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Mechanisms used by cancer cells to tolerate drug-induced replication stress

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ABSTRACT

Activation of oncogenes in cancer cells forces cell proliferation, leading to DNA replication stress (RS). As a consequence, cancer cells heavily rely on the intra S-phase checkpoint for survival. This fundamental principle formed the basis for the development of inhibitors against key players of the intra S-phase checkpoint, ATR and CHK1. These drugs are often combined with chemotherapeutic drugs that interfere with DNA replication to exacerbate RS and exhaust the intra S-phase checkpoint in cancer cells. However, drug resistance impedes efficient clinical use, suggesting that some cancer cells tolerate severe RS. In this review, we describe how an increased nucleotide pool, boosted stabilization and repair of stalled forks and firing of dormant origins fortify the RS response in cancer cells. Notably, the vast majority of the genes that confer RS tolerance are regulated by the E2F and NRF2 transcription factors. These transcriptional programs are frequently activated in cancer cells, allowing simultaneous activation of multiple tolerance avenues. We propose that the E2F and NRF2 transcriptional programs can be used as biomarker to select patients for treatment with RS-inducing drugs and as novel targets to kill RS-tolerant cancer cells. Together, this review aims to provide a framework to maximally exploit RS as an Achilles' heel of cancer cells.

1. Introduction: replication stress as vulnerability of cancer cells

DNA replication is a delicate process that ensures timely and error-proof genome duplication. However, oncogenes override this strictly controlled process. This allows excessive cancer cell proliferation, but comes at the costs of DNA replication stress (RS). An increasing body of evidence shows DNA damage as a result of oncogene-induced RS in the vast majority of tumors. Therefore, RS is a hallmark of cancer [1], which can be exploited as therapeutic target [2].

RS is defined as hindrance of replication fork progression and includes the stalling and collapsing of these forks. Oncogene-induced RS can predominantly be attributed to elevated CDK activity which augments origin firing in S-phase [3–5]. Subsequently, DNA replication is started at an abnormally high number of genomic sites, which leads to a shortage of supplies required for faithful DNA replication [6]. Additionally, elevated ongoing DNA replication and oncogene-induced transcription increases the chance of collisions between the two machineries and formation of potentially harmful RNA-DNA hybrids called R-loops [3,7,8]. Moreover, cancer cells rewire their metabolism which results in higher levels of reactive oxygen species (ROS) that induce RS by oxidizing nucleotides [9].

Since RS results in under-replicated DNA, mitotic errors and thus genomic instability [10], the intra S-phase checkpoint is in place to stabilize and repair stalled forks and prevent cell cycle progression in the presence of RS. In brief, stalling of replication forks results in aberrant DNA structures and uncoupling of the polymerase and helicase activities, exposing single-strand DNA (ssDNA). Single-strand DNA-binding protein Replication Protein A (RPA) binds this ssDNA, which recruits Ataxia Telangiectasia and Rad3 related kinase (ATR) and in turn activates the effector kinase Checkpoint Kinase 1 (CHK1) [11]. CHK1 delays S-phase progression by inhibiting CDK activity via degradation of CDC25A and activation of WEE1 G2 checkpoint kinase (WEE1) [12,13]. Simultaneously, ATR activates a signaling cascade to stabilize, repair and restart the stalled replication forks to rescue DNA replication [11].

Oncogene-induced RS in combination with dysfunctional surveillance mechanisms, such as P53 mutations, forces cancer cells to heavily rely on the intra S-phase checkpoint for survival [14–18]. This presents a novel therapeutic landscape in which ATR, CHK1, or WEE1 can be inhibited to force cell cycle progression in the presence of RS, resulting in mitotic catastrophe [16–18]. Currently, drugs inhibiting these three kinases are explored in clinical trials but resistance remains a challenge [2]. The recurrence of tumors, despite anti-cancer treatments that presumably induce severe RS, suggests that there exists a subset of cancer

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cells capable of resisting RS. Multiple studies over the past decade have shed light on the molecular underpinnings of RS tolerance in cancer cells. Moreover, an increasing body of evidence demonstrated a correlation between poor survival and the presence of resistance strategies.

With the great progression in our understanding of the RS response in malignant and non-malignant cells, the research field is currently at a crossroads where it is of critical importance to interconnect these studies. This will be essential to translate their findings into efficient therapeutic targeting of RS in cancer patients. Therefore, in this review we will provide an overview of RS tolerance avenues used by cancer cells to mitigate RS. Furthermore, we will evaluate the number of strategies a cell requires to survive despite RS and discuss how transcriptional programs can be used to adopt these strategies. Finally, we will elaborate on how a better understanding of RS tolerance may lead to efficient elimination of cancer cells.

2. Avenues towards RS tolerance

RS is not restricted to cancerous cells; it can also occur in normal cells. Hence multiple mechanisms to counteract RS have evolved in eukaryotic cells. Cancer cells can hijack these mechanisms to ameliorate RS. These strategies mainly occur around the DNA replication fork and can be classified into four RS tolerance avenues (Fig. 1). First, cells mitigate RS by securing the nucleotide pool, this allows DNA repair and diminishes the impact of nucleotide synthesis inhibitors such as gemcitabine. Second, stalled replication forks are stabilized to prevent DNA breaks and allow fork restart after RS. Third, mechanisms are in place to continue DNA replication despite RS. Fourth, back-up strategies ensure completion of DNA replication when initial replication forks fail. How cancer cells deploy these strategies to alleviate RS is delineated below.

2.1. Ensure sufficient nucleotides

In response to RS, the ATR-CHK1 axis initiates a signaling cascade that limits global origin firing and boosts nucleotide production [19,20]. In line with this, cells that lack functional ATR or CHK1 increase origin firing, but fail to induce nucleotide production [19]. This results in exposure of single-strand DNA (ssDNA), which is prone to breakage. Several lines of evidence show that DNA damage resulting from RS can be alleviated by exogenous supply of nucleosides [6,21,22]. Similarly, cancer cells can boost their nucleotide pool to tolerate RS.

