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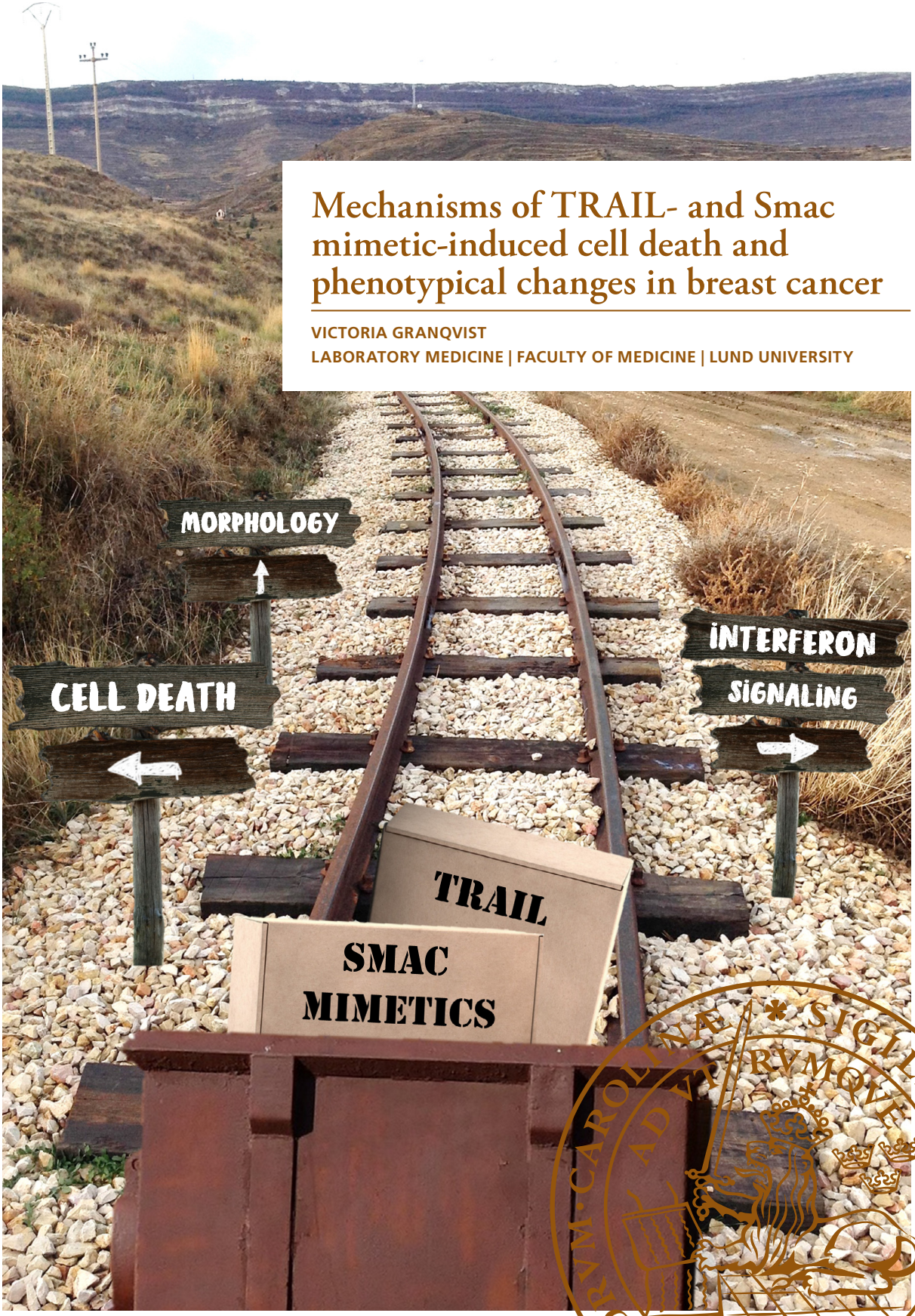

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Abstract

Breast cancer is the most common cause of cancer-related deaths in females. Despite a generally good prognosis, a substantial number of patients suffer from relapse. The acquired capacity of a cancer cell to resist apoptosis, a form of controlled cell death, can contribute to treatment resistance. One way to circumvent cell death resistance in cancer cells is to reactivate cell death. This can be achieved by treating with Smac mimetics, small molecule peptides which were developed to mimic the function of Smac. The function of Smac is to facilitate induction of apoptosis by inhibiting the inhibitor of apoptosis proteins (IAPs). This results in disinhibition of caspases, a family of proteins which can mediate the execution phase of apoptosis. However, the effect of Smac mimetics as a single treatment is limited and they are therefore also examined as part of a combination therapy. TRAIL, a death receptor ligand, preferentially induces apoptosis in cancer cells and can potentially be used together with Smac mimetics.

The first aim of this thesis, which was examined in Paper I, was to study if TRAIL together with the Smac mimetic LCL161 can induce apoptosis in breast cancer cells, and to investigate the underlying mechanism. We found that TRAIL and LCL161 induce cell death in one estrogen receptor (ER)-positive, CAMA-1, and one triple-negative, MDA-MB-468, breast cancer cell line. This was dependent on the activity of caspase-8. In CAMA-1, this was also found to be partially dependent on receptor-interacting protein kinase 1 (RIP1), but not its kinase activity, and suppressed by the caspase-8 inhibitor c-FLIP. In MCF-7, another ER-positive cell line, the combination of TRAIL and LCL161 could not induce apoptosis, but rather an alteration of morphology and gene expression.

In paper II, the mechanisms for the changes in MCF-7 cells, following treatment with LCL161 and TRAIL, were investigated. We found that the morphology was irreversible and that downregulation of caspase-8 blocked the alteration. However, we observed a slow and gradual processing of caspase-8, suggesting a low and long-term activity of caspase-8. We found upregulation of genes related to NF-κB and interferon (IFN) signaling after treatment, whereas the downregulated genes were enriched for genes found in a less ER-positive phenotype. The changes in morphology and gene expression are separate events since neither inhibition of IFN- nor NF-κB pathways affected the morphology change. We further wanted to examine the induction of IFN signaling since little is known about TRAIL-mediated induction of IFN signaling.

We studied the underlying mechanism, and potential mediators, of IFN-induction following treatment with TRAIL and LCL161 in Paper III and IV. Inhibition of Janus tyrosine kinases with Ruxolitinib blocked treatment-induced STAT1 phosphorylation and downregulation of the type I IFN receptor IFNAR1 suppressed the induction of IFN signaling. In addition there was a gradual increase in *IFNB1* mRNA levels following treatment, all of which indicate an autocrine IFN signaling. Ruxolitinib suppressed IFN-stimulated genes but could not block *IFNB1*-induction, which further supports the hypothesis of an autocrine IFN signaling. Downregulation of caspase-8 suppressed IFN signaling but inhibition of its activity did not, suggesting a scaffold role of caspase-8 in this context. Inhibition of TBK1 and IKKε, two proteins mediating transcription of *IFNB1*, with MRT67307 suppressed STAT1 phosphorylation but IFN signaling could not be blocked by downregulation of TBK1 or IKKε. NIK, a kinase which induces the non-canonical NF-κB pathway, could potentially be involved in mediating TRAIL- and LCL161-induced IFN signaling. The results presented in this thesis suggest that caspase-8 can mediate apoptosis if fully activated, stimulate morphology change if activated weakly and slowly, and induce IFN signaling if it acts as a scaffold in ER-positive breast cancer cells lines following treatment with TRAIL and Smac mimetic.

Key words

TRAIL, Smac mimetics, breast cancer, cell death, phenotypical changes, IFN, NF-κB

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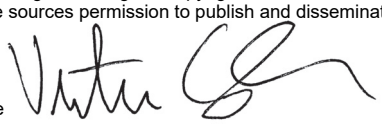
Signature  Date 2022-04-19

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Victoria Granqvist

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To my family and friends

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I. Induction of breast cancer cell apoptosis by TRAIL and Smac mimetics: Involvement of RIP1 and c-FLIP.

Holmgren C, Thörnberg ES, **Granqvist V**, Larsson C

Manuscript

II. The combination of TRAIL and the Smac mimetic LCL-161 induces an irreversible phenotypic change of MCF-7 breast cancer cells

Granqvist V*, Holmgren C*, Larsson C

Experimental and Molecular Pathology 125:104739 (2022)

III. Induction of interferon-β and interferon signaling by TRAIL and Smac mimetics via caspase-8 in breast cancer cells

Granqvist V, Holmgren C, Larsson C

PLoS One 26:e0248175 (2021)

IV. TRAIL- and Smac mimetic-mediated activation of TBK1 and NF-κB pathways in breast cancer cells and their roles in induction of interferon pathways

Granqvist V, Holmgren C, Larsson C

Manuscript

The asterisk (*) indicates equal contribution

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Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
AI	aromatase inhibitor
AP-1	activation protein 1
Apaf-1	apoptosis protease activating factor 1
BAFFR	B cell-activating factor receptor
BIR	baculovirus IAP repeat
CARD	caspase activation and recruitment domain
CBP	cAMP-response element binding protein (CREB)-binding protein
CD40	cluster of differentiation 40
c-FLIP	cellular FLICE-like inhibitory protein
cGAS	cyclic GMP-AMP synthase
cgDNA	cytosolic genomic DNA
cIAP	cellular IAP
CpG	cytosine-phosphate-guanine
DAI	DNA-dependent activator of IRFs
DAMP	damage-associated molecular pattern
DC	dendritic cell
DCIS	ductal carcinoma *in situ*
DcR	decoy receptor
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
DR	death receptor
dsRNA	double-stranded RNA
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FADD	Fas associated via death domain
FISH	fluorescent *in situ* hybridization
GAS	gamma-activated sequence
HAT	histone acetyltransferase
HER2	human epidermal growth factor receptor-2
HR	hormone receptor
IAP	inhibitor of apoptosis protein
ICI	immune checkpoint inhibitor
IDC	invasive ductal carcinoma
IDC-NST	invasive ductal carcinoma of no special type
IFITM1	IFN-induced transmembrane protein 1
IFN	interferon
IFNAR	IFN-alpha/beta receptor

IFNAR1	IFN-alpha/beta receptor subunit 1
IFNGR1	IFN-gamma receptor 1
IFNLR1	IFN-lambda receptor 1
IHC	immunohistochemical
IKKα	inhibitor of NF-κB kinase subunit alpha
IKKβ	inhibitor of NF-κB kinase subunit beta
IKKε	inhibitor of NF-κB kinase subunit epsilon
IL-10RB	interleukin-10 receptor subunit beta
IL-1R	interleukin-1 receptor
ILC	invasive lobular carcinoma
IRAK	interleukin-1 receptor associated kinase
IRF	IFN-regulatory factor
ISG	IFN-stimulated genes
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response elements
JAK	janus kinase
JNK	c-Jun N-terminal kinase
LCIS	lobular carcinoma *in situ*
LPS	lipopolysaccharide
LTβR	lymphotoxin β receptor
LUBAC	linear ubiquitin chain assembly complex
MDA5	melanoma differentiation-associated protein 5
MOMP	mitochondrial outer membrane permeabilization
MyD88	myeloid differentiation primary response 88
NAP1	NAK-associated protein 1
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-kappa B
NHG	Nottingham histological grade
NIK	NF-κB-inducing kinase
NK	natural killer
NOD	nucleotide-binding oligomerization domain-containing protein
OAS1	2′-5′-oligoadenylate synthase 1
PAMP	pathogen-associated molecular pattern
PD-1	programmed cell death protein 1
pDC	plasmacytoid dendritic cell
PR	progesterone receptor
PRD	positive regulatory domain
PRD-LE	PRD-like elements
RIG-I	retinoic acid-inducible gene I
RIP/RIPK	receptor-interacting kinase
SCAN-B	Sweden Cancerome Analysis Network – Breast
SERD	selective estrogen receptor degraders
SERM	selective estrogen receptor modifier
SH2	src homology 2
SLE	systemic lupus erythematosus
Smac	second mitochondria-derived activator of caspase
SOCS	suppressor of cytokine signaling
ssRNA	single-stranded RNA

STAT	signal transducer and activator of transcription
STING	stimulator of IFN genes
TAK1	transforming growth factor-β-activated kinase 1
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TDLU	terminal duct lobular unit
TIC	tumor-initiating cell
TIL	tumor-infiltrating lymphocyte
TLR	toll-like receptor
TN	triple-negative
TNFR	TNF receptor
TNF-α	tumor necrosis factor alpha
TRADD	TNFR-associated death domain protein
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
TRAM	translocating chain-associated membrane protein
TRIF	TIR-domain-containing adaptor-inducing IFN-β
TYK2	tyrosine kinase 2
XIAP	X-linked IAP

Populärvetenskaplig sammanfattning

Cancer omfattar en grupp sjukdomar och uppstår när celler delas och växer okontrollerat och stör funktionen hos det drabbade organet. Okontrollerad celldelning kan vara ett resultat av mutationer som uppstår vid normalt förekommande celldelning eller av mutationer som bildas av yttre faktorer så som UV-strålning. Oftast är mutationerna ofarliga men de kan ibland leda till en förändring hos cellen. Med tiden kan fler förändringar uppstå som tillslut gynnar en överdriven tillväxt av en cell. Detta kan då resultera i en tumör.

En typ av förändring som gynnar okontrollerad celldelning är en cells förmåga att undvika att gå igenom celldöd. Detta är en mekanism hos cellerna som normalt aktiveras när oönskade och skadade celler behöver elimineras. Genom att undvika celldöd kan cancerceller även stå emot olika cancerbehandlingar. En strategi för att kringgå detta, och därmed framkalla celldöd i cancerceller, är att provocera fram celldöd. Detta kan göras med hjälp av olika typer av nya behandlingsstrategier. Ett exempel är de så kallade Smac mimetics som imiterar effekten av Smac – ett protein som medverkar i processen att framkalla celldöd hos en cell. Dock har det påvisats att Smac mimetics har en begränsad effekt om de används som singelbehandling. Därför undersöks vilka behandlingsformer Smac mimetics kan kombineras med för att framkalla celldöd. Ett sådant exempel är proteinet TRAIL som främst stimulerar celldöd i cancerceller över normala celler.

I denna avhandling har vi undersökt om kombinationen TRAIL och Smac mimetics kan ge upphov till celldöd i bröstcancerceller. I **artikel I** fann vi att Smac mimetic gjorde cellerna känsligare mot celldöd stimulerad av TRAIL. Kombinationsbehandlingen ledde till celldöd i två olika typer av bröstcancerceller men en specifik celltyp förändrade istället både utseende och egenskaper. Kaspaser är viktiga proteiner i celldödsprocessen och aktiveras vid celldöd. Vi fann en snabb och kraftig aktivering av kaspaser i de celltyper som dog av behandlingen men inte i den celltyp som överlevde.

Förändringarna som uppstod efter behandling med TRAIL och Smac mimetic i en av bröstcancercelltyperna studerades närmre i **artikel II**. Vi kom fram till att förändringarna var bestående och beroende av en specifik kaspas, nämligen kaspas-8, som bara aktiverades svagt och långsamt. Men det ändrade utseendet och egenskaperna hos cellerna är två separata händelser. En av de förändrade egenskaperna påminner om vad som sker vid kroppens svar på virusinfektion. Detta

svar leder till produktion av så kallade interferoner som är en del av immunförsvaret och bidrar till elimineringen av virusinfektionen.

Hur produktionen av interferon uppstår efter behandling med TRAIL och Smac mimetic undersöktes i **artikel III** och **IV**. Vi fann att behandling med TRAIL och Smac mimetic även kan leda till produktion av interferon i en av celltyperna som dog efter behandling. Detta kunde dock enbart ske när celldöd förhindrades genom att blockera kaspaserna från att verka. I båda celltyperna som undersöktes var interferonsvaret beroende av närvaron av kaspas-8 men inte dess aktivitet. Produktionen av interferon ökade också med tiden av behandling eftersom interferon kan stimulera samma cell att producera mer interferon. Vi lyckades blockera stimuleringen av interferon med hjälp av en hämmare. Däremot kan de protein som är tänkta att hämmas inte kan fastställas ha en funktion i den produktion av interferon vi ser. Det finns dock indikationer på att ett annat protein, som tillhör en annan typ av inflammatoriskt svar, delvis medverkar i interferonsvaret som ses efter behandling med TRAIL och Smac mimetics. Sammanfattningsvis kan kombinationen av TRAIL och Smac mimetic stimulera celldöd i vissa typer av bröstcancercelltyper. Om kaspas-aktiviteten är låg kan detta istället leda till förändringar i utseendet hos cellerna. Även egenskaperna kan förändras hos cellerna då bland annat interferon produceras. Denna effekt är beroende av närvaron av kaspas-8 men inte dess aktivitet.

