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Research Article

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CDK2 Depletion Impairs Tumour Growth in the 3D Luminal Breast Cancer Model via CRISPR-CAS9 Gene Editing

CDK2 Geninin CRISPR-CAS9 ile Hedeflenmesi 3B Luminal Meme Kanseri Modellerinde Tümöral Büyümeyi Baskılar

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Abstract

Objective: Cyclin-Dependent Kinase 2 (CDK2) is a key regulator of the G1/S transition and genomic stability. While recent studies have linked CDK2 activity to therapy resistance in hormone receptor-positive breast cancer, its functional role under tumour-relevant conditions remains unclear. This study aims to elucidate the context-specific requirement of CDK2 in luminal breast cancer models.

Material and Methods: CDK2 was knocked out in T47D cells using CRISPR-Cas9. Phenotypic effects were evaluated in both 2D monolayer and 3D spheroid cultures. Quantitative PCR validated gene knockout. Colony formation and GFP-based competition assays assessed 2D proliferation, while 3D spheroid size was quantified using ImageJ.

Results: CDK2 depletion resulted in a 40% reduction in 2D colony formation and a 50% decrease in spheroid size. The fitness disadvantage was more pronounced in 3D cultures, suggesting increased dependency on CDK2 in complex microenvironments.

Conclusion: CDK2 plays a critical role in sustaining tumour growth and structural organization, particularly in 3D environments that mimic the tumour microenvironment. These findings highlight CDK2 as a potential therapeutic target in luminal breast cancer, especially in the context of anti-oestrogen resistance.

Keywords CDK2 · Luminal breast cancer · 3D culture · CRISPR-Cas9

Öz

Amaç: Siklin-Bağımlı Kinaz 2 (CDK2), G1/S geçişi ve genomik stabilitenin önemli bir düzenleyicisidir. Son çalışmalar, hormon reseptörü pozitif meme kanserinde CDK2 aktivitesini tedavi direnciyle ilişkilendirse de, tümörle ilişkili koşullarda fonksiyonel rolü net değildir. Bu çalışmanın amacı, luminal meme kanseri modellerinde CDK2'ye özgü bağlama-bağımlı gerekliliği ortaya koymaktır.

Gereç ve Yöntemler: CDK2, CRISPR-Cas9 sistemi kullanılarak T47D hücrelerinde devre dışı bırakıldı. Fenotipik etkiler hem 2D monolayer hem de 3D sferoid kültür modellerinde değerlendirildi. Gen silinmesi kantitatif PCR ile doğrulandı. 2D proliferasyon kolon oluşumu ve GFP-temelli rekabet analizleriyle incelendi. 3D sferoid boyutları ImageJ ile ölçüldü.

Bulgular: CDK2 silinmesi, 2D kolon oluşumunda yaklaşık %40, 3D sferoid boyutunda ise yaklaşık %50 oranında azalmaya neden oldu. Özellikle 3D ortamda CDK2'ye olan bağımlılık daha belirgin hale geldi.

Sonuç: CDK2, özellikle tümör mikroçevresini taklit eden 3D yapılarda tümöral büyüme ve yapısal bütünlüğün korunmasında kritik rol oynamaktadır. Bulgular, CDK2'nin luminal meme kanserinde, özellikle anti-östrojen direnci bağlamında potansiyel bir terapötik hedef olabileceğini göstermektedir.

Anahtar Kelimeler CDK2 · Luminal meme kanseri · 3B hücre kültürü · CRISPR-Cas9

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INTRODUCTION

Cyclin-dependent kinase 2 (CDK2) is a serine/threonine protein kinase that plays a central role in cell cycle progression, particularly at the G1/S transition, where it facilitates DNA replication (1). CDK2 becomes catalytically active through its association with cyclin E and cyclin A, enabling the phosphorylation of key substrates that regulate replication and genome stability, such as Retinoblastoma protein (Rb), members of the minichromosome maintenance (MCM) complex, and the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (2-7).

The activity of CDK2 is tightly regulated by cyclins and CDK inhibitors, including $p21^{Cip1}$ and $p27^{Kip1}$, which bind and suppress CDK2 function to maintain controlled cell proliferation (8, 9). Interestingly, CDK2 can phosphorylate $p27^{Kip1}$, promoting its degradation and contributing to a positive feedback loop that favours cell cycle progression (6).

Although CDK2 is not considered a primary oncogenic driver like CDK4/6, its overexpression has been reported in several malignancies, including bladder, cervical, and breast cancers (4, 10-13). In acute myeloid leukaemia (AML), the HDAC3-AKT-P21-CDK2 signaling pathway's activation is associated with poorer event-free and overall survival (10). Bladder cancer shows increased CDK2 levels alongside reduced expression of the CDK2-targeting microRNA, miR-3619, correlated with advanced tumour stages and grades (11). In breast cancer, higher MTHFD2 levels, which interact with CDK2, are linked to worse overall survival and higher tumour grades (12-14).

In breast cancer, CDK2 has emerged as a potential therapeutic target because of its elevated expression in aggressive tumour subtypes and its interaction with oncogenic pathways. Notably, CDK2-mediated phosphorylation of EZH2 at threonine 416 enhances histone methyltransferase activity, leading to transcriptional repression via H3K27me3—an epigenetic mechanism implicated in cancer progression (15, 16). In luminal breast cancer, CDK2 activity may play a subtype-specific role in sustaining proliferation and contributing to endocrine therapy resistance (17-20). Previous studies have shown that CDK2 is essential for the tumorigenic activity of the low-molecular-weight (LMW) isoform of cyclin E and that its inhibition can reduce the growth of anti-oestrogen-resistant breast cancer cells (21-23).

Understanding cancer development and improving therapeutic strategies increasingly depend on the use of advanced experimental models that better reflect in vivo conditions (24-26). While 2D cultures have long served as standard models for assessing gene function, three-dimensional (3D) systems provide a more physiologically relevant environment that mimics in vivo tumour architecture. These models are critical for uncovering context-specific dependencies that may not be evident in traditional monolayer cultures (27, 28).

In this study, we used CRISPR-Cas9 gene editing system to knock out CDK2 in the luminal breast cancer cell line T47D. By comparing the phenotypes in 2D monolayers and 3D spheroid cultures, we aimed to uncover the context-dependent functional role of CDK2 in breast cancer cell proliferation and structural integrity. Our findings demonstrate that CDK2 is a key regulator of tumour-like growth in complex microenvironments and a potential therapeutic target in hormone-responsive breast cancer.

