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Protracted p53-independent stimulation of p21$^{WAF1/Cip1}$ fuels genomic instability by deregulating the replication licensing machinery

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Abbreviations:

RLFs: Replication licensing factors, MUS81-EME1: methymethanesulphonate-MMS and ultraviolet-sensitive81-MUS81 and essential meiotic endonuclease1-EME1, EGF: epidermal growth factor, FGF2: fibroblast growth factor-2, TGF-β: transforming growth factor-β, HJ: Holliday Junction, NHEJ: non-homologous end joining, PIP: PCNA-Interacting-Protein motif

Abstract

The cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ is the prototype downstream effector of the tumor suppressor protein p53. Yet, evidence from human cancer and mice models, imply that $p21^{WAF1/Cip1}$, under certain conditions, can exercise oncogenic activity. The mechanism behind this behavior is still obscure. Within this context we unexpectedly noticed, predominantly in p53 mutant human cancers, that a subset of highly atypical cancerous cells expressing strongly $p21^{WAF1/Cip1}$ demonstrated also signs of proliferation. This finding suggests either tolerance to high $p21^{WAF1/Cip1}$ levels or that $p21^{WAF1/Cip1}$ *per se* guided a selective process that led to more aggressive off-springs. To address the latter scenario we employed $p21^{WAF1/Cip1}$-inducible p53-null cellular models and monitored them over a prolonged time period, using high-throughput screening means. After an initial phase characterized by stalled growth, mainly due to senescence, a subpopulation of $p21^{WAF1/Cip1}$ cells emerged, demonstrating increased genomic instability, aggressiveness and chemo-resistance. At the mechanistic level unremitted $p21^{WAF1/Cip1}$ production "saturates" the $CRL4^{CDT2}$ and SCF^{Skp2} ubiquitin ligase complexes reducing the turn-over of the replication licensing machinery. Deregulation of replication licensing triggered replication stress fuelling genomic instability. Conceptually, the above notion should be considered when anti-tumor strategies are designed, since $p21^{WAF1/Cip1}$ responds also to p53-independent signals, including various chemotherapeutic compounds.

Introduction

Accruing evidence point out that a number of molecules involved in key cellular processes display bimodality in cancer i.e. they can act either as tumor suppressors or as oncoproteins **(Supplemental Table 1)**. This odd phenomenon is attributed to the so called "cellular or environmental context" that configures their behavior. The mechanistic basis underlying this context-dependent duality is vague in most cases and its explication is essential for rationally designed therapeutic strategies.

The cyclin-dependent kinase inhibitor (CDKI) $p21^{WAF1/Cip1}$ is a pivotal downstream effector of the master tumor-suppressor protein p53, mediating mainly G1 growth arrest in response to various stimuli. This function is primarily dependent on the ability of $p21^{WAF1/Cip1}$ to inhibit cyclin-dependent kinase-2 (Cdk2)**(Abbas and Dutta 2009).** In spite of its profound p53-dependent role in halting cellular proliferation, several reports, in human cancer and mice models, suggest that $p21^{WAF1/Cip1}$ can manifest oncogenic activities **(Supplemental Table 1)**. In some of these studies the oncogenic function was credited to the non-conventional cytoplasmic localization of $p21^{WAF1/Cip1}$ which binds and inhibits the activity of proteins directly involved in apoptosis **(Roninson 2002; Pateras et al. 2009)**. However, in most cases the underlying mechanism remains speculative. It is also interesting that while p53 is frequently mutated in cancer **(Rivlin et al. 2011)**, $p21^{WAF1/Cip1}$ is rarely affected genetically **(Abbas and Dutta 2009; Warfel and El-Deiry 2013)**. The latter would be logical if $p21^{WAF1/Cip1}$ operated exclusively within the p53 signaling cascade. Nevertheless, $p21^{WAF1/Cip1}$ is activated by a wide range of p53-independent signals and stimuli, including growth factors, nuclear receptors, chemicals and drugs **(Abbas and Dutta 2009)(Supplemental Fig. S1)**.

We report that constitutive expression of $p21^{WAF1/Cip1}$, in a p53 loss of function environment, causes replication stress and triggers genomic instability by deregulating the replication licensing machinery. Replication licensing is a fundamental biological process that assures that replication takes place once per cell cycle **(Abbas et al. 2013).** The replication licensing factors (RLFs) ORC, Cdt1 and Cdc6 accumulate during late M and G1 phases forming together with the MCM2-7 helicase the pre-replication complex, licensing the genome for replication. Upon entry into S-phase Cdk activity increases, the replication origins are fired initiating the replication process, while the RLFs are targeted for degradation (unlicensed state) **(Takeda and Dutta 2005)**. Deregulation of the replication licensing process is linked with genomic instability and promotion of malignant behavior, mainly via a process termed re-replication **(Blow and Gillespie 2008; Negrini et al. 2010; Halazonetis et al. 2008)**. Aberrant expression of RLFs is reported in various common malignancies such as head and neck, lung and colon cancer **(Karakaidos et al. 2004; Liontos et al. 2007).**

Results

1. In advanced stage cancer, a subset of atypical cells expressing p21$^{WAF1/Cip1}$ demonstrated signs of proliferation: In an array of tumors, that have not undergone previous chemo- or radio-therapy, we observed an unusual relationship between p21$^{WAF1/Cip1}$ and the proliferation marker Ki67 that drove our attention. While the anticipated mutual exclusive p21$^{WAF1/Cip1}$/Ki67 expression pattern was evident, in line with p21$^{WAF1/Cip1}$'s growth inhibitory properties, we noticed that a number of large cancerous cells with giant nuclei co-expressed p21$^{WAF1/Cip1}$ and Ki67 **(Fig. 1)**. In pathology, identification of cancerous cells with such morphological features is considered a sign of adverse prognosis **(Rosai and Ackerman 2011)**. Notably, most of the carcinomas examined displayed p53 alteration **(Karakaidos et al. 2004; Liontos et al. 2007; Velimezi et al. 2013).** The irregular p21$^{WAF1/Cip1}$/Ki67 co-localization led us to hypothesize either tolerance to high p21$^{WAF1/Cip1}$ levels or that somehow constitutive p21$^{WAF1/Cip1}$ expression guided a selective process resulting in the emergence of a subpopulation of p21$^{WAF1/Cip1}$ expressing cancerous cells that regained their proliferative capacity and possibly during this process became more aggressive.

2. p53-independent stimulation of p21$^{WAF1/Cip1}$ up-regulates key replication licensing factors: To address the later scenario we employed a doxycycline-inducible p21$^{WAF1/Cip1}$ expression system (Tet-ON) introduced in the p53 null cells Saos2 (Tet-ON p21$^{WAF1/Cip1}$ Saos2)**(Bates et al, 1998)** and monitored, subsequent to p21$^{WAF1/Cip1}$ induction, their behavior over a prolonged period **(Supplemental Fig. S2)**. To avoid heterogeneous p21$^{WAF1/Cip1}$ expression from bulk cultures we isolated p21$^{WAF1/Cip1}$-inducible clones and experimented with those demonstrating the strongest induction.

Periodically (every 5 days) $p21^{WAF1/Cip1}$ expression was evaluated by *in situ* analysis. In parallel, the transcriptome and proteome landscapes were examined at distinct time-points (12hs, 48hs and 96hs) in an attempt to unravel pathways/networks that could exercise over time an "oncogenic" effect **(Supplemental Tables 2,3)**. Representative results obtained by the high-throughput assays were confirmed independently by quantitative real-time PCR and immune-blotting **(Fig. 2, Supplemental Fig. S3)**.

As expected and previously reported **(Bates et al. 1998),** the cells reduced their growth rate and progressively most of them acquired a senescent phenotype that peaked around day 10 **(Supplemental Fig. S4, Supplemental Video 1)**. In accordance with the phenotypical changes and growth kinetics, two-way Gene-Ontology biological-process enrichment analyses, followed by false discovery rate correction **(Supplemental Fig. S2)**, revealed at the transcriptomic and proteomic level, suppression of central factors involved in "mitosis" and "mitosis surveillance" **(Fig. 2a)**. Surprisingly, however, proteome analysis disclosed also a prominent up-regulation of the RLFs, Cdt1, Cdc6 and ORC **(**p=1.5 x 10^{-6}); with Cdt1 protein raise being the earliest biochemical alteration among all measured transcriptome and proteome changes **(Supplemental Tables 2,3)**. The protein abundance raise of the RLFs, was not accompanied by a significant mRNA increase, implying regulation at the post-transcriptional level **(Fig. 2)**. Since Cdt1 and $p21^{WAF1/Cip1}$ are targeted for degradation by the same ubiquitin ligase complexes, $CRL4^{CDT2}$ and SCF^{Skp2} **(Abbas et al. 2008; Kim et al. 2008; Havens and Walter 2011)**, we reasoned that unremitted $p21^{WAF1/Cip1}$ production could sequester their enzymatic activity leading eventually to Cdt1 accumulation **(Supplemental Fig. S5a)**. In support to this notion, SET8, an H4 methyltrasferase required for chromosome compaction in mitosis, and E2F1, targets

of $CRL4^{CDT2}$ **(Jorgensen et al. 2011)** and Skp2 **(Marti et al. 1999)**, respectively, were up-regulated after $p21^{WAF1/Cip1}$ induction **(Supplemental Fig. S5a,S6).** Moreover, transfection with a mutant $p21^{WAF1/Cip1}$ that harbors three substitutions in its PIP degron motif abrogating its interaction with PCNA, which is essential for $CRL4^{CDT2}$ to target $p21^{WAF1/Cip1}$, did not augment Cdt1 expression, strongly favoring the "saturation/competition" mechanism **(Fig. 2b)**. In addition, the cell cycle profile imposed by constitutive $p21^{WAF1/Cip1}$ expression, characterized by a G1-phase increase and an S-phase decline, could contribute to the reduced RLF protein turn-over observed **(Supplemental Fig. S5b)**. Along the same line, blocking protein synthesis with cyclohexamide, demonstrated a dramatic enhancement of Cdc6 protein stability, in $p21^{WAF1/Cip1}$ expressing cells **(Supplemental Fig. S5c)**. Reduced Cdc6 protein turn-over could be attributed to low activity of the E3-ligase APC^{Cdh1} that targets Cdc6 for degradation **(Duursma and Agami 2005)**. Indeed, the levels of the E3-ligase APC substrate recognition and activating modules Cdh1/Fzr1 were decreased, supporting the above assumption **(Supplemental Fig. S5c)**. Increased Cdc6 stability was also associated with Cdk2-mediated phosphorylation at Serine 54 (Cdc6-pS54) that protects it, as previously reported, from rapid destruction **(Supplemental Fig. S5c) (Duursma and Agami 2005)**. Despite the fact that $p21^{WAF1/Cip1}$ expression reduced Cdk2 activity **(Supplemental Fig. S7)** low Cdh1/Fzr1 levels appears to tilt the balance in favor of Cdc6-pS54 accumulation **(Supplemental Fig. S5c)**. Notably, in the presence of cycloheximide and the proteasome inhibitor MG132, the protein levels of Cdh1/Fzr1 were not restored implying regulation at the transcriptional level. To this end we examined the mRNA levels of Cdh1/Fzr1 and found them suppressed under conditions of $p21^{WAF1/Cip1}$ expression **(Supplemental Fig. S5d)**.

Since the p53 checkpoint was shown to protect from re-replication **(Vaziri et al. 2003)**, we reasoned that the status of p53 would define the ability of $p21^{WAF1/Cip1}$ to regulate the levels of Cdt1 and Cdc6. Indeed, challenging cellular systems bearing inactive p53, with $p21^{WAF1/Cip1}$, resulted in the up-regulation of these replication licensing factors (**Fig. 2c**). On the other hand, $p21^{WAF1/Cip1}$ expression in a cellular environment with wild-type p53 suppressed their expression (**Fig. 2c,d**). It was suggested, but not shown, that p53 shields the organism from cells undergoing re-replication by triggering apoptosis **(Vaziri et al. 2003)**. In line with this notion, we noticed that the $p21^{WAF1/Cip1}$-IPTG-ON HT1080 cells, harboring wtp53, experience massive apoptosis, after $p21^{WAF1/Cip1}$ induction, which was accompanied by a dramatic decrease of Cdt1 and Cdc6 expression, whereas silencing of p53 suppressed apoptosis, up-regulated Cdt1, and restored Cdc6 levels **(Vaziri et al. 2003)**.