Abstract

Breast cancer is the most common cause of cancer-related deaths in females. Despite a generally good prognosis, a substantial number of patients suffer from relapse. The acquired capacity of a cancer cell to resist apoptosis, a form of controlled cell death, can contribute to treatment resistance. One way to circumvent cell death resistance in cancer cells is to reactivate cell death. This can be achieved by treating with Smac mimetics, small molecule peptides which were developed to mimic the function of Smac. The function of Smac is to facilitate induction of apoptosis by inhibiting the inhibitor of apoptosis proteins (IAPs). This results in disinhibition of caspases, a family of proteins which can mediate the execution phase of apoptosis. However, the effect of Smac mimetics as a single treatment is limited and they are therefore also examined as part of a combination therapy. TRAIL, a death receptor ligand, preferentially induces apoptosis in cancer cells and can potentially be used together with Smac mimetics.

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We studied the underlying mechanism, and potential mediators, of IFN induction following treatment with TRAIL and LCL161 in Paper III and IV. Inhibition of Janus tyrosine kinases with Ruxolitinib blocked treatment-induced STAT1 phosphorylation and downregulation of the type I IFN receptor IFNAR1 suppressed the induction of IFN signaling. In addition there was a gradual increase in *IFNB1* mRNA levels following treatment, all of which indicate an autocrine IFN signaling. Ruxolitinib suppressed IFN-stimulated genes but could not block *IFNB1*-induction, which further supports the hypothesis of an autocrine IFN signaling. Downregulation of caspase-8 suppressed IFN signaling but inhibition of its activity did not, suggesting a scaffold role of caspase-8 in this context. Inhibition of TBK1 and IKKε, two proteins mediating transcription of *IFNB1*, with MRT67307 suppressed STAT1 phosphorylation but IFN signaling could not be blocked by downregulation of TBK1 or IKKε. NIK, a kinase which induces the non-canonical NF-κB pathway, could potentially be involved in mediating TRAIL and LCL161-induced IFN signaling. The results presented in this thesis suggest that caspase-8 can mediate apoptosis if fully activated, stimulate morphology change if activated weakly and slowly, and induce IFN signaling if it acts as a scaffold in ER-positive breast cancer cells lines following treatment with TRAIL and Smac mimetic.

Cancer

Cancer is the second-most common cause of death, both in Sweden and worldwide [1, 2]. As the life expectancy increases, the incidence of cancer cases does too. This is a result of damage and mutations of the DNA accumulating with age [3]. In 5-10% of all cancer cases, there is an inherited mutation in a cancer predisposition gene [4]. In addition, there are other risk factors than age and inherited genes for developing cancer, such as exposure to carcinogens, lifestyle factors (for example tobacco use, alcohol consumption, and overweight), as well as some viral or bacterial infections [5].

Cancer is not a single disease, but rather constitutes a group of diseases which are characterized by uncontrolled division, growth, and sometimes spread of cells. Traits that are acquired with time during the development of tumors are termed hallmarks of cancer and were summarized by Hanahan and Weinberg in 2000 [6]. The hallmarks of cancer include: sustaining proliferative signaling; evading growth suppressors; activating invasion and metastasis; enabling replicative immortality; inducing angiogenesis; and resisting cell death. In 2011, these were complemented with four additional hallmarks, namely: avoiding immune destruction; tumor promoting inflammation; genome instability and mutation; and deregulating cellular energetics [7].

DNA damage occurs naturally in cells during cell division, and can frequently be repaired. The cell can also go through cell death if a DNA damage cannot be repaired. However, if an incorrectly paired nucleotide escape proofreading this can result in mutation of a gene [8]. These mutations can affect oncogenes, which can drive transformation of cancer upon mutation, or suppressor genes, which are protective genes that can be inactivated following mutations [9]. Accumulation of these mutations in a cell can contribute to development of a cancer cell. This is followed by cellular proliferation, which leads to expansion and outgrowth of a population of clonally derived cells. Additional mutations may then follow, which facilitate rapid growth, increased survival, and perhaps invasion and metastasis, which increase the malignancy of the evolving tumor with time [10]. All hallmarks are potential targets for cancer therapeutics [7].

Breast cancer

Epidemiology

Breast cancer is the most commonly diagnosed cancer and the primary cause of cancer-related death among females [2, 11]. In 2019, 65 965 individuals were diagnosed with cancer in Sweden [12]. Of these were 8 288 women who were diagnosed with breast cancer [12]. In the same year, 1 353 females passed away due to breast cancer-related death [13]. Although breast cancer predominantly is diagnosed in women, approximately 60 men per year are affected by the disease in Sweden [12]. This thesis will focus on breast cancer in women. Looking at a broader perspective, 2.3 million females were diagnosed with breast cancer worldwide in 2020, while 685 000 deaths were reported, according to World Health Organization (WHO). However, of the women who have been diagnosed in the past 5 years, 7.8 million were still alive, making breast cancer the most prevalent cancer disease in the world [14]. The number of surviving patients are increasing due to early diagnosis [15] and treatment improvements [16]. However, this also results in more women being at risk of recurrence [17].

Risk factors

There are several different genetic and environmental factors that can increase the risk of developing breast cancer. Age is one of the most important mainly since the mutational burden in cells accumulates over time. Factors that influence the exposure to estrogen or substances that can stimulate estrogen receptor (ER) signaling also influence the risk of developing breast cancer. These include: early menarche, late menopause, nulliparity, high hormonal baseline levels, giving birth at a higher age, and the usage of oral contraceptives and hormonal replacements therapies [18-22]. In addition, having a high breast density [23] or a previous history of carcinoma *in situ* [24] are factors that result in an elevated risk of developing breast cancer. There is also a risk related to ethnicity, where Caucasian women are more prone to develop breast cancer as compared to African American or Hispanic women. However, there is a higher risk of developing more aggressive tumors with an African American ethnicity [25]. The majority of all breast cancers occur sporadically but family history seems to be related to 5-10% of all cases [25]. The

most classical example is the inactivation of the *BRCA1* and *BRCA2* genes, both involved in DNA repair [26], which leads to a prominent risk of breast cancer development, and accounts for approximately 25% of all hereditary breast cancer cases [27, 28]. Mutated *BRCA1* has been linked to an aggressive and less treatable form of malignancy [29]. Women with a family history of BRCA mutations are offered early prophylactic care.

In addition, there are many risk factors related to lifestyle habits, which include consumption of alcohol [30-32] and processed meat [33, 34], a high body fat level and obesity, both measured as high BMI and in a BMI-independent setting [35-39], lack of physical activity [40-42], and passive and active tobacco smoking [43, 44]. On the other hand, to have given childbirth and have breastfed, can be protective against breast cancer [45-47].

The development of breast cancer

The major development of the human breast starts at the beginning of puberty when hormones are produced which cause the breast epithelia to grow. A mature breast contains branches of terminal duct lobular units (TDLUs) in the surrounding fat tissue (Figure 1). These TDLUs contain several lobules, which are small cavities where the milk production occurs during lactation [48]. During pregnancy the mammary gland stem cells located in the breast start proliferating and differentiating. This gives rise to the different cell types necessary to constitute the expansion of the ductal network and causes the breast tissue to grow [49-51]. The duct of each TDLU is composed of apically positioned ductal cells and surrounding basal myoepithelial cells which contract during lactation when the milk is to be released from the nipple [52-54]. After terminated lactation, involution occurs, in which the breast tissue regresses and the organization of the mammary gland returns to a quiescent state [55, 56].

Breast cancer can arise in all compartments of the breast, but most often it starts as a benign alteration in the cells of the TDLUs [11] where it can progress from a hyperplasia into an atypical hyperplasia, which increases the risk of developing breast cancer [57, 58]. If the progression continues, it can ultimately develop into carcinoma *in situ*, which is a tumor consisting of abnormal cells but located only at the site of origin. This can be followed by tumor growth and subsequently tumor invasion where the cells can spread into the surrounding stroma and adjacent lymph nodes [59]. At this stage it is termed an invasive cancer. Tumor cells can also metastasize through invasion and intravasation of either the lymphatic system or blood vessels followed by extravasation to distant tissues and organs. The most common sites for breast cancer metastases are bone, liver, and lung [60, 61].

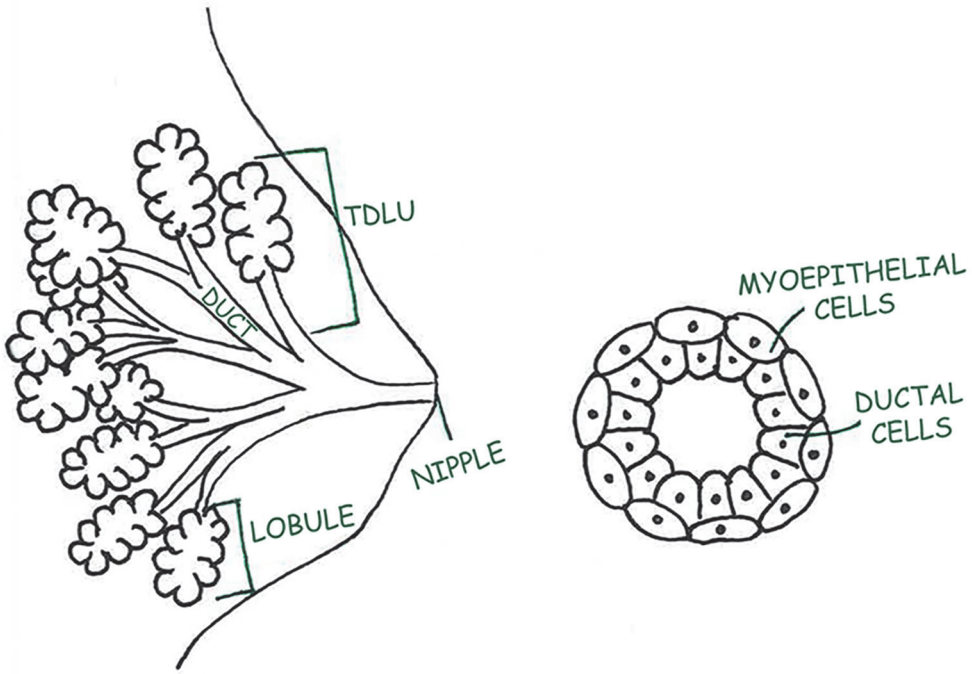

Figure 1. Schematic figure of the human mammary gland
The terminal duct lobular units (TDLUs) are composed of cavities, called lobules. The ductal cells line the duct and are surrounded by myoepithelial cells which contract during lactation to facilitate the flowing of the milk through the ducts and ultimately out through the nipple.

The so called tumor-initiating cells (TICs) in breast cancer have not been firmly established. According to one hypothesis, TICs originate from mammary stem cells. Intrinsic properties of the generally quiescent mammary stem cells, such as the ability of self-renewal, could benefit development of cancer cells [51, 62]. It has also been suggested that the breast cancer TICs derives from progenitor cells that have acquired the capacity of self-renewal [51, 62]. It is also under discussion if there is a common TIC that give rise to all breast cancer subtypes or if the TICs differ between subtypes. The clonal evolution model proposes that there is a common ancestor and that the oncogenic events determine the subtype [62]. On the other hand, a study by Keller *et al*. showed that transformation of luminal $EpCAM^+$ cells resulted in tumors with luminal features, such as ERα, whereas tumors derived from transformed $CD10^+$ cells showed squamous, metaplastic, and giant cell differentiation, and lack of ERα [63]. This could indicate that the TIC may determine the subtype. When it comes to *BRCA1* deletions in breast cancer, experiments in mice, where the *BRCA1* gene was deleted both in luminal and basal progenitor mammary epithelial cells, demonstrated that it was only the luminal

progenitor cells that gave rise to tumors resembling human BRCA1 breast cancers and basal-like tumors [64].

Breast cancer classification

Breast cancer can be classified by different means, taking distinctive features of the tumor into consideration. All classification systems provide information on prognosis and can be used as a guidance when selecting the most effective treatments.

Histological type

Histological types are determined by patterns related to the morphology and architectural characteristics of single tumor cells as well as the tumor bulk [65]. Invasive ductal carcinomas (IDCs) are the most common types of invasive breast cancer as they comprise approximately 80% of all breast cancer cases [66]. The IDC of no special type (NST, previously known as IDC-NST) accounts for approximately 75% of all IDC cases. NST includes tumors that do not harbor sufficient characteristics to be categorized into any of the special types [67, 68], which constitute for the remaining 25%. These include, for example, the medullary, tubular, apocrine, and metaplastic carcinomas. The tubular and medullary carcinomas have a more favorable prognosis as compared to the metaplastic and apocrine types [65]. Invasive lobular carcinoma (ILC) represents 5-15% of all invasive breast cancers and includes five different variants [69, 70], namely the classic type of ILC, pleomorphic lobular carcinoma, histiocytoid carcinoma, signet ring carcinoma, and tubulolubular carcinoma [65]. Since the majority of tumors do not present any specific characteristics, such as those belonging to NST, histological typing has a limited significance in a clinical setting [71, 72].

When a tumor is not considered invasive, it is called either ductal or lobular (depending on site of origin) carcinoma *in situ* (DCIS or LCIS), meaning that it has not invaded through the basement membrane. Tumors defined as DCIS constitute approximately 10% of all breast cancer cases in Sweden and are treated with surgery and post-operative radiation [11]. Hormone therapy can be used if the DCIS is ER-positive [11, 73]. LCIS is often hormone receptor (HR)-positive and human epidermal growth factor receptor-2 (HER2)-negative and is treated with surgery in combination with post-operative radiation or endocrine therapy [74]. A diagnosis of DCIS or LCIS confers an increased risk of developing invasive cancer at a later time point. Therefore, follow-ups are encouraged [11, 75].

Histological grade

Breast tumors are classified based on histological grade, which is a morphological marker where tumor differentiation, as compared to normal breast tissue, is evaluated. The Nottingham Grading System, based on Elston and Ellis histological grade system [76], is widely used in the clinic and has been shown to have prognostic value [72, 77]. By setting a score (1-3) of the degree of differentiation on three morphological features: tubule and gland formation (amount of normal structures), nuclear pleomorphism (variation in size and shape), and mitotic count (proliferation), it is possible to estimate the aggressiveness of the tumor. The three scores are added and when the sum is 3-5 (Nottingham Histological Grade (NHG) I) the tumor is morphologically similar to normal breast tissue. This correlates with good prognosis. On the contrary, tumors given a score of 8-9 (NHG III) are poorly differentiated and have the worst prognosis [11, 78].

TNM staging system

Another way of classifying breast cancer is by evaluating how advanced the tumor is. This can be done with the TNM staging system, where tumor size (T), nearby lymph node involvement (N), and distant metastasis (M), are assessed. Based on these three measures, patients are categorized into four stages (I-IV), where stage I has the most favorable prognosis and stage IV the worst [79].

Immunohistochemical classification

Immunohistochemical (IHC) staining of four protein biomarkers is used in the clinic to categorize tumors into clinical subtypes and thereby assess the best treatment option. These biomarkers includes the two hormone receptors (HRs), estrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and the proliferation marker Ki67. By counting the number of tumor cells positively stained with antibodies against these markers a percentage of stained cells is obtained. In Sweden, the threshold for HR positivity is set to 10% for ER and 20% for PR, whereas international guidelines set the threshold to 1% for both HRs. Staining of Ki67 is categorized as low (<10%), intermediate, or high (>20%) grade [11]. For HER2 it is more complex. Based on IHC, staining of HER2 (to determine protein levels) is graded 0-3+, where a tumor is considered HER2-negative if it is graded as 0-1+ (no or faint staining), and HER2-positive if graded as 3+ (strong staining in >10% of tumor cells). Those graded as 2+ (weak to moderate staining in >10% of tumor cells) are considered ambiguous and are therefore further evaluated with fluorescent *in situ* hybridization (FISH) to examine amplification of the *ERRB2* gene, which encode for HER2 [11, 80].

