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Granqvist, Victoria

2022

*Document Version:*

Publisher's PDF, also known as Version of record

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*Citation for published version (APA):*

Granqvist, V. (2022). *Mechanisms of TRAIL- and Smac mimetic-induced cell death and phenotypical changes in breast cancer*. [Doctoral Thesis (compilation), Department of Laboratory Medicine]. Lund University, Faculty of Medicine.

*Total number of authors:*

1

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# Mechanisms of TRAIL- and Smac mimetic-induced cell death and phenotypical changes in breast cancer

VICTORIA GRANQVIST

LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY





## FACULTY OF MEDICINE

Department of Laboratory Medicine

Lund University, Faculty of Medicine

Doctoral Dissertation Series 2022:75

ISBN 978-91-8021-236-6

ISSN 1652-8220



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cell death and phenotypical changes in breast cancer





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Victoria Granqvist



**LUND**  
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## DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 24<sup>th</sup> of May at 09.00 in Sharience, Spark building, Medicon Village, Scheeleorget 1, Lund, Sweden

*Faculty opponent*

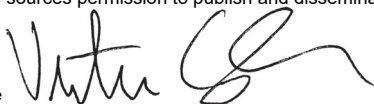
Prof. Bengt Hallberg

University of Gothenburg, Sweden

<b>Organization</b> LUND UNIVERSITY Faculty of Medicine Department of Laboratory Medicine Author: Victoria Granqvist		<b>Document name</b> Doctoral dissertation	
		<b>Date of issue</b> 24 <sup>th</sup> of May 2022	
		Sponsoring organization	
<b>Title</b> Mechanisms of TRAIL- and Smac mimetic-induced cell death and phenotypical changes in breast cancer			
<b>Abstract</b> <p>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.</p> <p>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.</p> <p>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-<math>\kappa</math>B 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-<math>\kappa</math>B 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.</p> <p>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 <i>IFNB1</i> mRNA levels following treatment, all of which indicate an autocrine IFN signaling. Ruxolitinib suppressed IFN-stimulated genes but could not block <i>IFNB1</i>-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 IKK<math>\epsilon</math>, two proteins mediating transcription of <i>IFNB1</i>, with MRT67307 suppressed STAT1 phosphorylation but IFN signaling could not be blocked by downregulation of TBK1 or IKK<math>\epsilon</math>. NIK, a kinase which induces the non-canonical NF-<math>\kappa</math>B 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.</p>			
<b>Key words</b> TRAIL, Smac mimetics, breast cancer, cell death, phenotypical changes, IFN, NF- $\kappa$ B			
Classification system and/or index terms (if any)			
Supplementary bibliographical information		<b>Language</b> English	
<b>ISSN</b> 1652-8220		<b>ISBN</b> 978-91-8021-236-6	
Recipient's notes		<b>Number of pages</b> 102	
		Price	
		Security classification	

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Victoria Granqvist



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Paper 4 © by the Authors (Manuscript unpublished)

Faculty of Medicine  
Department of Laboratory Medicine

ISBN 978-91-8021-236-6

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University  
Lund 2022



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*To my family and friends*

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# List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

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

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

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



# Populärvetenskaplig sammanfattning

Cancer omfattar en grupp sjukdomar och uppstår när celler delas och växer okontrollerat och stör funktionen hos det drabbade organet. Okontrollerad celledelning kan vara ett resultat av mutationer som uppstår vid normalt förekommande celledelning 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 celledelning 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 eliminering 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 bröstcancer celltyper. 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.

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- $\kappa$ B 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- $\kappa$ B 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 IKK $\epsilon$ , two proteins mediating transcription of *IFNB1*, with MRT67307 suppressed STAT1 phosphorylation but IFN signaling could not be blocked by downregulation of TBK1 or IKK $\epsilon$ . NIK, a kinase which induces the non-canonical NF- $\kappa$ B 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

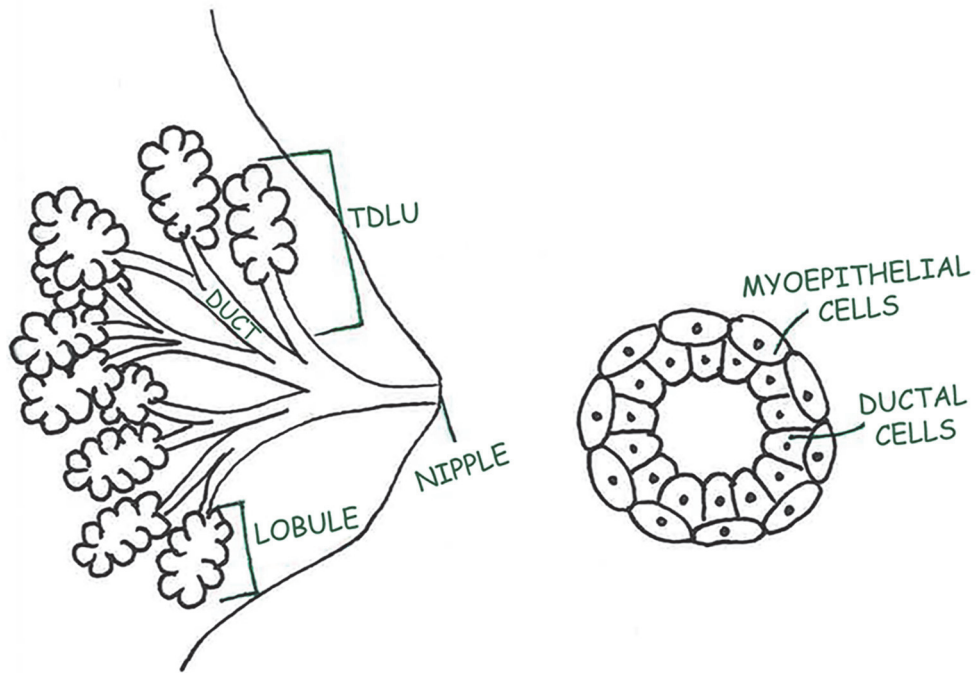
most classical example is the inactivation of the *BRCA1* and *BRCA2* genes, both involved in DNA repair [26], which leads to a prominent risk of breast cancer development, and accounts for approximately 25% of all hereditary breast cancer cases [27, 28]. Mutated *BRCA1* has been linked to an aggressive 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<sup>+</sup> cells resulted in tumors with luminal features, such as ER $\alpha$ , whereas tumors derived from transformed CD10<sup>+</sup> cells showed squamous, metaplastic, and giant cell differentiation, and lack of ER $\alpha$  [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 tubulolobular 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 *ERBB2* 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 *ERBB2* 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, ribociclib, 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-X<sub>L</sub>, 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**

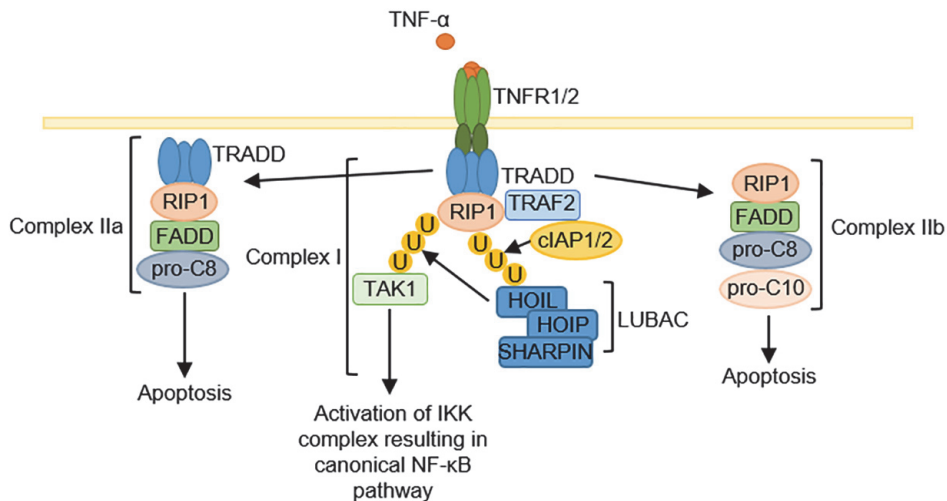
Initiation of the extrinsic pathway begins with binding of a ligand to its cognate transmembrane death receptor (DR) on the cellular membrane. The ligand FasL binds to Fas, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) binds to TNF receptor 1 and 2 (TNFR1 and TNFR2), and TNF-related apoptosis-inducing 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- $\alpha$ , 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- $\kappa$ B (NF- $\kappa$ B) 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- $\kappa$ B 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- $\beta$ -activated kinase 1 (TAK1) and the I $\kappa$ B kinase (IKK) complex, consisting of NF- $\kappa$ B essential modulator (NEMO), inhibitor of NF- $\kappa$ B kinase subunit  $\alpha$  (IKK $\alpha$ ), and IKK $\beta$ , to RIP1 [206, 212, 213]. This will lead to activation of the canonical NF- $\kappa$ B 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- $\kappa$ B signaling pathway [206].



**Figure 2. TNFR1/2 signaling pathway**

TNF- $\alpha$  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 ubiquitin chains to RIP1, causing recruitment of TAK1 and the IKK complex. Subsequently, TAK1 phosphorylates the IKK complex, leading to activation of the NF- $\kappa$ B pathway. The complex formed is called Complex I. The soluble Complex IIa cannot be formed until stimulation of the anti-apoptotic NF- $\kappa$ B 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.

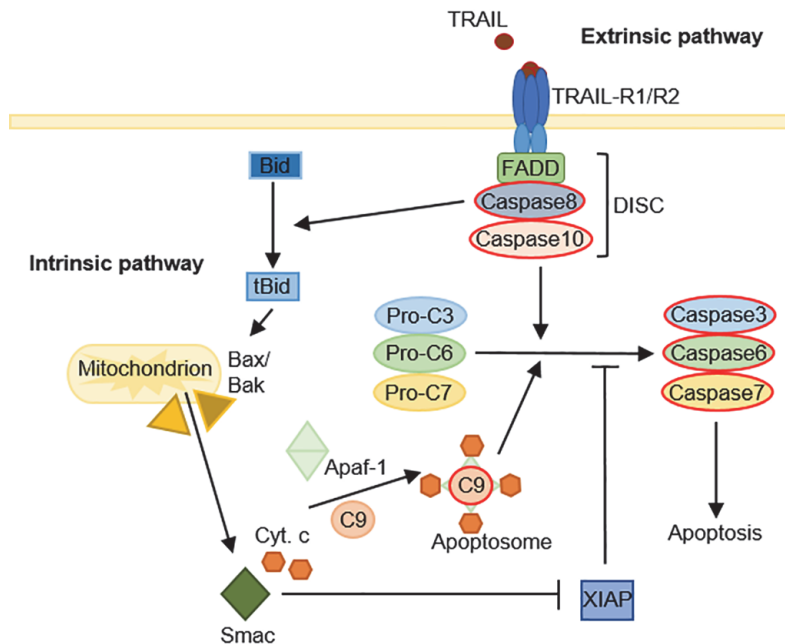
As long as Complex I is stimulated and continues to induce anti-apoptotic NF- $\kappa$ B signaling, the soluble cytoplasmic variants of Complex I, called Complex IIa/b, are blocked. However, following removal of K63- and M1-polyubiquitin chains of RIP1 by CYLD, which is induced by NF- $\kappa$ B, the NF- $\kappa$ B 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- $\kappa$ B 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**

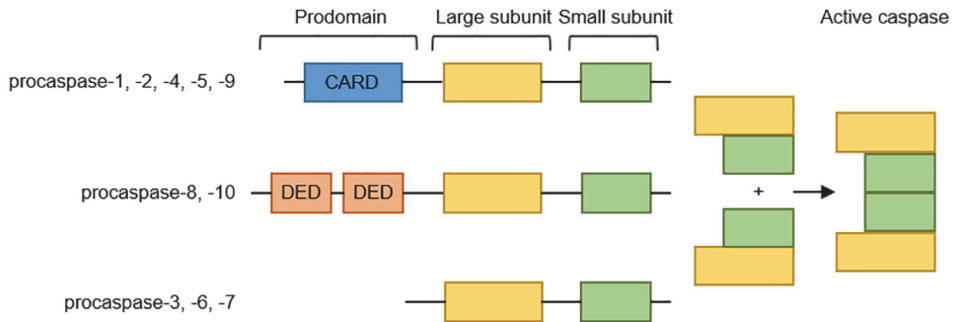
Binding of TRAIL to its receptor TRAIL-R1 (DR4) or TRAIL-R2 (DR5) results in association and formation of DISC, consisting of FADD, caspase-8, and -10. DISC can induce apoptosis through the extrinsic pathway by cleaving and activating the executioner caspases-3, -6, and -7. The intrinsic pathway is initiated 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- $\kappa$ B [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- $\beta$  (IFN- $\beta$ ) 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 proddomains. Upon activation the proddomains, 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- $\kappa$ B signaling pathways. In the canonical NF- $\kappa$ B 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- $\kappa$ B pathway by mediating a degradative ubiquitination of NF- $\kappa$ B-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 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- $\kappa$ B pathway, which in some cells is followed by induction of TNF- $\alpha$ , an NF- $\kappa$ B target gene [318, 319]. TNF- $\alpha$  can act in an autocrine manner and activate NF- $\kappa$ B as well as lead to caspase-8-dependent cell death in many tumor cells. By inhibiting TNF- $\alpha$ , caspase-8, or NF- $\kappa$ B-activated transcription it is possible to prevent apoptosis mediated by Smac mimetics [275, 320, 321]. However, it is not always that induction of TNF- $\alpha$  is sufficient to induce cell death. Chronic lymphocytic leukemia (CLL) cells are resistant to Smac mimetics, despite production of TNF- $\alpha$ , 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- $\kappa$ B signaling pathways

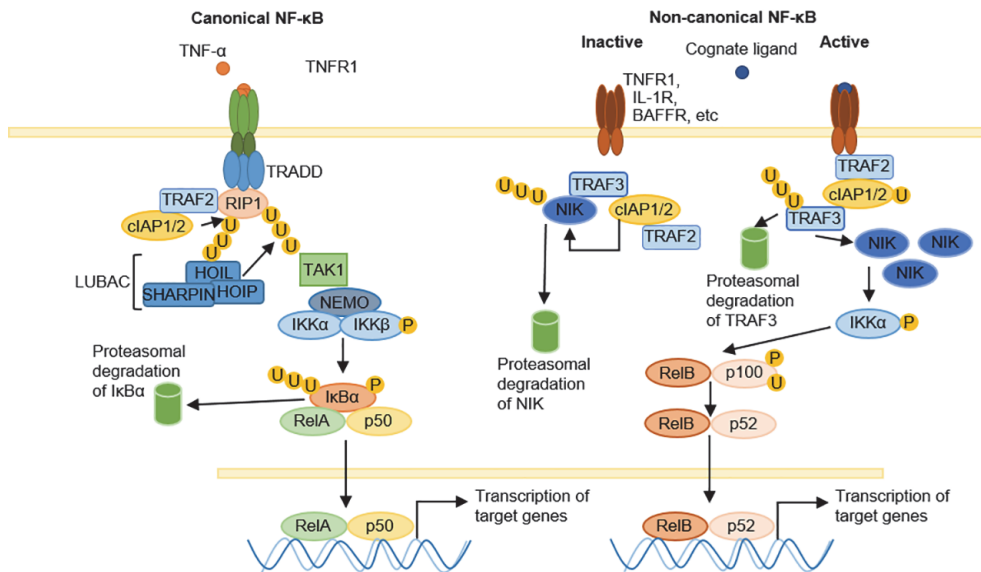
## Overview

There are five inducible transcription factors belonging to the nuclear factor- $\kappa$ B (NF- $\kappa$ B) family, called NF- $\kappa$ B/Rel proteins. They can all form either homo- or heterodimers. These transcription factors take part in either the canonical or the non-canonical NF- $\kappa$ B 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- $\kappa$ B signaling pathway

Induction of the canonical NF- $\kappa$ B 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- $\kappa$ B kinase subunit  $\alpha$  (IKK $\alpha$ ), IKK $\beta$ , and IKK $\gamma$  (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 I $\kappa$ B $\alpha$ , 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- $\kappa$ B 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 TNF-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- $\kappa$ B pathway can be induced upon binding of TNF- $\alpha$ , IL-1, lipopolysaccharide (LPS), or double-stranded RNA (dsRNA), to their cognate receptors TNFR, IL-1R, TLR, BAFFR (B cell-activating factor receptor), LT $\beta$ R (lymphotoxin  $\beta$  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 IKK $\alpha$  [344, 352, 353]. In contrast to the canonical NF- $\kappa$ B pathway, where IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$  (NEMO) are necessary, the non-canonical NF- $\kappa$ B pathway is solely dependent on phosphorylation of IKK $\alpha$  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- $\kappa$ B 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- $\kappa$ B pathways, which is mediated by RIP1. Here, RIP1 suppresses TNFR1-mediated activation of the non-canonical NF- $\kappa$ B [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- $\kappa$ B 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- $\kappa$ B 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- $\alpha$ , which in humans exists as 13 homologous subtypes [366], IFN- $\beta$ , IFN- $\delta$ , IFN- $\varepsilon$ , IFN- $\zeta$ , IFN- $\kappa$ , IFN- $\nu$ , IFN- $\tau$ , and IFN- $\omega$ . However, only IFN- $\alpha$ , IFN- $\beta$ , IFN- $\varepsilon$ , IFN- $\kappa$ , and IFN- $\omega$  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- $\alpha$

IFN- $\alpha$  is produced by plasmacytoid dendritic cells (pDCs) and leukocytes [373]. Depending on the IFN- $\alpha$  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- $\alpha/\beta$  receptor

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

## Interferon- $\beta$

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

## Type II interferons

There is only one type II IFN, namely IFN- $\gamma$ , and it is structurally different from the type I IFNs [383, 384]. The active form of IFN- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\lambda$ 1 (IL-29), IFN- $\lambda$ 2 (IL-28A), IFN- $\lambda$ 3 (IL-28B), and IFN- $\lambda$ 4 [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- $\alpha$  and - $\beta$  and not by IFN- $\gamma$ . On the other hand, IFN- $\gamma$  is the only IFN that can induce *IRF1* (IFN-regulatory factor 1) [398].