Sufficient nucleotide precursors and the enzymes to convert these into nucleotides are of critical importance to maintain the nucleotide pool. Ribonucleotide reductase (RNR), consisting of Ribonucleotide Reductase catalytic subunit M1 (RRM1) and Ribonucleotide Reductase regulatory subunit M2 (RRM2), is the rate-limiting enzyme for *de novo* nucleotide production. Interestingly, *in vitro* experiments showed that overexpression of RRM2 rescues RS and alleviates chromosomal instability induced by inhibition of ATR or CHK1 [19,23], whereas depletion of RRM2 enhanced the efficacy of chemotherapeutic drugs [24]. Since RRM2 is frequently upregulated in a wide range of human cancers and correlates with a poor response to gemcitabine, it is tempting to speculate that RNR-mediated upregulation of nucleotide production is deployed by cancer cells to tolerate RS [25,26].

In addition, metabolic rewiring can support nucleotide production. Specifically, cancer cells upregulate glutaminolysis which supplies the cell with carbon and nitrogen for *de novo* pyrimidine and purine synthesis [21,27]. Moreover, glutaminolysis increases the pool of available, non-damaged, nucleotides by providing the reactive oxygen species (ROS) detoxifying NADPH to ensure redox homeostasis [27,28]. Accordingly, glutaminolysis is boosted in chemoresistant tumors and high nucleotide levels correspond with poor prognosis [21,29].

Thus, cancer cells fuel their nucleotide pool by increasing RNR activity and glutaminolysis to mitigate RS.

2.2. Protection of stalled replication forks

Stalling of replication forks means in essence that the progression of DNA polymerase is blocked. However, the DNA polymerase activity of the replication fork is preceded by helicase activity, which can continue

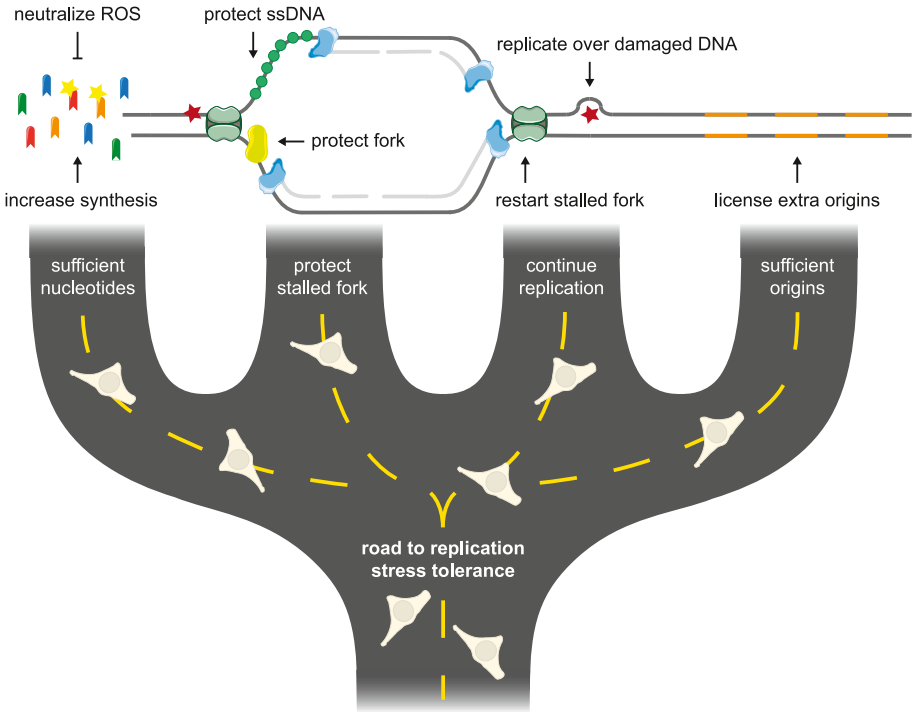

Fig. 1. Avenues towards replication stress tolerance.
Graphical representation of the potential avenues cancer cells can employ to confer RS tolerance. The main strategies that are part of these RS tolerance avenues are indicated on the ongoing replication fork.

to unwind DNA. Hence, a stalled replication fork results in the exposure of ssDNA. ssDNA is highly vulnerable to breakage and is therefore protected by the ssDNA binding protein RPA (Fig. 2A). However, RPA is rate-limiting: under conditions of severe RS, the presence of ssDNA exceeds the levels of RPA, resulting in replication fork collapse and DNA breaks [20]. Besides classical chemotherapeutics which induce replication fork stalling, inhibitors of the intra S-phase checkpoint unleash unrestricted origin firing which exhausts the pool of available RPA [20,30]. Interestingly, a 2- to 3-fold increase in RPA is already sufficient to make cells resilient to inhibition of ATR or CHK1 and nucleotide depletion [20,30].

To further prevent breakage of newly synthesized DNA, a stalled replication fork undergoes a process called fork reversal, in which remodeling allows annealing of the nascent DNA [31]. This requires the BRCA-mediated replacement of RPA by RAD51 (Fig. 2B). Consequently, BRCA-deficient tumors are highly sensitive to RS-inducing drugs, but they can be protected by increased RAD51 levels [31]. Similarly, the RAD51 antagonist RADX can be deleted to counteract the reduced RAD51 levels (Fig. 2C) and restore fork stability in BRCA-deficient cells [32]. Moreover, loss of fork remodelers such as SMARCAL1 or the exonuclease MRE11 prevents degradation of reversed forks (Fig. 2D) and thus confers chemoresistance [30,33–35].

Besides protection of the nascent DNA, the integrity of the stalled fork needs to be safeguarded to enable fork restart. The fork protection complex, consisting of CLASPIN, TIMELESS and TIPIN, interacts with the replication fork to elicit fork stabilization (Fig. 2E). The exact molecular mechanisms underlying fork stabilization by these proteins are unknown, but cells lacking components of this fork protection complex experience DNA damage and chromosomal instability [36]. Furthermore, overexpression of CLASPIN reduced RS and conferred radio resistance in a xenograft model [37]. It can be hypothesized that the increased CLASPIN and TIMELESS levels in human tumors compared to normal tissue are a mechanism to combat RS [36,38]. In line with this, high levels of CLASPIN or TIMELESS correlate with poor prognosis of non-small cell lung cancer (NSCLC) patients [36].

In summary, cancer cells can prevent fork collapse by ensuring the stability of stalled forks via upregulation of ssDNA binding or fork protection proteins.

