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# Senolytic drug discovery and mechanisms of action in BRAF-V600E oncogene-induced senescence

Valentin l'Hôte

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Valentin l'Hôte. Senolytic drug discovery and mechanisms of action in BRAF-V600E oncogene-induced senescence. Subcellular Processes [q-bio.SC]. Université Paris-Saclay, 2022. English. NNT : 2022UP-ASL042 . tel-03783600

**HAL Id: tel-03783600**

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# Senolytic drug discovery and mechanisms of action in BRAF-V600E oncogene- induced senescence

*Découverte de composés sénolytiques et caractérisation de leurs  
mécanismes d'action en sénescence induite par l'oncogène BRAF-V600E*

## Thèse de doctorat de l'université Paris-Saclay

École doctorale n°577, structure et dynamique des systèmes vivants (SDSV)  
Spécialité de doctorat : Biologie moléculaire et cellulaire  
Graduate School : Sciences de la vie et santé. Référent : Faculté des sciences d'Orsay

Thèse préparée dans l'unité de recherche **I2BC (Université Paris-Saclay, CEA, CNRS)**,  
sous la direction de **Carl MANN**, directeur de recherches CEA,  
et le co-encadrement de **Jean-Yves THURET**, directeur de recherches CEA

Thèse soutenue à Paris-Saclay, le 27 Juin 2022, par

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# Acknowledgments

First, naturally, I wholeheartedly thank Jean-Yves Thuret for trusting me in taking over this project, and for being such a great advisor. You always made yourself available, you trusted me in making my own choices for conducting this research, and you had at all times my own interest in mind, all while guiding me through the process. I gained invaluable insight from you on how to produce solid science. The balance in your management was perfect for me to develop as a scientist during this PhD. I am looking forward to future collaborations yielding exciting science – and pastry-filled coffee breaks!

Many thanks to Carl Mann for welcoming me in the team and being my PhD director in these four years. You also always made yourself available. You create a serene work environment which is utterly important in academia, and I felt like I always had a better grasp of things after discussing science with you.

I am very grateful to the members of my thesis jury Corinne Abbadie, Pascale Bertrand, Nadine Martin and Isabelle Petropoulos, who accepted to take the time to read and review my work, thus bringing crucial insight into it. It was a pleasure and an honor to discuss my work with you during my defense.

Many thanks to Guillaume Pinna and Marie Vandamme for designing and performing this first (challenging) chemical screen with me. Special thanks to Agnès Delaunay-Moisan and Gwenaëlle Le Pavec for guiding me into the world of ER stress. Thanks to Mélissa Rondon who went through long hours of Western blotting and RT-qPCR with me to profile ER stress in our cells, and to Sérine Sidia with whom we performed a first chemical screen on our own.

Thanks to everyone who was part of my thesis committee at some point, thus guiding me through the years: Jean-Christophe Aude, Pascale Bertrand, Marc Blondel, Eric Chevet, Jean-Christophe Cintrat, Agnès Delaunay-Moisan, Claude Gazin, Guillaume Pinna.

Being in the lab was an everyday pleasure thanks to the wonderful people who were part of our team at one time or another during my stay: Régis, Reem, Justine, Mélissa, Sirine, Adèle, Mathieu, Jorge,

Valentin, Donya, Timothée, Mauriane. And let's not forget traditions: *Régis c'est le plus fort, c'est le meilleur et on l'adore !*

Thanks to all the people in the department with whom I had the pleasure to share all those moments 'in between': Catherine, Gwen, Agnès, Jean-Christophe, Stéphane, Michel, Clément, Pascal, Rosa, Leo, Edern, Sylvain, Beata, Julien, Kris, Aurélie, Marina, Manon, Manu, Sigrid, Victoire, Célia, Emilie, Batoul, Marouane, Matthieu, Franck, Corinne, Benoît, Hélène... and everyone whose name I am shamefully forgetting to include.

*Merci* to my parents and sister for granting me with unconditional love and support. *Kiitos* to Miisa Häkkinen, petrologist *extraordinaire* – whom I know would have made an equally great cell biologist – for being there every single day during these years. I wish you the best for your PhD, and most importantly, for everything else. This thesis is dedicated to the four of you.

I want to give a special thanks to my dear friends Adam Kavanagh-Coyne and Francis Morgan, currently on a PhD journey of their own. Our discussions and study sessions were what made me fall in love with science in our year together in La Rochelle (*Propriétés Chimiques du Solide*, rings a bell?), and I want to acknowledge our professor Brahim Elouadi who that same year strongly influenced my choice to turn to scientific research.

Adam, Elise, Simon, you were with me nearly everywhere I have been this past decade. Thank you so much. Thanks to all the people whom I am lucky to call my friends, for giving me strength and making my life better: Axel, Sébastien, Jakub, Ronan, Patrycja, Valérian, Marc, Nicolas, Nini... Special thanks to my science buddies Eoghann, Melvin, and Maxime, with whom it's always a pleasure to share memes or discuss science for hours around a beer!

Finally, *tack så mycket* to Sjoerd Wanrooij and Mara Doimo for supervising my master's project and preparing me so well for the PhD. I am delighted to be back in Umeå as a post-doc!

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# List of abbreviations

BRafSen: BRAF-V600E-induced senescent

CDKI: cyclin-dependent kinase inhibitor

DDR: DNA damage response

ER: endoplasmic reticulum

EtoSen: etoposide-induced senescent

LSEC: liver sinusoidal endothelial cell

MAPK: mitogen-activated protein kinases

MOMP: mitochondrial outer membrane permeabilization

MEF: mouse embryonic fibroblast

MERCS: mitochondria-endoplasmic reticulum contact sites

MHC: major histocompatibility complex

NKA: Na,K-ATPase pump

PDGF-AA: platelet-derived growth factor AA

PROTAC: proteolysis-targeted chimera

OIS: oncogene-induced senescence

RasVSen: HRAS-V12-induced senescent

RIDD: regulated Ire1-dependent decay of mRNA

RT-qPCR: reverse transcription – quantitative polymerase chain reaction

ROS: reactive oxygen species

SA- $\beta$ Gal: senescence-associated  $\beta$ -galactosidase

SAHF: senescence-associated heterochromatin foci

SASP: senescence-associated secretory phenotype

siRNA: small interfering RNA

SSMD: strictly standardized mean difference

TGF $\beta$ : tumor growth factor  $\beta$

TIS: therapy-induced senescence

TPA: tissue plasminogen activator

UPR: unfolded protein response





# **Chapter 1. Senescence as a stress response, a cell fate, and a cell identity.**

Cellular senescence, or thereafter simply senescence, is a process that calls forth the notions of cellular stress response, cell fate and cell identity. In response to a variety of stresses including DNA damage, oncogene expression, loss of proteostasis, or redox imbalance, the cell is faced with the decision to undertake a path towards a given cell fate. Depending on the magnitude of the stress, homeostasis, and the cellular context, the cell may resolve the stress and resume proliferation, commit to apoptosis, or enter senescence. Senescence involves a highly stable, quasi-irreversible proliferative arrest, accompanied by important changes in transcriptional programs and cellular physiology, resulting in altered cell identity.

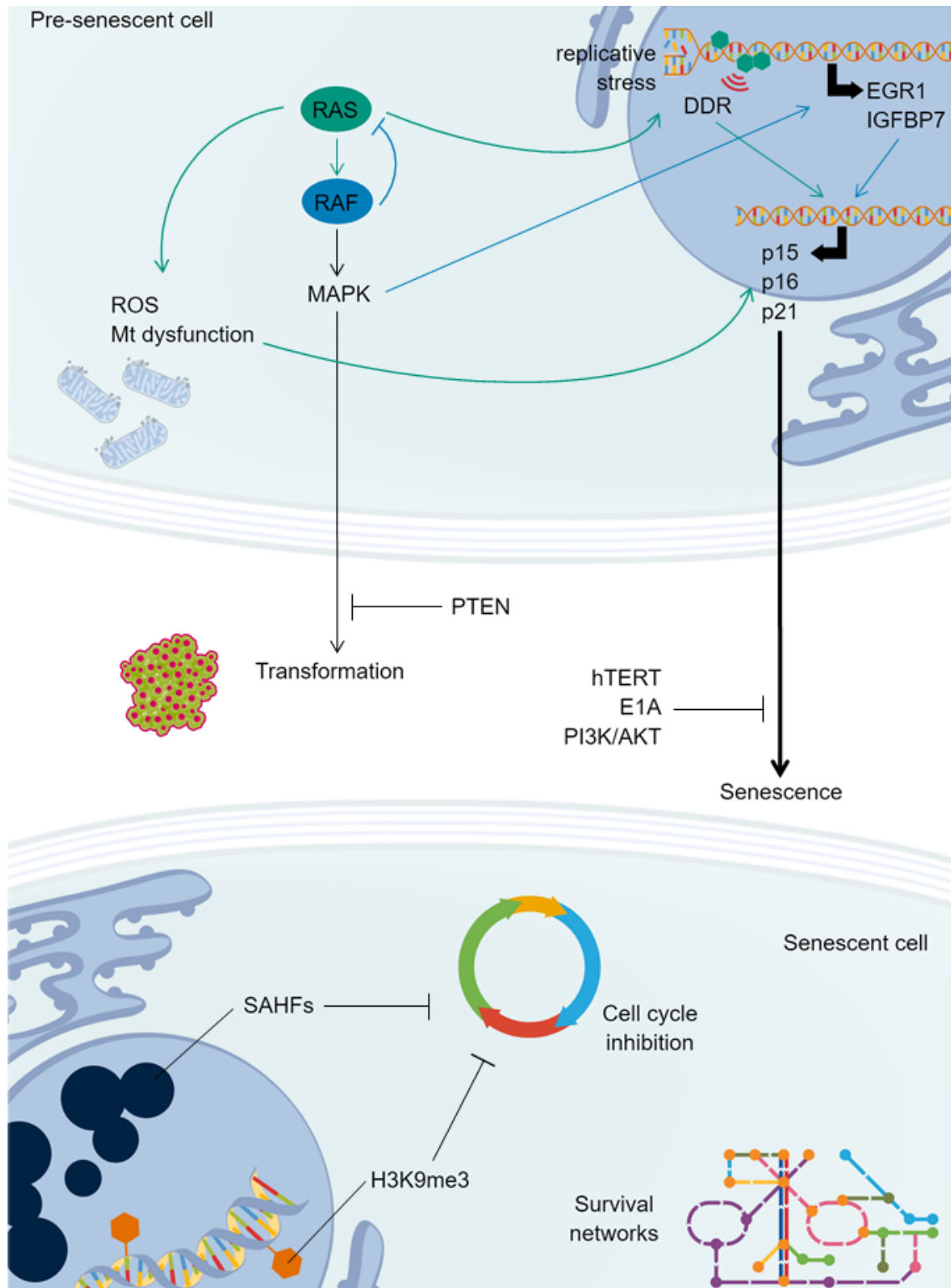
Importantly, most senescent cells secrete a complex collection of pro- and anti-inflammatory factors, matrix metalloproteases, and modulators of tumorigenesis, termed the senescence-associated secretory phenotype (SASP). Senescence is an essential tumor-suppressing mechanism because it prevents the proliferation of stressed cells potentially bearing pre-malignant potential. However, the abnormal accumulation of senescent cells is detrimental as the SASP causes chronic inflammation and tissue deterioration, and can promote adjacent tumor growth. Senescent cell accumulation is a driver of the aging process as well as multiple pathologies. There is therefore a strong clinical interest in unraveling the pathways that lead to the establishment of senescent phenotypes, and how these phenotypes differ from that of non-senescent cells in terms of metabolism, homeostasis, and regulation of cell survival, so as to selectively target senescent cells in the context of their accumulation with so-called senolytic drugs.

I start by introducing senescence as a stress response, a cell fate, and a change of cell identity (Figure 1.1). I notably focus on BRAF-V600E oncogene-induced senescence (OIS), as this was the main model used throughout the thesis for the identification of senolytic compounds and the characterization of their mechanisms of action.

## **1.1. Senescence as a stress response.**

Replicative senescence, which is the result of telomere shortening, was the first reported and is the most commonly known type of cellular senescence. It was observed 60 years ago that primary human fibroblasts in culture (including the strain that would become the widely used WI-38 cell line) eventually stopped proliferating after a certain number of population doublings, now known as the Hayflick limit. This seminal discovery challenged the long-lasting assumption that cultured cells were immortal and could be propagated ceaselessly, and Hayflick foresaw that this finite replication potential was the manifestation of aging at the cellular level (Hayflick, 1965; Hayflick & Moorhead, 1961). Since then and for decades, cellular senescence had been controversial with regards to its *in vivo* relevance. Some argued that it was an *in vitro* artifact resulting from imperfect culture conditions (S. He & Sharpless, 2017; Sherr & DePinho, 2000), until cells recapitulating hallmarks of senescence were finally detected in human skin samples and showed to accumulate with aging (Dimri et al., 1995; Jeyapalan et al., 2007). There is now a large body of evidence in favor of the *in vivo* occurrence of senescence, and a consensus that it plays major roles in aging and numerous pathologies (Gorgoulis et al., 2019; S. He & Sharpless, 2017).

Replicative senescence is an important barrier to cellular transformation, which is more likely to occur as the cell accumulates mutations through successive generations. As a matter of fact, reactivation of enzyme telomerase, which maintains telomere length and thus counteracts replicative senescence, is one of the most prominent genetic alterations in cancer (Akincilar et al., 2016). Independently of telomere attrition, other stimuli can trigger stress-induced premature senescence, such as oncogene activation or loss of tumor suppressor genes, DNA damage induced by ionizing radiation or genotoxic agents, and oxidative stress, among others (Hernandez-Segura et al., 2018). In all these contexts, the triggering of premature senescence plays a protective role by limiting the proliferation of potentially damaged or pre-neoplastic cells. This was notably evidenced by the increased susceptibility to tumorigenesis of a senescence-deficient mouse lacking p16, an important effector of the senescence-associated proliferative arrest (Sharpless et al., 2001).



**Figure 1.1. Mechanisms of OIS induction and maintenance.** Hyperactivated RAS and RAF can trigger a senescence-associated proliferative arrest through various routes. More stress pathways are implicated in RAS than in RAF senescence. Inactivation of tumor suppressors, or additional oncogenic events, may promote transformation over senescence in response to RAS or RAF. Besides CDKIs, the established senescent state is stabilized by chromatin reorganization. Senescent cells undergo profound transcriptional reprogramming, and actively suppress cell death through diverse strategies.

Beyond the tumor suppressive role of senescence triggering in itself, the physiological function of some senescent cell types was later established. p21-dependent programmed senescence was shown to be

implicated in tissue remodeling during mammalian embryonic development (Muñoz-Espín et al., 2013; Storer et al., 2013). Cells recapitulating senescence features were detected at various locations in the developing embryo and mediated their own removal through the recruitment of macrophages, probably via the SASP (Muñoz-Espín et al., 2013). Some of these developmentally programmed senescent cells were also ultimately eliminated through apoptosis (Storer et al., 2013). Senescence impairment was partially compensated by apoptosis and thus resulted in only mild developmental defects (Muñoz-Espín et al., 2013). It thus appears that senescence and apoptosis cooperate closely during development and are mutually compensatory. They also appear to be complementary responses to cellular damage and stress with their own perks and disadvantages with regards to tissue and organismal homeostasis (Childs et al., 2014; Kowald et al., 2020). Senescence is also required for optimal wound healing. PDGF-AA was secreted in the SASP of senescent cells located at cutaneous injury sites and induced myofibroblast differentiation. Accordingly, the clearance of p16-expressing cells in p16::3MR transgenic mice resulted in impaired wound healing (Demaria et al., 2014). Finally, whole-body systematic clearance of p16-expressing cells in the p16-Cre mouse allowed to identify an essential subpopulation of senescent cells. Liver sinusoidal endothelial cells (LSECs), which clear macromolecular waste from blood, became senescent with age and were not cleared by the immune system. Importantly, senescence induction increased their detoxifying capacity through the upregulation of endocytic receptors. They were not replaced following their genetic removal, which led to liver fibrosis and reduction of healthspan (Grosse et al., 2020).

Paradoxically, senescence and thus proliferative arrest can be induced by oncogene expression or loss of tumor suppressors depending on the cellular context, in what is an evident barrier to malignant transformation. Consequent literature focuses on the mechanisms of RAS-induced senescence. RAS is a family of proto-oncogenes encoding small membrane-bound GTPases regulating multiple signaling pathways notably involved in cell proliferation, such as the MAPK/ERK axis. Conditional transgenic expression of mutant HRAS-V12 in mice led to the formation of both pre-malignant and malignant tumors, with pre-malignant tumors only staining strongly for multiple senescence markers, showcasing the *in vivo* role of OIS to limit malignant progression (Collado et al., 2005). Overexpression of HRAS-

V12 in the mammary gland also triggered OIS (Sarkisian et al., 2007). Downstream of RAS within the MAPK/ERK pathway, constitutively active RAF kinases also trigger senescence. V600E is a frequent phosphomimetic mutation in BRAF's activation loop that renders the kinase constitutively active. BRAF-V600E is found in 80% of melanoma and 40% of thyroid cancers (Frasca et al., 2008; Pollock et al., 2003). BRAF-V600E is also expressed in 80% of nevi (commonly known as moles), which are benign, growth-arrested senescent neoplasms (Pollock et al., 2003). The current model for nevogenesis involves the clonal expansion of pre-neoplastic melanocytes acquiring the initial BRAF-V600E driver mutation, before OIS is triggered (Shain et al., 2015; Stark et al., 2018). Nevi may then evolve into malignant melanoma through a succession of additional mutational events. Ectopic expression of BRAF-V600E in primary human melanocytes induced an initial hyperproliferation phase of a few days to a week, followed by a robust senescence-associated proliferative arrest (Michaloglou et al., 2005). BRAF-V600E knock-in expression in melanocytes of mice led to senescent nevi formation, and later to melanoma (Dhomen et al., 2009). About a third of melanoma arise from a pre-existing nevus (Lin et al., 2015; Pampena et al., 2017). Sequencing of human primary melanoma and adjacent precursor lesions representing different stages of melanomagenesis showed that the vast majority of benign nevi were only positive for BRAF-V600E, whereas supplementary alterations such as hTERT promoter activation and NRAS mutations were found later in stages of intermediate neoplasia. TP53 and PTEN mutations, as well as homozygous deletion of p16, were found only in invasive melanoma (Shain et al., 2015). Ultraviolet radiation mutational signatures were evident in both benign nevi and malignant melanoma (Shain et al., 2015; Stark et al., 2018). Human melanocytic nevi stained strongly for senescence markers p16 and senescence-associated  $\beta$ -galactosidase activity (SA- $\beta$ Gal), and were not proliferative as shown by the absence of Ki-67 expression (Michaloglou et al., 2005). However, melanoma can arise de novo or from a nevus, showcasing instances of senescence bypass or escape.

Although it was established that aberrant oncogenic signaling induces senescence, the exact nature of the stress at the origin of the proliferative arrest was argued about. Hence, whether OIS was a direct response to oncogenic signaling itself that evolved as a tumor suppressor program (Storer et al., 2013), or in some contexts was rather analogous to replicative senescence as a consequence of oncogene-driven

hyperproliferation, had been debated (Bastian, 2003; Braig et al., 2005; Chandek & Mooi, 2010; Jones et al., 2000). Human thyroid epithelial cells ectopically expressing HRAS-V12 completed 20 population doublings before eventually entering senescence, which could suggest a role for telomere attrition in the proliferative arrest, but importantly this RAS-induced delayed growth arrest was unaffected by the expression of hTERT, which encodes the catalytic subunit of telomerase (Jones et al., 2000). The fact that hTERT promoter activation, which serves in bypassing replicative senescence, was selected early as a secondary event in melanomagenesis, strongly suggested that senescent melanocytes within pre-malignant nevi had undergone replicative exhaustion (Shain et al., 2015). This was however in apparent incompatibility with previous data showing that senescent melanocytes within human nevi retained telomeres, supporting a bona fide OIS response to BRAF-V600E expression independent of replicative senescence (Michaloglou et al., 2005). It was later demonstrated that hTERT does promote OIS escape by resolving chronic telomeric DNA damage contributing to proliferative arrest maintenance, thus providing a rationale for the early selection of hTERT in melanomagenesis in accordance with a telomere attrition-independent OIS response (Patel et al., 2016).

RAS induces multiple cellular stresses that are known to trigger senescence on their own. The DNA damage response (DDR) was essential in the establishment of RAS senescence, and its inhibition bypassed OIS (Di Micco et al., 2006). HRAS-V12 hyperproliferative signaling led to replicative stress and DNA damage, which was responsible for the initial growth arrest in early S phase, previously identified as a G1 arrest (Serrano et al., 1997). DNA damage was partly due to depletion of dNTP pools in RAS-senescent human fibroblasts (Mannava et al., 2013). DNA repair was inhibited by the dissociation of the BRCA1 complex from the chromatin of cells undergoing RAS senescence (Tu et al., 2011). RAS also induced the formation of mitochondrial reactive oxygen species (ROS) resulting in oxidative stress, which was required for the upregulation of p21 and the senescence arrest, and contributed to mitochondrial dysfunction (Lee et al., 1999; Moiseeva et al., 2009). In contrast, the DDR as well as oxidative stress were dispensable for RAF senescence establishment, and 50% of the cells were arrested without going through the S phase in response to CRAF activation (Jeanblanc et al., 2012). However, the cells that traversed the S phase following CRAF activation did exhibit markers of DNA

damage, evident of replicative stress. Up to 25% of CRAF-arrested cells were in G2/M (J. Zhu et al., 1998). In BRAF-V600E-induced senescent fibroblasts, we showed that hyperactivation itself of the MAPK pathway was sensed by transcription factor EGR1, which controlled the kinetics of the proliferative arrest by directly binding and activating the promoters of CDKN1A (encoding p21) and CDKN2B (encoding p15) (Carvalho et al., 2019). Nonetheless, MAPK/ERK signaling is essential for both RAS and RAF senescence induction, as genetic or pharmacological ablation of the pathway resulted in senescence bypass and transformation (Deschênes-Simard et al., 2013; J. Zhu et al., 1998).

## **1.2. Senescence as a cell fate.**

The decision to proliferate, engage apoptosis, or enter senescence in response to oncogene expression depends on the magnitude and duration of mitogenic signals, as well as on the presence of additional mutations that may bypass the senescence program. Controlled HRAS-V12 expression in the mammary gland of transgenic mice promoted proliferation and hyperplasia at low physiological levels, but induced OIS at higher levels (Sarkisian et al., 2007). Conversely, physiological expression levels of BRAF-V600E were sufficient to induce OIS in primary human fibroblasts, even in the absence of p16 (Michaloglou et al., 2005).

The initial proliferative arrest in response to stress, as well as the maintained restriction of cell cycle progression in senescent cells, are mediated by the expression of various cyclin-dependent kinase inhibitors (CDKIs). CDKIs may maintain the senescence arrest even upon withdrawal of oncogene signaling (Jeanblanc et al., 2012; Sarkisian et al., 2007; J. Zhu et al., 1998). The identity of CDKIs implicated as well as the mechanisms activating their expression vary depending on both the senescence-inducing stressor and the cell type. In many senescence settings, the p53/p21 pathway is responsible for the initial cell cycle exit in response to stress, and p16/Rb signaling later takes over the maintenance of the proliferative arrest through the repression of E2F-regulated genes implicated in cell cycle progression. Both the p53/p21 and the p16/Rb axes were required for induction of RAS senescence in mouse embryonic fibroblasts (MEFs), as RAS induced transformation of p53 or p16 deficient MEFs (Serrano et al., 1997). Moreover, lack of TP53 bypassed NRAS-G12V OIS in transgenic mice's



lymphocytes which resulted in invasive T cell lymphoma (Braig et al., 2005). In contrast, inhibition of either p53 or p16 alone did not suffice to bypass RAS-induced senescence in human fibroblasts, which thus appear more sensitive to RAS senescence (Serrano et al., 1997).

CRAF-induced senescence of human fibroblasts was maintained by both p21 and p16 (Jeanblanc et al., 2012), while the increase of p21 in replicative-senescent human fibroblasts was only transient and served only in the initial proliferative arrest (Alcorta et al., 1996). Senescence induced in human fibroblasts by ectopic expression of BRAF-V600E did not depend solely on p16, as although its expression was induced by BRAF-V600E, prior shRNA-mediated knockdown of p16 did not bypass OIS (Michaloglou et al., 2005). The ablation of p53 and p21 was also insufficient to bypass CRAF senescence in human fibroblasts (J. Zhu et al., 1998). However, TP53 knockdown bypassed BRAF-V600E senescence of primary human fibroblasts and melanocytes (Wajapeyee et al., 2008). p15 appears especially important in the induction and the maintenance of BRAF-V600E senescence (Carvalho et al., 2019; McNeal et al., 2015). BRAF-V600E induced senescence of human fibroblasts through a cooperation between p15 and p21, independently of p16 (Carvalho et al., 2019). Single ablation of p15 but not p16 bypassed BRAF-V600E senescence of human melanocytes, showing that p15 was necessary and sufficient for the proliferative arrest, although p16 participated as its depletion potentialized that of p15 (McNeal et al., 2015). Moreover, p16 inactivation was associated with melanomagenesis (Kamb et al., 1994), so although its absence is not sufficient for BRAF-V600E senescence bypass or escape, it participates in the robustness and stability of the proliferative arrest. Interestingly, p15 induction in melanocytes was triggered by a cell non-autonomous effect, through the secretion of TGF $\beta$  in response to BRAF-V600E expression. In fibroblasts, p15 induction was cell-autonomous and depended on EGR1 binding the CDKN1B promoter (Carvalho et al., 2019). Both the TGF $\beta$  and the EGR1 pathways may participate in p15 induction in either cell system. Other essential cell non-autonomous mechanisms were shown to be required for BRAF-V600E senescence in both fibroblasts and melanocytes, such as the secretion of IGFBP7, which functioned in autocrine and paracrine manner by eliciting a negative feedback loop downregulating BRAF/MEK/ERK signaling which participated in the proliferative arrest (Wajapeyee et al., 2008).

Senescence can be bypassed if RAS is activated in a context of other oncogenic events, leading to transformation. PI3K/AKT signaling has been shown to mediate OIS bypass, and is one of the effector pathways of RAS in addition to the MAPK/ERK axis (Sarkisian et al., 2007). Activation of PI3K/AKT concomitant with RAS or RAF activation bypassed senescence and led to proliferation and transformation (A. L. Kennedy et al., 2011; Vredeveld et al., 2012). Preliminary expression of the E1A oncogene bypassed senescence upon HRAS-V12 expression and induced transformation in human and mouse fibroblasts, even if HRAS-V12 was expressed at high levels (Serrano et al., 1997). Hyperactive CRAF triggered senescence notably through multiple negative feedback loops that resulted in the silencing of RAS signaling, and consequently of PI3K/AKT signaling too, thus participating in senescence onset (Courtois-Cox et al., 2006). Tumor suppressors appear to be essential for the induction and maintenance of OIS. Inactivation of tumor suppressors via co-expression of the SV40 large tumor antigen bypassed senescence. Consequently, human fibroblasts deficient for p53 and p16, and expressing the SV40 large tumor antigen and hTERT together with BRAF-V600E or HRAS-V12 exogenously introduced into mice led to tumor formation (Michaloglou et al., 2005). In benign nevi, copy number loss of tumor suppressors and oncogenes even out as they are mostly the result of loss of heterozygosity events encompassing large chromosomal regions, which has been proposed to prevent malignant transformation (Stark et al., 2018).

Cell fate decisions in the face of oncogenic stress are influenced even more broadly by the cellular context. The establishment of the DDR was essential for the triggering of senescence rather than transformation in response to RAS (Di Micco et al., 2006). In turn, the formation of senescence-associated heterochromatin foci (SAHFs) downregulated the DDR, which promoted apoptosis avoidance in RAS-senescent cells (Di Micco et al., 2011). Autophagy was shown to promote OIS over proliferation by facilitating the synthesis of SASP factors that participate in the spread of paracrine senescence (Young et al., 2009). Autophagy upregulation in OIS was congruent with the downregulation of the PI3K/AKT axis (Courtois-Cox et al., 2006).

The fact that senescence and apoptosis share common inducers and conceivably serve similar tumor-suppressing purposes, but that the SASP mediates deleterious effects damaging the surrounding tissue

as well as promoting tumorigenesis in some contexts, raised the question of why apoptosis had not been selected over senescence during evolution, as a seemingly more favorable cell fate (Childs et al., 2014). Moreover, and unlike apoptosis, as senescence escape mechanisms exist, OIS does not fully protect from the threat of cellular transformation imposed by the oncogene (Chandek & Mooi, 2010). However, besides the aforementioned physiological roles of senescence in embryonic development, wound healing, and liver function, senescence holds other advantages over apoptosis in tumor suppression. The SASP, although it may lead to tissue deterioration if senescent cells accumulate, allows the recruitment of the immune system and thus promotes immune surveillance of tumors and pre-malignant lesions (Kang et al., 2011). It also mediates paracrine induction of senescence in neighboring cells, thus extending protection from malignant transformation beyond the cell that is faced with the neoplastic threat (Rattanavirotkul et al., 2021; Wajapeyee et al., 2008). It was also proposed that OIS provides a fitter option than apoptosis for tissue homeostasis if triggered in cell types that are typically persistent and not replaced with high turnover (Chandek & Mooi, 2010). Interestingly, significant overlap between features of developmental senescence and OIS were reported (Storer et al., 2013), and it was thus proposed that OIS evolved and adapted from programmed developmental senescence.

### **1.3. Senescence as a cell identity.**

Although senescent cells are permanently withdrawn from the cell cycle, they remain viable and undergo profound transcriptional and metabolic changes. The hallmarks of cellular senescence have been reviewed and most notably include the proliferative arrest-associated expression of CDKIs, SA- $\beta$ Gal activity resulting from increased lysosomal content, resistance to apoptosis through reorganization of survival networks, and the SASP (Hernandez-Segura et al., 2018). None of the hallmarks of senescence is both universal and specific. The only universal feature shared by all senescent cells is permanent proliferative arrest, but other more or less stable forms of proliferative arrest exist, such as quiescence, terminal differentiation, and replicative exhaustion (S. He & Sharpless, 2017). Despite its acknowledged specificity, p16 expression is not exclusively restricted to senescent cells and is constitutively found in replicatively exhausted lymphocytes, and cancer cells with inactivated Rb (Sharpless & Sherr, 2015). The lack of a universal and specific marker for the unequivocal characterization of senescence is a

serious caveat to the *in vivo* identification of senescent cells and the characterization of so called senescent-like states; non-proliferative cells with classical features of senescence such as SA- $\beta$ Gal and SASP expression have been described that resume proliferation upon stress withdrawal, calling into question the relevance of our current definition of senescence. Despite checking multiple hallmarks of senescence, should these cells be denied the status of senescent because of the instability of their proliferative arrest, even though they may be selectively cleared by senolytics (Fleury et al., 2019; Malaquin et al., 2020)? Thus, it appears that senescence is a spectrum rather than one well-defined phenotype with immovable features.

OIS is arguably the type of senescence for which irreversibility of the proliferative arrest is the most controversial (Chandek & Mooi, 2010). However, OIS is certainly the senescence setting for which prospective escapes would be the most deleterious, and have thus been extensively studied. OIS is still exceptionally stable in many contexts. The vast majority of nevi never transform into malignant melanoma (Tsao et al., 2003). Moreover, in most OIS settings, maintenance of oncogene expression after the proliferative arrest is not even required for senescence (McNeal et al., 2015; Sarkisian et al., 2007; J. Zhu et al., 1998). p15 seems especially important for the maintenance of the established OIS arrest. HRAS-V12-induced senescent pre-malignant lung adenomas in mice stained for both p16 and p15 (Collado et al., 2005). Senescent melanocytes from human nevi expressed considerable levels of p15, and its knockdown mediated OIS escape (McNeal et al., 2015). Rendering CDK4 resistant to its inhibitors p16 and p15, or knocking down p15 expression, mediated senescence escape. Meanwhile, p16 knockdown resulted in only modest senescence escape. Recently, the stability of the BRAF-V600E growth arrest in melanocytes proved to depend upon their differentiation state and the presence of TPA, an additive to *ex vivo* melanocyte culture media (McNeal et al., 2021). It was suggested that melanocytes within a nevus were subject to phenotypic plasticity as they oscillate between arrested and proliferating states that depended on external factors.

We saw earlier that PI3K/AKT activity may bypass OIS, but it can also mediate escape from it. In melanoma, BRAF-V600E inhibited AKT through mTORC2, but AKT signaling was restored by the loss of tumor suppressor PTEN even in the presence of BRAF-V600E (Chen et al., 2012). Consequently,

PTEN depletion mediated OIS escape of established BRAF-V600E-induced senescent human fibroblasts and melanocytes through reactivation of PI3K and AKT (Vredeveld et al., 2012). PTEN depletion also promoted melanoma formation in a knock-in mouse model of melanocyte-restricted BRAF-V600E expression (Vredeveld et al., 2012). Moreover, BRAF-V600E and PI3K can also cooperate independently of AKT in melanoma to regulate protein synthesis and stimulate proliferation (Silva et al., 2014).

A functional chronic DDR was shown to be implicated in the maintenance of RAS senescence in human fibroblasts, as its abrogation mediated partial OIS escape, an effect that was further potentialized by p16 knockdown (Di Micco et al., 2006). However, because RAS-senescent cells are deficient in DNA repair due to BRCA1 inhibition, they are predisposed to additional mutations potentially promoting senescence escape and transformation (Tu et al., 2011).

The senescent cell identity is enforced by epigenetic changes. OIS is systematically characterized by SAHFs (Di Micco et al., 2011; Kosar et al., 2011). Exogenous expression of BRAF-V600E in human fibroblasts induced massive SAHFs staining for K9 trimethylation of histone 3 (H3K9me3), a transcriptional silencing epigenetic marker (Michaloglou et al., 2005). Interestingly, it was demonstrated that the spreading of repressive histone marks such as H3K9me3, and chromatin compaction into SAHFs, were discrete sequential events in senescence onset, independent from each other (Chandra et al., 2012). SAHFs are important for the stability of the senescence-associated proliferative arrest, by locking in the inactivation of E2F genes involved in cell cycle progression, and their formation is p16/Rb-dependent (Narita et al., 2003; L. Zhao et al., 2015). However, the double knockdown of p16 and p21 together with the inactivation of CRAF was sufficient to mediate senescence escape of CRAF-induced senescent fibroblasts in spite of the presence of SAHFs (Jeanblanc et al., 2012). SAHFs also form in some cancer cell types in response to genotoxic agents, and this is also dependent on Rb (Y. Zhang et al., 2013). SAHFs were not detected *in vivo* in RAS-induced senescent cells of the mouse mammary gland but chromatin underwent important condensation (Sarkisian et al., 2007). H3K9me3 was essential in inducing RAS OIS early in lymphoma development, as lymphocytes lacking methyltransferase Suv39h1 gave rise to invasive lymphoma (Braig et al., 2005).

It is now evident that cellular senescence is not a fixed phenotype but rather a dynamic process, with states of ‘light’ and ‘deep’ senescence that are more less irreversible. Differences in transcriptome trajectories during induction of senescence and quiescence were compelling, and illustrated the fact that senescence was a temporally dynamic state with an evolving transcriptional program, whereas quiescence resembled a fixed, dormant state (Martínez-Zamudio et al., 2020). Moreover, the important heterogeneity between different phenotypes of senescence, depending on the cell type and the senescence-inducing stressor, was evident at the transcriptome level (Hernandez-Segura et al., 2017).

Thus, senescence implies a clear change in cell identity, that renders senescent cells different enough from proliferative or quiescent cells to allow for their selective targeting by pharmacological means with senolytics. Phenotypical differences between senescent and non-senescent cells, in terms of apoptosis resistance, regulation of survival, and cellular homeostasis, and how to exploit them for senolysis, are later addressed in detail in Manuscript 1.



# Chapter 2. Senescent cell accumulation and senotherapeutics.

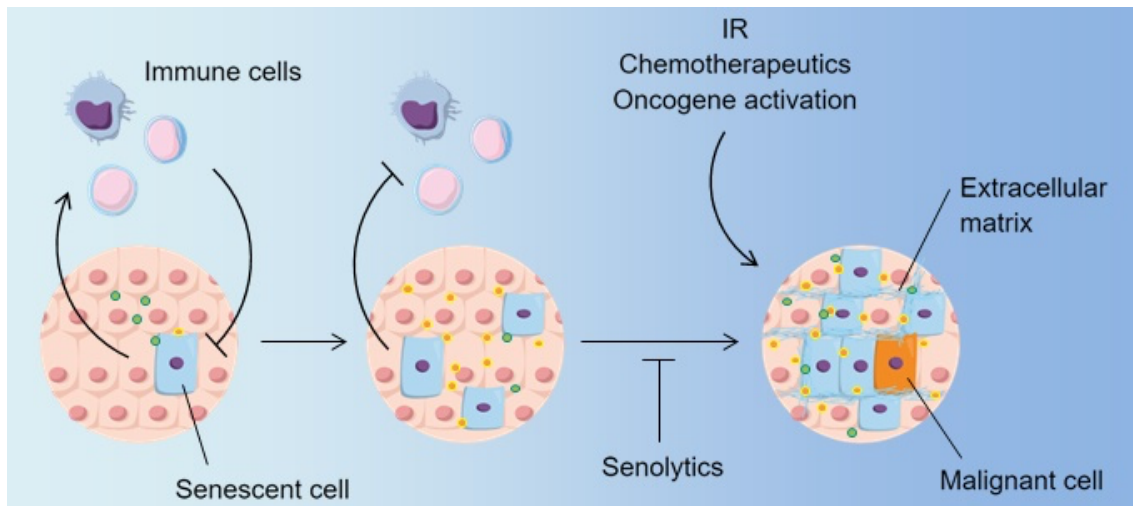
## 2.1. Persistence and accumulation of senescent cells.

One evident function of the SASP's inflammatory factors is to recruit the immune system to mediate the clearance of newly-senescent cells. The routes by which senescent cells are removed by the immune system are many and have been extensively reviewed by Prata and colleagues (Prata et al., 2018). The complex composition of the SASP allows senescent cells to coordinate the immune response leading to their removal, from the recruitment and activation of immune cells to the remodeling of the extracellular matrix to facilitate immune infiltration. Pre-malignant, oncogene-induced senescent hepatocytes ectopically expressing HRAS-V12 were eliminated *in vivo* by cooperating CD4<sup>+</sup> T cells and macrophages, and failure to carry out their immune clearance resulted in the development of hepatocellular carcinoma (Kang et al., 2011). Macrophages removed senescent cells in other instances (Egashira et al., 2017) and appear essential in senescent cell clearance, but the precise underlying mechanisms and whether phagocytosis or the secretion of cytotoxic factors are implicated, remain unclear and require further investigation (Prata et al., 2018). NK cells removed senescent hepatic stellate cells during liver fibrosis, through granule exocytosis. The Fas and TRAIL apoptotic pathway were inhibited by surface expression of Dcr2 by senescent cells (Sagiv et al., 2013). Adoptive NK cell infusion, i.e. transfusion of exogenous NK cells, was shown to clear senescent cells, and dopamine further enhanced the process by activating the D1-like receptors (Bai et al., 2022). In the long term, persisting senescent cells may limit immune invasion and thus their removal, because of tissue degradation mediated by the SASP. Moreover, senescent fibroblasts and other cell types suppressed their immune clearance by expressing the atypical MHC molecule HLA-E, which binds the NKG2A inhibitory receptor of NK and CD8<sup>+</sup> T cells (Pereira et al., 2019). Interestingly, HLA-E expression was higher in skin from aged donors, and systematically enriched in melanocytic nevi. This may partly explain the decades-long persistence of nevi (Green & Swerdlow, 1989).



