SUMMARY

Head and neck cancer (HNC) is the seventh most prevalent cancer worldwide (Mody et al., 2021). Unfortunately, approximately 40% of HNC patients treated with radiotherapy develop xerostomia due to the unintended irradiation of salivary glands (Sciubba and Goldenberg, 2006). These exocrine organs, located in and around the oral and pharyngeal regions, are responsible for saliva production, which is crucial for maintaining oral health, facilitating swallowing, and supporting speech (Miletich, 2010). Radiotherapy-induced damage to salivary gland acinar cells, along with the loss of functional salivary gland stem/progenitor cells, important for tissue turnover and regeneration, strongly compromises tissue function and repair. This leads to long-term gland dysfunction, severely impacting patients' quality of life (Sciubba and Goldenberg, 2006).

To address these challenges, recent advancements in radiation techniques, such as proton therapy, have been developed to improve targeting and minimize collateral damage (Leeman et al., 2017). Despite these improvements, significant harm to surrounding healthy tissues is often induced and may persist the rest of life.

Advanced *in vitro* models, such as salivary gland organoids, provide a promising platform for investigating radiation responses (Verstegen et al., 2025). These organoids, enriched with stem/progenitor cells and containing diverse cell populations, allow for detailed investigation of the signaling pathways triggered by irradiation (Soto-Gamez et al., 2024; Verstegen et al., 2025). Insights from such models could guide the development of novel therapeutic strategies that preserve normal tissue integrity while maximizing the effectiveness of cancer treatments.

Adult stem/progenitor cells are essential for maintaining tissue homeostasis and promoting regeneration (Beumer and Clevers, 2024). However, genotoxic stress can significantly impair their longevity and functional capabilities. Chapter 2 summarizes the key signaling pathways and events triggered by DNA damage, highlighting their influence on the microenvironment and function of stem/progenitor cells. Over the years, the dsDNA sensors cGAS has been recognized as a crucial player in initiating strong interferon responses and immune activation in both normal tissues and cancer cells. More recently, additional sensors, such as the dsRNA receptors RIG-I and MDA5, as well as the sensor ZBP1, have emerged as complementary upstream regulators of interferon activation.

DNA damage can also trigger cellular senescence, a process characterized by persistent cell cycle arrest and inflammatory phenotype. Radiation-induced senescence is particularly important in tissue degeneration and the loss of stem cell self-renewal potential. The secretion of cytokines during this process can recruit immune cells, contributing to an inflammatory response. Altogether these DNA

damage-mediated immunomodulatory responses have been shown to disrupt the homeostasis of tissue-specific stem cells, leading to degenerative conditions. Conversely, the release of specific cytokines can enhance the plasticity and regenerative capacity of tissue-specific stem cells, as well as promote the activity of cancer stem cells, potentially driving tumor progression.

Mitochondria are dynamic organelles essential for maintaining cellular metabolism and overall cell function. Dysfunctional mitochondria have been implicated in cellular senescence and the dysregulation of stem/progenitor cells, ultimately contributing to tissue dysfunction and disease. Chapter 3 explores how ionizing irradiation affects mitochondrial dynamics and its connection to senescence and stem/progenitor self-renewal. After photon irradiation, salivary gland organoids exhibited a strong impairment in mitophagy and mitochondrial dynamics, processes crucial for the effective clearance and turnover of damaged mitochondria and proper cellular function. Indeed, irradiated organoids showed a marked accumulation of long, dysfunctional mitochondria, which were associated with cellular senescence and stem/progenitor cell exhaustion. Pharmacological interventions using mitochondrial fission or mitophagy inducers were able to rescue these processes, significantly improving mitochondrial function, reducing senescence, and enhancing organoid self-renewal. This work highlights a potential new strategy for improving stem cell potential following irradiation by targeting mitochondrial dysfunction and senescence.

