## REVIEW ARTICLE

Cellular and Molecular Biology

# Cellular senescence in neuroblastoma

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Neuroblastoma is a tumour that arises from the sympathoadrenal lineage occurring predominantly in children younger than five years. About half of the patients are diagnosed with high-risk tumours and undergo intensive multi-modal therapy. The success rate of current treatments for high-risk neuroblastoma is disappointingly low and survivors suffer from multiple therapy-related long-term side effects. Most chemotherapeutics drive cancer cells towards cell death or senescence. Senescence has long been considered to represent a terminal non-proliferative state and therefore an effective barrier against tumorigenesis. This dogma, however, has been challenged by recent observations that infer a much more dynamic and reversible nature for this process, which may have implications for the efficacy of therapy-induced senescence-oriented treatment strategies. Neuroblastoma cells in a dormant, senescent-like state may escape therapy, whilst their senescence-associated secretome may promote inflammation and invasiveness, potentially fostering relapse. Conversely, due to its distinct molecular identity, senescence may also represent an opportunity for the development of novel (combination) therapies. However, the limited knowledge on the molecular dynamics and diversity of senescence signatures demands appropriate models to study this process in detail. This review summarises the molecular knowledge about cellular senescence in neuroblastoma and investigates current and future options towards therapeutic exploration.

## SENESCENCE, FROM AN IRREVERSIBLE ENDPOINT TO A DYNAMIC REVERSIBLE AMALGAM

Senescence was first described by Hayflick and Moorhead based on observations of in vitro cultured human fibroblasts, which exhibited a permanent loss of proliferative potential after a defined number of passages [1]. In the early 1970s, Olovnikov realised that the repeated shortening of the DNA molecule at each round of DNA replication might explain Hayflick and Moorhead findings [2, 3]. The first demonstration that human telomeres shorten as normal human fibroblasts divide in culture appeared in 1990 [4]. Around 40 years later from the first description of senescence by Hayflick and Moorhead, the observed growth arrest was shown to result from the shortening of telomeres with progressive replication rounds and hence was coined replicative senescence [5]. Over the years, evidence emerged that senescence plays a crucial role in normal development, wound healing and tumour suppression. In addition to telomere attrition, senescence can be triggered through other stressors including oxidative stress, oncogenic activation, and DNA damage (Fig. 1a). According to the classical paradigm, cells undergoing senescence are marked by a permanent cell-cycle arrest while remaining metabolically active in a so-called G0 phase [6, 7]. In addition, senescent cells

secrete a variety of bioactive cytokines including pro-inflammatory molecules, termed the senescence-associated secretory phenotype (SASP) [8]. SASP is considered as one of the hallmarks of senescence, along with epigenetic and metabolic reprogramming [9, 10]. In vitro, cells can also transiently enter G0 [11] upon restriction of growth factors or nutrients from the medium. However, this type of cell-cycle exit is reversible and is therefore referred to as quiescence. When normal growth conditions are restored, quiescent cells can re-enter the cell cycle and regain their proliferative potential [12] in contrast to senescent cells [13, 14].

Senescence is essential for tissue homeostasis as it limits the proliferative capacity of cells and has therefore been considered as a strategy for blocking tumour development [15] in conjunction with apoptosis, which eliminates unwanted or dysfunctional cells [16, 17]. Indeed, many chemotherapeutic agents induce irreparable DNA damage and prevent proliferation through induction of senescence, which has been termed therapy-induced senescence (TIS) [18] (Fig. 1b).

New observations have challenged our classical view on senescence as an irreversible process (Fig. 1c). The first of which was the analysis of gene-expression profiles in lymphoma-affected