Whereas the triggering of senescence is essential in suppressing tumorigenesis and should not be impeded, the abnormal accumulation of persisting senescent cells is detrimental (Figure 2.1). As a result of more cells becoming senescent and a decline in their immune clearance, senescent cells accumulate in tissues during aging (Dimri et al., 1995; Jeyapalan et al., 2007; Ovadya et al., 2018). Besides being a consequence of aging, this accumulation is also a driver of the process, and underlies various age-associated disorders and pathologies such as sarcopenia, atherosclerosis, and loss of adipose tissue (Childs et al., 2017). The causative implication of persisting senescent cells in the aging process was demonstrated in the INK-ATTAC transgenic mouse model, in which p16-expressing cells (thus probably most senescent cells) can be selectively removed. The lifelong, intermittent clearance of senescent cells in INK-ATTAC BubR1H/H progeroid mice delayed the onset of premature aging phenotypes, including decline in muscle function, cataracts, and loss of adipose tissue. Late-life clearance of senescent cells also rescued decline in muscle function and loss of adipose tissue (Baker et al., 2011). These findings were later strengthened with the elimination of senescent cells in naturally aging WT mice. The lifelong clearance of senescent cells extended both healthspan and median lifespan of INK-ATTAC WT mice, and delayed tumorigenesis, providing the demonstration for a causative role of cellular senescence in natural aging as well as a tumor-promoting role of established senescent cells secreting a SASP (Baker et al., 2016). Cellular senescence is thus a textbook case of antagonistic pleiotropy: it serves an essential role early in life by limiting transformation but later drives the organism's functional decline and aging through chronic inflammation and tissue degeneration.

Until recently, while it was known that the apparent number of senescent cells increased with age, the dynamics of this increase (a passive accumulation of senescent cells or an altered turnover) were not. Karin and colleagues demonstrated that the dynamics of senescent cell accumulation with age could be accurately described by a quantitative model in which the production of new senescent cells increased linearly with time, with senescent cells inhibiting their own removal (Karin et al., 2019). By comparing this model to longitudinal *in vivo* data of senescent cell abundance, they showed that the turnover of senescent cells greatly decreased with age, thus increasing senescent cell half-life and leading to an exponential increase in senescent cell abundance.



**Figure 2.1. Mechanisms of senescent cell persistence and accumulation.** Newly-senescent cells normally promote their own removal by recruiting the immune system via the SASP. However, senescent cell production increases with time and senescent cells eventually inhibit their own removal. Moreover, various endogenous and exogenous stresses may lead to an acute accumulation of senescent cells. This leads to deterioration of the tissue architecture, and senescent cells may promote malignant transformation via pro-tumorigenic components of the SASP. Senolytic compounds can be used to counteract senescent cell accumulation.

Persisting senescent cells are thought to accelerate aging primarily via secretion of the SASP. Chronic sterile inflammation, or inflammaging, is recognized as one of the pillars of aging that affect age-related diseases (Franceschi et al., 2018; B. K. Kennedy et al., 2014). The SASP may participate in inflammaging through its pro-inflammatory components. Matrix metalloproteases may degrade tissue architecture over time. Senomorphics are compounds that attenuate SASP expression, and could thus counteract the deleterious effects of accumulating senescent cells (Kim & Kim, 2019; L. Zhang et al., 2022). JAK inhibitors inhibited SASP expression of senescent preadipocytes, and importantly reduced systemic inflammation in aged mice, which improved their physical condition (M. Xu et al., 2015). mTOR is a master regulator of the SASP, and rapamycin was shown to function as a senomorphic and to inhibit SASP expression (Herranz et al., 2015; Laberge et al., 2015; R. Wang et al., 2017). Pro-longevity effects of rapamycin have been long known, and could be mediated partly by SASP inhibition, although it was shown to extend healthspan independently of inflammation modulation in NF- $\kappa$ B-enhanced mice (Correia-Melo et al., 2019). Likewise, comparing the effect of rapamycin on the lifespan of INK-ATTAC mice with and without senescent cell clearance may help retrieving the relative implications of SASP inhibition and modulation of aging-associated pathways in the mediation of its pro-longevity activity.

Beyond their lifelong exponential accumulation in tissues during aging, senescent cells may abnormally accumulate in other contexts, that fall into three main categories: in premalignant lesions as the result of OIS, at sites of chemotherapy or radiotherapy, and in pathologies involving senescence as a driver. The persistence of OIS cells within pre-malignant lesions, such as nevi or adenomas, was mentioned above (Collado et al., 2005; Michaloglou et al., 2005). In these contexts, eliminating senescent cells may or may not prevent malignant transformation depending on the pro- or anti-tumorigenic effects of the SASP, and the stage of neoplasia. There is however compelling evidence in favor of the one-two punch approach combining a senescence-inducing chemotherapeutic or radiotherapy with a senolytic drug, on the limitation of tumor recurrence (Fletcher-Sananikone et al., 2021; Guerrero et al., 2019; Triana-Martínez et al., 2019; C. Wang et al., 2019). Finally, persisting senescent cells were found to be implicated in numerous pathologies such as diabetes (Palmer et al., 2019; Sierra-Ramirez et al., 2020; Thompson et al., 2019), atherosclerosis (Childs et al., 2016, 2022; Roos et al., 2016), neurodegenerative disease (Bussian et al., 2018; Musi et al., 2018; Ogrodnik et al., 2021; P. Zhang et al., 2019), and fibrosis (Pan et al., 2017; Schafer et al., 2017; Wiley et al., 2019), and clearing senescent cells in these contexts was beneficial. There is therefore an immense interest to develop senolytic drugs that efficiently and selectively clear senescent cells in defined pathological contexts, with limited side effects. To this end, understanding how survival is differentially regulated in senescent and non-senescent cells, but also in different types of senescence, is key. These problematics are addressed below in Manuscript 1.

**2.2. Manuscript 1: From the divergence of senescent cell fates to mechanisms and selectivity of senolytic drugs.**

**Running title:** mechanisms and selectivity of senolytics

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## **Abstract**

Senescence is a cellular stress response that involves prolonged cell survival, a quasi-irreversible proliferative arrest and the expression of inflammatory factors. Senescent cells are resistant to apoptosis and if not eliminated by the immune system, can accumulate and lead to chronic inflammation and tissue dysfunction. Senolytics are recent drugs that selectively induce cell death in senescent cells but not in proliferative or quiescent cells, and they have proved a viable therapeutic approach in multiple preclinical models of pathologies in which senescence is implicated. As the catalog of senolytic compounds is expanding, novel survival strategies of senescent cells are uncovered, and variations in sensitivity to senolysis between different types of senescent cells emerge. We propose herein a mechanistic classification of senolytic drugs, based on the level at which they target senescent cells: directly disrupting BH3 protein networks, that are reorganized upon senescence induction; downregulating survival-associated pathways essential to senescent cells; or modulating homeostatic processes whose regulation is challenged in senescence. With this approach, we highlight the important diversity of senescent cells in terms of physiology and pathways of apoptosis suppression, and we describe possible avenues for the development of more selective senolytics.

## **Bullet points**

### *Facts*

- Senescent cells establish highly diverse strategies to suppress apoptosis, depending on the cell type and the senescence-inducing stressor
- Senolytic compounds target senescent cells at one of three levels: directly targeting apoptosis gatekeepers, modulating upstream survival pathways, or further dysregulating cellular homeostatic processes
- Indiscriminate clearance of senescent cells is undesirable, and calls for the development of senolytic drugs selective for specific types of senescent cells

### *Open questions*

- Can the combinatorial targeting of various senescence-associated anti-apoptotic pathways yield senolytic treatments with improved selectivity and efficacy?
- Can BH3 profiling accelerate the identification of context-specific BH3 mimetic senolytics?
- Can the regulation of selective autophagy networks be exploited for the development of novel precision senolytics?
- What are common regulators of multiple homeostatic processes in senescence, like MondoA?

Accumulating senescent cells are being found to drive a large number of pathologies. In the recent years, the therapeutic potential of senescent cell elimination has been demonstrated in pre-clinical models of diseases and disorders as diverse as pulmonary fibrosis (Pan et al., 2017; Schafer et al., 2017; Wiley et al., 2019), type 1 and 2 diabetes (Palmer et al., 2019; Sierra-Ramirez et al., 2020; Thompson et al., 2019), neurodegeneration (Bussian et al., 2018; Musi et al., 2018; Ogrodnik et al., 2021; P. Zhang et al., 2019), and atherosclerosis (Childs et al., 2016, 2022; Roos et al., 2016), among others. Pharmacological clearance of senescent cells is achieved by the means of drugs called senolytics, which exhibit a significant toxicity differential towards senescent cells, with no or limited effect on their proliferative or quiescent counterparts. Senolytics have been the object of intense research effort in the last five to ten years – the first demonstration of selective pharmacological elimination of senescent cells dating back to 2013 (Dörr et al., 2013), with the term senolytic being coined a few years later (Kirkland & Tchkonja, 2015; Zhu et al., 2015). The fast-moving senolytic research landscape considerably evolved in the last five years: many clinical trials are now under conduction, innovative pharmacological strategies are being explored, and the number of referenced senolytic drugs make up a dense catalog (Table 1); it is now time to take a step back and comprehensively review and conceptualize senolysis.

Cellular senescence is largely regarded as a cell fate in response to stress, characterized primarily by a highly stable proliferative arrest, and often accompanied by a complex secretome termed the senescence-associated secretory phenotype (SASP). Other non-obligatory though often encountered senescence hallmarks include senescence-associated  $\beta$ -galactosidase activity (SA- $\beta$ Gal) resulting from increased lysosomal content (Kurz et al., 2000), resistance to apoptosis, senescence-associated heterochromatin foci (SAHFs), and the expression of cyclin-dependent kinase inhibitors e.g. p16, p15, and p21 (Hernandez-Segura et al., 2018). The only universal feature shared by all types of senescent cells is permanent withdrawal from the cell cycle, though this is not exclusive to senescence as other stable forms of proliferative arrest exist such as terminal differentiation and replicative exhaustion. In this review, we propose a comprehensive conceptualization of senolysis in three possible routes, through the lens of inter-senescent cell type selectivity of senolytic drugs, emphasizing the plurality of senescence.

**Table 1. Notable senolytics: mechanisms and preclinical models.**

Senolytic compounds	Notable members	Class	Mechanism	Notable preclinical pathological models	References
<b>BH3 mimetics</b>	Navitoclax, ABT-737, venetoclax, A1331852, A1155463	1	BH1-4 anti-apoptotic factors inhibition	Diabetes, lung fibrosis, neurodegeneration, atherosclerosis, Covid-19, chemotherapy	(Aguayo-Mazzucato et al., 2019; Bussian et al., 2018; Childs et al., 2016, 2022; Fletcher-Sanankone et al., 2021; S. Lee et al., 2021; Pan et al., 2017; Shahbandi et al., 2020; Sierra-Ramirez et al., 2020; Thompson et al., 2019; Wiley et al., 2019)
<b>MDM2 and USP7 inhibitors</b>	P5091, UBX0101, nutlin-3a	2	p53 levels upregulation	Chemotherapy, osteoarthritis, macular degeneration	(Chae et al., 2021; Faust et al., 2020; He, Li, et al., 2020; Jeon et al., 2017)
<b>Dasatinib + quercetin</b>	-	2	Ephrin, AKT, PAI-2 inhibition	Lung fibrosis, neurodegeneration, diabetes, aging, Covid-19	(Camell et al., 2021; S. Lee et al., 2021; Musi et al., 2018; Ogrodnik et al., 2021; Palmer et al., 2019; Schafer et al., 2017; Sierra-Ramirez et al., 2020; M. Xu et al., 2018; P. Zhang et al., 2019)
<b>FOXO4/p53 disrupters</b>	FOXO4-DRI, ES2	2	p53 activity restoration	Chemotherapy, aging, lung fibrosis	(Baar et al., 2017; Le et al., 2021; Meng et al., 2021)
<b>HSP90 inhibitors</b>	Alvespimycin, ganetespib	2	AKT downregulation	Aging	(Fuhrmann-Stroissnigg et al., 2017)
<b>BET degraders and inhibitors</b>	ARV-825, JQ1	3	Autophagy (ferroptophagy) activation, ferroptosis, NHEJ inhibition	Obesity, chemotherapy	(Go et al., 2021; Wakita et al., 2020)
<b>Cardioglycosides</b>	Ouabain, digoxin, strophanthidin, bufalin	3	Autophagy inhibition, modulation of transmembrane potential and intracellular pH	Chemotherapy, lung fibrosis, pre-neoplastic lesions, aging	(Guerrero et al., 2019; L'Hôte et al., 2021; Triana-Martínez et al., 2019)
<b>Fibrates</b>	Fenofibrate	3	Autophagy activation	-	(Nogueira-Recalde et al., 2019)
<b>Autophagy blockers</b>	Chloroquine, bafilomycin A1	3	Autophagy inhibition	Chemotherapy	(Dörr et al., 2013; L'Hôte et al., 2021)
<b>Glutaminolysis inhibitors</b>	BPTES	3	Gutaminolysis inhibition, acidosis	Aging	(Johmura et al., 2021)
<b>Piperlongumine</b>	-	3	OXR1 inhibition, ROS production	-	(Y. Wang et al., 2016; X. Zhang et al., 2018)

Senolytics can be classified in three categories, as they target senescent cells at one of three levels. Class I senolytics target directly BCL-2 family proteins, which in senescence are rearranged in a network distinct from that of non-senescent cells, resulting in dependency on anti-apoptotic BCL-2 family members for survival; class II senolytics target upstream pathways that provide senescent cells with resistance to cell death, such as the USP7/MDM2/p53 axis, or AKT pro-survival signaling; and class III senolytics further disturb homeostatic processes that are already dysregulated in senescent cells, such as proteostasis maintenance or redox homeostasis.

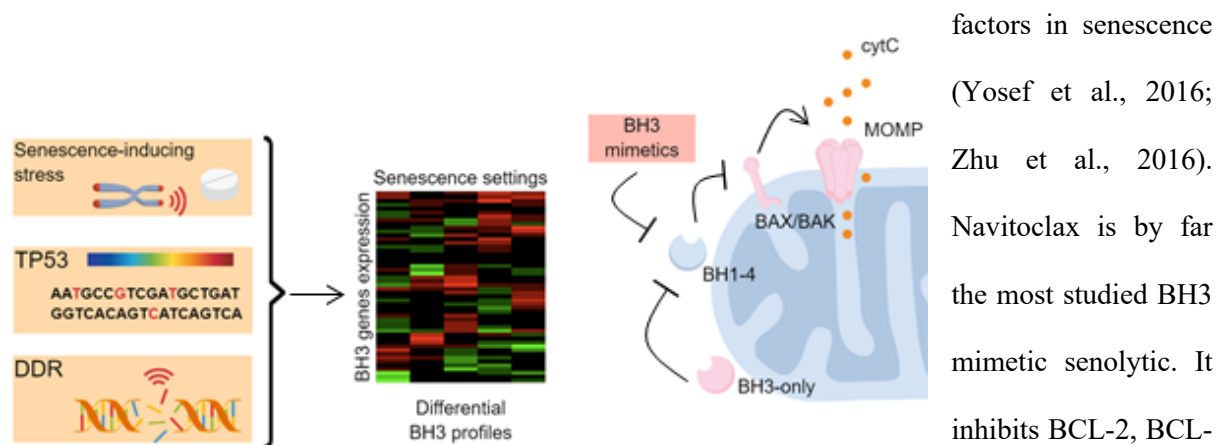


### **Class I senolytics: directly targeting apoptosis gatekeepers**

Commitment to apoptosis is directly controlled by the stoichiometry of BCL-2 family proteins, or BH3 proteins, that differentially interact with each other owing to their BCL-2 homology domains BH1 to 4 (Glab et al., 2017; Kale et al., 2018). Three distinct factions of BCL-2 family proteins can be distinguished, classified according to the BH domains they contain. Pro-apoptotic BH1-3 effectors BAX and BAK oligomerize to drive mitochondrial outer membrane permeabilization (MOMP), which is the irreversible tipping point leading to cell death orchestration. In unstressed conditions, MOMP is prevented by the sequestration of BH1-3 factors by anti-apoptotic BH1-4 guardians such as BCL-2, BCL-xL, or MCL-1. Finally, upstream pro-death or pro-survival signals translate into the modulation of the activity of BH3-only apoptotic inducers, such as NOXA, PUMA, or BIM. BH3-only proteins can either act as sensitizers that sequester pro-survival BH1-4 factors, or as activators by directly interacting with BAX/BAK and catalytically favoring their oligomerization (Delbridge et al., 2016; Lomonosova & Chinnadurai, 2009). Due to genetic variability in their BH3 sequences, individual members of each faction have their own profile of interactions with other BCL-2 family proteins in terms of affinity and selectivity, forming a complex but organized and finely tuned BH3 network (Timucin et al., 2019).

BCL-2 family proteins and notably BH1-4 anti-apoptotic guardians are essential in orienting cell fate towards senescence by suppressing apoptosis in response to stress. Commitment of triple-negative breast cancer cells to senescence rather than apoptosis in response to treatment with BET inhibitors was found to be largely determined by the upregulation of BCL-xL (Gayle et al., 2019). In different contexts of p53 activation, BCL-2 and MCL-1 were found to promote growth arrest over cell death (Kracikova et al., 2013; Rincheval et al., 2002). MCL-1 upregulation also promoted survival and senescence in IMR90 fibroblasts following aberrant mitosis caused by RASval12 expression (Dikovskaya et al., 2015). However, cell death avoidance in favor of senescence comes at the cost of the apoptotic priming of senescent cells, through the formation of stable complexes between BH1-4 proteins and pro-apoptotic BH3-only factors. The so-called one-two punch approach consists in suppressing tumors by first inducing senescence in cancer cells by the means of radiotherapy or chemotherapy, and then eliminating now-senescent cancer cells with senolytic compounds. The reorganization of the BH3 network inherited

from cell death avoidance singularly later provides senescent cells with sensitivity to so-called BH3 mimetics (Chong et al., 2020; Yosef et al., 2016; Zhu et al., 2016) (Figure 1). BH3 mimetics are small synthetic compounds that mimic the BH3 domain of pro-apoptotic BH3-only inducers. They bind to and inhibit anti-apoptotic BH1-4 guardians and prevent them from interacting with other BCL-2 family proteins, thus increasing the apparent stoichiometry of BH3-only proteins and shifting the equilibrium towards BAX/BAK oligomerization and apoptosis. The senolytic potential of BH3 mimetics was predicted from transcriptomic analyses revealing an increased expression of BH1-4 anti-apoptotic



**Figure 1. Class I senolytics target apoptosis-primed BH3 networks of senescent cells.** In response to stress, the cell fate decision to overgo senescence rather than apoptosis is accompanied by the reorganization of BH3 networks and the apoptotic priming of senescent cells. BH3 profiles evolve dynamically during senescence onset, and are dictated by the nature of the senescence-inducing stressor and its magnitude, expression levels and mutational status of TP53, as well as the establishment or lack thereof of a DNA damage response. The reorganization of BH3 networks in senescent cells renders them sensitive to the action of BH3 mimetics, that bind anti-apoptotic BH1-4 proteins to increase the apparent stoichiometry of pro-apoptotic BH3-only proteins and promote BAX/BAK oligomerization and mitochondrial outer membrane permeabilization.

factors in senescence (Yosef et al., 2016; Zhu et al., 2016). Navitoclax is by far the most studied BH3 mimetic senolytic. It inhibits BCL-2, BCL-xL, and BCL-w. It is considered a broad-spectrum senolytic, as it is efficient against a large panel of

senescent cells (Chang et al., 2016). In several models of DNA damage-induced senescent cancer cells selectively killed by navitoclax, the sole inhibition of BCL-xL was sufficient to induce senolysis. Hence, inhibition of BCL-xL but not BCL-2 was required for navitoclax-mediated senolysis of breast and lung cancer cells induced in senescence by genotoxic agents etoposide and doxorubicin (Saleh et al., 2020), and irradiation-induced senescent meningioma cells (Yamamoto & Kitanaka, 2021). Interestingly, prostate cancer cells were killed by navitoclax or BCL-xL specific inhibitors if induced in senescence by irradiation or genotoxic agents, but not if the proliferative arrest was triggered by antiandrogen enzalutamide, which does not damage DNA (Malaquin et al., 2020); the proliferative arrest induced by enzalutamide was however reversible upon withdrawal of the drug, calling into question the senescent

state of these cells. Other reports of navitoclax-resistant cancer cells induced in senescence by non-genotoxic chemotherapeutic agents such as alisertib or palbociclib (Guerrero et al., 2019) suggest that the DNA damage response may be important in reorganizing BCL-2 family factors into a navitoclax-sensitive BH3 network in senescent cancer cells. It is known that the DNA damage response differentially regulates the expression of BCL-2 family members (Zhan et al., 1997). TP53 mutational status may also affect the sensitivity of DNA damage-induced senescent cancer cells to BH3 mimetics, as the regulation of the expression of some BCL-2 family genes was found to be p53-dependent in response to genotoxicity (Miyashita et al., 1994; Widden & Placzek, 2021). Navitoclax resistance in wild-type TP53 therapy-induced senescent breast cancer cells expressing low levels of NOXA was overcome by dual treatment with a specific MCL-1 inhibitor (Shahbandi et al., 2020). Interestingly in this study, sensitivity to BCL-xL or BCL-xL/MCL-1 inhibition depended primarily on the cell line and was largely conserved for various senescence-inducing insults. This is consistent with recent work demonstrating that in senescent cancer cells, gene expression dynamics, SASP composition and sensitivity to BH3 mimetics correlated more with the cell type than with the nature of the senescence-inducing stressor (Jochems et al., 2021).

There are conflicting results on the ability of navitoclax to target etoposide-induced senescent primary lung fibroblasts. IMR90 cells exposed for 48 h to 20  $\mu\text{M}$  etoposide to induce senescence, were killed by 2.5  $\mu\text{M}$  navitoclax when added for 48 h after a 2-day etoposide withdrawal (Nogueira-Recalde et al., 2019), whereas 10  $\mu\text{M}$  navitoclax did not affect cell viability if added for 48 h after a longer 6-day etoposide withdrawal (Schafer et al., 2017). This may be underlain by a dynamic evolution of BCL-2 protein levels during senescence onset, from a navitoclax-sensitive to insensitive BH3 network. Furthermore, if IMR90 cells were induced in senescence with a higher concentration of 50  $\mu\text{M}$  etoposide for 48 h followed by a 5-day withdrawal, a 72-h exposure to as little as 1  $\mu\text{M}$  navitoclax was senolytic (Guerrero et al., 2019). This in turn suggests that beyond the cell type and the nature of the senescence-inducing insult, the magnitude of the stress may also influence the resulting evolution of BH3 profiles that underlies sensitivity to BH3 mimetics, possibly through the DNA damage response activation level. Similarly, while early senescent glioblastoma cells were killed by selective BCL-2 inhibition – although

no proliferating control cells were included in the assay (Schwarzenbach et al., 2021), late senescent glioblastoma cells were insensitive to BCL-2 inhibition and depended solely on BCL-xL for their survival (Rahman et al., 2022). In IMR90 cells, when compared to levels in proliferation, BCL-2, BCL-xL and BCL-w proteins all displayed a marked increase in etoposide and replicative senescence, whereas this increase was more important for BCL-2 than for BCL-xL and BCL-w in RASval12 senescence (Yosef et al., 2016). This may explain the efficiency of the specific BCL-2 inhibitor venetoclax in RASval12-induced senescent IMR90 only, while it appeared necessary to inhibit all BCL-2, BCL-xL and BCL-w with navitoclax or the related compound ABT-737 to kill etoposide- and replicative-senescent IMR90.

Neither navitoclax nor BCL-xL-specific inhibitors A1331852 and A1155463 are senolytic towards irradiation-senescent preadipocytes (Zhu et al., 2016, 2017). Upon senescence induction, whereas navitoclax- and A1331852/A1155463-sensitive human umbilical vein endothelial cells (HUVECs) and IMR90 fibroblasts showed a marked increase in BCL-xL and BCL-2 protein levels, preadipocytes exhibited steadier BCL-xL and even decreased BCL-2 protein levels, in contrast to significantly increased BCL-w protein levels (Zhu et al., 2016). Senescent preadipocytes may then rely preferentially on BCL-w for their survival. Although none is available at the moment (Hartman & Czyz, 2020), if selective BCL-w inhibitors were developed in the future, their senolytic potential in preadipocytes should be assessed. Preadipocytes may be among the most abundant senescent cell types in old age and mediate age-related metabolic disorders, making them targets of interest (Tchkonina et al., 2010).

In several studies, the sensitivity of senescent cells to different BH3 mimetics correlated well with the expression or protein levels of the various anti-apoptotic BCL-2 family factors; however, the levels of pro-apoptotic effectors and initiators were rarely assessed. This is unfortunate, because the potency of a given BH3 mimetic as a senolytic does not depend solely on the protein levels of its BH1-4 targets, but rather on the balance between these and their pro-apoptotic partners (Edlich, 2018). In a panel of soft-tissue sarcoma cell lines induced to senesce by irradiation, BCL-2 or BCL-xL were differentially induced depending on the cell line, but the cells were all comparably sensitive to senolysis by venetoclax or navitoclax, irrespectively of BCL-2 and BCL-xL expression levels (Lafontaine et al., 2021).

Similarly, while irradiation-induced senescent WI-38 fibroblasts upregulated BCL-xL and BAK but not BCL-2, they were only sensitive to dual BCL-xL/BCL-2 inhibition but not to that of either factor alone (Chang et al., 2016). Therefore, a more exhaustive characterization of BH3 networks in senescence models could guide the choice for better class I senolytics, for example through the BH3 profiling method (Del Gaizo Moore & Letai, 2013; Montero et al., 2015; Montero & Letai, 2018; Potter et al., 2021), which could significantly improve selectivity prediction of BH3 mimetics as senolytics.

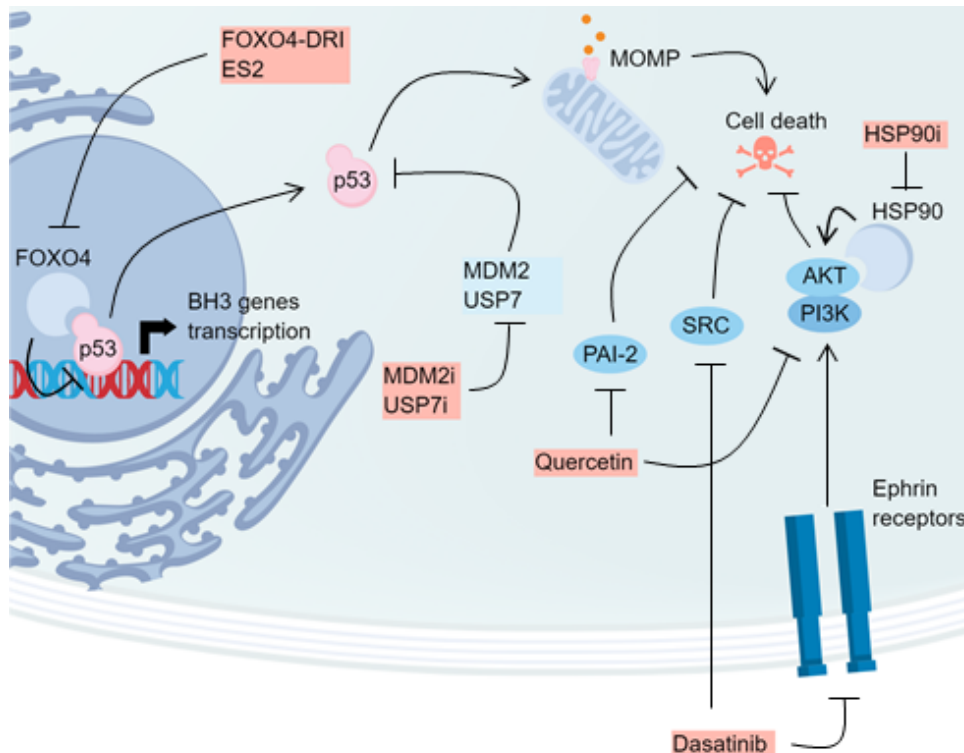
Both navitoclax and ABT-737 proved efficient senolytics in multiple preclinical models. Nevertheless, their translation into clinic as senolytics is impaired by their reported toxicity towards platelets and neutrophils due to the targeting of BCL-xL and BCL-2, respectively, leading to thrombocytopenia and neutropenia (Oltersdorf et al., 2005; Wilson et al., 2010). Given the high potential of BH3 mimetics as senolytics, various strategies are being designed to overcome this issue and improve their in vivo tolerability and therapeutic window, including conjugation of navitoclax to galactose for its specific release in senescent cells due to senescence-associated  $\beta$ -galactosidase activity (González-Gualda et al., 2020), synthesis of BCL-xL proteolysis-targeting chimera (PROTACs) taking advantage of low E3 ligase expression in platelets (He, Zhang, et al., 2020), and galactose-functionalized nanoparticle encapsulation (Galiana et al., 2020). In the future, IAP-based PROTACs could also hold promise as senolytics (Negi & Voisin-Chiret, 2022), especially in the light of recent work reporting on the overexpression of some IAP members in senescence (Schwarzenbach et al., 2021).

### **Class II senolytics: modulating upstream pro-survival pathways**

The SASP secreted by senescent cells comprises apoptosis-promoting factors as well as pro-inflammatory factors and proteases, together resulting in a harsh microenvironment (Coppé et al., 2010; Wajapeyee et al., 2008; Zhu et al., 2015). Independently of the reorganized BH3 networks, senescent cells also resist cell death through the upregulation of upstream pro-survival signaling pathways that can be targeted for senolysis (Figure 2).

Classically, p53 is transiently upregulated in response to stress to trigger senescence-associated cell cycle exit through the transcription of p21, and then returns to lower cellular levels to participate in the

regulation of other aspects of senescence such as SASP expression (Herranz et al., 2018; Johmura et al., 2016; Sheekey & Narita, 2021). When expressed at high levels, p53 can promote apoptosis by both upregulating the transcription of BH3-only genes and interacting with BCL-2 family proteins in the cytosol and at the mitochondrial outer membrane (Lomonosova & Chinnadurai, 2009). In cells with established senescence, p53 levels are maintained low through ubiquitination and its pro-apoptotic activity is repressed through nuclear segregation. Thus, restoration of p53 activity by either nuclear



**Figure 2. Class II senolytics inhibit survival pathways elicited by senescent cells.** Senescent cells promote survival through the downregulation of p53 activity via its nuclear segregation by FOXO4. Senolytic peptides FOXO4-DRI and ES2 suppress the FOXO4-p53 interaction, promote p53 nuclear exclusion and the subsequent triggering of apoptosis. Besides, p53 levels are actively kept low in senescent cells through MDM2 and USP7, the inhibition of which also results in apoptosis. The dasatinib and quercetin senolytic cocktail targets multiple survival-associated pathways including ephrins, PAI-2, SRC, and AKT signaling. In senescent cells, AKT signaling is sustained through the stabilization of phosphorylated AKT by HSP90 chaperones. HSP90 inhibitors downregulate AKT and induce senolysis.

(Bourgeois & Madl, 2018; Dankort et al., 2009; De Keizer et al., 2010). This interaction both prevents p53 from inducing the transcription of pro-apoptotic target genes, and restricts p53 localization to the nucleus so that it cannot interact with BCL-2 family proteins in the cytosol and at the mitochondrial outer membrane (Baar et al., 2017; Le et al., 2021). The senolytic peptide FOXO4-DRI, by binding p53 and relieving the p53-FOXO4 interaction, abrogated the nuclear sequestration of p53, that consequently

exclusion or suppression of its ubiquitination can lead senescent cells to apoptosis.

Transcription factor FOXO4 is upregulated in senescence to participate in proliferative arrest maintenance, and it physically interacts with p53 in the nucleus

migrated towards the cytosol and the mitochondria, where it triggered apoptosis (Baar et al., 2017). A computationally designed peptide, ES2, binds FOXO4 rather than p53, and like FOXO4-DRI, induced the nuclear exclusion of p53 and selectively elicited the death of senescent cells in vitro and in vivo (Le et al., 2021). Remarkably, the occurrence of the p53-FOXO4 nuclear interaction and the senolytic potential of its disruption was recapitulated in many different senescent models (Baar et al., 2017; Huang et al., 2021; Le et al., 2021; C. Zhang et al., 2020). The development of small synthetic compounds disrupting the p53-FOXO4 interaction is thus of interest to exploit this senolysis mechanism in a therapeutic context, as small molecule compounds bear more favorable pharmacokinetics properties than peptides (Lau & Dunn, 2018).

p53 protein levels are largely controlled and kept low by proteasomal degradation due to ubiquitination by the MDM2 E3 ligase. The stability of MDM2 is itself enhanced through its deubiquitination by USP7. Therefore, both USP7 and MDM2 negatively regulate the stability of p53 (S. K. Kwon et al., 2017). As predicted, USP7 inhibitors restored p53 activity in senescent cells, and selectively triggered senescent cell death (He, Li, et al., 2020). Surprisingly, USP7 inhibitors increased p53 levels in senescent cells only but not in proliferative cells, which suggests a higher dependency of senescent cells on the USP7/MDM2 axis for the regulation of p53 activity compared to their proliferative counterparts. Downstream of USP7, directly inhibiting MDM2 was also senolytic, but USP7 inhibitors reportedly exhibit fewer side effects than MDM2 inhibitors in mice (He, Li, et al., 2020; Jeon et al., 2017). Proprietary MDM2 inhibitor UBX0101 failed phase II clinical trial as a senolytic in patients with osteoarthritis (Dolgin, 2020). Interestingly, perturbation of the BH3 network and restoration of p53 activity appear to synergize to trigger senolysis, as a combination of navitoclax with USP7 or MDM2 inhibitors was more potent than either treatment alone (Faust et al., 2020; He, Li, et al., 2020). Recent work in cancer cells showed that apoptotic priming of BH3 networks favored cell death in response to p53 activity restoration (Sánchez-Rivera et al., 2021).

Moving away from p53, AKT signaling is essential to the survival of many senescent cells. AKT is a pleiotropic serine/threonine protein kinase that reportedly regulates over 100 downstream substrates, playing a central role in a complex network of signaling pathways comprising multiple positive and

negative feedback loops. AKT promotes survival notably through the inhibition of BH3-only protein BAD, the downregulation of p53 via MDM2, and the upregulation of anti-apoptotic BH1-4 protein MCL-1 (Manning & Toker, 2017; Osaki et al., 2004). Transcriptome analysis of irradiation-induced senescent preadipocytes highlighted the upregulation of various survival-associated pathways including ephrin-B-dependent suppression of apoptosis and the PI3K/AKT pathway, that can be targeted by dasatinib and quercetin, respectively (Zhu et al., 2015). Dasatinib is a broad-spectrum tyrosine kinase inhibitor that notably targets, besides SRC, various ephrin receptors that promote survival via stimulation of AKT signaling (Karaman et al., 2008; J. Li et al., 2010). Dasatinib as a senolytic is widely used in combination with quercetin, which targets notably PI3K and PAI-2. PI3K is directly activated by ephrin receptors, so quercetin can further participate in the dasatinib-induced downregulation of AKT signaling. On the other hand, pro-survival effects of PAI-2 are known but poorly understood. PAI-2 inhibition can result in the destabilization of p21, leading to apoptosis (Hsieh et al., 2017). Besides, due to Rb destabilization, PAI-2 inhibition results in the transcriptional activation of E2F-regulated pro-apoptotic genes (Tonnetti et al., 2008). While the senolytic activity of dasatinib was assigned to ephrin receptor inhibition, recent work demonstrated that SRC was an essential factor favoring survival and senescence over apoptosis in response to genotoxicity, through the downregulation of p53 (Anerillas et al., 2022). It is therefore plausible that dasatinib triggers senolysis in part through inhibition of survival-associated SRC signaling.

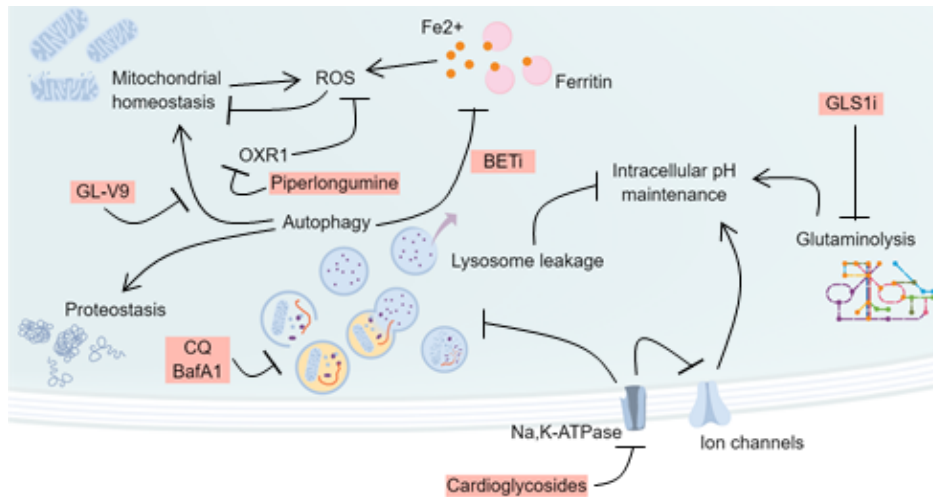
Senolysis by HSP90 inhibitors is thought to be mediated in part by the downregulation of AKT signaling. HSP90 chaperone proteins increased the stability of active, phosphorylated AKT, reinforcing pro-survival signaling in senescent cells. Inhibiting HSP90 chaperones led to the destabilization and degradation of active AKT and other client proteins. In oxidative stress-induced senescent MEFs, HSP90 inhibitors were senolytic, whereas specific AKT inhibitors were not. However, quercetin which targets other pathways in addition to PI3K/AKT, was senolytic in this model (Fuhrmann-Stroissnigg et al., 2017), suggesting that the essentiality of HSP90 proteins in senescence is imputable to the stabilization of not only AKT but other pro-survival factors.



Overall, many types of senescent cells rely on upregulated AKT signaling for apoptosis suppression, and upstream targeting of this pathway at the levels of ephrin receptors, PI3K, or HSP90 chaperones, proved efficient approaches for the selective clearance of senescent cells. Nevertheless, downregulating AKT is not always sufficient to induce senolysis. Dasatinib was senolytic in preadipocytes but not in HUVECs, which was consistent with the fact that the former but not the latter was sensitive to siRNA-mediated knockdown of ephrin genes. Conversely, quercetin which targets PI3K and PAI-2 was senolytic in HUVECs but not in preadipocytes (Zhu et al., 2015). The related flavonoid fisetin exhibited the same senolytic selectivity (Zhu et al., 2017). This suggests that while senescent preadipocytes rely primarily on ephrin signaling for survival, the inhibition of PAI-2 anti-apoptotic mechanisms is required to induce apoptosis of senescent HUVECs, in which targeting the PI3K/AKT axis is not sufficient to trigger senolysis. HSP90 inhibitors that downregulate AKT were not senolytic either in irradiation-induced senescent preadipocytes (Fuhrmann-Stroissnigg et al., 2017). This could indicate that the senolytic effect of dasatinib in these cells was mediated by SRC inhibition rather than the suppression of PI3K/AKT signaling, on which senescent preadipocytes do not seem to rely for survival. Whereas AKT activity is upregulated in many senescence models, it was shown to be reduced to levels even below those of proliferative cells in some forms of oncogene-induced senescence (OIS) (S. Courtois-Cox et al., 2008). Accordingly, HSP90 inhibitors and dasatinib were not senolytic in BRAF-V600E-induced senescent fibroblasts (L'Hôte et al., 2021). Downstream of AKT, inhibiting mTOR was senolytic in liver cancer cells induced in senescence by CDC7 inhibition, but not in OIS (C. Wang et al., 2019). Although lack of PI3K/AKT hyperactivity could explain the resistance of certain senescent cells to senolytics directly or indirectly targeting this pathway, more complex regulation and crosstalk are certainly at play.

### Class III senolytics: further disturbing cellular homeostatic processes

The last level on which senescent cells can be targeted for elimination, is through further disturbance of cellular homeostatic processes that are already dysregulated in senescence, often in a systemic manner, such as proteostasis, or mitochondrial and redox homeostasis (Figure 3).