The current lack of studies examining normal tissue responses to irradiation has left significant gaps in our understanding of the effects of photon and proton irradiation on normal tissues, particularly the salivary glands. In Chapter 4, we employed our salivary gland organoid model to investigate the molecular mechanisms activated by photon and proton irradiation. Both types of radiation induced a robust type-I interferon (IFN-I) signaling response, primarily mediated by shared micronuclei-induced cGAS activation at early time points and mtDNA recognition at later stages. These findings underscore the critical role of these mechanisms in shaping the inflammatory response in normal tissue. Interestingly, proton irradiation elicited a stronger interferon signaling response at later timepoints, driven by transposable element (TE)-derived dsRNA formation and subsequent RIG-I activation. This heightened IFN-I response was critical for the self-renewal capacity and proliferation of stem/progenitor cells, as interferon-beta (IFN-β) treatment significantly improved organoid growth and stem/progenitor cell proliferation in photon-irradiated samples. *In vivo* experiments further underscored the role of IFN-β in promoting stem/progenitor cell activity. This study not only elucidates the interconnected players driving IFN-I responses after irradiation but

also provides a foundation for developing innovative strategies to enhance radiotherapy outcomes and potentially improve normal tissue regeneration.

In Chapter 5, we employed a multi-omics approach to deepen our understanding of the cellular populations of salivary gland organoid models at various temporal stages and in response to radiation-induced damage. Single-cell RNA sequencing showed the presence of potential salivary gland stem/progenitor cell populations, which exhibited elevated stem cell properties following surface marker-specific cell sorting. Notably, we identified a novel cell population that may represent acinar precursor cells, distinguished by epithelial-mesenchymal-like features and an enhanced migratory phenotype. Further bioinformatic analyses and *in vitro* validations revealed that salivary gland stem/progenitor cells relied on Notch signaling to sustain their migratory capacity and self-renewal potential after irradiation. These results have been validated in other murine and human glandular tissues, including thyroid and mammary glands, highlighting the critical role of Notch signaling in regulating the stemness and differentiation potential of these cell populations.

In addition to Notch signaling, the Hippo pathway plays a pivotal role in regulating tissue growth and regeneration. In Chapter 6, we used an *in vivo* and *in vitro* model to investigate the role of YAP, a key component of the Hippo signaling pathway, in adult salivary gland homeostasis and regeneration. Through pharmaceutical and genetic modulation of YAP activity, we observed dynamic changes between homeostatic and injury conditions. While YAP activity remained low under homeostatic conditions, it increased markedly following injury. Indeed, activation of YAP nuclear translocation significantly enhanced the regeneration and proliferation of salivary gland cells. These findings highlight the critical role of YAP in regulating plasticity and the regeneration of damaged salivary glands, offering potential strategies to enhance salivary gland repair, particularly following radiation-induced damage.

TARGETING MITOCHONDRIA TO IMPROVE RADIOTHERAPY OUTCOMES

The results in Chapter 3 and 4 highlight the critical role of mitochondria in maintaining cellular function after irradiation and emphasize the need for a deeper understanding of radiation-induced mitochondrial damage (Figure 1). The significance of improving mitochondrial functionality after irradiation is further underscored by the involvement of mitochondria in various conditions, including autoimmune, metabolic, and neurodegenerative diseases, as well as cancer and aging (Michael T. Lin and M. Flint Be, 2006; Crow, 2019; Amorim et al., 2022; do Carmo Greier et al., 2022). For this reason, advanced *in vitro* models could provide

valuable insights into the intricate processes regulated by mitochondria leading to the development of new strategies to preserve their function. While several drugs are currently undergoing clinical trials to mitigate mitochondrial stress in different diseases (Singh et al., 2021), relatively few studies have explored approaches to repair mitochondrial damage caused by irradiation. Using clinically approved mitochondria-targeting drugs could open new avenues in radiotherapy, potentially enhancing cancer treatment and reducing radiation-induced damage.

