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## Reactive oxygen species in health and disease

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**General summary and future perspectives**

# Chapter 13

### **General summary and discussion**

While for a long time reactive oxygen species (ROS) were thought to be merely damaging agents that are detrimental for health, more and more research is now revealing their beneficial sides in signal transduction and cellular adaptation. In this thesis we aimed to look into both sides (detrimental vs beneficial) of ROS, in relation to epigenetics and cancer. However, as reactive oxygen species (ROS) are involved in many, both physiological and pathological, processes, the knowledge acquired in this thesis goes beyond just cancer. Indeed, many diseases, including diabetes (1) and neurodegenerative diseases (2), have been proposed to benefit from ROS-modulation therapies.

Gaining a better basic understanding of the actions of ROS will help us to obtain new avenues to prevent or treat diseases. Regarding this, an interesting avenue of exploration is the link between ROS and epigenetics; several papers now indicate that ROS modulate the epigenetic landscape in such a way that it may induce or promote tumorigenesis (3-5). Moreover, the reversible nature of epigenetic modifications makes this an interesting therapeutic target in ROS-related diseases such as cancer.

### **ROS as therapeutic target in cancer**

ROS-induced DNA mutations can contribute to both the initiation and progression of cancer, and as such ROS are, to a certain extent, beneficial for cancer cells. ROS levels are increased in cancer compared to normal cells (6). Several reasons may explain this, among which increased metabolic activity, mitochondrial dysfunction, oncogene activity, or through crosstalk with infiltrating immune cells (7-9).

In current cancer treatment, bleomycins (BLMs) are used for a variety of tumors (10). Interestingly, the oxidative DNA cleavage (i.e. via the production of ROS) in the presence of cellular metal ions and oxygen is believed to be the major source of BLM its antitumor activity (11-14). Therefore, we designed N4Py, a synthetic mimic of the metal-binding domain of BLM (15-17). N4Py acts as a transition metal catalyst that can convert primary ROS into highly reactive secondary ROS (18). As opposed to BLM, a natural antibiotic produced by *Streptomyces verticillus* (19), N4Py can be synthetically produced. This has as major advantages that it is a small, relatively easy to modify, molecule. As such, studying the effect of these modifications is more straight-forward in N4Py, and may give us insights into the mechanism of action of BLM.

Based on the higher baseline levels of ROS in cancer compared to normal cells, we hypothesized that cancer cells harbor increased sensitivity toward ROS-inducing agents, such as BLM and N4Py, and as such a therapeutic window would arise. In **Chapter 2**, we could indeed identify a therapeutic window in which the ROS-inducing agent resulted in toxicity only in the cancer and not the normal cells. However, to make a more general conclusion, and to exclude the possibility that toxicity arises in certain types of healthy cells, e.g. fast-dividing cells in for example the skin or the intestines, more cell types and other ROS-inducing agents should be included in further studies. Nevertheless, since N4Py is a synthetic molecule, the molecule can be easily further optimized in such a way that it targets cancer cells even more selectively. For example, N4Py could be conjugated to folate, and as a result may specifically target folate-receptor expressing cells. The folate receptor is a receptor that is commonly overexpressed in tumors (20, 21). Previously, it was shown for several folate-drug conjugates, not only in *in vitro* systems, but also in animal models and human clinical trials, that cancer cells can be more selectively targeted (22, 23).

Since N4Py may be an interesting molecule in the treatment of cancer (**Chapter 2**), it is important to understand the factors that determine its behavior inside the cell. Since it is known that the activity of BLM is dependent on the metal it is binding (12, 14, 24), we wondered how different metals within the N4Py molecule can affect the properties of this complex in living cells (**Chapter 3**). We showed that Fe(II)-N4Py is highly redox active, whereas the possible, if any, oxidizing power of Mn(II)-N4Py, Cu(II)-N4Py and Zn(II)-N4Py is too low to enable efficient DNA cleavage. Interestingly, from these studies it became evident that results obtained in cell free systems did not correlate well with results obtained in cell culture, highlighting the importance of determining *in vitro* activity early in the drug discovery process (25). These findings are in line with earlier reports in which the *in vitro* data of metallo-BLMs (26, 27) did not correlate well with the data obtained in cell-free systems (12, 14, 24); *in vitro*, the metallo-BLMs showed comparable levels of cytotoxicity, whereas in cell-free systems Fe-BLM is the most active compound.

