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Exploiting genomic instability as an Achilles' heel in cancer

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General introduction and outline of the thesis

Chapter 1

Introduction

Cell division involves the duplication and subsequent separation of the genetic material and all cellular organelles into two daughter cells. The progression through the different cell cycle phases is tightly regulated, and is in large part mediated by delicate control of the activity of cyclin-dependent kinases (CDKs) with their cognate Cyclins (1,2). During S-phase of the cell cycle, the DNA is replicated to fully equip each of the two nascent daughter cells with a complete copy of the genome during every round of cell division.

Replication is normally initiated at different genome locations called 'replication origins', and depends on the activation of MCM2-7 helicases. For this purpose, the GINS complex and Cdc45 are recruited to the MCM2-7 helicases to form the replicative helicase complex (also called CMG complex for GINS/MCM2-7/Cdc45), which leads to the firing of origins by unwinding the DNA strands (3). Replication origins are distributed throughout the genome, and depends on specific sequence motifs. The firing of the origins adheres to a specific program: some origins fire early in S phase ('early origins'), whereas others fire later ('late origins') (3, 4). The firing of early and late origins takes place in a controlled and progressive manner, whereas the so-called 'dormant origins' are exclusively activated to coordinate the completion of DNA replication when the firing of adjacent origins is compromised (5, 6). Once origins fire, a 'replication bubble' is formed and is flanked by two replication forks on each side, leading to bi-directional DNA synthesis by the ε polymerase on the leading strand, whereas the δ polymerase replicates the lagging strand (7, 8). Not surprisingly, the abundance of nucleotides is crucial to preserve the fidelity of replication dynamics (9).

Replication stress (RS) is defined as the stalling or slowing of DNA duplication (5). An important source of RS has been linked to expression of oncogenes, including Cyclin E1, Cdc25A and c-Myc (10, 11). Oncogene-induced RS is frequently observed in hard-to-treat cancers, including triple-negative breast cancers (TNBC) (12, 13). However, oncogene-induced RS may also create a window of opportunity in the therapeutic context for these cancer types, which are currently not treated with molecularly targeted drugs. A major cause of oncogene-induced RS is the accelerated activation of replication origins, which leads to the depletion of the nucleotide pool and interferes with progression of replication forks (9, 11, 14). Oncogene activation also negatively impacts replication dynamics through other effects, including collisions between the replication and transcription machineries (15).

As a consequence of oncogene activation, replication forks stall and potentially collapse, which leads to increased single-stranded DNA (ssDNA) and double-stranded DNA breaks (dsDSBs). In response to the generation of ssDNA stretched and dsDSBs, the DNA-damage response (DDR) is activated. Specifically, the DDR kinases ATR and ATM phosphorylate multiple downstream substrates, including checkpoint kinases CHK1 and CHK2 which have a fundamental role in halting cell cycle progression, to delay the ongoing replication process and allowing time to repair DNA damage before mitosis phase entry (16, 17). Genomically instable cancers, including TNBCs, have evolved mechanisms to cope with elevated levels of oncogene-induced RS (12).

These cancer cells normally depend on checkpoint kinases, including ATR and WEE1 to activate the DDR and allow cells to repair single or double-strand breaks produced by RS. Therefore, a strategy that has been developed to effectively target genomically instable cancers with high levels of RS is to inhibit ATR or WEE1. It has been shown that the cytotoxicity of ATR inhibition is related to oncogene activity, including Cyclin E1, Cdc25A and c-Myc (18-20). Similarly, the sensitivity to WEE1 inhibition in cells that harbor replication stress activity, such as HGSOC and TNBC cells is related to *CCNE1*

amplification and Cyclin E1 overexpression (21, 22). Specifically, WEE1 inhibition results in mitotic catastrophe by inactivating the G2/M checkpoint and resulting in mitotic catastrophe (23).

