DNA DAMAGE AFFECTING THYROIDAL IODIDE TRANSPORT: AN EXPLANATION TO THYROID STUNNING

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“Everything is possible but the impossible

– just do it”

To My Beloved Parents and Family,
Sten, Britt-Marie, Martin
and Jonathan

ABSTRACT

DNA DAMAGE AFFECTING THYROIDAL IODIDE TRANSPORT: AN EXPLANATION TO THYROID STUNNING

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^{131}I is widely used clinically in the treatment of Graves´ disease and differentiated thyroid cancer. However, cellular and molecular effects of ^{131}I irradiation in relation to absorbed dose are poorly documented. For instance, it is unknown what absorbed doses give rise to acute or delayed lethality, DNA damage that is fully restorable by DNA repair, or may cause permanent genomic instability. The phenomenon of thyroid stunning (i.e. inhibition of the iodide uptake in the thyroid gland after a diagnostic test dose of ^{131}I) indicates that further studies are needed to characterize the effects of radiation on the thyroid at the cellular and molecular levels. Elucidating the mechanism causing thyroid stunning was the aim of this thesis.

In papers I-II the effects of low absorbed doses of ^{131}I on TSH-stimulated iodide transport and NIS expression were investigated. Primary porcine thyroid cells cultured on filter in bicameral chambers were continuously exposed to ^{131}I for 48 h prior to analysis. A significant reduction of iodide transport was seen at absorbed doses $\geq$0.15 Gy, correlating to down-regulation of NIS mRNA expression. Notably, stimulation with IGF-I counteracted the effects of ^{131}I irradiation. DNA synthesis and total cell numbers were unchanged at doses $\leq$1 and 3 Gy, respectively, indicating that thyroid stunning is independent of radiation effects on cell cycle regulation.

In papers III-IV, a possible correlation between thyroid stunning and radiation induced DNA damage mediated by the ataxia telangiectasia mutated (ATM) kinase was investigated. The genotoxic agent calicheamicin γ1 was used to induce high amounts of DNA double strand breaks. Both iodide transport and NIS mRNA expression were significantly reduced by sub-lethal concentrations of calicheamicin γ1. This correlated with global formation of γ-H2AX and Chk2 nuclear foci activated by ATM. Blockage of DNA-PK enhanced genotoxic induced repression of NIS transcription and iodide transport, supporting the hypothesis that ^{131}I-induced thyroid stunning is a stress response to DNA damage. In addition, inhibition of ATM diminished the effect of calicheamicin γ1 on both iodide transport and NIS expression implying that ATM most likely is a mediator of DNA damage-induced thyroid stunning.

In conclusion, this thesis provides novel data indicating that thyroid stunning is due to down-regulation of NIS partially elicited by the ATM-dependent DNA damage response.

Keywords: thyroid, radioiodide, thyroid stunning, NIS, cell cycle, genotoxic stress, DNA damage, H2AX, ATM

LIST OF PUBLICATIONS

The thesis is based on the following papers, referred to in the text by roman numerals (I-IV)

I. **Lundh C*, Nordén M. M*, Nilsson M, Forsell-Aronsson E.** Reduced Iodide Transport (Stunning) and DNA Synthesis in Thyrocytes Exposed to Low Absorbed Doses from ^{131}I In Vitro. *J Nucl Med.* 2007; 48(3): 481-486.
* Contributed equally to this work.

II. **Nordén M. M, Larsson F, Tedelind S, Carlsson T, Lundh C, Forsell-Aronsson E, Nilsson M.** Down-regulation of the Sodium/Iodide Symporter Explains ^{131}I-Induced Thyroid Stunning. *Cancer Res*. 2007; 67: 7512-7517.

III. **Bhogal N*, Nordén M. M*, Karlsson J-O, Postgård P, Himmelman J, Forsell-Aronsson E, Hammarsten O, Nilsson M.** DNA Damage Represses Sodium/Iodide Symporter (NIS) Gene Expression. *Submitted Manuscript.*
* Contributed equally to this work

IV. **Nordén M. M, Ingeson C, Hammarsten O, Carlsson T, Nilsson M.** DNA Damage-induced Repression of NIS Expression and Iodide Transport is Mediated by ATM. *In Manuscript*

Paper **I** and **II** were reprinted by permission of the Society of Nuclear Medicine, and the American Association for Cancer Research, respectively.

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ABBREVIATIONS

AIT	Apical iodide transporter
ATM	Ataxia telangiestasia mutated kinase
cAMP	Cyclic adenosine mono-phosphate
Chk2	Checkpoint kinase 2
CLM	Calicheamicin γ 1
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DSB	Double-strand DNA breaks
EGF	Epidermal growth factor
γ–H2AX	Gamma-histone variant H2AX
^{125}I	Iodine-125
^{131}I	Iodine-131
IGF-I	Insulin like growth factor I
IR	Ionizing radiation
MAPK	Mitogen activated protein kinase
MMI	Methimazole
NIS	Sodium/Iodide symporter
NUE	NIS upstream enhancer
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
SSB	Single-strand DNA breaks
T_3	Triiodothyronine
T_4	Thyroxine
TG	Thyroglobulin
TPO	Thyroperoxidase
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor

INTRODUCTION

Although ^{131}I is widely used clinically in the treatment of thyroid diseases little is known about the cellular and molecular effects of ^{131}I irradiation in relation to absorbed dose. The phenomenon of thyroid stunning, implicating inhibition of the iodide uptake in the thyroid gland after a diagnostic test dose of ^{131}I indicates that further studies are needed to characterize these effects. With the aim to elucidate the mechanism causing thyroid stunning, this thesis presents cellular and molecular effects of ^{131}I irradiation on the thyroid.

The Thyroid Gland

The thyroid gland consists of two lateral lobes situated on either side of the trachea in the anterior part of the neck. The lobes, each being approximately 5 cm long and 3-4 cm wide in humans, are connected across the midline by the isthmus, reminiscent of the embryonic origin from a common median thyroid anlage. The functional units of the gland are the follicles, which are thousands of spherical structures each encircled by a single layer of epithelial cells resting on a basement membrane. Each follicle contains an inner follicular cavity called the lumen, which is filled with colloid, a proteinaceous fluid enriched of the thyroid prohormone thyroglobulin (TG) (Nilsson, 2001). The follicular cells produce the thyroid hormones, tetraiodothyronine (T_4) and triiodothyronine (T_3). Embedded within and between the follicles are also the calcitonin-producing C-cells, the second endocrine cell type of the thyroid.

The surface of the follicular cells is divided into apical and basolateral domains that possess distinct structural and biochemical features, as illustrated in figure 1. The basolateral plasma membrane facing the extrafollicular space exhibits all receptors to external signaling molecules as hormones, cytokines and growth factors. In the apical or luminal membrane the effector molecules of thyroid hormone synthesis, such as thyroperoxidase (TPO) and NADPH oxidase, reside. The polarized phenotype of the cells provides the fundamental basis of exteriorization of hormonogenesis to the extracellular (luminal) space. This is important not only to assemble the hormone constituents and enzymes involved in hormonogenesis but also to protect the interior of the cells from noxious hydrogen peroxide and other reactive oxygen species generated in large quantities at the apical surface with the purpose to oxidize iodide.

Tight junctions encircling the apical pole of the cells support the polarized distribution of membrane proteins by restricting lateral diffusion between the apical and basolateral plasma membranes (Nilsson, 2001). The tight junctions also maintain solute gradients established by active transport between the luminal and extrafollicular spaces. One such gradient is iodide, of central importance to the issues of this thesis.

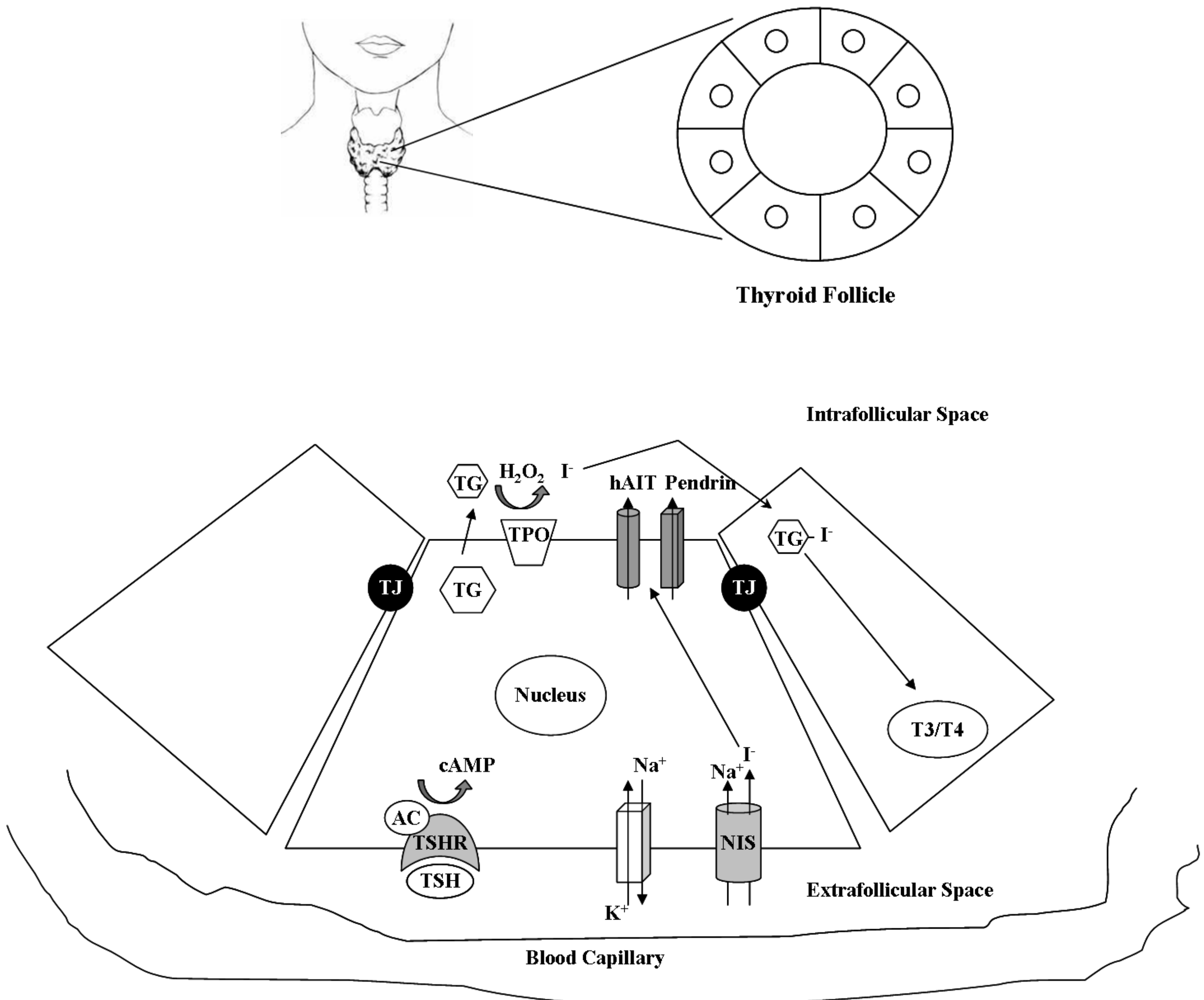

Figure 1. Top: *Illustration of the anatomical localization of the thyroid gland and the microscopic follicle structure.* ***Bottom:*** *An overview of the polarized distribution of molecules involved in the different steps of thyroid hormone synthesis in the thyroid epithelial cell. See text for further comments.*

Thyroid Hormone Synthesis

The thyroid hormones regulate developmental growth and metabolism in vertebrates. Ingested iodine has an essential role in thyroid physiology as it is incorporated in T_3 and T_4, without this component no thyroid hormones can be formed. Iodine, is absorbed as iodide in the intestine and concentrated 40-fold or more in the thyroid by active transport formerly known as the "iodide pump" (Wolff, 1964). Now we know that iodide uptake is mediated by a symport mechanism, further described below. The negatively charged iodide entering the follicular lumen is unable to bind to the tyrosine residues of TG and is therefore oxidized to iodine (I_2) by the action of TPO before organification, the enzymatic iodide-protein binding, can occur (Eskandari *et al.*, 1997; Smyth and Dwyer, 2002). In addition, this process involves TPO-mediated coupling of iodinated tyrosyls to form the thyronines still within the TG peptide chain. T_3 and T_4 are therefore stored in the follicular lumen, covalently bound to TG. The secretion of thyroid hormones is initiated by endocytosis of iodinated TG from the follicle lumen, followed by proteolytic cleavage of the prohormone by lysosmal enzymes releasing T_3 and T_4 into the circulation.

Iodide Transport

Accumulation of iodide within the gland is required for the efficient formation of thyroid hormone in the thyroid. This involves a two-step transport process in which iodide is efficiently extracted from the blood and delivered to the follicular lumen by sequentially basolateral uptake and apical efflux mechanisms. The most important aspects of thyroidal iodide transport will be summarized in the following sections (for more comprehensive reviews, see (Carrasco, 1993; Levy *et al.*, 1998; Nilsson, 1999; Nilsson, 2001; Riesco-Eizaguirre and Santisteban, 2006)).