2.3. Allow DNA replication despite fork stalling

Stalling of a replication fork does not necessarily result in failure of DNA replication. Instead, cells deploy multiple strategies to complete DNA replication upon RS. Firstly, a stalled replication fork can be restarted by the RECQ family of helicases such as RECQ1, WRN and BLM [39]. Accordingly, the absence of these helicases results in genomic instability and cancer predisposition. In contrast, overexpression of RECQ helicases is observed in a wide range of human malignancies and correlates with poor prognosis [40–43]. It is still unclear if the protective function of RECQ helicases can solely be attributed to its role in fork restart. Multiple lines of evidence show that the annealing activity of RECQ family members mediates fork reversal which subsequently allows restart of the stalled fork [44–46]. However, the helicase activity of RECQ1, BLM and WRN should not be overlooked, as it resolves secondary DNA structures that are formed in the ssDNA exposed upon RS. Furthermore, RECQ helicases play a role in the recruitment of RAD51 to allow fork stabilization [39]. Presumably these concerted mechanisms improved the recovery of cells with high levels of RECQ helicases from RS-inducing drugs [40,41].

In addition to RECQ-mediated fork restart, temporary replacement of regular polymerases by translesion synthesis (TLS) polymerases at stalled forks permit continued replication despite DNA lesions encountered

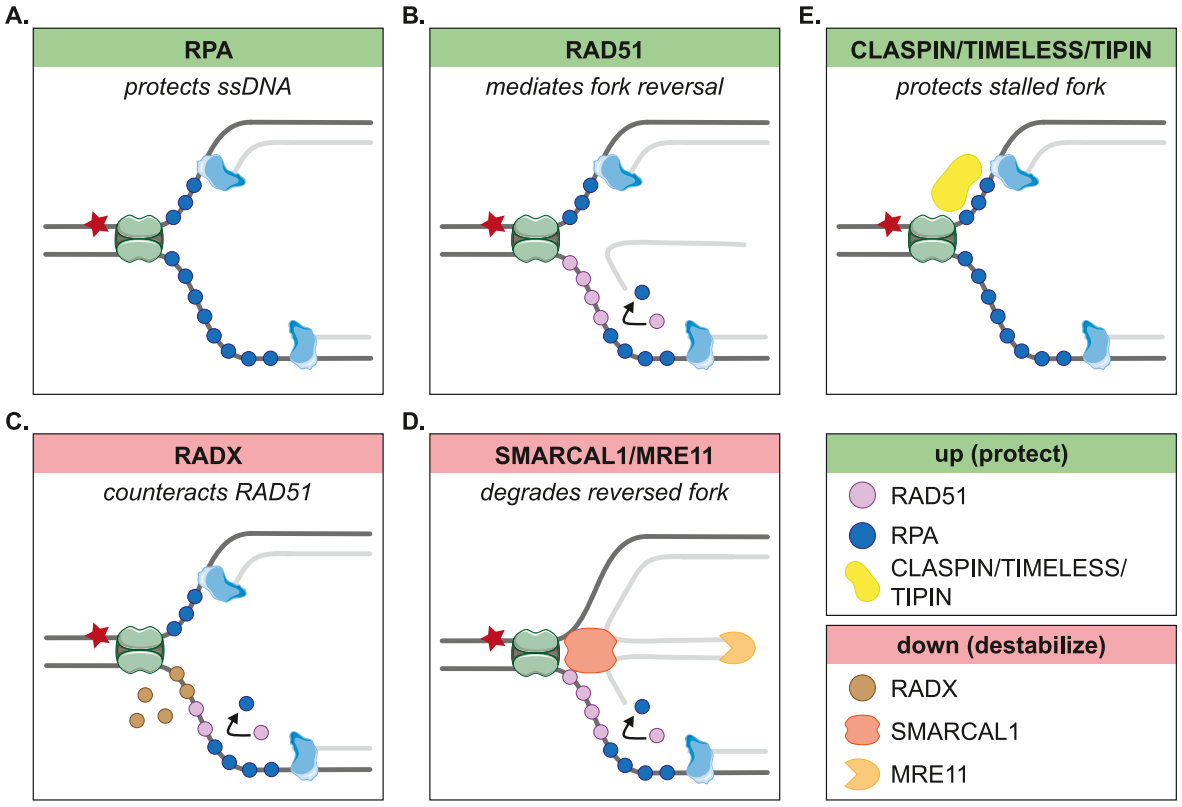

Fig. 2. Protection of a stalled replication fork.
Schematic representation of mechanisms to protect stalled forks. Note that these processes can occur simultaneously but are here depicted at individual replisomes for simplicity. Cells can boost replication fork stability by upregulation of RPA (A), RAD51 (B) or the fork protection complex (E) and downregulation of RADX (C), SMARCAL1 or MRE11 (D).

by the replication fork. The bigger catalytic pocket of TLS polymerases facilitates DNA replication over damaged DNA and allows S-phase progression but is inherently less accurate. TLS polymerases are found at high levels in tumors, and are linked to poor clinical outcome [47–51]. Specifically, a study by Peng and coworkers revealed enhanced levels of the TLS polymerase POLK in chemoresistant glioblastoma compared to paired treatment naïve samples [47]. In line with this, overexpression of the TLS polymerases POLH or REV3 protects cancer cells against damage induced by platinum-based therapies [47,52,53], whereas blockage of TLS polymerase activity provokes efficacy of cisplatin [47,52,54–56].

Besides TLS, the replication fork can be reinitiated behind the DNA lesion to allow continuation of DNA replication under RS. Such a restart event requires the primase activity of primase and polymerase PrimPol [57]. Of note, repetitive treatment of cancer cells with cisplatin resulted in increased levels of PrimPol in cells which continued DNA replication [35]. Moreover, augmented PrimPol levels decreased sensitivity of cells to an ATR inhibitor [35].

Thus, cancer cells hijack replication fork restart and progression mechanisms to guarantee DNA replication in the presence of RS, although this inevitably occurs at the cost of lower replication fidelity.