Molecular subtype classification

As a complementary tool to the histological and morphological classifications used in the clinic, analysis of mRNA expression can be applied [81, 82]. Based on microarray analyses, Perou *et al*. and Sørlie *et al*. were able to establish molecular (also called intrinsic) subtypes of breast cancer, based on similarities in gene expression [81-83]. Later, these studies were extended with qPCR analyses of a limited number of mRNAs. One example is the PAM50 assay which, in addition to classify breast cancer subtypes, can be used to assess the risk of recurrence when combined with other prognostic factors [11, 84-86]. There are five different intrinsic subtypes of breast cancers, namely: luminal A and B, HER2-enriched, basal-like, and normal-like [81, 82, 85].

Luminal A is the most common subtype as it comprises more than half of all newly diagnosed breast cancer cases [65, 87, 88]. Tumors of the luminal A subtype are in general ER- and PR-positive, lack amplification of HER2, and have a low to intermediate Ki67 expression. The luminal B tumors are in general ER-positive with low PR expression, high Ki67 expression, and lack HER2-amplification. They account for 10-20% of all breast cancer cases. The HER2-enriched subgroup represents 15-20% of all breast cancer cases [88, 89]. Tumors of this subtype are frequently HER2-positive and lack expression of the HRs. Tumors of the basal-like subtype account for 10-20% of all breast cancer cases. Their gene expression is similar to that of myoepithelial or basal cells, which explains the name [81, 88]. Tumors of this subtype generally lack expression of ER, PR, and HER2. Tumors which are negative for ER, PR, and HER2 are called triple-negative (TN). However, 20-30% of all basal-like tumors actually express one or more of these receptors [82, 88, 90-92]. To distinguish between TN and basal-like tumors, biomarkers such as cytokeratin 5/6 and/or epidermal growth factor receptor (EGFR), as well as negative staining for ER, PR, and HER2, can be used [91, 93-95]. The normal-like subtype comprises only 5-10% of all breast cancer cases. In general, tumors belonging to this subtype have a similar expression pattern to that seen in normal breast samples and fibroadenomas, which has given it its name [81, 88]. This subgroup is fairly uncharacterized, which could be explained by its low prevalence [88]. Moreover, it is still under debate whether this is a real subgroup or if it is a technical artefact due to normal tissue in the samples [88, 96]. Nevertheless, it presents variable expression of HRs, negative expression of HER2 and Ki67, as well as low/intermediate grade [88, 97].

Efforts are being made to improve current analyses and to develop new ones. For example, in 2010 the Sweden Cancerome Analysis Network – Breast (SCAN-B) was launched, as a collaboration between hospitals in the southern of Sweden, to identify new prognostic markers using whole transcriptome RNA sequencing [98].

Classification of breast cancer in the clinic

In the clinic, immunohistochemical analysis is used to approximate the molecular subtypes [11, 99]. This classification results in the following subgroups: luminal A-like, luminal B-like (HER2-positive), luminal B-like (HER2-negative), HER2-positive, and triple-negative (Table 1).

Tumors classified as luminal A-like are ER-positive, HER2-negative, have a low NHG (grade 1-2), and either a low or intermediate Ki67 expression together with high PR expression ($\geq$20%) [11]. The expression of PR could contribute to the low grade of luminal A tumors, since progesterone can counteract estrogen-dependent cell growth and division [100]. Tumors of this subgroup display many different histological variants of low grade, including IDC-NST and tubular carcinoma [101]. The prognosis of Luminal A-like cancers is favorable and the tumors are less prone to develop lymph node metastasis [102, 103].

Luminal B-like tumors are ER-positive and can be either HER2-negative or HER2-positive. A tumor that is ER-positive and HER2-negative is classified as luminal B-like if it has either a high expression of Ki67 together with NHG 2-3 or an intermediate Ki67 expression and low PR-expression (<20%) together with NHG 2-3 [11]. A HER2-positive tumor, which is ER-positive, is classified as luminal B-like independently of the level of PR and Ki67 [11, 99]. Compared to luminal A-like tumors, luminal B-like tumors are more aggressive and exhibit a worse prognosis [88]. As a result, they generally have an increased risk of locoregional relapse (recurrence at primary site or nearby lymph nodes) and metastatic spread [104-106]. Luminal B-like tumors encompass 10-20% of all breast tumors [88].

Tumors that are clinically characterized as HER2-positive have a high expression of HER2 protein levels and/or amplification of the *ERBB2* gene, which encode for HER2, in addition to being ER- and PR-negative [11]. However, amplification does not always correlate with increased protein levels [107, 108]. In addition to HER2 status, the tumors are associated with high expression of Ki67 [88, 99]. Tumors of this subtype are associated with a higher histological grade and poor prognosis [88]. However, with the development of targeted therapies such as trastuzumab, a monoclonal antibody targeting the HER2 receptor [109], these tumors have good response to treatments [110].

Triple-negative (TN) tumors are ER-negative, PR-negative, HER2-negative, and in most cases of a high histological grade [11]. The prognosis for this subtype is usually poor since the lack of receptors prevents the usage of targeted therapies [88, 111, 112].

Table 1. Approximated molecular subtypes for breast cancer

Approximated molecular subtypes	Luminal A-like	Luminal B-like		HER2-positive	Triple-negative
		HER2-	HER2+		
Frequency (%)	50-60	10-20		15-20	10-20
Biomarkers and histological grade	ER+ PR+ HER2- Ki67 low/interm. Low NHG	ER+ HER2- and either PR+ Ki67 high NHG interm. or Ki67 interm. PR low NHG interm.	ER+ HER2+ Indep. of PR and Ki67	ER- PR- HER2+ Ki67 high	ER- PR- HER2- NHG high
Prognosis	Good	Intermediate		Poor	Poor

Abbreviations: interm. = intermediate, indep. = independent

Treatment

The specific treatment given to a patient is decided based on the analyses and classifications described above. Before deciding the type of treatment, a biopsy sample of the tumor is collected to, by histological classification, decide the invasiveness and to analyze the expression of biomarkers (ER, PR, HER2, and Ki67) [11].

Surgery

Surgery is used to remove the tumor, and in some cases adjacent lymph nodes. It is either performed as a mastectomy, where the entire breast is removed, or as a breast-conserving surgery, in which cancerous tissue with margin is removed [113]. Surgery has been proven to be very effective since it results in lifelong absence of tumor disease in approximately 50% of all breast cancer cases when used either alone or in combination with local radiation therapy [11].

Radiation therapy

Local radiation therapy is often used as adjuvant therapy (post-operatively) to eradicate any remaining cancer cells after breast-conserving surgery, as it decreases the risk of recurrence and mortality [113]. However, it may also be used as a

neoadjuvant therapy (pre-operatively) to shrink the tumor before surgery. This has been shown to suppress local nodal recurrences [114].

Chemotherapy

The aim of chemotherapy is to shrink or eliminate the primary tumor and to eradicate tumor cells which potentially already have spread. Chemotherapy is given pre-operatively, if suitable for the patient, when the breast tumor is locally advanced, inoperable, or if it is accompanied with spread to lymph nodes. It is also given upon operable tumors > 2 cm which are either HER2-positive or TN [11]. Different types of chemotherapies are used for breast cancer. Anthracyclines, such as doxorubicin, primarily act by inhibiting topoisomerase II, resulting in DNA damage and apoptosis [115, 116]. Taxanes, which include paclitaxel and docetaxel, disrupt the microtubule dynamics causing halted cell division and cell death [117]. Cyclophosphamide is an alkylating agent and acts by cross-linking DNA and RNA strands, which prevents DNA replication, by adding an alkyl group to guanine bases [118]. Carboplatin is a platinum-based substance, which act by causing cross-linkage of DNA [119].

Endocrine therapy

Endocrine therapy can be given either as neoadjuvant or adjuvant therapy. Patients with HR-positive breast cancer are recommended post-operative endocrine therapy, where the treatment of choice is based on menopausal status, age, and lymph node status. The different types of endocrine therapies include selective estrogen receptor modifier (SERM), such as tamoxifen, selective estrogen receptor degraders (SERDs), such as fulvestrant, and aromatase inhibitors (AI) [11, 113, 120]. Tamoxifen is a complex drug as it acts both as a competitive ER antagonist by blocking the proliferative signaling of ER, and stimulates ER in some organs, making it a partial ER agonist as well [121, 122]. It is standard treatment for premenopausal women, who generally have high estrogen levels, and can be combined with a luteinizing hormone-releasing hormone (LHRH) agonist which suppresses the production of estrogen from the ovaries (ovarian suppression), but it also has an effect in postmenopausal patients [11]. Fulvestrant acts as an ER antagonist by competitively blocking the binding of estrogen to the receptor and causes impaired receptor dimerization which subsequently inhibits its nuclear localization [123-125]. In addition, the binding of fulvestrant to ER results in an unstable complex and therefore it is degraded [126]. It can be given to postmenopausal patients as a monotherapy, or in combination with a LHRH agonist and a CDK4/6 inhibitor in premenopausal patients [11, 127]. AIs function by inhibiting aromatase, the enzyme responsible for converting androgens to estrogens. They are given to postmenopausal women since they have been shown to be more efficient than tamoxifen for these patients [11, 128].

However, they cannot block the estrogen production in the ovaries prior to menopause and are therefore not given to premenopausal patients unless combined with ovarian suppression [11, 129].

Targeted therapy

Patients diagnosed with breast cancer harboring amplification of *ERRB2* and/or high expression of HER2 protein levels are offered targeted therapy with anti-HER2 drugs. One example is the monoclonal antibody trastuzumab, which is given either as neoadjuvant or adjuvant therapy [11, 80]. By blocking HER2 receptor dimerization, as well as inducing antibody-dependent cellular cytotoxicity (ADCC), trastuzumab induces cell-cycle arrest and apoptosis [130, 131].

Immunotherapy

The immune system can either suppress tumor growth by destroying cancer cells or promote its progression by creating a favorable microenvironment for tumor growth [132]. During immunotherapy, the goal is to utilize the patient's own immune system to identify and kill cancer cells. To avoid attacks on one's own normal cells, so-called self-tolerance, the immune system uses an immune checkpoint system. These checkpoints also function as immunosuppressive factors [133]. Programmed cell death protein 1 (PD-1) acts as an immune checkpoint inhibitor (ICI) and has been shown to inactivate the immune system in solid tumors when activated by its ligand PD-L1 [134, 135]. In breast cancer, the expression of PD-L1 is associated with large tumor size, high grade [136], and its expression correlates with a decreased survival [137]. To distinguish which breast cancer patients who will benefit from immunotherapy, different biomarkers are assessed, for example: the expression of PD-1 and its ligand PDL-1, tumor-infiltrating lymphocytes (TILs), and tumor mutation burden [132]. Inhibition of PD-1 has been proven efficient against TN breast cancer [138, 139] when combined with chemotherapy [11].

CDK4/6 inhibitors

Inhibitors have been developed to target CDK4 and CDK6, which together with the D-type cyclins regulate the G1-to-S phase cell cycle checkpoint. Treatment with these agents causes cell cycle arrest [140]. There are currently three approved CDK4/6 inhibitors: palbociclib, ribociblib, and abemaciclib, which are given to patients with advanced HR-positive/HER2-negative tumors. They have been proven to be most efficient when combined with either an aromatase inhibitor (AI) or endocrine therapy, such as fulvestrant [11, 141].

Cell death

Overview

In multi-cellular organisms, there is a requirement to remove cells through cell death in order to maintain the homeostasis between newly generated cells and cells that are either damaged or unwanted [142]. This process occurs as early as during development where, for example, fingers and toes are shaped from webbed limbs [143]. Another example is the development of cells within the adaptive immune system. Here cells with non-functional receptors for antigens and cells that strongly react with self-proteins are eliminated via cell death [144-146]. Thus, cell death can occur in various regulated situations. However, it can also occur accidentally, such as upon severe physical-, chemical-, or mechanical insults. This differs from regulated cell death which is controlled by a genetically encoded molecular machinery and takes part during tissue homeostasis and development [147].

In the course of tumor development, cell death may be stimulated to counteract some acquired features of the cancer cell, such as enhanced proliferation, or to deal with signaling imbalances due to increased oncogenic signaling. Enhanced proliferation can also result in DNA damage, which in turn may trigger cell death. However, it has been found that established tumor cells have acquired resistance to cell death [6, 7]. Thus, activation of cell death in cancer cells has emerged as a potential strategy to treat cancer. There are at least 11 different types of regulated cell death [148, 149]. One of them is apoptosis, which will be the main focus in this thesis.

Apoptosis

In 1972, the term “apoptosis” was used for the first time to describe controlled cell death with morphologically distinct features, such as cell shrinkage, nuclear fragmentation, and cytoplasmic and chromatin condensation. It results in extensive membrane blebbing, cellular fragmentation, and the release of spherical fragments called apoptotic bodies. The apoptotic bodies and the cellular fragments can in turn be engulfed by other cells and subsequently be degraded [142, 150, 151]. The dying cell will maintain the plasma membrane intact until it is obliterated [150], which contrasts the process of necrotic cell death. Necrosis involves cellular leakage and

swelling of the organelles and the cell (also called oncosis), resulting in damage of the surrounding tissue [142].

The morphological classifications of cell death are still used, but they are accompanied by some disadvantages. For example, the presence of a morphological feature does not explain the causative process. In addition, there are several subtypes of apoptosis which are morphologically similar to each other but are associated with different biochemical events, such as caspase cleavage and activation, phosphatidylserine exposure (which can be both caspase-dependent and –independent [152, 153]), and generation of reactive oxygen species (ROS). Moreover, different types of cell death can result in various immune responses. Apoptosis generally does not induce any immune reaction following engulfment of apoptotic bodies, but there are exceptions. For instance, an immune response has been observed during apoptosis during influenza A viral infection [154]. Necrosis and pyroptosis are two types of cell death which can provoke an immune response in which, for example, cytokines are released [148, 149, 155-157]. Therefore, biochemical methods have become more common to use when classifying cell death.

Based on biochemical events, apoptosis can be divided into the extrinsic and intrinsic pathway, which are brought together at the execution phase. Here the executioner caspases (caspase-3, -7, and -6) are cleaved and activated [149, 158] and can in turn cleave and activate different substrates, such as PARP, cytoplasmic endonuclease, gelsolin, ICAD, and proteases. This cause degradation of nuclear and cytoskeletal proteins, and lead to the characteristic morphology and biochemistry of apoptotic cells [151, 159-161]. Finally, phosphatidylserine is externalized which enables recognition by phagocytes and a non-inflammatory phagocytosis of the apoptotic cells [162, 163].

Intrinsic apoptosis

The intrinsic pathway is initiated upon different stimuli such as imbalance of growth factors, hormones or cytokines, or upon DNA damage, irradiation, free radicals, hyperthermia, or viral infection. All these stimuli can cause changes of the mitochondria [151] and lead to mitochondrial outer membrane permeabilization (MOMP), which releases cytochrome c into the cytosol [164, 165]. This event is controlled by the regulation of pro- and anti-apoptotic proteins of the Bcl-2 family. The pro-apoptotic proteins can be divided into multidomain effectors, which include Bax and Bak, and BH3-only proteins, which include Bim, Puma, Noxa, Bad, and Bid [166]. Bax and Bak can form the pores in the outer mitochondrial membrane causing the permeabilization of the membrane [167, 168]. The anti-apoptotic members of the Bcl2-family, such as Bcl-2 and Bcl-$_{XL}$, inhibit Bax and Bak through heterodimerization and thus suppress induction of apoptosis [169, 170]. The BH3-only proteins can facilitate apoptosis either by binding to and neutralizing the anti-apoptotic proteins or, as in the case for Bid following cleavage by caspase-8, directly

promote the pore formation by Bax and Bak [171-173]. The BH3-only proteins depend on the presence of Bax and Bak to promote apoptosis [173].