To find innovative methods to molecularly target tumor cells is necessary to increase the life span of patients that suffer from difficult-to-treat cancers. Genomically instable cancers such as TNBCs and high-grade serous ovarian cancers (HGSOCs) are known to display elevated levels of RS. Since a major source of RS is oncogene activation, it is crucial to identify which oncogenic alterations are associated with levels of RS in tumors and uncover therapeutic options to molecularly target RS.

How exactly oncogene-induced replication stress affects cancer cells remains unclear. Insight into these effects may reveal vulnerabilities of cancer cells with high levels of RS. The generation of inhibitors of cell cycle checkpoint kinases has provided a solution to target RS. However, validated technology for the identification of tumors with high levels of RS remain to be established, which precludes successful selection of patients for these drugs.

The aim of the thesis

The overall aim of this thesis is to uncover the cell biological effects of oncogene-induced replication stress on tumor cells and to uncover therapeutic opportunities to target cancer cells characterized by high levels of replication stress.

To address this aim, the work in this thesis was focused on answering the following questions:

1- Which oncogenic alterations are associated with levels of RS in tumors?

2- What are the cellular consequences of oncogene-induced RS, and do they offer therapeutic opportunities?

3- How can we identify tumors with high levels of RS?

Outline of the thesis

Treatment of genomic instable cancers including triple-negative breast cancer (TNBC) and high-grade serous ovarian cancer (HGSC) is limited by the lack of actionable targets. However, these tumors are frequently characterized by high levels of replication stress. Therefore, in **chapter 2** of this thesis, we perform a literature study to describe the regulation of unperturbed replication and summarize the mechanisms that can induce replication stress (RS) and consequently drive genomic instability. In addition, we discuss the processes that cells employ to cope with RS. Finally, we examine therapeutic strategies that could be exploited to target cancer cells with high levels of RS.

Replication stress has been extensively studied in experimental models, whereas the analysis of replication stress in clinical samples is very limited. To this end, in **chapter 3** we analyze RS levels in relation to oncogene expression and clinicopathological data in a series of breast cancer samples. Specifically, we immunohistochemically determine levels of RS using pRPA and γ-H2AX, two markers of replication stress, and we correlate these RS markers to expression levels of the oncogene Cyclin E1, c-Myc and Cdc25A in tissue microarrays. Finally, we associate mRNA expression levels of *CCNE1*, *MYC* and *CDC25A* in primary breast tumors obtained from the Gene Expression Omnibus and relate these expression levels with disease-free survival and overall survival.

Since overexpression of Cyclin E1 and Cdc25A overexpression is known to cause RS, in **chapter 4** we study the relation between oncogene-induced RS and mitotic progression, chromosome stability and the sensitivity to inhibitors of the WEE1 and ATR replication checkpoint kinases. To this end, we engineer cell line models to overexpress Cyclin E1 or Cdc25A and use DNA fiber analyses to measure replication kinetics. In these cells, immunofluorescence microscopy and live-cell microscopy are used to examine whether RS affects

mitotic fidelity. Additionally, single-cell whole genome sequencing is employed to investigate whether overexpression of Cyclin E1 or Cdc25A results in chromosome segregation defects and structural or numerical chromosome aberrations. Finally, we investigate the feasibility to target cancer cells with oncogene-induced RS using inhibitors of cell cycle checkpoint kinases.

Currently it is difficult to assess levels of RS in tumors to efficiently select patients for novel treatments that target RS. To this end, in **chapter 5** we develop an RNA expression-based signature for oncogene-induced replication stress. Specifically, we induce expression of oncogenes, including *CCNE1, MYC* and *CDC25A* in a panel of cell lines (RPE-1-TP53^{wt}, RPE-1-TP53^{mut}, MDA-MB-231, BT-549 and HCC-1806) and analyze the induction of replication stress in these models using DNA fiber analysis. RNA sequence analysis is performed to identify differentially expressed genes in response to perturbations (i.e. as Cyclin E1, Cdc25A or c-Myc). Subsequently, a combined replication stress signature is validated in breast cancer tissue microarrays and public tumor data.

Lastly, in **chapter 6** we summarize the findings of this thesis and discuss implications and perspectives for future studies.

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