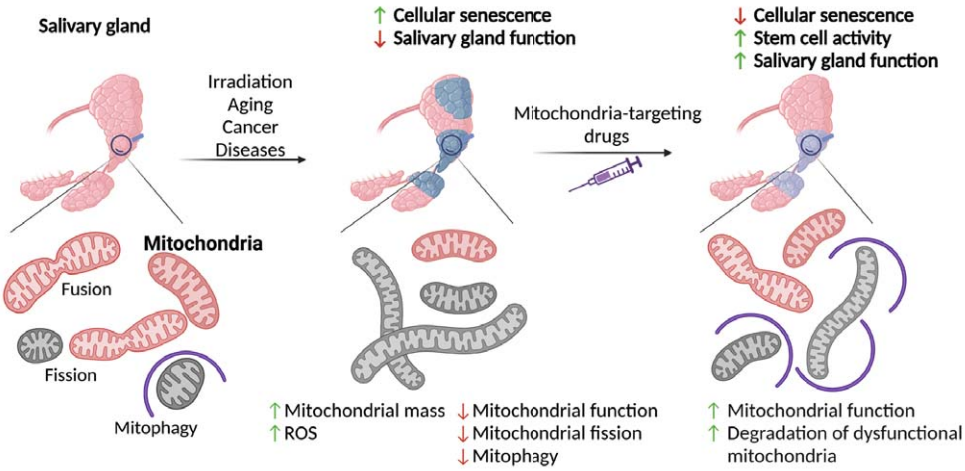

Figure 1. The role of mitochondria in salivary gland dysfunction and senescence.
Mitochondrial function is tightly regulated, and factors such as irradiation, aging, cancer, metabolic disorders, and autoimmune diseases can lead to mitochondrial dysfunction. This decline contributes to increased cellular senescence and salivary gland impairment. Targeting mitochondria with specific drugs may help mitigate these effects by restoring mitochondrial function, supporting salivary gland stem cell activity, and promoting tissue regeneration. Adapted from (Cinat et al., 2023). (Figure created with BioRender.com).

The selective degradation of damaged mitochondria through mitophagy is essential for preserving normal tissue homeostasis (Picca et al., 2023). Indeed, inhibition of mitophagy in proliferating cells is sufficient to induce premature senescence, a phenotype also observed in naturally aged cells (Kelly et al., 2024). In line with this, Chapter 3 shows a significant increase of senescence following radiation-induced mitophagy downregulation, consistent with previous *in vivo* findings (Peng et al., 2020). Our model, therefore, provides a valuable tool that can be used not only for studying radiation-induced damage but also for mimicking cellular senescence in the context of aging. Further improvements, such as

incorporating immune cells into an air-liquid-interface (ALI) organoid model, recently used to generate advanced intestinal, lung and tumor organoids (Neal et al., 2018; Salahudeen et al., 2020; Santos et al., 2024), could provide a more physiologically relevant system and enhance its utility in investigating both radiation responses and aging process.

In Chapter 3, we demonstrate that promoting mitophagy partially prevents radiation-induced senescence, suggesting a promising therapeutic strategy to prevent the side effects of irradiation (Figure 1). Intriguingly, increasing basal mitophagy levels in cancer cells, in combination with radiotherapy, has been shown to suppress tumor cell proliferation (Ren et al., 2023; Yang et al., 2023a), thereby enhancing the therapeutic effectives of radiation. Given its dual benefits (supporting regeneration of radiation-damaged healthy tissues while promoting cancer cell death) mitophagy could provide a compelling avenue for improving the therapeutic window of radiotherapy. Future studies integrating proton therapy and mitophagy inducers in *in vivo* models could provide valuable insights into the combined effects of mitophagy activation and irradiation on both tumor and healthy tissues, paving the way for more effective and targeted cancer therapies.

RADIATION-INDUCED INFLAMMATION AND TRANSPOSABLE ELEMENT UPREGULATION: FRIENDS OR FOES?