MATERIAL AND METHODS

Cell Culture

T47D (HTB-133™, ATCC) cell line was cultured with RPMI-1640 (Capricorn Scientific, Germany) supplemented with heat-inactivated 10% FBS (Biowest, France) and 100U/mL penicillin/streptomycin (Biowest, France). The HEK293T (CRL-3216™, ATCC) cell line was cultured with DMEM (Sigma, USA) supplemented with heat-inactivated 10% FBS (Biowest, France) and 100U/mL penicillin/streptomycin (Biowest, France). All cells were cultured at 37°C and 5% CO_2 concentration. To ensure their integrity and prevent contamination, all the lines were subjected to weekly mycoplasma testing.

Cloning of sgRNAs

CDK2 and non-targeting (NT) oligonucleotides were designed (Table 1) and cloned following the protocol recommended by the Zhang laboratory (29, 30). The pLenti Crispr-v2 plasmid (a gift from Feng Zhang (Addgene # 52961, USA) was digested with the enzyme BsmBI-v2 (NEB, USA) at 37°C for 30 minutes, and the resulting linearised backbone products were purified using the gel clean-up kit (MN NucleoSpin, Germany). Phosphorylation and annealing of complementary sgRNA oligos were carried out using 100 µM oligos, 1x T4 PNK Buffer (NEB), 1 mM ATP, and 5U T4 PNK (NEB, USA) under the following thermal conditions: incubation at 37°C for 30 minutes, denaturation at 95°C for 5 minutes, followed by gradual cooling at 5°C/min until reaching 25°C. Cloning was performed by T4 Ligase and confirmed through Sanger sequencing.

Packaging Using Lentiviral Vectors

HEK293T cells (4×10^6 cells/10 cm^2 dish) were transfected with 2.5 µg pRSV-Rev (Addgene #12253, USA), 3.5 µg pCMV-VSVG (Addgene #8454, USA), 5 µg pMDLg/pRRE (Addgene #12251, USA), and 10 µg target plasmids using polyethylenimine (PEI,

sigma) at a 1:3 DNA: PEI ratio and incubated for 18 hours. After adding fresh medium, the virus-containing supernatant was collected at 48- and 72-h post-transfection, filtered through a 0.45 µm PES filter, and stored at −80°C. pMDLg/pRRE, pRSV-Rev, and pCMV-VSVG plasmids are third-generation packaging plasmids containing the DNA elements required for lentiviral vector production.

Table 1. Sequences of DNA oligonucleotides used for the synthesis and cloning of single-guide RNA (sgRNA) constructs

	OLIGO 1 (FORWARD)	OLIGO 2 (REVERSE)
sgNT-1	CACCGGTCGTGAAGTGCATTCGATC	AAACGATCGAATGCACTTCACGACC
sgNT-2	CACCGGTAGCGAACGTGTCCGGCGT	AAACACGCCGGACACGTTCGCTACC
sgNT-3	CACCGGACCGGAACGATCTCGCGTA	AAACTACGCGAGATCGTTCCGGTCC
CDK2-1	CACCGAAGCAGAGAGATCTCTCGGA	AAACTCCGAGAGATCTCTCTGCTTC
CDK2-2	CACCGCATGGGTGTAAGTACGAACA	AAACTGTTCGTACTTACACCCATGC
CDK2-3	CACCGTCTGAGGTTTAAGGTCTCGG	AAACCCGAGACCTTAAACCTCAGAC

Lentiviral Transduction of T47D Cells

T47D cells were seeded into 10 cm² cell culture plates at a density of 4 million. The cells were then incubated for 24 hours at 37°C and 5% CO_2. On the following day, NT and CDK2 viruses were applied to the cells at a concentration of 8 µg/mL Polybrene (Sigma-Aldrich, Japan). The virus-treated cells were further incubated for 24 hours at 37°C and 5% CO_2. Subsequently, fresh medium was added to the cells. For antibiotic selection, the cells were incubated with a medium containing 4,5 µg/mL. puromycin for 48 hours, allowing for the selection of cells infected with the virus by the end of the 48 hours. At the end of this selection period, two separate cell lines were established: one infected with the CDK2-targeting virus and the other with the non-targeting (NT) control virus. The NT-infected cells were used as controls in all subsequent analyses.

RNA Isolation, cDNA Synthesis, and qPCR

RNA isolation was performed using Trizol (Invitrogen™, USA) following the manufacturer's protocol. cDNA synthesis from the isolated RNA was carried out using the iScript cDNA Synthesis Kit (Bio-Rad, USA). Quantitative PCR was performed using the LightCycler (Roche, Switzerland) qPCR instrument to measure changes in gene expression. All qPCR results were calculated using the ΔΔCt method relative to control cell populations and were normalised to the geometric mean of ß-actin levels, with 3 technical replicates per sample. The primers used for quantitative PCR (qPCR) are detailed in Table 2.

Table 2. Sequences of DNA oligonucleotides used for the synthesis and cloning of single-guide RNA (sgRNA) constructs

GENE NAME	PRIMER TYPE	SEQUENCE
ACTB	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT
CDK-2	Forward	ATGGATGCCTCTGCTCTCACTG
	Reverse	CCCGATGAGAATGGCAGAAAGC

GFP-based Competition Assay

To assess the relative proliferative capacity of the knockout (KO) cells compared with the wild-type (WT) control cells, a GFP-based competition assay was performed. Non-targeting (NT) control T47D cells were used as the WT population and labelled with GFP using the pLenti CMV GFP Puro plasmid (Addgene #17448, a gift from Eric Campeau & Paul Kaufman). These GFP-labelled NT control cells were then co-cultured with unlabelled CDK2 knockout (KO) T47D cells at a 1:3 ratio. The GFP fluorescence signal served as a marker to distinguish NT (WT) control cells from CDK2 KO cells within the mixed population. The cultures were analysed by flow cytometry every 3 days to determine the proportion of GFP-positive (NT control) and GFP-negative (KO) cells. Temporal changes in the GFP signal were used to evaluate the competitive growth dynamics between the two populations, thereby providing functional insights into the impact of CDK2 loss on cell proliferation.

Clonogenic Assays

Cells were initially seeded into 6-well plates at a density of 4×10^3 cells per well. Following seeding, the cells were allowed to proliferate for 15 days. After this incubation period, the experimental groups were fixed with methanol and stained with crystal violet. This staining process enables the visualisation and quantification of the colonies, as crystal violet binds to the cellular proteins and nucleic acids, resulting in a visible purple colour. The number and size of the colonies were then assessed to evaluate cell proliferation and clonogenic potential.

