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Genetic and epigenetic mechanisms in the atopic eczema associated *RAD50*-locus

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ABSTRACT

Atopic eczema is one of the most frequent chronic inflammatory skin diseases determined by multiple genetic and environmental risk factors and presumably shaped by epigenetic mechanisms. T helper cells 2 (Th2) expressing interleukin 4, 5, and 13 (IL4, IL5, and IL13) play a central role in atopic immune responses. Single nucleotide polymorphisms (SNPs) in the Th2 cytokine locus in intronic regions of the DNA-repair gene *RAD50* have been repeatedly associated with atopic eczema, asthma, atopy, and elevated levels of IgE. In mice, this particular region serves as an epigenetically regulated locus control region (LCR) consisting of several *Rad50* hypersensitive sites (RHS), which regulates the coordinate expression of *Il4* and *Il13*.

As the human Th2 cytokine LCR is not well characterized yet with respect to atopic diseases, investigations of each of the human conserved RHS sites RHS5, RHS6, as well as RHS7 with a special emphasis on atopy-associated SNPs regarding their regulatory potential on promoters and the effects on interleukin expression were performed. It was found that human RHS7 acts as a strong and T cell specific enhancer on a minimal promoter (593.6 % increase in luciferase activity), whereas human RHS5(II) and RHS6(I) were characterized as strong repressors in Jurkat and HeLa cells (16.1 % and 11.3 % decrease in luciferase activity for the RHS5(II) and the RHS6(I) respectively). One common atopy-associated SNP, the intronic human *RAD50* variant rs2240032, is located in the human conserved RHS7 site. Within this study, allele-specific binding of the transcription factors SMAD3, SP1 and additional protein complex partners, including SKI and EXOSC complex proteins, was found at rs2240032. With the atopy-risk allele being present, the SMAD3 and SP1 binding sites are altered, which significantly enhances luciferase activity by 266 % compared to the non-risk allele. Variant rs2240032 has a functional impact on a sub-regulatory region of the RHS7 within the LCR, which encompasses repressor activity. Further, elevated transcript levels of IL4 due to the risk allele in blood cells of the KORA F4 cohort were detected. In addition, preliminary results from T cell clones show genotype specific elevated levels of IL4, IL5, and IL13.

More atopy-associated variants, also featuring genotype specific promoter regulatory activity were identified, namely rs3798134 and rs3798135 within the RHS5(I) and rs2040704 within the RHS6(II). With the non-risk variants being present, those sites did not show promoter regulation *per se*, but promoter regulation was altered when the risk variants were tested. Furthermore, all of the named polymorphisms were identified to exhibit differential transcription factor binding and elevated IL4 expression levels with the risk allele being present.

Lastly, it was analyzed whether differences in the number and the extent of epigenetic sites in the LCR region of Th2 cells exist between atopic eczema cases and healthy controls exist. The multiplex ligation dependent probe amplification (MLPA) assay was successfully established to detect hypersensitive sites on the example of two cell lines. However, purity of the real samples, the T cell populations, remained a challenge and optimization will be subject to further research.

The here described identification of the regulatory role of human RHS sites on transcriptional activity and of functional relevant polymorphism on transcription factor binding and IL4 transcription provides important insights into molecular mechanisms involved in atopic diseases.

ZUSAMMENFASSUNG

Das atopische Ekzem ist eine der häufigsten chronischen Entzündungskrankheiten der Haut, welches durch eine Vielzahl an genetischen Komponenten und Umweltfaktoren hervorgerufen und vermutlich durch epigenetische Mechanismen beeinflusst wird. T Helferzellen 2 (Th2), welche die Interleukine 4, 5 und 13 (IL4, IL5, IL13) exprimieren, spielen eine zentrale Rolle bei atopischen Immunantworten. Einzelnukleotidpolymorphismen (SNPs) in Intronen des menschlichen DNA-Reparaturgens *RAD50* im Th2 Zytokin Lokus wurden wiederholt mit atopischem Ekzem, Asthma, Atopie sowie erhöhten IgE Werten assoziiert. Im Mausmodel wurde gezeigt, dass diese Region als epigenetische Lokus-Kontroll-Region (LCR) fungiert, welche aus mehreren *Rad50* hypersensitiven Stellen (RHS) besteht und die Expression von Il4 und Il13 koordiniert.

Bisher wurde die menschliche Th2 Zytokin LCR nicht in Bezug auf atopische Erkrankungen hin untersucht. In dieser Arbeit wurden die menschlichen, konservierten RHS Stellen RHS5, RHS6 und RHS7 mit besonderem Augenmerk auf Atopie-assoziierte SNPs und ihr Promotor-regulatorisches Potential sowie die Genotyp-spezifische Interleukin Expression hin untersucht. Es wurde herausgefunden, dass die menschliche RHS7 Stelle als starker T Zell-spezifischer Verstärker für Minimalpromotoren fungiert (593,6 % Zunahme der Luciferase-Aktivität), wohingegen die menschlichen RHS5(II) und RHS6(I) Regionen starke Repressoren in Jurkat und HeLa Zellen sind (16,1 % und 11,3 % Abnahme der Luciferase-Aktivität für je RHS5(II) und RHS6(I)). Ein Atopie-assoziierter SNP, die inronische *RAD50* Variante rs2240032, befindet sich in der menschlichen, konservierten RHS7 Stelle. In dieser Arbeit wurde eine Allel-spezifische Bindung der Transkriptionsfaktoren SMAD3, SP1 und weiterer Komplexproteine, einschließlich der Proteine SKI und EXOSC, an den SNP rs2240032 gefunden. Ist das Atopie-Risikoallel von rs2240032 vorhanden, hat dies eine Veränderung der SMAD3 und SP1 Bindestelle zur Folge, was zu einer signifikanten Steigerung der Luciferase-Aktivität auf 266 % im Vergleich zum Nicht-Risikoallel führt. Die Variante rs2240032 hat einen funktionellen Einfluss auf eine subregulatorische Region der RHS7 Stelle innerhalb der LCR, welche über eine Repressor-Funktion verfügt. Des Weiteren wurden erhöhte IL4 Transkript-Werte durch das Risikoallel in Blutzellen der KORA F4 Kohorte detektiert, welche durch vorläufige Ergebnisse mit T Zellklonen gestützt wird, bei denen eine Genotyp-spezifische Erhöhung der IL4, IL5 und IL13 Transkript-Werte gefunden wurde.

Auch weitere Varianten wie rs3798134 und rs3798135 innerhalb von RHS5(I) und rs2040704 innerhalb von RHS6(II) zeigen eine Genotyp-spezifische Promotorregulation. Bei dem Vorhandensein des nicht-Risikoallels ist für diese Stellen keine Promotorregulation zu sehen. Sind jedoch die

Risikoallele vorhanden, ist eine veränderte Promotorregulation zu beobachten. Beim Vorhandensein der Risikoallele für die genannten Polymorphismen wurden außerdem eine veränderte Transkriptionsfaktor-Bindung und eine erhöhte IL4 Expression festgestellt.

Der letzte Schwerpunkt dieser Arbeit lag auf der Analyse von Unterschieden in der Anzahl und Ausbreitung von epigenetischen Stellen innerhalb der LCR Region in Th2 Zellen zwischen Patienten mit atopischem Ekzem und gesunden Kontrollen. Hierfür wurde die „Multiplex Ligation dependent Probe Amplification" (MLPA) Methode erfolgreich in zwei Zelllinien etabliert. Die Gewinnung von reinen T-Zell-Populationen wird jedoch eine anspruchsvolle Aufgabe für zukünftige Projekte bleiben.

Die hier vorgestellten Regulationsmechanismen der RHS Stellen auf die Promotoraktivität sowie die Identifizierung funktionell relevanter Polymorphismen mit Einfluss auf die Transkriptionsfaktorbindung und die IL4 Transkription bieten wichtige Einblicke in die molekularen Mechanismen, welche bei atopischen Erkrankungen eine Rolle spielen.

ABBREVIATIONS

A	adenine
ADP	adenosine diphosphate
AE	atopic eczema
ARA	atopy-related auto-antigens
Bach	transcription regulator protein BACH1
Bp	base pairs
C	cytosine
Cbp	CREB-binding protein
CD	cluster of differentation
cDNA	complementary DNA
CGRE	conserved GATA3 response element
Chr	chromosome
ChIP	chromatin-immunoprecipitation
c-MYB	transcriptional activator Myb
CNS	conserved non coding sequence
CpG	cytosine-phosphate-guanine
CRTH2	G protein-coupled receptor
Cy5	cyanine 5
CXCR3	chemokine receptor CXCR3
DCODE	comperative genomics development
DNA	deoxyribonucleic acid
DNase1	deoxyribonuclease I
DNMT	DNA-methyltransferase
dNTP	desoxynucleotide triphosphate
E.coli	*Escherichia coli*
ECOSC	exosome complex exonuclease
e.g.	*exempli gratia*
ELISA	enzyme-linked immunosorbant assay
EMSA	electrophoretic mobility shift assay
ENCODE	ENCyclopedia of DNA Elements
et al.	*et alteri*
ETS1	transcription factor ETS1
FCER1A	alpha polypeptide of the high affinity receptor I for the Fc
G	guanine
GABRIEL	Consortium Large-Scale Genome-Wide Association Study of Asthma
Gata3	trans-acting T cell specific transcription factor 3
GM-CSF	granulocyte macrophage colony stimulation factor
GWAS	genome wide association studies
H	histone
HapMap	haplotype map
HAT	histone acetyl transferase
HBD	hemoglobin subunit delta

HDAC	histondeacetylase	**Mi**	minor
HeLa	Henrietta Lacks cell line	**MLPA**	multiplex ligation probe amplification
hGH	human growth hormone	**MS**	mass spectrometry
HS	hypersensitive site	**n**	number
IFNγ	interferon gamma	**NCBI**	National Center for Biotechnology Information
Ig	immunoglobulin	**Nfat**	nuclear factor of activated T cells
IL	interleukin	**NF-E2**	nuclear factor (erythroid-derived 2)
K	lysine	**NFkB**	nuclear factor kappa-light-chain-enhancer of activated B cells
kb	kilo base	**NK**	natural killer cells
kDa	kilo Dalton	**Nrf**	nuclear respiratory factor
KORA	Cooperative Health Research in the Region of Augsburg	**OCT**	pou domain class2 transcription factor
λ	wavelength	**OD**	optical density
LCR	locus control region	**OR**	odds ratio
LD	linkage disequilibrium	**ORMDL3**	orosomucoid1-like3
m	milli	**p**	short arm of the chromosome
Ma	major	**p300**	E1A binding protein
MACS	magnetic cell sorting	**PAGE**	polyacrylamide gel electrophoresis
MALDI-TOF- MS	matrix assisted laser desorption/ionisation time of flight mass spectrometry	**PCR**	polymerase chain reaction
Mb	mega base	**STAT6**	signal transducer and activator of transcription 6
MBD	methyl-CpG-binding domain protein	**pH**	logarithm of the hydrogen concentration
MHC-II	class II major histocompatibility complexes	**PMA**	phorbol 12-myristate 13-acetate

PRC	polycomb-repressing complex	**T**	thymine
p-value	likelihood	**T-bet**	T-box transcription factor
q	long arm of the chromosome	**TCR**	T cell receptor
r	correlation coefficient	**TENOR**	The Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimens study
RAD50	radiation induced protein 50	**TFAM**	transcription factor A, mitochondrial
RANTES	regulated on activation, normal T cell expressed and secreted	**TGFβ**	transforming growth factor beta
RHS	*Rad50* hypersensitive site	**Th**	T helper cell
RLU	relative light units	**TLR**	toll-like receptor
RNA	ribonucleic acid	**T_m**	melting temperature
rpm	revolutions per minute	**TSLP**	thymic stromal lymphopoietin
RT	room temperature	**U**	unit
RT-PCR	real time-polymerase chain reaction	**UCSC**	university of California Santa Cruz
S. aureus	*staphylococcus aureus*	**UTR**	untranslated region
SDS	sodium dodecyl sulfate	**V**	volt
SKI	nuclear protooncogen	**XIST**	X-inactive specific transcript
SMAD	mothers against decapentaplegic homolog	**YY1**	transcriptional repressor protein YY1
SNP	single nucleotide polymorphism	**µ**	micro
SP	specificity protein		

1 INTRODUCTION

1.1 ATOPIC DISEASES

1.1.1. Definition of Atopy

The term “atopy” is derived from the Greek word “atopos” (“not in the right place”) and was coined in 1923 by Coca and Cooke to describe some phenomena of hypersensitiveness in man (Coca, 1923). They considered “atopy” as a hereditary disorder, which is clinically characterized by asthma or hay fever, and which is associated with immediate-type (wheal-and-flare) skin. A few years later eczema (atopic eczema, atopic dermatitis) was included into the group of atopic diseases (Wise, 1933). As key serological factor of immediate-type hypersensitivity, immunoglobulin E (IgE), a trigger for immunological inflammation reactions in response to helminthic infections, was identified to be the key actor in atopy (Ishizaka and Ishizaka, 1970; Johansson, 1969). Elevated levels of total and specific IgE against environmental allergens are frequently found in individuals suffering from atopic diseases (Brown et al., 1979; Haupt et al., 1979). However, the role and temporal significance of IgE in the pathogenesis of atopic diseases is, at present, unresolved, and the definition of “atopy” and “atopic diseases” is a matter of ongoing controversy. The most widely used definition for atopy today is that proposed by a working group belonging to the World Allergy Organization (WAO) “… a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema.” (Johansson et al., 2004). The terminology of atopic eczema, atopic dermatitis, childhood eczema, atopiform dermatitis, and flexural dermatitis frequently used synonymously in the literature remains confusing. According to the WAO definition of atopy, “eczema” replaces the disease formerly called “atopic eczema” or “atopic dermatitis”, whereas the term “atopic eczema” is reserved for those patients with eczema and evidence for IgE involvement. However, this division might not adequately reflect the natural history of this disease, and it has to be considered that so far most studies, in particular genetic studies, were performed prior to these suggestions. Throughout this thesis the term “atopic eczema” is used.

1.1.2. Characterization of Atopic Eczema

Atopic eczema is one of the most common inflammatory skin disorders found worldwide (Catherine Mack Correa and Nebus, 2012). It is generally characterized by recurrent itchy rashes and crusted erosions with a typical age-related distribution of eczematous lesions, dry skin and intense pruritus, but shows a remarkably high clinical heterogeneity (FIGURE 1). One of the hallmarks of atopic eczema, the epidermal barrier dysfunction, affecting both lesional and non-lesional skin, results in transepidermal water loss and favors the penetration of allergens and microorganisms (Cork et al., 2009; Proksch et al., 2006). In 90 % of atopic eczema cases colonization by the toxin emitting *Staphylococcus aureus* (*S. aureus*) takes place, which increases severity of atopic eczema (Leyden et al., 1974). Secondary effects such as viral infections with *Herpes simplex* (*Eczema herpeticum*) can appear and are accompanied by widespread eruptions (Wollenberg et al., 2003). Until today no cure for atopic eczema exists but it can be treated very effectively by improving barrier function, control of microbial infections, and by suppressing inflammation.

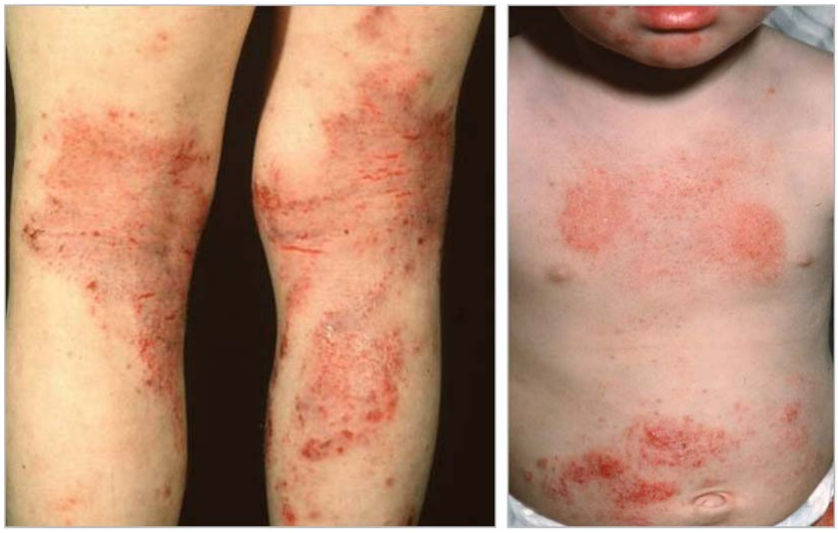

FIGURE 1. Flexural and abdominal eczema in a child and an infant, respectively (Weidinger, 2006)

The majority of patients show an onset of the disease in early childhood before the age of five (Williams and Strachan, 1998). It is estimated that approximately 60 % of patients with childhood eczema show a spontaneous remission in early adolescence, but up to 50 % may have recurrences in adulthood (Bieber, 2010; Williams and Strachan, 1998). The disease can persist into or start in adulthood, making it one of the most common skin disorders throughout all age groups. In addition,

the disease frequently co-occurs with other atopic disorders, and in a substantial amount of patients eczema proceeds the clinical manifestation of asthma and rhinitis (Bieber, 2008; Spergel, 2010; Spergel and Paller, 2003). Therefore, it has been hypothesized that a susceptible child commonly passes an overlapping series of phenotypes from atopic eczema and food allergy to asthma and subsequently allergic rhinitis ("atopic march") (Spergel, 2010; van der Hulst et al., 2007). However, the longitudinal nature of the "atopic march" is not easily reconciled with observations made in some cohort studies which suggest that the association between asthma and eczema may occur much earlier, i.e. early co-occurrence of eczema and early wheezers who progress to develop asthma (Illi et al., 2004). Recent research suggests that there may be multiple pathways to asthma and/or rhinitis with or without prior eczema (Zhang et al., 2009). Likewise, many patients with AE exhibit elevated levels of total IgE and specific IgE against food and aeroallergens (Akdis and Akdis, 2012; Kim, 2008). The exposure to the respective allergens might trigger flares (Akdis et al., 2006; Kim, 2008). For example, sensitization to chicken egg or cow milk in early years has been observed to be accompanied by condition-worsening or sensitization to inhaled allergens later in life (Hauk, 2008; Nickel et al., 1997). Aeroallergens such as house dust mite, moulds, pollen, and animal dander can contribute to the severity of the diseases (Adinoff et al., 1988; Rowe, 1959; Song, 2000; Tan et al., 1996). Autoreactive IgE can be found in a considerable fraction of patients with atopic eczema, especially in those with long-lasting and severe diseases (Tang et al., 2012). However, a significant proportion of patients with eczema is not "atopic", i.e. have normal total serum IgE concentrations and no specific IgE responses (Novak and Bieber, 2003). Furthermore, recent epidemiological research indicates that sensitization might simply be a shared epiphenomenon (Flohr et al., 2008; Williams and Flohr, 2006). Thus, both the role and temporal significance of elevated IgE in eczema and the link between eczema and respiratory atopic disease are still unclear.

1.1.3. Epidemiology of Atopic Eczema

Over the past decades, the prevalence for atopic diseases has shown a remarkable increase particularly in affluent countries (Beasley et al., 2000; Crater et al., 2001; Mannino et al., 2002; Saito, 2005; Stensen et al., 2008; Williams and Flohr, 2006). In industrialized countries, atopic eczema is now affecting 20 % of children and 5 % of adults and represents a major public health problem with an enormous burden on health care resources (Anderson et al., 2004; Asher et al., 2006; Toelle et al., 2004; Weiss and Sullivan, 2001; Williams et al., 2008). Approximately 45 % of all atopic eczema cases show first symptoms within the first 6 month of life, 85 % before the age of 5 and 70 % overcome atopic eczema before adolescence (Bieber, 2008). 50 % of children suffering from atopic eczema

before the age of 2 will also develop asthma (Akdis et al., 2006). Food allergies are observed in 35 % of children with atopic eczema (Kim, 2008). As atopic eczema has a higher prevalence in cities and affluent countries the hygiene hypothesis is frequently but controversially discussed, postulating that a lack of childhood exposure to microorganisms and parasites does not allow extensive immune system development (Okada et al., 2010; Strachan, 1989; Williams and Flohr, 2006).

1.1.4. Pathophysiology of Atopic Eczema and the Role of T cells

Atopic eczema is considered an extremely heterogenous disease with a multitude of factors that appear to influence the pathogenesis and clinical phenotype at different levels (Bieber, 2010). Hallmark features of atopic eczema include a disturbed epidermal barrier with lower hydration, modified lipid synthesis, and decreased epidermal differentiation and cutaneous and systemic immune abnormalities (Cork et al., 2009; Proksch et al., 2006). Both these features are heavily influenced by hereditary factors (see chapter 1.1.5). Current pathogenic models assume that antigens and allergens that pass the defect epithelial barrier encounter Langerhans cells and preferably induce Th2 cells to produce interleukins IL4 and IL13 (Callard and Harper, 2007). These cytokines induce IgE class switching and promote Th2 cell survival (Akdis and Akdis, 2012). The cytokines produced by Th2 cells (IL4, IL5, and IL13) in turn have direct effects on the epidermis, such as inducing keratinocytes to produce thymic stromal lymphopoietin (TSLP) (Soumelis et al., 2002). IL4 and IL13 also inhibit terminal differentiation and the production of antimicrobial peptides (AMPs) contributing to the disrupted epithelial barrier and increased rate of infections associated with atopic eczema (Ong et al., 2002; Schauber and Gallo, 2008). Further, cytokines and chemokines produced by Th2 cells and dendritic cells increase the number of eosinophils and mast cell precursors in the circulation (Bieber, 2008). However, whether skin barrier abnormalities precede the immune dysregulation or immunological abnormalities precede barrier changes is still unclear, and different mechanisms might dominate in distinct patient subsets (Boguniewicz and Leung, 2011).

CD4+ lymphocyte dependent immune responses are controlled by the balance of antigen-specific T helper cell subgroups (Sallusto and Lanzavecchia, 2009). In atopic eczema, an imbalanced serum concentration of Th1 (T helper cell 1)- and Th2 (T helper cell 2) derived chemokines contributes to the pathogenesis (Narbutt et al., 2009; Ong and Leung, 2006), which might partially be evoked by a polymorphism in interleukin 18 (Novak et al., 2005). IgE production is induced by allergens which trigger Th2 immune responses. When the epidermis is invaded by microorganisms, anti-microbial peptides like β-defensin HBD-2 and HBD-3 and cathelicidin hCAP18/LL-37 are activated (Izadpanah

and Gallo, 2005). Skin from cases of atopic eczema exhibits decreased level in expression of the anti-microbial peptides, which makes them more susceptible to microbial invasions (Ong et al., 2002). During the acute phase of atopic eczema, high levels of interleukin 4, 5, and 13 (IL4, IL5, and IL13) are expressed in the skin (Nomura et al., 2003), which are triggered by the antigen-driven cognate pathway leading to the transport of antigens to the lymph nodes. Dendritic cells bind the antigen via their class II major histocompatibility complexes (MHC-II) and present the antigens to the T cell receptors (TCR) of naïve CD4+ T cells (Novak and Bieber, 2005). Progenitor CD4+ T cells derive from the bone marrow and migrate via the blood stream into the lymph nodes. The dendritic antigen-presentation results in an activation of T cell differentiation processes predominantly towards the Th2 subtype. The majority of T cells in allergic responses are memory T cells secreting Th2 cytokines (O'Garra, 1998). Th2 cells have the ability to produce IL4, IL13, and the surface CD40 ligand. The CD40 ligand binds the CD40 of B cells and initiates class switching for IgE production, a central characteristic for atopic diseases (reviewed in: (Leung, 1993, 1998)). At the same time, Th2 cells produce IL5 which causes a release of eosinophilic inflammatory mediators. IL4 expression in T cells is promoted by B cells which collect the antigen and in return present it to naïve T lymphocytes (reviewed in: (Grammatikos, 2008; Skapenko et al., 2005; Vercelli, 2008)). In parallel, non-cognate mechanisms which are not driven by antigens, mast cells, basophils, and eosinophils express the surface IgE receptor FC-epsilon receptor (FCεRI) which bring together IgE and antigen and thereby induces CD40 ligand expression and IL4 secretion. IL4 sensitive B cells produce IgE and entail atopic diseases (reviewed in: (Grammatikos, 2008)). Failures in controlling the early allergic activation lead to disease progression (reviewed in: (Leung, 1998)). An overview of the Th2 mediated steps is given in FIGURE 2.

An opposing immunological mechanism is usually triggered by intracellular bacteria and the secretion of IL12 by naïve CD4+ T lymphocytes. This causes a differentiation of naïve CD4+ T cells into Th1 lymphocytes. The Th1-driven immunological signal cascade results in antibody dependent cell cytotoxicity. Loss of organ function and destruction of certain tissues during autoimmune diseases (e.g. rheumatoid arthritis, multiple sclerosis, psoriasis) are the outcome of deregulated Th1 mediated protective immunity against intracellular bacteria (Skapenko et al., 2005). An overview of the Th1 mediated steps is given in FIGURE 2.

Some lines of evidence support that autoimmune responses occur in atopic eczema, where patients show an IgE response to auto-allergens (Mittermann et al., 2004). Auto-allergens are proteins with a high homology to environmental allergens which trigger IgE production. For example skin colonization with the fungi *Malassezia sympodialis* can cause sensitization against the human manganese superoxide dismutase (MnSOD) due to high homology (Schmid-Grendelmeier et al.,

2005). Further autoimmune provoking proteins include the transcription factor LEDGF/DSF70 (Sugiura et al., 2007) and the atopy-related auto-antigens (ARA) Hom S1-S5 which are produced by keratinocytes (Valenta et al., 1998).

The here depicted Th1/Th2 paradigm was used to supply a simplified overview of the immunological steps that were essential for this study. Of note, recently more T cell subtypes like Th9, Th17, Th22 and Tregs have been identified to participate in multiple immunological pathways (Jutel and Akdis, 2011).

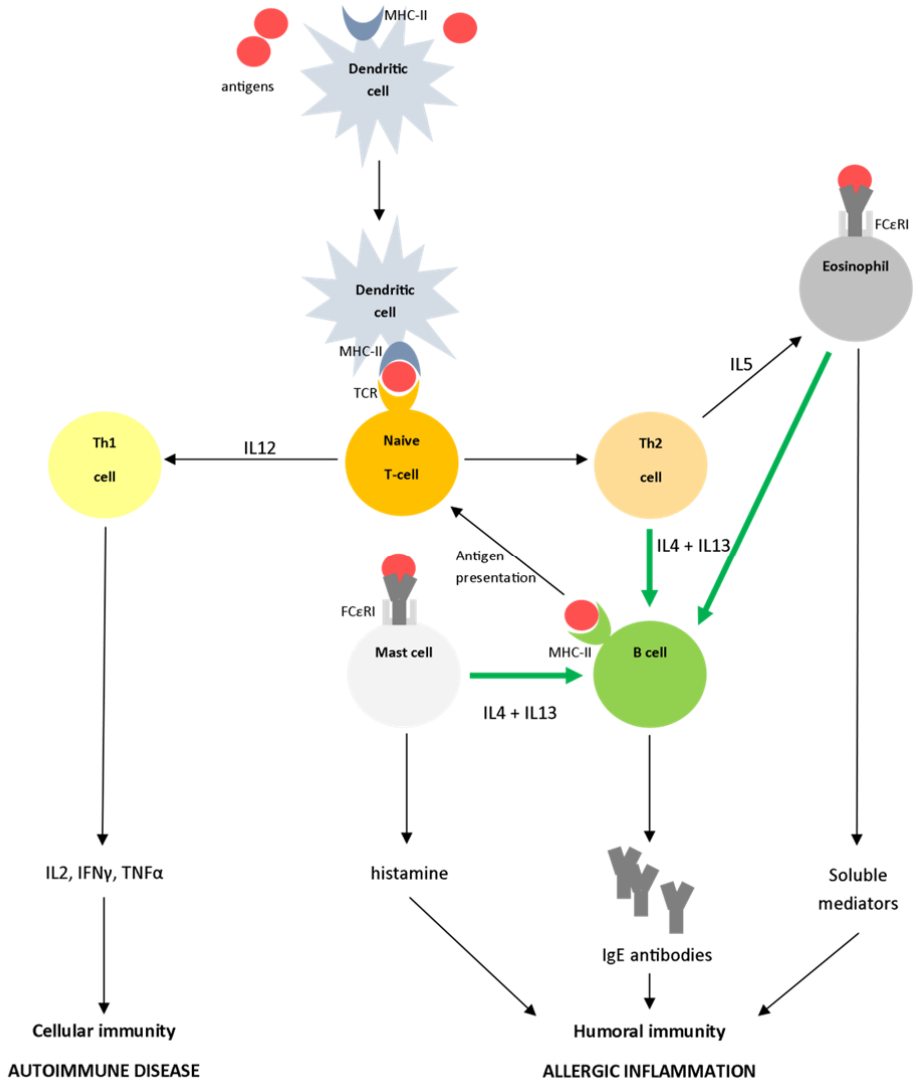

FIGURE 2. Complex network of Th2-mediated immunological steps causing atopy related traits (modified after (Grammatikos, 2008; Vercelli, 2008)). Dendritic cells bind and represent the antigen to naïve T cells which provokes a Th2-driven immunological network and a release of IgE which in turn results in allergic inflammation. The Th1-mediated pathway causes autoimmune diseases. Green arrows = activating effect.

1.1.5. Genetics in Atopic Eczema and Other Atopic Diseases

Genetic predisposition is a fundamental factor governing susceptibility to atopic diseases as evidenced by the clustering of asthma, eczema, and allergic rhinitis in families (Schaffer, 1966; Sneddon, 1951; Wadonda-Kabondo et al., 2004). A considerably higher concordance rate in monozygotic as compared to dizygotic twins has been observed (Larsen et al., 1986). It is increasingly being recognized that genetic factors act on different levels and influence both target organs and immune response patterns (Brown and McLean, 2009; Cookson, 1999, 2002). Atopic diseases are considered multifactorial disorders (Cookson, 2001; Hoffjan and Epplen, 2005) with a complex interplay between multiple genes, which is not following the classical Mendelian inheritance patterns (Glazier et al., 2002). Further, parent-of-origin effects have been observed for atopic eczema, where the maternally inherited allele is more likely to be risk-associated (Walley et al., 2001a; Weidinger et al., 2008a). In the past years, significant progress has been made in the field of atopic eczema and asthma genetics with the identification of numerous loci and candidate genes linked and associated (Barnes, 2010; Vercelli, 2008). While some of the identified gene variants appear to specifically influence the risk for atopic eczema and atopy, others are relevant across different conditions. The strongest known disease gene for atopic eczema is filaggrin, a major component of the protein-lipid envelope of the epidermis and a key player in epidermis differentiation (Marenholz et al., 2006; Morar et al., 2007; Palmer et al., 2006; Weidinger et al., 2006). Several genome-wide association studies (GWAS) further identified orosomucoid1-like3 (*ORMDL3*), *GM-CSF* (Granulocyte macrophage colony-stimulating factor), *RANTES* (Regulation of Activation, Normal T cell Expressed and Secreted), the IL4 receptor alpha, and the high affinity receptor for IgE (*FCER1A*) (Bratton et al., 1995; Forrest et al., 1999; Hershey et al., 1997; Kato et al., 2006; Weidinger et al., 2008c). Among the loci that are relevant across atopic diseases and other disorders is the Th2 cytokine locus, which comprises the genes encoding the Th2 cytokines *IL4*, *IL5*, and *IL13* and which is located in a 160 kb region on the human chromosome 5q31 (Walley et al., 2001b). This locus also contains the gene encoding *RAD50*, which is constitutively expressed during DNA repair (Dasika et al., 1999). It is well established that carriers of the risk allele of variants within the *IL13* locus exhibit increased total IgE levels and are susceptible to atopic eczema and asthma ((Cui et al., 2012; Li et al., 2010) reviewed in: (Vercelli, 2008)). The odds ratio (OR) as a measure of the effect size of these risk alleles ranges from 1.06 to 2.38 (Heinzmann et al., 2003; Heinzmann et al., 2000; Liu et al., 2000). Interestingly, recent GWAS for total IgE levels (representing a hallmark of atopic diseases), atopic eczema, and asthma provided strong signals from introns of *RAD50,* and it has been speculated whether the respective polymorphisms might exert long-range effects on Th2 cytokine expression via epigenetic mechanisms

(Li et al., 2010; Moffatt et al., 2010; Paternoster et al., 2012; Weidinger et al., 2008b). The observed OR values for the alleles of the *RAD50* intronic SNPs range from 1.1 - 1.17 (Moffatt et al., 2010). Interestingly, variants within *IL4*, *IL13,* and *RAD50* have also been associated with other immunological disorders like Crohn's disease (Onnie et al., 2006) and psoriasis (Elder, 2009). Furthermore, polymorphisms within the *IL13* gene have been associated with the susceptibility to the infectious malaria disease (Naka et al., 2009) and variants within the *IL4* gene have been associated with prostate cancer (Tindall et al., 2010) and type I diabetes (Nunez et al., 2008).

