THESIS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

(PHD)

TRANSCRIPTIONAL CONTROL OF

TRANSGLUTAMINASE 2 EXPRESSION IN MOUSE

APOPTOTIC THYMOCYTES

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LIST OF ABBREVIATIONS

3C	Chromosome conformation capture	CED	Caenorhabditis elegans cell-death gene
9cRA	9-cis retinoic acid	ChIA-PET	Chromatin Interaction Analysis by Paired-End Tag
A2AR	Adenosine 2A receptor	ChIP	Chromatin Immunoprecipitation
A3R	Adenosine 3 receptor	CREB	cAMP Response Element-Binding Protein
ADA	Adenosine Deaminase		
AMP	Adenosine-5'-monophosphate	CRTC1	CREB-regulated Transcription Coactivator 1
APO	Apoptotic thymocytes		
ATAC-seq	Assay for Transposase-Accessible Chromatin-sequencing	CTCF	CCCTC-binding factor
		dbcAMP	Dibutyryl-cAMP
ATP	Adenosine-5'- triphosphate	DHS	DNase I hypersensitive site
ATRA	All-trans Retinoic Acid	DMSO	Dimethyl sulfoxide
BMP	Bone Morphogenetic Protein	DN	Double negative
BSA	Bovine Serum Albumin	DNA	Deoxyribonucleic acid
CAD	Caspase Activated DNAse	DSG	Disuccinimidyl glutarate
cAMP	Cyclic Adenosine Monophosphate	ENCODE	Encyclopedia of DNA Elements
CBP	CREB-binding protein	EPAC	Exchange Factor Directly Activated by cAMP
CD	Cluster of Differentiation		
CD73	Ecto-5'-nucleotidase	ER	Endoplasmic reticulum
C/EBP	CCAAT-Enhancer-Binding Protein	eRNA	Enhancer RNA
		GAS 6	Growth arrest-specific 6

GR	Glucocorticoid receptor	NFR	Nucleosome Free Region
GRO-seq	Global Run-On Sequencing	NGS	Next-Generation Sequencing
GTP	Guanosine-5'-triphosphate	NPP	Ecto-nucleotide pyrophosphatase/phosphodi esterase
HDAC	Histone deacetylas		
HIF	Hypoxia-inducible factors	NR	Nuclear receptor
IGV	Integrative Genomics Viewer	PAF	Platelet-Activating Factor
IP3	Inositol Trisphosphate	PBS	Phosphate-buffered saline
KC	Neutrophil-attracting Chemokine	PGE2	Prostaglandin E2
		PKA	Protein Kinase A
LXR	Liver X Receptor	PPAR	Peroxisome Proliferator-Activated Receptor
MADP	Methyleneadenosine 5'-diphosphate		
		PS	Phosphatidylserine
MFG-E8	Milk Fat Globule-EGF Factor 8 protein	RAP1-GDS1	RAP1,GTP-GDP Dissociation Stimulator 1
MHC	Major Histocompatibility Complex	RAR	Retinoic acid receptor
MIP-2	Macrophage Inflammatory Protein-2	RNAPII	RNA Polymerase II
		RNA-seq	RNA-sequencing
MPh	Macrophages	ROR	Retinoid-related Orphan Receptor
MPT	Mitochondrial Permeability Transition pore		
		RXR	Retinoid X receptor
NECA	1,5'-N-ethylcarboxamidoadenosin	SMAD	Sma and Mad Related Family
NF-κB	Nuclear Factor κ-light-chain-enhancer of Activated B cells	SP	Single positive
		STAT	Signal Transducer and Activator of Transcription

TAF1	TATA Box Binding Protein (TBP)-Associated Factor	TNF	Tumor Necrosis Factor
TCR	T cell receptor	TR	Thyroid hormone receptor
TEC	Thymic epithelial cell	TSP	Thrombospondin
TF	Transcription factor	TSS	Transcriptional start site
TGM2	Tissue Transglutaminase	UTR	Untranslated region
TGF-β	Transforming Growth Factor β	VDR	Vitamin-D receptor
		WT	Wild type

1. INTRODUCTION

1.1 Apoptosis, history and basic characteristics

The apoptosis term was first used in 1972 to discriminate a morphologically different form of cell death (Kerr et al., 1972). The molecular details of apoptosis have been first described in the well-known nematode model organism, *Caenorhabditis elegans* (Shaham et al., 1999; Gumienny et al., 1999). In C. elegans, 1090 somatic cells are developed during the growth of the worm. Out of the 1090, 131 of these die due to the apoptotic program at certain points during development. This phenomenon is almost completely invariant between worms, showing a striking accuracy of the system. These observations unequivocally show the advantage of this model system, allowing the tracking of the faith of each individual cell. Horvitz using a series of elegant experiments proved that a genetic program exists in the nematode regulating cell death during development. In 1986, Horvitz and Ellis showed in a pioneering publication, that the two "death" genes, ced-3 and ced-4 are prerequisite for the execution of the cell death program, but they are not essential for the development of the nematode (Ellis and Horvitz, 1986). It was the first publication showing, that a programmable cell death pathway exists, which determines when, and which cell will die during development. Later on, he also discovered another gene called ced-9 which counteracts with ced-4 and ced-3 by a direct, physical interaction limiting cell death (Hengartner and Horvitz, 1994). Importantly, their studies also shed light on a ced-3 like gene in the human genome, which was the first step toward identifying the key players of the human apoptosis program. Today, we know that almost all of the genes that are crucial in the apoptosis program of *C. elegans* also exist in humans, supporting the importance of the findings in the model organism.

According to these findings, apoptosis normally occurs during development and aging in order to maintain normal cell populations in the body, functioning as a homeostatic mechanism. However, it has been also shown that it is also important as a defensive mechanism. During various immune reactions or when the cells are damaged in an irreversible manner by noxious chemical reagents, apoptosis takes place (Norbury and Hickson, 2001). It is important to note that plenty different agents can trigger apoptosis, but not all the cells will be responsive in the same way for a given stimuli. Cancer chemotherapy drugs and irradiation leads to apoptosis in many different cell types due to the serious DNA damage, which in turn activates the p53-dependent apoptosis pathway (Elmore, 2007). Interestingly, hormones like corticosteroids can trigger apoptosis via binding to the glucocorticoid receptor, known as a ligand activated

transcription factor (TF), leading to cell death in a very specific cell subset, known as thymocytes, however other cells are not sensitive for the stimulus in the thymic environment. It is important to note that corticosteroids can also affect the apoptosis program of the skeletal system, muscular system, circulatory system, nervous system, endocrine system and reproductive system, while exerting an anti-apoptotic response on others (Gruver-Yates and Cidlowski, 2013).

Other apoptosis pathways are initiated by the Fas or TNF cell surface receptors. Upon ligand binding, these receptors exert their apoptotic effects following protein cross-linking. An alternative mechanism for apoptosis initiation is interfering with the continuous blocking of the cell death pathway by a survival factor, which can be a hormone or various growth factors. These examples show that the apoptotic program can be achieved by different mechanisms of action, which is important for the precise, cell type-specific and spatio-temporal execution of programmed cell death. Due to the highly sophisticated nature of this process, apoptosis is very often an energy-dependent mechanism, involving the activation of the caspases (cysteine proteases) and the layered cascade processes achieved by these enzymes, which make the connections between the cell death initiating stimuli and the death of the cell (Elmore, 2007).

1.2 Apoptosis or Necrosis?

Several types of cell death have been described and classified based on different aspects as morphological appearance, enzymological criteria, functional aspects or immunological characteristics.
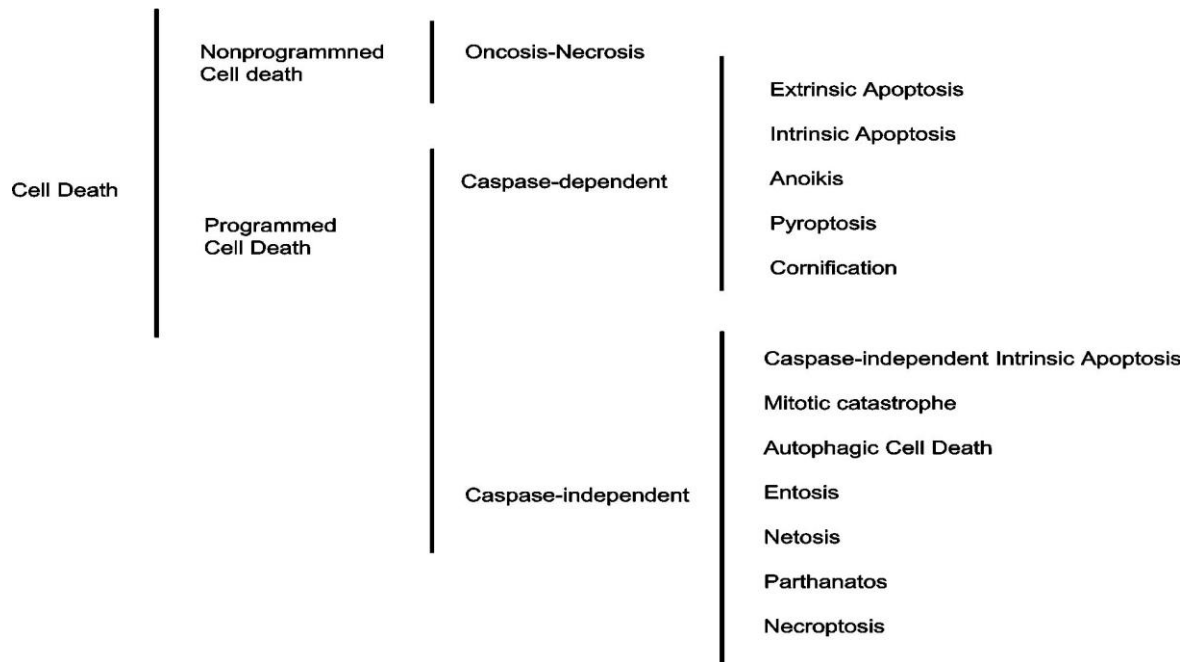
Figure 1. The classification of different types of cell death, in accordance with to the Nomenclature Committee on Cell Death (Galluzzi et al. 2012)

Historically, the classical methods to define cell death rely on morphological criteria. According to this, apoptosis was named programmed cell death type I, autophagy termed as programmed cell death type II, and necrosis as a type of death without characteristics of both type I and type II. The appearance of novel biochemical methods during the last decades helped the classification of cell death forms, providing a detailed view about these processes. Therefore, a more definite and concise classification has become necessary (Fig.1).
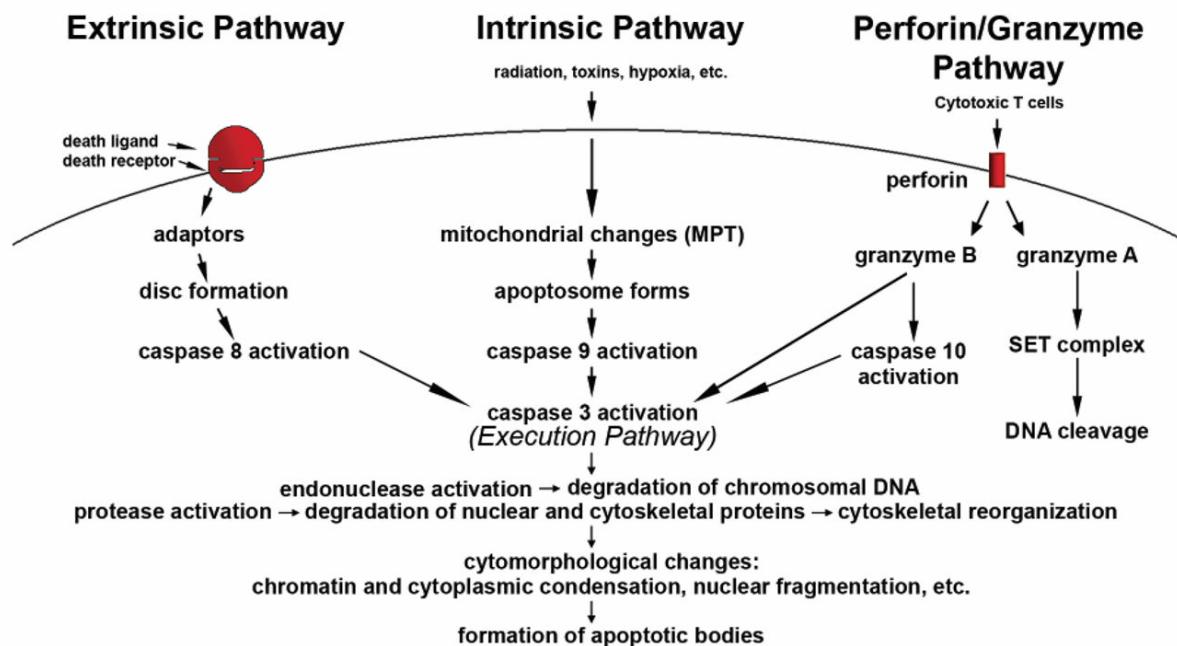
Necrosis serves as an alternative to apoptotic cell death, which is a passive, energy-independent process. The mechanisms and cell morphologies of the two processes are different, still there are common parts between the two. The "apoptosis-necrosis continuum" concept describes the commonalities of the two processes and provides evidence of a shared biochemical network

between the two (Zeiss, 2003; Leist et al., 1997; Denecker et al., 2001). The mode of how particular cells die is dependent on the nature of the cell death signal, the tissue milieu and the developmental stage (Fiers et al., 1999; Zeiss, 2003).

Necrosis is an uncontrolled, passive mode of cell death and usually affects big tissue areas, while apoptosis is extremely controlled, affecting individual cells in an energy-dependent manner. The two main initiators of necrotic cell death is the interference with energy supply of the cell or a direct, serious damage to the cell membrane. The major morphological changes occurring during necrosis include the followings: formation of cytoplasmic vacuoles, cell swelling, distended endoplasmic reticulum, formation of cytoplasmic blebs, ruptured mitochondria, disrupted organelle membranes and cell membrane (Kerr et al., 1972; Majno and Joris, 1995). The uncontrolled release of cytoplasmic content of the necrotic cell, initiate the recruitment of inflammatory cells leading to local inflammation. As opposed to necrosis, apoptosis does not lead to the release of the cytoplasmic content, thus it does not cause inflammation (Savill and Fadok, 2000). Pyknosis (chromatin condensation) and karyorrhexis (fragmentation of the nucleus) are not specific to apoptosis, because these can be observed also as part of the various cytomorphological changes during necrosis (Elmore, 2007).

1.3 Mechanisms of Apoptosis

From the mechanistic point of view, apoptosis is a highly complex, sophisticated process. Research in the last two decades show, that there are two main apoptotic pathways in the cells, the extrinsic or death receptor pathway and the intrinsic, or mitochondrial pathway (Elmore, 2007). Later on, it has been shown that the two pathways are interconnected and the regulators of one pathway can affect the other (Igney and Krammer, 2002). In addition, a completely different pathway also exists, which involves T-cell triggered cytotoxicity and the so called perforin-, granzyme-dependent execution of the cell. The above mentioned three apoptotic pathways will converge ultimately on the final, execution pathway, which is initiated by caspase3 leading to DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally phagocytic uptake by classical phagocytes (Elmore, 2007) (Fig. 2).
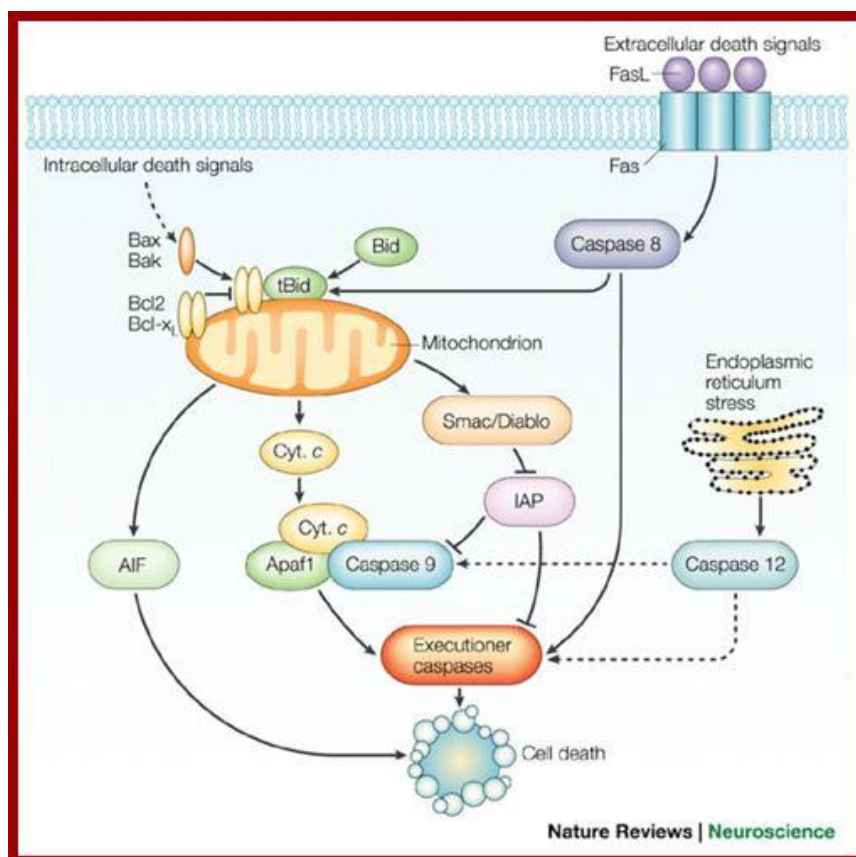
Extrinsic Pathway

death ligand →
death receptor →

adaptors

disc formation

caspase 8 activation

Intrinsic Pathway

radiation, toxins, hypoxia, etc.

mitochondrial changes (MPT)

apoptosome forms

caspase 9 activation

Perforin/Granzyme Pathway

Cytotoxic T cells

perforin

granzyme B   granzyme A

caspase 10 activation

SET complex

DNA cleavage

caspase 3 activation
(Execution Pathway)

endonuclease activation → degradation of chromosomal DNA
protease activation → degradation of nuclear and cytoskeletal proteins → cytoskeletal reorganization

cytomorphological changes:
chromatin and cytoplasmic condensation, nuclear fragmentation, etc.

formation of apoptotic bodies

Figure 2. Schematic representation of apoptotic events. The main pathways of apoptosis are extrinsic and intrinsic as well as a perforin/granzyme pathway. Each pathway activates its own initiator caspase (8, 9, 10) which in turn will activate the final so called execution caspase-3. Granzyme A works in a caspase-independent fashion (Elmore, 2007).

Extrinsic pathway of apoptosis is initiated in response to activation of the so-called death receptors, for example as CD95 (also known as FAS), tumor necrosis factor receptor 1 (TNFR1) or TNF-related apoptosis-inducing ligand (TRAIL) receptor. As a consequence, several proteins are recruited to the ligated receptors triggering the activation of the effector caspases. Caspases are very efficient cystein proteases, cleaving protein substrates in the cell, to activate the apoptotic program. These enzymes also able to cleave the B-cell lymphoma 2 (BCL-2) homology 3 (BH3)-only protein BID (BH3-interacting domain death agonist), which co-activates the intrinsic apoptosis pathway via its translocation to the mitochondria (Marino et al., 2014).

The intrinsic signaling pathway of apoptosis is thought to be more complex and also known as the mitochondrial pathway, involving non-receptor-mediated intracellular signals. Diverse stimuli are able to initiate alterations in the inner mitochondrial membrane, resulting in an opening of the mitochondrial permeability transition (MPT) pore, leading to the loss of mitochondrial transmembrane potential and release of two main pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens et al., 2004).

Members of the first group collects cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi (Cai et al., 1998; Du et al., 2000; Loo et al., 2002; Garrido et al., 2005). All of these proteins are acting via the induction of the caspase-dependent mitochondrial pathway. Cytochrome *c* activates Apaf-1 along with procaspase-9, forming an "apoptosome", which is established by the clustering of procaspase 9 (Chinnaiyan, 1999; Hill et al., 2004). The formation of the apoptosome leads to the activation of caspase-9. Smac/DIABLO and HtrA2/Omi are also described to promote apoptosis by attenuating the activities of IAPs (inhibitors of apoptosis proteins) (van Loo et al., 2002a; Schimmer, 2004) (Fig.3).

As a late event of the program, the second group of the released pro-apoptotic proteins can cause DNA fragmentation in a caspase-independent (AIF, endonuclease G) or caspase-dependent manner (Caspase Activated DNAse, CAD). (Marino et al, 2014).

Figure 3. *Intrinsic (mitochondrial) pathway of apoptosis (Kroemer, 2009)*

These complex apoptotic mitochondrial events are regulated by the members of Bcl-2 family of proteins (Cory and Adams, 2002) (Fig. 4). Bcl-2 proteins govern mitochondrial membrane permeability, thus they are crucial regulators of the release of cytochrome c and can be either pro- or anti-apoptotic.

The establishment of Bax or Bak-containing membrane channels are under negative regulation by the antiapoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-xL, and Mcl-1, while promoted by the BH3 only members of this family (Walensky, 2006)

Cellular stress, for example DNA damage, can lead to an increase in pro-apoptotic protein levels, which in turn allows pro-apoptotic BCL-2 proteins to induce the release of cytochrome c from mitochondria, activating the caspase cascade (Schlossmacher et al., 2011).
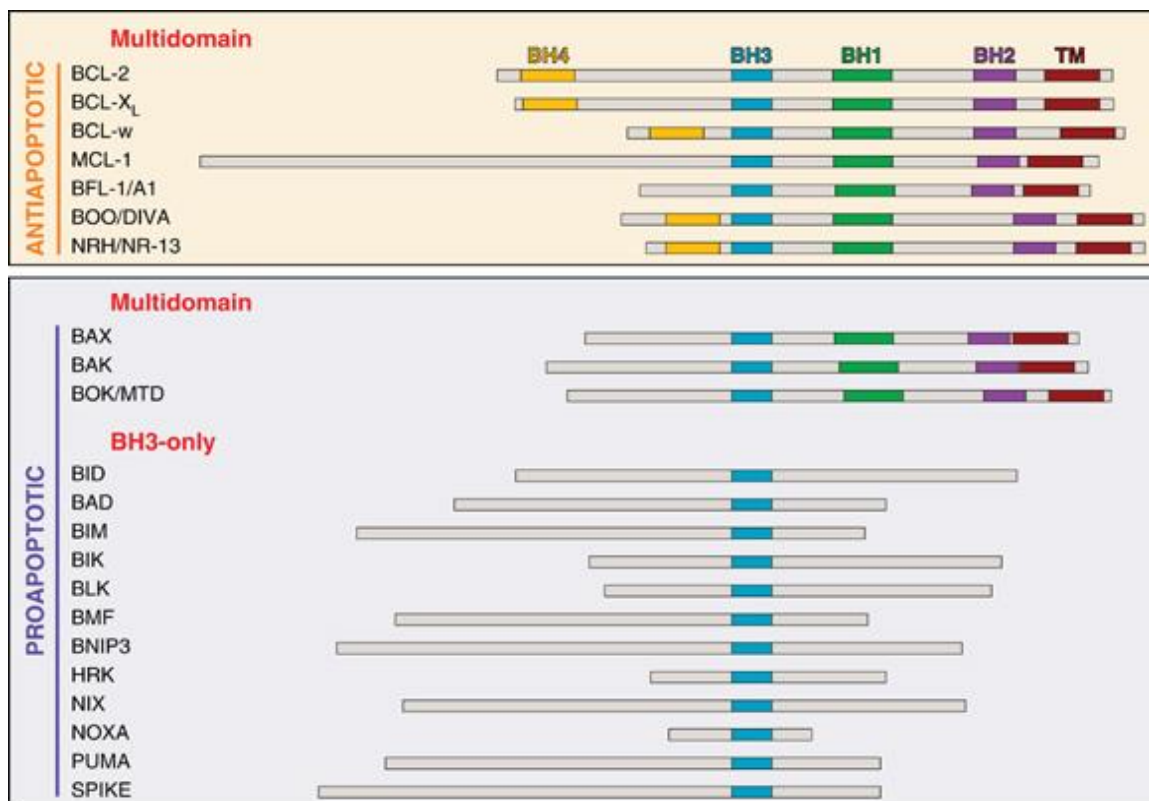
Figure 4. *The members of the Bcl-2 family. Antiapoptotic and proapoptotic protein members are shown. Bcl-2 proteins are also classified based on their domain structure (Walensky, 2006).*

Regardless of whether the intrinsic or extrinsic pathway is triggered by the apoptotic signals received by thymocytes, a common executor pathway then follows. This involves activation of the effector caspases, such as caspase-3, -6, and-7, by limited proteolysis catalyzed by the initiator caspases. The activated effector caspases then cleave at least 500 different proteins resulting in all the molecular and morphological changes that characterize apoptosis (Elmore, 2007).