2.4. Dormant origins as backup when initial replication forks fail

In G1 phase, pre-replicative complexes, consisting of MCM2-7, ORC1-6, CDC6 and CDT1, are loaded on the DNA. During normal S-phase only a subset of these replication forks is fired. But upon RS, replication forks can stall before reaching the opposing fork and dormant origins in the vicinity of the stalled fork are fired to ensure replication completion [58]. In line with this, lowering the expression of origin components does not affect normal proliferation, but reduces survival of cells challenged by RS [58]. Several lines of evidence suggest that licensing and firing of dormant origins is critical for cancer cell survival. Firstly, members of the pre-replicative complex are overexpressed in tumors [59,60]. Whether the overexpression of individual pre-replicative members is sufficient to boost origin licensing remains to be investigated. Nonetheless, oncogenes are shown to promote origin licensing and firing [5,61]. Although excessive origin firing results in RS, for example due to nucleotide shortage and more collisions, the importance of sufficient origins is supported by the finding that reduction of CDC6, MCM7 or ORC1 limits firing of dormant origins and sensitizes cancer cells to RS-inducing drugs [5,59,62,63].

Collectively, these data indicate that cancer cells counteract RS by ensuring sufficient origins which can serve as backup when initial DNA replication fails.

3. What is minimally required to combat RS?

As described above, a range of mechanisms is identified that support cancer cells to tolerate RS. This raises the question of whether a single adaptation is sufficient to confer resistance to RS-inducing drugs or if simultaneous activation of multiple RS tolerance avenues is required. Answering this question is crucial when designing novel therapeutic strategies to overcome resistance to drugs that target the RS response.

3.1. The lonely warrior

A recent CRISPR overexpression screen to identify single genes whose overexpression alleviated ATRi-induced cell cycle arrest suggested that one factor could be sufficient to grant tolerance to drug-induced RS [64]. This is consistent with several studies discussed above which find that boosting a single factor confers chemoresistance [20,35,36,40,41,47,65]. Surprisingly, factors previously described to ameliorate RS were not identified in the screen of Schleicher et al., which raises the question to what extent their findings can be translated to other experimental, and ultimately clinical, settings. Moreover, overexpression studies frequently evaluate specific short-term responses to RS-inducing drugs *in vitro*. For example, high levels of PrimPol are described to prevent fork reversal upon cisplatin treatment, but the effect on cell proliferation was not addressed [35]. This makes it difficult to draw clinically relevant conclusions from *in vitro* studies. Albeit long term *in vivo* effects remain scarcely evaluated, POLK overexpression resulted in tolerance to RS-inducing drugs in a xenograft model [47]. However, the extent to which artificial overexpression of POLK represents the expression in human tumors warrants further analysis.

When only one factor controls a cellular process, overexpression of this gene can achieve remarkable effects. A prime example is RRM2, the rate limiting component in the conversion of ribonucleotides to deoxyribonucleotides. As such, an extra allele of RRM2 increases RNR activity, reduces RS and confers resistance to nucleotide depletion and ATR inhibition [65]. In addition, cells with double the RPA expression compared to normal cells have less exposed ssDNA following ATR inhibition and are thus protected from DNA breaks [20].

To summarize, inconsistent experimental settings impede the correct evaluation of single factors on the RS response, but under rate-limiting conditions one factor can mitigate RS.

3.2. An army to fight RS

Whereas in specific situations one factor confers resistance to drug-induced RS, most processes controlling DNA replication are interconnected and regulated by several protein complexes. It is therefore conceivable that a multitude of factors needs to be augmented to create an environment that can resist RS. In line with this, simultaneous overexpression of *CLASPIN*, *TIMELESS* and *CHK1* in individual tumors was observed in multiple cancers [36]. Moreover, the authors show that, although ectopic expression of only *CLASPIN* or *TIMELESS* alleviated RS, the overexpression of both genes had a stronger effect [36].

While these data suggest a model in which multiple proteins are required to create a RS tolerance avenue, the number of tolerance avenues required to successfully combat RS remains enigmatic. During cancer progression and emergence of drug resistance multiple adaptations can be acquired via natural selection. An elegant study by Fumasoni and Murray employed a yeast model to mimic such evolution-like adaptation to RS [66]. The important, but non-essential, replisome component *Cft4* was deleted, which resulted in RS and forced the strain to evolve and acquire compensatory mechanisms. Interestingly, over 1000 generations, eight independent yeast strains underwent similar adaptations in three distinct processes. Namely, 1) mutations which inactivated the DNA damage checkpoint to allow proliferation, 2) amplification of chromosome regions encoding proteins that load cohesin on the DNA to ensure sister chromatid cohesion and 3) mutations to decelerate the replication machinery to improve replication fidelity. Whereas an adaptation in one of these pathways was sufficient to restore some replicative potential, adaptations in all three modules resulted in the strongest increase in fitness. This points towards a model where cancer cells employ multiple strategies to tolerate RS. Though Fumasoni and Murray model tumor evolution in a controlled laboratory setting and the mechanism of RS induction is not directly comparable to most chemotherapeutics, we discuss below how their findings translate to human cancer patients.

Firstly, yeast strains inactivate the G2-DNA damage checkpoint to allow cell proliferation despite RS. Similarly, loss of P53 confers chemoresistance in vertebrate cancer cells [67,68]. Conversely, we and others have shown that dampening of P53 signaling is detrimental for cancer cell survival when the intra S-phase checkpoint is inhibited [69,70]. We speculate that the intra S-phase checkpoint is required for complete but often erroneous, DNA replication in cancer cells [36,65], while the G2-DNA damage checkpoint must be weakened to allow cell cycle progression in the presence of mild damage and bypass apoptosis.

Besides attenuating the G2-DNA damage checkpoint, yeast strains adapt to RS by amplifying components of the cohesin loader complex. In contrast, mammalian cells remove cohesin from chromosomes to

facilitate DNA repair during RS. As a consequence of elevated endogenous RS, and thus cohesin removal, human cancer cells present reduced sister chromatid cohesion in mitosis [71]. Potentially, the amplification of cohesin loader complex components can restore the reduced cohesion after RS. Evidence supporting a pivotal role for cohesin in human cancer cells comes from studies which describe elevated levels of the cohesin component RAD21 in human breast cancer and Ewing sarcoma [72,73]. In these tumors, high levels of RAD21 increase cohesin loading, mitigate RS, confer chemoresistance and correlate with poor survival.