Once cytochrome c is released from the mitochondria into the cytoplasm, it mediates an ATP-dependent activation and oligomerization of apoptosis protease activating factor 1 (Apaf-1), which thereafter binds to and activate procaspase-9, and together they form the apoptosome (Figure 3) [164, 174]. Active caspase-9 can cleave and activate the executioner caspase-3 [175]. But a member of the inhibitor of apoptosis protein (IAP) family, X-linked IAP (XIAP), can inactivate caspase-9 by binding to its homodimerization surface and prevent activation of the executioner caspases by blocking their substrate cleft [176, 177]. Other proteins being released from the mitochondria are second mitochondria-derived activator of caspase (Smac), which can inhibit IAPs and thus facilitate apoptosis [178], and HtrA2, which contributes to apoptosis through either caspase-dependent or –independent mechanisms [179].

Extrinsic apoptosis

Initiation of the extrinsic pathway begins with binding of a ligand to its cognate transmembrane death receptor (DR) on the cellular membrane. The ligand FasL binds to Fas, tumor necrosis factor-α (TNF-α) binds to TNF receptor 1 and 2 (TNFR1 and TNFR2), and TNF-related apoptosis-inducing ligand (TRAIL) recognizes DR4 and DR5, all of which are examples of death receptors which can initiate a pro-apoptotic signaling cascade [180]. The death receptors consist of a cysteine-rich extracellular domain and a cytoplasmic death domain (DD) [181]. Upon ligation the receptor will trimerize to form signaling platforms, a process called capping [182-185]. This can be followed by recruitment of adaptor proteins and subsequent activation of procaspase-8 and/or -10 through their oligomerization and auto-catalytic activation [186, 187]. Caspase-8 can be negatively regulated by the protein c-FLIP (cellular FLICE-like inhibitory protein) [188-190]. Active caspase-8 and -10 can cleave and activate the executioner caspases-3, -6, and -7. However, caspase-8 may also cleave Bid to generate a truncated form (tBid), which in turn will cause the release of mitochondrial cytochrome c into the cytosol [171, 172, 191]. This describes two types of cells: type I and type II cells. For type I cells, death-induction relies solely on the extrinsic pathway and does not involve the mitochondria, whereas the intrinsic pathway, where Bid is an intermediate, is involved in type II cells [192].

In addition to death receptors mediating apoptosis, there are a number of decoy receptors which compete with the death receptors for binding of the ligand. They are not structurally capable of transmitting the signal, which is why ligand binding to decoy receptors results in a decreased activation of pro-apoptotic signaling [193]. There are several different decoy receptors belonging to the TNFR family. Decoy receptor 1 (DcR1) and DcR2, which bind TRAIL ligand, have both been found to be expressed in multiple normal tissues to a greater extent than in cancer cell lines

[194]. There are different reports of whether or not they are involved in TRAIL resistance [195-199]. DcR3, to which FasL binds, is a secreted decoy receptor whose gene was in one study reported to be amplified in at least half of the primary lung and colon tumors studied [200].

TNFR signaling pathways

Beside activation of the extrinsic pathway, death receptors can induce several other signaling pathways. Signaling through the TNF receptors TNFR1 and TNFR2 is initiated upon binding of its ligand TNF-α, which initiates a trimerization of the receptors [201, 202]. The two receptors differ from one another since TNFR2 does not harbor a death domain (DD) and is therefore unable to transmit apoptotic signaling. However, it can stimulate activation of the nuclear factor-κB (NF-κB) signaling pathway [203, 204], and activate c-Jun N-terminal kinase (JNK) [205]. TNFR1 contains a DD and can thereby trigger apoptosis but most often it activates the NF-κB signaling pathway [206, 207]. Following ligand binding, the adaptor molecule TNFR-associated death domain protein (TRADD) and receptor-interacting protein kinase 1 (RIP1/RIPK1) can bind to the DD of the receptor (Figure 2) [206-208]. This is followed by association of TNFR-associated factor 2 (TRAF2) together with the E3 ligases of the IAP family, cellular IAP1 (cIAP1) and cIAP2 [209]. cIAP1/2 have the potential to polyubiquitinate RIP1, via K63-linked ubiquitin chains [210], to enable a binding site for another E3 ligase, namely linear ubiquitin chain assembly complex (LUBAC), consisting of HOIP, HOIL-1, and SHARPIN [211]. LUBAC can further modify RIP1 by forming M1-linked ubiquitin chains which enables recruitment of transforming growth factor-β-activated kinase 1 (TAK1) and the IκB kinase (IKK) complex, consisting of NF-κB essential modulator (NEMO), inhibitor of NF-κB kinase subunit α (IKKα), and IKKβ, to RIP1 [206, 212, 213]. This will lead to activation of the canonical NF-κB pathway since TAK1 can phosphorylate the IKK complex [206, 214]. The complex formed, consisting of TRADD, RIP1, TRAF2, cIAP1/2, and LUBAC, is termed Complex I and stimulates the NF-κB signaling pathway [206].

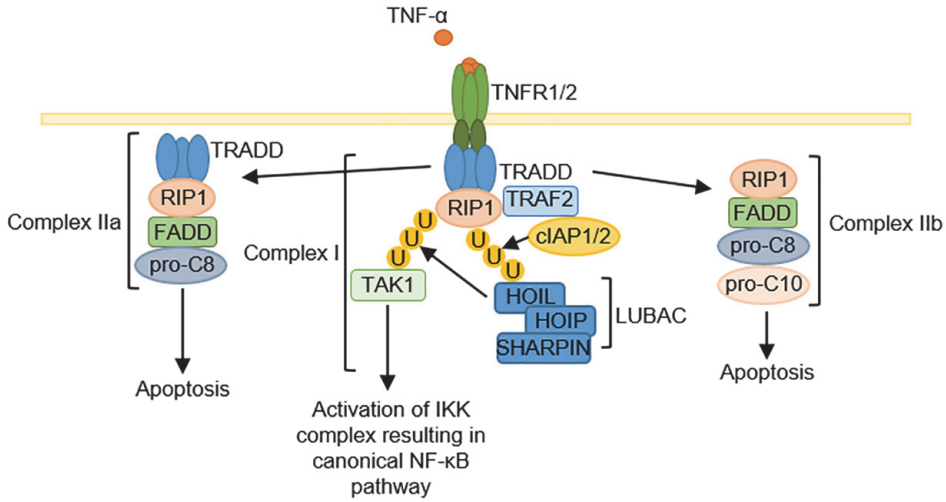

Figure 2. TNFR1/2 signaling pathway
TNF-α binds to its receptor TNFR1/2, and thereafter TRADD and RIP1 are recruited, which is followed by association of TRAF2 and cIAP1/2. Through their E3 ligase activity, cIAP1/2 can ubiquitinate RIP1, which results in binding of LUBAC, consisting of HOIP, HOIL, and SHARPIN. LUBAC can then add M1-linked ubiquitinin chains to RIP1, causing recruitment of TAK1 and the IKK complex. Subsequently, TAK1 phosphorylates the IKK complex, leading to activation of the NF-κB pathway. The complex formed is called Complex I. The soluble Complex IIa cannot be formed until stimulation of the anti-apoptotic NF-κB signaling ceases. Then TRADD, RIP1, FADD, and procaspase-8 can associate and induce apoptosis. Complex IIb (or the ripoptosome) can be formed following depletion of IAPs (XIAP and cIAP1/2) or inhibition of c-FLIP. It consists of RIP1, FADD, procaspase-8/-10, and can stimulate RIP1-dependent apoptosis.

As long as Complex I is stimulated and continues to induce anti-apoptotic NF-κB signaling, the soluble cytoplasmic variants of Complex I, called Complex IIa/b, are blocked. However, following removal of K63- and M1-polyubiquitin chains of RIP1 by CYLD, which is induced by NF-κB, the NF-κB signaling is inhibited. This results in dissociation of TRADD and RIP1 and subsequent formation of Complex IIa [215]. Complex IIa, consisting of TRADD, RIP1, Fas associated via death domain (FADD), and procaspase-8, can trigger apoptosis [216, 217]. Complex IIb (also known as the ripoptosome) is composed by RIP1, FADD, procaspase-8/-10, and is formed upon depletion of XIAP and cIAP1/2, following genotoxic stress, after treatment with Smac mimetics, which leads to either reduced or abolished K63-linked ubiquitination of RIP1 [215], or when c-FLIP is inhibited. Signaling through Complex IIb can stimulate RIP1-dependent apoptosis [218-220].

TRAIL signaling pathways

There are two TRAIL receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and both harbors DDs, making them able to induce apoptosis upon binding of their ligand TRAIL [221]. DR4 and DR5 are expressed in most human tissues [194, 222]. However, their expression can vary within a tissue and between cell types which

may affect through which receptor the apoptotic signaling is transmitted following ligand binding [223]. Upon homotrimeric ligand binding, the DR4/5 forms homotrimers (Figure 3) [224]. This results in recruitment of the death-inducing signaling complex (DISC) components FADD and procaspase-8, which can cause activation of caspase-8 and -10. This is followed by their subsequent activation of the executioner caspases, leading to apoptosis [223]. In addition to cell death, the NF-κB pathway has been shown to be induced upon ligation of DR4 and DR5 and subsequent recruitment of TRADD and FADD [225]. Furthermore, when investigating treatment with TRAIL in combination with Smac mimetics, a cytosolic complex consisting of RIP1, FADD, and procaspase-8 was identified, which can initiate the apoptotic cascade [226-228].

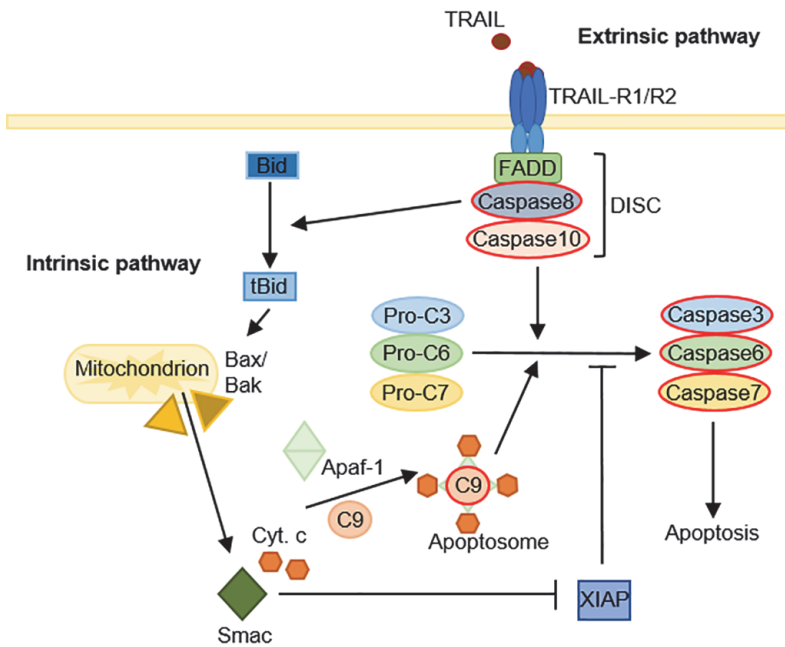

Figure 3. TRAIL signaling in the extrinsic and intrinsic apoptotic pathways
Binding of TRAIL to its receptor TRAIL-R1 (DR4) or TRAIL-R2 (DR5) results in association and formation of DISC, consisting of FADD, caspase-8, and -10. DISC can induce apoptosis through the extrinsic pathway by cleaving and activating the executioner caspases-3, -6, and -7. The intrinsic pathway is initiated upon permeabilization of the mitochondria. Here Bax and Bak causes the release of cytochrome c and Smac into the cytosol. Cytochrome c will bind to Apaf-1 and procaspase-9, inducing activation of the latter. This results in the formation of the apoptosome, which can cleave and activate the executioner caspases. Smac acts by inhibiting XIAP and can thereby facilitate induction of apoptosis. Caspase-8 can trigger the intrinsic pathway in some cells by cleaving Bid to tBid, which then may activate Bax and Bak. This is followed by the translocation of Bax/Bak to the mitochondrial membrane which they can permeabilize.

In addition to apoptosis, TRAIL signaling can induce production of inflammatory cytokines and chemokines, which can be mediated via either NF-κB [229], or FADD and caspase-8 [230, 231]. Together with caspase-8, FADD constitutes a cytosolic complex, called the FADDosome, in which caspase-8 is described to act as a scaffold protein, without utilizing any enzymatic activity. TRAIL has also been found to induce expression of interferon-β (IFN-β) and IFN-regulated genes [232].

Due to the ability of TRAIL to preferentially stimulate apoptosis in cancer cells over normal cells [233], it is a good candidate for anti-cancer therapy. This is thought to be a result of a more abundant expression of DcR1 and DcR2 in normal tissues than in tumor tissues [181, 194, 199]. However, some tumor types have been found to be resistant to TRAIL treatment, which could be mediated via different mechanisms. For example, insufficient transport of the receptors to the cell surface from the endoplasmic reticulum following their translation was reported to cause TRAIL-resistance in colon cancer cell lines [234]. In breast cancer cell lines, endocytosis of DR4 and DR5 leading to decreased surface expression has been shown as a desensitizing mechanism [235]. Mutations or deletions resulting in loss of both copies of the TRAIL receptors in some cancer cases, e.g. breast cancer and non-small cell lung cancer, could also result in TRAIL resistance [236]. In addition to effects at the receptor level, enhanced expression of the caspase-8 inhibitor c-FLIP, as well as inactivation of caspase-8, have been suggested to cause TRAIL resistance in cancer [237].

Caspases

The conserved enzyme family of caspases (cysteinyl aspartate proteinases) have well-defined roles in apoptosis and inflammation [238]. As the name implies, caspases are cysteine proteases which cleave their substrates at a site following an aspartate residue [239]. There are ten different caspases in humans which are categorized into initiator (caspase-2, -8, -9, and -10), executioner (caspase-3, -6, and -7), and inflammatory caspases (caspase-1, -4, and -5) [240]. In their inactive state, caspases exist as caspase zymogens (also called procaspases), and possess the ability to activate other procaspases once activated. However, some procaspases, such as the initiator caspases-8, -10, and -9, are also able to aggregate and autoactivate, [241-243]. Procaspases undergo proteolytic cleavage where a prodomain is removed and two subunits, one large and one small, are generated, which in turn comprise the active enzyme (Figure 4) [244, 245]. In addition to a catalytic domain, the initiator and inflammatory caspases also consist of protein-protein interaction domains: either a caspase activation and recruitment domain (CARD) or two death effector domain (DED), which are important for their activation as they provide for interaction with adaptor molecules [246].

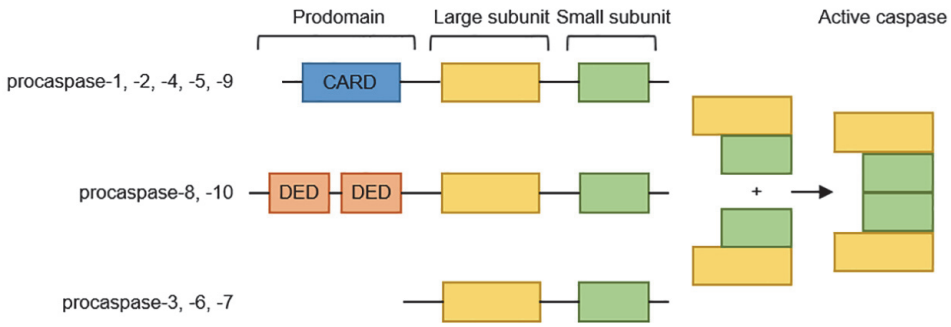

Figure 4. Structure of procaspases and active caspases
The initiator procaspases (2, 8, 9, and 10) and the inflammatory procaspases (1, 4, and 5) contain either a CARD or two DED prodomains. Upon activation the prodomains, as well as the linker regions between the domains, are cleaved. This causes the large and small units to dimerize before they associate with another dimer, resulting in an active caspase.

