Deciphering Splicing Regulation for TRAF3 Exon 8 Alternative Splicing – CELF2 and hnRNP C Regulate TRAF3 Exon 8 Skipping Upon T Cell Activation

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Science is endless fascination

Abstract

T cell activation is accompanied by dramatic changes in gene expression which mediate changes in functionality. These alterations in gene expression depend not only on transcriptional changes but also on alternative splicing. One of the inducible splicing events regulating T cell activation occurs in the TRAF3 gene in which exon 8 skipping increases upon T cell activation. It has already been shown that the full length TRAF3 isoform inhibits the non-canonical NFκB pathway while the short isoform activates this pathway and helps to trigger an immune response.

Here, the regulation of TRAF3 exon 8 splicing upon T cell activation was investigated. The *cis*-acting element was identified with minigenes. It is a sequence of 96 bp, which is located 336 bp upstream of TRAF3 exon 8. An siRNA screen and UV-Crosslink immunoprecipitation experiments identified CELF2 and hnRNP C as two *trans*-acting factors. UV-Crosslink assays confirmed a direct binding of both proteins to the *cis*-acting. While binding is antagonistic, both proteins function synergistic in inducing TRAF3 exon 8 skipping. TRAF3 exon 8 splicing induction is cell type-specific since it is missing in Hek cells and human B cells. These cell lines reveal that CELF2 is the driving force because its expression correlates with skipping induction. Ramos and Hek cells do not express CELF2 endogenously and do not induce exon 8 skipping but overexpression of exogenous CELF2 induces exon skipping. In contrast, hnRNP C is needed for exon 8 skipping but its expression is not sufficient.

Minigene experiments addressing a putative role of the *cis*-acting element to the TRAF3 exon 8 revealed that the distance is crucial for the strength of splicing induction. Changing the distance decreases TRAF3ΔE8 formation. The data proposed the following model: In resting T cells, hnRNP C is highly expressed and binds to the *cis*-acting element. In activated T cells, hnRNP C expression decreases. CELF2 expression increases and binds additionally to the *cis*-acting element. Binding of both proteins to the *cis*-acting element induce TRAF3 exon 8 skipping.

Furthermore TRAF3 splicing was investigated in the mouse T cell line El4. TRAF3 exon 8 alternative splicing is conserved between both species in resting T cells. In contrast, the splicing-induction of exon 8 skipping upon T cell activation is species-specific because it occurs in human but not in murine T cells. The complete regulatory mechanism needs further investigation but minigene experiments pointed to a *trans*-driven regulation. This is unusual because most species-specific splicing events are *cis*-driven regulated.

Abstract – German Translation

Es konnte bereits gezeigt werden, dass sowohl Änderungen der Transkription als auch das alternative Spleißen eine wichtige Rolle in der Umgestaltung zellulärer Prozesse während der T-Zellaktivierung darstellen. Eines der Gene, dessen alternatives Spleißen sich verändert, heißt TRAF3. Im Gegensatz zu ruhenden T-Zellen, die hauptsächlich die volle Länge TRAF3 Isoform (TRAF3vl) exprimieren, wird in aktivierten T-Zellen TRAF3 Exon 8, zu einem gewissen Prozentsatz, aus der mRNA ausgeschlossen (TRAF3ΔE8). Beide TRAF3 Isoformen übernehmen unterschiedliche Funktionen in T-Zellen. Während TRAF3vl den NFκB-Signalweg in ruhenden T-Zellen inhibiert und somit zur Verhinderung einer Immunabwehr beiträgt, aktiviert TRAF3ΔE8 diesen Signalweg. Obwohl die Funktion beider Isoformen bekannt war, war unklar wie diese Induktion von TRAF3Δ8 reguliert wird. Die Aufklärung des regulatorischen Mechanismus war Gegenstand dieser Arbeit.

Mit Hilfe von Minigen-Experimenten konnte eine Sequenz von 96 Basenpaaren identifiziert werden, die für die Regulation wichtig sind (ein sogenanntes „*cis*-acting element“). Diese Sequenz liegt 336 Basenpaare strangaufwärts von dem alternativ gespleißten Exon 8.
Eine Kombination aus siRNA-Screening und UV-Quervernetzungsversuchen, die an eine Immunpräzipitation gekoppelt waren, hat zwei Proteine identifiziert, die direkt an das *cis*-acting element binden und das Spleißen von TRAF3 regulieren. Sie heißen CELF2 und hnRNP C („*trans*-acting factors“). Die Versuche haben gezeigt, dass CELF2 und hnRNP C antagonistisch binden. Allerdings regulieren sie beide synergistisch, indem sie einen Exon 8 Ausschluss induzieren. Ein Vergleich von TRAF3 Spleißen in T-Zellen mit dem Spleißen in B-Zellen ergab, dass CELF2 die treibende Kraft für den Exon 8 Ausschluss ist. Dies wurde dadurch gezeigt, dass diese Zellen nach Aktivierung keinen Anstieg in der TRAF3ΔE8 Expression zeigen und das korreliert mit einer fehlenden endogenen CELF2 Expression. Das Einbringen eines CELF2-Plasmides in die B-Zellen konnte TRAF3ΔE8 Produktion induzieren. Im Gegensatz dazu exprimieren beide Zelllinien hnRNP C, dessen Expression nötig, aber nicht ausreichend für den Exon 8 Ausschluss ist.
Zusätzlich konnte gezeigt werden, dass die Position des *cis*-acting elements entscheidend ist für die Induktion von TRAF3ΔE8 ist. In Minigen-Versuchen, in denen die Position des *cis*-acting elements variierte, war die Fähigkeit das Exon 8 auszuschließen deutlich reduziert. Diese Positionsabhängigkeit und die Versuche, die eine Beteiligung von CELF2 und hnRNP C an der Regulation zeigten, führten zum Vorschlag des folgenden Modells: In ruhenden, humanen T-

Zellen wird CELF2 nur schwach exprimiert, während hnRNP C in großen Mengen vorhanden ist. In aktivierten T-Zellen, sinkt die hnRNP C Expression. Zusätzlich steigt die Expression an CELF2, das ebenfalls an das *cis*-acting element bindet. Die Bindung beider Proteine an das *cis*-acting element induzieren einen Ausschluss von Exon 8.

Des Weiteren wurde TRAF3-Exon 8-Spleißen in der Maus-T-Zelllinie El4 analysiert. Interessanterweise ist das Spleißmuster in ruhendem Zustand zwischen humanen und Maus-T-Zellen konserviert, jedoch ist die Induktion von TRAF3ΔE8 nach T-Zellaktivierung spezifisch für humane Zellen. Häufig wurde als Auslöser für eine spezies-spezifische Regulation eine Sequenzveränderung im *cis*-acting element beobachtet. Jedoch deuten die durchgeführten Minigen-Versuche darauf hin, dass in der Regulation von TRAF3-Exon 8-Spleißen die Expression von Proteinen (die *trans*-acting factors) ausschlaggebend ist.

Table of Content

1 Introduction

“Spliced Segments at the 5’ Terminus of Adenovirus 2 Late mRNA”

“An Amazing Sequence Arrangement at the 5’ Ends of Adenovirus 2 Messenger RNA”

These two citations are the titles of two publications printed in 1977 by the group of Phillip A. Sharp (Berget et al. 1977) and Richard J. Roberts (Chow et al. 1977). Both groups discovered independently that genes contain non-coding segments. This was at the time a scientific sensation and resulted in the award of a Noble Prize in Physiology or Medicine in 1993 (Nobel prize.org). This finding opened up a completely new field of research called RNA splicing that is still a research focus nowadays.

In the beginning it needs to be clarified where and when RNA splicing occurs. All the genetic information is stored in the DNA which is located in the nucleus. Several steps are essential to transform this information into proteins (Figure 1.1). It is known as the central dogma of molecular biology. In the first step the DNA is transcribed into a resultant pre-mRNA which contains the genetic information of a single gene. In the second step pre-mRNA is processed before it is exported into the cytoplasm. The processing comprises several steps: the addition of a 5’-Cap and a 3’-poly(A) tail as well as RNA splicing. The mature messenger RNA (mRNA) is then exported into the cytoplasm, where ribosomes translate the information into a protein. RNA-splicing will be the focus of this study.

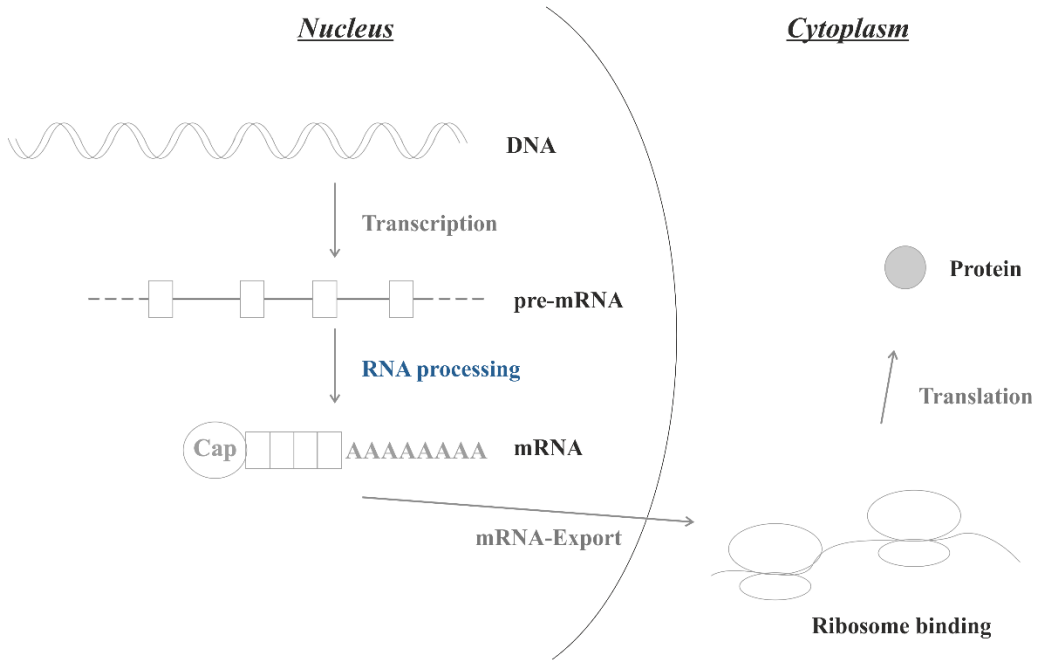

Figure 1.1: From DNA to protein.
The genetic information is stored in the DNA located in the nucleus. According to the central dogma of molecular biology the DNA is transcribed into a pre-mRNA. It then follows several processing steps including splicing. The mature mRNA is exported into the cytoplasm where it is translated into a protein.

1.1 Splicing, a Step In mRNA Processing

Splicing takes place in the nucleus and is an important step in RNA processing. Splicing is indispensable in higher eukaryotes because their pre-mRNAs contain non-coding regions (introns). Those introns need to be excised so that only the coding regions (exons) remain. This was first observed in 1977 (Berget et al. 1977; Chow et al. 1977). Introns in mammals are 10 to 100 fold longer than exons (Sakharkar et al. 2004) with an average size of around 5000 nucleotides (nt) (Matera and Wang 2014). Exons have an average length of around 145 nt (Kornblihtt et al. 2009). Therefore the spliceosome must recognize short exons between very long introns. Some additional short intronic regions are required to solve this problem: the 5'splice site (5'ss), the 3'splice site (3'ss) and the branch point (BP), which are all conserved nucleotides within introns (Figure 1.2). They all provide binding sites for the assembly of the spliceosome. The spliceosome is a large enzyme complex which catalyzes the splicing reaction (Figure 1.4). Two recognition mechanisms are conceivable: recognition of the long introns or the shorter exons. Mutational analyses lead to the conclusion that exon definition is the predominant

mechanism used in mammalian cells (Berget 1995). The recognition is done in the following way: U1 small nuclear ribonucleoproteins (snRNPs, complexes consisting of RNA and proteins) bind to the 5'ss. The splicing factor SF1 interacts with the branch point while U2AF, another splicing factor, associates with the polypyrimidine tract and the 3'ss (Wahl et al. 2009). The polypyrimidine tract is located between the branch point and the 3'ss. U2 snRNP then displaces SF1 and binds to the 3'ss (De Conti et al. 2013). U2 of intron B starts crosstalking with U1 from intron C across the exon and thereby they define the exon boundaries. Proteins that bind to the exon can additionally mediate this crosstalk (Berget 1995) (Figure 1.2).

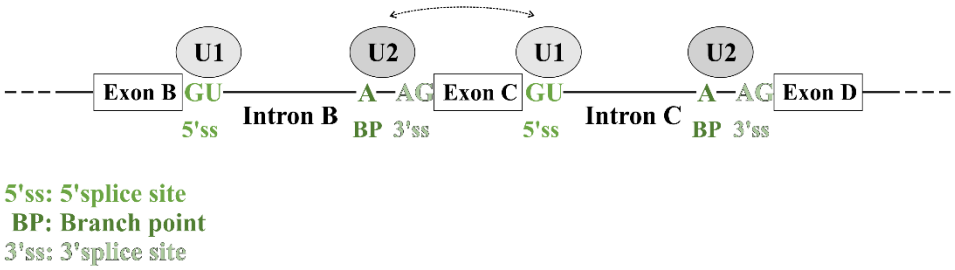

Figure 1.2: Exon definition.
The scheme shows a pre-mRNA: exons are shown in boxes and lines represent introns. The 5'splice site (5'ss), the branch point (BP) and the 3'splice sites (3'ss) are highlighted in several green shades as well as the nucleotides they refer to. These conserved sequence elements support the spliceosome assembly. In the exon definition process U1 snRNPs bind to 5'ss and U2 snRNPs recognize the BP as well as the 3'ss. U1 and U2 then communicate across the exon and define the exon boundaries.

Sharma et al. (2008) could show that exon definition is only the initial step in recognition. This exon definition state has then to be converted into an intron recognition state to allow spliceosome assembly. The spliceosome consists of five different snRNP subunits, namely U1, U2, U4, U5 and U6 as well as many associated proteins. The reaction catalyzed by this enzyme is a two-step reaction. The introns are removed from the pre-mRNA and then the exons are juxtaposed. Both reactions are transesterifications (Figure 1.3). First the 2'-hydroxyl-group of the branch point adenosine attacks the phosphodiester bond between the 5' exon and the intron. This leads to the formation of a free 5' exon and a lariat structure. Second the 3'-hydroxyl-group of the 5' exon attacks the phosphodiester bond between the intron and the 3' exon resulting in lariat excision and exon ligation (Papasaikas and Valcárcel 2016).

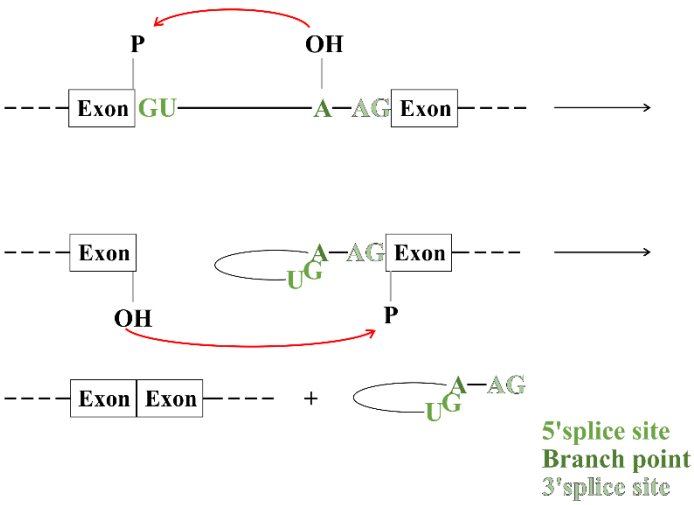

Figure 1.3: Two transesterification reactions excise an intron.
The hydroxyl group of the branch point attacks the phosphodiester bond in the upstream exon-intron boundary. This leads to the formation of a lariat structure. In the next transesterification reaction, the hydroxyl group of the free exon attacks the phosphodiester bond between the intron and the downstream exon. Both exons are ligated and the lariat structure is released.

The assembly of the spliceosome is complex and is a step-wise process as depicted in Figure 1.4. U1 recognizes the 5'ss, SF1 binds to the BP and the U2 snRNP auxiliary factor (U2AF) associates with the 3'ss, forming the E-complex. Then, the U2 snRNA base pairs with the BP of the pre-mRNA under ATP consumption and releases SF1, resulting in the A-complex. U4, U5 and U6 form a tri-snRNP and bind together to the already existing A-complex leading to the precatalytic spliceosomal complex (B-complex). The B-complex is still catalytically inactive and needs further remodeling. U1 and U4 are released and the activated spliceosome (B*-complex) is formed. This complex catalyzes the first transesterification and causes the formation of the C-complex. The C-complex undergoes internal remodeling and finally excises the intron and ligates the exons. Afterwards the post-spliceosomal complex dissociates from the spliced mRNA and the snRNPs are recycled in order to undergo a new assembly. (Wahl et al. 2009)

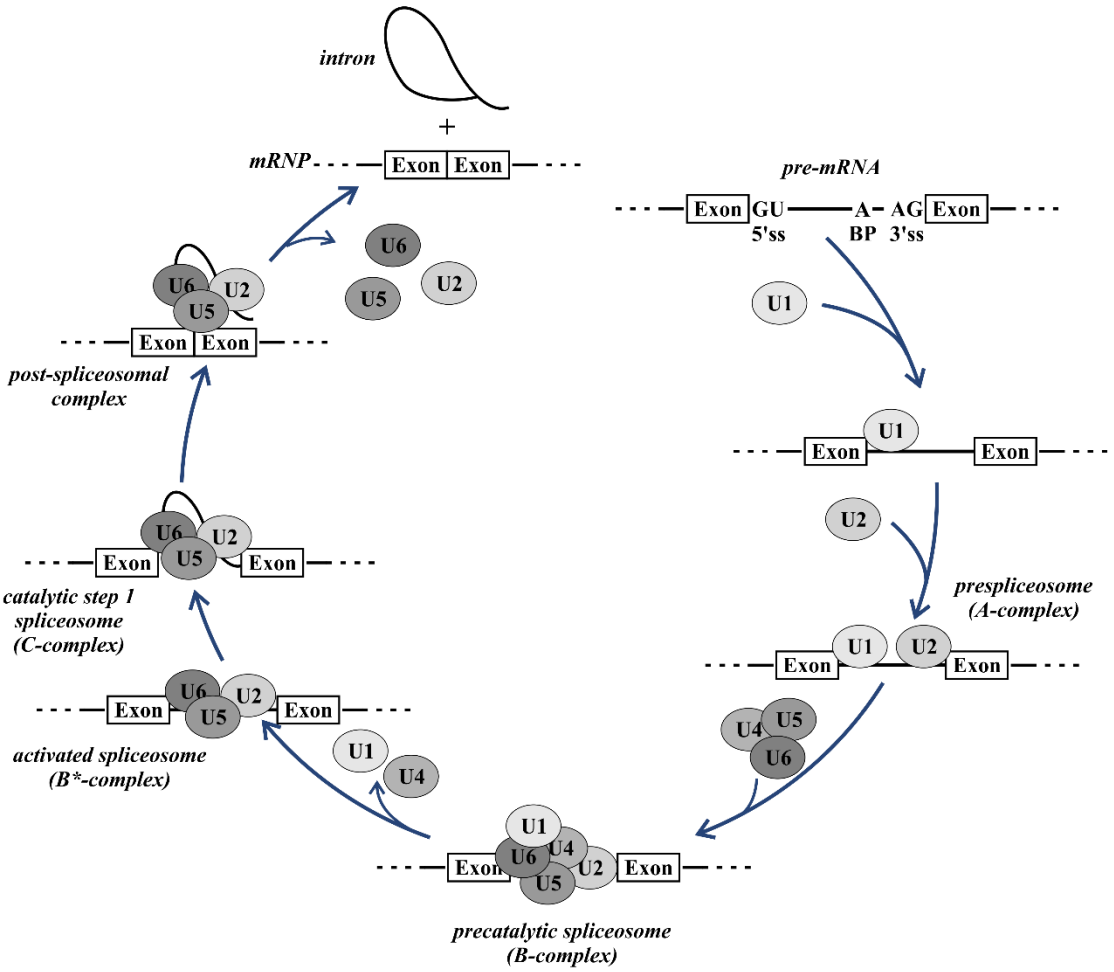

Figure 1.4: Spliceosome assembly.
The spliceosome assembles in several steps. First, U1 and U2 define the intron-exon boundaries and then the other snRNPs are recruited to the pre-mRNA. After several remodeling steps the catalytically active spliceosome is formed. It catalyzes the two transesterifications. Finally the snRNPs dissociate and leave behind the ligated exons and the intron lariat. (adapted from Wahl et al. 2009)

Exons span 1.1 % of the human genome whereas introns represent 24 %. The rest is intergenic DNA (Venter et al. 2001). Splicing is an essential mechanism to eliminate introns from the pre-mRNA. Notably the transcription of the human dystrophin gene takes 16 h of which 15 h and 54 min are needed for the synthesis of the introns (Papasaikas and Valcárcel 2016). This is an extreme example because in average the elongation of a single gene takes around 7 min. This is based on the finding that the average size of a human gene is 27 kbp (Venter et al. 2001) and elongation rate is around 3.8 kb/min (Ardehali and Lis 2009). It raises the question of how necessary are introns.

Introns do fulfill certain crucial functions. They form the basis for alternative splicing (chapter 1.2) and they are evolutionarily powerful. They contain cryptic splice sites, meaning sequences with high similarity to actual splice sites. Mutations in these sites can produce exonization of intronic sequences resulting in new exons. These new exons are included in minor amounts due to alternative splicing, and allow a co-existence with the original transcript. If the new exon leads to an evolutionary advantage mutations which will increase the splice site strength and the inclusion frequency will be favored over time (Sorek 2007).

1.2 Alternative Splicing

The genome of *Drosophila melanogaster* comprises approximately 14,000 protein-coding genes, the genome of *Caenorhabditis elegans* around 20,000 and the genome of mammals around 20,000 protein-coding genes (Nilsen and Graveley 2010). Although the complexity of these organisms differs widely, the number of protein-coding genes is very similar. The higher complexity can be yielded by a mechanism called alternative splicing. Alternative splicing can generate numerous transcripts from a single gene. The generated isoforms can have subtle or opposing functions. An extreme case of alternative splicing is found in *Drosophila melanogaster,* where the *DSCAM* gene is alternatively spliced and can generate more isoforms than the gene number of the entire genome (Graveley 2001). It is a cell surface protein that is involved in axon guidance in the brain during development and can be spliced into 38,016 isoforms (Matlin et al. 2005). This vast number of transcripts can only be obtained using alternative splicing. Different forms of alternative splicing exist (Figure 1.5).

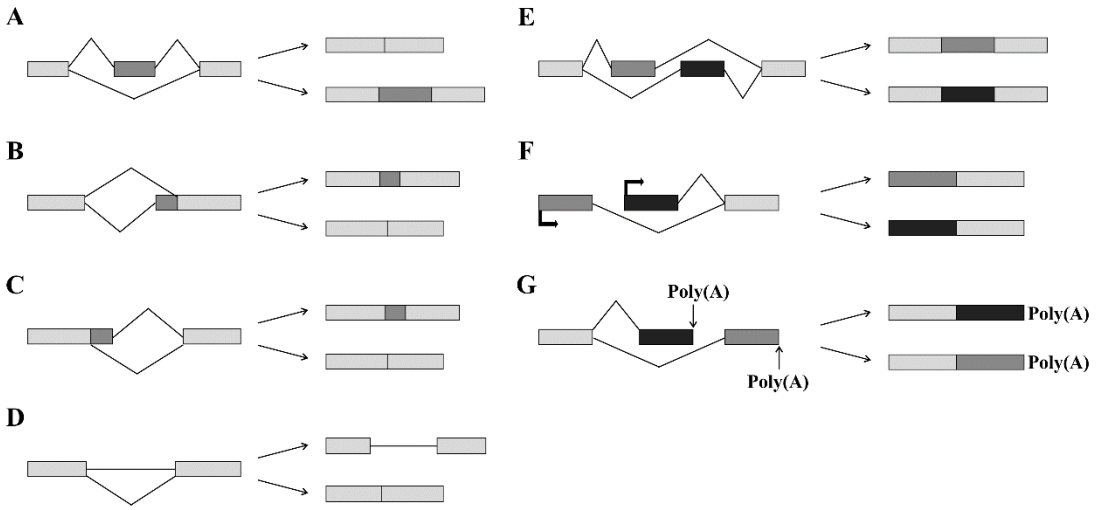

Figure 1.5: Mechanisms to generate multiple isoforms.
A: Exclusion or in inclusion of alternative exons
B: Alternative 3'ss
C: Alternative 5'ss
D: Intron retention
E: Mutually exclusive exon usage
F: Alternative promoter usage
G: Alternative poly(A)-site usage
(modified from Black 2003)

The most common alternative splicing pattern is the exclusion or inclusion of a single cassette exon (Figure 1.5A). This occurs in around 40 % of all alternative splicing events. There are splicing events known in which alternative 3'ss (18.4 %) (Figure 1.5B) or alternative 5'ss are used (7.9 %) (Figure 1.5C). Additionally introns can be included in the mature mRNA. This happens in less than 5 % of the alternative splicing events. (Keren et al. 2010)

Another mechanism is called mutually exclusive exons. Here only one of two or more exons are included in the mRNA but never both at the same time (Figure 1.5E). Finally alternative promoters (Figure 1.5F) as well as the usage of alternative poly(A) sites (Figure 1.5G) can result in different isoforms. Importantly, not only one type of alternative splicing can occur in a single transcript but combinations.

Next generation sequencing data indicate that around 95 % of human pre-mRNAs of multi-exon genes undergo alternative splicing (Nilsen and Graveley 2010). This emphasizes how important the mechanism is to expansion of the proteome. Alternative splicing is known to be an important switch in gene expression and influences various cellular and developmental processes like sex determination, apoptosis, axon guidance,

cell excitation and contraction (Black 2003). The importance of alternative splicing was further emphasized by a study demonstrating that isoforms generated by alternative splicing have different interaction profiles and share less than 50 % of their interaction partners (Yang et al. 2016).

Conclusively, alternative splicing has a high impact on many cellular processes and tight regulation is required to prevent malfunctions. The importance of proper regulation is emphasized by analysis of mutations that destroy functional splice sites or generate new ones. Such changes are responsible for 15 % of human genetic diseases (Smith and Valcárcel 2000).

1.3 Regulation of Alternative Splicing

As in constitutive splicing, alternatively spliced exons are flanked by 3' and 5'splice sites. These alternative splice sites are often weaker than sites from constitutive exons. The exon length can be suboptimal which complicates the exon definition process (Berget 1995). There are mechanisms which help the spliceosome to recognize these weak splice sites. This is done by *cis*-acting elements and *trans*-acting factors (chapter 1.3.2). 80 % of transcripts are known to be spliced co-transcriptionally. This indicates that all factors regulating transcription can also have an impact on alternative splicing (chapter 1.3.1).

1.3.1 Regulation by coupling splicing with transcription

The assumption that splicing takes place co-transcriptionally is based on two different observations. First splicing patterns of genes spliced *in vivo* with an ongoing transcription can differ from those spliced *in vitro*. Second the splicing outcome depends on the RNA polymerase II promoter (Naftelberg et al. 2015). Furthermore analysis of chromatin-associated RNAs showed that most human splicing events take place co-transcriptionally (Pandya-Jones and Black 2009; Tilgner et al. 2012). Consequently elements and factors regulating transcription can also have an influence on splicing, e.g. the promoter, transcription factors or chromatin remodeling complexes. Here the focus will be on histones, their modifications and the effect of the RNA polymerase II (RNAP II) elongation rate.

Transcription is regulated by the accessibility of the DNA to RNAP II which depends on nucleosome occupancy. A nucleosome is an octamer consisting of the four core histones

H2A, H2B, H3 and H4 that wraps 147 base pairs (bp) of DNA around itself (Naftelberg et al. 2015). These 147 bp are very close to the average length of an exon and indeed nucleosomes are preferentially positioned in exons (Andersson et al. 2009). The strength of binding between histones and DNA is regulated by histone modifications. The best-investigated modifications are acetylation and methylation. Histone acetylation leads to a decrease in positive charge of the histones and results in the formation of a more open chromatin structure. In this way the DNA is more accessible to the RNAP II. Histone methylation does not change the charge of the histones but can recruit remodeling complexes that change the accessibility of DNA to RNAP II (Bannister and Kouzarides 2011). Both modifications influence transcription and this affects constitutive and alternative splicing (Luco et al. 2010). Luco et al. (2010) provided first connections between both processes by directly investigating the impact of histone modifications on alternative splicing. By focusing on the *FGFR2* gene whose exons IIIb and IIIc are mutually exclusive and whose inclusion is cell type-specific regulated, it was demonstrated that depending on which histone methylations are enriched one exon is included or excluded. In explanation of how histone modifications influence the splicing outcome the first chromatin-adaptor complex consisting of MRG15 and PTB was proposed. MRG15 recognizes H3K36me3 and additionally recruits the splicing repressor PTB to the transcript. This results in exon exclusion (Luco et al. 2010). In subsequent studies more proteins that read histone modifications and translate the signal to downstream splicing events were found (Luco et al. 2011; Naftelberg et al. 2015).

Another important regulator of transcription is the RNAP II itself. Two models explain how RNAP II can regulate alternative splicing: recruitment coupling and kinetic coupling. In the recruitment coupling model the C-terminal domain of RNAP II recruits splicing factors to the transcript and these favor or prevent the usage of splice sites. The kinetic coupling assumes that the elongation rate determines the splice site choice. A slow elongation rate favors the usage of weak (alternative) splice sites because the spliceosome has more time to recognize them. A fast elongation rate results in alternative exon skipping because the strong splice sites are preferentially used. (Naftelberg et al. 2015)

1.3.2 Regulation by *cis*-acting elements and *trans*-acting factors

Another layer of regulation is the regulation by elements present in the transcript itself or factors that bind to it. Sequences that regulate spliceosome assembly are called *cis*-acting

elements and can be divided in two groups: sequences which improve splice site recognition called enhancers and sequences which block recognition named silencers. Depending on their position within the mRNA they are termed intronic or exonic. In total there are four different *cis*-acting elements: intronic splicing enhancer (ISE), intronic splicing silencer (ISS), exonic splicing enhancer (ESE) and exonic splicing silencer (ESS) (Black 2003). These *cis*-acting elements help to define the splice site by providing a platform for proteins involved in regulation (De Conti et al. 2013). These proteins are called *trans*-acting factors and can be divided into several groups such as SR proteins, hnRNPs and tissue-specific RNA binding proteins (Lee and Rio 2015).

The human SR protein family comprises 12 members that are preferentially located in the nucleus. They all have an RNA binding domain at their N-terminus, and a serine-arginine-rich (RS) domain at the C-terminus which is a platform for protein-protein and for RNA-protein interactions (Jeong 2017). Proteins of this family are involved in the recognition of the splice sites in constitutive as well as in alternative splicing (House and Lynch 2008) because they bind favorably to ESE elements. SR protein binding supports spliceosome assembly by recruiting U1 snRNP to the 5'ss and assists U2 snRNP association to the 3'ss as well as binding of the U2AF complex (Black 2003). The family members sometimes bind to introns and can then act as repressors or enhancers of spliceosome assembly depending on the position of binding. SR protein function depends not only on the position of binding but also on the phosphorylation state of the RS domain (Jeong 2017) and on the binding of interaction partners to this domain (Chen and Manley 2009).