3. Activation of $p21^{WAF1/Cip1}$, in p53 null cells, triggers replication stress in a Cdt1/Cdc6-dependent manner: Re-replication, is a form of replication stress and is considered, as mentioned, the basis of genomic instability driven by inappropriate expression of the RLFs, Cdc6 and Cdt1 **(Blow and Gillespie 2008; Abbas et al. 2013)**. Although various mechanistic aspects of how re-replication challenges the stability of the genome are still obscure, it is well ascertained that it leads to DNA damage and DNA damage response (DDR) activation (**Blow and Gillespie 2008; Abbas et al. 2013)**. Following $p21^{WAF1/Cip1}$ induction, MCM2-7 chromatin loading increased robustly indicating that Cdt1 and Cdc6 up-regulation is functional **(Supplemental Fig. S8)**. Flow cytometric analysis of propidium iodide stained cells (DNA content) and double co-stained cells for DNA content and DNA synthesis (Ethynyl-deoxyUridine: EdU) revealed a cellular sub-population with DNA content

greater than 4n, indicative of re-replication **(Fig. 2e; Supplemental Video 2).** Subsequently, DNA damage, as assessed by single cell gel electrophoresis (comet assay) under alkaline conditions (total number of DNA lesions) and pulse field gel electrophoresis (PFGE), was evident, tracked by signs of DDR, as depicted by increase recruitment of 53BP1 in foci and H2AX phosphorylation (γ-H2AX) **(Fig. 3a,b,c, Supplemental Fig. S6)**. Strikingly, re-replication, DNA damage, as well as the DDR, were Cdc6- and Cdt1-dependent further suggesting that re-replication was the source of replication stress **(Fig. 3b,c)**. Finally, re-replication and DNA damage was significantly lesser when the $p21^{PCNA}$ mutant was employed keeping in line with the proposed "saturation/competition" mechanism **(Supplemental Fig. S5,S9)**.

It seems paradoxical that $p21^{WAF1/Cip1}$ could trigger replication stress, given its role as a potent inhibitor of cell cycle progression. Nevertheless, fork progression analysis by DNA fiber spreading (DNA combing), using 20 min consecutive labeling pulses of CldU (red) and IdU (green), showed that replication fork progression did not cease, but its speed was significantly reduced **(Fig. 3d)**. In addition, replication fork asymmetry was observed possibly related with the presence of DNA lesions impeding symmetrical bi-directional movement **(Fig. 3d)**. Consistent with this notion, flow cytometry analysis with double co-staining for γ-H2AX, DNA content (Propidium Iodide: PI) and DNA synthesis (Ethynyl-deoxyUridine: EdU) showed that, following $p21^{WAF1/Cip1}$ induction, DNA damage accumulated mainly in cells incorporating EdU, whereas depletion of Cdc6 and Cdt1 profoundly suppressed the accumulation of DNA damage in S-phase **(Fig. 3e)**. Markedly, the cells expressing $p21^{WAF1/Cip1}$ demonstrated a focal PCNA pattern characterized by small dots that represents a trait of early S-phase **(Essers et al. 2005)(Supplemental Fig. S10)**. This finding suggests that DNA damage occurs at a time sensitive slot, as current data demonstrate the

presence of early replication fragile sites that are prone to breakage under conditions of replication stress **(Barlow et al. 2013)**.

4. $p21^{WAF1/Cip1}$-mediated replication intermediate lesions are processed by MUS81-EME1 and repaired by a Rad52-dependent mechanism: To further characterize $p21^{WAF1/Cip1}$-mediated replication stress we examined for single-stranded DNA (ssDNA) formation, a common intermediate at replication-associated lesions. To this end, $p21^{WAF1/Cip1}$ expressing cells were incubated with BrdU for 24hs, under non-denaturating conditions, allowing anti-BrdU staining to disclose ssDNA **(Beck et al. 2010)**. In line with our hypothesis, *in situ* analysis showed a strong co-localization between BrdU staining and $p21^{WAF1/Cip1}$ expression **(Fig. 4a)**, that was also associated with an increased number of foci formed by the ssDNA-binding protein RPA **(Fig. 4b)**. The ssDNA could be generated by replicative helicase – polymerase uncoupling (stalled fork), template-strand resection after double strand break (DSB) formation, unequal branch migration of stalled fork that yield reversed forks structures (chicken foot), or ssDNA gaps on template DNA **(Petermann and Helleday 2010; Neelsen et al. 2013a; Couch et al. 2013)(Supplemental Fig. S11)**. To further discriminate whether ssDNA is from the template or the newly synthesized (nascent) strands short BrdU pulses (20 min) were used. The analysis did not show BrdU staining, suggesting that the source of the ssDNA is the template strand **(Supplemental Fig. S12)**. Regardless of the way ssDNA is formed, it represents a structure of DNA replication intermediate lesions that need to be resolved for replication to restart. It has been reported that after long periods of replication inhibition double strand breaks (DSBs), generated by the structure-specific endonuclease complex of MUS81-EME1*, are required for restart **(Hanada et al. 2007; Petermann and Helleday 2010)**. Based on

this fact we hypothesized that sustained $p21^{WAF1/Cip1}$ expression phenocopies the latter state involving MUS81-EME1. Indeed, MUS81-EME1 depletion caused a significant decrease of the DNA damage inflicted by $p21^{WAF1/Cip1}$ expression, as well as reduction of the EdU positive cells harboring signs of DNA damage **(Fig. 4c,d; Supplemental Fig. S13)**. Recent reports present MUS81-EME1 as a central player in oncogene-induced genotoxicity **(Neelsen et al. 2013b; Murfuni et al. 2013)**, promoting homologous recombination (HR)-mediated repair of inactivated (collapsed) forks **(Petermann and Helleday 2010)**. Surprisingly, we noticed that silencing of the homologous repair recombinase Rad51 resulted in decreased γ-H2AX levels **(Fig. 4e)**. This finding implies a negative control over an alternative, Rad51-indepenent, repair process. Rad51 was reported to exert such an effect preventing Rad52-dependent DNA annealing and repair **(Wu et al. 2008)**. In fact, suppression of Rad52 was followed by increased γ-H2AX expression and cell death **(Fig. 4f,g)**, suggesting that Rad52 guided the repair process. Rad52 is possibly involved in microhomolgy-mediated repair pathways, which are considered mutagenic **(Ottaviani et al. 2014)** and according to latest data collapsed forks are recovered by error-prone replicative-based mechanisms challenging genome stability **(Iraqui et al. 2012)**. An interesting observation was that Rad51 levels were reduced upon $p21^{WAF1/Cip1}$ induction. Rad51 is in short supply and under certain stressogenic conditions such as hypoxia it is repressed by E2F4/p130 complexes. Given that p53-dependent gene repression via $p21^{WAF1/Cip1}$ is mediated by recruitment of such complexes **(Benson et al. 2013)**, we hypothesized that protracted $p21^{WAF1/Cip1}$ expression mimics such circumstances and examined whether the promoter of Rad51 is occupied by E2F4. Indeed it did **(Fig. 4h)**, providing a mechanistical explanation why Rad52 is the preferable repair choice in this setting.

5. Deregulated up-regulation of Cdt1 and Cdc6 links p53-independent activation of p21$^{WAF1/Cip1}$ with senescence: The results so far showed that sustained p21$^{WAF1/Cip1}$ expression triggered senescence, a well established anti-tumor response **(Bartkova et al. 2006; Halazonetis et al. 2008)** that nonetheless**,** in a p53 null environment, harbored the seeds of genomic instability, in the form of deregulated licensing factors. P21$^{WAF1/Cip1}$-imposed senescence was to a large extent mediated by Cdt1 and Cdc6, apparently as a result of the DNA damage checkpoints they activated **(Fig. 5a,b) (Bartkova et al. 2006; Liontos et al. 2007)**. In a previous study we noticed that p73, the p53 homologue, responds to the DDR pathway **(Liontos et al. 2009)**. As inappropriate expression of Cdt1 and Cdc6 caused replication stress and DNA damage we asked whether p73 could operate as the downstream effector of the emerging p21$^{WAF1/Cip1}$–RLFs–DDR–signaling route, stimulating senescence. We found that p21$^{WAF1/Cip1}$ induction elicited p73 up-regulation, in a Cdt1/Cdc6-dependent manner, that was essential for executing the senescent program **(Fig. 5a,b)**. According to the oncogene-induced DNA damage concept for cancer development continuous formation of DNA breaks exerts a selective pressure on the DDR-induced anti-tumor barriers, eventually breaching them, leading to genomic instability and tumor progression **(Halazonetis et al. 2008)**. If this holds true, then at some point the p21$^{WAF1/Cip1}$ expressing cells will bypass the senescence barrier generating more aggressive offsprings. In addition, apart from fueling genomic instability **(Sideridou et al. 2011)**, Cdc6 overexpression could confer to tumorigenesis by repressing the *INK4/ARF* locus **(Gonzalez et al. 2006)**. Indeed, p21$^{WAF1/Cip1}$ activation led to down-regulation of both products of the *INK4/ARF* locus, p16^{INK4A} and p14^{ARF}, apparently as a result of Cdc6 up-regulation **(Fig. 5c)**. Suppression of p16^{INK4A} removes an

indispensable factor for maintaining the senescent phenotype **(Beauséjour et al. 1999)**; thus rendering $p21^{WAF1/Cip1}$-mediated senescence possibly reversible.

6. Protracted expression of $p21^{WAF1/Cip1}$, in a p53 loss of function environment, bypasses senescence, fuels genomic instability, promoting aggressive behavior: Monitoring the behavior of the cells we interestingly observed that after the 10^{th} day of switching on $p21^{WAF1/Cip1}$ the senescent phenotype gradually declined. Concurrently, a sub-population of non-senescent $p21^{WAF1/Cip1}$ positive cells emerged **(Fig. 6a)** demonstrating, later than the 20^{th} day of induction, BdrU and EdU incorporation values similar to those seen in the control counterparts **(Fig. 6b,c; Supplemental Video 3)**. Likewise, around the same time period, the mutual exclusive relationship between cyclin A, an established late S/G2 marker **(Woo and Poon 2003; Pines and Hunter 1991)**, and $p21^{WAF1/Cip1}$ was reduced, and accompanied by a double $p21^{WAF1/Cip1}$/cyclin A positive sub-population **(Fig. 6d)**. These results strongly suggest that a fraction of $p21^{WAF1/Cip1}$ expressing cells evaded from $p21^{WAF1/Cip1}$-induced senescence and arrest, re-entering the cell cycle (from now on these cells are termed *"escaped"*). To support this observation, cdk2 activity as well as phosphorylation of the stimulatory site T160 of Cdk2 that were suppressed upon $p21^{WAF1/Cip1}$ induction, were restored **(Fig. 6e, Supplemental Fig. S14)**. Strikingly, the "escaped" cells showed a dramatic reduction of p73 expression that concurs with the decline of the senescent phenotype **(Fig. 6e)**. Another interesting finding was that most of their nuclei were significantly larger than those of the un-induced cycling control cells **(Fig. 6f, Supplemental Fig. S15, Supplemental Video 4)**, a feature that was also observed *in vivo* **(Fig. 1)**. DNA damage formation was also reduced in the "escaped" cells implying that an extensive repair process took place **(Fig. 6g)**. The