The Sodium Iodide Symporter (NIS)

Iodide uptake is mediated by the sodium iodide symporter (NIS) that co-transports one iodide and two sodium ions into the cell (Eskandari *et al.*, 1997). As shown in figure 2, NIS is an integral protein with 13 transmembrane segments, the NH_2 terminus facing extracellularly and

the COOH terminus facing the interior of the cell (Dohan *et al.*, 2003). The driving force of NIS-mediated iodide uptake is the inwardly directed Na^+ gradient generated by the sodium potassium adenosine triphosphatase (Na^+/K^+-ATPase). Hence, iodide transport is classically inhibited by the Na^+/K^+-ATPase blocking drug ouabain. In addition NIS activity is blocked by the competitive inhibitors thiocyanate and perchlorate. However, the mechanism by which perchlorate blocks NIS activity has been debated, and recently Dohán *et al.* (Dohan *et al.*, 2007) reported that NIS mediates electroneutral transport of perchlorate suggesting that NIS translocates different substrates with different stoichiometries.

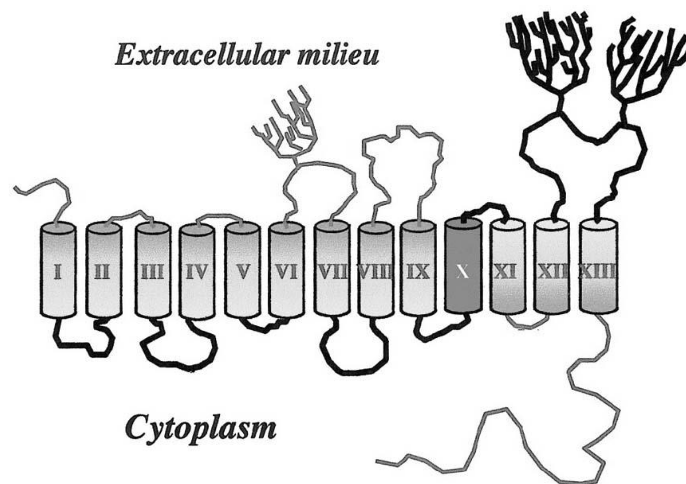

Figure 2. *Schematic representation of the 13 transmembrane segments of NIS. The amino terminus is located at the extracellular end whereas the carboxyl terminus faces the cytosol. Modified from (De La Vieja et al., 2000)*

The *NIS* gene was first cloned and characterized in rat *(rNIS*) by Carrasco and co-workers (Dai *et al.*, 1996). Subsequently *NIS* in human (*hNIS*) (Smanik *et al.*, 1996), mouse (*mNIS*) (Perron *et al.*, 2001; Pinke *et al.*, 2001) and pig (*pNIS*) (Selmi-Ruby *et al.*, 2003) were identified. The human *NIS* gene is mapped to chromosome 19 (19p12-13.2) and encodes a glycoprotein of 643 amino acids with a molecular mass of approximately 70-90 kDa (Smanik *et al.*, 1996). In humans, mice and rats a single *NIS* transcript varying in size between 2.9 to 3.7 kb is detected. However, p*NIS* consists of two transcripts of 3 kb and 3.5 kb, respectively (Selmi-Ruby *et al.*, 2003). The most abundant *pNIS* transcript is the full-length 3.5-kb

transcript encoding a protein composed of 643 amino acids similar to the hNIS protein. The reason for alternative splicing of *pNIS* is presently unknown. The pNIS protein presents 85% identity with the hNIS protein (figure 3) and about 79% with rNIS and mNIS proteins. Cultured porcine thyroid cells were used as an experimental model in all studies presented in this thesis. When pNIS expression was investigated by quantitative real time RT-PCR primers detecting both pNIS transcripts were used.

```
hNIS  1     MEAVETGERPTFGAWDYGVFALMLLVSTGIGLWVGLARGGQRSAEDFFTGGRRLAALPVG  60
            M  VE G R TFGAWDYGVFALMLLVSTGIGLWVGLARGGQRSAEDFFTGGRRL A+PVG
pNIS  1     MATVEGGARATFGAWDYGVFALMLLVSTGIGLWVGLARGGQRSAEDFFTGGRRLTAVPVG  60

hNIS  61    LSLSASFMSAVQVLGVPSEAYRYGLKFLWMCLGQLLNSVLTALLFMPVFYRLGLTSTYEY  120
            LSLSASFMSAVQVLGVP+EAYRYGLKFLWMCLGQLLNS+LTALLF+PVFYRLGLTSTY+Y
pNIS  61    LSLSASFMSAVQVLGVPAEAYRYGLKFLWMCLGQLLNSLLTALLFLPVFYRLGLTSTYQY  120

hNIS  121   LEMRFSRAVRLCGTLQYIVATMLYTGIVIYAPALILNQVTGLDIWASLLSTGIICTFYTA  180
            LE+RFSRAVRLCGTLQY+VATMLYTG+VIYAPALILNQVTGLDIWASLLSTGIICTFYT
pNIS  121   LELRFSRAVRLCGTLQYLVATMLYTGVVIYAPALILNQVTGLDIWASLLSTGIICTFYTT  180

hNIS  181   VGGMKAVVWTDVFQVVVMLSGFWVVLARGVMLVGGPRQVLTLAQNHSRINLMDFNPDPRS  240
            VGGMKAV+WTDVFQV+VML+GFWVVLARG +LVGGP +VL LA+NHSRINLMDF+ DPR
pNIS  181   VGGMKAVIWTDVFQVLVMLTGFWVVLARGTVLVGGPGRVLELAKNHSRINLMDFDLDPRR  240

hNIS  241   RYTFWTFVVGGTLVWLSMYGVNQAQVQRYVACRTEKQAKLALLINQVGLFLIVSSAACCG  300
            RYTFWTFVVGGTLVWLSMYGVNQAQVQRYVACRTEKQAKLALLINQVGLFLIVSSAA CG
pNIS  241   RYTFWTFVVGGTLVWLSMYGVNQAQVQRYVACRTEKQAKLALLINQVGLFLIVSSAAACG  300

hNIS  301   IVMFVFYTDCDPLLLGRISAPDQYMPLLVLDIFEDLPGVPGLFLACAYSGTLSTASTSIN  360
            IVMF  Y DCDPLL G ISAPDQYMPLLVLDIFEDLPGVPGLFLACAYSGTLSTASTSIN
pNIS  301   IVMFALYVDCDPLLAGHISAPDQYMPLLVLDIFEDLPGVPGLFLACAYSGTLSTASTSIN  360

hNIS  361   AMAAVTVEDLIKPRLRSLAPRKLVIISKGLSLIYGSACLTVAALSSLLGGGVLQGSFTVM  420
            AMAAVTVEDLIKPRL +LAPR+LVIISKGLSLIYGSACLTVAALSSLLGGGVLQGSFTVM
pNIS  361   AMAAVTVEDLIKPRLPNLAPRRLVIISKGLSLIYGSACLTVAALSSLLGGGVLQGSFTVM  420

hNIS  421   GVISGPLLGAFILGMFLPACNTPGVLAGLGAGLALSLWVALGATLYPPSEQTMRVLPSSA 480
            GVISGPLLGAF+LGMFLP+CNT GVL+GL AGLALSLWVA+GA+LYPPS Q+M VLPSSA
pNIS  421   GVISGPLLGAFVLGMFLPSCNTSGVLSGLAAGLALSLWVAVGASLYPPSAQSMGVLPSSA 480

hNIS  481   ARCVALSVNASGLLDPALLPANDSSRAPSSGMDASRPALADSFYAISYLYYGALGTLTTV 540
            A C   + NASGL DP +L  N SS A S   D  +P LA SFYAISYLYYGALGTL+T+
pNIS  481   AGCALPTANASGLQDP-VLAVNASSTASSLETDPEQPILAASFYAISYLYYGALGTLSTI 539

hNIS  541   LCGALISCLTGPTKRSTLAPGLLWWDLARQTASVAPKEEVAILDDNLVK-GPEELPTGNK 599
            LCGALISCLTGPTKRS L PGLLWWDL RQTASVAPKEEVA LDD+L+K G EELP   K
pNIS  540   LCGALISCLTGPTKRSALGPGLLWWDLTRQTASVAPKEEVAALDDSLMKQGAEELPLAIK 599

hNIS  600   KPPGFLPTNEDRLFFLGQKELEGAGSWTPCVGHDGGRDQQETNL 643
            KPP FL TNED L FLGQKE+ GA S TP   HD G D +ET+L
pNIS  600   KPPDFLSTNEDHLLFLGQKEVNGASSKTPGSEHDKGHDLRETDL 643
```

Figure 3. *Alignment of the human and porcine NIS amino acid sequences using BLAST at NCBI's webpage. The sequence of porcine NIS exhibits 85 % identity to that of the human NIS protein. + indicates similar amino acids, while – represents a gap.*

The cloning of the *NIS* gene opened up a new field of research that has lead to important discoveries regarding the role of NIS-mediated iodide transport in thyroid physiology and disease (Riesco-Eizaguirre and Santisteban, 2006). For instance, mutations of hNIS identified a novel cause of congenital hypothyroidism (De La Vieja *et al.*, 2000; Dohan *et al.*, 2003; Reed-Tsur *et al.*, 2008). The expression of functional NIS is fundamental to the successful use of iodine isotopes and other halides in diagnostic thyroid scintigraphy of various thyroid diseases and for radiotherapy of Graves' disease and thyroid cancer. It is also known that NIS is expressed in many extra-thyroidal organs, e.g. salivary gland, breast, gastric mucosa, placenta and kidney (Bidart *et al.*, 2000b; Lacroix *et al.*, 2001; Spitzweg *et al.*, 2001; Spitzweg *et al.*, 1998), although due to the lack of enzymes catalyzing iodination in these tissues the retention of radioiodine is much less than in the thyroid. Nevertheless, employing NIS-mediated iodide uptake in radiotherapy of non-thyroidal cancer is currently an issue of great interest (Spitzweg and Morris, 2002). In this respect, NIS is also suggested to be a novel therapeutic gene in genetic transfer, targeting radioiodine to tumors not expressing endogenous NIS (Riesco-Eizaguirre and Santisteban, 2006; Spitzweg and Morris, 2004).

Regulation of NIS Expression and Function

TSH and Signal Transduction Pathways

It has been known for many years that TSH stimulates iodide transport into the thyroid gland via an adenylate cyclase-cAMP-protein kinase A (PKA) mediated pathway (Carrasco, 1993; Dumont *et al.*, 1989). Accumulating evidence indicate that this is accomplished by both transcriptional and post-transcriptional regulation of NIS. As indicated in figure 4, TSH via cAMP stimulates the NIS promoter involving the NIS upstream enhancer (NUE), which is a unique promoter element found only in *NIS* among the thyroid-specific genes (Taki *et al.*, 2002). Ohno *et al.* (Ohno *et al.*, 1999) reported that cAMP regulation of NUE in rat requires binding of the thyroid transcription factor Pax8 at two sites to be fully functional, and Taki *et al.* (Taki *et al.*, 2002) reported that human NUE contains a Pax8 element and a cAMP responsive element (CRE)-like sequence, both necessary for full activity. This indicates that absence of either Pax-8 or CRE-like binding proteins (CREB) might explain the reduced *hNIS* gene expression in thyroid carcinomas.

The importance of cAMP in NIS gene regulation is illustrated by observations in chronically TSH-stimulated FRTL-5 cells in which the catalytic subunit of PKA is down-regulated. Under these conditions, acute stimulation with a cAMP agonist results in increased NIS transcriptional activity without PKA activation (Armstrong *et al.*, 1995). However, other pathways of TSH receptor (TSHR) signaling are also implicated in the transcriptional regulation of NIS (figure 4). Taki *et al*. (Taki *et al.*, 2002) showed that both PKA-dependent pathways and PKA-independent pathways are involved in the stimulation of NUE by observing an activation of NUE in response to forskolin treatment after chronic TSH stimulation without endogenous PKA, while over-expression of exogenous PKA increases the NUE activity without cAMP. Mitogen-activated protein kinase (MAPK) signaling is also regulating NIS expression. For instance, inhibition of MEK/Erk activated Rap1, a member of the Ras superfamily of small guanosine triphosphate (GTP)-binding proteins, (Tsygankova *et al.*, 2001), partially inhibits cAMP-induced NIS gene transcription (Taki *et al.*, 2002). However, the extent of MAPK activation in response to TSH/cAMP varies between the experimental system (different species and cell lines) and contradictory results have been reported (Medina and Santisteban, 2000; Vandeput *et al.*, 2003).

Activation of p38, another MAPK signaling pathway involving the small GTPase Rac1, may also participate in PKA-dependent regulation of NIS. It has been shown that application of the p38-MAPK inhibitor, SB203580, significantly decreased the cAMP-induced NIS mRNA expression in FRTL-5 cells (Pomerance *et al.*, 2000). The same authors reported activation of p38-MAPK by reactive oxygen species (Pomerance *et al.*, 2000) which is of interest to this thesis since ^{131}I generates free radicals. Moreover, Pax-8 can also be regulated by redox state (Puppin *et al.*, 2004). TSH stimulates the reduction of Pax-8, increases the expression of redox factor-1 (Ref-1, also known as apurinic apyrimidinic endonuclease, APE) as well as the translocation of Ref-1 into the nucleus (Tell *et al.*, 2000), events resulting in an up-regulation of NIS. Furthermore, Ref-1 acts as an apurinic/apyrimidinic endonuclease during the repair of cellular alkylation and oxidative DNA damages (Tell *et al.*, 2005).