3D Spheroid Assay

For spheroid generation, 7×10^5 breast cancer cells were resuspended in the culture medium supplemented with 10% methylcellulose (MC). MC stock solution was prepared by autoclaving 6 g of MC powder (Sigma-Aldrich, Japan) in a 500 mL flask. 20% MC RPMI containing 20% FBS (Biowest, France) and 200 U/mL penicillin/streptomycin (Biowest, France) was used for all assays. The cells were incubated at 37°C with 5% CO_2 for 6 days, and spheroid formation was monitored daily. Spheroid areas were quantified using ImageJ

(NIH, USA); at least 50 spheroids per replicate were manually traced and measured.

RESULTS

CDK2 Classified as a Strongly Selective Gene

The CDK2 gene, on chromosome 12q13, encodes a key enzyme that regulates the cell cycle and kinase-mediated processes (31) (Figure 1). The ATP-binding domain, characterised by a hydrophobic pocket, facilitates the ATP binding necessary for kinase function (32). Additionally, the peptide-binding (catalytic) domain enables the phosphorylation of substrate proteins when CDK2 complexes with cyclins A or E (32). Cyclin binding induces conformational changes, activating the ATP-binding site for efficient substrate phosphorylation (33). CDK2 is classified as a strongly selective gene in the Cancer Dependency Map (DepMap, https://depmap.org/portal/gene/CDK2?tab=overview), a distinction based on divergent findings between CRISPR and RNA interference (RNAi) approaches.

The RNA sequencing data from various breast cancer cell lines revealed that CDK2 expression was significantly elevated (Figure 2A). This overexpression is linked to aggressive tumour characteristics (34), demonstrating that CDK2 may play a pivotal role in tumour progression.

Given its association with cell cycle regulation (35), particularly during the G1/S phase transition, CDK2's heightened activity could lead to increased proliferation rates in breast cancer cells, contributing to more aggressive and invasive phenotypes.

CDK2 plays a pivotal role in the progression of breast cancer, with its expression often elevated in tumour tissues compared to normal tissues (34), correlating with more advanced tumour stages and poorer survival outcomes (34). CDK2 is a critical regulator in the progression of breast cancer, exhibiting significantly higher expression levels in tumour tissues compared to adjacent normal tissues (Figure 2B). This upregulation of CDK2 is frequently associated with more advanced stages of the disease, demonstrating its involvement in the transition to aggressive tumour phenotypes. Furthermore, clinical studies have demonstrated that elevated CDK2 expression correlates with poorer survival outcomes, indicating that it may serve as a prognostic biomarker for breast cancer progression (Figure 2C). The dysregulation of CDK2 is believed to enhance cell proliferation, disrupt cell cycle control, and contribute to tumour growth, thereby underscoring its potential as a therapeutic target in the management of breast cancer (34).

Impact of CDK2 Knockout on 2D Colony Formation Dynamics in Luminal Breast Cancer

To investigate the effect of the *CDK2* gene in T47D cells, it was initially targeted using the CRISPR-Cas9 system (29). The knockout (KO) was confirmed by quantitative PCR (qPCR), which showed a significant reduction (Difference between means ± SEM: −0.7135±0.01315; 95% CI: −0.7500 to −0.6770; p<0.0001) in *CDK2* gene level compared with the control cells (Figure 3A). Following this, functional assays were performed to further evaluate the consequences of CDK2 KO. In the competition assay, CDK2 KO cells exhibited a mild fitness disadvantage under competition, showing an approximately 20% decrease (Difference between means ± SEM: −21.56±5.670; 95% CI: −34.19 to −8.926; p=0.0035) in relative cell growth compared to control cells (Figure 3B). Moreover, CRISPR-Cas9-mediated knockout of CDK2 resulted in a 40% reduction (Difference between means ± SEM: −42.06±6.316; 95% CI: −59.59 to −24.52; p=0.0026) in the colony-forming ability in the 2D monolayer culture relative to the control cells (Figure 3C, Figure 3D). This finding suggests that while CDK2 plays a contributory role in promoting proliferation within the 2D monolayer culture, its absence does not completely abrogate growth. The observed reduction in colony-forming ability may be attributed to partial compensation by other cell cycle regulators, indicating a degree of redundancy in the regulatory networks governing cell proliferation.

CDK2's Crucial Role in 3D Spheroid Growth Mimics the Tumour Microenvironment

In contrast to the observations in 2D cultures, the knockout of CDK2 in T47D breast cancer cells exhibited a significant effect when these cells were cultured in three-dimensional (3D) spheroid models. The 3D culture system provides a more complex and biologically relevant microenvironment, closely resembling the in vivo tumour architecture, which allows cells to interact with each other and the extracellular matrix (ECM) in a way that is not possible in 2D (27, 28). These interactions in 3D cultures enable the formation of more structured, multicellular formations that better mimic tumour-like growth patterns (25). In this context, CDK2 knockout led to a marked reduction in the spheroid size. Quantitative analysis revealed that spheroids formed by CDK2 KO cells were markedly smaller in size, measuring approximately 50% less (Difference between means ± SEM: −0.4667±0.03480; 95% CI: −0.5633 to −0.3700; p=0.0002) (Figure 4A and Figure 4B). This substantial reduction in the spheroid area indicates that CDK2 plays a critical role in supporting tumour-like growth within 3D environments.

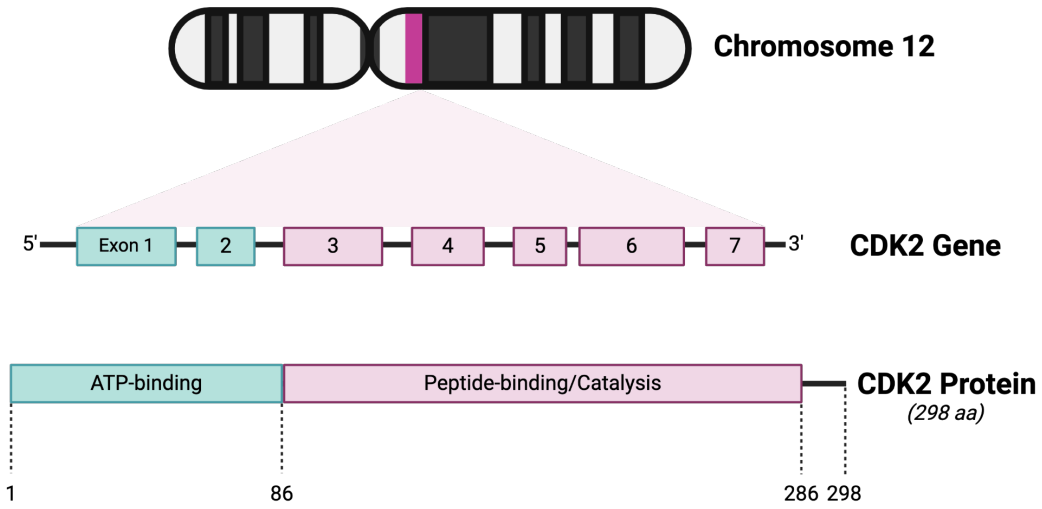

Figure 1. CDK2 Gene Structure and Functional Domains. The CDK2 gene located on chromosome 12q13 encodes a key cell cycle regulator. It has 7 exons encoding functional regions, including an ATP-binding domain (green) crucial for kinase activity and a catalytic domain (pink) responsible for substrate phosphorylation.

