HAKAN AYTAÇOĞLU

THE MOLECULAR REGULATION OF PREIMPLANTATION EMBRYO DEVELOPMENT AND UNDERLYING MECHANISMS OF ANEUPLOIDIES

PhD THESIS

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Approval

We certify that we have read the thesis submitted by Hakan Aytaçoğlu titled **"The Molecular Regulation of Preimplantation Embryo Development and Underlying Mechanisms of Aneuploidies"** and that, in our combined opinion, it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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Declaration

I hereby declare that all information, documents, analysis, and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of the Institute of Graduate Studies, Near East University. I also declare that, as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Hakan Aytaçoğlu

19/09/2025

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Faithfully yours,

Hakan Aytaçoğlu

Abstract

The Molecular Regulation of Preimplantation Embryo Development and Underlying Mechanisms of Aneuploidies

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Infertility is an ever growing concern. In correlation, use of Assisted Reproductive Technologies (ART) clinics has also seen a sharp rise over the years. However, success rates of these techniques remain far from ideal. One of the biggest detriments to ART is aneuploid cases as they are mostly incompatible with life. This thesis thus aimed to investigate gene expression level differences between euploid and aneuploid embryos, as well as complementing this with a mathematical model to understand aneuploidies better. Towards that goal, total RNA was isolated from euploid and aneuploid embryos for cDNA synthesis. Quantitative real time PCR reactions were then conducted to investigate gene expression level variations of key genes within the DNA damage response (DDR) pathway. For the mathematical model, parent parameters were associated with morphological and karyotypical outcomes with regression analyses. Results demonstrate that *BRCA1* levels are elevated in aneuploid embryos. Statistically insignificant variations among *ATM, MDM2* and *RAD51* levels were also observed between euploid and aneuploid embryos. Mathematical model was able to predict embryo morphology based on parent parameters to an extent comparable to image based artificial intelligence (AI) systems, but aneuploidies proved too complicated for the current model. Overall, *BRCA1* seems to be activated in aneuploid embryos irrelevantly of its roles in homologous recombination (HR). Moreover, mathematical models, with improvements, posit a promising technology as a widely available predictive software for ART clinics.

Keywords: Aneuploidy, Gene expression, DDR pathway, Mathematical modelling

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List of Abbreviations

IVF: In Vitro Fertilisation

ART: Assisted Reproductive Technologies

PCOS: Polycystic Ovary Syndrome

NGS: Next Generation Sequencing

PGT: Preimplantation Genetic Testing

PGT-A: Preimplantation Genetic Testing for Aneuploidy

SAC: Spindle Assembly Checkpoint

CIN: Chromosomal Instability

DDR: DNA Damage Response

GIN: Genomic Instability

ICSI: Intracytoplasmic Sperm Injection

PGT-M: Preimplantation Genetic Testing for Monogenic Disorders

PGT-SR: Preimplantation Genetic Testing for Structural Rearrangements

WHO: World Health Organization

FSH: Follicle Stimulating Hormone

hCG: Human Chorionic Gonadotropin

2PN: Two pronuclei

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

MCC: Mitotic Checkpoint Complex

DSB: Double Strand Break

HR: Homologous Recombination

NHEJ: Non-Homologous End Joining

UFB: Ultra-fine Bridges

UV: Ultraviolet

ROS: Reactive Oxygen Species

MAPT: Mutant Tau

FTLD: Frontotemporal Lobar Degeneration

LIF: Leukaemia Inhibitory Factor

BASC: BRCA1-Associated Surveillance Complex

SMC: Structural Maintenance of Chromosome

ssDNA: Single-stranded DNA

TERRA: Telomeric repeat-containing RNA

HER2: Human Epidermal Growth Factor Receptor 2

AI: Artificial Intelligence

Tm: Melting Temperature

Ct: Cycle Threshold

TAE: Tris-Acetic Acid-EDTA

COH: Controlled Ovarian Hyperstimulation

GnRH: Gonadotropin-releasing Hormone

LH: Luteinising Hormone

PVP: Polyvinylpyrolidone

TE: Trophectoderm

ICM: Inner Cell Mass

CHAPTER I

Introduction

This chapter contains information pertaining to the current state of practices in *In Vitro* Fertilisation (IVF) clinics as well as the growing concern of infertility worldwide. In addition, this section includes brief descriptions for aneuploidy and mathematical modelling techniques to prepare the setting for current research question and objectives.

Contemporary Situation in Infertility and Aneuploidy

Use of infertility clinics and Assisted Reproductive Technologies (ART) as a whole, have seen a steady incline since their inception. This elevation in the use of ART mimics the rise in infertility cases.

Infertility can be defined as the lack of conception despite frequent, unprotected intercourse for a period of minimum one year and represents a worldwide health concern that has been consistently growing with each passing year (World Health Organisation, 2023). Infertility can originate from female factors or male factors or from a culmination of both. Possible infertility causes of female origin include but not limited to; chromosomal and genetic aberrations, hormonal problems, zygote implantation disorders, damage to fallopian tubes and certain health conditions such as gonorrhoea or polycystic ovary syndrome (PCOS) (Brugo-Olmedo et al., 2001; Carson & Kallen, 2021; Zegers-Hochschild et al., 2017). Male aspect of infertility usually depends on altercations in sperm production and/or transport. This may be a result of a number of conditions such as chromosomal or genetic aberrations, hormonal imbalances, certain health conditions such as varicocele and heat and morphological conditions like undescended testes (Brugo-Olmedo et al., 2001; Carson & Kallen, 2021; Zegers-Hochschild et al., 2017). These factors can bring about infertility in various unique mechanisms. One such way is aneuploidy.

Aneuploidy can be described as an abnormal number of chromosomes within a cell that is not a multiplication of its native chromosome number (n), which separates this phenomenon from polyploidies. Aneuploidies can arise during meiotic or mitotic cell divisions via erroneous chromosome segregation. Under normal circumstances, genetic

integrity is of utmost importance for any cell and thus, it is normally under strict protection by various checkpoints and regulatory networks. However, even the most stringent and complex pathways are not infallible, hence aneuploidies can be observed.

Aneuploidy represents an intriguing and yet to be fully explored field. While typically associated with negative outcomes, in more recent years aneuploidy was shown to confer proliferative advantage under stress conditions (Kaya et al., 2020; R. Li & Zhu, 2022; Pavelka et al., 2010; Yona et al., 2012). Furthermore, this property of aneuploidies has been debated within the context of various cancers (Davoli et al., 2013; Gordon et al., 2012; G. Liu et al., 2015; Molina et al., 2021; Sack et al., 2018; van Leeuwen et al., 2020). Aneuploidy has additionally been of particular interest in the field of oncogenetics as most cancers involve aneuploid cells (Denko et al., 1994; Vasudevan et al., 2021; B. A. Weaver & Cleveland, 2006; Woo & Poon, 2004). Nevertheless, due to obvious experimental challenges, investigations regarding aneuploidies within the field of embryology remains pale in comparison. Despite this, aneuploidy posits a topic of great importance in the field of embryology as naturally, most aneuploidies are known to be incompatible with life in both natural conceptions and IVF clinics. Among trisomies, only three (trisomy 13, 18 and 21) are known to result in childbirth. However, trisomies of chromosomes 13 and 18 are typically associated with early death after birth (J. Wu et al., 2013). Such discoveries coupled with advances in next generation sequencing (NGS) technologies led to the emergence of preimplantation genetic testing (PGT) in IVF clinics around the world. More specifically, preimplantation genetic testing for aneuploidy (PGT-A) has become a very standard clinical practice to ensure the implanted embryos are free of aneuploidies with higher chances of successful pregnancies. Despite the commonality of PGT-A, its practical benefits remain a controversy within the field (Barad et al., 2022; Cornelisse et al., 2020; Pagliardini et al., 2020; Tong et al., 2022; Yan et al., 2021). While these studies show successful pregnancies and live births with aneuploid embryos, success rate in such cases remain low and birth defects are observed frequently (Barad et al., 2022; Cornelisse et al., 2020; Pagliardini et al., 2020;

Tong et al., 2022; Yan et al., 2021). Therefore, avoiding aneuploid embryo implantation suggests a safer approach so far.

Origin of Aneuploidy

In its simplest form, the cause of aneuploidies can be attributed to erroneous chromosome segregation. Conversely, the mechanisms leading to these segregation errors remain far from simple.

Fidelity of the genomic content is important and thus is tightly controlled. Regarding aneuploidies, the first line of defence is generally considered to be the spindle assembly checkpoint (SAC). SAC represents a well-established molecular network that is responsible for sensing and ensuring proper spindle attachment to kinetochores, prior to progression of the cell cycle (Musacchio, 2015; Musacchio & Salmon, 2007). Interactions between chromosomal instability (CIN), SAC and aneuploidies have been extensively researched and reviewed (Diaz-Rodríguez et al., 2008; Santaguida & Amon, 2015; Sotillo et al., 2007; B. A. A. Weaver et al., 2007). On the other hand, SAC is not the only mechanism related to aneuploidies. Many gene mutations have been associated with aneuploidies while the cells in question retained a functional SAC (McCulley & Petes, 2010; Narkar et al., 2021; Schvartzman et al., 2010; Vasudevan et al., 2021).

In more recent years, elements of DNA damage response (DDR) pathway have been associated with aneuploidy. Intriguingly, DDR association with aneuploidy can be independent of the DDR functions of the genes involved. As an example, ataxia telangiectasia Rad3 related (*ATR*) gene is a key sensor of DNA damage which then stimulates downstream effectors such as CHK1 as a response (Qian et al., 2012; Toledo et al., 2008). Recently, *ATR* and *CHEK1* were also implicated in regulating Aurora B kinase at centromeres, thus enhancing spindle stability and centromere-microtubule interactions, ultimately contributing to faithful chromosome segregation and cytokinesis. Moreover, this function appears independently of *ATR*'s role in DDR pathway (Kabeche et al., 2018; Zachos et al., 2007). In contrast, ataxia telangiectasia mutated (ATM) is another serine/threonine kinase that bears a lot of resemblance to ATR in many ways and is also associated with aneuploidy (Maréchal & Zou, 2013; Qian et al., 2012). Furthermore, ATM and

ATR are both considered important initiators of DNA damage response and they often work together intimately. However, despite their similarities, they have distinct duties and their roles are not redundant (Brown & Baltimore, 2003; Maréchal & Zou, 2013; H. Wang et al., 2004). Moreover, ATM was not detected at centromeres as opposed to ATR (Kabeche et al., 2018). This suggests that even the most intimately associated DDR elements can lead to aneuploidy in distinct but yet to be fully elucidated ways. However, the complexity communications between *ATM-ATR*, the other DDR member genes and their downstream effectors increase the challenge of understanding their roles in aneuploidy.

Hurdles preventing our understanding are not limited to the complexity of the suspected molecular networks. Another obstacle is the blurriness of the lines between causes and consequences of aneuploidy. For instance, disruptions in the DDR pathway can lead to genomic instability (GIN) which can ultimately lead to CIN and aneuploidies (Andriani et al., 2017; Burrell et al., 2013; Russo et al., 2015; Z. Shen, 2011). Conversely, aneuploidies can promote further CIN and GIN, as well as disruptions in a multitude of cellular mechanisms such as DDR (Andriani et al., 2017; Ganem & Pellman, 2012; Orth et al., 2012; Russo et al., 2015).

Despite all the challenges, aneuploidy remains an interesting and prominent area of investigation within the field of cancer genetics and reproductive genetics. In particular, DDR member genes are very commonly investigated in cancerous tissues and cell lines. However, in aneuploid embryos the literature is still lacking.

Understanding the role of these genes, their part as causes and consequences of aneuploidy can lead to important advances in infertility and cancer fields. Therefore, this thesis firstly investigates gene expression patterns for various DDR pathway genes and then compares expression levels between aneuploid and euploid human surplus embryos.

Embryo Quality Prediction in IVF Clinics

Increasing healthcare costs is an emerging problem in many countries (Bolongaita et al., 2023). This also reflects on IVF clinics. IVF and/or intracytoplasmic sperm injection (ICSI) procedures, especially when coupled

with NGS technologies for PGT purposes, can become extremely costly. Unfortunately, increased expenses do not warrant successful outcomes and thus patients can face economical and psychological loss in this endeavour. Additionally, success rates for IVF procedures remain relatively low, despite many major advances in the field (Kushnir et al., 2017; Qiao et al., 2014; M. R. Sadeghi, 2018; C. Thompson, 2016; Wong et al., 2014). Being able to predict success rates in IVF centres can enable the attendees to give a better informed decision about whether to commit to the entire procedure or not. To this end, resulting embryo quality while not perfect, can provide a good indicator as better quality embryos yield the highest chances of success.

At infertility clinics, various measurements are taken from both the female and male partners. Indeed, there is some variation between clinics, yet there are certain data points that are very commonly recorded. For females, some of this data include age, follicle number, number of oocytes that are in meiosis-1 (MI oocyte number) and finally number of oocytes that are halted in meiosis-2 (MII oocyte number). For males, typical measurements include age, sperm count, sperm motility and the morphology of sperm.

Embryo quality can be considered under two main categories. First is the morphological assessment of the embryo. Generally, embryo morphological qualities are assessed on day 3, following fertilisation and also on days 5 and 6 just before implantation. Intriguingly, day 5 and day 6 embryos can be graded by the same criteria as opposed to day 3 embryos. The latter requires a different grading system. To analyse the morphological qualities of day 3 embryos methodology described by Ciray and colleagues (2012) which consists of the number of blastomeres and their evenness, degree of fragmentation, nucleus details and characteristic of the cytoplasm can be utilised. For grading of day 5 and day 6 embryos, almost exclusively Gardner criteria is used as posited by Gardner and Schoolcraft (1999). In short, Gardner criteria consist of a grading system for the expansion status of the blastocyst as well as the quality of the trophectoderm and inner cell mass (Gardner & Schoolcraft, 1999).

The second quality marker for determining the embryo's overall quality is its genetic composition. Genetic analysis can be comprised of multiple

aspects. Firstly, preimplantation genetic testing for monogenic disorders (PGT-M) can allow the screening of any known single gene disorders that may be pre-existing in the family. Secondly, preimplantation testing for structural rearrangements (PGT-SR) may allow identification of structural chromosomal rearrangements such as translocations and inversions. Last but not least, as previously stated PGT-A can be utilised to examine for aneuploidies. While the former two PGT methods are usually used within more specific contexts, PGT-A possesses a broader range for its application.

Analysis and interpretation of large data sets have been an issue in the past. Fortunately, dawn of the computer age has changed this status quo. Particularly, the recent advances in AI technologies and predictive algorithms have enabled analyses of complex data with relative ease. These new technologies are rapidly becoming a part of a variety of fields such as infertility clinics (Gardner et al., 2015; Manna et al., 2013; Simopoulou, Sfakianoudis, Antoniou, et al., 2018; Simopoulou, Sfakianoudis, Maziotis, et al., 2018). Another alternative to these technologies can be mathematical modelling. Mathematical models can enable identification of complex biological interactions and may allow them to be understood in a simpler fashion (Hassan et al., 2020; Simopoulou, Sfakianoudis, Antoniou, et al., 2018; Simopoulou, Sfakianoudis, Maziotis, et al., 2018; Yenkie et al., 2013). Furthermore, mathematical models can ultimately lead to valuable predictive algorithms. In order to prepare predictive algorithms or softwares, it is imperative to understand all the contributing factors for the outcome. Regression analyses can help in determining which independent variables, if any, are related to the dependent variables. More specifically for the purposes of this thesis, mathematical modelling can be a tool to establish the relationship between parental parameters and the morphological quality and/or aneuploidy status of the embryo. Moreover, a mathematical model can be established to understand the complex interactions of the parental data with overall embryo quality, ultimately leading to a prediction software to assess the success chances of the IVF or ICSI procedure before it commences. Such a prediction can assist couples in deciding whether to commit to the entire procedure based on their success chances and

potentially allow aversion of psychological burdens that may arise from an unsuccessful attempt as well as saving from an economical obstacle.

Purpose of the Study

Aneuploidy presents a major obstacle for IVF success. Recent data suggests DDR elements may play a role in faithful chromosome segregation, but their parts and mechanisms remain unclear, particularly in embryos. Most research surrounding DDR is in cancerous tissues and contexts. Thus, this thesis aims to investigate gene expression patterns in euploid and aneuploid embryos comparatively to elucidate the role of DDR genes further in aneuploidies.

Secondly, predicting IVF success chances in a clinical setting can benefit patients immensely. Recent advances in computational tools and mathematical models may enable such predictions. However, complex interactions between parental parameters and embryo quality must be understood better before predictive algorithms can be written. Thus, the second aim of this study was to establish correlations between parental data and embryo quality in order to create a mathematical model of the IVF/ICSI procedure.

CHAPTER II

Literature Review

This chapter contains detailed information on the previous work done on infertility and aneuploidy as well as mathematical modelling which leads up to the current state of the above-mentioned fields. The detailed review of the literature in this section forms the basis of the thesis study.

Infertility and IVF

World Health Organisation (WHO) describes infertility as the absence of pregnancy despite frequent unprotected intercourse for a period of one year or more. Furthermore, infertility is a growing concern as its incidence increases every year (Fischer-Holzhausen & Röblitz, 2022; World Health Organisation, 2023). Causes of infertility may vary and can be genetic, hormonal or physiological in origin. Moreover, it can arise from the female or the male partner or both partners may play a part in the lack of conception (Brugo-Olmedo et al., 2001; Carson & Kallen, 2021). The most prominent methodology among our current health systems is the use of ART. In vitro fertilisation (IVF) constitutes the most well-known type of ART but by no means it is the only available technique in the entire ART catalogue (Table 1).

Table 1

Summary List of Available ART Methods

Methodology	**Abbreviation**
Intra-uterine Insemination	IUI
In Vitro Fertilisation	IVF
Gamete Intrafallopian Transfer	GIFT
Intracytoplasmic Sperm Injection	ICSI
Round Nuclei or Spermatid Injection	ROSNI

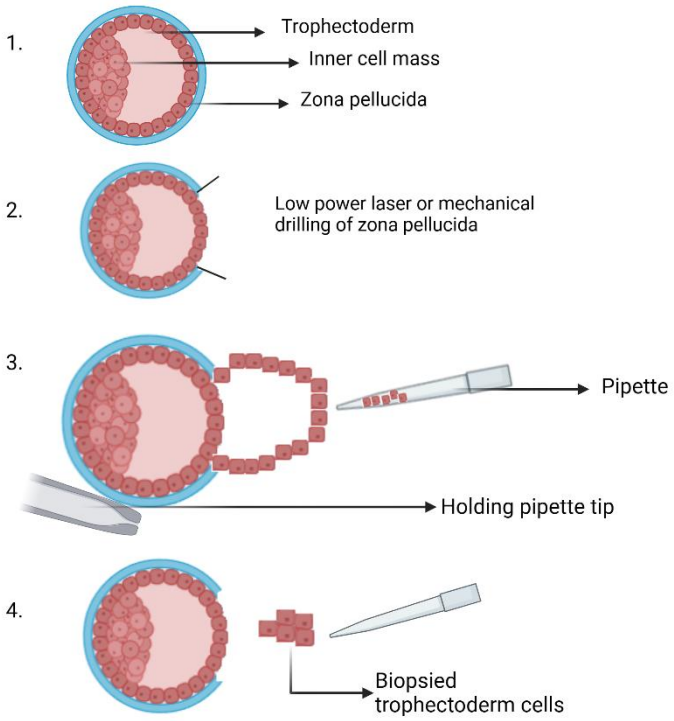
Patients can choose from various ART options, based on their clinician's guidance as well as the root cause of their infertility diagnosis among some other considerations such as economical concerns, female patient age, risk factors and the preference of the couple. Among those techniques, IVF and ICSI constitute the most popular options. Briefly, IVF describes a series of operations that initiates with controlled ovarian stimulation via recombinant follicle stimulating hormone (FSH) injections to achieve multiple follicle maturation which maximises the oocytes that will be gathered. When abundant follicle maturation is confirmed, typically after six days of hormonal injections, ovulation is triggered by administration of the hormone human chorionic gonadotropin (hCG). Follicle contents are then aspirated and oocytes are placed together with sperm from the paternal candidate in a petri dish and allowed to be fertilised in an incubator. Determination of the sperm quality is usually performed at this stage as well according to the latest WHO guidelines (World Health Organization, 2021). After fertilisation is confirmed by the presence of two pronuclei (2PN) under microscopic inspection, embryos are closely followed until day 3 post-fertilisation when the initial morphological quality of the developing embryo can be performed. At day 5 morphological quality of the embryos are re-evaluated according to the Gardner criteria (Gardner & Schoolcraft, 1999). Further details about these grading systems are covered in Chapter III: Materials and Methods section of this thesis. If the embryos do not show sufficient growth another assessment may be required the following day. Furthermore, when the embryos show adequate growth and reasonable morphology, a trophectoderm biopsy is commonly carried out for PGT analyses. Trophectoderm biopsy can be summarised by laser drilling of the zona via a laser and allowing trophectoderm herniation followed by collection of the herniated cells with the assistance of a needle (Figure 1). These cells can then be used for various PGT tests via different techniques ranging from real-time polymerase chain reaction (RT-PCR) to NGS (Zakaria, Al-Ibraheemi, et al., 2021). Most commonly this is used for aneuploidy screening via NGS. Indeed, classical karyotyping can also be used for the purposes of PGT-A, it would be a considerably slower process. Furthermore, partial and smaller aneuploidies could be missed depending on the

experience of the laboratory. While PGT tests are carried out, the blastocysts can be vitrified and stored in liquid nitrogen to prevent further embryo maturation as day 5/6 stage is the prime time for the embryo to embed itself into the mother's uterine wall and thus is the stage for embryo implantation in IVF as well.

Figure 1

An Overview of the Trophectoderm Biopsy Procedure

Main steps in trophectoderm biopsy involve drilling of zona pellucida, trophectoderm herniation and pipetting of the exposed trophectoderm cells with a specialised needle.



When the embryos with an euploid karyotype and high morphological quality are identified one or two of them are transplanted directly into the uterine lumen via ultrasound guided injection. When implantation is confirmed by blood hCG levels within a few days, pregnancy can then be followed closely by an obstetrician no different than a natural pregnancy. ICSI procedure is almost identical to the IVF counterpart with one major

difference. In ICSI, a single sperm cell is injected directly into the cytoplasm of the oocyte, hence the fertilisation process is slightly more artificial.

Despite the common use and the extensive history of ART, the success rates for remain relatively low (Kushnir et al., 2017; Qiao et al., 2014; M. R. Sadeghi, 2018; C. Thompson, 2016; Wong et al., 2014). There are a multitude of problems that can lead to IVF/ICSI failures and chromosomal errors belongs to this category as well. Literature consists of multiple studies that show chromosomal and/or spindle formation abnormalities in female oocytes and in male sperm can result in IVF failures (Carrell et al., 2003; Kilani & Chapman, 2014; Miyara et al., 2003; Puscheck & Jeyendran, 2007; Tilia et al., 2020). Furthermore, chromosomal or morphological aberrations of the resulting embryos can lead to unsuccessful attempts (Check et al., 2009; Fortin et al., 2019; Penzias, 2012; Uyar et al., 2013). In contrast, there are studies that suggest aneuploid embryo transplantation may result in successful pregnancy and childbirth (Barad et al., 2022; Cornelisse et al., 2020; Pagliardini et al., 2020; Tong et al., 2022; Yan et al., 2021). However, these studies suggest that indeed aneuploid embryos might bring about successful childbirth, yet the risk of failure is remarkably high. Additionally, childbirth in such cases often accompanied by various congenital defects (Barad et al., 2022; Cornelisse et al., 2020; Pagliardini et al., 2020; Tong et al., 2022; Yan et al., 2021).

Overall, aneuploidies represent a cause of infertility and can be an obstacle in both natural pregnancies and in artificially assisted pregnancies. Therefore, understanding the causes of aneuploidies in embryogenesis will be beneficial to multiple fields but primarily in reproductive genetics and oncogenetics.

Aneuploidy

Aneuploidy can be defined as one or more chromosomes being present in excess. Absence of such chromosomes also classifies as a case of aneuploidy. Moreover, partial aneuploidies also exist where only a part of a chromosome or few chromosomes are categorised under partial aneuploidies as well. It is important to note the difference between aneuploidies and

polyploidies, the latter referring to the addition or loss of complete sets of chromosomes.

Aneuploidy has always been of high interest due to its negative effects on overall cellular genomic integrity and tight association with almost all cancer cases (Duijf et al., 2013; Edwards et al., 1960; Patau et al., 1960; A. M. Taylor et al., 2018). More recent research however, showed that in yeast cells and some cancer cell lines aneuploidy could confer a proliferative advantage under stress conditions (Barja et al., 2017; Beaupere et al., 2018; Ben-David et al., 2014; Bussotti et al., 2018; G. Chen et al., 2012; Downing et al., 2011; Janbon et al., 1998; Kabir et al., 2005; Kaya et al., 2015; X. Liu et al., 1997; Lukow et al., 2021; Pavelka et al., 2010; Rancati et al., 2008; Rutledge et al., 2016; Ryu et al., 2016, 2018; A. Selmecki et al., 2006, 2008; A. M. Selmecki et al., 2009; Sionov et al., 2010; N. R. H. Stone et al., 2019; Su et al., 2021; Yona et al., 2012). Moreover, aneuploid cells have been observed in healthy human tissues at low frequencies as well (Coorens et al., 2021; Duncan et al., 2012; Knouse et al., 2014; McConnell et al., 2013; Terao et al., 2020; van den Bos et al., 2016). These observations led to the hypothesis of aneuploidies granting the cells a rapid adaptation mechanism for environmental stress (R. Li & Zhu, 2022). All in all, effects of aneuploidy appear to be context dependent and insufficiently understood. Another difficulty in isolating the consequences of aneuploidy comes from the fact that aneuploidies can confer phenotypical effects in two distinct contexts. Firstly, aneuploidy can cause karyotype specific effects. Naturally, depending on the specific chromosome or chromosomes affected, the expression levels of genes encoded on that chromosome is altered. This in turn leads to gene dosage imbalances and ultimately lead to transcriptomic alterations (Birchler & Veitia, 2012; R. Li & Zhu, 2022). In addition to such a direct effect, karyotype specific changes are disruptive to multiple regulatory networks. This is because many genes work in tandem and within complex regulatory networks in order to ensure homeostasis. For example, *MDM2* gene is situated on chromosome 12 and is involved in suppressing TP53 activity via ubiquitination. On the other hand, *TP53* is encoded on chromosome 17. An aneuploidy of chromosome 12 would alter expression levels of *MDM2* which would inadvertently affect the gene expression levels for *TP53* as well.

Depending on affected chromosomes, specific aneuploidies can also weaken spindle assembly checkpoint (SAC) and DDR pathways, thus can elevate chromosomal instability (CIN) which can ultimately result in further aneuploidy.

Secondly, aneuploidies can exert general effects on the cell regardless of the specific karyotype. In summary, aneuploid cells show slower cell cycle and proliferation, although these results are still under debate as isolating general effects of aneuploidy through the veil of karyotype-specific changes is challenging (R. Li & Zhu, 2022). Furthermore, aneuploid cells usually show high genomic instability (GIN) and CIN, nevertheless, these results were mostly observed in various cancer cell lines which complicates separating which alterations are the causes and which are the consequences of aneuploidy (Gordon et al., 2012; R. Li & Zhu, 2022). It is highly likely that the effects are a combination of both cause and consequence. Additional general effects of aneuploidies can include altered proteostasis and elevated reactive oxygen species (ROS) production and the resultant changes to cellular metabolism (R. Li & Zhu, 2022). Interestingly, these outcomes of aneuploidy, in particular increased ROS production can form a positive feedback loop that can cause further aneuploidy (Caracausi et al., 2018; G. Chen et al., 2012; Clemente-Ruiz et al., 2016; Dephoure et al., 2014; M. Li et al., 2010; Lim et al., 2020; Y. Liu et al., 2017; Mihalas et al., 2017; Niikura et al., 2006; Panagaki et al., 2019; Perkins et al., 2016; Técher et al., 2017; Valenti et al., 2011; G. F. Wang et al., 2017; Zuelke et al., 1997). For both general and karyotype-specific consequences of aneuploidy, the severity of the results is correlated with the sternness of the aneuploidy (Siegel & Amon, 2012). This can explain the worse and more unpredictable outcomes generally observed in complex and chaotic aneuploidies, which refer to aneuploidies affecting 3 or more chromosomes.

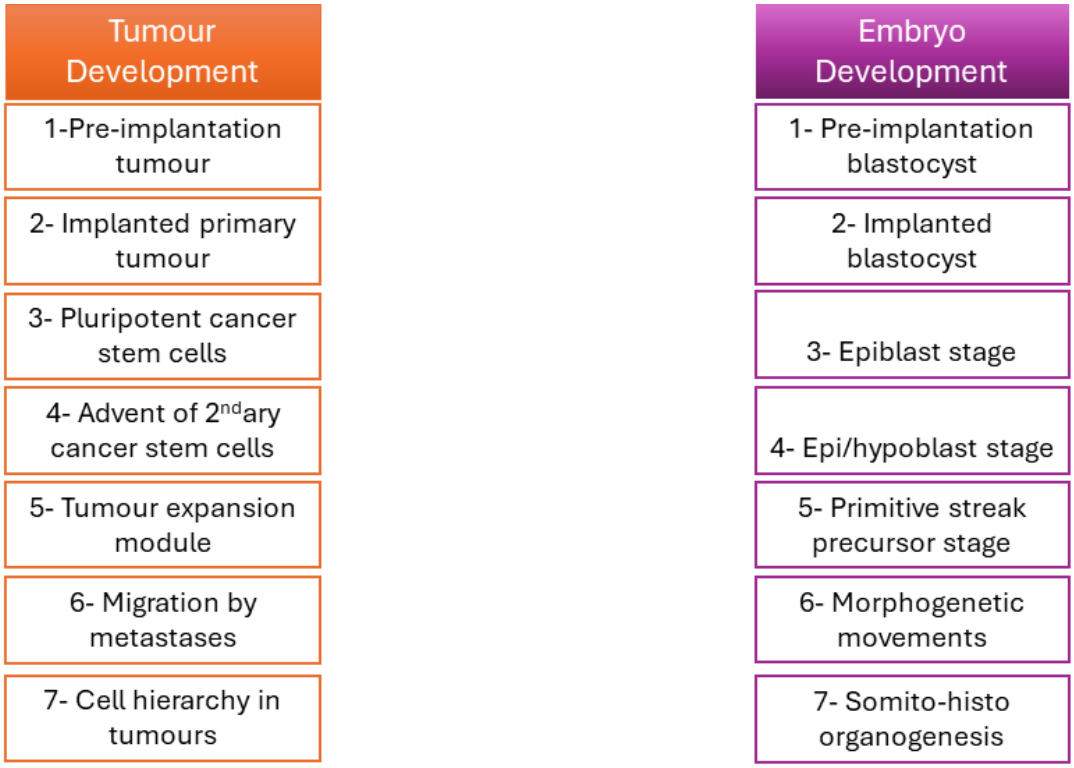
As previously stated, most of the molecular research surrounding aneuploidy was conducted on cancer cell lines. However, cancer cells can closely resemble embryo cells in certain aspects. Firstly, they share characteristics such as the capacity for initiating tumorigenesis and pluripotency. Furthermore, gene expression patterns between cancer cells and blastocyst cells share a lot of common ground in particular STAT3,

HSP70, CD44 which are regulators of pluripotency and fundamental growth pathways JAK/STAT, NOTCH, MAP-Kinase/ERK, PI3K/AKT, NFkβ, Wnt and TGFβ (Gnanavel et al., 2021; Manzo, 2019). Further similarities between blastocyst stage embryos and cancer cells are summarised in Figure 2.

Figure 2

Side by side listing of similar stages between embryogenesis and oncogenesis

Many stages of ontogenesis and cancer formation processes closely resemble each other. Steps that are hypothetically associated are summarised here.



Mechanisms of Aneuploidy

Under extreme stress conditions, aneuploidy can confer proliferative advantage (Pavelka et al., 2010). On the other hand, in a stress-free environment aneuploid cells are usually selected against in proliferation and/or removed by apoptosis, immune responses (R. Li & Zhu, 2022; Pavelka et al., 2010; B. R. Williams et al., 2008). There are multiple ways such defence mechanisms can be bypassed and aneuploidies can arise.

Despite the mechanisms discussed in this section are quoted as causes, in different contexts they can also be consequences of aneuploidy and in most cases, they are both.

Spindle Assembly Checkpoint (SAC)

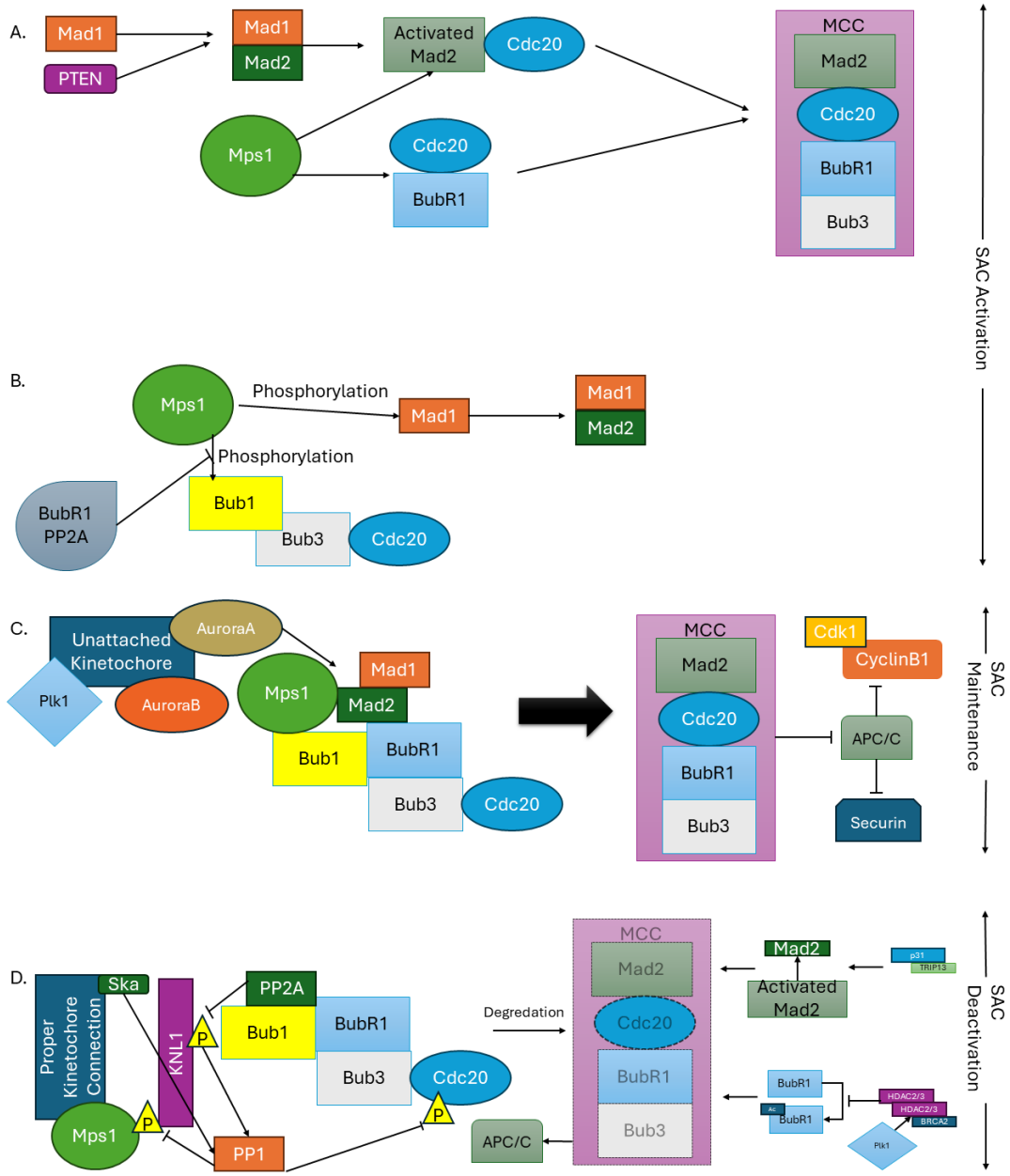
During meiosis and mitosis, accurate segregation of chromosomes into daughter cells is required to preserve the genetic integrity of the progenitor cells. During metaphase stage of the cell cycle, duplicated chromosomes align at the equator of the cell and are then required to attach to the microtubules generated by the centrosomes on opposite poles. Proper kinetochore attachment to spindles can be dependent on the duration of the procedure (B. Orr et al., 2015). The SAC network is the prime guardian of proper chromosome segregation. SAC is responsible for halting anaphase and cytokinesis until all spindles are correctly attached at kinetochores, thus guarding the fidelity of chromosome segregation. Moreover, failure to satisfy SAC for an extended duration may activate pro-apoptotic *BCL2*, *BAX* and *BAK* genes and trigger the mitochondrial apoptosis pathway by initiating cytochrome C release into the cytoplasm (Ruan et al., 2019).

