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Cell Cycle News & Views

Rapid and transient protein acetylation changes in response to DNA damage

Comment on: Bennetzen MV, et al. Cell Cycle 2013; 12:1688–95;
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Genotoxic stress arising from various endogenous and exogenous sources poses a constant threat to the genetic information of human cells. The preservation of genomic integrity, therefore, requires an instantaneous but concomitantly also well-orchestrated, fine-tuned and tightly controlled DNA damage response (DDR) in order to counteract deleterious consequences of DNA lesions.[1] Specific post-translational modifications (PTMs), including phosphorylation and poly(ADP-ribosyl)ation, are well known to occur within minutes after DNA damage to elicit DNA damage signaling events, allowing for efficient activation of DNA repair processes.[1]

Protein lysine acetylation is a PTM that has long been known to play a prominent role in the regulation of gene expression via modulation of chromatin structure involving modification of histone tails and non-histone proteins through lysine acetyltransferases (HATs/KATs) and deacetylases (HDACs/KDACs). Recent proteomic approaches identified a large number of protein acetylation sites in human cells.[2,3] Few examples exist for which a specific role of protein (de)acetylation has been described to regulate DDR-related processes.[1,4,5] However, a comprehensive picture of how acetylation events are modulated during the DDR is only beginning to emerge. In a recent quantitative proteomic screen, Beli and coworkers analyzed the regulation of protein acetylation in response to genotoxic stress.[3] They detected relatively minor acetylation changes at single time points after ionizing radiation (IR) or etoposide treatment. This stands in marked contrast to a more pronounced regulation of phosphorylation sites that also occur on a significantly larger set of proteins.[3]

In a similar approach utilizing quantitative mass-spectrometry Bennetzen et al. have now specifically focused on a more kinetic analysis of nuclear protein acetylation monitoring both very early (5 min) and late (1 h) changes in acetylation upon IR.[6] The authors made the striking observation that a transient deacetylation wave emerges very rapidly after DNA damage, which is reverted 1 h after the treatment (**Fig. 1**), with the vast majority of regulated sites being identified on histones and histone-modifying enzymes. Interestingly, the HAT p300 was also found to be rapidly deacetylated on two residues, potentially indicating an autoregulatory feedback loop for DNA damage-induced protein acetylation.

While the deacetylation wave was the most prominent observation in the screen by Bennetzen et al., an increased acetylation could be detected for some proteins. For example, the protein methyltransferase MLL3 was acetylated at several residues 1 h after IR, suggesting a functional link between methylation and acetylation in the epigenetic regulation of DNA damage-dependent gene expression.[6] The results from this screen are therefore likely to further initiate investigations on the hitherto poorly understood cross-talk between different PTMs happening on lysine residues (e.g., acetylation, methylation, ubiquitylation and SUMOylation) in response to DNA damage. All of these modifications can potentially compete for the same residue and thus need to be tightly regulated.

Interestingly, no consensus sequence motif could be identified for DNA damage-regulated acetylation sites in contrast to other studies, which revealed certain amino acid preferences in the vicinity of acetylation sites in unperturbed cells.[2] It would therefore be interesting to identify the HDACs responsible for this early deacetylation response. In light of the current clinical trials testing HDAC inhibitors for treatment of specific types of cancer, the results from the presented study could broaden up the possibilities for developing more effective, timely optimized combination therapies.[7]

Although the number of identified sites was relatively low in this proteomic screen compared with other studies, the results open up a new exciting view on early PTM regulation in the DDR and highlight the importance of performing further quantitative proteomic studies that focus more on the temporal dynamics of certain PTMs and their cellular consequences upon specific perturbations. Such studies could be complemented by quantitative fluorescence microscopy imaging using domains that recognize specific PTMs. For example, Bromodomains,[8] which are known to bind acetylated lysine residues, could be used to resolve temporal protein acetylation changes live and in a continuous

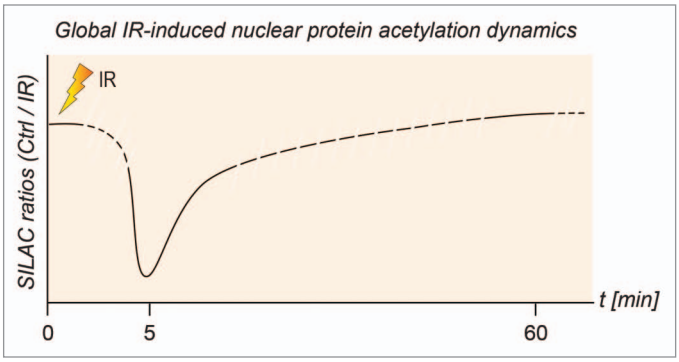

Figure 1. Schematic representation of protein deacetylation kinetics in response to ionizing radiation. The dotted line indicates that these time points have not been investigated yet.

way. This could also address whether changes in protein acetylation occur locally at sites of DNA damage, or whether they influence chromatin compaction in a widespread manner.