As highlighted in Chapter 2, inflammation, alongside mitochondrial dysfunction, is a key feature of radiation-induced damage and plays a crucial role in regulating the activity of stem/progenitor cells (Michael et al., 2016). In Chapter 4, we observed a heightened IFN-I response following proton irradiation, which correlated with increased stem/progenitor cell function and proliferation. This finding aligns with studies in other tissues, such as the intestine, where IFN-I promotes regeneration via the cGAS-STING pathway (Leibowitz et al., 2021b) (Figure 2). Despite these observations, whether IFN-I can be leveraged to improve radiotherapy outcomes remains an open question. While its effects on immune cells have been widely studied (Bolívar et al., 2018; Kumaran Satyanarayanan et al., 2019; Nishiyama et al., 2022), its direct impact on stem/progenitor cells and its role in modulating their interactions with the surrounding microenvironment remain less understood. For instance, given the pivotal role of macrophages in salivary gland regeneration (McKendrick et al, 2023), investigating how IFN-I-induced macrophage activation influences stem/progenitor cell activity after irradiation could provide valuable insights. To address this, *in vivo* models and patient-derived ALI organoids combined with single-cell and spatial transcriptomic techniques could help elucidate the impact of proton irradiation and IFN-I in a more physiological context. Moreover, the

use of patient-derived organoid or iPSC-derived organoid could help determine whether specific mutations in IFN-related genes influence the response to irradiation. This approach could pave the way for more personalized, targeted therapies, optimizing treatment strategies based on individual genetic profiles.

Critically, prolonged IFN-I signaling activation is linked to the development of senescence and stem cell dysfunction (Yu et al., 2015b), it is therefore crucial to determine whether transient IFN-I exposure promotes tissue repair while sustained signaling leads to adverse effects. If so, an optimal therapeutic strategy might involve leveraging its regenerative potential early while mitigating its harmful consequences over time. Additionally, a threshold may exist where excessive IFN-I levels induce cellular stress, whereas controlled, short-term exposure could awaken quiescent stem/progenitor cells and promote regeneration.

Intriguingly, the heightened IFN-I response detected following proton irradiation was driven by an exaggerated upregulation of TEs (Figure 2). Similar findings have been reported in the hematopoietic system, where chemotherapy-induced TE activation led to melanoma differentiation-associated protein 5 (MDA) signaling activation and enhanced regeneration (Clapes et al., 2021), as well as in cancer cells undergoing radiation treatment where TE-induced inflammation improved treatment outcomes (Du et al., 2023). These findings highlight the need for a deeper understanding of the complex regulatory network governing TE activity, as an increasing number of studies link TEs to inflammation as well as fundamental biological processes, including iPSC reprogramming, embryonic development, and differentiation (Ohnuki et al., 2014; Lu, 2023; Lyu et al., 2024).

Given the important role of TEs in activating immune responses (Kong et al., 2019; Du et al., 2023) and, in some cases, driving senescence (Liu et al., 2023; Di Giorgio et al., 2024), their targeted activation or suppression could have significant therapeutic potential. For instance, the activation of tumor- or stem cell-specific TEs may enhance immunotherapy and radiotherapy efficacy by inducing inflammation in the tumor microenvironment or promoting tissue regeneration following radiation-induced damage. However, further research and tools are needed to fully elucidate TE-associated mechanisms, as their activation has also been linked to oncogene activation and tumor formation (Jang et al., 2019), as well as to the onset of autoimmune and neurodegenerative conditions (Ye et al., 2020; Casale et al., 2022; Evering et al., 2023). Further research integrating chromatin analysis and single-cell ATAC sequencing techniques could help determine whether specific TEs are activated in a tissue- or treatment-dependent manner, paving the way for innovative therapeutic strategies, not only for improving radiotherapy outcomes but also for treating a wide range of currently untreatable conditions.