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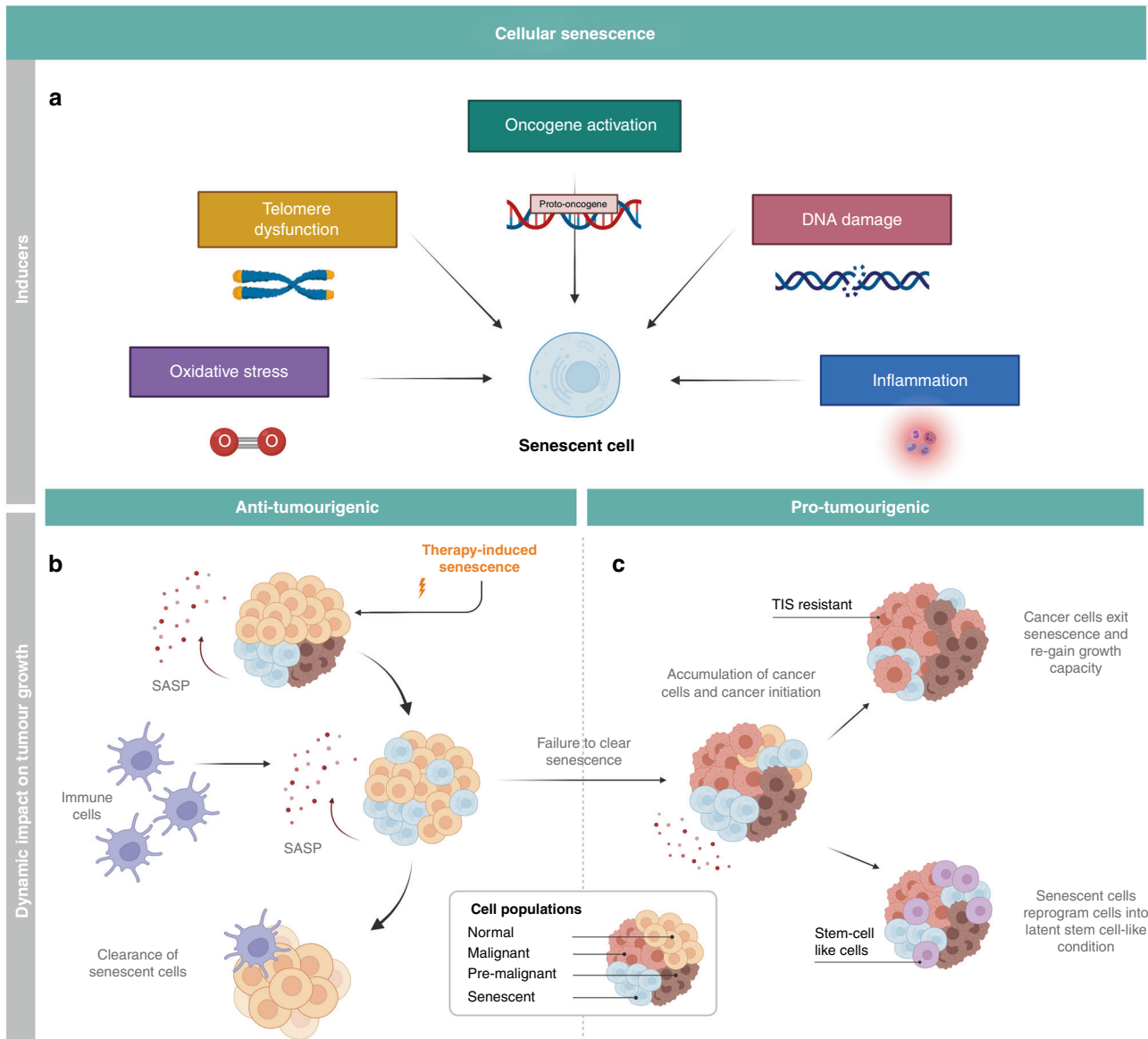
**Fig. 1 An overview of cellular senescence inducers and its dynamic impact on tumour growth. a** Telomere damage, epigenetic dysregulation, DNA damage and oxidative stress are primary drivers of that can induce senescence. **b** Senescence triggered by cellular inducers or therapeutically (TIS) halts the proliferation of premalignant cells and elicits immune surveillance of senescent cells via SASP secretion, which in turn mediates clearance of premalignant cells, conferring tumour suppression. **c** In contrast, failure to clear senescent cells may represent a stealth mode that can lead to repopulation. Chronic inflammation by SASP cultivates a pro-tumorigenic microenvironment that promotes proliferation and stemness of premalignant/malignant cells. Senescence escape may result in the re-emergence of malignant cells that may have higher aggressiveness. This population may be more robust toward TIS. Image created with BioRender.com.

mice which demonstrated that pathways activated during TIS are similar to those observed in stem cells, indicating that the senescent state may reprogramme cells into a latent stem cell-like condition with potential for re-entry into the cell cycle. Second, upon interruption of chemotherapy, cancer cells could exit senescence and even regain an enhanced growth capacity [19].

These new insights have forced us to revise our classical view of senescence as an irreversible endpoint towards a more dynamic process that contributes to the oncogenic process [20, 21].

Senescence, as we now come to understand, is a vastly complex, context-dependent and dynamic continuum that is dictated by its elicitors and cell type. Unsurprisingly, it has become clear that one single marker cannot cover the entire spectrum of the plastic senescence phenotype [22]. Senescence-associated β-galactosidase (SA-β-Gal) is arguably the most commonly used marker for senescence, although its specificity has been put into question.

Indeed, SA-β-Gal activity in confluent quiescent cells and in cells under serum starvation can also increase after prolonged cultivation cells [23–25]. Likewise, no single proliferation marker (nucleotide incorporation, Ki67, mitotic markers…) will unequivocally define the senescent cell state. DNA damage is frequently observed, but again not a unique feature of senescent cells and several senescence inducers do not provoke DNA damage. Similarly, senescence-associated heterochromatin foci (SAHFs), another broadly used marker for senescence, are not consistently found in senescent cells. Loss of lamin B1 and upregulation of p21 and p16 are commonly observed but are also noted in other unrelated conditions [26] such as age-related diseases [27], hepatocellular carcinoma and HPV infections [28]. Taken together, a common molecular definition for senescence is at present lacking, while heterogeneity of senescence across tissues and cell types demands a multi-parametric readout for appropriate identification.