1.4 T cell development, selection and apoptosis in the thymic environment

The thymus is the primary lymphoid, multi-lobed organ responsible for the maturation of immunocompetent T lymphocytes from CD4-CD8-double negative bone marrow-derived progenitors. This occurs via a series of differentiation and selection steps (Szondy et al., 2012). The thymic milieu directs these processes via the combinations of available molecules secreted by the dendritic cells, macrophages, endothelial cells, fibroblasts, and thymic epithelial cells (TECs) found in the thymus.

During differentiation, in the cortex, progenitor cells rearrange their TCR-β gene locus to express a pre-TCR complex consisting of the polymorphic β-chain, a nonpolymorphic pre-α chain, and CD3. Those thymocytes that are not able to create the preTCR undergo apoptosis is thought to be induced by the BH3 only Bcl-2 family proteins Bim and Bid (Szondy et al., 2012). Those thymocytes that express the pre-TCR, divide rapidly, acquire CD4 and CD8 molecules becoming CD4+CD8+ double positive (DP) cells, and undergo TCRα rearrangement. Cells that are able to successfully generate a functional α chain, replace their pre-TCR with mature TCRαβ.

During this period, expression of Bcl-xL and Mcl-1, two antiapoptotic Bcl-2 family members, allows the opportunity to test the newly exhibited TCR. The expression of Mcl-1 is continuous during thymocyte development, Bcl-xL - induced by retinoid-related orphan receptor (ROR)-γ - is specifically expressed on DP thymocytes, while express low levels of the antiapoptotic Bcl-2, due to a failure in IL-7 signaling (Szondy et al., 2012). The low level of Bcl-2 ensures, that these cells are more susceptible to apoptosis, which is needed for the proper selection of self-reacting thymocytes.

However, 90% of the DP thymocytes that are incapable of recognizing self-peptides presented on major histocompatibility complex (MHC) molecules present in the thymus undergo a default

death pathway named "death by neglect" (Chung et al., 2002; Szondy et al., 2012).The mechanism is unclear, but it has been proposed that, (1) in the absence of TCR signaling, binding of glucocorticoids (produced by TECs or the adrenal cortex), via the mitochondrial pathway involving Bcl-2 family members (Veis et al., 1993), especially via Puma and Bim (Erlacher et al., 2006; Villunger et al., 2003), or (2) the crosslinking of various cell surface receptors (such as CD8, CD24, CD45) will induce the cell death program in the neglected DP cell population.

Thymocytes harboring a TCR that strongly interacts with MHC molecules containing self-antigens are eliminated by negative selection via activation-induced apoptosis. This occurs via two molecular mechanisms: (1) Bim (Bouillet et al., 2002), a BH3 only protein that can neutralize the activity of each antiapoptotic Bcl-2 family members in a dose-dependent manner (Youle and Strasser, 2008), and (2) activation of Nur77, a transcription factor that can upregulate the initiators of the extrinsic cell death pathway (Rajpal et al., 2003). In addition, following translocation into the mitochondria, Nur77 can interact with Bcl-2, converting it into a proapoptotic protein (Thompson and Winoto, 2008).

Thymocytes having TCRs with low-to-moderate avidity for self-peptide/MHC are preserved by positive selection (Coutinho and Chapman, 2011) – expressing higher level of Bcl-2, that protect them from apoptosis – and continue to differentiate into CD4+ or CD8+ single positive cells (Kisielow and von Boehmer, 1995) and complete their maturation in the thymic medulla.

In the medulla, they will be further exposed to "self-specific" peripheral tissue antigens presented either by medullary TECs as a consequence of TEC expression of the autoimmune regulator transcription factor Aire (Anderson et al., 2002) or by thymic dendritic cells. Those cells that survive this second wave of negative selection will migrate into the periphery and form the peripheral T-cell repertoire. At the periphery, if T cells do not meet with antigen, they become eliminated by apoptosis.

1.5 Clearance of apoptotic cells

In vivo, the apoptosis program is completed by phagocytosis, with the majority of apoptotic thymocytes in mice being cleared by the thymic macrophages.

The clearance of apoptotic cells is a multistep process. First, at the early stages of apoptosis, cells that are engaged in the cell death program exhibit so called "find me" signals, which can

be recognized by professional phagocytes via their cell surface receptors. These find me signals can be: nucleotide triphosphates (ATP/UTP), lysophosphatidylcholine, CX_3CL1 chemokines, which can initiate the chemotaxis of professional phagocytes.

Second, with the help of the so called "eat me" signals, deposited on the cell surface, they can be phagocytosed by macrophages. Amongst the "eat me" signals phosphatydilserine (PS) is a key molecule. In normal cells, aminophospholipid translocase is responsible of keeping PS on the cytoplasmic side of the cell membrane via the so called flip-flop mechanism. Other molecules like the endoplasmic reticulum-specific calreticulin, or annexin I (Anx-I) are also exhibited on the cell surface of apoptotic cells, serving as "eat me" signals for phagocytes.

PS can be recognized by the phagocyte-specific Bai1, Tim-4 or Stabilin-2 receptors. In addition, other soluble molecules like thrombospondin-1 (TSP-1), milk-fat-globule-EGF factor-8 (MFG-E8), growth arrest-specific 6 (GAS6), β_2-glykoprotein I (β_2-GPI) and serum protein S can form bridges between the phosphatydilcholine found on the apoptotic cell surface and the phagocyte receptors, allowing the efficient uptake of apoptotic cells.

Finally, the phagocytosed cell/cell parts are digested via the phagolysosomal pathway. As a result, the ingested cell parts can be reused by the phagocyte and the indigestible cell part are excreted (Elliott and Ravichandran, 2010).

The clearance of apoptotic cells and cell parts by professional phagocytes represent an evolutionary conserved mechanism, preventing the release of the cytotoxic, or immunogenic cellular components. (Chung et al., 2006) In the absence of proper clearance, dying cells are able to undergo secondary necrosis, leading to inflammation, which is a suspected cause of autoimmunity (Hochreiter-Hufford and Ravichandran 2013). The most important feature of the phagocytosis of apoptotic cells, that distinguishes it from the phagocytosis of most bacteria or necrotic cells is the release of anti-inflammatory cytokines IL-10, TGF-β, platelet activating factor (PAF), prostaglandin E2 (PGE2) and adenosine (via A2A receptors). Another inherent characteristic of apoptosis is the inhibition of production of pro-inflammatory cytokines (including TNF-α), thus attenuating immune, or autoimmune responses (Chung et al., 2006; Hochreiter-Hufford and Ravichandran, 2013). Importantly, it has been shown that failure of apoptotic cell clearance leads to autoimmune disorders such as systemic lupus erythematosus or rheumatoid arthritis (Ravichandran and Lorenz, 2007), underlying the importance of the clearance of apoptotic cells during normal physiological responses.

1.6 Macrophage secreted molecules are inducers of apoptosis in the thymic environment

The phagocytosis process greatly affects the participating macrophages, which in turn become reprogrammed and release various signaling molecules and cytokines, affecting the apoptosis program of non-functional DP thymocytes.

It has been shown by our lab and others that various molecules, mediators are secreted by macrophages in the thymic environment during the engulfment, including transforming growth factor beta (TGF-β) (Fadok et al., 1998), adenosine (Koroskenyi et al., 2011) and retinoids (Garabuczi et al., 2013). These molecules can act on macrophages in an autocrine manner, but importantly, double positive thymocytes express receptors for all the above mentioned molecules. In addition, DP cells are also affected by glucocorticoids and retinoids produced by MHC presenting thymic epithelial cells. All of these contribute to the formation of the pro-apoptotic thymic milieu that ensures the induction of apoptosis of neglected thymocytes in the absence of TCR signaling (Chung et al., 2002; Szondy et al., 2003; Szondy et al., 2012).

Retinoids have been shown to induce apoptosis by activating retinoic acid receptor (RAR)γ (Szondy et al., 1997), and they also enhance glucocorticoid-induced apoptosis by facilitating the transcriptional activity of the glucocorticoid receptor acting on the RAR-α/retinoid X receptor heterodimers (Toth et al., 2011).

Adenosine has been also described as an apoptosis inducer in mouse thymocytes via the activation of the adenosine A2A receptor/adenylate cyclase pathway resulting in the upregulation of Bim (Kiss et al., 2006). PGE_2 also triggers adenylate cyclase activation, inducing cell death *in vivo* (Madstino et al., 1992).

Though TGF-β alone does not trigger apoptosis, it can ameliorate both glucocorticoid- and TCR-driven cell death (Szondy et al., 2003). So far, the underlying mechanism has not been investigated in thymocytes, but in B cells, TGF-β has been shown to regulate Bim (Ramesh et al., 2008). Moreover, both carbon monoxide (CO) and ATP have been described as thymocyte apoptosis-inducing signals, whereas ATP activating the mitochondrial intrinsic pathway of apoptosis (Turcano et al., 1998; Tsukimoto et al., 2006).

The majority of thymocytes dies and phagocytosed in the cortex (Surh et al., 1994). According to this, the cortex might contain bigger concentrations of these molecules if compared to the medulla. This presumption is supported by the study, showing that the enzymes of retinoid synthesis have higher levels in the thymic cortex (Kiss et al., 2008).

Based on these observations, it has been proposed that macrophages engulfing apoptotic cells continuously release engulfment-dependent molecules into the microenvironment of DP thymocytes that might initiate and tailor the apoptosis program of neglected cells (Fig. 5).
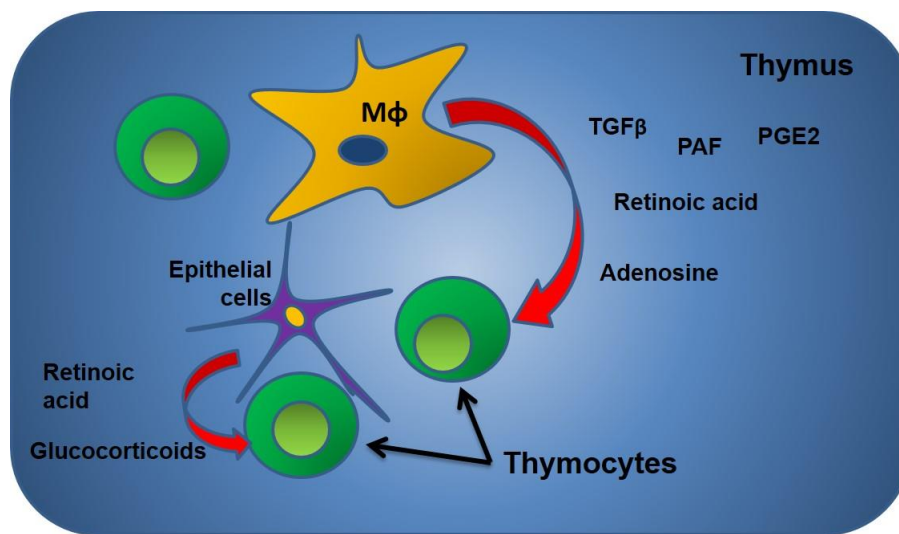
Figure 5. Signals initiating the apoptotic program of thymocytes are secreted by the cellular components of the thymus. TGFβ - transforming growth factor beta; PAF- Platelet-activating factor; PGE2- prostaglandin E2

Adenosine

Adenosine is build up of adenine and ribose and is generated by the degradation of ATP. It has widespread effects on the body, playing important roles in growth, differentiation and metabolism. Its excessive presence is an indicator of tissue injury and the absence of ATP resynthesis, which is indicative of starvation due to the lack of available nutrients. In accordance, it has been shown as an important mediator of anti-inflammatory and regeneration processes (Liu et al., 2015).

Adenosine is ubiquitously produced in almost all of the cells in our body under physiological condition and its level is highly elevated under stress condition. On the one hand, during normal

physiological conditions, both extracellular and intracellular adenosine levels are in the nanomolar range. On the other hand, under stress conditions, ATP is released from injured cells such as endothelial cells, neutrophils, and glial cells via transmembrane protein channels including pannexins or connexins and subsequently dephosphorylated to extracellular adenosine by ecto-nucleotidases including CD39, converting ATP to ADP/AMP and CD73, and converting AMP to adenosine.
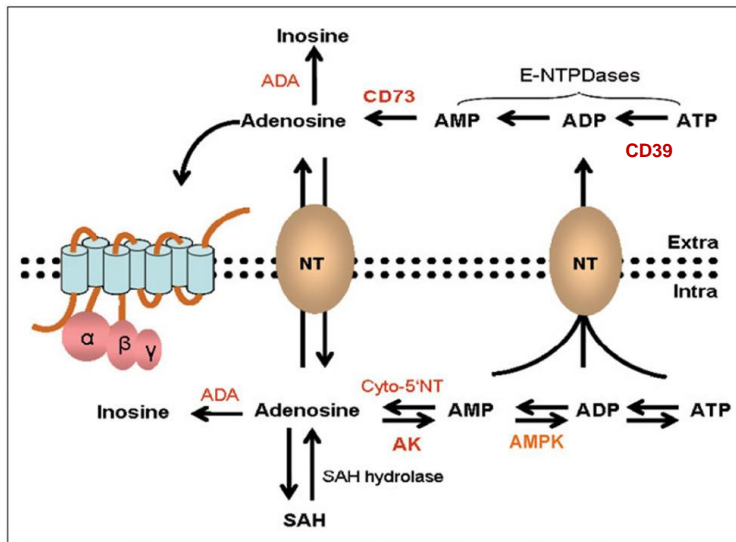
Figure 6. The pathways of adenosine synthesis and metabolism inside and outside the cell. Within the cell ATP and adenosine are continuously recycled depending on energy requirements via a series of dephosphorylation and phosphorylation steps mediated by enzymes such as cytosolic 5'-nucleotidase (cyto-5'NT), adenosine kinase (AK), and AMP kinase (AMPK). Under physiological conditions the rate limiting enzyme is AK. Adenosine can also be generated from the hydrolysis of S-adenosyl-homocysteine (SAH). In response to high metabolic activity ATP and adenosine is extruded to the outside of the cell via facilitated diffusion and secondary active transport. Upon stress conditions cells release ATP through connexins or pannexins channels. Further degradation of ATP to adenosine where it can activate adenosine receptors. Excess adenosine is irreversibly deamidated to inosine by the enzyme adenosine deaminase (ADA) (Ham et al., 2012).

Upon pathological conditions, extracellular adenosine concentrations can be highly elevated, almost reaching the millimolar range. Production of extracellular adenosine by these pathways is the main source of extracellular adenosine upon hypoxic condition-induced injury. Furthermore, extracellular adenosine is regulated by adenosine deaminase (ADA), which is capable of degrading extracellular adenosine to inosine. In addition, extracellular adenosine signaling is terminated by equilibrative nucleoside transporters (ENTs), which are the regulators of cellular adenosine uptake. After the transport, adenosine is metabolized by three enzymes in the intracellular environment: adenosine kinase (ADK), S-adenosylhomocytesine

hydrolase (SAHH), and adenosine deaminase (ADA). ADA catalyzes the irreversible conversion of adenosine to inosine. SAHH converts adenosine to adenosylhomocysteine (Ado-Hcy). ADK phosphorylates adenosine to AMP and is important in the regulation and maintenance of intracellular levels of adenosine and adenine nucleotides. The intracellular homeostasis of adenosine is also carried out by bidirectional equilibrative nucleoside transporters (ENTs) located on the plasma membrane via facilitated diffusion of adenosine according to the direction of concentration gradient (Fig. 6) (Liu et al., 2015).

Adenosine receptors

Adenosine acts via autocrine and paracrine mechanisms. It binds to specific seven-transmembrane receptors, coupled to G-proteins, but G-protein independent pathways were also identified. There are four adenosine receptors, all of which are G protein-coupled receptors. Based on their affinity ARs can divide into four subtypes: A1 (A_1R), A2A ($A_{2A}R$), A2B ($A_{2B}R$) and A3 (A_3R) (Haskó *et al.,* 2007; Table 1). All of these receptors are expressed on the surface of macrophages. A1 and A3 adenosine receptors are coupled to adenylyl cyclase by the inhibitory G-protein subunit (Gαi) and thereby can lower intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP), adenosine A_{2A} (A2AR), and A_{2B} receptors are commonly coupled to adenylyl cyclase by the stimulatory G-protein subunit (Gαs) and therefore can induce intracellular cAMP levels. Therefore, signaling through adenosine receptors plays important roles in the regulation of intracellular cAMP and thereby regulates multiple cellular functions, while adenosine A_3 receptors (A3Rs) mediate adenylate cyclase inhibition (Fredholm et al., 2001).

The **A_{2A} receptor** is expressed at high level on the immune cells participating in the organization of the immune response, for example macrophages, T cells, dendritic cells, mast cells, neutrophils or even on endothelial cells, showing its presumed, widespread regulatory role on different cell types.

Similarly to other Gs-coupled receptors, A_{2A} receptors communicate with the downstream signaling component via the adenylate cyclase enzyme. The activation of the enzyme leads to the increase of the cAMP secondary messenger, which in turn activate the protein kinase A (PKA) enzyme. Among others, PKA phosphorylates the cAMP response element binding transcription factor (CREB), leading to its recruitment to cAMP responsive elements in the genome, affecting the expression of the target genes. As an alternative pathway for the cAMP-

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PKA pathway, upon A_{2A} receptor activation it has been shown that the MAPK signaling can be also active. Out of this, the EPAC1-C/EBP (CCAAT/enhancer-binding proteins) transcription factor pathway can be also triggered by the receptor activation (Hasko and Pacher, 2008).

Subtype	Affinity * (EC(50))	Specific agonist	Specific antagonist	G protein	Effect of G protein coupling	High expression	Intermediate expression	Low expression
A_1	HIGH (0.31 μM)	CPA CCPA CHA CVT-510	CPX CPT N-0840 WRC-0571	$G_{1/2/3}$	↓ cAMP ↑ IP$_3$/DAG (PLC) ↑ arachidonate (PLA$_2$) ↓ Q, P, N-type Ca^{2+} channels ↑ K$^+$ channels ↑ choline, DAG (PLD) βγ-mediated	Brain (cortex, cerebellum, hippocampus) spinal cord, eye, adrenal gland, atria	Other brain regions, sceletal muscle, liver, kidney, adipose tissue, salivary gland, esophagus, colon, antrum, testis	Lung, pancreas
				G_0				
A_{2A}	HIGH (0.7 μM)	CGS21680 DPMA HE-NECA ATL-146E	KW6002 CAFFEINE SCH58261 ZM241,385	G_s G_{olf} $G_{15/16}$	↑ cAMP ↑ cAMP ↑ IP$_3$	Spleen, thymus, leukocytes, platelets, olfactory bulb, GABAerg neurons	Heart, lung, blood vessels, peripheral nerves	Other brain regions
A_{2B}	LOW (24 μM)	BAY60–6583 LUF5835	XAC MRS1754 ALLOXAZINE MRE2029F20	G_s $G_{q/11}$	↑ cAMP ↑ IP$_3$/DAG (PLC)	Cecum, colon, bladder	Lung, eye, blood vessels, mast cells, median eminence	Adipose tissue, adrenal gland, brain, kidney, liver, ovary, pituitary gland
A_3	HIGH (0.29 μM)	IB-MECA CI-IB-MECA	I-ABOPX MRS1191 MRS1220 MRS1292 MRS1523 VUF5574 MRE3008F20	$G_{i,2,3(o)}$ $G_{q/11}$	↓ cAMP ↑ IP$_3$/DAG (PLC) ↑ choline, DAG (PLD) ↑ K$^+$-ATP channels ↑ Cl$^-$ channels βγ-mediated ↑ IP$_3$/DAG (PLC)	Testis (rat), mast cells (rat)	Cerebellum, pippocampus, microglia, lung, spleen, pineal	Thyroid, most of brain, adrenal gland, spleen, liver, kidney, heart, intestine, testis

Table 1. Classification of adenosine receptors

(Fredholm et al., 2001)

It is important to note that adenosine A2A receptors also couple to other signaling pathways, including phospholipase C (PLC) and mitogen-activated protein kinase (MAPK) pathways (Fredholm et al., 2001).

Importantly, in mouse thymocytes A2ARs specifically activate the adenylate cyclase signaling pathway (Kiss et al., 2006) and it mediates apoptosis (Kizaki et al., 1990). Our group has shown that adenosine A2AR-induced death of mouse thymocytes requires Bim, and it occurs independently of "death receptor" signaling and is inhibited by Bcl-2 and Nur77 (Kiss et al., 2006).

TGFβ

Transforming growth factor-β (TGF-β) superfamily signaling plays a critical role in the regulation of cell growth, differentiation, and development in a wide range of biological systems.

TGF-β ligands act via complexes of type II and type I receptor serine/threonine kinases, inducing Smad and other alternative signaling pathways. The so called receptor-regulated Smads (R-Smads) are phosphorylated by type I receptors and form oligomers with Smad4 (CoSmad). In the nucleus, these R-Smads/Smad4 complexes regulate gene transcription. On the other hand, inhibitory Smads (I-Smads), like Smad6 and Smad7, are negative regulators that are able to block R-Smad phosphorylation and these can trigger the dephosphorylation of type I receptors, leading to proteasomal degradation (Moustakas, 2002).

SMADs recruit co-activators such as CBP (CREB-Binding Protein)/p300 that are responsible for the acetylation of nucleosomal histones and/or associated TF, leading to transcription initiation. Alternatively, nuclear SMADs also contribute to transcriptional repression via recruiting co-repressors that associate with HDACs (Histone Deacetylases) such as CTBP (C-Terminal Binding Protein) and Sin3, or proto-oncogene Ski and SnoN (Moustakas, 2002).

Interestingly, recognition of apoptotic cells via phosphatidylserine receptors, leads to TGF-β release from macrophages (Fadok et al., 1998). TGF-β mRNA expression is up-regulated by a PS-dependent manner with the participation of p38 MAPK, ERK and JNK (Park et al., 2004). In parallel, the protein translation is also enhanced via a newly defined PSRS/RhoA/PI3K/Akt/mTOR/eiF4E signaling pathway (Xiao et al., 2008). At the same time, phagocytes produce transglutaminase 2 (TGM2), which activates latent TGF-β (Upchurch et al., 1991). Apoptotic cells and macrophages also possess TGF-β receptors. In dying thymocytes, TGF-β alone does not trigger apoptosis, but promotes cell death induced by glucocorticoids and TCRs, while in macrophages TGF-β increases the phagocytic capacity and reduces the production of pro-inflammatory cytokines (Ritter and Davies, 1998; Szondy et al., 2003).

Moreover, in the thymus, the production of TGF-β, along with TCR engagement, induces *Foxp3* expression and ultimately drives the generation of CD4+ CD25+ Foxp3+ regulatory T cells that are crucial for immune homeostasis and self-tolerance (Liu et al., 2008).

In connection with this, thymic medulla is the area, where enriched TGF-β activity can be detected and intrathymic TGF-β activity gradually increases after birth over the neonatal time frame (Konkel et al., 2014). However, TGF-β activity can be also detected in the cortical region, where the majority of the thymocytes die (Surh and Sprent 1994).

As a summary, during apoptotic cell clearance, macrophages produce TGF-β, which in turn regulates T cell development and apoptosis in the thymus and at the periphery.

Retinoids

Retinoic acid (RA) is a morphogen derived from retinol (vitamin A), playing important roles in cellular growth, apoptosis, immune response, and epithelial growth (Duriancik et al., 2010).

Retinoids are highly oil-soluble and able to diffuse across the cell membrane. Due to the wide spectrum of RA functions, the metabolism and function of vitamin A have been extensively studied in the last 2 decades.

Retinoids are derived from diet in most animals, as there is no *de novo* synthesis pathway (Fig 7). The main circulating form of retinoid is retinol, which is bound to retinol-binding protein 4 (RBP4) and works as a carrier. Retinol homeostasis involves several proteins and enzymes that regulate its dietary uptake in intestinal cells and its storage mainly in liver hepatocytes and stellate cells (reviewed by D'Ambrosio et al., 2011)
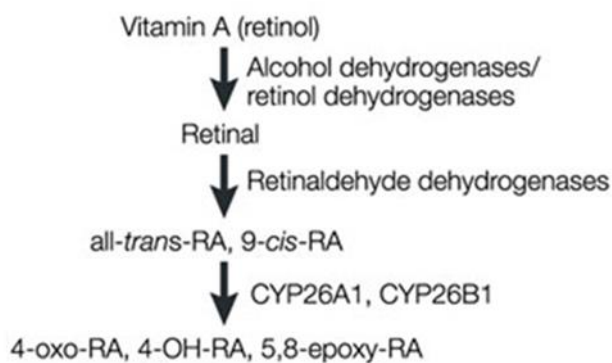
Figure 7. The metabolic pathway that converts vitamin A (retinol) into the various forms of retinoic acid (RA). (Maden, 2002.)

