CELL CYCLE-DEPENDENT REGULATION OF CXXC5 SYNTHESIS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ÖYKÜ DENİZ DEMİRALAY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

AUGUST 2022

Approval of the thesis:

CELL CYCLE-DEPENDENT REGULATION OF CXXC5 SYNTHESIS

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ABSTRACT

CELL CYCLE-DEPENDENT REGULATION OF CXXC5 SYNTHESIS

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Master of Science, Biology
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August 2022, 69 pages

17β-estradiol (E2) is the main estrogen in circulation and has many physiological effects on various tissues, including the mammary tissue. CXXC5 is an estrogen-responsive gene product that binds to nonmethylated CpG dinucleotides on DNA. CXXC5 synthesis shows fluctuation in the cell cycle. This led to our prediction that the level of CXXC5 synthesis is regulated through the cell cycle. To test this prediction, I investigated the synthesis of CXXC5 in cell cycle-synchronized cells for every 6h up to 36h. I found that the level of CXXC5 synthesis shows alterations with the cell cycle. To investigate cell cycle-dependent expression and/or synthesis of CXXC5, I in this thesis established experimental conditions and protocols. These findings lay the foundations for future studies that aim to further delve into mechanisms of CXXC5 expression, synthesis, and degradation in a cell cycle-dependent manner.

Keywords: Estrogen, Estrogen Receptor, CXXC5, Cell Cycle

ÖZ

CXXC5 PROTEİNİNİN HÜCRE DÖNGÜSÜNE GÖRE DÜZENLENMESİ

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Ağustos 2022, 69 sayfa

17β-östradiol (E2) dolaşımdaki ana östrojendir ve meme dokusu dahil olmak üzere çeşitli dokular üzerinde birçok fizyolojik etkiye sahiptir. CXXC5, DNA üzerindeki metillenmemiş CpG dinükleotitlerine bağlanan östrojene duyarlı bir gen ürünüdür. CXXC5 sentezi, hücre döngüsünde dalgalanma gösterir. Bu, CXXC5 sentez seviyesinin hücre döngüsü boyunca düzenlendiğine dair tahminimize yol açtı. Bu tahmini test etmek için, her 6 ila 36 saat arasında hücre döngüsü ile senkronize edilmiş hücrelerde CXXC5 sentezini araştırdım. CXXC5 sentezi seviyesinin hücre döngüsü ile değişiklikler gösterdiğini buldum. CXXC5'in hücre döngüsüne bağlı ekspresyonunu ve/veya sentezini araştırmak için bu tezde deneysel koşullar ve protokoller oluşturdum. Bu bulgular, hücre döngüsüne bağlı bir şekilde CXXC5 ekspresyonu, sentezi ve bozulması mekanizmalarını daha fazla araştırmayı amaçlayan gelecekteki çalışmaların temellerini atıyor.

Anahtar Kelimeler: Östrojen, Östrojen Reseptörü, CXXC5, Hücre Döngüsü

To all who supported me

ACKNOWLEDGMENTS

I would like to express my gratitude for my supervisor Prof. Dr. Mesut Muyan, without whose help this thesis would not actualize, he was always patient and aided me with his insight during the conduction of this work.

I also would like to thank the examining committee; for the time they spent and their much-appreciated suggestions.

Acknowledgements would not be enough for the valuable lessons as a person and a researcher Pelin Yaşar has equipped me with, she was always there for me both in lab and in my times of need. Here, I would also like to thank my lab mates Kerim Yavuz for his dedication and jokes, Gizem Kars for her relaxed yet tailormade advice, Gamze Ayaz Şen for her help during the earliest mornings, and Negin Razizadeh, Gizem Turan, Çağla Ece Olgun. I cannot express my gratitude for all that work at the basement floor, they were all like my lab mates who were there when I needed help and support.

I want to thank my family, İbrahim, Nüket and Bedia Demiralay, my aunts Serpil Dikbayır, Neslihan Kazazoğlu and Zerrin Kandemir Ünsal, they were always by my side, they never questioned and always trusted me. I am the person I am today because of them; they are the best family I could hope for, the ones I could turn to for guidance, they always listened to me. My dearest friend Damla Temel, who even without physically being near me made me feel her presence and assistance throughout my thesis research, I could always trust her love. Also, my roommate Hatice Asena Şanlı, who was supportive and listened to me about my troubles even when she was tired herself. My biggest help and my joy in life, Niyazi Mert Avcıoğlu, my anchor when I felt lost and unable to do much of the things in my life. I would like to thank him for the courage he provided and the love he gave.

This work is partially funded by Scientific and Technological Research Council of Turkey under grant number TÜBİTAK 118Z957.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

E2: 17β-Estradiol

CXXC5: Zinc-Finger CXXC5 domain Protein 5

CHX: Cycloheximide

Act D: Actinomycin D

Noc: Nocodazole

Aph: Aphidicolin

2,3-DCPE: 2-[[3-(2,3-Dichlorophenoxy)propyl]amino]ethanol hydrochloride

CHAPTER 1

INTRODUCTION

1.1 E2 Signaling

The main estrogen in circulation is the 17β-estradiol (E2), which is important for the regulation of many physiological and pathophysiological functions of tissues it affects, one of which is the breast tissue. [1] E2 functions through ligand dependent nuclear hormone receptors estrogen receptor α and β (ER α and β). ERs are transcription factors originating from different genes, ESR1 and ESR2, however they display structural and functional similarities. [1] At different tissues, ERs are present at varying levels, where ERα is the main one in the breast tissue. [1] There exists six different domains from the N-terminus to C-terminus in the ERα structure all of which have different functions. [2]

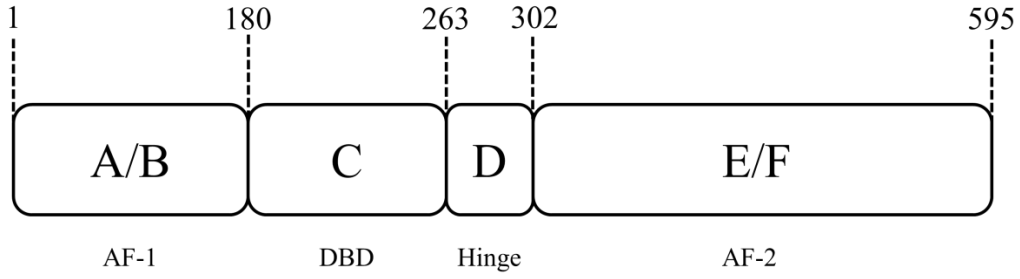

Figure 1: The domains of the ERα are represented N-terminus to C-terminus

The A/B domain at the N-terminus has a ligand-independent transactivation function. The C domain is the DNA Binding Domain (DBD), and the D region. The D region contains a hinge domain, which provides a flexibility between the N- and the C-termini. The D region also contains the nuclear localization signal. The E-F domain, also referred to as the ligand binding domain (LBD), is responsible for

the ligand binding, the ligand-dependent transactivation function (AF-2) and dimerization. [2]

The binding of E2 to ERα causes a conformational change in the receptor which causes the reactive surfaces to emerge for the interactions with the regulatory proteins and the transcription complex. [3 4]

The E2-ERα complex mediates the expression of the target genes via two different pathways: Estrogen Response Element (ERE)-dependent and ERE-independent signaling pathways. ERE sequences are the derivation of the 5'-GGTCA nnn TGACC -3' DNA sequence, where the palindromic sequence is divided by three nonspecific nucleotides in the middle. This sequence is the interaction point of the E2-ERα complex in the ERE-dependent pathway on the DNA, where target gene expression is controlled. Whereas in the ERE-independent pathway, the complex interacts with the transcription complexes already present on the DNA through their own response elements. [456]

1.2 CXXC Type Zinc Finger Protein 5 (CXXC5)

In our laboratory, we previously found that CXXC5 is an E2-responsive gene where its expression is controlled by ERE-dependent signaling pathway via the binding of ERα to a non-consensus ERE sequence GGTCAggaTGACA at the upstream of the first ATG of CXXC5. [7 5 8]

The CXXC5 gene found at 5q31.2 in forward position, and it covers about a 36 kb of the genomic DNA, with 11 exons that give rise to 16 transcript variants, where 14 are protein coding. [8 7] The main transcript variant, TV2, gives rise to a 322 amino acid protein around 33 kDa. [7] This protein belongs to CXXC-type zinc finger protein family, and carries two zinc finger motifs, with a total of eight cysteine residues that have two zinc ions in-between the amino acids 256 and 297. The CXXC5 protein is localized in the nucleus in MCF7 cells due to the nuclear localization signal on its C terminus. [7]

Limited number of studies on CXXC5 suggest that it has a role in transcriptional and epigenetic regulation gene expressions involved in a variety of cellular functions ranging from signal transduction, DNA damage response, energy metabolism, proliferation, differentiation, angiogenesis and apoptosis. ([9],[10] , [11], [12],, [13], [14], [15], [16])

1.3 The Cell Cycle

Cell cycle is the universal and complex process of every cell for the growth and proliferation. The process of cell cycle is thoroughly researched, and the cyclins and cyclin dependent kinases are the main components. There are stages of the cell cycle where individual events take place, these are called as G_1, S, G_2 (collectively known as the interphase), and the M (Mitosis) phase. ([17], [18]) (Figure 2)

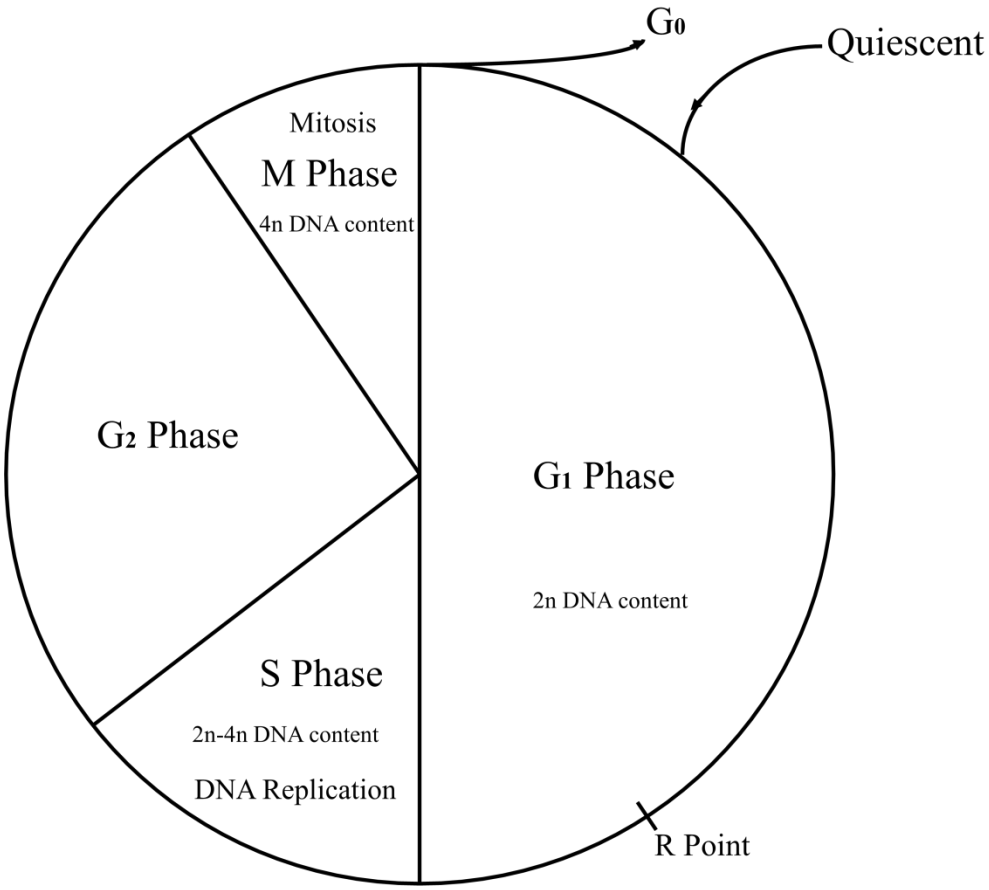

Figure 2: The normal cell cycle process.

The cyclin proteins which are periodically expressed in a cell cycle-dependent manner, are one of the main components of the control mechanism for the cell cycle. [19] The other component of the cell cycle control is the cyclin dependent kinases (CDKs), which are serine/threonine kinases that are activated at different phases of the cell cycle and form complexes with cyclins to control the events of the cell cycle.

In the G1 phase, retinoblastoma protein is one of the main targets of the cyclin complexes (pRb), the phosphorylation of pRb causes the dissociation of the protein from the transcription complex and the gene expression of the target genes starts. [20]

Some drugs have been known to arrest cells at certain phases of the cell cycle, some of which are aphidicolin, 2,3-DCPE, and nocodazole.

Aphidicolin is a mycotoxin that is a known inhibitor of the eukaryotic deoxyribonucleic acid synthesis, by inhibiting the DNA polymerase-α. [21 22] The use of Aphidicolin aids the investigation of cells accumulated at the S phase, where the DNA replication checkpoint is activated, and the cells are arrested due to incomplete replication.

