Vulnerabilities of cancer cells suffering from DNA replication stress

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Summary and general discussion

Requirements for mitogen-independent proliferation.

In normal cells, the different cell cycle checkpoints ensure tight regulation of cell cycle progression. Altered expression of tumor suppressors and oncogenes can diminish the functioning of these checkpoints and induce uncontrolled proliferation in cancer cells. *E.g.*, cancer cells often ignore the absence of growth factors or normal tissue context, and can thereby enter S phase prematurely. Premature S-phase entry can lead to so-called 'replication stress', a phenomenon described as the perturbation of error-free DNA replication that causes slowing, stalling or collapse of the replication fork hampering replication progression. In the first part of this thesis, we have studied the consequences of premature S-phase entry in a well-defined system of mouse embryonic fibroblasts (MEFs) in which the three retinoblastoma (Rb) genes were inactivated and in which the anti-apoptotic gene *Bcl2* was overexpressed. In **Chapter 2** we show that these triple knockout-Bcl2 (TKO-Bcl2) MEFs can enter S-phase in the absence of growth factors at the expense of severe replication stress manifested as decelerated DNA replication and reduced origin firing. This leads to the accumulation of DNA double-strand breaks (DSBs) and a G2-like arrest. Subsequently, we described that loss of p53 in TKO-Bcl2 MEFs (TKO-Bcl2-p53KO; TBP) enables proliferation without growth factors. Upon inactivation of p53 or its downstream target $p21^{Cip1}$, origin firing was restored and thereby DNA DSBs were reduced[1]. Therefore, we provided a novel rationale for the frequent co-occurrence of Rb and p53 pathway inactivation in the process of tumorigenesis; loss of p53 does not only attenuate cell cycle arrest or apoptosis, but also reduces replication stress-induced-DNA damage.

However, while restored origin firing upon ablation of the $p53/p21^{Cip1}$ axis is mechanistically plausible, we have not directly proven that DNA breakage as a consequence of replication stress was prevented by increased origin firing. To study additional factors involved in mitogen-independent proliferation, we performed a CRISPR/Cas9 screen in growth factor starved TKO-Bcl2 MEFs as described in **Chapter 3**. Remarkably, we identified disruption of *Tp53* as the only significant hit. Since it has been shown that DSBs induced by Cas9 activity can activate a p53-mediated DNA damage response[2,3], we hypothesized that inactivation of p53 during CRISPR/Cas9 screening induces growth arrest or apoptosis, thereby hampering the identification of gene disruptions that allow mitogen-independent proliferation.

Full blown tumors preserve proliferative capacity despite the presence of replication stress. Therefore, we hypothesize that tumor cells are highly dependent on 'replication rescue pathways'; mechanisms minimizing replication stress. A better understanding of these pathways could eventually provide novel therapeutic opportunities to restrain or revert tumor progression. To this aim, we used our well-defined system of MEFs lacking the G1/S phase checkpoint. Despite the fact that growth factor deprived TBP MEFs were able to

proliferate, they still suffered from replication stress revealed by a low replication fork speed. The work described in this thesis identified different replication rescue pathways, which potentially could be exploited in the future as therapeutic targets of cancer cells suffering from replication stress.

Balancing the level of origin firing.

The completion of DNA synthesis results from a balance between the overall number of active origins, the fork progression rate and the occurrence of fork stalling events. Eukaryotic cells license many origins, but only a subset of these is fired in S-phase. When progression of replication forks is slowed down or stalled, activation of dormant origins increases the number of replication forks and ensures completion of DNA synthesis[4]. The work delineated in this thesis emphasizes the importance of balancing the level of origin firing in replication stress conditions to prevent DNA damage and enable proliferation. As described in **Chapter 2**, we found that restoration of origin firing to normal levels by loss of p53 in G1/S-checkpoint-deficient cells reduced DNA DSBs and allowed proliferation in replication stress conditions[1]. Restored levels of origin firing could possibly rescue slow or stalled replication forks. This suggests that reduced origin firing in cells suffering from replication stress prolongs the vulnerability of stalled replication forks leading to DNA DSBs.