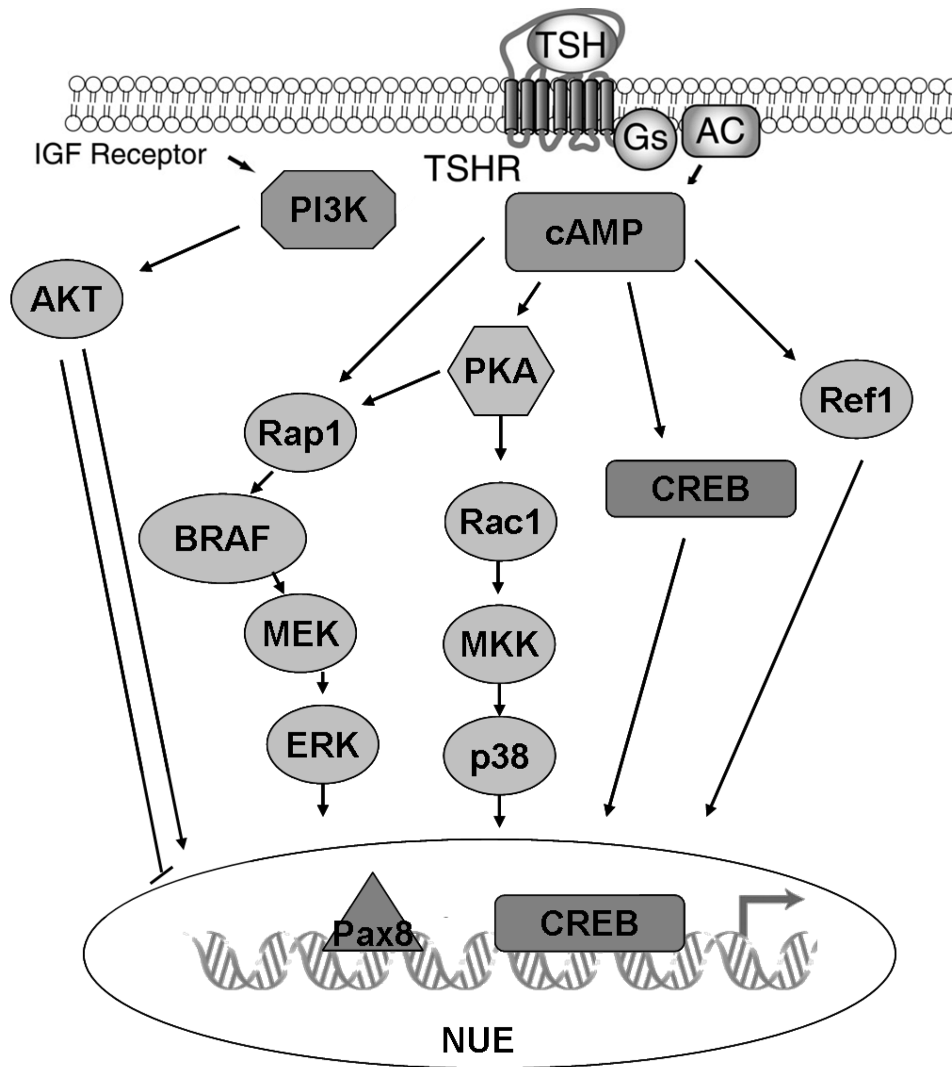

Figure 4. *Simplified schematic illustration of the signal transduction pathways involved in NIS regulation known to date. The figure shows the main TSH/cAMP stimulatory pathways both PKA-dependent and PKA-independent, and the IGF-I/PI3K pathway that either stimulates or inhibits NIS expression depending on species or cell type. See text for further details. BRAF, B-raf oncogene; CREB, cAMP responsive element binding protein; MEK, MAPK/Erk kinase; MKK, mitogen activated protein kinase kinsae. Modified from (Kogai et al., 2006; Riesco-Eizaguirre and Santisteban, 2006).*

Finally, PI3K signaling has been reported to regulate NIS expression. However, the resulting effect varies among species and cell types. Garcia and Santisteban (Garcia and Santisteban, 2002), showed that IGF-1 inhibits the cAMP-induced NIS expression through the PI3K pathway in FRTL-5 cells, and application of the PI3K inhibitor LY294002 significantly enhances NIS expression in these cells (Garcia and Santisteban, 2002; Kogai *et al.*, 2008b). On the other hand, it has been observed that activation of PI3K increases the NIS expression

in the MCF-7 human breast cancer cell line (Knostman *et al.*, 2004). Moreover, Kogai *et al.* (Kogai *et al.*, 2008a) recently reported that the IGF-I/PI3K pathway mediates retinoic acid-stimulated NIS expression in the same cells. Of particular importance to this thesis porcine thyroid cells respond to IGF-I with moderately increased iodide transport and, moreover, IGF-I markedly enhances TSH-stimulated iodide transport (Ericson and Nilsson, 1996). The delayed kinetics of this positive regulation suggests a permissive mode of action. However, it is yet unknown if IGF-I stimulates pNIS expression. Thus this was investigated in paper III along with evaluation of a putative radioprotective effect of IGF-I in irradiated cells (further commented below).

Iodine

Besides TSH the main regulating factor of NIS-mediated iodide uptake is iodine itself by a mechanism called auto-regulation. Already in 1948, Wolff and Chaikoff (Wolff and Chaikoff, 1948) showed that organification of iodide was blocked in rat thyroids *in vivo* when iodide plasma levels reached a critical high threshold. Since then this phenomenon is known as the Wolff-Chaikoff effect. A year later the same authors (Wolff *et al.*, 1949) reported an adaptation mechanism or escape from the effect appearing two days after its onset. Eventually this resulted in restored capacity of iodine organification. After all these years the Wolff-Chaikoff effect still remains poorly understood although it is believed that the inappropriate formation of iodolipids is an initiating event (Boeynaems *et al.*, 1995). Of central importance, inhibition of TPO activity with methimazole (MMI) abolishes the negative effect of excess iodine on iodide uptake (Grollman *et al.*, 1986), indicating that organification is required. MMI is used experimentally to avoid the Wolff-Chaikoff effect in iodide transport studies. *In vivo* experiments indicate that the escape from the Wolff-Chaikoff effect is manifested by down-regulation of NIS at the transcriptional level (Eng *et al.*, 1999; Spitzweg *et al.*, 1999). However, the precise mechanism by which the NIS expression is turned off has not been elucidated. It should also be mentioned that high doses of iodide decrease the NIS protein levels in a dose-dependent manner without affecting the NIS gene transcription in FRTL-5 cells (Eng *et al.*, 2001). This suggests that iodide may modulate NIS also post-transcriptionally.

Other Hormones and Cytokines

Transforming growth factor-beta (TGF-β), a potent inhibitor of thyroid cell proliferation has been shown to decrease TSH-induced NIS expression as well as NIS function in FRTL-5 cells (Pekary and Hershman, 1998). This may be part of general dedifferentiation induced by TGF-β in many epithelial cell types including thyrocytes (Grände *et al.*, 2002). As already mentioned, IGF-I decreases the NIS expression in the same cells (Garcia and Santisteban, 2002). rNIS transcription is also negatively regulated by estrogen (Furlanetto *et al.*, 2001). The authors hypothesized that the effect of sex steroids on NIS-mediated iodide uptake might contribute to the increased susceptibility to goiter in women (Furlanetto *et al.*, 2001).

Studies on cultured thyroid cells collectively indicate that NIS is negatively affected by pro-inflammatory cytokines. In fact, decreased iodide uptake is observed in response to the most important cytokines, i.e. tumor necrosis factor (TNF)-α, TNF-β, interferon (IFN)-γ, interleukin (IL)-1α, IL-1β and IL-6, implicated in autoimmune thyroid disease (Ajjan *et al.*, 1998; Rasmussen *et al.*, 1994; Spitzweg *et al.*, 1999). Moreover, Caturegli *et al.* (Caturegli *et al.*, 2000) showed significantly reduced NIS mRNA and protein levels in transgenic mice developing hypothyroidism following conditional expression of IFN-γ in the thyroid, indicating a direct role of NIS repression in the pathophysiology of autoimmune thyroiditis. As the cytokines affecting NIS act through distinct intracellular signaling pathways it is conceivable to assume that they trigger or deactivate a common down-stream regulatory mechanism, possibly shared by other stress responses in the thyroid cell.

Post-transcriptional Regulation of NIS

Riedel *et al*. (Riedel *et al.*, 2001) showed that NIS is also subjected to post-transcriptional regulation. It was demonstrated that NIS is a phosphoprotein and that the phosphorylation pattern is altered by TSH in a PKA-dependent manner. Furthermore, TSH increases the already long half-life of the NIS protein, and stimulates the trafficking of NIS to the plasma membrane. In subsequent studies it was shown that Glu 543, putatively located on the cytoplasmic side of transmembrane segment XIII of NIS, plays an important role for targeting intracellular NIS to the cell surface (De la Vieja *et al.*, 2005). Faulty trafficking of NIS likely explains the recognition of decreased iodide uptake activity in some thyroid cancers although

NIS is over-expressed (Arturi *et al.*, 2000; Dohan *et al.*, 2001; Saito *et al.*, 1997). Investigations of tissue extracts from hypofunctioning benign and malignant thyroid tumors have revealed that hNIS exhibiting a predominant intracellular localization is non-glycosylated (Trouttet-Masson *et al.*, 2004).

Epigenetic Events

Epigenetic modifications play an important role in the regulation of gene expression both normally and in disease, mainly recognized in cancer. The acetylation status of histones is determined by the activities of histone acetyltransferases and histone deacetylase (HDAC) (Marks *et al.*, 2001). HDAC inhibitors, e.g. depsipeptide, trichostatin A (TSA) and valproic acid (VPA) constitute a new group of anti-cancer drugs that reduce transformed cell growth both *in vitro* and *in vivo* and induce redifferentiation or apoptosis in tumor cells. Interestingly, HDAC inhibitors have been shown to reinduce NIS expression, suggesting means to improve the use of radioiodine therapy in poorly differentiated thyroid cancer.

DNA methylation of CpG islands in critical gene promoter regions results in heritable inhibition of gene expression, and abnormal patterns of DNA methylation are consistently observed in benign and malignant human thyroid tumors (Baylin, 1997; Matsuo *et al.*, 1993). The demethylating agent, 5-azacytidine, is able to restore NIS expression and radioiodide uptake in some thyroid cancer tumors (Neumann *et al.*, 2004; Venkataraman *et al.*, 1999).

Apical Iodide Efflux

In order to incorporate iodide into TG it must be delivered to the outer facet of the apical cell surface. This process is known as apical iodide efflux, executed by less well-characterized ion channels or carriers. The iodide permeability of the apical membrane varies depending on functional state of the thyroid cells, and TSH stimulates iodide efflux unidirectionally towards the apical compartment in cultured polarized thyrocytes (Nilsson *et al.*, 1990). Importantly, and in contrast to the effect of TSH on NIS expression and function, apical efflux of iodide is a rapid process noticed within minutes after the addition of TSH. The molecular correlate of

this TSH-regulated mechanism is not yet identified, although some candidates have been suggested (figure 1).

Pendrin is a chloride-iodide transport protein located at the apical end of the thyroid epithelium (Bidart *et al.*, 2000a; Royaux *et al.*, 2000), and functions by exchanging chloride with iodide (Scott *et al.*, 1999). Pendrin requires high concentrations of iodide in the cytoplasm in order to transport iodide hence its function depends on NIS (Yoshida *et al.*, 2004). Mutations in the gene encoding pendrin lead to Pendred's syndrome, a phenotype consisting mainly of congenital deafness and goiter. The thyroid of Pendred's patients is characterized by impaired transport and retention of iodide and enlarged follicles (Reardon and Trembath, 1996). The expression of pendrin is transcriptionally stimulated by TSH, but pendrin-mediated iodide efflux seems to be cAMP-independent involving protein kinase C (Muscella *et al.*, 2008).

The human apical iodide transporter (hAIT) sharing structural homology with NIS was originally proposed to mediate iodide efflux across the apical membrane (Lacroix *et al.*, 2004; Rodriguez *et al.*, 2002). However, it has now become clear that hAIT does not transport iodide, but instead transclocate monocarboxylic acids such as lactate, pyruvate, propionate, butyrate and nicotinate across membranes (Coady *et al.*, 2004; Miyauchi *et al.*, 2004; Paroder *et al.*, 2006). This molecule has therefore been renamed to Na^+/monocarboxylate transporter (SMCT).

For simplicity reasons and since the molecular mechanism still remains elusive, apical efflux was not directly monitored in the iodide transport experiments conducted in the present thesis. Data to be presented do not infer an effect on this parameter, although minor contributions cannot be excluded. This will not be further commented on other than stated here.