1.2 EPIGENETIC AND GENETIC ASPECTS AT THE TH2 CYTOKINE LOCUS

1.2.1. Introduction to Epigenetics

General Information to Epigenetics

Epigenetics is a key area of research that elucidates how genomes function. It combines genetics and the environment to address complex biological systems such as genome plasticity. An often cited definition of epigenetics from 1942 refers to epigenetic traits as a heritable phenotype resulting from chromosomal changes without alterations in the DNA sequence itself (reprinted: (Waddington, 2012)). Today we understand epigenetics as a mechanism that involves multiple steps causing meta-stable and heritable changes through either mitosis or meiosis in phenotype or gene expression which are not coded in the DNA sequence itself (Feinberg, 2007; Jirtle and Skinner, 2007). Epigenetic modifications such as DNA-methylation, histone modifications, nucleosome positioning, and RNA interference together orchestrate the epigenetic regulation (Portela and Esteller, 2010). Epigenetic mechanisms are involved in multiple biological processes such as tissue specific gene-regulation, cell differentiation, silencing of transposable elements, embryogenesis, X-chromosome inactivation, and genomic imprinting. The study of monozygotic twins revealed epigenetic marks that might explain the large diversity of phenotypes based on the same genotype (Fraga et al., 2005; Kaminsky et al., 2009). Currently, research is focussing on mechanisms involved in initiation, maintenance, and heritability of epigenetic conditions. Mechanistic reckoning proposes that the stably heritable epigenetic state is initialized by "epigenators" (e.g. protein-protein interaction, modification based event), which are environmental triggers that induce intracellular pathways. The "epigenetic initiator" (e.g. DNA-binding protein, non-coding RNA), triggered by the "epigenator", is necessary for

the definition of the exact position of where the local epigenetic chromatin is to be established by induction of the environment. The “epigenetic maintainer” (e.g. DNA-methylation, histone modifications, histone variants, nucleosome positioning) detects the chromatin environment and passes the information on to other mechanistic pathways (Berger et al., 2009).

DNA-Methylation

The most consistent epigenetic hallmark is methylation (reviewed in (Bjornsson et al., 2004)), the almost exclusively covalent addition of a methyl-group to a cytosine residue on a cytosine-phosphate-guanine (CpG) site. CpG sites tend to cluster in CpG islands, which hold a guanine/cytosine-content of more than 50 %, extend to more than 200 base pairs, and are found in most human promoters (Portela and Esteller, 2010). It is well established that their methylation states impact gene regulation and silencing, thus leading to differential allele expression (Jones and Baylin, 2002; Weber et al., 2007). Hypermethylation is involved in female X-inactivation and genomic imprinting causing monoallelic expression at one of the two parental alleles (Kacem and Feil, 2009; Reik and Lewis, 2005). Methylation mediated gene silencing takes place when the methylated DNA recruits methyl-CpG-binding domain proteins (MBD), which in turn recruit chromatin remodelling and histone modifying proteins (Lopez-Serra and Esteller, 2008; Reik and Lewis, 2005) (FIGURE 3). Transcriptional proteins are then inhibited to bind the DNA (Kuroda et al., 2009). Mammalian DNA methylation is mediated by DNA-methyltransferases (DMNT1, DNMT3a, DNMT3b), which catalyse the methyl-transfer from S-adenosyl methionine to the DNA (Portela and Esteller, 2010). CpG methylation is not exclusively observed in CpG islands, but also in widespread areas of about 2 kb surrounding CpG islands, the so called CpG island shores (Doi et al., 2009; Irizarry et al., 2009). Recently mammalian non-CpG site methylation has been observed in the context of CHG or CHH sites (H = adenine, cytosine, thymine) in stem cells (Laurent et al., 2010; Lister et al., 2009). Other DNA modifications like 5-hydroxymethyl-2’-deoxycytidine and 5-methylcytosine have been observed in Purkinje cells, but need to be investigated further in future projects (Kriaucionis and Heintz, 2009).

Histone Modifications

Dynamic posttranscriptional modifications on histone N-terminal tails (acetylation, methylation, ubiquitylation, phosphorylation, ADP-ribosylation, and SUMOylation) are another important epigenetic mechanism (Kouzarides, 2007; Rando and Chang, 2009). These modifications have a key role in transcriptional regulation, DNA repair, replication, alternate splicing, and chromosome condensation by regulating DNA accessibility (Kouzarides, 2007). Acetylated histone marks such as

H3K4, H3K36, and H3K79 predominantly tag accessible euchromatin, and are mediated by histone acetyltransferases (HATs). Acetyl residues are removable by histone deacetylases (HDACs). Condensed heterochromatin features methylated histone marks like H3K9, H3K27, and H4K20 (Li et al., 2007; Strahl and Allis, 2000) (FIGURE 3). In some cases, small non-coding RNAs were found in heterochromatic regions, which were shown to navigate histone modifications (Khalil et al., 2009). The human non-coding RNA *XIST* is involved in X-inactivation by recruiting the polycomb-repressing complex (PRC), which is able to ubiquitinate histones and has a methyltransferase activity (Chow and Heard, 2009). Histone modifications and DNA methylation are connected via the DNMT3L methyltransferase, which interacts with H3K4 causing DNA methylation by recruitment of DNMT3A (Ooi et al., 2007). Additionally, several histone methyltransferases interact with DNMTs and therefore induce DNA methylation at specific targets (Tachibana et al., 2008; Zhao et al., 2009).

Nucleosome Positioning

Nucleosomes represent a barrier for transcriptional proteins to directly interact with the DNA to transcribe the genes. Therefore, nucleosome positioning is an important feature for transcriptional initiation. Nucleosome-loss at the transcription start site is associated with gene activation, whereas nucleosome occlusion inhibits transcription (Schones et al., 2008) (FIGURE 3). Nucleosomes are built by histones and their function depends on the participating histone variants and their modifications. Histone variants are included into the DNA independent of DNA-replication and regulate nucleosome positioning (Li et al., 2007). The histone variant H2A.Z prevents DNA-methylation (Zilberman et al., 2008).

Epigenetics of Atopy

So far, epigenetic mechanisms are best studied in cancer and autoimmune diseases, whereas less is known about their role in other common diseases (Feinberg, 2007; Fraga et al., 2005; Javierre et al., 2010). However, epigenetic regulation is increasingly being recognized as a potentially important factor influencing the individual risk for complex diseases such as asthma (Bjornsson et al., 2004; Miller and Ho, 2008). The rise of allergies in industrialized countries over the past decades indicates that environmental changes affect immune function. In the uterus foetal reprogramming of function and response patterns of many systems takes place, allowing environmental factors to become effective modulators (Koldovsky, 1979; Waterland and Michels, 2007). Environmental exposures that modify immune function of the neonate towards the development of allergies later in life include maternal diet, microbial exposure, and cigarette smoke (Dunstan et al., 2003; Noakes et al., 2003;

Prescott et al., 2008). Immunological mechanisms have been developed which allow a coexistence of foetus and mother. The maternal immune system mainly adapts to produce Th2 cell driven

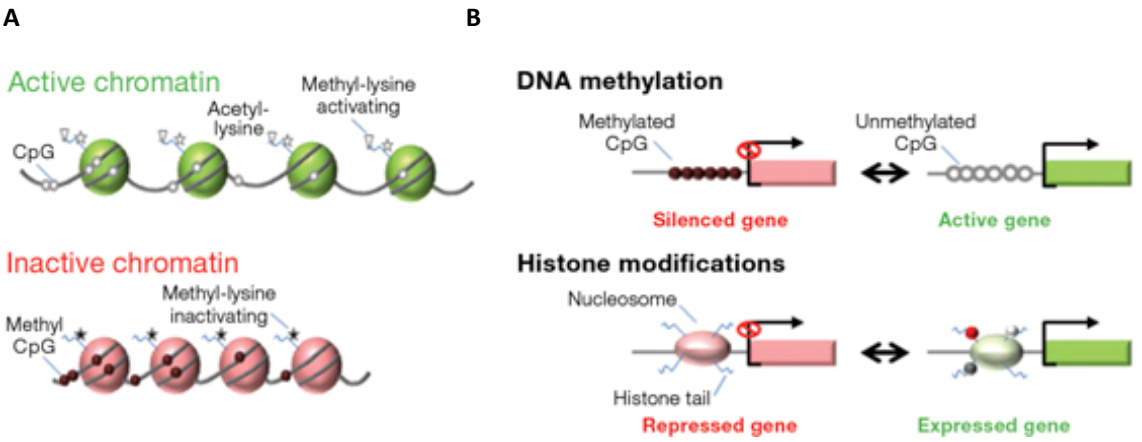

FIGURE 3. DNA methylation and histone modifications impact the transcriptional state of genes (Gudjonsson and Krueger, 2012). (A) Active chromatin is characterized by unmethylated CpG sites and specific euchromatic histone modifications, whereas inactive chromatin features DNA-methylation and heterochromatic histone-modifications. **(B)** DNA-methylation and heterochromatic histone-modifications inhibit gene transcription. Unmethylated DNA and euchromatic histone modifications allow gene transcription.

responses in order to down regulate Th1 cytokines which would attack the foetus (Breckler et al., 2008). Via epigenetic events, the foetal immune system also adapts the Th2 state (Zaghouani et al., 2009). The general differentiation processes for the development and the plasticity of the immune system are under epigenetic control. By vast changes in DNA methylation and histone modifications naïve CD4+ T cells differentiate towards Th1, Th2, Th17, and regulatory T cell as well as other subtypes (Janson et al., 2009). The sensitization to environmental factors, such as allergens, diet, respiratory viruses, air pollutants, tobacco smoke, endotoxins, house dust mite, and occupational exposure are risk factors for allergic diseases. The association of SNPs within TGFB1 is influenced by house dust mite, indicating a direct link between environment and gene interaction due to differential immune modulation (Hunninghake et al., 2008; Sharma et al., 2009). Latest results indicate an epigenetic role in the “hygiene hypothesis” and suggest that avoidance of microbial exposures causes modifications in epigenetic patterns of the immune system and therefore contributes to atopic diseases (Vuillermin et al., 2009).

Maternal allergy is a strong risk factor for the development of allergy in the offspring, which indicates maternal imprinting effects or direct maternal-foetus interactions. Allergic woman were observed to feature an even lower Th1 IFNγ response than healthy mothers (Prescott et al., 2010). It is likely, that

epigenetic changes induced by environmental factors are inherited to the next generation not just in animals (Hollingsworth et al., 2008; Prescott and Saffery, 2011).

1.2.2. Locus Control Regions as Epigenetic Operating Sites

Locus control regions (LCRs) are *cis*-regulatory elements and play an important role in long-range transactivation of mammalian genes. They are characterized by the ability to enhance the expression of linked genes to physiological levels in a copy number dependent and position-independent manner. Cell type specificity of the LCR is assured by epigenetic mechanisms. Single components of the LCR are colocalized with desoxyribonuclease 1 (DNase1) hypersensitive sites (HS) in the chromatin of the specific cell type. Euchromatic chromatin that is not protected by histones and other proteins and therefore accessible to endonuclease DNase1 is cleaved preferentially at phosphodiester linkages adjacent to pyrimidine nucleotides. Multiple transcription factors are able to bind to these HS within the LCR and promote LCR function (all reviewed in (Li et al., 2002a)). An LCR was first discovered in the human β-globin locus (Grosveld et al., 1987). The β-globin LCR, spanning five HS, is located 5-22 kb away from the first globin gene. HS1-4 are celltype-specifically pronounced in erythroid cells, while HS5 is present in multiple cell types (Greaves et al., 1989). Experiments in mice and human cell lines showed that a deletion of the LCR causes a reduction of β-globin expression down to 1 % (Magram et al., 1985; Reik et al., 1998). The enhancer activity of the β-globin LCR is based on the activity of a 200 bp to 300 bp core of HS2-4. These cores contain multiple binding sites for erythroid-specific transcription factors such as Maf homodimers, NF-E2, Nrf1-2, Bach1-2, and API (Moi et al., 1994; Oyake et al., 1996). It is suggested that RNA polymerase II binds to the LCR and p45/NF-E2 induces a transfer of the RNA polymerase II to the β-globin promoter and therefore initiates transcription (Johnson et al., 2001). For the functioning of HS3 and HS4 they need to be integrated into chromatin, implying a functional role for the chromatin structure. HS5 is characterized as an insulator element with chromatin opening activity (Ellis et al., 1996; Li et al., 2002b). There exist two favored models about how the β-globin LCR function is complemented: looping and tracking. Looping is thought to transpire by folding the HS core elements, which bind the transcription factors, and building physical loops with the promoter elements, therefore enhancing β-globin expression (Gribnau et al., 1998; Kadauke and Blobel, 2009). The tracking model proposes that the transcription factors bind to the LCR and that the protein-DNA complex migrates along the DNA-helix until it reaches and activates the promoter. At the same time, this serves to open the chromatin structure (Blackwood and Kadonaga, 1998; Kadauke and Blobel, 2009).

Further identified LCRs include those for the human cluster of differentiation 2 (CD2), human growth hormone (hGH), mouse T cell receptor (TCR), the mouse Th2 locus control region, and many more (Li et al., 2002a). Specific chromatin opening effects mediated by LCRs have been studied in different models. The hGH LCR has been observed to establish specific patterns of histone acetylation within the LCR and the regulated promoters by recruiting tissue-specific histone acetyltransferases (Ho et al., 2002). In the β-globin LCR, the acetylation pattern varies with different developmental stages, implying that dynamic histone modifications are important for developmental control of the β-globin locus (Forsberg et al., 2000). Within the TCR LCR induction of cell-type specific demethylation by its HS has been observed.

1.2.3. Genetic and Epigenetic Mechanisms at the Th2 Cytokine Locus

The Th2 cytokine locus consists of the genes *IL5, RAD50, IL13,* and *IL4* which cluster on the human chromosome 5q31 or the mouse chromosome 11, spanning over 140 kb. The Th2 cytokines IL4, IL5, and IL13 are predominantly expressed in Th2 cells, whereas the RAD50 protein is constitutively expressed in all tissues (Spilianakis and Flavell, 2004). It is well established that RAD50 is a major key-component in DNA-repair and cell cycle control (reviewed in: (Dasika et al., 1999)). For the protein there is no function in allergy-related responses known so far. The Th2 cytokine locus undergoes structural and epigenetic changes when cells differentiate from naïve CD4+ cells towards Th2 cells, allowing tissue-specific transcription factors to bind to accessible regulatory regions (Lee et al., 2002; Santangelo et al., 2002). Several regulatory regions within the Th2 cytokine locus were identified which orchestrate the expression of IL4, IL5, and IL13. Results from the mouse model give insights into epigenetic mechanisms occurring at the Th2 cytokine locus. Less is known for the human locus. However, functional variants at this locus were identified.

1.2.3.1 Evidence in the Mouse Model

Research has identified a remarkable number of DNase1 hypersensitive, regulatory sites within the Th2 cytokine locus over the past decades (FIGURE 4). The designation of those sites however is not uniform. Within the murine Th2 cytokine locus, intergenic DNase1 hypersensitive sites (HS) HSS1 and HSS2 between *Il4* and *Il13* were discovered, which were Th2 cell type specific (Takemoto et al., 1998). Another group identified the two highly conserved non-coding DNA elements CNS1

(containing HSS1 and HSS2) and CNS2 (containing HSVA and HSV) and showed that deletion of CNS1 resulted in a decreased production of Il4, Il5, and Il13 (Loots et al., 2000). They assumed that CNS1 acts as a coordinate regulator for Th2 cytokine genes. Further studies showed that CNS1 features enhancer activity and responds to Gata3 in chromatin remodelling (Lee et al., 2000; Takemoto et al., 2000). The highly conserved Th2-specific regulatory region HS1 is located 1.6 kb upstream of the *Il13* gene and was shown to enhance Th2 cytokine promoter activity by binding of Gata3, Cbp/p300 and RNA polymerase II (Agarwal and Rao, 1998; Kishikawa et al., 2001; Yamashita et al., 2002). At the 3'end of *Il4,* the Th2 specific hypersensitive sites HSV and HSVA (overlapping CNS2) were found to exhibit enhancer activity with HSVA binding Nfat and Gata3 (Agarwal et al., 2000; Agarwal and Rao, 1998). HSV/HSVA knockout mice revealed reduced levels of Il4, Il5, and Il13 (Solymar et al., 2002). The intronic enhancer HSII within *Il4* was observed in mast cells and Th2 cells (Henkel et al., 1992). In combination with CNS1 it exhibited Gata3 dependent Il4-production (Lee et al., 2001). The hypersensitive site HSIV, which is located at the 3'end of *Il4* as well, acted as a Th1-specific silencer and led to aberrant levels of Il4 and Il13 in HSIV-deficient mice (Ansel et al., 2004; Kiesler et al., 2010; Lee et al., 2001).

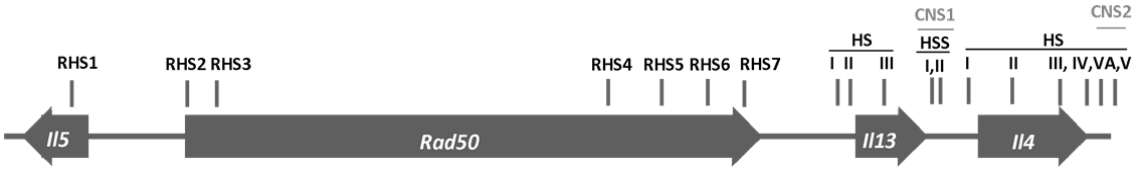

FIGURE 4. Schematic overview of the Th2 cytokine locus on murine chromosome 11 with identified important regulatory hypersensitive sites (HS). *Il5, Rad50, Il13*, *Il4,* and intergenic regions contain several HS, which were investigated by several groups and were not yet consistently entitled. Adapted from: (Lee et al., 2006). HS are indicated by vertical grey lines.

Within the introns at the 3' end of the *Rad50* gene, an LCR*,* which is constituted of four *Rad50* hypersensitive sites (RHS) in introns 21 (RHS4-6) and 24 (RHS7), controls the expression of its neighbouring genes *Il13* and *Il4* (Fields et al., 2004; Lee and Rao, 2004; Lee et al., 2003; Lee et al., 2005) (FIGURE 5). RHS4, RHS5, and RHS7 were shown to be present in Th2 cells, whereas RHS6 is also accessible in CD4+ and Th1, cells as shown by the group of R. Flavell (Fields et al., 2004). In contrast, A. Rao and colleagues showed that RHS5 is accessible in both Th1 and Th2 cells (Lee and Rao, 2004). Contradictory results might be due to the usage of primary cells in one and cell lines in the other study (Lee et al., 2006). As RHS5, RHS6, and RHS7 are highly conserved between species and combinations of specific mouse RHS can reconstitute LCR function in reporter gene assays, these RHS are regarded as the core of the LCR (Lee and Rao, 2004; Lee et al., 2006). RHS7 knockout mice

exhibited dramatic reduction in Th2 cytokine expression, demonstrating that the LCR has an essential function in Th2 cytokine production (Fields et al., 2004). The Th2 cytokine locus is not just a classical

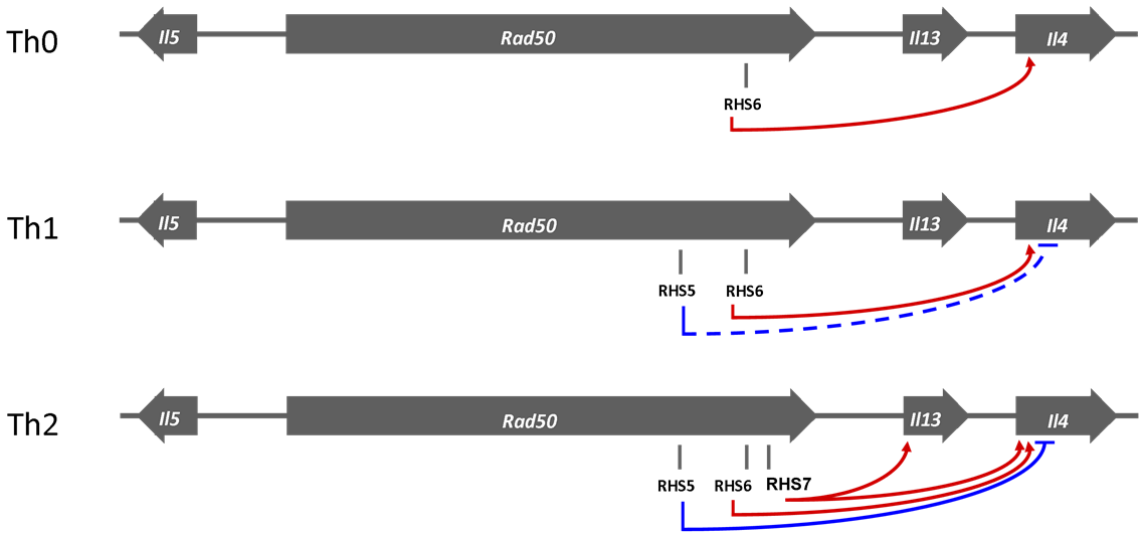

FIGURE 5. Schematic overview of the Th2 cytokine locus and regulating mechanisms acting between core RHS sites and interleukin promoters in T cells in mice. The repressor effect of RHS5 on *Il4* promoter activity is shown for Th1 and Th2 cells. RHS6 is present in naïve T cells (Th0), Th1, and Th2 cells and promotes Il4 expression. RHS7 enhances both Il13 and Il4 expression in Th2 cells. Blue line = repressor, red line = enhancer, dashed line = inconsistent results between (Fields et al., 2004) and (Lee and Rao, 2004), continuous line = consistent results between (Fields et al., 2004) and (Lee and Rao, 2004).

LCR according to the definitions but also undergoes cell-type specific epigenetic changes to allow binding of tissue-specific transcription factors and initiate chromatin changes for transcriptional activation. The entire Th2 locus becomes hyperacetylated and demethylated during the differentiation processes of naïve T cells into Th2 cells in mice (Fields et al., 2004; Lee et al., 2002). Additionally, it could be shown that the RHS are accessible in Th2 but methylated in Th1 cells (Fields et al., 2004). Evidence was provided that the RHS7 site is necessary for demethylation and thus for interactions with the Th2 cytokine promoters (Lee et al., 2005). The demethylation and hyperacetylation at the locus is dependent on the transcription factor Stat6, and not associated to any kind of cell division (Fields et al., 2004; Kim et al., 2007; Lee et al., 2002). The transcription factor Yy1 binds to the LCR in a Th2-specific manner and induces cytokine expression and chromatin remodelling by interaction with Gata3 (Hwang et al., 2013). Gata3-deficient Th2 cells exhibit impaired *Il4*, *Il5,* and *Il13* acetylation patterns and a reduction in Il5 and Il13 production, whereas Il4 is nearly not affected (Yamashita et al., 2004; Zhu et al., 2004). Furthermore, Gata3 is important for the

maintenance of the Th2 LCR and Th2 cytokine promoters (Lee et al., 2005). The RHS7 was found to be essential for the formation of long-range intrachromosomal interactions between the LCR and the Th2 cytokine promoters and therefore cytokine production (Lee et al., 2005). Furthermore, it was demonstrated that the formation of this core chromatin complex is connected with a looping-out of *Rad50* (Lee et al., 2006), which explains why there are no changes in *Rad50*-expression levels when comparing different cell-types (Spilianakis and Flavell, 2004) (FIGURE 6). The LCR strongly interacts with the *Il4* and *Il13* promoter but not with the *Il5* promoter, confirming earlier results where the LCR was shown to regulate Il4 and Il13 expression (Lee et al., 2003; Spilianakis and Flavell, 2004).

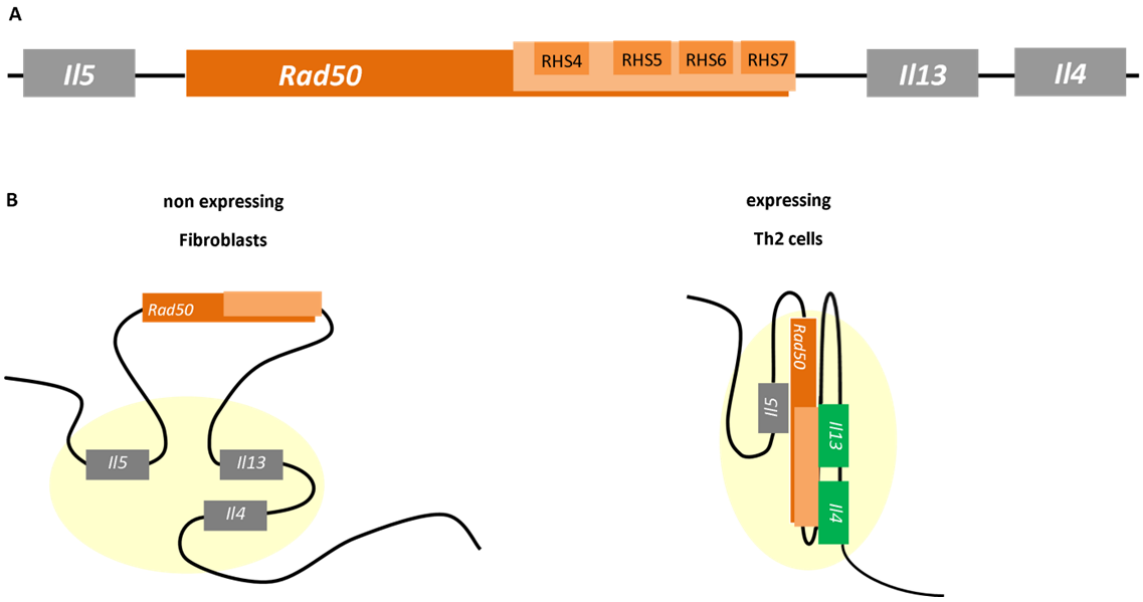

FIGURE 6. The Th2 LCR physically interacts with Th2 cytokine promoters of *Il4* and *Il13* in CD4+ lymphocytes by building a chromatin loop (modified after (Lee et al., 2006)). (A) Schematic representation of the Th2 cytokine locus consisting of the *Il5, Rad50, Il13,* and *Il4* gene. The *Rad50* gene contains the LCR constituted by RHS4-7. **(B)** In fibroblasts, no physical interaction between the *Rad50* LCR and the interleukin promoters is established, whereas in Th2 cells, the LCR containing RHS4-6, physically interacts with the Th2 cytokine promoters *Il4* and *Il13* and thereby enhances Th2 cytokine expression. Red = *Rad50* genes, orange = Th2 LCR within *Rad50*, green = genes controlled by Th2 LCR, yellow = active chromatin hub.

Groundbreaking results were obtained by experiments with knockout mice where the entire LCR was deleted, which led to a loss of histone acetylation, a dramatic reduction in expression levels of Th2 cytokines and IgE, and a loss of asthmatic symptoms in asthmatic mice (Koh et al., 2010). These results demonstrate the importance of the LCR for chromatin-remodelling, for the regulation of Th2 cytokine expression, and for the pathogenesis of atopic diseases in mice.

1.2.3.2 Evidence in Humans

Our understanding of regulatory mechanisms at the Th2 cytokine locus is mainly based on the analysis of the murine locus. However, some progress has lately been emerging in the characterization of the human Th2 cytokine locus. Besides disease-specific susceptibility loci, gene mapping approaches have identified loci shared between atopic diseases and other immune-mediated diseases, which could represent important checkpoints in branching pathways that lead to the development of related but distinct diseases (Heap and van Heel, 2009; Weidinger et al., 2010). Among such regions is the 5q31 region, where the *IL4*, *IL5*, and *IL13* genes are linked to atopy (Vercelli, 2008). Interestingly, genome-wide association studies (GWAS) for asthma, total IgE, and atopic eczema identified strong and apparently *IL13*-independent signals from SNPs within *RAD50* (Li et al., 2010; Moffatt et al., 2010; Paternoster et al., 2012; Weidinger et al., 2008c). Characteristic DNase1 HS and specific DNA demethylation have been observed at the Th2 cytokine locus during Th2 differentiation from naïve precursors towards Th2 cells, and changes of CpG methylation have been implicated in transcriptional regulation of Th2 cytokines (Santangelo et al., 2002; Webster et al., 2007). Specific DNase1 HS for naïve-, Th1, and Th2 cells have been identified at the Th2 cytokine locus with the data being available within the ENCODE project (Sabo et al., 2004; Sabo et al., 2006). With differentiation from naïve T cells towards Th2 cells the entire Th2 cytokine locus undergoes structural changes accompanied by extensive histone hyperacetylation (H3K9) and hypermethylation (H3K4), both representing markers for open chromatin. Moreover, an upregulation of GATA3 expression was observed (Kaneko et al., 2007). The transcription factors GATA3 and T-bet act as Th2 specific master regulators by binding to several regulatory elements all over the Th2 cytokine locus, with sites coinciding with DNase1 HS and regions of histone H3K4 hypermethylation which indicate euchromatic regions (Kanhere et al., 2012). At the conserved GATA3 response element (CGRE) 1.7 kb upstream of *IL13*, GATA3/c-MYB recruit the histone methyltransferase MLL during Th2 differentiation to allow chromatin accessibility and enhanced IL13 expression (Kozuka et al., 2011). Activation of *IL13* involves extensive chromatin remodelling and the formation of HS such as HS4 in the *IL13* promoter, which acts, mediated by the transcription factors NF45 and NF90, as an *IL13* regulator in human Jurkat T cells (Kiesler et al., 2010). HS4 and HS5 were shown to be present both in naïve and stimulated T cells (Webster et al., 2007). ETS1 contributes to Th2 cytokine regulation by binding to multiple *cis*-regulatory elements within the Th2 cytokine locus (Strempel et al., 2010). In the intergenic region between *IL4* and *IL13,* the two highly conserved regions CNS1 (analogue to the murine HSS1 and HSS2) and CNS2 are located which regulate the expression of IL4, IL5, and IL13 (Loots et al., 2000). It is well established that single nucleotide polymorphisms in the *IL13* locus are

associated with total IgE and atopic diseases (reviewed in:(Vercelli, 2008)). SNPs within HS4 in *IL13* create a binding site for OCT1 and enhance IL13 expression (Kiesler et al., 2009). Another allergy associated *IL13* polymorphism causes altered YIN-YANG1 binding and increased IL13 secretion (Cameron et al., 2006).

1.3 AIM OF THE STUDY

Atopic eczema and atopic diseases are influenced by multiple genes for the skin barrier function and for immune responses. In addition, an increasing body of evidence suggests an important role of epigenetic mechanisms. Among the loci most consistently associated with atopic diseases is the Th2 cytokine locus on human chromosome 5. In the mouse this region contains a locus control region (LCR) within *RAD50*. The LCR is built up by several conserved *RAD50* hypersensitive sites (RHS) which are epigenetically regulated and which coordinate the expression of the neighbouring genes *Il4* and *Il13* in Th2 cells. Of note, several studies observed strong signals from SNPs within these conserved RHS. So far, the RHS have been examined in mice only.

This study is aimed to contribute to the post-genome era, by integration of existing data from association studies and the ENCODE project together with own functional approaches to identify functional variants for an improvement of the mechanistic understanding of atopic eczema. So far, there have been no investigations in humans regarding conserved RHS and their regulatory impact. Also genotype specific effects of RHS have not been analyzed yet. However, it is of key interest to clarify whether there exist causative functional variants in this region, whether transcription factors will contribute to genotype specific effects, whether comparable mechanisms for the cytokine expression in humans exist and whether there are differences between atopic eczema cases and controls due to epigenetic mechanisms.

The first objective was to comprehensively study the regulatory effect of atopy-associated intronic *RAD50* variants within the LCR in human cells. After systematic review, promising SNPs within the LCR were functionally characterized, differentially binding transcription factors were identified, and their effects on interleukin expression in a population based cohort and T cell clones were analyzed.

The second objective was to explore if human conserved RHS of the described murine regulatory regions within *RAD50* introns might impact gene regulation in the human context as well. Therefore every conserved human core RHS was tested for its regulatory effects on promoter activation.

As epigenetic mechanisms at the Th2 cytokine locus were described to impact allergic immune response patterns in mice and humans, it was queried whether differences in the extent or number of epigenetic DNase1 HS exist when comparing the intronic *RAD50* regions of cases with atopic eczema and healthy controls. For this purpose, a new detection system for DNase1 hypersensitive sites was established and tested on human HeLa and Jurkat cells and afterwards applied to differentiated T cells.

This work has been dedicated to elucidate genetic and epigenetic mechanisms within the human atopy-associated Th2 LCR to contribute functional comprehension for personalized medicine and new pharmacological approaches.

2 RESULTS

This chapter is divided into three parts. The first part describes the functional impact of the atopy-associated intronic SNP rs2240032 within the human *RAD50* gene on differential protein binding, promoter activity, and interleukin expression. The second part analyzes the impact of human regulatory regions within the Th2 cytokine LCR in *RAD50* introns and also focusses on other atopy-associated SNPs located therein. The last part presents a method for the position-identification of regulatory regions in different tissues with the aim to identify differences in the patterns between atopic eczema cases and healthy controls.