This difference might be partly explained by the complexity of a whole cell. Moreover, we show that it is likely the exchange of metals within the cellular environment that contributes to the difference between the data obtained in cell free systems and *in vitro*. In other words, the N4Py-complex we treated our cells with, might not be the active complex in the cell due to metal exchange. In addition, many metals are important co-factors for cellular reactions. Therefore,

changing the intracellular concentration of these may have also changed the outcome. For example, Zn(II) acts as a co-factor for the X-linked inhibitor of apoptosis protein (XIAP), which is involved in the inhibition of apoptosis (28). N4Py can act as Zn-chelating agent, and as such, it has been shown that induction of apoptosis by N4Py correlates with inhibition of XIAP (29). In our results, N4Py indeed induces apoptosis. However, we have not looked into the contribution of XIAP inhibition herein. XIAP levels are often elevated in cancer, and this can contribute to chemoresistance (30). Consequently, it has been suggested that Zn-chelating agents such as N4Py may be useful in the treatment of apoptosis-resistant cancers (29).

Taken everything together, our study reveals the importance to realize that whatever is put inside a cell, does not always reflect the active complex intracellularly. Therefore, great care has to be taken into the interpretation of the data and the conclusion that can be drawn. As such, the XIAP depletion by Zn(II) chelation as described by Zuo et al., is not the only cause for N4Py-induced apoptosis, but more likely, the apoptosis can be contributed to a combination of multiple N4Py-metal complexes (29).

In addition to the type of metal N4Py forms a complex with, the intracellular activity of N4Py is affected by its intracellular trafficking. By fusing different fluorophores to N4Py, we could correlate the biological effects to the intracellular localization and dynamics of the molecule (**Chapter 4**). Despite having only a slight effect on the inherent activity, i.e. DNA cleavage activity on naked DNA, of N4Py, attachment of different fluorophores greatly changed the cellular behavior of the different N4Py variants. This was explained by the fact that the molecule could not enter the cell anymore (N4Py-fluorescein), the mode of transport was changed from passive to active (N4Py-Rhodamine B), or the molecule was targeted to the mitochondria (N4Py-Cy5). As a result of this, both N4Py-fluorescein and N4Py-Rhodamine B showed (almost) completely abolished intracellular activity. In contrast, despite being targeted to the mitochondria, the cytotoxicity of N4Py-Cy5 remained comparable to the parental molecule.

The knowledge obtained in this chapter may be used to gain more insights into how cells respond to differently localized N4Py molecules. Moreover, this knowledge may be useful when employing N4Py as anti-cancer agent. For example, the mitochondria-targeted N4Py-Cy5 variant changed the mode of cell death from an apoptotic to a non-apoptotic cell death pathway. Since many cancer cells are or will become resistant to apoptosis (31), induction of cell death via an alternative pathway may promote the anti-cancer activity of N4Py.

The development of molecular tools that can detect species-specific ROS molecules will help us to better understand the role of specific ROS in health and disease, and will contribute greatly to the successful implementation of ROS-modulating therapies. Current tools to detect primary ROS have many pitfalls, including the lack of selectivity and the ROS generation by the probe itself (32-35). One of the reasons that it is difficult to develop a probe that detects primary ROS, is the fact that primary ROS are not highly reactive by themselves, and therefore, require an additional catalyst to be detected. Given that each cell-type and organelle varies in the type and availability of catalysts, highly variable responses toward current ROS probes are obtained.

Since N4Py itself is a catalyst, it does not require an additional catalyst for the detection of primary ROS. By attaching a reduced fluorophore, i.e. profluorophore, to N4Py, N4Py may be transformed to a ROS probe for the detection of primary ROS; upon catalysis by N4Py of primary ROS into highly reactive secondary ROS, these secondary ROS molecules would preferentially react with the attached fluorophore resulting in fluorescence. As such, when in reduced form (as described before for Cy5 (36)), N4Py-Cy5 could be potentially used as a suitable ROS probe to monitor mitochondrial ROS (mtROS) levels.