This ends the overview of iodide handling in the thyroid. The following parts of the Introduction will focus on effects of ionizing radiation and cellular responses to DNA damage of relevance to the specific issues addressed in this thesis. Some clinical aspects of thyroid cancer and the use of radioiodine for diagnosis and therapy will also be highlighted.

Internal Radiation Effects on the Thyroid

Oncogenesis

Ionizing radiation is a well-known risk factor for the development of thyroid cancer but the initiating genetic aberrations leading to tumorigenesis are poorly understood (Mizuno *et al.*, 2000). The risk to develop thyroid cancer from internal ^{131}I irradiation accumulated in the thyroid is considered to be much higher in the growing gland illustrated by the high incidence of papillary thyroid cancer amongst children in the surrounding area of Chernobyl reported since the accident in 1986 (Williams, 2002). Conceivably, cells with a higher mitotic index are more susceptible to acquire permanent DNA lesions that can be propagated to progeny cells. Radiation-induced thyroid cancer was recently shown to exhibit a distinct genetic signature (Port *et al.*, 2007), contradicting previous reports (Detours *et al.*, 2005). It is also known that the presence of BRAF mutations in thyroid cancers from atomic bomb survivors correlates with the absorbed dose (Takahashi *et al.*, 2007), although this is also debated (Powell N, 2005). Presently, there are no reliable experimental models that allow study of early genetic aberrations generated by ^{131}I mimicking internal radiation. Nevertheless, it can be assumed that thyroid cells exposed to ionizing radiation will obtain DNA damage by free radicals eventually leading to genomic instability and permanent lesions (Hall and Holm, 1998).

In paper I and III we studied the cells responses to low absorbed doses of ^{131}I and if DNA lesions affect the iodide transport capacity.

Therapy

The discovery of X-rays in 1895 by Wilhelm Condrad Röntgen and of natural radioactivity a few months later by the physicist Henry Becquerel were two breakthroughs in medicine (Bernier *et al.*, 2004). Ever since discoveries in radiation physics, chemistry and biology has aimed to develop more accurate, efficient anticancer therapies that are less harmful to normal tissues. Medical uses of radiation include diagnosis of disease, therapy and research. Diagnosis covers a wide range of exams from routine x-rays to complex CT scans and the injections of radioactive material for nuclear medicine imaging. In general, radiation therapy involves delivering a large dose of radiation to a small area of the body. Therapy is mainly

directed to kill tumor cells as part of the treatment of cancer. The only exception to this in medicine today is the use of ^{131}I in the treatment of hyperthyroidism due to Grave's disease, with the aim to ablate the function of a hyperactive gland. ^{131}I is ingested orally as sodium iodine of high specific activity either as a liquid solution or as capsules. The radioiodine is absorbed in the upper intestine and circulates in the blood throughout the body. However, since iodine is rapidly and efficiently taken up by thyroid cells, there are few side effects of ^{131}I irradiation in other tissues (although other organs expressing NIS may be affected).

Malignant tumors arising from the thyroid epithelial cells are classified according to their histopathological features in papillary and follicular carcinomas, which may be highly, intermediate or poorly differentiated, and anaplastic thyroid carcinoma that have lost all characteristics of the thyrocyte (Miccoli *et al.*, 2007). Depending on the tumor type different treatment modalities comprising surgery, external radiation, radioiodine (^{131}I) therapy, and chemotherapy are employed (Spitzweg and Morris, 2004). The majority of thyroid cancers and its metastasis display a diminished iodide uptake compared to normal thyroid tissue. However, in many cases the iodide uptake is sufficient for radioiodine (^{131}I) treatment. Total loss of differentiation leads to inability of accumulating iodide and poor prognosis if the tumor is not detected early and radically removed by surgery.

There is a reduced expression of both NIS mRNA and protein in papillary and follicular thyroid cancer (Arturi *et al.*, 1998; Caillou *et al.*, 1998; Lazar *et al.*, 1999; Ringel *et al.*, 2001; Smanik *et al.*, 1997). A low or absent NIS expression may be due to silencing of the NIS promoter e.g. by hypermethylation (Matsuo *et al.*, 1993). To date several compounds have partially succeeded to reinduce endogenous NIS expression in thyroid cancer. Such agents are retinoic acid, peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, HDAC inhibitors and demethylating agents that are reviewed in (Kogai *et al.*, 2006; Riesco-Eizaguirre and Santisteban, 2006). Recent studies based on immunohistochemistry and reverse-transcriptase (RT)-PCR have revealed that in many thyroid cancer patients with a poor radioiodine uptake the tumor cells paradoxically over-express NIS, but the NIS protein is retained in the cytoplasm and not targeted to the cell surface (Dohan *et al.*, 2001; Saito *et al.*, 1998; Wapnir *et al.*, 2003). These observations indicate that loss of iodide uptake in differentiated thyroid cancer may be due to impaired targeting or insufficient retention of NIS in the plasma membrane rather than decreased expression of the *NIS* gene. Restoration of the

trafficking of NIS to the tumor cell surface, if possible, would be a nice strategy to increase the efficacy of ^{131}I therapy in a subset of thyroid cancer cases.

The observation that NIS is also expressed in extra-thyroidal tissues has proposed the use of NIS as a novel therapeutic gene in human cancer in order to apply radioiodide therapy to nonthyroidal cancers, especially breast cancer. Hydrocortisone, isobutylmethylxanthine and dexamethasone have all been reported to stimulate retinoic acid-induced NIS protein and plasma membrane targeting in MCF-7 cells (Dohan *et al.*, 2006; Kogai *et al.*, 2005; Unterholzner *et al.*, 2006). Gene transfer of NIS is one of the most promising and well-studied gene strategies in oncology today (Spitzweg and Morris, 2004).

Thyroid Stunning

After tumor surgery ^{131}I is used to destroy any possible thyroid or tumor remnants. If the ^{131}I uptake is sufficient this treatment has the potential to be curative. It is also important to ablate the normal thyroid tissue, allowing future relapse of cancer to be detected by routine monitoring of the TG level in blood. The amounts of remnants to be irradiated have traditionally been estimated with diagnostic scintigraphy using low amounts of ^{131}I administered a few days prior to radiotherapy. However, several studies have reported that the diagnostic exposure to ^{131}I may seriously diminish subsequent uptake of the therapeutic dose, a phenomenon known as thyroid stunning. Some authors claim that the stunning effect may eventually compromise the therapeutic efficiency and long-term outcome of the radioiodine treatment (Kalinyak and McDougall, 2004; Medvedec, 2005; Morris *et al.*, 2003; Park *et al.*, 1997). Whereas, other experts argue against a clinical significance of stunning and infer this as an early therapeutic effect of the ablative dose rather than being related to the preceding diagnostic procedure (Bajen *et al.*, 2000; Cholewinski *et al.*, 2000; McDougall, 1997). At the start of this thesis work thyroid stunning was still a controversial issue, augmented by the lack of experimental data of the mechanism. A short overview of the most important reports on the clinical observations and recent experimental data is therefore given below.

Although being recognized more than 50 years ago (Rawson *et al.*, 1951), the first clear clinical indication of thyroid stunning was published in 1986 by Jeevanram *et al.* (Jeevanram *et al.*, 1986). They studied 52 patients who received five different doses of ^{131}I for diagnostic imaging and found 25 % inhibition of the following therapeutic uptake of ^{131}I in patients who

delivered a dose less than 17.5 Gy. Additional 3-fold (75 %) reduction of the ablative ^{131}I uptake could be seen when a higher diagnostic dose estimated to deliver 35 Gy was used.

In 1994 Park and colleagues (Park *et al.*, 1994) showed an increasing incidence of thyroid stunning after administration of larger ^{131}I diagnostic activities. Specifically, a 40 % reduction in iodide uptake was observed after 111 MBq, and the corresponding figures were 67 % and 88 % after 185 MBq and 370 MBq, respectively. A later study from the same group (Park *et al.*, 1997) compared the outcome of ^{131}I treatment in patients who had diagnostic scans of 111-370 MBq of ^{131}I versus 11.1 MBq of ^{123}I. In this paper it was hypothesized that the lack of beta emissions from ^{123}I would eliminate the stunning phenomenon. Even though no statistical significant difference between the two groups was observed the data supported the concept of thyroid stunning with ^{131}I.

In another study the influence of diagnostic scans using either 37 MBq or 111 MBq ^{131}I, followed by a 3.7 GBq ablative dose were compared (Muratet *et al.*, 1998). Interestingly, ablation was successful in 76 % of patients receiving 37 MBq but only 50 % in those who received 111 MBq for diagnostic imaging.

Bajen *et al.* (Bajen *et al.*, 2000) reported a reduced uptake in 21 % of the post-therapy scans compared to the diagnostic ones (185 MBq ^{131}I) that were performed in average 8 weeks later. Despite this the authors claimed that no stunning could be observed, instead the decreased uptake of iodide was suggested to be due to a therapeutic effect.

One important factor that may explain conflicting results on the existence of thyroid stunning is the time-interval between the diagnostic and therapeutic administration of ^{131}I, which vary considerably in different reports. For instance, McDougall (McDougall, 1997) showed that the therapeutic uptake efficacy was only reduced 3.5 % after a 74 MBq ^{131}I diagnostic scan, given within a 24 hour time period. The possibility, that early treatment after the diagnostic scan may eliminate stunning is supported by others (Cholewinski *et al.*, 2000).

The first experimental study addressing the question of thyroid stunning was conducted in our laboratory (Postgård *et al.*, 2002). By using differentiated porcine thyrocytes growing on a filter in a bicameral chamber system, it was shown that the TSH-stimulated iodide transport was significantly reduced three days after ^{131}I irradiation, and that the level of transport inhibition was clearly dependent on the received absorbed dose. A 50% reduction of the iodide transport could be seen after an absorbed dose of 3 Gy, whereas at 80 Gy the transport

was almost untraceable. Importantly, under the experimental conditions irradiation did not influence the viability of the cells. Most likely due to that the cells were cultured to confluence before experiment, implicating that most cells had exited the cell cycle and were quiescent. This ruled out the possibility of cell death as an explanation for thyroid stunning at least *in vitro*.

In summary, today most researchers and clinicians agree that thyroid stunning is a real phenomenon. However, the mechanisms behind this phenomenon still remain elusive. The general aim of this thesis was to identify some of its characteristics at the molecular and cellular levels.

DNA Damage

Human cells are continuously exposed to a wide range of DNA-damaging agents. During every cell cycle the human genome is exposed to genotoxic events and it has been estimated that approximately 10 000 DNA lesions occur each day in a metabolically active mammalian cell. DNA-damage can also be induced exogenously by exposure to UV-light, ionizing radiation and chemical mutagens. In order to combat these attacks on the genome, the cell has evolved a response system that induces cell cycle arrest to allow sufficient time to repair the induced damages. The cellular response to genotoxic stress also activates the appropriate DNA repair pathway or, in the case of irreparable damage, induces apoptosis. Different types of DNA damage induce varying types of lesions, including strand breaks. Depending on whether only one or both DNA strands are broken they are described as single-strand breaks (SSBs) or double-strand breaks (DBSs), respectively. DSBs are considered to be the most lethal lesion since incorrect repair might lead to mutations or genomic instability (Jackson, 2001).

Next some general aspects of the DNA damage response and related details of particular relevance to this thesis will be outlined.

Ionizing Radiation

Ionizing radiation consists of highly-energetic particles or electromagnetic waves that can ionize at least one electron from an atom or molecule. These ionizations can damage living tissue by generating free radicals, break chemical bonds, produce new chemical bonds or cross-link macromolecules that regulate vital cell processes. Ionizing radiation mostly induces SSBs and only 2-5 % of the breaks are DSBs (Elmroth *et al.*, 2003). For over 50 years it has been known that exposure of cells to ionizing radiation delays the normal progression through the cell cycle (reviewed by (Iliakis *et al.*, 2003)). Early studies further showed that the cell cycle arrest reflects a window that provides time necessary for the irradiated cell to cope with the induced damage by facilitating DNA repair before mitosis (Lucke-Huhle, 1982; Tobey, 1975; Walters *et al.*, 1974). This notion was later supported in studies on cells from ataxia telangiectasia (A-T) patients, which in culture were prone to die after exposure to ionizing radiation due to a shortened delay in their cell cycle progression (Painter and Young, 1980).

Radioiodine Nuclides: ^{131}I and ^{125}I

The physical characteristics of radioiodine isotopes used in this study will be shortly described. ^{131}I is a beta-particle emitter, with the maximal beta-energy of 606 keV and the mean energy of 191 keV, with a physical half-life of 8.02 days (Robbins and Schlumberger, 2005). Except from emitting beta-particles the ^{131}I atom also emits gamma-rays the major being at 364 and 637 keV. These beta- and gamma-radiations account for 90% of the total radiation from ^{131}I, where gamma-radiation only contributes with 10%. Since the beta-particles are unable to penetrate deep into tissue, large doses of ^{131}I may be administered without damaging surrounding tissues. As mentioned earlier internal irradiation with ^{131}I is frequently used as therapeutics in thyroid diseases (Robbins and Schlumberger, 2005). In 1946 Seidlin *et al.* was the first one to report the use of radioiodine to treat thyroid cancer (Seidlin S, 1946). Ionizing radiation disrupts chemical bonds throughout the cell, causing DNA damage and triggering cellular arrest and ultimately cell death.