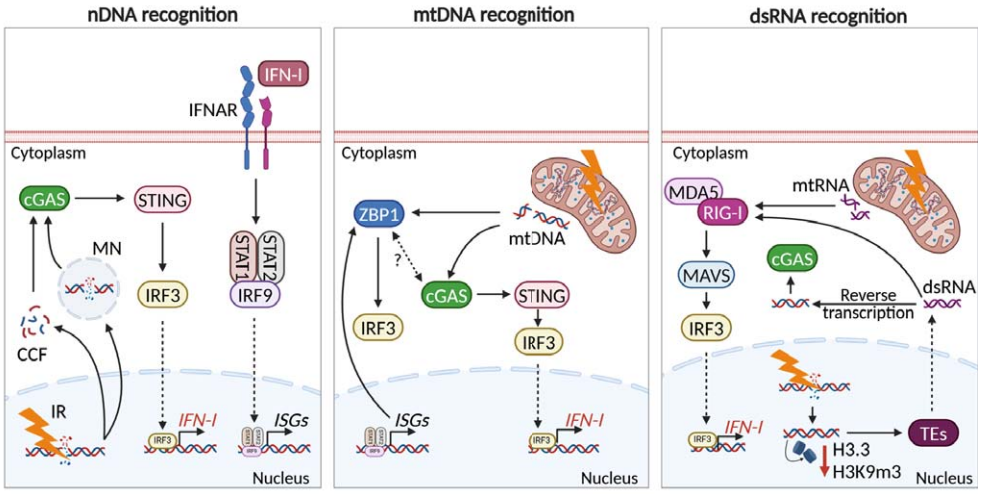

Figure 2. Radiation-induced IFN-I signaling activation.

This schematic summarizes the key pathways responsible for cytosolic nucleic acid recognition after irradiation (IR), based on findings from **Chapter 4** and existing literature (Miller et al., 2021). Radiation-induced DNA damage can lead to the release of cytoplasmic chromatin fragments (CCF) and the formation of micronuclei (MN). Upon binding dsDNA, cGAS activates STING, which in turn promotes IRF3 activation, leading to the expression of type I interferons (IFN-I). IFN-I then exits the cell and interacts with its receptor (IFNAR), triggering the formation of the STAT1/STAT2/IRF9 complex, which translocates into the nucleus to drive the transcription of interferon-stimulated genes (ISGs). In response to irradiation, mitochondria can also release DNA (mtDNA) and RNA (mtRNA) into the cytoplasm. cGAS and ZBP1 can recognize mtDNA and may interact to amplify the IRF3/IFN-I signaling axis. Meanwhile, cytoplasmic double-stranded RNA (dsRNA) and mtRNA are recognized by RIG-I and MDA5, activating the MAVS/IRF3/IFN-I pathway. Radiation-induced loss of histone H3.3 and H3K9me3 can lead to the derepression of transposable elements (TEs), resulting in dsRNA accumulation into the cytoplasm. This dsRNA can activate the RIG-I/MAVS/IRF3/IFN-I signaling cascade. Cytoplasmic dsRNA can be retrotranscribed into dsDNA, which is then recognized by cGAS. (Figure created with BioRender.com).

SALIVARY GLAND STEM CELLS: ADVANCES AND FUTURE CHALLENGES

The lack of well-defined salivary gland stem cell markers remains a major obstacle in advancing precision therapy techniques and personalized treatment strategies. Although efforts to spare the ductal region of salivary glands, where potential stem/progenitor cells reside, have shown promise in improving radiotherapy outcomes (Van Luijk et al., 2015; van Rijn-Dekker et al., 2024), and the use of organoids for regenerating damaged salivary glands (Maimets et al., 2016a; Zanten et al., 2024) have significantly advanced the field, a deeper understanding of stem/progenitor cell populations and their regulation is essential. While results in **Chapter 5** identify two populations with stem/progenitor cell features, a definitive

salivary gland stem/progenitor cell marker remains unidentified. Chapter 4 and 5 showed that cells with high SOX9 expression exhibited enhanced stem cell features and increased proliferation following irradiation, suggesting a compensatory response to radiation damage (Figure 3). However, SOX9 also marked intercalated duct cells in salivary gland tissue, indicating that this region may harbor these stem/progenitor cells. High SOX9 expression may specifically identify stem/progenitor populations, while lower SOX9 levels may define intercalated duct cells. This pattern is similar to endocrine tissues, where different SOX9 expression levels distinguish stem/progenitor cells (Formeister et al., 2009). To further characterize these populations, reporter mouse models carrying GFP-SOX9, combined with single-cell sequencing analysis of enriched GFP-SOX9-expressing cells, could help determine whether distinct SOX9 subpopulations exist. Additionally, bioinformatic tools could aid in identifying surface marker candidates for fluorescence-activated cell sorting (FACS), enabling the precise isolation and characterization of these subpopulations. Assessing the stemness and long-term post-autologous transplantation and regeneration potential of these cells could help determine whether high SOX9-expressing cells truly possess stem cell properties.