In addition to small-molecule fluorescent/chemiluminescent ROS probes, such as dichlorodihydrofluorescein (DCFH) and dihydroethidium (DHE), also several genetically-encoded fluorescent-protein based approaches have been developed in recent years to detect intracellular ROS levels (35, 37). This group consists of proteins such as redox-sensitive green fluorescent protein (roGFP) (38), circularly-permuted yellow fluorescent protein (cpYFP) (39) and HyPER (40). These proteins can be more easily targeted to any subcellular localization or cell-type, can be used for long-term monitoring of intracellular ROS levels and can measure ROS in a more quantitative fashion compared to the small-molecule based probes such as N4Py (37). However, also these biological approaches to detect ROS have their limitations, including their low dynamic range, slow reversibility and overexpression artifacts (37). Nevertheless, researchers are continuously working on further improvements of these proteins (41, 42). The combination of these genetically-encoded protein based approaches with high-resolution confocal imaging techniques has opened up the way to study defined processes in redox biology in living organisms. Currently, several transgenic species have already been used for this purpose, including zebrafish (43), mice (44) and *C. elegans* (45). Using all these new technologies, scientist may map the exact role of specific ROS molecules in many physiological and pathological

processes, and this could give us new insights into how to tackle malfunctioning of these biological systems in diseases such as cancer.

Increased ROS levels are a common feature in cancer cells (6). Despite the beneficial effects, even cancer cells can only handle a certain amount of oxidative stress. Therefore, they attempt to overcome increased ROS levels by increasing their antioxidant defense systems. One of the possibilities for cancer cells to do so, is by constitutively activating Nrf2, the master regulator of the antioxidant and cytoprotective responses (**Chapter 5 and 6**). Based on this, inhibition of Nrf2 could be an interesting option in the treatment of cancer. Indeed, it has been shown that inhibition of Nrf2 can inhibit tumor growth and increase the efficacy of chemotherapy in a variety of cancers (46, 47).

However, both normal and cancer cells make use of the Nrf2 defense system. Therefore, Nrf2 inhibitors might induce off-target effects in certain sensitive normal cell types, such as the rapidly dividing hematopoietic stem cells (48) and drug detoxifying hepatocytes (49, 50). In **Chapter 5** we looked into solutions to overcome this problem, and to enhance the therapeutic window in which Nrf2 inhibition might be useful as anti-ovarian cancer treatment.

So far, only a limited number of Nrf2 inhibitory compounds have been described, including brusatol from *Brucea javanica* (50, 51), procyanidins from *Cinnamomi Cortex* extract (52), retinoic acid receptor  $\alpha$  agonists (53), leutolin (54), and trigonelline (55). For the first two compounds the exact mechanism has not been elucidated yet. Nevertheless, brusatol is known to act as a transient Nrf2 inhibitor at the post-transcriptional level. Moreover, Keap1-, proteasomal- or autophagy-dependent mechanism have been excluded as well as a p38 MAPK-, AKT-, ERK1/2-, or JNK1/2-dependent signaling mechanism (50). For the other compounds, the mechanism of action has been elucidated; retinoic acid receptor  $\alpha$  agonists form a complex with Nrf2 that is unable to activate downstream genes (53); leutolin promotes *NRF2* mRNA degradation (54); and trigonelline inhibits the nuclear import of Nrf2 (55).

In our literature review (**Chapter 5**), we could identify several ovarian cancer specific factors being directly linked to the constitutive activation of Nrf2: mutations in *BRAF*, *KRAS* and *ERBB2*, and high levels of estradiol in combination with mutations in *BRCA1*. Based on this, we propose to use a combination strategy that combines direct Nrf2 expression inhibition with the blocking of ovarian cancer specific factors that cause aberrant activity of Nrf2. For the success of this strategy, it is essential to stratify the ovarian cancer patients in subgroups,

as each ovarian cancer subtype harbors its own set of mutations that is linked to the aberrant activation of Nrf2.

In **Chapter 6**, we took a closer look at a specific subtype of ovarian cancer, the high-grade serous epithelial ovarian cancers harboring a *BRCA1* mutation. This is a relatively common subtype of ovarian cancer that is often diagnosed at a late stage of the disease. Especially in these advanced stage patients, current first-line treatment options, including chemotherapeutics and surgery, are often inadequate (56). Therefore, better treatment options are urgently required for these patients.

