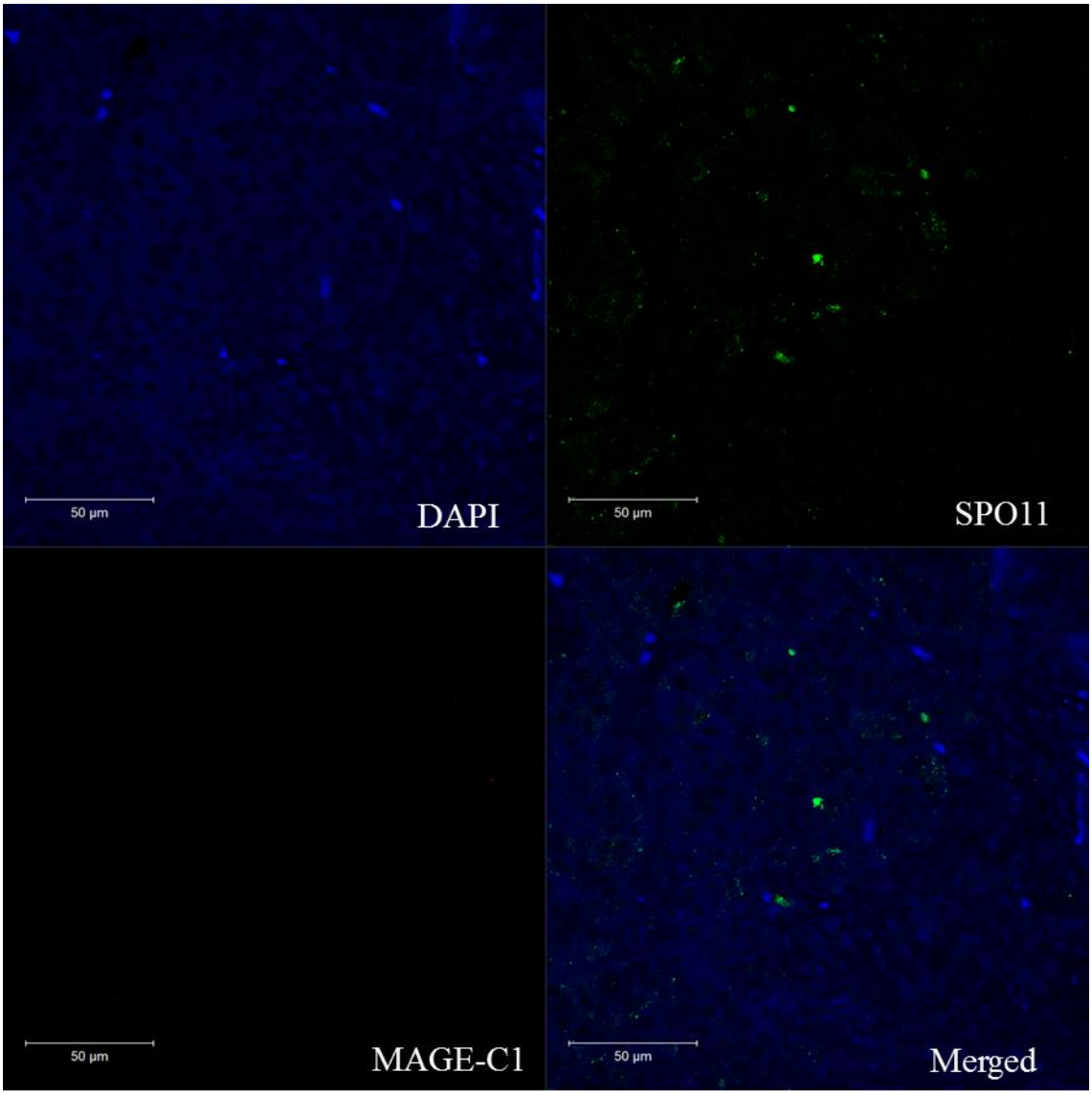



**Figure 3-21 IF staining analysis of the localisation of SPO11 in colorectal cancer tissues (X40).** The top left shows the blue staining (DAPI). The bottom left shows the staining of anti-MAGEC1 secondary antibody (red), whereas the top right shows the staining of anti-SPO11 (green) and secondary antibody (green) and was detected by the rabbit polyclonal anti-SPO11 antibody (Abcam, #ab81695). The bottom right shows the staining of DAPI, anti-MAGEC1 and anti-SPO11 antibodies. The images were taken with a ZEISS LSM 710 confocal microscope.

### 3.2.6 Cellular localisation of the SPO11 protein

The localisation of the SPO11 protein in proliferating cancer cells was determined by two different techniques: western blotting and the IF staining of fixed cells. Two cancer cell lines, HCT116 and Hela, were fractionated into nuclear and cytoplasm extracts in order to find the cellular localisation of SPO11 using the anti-SPO11 antibody (Ab81695). Whole cell extract was used for both cell lines. Lamin B was utilised as a nuclear positive control, whereas GAPDH was used as a cytoplasmic positive control. The western blot results show that SPO11 presented strongly in the nucleus, and no signal was observed in the cytoplasm in Hela cells. In contrast, in HCT116 (colon) cells, SPO11 localised mainly in the nucleus and weakly in the cytoplasm, as shown in Figure 3.22, although there is some lamin staining in the HCT116 cytoplasmic fraction, indicating there could be some nuclear contamination in this fraction.

In addition, IF staining was conducted using two cell lines, Hela and SW480 cells, in order to establish the cellular localisation of the SPO11 protein in the cells. The anti-α-tubulin antibody was used as a positive control to specify the cytoplasm region. The cells were fixed and stained with blue dye (DAPI).

The IF staining of only the secondary antibodies in Hela cells was used as a negative control (Figure 3.23). IF on Hela cells revealed the location of the SPO11 protein in the nucleus; the image in Figure 3.24 shows an anaphase cell. Secondary antibodies were used as a negative control for SW480 (Figure 3.25). Similarly, throughout anaphase, the SPO11 protein was observed exclusively in the nuclei of SW480 cells, as shown in Figure 3.26. Subsequently, the western blot results were consistent with the immunostaining results. These results correspond with the previously unpublished data obtained by the McFarlane group.

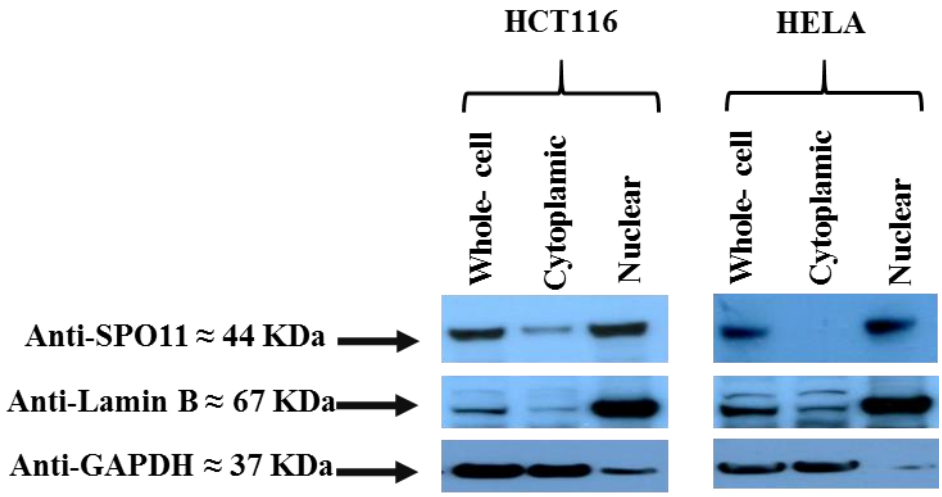



**Figure 3-22 Western blot analysis of SPO11 to determine the fractionations of HCT116 and Hela cells.**

This figure shows the cellular localisation of SPO11 in HCT116 and the Hela cell line. In the HCT116 cell line, SPO11 presents at high levels in the nucleus while, a very faint band was observed in the cytoplasm. SPO11 protein was detected by the rabbit polyclonal anti-SPO11 antibody (Abcam, #ab81695). However, in the Hela cell line, SPO11 was found only in the nucleus. Anti-GAPDH and anti-lamin B were used as cytoplasmic and nuclear controls, respectively, to determine the efficiency of the fractionation and gel loading.

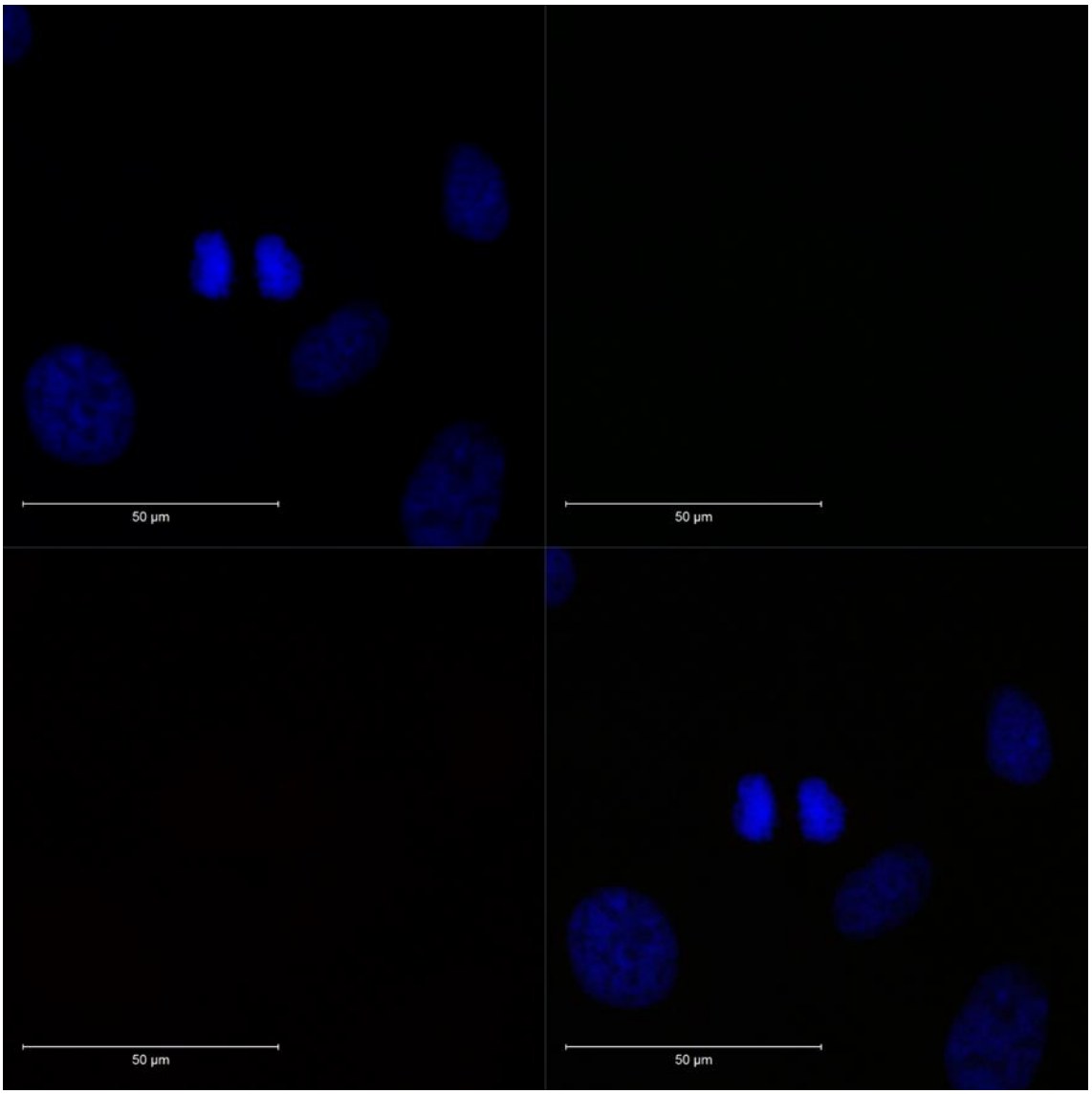



**Figure 3-23 Negative control staining in HeLa cells for IF analysis (X40).**
IF image staining of only secondary antibodies, which were used as a negative control. The top left and bottom right show the blue staining (DAPI). The top right shows the staining of anti-rabbit secondary antibody (green) only, whereas the bottom left shows the staining of anti-mouse secondary antibody (red) only. The images were taken with a ZEISS LSM 710 confocal microscope.

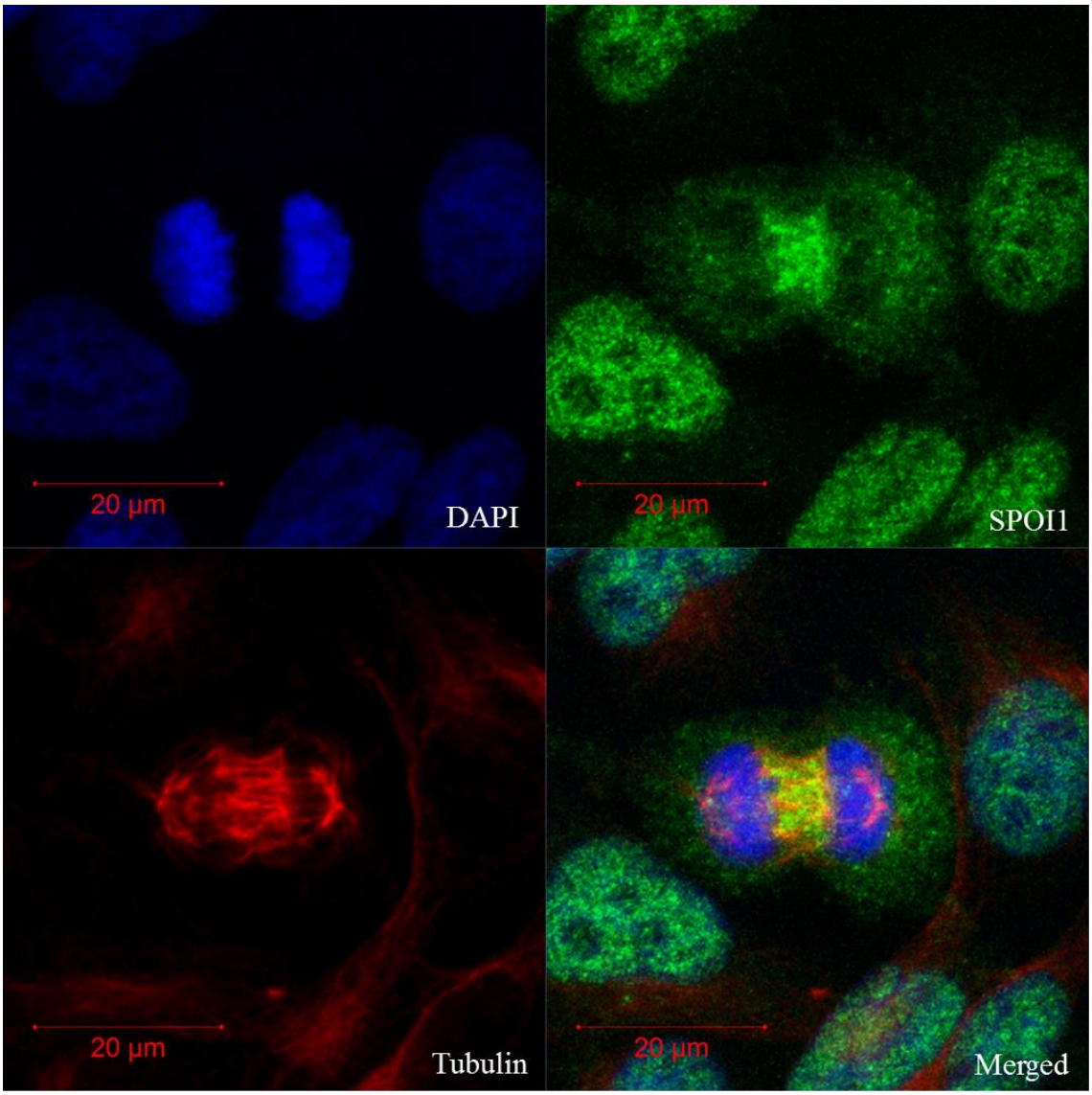



**Figure 3-24 IF staining of fixed HeLa cells with SPO11 and Tubulin (X40).**
The top left shows the blue staining (DAPI). The top right shows the staining of anti-SPO11 antibody (green) and was detected by the rabbit polyclonal anti-SPO11 antibody (Abcam, #ab81695), whereas the bottom left shows the staining of anti-Tubulin antibody (red). The bottom right shows the staining of DAPI, anti-SPO11 and anti-Tubulin antibodies during anaphase. The images were taken with a ZEISS LSM 710 confocal microscope.

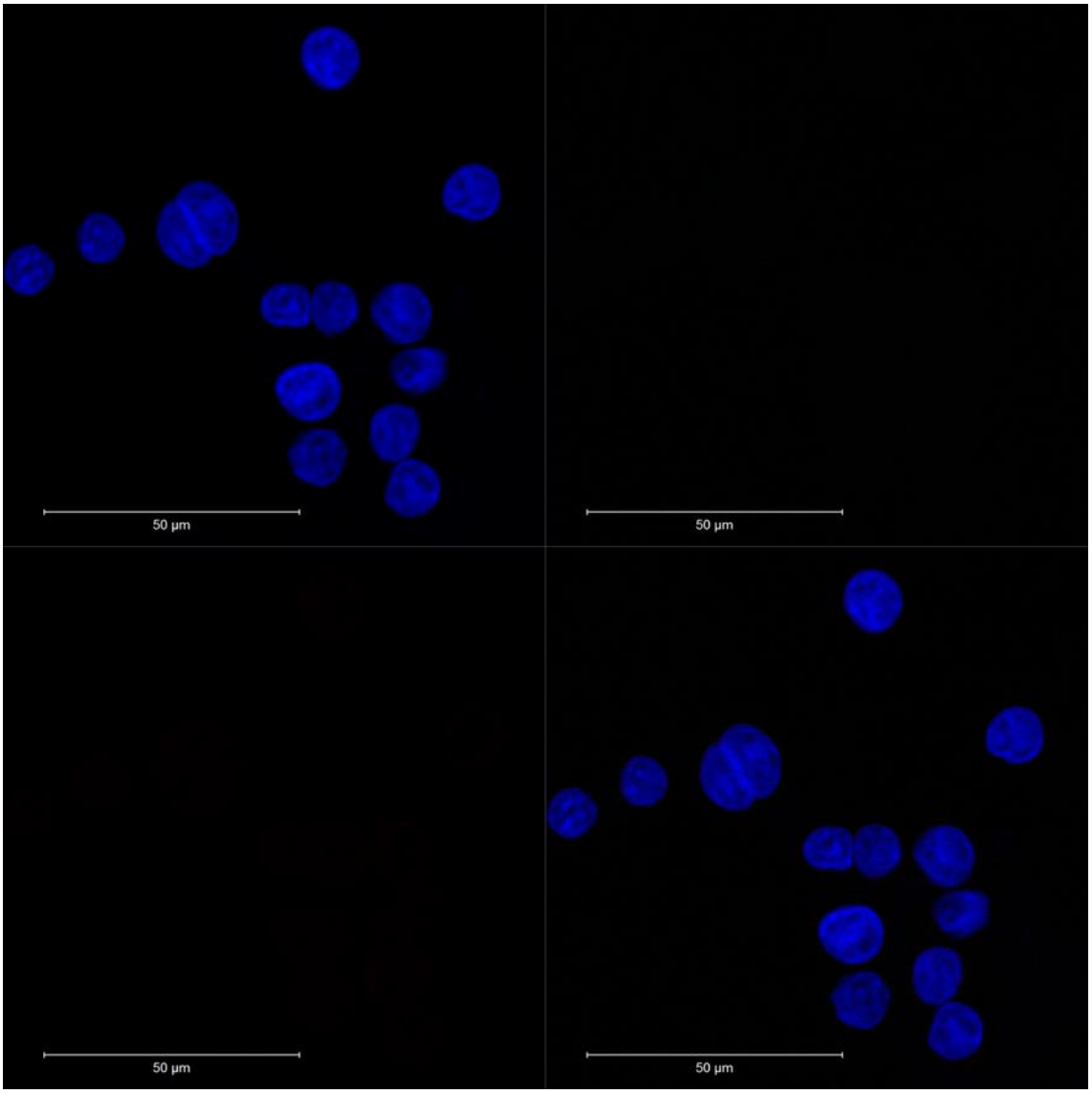



**Figure 3-25 Negative control staining in SW480 cells for IF analysis (X40).**
IF image staining with only secondary antibodies, which were used as a negative control. The top left and bottom right show the blue staining (DAPI). The top right shows the staining of anti-rabbit secondary antibody (green) only, whereas the bottom left shows the staining of anti-mouse secondary antibody (red) only. The images were taken with a ZEISS LSM 710 confocal microscope.

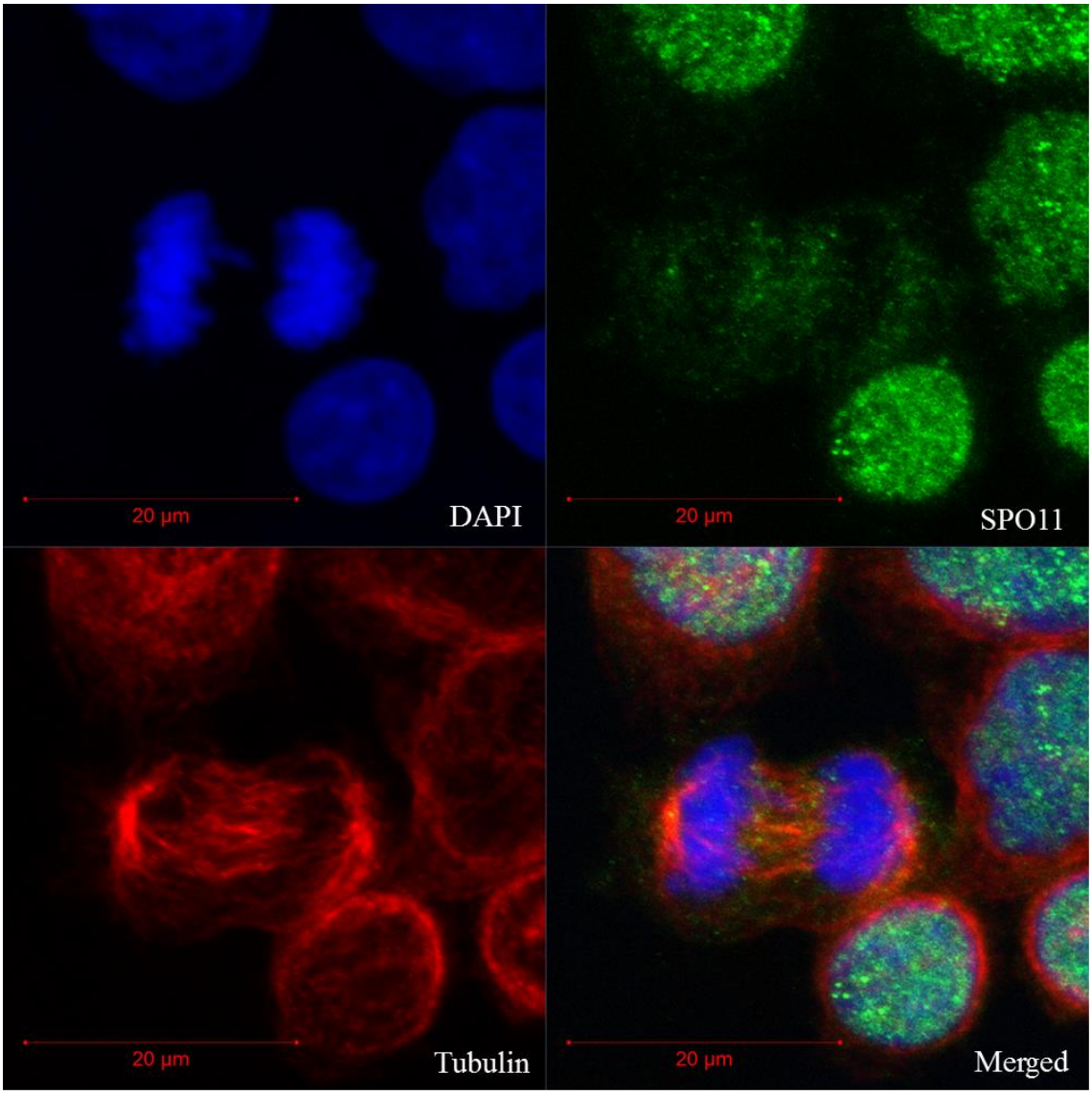



**Figure 3-26 IF staining analysis of SW480 cells fixed with SPO11 and Tubulin (X40).**
The top left shows the blue staining. The top right shows the staining of anti-SPO11 antibod (green) and was detected by the rabit polyclonal anti-SPO11 antibody (Abcam, #ab81695), whereas the bottom left shows the staining of anti-Tubulin antibody (red). The bottom right shows the staining of DAPI, anti-SPO11 and anti-Tubulin antibodies during anaphase. The images were taken with a ZEISS LSM 710 confocal microscope.

## 3.3 Discussion

### 3.3.1 Summary of findings

The SPO11 protein was detected only in normal testis tissues but not in other normal tissues when examined by western blotting and IF. Conversely, SPO11 was found at a detectable level in all the cancer cells and tumours tested. Moreover, cell fractionation and IF strongly indicated that the SPO11 protein is located in the nuclei of cancer cells. The depletion of the SPO11 protein in four different cancer cells was found to significantly inhibit cell proliferation and reduce cell survival. Our results suggest the potential function of SPO11 in cancer cells; therefore, SPO11 might be a suitable marker for cancer. SPO11 knockdown using several techniques will be performed in the following chapter. The aim of the work described in this chapter was to confirm SPO11 as CTA and to establish the effect of SPO11 knockdown on cell proliferation and viability in four different cancer cell lines. Additionally, we aimed to establish specific cellular localisation of *SPO11* in cancer cells and normal testis tissues.

### 3.3.2 SPO11 protein in normal tissues, cancer cell lines and tumour tissues

The adult tissue pattern of the human SPO11 protein was determined by western blotting and IF. Although clear results were obtained from the western blot, protein quantitation and protein transferring methods should be checked using different types of staining such as Ponceau staining. In addition, in order to quantify the full range of the presence of the protein of interest among the samples, a quality validated blot should be taken into account and the band intensity should be normalised. SPO11 was detected only in normal testis tissues and not in the other normal tissues included in this study. However, of the different cancer cells used in this study, including breast, lung, melanoma, leukaemia and colon cancer samples, SPO11 was present in all of them. Furthermore, SPO11 was present in some tumour tissues, such as ovary, liver and lung tumour tissues. On the other hand, SPO11 protein is significantly presented at lower level in thymus tissues as normal sample than in testis normal tissues. Thymus takes this importance since T cells, which play an critical role in cell-mediated immunity, are mature in such gland. Interestingly, this data is consistent with the results obtained by a previous study, which used northern blotting techniques to explore *SPO11* gene expression (Shannon et al., 1999). This study and ours confirmed that SPO11 is a CTA.

Hofmann et al. (2008) and Gjerstorff (2016) pointed out that SPO11 is not limited to male germ cells; therefore, it might have a function distinct from DSB initiation (Hofmann et al., 2008; Nielsen & Gjerstorff, 2016). Our results propose an oncogenic action of SPO11 protein in driving cancer proliferation, suggesting the possibility of using SPO11 as a cancer biomarker and treatment target.

### 3.3.3 SPO11 depletion inhibits cell proliferation in cancer cells

In normal and cancer cells, the balance of cell proliferation and cell death (apoptosis) has a vital role in the development of normal tissue homeostasis. Alterations to this balance are associated with cancer. For example, Zhou et al. (2010) found that CTA TSP50 is involved in the development of breast cancer. Further, it has been reported that reduced *TSP50* expression can affect cell proliferation, colony generation and migration and cell apoptosis promotion (Zhou et al., 2010). The metabolism of multiplying cells, including cancer cells, is modified to assist the absorption and incorporation of nutrients in order to initiate the production of new cells (Vander et al., 2009). A cell count curve assay was conducted in the current study to determine the effect of SPO11 on cell proliferation. In the assay, different parental cells, including SW480, A2780, HCT116 and Hela cells, were treated for several days with SPO11 siRNA 2 and 4.

The results suggested that SPO11 depletion inhibits cancer cell proliferation. Two siRNA molecules (2, 4) had the same effects on the cells, as there were no differences in SPO11 depletion; hence, the effects were not related to non-specific targeting. *SPO11* siRNA 2 and 4 are targeting exon 13 non-coding region. In fact, when comparing the total cell number after SPO11 knockdown to the cells treated with non-interfering, a significant difference was observed for all the tested cancer cells. The reduction in the SPO11 protein levels in the SW480 and Hela cells had a larger effect on proliferation than in A2780 and HCT116 due to the differences between the cancer cell lines in their genomes and mutations. The images of the cells taken after SPO11 knockdown confirmed the effect of SPO11 reduction on cell proliferation. These results may suggest the involvement of the SPO11 gene in cell cycle or cell division regulation, resulting in the inhibition of cell proliferation and reducing cell survival. Substantially, improving the comprehension of the mechanistic relationship between cellular metabolism and growth regulation may result in developing effective treatments for human tumours (Vander et al., 2009).