2,3-DCPE, like aphidicolin, arrests the cells at the S phase, but uses a different approach, via the activation of the p21 pathway, signaling a damage to the DNA by the p53 pathway and arrests the cells. [23]

Nocodazole is a benzimidazole type of inhibitor of the microtubule polymerization, therefore stopping the cells in G_2 or M phases, where the cells can be sorted by the flow cytometry. The differentiation of G_2 and M phase cells is not possible by only using the flow cytometer via DNA staining techniques, however the use of the shake-off technique allows the investigators to distinguish the G_2 and M phase cells. Cells in M phase round up and physically divides into two daughter cells, which are either has dislodged or loosely attached to the culture flask. The shake-off technique, which is the tapping of the cell culture flask slightly on a surface, allow the collection of loosely attached cells after dislodging from the base

of the flask. The remaining the attached cells at the bottom of the flask are considered cells that are the different cell cycle phases [24] [25] [26]

1.4 Half-Life Studies

In each cell, every protein and RNA molecules are constantly built and destructed, the half-life of a protein and RNA is an indicator of its structure, components, and functions. To understand these, the half-life of these biomolecules can be investigated. The half-life of a biomolecule is the time required for the elimination of half of the population of a given molecule, for this instance an RNA or a protein. [27]. Based on the observations from our laboratory that the expression of *CXXC5* is regulated by Rb1 and ELF1 transcription factors as key regulators of the G1/S transition of the cell cycle and that CXXC5 is involved in the cellular growth we predict that CXXC5 synthesis and degradation may occur in a cell cycle- dependent manner. Therefore, my aim has been to establish experimental approaches to test this prediction. For this, I utilized various chemicals and approaches described below.

1.4.1. Actinomycin D

Actinomycin D is a chemical that inhibits transcription by interacting with the guanine residues and inhibiting the DNA-dependent RNA polymerase activity [28] [29] [30] The inhibition of the transcription allows the measurement of the time a given RNA is present in a cell.

1.4.2. Cycloheximide

Cycloheximide is a glutarimide antibiotic produced by *Streptomyces griseus*, that interferes with the protein synthesis by inhibiting the movement of mRNA and the tRNAs in the elongation process, the elongation of the nascent peptides in the ribosome. [31] [32] [33]

The use of cycloheximide to inhibit the protein synthesis has been utilized to investigate the half-life of proteins. The inhibition of the new protein synthesis

allows the detection of the levels of a given protein by blotting techniques to assess the time of the said protein to be present in a cell.

1.5 Click Chemistry

The Click Chemistry is a branch of chemical synthesis to attach biomolecules with a luminescent molecule, to aid in the detection, localization and quantification of various organic molecules. [34] [35]

The classic click reaction is a copper-catalyzed reaction and occurs between an azide and an alkyne by a cycloaddition reaction (CuAAC) to form a cyclopentane. [36] [37] [38]

The reaction consists of an azide and an alkyne, and addition of an Cu^{+2} salt and a reducing agent such as sodium ascorbate, where the copper is converted to Cu^{+1} oxidation state and a catalyst, the most common being THPTA, that aids in the reaction at reduced Cu^{+1} levels, since the Cu^{+1} atoms in solution can go through reduction and the sufficient amounts of Cu^{+1} may not be reached. [39] [40] [41] [42]

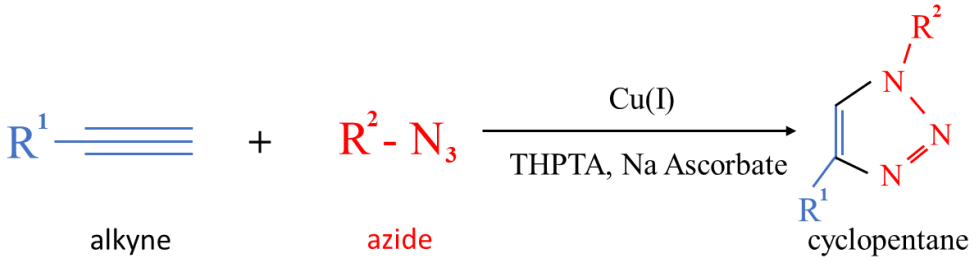

Figure 3: The cycloaddition reaction (CuAAC) by click reaction.

1.6 The Aim of This Study

Previously, our lab revealed that CXXC5 is an estrogen responsive gene product, [7] [43] and its expression is driven by a CpG island promoter [8]. Our promoter pull-down experiments for the CXXC5 also proved that ELF1 and RB1

transcription factors may be involved in the expression of CXXC5 gene [8]. Given the importance of the ELF1-RB1 interactions in the cell cycle regulation these observations suggest that the expression of CXXC5 may be cell cycle-dependent. We also previously described that CXXC5 is an unmethylated CpG binding protein that could interact with TRIM25 (Tripartite Motif Containing 25) and TRIM33 (Tripartite Motif Containing 33) that function E3 ubiquitin-protein ligases as well as USP15 (Ubiquitin Specific Peptidase 15) which acts as a deubiquitinase using a BioID approach. [44] Lastly, ongoing studies put forward that CXXC5 degradation may be dependent on proteasome degradation pathways (P Yaşar, unpublished observation). Based on these previous findings, the aim of my thesis project is to see whether the CXXC5 gene expression and the protein synthesis levels are modulated in a cell cycle-dependent manner or not.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines and Maintenance

MCF7 cells are maintained in high glucose (4.5 g/L) containing Dulbecco's Modified Eagle's Medium (DMEM, Biological Industries, Canada, BI01-053-1A) without phenol red supplemented with 10% Fetal Bovine Serum (FBS, Biological Industries, Canada, BI04-007-1A), 1.2% L-Glutamine (L-Glutamine, Biological Industries, Canada, BI03-020-1B), and 1% Penicillin-Streptomycin (Pen-Strep, Biological Industries, Canada, BI03-031-1B).

Cells are maintained for up to six passages in 95% humidified, 5% CO2, 37˚C cell culture incubator as monolayer and passaged or refreshed as needed every three days.

2.2 Synchronization of MCF7 Cells

MCF7 cells are collected using the trypsin detachment method (1x Trypsin, Biological Industries, Canada, BI03-053-1A and resuspended in normal growth medium, then the cells are pelleted at 300xg for 6 minutes, the medium is removed. The cells are washed once with 1x Phosphate Buffer Saline (PBS, Biological Industries, Canada, BI02-023-5A), then pelleted at 300xg for 7 minutes. After the removal of the 1x PBS, the cells are resuspended in the phenol red free medium with 10% charcoal-dextran (Dextran Coated Charcoal, Sigma, Germany, C6241-20G) treated FBS (CD-FBS), 1.2% L-Glutamine, and 1% Penicillin-Streptomycin. The cells are seeded as $2.5x10^6$ cells/T75 flask. After 48 hours, the medium is

changed with fresh medium containing 10% CD-FBS. 24 hours after medium renewal, (a total of 72 hours after plating), cells are collected by trypsinization, then the cells are counted and 1x 10^6 of the cells are washed once with 1xPBS and resuspended in 1xPBS containing 2% CD-FBS in a 15 ml centrifuge tube. The cells are continuously vortexed as 4 ml of 70% Ethanol is added dropwise onto the cells for fixation. The cells are then kept at -20°C, at least for 1 hour, up to a week, until flow cytometry.

The flow cytometry is for the quantification of cellular DNA in fixed cells as the DNA content for the dividing cells differ at each stage of the cell division. To stain the DNA, the fixed cells are centrifuged at 300xg for 6 minutes and the ethanol is removed, the cells are washed once with ice cold 1x PBS to remove any residual ethanol, then pelleted at 600xg for 7 minutes. The cells are kept on ice until stained with 100 µl of PBS containing propidium iodide (0.02 mg/ml; Sigma-Aldrich), 200 µg/ml RNase A (Thermo-Fisher Scientific) and 0.1% (v/v) Triton X-100 (AppliChem, Germany). The propidium iodide staining is performed with 200 µl at room temperature for 30 minutes at dark. After the incubation, 10x10^3 cells are analyzed with BD Accuri™ C6 Cytometer (BD Accuri™ C6 Cytometer; BD Biosciences, San Jose, CA, USA) in the FL2 channel, that is used for the PI reading, the components of the PI staining are given in Table 1.

Table 1: The Propidium Iodide Staining Solution Components

Component	**Amount**
0.4% Triton-X	50 µl
1 mg/ml Propidium Iodide	4 µl
10 mg/ml RNase A	4 µl
Ice Cold 1xPBS	142 µl

2.3 Hormone Treatments

After synchronization, we assessed the cell cycle distribution at time 0, and if the cells are accumulated at G1 phase about 75% or more, then we treat the cells with 17β-estradiol (E2) (E2, Sigma-Aldrich, St. Louis, MO, USA) or vehicle control (Ethanol). The treatments are 10^{-8} M for E2 treatment and 0.1% ethanol as vehicle control.

The cell cycle treatments were carried out as 6-hour intervals between each timepoints for a total of 36 hours, in the absence or presence of E2.

2.4 Cytoplasmic and Nuclear Protein Isolation

In order to obtain the cytoplasmic and nuclear fractions for protein isolation, NE-PER system is utilized (Nuclear and Cytoplasmic Protein Extraction Kit; Thermo Scientific, CA, USA). The protocol provided by the manufacturer is used. According to the volume of the pellet of the cells for protein isolation, different amounts of the three buffers of the kit is used, the amounts of the buffers are given in the table below. The CERI and NER buffers are prepared to have working concentration of protease inhibitors (Roche Applied Science, 12, Switzerland) and phosphatase inhibitors (). The proteins are kept at -80°C until use, when needed the proteins are always kept on ice. The protein concentrations were measured with Bradford assay (Quick Start Bradford Protein Assay, Bio-Rad, CA, USA).

Table 2: NE-PER kit buffer volumes for the protein isolation.

Cell Volume (µl)	CERI (µl)	CERII (µl)	NER (µl)
10	100	5.5	50
20	200	11	100
50	500	27.5	250
100	1000	55	500

2.5 Cycloheximide Treatment

To assess the protein half-life of CXXC5, we first optimized the concentration of L-Homopropargylglycine (L-HPG) for the nascent protein synthesis labelling for the microscopy imaging. MCF7 cells are seeded as $15x10^3$ cell/well to 48-well plates in normal growth medium, and 48 hours post-seeding, the medium is removed and refreshed with Methionine/Cysteine free medium for 1 hour to starve the cells. After the incubation, the medium is supplemented with 3, 6, 12 or 25 μM L-HPG for 6h. Then, Click-It reaction is applied for the labelling and samples are visualize under the microscope.

2.5.1 Optimizations

After we have optimized the L-HPG concentration, we then went on to optimize the treatment time and concentration of Cycloheximide to assess protein synthesis. We also wanted to assess whether the use of cycloheximide would affect the RNA amount and transcription was also observed.

As described previously, we have grown our MCF7 cells in normal growth medium for 48 hours and then refreshed the medium with Methionine/Cysteine free medium for 30 minutes, then add cycloheximide in 50 μg/ml onto the cells with 30 minutes pre-treatment and, at 45 minutes add cycloheximide in 50 μg/ml onto the cells with 15 minutes pre-treatment, and finally at 1 hour starvation end, add cycloheximide in 50 μg/ml onto the cells, at the end of the starvation period, 12 μM L-HPG and Cycloheximide for 6 hours. At the end of the treatment, the cells are fixed and stained with the Click-it reaction.

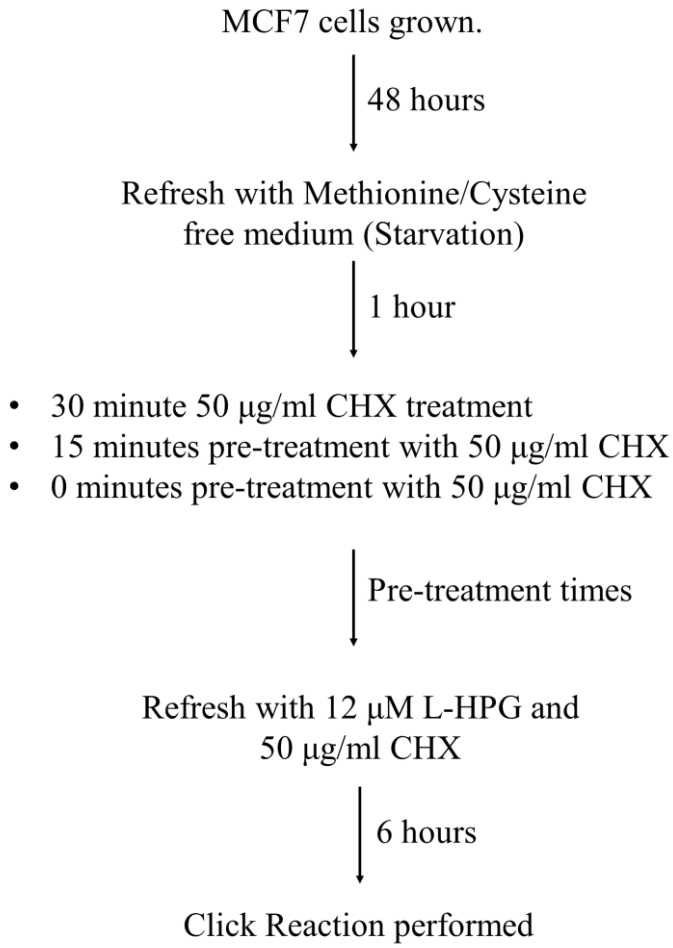

Figure 4: Click-it experimental procedure.

2.5.2 Click-It Reaction and Microscopy

The medium is removed, and the cells are washed once with 3 % BSA in 1xPBS, then the cells are fixed with 3.7 % Formaldehyde in 1x PBS (Applichem, A0936) for 15 minutes at room temperature while shaking. After incubation, we removed the fixation solution from the cells and added 0.5 % Triton-X in 1xPBS and incubate on shaker for 20 minutes at room temperature. Meanwhile, we prepare the Click reagent, which should be prepared fresh.

Table 3: CuSO4 and THPTA mixture preparation

Compound	Final Concentration	500 µl Reaction
100 mM $CuSO_4$ Stock	33.33 mM	10 µl
250 mM THPTA Stock	166.66 mM	20 µl

First the compounds from the table above were prepared, then the compounds below were mixed in the written order below.

Table 4: Click-It reaction buffer concentrations.