The treatment option we explored in this chapter, was the combination of poly ADP ribose polymerase (PARP) (57) and Nrf2 inhibition. PARP enzymes are involved in the repair of single strand DNA (ssDNA) breaks, which are the most common type of DNA breaks induced by ROS. Therefore, we hypothesized that inhibition of Nrf2, resulting in the accumulation of ROS, has the potential to synergize with PARP inhibitors; ROS-induced ssDNA breaks will accumulate at a high rate. Since these ssDNA breaks cannot be repaired, they are converted into double strand (dsDNA) breaks (58). *BRCA1* is an important protein involved in the repair of dsDNA breaks, and therefore, a *BRCA1* mutation will make these mutant cells extra sensitive to (ROS-induced) dsDNA damage (59, 60). In this chapter we could indeed show that this combination therapy is able to specifically kill the *BRCA1* mutant cancer cells. Moreover, as the combination acts synergistically, this would enable the use of a lower dosage and thereby decreasing the chance of side-effects, while remaining comparable efficacy as either monotherapy. In addition to inhibiting ssDNA break repair, certain PARP inhibitors, including olaparib, can also tighten the binding of PARP to the DNA. The resulting PARP-DNA complex is shown to prevent replication and transcription and can therefore contribute to the observed toxicity of these agents (61).

From the studies described in **Chapter 2-6** we can conclude that ROS modulating therapies are an interesting avenue to further explore for the treatment of cancer (9, 62). Moreover, the development of selective ROS probes could increase our understanding of the contribution of specific ROS molecules in cancer, and therefore, could aid in the rational development of new ROS modulating therapies. Work by others also indicates that one of the important mechanisms by which cancer cells acquire therapy resistance, such as resistance to radiation (63) and multidrug resistance (64), is by increasing their antioxidant capacity.

However, great caution should be taken when pursuing the path of ROS-modulating therapies; in contrast to previous beliefs, the use of antioxidants is less harmful after all (65, 66). On the other hand, increasing ROS levels is also not without any risk, and might even induce carcinogenic mutations in healthy cells (67, 68). Therefore, it is recommended to carefully monitor treatment efficacy not only on the short term, but also on the long term.

Alternatively, a different approach could be taken that targets the increased dependency of cancer cells on pathways clearing the ROS-induced damage (69); cancer cells do not only survive because they efficiently prevent ROS-induced damage, but also because they have an increased capacity to remove the ROS-induced damage, e.g. by increased capacity of the ubiquitin-proteasome pathway that regulates the degradation of misfolded and damaged proteins (70). Recently, the drug Bortezomib has been successfully implemented in the treatment of relapsed mantle cell lymphoma patients (71). Bortezomib inhibits the ubiquitin-proteasome pathway, and thereby increases the accumulation of ROS-induced protein damage, resulting in apoptosis. The advantage of such an approach compared to direct ROS modulation would be that interference with adaptive ROS responses in healthy cells (**Chapter 8**), being anti-tumorigenic in nature (72), is not expected.

### **Crosstalk between ROS and epigenetics**

Besides genetic mutations, epigenetic alterations are commonly found in cancer (73). Several examples point to a role for ROS herein (3-5). For example, H<sub>2</sub>O<sub>2</sub> treatment in colorectal cancer cells induced hypermethylation of the *RUNX3* promoter resulting in downregulation of Runx3 expression (3). *RUNX3* acts as a tumor suppressor gene in many cancers, among which colorectal cancer (74). As such, H<sub>2</sub>O<sub>2</sub>-induced silencing of *RUNX3* may contribute to the carcinogenic process. This silencing could be overcome by the antioxidant N-acetyl cysteine (NAC) or the DNA methyltransferase (DNMT) inhibitor 5-aza-2'-deoxycytidine (5-aza-dC). Moreover, this hypermethylation was associated with increased binding of DNMT1 to histone deacetylase 1 (HDAC1) and the *RUNX3* promoter. Such studies give us some indications on how ROS may affect epigenetics, but exact mechanisms remain largely unknown. Therefore, in **Chapter 7 and 8**, we revealed some of the mechanisms by which ROS may alter the nuclear epigenetic landscape. Besides having an effect on nuclear epigenetics, we hypothesize that ROS may also have an effect on mitochondrial epigenetics. In **Chapter 9**, we summarize current literature indicating the presence and functional relevance of

this previously overlooked epigenetic layer. Finally, in **Chapter 10**, we looked into the function of mitochondrial DNA methylation, as being one of the components of the mitochondrial epigenetic layer.