^{125}I is a radioisotope of iodine that emits gamma-rays with the maximal energy of 35 keV. Due to its suitable half-life of about 60 days, it is frequently used in *in vitro* experiments as a tracer to evaluate the cells capacity to accumulate or transport iodide, a technique applied in

this thesis. About 70% of the emitted energy comes from photons and 30% by electrons. The electron energy from ^{125}I is deposited locally with a very short range.

The susceptibility of proliferating thyroid cells to ionizing radiation from ^{131}I was investigated in paper I. The effect of ^{131}I on NIS-mediated iodide transport was mainly studied in papers I and II.

Genotoxic Drugs

Genotoxic drugs are best exemplified by chemotherapeutic agents used in cancer treatment. This is a heterogenous group of chemical compounds that affect nucleic acids and alter their functions by different mode of actions. The drugs may directly bind to DNA or they may indirectly lead to DNA damage by affecting enzymes involved in DNA replication. Hence, proliferating cells are more sensitive to genotoxic agents due to their active synthesis of new DNA than quiescent or senescent cells. If the damage is severe enough the cell undergoes apoptosis. The radiomimetic action of some of these drugs can be employed also in experimental studies. The features of one such agent used in papers III and IV are described in the next paragraph.

Calicheamicin γ 1

Calicheamicin γ 1 (CLM), figure 5, is an incredibly potent cytotoxic agent working by destroying the DNA in cells. It is an enediyne antibiotic naturally produced by the bacteria *Micromonospora echinospora ssp. calichensis*, discovered in the mid 80s during a field trip aiming to find new fermentation-derived antitumor antibiotics (Lee *et al.*, 1987; Lee *et al.*, 1991). CLM is an efficient inducer of DSB, with a DSB: SSB ratio of ~1:3, since the compound binds to double-stranded DNA with high affinity. Specifically, CLM contains two radical centers that become positioned close to the backbone of DNA in which the drug binds to the minor groove, as it reduces a trisulfide in the DNA helix (Elmroth *et al.*, 2003). The preference of inducing DNA DSBs is reflected by a strong DNA damage response typical for this kind of DNA lesions in cultured cells (Ismail *et al.*, 2005). The potency to induce DNA damage makes CLM an attractive chemotherapeutic agent. However, because of pronounced

side-effects of systemically administered CLM it is so far only used in antibody-targeted chemotherapy (Gemtuzumab Ozogamicin; Mylotarg, Wyeth-Ayerst, St Davids, PA, USA) of e.g. relapsed acute myeloid leukemia (Pagano *et al.*). The antibody conjugated-drug binds to CD33 on the leukemic cell surface after which the compound is internalized and released in the lysosomes before reaching nuclear DNA.

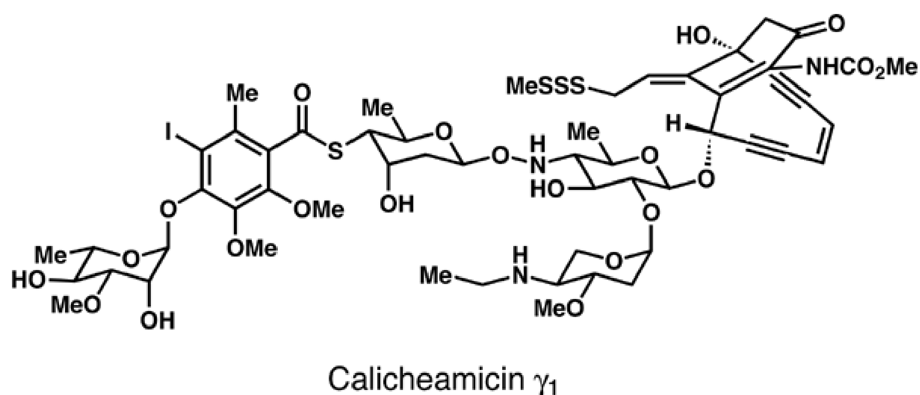

Figure 5. *Schematic picture of the enediyne antibiotic Calicheamicin γ 1 that efficiently induces double strand breaks in DNA. Modified from (Nicolaou et al., 1993).*

Cell Cycle

In order to understand the DNA damage response to DSB a short summary of the mechanisms controlling the cell cycle is warranted. The cell cycle comprises a range of sequential processes that eventually results in the duplication of a cell (Elledge, 1996). The four main stages of the cell cycle are: G_1 (presynthesis), S (DNA synthesis), G_2 (premitotic) and M (mitotic) phases. Quiescent cells are in a post-mitotic physiological state referred to as G_0.

To ensure the fidelity of cell division, cells have evolved regulatory circuits or checkpoints that monitor successful completion of distinct cell cycle events. There are two classes of such regulatory circuits, intrinsic and extrinsic. The intrinsic mechanisms act in each cell cycle to order the sequence of events ultimately leading to mitosis, whereas the extrinsic mechanisms are induced only when the cell encounter a defect (Elledge, 1996). The transition from one cell cycle stage to the next is dependent upon the activation of cyclin-dependent kinases (CDKs) that in turn are both positively and negatively regulated by other kinases and

phosphatases altering their phosporylation state. Moreover, binding to inhibitors and other proteins might also alter their activity (Sherr, 1996).

As described next, cell cycle checkpoints are activated by signals generated by DNA damage leading to delay or arrest of cell cycle progression.

DNA Damage Response Mechanisms

As depicted in figure 6, the DNA-damage response pathway is a signaling cascade, consisting of sensors, transducers and effectors that detects damaged DNA at the same time as it induces the transcription of DNA repair genes (Elledge, 1996). Depending upon the phase in which the DNA damage is sensed the cells arrests in the G_1 phase of the cell cycle (G_1-S checkpoint), slows down S phase (S phase checkpoint) or arrests cells in the G_2 phase (G_2-M) phase checkpoint). Some of the key signaling and effector molecules implicated in the DNA damage response have a central role in this thesis work and are therefore presented in more detail.

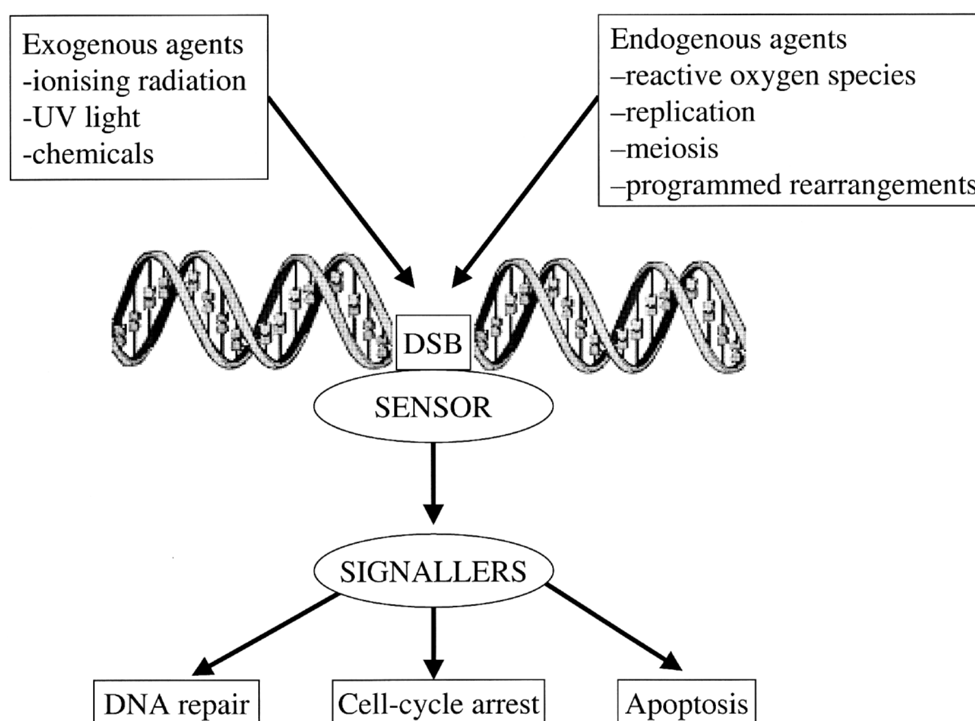

Figure 6. *Schematic picture of the DNA damage pathway consisting of sensors, transducers and effectors. See text for details. Modified from (Jackson, 2001).*

Ataxia-Telangiectasia Mutated (ATM)

The ATM protein is the product of the gene that is mutated in the rare, autosomal-recessive inherited human disorder ataxia-telangiectasia (A-T) characterized by amongst many organ dysfunctions, enhanced radiosensitivity and genomic instability (Morio and Kim, 2008). The *ATM* gene cloned in 1995 encodes a large (370 kDa) protein that belongs to the phosphoinositide 3-kinase (PI3K) like family (Savitsky *et al.*, 1995). ATM possesses serine/threonine kinase activity regulating cell-cycle checkpoints and is involved in DNA repair and recombination via protein phosphorylation of down-stream substrates, to be further discussed below (Abraham, 2004; Kastan and Lim, 2000; Shiloh, 2003). In fact, ATM is at the top of the DNA damage signaling cascade responding specifically to DNA DSBs. Cells from A-T patients show abnormal responses to ionizing radiation, checkpoint alterations in the cell cycle, and increased chromosomal breakage and telomere end fusions (Kastan and Lim, 2000). Conversely, the response of *ATM -/-* cells to ultraviolet irradiation and base damaging agents are relatively normal, further indicating that ATM is the initial and principal transducer in the response to DNA DSB (Shiloh, 2003). Additionally, the broad range of other abnormalities found in patients and cultured cells lacking ATM reveals the large number of substrates of ATM in various signaling pathways.

To date, the mammalian members of the PI3K-like family include five protein kinases: ATM, ataxia-telangiectasia- and Rad3-related (ATR), human SMG-1(hSMG-1), mammalian target of rapamycin (mTOR) and the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{cs}) of which all but mTOR are involved in the DNA-damage response (Shiloh, 2006). DNA-PK is similar to ATM in that it mainly responds to DSBs. ATR primarily transduces signals deriving from UV damage and blockage of replication forks (Nyberg *et al.*, 2002; Zhou and Elledge, 2000).

ATM Activation

Under normal conditions ATM is mainly located in the nucleus as an inactive dimer. In immediate response to double-stranded lesions ATM is converted into kinase-active monomers by an autophosphorylation on at least three sites: serine 367, serine 1893 and serine 1981 (Bakkenist and Kastan, 2003; Kozlov *et al.*, 2006; Shiloh, 2003). It is believed

that activated ATM exists both bound to the chromatin and freely movable throughout the nuclear matrix (Shiloh, 2003). As shown in figure 7, activated ATM phosphorylates a large number of key regulatory proteins e.g. p53, MDM2, BRCA1, CHK2, MDC1 and NBS1 that mediate down-stream signaling either by promoting or impairing the activity of their respective target molecules implicated in checkpoint activation.

As a part of the activation process in response to DNA damage ATM associates with the Mre11/Rad50/Nbs1 (MRN) multi-protein complex, which is known to tether and process the broken DNA ends as well as augment the DNA-damage signal (Lee and Paull, 2007; Shiloh, 2006). Mutations in the genes of two members of this complex NBS1 and MRE11 have been described in Nijmegen breakage syndrome and A-T-like disorder, respectively (Lavin, 2008), indicating the central importance to maintain genomic stability.