In conclusion, the paper provides a valuable resource for future investigations on the functional role of DNA damage-dependent changes in protein acetylation and highlights that both acetylation and deacetylation underlie a very rapid regulation.

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On the road to inflammation: Linking lysosome disruption, lysosomal protease release and necrotic death of immune cells

Comment on: Lima H Jr., et al Cell Cycle 2013; 12:1868–78; PMID:23708522; http://dx.doi.org/10.4161/cc.24903

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Lysosomes have long been recognized as the "suicide bags" of cells.[1] However, the exact mechanism by which the acidic endosomal-lysosomal cell compartment contributes to cell death is still under intense investigation. In terms of cell death modes, the classical dictum of unregulated necrosis vs. programmed caspase mediated-apoptosis has been recently extended by at least two necrotic pathways, namely caspase 1-dependent pyroptosis and RIP kinase-dependent necroptosis.[2,3] Importantly, these new concepts suggest that necrosis is actually highly regulated and represents another form of programmed cell death. Since the actively regulated cell death pathways are executed primarily in the cytosol, lysosomal contents, such as lysosomal proteases, i.e., cathepsins, can only interfere with those pathways after the lysosomal enzymes have entered the cytosol in a still-ambiguous process that is often described and termed as lysosomal membrane permeabilization. Once in the cytosol, lysosomal cathepsins have been shown to proteolytically process multiple key molecules of the apoptotic machinery, thereby ensuring efficient execution of the apoptotic cell death program.[4] Although cathepsin involvement in necrosis after lysosomal damage seems logical, there are no strong data concerning cathepsin substrates during necrosis. However, and to make matters more complex, cathepsin release from phagolysosomes of macrophages was implicated in Nlrp3 inflammasome activation and subsequent proteolytic maturation of interleukin 1β and its secretion as a physiological inflammatory cytokine.[5]

In the June 15, 2013 issue of *Cell Cycle*, Lima and colleagues[6] set out to dissect the role of cathepsins in these multiple processes in the immune system by treating murine macrophages with two types of lysosome disrupting agents: (1) Alum, which is widely used as adjuvant in immunizations, and (2) Leu-Leu-O-methylester (LLOMe), which needs to be polymerized by the acyl-transferase activity of cathepsin C in order to form the active membrane disrupting agent.[7] By using these agents in a previous work employing a haploid screen and knockout cell lines, the authors found a cathepsin-controlled necrotic cell death that is biochemically clearly distinct from pyroptosis or necroptosis.[8] They further demonstrated that cathepsins B and S control alum-mediated cell death, while LLOMe-mediated cell death is 100% controlled by cathepsin C, affirming the mechanism of LLOMe biotoxification. They also established that cathepsin C-dependent cell death was critical for induction of a strong adaptive immune response. In their recent study in *Cell Cycle*, the authors rigorously delineate this cathepsin-mediated cell death phenotype. They demonstrate that alum and LLOMe trigger distinct cellular pathways culminating in necrotic death. By addressing the lacking knowledge on cathepsin substrates during necrosis, the authors found that lysosome rupture and the associated release of lysosomal cathepsins causes a broad degradation of cytosolic proteins, including components of the Nlrp3 inflammasome. Though alum and LLOMe have been reported to activate the Nlrp3 inflammasome, the authors show that degradation of inflammasome components is consistent with a relative weak release of inflammasome-dependent cytokines and an Nlrp3/caspase-1-independent cell death.

In summary, the authors provide evidence that lysosome-disrupting agents trigger a unique form of programmed necrotic cell death, which is distinct from established necrotic pathways. More broadly these data provide a mechanism by which, upon adjuvant application, the lysosome-mediated necrotic cell death itself may be decisive for polarizing the immune response to the Th2 type. Because of its significance for regulating inflammation, it remains to be discovered which terms and conditions of lysosomal membrane damage (cell type, degree of lysosomal permeabilization, etc.) favor an apoptotic or a necrotic type of cell death.

