



Sveriges lantbruksuniversitet  
Swedish University of Agricultural Sciences

**Fakulteten för veterinärmedicin  
och husdjursvetenskap**

Institutionen för biomedicin och veterinär  
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# **The effect of differential phosphorylation of YB-1 on apoptosis and cell cycle regulation**

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*Uppsala  
2016*

*Examensarbete 30 hp inom veterinärprogrammet*

*ISSN 1652-8697  
Examensarbete 2016:19*



# The effect of differential phosphorylation of YB-1 on apoptosis and cell cycle regulation

## Effekten av olika fosforylerade former YB-1 på celldöd och reglering av cellcykeln

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*Examensarbete i veterinärmedicin*

*Omfattning: 30 hp*

**Nivå och fördjupning:** Avancerad nivå, A2E

**Kurskod:** EX0751

*Utgivningsort: Uppsala*

*Utgivningsår: 2016*

**Delnummer i serie:** Examensarbete 2016:19

**ISSN:** 1652-8697

**Elektronisk publicering:** <http://stud.epsilon.slu.se>

**Nyckelord:** YB-1, apoptos, fosforylering, cellcykel

**Key words:** YB-1, apoptosis, phosphorylation, cell cycle

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## **SUMMARY**

The purpose of this study was to investigate whether different phosphorylated forms of Y-box binding protein (YB-1) can affect the regulation of the cell cycle and/or apoptosis of cancer cells. Recent studies have suggested a key role for YB-1 in the regulation of cancer.

Just like many other oncoproteins YB-1 is required for vital processes in the cell and hence it is not possible to apply YB-1 in a therapeutic role. Therefore, my aim was to elucidate whether or not one or more phosphorylated forms of YB-1 play a decisive role in apoptosis.

Human colorectal cancer cells (HCT 116) were transfected with different phosphorylated forms of YB-1. These cells were subsequently analysed by flow cytometry in order to compare live/dead cells between the different mutants.

The results from this study suggest that one of the mutants (S176A) plays a more important role when it comes to protecting the cells from apoptosis and hence contribute to promoting sustained proliferation. Moreover, in opposite to S176A, the results indicate that another mutant (S167A) seems to have a more important role protecting the cells from sustained proliferation.

## **SAMMANFATTNING**

Syftet med denna studie var att undersöka huruvida olika fosforylerade former av Y-box binding protein-1 (YB-1) påverkar celldöd och/eller reglering av cell cykeln. Tidigare studier tyder på att YB-1 har en nyckelposition gällande reglering och uppkomst av cancer.

Precis som med många andra onkoproteiner krävs YB-1 för många vitala processer i cellerna och därför kan inte behandling riktas generellt mot YB-1. Med denna studie hoppas jag därför kunna finna en eller flera olika fosforylerade former av YB-1 som har en mer eller mindre avgörande roll gällande apoptos. Detta för att behandling av cancer i framtiden då skulle kunna riktas mot enbart ett eller flera av dessa fosforyleringsställen.

För att undersöka detta har humana colo-rektala cancerceller transfekterats med sex olika mutationer av YB-1, dessa har sedan analyserats med hjälp av flödescytometri för att jämföra levande/döda celler mellan de olika mutationerna.

Resultaten från denna studie tyder på att en av mutationerna har en mer betydande roll när det gäller att hjälpa cellerna att undvika apoptos och därmed främjar okontrollerad celledelning. Likaså tyder resultaten också på att en annan av mutationerna har mer betydande roll när det gäller att skydda cellerna från ohämmad tillväxt.

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## INTRODUCTION

Cancer has become an increasingly complex disease to fully understand. In the year 2000 Hanahan and Weinberg proposed that a “vast catalogue of cancer cell genotypes [could be organized into] a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth” (Hanahan and Weinberg, 2000). They denominated these alterations ”Hallmarks of Cancer”, defined as “...acquired capabilities” common to most cancers that “...incipient cancer cells... [must acquire to] enable them to become tumorigenic and ultimately malignant.”

The original hallmarks identified were:

- Self-sufficiency in growth signals (later renamed proliferative signalling) - cancer cells proliferate indefinitely.
- Insensitivity to anti-growth signals (evading growth suppressor signals) – cancer cells cannot respond to anti-proliferation signals or to the withdrawal of normal proliferation signals.
- Evading apoptosis (resisting cell programmed death) – cancer cells avoid the usual process whereby abnormal or redundant cells trigger internal self-destruction (as opposed to necrotic cell death).
- Limitless replicative potential (Enabling replicative immortality) - cancer cells do not become senescent cease to proliferate or die after a limited number of mitoses.
- Sustained angiogenesis (inducing angiogenesis) – cancer cells promote outgrowth of new blood vessels as part of the supporting stroma.



- Tissue invasion and metastasis (activating invasion and metastasis) - invasive tumors must be able to invade adjacent normal tissue.

Ten years later Hanahan and Weinberg expanded these Hallmarks of Cancer (Hanahan and Weinberg 2011). This expansion included two enabling characteristics namely:

- Genome instability and mutation, which allows changes in one cell to be inherited by daughter cells by mutation or even by epigenetic changes of parental cell DNA.
- Tumor-promoting inflammation in the microenvironment. Inflammation contributes to cancer cell survival, angiogenesis, metastasis, and the abrogation of adaptive immunity (Colotta et al 2009).

In this revised set of hallmarks they also included two “emerging” hallmarks:

- Avoidance of immune surveillance that would otherwise target them for destruction.
- Dysregulation of metabolism. This is one of the most prominent qualities of malignant disease (Warburg 1930, Eisenberg 1961). It is needed to support the increased anabolic and catabolic demands of rapid proliferation and most likely enables carcinogenesis.

Thus the hallmarks of cancer can be regarded as a prominent multilayered series of nested defences that must be overcome before cells can become immortalized. No less than seven of the eleven hallmarks comprise potent defensive mechanisms that have been resisted, overwhelmed or bypassed altogether in order for carcinogenesis to occur.

The first line of defence is the maintenance of antioxidant defences. Then DNA repair systems are in place to address a wide range of damage to maintain genome stability. Tumor suppressors such as p53 and other cell-cycle controls prevent cellular replication while repair is carried out. When this is not possible apoptosis and other forms

of cell death ensure that lineages of dysfunctional cells are not replicated, and properly functioning senescence prevents sustained proliferation and cellular immortalization.

Moreover, the genesis of cancer cells in tissues and organ systems is routinely monitored and arrested by the body's immune system through protective mechanisms of acute inflammation, or the maintenance of balance between apoptosis and wound healing, properties of immune and non-immune systems (e.g., vasculature, metabolic and neuroendocrine pathways) (Gajewski, T. *et al.*, 2013). This involves a host of immune system components (e.g., dendritic cells, T-cells, Natural killer (NK) cells and macrophages), and the hypothalamic-pituitary-adrenal axis output (i.e., cortisol) which invokes and suppresses the master immune/inflammatory cytokine macrophage migration inhibitory factor (MIF) to manage inflammation. Finally, adjacent tissues in the tumor microenvironment can also serve to normalize the behaviour of potentially cancerous cells to suppress tumor growth (Bissell et al 2011, Bizzarri et al 2014, Goetz et al 2011). However, in a fully developed cancer, most, if not all, of these lines of defence have been effectively muted, suppressed, bypassed or overcome.

In theory, it should not matter whether or not these lines of defence are rendered dysfunctional, overwhelmed or bypassed altogether by (a) a series of somatic mutations/copy number variations and epigenetic changes (in a Darwinian-style cellular evolution); (b) carcinogenic exposures to an indiscriminate mutagen; or (c) ongoing exposures to a mixture of disruptive chemicals. What is critical to recognize is that effective disablement of all of these nested defensive capabilities appears to be an integral part of carcinogenesis.

The aim of this study is to investigate the multipotent oncoprotein Y-box-binding protein 1 (YB-1) in greater detail. YB-1 has been shown to be involved in the regulation of a number of hallmarks involved in carcinogenesis, as detailed below. The mechanisms by which this occurs is unclear. One possible mechanism is that post-translational regulation may occur through differential phosphorylation of particular residues.

**Purpose**

Recent data have suggested a key role for YB1 in the context of carcinogenesis. Differentially phosphorylated forms of YB-1 may be involved in functionally different processes within the cell. Therefore, the aim of this study is to investigate the impact of phosphorylation of specific residues, on apoptosis and regulation of the cell cycle.

**Question at issue**

Do different phosphorylated forms of the oncogene YB-1 affect apoptosis and/or the regulation of the cell cycle?

## ABBREVIATIONS USED

Akt	RAC alpha serine/threonine protein kinase
CIP1	Cellulose induced protein 1
CSD	Cold shock domain
FACS	Flourescence activated cell sorter
FLICA	Fluorochrome labelled inhibitor of caspases
FS	Forward scatter
HA	Hemagglutinin influenza virus ep
HCT 116	Human Colorectal Carcinoma cells
IGF-1	Insulin like growth factor 1
IGF-2	Insulin like growth factor 2
MDR-1	Multidrug resistance gene 1
MCF 7	Human breast adenocarcinoma
MIF	Migration Inhibitory factor
mTOR	Mammalian target of rapamycin
NK-cells	Natural Killer cells
P21	Protein 21 Dalton
PI	Propidium Iodide
PI3K	Phosphoinoside 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
SS	Side scatter

WAF1	Wild type activating fragment 1
YB-1	Y-box binding protein 1

## LITERATURE REVIEW

### General

Y-box binding protein 1 (YB-1) is a multifunctional protein, which is a member of the cold shock protein family. It is mainly localized to the cytoplasm but in response to genotoxic stress it can accumulate in the nucleus (koicke et al 1997, Eliseeva IA, YB-1 and its functions). Its presence in the nucleus suggests that it plays a role in gene transcription. However, the fact that YB-1 is predominately localized to the cytoplasm indicates that its also involved in other processes.