2.1 FUNCTIONAL IMPACT OF THE INTRONIC SNP RS2240032 WITHIN THE HUMAN *RAD50* GENE

2.1.1. Computational Analyses and Motif Search

The human DNA-repair gene *RAD50* is located in the Th2 cytokine locus on human chromosome 5 (FIGURE 7). This study focuses on this gene because several GWAS reported associations of SNPs within with atopic eczema, asthma, and total IgE levels (Li et al., 2010; Moffatt et al., 2010; Paternoster et al., 2012; Weidinger et al., 2008c) although the RAD50 protein itself has no known function for the development of atopic diseases. Therefore, the repeatedly associated region was analyzed using the UCSC, ENCODE and DCODE databases and the literature concerning this region (FIGURE 8). The human *RAD50* intronic region possesses an evolutionary conservation between mammals (DCODE) and provides a high 7 x Regulatory Potential (regulatory potential scores computed from seven mammals; UCSC). In the past years, the working group of Richard Flavell at the Howard Hughes Medical Institute, Yale School of Medicine, had focused on this particular region. They identified a regulatory locus control region (LCR) containing intronic *Rad50* hypersensitive sites (RHS) for Th2 cytokine expression in mice and demonstrated a role for murine asthma development in dependency on this LCR (Koh et al., 2010; Lee et al., 2003). Data from the ENCODE project provided evidence for Th1 and Th2 DNase1 hypersensitive sites at the *RAD50* 3' introns which mark regulatory regions. These sites correspond to regions of high conservation and regulatory potential.

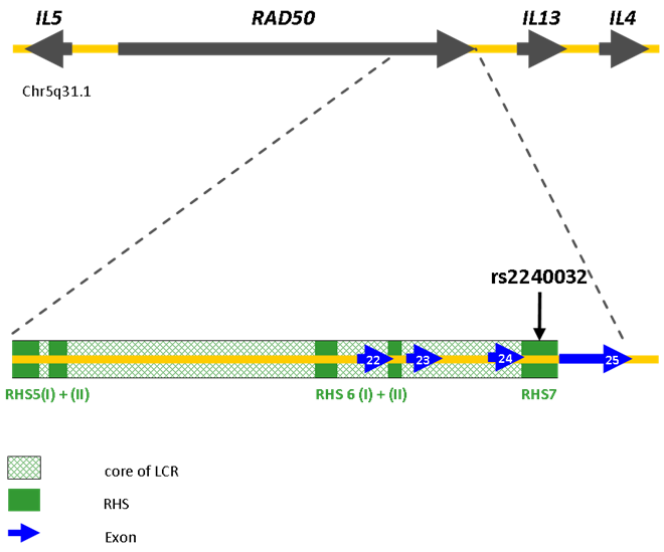

FIGURE 7. Schematic representation of the human Th2 cytokine locus and a zoom in of the conserved human LCR-core within *RAD50* containing the atopy-associated SNP rs2240032. RHS = *RAD50* hypersensitive site.

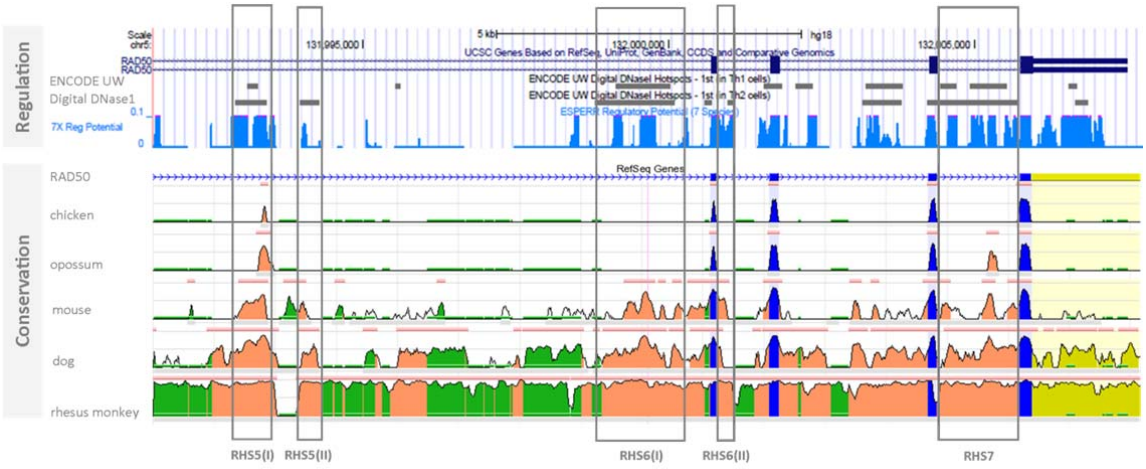

FIGURE 8. The human *RAD50* gene features DNase1 hypersensitive sites and a high 7 x Regulatory Potential at evolutionary conserved regions at the 3' end. Hotspots of DNase1 HS are indicted by grey bars. 7 x Regulatory Potential (UCSC, ENCODE) correspond to the medium blue curves. Grade of conservation in comparison to humans is pictured by the height of the curve. Dark blue = coding exon, green = repeats, orange = intronic region, yellow = UTRs (NCBI, DCODE). RHS sites are framed by grey boxes. Region shown: NCBI36/hg18 chr5:131,991,571-132,007,780.

This study focuses on the conserved RHS sequences that were published in mice (Fields et al., 2004) and combines them with the DNase1 hypersensitivity data that was published for human Th2 cells (Sabo et al., 2006). One of the SNPs repeatedly associated with atopic traits, rs2240032 (cytosine → thymine) (Li et al., 2010; Moffatt et al., 2010; Weidinger et al., 2008c), is the only SNP that is located

directly in the human conserved RHS7 site in Europeans. The RHS7 represents an essential functional part of the complete LCR in the mouse. Computational analysis predicted a disrupted SMAD3 core binding motif on the reverse complementary DNA strand when the minor risk allele of rs2240032 is present (**FIGURE 9**). SMAD3 and SP1 are known to act corporately in a complex (Poncelet and Schnaper, 2001) and furthermore, a conserved SP1 binding site is disrupted by rs2240032 on the reverse complementary DNA strand as well (FIGURE 9).

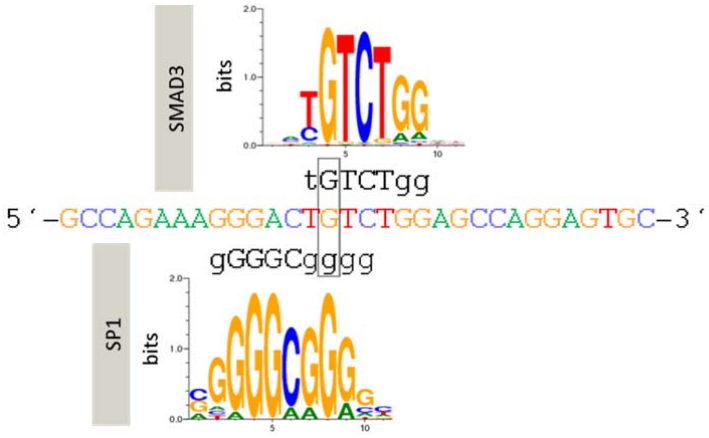

FIGURE 9. Binding motifs for SMAD3 and SP1 at rs2240032 are located inside the human RHS7. The reverse complementary DNA sequence containing the SNP at mid-position is depicted (GRCh37/hg19: chr5: 131,977,110-131,977,141). Position weight matrices (PWM) and IUPAC consensus sequences for SMAD3 and SP1 (black letters), identified with the SNPInspector- and MatInspector software, are shown. Box = position of rs2240032 [forward strand: C → T, complementary strand: G → A]. Black capital letters = core binding motif, small letters = conserved binding motif, blue = cytosine, green = adenine, red = thymine, yellow = guanine.

2.1.2. Identification of Differential DNA-Protein Binding Patterns and Transcription Factor Binding Candidates

To further investigate a potential DNA-protein interaction at rs2240032 electrophoretic mobility shift assays (EMSAs) with allele specific oligonucleotides for rs2240032 using Jurkat and HeLa nuclear protein extracts were performed. Jurkat cells are immortalized T lymphocytes and appropriate cells to study T cell specific mechanisms. HeLa cells, derived from a cervical cancer, were used in this study as negative controls to demonstrate T celltype specificity of the observed mechanisms in Jurkat cells. Western Blots with a specific SMAD2/3 antibody were performed in order to identify SMAD3 in Jurkat nuclear extracts. This ensures that SMAD3 is expressed in Jurkat nuclei and is therefore available as a binding transcription factor in the EMSA experiments. β-ACTIN antibody was used as an

internal loading control. To identify stimulation dependent SMAD3 availability, both stimulated and unstimulated nuclear extracts were used. SMAD2 and SMAD3 were detectable in both extracts, independent of stimulation status (FIGURE 10). β-ACTIN, which is available in every cell, was identified in the stimulated and unstimulated probes (FIGURE 10). As published, SMAD3 is also available in HeLa nuclear protein extracts (Gao et al., 2009). Furthermore, it has been shown that SP1 is available in both Jurkat and HeLa nuclear protein extracts (Griffin et al., 2003; Harrington et al., 1988).

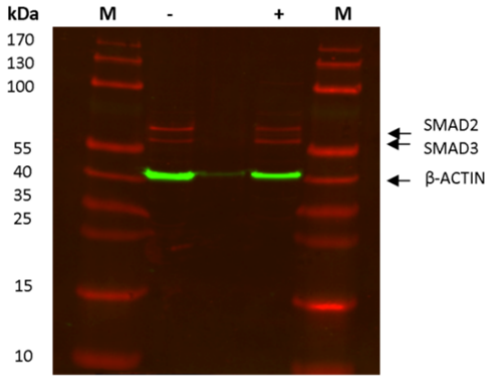

FIGURE 10. Detection of SMAD3 in Jurkat nuclear extracts. SMAD3 was detectable by Western Blot in unstimulated (-) and PMA/ ionomycine stimulated (+) nuclear extracts. M = Fermentas Page Ruler Marker.

EMSA experiments with Jurkat nuclear extracts revealed that the major allele of rs2240032 showed an additional strong band compared to the minor allele (FIGURE 11, lane 3 + 10). Competition with increasing concentrations of the unlabeled oligonucleotide containing the minor or the major allele led to a decrease in band intensity and this effect was stronger when competing with the major allele (FIGURE 11, lane 4-7, 11-14). The addition of the common transcription factor competitor OCT1 as negative control resulted in constant band intensities (FIGURE 11, lane 8+9, 15+16). These results indicate that a nuclear protein or a protein complex shows a lower binding capacity to the minor allele. EMSAs with HeLa nuclear extracts did not show visible pattern differences between the major and the minor allele (FIGURE 12A). The investigated band corresponding to the specific complex in Jurkat cells was only weakly visible in HeLa nuclear extracts. In an effort to elucidate whether the transcription factors SMAD3 and SP1 are contained in the protein complex binding to the sequence surrounding rs2240032, as predicted by the computational analysis, competition and supershift assays were performed. The addition of the SMAD3/4 competitor oligonucleotide led to a decrease in band intensity with increasing amounts of the competitor (FIGURE 11, lane 17-18). Comparable amounts of an OCT1 consensus sequence, as control competitor, did not modify band intensities

(FIGURE 12B), which indicates that the observed effect is specific. Supershift experiments using a SMAD2/3 antibody resulted in a decrease in band intensities without an additional supershift (FIGURE 11, lane 24). Competition with an SP1 oligonucleotide caused a strong decrease in band intensities even when low amounts of competitor were chosen (10 x) (FIGURE 11, lane 19-20) and supershift experiments with an SP1 antibody resulted in an additional band (FIGURE 11, lane 22).

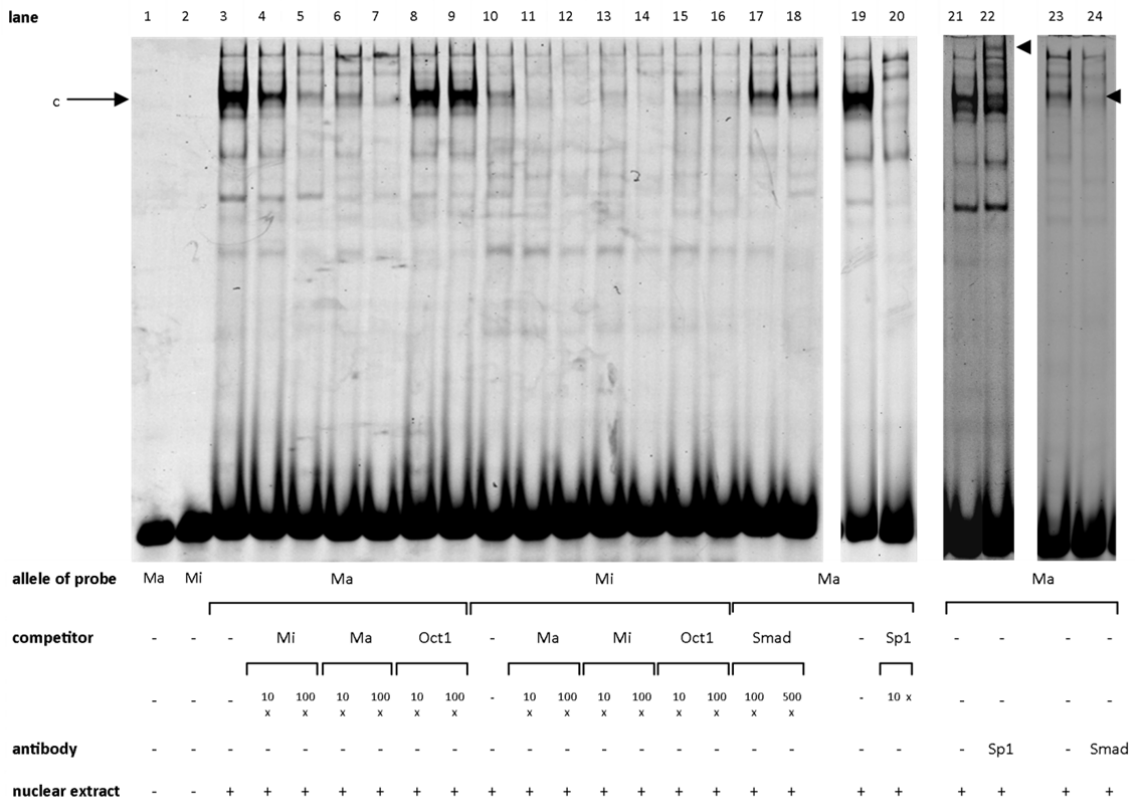

FIGURE 11. Allele-specific molecular interactions of rs2240032 within the *RAD50* intron 24 and identification of SMAD3 and SP1 as binding proteins to the major allele. EMSA Cy5-oligonucleotides, carrying the major or minor allele, revealed a differential protein binding in Jurkat T cell nuclear protein extracts. The unlabeled competitor DNA was added in indicated fold excess concentrations. Supershift with the SP1 antibody resulted in an additional band (arrow); Supershift with the SMAD2/3 antibody resulted in band decrease (arrow). c = specific complex, Mi = minor allele, Ma = major allele.

2.1.3. Verification of Transcription Factor Binding Candidates

To receive additional evidence for the involvement of the candidate proteins present in complex formation (SMAD3 and SP1) and to identify potential further proteins involved, Jurkat nuclear

proteins were affinity purified with oligonucleotides carrying the major or minor allele. Proteins binding to either the major or minor allele were eluted using increasing amounts of salt. The eluates were subsequently analyzed for DNA binding activity by EMSA. Eluates that showed differences when captured by the major or the minor allele were used for protein identification in mass spectrometry.

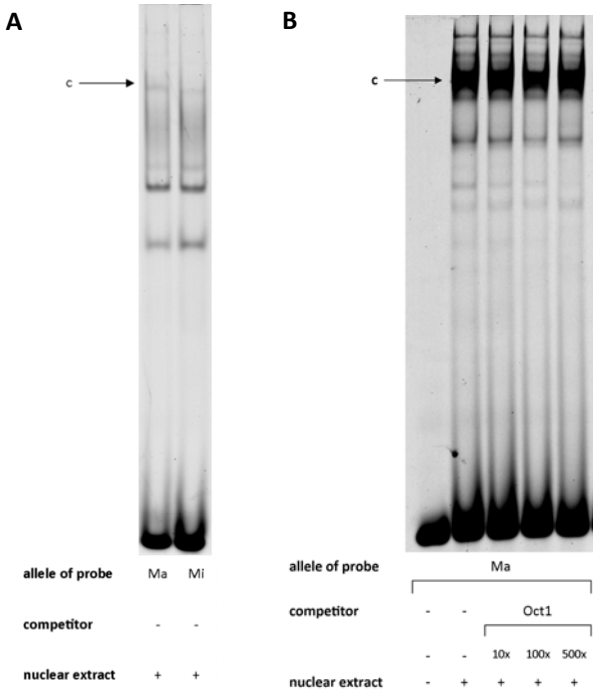

FIGURE 12. HeLa nuclear extract does not show allele-specific molecular interaction at rs2240032 and competition with a negative control does not affect band intensities of the specific DNA-protein complex in Jurkat nuclear extracts. (A) EMSAs carried out with HeLa nuclear protein extracts and Cy5-labeled oligonucleotides carrying either the major or minor allele of rs2240032 did not show visible differences in DNA-protein complex patterns. The investigated band corresponding to the SMAD3-complex in Jurkat cells was only weakly visible in HeLa nuclear extracts. **(B)** Competition EMSA was performed with increasing amounts (10 - 500 x) of an unlabeled consensus oligonucleotide for OCT1 with no effect on band intensities in Jurkat nuclear protein extracts. Ma = major allele, Mi = minor allele, c = specific complex.

EMSAs revealed an additional band for the 300 mM NaCl eluate (E300) when comparing the major to the minor allele (FIGURE 13). Analyzing eluate E300 with mass spectrometry (Stefanie Hauck, Research Unit Protein Science, Helmholtz Zentrum München) identified SMAD3 as one of the top hit proteins and confirmed the results obtained with EMSA competition and supershift experiments. Further identified proteins were the nuclear protooncogens SKI and SKIL, mitochondrial transcription factor A (TFAM), Krüppel-type zinc finger protein (ZNF48), nucleolin (NCL), exosome complex proteins EXOSC6,7,9, and the ribosome biogenesis protein WDR76 (TABLE 1). SKI, SKIL, SMAD3,

SMAD4, and TFAM provided the highest distinction between the major and minor allele binding. SP1 was not detectable by mass spectrometry in any of the fractions.

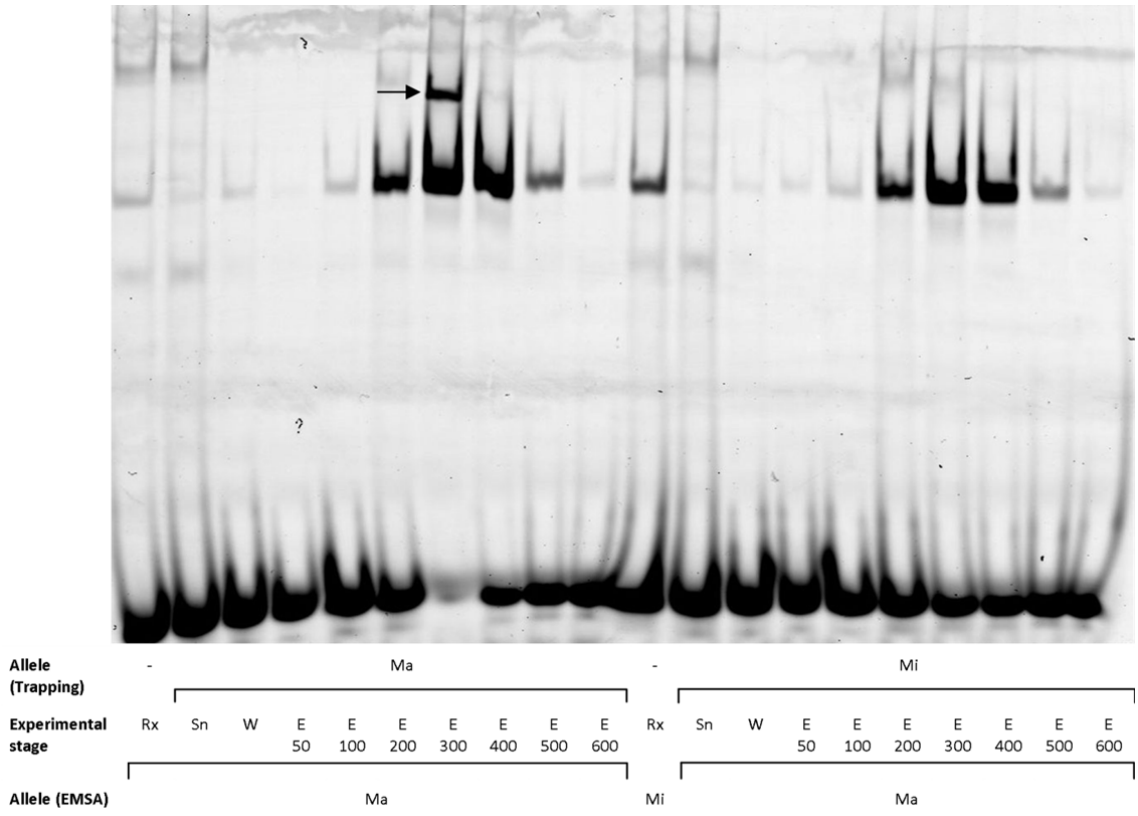

FIGURE 13. Trapping of differential binding proteins by affinity purification for mass spectrometric analysis. Differential rs2240032 binding proteins affinity purified by biotinylated oligonucleotides containing the major or minor allele of rs2240032 were visualized by EMSA with Cy5-oligonucleotides. Rx = trapping reaction reagent, Sn = supernatant, W = Washing steps 1-3, E50-600 = Elution steps with different NaCl concentrations, Mi = minor allele, Ma = major allele, arrow = additional band.

2.1.4. Genotype-Specific and Overall Effect of the RHS7 on Promoter Regulation

After having shown that the polymorphism rs2240032 influences the formation of a DNA-protein complex, potential alterations in transcriptional activity of the SNP-surrounding sequence were analyzed. For this purpose, one of three RHS7 fragments with different lengths was cloned in front of a minimal promoter, which controlled the luciferase gene of a luciferase vector. To detect differences in promoter activity due to the polymorphism rs2240032 a short RHS7 fragment (RHS7_150bp) containing the major (Ma) or the minor (Mi) allele was designed (FIGURE 14A). To identify whether

the human RHS7 has regulatory potential on promoter activity, as published for the murine RHS7, the entire conserved RHS7 containing the major allele (RHS7_1396bp_Ma) was cloned into the luciferase

TABLE 1. Putative complex members binding to the major allele of rs2240032: significant top hits of the mass spectrometry results

Accession	Peptide count	Peptides used for quantitation	Confidence score [§]	Anova (p)	Max fold change (major to minor)*	Description[#]
ENSP00000259119	4	4	110	<0.001	23.5	SKIL
ENSP00000341551	3	3	141	<0.001	25.7	SMAD4
ENSP00000367797	8	8	278	<0.001	4.2	SKI
ENSP00000332973	2	1	58	0.001	3.1	SMAD3
ENSP00000315476	2	2	69	0.006	1.3	EXOSC4
ENSP00000378776	6	1	159	0.007	76.6	TFAM
ENSP00000324056	1	1	18	0.013	1.8	ZNF48
ENSP00000318195	18	17	796	0.015	1.2	NCL
ENSP00000398597	5	5	205	0.016	1.3	EXOSC6
ENSP00000263795	2	2	84	0.024	1.6	WDR76
ENSP00000265564	7	7	247	0.024	1.3	EXOSC7
ENSP00000243498	4	4	181	0.032	1.3	EXOSC9
ENSP00000261692	2	2	42	0.034	1.3	CDK2AP1

(*) all significant top hit proteins that bound to the major allele are shown; (#) abbreviation according to ENSEMBL; (§) the confidence score of a protein is defined as the summed up peptide confidence scores of unique peptides passing the Mascot filter. The confidence score of unique peptides from the Mascot filter describe the likelihood that the detected sequence corresponds to the real protein sequence.

vector (FIGURE 14B). To receive additional information about the regulatory role of the short RHS7 region for the entire RHS7, the 150 base pairs containing the polymorphism rs2240032 were deleted from the entire RHS7 (RHS7Δ150bp_1246bp) and cloned into the luciferase vector (FIGURE 14C). The obtained luciferase vectors were then tested in reporter gene assays (FIGURE 15).

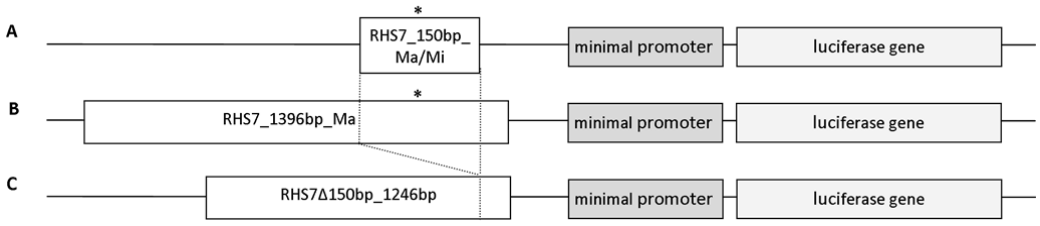

FIGURE 14. Overview of cloned luciferase constructs. Schematic representation of constructs (5' → 3' orientation) for the luciferase assays: **(A)** 150 bp fragment surrounding rs2240032 containing the major or the minor allele of rs2240032, **(B)** entire RHS7 (1396 bp) containing the major allele, **(C)** entire RHS7 without the 150 bp fragment (1246 bp). * = position of the SNP, Ma = major, Mi = minor.

Luciferase activity was significantly higher for constructs with the short RHS7_150bp fragment carrying the minor allele than for those with the major allele of rs2240032 in Jurkat cells (266.4 % increase in luciferase activity), whereas in HeLa cells, a genotype-specific effect was not significant (FIGURE 15A). Normalization was performed to the major allele of rs2240032 to demonstrate genotype specific effects.

The entire human RHS7 containing the major allele (RHS7_1396bp_Ma) has a strong and highly significant enhancing effect in Jurkat cells (593.6 % increase in luciferase activity) (FIGURE 15B). The short RHS7 (RHS7_150bp_Ma), however, had repressor function on the tested minimal promoter in Jurkat cells (30.5 % decrease in luciferase activity). In the HeLa cell line, the effect of the short and the entire RHS7 was independent of fragment length and without statistical relevance (184 % for RHS7_1396bp_Ma, 193 % for RHS7_150bp_Ma) (FIGURE 15B). For the overall effect of the fragments normalization to the empty vector was carried out.

After the deletion of the complete 150 bp region from the entire RHS7 (RHS7Δ150bp_1246bp), the remaining sequence led to a significant increase in minimal promoter activity when normalized to the entire RHS7 (RHS7_1396bp_Ma) in Jurkat cells (133.5 % increase in luciferase activity). As the entire RHS7 already had a strong enhancing effect, deletion of the 150 bp sequence increased the enhancing effect even more. In the HeLa cell line, the RHS7Δ150bp_1246bp fragment showed significantly reduced promoter activity (FIGURE 15C).

As luciferase assays are artificial *in vitro* procedures for the analysis of promoter regulation, it was of interest whether the insertion itself might have caused a change in promoter regulation and if the insert orientation had influenced promoter activity. Regulatory elements like LCRs are known to exhibit orientation dependent behavior and should therefore be investigated (reviewed in:(Maston et al., 2006)). To identify whether the insertion of any chosen fragment into the vector by itself caused altered minimal promoter activity, a 749 bp “desert site“, a non-coding, non-regulatory region according to UCSC (GRCh37/hg19: chr5:131,961,119-131,961,867) on the human chromosome 5 was cloned in front of the minimal promoter and tested in Jurkat cells. The „desert site“ had no significant effect on promoter regulation in Jurkat cells (FIGURE 16). Additionally, in order to test whether the orientation of RHS7 and “desert site” were essential for their capability to activate a minimal promoter, they were cloned reverse complementary orientated into the same vector that was used before, tested in Jurkat cells, and normalized to the empty vector. The forward oriented “desert site” exhibited no promoter regulation but when changing the insert orientation it turned into a strong repressor. The entire RHS7 site featured a very strong enhancer on a minimal promoter when oriented in the forward direction. The inverted construct however was a repressor.

The repressor effect of the 150 bp fragment of RHS7 was not orientation dependent (FIGURE 16).

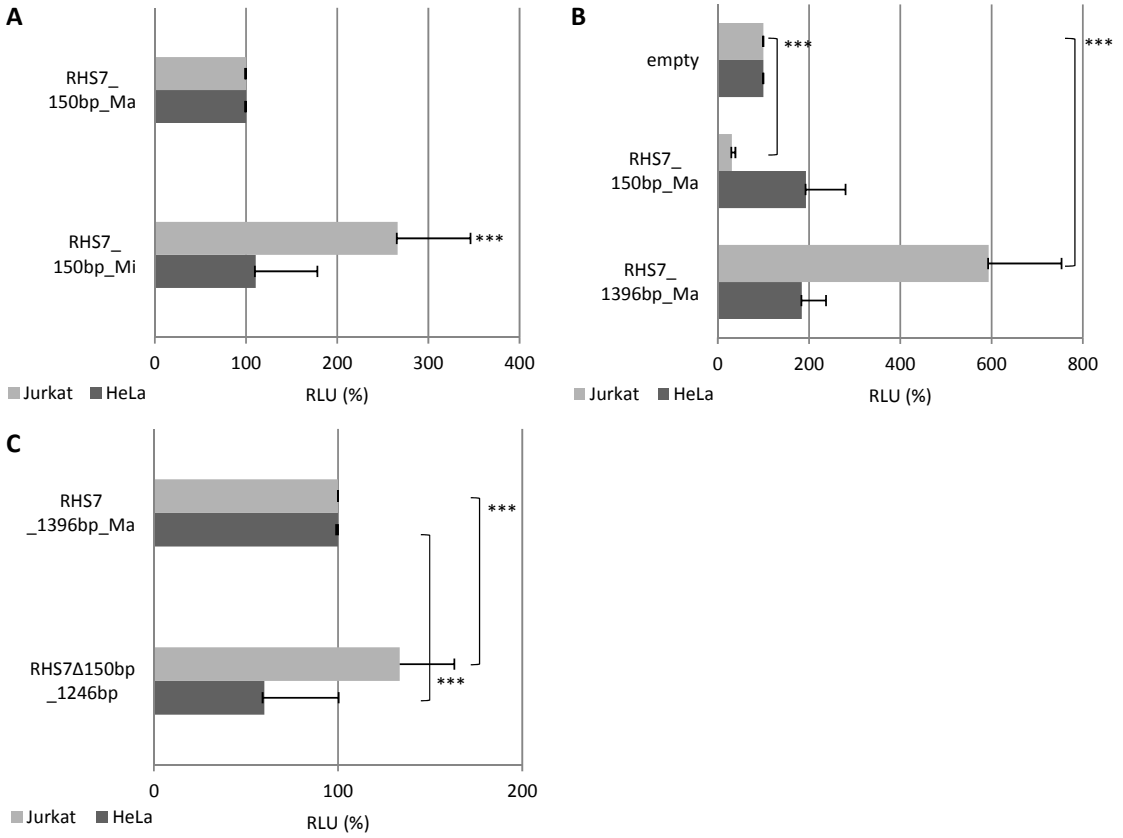

FIGURE 15. Genotype-specific regulation of minimal promoter activity depending on the rs2240032 variant located inside a regulatory sub-region of the human RHS7. Luciferase assays were performed in Jurkat T cells and HeLa cells in a minimal promoter vector with the following fragments: **(A)** RHS7_150bp_Ma/Mi, **(B)** RHS7_1396bp_Ma, RHS7_150bp_Ma or **(C)** RHS7Δ150bp_1246bp, RHS7_1396bp_Ma. Normalization was performed to **(A)** RHS7_150bp_Ma, **(B)** the empty vector, or **(C)** RHS7_1396bp_Ma. Significant effects: *** P<0.001. empty = minimal promoter vector without insert, Ma = major allele, Mi = minor allele, RLU = relative light unit.