The heterogeneous ribonucleoprotein (hnRNP) family is a very heterogeneous protein family containing 20 core members. Their structural features are an RNA binding domain and auxiliary domains that regulate RNA binding (Han et al. 2010). Since these proteins often prevent spliceosome assembly they are in general known as silencing factors. They bind to ESS and ISS apart from a few exceptions. Binding of hnRNPs to introns result in positive or negative regulation, e.g. the binding in proximity to intron boundaries was shown to activate splicing by bringing together both intronic ends (Chen and Manley 2009). Spliceosome assembly is mostly prevented by steric hindrance. The binding next to a splice site can block binding of spliceosomal components or the association of enhancers. Additionally hnRNPs can act from larger distances by multimerization along the RNA (Chen and Manley 2009). The function of hnRNPs depends on their post-translational modifications and their localization. Phosphorylation, methylation,

ubiquitylation and sumoylation can modify hnRNPs. HnRNPs mainly have a nuclear localization but can shuttle between nucleus and cytoplasm (Geuens et al. 2016).

The third group of *trans*-acting factors consist of the remaining RNA binding proteins that do not belong to the SR or hnRNP protein family. Most of them are known to regulate cell or tissue-specific splicing (chapter 1.4). Some well-studied examples are NOVA, the Rbfox family or the muscleblind/CELF family (Lee and Rio 2015).

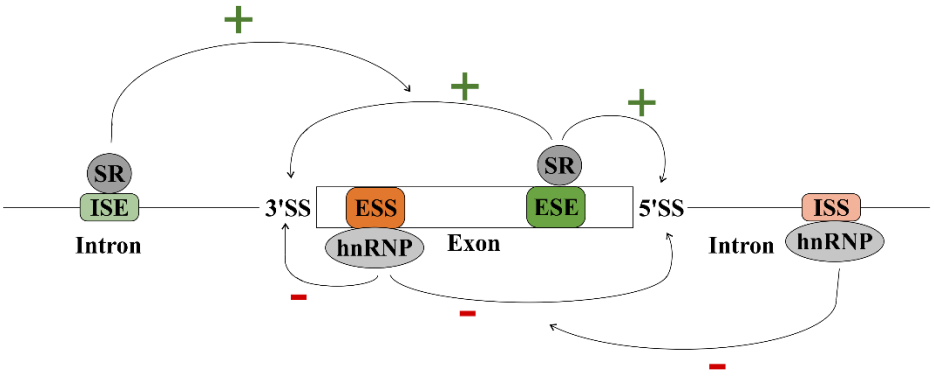

Figure 1.6: Splicing is regulated by a combination of regulators.
RNA sequences that regulate alternative splicing are called *cis*-acting elements. These are divided in four groups: exonic splicing enhancer (ESE), exonic splicing silencer (ESS), intronic splicing enhancer (ISE) and intronic splicing silencer (ISS). *Trans*-acting factors bind these elements. In general SR proteins are splicing enhancers whereas hnRNPs are inhibitors. The combination of all involved elements determines the final splice pattern. (modified from Lee and Rio 2015)

The splicing process is often regulated by more than one *cis*-acting element and/or several *trans*-acting factors. The balance of different factors determines the splicing outcome as indicated in Figure 1.6 (Fu and Jr 2014). The multitude of factors can lead to either competition or cooperation. If there is a competition between RNA binding proteins, the splice outcome is regulated by the concentration, the localization and the activity of the competitors. Such a competition was shown for hnRNP C and U2AF65 which compete for binding to cryptic splice sites resulting in the inclusion or exclusion of transposable elements (Zarnack et al. 2013). In contrast if several proteins work together they can form inhibitor- or activator-complexes. It is known that binding of SR proteins to ESEs is weak and can be easily prevented or enhanced by other proteins (House and Lynch 2008).

The regulatory feature can be position-dependent, meaning that binding to introns can result in differential splice patterns compared to binding of the same protein to exonic elements. For example the SR proteins promote splicing if they bind to exons but repress

it if they bind to intronic regions (Sahebi et al. 2016). Another example for position-dependency is CELF2 which was shown to induce exon skipping if it binds upstream of the alternative exon but leads to exon inclusion if it binds downstream (Ajith et al. 2016).

1.3.3 Regulation by RNA structures

RNA is often presented as line. This is highly simplified since it is well-known that RNAs form secondary structures. These secondary structures also impact splicing and have regulatory effects. Bioinformatic analyses focusing on RNA secondary structures of intron-exon junctions indicate a correlation between RNA secondary structure and splicing (Shepard and Hertel 2008; McManus and Graveley 2011). Shepard and Hertel (2008) found more RNA structures associated with alternative than with constitutive exons. These bioinformatic data are supported by several *in vivo* examples confirming this regulatory splicing mechanism. A more recent study found that only 15-40 % of RNA binding sites predicted *in vitro* are bound *in vivo* (Taliaferro et al. 2016). According to their findings an important regulator for this is RNA structure.

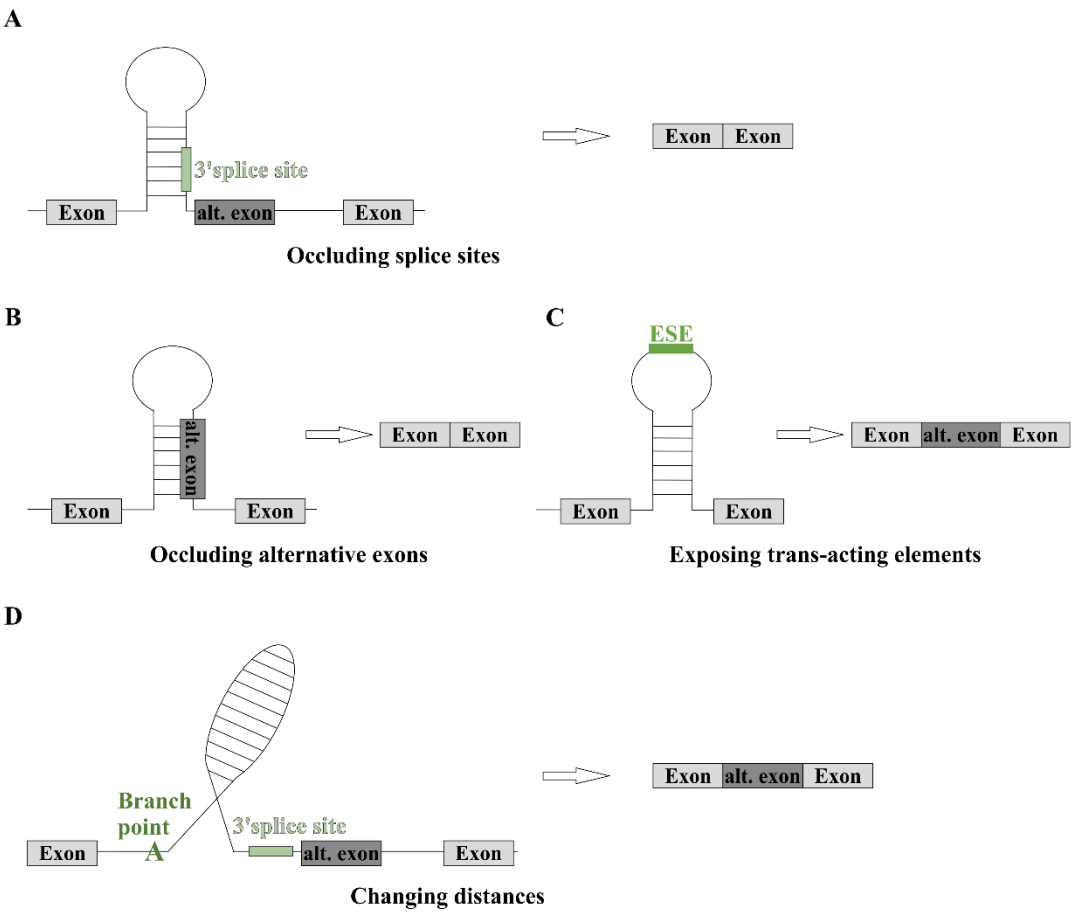

Figure 1.7: RNA structures regulate alternative splicing.
A/B: The formation of stem loops can occlude splice sites or mutually exclusive exons.
C: Stem loop formation can enhance splicing by exposing enhancers to the spliceosome.
D: RNA secondary structures can bring together regulatory elements which otherwise would be far apart.

The mechanism of a regulation by RNA structure works in the following way. *Trans*-acting factors which regulate splice site recognition and spliceosome assembly are known to bind to single stranded RNA (De Conti et al. 2013). Consequently RNA secondary structures can prevent binding by forming double stranded regions which cover splice sites or *cis*-acting elements (Figure 1.7A and B). Examples for this mechanism are found in a study of yeast with artificial short RNA hairpins that prevent splicing (Goguel et al. 1993) or in certain mutually exclusive splicing events in the Drosophila *DSCAM* gene (McManus and Graveley 2011). RNA structures can also increase splicing efficiency by exposing splice sites to the spliceosome (Figure 1.7C). This has been shown for the human and murine fibronectin EDA exons (Buratti and Baralle 2004). Moreover stem loop formation of RNA regions potentially brings factors or sequences in closer proximity to each other favoring splicing (Figure 1.7D). Such a mechanism is present in the yeast

ACTIN gene (Buratti and Baralle 2004). In 2015 a study identified a class of introns that needs RNA secondary structure for efficient splicing but does not need U2AF2, which is normally required to assemble the spliceosome (Lin et al. 2015). Lin et al. (2015) combined a bioinformatic approach with *in vivo* experiments using zebra fishes. Their bioinformatic data also indicate the presence of a similar mechanism in humans (Lin et al. 2015).

In conclusion, the interplay of multiple regulatory mechanisms and layers determine the alternative splicing pattern as summarized in Figure 1.8. Regulation depends on nucleosome occupancy, RNAP II elongation rate, *cis*- and *trans*-acting factors as well as on RNA secondary structures. The importance of correct regulation is emphasized by the fact that misregulation leads to many diseases including cancer (Chabot and Shkreta 2016).

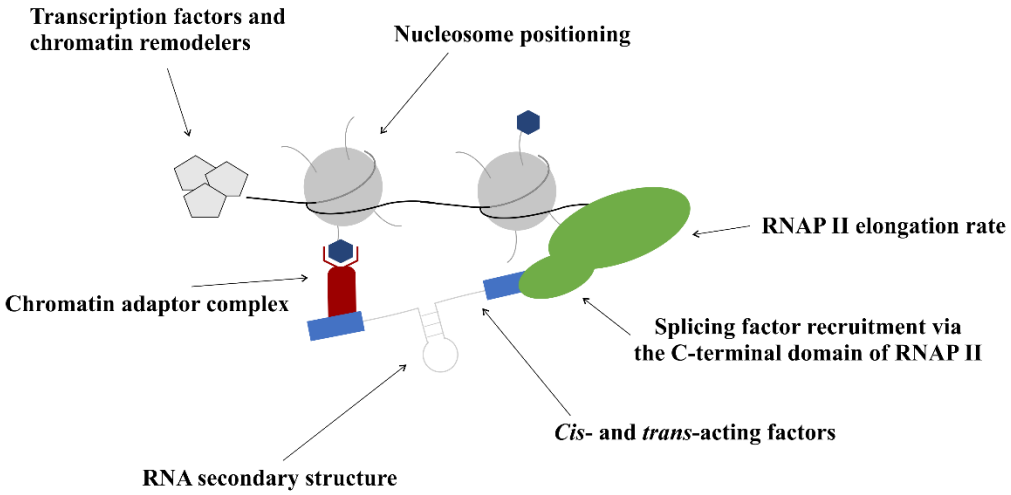

Figure 1.8: Complex regulation of alternative splicing.
Many factors regulate alternative splicing. Histone modifications influence the accessibility of DNA for the RNA polymerase II (RNAP II) and the elongation rate. Chromatin adaptor complexes can translate histone modification information to *trans*-acting factors. *Trans*-acting factors bind to *cis*-acting elements and prevent or support splice site recognition. The C-terminal domain of RNAP II is able to recruit splicing factors to the pre-mRNA. The structure of the pre-mRNA plays a role by occluding or exposing regulatory elements or exons. (modified from Luco et al., 2011)

1.4 Tissue and Species-Specific Splicing

Comparative analyses indicate that alternative splicing has significant roles in specifying tissues and species. As described previously (chapter 1.3.2) *cis*- and *trans*-acting elements are important regulators for splice site recognition. Since the sequence of *cis*-acting elements is identical in all cell types of an organism, cell- and tissue-specific alternative splicing cannot be regulated by changes in these elements. Consequently *trans*-acting factors are the key driver for a cell type- and tissue-specific regulation. Each cell type harbors a specific repertoire of SR proteins and hnRNPs. Their relative stoichiometry determines the alternative splicing pattern (Chen and Manley 2009). Additionally tissue-specific protein expression was observed. The best-known examples are NOVA, RBFOX, MBNLs, QKI and ESRPs. NOVA1 and NOVA2 are neuron-specific splicing factors that regulate splicing of transcripts involved in brain development and synapse formation (Ule et al. 2005). ESRP1 and ESRP2 regulate splicing events which are involved in epithelial to mesenchymal transition (Warzecha et al. 2010).
Tissue-specific splicing often affects exons in unstructured domains of the resulting protein. Often the ability to bind RNA is not changed but tissue-specific splicing provides a platform for post-transcriptional modifications and can change protein-protein-interaction profiles (Buljan et al. 2012; Ellis et al. 2012).

Comparative analyses which investigate species-specific splicing often focus on evolutionary aspects and try to understand how alternative splicing or splicing changes evolve. Studies investigating NOVA-dependent splicing events in different species reveal that *cis*-acting elements undergo more changes in evolution compared to *trans*-acting factors (Jelen et al. 2007; Brooks et al. 2011). Barbosa-Morais et al. (2012) published a genome-wide study in which they compared RNA sequencing data from 9 different tissues and 10 different species. They observed an increase in alternative splicing complexity along the phylogenetic tree with the highest complexity in primates. Most of these species-specific exons were regulated by *cis*-acting elements although some events might be additionally influenced by *trans*-acting factors. Alternative splicing in organs from different species was found to be more species-specific than organ-specific, meaning that the splicing within organs from one species is more related than the splicing of the same organ in different species (Barbosa-Morais et al. 2012). This strengthens the theory that alternative splicing has a crucial role in the establishment of species-specificity.

Species-specific splicing describes two different situations. The alternative exon is included in one species but is absent in the other or an exon is constitutively spliced in one species but alternatively spliced in another. Two possible scenarios explain absence of an exon in one species. New exons evolve mostly from exonization of intronic elements but they can also develop through exon shuffling (Zhang and Chasin 2006). Or exons can get lost during evolution although these events are rare (Wang et al. 2014). This can be caused by deletion of genomic sequences or by changes within the *cis*-acting element that block splice site recognition. An exon loss during evolution often increases the translation efficiency rather than changing the protein coding sequence (Wang et al. 2014). The fact that an exon is alternatively spliced in one species but constitutively in the other was analyzed by comparing mouse and human sequences. The results show that this case is true for at least 11 % of alternative splicing events (Pan et al. 2005). The different inclusion levels can change the function or the structure of the resulting protein. New constitutive exons mostly evolve from alternative exons but examples show that alternative exons can also be generated from constitutive exons (Lev-Maor et al. 2007).

Conclusively, tissue-specific splicing is often a result of tissue-specific expression of *trans*-acting factors. Driving forces behind the establishment of species-specific splicing are *cis*-acting elements (Barbosa-Morais et al. 2012; Gao et al. 2015).

1.5 T Cell Activation

The last chapters highlighted the importance of alternative splicing and its complex regulation. Alternative splicing research focuses on regulated events that respond to the cellular environment, developmental or environmental stimuli. T cell activation is an example in which the alternative splice patterns of many genes are changed. In response to an infection an immune response is triggered by changing the gene expression. In 2012 Martinez et al. published an involvement of alternative splicing in gene expression changes following T cell activation. They used an RNA-sequencing (RNA-seq) approach and identified 178 exons in 168 genes whose splice patterns changed in response to T cell activation. Many of these signal responsive exons are enriched in genes that are annotated to function in immune response (Martinez et al. 2012).

The immune system consists of two different arms, the innate and the adaptive immune system. The innate system is an unspecific response and acts immediately after infection.

The adaptive immune response is highly specific but T cell activation needs time as well as the recruitment of other cells of the immune system to set up a defense. Two different type of T cells can be distinguished, the T helper cells and the cytotoxic T cells. Both express T cell receptors (TCRs) on their surface but they differ in co-receptor expression. T helper cells have CD4 co-receptors while cytotoxic T cells have CD8s. The activation of a T helper cell in a simplified form is illustrated in Figure 1.9.

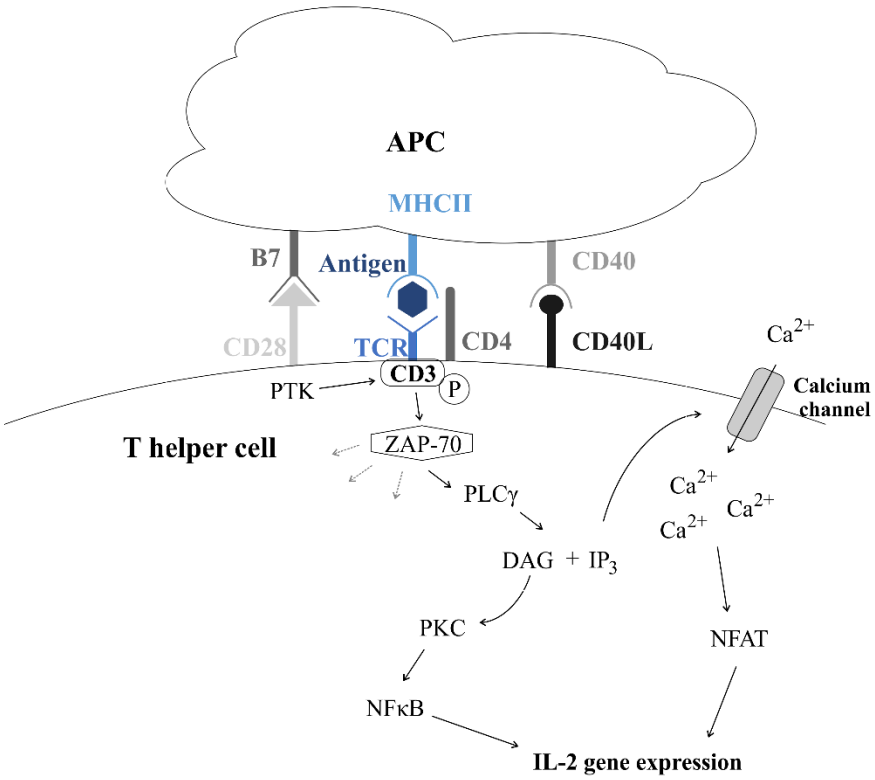

Figure 1.9: Simplified model of T cell activation.
The antigen presenting cell (APC) presents the antigen via its major histo-compatibility complex (MHCII) to the T cell receptor (TCR) of the T helper cell. Co-stimulations are needed for a successful T cell activation as indicated by B7-CD28 binding and CD40-CD40L binding. The TCR stimulation induces phosphorylation and translocation events that result in further cascade activations. Activated transcription factors (representative shown by NFκB and NFAT) are translocated into the nucleus where they induce gene expression of chemokines like IL-2. (Cronin and Penninger 2007; Smith-Garvin et al. 2009)

The first signal required for T cell activation is an antigen. An antigen is often a small peptide originating from a pathogen by its processing. TCRs cannot recognize these antigens by themselves but need antigen presenting cells (APCs). APCs can be various cell types, e.g. dendritic cells or B cells. These cells are able to present antigens with a major histo-compatibility complex (MHC) class. Two types of MHC classes can be

distinguished, the MHCI and MHCII. APCs with MHCI activate cytotoxic T cells while T helper cells are initiated by MHCII. Each activation is the result of a cooperation of two cells. The APC presents the antigen to the T cell and the T cell recognizes foreign peptides. The T cell-APC communication is enhanced by the stimulation of co-receptors. The best-known example is the binding of the B7 receptor to the CD28 receptor of the T helper cell. The recognition of a foreign antigen leads to the expression of CD40L by the T helper cell which binds CD40 from the APC and further strengthens the interaction of both cells. The region of interaction is called the immunological synapse. (Cronin and Penninger 2007)

Stimulation of the TCR then induces changes inside the T helper cell by transferring the signal to downstream signaling cascades (Figure 1.9). The TCR is associated with transmembrane proteins termed as CD3. These CD3 proteins have immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails, which are phosphorylated by protein tyrosine kinases (PTKs). The PTK activity is controlled by TCR stimulation. ITAM phosphorylation recruits ZAP-70, a protein that induces various kinases including PLCγ1. PLCγ1 produces two second messengers IP_3 and DAG from membrane-phospholipid-phosphatidyl-inositol-4,5-biphosphate. Both second messengers activate further signaling processes. DAG formation results for example in protein kinase C (PKC) activation and mobilization of NFκB.

IP_3 influences the cellular calcium flux by stimulating ion channels in the endoplasmic reticulum. This induces calcium transport from the ER to the cytosol and increases the influx of extracellular calcium. The high amount of calcium ions results in the activation of Ca^{2+}-calmodulin-dependent kinase and calcineurin. Calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), which is then transported into the nucleus. (Smith-Garvin et al. 2009)

In cell culture T cell activation can be mimicked by stimulation with phorbol myristate acetate (PMA). PMA activates the PKC (Castagna et al. 1982) and promotes IL-2 gene expression.

To conclude, TCR stimulation activates multiple signaling cascades which result in the activation of transcription factors. These transcription factors induce, among other genes, interleukin IL-2 expression and lead to T cell proliferation and differentiation. The T cell activation is additionally accompanied by cellular changes like cell polarization and rearrangement of the cytoskeleton. To achieve this gene expression has to be altered. This is mostly carried out by the different transcription factors activated upon stimulation

(Naito et al. 2011). Some of these changes are regulated by alternative splicing. Although Martinez et al. (2012) mapped the function of the alternative splicing events to immune response only a few single splicing events were investigated in more detail; e.g. LEF1 (Mallory et al. 2011), MKK7 (Martinez et al. 2015) and SEC16 (Wilhelmi et al. 2016). The function of most splicing events remains elusive.

One of the genes that was identified to be alternatively spliced in response to T cell activation is TRAF3 (chapter 1.6).

1.6 Tumor Necrosis Factor Receptor associated Factor 3 (TRAF3)

TRAF3 belongs to the TRAF family which has seven known members. It is a family of intracellular signaling molecules which bind to the cytoplasmic domains of Tumor Necrosis Factor Receptor (TNFR) and other receptors. This family takes part in the signaling cascades induced by these receptors (Yi et al. 2015). The domain organization of TRAF family members is shown in Figure 1.10. The N-terminus has a RING finger domain which is followed by five zinc fingers. A zipper domain connects the zinc fingers with the TRAF domain that can be divided into an N-terminal and a C-terminal part (van Eydnhoven et al. 1998). The TRAF domain is a platform for protein-protein interactions (Yi et al. 2015). The family members are involved in the activation of transcription factor NFκB. Two pathways activate NFκB, namely the canonical and the non-canonical NFκB pathway (Figure 1.11).

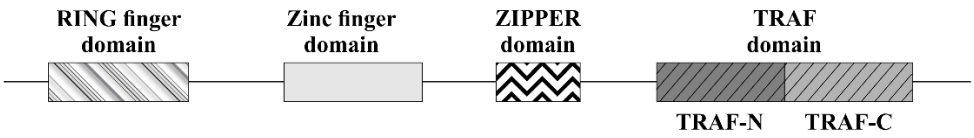

Figure 1.10: Conserved domains of TRAF family members.
All TRAF family members have a RING finger domain followed by a zinc finger and a ZIPPER domain. The C-terminus harbors the TRAF domain. (adapted from van Eydnhoven et al. 1998)

The canonical NFκB pathway is involved in innate immune response and inflammation induction (Häcker et al. 2011). It is activated if the receptors TNFR, Toll-like receptors (TLRs) or the interleukin-1 receptor (IL-1R) are stimulated. Stimulation activates the IKK complex, which consists of an α-, β- and γ-subunit. The IKK complex phosphorylates and ubiquitylates IκB that binds to the transcription factors p50 and RelB, both belonging to the NFκB family. This prevents their translocation into the nucleus. Ubiquitylation of IκB

results in its degradation and releases the nuclear localization signal (NLS) for p50 and RelB. These enter the nucleus where they activate gene expression of chemokines, cytokines and other genes (Bonizzi and Karin 2004). Most TRAF family members are involved in the activation of this canonical NFκB pathway.

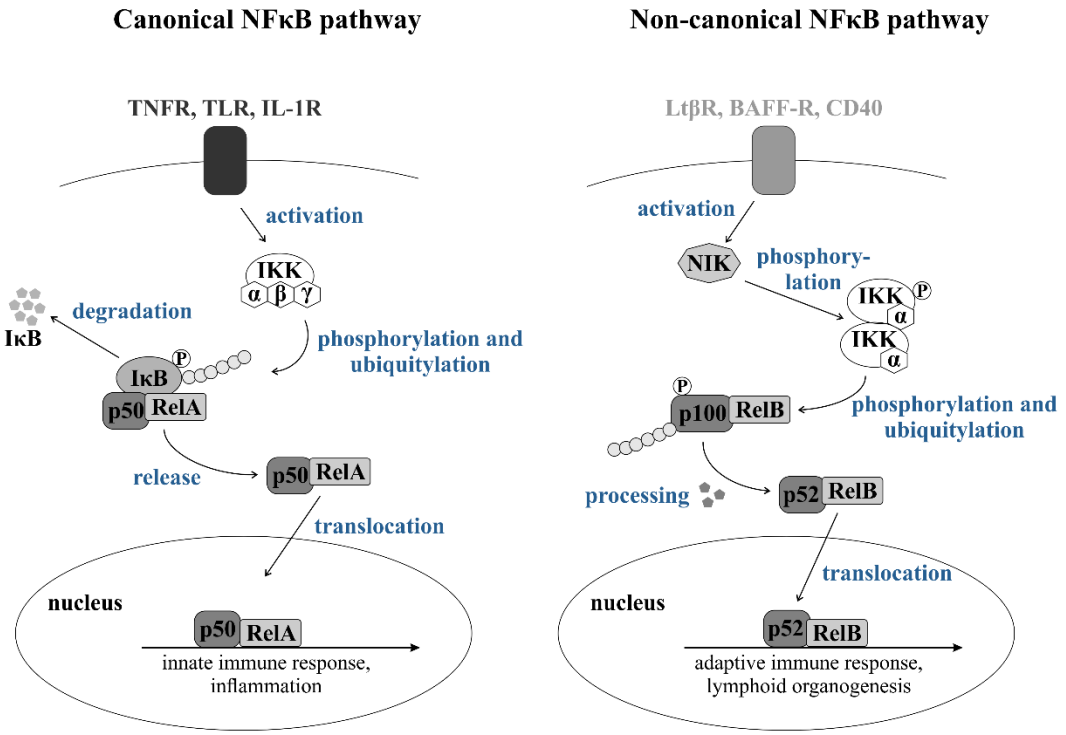

Figure 1.11: Activation of NFκB.
Two pathways exist that can activate NFκB, namely the canonical and the non-canonical NFκB pathway. (Häcker et al. 2011 and Bonizzi and Karin 2004)
Left: In the canonical NFκB pathway the receptor stimulation results in the activation of the IKK complex, the degradation of IκB and translocation of the transcription factors p50 and RelA into the nucleus.
Right: The non-canonical NFκB pathway is controlled by the kinase NIK and ends in the activation of the transcription factors p52 and RelB.

The non-canonical NFκB pathway (ncNFκB pathway) is activated by lymphotoxin-β-receptor (LtβR), B cell activating factor belonging to the TNF family receptors (BAFF-Rs) or CD40 receptor stimulation. The ncNFκB pathway is involved in adaptive immune response as well as in the regulation of lymphoid organ development (Häcker et al. 2011). The stimulated receptors transfer the signal to the NFκB inducing kinase (NIK). This results in phosphorylation of an IKKα-dimer that ubiquitylates p100 of NFκB family together with its binding partner RelB. As a result p100 is processed into p52, which is then transported into the nucleus together with RelB. Both activate expression of genes

for cytokine and chemokine production as well as lymphoid organogenesis genes in the nucleus (Bonizzi and Karin 2004). TRAF3 and TRAF2 are involved in the ncNFκB pathway.

In 1998, several TRAF3 isoforms were discovered. They are produced by alternative splicing of exon 7, exon 8 and exon 9 (van Eydnhoven et al. 1998). The isoforms differ in the number of zinc fingers. TRAF3 full length has five fingers while the shorter isoforms lack complete zinc fingers or parts of them. The TRAF3 full length isoform prevents NFκB activation whereas the shorter isoforms activate it (Van Eyndhoven et al. 1999). The mechanism of inhibition or activation as well as the function of the different isoforms was unknown.

In 2014 we brought together the existing knowledge of ncNFκB activation and alternative TRAF3 splicing. In the T cell line Jsl1 we observed TRAF3 exon 8 skipping in response to T cell activation (Figure 1.12A) (Michel et al. 2014). This splicing change was also observed in the RNA-seq data published by Martinez et al. (2012). The skipping of exon 8 results in the partial deletion of the fourth and the fifth zinc fingers (Figure 1.12B).

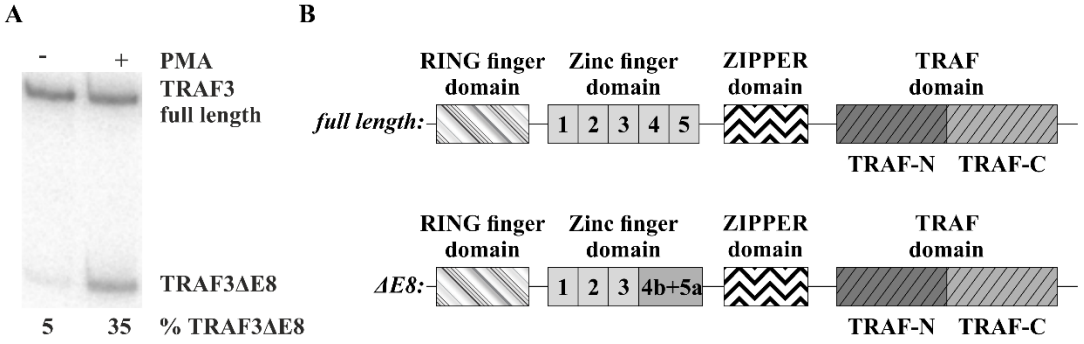

Figure 1.12: Alternative splicing of TRAF3 upon T cell activation.
A: TRAF3 RT-PCR was done with RNAs from resting (-PMA) and activated (+PMA) Jsl1 cells. The percentage of TRAF3ΔE8 isoform is given below the gel (n≥3). (also seen in (Michel et al. 2014; Schultz et al. 2017))
B: Domain organization of TRAF3 full length and TRAF3ΔE8. (modified from Van Eyndhoven et al. 1999)

The functional characterization of both TRAF3 isoforms in T cell activation by us conclude in the following model (Figure 1.13): in resting T cells TRAF3 interacts with TRAF2 which is mediated by the TRAF domains of both proteins and the fourth and fifth zinc finger of TRAF2 (He et al. 2004). TRAF3 interacts with NIK via its RING domain (He et al. 2007) while TRAF2 binds the E3 ubiquitin ligase cIAP. TRAF3 functions as a

platform that brings together cIAP, its substrate NIK and TRAF2. NIK becomes K48-linked ubiquitylated. This modification marks NIK for degradation. The low basal NIK expression level results in the inactivation of the ncNFκB pathway (Häcker et al. 2011).