SAC plays a part in both meiotic and mitotic divisions as its activation was shown to involve chromosome enrichment by BUB and MAD proteins in both (Maciejewska et al., 2009). SAC is a network comprised of a multitude of distinct genes that show complex interactions with each other. Some of the most popularly investigated ones include but not limited to *BUBR1*, *MAD2*, *CDC20*, *BUB3* which collectively form the mitotic checkpoint complex (MCC). Additionally, cyclin dependent kinases Cdk1 and 2, Aurora B kinase play critical roles in the SAC pathway as well. Ultimately, SAC complex seeks to inhibit APC/C complex until confirming proper and stable microtubule-kinetochore attachment to block mitotic exit (Musacchio, 2015; Ruan et al., 2019). Once correct spindle attachment is achieved, APC/C complex is activated to ubiquitinate Cyclin B and securin leading to their degradation ultimately activating separase and initiating anaphase. More detailed representation of the SAC pathway and its regulation of the cell cycle is shown in Figure 3.

Figure 3

Complex interactions of the SAC pathway elements

SAC is comprised of multiple genes that are associated tightly and regulate each other, in order to ensure faithful chromosome segregation. (A) represents nuclear SAC activation during interphase (B) shows Bub1 as the timer for SAC activation early in the mitotic process (C) summarises MCC maintenance and finally (D) shows the process involved in deactivation of MCC and eventual silencing of SAC to allow mitotic exit. P denotes phosphorylation and Ac stands for acetylation.



Despite its stringent regulation, SAC is still error-prone and can be disrupted by numerous ways. Mutations in key genes of the SAC pathway such as *BUBR1* are known to cause aneuploidies and increase cancer risk (Hanks et al., 2004; Snape et al., 2011; Yost et al., 2017). Nevertheless, SAC disruption is not necessary for its failure. Merotelic spindle attachments present a unique risk of avoiding detection by SAC (Gregan et al., 2011). Normally, Aurora B attempts to convert merotelic attachments to SAC-detectable monotelic attachments (R. Li & Zhu, 2022). Multipolar spindles can be observed as a direct consequence of centrosome overduplication and may also result in aneuploidies. Furthermore, centrosome overduplication can also elevate the incidence of merotelic attachments (Vitre & Cleveland, 2012). Centrosome overduplication is commonly observed in various cancers and is associated with aneuploidies as well (Brinkley, 2001; D'Assoro et al., 2002; Pihan et al., 2003). Under normal circumstances centrosome multiplication occurs once during a cell-cycle and is also a well-regulated process (Bettencourt-Dias & Glover, 2007; Hinchcliffe & Sluder, 2001; Mazia, 1987; Nigg, 2007; Tsou & Stearns, 2006). Intriguingly, Prosser and colleagues (2009) identified CDK2 activation as an early step that may lead to centrosome overduplication in the cytoplasm. Moreover, ATM/ATR induced TP53 and p21 pathway can inhibit CDK2 and prevent centrosome overduplication and the consequent aneuploidy. Additionally, CHK1 and CHK2 can inhibit CDC25A which has roles in regulating CDK2 expression. Together, these two pathways may form another connection between DDR genes and aneuploidies.

Genomic and Chromosomal Instability

Chromosomal instability refers to the condition where the chances of missegragation is elevated. Eventually, higher chances of chromosome segregation defects will result in chromosome number alterations in cells, which is the definition of aneuploidy. In other words, aneuploidy can be considered a direct result of CIN (Bakhoum & Cantley, 2018; R. Li & Zhu, 2022; Schukken & Foijer, 2018). CIN can be introduced in a cell by various ways. One most obvious is to disrupt SAC (Musacchio & Salmon, 2007). Furthermore, other alterations that affect centrosome duplication, spindle

formation and disruption of kinetochore-spindle attachments are all methods to provoke CIN formation. Finally, irradiating cells may also lead to CIN emergence (Bortoletto et al., 2001; Musacchio & Salmon, 2007; Simon et al., 2015). Irradiation in this context is interesting as irradiation is a potent mutagen as well as a prominent cause of double strand breaks (DSBs) in DNA. Increased frequency of mutations and double strand breaks is the description of GIN and GIN is a hallmark of aging and cancers (Giam & Rancati, 2015; López-Otín et al., 2013; Maslov & Vijg, 2009; Schukken & Foijer, 2018). In healthy cells, DNA damage is countered by various mechanisms such as mismatch repair, nucleotide-excision repair processes (N. Chatterjee & Walker, 2017). Moreover, homologous recombination (HR) (also known as homology-directed repair) and non-homologous end joining (NHEJ) are two types of repair pathways against DSBs. All the aforementioned repair pathways are regulated by genes of the DDR network. Additionally, CIN is considered a sub-type of GIN. Therefore, DDR network is intimately related to CIN which can eventually lead to aneuploidy.

Conversely, aneuploidy presence can occur independently of CIN as well which was observed in certain haematologic malignancies such as acute myeloid leukaemia (Paulsson & Johansson, 2007). The exact reason for this strange phenomenon is not determined yet however it was posited that it could arise due to low chromosome segregation rates or a possible selective advantage for trisomy 8 (Bakhoum & Cantley, 2018). Lastly, it is a recurrent theme in aneuploidies that causes can often be consequences as well. Adhering to this pattern, CIN can be a direct cause of aneuploidy but also it can be a consequence in a context-dependent manner (Schukken & Foijer, 2018).

Replication Stress and DNA Damage Response

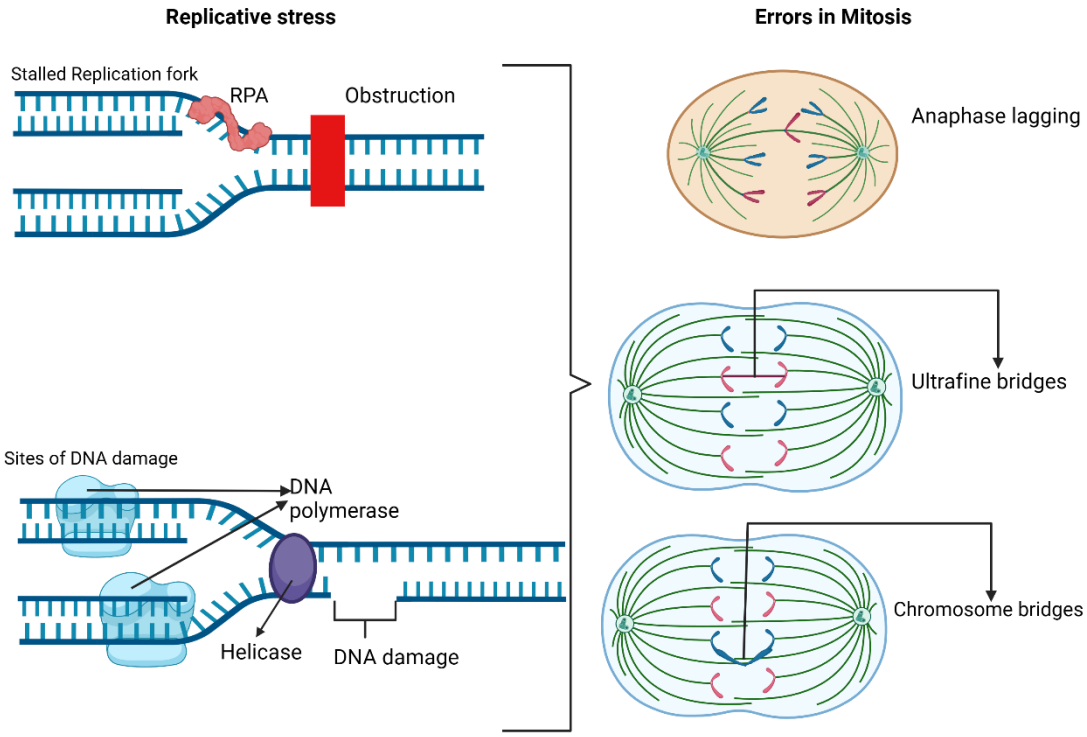
Cancers are very intimately associated with aneuploidies and indeed almost all cancers contain aneuploid cell populations (Duijf et al., 2013; A. M. Taylor et al., 2018). As previously stated, the major defender against aneuploidies is SAC. It is interesting then, that SAC mutations are rare occurrences in cancers (Schvartzman et al., 2010). In contrast, DDR genes are very commonly mutated in various cancers, most notorious examples

being *TP53*, *BRCA1* and *BRCA2* (Mendiratta et al., 2021; Shiovitz & Korde, 2015). DDR genes have a wide array of responsibilities within a cell. Beyond their primary role of sensing and acting upon DNA damage, they are also prominent regulators of the cell cycle and more recently they have been associated with faithful chromosome segregation (Kabeche et al., 2018; Stirling et al., 2011; D. J. Thompson et al., 2019). Furthermore, DDR genes may be involved in chromosome segregation and aneuploidy in different ways. Aside from more direct pathways, DNA replication stress mainly caused by stalled replication forks brought about by various sources of DNA damage or nucleotide depletion has been implicated in numerous aneuploidies (Burrell et al., 2013; Zeman & Cimprich, 2014). Additionally, replication stress may be provoked by replication forks that are progressing faster than normal or by disrupted replication origin firing (Kotsantis et al., 2018; Marchal et al., 2019; Maya-Mendoza et al., 2018). Replication stress and delayed forks eventually cause lagging chromosomes or anaphase bridging further down in the cell cycle (Figure 4). In particular, prolonged cell cycle halts were associated with increased frequency of anaphase bridges and ultra-fine bridges (UFBs), consequently increasing CIN (Ait Saada et al., 2017; Y. W. Chan et al., 2017; Y. W. Chan & West, 2018; Eykelenboom et al., 2013). Independently of anaphase bridging, microtubule defects and multipolar spindle formations are common outcomes of replication stress as well (Böhly et al., 2019; Wilhelm et al., 2019).

Figure 4

Summary of how replication stress may contribute to CIN

Replication stress induced by DNA damage may lead to anaphase bridges and ultimately can cause erroneous chromosome segregation and aneuploidy.



Anaphase bridges and UFBs mainly arise from unresolved homology directed repair during anaphase. Moreover, other inducers of end-to-end chromosome fusions such as telomere breaks, incomplete DNA replication or due to the presence of catenated chromosomes are considered instigators of UFBs (Carlton et al., 2012; Garsed et al., 2014; Janssen et al., 2011; Y. Li et al., 2014; Mardin et al., 2015). During mitosis these structures can cause chromosome ruptures which in turn can cascade into break-fusion-break cycles (Y. Li et al., 2014; Umbreit et al., 2020). More recently, it was also shown that these break-fusion-break cycles are complemented by chromothripsis, described as the rearrangement of a chromosome, to further elevate the mutation rates in a multitude of cancers (Umbreit et al., 2020). Same study also concluded that this combination is a primer of GIN and formation of micronuclei (Umbreit et al., 2020). Micronuclei can be formed by

lagging chromosomes during cell division and depicts small vesicles containing double stranded DNA within the cytoplasm. These vesicles are highly likely to rupture during S-phase of the cell cycle and release DNA into the cytoplasm, activating the anti-viral cGAS-STING pathway (Bakhoum & Cantley, 2018). Activity of cGAS-STING pathway may result in the establishment of senescence associated secretory pathway (SASP). SASP has the potential to affect the neighbouring cells in the microenvironment to provoke further aneuploidy in the surrounding population, which is a hallmark of aging (Freund et al., 2010; Q. He et al., 2018; Pawlikowski et al., 2013; Vasudevan et al., 2021).

DDR elements can also play a more direct role in aneuploidies. One of the most prominent DDR kinases, ATR, was recently discovered to interact with Aurora B kinase, a contributor to SAC and a defender of proper kinetochore attachments. Furthermore, this interaction was shown to be independent of its DDR functions (Kabeche et al., 2018). In addition, DDR activation during S-phase in yeast cells was shown to extend cell cycle to allow correct spindle assembly to take place (Pardo et al., 2017). Nevertheless, DNA damage and subsequent DDR activation during mitosis was shown to cause chromosome missegregation (Bakhoum et al., 2014). In tandem, these results suggest that DDR genes' involvement in chromosome segregation is complex and can rely on the diversity of the roles of the member genes in different contexts.

DNA is a relatively stable and durable molecule. However, endogenous and exogenous agents can still trigger deleterious modifications. Exogenous DNA damage can be brought about by different physical, chemical and biological perturbations. Some examples include ionising radiation, ultraviolet (UV) radiation, alkylating agents, certain toxins and pathogens. Endogenous agents include reactive oxygen species (ROS) and the intrinsic DNA replication errors during normal cell cycles. Each cell cycle accumulates single-base deletions and inversions with a frequency of approximately 10^{-8} per cell each generation (Kunkel, 2004, 2009). Susceptibility to damage is not an intrinsically negative feature of DNA as the ability to acquire mutations is crucial for evolution. In contrast, many alterations can exert a deleterious influence on the integrity of the DNA and

give rise to severe disorders and cancers. Therefore, protection of the genomic integrity is important for cells and is accomplished by different repair mechanisms collectively termed DNA damage response pathways. There are five main pathways that are commonly recruited, base excision repair, mismatch repair, nucleotide excision repair mechanisms are commonly used for single strand errors affecting one or a few bases only. The remaining two, HR and NHEJ are enrolled when double-strand breaks occur which poses a bigger threat for DNA integrity (N. Chatterjee & Walker, 2017).

Following DNA damage, DDR activation occurs in a sequential fashion (Polo & Jackson, 2011). Different repair mechanisms are tasked with correcting distinct DNA damage types. Base excision repair mechanism is involved in correcting single base level damage and is generally active in G1 stage of the cellular cycle (Dianov & Hübscher, 2013). When the damaged area involves multiple bases, nucleotide excision repair is generally employed. Mismatch repair on the other hand represents a highly conserved replication repair pathway that eliminates the majority of the nucleotide mismatches brought about by DNA polymerase errors post-replication (Arana & Kunkel, 2010). These errors usually affect a single strand of DNA and are generally easier to attenuate. On the other hand, double strand breaks present a tougher challenge for DDR and unattended DSBs are associated with multiple disorders, aneuploidies and cancers (Arnould & Legube, 2020; Jackson & Bartek, 2009). HR and NHEJ are the two major damage response mechanisms against DSBs. Initial sensor of the double strand break in both, is usually ATM which then cascades into downstream pathways phosphorylating histone variant H2AX and BRCA1 (Bhatti et al., 2011; Chapman, Taylor, et al., 2012; Chou et al., 2010; Gottschalk et al., 2009; C. Liu et al., 2014; J. Lukas et al., 2011; Rogakou et al., 1998; Rothkamm et al., 2003; Yu et al., 2006). In brief, NHEJ is mainly facilitated by Ku proteins which prevent resection at the broken ends and acts as a scaffold for other NHEJ proteins to dock (Mari et al., 2006; Mimitou & Symington, 2010; Pang et al., 1997; Soutoglou et al., 2007). As the broken genetic material is lost, NHEJ is a more error-prone correction of DSBs compared to HR. Conversely, HR pathway utilises DNA invasion to induce template directed repair and thus is a high-fidelity repair mechanism (X. Li & Heyer, 2008). Initiator of HR

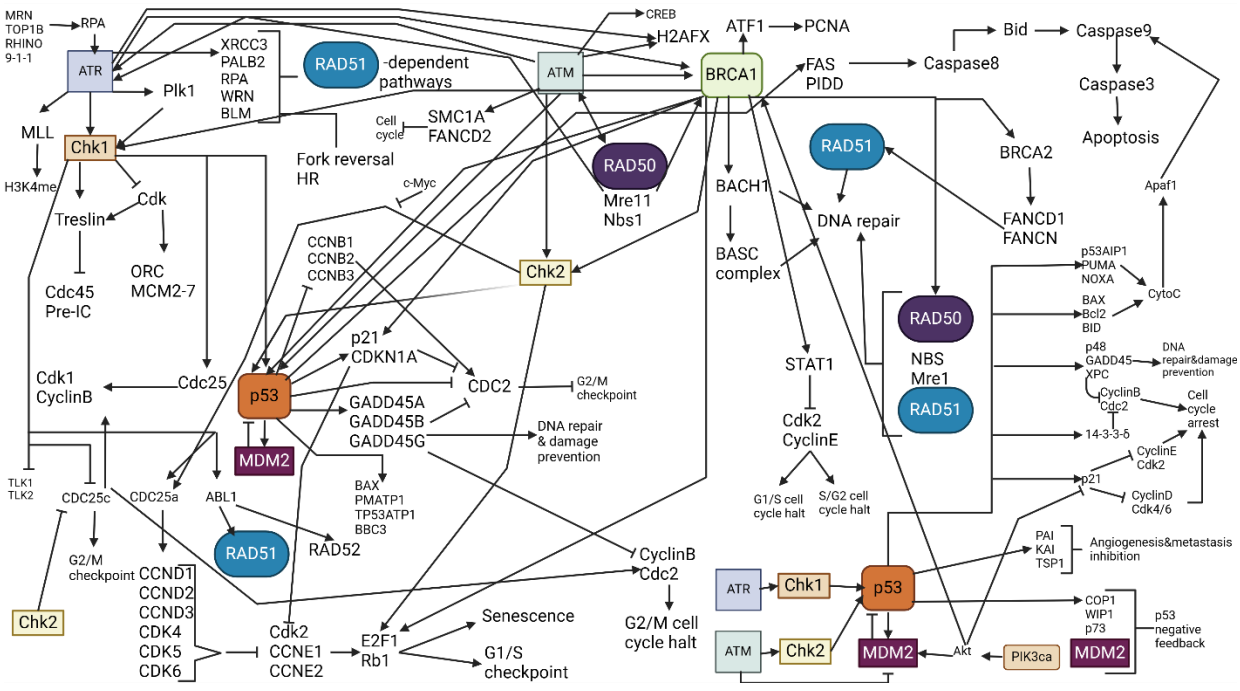
is the MRN complex, which is comprised of three proteins MRE11, RAD50 and NBS1. MRN complex then recruits ATM and downstream of ATM important HR proteins like BRCA1 and RAD51 (N. Chatterjee & Walker, 2017). As heavy DNA damage is incompatible with replication, most DDR genes are also regulators of the cell cycle. Many are tied to various cell cycle checkpoints which are activated to halt the cell cycle until DNA damage can be resolved or in extreme cases apoptosis is triggered (N. Chatterjee & Walker, 2017; Heeres & Hergenrother, 2007; W. Zhou & Doetsch, 1993). To accomplish these goals, DDR genes form very complex networks and interact with each other (Figure 5).

As the interactions of the entire network is too complex, in the following sub-sections only genes that are the direct focus of this thesis are introduced.

Figure 5

Complex DDR interactions form the core of cell cycle regulation and DNA repairs.

Damaging alterations to DNA structure initiate extremely complex pathways with different outcomes. Almost all members of the DDR grid are situated both upstream and downstream of each other concurrently, therefore complicating the pathway further. While this figure encapsulates most of the known interactions within the DDR pathway, it is important to note that it still lacks the entirety of the connections among DDR members. For ease of tracking, the genes that are directly the focus of this thesis are denoted with specific shapes and colours as opposed to other members within the network.



ATM and ATR. Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia Rad3-related (ATR) are serine/threonine kinases, belonging to PI3K-like kinase family, that act as the master conductors of the DDR network (Maréchal & Zou, 2013). While the pair often work intimately to govern the DDR, the prerequisites for their activation and their specific roles

are different and not redundant. They direct the DDR mainly by protein phosphorylation.

In summary, DNA damage or replication stress, such as stalled replication forks, is recognised by sensory proteins like the MRN complex which in turn recruits ATM and ATR to the site. ATM and ATR then instigate downstream signalling cascades that may trigger apoptosis, DNA repair and cell cycle arrest. Generally, *ATM* is primarily activated by DSBs, whereas *ATR* is activated in response to a broad spectrum of DNA damage as well as interferences with replication (Maréchal & Zou, 2013). First step in this pathway usually involves the recruitment of CHK1 and CHK2 kinases.

Double strand breaks present one of the biggest threats to the fidelity of the genome. Therefore, ATM activity is paramount in averting such catastrophes. This is further evidenced in ataxia telangiectasia patients where *ATM* perturbations lead to hypersensitivity to DNA damage via irradiation and also to defects in G1/S, S and G2/M cell cycle checkpoints. Normally, MRN complex would sense and bind to the site of DNA double strand breaks and recruits ATM to the site to initiate the DSB repair process. ATM, in turn, phosphorylates other DDR elements such as BRCA1, CHK2 and TP53 (Lavin, 2008; Shiloh, 2003). Furthermore, ATM also works closely with H2AX to induce the chromatin rearrangements that allow further recruitment of DDR elements (Maréchal & Zou, 2013; Meier et al., 2007; Savic et al., 2009). Interestingly, ATM-H2AX interaction is not compulsory to phosphorylate CHK2 and TP53, but it is necessary for further recruitment of DDR proteins and chromatin remodelling proteins (Fernandez-Capetillo et al., 2002; J. Kang et al., 2005). Moreover, ATM interaction with H2AX is a crucial initiator of ubiquitylation and sumoylation reactions that recruit BRCA1 and 53BP1 (Bekker-Jensen et al., 2009; Doil et al., 2009; Galanty et al., 2009; Huen et al., 2007; Kim et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Meerang et al., 2011; Morris et al., 2009; Sobhian et al., 2007; Stewart, 2009; B. Wang et al., 2007; B. Wang & Elledge, 2007). BRCA1 and 53BP1 antagonise one another and the balance between their levels is an important regulator of HR system of repairing DSBs (Bunting et al., 2010; Chapman, Sossick, et al., 2012; Y. Xu et al., 2017). Furthermore, BRCA1 and 53BP1 have been shown to amplify ATM activity in vitro, suggesting ATM boosts its

own activity for DSB repairs via H2AX-BRCA1-53BP1 expression (J. H. Lee et al., 2010). In addition, BRCA1 and 53BP1 assists in further recruitment of ATM substrates (DiTullio et al., 2002; Fabbro et al., 2004; Kitagawa et al., 2004; B. Wang et al., 2002). In addition, ATM seems to be further involved with mitochondrial pathways. Knockdown of Drp-1 show hyperfused mitochondria that lead to defects in chromosome segregation (Qian et al., 2012). Moreover, Drp-1 knockdown induced genomic instability was shown to trigger ATM-dependent G2-M arrest and the same study further showed that ATR deficiency in combination with Drp-1 knockdown further decreased stalled replication fork recovery leading to increased *ATM* activity, suggesting that the united efforts of ATM and ATR is important in ensuring genomic stability (Qian et al., 2012). ROS was also implicated in inducing ATM autophosphorylation (Z. Guo et al., 2010; Maréchal & Zou, 2013). Normally, the largest contributor to ROS in a cell are the mitochondria, suggesting that mitochondrial processes can induce *ATM* activity and indirectly affect genomic stability as well. Intriguingly, in many IVF centres, mitoscore is considered as a factor in selecting the embryos for implantation. While the exact effect of mitochondria is unknown on the quality of the embryo, the association between the two is clear (H. Arora et al., 2022; Bayram et al., 2017; Diez-Juan et al., 2015; Fragouli et al., 2015; May-Panloup et al., 2005; Viotti et al., 2017; Wai et al., 2010). This connection between the mitochondria and ATM might provide an answer to this mystery, thus elevating the interest in the research of ATM in a fertility context. ATM but not ATR is also a key contributor of NHEJ (H. Wang et al., 2004; Zha et al., 2011). ATM and ATR often work closely together and can assist in the recruitment of one another. ATR-ATRIP recruitment generally depends on the presence of RPA coated ssDNA sequences. In DSB cases ATM and MRN complex act as prominent drivers of resection which introduces ssDNA to the blunt ends. As resection process advances and ssDNA sequence elongates, ATM activation switches towards ATR activation instead (Shiotani & Zou, 2009).

ATR works in tandem with its effector kinase Chk1 to manipulate intra-S and G2/M checkpoints. Despite their close relationship, *ATR* but not *ATM* is essential to the survival of proliferating cells and its deletion leads to lethal

outcomes in embryogenic mice and in human cell lines (Brown & Baltimore, 2000, 2003; Cortez et al., 2001; Klein et al., 2000). ATR is a vigilant watcher of replication fork progression and is a protector against intrinsic replication stress (Casper et al., 2002; McNees et al., 2010). In contrast to ATM, ATR can also be activated by single strand DNA damage, in addition to DSBs and ATR has been implicated in nucleotide excision repair and mismatch repair responses (Ball et al., 2005; Byun et al., 2005; Lopes et al., 2006; Namiki & Zou, 2006; Shigechi et al., 2012; Zou & Elledge, 2003). ATR recruitment also depends on sensory proteins. RPA complex covers exposed or resected single strand DNA to initialise ATR-ATRIP recruitment (Maréchal & Zou, 2013; Menolfi & Zha, 2020). ATRIP refers to ATR interacting protein which is essential for ATR stability and function (Menolfi & Zha, 2020). However, full ATR activation pre-requisites at least three regulatory mechanisms to collaborate (Maréchal & Zou, 2013). ATR can also act in response to a compromised coordination between helicase and DNA polymerase as this would also induce stress on the replication fork (Byun et al., 2005; Lopes et al., 2006). Furthermore, ATR is involved in faithful origin firing during replication (Cimprich & Cortez, 2008; Flynn & Zou, 2011). As an important regulator of such eminent processes, ATR activation is tightly regulated to prevent premature initiation. Most notably, research showed that simple presence of ssDNA is insufficient for ATR activation and ss/dsDNA junctions are required. This prevents key ATR responses such as apoptosis, DNA repair and senescence from occurring spontaneously (Ellison & Stillman, 2003; Kumagai et al., 2006; J. Lee & Dunphy, 2010; S. Liu et al., 2011; Mordes et al., 2008; Mordes & Cortez, 2008; E. A. Nam et al., 2011; Yoo et al., 2009; Zou et al., 2003).

Another point of interest regarding ATR is that many of its downstream proteins are also direct substrates of ATR itself, further complicating the signalling network (Maréchal & Zou, 2013). Furthermore, ATM and ATR cross-talk at the substrate level as well (Matsuoka et al., 2007). For instance, ATR is also an inducer of H2AX, allowing downstream activation of INO80 and RAD51 in order to initiate DNA repair at stalled replication forks (Flynn & Zou, 2011). As previously mentioned, ATR generally exerts its influence via its effector kinase CHK1. One gene that is downstream of *CHEK1* is *CDC25*

which is inhibited in response to *ATR/CHEK1*. This *CDC25* inhibition prevents CDKs1 and 2 and cyclin B kinase functions to inhibit mitotic entry (Busino et al., 2003; Jin et al., 2003; Melixetian et al., 2009; C. Y. Peng et al., 1997; Sanchez et al., 1997). Another important CHK1 target is BRCA1, which aids in preventing the collapse of replication forks under replication stress (Arlt et al., 2006; Bartek & Lukas, 2003).

Both ATM and ATR are highly activated in various cancers and are thought to aid in the survival of cancer cells (Halazonetis et al., 2008). This theory is further supported by the observation that ATR is essential for sustained survival of proliferating cells and that even in unperturbed cell lines acts to safeguard the replication forks (Buisson et al., 2015; Simoneau & Zou, 2021). Interestingly, in tumours with numerous chromosomal abnormalities, commonly referred to as CIN tumours, SAC members are commonly mutated, but they cannot explain the degree of genome destabilisation on their own (Collura et al., 2005; Eun & Burke, 2008; Garber & Rine, 2002; Rajagopalan et al., 2003; Sugimoto et al., 2004). *ATM* and *ATR* are commonly mutated in CIN tumours and thus present two high-value candidates in that regard (McCulley & Petes, 2010). Furthermore, in yeast and CIN tumours mutations that elevate the number of gross chromosomal rearrangements are found to be in genes controlling S-phase DNA damage checkpoint, DNA replication, telomere length regulation and chromatin assembly (C. Chen & Kolodner, 1999; Craven et al., 2002; Myung, Chen, et al., 2001; Myung, Datta, et al., 2001; Myung et al., 2003). In contrast, SAC mutations do not elevate the levels of gross chromosomal rearrangements (Myung et al., 2004). In yeast *Tel1p* and *Mec1p* (orthologs of human *ATM* and *ATR* respectively) have been shown to have roles in DDR, S-phase checkpoint and telomere length regulation. Interestingly, McCulley and Petes also showed that even in the absence of DNA damage *Tel1p* and *Mec1p* mutations were associated with extensive aneuploidy and chromosomal rearrangements while the SAC remained intact (McCulley & Petes, 2010). Therefore, suggesting SAC independent influence of *ATM* and *ATR* on aneuploidies. Furthermore, correcting telomere defects reduced CIN but not the rate of aneuploidies, suggesting the role of ATM and ATR in aneuploidies

to be also independent of their role in telomere length regulation (McCulley & Petes, 2010).

ATR is a master regulator of DNA replication. Even in unperturbed cells, knockdown of *ATR* slows replication fork progression and increases the number of replication origins (Eykelenboom et al., 2013). Furthermore, such cells tend to undergo mitosis early, i.e., without sufficient control of proper DNA replication, as well as showed quicker division, leading to anaphase lagging and chromosome bridges, failed cytokinesis and cell death (Eykelenboom et al., 2013). ATR is considered to enable various cell cycle checkpoints to regulate the cell cycle. Firstly, activating S-M checkpoint minimises unreplicated DNA before mitosis (K. L. Chan et al., 2007, 2009; Eykelenboom et al., 2013; C. Lukas et al., 2011). Initialisation of intra S checkpoint inhibits replication origin firing, protects stalled replication forks from recombination, allows relaxation of chromosomal torsional stress and modification of proteins at the replication fork (Alabert et al., 2009; Bermejo et al., 2011; Boddy et al., 2003; De Piccoli et al., 2012; Santocanale & Diffley, 1998; Shirahige et al., 1998; Zegerman & Diffley, 2010). Furthermore, activation of the intra-S checkpoint induces dNTP production, which may aid in decreasing replication stress due to depleted dNTP reserves (Elledge et al., 1993). *ATR* is further involved in the balance of replication origin firing and dNTP production (Simoneau & Zou, 2021). In contrast, S/G2 checkpoint inhibits mitosis in the absence of complete DNA replication. However, as opposed to DDR, ATR activation during S/G2 transition is achieved by ETAA1 enabled by RPA (Bass et al., 2016; S. Feng et al., 2016; Haahr et al., 2016; Y.-C. Lee et al., 2016). Loss of *ATR* activity hastens S-phase progression albeit with increased levels of mitotic defects that ultimately lead to cell death. Furthermore, reduction in checkpoint regulation may lead to mitotic entry prior to total DNA replication which is then attempted to be corrected by the cells during mitosis. However, these attempts usually lead to chromosome missegregations and aneuploidies (Simoneau & Zou, 2021). In addition to its roles in checkpoint regulation, ATR also regulates the rate of DNA synthesis via controlling the number of replication origins that are active at any given time. ATR's influence on replication origins is dependent on

CDC25A phosphorylation via CHK1 (Buisson et al., 2015; H. Zhao et al., 2002).

Another interesting observation about *ATR* related aneuploidies is seen in neurodegenerative disorders. In Alzheimer patients, mosaic aneuploidy is observed (Arendt et al., 2010; Iourov et al., 2009, 2010; P. Thomas & Fenech, 2008). Similarly, in ataxia telangiectasia and Seckel syndromes, which are the results of *ATM* and *ATR* mutations respectively, mosaic aneuploidies are also a common occurrence (Potter et al., 2019). *ATM* and *ATR* mutation are associated with genomic instability, aneuploidy and increased cancer risk due to defective DNA repair in such patients. Moreover, loss of *ATR* function is a known cause of centrosome overduplication (Alderton et al., 2004; Collis et al., 2008; Stiff et al., 2016). Therefore, in addition to cancers, *ATM* and *ATR* mutations are also associated with aneuploidies in neurodegenerative disorders. However, similar observations in preimplantation embryos are currently lacking in the literature.

A recent study has highlighted that ATR but not ATM co-localises to the centromeres (Kabeche et al., 2018). Previously, CHK1 was considered to act independently of ATR in centromeres in tandem with Aurora B kinase (Zachos et al., 2007). Intriguingly, *CHEK1* inhibition seems to dampen SAC functions whereas a reduction in *ATR* activity appears to have no effect on the SAC (Kabeche et al., 2018; Zachos et al., 2007). However, this recent data suggests ATR induces Aurora B via CHK1 to prevent lagging chromosomes, thus providing defence against CIN and aneuploidy. Furthermore, results of this study also suggest this role of *ATR* is independent of its S-phase checkpoint activity and DDR functions (Kabeche et al., 2018). ATR is required for full Aurora B activation at centromeres which in turn allows correction of erroneous microtubule attachments at kinetochores (Cimini et al., 2006; Kabeche et al., 2018; Lampson & Cheeseman, 2011). Intriguingly, recruitment of ATR at centromeres is RPA-dependent, however ssDNA presence at centromeres is not confirmed. Instead, R-loop structures seem to act as RPA binding sites to conscript ATR (Kabeche et al., 2018). Interestingly, centromeric employment of ATR doesn't seem to affect CDDK1 and CDC25A, very common downstream kinases of

ATR in its DDR related pathways. Therefore, suggesting that the centromeric and DDR functions of ATR appear to be distinct (Kabeche et al., 2018).

Finally, *ATR* was also shown to associate with apoptosis directly. Interestingly, pro-apoptotic protein BID interacts with ATRIP and RPA to drive ATR activation during replicative stress (Y. Liu et al., 2011). Furthermore, ATR was shown to bind to BID to prevent apoptotic functions of BAK and BAX in order to give the cell time to correct DNA damage (Biswas et al., 2022; Hilton et al., 2015; Makinwa et al., 2020; Murga et al., 2009; Musich et al., 2017). Surprisingly, this function of cytoplasmic ATR is based on the cis- /trans-configuration of the protein and is independent of the nuclear function of ATR (Biswas et al., 2023). Usually, aneuploidies and other copy number variations are highly prone to apoptosis. Therefore, *ATR*'s involvement in both aneuploidies and apoptosis makes it a very interesting gene in this field.

CHEK1. CHK1 is a serine/threonine kinase that has roles in G2/M checkpoint, DNA replication, mitotic progression, DNA repair and overall cell cycle (Alderton et al., 2004; Neizer-Ashun & Bhattacharya, 2021). CHK1 is the protein encoded by the *CHEK1* gene situated on chromosome 11. CHK1 is the effector kinase of ATR and thus is integrated in many of its pathways (Alderton et al., 2004; Maréchal & Zou, 2013; Sørensen et al., 2005). Intriguingly, ATM and ATR have distinct pathways, yet their effector kinases, CHK2 and CHK1 respectively, show overlaps (Neizer-Ashun & Bhattacharya, 2021). Naturally, loss of ATR/CHK1 pathway functions either by loss of *ATR* and/or *CHEK1* activity is associated with defects in cell cycle and DDR. Furthermore, mutations of *TP53* and *Rb* genes were shown to render cells heavily reliant on ATR/CHK1 pathway. Suggesting, their cooperation in maintaining cell survival (Gaillard et al., 2015; Ma et al., 2012; Neizer-Ashun & Bhattacharya, 2021; Origanti et al., 2012). Additionally, in the absence of ATR/CHK1 pathway activity, replicative stress, nuclear fragmentation and micronuclei formations are common sights (Alderton et al., 2004). Conversely, hyper-expression of *CHEK1* can be detrimental to cellular and genomic homeostasis. Suggesting that strict regulation and oscillation of the expression levels of *CHEK1* is crucial to its function (Krystyniak et al., 2005; Mailand et al., 2006; Neizer-Ashun & Bhattacharya, 2021; Peschiaroli et al.,

2006; Tang et al., 2006; Zachos et al., 2007). *CHEK1* was also observed to be prominent in development and expansion of haemopoietic stem and progenitor cells (Schuler et al., 2019). Moreover, in mice deletion of *Chek1* completely abolished survival of embryos (Boles et al., 2010; Takai et al., 2000).

CHK1 forms different interactions with a multitude of proteins. As previously mentioned, ATR is considered to be the primary activator of CHK1. ATR phosphorylates CHK1 on its specific serine residues mainly as a response to replication stress, thus mediating G2/M and intra-S checkpoints as well as influencing SAC via Aurora kinases A and B (Dai & Grant, 2010; Gaillard et al., 2015; Krystyniak et al., 2005; Mailand et al., 2006; Peschiaroli et al., 2006; Petermann et al., 2010; Shaltiel et al., 2015; Syljuåsen et al., 2005; J. L. Wang et al., 2002; Zachos et al., 2007; Zeman & Cimprich, 2014; H. Zhao et al., 2002). As a prominent influencer of key cellular processes, *CHEK1* expression requires rigorous supervision. Furthermore, CHK1 protein was observed to exist in a constitutively inactive state prior to its phosphorylation due to its unique folding (Neizer-Ashun & Bhattacharya, 2021). Activation of CHK1 can involve varying pathways and the mode of its activation appears to depend on the root cause of its activation. As an example, CHK1 activation during DNA replication and its activation as a response to DNA damage rely on distinct mechanisms (Neizer-Ashun & Bhattacharya, 2021).