2.1.5. SNP-Dependent Candidate Gene Expression

2.1.5.1 Results from the KORA F4 Cohort

To test whether SNP rs2240032 influences the expression of interleukins IL4, IL5, and IL13, as well as the expression of the transcription factors SMAD3, SMAD4, and SP1 and the DNA repair gene RAD50 in whole blood of human probands, existing genotype and gene expression data of the KORA F4

cohort (n=740) was analyzed and correlated. A significant rs2240032-dependent difference in transcript levels was observed for the Illumina HT-12 v.3.0 probe ILMN_1669174 representing

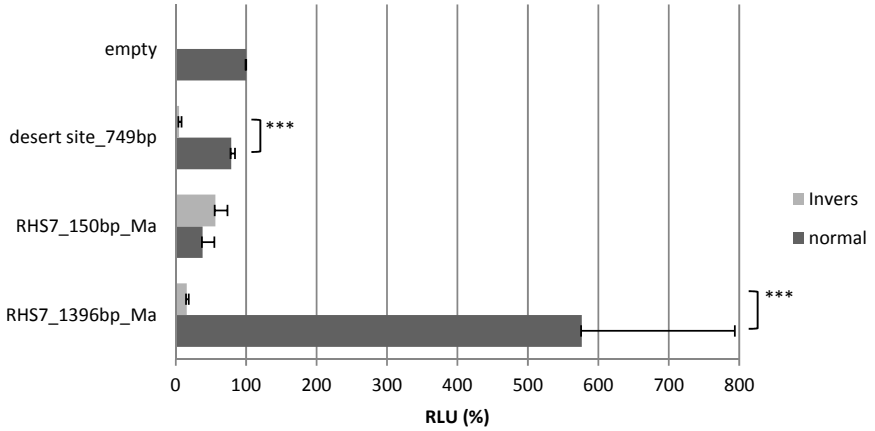

FIGURE 16. Orientation of fragments is essential for their capability to activate a minimal promoter. Luciferase assays were performed using Jurkat T cells with each fragment in both orientations for the RHS7_1396bp_Ma, RHS7_150bp_Ma and a site with no predicted function („desert site“) in a minimal promoter vector. Normalization was performed to the empty vector. Significant effects: *** P<0.001. empty = minimal promoter vector without insert, Ma = major allele, RLU = relative light unit.

interleukin 4 (IL4) (p=0.0184) (FIGURE 17). The second IL4 probe (ILMN_2389080) provided no genotype-dependent difference in transcription levels. After consideration of the UCSC data (GenBank), ILMN_1669174 was identified to bind to an IL4 exon, therefore covering all mRNA transcripts, whereas ILMN_2389080 binds to the 5’ UTR region and represents only one transcript. No significant SNP-dependent expression differences were observed for IL5 (ILMN_1709300, ILMN_2207190), IL13 (ILMN_2052511), SMAD3 (ILMN_1682738), SMAD4 (ILMN_1741477), SP1 (ILMN_1676010), and RAD50 (ILMN_1708789, ILMN_1755023). No genotype-dependent differences in transcription levels were obtained when narrowing the population down to allergy-cases and comparing allele-specific expression effects concerning interleukins and SMADs. To clarify whether IL4, IL5, IL13, SMAD3, and SP1 transcripts affect the expression of the other candidate transcripts (IL4, IL5, IL13, SMAD3, and SP1) in whole blood, an extended expression correlation analysis was carried out. The correlation analysis was performed for the whole KORA F4 population (n=740) and for the allergy-cases of the KORA F4 cohort (n=111). No significant correlations in expression among IL4, IL5, IL13, SMAD3 and SP1 were detectable (FIGURE 18).

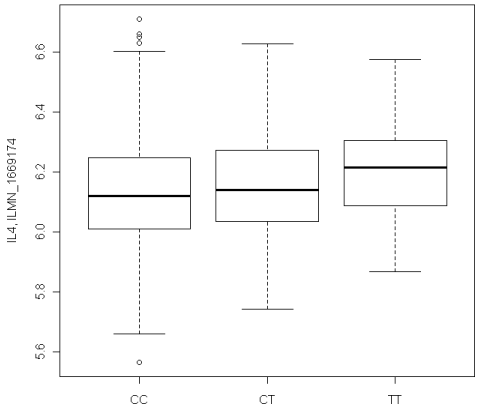

FIGURE 17. Boxplot depicts rs2240032 dependent significant difference of interleukin 4 (IL4) expression in whole blood cells of the KORA F4 cohort. Illumina HT-12 v.3.0 IL4 transcript ILMN_1669174 showed significant higher expression levels with the homozygous risk alleles of rs2240032 (p = 0.0184) (n = 740 samples). CC = homozygous non-risk alleles for rs2240032, CT = heterozygous alleles for rs2240032, TT = homozygous risk alleles for rs2240032.

2.1.5.2 Results from T Cell Clones

To specify the expression results observed in whole blood cells, genotype dependent effects on interleukin expression were investigated in 29 naïve T cell (Th0), Th1, and Th2 clones derived from the skin of 8 patients with atopic eczema, allergic contact dermatitis or psoriasis (TABLE 2). Significantly higher expression levels for IL4, IL5, and IL13 were observed with the heterozygous risk alleles being present (CT) compared to the homozygous major alleles (CC) of SNP rs2240032 (p_{IL4} = 0.003712, p_{IL5}=0.006935, p_{IL13} = 0.005177) (FIGURE 19).

TABLE 2. Overview of generated T cell clones from patients which were analyzed for their cytokine expression. CC = homozygous major alleles for rs2240032, CT = heterozygous alleles for rs2240032.

Patient ID	rs2240032 alleles	Number of clones			Phenotype
		Th0	Th1	Th2	
1	CC	-	3	-	atopic eczema
2	CC	-	-	2	allergic contact dermatitis
3	CC	1	-	-	psoriasis
4	CC	5	1	2	psoriasis
5	CC	-	9	2	psoriasis
6	CT	-	-	1	atopic eczema
7	CT	-	-	1	atopic eczema
8	CT	1	-	1	psoriasis

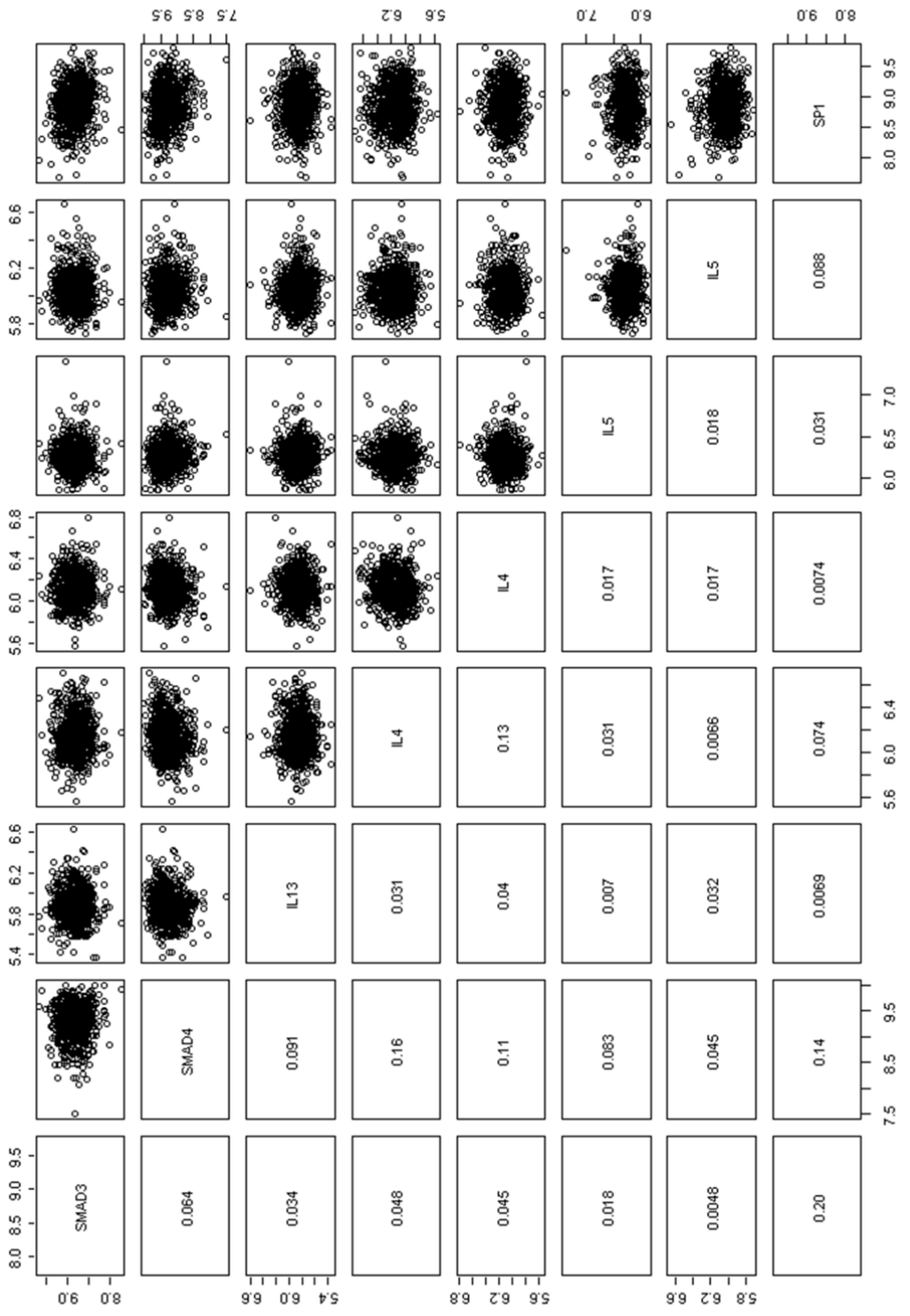

FIGURE 18. Missing correlations between SMAD3, SMAD4, IL4, IL5, IL13, and SP1 expression. A correlation blot depicts the correlation of two mRNAs in a scattered plot. Corresponding correlation coefficients (r) are shown (r>0.8 = highly correlated transcripts). X-axis and y-axis represent mRNA expression values for mRNA transcripts to be correlated (n = 740 samples).

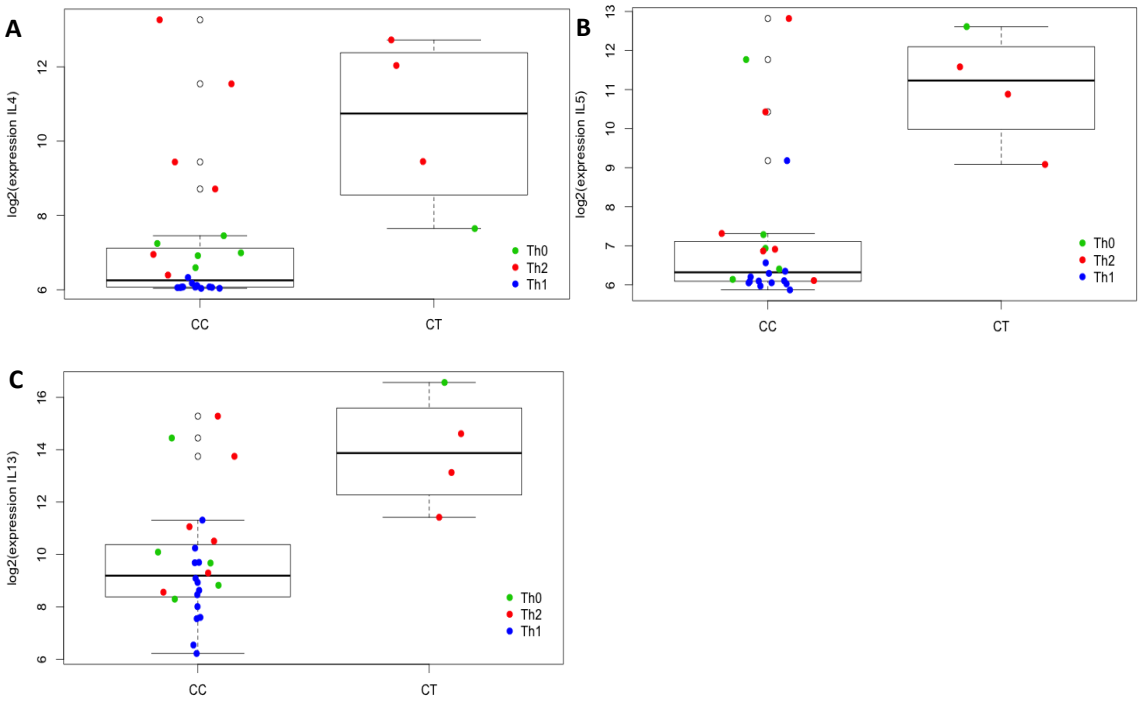

FIGURE 19. Basal mRNA expression levels of (A) IL4, (B) IL5 and (C) IL13 are genotype-specific for rs2240032 in T cell clones. Basal mRNA expression levels were obtained by the SurePrint G3 Human 8 x 60 K Microarray and revealed significantly higher expression levels with the heterozygous variant containing the associated risk allele being present (p_{IL4} = 0.003712, p_{IL5}=0.006935, p_{IL13} = 0.005177). Outliers are represented as grey circles. CC = homozygous non-risk alleles for rs2240032, CT = heterozygous alleles for rs2240032.

2.2 CHARACTERIZATION OF OTHER RHS SITES AND ATOPY-ASSOCIATED SNPS WITHIN *RAD50*

Besides the variant rs2240032 containing RHS7, the core of the entire Th2 LCR, consists of the RHS5(I), RHS5(II), RHS6(I), and RHS6(II) (reviewed in:(Lee et al., 2006)). Although the RHS7 is the best studied site in mice with a high impact on promoter regulation, the effect of each of the remaining sites in humans and atopy-associated functional variants on promoter regulation is of high interest. Four additional atopy-associated SNPs were identified by literature research (rs3798134, rs3798135, rs12653750, rs2040704). They are located inside the conserved RHS5(I), RHS6(I), and RHS6(II) in humans and thus being promising candidates for functional studies as well (FIGURE 20). Further associated SNPs between the RHS sites are probably worth looking at in future studies (FIGURE 20). All of them have predicted lost and gained sites for transcription factors. rs2074369 is predicted to have a high 7 x Regulatory Potential (UCSC) and is located in a Th2 specific DNase1 hypersensitive

site (ENCODE) that was not investigated in mice, which makes this variant a promising functional candidate. rs7737470 and rs2040703 feature a low 7 x Regulatory Potential. They are located in close proximity of a DNase1 hypersensitive site and might therefore be functional candidate SNPs.

2.2.1. Overall Effect of RHS Sites on Minimal Promoter Regulation

For the identification of the specific effect of each of the human core RHS sites (containing the major alleles of all included variants) every fragment was cloned into a vector containing a minimal promoter and used in a dual luciferase reporter gene assay both in HeLa- and Jurkat T cells. Normalization was carried out on the control plasmid pGL4.74 which constitutively expresses renilla luciferase. The empty vector was set to 100 % and all other constructs were normalized to the empty vector. The above mentioned “desert site” sequence was used as a control in order to show that it is not the insertion of any sequence into the vector itself which is responsible for altered promoter activation. The RHS7 was included as a positive control. The RHS7 exhibited the strongest enhancement of promoter regulation in a cell-type specific manner in Jurkat T cells, whereas the RHS5(II) and RHS6(I) were found to behave as strong repressors both in Jurkat- and HeLa cells. The RHS5(I) featured a moderate enhancing and the RHS6(II) a weak repressing activity in HeLa cells. The “desert site“ had no significant effect on promoter regulation in both cell lines (FIGURE 21).

2.2.2. Allele-Specific Effects within Specific RHS

The variant rs12653750 is located in the RHS6(I) site, which itself acts as a strong repressor on a minimal promoter. The RHS5(I) did not feature any remarkable effect on promoter regulation when investigating the site itself in Jurkat cells. Nevertheless, the existence of two atopy- associated SNPs (rs3798134, rs3798135) highlights this region. The variant rs2040704 is located within the RHS6(II) site, which did not show any regulatory effect on the activity of a minimal promoter. However, it is worth investigating this site because the associated SNP is located within. The variants might cause an enhancement of the LCR and therefore altered interleukin promoter regulation.

To identify SNP-dependent DNA-protein interaction, EMSAs were performed for both alleles of each of the SNPs followed by luciferase assays to clarify the functional impact on minimal promoter regulation. Genotype specific effects on cytokine expression were analyzed in existing KORA F4 mRNA expression data.

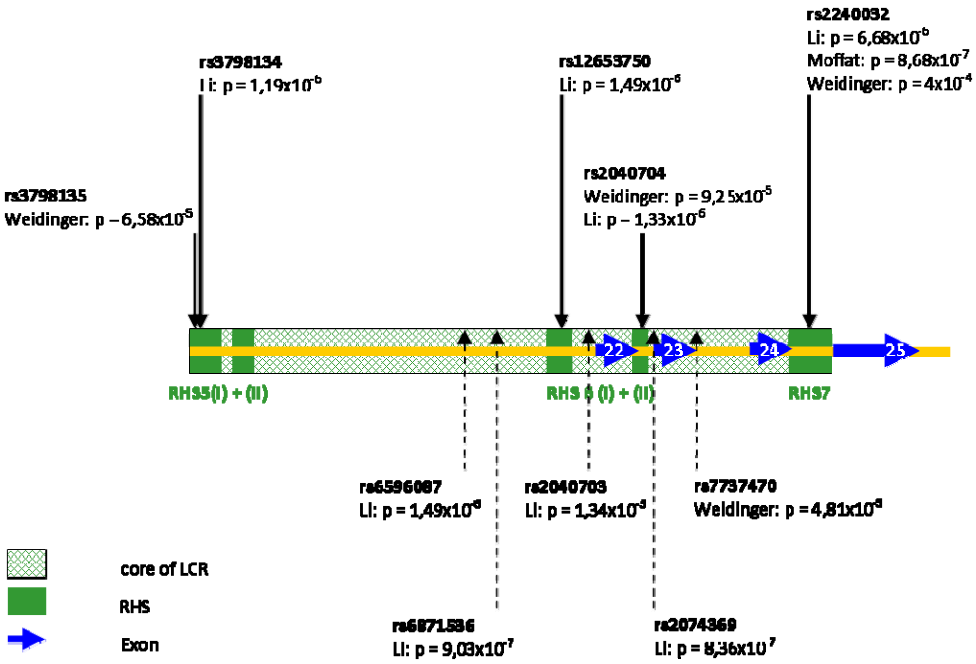

FIGURE 20. Schematic representation of the LCR within the *RAD50* gene consisting of several *RAD50* hypersensitive sites and which contains asthma and IgE associated SNPs (Li et al., 2010; Moffatt et al., 2010; Weidinger et al., 2008c). p = p-value mentioned in the listed studies; RHS = *RAD50* hypersensitive site.

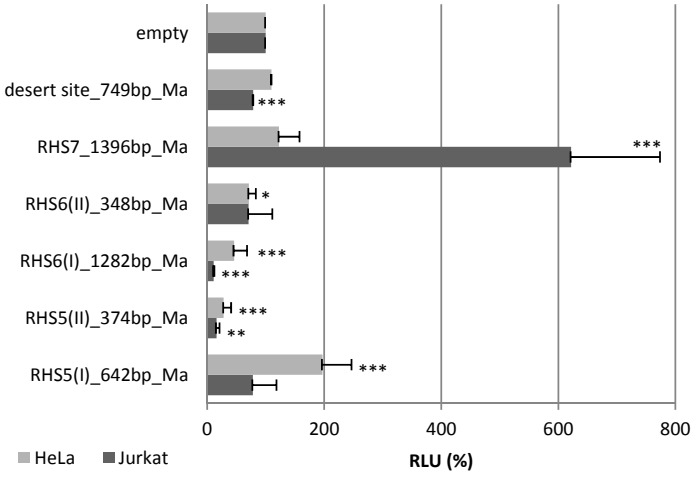

FIGURE 21. Luciferase assays with subunits of the LCR revealed cell-type specific and site specific effects on a minimal promoter. RHS7 showed a strong enhancing effect on a minimal promoter in Jurkat T cells compared to the empty vector. RHS5(II) and RHS6(I) have a repressing effect in Jurkat cells. RHS5(I) has a mild enhancing effect in HeLa cells, RHS5(II) and RHS6(I) are repressors. Normalization was performed to the empty vector. Significant effects: * P<0.05, ** P<0.01, *** P<0.001. empty = minimal promoter vector without insert, Ma = major allele, RLU = relative light unit.

2.2.2.1 Allele-Specific Effects within RHS5(I)

EMSAs revealed three allele-specific DNA-protein bands when comparing the major (cytosine) to the minor (thymine) allele of rs3798134 (FIGURE 22A.). Two complexes were bound exclusively to the major allele (C1 + C2) and one bound to a higher extent to the minor allele (C3). Polymorphism rs3798135 revealed two specific protein complexes that preferably bound to the major allele (guanine) compared to the minor allele (adenine) of the SNP (C1 + C2) (FIGURE 22B.). Band specificity

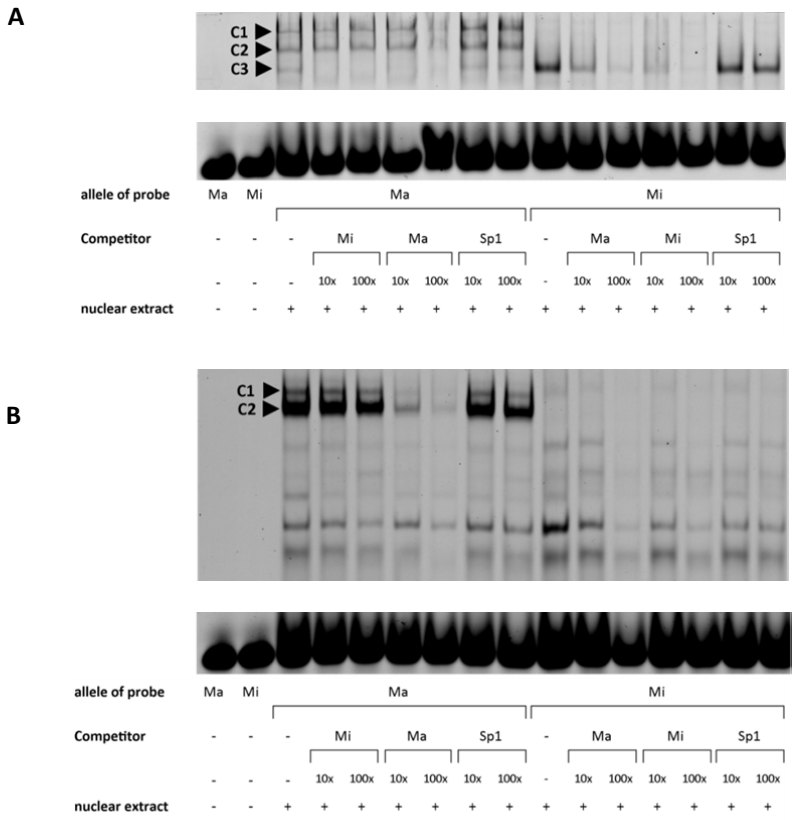

FIGURE 22. Allele-specific molecular interactions of SNPs (A) rs3798134 and (B) rs3798135 within the RHS5(I) of *RAD50* in Jurkat T cell nuclear protein extracts. EMSA Cy5-oligonucleotides carrying the major or minor allele revealed differential protein binding. An unlabeled competitor was added in indicated fold excess concentrations. Ma = major allele, Mi = minor allele, c = specific complex.

was assured by competing with increasing amounts of the unlabeled oligonucleotide containing the minor or major allele and also with oligonucleotides carrying the consensus sequence for SP1 as a negative control which did not cause a decrease in band intensity. The vector insert (642 bp) for the luciferase assay contained different combinations of the SNPs to test the effect of each SNP but also combinations of them on minimal promoter activity. After normalizing to the construct containing

both major alleles, it was observed that every combination containing at least one minor allele led to a slight increase in promoter activity in the Jurkat and HeLa cell line. Constructs containing both minor alleles for rs3798134 and rs3798135 caused significantly stronger promoter activity compared to the major allele in the Jurkat cell line (FIGURE 23A). To intensify the SNP-dependent effect and to diminish effects originated by other parts of the sequence, a shorter fragment (400 bp) containing both SNPs was cloned into the reporter vector and tested in the luciferase assay. Interestingly, a significant repressor effect was observed when at least one minor allele was present, adding up to a strong repressor when both minor alleles were tested in the Jurkat cell line. In the HeLa cell line, none of the constructs highly influenced promoter activity (FIGURE 23B).

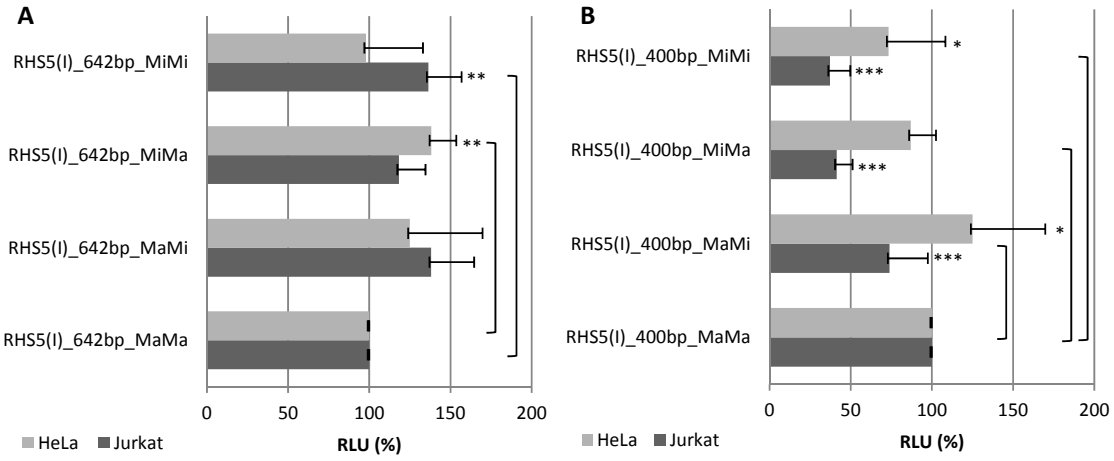

FIGURE 23. *In vitro* promoter activity is genotype-specifically regulated depending on rs3798134 and rs3798135 within RHS5(I). Luciferase assays were performed using Jurkat T cells and HeLa cells with **(A)** RHS5(I)_642bp_Ma/Mi and **(B)** RHS5(I)_400bp_Ma/Mi in a minimal promoter vector. Normalization was performed and p-values correspond to **(A)** RHS5(I)_642bp_MaMa or **(B)** RHS5(I)_400bp_MaMa. MaMa = rs3798135 major + rs3798134 major, MaMi = rs3798135 major + rs3798134 minor, MiMa = rs3798135 minor + rs3798134 major, MiMi = rs3798135 minor + rs3798134 minor, RLU = relative light unit. Significant effects: * P< 0.05, ** P<0.01, *** P<0.001.

A significant SNP dependent difference in transcript levels was observed with the Illumina HT-12 v.3.0 probe ILMN_1669174 for IL4 in probands of the KORA F4 cohort (n = 740) carrying both risk alleles of the variants (rs3798134: p = 0.0202; rs3798135: p = 0.0181) (FIGURE 24). For the second IL4 probe (ILMN_2389080), the IL5 probes (ILMN_1709300, ILMN_2207190), and the IL13 probe (ILMN_2052511) no difference in transcript levels were observed.

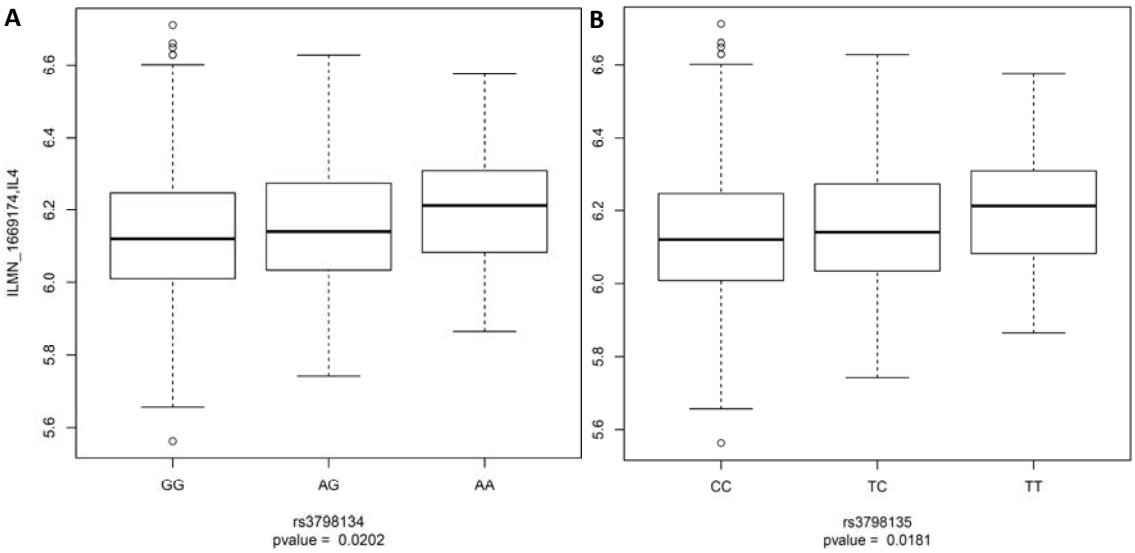

FIGURE 24. Genotype specific significant elevation of IL4 expression in whole blood cells of the KORA F4 cohort. Illumina HT-12 v.3.0 transcript ILMN_1669174 for IL4 shows significant SNP dependent higher expression in the boxplot for **(A)** rs3798134 (p = 0.0202) and **(B)** rs3798135 (p = 0.0181) (n = 740 samples). GG = homozygous non-risk alleles for rs3798134, AG = heterozygous alleles for rs3798134, AA = homozygous risk alleles for rs3798134, CC = homozygous non-risk alleles for rs3798135, TC = heterozygous alleles for rs3798135, TT = homozygous risk alleles for rs3798135.

2.2.2.2 Allele-Specific Effects within RHS6(I)

EMSA experiments revealed no differences in DNA-protein binding patterns in the Jurkat cell line for SNP rs12653750 (FIGURE 25). Therefore no further luciferase experiments were performed.

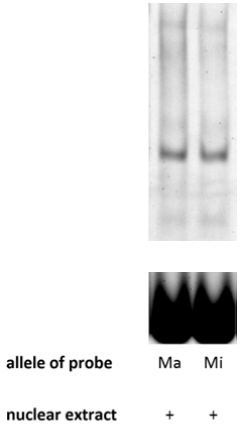

FIGURE 25. No allele-specific molecular interactions of rs12653750 within the RHS6(I) in Jurkat T cell nuclear extracts. EMSA Cy5-probes carrying the major or minor allele, revealed no differential protein binding. Ma = major allele, Mi = minor allele.

2.2.2.3 Allele-Specific Effects within RHS6(II)

EMSA experiments revealed two specific protein complexes that were formed preferably with the major allele (adenine) and not with the minor allele (guanine) of rs2040704 (C1 +C2). These results were underpinned by controls competing with the unlabeled oligonucleotide containing the minor or major allele as well as with the negative control SP1 (FIGURE 26).

A difficulty that occurred during the process of luciferase vector cloning was a point mutation (GRCh37/hg19: chr5: 131,973,154) in the major allele construct that always appeared and that could not be avoided by using QuikChange® mutagenesis or choosing other *E.coli* stains for cloning (BL21DE3, Jm107, Jm109). Clones containing the desired construct grew poorly and always carried the point mutation. However, according to UCSC (CRCH37/hg19) and the 1000 Genomes Project (released 13-December 2012) the mutation was not registered as known variant. To analyze whether this point mutation might be a not annotated SNP or a rare variant, 100 KORA F4 samples were sequenced. One individual carrying the C allele instead of the common T allele was identified (FIGURE 27). The calculated allele frequency according to the Hardy-Weinberg equilibrium for the C allele was 0.01 and for the T allele 0.99. The calculated genotype frequencies were for TT = 0.98,

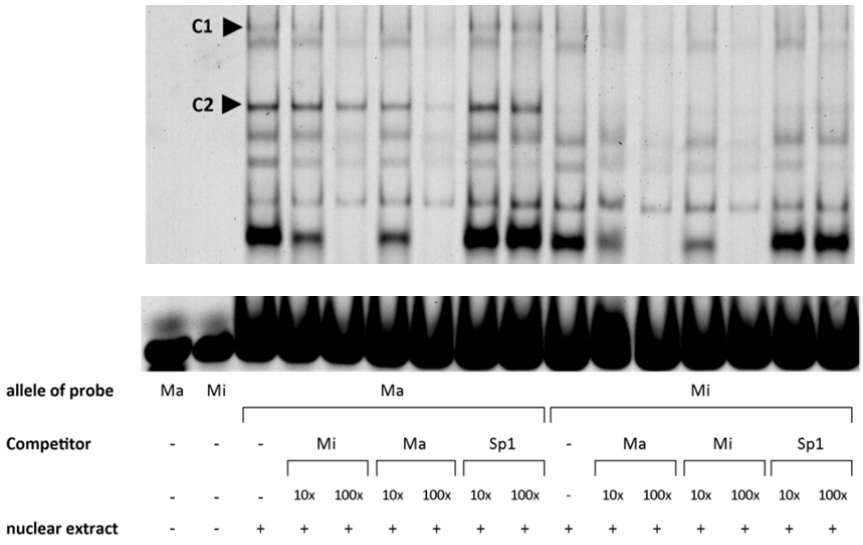

FIGURE 26. Allele-specific molecular interactions of rs2040704 within the RHS6(II) in Jurkat T cell nuclear extracts. EMSA Cy5-probes carrying the major or minor allele revealed differential protein binding. An unlabeled competitor was added in indicated fold excess concentrations. Ma = major allele, Mi = minor allele, c = specific complex.