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Complete lysosomal disruption: A route to necrosis, not to the inflammasome

Comment on: Lima H Jr., et al Cell Cycle 2013; 12:1868–78;
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Lysosomes and their most abundant hydrolases, the cathepsins, have been implicated in several modes of cell death, including necrosis and apoptosis.[1] Indeed, lysosomal membrane permeabilization with release of lysosomal enzymes into the cytosol is a feature in many cell death cascades and can either act as a primary trigger or as an amplifier of the death signaling.[2] What determines the mode of cell death, or if the cell ultimately survives the leakage of the lysosomal enzymes, is not completely understood, but it is likely that multiple factors influence the final outcome. For example, an extensive and complete lysosome rupture has been shown to induce necrosis, whereas a partial, selective lysosomal permeabilization leads to apoptosis. In addition, the presence of cytosolic endogenous cathepsin inhibitors (cystatins and some serpins) and the relative expression and stability of each cathepsin at neutral pH may all contribute to the preferential activation of one death pathway vs. another.[1] Recently, several studies have postulated that lysosomal disruption may also signal the activation of the NLRP3 inflammasome, resulting in secretion of the pro-inflammatory cytokines IL-1β and IL-18, and pyroptosis, a novel pro-inflammatory form of cell death initially described in macrophages ingesting microbes, especially Salmonella species.[3]

NLRP3 (also known as NALP3 or cryopyrin) is an intracellular surveillance receptor regulating immune signaling in response to a variety of ligands, including bacterial toxins, viral particles, organic crystals and inorganic particulate compounds. NLRP3 has been implicated in bacterial and viral pathogenesis, autoimmune disorders, chronic inflammatory conditions and vaccine adjuvant activities. Engagement of NLRP3 triggers the formation of a large cytoplasmic complex (inflammasome), resulting in activation of the cysteine protease caspase-1, which leads to caspase-1-mediated cell death (pyroptosis), and processing and release of the pro-inflammatory cytokines IL-1β and IL-18. Given the great structural diversity of NLRP3 inducers, it is unlikely that these agents interact directly with the receptor. Instead, these heterogeneous agents are thought to activate NLRP3 by inducing a common upstream stress signal. Based on the observations that the adjuvant alum and other particulate NLRP3 inducers effectively destabilize lysosomes, and that inhibitors of lysosomal cathepsins block NLRP3 signaling, it has been hypothesized that lysosomal disruption may be the common upstream trigger. However, a direct assessment of the role of lysosome rupture in NLRP3 activation has been lacking.

In the June 15, 2013 issue of *Cell Cycle*,[4] Lima et al. performed a side-by-side comparison of the effect of lysosome-disrupting agents (alum and LLOMe) and prototypical NLRP3 inducers (ATP and nigericin) on mouse macrophages. As expected, induction of the NLRP3 inflammasome resulted in caspase-1 activation, caspase-1-mediated pyroptosis and processing and secretion of IL-1β and IL-18. On the contrary, the lysosome-disrupting agents induced caspase-1-independent cell death with only minimal IL-1β release. The study provides evidence that alum and LLOMe trigger a cascade of events initiated by rapid and complete lysosome rupture, followed by cathepsin-dependent degradation of inflammatory proteins (including caspase-1) with inhibition of the NLRP3 signaling, and necrotic