Further, the influence of SPO11 depletion on cancer cell survival was detected using an ELDA essay. Reducing the SPO11 protein level in both the SW480 and HCT116 cells lead to decrease their proliferation frequency, and siRNA 2 had a significant effect on SPO11. This result was consistent with the previous observations of the growth curve and the cell viability assay, which both suggested that low proliferation activity might be a result of SPO11 silencing.

Interestingly, in *Drosophila melanogaster*, the oncogenic process requires expression of germline genes and the expression of the human orthologues of *Drosophila* genes are up-regulated in a variety of human tumours (Feichtinger et al., 2014; Janic et al., 2010). Thus, in human cancers, disruptions to cell proliferation and growth may result from a deregulating range of CT genes. For instance, CT *TACC3* and *MAD2* genes may play a role in tumour cell division (Cappell et al., 2012; Lindsey et al., 2013b).

Subsequently, CT genes may play a fundamental role in cancer progression, resulting in controlling cell division and proliferation (McFarlane et al., 2014; Rousseaux et al., 2013). For example, the *PRDM1* gene is expressed in a wide range of cancer cells and regulates p53 activity, as it is considered to be a transcription regulator of cell survival and proliferation. Further, apoptosis and cell division are triggered by *PRDM1* reduction in HCT116 (Yan et al., 2007). Furthermore, in melanoma therapy, the *NY-ESO-1* gene is targeted as an attractive adoptive therapy (Hunder et al., 2008). Interestingly, it is reported that meiotic regulatory such as SYCP3 can control cancer cells through the formation of a complex with BRCA2, which affects BRCA2 and RAD51 interaction, leading to the inhibition of HR (Hosoya et al., 2011). In fact, targeting such genes may affect tumours growth, minimise their symptoms or/and improving the effective of different cancer treatments (Almatrafi et al., 2014).

### 3.3.4 SPO11 protein localisation in cancer cells

Although the localisation of different proteins in the cytoplasm or nucleus depends on their functions, multifunctional DNA-binding proteins are found in both cytoplasmic and nuclear fractions (Wilkinson & Shyu, 2001). The location of SPO11 in the cell was determined by collecting the cell fractions and IF analysis. The western blotting analysis results shown here suggest that SPO11 is localised strongly in the nucleus. In order to demonstrate that SPO11 protein is functioning in normal testis as in cancer cells, IF was used on two cancer cell lines: SW480 and Hela.

The IF staining results showed that SPO11 is localised predominantly in the nucleus of SW480 and Hela cells. SPO11 protein is hugely presented in cancer cells tested in this study especially in Hela cells comparing to what would be expected in meiotic cells. The presence of SPO11 is concentrated mainly between new daughter cells during anaphase where the chromosomes are located, explaining an interesting and unknown role or/and function of this protein in cancer cells. Based on these results, the SPO11 protein may function in cancer cells in a similar manner as it does in normal testis tissues. Protein function and integration into functional biological networks correlate with an appropriate subcellular localisation of genes. Interestingly, abnormal protein localisation may arise from mutations, altered expressions of cargo proteins and/or transport receptors. These localisation abnormalities are considered a feature of some human diseases, including cancer (Hung & Link, 2011). The protein function can be affected by aberrant protein localisation, such as inactivation mechanisms in cancer, which may result from the mis-localisation of many tumour suppressors. Further, tumour progression, tumourigenesis and metastasis can be caused by the deregulation of the spatiotemporal signalling dynamic (Kau ET AL., 2004; Wang & Hung, 2005).

### 3.3.5 SPO11 protein in testis, normal and cancer tissues

The localisation pattern in testis tissues suggested that the SPO11 protein is a testis-specific protein. The MAGEA1 antibody serves as a spermatogonial marker of seminiferous tubules. The co-localisation of SPO11 and MAGEA1 proteins was identified at a very low level from our results, indicating that SPO11 may be found in spermatogonial cells. Interestingly, while SPO11 protein in normal colon tissues presented at lower level comparing to cancer colon tissues, it can be explained by the limitation of providing pure normal tissues. Therefore, the possibility of having cancer at too early stage should be considered. Subsequently, examining different samples from different providers with full sample details (ages, diseases, cause of death) should be addressed in the future studies. The presence of the SPO11 protein in cancer cells and tissues may support the possibility of an additional unknown function of this gene in cancer. Taken together, these results suggest that SPO11 might have an uncharacterised function in cancer cells, as differences in the survival between the SPO11-depleted cells compared to that of the controls were observed.

# Chapter 4

Identification and analysis of the biological role of *SPO11* in cancer cells

# 4. Identification and analysis of the biological role of *SPO11* in cancer cells

## 4.1 Introduction

A primary characteristic of cancer cells is the ability of those cells to divide uncontrollably. Different factors are considered as potential cause of cancer including, genetic or epigenetic changes, microenvironment interactions and metabolic alteration (Hirohashi et al., 2016). A feature of some human meiosis-specific genes, including *SPO11* is their expression in several types of human cancer cells. (Koslowski et al., 2002; LamKeeney, 2014). Germ-line or meiotic proteins can be recognised by the immune system in cancerous cells as specific antigens, which may have essential applications in cancer diagnosis and therapies (Grizzi et al., 2015). Although germ-line genes regulate meiotic cell division and are not expressed in non-meiotic normal cells, their expression in human cancer indicates a possible role in cancer progression (McFarlane et al., 2015). Therefore, genome instability, as a cancer hallmark, may result from the activation of a number of germ line/meiotic genes (Cho et al., 2014; Janic et al., 2010).

Interestingly, various studies have established an important correlation between CTA genes overexpression in cancer cells and tumour growth and cell proliferation. It has been reported that in different cancer cells, numerous CTAs play a critical role in tumour cell viability through targeting those genes via siRNA techniques (Maxfield et al., 2015). For example, use of such silencing methods demonstrated that the *CT45A1* gene a CTA gene drives cell proliferation and migration (Shang et al., 2014). Furthermore, cell proliferation is also thought to be stimulated by *MAGEC2* gene another CTA gene (Lajmi et al., 2015). Hence, since CTAs may promote tumour growth and progression, they postulate as biomarker in cancer diagnosis and cancer drug targets.

Protein knockdown is a technique that aims to determine the effect that a reduction in the levels of a specific protein may have on a particular cell. Additionally, it enables the validation of the antibody used in western blot and immunofluorescent. Protein knockdown can be achieved by using RNA interference (RNAi), which degrades mRNAs. The knockdown efficiency can be assessed by either quantitative real-time PCR (qRT-PCR) or by western blot (Agrawal et al., 2003).

The results presented in Chapter 3.0 suggested that cell proliferation of different cancer cells were significantly inhibited following the use of *SPO11* siRNA 2 and 4. Therefore, in the experiments conducted in the present chapter, SPO11 knockdown using different techniques such as siRNA and shRNA will be examined in order to confirm whether the level of SPO11 is reduced, and to establish the specificity of the SPO11 antibody (Abcam, #ab81695).

## 4.2 Results

### 4.2.1 SPO11 knockdown attempts

#### 4.2.1.1 Small interfering RNA (siRNA) depletion of SPO11

Various attempts to optimise SPO11 depletion with siRNA treatment were carried out using different altered transfection conditions. Knockdown experiments were performed using SW480 cells growing in media with or without serum (FBS) (Figure 4.1 A). Treatment was compared in early and late passage of SW480 cells initially using HiperFect transfection reagent (Figure 4.1 B). SPO11 knockdown in SW480 was attempted using various transfection reagents, increasing the number of siRNA 'hits' as well as siRNA concentration. The RNAiMAX transfection reagent was used at 10 μM siRNA concentration with three hits (Figure 4.1 C1), whereas viromer GREEN transfection reagent was used at 10 μM (Figure 4.1 C2) and 20 μM siRNA concentration with three hits (Figure 4.1 C3). The MCF7 cell line was transfected with HiperFect and viromer GREEN using SPO11 siRNA2 and 4 (Figure 4.2 A).

In this study, different cell lines, including Jurkat, K562, HCT116, NTERA2, Lovo and HeLa, were examined in order to attempt a reduction in SPO11 levels (Figure 4.2 B and Figure 4.3). Untreated cells are used as positive controls, whereas cells transfected with non-interfering siRNA are used as negative controls. Abcam (ab81695) anti-SPO11 antibody was used to determine SPO11 knockdown; the band obtained on western blots was of approximately 44 kDa. However, western blot results of all of the above attempts do not appear to show any significant knockdown of SPO11, despite these siRNA showing an inhibition of cell proliferation.

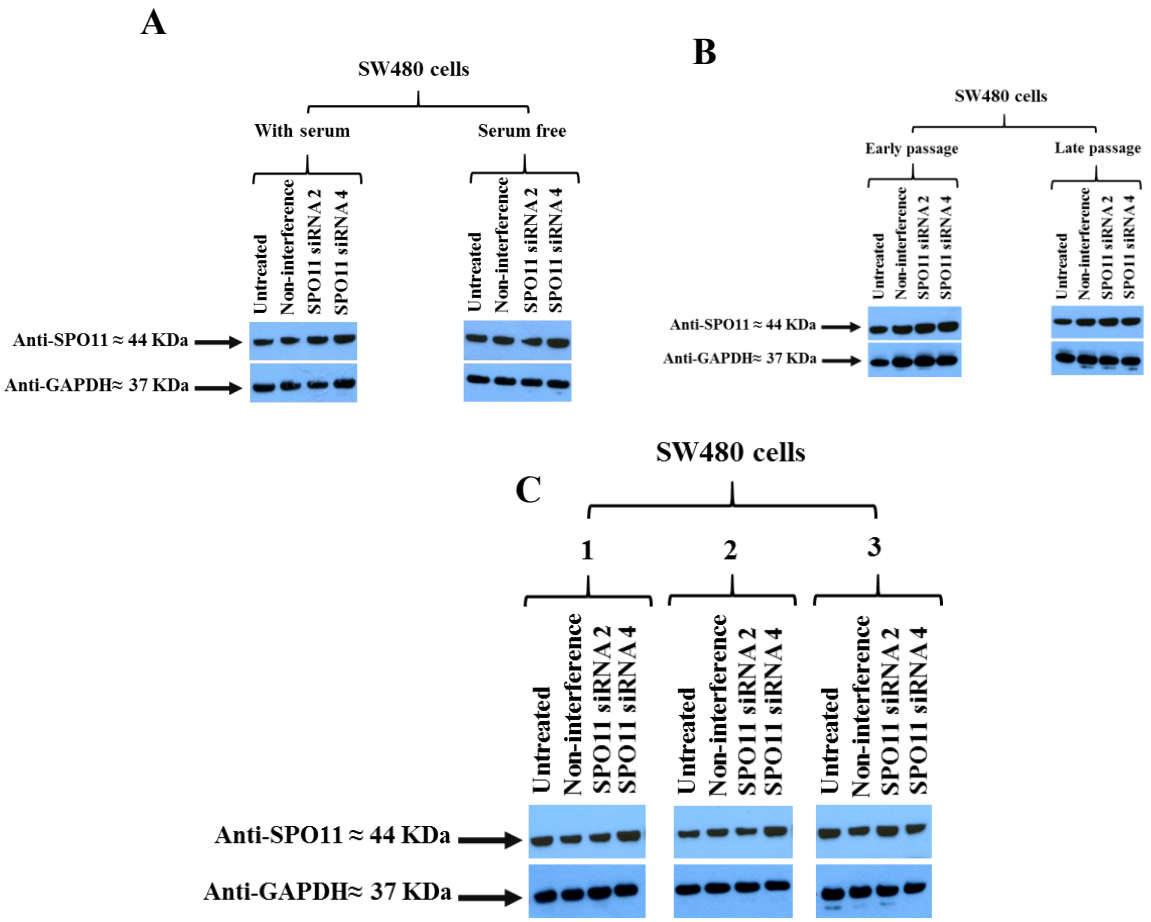



**Figure 4-1 Western blot analysis showing SPO11 siRNA knockdown attempts in SW480 cells using siRNA 2 and siRNA 4.**

Untreated SW480 cells were used as positive controls, while cells treated with non-interfering siRNA were utilised as negative controls. GAPDH protein levels were used as a loading control (bottom). (A) Media with and without serum were used during siRNA knockdown treatment. (B) Cells at early (P10) and late (P55) passage were utilised throughout siRNA courses. (C1) Using RNAiMAX transfection reagent with three hits of SPO11 siRNA 2 and siRNA 4. (C2) Using Viromer GREEN transfection reagent with three hits of SPO11 (10 µM) siRNA 2 and siRNA 4. (C3) Viromer GREEN transfection reagent with three hits of SPO11 (20 µM) siRNA 2 and siRNA 4 was used. Knockdown of SPO11 using these conditions was not detected when compared to the negative or positive controls.

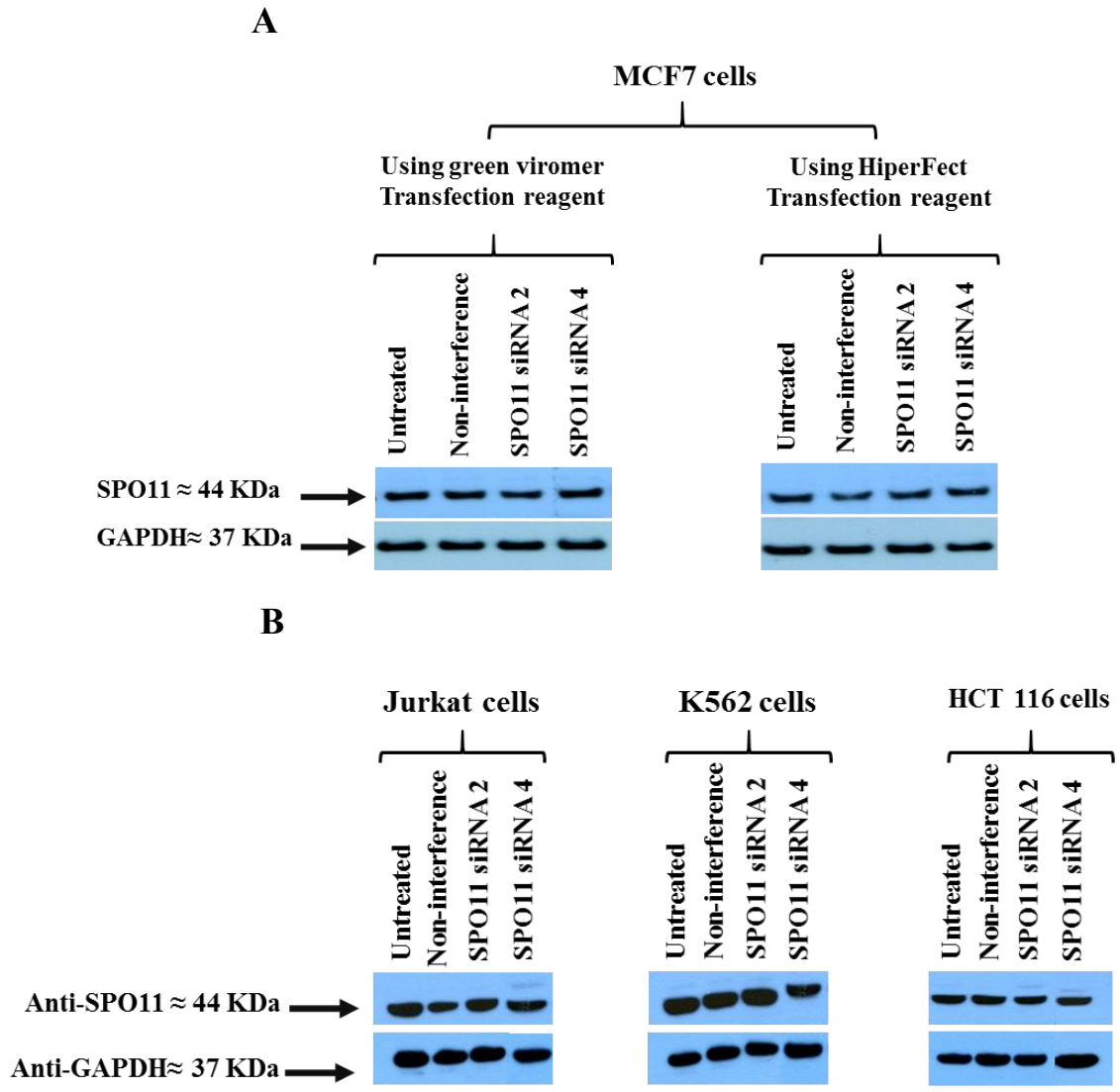



**Figure 4-2 Western blot analysis showing SPO11 siRNA knockdown attempts in various cells using siRNA 2 and siRNA 4.**
Untreated cells were used as positive controls, while cells treated with non-interfering siRNA were utilised as negative controls. GAPDH protein levels were used as a loading control (bottom). (A) SPO11 siRNA knockdown in MCF7 cells using HiperFect or viromer GREEN as transfection reagents. (B) SPO11 siRNA knockdown in Jurkat, K562 and HCT116 cells using HiperFect transfection reagent. Knockdown of SPO11 was not detected in those cells when compared with the negative or positive controls.

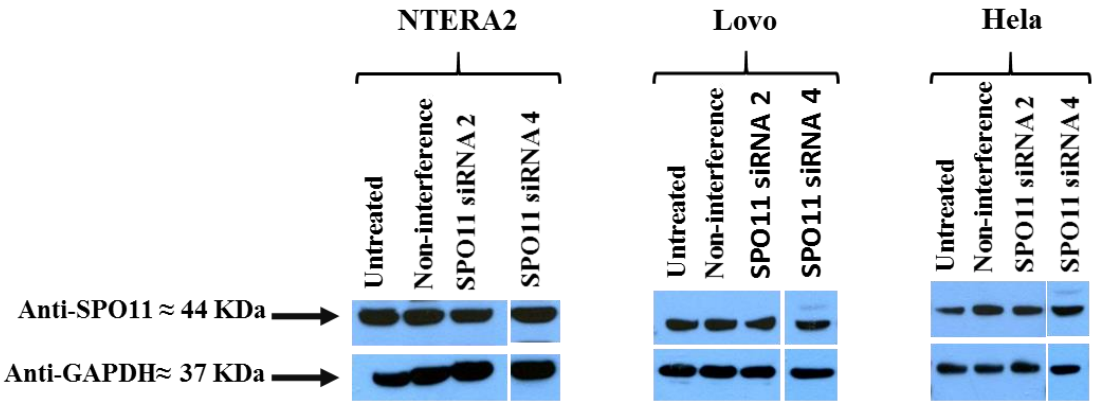



**Figure 4-3 Western blot analysis showing SPO11 siRNA knockdown attempts in NTERA2, Lovo and HeLa cells using siRNA 2 and siRNA 4.**
Untreated cells were used as positive controls, while cells treated with non-interfering siRNA were utilised as negative controls. GAPDH protein levels were used as a loading control (bottom). The image shows SPO11 siRNA experiments in NT2, Lovo and HeLa cells using HiperFect as the transfection reagent. Knockdown of SPO11 was not detected when compared with the negative or positive controls.

#### 4.2.1.2 Endonuclease-prepared siRNA (esiRNA) knockdown of SPO11

Silencing of a specific gene in order to reduce protein levels using a siRNA mechanism may trigger a non-specific interferon response in many organisms. Therefore, using esiRNA may increase the specificity of gene knockdown leading to effective reduction (Kittler et al., 2004). Herein, SPO11 knockdown using esiRNA was performed over 3 days and with cells harvested every day. In addition, untreated cells and positive esiRNA were used as positive controls whereas cells transfected with a non-interfering or lipofectamin were used as negative controls. GAPDH protein level was used as a control to test the quality of the WCE loading. Knockdown efficiencies for esiRNA were determined by western blot.

SPO11 knockdown using esiRNA was examined at days 1, 2 and 3. The predicted molecular weight of the SPO11 protein is approximately 44 kDa, which is what is observed when using anti-SPO11 Abcam antibody. The results indicate that the knockdown of SPO11 protein using this technique did not show any measurable reduction in the intensity of the 44 kDa band (Figure 4.4).

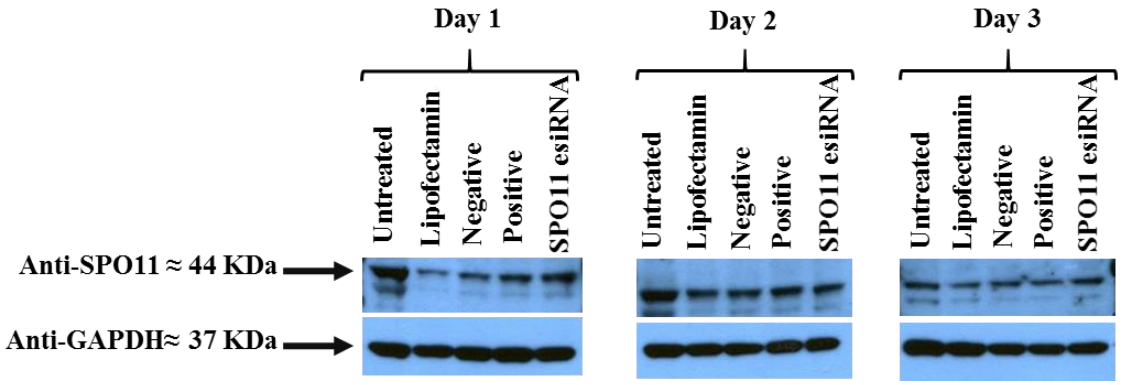



**Figure 4-4 Western blot analysis showing SPO11 knockdown attempts in SW480 cells using esiRNA.**
Untreated cells were used as positive controls, while cells treated with lipofectamin were used as negative controls. GAPDH protein levels were used as a loading control (bottom). Negative and positive panels are used as controls for this experiment. SPO11 Knockdown was not observed in esiRNA lane when compared with the negative or positive controls.

#### 4.2.1.3 Short hairpin RNA (shRNA) knockdown of SPO11

The study of gene function through RNA interfering (RNAi) is considered a powerful gene knockdown tool. Different RNAi techniques were applied, including siRNA, esiRNA and shRNA. The latter is a method designed to deliver small interfering into targeted mammalian cells more effectively than other knockdown techniques (Moore et al., 2010). Herein, SW480 cells were transfected with negative control shRNA vector or SPO11 silencing shRNA vector by my colleague, Dr. Ellen Vernon. In this study, cells were then treated with doxycycline in order to activate transcription of the shRNA. SW480 cells transfected with GAPDH shRNA were used as positive controls, while SW480 cells transfected with vector alone was served as the negative control. In addition, red fluorescent protein (RFP) antibody was used as a control for vector expression.

A cell count representing the total number of SW480 cells induced with doxycycline (3 µg/mL) over 7 days was plotted in order to show any change in cell number due to the induction of SPO11 shRNA (Figure 4.5 A). Whole cell extracts were prepared during doxycycline treatment to ensure that successful SPO11 knockdown was achieved using western blot (Figure 4.5 B). As a result, no significant SPO11 reduction was observed for SW480 transfected with SPO11 silencing shRNA vector (Figure 4.5 A and B). In addition, GAPDH shRNA (positive control) knockdown using SW480 transfected with shRNA was not successful (Figure 4.5 C and D).

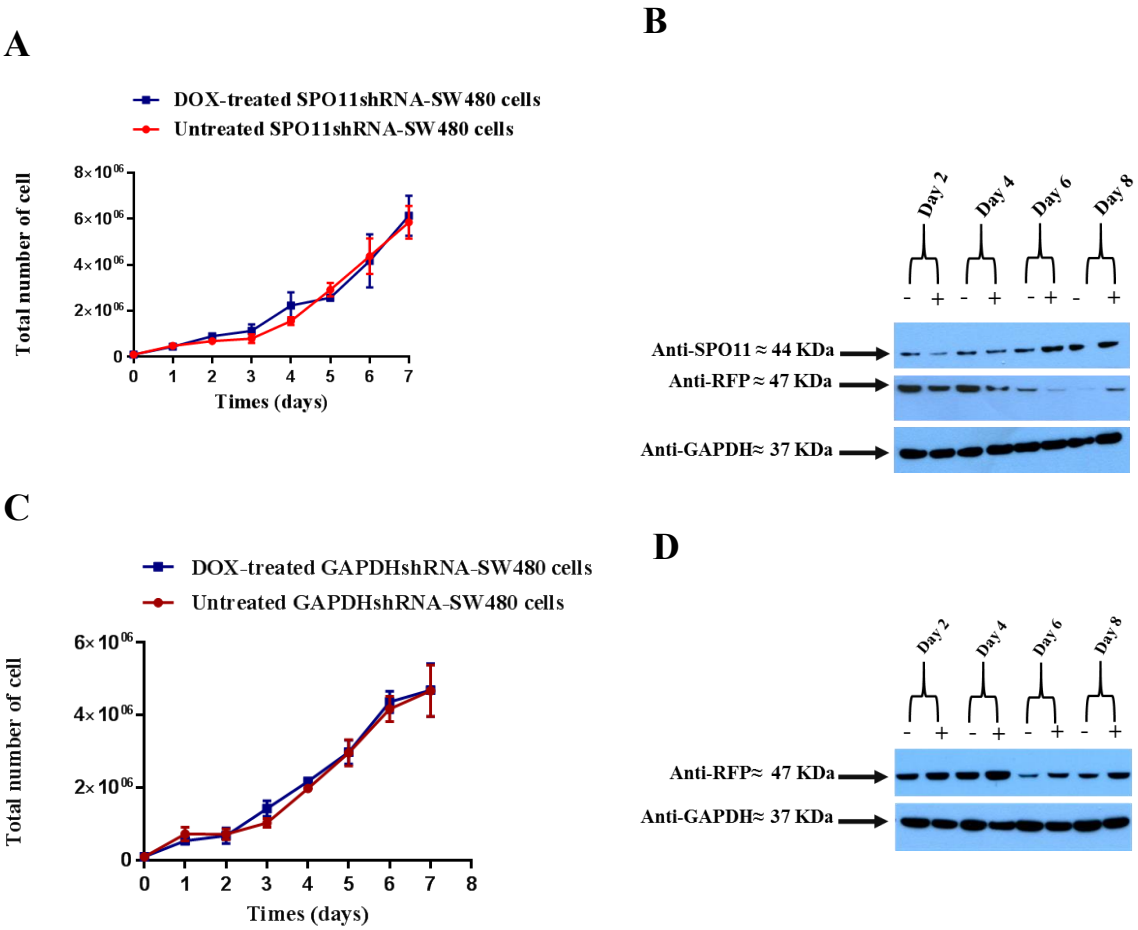



**Figure 4-5 Growth curves and western blot analysis showing SPO11 knockdown attempts in SW480 using shRNA.**

Untreated cells were used as control with no doxycycline, whereas GAPDH protein levels were used as a loading control. (A) Growth curves of SW480 cells transfected with SPO11 shRNA activated with 3 µg/mL of doxycycline (+) compared to untreated cells (no doxycycline) (-). (C) Growth curve of SW480 cells transfected with GAPDH shRNA activated with 3 µg/mL of doxycycline (+) compared with untreated cells (no doxycycline) (-). (B) and (D) western blot analysis showing the expression of SPO11 and RFP after activating SPO11 and GAPDH shRNA by doxycycline repectively to ensure a successful SPO11 knockdown. RFP was utilised as a transfection control. Knockdown of SPO11 was not observed when compared with the controls.

### 4.2.2 SPO11 knockdown: floating cells

#### 4.2.2.1 Attaching and floating cells

All attempts to reduce the level of SPO11 protein using various transfection reagents, different numbers of hits, varying concentrations of siRNA and different growing medium appeared to have no effect on the intensity of the 44 kDa band on western blot. Therefore, this suggested the siRNAs did not target SPO11 or the anti-SPO11 was not specific. However, we wished to explore this further, firstly, we varied cell numbers for transfections to determine whether this played a role. Secondly, we noticed SPO11-specific siRNA resulted in detachment of cells, with a large number of floating cells being generated. Protein extracts had previously been taken from attached cells only (not the floating cells). We hypothesised that active siRNA mediated depletion of SPO11 resulted in cell becoming detached (floating) and the remaining attached cells had not been successfully depleted for SPO11, so no depletion of protein levels was observed. Subsequently, this hypothesis is tested here.

A varying number of HeLa cells ($25 \times 10^3$, $50 \times 10^3$, $75 \times 10^3$, and $100 \times 10^3$ cells per well) were placed in culture wells prior to SPO11 siRNA transfection. After 24 hours post-transfection, three hits of SPO11 siRNA 2 and 4 were used to knockdown SPO11. Floating and attached cells were collected separately and the SPO11 protein level was checked using anti-SPO11 antibody by western blot. A reduction in SPO11 protein levels was observed in attached cells when lower cell concentrations were used, especially at $25 \times 10^3$ and $50 \times 10^3$ cells per well (Figure 4.6 A). Significant SPO11 knockdown was observed in floating cells for the various concentrations of HeLa cells (Figure 4.6 B). Interestingly, when the attached cells were harvested and counted after SPO11 siRNA 2 and 4 treatment, there were greatly reduced cell numbers compared to the controls. Moreover, hydroxyurea (HU) was used to create floating cells to be used as a positive control since these floating cells produced SPO11 protein (Figure 4.6 C), indicating detachment alone does not reduce SPO11 level.