When DNA damage is recognised by ATR, CHK1 is subsequently phosphorylated and activated in majority of cases. In turn, CHK1 acts to delay cell cycle progression to grant the cell more time for carrying out necessary repairs on the DNA (Manic et al., 2015; Shaltiel et al., 2015). CHK1 can then phosphorylate RAD51, which is a key step in its interaction with BRCA2 within the HR pathway. The S-phase checkpoint is triggered under replication stress and CHK1 usually activates cell-cycle checkpoints via CDC25 phosphatases (Donzelli et al., 2004; Melixetian et al., 2009; J. L. Wang et al., 2002; H. Zhao et al., 2002). CDC25 has three major isoforms that are downstream targets of Chk1. Firstly, CDC25A is phosphorylated by CHK1 leading to the inhibition of CDK1 and CDK2 and ultimately causing cell-cycle arrest at either intra-S or G2/M checkpoints (J. L. Wang et al.,

2002; H. Zhao et al., 2002). Secondly, CHK1 can phosphorylate CDC25C and enable its export from the nucleus. Consequently, nuclear cyclin B and CDK1 cannot be activated which prevents mitotic entry (Lopez-Girona et al., 1999; C. Y. Peng et al., 1997). Interestingly, third CDC25 isoform, CDC25B, is localised to centrosomes in unperturbed cell cycle and its phosphorylation by CHK1 prevents CDK1 activation at centrosomes (Krämer et al., 2004; Schmitt et al., 2006). On the other hand, CHK1's importance in unperturbed cells extends beyond centrosomes as it is critical for cell survival, DNA replication, G2/M transition and mitosis independently of its DDR functions (Wilsker et al., 2008). Furthermore, loss of *CHEK1* was shown to cause TP53 induced cell death via caspase 2 in undisturbed cells (Pan et al., 2009). During natural DNA replication, at S-phase, activated CHK1 phosphorylates CDC25A and mediates its degradation, thus promoting replication fork progression and inhibits excess origin firing. Conversely, knockdown of *CHEK1* was observed to result in elevated replication stress, DSBs, excessive replication origin firing and aberrant replication forks that may eventually result in aneuploidies (Petermann et al., 2010; Syljuåsen et al., 2005). In addition, CHK1 can also inhibit DNA replication via TRESLIN, a TOPBP1 binding protein (Qiu et al., 2018). Interestingly, CHK1 needs to be inactivated later in the cell cycle, to allow G2/M progression as otherwise nuclear export of CDC25C prevents cyclin B and CDK1 activation in the nucleus (Lopez-Girona et al., 1999; C. Y. Peng et al., 1997). Moreover, CHK1 is implicated in mitotic exit, just before cytokinesis. Cells that enter mitosis with damage will delay mitotic exit and tend to go through mitotic catastrophe and form binucleates. Binucleated cells are cleared via apoptosis in a TP53-dependent manner. Activation of CHK1 was shown to attenuate cyclinB1 degradation which in turn enforces mitotic catastrophe (Andreassen et al., 2001; X. Huang et al., 2005).

CHK1 is also a key in influencer of the SAC network. In particular, CHK1 regulates Aurora A and Aurora B kinases and thus is essential in kinetochore-microtubule attachments (Krystyniak et al., 2005; Zachos et al., 2007). Previously, these regulating roles of CHK1 was considered to be independent of ATR, however more recent data suggests ATR may play a role in this pathway after all (Kabeche et al., 2018; Zachos et al., 2007).

CHK1 also negatively regulates PLK1, an important regulator of SAC (Tang et al., 2006). Abrogation of *CHEK1* was further associated with chromosome misalignment in metaphase, lagging chromosomes and kinetochore defects during anaphase, all promising precursors of aneuploidies (Tang et al., 2006).

Chk1 was also shown to regulate SAC and DDR during early embryonic development in mice (Ju et al., 2020). Preimplantation embryos can acquire aneuploidies at any developmental stage, but the first two divisions are known to be the most susceptible to chromosomal aberrations (Mertzanidou et al., 2013). Moreover, cleavage stage embryos generally present more abnormalities compared to blastocyst embryos (Santos et al., 2010). Notably, loss of CHK1 has important implications on early embryo development. Firstly, loss of *Chek1* was shown to elevate DNA damage and oxidative stress which in turn induced early apoptosis and autophagy in mice embryos (Ju et al., 2020). In addition, abrogation of *Chek1* activity severely impedes the ability of embryos to develop to morula and/or blastocyst stages, while initially accelerating cell cycle progression at the first cleavage (Ju et al., 2020). Furthermore, SAC impairment in early embryogenesis, which can arise as a consequence of loss of *CHEK1* activity, results in micronuclei formation, chromosome misalignment and aneuploidies, thus decreasing implantation rates and delaying development (Dobles et al., 2000; Kalitsis et al., 2000; Q. T. Wang et al., 2004; Y. Wei et al., 2011). SAC disruption in early embryos due to *CHEK1* knockdown can be attributed to its active role in BUBR1 and Aurora B localisation and their recruitment or to CHK1's influence on MAD2, another well-known member of the SAC network (Carrassa et al., 2009; Chilà et al., 2013; Mackay & Ullman, 2015). As mentioned above, first cleavage divisions hasten in *CHEK1* knockdown cells, but the cells show abnormal spindle assembly and chromosome alignments. Moreover, lagging and scattered chromosomes were common observations in such cases alongside multipolar spindle formations (Ju et al., 2020). Furthermore, *CHEK1* elimination at the embryogenic stage results in increased *BAX* expression and subsequent apoptotic responses. Lastly, *CHEK1* loss was associated with an increase in H2AX activity which is an important marker of sensing DNA damage. In contrast, *RAD51* levels were shown to be decreased, suggesting that despite the DNA damage can be

detected, the cells may lack the restorative response in the absence of CHK1 (Dickey et al., 2009; Ju et al., 2020).

CHEK2. As CHK1 constitutes the immediate downstream effector kinase for ATR, CHK2 fulfils the same role for ATM. CHK2 is also a serine/threonine kinase encoded by the *CHEK2* tumour suppressor gene situated on chromosome 22 and has a part primarily in the DDR pathway (Tominaga et al., 1999). Following the recognition of DSB sites by the MRN complex, ATM is recruited which in turn phosphorylates and activates CHK2. CHK2 can in turn interact with other downstream substrates such as TP53, CDC25A, CDC25C and BRCA1 (Boonen et al., 2022). Through these substrates, CHK2 can be involved in various cellular processes as a moderator. For instance, through its interactions with BRCA1, CHK2 can be involved in DDR. Furthermore, via KAP1 it can influence heterochromatin relaxation. By interacting with CDC25A and CDC25C, checkpoint activation and cell cycle arrest can be regulated. Similarly, via its interaction with TP53, CHK2 can be prominent in cell cycle arrest and apoptosis (Boonen et al., 2022). Unsurprisingly, CHK2 has been associated with Li-Fraumeni syndrome much like TP53 (Bell et al., 1999; McBride et al., 2014; Schneider et al., 2019). Moreover, CHK2 has been shown to phosphorylate TP53 and drive its dissociation from MDM2, ultimately stabilising TP53, thereby elevating cellular responses against tumorigenesis (Chehab et al., 2000). Independently of these interactions, *CHEK2* is an eminent tumour suppressor gene by itself. Disruption of its activity has been extensively documented in breast cancers (Couch et al., 2017; Dorling et al., 2021; Hauke et al., 2018; Meijers-Heijboer et al., 2002; Weischer et al., 2007). Nonetheless, it is a cancer risk gene in multiple organs outside of breast cancers (Cybulski et al., 2004).

CHEK2 possesses an intriguing expression profile with regards to cellular activity. A past study has shown that *CHEK2* is highly expressed in proliferating, renewing cells whereas it was downregulated comparatively in terminally differentiated cells or resting cells (Zoppoli et al., 2012). Additionally, *CHEK2* mutations are one of the most frequent germline alterations (Stolarova et al., 2020). As a very actively expressed protein in

proliferating cells, it is not surprising that CHK2 holds duties in stem cell maintenance alongside circadian clock regulation duties and intracellular responses to viral infections (Stolarova et al., 2020). Since CHK2 exerts influence on a number of cellular pathways its expression necessitates tight regulation. Molecularly, in the absence of DNA damage stimuli, CHK2 remains as an inactive monomer. Upon activation of DNA damage signals, ATM phosphorylates CHK2, promoting its transient homodimerization (Matsuoka et al., 2000). CHK2 was also shown to be able to dimerise and self-activate independently of ATM when overexpressed. However, such an activation occurs partially and full activation of CHK2 requires phosphorylation by ATM (Schwarz et al., 2003). As such, CHK2 phosphorylation has been used extensively as a marker for ATM activation (Blackford & Jackson, 2017). Enigmatically, ATR has also been shown to be able to phosphorylate the same serine residue in vitro (Shang et al., 2014). Suggesting ATR could be a potential activator of CHK2.

In order to balance CHK2 activity, other molecules can antagonise its initiation. For instance, WIP1 can dephosphorylate CHK2 in DDR to suppress its activation (Freeman et al., 2010; Fujimoto et al., 2006; Oliva-Trastoy et al., 2007). Within undamaged cells WIP1, PP2A, PP1 phosphatases can antagonise ATM/CHK2 induction (Carlessi et al., 2010). When DNA damage demands enhanced CHK2 activity, it is suspected that phosphorylation by ATM brings forth full CHK2 activation and in this stage CHK2 is less sensitive to dephosphorylation by phosphatases such as WIP1. Ubiquitination of CHK2 is also important for its function as its turnover is reliant on it as well as presence of MDM2 (Kass et al., 2009). Phosphorylation of CHK2 on S456 prevents this ubiquitination, thus is needed for its activation in DDR (Bohgaki et al., 2013). Moreover, ubiquitination of CHK2 by CUL1 has been implicated in CHK2 mediated apoptosis (Stolarova et al., 2020). Another antagonist of CHK2 activation is PLK1. Together in a complex with 53BP1, PLK1 can phosphorylate CHK2 and prevent its activation in mitosis. Most curiously, CHK2 has been shown to colocalise to centromeres with PLK1 during mitosis (Chouinard et al., 2013). This suggests that CHK2 may have roles in faithful chromosome segregation, CIN and aneuploidy, yet the mechanistic significance of this colocalization remains under investigation. On the other

hand, a more recent paper suggests that the activity of CHK2 can be dispensable in aneuploidies (Zerbib et al., 2023).

Canonically, CHK2 is mentioned within the ATM-CHK2-TP53 pathway against DSBs (Falck et al., 2001; Matsuoka et al., 1998). MRN complex localises to the site of DSBs and recruits ATM which in turn activates CHK2 and cascades the DDR (Paull, 2015). CHK2 not only activates downstream effectors against DSBs but also shares substrates by ATM and thus can also amplify ATM signals and improve DDR regulation. Interestingly, ATR-CHK1 also has overlapping targets with CHK2, underscoring the intimacy between ATM-CHK2 and ATR-CHK1 networks (Bartek et al., 2007; Neizer-Ashun & Bhattacharya, 2021). Concurrently, other studies have shown that knockout of *Chek2* yield viable and fertile mice, insinuating that Chk2 duties are redundant and can be partially compensated by Chk1 (Niida et al., 2010; Takai et al., 2002). Further downstream in the pathway, CHK2 phosphorylates TP53 and activates it (Cheng & Chen, 2010). Additionally, CHK2 can phosphorylate MDM2 and disrupt its coupling with TP53 which enhances the stabilisation of TP53 further. Moreover, CHK2 can phosphorylate and inhibit CDC25A phosphatases, allowing checkpoint activation. However, for longer arrest of the cell cycle, senescence or apoptosis, TP53 activation is required (J. Chen, 2016). Phosphorylation of CDC25A induces dephosphorylation of CDK2 and halts cell cycle before S-phase (Falck et al., 2001). Furthermore, CHK2 can phosphorylate CDC25C which prevents CDK1-cyclinB activation, thus preventing mitotic entry (Matsuoka et al., 1998). On the other hand, normal levels of CHK2 were proven to be insufficient to cause G1 arrest and its overexpression is required to drive checkpoint activation (Jack et al., 2002). Lastly, CHK2 is also implicated in autophagy in response to oxidative stress via Beclin1 phosphorylation (Q. Guo et al., 2020). Overall, CHK2 exerts influence on many cellular processes, most notoriously in DSB repair and failing that, apoptosis via TP53. Intriguingly, it is highly expressed in proliferative cells such as embryogenic cells. Moreover, it has been shown to colocalise to centromeres, thus *CHEK2* presents an interesting gene for this study. While CHK2 has been shown to be dispensable in aneuploidies, lack of apoptosis

and/or senescence in aneuploid embryos warrant investigations into *CHEK2* expression levels.

BRCA1. *BRCA1*, alongside its close associate *BRCA2* represent two very common DDR genes. Together, they are the two most notorious genes mutated in breast and cervical cancers (Y. Chen et al., 1996). *BRCA1* plays a key role in various crucial cellular processes such as cellular growth, apoptosis, DDR, cell cycle progression and transcriptional regulation (Deng & Brodie, 2000; Gudmundsdottir & Ashworth, 2006).

BRCA1 is a gene comprised of 24 exons encoded on chromosome 17 (Hall et al., 1990). BRCA1 is a relatively large protein with many domains. Among those, its BRCT domain is conserved in many DDR networks. Furthermore, BRCA1 BRCT and RING domains are commonly altered in majority of the clinically significant *BRCA1* mutations as they are crucial in its contribution to HR in DSBs (Chabanon et al., 2021; Fu et al., 2022; B. Wang, 2012). BRCA1 works intimately with other DDR agents such as ATM, TP53, RAD50, RAD51 and many others to carry out most of its functions. For example, BRCA1-PALB2-BRCA2 complex is a prominent recruiter of RAD51 which in turn drives damage repair at DNA breaks (Ducy et al., 2019; Sy et al., 2009).

The balance between HR and NHEJ in the repair of DSBs can influence GIN within a cell (Chapman, Taylor, et al., 2012; Dever et al., 2011; Dimitrov et al., 2013; Y. Hu et al., 2011, 2014; Sonoda et al., 1998; Taouis et al., 2023; Vohhodina et al., 2020). While DSBs themselves can create GIN and chromosomal aberrations, too low or too high levels of HR can also cause GIN (Blackford & Jackson, 2017; Dever et al., 2011; Dimitrov et al., 2013; Her & Bunting, 2018; Y. Hu et al., 2011, 2014; Kieffer & Lowndes, 2022; Sonoda et al., 1998; Taouis et al., 2023; Vohhodina et al., 2020). BRCA1 is a regulator of HR in different ways. Firstly, BRCA1 and 53BP1 balance is a key regulator of HR response (Bunting et al., 2010). Secondly, BRCA1/CtIP complex is a promoter of DNA end resection which can allow ATR recruitment and in turn can enhance HR response (Adams et al., 2006; Fu et al., 2022; R. X. Huang & Zhou, 2020; Jazayeri et al., 2006; Maréchal & Zou, 2013; Myers & Cortez, 2006; G. Peng et al., 2012; Sartori et al., 2007;

Shiotani & Zou, 2009; Tomimatsu et al., 2012; Vohhodina et al., 2020). Interestingly, BRCA1 also prevents excess resection and thus unnecessary HR response. Therefore, BRCA1's role in HR appears to be as a regulator as opposed to being an enhancer or an inhibitor (R. X. Huang & Zhou, 2020; Vohhodina et al., 2020). Furthermore, mutations or absence of *BRCA1* has been shown to cause a shift towards NHEJ in the repair of DSBs (Narod & Foulkes, 2004; Zhong et al., 1999). As NHEJ constitutes an error-prone pathway, this fluctuation towards its use was posited to be a cause of chromosomal instability and a key step in carcinogenesis. Moreover, a combination of *BRCA2* and *RAD51* mutations were shown to result in similar phenotypic outcomes, suggesting that these three genes are part of the same pathway that regulate chromosome stability (Narod & Foulkes, 2004). In addition, BRCA1, BRCA2 and RAD51 levels were found to be elevated during S-phase, prompting a hypothetical role during or after DNA duplication and hence in preserving chromosome stability. Later, BRCA1 was confirmed to play a part in the maintenance of stressed replication forks as well as restart of the fork once necessary repairs have been carried out (Hatchi et al., 2015; Long et al., 2014; Morris & Solomon, 2004; Schlacher et al., 2012). On the other hand, BRCA1 can interact with a myriad of proteins apart from BRCA2 and RAD51. Very notably, ATM, CHEK2, RAD50, BLM helicase and MRN complex were all identified as members of the BRCA1 associated surveillance complex (BASC). Being comprised of many DNA repair proteins, BASC is involved in the detection and repair of DNA errors. Interestingly, the same study excluded BRCA2 and RAD51 from BASC (Y. Wang et al., 2000). Finally, BRCA1 has been implicated in R-loop induced DNA damage which are normal intermediates of RNA/DNA hybrid structures during transcription. While natural steps, unresolved R-loops can threaten the integrity of the genome (Gorthi et al., 2018; Hatchi et al., 2015; Herold et al., 2019). Increasing levels of R-loops at halted transcription sites were further observed in Ewing's sarcoma and neuroblastomas (Gorthi et al., 2018; Herold et al., 2019). BRCA1 was observed in the recruitment of senataxin and consequently resolving R-loops, effectively decreasing R-loop induced DNA damage (Hatchi et al., 2015). Association of BRCA1 in resolving R-loops may also suggest an indirect association with aneuploidy and ATR at

the centromeres which was shown to localise to the centromeres via R-loops (Kabeche et al., 2018). However, there is no conclusive evidence showing BRCA1 localisation at centromeres, thus this prospect remains a mere hypothesis at the moment.

Similar to ATM and ATR, BRCA1 is also a pertinent cell cycle checkpoint regulator. It has been shown to play active roles in controlling G1-S and G2-M checkpoints (Hakem et al., 1997; Larson et al., 1997; Ruffner & Verma, 1997; S. X. Shen et al., 1998; Somasundaram et al., 1997; Vaughn et al., 1996; X. Xu et al., 1999). Furthermore, its role in G2-M checkpoint may require phosphorylation by ATM and ATM deficiency appears to yield similar phenotypes as BRCA1 deficiency with regards to G2-M checkpoint and DNA damage (Barlow et al., 1997; X. Xu et al., 1999). Moreover, disruption of BRCA1 and BRCA2 show survival despite heavy GIN as checkpoint activity and apoptotic drive in these cells is heavily impaired similar to loss of TP53 and p21 (Gretarsdottir et al., 1998; Tirkkonen et al., 1997; Wessels et al., 2002). Survival of genomically unstable cells can be the precursor to carcinogenesis and aneuploidies. In addition to being a cell cycle checkpoint regulator BRCA1 is associated with centrosome duplication and thus may play a prominent role in aneuploidy cases (Deng & Brodie, 2000; Piemonte et al., 2021; Vodicka et al., 2023).

Lastly, *Brca1* mutations have been shown to be embryonically lethal in mice during gestation. All the more, embryos with *Brca1* deficiencies showed various structural and numerical chromosomal abnormalities (Gowen et al., 1996; Hakem et al., 1996; C. Y. Liu et al., 1996; Ludwig et al., 1997; S. X. Shen et al., 1998). These consequences of *BRCA1* loss may be dependent on its interactions with *BRCA2* and *RAD51* as nullification of these two genes result in phenotypically similar outcomes (Tavtigian et al., 1996; Wooster et al., 1995). Furthermore, a hypothesis suggests that mutations of *BRCA* genes allow survival in breast and ovarian cancer precursor cells. However, carcinogenesis initiation then necessitates oestrogen presence, which may in part explain why BRCA1 and BRCA2 are most commonly seen in those two cancers in comparison to other neoplasms (Elledge & Amon, 2002; Gompel et al., 2000; Narod & Foulkes, 2004; Scully & Livingston, 2000). Oestrogen has anti-apoptotic properties and is supplied in excess during most ART

procedures which may be a factor that enables the survival of aneuploid embryos.

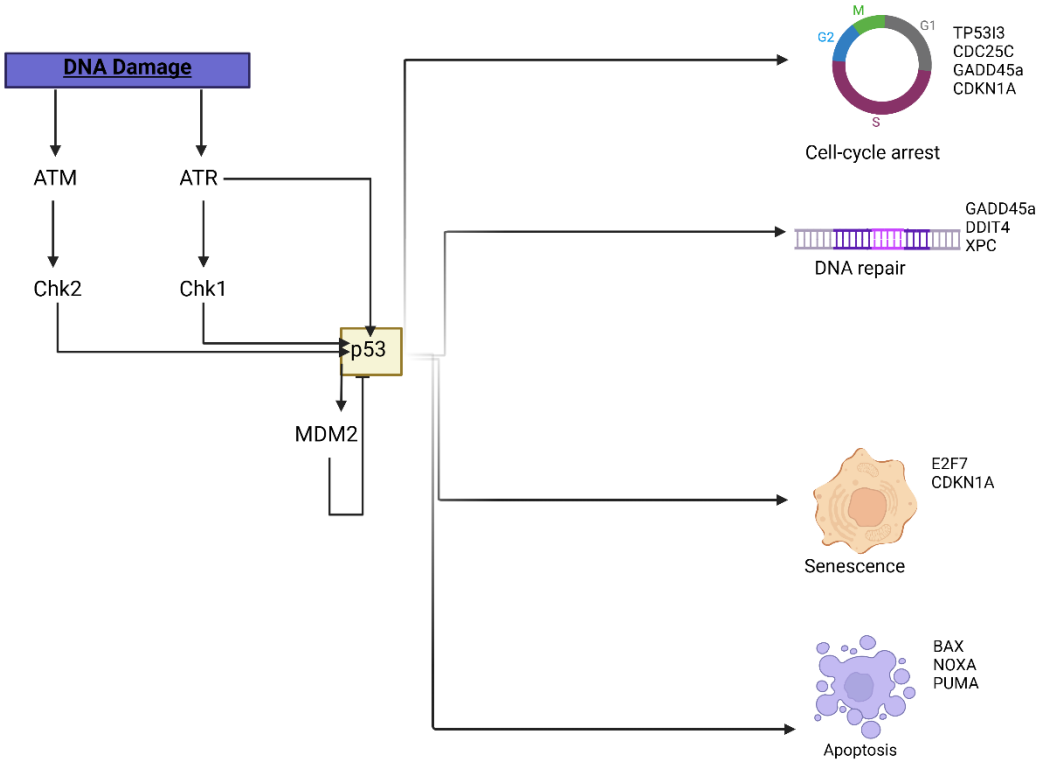
TP53. From a certain point of view, TP53 needs no introduction as it has been revered as the ‘’guardian of the genome’’ for many years (D. P. Lane, 1992) TP53, formerly called p53, has been researched in cancer genetics very extensively as it plays a major role in almost all cancers. Furthermore, *TP53* mutations were observed in all aneuploid tumours and such mutations are associated with poorer cancer prognosis, solidifying the tumour suppressor properties of TP53 (McClure et al., 2023; Narkar et al., 2021; Oltmann et al., 2018). Intriguingly, TP53 seems to cause aneuploidy without without disrupting the SAC, not unlike ATR (Schjølberg et al., 2009).

TP53 is a transcription factor that is best known as a tumour suppressor. However, it has more recently discovered roles in cell differentiation and aging as well (Hernández Borrero & El-Deiry, 2021; Jain & Barton, 2018). Moreover, TP53 is activated by various stress responses such as DNA damage, replication stress or instigation of oncogenic pathways within the cells (Kastenhuber & Lowe, 2017). In this TP53 activation process ATM, ATR, alongside their effector kinases CHEK2 and CHEK1 respectively, play prominent parts. Furthermore, MDM2 is a regulator and a target of TP53, thus regulating TP53 in a negative feedback loop manner (Hernández Borrero & El-Deiry, 2021). Collectively, these pathways enable TP53 to interact with downstream proteins such as CDC25C, E2F7, DDIT4 and leads to cell cycle arrest, DDR and senescence. Furthermore, TP53 can also interact with mitochondrial proteins such as BAX and BAK to trigger apoptotic responses (Hernández Borrero & El-Deiry, 2021) (Figure 6).

Figure 6

Summary of TP53 activation, and resultant cellular actions.

TP53 induction can occur under DNA damage conditions via ATM/ATR related pathways. In response, the cellular cycle is halted to allow DNA repair. However, when this fails, senescence and apoptosis of the cell are likely outcomes.



While the role of TP53 in tumorigenesis and aneuploid tumours is nothing de novo and is well-known, it is hardly the only field TP53 is implicated in. More recently, TP53 was shown to be associated with neurodegenerative disorders (Caneus et al., 2018). Mutant Tau (MAPT) is considered to be the main instigator of many neurodegenerative disorders such as frontotemporal lobar degeneration (FTLD) and Alzheimer's disease. In both, neuronal aneuploidy appears to be a common feature. However, in FTLD blocking of the cell cycle via activation of TP53 seems to alleviate aneuploidy (Caneus et al., 2018; Potter et al., 2019). Therefore, the role of TP53 in aneuploidies appears to be a preventative one. Mainly, TP53 is

considered to initiate apoptotic responses in opposition to a variety of cellular perturbations (Aylon & Oren, 2011; Hafner et al., 2019; Kastenhuber & Lowe, 2017; Mello & Attardi, 2018; Mijit et al., 2020; Narkar et al., 2021; Reinhardt & Schumacher, 2012). Interestingly, a more recent paper showed that TP53 does not act as a universal watcher for aneuploidies. Furthermore, the same study suggested that TP53 can influence faithful chromosome segregation directly or indirectly and mainly by minimising mitotic errors (Narkar et al., 2021). Inhibition of *TP53* seems to induce lagging chromosomes in anaphase and formation of multipolar spindles, both common precursors to CIN and aneuploidies (Artegiani et al., 2020; Drost et al., 2015; Kirsch-Volders et al., 2002; Lens & Medema, 2018; Narkar et al., 2021; Saunders et al., 2000; Shi & King, 2005; Steigemann et al., 2009). Moreover, this loss of *TP53* does not seem to alter SAC function. Coupled with prior research this suggests that TP53 influences aneuploidy independent of SAC (Narkar et al., 2021; Schjølberg et al., 2009). However, recent literature suggests that induction of aneuploidy does not always equate to TP53 activation and subsequent G1 arrest. Indeed, such responses can vary in a cell-line dependent manner (Narkar et al., 2021).

Finally, TP53 was established to have a direct impact on embryo implantation and pregnancy maintenance, thus can be significant in ART procedures. Relevance of TP53 in embryogenic contexts is thought to stem from its protective role in genomic stability (W. Hu, 2009). Interestingly, TP53's role in implantation appears to be independent of its tumour suppressor roles. In addition, TP53 within the context of embryo implantation was shown to be induced by leukaemia inhibitory factor (LIF) (Z. Feng et al., 2011; W. Hu et al., 2007; H. J. Kang & Rosenwaks, 2018). On the other hand, the effect of TP53 polymorphisms on IVF outcomes remains contradictory (Kay et al., 2006; Mohammadzadeh et al., 2019; Palomares et al., 2021). Additionally, there is a clear association between LIF induced TP53 response and embryo implantation, however the molecular mechanisms surrounding these interactions remain to be unveiled (J. R. Chen et al., 2000; Palomares et al., 2021).

MDM2. In the current literature, most information on MDM2 pertains to its capacity to moderate p53. MDM2 is an E3 ubiquitin ligase that is best known for ubiquitinating p53, thereby targeting it for degradation (Honda et al., 1997). As MDM2 mainly functions by downregulating TP53 activity, its overexpression and subsequent excessive inhibition of TP53 has been the perpetuator of various anomalies such as lymphomas, increased frequencies of chromosome and chromatid breaks and/or aneuploidies, elevated proliferation and loss of TP53-mediated apoptosis (P. Wang et al., 2007). MDM2 can also block the transactivation function of TP53 (Momand et al., 1992). MDM2 was further proven to facilitate SUMOylation and nuclear export of TP53 which in turn drives its degradation by the proteasome (Konopleva et al., 2020). Collectively, these studies show that MDM2 mediated inhibition of TP53 proceeds via two distinct pathways, on the one hand via proteosomal degradation and on the other, by blocking the transactivation domain of TP53 (Konopleva et al., 2020; Sciot, 2021). Furthermore, MDM2 is a direct transcriptional target of TP53, thus it forms an autoregulatory feedback loop to regulate the expression of TP53 (Barak et al., 1993; Karni-Schmidt et al., 2016; Vassilev, 2007; X. Wu et al., 1993). Activators of TP53, such as irradiation, hyperproliferative signals and hypoxia also upregulate MDM2, thereby triggering the feedback control that keeps TP53 response in check (Levine et al., 2006). Dampening of TP53 activity is critical for survival as *Mdm2* null mice were observed to show embryonic lethality which was rescued by simultaneous knockdown of *Tp53* (Shangary & Wang, 2009). Abolishing the MDM2- TP53 binding yielded identical results (Chinnam et al., 2022). In B-cells, elevated levels of MDM2 confer proliferative, survival advantage and CIN, paving the way for tumorigenesis (P. Wang et al., 2007). Interestingly, increased MDM2 expression in pre-B-cells also presaged aneuploidy and loss of chromosomes. On the other hand, substitute chromosomal abnormalities such as chromosomal breaks and fusions were not observed in these cells (P. Wang et al., 2007). Oddly, *MDM2* overexpression in mature B-cells caused a reversed profile, where aneuploidy levels failed to show a significant skew but chromosome/chromatid breaks were a lot more common (P. Wang et al., 2007). Other effects of *MDM2* overexpression mainly arise due to excess

TP53 inhibition, culminating in outcomes such as decreasing apoptotic responses, downregulation of other cell-cycle inhibiting genes like p21. In contrast, apoptotic pathways unrelated to TP53 activity showed no alterations (P. Wang et al., 2007). The exact mechanism of how *MDM2* overexpression leads to chromosome breaks and aneuploidy remains elusive but TP53 activity is one of the suspected pathways.

Even though, they are not as well-known as TP53- dependent functions, TP53-independent roles of MDM2 can be of paramount importance. For instance, MDM2 was shown to interact with NBS1 of the MRN complex and thus *MDM2* overexpression degenerates DSB repair (Alt et al., 2005). As discussed more in depth in the next section, this can be a potential path to GIN and aneuploidy. More recent studies have also pointed towards the importance of the TP53-independent roles of MDM2 in oncogenic contexts via the ubiquitination of various transcription factors like ATF, E2F and others (Fridman et al., 2003; S. N. Jones et al., 1998; Sciot, 2021; Sigalas et al., 1996). Other studies corroborate and expand on this further. MDM2-NBS1 interactions appear to stunt DSB repair leading to CIN and aneuploidy irrespective of TP53 yet in contrast NBS1 and ATM are indispensable in this context unlike the ubiquitin ligase activity of MDM2. Additionally, abolishing MDM2-Nbs1 interaction precludes MDM2 from delaying phosphorylation of H2AX and ATM-S/TQ sites, thereby delaying repair of DSBs and resolution of DNA foci (Bouska et al., 2008; St & Hall, 2020). Mutated *Mdm2* in mouse embryogenic fibroblasts presented G2-M transition anomalies and increased aneuploidy in the absence of TP53. Suggesting that MDM2 can influence cell-cycle and GIN separately from p53 (Chinnam et al., 2022). Interestingly, the E3 ligase activity of MDM2 was found to be indispensable both for TP53 driven G1 cell cycle arrest and for TP53 independent G2-M transition in the same study (Chinnam et al., 2022). Moreover, knockdown of *MDM2* was associated with elevated hyperploidy which was attributed to the weakened G2/M response which can propel re-replication of DNA (Chinnam et al., 2022). Finally, the same study also denoted that in the absence of *MDM2* embryonic fibroblasts fail to mount a TP53 mediated response against DSBs. Insinuating that MDM2 may not just

be a repressor of TP53 but is also crucial for a normal TP53 transcriptional response (Chinnam et al., 2022).

Other members of the DDR pathway can also interact with and/or influence MDM2 activity. For example, retinoblastoma (Rb) protein is a prominent gene in apoptosis and was shown to be inhibited by MDM2 to promote cell proliferation independent of TP53 (Z. X. Xiao et al., 1995). More recent studies also showed that ATM/ATR-CHK2/CHK1 activity can abolish or at least reduce TP53-MDM2 interactions, thus stabilising TP53 (Y. Zhou et al., 2023). Intriguingly, MDM2 was shown to suppress E2F1/DP1 driven apoptosis and stimulate DP1 induced DNA synthesis and proliferation (Loughran & Thangue, 2000). Overexpression of *Mdm2* in mice mammary glands was shown to promote S-phase without mitosis, thus generating polyploid cells (Lundgren et al., 1997). On the other hand, MDM2 is important for TP53 regulation as it can bind to TP53 mRNA and reinforce its translation together with ATM (Candeias et al., 2008; Chinnam et al., 2022; Gajjar et al., 2012).

Corroboratively, these studies suggest that MDM2 can temper TP53's growth supporting effects yet is also important for its normal translation and function. Furthermore, MDM2 can promote cell proliferation independently of TP53. TP53 is implicated in self-renewal, differentiation and reprogramming what is more loss of TP53 activity seems to induce stem cell reminiscent profiles in varying cancers (Mcconnell et al., 2016; Spike & Wahl, 2011). As a major modulator of TP53, MDM2 can indirectly lead to similar outcomes. Moreover, mono-ubiquitination of TP53 by MDM2 induces its translocation to mitochondria where HAUSP deubiquitinates TP53 to drive apoptosis, thereby drawing a connection between MDM2 and apoptosis (Marchenko et al., 2007). As previously stated, *MDM2* overexpression is associated with a delayed DSB repair response (Bouska et al., 2008; St & Hall, 2020). Therefore, *MDM2* is negatively correlated with DNA repair and genomic stability (Bouska & Eischen, 2009). Despite this, MDM2 and E2F1 were also shown to interact with the MRN complex via NBS1 and drive DSB repair (Manickavinayaham et al., 2021). MDM2 bound to NBS1 was shown to colocalise to the sites of DSBs, thus implying that MDM2 can be a promoter or a hindrance to DSB repair in a dosage-dependent manner. Moreover,

independently of TP53, MDM2 is associated with supplementing apoptosis, direct facilitation of DNA synthesis, cell proliferation and propagating invasiveness. Finally, ARF is posited to inhibit such tumorigenic properties by dampening the TP53-independent interactions of MDM2 (Y. Zhou et al., 2023).

Overall, MDM2 can influence DSB repair, apoptosis, CIN and aneuploidy via TP53 or irrespective of TP53. However, the precise pathways of MDM2's influence on such outcomes appear to be somewhat context-dependent and are not fully understood. Therefore, *MDM2* remains a gene of interest in a lot of research endeavours.

RAD50. As mentioned in previous sections, the MRN complex plays an important role in the repair of DSBs. MRN complex is comprised of MRE11, RAD50 and NBS1. Among the three, RAD50 is situated at the core of the complex and is held together by MRE11 (Y. Liu et al., 2016; Schiller et al., 2012; Sung et al., 2014). RAD50 itself is an ATPase belonging to the structural maintenance of chromosome (SMC)-like protein class (Hoencamp & Rowland, 2023; Kinoshita et al., 2009; Syed & Tainer, 2018). SMC-like proteins are typically involved in organising DNA to assure proper chromosome function. Furthermore, they also possess roles in organising DNA for repairs, which is a trait demonstrated by RAD50 (Kinoshita et al., 2009). Not surprisingly, RAD50 within the MRN complex is necessary for DSB repair. Indeed, disruption of any of the MRN complex members appears to abrogate cell viability and has been shown to induce embryonic lethality (G. Luo et al., 1999; Y. Xiao & Weaver, 1997; J. Zhu et al., 2001). Intriguingly, different members of the MRN complex can be more prominent in unique pathways. For instance, RAD50 appears to be more directly associated with DDR as opposed to cell cycle checkpoints where NBS1 shows a more intimate connection.

The MRN complex and in particular MRE11 and RAD50 are highly conserved among living organisms ranging from archaea to higher eukaryotes (Connelly & Leach, 2002). Moreover, RAD50 appears to be sufficient for DNA scanning and tethering, but MRE11 is required to recognise the DNA ends and exert exo-/endo-nuclease activity, supporting

both of their highly conserved nature (Reginato & Cejka, 2020; Zabolotnaya et al., 2020). The MRN complex is a key initiator is a key initiator of various pathways such as driving initial and sustained responses to DSBs, stalled replication forks, dysfunctional telomeres and viral DNA infections (Syed & Tainer, 2018). Interestingly, NBS1 of the MRN complex is only conserved in eukaryotes. However, any loss or mutations in *MRE11*, *RAD50* or *NBS1* lead to severe defects in humans like ataxia telangiectasia-like disease and/or Nijmegen breakage syndrome (Delia et al., 2004; Fernet et al., 2005; G. Luo et al., 1999; Matsumoto et al., 2011; Miyamoto et al., 2014; Ragamin et al., 2020; Stewart et al., 1999; Stracker & Petrini, 2011; Uchisaka et al., 2009; Y. Xiao & Weaver, 1997; J. Zhu et al., 2001). Presence of ataxia telangiectasia-like syndrome when MRN complex subunits are altered is unsurprising as the MRN complex is a prime recruiter of ATM during DSB response. Additional studies also showed that mutated RAD50 can't oligomerise, thus fails to activate Tel1 (orthologue of human ATM) (Kissling et al., 2022). Overall, the MRN complex is essential for genomic stability and it is frequently absent in epithelial, ovarian cancer patients (Brandt et al., 2017). The MRN complex has roles in DNA damage sensing, NHEJ and HR. Moreover, the activity of the MRN complex can determine the choice between NHEJ and HR pathways in the repair of DSBs. MRN achieves this level of action mainly by activating ATM and ATR cascades (Falck et al., 2005; Maréchal & Zou, 2013). Other studies have also shown that the MRN complex can interact with BRCA1 as well, further solidifying its role in DDR (Lafrance-Vanasse et al., 2015; G. J. Williams et al., 2010).