involvement of the MUS81-EME1 - Rad52 repair route **(Fig. 4c,d)** and the increased presence of micronuclei **(Fig. 7b)** that are considered surrogate markers of chromosomal instability, defective DDR and repair (**Terradas et al. 2009**), point out, as mentioned already above, that the latter was error-prone. To gain a more precise, genome wide, view of this likely outcome we performed array comparative genomic hybridization (aCGH) analysis, deep sequencing and M/FISH/SKY combined with inverted-DAPI banding between the "escaped" and the un-induced cells (**Fig. 7, Supplemental Fig. S16-S18; Supplemental Tables 4-5**). In total six independent biological replicates were performed and the duration of each experiment was 30 days to produce an adequate amount of "escaped" cells for the analysis. To avoid cell culture propagation effects that could mask the effect of $p21^{WAF1/Cip1}$ activation the OFF cells were cultured for the same period **(Supplemental Fig. S16a).** In aCGH the un-induced cells were used as reference; thus eliminating the noise showing only the differences between the two states (OFF vs ON). Cumulatively the results from all three experimental procedures clearly disclosed that the genome context of the "escaped" cells acquired chromosomal aberrations, in the form of gains and losses that ranged in size from approximately 1.75Kb to 92Mb (**Fig. 7a**, **Supplemental Figs S16b,c, S17, S18; Supplemental Tables 4-5**), as well as novel translocations (**Fig. 7c, Supplemental Fig. S16c**). Interestingly among the genetic lesions found were alterations concentrated in the same chromosome bands (3p14.3, 3p14.2, 10p15.3) consisting of alternating regions of gains or losses with retention regions in between (**Fig. 7a**, **Supplemental Table 4**), most probably representing events of chromoanasynthesis or chromothripsis, respectively (**Holland and Cleveland 2012**). One of the most noteworthy observations of this multifaceted experimental approach was the strong concordance between the findings of the cytogenetic, aCGH and deep

sequencing analyses **(Fig. 7d, Supplemental Fig. S16-S18).** Given that each experimental procedure took place at a different time period we can conclude that $p21^{WAF1/Cip1}$ steers a "deterministic" set of genetic events that may play a role in the behavior of the "escaped" cells. In line with this notion the transcriptome of the "escaped" cells **(Supplemental Fig S19,S20),** using Monte-Carlo simulation, demonstrated a specific non-random correlation with the genomic alterations found in these cells ($p<2.2*10^{-16}$). This finding suggests that a causality inference can be safely made since deregulation of the replication licensing machinery and particularly Cdt1 was the first biochemical event to occur after $p21^{WAF1/Cip1}$ stimulation; deducing that genomic instability led to the observed changes in the transcriptome of the "escaped" cells. Although $p21^{WAF1/Cip1}$ is not a transcription factor it has been reported to enhance transcription, in certain cases by modulating the activity of transcription cofactors **(Perkins et al. 1997)**. Nevertheless, the fact that only 42 (7.6%) of the 553 genes found differentially expressed in the "escaped" cells were detected in earlier time-points (12, 48 and 96 hrs) provides further evidence that $p21^{WAF1/Cip1}$-mediated transcription is most unlikely to be the driving force behind the emerged transcriptome of the "escaped" cells **(Supplemental Fig. S21)**.

The most important finding of the present study was that the descendent/"escaped" clones demonstrated significantly higher anchorage-independent growth and were more invasive when evaluated in comparison with the un-induced cells **(Fig. 6h,i)**. Even more they tolerated treatment with the genotoxic drugs, doxorubicin and cisplatin much more efficiently whereas, no significant difference in the response to taxol, a microtubule polymer stabilizer and mitotic inhibitor, was noticed **(Fig. 6j)**. The enhanced resistance to doxorubicin and cisplastin remained even when $p21^{WAF1/Cip1}$ activation was switched off in the "escaped" cells

for 10 days, clearly denoting that this feature was independent from a potential $p21^{WAF1/Cip1}$-mediated indirect transcriptional effect. As a final point, a number of the transcriptionally altered genes are reported to be functionally connected with aggressive behavior such as, invasion, metastasis and cancer "stemness" providing the ground to interpret the acquired aggressive phenotypic features **(Supplemental Fig. S19)**(**Supplemental Table 6**).

Discussion

The present report advances our understanding of how $p21^{WAF1/Cip1}$ can exhibit oncogenic activities. The key factor in tipping the balance from the tumor suppressor function to that of an oncogene was p53 inactivation. Our data suggest a novel function for p53; that of protecting the cells from the adverse effects of $p21^{WAF1/Cip1}$.

Dissecting $p21^{WAF1/Cip1}$ from the control of p53 demonstrated the ability of the former, when stimulated by p53-independent signals, to deregulate the replication licensing machinery. We provide evidence that continuous production of $p21^{WAF1/Cip1}$ saturates its degradation modules, $CRL4^{CDT2}$ and SCF^{Skp2}, leaving their other targets, including Cdt1 and E2F1/2 unabated to perform their function. Since Cdt1 and Cdc6 represent E2F targets **(Karakaidos et al. 2004; Schlisio et al. 2002)** a putative consequence of this mechanism could be the formation of positive feedback loops boosting Cdt1 and Cdc6 expression **(Fig. 7f)**. By up-regulating the pivotal replication licensing factors Cdt1, Cdc6 and Orc, the cells expressing $p21^{WAF1/Cip1}$ acquire the capacity to re-replicate. Our work sheds new light on an earlier mechanistic observation, noticing that $p21^{WAF1/Cip1}$ can promote "endo-reduplication" **(Niculescu et al**, **1998)**. It also goes one step further demonstrating experimentally that $p21^{WAF1/Cip1}$-mediated re-replication guides a selective process giving rise to descendant cells with more aggressive features compared to their progenitors **(Fig. 7f)**. Re-replication is a form of replication stress in which the temporal separation of origin licensing and firing is lost. This feature leads to replication fork collapse, DNA damage and eventually genomic instability, if not repaired in an error-free manner **(Blow and Gillespie 2008)**. In comparison to endo-reduplication (multiple consecutive S-phases with no passage through mitosis), which naturally takes place in certain types **(Porter 2008)**, re-replication does not appear to occur as part of any

physiological developmental program. Within this context, the p53 checkpoint was shown to hamper re-replication **(Vaziri et al. 2003)**. It was also suggested that cells undergoing re-replication could be eliminated by apoptosis **(Vaziri et al. 2003)**. This claim was clearly demonstrated in the $p21^{WAF1/Cip1}$-IPTG-ON HT1080 cellular model **(Fig. 2c)**, that harbors wt p53, further supporting our concept. The fact that the turn-over of $p21^{WAF1/Cip1}$ and Cdt1 is controlled by the same E3-ubiquitin ligases under-scores the significance of p53 since its inactivation confiscates a protective mechanism. Having in mind that $p21^{WAF1/Cip1}$ mutations are extremely rare events in cancer **(Abbas and Dutta 2009)**, it is apparent that human cancers, with loss of p53 function, are at risk of suffering additional deleterious genetic alterations by protracted operation of $p21^{WAF1/Cip1}$, activated through p53-independent signaling routes. Among the p53-independent stimuli shown to induce $p21^{WAF1/Cip1}$ are growth factors such as EGF, FGF2 and TGF-β frequently reported to be overexpressed in various types of human cancer **(Abbas and Dutta 2009)**.

The ensuing involvement of the HJ resolvase MUS81-EME-1 and the recombinase Rad52 points towards a replication-based error prone repair process. MUS81-EME-1 is a key player in oncogene-induced replication stress **(Neelsen et al. 2013b; Murfuni et al. 2013)**, whereas reduction of Rad51 generated the conditions for a switch from a high-fidelity homologous recombination to a lower fidelity process mediated by Rad52. Rad52-depedendent recombination requires much less homology (micro-homology) than Rad51 facilitating illegitimate events during repair **(Ottaviani et al. 2014; Hastings et al. 2009; Wu et al. 2008)(Fig. 7f)**. This notion was confirmed by aGCH and molecular cytogenetics unraveling an altered genome landscape in the “escaped” cells. Characteristically among the lesions observed some strongly resembled the recently described complex chromosomal rearrangement of

chromoanasynthesis that appears to result from replicative template-switching events **(Fig. 7)(Zhang et al. 2013)**. Chromothripsis was another complex chromosomal rearrangement viewed **(Fig. 7)**, considered to be the outcome of NHEJ **(Stephens et al. 2011)**, implying that other repair pathways, possibly non-replicative ones, may be involved in $p21^{WAF1/Cip1}$–driven genomic instability. In such a case, lesions that are not fixed during S-phase or the subsequent G2 by micro-homology-mediated procedures may be repaired in the next G1-phase by its preferred method, NHEJ **(Ottaviani et al. 2014)**. In parallel to re-replication, $p21^{WAF1/Cip1}$ can further promote genomic instability by suppressing, as reported, mismatch and nucleotide excision repair **(Abbas et al. 2009)**.

A question that always emerges when genomic alterations are acquired is whether they represented a passenger or a driver event. The fact that the transcriptomic changes showed a highly significant correlation ($p<2.2*10^{-16}$) with the genomic ones strongly supports the latter notion. Among the transcripts found deranged were that of IGF1, FGFR2, MMP13 and CD44 well established for their role in cancer progression, possibly accounting for the aggressive behavior of the "escaped" cells **(Supplemental Fig. S19,S20)**. Another up-regulated factor was ID2 shown to antagonize the suppressive effects of $p16^{INK4A}$ and $p21^{WAF1/Cip1}$ **(Sikder et al. 2003)**. That $p21^{WAF1/Cip1}$-driven genome changes constitute part of a selection trajectory to promote survival and long term evolution was further illustrated by the finding that the descendent/"escaped" clones tolerated DNA damage much more efficiently when exposed to the genotoxic drugs, doxorubicin and cisplatin. Even when exogenous stimulation of $p21^{WAF1/Cip1}$ was switched off the resistance to the DNA damaging agents remained impervious. This observation indisputably depicts that the "escaped" cells adapted to DNA damage inflicted by $p21^{WAF1/Cip1}$-dependent re-replication and

that this trait is independent from any potential $p21^{WAF1/Cip1}$-mediated indirect transcriptional effect.

Conclusively our results highlight a "dark side" of $p21^{WAF1/Cip1}$ that should be taken into consideration when designing therapeutic strategies, particularly in p53-null tumors, as a number of agents used in clinical oncology, such as dexamethazone, can activate $p21^{WAF1/Cip1}$ in a p53-independent manner **(Cha et al. 1998)** with potential detrimental effects to patients.

Material and Methods

Tumor specimens

Formalin-fixed, paraffin-embedded sections from 20 surgically removed head-neck carcinomas, 30 urothelial bladder carcinomas and 30 lung carcinomas were analyzed, after local ethical committee approval. Patients had not undergone chemo- or radiotherapy before surgical resection. The majority of clinical samples have been previously described **(Liontos et al. 2007; Evangelou et al. 2013)**.

Cell lines and culture treatments

Saos2 Tet-ON $p21^{WAF1/Cip1}$ (human osteosarcoma), H1299 $p21^{WAF1/Cip1}$–Ponesterone-ON (human lung carcinoma) and HT1080 $p21^{WAF1/Cip1}$-9 (human fibrosarcoma) carrying Tet-ON, ponesterone and isopropyl-b-D-thiogalactoside (IPTG)-inducible $p21^{WAF1/Cip1}$ respectively **(Bates et al. 1998; Chang et al. 1999; Wang et al. 1999)**, MCF7 (human breast cancer) and MDA-MB-231 (human breast cancer) cell lines were maintained in DMEM (Invitrogen) with 10% FCS (Invitrogen), 2mM l-glutamine (Invitrogen), and 100 μg/ml penicillin and streptomycin (Invitrogen) at 37°C and 5% CO_2. Growth and maintenance of Li-Fraumeni Syndrome (LFS) fibroblasts was as previously described (**Bischoff et al. 1990**).

For $p21^{WAF1/Cip1}$ expression induction, Saos2 $p21^{WAF1/Cip1}$ cells were treated with 2μg/ml Doxycycline (Applichem), while HT1080 $p21^{WAF1/Cip1}$-9 cells were treated with 100μM IPTG (Ambion). MCF7 and MDA-MB-231 cells were treated with 5 ng/ml TGF-β.