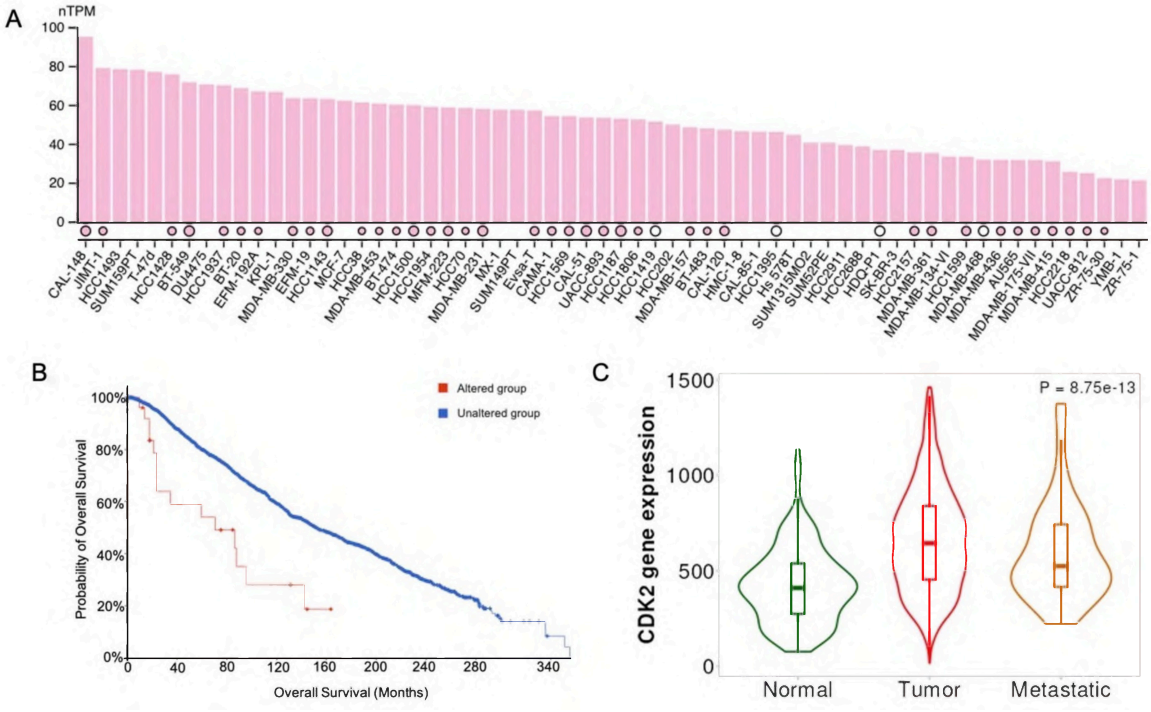

Figure 2. Elevated CDK2 Expression in Breast Cancer and Its Clinical Implications. (A) Bar plot showing CDK2 expression levels (measured in TPM, transcripts per million) across various breast cancer cells. The x-axis represents the cells, while the y-axis indicates CDK2 expression (The Human Protein Atlas). **(B)** Kaplan-Meier survival curve illustrating overall survival for patients with altered (mutated, red) and unaltered (wild-type, blue) CDK2 expression (cBioportal). **(C)** Violin plot comparing CDK2 expression levels in normal breast tissue, primary tumours, and metastatic samples (TNMPlot website).

The observed impairment in spheroid development indicates that the loss of CDK2 compromises the capacity of breast

cancer cells to effectively adapt to conditions that closely resemble the in vivo tumour microenvironment. In 3D cultures, cells experience distinct cell-cell and cell-matrix interactions, as well as variations in nutrient and oxygen gradients, which are pivotal for promoting cellular proliferation and survival (36, 37). The diminished spheroid size in CDK2 KO cells points to a disruption in these essential interactions, likely impacting not only cell proliferation but also the structural integrity and cohesiveness of the spheroids.

Furthermore, the findings imply that CDK2 is involved in key signaling pathways that facilitate the maintenance of cellular homeostasis within a 3D microenvironment. This role may involve the regulation of essential processes such as apoptosis, nutrient uptake, and the establishment of effective intercellular communication. The pronounced effect of CDK2 depletion in this model highlights its importance in enabling breast cancer cells to thrive in complex microenvironments, ultimately contributing to tumour growth and progression (25, 38)

Overall, the results underscore the necessity of CDK2 for sustaining tumour architecture and functionality in 3D cultures, demonstrating that targeting CDK2 could be a viable strategy for disrupting tumour growth in clinical settings. Further investigations are warranted to elucidate the specific molecular mechanisms through which CDK2 regulates spheroid formation and maintenance, as well as its broader implications for breast cancer therapy.

DISCUSSION

This study reveals a context-dependent requirement for CDK2 in luminal breast cancer, particularly within 3D culture systems that better recapitulate the in vivo tumour microenvironment. While CDK2 KO in 2D monolayer cultures resulted in only a moderate reduction in colony-forming ability, its depletion in 3D spheroid models caused a pronounced impairment in spheroid size and architecture. These findings underscore the significance of CDK2 not only in proliferation but also in maintaining structural integrity under physiologically relevant conditions.

Interestingly, our results are consistent with previous reports suggesting that CDK2 is not a universal driver of tumorigenesis but may be indispensable in specific cellular contexts, such as in hormone receptor-positive breast cancer and under therapeutic pressure. Previous studies have indicated that CDK2/cyclin E complexes are integral to the proliferation of oestrogen receptor-positive breast cancer cells, particularly those resistant to endocrine therapies (20). The reduction in spheroid size following CDK2 KO may reflect a decreased proliferative capacity among anti-oestrogen-resistant cells in the tumour microenvironment. This raises the intriguing possibility that targeting CDK2 could enhance the effectiveness of existing endocrine therapies by contributing to the survival of resistant subpopulations within the tumour mass.