The YB-1 protein is involved in several cellular processes, such as proliferation, differentiation and response to stress. In order to examine its physiological role *in vivo*, Lu *et. al* produced homozygous mice with a true null mutation in the YB-1 gene. They discovered that YB-1 is vital for the development of multiple organ systems. Moreover, they showed that YB-1 is required for accomplish a normal late embryonic development and survival (Lu *et. al.*, 2005).

Clinical studies have shown that cellular levels of YB-1 correlate with prognosis and tumour growth for ovary, lung, and breast cancers. 2006 Takeshi *et. al.* showed that YB-1 knockout embryos went through severe growth retardation and mortality after E10.5. In this experiment Takeshi *et al* also discovered that YB-1 plays a critical role in the early development of the mice, thus the targeted disruption resulted in a defect in the anterior neural tube and ended up being fatal in the late embryonic stage (Takeshi Uchiumi *et. al.*, 2006).

In earlier studies Bargou *et. al.*, expressed YB-1 in pre-neoplastic breast epithelial cells in order to explore its effects on the cell phenotype. This caused the cells to be doxorubicin resistant; a change that occurred in parallel with a simultaneous increase expression of a multidrug resistant gene (MDR1; Sutherland, B. W. *et al.*, 2005).

## Functions in the nucleus

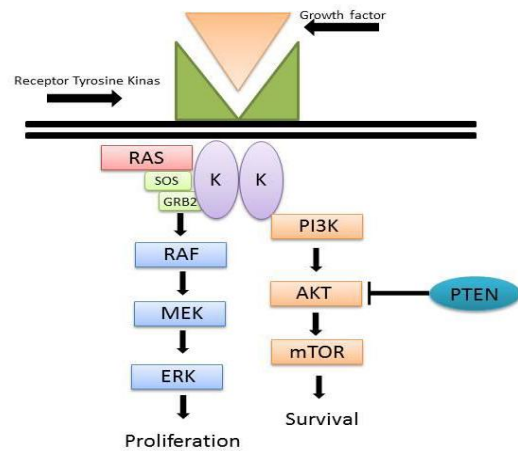
In the nucleus YB-1 can bind to both DNA and RNA and it is involved in almost all DNA and mRNA processes, as eg; DNA-replication, DNA-repair, transcription, mRNA splicing and translation (Eliseeva, I. A *et. al.*, 2011).

It has quite recently been demonstrated that YB-1 co-regulates E2F target genes and furthermore controls the expression of different E2F family members, including E2F2 and E2F5 promoters in MCF7 cells. In conclusion, YB-1 does not only promote expression of the E2F activator, but also inhibits the expression of the repressor of E2F's and by binding to several other E2F promoters it co-regulates their expression. This fact means that YB-1 might have an important, maybe crucial, role in the E2F regulated proliferation pathway (Lasham *et. al.* 2013).

## YB-1 and the Akt/PI3K Pathway

Except from E2F, other pathways can be regulated by YB-1. The PI3K/Akt/mTOR pathway is a well-known signalling pathway that is overactivated in several types of cancers (Fig. 1). The signal contains an inositol lipid, - phosphatidylinositol(3,4,5)triphosphate that is activated by phosphatidylinositol 3-kinase (PI3K). This pathway leads to a wide range of responses, including cell growth, proliferation and survival (Vivanco *et. al.* 2003).

Sutherland *et. al.*, (2005) reported that P-Akt binds to YB-1 at its cold shock domain (CSD) and thereby phosphorylates the serine (Ser) 102 site. They also showed that when Ser102 was substituted with an alanine (Ala) at this site the amount of YB-1 located in the nucleus decreased considerably, strongly suggesting that phosphorylation of Ser102 is required for the nuclear translocation of YB-1. This is important as YB-1 can only act as a transcriptional regulator when it is located in the nucleus.



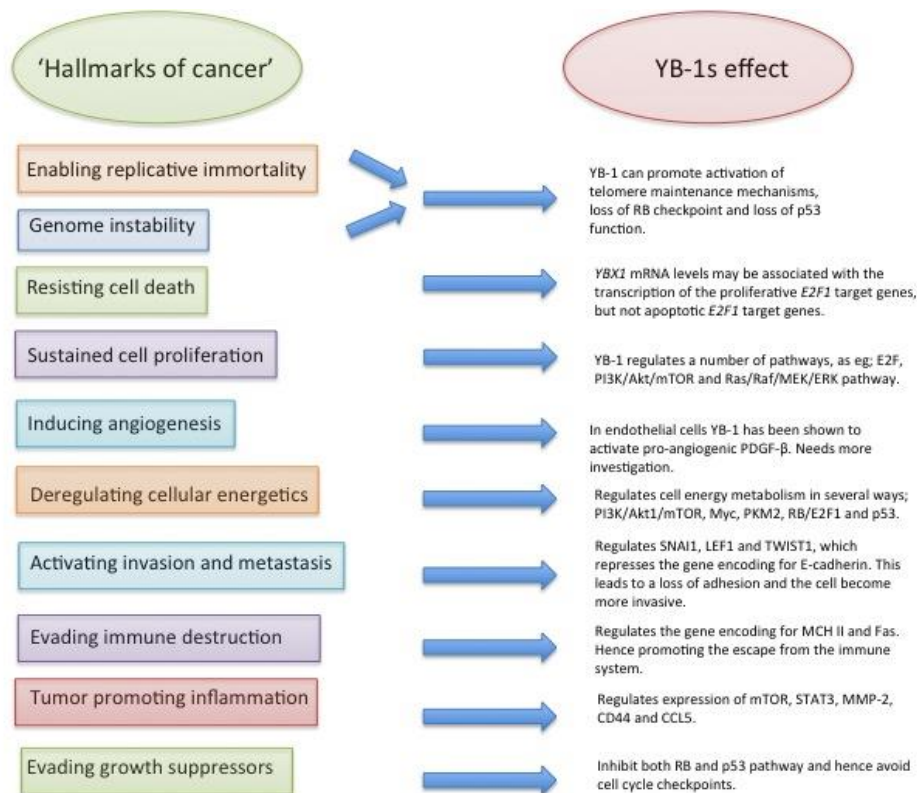
**Figure 1.** The Akt/PI3K signalling pathway.

Several different ligands (such as IGF1 and 2) can bind to the tyrosine kinase receptor which is the initial step in the activation of this pathway. Subsequent downstream phosphorylation, steps activate Akt. Once it is activated, a wide range of different proteins will in turn be phosphorylated leading to an alteration in function.

### **YB-1 and the 'Hallmarks of cancer'**

YB-1 regulates a wide variety of DNA and RNA events, and is involved in all ten 'Hallmarks of cancer' (Fig.2). The fact that Hanahan and Wienberg proposed that a cell must overcome all of these 10 hallmarks to become malignant points at a pivotal role for YB-1 in the oncogenic process.





**Figure 2. YB-1 and the hallmarks of cancer.** Illustrates how YB-1 affects and regulates the 10 Hallmarks of cancer (Lasham *et. al.* 2013).

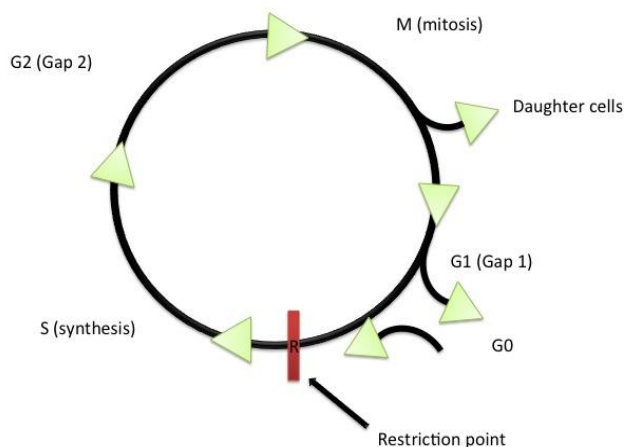
### YB-1, p53 and protection from apoptosis

YB-1 belongs to a protein family which regulates gene activity on a translational as well as transcriptional level. We know from earlier work that *tp53* is an important tumour suppressor gene which plays a pivotal role responding to DNA damage – hence the nickname – “guardian of the genome”. It is frequently mutated in many human cancers including cancers of the breast, colon, lung and liver (Hollstein *et al.*, 1991). People with an inherited loss of the *tp53* gene develop a syndrome known as Li Fraumeni syndrome (Li FP, Fraumeini JF, 1969, Li FP *et. al.*, 1992). Interestingly, Okamoto *et. al.*, (2000), showed that p53 directly interacts with YB-1 *in vitro* and it was also discovered that p53 and YB-1 can bind to each other *in vivo*.

In 2003 Zhang *et. al.*, demonstrated that functional p53 is required for YB-1 to translocate to the nucleus. They also showed that YB-1 inhibits p53 induced cell death when localized in the nucleus, providing further evidence that YB-1 is intricately linked to cell death via its interaction with p53.