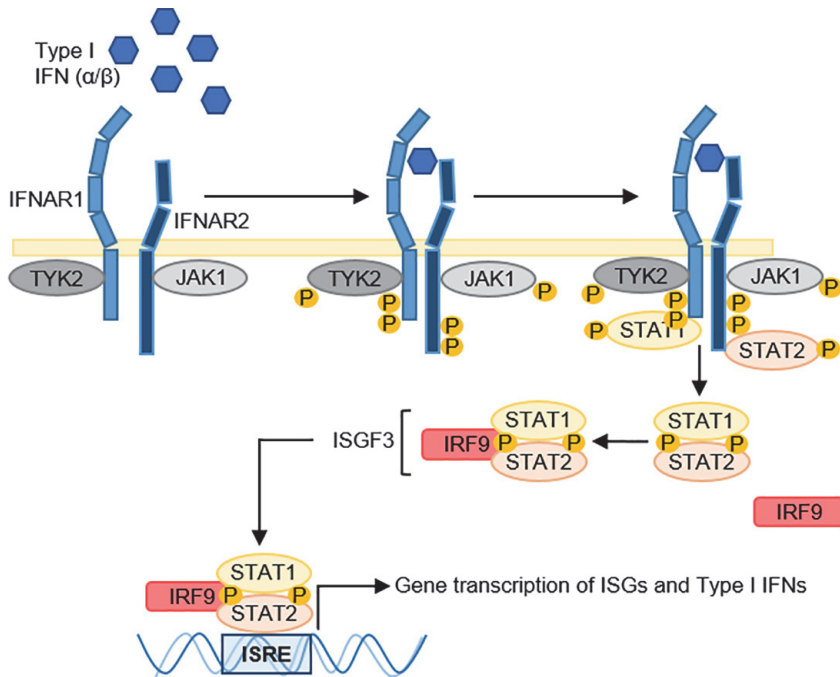
To enable induction of IFN-regulated genes, the IFNs must bind to their specific transmembrane receptors on the cell surface [399].

- 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- $\alpha/\beta$ /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- $\gamma$ /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- $\alpha/\beta$  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- $\beta$ , which

can act through an auto- or paracrine loop to further enhance IFN- $\beta$  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

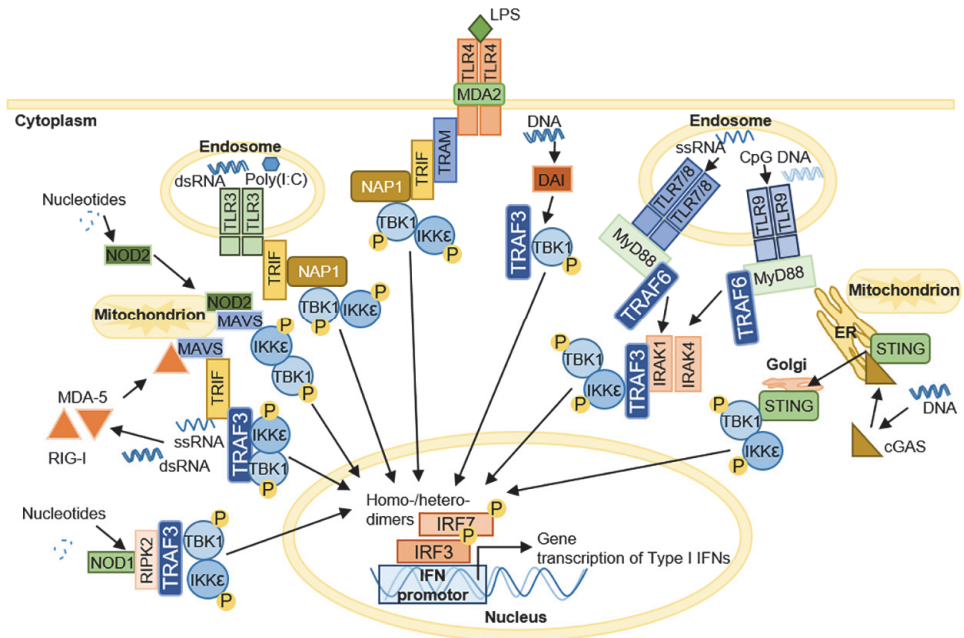
Type I IFNs are induced upon recognition of different pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), such as viral or bacterial double/single-stranded (ds/ss) RNA or DNA, cytosolic genomic DNA (cgDNA), or lipopolysaccharide (LPS) (Table 2). The different 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 IKK $\epsilon$  (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- $\beta$  (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 IKK $\epsilon$  [444, 447, 451, 452], resulting in activation of TBK1 and IKK $\epsilon$ . Both dsRNA and Poly(I:C), a structurally similar analogue to dsRNA, can stimulate TLR3 [455, 456].

TLR-7, -8, and -9-mediated IFN- $\alpha/\beta$ -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 IKK $\epsilon$  to cause activation of TBK1 and IKK $\epsilon$  [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/IKK $\epsilon$ , 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 IKK $\epsilon$  are activated following association of TRIF, TRAF3, TBK1, and IKK $\epsilon$  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 IKK $\epsilon$  [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 IKK $\epsilon$  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 IKK $\epsilon$  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- $\kappa$ B (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- $\beta$ ) [488]. The *IFNB1* promoter is controlled by four positive regulatory domains (PRDs) I-IV, where the IRFs bind to PRD I and III, NF- $\kappa$ B 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 IKK $\epsilon$

Although the IKK-related kinases (TBK1 and IKK $\epsilon$ ) today primarily are known to be essential for activation of IRF3 signaling [445], they were first identified as activators of NF- $\kappa$ B [499]. In this context, TBK1 and IKK $\epsilon$  can phosphorylate the canonical IKK complex [500] and thereby cause I $\kappa$ B degradation [501]. IKK $\alpha/\beta$  have also been found to activate TBK1 and IKK $\epsilon$  via phosphorylation [500]. Nevertheless, IKK $\epsilon$  and TBK1 and the canonical IKKs (IKK $\alpha/\beta$ ) were shown to be regulated by different pathways following stimulation of IL-1R, TLR3, and TLR4 [500]. Another effect of TBK1 and IKK $\epsilon$  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 IKK $\epsilon$  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 IKK $\epsilon$  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 IKK $\epsilon$  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 IKK $\epsilon$  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 IKK $\epsilon$  [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 IKK $\epsilon$  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- $\beta$ .



## TRAF3

TRAF3 is a protein which has been found to be important in many different pathways resulting in IFN- $\beta$ -induction. It is required for TRIF-dependent TLR-mediated induction of IFN- $\beta$  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 IKK $\epsilon$  [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<sup>+</sup> T-helper 1 (Th1) cells through enhancement of IFN- $\gamma$  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- $\alpha$  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- $\alpha$  to induce cell death in acute myeloid leukemia (AML) through induction of TNF- $\alpha$  and its subsequent signaling [539]. Treatment of renal cell carcinoma (RCC) with Smac mimetic and IFN- $\alpha$  also induced apoptosis [536]. This was found to be independent of TNF- $\alpha$  and RIP1 kinase activity, but dependent on a potential scaffold role of RIP1. One study showed that the combination of Smac mimetic and IFN- $\alpha$  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- $\alpha$  [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- $\alpha$ -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- $\kappa$ B 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- $\kappa$ B pathway. In addition, LCL161 enhanced cIAP2 mRNA levels both in CAMA-1 and MCF-7 cells. This was dependent on the non-canonical NF- $\kappa$ B pathway since knockdown of NIK suppressed this effect. Induction of NF- $\kappa$ B signaling following Smac mimetic treatment have previously been found to stimulate TNF- $\alpha$ -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- $\kappa$ B 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- $\kappa$ B 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<sub>s</sub>) and long (c-FLIP<sub>L</sub>). 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<sub>s</sub>, 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<sub>L</sub>, which can either inhibit or potentiate caspase-8 activation depending on high or low ratio of c-FLIP<sub>L</sub>: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<sub>L</sub> 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<sub>s</sub>, 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- $\alpha$ , but neither substance had any effect 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- $\kappa$ B- 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- $\kappa$ B 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- $\kappa$ B and IFN signaling was further examined. There was a tendency towards potentiation of the expression of IFN-inducible genes, but a suppression of NF- $\kappa$ B-inducible genes by zVAD-FMK. Similar to the morphology change, the induced expression of IFN- and NF- $\kappa$ B-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- $\beta$ 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- $\kappa$ B-inducible genes. This led us to study mediators and mechanisms behind induction of IFN signaling following LCL161 and TRAIL treatment. We examined the NF- $\kappa$ B and IFN signaling pathways and found increased levels of p52, indicating activation of non-canonical NF- $\kappa$ B 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- $\kappa$ B 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- $\beta$  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 *IFNBI* mRNA levels, suggesting that IFN- $\beta$  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 *IFNBI*, 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 *IFNBI* 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 *IFNBI* 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- $\beta$  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- $\kappa$ B signaling, which was dependent on FADD and caspase-8 [565], and to mediate production of IFN- $\beta$  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 IKK $\epsilon$ . The possible involvement of these and other proteins in IFN- $\beta$ -induction were investigated in Paper IV.

## Paper IV

### **TRAIL- and Smac mimetic-mediated activation of TBK1 and NF- $\kappa$ B 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- $\beta$  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- $\beta$  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 IKK $\epsilon$ , 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 IKK $\epsilon$  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 IKK $\epsilon$ , 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 IKK $\epsilon$  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<sup>-/-</sup> mice [569], suggesting a pathway independent of activation of TBK1, IKK $\epsilon$ , 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 IKK $\epsilon$ . 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<sup>-/-</sup>IKK $\epsilon$ <sup>-/-</sup> background [571], and to block CYLD phosphorylation following deletion of TBK1 and IKK $\epsilon$  with CRISPR/Cas9 [572].

NF- $\kappa$ B 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 I $\kappa$ B $\alpha$ , an indication of active canonical NF- $\kappa$ B pathway [573, 574], and downregulation of

p65 and IKK $\beta$  did not impact STAT1 phosphorylation. A critical involvement of the canonical pathway was therefore discarded.

TLR- and RLR-stimulated induction of IFN- $\beta$  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- $\beta$ . 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.

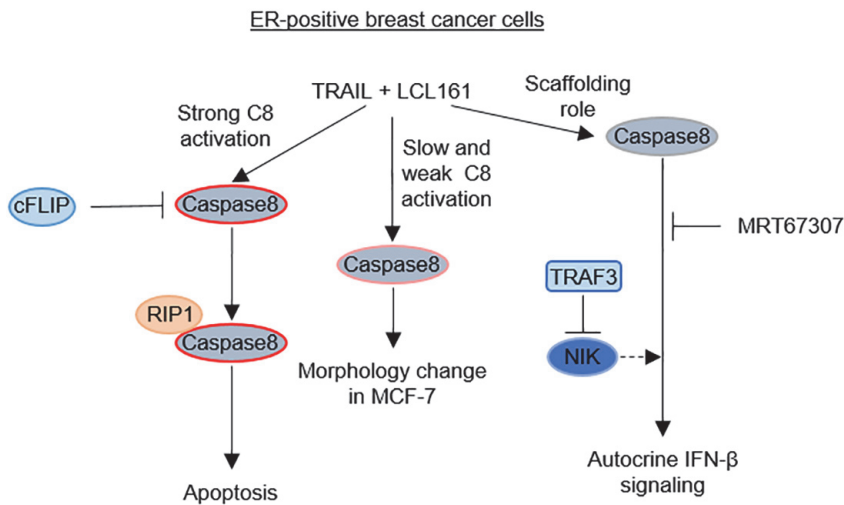
## Conclusions

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- $\kappa$ B and IFN signaling, in which caspase-8, independently of its enzymatic activity, is involved in mediating an IFN- $\beta$ -induction and autocrine IFN- $\beta$  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- $\kappa$ B and IFN signaling, which are separated from the induction of morphology change in MCF-7.
- LCL161 and TRAIL induce non-canonical NF- $\kappa$ B and IFN signaling in MCF-7 cells, and in CAMA-1 if caspases are inhibited. Non-canonical NF- $\kappa$ B does not mediate the IFN-induction.
- Caspase-8 has a non-apoptotic and non-enzymatic function in mediating autocrine IFN- $\beta$  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- $\kappa$ B pathway, is a potential mediator of the IFN signaling induced by TRAIL and LCL161, whereas the canonical NF- $\kappa$ B 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- $\beta$  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- $\beta$ -induction, whereas MRT67307 suppresses it.

# Acknowledgements

First of all, I would like to thank my supervisor **Christer** for taking me in as a master student during a summer project for almost seven years ago and for keeping me for a master project and finally a PhD project. It has definitely been a journey, a rollercoaster sometimes, which I will never forget and I have learned so much. I appreciate all the guidance you have given me and I will carry this knowledge with me. You are a true inspiration with your scientific mind.

I would also like to thank my co-supervisor **Alexander**, I have learned a lot from your scientific thoughts.

Thanks to my group members. **Annika**, I am glad you joined our group. **Christian**, you have taught me more or less everything I know about lab work and you can always answer my questions – no matter what they are about. I have enjoyed discussing sport and life with you, in between all science-stuff. You are a great tutor and a great companion at conferences.

**Margareta** and **Christina**, TCR would not be the same without you two keeping an eye (or two) on everything. Thanks for always spreading joy and for letting us come to you with questions and issues of any kind. **Elisabeth** and **Johanna** thanks for all administrative help over the years, I am truly grateful.

**Elinn**, you really are a true joy-spreader. I am glad for all our long and short chats about life and all that comes with it. **Rebecca**, I am sure that the rest of your PhD will be a success. **Elina**, thanks for making working in the cell lab fun and for always taking your time to talk. **Lina**, for sharing happiness and sorrow over Western blot with me and for constantly having a smile on your face. **Renée**, I could not have said it better than you did. It has been so much fun to go through the biomedicine programs and PhD studies with you.

To all other past and present members at TCR – you have all made these years fun and worth remembering. To those in the old Christmas committee – it was a pleasure.

Jag vill självklart också tacka min älskade **Erik** som, vad det känns som, alltid funnits vid min sida. Tack för att du stöttat mig i mitt studerande under alla dessa år. Jag är otroligt glad och tacksam över det liv och den tillvaro vi skapat oss. Nu väntar nya äventyr – det kommer bli fantastiskt. Jag älskar dig.

**Mamma, pappa** och min älskade syster **Rebecca**, utan er hade jag inte kunnat genomföra detta. Tack för att ni alltid trott på mig och för att ni motiverat, stöttat och ställt upp. Ni är mina klippor.

Äntligen **mormor**, som du sett fram emot detta. Du är och har alltid varit en trygg punkt i mitt liv. **Farmor**, jag önskar att du hade varit här och sett mig gå i mål. Min kära svärfamilj **Svenssons/Holmberg**, tack för all ert peppande och påhejande, det har varit guld värt.

Och tack till alla underbara vänner i **The Gang**, namnet är vedertaget och jag uppskattar er alla för ert stöd. **Amanda**, det har varit så kul att ha delat detta mig dig. Vi tog vara på alla tillfällen vi gavs för att fortsätta vara kursare. **Anna, Johanna, Josefine**, och **Lena**, jag är så glad att jag kunnat kalla er vänner i så många år. Och tack till alla **nära och kära**, ni är många som följt min doktorandtid.

# References

1. Socialstyrelsen: Statistics on Causes of Death 2020. 2021.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018, **68**(6):394-424.
3. White MC, Holman DM, Boehm JE, Peipins LA, Grossman M, Henley SJ: Age and cancer risk: a potentially modifiable relationship. *Am J Prev Med* 2014, **46**(3 Suppl 1):S7-15.
4. Hart SN, Polley EC, Yussuf A, Yadav S, Goldgar DE, Hu C, LaDuca H, Smith LP, Fujimoto J, Li S *et al*: Mutation prevalence tables for hereditary cancer derived from multigene panel testing. *Hum Mutat* 2020, **41**(8):e1-e6.
5. American Cancer Society medical and editorial content team: Cancer Facts & Figures 2022. 2022.
6. Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000, **100**(1):57-70.
7. Hanahan D, Weinberg RA: Hallmarks of cancer: the next generation. *Cell* 2011, **144**(5):646-674.
8. Chatterjee N, Walker GC: Mechanisms of DNA damage, repair, and mutagenesis. *Environ Mol Mutagen* 2017, **58**(5):235-263.
9. Martincorena I, Campbell PJ: Somatic mutation in cancer and normal cells. *Science* 2015, **349**(6255):1483-1489.
10. Cooper GM: The Cell: A Molecular Approach, 2 edn; 2000.
11. Regionala Cancercentrum i Samverkan: Nationellt vårdprogram - Bröstcancer. 2020.
12. Socialstyrelsen: Statistik om nyupptäckta cancerfall 2019. 2020.
13. Health and Medical Care: Statistics on Cancer Incidence 2019. 2020:4.
14. World Health Organization: Breast cancer. 2021.
15. Lu WL, Jansen L, Post WJ, Bonnema J, Van de Velde JC, De Bock GH: Impact on survival of early detection of isolated breast recurrences after the primary treatment for breast cancer: a meta-analysis. *Breast Cancer Res Treat* 2009, **114**(3):403-412.
16. Karim-Kos HE, de Vries E, Soerjomataram I, Lemmens V, Siesling S, Coebergh JW: Recent trends of cancer in Europe: a combined approach of incidence, survival and mortality for 17 cancer sites since the 1990s. *Eur J Cancer* 2008, **44**(10):1345-1389.
17. Spronk I, Schellevis FG, Burgers JS, de Bock GH, Korevaar JC: Incidence of isolated local breast cancer recurrence and contralateral breast cancer: A systematic review. *Breast* 2018, **39**:70-79.