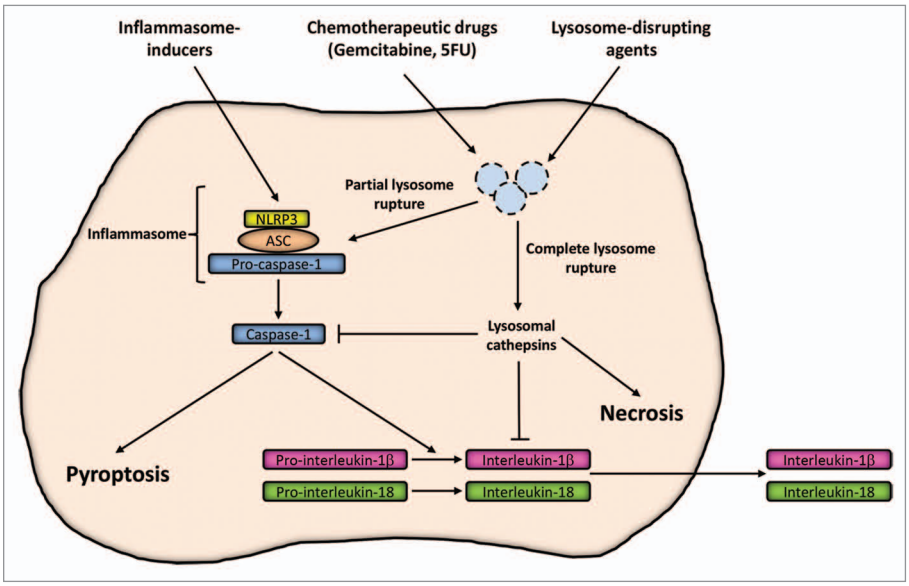

Figure 1. Involvement of lysosomes in different pro-inflammatory molecular pathways. Structurally different NLRP3-inducers stimulate the assembly of the NLRP3 inflammasome, which comprises the NOD-like receptor (NLR) NLRP3, the adaptor ASC and pro-caspase-1. Interaction of ASC with pro-caspase-1 leads to caspase-1 activation, which, in turn, results in (1) process and activation the pro-inflammatory cytokines pro-IL-1β and pro-IL-18 and extracellular secretion of mature IL-1β and IL-18 and (2) caspase-1-dependent cell death (pyroptosis). In this scenario, lysosome rupture is a late event that follows the induction of pyroptosis. Conversely, lysosome-disrupting adjuvants induce early and effective lysosome rupture with complete release of lysosomal cathepsins into the cytosol, which, in turn, leads to (1) inhibition of the NLRP3 inflammasome signaling through cathepsin-dependent degradation of pro-inflammatory proteins, including caspase-1, IL-1β and IL-18 and (2) cathepsin-mediated necrosis. Recent studies also suggest that selected chemotherapeutic drugs (i.e., gemcitabine, 5-fluorouracil) activate the NLRP3 inflammasome by causing limited lysosomal permeabilization and release of cathepsin B, which directly binds to NLRP3 and drives caspase-1 activation.[6]

cell death (**Fig. 1**). This was remarkably different from the cellular pathways mediated by ATP or nigericin, which triggered significant IL-1β release, caspase-1-dependent pyroptosis and no protein degradation. Importantly, in cells treated with ATP or nigericin, lysosome rupture occurred only after caspase-1 activation and induction of pyroptosis, suggesting that lysosome dysfunction is not required for inflammasome activation. Based on these results, the authors conclude that upstream signals, such as potassium efflux, are likely more effective stress signals for NLRP3 activation than lysosome disruption. Taken together, their findings confirm that complete lysosome rupture is a catastrophic event leading to necrotic cell death; this cell death is independent of NLRP3 signaling and distinct from pyroptosis triggered by inflammasome-inducers, and can therefore explain the different immune response associated with these compounds. These observations complement another recent article published by the same group, where the authors showed that alum and LLOMe trigger cathepsin-mediated, caspase-1 and RIP-1-independent necrosis that is essential for their function as immunologic adjuvants.[5] Together these papers provide insight into the mechanism by which the cell death phenotype of lysosome-disrupting agents contributes to the unique immunologic response generated by these compounds when used as adjuvants. More broadly, these studies provide strong proof for a danger theory of adjuvancy suggesting that our immune system has evolved to respond to agents that trigger cytotoxic events.

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Senescence-associated lysosomal α-L-fucosidase (SA-α-Fuc): A sensitive and more robust biomarker for cellular senescence beyond SA-β-Gal

Comment on: Hildebrand DG, et al. Cell Cycle 2013; 12:1922–7; PMID:23673343; http://dx.doi.org/10.4161/cc.24944

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Discovery and Origin of Cellular Senescence

Classical work by Hayflick and Moorhead[1] uncovered more than 50 y ago the biological and evolutionarily conserved phenomenon of cellular senescence. The authors demonstrated that primary fibroblasts exhibit only a finite proliferative capacity in culture, before they exit the cell cycle in a state known as replicative senescence ("Hayflick phenomenon").[1] This type of senescence is caused by progressive telomeric erosion associated with the accumulation of DNA damage. Alternatively, several stress factors, including hypermitogenic stimuli like oncogenic Ras, reactive oxygen species and cytotoxic drugs that cause DNA damage, as well as mitotic spindle dysfunction and aneuploidy can trigger an accelerated antiproliferative response, known as stress-induced, premature senescence (SIPS).[2]