Molecularly, MRE11 forms the core of the MRN complex, but the largest subunit in the complex is RAD50 (Hopfner et al., 2000; Lafrance-Vanasse et al., 2015). Upregulation of RAD50 has been shown to increase radio-resistance whereas its knockdown sensitises cells to radiation most likely due to changes in the DSB repair levels (Y. Wang et al., 2018). Zinc-hook and coiled coil domains are critical for functional RAD50-DNA tethering and subsequent ATM activation (Barfoot et al., 2015; J. He et al., 2012; Roset et al., 2014). ATM-MRN interactions are regulated by antagonistic balance of binding of ATMIN, MRNIP and PLK1 (Christopher Staples et al., 2016; Z. Li et al., 2017; T. Zhang et al., 2012, 2014). RAD50 undergoes conformational

changes in an ATP-dependent manner. These changes to its structure are necessary in order to make DNA available/unavailable to the MRN complex (Deshpande et al., 2014). Furthermore, correct interactions between RAD50-ATP-MRE11 are a must for DSB processing (Acharya et al., 2008; G. J. Williams et al., 2011). ATP hydrolysis opens RAD50 and allows MRE11 to bind to the DNA.

As mentioned previously, the activity of the MRN complex can alter the choice of DSB repair mechanism that will be employed between NHEJ and HR. Additionally, depending on the type of NHEJ that will be carried out, MRN may be required to employ different molecules and resection pathways (Biehs et al., 2017; Dutta et al., 2017; Mahaney et al., 2009; Sfeir & Symington, 2015; Sharma et al., 2015). On the other hand, the MRN complex can also initiate HR as well as interact with BRCA2, RAD51, ATR to halt cell cycle and repair replication forks. Strikingly, HR is implicated in replication fork recovery but in this context, it is considered to operate independently of DDR against DSBs (Carr & Lambert, 2013). During de-stressing of replication forks HR can restore robustness but can also induce GIN as the mechanism relies on a homologous sequence intrinsically (Carr & Lambert, 2013). Furthermore, uncoupling of helicase and polymerase at replication forks creates extended stretches of single stranded DNA (ssDNA) which triggers ATR induced checkpoint response (Zeman & Cimprich, 2014). ATR activation can also be induced by RAD50 (Gatei et al., 2014). Moreover, the MRN complex can assist in ATR-CHK1 phosphorylation and it has been shown that loss of MRN decreases but not completely eliminates CHK1 phosphorylation (J. Lee & Dunphy, 2013). ATR pathway can in turn regulate the temporal sequence of MRN activities at replication forks, thereby enabling a positive feedback loop that protects chromosome stability of cells. Defective MRN formation and resection at replication fork stress sites can lead to repair attempts via NHEJ which can contribute to GIN.

Telomeres are natural protectors of chromosome ends against DSB DDR and suppress replication instability (X. D. Zhu et al., 2000). Dysfunctional telomeres and/or shelterin defects at telomeres can enable MRN recruitment at chromosome ends and can lead to aneuploidy within a few cycles of replication (Déjardin & Kingston, 2009). Conversely, MRN

complex can also inhibit GIN at dysfunctional telomeres by initiating NHEJ. However, this inhibition appears to be context dependent as this NHEJ initiation was also shown to be able to cause GIN (Marcand, 2014).

Next, in the absence of RAD51, an important DSB repair member, MRE11 was shown to degrade DNA where the resulting fragments can be released into the cytoplasm and trigger innate immune responses (Bhattacharya et al., 2017; X. Liu et al., 2013). Furthermore, RAD50 is known to form a complex with the innate immune system adaptor protein CARD9 in dendritic cells, consequently activating NF-Kβ pathway (Roth et al., 2014). Studies in yeast also showed that the MRX complex (yeast equivalent to MRN in higher eukaryotes) can induce replicative senescence in conjunction with Tel1 (Ballew & Lundblad, 2013). In turn, ATM can promote MRX recruitment whereas molecules such as RIF2 can negatively regulate this process by ATP hydrolysis, thereby opening RAD50 and releasing the MRX complex from DNA (Cassani et al., 2016). In the more recent years, the MRX complex was shown to have a role in chromatin organisation and transcript regulation (Forey et al., 2021). Lastly, Simonetti and colleagues (2016) compared euploid and aneuploid acute myeloid leukaemia cells and reported higher mutation rates in *PLK1*, *CHK2*, *CDC20*, *BUB1B* and RAD50 in aneuploid cancers. The authors further argued that the disruptions in these key DDR and checkpoint genes alongside *TP53* mutations leads to forced progression through mitosis resulting in aneuploid daughter cells (Simonetti et al., 2016).

RAD51. RAD51 is best known for its strand invasion and exchange ability. Interestingly, while this canonical role of RAD51 is crucial for DDR, in particular the HR response, most *RAD51* mutations are not cancer-prone (M. Thomas et al., 2023). However, much like other HR genes such as *BRCA1*, *RAD51* is also observed in Fanconi anaemia which presents with spontaneous chromosomal instability (Ameziane et al., 2015; Auerbach, 2009; Geilmann et al., 2023; Takenaka et al., 2019; A. T. Wang et al., 2015). While most *RAD51* mutations do not show intrinsic tumorigenicity, in various cancers both increase and decrease in *RAD51* expression levels as well as levels of HR have been reported. Particularly in breast cancers a reduction in

RAD51 expression was observed (Yoshikawa et al., 2000). Nevertheless, in other cancers RAD51 levels appear to be elevated (Maacke, Jost, et al., 2000). In their study with mouse derived cell lines comparing overexpression of *Rad51* and reduced *RAD51* activity, Bertrand et. al. (2003) reported that hypo-*RAD51* cells showed higher tumorigenicity, centrosome duplication defects and subsequent aneuploidy compared to both hyper-*RAD51* and control groups. Moreover, dominant negative *RAD51* expression significantly elevated the levels of centrosome fragmentation irrespective of irradiation. The authors attributed these changes to RAD51's roles outside of the HR response yet associated with its canonical role in recombination (Bertrand et al., 2003). On the other hand, *RAD51* perturbations are more tightly associated with centrosome amplification rather than centrosome fragmentation (Date et al., 2006). Defects in centrosome amplification have long been correlated with aneuploidies seen in cancers and many tumour suppressor genes have been implicated in centrosome amplification defects and subsequent aneuploidies (Fukasawa et al., 1996; Harvey et al., 1993; Lingle et al., 2002; Pihan et al., 2001; Tutt et al., 1999; H. Zhou et al., 1998). Concurrently, BRCA1 shows localisation at centrosomes and ATM exerts influence on centromeric amplification in RAD51-deficient cells (Dodson et al., 2004; Hsu & White, 1998). These studies further underscore the importance of *RAD51* in aneuploidies and its close interactions with other DDR genes. The role of *RAD51* in DDR indeed is so prominent that formation of RAD51 foci is considered a marker of sites of DSBs and the ongoing repair processes (Lisby et al., 2003; Maser et al., 1997).

As a member of various pathways that are necessary for cell survival, it is not surprising that RAD51 is beholden to a multitude of regulatory mechanisms. Firstly, its expression can be regulated at the transcriptional level. Secondly, many post-translational modifications including the formation of active monomer structures, cellular localisation, phosphorylation, SUMOylation, ubiquitination, cleavage and degradation may alter the cellular activity of RAD51 (Daboussi et al., 2002). Other proteins can directly bind to and manipulate RAD51 activity, such as p51 (Buchhop et al., 1997; Stürzbecher et al., 1996). It has been posited that TP53 can downregulate RAD51-mediated HR reactions by de-escalating RAD51's strand exchange

and replication fork regression schemes (Yoon et al., 2004). Furthermore, ATM and other notable DDR members like BRCA1, BRCA2, ATR, RPA, Chk1 and Chk2 have been shown to be able to interact with and recruit RAD51 for HR (Richardson, 2005). As mentioned previously, *RAD51* gene and its expression levels were found to be altered in multiple cancers (Ben-Yehuda et al., 1996; Blasiak et al., 2003; Goode et al., 2002; Jakubowska et al., 2003; Raderschall, Bazarov, et al., 2002; Raderschall, Stout, et al., 2002; Schoenmakers et al., 1999; Seedhouse et al., 2004; W. W. Wang et al., 2001; Wick et al., 1996; Xia et al., 1997). In most of these carcinogenic formations, *RAD51* levels appear to be elevated. During unperturbed apoptosis, RAD51 is cleaved by caspase3. In tandem, elevated levels of *RAD51* in cancers may point towards an aversion of apoptotic signals (Flygare et al., 1998; Y. Huang et al., 1999; Kanamoto et al., 2002; Saintigny et al., 2001). Elevated levels of *RAD51* have been associated with other cellular anomalies as well. Firstly, increased *RAD51* levels were connected to interchromosomal HR-mediated repair that can result in translocation and aneuploidies (Richardson et al., 2004). Independently of HR, enhanced *RAD51* expression profile can lead to indirect disconcertion of DNA metabolism and consequent recombinogenic DNA lesions (Betti et al., 2001, 2003; Y. Huang et al., 1999). Moreover, increased *RAD51* levels can cause apoptotic escape in erroneously repaired cells due to its aberrant interactions with TP53, p21 and BCL2 (Raderschall, Bazarov, et al., 2002; Saintigny et al., 2001; Slupianek et al., 2001). In addition, *RAD51* overexpression has been shown to disrupt G2/tetraploid checkpoints, resulting in aneuploidies (Borel et al., 2002; Margolis et al., 2003). Even minute climbs in *RAD51* expression patterns during DSB repair can trigger crossovers which can result in GIN, CIN, translocations and aneuploidies. Furthermore, disturbed centrosome functions due to higher *RAD51* expression have been suggested as a contributor to CIN and aneuploidies (Richardson et al., 2004). Conversely, chromosomal aberrations can also cause *RAD51* overexpression. Besides the obvious chromosome 15 aneuploidies, the fusion protein Bcr-Abl which arises from Philadelphia chromosome, has been shown to lead to constitutive *RAD51* activation, thereby establishing another causal link between CIN and RAD51, as well as DDR and HR responses

(Slupianek et al., 2001). Another interesting contrast is that reduced *RAD51* levels can also present similar outcomes as aforementioned (Bertrand et al., 2003).

RAD51's significance is also demonstrated by the fact that total loss of RAD51 is embryonic lethal. This property of RAD51 can be attributed in part to its involvement in sister-chromatid exchange (Lambert & Lopez, 2001). Additionally, RAD51 defects have been associated with premature ovarian insufficiency (W. Luo et al., 2020; M. Thomas et al., 2023). Regardless, RAD51 also possesses various non-canonical roles in the cell that contributes to its cruciality. Protection of arrested replication forks, resistance against alternate DSB repair pathways, processing of R-loops can be listed among the more recently illuminated non-canonical roles of RAD51 (W. Luo et al., 2020; Mason et al., 2019; M. Thomas et al., 2023). Non-canonical NHEJ can be a hindrance to genomic stability. Addition of RAD51 on to the resected ssDNA at DSB sites favour the use of HR as opposed to non-canonical NHEJ, thereby preventing the use of alternate DSB repair pathways and protecting genomic stability (Ahrabi et al., 2016; Han et al., 2017; Mason et al., 2019; A. So et al., 2022). Furthermore, HR is usually limited to S-G2 phases of the cell cycle. However, RAD51 loading onto DNA at G1 phase was shown to prevent alternative NHEJ and enhance centromeric stability, thus preventing chromosomal rearrangements (Yilmaz et al., 2021). Moreover, RAD51 was shown to be recruited at centromeres in quiescent cells to shield centromeres from breakage (Saayman et al., 2023). RAD51 has also been implicated in the maintenance of stalled replication forks. It can promote fork reversal independently of its strand exchange function (Mason et al., 2019). Intriguingly, this RAD51-mediated drive towards fork reversal appears to perpetuate in a BRCA2-independent fashion, enforcing the idea that RAD51 plays a non-canonical role in this context (Berti, Teloni, et al., 2020; Chaudhuri et al., 2016; Mijic et al., 2017; Zellweger et al., 2015). Furthermore, MCM8 and MCM9 were shown to favour BRCA1 and RAD51 recruitment at the stalled sites of replication forks to protect them from excess degradation, implicating that BRCA1 but not BRCA2 works collaboratively with RAD51 to preserve the integrity of replication forks (Griffin et al., 2022). BRCA1's interaction with BARD1

appears to be imperative for RAD51 recruitment to stalled replication forks in order to prevent excess resection (Berti, Cortez, et al., 2020; Daza-Martin et al., 2019). Additionally, RAD51 has also been associated with post-replicative repair, either by translesion synthesis or by template switching (Giannattasio et al., 2014; Prado, 2021).

R-loops are RNA-DNA hybrid structures that may form naturally as intermediate products. R-loops can form in cis-conformation which happens co-transcriptionally or in trans-conformation which is a consequence of the RNA transcript binding to homologous DNA at a distant locus post-transcriptionally. In mutant yeast defective for RNA biogenesis, RNA-DNA hybrid formation is dependent on RAD51 and RAD52 activity. Moreover, these hybrids have been shown to lead towards chromosome loss and terminal deletion of chromosomes (Wahba et al., 2013). Furthermore, bacterial orthologue of *RAD51*, *RecA* is known to promote DNA-RNA hybridisation (Kirkpatirck et al., 1992; Kirkpatirck & Radding, 1992; Zaitsev & Kowalczykowski, 2000). Collectively, these studies show that RAD51 possesses a highly conserved role in the formation of R-loops. RAD51-originating R-loops can form in both cis and trans conformations, but cis R-loops present a bigger threat against genomic stability (M. Thomas et al., 2023). Telomeres can also be sites of R-loop formations. Telomeric repeat-containing RNA (TERRA) is a lncRNA capable of forming R-loops at the ends of telomeres and is involved in telomere maintenance (R. Arora et al., 2014; Azzalin et al., 2007; Graf et al., 2017). Recruitment of TERRA is perpetuated by RAD51 in humans (Feretzaki et al., 2020). Accumulation of ROS represents another pathway through which R-loops can be generated. Elevated levels of ROS can yield replication stress which in turn can prime formation of R-loops at transcription sites. ROS-driven R-loops are identified by a complex of comprised of RAD51, RAD52 and CSB (Teng et al., 2018; Wilhelm et al., 2016). In contrast, ROS-induced R-loops at telomeres do not recruit RAD51 (Tan et al., 2020). This is perplexing, as RAD51 has key roles in dissolving such R-loops independently of BRCA1, BRCA2 and the HR pathway (Teng et al., 2018). Finally, abrogated ATR-CHK1 pathway can induce the accumulation of cis R-loops during transcription (Barroso et al., 2019). As mentioned previously, cis R-loops can be deleterious to genomic

stability, thus resolving these structures is paramount. Studies show that in the absence of WRN, such as in Werner Syndrome cells, actions of WRNIP1 are crucial for the removal of these structures that are the consequences of ATR pathway defects and this deed of WRBIP1 necessitates RAD51 (Basile et al., 2014; Marabitti et al., 2020). Nevertheless, centromeric recruitment of ATR also appears to be reliant on R-loops and thus the relationship between ATR, R-loops, RAD51 and CIN remains to be fully elucidated (Kabeche et al., 2018). Overall, R-loops are drivers of many important cellular procedures like gene expression but are also instigators of GIN and are associated with many diseases such as cancers and Fanconi's anaemia (Crossley et al., 2019; Richard & Manley, 2017; Skourti-Stathaki & Proudfoot, 2014). RAD51's involvement with R-loops appear to be as a regulator, as its actions can either drive the formation of R-loops or their resolution.

RAD51 has five paralogues that have arisen from ancient gene duplications of *RAD51* itself, then proceeded to evolve into regulators and enhancers of *RAD51* function (Bonilla et al., 2020). These paralogs, namely *RAD51B, RAD51C, RAD51D, XRCC2* and *XRCC3* can promote RAD51 binding to ssDNA and stabilisation of the nucleoprotein filament (Masson et al., 2001; Sigurdsson et al., 2001). RAD51 paralogues are also intertwined with CIN and GIN (Loveday et al., 2011, 2012; N. Orr et al., 2012; Pierce et al., 1999; Takata et al., 2001). Paralogs of *RAD51* are as fundamental as their progenitor to the viability of cells. For instance, fully abrogated *RAD51B* ($RAD51B^{-/-}$) has been associated with early embryonic lethality (Shu et al., 1999). Partial knockdown of *RAD51B* and its subsequent haploinsufficiency have been shown to cause HR defects, centrosome fragmentation and aneuploidy (Date et al., 2006). RAD51B, similar to RAD51 itself, is required for chromosomal stability and is implicated in sister chromatid exchange which may account for the centromeric defects and aneuploidies observed in Date and colleagues' study (Date et al., 2006). Furthermore, haploinsufficiency of *RAD51B* causes a reduction in the formation of *RAD51* foci at DSB sites, debilitating the HR response (Date et al., 2006). Downregulation of *RAD51B* and *RAD51C* has also been alluded to G2/M arrest. *XRCC3* is another paralogue of *RAD51* that has been associated with checkpoint perturbations when inhibited. *XRCC3* is a paralogue with roles

later in the HR response in the resolution of Holliday junctions, thus having critical impact on preventing aneuploidies and aberrant mitotic divisions. Similar to *RAD51*, *XRCC3* knockdown also leads to centrosome aberrations, further connecting this gene with aneuploidies. Indeed, a study by Rodrigue and colleagues (2013) demonstrated that knockdown of *XRCC3* leads to chromosome misalignments, anaphase bridges and aneuploidies, albeit with a persistent SAC function in a striking fashion. Naturally, cells with *RAD51C*, *XRCC2* and *XRCC3* deficits all fail to fully propagate the HR response, the prime directive of RAD51, evidenced by the lack of damage induced RAD51 foci formation (Bishop et al., 1998; French et al., 2002; Godthelp et al., 2002; O'Regan et al., 2001). Therefore, insinuating that these paralogs are key in homology search and strand invasion actions of RAD51, much like RAD51B.

Reduction in the expression levels of *RAD51* paralogs might lead to similar outcomes. Nevertheless, abrogation of different paralogs may also bear distinct consequences. As an example, inhibition via siRNA knockdown of *RAD51B* and *RAD51C* triggers G2/M arrest whereas *XRCC3* knockdown did not alter G2/M arrested cell numbers. In contrast, treatment with siRNAs targeting *XRCC3* gave rise to several mitotic problems that could be attributed to failures in resolving Holliday junctions (Rodrigue et al., 2013). Moreover, knockout of *XRCC3* debilitates kinetochore/microtubule attachments, thus poses a highly likely candidate to cause aneuploidies despite the SAC function persisting (Rodrigue et al., 2013). Interestingly, the authors attribute the altercations observed in *XRCC3* knockdown to its RAD51 independent functions, such as the resolving of Holliday junctions. On the other hand, similar outcomes can be observed in *RAD51* and *RAD54* inhibited cells, necessitating further studies to fully understand these interactions (Dodson et al., 2004).

PIK3ca. Phosphoinositide 3-kinase (PI3k) pathway is a well-known proliferative pathway. It mainly activates in response to pro-growth signals transmitted via receptor tyrosine kinases and other growth factor receptors (P. Hu et al., 1992; Klippel et al., 1992; Paplomata & O'regan, 2014; Rasti et al., 2022). In return, PI3k mainly activates the AKT/mTOR pathway to support cellular growth and repress apoptosis (Chalhoub & Baker, 2009; Mora et al.,

2004). PI3k protein is formed of different subunits. One of the most notable among those is its catalytic subunit p110α, also known as the PI3k catalytic subunit α which is indispensable for normal PI3k function (Verret et al., 2019). This subunit is encoded by the *PIK3CA* gene situated on chromosome 3 and is notorious in various abnormalities and cancers such as human epidermal growth factor receptor 2 (HER2)-positive breast cancers (Utermark et al., 2012; Q. Wang et al., 2015). *PIK3CA* mutations primarily lead to activation of class I PI3k pathway which is a common happenstance in many cancers. Moreover, amplification of wild type *PIK3CA* has also been intimately tied to tumorigenic outcomes such as lung squamous carcinomas (McLendon et al., 2008; Vanhaesebroeck et al., 2019). As PI3k and its downstream pathway members AKT and two mTOR complexes mTORc1 and mTORc2 are drivers of anabolic reactions, migration, cell-cycle progression as well as survival, their infamy in diverse cancers is not surprising (Bilanges et al., 2019; Fruman et al., 2017). For healthy progression of cell cycle, PI3k levels need to oscillate (S. M. Jones et al., 1999; S. M. Jones & Kazlauskas, 2001; Marqués et al., 2008; Shtivelman et al., 2002). Constitutively activated PI3k can be detrimental to the cell cycle despite its pro-growth properties and can even lead to fatal consequences for the cell (Alvarez et al., 2001; Klippel et al., 1998). Other studies also associate elevated *PIK3CA* expression or mutations to the *PIK3CA* gene with stabilisation of microtubules which when prolonged can lead to GIN which is a hallmark of tumorigenesis (Gasic et al., 2019; Hanahan & Weinberg, 2011; Onishi et al., 2007). Interestingly, in breast cancers with duplicated genomes, *PIK3CA* mutations occur prior to the onset of aneuploidy (Vanhaesebroeck et al., 2019). Furthermore, in cancers *PIK3CA* mutations appear to transpire mutually exclusive with TP53 mutations (Berenjeno et al., 2017). Collaboratively, these studies imply that *PIK3CA* mutations might be more likely to be a cause of GIN than a consequence. Moreover, *PIK3CA* mutations may be bestowing properties to pre-tumorigenic cells to tolerate genome duplications and this facet of PIK3CA seems to act independently of TP53. While PI3k pathway is disrupted in many cancers, notably, *PIK3CA* is the second most altered gene in endometrial cancers (Millis et al., 2016; Oda et al., 2005; Thorpe et al., 2014). Nevertheless, *PIK3CA* mutations do not

readily warrant poor prognosis in endometrial cancer (Bredin et al., 2023). However, the close association with endometrial tumours could imply a faint connection with infertilities and oocyte aneuploidies which can then extend to the resulting embryo.

PI3k pathway is tightly intertwined with DDR. Upregulation of PI3k pathway member proteins have been shown to amplify DDR, reduce DNA damage and enhance cell proliferation in cancer scenarios (T. T. Huang et al., 2020; Kumar et al., 2010; Toulany et al., 2008). In addition, most PI3k inhibiting chemotherapeutics act via suppression of DDR to eliminate cancers (Gil Del Alcazar et al., 2014; Juvekar et al., 2012; Kirkpatrick et al., 2013; Shortt et al., 2013). Collectively, these studies point towards an undeniable regulatory connection between PI3k and DDR. Further studies established more concrete connections between PI3k/AKT and DDR pathways, in particular with members of the DDR pathway that belong to the PIKK protein family such as ATM, ATR and DNA-PKcs (Alemi et al., 2022; Karimian et al., 2019; Yap et al., 2020). A newer study concurs by showing that reduced PI3k phosphorylation downregulates DDR and increases apoptosis in non-small cell lung cancer. Moreover, in the same study quercetin intervention, a potent PI3k inhibitor, was shown to diminish phosphorylation levels of ATM, ATR, CHK1 and CHK2 (B. Zhou et al., 2023). Another investigation also identified a contraction in BRCA1 and BRCA2 following the inhibition of PI3k, strengthening the bond between PI3k and DDR (Ibrahim et al., 2012). Additional notable member of DDR and apoptotic pathways is TP53. Inhibition of the PI3k/AKT/mTOR pathway was proven to decelerate TP53 expression levels, asserting PI3k as an influencer of TP53 (Kojima et al., 2008). Interestingly, the same PI3k/mTOR pathway inhibition also enhanced TP53-mediated mitochondrial apoptotic pathway such as BAX and BAK (Kojima et al., 2008).

The PI3k pathway has been associated with CIN. Recent research suggests that when activated in an uncontrolled manner PI3k pathway might confer CIN tolerance and thereby facilitate oncogenic growth (Vanhaesebroeck et al., 2019). Based on the level of CIN, aneuploidy may lead to cell death, senescence or impaired proliferation (Andriani et al., 2016; Oromendia & Amon, 2014). PI3k/AKT/mTOR pathway was proposed to

induce CIN as well (Vanhaesebroeck et al., 2019). Amplification of wild type *PIK3CA* or mutations to the *PIK3CA* gene leads to centrosome amplification, enhanced microtubule stability and perturbations in microtubule/cell cortex interactions, all mechanisms that can eventually lead to CIN and aneuploidies. Within the PI3k pathway, AKT and CDK2 are considered alongside PIK3CA to be involved in centrosome amplification (Berenjeno et al., 2017; Vanhaesebroeck et al., 2019). The α subunit of PI3k is known to be activated at mitosis entry and is a regulator of early mitotic events such as prometaphase progression and spindle orientation (Silió et al., 2012). In oncogenic contexts, centrosome amplification appears to be reliant on the PI3k/AKT pathway but not the MAPK pathway (H. J. Nam et al., 2010). A latter study also showed that several PI3k pathway members and substrates are situated on centrosomes, further propagating PI3k's prominence in centrosome amplification (Vanhaesebroeck et al., 2019).

PIK3CA mutations can majorly affect embryonic development and adult life (Berenjeno et al., 2017; Hare et al., 2015). However, in tumorigenesis *PIK3CA* mutations appear to be insufficient by themselves but rather they exacerbate the process in collaboration with other factors (Berenjeno et al., 2017). On the other hand, *PIK3CA* mutation or overexpressed wild type *PIK3CA* may result in supernumerary centrosomes (Berenjeno et al., 2017). In particular, p110α^{H1047R} mutation appears to cause centrosome amplification in E8.5 embryos, suggesting *PIK3CA* mutations can impact embryonic development and aneuploidy (Berenjeno et al., 2017). The same mutation was confirmed to cause tetraploidies in mouse embryonic fibroblasts. Intriguingly, the researchers attributed these tetraploidies to DNA duplications as opposed to segregation errors (Berenjeno et al., 2017). Therefore, cell-cycle checkpoint genes are anticipated to play a larger role compared to SAC genes in the presence of *PIK3CA*^{H1047R} mutation. Increased tolerance to tetraploidy in such cells was also posited within the same study since the fibroblasts did not arrest as G1 tetraploids but achieved 8N DNA content (Berenjeno et al., 2017). Inactivation of TP53 is known to be an important mechanism resulting in tolerance to genome doubling (Aylon & Oren, 2011; S. L. Thompson & Compton, 2010). As stated earlier, PI3k repression may reduce *TP53* expression, further supporting this notion

(Kojima et al., 2008). Nevertheless, in this study tetraploidy tolerance appeared disjointed from TP53 (Berenjeno et al., 2017). Overall, mutations to p110α subunit of PI3k may lead to centrosome amplification, aneuploidy and tetraploidisation. Tetraploid cells possess unstable genetic structure and are prone to accumulating numerical and structural chromosomal abnormalities, aneuploidies that may have tumorigenic consequences (Davoli & De Lange, 2011; Ganem et al., 2007).

All in all, PI3k represents an important survival pathway for cells. Various studies associate PI3k pathway with cell cycle checkpoint genes such as *ATM, ATR, CHEK1, CHEK2, BRCA1* and TP53 (B. Zhou et al., 2023). Furthermore, *AKT* is downstream of *PI3k*. *AKT* is also downstream of *ATM* and *ATR* and is an upstream enabler of *BRCA1* and *RAD51*. Finally, mTORc1 and TP53 are downstream of Akt in apoptotic contexts. As the gene encoding one of the most critical PI3k catalytic subunits, *PIK3CA* is thus tightly associated with apoptosis, centrosome amplification, CIN and aneuploidies.

Modelling Techniques in ART

Technological developments have made it possible to use computational techniques to help comprehend extremely complex pathways and processes. Studies using mathematical modelling have been used in the past to improve discerning of the ART process (Fischer-Holzhausen & Röblitz, 2022; Hassan et al., 2020; Simopoulou, Sfakianoudis, Antoniou, et al., 2018; Simopoulou, Sfakianoudis, Maziotis, et al., 2018; Yenkie et al., 2013). Moreover, these investigations have furnished a foundation for the development of prediction models. Around the world, predictive studies have been used in a number of reproductive clinics as helpful tools to help the doctors (Simopoulou, Sfakianoudis, Antoniou, et al., 2018; Simopoulou, Sfakianoudis, Maziotis, et al., 2018). A few of these predicted characteristics can be summed up as time-lapse microscopy studies of morphokinetics, proteomic factors, and genetic analysis using NGS technology (Gardner et al., 2015). Image-based predictive artificial intelligence (AI) technologies also represent the more recent supportive techniques in ICSI with the emergence of the computer age (Manna et al., 2013). By making these models more

accurate and enhancing their predictive capability, ICSI failures may be prevented, saving couples from needless financial strain and possibly severe psychological damage. Additionally, it can give medical professionals and infertile couples more treatment options. In the realm of reproductive biology, mathematical models are still relatively young, thus further investigation is necessary before their full potential as prognostic tools is realised.

CHAPTER III

Materials And Methods

Within this chapter, detailed information on the methodology and the materials used while conducting this research is described. Furthermore, experimental details are provided to ensure the reproducibility of the presented work.

Embryo Collection

Vitrified surplus embryos collected from patients undergoing ICSI treatment at British IVF Centre were obtained for the purposes of this research with informed consent. The study was approved by the board of ethics of Near East University (Project no: YDU/2021/96-1432) and was conducted according to worldwide and local rules and regulations pertaining to human based research. All procedures were carried out by an expert embryologist at the IVF centre and the practical details employed are detailed henceforth.

Controlled ovarian hyperstimulation (COH) was performed with the assistance of Gonadotropin-releasing hormone antagonists (GnRH), preventing natural production of follicle stimulating hormone (FSH) and luteinising hormone (LH). Patients were then given recombinant FSH (150 - 300 IU, Gonal F, Serono) and hMG (75-150 IU, Merional, IBSA) at the start of their menstrual cycle. Response of the ovaries to the external stimulants was followed up on by biochemical screening of the blood serum for progesterone and estradiol levels as well as ultrasonography after the sixth day of ovarian stimulation. The day of ovulation triggering was determined via the size of the leading follicle which should exceed 13 mm, before ovulation can be initiated. Patients were given daily doses of 0.25 mg GnRH antagonist Cetrotide serono until this condition was met. To initialise ovulation, 250 mg hCG (Ovitrelle, serono) or 0.2 mg triptorelin (Gonapeptyle, ferrin) was administered to patients, so that oocytes could be retrieved approximately 35 hours later.

Ooocyte collection is carried out by follicle aspiration with a needle (Wego, 1816U, PRC) with aid from transvaginal ultrasonography. Aspirated follicle contents were then sent to the laboratory so that the cumulus-oocyte

complexes can be isolated which were then stored and cultured in bicarbonate buffered media (Continuous Single Culture, CSC, 90164, Irvine Scientific, USA) until denudation at 37˚C with 5% O_2 and 7% CO_2. Denudation was later carried out with modified HTF (mHTF with HEPES, 90126, Irvine Scientific, USA).

Methodology described previously by Coban et. al. (2020) was used to prepare semen samples. A single sperm cell detected via 10µl PVP (Polyvinylpyrrolidone Solution with HSA - 7%, 90121, Irvine Scientific, USA) was injected into the oocyte, completing the ICSI procedure. Fertilisation was confirmed roughly 16-18 hours after the ICSI process by the observation of two pronuclei. Day 3 embryo grading was performed using the criteria proposed by Ciray et. al. (2012) which incorporates number of blastomeres, degree of fragmentation, cytoplasmic properties, nuclear specifications and evenness of the cells of the blastocyst. For the analysis of day 5 and day 6 embryos Gardner specifications were utilised (Gardner & Schoolcraft, 1999). In brief, their criteria rank the expansion stage between 1-6 where 6 denotes the highest quality expansion. Furthermore, quality of the trophectoderm (TE) and inner cell mass (ICM) are graded in letters A-C where A indicates the best quality and C the poorest (Gardner & Schoolcraft, 1999).

Laser assisted hatching was employed for every embryo in order to elevate the number of herniating cells (10-15µm) (Octax EyeWare, Vitrolife, Sweden). Trophectoderm biopsies were taken from day5 or day6 blastocysts without disrupting the ICM. Approximately 5-8 cells were biopsied for each embryo with aspiration or flicking techniques with the additional help from laser shots.

For long term storage, embryos were vitrified and placed into liquid nitrogen. Vitrification was carried out using special vitrification media (Vit Kit-Freeze; 90133-SO, Irvine scientific, USA). Prior to their transport from the IVF centre, embryos were thawed utilising vitrification thawing media, as per the manufacturer’s guidelines (Vit Kit-Thaw; 90137-SO, Irvine Scientific, USA).

PGT-A Analysis and Sample Grouping

Biopsied trophectoderm cells were submitted to a private genetics laboratory by the IVF centre which conducted the NGS analyses to determine

numerical chromosomal abnormalities. The Ion ReproSeq PGS kit was used in each PGT-A test by the laboratory. The PGT-A results obtained from the IVF centre was used to separate experimental and control groups alongside morphological quality data. In particular, embryos with higher morphological quality were prioritised to be included in the research. Additionally, complex and chaotic aneuploidies were excluded from this study as their implications on the gene expression patterns would be too complex and too unpredictable to analyse. Finally, as the aneuploidy of the chromosomes where any genes of interest to this study are situated on would assuredly alter its gene expression levels, care was taken to avoid using any embryos possessing such aneuploidies. Namely, chromosome 17 is where *TP53* and *BRCA1* genes are located, chromosome 3 hosts *ATR* and *PIK3CA* genes, chromosome 11 contains *CHEK1* and *ATM*, *CHEK2* is situated on chromosome 22, *MDM2* is located on chromosome 12, *RAD50* and *RAD51* genes are located on chromosomes 5 and 15 respectively. Lastly, *ACTB* gene which acts as the housekeeping gene in this study is encoded on chromosome 7. Therefore, the embryos belonging to the aneuploid group all possessed euploid numbers for the chromosomes stated above and showed aneuploidies in other chromosomes. More detailed information on the experimental groups and the embryos allocated within them can be found in Table 2 below.

Table 2

Experimental groups and embryo details.

First ten samples belong to the aneuploidy group, whereas the final 10 samples belong to the euploid or the control group.

Embryo ID	Aneuploidy	Morphological Quality
B1	-10	3AA
B2	+4q	4AA
B3	-9q	4AA
B4	-19	4AA
B5	-20	4CC
B6	-16	5AB
B7	+14	5BB
B8	X0	5BB
B9	-4p	4AB
B10	-21	5BB
B11	Euploid	5BC
B12	Euploid	6BB
B13	Euploid	5AA
B14	Euploid	5BB
B15	Euploid	4AB
B16	Euploid	5AB
B17	Euploid	5BB
B18	Euploid	5AB
B19	Euploid	4AA
B20	Euploid	5BA

As optimisation took a great deal of time and numerous tries, eventually these cDNAs were depleted and new samples were acquired. In later stages, optimisations were completed with pooling of samples. From acquired blastocysts RNA isolations were carried out and cDNA synthesis was performed as outlined in the subsequent sections. Obtained cDNAs were later pooled in groups of 4 with the PGT-A results for each embryo taken into consideration. *RAD51* data was obtained with the use of cDNA pools as outlined in Table 3.

Table 3

Details of Pooled Embryos that was used in RAD51 qRT-PCR experiments
In every sample pool cDNAs from 4 distinct embryos were pooled in equal volumes. The list of all aneuploidies combined in each pool sample is given. Aneuploidies separated by ‘’/” refer to the unique aneuploidies contributed by each individual embryo comprising the pool.

Pool ID	Combined Aneuploidy
Aneuploid 1	-16 / +16 / -16 / +6, +18
Aneuploid 2	+4q / -4p / Partial -4q / -4
Aneuploid 3	-10 / +10 / -X / +X
Aneuploid 4	-19 / +19 / -13, -21 / -21
Aneuploid 5	Partial +6q, Partial -8p / +14 / -9q / -20
Euploid 1	Euploid
Euploid 2	Euploid
Euploid 3	Euploid
Euploid 4	Euploid
Euploid 5	Euploid

For the analyses of *ATM* and *MDM2*, similarly prepared yet different cDNA pools were used. Details regarding these sample pools are given in Table 4 below.