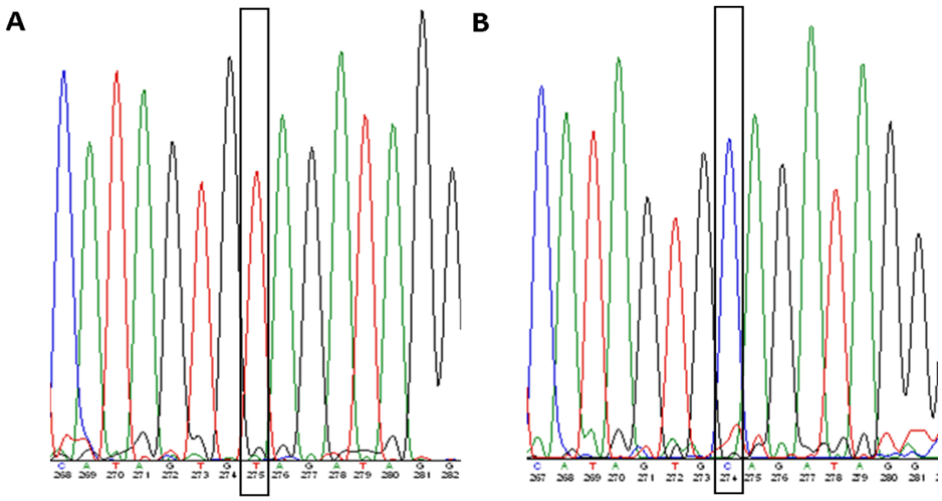

FIGURE 27. Electropherogram reveals a new rare variant in RHS6(II). (A) Electropherogram with the common T allele in 99 of 100 KORA F4 samples. **(B)** Electropherogram with the rare C allele in one of 100 KORA F4 sample. Black = G, blue = C, green = A, red = T. Black Box = position of rare variant.

for TC = 0.0198 and for CC = 0.0001. Due to the fact that clones without the point mutation could not be produced, experiments were continued with the obtained vectors. Nevertheless, it was found out that the rs2040704 minor allele caused a significantly enhanced promoter activity in the Jurkat cell line, whereas in the HeLa cell line, the minor allele exhibited a slight repressing effect (FIGURE 28). This result has to be considered with precaution due to the pointmutation in the RHS6(II).

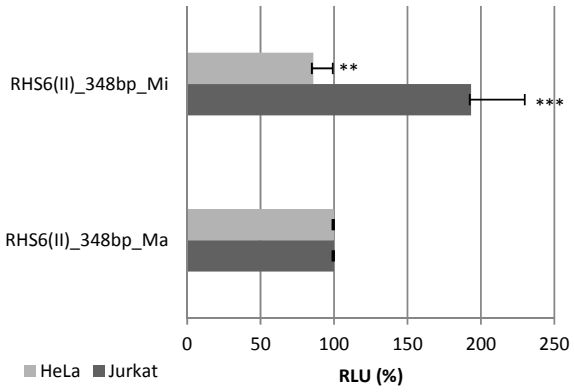

FIGURE 28. *In vitro* promoter activity is genotype-specifically regulated depending on rs2040704 within RHS6(II). Luciferase assays were performed using Jurkat T cells and HeLa cells with the full RHS6(II) fragment (348 bp) containing the major/minor SNP in a minimal promoter vector. Normalization was performed to RHS6(II)_348bp_Ma. Significant effects: ** P<0.01, *** P<0.001. Ma = major allele, Mi = minor allele, RLU = relative light unit.

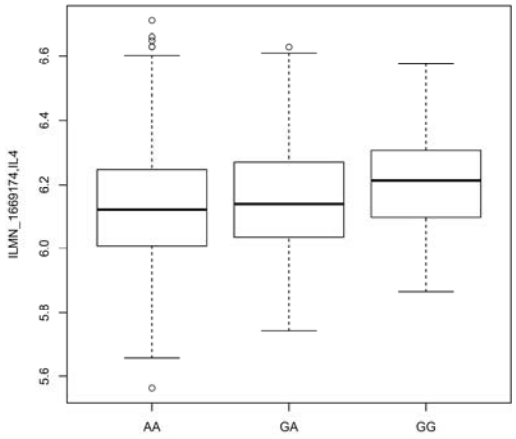

FIGURE 29. Genotype specific significant elevation of IL4 expression in whole blood of the KORA F4 cohort for SNP rs2040704. Illumina HT-12 v.3.0 transcript ILMN_1669174 for IL4 shows significant SNP dependent higher expression in the boxplot for rs2040704 (p = 0.0169). n = 740 samples. AA = homozygous non-risk alleles for rs2040704, GA = heterozygous alleles for rs2040704, GG = homozygous risk alleles for rs2040704.

A significant SNP dependent difference in cytokine transcript levels in the KORA F4 cohort was identified with the Illumina HT-12 v.3.0 probe ILMN_1669174 for IL4 in probands carrying the risk allele of rs2040704 (p = 0.0169) (FIGURE 29). For the second IL4 probe (ILMN_2389080), the IL5 probes (ILMN_1709300, ILMN_2207190), and the IL13 probe (ILMN_2052511) no differences in transcript levels were observed.

2.3 CHARACTERIZATION OF REGULATORY REGIONS BY DNASE1 HYPERSENSITIVE SITES

Genomic long range regulatory regions like enhancers, silencers, and LCRs are common sections of open chromatin, which are not protected by proteins and characterized by several DNase1 hypersensitive sites (Li et al., 2002a). The investigation of DNase1 hypersensitivity is therefore a frequently used tool to identify regulatory regions. Chromatin accessibility and the formation of hypersensitive sites is under epigenetic control during T cell development in mice (Fields et al., 2004) and in humans (Santangelo et al., 2002). Since the effect of the conserved RHS on promoter regulation in humans has been investigated in this study, it was now of interest whether there are extended or new Th2 DNase1 HS when comparing human atopic eczema cases with healthy controls. This might have the potential to lead to an even stronger activation of neighboring interleukin promoters by the use of the Th2 LCR.

2.3.1. Establishment of the MLPA-Method with HeLa Chromatin

To establish the MLPA system in our laboratory, published probes were used as controls, including those with target sites that are protected by proteins and therefore non-sensitive and those that are fully accessible and thus sensitive to DNase1 in HeLa cells (Ohnesorg et al., 2009). To investigate the effectiveness of the DNase1, the digested DNA probe was analyzed on a TBE-gel in parallel to each experiment. Usually, the genomic DNA was slightly digested when treated with 0.5 U of DNase1 for 30 min and strongly degraded after digestion with 2 U of DNase1 (FIGURE 30A). The bars represent the number of copies of each PCR-amplified DNA fragment normalized to the number of fragments that were obtained with the non-sensitive probes according to Ohnesorg et al. (Ohnesorg et al., 2009). For samples with non-sensitive probes a decrease or increase in bar height with increasing amounts of DNase1 was not expected. For the sensitive probes, however, a decrease in fragment number was anticipated. Evaluation of control probes revealed non-sensitivity for the non-sensitive probes and sensitivity for the sensitive probes according to the published results from Ohnesorg et al. (FIGURE 30B). After treatment with 2 U DNase1, the non-sensitive probes N1 and N5 showed a fragment number increase indicating the existence of even more PCR-copies than for the undigested probe, probably due to a higher PCR efficiency. Assuming that the conditions used for the experimental setup were reliable, they were applied to the experiments with Jurkat cells as well.

2.3.2. DNase1 Hypersensitive Sites in Jurkat Chromatin

To get closer to the conditions in the Th2 cell type, the establishment of a reliable DNase1 hypersensitivity detection system was continued by using Jurkat T cells. The advantage of Jurkat cells is that they are rather easy to cultivate and that experimental data for the presence of DNase1 hypersensitive sites exists, which can be transferred for the establishment of a new experimental system. Probes were designed for tests on the Jurkat chromatin. Afterwards, they could be used for the detection of RHS sites in differentiated T cell chromatin. The Jurkat chromatin was earlier shown to contain DNase1 hypersensitive sites within the RHS6(II) site (Sabo et al., 2004; Sabo et al., 2006) and was validated by MLPA (FIGURE 31 and TABLE 3). According to Ohnesorg et al., sensitive probes show a decrease in normalized peak heights < 75 % of the equivalent peak in undigested DNA (Ohnesorg et al., 2009). This DNase1 hypersensitivity criterion was also used for the here mentioned experiments. All of the other sites were shown to be not sensitive to DNase1 (FIGURE 31).

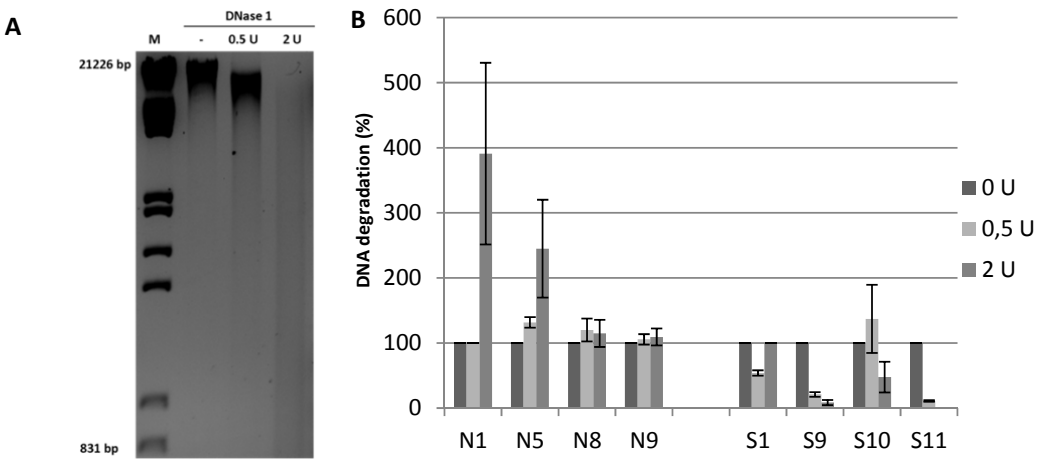

FIGURE 30. Results for the Multiplex Ligation dependent Probe Amplification Assay (MLPA) with HeLa chromatin using published probes for validation of the method. (A) The DNA-gel shows progressive DNA degradation with increasing amounts of DNase1. **(B)** The bar height in the graph indicates the degree of DNA degradation after incubating the nuclei with increasing amounts of DNase1. N = probe that targets a DNase1 non-sensitive region, S = probe that targets DNase1 sensitive region, U = Units, M = marker.

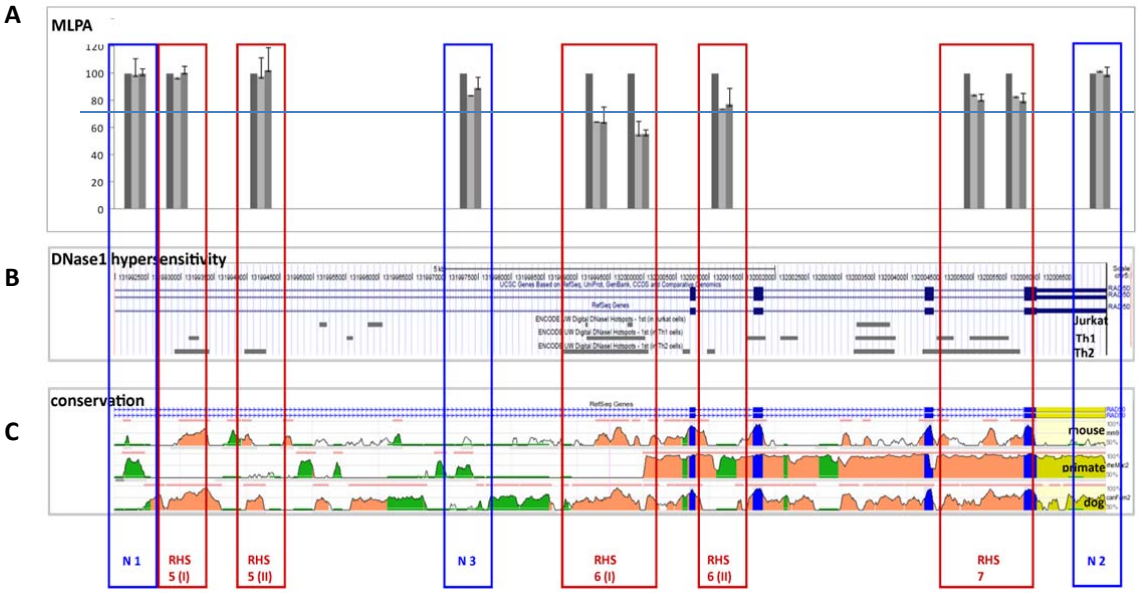

FIGURE 31. Results for the Multiplex Ligation dependent Probe Amplification Assay (MLPA) with Jurkat nuclei using self-designed probes. (A) Bars indicate the degree of degradation after incubating the nuclei with increasing amounts of DNase1. The blue line indicates the threshold between sensitive and non-sensitive probes (probes with bars below the threshold indicate sensitivity of the region). Besides, **(B)** published DNase1 hypersensitivity in the human Jurkat cell line and the human Th1 and Th2 cells and **(C)** conservation grade of different species according to UCSC and ENCODE are shown. The color code corresponds to (FIGURE 8). N = probe that targets DNase1 non-sensitive region, RHS = *RAD50* hypersensitive site. Region shown NCBI36/hg18: chr5:131,991,571-132,007,780.

TABLE 3. Same results for the detection of DNase1 hypersensitive sites in published data (ENCODE) compared to the results obtained with the MLPA for Jurkat nuclei. Sensitivities based on experimental results of DNase1 hypersensitive regions published within the ENCODE project (Sabo et al., 2004; Sabo et al., 2006) were compared to own results obtained with the MLPA system.

DNase1 HS (Jurkat)	N1	N2	N3	RHS5(I)	RHS5(II)	RHS6(I) _#1	RHS6(I) _#2	RHS6(II)	RHS7_ _#1	RHS7_ _#2
ENCODE project	-	-	-	-	-	+	+	-	-	-
MLPA results	-	-	-	-	-	+	+	-	-	-

(-) not sensitive, (+) sensitive

2.3.3. Differentiation of Human CD4+ Cells Towards Th1 and Th2 Cells

In order to obtain Th1- and Th2 cells that can be used for MLPA instead of Jurkat cells, CD4+ cells were derived from blood donated by healthy individuals and stimulated towards the Th1 or Th2 lymphocyte type. Differentiation was inspected and confirmed by RT-PCR (mRNA expression levels) and enzyme-linked immuno sorbent assay (ELISA) (protein secretion). Th1 cells are characterized to produce high amounts of IFNγ. Th2 cells predominantly produce IL4 and IL5. A clear differentiation towards the Th1 type was shown with both methods (FIGURE 32). Differentiation into the Th2 subtype was clearly shown with RT-PCR. For the secreted proteins measured by ELISA, higher levels for IL4 but not IL5 were observed in Th2 cells compared to Th1 cells.

2.3.4. DNase1 Hypersensitive Sites in Th1 and Th2 Cell Chromatin

Although effectiveness and purity of the “Th1” and “Th2” cell population was low, the next step towards the comparison of DNase1 hypersensitive sites in Th2 cell chromatin of atopic eczema cases and healthy controls was taken. To verify the reliability of the established MLPA system both Th1 and Th2 chromatin from healthy donors were used with the aim to compare the results to the already existing data (ENCODE project). Therefore, the same control probes as for the Jurkat nuclei were used to perform the experiments. In the chromatin of Th1 differentiated cells, two out of three non-sensitive control probes showed sensitivity to DNase1 (N1 + N3). The chromatin of Th1 cells was supposed to be non-sensitive in the RHS5(II) and RHS6(II) regions. This has been shown for RHS5(II) but not for the RHS6(II) region. RHS5(I) and also the Th1-specific site were described as sensitive

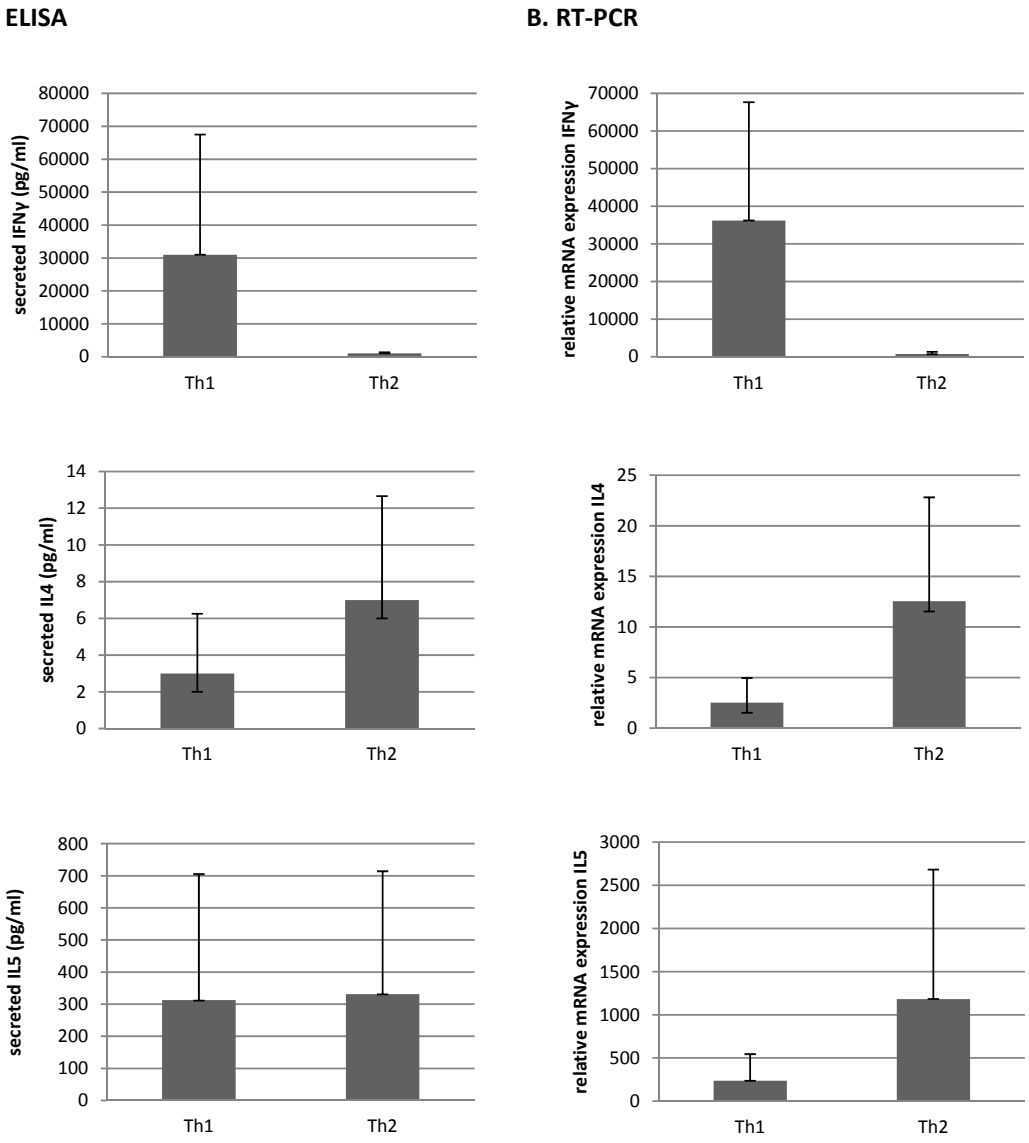

FIGURE 32. Verification of differentiation confirms T cell differentiation into the Th1 or Th2 subtype. (A) Secreted proteins were analyzed by ELISA and showed that the Th1 sample predominantly expresses IFNγ, whereas the Th2 sample produces more IL4 and IL5 than the Th1 sample. **(B)** Relative quantification of mRNA expression shows that the Th1 sample mainly produces IFNγ, whereas the Th2 sample produces higher levels of IL4 and IL5 than the Th1 sample.

regions in Th1 cell chromatin. This could not be shown with the MLPA experiments. All other sensitive probes featured DNase1 hypersensitivity as assumed (RHS6(I)_#1, RHS6(I)_#2, RHS7_#1, RHS7_#2). In the chromatin of Th2 differentiated cells, all non-sensitive probes (N1, N2, N3, Th1-specific) exhibited non-sensitivity to DNase1. Within the sensitive probes, RHS6(I)_#1, RHS6(I)_#2 and RHS7_#2 featured sensitivity, whereas the remaining probes were non-sensitive, which contradicts the ENCODE-data (FIGURE 33 and TABLE 4). Taken together, this experimental setup was

not used for further analysis of DNase1 hypersensitive sites of atopic eczema cases compared to healthy controls, due to the assumption that the results were not reliable in differentiated Th1 and Th2 cells.

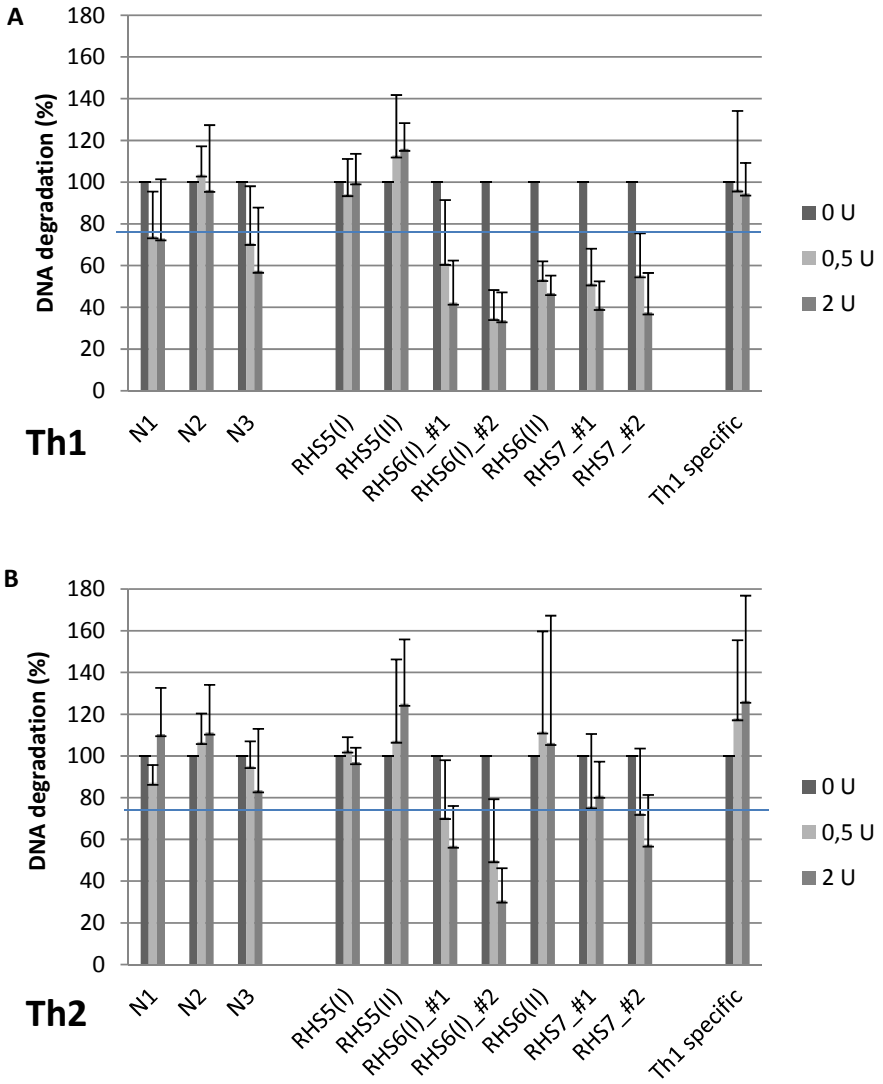

FIGURE 33. Results for the Multiplex Ligation dependent Probe Amplification Assay (MLPA) with chromatin of isolated human CD4+ and towards Th1/Th2 differentiated cells with self-designed probes, which are either located within or outside published hypersensitive sites. Bars indicate degree of DNA degradation after incubating the nuclei with increasing amounts of DNase1. The blue line indicates the threshold between sensitive and non-sensitive probes. N = probe that targets DNase1 non-sensitive region, RHS = *RAD50* hypersensitive sites, U = Units.

TABLE 4. Differing results for the detection of DNase1 hypersensitive sites in published data (ENCODE) compared to the results obtained with the MLPA method with differentiated Th1 and Th2 cell nuclei. Sensitivities based on experimental results of DNase1 hypersensitive regions, as published within the ENCODE project (Sabo et al., 2004; Sabo et al., 2006), were compared to own results obtained with the MLPA system. (-) not sensitive, (+) sensitive

DNase1 HS (Jurkat)	N1	N2	N3	RHS5(I)	RHS5(II)	RHS6(I) _#1	RHS6(I) _#2	RHS6(II)	RHS7_ _#1	RHS7_ _#2	Th1 specific
ENCODE Th1	-	-	-	+	-	+	+	-	+	+	+
MLPA Th1	+	-	+	-	-	+	+	+	+	+	-
ENOCDE Th2	-	-	-	+	+	+	+	+	+	+	-
MLPA Th2	-	-	-	-	-	+	+	-	-	+	-

3 DISCUSSION

Many genes have been identified to influence atopic eczema and other atopic diseases. This study focuses on the consistently with atopy associated Th2 cytokine locus on the human chromosome 5 (Li et al., 2010; Moffatt et al., 2010; Paternoster et al., 2012; Weidinger et al., 2008c). The Th2 cytokine locus contains the Th2 interleukin genes and plays a central role in the IgE driven immunological network and atopic diseases. Interestingly, in mice the Th2 cytokine locus was identified to contain an epigenetically regulated locus control region (LCR) within *Rad50*, which coordinates the expression of the interleukins *Il4* and *Il13* (Lee et al., 2003). This work has been dedicated to elucidate genetic and epigenetic mechanisms within the human atopy-associated Th2 LCR.

3.1 A COMMON ATOPY-ASSOCIATED VARIANT IN THE TH2 LOCUS CONTROL REGION IMPACTS TRANSCRIPTIONAL REGULATION AND ALTERS SMAD3 AND SP1 BINDING

The cytokine gene cluster on human chromosome 5q31 is one of the best established genetic susceptibility regions for atopic diseases (reviewed in: (Vercelli, 2008)). Functional work already focused on the cytokine genes, in particular human *IL13* polymorphisms (Cameron et al., 2006; Kiesler et al., 2010; Kiesler et al., 2009; Vladich et al., 2005; Webster et al., 2007). For example, SNPs within *IL13* create a binding site for OCT1, which enhances IL13 expression (Kiesler et al., 2009). Another variant causes altered YIN-YANG1 binding and an increased IL13 secretion (Cameron et al., 2006). Mechanisms at the murine Th2 locus control region suggest a regulatory role of *Rad50* intronic sequences for cytokine expression. Identification of a regulatory role of the intronic *RAD50* region in the human genome and the presence of a functional SNP within can add mechanistic knowledge to the genetic causes of atopy in humans. Indeed, the results presented here show that the risk allele of the common atopy-associated SNP rs2240032 within *RAD50* has a functional nature that causes diminished binding of the transcription factors SMAD3, SP1 and associated complex partners, modifies regulatory activity on a minimal promoter in an *in vitro* assay, and alters interleukin expression.

The Atopy-Associated SNP rs2240032 is Located in the Human Conserved RHS7 Site

Atopy-associated SNPs within the 3' region of the *RAD50* gene, which provide *IL13* independent signals, are in high linkage disequilibrium ($R^2 = 1$) with other polymorphisms (Weidinger et al., 2008c), making it hard to tell which of these variants are causal. Thus, functional studies are needed to narrow down association signals. The minor risk allele of the common SNP rs2240032 was associated with total IgE, a hallmark of Th2 driven atopy responses, and asthma (Li et al., 2010; Moffatt et al., 2010; Weidinger et al., 2008c). The studies of Weidinger et al. and Moffat et al. are considered robust, as they examined large numbers of participants in well characterized cohorts (GABRIEL consortium and KORA cohort) and extensively replicated their findings, which is considered the gold-standard for validation of association findings (Moffatt et al., 2010; Weidinger et al., 2008c). The smaller study of Li et al. investigated 473 asthma cases from the TENOR study and 1892 Illumina general population controls. It is less robust but confirms the previous findings (Li et al., 2010). The variant rs2240032 is the only SNP in Europeans located in the human *RAD50* hypersensitive site 7 (RHS7). RHS7 is a conserved site that was characterized in the mouse to be essential for the regulatory functionality of the Th2 LCR (Koh et al., 2010; Lee et al., 2005). According to the HapMap-database in Europeans (HapMap phase 3 -CEU) the genotype frequency of rs2240032, is 0.628 for the CC genotype, 0.336 for the CT genotype, and 0.035 for the TT genotype.

Jurkat Cell Line: An Appropriate Cell Line for Functional Studies at the Th2 Cytokine Locus?

To functionally characterize genetic mechanisms at the Th2 cytokine locus, it is essential to choose the most suitable cells for this quest. The group of R. Flavell, which comprehensively analyzed the murine Th2 LCR, isolated CD4+ cells from the spleen of mice and differentiated them towards the Th1 and Th2 subtype for this purpose (Lee et al., 2003). The information concerning the efficiency of differentiation towards the intended T cell subtype, however, was missing. As pure populations of Th1 and Th2 cells are the appropriate cells for the kind of analyses that were performed here, human CD4+ cells were isolated from whole blood and differentiated towards the Th1 and Th2 subtype (see chapter 2.3.3). Differentiation towards human Th1 and Th2 cells is only achievable up to 30 % (personal communications: Stefanie Eyerich, Zentrum Allergie Umwelt (ZAUM)). T cell clones, on the contrary, are pure populations (Stefanie + Kilian Eyerich, Zentrum Allergie Umwelt (ZAUM)). The disadvantage of T cell clones is that they are difficult to cultivate and more importantly, they are not suitable for transfection experiments with big luciferase plasmids. Thus cells were needed which were easy to cultivate, suitable for transfections, and still had the characteristics of T cells. Jurkat is an immortalized suspension cell line of T lymphocytes, which was established in the 1970s from T cells of a 14 year old boy with T cell leukemia (Schneider et al., 1977). Jurkat cells are frequently used

for Th2 interleukin promoter studies and for electrophoretic mobility shift assays investigating Th2 specific transcription factor binding (Kozuka et al., 2011; Ranganath et al., 1998; Rosenwasser et al., 1995). Nevertheless, the use of Jurkat cells represents a disadvantage for this study because the Jurkat cells consist of T lymphocytes rather than a pure Th2 specific population. Furthermore, these cells provide insufficient information about the effects of immortalization on epigenetic patterns. HeLa cells from human female cervix carcinoma (Scherer et al., 1953) were used as a control cell line to demonstrate cell type specific effects of Jurkat cells at the Th2 cytokine locus (Lavenu-Bombled et al., 2002; Liberman et al., 2009; Shin et al., 2005). Further, HeLa cells are missing T cell specific transcription factors like human GATA3 and can therefore be used to investigate effects due to the addition of those factors to the system (Lavenu-Bombled et al., 2002).

The rs2240032 Risk Allele Selectively Binds a Protein Complex Containing SMAD3 and SP1

Using the EMSA approach, specific differential binding of nuclear proteins from Jurkat, but not HeLa cells, to the polymorphic site rs2240032 were identified. In particular with the major allele oligonucleotide, a strong band appeared in the upper part of the EMSA-gel, probably indicating a big protein complex. Fewer complexes were formed when the minor risk allele was present. Computational analysis, competition, and supershift experiments showed SMAD3- and SP1 binding to the polymorphic site. However, SMAD3 competition experiments needed a high excess of unlabeled oligonucleotide competitor and supershift experiments led to a decrease in band intensity without an additional supershift, thus indicating that SMAD3 is probably involved in a complex and not freely available for DNA-binding. For the SMAD3 competition experiments, a commercially available SMAD3/4 competitor sequence was used whereas for the SMAD3 supershift experiments, a SMAD2/3 antibody was used. SMAD2 is not able to directly bind the DNA (Hoot et al., 2008; Yang et al., 2009), thereby the competitor consensus sequence is not detecting SMAD2. SMAD2 and SMAD3 possess a 90 % homology (Li et al., 2008). Therefore, most commercially available antibodies target both SMADs. The observation of both a competition and a supershift in the EMSAs indicates that SMAD3 was detected. SP1 was identified as a further member of the protein complex. Small amounts of SP1 unlabeled competitor oligonucleotide led to a dramatic band reduction with the labeled major allele oligonucleotide. The SMAD3 core binding motif is disrupted by the minor risk allele of rs2240032 (FIGURE 9), which is important for efficient protein-DNA binding. On the contrary, the minor allele of rs2240032 causes a disruption of a less important position within the SP1 conserved binding motif (FIGURE 9). This might indicate that SMAD3 is the protein which is affected by the SNP in a stronger fashion and which is therefore responsible for diminished complex formation when the risk allele is present.