The inflammatory caspases can activate cytokines in response to infection [247, 248] and mediate inflammatory cell death through pyroptosis, an infection-initiated cell death associated with water influx, cell swelling, and release of proinflammatory contents [249]. The initiator and executioner caspases have important roles in the apoptotic cascade [239], but some of them also possess the ability to induce other types of cell death than apoptosis. For example caspase-3 can induce cell death through pyroptosis via ROCK1 [250]. In addition to being mediators of different cell death signaling pathways, caspases have been shown to be involved in neural development and disease [251], cellular proliferation and differentiation [252], cytoskeleton rearrangement [253], erythroid differentiation [254], T cell development [255, 256], and spermatogenesis [257]. Especially caspase-8 is involved in several different cellular mechanisms. It can inhibit necroptosis, a controlled and inflammatory form of cell death, through cleavage of RIP1 and RIP3 [258], regulate proinflammatory cytokine release in response to activation of the inflammasome [259] and stimulation of toll-like receptor 3 (TLR3) and TLR4 [260], and act as a scaffold in inflammasome activation in response to viral RNA [261].

IAPs and Smac mimetics

The IAPs (inhibitor of apoptosis proteins) can regulate both the extrinsic and the intrinsic apoptotic pathways [262]. There are eight different IAPs in humans: XIAP, cIAP1, cIAP2, ILP-2, ML-IAP, NAIP, survivin, and apollon. They all contain at least one baculovirus IAP repeat (BIR) domain [263].

XIAP inhibits the activation of caspase-3, -7, and -9, an effect that is mediated by its three BIR domains (BIR1-3) [177, 264]. The linker region between BIR1 and BIR2 binds to and inhibits active caspase-3 and -7 [265-268], whereas binding of BIR3 prevents activation of caspase-9 by inhibiting its homodimerization [269, 270]. In addition, XIAP can inhibit the activation of the ripoptosome [218].

cIAP1/2, which possess E3 ubiquitin ligase activity [271], are important regulators of the NF-κB signaling pathways. In the canonical NF-κB pathway, cIAP1/2 act as positive regulators by promoting ubiquitination of RIP1, which subsequently results in activation of the pathway [210, 272-274]. However, cIAP1/2 blocks the non-canonical NF-κB pathway by mediating a degradative ubiquitination of NF-κB-inducing kinase (NIK), a protein kinase that initiates the non-canonical pathway [272, 275]. There are also indications that activation of JNK and p38 MAPK signaling pathways can be dependent on the activity of cIAP1 and cIAP2 [276]. Furthermore, cIAP1 and cIAP2 have been shown to inhibit the activation of the ripoptosome [218] and block Smac from inhibiting XIAP [277].

ILP-2 can inhibit apoptosis initiated through the intrinsic pathway as it binds to and inhibit caspase-9 [278], ML-IAP can inhibit Smac's ability to block XIAP [279], and NAIP inhibits the executioner caspases [280]. The role of survivin is somewhat wider as it, in addition to caspase-9 inhibition [281], also has a role in in cell division [282]. Apollon, finally, inhibits Smac-induced apoptosis by ubiquitinating Smac and caspase-9 for proteasomal degradation [283].

Many IAPs are overexpressed in several cancer types and they have been associated with tumor progression, poor prognosis, and decreased treatment efficacy [284-287]. This is exemplified by reduced sensitivity to chemotherapy in different types of lung and gastric cancer cells, and to TRAIL treatment in breast cancer cells and pancreatic carcinoma cells [288-293]. Nuclear expression of XIAP was found to be associated with poorer survival in breast cancer [294]. Higher expression of XIAP has also been correlated with a higher tumor grade [295], as well as larger tumor size and impaired overall survival [296], whereas downregulation of XIAP in triple negative and inflammatory breast cancer cells was associated with increased TRAIL-sensitivity [297-300]. One study has found that patients with basal-like breast cancer had higher levels of cytoplasmic XIAP, which correlated with increased risk of relapse [301]. The same study also showed that breast cancer tissues had higher expression of cytoplasmic XIAP than normal tissues. Smac has been shown to have a negative correlation with breast cancer tumor size [302]. Due to the mechanisms of action of the IAPs, they are potential targets for cancer therapies.

Smac mimetics are small molecule peptide-like mimetics of Smac and were developed to mimic the functions of Smac, which is to inhibit some of the IAPs, [285, 303]. Specifically, Smac mimetics mimic the tetrapeptide of the N-terminus, which binds to the BIR domains of cIAP1/2 and XIAP [304, 305]. Eight different

Smac mimetics have so far been tested in humans [285]. Some Smac mimetics are bivalent consisting of two monovalent units, which are connected via a chemical linker [306]. Smac mimetics can potentiate apoptosis both *in vitro* and *in vivo* [288, 307-310]. LCL161, which was used in the present investigation, is a monovalent Smac mimetic. It was developed by Novartis Pharmaceuticals as a structural analogue of another Smac mimetic (LBW242) and have progressed into clinical trials for myeloma and some solid tumors including, breast, lung, and colon [311-316]. The mechanism of action of LCL161 is to inhibit XIAP [317] and to bind to the BIR3 domains of cIAP1 and cIAP2, thereby causing their autoubiquitination and proteasomal degradation [318]. The result of this is activation of the non-canonical NF-κB pathway, which in some cells is followed by induction of TNF-α, an NF-κB target gene [318, 319]. TNF-α can act in an autocrine manner and activate NF-κB as well as lead to caspase-8-dependent cell death in many tumor cells. By inhibiting TNF-α, caspase-8, or NF-κB-activated transcription it is possible to prevent apoptosis mediated by Smac mimetics [275, 320, 321]. However, it is not always that induction of TNF-α is sufficient to induce cell death. Chronic lymphocytic leukemia (CLL) cells are resistant to Smac mimetics, despite production of TNF-α, which was shown to be due to an inability to form the ripoptosome [322]. Generally, the efficacy of Smac mimetics as a single agent has not been especially promising and they are therefore more commonly examined in the setting of a combination treatment [285, 323-325]. Smac mimetics in combination with TRAIL receptor agonists have been extensively studied in several cancer types, including breast-, bladder-, and pancreas cancer, glioblastoma, and multiple myeloma, where they were found to potentiate TRAIL-induced apoptosis [307, 326-329].

NF-κB signaling pathways

Overview

There are five inducible transcription factors belonging to the nuclear factor-κB (NF-κB) family, called NF-κB/Rel proteins. They can all form either homo- or heterodimers. These transcription factors take part in either the canonical or the non-canonical NF-κB pathway, where they bind to promoters of their target genes. This induces gene transcription and synthesis of proteins that are involved in several different processes, such as inflammatory response, cell proliferation, and differentiation [330].

Canonical NF-κB signaling pathway

Induction of the canonical NF-κB pathway may begin following signaling via different receptors, such as the TNFR, TLR, T cell receptor (TCR), and interleukin-1 receptor (IL-1R) [331]. Depending on the receptor, different adaptor molecules bind following ligation. All receptors have in common that they activate the IKK complex, via activation of TAK1 [332].

Upon TNFR1 stimulation, TRADD, RIP1, cIAP1/2, and LUBAC are recruited to the receptor. This is described in more detail under "TNFR signaling pathway". LUBAC ubiquitinates RIP1, which facilitates recruitment of TAK1, resulting in the subsequent recruitment of the IKK complex (Figure 5) [206, 212, 213]. The IKK complex consists of inhibitor of NF-κB kinase subunit α (IKKα), IKKβ, and IKKγ (also called NEMO), of which the two former are kinases whereas the latter is a nonenzymatic regulatory component [333]. Following its recruitment to TAK1 the IKK complex is either trans-autophosphorylated or phosphorylated by TAK1 [334, 335]. The IKK complex can thereafter mediate phosphorylation of the inhibitory IκBα, resulting in its subsequent polyubiquitination and proteasomal degradation [336]. The heterodimer RelA and p50, which is produced from its precursor p105 [337], will thereafter be free to translocate to the nucleus and activate transcription of NF-κB target genes [338-343].

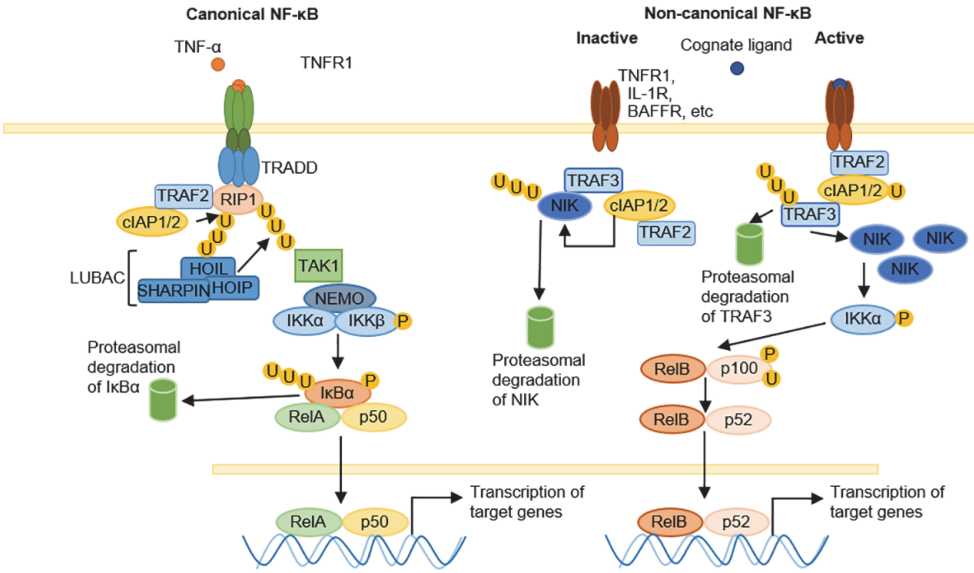

Figure 5. Canonical and non-canonical NF-κB pathway
The canonical NF-κB pathway is initiated upon ligand binding to its receptor, for example TFN-α to TNFR1 as shown in this figure. This leads to recruitment of TRADD, RIP1 and TRAF2, to which cIAP1/2 associates and thereafter ubiquitinate RIP1. This enables recruitment of LUBAC leading to further ubiquitination of RIP1, which thereby can bind TAK1 and the IKK complex, consisting of NEMO, IKKα, and IKKβ. This is followed by phosphorylation of the IKK complex by either TAK1 or through trans-autophosphorylation. Subsequently, the IKK complex can phosphorylate IκBα, which leads to its proteasomal degradation. RelA and p50 thus dissociates from IκBα and can translocate to the nucleus and act as transcription factors. In unstimulated cells, the non-canonical NF-κB is inactive as NIK is constantly degraded by the TRAF3-TRAF2-cIAP1/2 complex. However, upon stimulation of a receptor, for example TNFR1, IL-1R, or BAFFR, TRAF2 and TRAF3 assemble with the receptor, to which cIAP1/2 are recruited. They will in turn ubiquitinate TRAF3, causing its proteasomal degradation, after which NIK can accumulate and phosphorylate IKKα. This will lead to processing of p100 into p52, which will act as a transcription factor together with RelB.

Non-canonical NF-κB signaling pathway

The non-canonical NF-κB pathway is generally initiated by NF-κB-inducing kinase (NIK). In unstimulated cells, NIK is constantly degraded and the non-canonical NF-κB pathway is therefore not active (Figure 5). Newly synthesized NIK is rapidly bound to TRAF3 [344], which in turn binds to TRAF2. Together, TRAF2, TRAF3, and NIK form a complex with cIAP1/2, where TRAF2 binds to cIAP1/2 [345]. NIK will then be ubiquitinated by cIAP1/2, causing its proteasomal degradation [275, 345]. The importance of cIAP1/2 in this context is supported by the finding that treatment with Smac mimetics, which induce cIAP1/2 degradation, leads to NIK accumulation [275]. The role of TRAF3 in NIK degradation has been shown using TRAF3-deficient cells in which there is an accumulation of NIK [346].

Signaling through the non-canonical NF-κB pathway can be induced upon binding of TNF-α, IL-1, lipopolysaccharide (LPS), or double-stranded RNA (dsRNA), to their cognate receptors TNFR, IL-1R, TLR, BAFFR (B cell-activating factor receptor), LTβR (lymphotoxin β receptor), or CD40 (cluster of differentiation 40) [347-351]. This results in recruitment of the NIK-degrading complex consisting of TRAF2, TRAF3 and cIAP1/2 to the receptor, which is followed by ubiquitination and subsequent degradation of TRAF3 by cIAP1/2 [345]. This causes accumulation of NIK which phosphorylates and activates IKKα [344, 352, 353]. In contrast to the canonical NF-κB pathway, where IKKα, IKKβ, and IKKγ (NEMO) are necessary, the non-canonical NF-κB pathway is solely dependent on phosphorylation of IKKα and its subsequent phosphorylation of p100, leading to its processing into p52 [348, 352, 354, 355]. Together with RelB, p52 forms a heterodimer, which translocates to the nucleus and acts as a transcription factor [356, 357].

There are two main hypotheses explaining how activation of the non-canonical NF-κB is controlled. One theory is that only sub-optimal activity of cIAP1/2 is required to ubiquitinate NIK, causing its degradation, whereas the degradation of TRAF3, induced following receptor stimulation, requires stronger activities of cIAP1/2 [358, 359]. The other hypothesis is that TRAF3 degradation involves receptor internalization through the lysosomal pathway [359, 360].

There is an upstream crosstalk between the canonical and non-canonical NF-κB pathways, which is mediated by RIP1. Here, RIP1 suppresses TNFR1-mediated activation of the non-canonical NF-κB [351, 361], as it inhibits degradation of TRAF2 and cIAP1, in a kinase-independent manner [361]. On the contrary, upon depletion of RIP1, TRAF2 and cIAP1 are degraded, leading to activation of the non-canonical NF-κB pathway [361]. Another type of crosstalk between the two pathways involves NIK, which was shown to have an important role in activation of the canonical NF-κB signaling following CD40 and CD27 stimulation [362].

Interferons

Overview

Interferons were first described by Isaacs and Lindenmann in 1957 as they studied virus interference [363]. They discovered the release of a new factor with the ability to protect against viral infection, which they named interferon (IFN). In addition to protect against infections, IFNs have also been shown to be associated with autoimmune diseases, for example systemic lupus erythematosus (SLE), systemic sclerosis, and some cases of rheumatoid arthritis [364, 365]. Since their discovery, several IFNs have been identified and they are divided into three groups: type I, II, and III IFNs.

Type I interferons

The type I IFNs include IFN-α, which in humans exists as 13 homologous subtypes [366], IFN-β, IFN-δ, IFN-ε, IFN-ζ, IFN-κ, IFN-ν, IFN-τ, and IFN-ω. However, only IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω exist in humans [367]. Their genes are located on chromosome 9 [368]. Type I IFNs are produced upon viral and some bacterial infections where they have numerous effects on anti-pathogen immunity via their induction of IFN-stimulated genes (ISGs), which restrict pathogenic spread. In addition, type I IFNs can stimulate production of type II IFN by affecting macrophages [369]. However, they are not always protective against infections. A type I IFN response can result in impaired bacterial clearance for some infections [370, 371]. In addition to their role in infections, type I IFNs have been shown to induce cellular damage, inflammation, apoptosis, autophagy, migration and differentiation, and inhibit angiogenesis and proliferation [372].

Interferon-α

IFN-α is produced by plasmacytoid dendritic cells (pDCs) and leukocytes [373]. Depending on the IFN-α subtype, its binding can result in different signaling outcomes, which is thought to be due to differences in receptor-binding affinities [374]. However, one study showed that there are differences in IFN-α/β receptor

(IFNAR) expression, and that abundance of IFNAR on specific target cells can compensate for a weaker affinity [375].

Interferon-β

IFN-β is primarily produced by DCs, epithelial cells, and fibroblasts [373, 376], and is induced in response to viral infection [373]. IFN-β has been shown to induce an anti-proliferative response in some cancer types, such as glioma, retinoblastoma, and hepatocellular carcinoma [377-380]. Therefore, IFN-β can potentially be used as anti-cancer treatment [381, 382].