Table 4

Details of Pooled Embryos that was used in ATM and MDM2 qRT-PCR experiments

In every sample pool, cDNAs from 4 distinct embryos were pooled in equal volumes. The list of all aneuploidies combined in each pool sample is presented for each mix. Aneuploidies separated by ‘’/” refer to the unique aneuploidies contributed by each individual embryo comprising the pool.

Pool ID	Combined Aneuploidy
AY 1	+1, +5 / +16 / -16 / +6, +18
AY 2	-10 / +21, Partial -3q / Partial -4q / -4
AY 3	-17 / +10 / +X / -4, -14
AY 4	-6q / +19 / -13, -21 / +3p
AY 5	Partial +6q, Partial -8p / -9 / +19 / +5, -9q
EY 1	Euploid
EY 2	Euploid
EY 3	Euploid
EY 4	Euploid
EY 5	Euploid

DNA/RNA Isolation

For analysis of gene expression patterns, RNA extraction from the received embryos was imperative. This was accomplished via the use of a commercial kit, Hibrigen Total Nucleic Acid Isolation kit (Türkiye, Cat. Id: MG-TNA-01). Isolates were then tested for their quantity and purity by Nanodrop 2000 Spectrophotometer (ThermoScientific) and its relevant software. Data regarding these measurements can be found below in Table 5.

Table 5

Nanodrop data for RNA isolates

Isolates were measured with Nanodrop twice and the recordings presented here denote the average of the two measurements.

Embryo no	*Concentration (ng/µl)*	*260/280*
B1	7.3	1.80
B2	3.5	1.93
B3	15.6	1.52
B4	9.5	1.74
B5	3.5	1.97
B6	25.3	1.66
B7	15.1	1.58
B8	13.5	1.58
B9	17.5	1.65
B10	5.3	1.56
B11	4.7	1.52
B12	12.3	1.61
B13	18.2	1.56
B14	9.2	1.51
B15	8.8	1.60
B16	17.6	1.57
B17	4.3	1.62
B18	14.3	1.64
B19	30.1	1.64
B20	6.2	1.59

cDNA Synthesis

Before QRT-PCR could be carried out, RNA to cDNA conversion was needed to obtain a suitable template. This transition was achieved by the use of ThermoFisher High-capacity cDNA Reverse Transcription kit (ThermoFisher, Cat. ID: 4368814) where the manufacturer protocols were exactly adhered to.

QRT-PCR

QRT-PCR reactions were carried out to obtain gene expression data. All runs used SYBR green as the fluorescent reporter incorporated in a commercial kit, ThermoFisher Life Sciences PowerTrack SYBR Green Mastermix (ThermoFisher, Cat. ID: A46109). Later optimisations, namely the ones for *ATM*, *RAD51* and *MDM2* were carried using another commercial kit, Hibrigen 2x SYBR Green qPCR Master Mix (Hibrigen, Turkey; Cat. No: MG-SYBR-01-400). As previously mentioned, specific primers were designed for all genes of interest in an exon-exon spanning manner, using PrimerBlast software by NCBI, available online. Furthermore, possible off-target amplicons from genomic DNA or cDNA were ensured to be larger in size (>1500 bp) which renders them incompatible with SYBR green, decreasing confounding factors. Final primer sequences and their melting temperatures (T_m) are listed in Table 6. All primers were ordered from Bioligo (Türkiye) in lyophilised fashion. Preceding the QRT-PCR experiments, PCR runs were optimised and correct sized products were confirmed by agarose gel electrophoresis. 2% Tris-Acetic Acid-EDTA (TAE) gels were used in all gel runs and the amplicons were compared against a commercial 100 bp DNA ladder (Hibrigen, Turkiye) in each instance. Running buffer was also 1x TAE in all runs. Once optimised conditions for each run were determined, melting curve analysis was used in all final experiments as confirmation of proper amplification.

Table 6

Primer sequences and their respective melting temperatures as provided by the manufacturer.

To reiterate, the primers are specifically designed to span exon-exon junctions, suppressing possibilities for genomic DNA binding.

Primer Name	Sequence (5'->3')	T_m (˚C)
TP53 Forward	TCAGACCTATGGAAACTACTTCCTG	61
TP53 Reverse	CTGGGAAGGGACAGAAGATGA	60
ATM Forward	TGGGATGCTGTTTTTAGATTTTT	54
ATM Reverse	AGAGTTCTTGACATTTTAGCCT	55
ATR Forward	ATTGTGCCATTTCGCCTGAC	57
ATR Reverse	GAACATGGGTCTTGGCCTTTT	58
CHEK1 Forward	ATATGAAGCGTGCCGTAGAC	57
CHEK1 Reverse	ACCATGCAGATAAACCACCC	57
CHEK2 Forward	CCTTCAGGATGGATTTGCCA	57
CHEK2 Reverse	TTTAGGACCCACTTCCCTGA	57
RAD50 Forward	GCCTTGGATAGCCGAAAGAA	57
RAD50 Reverse	AGCTGTAGACGACCCTGTT	57
RAD51 Forward	ATTTCACGGTTAGAGCAGTGTG	58
RAD51 Reverse	TGACCGCCTTTGGTGGAAT	57
PIK3ca Forward	GCTCTACAGGGCTTTCTGTC	59
PIK3ca Reverse	CTTGCCGTAAATCATCCCCA	57
MDM2 Forward	GTGAGGAGCAGGCAAAT	53
MDM2 Reverse	ACCTCTTTCATAGTATAAGTGTCT	56
BRCA1 Forward	GATTTATCTGCTCTTCGCGT	56
BRCA1 Reverse	AGGTTCCTTGATCAACTCCA	59
ACTB Forward	GACGGCCAGGTCATCACTAT	59
ACTB Reverse	CGGATGTCAACGTCACACTT	58

Final experimental conditions used in QRT-PCR reactions following optimisation runs are stated in Table 7. Optimised QRT-PCR runs were designed to compare 10 aneuploid and 10 euploid samples and 2 biological replicates were included for each sample in all runs to improve data

accuracy. For *ATM*, *RAD50* and *MDM2* 5 aneuploid pools were compared with 5 euploid cDNA pools in 2 biological replicates again.

Table 7

Final experimental conditions for each QRT-PCR run to gather data on gene expression patterns.

Gene of Interest	Reaction Components	Thermal Cycler Conditions
BRCA1	5 µl ThermoFisher Life Sciences PowerTrack SYBR Green Mastermix 0.4 µM Forward Primer 0.4 µM Reverse Primer 2.2 µl Water 2 µl cDNA Template (water for Negative Control)	95˚C 5 Minutes X40 Cycles of: 95˚C 30 Seconds 57˚C 35 Seconds 72˚C 50 Seconds
RAD51	5 µl Hibrigen 2x SYBR Green qPCR Master Mix 0.4 µM Forward Primer 0.4 µM Reverse Primer 2.2 µl Water 2 µl cDNA Template (water for Negative Control)	95˚C 5 Minutes X40 Cycles of: 95˚C 20 Seconds 57˚C 55 Seconds 72˚C 30 Seconds
ATM	5 µl Hibrigen 2x SYBR Green qPCR Master Mix 0.4 µM Forward Primer 0.4 µM Reverse Primer 2.2 µl Water 2 µl cDNA Template (water for Negative Control)	95˚C 5 Minutes X40 Cycles of: 95˚C 20 Seconds 56˚C 55 Seconds 72˚C 30 Seconds
MDM2	5 µl Hibrigen 2x SYBR Green qPCR Master Mix 0.4 µM Forward Primer 0.4 µM Reverse Primer 2.2 µl Water 2 µl cDNA Template (water for Negative Control)	95˚C 5 Minutes X40 Cycles of: 95˚C 20 Seconds 54˚C 55 Seconds 72˚C 30 Seconds

Statistical Analysis

Cycle threshold (Ct) values obtained from QRT-PCR were analysed by the ΔΔCt method employing *ACTB* as the housekeeping gene in every instance. GraphPad Prism 8 software was used to conduct all analyses, in the form of student's t-tests to determine statistical significance.

Mathematical Modelling

For the mathematical modelling part of this thesis anonymised retrospective data obtained from British IVF Centre was used. The data belonged to patients attending this clinic between the years 2016-2021. Altogether, data from 765 different couples totalling up to 4123 embryos was collected. Data points included the ages of the parents, the number of follicles aspirated from the maternal candidate and the number of oocytes at the meiosis-I stage and the number of oocytes stalled at the meiosis-II stage. These oocytes are referred to as MI oocytes and MII oocytes from now on respectively. For paternal data, commonly measured sperm parameters were gathered such as sperm number, motility and morphology. To determine the ‘’good’’ and ‘’bad’’ thresholds for paternal parameters, the most recent WHO guidelines were referred to (World Health Organization, 2021).

The ages of parents ranged between 30-60 for females and for males the youngest data point was 30 whereas the eldest was 73. Conversely, for both genders the ages assembled within a scope of 30-40 years in the current dataset. Lastly, mathematical modelling part of this study was approved by the IRB committee of the university albeit separately from the first part of this thesis (Project no: NEU/2023/116-1766).

In order to investigate a potential connection between parent reproductive features and the quality of the resulting embryos, these parameters had to be allocated into two distinct groups. Measurements related to the quality of the embryos, namely follicle number, MI and MII oocyte numbers as well as factors pertaining to sperm quality such as number of sperm, sperm motility and morphology were termed the independent variables. In contrast, the parameters associated with embryo quality, such as morphological measurements as described by Ciray et. al. (2012) for day3 embryos and by Gardner and Schoolcraft (1999) for day5 and 6 embryos were considered as dependent variables. Additionally, the aneuploidy status of the embryos was categorised under dependent variables. A regression analysis was performed to understand the connection between the described independent variables and dependent variables. In general, a regression equation can be shown as:

$y = b_0 + b_1x_1 + b_2x_2 + ... + b_nx_n + e,$

in which b_0 stands for the intercept, b_x is the regression coefficient for the independent variable *x,* y denotes the dependent variable and finally *e* represents the error (S. Chatterjee & Hadi, 2006).

The specific regression equation to connect parental parameters with the aneuploidy status of the embryos was constructed as this:

Result = b_0+b_1Follicle No+b_2MI No+b_3MII No+b_4Sperm No+b_5Sperm Motility+b_6Sperm Morphology+*e*

where b_0, b_1, b_2, b_3, b_4, b_5 and b_6 stand for each of the regression coefficients for every independent variable, and *e* again for the error. Based on the outcome, specific equation is shown below:

Regression Equation: Result = −0.12267 + (−0.001018) × Follicle No + (−0.16904) × MI No + −0.014676 × MII No + 0.1018 × Sperm No + 0.0013029 × Sperm Motility + (−0.0012664) × Sperm Morphology

Table 8

Summary of the regression analysis results to draw out further equations.

Dependent Variable	Independent Variables	R-Squared	P-Value
2PN Number	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.695	0.002*
D3 Cell Number	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.481	0.033*
D3 Even/Uneven	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.405	0.067
D3	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.528	0.023*
D5 Expansion	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.592	0.015*
D5 TE	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.386	0.077
D5 ICM	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.509	0.026*
D6 TE	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.747	0.001*
D6 ICM	Follicle No, MI No, MII No, Sperm No, Sperm Motility, Sperm Morphology	0.636	0.006*

Using the values from Table 8, regression equations precise for each dependent variable was written out: D6 ICM: y = -1.195 + 0.040(Follicle Number) + 0.268(MI Number) + 0.332(MII Number) + 0.0002(Sperm Number) + 0.554(Sperm Motility) - 0.136(Sperm Morphology)

2PN Number: y = 0.386 + 0.014(Follicle Number) + 0.186(MI Number) + 0.175(MII Number) - 0.0002(Sperm Number) + 0.520(Sperm Motility) - 0.181(Sperm Morphology)

D3: y = -0.303 + 0.011(Follicle Number) + 0.114(MI Number) + 0.096(MII Number) + 0.0002(Sperm Number) + 0.199(Sperm Motility) - 0.068(Sperm Morphology)

D3 Even/Uneven: y = -2.088 + 0.062(Follicle Number) + 0.450(MI Number) + 0.145(MII Number) - 0.001(Sperm Number) + 0.437(Sperm Motility) - 0.223(Sperm Morphology)
D3 Cell Number: y = -23.127 + 0.649(Follicle Number) + 6.183(MI Number) + 4.034(MII Number) - 0.013(Sperm Number) + 4.822(Sperm Motility) - 2.694(Sperm Morphology)
D5 ICM: y = -0.769 + 0.035(Follicle Number) + 0.207(MI Number) + 0.233(MII Number) + 0.0002(Sperm Number) + 0.350(Sperm Motility) - 0.092(Sperm Morphology)

D5 Trophoectoderm: y = -2.901 + 0.092(Follicle Number) + 0.742(MI Number) + 0.048(MII Number) - 0.001(Sperm Number) + 0.607(Sperm Motility) - 0.435(Sperm Morphology)
D5 Expansion: y = -2.306 + 0.066(Follicle Number) + 0.632(MI Number) + 0.094(MII Number) + 0.001(Sperm Number) + 0.772(Sperm Motility) - 0.357(Sperm Morphology)
D6 TE: y = -5.390 + 0.140(Follicle Number) + 1.484(MI Number) + 0.344(MII Number) + 0.003(Sperm Number) + 1.480(Sperm Motility) - 0.986(Sperm Morphology)

CHAPTER IV

Results

This chapter aims to succinctly present the results of the study. The data is supplemented with figures to provide accurate representation of all findings.

Gene Expression Patterns are Altered Between Euploid and Aneuploid Blastocysts

Gene expression patterns were assessed between euploid and aneuploid embryos with the widely used β-actin (*ACTB*) gene as the normaliser in all experiments. The result show that the gene expression patterns show variation between the two experimental groups. Results for each gene investigated in this study are introduced individually in the following subsections.

BRCA1 Expression is Elevated in Aneuploid Embryos

Optimisation of PCR conditions was accomplished directly via qRT-PCR method followed by a melting curve analysis. Observation of a single peak under melting curve suggested that under the finalised conditions, as outlined in materials and methods (Table 7), no primer dimers and/or unintended products were present.

Quantitative Real-Time PCR reactions yielded two Ct values for each sample as one biological replicate per sample was employed (Figure 7). While primer dimerization cases were not expected, a melting curve analysis was employed at the end of PCR cycles. Melting curve results showed a single melting point common for all intended products and no additional peaks, confirming the lack of primer dimers or unintended products in the final reaction (Figure 8).

Figure 7

Real Time PCR Amplification Plot for BRCA1.

Sigmoidal shaped amplification curves suggest good expansion during the PCR reaction. Furthermore, lack of a signal in negative control instils confidence in the reaction quality.

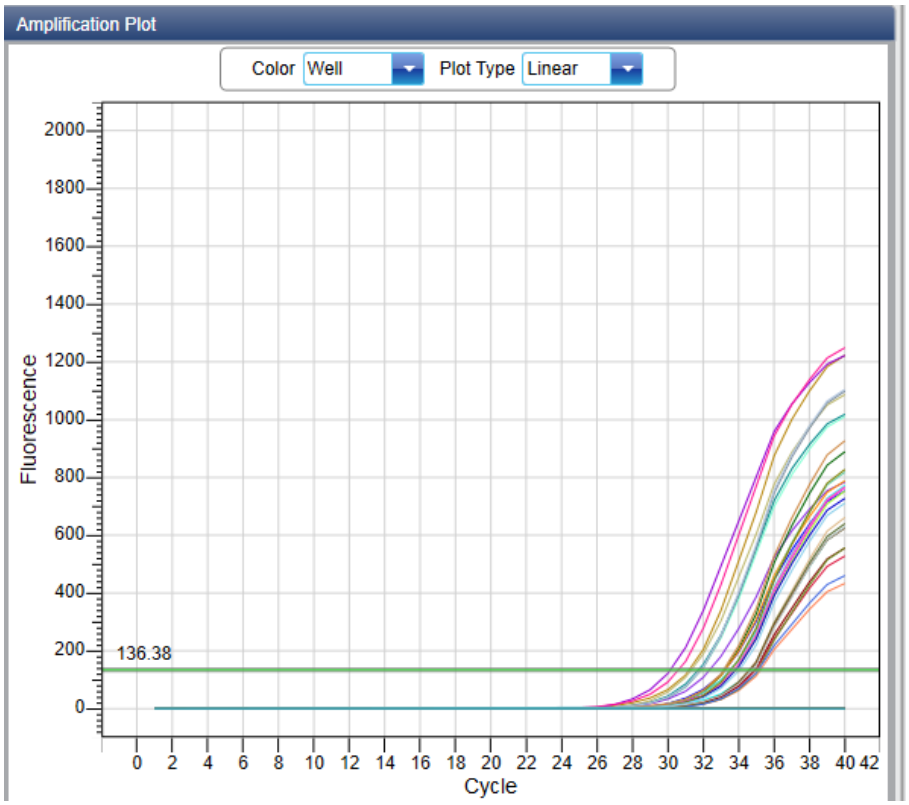
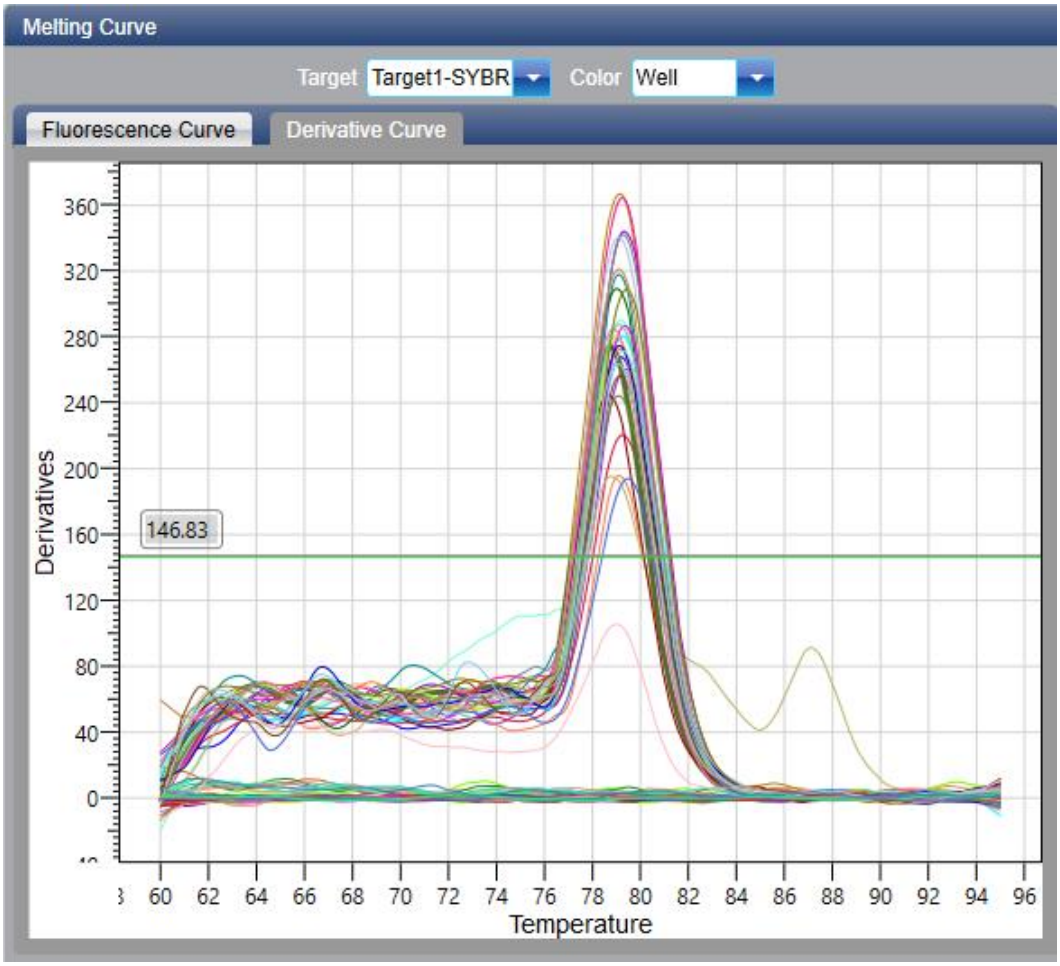


Figure 8

Melting Curve Analysis for BRCA1.

A single peak shared with all the samples suggested that *BRCA1* amplification was consistently obtained for all samples. Additionally, no signal was observed for negative control sample, eliminating the possibility of primer dimers.

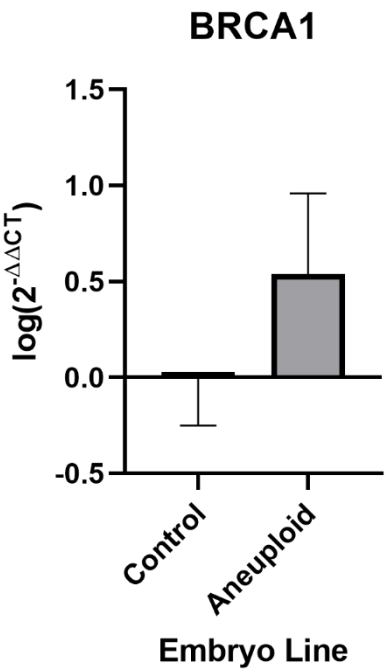


Averaged Ct values were evaluated via the ΔΔCt method and $2^{-(\Delta\Delta Ct)}$ values were log transformed prior to conducting a student's t-test to establish statistical significance. Results from the t-test revealed a notable increase in *BRCA1* expression (p=0.0046, t=3.294) in aneuploid samples versus euploid control group (Figure 9).

Figure 9

Student's t-test Results for BRCA1 Expression.

BRCA1 expression is significantly elevated in aneuploid embryos compared to euploid embryos.



ATM Expression Does Not Significantly Change Between Euploid and Aneuploid Preimplantation Embryos

PCR conditions outlined as (Table 7) was used to generate the data. Optimisation of runs were confirmed by agarose gel electrophoresis as depicted in (Figure 10). Quantitative real time PCR results successfully gave sigmoidal curves as well as corresponding Ct values as can be observed in (Figure 11).

Figure 10

Agarose Gel Electrophoresis Results for ATM Optimisation

2% TAE agarose gels were performed to confirm intended product formation for multiple genes in tandem. Optimisation runs were conducted with a single sample named Trial and a negative control lacking a cDNA template. From left to right, a commercial 100 bp ladder for reference, Trial sample for *ATR*, *TP53* and *ATM* followed by negative control samples for *ATR*, *TP53* and *ATM* can be observed in subsequent wells as labelled at the top of each well. Relevant size markers on the DNA ladder are also indicated by arrows. All wells clearly show dimer formations only, with the exception of *ATM* Trial, which also presents a ~193 bp size DNA band which is concurrent with the expected band size for the *ATM* primer pair. Observation of a single correct sized band for *ATM* and only primer dimers for the negative control suggested that the optimum conditions for these reactions were achieved.

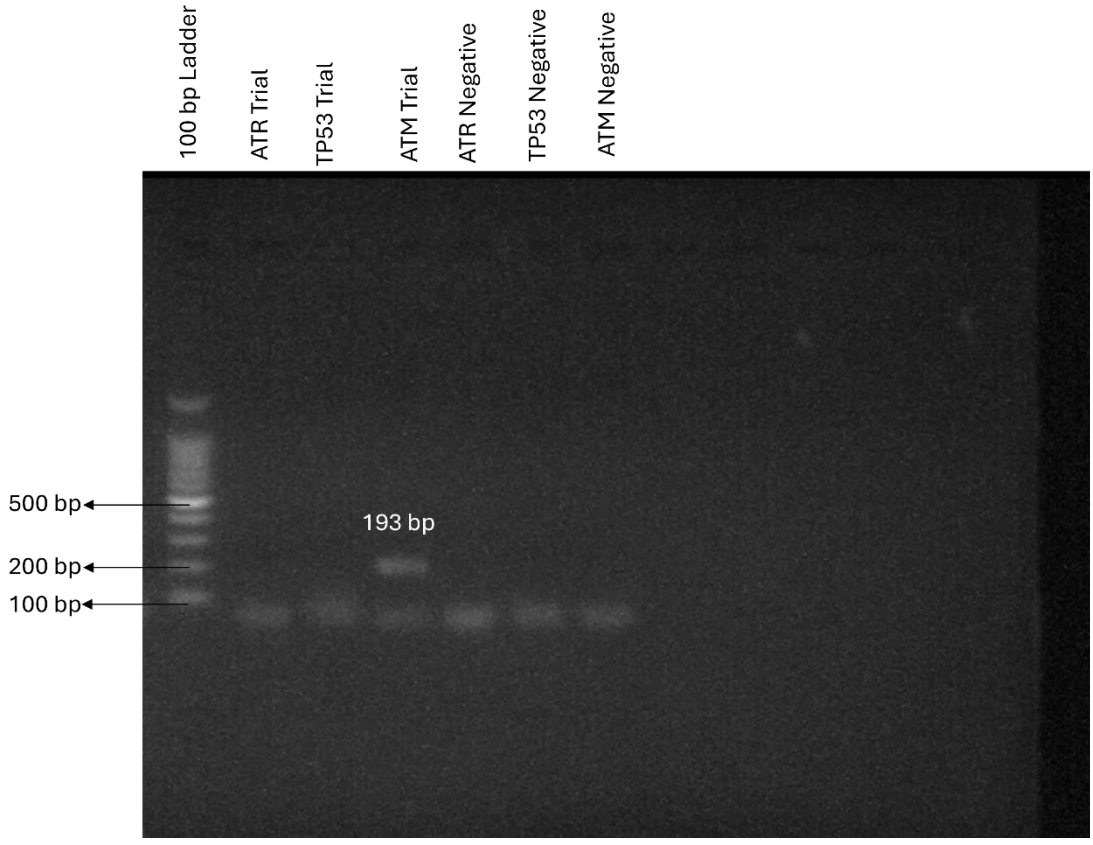
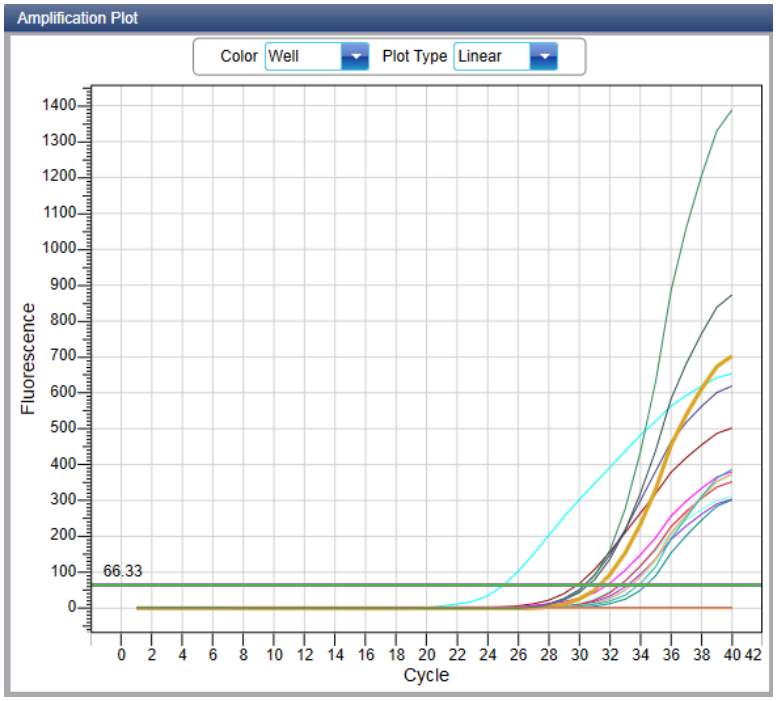


Figure 11

Real Time PCR Amplification Plot for ATM

Appearance of sigmoidal curves suggests no abnormal amplification occurred during the run.

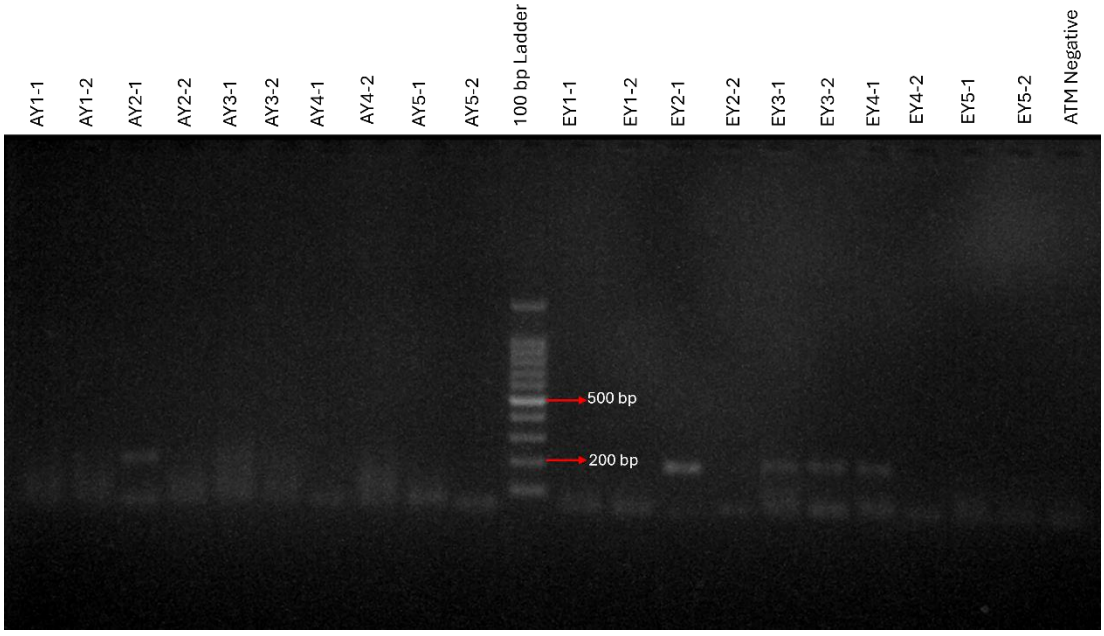


For *ATM* runs, primer dimers appeared to be unavoidable, particularly in negative controls. Moreover, these dimers were prominent enough to warrant Ct values in the qRT-PCR runs. To be absolutely certain that for the actual sample cDNA pools Ct values obtained arose from proper amplification of the intended product and not dimers, amplicons were ran on a secondary agarose gel (Figure 12). Indeed, few tubes only generated primer dimers during the reaction and hence those were excluded from further analyses even if the machine was able to generate a Ct value.

Figure 12

Agarose Gel Electrophoresis Results for the Data Acquisition qRT-PCR of ATM

A 2% agarose gel was utilised for all samples and their biological duplicates. From left to right in order, two duplicates of AY1, AY2, AY3, AY4 and AY5 were loaded onto the wells. To the adjacent well, a 100 bp ladder was loaded and then subsequent wells were populated with duplicates of EY1, EY2, EY3, EY4, EY5 similar to AY samples. The final well contained the negative control amplicon. All wells are labelled at the top and details about the sample pools can be found in Table 4. Ladder bands closer in size to expected *ATM* product size (193 bp) are indicated with red arrows.Confirmation of proper amplifications were made with observed correctly sized bands. Sample with excessive primer dimers and ones where no product band was observable were excluded from further analyses.

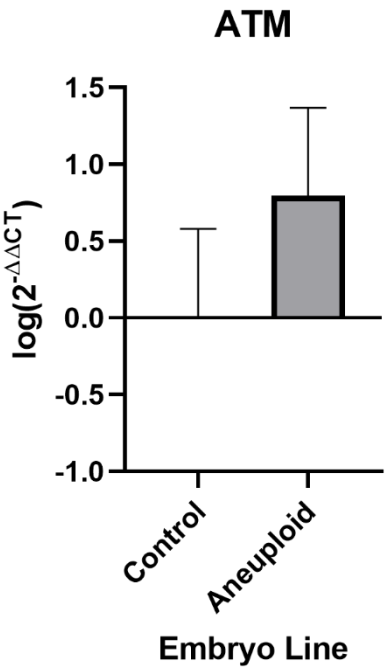


Finally, Ct values belonging to proper amplicons were subjected to ΔΔCt analysis and similarly to *BRCA1* obtained log($2^{-(\Delta\Delta Ct)}$) values were subsequently compared via student's t-test analysis. While there was an increase in *ATM* expression observed in aneuploid embryos, these differences failed to reach statistical significance (p=0.1641, t=1.701). (Figure 13).

Figure 13

Student's t-test results for ATM Expression

ATM expression was not significantly altered in aneuploid embryos



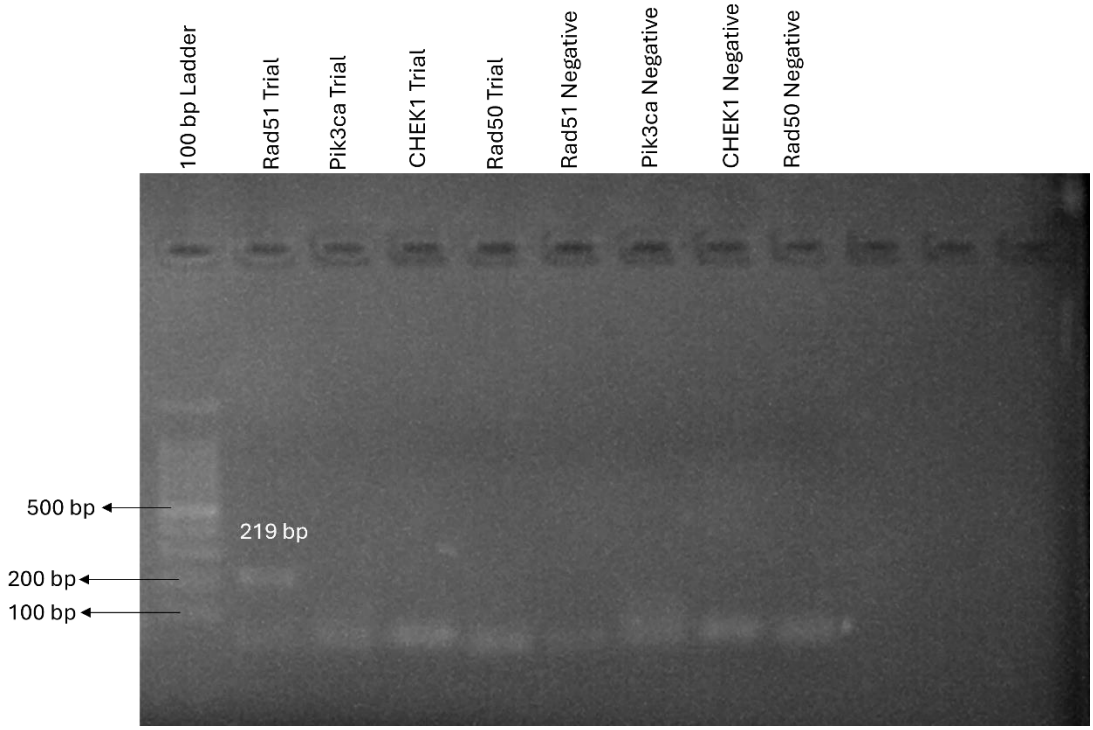
RAD51 Expression Remains Unchanged Between Euploid and Aneuploid Embryos

Similarly to *ATM* experiments, *RAD51* optimisation was also confirmed with agarose gel electrophoresis. Resembling the *ATM* results, *RAD51* also showed some primer dimers as well as a single, clear correct sized band (Figure 14).

Figure 14

Agarose Gel Electrophoresis Results for RAD51 Optimisation

Agarose gel electrophoresis was used during optimisations of multiple primer pairs. In order, first well contained 100 bp ladder for reference band sizes. Relevant band sizes on the ladder are highlighted with arrows. Subsequent wells were loaded with *RAD51* Trial, *PIK3CA* Trial, *CHEK1* Trial and *RAD50* Trial PCR amplicons. Final 4 wells contained the negative controls for these genes in the same order as indicated by the labels above each well. For *RAD51*, a single correct sized band was observed in the trial sample and only primer dimers in the negative control suggesting that the optimal reaction conditions were found.

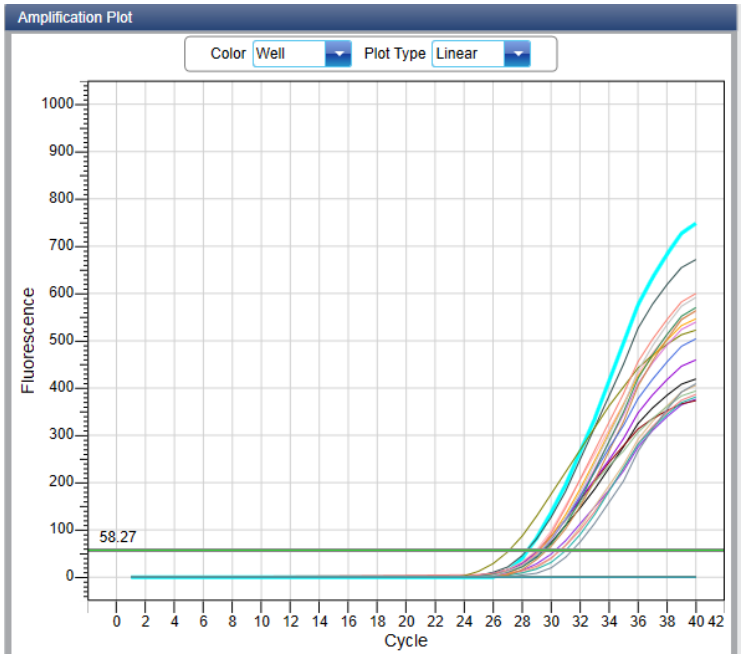


Quantitative PCR was then carried out by the determined optimum conditions and all readings appeared successful in light of the observed sigmoidal curves (Figure 15).