On the other hand, too much origin firing, far above normal levels, may also be detrimental for cells suffering from replication stress. As shown in **Chapter 4** cells suffering from replication stress were sensitive to inhibition of the ATR and CHK1 kinases; treatment with ATR or CHK1 inhibitors induced DNA DSBs and inhibited proliferation. Upon slowing or stalling of the replication fork, the DNA damage response kinases ATR and CHK1 are activated to inhibit global origin firing[5]. In line with this function, we and others have seen that inhibiting ATR or CHK1 increases the levels of origin firing (data not shown;[5]). It has been shown that the level of origin firing inversely affects the rate of replication fork progression, perhaps as a result of competition for deoxyribonucleotides[6]. Possibly, increasing the level of global origin firing in replication stress conditions depletes the limiting replication factors, already scarce due to premature entry into S-phase. This would hamper the rescuing effect of local dormant origin firing because of slow fork progression, more fork stalling, incomplete DNA synthesis and consequently DNA DSBs. Thus, a tide balance between replication fork initiation and fork speed is crucial during replication stress conditions. Disturbing this balance by either decreasing or increasing the level of origin firing induces DNA damage and inhibition of proliferation.

RECQL.

Upon stalling of the DNA polymerases, the replicative MCM2-7 helicase uncouples from the

polymerase and continuous to unwind the parental DNA creating stretches of single stranded DNA (ssDNA)[7]. The RPA protein binds ssDNA to prevent secondary structure formation and to protect it against nuclease activity. RPA binding to ssDNA activates the ATR-CHK1 pathway, which stabilizes the replication fork, *i.e.* keeping the replisome complex intact so that replication can restart as soon as the block to replication is alleviated[8]. When a large number of replication forks is stalled, excessive ssDNA accumulation leads to global exhaustion of RPA[9] and other rate-limiting replication factors[10]. Upon reduced binding of RPA, ssDNA at a stalled replication fork is vulnerable to cleavage by structure-specific nucleases through mechanisms yet to be fully understood. Cleavage of a stalled replication fork will result in a broken fork or in other words a one-ended DNA break[11]. We found, as shown in **Chapter 4**, that the RECQL helicase protects stalled replication forks from MRE11-dependent breakage[12]. However, whether RECQL inhibits MRE11 nuclease activity directly or indirectly still needs to be resolved.

We could envision different functions of RECQL at the replication fork. First, the helicase activity of RECQL could be involved in removing secondary structures formed in ssDNA upon replication fork stalling. For the polymerase to move on, these structures need to be resolved. We could hypothesize that in the absence of RECQL, secondary structures in ssDNA persist and are vulnerable for MRE11 cleavage, thereby generating broken replication forks. Second, it has been suggested that RECQL controls RPA dynamics[13]. We could speculate that upon depletion of RECQL, less RPA is recruited to ssDNA at stalled replication forks making them more vulnerable to MRE11 nuclease activity. Third, it has been proposed that RECQL is involved in the formation and resolution of so-called chicken foot structures, a regressed replication fork where the nascent strands anneal to each other and form a four-way Holliday-junction[14–16]. Regression of the replication fork has been described as mechanisms for fork stabilization and prevention of replication fork breakage[17]. Previously, it has been shown that the BRCA1 and BRCA2 proteins protects the annealed nascent DNA at the regressed replication fork against MRE11-dependent nucleolytic processing[18,19]. Maybe, RECQL has a similar role in protecting a chicken foot structure from degradation. Similar to loss of BRCA1 or BRCA2, mutations in *Recql* are associated with an increased risk to breast cancer development[20–22].

Removal of sister chromatid cohesion.

Prolonged stalling of a replication fork can eventually lead to replication fork collapse creating a broken replication fork[10,23]. How broken replication forks are repaired and restarted is not entirely understood in mammalian cells. It has been suggested that one-ended DSBs are repaired via a homology-mediated recombination mechanism related to break-induced replication[24,25]. At a broken replication fork, 5' end resection is necessary to

allow for RAD51-dependent 3' end strand invasion and restart of replication. In **Chapter 5**, we show that the cohesin antagonist WAPL is essential in this process. Upon loss of WAPL, broken replication forks cannot be repaired or restarted[26]. However, the molecular details behind the finding that cohesin needs to be removed from broken replication forks to allow repair and restart still need to be resolved. For example, we do not know at which step and at which location in the repair and restart process WAPL-dependent cohesin removal is essential. Possibly, cohesin complexes behind the replication fork might interfere with access of DSB repair factors or physically hinder the processes of homologous recombination at the fork. However, we could also speculate that cohesin removal is essential for the recruitment of SMC5/SMC6, a cohesin-related protein complex associated with replication through difficult to replicate regions and the recovery of stalled replication forks[27,28]. More mechanistic studies are necessary to elucidate the exact function of cohesin removal at the site of a broken replication fork.