In **Chapter 7**, we discussed recent literature showing that depending on its chromatin microenvironment, DNMTs may have opposite functions. Interestingly, high levels of ROS are one of the requirements for this unexpected function of DNMTs. We hypothesized that under specific (physiological) circumstances, these extremely high levels of ROS can be reached very locally and contribute to the rapid demethylation/remethylation cycles of extremely fast (“ultradian”) oscillating genes. In line with this, it is conceivable that under certain pathological circumstances, local ROS production is increased in such a way that it may alter the function of DNMTs. For other epigenetic enzymes similar phenomena have not yet been described, and are, at least in the case of “ultradian” oscillating genes, not expected to occur as activation and repression during oscillation co-occurs with the recruitment of active and repressive histone modifier proteins to the oscillating gene promoter (75). The recruitment of the ten-eleven translocation (TET) enzymes to these extremely fast oscillating genes has not been studied, since, at that time, the TET enzymes were not identified yet. However, it is possible that they may have functioned as the active DNA demethylases as suggested by other studies (32, 33). Nevertheless, H<sub>2</sub>O<sub>2</sub> has been shown to reduce the activity of the TET enzymes via a decrease of the Fe(II) pool, i.e. the co-factor for these enzymes (76). Therefore, it might be necessary for the DNMTs to take over the function of TET enzymes in specific local chromatin microenvironments with high levels of ROS.

As mentioned above there are several indications that ROS can modulate the nuclear epigenetic landscape. In **Chapter 8**, we wondered whether ROS-induced epigenetic changes can also explain the so-called mitohormetic response; a short-term increase in mtROS can be beneficial for health on the long-term by preventing a number of diseases, among which cancer and diabetes (72). We hypothesized that this (short-term) increase in mtROS levels induces epigenetic changes that can explain the health-benefits on the long-term. Previously, it was hypothesized that either via direct (77) or indirect (78) mechanisms mtROS may alter the epigenetic landscape.

In **Chapter 8**, we set up a system using the genetically-encoded SuperNova protein that would give us more insight in this topic. This fluorescent protein generates superoxide upon excitation with a specific wavelength (79). By targeting this protein to different locations within the mitochondria, we

generated a unique tool to reveal the role of mtROS produced at different locations within the mitochondria. Unfortunately, we were unable to successfully get the system up and running.

Nevertheless, current literature may give us some clues about the effect of mtROS on the nuclear epigenetic landscape. Most fascinating is the fact that mtROS seems to be somehow different from ROS produced at other sites; knockdown of the mitochondrial superoxide dismutase (SOD: converting  $O_2^{\cdot-}$  to  $H_2O_2$ ) increases longevity, whereas others even decrease the lifespan in worm (80). Moreover, in yeast, mitochondrial  $O_2^{\cdot-}$  is essential for the protection against  $H_2O_2$  (81). Such findings may point to some kind of undiscovered second messenger being only present in the mitochondria, and being only responsive to  $O_2^{\cdot-}$ . Further research is required to disentangle these unexplained observations.

In addition to modulating the nuclear epigenetic landscape, mtROS may also have a direct effect on components within the mitochondria itself, among which the epigenetic layer surrounding the mtDNA. Moreover, the so-called undiscovered second-messenger in the mitochondria that explains the longevity effects of mitochondrial  $O_2^{\cdot-}$  (80, 81) may be a component of the mitochondrial epigenetic layer.

For many decades, the presence of mitochondrial epigenetics (82-85), more specifically mitochondrial DNA (mtDNA) methylation, was under debate (86-88). In the last 5 years evidence has been accumulated that clearly points to the presence of mtDNA methylation that may influence physiological and pathological processes in the cell (reviewed in **Chapter 9**). Especially the identification of a mitochondrial-targeted DNMT1 variant that could alter the expression of two mitochondrial genes (mtND1 and mtND6), initiated the change of view on this topic (89). Since then, mtDNA methylation and hydroxymethylation have been linked to a wide variety of diseases, including colorectal cancer (90), cardiovascular disease (91), diabetes (92), Down Syndrome (93), amyotrophic lateral sclerosis (94), nonalcoholic fatty liver disease (95), Alzheimer's Disease and Parkinson's disease (96). However, from most of these studies it cannot be deduced whether the mtDNA methylation has any functional consequence or is merely there as a result of disturbed methionine metabolism, which is used as a building block for S-adenosyl methionine (SAM) – the co-factor required for DNA methylation.