Microphotographs were obtained on an inverted microscope (Axiovert S100; Carl Zeiss) with CP-Achromat objectives and a charge-coupled device IRIS high-

resolution color video camera (SSC-C370P; Sony), whereas the Image Pro Plus v3.0 (Media Cybernetics) was used for image acquisition.

siRNA transfections and retrovirus infections

Cdc6, Cdt1, Rad52 (Invitrogen), Rad51 (Thermo Scientific) and Mus81 (Santa Cruz) siRNA gene silencing was performed as previously described (**Liontos et al. 2009**). Briefly, 3×10^5 cells plated in 60mm dishes were transfected using Lipofectamine 2000 (Invitrogen) with the appropriate RNAi pool (set of three siRNAs from the manufacturer) or the corresponding RNAi negative control (Invitrogen) as per manufacturer's guidelines. Cells were harvested 48h after transfection for further analysis.

LFS cells were transiently infected with the pBabe-$p21^{WAF1/Cip1}$ or the corresponding control vector using the Phoenix helper-free retrovirus producer cell line as described before (**Sideridou et al. 2011**). H1299 cells were transiently infected with the pMSCV, pMSCV-$p21^{WAF1/Cip1}$ or pMSCV-$p21^{PCNA}$ (a mutant $p21^{WAF1/Cip1}$ harboring Q144, M147, F150 substitutions to A in its PIP degron motif) vectors using the Phoenix helper-free retrovirus producer cell line (**Abbas et al. 2008**).

Immunohistochemistry (IHC)

The following antibodies were used: $p21^{WAF1/Cip1}$ (1:200, Santa Cruz) and Ki67 (1:100, Dako). IHC was performed using the UltraVision LP Detection System (Thermo Scientific) as per manufacturer's instructions. Paraffin sections (4 μm) were deparaffinised in xylene and rehydrated in a graded series of ethanol-aqueous solutions. Antigen retrieval was carried out in 10mM citrate buffer (pH 6.0) by

heating the slides for 25min in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in TBS for 10min. Primary antibodies were incubated overnight. Evaluation and controls were as previously described **(Evangelou et al. 2013)**.

Indirect Immunofluorescence (IF)

Indirect IF analysis was performed as previously published **(Liontos et al. 2007)**. Briefly, cells grown on coverslips were fixed in 4% formaldehyde and permeabilized by 0.1% Triton X-100 in two 15 min consecutive steps, at RT. After washing with PBS, cells were blocked for 30min in 10% FCS. FFPE tissues sections were used after dewaxing and rehydration as described in the IHC paragraph. Primary antibodies used were: $p21^{WAF1/Cip1}$ (1:200, Santa Cruz), Ki-67 (1:300, Dako), histone H2A.X phosphorylated at serine 139 (1:300, Millipore), Cyclin A (1:200, Santa Cruz), 53BP1 (1:200, Abcam), PCNA (1:100, Santa Cruz), Rad51 (1:200, Santa Cruz,) and hRPA32 (1:1000, Genetex). Cells were then incubated with primary antibodies at 4°C overnight. Secondary antibodies Alexa Fluor® 488 goat anti-rabbit (1:500, Invitrogen) and Alexa Fluor® 568 goat anti-mouse (1:500, Invitrogen) were applied for 60min at RT, followed by final wash in PBS. Counterstaining was performed with 100 ng/ml of 4,6-diamidino-2-phenylindole (DAPI)(Sigma-Aldrich). Image acquisition of multiple random fields was automated on a ScanR screening station (Olympus) and analyzed by using ScanR (Olympus) analysis software, or a Zeiss Axiolab fluorescence microscope equipped with a Zeiss Axiocam MRm camera and Achroplan objectives while image acquisition was performed with AxioVision software 4.7.1.

Total protein extraction and immunoblotting

Total protein extracts. Total protein extraction was performed as described before **(Liontos et al. 2007)**. Briefly, cells were homogenized in 50 mmol/L HEPES pH 7.5, 150 mmol/L NaCl, 15 mmol/L ß-mercaptoethanol, 0.5 mmol/L phenylmethanesulfonyl fluoride (PMSF), 0.1% Nonidet P-40 (Sigma) and centrifuged at 1000x*g* at 4°C for 10min. The supernatant was collected and adjusted to 1μg/ml protease and phosphatase inhibitors (Pierce). The pellet was resuspended in 10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, and 1.5mM PMSF, incubated on ice for 30min and centrifuged at 1000x*g* at 4°C for 10min. The supernatant fraction containing histones was collected. Total protein and histone concentration was evaluated using the Bio-Rad Protein Assay (Bio-Rad).

Biochemical cell fractionation. Cells ($2\text{-}5x10^6$) were collected and re-suspended in 200μl buffer A (10mM Hepes pH7.9, 10mM KCl, 1.5mM MgCl2, 0.34M Sucrose, 10% glycerol, 1mM DTT, protease and phosphatase inhibitors)(**Supplemental Fig. S8a**). Following, 0,1% Triton X-100 was added and then incubated for 6min on ice. Next lysates were centrifuged at 1300g/4ºC for 5min. The supernatant (S1) fraction was clarified by centrifugation for 15min at 20000g/4ºC to remove cell debris and insoluble aggregates. The resultant supernatant, soluble fraction was used as cytosolic (S2) one. The pellet from the initial lysate was first washed with buffer A and then subjected to further lysis in buffer B (3mM EDTA, 0.2mM EGTA, 1mM DTT, protease and phosphatase inhibitors) for 30min on ice. Subsequently, a centrifugation of 5min at 1700g/4ºC was applied. The supernatant was collected as the soluble nuclear (S3) fraction. The pellet was washed in buffer A and then centrifuged for 5

min at 1700g/4ºC. Finally, the pellet was re-suspended in buffer A to obtain the chromatin fraction (P3)(**Supplemental Fig. S8a**).

Primary antibodies employed were: $p21^{WAF1/Cip1}$ (1:400, Santa Cruz), Cdc6 (1:1000, Millipore), Cdt1 (1:400, Santa Cruz), H2AX (1:1000, Abcam), γH2AX (1:1000, Millipore), p73 (1:1000, NeoMarkers), Mus81 (1:400, Santa Cruz), Cdk2 (1:1000, Santa Cruz), Cdk2-pT160 (1:1000, Cell Signalling), Cdk2-pY15 (1:1000, Santa Cruz), PLK1 (1:1000, Santa Cruz), TOP2A (1:1000, Santa Cruz), β-actin (1:1000, Cell Signalling), $p14^{ARF}$ (1:100, Abcam), Cyclin A (1:1000, Santa Cruz), histone H3-pS10 (1:1000, Abcam), Cdk1 (1:400, Santa Cruz), Rad51 (1:500, Santa Cruz), Rad52 (1:500, Santa Cruz), MCM7 (1:1000, Abcam) and p53 (1:1000, Santa Cruz).

Thirty μg of protein from total extracts or 1μg of histones from each sample were adjusted with Laemmli Buffer (Sigma) and loaded on PAGE gels. Gel electrophoresis and transfer to PVDF membrane (Millipore) were performed according to standard protocols. Blots were blocked for 1h in 5% non-fat milk in TBS-0.1% (v/v) Tween-20 solution at RT. Subsequently, membranes were incubated overnight with primary antibody solution in 0.5% non-fat milk in TBS with 0.1% (v/v) Tween-20 at RT, followed by a 45min incubation with alkaline phosphatase conjugated anti-mouse or anti-rabbit secondary antibody (1:5000 dilution)(Promega) at RT. Signal development was performed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) solution (Molecular Probes) as previously described **(Sideridou et al. 2011)**.

CDK2 kinase activity

For each sample, 50mg of total-cell protein extract was precleared for 2h at 4°C with 5mg of rabbit immunoglobulin G (anti-Cdk2) or 5 mg of mouse immunoglobulin G

(anti-cyclin B1) prebound to protein A-Sepharose (Millipore). Precleared lysates were transferred to new microcentrifuge tubes and incubated with anti-Cdk2, or anti-cyclin B1 with mixing for 2h at 4°C. Protein A-Sepharose was added and the samples were mixed for 2h at 4°C. The immunoprecipitates were washed twice with TBS and twice with kinase buffer (100mM Tris pH 7.4, 20mM MgCl2, 2mM dithiothreitol) before incubation with 5mg of histone H1 (Boehringer Mannheim) and 15nM ATP for 10min at RT. Samples were incubated with Malachite Green Reagent for 30min (Cdk2 and Cyclin B1). The kinase assay mixtures were quantified with optical density at 620nm by subtracting negative control.

Flow cytometric analysis (FACS)

For cell cycle analysis, cells were collected by trypsinization and fixed in 70% ethanol at 4^{o}C for 15min. After fixation, cells were washed with PBS, and the DNA was stained with propidium iodide (50μg/ml), in the presence of 5mM $MgCl_2$ and 10μg/ml RNAse A in 10mM Tris HCl pH 7.5. DNA content was assessed on a FACS Calibur (Becton-Dickinson).

For BrdU pulse-chase proliferation assays, cells were pulse labeled with 10μM BrdU (Roche) for 1h, fixed in 70% ethanol, and incubated in 2M HCl for 30min. Cells were incubated with mouse antibody against BrdU (1:100) for 1h. For EdU analysis, cells were either pulsed for 10min with 10μM EdU or 24h. Cells were fixed in 70% ethanol and incubated with mouse anti–g-H2AX (1:100, Millipore) or mouse anti-$p21^{WAF1}$ (1:100, SantaCruz) for 30min, followed by further 15min incubation with Alexa Fluor 488 anti–mouse IgG (1:100, Invitrogen) or anti-mouse E-Phycoerythrin (1:100, Invitrogen). EdU was detected with a Click-iT EdU Cell Proliferation Assay kit (Invitrogen). For MCM2 staining, cells were washed in wash buffer (1% (w/v) BSA

in PBS) and unbound MCM2-7 was extracted in CSK buffer (10 mM HEPES, 100mM NaCl, 3mM MgCl2, 1mM EGTA, 300mM sucrose, 1% BSA, 0.2% Triton-X100, 1mM DTT, 1mM PMSF, 10ng/ml Pepstatin, 10 ng/ml Leupeptin and 10 ng/ml Aprotinin) on ice for 10 min. Extracted cells were fixed in 2% (v/v) paraformaldehyde in PBS for 10 min at 37°C, washed twice and stored in wash buffer at 4°C until staining. CSK extracted fixed cells were permeabilised in 70% ethanol, washed and incubated for 1h at RT with mouse anti-human MCM2 (1:500, BD Biosciences). Next cells were washed and incubated for 30 min at RT in dark with Alexa Fluor 488 goat anti-mouse IgG (1:500, Life Technologies), before being washed twice and re-suspended in 50 µg/ml 7-AAD (7-aminoactinomycin D, Life Technologies) diluted in wash buffer. Samples were analysed using FACS Canto (Becton Dickinson) and BD FACS DIVA software (BD Biosciences). Data analysis was performed using Flowjo (v7.6.5, Tree Star Inc.).

Comet Assay

Comet assay was performed as previously described **(Sideridou et al. 2011)**. Briefly, 10^5 Saos2 $p21^{WAF1/Cip1}$ cells induced or non-induced with doxycyclin were seeded in 60mm dishes, respectively. Two days later cells were trypsinized, centrifuged, resuspended in 2ml PBS and kept on ice for 10min. Viable cells were counted using Trypan blue and PBS was added to adjust the number of cells to 10^5 in 500µl of PBS. 50µl of PBS containing cells were then mixed with equal volume of low-melting agarose 1,7% (w/v) and were embedded in plugs. Plugs were incubated in 50ml of lysis solution (100mM TrisHCl pH 10.0, 100mM EDTA, 2.5M NaCl, 1% (v/v) Triton X100, 10% (v/v) DMSO) for 1h on ice and in dark. After lysis, plugs were washed twice in 1xTBE for 1h on ice in dark. Finally, plugs were washed and incubated in

ice-cold alkaline denaturation buffer (300mM NaOH, 1mM EDTA, pH 13) for 45 min on ice in the dark. Plugs were mounted onto 1% agarose coated slides that were placed into a 30-cm horizontal constant-field gel electrophoresis chamber in ice-cold alkaline denaturation buffer for 20min at 0.7 V/cm and at 4ºC. After electrophoresis, slides were dehydrated in ice-cold ethanol (100%) for 10min and allowed to dry in the dark. After 24h slides were rehydrated in 5ml of deionized water for 10min and 40μl of 1x SYBR Green (Invitrogen) were applied on each plug. Cells were observed under a fluorescence microscope equipped with a monochrome CCD camera. Analysis was conducted using the Cometscore software (Tritek).