To ensure genetic stability p53 can induce either cell arrest or apoptosis (Fig. 3) by activating downstream regulators such as p21/CIP1/WAF1. Because p53 has such an important role regulating cell survival, p53 itself has to be tightly controlled which takes place on a transcriptional or translational level, via protein stability or by its subcellular localization (Okamoto *et. al.*, 2000).

Theoretically, there are a number of ways that YB-1 can protect cells from apoptosis, thus contributing to cancer development. One is by interacting with p53 and modulating its activity. In response to DNA damage, p53 normally activates two different types of genes; those regulating cell cycle arrest and those activating pro-apoptotic genes. YB-1 can regulate p53 directly at a transcriptional level and also indirectly by regulating the stability of p53 and therefore influencing its pro-apoptotic effect (Dmitry *et. al.*, 2013). In addition to affecting apoptosis through an interaction with p53 – there are also p53-independent ways that YB-1 may function to protect cells from apoptosis. One of the most obvious is by decreasing the transcription of the fas-receptor, which plays an important role in the pro-apoptotic signal pathway (Dmitry *et. al.*, 2013).



**Figure 3. The cell cycle.** Illustrating the different phases of the cell cycle and the restriction point where p53 plays an important role.

## **YB-1 and drug resistance**

As stated previously, YB-1 can function either in the cytoplasm or in the nucleus. Depending on its location, there are two models of how YB-1 responds to stress. In the nucleus it is believed that YB-1 can either modulate the functions of the transcription factors or even act as a transcription factor itself. One gene that YB-1 has been shown to transcriptionally regulate is the multiple drug resistance gene *MDR1* (Dmirty, N. *et. al.*, 2013).

*MDR1* encodes the multidrug resistance transporter P-glycoprotein. Manik *et. al.* discovered that “in multidrug resistant MCF-7 breast cancer cells, nuclear localization of YB-1 is associated with MDR-1 gene expression.” Moreover, they also showed that in drug-sensitive MCF-7 cells YB-1 was mainly located in the cytoplasm. In the same study they found a correlation between YB-1 levels in the nucleus and the presence of high levels of P-glycoprotein. Taken together, these data suggest that YB-1 plays an important role controlling transcription of the *MDR1* gene (Manik, *et al.*, 2008).

Taken together, YB-1 appears to be a *bona fide* oncoprotein with crucial roles in apoptosis and cell cycle regulation. YB-1 interacts with and regulates a wide range of important cell cycle components as p53, Akt and *MDR1*. Presumably, it is possible that YB-1's disordered structure can account for its multifaceted role. The activation of YB-1 is a truly complex process, not only by the phosphorylation-induced decrease in its affinity for mRNAs, but also through its many ‘partners’. It then follows that we would like to investigate whether phosphorylation of specific residues of this protein plays a critical role in its regulatory capacity.

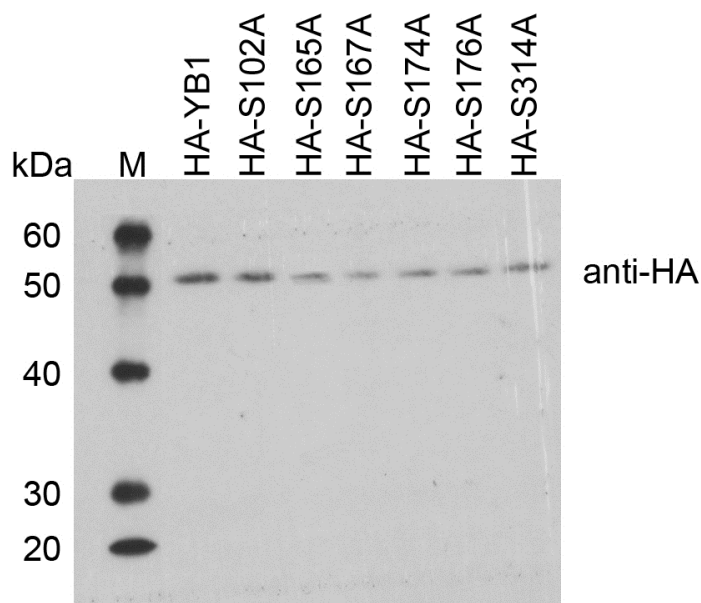
## **MATERIAL AND METHODOLOGY**

### **Cell culture and transfection**

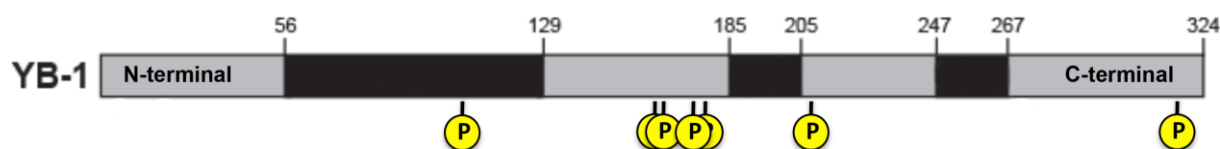
HCT 116 (Human Colorectal Carcinoma cells) were obtained from ATCC and cultured in Modified Eagle Medium (MEM) supplemented with 10% fetal calf serum, and 1% penicillin/streptomycin (50 ug/ml medium). When the cells reached 60-70% confluence they were transfected with a mutant form of the oncogene YB-1.

## Plasmids

The cells were transfected with plasmids containing a DNA construct where serine (S) had been mutated, using site-directed mutagenesis, to an alanine (A) at one site in the YB-1 gene (either S102A, S165A, S167A, S174A, S176A or S314A) as shown if figure 5. The substitution of a serine to an alanine prevents the ability of kinases to phosphorylate the residue, thus disabling potential signalling dependent on this phosphorylation. Each of these mutated plasmids also carried an HA-tag so that they could be labelled for subsequent analyses. To ensure that the presence of the HA-tag didn't obscure the result, control cells were transfected with a HA-YB-1 plasmid only. The plasmids also contained an ampicillin-resistant cassette *Escherichia Coli* and were grown in luria broth (LB)-agar. When an adequate concentration was reached in culture the bacteria were harvested and the antibiotic ampicillin added in order to ensure that only plasmid-expressing bacteria survived. The plasmid was then purified from the bacterial cultures by midi-preparation. Plasmid expression was confirmed by Western Blot (Figure 4).



**Figure 4.** Western blot of protein lysates from HCT 116 cells transfected with mutant HA-tagged YB1. Antibody is rabbit anti HA (1:1000) from Sigma Aldrich. HA-tagged YB-1 is present in all lanes.



**Figure 5. Linear representation of YB-1.** Showing phosphorylated serine residues (in yellow) 102, 165, 167, 174, 176, 314. These serines were mutated to alanine using site-directed cloning techniques. Alanine residues are not able to be phosphorylated.

## Transfection

Transfections were carried out using Lipofectamine 3000 (Intvitrogen) accordingly to the manufacturer's recommendations, for concentration see table below. Lipofectamine 3000 is a lipid-based method for transfection. By using lipid based reagents a lipid-DNA complex is formed, which is capable of crossing the cell membrane.

Transfection Amounts	Per well
6-well DNA	2500 ng
P3000™ Reagent	5 µL
Lipofectamine® 3000 Reagent	3.75 and 7.5 µL

**Table 1.** Concentrations used for transfection.

## Flow cytometry and staining

The transfected cells were incubated for either 12, 24 or 48 hours before harvesting and staining for flow cytometry. To determine whether the cells transfected with different phosphorylated forms of YB-1 have a higher rate of apoptotic cells we used Fluorescence-activated cell sorting (FACS). In the FACS – the ratio 633 nm live/dead cells was compared for the different plasmids and also if apoptosis could account for the cell increased death. Specific information concerning excitation, detectors, emission and fluorochromes, are summarised in Appendix one.

## FLICA, Annexin, Propidium Iodide, Zombie

Activation of a cascade of caspases (3, 8 and 9) signals apoptosis (see Parrish, AB, 2013 for review). To be able to detect their activation *in situ* Grabarek, *et. al.* used ‘applied fluorochrome labelled inhibitors of caspases (FLICA) as affinity labels of active centers of their enzymes’ (Grabarek, J *et. al.*, 2002). Hence, cells labeled with FLICA can be detected with the FACS and compared to live/dead cells.

Another early event of apoptosis includes translocation of a membrane phosphatidylserine from inside of the cell membrane to the surface (Segawa *et. al.*, 2015). Annexin V is a marker that will detect this and is therefore used as a back-up for the FLICA in this study.

A biotinylated anti-HA tag directly conjugated to the fluorophore (405nm) was used to detect cells that containing each of the plasmids, cells could be gated based on the expression of this tag. Another fluorescent dye (Zombie; 633 nm) was used to distinguish live cells from dead cells.