## ONCOGENE-INDUCED SENESCENCE IS A TWO-FACED RESPONSE

Activation of proto-oncogenes can induce premature senescence by triggering oncogene-induced cellular senescence (OIS). This mechanism was first described by Serrano et al. and is based on the finding that expression of oncogenic RAS resulted in a G1-like arrest accompanied by upregulation of p53 and p16 [29]. Whilst an unexpected observation at the time, considering the proliferation-promoting feature of oncogenes, it is now recognised that oncogene activation may trigger fail-safe mechanisms that halt cell-cycle progression. For example, activation of oncogenic RAS can result in *CDC6* overexpression and nucleotide metabolism inhibition. This triggers aberrant DNA replication, DNA damage and subsequent induction of the DNA damage repair pathway, eventually resulting in senescence [30, 31]. The OIS pathway relies on functional p53, as neoplastic lesions in mice with activated RAS display markers of cellular senescence while absent in a p53 deficient background [32, 33]. Hyperactive RAS also induces DNA damage and inhibits YAP/TAZ activity, the latter of which causes depletion of the dNTP precursor pool and is sufficient to induce OIS. Santinon et al. showed that inhibition of YAP/TAZ signalling downregulates target gene *RRM2*, which encodes the regulatory subunit of the ribonucleotide reductase enzyme that catalyzes the formation of deoxyribonucleotides. *RRM2* repression contributes to senescence maintenance by keeping dNTP levels low [34–37]. OIS has also been studied in the context of *BRAF* activation. BRAF activation causes an alteration within the pyruvate metabolism by suppressing the PDH-inhibitory enzyme pyruvate dehydrogenase kinase 1 (PDK1) and induction of the PDH-activating enzyme pyruvate dehydrogenase phosphatase 2 (PDP2). This subsequently drives an increase in pyruvate uptake during the tricarboxylic acid cycle which triggers increased respiration and redox stress and eventually leads to OIS activation [38]. The general aspects of senescence have been extensively covered in excellent reviews [7, 39, 40]. Here, we focus on the mechanisms and therapeutic relevance of senescence in the paediatric solid tumour neuroblastoma (NB) and how further insights into the induction of senescence in NB cells might offer novel therapeutic opportunities.

## NEUROBLASTOMA: CLINICAL AND GENETIC CHARACTERISTICS

NB is a neural-crest-derived embryonal malignancy and the most common extracranial tumour in children [41]. NB shows a highly heterogenous clinical behaviour [42], ranging from tumours curable by surgery alone or in combination with limited chemotherapy (L1 or L2) over patients with highly metastasised disease (M) to the special subcategory of NB that is metastatic at diagnosis but shows spontaneous regression (MS) with no therapeutic intervention, as described by the new International Neuroblastoma Risk Group (INRG) staging system [43]. Low-risk patients have a 95% chance of disease-free survival whilst high-risk patients have a 50% risk for disease relapse [44]. The clinical diversity of this disease is mirrored by distinct patterns of genetic aberrations, most notably high-level MYCN oncogene amplification and recurrent patterns of whole chromosome or segmental imbalances [45] including the gain of chromosome 17q, loss of chromosome 1p and loss of chromosome 11q [46]. A further exploration of genomic lesions in relation to tumour behaviour uncovered an important role for p53 mutational and hTERT activation status [47]. Of further notice, a large multicentric study also identified distal 6q-deletions as a marker of high risk for disease recurrence and poor survival [48].

The study of Ackermann et al. brought the role of telomere lengthening mechanisms under renewed attention. Early investigations performed on the characterisation of telomerase expression of 100 NB samples showed that tumours with poor prognosis displayed high telomerase activity [49]. Sequencing studies revealed elevated hTERT levels subsequent to direct MYCN activation in MYCN-amplified cases, *hTERT* activation through enhancer hijacking in high-risk MYCN non-amplified cases, and induction of alternative telomere lengthening (ALT), often in association with ATRX inactivating defects [47, 50].

*ATRX* encodes a chromatin remodeler that recognises guanine (G)-rich stretches of DNA and deposits H3.3 histone variant to prevent the formation of stable G-quadruplex (G4) structures which can block DNA replication or transcription [51]. Importantly, G4-rich repeats are enriched at telomeres and cells lacking ATRX face inefficient H3.3 depositions at telomeric G4s causing the stalling of replication forks and subsequent ALT [52]. Remarkably, MYCN amplification, hTERT rearrangement and ATRX alteration events are mutually exclusive events occurring in high-risk NBs. In patients with metastatic NB diagnosed with a spontaneous regression subtype (MS), the absence of a telomere maintenance mechanism and the presence of critically shortened telomeres are an absolute prerequisite for spontaneous regression or differentiation into benign ganglioneuroma. In line with this, transfecting NB cell lines with a dominant-negative hTERT mutant resulted in apoptosis and reduced tumorigenicity in vivo [53].