Retinol, after being taken up from the blood, binds to CRBP (cellular retinol-binding protein) in the cytoplasm. Retinol is metabolized to retinal and subsequently to RA – all-*trans* retinoic acid (ATRA), 9-cis-RA (9cRA) – by the retinaldehyde dehydrogenases (RALDHs: RALDH1, RALDH2 and RALDH3). RA is bound in the cytoplasm by CRABP (cellular RA-binding protein). RA enters the nucleus and binds to the RA receptors (RARs) and the retinoid X receptors (RXRs), which themselves heterodimerize and bind to a sequence of DNA known as the RARE (RA-response element).

Interestingly, RA can control the expression of its own metabolizing enzymes. RA treatments lead to rapid upregulation of the *Cyp26a1* gene, which contains two functional RAREs (Loudig et al., 2005). These data indicate that RA participates in autoregulatory negative-feedback loops by inducing the expression of its catabolizing enzyme.

It has become apparent that vitamin A deficiency leads to immune deficiency and is accompanied by susceptibility to various infectious diseases (Goodman, 1984), which is followed by remarkable atrophy of the thymus and the spleen (West et al., 1989). These symptoms could be prevented by addition of ATRA. After these discoveries, it has been suggested that the active metabolites of vitamin A are retinoic acids, which act on NRs, more specifically on the retinoic acid receptor family (Mendelsohn et al., 1994). Later on, it has been demonstrated that retinoids have an enhancing effect on the rate of glucocorticoid-induced cell death (Iwata et al., 1992; Fesus et al., 1995). On the other hand, retinoids are able to inhibit the TCR-mediated apoptosis of thymoctes and T cell hybridomas (Iwata et al., 1992; Fesus et al., 1995), without affecting the p53-dependent programmed cell death (Fesus et al., 1995). Interestingly, retionids were also found to be apoptosis inducers and it has been shown that 9-cis retinoic acid (9cRA) is a much potent inducer of T cell apoptosis than ATRA.

Surprisingly, most of the above mentioned biological effects of retinoids can be mimicked by glucocorticoids, which serve as ligands for GR. The activation of GR by glucocorticoids is a very efficient mode of apoptosis induction in T cells (Cohen, 1993; Szondy et al., 1997b). Other members of the NR superfamily, activated by thyroid hormones or vitamin D have no effect on the apoptosis program of T cells (Yang et al., 1995).

According to these, it seems that retinoids and glucocorticoids act in concert to fine-tune the positive and negative selection of thymocytes in a highly sophisticated manner.

ATRA and 9cRA are physiological ligands for the retinoic acid (RAR) and retinoid X receptors (RXR) (Chambon, 1994). These transcription factors are part of the nuclear receptor superfamily and they considered to be ligand-dependent, directly DNA-bound transcriptional regulators. ATRA and 9cRA are able to activate the RAR receptors, while ATRA is not a potent activator of RXR receptors, but it has been shown that its degradation to 9cRA can happen, which does serve as a potent activator of RXR (Heyman et al., 1992; Urbach and Rando, 1994). RXR acts as a promiscuous heterodmierization partner of several NRs, including RAR, but it can form homodimers (Zhang et al., 1992), however its physiological relevance is debated, because the homodimers are mainly detected in overexpression systems. The heterodimerization capacity of RXR is needed for the efficient binding of the partner, thus for the appropriate regulation of gene expression. An inherent characteristic feature of RXR heterodimers is whether the dimer can be activated from both sides, named as permissive dimer, or only from the partner's side, known as nonpermissive dimers. Permissive heterodimers are formed between RXR and peroxisome proliferator activated receptors (PPARs), liver X

receptors (LXRs), known as regulators a lipid- and cholesterol metabolism, while nonpermissive dimers contain RAR, thyroid receptors (TRs) or vitamin D receptor (VDR) (Evans and Mangelsdorf, 2014). The fact that there are multiple isoforms for these receptors, further increase the complexity of the system and explain the pleiotropic physiological action achieved by retinoids.

Retinoids contribute to TGFβ-induced peripheral regulatory T cell formation (Benoist and Mathis 2011), but it is not known whether they could also contribute to the formation of thymus derived regulatory T cells. Partly because RAs are known to improve the activation of the Foxp3+ locus (Benson et al. 2007) and partly because by antagonizing the biological activity of Nur77 by RAR in DP thymocytes they might promote the survival of those CD4+ thymocytes that have TCR specificities with intermediate affinity for self-antigens and express only immediate levels of Nur77 (Moran et al. 2011).

Recently, our group has shown that retinoids secreted from macrophages are also important pro-apoptotic molecules (Garabuczi et al., 2013) for immature thymocytes in a Nurr 77-dependent manner (Kiss et al., 2015). Retinoids produced by the engulfing macrophages contribute to the thymic selection processes as well, by inhibiting the negative selection of those thymocytes that have TCR specificities with intermediate affinity for self-antigens and thus perhaps contributing also to the thymic regulatory T cell formation.

Efficient execution of apoptotic cell death followed by efficient clearance mediated by professional and by nonprofessional neighboring phagocytes is a key process in maintaining tissue homeostasis. Though the two processes are subsequent, increasing evidence indicates that at least in the thymus molecules produced by macrophages during the engulfment process strongly influence the first step. Thus, they can induce the "death by neglect" of immature thymocytes (Szondy et al., 2012), or even affect the thymic selection and differentiation in the thymus by secreting the above summarized molecules (Sarang et al., 2014; Konkel et al., 2014).

1.7 Transglutaminase 2 (TGM2), a multifunctional enzyme regulating survival and death

Transglutaminases are a family of thiol- and Ca^{2+}-dependent acyl transferases that catalyze the formation of a covalent bond between the γ-carboxamide groups of peptide-bound glutamine residues and various primary amines, including the ε-amino group of lysine in certain proteins. The reaction results in post-translational modification of proteins by establishing ε-(γ-glutamyl)

lysine cross-linkages and/or covalent incorporation of mono- or polyamines and into proteins. The transglutaminase family has 9 members in higher vertebrates: TG1-7, band 4.2 and factor XIII. All, except band 4.2, retain the ability to catalyze transamidation reactions (Gundemir et al., 2012)

Transglutaminase 2 (TGM2) is a unique and most widely expressed member of the transglutaminase enzyme family, and almost all cell types in the body express TGM2 to varying extents; it therefore was previously called "tissue transglutaminase". Out of its primary enzymatic activity in Ca^{2+}-dependent transamidation of polypeptide chains, it also binds GTP (which blocks transamidation) and may act as a G protein (Fesus and Szondy, 2005). Moreover, it has been also revealed that TGM2 has a protein disulfide isomerase activity and may also function as a protein kinase (Fesus and Piacentini, 2002). In addition, TGM2 can also function in various biological settings as a protein/protein interaction partner (Gundemir et al., 2012). For example, it has a very high affinity for fibronectin (FN), and together they mediate many adhesion-dependent phenomena, such as cell migration, matrix assembly, and signaling. TGM2 can also bind many integrin receptors, via the extracellular domains of the β1 and β 3 integrin subunits, to maintain cell-extracellular matrix (ECM) interactions independently from fibronectin binding. In cell-matrix interactions, TGM2 can also function as a co-receptor for various isoforms of integrin and FN to form ternary complexes, where all the three proteins successfully interact with each other (Akimov et al., 2000).

TGM2 appears to be an important enzyme during apoptosis. It has been described, that TGM2 is induced and activated in apoptotic cells from the liver and thymus creating highly cross-linked protein polymers and proteinaceous particles, resistant to detergents as well as chaotropic agents (Fesus et al., 1987; Fesus et al., 1989; Fesus, 1993; Szondy et al., 2003; Fesus et al., 1996; Szondy et al., 1997a). Based on these studies, it has been proposed that the transamidation activity of TGM2 may be implicated as a mediator or facilitator of apoptosis, and may contribute to the stabilization of the cell death program (Piredda et al., 1997).

TGM2 has been known to be associated with the *in vivo* apoptosis program of T cells. TGM2 expression is induced in thymocytes dying *in vivo* following exposure to various apoptotic signals, and TGM2 also appears in the dying T lymphocytes of HIV-infected individuals (Gundemir et al., 2012)

The functional importance of TGM2 during programmed cell death was a major topic in the late 90s. For example it has been shown that in U937 cells, overexpression of the enzyme lead to a more efficient apoptosis, while inhibiting its expression showed significantly reduced

apoptosis (Oliverio et al., 1999). Similarly to the previous example, in neuroblastoma cells, overexpression of TGM2 leads to a more rapid apoptotic program (Melino et al., 1994). It is important to note, that the main apoptotic function of TGM2 is carried out by its crosslinking activity. The activation of the enzyme leads to highly cross-linked protein polymers in the apoptotic cell compartments, leading to the formation of protein aggregates, which in turn stabilizes and prevents the leakage of harmful intracellular components (Fesus et al., 1989). Our recent publication reports that overexpression of both the wild type (WT) and the cross-linking mutant of TGM2 (TGM2X) induced apoptosis in Jurkat T cells, but the WT was more effective. Overexpressed WT TGM2 cross-linked RAP1, GTP-GDP dissociation stimulator 1 (RAP1GDS1), an unusual guanine exchange factor acting on various small GTPases. Cross-linked RAP1GDS1 appeared in the ER and induced a yet uncharacterized signaling pathway to promote Ca^{2+} release from the ER via both inositol-triphopshate (IP3) and ryanodine sensitive receptors leading to enhanced mitochondrial Ca^{2+} uptake. Interestingly, TGM2 partly colocalized with mitochondria which contained increased amount of Ca^{2+}. This data indicate that TGM2 might act as a Ca^{2+} sensor in the mitochondria to amplify ER-derived Ca^{2+} signals, and we found a novel mechanism through which TGM2 can contribute to the induction of apoptosis in T cells (Hsieh et al., 2013).

As it has been mentioned above, under certain circumstances TGM2 possesses a protein kinase activity and can phosphorylate and stabilize p53, retinoblastoma protein and histones (Gundemir et al., 2012). It also functions as an accelerator of the mitochondrial cell death pathway by acting as a BH3-only protein. TGM2 has the ability to interact with the pro-apoptotic BCL-2 family members Bax and Bak, even before the cell encounters the apoptotic stimuli. Upon apoptosis initiation, the robust increase of calcium concentration in the mitochondria activates the cross-linking activity of TGM2, leading to the formation of BAX polymers, having stabilized pore-forming conformation resulting a more rapid apoptotic process due to the enhanced release of cytochrome C (Rodolfo et al., 2004). TGM2 can also support the migration of macrophages to the site of apoptosis by crosslinking the ribosomal S19 protein that functions as a chemoattractant (Gundemir et al., 2012). In addition, in the absence of TGM2, integrin β3 (which is involved in the uptake of apoptotic cells by macrophages and is a TGM2 interactor) cannot efficiently recognize the apoptotic cells to be engulfed, among other deficiencies in phagocytic portal formation. These deficiencies lead to an age-dependent autoimmunity in TGM2 knockout mice due to impaired clearance of apoptotic bodies. Other groups have also provided supporting evidence to the role of TGM2 in apoptotic cell clearance

by macrophages (Gundemir et al., 2012). These results suggest that TGM2 is rather a pro-apoptotic enzyme, not anti-apoptotic, however other studies showed that the picture is more complex.

In osteoblast and fibroblasts, it has been shown that TGM2 protects against anoikis cell death by an enzyme-fibronectin interaction (Verderio et al., 2003). Another example shows, that in breast cancer cells, epidermal growth factor (EGF) could inhibit doxorubicin-triggered apoptosis along with TGM2 upregulation (Antonyak et al., 2004).

These results clearly demonstrate that TGM2 can both facilitate and prevent apoptosis. It seems that depending on the actual apoptosis pathway and the cell type, its activities can be opposing. During thymocyte apoptosis, it has rather pro-apoptotic activity. These summarized findings confirm the Janus face of the enzyme in the initiation and regulation of apoptotic processes (Fesus and Szondy, 2005).

1.8 Macrophage secreted molecules are regulators of Tgm2 expression in dying thymocytes

As discussed above, TGM2 has been known for a long time to be associated with the *in vivo* apoptosis program of various cell types, including T cells (Fesus and Szondy, 2005). TGM2 expression is induced in thymocytes dying *in vivo* following exposure to various apoptotic signals (Szondy et al., 1997a). While, TGM2 is strongly induced in dying thymocytes *in vivo,* no induction of TGM2 was observed, when thymocytes were induced to die by the same stimuli *in vitro* (Szegezdi et al., 2000) indicating that signals arriving from the tissue environment contribute to the *in vivo* induction of the enzyme in apoptotic thymocytes. Later on, studies from our laboratory have demonstrated that in dying thymocytes the expression of TGM2 is induced by signals derived from engulfing macrophages, such as retinoids (Garabuczi et al., 2013), or TGF-β (Szondy et al., 2003).

In dying thymocytes, TGF-β promotes cell death, induced by specific signals and elevates the expression of TGM2. Induction of TGM2 by TGF-β in macrophages results in positive feedback loop leading to more TGF-β production and secretion (Fesus and Szondy, 2005).

Our research group has shown that retinoids also contribute to the induction of TGM2 during thymocyte apoptosis. Interestingly, 9cRA was more efficient in inducing the level of TGM2,

comparing to ATRA. Furthermore, the combination of retinoids with TGF-β appeared to be the most effective inducer of TGM2 (Garabuczi et al., 2013). However, it remained to be determined how other macrophage secreted molecules and their combinations affect the expression of TGM2 in thymocytes.

1.9 A promoter centric view on the transcriptional regulation of *Tgm2*

TGM2 is mainly regulated at the transcriptional level and it has been shown that the human TGM2 promoter is responsive to several signaling molecules (Lu et al., 1995). Retinoids are the most well-known activators of TGM2 via a tripartite response element in the human TGM2 promoter located approximately 1.7 kb upstream relative to the transcription start site (Nagy et al., 1996). The promoter also possesses a TGF-β response element, located approximately 0.9 kb upstream of transcription start site (Ritter and Davies, 1998). These studies identified the exact elements for retionid and TGF-β signaling in the promoter region of the gene, further supporting the direct regulation of TGM2 by these signaling pathways.

Other response elements were also revealed for two very important, stress-induced transcription factors in the promoter of TGM2. The inflammatory transcription factor NF-κB (Mirza et al., 1997) and the oxygen sensor, hypoxia inducible factor (HIF) (Jang et al., 2010) (Fig. 6). The TGM2 promoter not only functions as a positive regulator of gene expression, but it also has a role in repressing the expression of the gene. Interestingly, the expression of TGM2 generally correlates with the invasiveness of cancer cells (Mehta et al., 2006). In breast cancer cells, it has been shown that the CpG islands found in the promoter region of TGM2 are hypermethylated, thus the expression of TGM2 is silenced by an epigenetic mechanism, resulting in a less invasive cancer cell type (Ai et al., 2008). Surprisingly, a very similar epigenetic mechanism was observed in case of non-small cell lung cancer (Park et al., 2010) and also in glioma (Dyer et al., 2011). These studies shed light on a completely novel mechanism of action by which TGM2 is silenced. Other studies showed that histone deacetylases (HDACs) have a role in the repression of TGM2 (Liu et al., 2007). Altogether, various mechanisms are evolved to fine tune the expression of TGM2, which is generally considered to be a stress response gene (Ientile et al., 2007). According to this view, the upregulation of TGM2 is part of the cellular protective response, even if TGM2 is also facilitating cell death under certain circumstances. Strikingly, it has been shown that various stress inducers are able to switch between the expression of different isoforms of TGM2 (Lai

et al., 2007; Fraij et al., 1992). The mechanism is largely unknown how these splice variants are generated. Further studies are required to clarify the mechanisms by which these variants are generated and the role of each variant in normal physiology and pathology.

Altogether, the evolved gene regulatory mechanism that are discovered were all made on the promoter region of the gene (Fig. 8), but the regulation is probably even more complex and takes place on intergenic regulatory elements as well.
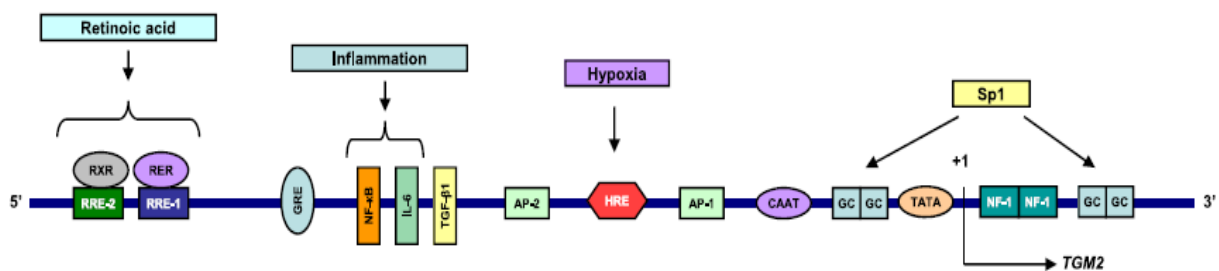
Figure 8. Identified regulatory elements in the promoter region of TGM2. Retinoic acid response elements: RRE-1 (−1731), RRE-2 (−1720), glucocorticoid response element: GRE (−1399), nuclear factor κB response element: NF-κB (−1338), interleukin-6 response element: IL-6 (−1190), tumor growth factor-β1: TGF-β1 (−900), activator protein-2: AP-2 (−634), hypoxia response element: HRE (−367), activator protein-1: AP-1 (−183), CAAT box (−96), GC box: Sp1 binding motifs (−54, −43, +59, +65), TATA box (−29), nuclear factor-1: NF-1 (+4, +12) (Gundemir et al., 2012).

1.10 Limitations of promoter centric analyses of gene regulation

Mammalian genes produce monocistronic RNA molecules, which suggest that each gene must have a specific regulatory element network. These networks can be reduced or extended depending on the cellular state, leading to altered gene expression. The discovery of enhancers supported evidence that genomic sequences can be located far away from the gene and still have effects on gene expression (Banerji et al., 1981), thus it was almost impossible to find them before the genomic era. It is important to note that many studies appeared in the literature using the so called "promoter bashing" approach in which a given DNA element was under investigation (Stanford et al., 2001). With the help of this approach more and more response element was discovered, but the distant regulatory elements remained elusive. Shortly the

weaknesses of these systems were recognized, but without the technical advances, it was almost impossible to get further insights into the location and function of distant cis-regulatory elements.

1.11 Genome-wide technologies revolutionize the regulatory principles of the genome

As a result of the genome programs and the major technological breakthroughs, the field of gene expression regulation is thoroughly revolutionized. During the last decade, sequencing technologies have greatly evolved and almost completely replaced the microarray-based methods, because of their increased sensitivity. The rapid evolution of sequencing technologies, called Next-Generation Sequencing (NGS) has revolutionized the mapping of transcription factor binding sites and also shed light on global transcriptomes. Connecting NGS with simple molecular biology technics proved to be very efficient and resulted in the generation of genome-wide datasets in a number of areas including whole genome sequencing, mRNA sequencing and revealing MNase (Micrococcal Nuclease) and DNase I hypersensitive sites (Park, 2009). Chromatin immunoprecipitation (ChIP) has been one of the first approaches linked to NGS leading to the identification of the typical histone acetylation and methylation patterns of gene regulatory elements, including gene promoters, enhancers, insulators and repressed chromatin territories (Barski et al., 2007). ChIP-seq also allows the determination of the sum of all the binding sites (cistrome) of a given transcription factor under certain circumstances. The first cistrome of a transcription factor was determined for Signal Transducer and Activator of Transcription (STAT) 1 (Robertson et al., 2007). These results were the first to show that cis-acting elements are widespread in the mammalian genomes and are mainly located in intergenic regions of the genome, very often located far away from the presumed target genes.

The next breakthrough was the development of the technology, called chromosome conformation capture (3C), allowing the detection of long-range chromatin interactions (Dekker et al., 2002). The 3C technology has greatly deepened our knowledge about the 3D folding of chromosomes and specific genomic loci (Dekker et al., 2002). The method is based on the cross-linking of chromatin loops stabilized by protein complexes on the DNA. After fixing the chromatin interactions, a restriction endonuclease is used to digest the genome into smaller pieces yielding sticky ends that can be ligated together under highly diluted conditions,

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favoring intramolecular ligation events, thus connecting the genomic regions in proximity (Dekker et al., 2002).

3C has been also linked to NGS in the last couple of years and also combined with the above mentioned ChIP which resulted in completely new technologies (3C-seq, 4C-seq, 5C, ChIA-PET, Hi-C). Hi-C has been one of the most powerful amongst these technologies by offering the unbiased way of detecting genome-wide chromatin interactions (Lieberman-Aiden et al., 2009). ChIA-PET (Chromatin interaction analysis followed by paired-end tag sequencing) (Fullwood et al., 2009) is the genome-wide version of the ChIP-loop method (Horike et al., 2005), in which ChIP and 3C is combined to reveal the interactions between any two loci bound by the protein of interest. These technological improvements have led to the determination of the principles of the folding of chromatin in 3D and showed that enhancers are communicating with their target genes from several hundred kilobase pairs away.

The extensive usage of the above mentioned approaches has led to the development of a very important concept, which is called the topologically associated domain (TAD) concept. The phenomenon of TADs is first observed when we first had the opportunity to visualize the genome-wide interaction profile of chromosomes, using Hi-C (Lieberman-Aiden et al., 2009). TADs are megabase sized genome architecture elements, thought to support the formation of a partly isolated genomic locus for gene-specific regulatory mechanism. However, the detailed structural components and associated functions are still remained elusive. Recently, a more detailed snapshot has been made about these TAD structures and the so called sub-TAD structures embedded in large TADs, revealing additional details about chromatin folding in 3D. These studies identified a very surprising fact which shapes the direction of looping from TAD borders, mediated by the association of CCCTC-binding factor (CTCF) and the cohesin multi-subunit protein complex (Parelho et al., 2008). Namely, that the direction of the CTCF motif determines chromatin topology (Rao et al., 2014). In addition, the cohesin complex seems to be following this logic and is localized always on the downstream side of the motif (Tang et al., 2015).
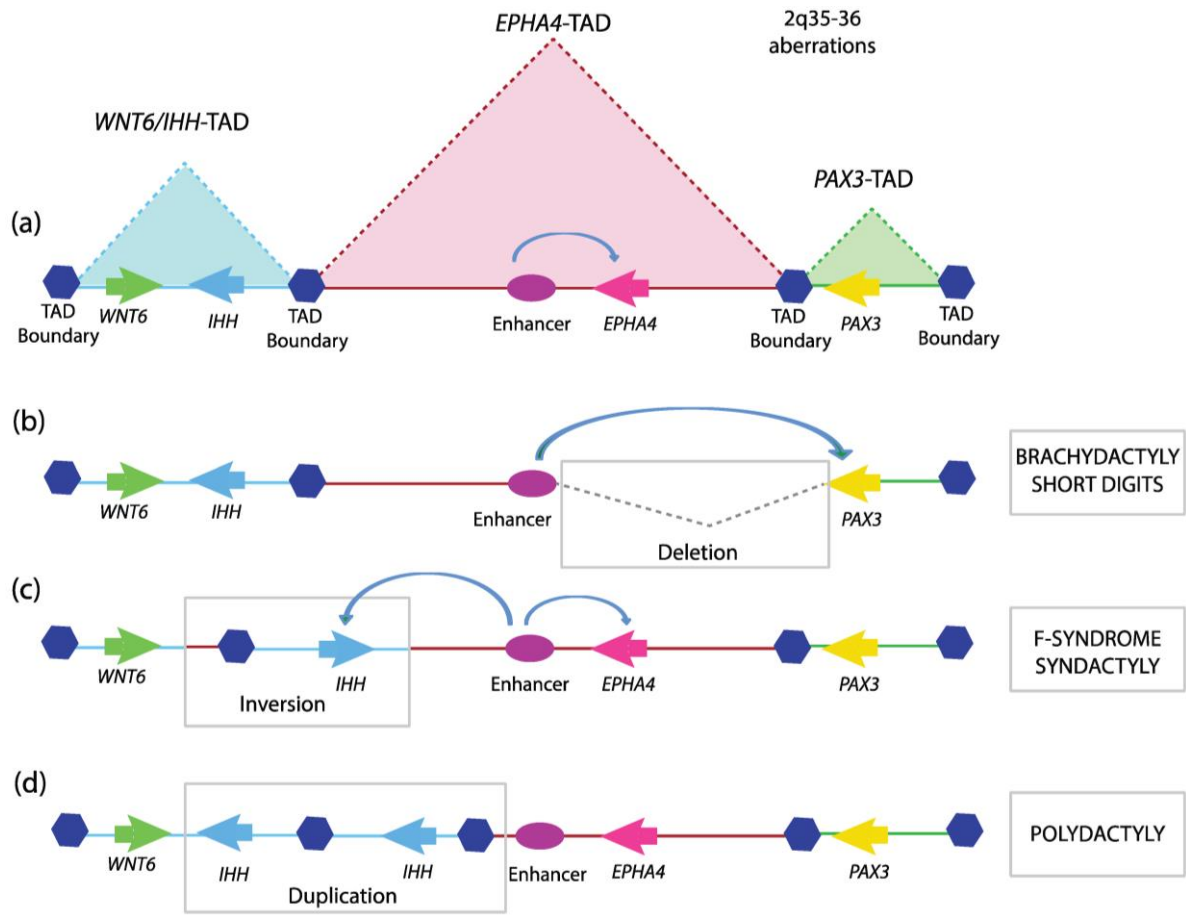
*Figure 9. The critical roles of topological associating domains (TADs) in regulating enhancer gene interactions. TAD disruption linked to limb malformations. Mutations encompassing the WNT6-IHH/EPHA4/PAX3 locus on human chromosome 2q arm disturb TAD structuring and are thus involved in limb phenotypes; (**a**) The WNT6-IHH/EPHA4/PAX3 locus is organized into three TADs with intra-TAD specific interactions playing a key role in setting up the transcriptional program during limb development; (**b**) A deletion encompassing the TAD boundary between EPHA4/PAX3 leads to mis-assignment of EPHA4 enhancer to PAX3 promoter, resulting short digits; (**c**) Inversion surrounding IHH and its nearest TAD boundary allows EPHA4 enhancer to drive the IHH promoter, which was prevented by structural hindrance posed by the TAD boundary in a wild-type scenario, hence leading to F-syndrome; (**d**) Duplication of IHH/TAD boundary results in extra TAD, which causes the polydactyly phenotype (Matharu and Ahanger, 2015).*

CTCF is an eleven Zn-finger containing chromatin binding factor, involved in transcriptional regulation as a chromatin insulator, preventing interactions between enhancers/silencers and genes. Cohesin is highly conserved in eukaryotes and has close homologs in bacteria. Its main molecular function is recognized in sister chromatid cohesion, but it is also required for efficient

DNA repair. Today we know that it has critical roles in gene regulation as a vital component of TAD-related protein complexes and enhancers (Peters et al., 2008) (Fig. 9).