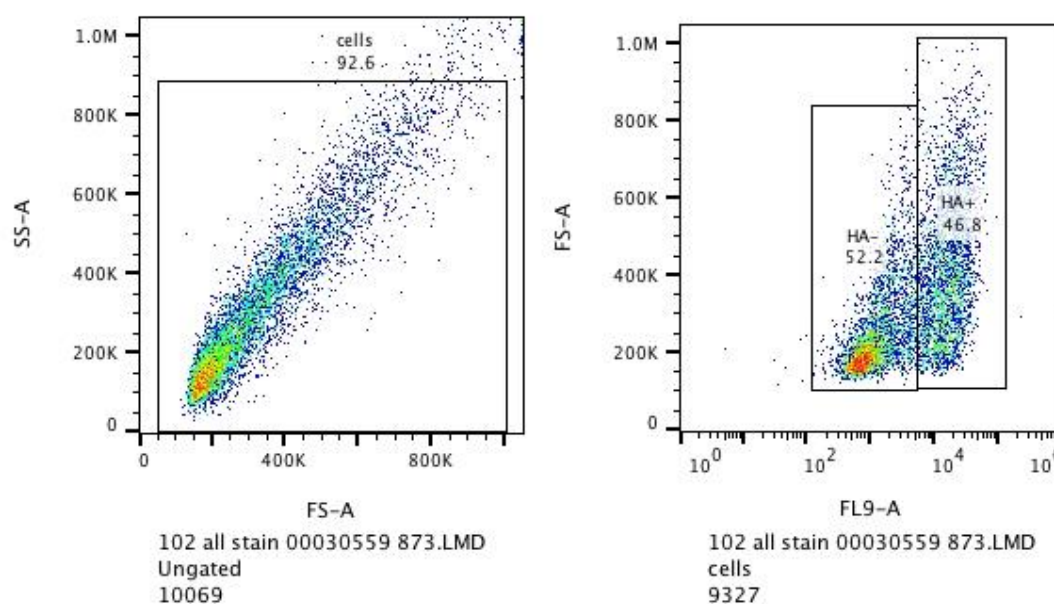
By using the nucleic acid intercalating dye Propidium Iodide (488 nm) it is possible to determine the momentary position of individual cells in the cell cycle by using the FACS.

By using flow cytometry analysis FLICA positive and FLICA negative, live/dead cells and transfected (HA+) / non transfected (HA-) cells could be separated and their relative numbers compared.

In flow cytometry, the cells are passed in front of a laser beam and detectors measure the forward scatter (FS) and side-scatter (SS). FS correlates with cell size and SS with cell granularity. Cells can also be gated, depending on the presence of a fluorescent tag.

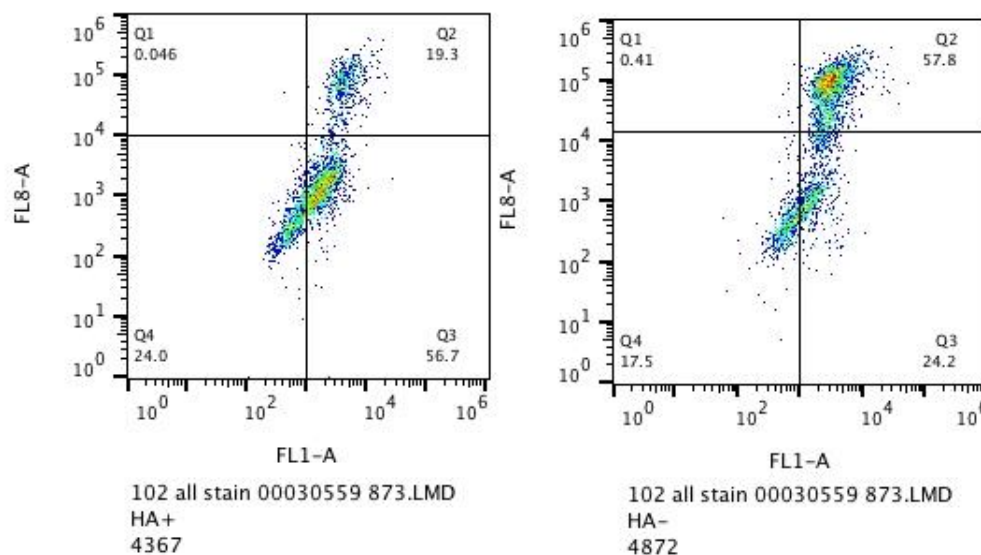
## **Analytical methodology**

The analyses were performed using the software *FlowJo data analysis software*.



**Figure 6a. Representation of HCT 116 cells as detected by FACS.** Cells expressing the plasmid S102A Each individual dot represents one cell.

The left hand graph (figure 6a) represents all cells separated by FACS. In the right hand graph these cells are separated based on the presence of the fluorescently-tagged HA (FL-9). HA+ cells make up 46,8% HA- cells 52,2%).



**Figure 6b. Percentage of apoptotic cells 24 hours post transfection.** Graphs for HA+ respectively HA- cells 24 hours after transfection. X-axis showing FLICA+/FLICA- and Y-axis live/dead cells. FL-8 is live/dead cells and represents the Zombie NIR dye and FL1 represents the FLICA+/FLICA- cells.

When the gates for transfected (HA+) and “non-transfected” (HA-) cells were set we were able to compare these populations to FLICA positive versus FLICA negative to our live/dead cells.

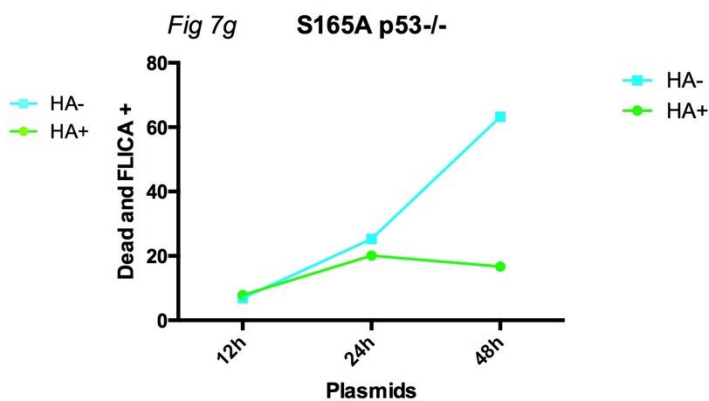
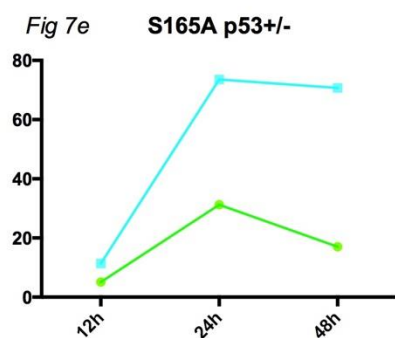
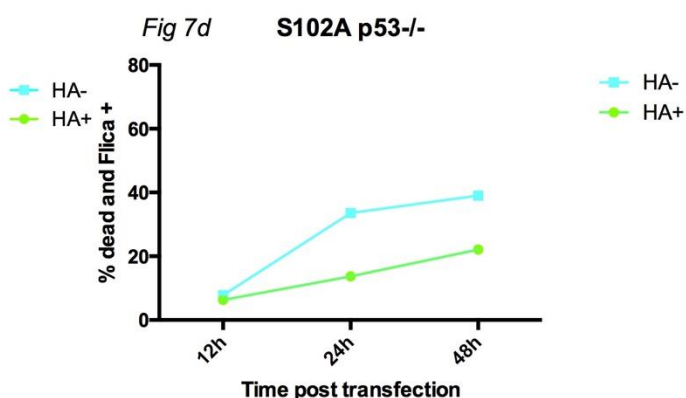
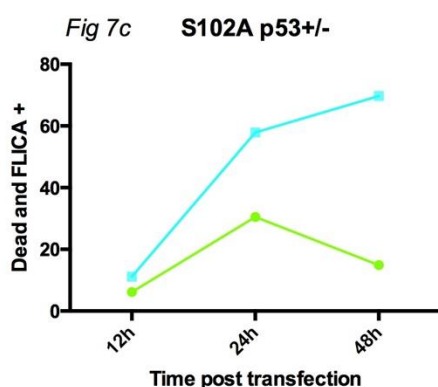
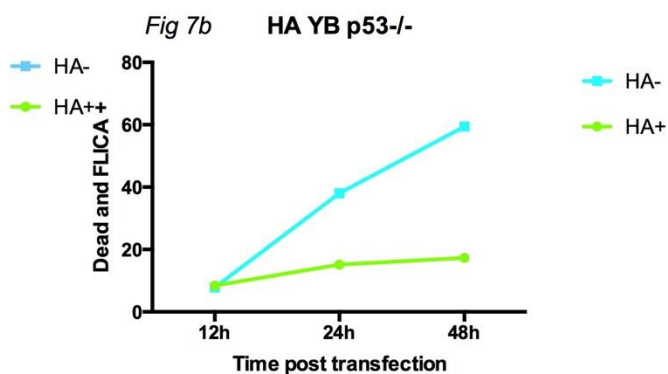
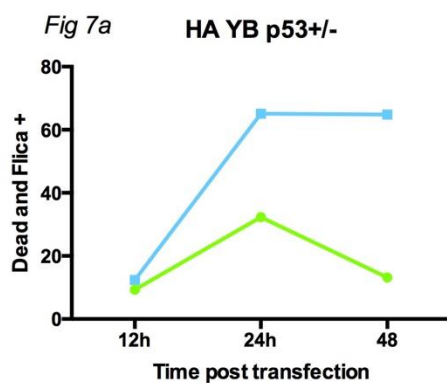
On the left graph above (Fig 6b), the transfected cells (HA+) (Quadrant (Q)1: Dead/FLICA neg. Q2: Dead/FLICA pos. Q3: Live/FLICA pos. Q4: Live/FLICA neg.) are shown. When we compare the results between HA+ and HA- cells in the HA – population about 58% of the cells are dead and almost all of them have been going through caspase-induced apoptosis. If we look at the HA+ cells about 19% of the cells were dead and FLICA positive.