The supershift experiments using an SMAD3- and an SP1 antibody needed extensive optimization to obtain results. Supershift experiments allow many starting points for adjustments such as the choice of antibody, antibody concentration, incubation conditions, and order of experimental steps. Many antibodies with different target epitopes are commercially available from different companies. As the proteins of interest are often involved in protein complexes, not every protein domain is accessible for antibody binding. Therefore, it is necessary to test different kinds of antibodies. Also for this study several SMAD3 antibodies were tested. To identify appropriate antibody concentrations, it is not sufficient to detect a supershift or band intensity loss. It is essential to use antibody class (isotype) controls as negative controls to assure that the observed binding effect is not originated by the isotype of the antibody. When adding high amounts of antibody, a band intensity loss was observed that was not antibody specific and based on the isotype. Therefore, isotype controls have to be used for every concentration and condition tested to assure antibody specificity. Appropriate incubation conditions for supershift experiments varied from experimental setup to experimental setup. In some cases nuclear extracts supplemented with antibodies needed to be kept on ice, probably due to protein complex instability. In other experiments supershifts were only detectable when the reaction was incubated at room temperature. Additionally, the incubation time had to be adjusted for each experiment. The order of the addition of all reaction components was crucial for the supershift formation or band intensity loss. In some cases the antibody had to be first incubated with nuclear extract before the addition of the labeled oligonucleotide. This approach facilitates the antibody binding to the protein of interest. Thus in turn, the protein-antibody complex might not be able to bind to the oligonucleotide due to a steric hindrance of the protein-antibody complex, which causes band intensity loss instead of a classical supershift. This was observed for the SMAD supershift experiments. For the SP1 supershift analysis, the protein was first allowed to bind to the labeled oligonucleotide before adding the antibody. The supershift is reliant on the availability of the protein epitope to the antibody after DNA-protein complex formation to produce an additional band visible on the EMSA gel. It was unfeasible to establish a standardized protocol for supershift experiments. However, it was assumed that the conditions used for the experiments produced reliable results, which needed verification by another EMSA paired method, e.g. by mass spectrometry (Carey et al., 2012; Hellman and Fried, 2007).

To verify the differential binding of the transcription factors that were identified by EMSA experiments and to identify further protein complex partners, mass spectrometry was performed. Based on the mass spectrometry and the STRING analysis results it was hypothesized that the complex that binds with a higher affinity to the major allele sequence than to the minor allele sequence consists of a SMAD-SKI-complex and an EXOSC-complex linked by SP1 and SKIV2L2 (FIGURE 34). This hypothetical network does not answer questions concerning the DNA binding chronology of

the proteins. TFAM, a protein identified by mass spectrometry, is not included in the hypothetical complex. Due to complete band intensity loss in the EMSA when SP1 was competed, it was assumed that SP1 stabilizes the entire protein complex. SP1 could not be detected by mass spectrometry, probably due to abundance below detection levels. Another explanation might be that SP1 strongly binds to the DNA, therefore requiring higher salt concentrations for the elution than SMAD3. SP1 might consequently be contained in another eluate.

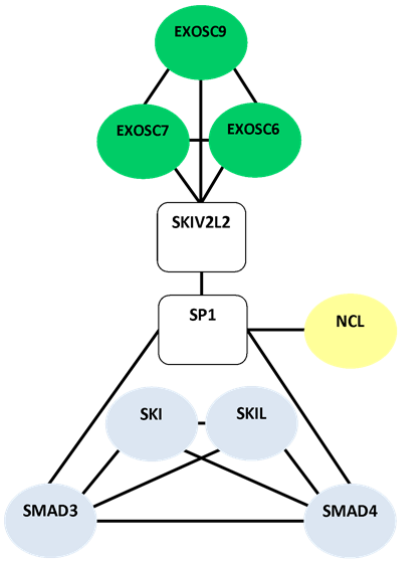

FIGURE 34. Hypothetical protein-network based on mass spectrometry results. The protein-network was created by using the significant mass spectrometry candidates that were binding to the major allele and formed DNA-protein complexes based on the protein network software STRING. Circle = identified proteins from mass spectrometry analysis; Square = Hypothetical proteins, that were not significantly detected but connect candidate proteins.

Candidate proteins from the hypothetical protein-network, based on the EMSA and mass spectrometry results, are feasible proteins in regard to atopic eczema and other atopic diseases. A connection between some of these proteins and atopic diseases has already been observed.

SMAD transcription factors are well described players in transforming growth factor-β (TGFβ) pathways which regulate growth, differentiation, and function of T cells, B cells, macrophages, and natural killer cells (Letterio and Roberts, 1998; Wahl, 1992). The SMAD family encompasses receptor specific SMADs (R-SMAD 1, 2, 3, 5, 8), the common SMAD (SMAD4), and inhibitory SMADs (I-SMAD6 and 7). After phosphorylation, the R-SMADs form heteromeric complexes with SMAD4, which translocate into the nucleus and initiate tissue specific target gene regulation (reviewed in (Derynck and Zhang, 2003)). Several lines of evidence point towards a role of SMAD transcription factors in

atopic eczema and other atopic diseases. In humans, SMAD3/4 mRNA expression is higher in the skin of healthy controls compared to atopic eczema lesional skin (Gambichler et al., 2006). SMAD3 polymorphisms have been associated with asthma (Moffatt et al., 2010; Noguchi et al., 2011) and atopic eczema (Otsuka et al., 2009). Furthermore, Smad3-knockout mice express elevated levels of Il4, Il5, and Il13 and are susceptible to asthma (Anthoni et al., 2007b) whereas the expression of Th1 chemokines is not changed (Anthoni et al., 2008). Additionally, these mice show enhanced allergen induced skin inflammation and IgE production (Anthoni et al., 2007a). SMADs must interact with SMAD-binding cofactors for high affinity and specific binding of target genes (reviewed in: (Schmierer and Hill, 2007)). It is assumed that at the site investigated here SMAD3 forms a complex with SMAD4 as described and interacts with other cofactors including SP1, probably due to specificity reasons. This might involve altered protein function or binding behavior of SMAD3 due to binding cofactors.

The human transcription factor SP1, which was only shown by EMSAs, is involved in many transcriptional processes and can directly interact with DNA but also with other proteins such as SMAD3 and SMAD4 (Holler et al., 1988; Poncelet and Schnaper, 2001). SP1 and SMAD proteins cooperate at promoter sequences to induce transcription of several genes in response to TGFβ signaling (Feng et al., 2000; Jungert et al., 2006; Poncelet and Schnaper, 2001). SP1 is regulated by nucleolin (NCL), which was also identified by mass spectrometry. NCL is present in the nucleus and possess helicase and chromatin decondensing activities (Tsou et al., 2008).

Further identified proteins like the SKI proteins (which were named after the institute where they were first discovered: Sloan-Kettering Institute) are transcriptional co-regulators and proto-oncogenes which are abundantly available in tumors (Vignais, 2000). They exist in the nucleus and the cytoplasm and are part of the SMAD pathway as they possess a SMAD-binding domain (Chen et al., 2007). SMAD3/4-SKI complexes are known to negatively regulate TGFβ-signaling (Liu et al., 2001; Stroschein et al., 1999). SKI proteins bind SMAD4 and repress the TGFβ promoter activity by recruitment of repressors when TGFβ is absent (Stroschein et al., 1999). As many regulatory proteins are involved in different pathways it is assumed that SKI-SMAD-complexes might also interfere in other pathways, which might be relevant for atopic diseases.

EXOSC proteins, which were identified to participate in the protein complex that is differentially binding to the major allele of rs2240032, are part of the multi protein exosome complex which possesses an exo- and endoribonucleolytic function for the degradation of all types of RNA in the nucleus and the cytoplasm. The core of the exosome consists of six proteins (EXOSC 4-9), but more proteins can be attached to regulate activity and specificity of the exosome complex (Schilders et al., 2006). The exosome complex interacts with single proteins and with other protein complexes such as the SKI family members, which includes the RNA helicase SKIV2L2 and which is involved in mRNA

degradation in yeast and humans (Lubas et al., 2011; Wang et al., 2005). EXOSC proteins were not described to be involved in atopy yet.

The rs2240032 Risk Allele Influences Human RHS7 Regulatory Function

Using luciferase assays, it was shown that the minor risk allele of rs2240032 resulted in significantly higher promoter activity in a short fragment (RHS7_150bp). Significance was lost when the entire RHS7 fragment (RHS7_1396bp) was tested, probably due to an overlay of the observed effect by the genomic context in the Jurkat cell line. Another explanation might be that the observed enhancer effect is strong enough for smaller SNP effects to disappear. When the short sub-regulatory region containing the SNP was deleted from the entire RHS7 (RHS7Δ150bp_1246bp), the promoter activation is even more pronounced in Jurkat cells, indicating that this region has regulatory potential for the entire RHS7 region in a SNP-dependent manner. In the HeLa cell line, the effects of the short (RHS7_150bp) and entire RHS7 fragment (RHS7_1396bp) were nearly the same. The deletion of the short fragment from the entire RHS7 (RHS7Δ150bp_1246bp) caused significantly reduced promoter activity compared to the entire fragment still containing the short sub-regulatory region (RHS7_1396bp), indicating that in the HeLa cell line, the region surrounding the short fragment possesses repressor activity. Transcriptional regulation is tissue dependent (Fu et al., 2012), probably due to tissue-specific transcription factor expression patterns. This explains why the results obtained in HeLa differ from the Jurkat outcome. Although SMAD3 and SP1, being two of the transcription factors, are present in both cell lines, other transcription factors from the hypothesized protein complex are most likely necessary for transcriptional regulation as well and might not be available in HeLa cells.

Luciferase based promoter assays are a useful tool to study promoter regulation in living cells. Some questions to estimate the effects of the experimental setup were answered. First question: Does the insertion of any arbitrary insert change promoter activity? To exclude promoter effects solely due to the insertion of an extra DNA-fragment into the vector, a so called “desert site”, which is located in an intronic and not annotated regulatory region (according to UCSC), was investigated. As desired, it did not alter promoter activity. Second question: Does the insert orientation inside the vector influence promoter regulation? It is well known that orientation-dependent insertion of a sequence can change promoter activity. For example, the human β-globin locus control region is described to possess orientation dependent effects (Tanimoto et al., 1999), a characteristic that was also shown for the entire RHS7 (RHS7_1396bp_Ma). However, reverse complementation of the short RHS7 fragment (RHS7_150bp_Ma) did not cause a significant change in promoter activity, allowing the interpretative approach that the site directed regulatory elements are not located within this region.

Surprisingly, the reverse complemented “desert site” turned into a repressor. After reverse complementation, a new genomic context was created, in which the “desert site” showed a regulatory effect. Third question: Does the distance between the promoter and the regulatory element influence promoter regulation? Especially studies of *cis*-regulatory elements like LCRs inside the luciferase vector, where the luciferase promoter and the regulatory element are just a few base pairs apart (45 bp), should be regarded with precaution. In this setup, transcription factors are in close proximity to the promoter and steric effects might prohibit proper gene activation. Additionally, *cis*-regulatory elements usually act over long distances (1 Mb pairs) by looping out the interjacent DNA to provide close proximity of transcription factors and promoter (Maston et al., 2006). However, LCRs are characterized as being position-independent, which means that the exact position within the genome does not contribute to LCR function (Li et al., 2002a). To answer whether position dependency of the LCR and regulatory elements exist, *in vivo* experiments would be required. It is highly arguable if “desert sites” or inverted fragments serve as reliable controls in luciferase assays. In this study it was shown that a site that had no function at its place of origin can acquire regulatory potential when added to a new genomic context, while other fragments keep their regulatory potential independently of their orientation.

Genotype Specific Interleukin Expression

SNP-dependent RNA expression and expression correlation analyses of interleukins, SMADs, SP1, and RAD50 in blood of the KORA F4 cohort were considered to illustrate implications about genotype specific promoter regulation of the RHS7 and the impact on IL4, IL5, and IL13 expression in whole blood cells. The analysis revealed a significant elevation for one of two IL4 transcripts by the risk allele of rs2240032 in the RHS7. This result has to be interpreted with caution because the other IL4 transcript did not show a significant association. Furthermore, the values for the detected transcripts were close to the background signal. However, the significantly regulated transcript represents most IL4 transcripts whereas the not regulated transcript represents only one transcript containing the 5’ UTR. Further correlation analyses did not reveal any correlation between all of the investigated transcripts. To verify the genotype specific difference in expression of IL4, replication in a large cohort is needed. Furthermore, it should be considered that whole blood is a heterogeneous tissue and might therefore be inappropriate for investigations of mechanisms in Th2 cells only. The overall T lymphocyte (Th1, Th2, Th9, Th17, Th22, Treg) amount in the peripheral blood is dependent on race, gender, medication, physical activity, environmental factors, and age (Blum and Pabst, 2007). As lymphocytes reside in primary lymphoid organs (thymus, bone marrow), secondary lymphoid organs (spleen, lymph nodes, tonsils), and non-lymphoid organs (blood, lung, liver, skin) the content in the

blood mirrors just a fraction (2 %) of the overall number and might also vary with differences in migration behavior (Blum and Pabst, 2007; Trepel, 1974). The amount of lymphocytes within white blood cells ranges from 14 % - 47 % (according to www.stemcell.com). The exact composition of T lymphocytes varies with infections or medical conditions. For example, helminthic infection increases the amount of Th2 cells whereas bacterial and viral infections cause elevated levels of Th1 cells (Scott and Kaufmann, 1991). Therefore, a pure Th2 population would be the best choice for further experiments and also represent a criterion for the choice of the cohort for the IL4 expression validation.

Using T cell clones from patients with atopic eczema, allergic contact dermatitis, and psoriasis it was shown that carriers of the heterozygous risk alleles (CT) had higher RNA expression levels of IL4, IL5 and IL13 than those with the homozygous non-risk alleles (CC). This illustrates that rs2240032 may have a regulatory function in the expression of the Th2 cytokines. It can be hypothesized that the regulatory mechanisms in humans might be comparable to the ones in mice. Therefore, it is proposed that SMAD3 and SP1 are present in the transcription factor complex that is involved in the looping of the LCR and control promoter activity. The loss of a SMAD3- and SP1 binding site due to the risk allele of rs2240032 might reinforce looping of the LCR containing RHS7. Thereby, physical proximity with the *IL4, IL5,* and *IL13* promoter may be established and enhancement of promoter activity might be achieved. However, there is a bias due to the use of naïve (Th0), Th1, and Th2 cells. The heterozygous risk genotype (CT) mainly contained Th2 clones whereas naïve, Th1, and Th2 cells contributed to the result obtained for the homozygous non risk allele (CC) carriers. It would be desirable to perform the analysis in only one T cell subtype population to get robust results that are not confounded by the mixture of cell types for the homozygous candidates on the one hand and the Th2 cell clones with heterozygous genotype on the other hand. Another improvement would be by using T cell clones from donors with the same kind of disease in order to eliminate a potential bias due to specific diseases. An appropriate number of homozygous risk allele carriers (TT) would additionally be desirable. Repetition of this experiment with higher numbers of Th2 cell samples from patients with atopic eczema only will help to get more robust results in future studies. Taken together, due to the risk allele rs2240032, genotype specific elevation of IL4, IL5, and IL13 mRNA levels are in line with atopic immune responses, as elevation of IL4, IL5, and IL13 are observed in atopic cases (Bieber, 2010; Callard and Harper, 2007).

Potential Interplay of Epigenetic Mechanisms and Genotype-Specific Effects at the RHS7

Regulation of Th2 cytokine expression and the looping-model are commonly discussed in literature in the context of epigenetic mechanisms. For the formation of regulatory active regions which in turn

loop out to control promoter activity, DNA regions that are accessible for transcription factors are necessary. In mice, it has been shown that CpG residues in the core Th2 LCR (including RHS5, RHS6, RHS7) are fully methylated in naïve CD4+ T cells, thereby probably suppressing cytokine transcription (Yoshimoto et al., 2013), and fully demethylated in Th2 cells associated with transcriptional competence (Fields et al., 2004). Methylation is not the only epigenetic mechanism. Additionally, extent histone modifications promote chromatin accessibility for transcription factors at the Th2 cytokine locus in Th2 cells (Fields et al., 2004; Lee et al., 2002). Exciting results were obtained by experiments with LCR-knockout mice which showed a loss of histone acetylation, a dramatic reduction in expression levels of Th2 cytokines and IgE, and a loss of asthmatic symptoms in asthmatic mice (Koh et al., 2010). Fewer studies have been carried out at the human Th2 cytokine locus. These studies indicate differences to the murine setting, i.e. the demethylation that occurs across the human Th2 cytokine locus during Th2 differentiation appears to be not as expanded, pronounced, and localized as in the murine locus (Lee et al., 2002; Santangelo et al., 2002). Histone modifications to establish the euchromatic state have been observed all over the Th2 cytokine locus (Kaneko et al., 2007). Data from the ENCODE project Digital DNase1 Methodology for human Th1 and Th2 cells reveals DNA accessibility for the RHS7 in both Th1 and Th2 cells at the position of SNP rs2240032. However, the RHS7 site in Th1 cells is smaller, than the one in Th2 cells (Sabo et al., 2004; Sabo et al., 2006) (FIGURE 8). The presence of the RHS7 DNase1 HS hints at the possibility of a looping out of the LCR containing RHS7 which might enable promoter activity control of neighboring genes. Probably exclusively due to the size of the RHS7 in human Th2 cells, this hypersensitive site allows efficient interleukin promoter enhancement whereas the RHS7 in Th1 cells might not be large enough to control promoter activity. Direct epigenetic mechanisms concerning interaction at rs2240032 are not obvious as the SNP is not located within a CpG site. The major allele of rs2240032 contains a non-CpG cytosine residue which is lost when the minor risk allele is present (GACAG → GATAG). In stem cells, it has been observed that cytosine residues followed by a thymine and adenine base can be epigenetically modified (Laurent et al., 2010; Lister et al., 2009), which allows speculations about epigenetic modifications at the here investigated variant. Furthermore, it might be possible that a SNP influences the methylation status of CpG sites that are located further away (the closest CpG site is located 42 bp away). In brain tissue, it was shown that the average distance between the SNP influencing methylation and the CpG sites is 81 kb (Gibbs et al., 2010). SNP dependent changes of distant CpG sites in the Th2 cytokine locus by epigenetic patterns might regulate the expression of IL4, IL5, and IL13. Future studies will include bisulfite sequencing to give insights into methylation status at non-CpG cytosine residues. Correlation analysis of variant rs2240032, CpG methylation, and IL4 and IL13 expression will further help to clarify underlying mechanisms. Apart from direct variant dependent consequences on the epigenetic status of the

RHS7, it is likely that the SNP affects the regulatory potential of the RHS7 only when the region is accessible for transcription factor binding. Chromatin immunoprecipitation (ChIP) represents a first approach to investigate polymorphism dependent aspects of the RHS7 epigenetic state concerning interactions with the *IL4, IL5,* and *IL13* promoter, as transcription factors are only able to bind to accessible DNA regions and in return potentially regulate promoter activity. Th2 cells containing the major or minor allele of rs2240032 are the best cell type for this investigation.

3.2 REGULATORY POTENTIAL OF *RAD50* HYPERSENSITIVE SITES AND ATOPY-ASSOCIATED SNPS

The murine Th2 locus control region has been intensively studied which provided a better comprehension of the regulatory mechanisms occurring at *Rad50* hypersensitive sites in mice (reviewed in: (Lee et al., 2006)). To my knowledge, no studies have investigated the effect of the conserved RHS in humans so far. Within the scope of this project each RHS located in the core of the human LCR (RHS5-7) has been characterized and rs3798134, rs3798135, and rs2040704 were identified as functional relevant SNPs within RHS5(I) and RHS6(II).

3.2.1. Characterization of RHS and Their Regulatory Role in Humans

The core of the highly conserved human LCR within *Rad50* encompasses RHS5 to RHS7 (Fields et al., 2004). In the mouse, combinations of these sites reestablish the regulatory potential of the LCR (Fields et al., 2004). Single RHS sites were reported to have *Il4*-promoter regulatory potential in the mouse model: RHS5 was shown to act as a repressor whereas RHS6 and RHS7 apparently cause enhanced *Il4* promoter activity (Fields et al., 2004; Lee et al., 2005). RHS7 is the best studied of those sites and was proven to physically interact with the *Il4* and *I13* promoters in T cells of mice (Spilianakis and Flavell, 2004). The results in the human Jurkat cell line and the results for the murine Th2 cells by other laboratories (Fields et al., 2004; Lee et al., 2005; Spilianakis and Flavell, 2004) both showed that the RHS7 acts as a cell type specific enhancer. Fields et al. published the conserved sequences for the RHS5(I), RHS5(II), RHS6(I), and RHS6(II) but missed to announce which sequences they used for their promoter studies (Fields et al., 2004). For the study of the regulation of the *Il4* promoter, they used fragments that differed in the lengths from the mentioned RHS5(I), RHS5(II), RHS6(I), and RHS6(II) sequences. Therefore, the exact sequence of the RHS5 and the RHS6 fragment

remains unknown. Both the RHS5 and RHS5(II) served as repressors on promoter regulation in both species and studies. The human RHS6(I) showed repressive activity in this study, which contradicts the results obtained in mice where the RHS6 acted as an enhancer (Fields et al., 2004). The different observations might be due to the use of RHS6 fragments with different lengths, performance in different species, or the use of a minimal promoter assay in this study, whereas Fields et al. tested the influence of RHS6 in an *Il4* promoter assay.

Apparently, RHS sites of the Th2 LCR region provide a regulatory potential in other cell types than immunological cells, like epithelial HeLa cells, which indicates that required transcription factors are available in this cell line. The RHS5(I) acted as a moderate enhancer in HeLa cells whereas in Jurkat cells no regulatory effect was detectable. The RHS5(II) and RHS6(I) were shown to be repressors independently of the tested cell type. However, it raises the question if this result is of biological relevance in HeLa cells because the Th2 LCR is not epigenetically accessible for transcription factors in HeLa cells according to the ENCODE project (Sabo et al., 2004; Sabo et al., 2006) (FIGURE 35). To verify T cell specific regulatory potential of the LCR, experiments with more immunological cell lines will be necessary.

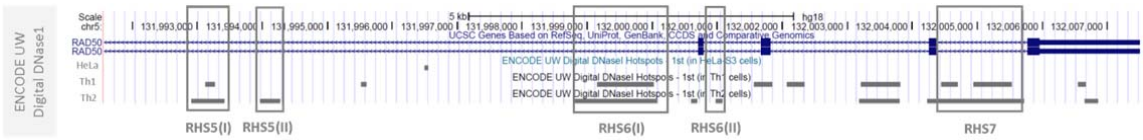

FIGURE 35. The existence of DNase1 hypersensitive sites differs between HeLa and T cells in the human *RAD50* gene. Hotspots of DNase1 hypersensitive sites are indicated by grey bars. RHS sites are indicated with grey boxes. Region shown: NCBI36/hg18: chr5:131,991,571-132,007,780.

It can be assumed that several RHS sites together orchestrate the human LCR as they do in the mouse (Fields et al., 2004; Spilianakis and Flavell, 2004; Spilianakis et al., 2005) and that the LCR might act as a *cis*-regulatory element on human *IL4* and *IL13* promoter activities. It is of high interest to address this assumption in future studies. Combinations of RHS could be examined by the established luciferase assays either testing on a minimal promoter or using specific human *IL4* and *IL13* core promoters. *In vivo*, physical interaction of the LCR with neighboring promoters within the genomic context could be investigated by chromatin immunoprecipitation (ChIP). This method would also help to clarify whether loop formation mechanisms comparable to the mouse model (Spilianakis and Flavell, 2004) are present in the human system. As Th2 cells are the cell type of interest, Th2 clones or other pure Th2 cell populations would be the best model for further experiments.

3.2.2. Functional SNPs within RHS5(I) and RHS6(II)

The common atopy-associated SNPs rs3798134, rs3798135, and rs2040704 are located within the RHS5(I) and RHS6(II) and in complete linkage disequilibrium ($R^2 = 1$) (Li et al., 2010; Weidinger et al., 2008c) (FIGURE 20). EMSAs performed in this study revealed SNP specific transcription factor binding, which was supported by genotype-specific and cell-type specific promoter regulatory potential in luciferase based assays.

To characterize potential genotypic effects within the RHS5(I), several combinations of the variants rs3798134 and rs3798135 were tested in promoter studies although *in vivo*, both major (MaMa) or minor (MiMi) alleles of the polymorphism are present. According to the HapMap database in Europeans (HapMap phase 3 -CEU) the genotype frequency of rs3798134 is 0.628 for the TT genotype, 0.336 for the CT genotype, and 0.035 for the CC genotype. The genotype frequency for rs3798135 is 0.627 for the GG genotype, 0.339 for the GA genotype, and 0.034 for the AA genotype. Luciferase results for the entire RHS5(I) site demonstrate a significant enhancing effect for the construct containing both minor alleles (MiMi) compared to the major allele (MaMa) construct whereas for the short RHS5(I) site both minor alleles (MiMi) caused a significant repressor effect. When investigating the effect of each SNP, it became clear that with the single minor allele of rs3798135 the repressor effect is nearly as pronounced as with both minor alleles being present. This might indicate that rs3798135 features a stronger regulatory potential than rs3798134. In HeLa cells, no clear regulatory effect was observed for both the long and the short fragment of the RHS5(I) region. It is well established that the LCR contains several regulatory sites, some of them being enhancers, insulators, or repressors, which orchestrate LCR function and physical interaction with the *Il4* and *Il13* promoters in the mouse (Fields et al., 2004; Lee et al., 2005; Spilianakis and Flavell, 2004; Spilianakis et al., 2005). The genotype-specifically regulated human RHS5(I) sequence investigated here might control other RHS regions of the LCR. It might be assumed that sequences surrounding the short core fragment of the RHS5(I) add cell-type specific enhancer potential to the entire site by genomic overlay. This effect becomes even more pronounced when the risk allele is present which might in turn increase IL4 and IL13 expression levels and therefore atopy. This assumption is in conformity with the KORA F4 expression results, which indicate a genotype specific elevation of IL4 expression in case of the risk alleles of both rs3798134 and rs3798135. However, validation experiments are required as discussed in chapter 3.1.

Genotype-specific characterization of the RHS6(II) revealed a cell-type specific enhancing regulatory potential on a minimal promoter with the minor risk allele of rs2040704 being present in Jurkat cells. In HeLa cells constructs carrying the minor allele acted as a weak repressor. The existence of the

atopy-associated minor allele of the SNP (Genotype frequency (HapMap phase 3 –CEU): 0.627 for the AA genotype, 0.339 for the AG genotype, and 0.034 for the GG genotype) in the human RHS6(II) might therefore contribute its enhancing effect to the entire LCR by interaction with other RHS. Thus, elevation of human IL4 and IL13 expression might occur as observed in the mouse (Fields et al., 2004; Lee et al., 2005; Spilianakis and Flavell, 2004; Spilianakis et al., 2005). This is supported by results of the KORA F4 cohort, where genotype specific IL4 expression levels were observed. The genotype specific enhancing effect detected in the luciferase assay has to be considered with high precaution due to a newly identified rare variant within the RHS6(II) at position GRCh37/hg19: chr5: 131,973,154, which might also contribute to the observed effect. The rare variant was detected within 100 KORA F4 samples but surprisingly, the SNP is not annotated in the 1000 Genomes Project (released 13-December 2012) where 1092 sample were genotyped. It can be speculated that the detected rare variant might be also found in the 1000 Genomes Project when more data is publicly available. A possible explanation for the missing annotation might be that this rare variant is mainly found in Europeans but not in other populations. The 1000 Genomes Project, however, includes subjects of several populations. As difficulties with the cloning of the RHS6(II) fragment containing the major allele of rs2040704 into the luciferase vector encountered, it is necessary to remove the newly identified rare variant within the RHS6(II) to get a clear picture about the functional potential of the SNP. Other cloning strategies will have to be considered to avoid bacteria mortality when carrying the construct without the rare variant. It can be assumed that the presence of the minor allele of rs2040704 helps to overcome mortality because the bacteria were only able to grow when they carried the vector containing the point mutation.

In this study, it was shown that the differential binding of transcription factors at certain RHS sites has a regulatory effect on a minimal promoter. Further studies to identify genotype specific binding of transcription factors like DNA affinity purification followed by mass spectrometry and supershift assays will help to clarify mechanisms due to the investigated SNPs. Mouse models can help to understand regulatory effects at the Th2 cytokine locus but luciferase assays testing the SNP effects within the RHS on *IL4* and *IL13* promoters as well as additional ChIP experiments will be necessary to clarify the mechanisms occurring at the human Th2 locus.

It remains open how the RHS sites and their polymorphisms converge with epigenetic mechanisms to effect changes in cytokine transcription. The analysis of polymorphisms influencing epigenetic marks is in the fledgling stage. It has been observed that CpG disruption by a polymorphism causes a change in DNA-methylation by a spread to adjacent sequences and thereby shaping promoter activity (Kerkel et al., 2008; Moser et al., 2009; Wang et al., 2008). Changes in methylation due to the disruption of a CpG by SNPs were also shown for several human cell lines (Shoemaker et al., 2010).

The here investigated SNPs rs3798134 and rs3798135 are not located in the CpG context. As studies also report DNA methylation at cytosine residues outside of CpGs (Laurent et al., 2010; Lister et al., 2009), even rs3798134 might be important in the context of epigenetic mechanisms. SNP rs3798134 loses its cytosine residue when the risk allele is present (TABLE 5). When the risk allele of rs2040704 is existent, a new CpG context is created (TABLE 5), which might alter epigenetic patterns as mentioned in the listed studies and therefore alter interleukin expression. Cross-action between polymorphisms and epigenetic patterns has also been observed regarding histone modifications. A cancer associated intronic SNP 335 kb away from the c-MYC gene genotype-specifically regulates the enhancement of gene expression by promoting the building of a loop by enhancer-like histone marks (Wright et al., 2010). Variants within epigenetic regulatory genes which are involved in writing, erasing, and reading histone modifications are correlated with cancer in several cell lines (Liu et al., 2012). Mechanisms for interactions between polymorphisms and epigenetics regarding atopy are still uninvestigated.

TABLE 5. Sequence information of investigated SNPs in RHS5(I) and RHS6(II). [Common major allele→ minor risk allele].

SNP	DNA sequence (5' → 3')
rs3798134	GTAAACAGCCTGGCTTTGTTCCTTAA[C→T]AAGCCTAAATTGCTAGAAAGCACTC
rs3798135	CCAGGCTCCACCAAGCTCCCTCATAG[G→A]TCCTCATTCTGCTCAGCATGCCTCT
rs2040704	GTGTAGATAGGGATAAGCCAAAATGC[A→G]ATAAGAAAAACCATCCAGAGGAAAC

Appropriate methods to analyze the SNP effect in interconnection with DNA accessibility will be of relevance in the future. Allele specific expression analysis allows determining the impact of genetic variants on gene expression but does not allow insights into epigenetic mechanisms (Ge et al., 2009). A method that would support SNP dependent and epigenetic analysis at the same time would be target-specific methylation dependent luciferase assays, which are currently not performable. As luciferase vectors are amplified within bacteria, the mammalian methylation code is changed and can therefore not be tested in luciferase assays yet. Indeed, target-unspecific plasmid methylation after amplification can be achieved by vector-treatment with the methylase SssI (DiNardo et al., 2001). This approach might still be used when investigating other questions.