Type II interferons

There is only one type II IFN, namely IFN-γ, and it is structurally different from the type I IFNs [383, 384]. The active form of IFN-γ is a dimer [385], which can be glycosylated. Glycosylation causes its existence in several different isoforms, but it is not necessary for its dimerization [386, 387]. IFN-γ is produced by immune cells, such as T cells and natural killer (NK) cells, but its receptor is expressed on multiple cell types and therefore it has a broad effect [388]. Like the other IFN types, IFN-γ is an important factor of the immune system in the response to infections. More specifically, it mediates activation of macrophages and affects T cell differentiation, which ultimately contribute to clearance of pathogens that reside intracellularly [388-390].

Type III interferons

This is the most recently identified group of IFNs and comprises of IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4 [391-394]. Their functions are similar to the type I IFNs [367, 395], but they have a limited activity since their receptors more or less are restricted to the surface of mucosal epithelial cells [396, 397].

Interferons and their receptors

Hundreds of genes are induced upon IFN signaling, some of which are regulated by specific IFNs and others by both type I and type II IFNs. For example, the expression of IFN-induced transmembrane protein 1 (*IFITM1*) and signal transducer and activator of transcription 1 (*STAT1*) can be induced by all IFNs, but *OAS1* (2′-5′-

oligoadenylate synthase 1) is induced by IFN-α and –β and not by IFN-γ. On the other hand, IFN-γ is the only IFN that can induce *IRF1* (IFN-regulatory factor 1) [398].

To enable induction of IFN-regulated genes, the IFNs must bind to their specific transmembrane receptors on the cell surface [399].

- The type I IFN receptor is composed of two components, IFNAR1 and IFNAR2, which together can recognize all type I IFNs [400]. It is assembled 1:1:1 (IFN-α/β/IFNAR1/IFNAR2) [401]. Since the type I IFN receptor does not harbor any intrinsic kinase activity it relies on association with Janus kinases. IFNAR1 is constitutively associated with Tyrosine kinase 2 (TYK2) and IFNAR2 with Janus kinase 1 (JAK1) [402, 403]. Following ligand binding TYK2 and JAK1 form a signaling unit, which activates TYK2 and JAK1 and in turn causes them to phosphorylate IFNAR1 and IFNAR2 [404].
- The type II IFN receptor is composed of IFN-gamma receptor 1 (IFNGR1) and IFNGR2 and the ligand-receptor complex assembles in a 1:2:2 IFN-γ/IFNGR1/IFNGR2 relation [401]. Similar to IFNAR1 and IFNAR2, IFNGR1 and IFNGR2 do not possess any intrinsic kinase activity, but they contain binding motifs for JAK1 and JAK2, which phosphorylate the receptor upon its ligation [390, 405, 406].
- The type III IFN receptor is composed of IFN lambda receptor 1 (IFNLR1 or IL28RA) and interleukin-10 receptor subunit beta (IL-10RB or IL10R2) [391], and assembles in a similar way as the type I IFN receptors, namely 1:1:1 [401]. Ligation results in activation of JAK1 and TYK2, both of which are associated with the receptor complex [407].

Upon binding of an IFN-ligand to its cognate receptor, the receptor-associated Janus kinases will become activated through trans- and autophosphorylation [390, 408] and subsequently phosphorylate the receptor tyrosine kinase residues of the IFN receptor. This allows for recruitment of STAT proteins, via their src homology 2 (SH2) domains. In unstimulated cells, STAT proteins are present in the cytosol as inactive monomers [409, 410]. Type I IFN signaling can lead to phosphorylation of several STAT proteins, such as STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6 [411, 412]. However, type II IFN signaling only involves STAT1 and STAT3, with STAT1 binding with higher affinity to the receptor. This results in a stronger and more persistent phosphorylation of STAT1 as compared to STAT3, and the STAT1 homodimer is therefore the most common STAT dimer in type II IFN signaling [413, 414]. In type III IFN signaling, STAT1 and STAT2 are involved [412].

Once bound to the receptor, STATs are phosphorylated, which enables their dissociation from the receptor as either homo- or heterodimers [408]. Phosphorylation occurs at Tyr701 of STAT1 [415]; at Tyr690 of STAT2 [416]; and at Tyr705 of STAT3 [417]. In addition to tyrosine phosphorylation, STAT1 and STAT3 are also

phosphorylated at Ser727 following IFN signaling, which is essential for full transcriptional activation but not required for nuclear translocation and binding to ISG promoters [418-420]. STAT2, on the other hand, can be phosphorylated at Ser287, which leads to negative regulation of type I IFN signaling [421].

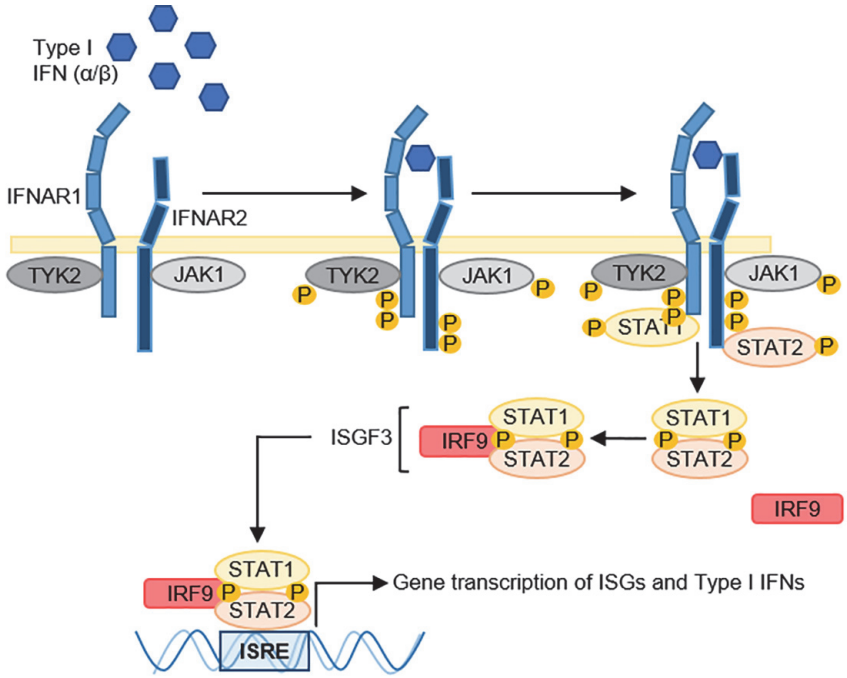

Figure 6. Type I IFN signaling
Type I IFN-α/β binds to its receptor, consisting of IFNAR1 and IFNAR2, to which TYK2 and JAK1 are bound. Upon ligation, JAK1 and TYK2 become phosphorylated and can phosphorylate the receptor subunits. This allows binding of STAT1 and STAT2, through their SH2 domains, resulting in their phosphorylation and subsequent dissociation from the receptor. Following their dissociation, they form a heterodimer which binds IRF9. Together they form the IFN-stimulated gene factor 3 (ISGF3), a transcription factor which binds to IFN-stimulated response elements (ISREs) to induce transcription of IFN-stimulated genes (ISGs) and type I IFNs.

In response to type I IFN signaling (Figure 6), STAT1 and STAT2 form a heterodimer which associates with IFN regulating factor 9 (IRF9). Together they form the transcription factor IFN-stimulated gene factor 3 (ISGF3) [408, 422, 423]. ISGF3 translocates to the nucleus where it interacts with co-activators which regulate transcription, such as p300 and CBP (cAMP-response element binding protein (CREB)-binding protein), [424, 425]. This complex binds to IFN-stimulated response elements (ISREs) of ISG promoter regions [408, 426]. In addition to inducing transcription of ISGs, this can also results in induction of IFN-β, which

can act through an auto- or paracrine loop to further enhance IFN-β signaling and synthesis [427-429].

Following type II IFN ligation, phosphorylated STAT1 is able to form a homodimer, which can bind to gamma-activated sequences (GAS) element in the promoter region of certain ISGs [430]. Stimulation of the type III IFN receptor activates the same JAK-STAT signaling pathway as mediated by type I IFN signaling [384, 431, 432]. Hence, STAT1 has a central role in the response to type I, II, and III IFN [433-435]. The essential role of STAT1 is exemplified by the finding that STAT1-deficient mice are unable to respond to type I and type II IFN signaling and are more sensitive to infections than wild-type mice [434]. JAK-STAT signaling declines upon dephosphorylation, nuclear export, or suppressor of cytokine signaling (SOCS) feedback inhibition [436-438].

Induction of type I interferon signaling

Type I IFNs are induced upon recognition of different pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), such as viral or bacterial double/single-stranded (ds/ss) RNA or DNA, cytosolic genomic DNA (cgDNA), or lipopolysaccharide (LPS) (Table 2). The different PAMPs/DAMPs associate with pattern recognition receptors (PRRs), in particular with members of the toll-like receptor (TLR) family. TLR4 is located on the cell surface, where it recognizes LPS [439, 440]. TLR3, -7, and -9 are localized on the cell surface and on the endosomal membrane and are activated upon ligation with viral dsRNA, ssRNA, and unmethylated cytosine-phosphate-guanine (CpG) DNA [439, 441, 442]. TLR8 is only expressed on the endosomal membrane and recognizes ssRNA [439]. Stimulation of these receptors ultimately results in activation of the protein kinases TANK-binding kinase 1 (TBK1) and IKKε (Figure 7) [443-448].

Following LPS stimulation of TLR4 the receptor oligomerizes, which is enabled by its association with MDA-2 [449]. Thereafter, translocating chain-associated membrane protein (TRAM) and TIR-domain-containing adaptor-inducing IFN-β (TRIF) bind to the receptor [450, 451]. NAK-associated protein 1 (NAP1) is recruited to TRAM and TRIF and mediates activation of TBK1 [452].

Activated TLR3, which in most cases is located in endosomes [453] but sometimes on the cell surface of epithelial cells [454], recruits TRIF, NAP1, TBK1, and IKKε [444, 447, 451, 452], resulting in activation of TBK1 and IKKε. Both dsRNA and Poly(I:C), a structurally similar analogue to dsRNA, can stimulate TLR3 [455, 456].

TLR-7, -8, and -9-mediated IFN-α/β-induction is, in contrast to TLR-3 and -4, dependent on the adaptor MyD88 [457], which associates with TRAF6, interleukin-

1 receptor associated kinase 1 (IRAK1)/IRAK4, TRAF3, TBK1 and IKKε to cause activation of TBK1 and IKKε [458, 459].

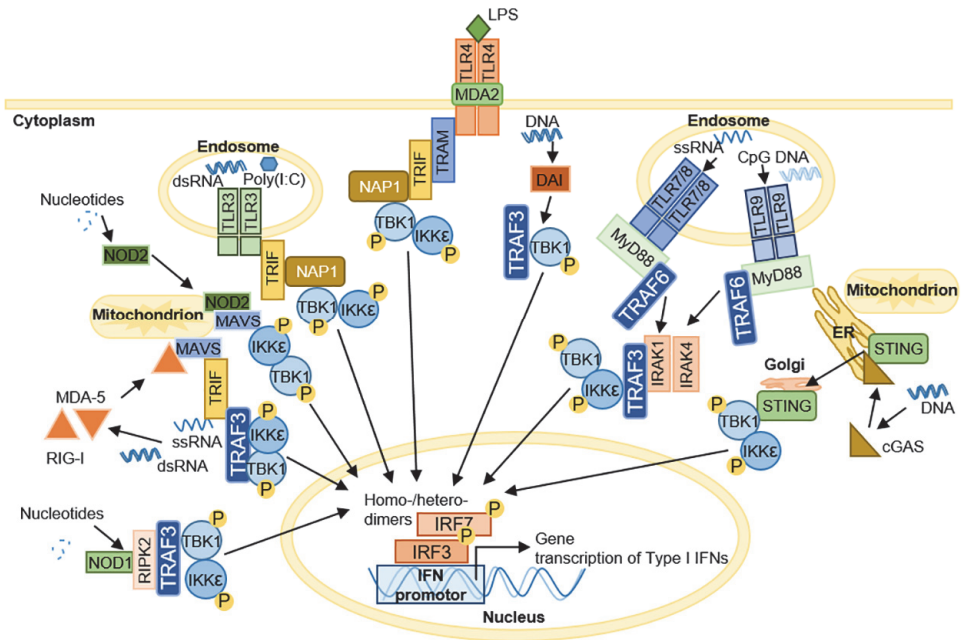

Figure 7. Induction of type I IFN following recognition of viral and cytosolic nucleic acids
Transcription of type I IFNs can be achieved through several different pathways responding to pathogenic RNA/DNA, circulating DNA and nucleic acids. Induction of these pathways begins upon ligand binding to a receptor in the cellular membrane (TLR4), in the endosomal membrane (TLR3, TLR7/8, and TLR9), or in the cytoplasm (NOD1, NOD2, MDA-5, RIG-I, DAI, and cGAS). This is followed by recruitment and association of different adaptor proteins and kinases, ultimately resulting in phosphorylation of TBK1/IKKε, which activate IRF3 and/or IRF7, causing their homo- or heterodimerization. They will then bind to the IFN promoter and induce transcription of type I IFNs.

In addition to TLRs, cytoplasmic receptors can mediate type I IFN-induction. Foreign or circulating DNA, RNA, and nucleic acids, which normally should not be found in the cytosol, can be recognized and lead to induction of type I IFNs. Cytosolic RNA binds to either retinoic acid-inducible gene I (RIG-I) or melanoma differentiation-associated protein 5 (MDA5), which in turn can associate with MAVS (also known as IPS-1 and CARDIF), an adaptor molecule located on the mitochondrial membrane, through its N-terminal caspase activation and recruitment domain (CARD) [460-463]. Thereafter, TBK1 and IKKε are activated following association of TRIF, TRAF3, TBK1, and IKKε to MAVS [464-466].

Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 recognize nucleotides in the cytoplasm. Ligation of NOD2 leads to its

association with MAVS and subsequent association with TBK1 and IKKε [467, 468]. Activation of NOD1 leads to its association with RIPK2/RICK through CARD-CARD interaction. Activated RIPK2/RICK recruits TRAF3, which leads to TBK1 and IKKε phosphorylation [467, 469].

Cytosolic genomic DNA (cgDNA) can be released from tumors or following different stress conditions, such as UV radiation, exposure to genotoxic agents, and radiotherapy [470-472]. The cgDNA motifs are recognized by DNA-dependent activator of IRFs (DAI) and cyclic GMP-AMP synthase (cGAS). cGAS is responsible for catalyzing the formation of cGAMP, which is recognized by STING (stimulator of IFN genes) [473]. STING, a scaffolding protein and an adaptor, is located in the endoplasmic reticulum and is translocated to the Golgi apparatus upon recognition of cytosolic DNA. There, it can recruit and activate TBK1 [474, 475].

Table 2. Location of receptors involved in type I IFN-induction following recognition of PAMPs or DAMPs

Receptor	Location	PAMP/DAMP recognition
TLR3	Cell surface, endosomes	dsRNA, poly(I:C)
TLR4	Cell surface	LPS
TLR7	Cell surface, endosomes	ssRNA, guanosine
TLR8	Endosomes	ssRNA
TLR9	Cell surface, endosomes	Unmethylated CpG DNA
RIG-I	Cytosol	RNA
MDA-5	Cytosol	RNA
DAI	Cytosol	DNA
cGAS	Cytosol	DNA
NOD1/NOD2	Cytosol	Viral and bacterial nucleic acids

Once activated, TBK1 and IKKε phosphorylate and activate the homologous IRF3 and -7 [443, 445, 446, 476]. IRF3 is ubiquitously expressed, whereas IRF7 is largely restricted to lymphoid cell types [477, 478]. Phosphorylation of IRF3 on serine 386 is critical for its activation [479] and results in its dimerization and subsequent removal of its autoinhibitory structure. This allows its translocation to the nucleus and subsequent association with CBP/p300 [480-485]. Together with NF-κB (RelA and p50) and activation protein 1 (AP-1), a dimer of ATF-2 and c-Jun [486, 487], they bind to the promoter of *IFNB1* (the gene encoding IFN-β) [488]. The *IFNB1* promoter is controlled by four positive regulatory domains (PRDs) I-IV, where the IRFs bind to PRD I and III, NF-κB binds to PRD II, and AP-1 to PRD IV [488-494]. Together with the high-mobility group protein (HMG-I(Y)) the former mentioned proteins form the enhanceosome [495, 496]. Formation of the enhanceosome is followed by binding of histone acetyltransferases (HATs), such as CBP and GCN5, which ultimately leads to induction of *IFNB1* gene expression [488, 497]. In contrast to the *IFNB1* promoter, the promoter of IFNA genes only contains PRD I and –III-like elements (PRD-LEs) [498].