(Patho)biological Role of Senescence

Senescence comes along as a (patho)biologically relevant, but two-edged, cellular stress response. On one hand, a protective and tumor-suppressive role of cellular senescence has been demonstrated at the pre-malignant level.[3] On the other hand, senescence exhibits detrimental effects at the cellular and organ level, including proliferative exhaustion of progenitor and stem cells or promotion of inflammatory processes linked to the so-called senescence-associated secretory phenotype (SASP) (**Fig. 1**).[4] Interestingly, elimination of accumulating senescent cells in vivo in the mouse effectively delays aging-associated disorders, thereby corroborating the causative link between cellular senescence and tissue dysfunction in age-related phenotypes.[5] Thus, cellular senescence seems to be ultimately connected to health and lifespan regulation during organismal aging.

Characteristics and Markers of Senescent Cells

In order to detect senescent cells in culture, and clearly more challenging under in vivo and in situ conditions, several markers with varying robustness are being used beyond the typical flattened cellular morphology and increased cell surface (**Fig. 1**). Overall, proliferative arrest, apoptosis resistance and altered gene expression and miRNA profiles (**Fig. 1**) represent general features of senescent cells.[2] At the nuclear architecture level, formation of DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence) and senescence-associated heterochromatic foci (SAHF)[4] mirror the repression of proliferative genes and therefore the lack of DNA synthesis as detected by a lack of BrdU incorporation and G_1 arrest. At the molecular checkpoint level, the p53-p21^{WAF1} pathway and the tumor suppressor p16INK4a, as well as rapamycin-sensitive mTOR (mammalian Target of Rapamycin) signaling take over synergistic roles in the induction and maintenance of the senescent phenotype (**Fig. 1**).[2,6] Lastly, at the biochemical and enzymatic level, a considerable expansion of the lysosomal compartment and, hence, increased granularity is typically observed in cells undergoing senescence. That is demonstrated by an increased senescence-associated β-galactosidase activity (SA-β-Gal; measured at pH 6), the classical marker that is widely used to detect senescent cells.[7]

Senescence-Associated Lysosomal α-L-Fucosidase (SA-α-Fuc): A New and Robust Senescence Marker

Several of the markers described above may work in a cell type- and senescence stimulus-dependent manner and are therefore not always reliable. For example, p16^{INK4a} increases during replicative aging. However, p16^{INK4a} is either not expressed or inactivated in certain tumor cells, which, however, are still prone to SIPS due to an intact p53 pathway.

Moreover, solely determining SA-β-Gal activity can potentially result in both wrong positive results (increased SA-β-Gal activity due to hyper-dense cell cultures), or wrong negative results, where SA-β-Gal is not or only weakly induced and therefore not sensitively enough detected upon cellular senescence. Taking in particular the latter observation into account, Hildebrand et al. investigated comprehensively several lysosomal hydrolases, besides SA-β-Gal, for their suitability as senescence markers.[8] Interestingly, the authors identified α-L-fucosidase, a glycosidase involved in the metabolism of certain glycolipids and glycoproteins, as a novel and promising biomarker for cellular senescence. Hildebrand et al. tested senescence-associated α-fucosidase activity (SA-α-Fuc) in various senescence models in cell culture, including replicative and oncogene-induced senescence, as well as SIPS. Unequivocally, both at the transcriptional and the enzymatic level SA-α-Fuc turned out as an at least equivalent, and in most cases an even more reliable, marker for the detection of cellular senescence as compared with SA-β-Gal.

Taken together, SA-α-Fuc represents a convenient, sensitive and robust senescence marker in cell culture experiments employing both rodent and human senescence models. Further characterization of SA-α-Fuc expression and activity under stringent in vivo and in situ settings could include the detection of rather sparsely occurring senescent cells at the cancer stem and progenitor level, as well as the analysis of senescent cells during tissue aging.