CDK2 facilitates the G1/S transition and promote efficient DNA replication. Its absence can disrupt these processes, particularly under stress conditions such as DNA damage (39). Research indicates that while other cyclin-dependent kinases (CDKs) may compensate for some functions of CDK2, they do not fully replace its role in orchestrating cell cycle responses during replication stress or DNA damage (40, 41) Specifically, CDK2 is essential for activating the ATR/Chk1 pathway, which is crucial for a robust DNA damage response (39) Without CDK2, cells may experience delayed progression through the S/G2 phases, leading to increased sensitivity to stressors and potentially resulting in premature cell cycle exit (13, 38).

Beyond its canonical role in the G1/S transition, CDK2 has been shown to phosphorylate epigenetic regulators such as EZH2 (15, 16), enhancing H3K27 trimethylation and repressing tumour suppressor gene expression. Although this study did not directly assess epigenetic changes, the profound effect of CDK2 loss in 3D cultures-where the chromatin state and microenvironmental signaling may differ substantially from 2D-suggests a possible epigenetic component to the observed phenotype. Future work should explore whether CDK2 inhibition alters histone modifications or transcriptional programs in 3D settings.

The observed impairment in spheroid development demonstrates that the loss of CDK2 compromises the capacity of breast cancer cells to effectively adapt to conditions that closely resemble the in vivo tumour microenvironment. This phenotype may also reflect a disruption in a stem- or progenitor-like subpopulation that plays a key role in maintaining tumour architecture and regenerative capacity. CDK2 has previously been implicated in supporting self-renewal and survival in specific cellular contexts, indicating that its loss may impair the function or maintenance of cancer stem-like cells in 3D cultures. Future studies should investigate whether CDK2 inhibition affects stemness-associated gene expression programs or lineage plasticity within tumour spheroids.

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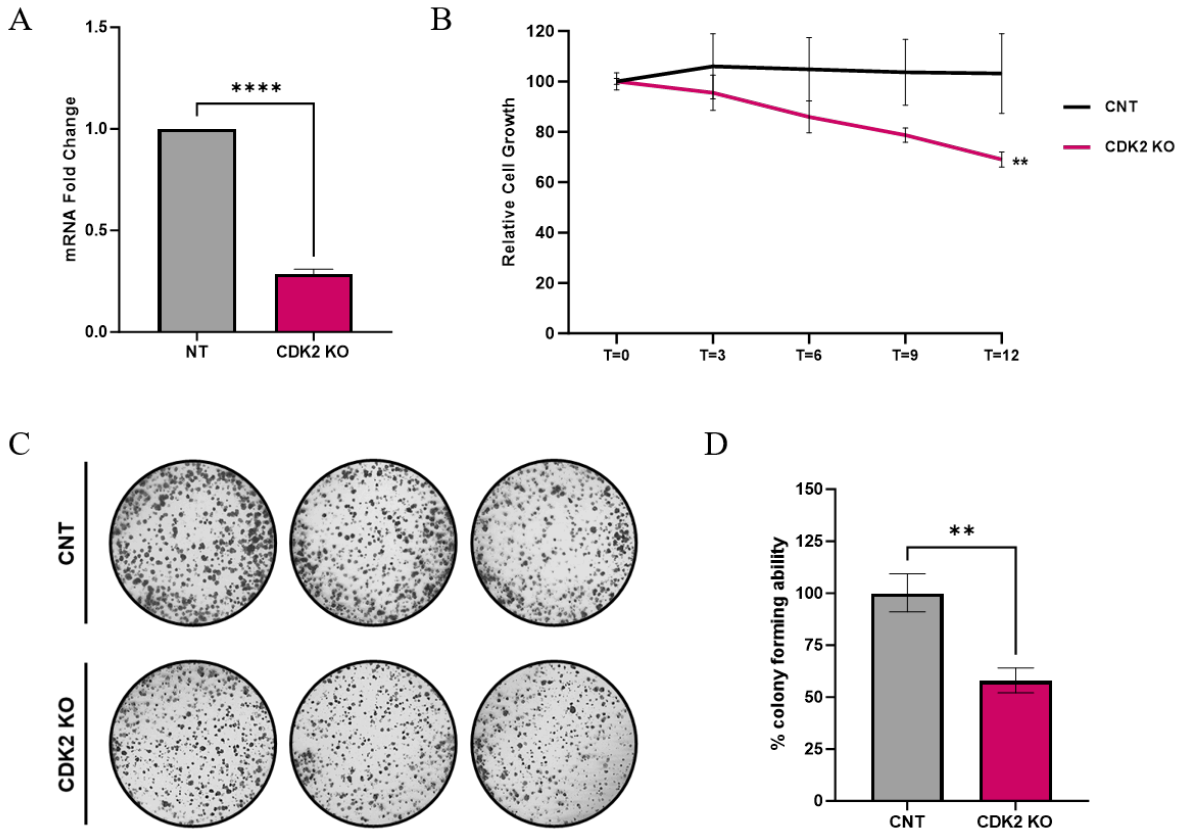

Figure 3. Validation of CDK2 Knockout and Its Impact on Proliferation in T47D Cells. (A) Quantitative PCR confirmed the effective knockout of CDK2 in T47D cells following CRISPR-Cas9-mediated gene editing (Difference between means ± SEM: −0.7135 ± 0.01315; 95% CI: −0.7500 to −0.6770; P<0.0001). **(B)** Relative cell growth was assessed using a competition assay, showing a significant reduction in proliferative capacity upon CDK2 depletion (Difference between means ± SEM: −21.56 ± 5.670; 95% CI: −34.19 to −8.926; p= 0.0035). **(C, D)** Colony formation assays revealed a marked decrease in clonogenic potential following CDK2 knockout, with the reduction in colony numbers compared to control (Difference between means ± SEM: −42.06 ± 6.316; 95% CI: −59.59 to −24.52; P=0,0026). All data are presented as mean ± standard deviation (SD) from at least three independent biological replicates. Statistical significance was determined using an unpaired two-tailed Student's t-test. p < 0.05 was considered statistically significant ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