18. Collaborative Group on Hormonal Factors in Breast C: Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. *Lancet Oncol* 2012, **13**(11):1141-1151.
19. Mørch LS, Skovlund CW, Hannaford PC, Iversen L, Fielding S, Lidegaard O: Contemporary Hormonal Contraception and the Risk of Breast Cancer. *N Engl J Med* 2017, **377**(23):2228-2239.
20. Collaborative Group on Hormonal Factors in Breast C: Type and timing of menopausal hormone therapy and breast cancer risk: individual participant meta-analysis of the worldwide epidemiological evidence. *Lancet* 2019, **394**(10204):1159-1168.
21. Beral V, Million Women Study C: Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003, **362**(9382):419-427.
22. Ewertz M, Duffy SW, Adami HO, Kvale G, Lund E, Meirik O, Møller A, Soini I, Tulinius H: Age at first birth, parity and risk of breast cancer: a meta-analysis of 8 studies from the Nordic countries. *Int J Cancer* 1990, **46**(4):597-603.
23. Pettersson A, Graff RE, Ursin G, Santos Silva ID, McCormack V, Baglietto L, Vachon C, Bakker MF, Giles GG, Chia KS *et al*: Mammographic density phenotypes and risk of breast cancer: a meta-analysis. *J Natl Cancer Inst* 2014, **106**(5).
24. Robinson D, Holmberg L, Møller H: The occurrence of invasive cancers following a diagnosis of breast carcinoma in situ. *Br J Cancer* 2008, **99**(4):611-615.
25. Coughlin SS: Epidemiology of Breast Cancer in Women. *Adv Exp Med Biol* 2019, **1152**:9-29.
26. Roy R, Chun J, Powell SN: BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer* 2011, **12**(1):68-78.
27. Antoniou A, Pharoah PD, Narod S, Risch HA, Eyfjord JE, Hopper JL, Loman N, Olsson H, Johannsson O, Borg A *et al*: Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. *Am J Hum Genet* 2003, **72**(5):1117-1130.
28. Nielsen FC, van Overeem Hansen T, Sørensen CS: Hereditary breast and ovarian cancer: new genes in confined pathways. *Nat Rev Cancer* 2016, **16**(9):599-612.
29. Atchley DP, Albarracin CT, Lopez A, Valero V, Amos CI, Gonzalez-Angulo AM, Hortobagyi GN, Arun BK: Clinical and pathologic characteristics of patients with BRCA-positive and BRCA-negative breast cancer. *J Clin Oncol* 2008, **26**(26):4282-4288.
30. Hamajima N, Hirose K, Tajima K, Rohan T, Calle EE, Heath CW, Jr., Coates RJ, Liff JM, Talamini R, Chantarakul N *et al*: Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. *Br J Cancer* 2002, **87**(11):1234-1245.



31. Romieu I, Scoccianti C, Chajes V, de Batlle J, Biessy C, Dossus L, Baglietto L, Clavel-Chapelon F, Overvad K, Olsen A *et al*: Alcohol intake and breast cancer in the European prospective investigation into cancer and nutrition. *Int J Cancer* 2015, **137**(8):1921-1930.
32. Garaycoechea JI, Crossan GP, Langevin F, Mulderrig L, Louzada S, Yang F, Guilbaud G, Park N, Roerink S, Nik-Zainal S *et al*: Alcohol and endogenous aldehydes damage chromosomes and mutate stem cells. *Nature* 2018, **553**(7687):171-177.
33. Inoue-Choi M, Sinha R, Gierach GL, Ward MH: Red and processed meat, nitrite, and heme iron intakes and postmenopausal breast cancer risk in the NIH-AARP Diet and Health Study. *Int J Cancer* 2016, **138**(7):1609-1618.
34. Anderson JJ, Darwis NDM, Mackay DF, Celis-Morales CA, Lyall DM, Sattar N, Gill JMR, Pell JP: Red and processed meat consumption and breast cancer: UK Biobank cohort study and meta-analysis. *Eur J Cancer* 2018, **90**:73-82.
35. Cheraghi Z, Poorolajal J, Hashem T, Esmailnasab N, Doosti Irani A: Effect of body mass index on breast cancer during premenopausal and postmenopausal periods: a meta-analysis. *PLoS One* 2012, **7**(12):e51446.
36. Key TJ, Appleby PN, Reeves GK, Roddam A, Dorgan JF, Longcope C, Stanczyk FZ, Stephenson HE, Jr., Falk RT, Miller R *et al*: Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *J Natl Cancer Inst* 2003, **95**(16):1218-1226.
37. Pearson-Stuttard J, Zhou B, Kontis V, Bentham J, Gunter MJ, Ezzati M: Worldwide burden of cancer attributable to diabetes and high body-mass index: a comparative risk assessment. *Lancet Diabetes Endocrinol* 2018, **6**(6):e6-e15.
38. Iyengar NM, Arthur R, Manson JE, Chlebowski RT, Kroenke CH, Peterson L, Cheng TD, Feliciano EC, Lane D, Luo J *et al*: Association of Body Fat and Risk of Breast Cancer in Postmenopausal Women With Normal Body Mass Index: A Secondary Analysis of a Randomized Clinical Trial and Observational Study. *JAMA Oncol* 2019, **5**(2):155-163.
39. Bjorge T, Haggstrom C, Ghaderi S, Nagel G, Manjer J, Tretli S, Ulmer H, Harlid S, Rosendahl AH, Lang A *et al*: BMI and weight changes and risk of obesity-related cancers: a pooled European cohort study. *Int J Epidemiol* 2019, **48**(6):1872-1885.
40. Shi Y, Li T, Wang Y, Zhou L, Qin Q, Yin J, Wei S, Liu L, Nie S: Household physical activity and cancer risk: a systematic review and dose-response meta-analysis of epidemiological studies. *Sci Rep* 2015, **5**:14901.
41. Wu Y, Zhang D, Kang S: Physical activity and risk of breast cancer: a meta-analysis of prospective studies. *Breast Cancer Res Treat* 2013, **137**(3):869-882.
42. Friedenreich CM, Neilson HK, Lynch BM: State of the epidemiological evidence on physical activity and cancer prevention. *Eur J Cancer* 2010, **46**(14):2593-2604.
43. Gaudet MM, Gapstur SM, Sun J, Diver WR, Hannan LM, Thun MJ: Active smoking and breast cancer risk: original cohort data and meta-analysis. *J Natl Cancer Inst* 2013, **105**(8):515-525.

44. Dossus L, Boutron-Ruault MC, Kaaks R, Gram IT, Vilier A, Fervers B, Manjer J, Tjonneland A, Olsen A, Overvad K *et al*: Active and passive cigarette smoking and breast cancer risk: results from the EPIC cohort. *Int J Cancer* 2014, **134**(8):1871-1888.
45. Ma H, Bernstein L, Pike MC, Ursin G: Reproductive factors and breast cancer risk according to joint estrogen and progesterone receptor status: a meta-analysis of epidemiological studies. *Breast Cancer Res* 2006, **8**(4):R43.
46. Collaborative Group on Hormonal Factors in Breast C: Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease. *Lancet* 2002, **360**(9328):187-195.
47. Chowdhury R, Sinha B, Sankar MJ, Taneja S, Bhandari N, Rollins N, Bahl R, Martines J: Breastfeeding and maternal health outcomes: a systematic review and meta-analysis. *Acta Paediatr* 2015, **104**(467):96-113.
48. Lanigan F, O'Connor D, Martin F, Gallagher WM: Molecular links between mammary gland development and breast cancer. *Cell Mol Life Sci* 2007, **64**(24):3159-3184.
49. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ: Purification and unique properties of mammary epithelial stem cells. *Nature* 2006, **439**(7079):993-997.
50. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE: Generation of a functional mammary gland from a single stem cell. *Nature* 2006, **439**(7072):84-88.
51. Yang X, Wang H, Jiao B: Mammary gland stem cells and their application in breast cancer. *Oncotarget* 2017, **8**(6):10675-10691.
52. Gudjonsson T, Adriance MC, Sternlicht MD, Petersen OW, Bissell MJ: Myoepithelial cells: their origin and function in breast morphogenesis and neoplasia. *J Mammary Gland Biol Neoplasia* 2005, **10**(3):261-272.
53. McGee SF, Lanigan F, Gilligan E, Groner B: Mammary gland biology and breast cancer. Conference on Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer Progression. *EMBO Rep* 2006, **7**(11):1084-1088.
54. McNally S, Stein T: Overview of Mammary Gland Development: A Comparison of Mouse and Human. *Methods Mol Biol* 2017, **1501**:1-17.
55. Milanese TR, Hartmann LC, Sellers TA, Frost MH, Vierkant RA, Maloney SD, Pankratz VS, Degnim AC, Vachon CM, Reynolds CA *et al*: Age-related lobular involution and risk of breast cancer. *J Natl Cancer Inst* 2006, **98**(22):1600-1607.
56. Watson CJ: Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. *Breast Cancer Res* 2006, **8**(2):203.
57. Hartmann LC, Degnim AC, Santen RJ, Dupont WD, Ghosh K: Atypical hyperplasia of the breast--risk assessment and management options. *N Engl J Med* 2015, **372**(1):78-89.
58. Myers DJ, Walls AL: Atypical Breast Hyperplasia. In: *StatPearls*. Treasure Island (FL); 2022.
59. Bombonati A, Sgroi DC: The molecular pathology of breast cancer progression. *J Pathol* 2011, **223**(2):307-317.

60. Weigelt B, Peterse JL, van 't Veer LJ: Breast cancer metastasis: markers and models. *Nat Rev Cancer* 2005, **5**(8):591-602.
61. Hunter KW, Crawford NP, Alsarraj J: Mechanisms of metastasis. *Breast Cancer Res* 2008, **10 Suppl 1**:S2.
62. Shah M, Allegrucci C: Keeping an open mind: highlights and controversies of the breast cancer stem cell theory. *Breast Cancer (Dove Med Press)* 2012, **4**:155-166.
63. Keller PJ, Arendt LM, Skibinski A, Logvinenko T, Klebba I, Dong S, Smith AE, Prat A, Perou CM, Gilmore H *et al*: Defining the cellular precursors to human breast cancer. *Proc Natl Acad Sci U S A* 2012, **109**(8):2772-2777.
64. Molyneux G, Geyer FC, Magnay FA, McCarthy A, Kendrick H, Natrajan R, Mackay A, Grigoriadis A, Tutt A, Ashworth A *et al*: BRCA1 basal-like breast cancers originate from luminal epithelial progenitors and not from basal stem cells. *Cell Stem Cell* 2010, **7**(3):403-417.
65. Makki J: Diversity of Breast Carcinoma: Histological Subtypes and Clinical Relevance. *Clin Med Insights Pathol* 2015, **8**:23-31.
66. American Cancer Society medical and editorial content team: About Breast Cancer - Breast Cancer Basics. 2019.
67. Sinn HP, Kreipe H: A Brief Overview of the WHO Classification of Breast Tumors, 4th Edition, Focusing on Issues and Updates from the 3rd Edition. *Breast Care (Basel)* 2013, **8**(2):149-154.
68. Weigelt B, Geyer FC, Reis-Filho JS: Histological types of breast cancer: how special are they? *Mol Oncol* 2010, **4**(3):192-208.
69. Rakha EA, El-Sayed ME, Menon S, Green AR, Lee AH, Ellis IO: Histologic grading is an independent prognostic factor in invasive lobular carcinoma of the breast. *Breast Cancer Res Treat* 2008, **111**(1):121-127.
70. Arpino G, Bardou VJ, Clark GM, Elledge RM: Infiltrating lobular carcinoma of the breast: tumor characteristics and clinical outcome. *Breast Cancer Res* 2004, **6**(3):R149-156.
71. Pereira H, Pinder SE, Sibbering DM, Galea MH, Elston CW, Blamey RW, Robertson JF, Ellis IO: Pathological prognostic factors in breast cancer. IV: Should you be a typer or a grader? A comparative study of two histological prognostic features in operable breast carcinoma. *Histopathology* 1995, **27**(3):219-226.
72. Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR *et al*: Breast cancer prognostic classification in the molecular era: the role of histological grade. *Breast Cancer Res* 2010, **12**(4):207.
73. Tomlinson-Hansen S, Khan M, Cassaro S: Breast Ductal Carcinoma in Situ. In: *StatPearls*. Treasure Island (FL); 2021.
74. Regionala Cancercentrum i Samverkan: Diagnos och behandling - Cancer i mjölkörtlarna, lobular bröstcancer. 2021.
75. Lobular Carcinoma in Situ (LCIS)
76. Elston CW, Ellis IO: Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991, **19**(5):403-410.

77. Rakha EA, El-Sayed ME, Lee AH, Elston CW, Grainge MJ, Hodi Z, Blamey RW, Ellis IO: Prognostic significance of Nottingham histologic grade in invasive breast carcinoma. *J Clin Oncol* 2008, **26**(19):3153-3158.
78. Frkovic-Grazio S, Bracko M: Long term prognostic value of Nottingham histological grade and its components in early (pT1N0M0) breast carcinoma. *J Clin Pathol* 2002, **55**(2):88-92.
79. Cserni G, Chmielik E, Cserni B, Tot T: The new TNM-based staging of breast cancer. *Virchows Arch* 2018, **472**(5):697-703.
80. Wolff AC, Hammond MEH, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, Bilous M, Ellis IO, Fitzgibbons P, Hanna W *et al*: Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. *Journal of Clinical Oncology* 2018, **36**(20):2105-+.
81. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA *et al*: Molecular portraits of human breast tumours. *Nature* 2000, **406**(6797):747-752.
82. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS *et al*: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 2001, **98**(19):10869-10874.
83. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S *et al*: Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* 2003, **100**(14):8418-8423.
84. Vieira AF, Schmitt F: An Update on Breast Cancer Multigene Prognostic Tests-Emergent Clinical Biomarkers. *Front Med (Lausanne)* 2018, **5**:248.
85. Prat A, Parker JS, Fan C, Perou CM: PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer. *Breast Cancer Res Treat* 2012, **135**(1):301-306.
86. Bastien RR, Rodriguez-Lescure A, Ebbert MT, Prat A, Munarriz B, Rowe L, Miller P, Ruiz-Borrego M, Anderson D, Lyons B *et al*: PAM50 breast cancer subtyping by RT-qPCR and concordance with standard clinical molecular markers. *BMC Med Genomics* 2012, **5**:44.
87. Acheampong T, Kehm RD, Terry MB, Argov EL, Tehranifar P: Incidence Trends of Breast Cancer Molecular Subtypes by Age and Race/Ethnicity in the US From 2010 to 2016. *JAMA Netw Open* 2020, **3**(8):e2013226.
88. Eroles P, Bosch A, Perez-Fidalgo JA, Lluch A: Molecular biology in breast cancer: intrinsic subtypes and signaling pathways. *Cancer Treat Rev* 2012, **38**(6):698-707.
89. Cho N: Molecular subtypes and imaging phenotypes of breast cancer. *Ultrasonography* 2016, **35**(4):281-288.
90. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X, Perou CM: Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res* 2010, **12**(5):R68.

91. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L *et al*: Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* 2004, **10**(16):5367-5374.
92. Rakha EA, Reis-Filho JS, Ellis IO: Basal-like breast cancer: a critical review. *J Clin Oncol* 2008, **26**(15):2568-2581.
93. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S *et al*: Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA* 2006, **295**(21):2492-2502.
94. Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, Perou CM, Nielsen TO: Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res* 2008, **14**(5):1368-1376.
95. Rakha EA, Ellis IO: Triple-negative/basal-like breast cancer: review. *Pathology* 2009, **41**(1):40-47.
96. Weigelt B, Mackay A, A'Hern R, Natrajan R, Tan DS, Dowsett M, Ashworth A, Reis-Filho JS: Breast cancer molecular profiling with single sample predictors: a retrospective analysis. *Lancet Oncol* 2010, **11**(4):339-349.
97. Dai X, Li T, Bai Z, Yang Y, Liu X, Zhan J, Shi B: Breast cancer intrinsic subtype classification, clinical use and future trends. *Am J Cancer Res* 2015, **5**(10):2929-2943.
98. Saal LH, Vallon-Christersson J, Hakkinen J, Hegardt C, Grabau D, Winter C, Brueffer C, Tang MH, Reuterswärd C, Schulz R *et al*: The Sweden Cancerome Analysis Network - Breast (SCAN-B) Initiative: a large-scale multicenter infrastructure towards implementation of breast cancer genomic analyses in the clinical routine. *Genome Med* 2015, **7**(1):20.
99. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, Senn HJ, Panel m: Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. *Ann Oncol* 2013, **24**(9):2206-2223.
100. Mohammed H, Russell IA, Stark R, Rueda OM, Hickey TE, Tarulli GA, Serandour AA, Birrell SN, Bruna A, Saadi A *et al*: Progesterone receptor modulates ERalpha action in breast cancer. *Nature* 2015, **523**(7560):313-317.
101. Vuong D, Simpson PT, Green B, Cummings MC, Lakhani SR: Molecular classification of breast cancer. *Virchows Arch* 2014, **465**(1):1-14.
102. Park S, Koo JS, Kim MS, Park HS, Lee JS, Lee JS, Kim SI, Park BW: Characteristics and outcomes according to molecular subtypes of breast cancer as classified by a panel of four biomarkers using immunohistochemistry. *Breast* 2012, **21**(1):50-57.
103. Hashmi AA, Aijaz S, Khan SM, Mahboob R, Irfan M, Zafar NI, Nisar M, Siddiqui M, Edhi MM, Faridi N *et al*: Prognostic parameters of luminal A and luminal B intrinsic breast cancer subtypes of Pakistani patients. *World J Surg Oncol* 2018, **16**(1):1.

104. Tsoutsou PG, Vozenin MC, Durham AD, Bourhis J: How could breast cancer molecular features contribute to locoregional treatment decision making? *Crit Rev Oncol Hematol* 2017, **110**:43-48.
105. Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H: Breast cancer subtypes and the risk of local and regional relapse. *J Clin Oncol* 2010, **28**(10):1684-1691.
106. Li ZH, Hu PH, Tu JH, Yu NS: Luminal B breast cancer: patterns of recurrence and clinical outcome. *Oncotarget* 2016, **7**(40):65024-65033.
107. Sircoulomb F, Bekhouche I, Finetti P, Adelaide J, Ben Hamida A, Bonansea J, Raynaud S, Innocenti C, Charafe-Jauffret E, Tarpin C *et al*: Genome profiling of ERBB2-amplified breast cancers. *BMC Cancer* 2010, **10**:539.
108. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P *et al*: Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res* 2005, **11**(16):5678-5685.
109. McKeage K, Perry CM: Trastuzumab: a review of its use in the treatment of metastatic breast cancer overexpressing HER2. *Drugs* 2002, **62**(1):209-243.
110. Llombart-Cussac A, Cortes J, Pare L, Galvan P, Bermejo B, Martinez N, Vidal M, Pernas S, Lopez R, Munoz M *et al*: HER2-enriched subtype as a predictor of pathological complete response following trastuzumab and lapatinib without chemotherapy in early-stage HER2-positive breast cancer (PAMELA): an open-label, single-group, multicentre, phase 2 trial. *Lancet Oncol* 2017, **18**(4):545-554.
111. Hennigs A, Riedel F, Gondos A, Sinn P, Schirmacher P, Marme F, Jager D, Kauczor HU, Stieber A, Lindel K *et al*: Prognosis of breast cancer molecular subtypes in routine clinical care: A large prospective cohort study. *BMC Cancer* 2016, **16**(1):734.
112. Fallahpour S, Navaneelan T, De P, Borgo A: Breast cancer survival by molecular subtype: a population-based analysis of cancer registry data. *CMAJ Open* 2017, **5**(3):E734-E739.
113. American Cancer Society: Breast Cancer Facts & Figures 2019-2020. 2019.
114. Riet FG, Fayard F, Arriagada R, Santos MA, Bourgier C, Ferchiou M, Heymann S, Delaloge S, Mazouni C, Dunant A *et al*: Preoperative radiotherapy in breast cancer patients: 32 years of follow-up. *Eur J Cancer* 2017, **76**:45-51.
115. Johnson-Arbor K, Dubey R: Doxorubicin. In: *StatPearls*. Treasure Island (FL); 2022.
116. Pommier Y, Leo E, Zhang H, Marchand C: DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol* 2010, **17**(5):421-433.
117. Abu Samaan TM, Samec M, Liskova A, Kubatka P, Busselberg D: Paclitaxel's Mechanistic and Clinical Effects on Breast Cancer. *Biomolecules* 2019, **9**(12).
118. PubChem Compound Summary for CID 2907, Cyclophosphamide
119. de Sousa GF, Wlodarczyk SR, Monteiro G: Carboplatin: molecular mechanisms of action associated with chemoresistance. *Brazilian Journal of Pharmaceutical Sciences* 2014, **50**(4):693-701.