Lastly, RS-tolerant yeast strains inactivate DNA replication factors to reduce fork speed. This reduces the amount of DNA that is exposed to RS per unit of time and thus potentially increases the portion of repair factors available per fork. Consequently, impeding DNA replication could ameliorate RS. Intriguingly, human cancer cells employ the opposite strategy to achieve the same effect, i.e. simply increasing the production of repair factors. DNA replication and repair factors such as RRM2B and CLASPIN are often overexpressed in cancer cells with oncogene-induced RS [25,36]. Similar to the situation in yeast this will elevate the amount of repair factors per fork, reducing RS [19,36].

Inevitably, cancer cells acquire properties to survive RS. However,

Table 1
Mechanisms of replication stress tolerance.

RS tolerance avenue	Process	Gene(s)	Transcription program[a]	Drugs	Background	Reference
Ensure sufficient nucleotides	increase RNR activity	RRM2	E2F	HU/gemcitabine/cisplatin	osteosarcoma/glioblastoma/ breast/pancreatic cancer cell lines	[19,24]
		RRM2B	P53	doxorubicin/γ-radiation	NSCLC/colon/breast cancer cell lines	[26]
	rewire metabolism to increase *de novo* nucleotide synthesis and detoxify ROS	ALDOA	E2F	doxorubicin/cisplatin	breast cancer cell lines	[21]
		GLUD1		hydrogen peroxide/ gemcitabine	pancreatic cancer cell lines/ KRAS mutant	[27–29]
		GOT1	E2F	hydrogen peroxide/ gemcitabine	pancreatic cancer cell lines/ KRAS mutant	hydrogen peroxide/ gemcitabine
		GLS1		gemcitabine	pancreatic cancer cell lines/ KRAS mutant	[28]
		SLC7A11	NRF2	gemcitabine	pancreatic cancer cell lines/ KRAS mutant	[28]
		AKR1C1		gemcitabine	pancreatic cancer cell lines/ KRAS mutant	[28]
		IDH1	NRF2	gemcitabine	pancreatic cancer cell lines/ KRAS mutant	[28]
		GCLM	NRF2	oncogene-inducedgemcitabine/	pancreatic cancer cell lines/ KRAS mutant	[28]
		HMOX1	NRF2	oncogene-induceddoxorubicin/	pancreatic cancer cell lines/ RAS/BRAF/MYC mutant	[9,28]
		NQO1	E2F/NRF2	cisplatin/ gemcitabinedoxorubicin/	pancreatic cancer/KRAS/ BRAF/MYC mutant	[9]
		TKT	NRF2	cisplatindoxorubicin/	breast/pancreatic/lung cancer cell lines	[21,29,92]
		TALDO1	NRF2	doxorubicin/cisplatin	breast cancer cell lines	[21]
		TKLT1		doxorubicin/cisplatin	breast cancer cell lines	[21]
		TKLT1		doxorubicin/cisplatin	breast cancer cell lines	[21]
		G6PG		doxorubicin/cisplatin	breast/lung cancer cell lines	[21,92]
Protection of stalled replication forks	protect ssDNA	RPA1		HU/ATRi/WEE1i/cisplatin	osteosarcoma/ovarian cancer cell lines	[20,30]
		RPA2	E2F	HU/ATRi/WEE1i/cisplatin	osteosarcoma/ovarian cancer cell lines	[20,30]
	prevent collapse of stalled fork	RAD51	E2F/NRF2	PARPi/cisplatin	BRCA-deficient	[31]
		CLSPN	E2F	radiation/oncogene-induced	osteosarcoma/colon/breast/ lungcancer lines/RAS/Cyclin E mutant	[36,37]
		TIMELESS	E2F	oncogene-induced	osteosarcoma/colon/breast cancer cell lines/RAS/Cyclin E mutant	[36]
		TIPIN	E2F	oncogene-induced	osteosarcoma/colon/breast cancer lines/RAS/Cyclin E mutant	[36]
Allow DNA replication despite fork stalling	restart stalled fork	PRIMPOL	P53	cisplatin/UV/PARPi	ovarian cancer cell lines/ BRCA deficient	[35]
		WRN	E2F	cisplatin	liver/gastric cancer cell lines	[41]
		BLM	E2F	HU/gemcitabine/ aphidicolin	bloom's syndrome	[45]
		RECQL4		cisplatin	gastric cancer cell lines	[42]
		RECQL	E2F	melphalan/PARPi/ camptothecin	osteosarcoma/myeloma cell lines	[40,44]
	ignore DNA lesion	POLK	P53	temozolomide	glioblastoma cell lines	[47]
		POLH	P53	cisplatin	ovarian cancer cell lines	[51]
		REV7		cisplatin	NSCLC	[55]
A backup when initial replication forks fail	license origins	MCM2-7	E2F	HU/aphidicolin/ATRi	osteosarcoma/breast cancer cell lines	[58,64]
		CDC6	E2F	CHK1/2i	prostate cancer cell lines	[59]

[a] Included in transcription programs based on indicated references, reference [80], CHEA database and TargetGeneRegulation Database [74,109].

the number of RS tolerance avenues minimally required for therapy resistance and cancer relapse remains unknown. Whereas under specific conditions one factor can counteract RS, we hypothesize, based on the following arguments, that most tumors require multiple factors to grant RS tolerance. First, proteins which contribute to RS tolerance, such as CLASPIN and TIMELESS, are overexpressed in a coordinated manner. Second, DNA replication and repair machineries consist of multiple protein complexes, rendering the upregulation of one component ineffective. Third, when tumor evolution is recapitulated, cells acquire multiple mechanisms to mitigate RS. Typically, transcription factors control sets of functionally related genes and can thus drive coordinated expression of multiple RS tolerance factors. In the following section, we discuss the crucial transcription factors involved in RS adaptation.

4. Fulfilling the requirements for RS tolerance

As outlined above, cancer cells challenged with chemotherapy require a variety of factors to support DNA replication. P53 mediates the primary response to drug-induced RS [70]. For example, it triggers the transcription of RRM2B, to stimulate nucleotide production, and the antioxidative sestrin proteins, which mitigate DNA damage caused by ROS [74]. Moreover, P53 transcriptionally activates DNA repair factors to ensure stabilization, restart and repair of stalled replication forks [74]. Thus, the activation of the P53 transcriptional program combats RS on multiple fronts. This corroborates with the idea that cancer cells require activation of multiple RS tolerance avenues to prevent excessive RS. Consequently, cells with a perturbed P53 response present increased sensitivity to CHK1 or ATR inhibition [69,70,75]. Paradoxically, P53 is mutated and transcriptionally inactive in the majority of the human tumors. As P53 is of central importance to accurately combat RS, it raises the question how P53 deficient cancer cells grant RS tolerance.