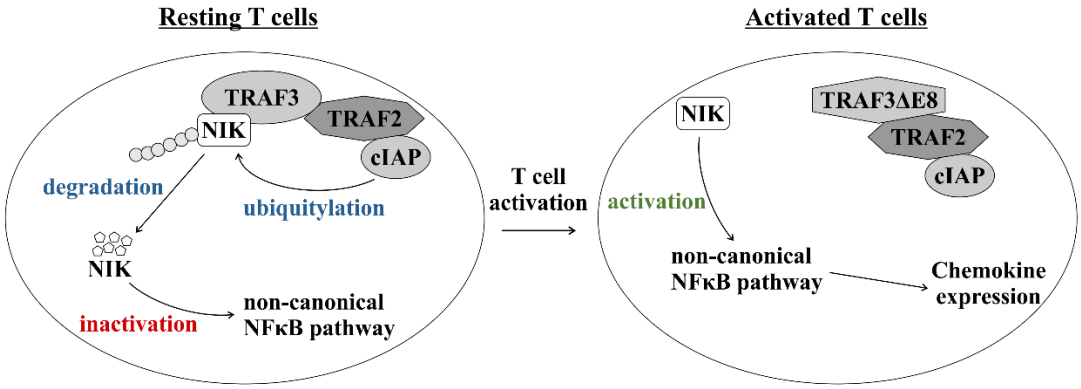

Figure 1.13: TRAF3 isoforms and their function in the ncNFκB pathway.
In resting T cells (left side) TRAF3 brings NIK and cIAP in close proximity. This results in ubiquitylation of NIK and its degradation followed by the inactivation of the ncNFκB pathway. T cell activation (right side) results in TRAF3ΔE8 induction which does not bind NIK. NIK is not degraded but activates the ncNFκB pathway. (adapted from Michel et al. 2014)

In activated T cells TRAF3 is alternatively spliced resulting in the accumulation of TRAF3ΔE8 isoform (Figure 1.12A). Although the zinc fingers do not directly mediate the interaction with NIK TRAF3ΔE8 loses its ability to bind NIK. Accordingly, NIK is not degraded and can activate the ncNFκB pathway (Michel et al. 2014).

In summary, TRAF3 alternative splicing upon T cell activation has a consequential function in immune response. It regulates the activation of the ncNFκB pathway which is needed to express components for an adaptive immune response. The exact regulation mechanism of this crucial splicing event remains elusive.

1.7 Aim of This Study

The previous chapters emphasize that alternative splicing is an important mechanism for proteome expansion generating several isoforms from a single transcript. The precise splicing process is highly regulated by *cis*- and *trans*-acting factors which facilitate or prevent the recognition of splice sites and the spliceosome assembly. Coupling the

splicing process to transcription and the secondary structure of the pre-mRNA can have an impact on regulation.

In 2012 Martinez et al. published a list of exons which change their inclusion levels upon T cell activation. This finding suggests a role for alternative splicing in immune response. One of these alternative spliced exons is TRAF3 exon 8 whose skipping increases in activated cells. We analyzed the function of the full length and the ΔE8 isoform and found them to regulate the ncNFκB pathway (Michel et al. 2014). While the full length TRAF3 isoform inhibits this pathway in resting T cells, TRAF3ΔE8 activates the ncNFκB pathway and triggers chemokine expression in response to T cell activation. The regulatory mechanism of this crucial splicing switch is not known and the unravelling of it was aim of this study.

In order to completely understand the regulation of alternative splicing events it is necessary to identify the *cis*- and *trans*-acting elements. The *cis*-acting element of TRAF3 exon 8 splicing was determined by minigene analyses. Several TRAF3 minigenes were cloned containing variations of TRAF3 exon 8 and surrounding intronic regions within two constitutive exons. Jsl1 cells were transfected with these constructs and splicing was analyzed by radioactive RT-PCR. In parallel, I searched for putative *trans*-acting factors with an siRNA-library screen. The siRNAs targeted many RNA binding proteins which are known to be involved in splicing. As soon as *cis*-acting elements were identified, the binding of putative *trans*-acting factors was validated by UV-crosslink analyses and immunoprecipitations. Next to *cis*- and *trans*-acting elements, splicing could be regulated by secondary structures of TRAF3 pre-mRNA. This was investigated using a structure prediction tool (RNA structure) as well as minigene approaches.

We did not observe TRAF3 exon 8 skipping in the human B cell lines Ramos and Raji indicating that exon 8 skipping is cell type-specific (Michel et al. 2014). A comparison of these human B cell lines with the human T cell line Jsl1 further helped to understand the regulation of TRAF3 exon 8 splicing. TRAF3 splicing was investigated in these cell lines by radioactive RT-PCR. Furthermore the expression of putative *trans*-acting factors was compared since it is known from literature that most cell-specific splicing events are regulated by cell type-specific expression of these regulators (Chen and Manley 2009). This was addressed by western blots. The overexpression of missing endogenous *trans*-acting factors revealed their sufficiency to induce TRAF3 exon 8 splicing.

Since 2012, numerous findings have highlighted the importance of species-specific splicing. To get a complete picture of TRAF3 exon 8 alternative splicing regulation it was investigated if this splicing event is species-specific by analyzing TRAF3 splicing in mouse T cell lines (El4 and Bw). Differences or similarities in TRAF3 exon 8 skipping in response to activation were studied in further detail. It is known that most species-specific splicing events are *cis*-element driven (Gao et al. 2015). To analyze this murine and human TRAF3 minigenes were cloned and murine and human cells were transfected with both. The result showed whether the *cis*-acting element regulates a putative species-specific splicing event or whether the cellular environment meaning the *trans*-acting factors might be crucial. Then the *cis*-acting elements of both species were analyzed in detail to understand why splicing occurs in one species but not in the other. Evolutionary changes in the sequence might destroy splice sites or splice site recognition could be prevented. In some cases, species-specific splicing is regulated by differences in *trans*-acting factors. Again, several scenarios are possible. The *trans*-acting factors might be differently expressed in both species or a specific post-translational modification might be unique for one species. Both scenarios were addressed by western blot analysis. Additionally, the *trans*-acting factors themselves might undergo alternative splicing. Putative splice isoforms can be detected with western blot or by radioactive RT-PCR.

2 Materials and Methods

2.1 Cell Lines and Their Cultivation

The cell lines used in this study are listed in table 2.1:

Table 2.1: Cell lines used in this study.

Name of the cell line	Cell type	Growing system
Jukat (Jsl1)	Human T lymphocytes	Suspension culture
Raji	Human B lymphocytes	Suspension culture
Ramos	Human B lymphocytes	Suspension culture
Ramos/empty vector	Human B lymphocytes	Suspension culture
Ramos/Celf2-myc-his	Human B lymphocytes over-expressing CELF2-MYC-HIS	Suspension culture
El4	Mouse T lymphocytes	Suspension culture
Hek293T	Human embryonic kidney cells	Adherent culture

Suspension cells were cultivated in RPMI-medium-1640 (1x) + GlutaMAX^{TMI} (+/+) (Biowest) containing 10 % heat-inactivated fetal bovine serum (FBS from Biochrome) and Penicillin/Streptomycin (Pen/Strep from Biowest) 1:100 (abbreviated: RPMI +/+).
Adherent cells were cultivated in DMEM (Biowest) containing heat-inactivated 10 % FBS and Pen/Strep 1:100 (DMEM +/+). Cell seeding was done in DMEM containing only 10 % FBS but no antibiotics (DMEM +/-).
Both suspension and adherent cells were grown at 37 °C with 5 % CO_2 in a Heraeus incubator from Thermo Scientific.

Suspension cells were stimulated where needed with PMA (20 ng/µl, Sigma) or the solvent control DMSO. For DMSO treatment $0.25x10^6$ cells were used, and $0.33x10^6$ for PMA treatment to compensate the different growth behavior post-stimulation. Transfected cells were split and stimulated 24 h post-transfection in the following way: one third of the transfected cells were treated with DMSO and the rest with PMA (20 ng/µl); cells were harvested 48 h after stimulation.

2.2 Transfection of Cells

Various methods for transfection were used in this study depending on the cell type as well as on the transfection efficiency. Two different approaches were used: transfection by electroporation and transfection using transfection reagents. In electroporation the cells are exposed to high-voltage electric shocks and thereby DNA can be introduced. The transfection reagents used in this study introduce exogenous DNA by a liposome-mediated mechanism.

2.2.1 Transfection via electroporation

Electroporation was done with two different protocols, Amaxa and Biorad electroporation. Amaxa method was used to transiently transfect siRNAs. Biorad electroporation generated transient and stable transfectants.

Transfection with Amaxa biosystems Nucleofector II (Lonza) was done according to the manufacturer's instructions for Jurkat (Clone E6-1), El4, Raji and Ramos cells. All protocols are similar but programs for electroporation and incubation times can be cell line-specific. General procedure in brief: $2x10^6$ cells were harvested per transfection and resuspended in 100 µl RPMI -/-. The siRNA (20 pmol final concentration) was diluted in 100 µl RPMI -/- and both cells and diluted siRNA were transferred into an electroporation cuvette (Amaxa Cell Line Nucleofector Kit V). The programs used for electroporation are listed in table 2.2.

Table 2.2: Amaxa programs used for electroporation.

Cell line	Program used for Amaxa electroporation
Jsl1	X-001
Raji	M-013
Ramos	O-006
El4	C-009

After incubation at room temperature 500 µl RPMI +/+ were added to the transfected cells and they were transferred into 12-well plates which already contained 1 ml preheated RPMI +/+. The cells were cultivated for 24 h before they were split and stimulated as described in 2.1.

Biorad electroporation was used to transfect Jsl1 with minigenes. Procedure in short: for each transfection $10x10^6$ cells were harvested and the cell precipitate was washed twice in RPMI -/- before it was resuspended in 400 µl RPMI -/-. The cells were transferred into an electroporation

cuvette (VWR Electroporation Cuvettes, 4 mm Gap) containing 5 µg minigene-DNA. Electroporation was done in a Bio-Rad Gene Pulser with 250 mV, 1000 Ω and 960 µF. The transfected cells were transferred to a 6-well plate containing 5 ml preheated RPMI +/+.

To create a Ramos cell line that stably overexpresses CELF2-MYC-HIS, the CELF2 plasmid was linearized with ScaI and then cells were transfected with this. Positive clones were selected 4-5 days post-transfection with 4 mg/ml Geneticin (G418; Sigma). The CELF2-MYC-HIS expression in single clones was verified by western blot analysis. Ramos/empty vector cells went through the same selection procedure but do not express CELF2-MYC-HIS. These cells were used as control.

2.2.2 Transfection with HiPerFect transfection reagent

HiPerFect transfection reagent was bought from Qiagen and the siRNA transfection was done as described for fast forward transfection in the manual. Procedure in short: one day before transfection cells were diluted to $3x10^5$ cells per ml to guarantee exponential cell growth. On the transfection day $1x10^6$ cells were harvested per transfection, resuspended in RPMI +/+ and transferred into a 24-well plate. The siRNA (20 µmol) was diluted in 100 µl RPMI -/- and then 9 µl HiPerFect were added. The mixture was incubated for 5 to 10 min to ensure the formation of transfection complexes before it was transferred to the cells. The transfected cells were cultured for 6 h under normal growth conditions (37 °C and 5 % CO_2) and then 400 µl RPMI +/+ were added. Cells were split and stimulated 24 h post-transfection as described in 2.1.

2.2.3 Transfection with RotiFect or Lipofectamine

Hek293T cells were transfected with the transfection reagents RotiFect (Roth) or Lipofectamine 2000 (Invitrogen). To this end $1.5x10^5$cells per 12-well or $4.5x10^5$ cells per 6-well were seeded in DMEM +/-. Transfection was done one day after seeding as described in the manufacturer's protocol. In short: plasmid and transfection reagent were individually diluted in OptiMEM (GIBCO by Life Technologies) and combined 5 min later for the formation of transfection complexes. After 20 min these complexes were transferred to the seeded cells. Cells were harvested 48 h post-transfection for protein and/or RNA extraction.

2.3 Nucleic Acids: Isolation, Analysis, Detection and Cloning

2.3.1 Isolation of RNAs

Cells for RNA extraction were harvested (7000 rpm, 1 min) and the cell precipitate was solved in 500 µl "RNA-Tri-flüssig" (Bio&Sell). Next 100 µl chloroform was added and the samples were vortexed and incubated on ice for 10 min. Phase separation was achieved by spinning the tubes for 15 min at 4 °C at full speed. The aqueous phase was transferred to a new tube containing 300 µl ice-cold isopropanol. The tubes were again vortexed and centrifuged for 15 min at 4 °C at full speed to pellet the precipitating RNA. The RNA precipitate was washed three times with 70 % ethanol and then dissolved in 12 µl Milli-Q-water. The concentration and the purity of the RNA were determined with the Nanophotometer P330 from Implen.

RNA extraction from Jsl1 cells which were transfected with the siRNA-library was done with the "GeneJET RNA Purification Kit" (Thermo Scientific) to ensure high purity.

2.3.2 Analysis using RNAs

RNAs were used to analyze gene expression on the RNA level with Reverse Transcriptase PCR (RT-PCR) or quantitative Realtime PCR (qRT-PCR). The binding of proteins to specific RNAs was investigated.

2.3.2.1 Reverse Transcriptase PCR

Reverse transcriptase PCR (RT-PCR) was used to convert mRNA into complementary DNA (cDNA) and amplify the resulting products. In this study all cDNA syntheses were done with gene-specific primers. Therefore 200-500 ng RNA were mixed with 1 µl hybridization buffer (1.5 M NaCl, 50 mM Tris pH 7.5, 10 mM EDTA) and 1 µl gene-specific primer (5 ng/µl) in a total volume of 4 µl. This mixture was heated in a PCR machine to 90 °C and then cooled down by increments (Δ1 K any 20 sec) to 43 °C to allow primer annealing. Reverse transcriptase (M-MuLV Reverse Transcriptase from Enzymatics) was added together with RT-mix (12.5 mM DTT, 12.5 mM. Tris pH 8.0, 7.5 mM $MgCl_2$, 1.25 mM dNTPs). The reaction was continued at 43 °C for 35 min. A reaction without reverse transcriptase served as control. Afterwards either traditional PCR or the more sensitive radioactive PCR was performed to amplify the generated cDNA. Both reaction procedures are very similar but the radioactive PCR includes a combination of radioactive labeled and unlabeled forward primer.

Primers and marker were radioactively labeled by transferring a radioactive phosphate group from ^{32}P-γ-ATP to the 5'-hydroxylgroup of the primer/marker with a T4 polynucleotide kinase (T4 PNK; Fermentas). The following components were mixed: 1x PNK buffer, 1 µl T4 PNK, 200 ng forward primer and 0.93 MBq ^{32}P-γ-ATP. The reaction was incubated for 30 to 45 min at 37 °C. Afterwards labeled DNA was extracted with Phenol/Chloroform/Isoamyl alcohol (PCI) by adding 200 µl PCI, 23 µl 3 M NaOAc (pH 5.0), 2 µl glycogen and 75 µl water. After vortexing and spinning the aqueous phase was transferred into a fresh tube and precipitated by ethanol. The precipitate was dissolved in 80 µl water (primers) and a combination of water and formamide loading dye (210.5 µg/ml bromophenol blue, 210.5 µg/ml xylene cyanol, 26.3 mM EDTA in formamide) (marker).

The subsequent PCR was done with Taq-Polymerase. In traditional PCR 100 ng forward and reverse primer were added. Radioactive PCR was done with 2.5 ng forward labeled and unlabeled primer (2.5 ng + 2.5 ng) and 5 ng unlabeled reverse primer (primers are listed in table 6.1 in the supplement). The reaction was covered with oil and the PCR was done using 60 to 65 °C annealing temperature and 1 min for elongation. PCR products were mixed with formamide loading dye and loaded to 5 or 10 % acrylamide gels for denaturing PAGE. Gels were detected with the phosphor imager Typhoon Fla7000 (GE Healtcare Life Science) and quantified with the Image Quant TL Software.

Traditional PCRs were loaded on 1 or 2 % agarose (Biozym) gels (depending on the product sizes) containing ethidium bromide. In general gels ran at 140 V for 15-20 min. Detection was done by UV-light using the ChemiImager^{TM}5500 from Alpha Innotech.

2.3.2.2 Quantitative Realtime PCR

The quantitative Realtime PCR (qRT-PCR) is used to analyze gene expression on RNA level in a quantitative way. The evaluation is done in the exponential phase of PCR in contrast to radioactive or traditional PCR where an end point detection is obtained. Template for qRT-PCR was cDNA that was generated as described above with 500 ng RNA and up to three different gene-specific primers. The qRT-PCR was performed in 96-well plates with Absolute qPCR SYBR green mix (Thermo Fischer) in a Stratagene MX3005p machine. Evaluation of the results was done with Excel (*GAPDH* served as reference gene). A list with all used primers is given in table 6.2 in the supplement.

2.3.2.3 In vitro transcription and UV-Crosslink with Immunoprecipitation

To analyze the binding of nuclear proteins to specific RNA sequences *in vitro* transcription was done followed by a UV-Crosslink analysis. Two complementary primers which include a T7 promoter as well as the sequence, which was analyzed for protein binding, were used as starting material. In the first step primers were annealed. Then the double strands were *in vitro* transcribed with a T7-polymerase (Fermentas) and radioactive ^{32}P-α-UTP (0.93 MBq per reaction).

After an incubation at 37 °C for 2 h DNAse was added for 15 min to degrade the template. The generated RNA transcript was precipitated with PCI and ethanol and resuspended in 40 µl water. The efficiency of the reaction was checked by loading 2 µl on a 10 % denaturing PAGE.

UV-Crosslink was performed as described in Motta-Mena et al. 2010. In short: nuclear extracts (preparation is described in chapter 2.4.2) and *in vitro* transcribed RNA were combined and incubated for 20 min at 30 °C. Proteins were crosslinked to RNA with short wave UV light for 20 min. The RNA was then digested using RNAse T1 (20 U) and RNAse A (20 µg) to end up with the remaining crosslinked RNA-Protein complexes. The complexes were directly loaded to an SDS-Page or an immunoprecipitation followed. For immunoprecipitation 3 identical samples were pooled and mixed with 2-15 µg antibody and RIPA buffer (10 mM Tris pH 8.1, 1 % NP-40, 5 mg/ml sodium deoxycholate, 2 mM EDTA and 200 mM NaCl). This mixture was rotated for at least 1 h at 4 °C and then protein A/G sepharose beads which were previously washed with RIPA buffer were added. After an overnight incubation beads were washed and loaded on a 10 % SDS gel. The gel was covered with a Fuji medical X-ray film ^{super}RX-N (FIJIFILM) for several hours or days before it was detected. The antibodies used for immunoprecipitation are listed in table 6.3 in the supplement.

2.3.3 Cloning

The template for cloning was either cDNA (human CELF2 variants and mouse TRAF3 minigenes) or plasmids (human TRAF3 minigenes and CD9 minigenes) which were already available in the lab and could be used to create shorter or mutated versions of the existing plasmid. The amplification was carried out with Phusion Polymerase (Thermo Scientifc or Molox). PCR products were separated on agarose gels and fragments with the correct sizes were cut out. Purification was performed via the "NucleoSpin® Gel and PCR Clean-up kit" from Macherey Nagel. The extracted DNA was digested with the appropriate restriction

enzymes. After an additional round of purification (with the above mentioned kit), ligation was done for 1 h at room temperature with T4-ligase (Fermentas). Chemically competent *E. coli* cells were transformed with ligations and plated on LB plates containing either ampicillin (50 µg/ml) or kanamycin (25 µg/ml). Single clones were grown in liquid cultures containing the appropriate antibiotic. Plasmids were extracted using the "Nucleo Bond Xtra Midi Kit" (Macherey Nagel). All cloned plasmids were verified by sequencing. A list of all cloned plasmids as well as the primers used for cloning is shown in the supplement, table 6.4.

2.4 Extraction and Analysis of proteins

2.4.1 Isolation of proteins

To extract proteins cells were harvested and washed with PBS. Cell lysis was carried out with Flag buffer (60 mM Tris pH 7.5, 30 mM NaCl, 1 mM EDTA, 1 % Triton X-100) containing the protease inhibitors PMSF, Vanadat, Aprotinin and Leupeptin. After incubation on ice for 10 min and spinning protein solution was transferred into a fresh tube. Protein concentrations were determined with Bradford solution at 600 nm in a photometer.

2.4.2 Preparation of nuclear extracts

Nuclear extract preparation started with cell harvest and cell washing with PBS. The cell precipitate was resuspended in 200 µl cytoplasma buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) containing protease inhibitors. After 5 min incubation on ice 200 µl cytoplasma buffer with 0.2 % NP-40 was added followed by another 5 min incubation. Nuclei were isolated by spinning (6500 rpm, 4 °C, 3 min) and then resuspended in 40 µl nuclear extract buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25 % glycerol). Three freezing (-80 °C) and thawing (37 °C) cycles were performed before the sample was again centrifuged (14,000 rpm, 4 °C, 20 min). The supernatant was transferred into a fresh tube and the concentration was measured by the Bradford method.

2.4.3 Gelelectrophoresis and visualization of proteins

Proteins were analyzed with SDS-Page followed by western blot analysis. 10-15 µg proteins were usually separated in a 10 % SDS gel using the Mini-PROTEAN®Tetra System from Biorad using 140 V. Afterwards semi-dry western blot was done to transfer the separated proteins on a PVDF membrane. The membrane was blocked with 2 % BSA in LS-TBST

(50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % Tween-20). Incubation with first antibodies was carried out overnight at 4 °C under shaking condition. All used antibodies are listed in table 6.5 in the supplement. The next morning the membrane was washed three times with HS-TBST (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 0.1 % Tween-20) before it was incubated with the second antibody (also listed in table 6.5) for at least 1 h at room temperature on a shaker. After another round of washing steps, the detection was performed with ECL solution (Thermo scientific) and an X-ray film.

2.5 Statistics

The number of independent experiments is always mentioned in the figure legend. Quantifications show the average of the independent experiments and the error bars represent standard deviation. The significance was calculated with unpaired student's t-test: $p^{*} > 0.05$, $p^{**} > 0.01$ and $p^{***} > 0.001$.

2.6 Sequences and Alignments

Sequences used in this study were extracted from the human GRCh37/hg19 assembly and the mouse NCBI37/mm9 assembly except where otherwise specified.

A putative RNA secondary structure in the TRAF3 gene was analyzed with RNA structure, a Web Server for RNA Secondary Structure Prediction (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html)

Sequence alignments were conducted with the EMBOSS Needle pairwise Sequencing Alignment tool provided by EMBL-EBI (http://www.ebi.ac.uk/Tools/psa/)

3 Results

We previously used the model T cell line Jsl1 and observed that TRAF3 exon 8 is skipped upon T cell activation. We analyzed the function of both isoforms and found a role in the regulation of the non-canonical NFκB pathway (ncNFκB pathway). The full length TRAF3 isoform has an inhibitory function whereas the TRAF3ΔE8 isoform whose expression increases in activated T cells activates this pathway. It is not known so far how this consequential splicing switch is regulated. This will be the focus of the work presented in the following chapters.

3.1 Cis- and Trans-Acting Factors Regulate TRAF3 Splicing

Alternative splicing events can be regulated on various levels as highlighted in the introduction. The central questions for understanding the regulation of a splicing event are: which sequences (*cis*-acting elements) are needed for regulation and what are the proteins (*trans*-acting factors) that bind to these elements and regulate splicing?

3.1.1 Identifying the *cis*-acting element in TRAF3 splicing

Alternative splicing of TRAF3 exon 8 could be regulated by one or more *cis*-acting elements that lie either within the alternatively spliced exon or in the surrounding introns upstream or downstream of it. To identify elements that are needed for regulation, minigenes that contain the alternative exon as well as the up- and downstream introns are used. Cells are transfected with these minigenes and the splicing is analyzed with minigene-specific primers. By systematically shortening the up- and downstream introns, it is possible to identify the *cis*-acting element because its removal causes a loss or a decrease in splicing.

In this study the minigene analyses were carried out with a vector that contains TRAF3 sequences in a context of constitutively spliced CD45 exons as done in (Motta-Mena et al. 2010). The first minigene contains TRAF3 exon 7, intron 7, exon 8, intron 8 and exon 9 (Figure 3.1A construct 1). Jsl1 cells were transfected with this construct and treated with the solvent control DMSO or PMA. The latter mimics T cell activation. The splicing pattern of the transfected minigene was analyzed by radioactive RT-PCR and the PMA-induced exon skipping value was calculated (% inclusion in stimulated cells subtracted by % inclusion in resting cells). The PMA-induced exon skipping is around 35 % for the first construct (Figure 3.1B) and is comparable to endogenous TRAF3 splicing (first bar in Figure 3.1B). This

shows that minigene construct 1 can mimic endogenous splicing and that further minigenes can be used to identify the *cis*-acting elements.

The minigene construct 1 was then systematically shortened. Construct 2 lacks exon 7 and exon 9 as well as 2100 bp of intron 7 and 404 bp of intron 8 (Figure 3.1A). It shows a slight reduction in PMA-induced exon skipping (Figure 3.1B). Interestingly a minigene containing only 336 bp of the upstream intron exon 8 and 436 bp of the downstream intron (construct 3, Figure 3.1A) loses the ability to respond to PMA (Figure 3.1B). This indicates that some of the truncated sequences are needed for the regulation. This further suggests a location of the *cis*-acting elements within the intronic regions rather than in the alternatively spliced exon itself. To narrow down the location construct 4, 5 and 6 contain 597 bp, 499 bp and 432 bp of the upstream intron (Figure 3.1A). All three constructs show a responsiveness to PMA but it is weaker than in the control (construct 1). The PMA responsiveness decreases with shorter sequences (Figure 3.1B). In conclusion a region between 336 bp and 597 bp is required for regulation of TRAF3 splicing. Construct 7 and 8 lack 160 bp or 96 bp in the 597 bp long upstream element of exon 8. Both constructs show little response to PMA stimulation demonstrating that the deleted 96 bp in construct 8 is the necessary core sequence for a response. The identified *cis*-acting element is depicted in Figure 3.1C. It is an intronic splicing silencer (ISS).

Construct 4 shows reduced PMA-induced exon skipping compared to the endogenous and control minigene (construct 1), indicating that downstream of exon 8 another *cis*-acting element might be present. However the effect of the upstream element is much stronger. This is the reason why further investigations will focus on the identified upstream positioned *cis*-acting element. Monika Michel and Florian Heyd did all the minigene cloning and many of the transfections.

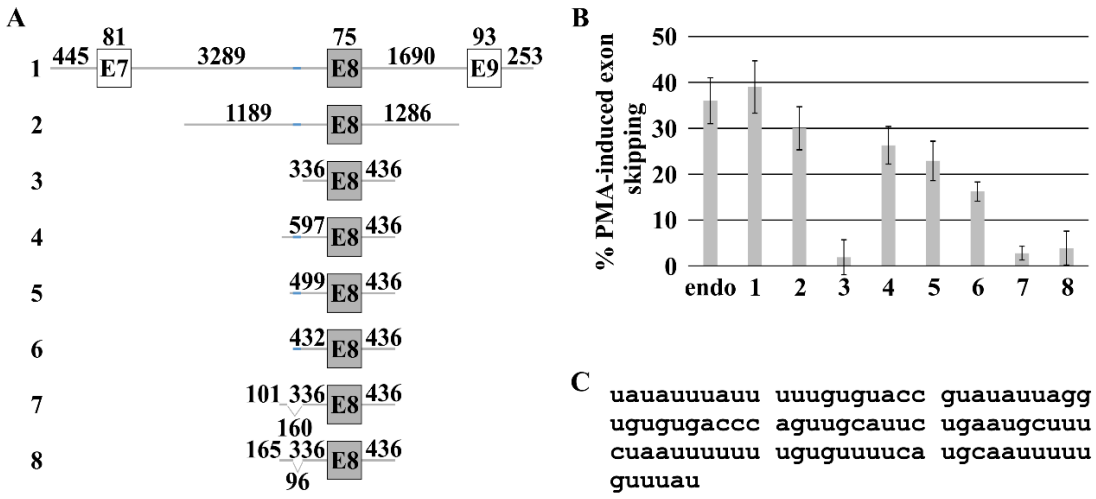

Figure 3.1: One *cis*-acting element mediates alternative splicing of TRAF3 exon 8.
A: The scheme presents the minigenes that were used to identify the *cis*-acting element. Introns are depicted as lines and exons as boxes. Both are not to scale. The intron and exon lengths are given on top of the respective fragments in base pairs. In construct 7 and 8 the number of deleted exons within the upstream intronic sequence is shown below the scheme. The bold blue line represents the *cis*-acting element.
B: Jsl1 cells were transfected with the minigene constructs shown in A and then treated with DMSO or PMA for 48 h. Radioactive RT-PCR was performed to analyze minigene splicing in the transfected cells. Endogenous TRAF3 splicing (first bar) serves as control. (n≥3; error bars: S.D.)
C: The 96 base pairs long identified *cis*-acting element is depicted.

3.1.2 Screening for *trans*-acting factors

With the identified *cis*-acting element the identification of the *trans*-acting factors can be achieved. A first impression of how many proteins bind to this sequence can be obtained by UV-Crosslink analyses. The *cis*-acting element was therefore *in vitro* transcribed and then incubated with nuclear extracts of either resting or stimulated Jsl1 T cells. The bound proteins were linked to the RNA by UV-light the unbound RNA was digested and the remaining RNA-protein complexes were separated on an SDS-gel. Many proteins bind to the *cis*-acting element but only two of them show a strong change in binding upon T cell activation (Figure 3.2A). A protein with a size of ~55 kDa binds stronger in PMA treated conditions compared to resting cells whereas a protein of around 40 kDa has a decreased binding in stimulated conditions. These observed changes in binding affinity to the *cis*-acting element lead to the assumption that both proteins are putative regulators in TRAF3 exon 8 splicing.

There are several approaches to identifying the bound proteins. A very straightforward method is to cut out the bands from the UV-Crosslink gel and identify its proteins by mass spectrometry.

A problem is that the UV-Crosslink is done with radioactive labeled RNA. Consequently it will be unlikely to find a facility which will do this. Another possibility is the use of siRNAs against RNA binding proteins in an siRNA screen and a third approach would be to test miscellaneous proteins in UV-Crosslink-immunoprecipitation based on literature.

In this study an siRNA-library screen was the chosen method for two main reasons. This screen can be performed in parallel with the identification of the *cis*-acting element because the *cis*-acting element itself is not needed for this approach and the generated siRNA-library can be used to identify *trans*-acting elements for further splicing events.