120. Patel HK, Bihani T: Selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) in cancer treatment. *Pharmacol Ther* 2018, **186**:1-24.
121. Osborne CK, Schiff R: Mechanisms of endocrine resistance in breast cancer. *Annu Rev Med* 2011, **62**:233-247.
122. Shang Y, Brown M: Molecular determinants for the tissue specificity of SERMs. *Science* 2002, **295**(5564):2465-2468.
123. Wakeling AE, Bowler J: Steroidal pure antioestrogens. *J Endocrinol* 1987, **112**(3):R7-10.
124. Fawell SE, White R, Hoare S, Sydenham M, Page M, Parker MG: Inhibition of estrogen receptor-DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. *Proc Natl Acad Sci U S A* 1990, **87**(17):6883-6887.
125. Dauvois S, White R, Parker MG: The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. *J Cell Sci* 1993, **106** ( Pt 4):1377-1388.
126. Nicholson RI, Gee JM, Manning DL, Wakeling AE, Montano MM, Katzenellenbogen BS: Responses to pure antiestrogens (ICI 164384, ICI 182780) in estrogen-sensitive and -resistant experimental and clinical breast cancer. *Ann N Y Acad Sci* 1995, **761**:148-163.
127. Tancredi R, Furlanetto J, Loibl S: Endocrine Therapy in Premenopausal Hormone Receptor Positive/Human Epidermal Growth Receptor 2 Negative Metastatic Breast Cancer: Between Guidelines and Literature. *Oncologist* 2018, **23**(8):974-981.
128. Dowsett M, Haynes BP: Hormonal effects of aromatase inhibitors: focus on premenopausal effects and interaction with tamoxifen. *J Steroid Biochem Mol Biol* 2003, **86**(3-5):255-263.
129. Hilborn E, Stal O, Jansson A: Estrogen and androgen-converting enzymes 17beta-hydroxysteroid dehydrogenase and their involvement in cancer: with a special focus on 17beta-hydroxysteroid dehydrogenase type 1, 2, and breast cancer. *Oncotarget* 2017, **8**(18):30552-30562.
130. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J: Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res* 2001, **61**(12):4744-4749.
131. Spector NL, Blackwell KL: Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol* 2009, **27**(34):5838-5847.
132. Bayraktar S, Batoo S, Okuno S, Gluck S: Immunotherapy in breast cancer. *J Carcinog* 2019, **18**:2.
133. Li Y, Miao W, He D, Wang S, Lou J, Jiang Y, Wang S: Recent Progress on Immunotherapy for Breast Cancer: Tumor Microenvironment, Nanotechnology and More. *Front Bioeng Biotechnol* 2021, **9**:680315.
134. Zielinski C, Knapp S, Mascaux C, Hirsch F: Rationale for targeting the immune system through checkpoint molecule blockade in the treatment of non-small-cell lung cancer. *Ann Oncol* 2013, **24**(5):1170-1179.

135. Jiang Y, Chen M, Nie H, Yuan Y: PD-1 and PD-L1 in cancer immunotherapy: clinical implications and future considerations. *Hum Vaccin Immunother* 2019, **15**(5):1111-1122.
136. Sabatier R, Finetti P, Mamessier E, Adelaide J, Chaffanet M, Ali HR, Viens P, Caldas C, Birnbaum D, Bertucci F: Prognostic and predictive value of PDL1 expression in breast cancer. *Oncotarget* 2015, **6**(7):5449-5464.
137. Muenst S, Soysal SD, Gao F, Obermann EC, Oertli D, Gillanders WE: The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. *Breast Cancer Res Treat* 2013, **139**(3):667-676.
138. Mittendorf EA, Philips AV, Meric-Bernstam F, Qiao N, Wu Y, Harrington S, Su X, Wang Y, Gonzalez-Angulo AM, Akcakanat A *et al*: PD-L1 expression in triple-negative breast cancer. *Cancer Immunol Res* 2014, **2**(4):361-370.
139. Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H, Dieras V, Henschel V, Molinero L, Chui SY *et al*: Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2020, **21**(1):44-59.
140. O'Leary B, Finn RS, Turner NC: Treating cancer with selective CDK4/6 inhibitors. *Nat Rev Clin Oncol* 2016, **13**(7):417-430.
141. Shah M, Nunes MR, Stearns V: CDK4/6 Inhibitors: Game Changers in the Management of Hormone Receptor-Positive Advanced Breast Cancer? *Oncology (Williston Park)* 2018, **32**(5):216-222.
142. D'Arcy MS: Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol Int* 2019, **43**(6):582-592.
143. Zakeri ZF, Ahuja HS: Cell death/apoptosis: normal, chemically induced, and teratogenic effect. *Mutat Res* 1997, **396**(1-2):149-161.
144. Kondo K, Ohigashi I, Takahama Y: Thymus machinery for T-cell selection. *Int Immunol* 2019, **31**(3):119-125.
145. ElTanbouly MA, Noelle RJ: Rethinking peripheral T cell tolerance: checkpoints across a T cell's journey. *Nat Rev Immunol* 2021, **21**(4):257-267.
146. Nemazee D: Mechanisms of central tolerance for B cells. *Nat Rev Immunol* 2017, **17**(5):281-294.
147. Galluzzi L, Bravo-San Pedro JM, Vitale I, Aaronson SA, Abrams JM, Adam D, Alnemri ES, Altucci L, Andrews D, Annicchiarico-Petruzzelli M *et al*: Essential versus accessory aspects of cell death: recommendations of the NCCD 2015. *Cell Death Differ* 2015, **22**(1):58-73.
148. Tang D, Kang R, Berghe TV, Vandenabeele P, Kroemer G: The molecular machinery of regulated cell death. *Cell Res* 2019, **29**(5):347-364.
149. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S *et al*: Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ* 2012, **19**(1):107-120.
150. Kerr JF, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972, **26**(4):239-257.



151. Elmore S: Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007, **35**(4):495-516.
152. Ferraro-Peyret C, Quemeneur L, Flacher M, Revillard JP, Genestier L: Caspase-independent phosphatidylserine exposure during apoptosis of primary T lymphocytes. *J Immunol* 2002, **169**(9):4805-4810.
153. Martin SJ, Finucane DM, Amarante-Mendes GP, O'Brien GA, Green DR: Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. *J Biol Chem* 1996, **271**(46):28753-28756.
154. Fujikura D, Miyazaki T: Programmed Cell Death in the Pathogenesis of Influenza. *Int J Mol Sci* 2018, **19**(7).
155. Nagata S, Tanaka M: Programmed cell death and the immune system. *Nat Rev Immunol* 2017, **17**(5):333-340.
156. Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, Kroemer G: Cell death modalities: classification and pathophysiological implications. *Cell Death Differ* 2007, **14**(7):1237-1243.
157. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR *et al*: Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 2009, **16**(1):3-11.
158. Galluzzi L, Lopez-Soto A, Kumar S, Kroemer G: Caspases Connect Cell-Death Signaling to Organismal Homeostasis. *Immunity* 2016, **44**(2):221-231.
159. Slee EA, Adrain C, Martin SJ: Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* 2001, **276**(10):7320-7326.
160. Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Kothe K, Kwiatkowski DJ *et al*: Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 1997, **278**(5336):294-298.
161. Sakahira H, Enari M, Nagata S: Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 1998, **391**(6662):96-99.
162. Bratton DL, Fadok VA, Richter DA, Kailey JM, Guthrie LA, Henson PM: Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *J Biol Chem* 1997, **272**(42):26159-26165.
163. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL: Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem* 2001, **276**(2):1071-1077.
164. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997, **91**(4):479-489.
165. Liu X, Kim CN, Yang J, Jemmerson R, Wang X: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996, **86**(1):147-157.

166. Doerflinger M, Glab JA, Puthalakath H: BH3-only proteins: a 20-year stock-take. *FEBS J* 2015, **282**(6):1006-1016.
167. Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC: Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci U S A* 1998, **95**(9):4997-5002.
168. Uren RT, O'Hely M, Iyer S, Bartolo R, Shi MX, Brouwer JM, Alsop AE, Dewson G, Kluck RM: Disordered clusters of Bak dimers rupture mitochondria during apoptosis. *Elife* 2017, **6**.
169. Oltvai ZN, Milliman CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993, **74**(4):609-619.
170. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ: Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 1995, **80**(2):285-291.
171. Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ: tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* 2000, **14**(16):2060-2071.
172. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X: Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998, **94**(4):481-490.
173. Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB: BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev* 2001, **15**(12):1481-1486.
174. Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G: Mechanisms of cytochrome c release from mitochondria. *Cell Death Differ* 2006, **13**(9):1423-1433.
175. Zou H, Li Y, Liu X, Wang X: An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 1999, **274**(17):11549-11556.
176. Silke J, Meier P: Inhibitor of apoptosis (IAP) proteins-modulators of cell death and inflammation. *Cold Spring Harb Perspect Biol* 2013, **5**(2).
177. Eckelman BP, Salvesen GS, Scott FL: Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep* 2006, **7**(10):988-994.
178. Du C, Fang M, Li Y, Li L, Wang X: Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000, **102**(1):33-42.
179. Vande Walle L, Lamkanfi M, Vandenabeele P: The mitochondrial serine protease HtrA2/Omi: an overview. *Cell Death Differ* 2008, **15**(3):453-460.
180. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, Alnemri ES, Altucci L, Amelio I, Andrews DW *et al*: Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ* 2018, **25**(3):486-541.
181. Ashkenazi A, Dixit VM: Death receptors: signaling and modulation. *Science* 1998, **281**(5381):1305-1308.
182. Guicciardi ME, Gores GJ: Life and death by death receptors. *FASEB J* 2009, **23**(6):1625-1637.

183. Legembre P, Daburon S, Moreau P, Moreau JF, Taupin JL: Modulation of Fas-mediated apoptosis by lipid rafts in T lymphocytes. *J Immunol* 2006, **176**(2):716-720.
184. Algeciras-Schimmich A, Shen L, Barnhart BC, Murmann AE, Burkhardt JK, Peter ME: Molecular ordering of the initial signaling events of CD95. *Mol Cell Biol* 2002, **22**(1):207-220.
185. Eramo A, Sargiacomo M, Ricci-Vitiani L, Todaro M, Stassi G, Messina CG, Parolini I, Lotti F, Sette G, Peschle C *et al*: CD95 death-inducing signaling complex formation and internalization occur in lipid rafts of type I and type II cells. *Eur J Immunol* 2004, **34**(7):1930-1940.
186. Ashkenazi A: Targeting the extrinsic apoptosis pathway in cancer. *Cytokine Growth Factor Rev* 2008, **19**(3-4):325-331.
187. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME: Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* 1995, **14**(22):5579-5588.
188. Irmeler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C *et al*: Inhibition of death receptor signals by cellular FLIP. *Nature* 1997, **388**(6638):190-195.
189. Kataoka T, Schroter M, Hahne M, Schneider P, Irmeler M, Thome M, Froelich CJ, Tschopp J: FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation. *J Immunol* 1998, **161**(8):3936-3942.
190. Scaffidi C, Schmitz I, Krammer PH, Peter ME: The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem* 1999, **274**(3):1541-1548.
191. Kluck RM, Esposti MD, Perkins G, Renken C, Kuwana T, Bossy-Wetzel E, Goldberg M, Allen T, Barber MJ, Green DR *et al*: The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. *J Cell Biol* 1999, **147**(4):809-822.
192. Ozoren N, El-Deiry WS: Defining characteristics of Types I and II apoptotic cells in response to TRAIL. *Neoplasia* 2002, **4**(6):551-557.
193. Kucka K, Wajant H: Receptor Oligomerization and Its Relevance for Signaling by Receptors of the Tumor Necrosis Factor Receptor Superfamily. *Front Cell Dev Biol* 2020, **8**:615141.
194. Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI *et al*: Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 1997, **277**(5327):818-821.
195. Petak I, Douglas L, Tillman DM, Vernes R, Houghton JA: Pediatric rhabdomyosarcoma cell lines are resistant to Fas-induced apoptosis and highly sensitive to TRAIL-induced apoptosis. *Clin Cancer Res* 2000, **6**(10):4119-4127.
196. Nimmanapalli R, Perkins CL, Orlando M, O'Bryan E, Nguyen D, Bhalla KN: Pretreatment with paclitaxel enhances apo-2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of prostate cancer cells by inducing death receptors 4 and 5 protein levels. *Cancer Res* 2001, **61**(2):759-763.

197. Lincz LF, Yeh TX, Spencer A: TRAIL-induced eradication of primary tumour cells from multiple myeloma patient bone marrows is not related to TRAIL receptor expression or prior chemotherapy. *Leukemia* 2001, **15**(10):1650-1657.
198. Sanlioglu AD, Dirice E, Aydin C, Erin N, Koksoy S, Sanlioglu S: Surface TRAIL decoy receptor-4 expression is correlated with TRAIL resistance in MCF7 breast cancer cells. *BMC Cancer* 2005, **5**:54.
199. O'Leary L, van der Sloot AM, Reis CR, Deegan S, Ryan AE, Dhami SP, Murillo LS, Cool RH, Correa de Sampaio P, Thompson K *et al*: Decoy receptors block TRAIL sensitivity at a supracellular level: the role of stromal cells in controlling tumour TRAIL sensitivity. *Oncogene* 2016, **35**(10):1261-1270.
200. Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P, Huang A, Donahue CJ, Sherwood SW, Baldwin DT *et al*: Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 1998, **396**(6712):699-703.
201. Zhao L, Fu Q, Pan L, Piai A, Chou JJ: The Diversity and Similarity of Transmembrane Trimerization of TNF Receptors. *Front Cell Dev Biol* 2020, **8**:569684.
202. Smith CA, Farrah T, Goodwin RG: The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 1994, **76**(6):959-962.
203. Rauert H, Wicovsky A, Muller N, Siegmund D, Spindler V, Waschke J, Kneitz C, Wajant H: Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). *J Biol Chem* 2010, **285**(10):7394-7404.
204. Marchetti L, Klein M, Schlett K, Pfizenmaier K, Eisel UL: Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway. *J Biol Chem* 2004, **279**(31):32869-32881.
205. Jupp OJ, McFarlane SM, Anderson HM, Littlejohn AF, Mohamed AA, MacKay RH, Vandenabeele P, MacEwan DJ: Type II tumour necrosis factor-alpha receptor (TNFR2) activates c-Jun N-terminal kinase (JNK) but not mitogen-activated protein kinase (MAPK) or p38 MAPK pathways. *Biochem J* 2001, **359**(Pt 3):525-535.
206. Wajant H, Siegmund D: TNFR1 and TNFR2 in the Control of the Life and Death Balance of Macrophages. *Front Cell Dev Biol* 2019, **7**:91.
207. Wajant H, Scheurich P: TNFR1-induced activation of the classical NF-kappaB pathway. *FEBS J* 2011, **278**(6):862-876.
208. Ting AT, Pimentel-Muinos FX, Seed B: RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J* 1996, **15**(22):6189-6196.
209. Samuel T, Welsh K, Lober T, Togo SH, Zapata JM, Reed JC: Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. *J Biol Chem* 2006, **281**(2):1080-1090.

210. Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, Gillard JW, Jaquith JB, Morris SJ, Barker PA: cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol Cell* 2008, **30**(6):689-700.
211. Ikeda F, Deribe YL, Skanland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, van Wijk SJ, Goswami P, Nagy V, Terzic J *et al*: SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis. *Nature* 2011, **471**(7340):637-641.
212. Peltzer N, Darding M, Walczak H: Holding RIPK1 on the Ubiquitin Leash in TNFR1 Signaling. *Trends Cell Biol* 2016, **26**(6):445-461.
213. Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ: Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol Cell* 2006, **22**(2):245-257.
214. Fan Y, Yu Y, Shi Y, Sun W, Xie M, Ge N, Mao R, Chang A, Xu G, Schneider MD *et al*: Lysine 63-linked polyubiquitination of TAK1 at lysine 158 is required for tumor necrosis factor alpha- and interleukin-1beta-induced IKK/NF-kappaB and JNK/AP-1 activation. *J Biol Chem* 2010, **285**(8):5347-5360.
215. Gough P, Myles IA: Tumor Necrosis Factor Receptors: Pleiotropic Signaling Complexes and Their Differential Effects. *Front Immunol* 2020, **11**:585880.
216. Muppidi JR, Tschoop J, Siegel RM: Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. *Immunity* 2004, **21**(4):461-465.
217. Micheau O, Tschoop J: Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 2003, **114**(2):181-190.
218. Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F, Zachariou A, Lopez J, MacFarlane M, Cain K *et al*: The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Mol Cell* 2011, **43**(3):432-448.
219. Feoktistova M, Makarov R, Brenji S, Schneider AT, Hooiveld GJ, Luedde T, Leverkus M, Yazdi AS, Panayotova-Dimitrova D: A20 Promotes Ripoptosome Formation and TNF-Induced Apoptosis via cIAPs Regulation and NIK Stabilization in Keratinocytes. *Cells* 2020, **9**(2).
220. Feoktistova M, Makarov R, Yazdi AS, Panayotova-Dimitrova D: RIPK1 and TRADD Regulate TNF-Induced Signaling and Ripoptosome Formation. *Int J Mol Sci* 2021, **22**(22).
221. von Karstedt S, Montinaro A, Walczak H: Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. *Nat Rev Cancer* 2017, **17**(6):352-366.
222. Ozoren N, El-Deiry WS: Cell surface Death Receptor signaling in normal and cancer cells. *Semin Cancer Biol* 2003, **13**(2):135-147.
223. van Roosmalen IA, Quax WJ, Kruijt FA: Two death-inducing human TRAIL receptors to target in cancer: similar or distinct regulation and function? *Biochem Pharmacol* 2014, **91**(4):447-456.
224. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, Ashkenazi A, de Vos AM: Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol Cell* 1999, **4**(4):563-571.