Figure 15

Real Time PCR Amplification Plot for RAD51

Sigmoidal curves indicated that nothing abnormal happened during the PCR experiment

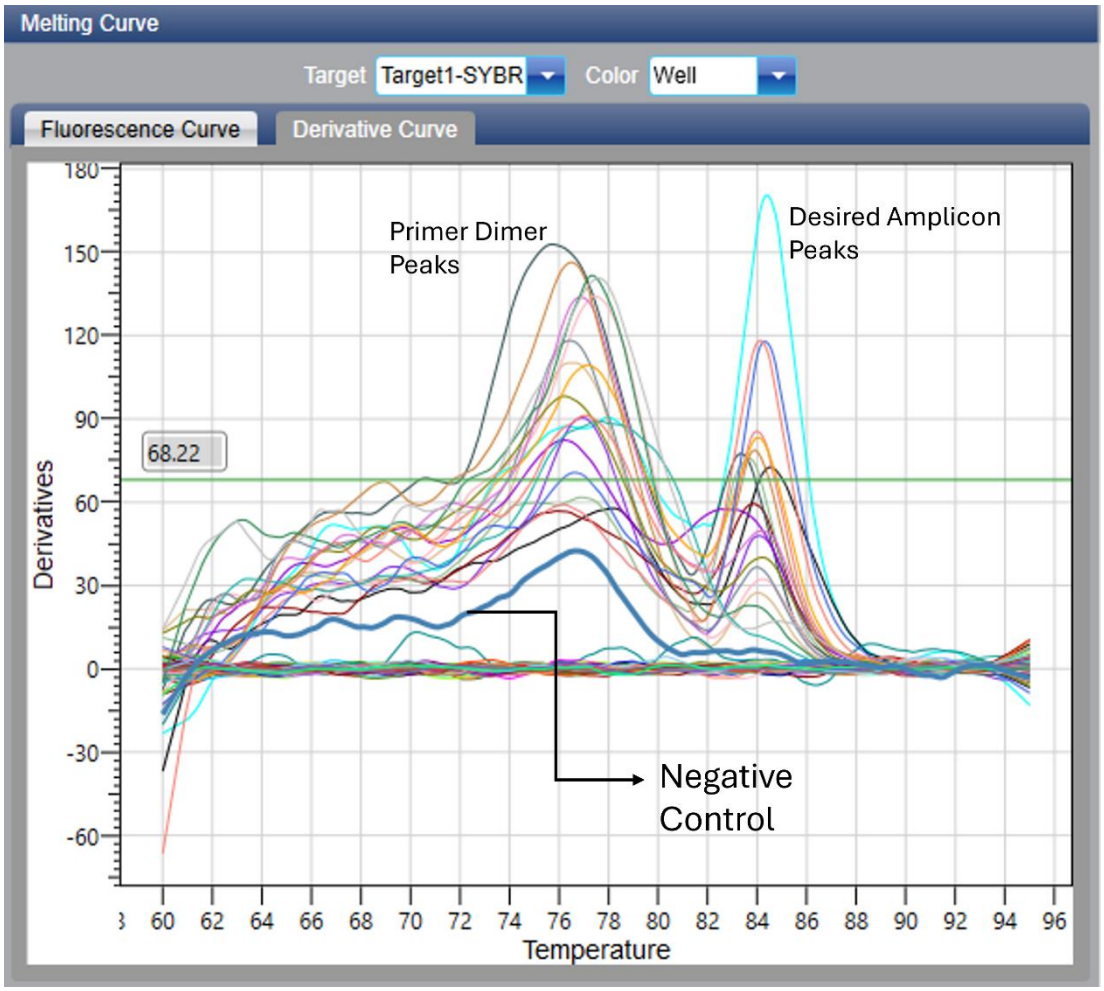


Once again, the PCR reaction was followed by a melting curve analysis to eliminate any interference of primer dimers as well as to exclude any amplicons that contained solely primer dimers from further analysis. Melting curve results are demonstrated in (Figure 16).

Figure 16

Melting Curve Analysis for RAD51

A single peak shared with all the samples suggested that *RAD51* amplification was consistently obtained for all samples. Primer dimers were identified by comparing the peaks with the negative control sample as indicated. Samples with heavy primer dimer influences or those that lacked the desired amplicon peaks were excluded from further analyses.

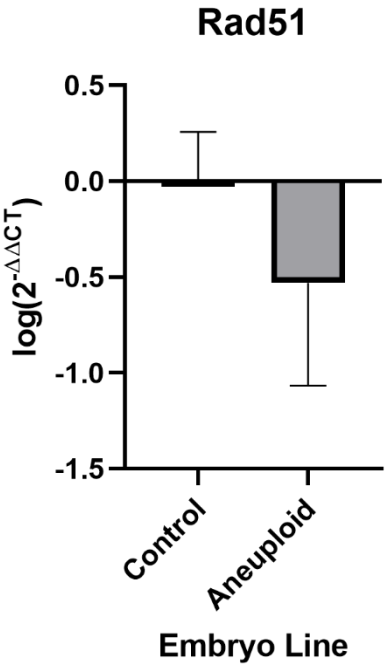


Once more, ΔΔCt values were calculated from the Ct values obtained and $\log(2^{-(\Delta\Delta Ct)})$ values were compared using t-test. Results of the statistical analysis revealed that *RAD51* levels were slightly lower in aneuploid embryos albeit in a statistically insignificant manner (p=0.0890, t=1.974) (Figure 17).

Figure 17

Student's t-test Results for RAD51 Expression

RAD51 transcription was slightly lower in aneuploid embryos, but this difference did not achieve statistical significance.



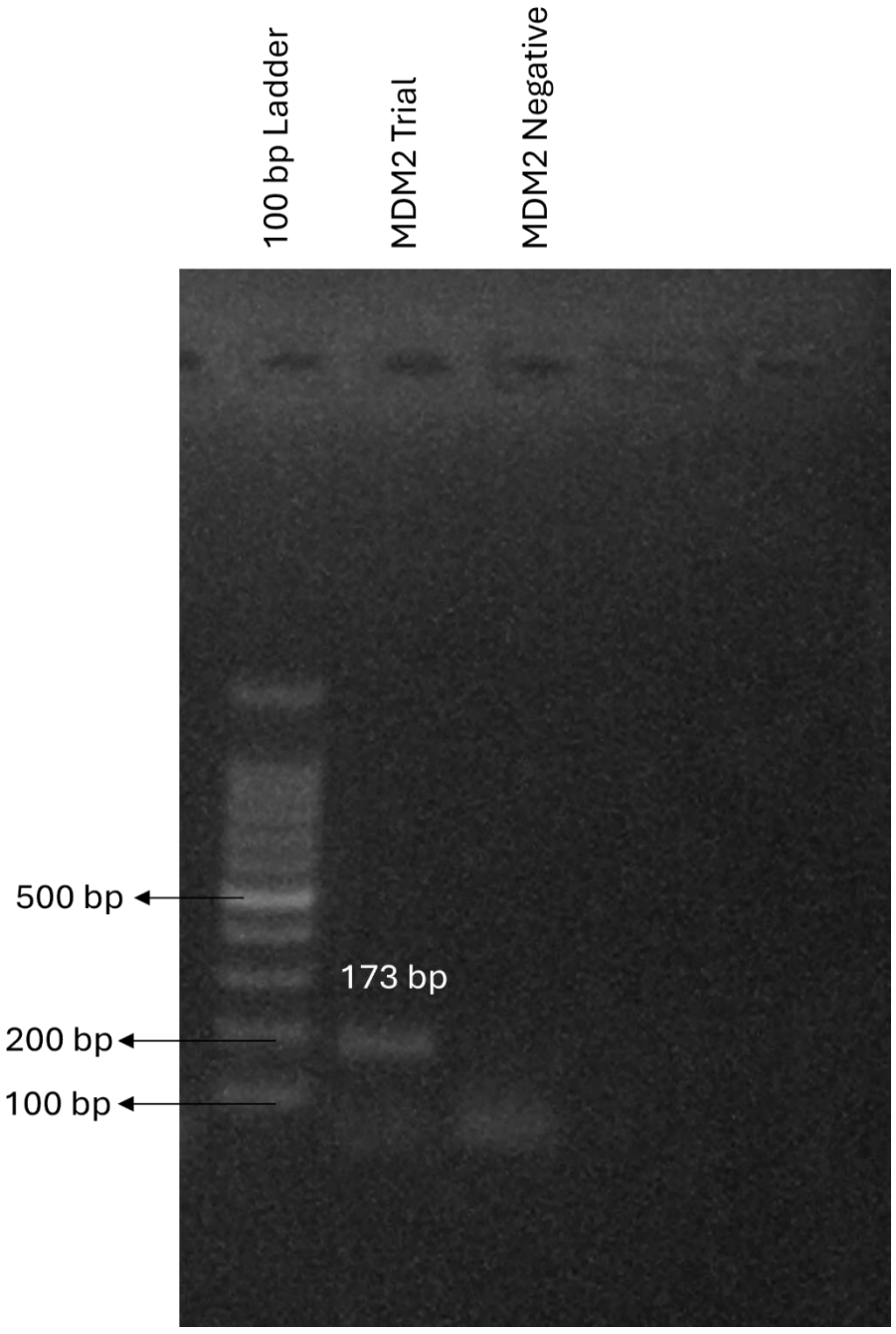
MDM2 Expression Levels are Similar between Euploid and Aneuploid Embryos

Experimentally similar procedures were followed for *MDM2* as well. Agarose gel electrophoresis was carried out to confirm optimisation. Gel results showed primer dimers as well as a single, correct sized band for the trial sample (Figure 18).

Figure 18

Agarose Gel Electrophoresis for MDM2 Optimisation

During *MDM2* optimisation a 2% agarose gel was employed for confirming proper amplification. Wells were loaded with a 100 bp ladder, the trial amplicon from the PCR reaction and the negative control as outlined by the labels above each well. 500, 200 and 100 bp reference bands are highlighted on the ladder with arrows. A negative control with only primer dimers and the presence of a single correct sized band in the trial sample inferred that the optimum conditions for this reaction was achieved.



Quantitative Real Time PCR reactions were then performed on full samples with the determined conditions as outlined in (Table 7). Sigmoidal curves were observed as shown in (Figure 19). Furthermore, melting curve analysis was performed after the qRT-PCR to separate primer dimers from proper amplifications as a Ct value was observed for the negative control. Melting curve analysis is presented in (Figure 20).

Figure 19

Real Time PCR Amplification Plot for MDM2

Presence of only sigmoidal curves showed that the data obtained came from reliable amplifications.

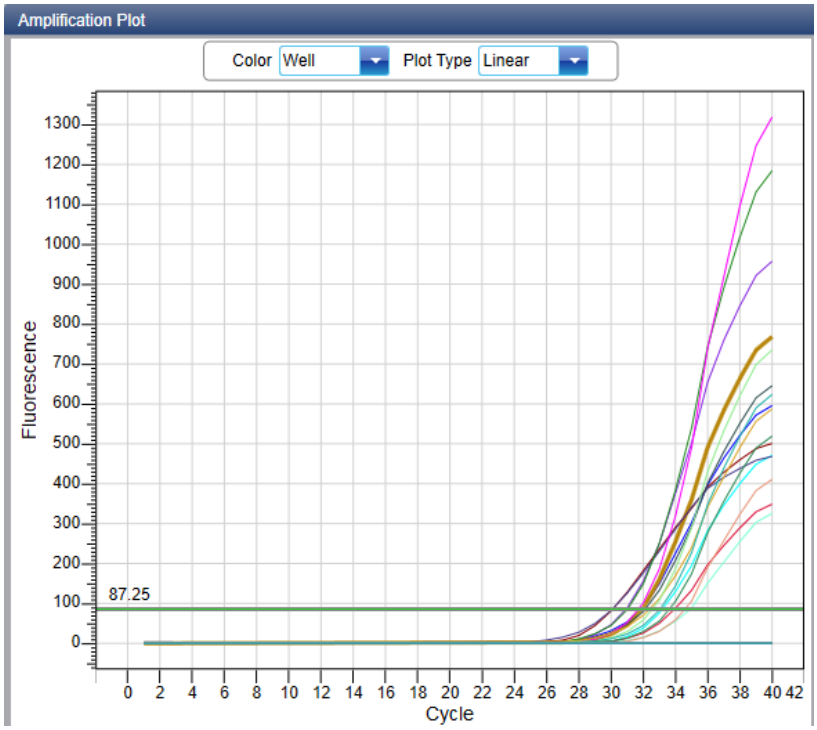
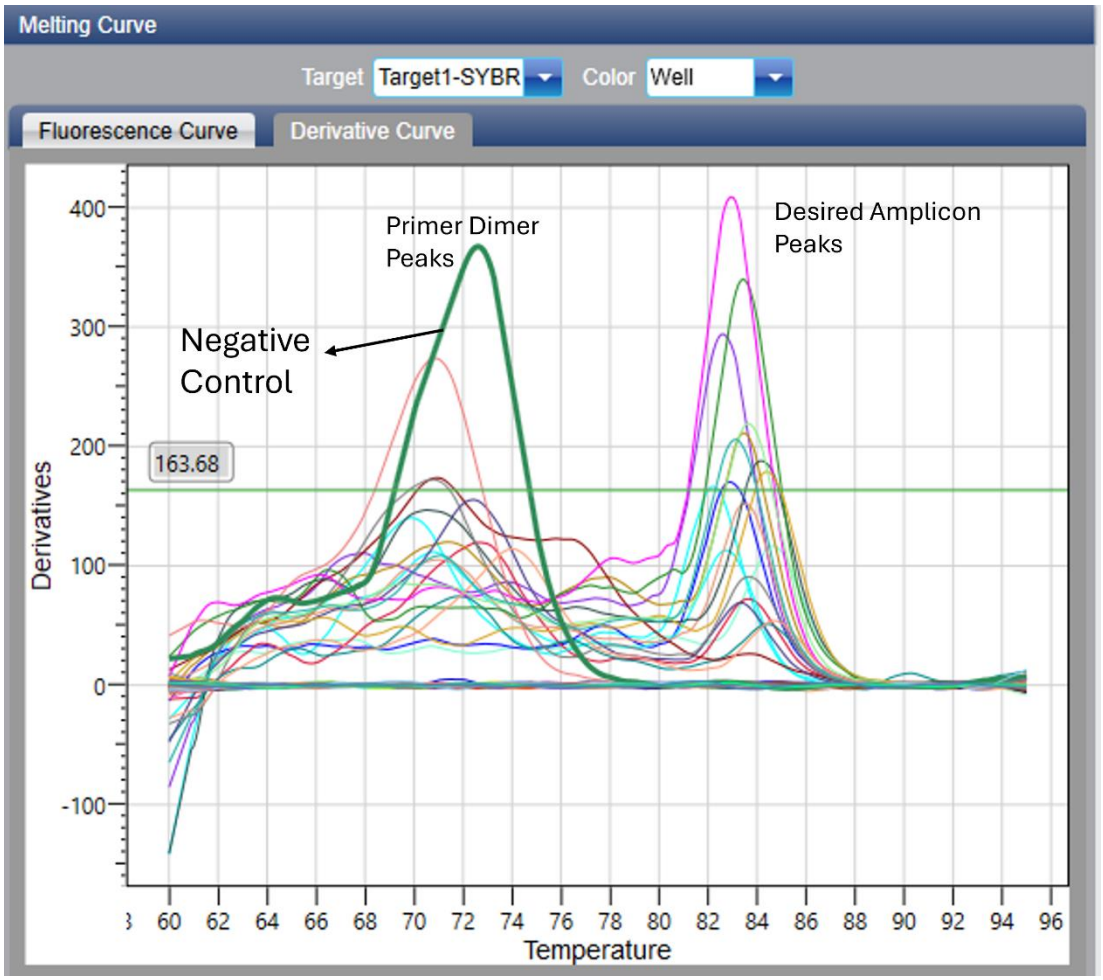


Figure 20

Melting Curve Analysis for MDM2

A single peak shared with all the samples suggested that *MDM2* amplification was consistently obtained for all samples. Primer dimers were identified by comparing the peaks with the negative control sample as indicated. Samples with heavy primer dimer influences or those that lacked the desired amplicon peaks were excluded from further analyses.

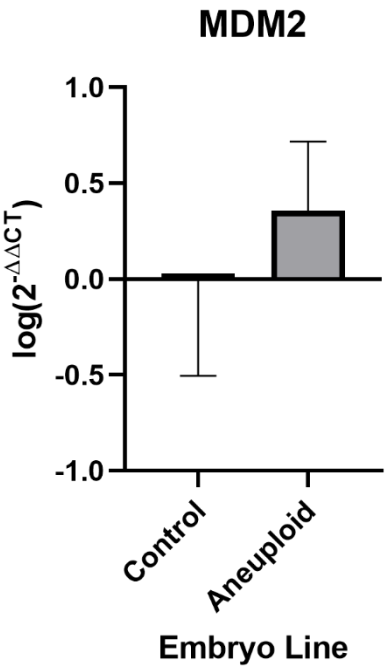


Obtained Ct values were then subjected to ΔΔCt analysis and $\log(2^{-(\Delta\Delta Ct)})$ values were used in student's t-test to investigate the statistical significance in differences of *MDM2* expression. *MDM2* values were slightly elevated in aneuploid embryos, nonetheless these differences were not statistically significant (p=0.2729, t=1.190) (Figure 21).

Figure 21

Student's t-test Results for MDM2 Expression

Expression levels of *MDM2* were slightly higher in aneuploid embryos, albeit this variation was not statistically significant.



Mathematical Modelling Can Partially Anticipate Embryo Quality

For ART purposes, two main characteristics of blastocysts can be considered to ensure only good quality embryos can be transplanted to patients. The first are the morphological aspects and the second is the aneuploidy status of the embryo. In this study, developed mathematical model proved successful in predicting the prior yet was unsuccessful in being informative about the latter. Detailed results about these investigations are presented within subsections below.

Parent Parameters Can Predict Embryo Morphology

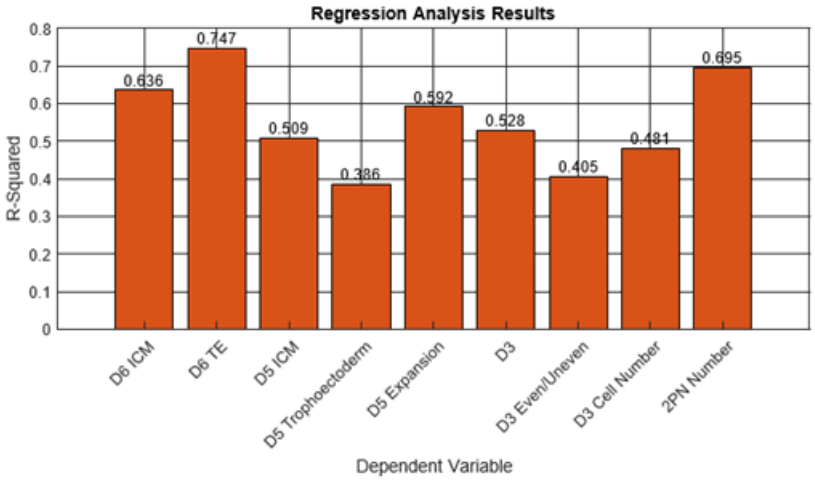
As detailed in the materials and methods section, a dataset consisting of information on 4123 blastocysts belonging to 765 couples undergoing ICSI treatment was investigated in its efficiency to offer a mathematical model capable of predicting the quality of the resulting embryos following the ICSI procedure. Associations between follicle numbers, MI oocyte numbers, MII oocyte numbers, sperm count, sperm motility and morphology, as the independent variables, and embryonic values constituting the dependent

variables were assessed via regression analyses. Outcomes of these queries showed significant correlations between all independent and dependent variables with the exception of D3 evenness status and the trophectoderm quality on day 5 (Table 8). R^2 values offer a representation of how good the regression model fits in explaining how much of the difference in dependent variables is tied to a variation in the independent variables. In the current study R^2 ranged between 0.386 – 0.747, showing that the model can explain 38.6% - 74.7% of the variance in dependent variables (Figure 22). Therefore, we conclude that the model can explain moderate – high variation.

Figure 22

Graph Depicting the R^2 Measurements of the Regression Analysis.

These values provide a measurement of how close the relationship is between the independent variables and each dependent variable. Typically, R^2 values between 0.3-0.5 denote low correlation, values ranging from 0.5-0.7 correspond to a moderate association and finally an R^2 value between 0.7-1 indicates a strong relationship between the assessed variables. Therefore, the current model can draw moderate to strong correlations between independent and dependent variables (Aytacoglu et al., 2025).



The results further suggest that the independent variables had a net positive correlation with the dependent variables (Figure 23). On the other hand, when considered individually this applied to majority of the independent variables but not all (Figure 24).

Figure 23

Graphical representation of the regression equations.

For each dependent variable, the current model predicts an overall positive correlation with the independent variables (Aytacoglu et al., 2025).

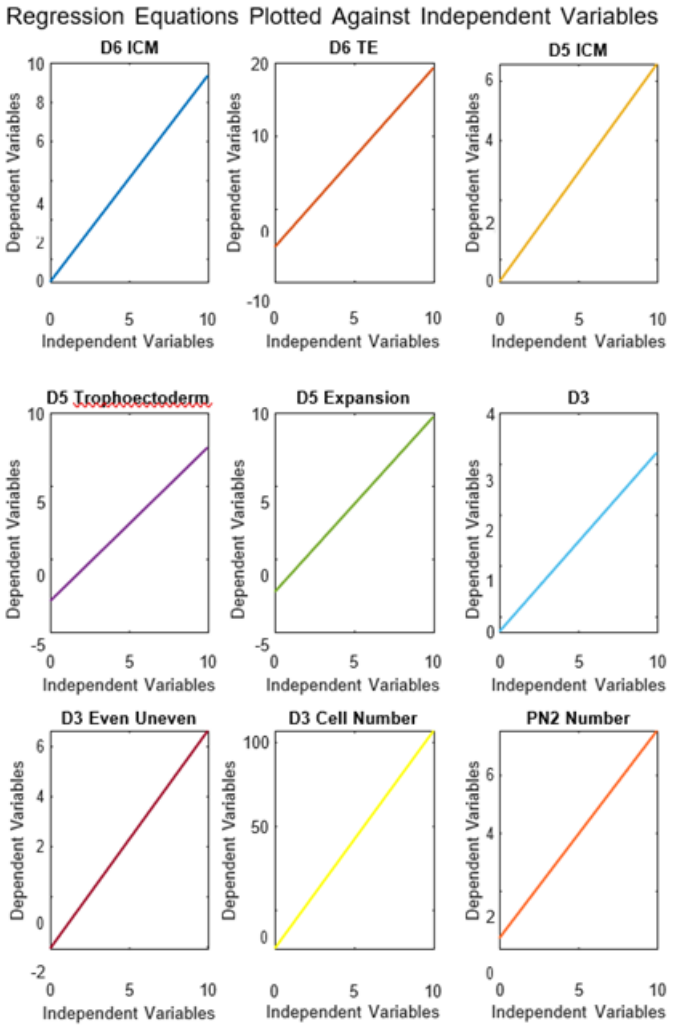
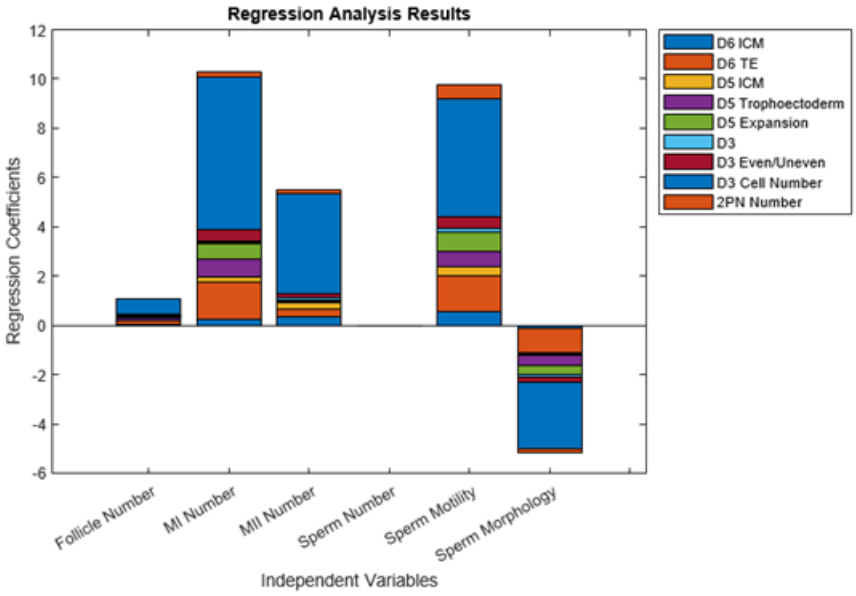


Figure 24

Graphical representation of regression coefficients of each independent variable.

Regression coefficients provide an indication on the degree of dependency between the variables. A positive denomination suggests a positive correlation and a negative value advocates an inverse correlation. Based on this, the current model draws positive correlations with most of the independent variables when they are evaluated individually. As opposed to this, sperm number by itself presented no direct association with any dependent variables and sperm morphology displayed an inverse correlation (Aytacoglu et al., 2025).



The associations drawn between the independent and dependent variables were statistically significant for almost all dependent variables, as underscored by a p value less than 0.05. Cell number evenness on day 3 and the trophectoderm status on day 5 were the only exceptions to this (Table 8).

The 2PN refers to the presence of two pronuclei in the embryo and thus proves successful fertilisation. Number of cells that showed 2PN presence was found to be influenced by independent variables. Firstly, the model drew a positive association between 2PN number and independent variables when considered as a whole (Figure 23). This correlation was

statistically significant (p=0.002). When taken individually, sperm motility, morphology, MI oocyte number were the top influencers on 2PN number. Conversely, sperm morphology showed an inverse correlation to 2PN number. Finally, the current model was able to explain roughly 70% of the variation observed for 2PN number, corresponding to an almost strong yet moderate association (R^2=0.695).

Next measurement was morphological parameters of day 3 following fertilisation. Such factors included D3, standing in for the number of nuclei within embryonic cells, D3 cell number, and lastly, D3 even/uneven denoting the evenness of the number of the cells on day 3 post-fertilisation. The model developed in this study was able to partially predict the outcome. Out of the three parameters stated, the model was able to predict the outcomes for D3 and D3 cell number (p=0.033 and p=0.023 respectively). The resolution of the associations for these two factors was low-moderate as established by the R^2 values 0.481 and 0.528 respectively. Overall independent variables showed a positive correlation with D3 and D3 cell number (Figure 23). When assessed individually, the most influential contributor of single nucleus presence within cells was found to be the sperm motility, closely followed by MI and MII oocyte number (Figure 24). For D3 cell number, the same independent variables retained their status as the most important contributors, however their order changed slightly. For D3 cell number, MI oocyte number showed the largest impact (Figure 24). On the other hand, day 3 evenness status could not be predicted by the current model, as any associations found were statistically insignificant (p=0.067).

The model also showed success in showing premonition potential about the morphology of the embryos 5 days after fertilisation. Day 5 characteristics under investigation included the quality of the expansion, trophectoderm and the inner cell mass. Among these variables, the model was unable to predict D5 TE, referring to the trophectoderm status (p=0.077). Conversely, relationships observed between the other two dependent variables were statistically significant, p=0.026 for D5 ICM and p=0.015 for D5 expansion. Furthermore, similar to day 3 cell number, MI oocyte number and MII oocyte number as well as sperm motility were the top influencers of

D5 ICM. Moreover, resembling day 3 cell number, MI oocyte number had the largest effect on D5 ICM (Figure 24). Regression coefficient or the R^2 value for D5 ICM was 0.509, indicating that the model can explain roughly 51% of the variation seen in D5 ICM by the independent variables. Therefore, the model can draw moderate associations between D5 ICM and independent variables. Expansion status on day 5 also showed a positive correlation with the independent variables (Figure 23). In contrast to D5 ICM, D5 expansion showed a similar profile to D3 in which sperm motility had the most substantial influence on. The model was found to have a moderate predictive potential for D5 expansion as well (R^2=0.592).

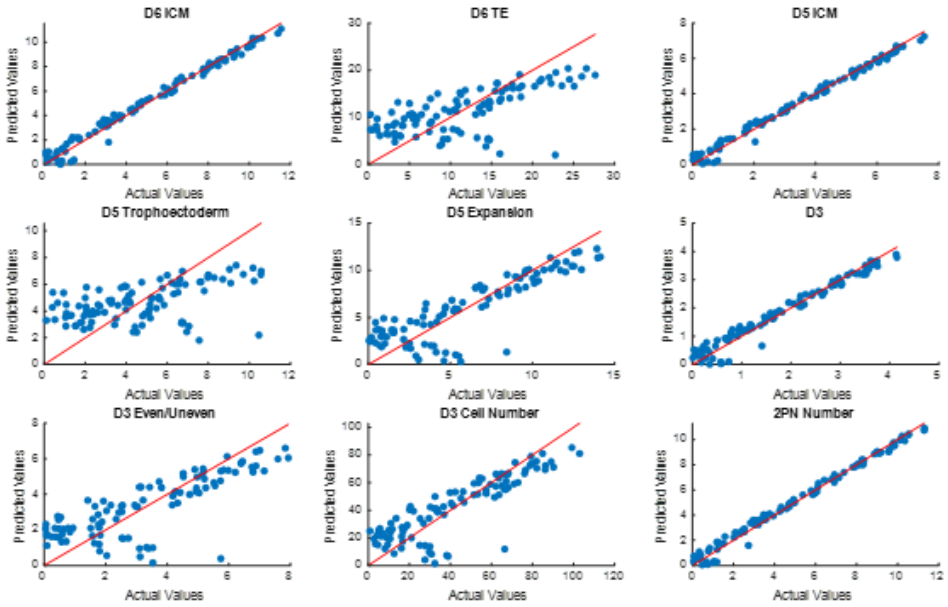
Lastly, morphological features of the blastocysts 6 days following fertilisation were investigated. The model was particularly successful at predicting day 6 outcomes. For both trophectoderm status and the quality of the inner cell mass positive correlations were observed with the combination of independent variable (Figure 23). For D6 TE, sperm motility, MI oocyte number and MII oocyte number were once again the biggest contributors with MI oocyte number ranking the top among the three (Figure 24). In addition, the model drew strong correlations between the dependent and independent variables, accounting for approximately 75% of the variance (R^2=0.747, p=0.001). On the other hand, D6 ICM had a moderate association (R^2=0.636, p=0.006). Furthermore, MI oocyte number was the biggest influencer of D6 ICM, followed closely by sperm motility and MII oocyte number (Figure 24).

The model was validated by plotting actual data obtained from the IVF centre against the predicted values. Validation plots further showed that the mathematical model developed had medium-strong accuracy (Figure 25).

Figure 25

Validation plot for the mathematical model.

Actual values plotted against the predicted values by the model shows that the model is particularly successful at predicting D6 ICM, D5 ICM, D3 and 2PN outcomes. Moreover, inconsistent distribution of actual values compared to predicted values for D5 TE and D3 even/uneven further support that the model was not able to successfully predict these variables (Aytacoglu et al., 2025).



Parent Parameters are Insufficient in Predicting Embryo Aneuploidy Status

The second main determinant of an embryo's quality is its aneuploidy status for ART purposes. Therefore, secondly this study aimed to predict aneuploidy status for resultant embryos with a mathematical modelling approach. Unfortunately, the current model proved insufficient in that endeavour as the regressions observed were statistically insignificant for every independent variable as can be observed in Table 9 ($p>0.05$). Moreover, Figure 26 also shows that the correlations between the independent variables and the aneuploidy status are weak. Lastly, figures Figure 27 and Figure 28 show that the error in the developed model is

minimal as evidenced by the high determination coefficient observed, despite the lack of significant associations observed concerning aneuploidy.

Table 9

Regression analysis results pertaining aneuploidy status of the resultant embryos.

All p-values are greater than 0.05 thus no significant associations could be made (Aytacoglu et al., 2025).

Variable	Coefficient	P-Value
Intercept	-0.12267	-
Follicle No	-0.001018	0.9791
MI No	-0.16904	0.2212
MII No	-0.014676	0.7594
Sperm No	0.1018	0.6301
Sperm Motility	0.0013029	0.8899
Sperm Morphology	-0.0012664	0.4456

Figure 26

Regression coefficients for independent variables against aneuploidy status of the resultant embryos.

The graph demonstrates that the correlations between independent variables and euploidy outcomes are weak (Aytacoglu et al., 2025).

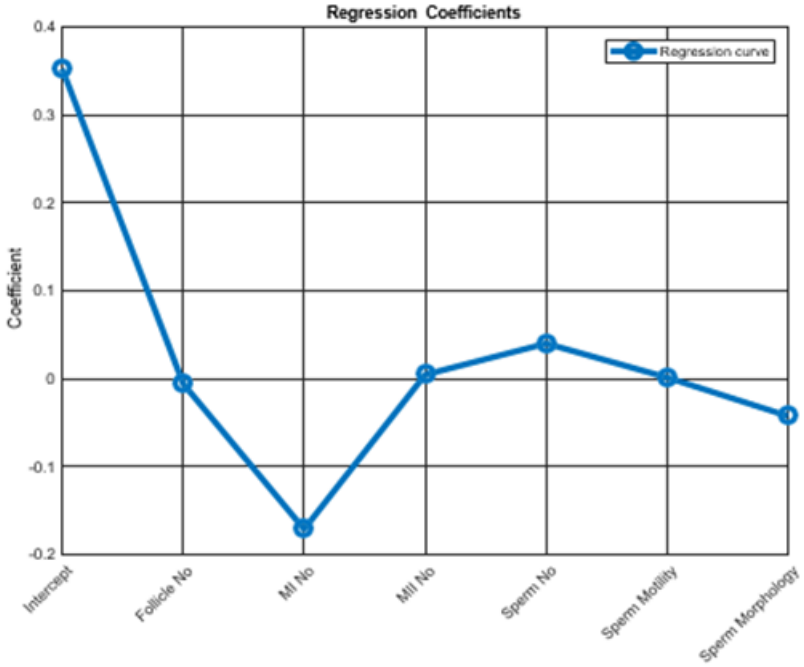


Figure 27

Representation of regression analysis combined with the actual data.

While no significant correlations were found, actual data fits the regression well, increasing confidence that the error in the mathematical model is minimal (Aytacoglu et al., 2025).

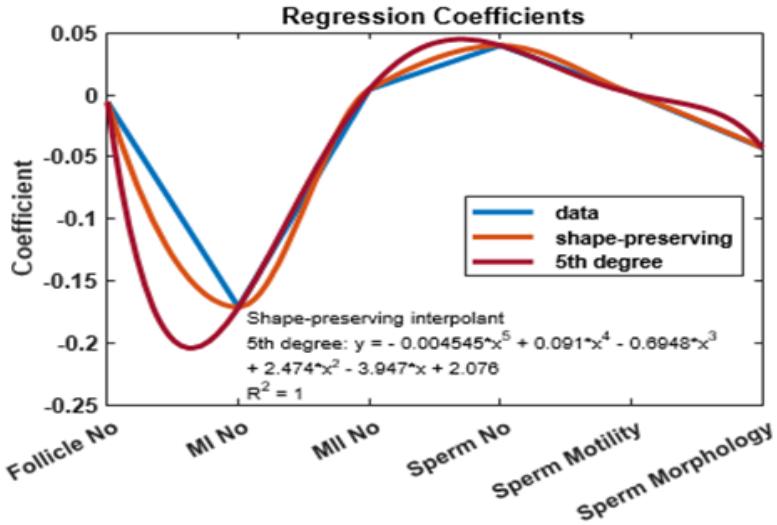
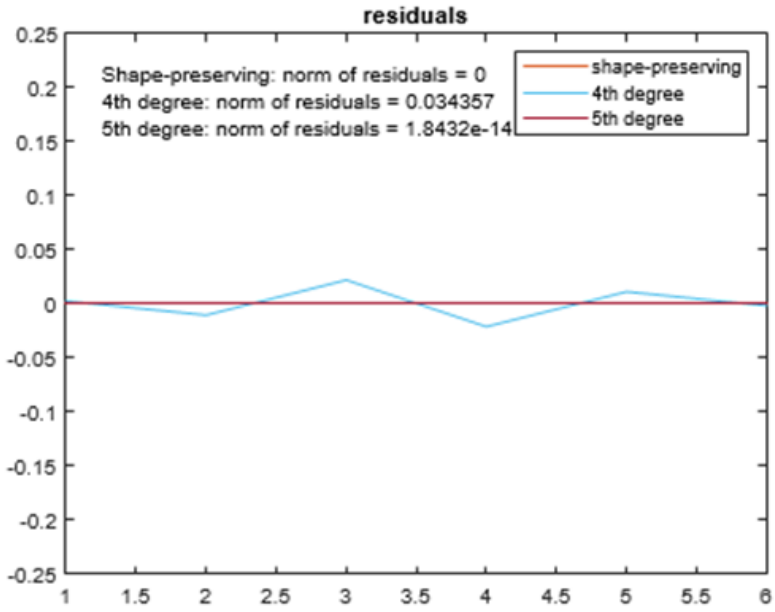


Figure 28

Graphical representation of the residual plot.