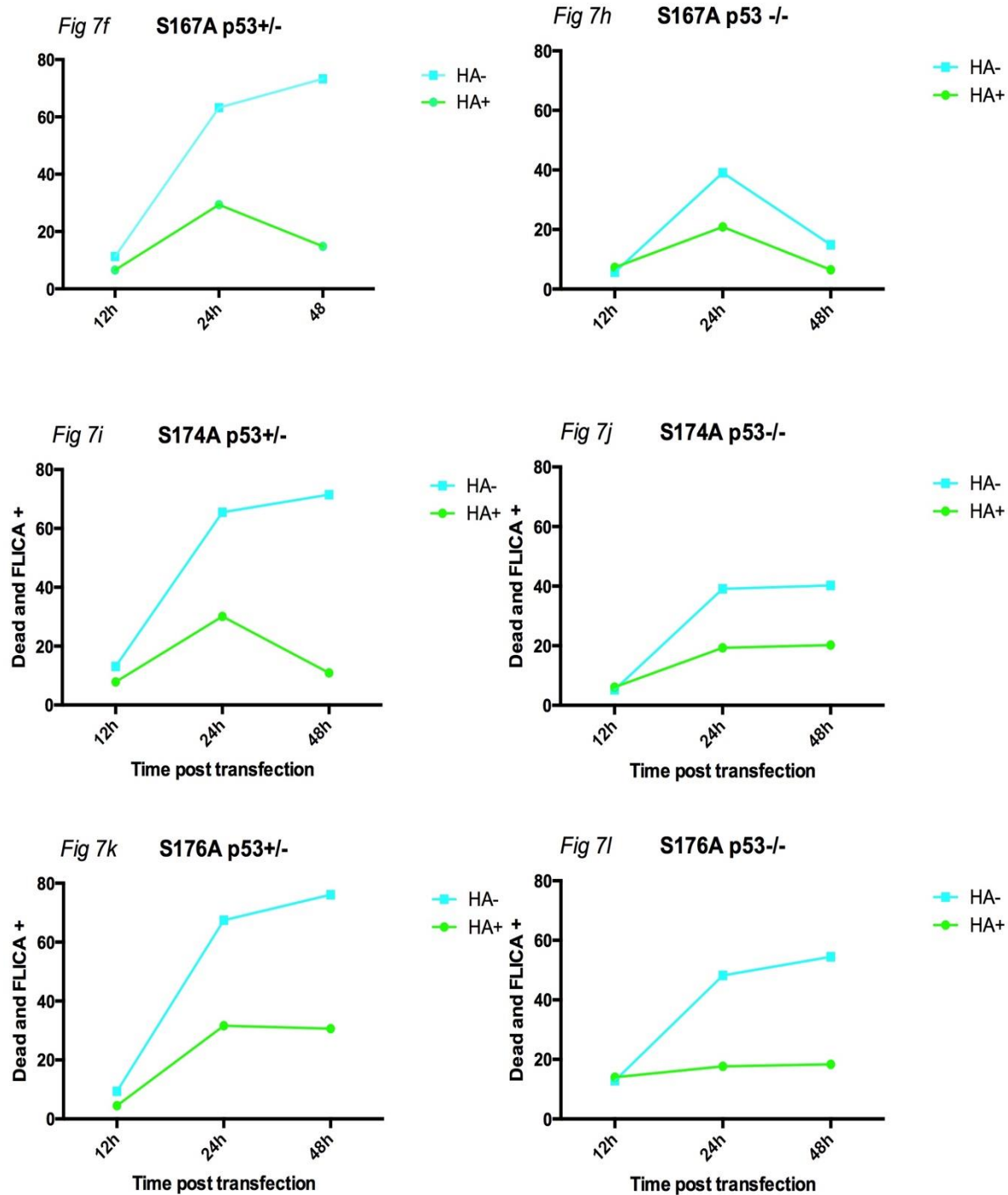


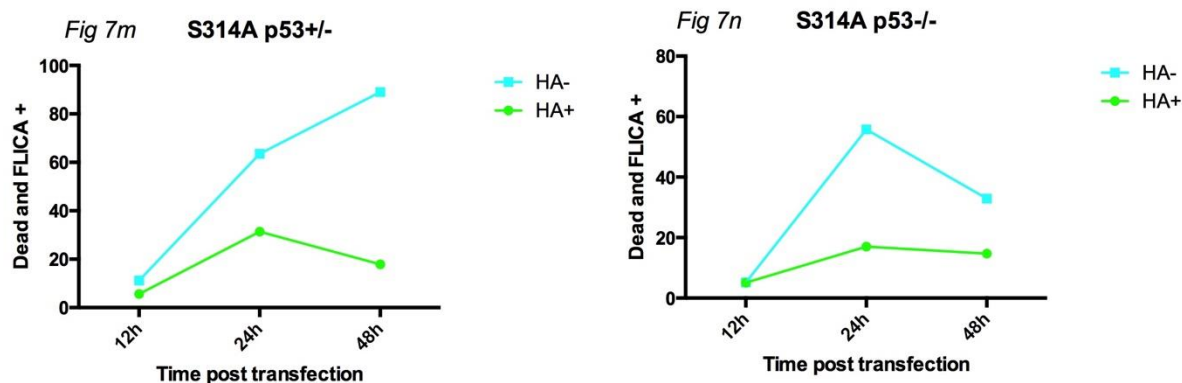
## RESULTS

### Temporal analysis of apoptotic response

The graphs displayed below (Figures 7a-n) is a comparison between the apoptotic outcome in untransfected cells (HA-) and transfected cells (HA+) containing the seven different plasmids over time.







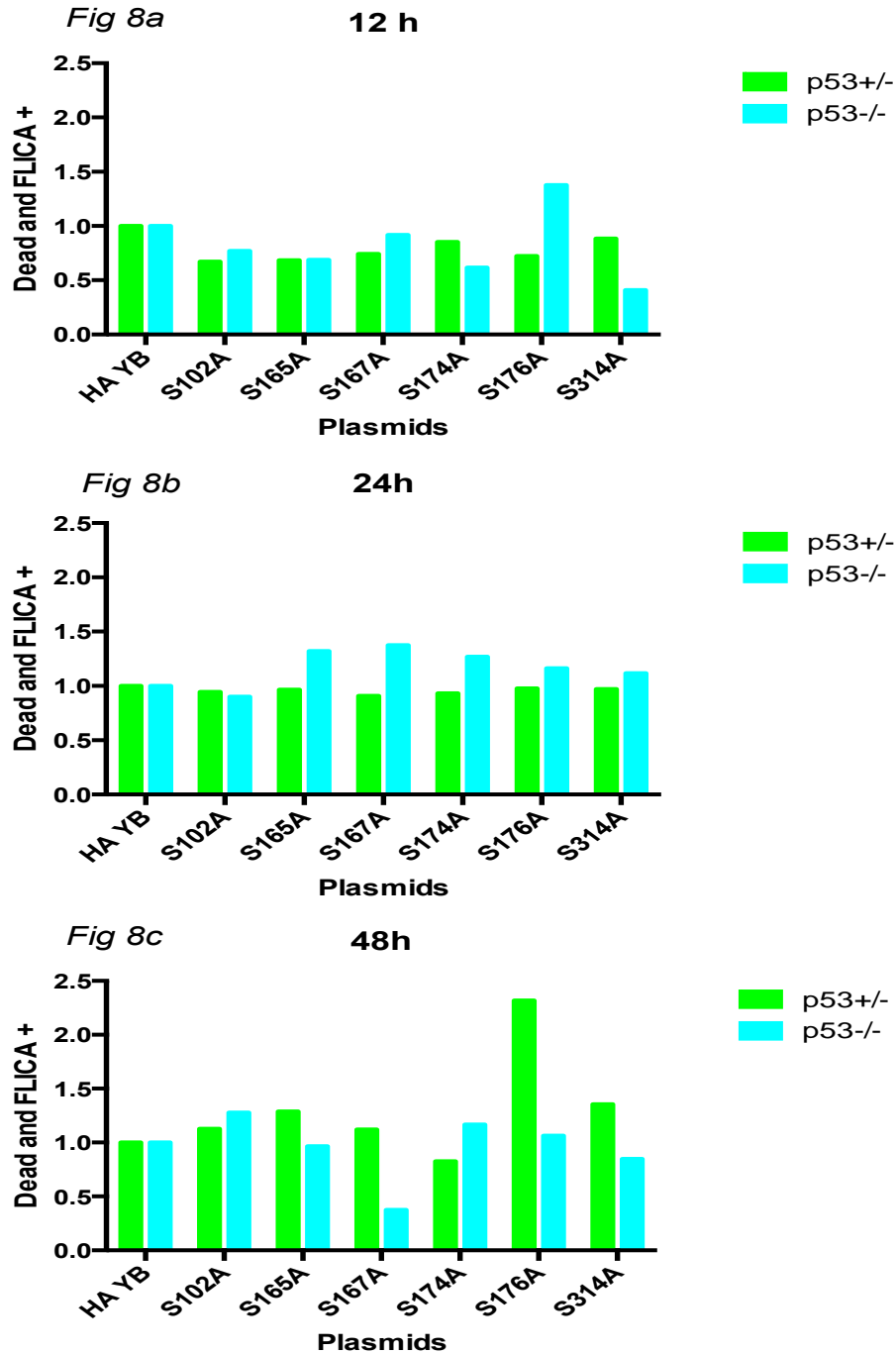
**Figure 7a-n.** Temporal analysis of apoptotic response in differentially phosphorylated forms of YB-1 showing a comparison between HA+ (transfected) and HA- (non transfected control cells) over time (12h, 24h and 48h). The ordinate represents FLICA positive (apoptotic) cells and the abscissa time post transfection.