225. Schneider P, Thome M, Burns K, Bodmer JL, Hofmann K, Kataoka T, Holler N, Tschopp J: TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. *Immunity* 1997, **7**(6):831-836.
226. Basit F, Humphreys R, Fulda S: RIP1 protein-dependent assembly of a cytosolic cell death complex is required for inhibitor of apoptosis (IAP) inhibitor-mediated sensitization to lexatumumab-induced apoptosis. *J Biol Chem* 2012, **287**(46):38767-38777.
227. Abhari BA, Cristofanon S, Kappler R, von Schweinitz D, Humphreys R, Fulda S: RIP1 is required for IAP inhibitor-mediated sensitization for TRAIL-induced apoptosis via a RIP1/FADD/caspase-8 cell death complex. *Oncogene* 2013, **32**(27):3263-3273.
228. Cristofanon S, Abhari BA, Krueger M, Tchoghandjian A, Momma S, Calaminus C, Vucic D, Pichler BJ, Fulda S: Identification of RIP1 as a critical mediator of Smac mimetic-mediated sensitization of glioblastoma cells for Drozitumab-induced apoptosis. *Cell Death Dis* 2015, **6**:e1724.
229. Tang W, Wang W, Zhang Y, Liu S, Liu Y, Zheng D: TRAIL receptor mediates inflammatory cytokine release in an NF-kappaB-dependent manner. *Cell Res* 2009, **19**(6):758-767.
230. Hartwig T, Montinaro A, von Karstedt S, Sevko A, Surinova S, Chakravarthy A, Taraborrelli L, Draber P, Lafont E, Arce Vargas F *et al*: The TRAIL-Induced Cancer Secretome Promotes a Tumor-Supportive Immune Microenvironment via CCR2. *Mol Cell* 2017, **65**(4):730-742 e735.
231. Henry CM, Martin SJ: Caspase-8 Acts in a Non-enzymatic Role as a Scaffold for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation. *Mol Cell* 2017, **65**(4):715-729 e715.
232. Kumar-Sinha C, Varambally S, Sreekumar A, Chinnaiyan AM: Molecular cross-talk between the TRAIL and interferon signaling pathways. *J Biol Chem* 2002, **277**(1):575-585.
233. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A *et al*: Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999, **104**(2):155-162.
234. Jin Z, McDonald ER, 3rd, Dicker DT, El-Deiry WS: Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. *J Biol Chem* 2004, **279**(34):35829-35839.
235. Zhang Y, Zhang B: TRAIL resistance of breast cancer cells is associated with constitutive endocytosis of death receptors 4 and 5. *Mol Cancer Res* 2008, **6**(12):1861-1871.
236. Zhang L, Fang B: Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer Gene Ther* 2005, **12**(3):228-237.
237. Fulda S: Tumor resistance to apoptosis. *Int J Cancer* 2009, **124**(3):511-515.
238. Lamkanfi M, Declercq W, Kalai M, Saelens X, Vandenabeele P: Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ* 2002, **9**(4):358-361.

239. Kumar S: Caspase function in programmed cell death. *Cell Death Differ* 2007, **14**(1):32-43.
240. Julien O, Wells JA: Caspases and their substrates. *Cell Death Differ* 2017, **24**(8):1380-1389.
241. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES: Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* 1998, **1**(7):949-957.
242. Shi Y: Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* 2002, **9**(3):459-470.
243. Salvesen GS, Dixit VM: Caspase activation: the induced-proximity model. *Proc Natl Acad Sci U S A* 1999, **96**(20):10964-10967.
244. Ramage P, Cheneval D, Chvei M, Graff P, Hemmig R, Heng R, Kocher HP, Mackenzie A, Memmert K, Revesz L *et al*: Expression, refolding, and autocatalytic proteolytic processing of the interleukin-1 beta-converting enzyme precursor. *J Biol Chem* 1995, **270**(16):9378-9383.
245. Yamin TT, Ayala JM, Miller DK: Activation of the native 45-kDa precursor form of interleukin-1-converting enzyme. *J Biol Chem* 1996, **271**(22):13273-13282.
246. Degterev A, Boyce M, Yuan J: A decade of caspases. *Oncogene* 2003, **22**(53):8543-8567.
247. Ghayur T, Banerjee S, Hugunin M, Butler D, Herzog L, Carter A, Quintal L, Sekut L, Talanian R, Paskind M *et al*: Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. *Nature* 1997, **386**(6625):619-623.
248. Jimenez Fernandez D, Lamkanfi M: Inflammatory caspases: key regulators of inflammation and cell death. *Biol Chem* 2015, **396**(3):193-203.
249. Shalini S, Dorstyn L, Dawar S, Kumar S: Old, new and emerging functions of caspases. *Cell Death Differ* 2015, **22**(4):526-539.
250. Bahatyrevich-Kharitonik B, Medina-Guzman R, Flores-Cortes A, Garcia-Cruzado M, Kavanagh E, Burguillos MA: Cell Death Related Proteins Beyond Apoptosis in the CNS. *Front Cell Dev Biol* 2021, **9**:825747.
251. Mukherjee A, Williams DW: More alive than dead: non-apoptotic roles for caspases in neuronal development, plasticity and disease. *Cell Death Differ* 2017, **24**(8):1411-1421.
252. Lamkanfi M, Festjens N, Declercq W, Vanden Berghe T, Vandenabeele P: Caspases in cell survival, proliferation and differentiation. *Cell Death Differ* 2007, **14**(1):44-55.
253. Nakajima YI, Kuranaga E: Caspase-dependent non-apoptotic processes in development. *Cell Death Differ* 2017, **24**(8):1422-1430.
254. Feinstein-Rotkopf Y, Arama E: Can't live without them, can live with them: roles of caspases during vital cellular processes. *Apoptosis* 2009, **14**(8):980-995.
255. Salmena L, Lemmers B, Hakem A, Matysiak-Zablocki E, Murakami K, Au PY, Berry DM, Tambllyn L, Shehabeldin A, Migon E *et al*: Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes Dev* 2003, **17**(7):883-895.
256. Kennedy NJ, Kataoka T, Tschopp J, Budd RC: Caspase activation is required for T cell proliferation. *J Exp Med* 1999, **190**(12):1891-1896.

257. Lei B, Zhou X, Lv D, Wan B, Wu H, Zhong L, Shu F, Mao X: Apoptotic and nonapoptotic function of caspase 7 in spermatogenesis. *Asian J Androl* 2017, **19**(1):47-51.
258. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P: Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol* 2014, **15**(2):135-147.
259. Allam R, Lawlor KE, Yu EC, Mildenhall AL, Moujalled DM, Lewis RS, Ke F, Mason KD, White MJ, Stacey KJ *et al*: Mitochondrial apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming. *EMBO Rep* 2014, **15**(9):982-990.
260. Maelfait J, Vercammen E, Janssens S, Schotte P, Haegman M, Magez S, Beyaert R: Stimulation of Toll-like receptor 3 and 4 induces interleukin-1 $\beta$  maturation by caspase-8. *J Exp Med* 2008, **205**(9):1967-1973.
261. Kang S, Fernandes-Alnemri T, Rogers C, Mayes L, Wang Y, Dillon C, Roback L, Kaiser W, Oberst A, Sagara J *et al*: Caspase-8 scaffolding function and MLKL regulate NLRP3 inflammasome activation downstream of TLR3. *Nat Commun* 2015, **6**:7515.
262. Deveraux QL, Roy N, Stennicke HR, Van Arsedale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC: IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* 1998, **17**(8):2215-2223.
263. Hrdinka M, Yabal M: Inhibitor of apoptosis proteins in human health and disease. *Genes Immun* 2019, **20**(8):641-650.
264. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC: Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J* 1999, **18**(19):5242-5251.
265. Huang Y, Park YC, Rich RL, Segal D, Myszka DG, Wu H: Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. *Cell* 2001, **104**(5):781-790.
266. Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, Shi Y: Structural basis of caspase-7 inhibition by XIAP. *Cell* 2001, **104**(5):769-780.
267. Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC, Salvesen GS: Structural basis for the inhibition of caspase-3 by XIAP. *Cell* 2001, **104**(5):791-800.
268. Silke J, Ekert PG, Day CL, Hawkins CJ, Baca M, Chew J, Pakusch M, Verhagen AM, Vaux DL: Direct inhibition of caspase 3 is dispensable for the anti-apoptotic activity of XIAP. *EMBO J* 2001, **20**(12):3114-3123.
269. Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, Lee RA, Robbins PD, Fernandes-Alnemri T, Shi Y *et al*: A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* 2001, **410**(6824):112-116.
270. Shiozaki EN, Chai J, Rigotti DJ, Riedl SJ, Li P, Srinivasula SM, Alnemri ES, Fairman R, Shi Y: Mechanism of XIAP-mediated inhibition of caspase-9. *Mol Cell* 2003, **11**(2):519-527.



271. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD: Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 2000, **288**(5467):874-877.
272. Lau R, Pratt MA: The opposing roles of cellular inhibitor of apoptosis proteins in cancer. *ISRN Oncol* 2012, **2012**:928120.
273. Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K, Fairbrother WJ, Vucic D: c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. *J Biol Chem* 2008, **283**(36):24295-24299.
274. Mahoney DJ, Cheung HH, Mrad RL, Plenchette S, Simard C, Enwere E, Arora V, Mak TW, Lacasse EC, Waring J *et al*: Both cIAP1 and cIAP2 regulate TNF alpha-mediated NF-kappa B activation. *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105**(33):11778-11783.
275. Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, Zobel K, Dynek JN, Elliott LO, Wallweber HJ *et al*: IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 2007, **131**(4):669-681.
276. Varfolomeev E, Goncharov T, Maecker H, Zobel K, Komuves LG, Deshayes K, Vucic D: Cellular inhibitors of apoptosis are global regulators of NF-kappaB and MAPK activation by members of the TNF family of receptors. *Sci Signal* 2012, **5**(216):ra22.
277. Hu S, Yang X: Cellular inhibitor of apoptosis 1 and 2 are ubiquitin ligases for the apoptosis inducer Smac/DIABLO. *J Biol Chem* 2003, **278**(12):10055-10060.
278. Richter BW, Mir SS, Eiben LJ, Lewis J, Reffey SB, Frattini A, Tian L, Frank S, Youle RJ, Nelson DL *et al*: Molecular cloning of ILP-2, a novel member of the inhibitor of apoptosis protein family. *Mol Cell Biol* 2001, **21**(13):4292-4301.
279. Vucic D, Franklin MC, Wallweber HJ, Das K, Eckelman BP, Shin H, Elliott LO, Kadkhodayan S, Deshayes K, Salvesen GS *et al*: Engineering ML-IAP to produce an extraordinarily potent caspase 9 inhibitor: implications for Smac-dependent anti-apoptotic activity of ML-IAP. *Biochem J* 2005, **385**(Pt 1):11-20.
280. Maier JK, Lahoua Z, Gendron NH, Fetni R, Johnston A, Davoodi J, Rasper D, Roy S, Slack RS, Nicholson DW *et al*: The neuronal apoptosis inhibitory protein is a direct inhibitor of caspases 3 and 7. *J Neurosci* 2002, **22**(6):2035-2043.
281. Dohi T, Beltrami E, Wall NR, Plescia J, Altieri DC: Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. *J Clin Invest* 2004, **114**(8):1117-1127.
282. Li F, Ling X: Survivin study: an update of "what is the next wave"? *J Cell Physiol* 2006, **208**(3):476-486.
283. Hao Y, Sekine K, Kawabata A, Nakamura H, Ishioka T, Ohata H, Katayama R, Hashimoto C, Zhang X, Noda T *et al*: Apollon ubiquitinates SMAC and caspase-9, and has an essential cytoprotection function. *Nat Cell Biol* 2004, **6**(9):849-860.
284. Mizutani Y, Nakanishi H, Li YN, Matsubara H, Yamamoto K, Sato N, Shiraishi T, Nakamura T, Mikami K, Okihara K *et al*: Overexpression of XIAP expression in renal cell carcinoma predicts a worse prognosis. *Int J Oncol* 2007, **30**(4):919-925.
285. Morrish E, Brumatti G, Silke J: Future Therapeutic Directions for Smac-Mimetics. *Cells* 2020, **9**(2).

286. Che X, Yang D, Zong H, Wang J, Li X, Chen F, Chen X, Song X: Nuclear cIAP1 overexpression is a tumor stage- and grade-independent predictor of poor prognosis in human bladder cancer patients. *Urol Oncol* 2012, **30**(4):450-456.
287. Esposito I, Kleeff J, Abiatari I, Shi X, Giese N, Bergmann F, Roth W, Friess H, Schirmacher P: Overexpression of cellular inhibitor of apoptosis protein 2 is an early event in the progression of pancreatic cancer. *J Clin Pathol* 2007, **60**(8):885-895.
288. Fulda S, Vucic D: Targeting IAP proteins for therapeutic intervention in cancer. *Nat Rev Drug Discov* 2012, **11**(2):109-124.
289. Vucic D, Fairbrother WJ: The inhibitor of apoptosis proteins as therapeutic targets in cancer. *Clin Cancer Res* 2007, **13**(20):5995-6000.
290. LaCasse EC, Mahoney DJ, Cheung HH, Plenchette S, Baird S, Korneluk RG: IAP-targeted therapies for cancer. *Oncogene* 2008, **27**(48):6252-6275.
291. Lee SH, Lee JY, Jung CL, Bae IH, Suh KH, Ahn YG, Jin DH, Kim TW, Suh YA, Jang SJ: A novel antagonist to the inhibitors of apoptosis (IAPs) potentiates cell death in EGFR-overexpressing non-small-cell lung cancer cells. *Cell Death Dis* 2014, **5**:e1477.
292. McManus DC, Lefebvre CA, Cherton-Horvat G, St-Jean M, Kandimalla ER, Agrawal S, Morris SJ, Durkin JP, Lacasse EC: Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. *Oncogene* 2004, **23**(49):8105-8117.
293. Tong QS, Zheng LD, Wang L, Zeng FQ, Chen FM, Dong JH, Lu GC: Downregulation of XIAP expression induces apoptosis and enhances chemotherapeutic sensitivity in human gastric cancer cells. *Cancer Gene Ther* 2005, **12**(5):509-514.
294. Zhang Y, Zhu J, Tang Y, Li F, Zhou H, Peng B, Zhou C, Fu R: X-linked inhibitor of apoptosis positive nuclear labeling: a new independent prognostic biomarker of breast invasive ductal carcinoma. *Diagn Pathol* 2011, **6**:49.
295. Jaffer S, Orta L, Sunkara S, Sabo E, Burstein DE: Immunohistochemical detection of antiapoptotic protein X-linked inhibitor of apoptosis in mammary carcinoma. *Hum Pathol* 2007, **38**(6):864-870.
296. Wang J, Liu Y, Ji R, Gu Q, Zhao X, Liu Y, Sun B: Prognostic value of the X-linked inhibitor of apoptosis protein for invasive ductal breast cancer with triple-negative phenotype. *Hum Pathol* 2010, **41**(8):1186-1195.
297. Strekalova E, Malin D, Rajanala H, Cryns VL: Metformin sensitizes triple-negative breast cancer to proapoptotic TRAIL receptor agonists by suppressing XIAP expression. *Breast Cancer Res Treat* 2017, **163**(3):435-447.
298. Finlay D, Vamos M, Gonzalez-Lopez M, Ardecky RJ, Ganji SR, Yuan H, Su Y, Cooley TR, Hauser CT, Welsh K *et al*: Small-molecule IAP antagonists sensitize cancer cells to TRAIL-induced apoptosis: roles of XIAP and cIAPs. *Mol Cancer Ther* 2014, **13**(1):5-15.
299. Allensworth JL, Aird KM, Aldrich AJ, Batinic-Haberle I, Devi GR: XIAP inhibition and generation of reactive oxygen species enhances TRAIL sensitivity in inflammatory breast cancer cells. *Mol Cancer Ther* 2012, **11**(7):1518-1527.

300. Allensworth JL, Sauer SJ, Lysterly HK, Morse MA, Devi GR: Smac mimetic Birinapant induces apoptosis and enhances TRAIL potency in inflammatory breast cancer cells in an IAP-dependent and TNF-alpha-independent mechanism. *Breast Cancer Res Treat* 2013, **137**(2):359-371.
301. Xu YC, Liu Q, Dai JQ, Yin ZQ, Tang L, Ma Y, Lin XL, Wang HX: Tissue microarray analysis of X-linked inhibitor of apoptosis (XIAP) expression in breast cancer patients. *Med Oncol* 2014, **31**(3):764.
302. Pluta P, Cebula-Obrzut B, Ehemann V, Pluta A, Wierzbowska A, Piekarski J, Bilski A, Nejc D, Kordek R, Robak T *et al*: Correlation of Smac/DIABLO protein expression with the clinico-pathological features of breast cancer patients. *Neoplasma* 2011, **58**(5):430-435.
303. Bai L, Smith DC, Wang S: Small-molecule SMAC mimetics as new cancer therapeutics. *Pharmacol Ther* 2014, **144**(1):82-95.
304. Wu G, Chai J, Suber TL, Wu JW, Du C, Wang X, Shi Y: Structural basis of IAP recognition by Smac/DIABLO. *Nature* 2000, **408**(6815):1008-1012.
305. Corti A, Milani M, Lecis D, Seneci P, de Rosa M, Mastrangelo E, Cossu F: Structure-based design and molecular profiling of Smac-mimetics selective for cellular IAPs. *FEBS J* 2018, **285**(17):3286-3298.
306. Sun H, Nikolovska-Coleska Z, Yang CY, Qian D, Lu J, Qiu S, Bai L, Peng Y, Cai Q, Wang S: Design of small-molecule peptidic and nonpeptidic Smac mimetics. *Acc Chem Res* 2008, **41**(10):1264-1277.
307. Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG: A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. *Science* 2004, **305**(5689):1471-1474.
308. Schimmer AD, Welsh K, Pinilla C, Wang Z, Krajewska M, Bonneau MJ, Pedersen IM, Kitada S, Scott FL, Bailly-Maitre B *et al*: Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. *Cancer Cell* 2004, **5**(1):25-35.
309. Oost TK, Sun C, Armstrong RC, Al-Assaad AS, Betz SF, Deckwerth TL, Ding H, Elmore SW, Meadows RP, Olejniczak ET *et al*: Discovery of potent antagonists of the antiapoptotic protein XIAP for the treatment of cancer. *J Med Chem* 2004, **47**(18):4417-4426.
310. Zobel K, Wang L, Varfolomeev E, Franklin MC, Elliott LO, Wallweber HJ, Okawa DC, Flygare JA, Vucic D, Fairbrother WJ *et al*: Design, synthesis, and biological activity of a potent Smac mimetic that sensitizes cancer cells to apoptosis by antagonizing IAPs. *ACS Chem Biol* 2006, **1**(8):525-533.
311. Weisberg E, Ray A, Barrett R, Nelson E, Christie AL, Porter D, Straub C, Zawel L, Daley JF, Lazo-Kallanian S *et al*: Smac mimetics: implications for enhancement of targeted therapies in leukemia. *Leukemia* 2010, **24**(12):2100-2109.
312. Pemmaraju N, Carter BZ, Bose P, Jain N, Kadia TM, Garcia-Manero G, Bueso-Ramos CE, DiNardo CD, Bledsoe S, Daver NG *et al*: Final results of a phase 2 clinical trial of LCL161, an oral SMAC mimetic for patients with myelofibrosis. *Blood Adv* 2021, **5**(16):3163-3173.