Additional roles of TBK1 and IKKε

Although the IKK-related kinases (TBK1 and IKKε) today primarily are known to be essential for activation of IRF3 signaling [445], they were first identified as activators of NF-κB [499]. In this context, TBK1 and IKKε can phosphorylate the canonical IKK complex [500] and thereby cause IκB degradation [501]. IKKα/β have also been found to activate TBK1 and IKKε via phosphorylation [500]. Nevertheless, IKKε and TBK1 and the canonical IKKs (IKKα/β) were shown to be regulated by different pathways following stimulation of IL-1R, TLR3, and TLR4 [500]. Another effect of TBK1 and IKKε is inhibition of cell death. One study demonstrated their recruitment to TNFR1 via TANK and NAP1, together with NEMO, following ubiquitination of RIP1 [502]. Here, TBK1 and IKKε act by phosphorylating RIP1, which leads to inhibition of cell death. In addition, TBK1 has been found to protect embryonic hepatocytes from apoptosis [503].

Both TBK1 and IKKε have been identified as oncogenes in several malignancies, including breast cancer, where they are frequently overexpressed and show enhanced activity [504-506]. The protein expression of IKKε was found to be higher in the ER-positive MCF-7 breast cancer cell line, as compared to the normal breast cell line MCF-10A [505]. The same study also found that MCF-10A lack *IKBKE* copy-number gain, whereas MCF-7 cells express low levels of *IKBKE* copy-number gain. Due to their ability to phosphorylate the ER, TBK1 and IKKε might have a role in resistance to therapies targeting ER, such as tamoxifen [507, 508]. This, in combination with the fact that they are structurally similar [509], and overexpressed in various cancer types [504-506], could make them suitable targets for pharmacological inhibition. One such inhibitor, called BX795, was found to block downstream IRF3 activation whereas it did not inhibit phosphorylation of TBK1 and IKKε [510]. This suggested that they are not activated through autophosphorylation. However, BX795 also targets PDK1, Aurora B, ERK8, and MARK3. Later, it was actually found that induction of autophosphorylation of TBK1 (at Ser172) and self-association are important for its activation [511], and that the activity of TBK1 is regulated through several posttranslational modifications such as phosphorylation, ubiquitination, modulation of kinase activity as well as prevention of formation of TBK1-containing complexes [512]. There are many similarities between IKKε and TBK1, for example their closely intertwined signaling properties. But they are expressed differently and exhibit differences in substrate specificity [445, 513]. Altogether, activation of TBK1 is important in the PRR-stimulated pathways where pathogenic or cyclic genome are recognized by its cognate receptors and results in induction of IFN-β.

TRAF3

TRAF3 is a protein which has been found to be important in many different pathways resulting in IFN-β-induction. It is required for TRIF-dependent TLR-mediated induction of IFN-β as it can interact with TRIF and TBK1. TRAF3 is also involved in MyD88-dependent TLR signaling since it is recruited via IRAK4 and IRAK1 and can, in turn, recruit TBK1 and IKKε [466, 514-516]. In addition, TRAF3 is recruited, either directly or indirectly, to cytoplasmic receptors, such as RIG-I, following their signaling [514]. However, TRAF3 may also act as a negative regulator of IFN production following activation of DNA pathways, as it suppresses NIK, which in turn is a positive regulator of the DNA pathway since it enhances STING signaling [517]. In the same study, NIK was found to be a negative regulator of RNA pathway.

Interferon signaling and breast cancer

In the late 80s it was found that treatment of breast tumor cells with low doses of type I IFNs induced expression of ER and made the cells, which were plated at a low density, more sensitive to tamoxifen [518-520]. This was later also confirmed in clinical studies, in which treatment with IFN increased ER expression [521-523]. However, the combination of IFN and tamoxifen reduced ER expression [522]. Estrogen signaling can also affect IFN signaling. For example, estrogen has been found to modulate $CD4^+$ T-helper 1 (Th1) cells through enhancement of IFN-γ expression [524]. In addition, activation of the PR can repress ISG expression in ER-positive breast cancer cells. This is due to impaired association of STAT2, and IRF9 to the ISG promoters following PR recruitment to ISG enhancers [525-527]. These examples suggest that there is a complex interplay between IFN signaling and endocrine therapy.

Since the 80s, additional studies on the effect of IFN signaling on breast cancer have been conducted. More recently, it was found that IFN signaling is associated with the aggressiveness of breast cancer. The association is dependent on the ER status of the breast tumor. ER-positive breast cancer, which have acquired resistance to different treatments, show an enhanced expression of ISGs and IFN signaling. For example, increased ISG expression is associated with radiotherapy- and hormonal therapy resistance [528, 529], as well as with resistance to CDK4/6 inhibitors [530]. Moreover, enhanced IFN-α signaling can result in ligand-independent ER signaling which can promote survival of AI-resistant breast cancer cells [531]. In addition, chronic activation of Fas in MCF-7 resulted in more stem cell like features of the cells. The effect was found to be driven by type I IFN and STAT1 signaling [532, 533]. In ER-negative breast cancer, on the other hand, IFN signaling has been found

to be associated with response to chemotherapy and longer distant metastasis-free survival following chemotherapy [534, 535].

Interferon signaling and Smac mimetics

Smac mimetics have been shown to cooperate with type I and type II IFNs to induce cell death in different cancer cells [536-539]. More specifically, a Smac mimetic was found to act synergistically with IFN-α to induce cell death in acute myeloid leukemia (AML) through induction of TNF-α and its subsequent signaling [539]. Treatment of renal cell carcinoma (RCC) with Smac mimetic and IFN-α also induced apoptosis [536]. This was found to be independent of TNF-α and RIP1 kinase activity, but dependent on a potential scaffold role of RIP1. One study showed that the combination of Smac mimetic and IFN-α induced RIP1-dependent cell death. Although, the induction of cell death differed depending on cell line, since A172 glioblastoma cells depended on subsequent production of TRAIL whereas HT29 colon adenocarcinoma cells required production of TNF-α [540].

Present investigation

Overview and aims

The overall aims of this thesis were to understand how TRAIL in combination with Smac mimetics influence the apoptotic machinery and phenotypic changes in breast cancer cells. The focus is on the induction of IFN signaling and how TRAIL and Smac mimetics can be used to suppress the malignancy of breast cancer cells. This was initiated in Paper I, where we studied the mechanisms of Smac mimetic- and TRAIL-induced cell death. The non-apoptotic effect by the same treatment was further investigated in Paper II, where we studied the effect on changed morphology and gene expression. The mechanisms underlying the changes in gene expression and induction of IFN signaling were further examined in Paper III and IV.

The specific aims of this thesis were:

I. To investigate if TRAIL in combination with the Smac mimetic LCL161 can induce apoptosis in breast cancer cells, and if so, examine the underlying mechanism.

II. To study the characteristics and underlying mechanisms of the irreversible change obtained following long-term treatment with LCL161 and TRAIL in MCF-7 cells.

III. To examine the mediators and mechanisms behind induction of IFN signaling following LCL161 and TRAIL treatment.

Paper I

Induction of breast cancer cell apoptosis by TRAIL and Smac mimetics: Involvement of RIP1 and c-FLIP

Overexpression of IAPs is associated with tumor progression and promotion of cancer cell survival due to their ability to suppress apoptosis [262, 284, 286-288]. Smac mimetics were developed to inhibit IAPs and thereby facilitate induction of apoptosis [285, 303]. The effect of Smac mimetics as single agents is limited, which is why they also are investigated as part of a combination treatment [285, 323-325].

The death receptor ligand TRAIL is a potential agent that can be used together with Smac mimetics since it preferentially stimulates apoptosis in tumor cells as compared to normal cells [233]. In Paper I, the aim was to investigate if the Smac mimetic LCL161 can potentiate TRAIL-induced apoptosis in breast cancer cells, and if so, examine the underlying mechanism. For this study, three different breast cancer cell lines were used, the TN MDA-MB-468, and the luminal and ER-positive CAMA-1 and MCF-7 cell lines. All three cell lines were resistant to LCL161 as single agent, but LCL161 potentiated TRAIL- or TNF-α-induced loss of cell viability in CAMA-1 and MDA-MB-468 cells. This was found to be caspase-dependent. MCF-7 cells differ from the two other cell lines since neither apoptosis nor activation of caspase-8 could be observed following the combination treatment.

Smac mimetics have the potential to degrade cIAP1/2, which in turn can activate the non-canonical NF-κB pathway [275]. Treatment with LCL161 caused cIAP1-degradation and induction of the processed p100 product p52 in all three cell lines, indicating activation of non-canonical NF-κB pathway. In addition, LCL161 enhanced cIAP2 mRNA levels both in CAMA-1 and MCF-7 cells. This was dependent on the non-canonical NF-κB pathway since knockdown of NIK suppressed this effect. Induction of NF-κB signaling following Smac mimetic treatment have previously been found to stimulate TNF-α-dependent cell death in breast cancer cells, among others [275, 321]. Knockdown of NIK had no effect on LCL161 and TRAIL-induced cell death in CAMA-1 cells, an indication that cell death occurs independently of the non-canonical NF-κB pathway.

Previous studies have found formation of a cell death-inducing cytosolic complex, consisting of FADD, procaspase-8, and RIP1, following treatment with TRAIL in combination with Smac mimetics. This complex is also known as the ripoptosome [226-228]. The ripoptosome can, in addition to apoptosis, stimulate NF-κB signaling [541, 542]. XIAP and cIAP1/2 have, in a previous study, been shown to inhibit the activity of the ripoptosome [218]. Therefore, we investigated the effects of Smac mimetic and TRAIL on this complex formation. The combination of LCL161 and TRAIL, but not the treatments alone, could stimulate association of RIP1 and caspase-8, both in MDA-MB-468 and CAMA-1 cells, but not in MCF-7 cells. Apoptosis could be suppressed in CAMA-1 following RIP1 downregulation, but not upon inhibition of RIP1 kinase activity, suggesting a role for RIP1 that is independent of its kinase activity. In contrast to CAMA-1, knockdown of RIP1 did not suppress the LCL161 and TRAIL-induced cell death in MDA-MB-468 cells and the amount of RIP1 co-immunoprecipitation to caspase-8 was lower in MDA-MB-468 than in CAMA-1 cells. A possible explanation of the different requirement of RIP1 in the two cell lines could be either lower amount of the complex formation or different roles of RIP1 in the cell lines. In CAMA-1, but not MCF-7 cells, TRAIL could stimulate cleavage of RIP1, which was further potentiated by LCL161 treatment. Inhibition of caspase activity blocked RIP1 cleavage.

An inhibitor of caspase-8 is c-FLIP [188-190], which can exist in two isoforms: short (c-$FLIP_S$) and long (c-$FLIP_L$). We speculated that there could be differences in c-FLIP levels and/or isoform expression that could explain the different responses in CAMA-1 and MCF-7 cells to stimulation with LCL161 and TRAIL. The expression of c-$FLIP_s$, which is known to inhibit caspase-8 activity [543, 544], was high in MCF-7, but it could not be detected in CAMA-1 cells. c-$FLIP_L$, which can either inhibit or potentiate caspase-8 activation depending on high or low ratio of c-$FLIP_L$:procaspase-8 [188, 543, 545, 546], was found to be highly expressed in both MCF-7 and CAMA-1 cells. Addition of TRAIL decreased c-$FLIP_L$ expression in both cell lines. Following downregulation of c-FLIP, LCL161 and TRAIL could induce caspase-7 cleavage in MCF-7 cells and there was also a tendency to cell death induction. The results suggest that c-FLIP could be an inhibitor of LCL161 and TRAIL-induced cell death in MCF-7. In CAMA-1, knockdown of c-FLIP potentiated TRAIL-induced cell death and cleavage of caspase-8 and -7. In this context, addition of LCL161 had barely any further potentiating effect. To investigate if the c-FLIP isoforms have specific roles in cell death induction following LCL161 and TRAIL treatment, the individual isoforms were downregulated. Neither in CAMA-1 nor in MCF-7 cells, did knockdown of the individual isoforms have any different effect on caspase cleavage and cell death as compared to simultaneous knockdown of both isoforms.

The expression of c-$FLIP_S$, an inhibitor of caspase-8, was found to be higher in MCF-7 cells than in CAMA-1 cells. In addition, MCF-7 cells lack expression of caspase-3 and -10, and express lower levels of caspase-8 than other breast cancer cell lines [547-549]. We found differences in caspase-8 activation between the cell lines. Following treatment with LCL161 and TRAIL, MCF-7 cells only showed minor cleavage of caspase-8, as compared to CAMA-1. Moreover, MCF-7 did not show any cleavage of the executioner caspase-7, an effect that was detected in CAMA-1 cells. The importance of caspase-8 for cell death-induction in CAMA-1 was demonstrated following knockdown of caspase-8 since this blocked apoptosis and caspase cleavage. Hence, hampered caspase-8 activation could be a plausible explanation to why MCF-7 breast cancer cells are not sensitive to treatment with Smac mimetic and TRAIL. Although we could not detect any cell death, the combination treatment did not leave the MCF-7 cells totally unaffected since we saw changes of cell morphology and growth pattern. These effects were further investigated in Paper II.

Paper II

The combination of TRAIL and the Smac mimetic LCL-161 induces an irreversible phenotypic change of MCF-7 breast cancer cells

In Paper I, we noticed that MCF-7 cells, instead of going through apoptosis, underwent a dramatic change in morphology and growth pattern following treatment with the Smac mimetic LCL161 and TRAIL. The cell morphology was characterized by a decreased size, a round shape, and lack of cell-cell contact. In addition, the cells were less adherent. The effect could also be obtained following LCL161 in combination with TNF-α, but neither substance had any affect when used alone. The cells undeniably resembled dead cells but cell viability and Annexin V assays confirmed that the cells were viable. In addition, the cells could be maintained in culture for several weeks with a maintained morphology and growth pattern, despite removal of treatment. The aim of this study was to investigate the characteristics and underlying mechanisms of the irreversible change obtained following treatment with LCL161 and TRAIL in MCF-7 cells.

We inhibited several different signaling pathways to study if any of them could mediate the morphology change. All pathways investigated have been found to potentially be activated following treatment with LCL161 and TRAIL. However, only inhibition of caspase activity with zVAD-FMK resulted in complete suppression of the morphology change. zVAD-FMK is a pan-caspase inhibitor and the importance of individual caspases were therefore investigated. Downregulation of caspase-8 completely blocked the change in morphology, indicating a non-apoptotic function of caspase-8 in the process. There was a gradual but fairly slow processing of caspase-8, perhaps indicating a low but long-term activity. Caspases have previously been associated with non-apoptotic roles in cell differentiation and proliferation [550-552]. Moreover, caspases have been shown to have morphological effects in the nervous system and during cell migration [553-556]. These findings could be in line with the caspase-dependent morphological changes we have observed.

Since MCF-7 cells do not express caspase-3 [547] we only investigated if the executioner caspase-6 and -7 had any effect in LCL161 and TRAIL-induced morphology change. Only downregulation of caspase-7 decreased the number of morphologically altered cells, but the effect was not complete. Similar to caspase-8, both caspase-6 and -7 were processed in a time-dependent manner. Caspase-7 has been shown to be necessary in detachment of cells [557].

The persistent alteration in morphology indicates a phenotypic change, where changes in gene expression may be involved. Global mRNA expression analysis confirmed that there is a considerable change in gene expression accompanying the change in morphology. Enrichment analysis of the upregulated genes demonstrated enrichment of NF-κB- and IFN-inducible genes after treatment with LCL161 and

TRAIL for 24h. This was also seen after LCL161 and TRAIL treatment for 4 days followed by 3 days of recovery (here called long-term treatment). However, inhibition of neither IFN nor NF-κB pathways influenced the morphology change, indicating that the changes in gene expression and morphology are independent events. Downregulated genes in long-term treated cells were enriched for ER-stimulated genes, genes typical for non-basal-like phenotype, and genes downregulated in cells resistant to anti-estrogen therapy. This result suggests that there has been a shift towards less ER-positive phenotype. The vast majority of genes that were up- or down-regulated after long-term treatment were not affected after 24h of treatment, indicating that they represent a more long-term alteration.