Collectively, these findings help us to imagine how certain genomic regions are folded in 3D and also relevant in predicting long-range enhancer-target gene interactions.

1.12 Mapping the location and features of intergenic enhancer elements

In metazoans, cis-acting elements are dispersed and can be located far away from their target genes (Nobrega et al., 2003). Enhancers are crucial regulators of gene expression and it has been shown in one of the earliest studies that their mutations may result in congenital diseases (Lettice et al., 2003).

According to these, it is obvious that the precise annotation of cis-elements has become a widely studied topic in the field of transcriptional regulation, and an obligatory requirement for determining genome function. Enhancers are DNA sequences with the capability to recruit various types of transcription factors for the interaction with the mediator complex, as well as with the members of the (pre-)initiation complex. Through chromatin looping mechanisms, the complexes assembled on the DNA can facilitate RNA-polymerase II (RNAPII) loading to the promoter, thus contributing to the initiation of gene transcription (Malik and Roeder, 2010). It has been also shown that transcription factors, acting on enhancers are able to remodel the surrounding chromatin structure to establish NFRs (nucleosome-free regions) by recruiting ATP-dependent chromatin remodelers, hence facilitating transcription factor binding, transcription initiation and elongation (Clapier and Cairns, 2009). The appearance of MNase-, DNase I-, ChIP-seq and recently ATAC-seq (Assay for transposase accessible region) (Buenrostro et al., 2013) have become the main tools to investigate open chromatin, transcription factor binding and epigenetic modifications, like histone landscapes. Studies using these technologies greatly impacted and improved our understanding about the main features of enhancers. Genome-wide studies mapping nucleosome occupancy indicate that histone replacement is more enhanced at cis-regulatory elements than at other genomic locations not harboring enhancer-like properties (Mito et al., 2007). Active promoters/TSSs are barely occupied by nucleosomes, thus they are nucleosome-free. These very intriguing observations suggested that nucleosome stability contributes to gene regulation (Heintzman et al., 2007). High-resolution co-activator CREB-binding protein (CBP) and P300 ChIP-seq experiments provided further insights into the chromatin signatures of enhancers, showing that P300 and

CBP are general markers of enhancers. These protein complexes interact with various transcription factors and harbor histone acetyl-transferase activity, which makes them capable to modify histones, affecting chromatin structure and gene expression (Bedford et al., 2010). Several studies showed that the appearance of P300 and/or CBP is a good indicator of enhancer functionality in a tissue specific manner (Lam et al., 2014). These observations indicate that CBP/P300 co-factors are key components of the enhancer binding complexes. Certain histone modifications also take part in cis-element function and it has been shown that active promoters are characterized by the presence of RNAPII and TBP-associated factor 1 (TAF1), marked by NFRs surrounded by trimethylated histone H3 at lysine 4 (H3K4me3), acetylated H3 (H3ac) and TFIID (Kim et al., 2005). As previously mentioned, P300 is one of the well-documented and accepted active enhancer mark along with the combination of H3K27ac and H3K4me1 or H3K4me2 (Kouzarides, 2007). These observations were evidenced in different cellular model systems resulting in the identification of enhancer landscapes in certain cell types (Lupien et al., 2008; Lin et al., 2010). Based on these studies, the identification of enhancers and their characteristic features are relatively straightforward (Fig 10), although the linking of enhancers to the target promoters remained largely elusive. Using total RNA-seq and the development of a very powerful technology called GRO-seq, allowed the identification of another enhancer feature. Studies utilizing these technologies described that active enhancers transcribe the so-called enhancer RNAs (Kim et al., 2010; Wang et al., 2011). The discovery of eRNAs added another layer to enhancer features, and it turned out that this can be one of the most reliable markers for demonstrating that the enhancer actively participates in a particular gene regulation (Wang et al., 2011).

These short RNAs are transcribed from active enhancers only and follow the induction profile of their target genes. Recently, it has been shown that the modified version of GRO-seq, enriching 5'-capped RNAs is a very powerful tool to identify active transcriptional regulatory elements based on its improved eRNA detection ability. The analysis of such datasets using a novel approach showed the enrichment of expression quantitative trait loci, disease-associated polymorphisms, H3K27ac and transcription factor binding sites at transcriptionally active regulatory elements (Danko et al., 2015). According to these, eRNAs represent an accepted
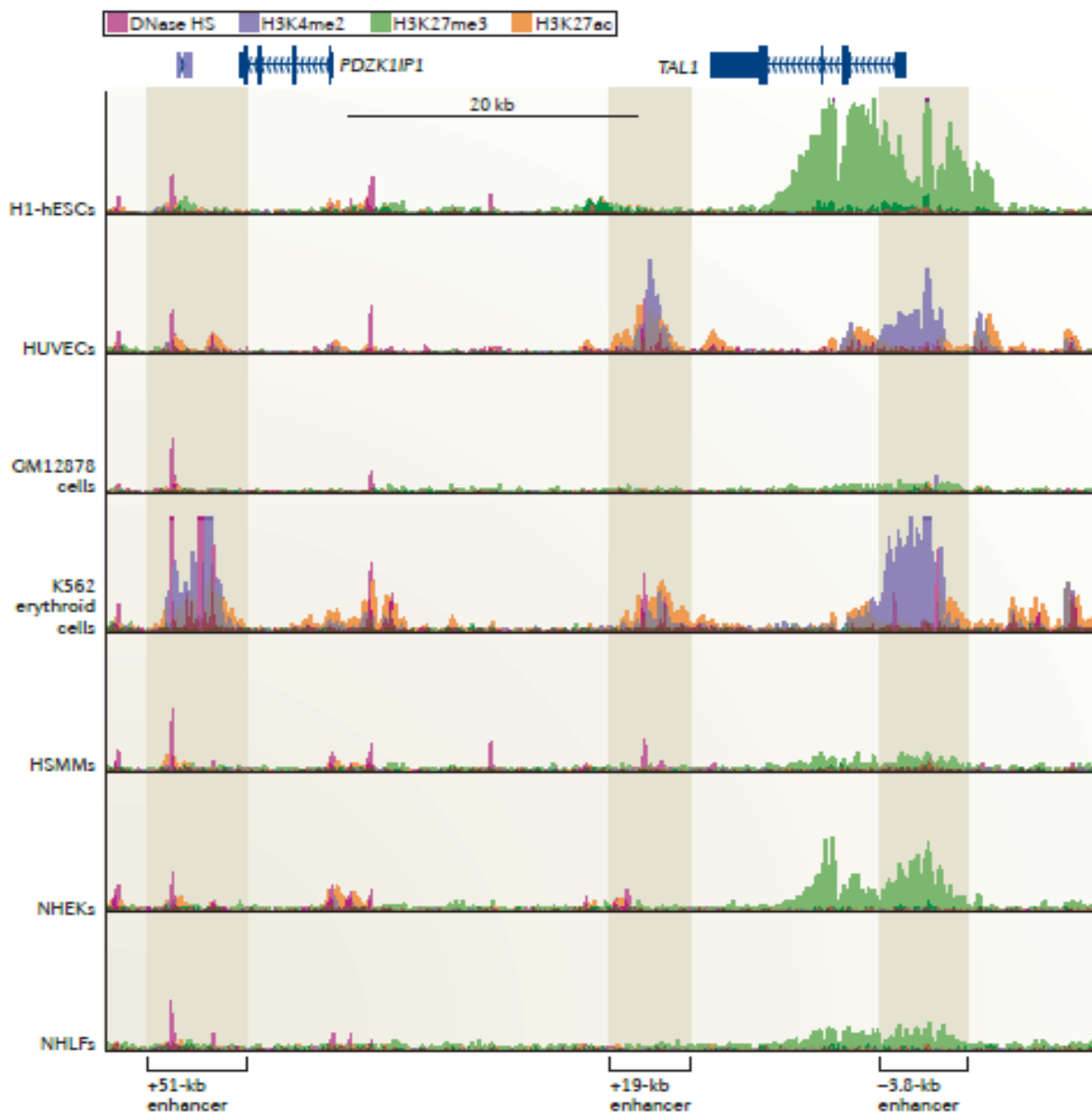
Figure 10. *Cell type-specific enhancer selection on the TAL1 locus (60kb). ENCODE generated datasets from 7 different human cell lines for DNase hypersensitive sites (DNase-seq=DNase HS) (pink) and ChIP-seq of H3K4me2 (purple), H3K27ac (orange), markers of transcriptionally permissive chromatin environment. ChIP-seq against H3K27me3 (green) is also shown, known as a repressive histone modification, not allowing transcription initiation. Functional enhancers are marked by the presence of H3K4me2 and H3K27ac along with highly accessible chromatin (DNase hypersensitive). TAL1 is not expressed in those cell types in which the gene promoter is covered with the H3K27me3 repressive histone mark (Heinz et al., 2015).*

functional enhancer feature (Lam et al., 2014). Hence, it has already been exploited to assign the active enhancer element repertoire of certain transcription factors, such as ESR1 (Hah et al., 2013) or RXR (Daniel et al., 2014b) to their target genes genome-wide.

Based on advanced sequencing technologies, in 2012 the ENCODE (Encyclopedia of DNA elements) Consortium has released genome-wide datasets in the form of 30 papers (Yue et al., 2014). These studies aimed to identify the cis-acting element repertoire of the human genome and also several mouse cell lines. In addition, they provided an enormous amount of genome-wide data also from various mouse tissues. These datasets are of great help to identify the putative enhancer elements repertoire of a given gene, knowing the basic features of active enhancers and how genome topology is shaped via the above mentioned organizers of 3D chromatin structure. We have successfully exploited these datasets from mouse thymus in order to predict the functional enhancer repertoire of the apoptotic regulator gene *Tgm2*.

2. AIMS

Previously our research group has shown that TGM2 is induced during *in vivo*, but not *in vitro* apoptosis of thymocytes indicating that factors present in the tissue environment are required for the process. Recently our laboratory has found that retinoids and transforming growth factor beta (TGF-β) released by engulfing macrophages are involved in this process. Since adenosine is also produced by macrophages during engulfment of apoptotic cells, we decided to

- investigate the possible involvement of adenosine in the TGM2 induction in apoptotic thymocytes.
- determine the mechanism of adenosine production during engulfment

Due to its ubiquitous appearance and multiple functions, the expression of the *Tgm2* gene must be tightly controlled. The *in vivo* appearance of TGM2 protein in dying thymocytes seems to be regulated at the level of transcription. Thus we decided to

- *identify* the putative enhancer elements of *Tgm2* in dying thymocytes
- and *characterize* them by measuring

  - ➢ **enhancer RNA (eRNA)** production
  - ➢ binding of **transcription factors** (RXR, RAR, CREB, SMAD4)
  - ➢ recruitment of **coactivators** (P300, CBP) and enrichment of H3K27ac,
    an **active enhancer marker**

3. MATERIALS AND METHODS

3.1 Materials

All reagents were obtained from Sigma-Aldrich (Budapest, Hungary) except indicated otherwise.

3.2 Experimental animals

Most of the experiments were carried out with 4-wk-old C57Bl/6 mice. In some experiments A3R deficient mice generated on a C57Bl/6 background (Lee et al., 2003) developed and provided to us by the Merck Co., NJ, USA, pannexin-1 null mice generated a C57Bl/6 background (Rigato et al., 2012) and A2AR deficient mice generated on an FVB background (Ledent et al., 1997) were also used. Mice were maintained in specific pathogen-free condition in the Central Animal Facility of our university, and all animal experiments were approved by the Animal Care and Use Committee of University of Debrecen (DEMÁB) /4/2010/DEMÁB/.

3.3 Macrophage isolation and culturing

Macrophages were obtained by peritoneal lavage with sterile physiological saline from 3- mo-old C57Bl/6 mice. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM Na-pyruvate, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 ug/ml streptomycin at 37°C in 5% CO_2 for 2 d before use. After 3-4 h incubation, the non-adherent cells were washed away. Before the experiments the cells were cultured for 2 d replacing media daily.

3.4 Thymocyte cultures

Isolated thymocytes (1×10^7/ml) were cultured in RPMI medium 1640 supplemented with 10% charcoal stripped FBS, 2 mM glutamine, 1 mM Na pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2. Cells were exposed to 5 µM NECA (5'-N-ethylcarboxamidoadenosine), a non-metabolisable adenosine analogue, to 1 µM CGS21680, an A2AR agonist (Tocris Bioscience, Eching, Germany), to 1 µM forskolin, an adenylyl cyclase activator, to 100 µM Rp-cAMPS triethylamine, a competitor of endogenous cAMP or to 100 µM db-cAMP, a cell permeable cAMP analogue, 1 µM AM580, an RAR agonist, 100 nM LG268, an RXR agonist alone, or in combination with 5 ng/ml recombinant human TGF-β1 (AbD Serotec, Kidlington, UK) and 0.3 µM 9-cis retinoic acid (9cRA) (Tocris Bioscience, Eching, Germany) for the indicated time periods.

3.5 Induction of thymic apoptosis *in vivo*

4-wk-old mice were injected intraperitoneally with 0.3 mg dexamethasone-acetate - dissolved in DMSO/physiological saline to induce thymic apoptosis. Controls received the same dose of DMSO/ physiological saline. Thymuses were removed at the indicated time points. Thymic apoptosis was evaluated by measuring the change in the amount of thymic weight.

3.6 Western blot analysis

Collected thymuses were homogenized in ice-cold lysis buffer containing 0.5% Triton X-100. Protein concentration of each samples were diluted to 2 mg/ml, then the samples were boiled with an equal volume of Laemmli buffer. 40 µg protein was run on an polyacrylamide gel, and blotted onto polyvinylidene difluoride membranes using the Bio-Rad electrophoresis and transfer system. The free binding sites of membranes were blocked with 5% non fat dry milk powder in 20 mM Tris, 0.1 M NaCl buffer with 0.1% Tween overnight at 4 °C. 2 µg anti-TGM2 antibodies (Santa Cruz Biotechnology) were added to the membranes. Equal loading of protein was

demonstrated with probing the membranes with anti-TGM2 and β-actin (Tocris Bioscience, Eching, Germany) antibody.

3.7 Determination of cytokine production

Peritoneal macrophages were plated onto 24-well plates at a density of 1 x 10^6 cells / well. To determine cytokine production by macrophages exposed to apoptotic cells, macrophages were exposed to apoptotic cells for 1 h in the presence or absence of the ecto-5'-nucleotidase inhibitor methylene adenosine-5'-diphosphate (50 µM) and NECA (5 µM). Apoptotic cells than were washed away, the compounds were readded and the macrophages were cultured for an additional five hrs. At the end of culture cell culture media were analyzed by Mouse Cytokine Array (Proteome Profile Array from R&D Systems). The pixel density in each spot of the array was determined by Image J software. Alternatively, cytokine-induced neutrophil-attracting chemokine (KC), and macrophage inflammatory protein-2 (MIP-2) cytokine levels were measured with R&D Systems ELISA kits.

3.8 ATP determination

Apoptotic cells were prepared from 4-wk-old wild-type or pannexin-1 null mice. The isolated thymocytes were cultured for 24 hrs. (10^7 cells/ml) in RPMI 1640 medium supplemented with penicillin/streptomycin in the absence of serum. The culture medium was then collected for ATP determination by the ATPlite Luminescence ATP Detection Assay System (PerkinElmer) according to the manufacturer's suggestions.

3.9 Real-Time Quantitative PCR (qRT-PCR) for detecting mRNA levels

Total RNA was isolated from control and treated thymuses or isolated thymocytes by TRI reagent according to the manufacturer's guidelines. The concentration and purity of RNA were measured by means of NanoDrop spectrophotometer (Thermo Scientific, Schwerte, Germany).

Total RNA (1 µg/samples) was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Budapest, Hungary) according to the manufacturer's instruction. QRT-PCR was carried out in triplicate using pre-designed FAM-MGB-labelled assays for TGM2 (Applied Biosystems, Budapest, Hungary) on an ABI Prism 7900. Relative mRNA levels were calculated using comparative C_T method and were normalized to cyclophilin mRNA. ABI Prism SDS2.1 software was used for data analysis (Applied Biosystems, Budapest, Hungary).

Tgm2

Fw: 5'-AGGACATCAACCTGACCCTG-3'

Rev: 5'-CTTGATTTCGGGATTCTCCA-3'

Rprd1b

Fw: 5'-GACGTGTCGCTGCTAGAGAA-3'

Rev: 5'-CCGCTAGTAACAGACATGCTTC-3'

3.10 qRT-PCR for detecting eRNA levels

For this purpose we designed eRNA specific primer pairs targeting the enhancer sequences upstream to their DNase hypersensitive sites, but on the enriched histone modifications. We used primer3 software to design the eRNA specific primers with standard conditions except the followings: amplicon length: 90-150bp; Tm: 59-60°C. RNA was isolated with Trizol Reagent according to the manufacturer's guidelines. For enhancer RNA measurements, RNA was isolated with the same Trizol based method, but samples were digested with DNaseI enzyme for 1 hour at 37C. RNA was then reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Budapest, Hungary) and quantification was performed with LightCycler® 480 SYBR Green I Master (Roche, USA). The following PCR program was used for enhancer RNA detection: 95°C-3 min; 95°C-30 sec; 60°C-1 min. The last two steps were repeated 40 times.

Transcript levels were normalized to *Ppia*. Primer sequences:

Tgm2 +30kb eRNA
Fw: 5'-CCACTGCAGCCTTCCTAGTT-3'
Rev: 5'-TCAGCCAGCACCTCTAGACA-3'

Tgm2 -7.9kb eRNA
Fw: 5'-CTCTAGGGCCCCAGTTGAG-3'
Rev: 5'-CAGGAGAGATGCCCTTCCAG-3'

Tgm2 -13kb eRNA
Fw: 5'-GTGAACTGTGCTGCTGAACA-3'
Rev: 5'-CAATCTCCAAAGCACCCCAC-3'

Tgm2 -20kb eRNA
Fw: 5'-AGCCTGAACCCACAAGTGTA-3'
Rev: 5'-TGTGTGTGCGTGTGTGTTTT-3'

Tgm2 -28kb eRNA
Fw: 5'-AGCTTTCCTGACCTGGTGTT-3'
Rev: 5'-TGCCTCACCACCATCATCA-3'

Prmt8 -1.7kb
Fw: 5'-CGTGAGCAGAGGTGAGGAGT-3'
Rev: 5'-GGTTAACCCAAGCTTCTTGCT-3'

3.11   Chromatin immunoprecipitation followed by qPCR

ChIP was performed as previously described (Daniel et al., 2014a) with minor modifications.

Cross-link and Harvest Cells

1. We utilized ~100 million cells as material for each ChIP, derived from 6 well plates ($1\text{x}10^7$ cells/well). Cells collected and double cross-linking was performed as the following:

 - Di(N-succinimidyl) glutarate (ProteoChem, USA) added for 30 minutes (2 mM final concentration) at room temperature.

 Cells were spun then resuspended in 10 ml PBS.

- formaldehyde added to achieve a final concentration of 1% and incubated at room temperature for 10 minutes.

2. The cross-linking agents were quenched by adding glycine (final concentration: 125 mM glycine). Incubated at room temperature for 5 minutes.

3. Cells were spined at 2000g x 5 minutes in a 4 ºC refrigerated microfuge then supernatant was removed.

Then samples were washed twice with cold PBS, and collected into microfuge tubes.

Lyse Cells and Shear Chromatin

1. 1 ml of cold Cell Lysis/Wash Buffer was added – containing protease inhibitors (a Roche Complete Protease Inhibitor EDTA-free mini tablet dissolved in 10 ml Cell Lysis/Wash

Buffer) – to the cell pellet. Then the homogenous cell slurry was pushed through a 1 ml insulin syringe   to   assist   in   breaking   apart   cell   membranes.
The cell slurry was centrifuged at 12,000g x 1 minute and the supernatant was aspirated.

2. Step 1 was repeated one additional time.

3. 1 ml of cold Shearing Buffer was added containing protease inhibitors to the nuclear pellet and resuspended

4. Using a Diagenode Bioruptor, we transferred the 1 ml solutions into 15 ml conical tubes (polystyrene) and placed the sonicator probes into the tubes.

5. The chromatin was sheared on 4 ºC. Using the Diagenode Bioruptor ® Standard model,

1×5 min long cycle is used, with 30 s on and 30 s off setup to generate 200-1000 bp fragments. The sonication cycle is carried out at low power setting.

Immunoprecipitation

1. Sheared chromatin was centrifuged at 12,000g x 10 minutes at 4 ºC. Then 900 µl supernatant was transferred to a new 15 ml conical tube and diluted tenfold with dilution buffer containing protease inhibitors. The diluted chromatin can be stored for 24 h at 4 °C.

2. 20 µl chromatin supernatant was set aside for input DNA to be used for normalize quantitative PCR measurements.

3. Immunoprecipitation was performed with antibodies against pre-immune IgG (Millipore, 12-370), RXR (sc-774), P300 (sc-585), CBP (sc-369X), CREB1 (ab31387), SMAD4 (sc-7154X), H3K27ac (ab4729) from Merck (Budapest, Hungary), Santa Cruz Biotechnology (Dallas, USA) or Abcam (Cambridge, UK).

2 µg antibody was added to each sample of diluted chromatin.

1 ml of diluted chromatin was set aside with 2 μg of pre-immune rabbit IgG, as a control of nonspecific binding.

4. Samples were incubated overnight at 4 °C, using an "end-over end" rotator.

5. Pre-block the Protein A-coated paramagnetic anti-rabbit beads (Life technologies, USA): For each IP, 190 microliters of beads were prepared. Beads were washed 3x with 1 ml of fresh filtered 0.5 % BSA/PBS using magnetic rack. Then beads were incubated in 1 ml of 0.5 % BSA/PBS on a rotating tube rack at 4 °C overnight.

6. Next day the 15 ml conical tubes containing antibody-chromatin complexes were spun in a refrigerated centrifuge at 4 °C for 20 minutes at ~3500 rpm.

7. Using a magnetic rack, paramagnetic beads were reconstituted to their original volume (190 μl) by replacing the 1 ml of 0.5 % BSA/PBS.

8. The top 90 % of the centrifuged antibody-chromatin mixtures (from Step 6) were aliquoted with 20 μl of blocked paramagnetic beads into 2.0 ml LoBind tubes. Samples were incubated on a rotating tube rack at 4 °C for 4 hours.

9. Prepare Cell Lysis/Wash buffer by adding an EDTA-free Roche Complete tablet to 50 ml of Cell Lysis/Wash buffer.

10. Using cooled magnetic rack, the samples – antibody-chromatin + paramagnetic bead mixes – were washed 6 times with 1 ml of cold Cell Lysis/Wash Buffer containing protease inhibitors (1 tablet dissolved in 50 ml Cell Lysis/Wash Buffer).

11. After that, samples were washed with 1 ml cold TE Buffer.

Bead Elution/De-Crosslinking/DNA Purification

1. 200 μl of Bead Elution Buffer was added to the beads at room temperature. After vortexing on moderate speed samples were placed on a thermomixer for 30 minutes @ 1000 rpm.

2. Using magnetic rack, the supernatants were collected into clean LoBind tubes yielding a total of 200 μl of eluted, ChIP'd chromatin.