**Figure 3. Class III senolytics disrupt homeostatic processes already challenged in senescent cells.** Multiple facets of homeostasis are interconnected in senescence and regulated differently than in proliferative cells. Autophagy is essential for proteostasis maintenance as well as mitochondrial homeostasis through mitophagy, which is inhibited by GL-V9. Mitochondrial dysfunction in senescent cells leads to the formation of ROS. Redox stress responses may be orchestrated by OXR1, which is inhibited by piperlongumine. The inhibition of ferritinophagy by BET inhibitors leads to ferroptosis and ROS formation. Intracellular pH of senescent cells is reportedly acidified by lysosome leakage, which is compensated for by ammonia production through glutaminolysis. Inhibitors of glutaminase 1 induce acidosis and are thus senolytic. Cardioliposides, by binding to the Na,K-ATPase, both alter membrane potential and intracellular pH regulation of senescent cells, as well as inhibit autophagy through signal

Proteostasis results from a tightly regulated balance between protein synthesis, folding, and degradation, that are coordinated to ensure a functional proteome and proper cell function. Mitochondrial homeostasis, redox homeostasis and

global proteostasis all influence each other. Proteostasis decline and mitochondrial dysfunction are hallmarks of aging and cellular senescence. Failure to restore proteostasis in the face of proteotoxic insults triggers senescence, as shown in keratinocytes in response to advanced glycation end products formation (Halkoum et al., 2022), or in post-mitotic neurons facing increasing proteotoxicity during long-term culture (Ishikawa & Ishikawa, 2020). Replicative-senescent fibroblasts experience a global decline in proteostasis, with dysregulated alternative splicing, and altered responses to proteotoxic stress including disruptions in the heat shock response and the unfolded protein response (UPR) (Sabath et al., 2020). Aged mice exhibit mild chronic endoplasmic reticulum (ER) stress and UPR activation, and trigger an exaggerated sustained response lacking fine tuning when subjected to further ER stress (Ward

et al., 2022). Senescent cells with ample SASP synthesis may mitigate proteotoxicity and the burden on their secretion systems by chronically upregulating the ubiquitin/proteasomal axis and the autophagy/lysosomal axis, and differentially activating UPR branches (Abbadie & Pluquet, 2020; Dörr et al., 2013). Meanwhile, improved protein translation fidelity results in extended lifespan in several model organisms (Martinez-Miguel et al., 2021). Different facets of cellular homeostasis share common regulators in aging. Transcription factor MondoA was found to delay replicative and DNA damage-induced senescence through the downregulation of autophagy suppressor Rubicon as well as maintenance of mitochondrial redox homeostasis through Prdx3 expression (Yamamoto-Imoto et al., 2022).

The facet of proteostasis that is the most targeted so far by known senolytics is autophagy. Autophagy is an adaptive cellular process in response to stress or energy deprivation, through which organelles and proteins are degraded (specifically as in selective autophagy, or non-specifically as in bulk autophagy) and broken down to small metabolic substrates that are recycled to maintain essential biosynthesis activities. Autophagy is first and foremost a survival process, but unimpeded autophagy can lead to apoptosis, necrosis, or even autophagic cell death in which the cell overwhelmed by the presence of autolysosomes is ‘eating itself to death’ (Bialik et al., 2018). Relationships between autophagy and senescence are seemingly paradoxical and yet to be fully understood. Basal autophagy in proliferative cells is considered anti-senescence because it decreases the cellular burden of potential senescence-inducing stressors, therefore acting as a preferred primary stress response; in this regard, inhibiting autophagy induces senescence in glioblastoma and bronchial epithelial cells (Fujii et al., 2012; Gammoh et al., 2016). Downregulating autophagy master regulator ULK1 induces senescence in a wide range of cancer cells and sensitizes them to navitoclax-induced apoptosis (Schepers et al., 2021). However, in some contexts of high-intensity cellular stress, autophagy rather suppresses apoptosis and promotes senescence induction (Gewirtz, 2013). Autophagy is especially important during OIS onset: inhibiting autophagy delays entry into senescence, and even allows the full bypass of BRAF senescence in melanocytes (Liu et al., 2013). Autophagy is often upregulated in senescent cells and is suspected to provide, through the recycling of cellular constituents, the small metabolites required for the massive

synthesis of SASP components, as well as to clear macromolecules damaged from increased metabolic fluxes and ROS production (Y. Kwon et al., 2017). Therefore, in low to mild stress conditions, autophagy suppresses senescence by mediating the return to homeostasis and proliferation, whereas in the face of higher intensity stress, autophagy favors senescence by suppressing apoptosis. Beyond bulk autophagy, the selective degradation of specific factors through selective autophagy via various ATG8 family receptors was shown essential in homeostasis maintenance of both replicative and DNA damage-induced senescence (Y. Lee et al., 2021). Selective autophagy of KEAP1, TNIP1, and NDP52, regulated redox homeostasis, SASP expression, and proteostasis, respectively, in senescent cells. Further exploration of selective autophagy networks in various types of senescent cells may foster the development of new-generation precision senolytics targeting autophagy, with improved proliferative versus senescent and inter-senescent selectivity.

Most senolytics that affect autophagy are inducers of the process. HSP90 inhibitors were identified in the rational screening of a small chemical library of compounds targeting autophagy, of which all senolytic hits were autophagy inducers (Fuhrmann-Stroissnigg et al., 2017). BET inhibitors are another group of senolytic drugs that exert their action through the upregulation of autophagy, together with the attenuation of non-homologous end-joining repair. They proved efficient *in vivo* in mice models of obesity and chemotherapy-induced senescence (Wakita et al., 2020). Their senolytic action was later assigned to the selective autophagy of ferritin (ferritinophagy) and the subsequent triggering of non-apoptotic, iron-dependent cell death ferroptosis, at least in therapy-induced senescent fibroblasts (Go et al., 2021). Indeed, replicative and irradiation-induced senescent fibroblasts and epithelial cells accumulate important amounts of intracellular iron, through the combined upregulation of iron-storage protein ferritin expression and inhibition of ferritinophagy (Masaldan et al., 2018). PPAR $\alpha$  agonist fenofibrate was senolytic through autophagy upregulation in IL-6-induced senescent chondrocytes (Nogueira-Recalde et al., 2019), but in TNF $\alpha$ -induced senescent synovial fibroblasts, it attenuated the senescent phenotype and inhibited SASP expression without exhibiting a differential toxicity towards senescent cells, a behavior corresponding to so-called senomorphic compounds (Del Rey et al., 2019).

Another senolytic strategy consists in depriving senescent cells from cytoprotective autophagy. In the first demonstration of selective elimination of senescent cells through pharmacological means, therapy-induced senescent lymphoma cells were shown to upregulate autophagy to cope with ER stress resulting from SASP production. The blockade of autophagy flux with bafilomycin A1 led to senolysis (Dörr et al., 2013). Autophagy flux also increased in BRAF-V600E oncogene-induced senescent fibroblasts, and inhibiting autophagy with chloroquine, bafilomycin A1 or low concentrations of cardioglycosides inducing Na,K-ATPase signal transduction and notably AKT, resulted in the specific senolysis of BRAF-senescent cells (L'Hôte et al., 2021). The essentiality of autophagy in some OIS models may correlate with the downregulation of basal AKT signaling in these cells. Whereas many senescent cells have upregulated AKT signaling and are sensitive to autophagy-inducing senolytics, in some instances OIS cells rather downregulate AKT signaling and increase basal autophagy, as concomitant oncogene and AKT activation favors senescence bypass and transformation (S. Courtois-Cox et al., 2008; Stéphanie Courtois-Cox et al., 2006; Kennedy et al., 2011).

Besides proteostasis and autophagy, other cellular homeostatic processes differentially regulated in senescence can be targeted for senolysis. Aforementioned cardioglycosides are broad-spectrum senolytics that target a myriad of senescent cell types. They proved efficient *in vivo* in models of oncogene-induced senescence, chemotherapy-induced senescence, natural aging, and irradiated mice (Guerrero et al., 2019). They bind the transmembrane Na,K-ATPase pump, which is involved in both membrane potential maintenance and signal transduction. While low doses of cardioglycosides induced the selective senolysis of BRAF-V600E-induced senescent fibroblasts through signal transduction and autophagy inhibition (L'Hôte 2021), their action in other senescence models was rather attributed to disturbance of membrane potential and the modification of intracellular pH (Guerrero et al., 2019; Triana-Martínez et al., 2019). Glutaminolysis inhibition was another senolytic strategy relying on differences in intracellular pH regulation between senescent and normal cells. Senescent cells were found to undergo intracellular acidosis because of lysosomal leakage and to rely on increased glutaminolysis-produced ammonia to neutralize their intracellular pH. Inhibiting glutaminase 1 consequently led to the selective clearance of senescent cells (Johmura et al., 2021).

Redox and mitochondrial homeostasis are intimately linked in senescence. Mitochondrial mass increases in senescence, but this is accompanied by mitochondrial dysfunction and the production of ROS, generating chronic oxidative stress (Moiseeva et al., 2009; Wu et al., 2017). ER-mitochondria contact sites appear critical in senescence regulation (Ziegler et al., 2021), and mitochondrial dysfunction and ROS production were shown to be mediated in many senescence models by increased IP3R-mediated mitochondrial calcium uptake from the ER following ITPR downregulation (Farfariello et al., 2022). Oxidative stress induces senescence in many settings (Ngoi et al., 2021; Zhong et al., 2019), but the upregulation of ROS detoxifying systems is also critical for the decision to undergo senescence over apoptosis (Wu et al., 2017). Consequently, targeting these detoxifying systems in established senescent cells can yield senolysis. The alkalization of lysosomes by GL-V9 was shown to impair mitophagy and further increase dysfunctional mitochondrial mass in senescent cells, leading to ROS overproduction and apoptosis (Yang et al., 2021). Piperlongumine is another senolytic causing redox imbalance in senescent cells through the targeting of oxidative stress sensor OXR1 (Y. Wang et al., 2016; X. Zhang et al., 2018). Procyanidin C1 was senomorphic at lower concentrations and senolytic at higher concentrations, and acted by further promoting ROS formation in senescent cells, leading to mitochondrial dysfunction (Q. Xu et al., 2021).

Still, how these various homeostatic processes are affected depending on senescent cell type largely remains to be explored. Mapping their interconnections, for example through the identification of common regulators like MondoA affecting both autophagy/proteostasis and mitochondrial function/redox homeostasis (Yamamoto-Imoto et al., 2022), shall yield novel avenues for selective senolysis.

## **Conclusions and perspectives**

Throughout this review, we proposed a conceptualization of senolysis based on mechanisms of action. This approach highlights the compelling diversity of senescent phenotypes in terms of apoptotic priming, cell death-suppressing strategies, and homeostatic regulation. The grouping of these seemingly heterogeneous cellular phenotypes under the one label of senescence calls into the question the relevance

of regarding all stress-induced quasi-irreversible proliferative arrests as senescence. The only universal, though not exclusive, feature of senescent cells is an irreversible or highly stable proliferative arrest; but even this is being challenged, as various stressors can induce so-called senescent-like states of reversible proliferative arrest that exhibit classical senescence hallmarks such as senescence-associated  $\beta$ -galactosidase activity, SASP expression and even sensitivity to senolytics (Fleury et al., 2019; Malaquin et al., 2020). We argue that challenging the paradigm conceptualizing senescence as one – heterogeneous – biological process would be beneficial as it would stimulate context-based senolytic drug development, yielding highly potent candidates more selective towards a subset of senescent cells. This is especially relevant considering the increasing awareness of the fact that indiscriminate, systemic removal of senescent cells may be harmful, as some senescent cell subpopulations appear to be beneficial (Grosse et al., 2020; Lujambio, 2016; Martin et al., 2021). Inter-senescent cell selectivity of senolytic compounds, rather than being a limitation of senolytic therapy, thus actually constitutes an opportunity for precision clearance of specific subsets of senescent cells in defined pathological contexts.

Innovative strategies like galacto-conjugation (Cai et al., 2020), or encapsulation in galactose-functionalized silica beads (Muñoz-Espín et al., 2018) can be used to bypass limitations caused by adverse side-effects of some drugs, such as BH3 mimetics. These strategies can also turn toxic compounds that do not discriminate between senescent and non-senescent cells into bona fide senolytics, through their release in senescent cells only. Nevertheless, bystanding non-senescent cells expressing high levels of  $\beta$ -galactosidase would also in principle be sensitive to their effects.

Mechanistic insights are lacking for some promising senolytic candidates that could lead to the identification of senolytic targets and the development of even more potent compounds. Curcumin analog EF24 is a senolytic in several models and induces the proteasomal degradation of BH1-4 anti-apoptotic factors via an unknown route (W. Li et al., 2019). It is however unclear whether curcumin, which itself is a controverted lead (Heger, 2017; Nelson et al., 2017), and the related compound o-vanillin, are bona fide senolytics or rather function as senomorphics (Cherif et al., 2019, 2020). Similar uncertainties exist about fisetin (Yousefzadeh et al., 2018; Zhu et al., 2017). This highlights the need,

especially *in vivo*, for unequivocally characterizing senolysis over senomorphism, which can be undesirable if it allows the reentrance of pre-neoplastic cells into the cell cycle. *In vivo* characterization of senolysis is now facilitated by the detection of a senescence-specific oxylipin released upon lysis of senescent cells (Wiley et al., 2021). It also remains a prime consideration to investigate and understand in details the molecular mechanisms underlying senolysis by novel compounds, notably by identifying with certainty the cellular target through which the compound exerts its activity and the signaling pathways leading to cell death, which has sometimes been neglected. Systematically characterizing the mechanisms of action of senolytics would yield opportunities to either develop optimized compounds for the identified target, or to discover even more interesting targets for senolysis within the same pathway.



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**Conflict of Interest Statement**

The authors declare no conflict of interest.

**Author Contribution Statement**

V.L., J.Y.T., and C.M. reviewed and analyzed the literature. V.L. and J.Y.T. conceptualized the article. V.L. wrote the manuscript and designed the figures. V.L., C.M., and J.Y.T. reviewed, edited, and validated the manuscript.

**Data Availability Statement**

This article does not include any original or unpublished research or data.

### **2.3. Current caveats and challenges in senolysis assessment, and distinction from senomorphism.**

Senolytics and senomorphics are grouped under the more general term of senotherapeutics. There is some confusion in the literature where senomorphic drugs or other compounds that for example delay senescence onset, such as rapamycin and glucocorticoids, are cited as senolytics (Cherif et al., 2019; Grezella et al., 2018). Senotherapeutic may be used as a blanket term for compounds that modulate senescence but for which exclusive senolytic or senomorphic activity is not evident (Nogueira-Recalde et al., 2019; Q. Xu et al., 2021; Yousefzadeh et al., 2018). Some newly described senotherapeutics such as olive polyphenols are labeled as senolytic in titles of papers, but are actually senomorphic with regards to the data presented (Varela-Eirín et al., 2020).

Properly assessing senolysis is challenging both in cell models and in vivo, and the need for careful experimental design and data interpretation has been stressed by others (Niedernhofer & Robbins, 2018). Studies aiming at demonstrating the senolytic activity of candidate drugs in senescent cell models sometimes lacked non-senescent control cells which are required for proving that senescent cell elimination is selective, a sine qua non for labeling a drug senolytic. This was the case for panobinostat (Samaraweera et al., 2017). A decrease in senescence markers such as p16 or SASP mRNAs in a mixed population of senescent and non-senescent cells following drug treatment (Varela-Eirín et al., 2020; Yousefzadeh et al., 2018) does not suffice to demonstrate senolysis, as such a reduction in senescence markers may indifferently result from senolysis or senomorphism. Further complications arise from the fact that some compounds appear to have both senomorphic and senolytic activities, such as curcumin and o-vanillin (Cherif et al., 2019, 2020) and gingerone A (Moaddel et al., 2022), sometimes in a concentration-dependent fashion like for procyanidin C1 (Q. Xu et al., 2021), whereas some senotherapeutics like pipelongumine appear to be senolytic in some senescent models and senomorphic in others (Malaquin et al., 2020; Y. Wang et al., 2016; X. Zhang et al., 2018). To unequivocally characterize senolysis in cell models, it is therefore required to demonstrate the selective induction of cell death in senescent cells. Some experimental designs for senolysis assessment consist in treating the cells with the senescence-inducing compound and the senolytic candidate at the same time, or adding the senolytic candidate in the first hours following exposure to the senescence-inducing stressor, which

does not yield information on the senolytic potential of the compound. Senolytic means that the compound selectively elicits the death of established senescent cells. Rather, studies with concomitant or closely spaced in time senescence induction and candidate drug treatment actually address the identification of pathways modulating the cell fate decision to undergo senescence or apoptosis in response to stress. This was the case for quercetin-functionalized nanoparticles MNPQ (Lewinska et al., 2020). These results do not preclude that MNPQ may be selectively toxic to established senescent cells, but as such, only demonstrate that MNPQ promote cell death over senescence in response to stress. This distinction is relevant since senescence is a highly dynamic state (Hernandez-Segura et al., 2017; Martínez-Zamudio et al., 2020), and that early and late senescent cells may be differently sensitive to senolysis (Nogueira-Recalde et al., 2019; Schafer et al., 2017). Drugs that synergize with senescence-inducing chemotherapeutic compounds to induce apoptosis when used conjointly are however still of great interest, and like bona fide senolytics may be employed in one-two punch approaches as they would eliminate cancer cells and prevent newly senescent cancer cells from persisting.

Senolytics have sparked tremendous research interest, with the number of new PubMed senolytic references nearly doubling each year since 2016. The apparent nearly invariable beneficial outcome of senolytic treatment in highly diverse pathological contexts, certainly contributes to the excitement but results in a fast-paced accumulation of data warranting caution. Pre-clinical studies reporting absence of improvements following senolytic treatment provide precious insight into the pathophysiological diversity of cell senescence (Kovacovicova et al., 2018), but are at present too few. The extent of actual reported outcomes of senolytic treatments in pre-clinical studies should be carefully assessed by the reader before citing these works as supporting an effect of senolytics on disease progression or prevention. For instance, an article titled ‘Dasatinib plus quercetin prevents uterine age-related dysfunction and fibrosis in mice’ (Cavalcante et al., 2020) actually reports an absence of anti-fibrotic effect of the senolytic cocktail. There was no difference in collagen deposition and uterine fibrosis between the control and the treated group. This is clearly stated in the text, and the authors even propose that ‘the specific D+Q protocol used may explain the absence of a uterine anti- fibrotic effect due to the short duration of the intervention’. They however report a downregulation of miR34a expression which

they hypothesize ‘could indicate a possible antifibrotic effect’, but is clearly insufficient grounds for the overextrapolation in the title. This study has been cited by others (including (Kulkarni et al., 2022; Partridge et al., 2020; Santin et al., 2020)) thus wrongfully supporting the claim that dasatinib and quercetin prevent uterine fibrosis in mice.

Parenthetically, there is surprisingly some confusion in the literature with claims that BH3 mimetic navitoclax/ABT-263 and MDM2 inhibitor UBX0101 are the same compound (Malaise et al., 2017; Tachikart et al., 2018; von Kobbe, 2019). The misunderstanding might come from the fact that in the first article describing UBX0101 as a senolytic in mice (Jeon et al., 2017), the authors referred to their previous studies of navitoclax to address the identification of UBX0101 as a senolytic (J. Chang et al., 2016; Y. Zhu et al., 2016), whereas there was no mention of UBX0101 in these papers. It should however be stressed that navitoclax and UBX0101 are two different compounds.

Given the diversity of senescence-associated pro-survival pathways, and the heterogeneity of senescent phenotypes, probably no universal senolytic will be discovered. This however may not be a problem, as selective senolytics are more desirable in order to limit side effects, spare essential senescent cell niches, and attain high drug potency. Importantly, some persisting senescent cell populations such as vascular endothelial cells were found not to be replaced following elimination in aged mice, which was compensated by fibrosis thus worsening the condition (Grosse et al., 2020). In these specific cases, senolytic clearance is not desirable and senomorphic treatment might be more appropriate. Classically senolytics are characterized by comparing their toxicity in senescent cells and in their proliferative or quiescent counterparts, and I stressed above the importance of that comparison to label a drug ‘senolytic’. For example, senolytics might possibly help preventing melanoma formation by eliminating BRAF-V600E-induced senescent melanocytes in nevi within patients at risk (L’Hôte et al., 2021). With the current paradigm of senolytic drug discovery, senolytics targeting senescent melanocytes would be sought so as not to target proliferative melanocytes. However, clearing all senescent melanocytes within a nevus while sparing potential BRAF-V600E-expressing non-senescent melanocytes would not be desirable as it may increase the risk of neoplastic transformation of these cells. In this context, a better drug candidate would clear all BRAF-V600E-expressing melanocytes, senescent or not, while sparing

other epidermal cell types in the vicinity of the nevus, namely, keratinocytes and dendritic Langerhans cells (Pils et al., 2021). Hence, a better approach to maximize chances of clinical translation might be context-driven senolytic drug discovery. Starting from a well-defined pathological condition in which accumulation of senescent cells is implicated, the toxicity of candidate drugs may be tested in culture in the relevant senescent cell type as well as in models of cell populations (possibly but not mandatorily including the proliferative counterparts of the targeted senescent cells) that may be exposed to the drug but should not be affected by it. Senolysis may then be validated *ex vivo* and *in vivo* with the detection of released oxylipin (Wiley et al., 2021).





# **Chapter 3. Mechanisms of senolysis by cardioglycosides.**

We previously characterized a human fibroblast model of BRAF-V600E senescence (Carvalho et al., 2019) Selective senolytics in BRAF-V600E senescence, which could hold clinical interest in the context of melanoma prevention, had not been described before. In a screen of the Prestwick repositioning library, we identified cardioglycosides as novel senolytics in a model of CRAF-senescent WI-38 fibroblasts. Cardioglycosides would later be described as broad-spectrum senolytics during our investigation (Guerrero et al., 2019; Triana-Martínez et al., 2019). Nevertheless, we found that cardioglycosides were remarkably potent senolytics in BRAF-V600E-senescent BJ fibroblasts. We thus sought to investigate their mechanisms of action in this senescence model, while characterizing new regulatory processes of BRAF-V600E-senescent cell survival. This study was published in 2021 as the following Article 2: Ouabain and chloroquine trigger senolysis of BRAF-V600E-induced senescent cells by targeting autophagy.

## **3.1. Article 2: Ouabain and chloroquine trigger senolysis of BRAF-V600E-induced senescent cells by targeting autophagy.**

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# Ouabain and chloroquine trigger senolysis of BRAF-V600E-induced senescent cells by targeting autophagy

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Fondation ARC pour la Recherche sur le Cancer; Ligue Contre le Cancer (Comité Val d'Oise)

**Abstract**

The expression of BRAF-V600E triggers oncogene-induced senescence in normal cells and is implicated in the development of several cancers including melanoma. Here, we report that cardioglycosides such as ouabain are potent senolytics in BRAF senescence. Sensitization by ATP1A1 knockdown and protection by supplemental potassium showed that senolysis by ouabain was mediated by the Na,K-ATPase pump. Both ion transport inhibition and signal transduction result from cardioglycosides binding to Na,K-ATPase. An inhibitor of the pump that does not trigger signaling was not senolytic despite blocking ion transport, demonstrating that signal transduction is required for senolysis. Ouabain triggered the activation of Src, p38, Akt, and Erk in BRAF-senescent cells, and signaling inhibitors prevented cell death. The expression of BRAF-V600E increased ER stress and autophagy in BRAF-senescent cells and sensitized the cell to senolysis by ouabain. Ouabain inhibited autophagy flux, which was restored by signaling inhibitors. Consequently, we identified autophagy inhibitor chloroquine as a novel senolytic in BRAF senescence based on the mode of action of cardioglycosides. Our work underlies the interest of characterizing the mechanisms of senolytics to discover novel compounds and identifies the endoplasmic reticulum stress-autophagy tandem as a new vulnerability in BRAF senescence that can be exploited for the development of further senolytic strategies.

**KEYWORDS**

cardioglycosides, cellular senescence, endoplasmic reticulum stress, melanoma, Na,K-ATPase, senolytic, Src

## 1 | INTRODUCTION

Cellular senescence is recognized as a driving factor in an increasing number of diseases and disorders and the accumulation of senescent cells in tissues is an important driver of aging (Van Deursen, 2014). Senescence is elicited in response to a variety of stresses

such as DNA damage, telomere attrition, or oncogene expression. It encompasses a diversity of phenotypes that are characterized notably by a stable—theoretically irreversible—proliferative arrest and the boosted secretion of inflammatory factors (Hernandez-Segura et al., 2018). Upon commitment to senescence, cells undergo profound epigenetic and transcriptional reprogramming that result in

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important physiological changes and the reliance on specific pathways for survival (Soto-Gamez et al., 2019). The differences between normal and senescent cells allow for the selective targeting of the latter by pharmacological means. Over the last few years, the specific elimination of senescent cells with so-called senolytic drugs has proven to be a ground-breaking new tool that facilitates the study of the *in vivo* impact of senescent cells and holds promise in therapeutic contexts in which senescence plays a major role.

The first demonstration of drug-mediated senolysis was in therapy-induced senescent (TIS) mouse lymphomas, which are more prompt to commit apoptosis than their non-senescent counterparts in response to the inhibition of autophagy and glucose metabolism (Dörr et al., 2013). The most studied senolytic treatment so far is the combination of dasatinib and quercetin, which in mice was shown to delay the onset of aging and to alleviate osteoporosis, idiopathic pulmonary fibrosis (IPF), and Alzheimer's disease (Farr et al., 2017; Musi et al., 2018; Schafer et al., 2017; Zhu et al., 2015). The senolytic catalog later grew to include notably BH3 mimetics (Yosef et al., 2016; Zhu et al., 2016), HSP90 inhibitors (Fuhrmann-Stroissnigg et al., 2017), and cardioglycosides (Guerrero et al., 2019; Triana-Martínez et al., 2019). Senolytics recently entered the clinic for pilot human trials in IPF and diabetic kidney disease (Hickson et al., 2019; Justice et al., 2019). The ongoing discovery and characterization of novel senolytics is yielding insights into the mechanisms of senescence and aging and drives the development of innovative therapies for age-related diseases.

BRAF-V600E is a hyperactive mutant form of the BRAF kinase whose expression can lead to oncogene-induced senescence (OIS) in normal fibroblasts and melanocytes (Carvalho et al., 2019; Michaloglou et al., 2005). This mutation is the initiator event in the formation of nevi (moles). Melanocytes acquiring the mutation initially proliferate, allowing the nevus to form, before entering senescence. Furthermore, BRAF-V600E is found in 50% of melanoma, and it is estimated that about a third of these cancers arise from a pre-existing nevus (Pampena et al., 2017; Shain et al., 2015). Employing senolytics to eliminate BRAF-senescent melanocytes within nevi in patients at risk of developing melanoma might then be an efficient strategy for preventing malignant transformation. We thus decided to screen the Prestwick chemical library for senolytics targeting BRAF-induced senescence, and we identified cardioglycosides, including ouabain, as extremely potent senolytics in BRAF-V600E-senescent human fibroblasts.

Cardioglycosides have been used in human medicine to treat congestive heart failure and atrial arrhythmias since the end of the 18th century. They increase the output force of the heart and regulate its contractions (Prassas & Diamandis, 2008). They inhibit the sodium potassium ATPase pump (Na,K-ATPase or NKA), which participates in membrane potential maintenance by importing K<sup>+</sup> ions and exporting Na<sup>+</sup> ions across the plasma membrane. The mechanisms by which cardioglycosides have beneficial effects in heart failure patients, however, remain a matter of controversy: cardioglycosides binding to NKA both inhibit ion transport and trigger signal transduction pathways (Askari, 2019). They also have other cellular targets (Campa et al., 2009; Wang et al., 2014). In this study, we demonstrated that senolysis of BRAF-senescent cells

by cardioglycosides was due to Na,K-ATPase signaling rather than membrane potential disruption. Ouabain induced signaling through Src, Akt, p38, and Erk in BRAF-senescent cells. Importantly, we showed that increased endoplasmic reticulum stress and autophagy flux induced by BRAF-V600E expression was a prerequisite for senolysis by ouabain that depended on inhibition of cytoprotective autophagy. Accordingly, we identified autophagy inhibitor chloroquine as a novel senolytic drug in BRAF-V600E-induced senescence.

## 2 | RESULTS

### 2.1 | Identification of cardioglycosides as senolytics in BRAF-senescent cells

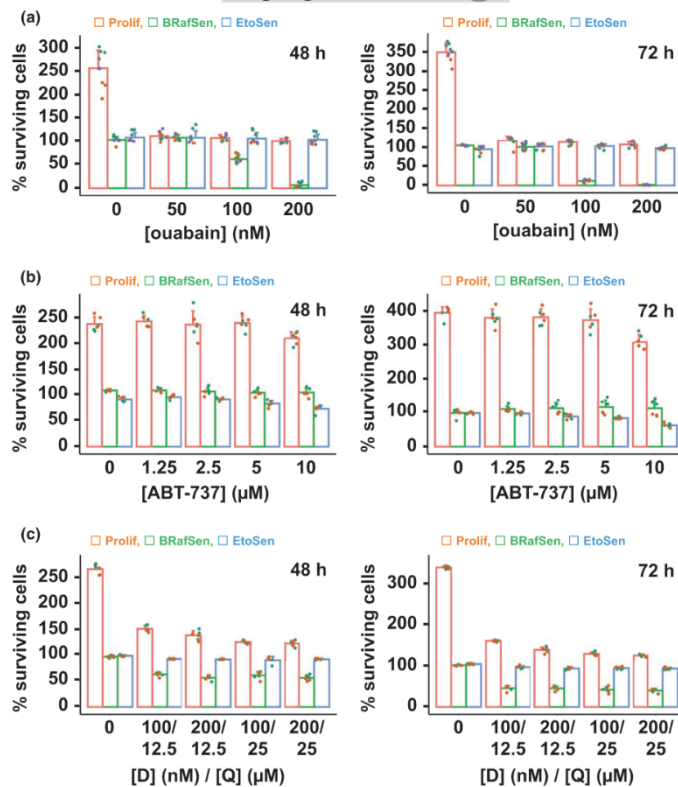
We screened the Prestwick drug repositioning library for molecules that would kill WI-38hTERT human fibroblasts induced in senescence by the expression of an activated form of the c-RAF kinase that we previously characterized (Jeanblanc et al., 2012). We identified several cardioglycosides as potential senolytics (digoxin, ouabain, strophanthidin, proscillaridin A; see Section 4), and we set out to investigate their effects on senescent cells.

First, we assessed the senolytic potential of ouabain, and other cardioglycosides, on a human fibroblast BJ hTERT cell line that we recently described, in which senescence can be triggered by the inducible expression of BRAF-V600E (Carvalho et al., 2019). In Figure 1a, we measured the toxicity of ouabain in proliferating cells (Prolif) versus cells rendered senescent by the expression of BRAF-V600E (OIS, BRafSen) or by etoposide treatment (DNA damage senescence, EtoSen). Briefly, we induced senescence for 1 week, and we incubated the cells in 96-well plates for 48 or 72 h with increasing concentrations of the drug. We counted surviving cells after fixation and nuclear staining as previously described (Carvalho et al., 2019). The graphs in Figure 1a show the percentage of cells counted after 48 and 72 h, relative to the initial number of cells at the time of drug addition (see Section 4). Toxicity on senescent cells is thus seen as a percentage of surviving cells below 100%. Remarkably, at concentrations where ouabain achieved near complete toxicity in BRafSen cells, EtoSen cells were not affected. At these concentrations, there was no increase in the number of proliferating cells (see for instance 100% surviving cells at 200 nM ouabain for 48 and 72 h), indicating that the drug was cytostatic, as previously described with cardioglycosides (Bloise et al., 2009). This proliferative arrest was partially reversible, as a fraction of the cells recovered the ability to carry out S-phase (Figure S1A), or to grow into colonies (Figure S1B), upon withdrawal of the drug. In BRafSen cells, ouabain triggered apoptosis, as evidenced by PARP1 cleavage (Figure S2A), loss of mitochondrial potential and nuclear condensation (Figure S2B). Hence, at low concentrations, ouabain was a potent senolytic in BRafSen but not EtoSen cells, while inducing reversible cytostasis in proliferating cells. Two other cardioglycosides, digoxin and strophanthidin, behaved similarly (Figure S3A-B).

We also compared the potential of ouabain to that of previously described senolytic drugs, namely navitoclax/ABT-263 (Zhu



**FIGURE 1** Ouabain is a potent senolytic in BRAF senescence. (a) Dose-response toxicity assay of cardioglycoside ouabain in BJ fibroblasts. Prolif: proliferating (red); BRafSen: BRAF-V600E senescent (green); EtoSen: etoposide senescent (blue). Survival expressed as the percentage of viable cells remaining attached to the well after incubation with the drug for 48 h (left) or 72 h (right), normalized to the initial number of cells at the time of drug addition. Note that vehicle-treated Prolif cells proliferated beyond 100%, whereas vehicle-treated senescent cells neither proliferated nor died and thus remained at ~100%. Data were aggregated from three independent biological replicates. (b) Dose-response toxicity assay of BH3 mimetic ABT-737 in BJ fibroblasts. Data were aggregated from two independent biological replicates. (c) Dose-response toxicity assay of the dasatinib (D) and quercetin (Q) combination in BJ fibroblasts. Data were aggregated from two independent biological replicates. For all panels, colored overlapping dots represent independent replicates



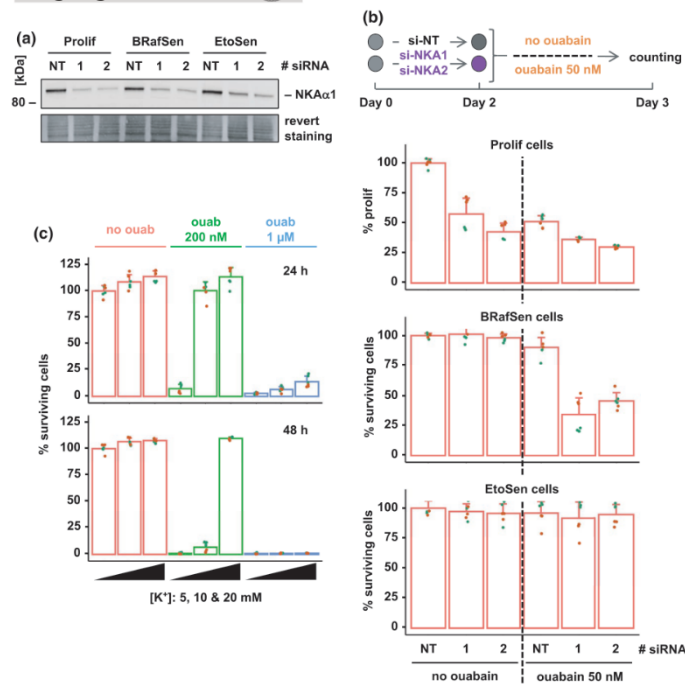
et al., 2016), ABT-737 (Yosef et al., 2016), alvespimycin/17-DMAG (Fuhrmann-Stroissnigg et al., 2017), dasatinib (Zhu et al., 2015), and the dasatinib and quercetin combination (D + Q, Zhu et al., 2015). As shown in Figure 1b–c, ABT-737 exhibited a senolytic effect in EtoSen but not BRafSen cells, while D + Q was senolytic in BRafSen cells, albeit to a lesser extent than ouabain. Navitoclax was also senolytic only in EtoSen cells (Figure S4A). The other drugs were not senolytic in our models (Figure S4B–C). Thus, in BJ cells, ouabain appeared to be more potent and specific of BRAF-V600E-induced senescence than any of the reference senolytics we tested. We therefore sought to decipher the mode of action of ouabain in BRafSen cells and to infer new senolytic drugs targeting the same cellular vulnerabilities.

## 2.2 | Senolysis by cardioglycosides is mediated by the Na,K-ATPase pump

Ouabain and the other cardioglycosides isolated in our screening of the Prestwick library bind to the alpha subunit of the Na,K-ATPase pump (NKA). NKA is composed of three subunits ( $\alpha$ ,  $\beta$  and FXYD-like) with transmembrane domains (Clausen et al., 2017). In BJ cells, transcriptomic data that we previously obtained (Carvalho et al.,

2019) indicated that of the four genes encoding  $\alpha$  subunit isoforms, ATP1A1 was the most expressed (Figure S5A). We observed a modest increase in ATP1A1 expression in RT-qPCR 7 days after senescence induction, the time at which cardioglycoside senolysis was assessed (Figure S5B). Accordingly, Western blotting did not reveal major changes in NKA $\alpha$ 1 protein levels (encoded by ATP1A1) in senescence (Figure 2a). We depleted ATP1A1 mRNA using two different siRNAs, si-NKA1 and si-NKA2, in proliferating, BRafSen, and EtoSen BJ cells. Two days after siRNA treatment, NKA $\alpha$ 1 levels were significantly reduced (Figure 2a and S5C). Depletion of ATP1A1 in itself (no ouabain) had no effect on the survival of BRafSen and EtoSen cells but slowed proliferation of non-senescent BJ cells (Figure 2b). However, we observed that reducing NKA $\alpha$ 1 levels sensitized BRafSen, but not EtoSen cells, to ouabain. 50 nM ouabain killed more than 50% of NKA $\alpha$ 1-depleted BRafSen cells in 24 h, with little toxicity in si-NoTarget control BRafSen cells. The potentiation of ouabain by the depletion of NKA $\alpha$ 1 in BRafSen cells strongly suggested that the drug acted by binding to the pump.

The Na,K-ATPase pump actively transports Na<sup>+</sup> and K<sup>+</sup> ions against their respective electrochemical gradients across the plasma membrane, thus participating in transmembrane potential maintenance. Extracellular potassium is imported into the cell by



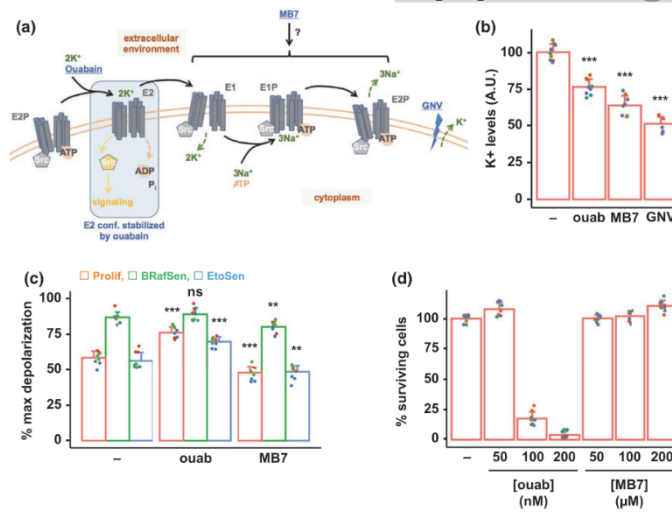
**FIGURE 2** Involvement of the Na,K-ATPase pump in senolysis by ouabain. (a) Protein levels of the Na,K-ATPase pump subunit  $\alpha$ 1 (NKA $\alpha$ 1) in BJ cells transfected for 48 h with a control no target siRNA ("NT") or ATP1A1 (gene encoding NKA $\alpha$ 1) siRNAs (si-NKA1 "1" or si-NKA2 "2"), assessed by Western blotting. Revert staining: total protein staining. Representative experiment of two independent biological replicates. (b) Toxicity and proliferation assay of 50 nM ouabain in Prolif, BRafSen, and EtoSen cells, following siRNA-mediated ATP1A1 depletion. As illustrated in the top diagram, cells were transfected with the siRNAs for 48 h and then further incubated with 50 nM ouabain or vehicle for 48 h. For Prolif cells, cell number was normalized to the final number of proliferating cells set as 100% (transfection with control si-NT, no ouabain). We observed no cell death in the wells. Data were aggregated from two independent biological replicates. (c) Protection of BRafSen cells from senolysis by KCl supplementation. Cells were treated for 24 h (top) or 48 h (bottom) with vehicle (red), 200 nM ouabain (green), or 1  $\mu$ M ouabain (blue). Treatments performed in the presence of increasing K<sup>+</sup> concentrations: 5 mM (left bar of a given color group), 10 mM (center bar of a given color group), 20 mM (right bar of a given color group). Data were aggregated from two independent biological replicates. For all panels, colored overlapping dots represent independent replicates

Na,K-ATPase. It competes with ouabain for binding to NKA $\alpha$ 1 (Noël et al., 2018). In Figure 2c, BRafSen cells were incubated with 200 nM or 1  $\mu$ M ouabain, with increasing concentrations of potassium. At 5 mM potassium (400 mg/L KCl, the normal concentration in culture media), ouabain killed nearly all BRafSen cells at both concentrations in 24 or 48 h. Doubling the concentration of potassium to 10 mM (800 mg/L KCl) completely protected the cells from 200 nM ouabain for 24 h, but not for 48 h. Although an even higher potassium concentration of 20 mM (1.4 g/L KCl) successfully protected the cells from 200 nM ouabain even for 48 h, increasing the concentration of ouabain to 1  $\mu$ M overcame the protective effect of supplemental KCl. These results reflected the competition between ouabain and K<sup>+</sup> ions for binding to the NKA $\alpha$ 1 subunit as reported, hence confirming that the effect of ouabain on BRafSen cells was mediated by the Na,K-ATPase. However, the toxicity of ouabain to BRafSen, but

not EtoSen or Prolif cells, could not be explained by a decrease in ATP1A1 expression levels (Figure 2a).