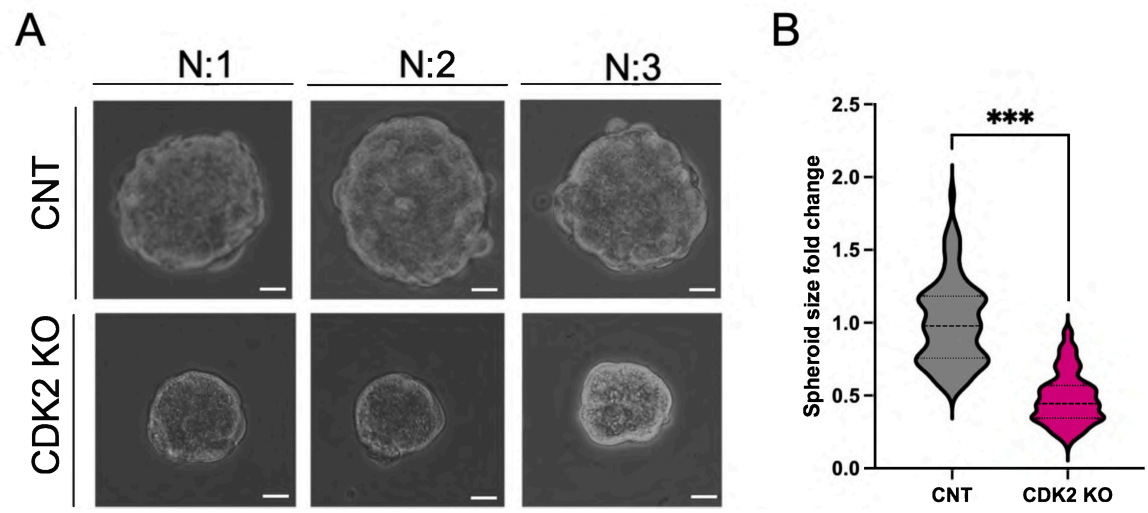

Figure 4. Impact of CDK2 Knockout on Spheroid Size in 3D Cultures of T47D Breast Cancer Cells. (A) Representative brightfield images of spheroids derived from control and CDK2 knockout T47D cells grown in a methylcellulose-based 3D culture for 6 days. **(B)** Quantification of spheroid size (projected 2D area) revealed a significant reduction in spheroid dimensions following CDK2 loss, indicating impaired 3D growth capacity (Difference between means ± SEM: −0.4667 ± 0.03480; 95% CI: −0.5633 to −0.3700; P=0,0002). Data represent the mean ± SD of at least three independent experiments. Statistical significance was assessed using an unpaired two-tailed Student's t-test. p < 0.05 was considered statistically significant ($p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***).

The diminished spheroid size in CDK2 KO cells points to a disruption in these essential interactions, likely impacting not only cell proliferation but also the structural integrity and cohesiveness of the spheroids.

Furthermore, the findings imply that CDK2 is involved in key signaling pathways that facilitate the maintenance of cellular homeostasis within a 3D microenvironment. This role may involve the regulation of essential processes such as apoptosis, nutrient uptake, and the establishment of effective intercellular communication. The pronounced effect of CDK2 depletion in this model highlights its importance in enabling breast cancer cells to thrive in complex microenvironments, ultimately contributing to tumour growth and progression (25, 38).

Overall, the results underscore the necessity of CDK2 for sustaining tumour architecture and functionality in 3D cultures, demonstrating that targeting CDK2 could be a viable strategy for disrupting tumour growth in clinical settings. Further investigations are warranted to elucidate the specific molecular mechanisms through which CDK2 regulates spheroid formation and maintenance, as well as its broader implications for breast cancer therapy.

Importantly, our findings highlight the need to integrate 3D models into functional genomics workflows, especially when evaluating candidate therapeutic targets (24, 42). Genes like CDK2, which may not appear essential in standard 2D cultures, could emerge as context-specific vulnerabilities in more complex systems.

From a translational standpoint, targeting CDK2 in luminal breast cancer-particularly along with endocrine therapies-may offer a strategy to overcome therapy resistance. Previous studies have shown that CDK2 inhibition sensitises resistant breast cancer cells to anti-oestrogens (20, 43-45), and our results support the notion that CDK2 function is critical for sustaining tumour-like growth in 3D.

Despite these insights, our study has limitations that warrant further exploration. Future research should aim to elucidate the specific molecular pathways affected by CDK2 loss, particularly those related to cell adhesion, migration, and invasion-the mechanisms underlying the 3D-specific dependency on CDK2.

Additionally, investigating the therapeutic potential of CDK2 inhibitors in combination with other targeted therapies may yield promising results for patients with luminal breast cancer exhibiting high CDK2 activity. Finally, it is important to evaluate whether CDK2-dependent epigenetic regulation contributes to survival in tumour spheroids.

In conclusion, our study highlights the critical role of CDK2 in luminal breast cancer cell proliferation and survival, especially within complex 3D tumour models (Figure 5). The significant impact of CDK2 KO on spheroid size underscores its importance in maintaining tumour architecture and growth in conditions that closely mimic the tumour microenvironment. These findings demonstrate that CDK2 represent a potential therapeutic vulnerability, particularly in the context of anti-oestrogen resistance. Further studies are warranted to elucidate its precise role in breast cancer progression and treatment response. Understanding these dynamics is crucial for developing effective therapeutic strategies aimed at enhancing patient outcomes in breast cancer treatment.

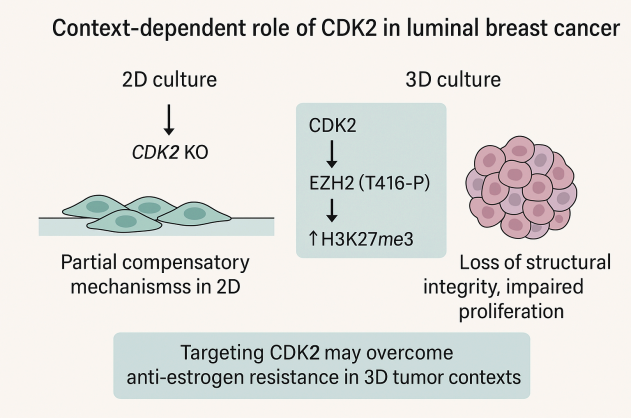

Figure 5. Context-dependent Role of CDK2 in Luminal Breast Cancer. CRISPR-Cas9-mediated knockout of CDK2 in T47D luminal breast cancer cells has a modest effect on colony formation in 2D cultures but leads to a significant reduction in spheroid growth and structural integrity in 3D cultures. This schematic summarises our findings and incorporates hypotheses based on previous studies indicating that CDK2 may regulate epigenetic factors such as EZH2 and H3K27me3. Although not directly tested here, these mechanisms may contribute to the observed 3D-specific phenotypes. Targeting CDK2 could represent a potential strategy to sensitise hormone receptor-positive breast cancers to therapy, particularly in resistant or tumour-like contexts.