## EARLY EVENTS DURING NEUROBLASTOMA FORMATION: BALANCING PROLIFERATION, DEATH OR SENESCENCE?

Our present understanding of the early steps of MYCN-driven NB initiation and the possible role of MYCN-induced senescence is limited. The TH-MYCN NB mouse model has been explored to some extent to gain insights into the earliest steps of malignant transformation. Perinatal overexpression of the MycN oncoprotein in neural-crest-derived precursor cells causes postnatal rests (referred to as hyperplastic ganglia) which are not observed in control mice. While these hyperplasias are often present shortly after birth, considerable latency in tumour formation can occur with little insight into which processes control proliferation. It is not yet fully understood which (epi-)genetic processes occur to trigger full-blown embryonic tumour formation. Hansford et al. [54] first demonstrated that inappropriate perinatal MycN expression in paravertebral ganglia cells from these mice, which initiated tumorigenesis through persistence of embryonal neural-crest cells, underwent further changes, such as MYCN amplification and repression of NGF receptor expression [54]. Using the same mouse model, Calao et al. showed that NB precursor cells are characterised by a repressed p53 signal following *MYCN* activation when compared to their adult counterpart cells. This is due to the activation of the adult stem cell maintenance factor and Polycomb group protein, Bmi1 [55]. In a recent study from our lab, the MEIS2 developmental transcription factor was identified as one of the putative early initiating factors [56]. Interestingly, a hitherto unknown link between PcG proteins and downregulation of retinoic acid (RA)-related signals were previously identified, which mediates the phase transition of Meis2 transcriptional status during the process of forelimb patterning [57]. Given the well-established role of RA signalling during adrenergic sympathetic nervous system development, it would be interesting to study this regulatory axis further with a focus on decisions for proliferation, differentiation and senescence during normal maturation and MYCN-induced NB formation. The MYCN gene encodes a protein that plays an important role in regulating cell growth and division in the formation of tissues and organs during development prior to birth. However, the constitutive and dysregulated expression of the transcription factor MYCN also has a central function in the pathogenesis of NB and other tumours, for example, retinoblastoma, brain tumours, leukaemia, neuroendocrine prostate cancers and pancreatic cancer [58]. The increased overexpression of the MYCN oncogene correlates with a worse prognosis.

To gain further insights into the early transcriptional and phenotypic effects of MYCN induction, we recently investigated the phenotypic and molecular events occurring immediately

**Table 1.** Frequently used antineoplastic agents that have been reported to induce senescence.

| Drug class or genetic perturbation | Name | Model/cell line | Senescence marker | Reference |
|---|---|---|---|---|
| **Anticancer therapies that induce senescence in NB in vitro and in vivo** | | | | |
| Topoisomerase inhibitors | | | | |
| Topoisomerase inhibitor I | Topotecan | STA-NB-10, CLB-Ma, STA-NB-10 mouse xenograft | Reduced DNA synthesis, morphology, SA-β-gal, growth arrest, p21 | Taschner-Mandl et al. [88] |
| Topoisomerase inhibitor I | Camptothecin | STA-NB-10, CLB-Ma, STA-NB-10 mouse xenograft | Reduced DNA synthesis, morphology, SA-β-gal, growth arrest, p21 | Taschner-Mandl et al. [88] |
| Topoisomerase inhibitor II | Doxorubicin | TET21N, SH-SY5Y, IMR5-75 and IMR-32 shMYCN | Enlarged nuclei, accumulation of the Cdt1 degron, SA-β-Gal | Ryl et al. [89] |
| Topoisomerase inhibitor II | Doxorubicin | SH-SY5Y | p21, low Ki67, growth arrest, SA-β-gal | Bojko et al. [90] |
| Topoisomerase inhibitor I | Irinotecan | SH-SY5Y | p21, low Ki67, growth arrest, SA-β-gal | Bojko et al. [90] |
| Antimetabolites | | | | |
| Nucleoside analogue | Bromodeoxyuridine | STA-NB-10, CLB-Ma, STA-NB-10 mouse xenograft | Reduced DNA synthesis, morphology, SA-β-gal, growth arrest, p21 | Taschner-Mandl et al. [88] |
| Ribonucleotide reductase inhibitor | Hydroxyurea | STA-NB-10, CLB-Ma, STA-NB-10 mouse xenograft | Reduced DNA synthesis, morphology, SA-β-gal, cell-cycle arrest, p21, DNA double-strand breaks | Taschner-Mandl et al. [88] |
| Ribonucleotide reductase inhibitor | Hydroxyurea | STA-NB-9, STA-NB-10 | Morphology, increased granularity, telomere length, SA-β-gal | Narath et al. [91] |
| Dihydrofolate reductase inhibitor | Methotrexate | SH-SY5Y | p21, low Ki67, growth arrest, SA-β-gal | Bojko et al. [90] |
| Microtubule inhibitor | Paclitaxel | SH-SY5Y | p21, low Ki67, growth arrest, SA-β-gal | Bojko et al. [90] |
| Aurora kinase inhibitors | | | | |
| AURKA inhibitor | MLN8237 | IMR-32 | p21, p53, growth arrest, SA-β-gal | Yang et al. [96] |
| AURKB inhibitor | Barasertib | SK-N-BE(2) | Morphology, SA-β-gal | Sun et al. [101] |
| INCENP knockdown | INCENP siRNA | SK-N-BE(2), NGP, SH-SY5Y | Morphology, p21, p53, hypophosphorylated RB, growth arrest, SA-β-gal | Sun et al. [101] |
| CDK4/6 inhibitors | | | | |
| CDK4/6 inhibitor | LEE011 | SK-N-BE(2), IMR5, SK-N-BE(2) mouse xenograft, NB-1643 mouse xenograft | Growth arrest, SA-β-gal, Low Ki67 | Rader et al. [104] |
| Ectopic expression | p19INK4d | SH-EP clones expressing p19INK4d | Morphology, SA-β-gal | Dreidax et al. [103] |
| Epigenetic permissive and repressive cellular states for senescence induction in NB cells | | | | |
| CDK4/6 inhibitor | Retinoic Acid | SK-N-SH-F | p16, morphology, granularity, growth arrest, SA-β-gal | Wainwright et al. [106] |
| Dicer knockdown | Dicer siRNA | differentiating SH-SY5Y | SA-β-gal | Jauhari et al. [107] |
| MECP2 knockdown | MECP2 shRNA | SK-N-BE(2) | SA-β-gal | Squillaro et al. [108] |