The comparison of apoptotic outcome in untransfected cells (HA-) and transfected cells (HA+) containing different plasmids, show that 30% of the HA+ cells are apoptotic after 24 hours for the HA-YB p53+/- (fig 7a), whereas after 48h the percentage had decreased to 15%. A similar pattern occurs for S102A (fig 7c), S165A (fig 7e), S167A (fig 7e) and S174A (fig 7i) in the p53+/-.

Plasmid S176A p53+/- displays 30% apoptotic cells after 24 hours (fig 7k) and the proportion of apoptotic cells stay similar after 48 hours, unlike the other plasmids. The transfected cells of the HA-YB plasmid population for p53 -/- cells contain approximately 20% apoptotic cells at 24 and 48 hours (fig 7b). For plasmid S102A (p53 -/-) 10% of the cells are apoptotic after 12 hours and 20% after 48 hours. The plasmids S165A, S174A and S176A show comparable results to those of HA-YB. In plasmid S167A (p53 -/-), the percentage of apoptotic cells were 20% after 24 hours and 7% after 48 hours. Moreover, cells containing p53 (+/-) have a higher rate of apoptotic cells (7a-n). The percentage apoptotic cells in S167A and S314A for HA- cells in the p53 +/- cell line is decreasing over time i.e. between 24 and 48 hours.

### **Comparison of mutations normalised to HA-YB**

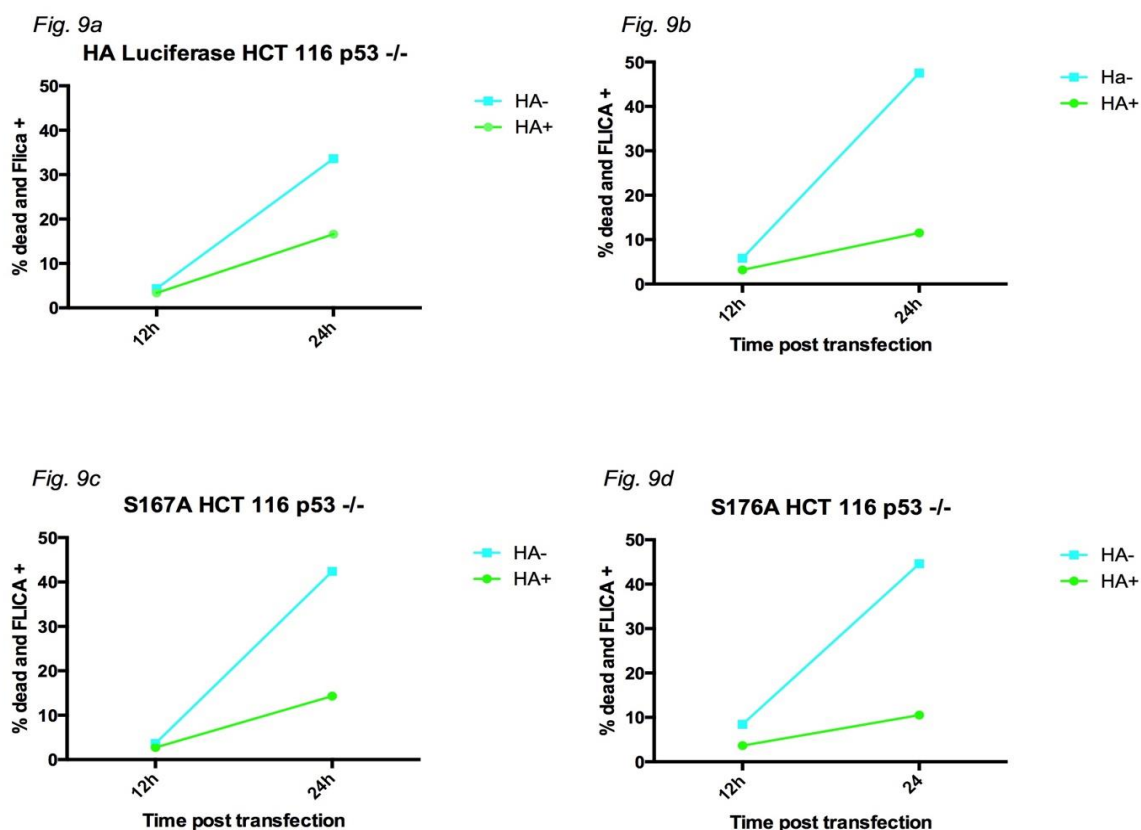
Figures shown on the next page illustrate the relative proportion of FLICA+ cells in all cells carrying an HA-tagged mutant YB-1 plasmid in comparison with a HA-YB-1 control plasmid. The figure clearly shows that for most of the plasmids, there was very little difference between any of the plasmids in comparison with the control. Some of the HA-tagged plasmids appear to have a slightly different effect on apoptosis. At 12 hours post transfection, there is an increase in the number of FLICA+ S176A p53<sup>-/-</sup> cells (fig 8a). There is a difference between cells with a mutation at residue 165,167 or 174 but to a lesser extent at residues 176 and 314. At 48 hours post transfection (fig 8c) p53 <sup>+/-</sup> cells containing the S176A plasmid have a higher percentage of FLICA + dead cells, which indicates that this residue might play a more significant role in protecting the cell from apoptosis in the presence of p53. When residue 176 is mutated, the number of FLICA+ cells increases.



**Figure 8a-c. Comparison of effects of different mutations on cell death, normalised to HA YB.** The ordinate represents the percentage of FLICA-positive (apoptotic) cells, and the abscissa represents each of the different plasmids and also p53 +/- respective p53 -/- cells for the HA+ cells only. In these graphs HA YB-1 is normalised to 1 (100%) and numbers of FLICA-positive cells adjusted accordingly to HA YB-1 control.

## Cells transfected with HA-luciferase plasmid

To complete the picture, we performed a final experiment where we transfected the same cell line with a non-YB-1 (Luciferase) HA-tagged plasmid. Figures 9a and b indicate that the Luciferase plasmid also protects the cells from apoptosis to some degree. To determine if there was any difference between the luciferase and the plasmids containing YB-1, the ratio for HA+: HA- for dead and FLICA positive cells at the certain time points (12h and 24h) were calculated. By then comparing the ratio of the Luciferase to the different plasmids (table 2 and 3) it is shown that the protective effect of the HA-tagged luciferase plasmid is less pronounced than that of the HA-YB-1. Luciferase has a ratio of 2 at 24 hours post transfection, while the HA YB-1 have a ratio at 4,1. Just like the previous experiments we can in this one see that S176A protects the cells from apoptosis, while the ratio for S167A is lower.



**Figure 9 Cells transfected with HA-luciferase plasmid show reduced protection from apoptosis.**

The ordinate represents percentage FLICA positive apoptotic cells and the abscissa time post transfection.