3. To de-crosslink the chromatin 8 μl of 5M NaCl was added to the 200 μl of eluted chromatin, then the tubes were heated at 65 °C overnight.

Input chromatin was processed in the same way, beginning at this step

4. 1 μl of RNase A (10 μg/μl) was added to the de-crosslinked chromatin and incubated at 37 °C for 30 minutes.

5. 4 μl of 0.5 M EDTA pH 8.0, 8 μl of 1 M Tris HCl pH 7.0, and 1 μl of proteinase K (10 μg/μl) were added then incubated at 45 °C on a thermomixer for 1 hour.

6. After these steps, DNA fragments were column purified (Qiagen, MinElute).

DNA was diluted and used for qPCR analysis.

The following primer sequences were used:

Tgm2 +30kb
Fw: 5'-TCTGCCTAAGATGTGCAGAGG-3'
Rev: 5'-CCCTCTCTGAGGCGTTCC-3'

Tgm2 -7.9kb
Fw: 5'-CCAGAATAGAGGAGTGCTGGTAA-3'
Rev:5'-CAGTATTTTCTTGGTTGACTGTGG-3'

Tgm2 -13kb
Fw: 5'-GCAGAGTTGGCTCTCGTCA-3'
Rev: 5'-GTGTCTGCCTGTCCTGTCTG-3'

Tgm2 -20kb
Fw: 5'-TGGCATACACACAAGGAAGTG-3'
Rev: 5'-TTCCTGATCTGGTTTGTCAGTG-3'

Tgm2 -28kb
Fw: 5'-GAAGTCACCCCCAATCTGAG-3'
Rev: 5'-ATGCAGGTTTGCTGACTGCT-3'

Prmt8 -1.7kb
Fw: 5'-CGTGAGCAGAGGTGAGGAGT-3'
Rev: 5'-GGTTAACCCAAGCTTCTTGCT-3'

3.12 Accession numbers for ChIP-seq experiments

ChIP-seq experiments performed by the ENCODE Consortium have been downloaded and visualized by the Integrative Genomics Viewer (IGV) genome browser. More specifically, ChIP-seq datasets were used against CTCF (GSM918734), H3K4me3 (GSM1000101), H3K4me1 (GSM1000102) and H3K27ac (GSM1000103). In addition DNaseI-seq results were also used (GSM1003827).

3.13 Chromosome conformation capture (3C)

Chromosome conformation capture experiments were carried out essentially as described previously (Dekker et al., 2002).

Cross-link and Lyse Cells

We utilized ~100 million cells as material for each sample, derived from 6 well plates (1×10^7 cells/well).

Thymocytes were collected then spun and resuspended in 10 ml PBS for cross-linking using 2% formaldehyde for 10 min. Cross-linking reaction was quenched with glycine (final concentration: 125 mM), and incubated for 5 min at room temperature. Cells were spun at 2000g x 5 minutes in a 4 ºC refrigerated microfuge then supernatant was removed.

Then samples were washed twice with cold PBS, then the pellet was resuspended in 5 ml cold lysis buffer and incubated for 10 min on ice. Samples were centrifuged for 5 min at 1800 RPM (750 g) at 4 ºC and the supernatant was removed then the pellet was taken up in 440 ml Milli-Q.

Restriction Enzyme Digestion of Cross-linked Chromatin

For digestion the six base pair-cutting enzyme HindIII was used:

1. Firstly, 60 µl of 10X RE buffer was added then tubes were placed at 37 ºC and 15 µl of 10% SDS was added and incubated for 1 hour, while shaking at 900 rpm.

2. After that, 75 µl of 20% Triton X-100 was added and incubated for 1 hour at 37 ºC, while shaking at 900 rpm.

3. Aliquot of "undigested control" was taken for latter investigation.

4. 200 U was added to the samples and incubated for 4 hours at 37 ºC while shaking at 900 RPM. Then 200 U was added again for overnight, and next day 200 U was added again for further 4 hours at 37 ºC, while shaking at 900 RPM.

5. Aliquot of "digested control." was taken for latter investigation.

6. Then enzyme was inactivated by incubating for 20 min at 65 ºC.

Ligation of cross-linked DNA fragments

1. 700 µl 10X ligation buffer was added to the samples then filled up with Milli-Q to 7 ml.

2. 50 U T4 DNA Ligase (Fermentas) was added and incubated overnight at 16 ºC

3. Aliquot of "ligation control" was taken for latter investigation

   Determine the ligation efficiency: 5 µl Prot K (10 mg/ml) was added and incubated for 1 h at 65 ºC. Then control samples was checked on 1,5 % agarose gel.

Reversal of cross-links

1. 30 µl Prot K (10 mg/ml) was added and samples were de-cross-linked overnight at 65 ºC. 2. Then 30 µl RNAse A was added and incubated for 45 min at 37 ºC.

3. Samples were mixed with 7 ml phenol–chloroform then centrifuged for 15 min at 3750 rpm at room temp.

4. The water phase was transferred then added: 7ml Milli-Q; 1.5 ml of 2 M NaAC pH 5.6; and 35 ml of 100% ethanol. After mixing, tubes were placed at -70 ºC for overnight, in order to completely frozen.

5. Samples were spun for 45 min at 3800 rpm at 4 ºC.

6. After the supernatant was removed, 10 ml of cold 70% ethanol was added then centrifuged for 15 min at 3750 rpm at 4 ºC.

7. The pellet was dried on room temp. and dissolved in 150 ml of 10mM Tris–HCl pH 7.5 at 37 ºC.

8. PCR reactions were performed by using approximately 100 ng 3C template.

The following primer sequences were used:

Tgm2 -20kb (BAIT)
Fw: 5'- CACACACACATACGGTTCTGTT -3'

Tgm2 +11kb
Fw: 5'- CAGTCCTAGGGAAGTGGAGC -3'

Tgm2 +13kb
Fw: 5'- TTATGGGTTTGGGGACTGCA -3'

Tgm2 +30kb
Fw: 5'- GGCAGACCTTGTTTGAATGTGA -3'

Loading control primers (chr10:74788584-74788803):

Fw: 5' - CTC CAC CAT GAT GTA CAC CG – 3'
Rev: 5'- CATGGTTTCGGGAGATGCAG - 3'

3.14 Statistical analysis

All data are presented as means +/-SD. We made three biological replicates in all the experiments and we performed paired (two tailed) t tests. Results were considered significant with $p < 0.05$.

4. RESULTS

4.1 Activation of the adenosine A2A receptor contributes to the upregulation of TGM2 in thymocytes dying *in vitro*

Since our previous studies indicated that macrophage-derived TGF-β and retinoids are involved in the regulation of TGM2 expression in dying thymocytes, we wanted to test whether adenosine, which is also produced during engulfment of apoptotic cells (Koroskenyi et al., 2011), could also affect TGM2 expression in thymocytes. Adenosine can be efficiently metabolized by adenosine deaminase of thymocytes, we applied NECA, a non-metabolisable analogue of adenosine known to act on all subtypes of adenosine receptors. For measuring TGM2 mRNA expression, thymocytes were exposed to NECA for 6 hours, as previous studies have indicated that the mRNA expression of TGM2 peaks at this time point in thymocytes (Garabuczi et al., 2013). Administration of NECA resulted in a 2 fold induction in the mRNA expression of TGM2 in wild type thymocytes implying that adenosine might be involved in the regulation of TGM2 expression (Fig. 11).

Thymocytes have been shown to express adenosine A2A (A2AR) and A3 (A3R) receptors (Streitova et al., 2010). In order to decide which adenosine receptors are involved in the adenosine-mediated induction of TGM2 at the mRNA level, we exposed thymocytes isolated from A2AR (Ledent et al., 1997) or A3R (Lee et al., 2003) null mice to NECA. NECA could significantly enhance the mRNA expression of TGM2 in A3R null thymocytes, however it had no effect in A2AR null cells on the expression of TGM2 at the mRNA level. These data indicate that adenosine mediates its effects on TGM2 mRNA expression via adenosine A2A receptors.

Next, we focused on the effect of selective A2AR activation on TGM2 mRNA expression alone or in combination with TGF-β and retinoic acids (RAs) by using CGS21680, a synthetic A2AR agonist. Since in our previous studies indicated that 9-*cis* retinoic acid (9cRA) is the most effective retinoid in inducing TGM2 at the mRNA level (Garabuczi et al. 2013), 9cRA was selected to be used in this study. 9cRA and TGF-β were applied in a concentration that was shown to be effective to affect apoptosis of thymocytes (Szondy et al., 1997b; Szondy et al., 2003), while CGS21680 was administered at 1 µM concentration, because we have shown previously by using A2AR null cells that CGS21680 administered at this concentration selectively activates A2ARs (Koroskenyi et al., 2011).
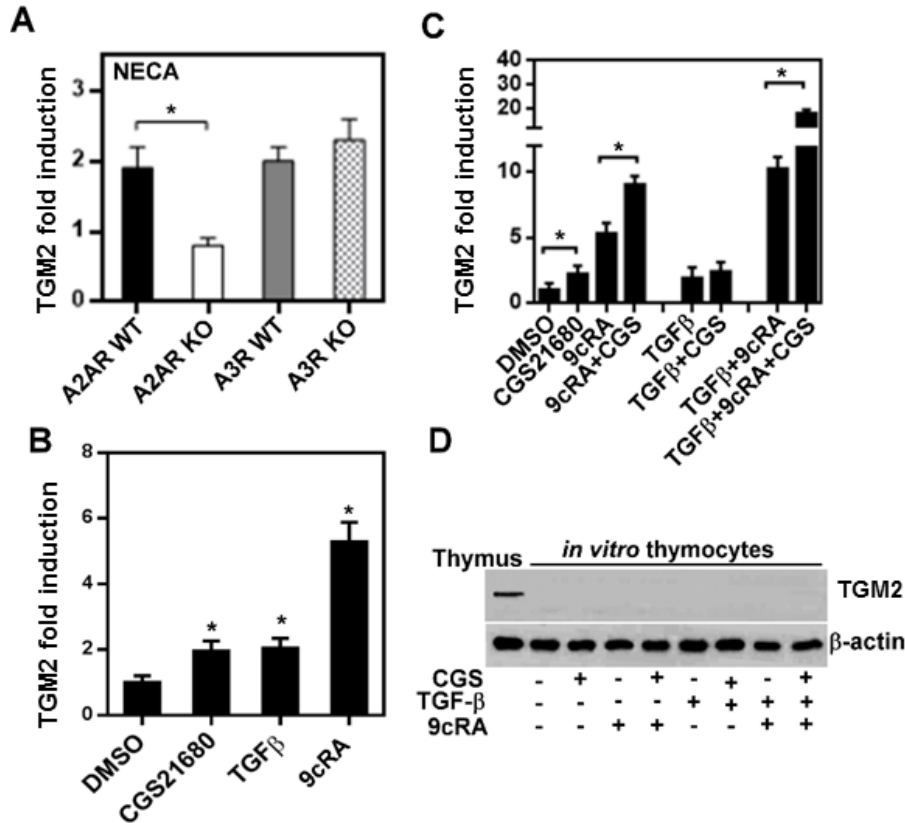
Figure 11. *Adenosine analogues acting on A2ARs applied alone, or in combination with TGF-β and 9-cis retinoic acid significantly enhance TGM2 mRNA expression in cultured thymocytes. (A) Wild type, A2AR null or A3R null thymocytes were exposed to 5 µM NECA, a non-metabolisable adenosine analogue for 6h. Inductions in TGM2 mRNA levels were compared to the DMSO vehicle exposed controls. **Performed by co-authors**. (B) Wild type thymocytes were exposed to 1 µM CGS21680, an A2AR agonist, to 5 ng/ml rTGF-β or to 0.3 µM 9cRA for 6h. (C) Wild type thymocytes were exposed to 1 µM CGS21680 [CGS], 5 ng/ml rTGF-β or 0.3 µM 9cRA in various combinations for 6h. Inductions in TGM2 mRNA levels were compared to the non-treated controls. TGM2 mRNA expression levels were measured by qRT-PCR using cyclophillin as a control. Results are expressed as mean ± S.D. of four independent experiments (*p<0.05). (D) Wild-type thymocytes were exposed to 1-µM CGS21680, 5-ng/ml rTGF-β, or 0.3-µM 9cRA in the indicated combinations for 12 h in the presence of 2-µg/ml ac-DECD-CHO, a caspase 3 inhibitor. TGM2 protein levels were determined by Western blot analysis using β-actin as loading control. Dexamethasone-treated thymus sample was used as positive control for TGM2 protein expression.*

Exposure to TGF-β 9cRA or CGS21680 alone all increased the mRNA expression of TGM2 in cultured thymocytes, while no increase was found in the endogenous mRNA expression of TGM2 during the 6h culture period (Fig. 11B). Among the compounds tested alone, 9cRA was the most effective in inducing the mRNA expression of TGM2 resulting in a robust 5 fold increase as compared to the DMSO-treated control.

Activation of A2ARs had no significant effect on TGF-β-induced TGM2 expression at the mRNA level, but significantly enhanced the 9cRA-induced TGM2 mRNA expression. As we reported previously (Garabuczi et al., 2013) TGF-β could also significantly enhance the 9cRA-induced TGM2 expression. However, the most significant (about 20 fold) induction was found, if the three compounds were applied together (Fig. 11C). While the induction of TGM2 mRNA could be well detected in thymocytes dying *in vitro* exposed to the above compounds, the TGM2 protein remained still invisible (Fig. 11D), even if we performed the experiments in the presence of the caspase 3 inhibitor Ac-DECD-CHO to prevent a possible degradation by caspase 3 (Fabbi et al., 1999). Altogether these data indicate that *in vitro* retinoids, adenosine and TGF-β, compounds all known to be produced by engulfing macrophages, can work together to significantly induce the levels of TGM2 in dying thymocytes.

4.2 Adenosine A2A receptors affect the expression of TGM2 in thymocytes via the adenylate cyclase pathway

Previous studies from our laboratory have shown that though A2ARs can signal through both the adenylate cyclase and the phospholipase C system in other cell types (Fredholm et al., 2001), A2ARs in mouse thymocytes specifically activate the adenylate cyclase signaling pathway (Kiss et al., 2006). Thus we administered 100 μM Rp-cAMPS triethylamine, a specific membrane-permeable competitor of intracellular cAMP (Van Haastert et al., 1984), to test whether adenylate cyclase signaling indeed contributes to TGM2 induction. As shown in Figure 12A, in the presence of Rp-cAMPS triethylamine activation of A2ARs by CGS21680 alone or in combination could not enhance TGM2 mRNA expression in thymocytes.

Then we further tested whether activation of the adenylate cyclase pathway alone is sufficient to induce the expression of TGM2 at the mRNA level in mouse thymocytes. As shown in Figure 12B and C, exposure of thymocytes to forskolin, an adenylate cyclase activator (Seamon et al., 1981), or to dibutyryl cAMP, a membrane permeable analogue of cAMP, alone or together with 9cRA or 9cRA and TGF-β resulted in enhanced TGM2 mRNA expression. Altogether these

data indicate that compounds increasing intracellular cAMP levels can contribute to the TGM2 mRNA expression in mouse thymocytes, and A2AR signaling enhances the expression of TGM2 by triggering the adenylate cyclase pathway.
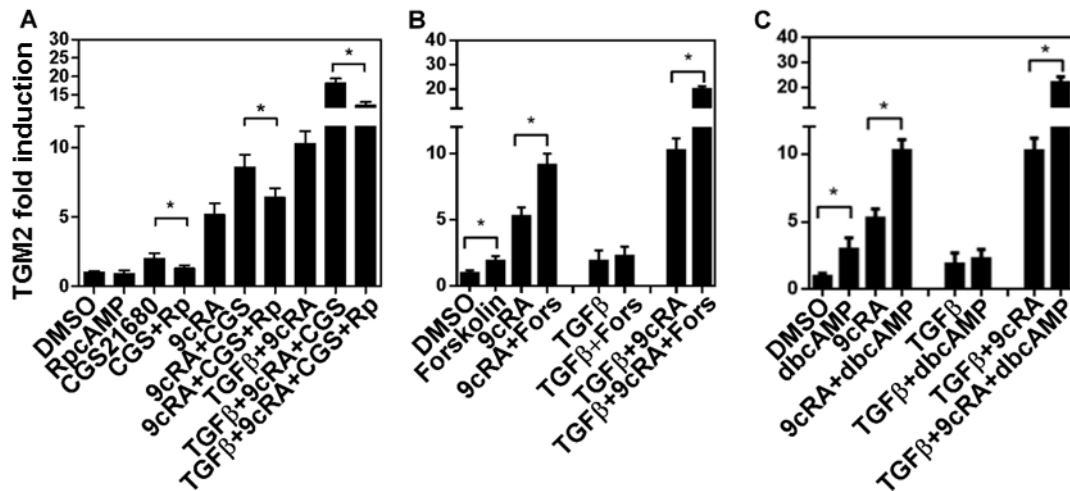
Figure 12. A2AR activation regulates TGM2 mRNA expression in thymocytes by triggering the adenylate cyclase signaling pathway. (A) Rp-cAMPS trimethylamine [Rp], a competitive inhibitor of endogenously produced cAMP, prevents A2AR agonist induced TGM2 mRNA expression in cultured thymocytes. Wild type thymocytes were exposed to 1 μM CGS21680, 5 ng/ml rTGF-β or 0.3 μM 9cRA in various combinations in the presence and absence of 100 μM Rp-cAMPS trimethylamine for 6h. (B) Exposure of thymocytes to forskolin, an adenylate cyclase activator, or (C) to dibutyryl cAMP, a membrane permeable analogue of cAMP, alone or together with 9cRA or 9cRA and TGF-β also enhances TGM2 mRNA levels in cultured thymocytes. Wild type thymocytes were exposed to 5 ng/ml rTGF-β or to 0.3 μM 9cRA in various combinations in the presence and absence of 1μM forskolin [FORS] or 100 μM db-cAMP for 6h. TGM2 mRNA expression levels were measured by qRT-PCR using cyclophillin as a control. Results are expressed as mean ± S.D. of three independent experiments (*p<0.05).

4.3 Specific activation of the adenosine A2A receptor contributes to the upregulation of TGM2 in dying thymocytes *in vivo*

In the following experiments we wanted to test the idea whether adenosine produced in the context of the *in vivo* apopto-phagocytosis program of thymocytes could also contribute to the upregulation of TGM2.
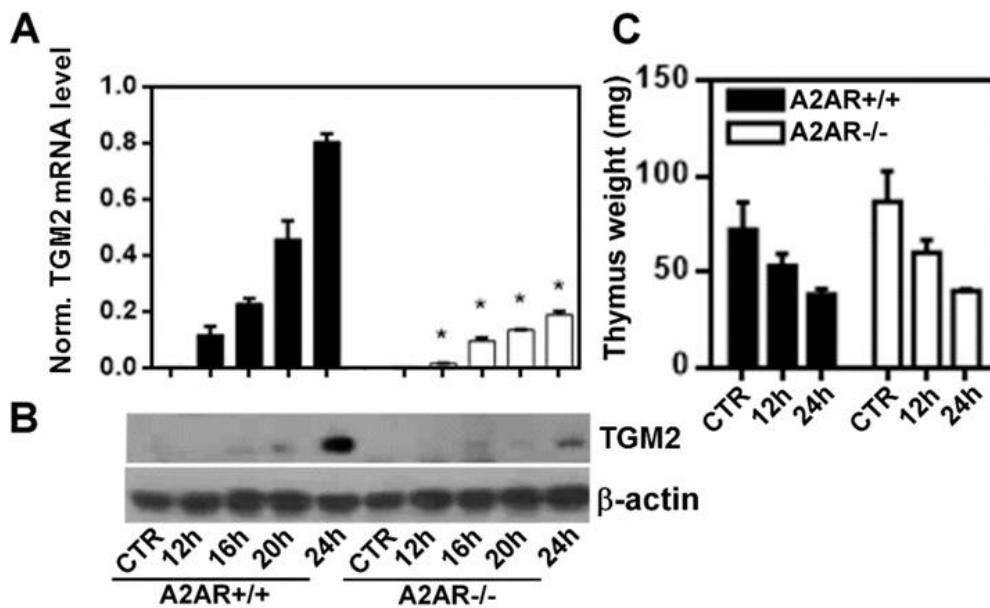
Figure 13. Dexamethasone-induced Tgm2 mRNA and protein expression is attenuated in the thymus of A2AR null mice. In vivo thymic apoptosis was induced in 4-wk-old wild type and A2AR null mice by intraperitoneal injection of 0.3 mg dexamethasone acetate. Thymuses were collected at the indicated time periods and (A) Tgm2 mRNA levels were determined by qRT-PCR using cyclophillin as a control, while (B) TGM2 protein levels were determined by Western blot analysis using β-actin as loading control. (C) Changes in the thymic weight following dexamethasone injection in wild type and A2AR null mice. Results are expressed as mean ± S.D. of three independent experiments (*Significantly different from the wild type control, $p<0.05$).

For this purpose apoptosis was induced by intraperitoneal injection of dexamethasone in the thymus of both A2AR null mice and their wild type counterparts. Dexamethasone is a powerful apoptosis inductor of thymocytes, and if adenosine is produced under *in vivo* conditions and contributes to the *in vivo* upregulation of TGM2, we should detect an altered TGM2 expression in A2AR null mice. Indeed, in the thymus of A2AR null mice, the dexamethasone-induced TGM2 expression was significantly attenuated at both mRNA (Fig. 13A) and protein (Fig. 13B) levels. The failure of inducing TGM2 fully in the thymus of A2AR null mice was not related to a failure of thymic apoptosis induction, as no significant difference in the loss of thymic weight was detected between the two mouse strains (Fig. 13C). This is in line with our previous results, which demonstrated that A2AR null thymocytes show similar apoptotic sensitivity to dexamethasone, as their wild type counterparts (Kiss et al., 2006). Altogether these data indicate

that A2AR signaling is the third pathway that contributes to the upregulation of TGM2 in dying thymocytes under *in vivo* conditions.

4.4 Adenosine is produced extracellularly during phagocytosis of apoptotic cells

Our previous studies indicated that neither macrophages nor apoptotic thymocytes produce detectable amount of adenosine, when cultured alone. Adenosine formation was detected only when these cells were incubated together (Koroskenyi et al., 2011). Thus adenosine formation could be the result of either intracellular degradation of apoptotic cell-derived nucleotides by engulfing macrophages, or that of extracellular breakdown of adenine nucleotides released by either apoptotic cells or engulfing macrophages. The rate limiting step in extracellular adenosine formation is catalyzed by either ecto-nucleoside triphosphate diphosphohydrolases, such as CD39 that phosphohydrolyses ATP, and less efficiently ADP, in a Ca^{2+}- and Mg^{2+}-dependent fashion, to yield AMP (Heine et al., 2001), or by members of the ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family, such as NPP1 and 3, which are also located on the cell surface, but produce AMP directly (Stefan et al., 2006). AMP in turn, is rapidly degraded to adenosine by soluble or membrane-bound ecto-5′-nucleotidases, such as CD73 (Resta et al., 1998).