313. Pemmaraju N, Carter BZ, Kantarjian HM, Cortes JE, Bose P, Kadia TM, Garcia-Manero G, Bueso-Ramos CE, DiNardo CD, Bledsoe S *et al*: Final Results of Phase 2 Clinical Trial of LCL161, a Novel Oral SMAC Mimetic/IAP Antagonist, for Patients with Intermediate to High Risk Myelofibrosis. *Blood* 2019, **134**.
314. A Randomized, Phase 2, Neoadjuvant Study of Weekly Paclitaxel With or Witout LCL161 in Patients With Triple Negative Breast Cancer [<https://clinicaltrials.gov/ct2/show/results/NCT01617668?term=LCL161&draw=1&rank=6>]
315. Infante JR, Dees EC, Olszanski AJ, Dhuria SV, Sen S, Cameron S, Cohen RB: Phase I dose-escalation study of LCL161, an oral inhibitor of apoptosis proteins inhibitor, in patients with advanced solid tumors. *J Clin Oncol* 2014, **32**(28):3103-3110.
316. A Study of PDR001 in Combination With LCL161, Everolimus or Panobinostat [<https://clinicaltrials.gov/ct2/show/NCT02890069?term=LCL161&draw=2&rank=10>]
317. Ramakrishnan V, Painuly U, Kimlinger T, Haug J, Rajkumar SV, Kumar S: Inhibitor of apoptosis proteins as therapeutic targets in multiple myeloma. *Leukemia* 2014, **28**(7):1519-1528.
318. Chang Y-CC, Chun Hei Antonio.: An Updated Review of Smac Mimetics, LCL161, Birinapant, and GDC-0152 in Cancer Treatment. *Applied Sciences* 2021, **11**(1), 335.
319. Probst BL, Liu L, Ramesh V, Li L, Sun H, Minna JD, Wang L: Smac mimetics increase cancer cell response to chemotherapeutics in a TNF-alpha-dependent manner. *Cell Death Differ* 2010, **17**(10):1645-1654.
320. Petersen SL, Wang L, Yalcin-Chin A, Li L, Peyton M, Minna J, Harran P, Wang X: Autocrine TNFalpha signaling renders human cancer cells susceptible to Smac-mimetic-induced apoptosis. *Cancer Cell* 2007, **12**(5):445-456.
321. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, Benetatos CA, Chunduru SK, Condon SM, McKinlay M *et al*: IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007, **131**(4):682-693.
322. Maas C, Tromp JM, van Laar J, Thijssen R, Elias JA, Malara A, Krippner-Heidenreich A, Silke J, van Oers MH, Eldering E: CLL cells are resistant to smac mimetics because of an inability to form a ripoptosome complex. *Cell Death Dis* 2013, **4**:e782.
323. Lalaoui N, Hanggi K, Brumatti G, Chau D, Nguyen NN, Vasilikos L, Spilgies LM, Heckmann DA, Ma C, Ghisi M *et al*: Targeting p38 or MK2 Enhances the Anti-Leukemic Activity of Smac-Mimetics. *Cancer Cell* 2016, **30**(3):499-500.
324. Fulda S: Smac mimetics as IAP antagonists. *Semin Cell Dev Biol* 2015, **39**:132-138.
325. Cheung HH, Mahoney DJ, Lacasse EC, Korneluk RG: Down-regulation of c-FLIP Enhances death of cancer cells by smac mimetic compound. *Cancer Res* 2009, **69**(19):7729-7738.
326. Fulda S, Wick W, Weller M, Debatin KM: Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. *Nat Med* 2002, **8**(8):808-815.

327. Bockbrader KM, Tan MJ, Sun Y: A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL- and etoposide-induced apoptosis in breast cancer cells. *Oncogene* 2005, **24**(49):7381-7388.
328. Metwalli AR, Khanbolooki S, Jinesh G, Sundi D, Shah JB, Shrader M, Choi W, Lashinger LM, Chunduru S, McConkey DJ *et al*: Smac mimetic reverses resistance to TRAIL and chemotherapy in human urothelial cancer cells. *Cancer Biology & Therapy* 2010, **10**(9):889-896.
329. Chauhan D, Neri P, Velankar M, Podar K, Hideshima T, Fulciniti M, Tassone P, Raje N, Mitsiades C, Mitsiades N *et al*: Targeting mitochondrial factor Smac/DIABLO as therapy for multiple myeloma (MM). *Blood* 2007, **109**(3):1220-1227.
330. Oeckinghaus A, Ghosh S: The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol* 2009, **1**(4):a000034.
331. Yu H, Lin L, Zhang Z, Zhang H, Hu H: Targeting NF-kappaB pathway for the therapy of diseases: mechanism and clinical study. *Signal Transduct Target Ther* 2020, **5**(1):209.
332. Sun SC: The non-canonical NF-kappaB pathway in immunity and inflammation. *Nat Rev Immunol* 2017, **17**(9):545-558.
333. Karin M, Delhase M: The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Semin Immunol* 2000, **12**(1):85-98.
334. Hayden MS, Ghosh S: Regulation of NF-kappaB by TNF family cytokines. *Semin Immunol* 2014, **26**(3):253-266.
335. Hinz M, Scheidereit C: The IkappaB kinase complex in NF-kappaB regulation and beyond. *EMBO Rep* 2014, **15**(1):46-61.
336. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S: Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* 1995, **9**(22):2723-2735.
337. Fan CM, Maniatis T: Generation of p50 subunit of NF-kappa B by processing of p105 through an ATP-dependent pathway. *Nature* 1991, **354**(6352):395-398.
338. Urban MB, Baeuerle PA: The role of the p50 and p65 subunits of NF-kappa B in the recognition of cognate sequences. *New Biol* 1991, **3**(3):279-288.
339. Urban MB, Schreck R, Baeuerle PA: NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J* 1991, **10**(7):1817-1825.
340. Trares K, Ackermann J, Koch I: The canonical and non-canonical NF-kappaB pathways and their crosstalk: A comparative study based on Petri nets. *Biosystems* 2022, **211**:104564.
341. Baeuerle PA, Baltimore D: I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 1988, **242**(4878):540-546.
342. Baeuerle PA, Baltimore D: A 65-kappaD subunit of active NF-kappaB is required for inhibition of NF-kappaB by I kappaB. *Genes Dev* 1989, **3**(11):1689-1698.
343. Ghosh S, Baltimore D: Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature* 1990, **344**(6267):678-682.
344. Liao G, Zhang M, Harhaj EW, Sun SC: Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *J Biol Chem* 2004, **279**(25):26243-26250.

345. Vallabhapurapu S, Matsuzawa A, Zhang W, Tseng PH, Keats JJ, Wang H, Vignali DA, Bergsagel PL, Karin M: Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. *Nat Immunol* 2008, **9**(12):1364-1370.
346. He JQ, Zarnegar B, Oganessian G, Saha SK, Yamazaki S, Doyle SE, Dempsey PW, Cheng G: Rescue of TRAF3-null mice by p100 NF-kappa B deficiency. *J Exp Med* 2006, **203**(11):2413-2418.
347. Xiao G, Harhaj EW, Sun SC: NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* 2001, **7**(2):401-409.
348. Claudio E, Brown K, Park S, Wang H, Siebenlist U: BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nat Immunol* 2002, **3**(10):958-965.
349. Gardam S, Brink R: Non-Canonical NF-kappaB Signaling Initiated by BAFF Influences B Cell Biology at Multiple Junctions. *Front Immunol* 2014, **4**:509.
350. Mordmuller B, Krappmann D, Esen M, Wegener E, Scheidereit C: Lymphotoxin and lipopolysaccharide induce NF-kappaB-p52 generation by a co-translational mechanism. *EMBO Rep* 2003, **4**(1):82-87.
351. Kim JY, Morgan M, Kim DG, Lee JY, Bai L, Lin Y, Liu ZG, Kim YS: TNFalpha induced noncanonical NF-kappaB activation is attenuated by RIP1 through stabilization of TRAF2. *J Cell Sci* 2011, **124**(Pt 4):647-656.
352. Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun SC *et al*: Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* 2001, **293**(5534):1495-1499.
353. Sun SC: Controlling the fate of NIK: a central stage in noncanonical NF-kappaB signaling. *Sci Signal* 2010, **3**(123):pe18.
354. Dejardin E, Droin NM, Delhase M, Haas E, Cao Y, Makris C, Li ZW, Karin M, Ware CF, Green DR: The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity* 2002, **17**(4):525-535.
355. Coope HJ, Atkinson PG, Huhse B, Belich M, Janzen J, Holman MJ, Klaus GG, Johnston LH, Ley SC: CD40 regulates the processing of NF-kappaB2 p100 to p52. *EMBO J* 2002, **21**(20):5375-5385.
356. Solan NJ, Miyoshi H, Carmona EM, Bren GD, Paya CV: RelB cellular regulation and transcriptional activity are regulated by p100. *J Biol Chem* 2002, **277**(2):1405-1418.
357. Bonizzi G, Bebién M, Otero DC, Johnson-Vroom KE, Cao Y, Vu D, Jegga AG, Aronow BJ, Ghosh G, Rickert RC *et al*: Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *EMBO J* 2004, **23**(21):4202-4210.
358. Gardam S, Turner VM, Anderton H, Limaye S, Basten A, Koentgen F, Vaux DL, Silke J, Brink R: Deletion of cIAP1 and cIAP2 in murine B lymphocytes constitutively activates cell survival pathways and inactivates the germinal center response. *Blood* 2011, **117**(15):4041-4051.
359. Yang XD, Sun SC: Targeting signaling factors for degradation, an emerging mechanism for TRAF functions. *Immunol Rev* 2015, **266**(1):56-71.

360. Ganeff C, Remouchamps C, Boutaffala L, Benezech C, Galopin G, Vandepaer S, Bouillenne F, Ormenese S, Chariot A, Schneider P *et al*: Induction of the alternative NF-kappaB pathway by lymphotoxin alphabeta (LTalphabeta) relies on internalization of LTbeta receptor. *Mol Cell Biol* 2011, **31**(21):4319-4334.
361. Gentle IE, Wong WW, Evans JM, Bankovacki A, Cook WD, Khan NR, Nachbur U, Rickard J, Anderton H, Moulin M *et al*: In TNF-stimulated cells, RIPK1 promotes cell survival by stabilizing TRAF2 and cIAP1, which limits induction of non-canonical NF-kappaB and activation of caspase-8. *J Biol Chem* 2011, **286**(15):13282-13291.
362. Ramakrishnan P, Wang W, Wallach D: Receptor-specific signaling for both the alternative and the canonical NF-kappaB activation pathways by NF-kappaB-inducing kinase. *Immunity* 2004, **21**(4):477-489.
363. Isaacs A, Lindenmann J: Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* 1957, **147**(927):258-267.
364. Meyer O: Interferons and autoimmune disorders. *Joint Bone Spine* 2009, **76**(5):464-473.
365. Postal M, Vivaldo JF, Fernandez-Ruiz R, Paredes JL, Appenzeller S, Niewold TB: Type I interferon in the pathogenesis of systemic lupus erythematosus. *Curr Opin Immunol* 2020, **67**:87-94.
366. Durbin RK, Kotenko SV, Durbin JE: Interferon induction and function at the mucosal surface. *Immunol Rev* 2013, **255**(1):25-39.
367. Levy DE, Marie IJ, Durbin JE: Induction and function of type I and III interferon in response to viral infection. *Curr Opin Virol* 2011, **1**(6):476-486.
368. Diaz MO, Pomykala HM, Bohlander SK, Maltepe E, Malik K, Brownstein B, Olopade OI: Structure of the human type-I interferon gene cluster determined from a YAC clone contig. *Genomics* 1994, **22**(3):540-552.
369. Decker T, Muller M, Stockinger S: The yin and yang of type I interferon activity in bacterial infection. *Nat Rev Immunol* 2005, **5**(9):675-687.
370. Monroe KM, McWhirter SM, Vance RE: Induction of type I interferons by bacteria. *Cell Microbiol* 2010, **12**(7):881-890.
371. Carrero JA: Confounding roles for type I interferons during bacterial and viral pathogenesis. *Int Immunol* 2013, **25**(12):663-669.
372. Trinchieri G: Type I interferon: friend or foe? *J Exp Med* 2010, **207**(10):2053-2063.
373. Li SF, Gong MJ, Zhao FR, Shao JJ, Xie YL, Zhang YG, Chang HY: Type I Interferons: Distinct Biological Activities and Current Applications for Viral Infection. *Cell Physiol Biochem* 2018, **51**(5):2377-2396.
374. Lavoie TB, Kalie E, Crisafulli-Cabatu S, Abramovich R, DiGioia G, Moolchan K, Pestka S, Schreiber G: Binding and activity of all human alpha interferon subtypes. *Cytokine* 2011, **56**(2):282-289.
375. Moraga I, Harari D, Schreiber G, Uze G, Pellegrini S: Receptor density is key to the alpha2/beta interferon differential activities. *Mol Cell Biol* 2009, **29**(17):4778-4787.

376. Jamaluddin M, Wang S, Garofalo RP, Elliott T, Casola A, Baron S, Brasier AR: IFN-beta mediates coordinate expression of antigen-processing genes in RSV-infected pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2001, **280**(2):L248-257.
377. Yoshida J, Mizuno M, Fujii M, Kajita Y, Nakahara N, Hatano M, Saito R, Nobayashi M, Wakabayashi T: Human gene therapy for malignant gliomas (glioblastoma multiforme and anaplastic astrocytoma) by in vivo transduction with human interferon beta gene using cationic liposomes. *Hum Gene Ther* 2004, **15**(1):77-86.
378. Wakabayashi T, Natsume A, Hashizume Y, Fujii M, Mizuno M, Yoshida J: A phase I clinical trial of interferon-beta gene therapy for high-grade glioma: novel findings from gene expression profiling and autopsy. *J Gene Med* 2008, **10**(4):329-339.
379. Shih CS, Laurie N, Holzmacher J, Spence Y, Nathwani AC, Davidoff AM, Dyer MA: AAV-mediated local delivery of interferon-beta for the treatment of retinoblastoma in preclinical models. *Neuromolecular Med* 2009, **11**(1):43-52.
380. Enomoto H, Tao L, Eguchi R, Sato A, Honda M, Kaneko S, Iwata Y, Nishikawa H, Imanishi H, Iijima H *et al*: The in vivo antitumor effects of type I-interferon against hepatocellular carcinoma: the suppression of tumor cell growth and angiogenesis. *Sci Rep* 2017, **7**(1):12189.
381. Strander H, Einhorn S: Interferons and the tumor cell. *Biotherapy* 1996, **8**(3-4):213-218.
382. Borden EC: Interferons alpha and beta in cancer: therapeutic opportunities from new insights. *Nat Rev Drug Discov* 2019, **18**(3):219-234.
383. Pestka S, Langer JA, Zoon KC, Samuel CE: Interferons and their actions. *Annu Rev Biochem* 1987, **56**:727-777.
384. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD: How cells respond to interferons. *Annu Rev Biochem* 1998, **67**:227-264.
385. Lunn CA, Davies L, Dalgarno D, Narula SK, Zavodny PJ, Lundell D: An active covalently linked dimer of human interferon-gamma. Subunit orientation in the native protein. *J Biol Chem* 1992, **267**(25):17920-17924.
386. Rinderknecht E, O'Connor BH, Rodriguez H: Natural human interferon-gamma. Complete amino acid sequence and determination of sites of glycosylation. *J Biol Chem* 1984, **259**(11):6790-6797.
387. Sareneva T, Pirhonen J, Cantell K, Kalkkinen N, Julkunen I: Role of N-glycosylation in the synthesis, dimerization and secretion of human interferon-gamma. *Biochem J* 1994, **303** ( Pt 3):831-840.
388. Schoenborn JR, Wilson CB: Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol* 2007, **96**:41-101.
389. Hu X, Ivashkiv LB: Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. *Immunity* 2009, **31**(4):539-550.
390. Schroder K, Hertzog PJ, Ravasi T, Hume DA: Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004, **75**(2):163-189.



391. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP: IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003, **4**(1):69-77.
392. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J *et al*: IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 2003, **4**(1):63-68.
393. Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, Hergott D, Porter-Gill P, Mumy A, Kohaar I *et al*: A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet* 2013, **45**(2):164-171.
394. Pestka S, Krause CD, Walter MR: Interferons, interferon-like cytokines, and their receptors. *Immunol Rev* 2004, **202**:8-32.
395. Zheng YW, Li H, Yu JP, Zhao H, Wang SE, Ren XB: Interferon-lambdas: special immunomodulatory agents and potential therapeutic targets. *J Innate Immun* 2013, **5**(3):209-218.
396. Witte K, Witte E, Sabat R, Wolk K: IL-28A, IL-28B, and IL-29: promising cytokines with type I interferon-like properties. *Cytokine Growth Factor Rev* 2010, **21**(4):237-251.
397. Meager A, Visvalingam K, Dilger P, Bryan D, Wadhwa M: Biological activity of interleukins-28 and -29: comparison with type I interferons. *Cytokine* 2005, **31**(2):109-118.
398. Der SD, Zhou A, Williams BR, Silverman RH: Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A* 1998, **95**(26):15623-15628.
399. de Weerd NA, Nguyen T: The interferons and their receptors--distribution and regulation. *Immunol Cell Biol* 2012, **90**(5):483-491.
400. de Weerd NA, Samarajiwa SA, Hertzog PJ: Type I interferon receptors: biochemistry and biological functions. *J Biol Chem* 2007, **282**(28):20053-20057.
401. Walter MR: The Role of Structure in the Biology of Interferon Signaling. *Front Immunol* 2020, **11**:606489.
402. Plataniias LC: The p38 mitogen-activated protein kinase pathway and its role in interferon signaling. *Pharmacol Ther* 2003, **98**(2):129-142.
403. Seif F, Khoshmirsafa M, Aazami H, Mohsenzadegan M, Sedighi G, Bahar M: The role of JAK-STAT signaling pathway and its regulators in the fate of T helper cells. *Cell Commun Signal* 2017, **15**(1):23.
404. Zanin N, Viaris de Lesegno C, Lamaze C, Blouin CM: Interferon Receptor Trafficking and Signaling: Journey to the Cross Roads. *Front Immunol* 2020, **11**:615603.
405. Bach EA, Aguet M, Schreiber RD: The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 1997, **15**:563-591.
406. Wei J, Lian H, Zhong B, Shu HB: Parafibromin Is a Component of IFN-gamma-Triggered Signaling Pathways That Facilitates JAK1/2-Mediated Tyrosine Phosphorylation of STAT1. *J Immunol* 2015, **195**(6):2870-2878.