A tantalizing hypothesis is that alternative transcriptional programs are activated to combat RS. Though the introduction of new mutations promotes tumor evolution and can give rise to permanent drug resistant clones, cancer cells first need to resist initial treatment. It is tempting to speculate that non-genetic or stochastic upregulation of protective gene expression programs provides temporal resistance, which gives time for permanent resistant clones to arise, a concept put forth to explain resistance to BRAF inhibitors [76].

Similar to the P53 transcriptional program, specific transcription factor programs would allow simultaneous activation of multiple mechanisms to curb RS. Since a large subset of known RS tolerance genes are either E2F or NRF2 targets (Table 1), the E2F and NRF2 transcription factor networks appear to fulfill such a role. Interestingly, elevated expression of these transcription programs is correlated with poor survival of cancer patients [28,77,78]. Moreover, E2F and NRF2 are previously described as key players in the RS response [28,79]. Therefore, we discuss below the evidence that these two transcriptional programs underly resistance to RS-inducing drugs in cancer patients.

4.1. The E2F program

The E2F family of transcription factors is tightly regulated, and consists of activators (E2F1-3) and repressors (E2F4-8). This allows the timely expression of an extensive gene set required for DNA replication and repair during the cell cycle [80]. There is mounting evidence that the expression of E2F target genes (referred to as E2F transcription) is elevated in tumors. Importantly, the elevated levels of E2F target genes in tumors cannot only be attributed to an overall increase in proliferation in cancer cells. Instead, individual cycling cancer cells can display excessive levels of E2F transcription independent of their proliferative status [81].

Upon RS, E2F transcription is boosted to provide sufficient DNA replication and repair factors [79,82]. The ATR-CHK1 axis governs this response by stabilizing the transcriptional activator E2F1, and inactivating the repressors E2F6-8 [19,82–84]. Moreover, cyclin F could fulfil an important role in the control of E2F transcription during RS by mediating the degradation of both activating and repressor E2Fs [85–87]. ATR can inactivate cyclin F and thereby potentially elevate the levels of multiple E2Fs [88]. It is still unclear whether the activator E2Fs (E2F1/2/3) and repressors (E2F7/8) are degraded simultaneously, or in timely order. This makes it difficult to predict how perturbations in cyclin F activity affect overall E2F transcriptional output. Nonetheless, several studies revealed that sufficient E2F transcription is crucial for accurate DNA replication. Dampening E2F transcription by overexpressing inhibitory E2Fs results in severe RS [77,79,80], while enhanced E2F transcription limits DNA damage and cell death when CHK1 is depleted [79,82]. Furthermore, a growing body of evidence shows that cells resistant to oncogene or drug-induced RS are characterized by hyperactivation of E2F transcription [31,36,89,90].

Congruent with the key role for E2F transcription to mitigate RS, loss of RB1 and amplification of cyclin E and activator E2Fs are described as mechanisms to unleash E2F transcription in cancer cells [91]. Moreover, 14-3-3 isoforms, which inhibit the E2F7/8 repressors, are overexpressed in liver cancer and correspond with elevated E2F transcription [83]. These oncogenic alterations to boost E2F transcription are potentially of critical importance when intra S-phase inhibitors are deployed and CHK1 can no longer facilitate E2F transcription (Fig. 3A).

Thus, multiple genetic alterations can stimulate E2F transcription in cancer patients. The subsequently increased E2F transcription mitigates RS and is an important determinant in RS tolerance. As a result, high E2F transcription predicts poor prognosis in multiple cancers, including primary liver cancer [77].

4.2. The NRF2 program

NRF2 is a well-known master regulator of the antioxidant response. It controls the expression of classical ROS-detoxifying genes by binding the antioxidant response element (ARE) in their promotor region [9]. However, NRF2 has a role beyond ROS detoxification. It promotes glutaminolysis, the pentose phosphate pathway and serine biosynthesis which fuel nucleotide synthesis and provide the reductive NADH [92,93]. Moreover, *BRCA1* and *RAD51* were recently identified as NRF2 target genes [94]. As a result, cells lacking NRF2 do not only display increased ROS levels and impaired nucleotide production, but also have reduced DNA repair capacity [94]. Thus, multiple NRF2-stimulated pathways can coordinately contribute to RS tolerance.

In line with this, overexpression of NRF2 bolsters chemoresistance *in vitro* [28,95] whereas suppression of NRF2 in xenografts increases the efficacy of RS-inducing drugs [92,95]. There is mounting evidence that NRF2 levels are also relevant in human cancer as high levels of NRF2 correlate with poor therapy response and survival [28,96].

Although NRF2 is rarely mutated in cancer, several alternative mechanisms are described to boost NRF2 transcription in cancer cells (Fig. 3B). For example, inactivating mutations in *KEAP1* and *CUL3*, which target NRF2 for degradation, increase NRF2 activity and confer chemoresistance [97,98]. In addition, NRF2 expression is directly controlled by several oncogenes including *KRAS* and *c-MYC* [9,28]. It is noteworthy that mutations in *KEAP1* frequently co-occur with *KRAS* mutations. While oncogenic KRAS induces endogenous RS and renders cells sensitive to RS-inducing drugs, this increased sensitivity is counteracted by KEAP1 inactivation [99]. Together, this illustrates the importance of the NRF2 transcription program in cancer cells to combat RS.