Ethics Committee Approval: Since the study was not conducted on animals or humans, ethics committee approval is not required.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- G.K., E.G.; Data Acquisition- G.K., E.G.; Data Analysis/Interpretation- G.K., E.G.; Drafting Manuscript- G.K.; Critical Revision of Manuscript- G.K., E.G.; Final Approval and Accountability- G.K.

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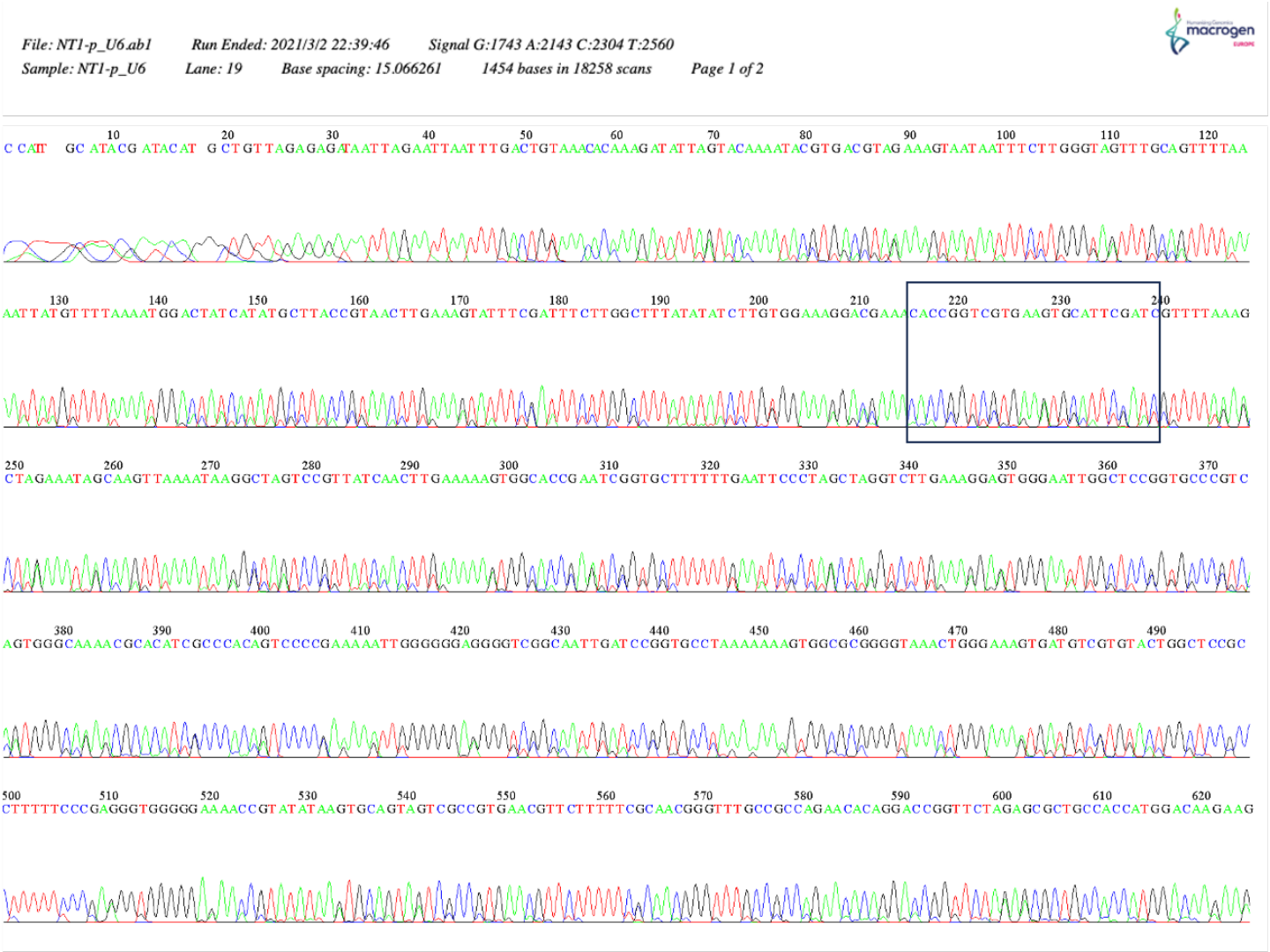
Appendix

Supplementary Figure 1. Validation of sgRNA cloning into the pLentiCRISPRv2 vector by Sanger sequencing. Chromatogram confirms the correct insertion of the target sgRNA sequence into the pLentiCRISPRv2 backbone.



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File: NT2-p_U6.ab1 Run Ended: 2021/3/2 22:39:46 Signal G:91 A:127 C:137 T:155
Sample: NT2-p_U6 Lane: 17 Base spacing: 15.185815 1139 bases in 16301 scans Page 1 of 2
macrogen

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File: C1-1_U6.ab1 Run Ended: 2024-02-23 17:06:44 Signal G:1142 A:1412 C:1631 T:1736
Sample: C1-1_U6 Lane: 21 Base spacing: 15.3542385 1426 bases in 18009 scans Page 1 of 2
macrogen
EUROPE