Cell viability counts were carried out using trypan blue staining for attached cells as well as floating cells after SPO11 siRNA 2 treatment. The live attached cell number was seen to be greater than the dead attached cell number (Figure 4.7 A and B). However, the number of live floating cells after SPO11siRNA 2 knockdown was significantly reduced compared to dead cells (Figure 4.8 A and B).

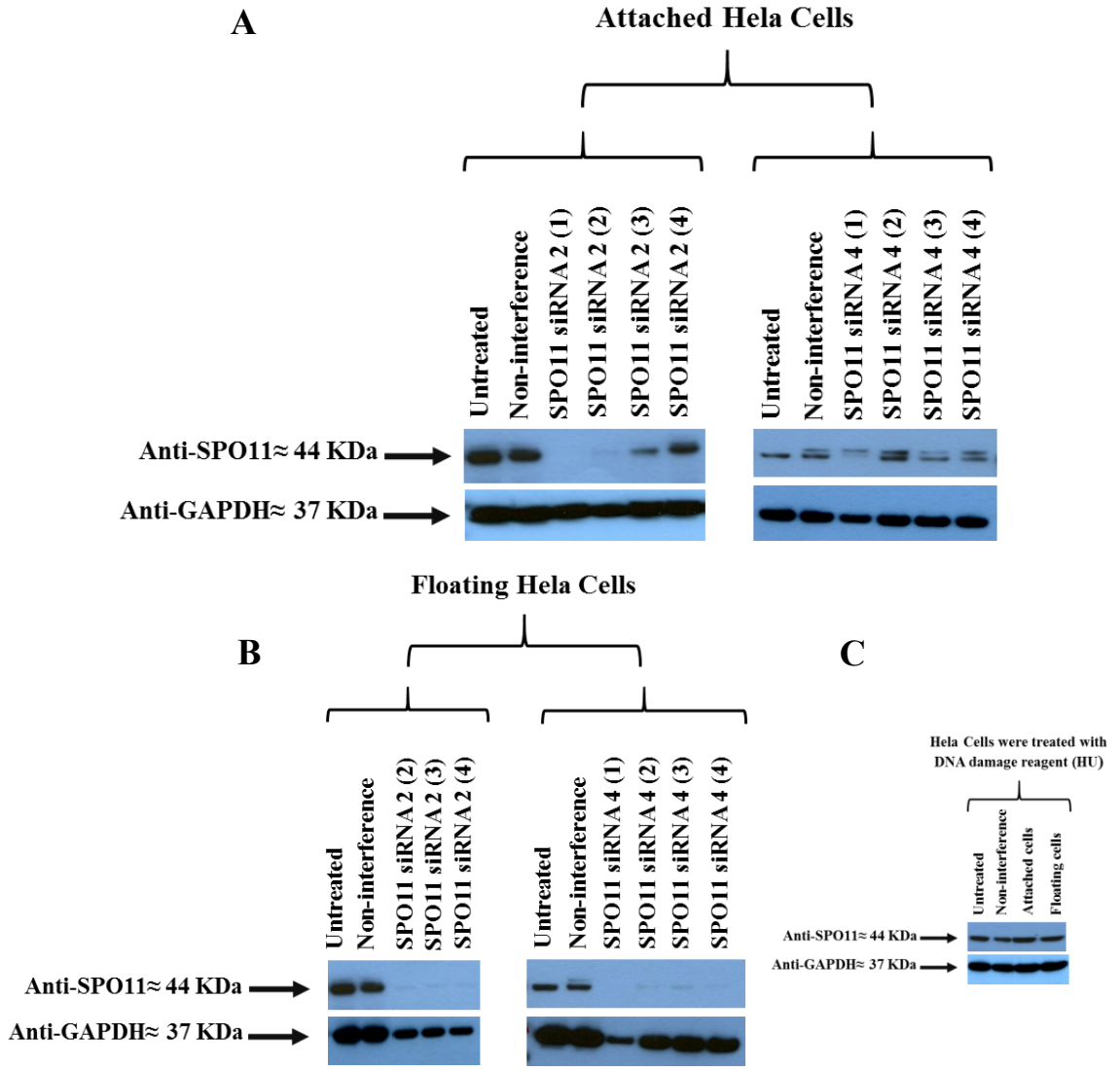



**Figure 4-6 Western blot analysis showing siRNA knockdown of SPO11 in attached and floating HeLa cells using siRNA 2 and siRNA 4.**
Untreated cells were used as positive controls, while cells treated with non-interfering siRNA were used as negative controls. GAPDH protein levels were used as a loading control (bottom). Different cell concentration per well in each 6-well plate was used: (1) 25,000 cells/ml, (2) 50,000 cells/ml, (3) 75,000 cells/ml, and (4) 100,000 cells/ml. (A) SPO11 siRNA experiment in attached Hela cells using HiperFect transfection reagent (3 hits). (B) SPO11 siRNA experiment in floating Hela cells using HiperFect transfection reagent (3 hits). Successful SPO11 knockdown was observed when siRNA 2 was used in 25,000 and 50,000 cells/ml in attached Hela cells. Full deletion of SPO11 was obtained when the protein was collected from floating cells. (C) Floating cells were induced with hydroxyurea (HU) reagent and SPO11 level was compared with that of the attached cells.

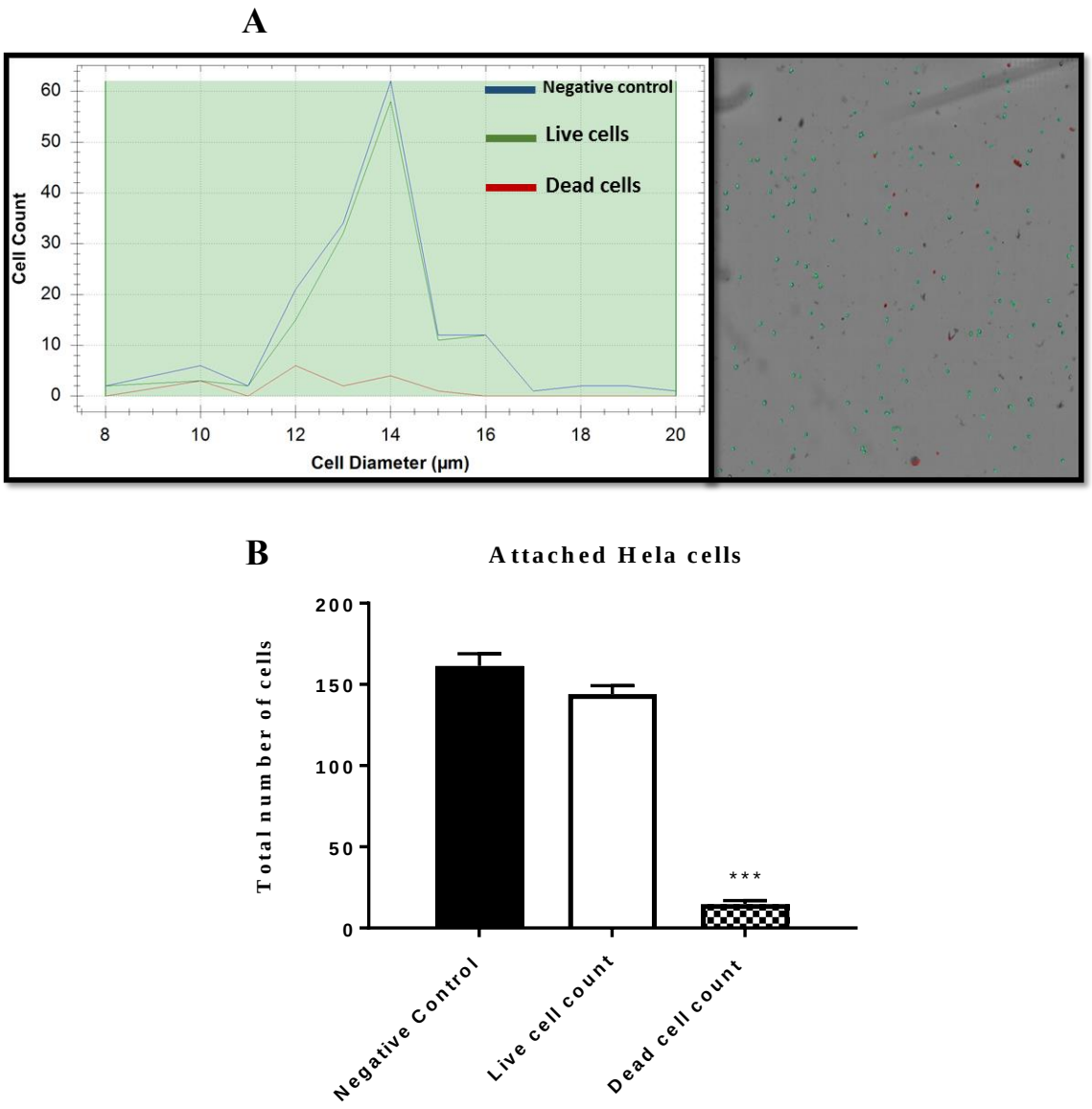



**Figure 4-7 The influence of knocking down SPO11 protein on attached HeLa cells using siRNA2.**

Knockdown was carried out using siRNA 2 in attached HeLa cells. Protein was then collected from attached cells followed by trypsinization with Biorad's TC 20 automated cell counter with trypan blue staining to take images and count cells. (A) Top left, graph compares live and dead cells to total cells. (A) Top right, the image shows live (green) and dead (red) cells. (B) Graph shows cell viability of attached HeLa cells after SPO11 depletion. The error bars represent the standard error for the total number of cells, calculated for three repeats (***P < 0.001). The number of attached dead cells was less than live ones when compared with negative control siRNA.

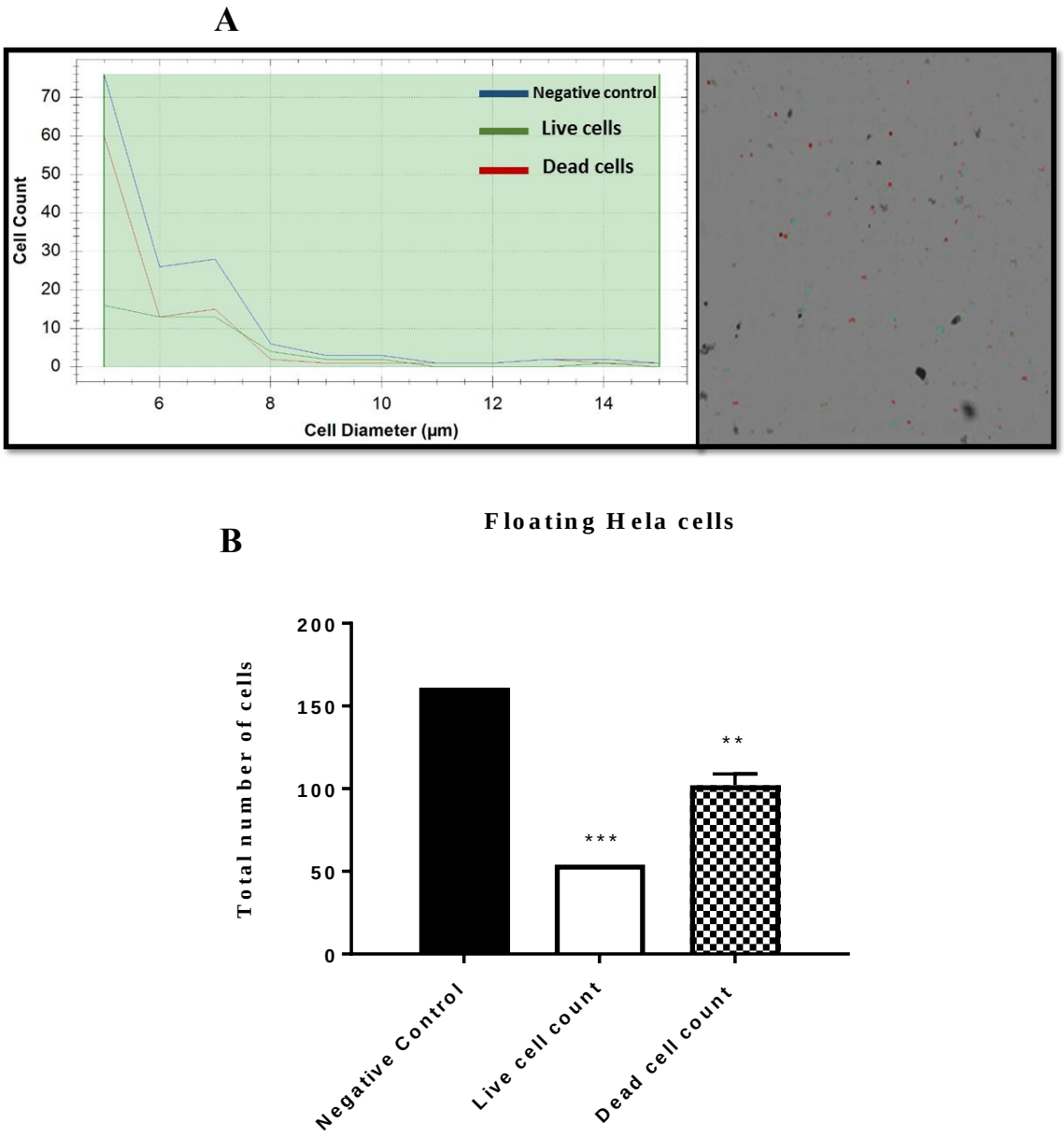



**Figure 4-8 The influence of knocking down SPO11 protein on floating HeLa cells using siRNA2.**

Knockdown was carried out using siRNA 2 in floating HeLa cells. Protein was then collected from floating cells followed by trypsinization with Biorad's TC 20 automated cell counter with trypan blue staining to take images and count cells. (A) Top left, the graph compares live and dead cells with total cells. (A) Top right, the image shows live (green) and dead (red) cells. (B) The graph shows cell viability of floating HeLa cells after SPO11 depletion. The error bars represent the standard error for the total number of cells, calculated for three repeats (**P < 0.01, ***P < 0.001). The number of floating dead cells was more than live ones when compared with negative control siRNA.

#### 4.2.2.2 Growing floating cells in different flasks

Floating HeLa cells appeared to have undergone SPO11 depletion after transfection with siRNA 2 and 4. To assess whether the floating cells were dead or undergone temporarily proliferation arrest, cells were transferred into fresh media using attached and a suspended T75 flasks, after washing to remove SPO11 siRNA. After 6 days, no proliferation was observed in either flasks when compared with the positive controls (untreated cells), therefore suggesting that the lack of SPO11 was probably affecting cell viability. In addition, it is likely that the reduction in proliferation due to SPO11 depletion is not reversible.

#### 4.2.2.3 Cell viability assays

Cell viability and cell proliferation of SPO11 depleted cells was monitored with various assays. In this section, two cell viability assays were performed, RealTime-Glo$^{TM}$ MT cell viability Assay and CellTiter 96 AQueous One Solution Assay. Firstly, absorbance (CPS) luminescence was utilised to determine the number of viable floating cells in culture by measuring the reducing potential of cells when the RealTime-Glo$^{TM}$ MT cell viability assay was used. Secondly, when CellTiter 96 AQueous One Solution Assay was performed, the absorbance at 490 nm was recorded to determine the number of viable floating cells.

Knockdown of SPO11 using siRNA 2 triggered HeLa cells to lose their ability to remain attached to the flasks, leading cells to float. Non-interfering siRNA and media only with no cells was served as the negative control. Cell viability analysis using both assays for floating HeLa cells had reduced viability when compared to negative controls (Figure 4.9 A and B).

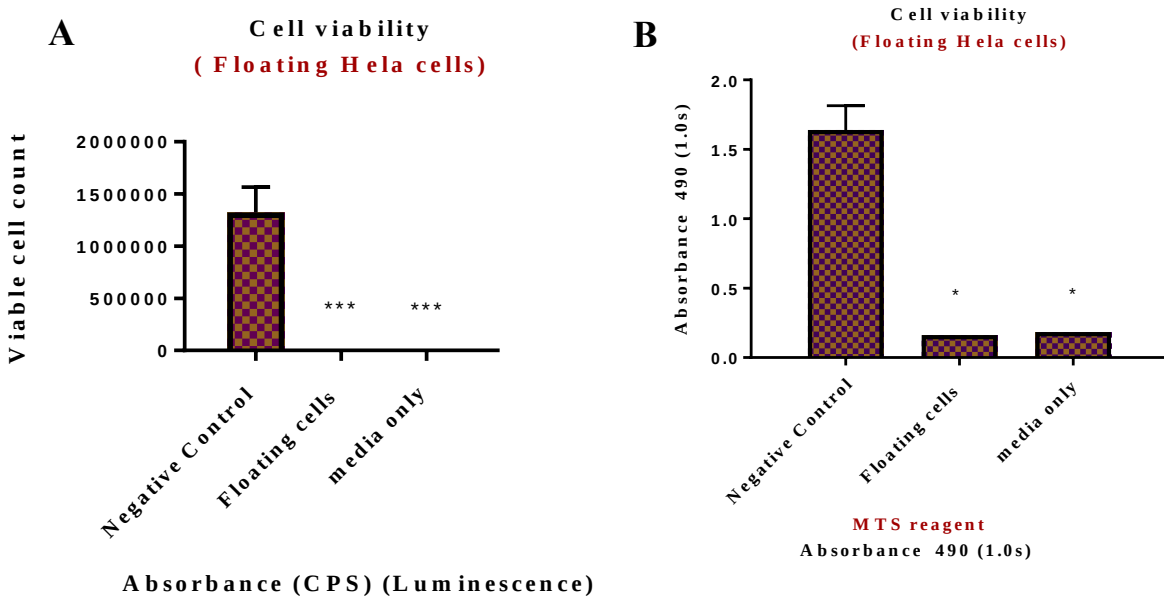



**Figure 4-9 HeLa cell viability after SPO11 knockdown.**
Media only (no cells) was used as the negative control. Graphs show cell viability of attached and floating cells subjected to three siRNA 2 hits using HiperFect transfection reagent using a 96-well plate reader. Floating cells were transferred to 96-well plates to carry out this experiment. In panel A, absorbance (CPS) luminescence was used to determine the number of viable cells in culture by measuring the reduction potential of cells in the RealTime-Glo$^{TM}$ MT cell viability assay. However, in panel B, absorbance at 490 nm was recorded to determine the number of viable cells in the CellTiter 96 AQueous One Solution proliferation assay. The error bars represent the standard error for the total number of cells, calculated for three repeats (*P < 0.05, ***P < 0.001). There is a clear reduction in floating cells viabilty when compared with negative control siRNA or media with no cells.

#### 4.2.2.4 Knockdown of SPO11 in floating cells does not induce cellular senescence

HeLa cells (50 × $10^3$ cell/mL) were seeded into 6-well plates and, 24 hours later, cells were treated with three hits of SPO11 siRNA 2 and 4. Non-interfering siRNA cells were used as negative controls while untreated cells served as positive controls. Three days after transfection, floating cells were collected and placed into new 6-well plates in order to perform a senescence assay for both floating and attached cells. The results suggest that knockdown of SPO11 using HeLa cells does not activate the senescent state as evaluated by lysosomal senescence-associated β-galactosidase activity at pH 6 (Figure 4.10).

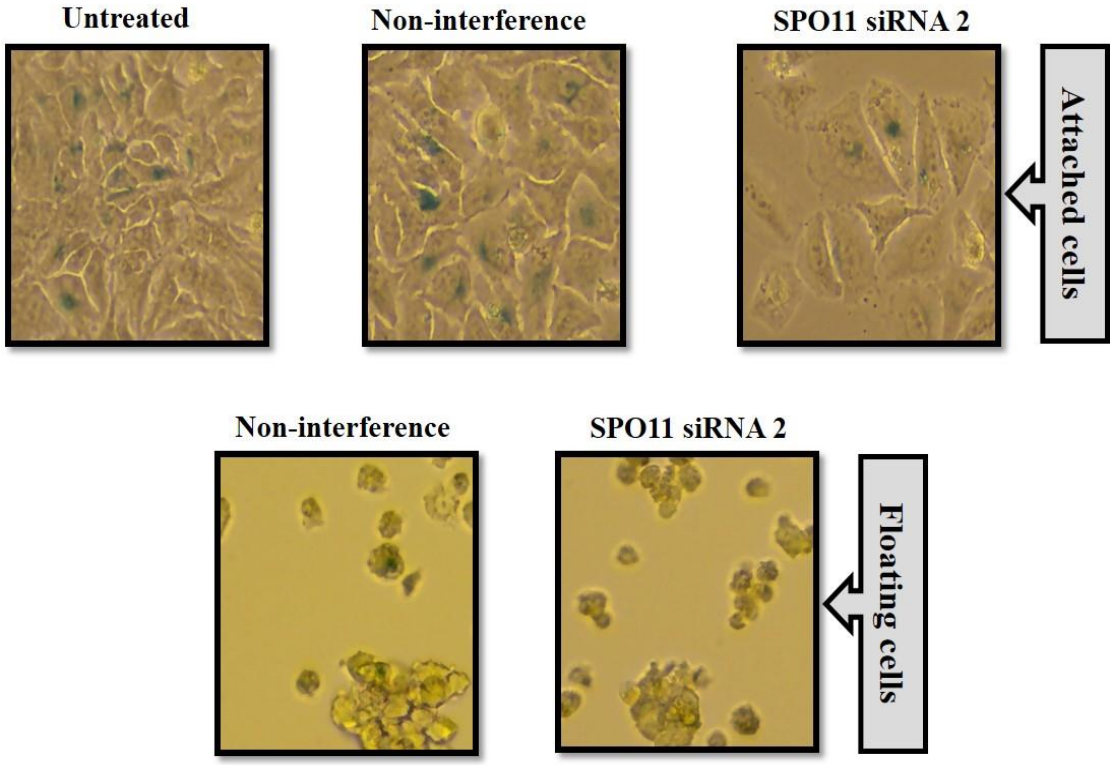



**Figure 4-10 Senescence β-galactosidase staining of attached and floating HeLa cells.**
The images show positive staining for β-galactosidase for untreated (positive controls) and non-interfering (negative controls) cells when attached and floating cells were used. No strong staining was detected in attached and floating HeLa cells after SPO11 siRNA 2 transfection (three hits).

## 4.3 Discussion

### 4.3.1 Summary of findings

The work in this chapter focused on SPO11 depletion using various techniques. The protein level of SPO11 was significantly reduced in floating cells using siRNA 2 and 4. Therefore, cell viability was affected in response to SPO11 protein reduction in floating cells compared to attached cells. The results also suggested that the inhibition of cell proliferation after SPO11 knockdown is not due to the cellular senescence state. In Chapter 5, the effective of SPO11 depletion on cell cycle and apoptosis in cancer cells will be analysed in order to determine the postulated function or role of the gene of interest in cell division regulation.

Taken together, these results suggest that SPO11 may have uncharacterised functions in cancer cells since differences in survival between SPO11-depleted cells compared to the controls were evident. These results also indicate that SPO11 might be required for cell proliferation and therefore it may become essential for cancerous cells, enabling SPO11 to serve as potential cancer biomarker and drug targets. Therefore, the possible function of SPO11 in cancer cells remains unclear and that this will be addressed in the final Chapter. In the next chapter, new experiments will be undertaken to assess the potential effect of SPO11 depletion on the cell cycle and on the induction of apoptosis in cancer cells.

### 4.3.2 SPO11 protein level in not reduced in attached cancer cells

SPO11 is an important protein in meiotic homologous recombination as it is required for the initiation of DNA double-strand breaks (DSBs). The expression of the *SPO11* gene has been detected in various cancer tissues, including melanoma, lung and cervical cancer tumours, but not in somatic tissues. Therefore, *SPO11* has been identified as a CTA gene (CT35) (Koslowski et al., 2002; Shannon et al., 1999). The localisation of human *SPO11* gene is on chromosome 20q13.2–13.3, an area which links to genomic instability and is amplified in different breast cancers such as aneuploidy (Shannon et al., 1999; Tanner et al., 1994), potentially linking it to oncogenesis.

In order to examine the function of a specific gene in distinct cell types, gene expression must be depletion or its genome mutated; however, the normal activity of the silenced gene in other cells must remain unaffected (Meneely & Bloom, 2013). Human gene function can be studied through an RNAi process known as post-transcriptional gene silencing. In this process, a sequence specific to the gene of interest is induced by double-stranded siRNA, leading to gene knockdown or silencing through mRNA cleavage. Since this evolutionarily conserved process has a high efficiency, noticeable specificity, and is easy applicable, it is considered to have valuable applications in therapeutic approaches both *in vitro* and *in vivo* (Behlke, 2006; Fire et al., 1998; Hannon, 2002; Rutz & Scheffold, 2004). Importantly, the effectiveness of siRNA knockdown varies and depends on the siRNA sequences and targeted genes (Ma et al., 2010). Recently, dependable RNAi methods involving RNA molecule usage or shRNA have been improved in order to target and silence the expression of specific genes (Owens et al., 2013).

Since the results obtained from chapter 3 suggested that the depletion of SPO11 affected cancer cell proliferation, addressing the validity of this depletion was determined herein. Therefore, different techniques such as siRNA, esiRNA and shRNA were used to downregulate the level of expression of the human *SPO11* gene in a range of cancer cell lines. Initially, no notable SPO11 knockdown was measured using WB after various time points and conditions, including the use of SPO11 siRNA molecules 2 and 4 and different transfection reagents in a wide range of human cancer cells such as SW480, HCT116, McF7 and Lovo cells. These results are consistent with previous results achieved by a colleague Dr. Ibrahim Aleailej, wherein depletion of SPO11 protein was not observed in attached cells following siRNA treatment that reduced proliferation.

### 4.3.3 SPO11 protein level is reduced in floating cancer cells

One possible explanation for previous outcomes is that cancer cell survival requires SPO11; therefore, cells die when SPO11 is depleted and so only cells gaining SPO11 remain viable / attached (I. Aldeailej, PhD. Thesis, Bangor University). Of note, attached cells were used to assess SPO11 knockdown by western blot, whereas the floating cells in the media, which may have reduced SPO11, were washed off. Therefore, it was assumed that SPO11 levels were not reduced in attached cells, whereas SPO11 was depleted and resulted in floating cells, with these losing their ability to remain attached to the plate, and dying.

For further investigation, floating cells were used to assess SPO11 knockdown in order to determine whether the loss of this protein caused cell death under these conditions. These results indicate that the SPO11 antibody used in this study is indeed identifying SPO11. Furthermore, SPO11 deletion experiments approves the expected size of this protein is approximately 44 KDa.

A cell viability count assay was performed using attached and floating cells after 72 hours of SPO11 siRNA treatment. No significant differences between total and live cells were observed for attached cells, whereas in floating cells, a significant difference was observed. SPO11 was still present in cells treated with hydroxyurea (HU), a DNA damage reagent that also causes cell flotation. This result confirms that floating alone does not cause SPO11 depletion.

### 4.3.4 Reducing SPO11 protein level is not inducing senescence state

Due to the success of reducing the level of SPO11 protein in floating cells, the influence of this reduction on cellular senescence was tested. The SPO11-depleted cells did not appear to undergo senescence, as assessed by β-galactosidase assay, since HeLa SPO11siRNA treated cells were negative for this assay. Detached cells were grown under normal growth conditions for an extended period of time, and they were unable to recover from proliferation arrest and were thought to be in a dormant and/or inactive state. Therefore, the knockdown of SPO11 in HeLa cells may trigger cells to enter a quiescent-like state, which indicates that SPO11-depleted HeLa cells might remain viable, but are no longer dividing.

# Chapter 5

## Cell cycle perturbation following SPO11 depletion

# 5. Cell cycle perturbation following SPO11 depletion

## 5.1 Introduction

In eukaryotic organisms, cell division occurs through meiosis and mitosis. Meiosis is a specialised form of cell division, leading to the generation of gametes. In contrast, mitosis occurs in somatic cells and germ cells, leading to the replacement of damaged tissues, and is required for tissue homeostasis and germ cell proliferation (Silkworth & Cimini, 2012; Walczak et al., 2010; Prieler et al., 2005). The mitotic cell cycle is sectioned into two major phases, the interphase and mitosis (M). Interphase is classified into three main stages, namely Gap-1 (G1), synthesis (S) and Gap-2 (G2) (Kronja et al., 2011). While the interphase includes DNA replication, the mitotic phase separates the cell into two daughter cells (Figure 5.1) (Deweerdt., 2012).

Cell cycle processes are strictly controlled by a series of checkpoints and specialised proteins, namely cyclins and cyclin-dependent kinases (CDKs) (Ziegler & Behl, 2014). Since CDKs are not active in their monomeric form, the association with a cyclin subunit, such as cyclin A or B, is required for their activation (Harper & Adams, 2001). CDKs, together with their cyclin partner, regulate cell cycle progression through protein phosphorylation (Figure 5.1) (Morgan, 1997; Russo, 1997; Solomon et al., 1992). Interestingly, as a response to any change in the extracellular microenvironment, such as a lack of growth factors, cells may enter a reversible resting state, G0, or the quiescence phase (Ziegler & Behl, 2014), whereas other cells may undertake programmed cell death or apoptosis. Apoptosis is one of the main cell death mechanisms and is considered as a selected death pathway, involving metabolic and genetic alterations with the aim to maintain cell proliferation (Wyllie et al., 1980; Formigli et al., 2000; Sperandio et al 2000; Debnath et al., 2005). Additionally, cells enter apoptosis as a result of physiological and pathological stimulations such as aging, disease, irradiation and cancer drugs (Norbury & Hickson, 2001). The activation of cysteine protease groups, known as caspases, and the stimulation of proteins such as Bcl-2, may lead to apoptotic death in some cells (Elmore., 2007).