The study of Mishra et al. gave us first insights in the functionality of mtDNA methylation (92). In this study, high glucose treatment of bovine retinal endothelial cells was shown to induce mtDNA methylation in specific regions of

the mtDNA. Similar data were generated in human cells from the retinal microvasculature of patients with diabetic retinopathy versus healthy controls. These increased levels of mtDNA methylation co-occurred with increased levels of DNMT1, increased binding of DNMT1 to these higher methylated regions in the mtDNA, decreased mitochondrial gene expression and increased apoptosis. Moreover, the causal relationship was further proven as each of these effects could be reversed using DNMT1 siRNA or the DNMT inhibitor 5-aza-dC. Nevertheless, since both approaches to induce DNA demethylation target both the nuclear and mitochondrial genome, it cannot be excluded that above findings are the result of nuclear (and not mitochondrial) DNA demethylation.

Spurred by all these findings, in **Chapter 10** we aimed to obtain a greater understanding in the functional effect of mtDNA methylation. To this end, we targeted several DNA methyltransferases, each with its own characteristics, to the mitochondria. In both tested cell lines, highly efficient mtDNA methylation was induced by the bacterial CpG methyltransferase M.SssI. Unexpectedly, this did not alter mitochondrial gene expression, 7S DNA primer formation (suggested to be involved in DNA replication (97)), mitochondrial metabolism, mtROS production, sensitivity to apoptosis or cell viability. MtDNA copy number was the only factor that was reduced in one out of two cell lines. Interestingly, mtDNA copy number has been shown to influence DNA methylation levels in a number of nuclear genes, including BACH2 and PRKC1B, genes that are both involved in the regulation of apoptosis in response to oxidative stress (98). Therefore, by altering mtDNA copy number, mtDNA methylation may be a way for the mitochondria to communicate to the nucleus in case of environmental stress (99, 100).

In addition to CpG methylation, non-CpG methylation seems to be also a relatively important modification of the mtDNA (96, 101, 102). Therefore, we also targeted the viral GpC methyltransferase M.CviPI to the mitochondria. The resulting induction of mtDNA methylation was less efficient compared to M.SssI, but may actually be a better representation of the variation in physiological levels. In contrast to the CpG methylation, GpC methylation did not affect mtDNA copy number but resulted in a decrease of some of the mitochondrial genes. Interestingly, depending on the cell line, different mitochondrial genes were repressed, being transcribed from different mitochondrial promoters. This may point to cell type specific factors that can affect the outcome of mtDNA methylation. Moreover, this may indicate that different levels of mtDNA methylation, hemi-methylated DNA (non-CpG methylation is by definition hemi-methylated) or specific positions may have a different effect.

In summary, the exact contribution of mtDNA methylation remains largely a “black-box”. Since, besides mtDNA methylation, also mtDNA hydroxymethylation and post-translational modifications of TFAM have been described (**Chapter 9**), studying the effect of these mitochondrial epigenetic features is essential for the greater understanding of the function of mitochondrial epigenetics.

Mitochondrial dysfunction is a common feature of normal aging and (mitochondrial) diseases, including cancer and diabetes (103). Possibly, mtDNA methylation or other mitochondrial epigenetic modifications may contribute to the mitochondrial dysfunction observed in these conditions. Since epigenetic modifications are reversible, this could provide us with new therapeutic targets for many of those diseases. Therefore, it is essential that future efforts should give us a greater insight in this previously unappreciated level of regulation.

### **The local epigenetic environment as therapeutic target in cancer**

As described in the above, epigenetic alterations are commonly found in cancer (73), and can be induced by ROS (3-5). Since epigenetic changes are reversible, they can be interesting targets for therapeutic intervention. One of these pro-tumorigenic epigenetic alterations induced by ROS is the DNA hypermethylation of specific tumor suppressor genes (TSGs) (3-5).