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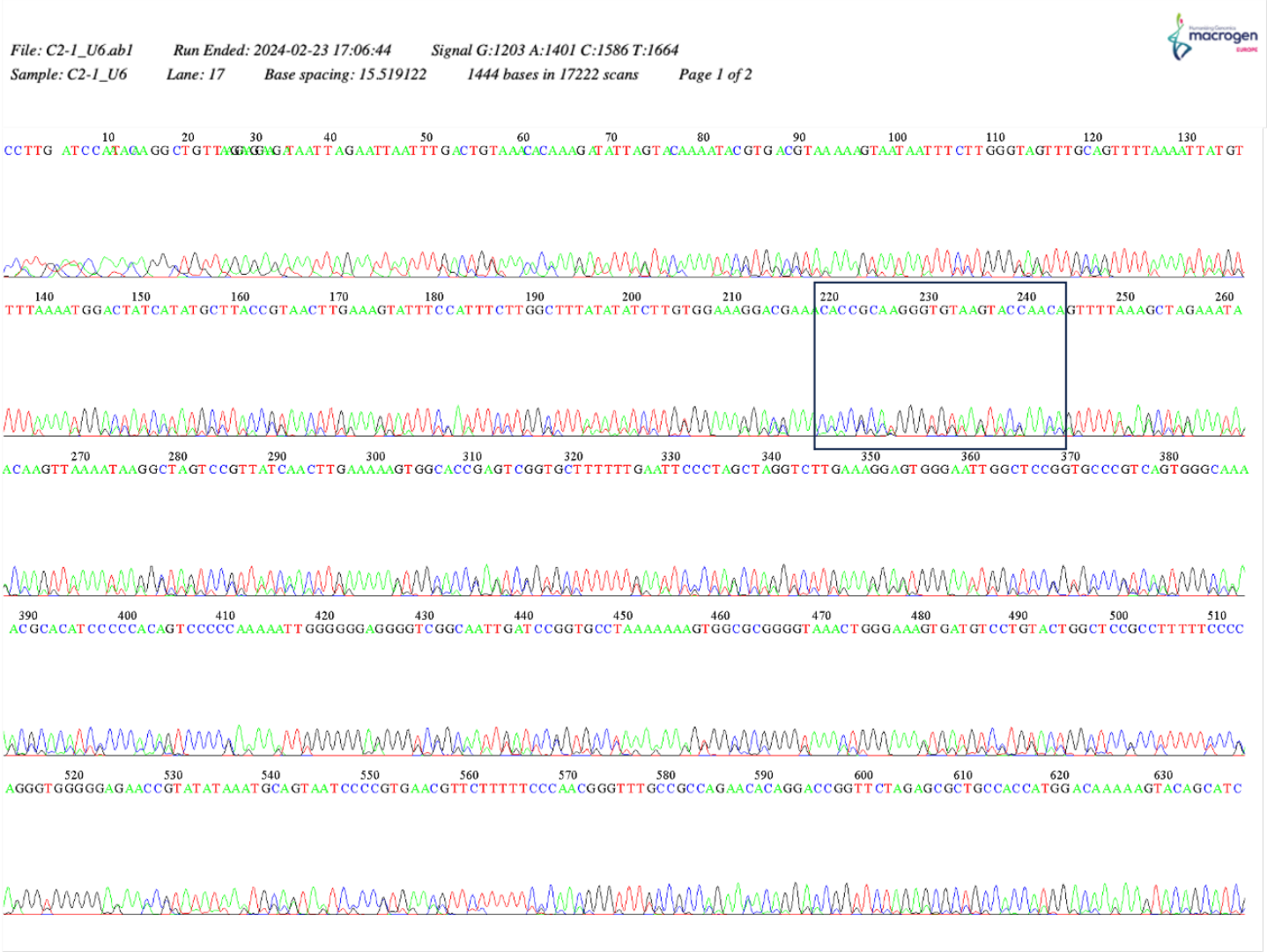
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Sample: C2-1_U6 Lane: 17 Base spacing: 15.519122 1444 bases in 17222 scans Page 1 of 2
macrogen

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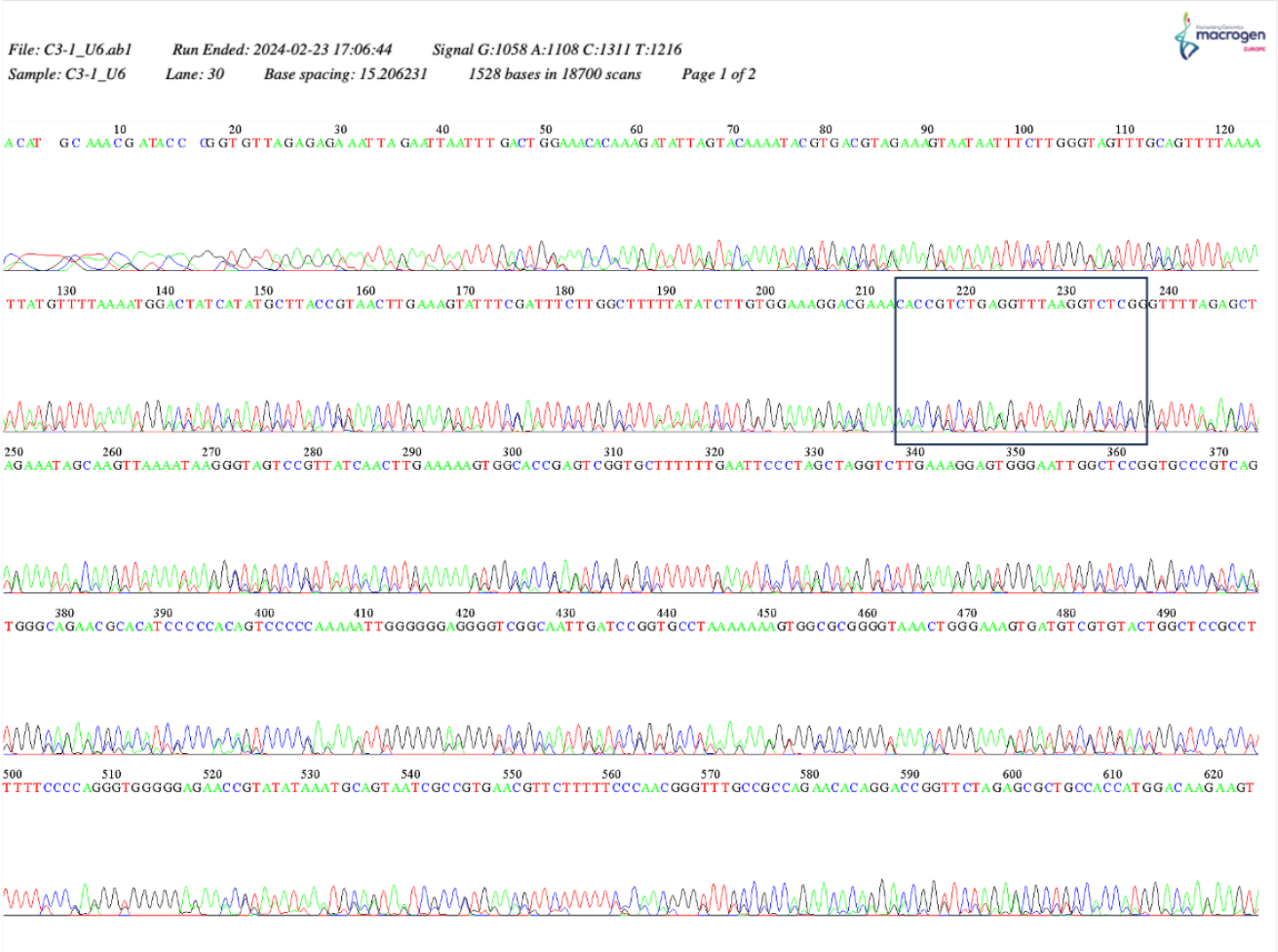
File: C3-1_U6.ab1 Run Ended: 2024-02-23 17:06:44 Signal G:1058 A:1108 C:1311 T:1216
Sample: C3-1_U6 Lane: 30 Base spacing: 15.206231 1528 bases in 18700 scans Page 1 of 2
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