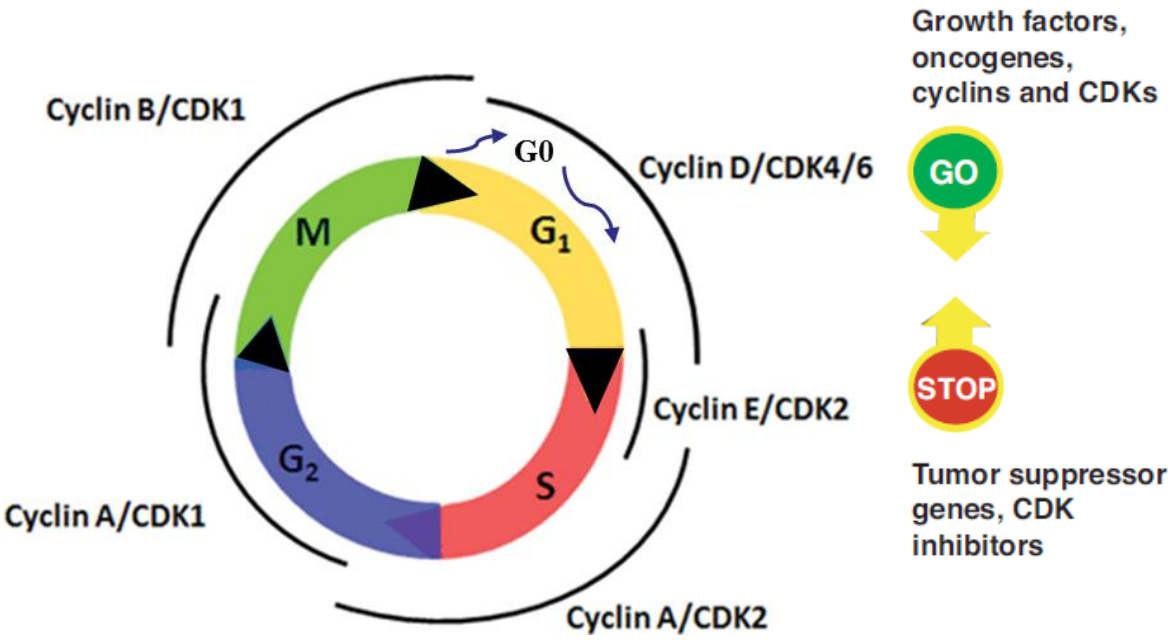



**Figure 5-1 Cell cycle progression and cyclin–CDK regulation.**
Several cyclins, such as A, B, C and D, form cyclin–CDK complexes that control the cell progression during the different stages of the cell cycle. Cell cycle progression is highly regulated by various checkpoints (black arrows) at the end of G2, M and G1 (Suryadinata et al., 2010). G0 indicates resting state (non-dividing stsate). Additionally, cell cycle processes are promoted via growth factors, oncogenes and cyclins (indicated by the 'Go' green arrow), whereas tumour suppressor genes and CDK inhibitors inhibit cell cycle progression (indicated by the 'stop' red arrow) (Sandal., 2002).

**Table 5-1 Panel of proteins used to determine apoptosis following SPO11 knockdown**

| Protein | Function | Reference |
|---|---|---|
| **Caspase-3** | Control cell death | (McIlwain et al., 2013) |
| **Bak** | Control caspase activation | (Karch et al., 2013) |
| **Bcl-2** | Control cell death | (Hardwick & Soane, 2013) |
| **PARP** | Control DNA repair, cell death, chromatin functions and genomic stability | (Chaitanya,. et al 2010; Herceg & Wang, 2001) |

SPO11 function is functionally associated with various proteins in meiosis such as eukaryotic, RAD51 and DMC1, the eukaryotic RecA orthologues. The RAD51 protein plays a role in meiotic and mitotic recombination, while DMC1 only plays a role in meiosis (Tsai & McKee, 2011; Neale & Keeney, 2006). Alteration in meiotic DSB levels can be measured through the association of RAD51/DMC1 with the breaks (Cole et al., 2012). Interestingly, elimination of foci formation of DMC1 and RAD51 can occur by a reduction in SPO11 levels, leading to inhibition of the formation of meiotic recombination and synapsis (Baudat et al., 2000; Romanienko et al., 2000).

Since apoptosis defects is a major hallmark of cancer cells, the response of casepase-3 cleavage and Bak, Bcl-2 and Parp proteins to SPO11 depletion (Table 5.1) will be used in the present chapter to investigate whether SPO11 knockdown leads to apoptotic cell death. Moreover, since the cell cycle is an ordered set of events, controlled by CDKs in conjunction with cyclins, the effectiveness of SPO11 knockdown on several cell cycle proteins (Table 5.2) will also be determined. Furthermore, the relationship between SPO11 depletion and the levels of RAD51 and DMC1 will be assessed to determine whether SPO11 activity in cancer cells has any link to recombinase activity.

**Table 5-2 Panel of proteins used to determine cell cycle changes following SPO11 knockdown**

| Protein | Function | Reference |
|---|---|---|
| **Cyclin A** | S phase regulation | (Chen et al., 2009; Marais et al., 2010) |
| **Cyclin B** | M phase regulation | (Chen et al., 2009; Marais et al., 2010) |
| **Cyclin E** | G1-S phase regulation | (Odajima et al., 2011) |
| **CDK2** | G1-S phase regulation | (Chen et al., 2009; Marais et al., 2010) |
| **P21** | CDK/cyclin complex inhibition | (Marques et al., 2013) |
| **P27** | CDK/cyclin complex inhibition | (Pippa et al., 2012) |
| **Rb** | S phase entry and cell growth obstruction | (Weinberg, 1995; Vermeulen et al., 2003) |
| **PRb** | S phase entry when phosphorylated | (Weinberg, 1995; Vermeulen et al., 2003) |
| **c-myc** | Monitoring of several cell cycle regulators such as CDKs and cyclins and control cell proliferation | (Dang et al., 1999; Lutz et al., 2002; Meyer & Penn, 2008) |

## 5.2 Results

### 5.2.1 SPO11 knockdown in Hep-G2, SW480 and HeLa cells: cell cycle regulation

To assess whether SPO11 depletion influences cell cycle regulation or induces apoptosis we depleted SPO11 in three cancer cell lines, Hep-G2, SW480 and Hela cells. We then used protein extracts from attached depleted cells to assess cell cycle regulators / apoptosis. Firstly, we confirmed SPO11 depletion in these cells. Knockdown of SPO11 was performed using siRNA 2 in three different cancer cell lines, namely Hep-G2, SW480 and HeLa (Figure 5.2, Figure 5.3 and Figure 5.4 respectively). HiperFect (10 µL) was used as the transfection reagent and non-interfering and untreated cells served as the controls. GAPDH protein level was used as a control to test the quality of whole cell extract (WCE) loading; the correct predicted size of approximately 44 kDa was obtained using the SPO11 Abcam (ab81695) antibody.

Western blot results in Hep-G2, SW480 and HeLa cells showed a significant level of SPO11 knockdown after three hits in attached cells, compared with the negative and positive controls. GAPDH levels appeared to be relatively equal, suggesting that differences in SPO11 levels were due to successful SPO11 knockdown (Figure 5.2 A, Figure 5.3 A and Figure 5.4 A respectively). According to the results in Chapter 4, siRNA 2 and 4 reduced SPO11 protein levels to the same degree using low cell number, and therefore siRNA 2 was used herein.

Interestingly, all cells displayed signs of cell proliferation reduction following SPO11 siRNA treatment (Figure 5.2 B, Figure 5.3 B and Figure 5.4 B), compared with negative controls. In addition, cell viability was significantly affected by SPO11 knockdown compared with non-interfering cells as observed in cell viability counts with trypan blue (Figure 5.2 C, Figure 5.3 C and Figure 5.4 C).

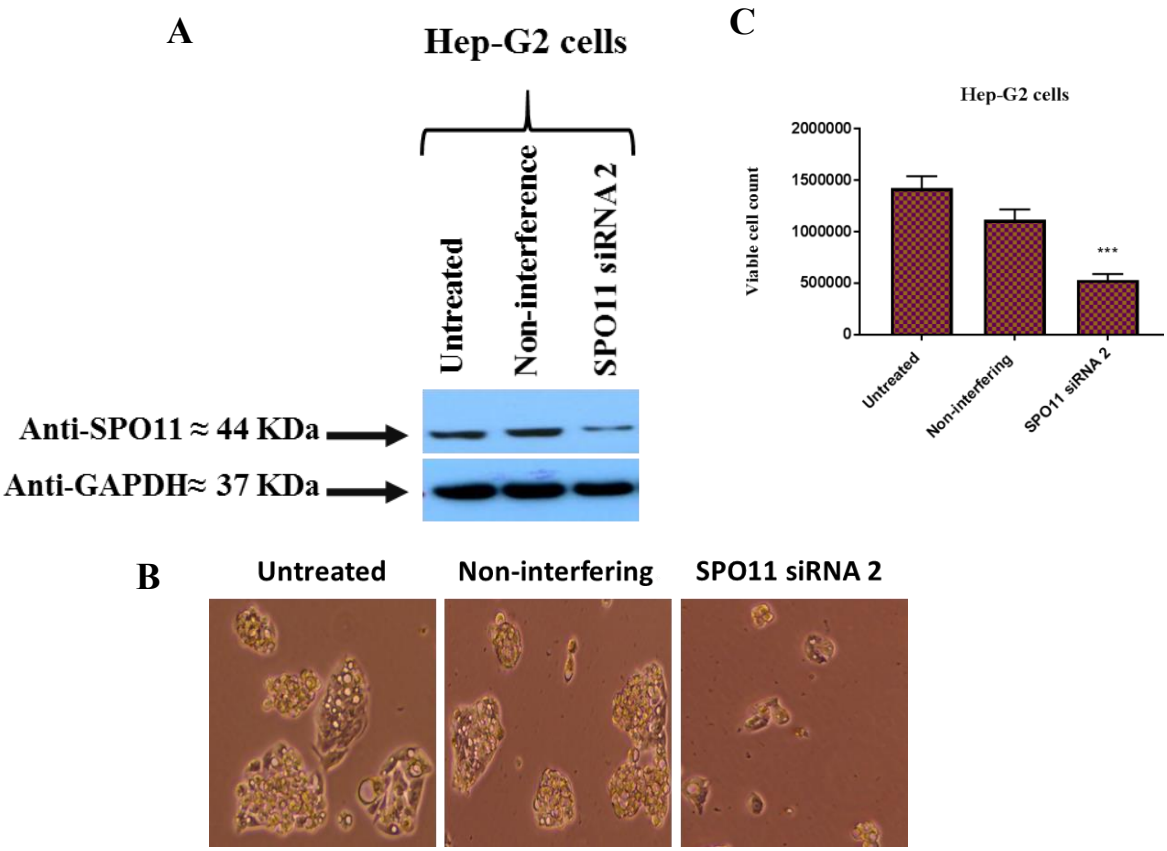



**Figure 5-2 SPO11 knockdown in Hep-G2 cells using siRNA 2.**
Untreated Hep-G2 cells were used as positive controls, while cells treated with non-interfering siRNA were used as negative controls. GAPDH protein levels were used as a loading control (bottom). (A) Western blot analysis confirm that SPO11 knockdown at the protein level in Hep-G2 cells was achieved using siRNA 2. (B) Cell images taken before trypsinisation to assess cell density using an ECLIPSE inverted microscope (5× lens). (C) Plot showing cell viability of Hep-G2 cells subjected to siRNA 2. Error bars represent the standard error for the total number of cells, calculated for three repeats (***P < 0.001). Knockdown of SPO11 using siRNA 2 significantly affected cell viability compared to non-interfering or untreated cells.

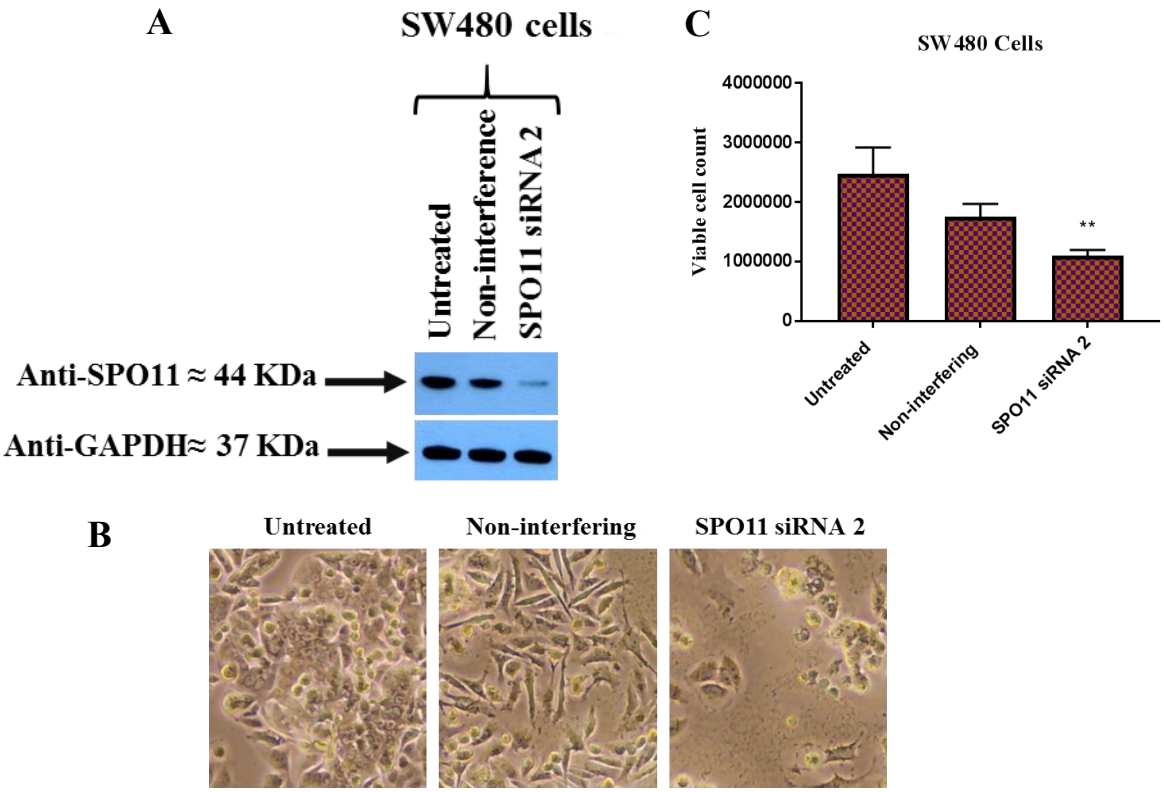



**Figure 5-3 SPO11 knockdown in SW480 cells using siRNA 2.**
Untreated SW480 cells were used as positive controls, while cells treated with non-interfering siRNA were used as negative controls. GAPDH protein levels were used as a loading control (bottom). (A) Western blot analysis confirmed that SPO11 knockdown at the protein level in SW480 cells was achieved using siRNA 2. (B) Cell images taken before trypsinisation to assess cell density using an ECLIPSE inverted microscope (5× lens). (C) Plot showing cell viability of SW480 cells that were subjected to siRNA 2. The error bars represent the standard error for the total number of cells, calculated for three repeats (**P < 0.01). Knockdown of SPO11 using siRNA 2 significantly affected cell viability compared to non-interfering or untreated cells.

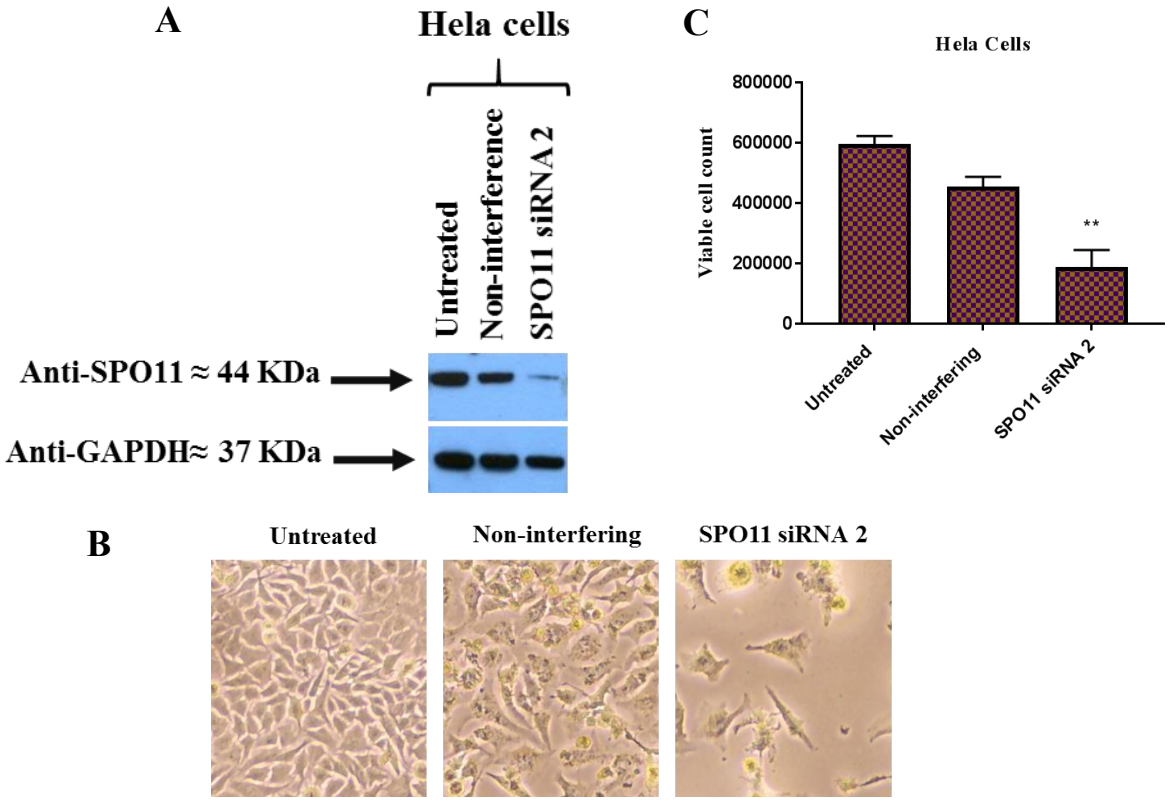



**Figure 5-4 SPO11 knockdown in HeLa cells using siRNA 2.**
Untreated HeLa cells were used as positive controls, while cells treated with non-interfering were used as negative controls. GAPDH protein levels were used as a loading control (bottom). (A) Western blot analysis confirmed SPO11 knockdown at the protein level in HeLa cells using siRNA 2. (B) Cell images taken before trypsinisation to assess cell density using an ECLIPSE inverted microscope (5× lens). (C) Plot showing cell viability of HeLa cells subjected to siRNA 2. Error bars represent the standard error for the total number of cells, calculated for three repeats (**P < 0.01). Knockdown of SPO11 using siRNA 2 significantly affected cell viability compared to non-interfering or untreated cells.

### 5.2.2 SPO11 knockdown does not induce apoptosis

Apoptosis, or programmed cell death, is considered a controlled regulated process and a normal component of the healthy development of organisms. Moreover, when cells are about to undergo apoptosis, several changes are initiated, including the activation of caspases, Bak, Bcl-2 and Parp proteins. Therefore, the degree of apoptosis can be assessed through evaluation of the above protein levels (Choudhury et al., 2012). Herein, caspase-3, Bak, Bcl-2 and Parp protein levels were assessed following SPO11 depletion, with GAPDH protein levels used as a control to test the quality of WCE loading.

The response to apoptosis was assessed using caspase-3 cleavage in Hep-G2 and SW480 attached cells following SPO11 depletion (Figure 5.5 and Figure 5.6). The results show that no apoptosis response was observed, since no cleavage could be detected in SPO11 knockdown (siRNA 2) lanes in both cell lines, as compared to the Jurkat cell line positive control.

Three apoptotic proteins, namely Bak, Bcl-2 and Parp, were examined in order to assess changes in their levels when SPO11 protein levels were reduced due to SPO11 siRNA 2 knockdown in Hep-G2 cells (Figure 5.7). The results indicate that the apoptotic proteins Bak, Bcl-2 are not affected by the reduction of SPO11 levels in Hep-G2. Additionally, there is no cleavage was detected when Parp protein was examined.

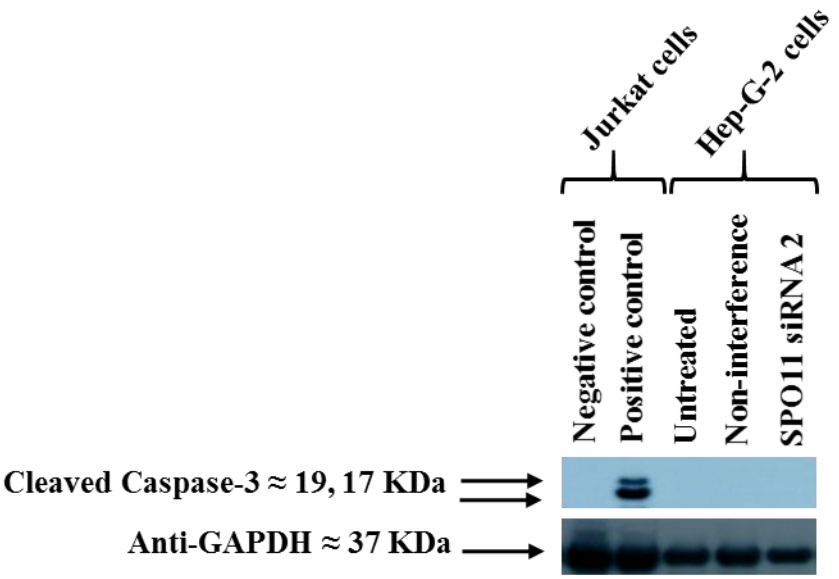



**Figure 5-5 Western blot detection of cleaved caspase-3 in Hep-G2 cells.**
Cytochrome C-treated Jurkat cell extract was considered as the apoptotic positive control, while untreated Jurkat cells were used as negative controls. GAPDH protein levels were used as a loading control (bottom). Knockdown of SPO11 using siRNA 2 in Hep-G2 did not induce apoptosis as determined by detection of any cleaved caspase-3.

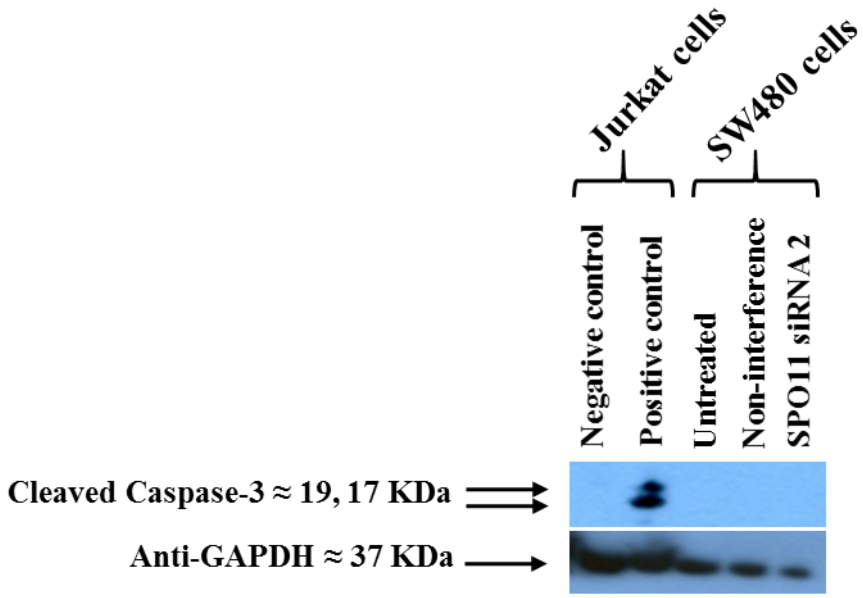



**Figure 5-6 Western blot detection of cleaved caspase-3 in SW480 cells.**
Cytochrome C-treated Jurkat cell extract was considered as the apoptotic positive control, while untreated Jurkat cells were used as negative controls. GAPDH protein levels were used as a loading control (bottom). Knockdown of SPO11 using siRNA 2 in SW480 cells did not induce apoptosis as determined by detection of any cleaved caspase-3.

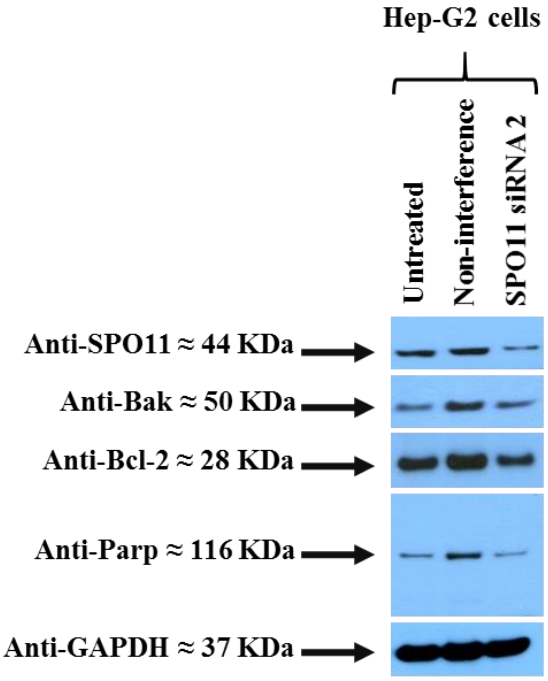



**Figure 5-7 Western blot analysis for some apoptotic proteins in response to siRNA 2 knockdown of SPO11 in Hep-G2 cells.**
Untreated Hep-G2 cells were used as positive controls, while cells treated with non-interfering were used as negative controls. GAPDH protein levels were used as a loading control (bottom). No change can be seen in anti-Bak and anti-Bcl-2 proteins and no cleavage in anti-Parp (cell signalling #9542s) protein in terms of SPO11 knockdown using siRNA 2 in Hep-G2 cells.

### 5.2.3 A reduction in SPO11 protein levels results in a decrease in the level of several cell cycle proteins

Western blot analysis was used to assess different cell cycle regulations following SPO11 knockdown using siRNA 2 in Hep-G2 and SW480 attached cells. Antibodies against cyclin A2, B1, E1 and CDK2 as well as P21, P27, PRb, Rb and c-Myc were used in order to assess any potential arrest or changes in the cell cycle progression due to SPO11 depletion. GAPDH protein levels were used as controls to test the quality of WCE loading.

Western blot analysis showed a significant decrease in cyclin A2, cyclin B1 and CDK2 following SPO11 depletion in both Hep-G2 and SW480 cell lines (Figure 5.8 and Figure 5.9 respectively). Additionally, cyclin E1 was significantly affected in Hep-G2 cells (Figure 5.8). Also P21, P27, PRb, and Rb protein levels in both cell lines were reduced as a result of SPO11 knockdown with siRNA 2 (Figure 5.10 A and Figure 5.11 A). Furthermore, the reduction in SPO11 protein levels through siRNA 2 knockdown led to a reduction in c-Myc protein levels in both cell lines, Hep-G2 and SW480 (Figure 5.10 B and Figure 5.11 B). The reduction of those cell cycle proteins were compared to non-interfering cells (negative controls) and untreated cells (positive controls).

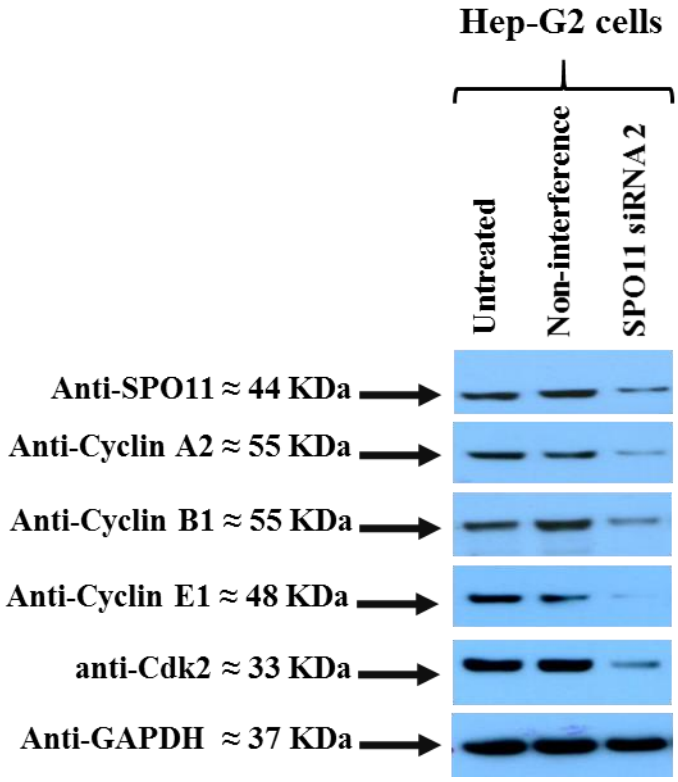



**Figure 5-8 Western blot analysis of cell cycle proteins in response to siRNA 2 knockdown of SPO11 in Hep-G2.**
Untreated Hep-G2 cells were used as positive controls, while cells treated with non-interfering were used as negative controls. GAPDH protein levels were used as a loading control (bottom). Changes can be seen in cell cycle protein levels in response to SPO11 knockdown using siRNA2 in Hep-G2 cells.