Large scale analyses of the “cancer methylome” have identified many cancer-specific hypermethylated promoters (104). However, the individual contribution of these hypermethylated promoters to the carcinogenic process is largely unknown; one can distinguish between driver and passenger epimutations, i.e. mutations that are causally implicated in the disease versus those that are random and do not directly contribute to the disease (105). In order to distinguish between drivers and passengers within the “cervical cancer methylome”, we set out to re-express a set of putative hypermethylated TSGs in cervical cancer that were identified in these large scale screenings, including *C13ORF18*, *CCNA1*, *EPB41L3* and *TFPI-2* (104, 106).

Several tools are now available to re-express genes, including artificial transcription factors (ATFs) (107), “engineered epigenetic editors” (EEE) (108) and epigenetic drugs (109). Especially the gene-targeted approaches, i.e. ATFs and EEE, are useful to distinguish driver from passenger epimutations, as these allow for gene re-expression in a more targeted fashion (110-112). Moreover, these approaches may also be very suitable for the re-expression of ROS-induced hypermethylated genes.

In **Chapter 11**, zinc-finger-based ATFs were used to identify *EPB41L3* as a TSG in cervical cancer, making it an interesting target to re-express in this malignancy. For this ultimate goal, several approaches were explored: zinc-finger protein (ZFP) based ATFs, EEE using enzymes involved in the active DNA demethylation (TET2 and TDG), and epigenetic drugs (HDAC inhibitor: trichostatin A (TSA), DNMT inhibitor: 5-aza-dC).

In this study, the most ideal intervention, inducing sustained, gene-specific and effective gene re-expression, could not be identified; epigenetic drugs are efficient but act genome-wide, which may induce many side-effects. On the other hand, ATFs act only transiently but may allow for gene re-expression in a more specific fashion (110-112). EEE are believed to act in a more sustained fashion, but relatively low delivery, as a result of their relatively large size, hampered their effectiveness in our study.

Interestingly, by combining ATFs and epigenetic drugs, we could induce sustainable and more efficient gene re-expression than monotherapy. Possibly, this combination changed the local epigenetic environment of *EPB41L3* in such a way that otherwise transient re-expression was maintained. The finding that the combination of ATFs and epigenetic drugs improves monotherapy, has been described before for other genes, including *C13ORF18* (113), *Maspin* (114) and *Oct4* (115), but was not effective for *frataxin* (116). This combination therapy could be exploited in a therapeutic intervention as it might offer merely the advantages and not the disadvantages of both tools; the combination offers sustainability, the ATFs offer increased gene-specificity, while genome-wide side-effects effect may be limited by a lower dose of the epigenetic drugs. Nevertheless, when problems associated with the EEE approach, such as difficulties to obtain sufficient delivery (**Chapter 11**, (117)) and underlying rules allowing epigenetic reprogramming have been deciphered, EEE will be the most ideal approach to sustainably re-express hypermethylated genes.

Therefore, we aimed to take this approach to the next level in **Chapter 12**; by further improving the total cellular delivery of the EEE (using cell sorting and superinfections), we could proof that TET2-mediated targeted re-expression can be sufficient to translate into decreased cancer growth and increased apoptosis. Using this approach, in addition to cDNA overexpression and ATF-mediated re-expression, we could identify *C13ORF18* (113) and *TFPI2* as TSGs in cervical cancer.

Current efforts in our group indicate that the epigenetic factors required for sustainable gene re-activation are dependent on the chromatin

microenvironment (Cano Rodriguez et al., Nature Communications, in press). In the studied hypermethylated genes, a combination of at least H3K4me3, H3K79me2 and DNA demethylation was required to achieve long-term sustainable gene re-activation. However, this is a young field and many more insights are needed, including the importance of timing and the exact role of the chromatin microenvironment on the outcome of epigenetic editing. When underlying rules allowing epigenetic reprogramming have been deciphered, we can start translating this “state-of-the-art” method to clinically relevant therapies. This would allow for a “hit-and-run” approach, as opposed to currently available epigenetic drugs that require long-term treatment to be efficient (118), in which short-term treatment can permanently change the (local) epigenetic environment.