407. Stanifer ML, Pervolaraki K, Boulant S: Differential Regulation of Type I and Type III Interferon Signaling. *Int J Mol Sci* 2019, **20**(6).
408. Schindler C, Levy DE, Decker T: JAK-STAT signaling: from interferons to cytokines. *J Biol Chem* 2007, **282**(28):20059-20063.
409. Shuai K, Schindler C, Prezioso VR, Darnell JE, Jr.: Activation of transcription by IFN-gamma: tyrosine phosphorylation of a 91-kD DNA binding protein. *Science* 1992, **258**(5089):1808-1812.
410. Mertens C, Zhong M, Krishnaraj R, Zou W, Chen X, Darnell JE, Jr.: Dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the monomers facilitated by the N-terminal domain. *Genes Dev* 2006, **20**(24):3372-3381.
411. Ivashkiv LB, Donlin LT: Regulation of type I interferon responses. *Nat Rev Immunol* 2014, **14**(1):36-49.
412. Castro F, Cardoso AP, Goncalves RM, Serre K, Oliveira MJ: Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. *Front Immunol* 2018, **9**:847.
413. Qing Y, Stark GR: Alternative activation of STAT1 and STAT3 in response to interferon-gamma. *J Biol Chem* 2004, **279**(40):41679-41685.
414. Ramana CV, Kumar A, Enelow R: Stat1-independent induction of SOCS-3 by interferon-gamma is mediated by sustained activation of Stat3 in mouse embryonic fibroblasts. *Biochem Biophys Res Commun* 2005, **327**(3):727-733.
415. Pilz A, Ramsauer K, Heidari H, Leitges M, Kovarik P, Decker T: Phosphorylation of the Stat1 transactivating domain is required for the response to type I interferons. *EMBO Rep* 2003, **4**(4):368-373.
416. Steen HC, Gamero AM: STAT2 phosphorylation and signaling. *JAKSTAT* 2013, **2**(4):e25790.
417. Levy DE, Lee CK: What does Stat3 do? *J Clin Invest* 2002, **109**(9):1143-1148.
418. Wen Z, Zhong Z, Darnell JE, Jr.: Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* 1995, **82**(2):241-250.
419. Sadzak I, Schiff M, Gattermeier I, Glinitzer R, Sauer I, Saalmuller A, Yang E, Schaljo B, Kovarik P: Recruitment of Stat1 to chromatin is required for interferon-induced serine phosphorylation of Stat1 transactivation domain. *Proc Natl Acad Sci U S A* 2008, **105**(26):8944-8949.
420. Wen Z, Darnell JE, Jr.: Mapping of Stat3 serine phosphorylation to a single residue (727) and evidence that serine phosphorylation has no influence on DNA binding of Stat1 and Stat3. *Nucleic Acids Res* 1997, **25**(11):2062-2067.
421. Steen HC, Nogusa S, Thapa RJ, Basagoudanavar SH, Gill AL, Merali S, Barrero CA, Balachandran S, Gamero AM: Identification of STAT2 serine 287 as a novel regulatory phosphorylation site in type I interferon-induced cellular responses. *J Biol Chem* 2013, **288**(1):747-758.
422. Mazewski C, Perez RE, Fish EN, Plataniias LC: Type I Interferon (IFN)-Regulated Activation of Canonical and Non-Canonical Signaling Pathways. *Front Immunol* 2020, **11**:606456.

423. Fu XY, Schindler C, Improtta T, Aebersold R, Darnell JE, Jr.: The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proc Natl Acad Sci U S A* 1992, **89**(16):7840-7843.
424. Zhang JJ, Vinkemeier U, Gu W, Chakravarti D, Horvath CM, Darnell JE, Jr.: Two contact regions between Stat1 and CBP/p300 in interferon gamma signaling. *Proc Natl Acad Sci U S A* 1996, **93**(26):15092-15096.
425. Bhattacharya S, Eckner R, Grossman S, Oldread E, Arany Z, D'Andrea A, Livingston DM: Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha. *Nature* 1996, **383**(6598):344-347.
426. Schindler C, Shuai K, Prezioso VR, Darnell JE, Jr.: Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. *Science* 1992, **257**(5071):809-813.
427. Kawabata K, Okamoto S, Takakura Y, Hashida M, Hashimura T, Watanabe Y: A physiological role of interferon (IFN)-beta derived from tumor: tumor growth of a mouse bladder carcinoma line MBT-2 is partially suppressed by autocrine IFN-beta. *Cancer Lett* 1997, **113**(1-2):159-164.
428. Resnitzky D, Yarden A, Zipori D, Kimchi A: Autocrine beta-related interferon controls c-myc suppression and growth arrest during hematopoietic cell differentiation. *Cell* 1986, **46**(1):31-40.
429. Lazear HM, Schoggins JW, Diamond MS: Shared and Distinct Functions of Type I and Type III Interferons. *Immunity* 2019, **50**(4):907-923.
430. Michalska A, Blaszczyk K, Wesoly J, Bluysen HAR: A Positive Feedback Amplifier Circuit That Regulates Interferon (IFN)-Stimulated Gene Expression and Controls Type I and Type II IFN Responses. *Front Immunol* 2018, **9**:1135.
431. Dumoutier L, Tounsi A, Michiels T, Sommereyns C, Kotenko SV, Renauld JC: Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-lambda 1: similarities with type I interferon signaling. *J Biol Chem* 2004, **279**(31):32269-32274.
432. Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P *et al*: Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. *Hepatology* 2006, **44**(4):896-906.
433. Durbin JE, Hackenmiller R, Simon MC, Levy DE: Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 1996, **84**(3):443-450.
434. Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D *et al*: Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 1996, **84**(3):431-442.
435. Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, Hartmann R: Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. *J Virol* 2007, **81**(14):7749-7758.
436. Shuai K: Modulation of STAT signaling by STAT-interacting proteins. *Oncogene* 2000, **19**(21):2638-2644.

437. Tanaka T, Soriano MA, Grusby MJ: SLIM is a nuclear ubiquitin E3 ligase that negatively regulates STAT signaling. *Immunity* 2005, **22**(6):729-736.
438. O'Shea JJ, Murray PJ: Cytokine signaling modules in inflammatory responses. *Immunity* 2008, **28**(4):477-487.
439. Mielcarska MB, Bossowska-Nowicka M, Toka FN: Cell Surface Expression of Endosomal Toll-Like Receptors-A Necessity or a Superfluous Duplication? *Front Immunol* 2020, **11**:620972.
440. Perry AK, Chen G, Zheng D, Tang H, Cheng G: The host type I interferon response to viral and bacterial infections. *Cell Res* 2005, **15**(6):407-422.
441. Moynagh PN: TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. *Trends Immunol* 2005, **26**(9):469-476.
442. Karapetyan L, Luke JJ, Davar D: Toll-Like Receptor 9 Agonists in Cancer. *Onco Targets Ther* 2020, **13**:10039-10060.
443. McWhirter SM, Fitzgerald KA, Rosains J, Rowe DC, Golenbock DT, Maniatis T: IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proc Natl Acad Sci U S A* 2004, **101**(1):233-238.
444. Hemmi H, Takeuchi O, Sato S, Yamamoto M, Kaisho T, Sanjo H, Kawai T, Hoshino K, Takeda K, Akira S: The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *J Exp Med* 2004, **199**(12):1641-1650.
445. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T: IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 2003, **4**(5):491-496.
446. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J: Triggering the interferon antiviral response through an IKK-related pathway. *Science* 2003, **300**(5622):1148-1151.
447. Perry AK, Chow EK, Goodnough JB, Yeh WC, Cheng G: Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection. *J Exp Med* 2004, **199**(12):1651-1658.
448. Zhou R, Zhang Q, Xu P: TBK1, a central kinase in innate immune sensing of nucleic acids and beyond. *Acta Biochim Biophys Sin (Shanghai)* 2020, **52**(7):757-767.
449. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, Kimoto M: MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999, **189**(11):1777-1782.
450. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, Akira S: TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol* 2003, **4**(11):1144-1150.
451. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K *et al*: Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003, **301**(5633):640-643.

452. Sasai M, Oshiumi H, Matsumoto M, Inoue N, Fujita F, Nakanishi M, Seya T: Cutting Edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *J Immunol* 2005, **174**(1):27-30.
453. Funami K, Matsumoto M, Oshiumi H, Akazawa T, Yamamoto A, Seya T: The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. *Int Immunol* 2004, **16**(8):1143-1154.
454. Bugge M, Bergstrom B, Eide OK, Solli H, Kjonstad IF, Stenvik J, Espevik T, Nilsen NJ: Surface Toll-like receptor 3 expression in metastatic intestinal epithelial cells induces inflammatory cytokine production and promotes invasiveness. *Journal of Biological Chemistry* 2017, **292**(37):15408-15425.
455. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA: Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001, **413**(6857):732-738.
456. Schulz O, Diebold SS, Chen M, Naslund TI, Nolte MA, Alexopoulou L, Azuma YT, Flavell RA, Liljestrom P, Reis e Sousa C: Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 2005, **433**(7028):887-892.
457. Wagner H: The immunobiology of the TLR9 subfamily. *Trends Immunol* 2004, **25**(7):381-386.
458. Honda K, Yanai H, Mizutani T, Negishi H, Shimada N, Suzuki N, Ohba Y, Takaoka A, Yeh WC, Taniguchi T: Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proc Natl Acad Sci U S A* 2004, **101**(43):15416-15421.
459. Thompson AJ, Locarnini SA: Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response. *Immunol Cell Biol* 2007, **85**(6):435-445.
460. Onomoto K, Onoguchi K, Yoneyama M: Regulation of RIG-I-like receptor-mediated signaling: interaction between host and viral factors. *Cell Mol Immunol* 2021, **18**(3):539-555.
461. Horner SM, Liu HM, Park HS, Briley J, Gale M, Jr.: Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. *Proc Natl Acad Sci U S A* 2011, **108**(35):14590-14595.
462. Liu HM, Loo YM, Horner SM, Zornetzer GA, Katze MG, Gale M, Jr.: The mitochondrial targeting chaperone 14-3-3epsilon regulates a RIG-I translocon that mediates membrane association and innate antiviral immunity. *Cell Host Microbe* 2012, **11**(5):528-537.
463. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T: The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004, **5**(7):730-737.
464. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S: IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 2005, **6**(10):981-988.
465. Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB: VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* 2005, **19**(6):727-740.

466. Saha SK, Pietras EM, He JQ, Kang JR, Liu SY, Oganessian G, Shahangian A, Zarnegar B, Shiba TL, Wang Y *et al*: Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif. *EMBO J* 2006, **25**(14):3257-3263.
467. Moreira LO, Zamboni DS: NOD1 and NOD2 Signaling in Infection and Inflammation. *Front Immunol* 2012, **3**:328.
468. Keestra-Gounder AM, Tsois RM: NOD1 and NOD2: Beyond Peptidoglycan Sensing. *Trends Immunol* 2017, **38**(10):758-767.
469. Watanabe T, Asano N, Fichtner-Feigl S, Gorelick PL, Tsuji Y, Matsumoto Y, Chiba T, Fuss IJ, Kitani A, Strober W: NOD1 contributes to mouse host defense against *Helicobacter pylori* via induction of type I IFN and activation of the ISGF3 signaling pathway. *J Clin Invest* 2010, **120**(5):1645-1662.
470. Gehrke N, Mertens C, Zillinger T, Wenzel J, Bald T, Zahn S, Tuting T, Hartmann G, Barchet W: Oxidative damage of DNA confers resistance to cytosolic nuclease TREX1 degradation and potentiates STING-dependent immune sensing. *Immunity* 2013, **39**(3):482-495.
471. Vanpouille-Box C, Alard A, Aryankalayil MJ, Sarfraz Y, Diamond JM, Schneider RJ, Inghirami G, Coleman CN, Formenti SC, Demaria S: DNA exonuclease Trex1 regulates radiotherapy-induced tumour immunogenicity. *Nat Commun* 2017, **8**:15618.
472. Shen YJ, Le Bert N, Chitre AA, Koo CX, Nga XH, Ho SS, Khato M, Tan NY, Ishii KJ, Gasser S: Genome-derived cytosolic DNA mediates type I interferon-dependent rejection of B cell lymphoma cells. *Cell Rep* 2015, **11**(3):460-473.
473. Liu X, Wang C: The emerging roles of the STING adaptor protein in immunity and diseases. *Immunology* 2016, **147**(3):285-291.
474. Shu HB, Wang YY: Adding to the STING. *Immunity* 2014, **41**(6):871-873.
475. Ding C, Song Z, Shen A, Chen T, Zhang A: Small molecules targeting the innate immune cGAS/STING/TBK1 signaling pathway. *Acta Pharm Sin B* 2020, **10**(12):2272-2298.
476. Antonia RJ, Hagan RS, Baldwin AS: Expanding the View of IKK: New Substrates and New Biology. *Trends Cell Biol* 2021, **31**(3):166-178.
477. Au WC, Moore PA, LaFleur DW, Tombal B, Pitha PM: Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. *J Biol Chem* 1998, **273**(44):29210-29217.
478. Au WC, Moore PA, Lowther W, Juang YT, Pitha PM: Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. *Proc Natl Acad Sci U S A* 1995, **92**(25):11657-11661.
479. Mori M, Yoneyama M, Ito T, Takahashi K, Inagaki F, Fujita T: Identification of Ser-386 of interferon regulatory factor 3 as critical target for inducible phosphorylation that determines activation. *J Biol Chem* 2004, **279**(11):9698-9702.
480. Wathlet MG, Lin CH, Parekh BS, Ronco LV, Howley PM, Maniatis T: Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. *Mol Cell* 1998, **1**(4):507-518.

481. Lin R, Heylbroeck C, Pitha PM, Hiscott J: Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol Cell Biol* 1998, **18**(5):2986-2996.
482. Yoneyama M, Suhara W, Fukuhara Y, Fukuda M, Nishida E, Fujita T: Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J* 1998, **17**(4):1087-1095.
483. Weaver BK, Kumar KP, Reich NC: Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAFI. *Mol Cell Biol* 1998, **18**(3):1359-1368.
484. Sato M, Tanaka N, Hata N, Oda E, Taniguchi T: Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-beta gene. *FEBS Lett* 1998, **425**(1):112-116.
485. Yang H, Ma G, Lin CH, Orr M, Wathelet MG: Mechanism for transcriptional synergy between interferon regulatory factor (IRF)-3 and IRF-7 in activation of the interferon-beta gene promoter. *Eur J Biochem* 2004, **271**(18):3693-3703.
486. Falvo JV, Parekh BS, Lin CH, Fraenkel E, Maniatis T: Assembly of a functional beta interferon enhanceosome is dependent on ATF-2-c-jun heterodimer orientation. *Mol Cell Biol* 2000, **20**(13):4814-4825.
487. Honda K, Taniguchi T: IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 2006, **6**(9):644-658.
488. Honda K, Takaoka A, Taniguchi T: Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity* 2006, **25**(3):349-360.
489. Harada H, Fujita T, Miyamoto M, Kimura Y, Maruyama M, Furia A, Miyata T, Taniguchi T: Structurally Similar but Functionally Distinct Factors, Irf-1 and Irf-2, Bind to the Same Regulatory Elements of Ifn and Ifn-Inducible Genes. *Cell* 1989, **58**(4):729-739.
490. Kawakami T, Matsumoto M, Sato M, Harada H, Taniguchi T, Kitagawa M: Possible Involvement of the Transcription Factor Isgf3-Gamma in Virus-Induced Expression of the Ifn-Beta Gene. *Febs Letters* 1995, **358**(3):225-229.
491. Yoneyama M, Suhara W, Fukuhara Y, Sato M, Ozato K, Fujita T: Autocrine amplification of type I interferon gene expression mediated by interferon stimulated gene factor 3 (ISGF3). *J Biochem* 1996, **120**(1):160-169.
492. Hiscott J, Alper D, Cohen L, Leblanc JF, Sportza L, Wong A, Xanthoudakis S: Induction of human interferon gene expression is associated with a nuclear factor that interacts with the NF-kappa B site of the human immunodeficiency virus enhancer. *J Virol* 1989, **63**(6):2557-2566.
493. Lenardo MJ, Fan CM, Maniatis T, Baltimore D: The involvement of NF-kappa B in beta-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell* 1989, **57**(2):287-294.
494. Du W, Maniatis T: An ATF/CREB binding site is required for virus induction of the human interferon beta gene [corrected]. *Proc Natl Acad Sci U S A* 1992, **89**(6):2150-2154.