Columns indicate the experimental model, marker(s) used to establish senescence induction and references to the corresponding research article. Key: SA-β-gal: Senescence-Associated β-galactosidase. NB cell lines are STA-NB-9, STA-NB-10, TET21N, IMR5-75, CLB-MA, SH-SY5Y, SK-N-BE(2), IMR5, SH-EP, SK-N-SH-F, IMR-32, NGP. Mouse xenografts were established from STA-NB-10, SK-N-BE(2) and NB-1643. Figure integrated from Saleh et al. [86].

following MYCN activation in hTERT immortalised retinal RPE cells [59]. Following attenuation of the initially induced MYCN gene-expression signature, we found an unexpected growth reduction that was accompanied by upregulation of pivotal cell-cycle regulators such as *p53* and *CDKN1A* at transcriptional and protein levels. Interestingly, we observed the induction of several previously reported senescence-induced gene signatures and a robust downregulation of *LMNB1* but, remarkably, in the absence of other canonical hallmarks of senescence (such as β-galactosidase activity and SAHFs), suggesting that cells were not fully committing to the senescent programme. Transcriptional and phenotypic evidence of nucleolar stress was noted, suggesting translational overload. The phenotypic changes observed included nucleolar coalescence and cytoplasmic granularity which aligned with transcriptional evidence for upregulation of ribosome biogenesis. The impact of MYCN on ribogenesis and translation is well described, as MYCN activation can trigger the activation of the IRBC (impaired ribosome biogenesis checkpoint) [60] and is implicated in upregulation of RPL (ribosomal protein large) and RPS (ribosomal protein small) proteins in NB cells [61]. Supported by both population-level and single-cell transcriptomics, we proposed that MYCN-induced nucleolar stress drives cells into a pre-senescent state. We observed that MYCN-activated cells are not fully committed to senescence, possibly due to an inadequate checkpoint response. The transient nature of this complex response mechanism might result difficult to entirely recapitulate in in vivo models. Nevertheless, future studies could include live-cell imaging using RPE1-MYCN-ER cells or attempt to assess in vivo single-cell analysis of early emerging hyperplastic lesions in MYC(N) transgenic animal tumour models. c-MYC is classically viewed as a pro-apoptotic and anti-senescence oncogene [62–68], and this was highly reinforced by studies that described its close cooperation with *RAS* during tumorigenesis [29, 69–72]. It was subsequently shown that both c-MYC and MYCN do not directly induce apoptosis but rather act to sensitise cells to pro-apoptotic insults [73, 74]. In addition, our and other researches [59, 75–78], show how MYC can also sensitise cells to senescence. Caspase activity was not significantly increased, suggesting that there was no induction of apoptosis. Although MYCN-activated cells grew more slowly, the response was also accompanied by a robust induction of *CDKN1A* (encoding p21). Studies have pointed out that in addition to being an inhibitor of cell proliferation, p21 acts as an inhibitor of apoptosis in several systems, and this may counteract its tumour-suppressive functions. In addition, a so-called "Goldilocks" zone for p21 levels was identified to control the proliferation-senescence cell-fate decision after drug treatment [79]. Either a delayed or acute drug-induced p21 response led to senescence, while an intermediate p21 pulse enabled sustained proliferation. The cell-cycle dependent p21 overshoot that we witnessed in MYCN-activated cells may thus reflect an attempt to initiate cell-cycle arrest, which was not completely successful, either by having insufficient intensity or improper timing. As the previously MYC-driven senescence-related studies were not all specific to the NB field, we speculate that this response depends upon specific, yet undefined genetic backgrounds. Different MYC proteins (c-MYC, MYCN and MYCL in humans) bind to the same sites in DNA and functional differences between the proteins has been primarily attributed to their differential expression in different cells. For example, dysregulated expression of c-MYC is involved in the development of many cancers, MYCN over-expression is primarily associated with the development of NB and some other childhood cancers [80, 81] while MYCL is often deregulated in small lung cell carcinoma [80]. In addition, c-MYC and MYCN but not MYCL are essential for embryonal development [82]. In response to treatment, differential MYC protein expression also modulates response to therapy: whereas c-MYC overexpression generally sensitises cells to cytotoxic drugs, N-MYC and L-MYC overexpression produces resistance [83]. Hence, the different MYC variants may trigger very different senescence-related responses as well.