Pulse-field gel electrophoresis (PFGE)

PFGE was performed as previously described (**Beck et al, 2010**). Briefly cells were embedded in a 0.8% agarose plugs ($2.5x10^5$ cells/plug), digested in lysis buffer [100mM EDTA, 1%(w/v) sodioum lauryl sarcosyne, 0.2% (w/v) sodium deoxycholate and 1mg/ml proteinase K] at 37^0C for 48h and washed in 10mM Tris-HCl pH8.0 and 100mM EDTA. Electrophoresis was performed at 15^0C in 0.8% (w/v) PFGE certified agarose (Bio-Rad Laboratories) with Tris-borate/EDTA buffer employing a CHEF DR III apparatus (9h, 120^0), 5.5V/cm, 30-18s switch time)(Bio-Rad). The gel was stained with ethidium bromide.

DNA fiber spreadings

DNA fiber assay was performed as previously described with slight modifications (**Bartkova et al. 2006**). Briefly, Saos2 $p21^{WAF1/Cip1}$ cells were grown in the presence or absence of doxycyclin for 4 days and then pulsed-labeled with 25μM CldU for

20min, and then labelled with 250μM IdU for 20min. The cells were then harvested and lysed on glass slides in spreading buffer. The DNA was denatured and stained with rat anti-BrdU/CldU (1:1000, Immunologicals Direct) and mouse anti-IdU/BrdU (1:500, Becton Dickinson) primary antibodies.

Senescence staining

Saos2 $p21^{WAF1}$ cells were Doxycyclin induced at the time points indicated (**Fig. 5**), as described above. Subsequently, cells grown on coverslips were transfected with control siRNA, or anti-Cdc6 and/or anti-Cdt1 siRNAs. Control (OFF) and induced cells were fixed in 1% paraformaldehyde and then processed for Sa-β-gal or Sudan Black B staining and counterstained with nuclear fast red, as described elsewhere **(Georgakopoulou et al, 2013)**. Only cytoplasmic staining was scored as positive signal.

MTT Assay

Cytotoxicity was estimated by the MTT assay (**Eliades et al. 2009**). Briefly, cells were plated in 96-well, flat-bottomed microplates at a density of approximately 15,000 cells/cm^2, in DMEM containing 10% FBS. 24h after plating, the medium was replaced containing the chemotherapeutic agents at the concentrations indicated. After 72h of incubation, the medium was replaced with MTT dissolved at a final concentration of 1 mg/ml in serum-free, phenol-red-free DMEM, for further 4h incubation. Then, the MTT formazan was solubilized in isopropanol, and the optical density was measured at a wavelength of 550nm and a reference wavelength of 690nm.

Isolation of nucleic acids

DNA extraction: cells were lysed in 50mM Tris-HCl (pH 8.0), 150mM NaCl, 5mM EDTA, 1% SDS in the presence of proteinase K (0.1mg/ml) until completely dissolved. DNA was extracted with phenol/chloroform and RNase (Sigma) digestion, followed by ethanol precipitation **(Liontos et al. 2007)**.

RNA was extracted with the RNeasy Mini Kit (Qiagen). cDNA was generated with Superscript® II Reverse Transcriptase (Invitrogen) and oligo-dT (Invitrogen).

cDNA preparation and real time RT-PCR

Real-time reverse transcription PCR (RT-PCR) analysis employed the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and was run on an ABI Prism 7300 cycler (Applied Biosystems)(**Evangelou et al. 2013)**. Primer sequences and annealing temperatures are provided in **Supplemental Table 7**. Results are presented as n-fold changes for the various time points after $p21^{WAF1/Cip1}$ induction versus the values of the non-induced sample. Mean value was calculated from three independent measurements.

Soft agar assay

Dishes of 60mm were layered with 3ml 0.7% (w/v) SeaPlaque Low Melting Point Agarose (Lonza) dissolved in serum-containing medium **(Sideridou et al. 2011)**. Saos2 Tet-ON $p21^{WAF1/Cip1}$ cells (25×10^3) were then mixed with 1.6 ml of 0.35% (w/v) warm agar (42^oC) in serum-containing medium and plated on the solidified

agarose layer. Agar was added weekly and foci were enumerated on day 45. Experiments were performed in three independent replicates.

Invasion assay

Saos2 Tet-ON $p21^{WAF1/Cip1}$ cells were seeded on top of matrigel-coated transwell (Beckton Dickinson) at 5,000 cells per 24-well with serum-free medium **(Sideridou et al. 2011)**. Transwells were embedded into complete (full-serum) culture medium and cells were allowed to invade for 24h. Finally, cells at the top side of the well were scraped, and cells (invading) on the bottom side of the well were stained with Giemsa, photographed and counted. Three independent measurements were averaged and the corresponding s.e. is also reported.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously described (**Sideridou et al. 2011**). A 100 bp fragment in the *Rad51* promoter and a 110bp amplicon, located approx 1000bp from the transcription start site (**Fig. 4h**), were amplified. Primers and annealing temperatures are provided in **Supplemental Table 7**. As inputs we used products that corresponded to PCR reactions containing 1% of the total chromatin extract used in the immunoprecipitation reactions.

Proteomics

Protein Digestion and iTRAQ Labeling. Cell pellets were dissolved in 200uL dissolution buffer [0.5M triethylammonium bicarbonate (TEAB), 0.05% SDS] with

vortex mixing, 5min heating at 90°C and pulsed probe sonication for 20sec. Non dissolved cellular debris was separated from the protein solution with centrifugation at 13,000 rpm for 10min. For each sample a total protein amount of 100 μg was measured with Bradford assay (Bio-Rad Protein Assay) and diluted with the addition of dissolution buffer up to a final 20μL volume. The reduction, cysteine blocking and iTRAQ labeling were conducted in accordance to manufacturer protocols (AB Sciex) as reported before **(Delehouze et al. 2013; Papachristou et al. 2013)**. The pooled whole sample mixture was split in two equal parts, lyophilized and stored at -20°C.

High-pH Reverse Phase (RP) Peptide Fractionation. To one-half of the iTRAQ labeled sample described above, peptide fractionation was performed with reverse phase C_{18}-liquid chromatography (XBridge, 150x4.6 mm, 3.5 μm; Waters) under alkaline conditions using the Dionex P680 pump equipped with a PDA-100 photodiode array detector as described before **(Papachristou et al. 2013)**. Fractions were collected in 1min intervals and lyophilized to dryness and stored at -20°C until LC-MS analysis.

Hydrophilic Interaction Chromatography (HILIC) peptide fractionation. The second half of the iTRAQ labeled peptides was fractionated with Hydrophilic Interaction Chromatography (Core-Shell Kinetex, 150×2.1 mm, 2.6 μ, 100 Å column) (Phenomenex) under acidic conditions using the Dionex P680 pump equipped with a PDA-100 photodiode array detector using gradient conditions as described by the authors **(Garbis. et al. 2011; Delehouze et al. 2013)**. Fractions were collected in a peak dependent manner based on the absorbance signal response at 280nm, lyophilized to dryness and stored at -20°C until LC-MS analysis.

LC-MS Analysis. All LC-MS experiments were performed with nano-Ultra-high performance liquid chromatography (Ultimate 3000, Thermo Fisher Scientific)

hyphenated with a nanospray ionization / ultra high-resolution LTQ FT-Orbitrap-Elite mass spectrometer (Thermo Fisher Scientific) system. Individual high-pH RP and HILIC peptide fractions were reconstituted in 30μL loading solution (aqueous 0.1% formic acid, 2% acetonitrile). Sample volumes of 2μL volume were injected and loaded for 8min on the Acclaim PepMap 100, 100 μm × 2 cm C18, 5 μm, 100 Å trapping column with the loading pump at a flow rate of 5 μL/min. The trapped peptides were then gradient separated with reverse phase C_{18}-nano-capillary liquid chromatography (Acclaim PepMap RSLC, 75 μm×25 cm, 2μm, 100 Å) (Thermo Fisher Scientific) under acidic conditions, and nano-spray ionized with the PicoTip emitter (FS360-20-10-D-20-C7, New Objective), as previously described (**Papachristou et al. 2013**). Gaseous phase transition of the separated peptides was achieved with positive ion electrospray ionization applying a voltage of 2.5 kV. For every MS survey scan, the top 10 most abundant multiply charged precursor ions between m/z ratio 350 and 1900 and intensity threshold 500 counts were selected with FT mass resolution of 240,000 and subjected to HCD fragmentation with an isolation window of 1.2 Da. Tandem mass spectra were acquired with FT resolution of 15,000 within m/z range of 100-1900. Normalized collision energy was set to 35 and already targeted precursors were dynamically excluded for further isolation and activation for 30sec with 5ppm mass tolerance.

Database search. The HCD tandem mass spectra collected from RP and HILIC fractions were submitted to Sequest search engine implemented on the Proteome Discoverer software v1.3.0.339 for peptide and protein identifications. All spectra were searched against a UniProt Fasta file containing 20,200 human reviewed entries. The Sequest node included the following parameters: Precursor Mass Tolerance 10 ppm, Fragment Mass Tolerance 20 mmu, Dynamic Modifications were Oxidation of

M (+15.995 Da), Deamidation of N, Q (+0.984 Da), Phosphorylation of S (+79.966 Da) and Static Modifications were iTRAQ8plex at any N-Terminus, K, Y (+304.205 Da) and Methylthio at C (+45.988 Da). The Percolator node was used for the determination of the confidence level for peptide identifications with decoy database searching. Strict FDR was set to 0.01, relaxed FDR was set to 0.05 and validation was based on q-Value. The Reporter Ion Quantifier node included a custom iTRAQ 8plex (Thermo Scientific Instruments) Quantification Method, integration window tolerance 20 ppm and integration method Most Confident Centroid. Protein ratios were normalized to protein median and peptides with missing iTRAQ channels were excluded from relative protein quantification. Phosphorylation localization probability was estimated with the phosphoRS node.

High-throughput whole genome analyses

aCGH analysis. Genomic DNA from 30days induced and non-induced Saos2 $p21^{WAF1/Cip1}$ cells was extracted using the BioRobot® M48 System (Qiagen) and the MagAttract® DNA Blood Midi M48 Kit (Qiagen). Quality and quantity of the DNA samples was determined on a NanoDrop ND-1000 spectrophotometer.

Agilent Human Genome CGH 4x180K and 1x1MK microarrays were used (Agilent Technologies). The 4x180K platform is composed of more than 170,000 60-mer oligonucleotide probes with average spatial resolution of 13-25 Kb (NCBI build 37, hg19). The 1x1MK platform is composed of more than 963,000 60-mer oligonucleotide probes for the mapped genes or unique DNA sequences with an average spatial resolution of 2.1 Kb (NCBI build 37, hg19). Labelling and hybridization was carried out according to manufacturer's guidelines. Data were processed using Feature Extraction 10.7.3.1 and analysed using Cytogenomics

2.7.22.0 software (Agilent) with the following settings: Algorithm: ADM-1, Threshold: 6.7, with a minimum of 4 probes for a region to be included. Centralization and fuzzy zero corrections were applied to remove putative variant intervals with small average $\log_2$ ratios.

Deep (next generation) sequencing (NGS). Genomic DNA from 30 days induced and non-induced Saos2 $p21^{WAF1/Cip1}$ cells was used for whole genome sequencing. The library preparation and the whole genome sequencing were carried out in EMBL Genecore facility according to the Illumina platform.