Compound	Final Concentration	500 µl Reaction
100 mM Sodium Phosphate Reaction Buffer, pH: 7		419 µl
10 mM Sulfo-Cy5 Azide	20 µM	1 µl
$CuSO_4$/THPTA Mixture	2 mM / 10 mM	30 µl
1 M Na-Ascorbate Solution	100 mM	50 µl

When the incubation was finished, we removed the permeabilization solution and washed cells twice with 3% BSA in 1xPBS, then added the Click reaction mixture, and incubated for 45 minutes at room temperature on shaker. At the end of the labeling, we removed the solution and washed cells once with 3 % BSA in 1xPBS, then stained the nuclei with 2 nM DAPI solution in 1xPBS for 10 minutes on shaker at room temperature. We then washed cells twice with 1% BSA in 1xPBS and visualized in the washing solution under the microscope.

2.5.3 Western Blotting

The NE-PER protocol was applied to extract nuclear and cytoplasmic proteins, the extracted proteins were added 6x Laemmli Buffer for denaturation and boiled at 95 °C for 10 minutes.

Table 5: 6x Laemmli Buffer components

Component	Final Concentration
Tris-base	0.375 M Tris (pH 6.8)
Glycerol	60%
β-mercaptoethanol	30%
SDS	12%
Bromophenol Blue	0.012%

The samples in the buffer described above were then loaded onto the 10% SDS-PAGE according to the target protein size and electrophoresed with 100-volt power for an hour. After the separation of the proteins, the samples were transferred to a PVDF membrane (Polyvinylidene Fluoride, Advansta, USA, L-08008-001) by wet-transferring method. The membrane was blocked in 5% skim milk in 0.1% Tween containing Tris Buffered Saline (TBS-T) for an hour at room temperature while shaking. The CXXC5 (CXXC5 antibody, Abcam, USA, ab106533) in 1:500 dilution, the ERα (Estrogen Receptor α antibody, Santa Cruz, USA, sc-543) in 1:1000 dilution, Cyclin B1 (Cyclin B1 antibody, Santa Cruz, USA, sc-245), c-MYC (c-MYC antibody, Cell Signaling, USA, 9402), HDAC1 (HDAC1 antibody, Abcam, USA, ab19845) and the membrane was incubated with the primary antibody for an hour at room temperature while shaking. After incubation, the membrane was washed thrice with 0.1% TBS-T (15 minutes in total), and the secondary antibody incubation for CXXC5, Era, p-RB1, c-MYC and HDAc1 a secondary goat anti-rabbit horseradish peroxidase, HRP, conjugated

(Advansta, USA, R-05072-500) antibody was used with a dilution of 1:5000, while for Elf-1, E2F1, RB1 and Cyclin B1, a secondary goat anti-mouse horseradish peroxidase, HRP, conjugated (Advansta, USA, R-05071-500) antibody is used with a dilution of 1:5000, and incubated an hour at room temperature on shaker. After incubation, the membrane was washed thrice with 0.1% TBS-T and incubated in Enhanced Chemiluminescence solution (ECL, Advansta, USA, K-12045-D50) the membrane for 2 minutes in dark and visualized using ChemiDoc™ XRS+ System (Molecular Imager ® ChemiDoc™ XRS+ System, BioRad, USA, 170-8265). The ImageLab software (ImageLab software, BioRad, USA, 170-9690) was used to obtain the membrane images.

2.6 Actinomycin D Treatment

To assess the half-life of CXXC5 mRNA, we first optimized the concentration of 5-Ethynyl Uridine (5-EU) for the nascent protein synthesis labelling for the microscopy imaging. MCF7 cells were seeded as $15x10^3$ cell/well to 48-well plates in normal growth medium. After the incubation, the medium was supplemented with 0.2, 0.5, 1 and 2 mM 5-EU for 6h. Then, Click-It reaction was applied for the labelling and visualization under the microscope.

2.6.1 Optimizations

After we have optimized the 5-EU concentration for the experiments. Then, the treatment time and the dose for Actinomycin D should be optimized, and whether it would affect the protein levels and the synthesis should be observed.

As described previously, we have grown our MCF7 cells in normal growth medium for 48 hours and started adding the actinomycin D in 8 μM concentrations, with 30 minutes, 15 minutes and 0-minute pre-treatments. At 0-hour, 1 mM 5-EU is added onto the cells, and at the end of the treatment, the cells are fixed and stained with the Click-it reaction.

2.6.2 Click-It Reaction and Microscopy

The procedure for the click-it reaction was the same as described for cycloheximide and actinomycin D treatments.

Western Blotting

The identical protocol is applied for the western blotting of Actinomycin D samples.

2.7 Cell Cycle Arrest at Other Phases

While cell cycle analysis using flow cytometry provides an effective approach for cell cycle phase distributions, the conduction of various biochemical endpoints requires phase arrests to augment the cell population at a given phase. Aphidicolin through the specific inhibition of DNA polymerase is used to arrest the cell-cycle at the G_2/M phase.

2.7.1 Aphidicolin

To accumulate the cells in S phase, $4x10^5$ cells/T25 flask MCF7 cells were seeded in CD-FBS containing medium for synchronization and then the cells were treated in the presence and absence of E2 treatment, which was carried out for 12 hours as previously described. After 12 hours, the cells were co-treated with Aphidicolin in 10 μM concentration and E2 as described previously. At the end of the 6-hour treatment, cells were trypsinized as normal and ethanol fixation was carried out for subsequent flow cytometric analyses as described previously.

From 3h to 24h times and from 1 to 15 μM concentration is applied.

2.7.2 2,3-DCPE

After the failure to arrest cells with Aphidicolin, to accumulate the cells in S phase, $4x10^5$ cells/T25 flask MCF7 cells were seeded in CD-FBS containing medium for synchronization and then the cells were treated in the presence and absence of E2 treatment, which was carried out for 12 hours as previously described. After 12 hours, the cells were co-treated with 2,3-DCPE in 20 or 40 μM concentration and E2 as described previously. At the end of the 6- or 12-hour treatment, cells were trypsinized as normal and ethanol fixation was carried out for subsequent flow cytometric analyses as described previously.

2.7.3 Nocodazole

To get the cells to accumulate more in G_2/M phases, the cells were seeded $4x10^5$ cells/T25 flask for synchronization and then in the presence and absence of E2 treatment, which was carried out for 12 hours as previously described, where the cells started to go into the S phase. Then the cells are co-treated with Nocodazole in 0.3 μM concentration with E2 for 6 hours. At the end of the treatment, the cells were collected and fixed for flow cytometry as described before.

2.7.3.1 Mitotic Shake-Off

The Nocodazole treatment collects cells in G_2/M phase, the G_2 phase cells are still attached while the M phase cells are floating. (Tobey, Anderson, and Petersen, 1967) The floating cells are collected by gently tapping the flask and detaching the loosely attached cells from the bottom of the flask. We collected also these floating cells and fixed as well, as described before. [26]

CHAPTER 3

RESULTS AND DISCUSSION

Previous studies in our lab indicate that the CXXC5 protein is encoded by an estrogen responsive gene [7 4 3] whose expression is driven by a CpG island promoter. [8] CXXC5 promoter pull-down approach further revealed that ELF1 and RB1 transcription factors may be involved in the expression of CXXC5 gene. [8] Given the importance of ELF1-RB1 interactions in the transcriptional regulation of genes involved in the cell cycle regulation, these observations suggest that CXXC5 expression may be regulated in a cell cycle-dependent manner. Our laboratory also reported using a BioID approach that CXXC5 as an unmethylated CpG binding protein could interact with TRIM25 (Tripartite Motif Containing 25) and TRIM33 (Tripartite Motif Containing 33) that function E3 ubiquitin-protein ligases as well as USP15 (Ubiquitin Specific Peptidase 15) which acts as a deubiquitinase. [8] Moreover, ongoing studies suggest that CXXC5 undergoes proteasome-dependent degradation (P Yaşar, unpublished observation). Thus, it is likely that not only the CXXC5 gene but also the intracellular levels of the CXXC5 protein is modulated in a cell cycle-dependent manner.

Since exponentially growing cells in cell culture systems are asynchronous with respect to the cell cycle stage, a better understanding of cell cycle-related events is dependent upon the enrichment of cells at a given cell cycle stage. To address whether the CXXC5 gene expression and/or CXXC5 synthesis/degradation occur cell cycle dependent manner, we initially wanted to establish an effective cell-cycle profiles of our model cells: MCF7 cells.

3.1 Cell Synchronization and cell cycle stages of MCF7 cells

Estrogen drives the cell cycle progression in experimental cell models, including MCF7 cells that synthesize ER. [46] During the cell cycle, the cells replicate their DNA and then at the end of the cycle the cell divides into two daughter cells, which can be followed with flow cytometry. [47] Since the stages of the cell cycle can be followed by the flow cytometry (as explained in Section 2.2), we first wanted to see the timepoints which corresponds to the stage of the cell cycle phase enriched at that time of the treatment with the E2 (Figure 5). Although there are a number of approaches for cell cycle synchronization, the most effective cell cycle synchronization of MCF7 cell is the charcoal-dextran treated (CD)-FBS treatment (User, S.D, MSc Thesis, METU-Biological Sciences, Ankara, Turkey). CD-FBS is absorbed with activated carbon that removes non-polar material such as lipophilic materials including steroid hormones as well as viruses, certain growth factors and cytokines. [48] To synchronize MCF7 cells, we cultured MCF7 cells in medium containing CD-FBS for 72h. Under these conditions, cells primarily accumulate in G_1 phase thereby allowing cell cycle synchronization. We then incubated cells in CD-FBS containing medium supplemented with 10^{-8} M E2 at 6h intervals for 36h. As shown by the time interval flow cytometry experiments, we see the transition of the cells throughout the phases of the cell cycle by the DNA content analysis. With the start of the E2 treatment (10^{-8} M) cells at 6h to 12h, cells start to enter the S phase, then at 18h E2 treatment the cells complete their DNA synthesis and enter the G_2/M phase, the percentage of the S phase then decrease and at 24h the G_2/M phase increase in percentage. After the 30h E2 treatment a part of the population re-enters the cell cycle by the G_1 phase and after 36h E2 treatment cells are completely desynchronized. (Figure 5; also, Supplemental Data Figure S1). Observing an effective synchronization procedure, we then assessed the changes in protein levels in cell cycle phases. We isolated the nuclear and cytoplasmic extracts and performed western blotting to see the levels of our proteins of interest, such as CXXC5, ERα, Cyclin B1.

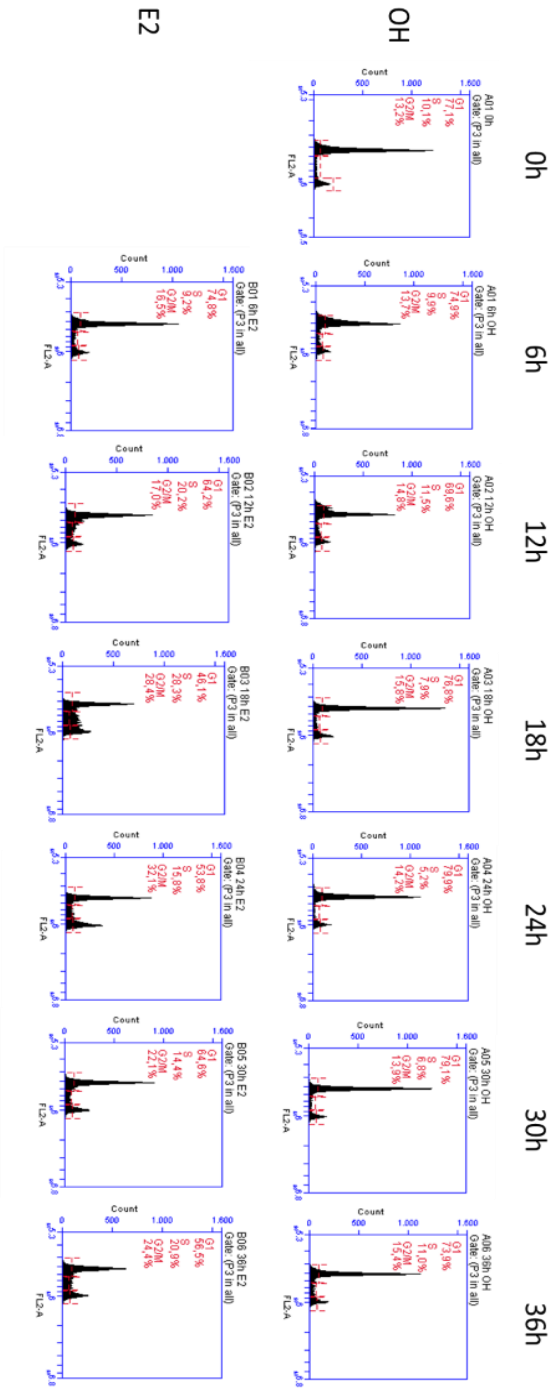

Figure 5: Cell synchronization and cell cycle stages of MCF7 cells. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%) for a total of 36h, with 6h intervals. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The result of at least two independent experiments. In Figure S1, the data is given in table and graph form.