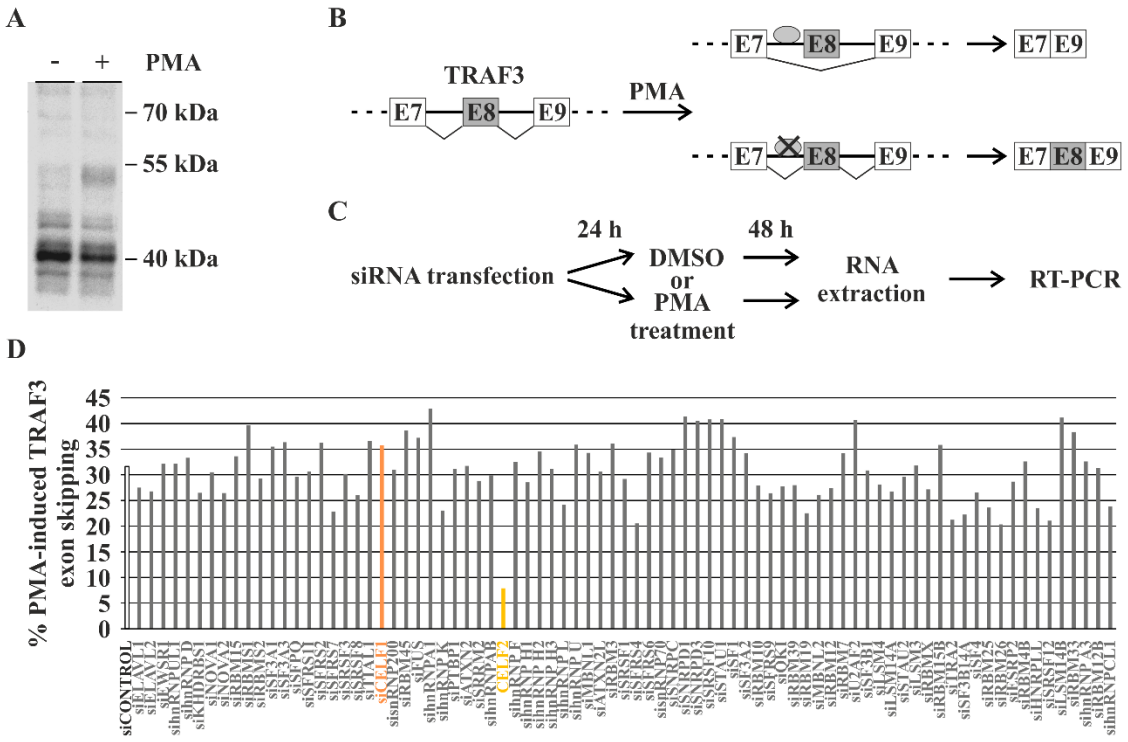

Figure 3.2: Screening for *trans*-acting factors that regulate TRAF3 exon 8 skipping.

A: UV-Crosslink analysis was performed with the *in vitro* transcribed *cis*-acting element and nuclear extracts from resting (-PMA) and stimulated (+PMA) Jsl1 cells.

B: Scheme shows the rationale of the siRNA screen. The knockdown of a protein involved in splicing regulation should result in the loss of exon 8 skipping.

C: Screening strategy: Jsl1 cells were transfected with siRNAs and 24 h later these cells were split and treated with either DMSO or PMA. RNA extraction was done 48 h post-stimulation and TRAF3 splicing was investigated with radioactive RT-PCR.

D: Jsl1 cells were transfected with around 100 siRNAs (each siRNA is a pool of four individual siRNA targeting the same gene) and treated as described in C. The first bar is the control. CELF2 knockdown shows the strongest effect on TRAF3 splicing and is highlighted in yellow. CELF1 belongs to the same protein family as CELF2 and is colored in orange. The results shown were consistent in two independent RT-PCRs.

The basic principle of the siRNA screen for TRAF3 splicing is shown in Figure 3.2B: in untransfected resting cells, TRAF3 exon 8 is included in the mRNA. PMA stimulation leads to binding of at least one protein to a *cis*-acting element and induces exon 8 skipping. If an siRNA targets this protein and causes its knockdown the bound protein amount decreases and the ability to skip TRAF3 exon 8 will be disappear or be impaired. This can be visualized by RT-PCR. In contrast cells transfected with siCONTROL will be able to skip exon 8 as observed in untransfected cells.

Jsl1 cells were transfected with 109 siRNAs. One siRNA consists of a pool of four siRNAs against the same target. The transfectants were split and stimulated either with the solvent control DMSO or PMA 24 h post-transfection. After 48 h RNAs were extracted and TRAF3 splicing was analyzed by radioactive RT-PCR (Figure 3.2C) using primers which bind in exon 7 and exon 9. The RT-PCR was done twice for each RNA and only if both reactions led to consistent results were they considered for further analyses.

Most of the samples show a PMA-induced exon skipping of around 30 % similar to the control (first bar) (Figure 3.2D). Some have a lower or higher skipping rate than the control. The most striking candidate is CELF2 with 8 % PMA-induced exon skipping (Figure 3.2D, highlighted in yellow). CELF1 from the same protein family (colored in orange) was also analyzed but was not seen to have an impact on TRAF3 splicing. This indicates that the effect of CELF2 is highly specific. The CELF family in general is known to regulate alternative splicing events (Vlasova-St. Louis et al. 2013).

3.1.3 CELF2 is *trans*-acting factor in TRAF3 splicing

The siRNA screen identified CELF2 as a putative candidate for regulation of TRAF3 splicing because its knockdown shows the strongest effect on splicing. Further analyses focused on CELF2. Due to the fact that the screen data are based on a single transfection the data from the screen were validated. Jsl1 cells were again transfected with CELF2 siRNA in at least three independent transfections. The knockdown of CELF2 expression was analyzed on the protein level (Figure 3.3A). CELF2 knockdown is very efficient as the western blot shows almost no remaining CELF2. This was supported by qRT-PCR data that revealed a remaining CELF2 mRNA level of around 20 % (data not shown). The results of the RT-PCRs investigating TRAF3 splicing upon CELF2 knockdown are shown in Figure 3.3B and 3.3C.

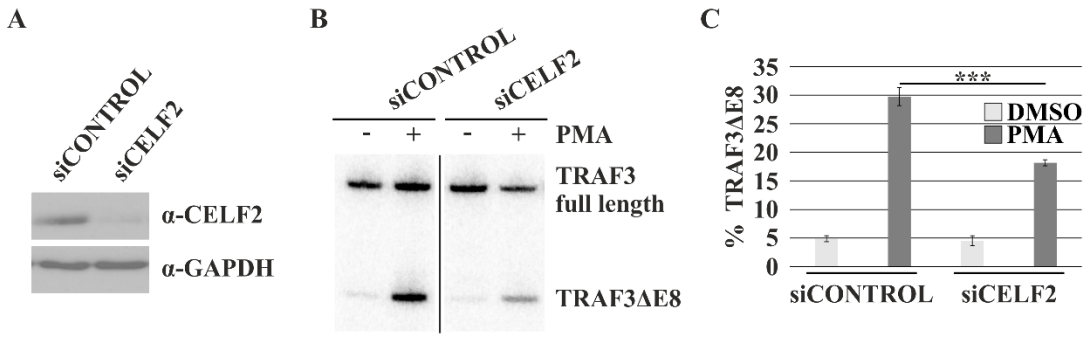

Figure 3.3: CELF2 is *trans*-acting factor in TRAF3 exon 8 splicing.
Jsl1 cells were transfected with the indicated siRNAs, split and stimulated with DMSO or PMA for 48 h.
A: CELF2 knockdown was validated by western blot.
B: Representative gel of a TRAF3 RT-PCR
C: Quantification of TRAF3 RT-PCRs (n=4; error bars: S.D.; ***p<0.001).

In DMSO treated cells CELF2 knockdown does not have an effect on TRAF3 exon 8 skipping but in PMA stimulated cells it leads to a significant decrease in TRAF3ΔE8 formation (from ~30 % to ~18 %, Figure 3.3C). The direct compare of the screen data with the validation data reveal a less strong effect of TRAF3 exon 8 skipping in the validation data (PMA-induced exon skipping rate is 13 % in the validation and 8 % in the siRNA screen). The weaker effect on splicing can be explained by the use of different transfection methods. In the screen cells were transfected via electroporation while a transfection reagent was used in the validation experiment. Nevertheless the validation strongly indicates that CELF2 regulates TRAF3 exon 8 skipping. CELF2 is known to regulate alternative splicing of cTNT exon 5 (Ladd et al. 2001) and its expression is crucial in embryonic (Blech-Hermoni et al. 2013) and heart development (Kalsotra et al. 2008). It furthermore regulates LEF1 exon 6 splicing in response to T cell activation (Mallory et al. 2011) and was found to be involved in the regulation of several alternative splicing events in activated T cells (Mallory et al. 2015).

The observation that CELF2 regulates TRAF3 exon 8 splicing raises the question if it directly binds to the identified *cis*-acting element. CELF2 has a size of 52 kDa and might correspond to the ~55 kDa band observed in the UV-Crosslink analyses (Figure 3.2A). To validate this possibility new UV-Crosslink analyses were done and coupled to immunoprecipitations. CELF2 antibody precipitates the 55 kDa band (Figure 3.4A). An immunoprecipitation with an antibody against CELF1 results in no band (Figure 3.4B) showing that CELF2 binding is specific. These results strongly suggest direct and specific binding of CELF2 to the *cis*-acting element and this binding has a regulatory function in TRAF3 exon 8 splicing.

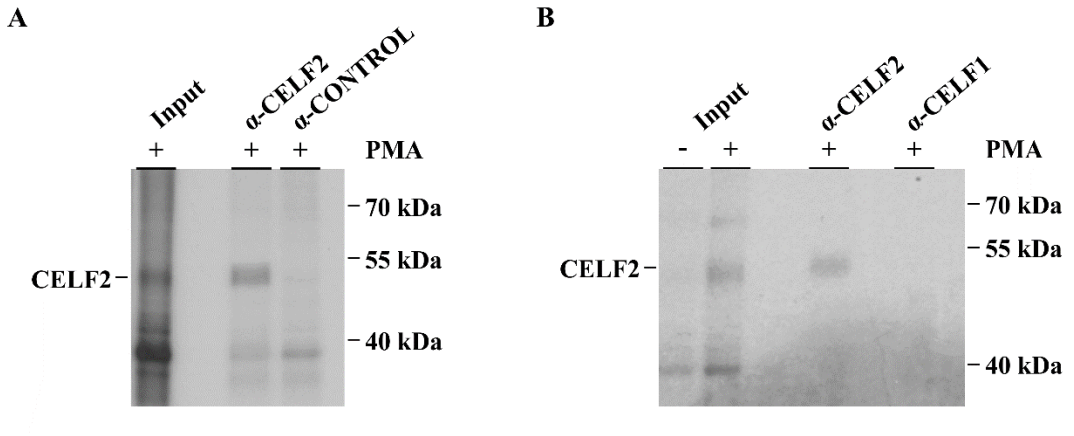

Figure 3.4: CELF2 directly binds to the *cis*-acting element.
UV-Crosslink immunoprecipitations were carried out with the TRAF3 *cis*-acting element and nuclear extracts from unstimulated (-PMA) and stimulated Jsl1 cells (+PMA). In both cases a representative gel is shown from at least three independent experiments.
A: Immunoprecipitation was done with antibodies against CELF2 or a control (α-HA).
B: Antibodies against CELF2 or CELF1 were used.

3.1.4 CELF2 expression regulates TRAF3ΔE8 formation and the activation of the ncNFκB pathway

Consistent with an increased binding of CELF2 to the ISS in activated T cells the expression of CELF2 is upregulated in PMA treated cells (Figure 3.5A) (Mallory et al. 2015). Together with our results published in Michel et al. (2014) which stated an activation of the ncNFκB pathway by TRAFΔE8 the following model can be proposed (Figure 3.5B): in resting T cells CELF2 expression is low and TRAF3 exon 8 is mainly included in the mRNA. In response to T cell activation CELF2 expression increases. Its binding to the *cis*-acting element induces exon 8 skipping. The resulting TRAF3ΔE8 isoform activates the ncNFκB pathway and chemokine expression is upregulated.

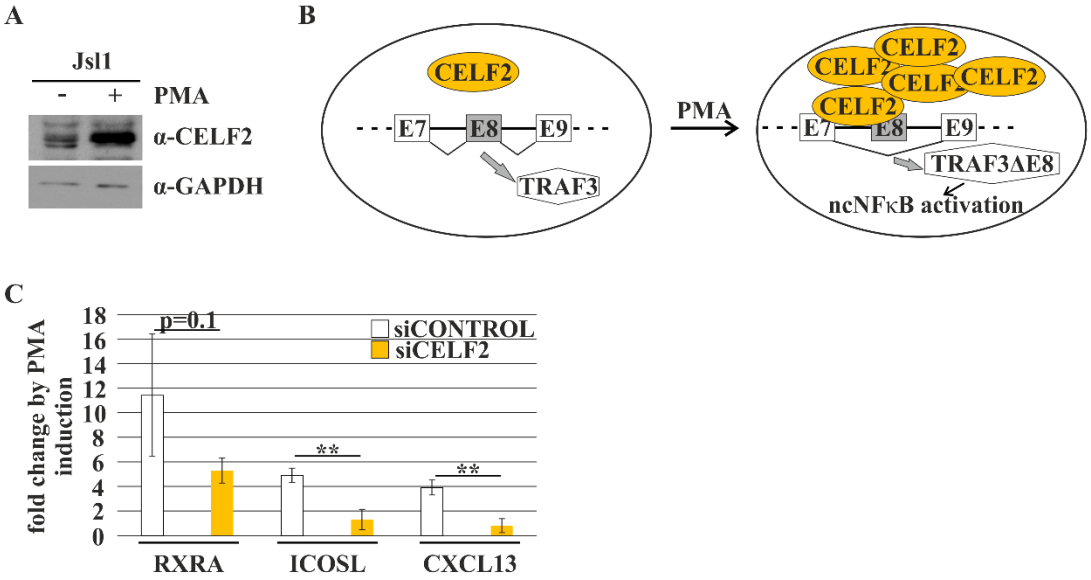

Figure 3.5: CELF2 expression induces TRAF3 exon 8 skipping and activates the ncNFκB pathway.

A: Jsl1 cells were either treated with DMSO or PMA for 48 h. Lysates were used for western blot analysis with CELF2 and GAPDH antibodies. GAPDH is loading control.

B: Model summarizing the previously shown data: T cell activation upregulates CELF2 expression and leads to its binding to the TRAF3 *cis*-acting element. This results in TRAF3ΔE8 formation and activates the ncNFκB pathway.

C: Jsl1 were transfected with the indicated siRNAs and grown in the presence or absence of PMA for 48 h. Induction of ncNFκB target genes was measured by qRT-PCR. Expression was normalized to GAPDH and a fold induction upon PMA stimulation was calculated (n=3; error bars: S.D.; **p<0.01).

According to the proposed model CELF2 knockdown should prevent the activation of the ncNFκB pathway. To check this hypothesis and verify this model Jsl1 cells were either transfected with siCONTROL or siCELF2. Cells were treated as described before. As a read out for ncNFκB pathway activation the expression of three different chemokines was analyzed by qRT-PCR. These chemokines were chosen based on our previously work (Michel et al. 2014). The induced expression of RXRA, ICOSL and CXCL13 is reduced in CELF2 knockdown cells compared to the control cells (Figure 3.5C). The effect is significant for ICOSL and CXCL13. The result strengthens the proposed model.

3.1.5 *Cis*-acting element might be sufficient to induce splicing

The minigene analyses which were conducted to identify the *cis*-acting element revealed the necessity of the 96 nt long element but it does not address the question of wether this element is sufficient to induce splicing. An element is sufficient if it can induce splicing of unrelated

exons. To address this the TRAF3 *cis*-acting element was cloned into a vector with a CD45 background. This vector contains the CD45 exon 9 as an alternative exon which is surrounded by the constitutive CD45 exon 3 and 7 (Figure 3.6A). The same vector without the TRAF3 *cis*-acting element was used as a control.

Jsl1 cells were transfected with both constructs and exon 9 skipping was analyzed using radioactive RT-PCR. In cells transfected with the control minigene, exon 9 was fully included (data not shown). This indicates that the splice sites are too strong for this approach and these constructs cannot be used to investigate the sufficiency of the TRAF3 *cis*-acting element. New constructs were cloned in which either the 5'splice site, the 3'splice site or both splice sites were weakened. Jsl1 cells were transfected with these constructs and their splicing was analyzed using RT-PCR. The weaken splice sites result in almost 100 % exon skipping. An effect of the *cis*-acting element could not be observed due to the already strong exon skipping rate in control cells (data not shown).

The skipping rate was lower when Hek cells were transfected with these minigenes. That is why Hek cells were used as a model system for this experiment. The advantage of using Heks is a higher transfection efficiency compared to Jsl1 cells. However a critical point is the missing endogenous CELF2 expression (Figure 3.6B, empty vector control). This means the cellular environment is different in Heks compared to Jsl1 cells and CELF2 has to be overexpressed. Hek cells were co-transsfected with a plasmid encoding CELF2-MYC-HIS and either a minigene containing the TRAF3 *cis*-acting element (CD9T3) or a control without this element (CD9) (Figure 3.6A). The expression of CELF2-MYC-HIS was measured by western blot analysis (Figure 3.6B). The minigene splicing was visualized in radioactive RT-PCR using primers that bind in the minigenes. CELF2-MYC-HIS expression increases CD45 exon 9 skipping in cells transfected with CD9 (Figure 3.6C). In cells transfected with CD9T3 the exon skipping increases by 2-fold compared to the control (Figure 3.6C). Although only the sufficiency of the *cis*-acting element was addressed in these minigene experiments the expression of CELF2 itself shows an impact on exon skipping.

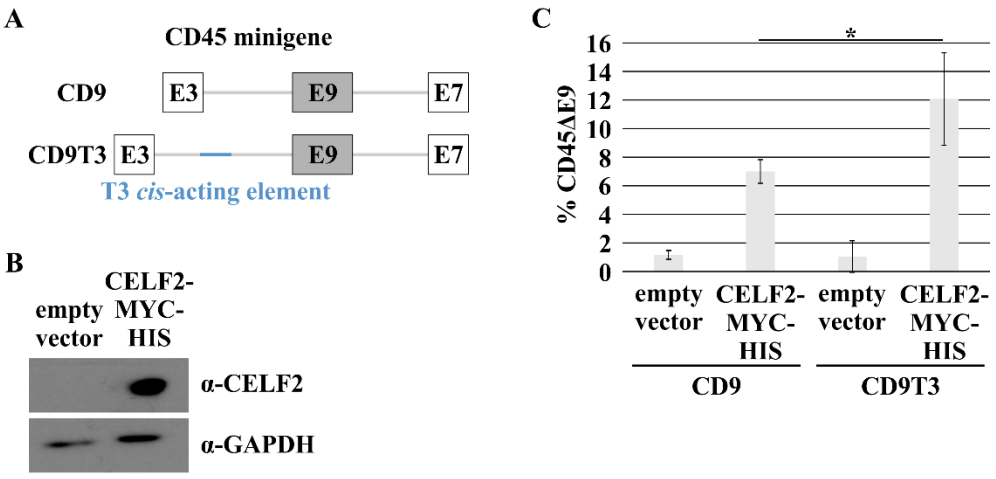

Figure 3.6: TRAF3 exon 8 *cis*-acting element can induce CD45 exon 9 splicing.
A: Schematic presentation of the used minigene constructs. Construct CD9T3 contains the TRAF3 *cis*-acting element approximately 200 bp upstream of exon 9 (shown in blue). Boxes represent exons and lines depict intronic sequences.
B: Hek cells were co-transsfected with CELF2 or an empty vector control and the minigenes shown in A. Cells were harvested 48 h post-transfection and western blot was performed with the indicated antibodies. GAPDH served as loading control. A representative blot is shown.
C: Cells were transfected as described in B. Minigene splicing was analyzed with RT-PCR and was quantified (n=3; error bars: S.D.; $p<0.05$).

In summary the data from Hek cells indicate that the TRAF3 *cis*-acting element is sufficient to induce splicing although the observed effect is small. The observation that CELF2-MYC-HIS overexpression induces exon skipping in the control cells might indicate a CELF2 binding to already existing intronic sequences in the minigene. It raises the question, to which sequences does CELF2 bind.

3.1.6 CELF2 binds to the U-stretches in the TRAF3 *cis*-acting element

The binding of CELF2 to the *cis*-acting element was investigated in more detail. To narrow down the CELF2 binding site the *cis*-acting element was divided into three parts with lengths of 34 nt, 31 nt and 31 nt (Figure 3.7A). All three parts were individually *in vitro* transcribed and UV-Crosslink analyses were done with nuclear extracts from stimulated and unstimulated Jsl1 (Figure 3.7B).

Figure 3.7: CELF2 binds to U-stretches within the *cis*-acting element.
A: The 96 nucleotides long *cis*-acting element was divided into three parts as shown in the overview. Capital letters mark the U-stretches.
B: UV-Crosslink analyses were done with sequences illustrated in A and nuclear extracts from unstimulated (DMSO) and stimulated (PMA) Jsl1 cells. A representative gel is shown.

As previously observed CELF2 binding increases in response to PMA stimulation. Notably part 1 as well as part 3 bind CELF2, but not part 2 and CELF2 binding is stronger in part 3 compared to part 1. A closer look at the sequences reveals that part 1 and part 3 contain U-stretches (5 uridines in a row) which are absent in part 2 (Figure 3.7A, marked with capital letters). Consistent with a stronger CELF2 binding in part 3, this sequence part has two U-stretches whereas part 1 contains only one. To prove a dependency of CELF2 binding on the presence of U-stretches UV-Crosslink experiments were carried out with mutated sequences. Since part 3 shows the strongest CELF2 binding the mutational analyses focused on this part (from now on called Wt). The sequences are shown in Figure 3.8A. In the sequence called U1 the first U-stretch is disrupted by adenines. In contrast U2 has nucleotide changes in both U-stretches. In the third sequence (G2) the guanosines which follow the uridine rich sequence are converted to cytosines. All these constructs were *in vitro* transcribed and incubated with Jsl1 nuclear extracts. UV-Crosslink analyses reveal that mutations within the U-stretches have no effect on CELF2 binding or might lead to an even stronger binding (Figure 3.8B). The guanosine-to-cytosine mutated sequence (G2) completely loses the ability to bind CELF2. These experiments narrow down the CELF2 binding to a single nucleotide, precisely the guanosine following the U-stretches (underlined in Figure 3.8A).

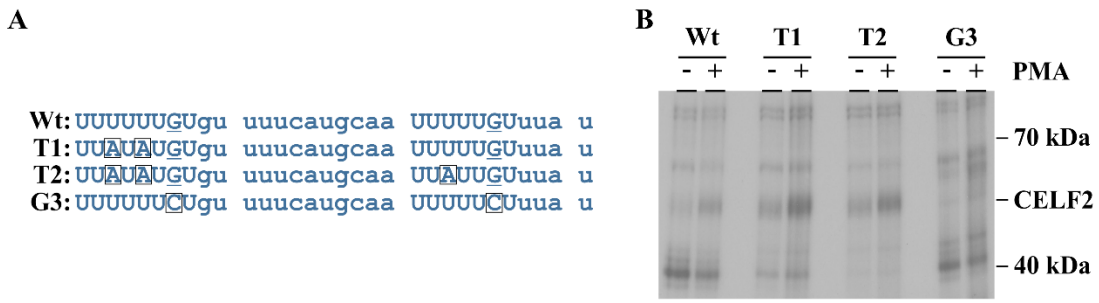

Figure 3.8: CELF2 binds specifically to a single guanosine within UUUUUGU-motifs.
A: Depicted are sequence part 3 and different mutated sequences used for UV-Crosslinks in B. Nucleotides, which are mutated from the wild type sequence are marked with boxes. U-stretches are highlighted with capital letters and the guanosines important for CELF2 binding are underlined.
B: UV-Crosslink analysis with the sequences shown in A and nuclear extract from unstimulated (-PMA) and stimulated (+PMA) Jsl1 cells. Shown is a representative gel from at least three independent experiments.

With the new finding that the guanosine within the UUUUUGU-motif is crucial for CELF2 binding the observed data in Heks which investigated a putative sufficiency of the *cis*-acting element can be explained (chapter 3.1.5). The minigenes used in these experiments contain CD45 intronic regions that have UUUUUGU-motifs. CELF2 may bind to these motifs and induces exon skipping without the presence of the TRAF3 *cis*-acting element. The insertion of the *cis*-acting element increases the number of these motifs and strengthens the effect on exon 9 skipping. The Hek data provide a hint for a sufficiency of the TRAF3 *cis*-acting element but new minigene experiments are needed to proof a sufficiency.

3.1.7 CELF2 expression is sufficient to induce TRAF3 splicing

The CELF2 knockdown experiments demonstrated that CELF2 expression is necessary for TRAF3 splicing but the remaining question is whether it is also sufficient to induce TRAF3 splicing. This question can be addressed by overexpressing CELF2 in cells, which neither express CELF2 endogenously nor skip TRAF3 exon 8. Hek cells lack CELF expression as shown in the minigene experiments in Figure 3.6.
Hek cells were transfected with GFP-tagged CELF2 and harvested 48 h later. CELF2-GFP is expressed (Figure 3.9A). TRAF3 splicing was analyzed with radioactive RT-PCR (Figure 3.9B and 3.9C). Cells transfected with GFP control have almost no TRAF3ΔE8 but CELF2-GFP overexpression significantly induces exon 8 skipping to 23 %.

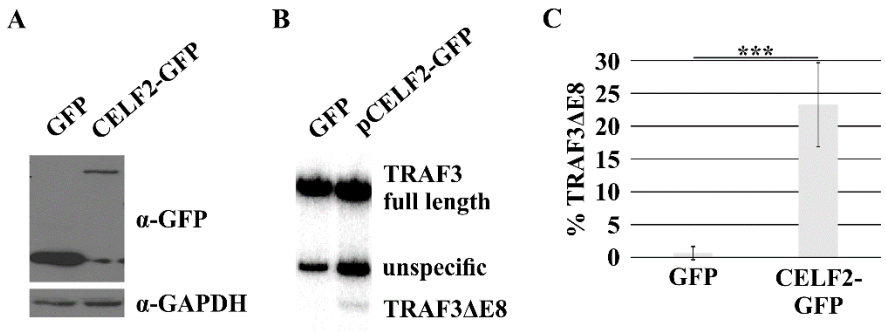

Figure 3.9: CELF2 is sufficient to induce TRAF3 splicing.
Hek cells were transfected with CELF2-GFP or GFP and were harvested 48 h post-transfection.
A: The representative blot verifies CELF2-GFP expression.
B: TRAF3 splicing was analyzed by RT-PCR. A representative gel is shown.
C: RT-PCRs were quantified (n=6; error bars: S.D.; $^{***}p<0.001$).

The same experiment was repeated with the human B cell line Dg75. These cells were also transiently transfected. The results show a similar trend (data not shown) although the effect was smaller. Since transfection efficiency in Dg75 cells is considerably lower than in Hek cells the results indicate an influence of transfection efficiency on the result. A better system to study CELF2 overexpression is a stable transfection where the foreign DNA is integrated into the genome. Raji and Ramos cells, both human B cell lines were used for such a stable transfection. Both cell lines are suitable for these experiments because both have no TRAF3 exon 8 skipping upon PMA stimulation (Figure 3.10A and 3.10B) (Michel et al. 2014). The model proposed in Figure 3.5B states that CELF2 binding is necessary to induce TRAF exon 8 skipping and activation of the ncNFκB pathway. Since both B cell lines do not express CELF2 (western blot in 3.10C) these cells are a good system to investigate if CELF2 expression is sufficient to induce TRAF3 exon 8 skipping or if further signals are needed.

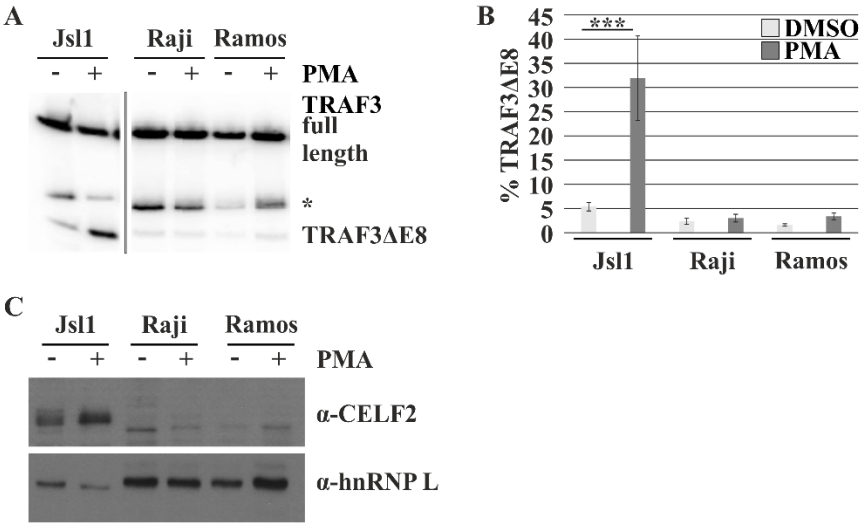

Figure 3.10: TRAF3 exon 8 splicing in Raji and Ramos cells.
A: Jsl1, Raji and Ramos cells were treated with DMSO or PMA for 48 h and RT-PCR was carried out to analyze TRAF3 exon 8 splicing. A representative gel is shown. An asterisk marks an unspecific band that comigrates with the loading dye front.
B: Quantification of RT-PCRs as shown in A (n≥5; error bars: S.D.; ***p<0.001).
C: Cells were treated as described in A and a western blot was done with the indicated antibodies. HnRNP L served as loading control. A representative blot of at least three independent experiments is illustrated.

For the generation of stable cell lines CELF2 was cloned into the pEF-MYC-HIS vector resulting in a MYC-HIS tagged protein. The reason for the additional cloning step is the presence of a CMV-promoter in the CELF2-GFP plasmid. This promoter is inducible by PMA. This would lead to an increased CELF2 expression in response to stimulation and this might influence the results. In contrast the MYC-HIS plasmid has a T7-promoter which does not respond to PMA. Raji and Ramos cells were transfected with the linearized CELF2-MYC-HIS plasmid and geneticin was used to select for positive clones. Transfection of Raji cells did not result in positive clones but one Ramos cell line was generated that expresses CELF2-MYC-HIS (western blot in 3.11A). The empty vector control are cells which went through the same procedure as the cells expressing CELF2-MYC-HIS but do not express it. A second control are parental Ramos cells that were not transfected. TRAF3 splicing was analyzed in the newly generated cells by radioactive RT-PCR. CELF2-MYC-HIS overexpression induces exon 8 skipping (Figure 3.11B) by increasing it from around 8 % to approximately 55 %. Stimulation with PMA has little effect on the skipping level. CELF2 expression is sufficient to induce TRAF3 exon 8 splicing.