### 2.3 | Senolysis by cardioglycosides in BRAF-senescent cells is not due to ion transport inhibition

The molecular mechanism by which NKA functions as an ion pump is classically modeled as the Albers-Post cycle depicted in Figure 3a, in which the pump adopts a series of different conformations (Cui & Xie, 2017). Besides its role as an ion transporter, the Na,K-ATPase is also a receptor for signal transduction. NKA $\alpha$ 1 notably interacts with Src, keeping it inactive in protein complexes. Upon binding, ouabain stabilizes Na,K-ATPase in the E2 conformation, which both inhibits the ion transport function of the pump and releases Src (Figure 3a),



**FIGURE 3** Senolysis by ouabain is not mediated by inhibition of ion transport. (a) Diagram of the Na,K-ATPase's (NKA) Albers-Post cycle. The various conformations adopted by the pump are indicated in gray. Compounds used in the study are in blue and underlined. In basal conditions, NKA binds Src and keeps it inactive. The NKA-Src interaction is relieved when the pump adopts the E2 conformation, bound and stabilized by ouabain. GNV: gramicidin + nigericin + valinomycin. GNV is a combination of ionophores creating pores in the plasma membrane, resulting in an increased efflux of potassium from the intracellular to the extracellular medium. (b) Relative intracellular K<sup>+</sup> levels of BRafSen cells after a 7-h incubation with vehicle, 200 nM ouabain, or 200 μM MB7. GNV: positive control, treatment for 30 min with GNV 10 μM each. Following treatment with the compounds, cells were incubated with ION Potassium Green-2 AM, a fluorescent potassium indicator. Fluorescence of live cells was measured in whole wells. The fluorescence of the indicator is proportional to intracellular K<sup>+</sup> levels. Data were aggregated from three independent biological replicates. (c) Relative plasma membrane depolarization measurement in Prolif, BRafSen, and EtoSen cells after a 7-h incubation with vehicle, 200 nM ouabain, or 200 μM MB7. Cells were incubated with 500 nM DISBAC, whose fluorescence increases upon depolarization. Results were normalized to fluorescence intensity of cells in which maximum depolarization was achieved with 80 mM potassium gluconate. Data were aggregated from three independent biological replicates. (d) Dose-response toxicity assay of MB7 and ouabain in BRafSen cells. Cells were treated with increasing concentrations of MB7 and ouabain for 72 h. Note that 200 μM MB7 was not toxic in BRafSen cells despite inducing a similar K<sup>+</sup> depletion as 200 nM ouabain, which conversely killed virtually all cells in 72 h. Data were aggregated from three independent biological replicates. For all panels, colored overlapping dots represent independent replicates. Results from statistical tests performed as described in the Section 4 are indicated as follows: \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001

resulting in the activation of various signaling pathways (Nie et al., 2020; Tian et al., 2006). Ouabain-induced NKA signaling has been detected at concentrations that were too low to detect measurable changes in ion transport resulting from pump inhibition at the level of the cell (Prassas & Diamandis, 2008). Therefore, we next asked whether senolysis of BRafSen cells resulted from pump inhibition and/or Na,K-ATPase signaling.

To distinguish between the two possibilities, we employed a distinct small molecule inhibitor of the Na,K-ATPase pump which does not trigger signaling, MB7 (3,4,5,6-tetrahydroxanthone). The affinity of MB7 for the various NKA conformations is not known. However, based on *in vitro* experiments, it was suggested that MB7 does not stabilize the E2 conformation (Zhang et al., 2010), and MB7 failed to trigger the activation of several signaling pathways reportedly triggered by ouabain binding to the Na,K-ATPase (Cui & Xie, 2017). Inhibition of the pump by MB7 has been characterized *in vitro* with the purified protein, but never in cultured cells. NKA inhibition leads to a drop in

intracellular K<sup>+</sup> levels. Indeed, using a fluorescent potassium indicator (ION Potassium Green-2 AM, see Section 4), we observed a similar decrease in the levels of cytoplasmic potassium upon treatment with 200 nM ouabain or 200 μM MB7 (Figure 3b). As a positive control, we used a combination of ionophores gramicidin, nigericin, and valinomycin to induce membrane pore formation and potassium efflux. Using the plasma membrane potential indicator DISBAC2(3), whose fluorescence correlates with depolarization, we measured that the plasma membrane of BRafSen cells was constitutively depolarized compared to proliferating and EtoSen cells (Figure 3c). 200 nM ouabain induced a slight, non-significant further depolarization of BRafSen cells, while treatment with 200 μM MB7 resulted in a slight membrane hyperpolarization. The effect of both drugs on membrane potential was more pronounced in proliferating and EtoSen cells whose plasma membranes were more polarized. Importantly, 200 μM MB7 was not toxic to BRafSen cells even if incubated for 72 h (Figure 3d), despite inhibiting potassium import to the same extent as 200 nM ouabain.



## 2.4 | Cardioglycoside-induced Na,K-ATPase signaling is required for senolysis

Since ion transport inhibition appeared to be insufficient for the senolysis of BRafSen cells, we next investigated signaling pathways reportedly activated by cardioglycosides (Cui & Xie, 2017; Prassas & Diamandis, 2008). Ouabain, but not MB7, rapidly induced the phosphorylation of Akt (T308), p38 (T180/Y182), and Erk (T202/Y182; Figure 4a). Phosphorylation of Src (Y530) was already high in untreated BRafSen cells. Quantification showed that this phosphorylation was sustained by ouabain treatment, but MB7 decreased the abundance of phospho-Src (Figure 4a). Immunoblots of total proteins are provided in Figure S6A.

A subset of cellular Src is located in signaling complexes with NKA, and its activation can often be the first step in ouabain-induced signaling. Akt has also been described to be activated by cardioglycoside binding to NKA in a Src-independent manner (Wu et al., 2013). Src inhibitor dasatinib decreased ouabain-induced activation of Src and p38, but not Akt and Erk (Figure 4b). This suggested that cardioglycosides triggered the activation of a Src/ p38 axis in parallel to Src-independent Akt and Erk axes. Immunoblots of total proteins are provided in Figure S6B.

To determine whether senolysis was actually dependent on the signaling triggered upon binding of cardioglycosides to NKA, we performed a time course rescue experiment with inhibitors. We treated BRafSen cells with ouabain, before introducing signaling inhibitors at various time points. We assessed cell survival 24 h after ouabain addition (Figure 4c). Dasatinib (Src inhibitor), MK-2206 (Akt inhibitor), SB203580 (p38 inhibitor), and ulixertinib (Erk inhibitor) were effective in protecting BRafSen cells from senolysis if introduced in the first hours of incubation with ouabain. Their potency decreased with time, suggesting that the signaling events underlying cell death induction with 200 nM ouabain took place in the first 8 h of contact with the drug. The fact that inhibiting pathways associated with NKA transduction protected the cells from death was a further indication that senolysis of BRafSen cells by ouabain was primarily due to signaling rather than inhibition of ion transport, although we cannot exclude that a further slight depolarization of BRafSen cells by ouabain may participate in cell death.

## 2.5 | BRAF-V600E sets the stage for cardioglycoside senolysis

We then sought an explanation for the selectivity of ouabain for BRafSen cells over EtoSen and Prolif cells. To gain insight into the implication of BRAF-V600E in the susceptibility to cardioglycosides, we induced expression of BRAF-V600E with doxycycline for 1 week in EtoSen cells (EtoSenDox cells) and assessed their survival in the presence of ouabain. We compared their survival to control EtoSen cells treated with ethanol, the doxycycline vehicle (EtoSenEtOH). EtoSenEtOH cells were unaffected by ouabain within the concentration range tested, while EtoSenDox cells became sensitive to it, although to a lesser extent than BRafSen cells (Figure 5a). We concluded

that BRAF-V600E expression conferred susceptibility to senolysis by ouabain. Moreover, ouabain's toxicity did not depend on sustained expression of BRAF-V600E in BRafSen cells and acute overexpression of BRAF-V600E in proliferating cells failed to sensitize them to ouabain (Figure S7). Thus, senolysis by ouabain required the cells to be senescent or to express BRAF-V600E for a prolonged time.

In melanoma cell lines, BRAF-V600E was shown to induce chronic endoplasmic reticulum (ER) stress and subsequent upregulation of autophagy flux (Corazzari et al., 2015). It is also known that autophagy is important for the onset of senescence induced by BRAF-V600E (Liu et al., 2014), but the status of ER stress and autophagy flux in cells once OIS is established has been less studied. The unfolded protein response (UPR) is one of the processes triggered as a consequence of ER stress and can thus be monitored as an indicator of ER stress magnitude. BRafSen cells exhibited a heightened rate of XBP1 mRNA splicing, indicative of constitutive UPR (Figure 5b). Interestingly, the splicing of XBP1 mRNA was also increased in EtoSenDox cells compared to EtoSenEtOH cells.

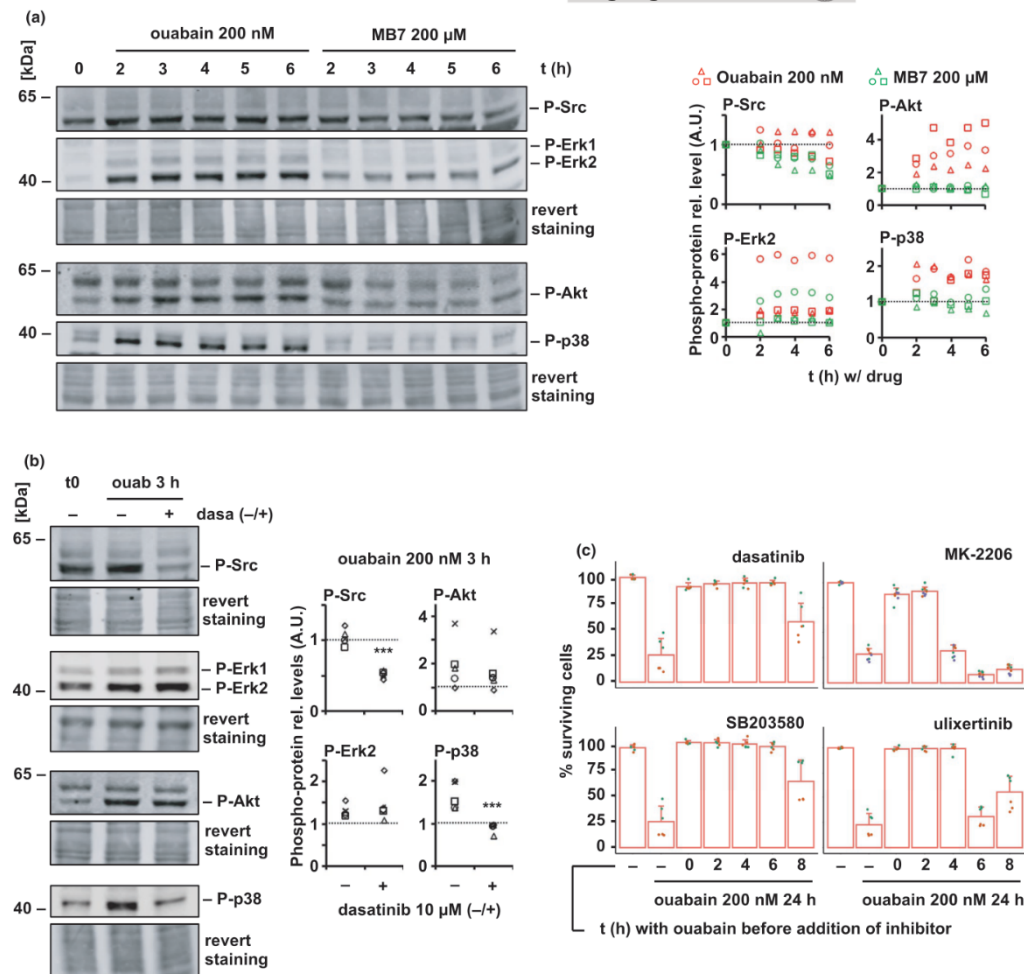
Variations in autophagy flux between conditions can be assessed by comparing the levels of autophagy proteins in these conditions when there is flux (CQ-) and when flux is blocked during a short period of time with chloroquine (CQ+), which inhibits autophagosome degradation (Klionsky et al., 2016). LC3 is a protein that is converted from its LC3-I form to its LC3-II form upon incorporation into autophagosomes and as such is one of the most used markers for autophagy. An increase in LC3-II levels can result from an upregulation of autophagosome formation (stimulated flux) as well as from a blockade of autophagosome degradation (interrupted flux). An increase in LC3-II levels between conditions when flux is blocked shortly with chloroquine is indicative of upregulated autophagy. Another common indicator of autophagy flux is p62, which mediates the recruitment of ubiquitinated substrates into autophagosomes, and whose degradation is primarily controlled through autophagy, though the correlation of p62 levels to autophagy flux is less straightforward than with LC3 (Klionsky et al., 2016). In BRafSen and EtoSenDox cells, the accumulation of LC3-II in the presence of CQ was more important than in Prolif and EtoSenEtOH cells, respectively (Figure 5c). Hence, BRAF-V600E expression resulted in augmented basal autophagy flux. To assess the relevance of autophagy in senolysis by cardioglycosides, we treated BRafSen cells with ouabain for various durations, introducing CQ in the last 2 h before protein extraction (Figure 5d). The accumulation of LC3-II during CQ treatment declined over time with ouabain, indicating that ouabain decreased the rate of autophagosome formation and autophagy flux in BRafSen cells.

Collectively, these data showed that BRAF-V600E expression resulted in chronic ER stress and increased autophagy flux, which was disrupted by ouabain treatment.

## 2.6 | Autophagy inhibitors are senolytic in BRAF senescence

BRAF-V600E-induced autophagy in response to increased ER stress is often a cytoprotective process (Rather et al., 2020), and pharmacological

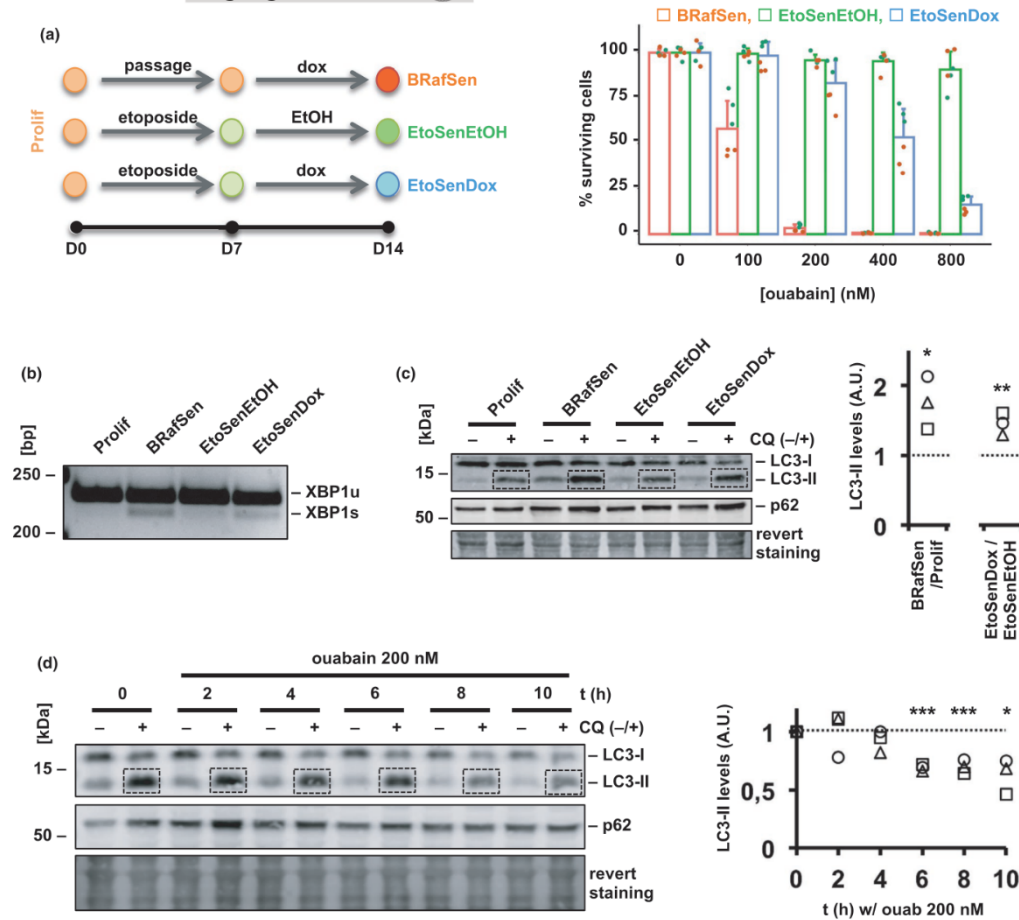




**FIGURE 4** Senolysis by ouabain is mediated by Na,K-ATPase signal transduction. (a) Phosphorylation of Src, Akt, p38, and Erk, in BrafSen cells treated with 200 nM ouabain or 200 μM MB7 in BrafSen cells, as assessed by Western blotting. Revert staining: total protein staining. Representative blot of three independent biological replicates. Quantified bands were normalized to revert staining within each lane then to t0. Shaped data points represent independent replicates. (b) Phosphorylation of Src, Akt, p38, and Erk, after 200 nM ouabain for 3 h in the presence or absence of Src inhibitor dasatinib. Revert staining: total protein staining. Representative blot of five independent biological replicates. Quantified bands were normalized to revert staining within each lane then to t0. (c) Time course rescue experiment of senolysis in BrafSen cells by signaling inhibitors of Src (10 μM dasatinib), p38 (25 μM SB203580), Akt (2 μM MK-2206), and Erk (1 μM ulixertinib). Cells were treated with 200 nM ouabain, and inhibitors were added at various time points thereafter. Survival was assessed after 24 h incubation with ouabain. Data were aggregated from two independent biological replicates. For all panels, colored overlapping dots represent independent replicates. Results from statistical tests performed as described in the Section 4 section are indicated as follows: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

inhibition of autophagy was shown to improve the potency of anti-cancer drugs in melanoma harboring the BRAF-V600E mutation (Goodall et al., 2014; Ma et al., 2014). We hypothesized that autophagy flux inhibition may underlie the specific senolysis of BrafSen cells by cardioglycosides and that, therefore, other autophagy inhibitors should behave similarly.

We thus assayed the toxicity of autophagy inhibitors CQ and bafilomycin A1 (Baf A1) on proliferating, BrafSen and EtoSen BJ fibroblasts. Strikingly, these drugs were senolytic with toxicity profiles similar to that of cardioglycosides. 50 μM CQ killed virtually all BrafSen cells in 24 h, induced cytostasis in Prolif cells, and did



**FIGURE 5** BRAF-V600E expression induces ER stress and autophagy and sensitizes cells to ouabain. (a) Dose-response toxicity assay of ouabain in BJ cells with and without expression of BRAF-V600E. As shown in the diagram, senescence was induced in BJ cells by incubation with etoposide (20  $\mu$ M for 1 week, EtoSen cells). EtoSenEtOH cells (green): as a negative control, EtoSen cells were incubated with doxycycline vehicle (0.01% ethanol) for 1 week. EtoSenDox cells (blue): etoposide was withdrawn from EtoSen cells that were further incubated with 1  $\mu$ g/ml doxycycline for 1 week to induce the expression of BRAF-V600E. BRafSen cells (red): OIS in BJ cells was induced by incubating the cells with 1  $\mu$ g/ml doxycycline for 1 week. Survival was assessed following a 48-h incubation with increasing concentrations of ouabain. Data were aggregated from two independent biological replicates. (b) XBP1 mRNA splicing in Prolif, BRafSen, EtoSenEtOH, and EtoSenDox cells, assessed by RT-PCR (reverse transcription coupled with polymerase chain reaction). Total RNA was extracted and reverse transcription was performed on an equal amount of RNA across all four cell types. XBP1 cDNA was then amplified by PCR, and PCR products loaded on a 3.5% agarose gel to separate the unspliced (u) and the spliced (s) XBP1 products. The detection of XBP1s is associated with endoplasmic reticulum (ER) stress. Representative experiment of two independent biological replicates. (c) Autophagy flux in Prolif, BRafSen, EtoSenEtOH, and EtoSenDox cells, assessed by Western blotting. Cells were treated with 50  $\mu$ M chloroquine (CQ) or vehicle for 2 h prior to protein extraction. CQ blocks autophagosome degradation, thus allowing to assess the rate of autophagosome formation during the treatment 2-h time window. Representative blot of three independent biological replicates. Quantified LC3-II bands in CQ+ samples were normalized to revert staining within each lane then to control. (d) Effect of ouabain on autophagy flux in BRafSen cells, assessed by Western blotting. Cells were treated with 200 nM ouabain for increasing periods of time. 50  $\mu$ M CQ or vehicle added during the last 2 h of incubation prior to protein extraction. Representative experiment of three independent biological replicates. Quantified LC3-II bands in CQ+ samples were normalized to revert staining within each lane then to t0. For all panels, colored overlapping dots represent independent replicates. Results from statistical tests performed as described in the Section 4 are indicated as follows: \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001



not affect EtoSen cells (Figure 6a). Baf A1 achieved results of similar trend in 48 h, though with lesser potency (Figure S8). This resemblance in the selectivity of cardioglycosides and autophagy inhibitors for BRafSen cells further reinforced the hypothesis that ouabain senolysis was ultimately mediated by autophagy inhibition. CQ indeed inhibited autophagy flux in BRafSen cells, although at a different step than ouabain, as shown by the accumulation of LC3-II and p62 due to blockade of autophagosome–lysosome fusion (Figure 6b).

To finally demonstrate that autophagy inhibition happened subsequently to cardioglycoside-induced NKA signaling, we monitored autophagy flux in BRafSen cells treated with ouabain and signaling inhibitors. Autophagy flux was decreased by ouabain and significantly restored in the presence of Src or Akt inhibitors (Figure 6c). Inhibiting p38 did not significantly rescue the decrease in autophagy flux induced by ouabain. Inhibiting Erk partially restored autophagy flux, with lesser statistical significance.

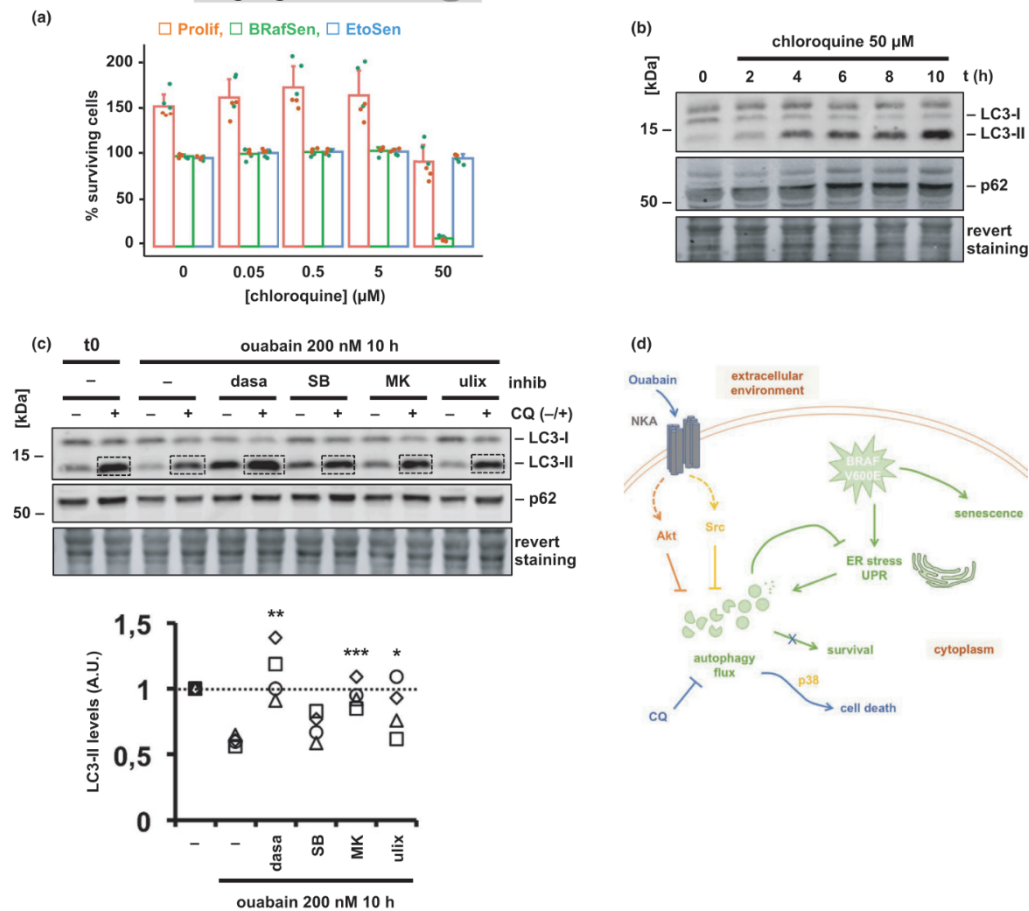
Together, these data suggested that ER stress-induced autophagy was the essential mechanism targeted in BRafSen cells during senolysis, inhibited either directly by CQ, or consequently to ouabain-induced NKA signaling. CQ is a novel senolytic in BRAF senescence described herein for the first time.

### 3 | DISCUSSION

Since Dörr and colleagues identified the first drugs in 2013, and Zhu and colleagues coined the term "senolytic" in 2015, the catalog of molecules identified as such keeps growing (Dörr et al., 2013; Zhu et al., 2015). Unfortunately, the molecular and cellular mechanisms underlying senolysis are often overlooked. Yet, determining the mode of action of a novel active compound and the series of events leading to cell death is likely to yield valuable information for the development of possibly even more potent drugs. We identified ouabain and other cardioglycosides during a screening for compounds modulating RAF-induced senescence. Compared to reference senolytics, cardioglycosides appeared to be remarkably potent on BRAF senescence, which prompted us to try to decipher the mechanisms underlying their activity. During our investigation, two articles were concomitantly published introducing cardioglycosides as broad-spectrum senolytics targeting a large range of senescent cells (Guerrero et al., 2019; Triana-Martínez et al., 2019). BRAF senescence was not investigated in these studies. Here, we reported an important selectivity of ouabain for BRafSen cells in the 10–7 M range, but we also recently identified cardioglycosides as hit senolytic compounds in a new 20  $\mu$ M screening of the Prestwick library including EtoSen cells (Figure S9A). In IMR90 cells induced in senescence by the expression of RasVal12 or by etoposide treatment, we measured a similar toxicity of cardioglycosides to what Guerrero et al. reported (Figure S9B–C). Therefore, our data are in accordance with cardioglycosides being broad-spectrum senolytics. Although they did not exclude a potential implication of other mechanisms, Guerrero and colleagues assigned senolysis by cardioglycosides to ion transport inhibition. Their investigation was albeit performed at 1  $\mu$ M ouabain. They also reported ouabain-mediated activation of Akt and p38. Triana-Martínez and colleagues proposed a model in which the blocking

of NKA by digoxin results in the inhibition of the Na,H-exchanger, leading to a drop in pH and the death of senescent cells. Both studies interpreted the rescue of senolysis by supplemental KCl as intracellular K<sup>+</sup> levels being replenished. Based on the analysis of the literature and our results, we suggest that this rescue, at least in BRafSen cells, is due to the competition between K<sup>+</sup> and cardioglycosides for NKA binding. Depending on the molecule, the concentration, and the cell type considered, we do not exclude that senolysis by cardioglycosides might be mediated by ion transport inhibition. We propose however that our model is applicable to the remarkably potent senolysis of BRAF-senescent cells by cardioglycosides.

Cardioglycosides primarily target the Na,K-ATPase pump, even though they have been shown to interact with and regulate the activity of other proteins, such as SREBP2/SCAP (Sterol Regulatory Element-Binding Protein-2/SREBP Cleavage-Activating Protein) and SRC-3 (Steroid Receptor Coactivator-3; Campia et al., 2009; Wang et al., 2014). Nevertheless here, depleting ATP1A1 potentiated the toxicity of ouabain in BRafSen cell, and complementing the culture medium with extra potassium protected the cells from apoptosis, indicating that NKA was indeed the target of cardioglycosides involved in senolysis. Although it is best known for transporting Na<sup>+</sup> and K<sup>+</sup> ions across the plasma membrane, NKA also has a well-described role in signal transduction. In basal conditions, NKA physically interacts with and inhibits the tyrosine-protein kinase Src within protein complexes. Src is released and activated when the Na,K-ATPase pump is stabilized in the E2 conformation following cardioglycoside binding (Nie et al., 2020; Tian et al., 2006). Here, in BRafSen cells where NKA $\alpha$ 1 was depleted, we observed a sensitization to ouabain-induced apoptosis (Figure 2b). This was compatible with a role of NKA signaling, as an increase in basal Src activity has been described upon Na,K-ATPase knockdown (Banerjee et al., 2018). MB7 (3,4,5,6-tetrahydroxyxanthone) was developed to block the Na,K-ATPase pumping function without activating signaling (Zhang et al., 2010). To our knowledge, inhibition of ion transport by MB7 had never been assayed in intact cells. Here, we demonstrated for the first time the ability of MB7 to inhibit the ion transport function of the Na,K-ATPase in vivo. At concentrations where both compounds induced a similar inhibition of NKA-mediated potassium import, ouabain was toxic to BRafSen cells while MB7 was not. On the other hand, resulting modulations of plasma membrane potential in BRafSen cells by ouabain were insignificant, while MB7 induced a slight hyperpolarization. Ouabain, but not MB7, rapidly induced signaling through Src, Akt, p38, and Erk. Moreover, inhibiting these pathways protected the cells from senolysis. These results indicated that the lethality of cardioglycosides on BRafSen cells was due to NKA signal transduction rather than inhibition of ion transport. However, BRafSen cells were constitutively much more depolarized than Prolif and EtoSen cells, which made it technically difficult to measure a significant further depolarization. Therefore, we cannot formally exclude that ouabain may further depolarize BRafSen cells and that this, through signaling induction, may play a role in cell death. Only a subset of Src is bound to NKA and activated by cardioglycosides. Given that phospho-Src was already abundant



**FIGURE 6** Autophagy inhibitor chloroquine is senolytic in BRAF senescence. (a) Dose-response toxicity assay of chloroquine in BJ cells. Cells were incubated with chloroquine for 24 h before survival assessment as described above. Data were aggregated from two independent biological replicates. (b) Effect of chloroquine on autophagy flux in BRAfSen cells, assessed by Western blotting. Cells were treated with 50  $\mu\text{M}$  chloroquine for increasing periods of time. Representative blot of two independent biological replicates. (c) Effect of signaling inhibitors on ouabain-induced autophagy flux decrease in BRAfSen cells, assessed by Western blotting. Cells were treated for 10 h with 200 nM ouabain in combination with an inhibitor of Src (10  $\mu\text{M}$  dasatinib, dasa), p38 (25  $\mu\text{M}$  SB203580, SB), Akt (2  $\mu\text{M}$  MK-2206, MK), or Erk (1  $\mu\text{M}$  ulixertinib, ulix), or with vehicle. 50  $\mu\text{M}$  CQ or vehicle added during the last 2 h of incubation, prior to protein extraction. Revert staining: total protein staining. Representative blot of four independent biological replicates. Quantified LC3-II bands in CQ+ samples were normalized to revert staining within each lane then to t0. (d) Model of the senolysis of BRAF-senescent cells by ouabain and chloroquine. BRAF-V600E expression induces ER stress and a subsequent upregulation of autophagy flux, on which the cell depends for its survival. Ouabain triggers Na,K-ATPase signaling resulting in autophagy inhibition, thus specifically killing BRAfSen cells. Chloroquine, a blocker of autophagosome degradation, is also a senolytic in BRAF senescence. For all panels, colored overlapping dots represent independent replicates. Results from statistical tests performed as described in the Section 4 are indicated as follows: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

in BRAfSen cells, we assume that the activation of the NKA-bound Src pool was probably not sufficient to see a significant increase in phospho-Src abundance at the level of the cell. However, the fact that inhibiting Src protects cells from death, prevents p38 activation, and rescues autophagy flux shows that Src activity is indeed required for ouabain-induced NKA signaling.

Autophagy and senescence share a variety of common inducing stressors. Oncogene activation leads to both autophagy and senescence, but the relationships between the two are yet to be fully understood, with reports of both positive and negative regulation of one by the other (Kwon et al., 2017). Autophagy mediates OIS onset, and its inhibition delays the proliferative arrest following oncogene expression



(Young et al., 2009). TIS mouse lymphomas increase autophagy flux in response to proteotoxic stress caused by SASP production and are consequently more susceptible to bafilomycin A1-induced apoptosis than their non-senescent counterparts (Dörr et al., 2013). In melanoma, high levels of BRAF-V600E concomitantly trigger a senescent-like phenotype and autophagy through mTOR downregulation; inhibiting autophagy allowed cells to resume proliferation (Maddodi et al., 2010). Autophagy inhibitors can sensitize melanoma cells to chemotherapy (Goodall et al., 2014). Given that ouabain was remarkably potent in BRAF senescence and that it strongly activated Akt, upstream of mTOR, we hypothesized that cardioglycosides functioned as senolytics by disrupting autophagy, to which BRafSen cells would be more susceptible than proliferating and EtoSen cells. Senolysis by cardioglycosides was indeed dependent on BRAF-V600E activation, which entailed increased ER stress and autophagy. The molecular mechanisms by which BRAF-V600E leads to ER stress and autophagy in senescent cells are still to characterize. In melanoma, BRAF-V600E-induced ER stress can be mediated by the sequestration of ER chaperone GRP78 or the activation of p38 (Corazzari et al., 2015; Ma et al., 2014; Rather et al., 2020). Strikingly, autophagy inhibitor chloroquine exhibited the same toxicity profile as ouabain, killing virtually all BRafSen cells while not affecting EtoSen cells, and being cytostatic in proliferating cells. This strengthened the idea that autophagy was the fundamental process underlying the specific senolysis of BRafSen cells by cardioglycosides. We showed that distinct Src and Akt axes were implicated in the inhibition of autophagy flux by ouabain. p38, despite being activated downstream of Src, was not required for autophagy inhibition, and might thus be implicated in the subsequent steps leading to apoptosis following autophagy flux reduction. As such, the role of p38 in senolysis should be characterized in greater detail to better understand how inhibiting autophagy leads to BRafSen cell death. Although Erk appears to be required for senolysis, its implication in autophagy modulation remains partial, and its activation was not dependent on Src. Deciphering its precise role in the senolysis of BRafSen cells would however be complicated by the fact that it is a downstream target of BRAF-V600E as well as being activated by cardioglycosides.

Taken together, our data suggest the following model for senolysis of BRAF-senescent cells by ouabain (Figure 6d): the expression of BRAF-V600E induces ER stress in BRafSen cells, in response to which autophagy flux is augmented and required for survival; ouabain binds to the Na,K-ATPase pump, both inhibiting ion transport and triggering signal transduction, the latter being primarily relevant to senolysis; NKA-mediated activation of Src and Akt leads to a decrease of autophagy flux. Due to BRAF-V600E-induced ER stress, a larger autophagy flux is required for the survival of BRafSen cells than that of Prolif and EtoSen cells. Consequently, ouabain preferentially induces apoptosis in senescent cells that express BRAF-V600E, potentially through p38. Blocking autophagy flux at a different step such as autophagosome-lysosome fusion using chloroquine, also results in senolysis.

Depending on the cell type and the inducing stressor, senescent cells rely on various survival mechanisms to resist apoptosis, such as ephrin signaling or changes in BCL2 family protein expression patterns (Soto-Gamez et al., 2019; Yosef et al., 2016; Zhu et al., 2015). HSP90 inhibitors, azithromycin, fenofibrate, and

quercetin-functionalized MNPQ nanoparticles are senolytic drugs that all increase autophagy flux (Fuhrmann-Stroissnigg et al., 2017; Lewinska et al., 2020; Nogueira-Recalde et al., 2019; Ozsvari et al., 2018). We identify here cardioglycosides and chloroquine as senolytics that function by suppressing rather than inducing autophagy.

In conclusion, by deciphering the mechanisms of senolysis by cardioglycosides, and as previously shown in TIS (Dörr et al., 2013), we have identified autophagy as an essential process for the survival of BRAF-senescent cells. The interplay between ER stress and autophagy and how it regulates the survival of BRAF-senescent cells are yet to understand.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Materials

### 4.2 | Screening of the Prestwick chemical library

### 4.3 | Cell lines, cell culture, and senescence induction

### 4.4 | Protein extraction and Western blotting

### 4.5 | EdU incorporation

### 4.6 | Clonogenicity assay

### 4.7 | Fluorescence microscopy

### 4.8 | Relative plasma membrane potential measurement

### 4.9 | siRNA knockdown experiments

### 4.10 | RNA extraction, RT-qPCR, and RT-PCR

See Supporting Experimental Procedures.

#### 4.11 | Dose–response toxicity assay

We seeded 4000 proliferating or 10,000 senescent cells per well in CostarAssay 96-well plates (Corning 3904, Sigma-Aldrich). The day after seeding, we treated cells with compounds of interest at various concentrations with at least three wells per condition (technical triplicates). In negative control wells, we treated cells with vehicle. In baseline control wells, we treated cells with 20  $\mu$ M etoposide which very rapidly stops proliferation (Carvalho et al., 2019). The number of cells in these wells at the end of the incubation period was used as a surrogate for the initial cell number at the time of drugs addition and used for normalization. Cells were fixed and their nuclei stained by incubation in 1% paraformaldehyde (P6148, Sigma-Aldrich), 0.1% Triton X-100 (T8787, Sigma-Aldrich), and 10  $\mu$ g/ml Hoechst 33342 (B2261, Sigma-Aldrich) in PBS, for 30 min at room temperature, then washed with PBS. Images were acquired on a CellInsight CX5 (Thermo Fisher Scientific) screening microscope with a 4x objective or on an Operetta (Perkin-Elmer) screening microscope with a 10x objective. Surviving cell numbers in each well were determined by automated nucleus segmentation and counting.

#### 4.12 | Relative intracellular potassium content measurement

We seeded 10,000 BRAfSen cells per well in 96-well plates. 24 h after seeding, we applied various treatments to cells in triplicate wells. At the endpoint, we incubated cells with 5  $\mu$ M ION Potassium Green-2 AM K+ indicator (Abcam) for 40 min at 37°C. We withdrew the indicator and incubated the cells in fresh medium for 20 min at 37°C. Live cells were then imaged with a CellInsight CX5 microscope. We analyzed the total fluorescence of each well.

#### 4.13 | Statistical analyses

Statistical significance was assessed for all datasets by performing a bilateral unpaired Student's *t* test assuming equal variances, except for rescue experiments in which we performed a unilateral unpaired Student's *t* test assuming equal variances. A *p*-value < 0.05 was considered significant. For intracellular potassium levels and membrane potential measurements, treated cells were compared to vehicle. For time course Western blotting experiments, quantified bands were compared to t0. For other Western blotting experiments, quantified bands were compared to control.

#### ACKNOWLEDGMENTS

This work was funded by the Fondation ARC pour la Recherche sur le Cancer and the Ligue Contre le Cancer (Comité Val d'Oise). We warmly thank Jean-Christophe Aude, Pascale Bertrand, Marc Blondel, and Claude Gazin, for fruitful discussions.