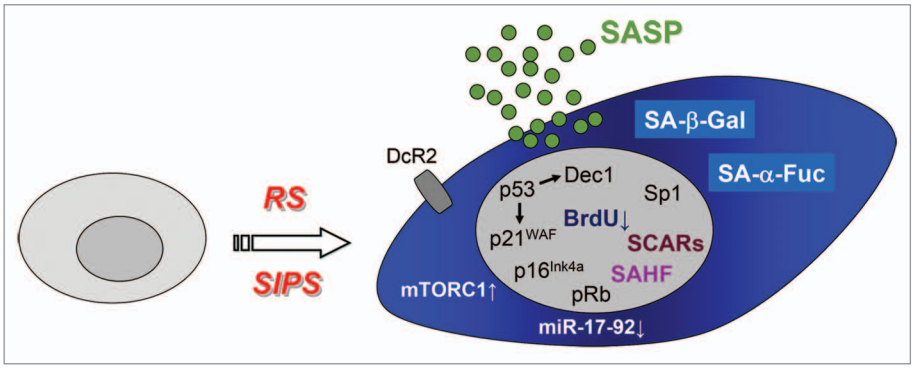

Figure 1. Characteristic features of senescent cells. Cells undergoing replicative senescence (RS) or stress-induced premature senescence (SIPS) are distinguished by an enlarged and flattened morphology and several molecular and subcellular changes, including activation of tumor suppressor pathways (p53-p21^{WAF}, p53-Dec1, p16^{Ink4a}), chromatin alterations (DNA-SCARS, DNA segments with chromatin alterations reinforcing senescence; SAHF, senescence-associated heterochromatic foci) and activation of certain transcription factors (Sp1) as well as production of secreted factors (SASP, senescence-associated secretory phenotype). Moreover, cell surface expression of decoy receptor 2 (DcR2) typically increases during senescence. Lastly, the lysosomal compartment expands considerably in cells undergoing cellular senescence. Here, as demonstrated by Hildebrand et al.,[8] the classical and widely used senescence-associated β-galactosidase activity (SA-β-Gal)[7] is now joined by a novel and more robust lysosomal (bio)marker for cellular senescence, SA-α-Fuc (senescence-associated α-fucosidase activity).

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INK4a/ARF-dependent senescence upon persistent replication stress

Comment on: Monasor A, et al. Cell Cycle 2013; 12:1948–54; PMID:23676215; http://dx.doi.org/10.4161/cc.25017

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One outcome of persistent activation of the DNA damage response (DDR) is cellular senescence; a programmed, permanent, cell cycle exit. DDR activation serves as a block to malignant transformation with senescence as a core feature of this effect. Diverse conditions that induce senescence, for example ionizing radiation, oncogene activation and telomere attrition, themselves induce DDR activation. In virtually all of these instances, senescence can be rescued by ablation of p53 function, thereby allowing the cells to re-enter the cell cycle in the presence of DNA damage. In this issue, Monasor and colleagues provide new evidence suggesting that the well-known tumor suppressor INK4a/ARF enables replicative stress (RS)-induced senescence.[1] This is notable, since, in contrast to p53, INK4a/ARF has generally been thought not to play a role in DNA damage-induced senescence. Loss of the *INK4a/ARF* locus on chromosome 9p21 is one of the most frequent abnormalities observed in human tumors, second only to loss and/or mutation of *TP53* (p53). The *INK4a/ARF* locus encodes two proteins, p16(INK4a) and ARF (p16^{Ink4A} and p19^{ARF} in mice), which regulate the Retinoblastoma and p53 pathways respectively.[2] In normal cells, INK4a/ARF levels are usually barely detectable. Monasor and colleagues report that persistent RS is also associated with elevated INK4a/ARF expression, and that INK4a/ARF deficient cells can sustain growth in the presence of substantial levels of RS. Furthermore, upon interrogation of the Cancer Cell Line Encyclopaedia Project (http://www.broadinstitute.org/ccle/home),