The plot both confirms that the data set is suitable for a regression analysis (Aytacoglu et al., 2025).



Embryo Morphology and Embryo Aneuploidy Status Share an Intricate Correlation

As the first part of the mathematical model has established, embryo morphology can be inferred from parental parameters. Following this, the final part of the model investigated whether embryo morphology can be indicative of its euploidy status. If so, parent parameters could then be utilised to infer euploidy status of the embryo in an indirect manner. For this third analysis, a logistic regression model was utilised. All p-values in this analysis were less than 5% ($p<0.05$) and thus results were deemed statistically significant. Among the morphological criteria, D3 cell no, D3 nucleus, D3 light/dark, D5 TE, D6 TE and D6 expansion correlated positively with euploid outcomes. Conversely, D3 fragmentation, D3 evenness, D5 expansion, D5 ICM and D6 ICM parameters associated inversely with euploid consequences. All these results are summarised in Table 10 and Figure 29.

Table 10

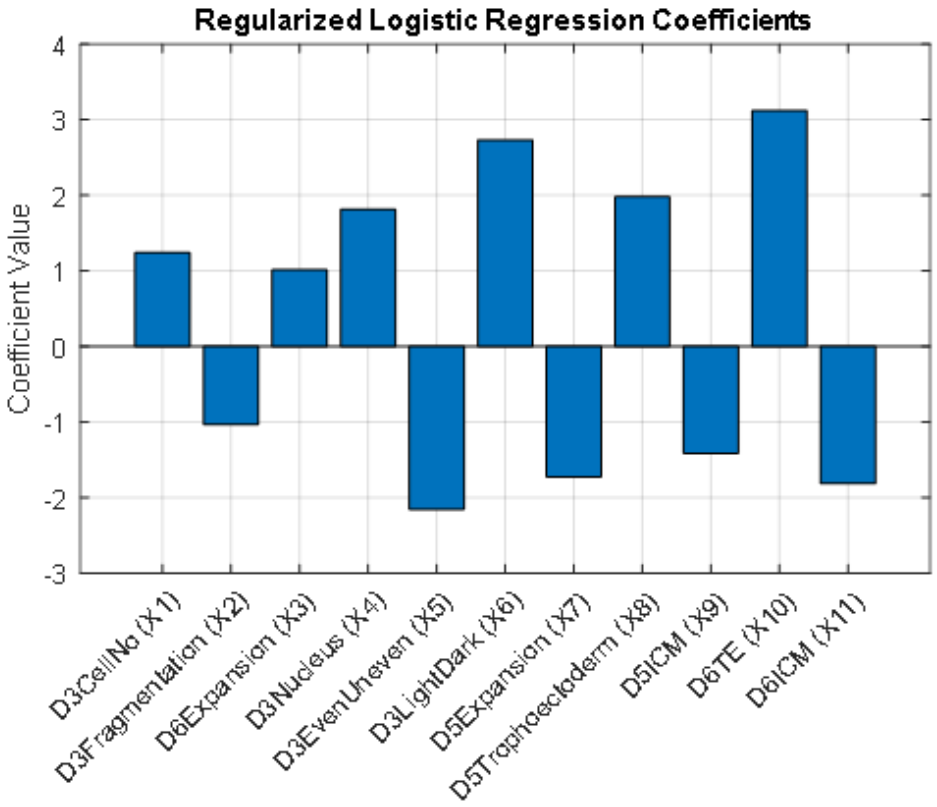
Detailed output of the results obtained from the logistic regression analysis. Statistical significance of the results was evaluated based on p-values where $p < 0.05$ denotes a statistically significant result. Coefficient values are indicators of the relationship between the dependent and independent variables. A positive denomination indicates a positive association whereas a negative value points towards an inverse correlation (Aytacoglu et al., 2025).

Predictor	Coefficient	Std. Error	t-Statistic	p-Value
Intercept	-1.50	0.50	-3.00	0.003
D3CellNo	0.02	0.008	2.50	0.012
D3Fragmentation	-0.04	0.009	-4.44	0.0001
D6Expansion	0.08	0.015	5.33	0.00001
D3Nucleus	0.50	0.12	4.17	0.0002
D3EvenUneven	-0.30	0.10	-3.00	0.003
D3LightDark	0.40	0.13	3.08	0.002
D5Expansion	-0.25	0.11	-2.27	0.02
D5Trophoectoderm	0.60	0.14	4.29	0.0001
D5ICM	-0.20	0.09	-2.22	0.025
D6TE	0.35	0.11	3.18	0.0015
D6ICM	-0.40	0.12	-3.33	0.001

Figure 29

Regularised Logistic Regression Model.

This figure presents the effect of regularization on model coefficients, demonstrating how the penalty term prevents overfitting and improves generalization by shrinking some coefficients (Aytacoglu et al., 2025).



Quality control for this model was achieved via the construction of a ROC curve, demonstrating how well the model fits to the actual data, which is presented in Figure 30. Additional performance measurements for the model's precision, accuracy, F1 score and recall are presented in Table 11.

Figure 30

ROC curve for the logistic regression model.

The ROC curve provides an illustration of the trade-off between sensitivity and specificity, thus presenting an evaluation of model performance. A higher area under the curve (AUC) corresponds to better classification capabilities for the model (Aytacoglu et al., 2025).

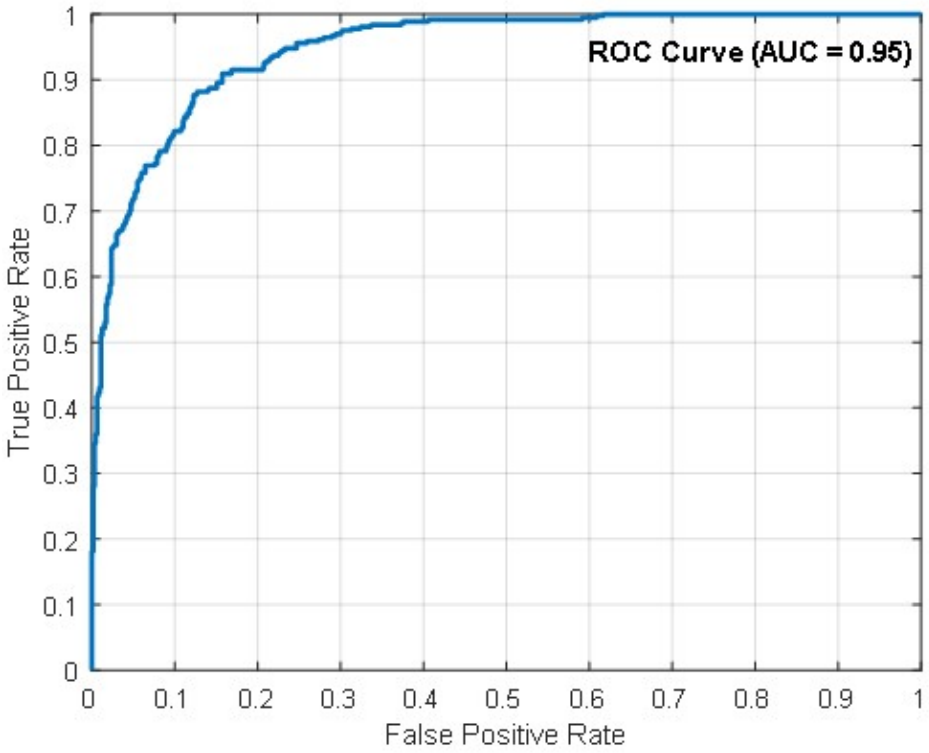


Table 11

Performance metrics of the model.

Accuracy represents the overall correctness of predictions, Precision indicates the proportion of positive identifications that were actually correct, Recall measures the ability to identify all relevant instances, and the F1 Score provides a balanced evaluation of Precision and Recall (Aytacoglu et al., 2025).

Metric	Value
Accuracy	88.00%
Precision	85.00%
Recall	90.00%
F1 Score	0.87

CHAPTER V

Discussion

This chapter aims to evaluate obtained results that have been presented in the previous chapter in great detail. Providing possible explanations for the observations made with relevant comparisons to the current literature and the body of knowledge.

For the first part of this thesis, 10 DDR and cell cycle checkpoint genes were investigated on a transcriptional level between euploid and aneuploid embryos. Namely, *ATR, ATM, CHEK1, CHEK2, RAD50, RAD51, TP53, PIK3CA, BRCA1* and *MDM2* genes were researched using the quantitative real time PCR. Among these, qRT-PCR has failed to yield data on *ATR, CHEK1, CHEK2, RAD50, TP53* and *PIK3CA*. There are two main explanations for this. First is the very simple explanation being that despite the numerous attempts at optimising the PCR conditions, these efforts have failed and thus the cDNA was not amplified whether it was present or not. In other words, PCR experiments failed to confirm or deny the presence or absence of these genes. The second reason could be that these six genes are not expressed in embryos. In the following paragraphs these two reasonings are mused on in broader detail.

First and foremost, qRT-PCR reactions may have been unsuccessful. To briefly reminisce the experimental procedure, total RNA isolation was carried out for all embryos, which were subsequently transformed into cDNA via reverse transcription reactions. Any errors or imperfections during these steps may have compromised the final PCR run. However, the earlier two steps incurring such detrimental effects to final PCR outcomes is unfeasible as the same cDNAs were successful in providing proper amplifications for other genes of interest such as *BRCA1, ATM, RAD51* and *MDM2.* However, one argument can be made for RNA isolation, where low amounts of RNA obtained may explain these results. Indeed, relatively low number of cells within blastocysts presents practical challenges in obtaining abundant and high quality RNA for the subsequent reactions. In isolations from primary tissue samples or organs this represents a lesser issue. On the other hand, lower number of available cells in blastocysts lowers the quantity of RNA

obtained significantly. Most DDR genes in healthy cells are tightly regulated at transcriptional and post-translational level as their over- or under-expression is associated with cancers and other various cellular perturbations (Bakkenist & Kastan, 2003; Batchelor et al., 2008; Bond et al., 2004; Brooks & Gu, 2006; Ellison & Stillman, 2003; Kumagai et al., 2006; J. Lee & Dunphy, 2010; S. Liu et al., 2011; Mordes et al., 2008; Mordes & Cortez, 2008; E. A. Nam et al., 2011; Richardson et al., 2004; Sun et al., 2007; Yoo et al., 2009). Furthermore, not all genes are expressed at the same level in cells. For instance, *B-ACTIN* or *ACTB*, a very commonly used housekeeping gene in many studies is expressed at a much higher level in cells compared to DDR genes. In fact, this is observable in the results of this study as well where CT values obtained for the housekeeping gene is lower compared to the gene of interest within the same cDNA template for analysis. A similar phenomenon exists among the DDR genes as well where not all are expressed at the same level. Therefore, it is possible that *ATR, CHEK1, CHEK2, RAD50, TP53* and *PIK3CA* are transcriptionally less active compared to *ATM, BRCA1, RAD51* and *MDM2*. In combination with RNA isolation challenges this could provide an explanation for why certain DDR genes were detected via qRT-PCR approach and others did not. On the other hand, main purpose of PCR is to amplify low amounts of a desired DNA template and thus it is inherently designed to amplify low levels of isolates. Indeed, there are many stories that exist in the scientific literature where isolates from even single cells have been successfully amplified by PCR. Moreover, *ATM*'s detectability in light of *CHEK2*'s inability to be amplified is surprising within this context. As outlined in the introduction section, *ATM* interacts with a multitude of downstream elements, though *CHEK2* is the main kinase it induces especially in a DDR pathway (Boonen et al., 2022). Hence, while not impossible, it is puzzling for *ATM* to be expressed at a level detectable by PCR whereas *CHEK2* would be expressed in a lesser degree to the extent that it couldn't be detected by the same methodology. Together these two reasonings undermine the confidence in this first explanation yet does not discredit it completely. On the qRT-PCR side of the experiment, typically optimisation tries involve changing of varying parameters. Over the course of this thesis, many adjustments were tried for optimising reaction

conditions. Some of which were, varying concentrations of primers (ranging from 0.3 µM – 0.6 µM), varying cDNA template volumes (ranging from 0.5 µl – 4 µl), adjustments to extension times within each cycle (ranging from 15 seconds – 45 seconds) as well as a range of annealing temperatures (± 6˚C of the Tm for each gene). In addition, differing annealing times per cycle was also tested (varying from 15 seconds – 1 minute). Furthermore, different SYBR Green reaction ready mixes from different brands were also utilised with these varying conditions. Unfortunately, despite all these efforts PCR runs for *ATR, PIK3CA, RAD50, TP53, CHEK1* and *CHEK2* were unable to generate amplifications. Results varied from primer dimers to null outcomes that were presented as blank both in real time PCR and in agarose gels that were ran for confirmation. Therefore, an alternative explanation for the lack of results must be considered.

Secondly, it is plausible that while some genes are expressed in the preimplantation embryos, some genes are yet to be activated. This phenomenon is not new to preimplantation embryos as it is well-known that the embryonic genome is inactive initially which is then activated post-blastocyst stage. For example, apoptotic gene expression was shown only to become prominent after embryonic genome activation (Regin et al., 2024). It is possible thus, that at the time of RNA isolation not all genes within the embryonic genome were activated and methylation pattern structuring was still ongoing. It is noteworthy that both *ATR* and *CHEK1* are among the genes that were inactive and their close relationship is in support of this hypothesis. In contrast, *ATM* being activated when *CHEK2* was not once again casts doubts on this notion. One counter-argument for such concerns could be that, *ATM* and *ATR* are capable of diverse interactions independent of *CHEK2* and *CHEK1* (Bekker-Jensen et al., 2009; Biswas et al., 2022; Doil et al., 2009; Flynn & Zou, 2011; Huen et al., 2007; Kolas et al., 2007; Y. Liu et al., 2011; Mailand et al., 2007; Meerang et al., 2011; Morris et al., 2009; Sobhian et al., 2007; Stewart, 2009; B. Wang et al., 2007; B. Wang & Elledge, 2007). Therefore, *ATM* and *ATR* activity may insinuate yet not necessitate *CHEK1* and *CHEK2* transcription. Nevertheless, these ideas need to be confirmed by further experiments. One improvement in this regard

would be to use high fidelity kits for RNA isolation that are more suited for lower cell numbers.

Among the genes that were successfully amplified by qRT-PCR reactions, *ATM, RAD51* and *MDM2* were found to be slightly altered in their relative expression levels although these differences were not statistically significant. On the other hand, *BRCA1* portrayed prominently increased transcription levels in aneuploid embryos compared to their euploid counterparts. In the subsequent passages possible causes for these observations will be investigated.

Initially, albeit less likely explanations, experimental causes must be reviewed. First and foremost, there were unfortunate yet unavoidable experimental differences between *BRCA1* and the other three genes. Most notably, *BRCA1* reaction was optimised ThermoFisher Life Sciences PowerTrack SYBR Green Mastermix (ThermoFisher, Cat. ID: A46109) whereas *ATM, RAD51* and *MDM2* were optimised with Hibrigen 2x SYBR Green qPCR Master Mix (Hibrigen, Turkey; Cat. No: MG-SYBR-01-400). Moreover, for *BRCA1* it was possible to obtain data from individual cDNA templates, whereas for the remaining three genes reactions could only be optimised with pooling of four cDNA templates together as explained in the materials and methods section of this work. While each reaction was normalised with their own SYBR using the Ct values obtained for *ACTB*, these differences may cause significant considerations for the reliability and comparability of data. The first observation regarding these considerations was that in experiments for *ATM, RAD51* and *MDM2*, there were primer dimers clearly observable alongside correct sized product bands. Further optimisation tests were run prior to data acquisition runs, yet the dimers were unavoidable and were prominent enough to give CT values in negative control. The levels of dimers appear to be similar across samples in all runs; hence it is not expected for the dimers to have an impact on the overall results. On the other hand, the situation is not ideal and it is impossible to rule out the chance of interference from those primer dimers on the analysis, regardless of its low probability. Secondly, there are different conclusions that can be drawn from the use of pooled samples. For instance, inability to optimise the qRT-PCR runs without a collective cDNA pool can indicate that

the initial RNA and cDNA levels were too low for *ATM, RAD51* and *MDM2*. Additionally, despite pooling cDNA amount could still be less than desired for the PCR reaction, at least in certain tubes which can explain why in certain mixes no data could be obtained despite the use of optimised conditions. Another impact of using a collective sample mix as template is that different cDNAs could have opposing effects ultimately masking the changes that could be present in individual embryos. Particularly for aneuploid sample pools this poses a threat. While care was taken to pool same aneuploidies into the same mix, in most cases this was practically not possible and varying aneuploidies had to be colluded together. Therefore, those pools when used as templates would in essence have effects that mimic complex or chaotic aneuploidies. Indeed, this complicates commenting on the findings as varying aneuploidies can show opposing effects as stated. Furthermore, *BRCA1* data was obtained by single cDNAs. Consequently, it can be questionable to compare the results of *BRCA1* with *ATM*, *RAD51* and *MDM2* despite in each analysis samples were normalised within themselves with the use of ΔΔCt analyses. Indeed, the use of ΔΔCt analysis improves confidence in the analyses despite the shortcomings, yet more prominent hypotheses are needed to explain the data acquired.

ATM, BRCA1, RAD51, MDM2 usually work together intimately especially in the repair of DSBs. As outlined in the introduction, BRCA1 cooperates with the MRN complex to promote end resection at DSB sites (Reczek et al., 2013). In addition, BRCA1 acts as a scaffold for BRCA2 and PALB2 to recruit RAD51 to location of the breaks under HR response which appears dependent on CHEK2 mediated phosphorylation of BRCA1 (Roy et al., 2011). However, this does not mean they cooperate exclusively. Concurrently, different signals and pathways can also recruit these proteins. One example of such an event is that BRCA1 involvement in S-phase checkpoint activity is induced by CHK1 which is the downstream kinase of ATR (F. Sadeghi et al., 2020). In contrast, this association appears to necessitate ATM to phosphorylate BRCA1 as well as MRN complex's influence (J. H. Lee & Paull, 2004, 2005; B. Xu et al., 2001). This is not the only pathway of BRCA1 recruitment. RAP80 complex can act as another recruiter of BRCA1 in the activation of G2/M checkpoint which later induces

CHK1 (Kim et al., 2007). Another key element of this pathway is CtIP-BRCA1, a complex formed by the namesake proteins as this complex is responsible for the phosphorylation of CHK1 (Escribano-Díaz et al., 2013; Yu & Chen, 2004). On the other hand, G2 accumulation checkpoint brought on by DNA damage is regulated by the BRCA1-BACH1 complex as opposed to CtIP-BRCA1 (Yu & Chen, 2004). Which is consistent with prior studies that demonstrated that BRCA1's role in HR repair is quite different compared to its other roles in checkpoint regulation and utilise varying pathways (J. Zhang et al., 2004). Furthermore, even same proteins may play diverse roles in different pathways leading to distinct outcomes. For example, BRCA1 prevents MRE11 from initiating degradation of stalled replication forks (Bunting et al., 2012). In contrast, in a DSB repair context, BRCA1 needs to cooperate with CtIP as well as the MRN complex to promote resection at the broken DNA ends (L. Chen et al., 2008). BRCA1 also has been shown to have roles in G1/S checkpoint, working together with ATM which phosphorylates TP53 to induce p21 (Fabbro et al., 2004; Shorrocks et al., 2004). Collectively these studies indicate that these DNA repair and cell cycle checkpoint genes can have diverse and complex interactions that are heavily context dependent. Therefore, it is plausible that *BRCA1* can have elevated transcription independent of *ATM*, *RAD51* or *MDM2*. One example of its alternative activation could be via *CHEK1* activity which is generally induced by *ATR* (Businо et al., 2003; Melixetian et al., 2009). Although, *ATR* and *CHEK1* transcription were not detectable by qRT-PCR in the current study as discussed previously. This lack of transcriptional confirmation can be suggestive of their inactivity which casts doubts on this theory.

There are further mechanisms that may explain elevated expression levels of *BRCA1* independent of *ATM* and its downstream kinases. Oestrogen is a known inducer of BRCA1. Previous studies have demonstrated that oestrogen induction increases *BRCA1* levels both on a transcriptional level and a translational level (Gorski et al., 2009). Experiments in mice showed similar results where *Brca1* levels elevated at the time of puberty as well as pregnancy, following a positive association with rising oestradiol levels. Oestradiol was able to increase *BRCA1* levels even in ovariectomised animals (T. F. Lane et al., 1995; Marquis et al., 1995).

Oestrogen levels may pose as a viable explanation for why *BRCA1* levels may have risen in aneuploid embryos without altering *ATM*, *RAD51* and *MDM2* transcription. If this is the case, the differences in oestradiol levels are unlikely to come from incubation conditions following ICSI procedure as the media and incubators would be the same standardised protocol within the same clinic. However, evidence exists within the literature that embryos are reactive towards oestradiol levels (Logsdon et al., 2023; Melnick et al., 2015; Tsutsui et al., 1987) yet there is no conclusive evidence that oestrogen levels differ between aneuploid and euploid embryos. *BRCA1* results are insinuating that this could be an interesting research area to be pursued.
An additional BRCA1 regulator is retinoblastoma protein commonly known as Rb. Rb achieves its influence on *BRCA1* levels by adjusting E2F activity (A. Wang et al., 2000). Furthermore, BRCA1 itself can form complexes with E2F-1 as well as Rb to form a negative autoregulatory loop to inhibit its own transcription. In cases of genotoxic stress however, BRCA1 can be displaced from the *BRCA1* promoter to counter the incoming DNA damage thus re-allowing the upregulation of *BRCA1* (De Siervi et al., 2010). As opposed to this, Rb-E2F pathway is also known to activate *ATM*, *CHEK1* and *RAD51* (Berkovich & Ginsberg, 2003; Iwanaga et al., 2006; Verlinden et al., 2007). Furthermore, inactive Rb pathway has been shown to promote cellular proliferation which can be TP53-dependent or independent (Macleod et al., 1996; Morgenbesser et al., 1994; A. Wang et al., 2000). Another activator of BRCA1 is c-Myc (Y. Chen et al., 2011). It also can have indirect effects on gene expression patterns as it has been connected with DNA methyltransferases which suggests it can have epigenetic influences on gene expression patterns (Brenner et al., 2004). Indeed, epigenetic regulation has also been demonstrated by previous studies to influence *BRCA1* levels in breast cancer cells (Butcher & Rodenhiser, 2007; Esteller et al., 2000; Seery et al., 1999; C. Stone et al., 2003; M. Wei et al., 2005; J. Xu et al., 2009). Nevertheless, c-Myc is also associated with *ATM, ATR, CHEK1* and *RAD51*. Specifically, ATM has the capacity to suppress overexpression of c-Myc (Najnin et al., 2023). In addition, c-Myc can activate ATR/CHK1 pathway to confer tolerance to stalled replication forks as well as activating RAD51 to resolve them (Krüger et al., 2018; Luoto et al., 2010). Moreover, two other

mechanisms for *BRCA1* expression control have been identified. First the mechanism of nucleosome occupancy, referring to the chance of a specific DNA region to be entangled by histone proteins in a population of cells (J. C. Lin et al., 2007). A high occupancy indicating highly condensed regions that are mostly unavailable to transcription factors, whereas low occupancy suggests a structure mainly open for transcription. Secondly, microRNAs have been shown to regulate *BRCA1* activity (Iorio et al., 2005). Last but not least, both Rb/E2F pathway and c-Myc have associations with other genes of interest of this study such as *ATM* and *RAD51*. Combined with the fact that the expression levels for these were unchanged unlike *BRCA1*, stimulation by Rb or c-Myc seems unlikely to explain the single upregulation observed for *BRCA1*, though it is important to consider that based on different contexts varying pathways can be induced as stated before. In contrast, methylation appears to be a more likely candidate for an explanation. Specifically, *BRCA1's* epigenetic regulation in preimplantation embryos has already been demonstrated (Tulay et al., 2016). Intriguingly, throughout the development of an embryo it was shown that methylation patterns can follow stage-specific changes and certain genes such as regulatory genes, pluripotency genes and differentiation genes show specific methylation patterns underlining the importance of epigenetics in embryonic gene expression (Paranjpe & Veenstra, 2015; Tomizawa et al., 2011; M. T. Zhao et al., 2013).

When investigating gene expression patterns, an understanding of how the genes are regulated on a transcriptional level can be obtained. Nevertheless, transcription is only one part of the proverbial equation. Translational controls and post-translational modifications also play an important role. Particularly in the case of critical DNA damage repair genes, most activity control is accomplished post-translationally. ATM as an example, is constantly present in the cells as a dimer which is then activated by the MRN complex by monomerization brought on by proper phosphorylation and acetylation modifications (Bakkenist & Kastan, 2003; S. So et al., 2009; Sun et al., 2007). Interestingly, ATM also has autoregulatory functions which is prominent for its sustained localisation at the DSB sites which requires the assistance of complexes MDC1 and MRN (Dupré et al., 2006; S. So et al., 2009). Furthermore, it is important to stress that ATM is

known to possess a multitude of phosphorylation sites, which makes defining its exact post-translational mechanisms an enormous and difficult task as not all of these phosphorylation sites are associated with its roles in the DDR mechanism (Kozlov et al., 2011). It is highly likely ATM's unique phosphorylation patterns whether by itself in an autoregulatory fashion or by other interactions may have varying consequences that are dependent on the cellular context its being utilised in. A similar phenomenon can be observed for RAD51. Among normal cells, expression as well as activity levels of *RAD51* are under strict controls to avoid erroneous DNA recombination (Richardson et al., 2004). In fact, *RAD51* levels were found to be significantly elevated in various cancers (Maacke, Opitz, et al., 2000; Sarwar et al., 2017; X. Zhang et al., 2019). RAD51 is usually constitutively present in cells which is then recruited to DSB sites with the actions of BRCA1, BRCA2 and PALB2 instead of being transcriptionally activated (Orhan et al., 2021; Roy et al., 2011; F. Sadeghi et al., 2020). In contrast, other studies have demonstrated that DNA damage strengthens *RAD51* transcription (Aboussekhra et al., 1992; Barrington et al., 1999; Basile et al., 1992; Shinohara et al., 1992). There are also studies that have identified several transcriptional regulators for *RAD51*. For instance, E2F1, EGR1 as well as CDK12 were identified as positive regulators of *RAD51* transcription whereas E2F4, E2F7 and TP53 are transcriptional repressors of *RAD51* (Arias-Lopez et al., 2006; Orhan et al., 2021). Therefore, regulatory mechanisms for *RAD51* appear to be multilayered and complex. Intriguingly, CDK12 is known to have the capacity for inducing *BRCA1, ATM* and other DDR genes' transcription as well (Blazek et al., 2011; Krajewska et al., 2019). E2F1, E2F4 and E2F7 were also shown to be phosphorylated under DNA damage conditions which was hypothetically attributed to *ATM, ATR* and *CHEK2* (W.-C. Lin et al., 2001; Orhan et al., 2021; Stevens et al., 2003). Considering the unchanged mRNA levels for *ATM* and *RAD51* as well as null mRNA observed for *ATR* and *CHEK2*, our findings suggest that activation of *BRCA1* most likely comes from pathways independent of *RAD51* regulators such as CDK2, E2F1, E2F4 or E2F7. Similarly, MDM2 also appears to be ever-present in cells as a regulator of TP53 activity. Naturally, *TP53* levels within a cell is regulated very carefully and similar to *ATM* and *RAD51*, TP53 is also constantly present at

low levels. Definitive machinations that lead to the activation of TP53 are yet to be elucidated, yet they are thought to involve mainly post-translational changes (Brooks & Gu, 2006). This poses a great significance for MDM2 as well as its activity is to assist TP53 to form a negative feedback loop to guide its own expression levels (X. Wu et al., 1993). On the other hand, regulation of *BRCA1* levels within cells are relatively more transcription oriented. In particular, how genotoxic stress affects *BRCA1* expression levels is still a topic of debate in the field. Certain studies have identified that genotoxic stress downregulate the expression of *BRCA1* in a TP53-dependent manner (Andres et al., 1998; Fan et al., 1998). Other investigations have concluded that levels of *BRCA1* fluctuate over time where there is an initial peak of *BRCA1* levels after encountering a genotoxic stress which is then gradually lost as TP53 activation ensues (Clarkin et al., 2000; MacLachlan et al., 2000). Although, TP53 was deemed not essential for inhibition of BRCA1 as well (Andres et al., 1998). *BRCA1* also possesses a capacity for autoregulation where it can bind to its own promoter to suppress its own transcription as stated above (De Siervi et al., 2010). In the current study protein levels were unable to be analysed. Hence, it is not possible to gauge the extent of post-translational alterations on the overall activity levels of *ATM, RAD51, MDM2* AND *BRCA1*. However, *ATM*, *RAD51* and *MDM2* are more likely to be post-translationally regulated as opposed to transcriptionally. This could pose a very prominent reason why *BRCA1* was found to be significantly elevated when *ATM*, *RAD51* and *MDM2* mRNA levels showed no significant alterations between euploid and aneuploid embryos. Therefore, it would be paramount to investigate protein levels in future studies to understand clearly if there is an increase in the activity of *ATR, RAD51* and *MDM2* despite no transcriptional changes were observed.

MDM2 have additional pathways it is involved in that merit further considerations. Firstly, MDM2 is known to impact cell cycle progression by interacting with pRb, a Rb-family protein (Castéra et al., 2010). MDM2 can degrade pRb and eliminate its repression of E2F allowing the cell cycle to continue (Freitas, 2019). In addition, MDM2 was shown to have high correlation with steroid regulation, specifically for oestrogen (Portman et al., 2020; Zafar et al., 2023). MDM2 together with TP53 regulates the turnover of

ERα (Duong et al., 2007; Leclercq et al., 2006). Moreover, *MDM2* overexpression enforces the growth of ERα-positive breast cancer cell lines and has been shown to form a positive feedback loop to feed its own overexpression thus perpetuating this growth (Brekman et al., 2011; Caldon, 2014). As stated previously, Rb/E2F as well as oestrogen are regulators of BRCA1 activity as well. In combination, however, these two pathways do not seem to be the reason behind *BRCA1*'s increased transcription in aneuploidies as *MDM2* expression was not affected. Nevertheless, as Rb/E2F and ERα also belong to various other pathways it is not easy to disregard their possible involvement.

ATM/CHEK2 pathway usually recruits BRCA1 downstream which in turn recruits RAD51 to induce HR (Roy et al., 2011; San Filippo et al., 2008). Considering this in combination with the findings of this study where *ATM* and *RAD51* levels remained unchanged as opposed to an elevation of *BRCA1* in aneuploid embryos, it can be theorised that the elicited response in these blastomeres is not HR. If only *ATM* mRNA levels remained constant it may not have been sufficient to rule out HR by itself as it was demonstrated that BRCA1 is essential in HR mediated repair of DSBs whereas ATM was dispensable (Kass et al., 2013). Nevertheless, steady mRNA levels in both *ATM* and *RAD51* suggests an alternative response may have been activated in aneuploid blastomeres. An exciting possibility is the attempt at recovering SAC function. Spindle Assembly Checkpoint is one of the most prominent defences a cell has against chromosomal anomalies during cell division. Moreover, in a previous study BRCA1 has been shown to be essential for MAD2 function which is a key member of the SAC system (R. H. Wang et al., 2004).

Numerous ART systems have evolved over the years to treat various infertilities and/or to help people conceive. While revolutionary advancements have been made in the field, success rates of these techniques remain lower than ideal thus leaving many bereft of any hopes of parenthood (Kushnir et al., 2017; Qiao et al., 2014; Sadeghi, 2018; C. Thompson, 2016; Wong et al., 2014). One of the methods for improving success rates in ART applications like IVF and ICSI procedures involves next generation sequencing technologies for identifying aneuploidies albeit while incurring heavier

economical burdens on an already relatively expensive procedure (Coates et al., 2017; Doroftei et al., 2022; Fishel et al., 2010). Emerging technologies have allowed computers to assist clinicians in infertility clinics with making the correct treatment decisions. However, these options so far have only attained supporting roles and lack prognostic capabilities (Simopoulou, Sfakianoudis, Antoniou, et al., 2018; Simopoulou, Sfakianoudis, Maziotis, et al., 2018). Enabling prognostic or predictive capabilities in such novel tools can allow patients to give more informed decisions on whether or not to commit to such arduous and expensive pursuits in the future. The first step in such an endeavour is to find a system that can make accurate predictions as to the resulting embryos quality. While embryo quality does not warrant successful pregnancies, it improves the chances greatly and embryo quality is used as the main criterion for choosing embryos currently (Hossain et al., 2016; Niinimäki et al., 2015). Embryo quality typically pertains to two aspects, first one being the morphological quality of the embryos and the second being the chromosomal composition mainly assessed as euploidy versus aneuploidy. As mentioned previously in this thesis, there are studies on assessing embryo quality with the use of artificial intelligence and imaging systems. However, these methodologies' main drawback comes from their reliance on imaging systems which may prove expensive and not widely accessible across all clinics. Furthermore, the process necessitates the formation of the embryo to be imaged and thus even improved it possesses more potential as a diagnostic approach as opposed to a predictive one. Therefore, in this thesis we aimed to use a mathematical modelling approach which has the capability to be transformed into a computer software that can predict possible success rates allowing clinicians to choose better suited treatments for each patient as well as allowing the patients to decide whether to pursue one treatment option or not in a better-informed manner.

The first step towards a predictive algorithm, was to investigate possible connections between parent parameters (as outlined in the materials and method section of this thesis) and the morphological quality of the resulting embryos. Briefly, parent parameters appear to be medium to strong predictors of embryo quality. For all parameters except for D3 even/uneven and D5 TE, statistically significant correlations were drawn

($p<0.05$). Strongest association was found between the parent parameters and D6 TE quality where approximately 75% accuracy was observed ($R^2 = 0.747$). Other studies that utilised artificial intelligence in tandem with embryo imaging reported various rates of success (Kromp et al., 2022). The best performance observed with such tools were 72% accuracy in predicting ICM quality, 73% in trophectoderm and 77% for expansion status. Interestingly, among various AI models, trophectoderm accuracy was regularly the lowest (Kromp et al., 2022). Taken together, these suggest that mathematical modelling not only can be a viable substitute for AI based systems but can also be superior in some aspects, as it shows higher accuracy in trophectoderm predictions. Notably, current AI studies are generally coupled with expensive time-lapse imaging systems that may not be widely available. Therefore, mathematical models that can predict embryo quality even before their inception based on parent parameters show additional advantages over such systems. Evaluating the prediction accuracy for individual embryogenic morphology criteria, the developed model appears to be capable of making moderate to high accuracy predictions for most of the parameters. For D3 even/uneven and D5 TE, the model shows low accuracy predictions ($0.3 < R^2$ value < 0.5) albeit these predictions appear statistically insignificant ($p>0.05$). In the case of D3 even/uneven, prediction may be complicated by the inherent differences in the cell division rates between different embryos. Inability to predict trophectoderm status on day 5 is more intriguing as the same model shows the biggest success in predicting day 6 trophectoderm quality. This phenomenon may have been brought about by the inherent weaknesses of day 6 embryos by making them more susceptible to other factors such as parent parameters thus making them easier to predict. Although the data regarding the use of day 5 or day 6 embryos remain contradictory in the literature, later developing embryos, such as day 6 embryos, are typically associated with higher rates of chromosomal abnormalities when compared to day 5 blastocysts (Park et al., 2020; Zakaria, Steven, et al., 2021). Furthermore, day 6 blastocysts have been shown to have up to 10% more aneuploidies (Davie et al., 2017; T. H. Taylor et al., 2014). Embryos with lesser developmental haste also show abnormalities in mitotic spindle formations as well as other molecular

abnormalities (Hashimoto et al., 2013; Wood et al., 2007). Anomalies with the spindle formation can also be attributed to their dampened expression in day6 blastocysts (Hsieh et al., 2004). An extended time period spent in *in vitro* culture conditions have also been shown to exacerbate negative effects on development and debilitate cleavage in relation to the laboratory conditions as opposed to innate deficiencies attributed to the embryos (Abdelmassih et al., 2001). Collectively, these studies may indicate that as the embryos grow more susceptible to different effectors like spindle anomalies, molecular aberrations and/or laboratory conditions, their susceptibility to parent factors also increase and thus their morphology particularly the trophectoderm, in light of being the site of the biopsies, becomes easier to predict. Another interesting notion could be that normally developing blastocysts could be activating the embryonic genome faster compared to day 6 or later blastocysts. This may suggest that they are at least in part shielded against inherited anomalies from the maternal oocyte and/or the paternal sperm. While not experimentally proven yet, this may present an explanation for a weaker predictive potential for day 5 embryos in comparison with day 6 embryos. On the other hand, embryos that reach blastocyst stage on day6 have been observed to have similar implantation rates with their day5 counterparts, under the condition they show same or similar morphological quality as them (Sunkara et al., 2010). Significance of this remark is two-fold. Firstly, if similar morphological qualities warrant similar implantation outcomes, the model's inability to predict day 5 TE when showing immense prediction potential for day 6 TE raises new questions with regards to its reliability. In contrast, if day 5 and day 6 embryos behave similarly with the same morphological qualities then being able to predict day 6 TE status can be equated with day 5 TE status with regards to implantation potential. In other words, in a practical case, day 6 TE prediction may prove sufficient for indicating implantation potential of the resulting embryo. However, these require further experiments to firmly delineate such implications.