Validating the presence and functionality of SOX9-expressing cells in patient-derived tissue samples is crucial for clinical translation. This could contribute to the development of personalized therapies, including the potential autologous transplantation of SOX9-expressing stem/progenitor cells to promote salivary gland regeneration. However, thorough preclinical *in vitro* and *in vivo* studies are necessary, as sustained SOX9 expression has been implicated in oncogenic transformation of follicle stem cells (Yang et al., 2023b) and has been linked to tumor initiation and invasion (Larsimont et al., 2015; Kawai et al., 2016; Panda et al., 2021). Therefore, ensuring that transplanted cells do not contribute to tumorigenesis will be a critical step in developing safe and effective regenerative therapies.

While *Sox9*-expressing cells in mouse salivary gland responded positively to IFN-β treatment by exhibiting increased stemness, *Itgb1/Cd44*-expressing cells showed unique pro-acinar features, migratory properties, and upregulated Notch signaling at early time points post-irradiation (Figure 3). Staining of mouse salivary gland tissue confirmed CD44 expression in acinar cells (data not shown), suggesting that CD44 could serve as both a pro-acinar and acinar marker. However, similarly to SOX9, further analysis is necessary to determine whether a distinct CD44 subpopulation characterizes pro-acinar stem/progenitor cells *in vivo*. Notably, what stands out from these findings is the unique migratory capacity of CD44-expressing cells. Stem cells inherently migrate to maintain tissue homeostasis and regeneration

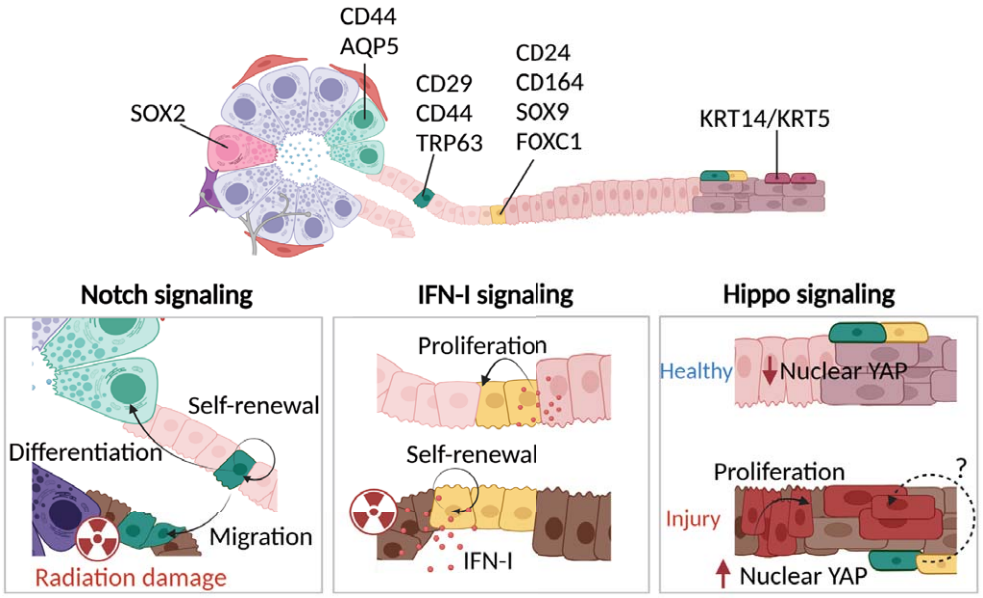

Figure 3. Stem/progenitor cell markers and signaling pathways regulating stem/progenitor cell activity in the salivary gland.

This figure presents an updated overview of stem/progenitor cell markers and signaling pathways in the salivary gland, integrating findings from **Chapters 4**, **5**, and **6** and existing literature. **Chapter 5** highlights the essential role of Notch signaling in regulating self-renewal and differentiation of salivary gland organoids under regenerative conditions, as well as its role in controlling the migration of CD29/CD44-expressing cells. **Chapter 4** demonstrates the pivotal role of the IFN-I signaling pathway in enhancing SOX9-expressing stem/progenitor cell activity after irradiation, both *in vitro* and *in vivo*. **Chapter 6** elucidates the importance of Hippo signaling and YAP nuclear translocation in coordinating ductal cell proliferation following salivary gland injury and promoting organoid self-renewal after irradiation. (Figure created with BioRender.com).