Whole genome sequencing was performed in "OFF" and "ON" cells achieving a 30x coverage of the human genome. Paired-end 2x100 bp was performed with the use of Illumina Hi-seq 2000. Quality control of the reads was performed with the use of fastqc software (**Ramirez et al. 2013**) and alignment to the human genome (GRCh37/hg19 version) was performed with bowtie 2 algorithm (**Langmead and Salzberg 2012**) with the use of the following code (-D 5 -R 1 -N 1 -L 22 -i S0,2,.50 –trim3 25 -I 0 -X 500). Samtools (**Li et al. 2009**) was used to convert sam files to bam and for sorting bam files. Breakdancer software (**Chen et al. 2009**) was utilized in order to identify SV (intra- & inter- chromosomal translocations, deletions, insertions and inversions). Taking into consideration the spacing between the two paired ends in the sequencing process we filtered out SVs with less than 500 bp length (deletion, insertion, inversion).

Use of breakdancer with default parameters led to the identification of 103 new inter-chromosomal translocations in "ON" cells versus "OFF" cells.

In order to identify microhomology regions in the 103 inter-chromosomal translocations we used the coordinates from the breakdancer output and extented 30bp

on both sides of the breakpoint-junction. Clustal W was used for aligning the regions around the 103 breakpoint junctions. Microhomology regions identified on the breakpoint spanned from 1-9 bp.

Expression microarray analysis. Total RNA was isolated from non-induced and 12h, 48h and 96h induced Saos2-p21$^{WAF1/Cip1}$ Tet ON cells, using RNeasy Total RNA kit (Qiagen). Microarray analysis was performed by the microarray unit of CBM Core Facility Italy (http://www.cbm.fvg.it) using Illumina's Whole-Genome Expression Beadchip. This platform targets more than 47,000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 and other sources. Integrity of total RNA was evaluated using capillary electrophoresis (Bioanalyzer 2100, Agilent) and quantified using a Nanodrop 1000. Aliquots of RNA (250 ng) samples were amplified according to the specifications of the Illumina® TotalPrep™ RNA Amplification Kit (Ambion) to produce a pool of biotin-labeled RNA corresponding to the polyadenylated (mRNA) fraction. The cRNA samples were applied to whole-genome HumanHT-12 v4.0 arrays (Illumina) and hybridized according to manufacturer's specification. Each array on the BeadChip targets over 29.000 transcripts using 3-micron beads bearing covalently attached 50-base oligonucleotide probes. Each probe interrogates a single gene, and each bead type is represented with an average 30-fold redundancy on every array. The BeadChips were scanned with the Illumina's Beadarray system scanner (Illumina). The hybridization images signal intensity was extracted and background subtracted using Illumina Inc. BeadStudio software v3.3.7. Data were checked for the Illumina internal quality control.

To compare the non-induced and "escaped" Saos2-p21$^{WAF1/Cip1}$ Tet-ON cells gene expression profile, total RNA was extracted using Trizol (Life Technologies) and

choloroform/isoamyl alcohol (49:1). All RNA samples had a 28S/18S rRNA ratio close to 2 on 1.5% agarose gels, and absorbance ratios 260/280 nm between 1.9 and 2.1. Targets were prepared using the GeneChip Whole Transcriptome (WT) Plus reagent kit and hybridized to Affymetrix GeneChip Human Transcriptome array 2.0 (HTA 2.0) that contains over 6 million probes representative of 245,000 coding transcripts and 40,000 non-coding transcripts, as well as 339,000 probe sets covering exon-exon junctions.

Molecular Cytogenetics

Saos2 Tet-ON $p21^{WAF1/Cip1}$ cell cultures were exposed to colcemid (0.1μg/ml) (Gibco) for 12h, at 37°C in 5% CO_2. Cells were harvested by trypsinization (Gibco), suspended in medium, and spun down (10min, 1000 rpm). Supernatant was removed and 5ml of 0.075M KCl at RT was added drop by drop. Cells were incubated for 20min at RT, and then 1ml of fixative [3×methanol (Applichem)/1×CH_3COOH (Merck)] was added. Cells were spun down (10min at 1000 rpm), supernatant was removed, fixative was added and the cells were re-centrifuged for 10min at 1000 rpm. Finally, cells were dropped onto wet microscope slides and left to air-dry. For analysis, we combined inverted 4′,6′-diamidino-2-phenylindole (DAPI) Banding, and molecular karyotyping by M-FISH (MetaSystems). Multicolor FISH was performed according to manufacturer's protocols (MetaSystems). For inverted DAPI banding, slides were counterstained and mounted with 0.1μg/ml DAPI in Vectashield antifade medium (Vector Laboratories). Analyses were performed using a 63× magnification lens on a fluorescent Axio-Imager Z1, Zeiss microscope, equipped with a MetaSystems charge-coupled device (CCD) camera and the MetaSystems Isis software.

Bioinformatic analysis

Transcriptome and Proteome analyses from non-induced, 12h, 48h and 96h induced Saos2-p21$^{WAF1/Cip1}$ Tet-ON cells. Protein and gene ratios (time-point/time-0) were log(2) transformed and centered. The 95% confidence intervals of the averaged log2-ratios were calculated for each time-point to determine the statistically significant thresholds of over-expression and suppression. Time-series log2-ratio sets for each protein and gene where analyzed by ANOVA to detect a statistically significant change anywhere within the three time-points taking into account that each time point had 2 replicates for proteomics and 3 replicates for micro-arrays. Proteins and genes whose log-2-ratio was above or below the aforementioned over-expression and suppression thresholds respectively and their ANOVA p-value was <0.05 were considered regulated. All calculations were performed with R. The "Gene-Set Enrichment Analysis" algorithm with False-Discovery-Rate (FDR) correction was applied on the Gene-Ontology Biological-Process sets through the Ariadne Genomics Pathway Studio v9.0 to detect which functional groups where non-randomly impacted (FDR adjusted p-values <0.05 where considered significant). Pathway visualization for all data-sets was performed with Ariadne Genomics Pathway Studio v9.0.

Transcriptome analysis from non-induced and "escaped" Saos2-p21$^{WAF1/Cip1}$ Tet-ON cells. The Affymetrix .CEL files generated were analyzed using Partek Genomics Suite. Following normalization with the Robust Multi-array Average (RMA) algorithm, a full set of quality control analysis (which led to the exclusion of one sample) was performed followed by Power Analysis, Principal Component Analysis, and one-way ANOVA analyses for a range of different fold (2-5) and false discovery rate (0.001-0.05) thresholds. For the Hierarchical Clustering of significantly changed

transcripts the level of each transcript was standardized to mean 0 and standard deviation of 1.

The significantly changed transcripts identified for thresholds of $\geq$ 2fold and $\leq$ 0.01FDR were analyzed using the Ingenuity Pathway Analysis software. Specifically, all direct and indirect relationships were considered, all data sources, and only experimentally observed information. Five levels of analysis were performed: Functional Categorization, Canonical Pathway analysis, Network analysis, Regulator Effects analysis, and Upstream Regulator analysis.

Deep (next generation) sequencing (NGS) and aCGH data comparison. To compare the NGS data with the aCGH one regarding DNA copy number aberrations in "escaped" (ON) cells versus control (OFF) cells, the NGS data were processed as follows: a) Genomic regions presenting less than 10-times coverage were filtered out to ensure data high-quality, b) The log2 of the ratio of the normalized reads in the "escaped" cells over the normalized number of reads in the control-cells was calculated, c) For each chromosome the aforementioned log2-ratios underwent DNA copy-number segmentation analysis utilizing the circular binary segmentation algorithm through the Bioconductor package "DNAcopy" (**Venkatraman and Olshen 2007**). All statistical analysis was carried out with R.

Pathway Analysis. Proteins and genes from proteomics and transcriptomics respectively were imported into Ariadne Pathway Studio v9.0 and analyzed for biological context against Ariadne's canonical cell-process pathways utilizing the in-built Gene Set Enrichment Analysis procedure which uses the Mann-Whitney U-Test to evaluate the pathways enrichment p-values that where adjusted according to the FDR correction for multiple testing. The confidence-level was 95%; thus p-values <0.05 where considered significant.

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In the memory of Ioannis Terrovitis

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Figure legends

Figure 1. The p21$^{WAF1/Cip1}$ and Ki67 are co-expressed in a subset of atypical cells of high grade/poorly differentiated, advanced human carcinomas. Serial-section immunohistochemical (IHC) analysis and double immunofluorescent (IF) analysis showed co-expression of p21$^{WAF1/Cip1}$ and the mitotic marker Ki67 in a subset of large cancer cells with giant nuclei in **a)** head and neck squamous cell carcinomas (HNSCC) (N=20), **b)** lung squamous cell carcinomas (N=30) (inset depicts a large atypical cell with p21$^{WAF1/Cip1}$/Ki67 co-expression) and **c)** urothelial carcinomas (N=30). IHC and IF: Black and white thin arrows denote p21$^{WAF1/Cip1}$ and Ki67 co-expressing cells, respectively; IF: white and yellow thick arrows depict cells with mutual exclusive p21$^{WAF1/Cip1}$ and Ki67 expression, respectively. Bars in IHC panels: 50 μm, IF panels: 50 μm.

Figure 2 Prolonged stimulation of p21$^{WAF1/Cip1}$ in p53 null environment up-regulates the Replication Licensing Factors (RLFs) at the protein level. a. Heat-maps from the transcriptomic and proteomic analyses, at days 2 and 4, after p21$^{WAF1/Cip1}$ induction in Saos2 p21$^{WAF1/Cip1}$ Tet-ON cells. Set of significant genes that are overexpressed and underexpressed are shown, respectively. Schematic representation depicting significant genes that are up-regulated and down-regulated, along with their biochemical function, at day 4 upon p21$^{WAF1/Cip1}$ induction in Saos2 p21$^{WAF1/Cip1}$ TetON cells. **b.** A specific p21$^{WAF1/Cip1}$ mutant (p21^{PCNA}: harboring Q144, M147, F150 substitutions to A in its PIP degron motif) abrogating its interaction with PCNA shifts CRL4^{CDT2}-mediated degradation towards Cdt1. Lysates from Saos2 cells, after transductions with corresponding retroviral-vectors, were separated by

SDS-PAGE and immunoblotted to detect the indicated proteins. (Mock: pMSCV, $p21^{WAF1}$: pMSCV-$p21^{WAF1/Cip1}$, $p21^{PCNA}$: pMSCV-$p21^{PCNA}$) **c.** The status of p53 defines the ability of $p21^{WAF1/Cip1}$ to regulate the levels of Cdt1 and Cdc6. Immunoblots (IBs) for Cdt1 and Cdc6 in Li-Fraumeni fibloblasts, H1299 $p21^{WAF1/Cip1}$–Ponesterone-ON and HT1080 $p21^{WAF1/Cip1}$–IPTG-ON cells challenged with $p21^{WAF1/Cip1}$. Actin serves as a loading control. **d.** Flow cytometry analysis (FACS) of HT1080 $p21^{WAF1/Cip1}$–IPTG-ON cells showed that induction of apoptosis as well as Cdt1 and Cdc6 expression are p53-dependent upon $p21^{WAF1/Cip1}$ induction. Corresponding IBs for p53, Cdt1 and Cdc6 in the manipulated HT1080 $p21^{WAF1/Cip1}$–IPTG-ON cells. Actin serves as a loading control. **e.** FACS analysis of Saos2 $p21^{WAF1/Cip1}$ Tet-ON cells showed an accumulation of cells with >4N DNA content (re-replication) after $p21^{WAF1/Cip1}$ induction that is Cdt1 and Cdc6 dependent.