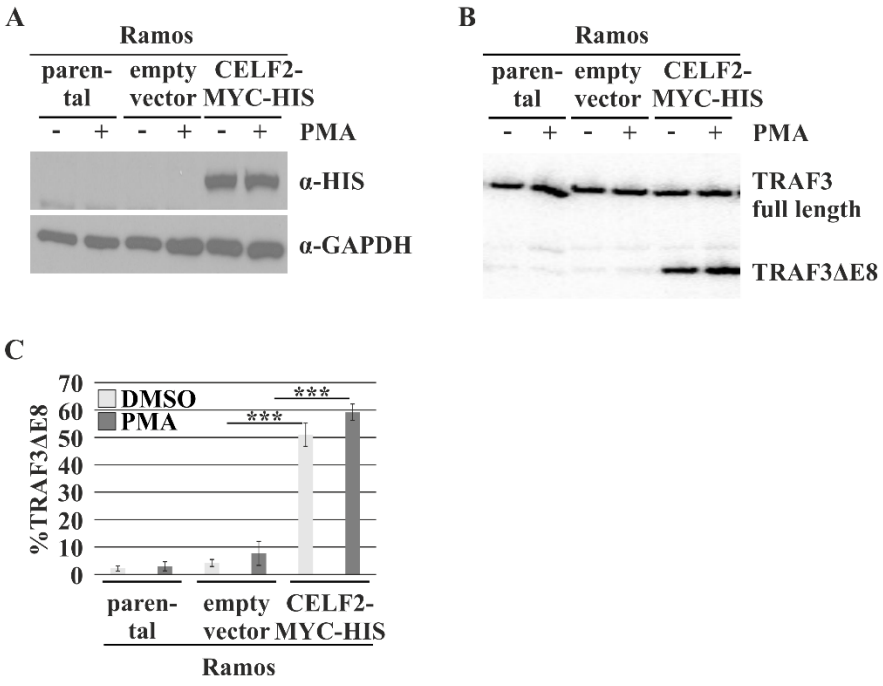

Figure 3.11: Stable expression of CELF2-MYC-HIS in Ramos cells induces TRAF3 exon 8 skipping.

A: Lysates of various Ramos cell lines that were treated with DMSO or PMA for 48 h were used for western blots with the indicated antibodies. GAPDH served as loading control.

B: TRAF3 exon 8 splicing in cells treated as described in A was analyzed with RT-PCR. A representative gel is shown.

C: Quantification of RT-PCRs is depicted (n≥4; error bars: S.D.; ***p<0.001).

CELF2 regulates several other splicing events in Jsl1 in response to T cell activation (Mallory et al. 2011; Martinez et al. 2015; Mallory et al. 2015). To explore whether a similar effect were observed three other targets were investigated, namely PPP1R12A, BRD8 and NAB2. PPP1R12A and BRD8 showed a significant increase in exon skipping when CELF2-MYC-HIS was overexpressed in Ramos cells compared to the controls (Figure 3.12).

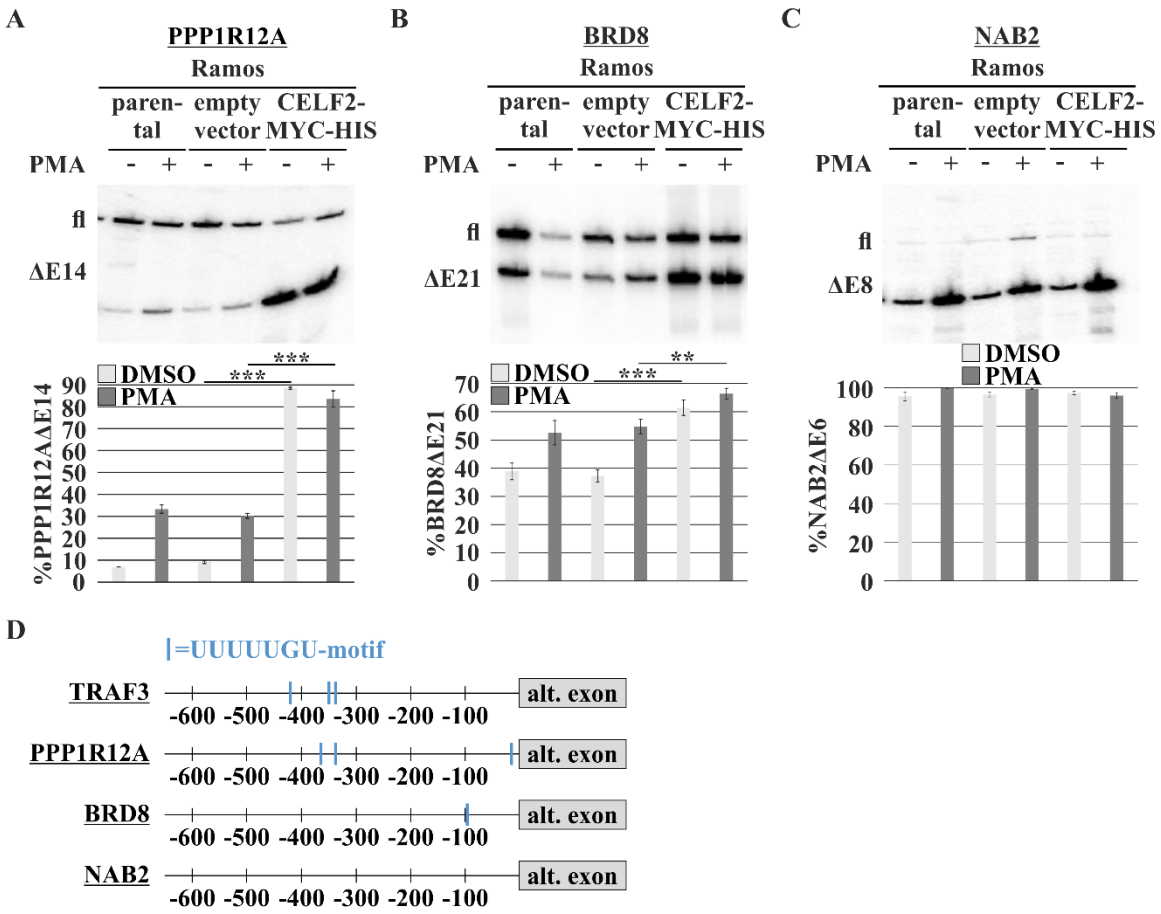

Figure 3.12: CELF2-MYC-HIS expression induces skipping of several PMA responsive exons.
A: RNAs from resting (DMSO) and activated (PMA) Ramos cell lines were analyzed with regard to PPP1R12A splicing by RT-PCR. A representative gel is shown on top and the quantification below (fl = full length; n≥3; error bars: S.D.; ***p<0.001).
B: Same cells as in A were used for RT-PCR to analyze BRD8 splicing. A representative gel and a quantification is shown (fl = full length; n≥3; error bars: S.D.; **p<0.01, ***p<0.001).
C: Same as in A and B but NAB2 splicing was investigated (fl = full length; n≥3; error bars: S.D.).
D: A schematic presentation of the 600 nt long intronic region upstream of the alternative spliced exons of TRAF3, PPP1R12A, BRD8 and NAB2. A blue dash indicates the presence of an UUUUUGU-motif. Sizes are not to scale.

The effect of CELF2-MYC-HIS expression is stronger for PPP1R12A, where skipping increases from around 10 % to 90 % in unstimulated cells (Figure 3.12A) compared to BRD8. In BRD8-PCR the short isoform rises from 40 % to 62 % in unstimulated cells (Figure 3.12B). NAB2 splicing is not affected by CELF2-MYC-HIS expression (Figure 3.12C). The analyses of CELF2 binding to the *cis*-acting element that was previously shown already demonstrated that CELF2 binds to a single nucleotide within a UUUUUGU-motif. The intronic sequences of all investigated targets were examined with respect to the presence or absence of such an

element. Indeed PPP1R12A and BRD8 have an UUUUUGU-motif but not NAB2 (Figure 3.12D). This could explain the missing effect of CELF2-MYC-HIS expression on NAB2 splicing. PPP1R12A has three of these sequence motifs and BRD8 only one. This could give an explanation for the weak effect observed in exon skipping in BRD8.

To test the hypothesis that an UUUUUGU-motif can predict an alternative splicing regulation by CELF2 further targets were analyzed. Two targets have such a motif, MACF1 and REPS1 (Figure 3.13A). Three other genes ARMC10, MYL6 and FAM33A were investigated that do not have a UUUUUGU-motif within a length of 600 nt upstream of the intron (Figure 3.13A). All chosen targets were previously shown to be alternatively spliced upon T cell activation (Martinez et al. 2012). MACF1 and REPS1 show more exon skipping in Ramos cells expressing CELF2-MYC-HIS compared to control cells (Figure 3.13B). In contrast ARMC10, MYL6 and FAM33A, do not change skipping rates in cells expressing CELF2-MYC-HIS (Figure 3.13C). Although the number of investigated genes is low the general trend is that the presence of an UUUUUGU-motif may be a first hint for a regulation by CELF2.

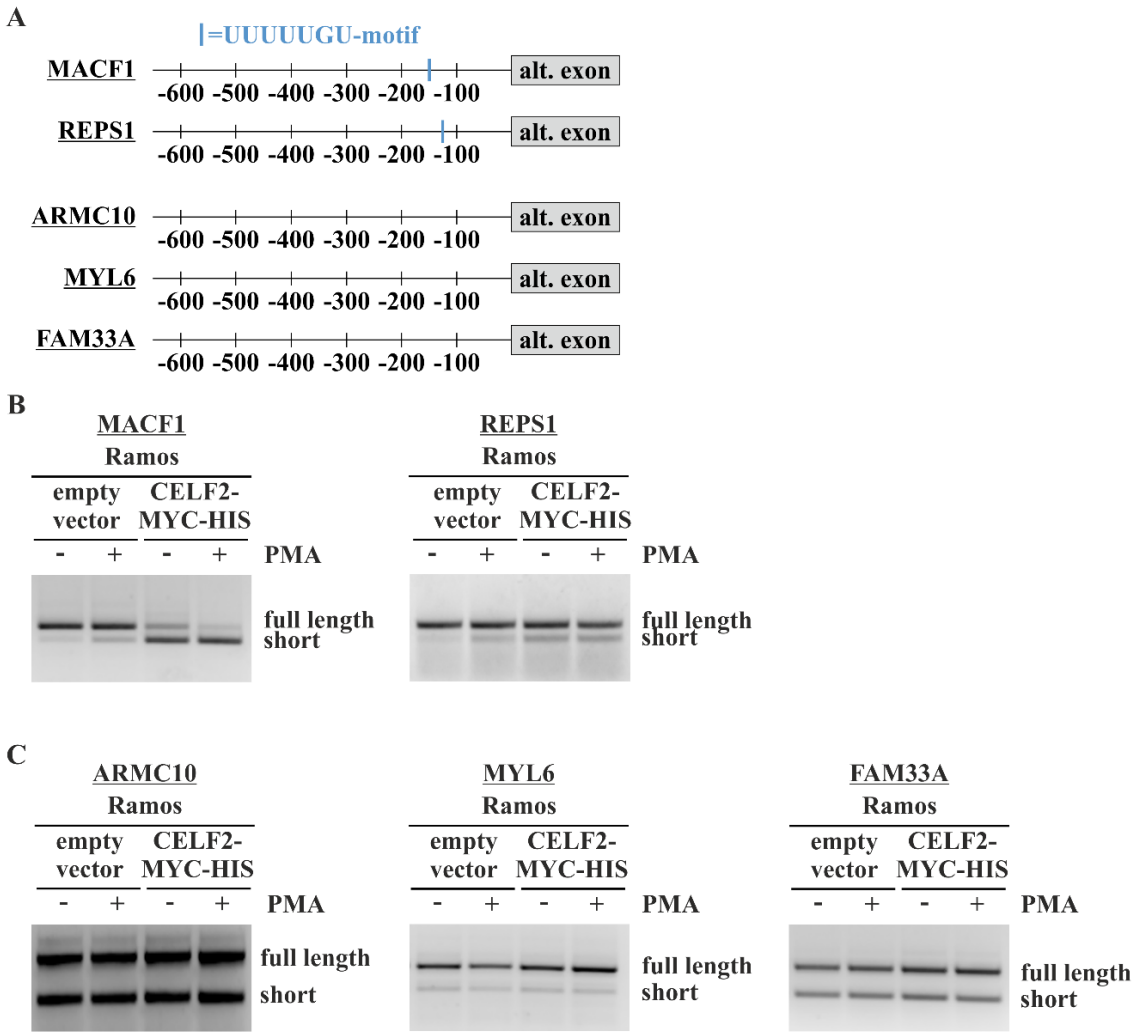

Figure 3.13: The presence of an UUUUUGU-motif is a first indicator for a response to CELF2 overexpression.

Ramos-CELF2-MYC-HIS cells and control cells were treated with DMSO or PMA and alternative splicing of different exons was investigated with traditional non-radioactive RT-PCR (n≥3).

A: Scheme showing the 600 nt intronic region upstream of the alternatively spliced exons of several genes. The blue dash highlights the presence of an UUUUUGU-motif. Sizes are not to scale.

B: Exons that respond to CELF2-MYC-HIS overexpression with more exon skipping are shown.

C: CELF2-MYC-HIS overexpression has no influence on alternative splicing of these exons.

3.1.8 Other *trans*-acting factor(s)

There are several indications that CELF2 is not the only *trans*-acting factor involved in the regulation of TRAF3 splicing. 1) There are more knockdowns which had an impact on TRAF3 splicing in the siRNA screen. 2) A CELF2 knockdown leads to a decrease in exon 8 skipping but not to a total loss, indicating that more factors are needed. 3) Multiple proteins bind to the

cis-acting element in the UV-Crosslinks and at least one protein, which has a size of around 40 kDa changes its binding affinity upon stimulation.

In UV-Crosslink experiments which further narrowed-down the binding site of CELF2 it was observed that mutation of the uridines within the U-stretches lead to a complete loss of binding of the 40 kDa protein (Figure 3.8B) and a slight increase in CELF2 binding. This indicates that this protein binds antagonistically to CELF2 and might be involved in the regulation of TRAF3 exon 8 splicing. To test this hypothesis the 40 kDa protein has to be identified.

The siRNA screen includes two 40 kDa proteins which are known to bind to uridine sequences and regulate alternative splicing events, TIA1 and PTB (Izquierdo et al. 2005). TIA1 popped up in the screen as a putative protein involved in TRAF3 splicing but the screen data could not be reproduced. TIA1 was a false positive in the screen. PTB knockdown did not show any effect on TRAF3 exon 8 skipping and it was not further considered.

HUR is a 40 kDa protein which is well-known to bind to U-stretches (Ma et al. 1996). To examine if HUR binds to the TRAF3 *cis*-acting element UV-Crosslinks were coupled to immunoprecipitation experiments. The protein could not be precipitated (data not shown). Another putative protein with similar size and binding preference could be hnRNP C (Görlach et al. 1992). hnRNP C is known to regulate splicing by competing with U2AF65 for cryptic splice sites (Zarnack et al. 2013). A further UV-Crosslink immunoprecipitation experiment was performed and in this case hnRNP C could be precipitated with anti-hnRNP C (Figure 3.14A). This verified a direct binding of hnRNP C to the TRAF3 *cis*-acting element.

To confirm that the binding of hnRNP C is lost upon mutations in the U-stretches the same transcripts were used as in Figure 3.8 (Figure 3.14B, bottom). Neither in the transcript in which two Us are mutated (T1) nor in the transcript with more mutations (T2) could hnRNP C be precipitated. These data demonstrate that hnRNP C binds to the U-stretches within the TRAF3 *cis*-acting element. Consistent with the observed decline in binding upon stimulation in first the UV-Crosslink experiments (Figure 3.2A), hnRNP C expression decreases in activated T cells compared to resting cells (Figure 3.14B). The hnRNP C expression is not influenced by CELF2 knockdown (Figure 3.14C).

The UV-Crosslink experiments additionally showed that CELF2 binding increases upon T cell activation whereas hnRNP C binding decreases. The antagonistic binding of hnRNP C might indicate a competition between CELF2 and hnRNP C for binding sites. A competitive binding was already shown for hnRNP C and U2AF65 (Zarnack et al. 2013) as mentioned earlier.

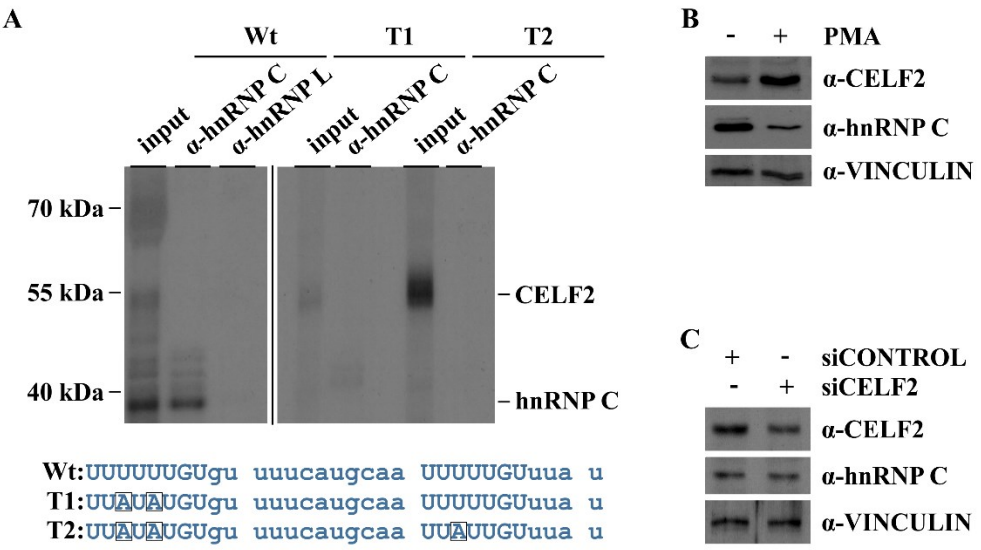

Figure 3.14: hnRNP C binds to the TRAF3 *cis*-acting element.

A: The shown sequences were *in vitro* transcribed and UV-Crosslink immunoprecipiations were performed with the indicated antibodies and nuclear extracts from unstimulated Jsl1 cells. HnRNP L served as control.

B: Lysates from resting (-PMA) and stimulated (+PMA) Jsl1 cells were used for a western blot analysis with the indicated antibodies.

C: Jsl1 cells were transfected with siCONTROL or siCELF2, split and stimulated with either DMSO or PMA 24 h post-transfection. Cells were harvested 48 h post-stimulation. A western blot was carried out with the indicated antibodies.

Representative blots from at least three independent experiments are shown. VINCULIN served as loading control in B and C.

To investigate a putative role of hnRNP C in regulation of TRAF3 exon 8 splicing and a putative antagonistic function to CELF2, a knockdown experiment was performed.

To this end Jsl1 cells were transfected with sihnRNP C, stimulated 24 h later and harvested 72 h post-transfection. Although several siRNAs against hnRNP C were tested as well as various transfection methods, no knockdown could be detected via qRT-PCR. The controls siGAPDH and siCELF2 led to a knockdown (data not shown). It was concluded that the transfection itself is efficient but a knockdown is not viable in Jsl1 cells. Consequently another system was used to investigate the influence of hnRNP C on TRAF3 splicing. Transfection of Hek cells is very efficient and Zarnack et al. (2013) published hnRNP C knockdowns demonstrating that these cells are viable.

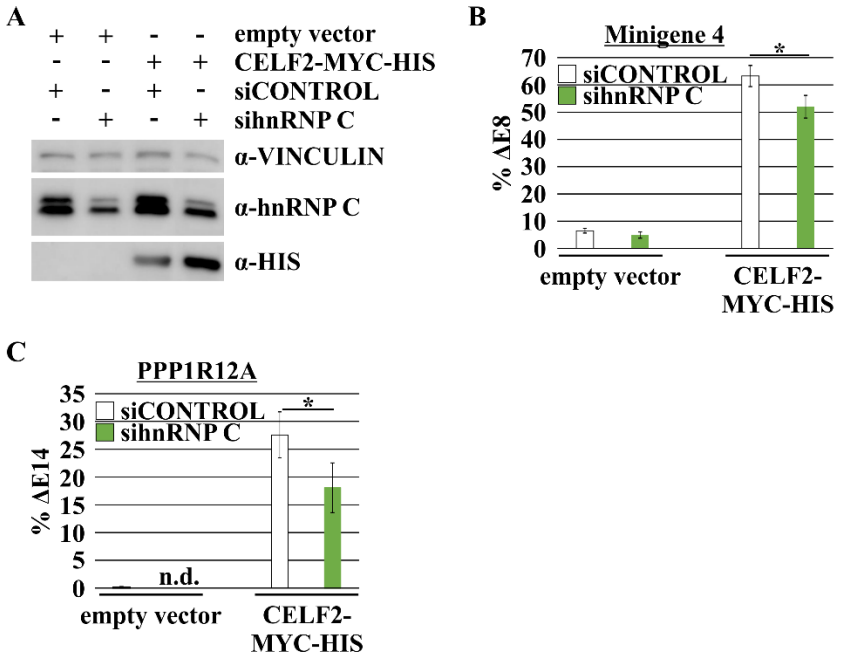

Figure 3.15: hnRNP C knockdown in Hek cells.

A: Hek cells were co-transsfected with sihnRNP C (or a control), CELF2-MYC-HIS (or empty vector) and minigene 4. 48 h post-transfection cell lysates were prepared and a western blot was performed with the indicated antibodies. VINCULIN served as a loading control and a representative blot is shown.

B/C: TRAF3 minigene splicing (B) or PPP1R12A exon 14 splicing (C) of cells transfected as described in A (n.d. = not detectable; n=3; error bars: S.D. *p<0.05).

Hek cells were co-transfected with CELF2-MYC-HIS or an empty vector control, sihnRNP C or siCONTROL and minigene construct 4, which exhibits a splicing comparable to endogenous TRAF3 exon 8 splicing. The transfection of Hek cells results in an efficient knockdown in hnRNP C expression (Figure 3.15A). The western blot shows two bands for hnRNP C that correspond to the isoforms hnRNP C1 and hnRNP C2. CELF2-MYC-HIS co-transfection was successful (western blot in Figure 3.15A). Splicing of minigene 4 was analyzed by radioactive RT-PCR (Figure 3.15B). In the empty vector control knockdown of hnRNP C has no impact on exon 8 skipping. Cells which overexpress CELF2-MYC-HIS, show a decrease in % ΔE8 of about 10 % in response to hnRNP C knockdown. This shows that hnRNP C does not act antagonistically to CELF2 but induces exon skipping, meaning both work synergistically in exon 8 skipping. Additionally splicing of PPP1R12A was analyzed and validated this finding as an independent target (Figure 3.15C). Exon skipping is decreased when cells are co-transsfected with CELF2-MYC-HIS and sihnRNP C. Consistent with the observation that CELF2 and hnRNP C are needed for efficient TRAF3 exon 8 splicing, Ramos cells which were used to analyze the sufficiency of CELF2 overexpression (3.1.7), express hnRNP C

(Figure 3.16). The expression of hnRNP C in Ramos cells additionally indicates that hnRNP C is not sufficient for TRAF3 splicing because parental Ramos cells do not skip TRAF3 exon 8. These data point to an antagonistic binding of hnRNP C and CELF2 and to a synergistic action of both proteins in inducing TRAF3 exon 8 skipping.

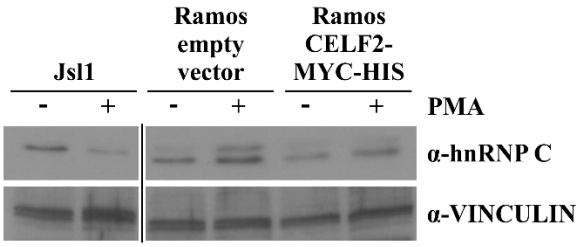

Figure 3.16: hnRNP C expression in Jsl1 and Ramos cell lines.
Jsl1 and Ramos cell lines were treated with DMSO or PMA and cell lysates were prepared 48 h post-stimulation. Western blots were conducted with the indicated antibodies. VINCULIN served as loading control. A representative gel from at least three independent experiments is shown.

3.1.9 CELF2 and hnRNP C in TRAF3 splicing

The previous data suggest a synergistic regulation by CELF2 and hnRNP C. However the situation in Heks is artificial because two different plasmids and an siRNA have to be co-transfected to simulate a *trans*-acting environment in Hek cells that is comparable to the situation in Jsl1 cells. A knockdown of hnRNP C is not viable in Jsl1 cells leading to the requirement of another model to analyze the impact of CELF2 and hnRNP C on TRAF3 exon 8 splicing in these cells. A minigene approach was used that took advantage of the binding assay results, which uncovers the CELF2 and hnRNP C binding sites. This means TRAF3 minigenes which have mutations in CELF2 or hnRNP C binding sites were cloned. CELF2 or hnRNP C cannot bind to this *cis*-acting element comparable to a knockdown condition in which not enough protein is expressed to bind to the element.

Controls are again minigene 4 which mimics endogenous TRAF3 splicing, and minigene 3 which lacks the *cis*-acting element and lost the ability to respond to PMA. Jsl1 cells were transfected with all these minigenes (Figure 3.17) and stimulated with PMA or DMSO one day later. Cells were harvested 48 h post-stimulation. Splicing of the minigenes was again analyzed by radioactive PCR with primers that specifically bind to the minigene. All minigenes that have mutations in CELF2 or hnRNP C binding sites cannot respond to PMA and show the same splice pattern as the control, which does not have the *cis*-acting element. Splicing of the control

minigene 4 demonstrates a significant increase in TRAF3ΔE8 (Figure 3.17B) upon stimulation as observed before. This leads to the conclusion that hnRNP C and CELF2 are the main factors involved in the regulation of TRAF3. It should be remembered that other proteins may bind to the same sequence motifs. Both *trans*-acting factors work synergistically in inducing exon skipping.

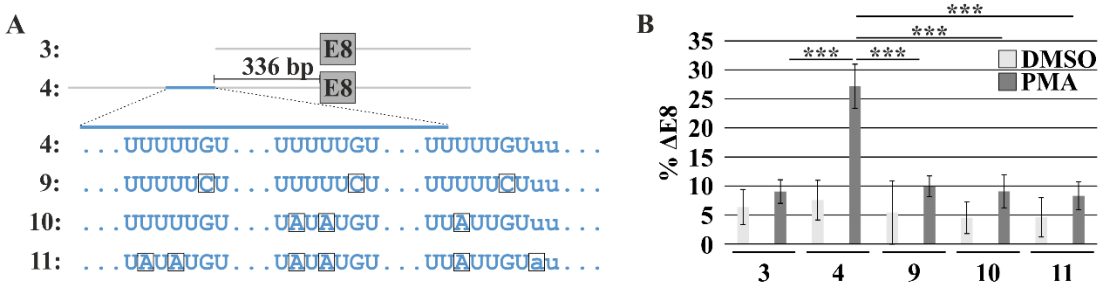

Figure 3.17: Minigenes lacking CELF2 or hnRNP C binding do not respond to PMA stimulation.

A: Scheme showing the TRAF3 minigenes that were used for Jsl1 transfection. Mutations within the *cis*-acting element are highlighted with boxes.

B: Jsl1 cells were transfected with the minigenes shown in A and cells were treated with DMSO or PMA for 48 h. The RT-PCR results were quantified (n≥3; error bars: S.D.; ***p<0.001).

A remaining question is, how are these proteins involved in exon skipping. It is also curious that hnRNP C is needed for exon skipping but its expression decreases in T cell activation. The position of the ISS located 336 bp upstream of exon 8 is unusual. Most of the *cis*-acting elements are located closer to the exons. Most studies inspected only 300 bp around the exons when *cis*-acting elements were identified e.g. (Barash et al. 2010; Ajith et al. 2016). *Cis*-acting elements that are located further away may indicate the involvement of an RNA structure in splicing regulation.

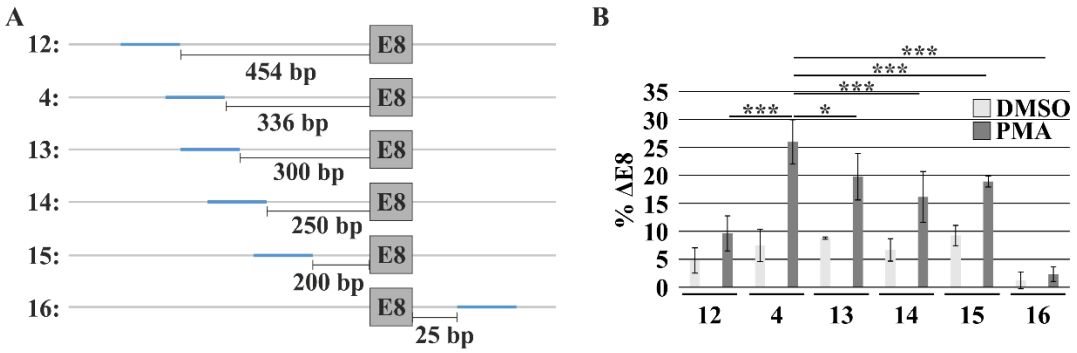

Figure 3.18: TRAF3 exon 8 skipping induction depends on the location of the ISS.
A: Scheme of minigenes in which the distance of the ISS to exon 8 was altered.
B: Jsl1 cells were transfected with the minigenes shown in A and stimulated with PMA or the solvent control DMSO for 48 h. Minigene splicing was analyzed by RT-PCR (n≥3; error bars: S.D.; *p<0.05, ***p<0.001).

To analyze if the position of the *cis*-acting element is crucial for the splicing outcome, TRAF3 minigenes that have different positions of the *cis*-acting element were cloned. Minigene 4 served as control and mimics endogenous TRAF3 splicing. Minigene 12 has an enlarged distance of the *cis*-acting element to exon 8 (454 bp instead of 336 bp; Figure 3.18A). In minigene 13, 14 and 15 the *cis*-acting element moves closer to the alternatively spliced exon (300 bp, 250 bp and 200 bp; Figure 3.18A). In minigene 16, the element is shifted to the downstream region of exon 8 (Figure 3.18A). Jsl1 cells were transfected with all these minigenes and stimulated 24 h post-transfection for another 48 h. Splicing of the minigenes was analyzed with radioactive RT-PCRs with minigene-specific primers.
All minigene constructs show a similar exon 8 skipping in resting Jsl1 cells (treated with DMSO) of 3 to 9 %. In stimulated cells the percentage of exon 8 skipping differs. Minigene 4 the control, has the highest amount of exon skipping (25 %). All other minigene constructs have a decreased responsiveness to PMA. Additionally minigene 16, whose *cis*-acting element was shifted to the downstream region of exon 8 lost exon skipping upon stimulation. These results demonstrate that shifting the ISS influences PMA responsiveness. This means the position of the element is crucial for the level of TRAF3ΔE8 induction.

The data presented in this chapter show that CELF2 and hnRNP C are both *trans*-acting factors which bind directly to the identified ISS. Both proteins bind antagonistically to UUUUUGU-motifs within the *cis*-acting element but work synergistically in the induction of TRAF3 exon 8 skipping in response to T cell activation. In this regard CELF2 is the driving force. CELF2 is

sufficient to induce exon 8 splicing in presence of hnRNP C while hnRNP C expression alone is not enough to induce exon skipping. The position of the *cis*-acting element is crucial for the level of TRAFΔE8 formation since relocation up- or downstream of the original position decreases responsiveness to PMA.

3.2 Species-Specific TRAF3 Splicing

An ISS and two *trans*-acting factors, CELF2 and hnRNP C are seem to regulate TRAF3 splicing in Jsl1 cells. This regulation happens in a cell type-specific manner as several cell lines (Hek and Ramos cells) showed no TRAF3 splicing. TRAF3 splicing will be investigated in a species-specific context by analyzing its splicing in murine cells.