#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

#### AUTHOR'S CONTRIBUTIONS

VL, RC, GP, and JYT, performed the experiments. GP designed the Prestwick library screening strategy. JC prepared the Prestwick library for screening. GLP and ADM optimized XBP1 splicing analysis. CM and JYT conceptualized and supervised the study, and secured funding. VL, ADM, and JYT, analyzed the data. VL and JYT wrote the manuscript. All authors reviewed, edited, and validated the manuscript.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** L'Hôte, V., Courbeyrette, R., Pinna, G., Cintrat, J.-C., Le Pavec, G., Delaunay-Moisan, A., Mann, C., & Thuret, J.-Y. (2021). Ouabain and chloroquine trigger senolysis of BRAF-V600E-induced senescent cells by targeting autophagy. *Aging Cell*, 20, e13447. <https://doi.org/10.1111/acer.13447>



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**Supporting information**

## **Supporting experimental procedures**

### **Materials**

The drug repositioning library was purchased from Prestwick Chemical (Illkirch, France). The following drugs were used in the study: doxycycline (D3447, Sigma-Aldrich), etoposide (E1383, Sigma-Aldrich), ouabain (O3125, Sigma-Aldrich), digoxin (D6003, Sigma-Aldrich), strophanthidin (S6626, Sigma-Aldrich), ABT-737 (S1002, Selleckchem), navitoclax / ABT-263 (S1001, Selleckchem), dasatinib (D074, TOKU-E), quercetin (Q0125, Sigma-Aldrich), alvespimycin / 17-DMAG (S1142, Selleckchem), MB7 / 3,4,5,6-tetrahydroxyxanthone (T5955, Sigma-Aldrich), ulixertinib (S7854, Selleckchem), SB203580 (S1076, Selleckchem), MK-2206 (S1078, Selleckchem), chloroquine diphosphate (C6628, Sigma-Aldrich), bafilomycin A1 (B1793, Sigma-Aldrich), 4-hydroxytamoxifen (H6278, Sigma-Aldrich), valinomycin (V0627, Sigma-Aldrich), gramicidin (C5502, Sigma-Aldrich), nigericin (481990, Merck Millipore).

### **Screening of the Prestwick chemical library**

WI-38-hTERT/C-BRAF-ER cells in which senescence was induced with 20 nM 4-hydroxytamoxifen (Jeanblanc et al. 2012) were incubated with 1,030 molecules of the Prestwick drug repositioning library. The final drug concentration was 35  $\mu$ M in 0.35% DMSO. Cells were incubated with drugs for 4 days before cell number was evaluated as described in Carvalho et al. (Carvalho et al. 2019). The primary screen was carried out in duplicate plates. For each plate, the mean number of cells and the corresponding standard deviation were calculated in 8 negative control wells (DMSO only). From this, we calculated the coefficient of variation for the negative control (CV, standard deviation as a percentage of the mean), and the plate was considered valid only if the CV was less than 5%. For every well, we calculated a score corresponding to the number of cells in the well minus the mean number of cells of the negative controls, divided by the standard deviation of the negative controls. For each molecule, we thus had two z-scores, corresponding to the difference in the number of cells with the molecule assayed and in the negative control, expressed as the number of standard deviations of the negative control. A molecule was determined a hit if a decrease in the number of cells was measured, with scores higher than 3 in both replicates. Of the 1,030 molecules assayed, 78 (8%) were positive. Of these hits, 25 molecules killed more than 50% of the cells. The 78 molecules were assayed at 20  $\mu$ M and 8  $\mu$ M, again on C-BRAF-ER-senescent

WI-38-hTERT cells and on quiescent WI-38-hTERT cells (kept in medium with 1% fetal bovine serum). We further selected molecules with senolytic potential when their lethality score compared to the negative control was 3 times higher in senescent cells compared to the quiescent cells ( $\Delta Z = Z_{\text{senescent}} - Z_{\text{quiescent}} > 3$ ). 7 molecules were retained as bona fide candidates at 20  $\mu\text{M}$ , among which digoxin ( $\Delta Z=4.8$ ), strophanthineoctahydrate ( $\Delta Z=4.8$ ), strophanthidin ( $\Delta Z=4.7$ ), digoxigenin ( $\Delta Z=4.3$ ), proscillaridin A ( $\Delta Z=4.5$ ) and digitoxigenin ( $\Delta Z=3.5$ ). 4 passed at 8  $\mu\text{M}$ , strophanthidin ( $\Delta Z=3.8$ ), digoxin ( $\Delta Z=3.2$ ), strophanthine octahydrate ( $\Delta Z=3.2$ ) and proscillaridin A ( $\Delta Z=3.2$ ). We used ouabain (strophanthine octahydrate) for further experiments to characterize the mechanisms of senolysis by glycosides.

#### **Cell lines, cell culture and senescence induction**

The BJ1-hTERT cell line was purchased from Clontech, and the BJ1-hTERT/pTRIPz-3HA-BRAF-V600E (BJ) cell line was generated as described in (Carvalho et al. 2019). Cells were cultured in MEM (Gibco #51200, Thermo Fisher Scientific) with FBS 9% (Eurobio), Glutamax 2 mM (A12860, Gibco), MEM non-essential amino acids 1X (M7145, Sigma-Aldrich), sodium pyruvate 1 mM (S8636), and penicillin-streptomycin 1X (P0781, Sigma-Aldrich), at 37°C and 5% CO<sub>2</sub> in ambient oxygen. Routine culture included splitting cells before confluence and regularly checking for mycoplasma infection. For oncogene-induced senescence (BRafSen), BRAF-V600E overexpression was triggered by incubating the cells with 1  $\mu\text{g}/\text{mL}$  doxycycline for one week, renewing medium with doxycycline every 3 to 4 days. DNA damage senescence (EtoSen) was induced by incubating the cells with 20  $\mu\text{M}$  etoposide for one week, renewing medium with etoposide every 3 to 4 days. Etoposide was withdrawn for subsequent experiments.

The IMR90/ER-RasVal12 cell line was a kind gift from Masashi Narita (Cambridge Research Institute, CRUK, Cambridge, UK), and the immortalized IMR90-hTERT/ER-RasVal12 cell line was generated as described in (Jeanblanc et al. 2012). Cells were cultured in high glucose DMEM (Gibco D6429, Thermo Fisher Scientific) with FBS 9% (Eurobio), at 37°C, 5% CO<sub>2</sub> and 5% oxygen. Routine culture included splitting cells before confluence and regularly checking for mycoplasma infection. For oncogene-induced senescence (RasVSen), RasVal12 overexpression was triggered by incubating the cells with 100 ng/mL 4-hydroxytamoxifen for one week, renewing medium with 4-hydroxytamoxifen every 3 to 4 days. DNA damage

senescence (EtoSen) was induced by incubating the cells with 50  $\mu$ M etoposide for 48 h, then withdrawing the drug for 5 days before starting experiments.

#### **Protein extraction and western blotting**

We scraped cells on ice in culture media, collected and centrifuged them at 1000 g for 5 min at 4°C. We resuspended cells in ice-cold PBS and centrifuged them again at 1000 g for 5 min at 4°C. We carefully eliminated supernatant, and we resuspended cells in 2 volumes of PBS with protease and phosphatase inhibitors (B14001 and B15001A/B, Bimake) and 1 volume of 3X Sample Buffer. Final 1X Sample Buffer composition was: 250 mM Tris (T1503, Merck), 70 mM sodium dodecylsulfate (SDS) (1066934, Gibco), 0.4 mM ethylenediaminetetraacetic acid (EDTA) (8418, Merck), 1 M glycerol (24388.320, VWR Chemicals), 0.015% Serva Electrophoresis Serva Blue G (3505002, Thermo Fisher Scientific), 2.5% 2-mercaptoethanol (805740, Merck). We incubated samples at 70°C for 10 min, briefly sonicated them until viscosity was broken, and loaded them onto Bis-Tris (B7535, Sigma) acrylamide (A7802, Sigma) gels for separation by electrophoresis. We transferred proteins onto Amersham Protan 0.2  $\mu$ m nitrocellulose blotting membranes (10600001, GE Healthcare Life Sciences). We blocked membranes in Intercept PBS Blocking Buffer (927-70001, Li-Cor) diluted  $\frac{1}{2}$  in PBS, or in Odyssey TBS Blocking Buffer (927-50000, Li-Cor) diluted  $\frac{1}{2}$  in TBS, for 1 h at room temperature with gentle shaking. We then incubated membranes overnight at 4°C with primary antibodies in blocking solution. We washed membranes in PBS or TBS with 0.1% Tween-20, and incubated them with NIR-secondary antibodies (Li-Cor) diluted in 0.1% Tween-20 PBS or TBS. We washed membranes in 0.1% Tween-20 PBS or TBS, and imaged them on a Li-Cor Odyssey CLx scanner. For detection of NKA $\alpha$ 1, cells were harvested as described above, then resuspended in an appropriate volume of RIPA buffer with protease and phosphatase inhibitors. After a 30-min incubation on ice, we centrifuged samples at 17000 g for 15 min at 4°C. The supernatant was collected and the required volume of 3X Sample Buffer was added. Samples were incubated at 37°C for 15 min, then loaded on Bis-tris acrylamide gels for electrophoresis. Western blotting was performed as above.

For signaling and autophagy studies, cells were flash-frozen after washing with PBS. PBS was removed, and plates plunged in a dry ice ethanol bath for 1 min (cells were not in contact with ethanol). Plates were incubated at -80°C overnight then proteins were extracted by scraping cells on ice in 1X Sample Buffer containing protease and phosphatase inhibitors. Samples were

incubated at 70°C for 10 min and sonicated until viscosity was broken. We loaded proteins onto gels and carried out western blotting as above. See table S1 for the list of antibodies and their dilutions.

Secondary antibodies were IRDye Goat anti-mouse / anti-rabbit IgG (H+L) 680RD / 800CW (Thermo Fisher Scientific).

#### **EdU incorporation**

We seeded 4000 proliferating cells per well in 96-well plates and incubated them with 25 nM EdU (Molecular Probes, Invitrogen, Thermo Fisher Scientific) for 48 h. Cells were fixed with 1% formaldehyde (F1635, Sigma-Aldrich) for 15 min at room temperature. We detected incorporated EdU by click chemistry using the Click-iT EdU Imaging kit (Molecular Probes, Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions. Images were acquired on a CellInsight CX5 microscope. The percentage of EdU-positive cells in each well was determined after automated nucleus segmentation as described above.

#### **Clonogenicity assay**

We seeded 1000 cells per well in a 6-well plate (Corning 353046, Sigma Aldrich) and cultured for two weeks, renewing medium every 3 to 4 days. We used 20  $\mu$ M etoposide as a negative control to block clone formation. Cells fixed with 2% formaldehyde for 10 min at room temperature, washed once with PBS and incubated with 0.1% crystal violet for 1 h at room temperature. Wells were washed 4x with PBS, and images acquired with a LiCor Odyssey CLx scanner at 700 nm.

#### **Fluorescence microscopy**

Cells were seeded in 96-well plates and treated with compounds of interest. Live cells were treated with 200 nM MitoTracker Red CMXRos (M7512, Invitrogen, ThermoFisher Scientific) for 15 min before fixation according to the manufacturer's protocol. Plates fixed with 1% formaldehyde for 15 min at room temperature. Cells permeabilized with 0.5% Triton X-100 for 10 min at room temperature. Blocking with 5% bovine serum albumin (BSA) (A7906, Sigma-Aldrich) and 0.1% Tween-20 (P1379, Sigma-Aldrich) in PBS, 1 h at room temperature. Nuclei stained with 500 ng/mL DAPI (D9542, Sigma-Aldrich) for 10 min at room temperature. Images acquired on a CellInsight CX5 microscope.

#### **Relative plasma membrane potential measurement**

We seeded 10000 cells per well in 96-well plates in serum-free MEM containing 500 nM DiSBAC2(3) (Thermo Fisher Scientific) and the various drugs to assess. The plates were incubated for 7 h. 10 µg/mL Hoechst 33342 was added to the cells 30 min before imaging. 10 min before imaging, maximum depolarization was achieved by adding 80 mM potassium D-gluconate (Sigma-Aldrich) in half the wells for each treatment. We imaged cells live with an Operetta screening microscope with a 10X objective. We calculated in each well the mean integrated DiSBAC fluorescence intensity per cell. Within each treatment, this value was normalized to the mean integrated DiSBAC fluorescence intensity in totally depolarized cells with potassium gluconate.

#### **siRNA knock-down experiments**

We incubated siRNAs (Ambion Life Technologies, Thermo Fisher Scientific) and Lipofectamine RNAiMAX Reagent (Invitrogen, Thermo Fisher Scientific) in Opti-MEM (Gibco #31985070, Thermo Fisher Scientific) for 25 min at room temperature. Cells were added to the siRNA mix then seeded in 96-well plates (5000 cells/well), or 6-well plates (250000 cells/well). Final reagent concentrations were 15 nM siRNA, 0.1% Lipofectamine RNAiMAX, and 25% OptiMEM. Target knock-down was assessed 48 hours later by RT-qPCR and/or western blotting. Further treatments were applied as described. Surviving cells in 96-well plates were fixed and counted as described. ATP1A1 siRNAs were purchased from Ambion/Life Technologies (si-NKA1: s1719; si-NKA2: s1720).

#### **RNA extraction, RT-qPCR, and RT-PCR**

Cells were scraped on ice in culture media, collected, centrifuged at 1000 g for 5 min at 4°C, resuspended in ice-cold PBS and centrifuged again. Supernatant was eliminated and RNA extraction was done using the NucleoSpin RNA Plus kit (Macherey-Nagel, Hoerd, France) following the manufacturer's protocol. RNA was eluted in ddH<sub>2</sub>O and quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

For ATP1A1 RT-qPCR, reverse transcription was done on 500 ng RNA with 10.2 ng/µL random hexamer primers (Invitrogen, Thermo Fisher Scientific), 250 µM dNTP, and 4 U/µL Maxima Reverse Transcriptase (Thermo Fisher Scientific) in M-MLV Reverse Transcriptase buffer (Thermo Fisher Scientific) with 10 min at 25°C, 30 min at 50°C, 5 min at 85°C. The cDNA was

diluted 1/5 in ddH<sub>2</sub>O and qPCR performed with a Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific) with 300 nM primers, monitored on an IQ5 apparatus (Bio-Rad). Thermal cycling was: 2 min at 50°C, 10 min at 95°C; 45 cycles of 15 s at 95°C and 1 min at 60°C. GAPDH was used for normalization. qPCR primers were synthesized by Sigma-Aldrich. qPCR primer sequences were: ATP1A1 forward: 5'-GGTCCCAACGCCCTCACTC-3'; ATP1A1 reverse: 5'-ACCACACCCAGGTACAGATTATCG-3'; GAPDH forward: 5'-ATGGGGAAGGTGAAGGTCG-3'; GAPDH reverse: 5'-GGGGTCATTGATGGCAACAATA-3'.

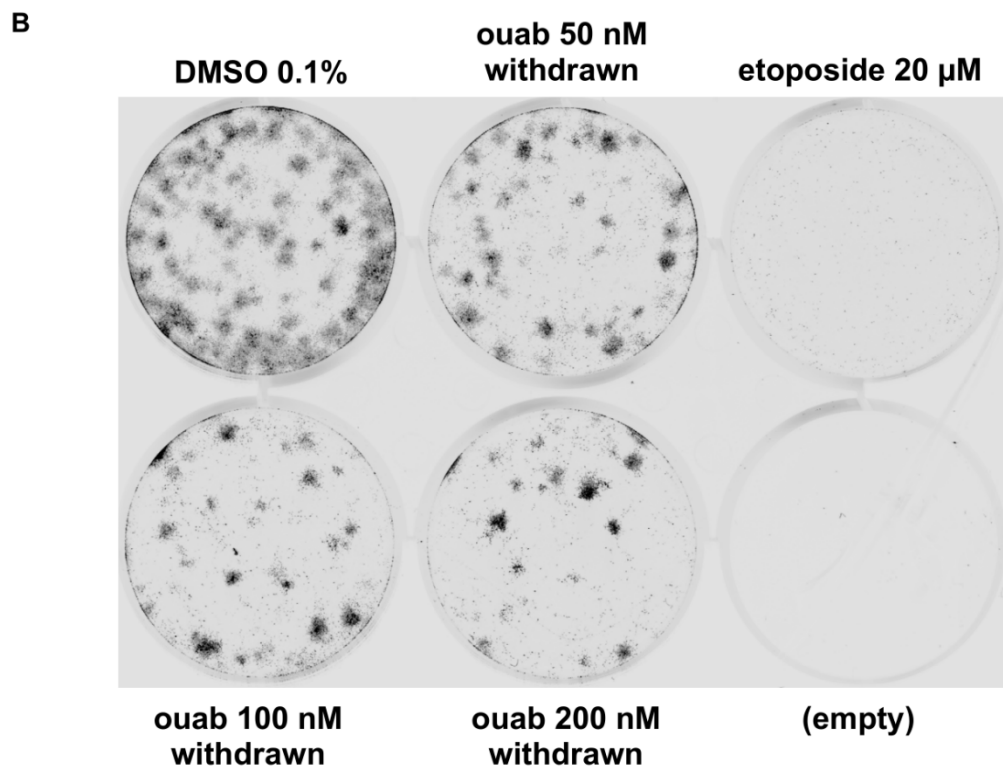
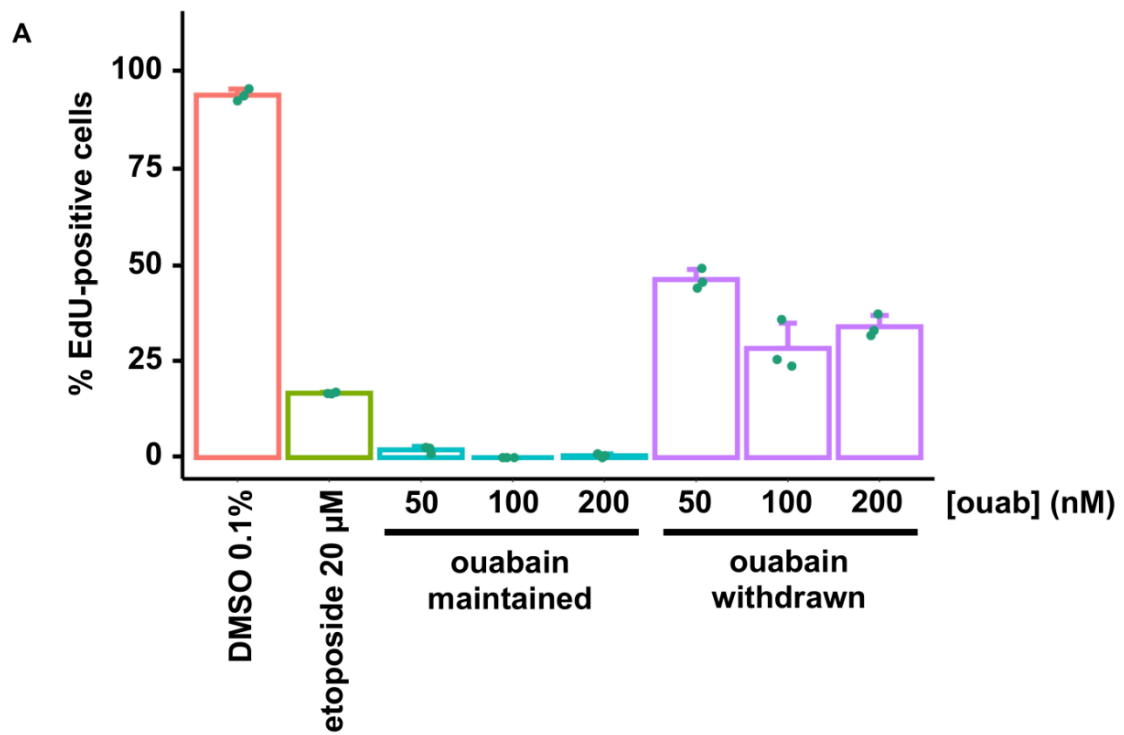
For XBP1 RT-PCR, reverse transcription was done on 500 ng RNA with 1.25 μM oligo-dT (Sigma-Aldrich), 500 μM dNTP, 10 mM dithiothreitol (DTT) (Thermo Fisher Scientific), and 5 U/μL M-MLV Reverse Transcriptase (Thermo Fisher Scientific) in M-MLV Reverse Transcriptase buffer (Thermo Fisher Scientific) with 10 min at 70°C, 2.5 min at 62°C, 90 min at 42°C, 10 min at 95°C. We added DTT and M-MLV reverse transcriptase only during the 42°C step. We diluted cDNA 1/2 in ddH<sub>2</sub>O and then 1/5 in PCR reaction buffer. We performed PCR with 300 nM of each XBP1 primer, 125 μM dNTP (Thermo Fisher Scientific), 1.2 mM MgCl<sub>2</sub> (Thermo Fisher Scientific), and 50 mU/μL Taq polymerase (Thermo Fisher Scientific) in MgCl<sub>2</sub> Buffer (Thermo Fisher Scientific). Thermal cycling was: 4 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 60°C, and 50 s at 72°C; then 7 min at 72°C. PCR primers were synthesized by Sigma-Aldrich. PCR primer sequences were: XBP1 forward: 5'-GGAACAGCAAGTGGTAGA-3'; XBP1 reverse: 5'-CTGGAGGGGTGACAAC-3'. We loaded PCR products on a 3.5% agarose gel with 0.00005% ethidium bromide (Gen-Apex) for electrophoresis. We imaged the gel with a Fusion Solo S apparatus (Vilber).

**Supporting Table 1: list of antibodies used in the study.**

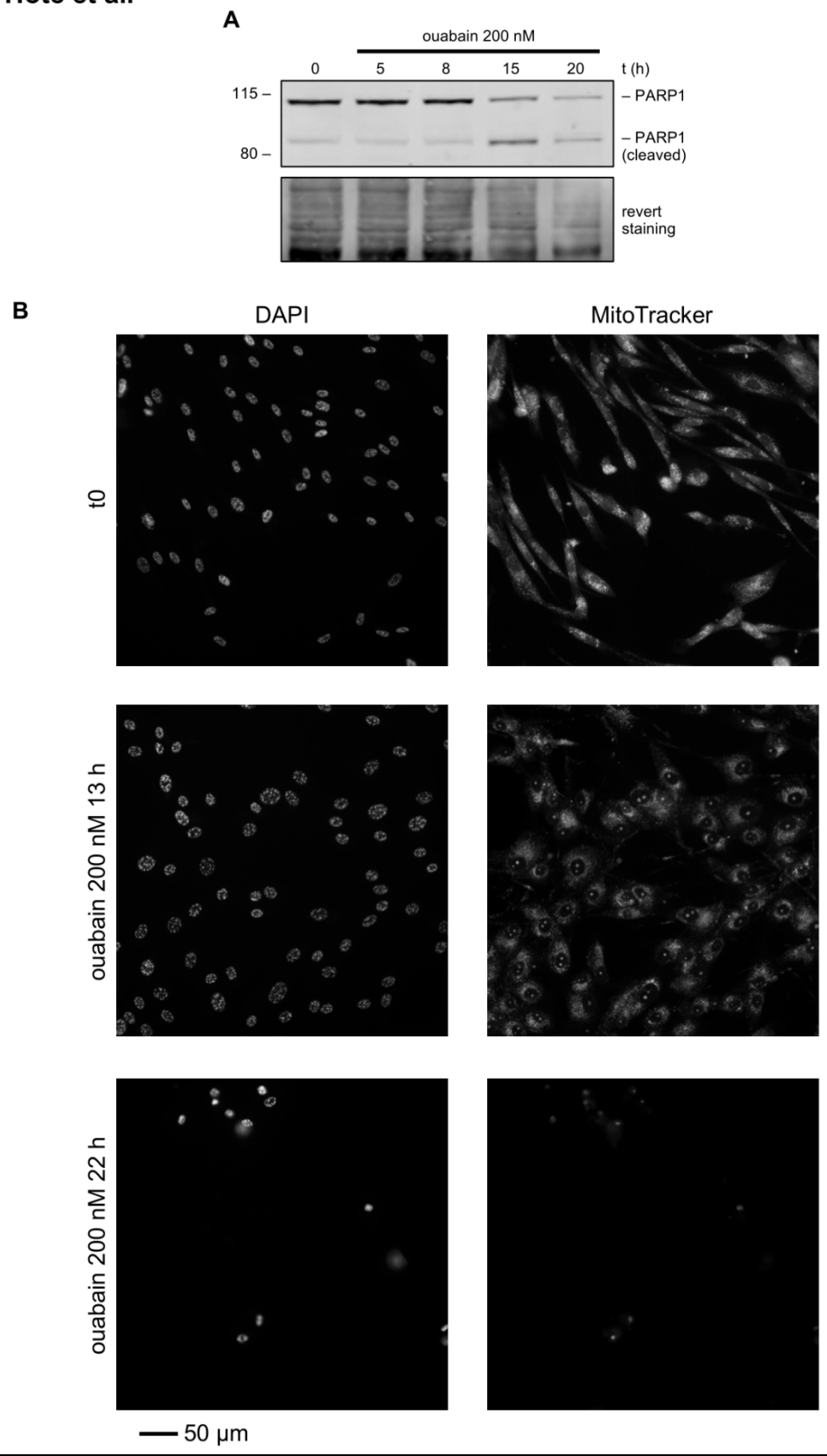
Protein target	Manufacturer	Reference	Dilution
NKA $\alpha$ 1	Santa Cruz	sc-21712	1/500
SRC	Proteintech	60315	1/10000
phospho-SRC	Santa Cruz	sc-166860	1/200
AKT	Cell Signaling Technology	2920S	1/1000
phospho-AKT	Cell Signaling Technology	4060P	1/500
ERK	Cell Signaling Technology	4695	1/1000
phospho-ERK	Cell Signaling Technology	9106S	1/1000
p38	Cell Signaling Technology	9218	1/1000
phospho-p38	Cell Signaling Technology	9216	1/500
LC3	Sigma-Aldrich	L8918	1/500
p62	Sigma-Aldrich	P0067	1/1000
PARP1	Santa Cruz	sc-7150	1/1000



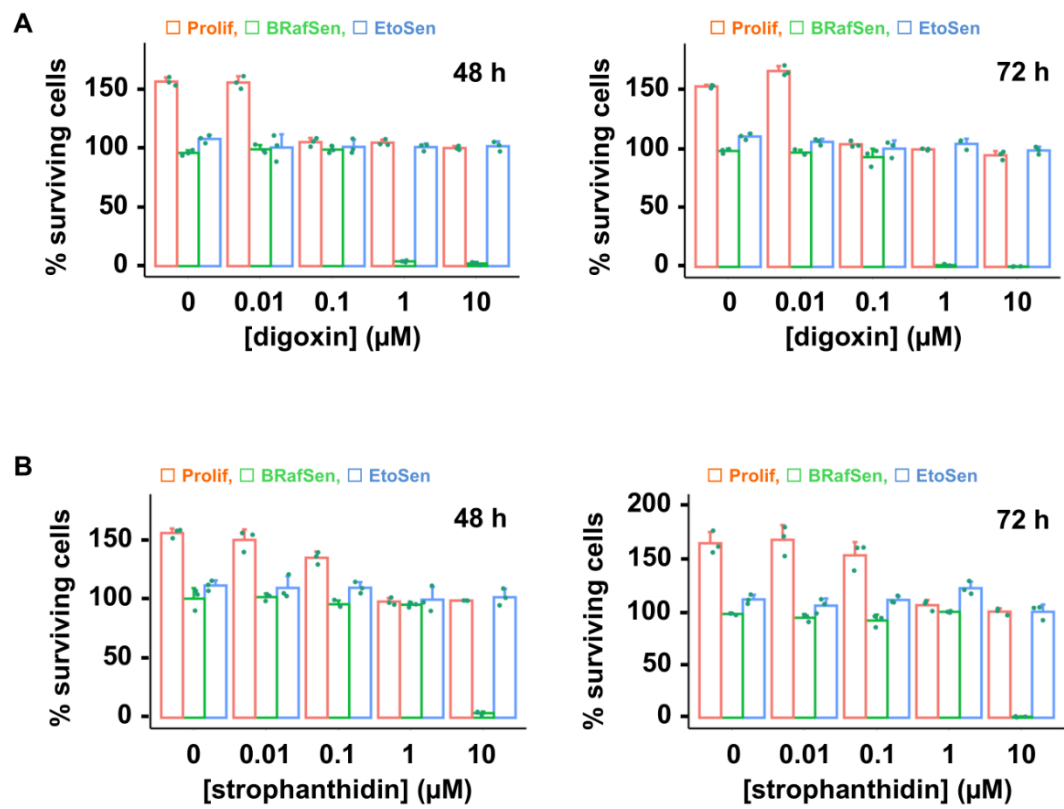
**Figure S1.** Reversibility of ouabain-induced cytostaticity in Prolif cells.  
L'Hôte et al.



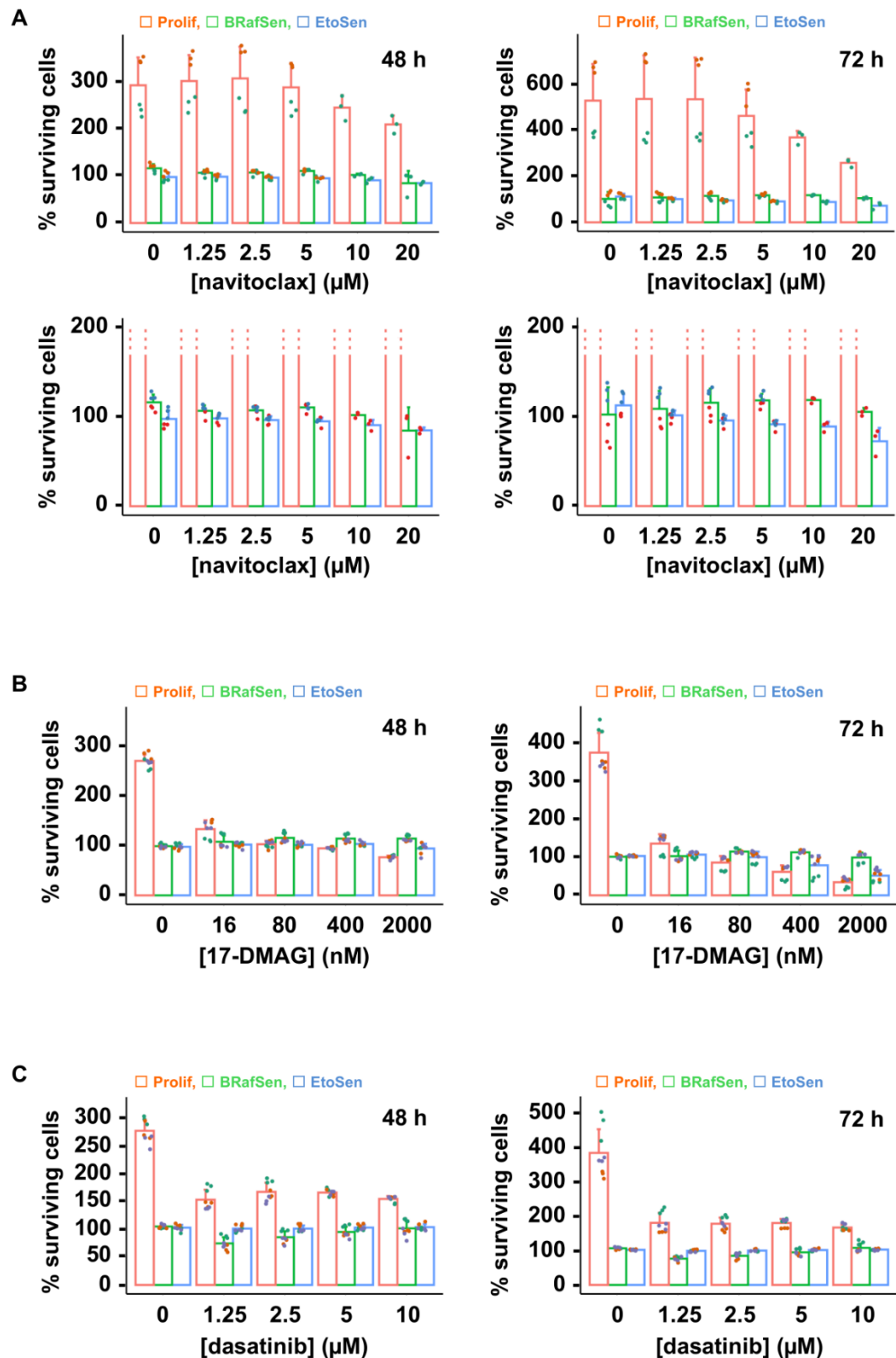
**Figure S2.** Ouabain-induced apoptosis hallmarks in BRafSen cells.  
**L'Hôte et al.**



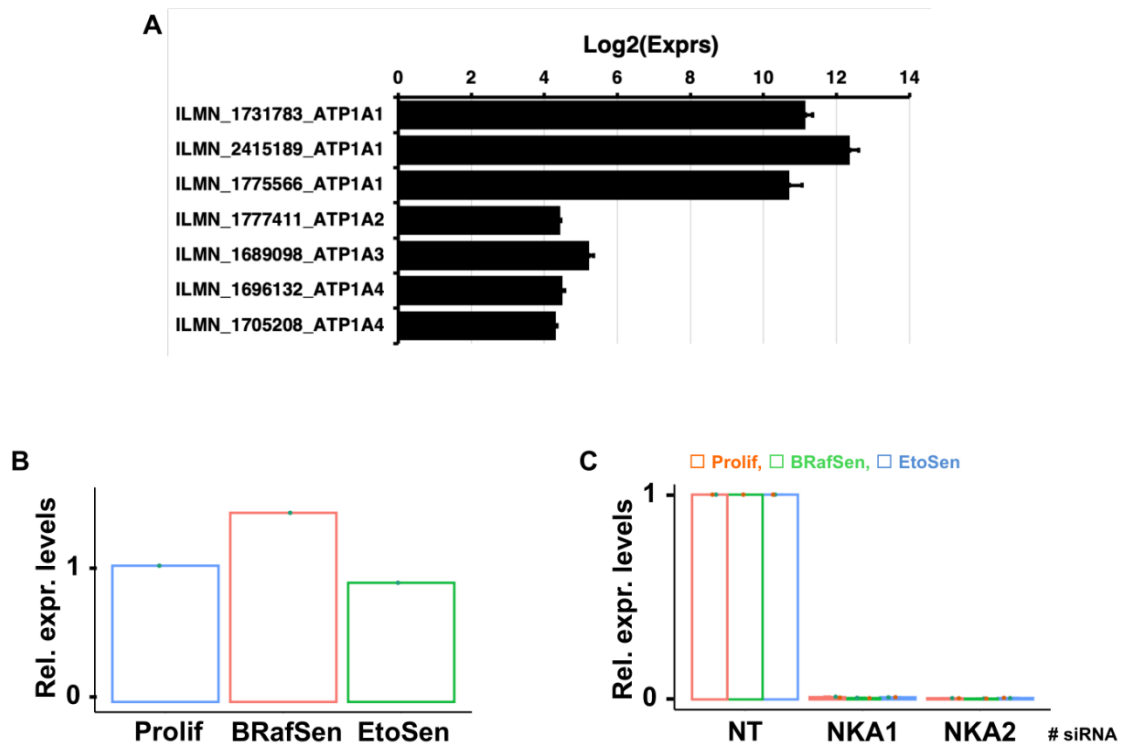
**Figure S3.** Cardioglycosides digoxin and strophanthidin exhibit the same toxicity profile as ouabain in BJ cells.  
L'Hôte et al.



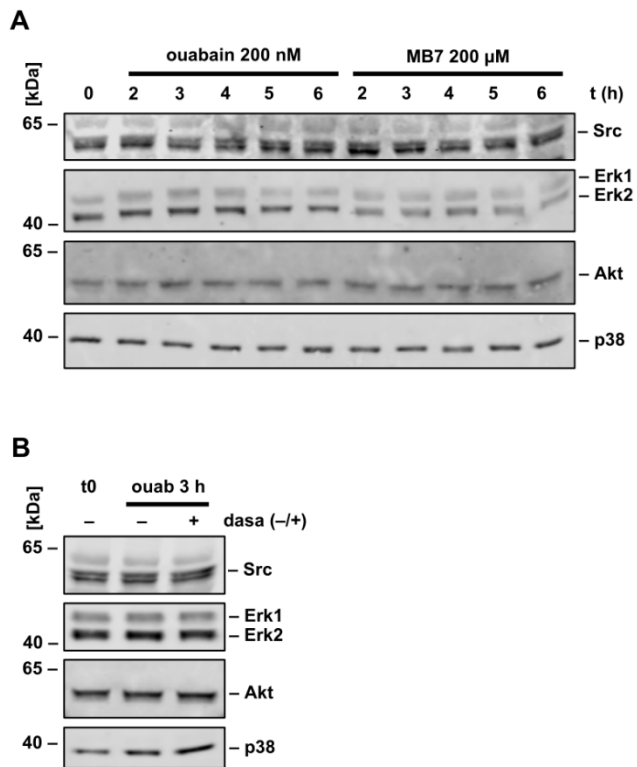
**Figure S4.** Senolytic activity of reference drugs in BJ cells.  
L'Hôte et al.



**Figure S5.** siRNA-mediated knockdown of ATP1A1.  
L'Hôte et al.

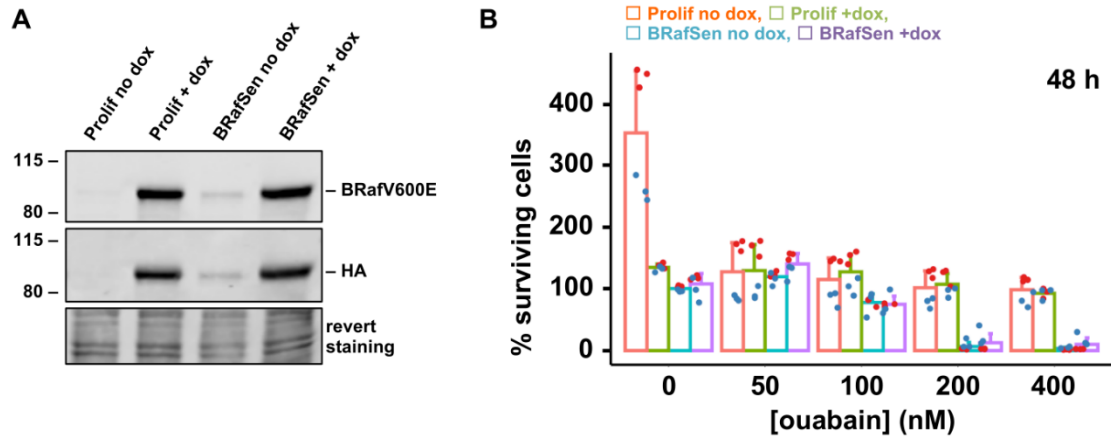


**Figure S6.** Total protein levels of Src, Erk, Akt, and p38, during ouabain and MB7 treatment, and ouabain and dasatinib treatment.  
**L'Hôte et al.**

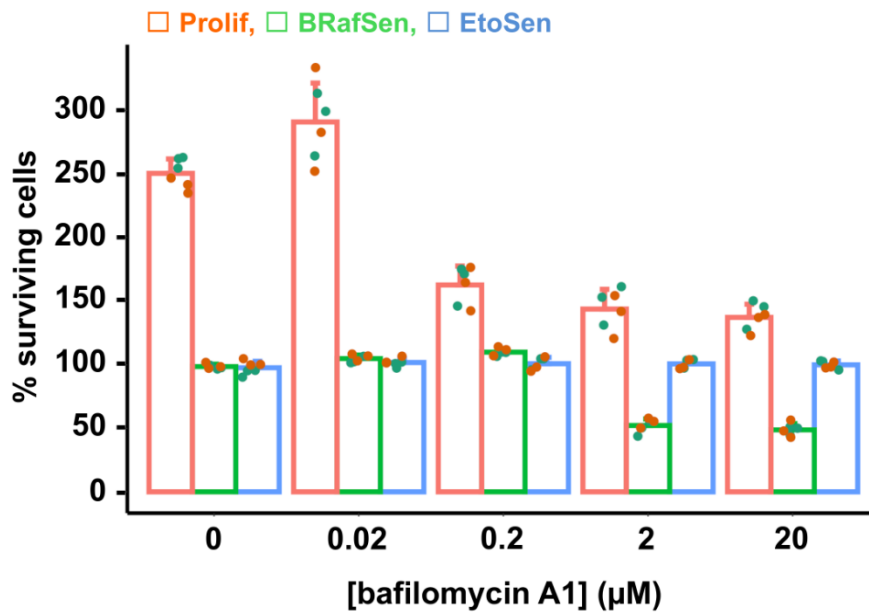


**Figure S7.** Ouabain differentiates between senescent and non-senescent cells overexpressing BRAFV600E, and kills BRafSen cells regardless of BRAFV600E expression level.