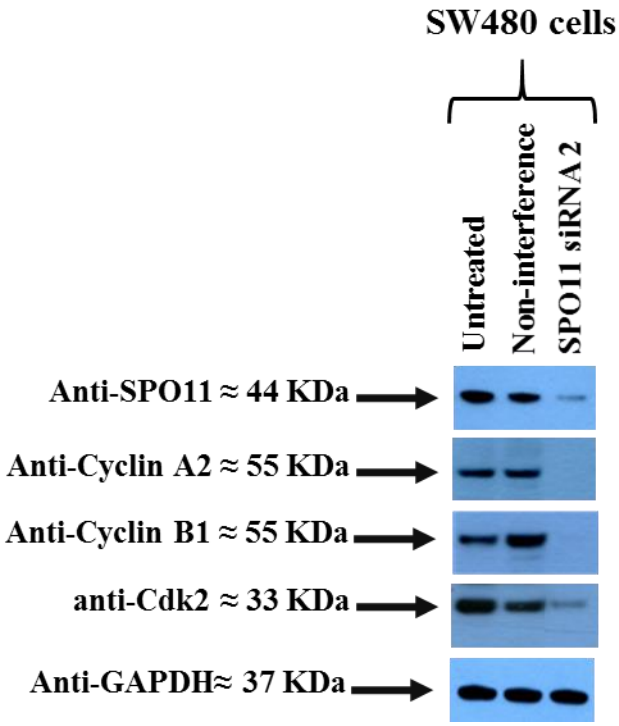



**Figure 5-9 Western blot analysis of cell cycle proteins in response to siRNA 2 knockdown of SPO11 in SW480.**
Untreated SW480 cells were used as positive controls, while cells treated with non-interfering were used as negative controls. GAPDH protein levels were used as a loading control (bottom). Changes can be seen in protein levels in response to SPO11 knockdown using siRNA2 in SW480 cells.

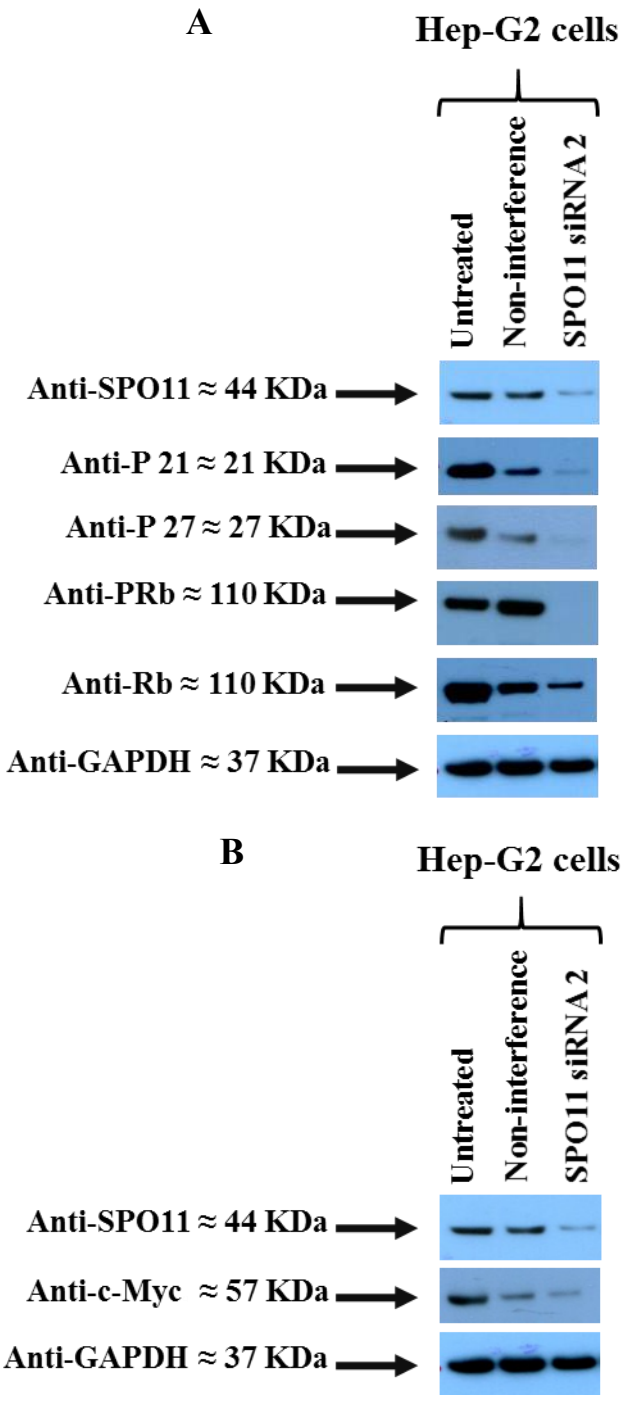



**Figure 5-10 Western blot analysis of cell cycle proteins in response to siRNA 2 knockdown of SPO11 in Hep-G2.**
Untreated Hep-G2 cells were used as positive controls while cells treated with non-interfering were used as negative controls. GAPDH protein levels were used as a loading control (bottom). (A) and (B) show the reduction of cell cycle proteins in response to SPO11 knockdown.

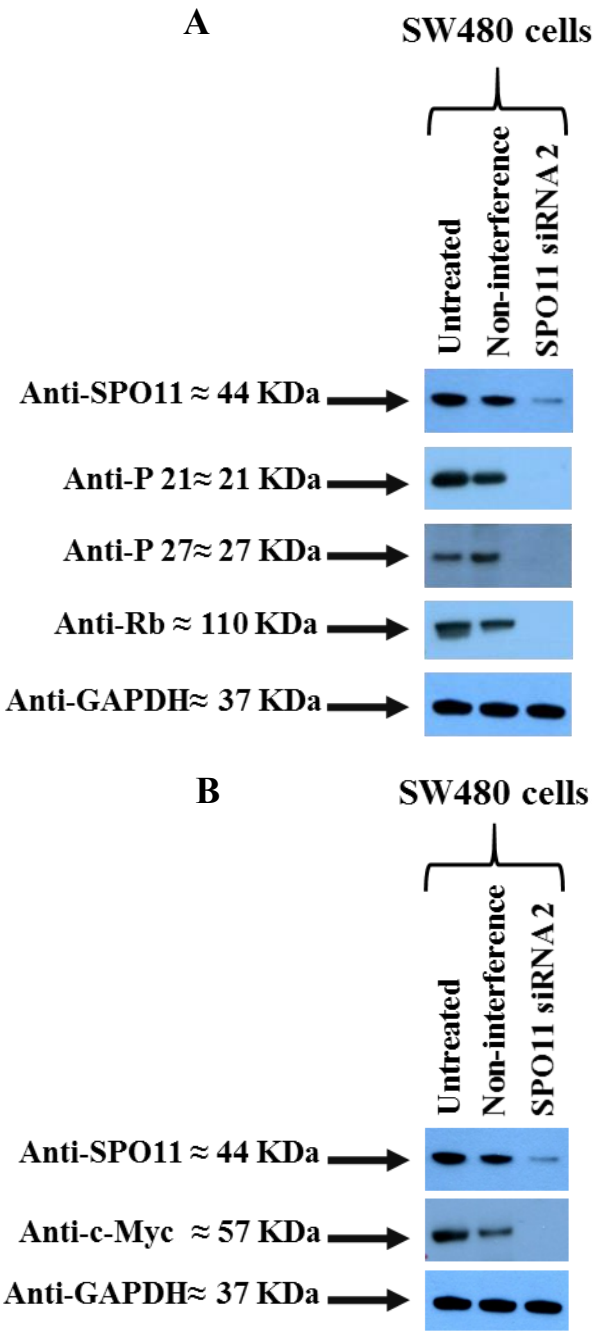



**Figure 5-11 Western blot analysis of cell cycle proteins in response to siRNA 2 knockdown of SPO11 in SW480.**
Untreated SW480 cells were used as positive controls, while cells treated with non-interfering were used as negative controls. GAPDH protein levels were used as a loading control (bottom). (A) and (B) show the reduction of cell cycle proteins in response to SPO11 knockdown.

### 5.2.4 The influence of SPO11 knockdown on the RecA family of proteins (RAD51 and DMC1)

The RecA family of proteins in mammals includes RAD51 and DMC1 proteins, which play an essential role in DNA repair and maintenance (Kawabata et al., 2005). Western blot was performed in order to evaluate the effectiveness of SPO11 knockdown using siRNA 2 in Hep-G2 and SW480 cells on RAD51 and DMC1 proteins. Untreated and non-interfering cells served as positive and negative controls, respectively. GAPDH protein level was used as control to test the quality of WCE loading.

The depletion of SPO11 protein levels due to siRNA 2 knockdown in Hep-G2 and SW480 cells led to reduced RAD51 protein level compared with the negative and positive controls (Figure 5.12 A and Figure 5.13 respectively). The GAPDH protein appears to be relatively equal, thus suggesting that gel loading is comparative. However, no significant effect on DMC1 protein levels was observed following SPO11 depletion in Hep-G2 cells (Figure 5.12 B).

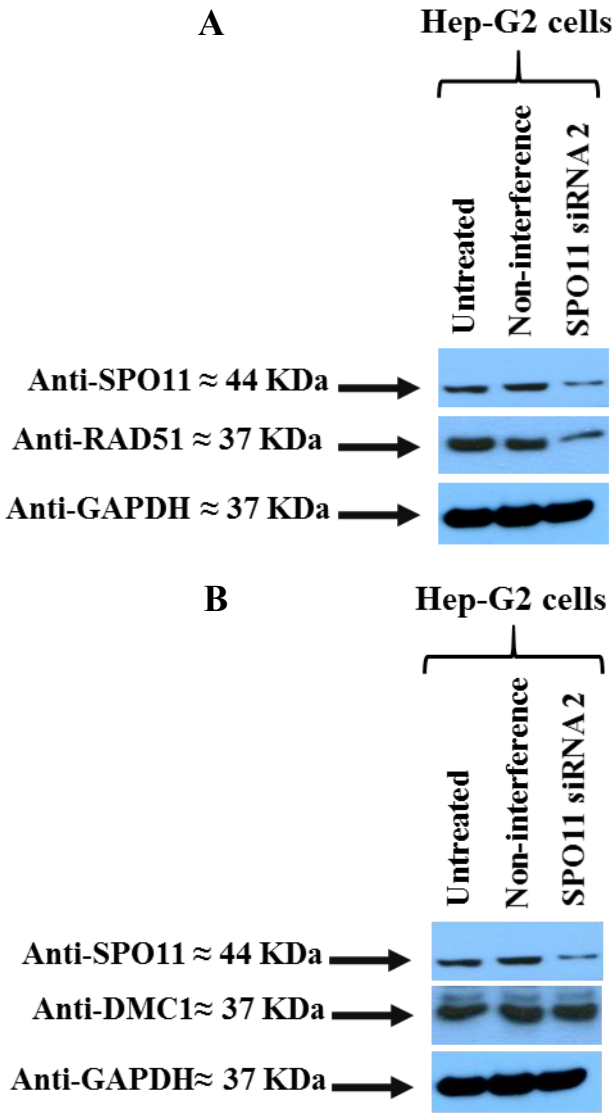



**Figure 5-12 Western blot analysis of RAD51 and DMC1 proteins against siRNA 2 knockdown of SPO11 in Hep-G2 cells.**
Untreated Hep-G2 cells were used as positive controls, while cells treated with non-interfering were used as negative controls. GAPDH protein levels were used as a loading control (bottom). (A) and (B) show the levels of RAD51 and DMC1 proteins in response to SPO11 knockdown. Knockdown of SPO11 reduced RAD51 protein levels, whereas it did not affect DMC1 protein levels.

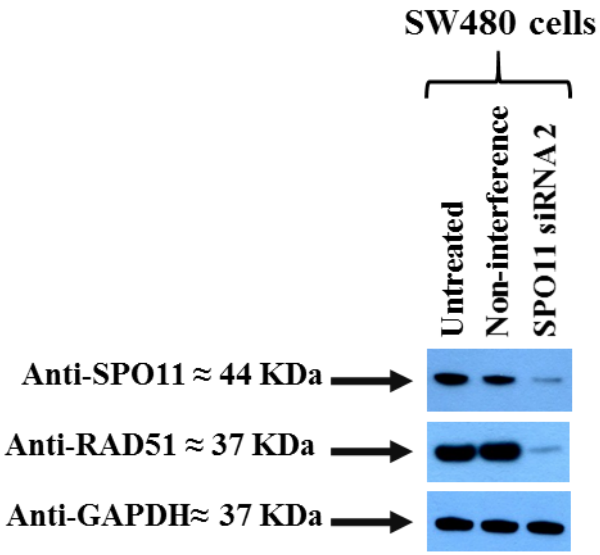



**Figure 5-13 Western blot analysis showing changes in RAD51 protein levels in response to siRNA 2 knockdown of SPO11 in SW480 cells.**
Untreated SW480 cells were used as positive controls, while cells treated with non-interfering were used as negative controls. GAPDH protein levels were used as a loading control (bottom). The image shows a reduction in RAD51 protein levels in response to SPO11 knockdown.

## 5.3 Discussion

### 5.3.1 Summary of findings

The reduction of SPO11 protein levels does not induce apoptosis, but may alter the level of cell cycle regulatory proteins such cyclin A2, B1, E1 and CDK2. Therefore, SPO11 may be essential for the completion of cell cycle. Furthermore, the level of RAD51 protein was significantly affected due to the depletion of SPO11. Taken together, these data indicate that the anti-proliferative effect of SPO11 depletion may be used as an effective cancer treatment. Thus, results presented herein are therefore useful in the selection of proper target molecules for cancer therapies using SPO11. Ultimately, functional investigation is required in order to firmly establish the potential role of SPO11 in the cell cycle process. The aim of the present chapter was to determine whether SPO11 depletion is sufficient to affect apoptosis and cell cycle regulators; this was assessed in two cancer cell lines, Hep-G2 and SW480 cells. Moreover, the effectiveness of SPO11 depletion on RAD51 and DMC1 proteins was also examined to assess whether recombination was influenced, and whether the meiosis-specific DMC1 functions in cancer cells alongside SPO11.

### 5.3.2 SPO11 depletion in three different cancer cell lines

Based on the results from the previous chapter regarding the successful knockdown of SPO11 with siRNA 2 and 4 molecules in attached cells, the effect of SPO11 knockdown in three different cell lines (Hep-G2, SW480 and HeLa) was assessed herein. SPO11 protein levels were significantly reduced in all three cell lines using siRNA 2 when compared to the negative controls. Furthermore, following the reduction of SPO11 protein levels, cell viability and cell density were significantly affected. Therefore, SPO11 depletion achieved in Hep-G2 and SW480 attached cells were used in further experiments, including the assessment of apoptosis and cell cycle processes.

### 5.3.3 SPO11 depletion is not inducing apoptosis

Since apoptosis resistance is considered a hallmark of various tumour cells, anti-apoptotic factors may provide attractive therapeutic strategies for cancer treatment. Indeed, the action of traditional cancer therapeutic methods, such as chemotherapy and radiotherapy, depends on the

stimulation of apoptosis; therefore, the most common reason for therapeutic resistance is apoptosis deficiency (Teodoro et al., 2012). Caspases, or cysteine protease enzymes that are usually present in cells in their inactive state, can be used to assess the apoptotic process. Cleaving of these enzymes can lead to their activation, resulting in the triggering of apoptosis (Dash., 1994). Caspase-3 was used herein to examine whether SPO11 depletion induces apoptosis through this pathway. Moreover, the response to apoptosis may also be evaluated by assessing other protein levels, including proteins in the Bcl-2 family for instance Bak, which are thought to be activated via apoptosis signals such as cell stress and growth factor deficiency (Table 5.1). The Bcl-2 family controls apoptosis through regulation of mitochondrial permeability (Choudhury et al., 2012). Apoptosis can be characterised by an increase in Bcl-2/Bak levels, caspase activation and PARP protein cleaving (Tzifi et al., 2012). Although PARP protein has many members (See Section 1.1.4), anti-PARP tested in this chapter (cell signalling #9542s) is presented almost all of PARP members. We observed that caspase-3 was not activated and there was no significant change in the level of Bcl-2, Bak proteins and no cleavage of PARP protein, which serves as apoptotic marker and maintained cell viability was observed, thereby demonstrating that SPO11 knockdown did not induce apoptosis. However, although there was no apoptosis evident in these attached cells, it is possible that apoptotic cells had detached and were not assessed here. Further experiment should be performed in order to examine apoptosis in floating cell with depleted SPO11.

### 5.3.4 SPO11 depletion is affecting cell cycle proteins

Cancer progression involves multiple mechanisms and processes, all of which play a vital role in tumour development, which may be exploited in the diagnosis and therapy of such a complicated disease. Cancer hallmarks include unlimited cell division caused by the dysfunction of genes responsible for cell cycle regulation. Importantly, cell cycle processes are tightly regulated, resulting in an accurate balance between apoptosis and proliferation in normal tissues in order to avoid tumour development (Sandal., 2002). Nevertheless, cancer cells have the ability to escape from such regulation, resulting in uncontrolled cell division (Sherr., 1996).

In mouse testis, phosphorylation of HORMAD1, HORMAD2 and SMC3, which play an important role in meiotic chromosome synapsis, requires SPO11. Additionally, the involvement of SMC3 phosphorylation in the pachytene checkpoint has been suggested. Recombination and synapsis or DNA damage are regulated via this checkpoint (Fukuda et al., 2012). However, if

a pachytene-like checkpoint was applied to cancer cells, it could have an effect on the cell cycle. Therefore, the reduction in SPO11 protein levels observed herein would be sufficient to effect the physiological function of the cell cycle.

Lindesy and Scott (2013), showed that SPO11 and another three proteins, including SCPI (homologous chromosome paring), HORMAD1 (meiotic synapse regulation) and REC8 (meiosis cohesion protein) were found in melanoma cells. The potential role of meiotic proteins in chromosomal instability during mitosis has been reported, but still needs further research. Furthermore, during meiosis in yeast, Rad51 and Dmc1 mediated recombination are stimulated by the meiotic Hop2-Mnd1 heterodimer, which also plays important role in the clustering of alternative lengthening of telomeres (ALT) (Petukhova et al., 2003; Petukhova et al., 2005). Interestingly, Hop2-Mnd1 have been shown to be needed for ALT in cancer cell lines (Cho et al., 2014).

Cyclin–CDK complexes control cell cycle processes, including the order, the metabolic and synthetic effort of each phase and cellular function. The phosphorylation and dephosphorylation states are considered the main control signals at every cell cycle phase. Eight cyclin (cyclin A–H) and nine CDK (CDK1–9) proteins are known, whereas only CDK1, 2, 4 and 6 are known to have an impact on the cell cycle (Behl & Ziegler, 2014b; Mariaule & Belmont, 2014). Generally, cyclins are classified into three groups: G-S cyclins, S-phase cyclins, and M cyclins (Finn et al,. 2016; Sandal, 2002). Uncontrolled proliferations, a hallmark of cancer, may be caused by any defect in these proteins. Furthermore, the activity of the CDKs can be regulated either via phosphorylation or CDK inhibitor proteins such as p21 (Behl & Ziegler, 2014b). The transition from one phase of the cell cycle to the next and the regulation of the cellular environment are monitored by sensing mechanisms, known as checkpoints (Figure 5.1). Therefore, roles of such checkpoints during tumour progression should be considered since they regulate cell division (Giacinti & Giordano, 2006).

Several cyclin proteins were analysed in the present study, including cyclin A2 (presents in growing somatic cells), cyclin B1 (regulates other cyclins) and cyclin E1 (overexpresses in human tumours) (Table 5.2). CDK2 can be activated through the production of cyclin A and E proteins, forming a complex (Giacinti & Giordano, 2006).

The results herein showed that the protein levels of cyclin A (marker of S phase), cyclin B (G2 to M transition), and cyclin E (G1 to S transition) (Figure 5.14) were dramatically decreased following SPO11 depletion. The levels of CDK2 protein, which regulates the G1-S transition, were significantly reduced due to the depletion of SPO11. In the late stage of G1, the synthesis of cyclin A has an important role in the G1-S transition, and therefore cyclin A reduction may lead to blocking of the S phase (Girard et al., 1991). Furthermore, in yeast, Cdk2 regulates both the G-S and G2-M transition, hence inhibition of the activity of Cdk2 might result in the prevention of mitotic cell division (Figure 5.15). It has been suggested that CDK2 is a positive regulator of the CDK2–cyclin B complex (Guadagno & Newport, 1996; Tong et al., 2010). During tumorigenesis, abolition of the G1-S checkpoint may result from altered expression of cyclins D, E and A in some cancers (Hartwell & Kastan, 1994). Remarkably, it has been suggested that repression of CDKs, as cell cycle regulators and RNA transcription factors, may have an application in cancer therapy, yet further work is needed (Geleta et al,. 2016). This finding may add further evidence to support the hypothesis that SPO11 inhibits the proliferation of cancer cells through a mechanism involving the regulation of cell cycle proteins. Thus, the present results suggest that the SPO11 protein may have an interesting function in mitotic cell division in cancer cells. This possible role in cancer progression may be related to the regulation of the cell cycle. Other possibility that the clear reduction in these regulators may indicate that these cells have left cell cycle into a quiescent/quiescent-like state or the time point all are reduced in metaphase-anaphase, leading to cells failure at this point (Figure 5.14). Therefore, this finding may indicate that the SPO11 protein is essential for cancer cells, supporting the idea of using this gene as an attractive target for anti-cancer agents.

Rb plays a key role in the connection of cell cycle control to the transcriptional machinery through the interaction with E2F protein, resulting in cell growth control (Sandal., 2002). The regulation of a major G1 checkpoint, S phase entry and cell proliferation are considered additional functions of PRb (phosphorylated Rb). The E2F protein participates in DNA replication during the S phase. In addition, it is prevented from working as a transcription factor when interacting with Rb, which should be in the non-phosphorylated state.

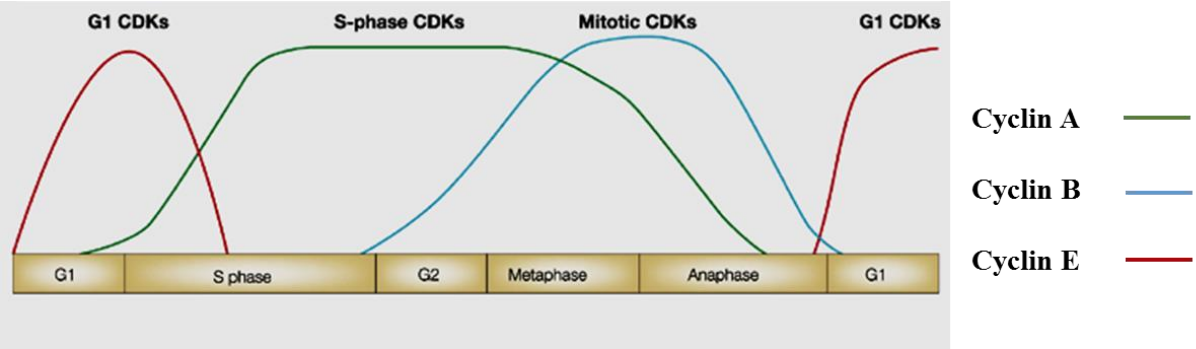



**Figure 5-14 Graphical model illustrating the expected cell cycle profile of various cyclins, including cyclin A (green), cyclin B (blue) and cyclin E (red) (Bardin & Amon, 2001).**

Interestingly, mutated Rb leads to phosphorylation of Rb, and therefore it cannot bind to E2F, resulting in unlimited cell division at S phase (Hatakeyama & Weinberg, 1995; Ewen, 1994). Moreover, loss of function of Rb and/or Rb mutation may derive tumorigenesis in several human cancers. Thus, deletion of Rb may lead to release of E2F protein, resulting in cancer development (Sandal, 2002; Macaluso, et al. 2005; Weinberg, 1995). The main cell cycle regulatory genes affected in tumours are G-S transition genes and Rb pathway regulation (Sherr., 1996). In the present study, the depletion of SPO11 significantly decreased the levels of PRb, whereas total Rb was slightly affected. This observation suggests that the SPO11 protein may regulate or de-phosphorylate Rb, leading to promotion of the interaction between E2F and Rb. As a result, this interaction may inhibit cell proliferation at the S phase.

Anti-cancer agents that reduce p21 levels in cells may lead to an effect on the dependence of DNA synthesis just before the M phase, causing cells to synthesise DNA but without mitosis (Waldman et al., 1996). In addition, G2/M and G1 cell cycle checkpoints are regulated by p21 protein (Harris & Levine, 2005). The present results demonstrated that the depletion of SPO11 protein led to decrease the protein levels of p21. It has been reported that p21 may play a critical role in supporting cell cycle progression (LaBaer et al., 1997). Therefore, cell proliferation in cancer cells may be affected by the reduction of p21 protein levels.

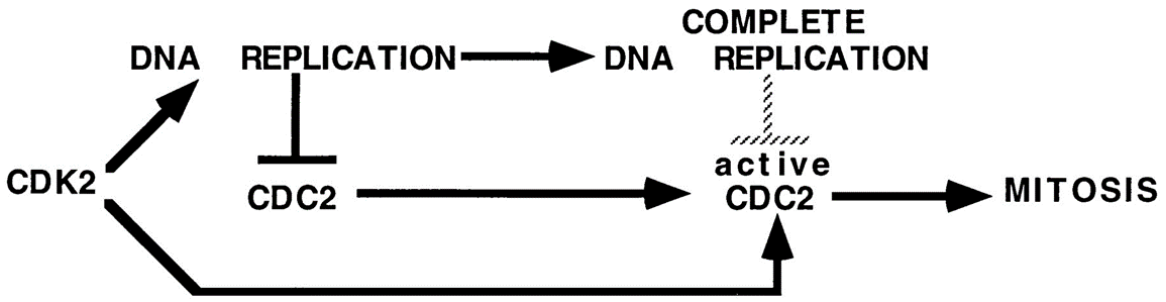



**Figure 5-15 Schematic model showing how CDK2 activity controls DNA replication at the S phase.**
The activity of CDK2 is inhibited via a specific pathway, activated by ongoing DNA replication. As a result, inhibition of CDK2 can lead to prevention of mitotic cell division until complete DNA replication during the S phase. The negative feedback signal is indicated by the black and white striped symbol (Guadagno & Newport, 1996).

The human *Myc* (*c-Myc*) gene encodes an oncogenic transcription factor. *MYC* is deregulated in nearly half of human solid tumours and in leukaemia and is found to be associated with tumor progression. Further, the association of this protein with tumour development has been suggested (Bretones et al,. 2015). Myc has important roles in cell cycle regulation in some cell types and, in conjunction with other genes, encodes most of the cell cycle regulators, including CDKs and cyclins. A study carried out in rat fibroblasts reported that 37 out of 87 cell cycle-related genes are controlled by *Myc* overexpression in the Kyoto Encylopedia of Gene and Genomes (Yap et al., 2011). Moreover, cell cycle inhibitors, for instance p21 and p27, are antagonised or controlled by Myc. In addition, DNA replication can be induced through Myc, which also controls some mitotic genes (Bretones et al., 2015).

Interestingly, cell proliferation rates are correlated with *MYC* mRNA and protein expression. Therefore, faulty cell cycle re-entry of quiescent cells may result from MYC downregulation. A reduction in MYC levels via antisense RNA may lead to slowing down of the rate of cell proliferation (Bretones et al,. 2015; Wang et al., 2008). Additionally, Myc plays an important role in inducing apoptosis; for example, downregulation of Myc in interleukin-3-dependent can lead to G0/G1 accumulation and growth arrest (Askew et al., 1991; Hoffman & Liebermann, 2008). In this study, and based on the idea that active growth depends on Myc levels, the depletion of SPO11 had a significant effect on cell proliferation, as demonstrated by decreasing Myc protein levels using western blot, suggesting that SPO11 might regulate, to some extent,

the production of Myc protein, leading to a reduction in cell growth and cell viability rates. The possible action of SPO11 in controlling Myc protein in cancer cells may support the proposal of considering SPO11 as a drug target.