Our previous studies have demonstrated that adenosine produced during engulfment of apoptotic cells selectively prevents the formation of a group of pro-inflammatory cytokines by macrophages, such as the chemokines KC and MIP-2 (Koroskenyi et al., 2011). Thus to determine whether during engulfment of apoptotic cells adenosine is released from an intracellular store or is generated extracellularly from nucleotides, we decided to detect the cytokine profile of engulfing macrophages in the presence of the ecto-5'-nucleotidase (CD73)

A

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
A	+control											+control
B	BLC	C5/C5a	G-CSF	GM-CSF	I-309	Eotaxin	sICAM-1	IFN-γ	IL-1α	IL-1β	IL-1ra	IL-2
C	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-13	IL-12p70	IL-16	IL-17	IL-23	IL-27
D	IP-10	I-TAC	KC	M-CSF	JE	MCP-5	MIG	MIP-1α	MIP-1β	MIP-2	RANTES	SDF-1
E	TARC	TIMP-1	TNF-α	TREM-1								
F	+control											-control
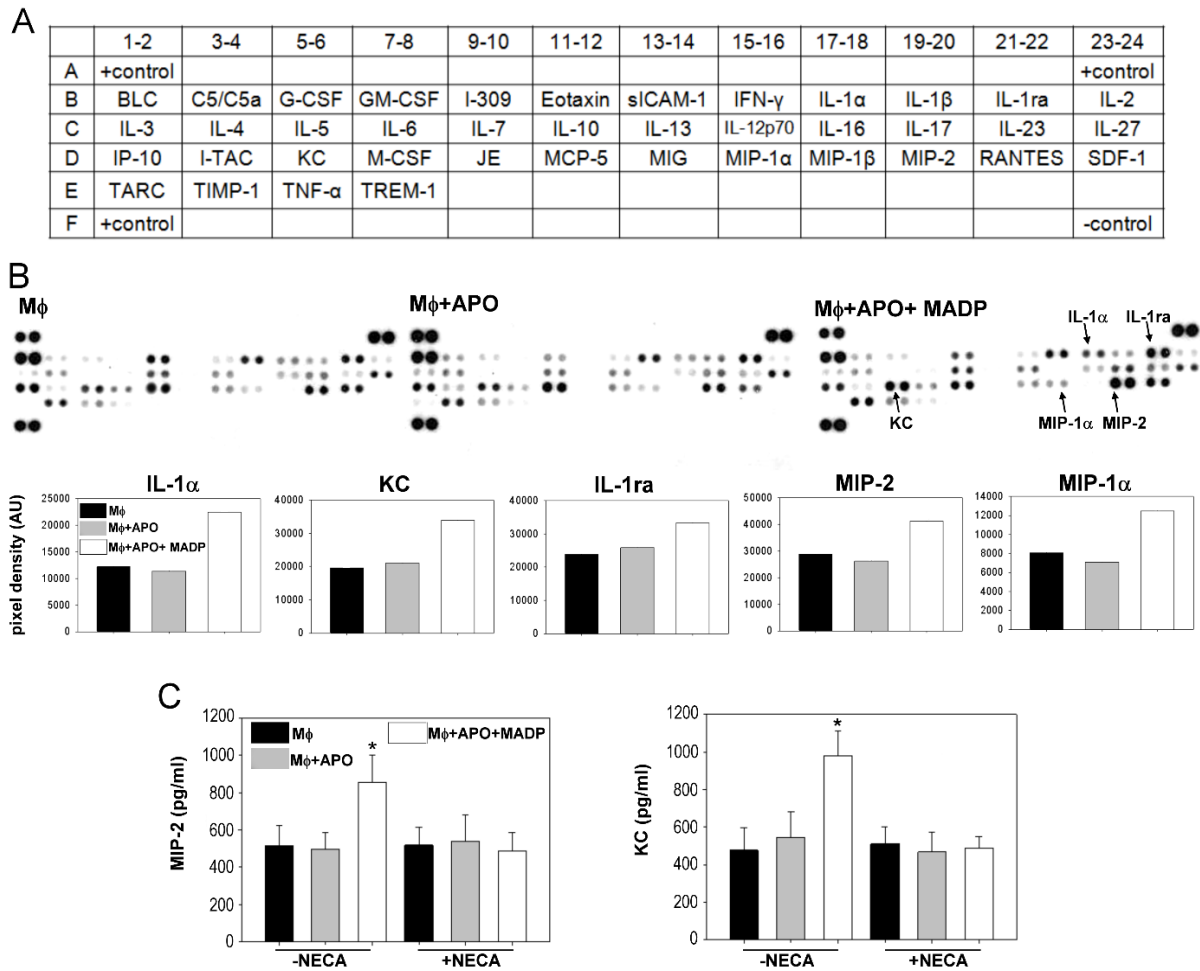
Figure 14. As compared to controls, MIP-2 and KC production is enhanced by ecto-5'-nucleotidase inhibitor-treated peritoneal macrophages (MPh) engulfing apoptotic cells. (A) The map of the 40 cytokines detected on the membranes. (B) Cytokine panel of control, apoptotic cell- exposed, or apoptotic cell-exposed, methylene adenosine-5'diphosphate-treated macrophages. Peritoneal macrophages were pre-incubated with 50 μM MADP for 30 min, then they were exposed to apoptotic thymocytes (APO) for 1 h (MPh:APO=1:10). 5 h after the removal of apoptotic cells supernatants were collected and cytokine levels were determined by cytokine array. Arrows highlight cytokines overproduced by MADP-treated macrophages. One experiment of two is shown. (C) MIP-2 and KC production of control, apoptotic cell-exposed or apoptotic cell-exposed, MADP-treated macrophages in the presence and absence of 5 μM NECA determined by ELISA. Results are expressed as mean ± S.D. of five independent experiments (*Significantly different from macrophages engulfing apoptotic cells in the absence of MADP, p<0.05). **Performed by co-authors.**

inhibitor methyleneadenosine 5'-diphosphate (MADP). Evaluation of the cytokine secretion profile of macrophages was performed using a highly sensitive cytokine antibody array method, enabling the simultaneous detection of low concentrations of multiple cytokines in one assay (picogram per milliliter range). The map of the 40 cytokines detected on the membranes is diagrammed in Figure 14A. As shown in Figure 14B and C, while engulfment alone did not alter the cytokine pattern released by peritoneal macrophages, inhibition of the ecto-5'-nucleotidase activity significantly enhanced the MIP-2 and KC production of engulfing macrophages. However, MADP did not the production of these cytokines during engulfment of apoptotic cells, if NECA, a non-metabolisable analogue of adenosine, was present (Fig. 14C) proving that addition of MADP indeed interfered with the extracellular adenosine formation. These data indicate that adenosine, a suppressor of pro-inflammatory cytokine formation during engulfment, is produced extracellularly.

Cells undergoing apoptosis release ATP which is required for macrophage migration towards the apoptotic cells (Elliott et al., 2009). The channel, which opens in a caspase-dependent manner during apoptosis, has been identified as the pannexin channel (Chekeni et al., 2010). Previous studies using supernatants of pannexin-1 channel-overexpressing W3 apoptotic cells have shown that adenine nucleotides released via pannexin-1 channels can be converted to adenosine by macrophages to trigger A2AR signaling in macrophages (Yamaguchi et al., 2014). Since thymocytes express and activate pannexin-1 channels during apoptosis (Qu et al., 2011), to decide whether the adenine nucleotide released by apoptotic cells alone determines adenosine formation required for the inhibition of pro-inflammatory cytokine formation or engulfing macrophages also contribute, we determined the pro-inflammatory cytokine formation by macrophages engulfing wild type or pannexin-1 null (Rigato et al., 2012) apoptotic thymocytes. In agreement with previous findings (Qu et al., 2011), loss of pannexin-1 channels did not affect significantly the rate of apoptosis (Fig. 15A). Prior to phagocytosis, we placed apoptotic thymocytes into a new medium and then co-cultured them with peritoneal macrophages for 1 h. 5 h after the removal of apoptotic cells supernatants of macrophages were collected, and cytokine levels were determined by cytokine array. Exposure of pannexin-1 null apoptotic thymocytes to macrophages resulted in the same cytokine profile as exposure to wild type thymocytes (data not shown) indicating that even in the absence of pannexin-1 channels on apoptotic cells sufficient amount of adenosine was formed to suppress the formation of pro-inflammatory cytokines by macrophages. Thus we checked whether pannexin-1 null thymocytes release ATP, and found that though less, but ATP was released from pannexin-1

null cells as well (Fig. 15B). Incomplete inhibition of the ATP release by the pannexin-1 null thymocytes may be due to the expression of connexins, another ATP channel type, in mouse thymocytes (Fonseca et al., 2004), or because a significant fraction of the thymocytes had undergone secondary necrosis (Fig 15A). Thus we could not decide whether adenosine was generated from nucleotides released only by the apoptotic thymocytes, or also by engulfing macrophages, which are also capable of releasing ATP upon certain stimuli (Cohen et al., 2013).
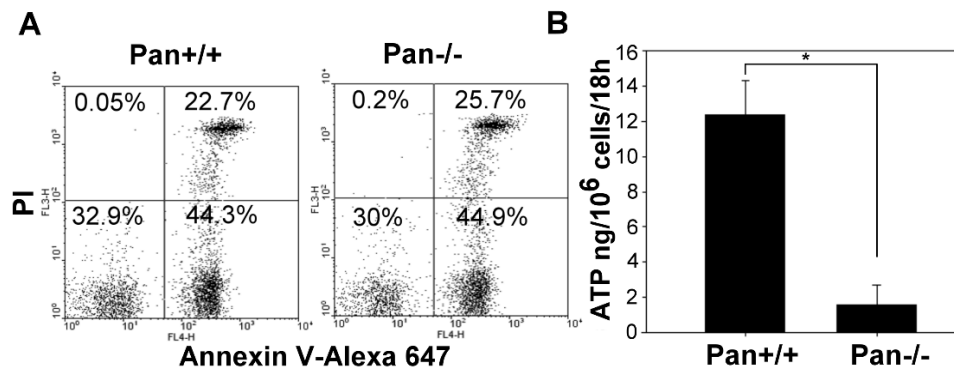
Figure 15. *ATP production and apoptosis of wild-type and pannexin-1 null thymocytes during 18 h culture in serum free medium. (A) Loss of pannexin-1 channels does not alter the rate of apoptosis of mouse thymocytes. (B) ATP released from wild-type or pannexin-1 null thymocytes during 18 h culture in serum free medium. Results are expressed as mean ± S.D. of three independent experiments (*p<0.05).* **Performed by co-authors.**

The above presented data provide evidence that the A2AR signaling pathway act via the activation of adenylate cyclase and contributes to the expression of *Tgm2* in dying thymocytes. To date there are at least three different signaling pathways that can regulate the expression of *Tgm2* at the mRNA level, but how these act together and what kind of intergenic regulatory elements are available serving as molecular beacons for these around the gene, remained completely elusive.

In our next study, we aimed to determine the functional enhancer element repertoire through which the described activator molecules and their downstream transcription factors act in order to regulate the level of *Tgm2* in dying thymocytes.
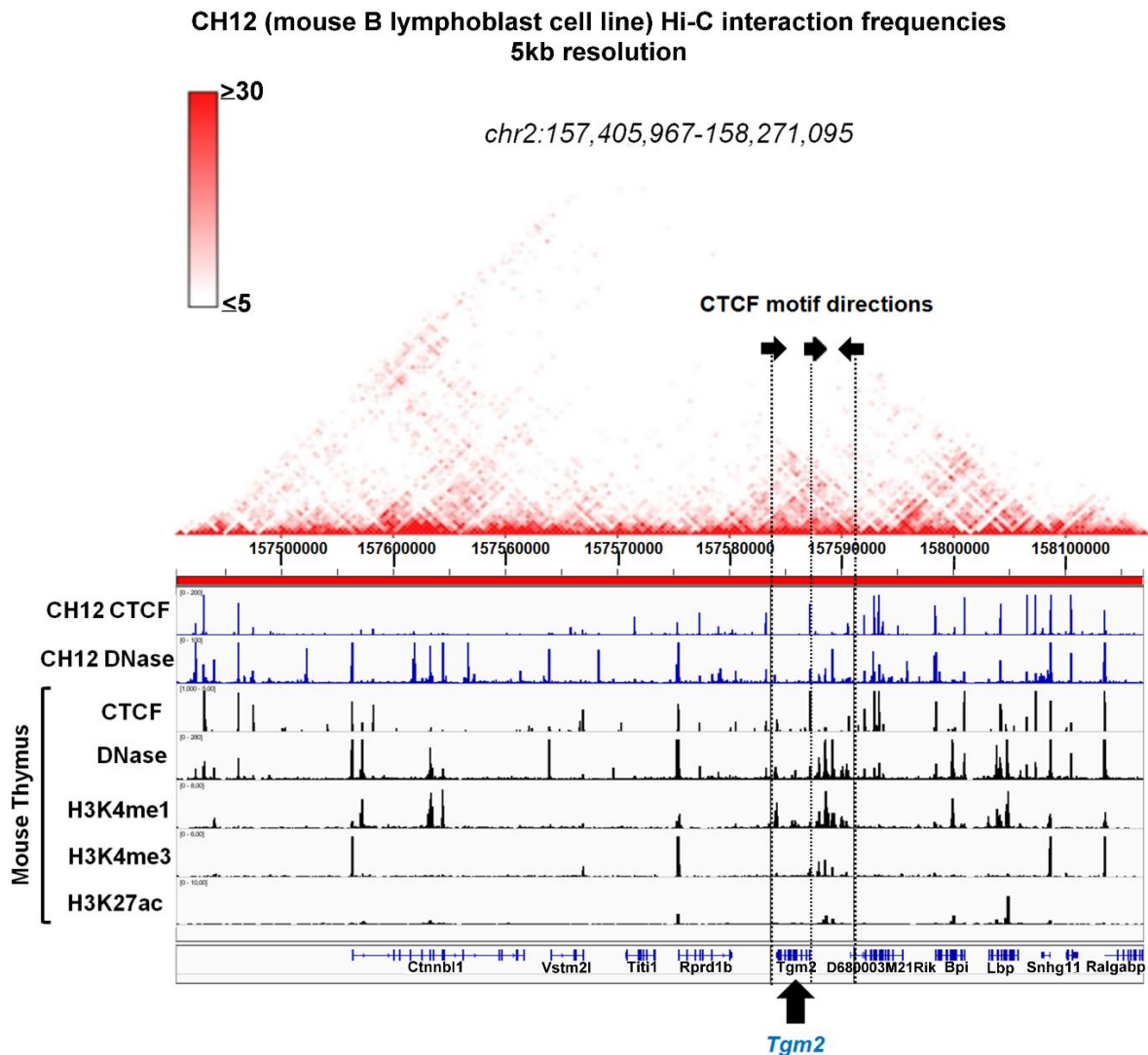
4.5 The combination of DNase-seq and ChIP-seq allows the identification of the putative enhancer set of *Tgm2*

In order to identify the putative enhancer elements, which might play a role in the regulation of *Tgm2* in mouse thymocytes, we decided to use several mouse thymus derived datasets generated by the ENCODE Consortium. More specifically, we utilized DNase-seq data, ChIP-seq datasets for H3K4me1, H3K4me3, and H3K27ac for enhancer identification and CTCF ChIP-seq to determine the putative gene domain for *Tgm2* in which the regulation might take place. As a very first step, we tried to exploit the recently described feature about the motif orientation of CTCF and its effect on long-range chromatin looping and demarcating functionally different genomic regions. Tandem orientation of the CTCF motifs has been proposed to play role in gene regulation by allowing the enhancers to find their targets, while convergent motifs are more likely to be implicated in the formation of higher-order chromatin structure (Rao et al., 2014; Tang et al., 2015). We identified three high affinity CTCF peaks flanking a 157kb wide genomic region around *Tgm2* (Fig. 14). Analysis of the orientation of the motifs showed one convergent motif pair located at the borders of the 157kb genomic area, while the third, transcription start site (TSS) specific motif constitutes a tandem motif pair with the upstream CTCF border (Fig. 16). These data suggest that the regulation of the gene might take place in a genomic region spanning 157 kb pairs.

While convergent motifs are likely to function in establishing topologically associated domain borders, there are still many exceptions in which case potential enhancers could be overlooked. To avoid this possibility, we overlapped the interaction profile of a genomic loci of ~800kbp determined by Hi-C, containing the *Tgm2* locus with the available CTCF ChIP-seq and DNase-seq from the CH12.LX mouse B lymphoblast cells (Rao et al., 2014) and the same datasets from thymus along with the histone modification ChIP-seq profiles (Fig. 17). We found that interactions between the two proposed boundaries are clearly enriched suggesting that the regulation of *Tgm2* most probably takes place in this genomic unit and the enhancers do not communicate with the neighboring genes. In addition, we also measured the mRNA expression of the neighboring gene (*Rprd1b,* chr2:157854530-157903056), which seems to be active based on the H3K4me3 histone modification present at the transcription start site of the gene. However, we could not detect any difference in its expression level in the presence of those compounds which affected the expression *Tgm2*.
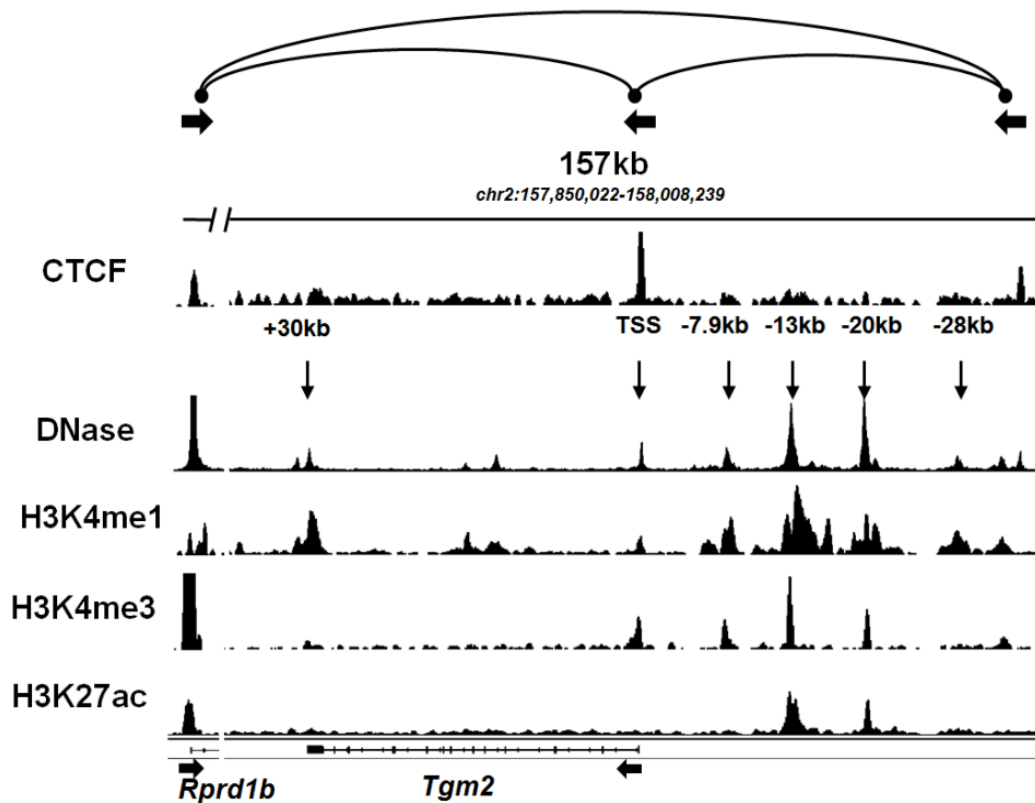
Figure 16. *Finding putative enhancers for the Tgm2 gene in mouse thymocytes. IGV genome browser view about the Tgm2 locus. ChIP-seq results against CTCF, H3K4me1, H3Kme3 and H3K27ac along with the DNase hypersensitive sites are shown from mouse thymus. CTCF motif orientations are indicated with black horizontal arrows and the presumable interactions are depicted as connecting lines between them (top). Intergenic DNase hypersensitive sites and the TSS of Tgm2 are shown with vertical arrows, and their relative distance to the TSS is also highlighted (under the CTCF track). TSSs of Tgm2 and Rprd1b genes are also shown with the direction of the gene highlighted by horizontal arrows.*

CH12 (mouse B lymphoblast cell line) Hi-C interaction frequencies 5kb resolution

chr2:157,405,967-158,271,095

Figure 17. *Integration of Hi-C interaction data, CTCF ChIP-seq and DNase-seq from CH12.LX cells along with mouse thymocyte data for DNase-seq and ChIP-seq against CTCF, H3K4me1, H3K4me3 and H3K27ac. Contact matrix heatmap was generated using the Interactive Hi-C Data Browser available at http://www.3dgenome.org. Hi-C data was generated by Rao et al. from the CH12.LX mouse B lymphoblast cell line. Data is shown with 5kb resolution on the indicated genomic region (top). IGV browser representation of ChIP-seq and DNase-seq experiments from the indicated cell types. CH12.LX-derived datasets are available under accessions: GEO:GSM912909 (CTCF ChIP-seq), GEO:GSM1014153 (DNase-seq) The Tgm2 locus is marked with dashed lines and the motif direction of the two CTCF anchors (black arrows) are also shown used by us to predict the functional domain of the gene in which the regulation might take place (bottom).*

Next we took advantage of the available DNase-seq dataset and identified several hypersensitive sites around the *Tgm2* gene located between the tandem CTCF sites, and an additional one located between the TSS-specific and the downstream CTCF site, which show convergent motif orientation (Fig 16). Since integration of DNase-seq with ChIP-seq against H3K4me3, H3K4me1 and H3K27ac proved to be useful in the identification of putative functional enhancers (Heintzman et al., 2007; Creyghton et al., 2010; Visel et al., 2009a), we applied this approach and found six (+30kb, TSS, -7.9kb, -13kb, -20kb, -28kb) hypersensitive regions which showed enrichment for H3K4me3 and for H3K4me1. H3K4me3 is a hallmark of actively transcribed protein coding gene promoters (Visel et al., 2009b), but can be detected occasionally together with enhancers due to a strong enhancer/promoter interaction. H3K4me1 is associated with most of the accessible regulatory elements in the genome and represents active, poised or primed enhancers depending on the presence of other histone modifications (Heintzman et al., 2007; Creyghton et al., 2010). Out of the six elements, only two (-13kb and -20kb) showed enrichment for H3K27ac, which is a widely accepted functionally active enhancer marker (Creyghton et al., 2010). In summary. by using the ENCODE generated genome-wide datasets from mouse thymus we have identified five intergenic enhancers for detailed analysis excluding the TSS/promoter region, which has been studied thoroughly.

4.6 Whole thymus tissue derived acetylation patterns correlate with the acetylation pattern of *in vitro* cultured thymocytes

The thymus is composed of various types of cells. Although thymocytes constitute the majority of cells in the thymus (Bellamy and Alkufaishi, 1972), one should be careful of using whole thymus for enhancer mapping, because other cell types, such as macrophages, dendritic cells and epithelial cells are also present. Therefore, signals are not reliably originated from the cell type of interest. To make sure that we have a pure population of thymocytes we isolated them from the mouse thymus to check whether the selected putative enhancers of *Tgm2* in these cells show a similar pattern of H3K27ac.

As shown in Figure 18, our ChIP-qPCR experiments demonstrated that similar to the ENCODE data originated from the whole thymus (Fig.16), in *in vitro* cultured thymocytes the two (-13kb and -20kb) enhancer elements are much stronger acetylated as compared to the other putative enhancers of *Tgm2* (Fig. 16). In accordance we found that these two highly acetylated enhancers have a concomitant enrichment also for the two well-known histone acetyltransferases P300

and CBP. These results proved the adaptability of the two systems showing positive correlation between the whole thymus derived genome-wide datasets and our ChIP experiments on individual enhancers from thymocytes cultured *in vitro*.
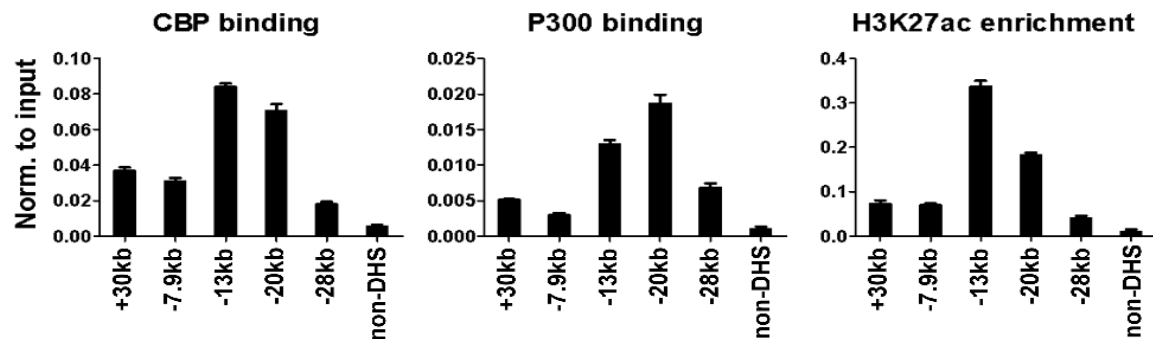
Figure 18. ChIP-qPCR measurements against CBP, P300 and H3K27ac from in vitro cultured mouse thymocytes are shown on the indicated intergenic regulatory elements. As a control, we also performed the same ChIP-qPCRs on the neuron specific Prmt8 gene -1.7kb region, which in the thymic genome appears as a non-DNase hypersensitive region (non-DHS). Data represent mean±SD of triplicate measurements.
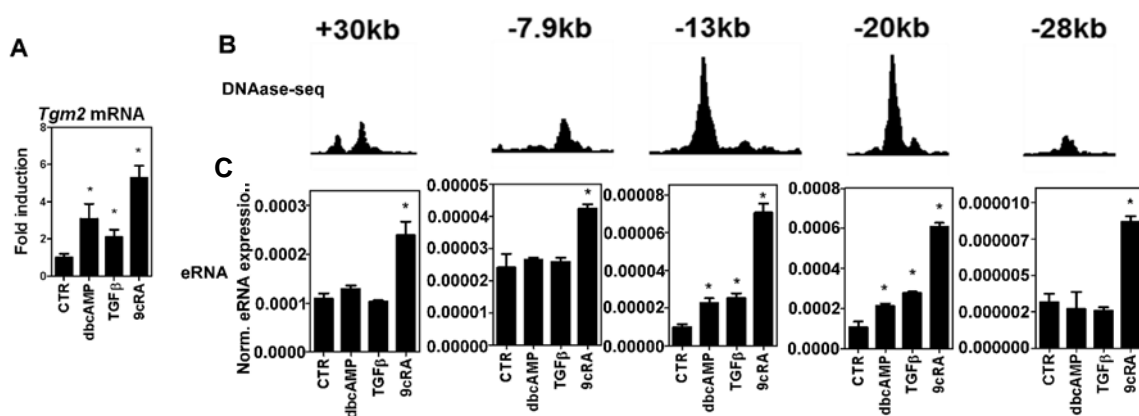
4.7 Enhancer RNA measurements identify functional enhancers for the *Tgm2* gene

Previous studies from our laboratory have shown that in thymocytes *Tgm2* expression is regulated by external signals derived from engulfing macrophages, such as retinoids (Garabuczi et al., 2013), TGF-β and adenosine, the latter activating adenosine A2A receptors and the consequent adenylate cyclase pathway (Sandor et al., 2016). Among these signals 9cRA was the most effective in inducing *Tgm2* mRNA levels, resulting in a 5 fold induction. Activation of both the adenylate cyclase and the TGF-β signaling pathway synergized with the retinoid signaling pathway in inducing *Tgm2* mRNA levels.