L'Hôte et al.



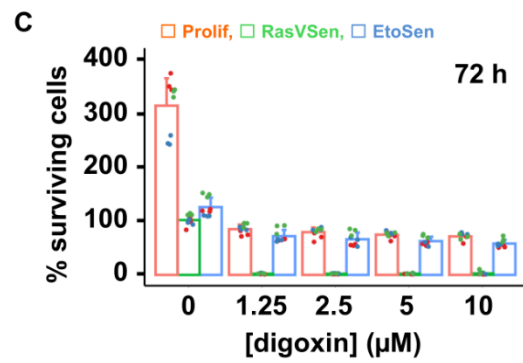
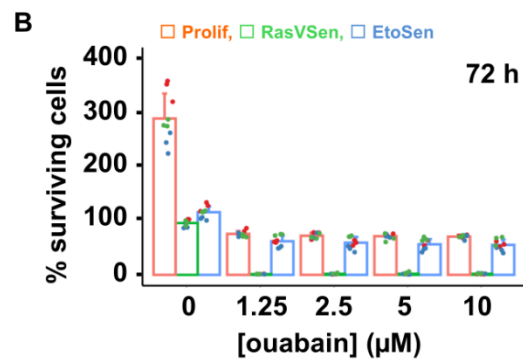
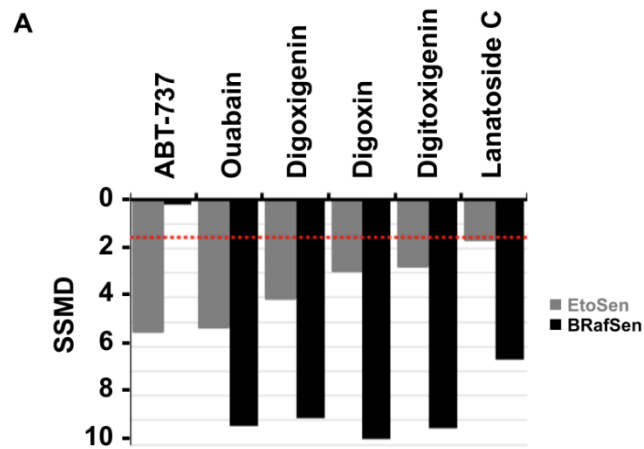
**Figure S8.** Autophagy inhibitor bafilomycin A1 is a senolytic in BRAF senescence.  
L'Hôte et al.





**Figure S9.** Cardioglycosides are broad-spectrum senolytics at higher concentrations.

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**Supporting figure legends**

**Supporting Figure 1: Reversibility of ouabain-induced cytostaticity in proliferating cells.**

**A.** EdU incorporation assay of Prolif BJ cells after ouabain withdrawal. We incubated Prolif cells with 50, 100, or 200 nM ouabain for 72 h. Then, we washed the wells with PBS and further incubated the cells with either the same concentration of ouabain or without ouabain, in the presence of 25 nM EdU for 48 h. We then fixed the cells and performed click-chemistry to measure the percentage of cells that carried out S phase (EdU-positive cells) during EdU incubation in various conditions. We used 20  $\mu$ M etoposide as a negative control for proliferation. Representative experiment of two independent replicates.

**B.** Clonogenicity assay of Prolif cells after ouabain withdrawal. We incubated Prolif cells with 50, 100, or 200 nM ouabain for 72 h. Then, we washed and trypsinized the cells, and reseeded them at low density without ouabain. We used 20  $\mu$ M etoposide as a negative control for proliferation. We cultured the cells for two weeks, renewing medium every 3 to 4 days, then fixed the cells and stained the colonies with crystal violet. Representative experiment of three independent replicates.

**Supporting Figure 2: Ouabain-induced apoptosis hallmarks in BRAF-senescent cells.**

**A.** PARP1 cleavage in BRafSen cells, as assessed by western blotting following incubation with 200 nM ouabain for various periods of time. Revert staining: total protein staining.

**B.** Fluorescence microscopy imaging of BRafSen cells treated with 200 nM ouabain for various periods of time, showing loss of mitochondrial potential (MitoTracker, 13 h), and nuclear condensation (DAPI, 22 h). Representative experiment of two independent replicates.

**Supporting Figure 3: Cardioglycosides digoxin and strophanthidin exhibit the same toxicity profile as ouabain in BJ cells.**

**A.** Dose-response toxicity assay of digoxin in BJ fibroblasts. Prolif: proliferating (red), BRafSen: BRAF-V600E senescent (green), EtoSen: etoposide senescent (blue). We expressed survival as the percentage of viable cells remaining attached to the well after incubation with the drug for 48 h (left) or 72 h (right), normalized to the initial number of cells at the time of drug addition.

**B.** Dose-response toxicity assay of strophanthidin in BJ fibroblasts.

For all panels, colored overlapping dots represent independent replicates.

**Supporting Figure 4: Senolytic activity of reference drugs in BJ cells.**

**A.** Dose-response toxicity assay of navitoclax / ABT-263 in BJ cells. Prolif: proliferating (red), BRafSen: BRAF-V600E senescent (green), EtoSen: etoposide senescent (blue). We expressed survival as the percentage of viable cells remaining attached to the well after incubation with the drug for 48 h (left) or 72 h (right), normalized to the initial number of cells at the time of drug addition. Zoomed-in graphs are provided in the lower row. Data were aggregated from two independent biological replicates.

**B.** Dose-response toxicity assay of alvespimycin / 17-DMAG in BJ cells. Data were aggregated from three independent biological replicates.

**C.** Dose-response toxicity assay of dasatinib in BJ cells. Data were aggregated from three independent biological replicates.

For all panels, colored overlapping dots represent independent replicates.

**Supporting Figure 5: siRNA-mediated knockdown of ATP1A1 in BJ cells.**

**A.** Transcriptomics analysis of Illumina beadarray data reveals that  $\alpha 1$  is the most expressed  $\alpha$  subunit isoform of the Na,K-ATPase pump in BRafSen BJ cells. The NKA $\alpha 1$  protein subunit is encoded by the ATP1A1 gene.

**B.** ATP1A1 mRNA levels in Prolif, BRafSen, and EtoSen cells, as measured by RT-qPCR. Cells had been induced in senescence for 7 days before RNA extraction.

**C.** ATP1A1 mRNA levels in BJ cells following transfection with ATP1A1-targeting siRNAs (si-NKA1 and si-NKA2) for 48 h, as measured by RT-qPCR. Virtually 100% of ATP1A1 mRNA levels are effectively depleted by both siRNAs in all cell lines. NT = no target control siRNA. Data were aggregated from two independent biological replicates.

**Supporting Figure 6: Total protein levels of Src, Erk, Akt, and p38, during ouabain and MB7 treatment, and ouabain and dasatinib treatment.**

**A.** As assessed by western blotting. Protein extracts are identical to those displayed in Figure 4A. Cells were treated with 200 nM ouabain or 200  $\mu$ M MB7. Revert staining: total protein staining.

**B.** As assessed by western blotting. Protein extracts are identical to those displayed in Figure 4B. Cells were treated with 200 nM ouabain with or without 10  $\mu$ M dasatinib for 3 h. Revert staining: total protein staining.

**Supporting Figure 7: ouabain differentiates between senescent and non-senescent cells overexpressing BRAFV600E, and kills BRafSen cells regardless of BRAFV600E expression level.**

**A.** Protein levels of BRafV600E/HA in proliferating and 1-week BRafSen cells seeded 24 h before with (+dox) or without (no dox) 1  $\mu$ g/mL doxycycline, as assessed by western blotting. Revert staining: total protein staining. Representative experiment of four independent replicates.

**B.** Dose-response toxicity assay of ouabain in proliferating and 1-week BRafSen cells seeded 24 h before drug addition with (+dox) or without (no dox) 1  $\mu$ g/mL doxycycline. The cells were treated with ouabain for 48 h. Prolif: proliferating (red), BRafSen: BRAF-V600E senescent (green). We expressed survival as the percentage of viable cells remaining attached to the well after incubation with the drug for 48 h normalized to the initial number of cells at the time of drug addition. Data were aggregated from two independent biological replicates. Colored overlapping dots represent independent replicates.

**Supporting Figure 8: Autophagy inhibitor bafilomycin A1 is a senolytic in BRAF senescence.**

Dose-response toxicity assay of bafilomycin A1 in BJ cells. Prolif: proliferating (red), BRafSen: BRAF-V600E senescent (green), EtoSen: etoposide senescent (blue). We expressed survival as the percentage of viable cells remaining attached to the well after incubation with the drug for 48 h, normalized to the initial number of cells at the time of drug addition. Data were aggregated from two independent biological replicates. Colored overlapping dots represent independent replicates.

**Supporting Figure 9: Cardioglycosides are broad-spectrum senolytics at higher concentrations.**

**A.** Cardioglycosides as senolytic screening hits of the Prestwick library (ouabain: supplementary control) in both BRafSen and EtoSen BJ cells. Cells were incubated with drugs at 20  $\mu$ M for 72 h. Surviving cell count was determined after fixation and nuclei staining using

a CX5 screening microscope. Strictly standardized mean difference (SSMD) was calculated for each compound, and a score of  $SSMD < 1.645$  (red dotted line) was considered a hit.

**B.** Dose-response toxicity assay of ouabain in IMR90 cells. Prolif: proliferating (red), RasVSen: RasVal12 senescent (green), EtoSen: etoposide senescent (blue). We expressed survival as the percentage of viable cells remaining attached to the well after incubation with the drug for 72 h, normalized to the initial number of cells at the time of drug addition. Data were aggregated from three independent biological replicates.

**C.** Dose-response toxicity assay of digoxin in IMR90 cells. Data were aggregated from three independent biological replicates.

For all panels, colored overlapping dots represent independent replicates.

### 3.2. Extended discussion on Article 2.

In Article 2, as previously in (Carvalho et al., 2019), we demonstrated the validity of the approach consisting in screening for bioactive compounds modulating a phenotype of interest, and then deciphering their mechanisms of action in order to uncover regulatory processes. Hence, in (Carvalho et al., 2019), we first found that glucocorticoids delayed or bypassed the entry into BRAF-V600E senescence, and investigating this effect allowed us to identify EGR1 as a sensor of MAPK activity implicated in the kinetics of the senescence-associated proliferative arrest. In Article 2 (L'Hôte et al., 2021), by asking what underlay the remarkable sensitivity of BRafSen cells to senolysis by cardioglycosides, we uncovered an essential role for increased autophagy flux in the survival of these cells.

Our data strongly suggested that although cardioglycosides acted by binding their canonical target NKA, signal transduction rather than ion transport inhibition was responsible for senolysis. We showed that ouabain-induced activation of Src, p38, Akt, and Erk were implicated in senolysis. We demonstrated that p38 activation depended on Src, but the remaining interactions between these pathways and how they inhibit autophagy is yet to be determined. Cardioglycosides activate Src, which physically interacts with NKA (Cui & Xie, 2017). Following release from NKA, Src mediates the activation of the MAPK pathway, including Erk, which can transactivate the PI3K/Akt/mTOR axis (Dai et al., 2013). However, ouabain can also activate PI3K in a Src-independent manner (Jian Wu et al., 2013). There are reports of NKA-mediated p38 activation, but this has been less studied than the Src/MAPK and PI3K/Akt axes (Akimova et al., 2009; Garcia et al., 2015). Src, Akt, p38, and Erk, have all been implicated in context-dependent regulation of autophagy. The thoroughly described Akt-mediated suppression of autophagy, notably via mTOR, can be triggered by Src (Pal et al., 2014; van Grol et al., 2010; G. S. Zhao et al., 2018). Conversely, the activation of p38 can lead to autophagy inhibition through various mTOR-independent mechanisms (Y. He et al., 2018; Henson et al., 2014; Jiang et al., 2014). Interestingly, sustained p38 signaling was also shown to promote autophagy and favor therapy-induced senescence over apoptosis in cancer cells (Slobodnyuk et al., 2019). Finally, how the MAPK pathway modulates

autophagy has been less investigated, but Erk typically mediates autophagy suppression (Bryant et al., 2019; Corcelle et al., 2006).

Although signaling inhibitors 1) restored autophagy flux and 2) prevented senolysis, we did not demonstrate per se a causal implication of autophagy inhibition in senolysis by ouabain independently of NKA signaling, although the aforementioned results together with the phenocopy of ouabain senolysis by chloroquine and bafilomycin A1 strongly suggest that ouabain ultimately exerts its senolytic effect via autophagy inhibition. To definitely demonstrate this point, we could increase autophagy flux in ouabain-treated BRafSen cells through genetic approaches, and show that this prevents senolysis.

In the context of pre-malignant nevi, the relevance of autophagy in cell survival, as well as the senolytic potential of cardioglycosides and autophagy inhibitors, should be assessed in BRafSen melanocytes, as well as in ex-vivo nevi.

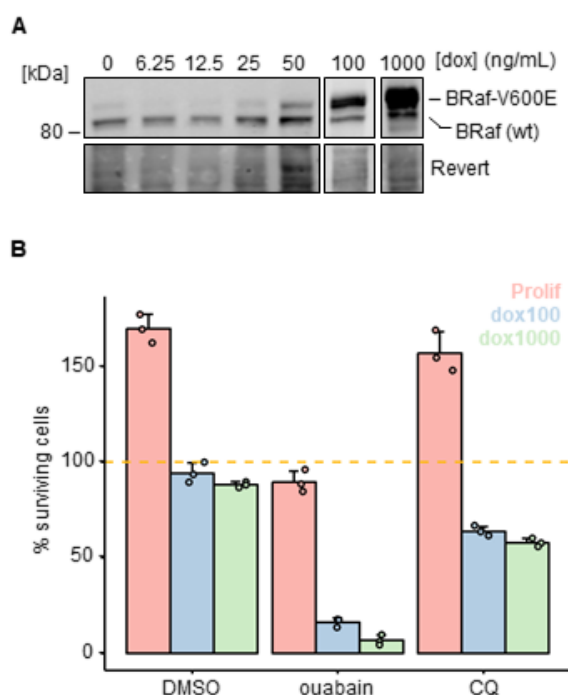
## Chapter 4. Exploring the role of Ire1 in senescent cell survival.

In Article 2 (L'Hôte et al., 2021), we showed that BRAF-V600E-induced senescent (BRafSen) cells had an increased autophagy flux compared to their proliferative counterparts, and that downregulating autophagy with cardioglycosides or blocking autophagosome degradation with chloroquine or bafilomycin A1 led to selective senolysis. We then sought to unravel what physiologically underlay BRafSen cells' dependency to autophagy, and what were the consequences of autophagy blockade or downregulation that led to cell death. In BRAF-V600E-expressing melanoma, chronic endoplasmic reticulum (ER) stress due to increased secretory demands resulted in an increased autophagy flux on which the cells depended for their survival (Corazzari et al., 2015). We thus decided to characterize ER stress and the unfolded protein response (UPR) in various senescence settings.

An important increase in secretory load can overwhelm the folding capacities of the ER, resulting in an accumulation of misfolded proteins in the ER lumen, known as ER stress (Almanza et al., 2019). Other insults to ER homeostasis, such as a depletion in ER calcium stores, may also cause ER stress (Mekahli et al., 2011). In order to return to homeostasis, a signaling response known as the UPR is elicited from the ER, that modulates the expression of genes associated with proteostasis, and can trigger apoptosis if it fails to resolve ER stress. In mammalian cells, the UPR consists of three parallel branches that mediate distinct signaling outcomes, issuing from sensors Ire1 $\alpha$  (thereafter Ire1), Perk, and ATF6. Ire1 mainly promotes adaptation to ER stress, by upregulating genes involved in protein folding and secretion, and decreasing the ER load by upregulating autophagy and degrading ER-targeted transcripts. Perk rather promotes apoptosis, and may be activated after Ire1-mediated adaptation to stress has failed (T. K. Chang et al., 2018; Verfaillie et al., 2012), although it also serves an adaptive role by decreasing the rates of global translation through eIF2 $\alpha$  activation. Finally, ATF6 is thought to coordinate the adaptive and cytotoxic facets of the UPR (Walter et al., 2018; Jun Wu et al., 2007; Yang et al., 2020).



In (L'Hôte et al., 2021), BRAF-V600E expression in BJ fibroblasts was induced with a high dose of 1  $\mu\text{g}/\text{mL}$  doxycycline which results in high protein levels of BRaf-V600E (Carvalho et al., 2019). Since protein overexpression may cause ER stress by itself, in all subsequent studies we switched to a lower dose of 100 ng/mL doxycycline that resulted in BRaf-V600E levels closer to those of endogenous wild-type BRaf (Figure 4.1A). Importantly, we verified that senescent BJ cells induced with either 1  $\mu\text{g}/\text{mL}$  or 100 ng/mL doxycycline had similar sensitivities to senolysis by ouabain or chloroquine (Figure 4.1B). To better mimic the transient exposure to chemotherapeutics in the clinic, we also adapted our protocol for inducing senescence with etoposide. Instead of treating the cells with 20  $\mu\text{M}$  etoposide continuously for 1 week before withdrawing the drug, we treated the cells for 48 h with 50  $\mu\text{M}$  etoposide followed by a 5-days withdrawal period before proceeding with experiments.



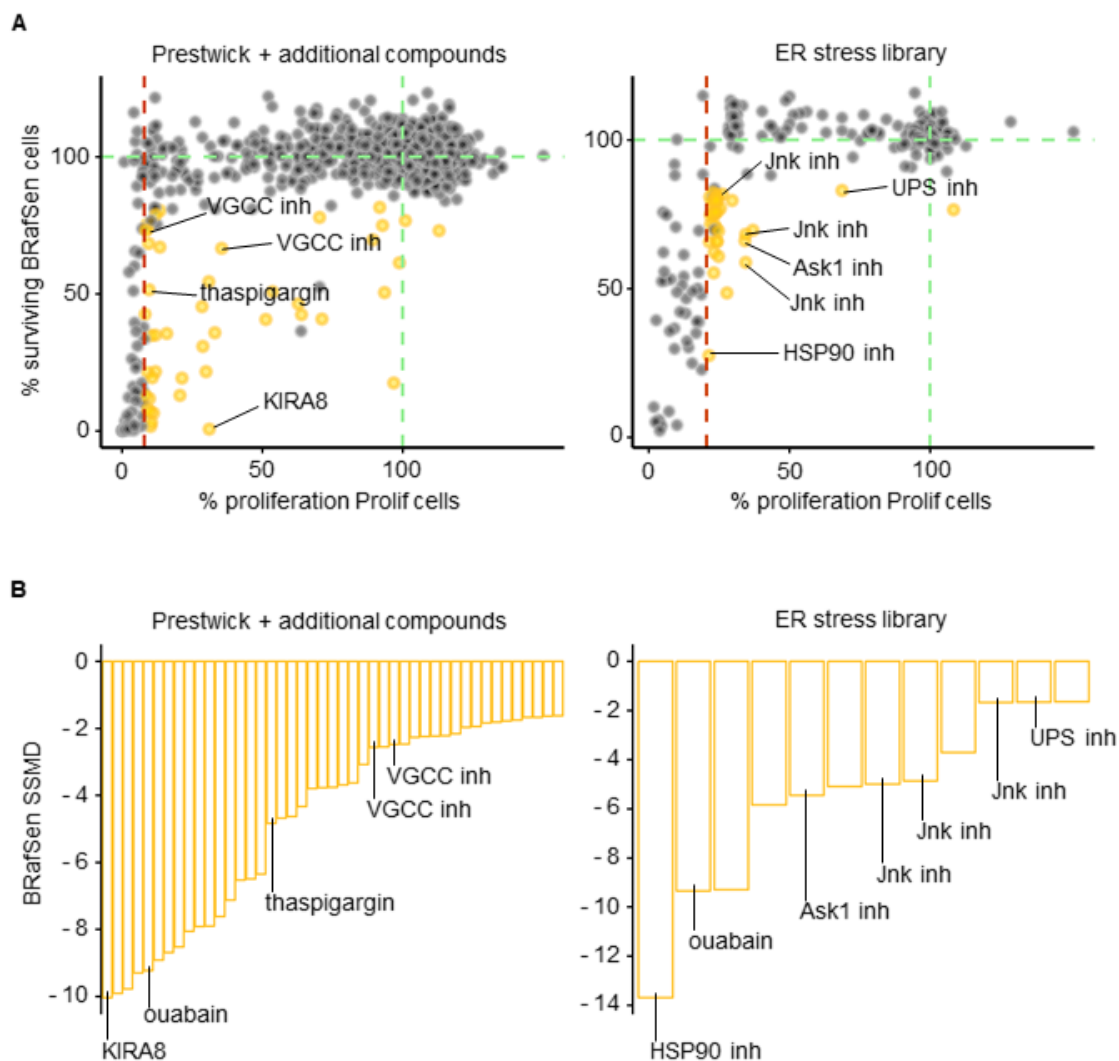
**Figure 4.1. Senescent cells induced with 100 ng/mL doxycycline are still sensitive to senolysis by ouabain and chloroquine.** **A.** Expression of wild-type BRaf and BRaf-V600E after 48 h with doxycycline, as assessed by Western blotting. At 100 nM, the ratio of V600E to WT was 2.3. **B.** Toxicity assay of 400 nM ouabain and 50  $\mu\text{M}$  chloroquine for 24 h in BRafSen cells induced with different doxycycline concentrations.

#### 4.1. Chemical library screening identifies Ire1 pathway inhibitors and compounds affecting proteostasis maintenance as senolytic hits in BRafSen cells.

In parallel to our investigation of ouabain as a senolytic, we decided to search for novel senolytics in BRafSen cells by screening two small molecules libraries: the Prestwick repositioning library (1,520 compounds) to which we added seven inhibitors involved in ER stress transactions, as well as the TargetMol ER stress compound library (155 compounds). Proliferative and BRafSen cells were exposed for 3 days to 20  $\mu\text{M}$  of compounds from both libraries and various

concentrations of the additional inhibitors (see materials and methods), before fixing the plate and assessing survival as described previously (L'Hôte et al., 2021). Of the seven inhibitors added to the Prestwick library, KIRA8 and thapsigargin were identified as hits (Figure 4.2A). KIRA8 is an inhibitor

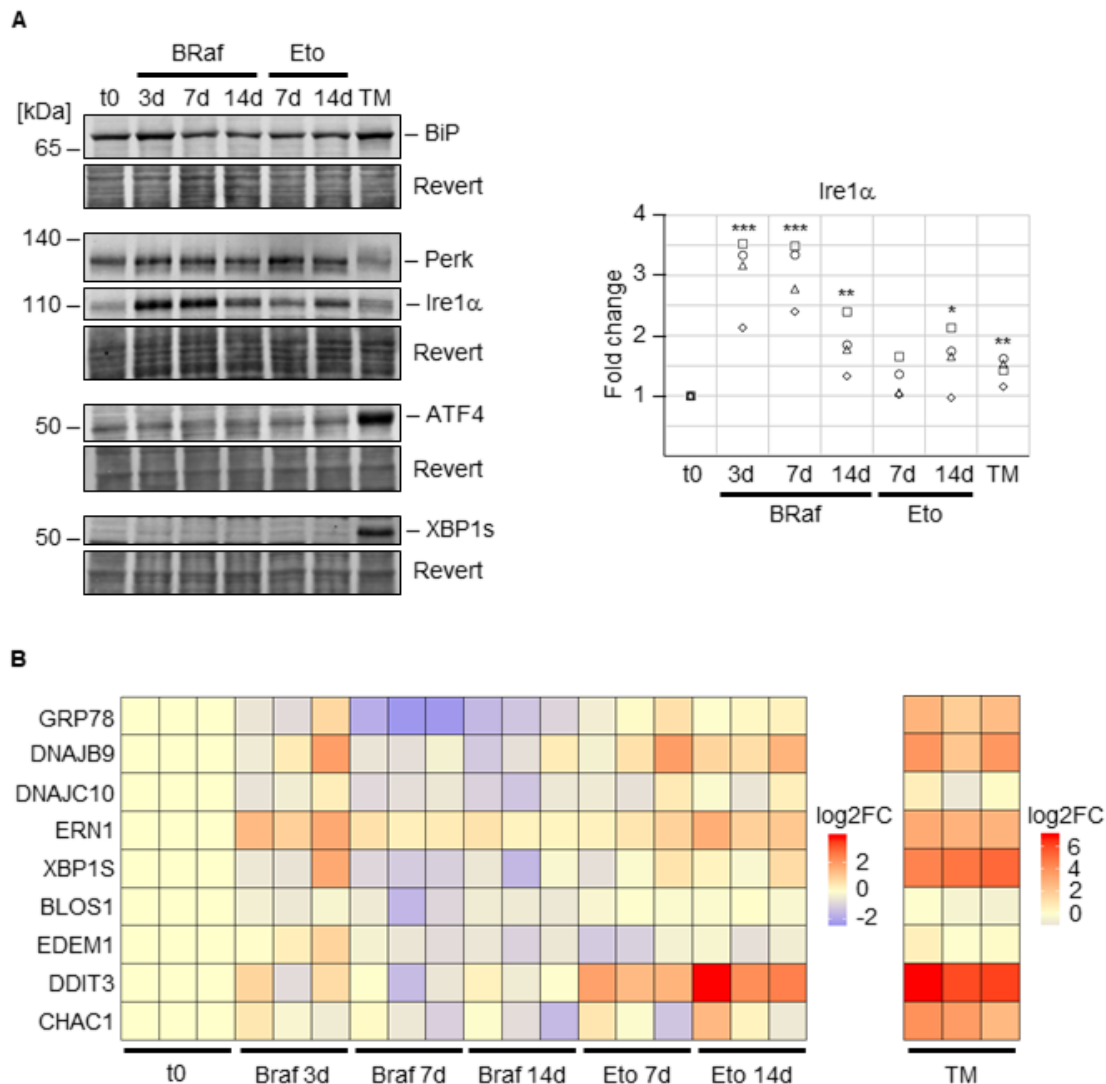
of UPR sensor Ire1, and thapsigargin is an inducer of ER stress through inhibition of the sarco/endoplasmic reticulum Ca<sup>2+</sup> ATPase. Remarkably, KIRA8 killed 100% of BRafSen cells in 72 h, and its SSMD was even lower than that of ouabain (see Materials and Methods) (Figure 4.2B). Interestingly, we identified multiple inhibitors of Jnk as senolytics from the ER stress library, as well as an inhibitor of Ask1. The Traf2/Ask1/Jnk axis is one of the pathways elicited by Ire1 (Zeng et al., 2015). We also identified compounds targeting different facets of proteostasis, such as an inhibitor of HSP90 (other HSP90 inhibitors were previously identified as senolytics in (Fuhrmann-Stroissnigg et al., 2017)) and an inhibitor of the ubiquitin-proteasome system. From the Prestwick library, multiple cardioglycosides as well as two Ca<sup>2+</sup> channel blockers were identified as senolytics. These newly identified candidate senolytics formed a coherent group of drugs targeting proteostasis and/or the endoplasmic reticulum at various levels.



← **Figure 4.2. Senolytic screening of the Prestwick library, an ER stress library, and additional compounds.** **A.** Prolif vs BRafSen surviving cell count for each compound. Cell counts were normalized to vehicle (DMSO, green dotted line). The red dotted line represents the initial number of Prolif cells (treated with etoposide, no proliferation). Senolytic hits are in yellow. Compounds in grey were either not toxic enough in BRafSen cells (SSMD>-1.608), or decreased Prolif cell number below etoposide. **B.** SSMD of all senolytic hits. A compound that was not toxic on Prolif cells was considered a senolytic hit if its associated SSMD for BRafSen cells was <-1.608. Lower SSMDs correspond to increased potency and/or statistical significance.

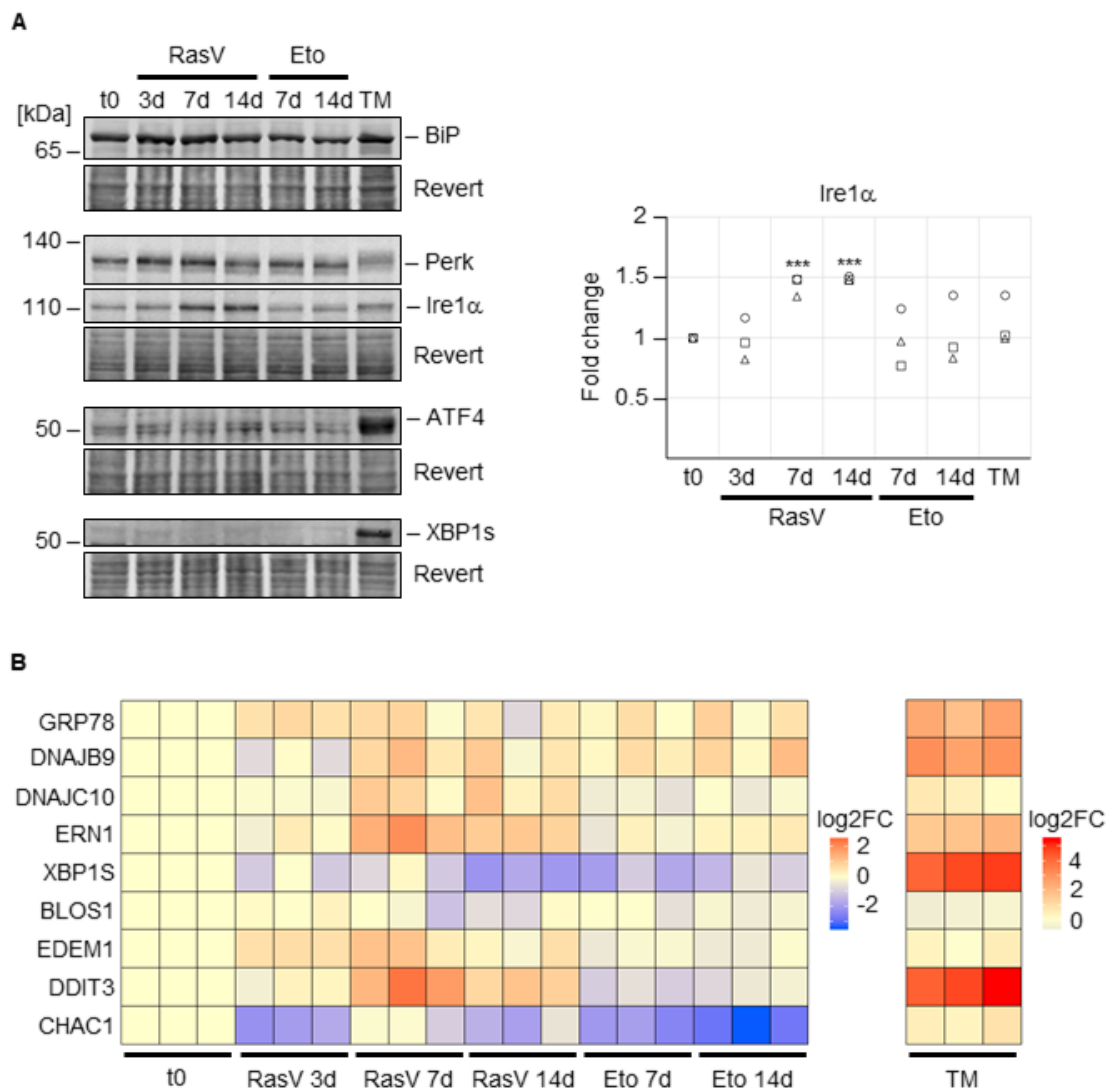
## 4.2. Ire1 protein levels systematically increase in oncogene-induced senescence

We then profiled ER stress and the UPR at various time points during senescence induction in BJ fibroblasts induced by BRAF-V600E or etoposide (EtoSen) (Figure 4.3), in IMR90 fibroblasts induced by HRAS-V12 (RasVSen) or etoposide (Figure 4.4), and in Hermes melanocytes induced by BRAF-V600E (Figure 4.5). Tunicamycin was used as a positive control to induce ER stress and the UPR via inhibition of N-glycosylation. We characterized the UPR at the protein level (Figure 4.3A, Figure 4.4A, Figure 4.5A), and we measured the levels of various transcripts associated with ER stress or with the UPR (Figure 4.3B, Figure 4.4B, Figure 4.5B). Whereas we managed to explore the Ire1 and Perk branches of the UPR at the protein level, we were not able to detect ATF6 by Western blotting. Strikingly, Ire1 $\alpha$  (encoded by ERN1) was systematically upregulated at both protein and mRNA levels in all instances of oncogene-induced senescence (OIS). Activated Ire1 catalyzes the splicing of XBP1u (unspliced) mRNA owing to its RNase activity, uncovering an alternative reading frame and yielding a novel XBP1s (spliced) mRNA which will be translated to a transcription factor that promotes adaptation and survival through the upregulation of autophagy and secretory capacities. Unhindered Ire1 RNase also mediates a process known as regulated Ire1-dependent decay of mRNA (RIDD), in which it degrades ER-targeted mRNAs. Ire1 upregulation was accompanied by an increased basal splicing of XBP1 mRNA in melanocytes but not in fibroblasts. In contrast, RIDD seemed activated in OIS fibroblasts as indicated by decreased BLOS1 mRNA levels, but not in melanocytes. BiP (encoded by GRP78) is an important ER-resident chaperone that plays a role in UPR triggering by binding and keeping UPR sensors inactive in the absence of ER stress, and binding misfolded proteins when they accumulate, thus detaching from UPR sensors allowing them to activate. Variations in the levels of BiP



**Figure 4.3. ER stress and UPR profiling during senescence of BJ fibroblasts.** **A.** Protein levels of UPR components as assessed by Western blotting (left). Representative blots or three or four independent biological replicates. Revert: total proteins. Ire1 levels were normalized to total proteins and then to t0 for densitometric quantification (right). **B.** mRNA levels of ER stress and UPR factors as assessed by RT-qPCR. Three independent biological replicates per condition are shown.

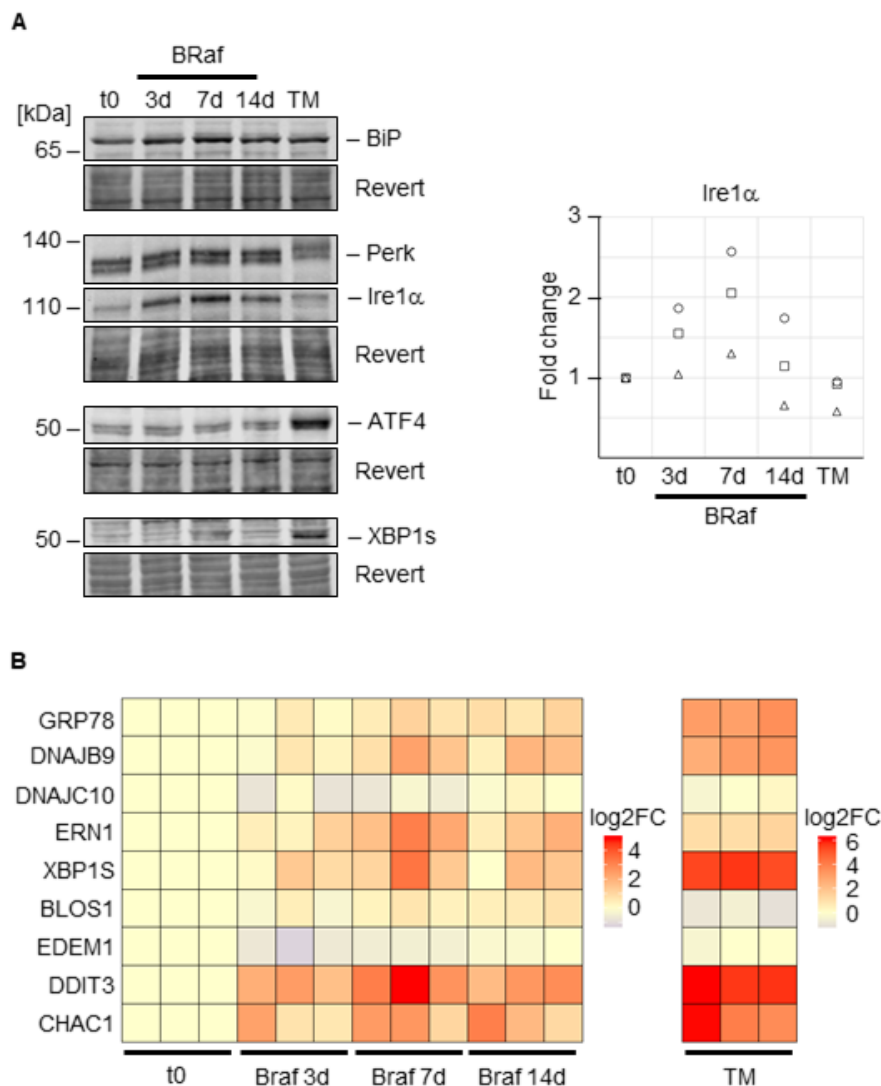
(encoded by GRP78) and its partners ERdj4 (encoded by DNAJB9) and ERdj5 (encoded by DNAJC10) (Pobre et al., 2019) appeared both cell line- and senescence-inducing stress-dependent. The pro-apoptotic DDIT3 (encoding CHOP)/CHAC1 axis was strongly upregulated in BRafSen Hermes cells. Therefore, multiple components of ER stress and the UPR during senescence induction were differentially modulated depending on the cell line and the senescence-inducing stressor. Nevertheless, upregulation of Ire1 on the other hand was a constant in OIS, even though downstream pathways, namely XBP1 splicing and RIDD, were differentially affected between cell lines.



**Figure 4.4. ER stress and UPR profiling during senescence of IMR90 fibroblasts.** **A.** Protein levels of UPR components as assessed by Western blotting (left). Representative blots or three independent biological replicates. Revert: total proteins. Ire1 levels were normalized to total proteins and then to t0 for densitometric quantification (right). **B.** mRNA levels of ER stress and UPR factors as assessed by RT-qPCR. Three independent biological replicates per condition are shown.

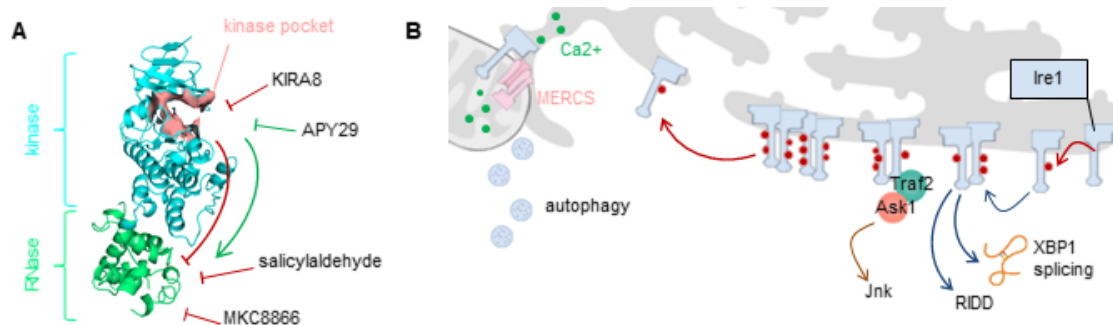
#### 4.3. Modulating Ire1 activity is a novel senolytic strategy.

Ire1 activity is finely tuned by elements of both cis- and trans-regulation (Figure 4.6). Accumulation of misfolded proteins in the ER is sensed by Ire1's luminal domain, whereas Ire1's cytoplasmic domain is responsible for triggering downstream responses. Ire1's cytoplasmic domain has both RNase and kinase activity (Figure 4.6A). Ire1 kinase catalyzes its trans-autophosphorylation, which promotes Ire1 oligomerization and RNase activation (Figure 4.6B). Ire1 phosphorylation may also regulate the termination of its RNase activity (Rubio 2011). Besides its canonical RNase activity, Ire1 acts as a scaffold protein for the triggering of the Traf2/Ask1/Jnk axis (Nishitoh et al., 2002; Zeng et al., 2015).