Before being able to translate these findings to clinically relevant therapies, scientist have to work hard to overcome the last hurdles, including delivery, specificity and safety of the approach (119-122). Nowadays, three DNA targeting moieties are available: 1. ZFPs; 2. Transcription activator-like effector (TALEs); 3. Clustered, regularly interspaced, short palindromic repeats-associated protein 9 (CRISPR/Cas9) (109, 121). These DNA targeting moieties can be fused to transcriptional activators (e.g. VP64 – **Chapter 11, 12**) or repressors (e.g. SKD – **Chapter 6**), epigenetic enzymes (e.g. TDG and TET2 – **Chapter 11, 12**) or Fok1 nuclease (“scissors”). So far, only the first (123) and the latter application (124) ([www.sangamo.com](http://www.sangamo.com)) entered clinical trials using ZFPs as DNA targeting moiety. In the first approach, a transcriptional activator was fused to a *VEGFA* targeting ZFP to treat patients with diabetic neuropathy (123). Only minimal adverse effects were observed. Though, unfortunately, treatment did not significantly improve disease outcome compared to placebo controls. Several clinical trials are now ongoing using the latter approach; either trying to prevent HIV infection by cutting out CCR5 on CD4+ T-cells, or by gene-correction (cut-and-paste) of the mutant clotting factor VIII or IX in hemophilia patients ([www.sangamo.com](http://www.sangamo.com)). Despite, this head-start of ZFPs, the other technologies are catching up fast; the first person is already successfully treated with TALE nucleases (TALEN) to overcome a graft-versus-host disease by multiplex targeting of both the T cell receptor alpha constant chain locus, and the CD52 gene locus (125) (Cellectis, Pfizer). Moreover, last year, the first biotech-pharma (Intellia-Novartis) deal was disclosed in which Novartis is planning to move on the CRISPR/Cas9 technology into clinical trials (126). In addition, Editas Medicine intends to start up a clinical trial in 2017 to treat a rare eye disease called Leber congenital amaurosis using

the CRISPR/Cas9 technology (127). So in the near future, many more of these clinical trials, so far mainly focusing on the nuclease approach, are expected to come.

Specifically for the re-activation of hypermethylated TSGs, ZFPs have the advantage to be small enough to enable binding within hypermethylated CpG regions (**Chapter 11, 12**) (113), whereas for CRISPR/Cas9 it seems that dense hypermethylation in CpG islands impairs target DNA binding (Cano Rodriguez et al., Nature Communications, in press) (128). However, to circumvent this issue, several studies have achieved successful gene re-activation of hypermethylated genes using CRISPR/dCas9 (129-131) or TALEs (132) by targeting a gene outside its CpG islands. Moreover, despite being more specific than epigenetic drugs, specificity can still be an issue for all (epi-)genome editing tools: ZFPs (**Chapter 11, 12**) (133), CRISPR/Cas9 (134, 135), and TALEs (136, 137). Nevertheless, in some cases, indications of single gene specificity are obtained for CRISPR/dCas9 (138, 139). Moreover, scientist are continuously trying to improve these genome editing tools (140, 141) or strategies (142) in order to ultimately exclude off-target effects. So each approach has its own pros and cons that may be overcome using smart solutions. The future will have to tell which of these will finally be most successful in the clinic.

## Conclusion

In conclusion, this thesis describes the complex relationship between ROS in health and disease (with a specific focus on cancer) (**Part 1**), between ROS and epigenetics (**Part 2**) and epigenetics and cancer (**Part 3**). By combining all knowledge gained in these studies, we provide novel avenues that can be pursued for cancer therapy.

In summary, at low doses, ROS act as signaling molecule and as such they can have an impact on many intracellular processes. In addition, ROS can induce long-term changes/adaptations by modulating the epigenetic landscape, in the nuclear and possibly also mitochondrial DNA. At high doses, ROS can destroy all kinds of biomolecules, including DNA and proteins. This damage contributes to normal aging but also diseases such as cancer. Here, we show that modulation of intracellular ROS levels, either using the BLM-mimic N4Py or by inhibiting the activity of Nrf2 using ZFPs, turns out to be a successful strategy to preferentially kill cancer cells. Moreover, we provide a state-of-the-art approach (“Epigenetic Editing”) that might reverse unwanted ROS-induced epigenetic changes. The development of molecular tools that can detect site-specific and/or species-

specific ROS molecules, such as was initiated in our N4Py-fluorophores studies, will greatly aid in the detailed understanding of specific ROS in health and disease, and will be essential for the successful implementation of above mentioned therapeutic strategies.

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