To determine whether the putative enhancers respond to these signals, we decided to measure the amount of eRNAs transcribed from them. To detect the response of the individual enhancers, primary thymocytes cultured *in vitro* were exposed for 1 h to 0.3 uM 9cRA, a ligand of the nuclear retinoic acid receptors (RAR/RXR) (Heyman et al., 1992), to 5 ng/ml rTGF-β leading to trimerization of SMAD4 with phosphorylated SMAD2 or 3 and its nuclear transport

(Massague, 2012), or to 100 µM dbcAMP, a membrane permeable analogue of cAMP, resulting in the activation of protein kinase A and in subsequent phosphorylation of CREB (Manna et al., 2014). However, phosphorylation and activation of CREB can occur also via activation of both the TGF-β signaling pathway, which activates protein kinase A in a cAMP-independent manner leading to the release of its regulatory subunit (Al-Wadei and Schuller, 2006) and the retinoid signaling pathway, which can lead to CREB phosphorylation in a protein kinase A-dependent (Canon et al., 2004; Aggarwal et al., 2006) and independent manner (Aggarwal et al., 2006).

Interestingly, all of the enhancers showed elevated eRNA expression in response to 9-cRA, but only two of them, located -13kb and -20kb, appeared to be regulated also by TGF-β or dbcAMP (Fig. 19 A, C). In addition, these two enhancers showed clear differences in their retinoid response showing a much robust eRNA upregulation as compared to the +30kb, -7.9kb and -28kb enhancers.

Figure 19. Combined eRNA measurements and determination of transcription factor binding identify functional enhancers for the Tgm2 gene. (A) Tgm2 mRNA levels determined by RT-qPCR analysis in the presence of the indicated compounds (0.3 µM 9cRA, 5 ng/ml rTGF-β or 100 µM dbcAMP). (B) Intergenic DNase hypersensitive sites and their relative distance to the TSS of Tgm2 are shown. (C) Enhancer RNA levels determined by RT-qPCR analysis on the indicated Tgm2 enhancers in the presence of the indicated molecules (0.3 µM 9cRA, 5 ng/ml rTGF-β or to 100 µM dbcAMP) in in vitro cultured thymocytes after 1 h treatment. CTR: vehicle treated control.

Our knowledge about these signaling pathways allowed the investigation of the binding of the signal specific transcription factors on these enhancers. We applied ChIP-qPCR to measure the

enrichment of CREB, SMAD4, RAR and RXR in the presence of their activators on the identified enhancer elements (Fig. 20).
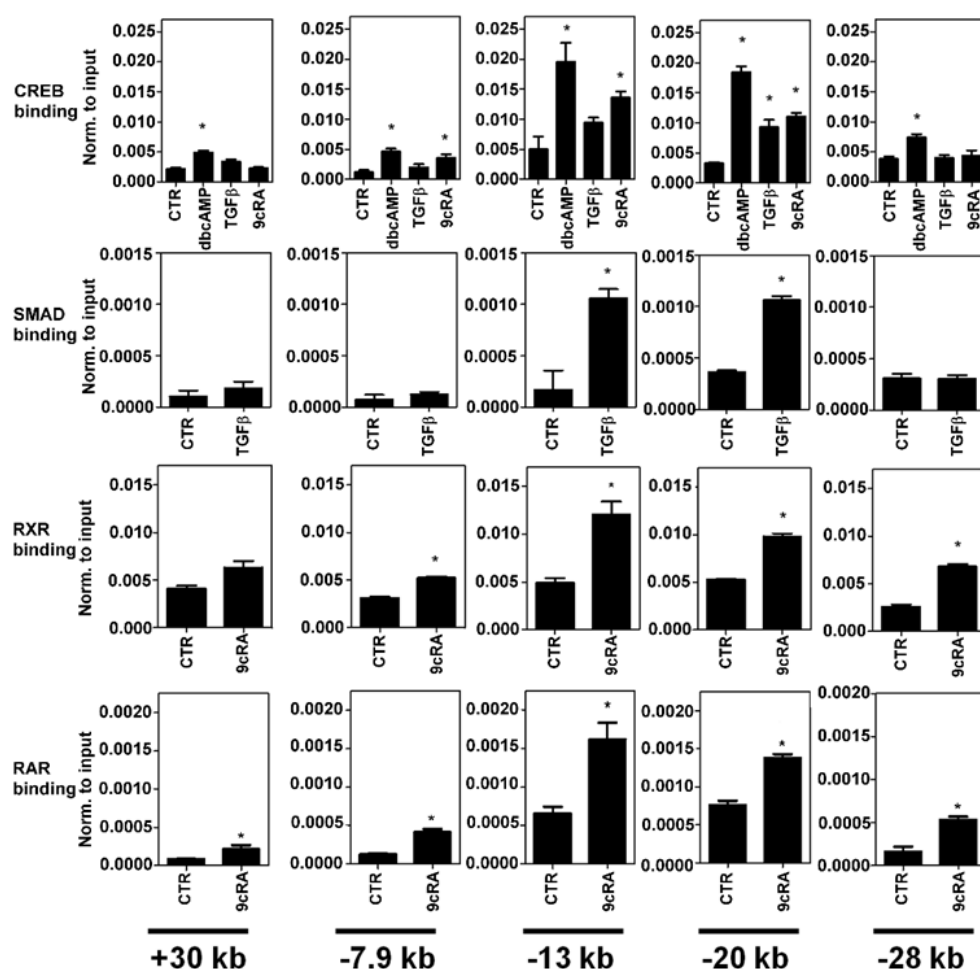
Figure 20. CREB, SMAD4, RXR and RAR binding on the indicated enhancer elements determined by ChIP-qPCR analysis in the presence of the indicated molecules after 1 h exposure. Background was determined by using rabbit IgG and subtracted from the values presented on the graphs.

Exposure to dbcAMP efficiently recruited CREB to each enhancer elements, but the occupancy of CREB appeared to be the highest when we tested its binding on enhancers located at -13kb and -20kb. In accordance with reports, which demonstrated CREB activation in the retinoid and TGF-β signaling pathways, enhanced CREB binding could be detected also following exposure to 9cRA or rTGF-β, but again the occupancy of CREB appeared to be the highest when we tested its binding on the enhancers located at -13kb and -20kb (Fig. 20). Similarly, when we assessed the binding of SMAD4 in the presence of rTGF-β, we found a dynamic recruitment to

the -13kb and -20kb enhancers. ChIP-qPCR experiments performed against RAR or RXR in the presence of 9cRA did not show robust recruitment on the investigated enhancers. Still, an enhanced binding of RAR and RXR, as a 9cRA response, was found on all the investigated enhancers. In line with the eRNA measurements, these results indicate that the majority of RARs and RXRs are already bound to all of the investigated sites even in the absence of the externally added agonist, as it was previously shown by others (Daniel et al., 2014b).

Our results demonstrate that the strategy developed by us proved to be efficient in the identification of signal selective enhancers. By measuring the synthesized eRNA species from individual enhancers and combining the results with transcription factor binding data, we identified five signal selective enhancers of *Tgm2* in mouse thymocytes.

4.8 The signaling pathway induced by TGF-β selectively potentiates the retinoid signaling on *Tgm2* associated enhancer elements

Recently, it has been shown by our group that the adenylate cyclase and TGF-β mediated signaling synergize with the retinoid signaling on TGM2 leading to a more pronounced induction of Tgm2 at mRNA level (Garabuczi et al., 2013). Based on these results we decided to test, whether the activation of TGF-β signaling could potentiate the retinoid-mediated signaling pathways in the context of *Tgm2* regulation. To identify those enhancers on which the collaboration between the two signaling pathways might take place, we determined enhancer specific eRNA levels in the presence of various signal combinations. As shown in Figure 21 B, enhancer elements located at +30kb and at -28kb did not show any sign of collaboration in agreement with our previous results, which indicated that on these elements no significant TGF-β-induced SMAD4 binding was found. On the other hand, we could detect significantly increased eRNA expression on the -7.9kb, -13kb and -20kb enhancers if both signals were present, indicating that the TGF-β and the retinoid pathways crosstalk on these enhancers.
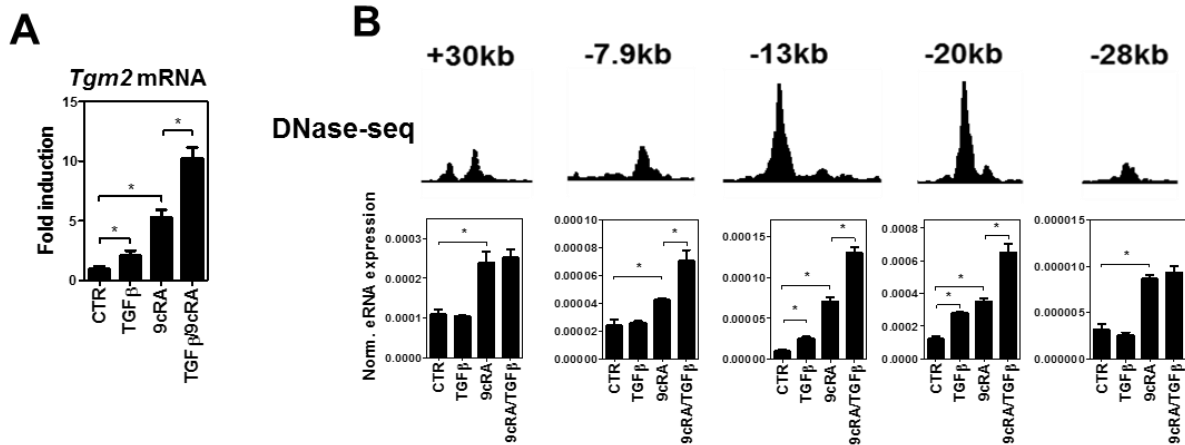
Figure 21. The signaling pathway induced by TGF-β selectively potentiates the retinoid signaling on Tgm2 associated enhancer elements. (A) Tgm2 mRNA levels determined by RT-qPCR analysis in the presence of the indicated compounds (0.3 µM 9cRA, 5 ng/ml rTGF-β or 0.3 µM 9cRA/5 ng/ml rTGF-β) after 6 h treatment. mRNA levels are expressed as fold induction as compared to the DMSO (0.5%) exposed samples. (B) Enhancer RNA levels determined by RT-qPCR analysis on the indicated Tgm2 enhancers in the presence of the indicated molecules in in vitro cultured thymocytes after 1 h treatment.

To analyze this crosstalk in more detail, we performed ChIP-qPCR experiments using RXR specific antibodies. While 9-cRA alone resulted only in a slight increase in the RXR recruitment, a significant increase in the recruitment of RXR to the enhancers located -7.9kb, -13kb and -20kb could be detected, when TGF-β was also administered (Fig. 22). Interestingly, in the presence of both signals the SMAD4 recruitment increased also. Moreover, the recruited P300 and CBP showed also elevated levels on these enhancer elements in response to TGF-β/9cRA co-treatment as compared to 9cis-RA alone. These results suggest that in the presence of both signals the accessibility of these sites increases for both transcription factors, which ultimately leads to an increased coactivator binding and more efficient transcription. Interestingly, in the case of the -7.9 kb enhancer the interaction existed between the two pathways despite the lack of significant binding of SMAD4 to this enhancer in the presence of TGF-β alone.
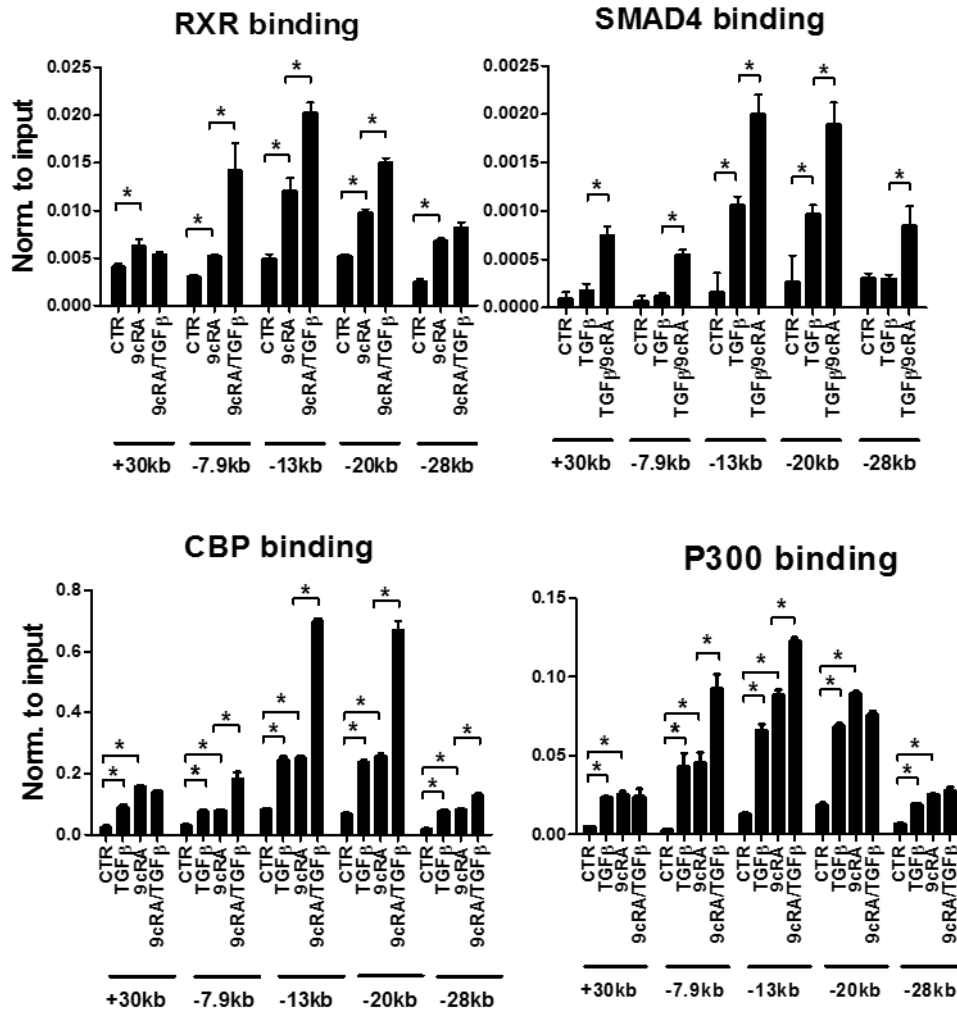
Figure 22. *RXR, SMAD4, P300 and CBP binding on the indicated enhancer elements determined by ChIP-qPCR analysis in the presence of the indicated compounds after 1 h treatment. Background was determined by using rabbit IgG and subtracted from the values presented on the graphs.*

Knowing the fact that 9cRA is able to activate various RXR heterodimers including RAR/RXR, we applied a selective synthetic agonist of RARα (AM580) and that of RXR (LG268) to determine the target of interaction between the retinoid and the TGF-β signaling pathways on the signal integrator enhancers (-13kb and -20kb). We found that both the RARα and the RXR activator alone could significantly induce the level of enhancer transcripts at the signal integrator enhancers. Interestingly, when we measured the activation of these enhancers in the presence of the signal combinations of TGF-β/AM580 or TGF-β/LG268, we found a more pronounced eRNA expression in both cases and on both enhancers (Fig. 23). These data indicate

that TGF-β signaling is able to potentiate the transcriptional activity of the RXR/RARα heterodimer, in line with the fact that we detected both RAR and RXR binding on these enhancers. In addition, administration of the pan RAR antagonist AGN194310 reduced LG268/TGF-β-induced eRNA levels to the TGF-βAGN194310-induced eRNA levels, and the AM580/TGF-β and the LG268/TGF-β-induced eRNA levels to that of the AM580/AGN194310- and the LG268/AGN194310-induced eRNA levels, respectively, indicating that only RAR/RXR heterodimers interact with the TGF-β signaling on these enhancers.
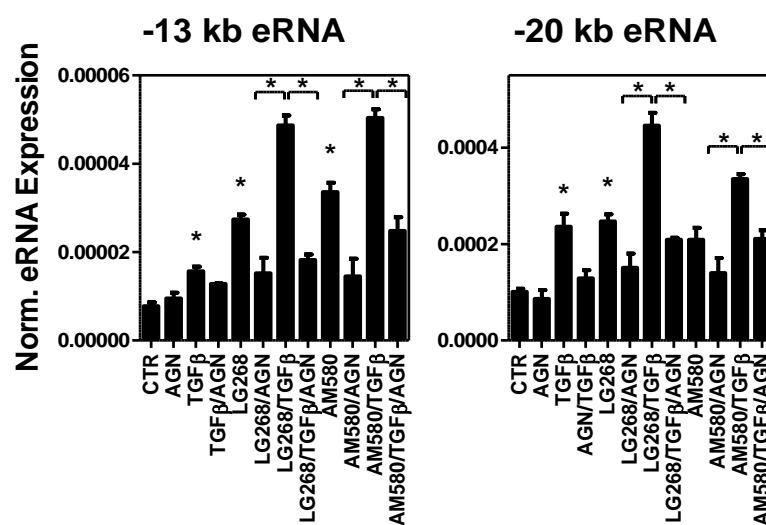
Figure 23. Enhancer RNA levels determined by RT-qPCR analysis on the indicated *Tgm2* enhancers in the presence of 1 μM AM580, an RARα agonist, 100 nM LG268, an RXR agonist, 5 ng/ml rTGF-β, 100 nM AGN194310, a pan RAR agonist in in vitro cultured thymocytes after 1 h treatment. CTR: vehicle treated control. As a negative control, we also performed the same eRNA and ChIP-qPCR measurements on the neuron specific Prmt8 gene -17kb region, which in the thymic genome appears as a non-DNase hypersensitive region. Data represent mean±SD of triplicate determinations. *Significantly different (P<0.05).

4.9 The adenylate cyclase pathway also selectively potentiates retinoid signaling on *Tgm2* associated enhancer elements

Above indicated that the adenylate cyclase pathway also enhances TGM2 protein levels in the thymocytes *in vivo* (Sandor et al., 2016). As shown in Figure 24A exposure to dbcAMP alone

enhanced the steady state levels of *Tgm2* mRNA, and when dbcAMP was applied together with 9cRA, it synergized with retinoid signaling in regulating *Tgm2* mRNA levels, though less efficiently as TGF-β did. Next by measuring eRNA levels on the various enhancers, we examined whether the two signaling pathways synergize also in the context of *Tgm2* regulation. As shown in Figure 24B, enhancer elements located at +30kb, -7.9kb and -28kb did not show any sign of collaboration in agreement with our previous results, which indicated that on these elements no significant dbcAMP-induced CREB binding was found. On the other hand, we could detect significantly increased eRNA expression on the -13kb and -20kb enhancers if both signals were present, indicating that the adenylate cyclase and the retinoid signaling pathways crosstalk on these enhancers.
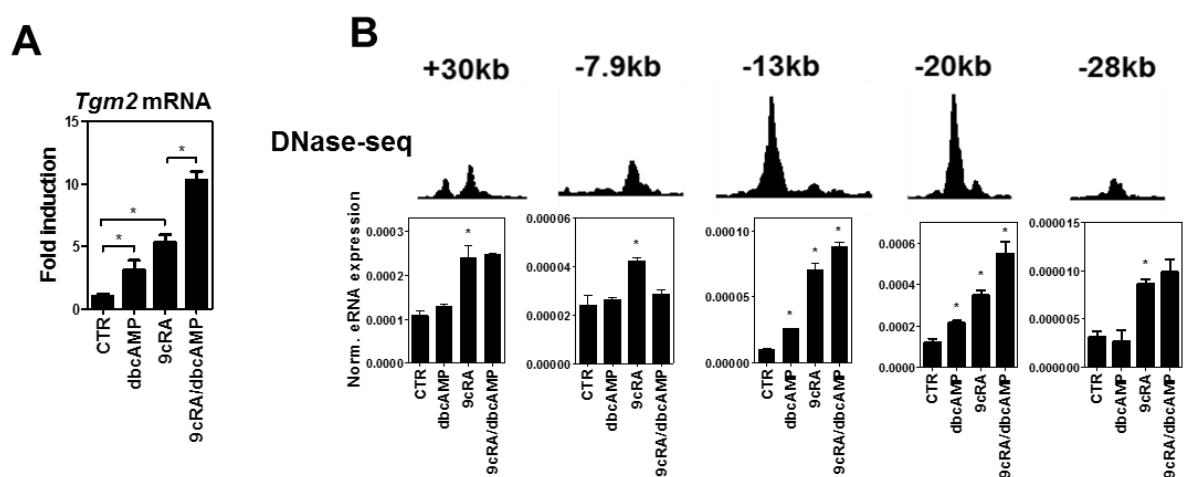
Figure 24. The adenylate cyclase pathway also selectively potentiates retinoid signaling on Tgm2 associated enhancer elements. (A) Tgm2 mRNA levels determined by RT-qPCR analysis in the presence of the indicated compounds (0.3 μM 9cRA, 100 μM dbcAMP or 0.3 μM 9cRA/100 μM dbcAMP) after 6 h treatment. mRNA levels are expressed as fold induction as compared to the DMSO (0.5%) exposed samples. (B) Enhancer RNA levels determined by RT-qPCR analysis on the indicated Tgm2 enhancers in the presence of the indicated molecules in in vitro cultured thymocytes after 1 h treatment.

To analyze this crosstalk in more detail, we performed similar ChIP-qPCR experiments for transcription factor binding, as we did for studying the TGF-β/retinoid signaling crosstalk. However, as seen in Figure 25, exposure to dbcAMP did not enhance the 9cRA-induced RXR recruitment to any of the enhancers. The recruitment of CREB, on the other hand, on most

enhancers was higher in the presence of the two signals, than in the presence of 9cRA alone, but not higher than in the presence of dbcAMP alone.
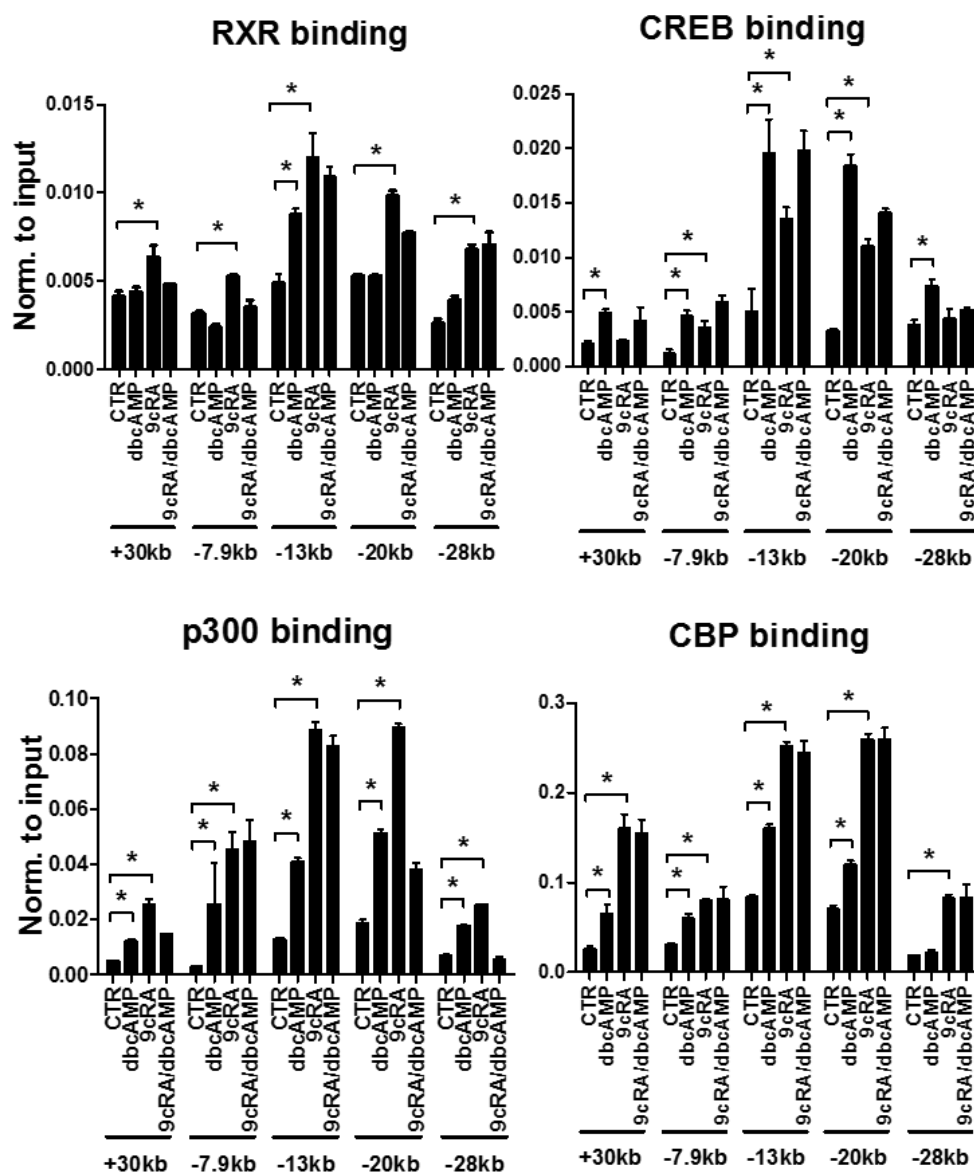
Figure 25. *RXR, CREB, P300 and CBP binding on the indicated enhancer elements determined by ChIP-qPCR analysis in the presence of the indicated compounds after 1 h treatment. Background was determined by using rabbit IgG and subtracted from the values presented on the graphs. CTR: vehicle treated control.*

Thus, while in the case of the TGF-β/retinoid crosstalk we detected increased enhancer accessibility for RXR, when the two signals were present together, no such an enhancement for

RXR binding was detected in the adenylate cyclase/retinoid signaling crosstalk. In addition, despite the fact that more CREB was recruited to most of the enhancers in the presence of the two signals, neither more CBP, nor more P300 coactivator binding were detected in the presence of dbcAMP, as compared to 9cRA alone.