3.2.1 TRAF3 splicing in El4 cells

TRAF3 splicing was monitored in Bw and El4 cells which are both murine T cell lines. To analyze species-specific splicing two processes must be distinguished. In a classical species-specific analysis splicing in resting T cells is compared between human and murine cells. Here the situation in activated T cells is additionally investigated. To this end cells were treated with PMA or the solvent control DMSO and harvested 48 h later. In resting El4 cells, TRAF3 exon 8 is mainly included in the mRNA (Figure 3.19A and 3.19B). The isoform which lacks exon 8 is weak (~6 %). The skipping rate in resting T cells in human and murine cells is very similar. The splicing in resting T cells is conserved in both species.

In human T cell stimulation with PMA leads an activation-dependent induction of TRAF3ΔE8 but in murine El4 cells PMA stimulation does not induce TRAF3ΔE8 (Figure 3.19A and 3.19B). The activation-dependent splicing induction in human cells seems to be not conserved in El4 cells. Bw cells showed a similar result (data not shown) but the PCRs were less stable. All further studies were done in El4 cells. To confirm a missing TRAF exon 8 skipping induction upon stimulation at the protein level, a western blot was performed. In Jsl1 cells the TRAF3 full length and TRAF3ΔE8 isoform was detected upon stimulation (Figure 3.19C, arrow marks weak ΔE8 band). El4 cells express only the full-length isoform in detectable amounts in resting and activated conditions (Figure 3.19C). This indicates that TRAF3 exon 8 skipping is not induced in this murine T cell line in response to T cell activation.

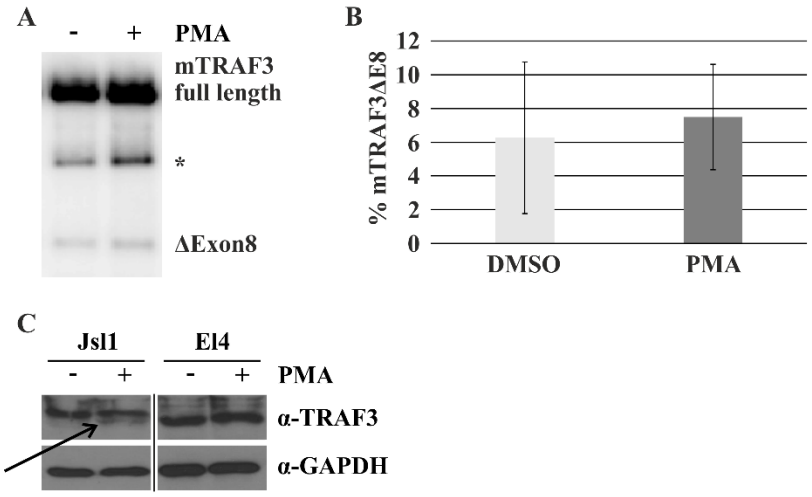

Figure 3.19: TRAF3 splicing in the murine T cell line El4.

A: El4 cells were stimulated with PMA or the solvent control DMSO for 48 h. Splicing of the murine TRAF3 was analyzed with radioactive RT-PCR. A representative gel from at least three independent experiments is shown. An asterisk marks an unspecific band that comigrates with the loading dye front.

B: Quantification of the RT-PCRs conducted in A (n≥3).

C: Jsl1 and El4 lysates of cells that were stimulated as described in A. A western blot analysis was performed with the indicated antibodies. GAPDH served as a loading control. The arrow directs to the TRAF3ΔE8 isoform present in stimulated Jsl1 cells.

3.2.2 Murine T cell activation in response to PMA stimulation

Radioactive RT-PCR and western blot analyses demonstrate no change in TRAF3 exon 8 skipping in El4 cells upon PMA stimulation in contrast to Jsl1 cells. Several explanations are conceivable.

First it might be that PMA stimulation does not activate these cells. To address this chemokine expression was analyzed and compared in resting and activated cells. T cell activation leads to stable expression of NIK in human T cells which induces the ncNFκB pathway and results in the expression of several chemokines, e.g. CXCL12 and CXCL13. In El4 cells chemokine expression was analyzed with qRT-PCR. CXCL12 expression significantly increases in response to PMA stimulation (Figure 3.20A). CXCL13 expression shows a similar trend but the error bar is large. Conclusively PMA stimulation leads to T cell activation in El4 cells and this is not the reason for the absence of a change in TRAF3 exon 8 skipping. As a second control CD45 splicing was analyzed. CD45 is very-well studied example of alternative splicing in mice. It has three exons that are alternatively spliced: exon 4, exon 5 and exon 6. In response to T cell activation isoforms lacking exon 6 accumulate (Birkeland et al. 1989). CD45-RT-PCR was

tested in El4 cells but the resulting products have sizes that were smaller than predicted and not all expected isoforms were detected (data not shown). The results were not conclusive although they indicate a general trend towards T cell activation.

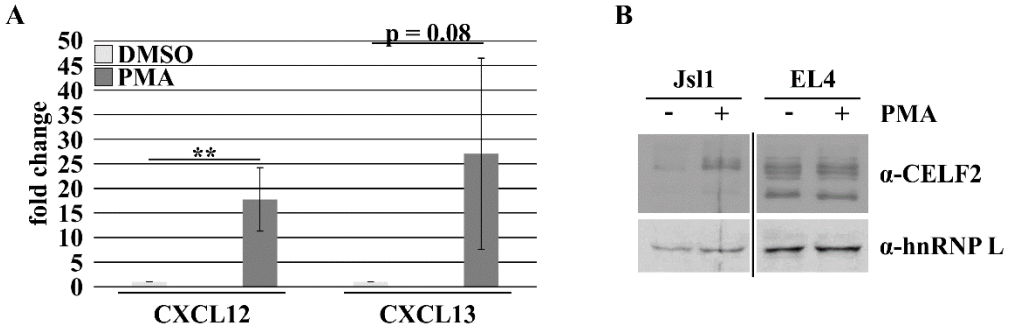

Figure 3.20: Chemokine and CELF2 expression in El4 cells.
A: El4 cells were stimulated with PMA or the solvent control DMSO for 48 h. Expressions of the chemokines CXCL12 and CXCL13 were analyzed by qRT-PCR. Expression was normalized to GAPDH (n=3; error bars: S.D.; **p<0.01).
B: Representative western blots of resting (-PMA) and activated (+PMA) Jsl1 and El4 cells are shown with the indicated antibodies. HnRNP L serves as loading control.

Second a missing splicing induction in TRAF3 might be caused by absent CELF2 expression as observed in Hek and Ramos cells. To address this a western blot was performed with α-CELF2. In parallel a western blot with Jsl1 cells was done to serve as positive control. Jsl1 cells show an increase in CELF2 expression upon activation as previously observed (Figure 3.20B). In El4 cells CELF2 is expressed and the expression does not change after PMA treatment. Notably the basal CELF2 expression level in El4 cells is comparable to the expression of CELF2 in activated Jsl1 cells. Additionally CELF2 antibody detects several bands in El4 cells (3.2.4).
Third the *cis*-acting element might not be conserved between both species (see 3.2.3).

3.2.3 TRAF3 *cis*-acting element in murine cells

The *cis*-acting elements and the cellular environment (*trans*-acting factors) regulate species and cell type-specific alternative splicing events (1.4). Since the *cis*-acting element does not change in cells of the same species cell type-specific splicing is mainly achieved by differential expression of *trans*-acting factors as observed in Ramos and Hek cells that lack endogenous CELF2 expression. In contrast *cis*-acting elements can play a role in species-specific splicing.

To address this, the mRNA sequences of TRAF3 from mouse and human were compared (Figure 3.21A). In both species TRAF3 is encoded by twelve exons. Exon 8 has the highest sequence identity with 98.7 %, determined with a pairwise sequence alignment with the EBOSS needle algorithm provided by the EMBL-EBI. Exon 7 is 88.9 % conserved between human and mouse and exon 9 91.4 %. The human intronic splicing silencer for TRAF3 splicing is a 96 nt long sequence which is located 336 nt upstream of exon 8. In mouse there exists a similar sequence 343 nt upstream of exon 8 which shows 83.7 % conservation (Figure 3.21A). Since the UUUUUGU-motif includes the binding sites for human CELF2 (hCELF2) and human hnRNP C it was analyzed if this motif also exists in the murine sequence.

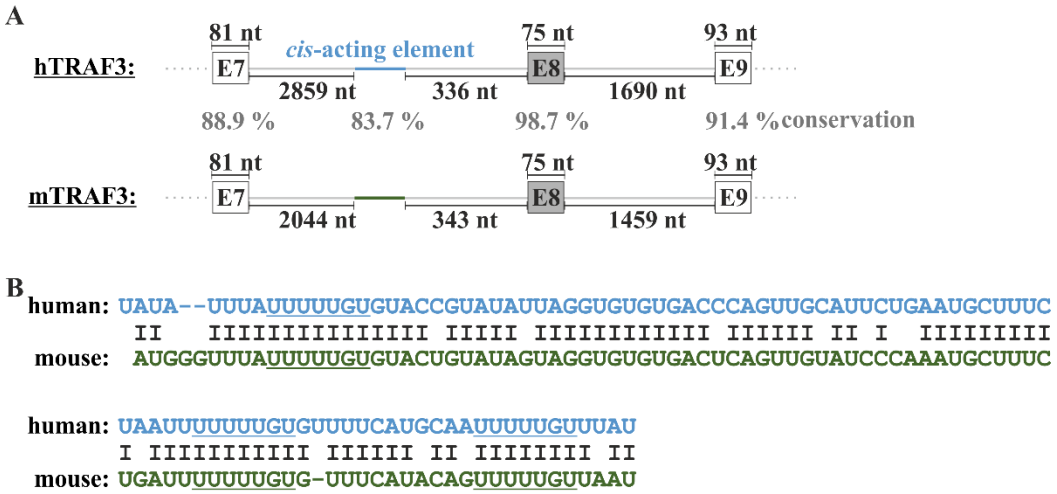

Figure 3.21: TRAF3 *cis*-acting element in human and murine cells.
A: Scheme illustrating human and murine TRAF3 exon 7, exon 8 and exon 9 as well as the in introns lying in between. Length of exons are given above them. The length of introns is depicted beneath. Conservation of the different exons and the *cis*-acting element is shown in dark grey between both plots.
B: Alignment of the human and the murine TRAF3 *cis*-acting element. UUUUUGU-motifs that contain the binding sites for CELF2 and hnRNP C are underlined.

The human and the murine TRAF3 *cis*-acting elements were aligned (Figure 3.21B). The murine sequence like the human sequence has three U-stretches following a guanosine showing a conservation of these elements. Since the U-stretches are conserved but no effect upon stimulation is present, it might indicate that next to the U-stretches other sequence elements might be necessary for TRAF3 exon 8 induction. The *trans*-acting environment might also be crucial. To investigate this minigenes that contain either the human or the murine exon 8 and parts of the surrounding introns between two constitutive spliced exons were used

(Figure 3.22A). The human minigene 4 (hMG4) is the same minigene that was used as control in all previous analyses.

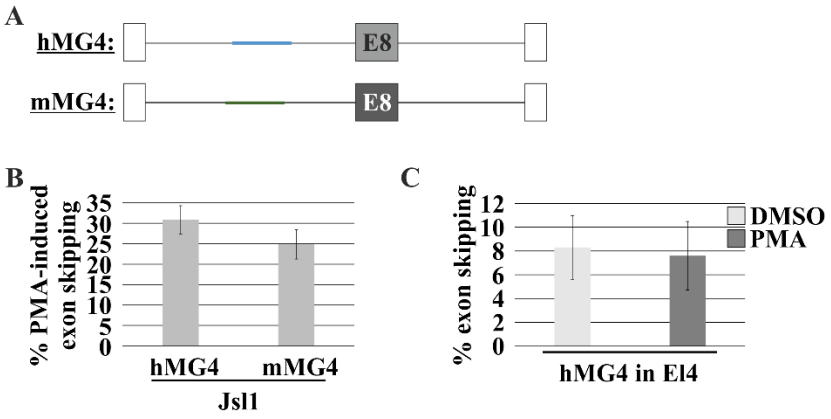

Figure 3.22: Splicing of human and murine TRAF3 minigenes.
A: Scheme representing the structures of the human and the murine TRAF3 minigenes. The minigenes contain TRAF3 exon 8 and the surrounding introns from human (light grey) or mouse (dark grey) and both are flanked by the same constitutive spliced exons (white boxes). The TRAF3 *cis*-acting element is shown in blue (human) or green (murine).
B: Jsl1 cells were transfected with the minigenes shown in A. The transfectants were split and stimulated with DMSO or PMA for 48 h. Minigene splicing was investigated with radioactive RT-PCR (n≥3; error bars: S.D.).
C: El4 cells were transfected with hMG4 and treated as described in B (n=3; error bars: S.D.).

First Jsl1 cells were transfected with the human hMG4 and murine minigenes mMG4 respectively. Transfectants were split as previously described. The splicing of the minigenes was analyzed with radioactive RT-PCR. Both minigenes are spliced in Jsl1 cells: the hMG4 exhibits around 30 % PMA-induced exon skipping and the mMG4 less with around 25 % (Figure 3.22B). Both minigenes show a similar % PMA-induced exon skipping rate in human cells. Second El4 cells were transfected with hMG4. The radioactive RT-PCR reveals that hMG4 is not differentially spliced in murine cells in response to stimulation. Aaround 8 % exon skipping is detectable which does not change upon PMA stimulation (Figure 3.22C). The western blot showed already a high basal CELF2 expression in unstimulated El4 cells. Together these data strongly indicate a dependency of TRAF3 exon 8 splicing on the cellular environment.

3.2.4 Murine and human *trans*-acting factors

The findings from Jsl1 and Ramos cells show that TRAF3 exon 8 is regulated by CELF2 and hnRNP C in the human system where CELF2 is the driving force. The western blot in El4 cells indicates to the presence of different CELF2 bands which might indicate post-translational modifications or splicing of murine CELF2 (mCELF2). T cell activation is accompanied by the activation of phosphorylation cascades so phosphorylation of mCELF2 seems a reasonable assumption. The hypothesis of phosphorylation was tested by preparation of lysates (from Jsl1 and El4), which were either treated with the alkaline phosphatase CIP or with a control solution. The samples were separated on an SDS gel and a western blot was performed to detect CELF2. The CIP treatment has no influence on the bands. There are still at least two bands detectable and the strength of both bands is not changed upon CIP treatment (data not shown). However a positive control is missing. It cannot be excluded that CIP treatment in general was insufficient. Another hypothesis which could explain the detection of several bands in western blot analysis is an alternative splicing of CELF2 itself. The detected bands might correspond to different CELF2 isoforms. It has been published that CELF2 is alternatively spliced in mouse and human cells (Barreau et al. 2006). Referring to hg19 four human isoforms exist whereas in mouse eight CELF2 isoforms are known (referring to mm9) (Figure 2.23).

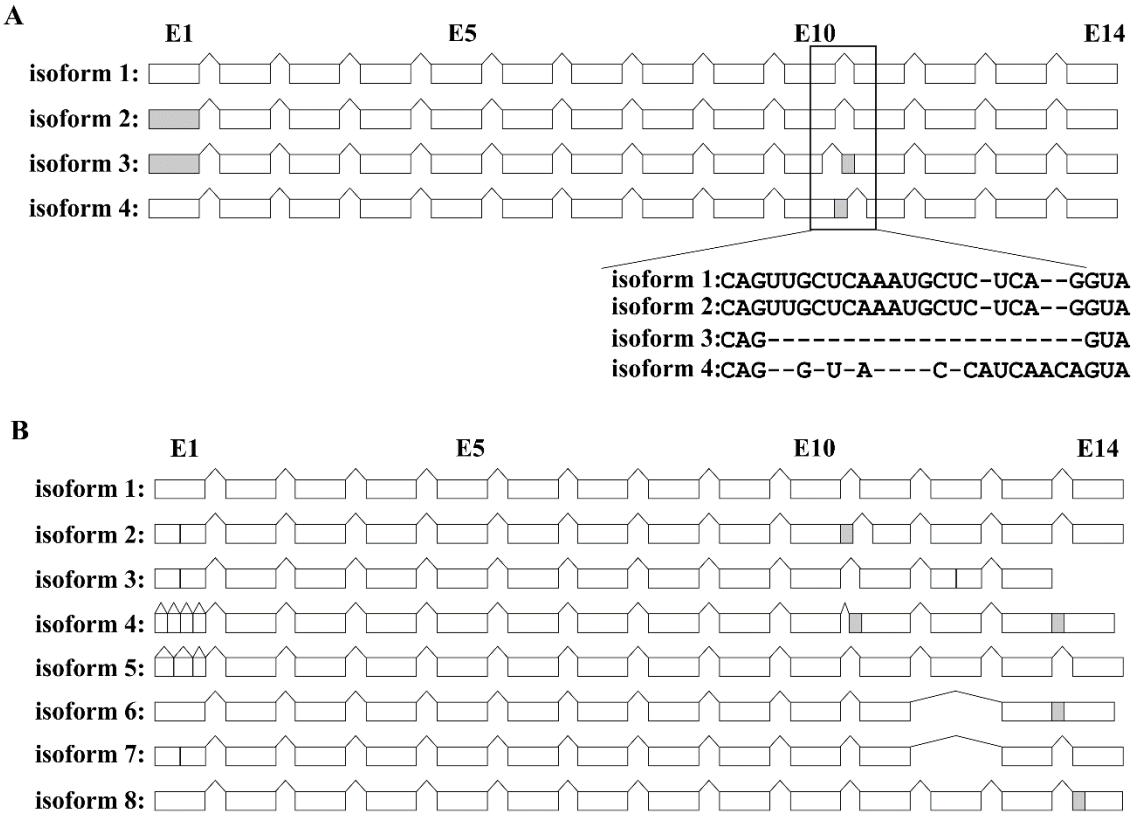

Figure 3.23: Human and murine CELF2 isoforms.
A: Scheme presenting the four known human CELF2 isoforms (based on hg19). An alignment of parts of exon 10 and exon 11 is shown beneath. Regions marked in grey show low sequence similarity to isoform 1.
B: Overview of the known murine CELF2 isoforms (based on mm9)

It was hypothesized that in human cells one CELF2 isoform is upregulated upon stimulation, which is lacking in murine cells. A closer look at the human CELF2 isoforms shows a region in exon 10 and 11 which differs in three of the four isoforms (highlighted in Figure 3.23A with a box and an alignment is shown). This specific region can be amplified by RT-PCR and it can be investigated if the expression on the mRNA level of one of these isoforms is upregulated in response to PMA stimulation. All CELF2 isoforms are upregulated on the mRNA level upon T cell activation indicating that not a single CELF2 isoform is needed for PMA responsiveness but CELF2 expression in general is required at least in human cells (Figure 3.24).

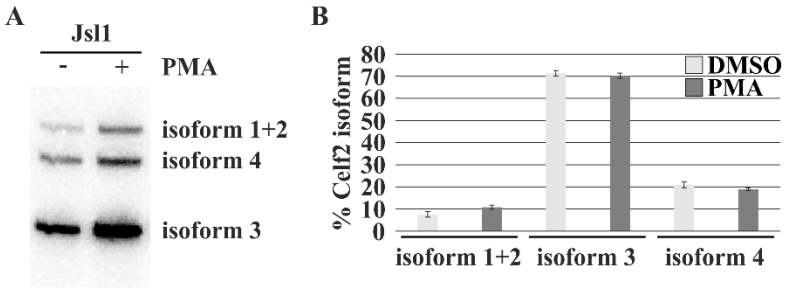

Figure 3.24: CELF2 isoform expression in Jsl1 cells.
A: Resting (DMSO) or activated (PMA) Jsl1 cells were used for RT-PCR in which different CELF2 isoforms can be distinguished. A representative gel is shown.
B: Quantification of RT-PCRs done in A (n≥3; error bars: S.D.).

The hCELF2 protein encoded by human isoform 1 is 100 % conserved to the mCELF2 encoded by murine isoform 4. All experiments previously performed in this thesis were done with the hCELF2 protein encoded by the human isoform 4 (hCELF2 4), which is not present in mice. Subsequently the CELF2 protein that is conserved in both species (hCELF2 1) was cloned and overexpressed in Heks to check if both isoforms are able to induce TRAF3 exon 8 skipping. Expression of hCELF2 1 results in the same percentage of TRAF3ΔE8 as hCELF2 4 (Figure 3.25). This indicates that mice have the same CELF2 isoform as humans but this isoform does not induce TRAF3 exon 8 skipping in El4 cells.

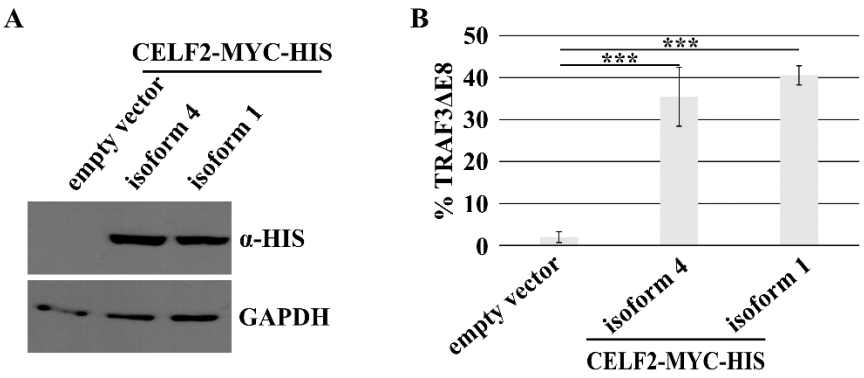

Figure 3.25: CELF2 isoform overexpression in Hek cells and TRAF3 splicing.
A: Hek cells were transfected with plasmids encoding several CELF2 isoforms and harvested 48 h later. A representative western blot is depicted (n≥3). GAPDH served as loading control.
B: TRAF3 RT-PCR with Hek cells transfected as described in A (n≥3; error bars: S.D.; ***p<0.001).

To analyze this in more detail, the binding of either hCELF2 or mCELF2 to the human and murine *cis*-acting element was investigated. The focus was set on the third part of the *cis*-acting

element which showed the strongest CELF2 binding in the previous experiments. The human (hISS-Wt) as well as the corresponding murine sequence (mISS-Wt) was *in vitro* transcribed (Figure 3.26A). Two sequences with some nucleotide changes were additionally prepared: 1) the human sequence which has mutated guanosines and consequently is not able to bind CELF2 (hISS-GtoC) (Figure 3.26A, mutation is highlighted with a box) and 2) a mutated murine sequence in which an adenosine was exchanged by an uracil (mISS-AtoU). This nucleotide exchange results in a sequence which mimics the human sequence (Figure 3.26A, mutation is highlighted). These transcribed sequences were incubated with nuclear extract of unstimulated and stimulated Jsl1 cells and UV-Crosslinks experiments were carried out (Figure 3.26B).

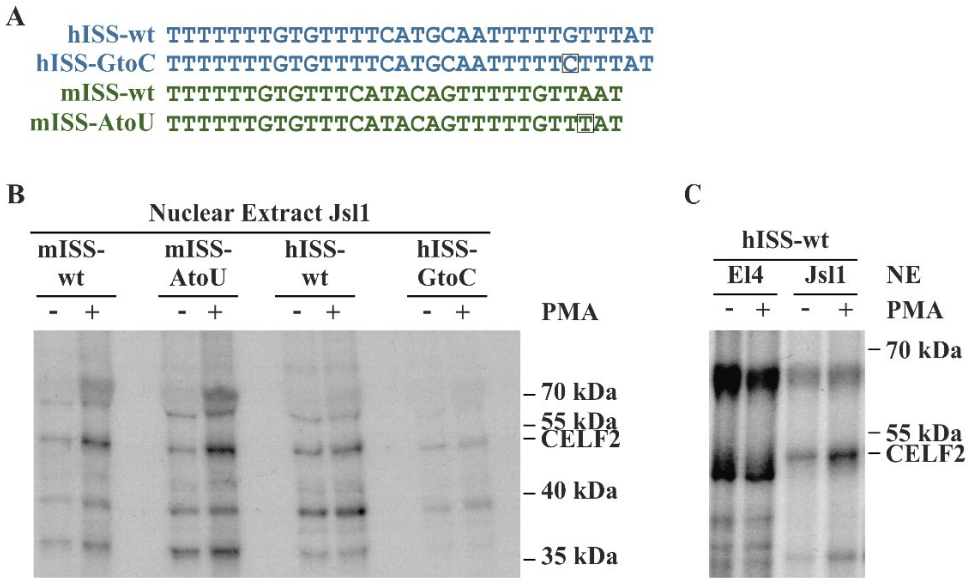

Figure 3.26: CELF2 binding to the human and the murine *cis*-acting element.
A: The overview shows the sequences that were *in vitro* transcribed and used for UV-Crosslinks.
B: Transcripts shown in A were incubated with nuclear extract from resting (-PMA) and activated (+PMA) Jsl1 cells and UV-Crosslinks were done.
C: hISS-Wt was incubated with nuclear extracts from Jsl1 and El4 cells treated with either DMSO or PMA.
In B and C, representative blots are shown from at least three independent experiments.

As observed before hCELF2 binds to the hISS-Wt sequence and the binding increases in response to stimulation but hCELF2 binding is lost in the hISS-GtoC transcript. Notably hCELF2 also binds to the mISS-Wt and its binding increases in stimulated extracts. In the mISS-AtoU the hCELF2 binding seems even stronger. This indicates that the *cis*-acting element alone cannot be the reason for a missing TRAF3 exon 8 skipping in PMA treated El4 cells but it strongly indicates that the cellular environment is crucial. To further investigate this

assumption new UV-Crosslinks were performed with hISS-Wt which was incubated with nuclear extracts from either Jsl1 or El4 cells (Figure 3.26C). As previously observed hCELF2 binds to the hISS-Wt. In contrast UV-Crosslinks with murine nuclear extracts result in a band which runs below the hCELF2 band. It is not yet clear if this band corresponds to another mCELF2 isoform or if it is another protein.

The data from human TRAF3 exon 8 splicing showed an involvement of hnRNP C in this regulation. The UV-Crosslink analysis in Figure 3.26B indicates binding of a protein with a similar size to the mISS-Wt sequence. To validate hnRNP C binding a western blot was performed. The used antibody detects hnRNP C in Jsl1 cells but not in El4 cells (Figure 3.27). The human and the murine hnRNP C protein have a conservation of over 90 % (according to EMBOSS Needle pairwise Sequencing Alignment tool) but the used antibody is stated to detect human hnRNP C. Consequently a missing detection might be caused by the antibody and does not prove a missing hnRNP C expression.

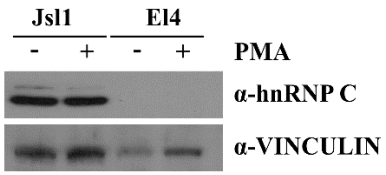

Figure 3.27: hnRNP C expression.
Jsl1 and El4 cells were treated with DMSO or PMA and cells were harvested 48 post-stimulation. Proteins were extracted and a western blot was performed with the indicated antibodies. VINCULIN served as loading control. A representative blot is shown.

The data investigating species-specific aspects of TRAF3 exon 8 alternative splicing resulted in different findings. A comparison of human and murine T cells in resting conditions showed a conservation of TRAF3 exon 8 splicing. In resting T cells exon 8 is mainly included in the mRNA. A comparison of both cell lines in activated conditions revealed that human cells have an activation-dependent induction of TRAFΔE8 isoform which is absent in murine cells formation is species-specific. The experiments strongly indicate that the *trans*-acting environment is responsible for this missing splicing induction in TRAF3ΔE8 in El4 cells. The differences in *trans*-acting environments need further investigation to understand the regulatory effect on TRAF3 splicing in El4 cells. It is clear that mice express multiple CELF2 isoforms and their binding to the *cis*-acting element needs to be verified. Post-translational modification of CELF2 cannot be excluded.

4 Discussion

Alternative splicing is well-known as a process that expands the proteome by generating several isoforms from one single pre-mRNA (Graveley 2001; Nilsen and Graveley 2010). Often these isoforms have different functions due to changes in protein-protein interaction profiles (Yang et al. 2016). Additionally alternative splicing can influence gene expression by changing the stability of the resulting protein or its localization. This ability to change gene expression is used to regulate developmental processes and to face cellular changes. One important transition that is accompanied by alternative splicing is immune response, more precisely T cell activation (Ip et al. 2007; Martinez et al. 2012). The transition from resting to activated T cells includes many changes in the cell such as induction of chemokine expression, increase of cellular growth and proliferation, triggering inflammatory response and upregulation of trafficking. At least some of these processes are regulated by alternative splicing (e.g. Sec16 alternative splicing increases COPII transport in response to T cell activation (Wilhelmi et al. 2016); (Martinez et al. 2012)). One gene whose alternative splicing changes in T cell activation is TRAF3. In resting T cells TRAF3 full length isoform facilitates the interaction of TRAF2 and cIAP (an E3 ubiquitin ligase) with NIK. This interaction leads to ubiquitination and degradation of NIK which results in an inactive non-canonical NFκB pathway. In response to stimulation TRAF3 exon 8 is skipped leading to a shorter isoform (TRAF3ΔE8). TRAF3ΔE8 can no longer interact with NIK. NIK is not targeted for degradation and activates the non-canonical NFκB pathway leading to chemokine expression (Michel et al. 2014). Although the function of both TRAF3 isoforms is well-understood the regulatory mechanism for this alternative splicing event remains elusive. The aim of this study is to analyze the regulatory mechanism behind the skipping of TRAF3 exon 8 in T cell activation. The regulation of alternative splicing can happen on different layers (summarized in Figure 1.8) and are the focus of this thesis.

4.1 Regulation by Histone Modification

T cell activation is accompanied by alternative splicing changes of 178 exons (Martinez et al. 2012). How most of these splicing changes are regulated remains elusive since only some examples have been studied in greater detail (e.g. Lef1 (Mallory et al. 2011) and (Martinez et al. 2015)). A publication of Luco et al. (2010) showed a regulation of alternative splicing events by histone modification. A putative regulation of TRAF3 exon 8 splicing by histone modification has not yet been analyzed but I have begun to investigate how many of these 178

alternative splicing events are regulated by H3K4me3 and H3 acetylation. To this end both resting and activated cells were harvested and chromatin immunoprecipitation experiments were performed followed by qRT-PCR. 36 exons were investigated and only one target depends on histone methylation and one on histone acetylation (unpublished results). This indicates a more particular mechanism for these single targets and not a common mechanism of splicing regulation in response to T cell activation. TRAF3 exon 8 was not part of the investigated exons but the results presented in this study pointed to a regulation independent of histone modifications. One main reason for this assumption is the identification of two *trans*-acting factors which regulate TRAF3 splicing. The published chromatin-adaptor complexes that translate a histone modification to the regulation of an alternative splicing event consisting of two proteins: one protein recognizes the histone modification and the other transfers the signal to the splicing process. This second protein is a *trans*-acting factor (Luco et al. 2011). Since two auxiliary *trans*-acting factors are needed for efficient TRAF3 exon 8 skipping it does not agree with the already published chromatin adaptor complexes. More experiments need to be done to exclude an influence of histone modification on TRAF3 exon 8 splicing upon T cell activation since this is only an indication.