3.2 Cell Cycle Western Blot

The nuclear extracts (50 ug) which were isolated as described in Section 2.7., were subjected to WB analysis (Figure 6)

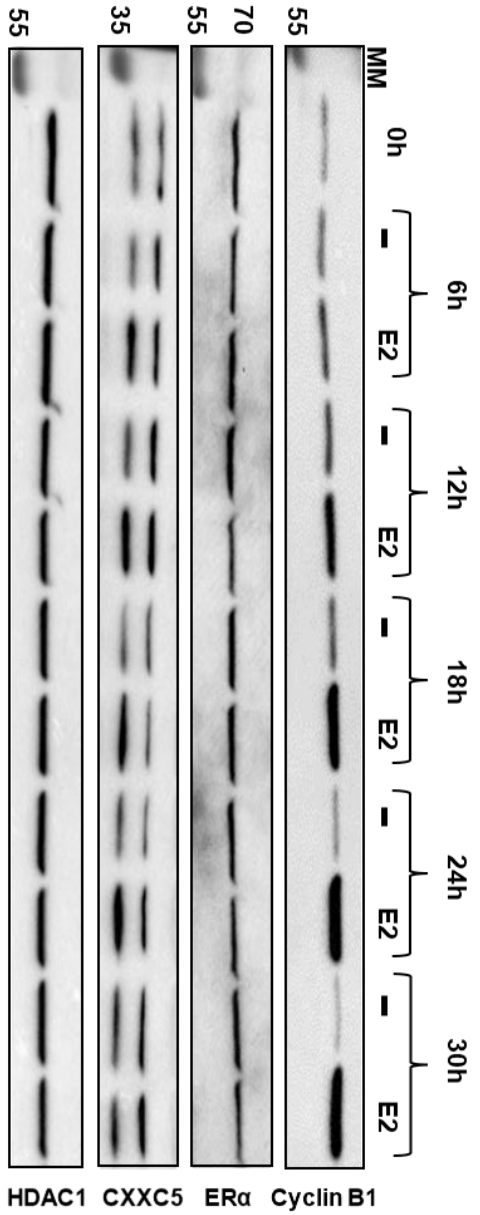

Figure 6: Cell cycle western blotting of MCF7 cells. The changes in the levels of the proteins with the timepoints of the cell cycle were detected by WB analysis. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%) for a total of 36h, with 6h intervals. Then the

cells are collected and separated for RNA isolation, Cytoplasmic and Nuclear Protein Extraction and Flow cytometry.

Results revealed that the protein levels of our protein of interest, CXXC5, is increasing as the cells commence into the S phase in 6h as shown in the flow cytometric analysis, and the levels stay high as the cells complete the cell cycle at 30h. The protein level of the ERα is not significantly affected by the E2 treatment or the cell cycle. Cell cycle related protein Cyclin B1, increases as the S phase continues towards the end and increases until the end of the G_2/M phase, the levels of the proteins are repressed with respect to the start of the cycle in the vehicle treated sample.

These results suggest that the levels of the CXXC5 protein is modulated by cell cycle phases.

3.3 Aphidicolin Optimization via Flow Cytometry

To assess whether we could enrich cell cycle phases in order to address cell cycle dependent expression and/or synthesis of CXXC5 in MCF7 cells, we utilized chemical synchronization approaches.

3.3.1 Aphidicolin Dose Trials

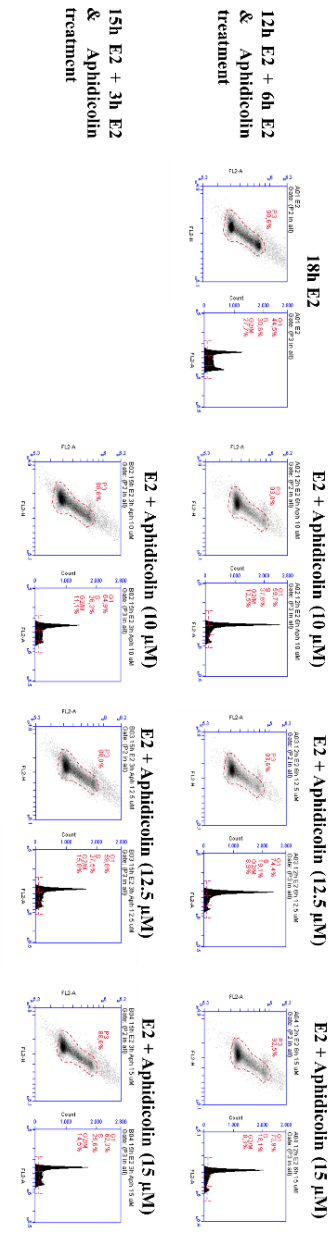

Figure 7: The optimization of Aphidicolin dose for S-phase synchronization. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) for 12-hours or 15-hours, then they are co-treated with 6-hours or 3-hours, for a total treatment time of 18-hours, with doses of 10, 12.5, and 15 µM of Aphidicolin. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The result of at least two independent experiments. In Figure S2, the data is given in table and graph form.

Aphidicolin is a drug that intervenes with the eukaryotic process of DNA replication by the inhibition of the DNA polymerase α, δ and ε thereby blocking the cell cycle at early S phase leading to an accumulation of cells in S phase (10.1007/978-1-61779-182-6_6). Since MCF7 cells enters the S-phase about 6h after the E2 treatment and the population in S-phase peaks at 18h after treatment, we reasoned that the co-treatment with E2 could enrich the population in S phase dependent upon the duration of E2 treatment. For this, we used various amount of aphidicolin (10, 12.5 and 15 μM) at 12-hour or 15-hour after E2 treatment of synchronized cells in CD-FBS containing medium, according to our findings in Figure 1, where the cells transition to S-phase in these timepoints. The Aphidicolin treatment did not arrest cells significantly at S-phase in the dose trials (Figure 7, also Supplemental Data, Figure S2.).

3.3.2 Aphidicolin Time Trial

After the dose optimization for Aphidicolin treatment (Figure 7, also Supplemental Data, Figure S2), we tried to optimize the time of the E2 treatment, to yield a significant accumulation of the cells in the S-phase. (Figure 8, also Supplemental Data, Figure S3)

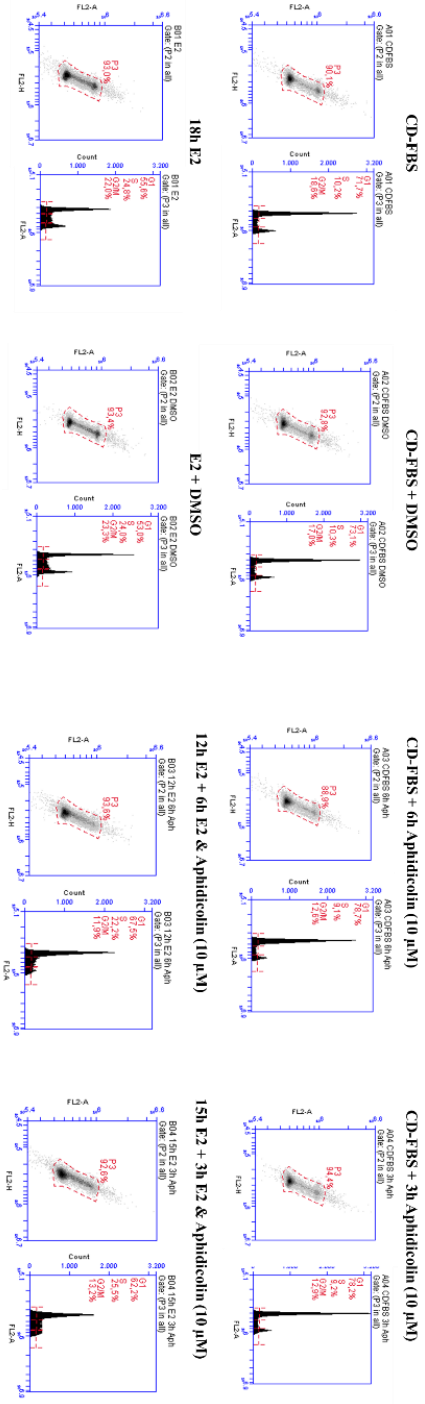

Figure 8: The optimization of Aphidicolin timepoints for S-phase synchronization. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) for various times, then they are co-treated with 6-hours or 3-hours, for a total treatment time of 18-hours, with 10 µM of Aphidicolin. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The result of at least two independent experiments. In Figure S3, the data is given in table and graph form.

The Aphidicolin treatment with the longer timepoints, did not give a significant increase in the S-phase population either, the cells rather accumulated in the G1 phase more than the S-phase anticipated, or the percentage of cells were like the E2 treatment alone as the results in Figure 1, also Supplemental Data, Figure S2, cell cycle phases of the untreated MCF7 cells. (Figure 8, also Supplemental Data, Figure S3)

Finally, to see if the cells were not transitioning to S-phase at all or the cells are dividing after the DNA replication has stopped, we treated the cells with Nocodazole, a microtubule poison that stops the cells in the M phase. [49] We treated the cells with either Aphidicolin or co-treated the cells with Aphidicolin and Nocodazole together. (Figure 9, also Supplemental Data, Figure S4)

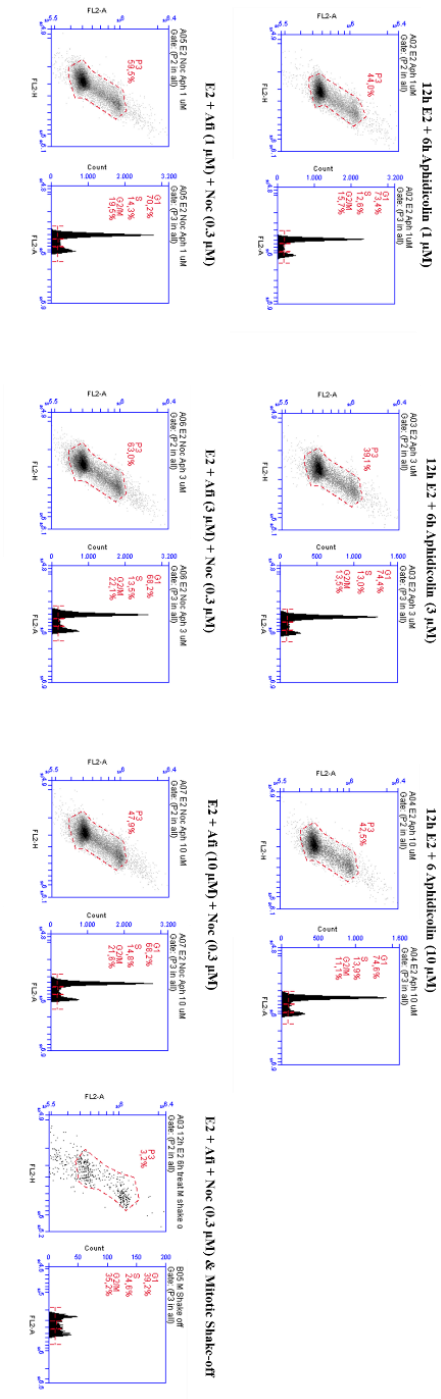

Figure 9: The optimization of Aphidicolin timepoints for S-phase synchronization, with nocodazole. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) for 12 hours, then they are co-treated with E2 and Aphidicolin and/or E2 and Aphidicolin and Nocodazole for 6 hours, for a total treatment time of 18-hours, with 1 μM of Aphidicolin and 0.3 μM. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The result of at least two independent experiments. In Figure S4, the data is given in table and graph form.

The cells when treated with Aphidicolin, did not yield any significant accumulation of the cells in S-phase, and interestingly, when treated with Nocodazole, the cells were accumulated at G_1-phase, this suggests that the cells do not go through the S-phase at all, the cells do not go through the G_1/S boundary and the cells cannot be synchronized using this drug for the S-phase (Figure 9, also Supplemental Data, Figure S4). After this conclusion, we decided to use another drug to increase the cell population in the S-phase.

3.4 2,3-DCPE Optimization via Flow Cytometry

3.4.1 2,3-DCPE Dose Trial

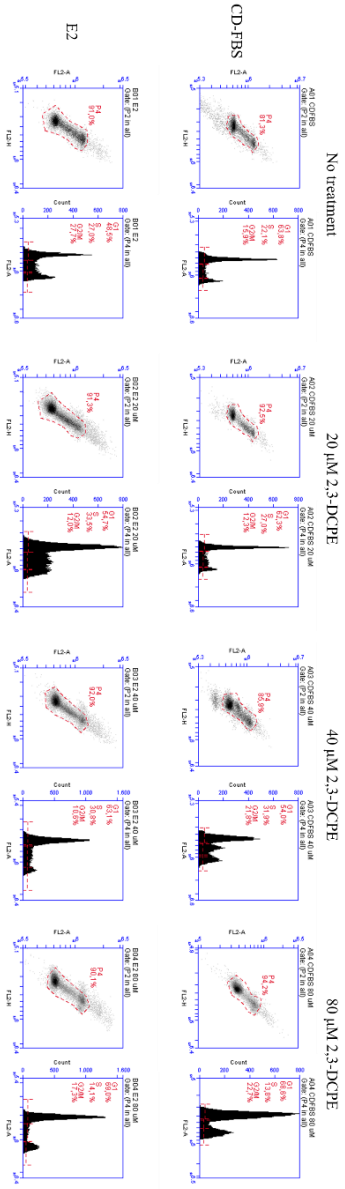

Figure 10: The dose trial for 2,3-DCPE for S phase enrichment. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%), with or without 2,3-DCPE in the indicated concentrations for 18 hours. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The DNA content is then assessed by flow cytometry. The result of at least two independent experiments. In Figure S5, the data is given in table and graph form.