Figure 3. Sustained $p21^{WAF1/Cip1}$ expression triggers replication stress and accumulation of DNA damage in a Cdt1/Cdc6-dependent manner in S-phase. a. Prolonged $p21^{WAF1/Cip1}$ expression causes DNA damage. Lysates from Saos2 $p21^{WAF1/Cip1}$ TetON cell, after treatment with 1μg /ml Doxycycline for 96h, were separated by SDS-PAGE and immunoblotted to detect the indicated proteins and phosphorylation levels. To detect DNA double strand breaks, cells were collected and subjected to pulsed field gel electrophoresis (PFGEs). **b.** $p21^{WAF1/Cip1}$ – dependent Cdc6 and Cdt1 overexpression produces DNA damage. Comet assays showed DNA breaks in cells induced for the indicated time points and after siRNA treatments against Cdc6, Cdt1 or both. Red lines in magnifications of insets label comet (moment) tails for length comparison. Bars: 50μm. **c.** $p21^{WAF1/Cip1}$ expression, in cells with non-functional p53, activated the DDR pathway in a Cdc6 and Cdt1-dependent

manner. Immunofluorescence and immunoblot analyses of the status of DNA damage response markers (53BP1, γH2AX) in $p21^{WAF1/Cip1}$ induced cells for the corresponding time points and treated with siRNAs against Cdc6, Cdt1 or both. Bars: 5μm, except in magnifications (circled or elipsoid shapes): 7.5 μm. **d.** Sustained $p21^{WAF1/Cip1}$ expression reduced replication fork speed and affected replication fork symmetry. Saos2 $p21^{WAF1/Cip1}$ Tet-ON non-induced cells versus induced ones for 96h and after 20min consecutive labeling pulses of CIdU (red) and IdU (green) were subjected to DNA fiber spreading analysis. **e.** Protracted $p21^{WAF1/Cip1}$ expression inflicts DNA damage in S-phase. Flow cytometry analysis (FACS) of $p21^{WAF1/Cip1}$ induced cells for 96h and co-stained for γH2X/Propidium Iodide (PI), with or without anti-Cdc6/Cdt1 siRNA targeting, and $p21^{WAF1/Cip1}$ induced cells for the indicated time points and co-stained for EdU/γH2X.

Figure 4. Extended $p21^{WAF1/Cip1}$ over-expression mediates replication intermediate lesions accumulation that are processed by MUS81-EME1 and repaired by a Rad52-dependent mechanism. a-b. Single-strand DNA (ssDNA) production in $p21^{WAF1/Cip1}$ over-expressing cells. $p21^{WAF1/Cip1}$ expression was induced for 96h with 1μg/ml Doxycycline. To detect ssDNA, cells were cultured in medium containing 10 μM BrdU for 24h, fixed and stained with antibodies against BrdU without DNA denaturation (a). Induced cells at 96h were also incubated with anti-RPA antibody (b). **c-d.** $p21^{WAF1/Cip1}$ mediated DNA damage is processed by MUS81 resolvase. Comet assays showed DNA breaks in $p21^{WAF1/Cip1}$ cells induced for 96h and after siRNA treatments against MUS81 (c). Red lines in magnifications of insets label comet (moment) tails for length comparison. Bars: 50μm. IB analysis depicts the efficiency of MUS81 siRNA treatment (c). Flow cytometry analysis (FACS) of

$p21^{WAF1}$ induced cells for 96h and co-stained for EdU/γH2X, with or without anti-MUS81 siRNA targeting (d). **e.** Silencing of the homologous repair recombinase Rad51 resulted in decreased γ-H2AX levels. Lysates from Saos2 $p21^{WAF1/Cip1}$ Tet-ON cell, after treatment with 1μg /ml Doxycycline for 96h, were separated by SDS-PAGE and immunoblotted to detect the indicated proteins and phosphorylation levels. **f-g.** Suppression of Rad52 was followed by increased γ-H2AX expression (f,g) and cell death (g). Lysates from Saos2 $p21^{WAF1/Cip1}$ Tet-ON cell, after treatment with 1μg /ml Doxycycline for 96h, were separated by SDS-PAGE and immunoblotted to detect the indicated proteins and phosphorylation levels. FACS depicted increased γ-H2AX expression and cell death. **h.** Rad51 promoter is occupied by the E2F4 repressing transcriptional factor upon $p21^{WAF1/Cip1}$ induction. Chromatin immunoprecipitation (ChIP) for E2F4 binding to the Rad51 promoter in non-induced and induced $p21^{WAF1/Cip1}$ cells.

Figure 5. Deregulated up-regulation of Cdc6 / Cdt1 links p53-independent activation of $p21^{WAF1/Cip1}$ with senescence. a. Sustained $p21^{WAF1/Cip1}$ expression triggers senescence. $p21^{WAF1/Cip1}$ expression was induced for 96h with 1μg/ml doxycycline. Cells grown on coverslips were stained to assess the senescent phenotype applying the Sudan Black-B protocol (**Georgakopoulou et al. 2013**) and SA-b-gal (**Dimri et al. 1995**). Cells were treated with siRNAs against Cdc6, Cdt1, Cdc6/Cdt1 and p73. **b.** IBs depict p73 status upon siRNAs targeting Cdc6 and Cdt1, as well as the efficiency of anti-p73 treatment. **c.** Sustained $p21^{WAF1/Cip1}$ expression reduces $p14^{ARF}$ and $p16^{INK4A}$ protein levels. Bars: 20 μm

Figure 6. Prolonged p21$^{WAF1/Cip1}$ expression, in cells with p53 loss of function, overrides the senescence barrier leading to aggressive behavior. a. Morphological features observed by reverse-phase contrast microscopy of escaped cells (20 days of p21$^{WAF1/Cip1}$ expression). Bars: 15μm. **b.** BrdU incorporation is restored to almost similar levels to non-induced cells after bypass of senescence. Doxycyclin (1μg/ml) treated cells, up to 20 days, were cultured in medium complemented with 10 μM BrdU at 24h before collection, fixed and IF stained with antibodies against BrdU and p21$^{WAF1/Cip1}$. **c.** EdU incorporation increases in p21$^{WAF1/Cip1}$ expressing cells after 20 days of continuous induction. FACS analysis of p21$^{WAF1/Cip1}$ induced cells for 96h and 20 days that were co-stained for EdU and p21$^{WAF1/Cip1}$. **d.** Appearance of a significant sub-population of Cyclin A and p21$^{WAF1/cip1}$ positive cells at 20 days of induction. Double IF analysis of induced cells for Cyclin A and p21$^{WAF1/Cip1}$ at indicated time points. Bars: 50μm. **e.** Restoration of Cdk2 activity and reduction of p73 levels in cells "escaping" senescence (see also **Supplemental Fig. S14**). Cell lysates were separated by SDS-PAGE and immunoblotted to detect the indicated proteins and phosphorylation levels at 4 and 20 days of induction. **f.** Escaped cells depict larger nuclei than non-induced ones. Nuclei stained with DAPI. Histogram depicts average values in the two groups: OFF versus 20 days ON. Bars: 7.5μm. **g.** DNA damage was significantly reduced in escaped cells. Comet assays showed DNA breaks in cells induced for the indicated time points. Red lines in magnifications of insets label comet (moment) tails (TM) for length comparison. Bars: 50μm. **h-i.** Escaped Saos2 p21$^{WAF1/Cip1}$ TetON cells (45 days of p21$^{WAF1/Cip1}$ expression) form more and larger colonies than the cells with non-induced (OFF) p21$^{WAF1/Cip1}$ in soft agar assay ($P < 0.001$) (h). They also exhibit increased invasion capability (i). **j.** Escaped cells (20d)

exhibit increased genotoxic drug tolerance. Histogram depicting increased IC_{50} values by escaped cells upon treatment with Doxorubicin and Cis-platinum.

Figure 7. $p21^{WAF1/Cip1}$ expressing cells that have overridden (escaped) the senescence barrier demonstrate genomic instability. a. High resolution array-Comparative Genomic Hybridization (array-CGH) analysis (Agilent G3 CGH 1M arrays) between "escaped" $p21^{WAF1/Cip1}$ expressing cells and non-induced ones at the time point of 30 days. Gains and losses in the genome are depicted as blue and red colored regions along the chromosome ideograms, respectively (upper panel). Enlarged inset on left depicts two narrow subchromosomal areas exhibiting alternating regions of gains or losses with retention regions in between (highlighted in blue and red shadowed rectangles, respectively), that possibly indicate events of chromoanasynthesis and chromothrypsis, respectively. Along these areas there are large blocks of chromosomal losses located in the fragile site *FRA3B*. Inset on right denotes a representative result for a large scale subchromosomal loss. **b.** Prolonged $p21^{WAF1/Cip1}$ expression leads to increase frequency of micronuclei in "escaped" cells. Arrows depict micronuclei. Histogram shows quantification of micronuclei in $p21^{WAF1/Cip1}$ induced cells. **c.** Circos diagram depicting novel (N: 103) chromosomal rearrangements in "escaped" $p21^{WAF1/Cip1}$ expressing cells revealed by deep sequencing (human chromosomes are located at the perimeter). **d.** Representative results showing the high correlation between the aCGH, deep sequencing (Next Generation Sequencing: NGS) and cytogenetic analyses results in "escaped" $p21^{WAF1/Cip1}$ cells. **e.** Proposed model depicting prolonged p53-independent $p21^{WAF1/Cip1}$ oncogenic action (see text for details).

Supplemental Figure legends

Suppl. Figure 1. p53-independent induction of p21$^{WAF1/Cip1}$. Cell lysate of MCF7 and MDA-MB-231 treated with TGF-β (5 ng/ml) for the time points indicated were immunobloted for p21^{WAF1} expression. GAPDH serves as loading control.

Suppl. Figure 2. Timeline and bioinformatic algorithm employed in the analysis of the results obtained from the high-throughput transcriptome and proteome assay. a. Timeline of p21$^{WAF1/Cip1}$ induction in Saos2 p21$^{WAF1/Cip1}$ Tet-ON cells. **b.** Bioinformatic algorithm employed for the results obtained from the high-throughput transcriptome and proteome analyses at the indicated time points. Statistical analysis of the signaling outputs was followed by an adjusted Gene Set Enrichment analysis based on False Discovery Rate (FDR) correction.

Suppl. Figure 3. Representative factors affected by p21$^{WAF1/Cip1}$ induction at transcriptional and translational level. Representative real-time RT-PCR analyses to validate the high-throughput expression results (see also **Fig. 2**). **a.** Mitotic factors: PLK1, AURKB, BUB1, BUB1B, KIF23 and the pro-apoptotic factor GLIPR1 along with the suppressor of the p21$^{WAF1/Cip1}$ mediated effects ID1 are transcriptionally downregulated at the indicated time points in Saos2 p21$^{WAF1/Cip1}$ Tet-ON induced cells. Growth factor IGFBP5, the ion channel encoding gene TRPM8 and the poly-A binding protein PABPC1L are upregulated. PBGD: Porphobilinogen deaminase (house-keeping gene) **b.** Representative immunoblots that validate the proteome.

Actin serves as a loading control. (PLK1: Polo-like kinase-1; AURKB: Aurora kinase B; BUB1: budding uninhibited by benzimidazoles 1 homolog; KIF23: kinesin family member 23; GLIPR1: Glioma pathogenesis related 1; ID1: inhibitor of DNA binding 1; IGFBP5: insulin-like growth factor binding protein 5; TRPM8: transient receptor potential cation channel subfamily M member 8; PABPC1L: poly(A) binding protein, cytoplasmic 1-like; TOP2A: topoisomerase 2A)

Suppl. Figure 4. Timeline of senescence appearance in Saos2 p21$^{WAF1/Cip1}$ Tet-ON induced cells. Activation of the senescence barrier occurs at day 3 of induction in Saos2 p21$^{WAF1/Cip1}$ Tet-ON cells and increases gradually, reaching its highest value at day10, while no signs of senescence are evident in untreated cells (as corresponding graphs depict). p21$^{WAF1/Cip1}$ was confirmed by western blot (upper right panel). Actin served as a loading control.