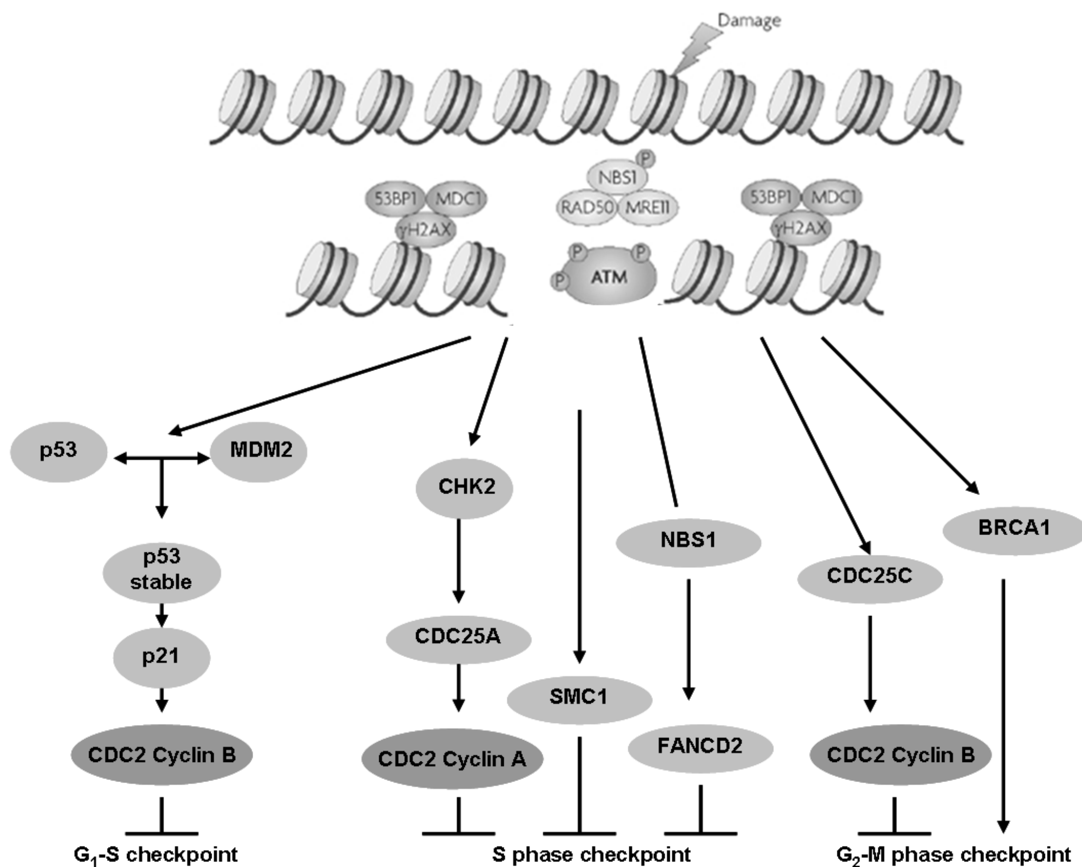

Figure 7. *Simplified scheme of ATM activation and signaling to downstream substrates in response to DNA double-strand breaks. ATM phosphorylates or mediates the phosphorylation of many different proteins to ensure the regulation of one specific checkpoint. SMC1, structural maintenance of chromosomes protein-1; BRCA1, breast cancer susceptibility protein-1; FANCD2, Fanconi anaemia complementation group D2. Modified from (Lavin, 2008).*

ATM has also been found in the cytoplasm, associated with peroxisomes and endosomes (Lim *et al.*, 1998; Watters *et al.*, 1999) and several of the abnormalities seen in ATM-deficient cells provide evidence for a more general signaling role of ATM besides direct involvement in DNA damage. These abnormalities include reduced internalization of cell surface-bound molecules, defective Ca^{2+} mobilization, depolarization in response to extracellular ions, and defective signaling through the epidermal growth factor (EGF) receptor (Lavin, 2008). In addition, ATM seems to participate in the regulation of insulin signaling through activation of the PKB/Akt pathway, which may explain the insulin resistance seen in some A-T patients (Viniegra *et al.*, 2005).

ATM Key Substrates

The diversity of ATM-mediated responses to DSBs verifies that it has many substrates, some of which will be briefly mentioned below. However, it should be emphasized that more ATM targets are yet likely to be discovered. In controlling a specific process ATM directly phosphorylates target proteins or activates other kinases that act as downstream mediators. For instance, p53, being the main regulating component of the G_1 DNA damage checkpoint, regulates the transcription of the gene encoding the CDK2-cyclin-E inhibitor p21, also known as WAF1 or CIP1 (Kastan and Lim, 2000). Activated ATM phosphorylates p53 at serine 15 as well as two proteins involved in controlling p53 function and expression levels; MDM2 and Chk2. The oncogenic protein MDM2 is an important regulator of p53 degradation and has been shown to be phosphorylated on serine 395 by ATM in response to ionizing radiation resulting in accumulation of p53 in the nucleus (Khosravi *et al.*, 1999). Chk2 is a serine/threonine kinase that is phosporylated by ATM in response to ionizing radiation. Chk2 is structurally unrelated to Chk1, which is phosphorylated by ATR, but the two checkpoint kinases exhibit overlapping substrate specificity (Bartek *et al.*, 2001; Matsuoka *et al.*, 1998). In mammals Chk2 is phosphorylated on threonine 68 (Thr68) by ATM which probably induces a conformational change of the protein. Chk2 in turn phosphorylates p53 on serine 20. In addition, activated Chk2 induces G_1/S cell cycle arrest and/or S-phase delay by phosphorylating Cdc25A and contributes to the G_2/M block by phosphorylating the mitosis-promoting phosphatase Cdc25C (Bartek *et al.*, 2001)

In paper III we analyzed Chk2 phosphorylation and foci formation to determine ATM-dependent cell cycle arrest following ^{131}I irradiation and exposure to CLM. In addition, $p53^{Ser15}$ phosphorylation was analyzed in papers III and IV to monitor the levels of ATM activation in response to CLM in the presence or absence of ATM inhibitor.

In most cases ATM ends up regulating gene expression, whereby p53 and NF-κB mediates the transcriptional response. Another transcription factor recently found to be regulated by ATM is the Ca^{2+}/cAMP response element binding protein (CREB), which is involved in various cellular growth pathways (Shi *et al.*, 2004). Interestingly, ATM modulates the phosphorylation of CREB in response to ionizing radiation and oxidative stress, leading to inactivation of CREB. As the NIS expression in thyroid cells is regulated by CRE, the relationship between ATM and CREB may be of relevance to the interpretation of some of the data presented in this thesis (Chun *et al.*, 2004).

γ-H_2AX

ATM also phosphorylates structural components of chromatin in response to DNA damage. One well-studied target is the histone variant H2AX that is intimately involved in the recognition of bi-stranded DNA lesions (Rogakou *et al.*, 1998). The nucleosome is a subunit of the eukaryotic chromatin composed of approximately 145 bp DNA wrapped around a core of eight histone proteins. There are four histone protein families, H4, H3, H2B and H2A. H2A consist of three subfamilies, H2AX being one of them comprising 2-10% of the total H2A constituent in mammalian tissues. H2AX is rapidly phosphorylated by ATM on serine 139, giving rise to γ-H2AX, in response to DNA DSBs induced by genotoxic insults. The phosphorylation of H2AX is apparent within a minute and reaches a maximum in 10 minutes (Redon *et al.*, 2002). In DNA damaged cells antibodies specific for γ-H2AX recognizes immunofluorescent foci in interphase nuclei and bands on metaphase chromosomes that can be counted as a very sensitive measure of the amount of individual DSBs (Rogakou *et al.*, 1999; Rothkamm and Lobrich, 2003). γ-H2AX is dephosphorylated with a half-life of ~2 h, similar to the kinetics of DNA repair, resulting in a decrease of the number of foci provided that the cell are no longer irradiated or exposed to genotoxic drugs. Thus, monitoring the resolution of γ-H2AX foci is a method to roughly estimate recovery from DNA damage.

In papers III-IV γ-H2AX phosphorylation and foci formation were determined to characterize the ATM-mediated DNA damage response and recovery following irradiation with ^{131}I and exposure to CLM.

ATM Inhibitors

It is well established that malfunction of DNA checkpoints are important in human pathology, especially cancer (Nyberg *et al.*, 2002). As cancer cells often lack one or more checkpoints, inhibition of the remaining checkpoints could sensitize the tumor cells to anticancer therapies, such as γ-irradiation or DNA-damaging drugs (Bartek *et al.*, 2001). Normal cells would still be able to activate the other checkpoints and after DNA repair recover from the temporary cell-cycle arrest. The fact that cells with defective ATM are hypersensitive to ionizing radiation and deficient in repairing all acquired DNA DSBs (Foray *et al.*, 1997) has lead to the proposal that ATM might be an attractive target for the development of new radio-sensitizing agents (Sarkaria and Eshleman, 2001). Amongst the earliest reported ATM inhibitors were wortmannin and caffeine. However, due to their unspecific binding to ATM and systemic toxicity the identification and development of more potent and tolerable pharmacologic agents were needed (Sarkaria *et al.*, 1998).

KU-55933

In 2004 Hickson *et al*. (Hickson *et al.*, 2004) characterized a novel specific inhibitor of the ATM kinase named KU-55933, depicted in figure 8. This compound exhibits an IC_{50} of 12.9 nmol/L against the ATM kinase activity, acting through competitive binding to its ATP-binding site. KU-55933 is at least 100-fold more potent inhibiting ATM than the other PI3K family members. KU-55933 is able to prevent phosphorylation of p53 on serine 15 (Hickson *et al.*, 2004), which is an early event executed by ATM in response to DNA DSB (Shiloh, 2003).

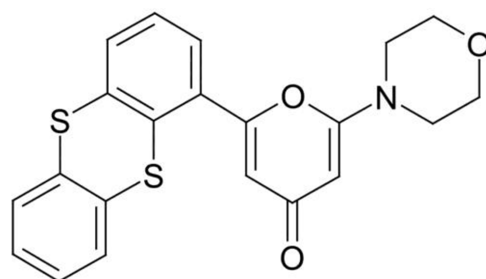

Figure 8. *Chemical structure of KU-55933 [morpholin-4-yl-6-thianthren-1-yl-pyran-4-one]. Modified from (Hickson et al., 2004).*

In paper IV KU-55933 was employed to elucidate the possible role of ATM in DNA damage-induced changes of NIS expression and iodide uptake in thyrocytes.

DNA Repair

As mentioned earlier, DSBs are considered the most lethal form of DNA damage in eukaryotic cells. This is due to the increased risk of incorrect repair that may lead to permanent mutations or even chromosomal translocations due to the concurrent genomic instability state. DNA DSBs can be induced by exogenous genotoxic exposures as ionizing radiation and radiomimetic chemicals, or can arise within the cell by the action of endogenously produced reactive oxygen species or when the replication fork encounters other DNA lesions such as SSBs (Jackson, 2001). DSBs differ from most other types of DNA lesions in that they affect both strands of the DNA duplex and therefore prevent use of the complementary strand as a template for repair. In order to maintain genomic integrity, higher eukaryotes have evolved multiple pathways for the repair of DSBs including homologous recombination (HR), non-homologous end-joining (NHEJ), outlined in figure 9 (for a review see (Khanna and Jackson, 2001)). NHEJ repairs predominately DSBs in G_1 and G_0 phases whereas HR is most active in S and G_2 phase, although, an overlap and competition between the two repair mechanisms seem to exist (Burma and Chen, 2004). In HR a homologous DNA strand is used as a template for repair of DSB. In NHEJ, on the other hand, double stranded DNA is directly joined together and often leads to small DNA sequence deletions, see figure 8. A range of proteins are critically involved in NHEJ i.e. Ku, DNA-PKcs, Xrcc4 and DNA-

ligase IV. The Rad50-Mre11-Nbs1 (RMN) complex, comprising helicase and exonuclease activities, is involved in NHEJ if the DNA ends require processing before ligation. HR requires the proteins Rad52 and Rad51 that respectively binds DNA ends and facilitates strand invasion, before the DNA strand is extended by DNA polymerase.

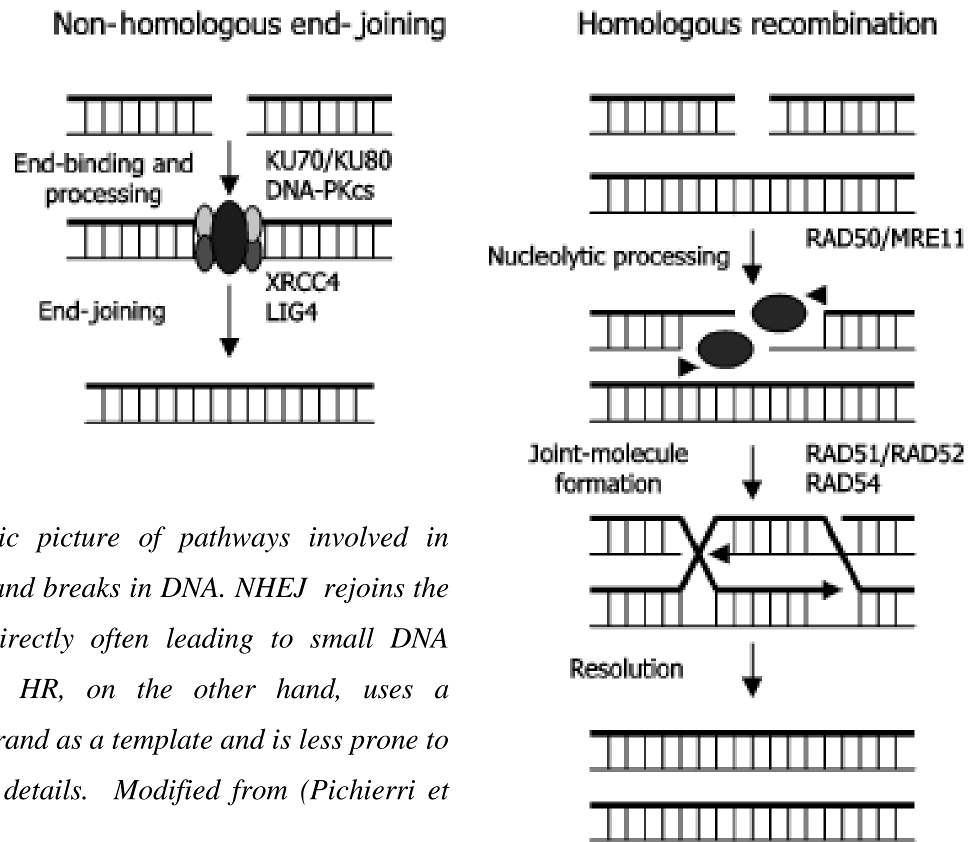

Figure 9. *Schematic picture of pathways involved in repair of double-strand breaks in DNA. NHEJ rejoins the two broken ends directly often leading to small DNA sequence deletions. HR, on the other hand, uses a homologous DNA strand as a template and is less prone to errors. See text for details. Modified from (Pichierri et al., 2000)*