Aphidicolin for the S phase enrichment failed (Figure 7-9, also Supplemental Data, Figure S2-4), therefore we used 2,3-DCPE that is suggested to arrest cells at S phase via the p21 pathway and the Chk1 and Chk2 proteins. [50] [51] [52] First, we optimized the concentration of 2,3-DCPE, by using 5, 10, 20, 40 and 80 μM concentrations and concluded that 20 μM concentration for E2 treated samples and 40 μM concentration for vehicle treated samples yielded the highest percentages for the S phase enrichment. Then, the treatment time was optimized, to increase the percentage of the cells in the S phase. (Figure 10, also Supplemental Data, Figure S5)

3.4.2 2,3-DCPE Time Trial

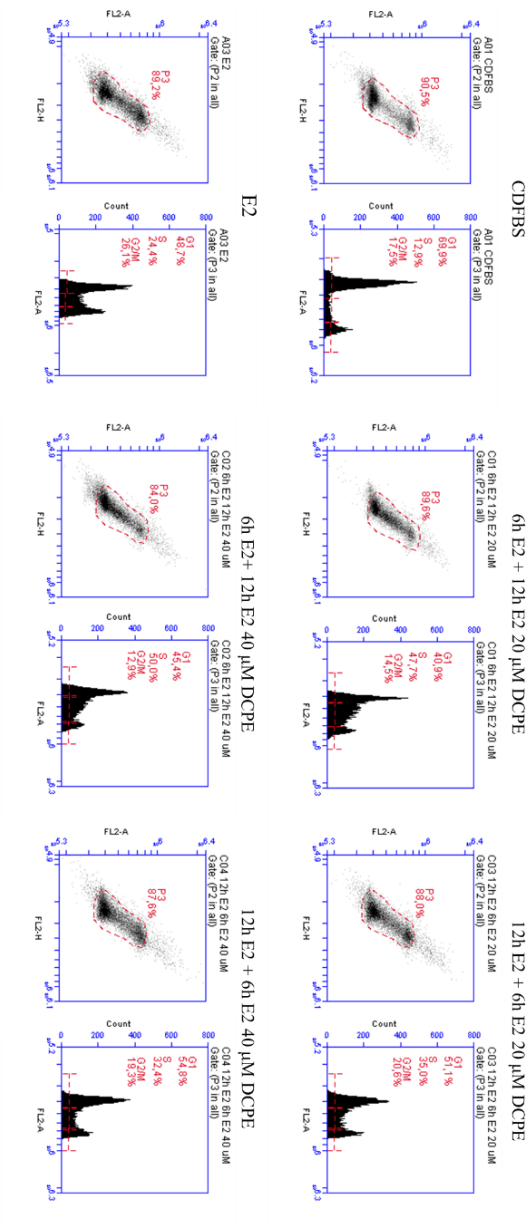

Figure 11: The timepoint optimization for the 2,3-DCPE treatment. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%), with or without 2,3-DCPE in the indicated concentrations and times for 18 hours. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The DNA content is then assessed by flow cytometry. The result of at least two independent experiments. In Figure S6, the data is given in table and graph form.

The cells are treated with 2,3-DCPE as explained in Section 2.8., and the optimal doses for the S phase enrichment was decided to be 20 and 40 μM (Figure 10, also Supplemental Data, Figure S5). Based on this conclusion, we examined the duration for the treatment of MCF7 cells with 2,3-DCPE. From our previous synchronization experiments, Figure 1, we knew that the cells were entering the S phase in 6h however there were no visible increase in the population and at 12 hours, we observed an increase in the S phase percentage but as the working principle of the 2,3-DCPE, p21 pathway is only effective at the beginning of the S phase and the Restriction point of the G_1/S boundary. We therefore tried 6, 8, 10 and 12 hours of either E2 or vehicle treatment and then treated the cells with 2,3-DCPE completing the treatment time to 18 hours in total. We also tried to treat the cells with first 2,3-DCPE and then co-treat these cells with E2 and 2,3-DCPE due to the results obtained in CD-FBS 40 μM 18-hour treatment we performed previously (Figure 10, also Supplemental Data, Figure S5). The best treatment for the S phase enrichment using the chemical 2,3-DCPE was determined to be 6-hour E2 treatment followed with a 12-hour co-treatment with E2 and 20 μM 2,3-DCPE. In future experiments, this regime will yield the healthiest and most enriched cell population in S phase (Figure 11, also Supplemental Data, Figure S6).

3.5 Nocodazole Optimization via Flow Cytometry

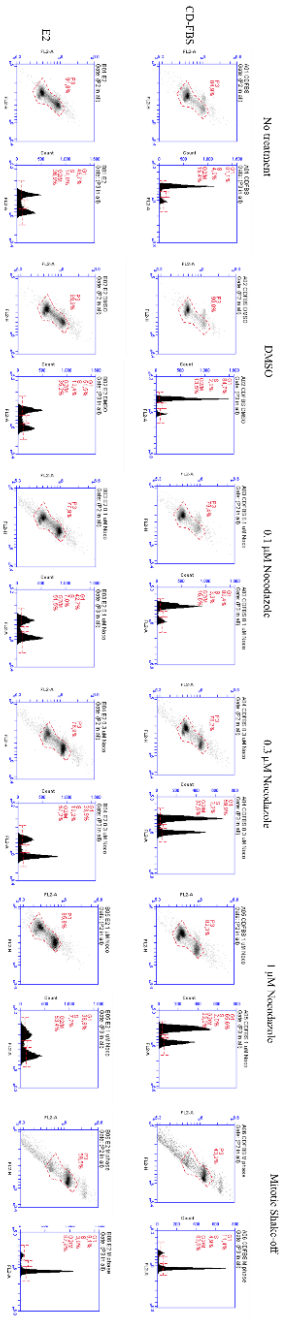

Figure 12: The dose trial for Nocodazole for G2/M-phase enrichment. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%) for 18 hours, with or without Nocodazole in the indicated concentrations for 6 hours. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The DNA content is then assessed by flow cytometry. The result of at least two independent experiments. In Figure S7, the data is given in table and graph form.

Nocodazole is a microtubule poison that arrests the cells in G_2/M phase. In our synchronization experiments we were able to synchronize the cells but not sufficiently enrich the population of the G_2/M phase cells, therefore our aim was to obtain an enriched population of these cells, to see the protein levels at this phase. As described in Section 2.10.2, we have synchronized cells and treated the cells with first E2 and then co-treated these cells with E2 and Nocodazole. According to the literature, the attached cells after the treatment are the G_2 phase, as the floating or loosely attached cells are the M phase. [49] Trails with 0.1, 0.3 and 1 μM Nocodazole concentrations, the cells in the 0.3 μM Nocodazole treated sample yielded a very significant percentage of cells in G2/M phase, in both attached cells which are considered the G_2-population and the Mitotic Shake-off cells which are considered the M-phase population (Figure 12, also Supplemental Data, Figure S7). We then replicated the experiment with the 0.3 μM Nocodazole concentration (Figure 13, also Supplemental Data, Figure S8), which were conducted using the same protocol (Figure 13, also Supplemental Data, Figure S8).

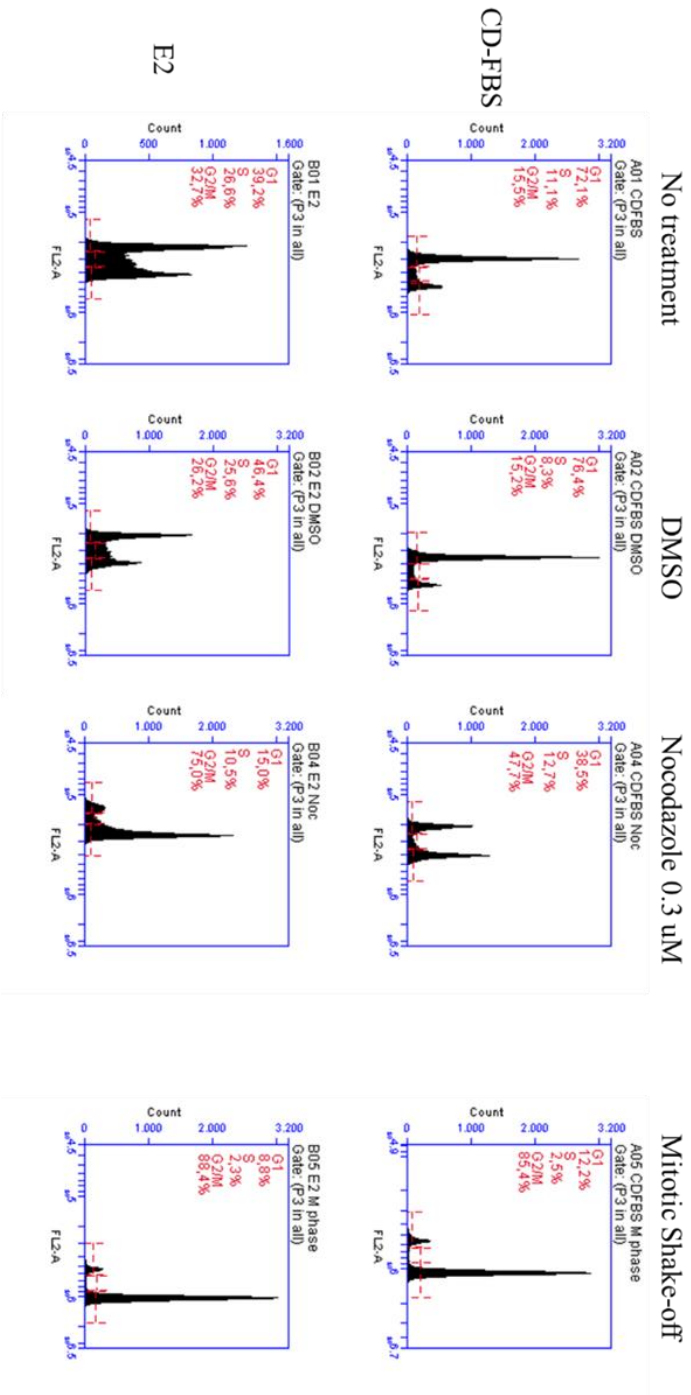

Figure 13: Cell synchronization of MCF7 cells by Noc treatment at G2/M phase. MCF7 cells are seeded as $4x10^5$ cells/T25 flask and synchronized by hormone deprivation in T25 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%) for 18h, then the cells are treated with Noc for 6 hours. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide and the DNA content is then assessed by flow cytometry. The DNA content is then assessed by flow cytometry. The result of at least two independent experiments. In Figure S8, the data is given in table and graph form.

Our observations align with the previous results of the Nocodazole treatment, and we were able to enrich the G_2-phase to 75% in E2 treated cells and the M-phase in Mitotic Shake-off samples in the E2 treated cells to 88.4%. (Figure 13, also Supplemental Data, Figure S8)

3.6 Cycloheximide and Actinomycin D Optimizations

To assess whether the CXXC5 gene expression correlates with the CXXC5 protein levels, we wanted initially to assess the optimal concentrations of drugs that affectively repress the transcription or translation. Actinomycin D (Act D), a polypeptide antibiotic isolated from the genus Streptomyces, prevents the progression of RNA polymerases by intercalating into DNA. [53] We used ActD as an effective transcription inhibitor. We also used cycloheximide (CXH) which inhibit translation elongation through binding to the E-site of the 60S ribosomal unit and interfering with deacetylated tRNA and thereby is used as an effective protein synthesis inhibitor in eukaryotes.

3.6.1 5-EU Optimization

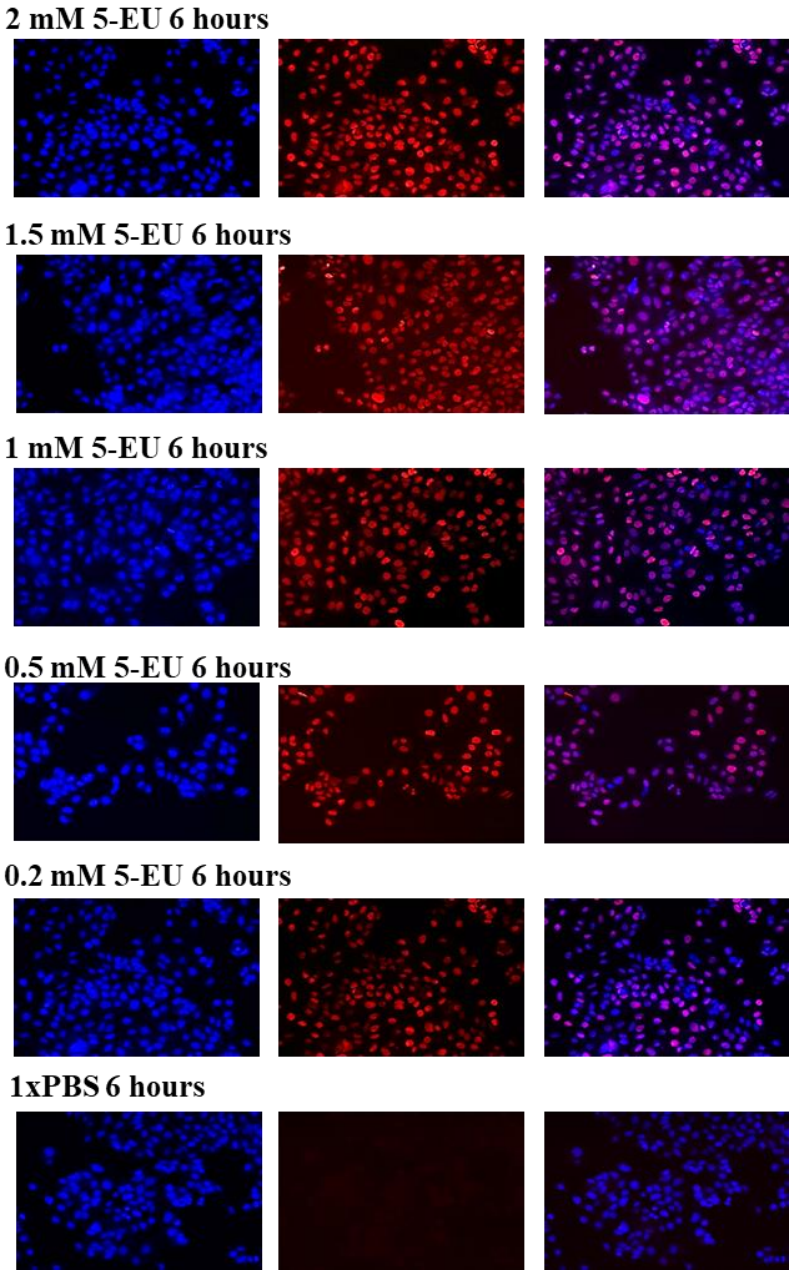

Figure 14: Optimization of the 5-EU treatment concentration for RNA synthesis visualization. MCF7 cells are seeded as $15x10^3$ cell/well in 48-well plates in normal growth medium and after 48 hours, the medium is refreshed using 0.2, 0.5, 1, 1.5 and 2 mM 5-EU for 6h. The vehicle control is 1xPBS as the 5-EU is dissolved in it, and the control is carried out as maximal dose. After the labelling, the cells are subjected to Click-It reaction and then visualized under the microscope.