Suppl. Figure 5. Potential mechanisms leading to increased Cdc6 and Cdt1 protein abundance upon sustained p21$^{WAF1/Cip1}$ expression. a. Cdt1 stabilization via saturation of CRL4^{CDT2} and SCF^{Skp2} ubiquitin ligase complexes due to overabundance of p21$^{WAF1/Cip1}$ (see text for details). Lysates from Saos2 p21$^{WAF1/Cip1}$ Tet-ON cell, after treatment with 1μg /ml Doxycycline for the depicted time points, were separated by SDS-PAGE and immunoblotted to detect the indicated proteins. **b.** Cdt1 and Cdc6 reduced turnover due to the cell cycle profile imposed by constitutive p21$^{WAF1/Cip1}$ expression. Flow cytometry analysis (FACS) to assess DNA content (Propidium iodide) was performed at the indicated time points. **c.** Stabilization of Cdc6 by p21$^{WAF1/Cip1}$ over-expression. p21$^{WAF1/Cip1}$ expression was induced for 96h

with 1μg/ml doxycycline. Cells were treated with 100μg/ml cycloheximide (CHX) and 10μM MG132 for the indicated time points and depicted proteins and phosphorylation levels were analyzed by immunoblotting. Actin serves as loading control. **d.** Real-time RT-PCR assessment of Cdh1/Fzr1 in induced and non-induced Saos2 $p21^{WAF1/Cip1}$ TetON cells. GAPDH served as a normalizing housekeeping gene.

Suppl. Figure 6. E2F1 is upregulated while Chk1 is activated upon prolonged $p21^{WAF1/Cip1}$ induction. Lysates from Saos2 $p21^{WAF1/Cip1}$ Tet-ON cell, after treatment with 1μg /ml Doxycycline for the depicted time points, were separated by SDS-PAGE and immunoblotted to detect the indicated proteins. Actin serves as a loading control.

Suppl. Figure 7. A decline of Cdk2 activity is observed following $p21^{WAF1/Cip1}$ induction. Histogram depicting decreased Cdk2 activity at days 4 after $p21^{WAF1/Cip1}$ induction.

Suppl. Figure 8. MCM2-7 chromatin loading is increased following $p21^{WAF1/Cip1}$ induction. **a.** Diagram describing cell fractionation experimental algorithm. **b.** All fractions were separated by SDS-PAGE and were analyzed by IB. Lamin-B served as fractionation control, while β-tubulin as loading control. **c.** FACS analysis of MCM2 chromatin loading in induced Saos2 $p21^{WAF1/Cip1}$ Tet-ON cells versus non-induced (-ve: control experiment with no MCM2 antibody; +ve: experiment with MCM2 antibody).

Suppl. Figure 9. Re-replication and DNA damage was significantly lesser in Saos2 cells infected with p21^{PCNA} mutant. a. Comet assays showed DNA breaks in cells infected with the indicated constructs (see also **Fig. 2b**). Red lines in magnifications of insets label comet (moment) tails for length comparison. **b.** FACS analysis of the corresponding treatments. (p21^{PCNA}: mutant p21$^{WAF1/Cip1}$ harboring Q144, M147, F150 substitutions to A in its PIP degron motif)

Suppl. Figure 10. PCNA staining patterns reveal that sustained p21$^{WAF1/Cip1}$ expression, in cells with non-functional p53, "traps" cells mainly in early S-phase. IF analysis for assessing PCNA staining patterns in non-induced and 96h induced cells. Histograms depict average of observed patterns in the induction conditions employed. Bars: 10 μm.

Suppl. Figure 11. Replication intermediate lesions harboring single stranded DNA (ssDNA). a. Uncoupling of the replicative helicase and polymerases results in generation of template ssDNA due to excessive unwinding of the template (stalled fork). **b.** Deregulated firing of clustered origins leads to replication stress and accumulation of gaps in the nascent strands, leaving template ssDNA. **c.** A stalled replication fork may undergo remodeling by creation of a Holliday junction-like structure termed "chicken foot": **i**) Direct CtIP processing of a reversed fork may lead to nascent strand ssDNA formation. **ii**) Cleavage by SLX4-docking nucleases generates DNA double strand break that is subsequently followed by resection resulting into nascent strand ssDNA generation. **d.** Unequal branch migration or resection (by CtIP) of a reversed fork can also lead to generation of template ssDNA.

Suppl. Figure 12. Absence of nascent ssDNA in p21$^{WAF1/Cip1}$ expressing cells. p21$^{WAF1/Cip1}$ expression was induced for 96h with 1μg/ml doxycycline. The newly synthesized DNA was labeled for 20 min with 10 μM BrdU. 2 mM HU and 5 μM ATRi were added after the BrdU pulse as indicated for 2 h. After the indicated treatments, cells were fixed and stained with antibodies against BrdU without DNA denaturation to selectively detect nascent-strand ssDNA.

Suppl. Figure 13. P21$^{WAF1/Cip1}$ mediated DNA damage is processed by MUS81 resolvase. IF staining of DDR markers (53BP1 and γH2AX) in p21^{WAF1} induced cells for 96h, with or without anti-MUS81 siRNA targeting. Histogram depicts quantification of 53BP1 and γH2AX foci/cell. Bars: 10μm.

Suppl. Figure 14. Sustained expression of p21$^{WAF1/Cip1}$ in cells with non-functional p53 leads to restoration of Cdk2 activity in "escaped" cells**.** Following an initial decline (days 2-12) Cdk2 activity is increased in "escaped" cells (after day 20).

Suppl. Figure 15. Continuous p21$^{WAF1/Cip1}$ expression affects nuclei area of escaped cells. Escaped (20 days) p21$^{WAF1/Cip1}$ induced cells exhibit increased nuclear area.

Suppl. Figure 16. "Escaped" Saos2 p21$^{WAF1/Cip1}$ cells exhibit increased genomic instability relative to non-induced cells. a. Timeline of experimental planning of genomic analyses. **b.** Overview of all array-CGH (aCGH) analyses results. In total 41 aberrations were found involving all chromosomes (except 9, 12, 14 and 15). The aberrations included 19 gains and 22 losses (**Supplemental Table 5**). The majority of aberrations were concentrated in chromosomes 3, 10 and X (**Supplemental Table 5**). [reference (Ref) genome is from un-induced (0 d) Saos2 p21$^{WAF1/Cip1}$ cells] **c.** Novel clonal rearrangements distinguish the "escaped" Saos2 p21$^{WAF1/Cip1}$ (ON) from OFF cells [arrows indicate lost (in OFF cells) or rearranged (in "escaped"-ON cells) chromosomes]. The p21$^{WAF1/Cip1}$-OFF cells (control), were mainly hypotriploid (51-56 chromosomes) and shared most of the characteristic structural chromosome aberrations of the parental Saos2 cell line **(Sakellariou et al. 2013).** Compared to these cells, the "escaped" ones remained hypo-triploid but displayed at least 10 novel clonal structural or numerical aberrations affecting chromosomes 2, 3, 5, 8, 11, 13, 14, 20, 21 and X. Large portions of chromosomes X and 13 were lost in 90% of the "escaped" cells, confirming the aCGH findings. Furthermore, differential imbalances of chromosomes 5 and X between Saos2 p21$^{WAF1/Cip1}$ ON cells and the controls were observed. In "escaped" (ON) cells, an additional inverted duplication of 5p was also present in 90% of the examined nuclei. **d.** The Saos2 p21$^{WAF1/Cip1}$ ON cells exert significantly higher rates (two fold) of random structural CIN/chromosome as compared to controls. (CIN:chromosomal instability) **e.** Genomic distribution of breakpoints of random structural chromosome anomalies. Telomeric regions were found to be most frequently affected by fusions, translocations and tandem duplications of large chromosome segments. As unidentified ones were categorized the non-telomeric, non centromeric genomic rearrangements in which the cytogenetic

bands of their breakpoints remained obscure. **f.** "Escaped" Saos2 p21$^{WAF1/Cip1}$ cells exhibit increased karyotypic aberrations relative to non-induced cells. Comparative pseudo-colored M-FISH/SKY karyograms of 10 non-induced (OFF) Saos2 p21$^{WAF1/Cip1}$ cells (588 chromosomes) and 10 "escaped" (ON) ones (639 chromosomes), for the evaluation of whole genome structural CIN at the 350 chromosome band level. Arrows (and dashed rectangles) indicate representative non-clonal random structural rearrangements (unique anomalies encountered in a single cell). The "escaped" p21$^{WAF1/Cip1}$ expressing cells (ON) displayed significantly higher rates of genome wide, random structural chromosomal rearrangements. *ON cells (upper panel):* Cells #1 and #7, from the Saos2 p21$^{WAF1/Cip1}$ OFF panel, represent a minor subclone (20%) of this population because they share a distinctive clonal rearrangement affecting a derivative chromosome X and a deletion of 12p. Cells #3 and #5, belong to a second subclone of the control cells that is characterized by a deletion of a rearranged chromosome 19. The remaining non-induced (OFF) p21$^{WAF1/Cip1}$ cells #2, #4, #6, #8 and #10, display a homogeneous karyotypic constitution and represent the major clone. Cell #9 is a polyploid product of whole genome endoreduplication of the major clone of Saos2 p21$^{WAF1/Cip1}$ OFF cells. *"Escaped"-OFF cells (lower panel):* Cells #1 and #6 differ from the majority of the "escaped" (ON) population as they share a clonal inverted duplication of the long arm of chromosome 21. In addition, cells #2, #4 and #9, have lost a marker translocation der(9)t(5;9) that was replaced by a deletion 9p and acquired clonally an extra translocated der(22)t(20;22). A unique subclonal finding in Cells #3 and #10, of the "escaped" (ON) cells is the persistence of der(9)t(5;9). Cells #5 and #7 represent two different endoreduplicated ON subclones, characterized by unique structural abnormalities of chromosomes 7, 15 and 6 respectively. The karyotypic constitution

of cell #8, resembles that of the control population and justifies the presence of an additional subclone that does not exceed the 10% of the "escaped" (ON) cells. (CIN:chromosomal instability)

Suppl. Figure 17. Correlation between aCGH replicates and corroboration with the cytogenetically detectable novel clonal alterations (* see also Supplemental Fig. S16c). [reference (Ref) genome is from un-induced (0 d) Saos2 $p21^{WAF1/Cip1}$ cells]

Suppl. Figure 18. Correlation between aCGH and deep sequencing (Next Generation Sequencing: NGS) analyses.

Suppl. Figure 19. Differentially expressed genes whose expression status affects cancer according to literature. Expression status of genes associated with cancer progression**. a.** Timeline of experimental planning of transcriptome analyses. **b.** Principal Component Analysis (PCA) of the differentially expressed genes depicting the majorly different gene expression signatures over 285,000 transcripts analysed. **c.** Heat maps of differentially expressed genes. **d.** Relative expression levels given as log-2-ratios of differentially expressed genes ($p < 0.05$) of the "escaped" vs OFF-cells, whose expression status (up or down-regulated) is reported to promote carcinogenesis. **e.** Differentially expressed genes whose expression status either promotes or suppresses cancer according to literature. Relative expression levels

given as log-2-ratios of differentially expressed genes ($p < 0.05$) of the "escaped" vs OFF-cells. The lengths of the "encircled" lines depict the intensity of expression.

Suppl. Figure 20. Validation of representative factors in "escaped" (ON) cells versus non-induced (OFF) cells. Representative real-time RT-PCR analyses, validating the high-throughput expression analysis (see also **Fig. 8**).

Suppl. Figure 21. Relative gene expression levels (log-2 ratios) at 12, 48, 96-hs after p21$^{WAF1/Cip1}$ -induction as well as "escaped" versus OFF cells. a. Relative expression of all measured genes (19540) at each depicted time-point as compared to non-induced cells (OFF). The correlogram at the bottom which presents the Pearson correlation coefficient among the 4 datasets illustrates that the overall gene-expression of the "escape" population is non-correlated (~0 correlation coefficient) to the three prior time points, which amongst them present a high degree of correlation. **b.** Relative expression of genes presenting differential expression ($p < 0.05$) in the "escaped" cells in relation to OFF (553 genes). The correlogram at the bottom illustrates the absence of correlation between the "escaped" population with the three early time points (12, 48, 96hs). **c.** Relative expression of commonly differentially expressed genes (42) ($p < 0.05$) at each time-point versus OFF. Special interest present the 16 out of 42 marked genes whose expression levels are reversed at the "escape" population in comparison to the previous time-points.