### 5.3.5 SPO11 depletion is affecting RAD51 protein level

Carofiglio et al. (2013) showed that the number of SPO11-dependent meiotic DSBs in oocytes and spermatocytes was reduced in the case of point mutation in the *SPO11* gene. Therefore, the present results may indicate that the depletion of SPO11 protein decreased the number of formed DSBs. Furthermore, the accumulation of RAD51 and DMC1 proteins in the absence of DSBs is not predictable, since they play an important role in the repair of DSBs through the initiation of recombination filaments (Carofiglio et al., 2013). The results presented in this chapter reveal that RAD51 protein levels were reduced when SPO11 was depleted. However, unexpectedly, DMC1 protein levels remained unaffected. SPO11 knockdown may have a topo-like activity in S-phase or metaphase and anaphase, leading to significant effect on the RAD51 protein, thus confirming the interesting correlation between SPO11 and RAD51 proteins.

# Chapter 6

## Cloning of *SPO11*::*N-HA* and *SPO11*::*C-Myc* into the mammalian expression system Tet-on 3G

# 6. Cloning of SPO11::N-HA and SPO11::C-Myc into the mammalian expression system Tet-on 3G

## 6.1 Introduction

Based on the findings presented in the previous chapters, SPO11 protein seems to play a major role in regulating the cell cycle at some point and affects cell proliferation in different cancer cells. To identify the possible function of SPO11 in cancer cells, a specific, validated antibody needs to be identified. Recombinant proteins are highly recommended for use in determining the specificity of antibodies and studying their function / location (Terpe, 2003).

The production of recombinant proteins is considered an indispensable molecular tool, and different strategies have been established to accomplish this (Morlacchi et al., 2012). The most common method is to add a small peptide sequence of 3–12 amino acids to a target protein using recombinant DNA technique. Detecting a fusion protein can be achieved by using the tag-specific monoclonal antibody for the tag peptide. Antibodies of such tags have been used in many assays, such as western blot, immunofluorescent and immunoprecipitation assays. The major advantage of using extremely small sequences in these tags is that they are less likely to interfere with the biological function of proteins of interest (Bucher et al. 2002; Gill et al. 1996).

Of the more recognised epitope tags, hemagglutinin (HA) and Myc tags are considered here. The HA tag is produced in the human influenza virus hemagglutinin protein and contains nine amino acids, whereas the Myc tag is derived from the C-Myc protein and has 10 amino acids (Table 6.1).

Furthermore, these tags can be designed for either the C-terminus or the N-terminus. In addition, HA and Myc tags are detected by the highly specific anti-HA and anti-Myc monoclonal antibodies respectively (Gill et al. 1996; Morlacchi et al. 2012; Kramer et al. 1999).

**Table 6-1 Protein and DNA Sequences of HA and Myc Tags**

| Tags | Protein sequence | DNA sequence |
|---|---|---|
| HA | YPYDVPDYA | TAC CCA TAC GAT GTT CCA GAT TAC GCT |
| Myc | EQKLISEEDL | GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG |

The aim of the research described in this chapter is to clone *SPO11*::*N-HA* and *SPO11*::*C-Myc* into the pTRE-3G plasmid to visualise and investigate the effectiveness of overexpression of SPO11 in cancer cells and to determine SPO11 antibody specificity. In addition, immunofluorescent assay is used to test any potential co-localisation of SPO11 protein and its tags with N-HA and C-Myc.

## 6.2 Results

### 6.2.1 Preparation of pTRE-3G vector, *SPO11*::*N-HA* and *SPO11*::*C-Myc*

The Tetracycline-inducible Gene Expression System (Tet-On-3G) is commonly used in mammalian cells for inducible gene expression. In addition, in the tested cells, a high level of transgenes can be expressed when controlling Tet-On 3G transactivator protein. The level of target gene expression is regulated by the TRE3G promoter ($P_{TRE3G}$) in the presence or absence of doxycycline (DOX) (Gossen & Bujard 1992; Vigna et al. 2002; Loew et al. 2010). Figure 6.1 shows the pTRE-3G map and its restriction enzyme site. HeLa and HCT116 Tet-on 3G stable cell lines, which are used in this experiment, were created in our lab by my colleague Mariam Alahdal.

The *SPO11* gene was cloned into both pCMV MCS N-HA (Figure 6.2) and pCMV MCS C-Myc (Figure 6.3) vectors in our laboratory D7 by Dr. Ellen Vernon then *SPO11*::*N-HA* and SPO11::*C-Myc* cDNA was confirmed by sequencing. In this study, *SPO11*::*N-HA* and SPO11::*C-Myc* was cloned into pTRE-3G vector and then integrated into HCT116 and HeLa Tet-on 3G stable cell lines in order to tag and overexpress *SPO11*, as well as to test the specificity of anti-SPO11 antibody.

The digested and purified pTRE-3G and *SPO11*::*N-HA* with *Nde*I and *Pst*I restriction enzymes were used to clone *SPO11*::*N-HA* (Figure 6.4). Undigested pTRE-3G vector was used as a control. In contrast, *Bam*Hl and *Pst*I restriction enzymes were used to digest and purify pTRE-3G vector to clone *SPO11*::*C-Myc* (Figure 6.5). Both inserts *SPO11*::N-HA and SPO11::C-Myc were also separated on the gel after digestion and purification to show a single band of approximately 1200 bp as shown in Figure 6.4 and Figure 6.5 respectively. Agarose gel was used before and after digestion enzymes to verify the digestion efficiency of the vector. After separating the samples on agarose gel, vector and insert bands were cut out under long-wave UV light. DNA purification for those bands was carried out to purify DNA to be ready for cloning. The purified *SPO11*::*N-HA* and *SPO11*::*C-Myc* fragments were ligated into the purified, digested pTRE-3G plasmid. Ampicillin resistance was used for selection of the recombinant plasmids after transformation into the *E. coli*.

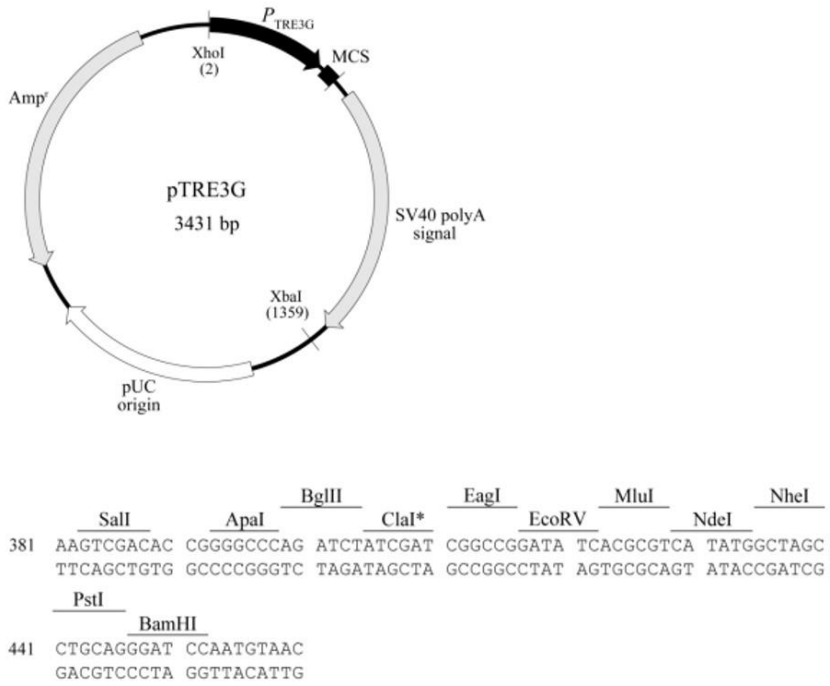



**Figure 6-1 Map of pTRE-3G vector.**
PTRE-3G is a 3431-bp Inducible Expression Plasmid System designed for use with the Tet-On 3G System and contains the following components: $P_{TRE3G}$ (third generation Tet-responsive promoter), multiple cloning site (MCS), SV40 polyA signal, pUC origin of replication and ampicillin resistance gene (Ampr; β-lactamase; adapted from Clontech, http://www.clontech.com).

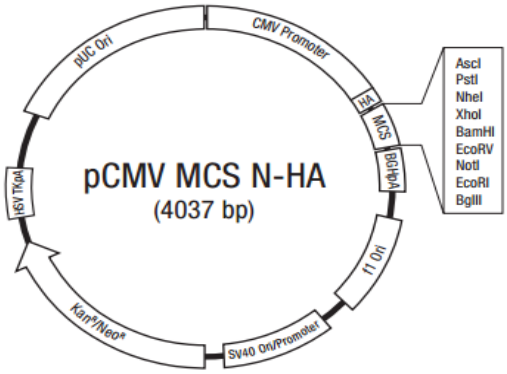



**Figure 6-2 Map of pCMV MCS N-HA.**
pCMV MCS N-HA is a 4037-bp, epitope-tagged, multiple-cloning-site (MCS) mammalian expression vector and contains the following components: cytomegalovirus (CMV) promoter, MCS, N-terminal human influenza hemagglutinin (HA) epitope tag and kanamycin/neomycin marker for drug selection (adapted from ThermoFisher; https://www.thermofisher.com).

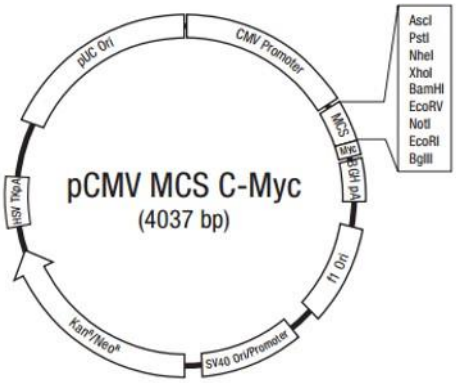



**Figure 6-3 Map of pCMV MCS C-Myc.**
pCMV MCS C-Myc is a 4037-bp, epitope-tagged, multiple-cloning-site (MCS) mammalian expression vector and contains the following components: cytomegalovirus (CMV) promoter, MCS, C-terminal Myc epitope tag and kanamycin/neomycin marker for drug selection (adapted from ThermoFisher; https://www.thermofisher.com).

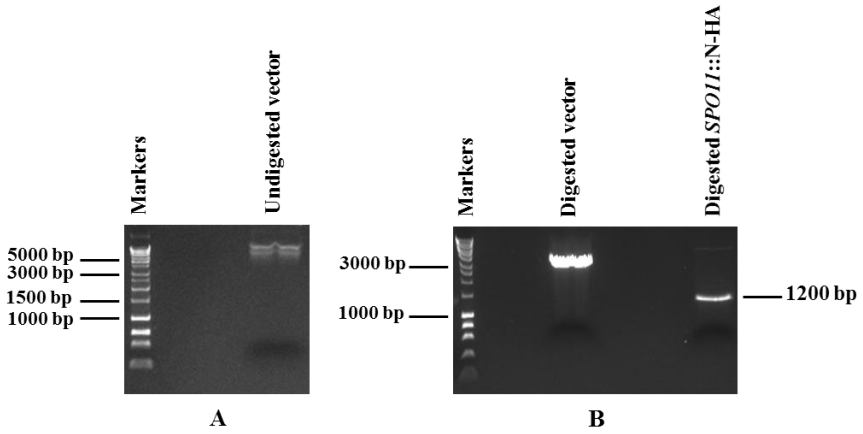



**Figure 6-4 Amplification of pTRE-3G vector and digestion of the *SPO11*::N-HA construct with *Nde*I and *Pst*I restriction enzymes.**
The image of 1% agarose gel shows undigested pTRE-3G vector as control to be compared with the digestion vector (panel (A)). Panel (B) (left) displays the vector after digestion by *Ned*I and *Pst*I restriction enzymes and purification. The enzymes linearize the 3431bp plasmid into one single fragment. Panel (B) (right) shows the amplification of the *SPO11*::*N-HA* sequence from the pCMV MCS N-HA vector, which was already cloned with the *SPO11* cDNA. This band was digested with the same restriction enzymes, *Nde*I and *Pst*I, and then underwent purification. HyperLadder 1 kb plus (5 µl) was used as marker.

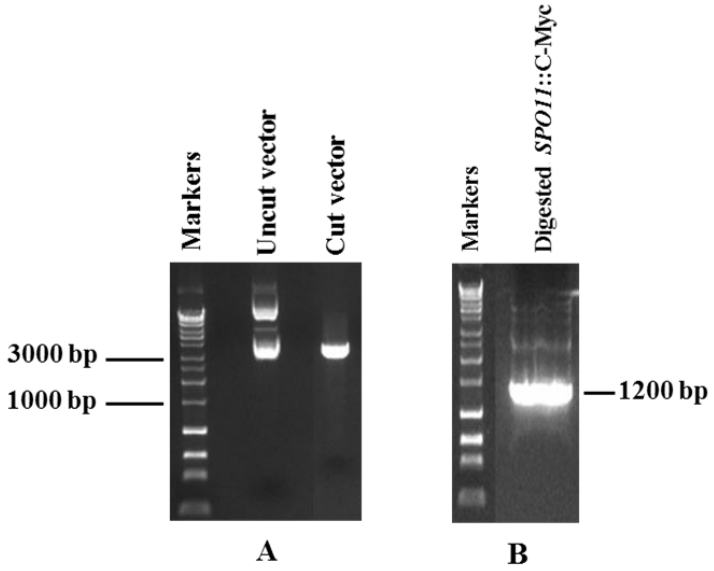



**Figure 6-5 Amplification of pTRE-3G vector and digestion *SPO11::* C-Myc construct with *Bam*Hl and *Pst*I restriction enzymes.**
The image of 1% agarose gel shows undigested (uncut) pTRE-3G vector as control to be compared with the digestion vector (panel (A)) (left). Panel (A) (right) displays the vector after digestion by *Bam*Hl and *Pst*I restriction enzymes and purification. The enzymes linearize the 3431bp plasmid into one single fragment. Panel (B) shows the amplification of the *SPO11*::C-Myc sequence from the pCMV MCS C-Myc vector, which was already cloned with the length of *SPO11*cDNA. This band was digested with the same restriction enzymes, *Bam*Hl and *Pst*I, and then underwent purification. HyperLadder 1 kb plus (5 µl) was used as marker.

To analyse the clones after transformation, 10 *E. coli* colonies were selected randomly for each clone. PCR screening was performed on those colonies using internal primers for *SPO11*. The result suggested that all *E. coli* colonies except Colony G had the insert *SPO11::N-HA*; (Figure 6.6). In addition, 8 out of 10 *E. coli* colonies had the insert *SPO11::C-Myc*, as shown in Figure 6.7. A sample including only water was used as a negative control. Positive PCR colonies were grown overnight to be investigated further.

Recombinant plasmids containing *SPO11* with N-HA and C-Myc tags were isolated from *E. coli* cells. Restriction enzyme digestion (*Nde*I and *Pst*I) was used to assess the presence of *SPO11::N-HA* in six colonies (Figure 6.8). In contrast, *Bam*Hl and *Pst*I restriction enzymes were utilised in the case of *SPO11::C-Myc* cloning in 10 colonies, as shown in Figure 6.9. Producing two bands in each digested plasmid indicated successful cloning of each fragment.

Colony F was chosen for the pTRE-3G::SPO11::N-HA clone, then *Nde*I and *Pst*I restriction enzymes were used, both together and separately, to confirm the correct cloning. While *Bam*Hl and *Pst*I restriction enzymes were used for pTRE-3G::SPO11::C-Myc (Colony No. 4). The results are shown in Figure 6.10 and Figure 6.11 respectively. It was found that using one restriction enzyme, such as *Nde*I or *Bam*Hl, yielded a single band, whereas using two restriction enzymes produced double bands, indicating the vector and the insert.

Sending Colony F and Colony No. 4 for DNA sequencing allowed further confirmation of correct orientation and the assessment of unwanted mutations. As Colony F and Colony No. 4 exhibited no mutations, further analysis was carried out in this study.

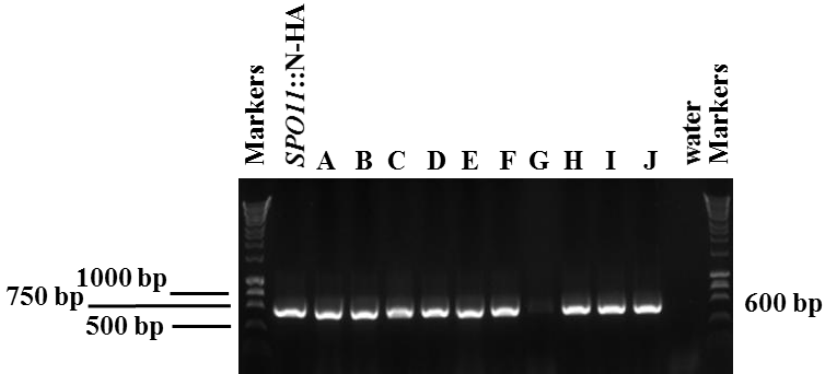



**Figure 6-6 Polymerase chain reaction (PCR) screening of colonies for the cloning of *SPO11*::N-HA into the pTRE-3G vector.**
Agarose gel picture showing PCR screening of *E. coli* colonies for the cloning of *SPO11*:: N-HA into the pTRE-3G using an internal primer for the *SPO11* gene. The approximately The 600 bp bands show the presence of *SPO11* in the *SPO11*::N-HA to confirm that the primer is working while water was utilised as negative control. The results show that all the samples in lanes A-J contain a *SPO11* insert of the expected size, 600 bp, except for lane G. 5 µl of HyperLadder 1 kb was used as marker.

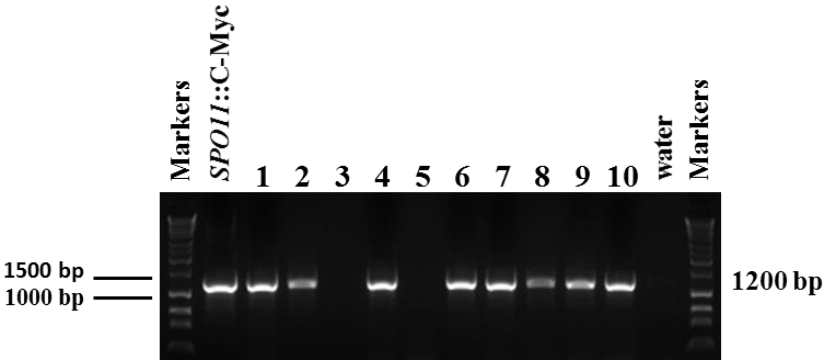



**Figure 6-7 Polymerase chain reaction (PCR) screening of colonies for the cloning of *SPO11*::C-Myc into pTRE-3G vector.**
Agarose gel picture showing PCR screening of *E. coli* colonies for the cloning of *SPO11*::C-Myc into the pTRE-3G using the primer for the *SPO11* gene. The approximately 1190 bp bands show the presence of *SPO11* in the *SPO11*::C-Myc to confirm that the primer is working; water was utilised as negative control. The results show that all the samples in lanes 1–10 contain a *SPO11* insert of the expected size, 1200 bp, except for lanes 3 and 5. HyperLadder 1 kb plus (5 µl) was used as marker.

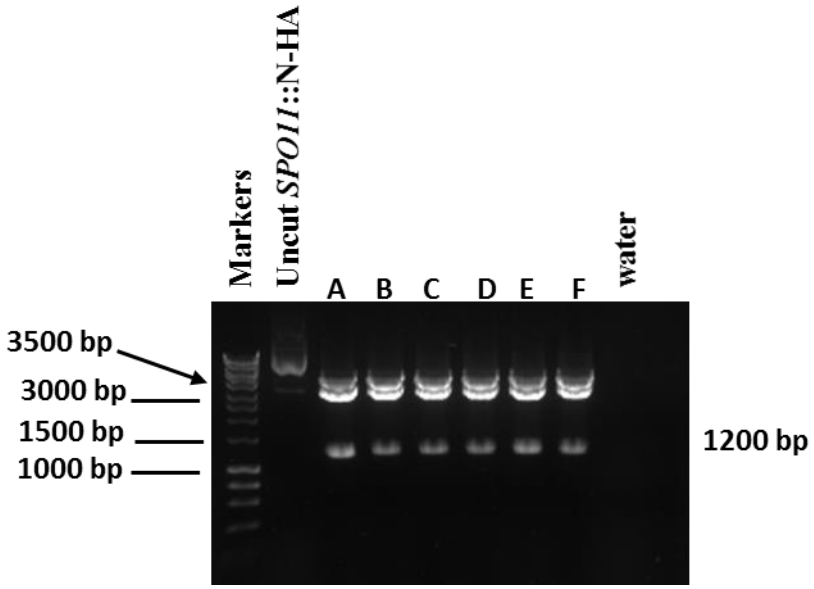



**Figure 6-8 Digestion of recombinant plasmids (pTRE-3G::*SPO11*::N-HA).**
Agarose gel presenting the digestion of the recombinant purified pTRE-3G::*SPO11*::N-HA vectors by *Nde*I and *Pst*I restriction enzymes. Two bands were obtained in all selected colonies. The higher band belongs to the pTRE-3G vector, with approximately 3431 bp, while the lower band is the *SPO11* gene, with approximately 1200 bp. All colonies in lanes A–F show successful cloning of the *SPO11* gene. HyperLadder 1 kb (5 µl) was used as marker.

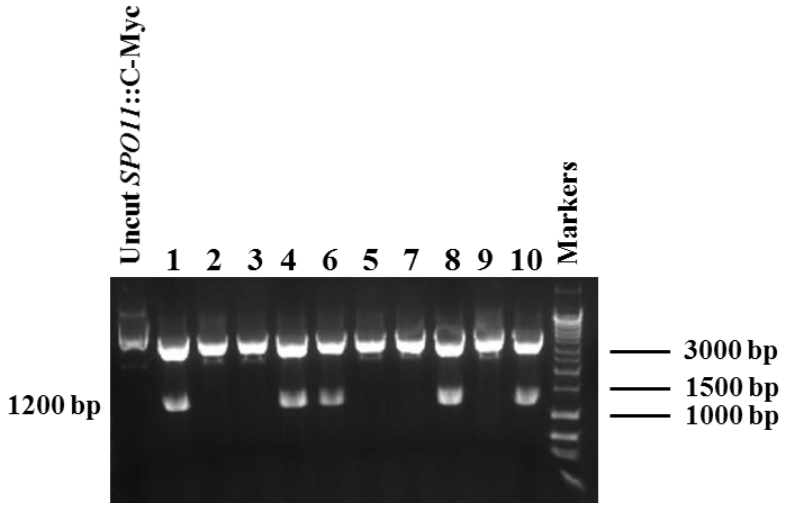



**Figure 6-9 Digestion of recombinant plasmids (pTRE-3G::*SPO11*::C-Myc).**
Agarose gel presenting the digestion of the recombinant purified pTRE-3G::*SPO11*::C-Myc vectors by *Bam*Hl and *Pst*I restriction enzymes. Two bands were obtained in all selected colonies. The higher band belongs to the pTRE-3G vector, with approximately 3431 bp, while the lower band is the *SPO11* gene, with approximately 1200 bp. Colonies No. 1, 4, 6, 8 and 10 show successful cloning of the *SPO11* gene, while 2, 3, 5, 7 and 9 do not. HyperLadder 1 kb plus (5 µl) was used as marker.

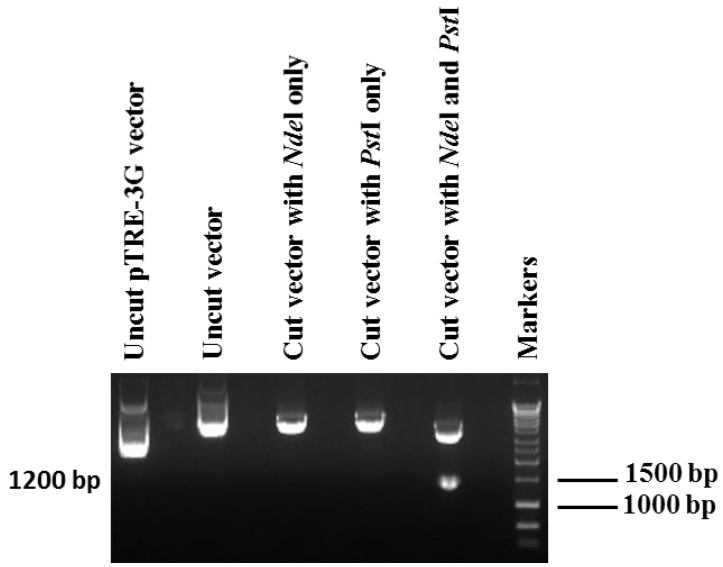



**Figure 6-10 Digestion of recombinant plasmid (Clone F; pTRE-3G::*SPO11*::N-HA).**
The 1% agarose gel shows the digestion of the recombinant purified PTRE-3G::*SPO11*::N-HA vectors (clone F) by *Ned*l and *Pst*l restriction enzymes. Two bands were obtained in all selected colonies. The higher band belongs to the pTRE-3G vector, with approximately 3431 bp, while the lower band is the *SPO11* gene, with approximately 1200 bp. Clone F (the selected clone) confirms successful cloning of the *SPO11* gene. HyperLadder 1 kb (5 µl) was used as marker.

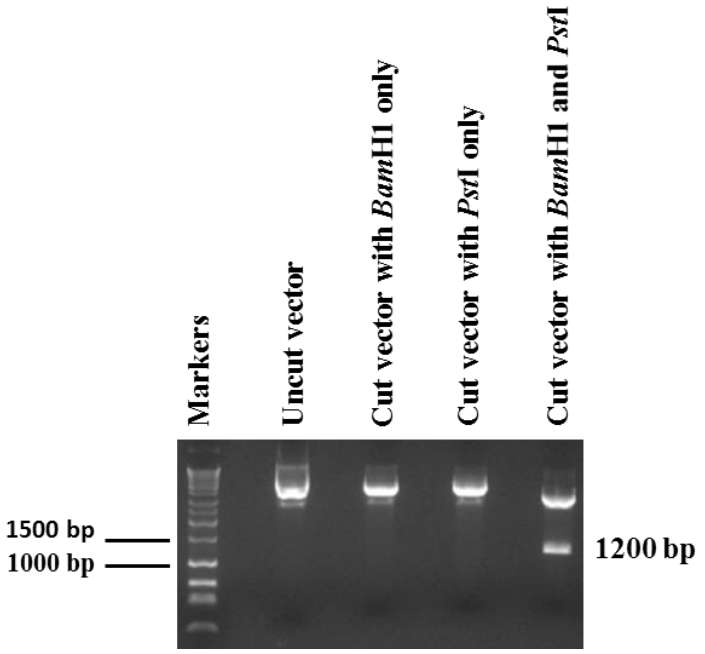



**Figure 6-11 Digestion of recombinant plasmid (Clone No. 4; pTRE-3G::*SPO11*::C-Myc).**
The 1% agarose gel shows the digestion of the recombinant purified PTRE-3G::*SPO11*::C-Myc vectors (Clone No. 4) by *Bam*Hl and *Pst*l restriction enzymes. Two bands were obtained in all selected colonies. The higher band belongs to the pTRE-3G vector, with approximately 3431 bp, while the lower band is the *SPO11* gene with approximately 1200 bp. Clone No. 4 (the selected clone) confirms successful cloning of the *SPO11* gene. HyperLadder 1 kb plus (5 µl) was used as marker.

### 6.2.2 Establishment of a double-stable Hela Tet-On 3G cell line

In this study, double-stable Tet-On 3G cell lines containing either *SPO11::N-HA* or *SPO11::C-Myc* were generated. Although SPO11 protein is present in almost all cancer cell lines, HeLa and HCT116 cells were chosen to be transfected with N-HA and C-Myc tags since they have Tet-On3 G inducing system ready in our lab.

#### 6.2.2.1 Selection of double-stable Hela and HCT116 Tet-On 3G cells

In order to optimise an optimal killing concentration, both cell lines HeLa and HCT116 cloning the Tet-On system were grown for 48 hours in media without puromycin antibiotic. HeLa and HCT116 cells were then exposed to different concentrations of puromycin antibiotic (0.0–0.8 µg/ml) for 3–5 days, as shown in Figure 6.12 and Figure 6.14, respectively. HeLa and HCT116 Tet-On 3G were co-transfected individually with pTRE-3G::SPO11::N-HA and pTRE-3G::SPO11::C-Myc plasmids, along with a linear selection marker for puromycin antibiotic. The cells were also individually transfected with empty pTRE-3G plasmid as a control to evaluate the gene inductions. In addition, cells were grown for 4 days in an antibiotic free media. The HeLa cells were then treated with 100 µg/ml of G418 and 0.8 µg/ml of puromycin, while 100 µg/ml of G418 and 2.0 µg/ml of puromycin were used with HCT116 cells. After 4 days of this treatment, most cells died except for a few single cells, which may have been successfully transfected. After 2 weeks, large and healthy colonies (Figure 6.13 and Figure 6.15) were collected and grown separately, first in 6-well plates and then in 10 cm plates; then, they were transferred to T75 flasks.