To visualize the nascent RNA synthesis, we first optimized the 5-EU concentration for the labelling. 0.2, 0.5, 1, 1.5 and 2 mM 5-EU doses were applied onto the cells for 6 hours and then the Click reaction was carried out, as explained in Section 2.8.2. Results revealed that 1 mM of 5-EU labels all cells with a sufficient fluorescence intensity thereby allowing us to conclude that 1 mM of 5-EU is the concentration to effectively assess the nascent RNA synthesis for the future experiments (Figure 14).

3.6.2 L-HPG Optimization

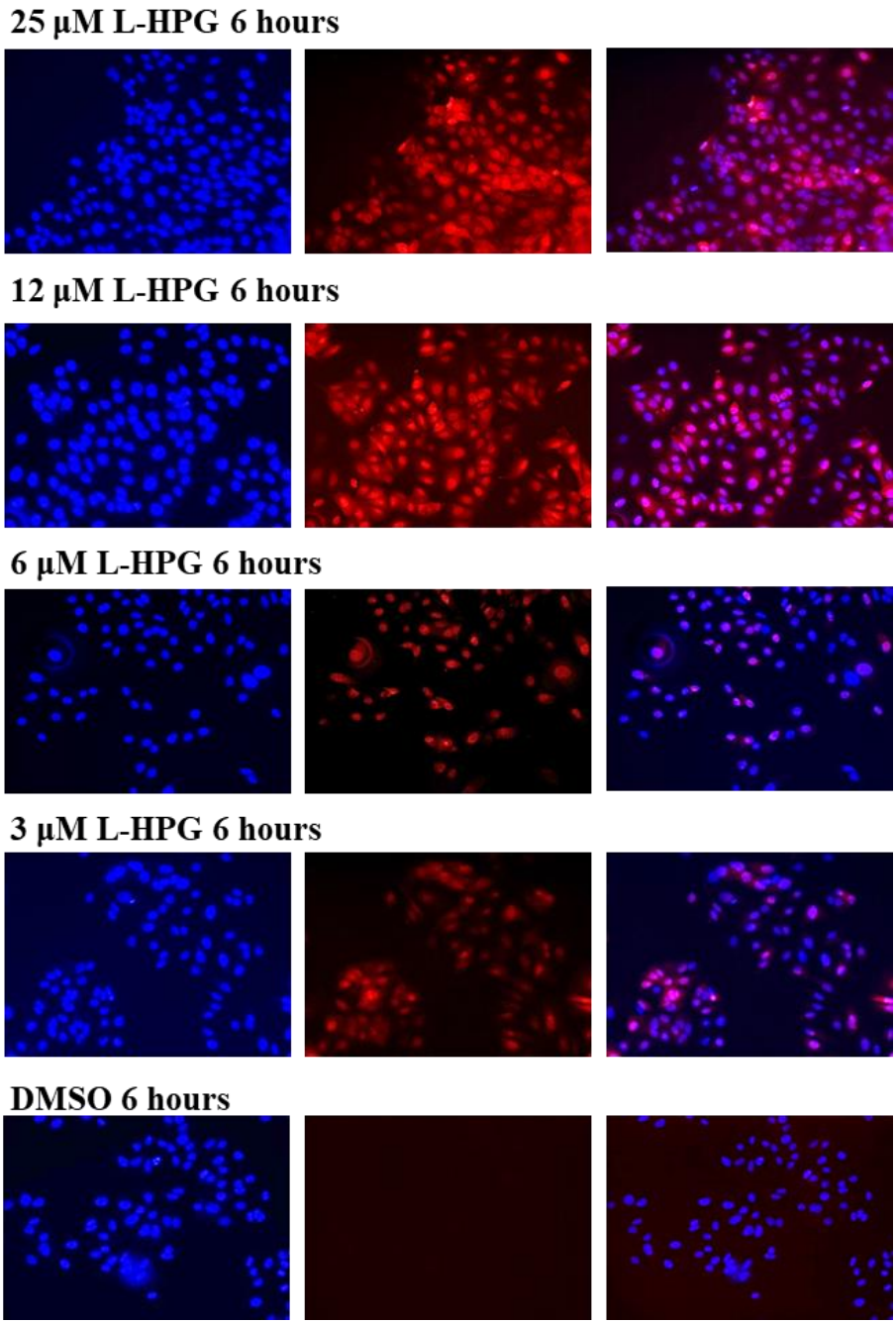

Figure 15: Optimization of the L-HPG treatment concentration for protein synthesis visualization. MCF7 cells are seeded as $15x10^3$ cell/well in 48-well plates in normal growth medium and after 48 hours, the medium is refreshed with Methionine/Cysteine free medium for 1 hour, and then the medium is supplemented with 3, 6, 12 and 25 µM L-HPG for 6h. The vehicle control is applied as DMSO as the L-HPG is dissolved in DMSO After the labelling, the cells are subjected to Click-It reaction and then visualized under the microscope.

Similar to the RNA transcription visualization, the L-HPG treatment concentration was optimized before the experiments, the doses 3, 6, 12 and 25 μM L-HPG was used for 6 hours as described in Section 2.8.1 by using Click Chemistry approach, the optimal dose is determined as 12 μM for 6 hours for future experiments (Figure 15).

3.6.3 Pre-Treatment Time Optimization

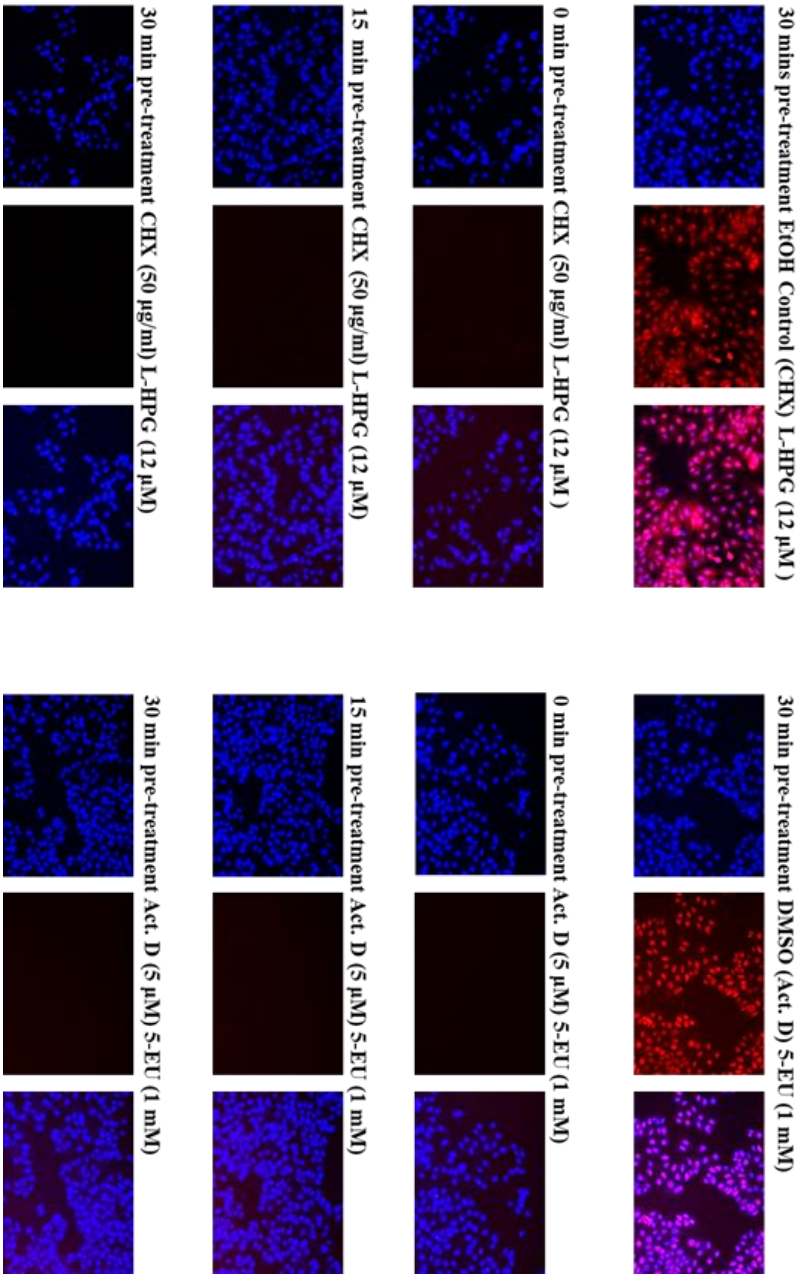

Figure 16: Optimization of pre-treatment times of CHX and Act. D. MCF7 cells are seeded as $15x10^3$ cell/well in 48-well plates in normal growth medium and after 48 hours, the labelling is carried out as starvation for 1 hour in Methionine/Cysteine free medium for L-HPG setup and then pre-treatments for 30 minutes, 15 minutes and co treatment with labelling reagents and the inhibitors CHX and Act.D is carried out. After the pretreatments, 6 hours of labelling is completed, and Click Chemistry is applied.

Since protein levels in cells is either a direct reflection of the encoding gene expression and/or of the rate of protein synthesis/degradation, we wanted to assess whether we could effectively block RNA synthesis and/or protein synthesis using chemical approaches. Actinomycin D (ActD) acts as a transcription inhibitor by binding to DNA duplexes at the transcription initiation complex and preventing RNA polymerase elongation. [54] On the other hand, CHX blocks the elongation phase of translation by binding to the ribosome and thereby inhibitsingeEF2-mediated translocation. [55] Our aim is to assess the half-life of the CXXC5 transcript and protein to examine whether cell-cycle dependent changes in the level of CXXC5 protein is due to change in the expression and/or protein degradation. Since half-life of RNA transcripts or proteins vary dramatically (from very short to very long half-life), it was imperative that we could establish an effective pre-treatment of cells with ActD and/or CHX. After the doses for the labelling reagents were determined (Figure 14 and 15), the optimal pre-treatment times for the CHX and Act D were investigated. The same protocol as explained in Section 2.8.1 was conducted with 30-, 15- and 0-minutes drug-treatments. Then the labelling was carried out for 6 hours using the previously determined 12 μM L-HPG and 1 mM 5-EU doses (Figure 14 and 15). According to the results, the pre-treatment time does not affect the efficiency of the inhibition of neither translation nor transcription, that is the co-addition of CHX, or Act D effectively blocks the incorporation of L-HPG or EdU respectively. These finding indicates that we can effectively assess the RNA transcription and/or protein synthesis without preincubation. Based on these findings, we have decided to carry out the future experiments without performing a pre-treatment with the CHX and Act. D. (Figure 16).

3.6.4 The Control Staining for the RNA and Protein Synthesis

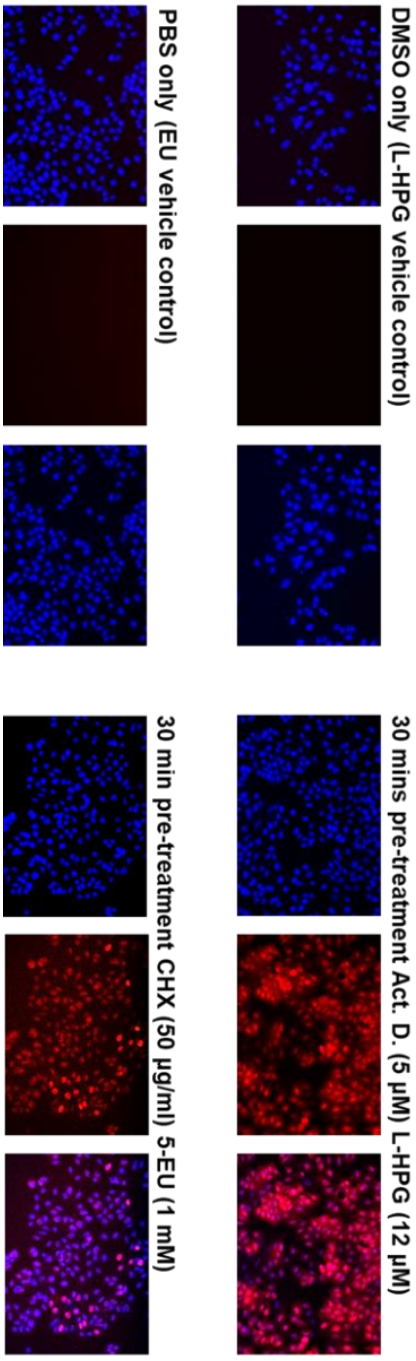

Figure 17: The control staining results of RNA and protein synthesis visualizations. As previously described, the nascent protein synthesis labelling with L-HPG (12 µM) and RNA synthesis inhibitor Act. D (5 µM), and the nascent RNA synthesis labelling with 5-EU (1 mM) and protein synthesis inhibitor CHX (50 µg/ml) is carried out.