3.3 MLPA – AN APPROPRIATE METHOD FOR THE DETECTION OF DNASE1 HYPERSENSITIVE SITES?

The here investigated Th2 cytokine locus is known to underlie epigenetic regulation and thereby mechanisms influencing DNA accessibility for transcription factors. In the mouse model, complete DNA demethylation at the RHS7 and other RHS sites (Fields et al., 2004; Kim et al., 2007) is accompanied by histone H3 hyperacetylation in Th2 but not in Th1 cells (Fields et al., 2004). In humans, changes in DNA methylation coincides with the formation of DNase1 hypersensitive sites in Th2 cells (Santangelo et al., 2002). As atopy-associated SNPs in *RAD50* introns (Li et al., 2010; Moffatt et al., 2010; Paternoster et al., 2012; Weidinger et al., 2008c) also support the regulatory importance for this region, it is hypothesized that the number of regulatory DNase1 hypersensitive sites or their sizes differs within *RAD50* introns in Th2 cells from atopic eczema cases compared to healthy controls and might as well be regulated by functional DNA variants. This might explain atopy development by elevated IL4 and IL13 expression levels. Therefore, it was aimed to establish a robust system for the detection of DNase1 hypersensitive sites in Th2 cells. The MLPA method was successfully established in our laboratory. Reliable results were produced for the detection of DNase1 hypersensitive sites for HeLa and Jurkat cells. However, results with Th1 and Th2 populations were not consistent with published data. The MLPA method was chosen for the detection of DNase1 hypersensitive sites because it features high sensitivity together with a small amount of cells needed (10^5 cells) compared to other techniques like gel based detection of DNase1 hypersensitive sites (10^8 cells (Sambrook, 2001)) and microarrays ($3x10^7$ cells (Dorschner et al., 2004)). This consideration was essential as CD4+ cells represent a minor fraction in the whole blood of humans and still had to become differentiated towards Th2 cells. To earn high amounts of CD4+ cells, it is necessary to withdraw high volumes of blood which represents a hazard to potential donors. Also, purification of CD4+ cells and differentiation towards Th2 cells is very time and money consuming. The MLPA method is based on the determination of amplified DNA copies after DNA digest with DNase1. When performing the MLPA assay in HeLa cells more PCR-copies were observed after DNase1 digestion with 2 U DNase1 compared to the non-digested DNA for the non-sensitive control probes N1 and N5. This phenomena can also be seen in the published data, but to a lesser extend (Ohnesorg et al., 2009). It is assumed that by the DNA digest with DNase1, smaller fragments were created that might facilitate amplification by resolving secondary structures. Strong secondary structures, as for example induced by GC-rich sequences, tend to resists DNA double strand denaturation even at high temperatures, preventing annealing of probes and therefore exacerbating amplification steps (Veal

et al., 2012). Digestion with restriction enzymes prior to amplifications improve the amplification outcome (Veal et al., 2012). The MLPA system did not prove to be a robust method for the detection of DNase1 hypersensitive sites in human differentiated Th1 and Th2 cells. Within the ENCODE project, DNase1 hypersensitive sites were determined at high resolution and genome scale by using DNase1 microarrays, whereas the MLPA system applied here is a PCR-based technique for the detection of DNase1 hypersensitive sites and allows multiplexing of up to 50 loci at the same time only. While the ENCODE reference data (Sabo et al., 2004; Sabo et al., 2006) supported the results obtained for the MLPA system in HeLa and Jurkat cells, they were contrary to those in differentiated T cells. However, it was found that differentiation can be only achieved to an extent of about 30 % of the desired T cell subtype (personal communications Stefanie Eyerich, Zentrum Allergie & Umwelt). The remaining ~70 % cells cover different types of T cells. It is assumed that due to limited purity in Th1 and Th2 cell populations the MLPA results were not identical to the ENCODE results. It was decided not to continue with the analysis of DNase1 hypersensitive sites in Th2 cells from atopic eczema cases because the results were assumed not to be trustworthy.

As the purity of the Th2 cell population was the major difficulty, it will be inevitable to optimize T cell differentiation protocols to earn higher amounts of the desired T cell population. An elegant method to earn pure cell populations would be FACS-sorting of whole blood. Technically, however, this is not possible due to the lack of surface markers for the separation of Th1 from Th2 cells. Although Th1 cells express the chemokine receptor CXCR3, selection on the basis of this surface protein leads to a Th1 cell population that is enriched with other T cell subtypes (Xie et al., 2003). Th2 cells exclusively express the receptor CRTH2, but only a small subpopulation of Th2 cells expresses this surface protein. Therefore, no clean and efficient dissection of Th1 and Th2 cells can be performed at this time (Cosmi et al., 2000; Otten et al., 2003). More research is necessary to identify methods for an enrichment of specific T cell population in order to use them for functional studies.

3.4 OUTLOOK

Atopic eczema and other atopic diseases are characterized by a dysregulation of the immune response with increased Th2 cytokine expression and elevated IgE serum levels. In mice, the regulatory mechanisms, including a complex epigenetic regulation, of an intronic LCR within *RAD50* are well known. Genome wide association approaches identified numerous atopy-associated variants within human *RAD50* introns but so far, potential functional mechanisms altered by these polymorphisms are unknown.

In this study, it was shown that human conserved RHS sites within the LCR exist, which exhibit an atopy associated variant specific regulatory potential on minimal promoters and differential transcription factor binding. The risk allele of rs2240032 has an eased SMAD3- and SP1 binding capability and causes a higher activity of a minimal promoter. These mechanisms might contribute to atopy susceptibility for carriers of the risk allele. Another attempt of this thesis was the establishment of a robust system for the detection of epigenetic sites, such as DNase1 hypersensitive sites. DNase1 hypersensitive sites were detected in HeLa and Jurkat cells. Due to technical limitations, the question about atopic eczema specific differences in epigenetic patterns at the Th2 cytokine locus remains to be solved in future projects. The here presented work provides a starting point for future studies to decode genetic and epigenetic mechanisms at the Th2 cytokine locus, which contribute to atopic diseases. Future studies should focus on further functional characterization of the LCR and its regulatory potential on the *IL4*, *IL5,* and *IL13* promoter activity in regard to associated polymorphisms. As epigenetic mechanisms at the Th2 cytokine locus were observed in mice and humans, epigenetic control of interleukin expression should be addressed and characterized. An exciting question to be solved remains the interplay between functional variants and epigenetic mechanisms acting at the Th2 LCR. This might allow an understanding of the elaborate genetic and epigenetic mechanisms contributing to the complex atopic phenotype and might also identify the Th2 cytokine locus as central regulatory region for other common diseases and thus enable pharmacological approaches. The identification of new atopic disease markers might allow early diagnosis to enable fast treatment and could thereby contribute to a sensible management of health care resources.

The field of high throughput methods for the characterization of the human genome is evolving rapidly, generating tremendous amounts of data, e.g. by RNA-Seq, 450 K DNA-methylation analyses, and exome sequencing. New research areas such as integromics (integration of numerous types of data from different experimental platforms) and interactomics (study of complex interactions between biological molecules) arise to link different high throughput fields to merge in a broader view on the molecular basis of common diseases. Integrating genomics and epigenomics data to get from SNP association to function is increasingly being recognized as future quest. One of the so called post-genome era projects, the ENCODE project, addresses this issue. Although the benefit of the ENCODE results is controversially discussed, incorporation of ENCODE data is valuable for the regeneration of functional questions. Data integration of different research fields is already beginning to improve the functional understanding and will most probably entail exciting new mechanistic insights into the development and onset of the disease and enable treatment of atopic eczema and other atopic diseases.

4 METHODS

4.1 WORKING WITH ESCHERICHIA COLI

4.1.1. Culture Media

LB medium (Bertani 1951):	10 g casein hydrolysate
	5 g yeast extract
	4 g NaCl
	H_2O_{bidest} add 1 l, pH 7.4
for solid agar plates add:	1,5 % (w/v) agar-agar

After preparation, media have been autoclaved for 20 min at 121 °C. Ampicillin (50 µg/ml) was added as selection antibiotic after autoclaving/heating (c < 45°C).

4.1.2. Growing Bacteria

For inoculation of liquid media (from colonies on solid agar plates or glycerol stocks) sterile pipet tips were used. Cultures were grown at 37 °C overnight. Additionally, liquid cultures were kept under vigorous shaking (200 rpm).

4.1.3. Short- and Long-Term Storage

For short-term-storage, agar-plates were sealed with Parafilm M® and kept for several months at 4°C. For long-term storage, glycerol stocks were prepared by adding 80 % sterile glycerol 1:1 (v/v) to overnight grown liquid culture and kept at -80 °C in Cryo Tubes™.

4.1.4. Production of Chemocompetent *E.coli*

2.5 ml overnight culture were grown in 250 ml LB-medium, supplemented with 20 mM $MgSO_4$ and incubated under vigorous shaking at 37 °C to an OD_{600} of 0.6-0.8. Bacteria were centrifuged at 4000 x g for 15 min at 4 °C. The pellet was resuspended in 100 ml ice-cold buffer TFB1 pH 5.8 (30 mM KAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15 % Glycerol) and incubated for 5 min on ice. Afterwards the cells were pelleted by centrifugation again. After addition of TFB2 buffer pH 6.5 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15 % Glycerol) and incubation for 30 min on ice, 100 µl aliquots were prepared and stored at -80 °C.

4.1.5. Transformation of Chemocompetent *E.coli* by Heat Shock and PCR-Based Colony Screen

Chemocompetent *E.coli* bacteria were thawed on ice. 200 ng of plasmid DNA e.g. from the ligation reactions were added and incubated for 20 min on ice. Cells were heat-shocked at 42°C for 45 sec and incubated on ice for another two minutes. 150 µl LB-medium without any antibiotics were added and bacteria were kept under vigorous shaking for one hour at 37 °C. 50 µl were plated on LB agar plates supplemented with ampicillin or other appropriate antibiotics and incubated overnight at 37 °C. Several clones were picked and tested via PCR with vector primers for the existence of the transformed plasmid and the insertion of fragments with the right size into the vector. If the clones carried the insert, sequencing reactions followed.

4.2 WORKING WITH EUCARYOTIC CELL LINES

4.2.1. Cultivation

HeLa

HeLa is an adhesive human cervix carcinoma cell line. HeLa cells were cultured in modified eagle medium (MEM) supplemented with 10 % FBS, 100 U/ml penicillin, and 100 µg/µl streptomycin at 37 °C and 5 % CO_2 in a humidified atmosphere.

Jurkat

Jurkat is an immortalized suspension cell line of T lymphocytes. Jurkat cells were grown in RPMI-1640 medium supplemented with 10 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/µl streptomycin at 37°C and 5 % CO_2 in a humidified atmosphere.

4.2.2. Maintenance of Cell Culture

Frozen eukaryotic cell aliquots were thawed quickly in a water bath and diluted in 10 ml fresh medium. After centrifugation for 2 min at 1000 x g, the cell pellet was resuspended in 6 ml of medium and incubated overnight in a T25 cell culture flask. On the following day, cells were transferred to a T75 culture flask.

Both HeLa and Jurkat cells were split three times a week. Before splitting, the adherent HeLa cells were washed with phosphate buffered saline solution (PBS) (10 mM sodium-phosphate buffer (pH 7.4), 150 mM NaCl) and trypsinized with 1 ml trypsin. After a short incubation at 37 °C, trypsinization was stopped by addition of fresh medium, cells were suspended in fresh medium, and a part was transferred to a new flask (Splitting). Splitting ratio was typically 1:3 - 1:4. For the splitting of suspended Jurkat cells, 20 ml medium was removed. The remaining medium containing the cells was split into two halves, transferred to two T75 culture flasks, and filled up with fresh medium to reach a final volume of 30 ml.

To freeze cells for storage, 7 x 10^6 cells were suspended in appropriate medium supplemented with 20 % FBS and 10 % DMSO in Cryo Tube™ vials and cooled down to -80 °C in Mr. Frosty Freezing Container. For long-term storage, aliquots were kept in liquid nitrogen tanks.

4.2.3. Transfection of Eukaryotic Cell Lines

For reporter gene assays, cells were lipo-transfected to deliver plasmids into the cells. Based on the manufacturer's protocol, transfection procedure was optimized for both cell lines. HeLa cells were transfected using the FuGene6 DNA transfection reagent. 500 ng of test vector and 50 ng of control vector together with 1.5 µl transfection reagent were incubated in 50 µl MEM without supplements for 20 min at room temperature. After the incubation step, the reaction mixture was added to the cells. Jurkat cells were transfected using the Mirus TransIT® Jurkat transfection reagent. 2000 ng test vector and 50 ng control vector together with 3 µl transfection reagent were incubated in 100 µl

MEM-medium without supplements for 20 min at room temperature. The reaction mixture was then added to the cells. Cells were grown for 30 h at 37 °C and 5 % CO_2 in a humidified atmosphere.

4.3 WORKING WITH PRIMARY CELLS

4.3.1. Blood Collection

150-300 ml blood was withdrawn from donors at the „Klinik und Poliklinik für Dermatologie und Allergologie" and mixed with 20 µl EDTA according to clinics standard procedures.

4.3.2. Isolation of CD4+ Cells

For isolation of naïve T cells from EDTA-blood, it was mixed with PBS (1:2). 25 ml of the mixture were given on to 15 ml Lymphoprep™ in a 50 ml Falcon tube. Centrifugation, with the brake being out of action, was performed at 2200 rpm (Heraeus, Megafuge 1.0R) for 15 min at 15 °C. Peripheral blood mononuclear cells (PBMCs) were visible as a white layer, which was recovered and mixed with 40 ml 5 mM EDTA-PBS. After centrifugation at 1600 rpm (Heraeus, Megafuge 1.0R) for 10 min, the cells were washed with 40 ml PBS and centrifugation was repeated. The cells were counted to prepare the appropriate aliquots for the Naïve $CD4^+$ T cell Isolation Kit II. CD4+ cells were sorted according to the manufacturer's protocol using the AutoMACS® Pro Seperator.

4.3.3. Cultivation

Isolated naïve CD4+ cells were cultured in AIM V® medium supplemented with differentiation specific interleukins and antibodies at a density of 10^6 cells/ml in 24-well plates. After stimulation, the cells were split every three days to a densitiy of 10^6 cells/ml in 24-well plates. All procedures have been performed according to the standard methods of the "Zentrum Allergie und Umwelt" (ZAUM, Technical University München).

4.4 DNA-BASED MOLECULAR METHODS

4.4.1. Plasmid-Production

DNA inserts for luciferase-vectors were obtained by PCR amplification of fragments from human genomic DNA or bacterial *RAD50* clones. The PCR products were cloned via two restriction sites into the pGL4.23-vector which contains a minimal promoter. All constructs were verified by sequencing.

4.4.2. Isolation and Purification Procedures

Mini Preparation for Small Amounts of plasmid DNA

Isolation of plasmid DNA from *E.coli* was carried out by using 5 ml over-night culture with the NucleoSpin-System Kit according to the manufacturer's protocol. Elution was performed with 50 µl ml H_2O_{bidest}.

Midi Preparation for Medium Amounts of DNA

Isolation of plasmid DNA from *E.coli* was carried out by using 50 ml over-night culture with the NucleoBond® Xtra Midi/Maxi Kit according to manufacturer's protocol. The DNA was dissolved in 100-200 µl ml H_2O_{bidest}.

Purification of PCR- or Restriction Products

Purification of PCR- or restriction-products was performed with the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions. Elution was performed with 50 µl H_2O_{bidest}.

4.4.3. Measurement of DNA Concentrations and Purity

DNA concentrations were measured with a Nanodrop. For good DNA purity, the samples had to feature an A_{260}/A_{280} ratio of 1.7-2.0 (values ensure exclusion of protein contamination) and an A_{260}/A_{230} ratio of more than 1.5 (values ensure elimination of salts and phenol).

4.4.4. Cloning Strategies

PCR-amplified DNA-fragments were inserted via two restriction sites into the vector pGL4.23 which provides a minimal promoter. Digestion of insert and vector with Acc651 and Xho1 resulted in complementary DNA ends ready for site-directed ligation. 1 µg of DNA was digested in 20-30 µl reaction volume together with the appropriate amounts of buffer 3, BSA, and 10-20 U enzymes at 37 °C overnight. Heat inactivation was performed for 20 min at 65 °C followed by clean up with the Wizard SV Gel and PCR Clean-Up System, if required. For the ligation, insert and vector were mixed at a ratio of 5:1 and incubated with 1 x ligase buffer and T4-DNA-Ligase for 3 h at room temperature. Ligated products were then used for transformation in chemocompetent cells.

4.4.5. Dual Luciferase Assay

Jurkat cells were seeded at a density of 4 x 10^5 cells/well in a 12-well plate in RPMI medium supplemented with L-glutamine, 10 % FBS, and 1 % penicillin/streptomycin and were incubated overnight. HeLa cells were seeded at a density of 1 × 10^5 cells/well in 12-well plates in MEM medium with stable L-glutamine, containing 10 % FBS and 1 % penicillin/streptomycin and incubated overnight. Jurkat cells were transfected with 2000 ng of plasmid per well using Mirus Jurkat TransIT®. HeLa cells were transfected with 500 ng of plasmid per well using FuGene6. For normalization 50 ng of the pGL4.74 vector, which constitutively expresses renilla luciferase, was co-transfected. Transfected cells were incubated for 30 h at 37 °C in a 5 % CO_2 atmosphere. Cells were washed in PBS buffer once before lysis in 200 µl of 1× passive lysis buffer. After shaking for 30 min at room temperature, the lysates were frozen at −80 °C overnight. Luciferase activity was measured in 20 µl samples with the GloMax®-Multi Detection System by using 50 µl reagents from the Dual Luciferase Reporter Assay System according to the manufacturer's instructions. Experiments were performed at least three times in analytical triplicates. Calculation of the intensity ratios of firefly-renilla luciferase activity resulted in the relative promoter activity of the constructs. Normalization on the major allele was applied for characterization of allele specific effects, whereas normalization on the empty control vector was performed to display the overall effect of the tested sequence on the minimal promoter. Kolmogorov-Smirnov-tests showed no significant deviation from normal distribution. To identify statistically significant differences in promoter activity between the constructs, linear mixed-effects models (LME) with random intercept were used (R-software). LME models are the

appropriate statistical method because they consider the correlation among the technical replicates, which is not assured when using the Student's t-test or comparable statistical models.

4.5 PCR-BASED METHODS

4.5.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify specific DNA-fragments. PCR reaction in a 50 µl volume contained 200 nM dNTP-mix, 0.5 µM each of the forward and reverse primer, and 1 U of Pfu Turbo DNA polymerase in supplied 1 x PCR buffer. Templates included human genomic DNA, plasmid DNA, primary PCR, or bacterial culture. For PCR reactions with bacterial cultures, the Taq-polymerase together with the PCR-buffer (100 mM Tris-HCl (pH9), 500 mM KCl, 15 mM $MgCl_2$), both produced in-house, were used. Cycler-conditions started with an initial denaturing step of 5 min at 95 °C followed by 35 cycles with 30 sec at 95 °C, 35 sec with the appropriate annealing temperature according to Metabion calculator, and 1 min for each amplified kb at 72 °C, followed by one final cycle with 10 min at 72 °C and hold on 6 °C. For difficult to amplify fragments, the Phusion Polymerase was used according to manufacturer's protocol. The PCR-product was analyzed by electrophoresis on a 1.5 % agarose-gel (89 mM Tris, 89 mM Boric Acid, 2 mM EDTA (pH8.0)) together with a DNA marker for the appropriate fragment length.

4.5.2. *Real-Time* PCR (RT-PCR)

To verify successful T cell-differentiation, *real-time* PCR was performed. RNA was isolated from frozen cell pellets with the RNeasy® Mini Kit according to the manufacturer's instructions. Reverse transcription was performed with the iScript™ cDNA synthesis kit. Primers were designed by the Primer Express software (Applied Biosystems). For pipetting in 384-well plates, the robot EP Motion 5075 was used. The gained cDNA was amplified with the SYBR Green Mastermix and the ViiA7 Real-Time PCR System. Relative expression for IL4, IL5, IL13, IL17, IL22, and IFNγ was conducted by subtraction of the measured CT of the housekeeper gene EF1A from the measured CT-value of the probes of interest (ΔCT). ΔΔCT was determined by subtracting the ΔCT-value of the stimulated probe

from the ΔCT-value of the unstimulated probe. The relative expression corresponds to $2^{\Delta\Delta CT}$ and explains the relative upregulation of the cytokine of interest.

4.5.3. QuikChange® Site-Directed Mutagenesis

For site-directed insertion of point-mutations into plasmid DNA, the QuikChange® kit was used according to manufacturer's instructions.

4.5.4. Simple PCR Mutagenesis

Single point mutations that were located closely to the fragment-ends were inserted during the cloning process. Primers were designed to contain the desired point-mutation. PCR reactions were carried out as described above (chapter 4.5.1).

4.5.5. Fusion PCR

Fusion-PCR was used in order to gain an RHS7 fragment that was missing a central 150 bp region. Therefore, primers were designed to bridge the 150 bp which were supposed to be missing in the final product. Two separate PCR reactions were carried out, one using the general forward primer together with the reverse bridge primer, the other using the forward bridge primer together with the general reverse primer. The gained products were analyzed on a 1.5 % agarose-gel and were purified using the Wizard® SV PCR Cleanup Kit. To receive the final product, a second PCR reaction was carried out using the general PCR primers and the two gained products of the first PCR as templates (FIGURE 36). All PCR reactions were performed as described above (chapter 4.5.1).

4.5.6. DNA Sequencing

Identification and verification of DNA sequences was carried out by Sanger sequencing with the ABI PRISM 3730 DNA Analyzer. For the sequencing reaction, the BigDye Terminator v3.1 Cycle

Sequencing Kit was used. The probes were cleaned by the Montage™ Seq_{96} Sequencing Reaction Cleanup Kit. Both kits were used according to the manufacturer's instructions.

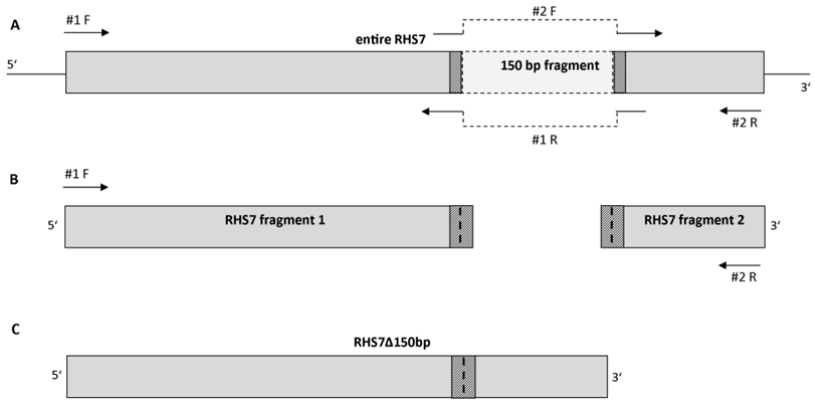

FIGURE 36. Fusion PCR produces a RHS7 fragment without the short 150 bp sub-region. (A) Two primer pairs were used in separate PCR reactions, each pair containing a bridge-primer, which excludes the 150 bp fragment and contained an overlapping region (dark grey). **(B)** Two RHS7 fragments (1 and 2) were obtained which contained the overlapping region, and introduced in a second PCR reaction containing the normal RHS7 forward and reverse primer. **(C)** The product RHS7Δ150bp features a deleted 150 bp region. #1 = primer pair 1, #2 = primer pair 2, F = forward, R = reverse.

4.5.7. Multiplex Ligation Dependent Probe Amplification (MLPA)

Before starting with the Multiplex Ligation dependent Probe Amplification (MLPA), nuclei had to be derived from HeLa, Jurkat, and CD4+ cells and treated with DNase1 for the detection of hypersensitive sites in the genome. For the isolation of nuclei from HeLa cells, nuclei from 2 x 10^6 cells were isolated with the NE-PER Nuclear and Cytoplasmic Extraction Kit according to manufacturer's instructions. After the isolation, nuclei were kept intact on ice and not further lysed. For Jurkat- and CD4+ nuclei isolations, 3 x 10^6 cells were isolated with the ProteoJet™ Kit according to manufacturer's instructions. Nuclei were washed in 500 µl and incubated in 75 µl 1 x DNase1 Reaction Buffer. The reaction was split into tree tubes and was incubated with different amounts of DNase1 (0 U, 0.5 U, 2 U) for 30 min at constant 23 °C. The reaction was stopped by incubation for 10 min at 65 °C. DNA was purified using the Dneasy® Blood & Tissue Kit according to the manufacturer's protocol. To control the quality of the DNase1 digest, 100 ng of each digest was run on a 1.5 % agarose-gel at 100 V for 90 min. A probe-mix containing all self-designed primers for the chosen DNase1 sites of interest or as controls to regions with no DNase1 hypersensitivity at concentrations of 4 nM (multiplexing of a maximum of 50 sites at one run) was prepared. All probes were combined

in one single mix and could be distinguished by their final product length. 50 ng of each DNA sample in a volume of 5 µl was denatured for 5 min at 98 °C. For the following procedure, the SALSA® MLPA® Reagent-Kit was used. 1.5 µl probe-mix and 1.5 µl SALSA MLPA-buffer were added to the DNA sample, heated for 1 min at 95 °C, and kept for 16 h at 60 °C. On the next day, the ligation mix was prepared by mixing 3 µl Ligase-65 Buffer A, 3 µl Ligase-65 Buffer B, 0.2 µl dNTPs, 0.1 µl Stoffel fragment, 1 µl Ligase-65, and 25 ml H_2O_{bidest}. 32 µl Ligation-Mix were given to the DNA-probe mix and the temperature was lowered to 54 °C for 20 min. The inactivation was performed by heating for 5 min at 98 °C. A PCR reaction followed containing 2 µl SALSA-PCR Buffer, 1 µl SALSA ENZYME Buffer, 1 ml SALSA-PCR-Primers, 0.25 µl SALSA Polymerase, 15.75 µl H_2O_{bidest}, and 5 µl ligation mix. PCR-cycler conditions were as follows: 1 min 95 °C; 35 cycles with 30 sec at 95 °C; 30 sec at 58 °C, and 60 sec at 72 °C; 20 min at 72 °C. Afterwards, the fragments were analyzed by the ABI PRISM 3730 DNA Analyzer. The principle of the MLPA method is shown in FIGURE 37.

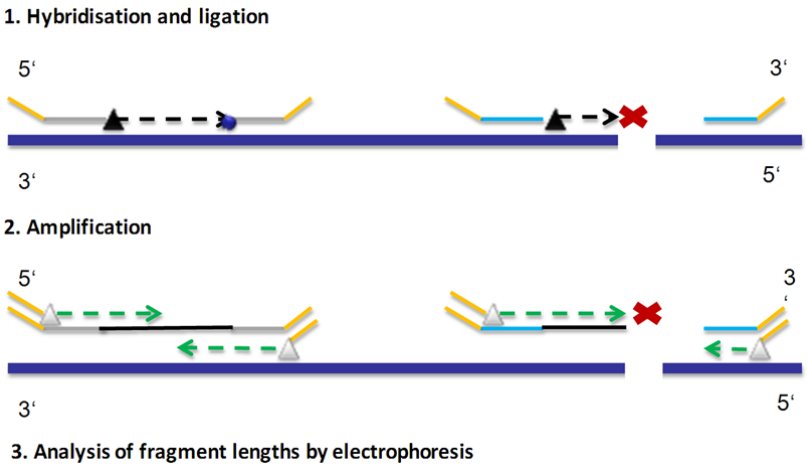

FIGURE 37. Principle of MLPA. (1) Site-specific probes are hybridized; a polymerase elongates according to the genomic DNA followed by ligation when the DNA is intact. **(2)** Amplification occurs when the DNA is intact. **(3)** Obtained fragment lengths are analyzed by electrophoresis.

4.6 PROTEIN CHEMISTRY

4.6.1. Preparation of Nuclear Protein Extracts

Jurkat cells were stimulated for 6 hours with 25 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin. Nuclear extracts were prepared from stimulated Jurkat cells using the Nuclear

Extraction Kit and from HeLa cells using the NE-PER Kit® Nuclear and Cytoplasmic Extraction reagents. Protein concentration was measured by the BCA Protein Assay Reagent.

4.6.2. Electrophoretic Mobility Shift Assay (EMSA)

Cy5-labeled and unlabeled oligonucleotides containing the major or minor allele of SNPs of interest or the consensus sequences for SP1, OCT1, or SMAD3/4 were annealed and purified by 12 % polyacrylamide gel electrophoresis to obtain double-stranded DNA probes. Protein binding reaction was carried out with or without different concentrations of unlabeled competitor oligonucleotides using 5 µg of nuclear extract in 1x binding buffer (4 % v/v Glycerol, 1mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM TrisHCl pH7.5), 0.5 µg poly dI-dC, and 1 ng of labeled probe in a total volume of 10 µl for 20 min at 4°C. Protein-DNA complexes were separated on a 5.3 % polyacrylamide gel by electrophoresis in 0.5 x tris-borate-EDTA (TBE) buffer. Band patterns were visualized by scanning the gel with the Thyphoon Trio + (λ=633 nm). Experiments were performed at least twice. EMSA-supershifts were carried out as described above with the following adjustment: For SP1-supershifts, 0.1µg of SP1 antibody was added to the reaction mixture following the standard binding reaction and incubated for another 45 min at 4 °C. For SMAD3-supershifts, nuclear extract and 0.75µg SMAD2/3 antibody were incubated first for 30 min at room temperature before the binding reaction was carried out. To assure specificity of antibody binding, isotype antibodies were used as controls. Experiments were performed at least twice.

4.6.3. DNA Affinity Purification

For DNA affinity purification of differential binding proteins at SNP rs2240032, 200 µl Dynabeads® M-280 Streptavidin were prepared according to manufacturer instructions and incubated over night at 4°C with the same oligonucleotides that were used for the EMSAs before, but biotin- instead of Cy5-labeled. After washing with Bind and Wash-Buffer (5 mM Tris-HCl, 0.5 mM EDTA, 1 M NaCl), the beads were incubated for 1 h with 2 ng/µl biotin and resuspended and incubated in 1 x EMSA-Buffer without salt (4 % v/v Glycerol, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM TrisHCl pH7.5) for 20 min together with 2100 µg Jurkat nuclear extract, 2.5 % Chaps, and poly dI-dC. After washing three times with Wash-Buffer (1x EMSA binding buffer without salt, 10 mM NaCl), elution steps were performed with increasing amounts of NaCl (50 mM-600 mM). EMSAs were performed with each

eluate to identify the eluate with purified binding proteins that can be isolated for analysis in mass spectrometry. The experiment was repeated four times.

4.6.4. Mass Spectrometry

Mass spectrometry was carried out by Stefanie Hauck (Research Unit Protein Science, Helmholtz Zentrum München). Samples were prepared according to the FASP approach (Wisniewski et al., 2009) using Microcon devices YM-30 and foregoing the salt elution step. LC-MS/MS analysis was performed on a LTQ-Orbitrap XL as described previously (Hauck et al., 2010) with the following adjustments: a nano trap column was used (300 µm inner diameter × 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100 Å; LC Packings), before separation by reversed phase chromatography (PepMap,25 cm,75 µm ID, 2µm/100 Å pore size, LC Packings) operated on a RSLC (Ultimate 3000, Dionex) with a nonlinear 300 min gradient using 2 % acetonitrile in 0.1 % formic acid in water (A) and 0.1 % formic acid in 75 % acetonitrile (B) at a flow rate of 300 nl/min. The gradient settings were: 5–270 min: 5–50 % B, 270-275 min: 50–95 % B, 280-285 min: 95 % B, followed by equilibration for 15 min to starting conditions. From the MS pre-scan, the 10 most abundant peptide ions were selected for fragmentation and the dynamic exclusion was set to 60 seconds.

4.6.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For the separation of denaturized proteins according to their electrophoretic mobility, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was applied. The electrophoretic mobility of proteins is a function of protein charge and length. 10 µl of protein extract was mixed with 10 µl of 5 x Laemmli buffer, heated for 5 min at 95 °C, and separated on a SDS-PAGE. The gel consists of a stacking gel where the proteins become concentrated and a resolving gel where the proteins become separated. Gels were run for 100 min at 80 V.

Stacking Gel (10 %):	1.65 ml 30 % acrylamide
	1.5 ml gel buffer
	1.25 ml 50 % glycerol
	500 µl H_2O

	10 µl TEMED
	25 µl 10 % APS
Resolving Gel (4 %):	0.335 ml 30 % acrylamide
	0.335 ml gel buffer
	1.835 ml H_2O
	5 µl TEMED
	20 µl 10 % APS
10 x Running Buffer:	30 g Tris
	144 g Glycin
	1 % SDS
	with H_2O up to 1 l
Gel Buffer (Schagger and von Jagow, 1987):	3 M Tris/HCl pH8.45
	0.3 % (W/V) SDS
Stacking Gel Buffer (pH 6.8):	0.5 M Tris
	2 g SDS
	with H_2O up to 500 ml
Resolving Gel Buffer (pH 8.8):	1.5 M Tris
	2 g SDS
	with H_2O up to 500 ml
5 x Laemmli Buffer:	50 % glycerol
	0.16 Tris HCl (pH 8.6)

2 % SDS

5 % β-Mercaptoethanol

0.1 % Comassie Brilliant Blue G25

4.6.6. Western Blot

Western Blots were carried out in order to detect specific proteins with polyclonal antibodies within nuclear protein extracts. 20 µg of nuclear proteins extracts were separated on a denaturing polyacrylamide gel (PAGE) (chapter 4.6.5) and transferred to a PVDF-membrane by semi-dry blotting with a blotter at 20 V for 40 min. Before blotting, the gel was equilibrated for 10 min in buffer and the membrane was activated in methanol. After Blotting, the membrane was blocked in ODYSSEY Blocking Buffer for 1 hour at room temperature. Protected from light the membrane was then incubated overnight with primary antibodies for β-ACTIN (1:5000) and SMAD2/3 (1:2000) in ODYSSEY Blocking Buffer at the same time. The membrane was rinsed three times for 5 min in 0.05% PBS-Tween 20 and incubated with the secondary antibodies (1:20000) for 1 h at room temperature. The secondary antibodies were labeled either with IRDye680 (anti-rabbit for the SMAD2/3 antibody) or IRDye800CW (anti-mouse for the β-ACTIN antibody) which allows detection of two different proteins at the same time. The membrane was rinsed three times in 0.05 % PBS-Tween 20 and analyzed in the ODYSSEY® Infrared Imaging System.