DNA-PK

The DNA-dependent protein kinase (DNA-PK) is a key enzyme of DNA damage recognition, repair and signaling pathways, activated by DNA strand breaks. Mammalian DNA-PK is an important component of both the NHEJ pathway for DSB repair and the *V(D)J* recombination (Smith and Jackson, 1999). It is a nuclear serine/threonine kinase comprised of a regulatory subunit, containing the Ku70/80 heterodimer, and the 460 kDa catalytic subunit, DNA-PKcs (Collis *et al.*, 2004). Like ATM DNA-PK belongs to the PI3K-like family (Shiloh, 2003). It is believed that Ku first binds to the DNA ends, before recruiting DNA-PKcs which stabilizes its binding to DNA. DNA-PK-deficient cells are defective in the repair of DNA DSBs and

display a radiosensitive phenotype (Smith and Jackson, 1999). Additionally, the increased susceptibility of the immunodeficient SCID mouse strain to genotoxic insults has been shown to be due to a mutation in the gene for DNA-PKcs (Blunt *et al.*, 1995; Lee *et al.*, 1997). DNA-PK inhibitors may therefore have clinical utility as radio- and chemo-potentiating agents in the treatment of cancer. In the search of new potent and selective DNA-PK inhibitors suitable for clinical purposes Hollick *et al*. (Hollick *et al.*, 2003) synthesized NU7026, using LY294002 as a structural guide. The molecular structure of NU7026 is depicted in figure 10. NU7026 is approximately 6-fold more potent than LY294002, which is a selective ATP-competitive PI3K inhibitor, inhibiting DNA-PK activity (Izzard *et al.*, 1999). NU7026 has been shown to have a radiosensitizing effect also in quiescent (G_0) cells by preventing potentially lethal damage recovery (Veuger *et al.*, 2003), indicating that DNA-PK is important for DNA repair also in quiescent (G_0) cells.

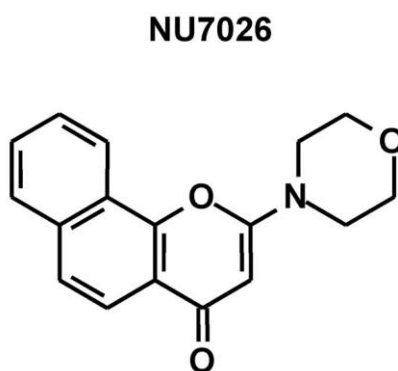

Figure 10. *Chemical structure of NU7026 [2-(morpholin-4-yl)-bezo[h]chomen-4-one]. Modified from (Veuger et al., 2003).*

In paper III we investigated the possible contribution of DNA repair mechanisms for the susceptibility of NIS-mediated iodide transport to DNA damage by using the DNA-PK inhibitor NU7026.

AIMS

With the aim to elucidate possible molecular mechanisms involved in the thyroid stunning phenomenon we investigated the following questions using primary cultured normal thyroid cells:

- Is thyroid stunning related to radiation-induced cell cycle arrest?
- Is NIS-mediated iodide uptake targeted in cells exposed to ^{131}I? If so, is it a transcriptional response to irradiation?
- Is DNA damage and specifically DNA DSBs a primary event inducing thyroid stunning? If so, can DNA repair mechanisms modulate the response?
- Provided that thyroid stunning can be linked to DNA damage, is the down-stream signaling leading to impaired iodide transport ATM-dependent?

RESULTS AND DISCUSSION

The mechanism causing thyroid stunning remained elusive for many years since the discovery more than 50 years ago. However, previous studies in our laboratory (Postgård *et al.*, 2002) provided the first experimental evidence indicating that thyroid stunning is a real phenomenon and not an artifact related to the methodological difficulties estimating specific radioiodine uptake in thyroid tissues from scan images (Brenner, 2002). In the original paper by Postgård *et al.* (Postgård *et al.*, 2002), an absorbed-dose dependent reduction of TSH-stimulated iodide transport in ^{131}I-irradiated porcine thyroid cells was unequivocally proven. It was further found that an absorbed dose of 3 Gy, which is in the dose range received in clinical dosimetric scanning with ^{131}I, was sufficient to cause a 50% transport inhibition compared to the transport in non-irradiated control cultures. Papers I-IV in this thesis are the result of our efforts to understand the stunning mechanism in more detail, the ultimate goal being to suggest possible means of minimizing this unwanted side effect. However, the summarized data presented here will be discussed mainly from a biomedical, mechanistic point of view.

Effect of Low Absorbed Doses of ^{131}I on Iodide Transport (Paper I)

No information on the lowest absorbed dose from ^{131}I required to negatively influence iodide transport was available. In paper I we therefore investigated the effect of low absorbed doses from ^{131}I on iodide transport in primary porcine thyrocytes cultured on filter in bicameral Transwell chambers. According to previous transport studies (Nilsson *et al.*, 1990; Nilsson and Ericson, 1994; Postgård *et al.*, 2002), the cells were pre-stimulated with TSH for 48 h in order to up-regulate NIS gene expression and protein expression (Riedel *et al.*, 2001). Cultures were thereafter continuously exposed to ^{131}I for additionally 48 h, during ongoing TSH-stimulation, after which the basal to apical transepithelial transport of ^{125}I (used as a tracer in all transport experiments throughout the thesis) was monitored. Transepithelial resistance was measured both before the addition of ^{131}I and the ^{125}I$^-$-transport study, confirming that a tight monolayer was maintained throughout the study period lasting for up to 5 d after start of irradiation. The novel result from this part of paper I was that as low absorbed doses as 0.15 Gy from ^{131}I showed a 20 % inhibition of the iodide uptake. Although it is difficult to translate these *in vitro* dose response findings to a putative clinical situation,

paper I suggests that stunning might be recognized at lower absorbed doses than previously expected. This in turn indicates that stunning may occur also in ^{131}I therapy of benign diseases such as thyrotoxicosis or Graves' as patients generally receive a test activity of 0.5 MBq corresponding to an absorbed dose of approximately 0.1-0.5 Gy to the thyroid (Berg *et al.*, 1996).

Effect of ^{131}I on Cell Proliferation (Paper I)

Irradiation was conducted on confluent growth-arrested cells in order to avoid confounding effects of reduced cell numbers due to radiation-induced cell damage. Loss of cells due to a therapeutic effect of ^{131}I in the experimental model is likely not occurring since no change in total DNA content was evident between irradiated and non-irradiated cells in previous studies (Postgård *et al.*, 2002). However, whether there is an increased cell turnover rate in irradiated cultures has not been investigated, although preliminary findings of only small changes in DNA synthesis (measured by [^{3}H]thymidine incorporation) argue against this possibility (data not shown). To further corroborate this, we in paper I determined the lowest absorbed doses of ^{131}I leading to cell cycle arrest. For this purpose we used subconfluent growing thyroid cells and compared the effect of ^{131}I on cell proliferation with that on iodide transport on confluent cells. We found that absorbed doses $\geq$ 1 Gy produced a dose dependent reduction in the incorporation of [^{3}H]thymidine and an absorbed dose of about 4 Gy was required to enhance a reduction greater than 50%. Quantification of total DNA showed no significant change in cell numbers at doses $\leq$ 3 Gy. It can therefore be excluded that the inhibition of iodide transport observed in cultures irradiated with lower absorbed doses is due to altered cell number. These data further support the notion that confluent thyroid cell cultures, in which the vast majority of cells are G_0, can be safely used to investigate cellular responses to ^{131}I uncoupled from the mechanisms by which ionizing radiation provokes cell cycle arrest.

The finding that low absorbed doses 0.01 and 0.1 Gy significantly decreased the [^{3}H]thymidine incorporation whereas the slightly higher absorbed doses 0.15 and 0.3 Gy did not suggest that proliferating cells might be exceedingly radiosensitive to very low absorbed doses of ^{131}I. It is previously shown that radiation doses $\leq$ 0.5 Gy are more effective per unit in killing cancer cells *in vitro* than higher doses (Joiner *et al.*, 2001). However, alternative explanations to small changes in [^{3}H]thymidine incorporation e.g. reduced nucleotide

transport, cannot be excluded. Further studies are therefore required before a putative hyper-radiosensitivity interval towards ^{131}I irradiation can be proven for normal thyroid cells.

Effect of ^{131}I on NIS mRNA Expression (Paper II)

To date NIS in the basolateral membrane and AIT/SMCT and Pendrin, in the apical membrane are the only identified iodide transporting molecules in thyroid cells. Defects in either one of these proteins alone or in combination could be possible mediators of impaired iodide transport explaining thyroid stunning. However, since all iodide transport is governed by NIS-mediated uptake, it can be hypothesized that the expression and/or function of NIS is the most likely target of ionizing radiation. This possibility was addressed in paper II, in which the effect of a moderate high absorbed dose of ^{131}I (7.5 Gy) on NIS mRNA expression in primary cultured thyrocytes was investigated. TSH-stimulated up-regulation of NIS was monitored with real time RT-PCR using primers against pNIS according to previous a protocol (Tedelind *et al.*, 2006). Based on the fact that the peak levels of increased NIS mRNA occurs 48 h after addition of TSH the cells were, in contrast to earlier transport studies (Lundh *et al.*, 2007; Nilsson *et al.*, 1990; Nilsson and Ericson, 1994; Postgård *et al.*, 2002) irradiated with ^{131}I from the start of TSH stimulation. This allowed analysis of the effects of irradiation on the neosynthesis of NIS rather than being confounded by possible effects on its turnover at steady state (Riedel *et al.*, 2001).

The data showed that ^{131}I (7.5 Gy) repressed the NIS mRNA expression by 60-80%, and that the reduced transcript levels correlated with a corresponding decrease of iodide transport in similarly treated cultures. This implies that the major probable cause of thyroid stunning is down-regulation of the *NIS* gene. Exactly how the NIS transcript level is modulated in response to ionizing radiation was however, not possible to determine in this study. But since ionizing radiation induces genotoxic stress it is possible that NIS is affected by a stress response to DNA damage. As previously mentioned, several signaling pathways converge on the NIS promoter regulating its transcriptional activity. These include apart from the TSHR-cAMP the IGF-I-PI3K/Akt and several MAPK pathways (Pomerance *et al.*, 2000; Taki *et al.*, 2002). In addition, it can be mentioned that Ref-1, a factor readily activated in response to genotoxic stress, is involved the regulation of the NIS promoter (Fritz *et al.*, 2003; Puppin *et al.*, 2004).

Effect of IGF-I on NIS-mediated Iodide Transport after ^{131}I Irradiation (Paper II)

In paper II, we further investigated whether the NIS gene transcription stimulated by another signaling pathway than TSHR-cAMP also might be radiosensitive. This was based on the knowledge from earlier studies performed in our laboratory group showing that IGF-I are able to stimulate the transepithelial iodide transport (Ericson and Nilsson, 1996).

IGF-I alone was found to stimulate NIS expression and iodide transport although much less efficiently than TSH. Moreover, the stimulating effect on NIS expression was delayed several days implicating that IGF-I probably regulates NIS indirectly. However, together with TSH IGF-I accelerated the NIS expression and also counteracted the ^{131}I-induced loss of NIS. In fact, in the presence of IGF-I the magnitude of iodide transport in irradiated cultures were greater than that in non-irradiated TSH-stimulated cultures.

Since IGF-I is known to be mitogenic to confluent cells (Ericson and Nilsson, 1996) it was necessary to exclude that the effects of IGF-I was not due to altered cell number. This was accomplished by normalizing all NIS mRNA quantifications to the housekeeping gene *18S*. Moreover, irradiation blocked the IGF-I stimulated increase in total DNA, verifying that the observed effects on iodide transport were not due to the relatively small changes in cell number.

Another important issue to keep in mind is that IGF-I *per se* are able to modulate the radiation response that in turn could influence cell behavior. For instance, activation of the IGF-I receptor has been reported to induce radioresistance (Yu *et al.*, 2003) and several studies (Macaulay *et al.*, 2001; Perer *et al.*, 2000; Wen *et al.*, 2001) have shown that inhibition of IGF-I receptor signaling increases the radiosensitivity of tumor cells. Furthermore, IGF-I seems to be linked to the early DNA damage response mediated by ATM, which are able to stimulate the IGF-I receptor expression (Peretz *et al.*, 2001; Shahrabani-Gargir *et al.*, 2004) and to induce effective DNA repair (Heron-Milhavet *et al.*, 2001).

The co-stimulating effect of IGF-I and TSH on the ^{131}I-induced loss of NIS suggests that IGF-I could be used clinically. However, the diversity of IGF-I effects on NIS among different species must be further examined before a possible clinical protocol could be discussed. Garcia and Santisteban (Garcia and Santisteban, 2002) have shown that IGF-I suppresses NIS

transcription in the rat thyroid cell line, FRTL-5. On the other hand, IGF-I receptor signaling is implicated in retinoic acid-stimulated NIS expression in the human breast cancer cell line MCF-7 (Kogai *et al.*, 2008a).