(de Lucas et al., 2018). To validate this behavior, *in vivo* analyses using reporter mice with inducible markers could allow the tracking of CD44-expressing cells movement and their regenerative role. Additionally, selective irradiation or partial gland damage would further clarify whether these cells become activated and migrate in response to injury. Interestingly, a study in mouse and human mammary gland tissue showed that co-expression of epithelial-mesenchymal transition (EMT)-related genes and *Sox9* induces a stem cell state (Guo et all., 2009). Since bioinformatic analysis in **Chapter 5** identified strong EMT features in *Itgb1/Cd44*-expressing cells, it would be interesting to investigate whether a subpopulation of these cells also expresses *Sox9*, potentially leading to enhanced stemness and migratory capacity. Building on previous research (Van Luijk et al., 2015), irradiating different portions of the salivary

gland and integrating single-cell RNA sequencing with spatial transcriptomics at different timepoints post-irradiation could provide deeper insights into which regions should be spared to enhance salivary gland regeneration. This approach would also help determine the presence of a mixed *Sox9/Itgb1/Cd44* stem/progenitor population, assess its location, potential migration in response to damage, and identify the signaling pathways regulating this process.

Chapters 5 and 6 highlight Notch and Hippo signaling as critical pathways involved in salivary gland regeneration (Figure 3). Investigating their potential crosstalk with the IFN-I response observed in Chapter 4 could provide valuable insights for enhancing post-radiation recovery. *In vivo* models and knockout systems could help elucidate the regulatory network governing these pathways and determine their interconnections. For instance, Chapter 5 shows a higher upregulation of Notch signaling following proton irradiation compared to photon irradiation, aligning with the increased IFN-I response observed in Chapter 4. This raises the question whether a connection between these two pathways mediates the heightened stem cell activity observed after proton irradiation. Interestingly, recent studies have linked the DNA damage response protein ATM to Notch signaling (Adamowicz et al., 2016; Giuranno et al., 2020). Additionally, KAP1, a key regulator of TE expression (Stoll et al., 2019), is a known ATM substrate involved in chromatin relaxation following DNA damage (White et al., 2012). This suggests that Notch signaling activation in response to DNA damage could have broader implications by influencing KAP1 activation, altering specific TE expression patterns, and modulating inflammatory and immune responses. Therefore, Notch signaling may serve as a central regulator not only in stem cell function and differentiation but also in immunity and inflammation after irradiation. Notably, Notch signaling is essential for embryonic development (Yu et al., 2008), with its activity tightly regulated during salivary gland formation and branching morphogenesis (Chatzeli et al., 2023). Interestingly, KAP1 has been shown to regulate endogenous retrovirus (ERV) expression, a type of TE, during embryogenesis (Rowe et al., 2010). This suggests a potential interconnection between Notch signaling, KAP1, and TE expression, which may play a role not only in tissue homeostasis and repair but also during cell fate decisions and tissue formation. Furthermore, KAP1-mediated ERV regulation appears to be driven by histone methylation patterns, such as H3K9me3 (Rowe et al., 2010). Similar changes have been observed in Chapter 4 following irradiation, suggesting that shared regulatory mechanisms could govern both embryonic development and adult stem cell responses during regeneration.

Chapter 6 reveals increased proliferation and YAP nuclear translocation in damaged excretory and striated ducts (Figure 3). Moreover, modulation of Hippo

signaling strongly influenced stem/progenitor cell activity *in vitro*. However, it remains unclear whether specific stem/progenitor cells modulate Hippo signaling to drive proliferation and tissue regeneration. A combined spatial and single-cell RNA sequencing approach in healthy and damaged tissue could help determine whether regeneration originates from cells within the striated/excretory compartment or from migratory stem/progenitor cells from other locations. Furthermore, since YAP nuclear translocation was observed in ligated salivary glands, irradiation studies are needed to evaluate the role of Hippo signaling in the regeneration of irradiated salivary glands.