5. Challenges and opportunities: RS tolerance in cancer patients

5.1. Biomarkers of RS tolerance

E2F and NRF2 transcription factors drive key transcriptional programs that mitigate RS. This provides a rationale to employ NRF2 and E2F activity as biomarkers to select patients who will benefit from

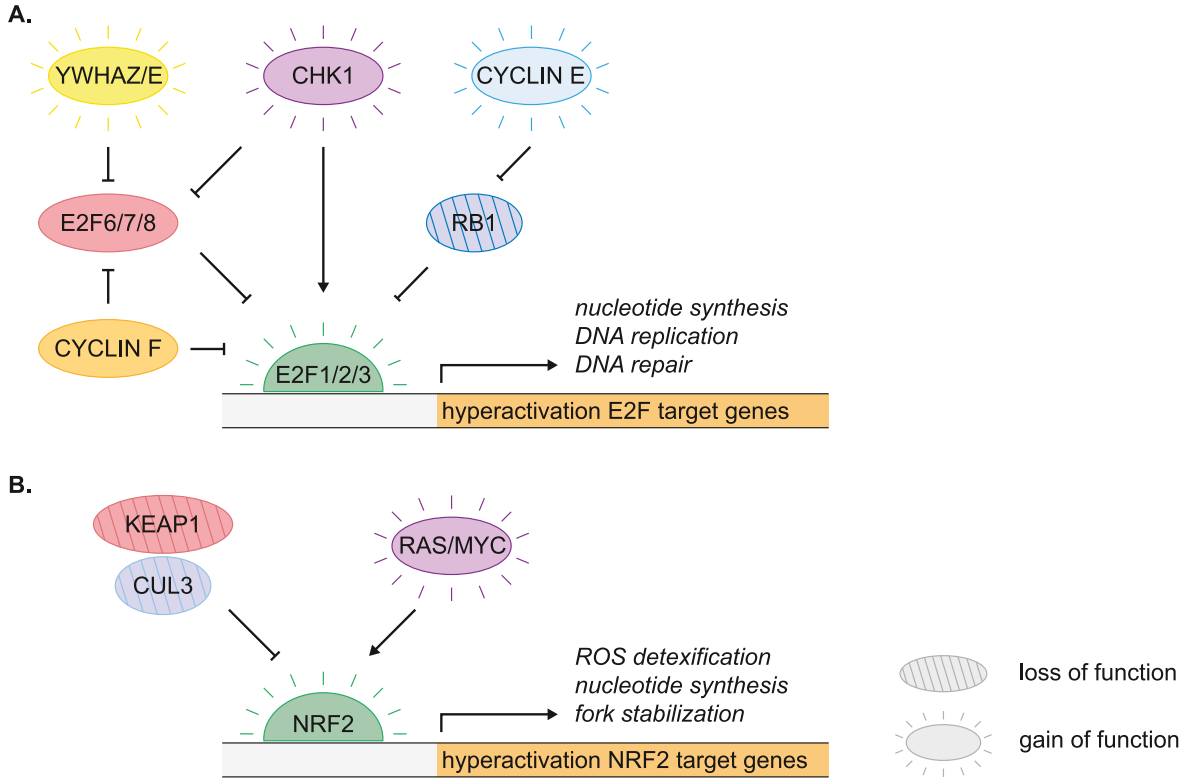

Fig. 3. Activation of E2F and NRF2 transcriptional program to curb replication stress.
A Simplified model of the regulation of E2F target genes. Loss- or gain-of-function mutations can hyperactivate the transcription of target genes resulting in RS tolerant cancer cells.
B Same as in A, but now for NRF2 target genes.

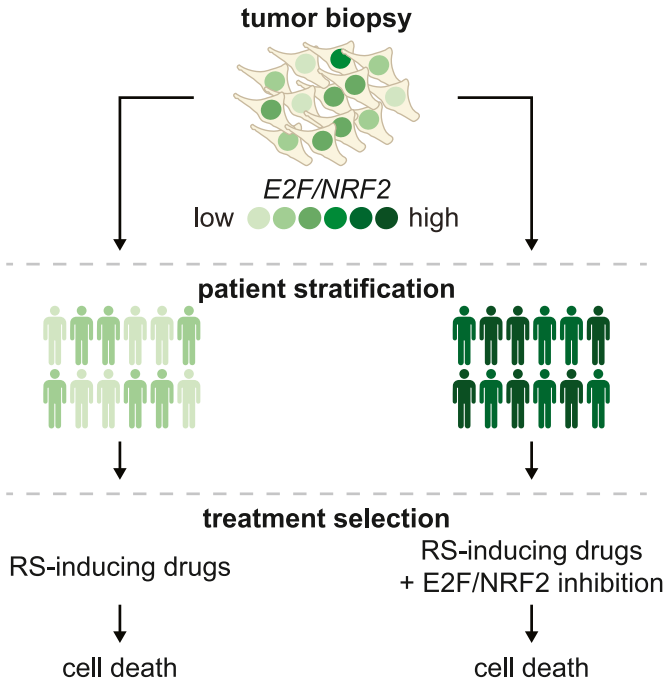

Fig. 4. Dealing with replication stress tolerance in cancer patients.
Schematic overview summarizing how the E2F and NRF2 transcriptional activity can be used to stratify patients. We propose that patients with low levels of E2F/NRF2 transcription can efficiently be treated with RS-inducing drugs (left), whereas this treatment in patients with high levels of E2F/NRF2 transcription would need to be supplemented with E2F/NRF2 inhibitors (right).

treatment with RS-inducing drugs (Fig. 4). The clinical benefit of such strategy is shown for *BRCA*-deficient tumors which are extremely sensitive to PARP inhibitors. To create a robust biomarker for E2F and NRF2 activity, the expression across multiple NRF2 and E2F target genes can be used as read-out. Although gene expression is inherently noisy compared to mutation analysis, this problem can be in part tackled by analyzing a panel of genes. The advantage of such transcriptomic analysis is that the net effect of a wide range of mutations as well as transcriptional (e.g. promotor methylation) and post-transcriptional alterations (e.g. impaired degradation) boosting E2F or NRF2 transcription are taken into account. However, care should be taken when evaluating the levels of E2F target genes as their expression oscillates throughout the cell cycle. As a consequence, highly proliferative tumors present high levels of E2F target genes. In line with this, high E2F target gene expression in a tumor biopsy may simply reflect high proliferation rates, and thus correlates with chemosensitivity [100]. To overcome this confounding effect, E2F transcription in single cells must be evaluated. Next, proliferating cancer cells can be selected using a machine learning classifier after which an E2F biomarker score can be calculated independent of the proliferation rate of a tumor [81,101].