## Effect of individual HA-tagged mutants on apoptosis compared to Luciferase

### 12 hours post transfection

Plasmids	HA-:HA+
HA Luciferase	1,2
HA YB-1	1,8
S167A	1,3
S176A	2,3

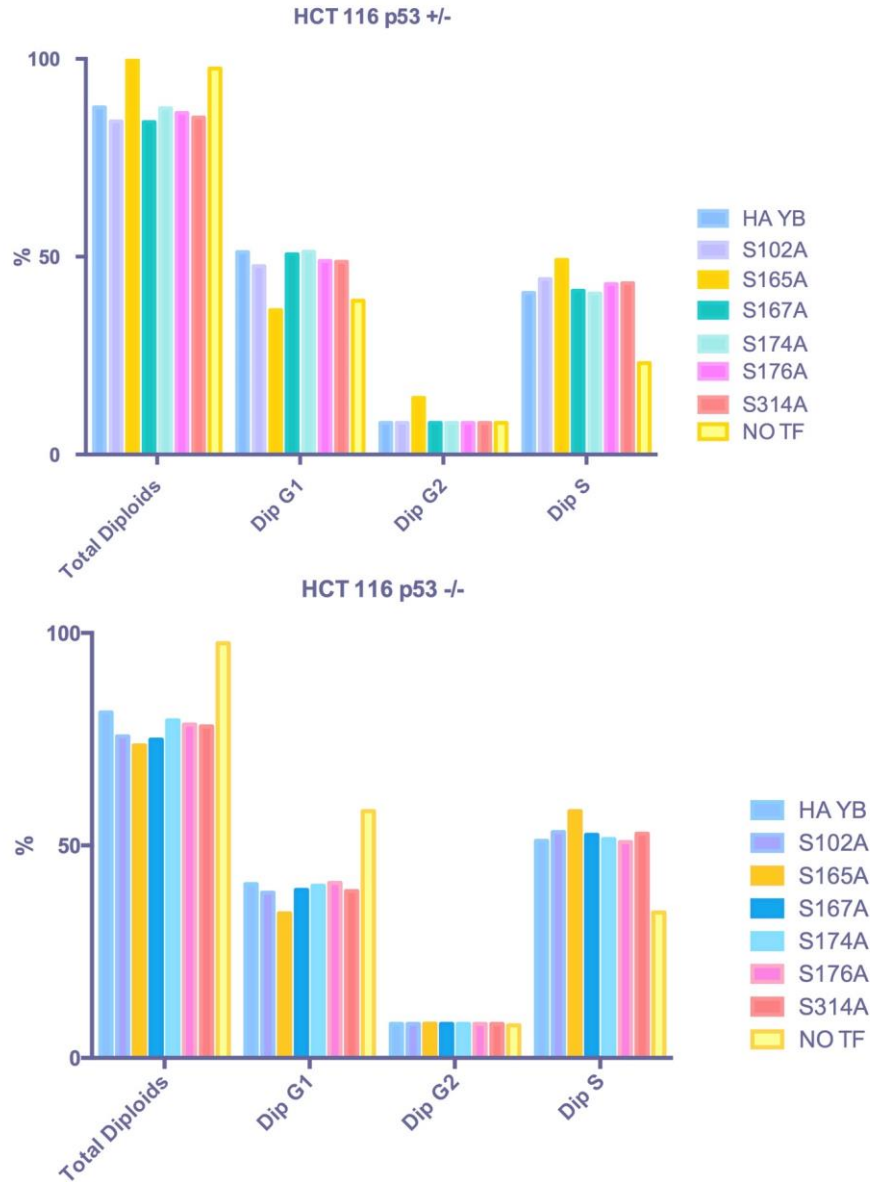
### 24 hours post transfection

Plasmids	HA-:HA+
HA Luciferase	2
HA YB-1	4,1
S167A	2,9
S176A	4,2

**Table 2 and 3.** shows the ratio for HA+: HA- cell over 12h respective 24h for the different phosphorylated forms of YB-1.

## Effects of different mutants on cell cycle position

By using the nucleic acid intercalating dye Propidium Iodide, it is possible to determine the momentary position of individual cells in the cell cycle by using the FACS (for graphs, see appendix 2). A disadvantage of this method is that we were unable to gate for HA+ / HA- cells. However, on the basis of the transfection rate from earlier experiments I estimated the transfection rate to be at least 40% for this cell line. Bearing this in mind, it was anticipated that a clear effect should be seen on the cell cycle.



**Figure 10. Variation in the cell cycle phases.** The percentage of cells in each phase of the cell cycle for each plasmid, 24 hours post transfection.

The effects of different mutants on the cellular distribution in the cell cycle 24 hours post transfection is summarized in figure 10. The figure shows that for mutation S165A (p53+/-) an increased proportion of cells is diploids and hence mostly of the cells remain in the S-phase.



## DISCUSSION

### Overexpression of YB-1 and apoptosis

The key question has been whether differentially phosphorylated forms of YB-1 affect apoptosis and/or are capable of dysregulating the cell cycle.

The main finding in this study was that untransfected (HA-) cells showed an increased percentage of FLICA+ (i.e. apoptotic) cells. From this result it was concluded that the transfection with Lipofectamine 3000 stresses the cells and therefore forces the cells towards apoptosis. This suggests that the presence of YB-1 (regardless of differentially phosphorylated sites) protects the cells from apoptosis following exposure to the transfection reagent.

This finding is consistent with previous observations. The overexpression of YB-1 has been shown to protect cells from p53-induced apoptosis (Lasham 2013, Zhang 2003). YB-1 can protect cells from apoptosis in more than one way. It can act by interfering with the p53 modulation of gene transcription and therefore ‘control the regulation’ of cell cycle arrest and pro-apoptotic genes. YB-1 can also reduce the transcription of the *fas-receptor gene*, which plays an important role in the pro-apoptotic signalling pathway (Dmitry, N et al. 2013).

Moreover, all cells used in these experiments contain an endogenous form of YB-1. It is known that cancer cells exposed to drugs or other stress stimuli will overexpress YB-1, which protects the cells from apoptosis.

In summary, YB-1 protects the cells from undergoing apoptosis and hence promoting sustained proliferation and cancer. The main question however remains -, whether or not differentially phosphorylated forms differ from each other in their capacity of repressing apoptosis in these cells. In figure 7a-n we can see that all of the mutant forms of YB-1 respond to the transfection in a similar way.

It thus seems that two of the plasmids play a more important role. Plasmid S176A seems to have a more potent effect on counteracting apoptosis than the other forms. In opposite it seems like plasmid S167A seems to have a less anti apoptotic effect. This suggests that residual S176A plays a pivotal role in preventing the cells from undergoing apoptosis following cellular stress, and thereby helps to maintain their neoplastic phenotype. It also suggests that S167A plays a less protective role.

To ensure that it was the transfection that induced the cellular stress and the overexpression of YB-1 we performed a final experiment where we transfected the same cell line with a non-YB-1 (Luciferase) HA-tagged plasmid. This experiment showed that the Luciferase plasmid itself protected the cells from apoptosis, but to a lesser extent than the plasmids containing YB-1.

### **The effect of differentially phosphorylated forms of YB-1 on cell proliferation**

It has been demonstrated that two mutated forms of YB1; S176A and S167A, act in a different way compared to other plasmids and also compared to HA-YB on apoptosis.

Moreover, mutation S165A, showed an increased proportion of cells in the S-phase. One reasonable explanation is that phosphorylation on 165 promotes senescence and when the Serine is changed to an Alanine, (which abrogates the phosphorylation effect), the cells will divide more frequently, suggesting that residual 165 possibly protects the cells from sustained proliferation.

In conclusion, a higher percentage (100%) of the cells mutated at residual S165A seems to remain in DNA-synthesis (S-phase). It is not unreasonable to assume that S165A plays a more important role than hitherto assumed in senescence.

To confirm this conclusion, we would need to repeat this experiment at each of the time points (12h, 24h, and 48h). Temporal cell cycle data are required and thus, we would need to analyze the cells over time and not just a single time point. We would also need to repeat this experiment for different cell lines.

One of the limitations in the PI-based cell cycle analysis is that the use of PI prevents us from gating a fluorescently tagged HA population of cells. To be able to tag the cells with HA cells must be fixed, which can't be done if the cells are going to get stained with PI.

YB-1 is not only important in counteracting programmed cell death and developing cancer. Like many other oncoproteins, it plays different roles in various types of essential cell events. Therefore, it's not possible to directly target YB-1 for therapeutic developments, but if one or more of the different phosphorylated forms of YB-1 play a certain role in evading apoptosis or promoting drug resistance we could possibly, in the future, pinpoint the specific kinase (using a kinase inhibitor) and hence prevent phosphorylation of this specific residue.

It then follows that if S176A plays a more important role to abrogate apoptosis, which seems likely in these experiments, it would be possible to target this specific residue in a therapeutic quest.

Moreover, as shown by Bargou *et al.*, 1997, YB-1 is capable of up-regulating the *MDR1* gene and hence promoting drug resistance. Except for avoiding apoptosis by regulating p53 that is another possible reason to why these cells are protected in this experiment. The Lipofectamine is a stress to the cells, which possibly make YB-1 to relocate from the cytoplasm to the nucleus.

Like S176A seems to have a more crucial role in avoiding apoptosis, other residuals might have a similar role in affecting drug resistance which in theory at least suggests novel routes for the development of therapies for important groups of human cancer.

## **Conclusions**

This study aimed to investigate whether different phosphorylated forms of YB-1 can affect the regulation of the cell cycle and/or apoptosis of cancer cells. In conclusion, two different mutations seem to play a more important role for programmed cell death. The first mutation, S176A seems to protect the cancer cells from apoptosis whereas S167A seems to play a less protective role. Moreover, we wanted to determine whether the different forms have any impact on the cell cycle parameters and if any of the plasmids trapped the cells in any particular cell cycle phase. From this experiment we could tell that S165A accumulates an increased proportion of diploid cells, as compared to the other plasmids. Thus suggesting, that S165A plays a more important role in the cellular decision to enter senescence.

## **Future directions**

This project has a clear potential for further development. One such future research line would involve the other cancer hallmarks. Hitherto, the interest has focused on evasion of apoptosis and sustained proliferative signalling. But a broader, “holistic”, view of the role of YB1 would be of interest for the detailed understanding of its role in carcinogenesis. One striking example is how the different phosphorylated forms of YB-1 affect drug resistance.

Furthermore, we could envisage that the residuals located adjacent to each other, for example S174A and S176A, act in a similar direction. Another interesting experiment would therefore involve transfection of the cells with a plasmid mutated for both these loci and see how it affects apoptosis and cell proliferation.

## ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my New Zealand supervisor Dr. Adele Woolley for your motivation, support and for being the best supervisor I could have had.

Also, thanks to Professor Antony Braithwaite and the Department of pathology for having me and for being a wonderful host.

My sincere thanks also goes to Katie Young for your patience and for helping me whenever I needed it.

I would also like to thank Dr. Linda Gulliver, without you this thesis would never have happened. Thank you!