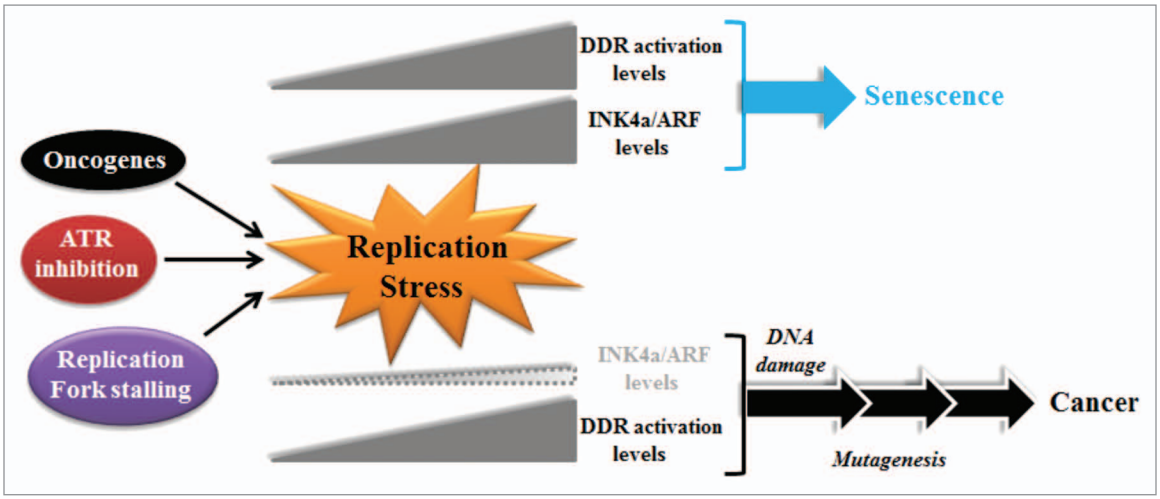

Figure. 1. A schematic summarizing the contrasting outcomes of elevated replicative stress in the presence or absence of INK4a/ARF, as proposed by Monasor and colleagues. Prolonged replicative stress, which can occur under various non-mutually exclusive circumstances, results in elevated DDR activation. In the presence of functional INK4a/ARF, its expression is also increased, and this usually drives the cells toward senescence. In the absence of functional INK4a/ARF, cells can replicate even in the context of an elevated DDR, thereby creating the environment

they found a correlation between copy number variation (CNV) and $p16^{INK4A}$ levels; cell lines with high levels of CNVs (RS has been shown to cause CNV[3]) exhibited low levels of $p16^{INK4A}$. The authors consequently propose INK4a/ARF as a bona fide RS-checkpoint activator.[1]

ATR is an activating protein kinase of the DDR. Persistent ATR activation can drive cells to senescence, although independently of INK4a/ARF.[4] Previous work from this team in modeling ATR-defective Seckel syndrome in mice ($Atr^{S/S}$) showed that impaired ATR function also results in RS-induced DNA damage, senescence and cell death.[5] $Atr^{S/S}$ mice exhibit severe embryo-wide cellular attrition with the surviving postnatal animals exhibiting a profound growth restriction, progeria and early death. $Atr^{S/S}$ MEFs fail to cycle, instead undergoing premature senescence. They do not undergo spontaneous immortalization and are even impervious to oncogene-induced immortalization. Genetic ablation of *TP53* did not rescue senescence in either the $Atr^{S/S}$ animal or their derived MEFs.[5] In fact, synthetic lethality was observed, a serendipitous observation now being actively pursued in the context of ATR kinase inhibitors (ATRi) as an anticancer therapy.[6]

The characteristic premature senescence seen in $Atr^{S/S}$ MEFs is overcome by reduced INK4a/ARF, a rescue not concomitant with a restoration of ATR expression or a reduction of RS.[1] Indeed, $ATR^{S/S}$ + $INK4a/ARF^{-/-}$ cells effectively cycle with persistent RS. In contrast to these MEFs, deletion of *INK4a/ARF* does not rescue the phenotype of the $Atr^{S/S}$ mouse. The authors argue that INK4a/ARF-dependent senescence is the likely outcome of modest RS, while in the context of high levels of RS (e.g., during $Atr^{S/S}$ embryonic development and/or following high doses of ATRi), significant cell death occurs. This is significant, as it implies that ATR inhibitors would still be toxic toward INK4a/ARF-deficient tumors, as, indeed, demonstrated by the authors here using a mouse pancreatic cancer cell model.

While INK4a/ARF deletion cannot rescue the $ATR^{S/S}$ phenotype, the authors suggest that this does not exclude the fact that the RS-checkpoint defined here could be operative in other contexts associated with more modest levels of RS. One situation where low but persistent RS likely occurs, as discussed by Monasor and colleagues, is oncogene-induced senescence. Oncogene-induced RS, which has long been associated with $p16^{INK4a}$ expression,[7] represents a barrier, characterized by DDR activation,[8] that must be overcome on the route to frank malignant transformation. The model proposed by Monasor and colleagues is that a key role of the $p16^{INK4a}$-Rb pathway would be to promote senescence in cells experiencing chronic RS, thus stopping them on the march toward transformation (**Fig. 1**). Still, how persistent RS activates INK4a/ARF remains a provocative, open question.

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