4.7 IMMUNOLOGICAL METHODS

4.7.1. Differentiation of Naïve T Cells Towards Th1-/Th2-Conditions

For differentiation of CD4+ cells towards Th1- or Th2-cells, 24-well plates were coated with 1 µg/ml anti-CD3 antibodies in PBS for 2h at 37 °C. After removal of the liquid, 1 ml containing 10^6 cells was added to the wells together with the desired T cell-subunit stimulation mixture.

Th1 stimulation mixture: 20 U IL2

25 ng IL12

5 µg anti-IL4

	1 µg anti-CD28
Th2 stimulation mixture:	20 U IL2
	25 ng IL4
	5 µg anti-IL12
	5 µg anti-IFN-gamma
	1 µg anti-CD28

The differentiation-success was controlled by intracellular staining, ELISA (chapter 4.7.2), and RT-PCR (chapter 4.5.2). Cells were harvested after seven days.

4.7.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a tool to verify the successful T cell-differentiation by analyzing the concentrations of INFγ, IL4, IL5, IL10, IL17, and TNFα in cell free cell culture supernatant. This was performed by using sandwich ELISA kits according to manufacturer's protocols. Plates were analyzed with the ELISA reader.

4.8 POPULATION- AND PATIENT BASED APPROACHES

4.8.1. KORA F4 Cohort

The KORA (Cooperative Health Research in the Region of Augsburg) (Holle et al., 2005) F4 cohort is the follow-up survey of the survey number 4 (S4) (1999 to 2001) which was conducted from 2006-2007 and encompasses 3080 individuals from the KORA S4 study. The study includes interviews, laboratory measurement, and anthropometric measurements (Meisinger et al., 2010; Rathmann et al., 2009). Genotyping- and imputated data (Affymetrix 1000K chip, Illumina 550K chip), methylation-(Illumina 450K chip), metabolite concentration profile (Illumina Metabo-chip), and gene-expression-data (Illumina HumanHT-12 v3 Expression BeadChip) are available for KORA F4 upon request (Institute for Epidemiology II, Helmholtz Zentrum München).

4.8.2. T Cell Clones

T cell clones from patients with atopic eczema, allergic contact dermatitis and psoriasis were produced and the levels of IL4, IL13, and IFNγ were measured using SurePrint G3 Human 8x60K Microarray (Agilent) (mRNA) and ELISA (protein levels) in order to classify the obtained T cell clones (in cooperation with Stefanie & Kilian Eyerich (ZAUM)). Genotyping was performed by sequencing. The correlation between genotype and basal cytokine expression was analyzed in cooperation with Hansjörg Baurecht (Klinikum Schleswig-Holstein, Kiel). For the calculation of the p-value, the Mann-Whitney-U Test was applied.

4.9 BIOINFORMATICS

4.9.1. DNA Sequence Analysis (Vector NTI, ENCODE, UCSC)

The Vector NTI component AlignX was used to analyze similarities between nucleotide sequences. A template sequence containing the desired nucleotide arrangement was compared to a sequenced product e.g. to verify cloning of a nucleotide sequence of interest.

DNase1 hypersensitive sites and sequence similarity between species were analyzed by using the "ENCyclopedia of DNA Elements" (ENCODE) (Feingold, 2004) and UCSC Genome Browser (Miller et al., 2007).

4.9.2. MatInspector and SNPInspector

The prediction of gained or lost transcription factor binding sites (TFBS) at SNP rs2240032 was performed by using the Genomatix SNPInspector and MatInspector software (Cartharius et al., 2005).

4.9.3. GeneMapper (MLPA-Fragment Analysis)

For the evaluation of the obtained fragment sizes with the MLPA-method, the peak data from the ABI PRISM 3730 DNA Analyzer were analyzed by the GeneMapper® Version 3.5 software and exported to Excel for further analysis. Basic data analysis was applied as described earlier (White et al., 2004). As every probe had an allocated fragment size, the amount of each fragment could be determined by peak height. Reactions were normalized to all undigested DNA and to all non-sensitive probes.

4.9.4. Evaluation of Mass Spectrometry Data (Progenesis LC-MS Software, Mascot Software, STRING-Database)

The RAW files (Thermo Xcalibur file format) were further analyzed using the Progenesis LC-MS software (version 4.0, Nonlinear) by the Institute for Human Genetics (Helmholtz Zentrum München) as described previously (Feingold, 2004; Merl et al., 2012). Briefly, for retention time alignment, 5-10 manual landmarks were set, followed by automatic alignment. Features with one charge or ≥ 8 charges were masked and excluded from further analyses. For peptide identification, all features were exported and the Mascot (Matrix Science, version 2.3) was set up to search with one missing cleavage allowed, a fragment ion mass tolerance of 0.6 Da, and a parent ion tolerance of 10 ppm. Carbamidomethylation was set as fixed modification; methionine oxidation and asparagine or glutamine deamidation were allowed as variable modifications. Spectra were searched against the ENSEMBL human database (Release 66; 96556 sequences) and a Mascot-integrated decoy database search using the Percolator algorithm calculated an average peptide false discovery rate of < 1 % when searches were performed with a Percolator score cut-off of 15 and a significance threshold of $p < 0.05$. Peptide assignments were re-imported into Progenesis LC-MS. Normalized abundances of all unique peptides were summed up and allocated to the respective protein. Proteins with less than 2 peptides were excluded for quantification. The hypothetical protein-network was built by using the publicly available STRING-database.

5 MATERIAL AND ORGANISMS

5.1 BACTERIA

E. coli BL21(DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS* *λ DE3 = λ sBamHIo ΔecoRI-B int:lacI::PlacUV5::T7 gene1) i21* *Δnin5*	New England Biolabs
E. coli DH5α	*F-, Φ80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-,mk+), phoA,* *supE44, λ-thi-1, gyrA96, relA1*	Stratagene
E. coli Jm107	*endA1 glnV44 thi-1 relA1 gyrA96 Δ(lac-proAB) [F' traD36 proAB^{+} lacI^{q} lacZΔM15] hsdR17(R_K^- m_K^+) λ$^-$*	Sigma
E. coli K12 Jm109	*F´traD36 proA+B+ lacIq Δ(lacZ)M15/* *Δ(lac-proAB) glnV44 e14- gyrA96 recA1 relA1* *endA1 thi hsdR17*	New England Biolabs
E. coli Top 10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Δ80lacZΔM15, ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*	Stratagene

5.2 GENOMIC CLONE

IMGSB737F092008D	Imagenes

5.3 CELL LINES

Cell line	Company	Accession number
HeLa	DMSZ	Acc57
Jurkat	DMSZ	ACC282

5.4 VECTORS

pGL4.23	Promega
pGL4.74	Promega

5.5 ENZYMES

Acc651	New England Biolabs
DNase1	Promega
Pfu Turbo DNA Polymerase	Agilent Technologies
Phusion Polymerase	New England biolabs
Stoffel Fragmet	Applied Biosystems
T4-DNA-Ligase	New England Biolabs
Trypsine	Sigma
Xho1	New England Biolabs

5.6 ANTIBODIES

Human antibody	Company	Number
CD3 (mouse IgG1)	Beckton Dickinson	555329
CD28 (mouse IgG1)	Beckton Dickinson	555725
Goat IgG	Santa Cruz	Sc-2028X
Smad2/3 (goat IgG)	Santa Cruz	SC-6033
SP1 (goat IgG)	Santa Cruz	SC-59
Anti-rabbit IRDye680 (Goat)	LI-COR	926-3221D
Anti-mouse IRDye800CW	LI-COR	926-32210

5.7 MARKER

Page Ruler #26616	Fermentas
GeneRuler 100 bp DNA Ladder	Fermentas

5.8 CHEMICALS, SUPPLEMENTS, MEDIA

Acetic acid	Merck
Agarose	Biozym
AIM V® medium	Life Technologies
Ampicillin	Sigma
Ammonium peroxodisulfate (APS)	Biozym
Bacto-Agar	BD Biosciences
Biotin	Roth
Boric acid	Alfa Aesar

Bovine serum albumin (BSA) fraction V	Roth
Brefeldin A	eBiosciences
Casein hydolysate	BD Difco
Chaps	Roth
Dimethylsulfoxide (DMSO)	Sigma
dNTPs	Fermentas
Dithiothreitol (DTT)	Fermentas
Dynabeads® M-280 Streptavidin	Invitrogen
EDTA	Merck
Ethanol	Merck
Ethidium bromide	Sigma
Fetal bovine serum (FBS)	PAA Laboratories
Calcium chloride	Merck
Comassie Brilliant Blue G25	Biomol
Difco™ Yeast extract	BD Biosciences
Fugene6 Transfection Reagent	Promega
L-Glutamine	Invitrogen
Glycerol	neoLab
Glycine	Biomol
Hepes	Biomol
Human serum	Lonza
Hydrogen chloride	Sigma
Ionomycine	Sigma
Jurkat TransIT™	Mirus
6x Loading buffer	Fermentas
Lymphoprep™	PROGEN Biotechnik GmbH
Magnesium chloride	Roth

Magnesium sulfite	Serva
Manganese chloride	Sigma
MEM	PAA
β-Mercaptoethanol	Sigma
Monensin	eBiosciences
3-(N-morpholino)propanesulfonic acid (MOPS)	Sigma
MEM Non-essential amino acids	Invitrogen
ODEYSSEY Blocking Buffer	LI-COR
Orange G	Sigma
Penicillin-Streptomycin	Gibco
Phorbol mystate acetate (PMA)	Sigma
Poly[d(I-C)]	Roche Diagnostics
Potassium chloride	Merck
Potassium acetate	Merck
2-Propanol	Merck
Rotriphorese® Gel 40 (37,5:1)/30 (37,5:1)	Roth
RPMI1640	Gibeco
Rubidium chloride	Sigma
SDS	Roth
Sodium chloride	Merck
Sodium phosphate	Merck
Sodium pyruvate MEM	Invitrogen
Tetramethylethylenediamine (TEMED)	Sigma
Tris	Merck

5.9 CONSUMABLES

Cellstar plastic pipettes 5 ml/ 10 ml/ 25 ml	Greiner bio-one
Cryo Tube™ Vials	Nunc
Dishes 94/16 with vents (100 mm)	Greiner Bio One
DNA LoBind® tubes	Eppendorf
Falcons 15 ml/ 50 ml	BD
Falcons 15 ml/ 50 ml	Sardtedt
Immobilon FL	Millipore
Millex Syringe Driven Filter Unit sterile GP 0.22 µm	Millipore
Parafilm M® Laboratory Film	Pechiney Plastic Packaging
PCR softstrips 0.2 ml	Biozym
Protein LoBind® tubes	Eppendorf
StableStak Racks (20µl, 200µl, 1000µl)	Mettler Toledo
Safe-Lock Tubes 1.5 ml/ 2.0 ml	Eppendorf
Safe-Lock Tubes 1.5 ml, black	A. Hartenstein
TC dish 140x20 Vents nunclons (150mm)	Nunc
TC Flask (T25/T75)	Greiner Bio One
96 F Nunclon® MicroWell plates white	Nunc

5.10 LABORATORY EQUIPMENT

ABI PRISM® 3730 DNA analyzer	Applied Biosystems
AutoMACS® Pro Seperator	Miltenyi
Centrifuge Universal 32R	Hettich Centrifuges
Epoch Microplate Spectrophotometer	BioTek

EP Motion 5075	Eppendorf
GloMax® 96 Microplate Luminometer	Promega
Haake K20 + Haake DC10 Kühlbad	ThermoHaake
Heater TR-L 288	Liebisch
Heraeus, Megafuge 1.0R	Thermo Scientific
Incubator Function line B6	Heraeus instruments
Incubator KB53	Binder
Incubator	Heraeus Instruments
Linear quadrupole ion trap-orbitrap (QTL orbitrap XL) MS	ThermoFisher
MagnaRack™	Invitrogen
Maxigel System	Biometra
Microflow	Nunc
Microcon devices YM-30	Millipore
Micropipettes	Rainin
Mikro200	Hettich Centrifuges
Microscope	Zeiss
Mr. Frosty Freezing Container	Thermo Scientific
NanoDrop ND 1000 Spectrophotometer	NanoDrop Technologies
Nano-ESI source	ThermoFisher
ODYSSEY® Infrared Imaging System	LI-COR
P25T Standard Power Pack	Biometra
pH Meter 766 calimatric	Knick
Power PAC 200 and 300	BioRad
Proteomics Analyzer 4700 (MALDI-TOF/TOF)	Applied Biosystems
Robocycler 96	Stratagene
Rotania	Hettich Centrifuges
Semidry Blotter	Biorad

Shaker Innova 4230	New Brunswick Scientific
Special accuracy weighting machine BP2215	Satorius
Thermomixer comfort	Eppendorf
Thermomixer compact	Eppendorf
Typhoon Trio +	GE Healthcare
UV-VIS Spectrophotometer DU530	Beckman
ViiA7 Real-Time PCR System	Applied Biosystems

5.11 USED KITS

BCA™ Protein Assay Reagent	Thermo Scientific
BD OptEIA Human IL-10 ELISA Set	BD Biosciences
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
DNeasy® Blood & Tissue Kit	Qiagen
Dual-Luciferase® Assay Kit	Promega
DuoSet Human IFN-g	R&D systems
DuoSet Human IL-4	R&D systems
DuoSet Human TNFa	R&D systems
iScript™ cDNA Synthesis Kit	Bio-Rad
Montage^{TM} Seq_{96} Kit	Milipore
Naïve $CD4^+$ T cell Isolation Kit II human	Miltenyi Biotec Macs
NE-PER Nuclear and Cytoplasmic Extraction Reagents	Thermo Scientific
Nuclear Extraction Kit	Active Motif
NucleoSpin® Plasmid	Macherey Nagel
NucleoBond® Xtra Midi/Maxi	Macherey Nagel
ProteoJet™ Kit	Fermentas

QuikChange® Site-directed mutagenesis PCR	Stratagene
RNeasy® Mini kit	Qiagen
SALSA® MLPA® Reagent-Kit	MRC-Holland
Wizard® SV PCR Clean-UP System	Promega

5.12 COMPUTER SOFTWARE AND PROGRAMS

5.12.1. Online Tools, Databases and Free Programs

BLAST (Basic Local Alignment Search Tool): http://www.ncbi.nlm.nih.gov/BLAST

ClustalW (multiple sequence alignments): http://www2.ebi.ac.uk/clustalw

DCODE (evolutionary conservation database): http://www.dcode.org/

Ensemble Genome Browser: http://www.ensembl.org/index.html

ENCODE (Encyclopedia of DNA elements): http://genome.ucsc.edu/ENCODE/

Genomatix (MatInspector, etc.): http://www.genomatix.de/

HapMap Project: http://hapmap.ncbi.nlm.nih.gov/

Mascot (search database for MS): http://www.matrixscience.com

NCBI (National center of Biotechnology Information): http://www.ncbi.nlm.nih.gov/

UCSC Genome Bioinformatics: http://genome.ucsc.edu/

UniProt (Universal Protein Resource): http://www.uniprot.org/

STRING (functional protein association network): http:// http://string-db.org/

1000 Genomes: http://www.1000genomes.org/

5.12.2. Computer Software

Adobe Photoshop (Adobe)

BioEdit Sequence Alignment Editor (Ibis Biosciences)

GeneMapper® Version 3.5 (Applied Biosystems, USA)

Gen5 Data Analysis Software (GenTek)

GloMax® 96 Microplate Luminometer Software version 1.9.2 (Promega)

ImageQuant TL (Amersham Biosciences)

LI-COR ODYSSEY application software v3.0 (LI-COR)

Microsoft Office 2010

NanoDrop version 3.1.0 NanoDrop Technologies

R software

Scaffold version 2.02.03 Proteome Software Inc.

Vector NTI Invitrogen

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APPENDIX

5.12.3. List of Used Oligonucleotides and Primers

All primers and oligonucleotides were purchased from Metabion.

primer code	5'→ 3' sequence	product length (bp)	purpose
9 RV-Primer Forw	CTAGCAAAATAGGCTGTCCC	-	Sequencing Inserts pGL4.23
11 pGL4.23 Rev	CATAGCTTCTGCCAGCCGAAC	-	Sequencing Inserts pGL4.23
N1 left	GGGTTCCCTAAGGGTTGGAAAGACAGAGTCAGCACCAAG CAACCTG	34	MLPA HeLa
N1 right	[PHOS]GAGCGGCTGCTTCTTTCTCTCTTGCTCTAGATTGG ATCTTGCTGGC		MLPA HeLa
N5 left	GGGTTCCCTAAGGGTTGGACAAATAGTCGAGTGGTACCT GTTCAGCC	154	MLPA HeLa
N5 right	[PHOS]GACAGAACTAGGAAACAAATACCTCCTCATTCTAT ATGGCTCTAGATTGGATCTTGCTGGC		MLPA HeLa
N8 left	GGGTTCCCTAAGGGTTGGACTAAGGTGGCCATGCTTCTCT GGATTTGCGGATTTGC	180	MLPA HeLa
N8 right	[PHOS]CAGCTCATCCCGCGTCGATTCCTGGAAGTGTTATC TCTAGATTGGATCTTGCTGGC		MLPA HeLa
N9 left	GGGTTCCCTAAGGGTTGGAGGAAAGAGCAGGAGAAAGG GAATCTTGG	192	MLPA HeLa
N9 right	[PHOS]GTCTCTTCAGCTTGTGGGAACAAACGAGTCTAGAT TGGATCTTGCTGGC		MLPA HeLa
S1 left	GGGTTCCCTAAGGGTTGGAGTTTTGTACTGTGGGAGTCT GAGAGCGAG	96	MLPA HeLa
S1 right	[PHOS]GAGGTCCGAAAGCCGAATCACAGTCTCTAGATTG GATCTTGCTGGC		MLPA HeLa
S9 left	GGGTTCCCTAAGGGTTGGACCGAGAGTGGGAGCTACTCA TTTTGAGG		MLPA HeLa
S9 right	[PHOS]CCCTTTAATTAAAGTCGCAGGCACCTAGGTCTAGA TTGGATCTTGCTGGC	154	MLPA HeLa
S10 left	GGGTTCCCTAAGGGTTGGACTCTGACGTAGTGTGACCTTG CTCATCC	183	MLPA HeLa
S10 right	[PHOS]CGAATTCAGCTCTGCTAGGACTGTTGGTCTAGATT GGATCTTGCTGGC		MLPA HeLa
S11 left	GGGTTCCCTAAGGGTTGGAGCTCTTTGCATCGCTCTCTGTCGG	207	MLPA HeLa

S11 right	[PHOS]CGTCTTCGCACTTACGCGGAGCGGTAATCTAGATTGGATCTTGCTGGC		MLPA HeLa
N1 left	GGGTTCCCTAAGGGTTGGACAACTTCTCCTGTGACGGGGAGC	120	MLPA T cells
N1 right	[PHOS]CCAGGGACCAAGTTAAATAGGCAGGTTGTCTAGATTGGATCTTGCTGGC		MLPA T cells
N2 left	GGGTTCCCTAAGGGTTGGACCATGTGGAAGGCAAGCCAGGCTCAC	161	MLPA T cells
N2 right	[PHOS]CTCTTCAGATGCCACTTCTAAGAAAAGCCTCTAGATTGGATCTTGCTGGC		MLPA T cells
N3 left	GGGTTCCCTAAGGGTTGGACCTAAGTAACTCTCACCTATTACCGTGGC	134	MLPA T cells
N3 right	[PHOS]CCACCTCCACCCATTATACTTCTGCCTCTAGATTGGATCTTGCTGGC		MLPA T cells
RHS5(I) left	GGGTTCCCTAAGGGTTGGAGGTAACACAGGAAGTCAGCAGTGCATTTTC	143	MLPA T cells
RHS5(I) right	[PHOS]CATCACTAAGTATAAGCATTGGCAGTGGTCTCTAGATTGGATCTTGCTGGC		MLPA T cells
RHS5(II) left	GGGTTCCCTAAGGGTTGGAGGAGCTTCAGATGAGGGCTTTATTCAG	128	MLPA T cells
RHS5(II) right	[PHOS]CCATGGATGCAGTAAGGTTTCACAGTAGCTCTAGATTGGATCTTGCTGGC		MLPA T cells
RHS6(I)_#1 left	GGGTTCCCTAAGGGTTGGACGACCTTAGTCATCCCCATCTCTTACAC	210	MLPA T cells
RHS6(I)_#1 right	[PHOS]CTCGCATATGGTAAATGGTCATCAATAATAATCTCTAGATTGGATCTTGCTGGC		MLPA T cells
RHS6(I)_#2 left	GGGTTCCCTAAGGGTTGGACTAAGGGCTTTTTGATGAAAGGTGACTGTG	214	MLPA T cells
RHS6(I)_#2 right	[PHOS]CATGTCTGGACTAAGTGGAATTTAGTGCTTAGAGTCTAGATTGGATCTTGCTGGC		MLPA T cells
RHS6(II) left	GGGTTCCCTAAGGGTTGGAGTTCCAGAGCCTAACAGGACTTACATATTTGAC	139	MLPA T cells
RHS6(II) right	[PHOS]GGTTCTGTCATAGTGTAGATAGGGATAAGCCTCTAGATTGGATCTTGCTGGC		MLPA T cells
RHS7_#1 left	GGGTTCCCTAAGGGTTGGACACATGCTCTGGTTGAGTAAGTATCTCTTGC	172	MLPA T cells
RHS7_#1 right	[PHOS]CTGTGATGAAAACGTTCTCTAGCTGTGTTCTCTAGATTGGATCTTGCTGGC		MLPA T cells
RHS7_#2 left	GGGTTCCCTAAGGGTTGGACCTACTGTCTGGAGAGGAGAAGAGACTC	175	MLPA T cells
RHS7_#2 right	[PHOS]CATGTGTCTGACAAGGTTTGCGGTGACTCTAGATTGGATCTTGCTGGC		MLPA T cells
Th1 specific left	GGGTTCCCTAAGGGTTGGACTAGAATGAATATTCCAGTATAGTAAGGAGTCTC	117	MLPA T cells
Th1 specific right	[PHOS]CCTCATAGAACTAAGTCCCTACCACAGCTCTAGATTGGATCTTGCTGGC		MLPA T cells
Forw_rs2240032_Ma	CY5-GCACTCCTGGCTCCAGACAGTCCCTTTCTGGC	32	EMSA
	GCACTCCTGGCTCCAGACAGTCCCTTTCTGGC		EMSA

	BIOTIN-GCACTCCTGGCTCCAGACAGTCCCTTTCTGGC		DNA affinity purification
Rev_rs2240032_Ma	GCCAGAAAGGGACTGTCTGGAGCCAGGAGTGC		EMSA
Forw_rs2240032_Mi	CY5- GCACTCCTGGCTCCAGATAGTCCCTTTCTGGC	32	EMSA
	GCACTCCTGGCTCCAGATAGTCCCTTTCTGGC		EMSA
	BIOTIN-GCACTCCTGGCTCCAGATAGTCCCTTTCTGGC		DNA affinity purification
Rev_rs2240032_Mi	GCCAGAAAGGGACTATCTGGAGCCAGGAGTGC		EMSA
Forw_rs3798134_Ma	CY5-TAGCAATTTAGGCTTGTTAAGGAACAAAGC	30	EMSA
	TAGCAATTTAGGCTTGTTAAGGAACAAAGC		EMSA
Rev_rs3798134_Ma	GCTTTGTTCCTTAACAAGCCTAAATTGCTA		EMSA
Forw_rs3798134_Mi	CY5-TAGCAATTTAGGCTTATTAAGGAACAAAGC	30	EMSA
	TAGCAATTTAGGCTTATTAAGGAACAAAGC		EMSA
Rev_rs3798134_Mi	GCTTTGTTCCTTAATAAGCCTAAATTGCTA		EMSA
Forw_rs3798135_Ma	CY5- GAGCAGAATGAGGACCTATGAGGGAGCTTG	30	EMSA
	GAGCAGAATGAGGACCTATGAGGGAGCTTG		EMSA
Rev_rs3798135_Ma	CAAGCTCCCTCATAGGTCCTCATTCTGCTC		EMSA
Forw_rs3798135_Mi	CY5- GAGCAGAATGAGGATCTATGAGGGAGCTTG	30	EMSA
	GAGCAGAATGAGGATCTATGAGGGAGCTTG		EMSA
Rev_rs3798135_Mi	CAAGCTCCCTCATAGATCCTCATTCTGCTC		EMSA
Forw_rs12653750_Ma	CY5-ATTAGTTCATAAACCCGCTTCCAAAGAACC	30	EMSA
	ATTAGTTCATAAACCCGCTTCCAAAGAACC		EMSA
Rev_rs12653750_Ma	GGTTCTTTGGAAGCGGGTTTATGAACTAAT		EMSA
Forw_rs12653750_Mi	CY5-ATTAGTTCATAAACCTGCTTCCAAAGAACC	30	EMSA
	ATTAGTTCATAAACCTGCTTCCAAAGAACC		EMSA
Rev_rs12653750_Mi	GGTTCTTTGGAAGCAGGTTTATGAACTAAT		EMSA
Forw_rs2040704_Ma	CY5-GATAAGCCAAAATGCAATAAGAAAAACCATC	31	EMSA
	GATAAGCCAAAATGCAATAAGAAAAACCATC		EMSA
Rev_rs2040704_Ma	GATGGTTTTTCTTATTGCATTTTGGCTTATC		EMSA
Forw_rs2040704_Mi	CY5-GATAAGCCAAAATGCGATAAGAAAAACCATC	31	EMSA
	CY5-GATAAGCCAAAATGCGATAAGAAAAACCATC		EMSA
Rev_rs2040704_Mi	GATGGTTTTTCTTATCGCATTTTGGCTTATC		EMSA
Forw_SMAD3/4 (Dennler et al., 1998)	CY5-TCGAGAGCCAGACAAAAAGCCAGACATTTAGCCAGACAC	39	EMSA
	TCGAGAGCCAGACAAAAAGCCAGACATTTAGCCAGACAC		EMSA
Rev_SMAD3/4	GTGTCTGGCTAAATGTCTGGCTTTTTGTCTGGCTCTCGA		EMSA
Forw SP1 (Briggs et al., 1986)	CY5-ATTCGATCGGGGCGGGGCGAGC	22	EMSA
	CY5-ATTCGATCGGGGCGGGGCGAGC		EMSA
Rev SP1	GCTCGCCCCGCCCCGATCGAAT		EMSA
Forw_Oct1 (O'Neill et al., 1988)	CY5-TGTCGAATGCAAATCACTAGAA	22	EMSA

	CY5-TGTCGAATGCAAATCACTAGAA		EMSA
Rev_Oct1	TTCTAGTGATTTGCATTCGACA		EMSA
RHS 5 (I) forw (Acc65I)	TTTTGGTACCTTGTCAGGCTCTGGGAAAACAGGA	642	Insert Luciferase Assay
RHS 5 (I) rev (XhoI)	TTTTCTCGAGCTATGTGTCTCTGACCACTGCCAAT		Insert Luciferase Assay
F RHS5(I)rs3798135Mi	TGAGCAGAATGAGGATCTATGAGGGAGCTT	642	Mutagenesis Primer Luciferase-Insert with rs3798135Mi
R RHS5(I)rs3798135Mi	AAGCTCCCTCATAGATCCTCATTCTGCTCA		Mutagenesis Primer Luciferase-Insert with rs3798135Mi
RHS 5 (II) forw (Acc65I)	TTTTGGTACCCCTGGCCAGGTCATGCTTCTTTTAA	374	Insert Luciferase Assay
RHS 5 (II) rev (XhoI)	TTTTCTCGAGTAGTTTTCTTTAGCTAGACTCCCAAACAT		Insert Luciferase Assay
RHS 6 (I) forw (Acc65I)	TTTTGGTACCATGGTAAATAGAGATAACAGTA	1282	Insert Luciferase Assay
RHS 6 (I) rev (XhoI)	TTTTCTCGAGAAGCTGAACTGACATCAAAATT		Insert Luciferase Assay
RHS 6 (II) forw (Acc65I)	TTTTGGTACCTGACCTGTGGCGAAGTACCTATCG	348	Insert Luciferase Assay
RHS 6 (II) rev (XhoI)	TTTTCTCGAGAAAAGAGTTTCCTCTGGATGGTTTTTCT		Insert Luciferase Assay
Rev_RHS6(II)rs2040704_Mi Mutagenese	TTTTCTCGAGAAAAGAGTTTCCTCTGGATGGTTTTTCTTATCGCATTTTG	348	Mutagenesis Primer Luciferase-Insert with rs2040704Mi
6(II) Forw Quickchange	GACATAATAGGTTCTGTCATAGTGTAGATAGGGATAAGCCAAAATGC		Quickchange mutagenesis
6 (II) Rev Quickchange	GCATTTTGGCTTATCCCTATCTACACTATGACAGAACCTATTATGTC		Quickchange mutagenesis
RHS 7 for (Acc651)	TTTTGGTACCGGTTGAGTAAGTATCTCTTGCACA	1396	
RHS 7 rev (XhoI)	TTTTCTCGAGGAGCGACTTTTTATTATCCTGCAACAAT		Insert Luciferase Assay
RHS 7 INVERS forw (Xho1)	TTTTCTCGAGGGTTGAGTAAGTATCTCTTGCACA	1396	Insert Luciferase Assay
RHS 7 INVERS rev (ACC65I)	TTTTGGTACCATTATCCTGCAACAATATATTCAGAAC		Insert Luciferase Assay
RHS7_Forw 150bp (Acc651)	TTTTGGTACCGGACTATGCTGGCAGAGAAAAAG	150	Insert Luciferase Assay
RHS7_Rev 150bp (Xho1)	TTTTCTCGAGCTTGTGATGATGGCTGCAGAAAG		Insert Luciferase Assay
RHS7_Forw 150bp INVERS (Xho1)	TTTTCTCGAGGGACTATGCTGGCAGAGAAAAAG	150	Insert Luciferase Assay
RHS7_Rev 150bp INVERS (Acc651)	TTTTGGTACCCTTGTGATGATGGCTGCAGAAAG		Insert Luciferase Assay
RHS7Δ150bp_Rev	AAGCACTCGCCCTTATTATGCAAGAGCTGC	1246	Fusion-PCR for RHS7 without 150bp (Insert Luciferase Assay)

RHS7Δ150bp_For	GCAGCTCTTGCATAATAAGGGCGAGTGCTT	1246	Fusion-PCR for RHS7 without 150bp (Insert Luciferase Assay)
„desert site“ Forw (Acc65I)	TTTTGGTACCGGCTATATAGCCGGTATATTTATGTGGTACG	749	Insert Luciferase Assay
„desert site“ Rev (Xho1)	TTTTCTCGAGGTGGTTCCTCTCGGGCGCGGTG		Insert Luciferase Assay
„desert site“ INVERS Forw (Xho1)	TTTTCTCGAGGGCTATATAGCCGGTATATTTATGTGGTACG	749	Insert Luciferase Assay
„desert site“ INVERS Rev (Acc651)	TTTTGGTACCGTGGTTCCTCTCGGGCGCGGTG		Insert Luciferase Assay
RHS7 Forw Genotyp	GCACACCTTTTACCACATTCACC	400	Genotypisierung rs2240032
RHS7 Rev Genotyp	GAATTCTGCCACCACCAGTCAC		Genotypisierung rs2240032
IFNg Forw	TCAGCCATCACTTGGATGAG		RT-PCR
IFNg Rev	CGAGATGACTTCGAAAAGCTG		RT-PCR
IL4 Forw	GTGTCCTTCTCATGGTGGCT		RT-PCR
IL4 Rev	CAGACATCTTTGCTGCCTCC		RT-PCR
IL17-A Forw	CCATCCCCAGTTGATTGGAA		RT-PCR
IL17-A Rev	CTCAGCAGCAGTAGCAGTGACA		RT-PCR
IL22 Forw	ACAGCAAATCCAGTTCTCCAA		RT-PCR
IL22 Rev	TCCAGAGGAATGTGCAAAAG		RT-PCR
IL13 Forw	GATTCCAGGGCTGCACAGTA		RT-PCR
IL13 Rev	GGTCAACATCACCCAGAACC		RT-PCR
IL5 Forw	TCTCCAGTGTGCCTATTCCC		RT-PCR
IL5 Rev	CGAACTCTGCTGATAGCCA		RT-PCR

Abbrevations: Forw = Forward, Rev = Reverse, Mi = Minor, Ma = Major, Cy5 = Cyanine5, [PHOS] = Phosphorylation.

PUBLICATIONS

Steininger S, Ahne F, Winkler K, **Kleinschmidt A,** Eckardt-Schupp F, Moertl S. A novel function for the Mre11-Rad50-Xrs2 complex in base excision repair. *Nucleic Acids Res* 38:1853-1865, 2010.

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