Effect of DNA Damage on NIS Expression and Iodide Transport (Paper III and IV)

From the experimental data obtained so far we believe that thyroid stunning is the result of a transcriptional blockage of NIS expression in metabolically active cells subjected to low-to-moderately high absorbed doses of ^{131}I. Still, it is unknown what molecular changes in irradiated thyrocytes lead to repressed NIS activity and loss of iodide uptake. As mentioned previously it is conceivable that NIS might be affected by a stress response to DNA damage. In paper III we investigated this and particularly whether the formation of DNA DSBs is able to trigger and thereby mimic radiation-induced thyroid stunning. The overall DNA damage induced by ionizing radiation is primarily SSB and base damage, and only 2-5% of the induced damage is DSB (Elmroth *et al.*, 2003). In these experiments we therefore employed calicheamicin γ 1 (CLM) to induce high ratio of DSBs. The results unequivocally showed that both iodide transport and NIS transcription were strongly suppressed by the genotoxic drug administered at subnanonomlar concentrations.

That these very low CLM concentrations induced a typical DNA damage response was evidenced by the formation of γ-H2AX foci.

Moreover, involvement of a genotoxic stress response was further corroborated by the observation that the DNA-PK inhibitor NU7026 augmented the response to CLM and even more interestingly that the NIS mRNA expression and iodide transport were significantly reduced by NU7026 also in the absence of endogenously induced DNA damage. Again, observations of γ-H2AX foci in the nuclei of treated cells indicated that the inhibitor in all probability interfered with DNA repair, thus arguing against an off-target effect. This notion is further supported by earlier studies showing that DNA-PK inhibition potentiates *in vitro* cytotoxicity of ionizing radiation (Jackson, 1997; Rosenzweig *et al.*, 1997) and anticancer drugs (Boulton *et al.*, 2000; Kim *et al.*, 2002; Nuno G. Oliveira, 2002).

Paper III provided the first causal evidence of thyroid stunning, linking formation of the most serious form of DNA lesion, DSBs, to subsequent repression of NIS gene transcription in affected thyroid cells. The last paper of this thesis therefore focused on the major DNA damage response pathway known to be elicited by DNA DSBs.

Role of ATM in DNA Damage-induced Repression of NIS-mediated Iodide Transport (Paper IV)

ATM is the most important key player in the early response to DNA DSBs induced by ionizing radiation (Ismail *et al.*, 2005). The possible involvement of ATM in CLM-induced inhibition of NIS expression and iodide transport was therefore investigated in paper IV, adopting the experimental approach used in paper II. Cells were pre-treated with the novel ATM inhibitor, KU-55933 (Hickson *et al.*, 2004) overnight and then exposed to CLM for 1-2 h in the continued presence of inhibitor. This showed that KU-55933 dose dependently reduced ATM-mediated activation of p53 and also counteracted the CLM-induced formation of γ-H2AX foci, indicating drug specificity. Pre-incubation with the same concentrations of KU-55933 were found to significantly counteract the blocking effect of CLM on both iodide transport and NIS expression, although restoration was not complete. In contrast, prolonged exposure to KU-55933 for 48 h or more reduced the NIS transcript levels giving an opposite effect to that monitored after shorter incubations of the drug.

Putative down-stream targets of ATM repressing *NIS* gene expression in DNA damaged thyroid cells remain to be investigated. However, since ATM is implicated in a number of signaling cascades several possible mechanisms can be envisaged. Of particular interest is the notion that the PKB/Akt protein, commonly known as Akt, plays a key role in the pathway related to survival by inhibition of apoptotic signals and promotion of cell cycle progression. Akt signaling mediates effects of several growth factors e.g. insulin or IGF-I, and is aberrantly activated in cancer cells (Alessi *et al.*, 1996). Recent findings also indicate that Akt participates in the DNA damage response (Brognard *et al.*, 2001). Viniegra *et al.* (Viniegra *et al.*, 2005) found that ATM is an upstream activator of Akt activity, which could explain some of the biological symptoms of A-T patients e.g. radiosensitivity and resistance to insulin.

In the PCCl3 thyroid cell line, Zaballos *et al.* (Zaballos *et al.*, 2008) recently found that TSH stimulates Akt phosphorylation in a PI3K-dependent but cAMP-independent manner, mediated by Gβγ dimers released from G_s subfamily upon TSHR activation. Moreover, activation of this pathway was found to negatively regulate *NIS* gene expression by decreasing Pax8 binding to the NIS promoter. This suggests a possibility that ATM-mediated activation of the PI3K/Akt pathway might provide a direct link between DNA damage and inhibited NIS transcription. In line with this hypothesis is the observation that PI3K mediates NIS down-regulation in response to IGF-I in FRTL-5 thyroid cells (Garcia and Santisteban, 2002). However, this possibility is contradicted by to the present findings of a stimulatory effect of IGF-I on *pNIS* (Paper II). A positive regulation of NIS by IGF-I via PI3K is also reported for thyroid and breast cancer cells (Knostman *et al.*, 2007; Knostman *et al.*, 2004; Kogai *et al.*, 2008b). Thus, involvement of the PI3K/Akt pathway in thyroid stunning requires further studies to be elucidated.

The paradoxical inhibition of pNIS transcription in cells subjected to prolonged exposure to KU-55933 might be related to secondary long-term effects of inhibited ATM activity. ATM is likely required for the continuous surveillance of DNA integrity, given that if absent DNA repair in response to endogenously generated lesions are repaired less efficiently. Thus, analogous to the effect of DNA-PK inhibition (Paper III), impaired ATM signaling beyond a critical time point is assumed to increase yet poorly understood components of the DNA damage response, which may also affect the transcriptional control of NIS expression. Involvement of multiple pathways is supported by the fact that KU-55933 only partially reduced the NIS expression even though ATM kinase activity reflected by p53 phosphorylation was nearly completely blocked. It cannot be ruled out, however, that off-target effects of KU-55933 might contribute at the higher drug concentrations.

SUMMARY AND CONCLUSIONS

The work in this thesis was initiated to unravel the mechanism of thyroid stunning, a clinical phenomenon that has confused radiation physicists and thyroid oncologist for decades. Using an *in vitro* porcine thyroid cell culture model that allows mimicking of internal radiation with ^{131}I, we in papers I and II show that:

1. Transepithelial iodide transport is decreased by ^{131}I-irradiation, confirming previous findings in our laboratory (Postgård *et al.*, 2002), and that significant transport inhibition is recognized already at absorbed doses ≤ 1 Gy.

2. Absorbed doses of ^{131}I ≥ 1 Gy are required to inhibit cell proliferation.

3. ^{131}I-induced loss of TSH-stimulated iodide transport is paralleled by decreased pNIS mRNA levels.

4. IGF-I stimulates both NIS mRNA expression and iodide transport in irradiated cells.

These experimental findings suggest that thyroid stunning likely is due to diminished *NIS* gene transcription as part of the stress response to ionizing radiation. This can partially be counteracted by co-stimulation with TSH and IGF-I, possibly representing a radioprotective effect. Furthermore, iodide transport appears to be more radiosensitive than checkpoints regulating cell cycle progression in normal thyroid cells.

In subsequent studies concerning the mechanisms leading to suppressed NIS-mediated iodide uptake, we in papers III and IV show that:

1. DNA damage and preferably DSBs, induced by the genotoxic drug calicheamicin γ1, lead to down-regulation of NIS at the transcriptional level and inhibition of TSH-stimulated iodide transport.

2. DNA-damage induced decrease in iodide transport is augmented by a specific DNA-PK inhibitor (NU7026).

3. Specific blocking of ATM kinase activity with KU-55933 partially prevents the negative effects of DNA damage on NIS expression and iodide transport.

4. Normal thyroid cells lacking exogenous DNA insults nearly abolished the NIS-mediated iodide transport in response to prolonged exposure to ATM inhibitor.

This provides the first evidence that radiation-induced thyroid stunning in all probability is due to DNA DSBs. The notion is supported by the observation that impaired DNA repair enhances the stunning effect *in vitro*. The signaling pathway(s) leading to DNA damage-induced repression of NIS expression is at least partly dependent of ATM kinase activity. The current model of the stunning mechanism, considered to be part of the transcriptional response to DNA damage in thyroid cells, is depicted in figure 11. This encourages further research aiming to identify signaling molecules down-stream of ATM that negatively regulates the NIS promoter. Knowledge of this will eventually tell us how thyroid stunning might be avoided e.g. by pharmacological means in the clinical use of radioiodine. It can also be envisaged that identification of signaling intermediates might uncover novel autoregulatory mechanisms putatively acting to secure that the NIS expression and iodide uptake is sufficient in the normal thyroid gland, even though it throughout life is continuously exposed to very high levels of endogenous metabolites e.g. reactive oxygen species harmful to DNA.

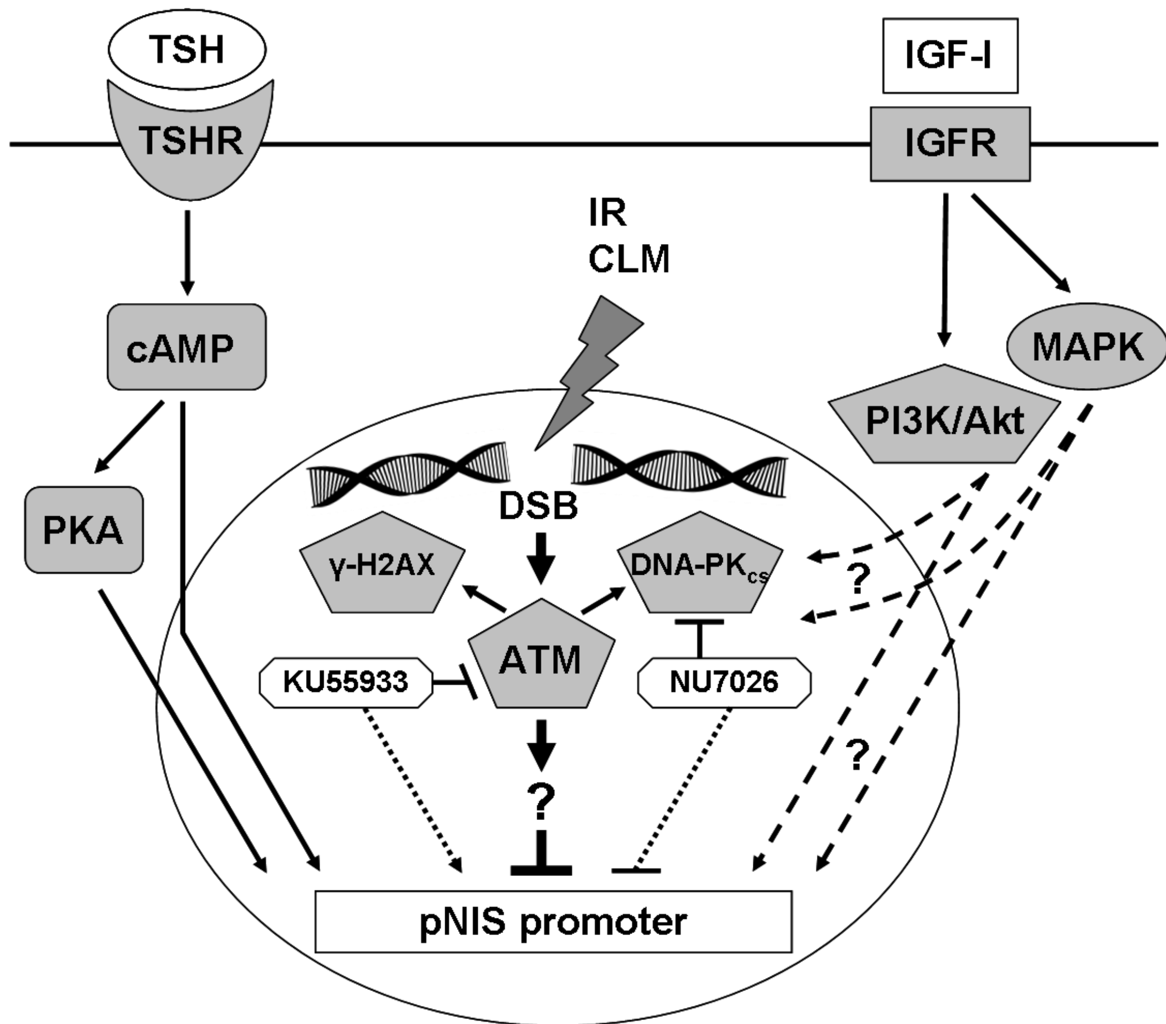

Figure 11. *Summary of present findings indicating the putative signaling pathway(s) involved in DNA damage-induced repression of the pNIS gene expression. In addition, the effects of ATM and DNA-PK inhibitors are indicated: dotted lines indicate outcome of kinase inhibition, although the precise molecular mechanisms are yet unknown. The signaling pathway mediating the radioprotective action of IGF-I counteracting down-regulation of NIS is also hypothetical. Not indicated in graph are putative ATM-independent signals that may contribute to loss of NIS-mediated iodide transport in DNA-damaged thyroid cells. IR – ionizing radiation; CLM – calicheamicin (for other abbreviations, see text of this thesis)*

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