As the treatments were carried out, to see if the inhibitors have non-specific effects, we have carried out the experiments with the opposite labelling reagents with the protein and RNA synthesis. Previously, the labelling for the CHX treatment was carried out with L-HPG, for nascent protein synthesis, whereas the labelling for the Act. D treatment was carried out with 5-EU (Figure 14-17). To see whether the CHX treatment has an effect in the RNA synthesis, we stained the treated cells with 5-EU and visualized for the nascent RNA synthesis. Conversely, to assess the effect of the Act D treatment on protein synthesis, we stained the treated cells with L-HPG and visualized for the nascent protein synthesis. According to the results, the Act D treatment does not interfere with the protein synthesis, as the CHX treatment does not interfere with RNA synthesis (Figure 17).

3.6.5 The Co-treatment Effect on Transcription and Translation

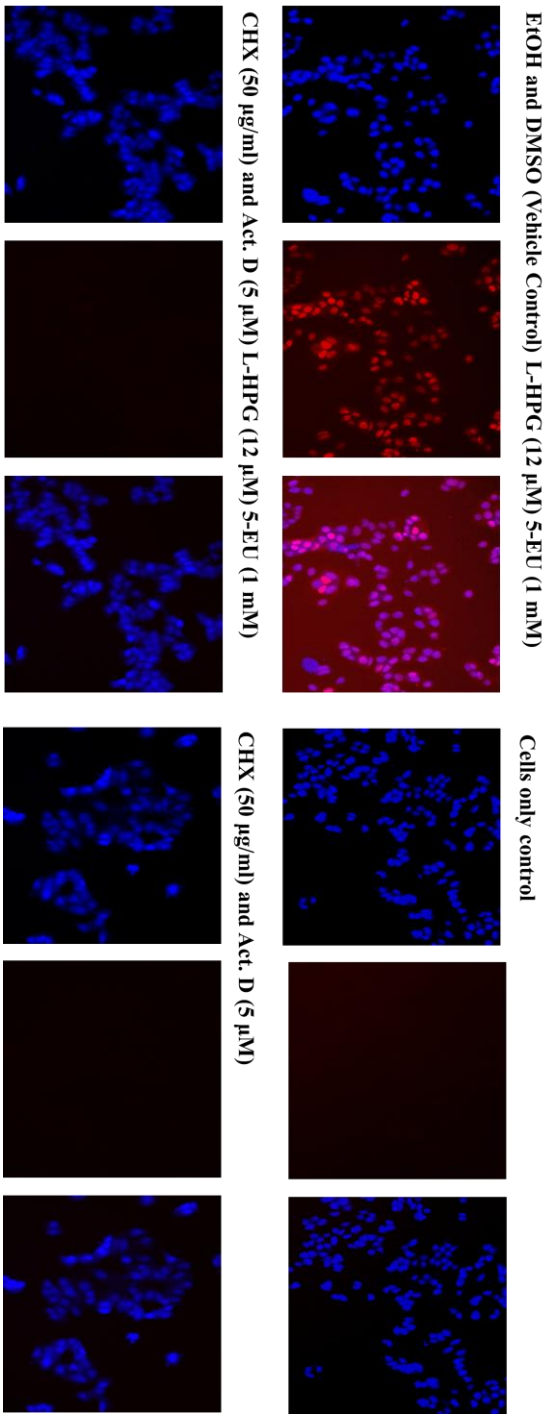

Figure 18: The Co-treatment effect on transcription and translation. MCF7 cells are seeded as $15x10^3$ cells/well in normal growth medium for 48 hours, then the cells are refreshed with Methionine/Cysteine free medium for 1 hour, and the cells are treated with both CHX (50 μg/ml) and Act. D (5 μM) with 0-minute pre-treatment for 6 hours, with both L-HPG (12 μM) and 5-EU (1 mM).

After confirming that the inhibitors do not cross-effect the processes, we then wanted to see if individual doses of them were sufficient to stop both translation and transcription. The cells are co-treated with both inhibitors CHX and Act D, and the cells were labelled with both nascent protein and RNA synthesis, the results showed no staining in the inhibitor treated sample, the inhibitors inhibited both the protein and the RNA synthesis completely (Figure 18).

As the microscopy images show, the nascent protein and RNA synthesis can be inhibited with no pre-treatment. The CHX treatment does not affect the RNA synthesis nor does the Act D treatment affect the protein synthesis. The co-treatment inhibits both procedures completely, without any pre-treatment, the doses are sufficient to stop the processes altogether and rapidly.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Previously, our laboratory established that CXXC5 is an estrogen dependent gene product, whose expression is driven by a CpG island promotor. Our promoter pull-down studies revealed that RB1 and ELF1 proteins are involved in the expression of *CXXC5*. Based on these previous results, our prediction is that the expression and/or synthesis of CXXC5 is cell cycle dependent. To test this prediction, we are in the need of establishing effective experimental approaches. My thesis, therefore aimed to establish experimental systems to begin to investigate the cell cycle dependent synthesis and expression of CXXC5 in MCF7 cells, an ERα-positive breast cancer cell line. To accomplish this, I have established experimental regiments for cell cycle synchronization, the doses and visualization methods for the cell cycle drugs that accumulate cells in S-phase and G2/M phases and lastly, doses and treatment durations half-life experiments for translation and transcription.

1. **The cell synchronization and cell cycle stages of MCF7 cells:** MCF7 cells are synchronized by hormone deprivation for 72 hours in Charcoal-dextran treated Fetal Bovine Serum (CD-FBS), then treated with E2 (10 nM) or vehicle control EtOH (0.1%) for a total of 36h, with 6h intervals. At each timepoint, the cells are collected for flow cytometry, protein isolation and total RNA isolation.
    i. The flow cytometric analyses reveal that the cells are accumulated at the G1 phase, prior to the E2 treatment and subsequently go through the cell cycle phases, which can be followed by the flow

cytometry. The cell percentages at the S and G2/M phases, even though lower in accumulation.

ii. The proteins isolated from these cells which are subjected to western blotting reveal an increased level of CXXC5 through the starting of the S phase until the end of the cell cycle where the cells become asynchronized.

iii. The total RNAs isolated were converted to cDNAs for the assessment of the CXXC5 expression, however the normalization controls used in the RT-qPCR reaction were changing in the treatment and the vehicle controls. Under the same conditions, the expressions of RPLP0 and HDAC1 genes were used, as well as PUM1 gene. Due to the differences in the Ct values of these genes in the RT-qPCR, we cannot use these data. We have to re-do these experiments to look at the expression change of the CXXC5 gene.

2. **S-phase and G2/M phase enrichment for the analyses:** The cell synchronization studies reveal that the MCF7 cells go through the cell cycle and the phases and these phases can be followed experimentally, though the enrichment of the cell population in the S-phase and G2/M phase may not be sufficient. Therefore, we conducted the optimization for three drugs, Aphidicolin and 2,3-DCPE for the S-phase, and Nocodazole for the G2/M phase.

   i. The Aphidicolin treatment yielded little to no accumulation of cells in the S-phase, despite the various doses and timepoints used to treat the cells. Also, the Aphidicolin and Nocodazole co-treatment showed that the cells were still in the G1 phase and did not enter the S-phase. Therefore, we cannot use this drug to enrich the S phase cell population.

   ii. The 2,3-DCPE treatment enriched the S-phase population percentage up to 50% at 6 hours E2 (10 nM) treatment followed by 12 hours E2 (10 nM) and 20 μM 2,3-DCPE co-treatment.

iii. The Nocodazole treatment caused an 80% cell accumulation in the G2 phase, as well as up to 90% accumulation in the M phase when the cells are treated for 18 hours with E2 (10 nM) and then co-treated with 6 hours with E2 (10 nM) and 0.3 μM Nocodazole.

3. **The optimization studies for the Cycloheximide and Actinomycin D treatments for Click reaction:** We wanted to establish a way where the transcription and translation processes could be visualized in a cell to optimize the concentrations and treatments of the inhibitors of these mechanisms.
   i. The labelling reagent for the translation, L-HPG, is optimized as 12 μM and the labelling reagent for the transcription, 5-EU, is optimized as 1 mM. The labelling reactions were conducted for 6 hours in total.
   ii. The optimal dose for cycloheximide, the translation inhibitor, is 50 μg/ml where no fluorescence is detected, and the optimal dose for the actinomycin D, the transcription inhibitor, is 5 μM.
   iii. The treatments where translation is labelled in the presence of Actinomycin D, the inhibitor of transcription, shows us fluorescence signaling suggesting that the protein synthesis continues. The treatments where transcription is labelled when the cells are treated with Cycloheximide, the inhibitor of translation, shows the continuing translation by the fluorescence signaling.
   iv. Findings that the co-treatment with Actinomycin D and Cycloheximide results in no fluorescence staining conclude both the translation and transcription processes have stopped.

Overall, our study reveals that MCF7 cell cycle can be followed by flow cytometry and the CXXC5 synthesis is cell cycle dependent. The cell percentages in the S- and G2/M-phase can be enriched using 2,3-DCPE and Nocodazole, respectively, in the explained doses and treatment in this thesis. Also, the

transcription and translation processes can be followed by the Click-it method, and the inhibition doses for these processes are as explained here.

In the future studies, we intend to further study the synthesis of CXXC5 in the cell cycle stages of MCF7 cells, where future partners in these stages, as well as the degradation mechanism for the CXXC5 protein will be investigated, regarding the previously established partners of it which are also related to the ubiquitination pathway. Lastly, as the optimization for the doses of Cycloheximide and Actinomycin D are completed, we will analyze the half-life of the mRNA and the protein of CXXC5.

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APPENDICES

A. Charcoal Dextran Treatment of Fetal Bovine Serum

Into the bottle of a 500 ml Fetal Bovine Serum, 10 g of Dextran coated charcoal (C6241, Sigma Aldrich, Germany) is added. The solution is mixed at 4°C overnight with an autoclaved magnetic fish. After the mixing, the FBS is aliquoted into two sterile Nalgene bottles which have equal weights, which are centrifuged at 10800xg (8000 rpm for Sorvall SLA-3000 rotor) for 30 minutes at 4°C, the supernatants are decanted into a 0.45 μm sterile filter unit (Corning, polystyrene, cellulose acetate membrane, low protein binding), and filtered. Then, 10 g of Charcoal, dextran coated is added and the solution is mixed with a sterile magnetic fish for 4-6 hours at 4°C. After the mixing, the supernatant is decanted into a new filter unit, which is filtered in the biological safety cabinet. The charcoal dextran treated fetal bovine serum (CD-FBS) is obtained and aliquoted as 40 ml into 50 ml centrifuge tubes and the aliquots are stored at -20°C until use.

B. Gating Strategy for Flow Cytometry

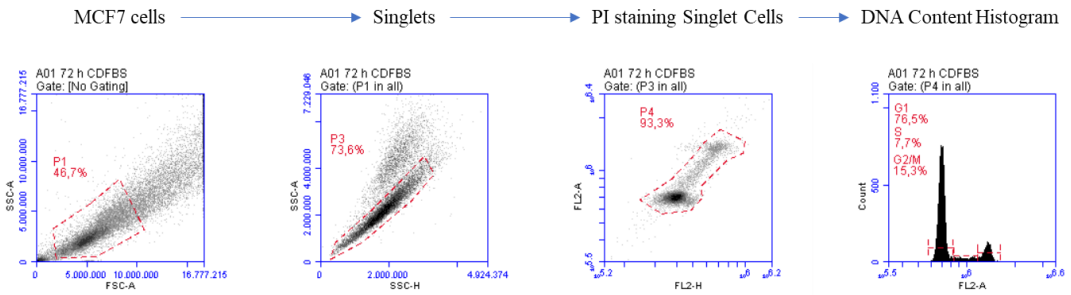
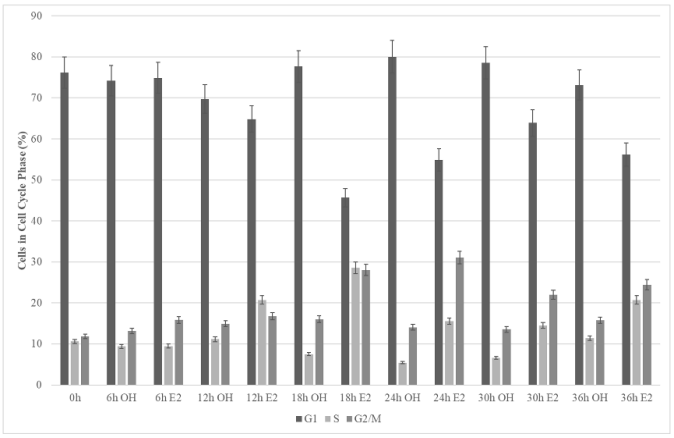


Figure 19: A representative image of the Flow cytometry gating strategy and the analysis of the DNA content of the MCF7 cells synchronization.

C. Supplemental Data

a.

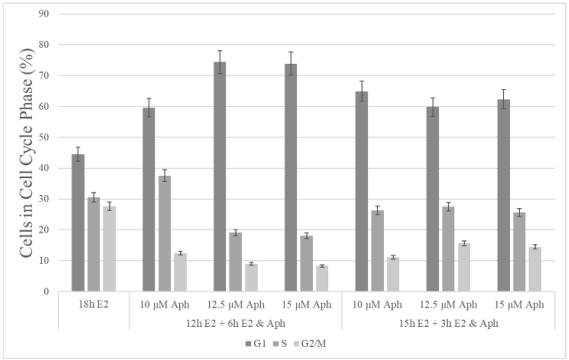


b.

	G1	S	G2/M
0h	76.15	10.6	11.85
6h OH	74.15	9.4	13.15
6h E2	74.9	9.5	15.85
12h OH	69.7	11.15	14.95
12h E2	64.85	20.75	16.75
18h OH	77.65	7.5	16.05
18h E2	45.65	28.6	28.05
24h OH	80	5.45	14.05
24h E2	54.9	15.55	31.05
30h OH	78.6	6.65	13.55
30h E2	63.9	14.5	22
36h OH	73.15	11.4	15.8
36h E2	56.2	20.7	24.45

Figure S 1: Cell synchronization and cell cycle stages of MCF7 cells. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%) for a total of 36h, with 6h intervals. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The result of at least two independent experiments. The average of two replicates has been represented in **a.** graph form, **b.** table form.

a.