## NEUROBLASTOMA: CURRENT THERAPY AND THERAPY-INDUCED SENESCENCE

Treatment schemes for NB patients are largely guided by risk group stratification (see above). Observation only is applied to children younger than 1-year-old without risk factors. For patients in the low-risk group, surgery will be performed if possible. If life-threatening symptoms are present and/or the tumour cannot be removed, chemotherapy is applied and followed by surgery or in rare cases radiotherapy [45]. High-risk patients undergo multi-modal treatments including a combination of high-dose che-motherapy, surgery, radiotherapy, monoclonal antibody therapy, maturation therapy and autologous stem cell transplant. However, these therapeutic approaches demonstrate low effectiveness in high-risk cases with many patients showing resistance. They also fail to clear minimal residual disease (MRD) in good responders as many relapses occur. MRD persistence mechanisms are presumed to include evasion of the immune system and intracellular heterogeneity, causing these subsequent relapses [84]. Moreover, many of the surviving patients suffer from secondary conse-quences from their intensive treatment warranting the need for less toxic therapies. Hence, more effective therapies are urgently needed. Senescence is much more frequently encountered after treatment with chemotherapy (TIS), which has been demonstrated both in vitro and in vivo [85]. Table 1 summarises senescence-inducing agents (by either FDA-approved drugs or genetic perturbations, i.e., sh/siRNAs) tested against NB [86]. In essence, TIS compounds act by interfering with replication (i.e., topoisome-rase inhibitors), nucleic acid synthesis (i.e., antimetabolites), chromosomal segregation (i.e., aurora kinase inhibitors) or by blocking G1/S-phase transition (i.e., CDK inhibitors) and telomere maintenance.

### Topoisomerase inhibitors
The most promising class of anticancer drugs that induce senescence in NB are topoisomerase inhibitors. These agents damage the DNA as they prevent the topological strain release that is exerted by topoisomerase I (camptothecin, irinotecan, topotecan) or II (doxorubicin) [87] during DNA and RNA synthesis. Topotecan and camptothecin treatments administered in low doses at regular intervals over an extended period of time lead to DNA damage, *CDKN1A* upregulation, senescence and tumour regression in aggressive MYCN-amplified NB cell lines and mouse MYCN-amplified NB xenografts. Senescent cells had a strong reduction in DNA synthesis rate as measured by decreased EdU integration levels, an enlarged, flat morphology, and high SA-β-Gal positivity [88]. An additional study characterised the response of individual, patient-derived NB cells driven by the prominent oncogene MYCN to doxorubicin treatment. Upon doxorubicin administration, MYCN-activated cells entered cell-cycle arrest, which was marked by enlarged nuclei and expression of SA-β-Gal [89]. Moreover, a single treatment of a panel of NB cell lines, including *MYCN* single-copy SH-SY5Y cells, with irinotecan and doxorubicin, resulted in increased *CDKN1A* expression and cellular growth arrest, indicating the activation of the senescent programme [90].

### Antimetabolites
Compounds of this class act by interfering with indispensable metabolic pathways necessary for nucleic acid synthesis. For example, treatment with hydroxyurea, a drug that selectively inhibits ribonucleotide reductase thereby inducing G1/S-phase arrest, or bromodeoxyuridine, a nucleoside analogue for thymine which pairs with guanine, can both induce senescence in NB [88]. Low-dose hydroxyurea treatment in two MYCN-amplified NB cell

lines also triggered the activation of senescence [91]. In addition, single treatment of the *MYCN* single-copy SH-SY5Y cells with methotrexate, a dihydrofolate reductase inhibitor [92], or paclitaxel, which stabilises microtubules, resulted in proliferation arrest characterised by upregulation of p21 and thereby suggesting senescence induction [93].

### Aurora kinase inhibitors

Aurora kinases, a family of serine/threonine kinases, consisting of Aurora A (AURKA), Aurora B (AURKB) and Aurora C (AURKC), are essential kinases that regulate progression through the cell cycle, especially in the process of chromosome segregation. Their inhibition was found to interfere with tumour progression and novel compounds are currently being investigated as potential anticancer drugs. The most novel antineoplastic agent reported to induce senescence in NB is MLN8237, an AURKA inhibitor. The protein encoded by *AURKA* is a cell-cycle-regulated kinase that appears to be involved in many biological processes, including MYCN stabilisation during S-phase and microtubule formation and/or stabilisation at the spindle pole during chromosome segregation in M-phase [93]. In NB cells, the MYCN-AURKA complex prevents degradation, controls promoter escape and pauses the release of RNA polymerase II during the cell cycle [94]. AURKA inhibition has shown potential anticancer effects in preclinical studies with fewer side effects compared to traditional chemotherapy drugs [95]. MLN8237 induced G2/M cell-cycle arrest and cellular senescence in vitro in the NB cell line IMR-32, mediated by the p53/p21 pathway and degradation of MYCN. The antitumor effects of MLN8237 were further examined using IMR-32 mouse xenografts. Results demonstrated that MLN8237 can inhibit the growth of transplanted NB tumours in mice, inducing G2/M cell-cycle arrest and cell senescence in vivo [96]. AURKB mediates chromosome condensation and is regulated by its complex partners' inner centromere protein (*INCENP*) and survivin (encoded by *BIRC5*). Inhibition of AURKB, INCENP or survivin significantly blocks NB tumour cell growth in vitro and xenograft growth in vivo [97–100]. A critical report by Sun et al. demonstrated the ability of Barasertib, an AURKB inhibitor, to induce senescence in SK-N-BE(2) NB cells as evidenced by a significantly increased SA-β-gal activity. Similarly, *INCENP* knockdown in SK-N-BE(2), NGP and SH-SY5Y NB cells also induced an increase in the fraction of cells with SA-β-gal activity, increased p21 protein levels while levels of pRB decreased [101]. These findings suggest that strategies aimed at inhibiting Aurora kinases inhibit NB tumour growth through TIS.