4.2 Regulation by Cis-Acting Element

A *cis*-acting element is a sequence which regulates splicing. It provides a platform for proteins (*trans*-acting factors) that are involved in the regulation of the splicing events. To identify the *cis*-acting element, which is needed for the regulation of TRAF3 exon 8 alternative splicing in response to T cell activation, a minigene approach was used. The first minigene contained intronic sequences flanking TRAF3 exon 8. By shortening these intronic elements in other minigenes and using TRAF3 exon 8 skipping as read out, an intronic splicing silencer (ISS) was identified with a length of 96 bp. The ISS is located 336 bp upstream of TRAF3 exon 8. The exclusion of this element leads to a loss in PMA responsiveness. There may also be a second *cis*-acting element downstream of exon 8. This was not further investigated because its deletion had a less strong effect on PMA responsiveness whereas the effect of the upstream identified element was stronger. It has to be emphasized that minigenes are artificial systems and they might not represent the situation as found under endogenous conditions. The minigene regulation might differ from the regulation of the full length isoform and the regulation might depend on its expression via the endogenous promoter (Fu and Jr 2014). One example for a difference in splicing regulation has been found in a gene called cTNT in which exon 5 is

alternatively included. Minigene experiments identified CELF1 and CELF2 as regulator for cTNT exon 5 splicing (Ladd et al. 2001). Later experiments in transgenic mice revealed that endogenous regulation is independent from CELF1 (Dasgupta and Ladd 2012). Nevertheless minigenes are an established approach to identify *cis*-acting elements. The control minigene (construct 4) also shows a splice pattern which is very similar to the endogenously observed pattern. This indicates that the minigene undergoes the same regulatory mechanism. A promising improvement for the minigene approach is the use of the CRISPR/Cas9 technology. This technology can be used to induce changes in the genome. Using this approach the endogenous TRAF3 could be changed and subsequent influences could be analyzed under endogenous conditions.

Next to the necessity of the ISS element for TRAF3 exon 8 skipping its sufficiency was tested by using CD45 minigenes. If the ISS is sufficient it should induce skipping of an unrelated exon. Two CD45 minigenes were used of which one contains the TRAF3 *cis*-acting element. The experiments indicate a sufficiency without ultimately proving it. The reason for this was a missing knowledge about the binding sites of the *trans*-acting factors. It was later discovered that the control CD45 minigene (without TRAF3 *cis*-acting element) already contained these binding sites. This explains a response to overexpression of the *trans*-acting factor CELF2 without the TRAF3 ISS. Additionally the distance of the *cis*-acting element to the alternatively spliced exon was found to be crucial in TRAF3 splicing and this was not taken into account in the CD45 minigene experiments. Nevertheless the experiments point to a sufficiency of the ISS but new minigene experiments needs to be done. These new experiments must use minigenes which do not contain any binding sites for the identified *trans*-acting factors next to the ISS and which do include the relevant distance of 336 bp to the exon.

4.3 Identification of Trans-Acting Factors

Trans-acting factors are proteins which bind to *cis*-acting elements and which are involved in the regulation of alternative splicing events. In this study two *trans*-acting factors that regulate skipping of TRAF3 exon 8 in response to T cell activation were identified, CELF2 and hnRNP C. CELF2 seems to be the driving force but hnRNP C is additionally needed.

4.3.1 CELF2

CELF2 was identified as a *trans*-acting factor by an siRNA screen and UV-Crosslink immunoprecipitation assays. First the siRNA screen was performed. The advantage of using such a screen is that these experiments can be conducted in parallel with the identification of *cis*-acting elements. Consequently siRNA screens have been used in various publications before e.g. Oberdoerffer et al. 2008; Tejedor et al. 2015; Meininger et al. 2016. The screen identified CELF2 to be a regulator of TRAF3 splicing since its knockdown has the strongest effect in reducing exon 8 skipping. Next these results were also validated in further CELF2 knockdown experiments although the effect was not as strong as in the screen. This is most likely caused by the usage of different transfection methods. In this study different transfection methods were used for Jsl1 cells because transfection efficiency differs depending on the transfected reagent. To improve the transfection efficiency and prevent the usage of different methods, a virus-mediated transfection (transduction) should be established for future studies. Transduction is well known to be highly efficient and easy to use (Kim and Eberwine 2010).

CELF2 belongs to the CELF family. This family is involved in gene expression regulation and splicing (Dasgupta and Ladd 2012). The best studied members are CELF1 and CELF2, which both are widely expressed. Several studies showed that these proteins have an important role in developmental processes, e.g. expression of CELF1 and CELF2 decreases during heart development (Kalsotra et al. 2008). CELF2 is also differentially expressed during embryonic and nervous system development (Blech-Hermoni et al. 2013; Ladd 2013). CELF2 was already shown to be involved in the regulation of alternative splicing changes in response to T cell activation (Mallory et al. 2015) where CELF2 was found to regulate splicing of LEF1 (Mallory et al. 2011) and MKK7 (Martinez et al. 2015). Mallory et al. (2015) published RNA sequencing data that investigated CELF2 dependent splicing switches in T cells by comparing splicing in wild type cells and CELF2 depleted cells. This analysis resulted in 72 exons whose splicing depends on CELF2. 72 exons are approximately one-third of the exons which are known to undergo alternative splicing in response to T cell activation (Martinez et al. 2012). Unexpectedly the list of the 72 exons does not include TRAF3, LEF1 and MKK7. LEF1 is mentioned in the paper as a target that is known to be regulated by CELF2 but it is not discussed why it is not included in the list. MKK7 should also be included since the same group investigated its regulation and they showed a CELF2-dependent regulation. The data indicate a CELF2-dependent splicing regulation for many alternative splicing changes in T cell activation.

Binding of CELF2 to the *cis*-acting element was further mapped to a UUUUGU-motif, specifically to the guanosine within this motif. SELEX data published previously showed a CELF2 binding to UGUU or UG repeats (Faustino and Cooper 2005). Faustino and Cooper (2005) observed that minigenes containing these repeats not only respond to CELF2 but also to CELF1 and CELF4. This indicates a usage of the same binding motif by several family members. The *cis*-acting element of LEF1 is bound by CELF1 and CELF2 but CELF1 binding does not change in response to T cell activation (Mallory et al. 2011). In contrast the TRAF3 *cis*-acting element seems only to bind CELF2 because the antibody against CELF1 was unable to precipitate it in UV-Crosslink immunoprecipitation experiments. Conclusively CELF2 binding is highly specific within the TRAF3 ISS. The SELEX data showed additionally that an efficient CELF2 binding requires more than one U-rich binding motif (Faustino and Cooper 2005). This fits well to the three repetitive UUUUUGU-motifs in the TRAF3 *cis*-acting element. The exchange of all three guanosines in a TRAF3 minigene mimics the same decrease in TRAF3ΔE8 formation as observed in CELF2 knockdown cells.

Additional experiments were performed to analyze the effect of CELF2 overexpression and a putative sufficiency of CELF2. CELF2 is sufficient if its expression induces exon skipping in cells which do not express endogenous CELF2. The experiments revealed that CELF2 expression is sufficient to induce TRAF3 exon 8 skipping. Sufficiency was investigated by overexpression of CELF2 in several cells lines. In Hek cells, the overexpression strongly increases TRAF3 exon 8 skipping. However it cannot be excluded that overexpression in general leads to changes in the cell. Accordingly a Ramos cell line that stably overexpresses CELF2-MYC-HIS was generated. Ramos cells either do not express endogenous CELF2 or express a smaller CELF2 isoform as a slight band which runs faster than the CELF2 band in Jsl1 appears in western blot. The Ramos/CELF2-MYC-HIS cell line shows a high enrichment of TRAF3 exon 8 skipping. PMA stimulation increases neither CELF2-MYC-HIS expression (as observed in Jsl1) nor does it have a big influence on TRAF3 exon 8 skipping. In Jsl1 cells PMA stimulation results in an increase in CELF2 transcription and the transcript is more stable (Mallory et al. 2015). Most likely this is a cell type-specific mechanism. Additionally basal CELF2-MYC-HIS expression in this Ramos strain is already high and it is questionable if a further induction is possible. CELF2 is known to regulate its own expression by binding to its own mRNA and preventing binding of U2 snRNP. This results in exon 6 skipping and is followed by a frame-shift which targets the mRNA to nonsense-mediated decay (Dembowski and Grabowski 2009). This negative feedback loop was also observed in the study of Mallory

et al. (2015) in which they found only a threefold induction in CELF2 protein but a 10-12 fold induction in CELF2 mRNA in Jsl1 cells in response to T cell activation.

CELF2-dependent splicing of several other targets next to TRAF3 exon 8 was investigated using the generated Ramos cell line. The chosen targets are known to be alternatively spliced in response to T cell activation (Martinez et al. 2012). From the genes analyzed, several showed more exon skipping in cells overexpressing CELF2, PPP1R12A, BRD8, MACF1 and REPS1. Four of the tested genes displayed unchanged alternative splicing, NAB2, ARMC10, MYL6 and FAM33A. RNA sequencing analyses revealed that 72 exons are CELF2-dependent regulated in T cell activation (Mallory et al. 2015). These 72 exons include PPP1R12A and BRD8 but none of the other genes. MACF1 and REPS1 have one CELF2 binding motif at around 150 nt upstream of the alternatively spliced exon. It might be that a single U-rich element is not sufficient for CELF2 binding since a requirement for more U-rich motifs for efficient binding has been published (Faustino and Cooper 2005). In contrast to that CELF2 regulates BRD8 alternative splicing which has only one UUUUUGU-motif. The data indicates that an UUUUUGU-motif might be an indicator for a CELF2-dependent regulation.

It has been published that the outcome of CELF2-regulated alternative splicing depends on the position of CELF2 binding. If CELF2 binds in the upstream intronic region it induces exon skipping. Binding in the downstream intron is followed by exon inclusion (Ajith et al. 2016). In the minigene experiments which were performed to identify the *cis*-acting element, a strong effect on PMA responsiveness was observed for an upstream element. This element was the focus of all the other experiments but there were some indications for a second *cis*-acting element in the downstream intronic sequence. There is a UUUUGU-motif 581 nt downstream of TRAF3 exon 8. It is only a single motif. This leads to the hypothesis that CELF2 preferentially binds to the further investigated upstream *cis*-acting element which contains three U-rich motifs. The single U-rich motif might explain the small effect observed for the downstream intronic element in the minigene experiments which identified the *cis*-acting element.

CELF2-dependent TRAF3 exon 8 splicing was identified by CELF2 knockdown. Consequently it is interesting to analyze which effect a CELF2 overexpression might have on Jsl1 cells. This was addressed by the attempt to generate Jsl1 cells, which stably overexpress CELF2 (data not shown). These cells were not viable. CELF2 was originally found in a screen, in which it was shown to be involved in apoptosis (Ladd 2013). It might be that a permanent expression of

CELF2 induces apoptosis in Jsl1 cells. This might explain why no viable stable cell lines could be generated.

4.3.2 hnRNP C is a second *trans*-acting factor in TRAF3 exon 8 splicing

The UV-Crosslink analyses which narrowed down the binding of CELF2 to a single guanosine within the UUUUUGU-motif additionally revealed the binding of the U-stretches by another protein. This protein binds antagonistically to CELF2, has a size of around 40 kDa and was later identified as hnRNP C. hnRNP C was identified by UV-Crosslink immunoprecipitation experiments in which different 40 kDa proteins were tested. Several proteins are known to be involved in splicing regulation with a similar size to hnRNP C and these were putative candidates for TRAF3 exon 8 splicing regulator. PTB is a well-studied splicing repressor but it was not considered because it was included in the siRNA screen and showed no effects there. TIA1 and HUR are both known to bind to U-stretches and to compete with CELF proteins (Vlasova-St. Louis et al. 2013). Both of them could not be precipitated in UV-Crosslink immunoprecipitation experiments which demonstrated that they do not bind the TRAF3 *cis*-acting element. A protein called MBNL was identified to regulate alternative splicing together with CELF2. For example, CELF1 and CELF2 expression decreases in heart development whereas MBNL expression is upregulated (Kalsotra et al. 2008). The binding of CELF proteins and MBNL is independent of each other and both function antagonistically (Wang et al. 2015). In the performed UV-Crosslink analyses the binding of CELF2 and the 40 kDa proteins seemed to be antagonistic since a mutation resulting in a loss of CELF2 binding is accompanied by a stronger binding of the other protein. This observation is a first hint for a TRAF3 splicing regulation which is independent of MBNL. Furthermore CELF and MBNL are known to function in opposite directions (Faustino and Cooper 2005; Vlasova-St. Louis et al. 2013; Wang et al. 2015) and the minigene experiments indicate a synergistic function of both proteins (hnRNP C and CELF2) in TRAF3 splicing. Mutations that prevent binding of CELF2 or hnRNP C lead to a decrease in exon 8 skipping. In summary several proteins were tested and hnRNP C was the only one which showed a direct binding to the TRAF3 ISS. The hnRNP C monomer binding is weak while oligomerization is generally stronger (Geuens et al. 2016). The three U-stretches within the ISS might enhance its binding. Nevertheless many proteins bind to the *cis*-acting element and the involvement of further proteins in the regulation of TRAF3 exon 8 splicing cannot be excluded.

In general hnRNP proteins are known to have multiple functions. They are involved in mRNA maturation, mRNA stabilization, mRNA transport into the cytoplasm and they control translation (Geuens et al. 2016). Most hnRNPs function as splicing silencers. The identified hnRNP C protein was the first found member of the hnRNP family and the first shown to be involved in splicing (Han et al. 2010). hnRNP C also increases mRNA translation (Han et al. 2010) showing that hnRNP C has crucial functions in the cell. This might explain why an hnRNP C knockdown in Jsl1 cells was not viable but it does not answer the question why a knockdown in Hek cells is viable. Hek cells could be used as a model system to study effects of hnRNP C knockdown on the splicing of TRAF3 minigenes. The results revealed that its knockdown decreases CELF2-dependent TRAF3ΔE8 formation. This finding was also validated in Jsl1 cells with minigene experiments in which the hnRNP C binding sites were exchanged. The effect of hnRNP C knockdown in Hek cells is not very strong and Ramos/CELF2-MYC-HIS cells also have a lower hnRNP C expression compared to Jsl1 cells. These results demonstrated that hnRNP C expression alone is not able to induce TRAF3 splicing. It strongly indicates that hnRNP C is needed for TRAF3ΔE8 formation but the driving force is CELF2. Moreover these experiments imply a synergistic function of CELF2 and hnRNP C since both are needed to induce TRAF3 exon 8 skipping. hnRNP C was also detected in the CELF2-dependent regulation of MKK7 (Martinez et al. 2015). In this publication, hnRNP C binding to the *cis*-acting element was observed but its knockdown led only to subtle changes. Consequently it was concluded that CELF2 is the main regulator without performing further experiments to address the function of hnRNP C. The MKK7 *cis*-acting element is additionally located closer to the exon (the flanking 100 nt are needed for regulation) compared to the *cis*-acting element in TRAF3 splicing.

CELF2 and hnRNP C both work synergistically but the UV-Crosslink analyses showed an antagonistic binding of both proteins to the ISS. In line with the antagonistic binding hnRNP C expression and its binding decrease upon T cell activation. hnRNP C was already found to compete for binding sites with the splicing factor U2AF65. It was discovered that hnRNP C prevents binding of U2AF65 and thereby inhibits the usage of cryptic splice sites and the exonization of Alu-elements (Zarnack et al. 2013). This points up the contradiction of an involvement of hnRNP C in TRAF3 exon 8 skipping and its decreased expression and binding.

4.4 Regulation of TRAF3 Exon 8 Splicing by RNA Structure

The distance between the ISS and exon 8 in TRAF3 splicing is more than 300 nt. Most of the studies investigating the regulation of splicing events focus on flanking intronic regions of 200 to 300 nt (Nilsen and Graveley 2010; Fu and Jr 2014). There are also examples in which distant elements regulate splicing for example, the Drosophila DSCAM gene is well studied and targeted by many different regulatory mechanisms including regulation via a long-range RNA structure (Graveley 2005). Graveley et al. (2005) studied the alternative splicing of exon 6 and found that two different RNA structures are formed which determine the inclusion rate of exon 6. Later such long-range structures were shown to be a common mechanism in Drosophila (Raker et al. 2009). Another example is the *trans*-acting factor RBFOX in mammalian cells. It binds to sequences that are more than 500 nt away from the splice site they regulate. The formation of a loop then brings the RBFOX protein in close proximity to the splice site and splicing can be regulated (Lovci et al. 2013). The long distance between the TRAF3 *cis*-acting element and exon 8 might indicate a regulation by RNA structure. A query using structure prediction tools did not identify any secondary structures but the minigene experiments with different distances from the *cis*-acting element to exon 8 clearly demonstrate a distance dependency. Changing the distance by moving it further away or closer to the exon results in a decrease in exon skipping. Shifting the *cis*-acting element downstream of exon 8 should result in more inclusion according to the model predicted by Ajith et al. (2016). Since exon 8 inclusion rate is around 95 % in resting T cells it is unlikely that a significant increase in exon inclusion in unstimulated cells could be observed. In stimulated cells we observe the expected decrease in exon exclusion and inclusion level increases. To come back to the observation that the distance is crucial for TRAF3 splicing it is interesting to see an involvement of hnRNP C in TRAF3 exon 8 splicing. hnRNP C is known to function as a molecular ruler because it wraps RNA around itself similar to nucleosomes which wrap DNA. The wrapping by hnRNP C determines the length of the mRNA and controls the export pathway used for the transport into the cytoplasm. According to the published model hnRNP C binds co-transcriptionally to RNAs which are longer than 200 to 300 nt. The binding of hnRNP C recruits export factors and induces export via the mRNA export pathway. Shorter RNAs are not bound by hnRNP C and these will be exported by the U-snRNA pathway (McCloskey et al. 2012). HnRNP C can only wrap RNA in the case it forms oligomers. In human two hnRNP C splice isoforms exist, C1 and C2 (Han et al. 2010). Three C1 and one C2 monomer can form a tetramer which then binds 230 to 240 nt long pre-mRNAs (Huang et al. 1994) and wraps it around itself. A wrapping of the TRAF3 pre-mRNA would explain why the distance of the *cis*-acting element is important

for the regulation of TRAF3 splicing and how the *cis*-acting element comes close enough to the 3'splice site to prevent its recognition.

4.5 Regulation of TRAF3 Exon 8 Splicing – A Model

The findings from this study together with already published data can be summarized as follows (Figure 4.1). In resting T cells hnRNP C is highly expressed and tetramers bind to the *cis*-acting element of TRAF3. This leads to the wrapping of the pre-mRNA around the hnRNP C tetramers but has no further effect on splice site recognition. CELF2 expression is low and binding to the *cis*-acting element is weak. U2AF binds to the 3'splice site and exon 8 is included in the resulting mRNA.

In response to T cell activation hnRNP C expression as well as its binding decreases but the remaining hnRNP C is still able to wrap the TRAF3 pre-mRNA. CELF2 expression is upregulated because the transcription factors NFκB and RelA bind to sites close to the CELF2 promoter (Mallory et al. 2015). CELF2 regulates its own expression by inducing skipping of one of its exons which results in nonsense-mediated decay (Dembowski and Grabowski 2009). This means only a moderate expression of CELF2 can be reached. Since CELF2 and hnRNP C bind antagonistically to the U-stretches they most likely compete for binding sites. To ensure that CELF2 expression is high enough to bind, hnRNP C expression is downregulated. It might also be crucial in which order both proteins bind but this was not addressed. CELF2 binds to the TRAF3 *cis*-acting element which is wrapped by hnRNP C tetramers or vice versa (first CELF2 binds and then hnRNP C). This brings CELF2 within close proximity to the 3'splice site and it prevents binding of U2AF (Dasgupta and Ladd 2012). As a result the splice site cannot be recognized and TRAF3 exon 8 is skipped. TRAF3ΔE8 then activates the non-canonical NFκB pathway (Michel et al. 2014).

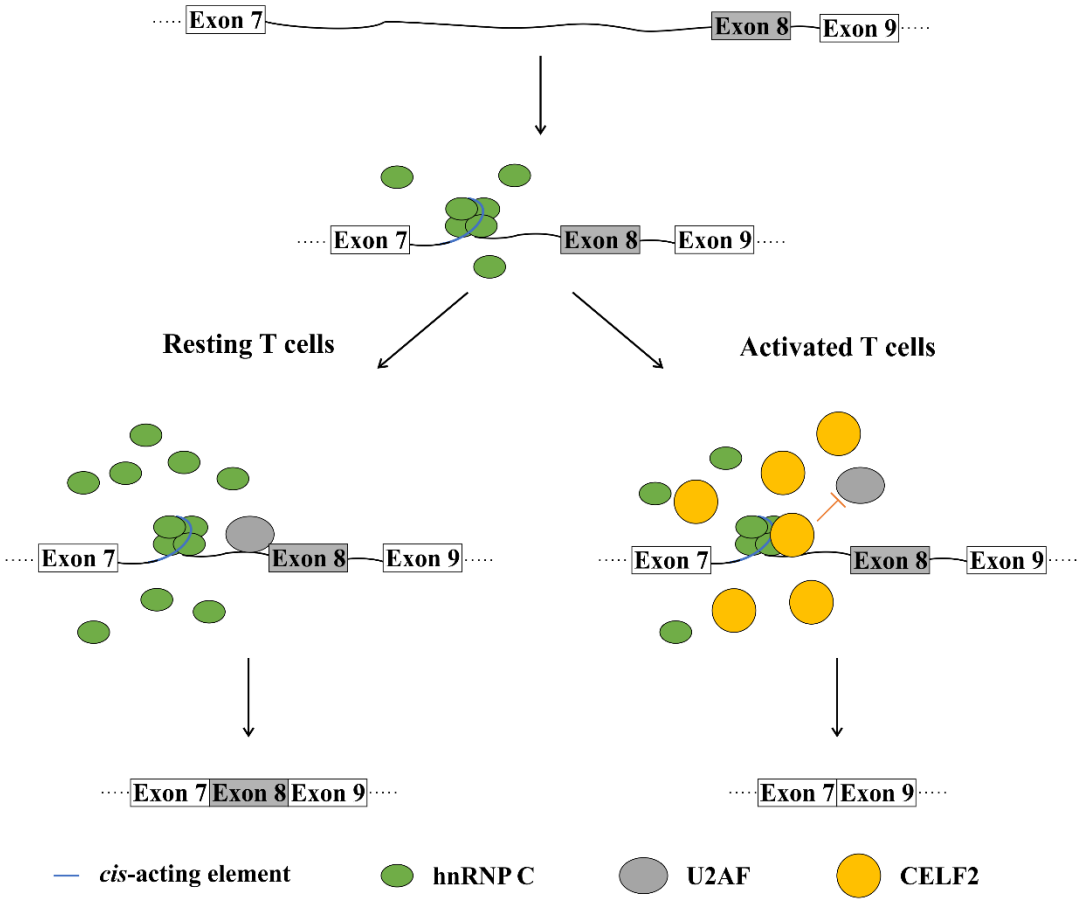

Figure 4.1: Model describing the regulation of TRAF3 exon 8 skipping.
In resting T cells an hnRNP C tetramer binds to the *cis*-acting element and wraps the pre-mRNA around itself. U2AF recognizes the 3'splice site resulting in TRAF3 exon 8 inclusion. In activated T cells hnRNP C expression decreases and CELF2 expression is upregulated. There are still enough hnRNP C tetramers to wrap the pre-mRNA but CELF2 binds additionally. The wrapping brings CELF2 in close proximity to the 3'splice site where it prevents the binding of U2AF. TRAF3 exon 8 is skipped.

The proposed model explains why a knockdown of either CELF2 or hnRNP C leads to decreased exon skipping. HnRNP C is needed to bring CELF2 within close proximity to the splice site and CELF2 blocks splice site recognition. This is an agreement with the observation that the position of the *cis*-acing element is crucial. If the *cis*-acting element is moved further away from the exon hnRNP C still wraps the RNA but CELF2 is not close enough to the splice site and cannot prevent binding of U2AF. A movement of the *cis*-acting element closer to exon 8 results again in a suboptimal distance between 3'splice site and CELF2 leading to exon inclusion. TRAF3 exon 8 splicing is therefore regulated by a distinct balance of hnRNP C and CELF2.

4.6 Species-Specific Regulation of TRAF3 Exon 8 Splicing

TRAF3 exon 8 splicing was additionally investigated in the mouse T cell line El4. Two different situation were investigated: conservation between resting T cells and conservation in activated T cells. TRAF3 exon 8 splicing is conserved in resting T cells but the TRAF3ΔE8 induction in activated cells is species-specific since it occurs in human but not in murine T cells. The regulation of the species-specific splicing induction needs further investigation but the experiments point to a crucial effect of the *trans*-acting environment in murine cells.

4.6.1 TRAF3 in mice

In murine T cells no induction of TRAF3 exon 8 skipping was detected in response to T cell activation, neither in RT-PCR nor in western blot analyses. This raises the questions: what is known about murine TRAF3 (mTRAF3)? Are any functional or alternative splicing data available? The literature focuses on the analysis of TRAF3 full length in murine cells. There is a TRAF3 deficient mouse model. These mice are very small, have an impaired immune system and die 10 days after birth (Xu et al. 1996). This model demonstrates that mTRAF3 has an important function in postnatal development and in the establishment of a competent immune system. In the nucleotide database of NCBI, two murine TRAF3 isoforms are listed, NM_011632.3 and NM_001286122.1. The shorter isoform lacks exon 2 as well as exon 8 which proves the existence of an isoform lacking exon 8. This is consistent with the observation of the RT-PCR results that show a low basal exon 8 skipping in murine cells which does not change in response to T cell activation. Exon 2 lies outside of the coding region and its skipping has no impact on the encoded protein.

In humans, exon 7, exon 8 and exon 9 are alternatively spliced in TRAF3 and in response to T cell activation in Jsl1 only exon 8 skipping is induced. All of the human TRAF3 short isoforms are known to activate the transcription factor NFκB (Van Eyndhoven et al. 1999). There are more than the two already mentioned isoforms predicted in mice which raises the possibility that exon 7 and exon 9 might also be alternatively spliced. One of the other short isoforms could take over the job of the hTRAF3ΔE8 isoform in mice in T cell activation but this is speculative. The primers used in this study bind to exon 7 and exon 9 and thereby only amplify transcripts which contain both exons. This hypothesis was not addressed in this study.

In order to find an explanation for the missing induction of TRAF3 exon 8 skipping in stimulated El4 cells it was tested if PMA induces T cell activation. CXCL12 expression

increases significantly indicating that stimulation results in activation. However this is based on a single cytokine. T cell activation can be enhanced by a combined treatment of PMA and ionomycin. Ionomycin treatment acts on the calcium channels and thereby activates NFAT which is translocated in the nucleus and triggers gene expression changes leading to adaptive immune response (chapter 1.5). Additionally ionomycin was shown to induce PKC activation (Chatila et al. 1989) which is also activated by PMA treatment.

4.6.2 Conservation and sequence identity

Bioinformatic studies comparing orthologous genes from humans and mice reveal a high conservation in both species in respect to the number of exons, the exon lengths, the exon boundaries and the sequence identity (Modrek and Lee 2003). In TRAF3, exon 4 to exon 11 are conserved in length and show a sequence identity greater than 80 % (Table 4.1). Modrek et al. (2003) found in their study that 90 % of the analyzed exons are conserved between both species where conservation is defined as having the same exon boundaries in both genomes. These exons have sequence identities of around 87 % which fits very well to the found conservation between human and murine TRAF3. Modrek et al. (2003) observed a high similarity between alternatively spliced exons. They argue that the high conservation can be explained by a similar regulation of these exons. Supporting this assumption TRAF3 exon 8 is the exon with the highest conservation (98.7 %) and the RT-PCRs show exon 8 skipping in both cell lines. In contrast to human cells murine cells do not show an increase in TRAF3ΔE8 isoform expression upon stimulation. This strongly indicates the presence of a different regulation mechanism in human over murine cells in contrast to the postulation from Modrek et al. (2003). Previous studies analyzing species-specific splicing mainly compared orthologous genes and distinguished three cases. 1) an exon is present in both species and consequently constitutive. 2) alternatively spliced exons are exons that are alternatively spliced in both species. 3) an alternatively spliced exon is only present in one species but not in the other. In this study TRAF3 exon 8 splicing was analyzed in human and murine cells. TRAF3 exon 8 is alternatively spliced in both species in a low basal level (~5 % exon skipping in resting conditions) but an additional activation-dependent induction of exon 8 skipping was investigated which was only observed in human and not in murine T cells. This study adds a further layer to the analysis of species-specific splicing which is rarely found in the literature. A comparison of resting T cells as usually done in literature ends up with a conserved splicing event. Here a species-specific splicing induction was additionally discovered. Considering that this phenomenon is probably not a unique event it indicates that investigation of species-

specificity demands more than just comparing RNA sequences and resting conditions only since this is a limited view on species-specificity and will not give a complete picture.

Table 4.1: Human and murine TRAF3 exon lengths and their sequence identities.
Exon numbers written in bold mark exons that have the same length in human and mouse.

Exon number	Human exon length [nt]	sequence identity [%]	Murine exon length [nt]	Exon number	Human exon length [nt]	sequence identity [%]	Murine exon length [nt]
1	197	73.7	233	**7**	81	88.9	81
2	139	37.9	111	**8**	75	98.7	75
3	262	83.6	259	**9**	93	91.4	93
4	52	84.6	52	**10**	141	83.0	141
5	105	90.5	105	**11**	175	90.3	175
6	168	82.1	168	12	6305	63.5	5671

4.6.3 *Cis*-acting element

Most species-specific splicing events are regulated by *cis*-acting elements (Barbosa-Morais et al. 2012; Gao et al. 2015). A closer look at the *cis*-acting element which is necessary for activation-dependent TRAF3 exon 8 skipping revealed a conservation of 83.7 %. The binding of CELF2 and hnRNP C depends on the presence of an UUUUUGU-motif in human T cells. These binding sites are 100 % conserved by both species. Moreover the minigene data strongly points to a dependency on the cellular environment but not to a *cis*-directed splicing regulation. Barbosa-Morais et al. (2012) stated in their publication that *trans*-dependent species-specific splicing regulation is a minor mechanism. A study from 2014 demonstrated differential expression of RNAs in different species. The authors proposed a contribution by this to a higher complexity in splicing regulation (Lin et al. 2014). This shows that species-specific regulation by *trans*-acting factors is known but more as an exception than a rule. The results from this thesis strongly indicate a regulation of the species-specific splicing induction by the *trans*-acting environment which is a rarely-found regulatory mechanism.

4.6.4 *Trans*-acting environment

The minigene experiments indicated a regulation by the *trans*-acting environment. This was investigated in more detail. In the human cells lines Hek and Ramos no TRAF3 exon 8 skipping was observed. This was the result of a missing CELF2 expression and led to the hypothesis of a missing CELF2 expression in El4 cells. A western blot showed a high CELF2 expression in El4 cells which is even higher than in activated human T cells. Several bands were detected which indicates that CELF2 might be a target for post-translational modifications or undergo splicing. CELF2 can be post-translationally modified by phosphorylation or acetylation (Vlasova-St. Louis et al. 2013). Phosphorylation happens on serine-threonine rich elements in the linker domain of CELF2 that separates the second and the third RNA recognition motifs. Phosphorylation of these sites modulates CELF2 binding to RNA. Acetylation has an impact on protein-protein interaction profiles (Vlasova-St. Louis et al. 2013). The hypothesis that the multiple CELF2 bands in western blots correspond to CELF2 subjected to post-translational modifications could not be confirmed in further experiments. It is unlikely that all the different mCELF2 bands refer to post-translational modifications because most of these modifications lead to small size changes, which are often not detectable by western blot. Alternative splicing of CELF2 is an additional explanation for the detection of different bands. Both human and mouse CELF2 are alternatively spliced (Figure 3.23). These isoforms can have different localizations (Vlasova-St. Louis et al. 2013). To exclude the possibility that humans encode a CELF2 isoform which is not present in mice hCELF2 1 was cloned. All other experiments were done with hCELF2 4 which is not expressed in murine cells. In contrast hCELF2 1 is 100 % conserved in both species and induces TRAF3 exon 8 skipping in Hek cells as previously observed for hCELF2 4. Although hCELF2 1 exists in mice it might have a cytoplasmic localization and be unable to induce TRAF3 exon 8 skipping. This is more unexpected as it is 100 % conserved to the human CELF2 isoform but it is known that the localization of the different CELF2 isoforms can differ. The expression of the CELF2 isoform might also be too low in mice to induce TRAF3 exon 8 skipping. New annotations reveal the existence of further CELF2 isoforms in both organisms (Figure 4.2). The localization of the different isoforms as well as their impact on TRAF3ΔE8 induction should be analyzed in more detail (4.7).