Since the change of morphology can be blocked by inhibition and downregulation of caspases, their role for the changes in gene expression was investigated using RNA-seq analysis. A principal component analysis of the expression data demonstrated that caspase inhibition had minor effects on the expression pattern induced by LCL161 and TRAIL. The effect of caspase inhibition on individual genes related to NF-κB and IFN signaling was further examined. There was a tendency towards potentiation of the expression of IFN-inducible genes, but a suppression of NF-κB-inducible genes by zVAD-FMK. Similar to the morphology change, the induced expression of IFN- and NF-κB-related genes was maintained following removal of LCL161 and TRAIL. We have not encountered or observed any similar phenotypic change in any other breast cancer cell line following treatment with TRAIL and Smac mimetic, but a similar phenotypic change which involved IFN signaling has been demonstrated in breast cancer (including MCF-7) and squamous cell lines following treatment with Fas [532, 533]. IFN signaling has been shown to be associated with treatment resistance and enhanced aggressiveness in ER-positive breast cancer [528, 529]. Furthermore, TRAIL-mediated induction of IFN signaling is not well characterized. Therefore, the mechanisms mediating the induction of IFN signaling were investigated in Paper III and IV.

Paper III

Induction of interferon-β and interferon signaling by TRAIL and Smac mimetics via caspase-8 in breast cancer cells

Treatment of MCF-7 cells with TRAIL and the Smac mimetic LCL161 led to expression of IFN- and NF-κB-inducible genes. This led us to study mediators and mechanisms behind induction of IFN signaling following LCL161 and TRAIL treatment. We examined the NF-κB and IFN signaling pathways and found increased levels of p52, indicating activation of non-canonical NF-κB pathway, as well as phosphorylation of STAT1, suggesting activation of type I IFN signaling pathway. In MCF-7 cells treatment with LCL161 alone resulted in a slight induction

of p52 levels, which was further enhanced upon combination with TRAIL. TRAIL alone was sufficient for induction of STAT1 phosphorylation, whereas LCL161 suppressed this effect of TRAIL. In CAMA-1, another ER-positive breast cancer cell line, the combination of LCL161 and TRAIL induces caspase-dependent cell death. A caspase inhibitor was therefore included to enable analysis of the signaling pathways in these cells. Both non-canonical NF-κB and IFN signaling pathways were activated by LCL161 and TRAIL in CAMA-1 cells. Here, LCL161 was sufficient to induce p52, whereas both LCL161 and TRAIL were necessary for STAT1 phosphorylation. In MCF-7 cells, downregulation of NIK did not inhibit the induction of STAT1 phosphorylation. Inhibition of Janus tyrosine kinases with Ruxolitinib on the other hand blocked STAT1 phosphorylation.

Type I IFN signaling is activated following ligation of IFN-β to its receptor complex consisting of IFNAR1 and IFNAR2. This results in activation of the Janus tyrosine kinases, with subsequent phosphorylation of STAT1/2 [558]. In turn, STAT1/2 dimerize and form a complex with IRF9 called ISGF3 which can bind to ISREs and induce transcription of ISGs [559]. Our results raise the possibility of the induction of autocrine type I IFN signaling. This is supported by the RNA-seq analysis as type I and type III IFNs were induced in MCF-7 cells following LCL161 and TRAIL treatment. Further examination showed that mRNA of both type I receptors IFNAR1 and IFNAR2, and one of the type III receptors were detected in MCF-7 cells. LCL161 and TRAIL induce a gradual increase of *IFNB1* mRNA levels, suggesting that IFN-β production increases with time. Moreover, downregulation of IFNAR1 suppressed both LCL161 and TRAIL-mediated STAT1 phosphorylation and the increase in total STAT1 levels, indicating the involvement of signaling via the type I IFN receptor. Our hypothesis was further supported by the finding that Ruxolitinib could not block the induction of *IFNB1*, whereas the downstream ISGs *IRF9* and *STAT1* were suppressed. Autocrine type I IFN signaling has previously been described in other cell types following different types of stimuli, as demonstrated following silencing of IFNAR1/2 and the ligands [560-562].

Depending on the ER-status of a breast cancer, IFN signaling has been suggested to have different effects on cancer aggressiveness. IFN signaling in ER-positive breast cancers is associated with resistance to radiotherapy and hormonal therapy [528, 529], whereas in ER-negative breast cancers it is related to response to chemotherapy and longer distant metastasis-free survival [534, 535]. The IFN expression was examined in MCF-7 cells following treatment with LCL161 and TRAIL and in breast tumors using public RNA-seq data from the SCAN-B cohort of consecutive breast cancers. The type II IFN (which is generally found on leukocytes) was highly expressed in breast tumors, which probably reflects immune cell infiltration but it was not expressed in MCF-7 cells. Type III IFN genes were expressed in some of the breast cancer samples and in treated MCF-7 cells, whereas only *IFNB1* of the type I IFNs was expressed in MCF-7. In breast cancers, type I IFNs were expressed in some tumors, and the expression of *IFNB1* was higher than

any of the other type I IFNs. We also studied the expression of the IFN receptor genes in MCF-7 cells following treatment and in the SCAN-B cohort. The pattern was similar in both MCF-7 cells and in SCAN-B where both type I IFN receptors were expressed. In MCF-7 cells, only one of the type III IFN receptors were expressed, whereas both were expressed in tumors, but at different levels. We also examined the association of prognosis with the number of *IFNB1* and IFNL genes with an expression level above baseline. The prognosis was found to be worse for ER-positive cancers if more than two genes were expressed, as compared to expression of fewer genes. Since TRAIL stimulation is a potential strategy to suppress breast cancer growth [563, 564], it could be important to also assess induction of IFN-β signaling in ER-positive breast cancers.

TRAIL stimulation has been found to induce inflammatory signaling which is dependent on a scaffolding and non-apoptotic role of caspase-8 [230, 231]. Downregulation of caspase-8 completely abolished TRAIL-induced STAT1 phosphorylation in MCF-7 cells and suppressed it following treatment with zVAD-FMK, LCL161, and TRAIL in CAMA-1 cells. Moreover, downregulation of caspase-8 decreased treatment-induction of ISGs in both cell lines. However, inhibiting caspase activity by either zVAD-FMK or the caspase-8-specific inhibitor zIETD-FMK could not suppress TRAIL-mediated STAT1 phosphorylation, which contrasts a previous study where zIETD-FMK treatment inhibited Fas-induced STAT1 phosphorylation in MCF-7 [533]. Previous studies suggest that stimulation of death receptors can induce formation of a complex called the FADDosome, where caspase-8, through its scaffolding role, associates with FADD [230, 231]. We could determine that FADD and caspase-8 are co-immunoprecipitated following TRAIL stimulation. However, downregulation of FADD did not inhibit STAT1 phosphorylation as efficient as knockdown of caspase-8. RIP1 has been described to induce NF-κB signaling, which was dependent on FADD and caspase-8 [565], and to mediate production of IFN-β in macrophages [566]. The protein c-FLIP can either block or promote the activity of caspase-8. Therefore, these proteins were examined for a potential role in IFN-induction, but downregulation of RIP1 or c-FLIP did not have any effect on TRAIL-mediated phosphorylation of STAT1. This contrasts previous results where TRAIL-mediated cytokine production was dependent on RIP1 [230, 231]. IRF3 and IRF7 are well established activators of *IFNB1* transcription [480, 484] and can be regulated by TBK1 and IKKε. The possible involvement of these and other proteins in IFN-β-induction were investigated in Paper IV.

Paper IV

TRAIL- and Smac mimetic-mediated activation of TBK1 and NF-κB pathways in breast cancer cells and their roles in induction of interferon pathways

The combination of TRAIL and the Smac mimetic LCL161 can stimulate a caspase-8-dependent induction of IFN-β signaling in ER-positive breast cancer cell lines. The aim of Paper IV was to elucidate which other mediators that contribute to the effect. IFN-β is in general induced following viral infection where either TLRs or RLRs are activated [445, 515, 516, 567, 568]. This results in phosphorylation and activation of TBK1 and IKKε, which in turn can phosphorylate IRF3 and -7 [445, 446]. Phosphorylated IRF3/7 can then dimerize and translocate to the nucleus where it induces transcription of *IFNB1* [480, 481, 484, 485]. Our results both support and discard an involvement of TBK1 and IKKε in the induction of IFN signaling following treatment with LCL161 and TRAIL in CAMA-1 cells. The results supporting their involvement include the finding that pretreatment with an inhibitor against TBK1 and IKKε, MRT67307, blocks STAT1 phosphorylation and induction of ISG mRNA expression in CAMA-1 cells. Furthermore, treatment with TRAIL and LCL161 resulted in phosphorylation of TBK1. In addition, TBK1 activation was found to be caspase-8-dependent. Similar results were found in MCF-7 cells stimulated with TRAIL alone. Treatment with TRAIL led to TBK1 phosphorylation and MRT67307 suppressed the TRAIL-induced STAT1 phosphorylation. Downregulation of caspase-8 also reduced TRAIL-mediated phosphorylation of TBK1 in MCF-7 cells. The results speaking against an involvement of TBK1 and IKKε includes the facts that downregulation of TBK1 or its downstream mediator IRF3 could not suppress STAT1 phosphorylation in CAMA-1 cells. In addition, the combination treatment could not induce phosphorylation of IRF3, which the TLR3 agonist Poly(I:C) could.

A type I IFN response has previously been demonstrated following dsRNA stimulation in IRF3$^{-/-}$ mice [569], suggesting a pathway independent of activation of TBK1, IKKε, and IRF3. Therefore, one possible theory to the conflicting results regarding the involvement of TBK1 is that MRT67307 has off-target effects and acts by other mechanisms than suppressing TBK1 and IKKε. For example, ULK1/2, two proteins involved in autophagy, have been found to be inhibited by MRT67307 [570]. MRT67307 has also been found to affect the AMP-dependent kinase family in a TBK1$^{-/-}$IKKε$^{-/-}$ background [571], and to block CYLD phosphorylation following deletion of TBK1 and IKKε with CRISPR/Cas9 [572].

NF-κB can facilitate transcription of *IFNB1* together with c-Jun and ATF-2 following their binding to the *IFNB1* promoter [495, 496]. However, MRT67306 further potentiated LCL161 and TRAIL-induced phosphorylation of IκBα, an indication of active canonical NF-κB pathway [573, 574], and downregulation of

p65 and IKKβ did not impact STAT1 phosphorylation. A critical involvement of the canonical pathway was therefore was discarded.

TLR- and RLR-stimulated induction of IFN-β includes involvement of TRAF3 [515, 516], an adaptor molecule which we investigated through knockdown. This actually enhanced LCL161 and TRAIL-mediated STAT1 phosphorylation, which excludes it as a mediator of the effect. Expression of NIK has been found to be enhanced in TRAF3 knockout MEFs [517]. In the same study, NIK was shown to interact with STING and induce IFN-β. TRAF3 is known to be critical for the degradation of NIK [344, 345, 575], and our results showed that downregulation of TRAF3 caused enhanced phosphorylation of STAT1. Therefore, we investigated NIK. Knockdown of NIK diminished the treatment-induced STAT1 phosphorylation, proposing a role for NIK in the LCL161 and TRAIL-induced IFN response.

Conclusions

In this thesis, we have found that the combination of the Smac mimetic LCL161 and TRAIL can induce cell death in some breast cancer cell lines. This was dependent on caspase-8 activity. But in ER-positive MCF-7 cells the combination induces a caspase-8-dependent and non-apoptotic change of morphology and gene expression. A slow processing of caspase-6, -7, and -8 was observed upon the morphology change. LCL161 and TRAIL specifically upregulated and activated NF-κB and IFN signaling, in which caspase-8, independently of its enzymatic activity, is involved in mediating an IFN-β-induction and autocrine IFN-β signaling. We have studied the underlying mechanisms of induction of cell death, the morphology change, and IFN signaling by investigating several different pathways and potential mediators.

We can conclude that:

- The combination of Smac mimetic LCL161 and TRAIL induces caspase-dependent apoptosis in the ER-positive CAMA-1 and TN MDA-MB-468 breast cancer cell lines. This is accompanied by a strong caspase-8 activation. In CAMA-1, induction of apoptosis was dependent of RIP1, but independent of RIP1 kinase activity.
- Downregulation of the caspase-8 inhibitor c-FLIP showed a tendency towards enhancing the sensitivity of MCF-7 cells to LCL161 and TRAIL treatment. This also correlated with increased cleavage of caspase-7. In CAMA-1 cells, downregulation of c-FLIP potentiated TRAIL-induced cleavage of caspases and cell death.
- Instead of apoptosis, treatment with LCL161 and TRAIL induces a caspase-8-dependent irreversible change of morphology in MCF-7 cells.

- Long-term treatment with LCL161 and TRAIL induces downregulation of ER-related genes and upregulation of genes related to NF-κB and IFN signaling, which are separated from the induction of morphology change in MCF-7.
- LCL161 and TRAIL induce non-canonical NF-κB and IFN signaling in MCF-7 cells, and in CAMA-1 if caspases are inhibited. Non-canonical NF-κB does not mediate the IFN-induction.
- Caspase-8 has a non-apoptotic and non-enzymatic function in mediating autocrine IFN-β signaling in MCF-7 cells following TRAIL stimulation.
- Although LCL161 and TRAIL induces activation of TBK1 and inhibition of TBK1 suppresses IFN signaling, downregulation of TBK1 and its downstream protein IRF3 does not affect IFN signaling, suggesting involvement of other proteins sensitive to the inhibitor MRT67307.
- NIK, a protein that induces the non-canonical NF-κB pathway, is a potential mediator of the IFN signaling induced by TRAIL and LCL161, whereas the canonical NF-κB pathway seems to be redundant.

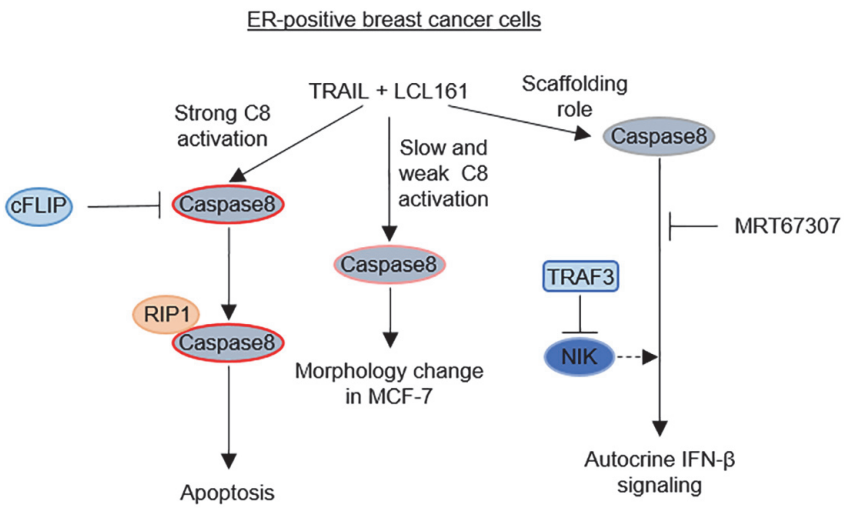

Figure 8. Summary figure
Treatment of ER-positive breast cancer with TRAIL and the Smac mimetic LCL161 results in apoptosis when caspase-8 is strongly activated. RIP1 associates with caspase-8 and can potentially further promote apoptosis induction. Apoptosis can be suppressed by c-FLIP. In MCF-7 cells slow and weak caspase-8 activation, which is induced by TRAIL and LCL161, results in a morphology change. The combination treatment can also result in autocrine IFN-β signaling, which is independent of caspase-8 activity. Here caspase-8 conceivably has a scaffold role. NIK, which is inhibited by TRAF3, is a potential mediator of IFN-β-induction, whereas MRT67307 suppresses it.

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