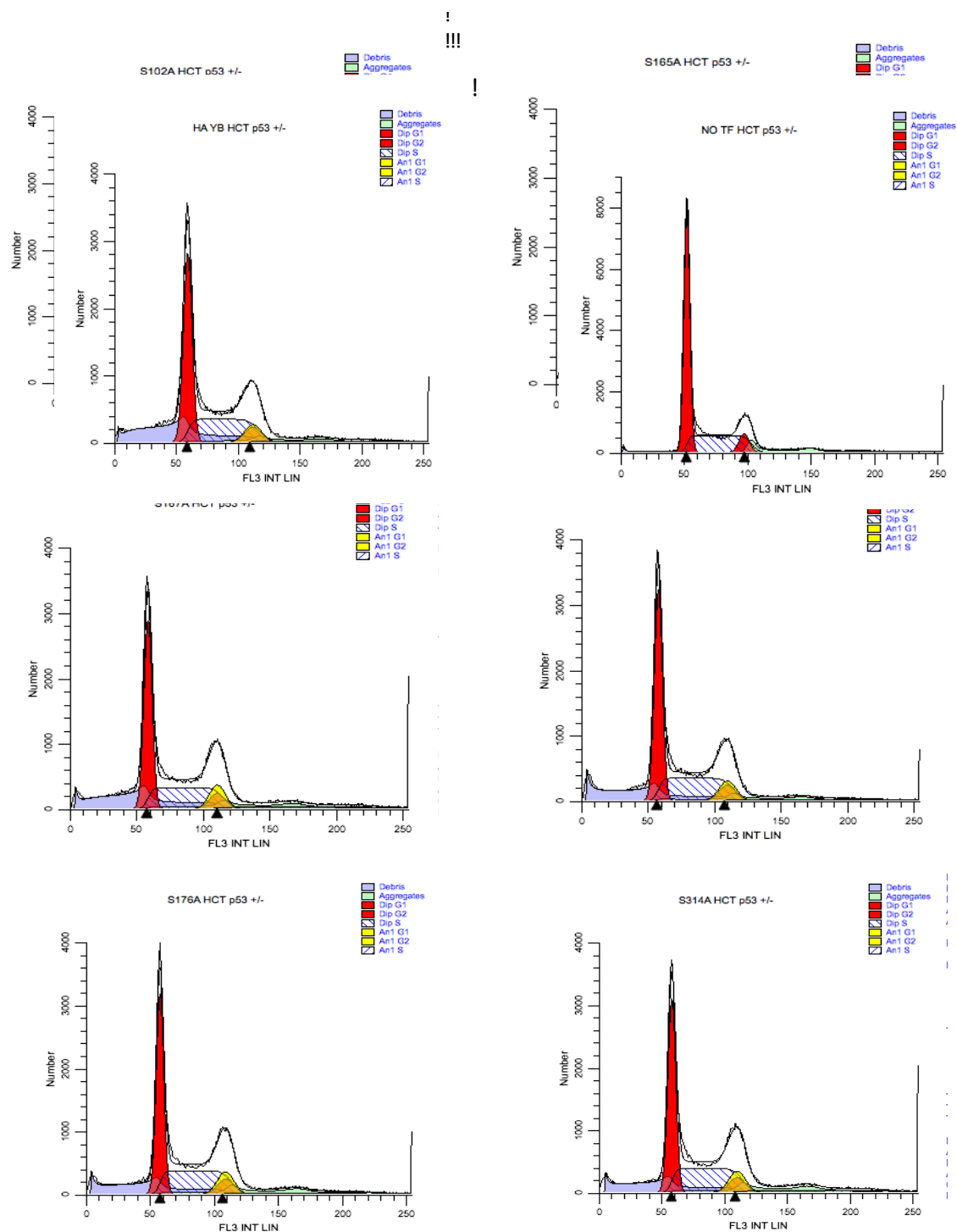
Last, but not least, thanks to my supervisor Wilhelm Engström for guidance in science as well as in life.

## Appendix 1:

Excitation	Detectors	Emission	Fluorochromes
488nm	FL1	525/40 nm	FITC, AlexaFluor 488, GFP, CFSE, Sytox Green
488nm	FL2	575/30 nm	PE, DsRed,
488nm	FL3	620/30 nm	PI, PE-CF594, PE-Texas Red
488nm	FL4	695/30 nm	PE-Cy5, PerCP, 7AAD, PE-Cy5.5
488nm	FL5	755 LP nm	PE-Cy7
633nm	FL6	660/20 nm	APC, AlexaFluor 647, Sytox Red
633nm	FL7	725/20 nm	AlexaFluor700
633nm	FL8	755 LP nm	APC-Cy7, Fixable L/D Near IR
405nm	FL9	450/40 nm	Pacific Blue, BV421, VPD, Sytox Blue
405nm	FL10	550/40 nm	Pacific Orange, AlexaFluor430, Fixable L/D Yellow

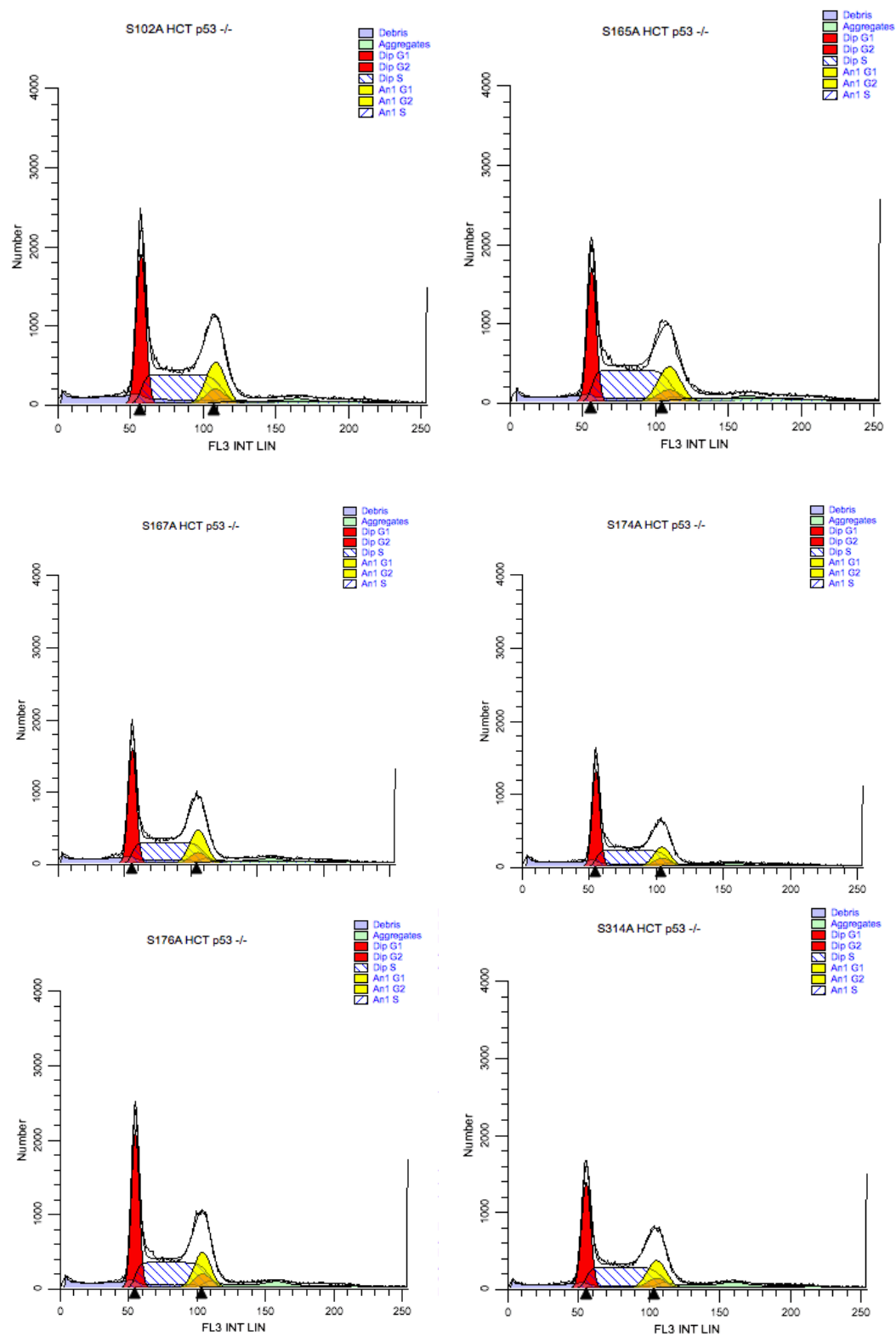
## Appendix 2:

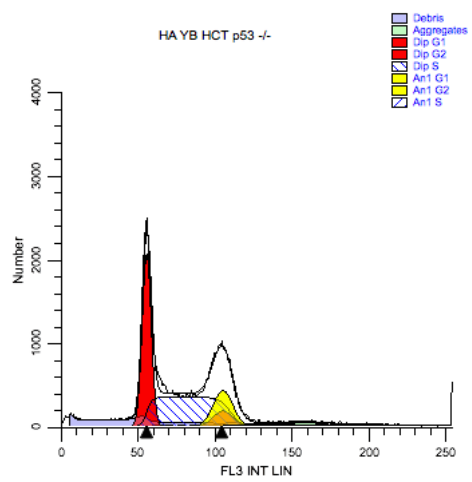
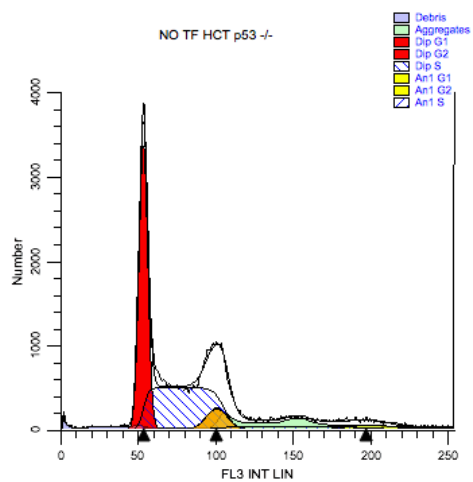
### PI experiment HCT 116 p53 -/-



## Appendix 3:

### PI experiment HCT 116 p53 -/-





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