SPECULATIONS ON THE EFFECTS OF PROTON IRRADIATION ON CHROMATIN DYNAMICS

Chapter 4 reveals a significant loss of heterochromatin regulators following irradiation, with a more pronounced effect observed after proton irradiation compared to photon irradiation. This aligns with a recent study showing decreased methylation patterns in tumors exposed to proton therapy (Schniewind et al., 2022). However, the underlying mechanisms that drive these effects remain unknown. A more physics modelling-based approach is needed to determine whether the distinct physical properties of protons are responsible for the observed differences in Chapter 4 and whether these effects are unique to protons or shared with other high-LET particle therapies, such as carbon ions.

Protons, unlike photons, are particles with a mass and positive charge, which fundamentally influence the way they interact with biological tissue and DNA (Vitti and Parsons, 2019). Given this, it is not surprising that since the advent of proton therapy, research has explored whether there are differences in DNA damage foci formation and the activation of DNA damage repair signaling pathways compared to conventional X-ray irradiation (Giedzinski et al., 2005; Alan Mitteer et al., 2015; Vitti and Parsons, 2019; Schniewind et al., 2022). However, while DNA damage-related mechanisms are typically transient and observed at very early time points after irradiation, the findings in Chapter 4 reveal differences at 6 days post-irradiation. By this time, DNA repair mechanisms should have been largely completed, with cells either repairing the damage, undergoing cell death, or entering senescence. This suggests that proton irradiation may induce lasting cellular changes beyond the initial DNA damage response.

Protons have been shown to induce complex DNA double-strand breaks, particularly at the distal edge of the Bragg peak, which are more difficult to repair (Horendeck et al., 2021). It would be intriguing to investigate whether similar damage may occur at the plateau region of protons inducing complex yet reparable DNA

lesions, potentially leaving behind a form of epigenetic memory. This phenomenon has been observed in skin fibroblasts, where photon-irradiated cells retained a long-term radiation memory in form of epigenetic modifications, leading to the upregulation of specific signaling pathways, such as THBS1 (Bian et al., 2023). Similarly, mouse studies have shown persistent changes in DNA methylation patterns in proton-irradiated brains (Impey et al., 2016). This raises the possibility of an "epigenetic scar" following the interaction of charged proton particles with DNA structures. To explore this, in-depth chromatin analysis using bulk and single-cell ATAC sequencing, along with more fundamental physics approaches, could be employed to investigate the presence of such an epigenetic signature. Additionally, this could help determine whether proton particles interact differently with compact chromatin regions, potentially explaining the greater loss of heterochromatin regulators, such histone variant H3.3 and H3K9me3, and the upregulation of transposable elements (TEs) after proton irradiation. Moreover, it would be interesting to show how multiple irradiation sessions influence this response, mimicking the fractionated plan used in the clinic.

Interestingly, a recent study showed no differences between photon and proton irradiation on irradiated brain tissue at the level of the microglial immune response (Voshart et al., 2024). This raises the important question of whether the effects observed in salivary gland organoids are tissue- or model-dependent. Extending these studies to different models and tissues would help further elucidate the effects of proton therapy on normal tissue.

CONCLUSION

In conclusion, this thesis demonstrates how organoid models can provide valuable insights into the molecular mechanisms affected by photon and proton irradiation. Translating these findings into (pre-)clinical settings is crucial for refining HNC treatment options and improving patients' quality of life and recovery. Incorporating immune cells by using *in vivo* models or more advanced *in vitro* systems would allow for the evaluation of drug-radiotherapy combinations and immunotherapy in more physiologically relevant systems, paving the way for more effective treatment strategies. Furthermore, a deeper understanding of the stem/progenitor cell populations within the salivary gland and their complex regulatory networks is essential, as it would significantly contribute to the development of personalized treatment approaches. Additionally, the integration of advanced sequencing techniques and chromatin studies would help validate the differential responses and chromatin interactions between proton and photon irradiation, further enhancing therapeutic strategies.