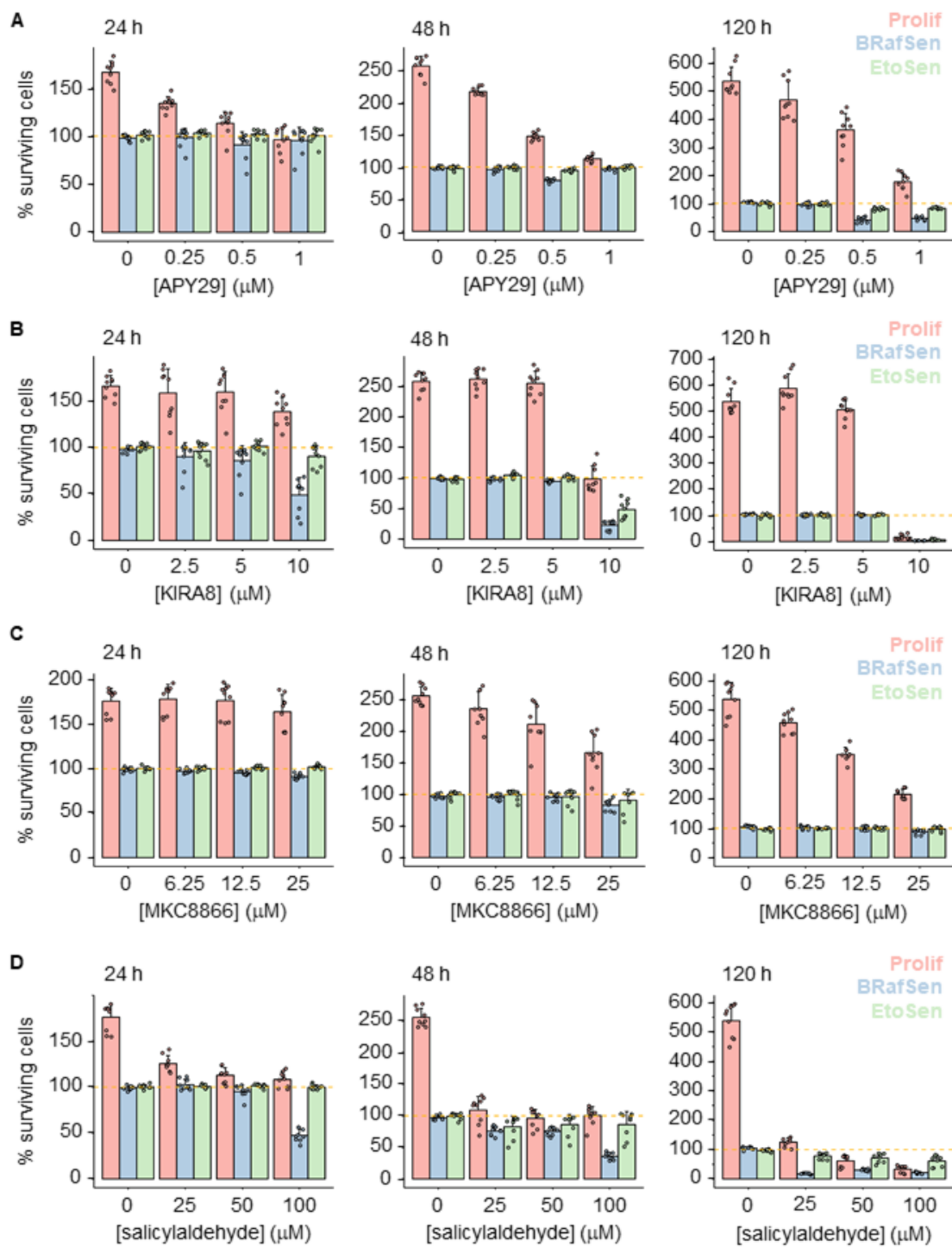
**Figure 4.5. ER stress and UPR profiling during senescence of Hermes melanocytes. A.** Protein levels of UPR components as assessed by Western blotting (left). Representative blots or three independent biological replicates. Revert: total proteins. Ire1 levels were normalized to total proteins and then to t0 for densitometric quantification (right). **B.** mRNA levels of ER stress and UPR factors as assessed by RT-qPCR. Three independent biological replicates per condition are shown.

Ire1 is also essential in the assembling of mitochondria-endoplasmic reticulum contact sites (MERCs) where it regulates calcium homeostasis (Carreras-Sureda et al., 2019). Interestingly, MERCs are essential in autophagosome biogenesis (Hamasaki et al., 2013). In the last ten years, many Ire1 small compound modulators were developed, that differentially affect Ire1 kinase and RNase. 3-ethoxy-5,6-dibromosalicylaldehyde (thereafter salicylaldehyde) and MKC8866 are both hydroxy-aryl-aldehyde (HAA) derivatives that non-competitively inhibit the RNase. MKC derivatives were shown to covalently bind the RNase active site (Sanches et al., 2014), whereas the precise binding site of salicylaldehyde could not be determined (Langlais et al., 2021; Volkmann et al., 2011). KIRA8 is a type II ATP-



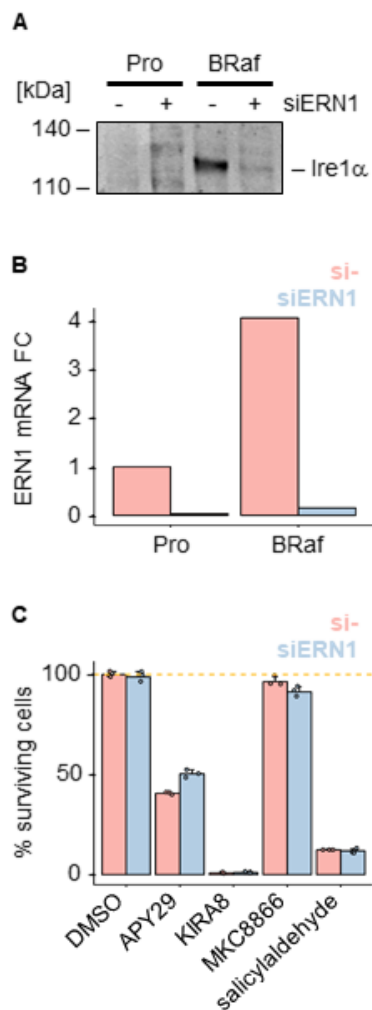
**Figure 4.6. Regulation of Ire1 activity.** **A.** Ire1 cytoplasmic domain (PDB: 4z7g). The kinase is in blue with the kinase pocket in pink. The RNase is in green. **B.** Mechanisms of Ire1 activation. Phosphorylations are in red. Trans-autophosphorylation promotes dimerization and oligomerization, which activates the RNase. Activated Ire1 also serves as a scaffold protein for the activation of the TRAF2/ASK1/JNK axis. Phosphorylation may also be required for the termination of Ire1 activity. Ire1 phosphorylation and oligomerization may regulate its subcellular localization, such as at MERCS.

competitive kinase inhibitor that allosterically inhibits RNase activity by stabilizing an inactive conformation of the kinase pocket (Feldman et al., 2016; Morita et al., 2017). Finally, APY29 is a type I ATP-competitive kinase inhibitor which conversely to KIRA8 stabilizes the kinase pocket in an active conformation, thus allosterically activating the RNase despite preventing phosphorylation (Korennykh et al., 2009; L. Wang et al., 2012). Given the systematic upregulation of Ire1 across different OIS models (Figure 4.3, Figure 4.4, Figure 4.5) and the identification of KIRA8 as a senolytic hit in our screen (Figure 4.2), we decided to profile the toxicity of APY29, KIRA8, MKC8866, and salicylaldehyde in proliferative and senescent BJ cells (Figure 4.7). KIRA8 and salicylaldehyde were both senolytics in the short term (24 and 48 h). After 120 h of incubation, 10  $\mu\text{M}$  KIRA8 eventually indiscriminately killed all cells, while 5  $\mu\text{M}$  did not affect any population. In the screen in which KIRA8 was identified as a hit, the cells were exposed to the drugs for 72 h only, which is therefore not incompatible with an absence of selectivity at 120 h. Whereas 100  $\mu\text{M}$  salicylaldehyde was selectively toxic towards BRafSen cells at 24 and 48 h, it was toxic for all populations at 120 h, but a lower dose of 25  $\mu\text{M}$  was still senolytic for BRafSen cells at 120 h. APY29 was selectively toxic in BRafSen cells too, but the effect only manifested in the long-term after 120 h. Interestingly, MKC8866 was not toxic for either senescent cell population, and only slowed the proliferation of non-senescent cells. Thus, some but not all Ire1 modulators are senolytic in BRafSen cells.



**Figure 4.7. Dose-response toxicity assay of Ire1 modulators in BJ cells.** Survival of Prolif (pink), BRafSen (blue), and EtoSen (green) BJ cells in the presence of APY29 (A), KIRA8 (B), MKC8866 (C), and salicylaldehyde (D), for 24, 48, or 120 h. Surviving cell number at the end-point was normalized to the initial cell number at the time of drug addition.  $n=3$  independent biological replicates.



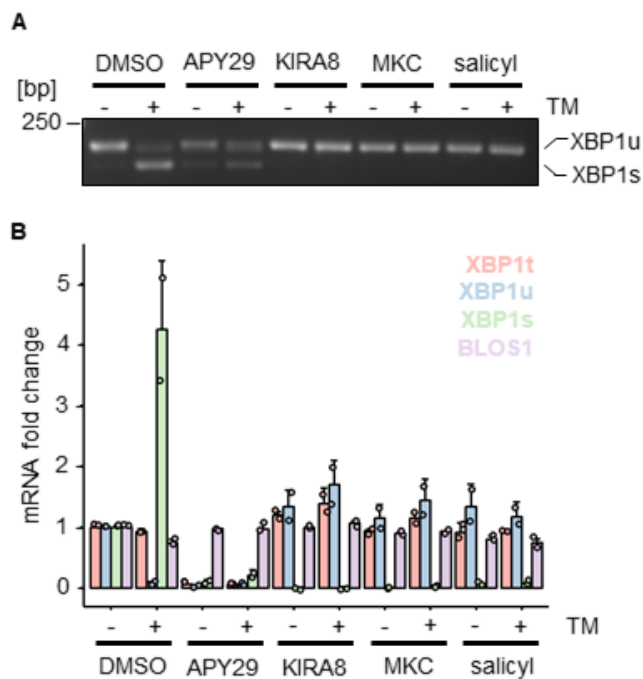


**Figure 4.8. Effect of ERN1 siRNA on senolysis by Ire1 modulators.** Proliferative and BRafSen BJ cells were transfected with the siERN1, and depletion at the protein (A) and RNA (B) levels were checked 48 h later. C. Toxicity assay of 1  $\mu$ M APY29, 10  $\mu$ M KIRA8, 25  $\mu$ M MKC8866, and 100  $\mu$ M salicylaldehyde, for 96 h, 48 h after siRNA transfection.

#### 4.4. The siRNA-mediated knockdown of Ire1 is not senolytic and does not affect senolysis by Ire1 modulators.

Since the inhibition of Ire1 RNase with KIRA8 or salicylaldehyde was senolytic, we asked whether decreasing expression levels of Ire1 would be toxic to BRafSen cells or potentiate the effect of inhibitors. We transfected proliferative and BRafSen BJ cells with an siRNA targeting ERN1 (siERN1) or a no-target control siRNA. 48 h later, the knockdown was evident both at the protein (Figure 4.8A) and the mRNA levels (Figure 4.8B). BRafSen cells were then treated with Ire1 modulators for 96 h (Figure 4.8C). The knockdown of Ire1 in itself was not toxic to BRafSen cells. Surprisingly, the knockdown had no measurable effect on the senolysis by inhibitors. This could be indicative of off-target effects, but the fact that multiple Ire1 modulators are senolytic in BRafSen cells, and that we identified the Ask1/Jnk pathway downstream of Ire1 as a strong candidate target for senolysis in our screen, made it unlikely that all senolytic Ire1 modulators acted through off-target effects. One plausible alternative target of modulators was paralog Ire1 $\beta$  (encoded by ERN2), but its expression is restricted to the gastrointestinal tract and the bronchial epithelium (Bertolotti et al., 2001; Martino et al., 2013), and accordingly we were not able to

detect ERN2 expression by RT-qPCR or Ire1 $\beta$  by Western blotting in our cells. Although we cannot, at this point, exclude that the aforementioned results are independent of Ire1, our working hypothesis is that a small subset of Ire1 $\alpha$  that is not depleted by the siRNA is relevant for senolysis. It may correspond to the band seen in BRafSen + siERN1 extracts (Figure 4.8A). Ire1 molecules from this subset may have a higher half-life, for example due to their integration in protein complexes such as Traf2/Ask1 platforms or MERCS.

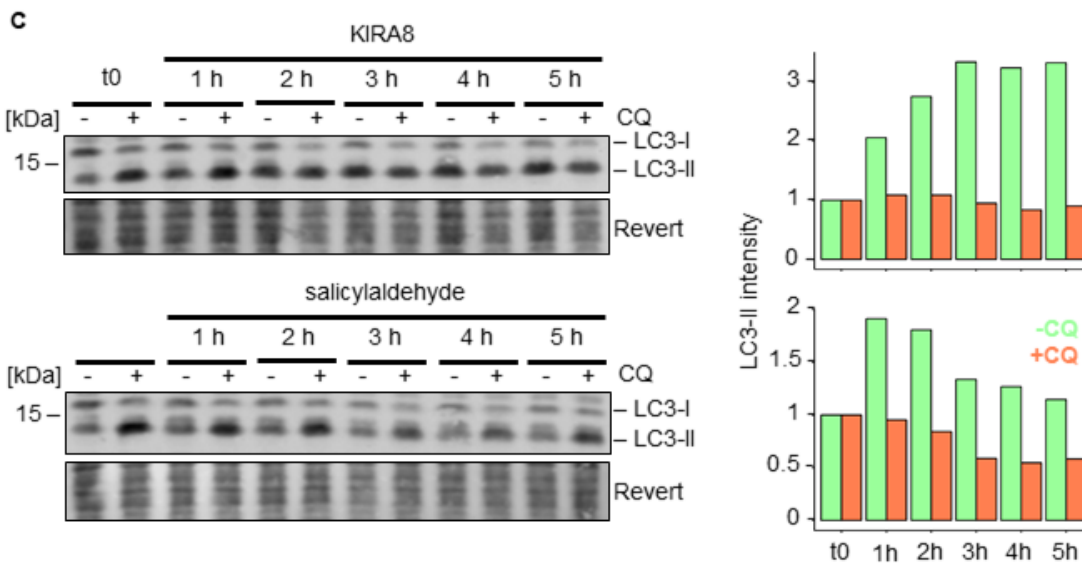
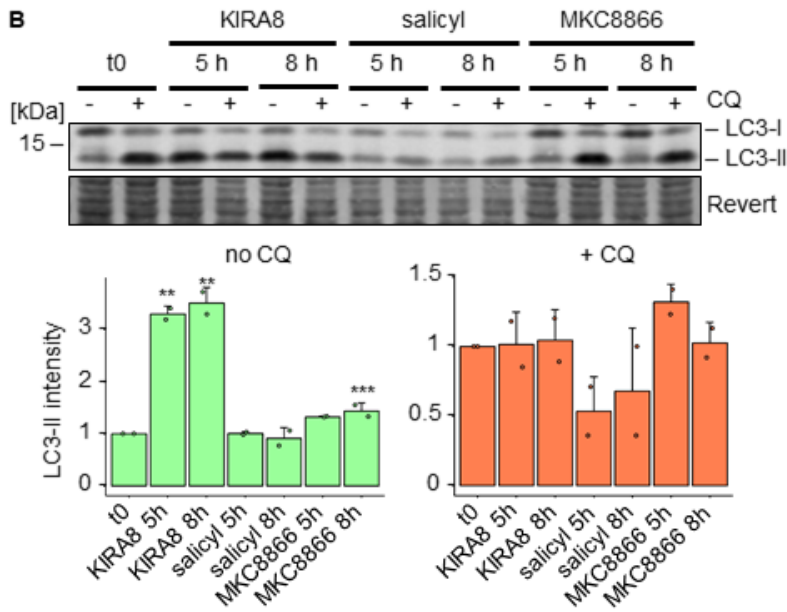
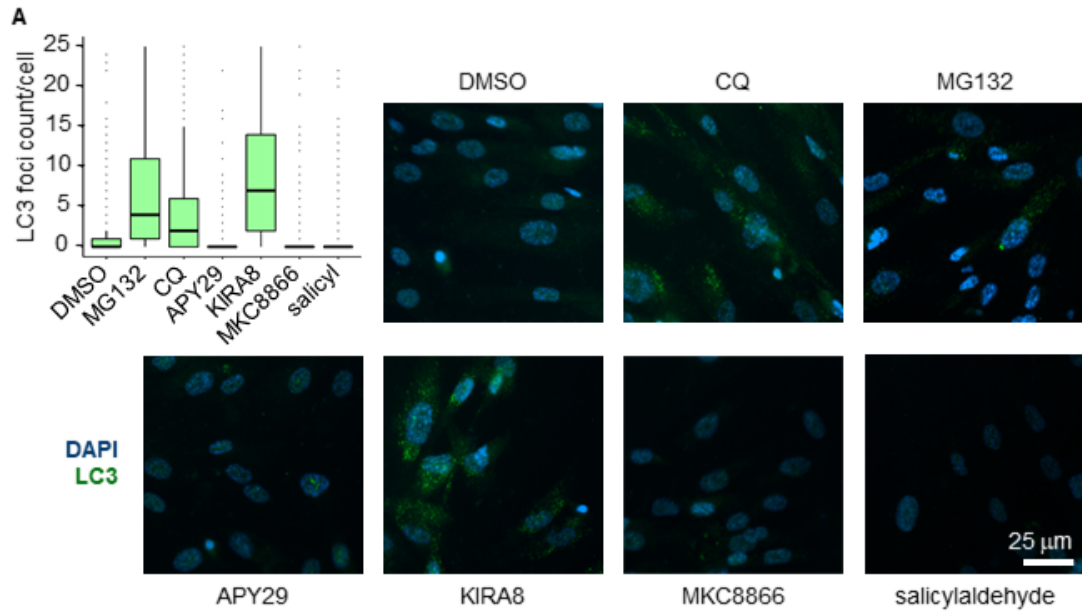


**Figure 4.9. Characterization of the effects of Ire1 modulators on the RNase in BRafSen cells.** The cells were treated with 500 nM APY29, 10  $\mu$ M KIRA8, 25  $\mu$ M MKC8866 or 100  $\mu$ M salicylaldehyde. Tunicamycin was used at 10  $\mu$ g/mL. **A.** XBP1 splicing as assessed by RT-PCR. XBP1u: unspliced; XBP1s: spliced. Representative gel of three independent biological replicates. **B.** Relative mRNA levels of XBP1u, XBP1s, XBP1t (XBP1u+s), and BLOS1 by RT-qPCR. n=2 independent biological replicates.

#### 4.5. Senolysis by Ire1 modulation does not depend on the RNase.

Since the mode of action of Ire1 inhibitors varies, we verified the effects of each compound on XBP1 splicing and RIDD in BRafSen cells. We pre-treated the cells for 2 h with Ire1 modulators at senolytic concentrations (25  $\mu$ M for MKC8866 which was not senolytic for any dose explored) before adding 10  $\mu$ g/mL tunicamycin for 5 h to induce ER stress and the UPR, still in the presence of compounds. As expected, tunicamycin treatment resulted in important XBP1 splicing (Figure 4.9A). Surprisingly, RNase activator APY29 did not induce

XBP1 splicing, and even attenuated the effect of tunicamycin on Ire1 activation. This may be explained by the fact that while it allosterically activates the RNase, APY29 inhibits Ire1 phosphorylation, which is required for full activation of the protein. All other compounds completely prevented the effect of tunicamycin on XBP1 splicing, showing that they all effectively inhibit the RNase. Using recently designed primers (Yoon et al., 2019), we performed a finer analysis of XBP1 splicing by qPCR as well as an assessment of RIDD by measuring BLOS1 mRNA levels (Figure 4.9B). This analysis allowed us to quantify the fold change in the levels of total XPB1 (XBP1t), XBP1u, and XBP1s. Tunicamycin greatly increased XBP1s levels, completely depleted XBP1u levels, while not affecting global XBP1t levels. It mildly activated RIDD. To our surprise, APY29 completely abolished the levels of all XBP1 species while not affecting RIDD, which to our knowledge has not been described before. Once again, KIRA8, MKC8866, and salicylaldehyde, all suppressed XBP1 splicing in the absence or presence of

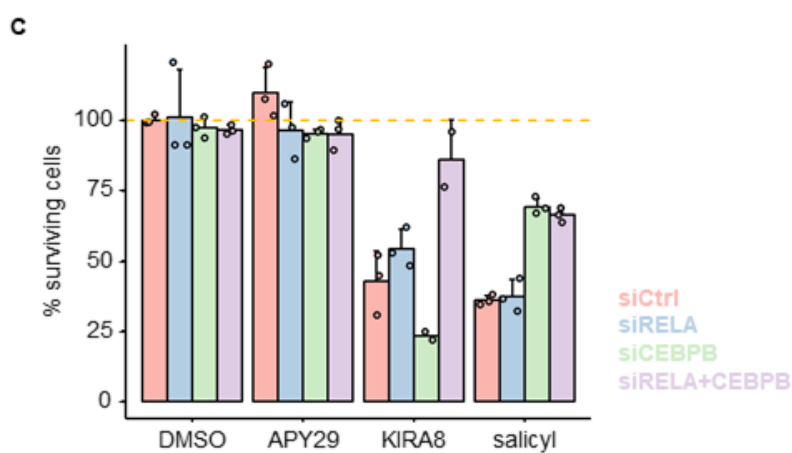
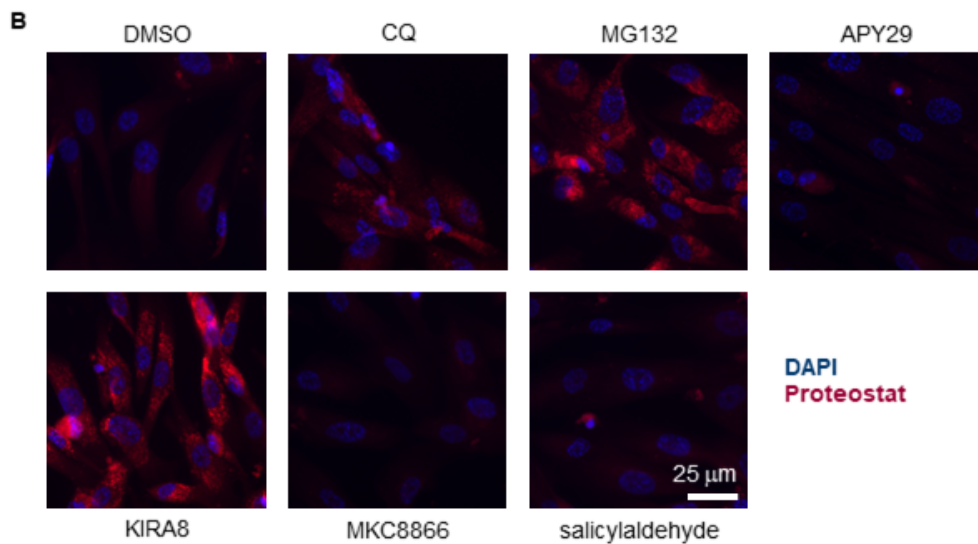
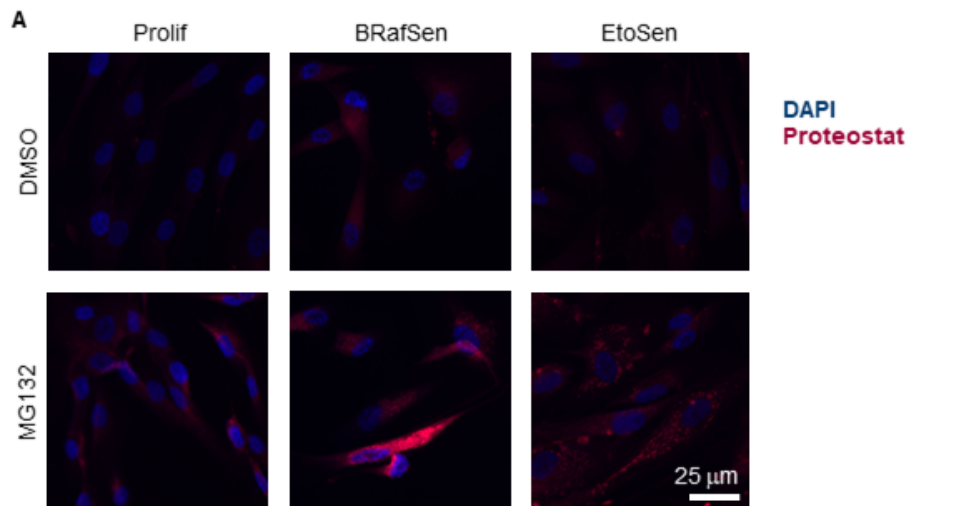


← **Figure 4.10. Exploratory assessment of the effect of Ire1 modulators on autophagy: KIRA8 and salicylaldehyde affect autophagy flux.** **A.** LC3 immunofluorescence in BRafSen treated with Ire1 modulators. Positive controls: chloroquine (CQ) 50  $\mu$ M, MG132 10  $\mu$ M. Quantification: LC3 foci count per cell ( $n > 1,500$  cells per condition). **B.** Autophagy flux of BRafSen cells treated with KIRA8, salicylaldehyde or MKC8866, assessed by LC3 Western blotting in the presence or absence of CQ 50  $\mu$ M in the last 2 h of incubation. Representative blot of two independent biological replicates. LC3-II densitometric quantification: intensity was normalized to total proteins then to t0 (with or without chloroquine). **C.** Autophagy flux of BRafSen cells treated with KIRA8 or salicylaldehyde assessed by Western blotting in the presence or absence of CQ 50  $\mu$ M in the last 1 h of incubation. LC3-II densitometric quantification: intensity was normalized to total proteins then to t0 (with or without chloroquine).

tunicamycin. Salicylaldehyde induced a modest activation of RIDD. Taken together with our senolytic profiling, these results show that senolysis by Ire1 modulators is not mediated by the XBP1 pathway or RIDD. This is consistent with a previously hypothesized non-canonical role of Ire1 targeted for senolysis.

#### **4.6. Exploratory investigation of the role of autophagy in senolysis by Ire1 modulation.**

The difference in senolytic potential between Ire1 modulators could be explained by differential effects on Ire1 phosphorylation, oligomerization dynamics, and subcellular localization, thus modulating processes such as autophagy. Given the long incubation time required for senolysis by APY29, and its strikingly different effects on XBP1 compared to other Ire1 modulators, we supposed that it mediated senolysis through a different mechanism than others, and decided to focus on KIRA8, MKC8866, and salicylaldehyde to understand the mechanisms of short-term senolysis by Ire1 modulation. Among Ire1 modulators, only KIRA8 led to the formation of LC3 foci in BRafSen cells, indicating an accumulation of autophagosomes, that could be due either to increased autophagy flux or blockade of autophagosome degradation (Figure 4.10A). Accordingly, LC3-II levels greatly increased in KIRA8-treated cells as assessed by Western blotting (Figure 4.10B, lower left green barplot). Because of significant cell death as soon as 5 h post-treatment, less proteins were collected from salicylaldehyde-treated cells. We also blocked autophagosome degradation with chloroquine during the last 2 h of each treatment to assess the relative rate of autophagosome formation (Figure 4.10B, lower right orange barplot). Autophagosome formation was not increased with KIRA8. Additive toxic effects of chloroquine and salicylaldehyde on BRafSen cells unfortunately increased variability. We decided to explore more in details the dynamics of autophagy modulation by KIRA8 and salicylaldehyde in the first hours of treatment (Figure 4.10C). KIRA8 never increased autophagosome biogenesis, but quickly induced an accumulation of



autophagosomes in the cell, showing that it blocked autophagosome degradation. Salicylaldehyde also blocked autophagosome formation early on, but later decreased autophagosome biogenesis, resulting in steady-state levels of autophagosomes returning near normal, however with a diminished autophagy

← **Figure 4.11. Exploratory assessment of the effect of Ire1 modulators on proteostasis.** **A.** Visualization of protein aggregates in Prolif, BRafSen, and EtoSen BJ cells using the Proteostat fluorescent probe. Positive control: MG132 10  $\mu$ M. **B.** Visualization of protein aggregates in BRafSen cells treated with Ire1 modulators. **C.** BRafSen cell survival after 24 h treatment with 500 nM APY29, 10  $\mu$ M KIRA8, or 100  $\mu$ M salicylaldehyde, 48 h post-transfection with 5 nM siRNA targeting RELA or CEBPB.

flux. Therefore, both KIRA8 and salicylaldehyde, which are senolytic, decreased or blocked autophagy flux, whereas MKC8866, which is not senolytic, did not.

#### **4.7. Exploratory investigation of the role of proteostasis in senolysis by Ire1 modulation.**

Finally, we assessed the consequences of treatment with Ire1 modulators on proteostasis by visualizing protein aggregates with fluorescent indicator Proteostat. BRafSen BJ cells seemed to have higher basal levels of protein aggregates than Prolif and EtoSen cells, and both BRafSen and EtoSen cells more readily accumulated aggregates upon proteasome blockade, which might reflect higher protein synthesis rates or challenged proteostasis in senescent cells (Figure 4.11A). KIRA8 treatment resulted in a massive accumulation of protein aggregates in BRafSen cells, while salicylaldehyde did not result in protein aggregates formation (Figure 4.11B). Complete blockade of autophagy flux in KIRA8-treated cells might result in the accumulation of aggregates in autophagosomes. Salicylaldehyde-treated cells eventually resolved autophagosome degradation, potentially explaining the absence of protein aggregates at 5 h. We reasoned that decreasing expression of the senescence-associated secretory phenotype (SASP), which may impose a burden on the ER folding capacities and the secretory pathway, might prevent senolysis by KIRA8 or salicylaldehyde. We depleted the expression of RELA and CEBPB, which are master regulators of SASP, using siRNAs, before treating the cells with Ire1 modulators (Figure 4.11C). The double knockdown of RELA and CEBPB improved survival of BRafSen cells upon both KIRA8 and salicylaldehyde treatment. This suggests that SASP synthesis might underlie the sensitivity to senolysis by Ire1 modulation.

#### **4.8. Discussion.**

In Article 2 (L'Hôte et al., 2021), we uncovered an essential role for autophagy in the survival of BRafSen cells. A low-grade basal XBP1 splicing in BRafSen cells suggested a role for chronic ER stress in the requirement for a higher autophagy flux. Simultaneously, we identified KIRA8, an inhibitor of

Ire1, and thapsigargin, an inducer of ER stress, as senolytic hits from the screen of a small collection of compounds affecting ER stress responses. Together, these preliminary results prompted us to investigate the status of ER stress and the UPR in BRAF-V600E senescence.

First, we profiled the UPR at various time points during senescence induction in various models. The most striking result was the upregulation of Ire1 in BRAF-V600E senescence in BJ fibroblasts and in HRAS-V12 senescence in IMR90 fibroblasts. Ire1 seemed to be upregulated too in BRAF-V600E senescence in Hermes melanocytes, but this was not statistically significant. Ire1 levels also increased modestly in late etoposide-induced senescence in BJ fibroblasts. Although Ire1 protein levels remained high in OIS during the time span investigated, the dynamics of this upregulation differed between senescence models at both the RNA and the protein levels. BRafSen BJ cells strongly upregulated Ire1 until 7 days post-oncogene activation, and protein levels decreased at 14 days, although they were still significantly higher than in proliferation. Changes in mRNA levels seemed to precede changes in protein levels. Meanwhile, Ire1 increased continuously during HRAS-V12 induction in IMR90 cells, but the magnitude of the fold change was never as high as in BRafSen BJ cells. This suggests that Ire1 might play distinct roles in the onset of senescence, and in the physiology of established senescent cells. These roles may be uncoupled for investigation by modulating Ire1 activity before inducing senescence, and once senescence is established. The balance between XBP1 splicing and RIDD was previously shown to dictate the entry into RAS senescence in keratinocytes (Blazanin et al., 2017). Moreover, Ire1 was reported to cooperate with ATF6 to shape several facets of the replicative senescent phenotype including the SASP, via upregulation of the COX2/PGE2/EP3 intracrine pathway (Cormenier et al., 2018).

In both BRafSen BJ and RasVSen IMR90, the increase in Ire1 was surprisingly accompanied by a decrease in XBP1 splicing and a mild activation of RIDD. This is in contradiction with our previously reported basal splicing of XBP1 in BRafSen cells versus proliferating cells. This discrepancy could be due to differences in experimental conditions: for the UPR profiling experiment, the cells were seeded and remained plated until proteins and RNA were extracted at a given time point. However, for the assessment of XBP1 splicing in Article 2, proliferative and established BRafSen cells were trypsinized and seeded at similar densities, and proteins and RNA were collected the day after, possibly generating

stress associated with trypsinization and re-plating. A similar experimental design was used for evaluating the effects of Ire1 inhibitors on XBP1 splicing, where established BRafSen BJ cells were trypsinized and seeded at a given density, and were treated the following day with inhibitors. In Figure 4.9A, a modest basal XBP1 splicing that was suppressed by inhibitors was seen in vehicle-treated cells in the absence of tunicamycin, similar to what was observed in Article 2. Thus, it appears from Figure 4.9 that BRafSen cells may not exhibit a basal XBP1 splicing, but that they more readily trigger an UPR in response to stress such as trypsinization and re-plating, probably because of the upregulation of Ire1, explaining the small amounts of XBP1s detected in Article 2 and in Figure 4.9A.

Overall, the one unequivocal common trend from this series of experiments was an important upregulation of Ire1 in OIS. We thus decided to assess the senolytic potential of various Ire1 modulators that differentially affect the RNase and/or kinase activity in BJ cells (Figure 4.6A and Figure 4.7). We confirmed the senolytic activity of KIRA8 that killed most BRafSen cells and about half of EtoSen cells in 48 h. A longer treatment of 120 h however resulted in the indiscriminate death of senescent and proliferating cells. 3-ethoxy-5,6-dibromosalicylaldehyde (salicylaldehyde) exhibited a similar profile, although at the concentration of 25  $\mu$ M, it was selectively senolytic for BRafSen cells in the long term without being toxic to proliferative cells. However, MKC8866 was not senolytic for any condition tested. Finally, APY29 was selectively senolytic in BRaf senescence at 120 h. These results were puzzling, notably because salicylaldehyde and MKC8866 both inhibit the RNase without affecting the kinase, but only salicylaldehyde was senolytic. However, although they are derived from the same scaffold, it is not certain that they bind to the same site (Langlais et al., 2021), and may therefore hold different effects with regards to Ire1 oligomerization dynamics.

Another surprising result was the absence of senolytic effect or potentiation of Ire1 modulators by the siRNA-mediated depletion of the protein. As detailed above, it seems unlikely that senolysis by the different Ire1 modulators would be imputed only to off-target effects. We hypothesize that a small subset of Ire1 molecules persist following siRNA transfection, and that only this subset is relevant for senolysis. The persistence of this essential Ire1 subset could be due to increased protein half-life, for example due to incorporation in protein complexes. An essential step to test this hypothesis will be to generate an



ERN1 knock-out (KO) cell line. The induction of BRAF-V600E in the ERN1-KO cell line will allow to study the implication of Ire1 in senescence establishment, as well as to test its essentiality. An alternative possibility would be to knock-down ERN1 with siRNAs before and during senescence induction, aiming at blocking the emergence of the long half-life Ire1 fraction. It would also be helpful to visualize Ire1's subcellular localization in fluorescence microscopy. Unfortunately, we have not been able to do so as our anti-Ire1 antibody was non-specific in immunofluorescence, so we might have to circumvent this issue by generating a tagged-Ire1 cell line or alternatively assess Ire1, in MERCS for instance, by subcellular fractionation and Western blotting (Mori et al., 2013).

We still decided to verify the effect of inhibitors on Ire1 RNase activity in BRafSen cells. As expected, KIRA8, MKC8866, and salicylaldehyde, potently inhibited XBP1 splicing. Salicylaldehyde modestly activated RIDD. Therefore, these results demonstrated that inhibition of the RNase did not underlie senolysis by Ire1 modulation. We reported an unexpected striking effect of APY29, which dramatically degraded all XBP1 RNA species. To our knowledge, this has not been described before, but XBP1 levels may not have been relatively quantified by RT-qPCR in previous studies following APY29 treatment, like we did here. Since APY29 stabilizes the kinase in its active conformation, it allosterically activates the RNase, which in BRafSen cells may then promote XBP1 degradation in a RIDD fashion instead of mediating the specific splicing of XBP1<sub>u</sub> to XBP1<sub>s</sub>. Of note, APY29 did not induce the degradation of RIDD target BLOS1, and its effect thus seems specific of XBP1. APY29 however prevents phosphorylation, which precludes full activation of Ire1 and may alter its oligomerization. We should assess the effect of APY29 in proliferating and EtoSen cells, to determine whether this XBP1-degrading effect is dependent on a specific regulation of Ire1 in BRaf senescence. It is possible that APY29 induces the senolysis of BRafSen cells because of the long-term suppression of XBP1 signaling, although we need to assess the levels of XBP1 species at later time points. Beyond senolysis, the strong effect of APY29 on XBP1 degradation in BRafSen cells provides an original starting point to better characterize the intracellular regulation of Ire1 activity.

Of note, from this point onwards the rest of the study was exploratory. I chose to include these data in the thesis because they provide stimulating grounds for discussion and draw promising perspectives, but

please note that some of these experiments were performed only once and should therefore be repeated before reaching firm conclusions. Ire1 may regulate autophagy through various routes. RIDD-mediated degradation of BLOS1 mRNA promotes the microautophagy of protein aggregates (Bae et al., 2019). However, we showed that neither XBP1 nor RIDD was relevant for senolysis. Our main hypothesis was that Ire1 modulators triggered senolysis depending on their effect on Ire1 oligomerization and subcellular localization, and that therefore only a small subset of Ire1, for example at the MERCS (Carreras-Sureda et al., 2019), would be relevant for senolysis. MERCS are important structures for autophagosome formation (Hamasaki et al., 2013). Ire1 architectural roles also extend to its integration in signaling platforms for the activation of Traf2/Ask1/Jnk (Nishitoh et al., 2002; Zeng et al., 2015), and we identified several inhibitors targeting Ask1 and Jnk in our screen. Ire1 was recently shown to increase autophagy through the Traf2/Ask1/Jnk pathway (Liu et al., 2020). We thus decided to explore the effects of Ire1 modulators on autophagy. Both short-term senolytics KIRA8 and salicylaldehyde decreased or blocked autophagy, and MKC8866 which is not senolytic did not. The blockade of autophagy by KIRA8 was accompanied by a strong accumulation of intracellular protein aggregates. Still, the effect of Ire1 modulators on autophagy might be correlated to senolysis but not causative, and Ca<sup>2+</sup> transfer at MERCS (Carreras-Sureda et al., 2019; Wiel et al., 2014; Ziegler et al., 2021) or inhibition of Jnk pro-survival signaling (Q. Wu et al., 2019) might be relevant for senolysis, especially given that KIRA8 and salicylaldehyde seem to affect autophagy in different ways. Therefore, the main hypotheses that we will explore, are that senolysis by Ire1 modulation is mediated by:

- The perturbation of Ire1 integration in signaling platforms for the activation of the Traf2/Ask1/Jnk pathway. We will investigate this by modulating Ask1 and Jnk, and assessing the interactions of Ire1 with Traf2/Ask1 by co-immunoprecipitation and proximity ligation assay.
- The perturbation of Ire1 integration in MERCS. This would result in alteration of autophagy and/or calcium homeostasis. We identified several calcium channel blockers as senolytic hits in our screen.

Furthermore, we should assess the senolytic potential of Ire1 modulators in senescent models of IMR90 fibroblasts and Hermes melanocytes. The Jnk pathway should be explored, especially because NF- $\kappa$ B and CEBPB were showed to be modulated by Ire1 too. Of note, ERdj4, whose expression was modulated during senescence induction (Figure 4.2, Figure 4.3, Figure 4.4) negatively regulates Ire1 activation by favoring its monomerization (Amin-Wetzel et al., 2017), and could therefore be implicated in the sensitivity of senescent cells to senolysis by Ire1 modulation.