### CDK4/6 inhibitors

Cyclin-dependent kinases (CDKs) are a family of multifunctional enzymes that can modify various protein substrates involved in cell-cycle progression. Specifically, CDK4 and CDK6 are key regulators of the G1/S transition, acting via the phosphorylation and consequent inactivation of RB1. The upregulation of CDK inhibitors, such as p21 and p16, is a feature of the senescent response, which blocks the activity of CDKs and thus allows RB1 to enable growth arrest [102]. Indeed, ectopic expression in SH-EP NB cells of p19INK4d, which prevents the activation of CDK4/6 kinases, caused a shift towards a senescent phenotype with enlarged flattened cells positive for SA-β-gal activity [103]. A wide variety of compounds were recently developed to interfere with CDKs and consequently induce growth arrest and senescence in NB. Specifically, the CDK4/6 inhibitor LEE011 triggered growth arrest and senescence in SK-N-BE(2) and IMR5 NB cell lines as evidenced by positive SA-β-gal staining, as well as in SK-N-BE(2)- or NB-1643-mouse xenografts verified by low Ki67 expression [104]. Overall, CDK inhibitors are now recognised as reliable senescence inducers and are becoming more frequently used for preclinical studies of TIS.

### Epigenetic permissive and repressive cellular states for senescence induction in NB cells

Recent studies have revealed the existence of two cellular identities amongst NB cell lines, i.e., adrenergic and immature neural crest or mesenchymal cell types, marked by distinct epigenetically controlled core-regulatory circuitries of multiple transcription factors. This biphasic phenotype was already observed in the SK-N-SH cell line many years ago as distinct morphologic appearances between neuroblast-like and epithelial cells [105]. Not unexpectedly, these distinct cell types respond differentially to RA induced CDK inhibition. RA-treated adrenergic SK-N-SH (neuroblast-like) cells acquired a more differentiated neuronal-like phenotype with neurite-like extensions and expression of neuronal markers, whereas the mesenchymal SK-N-SH (epithelial-like) cells acquired a senescence-like phenotype with flattened morphology, SA-β-gal activity and increased p16 expression [106]. Further experiments using RA for induction of differentiation show that modulation of the epigenetic landscape NB cells can alter the normal route of differentiation towards senescence induction [107, 108]. Key genetic factors of the cell lines presented in Table 1 are summarised in Table 2. Taken together, these studies indicate that senescence induction depends on the genetic background of NB cells and differentiation status.

### Novel senescence-inducing therapeutic strategies

As pointed out above, telomerase or ALT activating mechanisms are an important feature of high-risk NB. Therefore, telomerase inhibitors hold promise as therapeutic agents that favour the activation of the senescent programme in a large subset of NB cases. However, traditional compounds targeting telomerase, such as Imetelstat [109], BIBR1532 [110], KML001 [111] were evaluated and subsequently discontinued due to toxicity constraints. The 6-thio-2′-deoxyguanosine (6-thio-dG) compound is a nucleoside analogue, for which preclinical efficacy has been demonstrated in a subset of cancer entities but has yet to be tested in clinical trials. Nevertheless, data revealed reduced toxicity compared to traditional telomerase inhibitors [112]. Both ATM and ATR are necessary for full telomerase recruitment to telomeres in human cell lines [113], and in vitro sensitivity to ATR inhibition in ALT cancer cell lines has been previously demonstrated [114]. However, when ATR inhibition using AZD6738 was tested in ALT NB cells, it unexpectedly resulted in a resistant phenotype as compared to other NB subtypes [115]. More recent ATR inhibitors

**Table 2.** An overview of the key genetic features associated with NB cell lines.

| Sample ID | MYCN | ALK | p53 |
|---|---|---|---|
| IMR5-75 | A | A | Undetermined |
| IMR-32 | A | partial A | wt |
| STA-NB-9 | A | wt | wt |
| SH-SY5Y | NA | F1174L | wt |
| SK-N-SH | NA | F1174L | wt |
| SK-N-BE(2) | A | wt | 404 G > T (C135F) |
| NGP | A | wt | wt |
| SH-EP | NA | F1174L | wt |
| NB-1643 | A | wt | wt |
| SH-EP TET21N | Tet inducible | F1174L | wt |
| STA-NB-10 | A | wt | wt |
| CLB-MA | A | wt | Undetermined |

*wt* wild-type, *NA* non-amplified, *A* amplified.
key genetic features associated with NB cell lines.

have shown selective sensitivity for ALT cancers but have not been assessed in NB thus far [116].

## THERAPY-INDUCED SENESCENCE FOLLOWED BY NEUROBLASTOMA DISEASE RECURRENCE

Whilst senescence induction is part of the chemotherapeutic *modus operandi*, it has become clear that this may not be the desired endpoint in NB. For one, it may add to the carcinogenic process since chronic secretion of pro-inflammatory cytokines (SASP) can induce de-differentiation and cell division in neighbouring cells [117]. Secondly, viable senescent cells harbour the potential of cell-cycle re-entry, therefore compromising treatment efficacy in the long term [89]. Dörr and coworkers found that inducing senescence in lymphoma cells resulted in higher glucose- and energy demand as well as proteotoxic stress. Experimental depletion of mitochondrial content (by mTORC1 inhibition or PGC-1β deletion), successfully reduced SASP [118, 119]. Thus, this specific metabolic condition of senescent cells, could also be explored in the context of NB to design a novel class of senomorphics, small molecules able to suppress senescence. These findings unveil the crucial role of SASP which can be therapeutically exploitable by synthetic lethal metabolic targeting.