4.10 Chromosome conformation capture experiments reveal interactions between the -20kb enhancer and the *Tgm2* gene

As a control for our approach, we performed chromosome conformation capture experiments (Dekker et al., 2002) to prove that the enhancers, we identified, interact with *Tgm2* gene. For this purpose we selected the +11kb and the +30 kb (-3' UTR) regions of the gene, which are DNase hypersensitive and as a control the +13kb region, which is a lowly accessible region. The limitation of the method related to the HindIII restriction sites allowed us to investigate the interaction of these regions with the potential -20kb enhancer. As shown in Figure 26, we were able to demonstrate the interaction between the enhancer located -20kb and the intronic region of the gene. Interestingly, we also found an interaction with the 3' UTR of the gene, where we also identified a DNase hypersensitive site, but could not show the interaction with the intervening sequence (+13kb region). Importantly, none of these contacts appeared to show induced interactions in the presence of the applied agonists, indicating that these are stable, pre-formed loops between the enhancer and the gene.
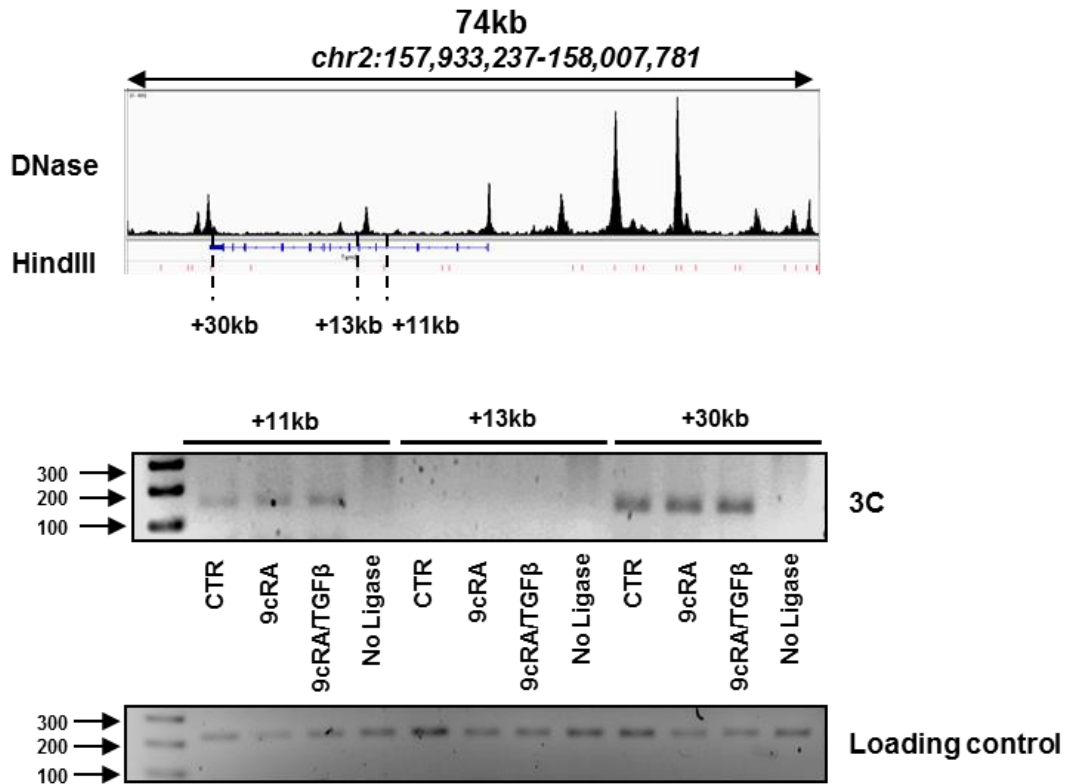
Figure 26. *Chromosome Conformation Capture (3C) experiments reveal interactions between the -20kb enhancer and the intronic-, 3'UTR region of the Tgm2 gene. (A) IGV genome browser representation of DNase hypersensitive regions on the Tgm2 locus in mouse thymocytes. HindIII cutting sites are indicated all over the locus, along with the used restriction fragments for 3C analysis. BAIT represents the starting point of the analysis overlapping with the -20kb enhancer. (B) Gel (1.5% agarose) image of 3C-PCR products combining BAIT-specific with the +11kb-, +13kb-, +30kb-specific primers. Thymocytes were treated with the indicated compounds for 1 h before fixation. No ligase controls are also carried out and indicated (middle). Gel image (1.5% agarose) of PCR products using primers specific to* chr10:74788584-74788803 *genomic region as a loading control (bottom).*

5. DISCUSSION

Efficient execution of apoptotic cell death followed by efficient clearance mediated by professional and by nonprofessional neighboring phagocytes, is a key mechanism in maintaining tissue homeostasis. Though the two processes are subsequent, increasing evidence indicate that at least in the thymus molecules produced by macrophages during the engulfment process strongly influence the first step. Thus they can induce the "death by neglect" of immature thymocytes (Szondy et al., 2012), or even affect the thymic selection and differentiation processes in the thymus (Sarang et al., 2013; Konkel et al., 2014). Previous studies coming from our laboratory have shown that engulfment-related molecules, such as TGF-β or retinoids, might also contribute to the appearance of TGM2 in apoptotic thymocytes (Szondy et al., 2003; Garabuczi et al., 2013). Since thymocyte apoptosis is a continuously ongoing process in the thymus, macrophages engulfing these apoptotic cells continuously release engulfment-dependent molecules, thus they contribute to the formation of a thymic microenvironment for immature thymocytes which promotes both cell death and appearance of TGM2 in dying thymocytes. Though *in vitro* thymocytes die efficiently in the absence of TGM2 (Szondy et al., 2003), *in vivo* TGM2 is needed to form a "find me" signal by apoptotic cells for initiating migration of macrophages towards the dying cells, and to prevent the leakage of the harmful cell content by forming protein crosslinks during apoptosis. Thus TGM2 *in vivo* is required to be present for the silent removal of apoptotic cells (Szondy et al., 2011).

In the present study we provide evidence for the regulation of TGM2 expression in dying thymocytes by a third signal, adenosine, which is also produced during engulfment of apoptotic cells. Adenosine mediates upregulation of TGM2 in thymocytes via A2ARs, which trigger the adenylate cyclase pathway. Since other molecules known to activate the adenylate cyclase pathway, such as forskolin or dbcAMP, could also increase TGM2 mRNA levels in thymocytes, it can be predicted that other molecules released by engulfing macrophages, such as prostaglandin E_2 (Fadok et al., 1998), which can also activate the adenylate cyclase pathway, might also contribute to the appearance of TGM2 in dying thymocytes *in vivo*. In addition, we also demonstrate that in the regulation of TGM2 expression adenosine strongly synergizes with other signals already known to upregulate TGM2 in dying thymocytes. How the interaction among the three signals is mediated at transcriptional level is under current investigation in our laboratory.

Previous studies from our laboratory have shown that neither macrophages nor dying thymocytes produce detectable amount of adenosine, if they are cultured alone: adenosine was produced during the engulfment process (Koroskenyi et al., 2011). Adenosine formation can be the result of intracellular degradation of apoptotic cell-derived nucleotides by engulfing macrophages, as well as it can be formed extracellularly. Previous studies using supernatants of W3 apoptotic cells have already shown that adenine nucleotides released via pannexin-1 channels can be converted to adenosine by macrophages to induce suppression of inflammation (Yamaguchi et al., 2014). We confirm that dying thymocytes release adenine nucleotides, partially in a pannexin-1 channel dependent manner. In addition, our data coming from mixed cell culture also indicate that whichever cell the source of adenine nucleotides is, suppression of inflammation is fully dependent on the activity of 5'-ecto-nucleotidases indicating that all the adenosine is formed extracellularly. Though we tried to answer by applying pannexin-1 null thymocytes whether solely the apoptotic cells are the source of adenine nucleotides or the engulfing macrophages also contribute with ATP release, because the supernatant of pannexin-1 null thymocytes was not ATP free, our experiments could not provide a clear answer.

CD39 is constitutively expressed in the thymus and is associated primarily with macrophages and Treg cells (Antonioli et al., 2013), while NPP1 was shown to be expressed by thymocytes (Petersen et al., 2007). Thus during the engulfment process, when both thymocytes and macrophages are present, both cell types can contribute to the extracellular degradation of ATP to AMP. However, while CD73 activity is constantly expressed by tissue resident macrophages (Eichin et al., 2015), it changes substantially during T cell development with CD73 being virtually absent in cortical thymocytes (Resta et al., 1997). Thus in the thymic cortex, where the majority of immature thymocytes die, adenosine formation must be fully dependent on the CD73 activity of macrophages. Altogether our data provide an additional example for the existence of a crosstalk between apoptotic thymocytes and their engulfing macrophages. The interaction between the two cells results in extracellular adenosine formation, which acting on macrophage A2ARs suppresses inflammation (Koroskenyi et al., 2011; Yamaguchi et al., 2014), while triggering simultaneously A2ARs on the surface of surrounding thymocytes, it contributes to the appearance of TGM2 in cells entering apoptosis.

Based on these data it seems that the expression of the TGM2 protein is associated with the *in vivo* apoptosis of thymocytes and is regulated transcriptionally by at least three extracellular signals arriving from the neighboring engulfing macrophages (Szegezdi et al., 2000; Garabuczi

et al., 2013; Szondy et al., 2003; Sandor et al., 2016). However, the regulation of TGM2 expression must be very complex, since the protein is ubiquitously expressed and participates in many biological processes (Fesus and Piacentini, 2002). During the years several publications appeared in the literature about the regulation of the *Tgm2* gene (Nagy et al., 1996; Ritter and Davies, 1998; Brown, 2013; Penumatsa et al., 2014). However, these studies were limited to the investigation of one single promoter element, and did not address the cell type-specific transcriptional regulation of the gene. With the help of the next-generation sequencing coupled approaches we had the opportunity to specifically map and identify the cell type- and gene-specific regulatory elements genome-wide as shown by others (Visel et al., 2009a; Visel et al., 2009b; Heintzman et al., 2007). In the present study, we took advantage of the ENCODE generated datasets from mouse thymus. Using the integration of DNase-seq and ChIP-seq approaches we revealed the putative regulatory element repertoire of *Tgm2*. We have exploited the recently described phenomenon about CTCF motif orientation (Rao et al., 2014; Tang et al., 2015) in order to find the most probable, functional enhancer set for *Tgm2*. These approaches lead to the identification of five intergenic elements (+30kb, -7.9kb, -13kb, -20kb, -28kb) as potential regulators of the gene in dying thymocytes. The functional contribution of these elements to transcription regulation could be studied with the well-known, though sometimes artificial luciferase based reporter assays. However, if the cell type of interest is not a good subject for modification, such as the fast dying thymocytes, it is almost impossible to get reliable information about the function of a given enhancer element under the cell type specific setting. Several studies have recently described that activation of a signal specific transcription factor leads to a similar induction profile on the target genes as on the contributing enhancer elements at the nascent RNA level (Wang et al., 2011; Hah et al., 2013; Daniel et al., 2014b). As a result, eRNA production from an enhancer has been accepted as one if not the best marker for enhancer activation (Lam et al., 2014). Although, their function in transcription regulation is debated, more and more studies report eRNA specific functions in gene regulation (Lam et al., 2013; Li et al., 2013; Melo et al., 2013). In addition, the presence of eRNAs is indicative of loop formation between the enhancer and gene promoter. Thus, as a novel possible tool, so far not applied for clarification of the functional contribution of the individual enhancer elements to the regulation of a gene, we decided to determine the production of eRNAs (Kim et al., 2010) synthesized from active enhancers of the *Tgm2* gene in the presence of TGF-β, dbcAMP or retinoids. These measurements revealed three retinoid selective enhancers located +30kb, -7.9kb and -28kb respectively. Surprisingly, we found two distant enhancer elements located at -13kb and -20kb away from the gene, at which we detected eRNA transcription in

response to all of the above mentioned activators. Actually, these two regions show the highest DNase hypersensitive profile in agreement with their histone acetylation level, histone acetyltransferase enrichment determined by the presence of P300 and CBP, and the occupancy of the signal specific transcription factors in response to stimulation. In addition, combinations of 9cRA with dbcAMP or TGF-β showed a potentiated retinoid response on these enhancers. Furthermore, we were also able to show that the more distant -20kb enhancer interacts with the intronic- and 3'UTR-region of the gene using 3C approach. These data indicate that these enhancers might act as integrators, and might function also as shadow enhancers (Hong et al., 2008) in the regulation of the *Tgm2* gene expression (Fig. 27).
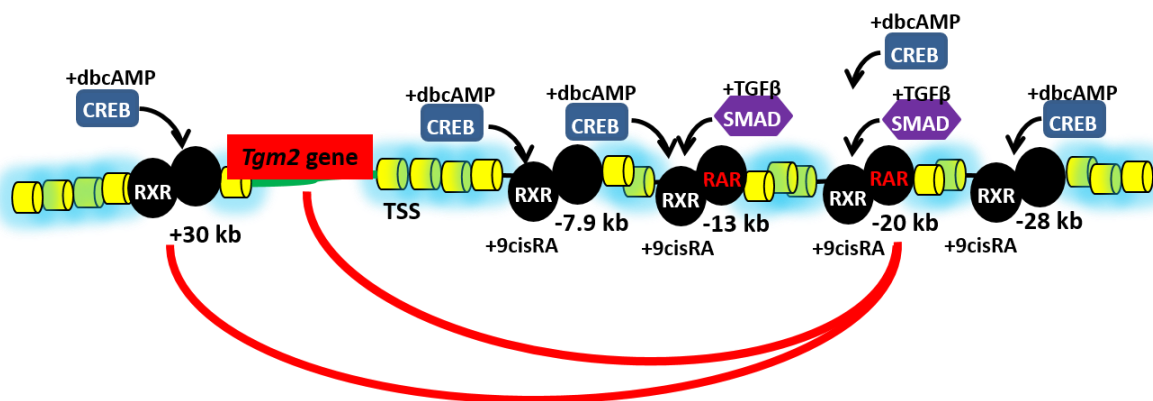
Figure 27. The revealed regulatory circuit on the Tgm2 locus in mouse apoptotic thymocytes.

In the case of TGF-β and retinoid synergism the enhanced eRNA transcription at these sites was attributable to more RXR, SMAD4, P300 and CBP recruitment in the presence of the two signals. Interestingly, though we detected a crosstalk between the adenylate cyclase and retinoid signaling pathways as well, this latter interaction did not result in enhanced CBP or P300 binding, despite the fact that an enhanced CREB binding was also detected. However, CREB works in concert not only with the coactivator CBP, but depending on the cellular context, also with a family of coactivators called CREB-regulated transcription coactivators (CRTCs) (Altarejos and Montminy, 2011). Of the CRTC family members, CRTC2 is thought to be the major mediator of regulated CREB target gene expression (Conkright et al., 2003) and is known to be expressed by T cells as well (Hernandez et al., 2015). Thus the crosstalk between the

adenylate cyclase and the retinoid signaling pathways might be mediated via proteins that we did not investigate in our study.

In summary, in the present study, we dissected the regulation of an apoptosis related gene in mouse thymocytes in a so far unprecedented manner. Our strategy to map and study the contribution of individual enhancers in the regulation of *Tgm2* serves as a proof of concept, thus can be applied to solve similar problems. However, we would like to emphasize that the usage of genome engineering is inevitable in order to support direct evidence about the contribution of a single enhancer element in gene regulation. Our results shed light on a very intriguing regulatory process at the level of individual enhancers and show that such enhancer clusters are able to integrate various signaling pathways, as it has been proposed recently by the super-enhancer concept (Hnisz et al., 2015). This theory suggests that members of the super-enhancers can physically and functionally interact with each other in order to fine tune the activity of the target gene. We think that the enhancers of *Tgm2* in thymocytes serve a similar function by enabling the dying cells to respond very precisely to the environmental signals in the thymus. Among these signals macrophage-derived TGF-β has been shown to contribute also to the differentiation of regulatory T cells which, as a result, depends on the constantly ongoing apoptosis and engulfment of the improperly produced thymocytes in the thymus after birth (Konkel et al., 2014).

6. SUMMARY

Apoptosis is a physiological process, critical for the elimination of cell types that are not needed or represent threat to the body. Day by day, billions of cells undergo apoptosis and cleared by professional phagocytes, without any inflammatory response. An important component of the cell death program is transglutaminase 2 (TGM2), which is a multifunctional enzyme, critical for proper apoptotic cell recognition and clearance and it also prevents the release of harmful, cytotoxic cell contents via its protein crosslinking activities. The enzyme is highly upregulated during T cell apoptosis in the thymus. The mouse thymus is very active apoptotically, because above 90% of the T cells die in the thymus during their maturation and selection. Based on this, mouse thymus-derived apoptotic thymocytes represent an excellent model system for studying the regulation of the enzyme by the molecules present in the thymic milieu *in vivo*.

In the first part of my thesis, I present how thymus-specific molecules, known to be secreted by macrophages, for example TGF-β, adenosine and retinoids contribute to the upregulation of TGM2 at the gene expression level. We found that adenosine work in concert with TGF-β and retinoic acid to significantly enhance the level of *Tgm2* in dying thymocytes *in vitro*. Moreover, adenosine acts via the adenosine A2A receptor – adenylate cyclase pathway and contributes to the upregulation of TGM2 *in vivo*. We also demonstrate that adenosine is produced extracellularly during the clearance of apoptotic thymocytes, partly from adenine nucleotides released by pannexin-1 channels, revealing a novel crosstalk between macrophages and apoptotic cells.

In the second part of my work, I present how thymus-specific mediators regulate the expression of *Tgm2* using different intergenic regulator elements. Due to technical limitation, so far only the promoter region of the gene has been investigated in detail with the mostly artificial reporter assays. The role of intergenic regulatory elements remained elusive in the context of TGM2 in thymocytes. We overcome this limitation by exploiting genome-wide datasets of ChIP-seq and DNase-seq from the ENCODE consortium. We revealed the putative regulatory elements of the gene by the combination of these technologies and identified the ones that might be functionally important for gene regulation, measuring enhancer-specific RNA transcripts (eRNAs) by mimicking the activation of TGF-β, retinoic acid and adenosine triggered signaling pathways. Our study describes a novel strategy to identify and characterize the signal-specific functional enhancer set of a gene by integrating genome-wide datasets and measuring the production of eRNA molecules.

7. ÖSSZEFOGLALÁS

Az apoptózis egy fiziológiás folyamat, kritikus a feleslegessé vált, vagy veszélyt jelentő sejtek eliminálásában. Nap mint nap több milliárd sejt hal el apoptózissal és takarítódik el a professzionális fagociták által, anélkül, hogy gyulladás alakulna ki. A sejthalál program egyik fontos résztvevője a transzglutamináz 2 (TGM2), amely egy multifunkcionális enzim, kritikus szerepet játszik az apoptótikus sejtek megfelelő felismerésében, eltakarításában, továbbá megakadályozza a gyulladáskeltő sejttartalom kijutását. Az enzim kifejetődése erősen megnövekszik a T-sejtek apoptózisa során a tímuszban. Az egér tímusz apoptótikusan meglehetősen aktív, mivel a sejtek több mint 90%-a elhal érésük és differenciálódásuk során. Emiatt az egér tímusz eredetű apoptótikus timociták kiváló modellrendszert nyújtanak a TGM2 enzim szabályozásának tanulmányozásához olyan molekulák részvételével, amelyek jelen vannak a timikus környezetben *in vivo*.

A disszertáció első részében azt vizsgáltam, hogy a tímuszban jelen lévő, makrofágok által szekretált molekulák, mint például a TGFβ, adenozin és retinoidok hogyan járulnak hozzá a TGM2 kifejeződéséhez génexpressziós szinten. Azt találtuk, hogy az adenozin együttműködik a TGFβ és a retinsav által aktivált jelátviteli utakkal, ezáltal szignifikánsan növeli a *Tgm2* gén kifejeződését az elhaló timocitákban *in vitro*. Továbbá, az adenozin az adenozin A2A recetor – adenilát cikláz útvonalon keresztül fejti ki hatását, hozzájárul a TGM2 gén és fehérje szintű kifejeződéséhez *in vivo*. Azt is megmutattuk, hogy az adenozin exrtacellulárisan keletkezik az apoptótikus timociták eltakarítása során, részben adenin nukleotidokból, amelyek a pannexin-1 csatornákon keresztül jutnak ki, mely egy újfajta együttműködésre világít rá a makrofágok és az apoptótikus sejtek között.

A munkám második részében bemutattam, hogy a tímuszban jelenlévő mediátorok hogyan regulálják a Tgm2 gén kifejeződését különböző intergenikus regulátor elemeken. Az intergenikus szabályozó elemek szerepe eddig tisztzatlan volt a Tgm2 esetében a timocitákban. Hogy túllépjünk a technikai korlátokon, az ENCODE konzorcium által generált teljes genom szintű ChIP-seq és DNase-seq adatokat használtunk, melyek kombinálásával feltártuk a gén lehetséges regulátor elemeit illetve enhanszer-specifikus RNS transzkriptek (eRNS) mérésével azonosítottuk azokat, amelyek funkcionálisan fontosak lehetnek a gén regulációja szempontjából. Munkánk egy újfajta stratégiát nyújt arra, hogyan azonosítsuk és karakterizáljuk egy gén szignál specifikus, funkcionális enhanszer készletét, teljes genom szintű adatokat használva és eRNS moleulák produkcióját mérve.

8. LIST OF KEYWORDS

Apoptosis; Transglutaminase 2; Adenosine; T cells; Macrophages; Phagocytosis; Gene regulation; ChIP-seq; Enhancer RNA;

9. KULCSSZAVAK

Apoptózis; Transzglutamináz 2; Adenozin; T-sejtek; Makrofágok; Fagocitózis; Génszabályozás; ChIP-seq; Enhanszer RNS

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List of publications related to the dissertation

1. **Sándor, K.**, Dániel, B., Kiss, B., Kovács, F., Szondy, Z.: Transcriptional control of transglutaminase 2 expression in mouse apoptotic thymocytes.
   Biochim. Biophys. Acta, Gene Reg. Mechan. "Accepted by Publisher" (2016)
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2. **Sándor, K.**, Pallai, A., Duró, E., Legendre, P., Couillin, I., Sághy, T., Szondy, Z.: Adenosine produced from adenine nucleotides through an interaction between apoptotic cells and engulfing macrophages contributes to the appearance of transglutaminase 2 in dying thymocytes.
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List of other publications

3. Hsieh, Y., Liu, G., Lee, Y., Yang, J., **Sándor, K.**, Sarang, Z., Bononi, A., Pinton, P., Tretter, L., Szondy, Z., Tsay, G.J.: Transglutaminase 2 Contributes to Apoptosis Induction in Jurkat T Cells by Modulating Ca2+ Homeostasis via Cross-Linking RAP1GDS1.
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