495. Kim TK, Maniatis T: The mechanism of transcriptional synergy of an in vitro assembled interferon-beta enhanceosome. *Mol Cell* 1997, **1**(1):119-129.
496. Thanos D, Maniatis T: Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. *Cell* 1995, **83**(7):1091-1100.
497. Agalioti T, Lomvardas S, Parekh B, Yie J, Maniatis T, Thanos D: Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. *Cell* 2000, **103**(4):667-678.
498. Ryals J, Dierks P, Ragg H, Weissmann C: A 46-nucleotide promoter segment from an IFN-alpha gene renders an unrelated promoter inducible by virus. *Cell* 1985, **41**(2):497-507.
499. Pomerantz JL, Baltimore D: NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. *EMBO J* 1999, **18**(23):6694-6704.
500. Clark K, Peggie M, Plater L, Sorcek RJ, Young ER, Madwed JB, Hough J, McIver EG, Cohen P: Novel cross-talk within the IKK family controls innate immunity. *Biochem J* 2011, **434**(1):93-104.
501. Tojima Y, Fujimoto A, Delhase M, Chen Y, Hatakeyama S, Nakayama K, Kaneko Y, Nimura Y, Motoyama N, Ikeda K *et al*: NAK is an IkappaB kinase-activating kinase. *Nature* 2000, **404**(6779):778-782.
502. Lafont E, Draber P, Rieser E, Reichert M, Kupka S, de Miguel D, Draberova H, von Massenhausen A, Bhamra A, Henderson S *et al*: TBK1 and IKKepsilon prevent TNF-induced cell death by RIPK1 phosphorylation. *Nat Cell Biol* 2018, **20**(12):1389-1399.
503. Bonnard M, Mirtsos C, Suzuki S, Graham K, Huang J, Ng M, Itie A, Wakeham A, Shahinian A, Henzel WJ *et al*: Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. *EMBO J* 2000, **19**(18):4976-4985.
504. Shen RR, Hahn WC: Emerging roles for the non-canonical IKKs in cancer. *Oncogene* 2011, **30**(6):631-641.
505. Boehm JS, Zhao JJ, Yao J, Kim SY, Firestein R, Dunn IF, Sjostrom SK, Garraway LA, Weremowicz S, Richardson AL *et al*: Integrative genomic approaches identify IKBKE as a breast cancer oncogene. *Cell* 2007, **129**(6):1065-1079.
506. Korherr C, Gille H, Schafer R, Koenig-Hoffmann K, Dixelius J, Eglund KA, Pastan I, Brinkmann U: Identification of proangiogenic genes and pathways by high-throughput functional genomics: TBK1 and the IRF3 pathway. *Proc Natl Acad Sci U S A* 2006, **103**(11):4240-4245.
507. Wei C, Cao Y, Yang X, Zheng Z, Guan K, Wang Q, Tai Y, Zhang Y, Ma S, Cao Y *et al*: Elevated expression of TANK-binding kinase 1 enhances tamoxifen resistance in breast cancer. *Proc Natl Acad Sci U S A* 2014, **111**(5):E601-610.
508. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR *et al*: Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Mod Pathol* 2011, **24**(2):157-167.



509. Kishore N, Huynh QK, Mathialagan S, Hall T, Rouw S, Creely D, Lange G, Carroll J, Reitz B, Donnelly A *et al*: IKK-i and TBK-1 are enzymatically distinct from the homologous enzyme IKK-2: comparative analysis of recombinant human IKK-i, TBK-1, and IKK-2. *J Biol Chem* 2002, **277**(16):13840-13847.
510. Clark K, Plater L, Pegg M, Cohen P: Use of the pharmacological inhibitor BX795 to study the regulation and physiological roles of TBK1 and I $\kappa$ B kinase epsilon: a distinct upstream kinase mediates Ser-172 phosphorylation and activation. *J Biol Chem* 2009, **284**(21):14136-14146.
511. Ma X, Helgason E, Phung QT, Quan CL, Iyer RS, Lee MW, Bowman KK, Starovasnik MA, Dueber EC: Molecular basis of Tank-binding kinase 1 activation by transautophosphorylation. *Proc Natl Acad Sci U S A* 2012, **109**(24):9378-9383.
512. Zhao W: Negative regulation of TBK1-mediated antiviral immunity. *FEBS Lett* 2013, **587**(6):542-548.
513. Yu T, Yi YS, Yang Y, Oh J, Jeong D, Cho JY: The pivotal role of TBK1 in inflammatory responses mediated by macrophages. *Mediators Inflamm* 2012, **2012**:979105.
514. Saha SK, Cheng G: TRAF3: a new regulator of type I interferons. *Cell Cycle* 2006, **5**(8):804-807.
515. Oganessian G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, Perry A, Cheng G: Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature* 2006, **439**(7073):208-211.
516. Hacker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Hacker G *et al*: Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 2006, **439**(7073):204-207.
517. Parvatiyar K, Pindado J, Dev A, Aliyari SR, Zaver SA, Gerami H, Chapon M, Ghaffari AA, Dhingra A, Cheng G: A TRAF3-NIK module differentially regulates DNA vs RNA pathways in innate immune signaling. *Nat Commun* 2018, **9**(1):2770.
518. Sica G, Natoli V, Stella C, Del Bianco S: Effect of natural beta-interferon on cell proliferation and steroid receptor level in human breast cancer cells. *Cancer* 1987, **60**(10):2419-2423.
519. van den Berg HW, Leahey WJ, Lynch M, Clarke R, Nelson J: Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitizes them to the anti-proliferative effects of tamoxifen. *Br J Cancer* 1987, **55**(3):255-257.
520. Sica G, Angelucci C, Iacopino F, Lama G, Robustelli della Cuna G, Serlupi-Crescenzi O, Donini S: Effect of natural beta-interferon on estrogen receptor mRNA of breast cancer cells. *Anticancer Res* 1992, **12**(6B):2061-2064.
521. Sica G, Iacopino F, Lama G, Amadori D, Baroni M, Lo Sardo F, Malacarne P, Marchetti P, Pellegrini A, Zaniboni A *et al*: Steroid receptor enhancement by natural interferon-beta in advanced breast cancer. *Eur J Cancer* 1993, **29A**(3):329-333.
522. Seymour L, Bezwoda WR: Interferon plus tamoxifen treatment for advanced breast cancer: in vivo biologic effects of two growth modulators. *Br J Cancer* 1993, **68**(2):352-356.

523. Di Martino L, Demontis B, Saccani Iotti G, Murenu G: In vivo effect induced by interferon beta on steroid receptor status, cell kinetics and DNA ploidy in operable breast cancer patients. *Anticancer Res* 1995, **15**(2):537-541.
524. Grasso G, Muscettola M: The influence of beta-estradiol and progesterone on interferon gamma production in vitro. *Int J Neurosci* 1990, **51**(3-4):315-317.
525. Goodman ML, Trinca GM, Walter KR, Papachristou EK, D'Santos CS, Li T, Liu Q, Lai Z, Chalise P, Madan R *et al*: Progesterone Receptor Attenuates STAT1-Mediated IFN Signaling in Breast Cancer. *J Immunol* 2019, **202**(10):3076-3086.
526. Walter KR, Goodman ML, Singhal H, Hall JA, Li T, Holloran SM, Trinca GM, Gibson KA, Jin VX, Greene GL *et al*: Interferon-Stimulated Genes Are Transcriptionally Repressed by PR in Breast Cancer. *Mol Cancer Res* 2017, **15**(10):1331-1340.
527. Walter KR, Balko JM, Hagan CR: Progesterone receptor promotes degradation of STAT2 to inhibit the interferon response in breast cancer. *Oncoimmunology* 2020, **9**(1):1758547.
528. Post AEM, Smid M, Nagelkerke A, Martens JWM, Bussink J, Sweep F, Span PN: Interferon-Stimulated Genes Are Involved in Cross-resistance to Radiotherapy in Tamoxifen-Resistant Breast Cancer. *Clin Cancer Res* 2018, **24**(14):3397-3408.
529. Choi HJ, Lui A, Ogony J, Jan R, Sims PJ, Lewis-Wambi J: Targeting interferon response genes sensitizes aromatase inhibitor resistant breast cancer cells to estrogen-induced cell death. *Breast Cancer Res* 2015, **17**:6.
530. De Angelis C, Fu X, Cataldo ML, Nardone A, Pereira R, Veeraraghavan J, Nanda S, Qin L, Sethunath V, Wang T *et al*: Activation of the IFN Signaling Pathway is Associated with Resistance to CDK4/6 Inhibitors and Immune Checkpoint Activation in ER-Positive Breast Cancer. *Clin Cancer Res* 2021, **27**(17):4870-4882.
531. Escher TE, Dandawate P, Sayed A, Hagan CR, Anant S, Lewis-Wambi J: Enhanced IFNalpha Signaling Promotes Ligand-Independent Activation of ERalpha to Promote Aromatase Inhibitor Resistance in Breast Cancer. *Cancers (Basel)* 2021, **13**(20).
532. Qadir AS, Ceppi P, Brockway S, Law C, Mu L, Khodarev NN, Kim J, Zhao JC, Putzbach W, Murmann AE *et al*: CD95/Fas Increases Stemness in Cancer Cells by Inducing a STAT1-Dependent Type I Interferon Response. *Cell Rep* 2017, **18**(10):2373-2386.
533. Qadir AS, Stults AM, Murmann AE, Peter ME: The mechanism of how CD95/Fas activates the Type I IFN/STAT1 axis, driving cancer stemness in breast cancer. *Sci Rep* 2020, **10**(1):1310.
534. Legrier ME, Bieche I, Gaston J, Beurdeley A, Yvonnet V, Deas O, Thuleau A, Chateau-Joubert S, Servely JL, Vacher S *et al*: Activation of IFN/STAT1 signalling predicts response to chemotherapy in oestrogen receptor-negative breast cancer. *Br J Cancer* 2016, **114**(2):177-187.
535. Lan Q, Peyvandi S, Duffey N, Huang YT, Barras D, Held W, Richard F, Delorenzi M, Sotiriou C, Desmedt C *et al*: Type I interferon/IRF7 axis instigates chemotherapy-induced immunological dormancy in breast cancer. *Oncogene* 2019, **38**(15):2814-2829.

536. Reiter M, Eckhardt I, Haferkamp A, Fulda S: Smac mimetic sensitizes renal cell carcinoma cells to interferon-alpha-induced apoptosis. *Cancer Lett* 2016, **375**(1):1-8.
537. Cekay MJ, Roesler S, Frank T, Knuth AK, Eckhardt I, Fulda S: Smac mimetics and type II interferon synergistically induce necroptosis in various cancer cell lines. *Cancer Lett* 2017, **410**:228-237.
538. Hao Q, Tang H: Interferon-gamma and Smac mimetics synergize to induce apoptosis of lung cancer cells in a TNFalpha-independent manner. *Cancer Cell Int* 2018, **18**:84.
539. Bake V, Roesler S, Eckhardt I, Belz K, Fulda S: Synergistic interaction of Smac mimetic and IFNalpha to trigger apoptosis in acute myeloid leukemia cells. *Cancer Lett* 2014, **355**(2):224-231.
540. Roesler S, Eckhardt I, Wolf S, Fulda S: Cooperative TRAIL production mediates IFNalpha/Smac mimetic-induced cell death in TNFalpha-resistant solid cancer cells. *Oncotarget* 2016, **7**(4):3709-3725.
541. Azijli K, Weyhenmeyer B, Peters GJ, de Jong S, Kruyt FA: Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family. *Cell Death Differ* 2013, **20**(7):858-868.
542. Siegmund D, Lang I, Wajant H: Cell death-independent activities of the death receptors CD95, TRAILR1, and TRAILR2. *FEBS J* 2017, **284**(8):1131-1159.
543. Hughes MA, Powley IR, Jukes-Jones R, Horn S, Feoktistova M, Fairall L, Schwabe JW, Leverkus M, Cain K, MacFarlane M: Co-operative and Hierarchical Binding of c-FLIP and Caspase-8: A Unified Model Defines How c-FLIP Isoforms Differentially Control Cell Fate. *Mol Cell* 2016, **61**(6):834-849.
544. Krueger A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S: Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J Biol Chem* 2001, **276**(23):20633-20640.
545. Micheau O, Thome M, Schneider P, Holler N, Tschopp J, Nicholson DW, Briand C, Grutter MG: The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem* 2002, **277**(47):45162-45171.
546. Humphreys LM, Fox JP, Higgins CA, Majkut J, Sessler T, McLaughlin K, McCann C, Roberts JZ, Crawford NT, McDade SS *et al*: A revised model of TRAIL-R2 DISC assembly explains how FLIP(L) can inhibit or promote apoptosis. *EMBO Rep* 2020, **21**(3):e49254.
547. Janicke RU, Sprengart ML, Wati MR, Porter AG: Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 1998, **273**(16):9357-9360.
548. Engels IH, Totzke G, Fischer U, Schulze-Osthoff K, Janicke RU: Caspase-10 sensitizes breast carcinoma cells to TRAIL-induced but not tumor necrosis factor-induced apoptosis in a caspase-3-dependent manner. *Mol Cell Biol* 2005, **25**(7):2808-2818.
549. Rahman M, Davis SR, Pumphrey JG, Bao J, Nau MM, Meltzer PS, Lipkowitz S: TRAIL induces apoptosis in triple-negative breast cancer cells with a mesenchymal phenotype. *Breast Cancer Res Treat* 2009, **113**(2):217-230.

550. Bell RAV, Megeney LA: Evolution of caspase-mediated cell death and differentiation: twins separated at birth. *Cell Death Differ* 2017, **24**(8):1359-1368.
551. Dick SA, Megeney LA: Cell death proteins: an evolutionary role in cellular adaptation before the advent of apoptosis. *Bioessays* 2013, **35**(11):974-983.
552. Connolly PF, Jager R, Fearnhead HO: New roles for old enzymes: killer caspases as the engine of cell behavior changes. *Front Physiol* 2014, **5**:149.
553. Kuo CT, Zhu S, Younger S, Jan LY, Jan YN: Identification of E2/E3 ubiquitinating enzymes and caspase activity regulating Drosophila sensory neuron dendrite pruning. *Neuron* 2006, **51**(3):283-290.
554. Williams DW, Kondo S, Krzyzanowska A, Hiromi Y, Truman JW: Local caspase activity directs engulfment of dendrites during pruning. *Nat Neurosci* 2006, **9**(10):1234-1236.
555. Helfer B, Boswell BC, Finlay D, Cipres A, Vuori K, Bong Kang T, Wallach D, Dorfleutner A, Lahti JM, Flynn DC *et al*: Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. *Cancer Res* 2006, **66**(8):4273-4278.
556. Senft J, Helfer B, Frisch SM: Caspase-8 interacts with the p85 subunit of phosphatidylinositol 3-kinase to regulate cell adhesion and motility. *Cancer Res* 2007, **67**(24):11505-11509.
557. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH: Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biol* 2013, **14**:32.
558. Darnell JE, Jr., Kerr IM, Stark GR: Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 1994, **264**(5164):1415-1421.
559. Harada H, Matsumoto M, Sato M, Kashiwazaki Y, Kimura T, Kitagawa M, Yokochi T, Tan RS, Takasugi T, Kadokawa Y *et al*: Regulation of IFN-alpha/beta genes: evidence for a dual function of the transcription factor complex ISGF3 in the production and action of IFN-alpha/beta. *Genes Cells* 1996, **1**(11):995-1005.
560. Song J, Guan M, Zhao Z, Zhang J: Type I Interferons Function as Autocrine and Paracrine Factors to Induce Autotaxin in Response to TLR Activation. *PLoS One* 2015, **10**(8):e0136629.
561. Gautier G, Humbert M, Deaudeau F, Scuiller M, Hiscott J, Bates EE, Trinchieri G, Caux C, Garrone P: A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J Exp Med* 2005, **201**(9):1435-1446.
562. Silginer M, Nagy S, Happold C, Schneider H, Weller M, Roth P: Autocrine activation of the IFN signaling pathway may promote immune escape in glioblastoma. *Neuro Oncol* 2017, **19**(10):1338-1349.
563. Greer YE, Gilbert SF, Gril B, Narwal R, Peacock Brooks DL, Tice DA, Steeg PS, Lipkowitz S: MEDI3039, a novel highly potent tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor 2 agonist, causes regression of orthotopic tumors and inhibits outgrowth of metastatic triple-negative breast cancer. *Breast Cancer Res* 2019, **21**(1):27.

564. Dine JL, O'Sullivan CC, Voeller D, Greer YE, Chavez KJ, Conway CM, Sinclair S, Stone B, Amiri-Kordestani L, Merchant AS *et al*: The TRAIL receptor agonist drozitumab targets basal B triple-negative breast cancer cells that express vimentin and Axl. *Breast Cancer Res Treat* 2016, **155**(2):235-251.
565. Kreuz S, Siegmund D, Rumpf JJ, Samel D, Leverkus M, Janssen O, Hacker G, Dittrich-Breiholz O, Kracht M, Scheurich P *et al*: NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. *J Cell Biol* 2004, **166**(3):369-380.
566. Saleh D, Najjar M, Zelic M, Shah S, Nogusa S, Polykratis A, Paczosa MK, Gough PJ, Bertin J, Whalen M *et al*: Kinase Activities of RIPK1 and RIPK3 Can Direct IFN-beta Synthesis Induced by Lipopolysaccharide. *J Immunol* 2017, **198**(11):4435-4447.
567. Goubau D, Deddouch S, Reis e Sousa C: Cytosolic sensing of viruses. *Immunity* 2013, **38**(5):855-869.
568. Paludan SR, Bowie AG: Immune sensing of DNA. *Immunity* 2013, **38**(5):870-880.
569. Bourne N, Scholle F, Silva MC, Rossi SL, Dewsbury N, Judy B, De Aguiar JB, Leon MA, Estes DM, Fayzulin R *et al*: Early production of type I interferon during West Nile virus infection: role for lymphoid tissues in IRF3-independent interferon production. *J Virol* 2007, **81**(17):9100-9108.
570. Petherick KJ, Conway OJ, Mpamhanga C, Osborne SA, Kamal A, Saxty B, Ganley IG: Pharmacological inhibition of ULK1 kinase blocks mammalian target of rapamycin (mTOR)-dependent autophagy. *J Biol Chem* 2015, **290**(18):11376-11383.
571. Clark K, MacKenzie KF, Petkevicius K, Kristariyanto Y, Zhang J, Choi HG, Peggie M, Plater L, Pedrioli PG, McIver E *et al*: Phosphorylation of CRTC3 by the salt-inducible kinases controls the interconversion of classically activated and regulatory macrophages. *Proc Natl Acad Sci U S A* 2012, **109**(42):16986-16991.
572. Lork M, Kreike M, Staal J, Beyaert R: Importance of Validating Antibodies and Small Compound Inhibitors Using Genetic Knockout Studies-T Cell Receptor-Induced CYLD Phosphorylation by IKKepsilon/TBK1 as a Case Study. *Front Cell Dev Biol* 2018, **6**:40.
573. Zabel U, Baeuerle PA: Purified human I kappa B can rapidly dissociate the complex of the NF-kappa B transcription factor with its cognate DNA. *Cell* 1990, **61**(2):255-265.
574. Whiteside ST, Israel A: I kappa B proteins: structure, function and regulation. *Semin Cancer Biol* 1997, **8**(2):75-82.
575. Zarnegar BJ, Wang Y, Mahoney DJ, Dempsey PW, Cheung HH, He J, Shiba T, Yang X, Yeh WC, Mak TW *et al*: Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. *Nat Immunol* 2008, **9**(12):1371-1378.