Senescent NB cells have also been found to reboot from TIS upon drug withdrawal [89]. When exposed to doxorubicin, low- and high-MYCN expressing NB cells enter TIS, but when cell-cycle arrest was sustained in low-MYCN expressing cells, a large fraction of high-MYCN cells lost SA-β-gal expression and resumed proliferation after doxorubicin withdrawal. Cell-cycle re-entry after TIS was either followed by cell death or gave rise to the sustained proliferation of a cell clone. The authors suggest that after treatment, the viability of high-MYCN cells depends on their cell-cycle position during treatment: it is the transient G1 arrest of cells born at the start of chemotherapeutic treatment that ultimately supports therapy resistance.

In human lung carcinoma cells, recovery from TIS was found to be promoted by high levels CDC2/CDK1 and survivin after release from chemotherapy [120, 121]. Survivin prevents apoptotic cell death and is associated with chemoresistance and decreased patient survival in other cancers [122, 123]. Of further interest, survivin is expressed during mouse development in neural-crest-derived cells, including dorsal root ganglion neurons [124]. The BIRC5 locus encoding survivin is located on chromosome 17q and often affected by gains as part of large segmental imbalances. Therefore, elevated survivin levels could be implicated in TIS escape within NB entities.

The variability in gene-expression dynamics and a potential senescent fate were also observed in the context of *CDKN1A*. In non-small cell lung cancer cells, the dynamics of *CDKN1A* expression before, during, and days after chemotherapy were linked to the final cell fate [79]. During drug treatment, cells characterised by low or high *CDKN1A* expression entered a senescent fate, while intermediate p21 levels promoted cell proliferation. In NB cells, p21 is found to be dysfunctional as its activation does not result in a complete arrest but rather a G0-G1 checkpoint attenuation. This particular p21 dysfunction relies on the inability of p21 to bind to or inhibit the activity of Cdk2 [125].

The NB-dependency gene *TBX2* is also located on chromosome 17q gene. TBX2 acts as the dimerisation partner for the DREAM complex, a conserved protein complex considered as one of the master regulators of the cell cycle, and together with p21 are known to be implicated in senescence [126]. Interestingly, *TBX2* overexpression allows cells to bypass senescence in other tumour entities, for example in human breast cancer through the repression of the p14 pathway [127] or in melanoma cells via the inhibition of p21 [128], suggesting that it might be a putative target for senescence induction in NB.

BCL-XL inhibitors show senolytic properties as they can reduce senescent cells by promoting apoptosis. An example is given by the compound A1331852 which was recently tested in a panel of NB cell lines and primary patient-derived cells. Inhibition of BCL-XL induced apoptosis in all samples, and thus treatment with A1331852 may have the additional benefit of targeting senescent cells [129].

In view of the above, future therapies could be more effective by combining TIS-chemotherapeutics with senolytics or senomorphics. A "first punch" using chemotherapy could induce cell death and TIS while the "second punch" could then consist of senotherapy, selectively killing senescent cells induced by therapy [18, 130].

## CONCLUDING REMARKS

Senescence is an essential mechanism for tissue homeostasis as it limits the proliferative capacity of cells and for this reason, it represents an effective strategy to block tumour development. In recent years our view on senescence has evolved from a rigid, terminal, non-active state to a much more dynamic and transient cell state that plays an important role in cancer development. Initially considered as a roadblock for uncoordinated cell proliferation, it is now clear that senescence induction is an intrinsic part of the oncogenic transformation process, representing a protective measure to avoid damage accrual under chemotherapeutic pressure. Of note, senescence may even promote carcinogenesis by the escape of toxicity of therapeutic regiment and promoting relapse through paracrine signalling. While complicating the picture of both tumour initiation and relapse, senescence also offers a new window for therapeutic (combination) approaches such as the previously described "one-two punch" strategy. The heterogeneity of both NB and the senescence programme, however, emphasises the difficulties encountered when aspiring to use broad-spectrum senolytic compounds for the treatment of NB-driven entities. In order to maximally exploit this opportunity, it will be crucial to identify a unique biomarker for senescent cells in NB and better understand their dynamics in the context of realistic treatment regimen.

To summarise, the molecular mechanisms of TIS, as well as NB, are gradually being unveiled, which should contribute to a better understanding of commonalities in their potential as anticancer agents. This in turn, may be exploited to develop and refine therapeutic targeting strategies in the context of NB.

## DATA AVAILABILITY
Not applicable.

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## AUTHOR CONTRIBUTIONS
SZ, WHDV and FS: conceptualisation. SZ, BD and SV: literature research and preparation of the first draft of the paper. SZ: table content and figure design. BD, BDW, WHDV and FS: critical revision and editing. All authors have read and agreed to the published version of the paper.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Not applicable.

## CONSENT TO PUBLISH
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The authors declare no competing interests.

## ADDITIONAL INFORMATION
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