The requirement for cohesion removal in cells suffering from replication stress that need to restart broken replication forks is eventually visible as reduced sister chromatid cohesion at the start of mitosis. Indeed, we observed that cancer cell lines of different origin suffer from sister chromatid cohesion loss independent of mutations in cohesin-related genes[26]. Interestingly, similar to WAPL, we have seen in the **Chapter 6** that the cohesin loading proteins NIPBL and MAU2 were also essential for proliferation of cells in replication stress conditions. However, in **Chapter 5** and **Chapter 6** we showed that NIPBL and MAU2 were not crucial for the repair of broken replication forks. We therefore envisage that reduced sister chromatid cohesion caused by either cohesin removal at a broken replication fork or knockdown of one the cohesin loaders does not induce problems during chromosome segregation on its own. However, when a cell experiences a combination of reduced cohesin binding and replication stress, and thereby removal of the remaining cohesin rings from the DNA to be able to restart broken replication forks, the amount of cohesion may become too low for proper chromosome segregation.

Other causes of replication stress.

In the work described in this thesis, we mainly studied consequences of replication stress induced by lack of the G1/S phase checkpoint and absence of growth factors. Another cause of replication stress in cancer cells can be the expression of oncogenes such as c-MYC, Cyclin E or CDC25A[29]. In contrast to loss of the G1/S phase checkpoint where replication stress is induced by a reduction in the number of origins fired, untimely S-phase initiation resulting from constitutive oncogenic signaling is accompanied with increased origin firing[30,31]. These seemingly different forms of DNA replication stress may include more distinctive features, but do they depend on similar pathways to rescue replication? It would be interesting to

study whether the 'replication rescue' genes (*i.e.*, RECQL, WAPL) found to be essential for proliferation of mitogen-deprived TBP MEFs, are also essential in oncogene-induced replication stress conditions. Preliminary results shown in **Chapter 4**, show that human retinal pigment epithelial cells (RPE1s) overexpressing c-MYC were slightly dependent on RECQL expression to minimize MRE11-dependent DNA DSBs[12], suggesting a role for RECQL in protecting replication forks during oncogene-induced replication stress.

Targeting replication stress.

Collectively, the findings described in this thesis divulge the presence of many different pathways involved in maintaining proper replication during replication stress conditions. Targeting these pathways individually may induce specific lethality in cancer cells. Inhibitors of ATR or CHK1 already show promising results in clinical trials either as monotherapy or combined with conventional therapies[32,33]. Furthermore, it has already been shown that silencing of RECQL significantly reduced proliferation of cancer cells as compared to normal cells in cell culture models[34–36]. Also, depletion of RECQL inhibited tumor growth in mouse xenograft models[37,38]. Although these studies suggest a relation between RECQL and tumor growth, the molecular function of RECQL is not properly understood. As described in **Chapter 4**, we now suggest that inhibition of RECQL could potentially induce DNA damage by inducing replication fork breakage[12]. Unfortunately, to our knowledge, there are no inhibitors for the RECQL helicase developed yet.

Inhibiting WAPL-dependent cohesin removal could also be an option to treat cancer cells suffering from replication stress. Inhibition of WAPL hampers the repair of broken replication forks[26], as described in **Chapter 5**. However, depletion of WAPL induces chromosome segregation errors and DNA damage in normal cells and inhibits proliferation[39], disfavoring WAPL as therapeutic target. To inhibit cohesin removal specifically at broken replication forks, more mechanistic research is necessary into the specific players and pathways involved in this process.

Also, inhibiting different replication rescue pathways at the same time could be exploited as therapy to target cancer cells. For example, stimulating the level of global origin firing in cells suffering from replication stress using ATR or CHK1 inhibitors may aggravate replication fork stalling and increase RECQL dependence. Thus, combining an ATR or CHK1 inhibitor with inhibition of RECQL will increase replication fork collapse, inducing DNA damage and inhibiting proliferation. Along similar lines, inhibition of both RECQL as well as cohesin removal from collapsed forks will increase the level of broken replication forks that remain unrepaired eventually blocking proliferation.

In conclusion.

This thesis provides a series of novel molecular insights regarding the mechanisms of cells to minimize replication stress. A better understanding of these processes is essential to explore vulnerabilities of cancer cells suffering from replication stress. This could lead to the development of novel therapeutic strategies that benefit from the presence of DNA replication stress in tumors, but not in normal cells.

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