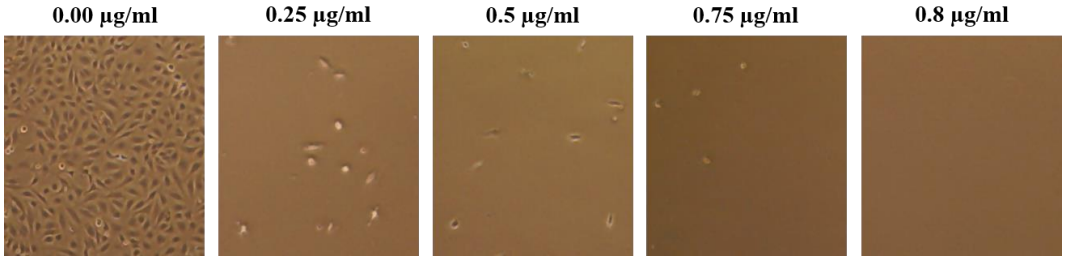



**Figure 6-12 Untransfected Hela Tet-On 3G cells were exposed to different concentrations of puromycin antibiotic to generate the optimal killing concentration.**
Applicable concentrations of puromycin antibiotic were determined by growing untransfected Hela cells in normal media (no antibiotic) for 48 hours. Four doses of puromycin (0–0.8 µg/ml) were optimised as the minimum dose that would kill all cells after 3–5 days. Untreated cells (0.00 µg/ml) were used as control to compare the effect of puromycin on treated cells, and 0.8 µg/ml was the optimum dose based on colony selection.

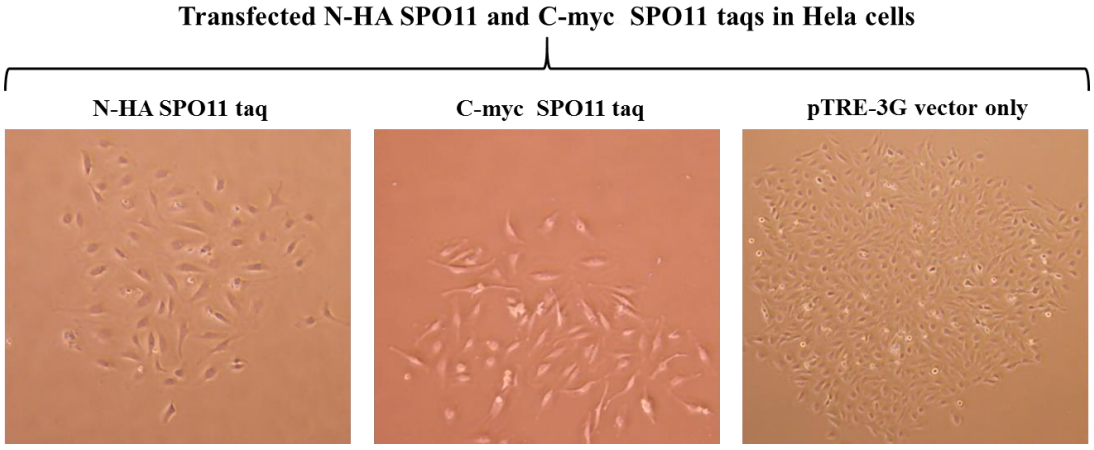



**Figure 6-13 Example of individual puromycin resistance colonies in HeLa cells.**
The image demonstrates that after 4 days, a single colony started to grow and survive after adding 0.8 µg/ml of puromycin antibiotic. These cells were integrated with *SPO11*::N-HA tag, *SPO11*::C-Myc tag and pTRE-3G only, which served as *SPO11*-negative control.

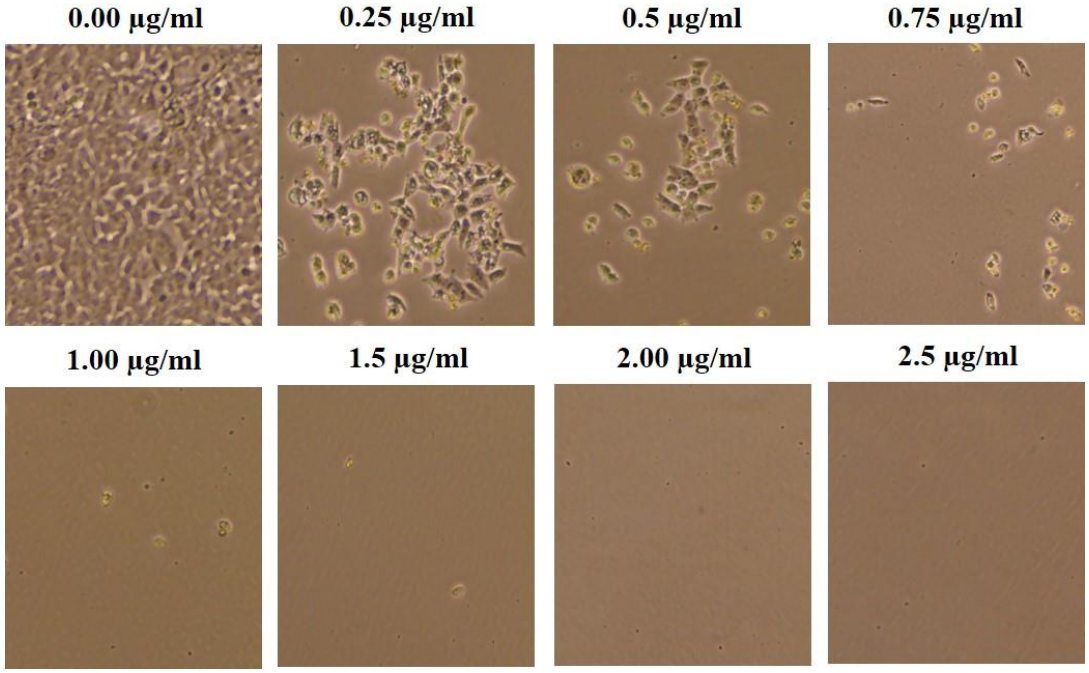



**Figure 6-14 Untransfected HCT116 Tet-On 3G cells were exposed to different concentrations of puromycin antibiotic to generate the optimal killing concentration.**
Applicable concentrations of puromycin antibiotic were determined by growing untransfected HCT116 cells in normal media (no antibiotic) for 48 hours. Seven doses of puromycin (0–2.5 µg/ml) were optimised as the minimum dose that would kill all cells after 3–5 days. Untreated cells (0.00 µg/ml) were used as control to compare the effect of puromycin on treated cells, and 2 µg/ml was the optimum dose based on colony selection.

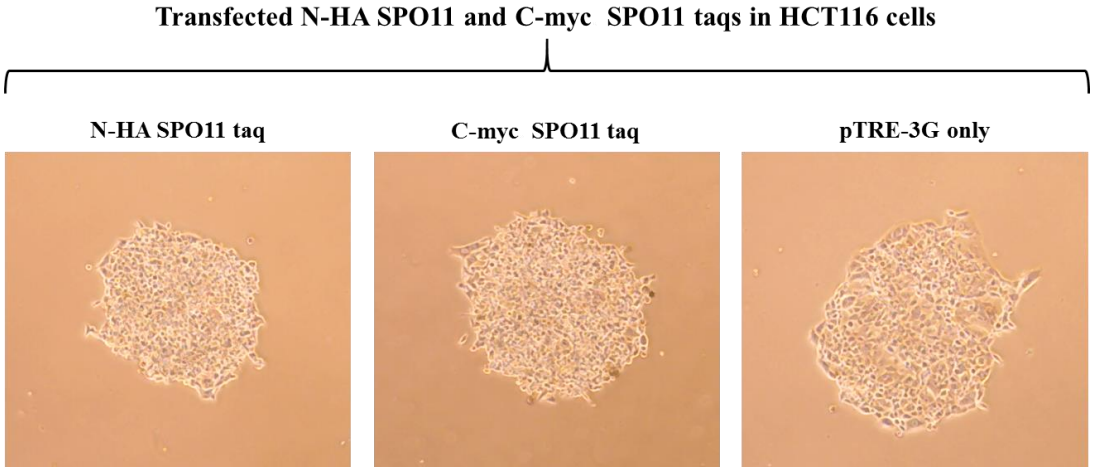



**Figure 6-15 Example of individual puromycin resistance colonies in HCT116.**
The image demonstrates that after 4 days, a single colony started to grow and survive after adding 2 µg/ml of puromycin antibiotic. The cells integrated with *SPO11*::N-HA tag, *SPO11*::C-Myc tag and pTRE-3G only, which served as negative control.

#### 6.2.2.2 Evaluation of the successful integrant double-stable Hela and HCT116 cell lines

Individual colonies with possible tags were assessed by comparing the presence of N-HA and C-Myc tags with or without adding 1 µg/ml of DOX. High-quality Tet system FBS medium was used to grow cloned cells, since it is free of tetracycline. Analysis of the successful integration of pTRE-3G::SPO11::N-HA and pTRE-3G::SPO11::C-Myc into the genome of HeLa and HCT116 cells was carried out using two methods, namely western blot and immunofluorescence (IF). The first method involved screening for N-HA and C-Myc tags using their specific commercial antibodies through the western blot technique.

In Hela cells, four colonies (H1, H2, H3 and H4) were successfully cloned with the pTRE-3G::SPO11::C-Myc plasmid, whereas only one colony (N1) was cloned with pTRE-3G::SPO11::N-HA plasmid; the results are shown in Figure 6.16 A and B. In contrast, in HCT116 cells, three colonies (D1, D2 and D20) were successfully integrated with pTRE-3G::SPO11::C-Myc plasmid and three colonies (C23, C29 and C18) were also cloned with pTRE-3G::SPO11::N-HA plasmid (Figure 6.17 A and B). All these colonies present N-HA or C-Myc tags compared to the plasmid with only pTRE-3G (negative control).

IF was also undertaken using cloned HeLa cells (N-HA and C-Myc) to establish the specificity of the anti-SPO11 antibody used in this study and in order to determine the co-localisation between anti-SPO11 antibody and N-HA/C-Myc antibodies, since they should detect the same protein. Cells were fixed and stained with DAPI, which stains DNA. HeLa cells stained with C-Myc tag indicated quite good co-localisation between anti-C-Myc and anti-SPO11 since yellow colour is presented; the results are shown in Figure 6.18. Using N-HA tag suggested that SPO11 also localises to the N-HA tag (Figure 6.19).

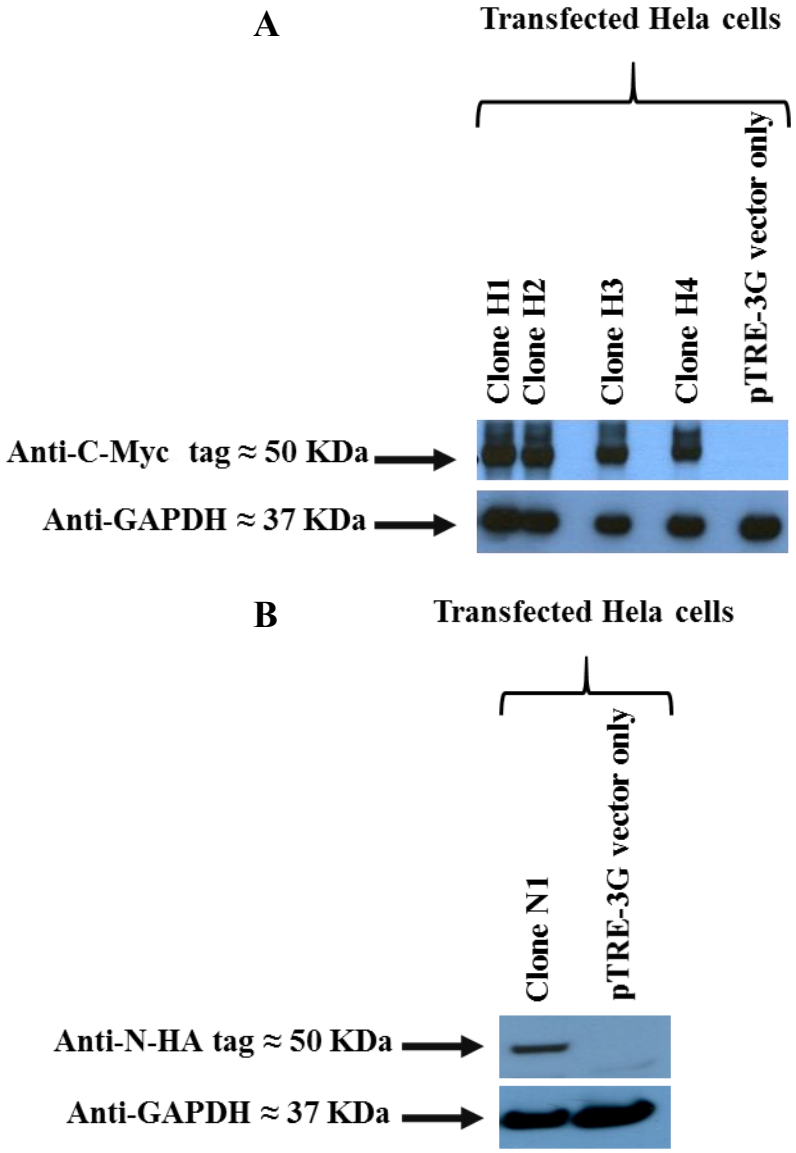



**Figure 6-16 Western blot analysis confirming the presence of N-HA and C-Myc tags after inducing Hela Tet-On stable cell line.**
Anti-GAPDH protein levels were used as a loading control (bottom). (A) Four independent colonies (H1, H2, H3, H4) of *SPO11*::C-Myc were induced with 0.8 µg/ml of doxycycline (DOX) for 24 hours. Anti-C-Myc tag antibody was used to check for the presence of the *SPO11*::*C-Myc* into each colonies. (B) One independent colony (N1) of *SPO11*::N-HA was induced with 0.8 µg/ml DOX for 24 hours. The anti-N-HA tag antibody was used to check for the presence of *SPO11*::*N-HA* in this colony. C-Myc and N-HA band sizes of 50 kDa were observed in all colonies compared with the pTRE-3G vector only as negative control.

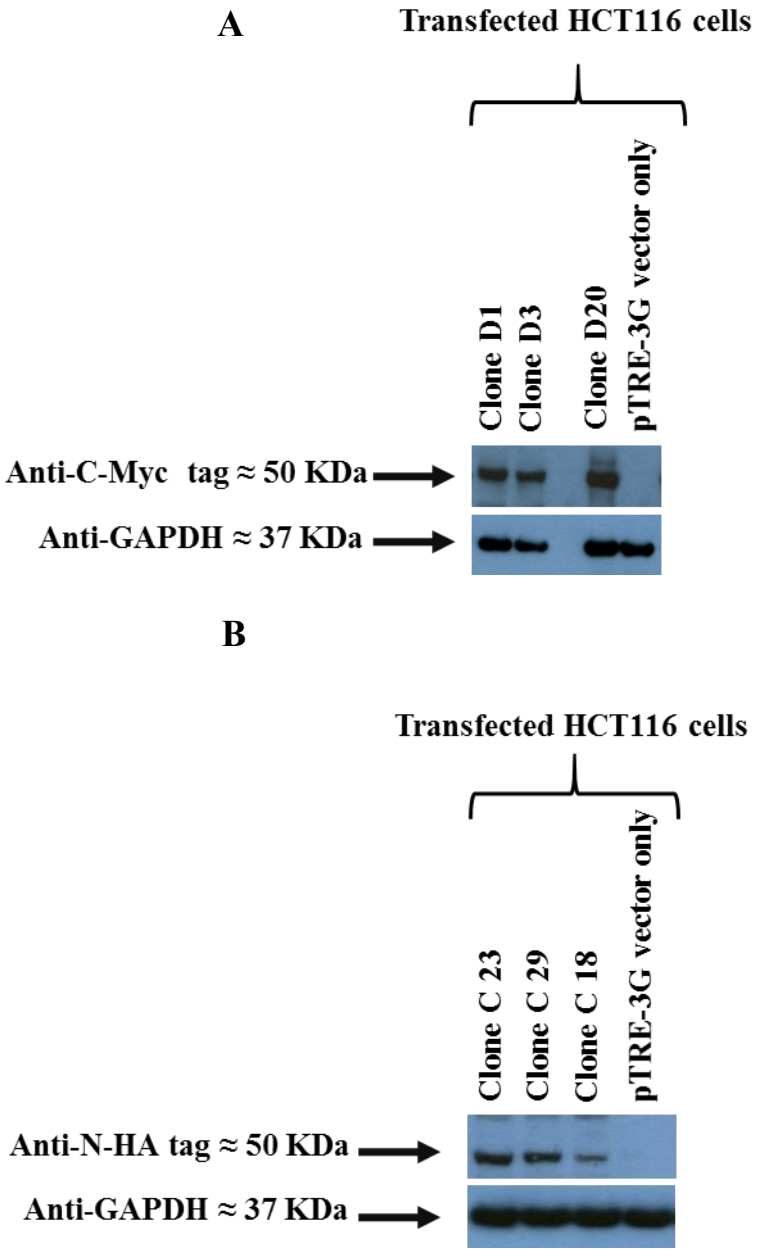



**Figure 6-17 Western blot analysis confirming the present of N-HA and C-Myc tags after inducing HCT116 Tet-On stable cell line.**
Anti-GAPDH protein levels were used as a loading control (bottom). (A) Three independent colonies (D1, D3, D20) of *SPO11*:: C-Myc were induced with 2 µg/ml of doxycycline (DOX) for 24 hours. The anti-C-Myc tag antibody was used to check for the presence of *SPO11*::*C-Myc* clone. (B) Three independent colonies (C23, C29, C18) of *SPO11*::N-HA were induced with 2 µg/ml of DOX for 24 hours. The anti-N-HA tag antibody was used to check the presence of *SPO11*::*N-HA* clone. C-Myc and N-HA band sizes of 50 KDa were observed in all colonies compared with the pTRE-3G vector only as negative control.

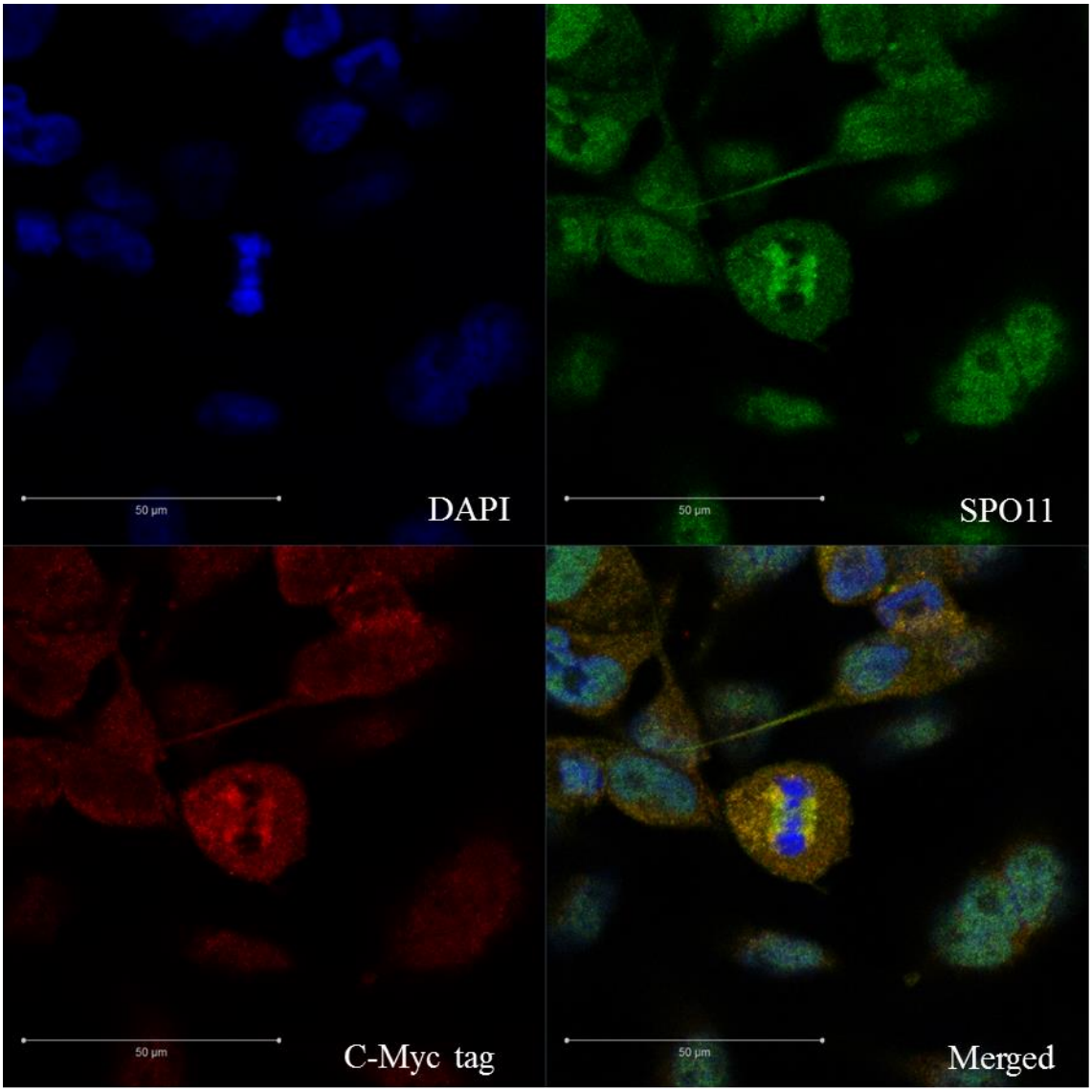



**Figure 6-18 Immunofluorescence (IF) staining showing subcellular SPO11 in Hela transfected with the SPO11–C-Myc tag (×40).**
Top left, DAPI staining (blue). Top right, staining of the anti-SPO11 (green) antibody. Bottom left, staining of the anti-C-Myc (red) antibody for C-Myc::SPO11. Bottom right, DAPI, anti-SPO11 and anti-C-Myc antibodies staining. The image shows co-localisation between SPO11 and with C-Myc tag during metaphase. The images were viewed using a ZEISS LSM 710 confocal microscope.

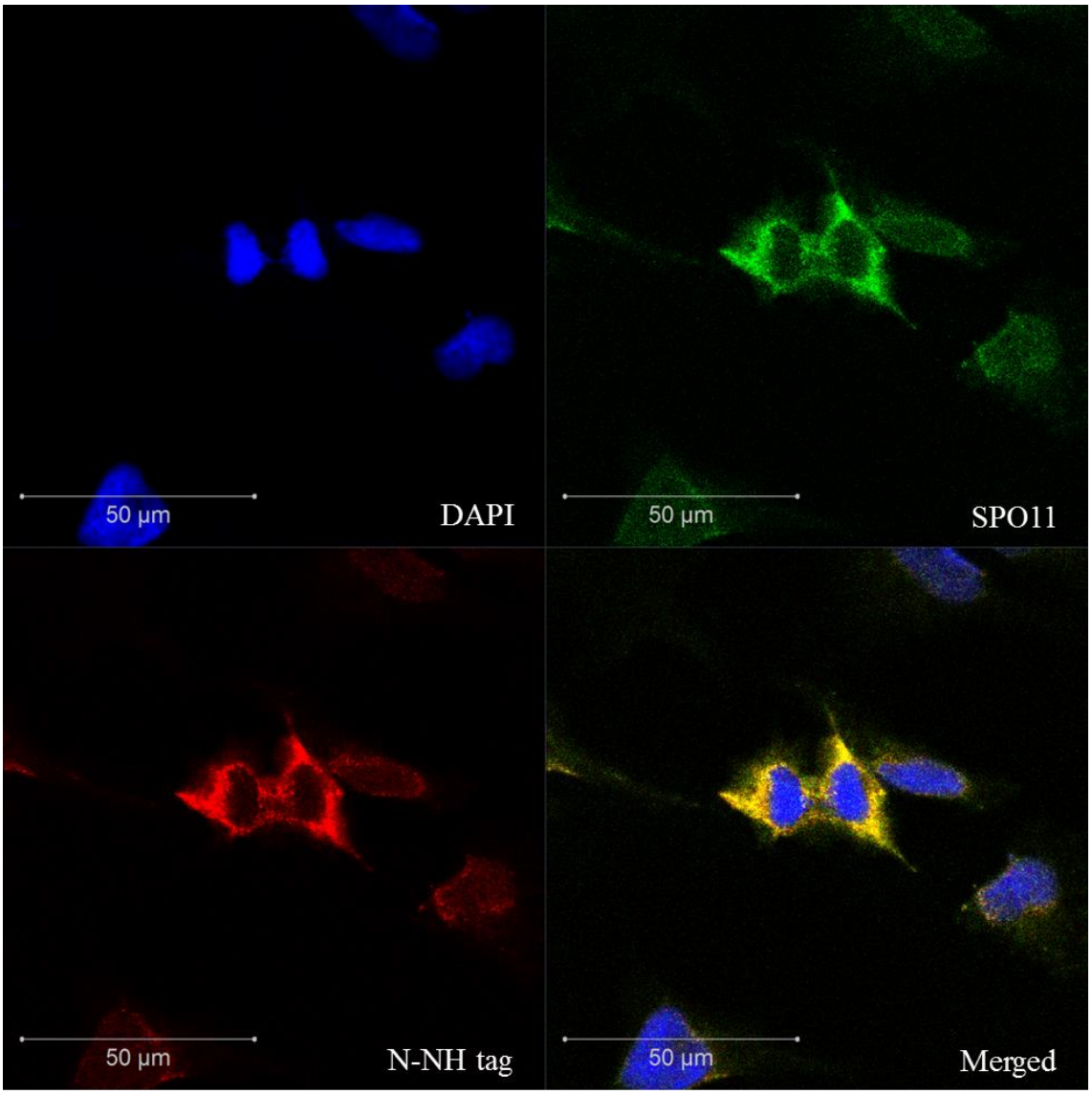



**Figure 6-19 Immunofluorescence (IF) staining showing subcellular SPO11 in Hela transfected with SPO11–N-HA tag (×40).**
Top left, DAPI staining (blue). Top right, staining of the anti-SPO11 (green) antibody. Bottom left, staining of the anti-N-HA (red) antibody for N-HA::SPO11. Bottom right, DAPI, anti-SPO11 and anti-N-HA antibodies staining. The image indicates strong co-localisation between SPO11 and with N-HA tag during anaphase. The images were viewed using a ZEISS LSM 710 confocal microscope.

### 6.2.3 Evaluation SPO11 protein level in Hela and HCT116 Tet-on 3G stable cell lines

The production of SPO11 protein was analysed using western blot analysis. SPO11-tag protein was induced by the addition of DOX to culture cells (1 μg/ml). Different colonies of Hela and HCT116 Tet-on 3G stable cell lines, which had been cloned with either *SPO11::N-HA* and *SPO11::C-Myc* tags, were examined. Cells with no DOX (-) were used as un-induced controls. The results from western bolt showed that there were no significant differences between Hela and HCT116 stable cell lines induced with or without DOX when anti-C-Myc and anti-HA antibodies were assessed as presented in Figure 6.20 and Figure 6.21 respectively.

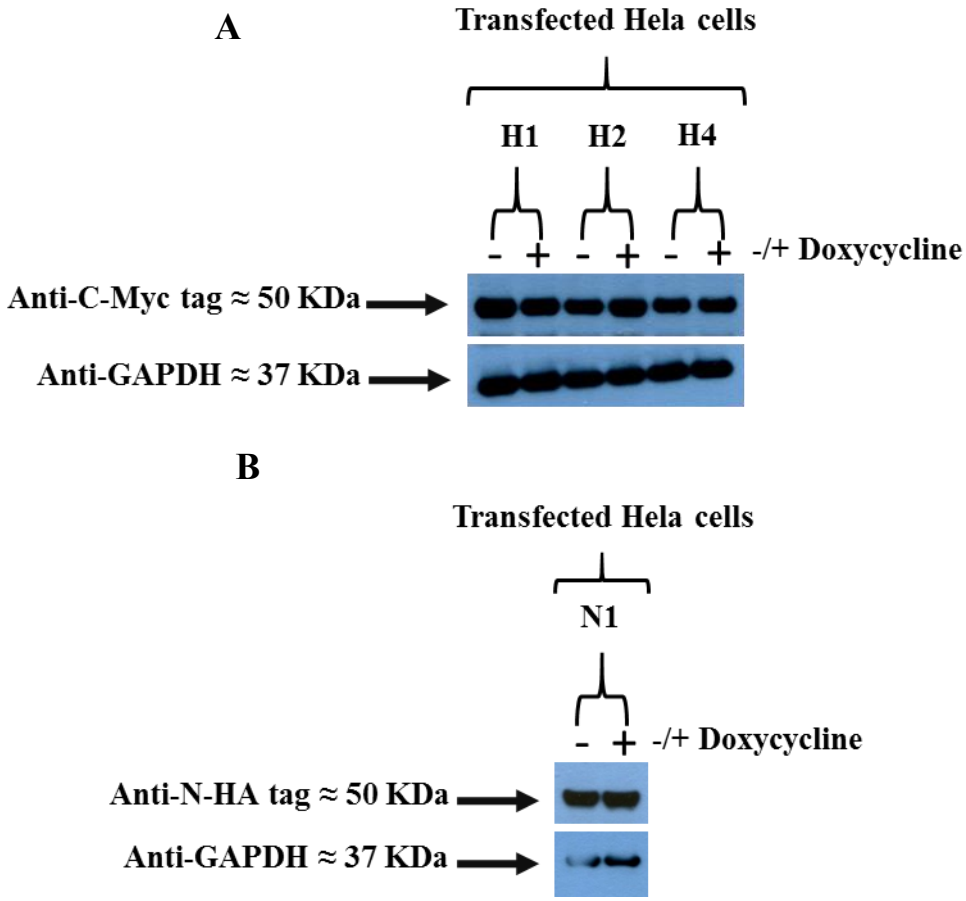



**Figure 6-20 Western blot analysis showing the induction of HeLa Tet-On stable cell line cloned with *SPO11::C-Myc* and *SPO11::N-HA* using doxycycline (DOX).**
Anti-GAPDH protein levels were used as a loading control (bottom). (A) Three different colonies (H1, H2 and H4) of *SPO11*::*C-Myc* indicated the production of SPO11 protein in the presence or absence of DOX using the anti-C-Myc tag antibody. (B) One colony (N1) of *SPO11*::*N-HA* indicated the production of SPO11 protein in the presence or absence of DOX using the HA tag antibody. The cells were harvested 24 hours post-induction. No significant differences were observed between induced and non-induced cells when both C-Myc and HA tag antibodies were used.