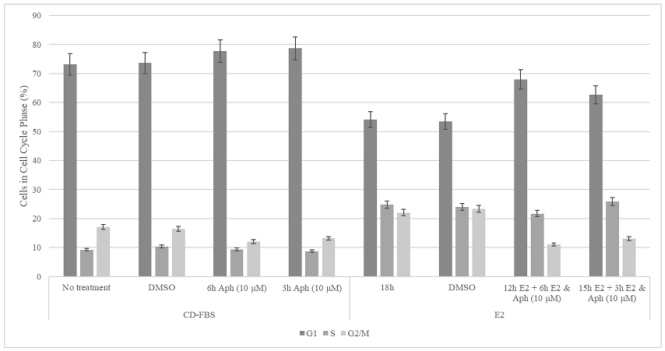


b.

		G1	S	G2/M
	18h E2	45	30	27.3
12h E2 + 6h E2 & Aph	**10 µM Aph**	59	37.2	13.3
	12.5 µM Aph	74	19	9.1
	15 µM Aph	74	18.6	8.5
15h E2 + 3h E2 & Aph	**10 µM Aph**	64.2	26.4	11.6
	12.5 µM Aph	59.4	26.8	15.95
	15 µM Aph	62.7	25.9	14.55

Figure S 2: The optimization of Aphidicolin dose for S-phase synchronization. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) for 12-hours or 15-hours, then they are co-treated with 6-hours or 3-hours, for a total treatment time of 18-hours, with doses of 10, 12.5, and 15 µM of Aphidicolin. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The result of at least two independent experiments. The average of two replicates has been represented in **a.** graph form, **b.** table form.

a.

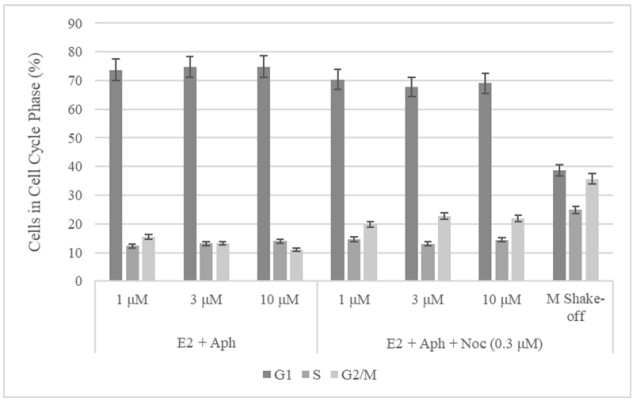


b.

		G1	S	G2/M
CD-FBS	No treatment	73.2	9.25	17.1
	DMSO	73.6	10.4	16.4
	6h Aph (10 µM)	77.7	9.35	12.05
	3h Aph (10 µM)	78.7	8.7	13.15
E2	18h	54.2	24.8	22
	DMSO	53.5	24	23.3
	12h E2 + 6h E2 & Aph (10 µM)	67.9	21.7	11.05
	15h E2 + 3h E2 & Aph (10 µM)	62.7	25.9	13

Figure S 3: The optimization of Aphidicolin timepoints for S-phase synchronization. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) for various times, then they are co-treated with 6-hours or 3-hours, for a total treatment time of 18-hours, with 10 µM of Aphidicolin. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The result of at least two independent experiments. The average of two replicates has been represented in **a.** graph form, **b.** table form.

a.

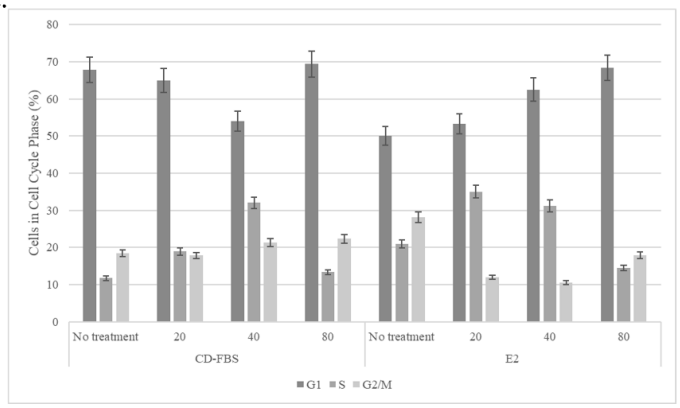


b.

		G1	S	G2/M
E2 + Aph	1 µM	73.8	12.3	15.5
	3 µM	74.8	13.1	13.2
	10 µM	74.9	14	11
E2 + Aph + Noc (0.3 µM)	1 µM	70.4	14.6	19.8
	3 µM	67.7	13.1	22.8
	10 µM	69.1	14.5	21.9
	M Shake-off	38.8	24.9	35.7

Figure S 4: The optimization of Aphidicolin timepoints for S-phase synchronization. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) for 12 hours, then they are co-treated with E2 and Aphidicolin and/or E2 and Aphidicolin and Nocodazole for 6 hours, for a total treatment time of 18-hours, with 1 µM of Aphidicolin and 0.3 µM. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The result of at least two independent experiments. The average of two replicates has been represented in **a.** graph form, **b.** table form.

a.

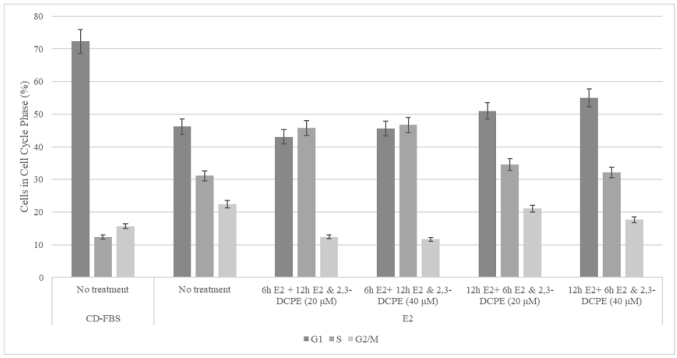


b.

		G1	S	G2/M
CD-FBS	No treatment	67.85	11.7	18.45
	20 µM	65	18.9	17.8
	40 µM	53.9	32	21.35
	80 µM	69.35	13.35	22.3
E2	No treatment	50.1	21	28.1
	20 µM	53.25	35	11.95
	40 µM	62.45	31.15	10.5
	80 µM	68.3	14.5	17.9

Figure S 5: The dose trial for 2,3-DCPE for S phase enrichment. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%), with or without 2,3-DCPE in the indicated concentrations for 18 hours. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The DNA content is then assessed by flow cytometry. The result of at least two independent experiments. The average of two replicates has been represented in **a.** graph form, **b.** table form.

a.

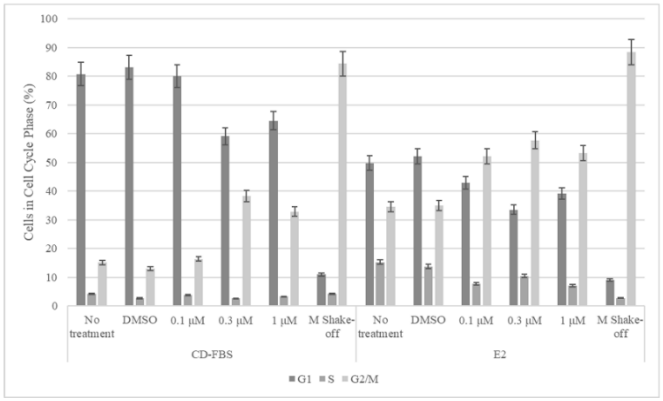


b.

		G1	S	G2/M
CD-FBS	No treatment	72.25	12.4	15.7
E2	No treatment	46.2	31.1	22.5
	6h E2 + 12h E2 & 2,3-DCPE (20 µM)	43.05	45.7	12.45
	6h E2+ 12h E2 & 2,3-DCPE (40 µM)	45.65	46.7	11.7
	12h E2+ 6h E2 & 2,3-DCPE (20 µM)	51	34.6	21.1
	12h E2+ 6h E2 & 2,3-DCPE (40 µM)	55	32.2	17.65

Figure S 6: The timepoint optimization for the 2,3-DCPE treatment. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%), with or without 2,3-DCPE in the indicated concentrations and times for 18 hours. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The DNA content is then assessed by flow cytometry. The result of at least two independent experiments. The average of two replicates has been represented in **a.** graph form, **b.** table form.

a.

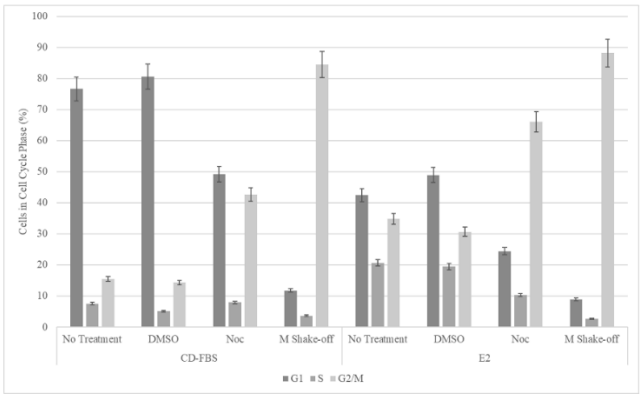


b.

		G1	S	G2/M
CD-FBS	No treatment	80.8	4.15	15.15
	DMSO	83.1	2.65	13.1
	0.1 µM	80	3.7	16.35
	0.3 µM	59.1	2.6	38.25
	1 µM	64.5	3.25	32.85
	M Shake-off	11	4.15	84.35
E2	No treatment	49.75	15.3	34.5
	DMSO	52.1	13.75	35.05
	0.1 µM	42.9	7.7	52.05
	0.3 µM	33.5	10.4	57.7
	1 µM	39.1	7.05	53.15
	M Shake-off	9	2.7	88.3

Figure S 7: The dose trial for Nocodazole for G2/M-phase enrichment. MCF7 cells are synchronized by hormone deprivation in T75 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%) for 18 hours, with or without Nocodazole in the indicated concentrations for 6 hours. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide. The DNA content is then assessed by flow cytometry. The result of at least two independent experiments. The average of two replicates has been represented in **a.** graph form, **b.** table form.

a.



b.

		G1	S	G2/M
CD-FBS	No Treatment	76.6	7.55	15.45
	DMSO	80.55	5.15	14.35
	Noc	49.2	7.95	42.65
	M Shake-off	11.8	3.7	84.5
E2	No Treatment	42.45	20.7	34.8
	DMSO	48.95	19.5	30.7
	Noc	24.45	10.35	66.1
	M Shake-off	8.95	2.65	88.15

Figure S 8: Cell synchronization of MCF7 cells by Noc treatment at G2/M phase. MCF7 cells are seeded as $4x10^5$ cells/T25 flask and synchronized by hormone deprivation in T25 flasks containing charcoal-dextran treated fetal bovine serum (CD-FBS) for 72 hours, and then treated with E2 (10 nM) or vehicle control EtOH (0.1%) for 18h, then the cells are treated with Noc for 6 hours. Cells are collected and fixed with 70% Ethanol and stained with Propidium Iodide and the DNA content is then assessed by flow cytometry. The DNA content is then assessed by flow cytometry. The result of at least two independent experiments. The average of two replicates has been represented in **a.** graph form, **b.** Table form.

D. Buffers and Solutions

25x PI

Dissolve 1 tablet in 2 ml distilled water and filter sterilize with 0.22 μm filter, store at -20°C.

10x PhosSTOP

Dissolve 1 tablet in 1 ml distilled water, store at -20°C.

6X Laemmli Buffer (10 ml)

Tris-base	3.5 mL (1 M Tris, pH 6.8)
SDS	1.2 g
Glycerol	6 ml (100%)
Bromophenol Blue	1.2 mg

To this mixture, the β-mercaptoethanol (30%) is added freshly before use.

Tris Buffer Saline (TBS) pH: 7.6

20 mM Tris

137 mM NaCl

Dissolved in 700 ml distilled water, then pH is adjusted to 7.6 with HCl, and the volume is completed to 1 L. The Tween-20 percentage is arranged as 0.05% or 0.1%, as required by the antibody working conditions.

Protein Running Buffer

250 mM Tris

2500 mM Glycine

1% SDS

The volume is completed to 1 L with distilled water.

10x Transfer Buffer

Tris 30.3 g

Glycine 144.1 g

Then complete the volume to 1 L with distilled water.

For 1x Transfer Buffer:

100 ml 10x Stock

700 ml distilled water

200 ml methanol

Then store the buffer at 4°C

Blocking buffer:

5% w/v Skimmilk (non-fat dry milk) and TBS+ 0.1 or 0.05% Tween-20

SDS gel (Separating) (10%):

Table 6: SDS gel (Separating) (10%).

	Amount of the Chemical
dH_2O	3.97 ml
30% Acrylamide: Bisacrylamide sokution (29:1)	3.33 ml
1.5 M Tris Buffer (pH 8.8)	2.5 ml
10% SDS	100 ul

10% Ammonium Persulfate	100 ul
TEMED	4 ul

SDS gel (Stacking) (5%):

Table 7: SDS gel (Stacking) (5%).

	Amount of the Chemical
dH_2O	5.44 ml
30% Acrylamide: Bisacrylamide sokution (29:1)	1.36 ml
1 M Tris Buffer (pH 6.8)	1 ml
10% SDS	80 ul
10% Ammonium Persulfate	80 ul
TEMED	8 ul