For ICM prediction, the model was able to successfully predict both day 5 and day 6 ICM quality with moderate strength. Similarly to TE, the model was able to infer slightly stronger associations with day 6 ICM as

opposed to day 5 ICM. To explain this relative superiority, same principles as the trophectoderm case can be put forth. Namely, weaknesses associated with day 6 embryos previously such as spindle anomalies, and molecular anomalies which were presented in the previous paragraph may be causing the later developing embryos to show a higher sensitivity to other external factors like parent parameters investigated here. This observation holding true for both trophectoderm and ICM comparisons between day 5 and day 6 embryos is constituting a more compelling argument for this hypothesis. Furthermore, the longer an embryo is kept under culture conditions *in vitro*, a higher risk of developmental debilitations is incurred as stated in the previous paragraph (Abdelmassih et al., 2001; Zakaria, Steven, et al., 2021). Finally, if embryos with a normal development rate have earlier embryonic genome activation as opposed to their slower developing counterparts, they may be shielded against inherited deficiencies via activated repair mechanisms. While these require further research to confirm, consistency of better predictions for day 6 embryos when compared to day 5 embryos increases the possibility of such hypotheses as well as strengthening the confidence in the current model's performance.

Among the remaining outcomes regarding embryo morphology, the model was able to account for 48.1% of D3 cell number corresponding to a weak association. This may indicate that the replication speed at the cleavage stage may be affected by other factors such as environmental influences more than the innate characteristics of the oocyte or the sperm cells. On the other hand, nuclei status of day 3 embryos as well as expansion status on day 5 were managed to be moderately predictable by the model. Finally, 2PN number was also predicted at a moderate strength (69.5%) and was close to high levels of association. Fertilisation success' intimate connection to parent parameters is not very surprising, however the missing approximately 30% is stressing of other factors playing a role in fertilisation yet still. Indeed, with the current model three parameters showed high or higher than 60% predictability. Aside from the 2PN number, the other parameters both belonged to day 6 datapoints. Stronger associations made in day 6 observations when compared to slightly lower R^2 values seen for day 3 and day 5 parameters is supportive of later developing embryos having

a higher susceptibility to the effects of parent factors as discussed previously. The model also holds potential for further improvement with the addition of other parameters like the parent ages. In particular, maternal age has been associated with blastocyst quality for a long time (Janny & Menezo, 1996). In contrast, preliminary results from another study of our group showed maternal age to possess a non-significant association with embryo morphology when using a revised model.

In the current literature, there are only 5 aneuploidies that are known to associate with live births. Namely these are the aneuploidies of chromosomes 13, 18, 21, X and Y. Therefore, many clinics around the world has adopted an embryo selection process that includes an aneuploidy analysis based on biopsied cells, PGT-A. The necessity for taking biopsies to conduct the test, however, is considered to be the culprit behind the lower-than-expected success rates achieved by the employment of PGT-A in embryo selection (Awadalla et al., 2022; Paulson, 2017). Therefore, being able to predict an embryo's aneuploidy status without compromising its integrity can be paramount in elevating the success rates for PGT-A and consequently all ART endeavours. Thus, the second part of this project aimed to predict aneuploidy status of embryos based on the parent parameters. This evaluation was once again conducted by employing regression analyses. While the devised mathematical model was successful at predicting embryo morphology at a moderate to high level, it lacked the same accomplishment in predicting the aneuploidy status of the embryos. In this study, aneuploidies were categorised uniformly without considering either varied single aneuploidies or multiple aneuploidies. Inability to separate aneuploidies despite this generalised classification is highly indicative that aneuploidy is a very complex phenomenon that is not solely dependent on oocyte and/or sperm quality. Indeed, cleavage anomalies depending on factors other than inherent characteristics of the embryos have been documented in the literature (Abdelmassih et al., 2001). A time-lapse imaging AI learning module was tested in a similar capacity in another study which achieved high (approximately 70%) accuracy (Chavez-Badiola et al., 2020). Undoubtedly impressive, the biggest drawback of this study was the obligatory use of time-lapse imaging which reduces its wide-scale

applicability across world-wide clinics. Therefore, a mathematical model could prove a worthy substitute that can have a farther reach. Regrettably, all parameters showed very minor associations with regards to aneuploidy prediction as indicated by coefficients (Table 9). In addition, regardless of the coefficients none of the parameters yielded p values lesser than 0.05 and thus any effects the parent parameters may exert on the embryos does not appear to be sufficient to alter their euploidy outcomes. On the other hand, higher calibre models that utilise machine learning approaches may be able to identify more complex relationships between parent parameters and euploidy status of resulting embryos and hence presents an exciting avenue of investigation. Curiously a recent study has attempted to guess euploidy outcomes based on blastocyst morphology with assistance from maternal age and embryo imaging technologies with an accuracy of almost 70% (Barnes et al., 2023). While the imaging necessity in this study raises the same concerns as Chavez-Badiola's work (2020), blastocyst score was found to be one of the most influential factors on predicting euploidies (Barnes et al., 2023). As mathematical model developed in this thesis can predict embryo morphology, it was plausible to try and combine the two findings together to try predicting the euploidy status of embryos based on an initial prediction of embryo morphology. Undoubtably, making predictions in succession like this would be compromising to the integrity and accuracy of the final prediction. Nevertheless, if the accuracy remains high between blastocyst morphology and euploidy status, this could still present a viable strategy. In order to test this, the third and final part of this modelling study aimed to find correlations between the morphological quality parameters of an embryo and outcomes regarding its euploidy. Unlike Barnes and colleagues (2023), in our investigation maternal age and embryo images were not incorporated into the predictor pool. In a case high accuracy of predictions could be achieved like such an approach, then the first model used to predict embryo morphology based on parent parameters could be integrated into an euploidy prediction without the need for any additional measurements or equipment such as a time-lapse imaging incubator hence such a methodology was chosen. To conduct this investigation a logistic regression model was developed. This model demonstrated that all

morphology parameters showed significant influence on the embryo euploidy status ($p<0.05$). D3 cell number, D3 nucleus, D3 light/dark, D5 trophectoderm, D6 trophectoderm as well as D6 expansion were positively correlated with euploidies evidenced by the positive coefficients put out by the model in Table 10. These associations were somewhat expected as better quality morphology insinuates healthier genetic components thus lesser chances of aneuploid observations provides a straightforward assumption. Specifically, a better quality trophectoderm corresponding to euploid PGT-A results is quite elementary as trophectoderm is the site where biopsies are taken for the aneuploidy test. Intriguingly, not all independent variables showed a positive association with euploidies. Namely, D3 fragmentation, D3 evenness, D5 expansion, D5 ICM as well as D6 ICM demonstrated an inverse correlation with euploidy. As stated before, better morphological qualities bringing about higher chances of euploidies is an easy assumption to make. Therefore, these inverse correlations are perplexing. For D3 fragmentation an inverse correlation can be considered natural, and it has been documented in the literature previously that highly fragmented cells often bore aneuploid results (Cecchele et al., 2022; Lagalla et al., 2020). Thus, the devised model concurs with the current literature on higher fragmentation rates indicating higher incidences of aneuploidies. Despite this, contradictory evidence remains in the literature and thus this point may be open for debate still. In 2018, one study concluded that fragmentation percentages for day 3 embryos were not significant indicators of aneuploidies (Pasternak et al., 2018).

Next, evenness of the embryos appears to negatively impact their chances of turning out euploid. An even appearance during the morula stage is considered to be morphologically superior. Thus, having such a morphological advantage causing aneuploid outcomes is surprising. Morula evenness is tightly linked to the number of cells at that stage shown by multiple studies. For instance, one recent study has concluded that 8-cell embryos possess the best general evenness and higher or lower cell numbers than 8 showed a comparatively more uneven structure (J. Wang et al., 2022). An earlier paper also demonstrated that embryos with six to nine cells have better chances for euploidy as opposed to their counterparts that

possess 9 or more cells (Kroener et al., 2015). While this finding concurs with our model's output for cell number, it may also suggest that having a cell number closer to 8 may have a positive influence on euploidy via promoting evenness in addition to having a favourable cell number in the morula. On the other hand, having an excess of 9 cells declines the rate of aneuploidies, insinuating that the relationship between cell number, embryo evenness and euploidies may not be as directly correlated as it may initially appears. Indeed, another research project has concluded that embryos that are constituted of 11 or more blastomeres imitate 8-cell morulas more with regards to aneuploidies when compared with 9-11 cell morulas (Pons et al., 2019). Collectively, these findings indicate that the relationship between cell number on day 3, evenness on day 3 and the euploidy result may have a more complex nature. Currently developed model outputting a direct correlation with cell number and an inverse relationship with day 3 evenness may be a result of their antagonistic effects as evidenced in the literature. A balance of these opposite forces may be the real influencer of euploidies. Furthermore, an improvement to future modelling endeavours could be implemented that analyses cell number in distinct groups where embryos that have less than 9 cells, 9-11 cells and more than 11 cells are segregated into different categories to be investigated separately with respect to their evenness as well as PGT-A outcomes. While such an approach may be difficult to achieve with simple regression analyses, stronger modelling toolkits like machine learning assisted models may accomplish such a feat. Such an approach may broaden our understanding of the complex interactions between cell number, morula evenness and euploidy.

Another interesting outcome of the logistic regression analyses was the opposing coefficients for day 5 expansion and day 6 expansion. To reiterate, a positive coefficient corresponds to a positive correlation whereas a negative coefficient value indicates an inverse relationship. Therefore, day 5 expansion and day 6 expansion are showing antagonistic effects on euploidy which is perplexing. Previous discoveries have shown that expansion rates and euploidy usually take a concurrent path (T. T. Huang et al., 2019). Thus, the result obtained from D6 expansion was not surprising. Nevertheless, D5 expansion demonstrated the opposite effect with euploid

outcomes. While unexpected, there can be a few explanations for this observation. Firstly, perhaps, increased cell division rate posits a reason. In the cases where expansion score is too high too early during the blastulation process, due to excess cell division, lesser cell cycle checkpoint activity at this stage could lead to higher aneuploidy incidences. Indeed, in the first part of this thesis, many DNA repair pathway genes were not have been able to be detected amongst euploid or aneuploid embryos using a qRT-PCR approach as discussed before. While this may certainly be brought on by the inabilities of the methodology, it could also indicate that these genes are not significantly expressed in early stage blastocysts. Which can bolster this theory. Granted, more studies to are required for precise confirmation and understanding in this regard. Secondly, patient group profile may have had played a role in this finding. Namely, one study had found that embryos develop into blastocysts significantly slower, especially in 35 year old or older patients. This is of particular note as in our dataset, mean ages for both genders clustered around 30-40 as well. The same study also concluded that if waited longer than the common 5-6 day period, a little over 43% of usable embryos would demonstrate an euploid profile (Tiegs et al., 2019). Therefore, this study is in line with our observation that slower blastulation on day 5 might still be capable of becoming euploid embryos, or in other words, it points towards a possibility of an inverse correlation between day 5 expansion and euploidies. In addition, this outlook can also explain the reversal of the correlation direction for day 6 expansion, as it shows that further development can indeed result in euploid embryos.

By far the most interesting finding of the logistic regression analyses was the inverse correlation observed between both day5 and day6 ICM quality and euploidies. Inner cell mass, is where the embryo proper originates from, hence a better quality ICM should be heralding greater quality embryos with better chances of becoming euploid. Though these results are surprising, there is evidence in the current literature that may support our findings regarding ICM. For instance, one theory exists within the field that embryos possess self-correcting mechanisms resembling extrusions of the blastomere where aneuploid blastomeres are shunted to the trophectoderm (Lagalla et al., 2020). This exile would concurrently enrich the trophectoderm

with aneuploid cells and improve the quality of the ICM. PGT-A analyses use cells biopsied from the trophectoderm and therefore this phenomenon can explain how the ICM can remain in good quality and develop into euploid embryos despite an elevation in observed aneuploidies, thus creating a contrary association. On the other hand, findings from diverse studies within the current literature also indicate that the connection between ICM quality and euploid results by PGT-A may not be as straightforward as it might initially assumed. Moreover, this statement appears particularly applicable for patients aged over 30. A prior study had demonstrated that in patients aged less than 35 years of age as well as patients aged 35-39 ICM quality was not significantly correlated with euploid results (Coates et al., 2015). In addition, for the age groups younger than 35 years of age, overall embryo quality was not significantly associated with euploidies in the same study (Coates et al., 2015). One other study had shown that individual ICM grades may not share a relationship with positive foetal heartbeat. Specifically, the study identified that grade B ICM scores didn't correlate with a positive foetal heartbeat. In this particular research venture, mean ages for patient groups were 33.4 and 34.4 (Abdala et al., 2022). Though a comparison with foetal heartbeat and euploidy is difficult to draw, this study still enforces the idea that for ages above 30, ICM grade may not necessarily be an indication of clinically beneficial outcomes. While non-significant findings can support the notion that ICM quality may be independent than euploid results, more convincingly other studies exist where negative associations between morphology and euploid outcomes were observed. One body of work had demonstrated that excellent grade embryos would show a higher rate of aneuploidies. Researchers had attributed their findings to the higher tendency of better embryos' to be biopsied in contrast to low quality embryos (Salame et al., 2024). A facsimile of this principle also applies to our dataset as most embryos tested by PGT-A were of high quality and were being considered for implantation. Further evidence is available in the literature. One other body of research has demonstrated that when comparing excellent, good and average grade embryos observed euploidy rates were 56.4%, 39.1% and 42.8% in each group respectively (Capalbo et al., 2014). Indeed, excellent grade embryos showed the highest euploidy rates but when comparing good

and average quality embryos a clear negative correlation is observed between embryo grade and its euploidy status. Thus, it is inferable that the connections between embryo quality and an euploid PGT-A result is more complex than initially assumed. All in all, this complexity appears to be exceptionally prominent when maternal age is between 30 and 40. As the data used for the mathematical model has a majority of patients within this age group, this can posit a possible explanation for the surprising results observed for day5 and day6 ICM quality and euploidies. As discussed above, non-significant or inverse correlations between euploidies and embryo morphology is evident within the current literature. However, other confounding effects may have been the cause for the intriguing results obtained from our model as well. Two prominent examples can be named as the causes of infertility in the couple and the day of the biopsy, as both have been shown to alter these associations (Salame et al., 2024; Thang et al., 2024). Another consideration that is essential is the potential differences in embryo grading criteria and variations in laboratory procedures between different clinics and studies. Therefore, any comparisons and generalisations that may be made between different studies must be done with caution which was suggested previously (Thang et al., 2024). Nonetheless, overall, developed model seems to concur with the current literature.

The developed model was successful in drawing correlations between parent parameters and embryo morphology but was unsuccessful in associating the same parent parameters with PGT-A results. Finally, it was able to correlate embryo morphology with euploidy. Consequently, combining these two predictions to estimate the euploidy status of an embryo can prove to be a viable strategy. Certainly, predictions made in succession in such a way could undermine its ultimate accuracy. While this relatively simple modelling technique can provide a basis for decisive prediction models, integrating similar strategies with higher forms of modelling methods such as machine learning models or AI calculations can also posit a viable improvement in future research.

All in all, mathematical model presents an acceptable substitute for time-lapse imaging/AI based arrangements. Not necessitating any additional equipment or data collection, mathematical models have the unparalleled

capacity for cheaper and a much wider-scale applicability over such systems. Furthermore, the model can prepare a basis for a more structured computer software where a clinician can simply enter the relevant data and embryo viability predictions are calculated by the machinery. In addition to these practical advantages, results obtained via analyses of this model once again highlights the complexity of the factors governing the morphology and euploidy of embryos, thus contributing to the current literature on this topic. An embryo's overall quality and hence it's viability naturally depends on both its morphology and it's euploidy. Therefore, inverse correlations observed with this model again highlights the complexities of creating a prognostic software in ART clinics. In particular, developed mathematical model shows a strong affinity for quality prediction of day5 and day6 embryos. In clinic, embryos are most commonly transplanted to the mother candidate at these developmental stages which makes this observation a very eminent one.

As with many studies, this one is also not devoid of limitations. Firstly, the model's prediction of a negative association among sperm morphology and embryo quality remains startling and opposing to the current literature. In addition to the above discussions, it is also pertinent to point out that mathematical models generally identify stochastic associations between variables and thus there is always a possibility of random influences affecting the outcome. More powerful modelling tools such as ones that utilise machine learning approaches may assist in reducing this randomness as well as drawing a generally better fitting model. Secondly, this model was derived from a dataset obtained from a single clinic and thus is composed of data that followed a uniform lab procedure across all patients. This uniformity can be a strength as well as a hindrance. Data obtained from a consistent treatment and laboratory practice regiment can alleviate randomness within the data, hence reducing the effects of confounding variables. On the other hand, it lessens the generalisation capacity of the findings as well as hindering the analysis strength of certain variables. Notably, ICSI uses a single sperm cell for insemination of the oocytes. This can have an invalidating effect on sperm number and its association with embryo morphology as regardless of the total sperm number observed a single sperm will be injected into the oocyte. Consequently, this impacts the

applicability of the findings in other clinics with diverse laboratory practices as well as its generalisability across various ART techniques even within the same clinic. Last but not least, developed mathematical model was incapable of predicting aneuploid embryos strictly from parent parameters.

Historically, aneuploidies of chromosomes 13, 18, 21, X and Y are the sole ones that have been documented to result in live births. Despite the successful birth, such individuals are typically afflicted with serious conditions in their postnatal life, the most well-known example being trisomy 21 also known as Down syndrome. In light of that, many ART clinics chose to utilise PGT-A tests to choose euploid embryos for implantation. However, PGT-A testing is widely considered to have been unsuccessful in attaining the projected success rates it would bring. It has been considered that the obligatory biopsies to perform the test is also hindering its success rates due to its damaging nature (Wells, 2010). Lack of success has led many IVF centres to abandon this practice disharmonising the treatment approaches across different clinics. Nonetheless, the power of being able to predict aneuploidies prior to implantation remains undeniable and thus it appears imperative to find alternative methods to accomplish this goal. For this purpose, in this study the potential of parent parameters to predict embryo morphology and aneuploidies were being investigated which the latter was an unsuccessful aspect of this venture. The dataset in this study was derived from 4123 embryos from 765 diverse couples allowing for distinct yet comprehensive data to be examined for developing a strong model. Overall, the model was successful in providing a foundation for a precise prediction software as well as supporting many long-theorised phenomena in the field that are difficult to investigate experimentally. The model also holds potential for further improvements in the form of addition of further parameters. A few potential candidates are ages for maternal and paternal candidates, as well as at which day the biopsies were obtained from the embryos. Specifically for maternal age, prior data is highly suggestive of a strong influence on embryo morphology as well as its euploidy chances (Barnes et al., 2023; Zhan et al., 2020).

All in all, while not perfect, mathematical modelling approach was successful in predicting embryo morphology and showed the potential for

predicting euploidy status based on this morphology. Indeed, not being able to separate euploid and aneuploid embryos directly from parent parameters presents a weakness for this approach, however the findings are sufficient for forming a baseline for development of a prognostic software to be used in ART clinics worldwide. It is envisioned that specifically strong predictions in day 6 criteria to have important implications for improving success rates in ICSI endeavours.

CHAPTER VI

Conclusions and Suggestions

In this thesis, it was aimed to understand the roles of DDR genes under aneuploidy conditions. According to the results of this study, HR mechanism seems to be inactive in aneuploid embryos and increased transcription of *BRCA1* must be attributed to its roles independent of HR mediated repair mechanisms. There are two significant improvements that can be done for this part of the study. Firstly, findings regarding gene expression profiles should also be investigated on a protein level to understand the impact of post-translational modifications. Secondly, though the difficulty of limited cell numbers make experimentation difficult, if possible same samples and preferably not pooled cDNA templates should be used to acquire more accurately comparable data. Nevertheless, challenges will remain as it will be difficult to isolate proteins and RNA from the same blastocyst for downstream analyses.

A mathematical model was also developed to understand how parent parameters can affect embryo morphology and karyotype. Developed model was successful at predicting embryo morphology to the extent that it can compete with image based AI systems on trial for similar purposes. However, ease of access to mathematical models can confer an edge to such a model in this context. Unfortunately, the same model failed to predict aneuploidies directly based on parent data but results suggest an indirect prediction may be possible. These findings also reinforce the complexity of mechanisms behind aneuploidies. With expanding knowledge in the field, however, the model can easily be expanded upon to make it stronger in aneuploidy predictions. Furthermore, stronger modelling methods such as AI or machine learning based systems can increase predictive power.

Overall, this body of work extends the knowledge on aneuploidies, one of the biggest problems faced in IVF clinics. An elevation in *BRCA1* levels is particularly interesting as it could suggest a role in aneuploidy corrections, though more experiments are required to precisely delineate this role. Furthermore, the mathematical model successfully provides a basic

background for development of predictive and diagnostic softwares that can improve the quality of care in ART clinics around the world.

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APPENDICES

Appendix 1. Board of Ethics Approvals

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi :28.10.2021
Toplantı No : 2021/96
Proje No :1432

Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Pınar Tulay'ın sorumlu araştırmacısı olduğu, YDU/2021/96-1432 proje numaralı ve **"The Molecular Regulation of Oocyte Formation and Preimplantation Embryo Development"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Prof. Dr. Şanda Çalı
Yakın Doğu Üniversitesi
Bilimsel Araştırmalar Etik Kurulu Başkanı

Kurul Üyesi	Toplantıya Katılım	Karar
	Katıldı(✓)/ Katılmadı(X)	*Onay(✓)/ Ret(X)*
Prof. Dr. Tamer Yılmaz	X	—
Prof. Dr. Şahan Saygı	✓	✓
Prof. Dr. Nurhan Bayraktar	✓	✓
Prof. Dr. Mehmet Özmenoğlu	X	—
Prof. Dr. İlker Etikan	X	—
Doç. Dr. Mehtap Tınazlı	✓	✓
Doç. Dr. Nilüfer Galip Çelik	✓	✓
Doç. Dr. Emil Mammadov	✓	✓
Doç. Dr. Ali Cenk Özay	✓	✓

https://etikkurul.neu.edu.tr/

NEAR EAST UNIVERSITY
SCIENTIFIC RESEARCH ETHICS COMMITTEE

RESEARCH PROJECT EVALUATION REPORT

Meeting date :28.09.2023
Meeting Number :2023/116
Project number :1766

The project entitled **"The Molecular Regulation of Oocyte Formation and Preimplantation Embryo Development: Mathematical Modelling"** (Project no: NEU/2023/116-1766) has been reviewed and approved by the Near East University Scientific Research Ethical Committee.

Prof. Dr. Şanda Çalı
Near East University
Head of Scientific Research Ethics Committee

Committee Member	*Decision*	*Meeting Attendance*
	Approved (✓) / Rejected (X)	*Attended (✓) / Not attended(X)*
Prof. Dr. Tamer Yılmaz	✓	✓
Prof. Dr. Şahan Saygı	✓	✓
Prof. Dr. İlker Etikan	✓	✓
Doç. Dr. Mehtap Tınazlı	X	X
Doç. Dr. Nilüfer Galip Çelik	X	X
Doç. Dr. Dilek Sarpkaya Güder	✓	✓
Doç. Dr. Gulifeiya Abuduxike	✓	✓
Doç. Dr. Burçin Şanlıdağ	✓	✓

Appendix 2. Similarity Report

Hakan Aytacoglu PhD Thesis

ORIGINALITY REPORT

9%	3%	8%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Hakan Aytacoglu, David Amilo, Bilgen Kaymakamzade, Evren Hincal, Onder Coban, Pinar Tulay. "Influence of oocyte and sperm parameters on the morphological quality and the aneuploidy status of the ICSI embryos: a mathematical modelling approach", Zygote, 2025 Publication	4%
2	theses.hal.science Internet Source	1%
3	"Targeting the DNA Damage Response for Anti-Cancer Therapy", Springer Science and Business Media LLC, 2018 Publication	<1%
4	"DNA Replication", Springer Science and Business Media LLC, 2017 Publication	<1%
5	Ebru Emekli-Alturfan. "Zebrafish Models of Neurodegenerative Disorders", CRC Press, 2025 Publication	<1%

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7	Dimitris J. Panagopoulos. "Electromagnetic Fields of Wireless Communications: Biological and Health Effects", CRC Press, 2022 Publication	<1 %
8	Iwakun, Oluwasegun Abraham. "Investigating the Role of NEIL3 in Colorectal Cancer Resistance to Oxaliplatin", University of Salford (United Kingdom), 2022 Publication	<1 %
9	Sajal Chakraborti. "Handbook of Proteases in Cancer - Cellular and Molecular Aspects", CRC Press, 2024 Publication	<1 %
10	"Overcoming Breast Cancer Therapy Resistance", Springer Science and Business Media LLC, 2024 Publication	<1 %
11	repository.upenn.edu Internet Source	<1 %
12	Sajal Chakraborti. "Handbook of Proteases in Cancer - Therapeutic Aspects", CRC Press, 2024 Publication	<1 %

13	Yongtai Bai, Weibin Wang, Jiadong Wang. "Targeting DNA repair pathways: mechanisms and potential applications in cancer therapy", Genome Instability & Disease, 2020 Publication	<1 %
14	Rosalva Mora-Escobedo, Gloria Dávila-Ortiz, Gustavo F. Gutiérrez López, José J. Berrios. "Health-Promoting Food Ingredients during Processing", Routledge, 2024 Publication	<1 %
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16	studenttheses.uu.nl Internet Source	<1 %
17	Plasencia, Jessel Ayra. "Study of the Cellular Response, Signaling, and Repair Pathways to Confront DNA Double-strand Breaks in Telophase", Universidad de La Laguna (Canary Islands, Spain), 2022 Publication	<1 %
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Contemporary Understanding", Routledge, 2024
Publication

20	Haroon Khan, Muhammad Ajmal Shah, Tarun Belwal, Eduardo Sobarzo-Sánchez. "Phytonutrients in the Treatment of Breast Cancer - Molecular Aspects and Therapeutic Potential", CRC Press, 2025 Publication	<1 %
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23	Kim, HaEun. "Investigating the Role of mTor in Histone Methylation", McGill University (Canada), 2024 Publication	<1 %
24	Abraham Fainsod, Sally A. Moody. "Xenopus - From Basic Biology to Disease Models in the Genomic Era", CRC Press, 2022 Publication	<1 %
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	Embryonic Stem Cells", Cold Spring Harbor Laboratory, 2024 Publication	
26	Knoblochova Lucie, Duricek Tomas, Vaskovicova Michaela, Zorzompokou Chrysoula et al. "CHK1-CDC25A-CDK1 regulate cell cycle progression in early mouse embryos to protect genome integrity", Cold Spring Harbor Laboratory, 2022 Publication	<1 %
27	Fu, Richard Z.. "Investigating Oscillatory Gene Expression in Glioblastoma.", The University of Manchester (United Kingdom) Publication	<1 %
28	Michels, David C. R.. "Endothelial Cell-Specific Loss of Breast Cancer Susceptibility Gene 2 Exacerbates Atherosclerosis", The University of Western Ontario (Canada), 2022 Publication	<1 %
29	Fonseca, Irina Suheila Martins Leal. "Understanding PLK4's Role in Cancer", Universidade NOVA de Lisboa (Portugal), 2024 Publication	<1 %
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40	Adesuwa, Iseghohimen J.. "Overcoming Drug Resistance: Targeting the BCL-2 Family and the Long Non-Coding RNA HCP5 in Medulloblastoma and Colorectal Cancer", University of Salford (United Kingdom), 2024 Publication	<1 %
41	Colin Stok, Nathalie van den Tempel, Marieke Everts, Elles Wierenga et al. "The FIGNL1-interacting protein C1orf112 is synthetic	<1 %

lethal with PICH and mediates RAD51 retention on chromatin.", Cold Spring Harbor Laboratory, 2022
Publication

42	Fabian-Kolpanowicz, Kim Jadwiga. "Reversal of Centrosome Amplification to Reduce Oncogenicity of Metastatic Uveal Melanoma", Lancaster University (United Kingdom), 2025 Publication	<1 %
43	orcid.org Internet Source	<1 %
44	westminsterresearch.westminster.ac.uk Internet Source	<1 %

Exclude quotes	Off	Exclude matches	< 5 words
Exclude bibliography	On		

Appendix 3. Curriculum Vitae

CURRICULUM VITAE

1. **Name Surname** : Hakan Aytaçoğlu
2. **Date of Birth** : 09 August 1994
3. **Title** : MSc.
4. **Educational Background :**

Degree	Field	University	Year
Bachelor's	Genetics (Hons)	University of Manchester	2016
Master's	Cancer Research and Molecular Biomedicine	University of Manchester	2017
PhD.	Medical Genetics	Near East University	2019-Current

5. Certificates Received:

6. Institutions Worked :

MISSION TITLE	INSTITUTION	YEAR
INTERN	**MERSIN UNIVERSITY, PLANT PHYSIOLOGY LABORATORY**	**AUGUST 2014**
INTERN	**MIDDLE EAST TECHNICAL UNIVERSITY, INSTITUTE OF MARINE SCIENCES, GENETICS LABORATORY**	**JULY-AUGUST 2015**
FULL TIME EMPLOYEE	**NEAR EAST UNIVERSITY, MEDICAL GENETICS DEPARTMENT, SARS-COV2 DIAGNOSIS LABORATORY**	**JULY 2021 – FEBRUARY 2022**
RESEARCH ASSOCIATE	**NEAR EAST UNIVERSITY, FACULTY OF MEDICINE, DEPARTMENT OF MEDICAL GENETICS**	**SEPTEMBER 2021 - CURRENT**

7. Academic Titles

Assistant Professorship Date :

Associate Professorship Date :

Professorship Date :

8. Supervised Master's and Doctoral Theses

8.1. Master's Theses

8.2. Doctoral Theses

9. Publications

9.1. Articles published in journals covered by SCI, SCI-EXP, SSCI, AHCI

- **Hakan Aytacoglu**; David Amilo; Bilgen Kaymakamzade; Evren Hincal; Onder Coban; Pinar Tulay (2025). Influence of oocyte and sperm parameters on the morphological quality and the aneuploidy status of the ICSI embryos: a mathematical modelling approach. Zygote, Volume 33, Issue 3, June 2025, pp. 163 – 178. DOI: https://doi.org/10.1017/S0967199425100099.

- Benedict Marshall; **Hakan Aytacoglu**; Onder Coban; Pinar Tulay (2024). Assessment of the expression levels of two long non-coding RNAs, lnc-CYP11A1-1 and RP11573D15.8, in human aneuploid and euploid embryos. Zygote , Volume 33 , Issue 1 , February 2025 , pp. 19 – 22. DOI: https://doi.org/10.1017/S0967199424000492

- M. Aktan**, H. Aytaçoğlu**, B. Özbakır & P. Tulay (2023). Regulation of CYP11A1 Gene by lncRNAs in Human Oocytes Obtained from Patients with Polycystic Ovaries. Russian Journal of Genetics Volume 59, pages 106–109. DOI: https://doi.org/10.1134/S102279542313001X

- Ahmed, M.; **Aytacoglu, H**.; Coban, O.; Tulay, P. (2023). Investigation of *BAK*, *BAX* and *MAD2L1 g*ene expression in human aneuploid

blastocysts. Zygote , Volume 31 , Issue 6 , December 2023 , pp. 605 – 611. DOI: https://doi.org/10.1017/S0967199423000539

9.2. Articles published in journals covered by at least one international index

- ABDULMOHSIN, D., **AYTACOGLU, H.**, KOCAMAZ, G., KUKNER, A., & TULAY, P. (2025). Does taking L-Carnitine and Cadmium have an impact on gene expression of the liver tissues from rat models?. *Bangladesh Journal of Medical Science*, *24*(2), 614-620. DOI: https://doi.org/10.3329/bjms.v24i2.81728

- Rai, W., **Aytaçoğlu, H**., Özbakır, B., Özverel, C. S., Kandemis, E., & Tulay, P. (2024). LncRNA regulation in human oocytes obtained from patients with polycystic ovaries. *Academic Journal of Health Sciences*, *39*(4), 149-153. DOI: 10.3306/AJHS.2024.39.04.149

9.3. Papers Presented at International Scientific Conferences and Published in Proceedings

- Mihad Ahmed, **Hakan Aytacoglu**, Ozgur Coban, Pınar Tulay. 'Investigation of BAK, BAX, and MAD2L1 gene expression in human aneuploid blastocysts'. European Biotechnology Congress 2022, Prague-Czechia, October 5-7, 2022 – Presentation.
- Duaa Abdulmohsin, **Hakan Aytacoglu**, Gamze Kocamaz, Aysel Kukner, Pınar Tulay. 'Investigation Of L-Carnitine and Cadmium Impact on mTOR Pathway-Related Genes Obtained From Rats Liver'. European Biotechnology Congress 2022, Prague-Czechia, October 5-7, 2022 – Presentation.
- Yussuf M. Abdi, **Hakan Aytacoglu**, Onder Coban, Emine Kandemiş, C. Serhan Özverel, Pınar Tulay. 'Role of LncRNAs in Human Aneuploid Blastocysts'. European Biotechnology Congress 2022, Prague-Czechia, October 5-7, 2022 – Presentation.

- **Aytacoglu, H.**, Ozbakir, B., Tulay, P. ‘Effects of Cigarette Smoking on Sperm Quality and Quantity’ European Biotechnology Congress 2021, Hybrid Conference, September 23-25, 2021 – Online Presentation

9.4. International Books or Book Chapters

9.5. Articles published in national peer-review journals

- **AYTAÇOĞLU, H.**, ÖZGE, A., KÖSTEKÇI, İ., TAŞDELEN, B., ÖKSÜZ, N., & TOROS, F. Research Article The Effects of Daily Variables on Primary Headache Disorders in High-School Children; A Proposal For A Cut-off Value For Study/Leisure Time Regarding Headache Types. *headache*, *10*(20), 38.

9.6. Papers Presented at National Scientific Conferences and Published in Proceedings

- Warda Rai, Hakan Aytaçoğlu, Mehmet Aktan, Burcu Özbakır, Pınar Tulay. ‘Polikistik Over Tanısı Konulan Hastaların Oositlerinde lncRNA Regülasyonu’. 7. Erciyes Tıp Tıbbi Genetik Kongresi, Kayseri – Türkiye, Mayıs 26-28, 2022 – Sunum

- Çiftçi, Ozan; Karahan, Arzu; Tutar, Özge; **Aytaçoğlu, Hakan**; Kıdeyş, Ahmet ErkanKuzeydoğu Akdeniz’deki iki kıyı bitki türünün (Posidonia Oceanica ve Pancratium Maritimum) DNA barkod ve genetik çeşitlilik analizleri. I. Ulusal Denizlerde İzleme ve Değerlendirme Sempozyumu (2016)

9.7. Other Publications

- Mehmet Aktan, **Hakan Aytacoglu**, Burcu Ozbakir, Pınar Tulay, ‘Investigating lncRNA expression patterns in human oocytes from patients with polycystic overies (PCO)’. European Human Genetics Conference, Glasgow- UK June 10-13, 2023 – Online Poster

10. Projects

11. Administrative Duties

- Turkish Cypriot Association of Human Genetics Executive Committee…… 2022-2025

- Near East University, Cancer and Genetics Research Centre Executive Committee……...2025-Currently

11. Memberships in Scientific and Professional Associations

- Turkish Cypriot Association of Human Genetics…… 2022-2025

- Near East University, Cancer and Genetics Research Centre……2025-Current

12. Awards

13. Undergraduate and Graduate Courses Taught in the Last Three Years

Academic Year	Semester	Course Name	Weekly Hours		Number of students
			Theo.	Prac.	
2022-2023	FALL	TBG403-Prenatal Gelişimde Teratoloji ve Danışma	3	0	6
2022-2023	FALL	MBG403-Teratology in Prenatal Development and Counselling	3	0	9
2022-2023	SPRING	MBG208-Embryology,	3	0	16

		Developmental Genetics and Teratology			
2023-2024	SPRING	MBG312-Embryology and Reproduction	3	0	2
2024-2025	SPRING	MBG312-Embryology and Reproduction	3	0	19
2024-2025	SPRING	TMG312-Embryoloji ve Üreme	3	0	4