#### **4.9. Materials and methods.**

##### **Materials and reagents**

We used the following drugs: KIRA8 (HY-114368, MCE), MKC8866 (S8875, Selleckchem), APY29 (SML2381, Sigma-Aldrich), 3-ethoxy-5,6-dibromosalicylaldehyde (SML0149, Sigma-Aldrich), chloroquine diphosphate (C6628, Sigma-Aldrich), ouabain (O3125, Sigma-Aldrich), etoposide (E1383, Sigma-Aldrich), doxycycline (D3447, Sigma-Aldrich), 4-hydroxytamoxifen (H6278, Sigma-Aldrich), tunicamycin (T7765, Sigma-Aldrich).

##### **Cell culture and senescence induction**

BJ1-hTERT fibroblasts were purchased from Clontech, and the BJ1-hTERT/pTRIPz-3HA-BRAFV600E (BJ) was generated as described previously (Carvalho et al., 2019). BJ cells were cultured in MEM (Gibco 51200, Thermo Fisher Scientific) with 9% FBS (Eurobio), 2 mM Glutamax (A12860, Gibco), 1X MEM non-essential amino acids (M7145, Sigma-Aldrich), 1mM sodium pyruvate (S8636), at 37°C and 5% CO<sub>2</sub> in ambient oxygen. IMR90/ER-HRASV12 fibroblasts were a kind gift from Masashi Narita (Cambridge Research Institute, CRUK, Cambridge, UK), and the immortalized IMR90-hTERT/ER-HRASV12 (IMR90) cell line was generated as described in (Jeanblanc et al., 2012). IMR90 cells were cultured in high glucose DMEM (Gibco D6429, Thermo Fisher Scientific) with FBS 9%, at 37°C, 5% CO<sub>2</sub>, and 5% O<sub>2</sub>. Hermes1-hTERT melanocytes were a kind gift from Dot Bennett (St George's University of London, London, UK). Hermes1-hTERT cells were transduced with pTRIPz-3HA-BRAFV600E. However, since Hermes1 had been immortalized with a pBabe-puro-hTERT vector, we could not further select for puromycin resistance. Clones were thus picked by seeding the cells

previously transfected with pTRIPz-3HA-BRAFV600E at low density and locally trypsinizing colonies. Hermes were cultured in RPMI (Gibco 61870036, Thermo Fisher Scientific) supplemented with 9% FBS, 1X penicillin-streptomycin (P0781, Sigma-Aldrich), 200 pM cholera toxin (C8052, Sigma-Aldrich), 200 nM TPA (P1585, Sigma-Aldrich), 176 pM  $\beta$ -FGF (SRP3043, Sigma-Aldrich), and penicillin-streptomycin 1X (P0781, Sigma-Aldrich), at 37°C and 5% CO<sub>2</sub> in ambient oxygen. All cells were regularly passaged before reaching confluence and checked for mycoplasma contamination.

BRAF-V600E senescence was induced in BJ fibroblasts and Hermes melanocytes with 100 ng/mL doxycycline renewed every 2 to 3 days for 1 week before proceeding with subsequent experiments. Doxycycline was kept in the media for the whole duration of experiments. HRAS-V12 senescence was induced in IMR90 fibroblasts with 100  $\mu$ M 4-hydroxytamoxifen renewed every 2 to 3 days for 1 week before proceeding with subsequent experiments. 4-hydroxytamoxifen was kept in the media for the whole duration of experiments. DNA damage-induced senescence was triggered in BJ and IMR90 fibroblasts with 50  $\mu$ M etoposide for 48 h. The drug was removed and the cells were allowed to recover for 5 more days before proceeding with subsequent experiments. Thus, etoposide was not kept in the media during experiments with established senescent cells.

### **Screening of small compound libraries**

We induced BRAF-V600E expression for 1 week in BJ cells prior to screening. For the Prestwick repositioning library screening, we had induced senescence 1  $\mu$ g/mL doxycycline. For the TargetMol ER stress compound library (L9700) screening, we induced senescence with 100 ng/mL doxycycline. We renewed doxycycline every 2 to 3 days. We trypsinized proliferative and senescent BJ cells and seeded them in multi-well plates. Doxycycline was kept in the media of senescent cells during the screen. The day after seeding, we treated the cells with compounds from the library in triplicate wells. The final concentration of compounds from both libraries was 20  $\mu$ M. We added the following compounds to the Prestwick library at the indicated final concentrations: 10  $\mu$ M KIRA8, 10  $\mu$ M MKC8866, 20  $\mu$ M STF-083010, 1  $\mu$ M thapsigargin, 8  $\mu$ M HA15, 10  $\mu$ M PDD005, 10  $\mu$ M roscovitine. We also treated the cells with 200 nM ouabain (positive senolytic control) and 20  $\mu$ M etoposide (negative proliferation control).

For both screens, the final concentration of DMSO in all wells was 0.2%. The cells were incubated with the compounds for 3 days, then we fixed surviving cells and stained their nuclei in 1% formaldehyde, 0.1% Triton X-100, 10 µg/mL Hoechst-33342 for 30 min at room temperature. We then replaced the fixation solution with PBS, and the number of surviving cells per well was automatically counted with a CellInsight CX-5 high-content microscope.

The number of cells in each well was expressed as a percentage of the mean number of cells treated with DMSO (vehicle). For each compound, including controls, in each cell population, we calculated the mean and the standard deviation (SD) of surviving cell counts. We first eliminated all compounds from hit selection that resulted in a final count of proliferative cells below their initial number (etoposide control) minus 1 SD and below the final number of proliferative cells treated with ouabain minus 1 SD (ouabain is cytostatic and may result in a slightly quicker proliferative arrest than etoposide). Hit selection was then performed in senescent cells from the remaining compounds. Selection was based on the strictly standardized mean difference (SSMD) assuming unequal variances, comparing the effect of compounds from the library to the DMSO negative control. The base rate of false negatives was set to 2.5%, corresponding to a SSMD threshold of -1.608, a standard for screening of chemical or siRNA libraries (X. D. Zhang, 2007, 2008; X. D. Zhang et al., 2010). Thus, a compound was considered a hit if its SSMD was < -1.608. The SSMD for a given compound was calculated as the difference between the mean cell count for the compound and the mean cell count for DMSO, divided by the square root of the sum of the squares of cell count SDs of the compound and the DMSO control:

$$SSMD = \frac{mean_{compound} - mean_{DMSO}}{\sqrt{SD_{compound}^2 + SD_{DMSO}^2}}$$

### **Dose-response toxicity assays**

We performed dose-response toxicity assays in proliferative and senescent cells as described previously (L'Hôte et al., 2021). We seeded the cells in 96-well plates the day before treatment. Proliferative cells were seeded at a density 4,000 cells/well, and senescent cells were seeded at a density of 10,000 cells. The day after, we treated the cells with inhibitors, vehicle (often DMSO) and 20 µM etoposide in

triplicate wells. Etoposide induced an instant proliferative arrest of non-senescent cells but no apoptosis, and had no effect on senescent cells. Thus, counting cells treated with etoposide at the end of the experiment allowed us to retrieve the initial number of cells at the time of drug addition. At the end of the incubation, we fixed the cells and stained their nuclei in 1% formaldehyde, 0.1% Triton X-100, 10 µg/mL Hoechst-33342 for 30 min at room temperature. We then replaced the fixation solution with PBS, and the number of surviving cells per well was automatically counted with a CellInsight CX-5 high-content microscope. Final cell numbers were expressed as a percentage of the initial cell number.

### **Protein extraction and Western blotting**

To analyze UPR proteins as well as BRaf expression, we trypsinized the cells and washed them in PBS, before extracting proteins in RIPA buffer containing protease and phosphatase inhibitors (B14001 and B15001A/B, Bimake) for 30 min on ice. We cleared protein lysates by centrifugation at 17,000 g for 15 min at 4°C, and we added Sample Buffer. Final 1X Sample Buffer composition was: 250 mM Tris (T1503, Merck), 70 mM sodium dodecylsulfate (SDS) (1066934, Gibco), 0.4 mM ethylenediaminetetraacetic acid (EDTA) (8418, Merck), 1 M glycerol (24388.320, VWR Chemicals), 0.015% Serva Electrophoresis Serva Blue G (3505002, Thermo Fisher Scientific), 2.5% 2-mercaptoethanol (805740, Merck). We heated the samples at 37°C for 5 min, aliquoted them and stored them at -80°C until loading onto Bis-Tris (B7535, Sigma) acrylamide (A7802, Sigma) gels for electrophoresis.

For autophagy studies, we flash-froze the cells in an ethanol-dry ice bath and put the plates at -80°C at least overnight. Then, we extracted proteins by directly scraping cells on ice in Sample Buffer 1X. We heated the samples at 70°C for 5 min, aliquoted them and stored them at -80°C until loading onto Bis-Tris acrylamide gels for electrophoresis.

We then transferred proteins onto nitrocellulose membranes (10600001, GE Healthcare Life Sciences). We blocked membranes in Intercept TBS Blocking Buffer (Li-Cor) diluted 1:1 in TBS for 1 h at room temperature and incubated membranes overnight with the following primary antibodies diluted in blocking buffer at the indicated concentrations: mouse anti-Irel $\alpha$  (SCBT sc-390960, 1/500), rabbit anti-

Perk (CST 3192S, 1/500), rabbit anti-BiP (CST 3177S, 1/1,000), rabbit anti-ATF4 (CST 11815S, 1/500), rabbit anti-XBP1S (CST 12782S, 1/500), rabbit anti-LC3B (CST 2775S, 1/500), mouse anti-BRaf (SCBT sc-5284, 1/1,000). The day after, we washed the membranes in TBS containing 0.1% Tween-20, and we performed NIR-secondary antibody (IRDye goat anti-mouse / anti-rabbit IgG (H+L) 680RD / 800CW, Thermo Fisher Scientific) incubation in TBS containing 0.1% Tween-20, for 1 h at room temperature. We washed the membranes again and imaged them with an Li-Cor Odyssey CLx scanner. We then stained total proteins with Revert Staining (Li-Cor) according to the manufacturer's instructions. For protein quantification by densitometry, the band intensity (after local background subtraction) within a lane was normalized to total proteins (Revert), and then normalized to control or t0.

### **siRNA transfection**

We performed reverse transfection, ie, the cells were seeded and transfected with the siRNA at the same time. We incubated the siRNAs (Ambion Life Technologies) with Lipofectamine RNAiMAX Reagent (Invitrogen, Thermo Fisher Scientific) in Opti-MEM (31985070, Gibco) for 25 min at room temperature before adding the transfection mix to cell suspensions. The final concentrations of reagents with cells were: 5 nM siRNA (for multiple depletions, 5 nM of each siRNA), 0.1% RNAimax, and 25% OptiMEM. siRNAs were used against: ERN1 (s200430), RELA (s11914), CEBPB (s2891). The cells were incubated with siRNAs for 48 h before assessing knockdown by Western blotting or RT-qPCR, or performing further treatments.

### **RNA extraction, reverse transcription, qPCR, and XBP1 PCR**

We trypsinized the cells and washed them in PBS, before extracting RNA with the NucleoSpin RNA Plus kit (Macherey-Nagel) according to the manufacturer's protocol. We eluted RNA in ddH<sub>2</sub>O and quantified RNA with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). We performed reverse transcription (RT) on 500 ng RNA per sample with 10.2 ng/ $\mu$ L random hexamer primers (Invitrogen, Thermo Fisher Scientific), 250  $\mu$ M dNTP, and 4 U/ $\mu$ L Maxima Reverse Transcriptase

(Thermo Fisher Scientific) in M-MLV Reverse Transcriptase buffer (Thermo Fisher Scientific) for 10 min at 25°C, 30 min at 50°C, 5 min at 85°C.

For qPCR, cDNA were diluted 1/5 in ddH<sub>2</sub>O and then 8/25 in qPCR reaction mix. qPCR performed with a Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific) with 300 nM primers, monitored on an IQ5 apparatus (Bio-Rad). Thermal cycling was: 2 min at 50°C, 10 min at 95°C; 45 cycles of 15 s at 95°C and 1 min at 60°C. GAPDH was used for normalization. We calculated fold change relative to control with the ddCt method. All conditions were run in technical triplicates, and we checked that the Ct dispersion within a triplicate was not >0.5. qPCR primers were synthesized by Sigma-Aldrich. qPCR primer sequences were: GRP78 forward: 5'-CACAGTGGTGCCTACCAAGA-3'; GRP78 reverse: 5'-TGTCTTTTGTGAGGGGTCTTT-3'; GAPDH forward: 5'-ATGGGGAAGGTGAAGGTCG-3'; GAPDH reverse: 5'-GGGGTCATTGATGGCAACAATA-3'; DNAJB9 forward: 5'-AAGGCCTTTCACAAGTTGGC-3'; DNAJB9 reverse: 5'-ACGCTTCTTGGATCCAGTGTT-3'; ERN1 forward: 5'-GCATAGTCAAAGTAGGTGGCA-3'; ERN1 reverse: 5'-GATAGTCTCTGCCCATCAACC-3'; EDEM1 forward: 5'-TTCCCTCCTGGTGGAAATTTG-3'; EDEM1 reverse: 5'-AGGCCACTCTGCTTTCCAAC-3'; CHAC1 forward: 5'-GAACCCTGGTTACCTGGGC-3'; CHAC1 reverse: 5'-CGCAGCAAGTATTCAAGGTTGT-3'; BLOS1 forward: 5'-CCCAATTTGCCAAGCAGACA-3'; BLOS1 reverse: 5'-CATCCCCAATTCCTTGAGTGC-3'; DDIT3 forward: 5'-AGAACCAGGAAACGGAAACAGA-3'; DDIT3 reverse: 5'-TCTCCTTCATGCGCTGCTTT-3'; DNAJC10 forward: 5'-TCATGTTACCACGCTTGGACC-3'; DNAJC10 reverse: 5'-GTAAAGCTCGACATGGTGGACAC-3'; XBP1s forward (Figures 4.3/4/5): 5'-TGCTGAGTCCGCAGCAGGTG-3'; XBP1s reverse (Figures 4.3/4/5): 5'-GCTGGCAGGCTCTGGGGAAG-3'; XBP1t forward: 5'-TGAAAAACAGAGTAGCAGCTCAGA-3'; XBP1t reverse: 5'-CCCAAGCGCTGTCTTAACTC-3'; XBP1u forward: 5'-CAGACTACGTGCACCTCTGC-3'; XBP1u&s reverse (Figure 4.9): 5'-CTGGGTCCAAGTTGTCCAGAAT-3'; XBP1s forward (Figure 4.9): 5'-GCTGAGTCCGCAGCAGGT-3'.



For XBP1 PCR, we diluted cDNA directly from the RT 1/5 in PCR reaction buffer. We performed PCR with 300 nM of each XBP1 primer, 125  $\mu$ M dNTP (Thermo Fisher Scientific), 1.2 mM MgCl<sub>2</sub> (Thermo Fisher Scientific), and 50 mU/ $\mu$ L Taq polymerase (Thermo Fisher Scientific) in MgCl<sub>2</sub> Buffer (Thermo Fisher Scientific). Thermal cycling was: 4 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 60°C, and 50 s at 72°C; then 7 min at 72°C. PCR primers were synthesized by Sigma-Aldrich. PCR primer sequences were: XBP1 forward: 5'-GGAACAGCAAGTGGTAGA-3'; XBP1 reverse: 5'-CTGGAGGGGTGACAAC-3'. We loaded PCR products on a 3.5% agarose gel with 0.00005% ethidium bromide (Gen-Apex) for electrophoresis. We imaged the gel with a Fusion Solo S apparatus (Vilber).

### **Fluorescence microscopy and immunofluorescence**

For fluorescence microscopy, we seeded the cells in 96-well plates and performed various treatments. We fixed the cells in 1% formaldehyde (F1635, Sigma-Aldrich) for 15 min at room temperature. We washed the cells twice with PBS, and performed permeabilization in 0.2% Triton-X100 for 10 min at room temperature. If looking at protein aggregates, we added 3 mM EDTA in the permeabilization solution to limit non-specific staining of lipids by the Proteostat. We washed the cells three times with PBS.

For LC3 immunofluorescence, we incubated the primary antibody, rabbit anti-LC3B (CST 3868S, 1/1,000), diluted in 5% BSA (A7906, Sigma-Aldrich) 0.1% Tween-20 (P1379, Sigma-Aldrich) PBS, overnight at 4°C. We washed the cells three times in 0.1% Tween-20 PBS, and incubated the secondary antibody goat anti-rabbit Alexa 488 (A48282, Invitrogen) diluted 1/500 in 5% BSA 0.1% Tween-20 PBS for 1 h at room temperature. We washed the cells three times with 0.1% Tween-20 PBS, and stained nuclei with 500 ng/mL DAPI (D9542, Sigma-Aldrich) for 10 min at room temperature. Images were acquired on the CellInsight CX5 microscope, and the number of LC3 spots per cell were automatically counted. At least 1,500 cells per condition were considered for analysis.

For staining of protein aggregates, we used the Proteostat Aggresome detection kit (51035, Enzo), following the manufacturer's instructions. We stained the nuclei with DAPI as described above. Images were acquired on the CellInsight CX5 microscope.

### **Statistical analyses**

All error bars in the figures represent the standard deviation.

In Figures 4.3/4/5A, to assess the statistical significance of Ire1 $\alpha$  protein level variations, we performed a bilateral unpaired Student's t-test assuming equal variances. Each condition was tested against t0. A difference was considered significant if the p-value was <0.05. \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.



## Chapter 5. Contributions and perspectives

To conclude this dissertation, I outline here the main contributions of my thesis work in context.

- In Manuscript 1, I proposed a novel mechanism-based conceptualization of senolysis, arguing that senolytics can target senescent cells at three distinct levels: directly disrupting BH3 networks, modulating upstream pro-survival pathways, or further dysregulating homeostatic processes. By reviewing the senolytic literature from this angle, I highlighted important differences in pro-survival strategies of senescent cells, and I argued that inter-senescent cell selectivity would be a desirable feature of senolytic candidates in development, as well as an incentive to design highly efficient precision senolytic drugs.
- In Article 2, I investigated a striking instance of such inter-senescent cell selectivity of senolytic drugs. I showed that BRAF-V600E-senescent cells were exceptionally sensitive to senolysis by cardioglycosides, and that this was due to an inhibition of autophagy flux by these compounds. At the time of publication, mechanism-focused senolysis articles were few. Importantly, this approach allowed me to uncover autophagy upregulation as a survival mechanism of BRAF-V600E-senescent cells, which guided subsequent investigations.
- In Chapter 4, I interrogated the status of ER stress and the UPR in various senescence models. My results showed a marked upregulation of Ire1 in OIS. Modulating Ire1 activity seems a promising novel senolytic strategy, although we need to deepen our understanding of mechanisms at play. The potential role of Ire1 in senescent cell survival is exciting, because it is a highly druggable target that is already being exploited in the development of novel cancer therapies.

From my results, autophagy and the UPR appeared of interest in senolysis. In our screen in Chapter 4, we also identified proteasome and HSP90 inhibitors as potential senolytics. It thus seems that the multi-level targeting of proteostasis may provide many new avenues for senolysis that have been under-explored so far. We briefly discuss this perspective in the following and final Article 3 (L'Hôte et al., 2022).

## 5.1. Article 3: Targeting proteostasis maintenance and autophagy in senescence.

### Targeting proteostasis maintenance and autophagy in senescence

Valentin L'Hôte, Carl Mann, Jean-Yves Thuret

The pharmacological elimination of senescent cells by the means of selectively toxic compounds, termed senolytics, is a strategy that has gained increasing interest in the last ten years as a potential means to alleviate age-related diseases, as well as to potentiate chemotherapy and to prevent malignant transformation. Senescence is a cellular response to stress, alternative to apoptosis, in which the cell undergoes a quasi-irreversible proliferative arrest accompanied by important modifications of its transcriptional programs, and often secretes a collection of inflammatory factors termed the senescence-associated secretory phenotype (SASP). Senescence-inducing stressors are various and notably include telomere attrition, chemotherapeutic agents, ionizing radiations, oxidative stress, and oncogene activation. Senescent cells accumulate during aging and in many pathological contexts, where they play a detrimental role often owing to SASP-mediated chronic inflammation. In many pathological conditions in which accumulating senescent cells are implicated, their elimination by senolytics was shown to be highly beneficial.

The oncogenic V600E mutation in the BRAF kinase induces senescence in melanocytes and leads to the formation of nevi (moles). Melanoma can arise from BRAF-V600E melanocytic nevi, suggesting mechanisms of senescence escape at play. One strategy to prevent melanoma formation in patients at risk would be to eliminate pre-malignant, senescent melanocytes, with senolytics.

In a recent study, we sought to uncover novel vulnerabilities of BRAF-V600E-induced senescent cells (BRafSen cells) that could be targeted by senolytics to prevent melanoma formation [1]. To this end, we employed a fibroblast senescence model of ectopic BRAF-V600E expression. We found that cardioglycosides, such as ouabain, showed exceptional senolytic potency in BRafSen fibroblasts. Interestingly, we found that BRAF-V600E expression induced endoplasmic reticulum stress and a subsequent increase in basal autophagy flux in senescent cells. We demonstrated that ouabain inhibited autophagy through Na,K-ATPase signal transduction, and that BRafSen cells required this heightened autophagy flux for survival, explaining their high sensitivity to cardioglycosides. Accordingly, inhibiting autophagy through

other routes, such as with chloroquine or bafilomycin A1, also led to senolysis in BRafSen cells.

The relationship between cellular senescence and autophagy is regarded as paradoxical. Autophagy activation in response to stress can successfully resolve it and thus spare the cell from entering senescence. Aiming to identify ways of specifically eliminating cancer cells, Schepers et al. recently showed that targeting macroautophagy by ULK1 inhibition induces senescence in a panel of cancer cells (but not in normal BJ fibroblasts), and renders cells sensitive to senolysis by BH3-mimetic ABT-263 [2]. However, if a cell does commit to senescence by other ways, autophagy becomes essential for cell survival and senescence establishment. Indeed, SASP production imposes its burden on the secretory pathway, calling for increased proteostasis maintenance. In this regard, the first-ever demonstration of selective pharmacological elimination of senescent cells consisted in depriving therapy-induced senescent lymphoma of adaptive autophagy, leading to proteotoxic stress overload due to SASP expression [3]. Senolysis can actually be achieved by modulating autophagy in either direction: inhibiting autophagy can lead to proteotoxic stress in senescent cells producing an abundant SASP [1] [3], and conversely, further activating autophagy can selectively kill senescent cells through type II autophagic cell death, i.e. excessive "self-eating" [4]. On the other side of the proteostasis network, inhibitors of HSP90 chaperone proteins were found to be senolytic early on. HSP90 activity was essential in senescence to stabilize activated phospho-AKT, which is a master regulator of senescent cell survival [5].

Beyond bulk autophagy flux modulations to cope with increased secretory demands, finer processes appear to be at play in regulating proteostasis in senescence. Recently, it was shown that the stability of a defined set of proteins was regulated by selective autophagy in senescence through differential interactions with ATG8 family receptors. This selective autophagy network was fundamental in shaping several facets of the senescent phenotype, including redox homeostasis (through KEAP1 degradation), SASP production (through TNIP1 degradation), and proteostasis (through eIF3 degradation) [6]. In another study, nuclear selective autophagy was shown to mediate the degradation of

SIRT1 in aging and in senescence via LC3, participating in the expression of SASP components otherwise downregulated by SIRT1 [7]. These studies thus pave the way for a more precise understanding of autophagy regulation in the physiology and the proteostasis of senescent cells, and the discovery of potentially more potent senolytic strategies through the targeting of distinct components of the selective autophagy network (Figure 1). The generality of these

autophagic pathways in different senescent states also remains to be explored. Variable levels of proteostatic stress in different senescent states, and potential specificity in the use of particular autophagy pathways in different cell types may allow selective elimination of specific types of senescent cells. This may be beneficial in the light of recent work suggesting that indiscriminate removal of senescent cells may be harmful [8].

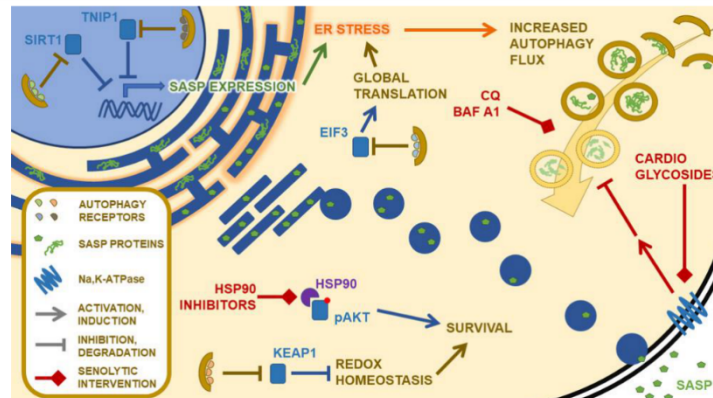


Figure 1. Targeting the proteostasis network in senescence.

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**Keywords:** cellular senescence, senolytics, proteostasis, selective autophagy, BRAF-V600E

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**Received:** February 26, 2022

**Published:** March 7, 2022



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## Résumé substantiel en français

La sénescence cellulaire, ou simplement la sénescence, est un processus faisant appel aux notions de réponse au stress cellulaire, de destin cellulaire et d'identité cellulaire. En réponse à une variété de stress, dont les dommages à l'ADN, l'expression d'oncogènes, la perte de la protéostasie ou le déséquilibre redox, la cellule est confrontée à la décision d'entreprendre un chemin vers un destin cellulaire donné. Selon l'ampleur du stress, l'homéostasie et le contexte cellulaire, la cellule peut résoudre le stress et reprendre la prolifération, engager l'apoptose ou entrer en sénescence. La sénescence implique un arrêt de la prolifération très stable et quasi-irréversible, accompagné de changements importants dans les programmes de transcription et la physiologie cellulaire, entraînant une altération de l'identité cellulaire.

La plupart des cellules sénescents sécrètent un ensemble complexe de facteurs pro- et anti-inflammatoires, de métalloprotéases matricielles et de modulateurs de la tumorigenèse, appelé phénotype sécrétoire associé à la sénescence (SASP). La sénescence est un mécanisme essentiel de suppression des tumeurs car elle empêche la prolifération de cellules stressées potentiellement porteuses d'un potentiel pré-malin. Cependant, l'accumulation anormale de cellules sénescents est préjudiciable car le SASP provoque une inflammation chronique et une détérioration des tissus, et peut favoriser la croissance des tumeurs adjacentes. L'accumulation de cellules sénescents est un moteur du processus de vieillissement ainsi que de multiples pathologies. Il y a donc un fort intérêt clinique à déterminer les voies qui conduisent à l'établissement des phénotypes sénescents, et comment ces phénotypes diffèrent de ceux des cellules non sénescents en termes de métabolisme, d'homéostasie et de régulation de la survie cellulaire, afin de cibler sélectivement les cellules sénescents dans le contexte de leur accumulation, avec des composés dits sénolytiques.

Je commence dans l'introduction bibliographique par présenter la sénescence comme une réponse au stress, un destin cellulaire et un changement d'identité cellulaire. Je me concentre notamment sur la sénescence induite par l'oncogène BRAF-V600E, le principal modèle utilisé tout au long de la thèse pour l'identification de composés sénolytiques et la caractérisation de leurs mécanismes d'action.



Alors que le catalogue des composés sénolytiques s'élargit, de nouvelles stratégies de survie des cellules sénescents sont découvertes, et des variations de sensibilité à la sénolyse entre différents types de cellules sénescents apparaissent. Je propose dans un manuscrit de revue de la littérature une classification mécanistique des composés sénolytiques, basée sur le niveau auquel ils ciblent les cellules sénescents : la perturbation directe des réseaux de protéines BH3, qui sont réorganisés lors de l'induction de la sénescence ; la régulation négative des voies associées à la survie, essentielles aux cellules sénescents ; ou la modulation des processus homéostatiques dont la régulation est remise en cause lors de la sénescence. Grâce à cette approche, je souligne l'importante diversité des cellules sénescents en termes de physiologie et de voies de suppression de l'apoptose, et je décris des pistes possibles pour le développement de sénolytiques plus sélectifs.

Nous avons précédemment caractérisé un modèle de fibroblastes humains en sénescence BRAF-V600E. Des sénolytiques sélectifs en sénescence BRAF-V600E, qui pourraient présenter un intérêt clinique dans le cadre de la prévention du mélanome, n'ont pas été décrits auparavant. Mon projet de thèse visait à identifier de nouvelles voies de régulation de la survie des cellules sénescents induites par BRAF-V600E (BRafSen), et par le même temps à identifier de nouveaux composés sénolytiques efficaces et sélectifs. Lors d'un crible de la chimiothèque de repositionnement de Prestwick, nous avons identifié les cardioglycosides comme de nouveaux sénolytiques dans un modèle de fibroblastes WI-38 en sénescence induite par CRAF. Les cardioglycosides seraient plus tard décrits comme des sénolytiques à large spectre au cours de notre investigation. Néanmoins, nous avons constaté que les cardioglycosides étaient des sénolytiques remarquablement puissants dans les fibroblastes BJ sénescents BRAF-V600E. Nous avons donc cherché à étudier leurs mécanismes d'action dans ce modèle de sénescence, tout en caractérisant de nouveaux processus de régulation de la survie cellulaire en sénescence BRAF-V600E. Cette étude a été publiée sous forme d'une publication scientifique en 2021. Dans cet article, nos données suggèrent le modèle suivant pour la sénolyse des cellules BRafSen par la ouabaïne, un cardioglycoside : l'expression de BRAF-V600E induit un stress du réticulum endoplasmique (ER) dans les cellules BRafSen, en réponse auquel le flux autophagique est augmenté et nécessaire à la survie ; la ouabaïne se lie à la pompe Na,K-ATPase, inhibant à la fois le transport ionique et déclenchant la

transduction du signal, cette dernière étant principalement pertinente pour la sénolyse ; l'activation de Src et Akt médiée par la NKA conduit à une diminution du flux autophagique. En raison du stress ER induit par BRAF-V600E, un flux autophagique plus important est nécessaire pour la survie des cellules BRafSen. Par conséquent, la ouabaïne induit préférentiellement l'apoptose dans les cellules sénescents qui expriment BRAF-V600E, potentiellement par l'intermédiaire de p38. Le blocage du flux autophagique à une étape différente, comme la fusion autophagosome-lysosome à l'aide de la chloroquine, entraîne également la sénolyse.

Nous avons ensuite cherché à comprendre ce qui sous-tend physiologiquement la dépendance des cellules BRafSen à l'autophagie, et quelles sont les conséquences du blocage ou de la régulation négative de l'autophagie qui conduisent à la mort cellulaire. Dans les mélanomes exprimant BRAF-V600E, le stress chronique du réticulum endoplasmique dû à une demande accrue de sécrétion entraîne une augmentation du flux autophagique dont les cellules dépendaient pour leur survie. Nous avons donc décidé de caractériser le stress ER et la réponse aux protéines mal repliées (UPR) dans différents contextes de sénescence. Tout d'abord, nous avons établi le profil UPR à différents moments de l'induction de la sénescence dans divers modèles. Le résultat le plus frappant fut la régulation à la hausse d'Ire1 en sénescence induite par les oncogènes. Bien que les niveaux protéiques d'Ire1 soient restés élevés pendant la période étudiée, la dynamique de cette régulation ascendante différait selon les modèles de sénescence, tant au niveau de l'ARN que des protéines. Nous avons ensuite décidé d'évaluer le potentiel sénolytique de divers modulateurs d'Ire1 qui affectent de manière différentielle l'activité RNase et/ou kinase dans les cellules BJ. Les principales hypothèses que nous devons explorer dans le futur, sont que la sénolyse par la modulation d'Ire1 est médiée par :

- La perturbation de l'intégration d'Ire1 dans les plateformes de signalisation pour l'activation de la voie Traf2/Ask1/Jnk.
- La perturbation de l'intégration d'Ire1 dans MERCS. Résultant en une altération de l'autophagie et/ou de l'homéostasie calcique.

Pour conclure, suivent les principales contributions de mon travail de thèse.

J'ai proposé une nouvelle conceptualisation mécanistique de la sénolyse, en argumentant que les sénolytiques peuvent cibler les cellules sénescents à trois niveaux distincts : en perturbant directement les réseaux de BH3, en modulant les voies pro-survie en amont, ou en perturbant davantage les processus homéostatiques. En examinant la littérature des sénolytiques sous cet angle, j'ai mis en évidence des différences importantes dans les stratégies pro-survivantes des cellules sénescents, et j'ai fait valoir que la sélectivité inter-sénescence serait une caractéristique souhaitable des candidats sénolytiques en développement, ainsi qu'une incitation à concevoir des médicaments sénolytiques de précision très efficaces.

J'ai étudié un exemple frappant de cette sélectivité inter-sénescence des composés sénolytiques. J'ai montré que les cellules BRafSen étaient exceptionnellement sensibles à la sénolyse par les cardioglycosides, et que cela était dû à une inhibition du flux autophagique par ces composés. Au moment de la publication, les articles sur la sénolyse axés sur les mécanismes d'action étaient peu nombreux. Il est important de noter que cette approche m'a permis de découvrir la régulation positive de l'autophagie comme mécanisme de survie des cellules sénescents BRAF-V600E, ce qui a orienté les recherches ultérieures.

J'ai ensuite étudié le statut du stress ER et de l'UPR dans différents modèles de sénescence. Mes résultats ont montré une forte régulation à la hausse d'Ire1 dans les OIS. La modulation de l'activité d'Ire1 semble être une nouvelle stratégie sénolytique prometteuse, même si nous devons approfondir notre compréhension des mécanismes en jeu. Le rôle potentiel d'Ire1 dans la survie des cellules sénescents est excitant, car il s'agit d'une cible hautement médicamenteuse qui est déjà exploitée dans le développement de nouvelles thérapies contre le cancer.

D'après mes résultats, l'autophagie et l'UPR apparaissent d'intérêt dans la sénolyse. Dans notre crible, nous avons également identifié des inhibiteurs du protéasome et des HSP90 comme des sénolytiques potentiels. Il semble donc que le ciblage multi-niveaux de la protéostase puisse fournir de nombreuses nouvelles voies pour la sénolyse qui ont été sous-explorées jusqu'à présent.

**Titre :** Découverte de composés sénolytiques et caractérisation de leurs mécanismes d'action en sénescence induite par l'oncogène BRAF-V600E

**Mots clés :** Sénescence cellulaire, Criblage de chimiothèques, Mécanismes d'action, Sénolytiques, Cardioglycosides, Ire1

**Résumé :** En réponse à l'expression d'oncogène (tel que BRAF-V600E), à des traitements génotoxiques ou à d'autres stress, les cellules eucaryotes peuvent éviter l'apoptose et déclencher la sénescence. La sénescence est un destin cellulaire caractérisé par un arrêt prolifératif quasi-irréversible et une reprogrammation transcriptionnelle profonde, conduisant notamment à une sécrétion importante de facteurs inflammatoires collectivement appelés phénotype sécrétoire associé à la sénescence (SASP). En raison de l'augmentation de la demande sécrétoire et de stress chroniques, la protéostase peut être perturbée en sénescence. Comme elle limite la prolifération de cellules susceptibles de présenter un potentiel pré-néoplastique, la sénescence est un processus essentiel de suppression tumorale ; cependant, l'accumulation de cellules sénescents au cours du vieillissement, dans des contextes pathologiques, ou après chimiothérapie ou radiothérapie, est préjudiciable et entraîne un dysfonctionnement tissulaire. Les sénolytiques sont des composés qui induisent sélectivement l'apoptose dans les cellules sénescents tout en épargnant les cellules normales, et leur application thérapeutique s'est avérée une stratégie pharmacologique efficace dans différents contextes pathologiques où la sénescence joue un rôle moteur. Le but de ce projet était d'identifier de nouveaux composés sénolytiques, notamment dans la sénescence induite par BRAF-V600E, et de caractériser leurs mécanismes d'action, ajoutant ainsi à la compréhension de la régulation des voies de survie cellulaire en sénescence. Les cardioglycosides constituent une classe de composés qui ont été identifiés comme de puissants sénolytiques dans le criblage d'une chimiothèque de repositionnement. Nous avons montré que les cellules sénescents BRAF-V600E étaient remarquablement sensibles à la sénolyse induite par les cardioglycosides. Nous avons démontré que les cellules sénescents BRAF-V600E ont un flux autophagique accru, essentiel à leur survie, et que les cardioglycosides agissent comme sénolytiques en inhibant l'autophagie via la transduction du signal par la Na,K-ATPase. En conséquence, le blocage de l'autophagie par d'autres voies, comme avec la chloroquine, était également sénolytique. Pour mieux comprendre la régulation de l'autophagie et de la protéostase en sénescence et identifier de nouvelles cibles sénolytiques, nous avons ensuite évalué le stress du réticulum endoplasmique et la réponse aux protéines mal repliées (UPR) dans différents modèles de sénescence. En parallèle, nous avons criblé diverses chimiothèques, dans lesquelles nous avons identifié des sénolytiques potentiels ciblant différentes facettes de la protéostase. Notamment, nous avons découvert que le senseur de l'UPR Ire1 était régulé à la hausse en sénescence induite par l'expression d'oncogènes. Ire1 régule le destin cellulaire par plusieurs voies, et de nombreux composés qui modulent de manière différentielle son activité sont disponibles. Nous avons donc utilisé un panel de modulateurs d'Ire1 pour commencer à caractériser son rôle en sénescence, et établir de nouvelles stratégies sénolytiques. En résumé, nos résultats soulignent le potentiel sénolytique du ciblage de l'autophagie et de la protéostase dans la sénescence induite par l'expression d'oncogènes, et l'importance de caractériser en détails les mécanismes d'action des sénolytiques pour identifier de nouvelles cibles et voies de régulation.

**Title :** Senolytic drug discovery and mechanisms of action in BRAF-V600E oncogene-induced senescence

**Keywords :** Cell senescence, Chemical library screening, Mechanisms of action, Senolytics, Cardioglycosides, Ire1

**Abstract:** In response to oncogene expression (such as BRAF-V600E), genotoxic insults, or other stresses, eukaryotic cells can suppress apoptosis and enter senescence. Senescence is a cell fate characterized by a quasi-irreversible proliferative arrest and deep transcriptional reprogramming, notably leading to an important secretion of inflammatory factors collectively termed the senescence-associated secretory phenotype (SASP). Due to increased secretory demands and chronic stress, proteostasis may be challenged in senescence. As it limits the proliferation of cells possibly bearing pre-neoplastic potential, senescence is an essential tumor suppressing process; however, the accumulation of senescent cells during aging, in pathological contexts, or following chemotherapy or radiotherapy, is detrimental and leads to tissue dysfunction. Senolytics are drugs that selectively induce apoptosis in senescent cells while sparing normal cells, and their therapeutical application has proved a valuable pharmacological strategy in pathological contexts in which senescence plays a driving role. The aim of this project was to identify novel senolytic compounds, notably in BRAF-V600E-induced senescence, and to characterize their mechanisms of action, thereby adding to the understanding of cell survival pathways regulation in senescence. Cardioglycosides constitute a class of drugs that were identified as potent senolytics in the screen of a repurposing library. We showed that BRAF-V600E

senescent cells were remarkably sensitive to senolysis induced by cardioglycosides. We demonstrated that BRAF-V600E senescent cells have a heightened autophagy flux that is essential to their survival, and that cardioglycosides acted as senolytics by inhibiting autophagy through Na,K-ATPase signal transduction. Accordingly, blocking autophagy through other routes such as with chloroquine was also senolytic. To gain insight into the regulation of autophagy and proteostasis in senescence and identify new senolytic targets, we then assessed endoplasmic reticulum stress and the unfolded protein response (UPR) in different senescence models. In parallel, we screened various chemical libraries, in which we identified potential senolytics targeting different facets of proteostasis. Interestingly, we found that UPR sensor Ire1 was upregulated in oncogene-induced senescence. Ire1 regulates cell fate through several pathways, and many small compounds that differentially modulate its activity are available. We thus employed a panel of Ire1 modulators to begin characterizing its role in senescence, and establish novel senolytic strategies. Collectively, our results highlight the senolytic potential of targeting autophagy and proteostasis in oncogene-induced senescence, and the importance of deciphering the mechanisms of action of senolytics to identify new targets and regulatory pathways.