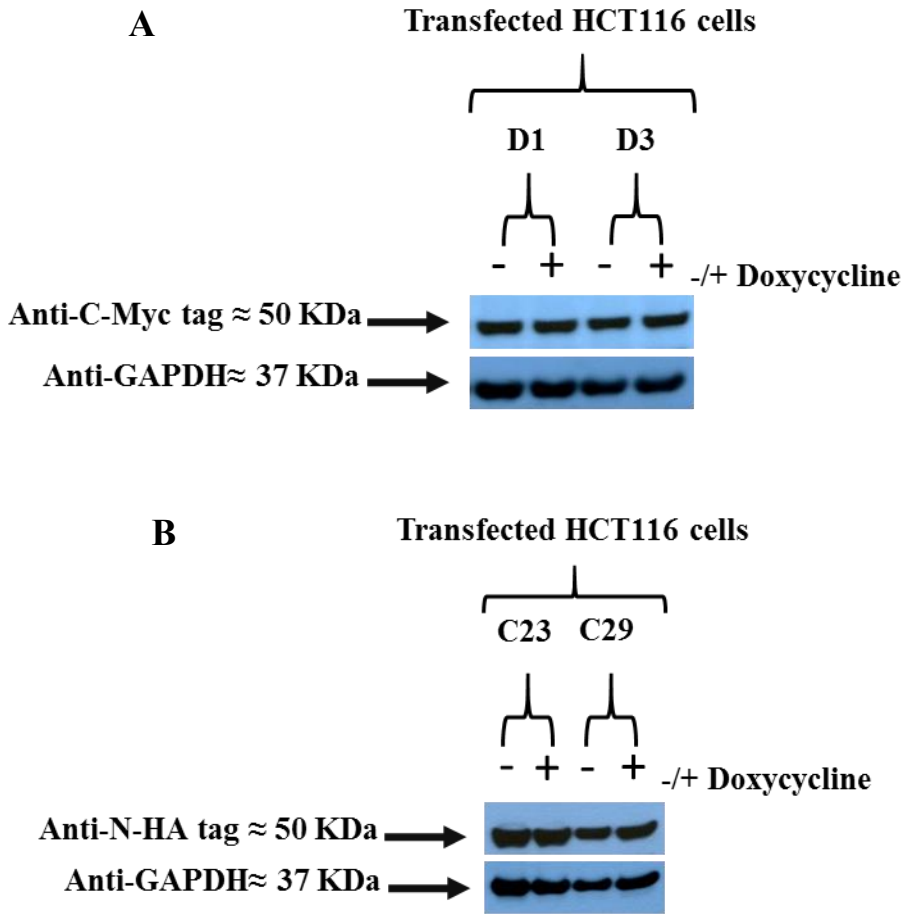



**Figure 6-21Western blot analysis showing the induction of HCT116 Tet-On stable cell line cloned with *SPO11::C-Myc* and *SPO11::N-HA* using doxycycline (DOX) in the Tet-On 3G.** Anti-GAPDH protein levels were used as a loading control (bottom). (A) Two different colonies (D1 and D3) of *SPO11::C-Myc* indicated the production of SPO11 protein in the presence or absence of DOX using anti-C-Myc tag antibody. (B) Two colonies (C23 and C29) of *SPO11::N-HA* indicated the production of SPO11 protein in the presence or absence of DOX using anti-HA tag antibody. The cells were harvested 24 hours post-induction. No significant differences were observed between induced and non-induced cells when both C-Myc and HA tag antibodies were used.

## 6.3 Discussion

### 6.3.1 Summary of findings

The work conducted in this chapter was mainly carried out to validate the SPO11 antibody and confirm the interesting observations that emerged during this project. The specificity of the SPO11 antibody was confirmed using two different tags, namely N-HA and C-Myc. The western blot results suggested that SPO11 co-localised with N-HA and C-Myc tags cloned with SPO11, whereas IF results showed evident coloration with only C-Myc tag. These results suggest that the SPO11 antibody recognises the correct protein at the correct size, which gives more support for the use of this antibody. SPO11 protein may be considered a promising therapeutic target in cancer cells, since the depletion of SPO11 seems to inhibit cell proliferation, affect cell viability and disrupt some cell cycle proteins, as suggested by our results from previous chapters. Our finding is based on the results for the SPO11 antibody, which was used in this study. Therefore, this chapter has focussed on the conformation of SPO11 antibody using two types of tags, namely N-HA and C-Myc. *SPO11::N-HA* and *SPO11::C-Myc* were cloned into the mammalian expression pTRE-3G vector (Tet-On-3G system) to determine the effective of SPO11 overexpression on cell proliferation and to validate the SPO11 antibody.

### 6.3.2 Examining N-HA and C-Myc tags

The immune system produces specific proteins known as antibodies that bind to unique epitopes on antigens with high specificity to destroy any foreign molecules. In this research area, antibodies represent attractive tools, as they can detect a particular protein in an assay, such as western blot, while avoiding binding to unrelated proteins (Mian et al. 1991; Wang et al. 2007). Most important, since IF assay used chemical reagents to fixate cells, this may affect the interaction of the antibody with its protein. Thus, cross-linking or un-specific binding may occur. Therefore, validation of the antibody specificity might be different when using IF assay or western blot. In the present study, N-HA and C-Myc were cloned into *SPO11* (*SPO11::N-HA* and *SPO11::C-Myc*) to validate and examine the anti-SPO11 antibody against those tags.

The sequence of those tags can be added to proteins of interest via recombinant DNA to validate the antibody and to study new proteins with no specific antibodies. The tagged gene product can be easily identified using commercial tag-specific antibodies. The observations from western blot and IF analysis in this chapter suggested that C-Myc tags were co-expressed with *SPO11*, while N-HA may not detect the correct protein since they are detecting cytoplasmic protein, whereas SPO11 is localised in nucleus. For further investigation, other transfected colonies should be examined.

Regarding western blot analysis, N-HA and C-Myc antibodies were detected at a SPO11 band size of approximately 50 kDa. N-HA and C-Myc tags have an extremely short sequence; therefore, they should detect the SPO11 protein size because they have already cloned with the SPO11 sequence when using their antibodies. Importantly, since the sequences of these tags are small, they are unlikely to overlap with the function of the target protein. In contrast, the IF results indicated that N-HA, C-Myc and SPO11 antibodies selected the same protein, namely SPO11. Interestingly, these results confirm the anti-SPO11 antibody's specificity. In fact, the development and success of the immunoassay may depend on the affinity and specificity of existing antibodies.

### 6.3.3 Examining Tet-On 3G induction system

Ultimately, antibody specificity play a critical role in recognising the target protein accurately while limiting cross-reaction with non-specific proteins (Spinks 2000; Wang et al. 2007). To study the function of human SPO11, it should be integrated into a cell line that does not already express *SPO11* to determine the effectiveness of the overexpression of gene of interest on cell proliferation and cell survival. Therefore, since the *SPO11* gene is expressed in almost all cancer cells (tested in this study), it is considered a big challenge to perform such experiment. Consequently, other approaches, such as induced systems (the Tet-on 3G system) were examined in this project. In mammalian cells, the Tet-on 3G system is considered a powerful inducible gene expression system in the presence of DOX. The expression of gene of interest can be induced using Tet-On-3G system when growing in medium containing DOX. Tet-on 3G provides useful tools for gene expression and gene regulation after cloning the gene of interest in this system (Gossen & Bujard 1992; Urlingeret al. 2000). In our study, many colonies of Hela and HCT116 stable cell lines have been established for both the N-HA and C-Myc tags.

The influence of *SPO11* overexpression on cell growth and cell phenotype can then be measured using the stable HeLa and HCT116 Tet-on 3G system with and without DOX. Using this technique will enable us to further confirm that the reduction of the SPO11 protein level in cancer cells may lead to inhibition of cell proliferation and regulation of the cell cycle, as demonstrated from previous chapters. Our results suggested that SPO11 overexpression using the Tet-on 3G system was not successful, and SPO11 did not appear to induce using Hela and HCT116 stable cell lines; hence, there were no significant observations and the cells grew normally.

As seen in Figure 6.20 and 6.21 the lysate without DOX showed that SPO11 protein could clearly be detected suggesting that the Tet-on 3G system may not have worked. This may indicate that the Tet-on 3G system 'is leaky' therefore; SPO11 protein is present even without DOX (system is always 'on'). To date, this experiment has been limited due to time constraints, and further investigation is required. Although DNA extracted from selected colonies was successfully sequenced after cloning, the sequence on the genomic after transfection should be taken into account. In addition, Performing CO-IPs (co-immunoprecipitations) with anti-tags vs. western blot with anti-SPO11 is also suggested.

# Chapter 7

## Summary and General Discussion

# 7. Summary and General Discussion

## 7.1 Summary of findings

The obvious effectiveness of the reduction of SPO11 protein observed herein on cell proliferation, cell viability and cell cycle processes suggest that SPO11 is essential for cancerous cells. This finding is supported by our observations that SPO11 protein depletion was detected mostly in floating cells generated due to SPO11 knockdown. According to the results presented in this work, cell viability and survival of floating cells were significantly affected compared to attached cells as a result of SPO11 knockdown. In contrast, the effect of SPO11 knockdown on cell proliferation did not induce apoptosis. Likewise, the cell senescence state was not triggered by SPO11 depletion. However, SPO11 depletion has a significant effect on cell proliferation. This interesting result support the idea that SPO11 protein may be considered a promising therapeutic target in cancer cells.

## 7.2 SPO11 as a CTA gene

Likely due to changes in modern lifestyle, cancer is the major cause of death on a global scale. A distinct characteristic of cancer is the disruption of normal division and cellular activity due to mutations and/or alterations in gene regulation, resulting in unlimited cell division and cell invasion (Jayashree et al., 2015; Larsson. 2011). Cancer treatment and diagnostic techniques are being rapidly developed; these range from traditional methods such as surgery, radiotherapy and chemotherapy, to more modern techniques like immunotherapy. In terms of the latter, new human-specific antigens, known as CTAs, have been identified as potential targets for immunotherapy given their presence in numerous malignant tumours but not in somatic adult tissues except testis. Moreover, CTAs are also considered a promising group of cancer-specific biomarkers and direct drug targets (Caballero & Chen, 2009; Costa et al., 2007).

While CTA genes appear to be an interesting biomarker for cancer diagnosis and/or therapy, identifying the specific functions of these genes in tumours could improve the effectiveness of cancer immunotherapy and for the development of cancer-specific drugs. In the current study, the function and involvement of the meiosis-specific gene *SPO11* was investigated in cancer cells.

Koslowski et al. (2002) reported that *SPO11* (CT35) is a CTA gene since its expression was detected in testis and in various cancer cells, including melanoma, lung and cervical cancer tissues, but not in normal tissues. Indeed, in this study, SPO11 protein was found to be present in the testis but not in other normal tissues via western blot analysis. Moreover, the presence of SPO11 protein was also detected in all cancer cell lines and cancer tissues tested in this project. Indeed, the Abcam (ab81695) SPO11 antibody was used following full validation via knockdown experiments and cloning-specific tags, including HA and Myc tags and the present results confirm the identification of *SPO11* as a CTA gene.

## 7.3 SPO11 protein localisation

Our data from western blot analysis and immunofluorescence staining indicated that the localisation of SPO11 protein in cancer cells seems to be mainly nucleic. In addition, using immunofluorescence, SPO11 protein was detected in the nucleus of spermatogonia in the normal adult testis, which is consistent with the formation of meiotic recombination, the main known biological function of SPO11.

## 7.4 SPO11 depletion

Many attempts were performed in order to knockdown SPO11 protein or at least reduce its level and different techniques such as siRNA, esiRNA and shRNA were used with different timeframe and various conditions, including different types of transfection reagents. In addition, two different molecules of siRNA, 2 and 4, were utilised as well in this project and both of them seem to reduce the level of SPO11 protein, reducing the possibility of being un-specific. All these silencing post-transcriptional methods are being tested to produce an effective siRNA in order to target different regions of mRNA. Furthermore, siRNA 2 and 4 are overlapping and targeting exon 13, which in fact may consider as suitable gene target sites, whereas shRNA sequences target exon 13 (Figure 7.1). SPO11 depletion using two siRNAs (2 and 4) was confirmed in floating cells and in attached cells with low cell concentrations. Obtaining such result indicates that these siRNAs are specific and targeting SPO11 mRNA. Most importantly, cells treated with non-interference (negative control) was slightly affected comparing to cells treated with siRNA because of the cell toxicity of some transfection reagents. These negative effects vary depending on the type of cancer cells, cell density, transfection reagents and number of hits (treatments).

```
SPO11-1      CCGCTCAGAAAGCGCGGGAAAGGCACGCAGCCACGCCCCAAGGGCGCAGCCTAGGACAGG
SPO11-2      CCGCTCAGAAAGCGCGGGAAAGGCACGCAGCCACGCCCCAAGGGCGCAGCCTAGGACAGG
             ************************************************************

SPO11-1      GGCTTCTGGAGCTTCTGGCAGCCGTCTGCCCTCATGGCCTTTGCACCTATGGGGCCCGAG
SPO11-2      GGCTTCTGGAGCTTCTGGCAGCCGTCTGCCCTCATGGCCTTTGCACCTATGGGGCCCGAG
             ************************************************************

SPO11-1      GCCTCGTTCTTCGACGTTTTGGACCGACACAGGGAGTCCCTGCTGGCTGCCCTGAGGAGA
SPO11-2      GCCTCGTTCTTCGACGTTTTGGACCGACACAGGGAGTCCCTGCTGGCTGCCCTGAGGAGA
             ************************************************************

SPO11-1      GGTGGCAGGGAGCCCCCAACTGGGGGAAGCCGCCTGGCCTCCAGTTCTGAGGTTCTTGCA
SPO11-2      GGTGGCAGGGAGCCCCCAACTGGGGGAAGCCGCCTGGCCTCCAG----------------
             *****************************************

SPO11-1      TCTATAGAAAATATTATCCAAGACATAATCACAAGCTTGGCAAGAAATGAAGCACCTGCA
SPO11-2      ------------------------------------------------------------


SPO11-1      TTCACGATAGACAACAGATCAAGCTGGGAAAACATAAAGTTTGAAGATTCTGTGGGTCTT
SPO11-2      ------------------------------------GTTTGAAGATTCTGTGGGTCTT
                                                  **********************

SPO11-1      CAGATGGTATCCCATTGCACCACCAGAAAGATCAAAAGTGATTCACCAAAATCAGCTCAA
SPO11-2      CAGATGGTATCCCATTGCACCACCAGAAAGATCAAAAGTGATTCACCAAAATCAGCTCAA
             ************************************************************

SPO11-1      AAATTTTCTCTAATCCTTAAAATATTGTCCATGATTTATAAATTAGTACAGAGCAACACT
SPO11-2      AAATTTTCTCTAATCCTTAAAATATTGTCCATGATTTATAAATTAGTACAGAGCAACACT
             ************************************************************

SPO11-1      TATGCAACCAAAAGGGACATATATTACACTGACAGTCAACTCTTTGGTAACCAGACTGTC
SPO11-2      TATGCAACCAAAAGGGACATATATTACACTGACAGTCAACTCTTTGGTAACCAGACTGTC
             ************************************************************

SPO11-1      GTCGACAATATTATCAATGACATTTCTTGCATGTTAAAAGTGTCAAGGAGGAGTCTACAT
SPO11-2      GTCGACAATATTATCAATGACATTTCTTGCATGTTAAAAGTGTCAAGGAGGAGTCTACAT
             ************************************************************

SPO11-1      ATATTATCTACATCAAAAGGTTTAATTGCTGGCAACTTAAGATACATCGAGGAAGATGGC
SPO11-2      ATATTATCTACATCAAAAGGTTTAATTGCTGGCAACTTAAGATACATCGAGGAAGATGGC
             ************************************************************

SPO11-1      ACCAAAGTGAATTGTACCTGTGGTGCAACGGCTGTTGCTGTGCCATCGAATATTCAAGGA
SPO11-2      ACCAAAGTGAATTGTACCTGTGGTGCAACGGCTGTTGCTGTGCCATCGAATATTCAAGGA
             ************************************************************

SPO11-1      ATTCGGAATTTAGTTACAGATGCAAAGTTTGTATTAATTGTAGAAAAAGATGCAACATTT
SPO11-2      ATTCGGAATTTAGTTACAGATGCAAAGTTTGTATTAATTGTAGAAAAAGATGCAACATTT
             ************************************************************

SPO11-1      CAGCGGCTCCTAGATGACAACTTTTGCAACAAATTGTCTCCTTGCATCATGATTACGGGA
SPO11-2      CAGCGGCTCCTAGATGACAACTTTTGCAACAAATTGTCTCCTTGCATCATGATTACGGGA
             ************************************************************

SPO11-1      AAGGGAGTTCCTGATCTAAACACAAGACTTTTAGTCAAGAAACTGTGGGATACATTTCAT
SPO11-2      AAGGGAGTTCCTGATCTAAACACAAGACTTTTAGTCAAGAAACTGTGGGATACATTTCAT
             ************************************************************

SPO11-1      GTTCCTGTTTTCACTCTTGTAGATGCTGATCCACATGGCATAGAAATAATGTGCATCTAT
SPO11-2      GTTCCTGTTTTCACTCTTGTAGATGCTGATCCACATGGCATAGAAATAATGTGCATCTAT
             ************************************************************

SPO11-1      AAGTATGGATCTATGTCTATGTCTTTTGAAGCTCATCATCTCACAGTTCCAGCTATTAGA
SPO11-2      AAGTATGGATCTATGTCTATGTCTTTTGAAGCTCATCATCTCACAGTTCCAGCTATTAGA
             ************************************************************
```

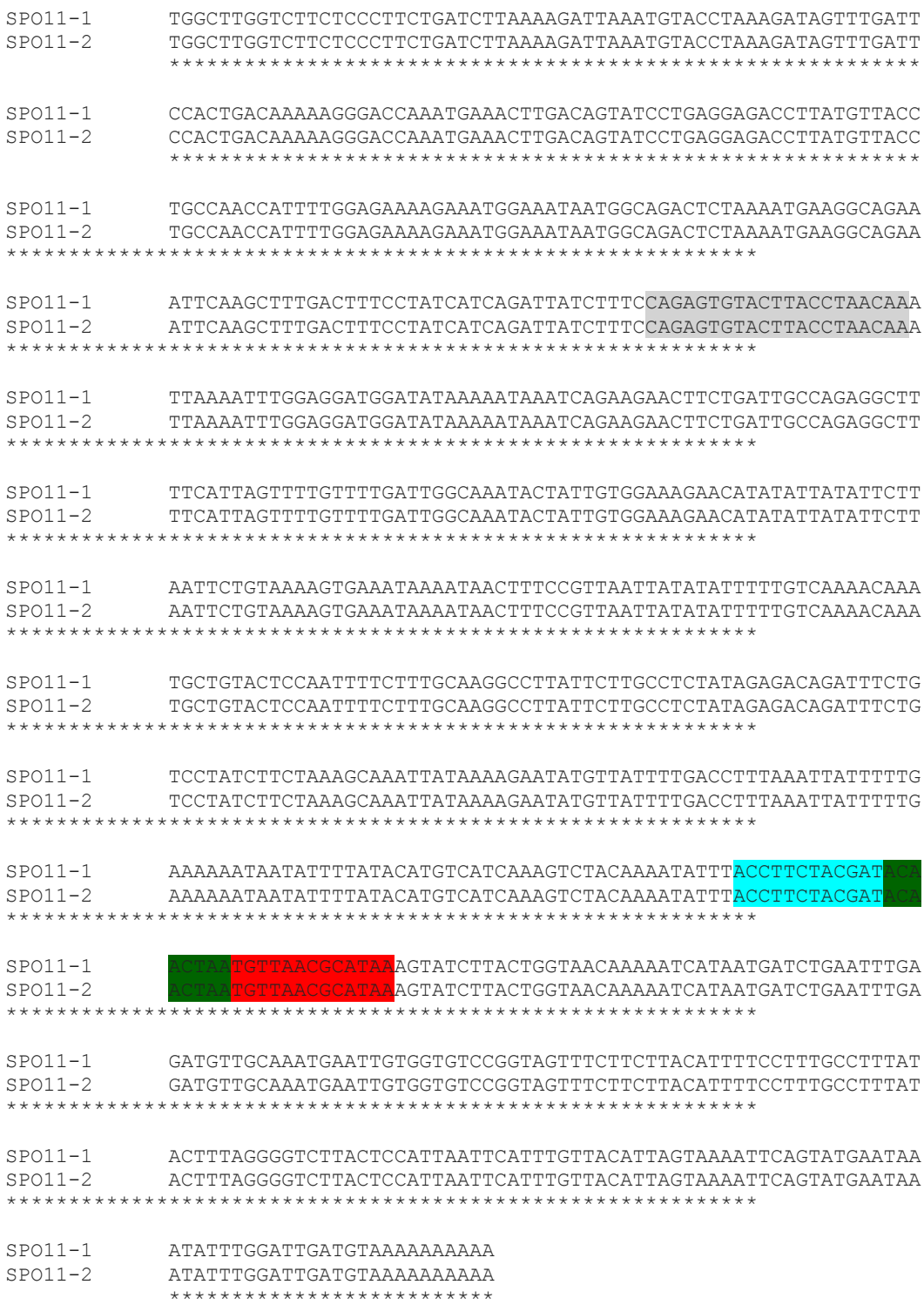



**Figure 7.1 Schematic representation of two transcript variants of *SPO11* (Adapted from NCBI accession and BLAST search).**

SPO11-1 indicates transcript variant 1 (NM_012444.2), while SPO11-2 refers to transcript variant 2 (NM_198265.1). The figure shows the two transcript variants 1 and 2, mRNA of *SPO11* gene. Blue colour displays siRNA 2 target sites, whereas red colour indicates siRNA 4 target sites. In addition, green colour points out overlapping of siRNA 2 and 4. Grey colour shows the shRNA target sites on the sequence.

## 7.5 Functional analysis of the Human SPO11 protein

The DSBs formation and repair during cell division is considered a serious and toxic mechanism due to the association of this mechanism with several immunological, developmental and neurological disorders. Additionally, driving genetic instability and cancer development can be the outcome of disrupting DSB repair (Mc & Kinnon, 2009; Jackson & Bartek, 2009). SPO11 plays a major role in meiotic recombination via initiating DNA double-strand breaks (DSBs) during meiotic prophase I (Atcheson et al., 1987; Keeney, 2001). Disrupting *SPO11* in *S. cerevisiae* affects the formation of DSBs and Holliday junctions (Cao et al., 1990; Schwacha & Kleckner, 1994). However, in mice, *Spo11* knockout may affect progression of prophase I, leading to arrest and spermatocyte apoptosis (Romanienko et al., 2000b). In addition, pre-meiotic DNA replication is altered in *spo11* mutations of *S. cerevisiae* , which is not dependent on the DSB function (Cha & Kleckner, 2000). Therefore, the presence of SPO11 protein in a wide range of cancer cells may indicate a possible additional role of SPO11 as topo-like function. In meiotic DSB formation, SPO11 acts with many partner proteins, for example, TopoVIB-like protein, which might suggest the topoisomerisation function of SPO11, there is no evidence for their activity or presence in cancer cells (Robert et al., 2016; Vrielynck et al., 2016).

Many CTAs antigens for instance SPO11 and SYCP1 have a specific function in normal gamete development (Tureci et al., 1998). Thus, meiotic-like cell division program can be induced via up-regulation of meiotic proteins in somatic cells, leading to oncogenic genetic alterations. In cancer cells, inappropriate inter-chromosome recombination and inter-homoluge recombination may result from up-regulation of meiosis-specific proteins (Caballero & Chen, 2009). Furthermore, forming the meiotic-like complex might lead to genomic instability and/or promote cell proliferation in mitotically dividing cancer cells. This has been previously demonstrated as the activation of the meiosis-specific *SYCP3* gene in cancer cells causes disruption to BRCA2-mediated recombination (Hosoya et al., 2011). It is also interesting to note is that while SPO11 is found in all cancer cell lines and most tumour tissues, it may have meiotic-like functions leading to chromosome rearrangement and/or mis-segregation. In order to further investigate the proposal that SPO11 plays a role in a mitotic chromosome dynamics, the co-staining with Bloom (BLM) protein should be taken into account. In fact, BLM has an important role in controlling homologous recombination (HR), aiming to repair DSBs and dissolving double Holliday junctions (dHJs) (Kikuchi et al., 2009; Wu & Hickson, 2003).

However, it is also possible that SPO11 depletion causes chromosome rearrangement during mitotic cell division because of incorrect replication and/or initiation of DSBs. The notion that DNA may bind covalently to SPO11 is supported by preliminarily analysis of intermediates in mitotic cells (Aldeailej, PhD. Thesis, Bangor University), although further analysis is required.

The result presented here provide further evidence to suggest that the hypothesized function of SPO11 in cancer cells may relate to the involvement in pre-meiotic DNA replication. Spo11 and Rad50 have been shown an indirect implication in processes associated with DNA replication during S phase-related events in *S. cerevisiae*. Since these proteins are required in the formation of meiotic recombination and chromosome synapsis, their function during DNA replication may be relevant to that (Merino et al., 2000). Additionally, these proteins may have an important part in DNA repair through their potential participation in sister chromatid association (Bressan et al., 1998; Ivanov et al., 1992). Interestingly, it has been proved that the absence of Spo11 function can result in shortening the length of the pre-meiotic replication program during S phase. Therefore, despite the main described function of SPO11 protein in meiosis is related to the formation of DSBs, its task is not limited to this function (Cha & Kleckner, 2000).

The effects of SPO11 depletion on cell count and self-renewal can be explained by the behaviour of cancer cells following SPO11 protein reduction. In this study, different cell cycle proteins, such as cyclin A2, B1, P21, c-Myc and Cdk2, were analysed. Despite the tight regulation of cell cycle processes through specific proteins known as cyclins, any changes in the level of these proteins may lead to uncontrolled growth, a well-known cancer hallmark (Behl & Ziegler, 2014a). Indeed, cell cycle protein levels assessed in this study were significantly affected by the reduction of SPO11 protein. We further characterised treated cells, which showed SPO11 depletion, against P21, P27 and c-Myc proteins, resulting in protein level reductions. It is highly likely that the disruption in SPO11 protein levels in cancer cells affects cell cycle protein regulators, such as those regulating the G1-S and G2-M transitions, leading to inhibition of cell proliferation and self-renewal. Cha et al. (2000) reported that SPO11 knockout may lead to a reduction in the length of the pre-meiotic S-phase by 25%. Our results also suggest the involvement of SPO11 protein as a CTA in mitotic cell division in cancer cells. The results presented here also suggest that the hypothesis of the involvement of SPO11 in DNA replication in cancer cells is supported by the obvious influence of SPO11 deletion on

several cell cycle proteins such as cyclin A, which is responsible for S phase regulation (see Chapter 5).

It is worth mentioning that RAD51 levels were significantly reduced due to SPO11 knockdown. These observations are in line with the finding that, in mice, the initiation of DSBs can be prevented through the absence of Spo11, leading to a reduction in the levels of Rad51 and Dmc1 protein, which are responsible for the formation and repair of DSBs (Carofiglio et al., 2013). This may suggest that SPO11 plays an important role in minimising the number of DSBs, which is linked to the regulation of RAD51 protein. Taken together, the present results provide a platform for further studies investigating the postulated function of SPO11 protein as an oncogenic driver in cell proliferation and cell cycle regulation in cancer cells, making *SPO11* an attractive and effective cancer biomarker and tumour-specific drugs target.

## 7.6 Closing remarks

The results and findings obtained from this thesis shed light on the essential and uncharacterised role(s) of SPO11 protein in cancer cells. For further studies, a stable SPO11 knockout cell line could be created using, for instance, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9), which may aid in the functional analysis of SPO11 in mitotic cells. A Tet-On 3G inducing system stable cell line with SPO11 and tags (N-HA and C-Myc) should be established and tested at different doxycycline concentrations in order to demonstrate the effect of *SPO11* overexpression on cell proliferation. Further investigations are required to confirm the influence of SPO11 knockdown on cell proliferation *in vivo* (mouse model) using shRNA techniques. Ultimately, confirmation of SPO11 as a potential cancer drug target could be achieved through the design and testing of small molecular SPO11 inhibitors.

# Chapter 8

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# 8. References

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