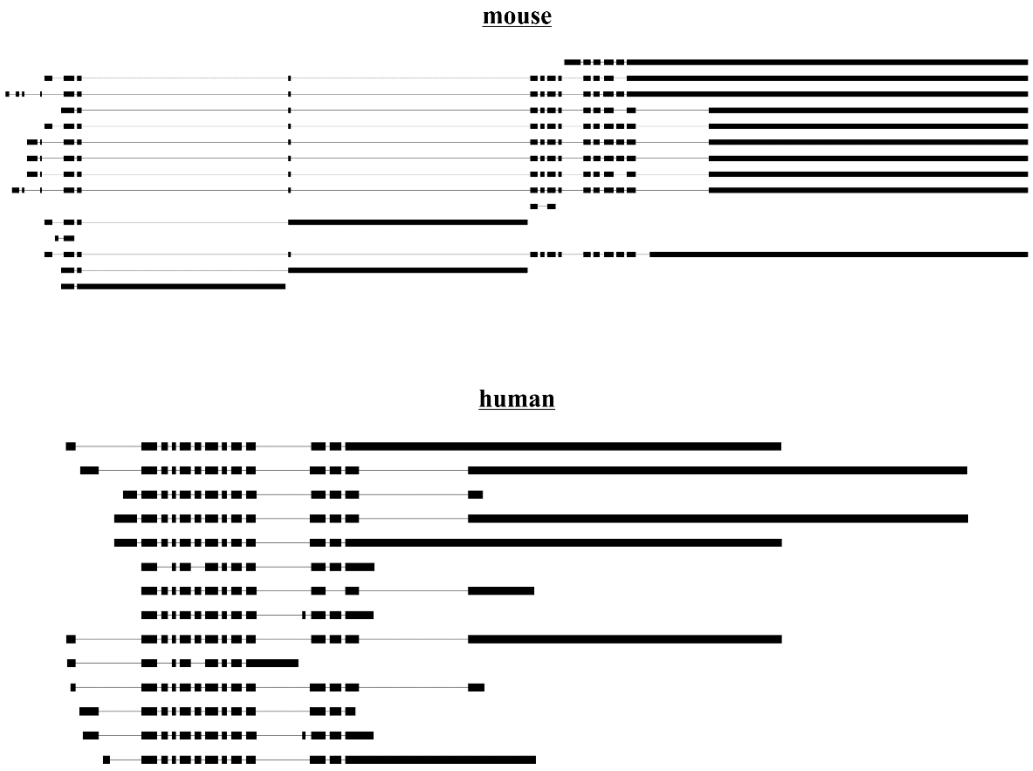

Figure 4.2: Annotated mouse and human CELF2 isoforms.
Schematic presentation of human and mouse CELF2 isoforms (referring to human GRCh37/hg38 and mouse GRChm38/mm10). Exons are to scale and introns are not to scale. Schemes were provided by Alexander Neumann.

The investigations of the regulation of TRAF3 exon 8 splicing in humans resulted in a second *trans*-acting factor which is needed for regulation, hnRNP C. A proposed model in human T cells stated that hnRNP C is needed to form tetramers that wrap the pre-mRNA around itself. This wrapping brings CELF2 into close proximity to the 3'splice site. In murine cells the distance of the *cis*-acting element is similar to the distance of the *cis*-acting element within humans (343 nt versus 336 nt). This raises the possibility of a similar wrapping in murine cells. The wrapping of pre-mRNA by hnRNP C tetramers was detected in human cells and it remains undetermined whether a similar mechanism exists in mice. Mice have four different hnRNP C isoforms instead of the two in humans (Han et al. 2010). This opens the possibility of a more complex regulation in mice. These isoforms shuttle between the nucleus and the cytoplasm and can be targeted for post-translational modification. Known modifications are methylation, phosphorylation, ubiquitination and sumoylation (Han et al. 2010). Based on this a missing activation-dependent Traf3 exon 8 skipping in mice might be caused by different localizations of hnRNP C isoforms and other post-translational modifications compared to human cells. The

results in human T cells revealed the importance of a distinct balance of CELF2 and hnRNP C expression. The hnRNP C expression must be high enough to wrap the TRAF3 pre-mRNA and enough CELF2 must bind to prevent the recognition of the 3'splice site by U2AF65. Further experiments are needed to investigate if such a balanced expression is present in murine cells.

In summary the experiments performed to address species-specific TRAF3 exon 8 alternative splicing revealed that exon 8 skipping is conserved in resting T cells for humans and mice. A species-specific splicing induction was additionally found in response to T cell activation since this induction happens in human but not in murine cells. Minigene experiments directed to a regulation by the *trans*-acting environment and not to a *cis*-directed regulation which is often observed in species-specific splicing. The reason why an induction of TRAF3ΔE8 in response to T cell activation is only present in humans but not in mice needs further elucidation.

4.7 Future Perspectives

The aim of this study was to explore the regulation of TRAF3 exon 8 skipping in response to T cell activation. In human T cells TRAF3 exon 8 is regulated by a combination of *cis*- and *trans*-acting factors as well as the TRAF3 pre-mRNA structure. The *cis*-acting element was shown to be necessary but the sufficiency to induce splicing of an unrelated exon needs new minigene experiments. The used minigenes have CELF2 binding sites next to the binding sites within the *cis*-acting element. CELF2 overexpression alone induces exon skipping in these minigenes. New minigenes need to be cloned which contain UUUUUGU-motifs only within the *cis*-acting element. Later experiments showed that the position of the *cis*-acting element is crucial and this also was not included in the minigenes experiments addressing the sufficiency.

CELF2 and hnRNP C were identified as *trans*-acting factors but CELF2 was determined to be the driving force. Mallory et al. (2015) analyzed why CELF2 expression increases in activated T cells and found an increased transcription and a higher stability of the CELF2 mRNA. They could not explain why the mRNA became more stable because the hypothesis that nonsense mediated decay is involved could not be proven. CELF1, another CELF family member is known to be involved in gene expression changes in T cell activation. In resting T cells it binds to the 3'UTR of transcripts which have GU-rich elements and facilitates their degradation. In response to T cell activation CELF1 becomes phosphorylated and this lowers its binding affinity to these elements. The target transcripts become more stable (Vlasova-St. Louis et al. 2013). In accordance with this it would be of interest to investigate if CELF2 stability also

depends on CELF1. A CELF1 binding to CELF2 transcript could be explored with UV-Crosslink immunoprecipitation experiments.

The UV-Crosslink experiments demonstrated binding of many proteins to the *cis*-acting element. It might be that more proteins which have not yet been taken into account are additionally involved in the regulation of TRAF3 exon 8 skipping. A first indication for the participation of other proteins would be provided by a double knockdown of CELF2 and hnRNP C. Since a knockdown of hnRNP C is not viable in Jsl1 minigenes would have to be cloned in which binding sites for both proteins were replaced. If the minigene still showed some exon skipping other proteins might be involved in the regulation.

Mallory et al. (2015) published that 72 exons undergo CELF2-dependent splicing in response to T cell activation. Since these 72 exons account for a third of the activation-dependent splicing changes observed in activation, regulation by CELF2 seems to be a more general mechanism. It would be interesting to check if there are more exons whose splicing is regulated by a combination of hnRNP C and CELF2. The best way to investigate this would be the usage of double knockdown cells but hnRNP C knockdown is not viable in Jsl1 cells. This means Hek cells would have to be used again as model system. All 72 exons which undergo CELF2-dependent splicing (Mallory et al. 2015) would have to be checked for a response to hnRNP C knockdown.

The assumption that CELF2 and hnRNP C bind antagonistically is based on two observations. First UV-Crosslinks with disrupted CELF2 binding sites show a decrease in CELF2 binding but a stronger binding of hnRNP C. Second hnRNP C expression decreases and CELF2 expression is induced in activated T cells. A further means of validating the antagonistic binding are UV-Crosslink experiments in which the concentration of one protein (e.g. hnRNP C) is set but the concentration of the second (e.g. CELF2) is increased by addition of recombinant protein. At some threshold the added recombinant protein will replace the other (hnRNP C). This can also be done vice-versa. Such an experiment would provide a better knowledge of the needed ratio of both proteins since a distinct balance of both is necessary to ensure an efficient TRAF3 exon 8 skipping. According to the proposed model a specific ratio of hnRNP C and CELF2 is important because there must be enough hnRNP C to wrap the pre-mRNA around itself and CELF2 must bind to prevent 3'splice site recognition. The wrapping of TRAF3 pre-mRNA by hnRNP C was not directly shown. The assumption is based on findings from literature and the observation that the distance of the *cis*-acting element to exon 8 is crucial for exon skipping. The original publication which showed the wrapping of hnRNP C

tetramers to 230 to 240 nt long of pre-mRNAs, used a combination of electron micrographs and glycerol gradients (Huang et al. 1994). In addition a structural approach could be used to prove the wrapping model. There is a crystal structure from an hnRNP C monomer bound to RNA (Cieniková et al. 2014) and an NMR structure of the hnRNP C tetramerization domain (Whitson et al. 2005). In a minigene experiment the distance of the *cis*-acting element to TRAF3 exon 8 could also be enlarged by 240 nt. Possibly this might add another round of wrapping. The binding of a second tetramer might also prevent exon skipping because no binding site for CELF2 might be left. CELF2 binding could also be blocked by steric hindrance.

In mice the remaining questions are why no PMA-induced skipping can be observed and how the ncNFκB pathway is activated in these cells if TRAF3ΔE8 is not induced. As already indicated more investigations on both *trans*-acting factors CELF2 and hnRNP C should be pursued to verify a direct binding of both proteins to the *cis*-acting element. This can be done with UV-Crosslink immunoprecipitation experiments. The isoform hCELF2 1 induces exon skipping in Hek cells and although this isoform is 100 % conserved in mice El4 cells show no skipping. It was discussed that the localization of this isoform might be not nuclear but cytoplasmic in El4 cells. To check this possibility a nuclear localization signal could be added and El4 cells could be transfected with the corresponding construct. A radioactive PCR would show if the addition of the nuclear localization signal induces exon 8 skipping. The different CELF2 bands observed in the western blot should also be identified. A better resolution could be reached through the usage of gradient gels. Then single bands could be cut out and analyzed by mass spectrometry. Mass spectrometry should provide information about putative post-translational modifications and different isoforms. The effects of the identified isoforms on TRAF3 exon 8 splicing could be assessed in more detail. A possible first step would be to discover if one isoform is located only in the nucleus or only in the cytoplasm. To this end nuclear and cytoplasmic extracts could be prepared. Both could be separated on gradient gels to have a higher resolution. Similar analyses can also be performed with hnRNP C to identify putative differences between the mouse and the human protein. If all experiments pointed to the presence of the same CELF2 and hnRNP C isoforms as in human cells, it might be that the ratio of both proteins is suboptimal. The suggested experiment in human cells to analyze the needed ratio of both proteins by adding recombinant protein might help here. The ratio could be manipulated by testing several concentrations of siRNAs and knocking down one of the proteins.

The last remaining question is how the ncNFκB pathway is activated in El4 cells because full length TRAF3 inhibits this pathway in humans. The activation of the ncNFκB pathway results in chemokine expression and triggers an adaptive immune response. Since chemokine expression was measured in PMA treated El4 cells, these cells seems to be activated T cells. In human cells TRAF3 exon 7, exon 8 and exon 9 can be alternatively spliced and all three of them activate NFκB (Van Eyndhoven et al. 1999). In human T cells, only exon 8 is skipped in response to T cell activation. It is possible that in mice one of the other exons is skipped. The resulting isoform could be substituting the job of TRAF3ΔE8 in murine cells.

The presented work deciphers the splicing regulation for TRAF3 exon 8 alternative splicing in response to T cell activation in human cells. The intronic splicing silencer is located 336 nt upstream of TRAF3 exon 8. It is bound by the two *trans*-acting factors CELF2 and hnRNP C. CELF2 might prevent the recognition of the 3'splice site and hnRNP C might wrap TRAF3 pre-mRNA around itself to bring CELF2 close to the 3' splice site.
Since CELF1 is already known to regulate gene expression in response to T cell activation, this work combined with other recent CELF2 publications add a further CELF family member to the regulators of T cell activation. This is also the first study showing that CELF2 and hnRNP C work synergistically in the induction of exon skipping.
The investigations in murine T cells revealed a new species-specific splicing induction. The species-specific splicing induction is *trans*-driven regulated which is a rarely observed phenomenon.

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6 Supplemental material

6.1 Frequently Used Abbreviations

3'ss	3' splice site
5'ss	5' splice site
APC	Antigen presenting cell
BAFF-Rs	B cell activating factor belonging to the TNF family receptors
BP	branch point
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
DMEM	Dulbecco's modified eagles's medium
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetatic acid
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
et al.	et alii
FBS	Fetal bovine serum
GFP	green fluorescence protein
hnRNP	heterogeneous ribonucleoprotein particle
IL-1R	interleukin-1 receptor
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
Jsl1	Jurkat
LtßR	lymphotoxin-β-receptor
MHC	major histo-compatibility complex
mRNA	messenger RNA
ncNFκB	non-canonical NFκB
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NFκB inducing kinase
NLS	nuclear localization signal
nt	nucleotides

PCR	polymerase chain reaction
PKC	protein kinase C
PMA	phorbol myristate acetate
PNK	polynucleotide kinase
qRT-PCR	quantitative realtime PCR
RNA	ribonucleic acid
RNAP II	RNA polymerase II
RNA-seq	RNA sequencing
RPMI	Roswell park memorial institute
RS	serine-arginine rich
RT-PCR	Reverse transcriptase PCR
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
snRNPs	small nuclear ribonucleoproteins
TCR	T cell receptor
TLRs	toll-like receptors
TNFR	tumor necrosis factor receptor
TRAF3	tumor necrosis factor receptor associated factor 3
UTR	Untranslated region
UV	ultraviolet
Wt	Wild type
Δ	delta

Common international system of units (SI) units were not considered in the list.

6.2 Used Primers and Antibodies

Table 6.1: Primers used in RT-PCR.

Internal number	Primer name	Primer sequence
1131	ARMC10_for	GCTATTATTCGTGAATTGGGTGGTATTCCAATTGTTGC
1132	ARMC10_rev	GGCACGGAGAAGTCCTTCTGTCATGG
1065	BRD8_for	GCAAACATCCGAGTCTGGGATCAGTGC
1066	BRD8_rev	CCTCTTCTGTAACTTCCACCTCTGGAACTGG
1662	CELF2mRNA_for	GGACTCTGCAAGGACTGGCTGG
1663	CELF2mRNA_rev	GGCGCCAAGTCCTCCATTCAGAG
2878	E7R1 (Minigene primer)	CAGCGCTTCCAGAAGGGCTCAGAGT
1133	FAM33A_for	CCAGAAAGCTGAGTCTGATCTGGATTAC
1134	FAM33A_rev	GTGAAATTTGAATTGCTCTGCCGCAG
1073	MACF1_for	GTCTACTAGATGACTGGGCAAGTAAGGG
1074	MACF1_rev	GAACCTCCTCCATTCTGTTGAGGATAGCC
1221	MouseTRAF3_for	CATGAAGACACAGATTGTCCCTGT
1222	MouseTRAF3_rev	CCAGGGAGTTGCTCCACTCCTTCAGC
1121	MYL6_for	GTGACTTCACCGAAGACCAGACCGC
1122	MYL6_rev	GTGACAAGAACATGCCGGATTTCAGCACC
1668	NAB2_for	GGAGAACAGAGTCACCCTGAAATCC
1669	NAB2_rev	GAGGTCCTGGGTCTGAAGACACC
1071	PPP1R12A_for	CCAAGCACCACATCAACACCAACAG
1072	PPP1R12A_rev	CTGTGTTGATCTTCTAGATTGTCTTGC
1075	REPS1_for	CCAGGTGAGGTAGGTTATTCAGGCTCTCC
1076	REPS1_rev	GCTTGGAGTCATACGAATGGGAACTGG
2877	T7Mlu (Minigene primer)	GGGAGCTTGGTACCACGCGTCGACC
442	TRAF3for	CACGAAGACACCGACTGTCCCTGC
443	TRAF3rev	CGAGCGAGTTGCTCCACTCCTTCAGC

Table 6.2: Primers used in qRT-PCR.

Internal number	Primer name	Primer sequence
51	CXCL12mousefor	CTGGCCGCGCTCTGCATCAGTG
52	CXCL12mouserev	GTCTGTTGTTGTTCTTCAGCCGTGC
17	CXCL13for	GGTCAGCAGCCTCTCTCCAGTCC
53	CXCL13mousefor	CCTGGGAATGGCTGCCCCAAAAC
54	CXCL13mouserev	GGAGCTTGGGGAGTTGAAGACAGAC
18	CXCL13rev	CTTGGACAACCATTCCCACGGG
1183	hRXRA-q-fw	AGAAGGTCTATGCGTCCTTGG
1184	hRXRA-q-rev	CAGGCATTTGAGCCCGATG
89	ICOSL1for	CACCATGCGGCTGGGCAGTC
90	ICOSL1rev	GCTTCCTTCAGGGCAAGCGC

Table 6.3: Antibodies used in UV-Crosslink immunoprecipitations.

Antibody	Company
Mouse-α-CELF2 (#C9367)	Sigma
Mouse-α-HA (#sc-7392)	Santa Cruz
Mouse-α-hnRNP C (#sc-32308)	Santa Cruz
Mouse-α-hnRNP L (#sc-32317)	Santa Cruz
Rabbit-α-CELF1 (#GTX114129)	GeneTex

Table 6.4: Primers and backbones for cloning.

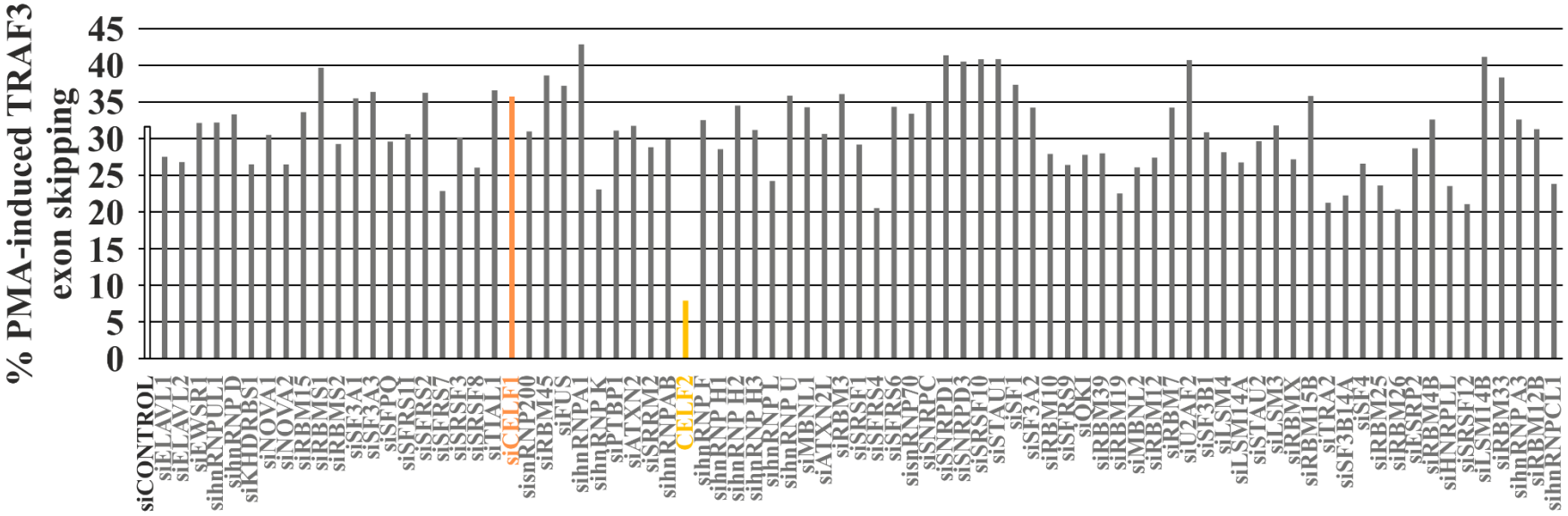
Cloned plasmid name (internal number)	Plasmid name in this study	Primer used to generate insert (internal number)	Template (internal number)	Plasmid used as backbone	Used restriction enzymes	Antibiotic resistance kanamycin (kan) or ampicillin (amp)
CD9 Pst weak without RS (374)	CD9	**1685:** AATTAGGTCC TTGTTAGGGC AGTAA **1686:** TTGCATATTT TTCATCTATT AGATTATAGT ACATTGAGTG TTATTTTTCC **1687:**GGAAAAATAA CACTCAATGT ACTATAATCT AATAGATGAA AAATATGCAA **1688:** GAAATAAATT GATATTTACC AAGTGGCACA TCTAATATTA ATGTC **1689:** GACATTAATA TTAGATGTGC CACTTGGTAA ATATCAATTT ATTTC **1690:** AATTAAGCTT GGATCTGTCG ACGG	MG24 (453)	pCAT-CD	PpuMI, HindIII	amp
CD9 Pst Traf3 reg' weak (375)	CD9T3	**1685:** AATTAGGTCC TTGTTAGGGC AGTAA **1686:** TTGCATATTT TTCATCTATT AGATTATAGT ACATTGAGTG TTATTTTTCC **1687:** GGAAAAATAA CACTCAATGT ACTATAATCT AATAGATGAA AAATATGCAA **1688:** GAAATAAATT GATATTTACC AAGTGGCACA TCTAATATTA ATGTC **1689:** GACATTAATA TTAGATGTGC CACTTGGTAA ATATCAATTT ATTTC **1690:** AATTAAGCTT GGATCTGTCG ACGG	MG25 (454)	pCAT-CD	PpuMI, HindIII	amp
CELF2-GFP (365)	CELF2-GFP	**863:** GCCTCGAGAT GAACGGAGCT TTGGATCACT CAGACC **864:** GCAGATCTGT AAGGTTTGCT GTCGTTTTTG GAACG	cDNA JSL1	pEGFP-N3	XhoI, BglII	kan
CELF2-MYC-HIS (370)	CELF2-MYC-HIS	**1359:** GCACTAGTAT GAACGGAGCT TTGGATCACT C	CELF2-GFP (365)	pEF-MYC-HIS B	SpeI, EcoRV	amp

		1364: GCGATATCGT AAGGTTTGCT GTCGTTTTTG G				
MG18 (369)	9	**28:** GCACCATATG CAGTAGTAAG AGTGTATGGT TAGAC **Traf3genref:** GCAGATCTGC TAAAATTTCC GTAACA	cDNA JSL1	pCAT-CD	NdeI, BglII	amp
MG19 (448)	10	**2867:** GCACCATATG CAGTAGTAAG AGT **2872:** GCAGATCTGC TAAAATTTCC GTA **2519:** CTATTTATAT GTGTTTTCAT GCAATTATTG TTTATG **2520:** CATAAACAAT AATTGCATGA AAACACATAT AAATAG	MG7 (9)	pCAT-CD	NdeI, BglII	amp
MG20 (449)	11	**2867:** GCACCATATG CAGTAGTAAG AGT **2872:** GCAGATCTGC TAAAATTTCC GTA **2517:** GCTTGCTTTG AAAATATATT TATATATGTG TAC **2518:** GTACACATAT ATAAATATAT TTTCAAAGCA AGC **2521:** CTATTTATAT GTGTTTTCAT GCAATTATTG TATATG **2522:** CATATACAAT AATTGCATGA AAACACATAT AAATAG	MG7 (9)	pCAT-CD	NdeI, BglII	amp
MG21 (450)	12	**2867:** GCACCATATG CAGTAGTAAG AGT **2872:** GCAGATCTGC TAAAATTTCC GTA **2524:** GAAAAGCTAC CTTATATTTA TTTTTGTGTA CCGTATATTA GG **2525:** GCCAGAAACT CATAAACAAA AATTGCATGA AAACAC **2526:** AAAATAAATA TAAGGTAGCT TTTCAAAGAC C	MG7 (9) MG16 (18)	pCAT-CD	NdeI, BglII	amp

		2527: CAATTTTTGT TTATGAGTTT CTGGCCCTTA CA				
MG28 (457)	13	**2867:** GCACCATATG CAGTAGTAAG AGT **2872:** GCAGATCTGC TAAAATTTCC GTA **2747:** CAGTTTATGT TTTTTCTTAT ATTTATTTTT GTGTACCGTA TATTAGG **2748:** AGAATTTAAA AAAGAAATAA ACAAAAATTG CATGAAAACA C **2749:** GCAATTTTTG TTTATTTCTT TTTTAAATTC TGGCACTAC **2750:** CACAAAAATA AATATAAGAA AAAACATAAA CTGAAATGCA	MG7 (9) MG16 (18)	pCAT-CD	NdeI, BglII	amp
MG27 (456)	14	**2867:** GCACCATATG CAGTAGTAAG AGT **2872:** GCAGATCTGC TAAAATTTCC GTA **2743:** CACATTGAAC AATTTTATAT TTATTTTTGT GTACCGTATA TTAGG **2744:** CCAGTTTCTT TAGAACATAA ACAAAAATTG CATGAAAACA C **2745:** CAATTTTTGT TTATGTTCTA AAGAAACTGG AAGTTTAAG **2746:** CACAAAAATA AATATAAAAT TGTTCAATGT GACATGC	MG7 (9) MG16 (18)	pCAT-CD	NdeI, BglII	amp
MG26 (455)	15	**2867:** GCACCATATG CAGTAGTAAG AGT **2872:** GCAGATCTGC TAAAATTTCC GTA **2739:** GTAAATGTAT TCTTGTATAT TTATTTTTGT GTACCGTATA TTAGG **2740:** CATATACGCC AATATAATAA ACAAAAATTG CATGAAAACA C **2741:** TCATGCAATT TTTGTTTATT ATATTGGCGT ATATGTGCAT AC **2742:** CAAAAATAAA TATACAAGAA TACATTTACT ATAACATTTC TTAAAC	MG7 (9) MG16 (18)	pCAT-CD	NdeI, BglII	amp

MG23 (452)	16	**2867:** GCACCATATG CAGTAGTAAG AGT **2872:** GCAGATCTGC TAAAATTTCC GTA **2532:** ATTTGTCCTT CCTATATTTA TTTTTGTGTA CCGTATATTA GG **2533:** TGTCAGTGAC TGATAAACAA AAATTGCATG AAAACAC **2534:** AAAATAAATA TAGGAAGGAC AAATGTCGGA TAC **2535:** ATTTTTGTTT ATCAGTCACT GACATTCTGC C	MG7 (9) MG16 (18)	pCAT-CD	NdeI, BglII	amp
CELF2-v1-MYC-HIS (373)	CELF2-v1-MYC-HIS	**1359:** GCACTAGTAT GAACGGAGCT TTGGATCACT C **1364:** GCGATATCGT AAGGTTTGCT GTCGTTTTTG G **1660:** CTGAGAGCAT TTGAGCAACT GCTAGTGCAT TAATATTATT CAGTCCAAC **1661:** TTGCTCAAAT GCTCTCAGGT ATGGCGGCTC TGAATGGAGG	pCELF2-MYC-HIS (370)	pEF-myc-his B	SpeI, EcoRV	amp
mTRAF3 (366)	mMG4	**1321:** GCCATATGTG TGAGCTTGCT TTGGAAATGG G **1322:** GCAGATCTCC AGCTGCACGC ATGTGGC	cDNA El4	pCAT-CD	NdeI, BglII	amp

6.3 Figure 3.2D in Large



Jsl1 cells were transfected with around 100 siRNAs (each siRNA is a pool of four individual siRNA targeting the same gene) and treated as described in C. The first bar is the control. CELF2 knockdown shows the strongest effect on TRAF3 splicing and is highlighted in yellow. CELF1 belongs to the same protein family as CELF2 and is colored in orange. The shown results were consistent in two independent RT-PCRs.

Table 6.5: List of all antibodies used in for western blot analysis in this study.

Antibody	Company	Used dilution	Kind of antibody
Goat-α-rabbit (#7074)	Cell Signaling Technology	1:5000	secondary
Horse-α-mouse (#7076)	Cell Signaling Technology	1:5000	secondary
Mouse-α-CELF2 (#C9367)	Sigma	1:1000	primary
Mouse-α-GAPDH (#GTX239)	GeneTex	1:5000	primary
Mouse-α-GFP (#sc-9996)	Santa Cruz	1:500	primary
Mouse-α-hnRNP C (#sc-32308)	Santa Cruz	1:500	primary
Mouse-α-hnRNP L (#sc-32317)	Santa Cruz	1:1500	primary
Rabbit-α-HIS (#3735)	Roth	1:5000	primary
Rabbit-α-TRAF3 (#4726)	Cell Signalling Technology	1:1000	primary
Rabbit-α-VINCULIN (#sc-5573)	Santa Cruz	1:500	primary

6.4 List of Figures

6.5 List of Tables

6.6 Acknowledgment

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6.8 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Publications:

A.-S. Schultz, M. Preußner, M. Bunse, R. Karni, F. Heyd, **Activation-dependent TRAF3 exon 8 alternative splicing is controlled by CELF2 and hnRNP C binding to an upstream intronic element**. *Mol. Cell. Biol.* (2016) (Accepted manuscript)

M. Preußner, I. Wilhelmi, A.-S. Schultz, F. Finkernagel, M. Michel, T. Möröy, F. Heyd, **Rhythmic U2af26 alternative splicing controls PERIOD1 stability and the circadian clock in mice**. *Mol. Cell*. **54**, 651–662 (2014)

M. Michel, I. Wilhelmi, A.-S. Schultz, M. Preussner, F. Heyd, **Activation-induced tumor necrosis factor receptor-associated factor 3 (Traf3) alternative splicing controls the noncanonical nuclear factor κB pathway and chemokine expression in human T cells**. *J. Biol. Chem.* **289**, 13651–13660 (2014).

6.9 Statutory Declaration

I, Astrid-Solveig Schultz, declare that I have developed and written the enclosed dissertation entitled

"*Deciphering the splicing regulation for TRAF3 exon 8 alternative splicing – CELF2 and hnRNP C regulate TRAF3 exon 8 skipping upon T cell activation*"

by myself. I have not used sources or means without declaration in the text. Any thought from others or literal quotations are clearly marked. The thesis was not used in the same or in a similar version to achieve an academic grading.

Berlin, 16.05.2017

Location, Date

Astrid-Solveig Schultz