We propose that such cell cycle phase-corrected E2F or NRF2 transcription biomarkers could be particularly useful for patients suffering from muscle-invasive bladder cancer (MIBC) because elevated E2F transcription in cycling cells is expected in a substantial number of these patients caused by amplification of *E2F1* or *E2F3* [102]. Furthermore, DNA methylation can underlie altered expression of E2F family members [103]. Another cancer type for which an E2F biomarker could be of specific interest is ovarian cancer. High-grade ovarian carcinomas show *E2F3* amplification, but even more frequently amplification of *CCNE1*, the gene that encodes cyclin E1 [104]. *CCNE1* amplification can unleash E2F-dependent transcription via activation of CDK2. Ovarian carcinomas often show high levels of replication stress, and encouraging

results in clinical trials with the WEE1 inhibitor adavosertib were recently reported [105]. Likewise, NSCLC patients are of interest due to the high prevalence of mutations in the negative regulator of NRF2, *KEAP1*, and transcriptional activation of NRF2 via oncogenic RAS [9,96,99]. Since MIBC, ovarian cancer and NSCLC are often, but not always, characterized by alterations in pathways that affect E2F and NRF2, a biomarker that can differentiate patient populations with low versus high E2F and NRF2 transcriptional activity could better predict treatment success. Furthermore, RS-inducing drugs, such as cisplatin and gemcitabine, are part of the first-line treatment regimens for MIBC, ovarian cancer and NSCLC [102]. Because elevated E2F and NRF2 transcription protect against numerous RS-inducing drugs in different clinical backgrounds [28,31,79,82,95,98] (Table 1), we suggest that the E2F-NRF2 transcription biomarker is widely applicable to drugs that interfere with DNA-replication, including RS-inducing drugs currently used to treat MIBC, ovarian cancer and NSCLC patients.

5.2. Targeting RS tolerance

The different RS tolerance avenues described here provide a new strategic framework for cancer treatment. For example, cancer cells heavily rely on glutaminolysis to synthesize sufficient nucleotides to combat RS. Hence, co-administration of glutaminolysis inhibitors and RS-inducing drugs is an attractive treatment strategy. Recent efforts have been made to manipulate glutamine metabolism. Specifically, glutaminase, the rate-limiting enzyme for glutaminolysis can be pharmaceutically inhibited by CB-839 [28]. CB-839 has sparked interest since it was shown to potentiate the effect of gemcitabine in xenograft experiments and is currently undergoing clinical trials [28]. Moreover, the fact that sensitization to glutaminase inhibition was dependent on hyperactivation of the NRF2 pathway holds promise for patients with RS tolerant tumors [96].

Besides glutaminolysis, cancer cells require TLS polymerases to deal with RS. Therefore, several studies evaluated the potential of targeting TLS polymerases. For example, the small-molecule JH-RE-06 was shown to bind the surface of REV1 and thereby disrupt its interaction with the POLZ TLS subunit REV7. As a result, JH-RE-06 potentiated the effect of cisplatin in *in vitro* and xenograft experiments [54]. Moreover, another TLS inhibitor targeting REV1 increased the RS induced by ATR inhibition [106]. Besides an effective anti-cancer strategy, TLS polymerase inhibitors are shown to limit cisplatin induced mutagenesis which potentially prevents secondary drug-induced malignancies [54].

So far, we highlighted the potential to target specific RS tolerance avenues. Nonetheless, a tumor can be depicted as multiheaded dragon which might easily adopt alternative strategies. Therefore, we propose to inhibit the E2F and NRF2 transcription program to completely ablate RS tolerance (Fig. 4). Although targeting proteins without enzymatic functions, such as transcription factors, is technically challenging, several promising candidate strategies are on the rise [107]. For example, PROTAC in which the protein of interest is brought into proximity of an E3 ligase, may be an attractive strategy to restrain E2F and/or NRF2 activity [108].

6. Concluding remarks

As our understanding on the basic biology of cancer cells deepens, more mechanisms exploited by cancer cells to escape anti-cancer therapies are revealed. Specifically, detailed analysis of the replication fork has shed light on the molecular processes that protect and repair stalled replication forks. Importantly, these experiments are accompanied by large scale transcriptomic studies to validate *in vivo* relevance. The challenge for cancer researchers in the coming decade is to interconnect all these findings, identify biomarkers and develop drugs against novel therapeutic targets. This will allow a personalized approach in which effective anti-cancer therapies for every patient can be selected.

Author contributions

H.A.S.: Conceptualization, Visualization and Writing - original draft. B.W.: Conceptualization, Writing - review and editing, Supervision and Funding acquisition.

Declaration of competing interest

None.

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List of abbreviations

ARE	Antioxidant Response Element
ATR	Ataxia Telangiectasia and Rad3 related kinase
BLM	Bloom syndrome RecQ like helicase
BRCA1	BRCA1 DNA repair associated
BRCA2	BRCA2 DNA repair associated
CCNE1	Cyclin E1
CDC6	Cell Division Cycle 6
CDK	Cyclin Dependent Kinase
CDT1	Chromatin licensing and DNA replication factor 1
CHK1	Checkpoint Kinase 1
CUL3	Cullin 3
KEAP1	Kelch-like ECH-associated protein 1
KRAS	KRAS proto-oncogene, GTPase
MCM2-7	Mini-Chromosome Maintenance complex component 2-7
MIBC	Muscle-Invasive Bladder Cancer
NRF2	Nuclear factor erythroid 2-related factor 2
NSCLC	Non-Small Cell Lung Cancer
ORC1-6	Origin Recognition Complex subunit 1-6
POLH	DNA polymerase eta
POLK	DNA polymerase kappa
POLZ	DNA polymerase zeta
RAD21	RAD21 cohesion complex component
RAD51	RAD51 recombinase
RADX	RPA1 related single stranded DNA binding protein
RECQ	ATP-dependent DNA helicase RecQ
REV1	REV1 DNA directed polymerase
REV3	DNA polymerase zeta, Rev3 catalytic subunit
REV7	DNA polymerase zeta, Rev7 subunit
RNR	Ribonucleotide Reductase
RPA	Replication Protein A
RRM1	Ribonucleotide Reductase catalytic subunit M1
RRM2	Ribonucleotide Reductase regulatory subunit M2
RRM2B	Ribonucleotide Reductase regulatory TP53 inducible subunit M2B
RS	Replication Stress
ROS	Reactive Oxygen Species
SMARCAL1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin
ssDNA	single-stranded DNA
TIMELESS	timeless circadian regulator
TIPIN	TIMELESS interacting protein
TLS	Translesion Synthesis
WEE1	WEE1 G2 checkpoint kinase
WRN	Werner RecQ like helicase

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