

An IgE-associated polymorphism in *STAT6* alters NF- κ B binding, *STAT6* promoter activity, and mRNA expression

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Background: The IL-4/IL-13 pathway is central for IgE regulation. Signal transducer and activator of transcription 6 (*STAT6*) is the major transcription factor within this pathway. *STAT6* polymorphisms were recently associated with elevated total IgE levels in a genome-wide association study.

Objective: This study aimed to assess biological mechanisms by which an IgE-associated genetic variation in *STAT6* may potentially influence gene expression.

Methods: *STAT6* intron 2 carrying either the wild-type C or the polymorphic T allele of the putatively causal single nucleotide polymorphism rs324011 was cloned into *STAT6* promoter vectors to investigate their influence on *STAT6* promoter activity by *in vitro* luciferase assays. Transcription factor binding depending on rs324011 was examined by electrophoretic mobility shift assays in Jurkat T cells and primary CD4⁺ T cells. Allele-specific *STAT6* gene expression of 3 splice variants was studied *ex vivo* by real-time PCR in 239 individuals.

Results: *STAT6* intron 2 acts as a silencer regulatory element. The polymorphic T allele at rs324011 (in linkage disequilibrium with the genome-wide association signal and consistently associated with elevated IgE levels in 3 previous studies) increases *STAT6* promoter activity significantly *in vitro* ($P < .00001$) and gene expression of *STAT6* splice variants *ex vivo* ($P < .01$) compared with the wild-type C allele. These effects correlate with the creation of a novel, T-allele-specific binding site for the transcription factor nuclear factor- κ B in T cells.

Conclusion: The consistently replicated effects of genetic variance in *STAT6* on IgE regulation may be explained in part by allele-specific alterations in nuclear factor- κ B binding at rs324011 and consecutive changes in *STAT6* gene expression. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *STAT6*, NF- κ B binding, regulatory element, mRNA gene expression, splice variants

Signal transducer and activator of transcription (STAT)-6 is known to be a key mediator in the activation of the IL-4/IL-13 pathway essential for the class-switch to IgE. *STAT6* affects the development of atopic diseases in many ways, and the Janus tyrosine kinase-STAT signaling pathway is involved in numerous physiologic events that are dysregulated in asthma.¹⁻³ It was shown that *STAT6*-deficient mice failed to develop bronchial hyperreactivity and airway eosinophilia after allergen provocation.^{4,5}

Three major genetic determinants of IgE regulation have recently been identified by a genome-wide association study and replications in white populations.⁶ The *STAT6*-associated polymorphism rs12368672 relates to one of the genome-wide association signals. It is in strong linkage disequilibrium ($r^2 = 0.8$) with a single nucleotide polymorphism (SNP) in *STAT6* intron 2 (rs324011; see this article's Fig E1 in the Online Repository at www.jacionline.org), which had been associated with elevated IgE levels in 3 independent previous populations.⁷⁻⁹ The same SNP in intron 2 strongly influenced IgE regulation and asthma susceptibility in combination with other polymorphisms of the IL-4/IL-13 pathway.¹⁰

Although *STAT6* is a central transcription factor in an important immunological pathway, few data on *STAT6* gene regulation and even less information on genetically determined changes in *STAT6* gene function are available. A putative functional role of rs324011 was previously suggested by the high degree of phylogenetic conservation of the SNP harboring region among apes and primates.⁸ *In silico* sequence analysis implied that the polymorphic T allele of rs324011 may create a novel nuclear factor- κ B (NF- κ B) transcription factor binding site not detectable with the C allele. Thus, in the current study, the functional role of intron 2 *per se* was analyzed. Also investigated was how polymorphism rs324011 located in this intronic region may affect *STAT6* regulation, explaining the highly consistent associations with IgE across different populations.

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Abbreviations used

EMSA:	Electrophoretic mobility shift assay
GMR:	Geometric mean ratio
kb:	Kilo base pair
NF- κ B:	Nuclear factor- κ B
PARSIFAL:	Prevention of Allergy-Risk factors for Sensitization In Children related to Farming and Anthroposophic Lifestyle
PMA:	Phorbol 12-myristate 13-acetate
PO:	Polymorphic allele
SNP:	Single nucleotide polymorphism
SP:	Specificity protein
STAT:	Signal transducer and activator of transcription
WT:	Wild-type allele

METHODS**STAT6 reporter constructs**

Three pGL3-basic vector constructs containing *STAT6* promoters of different sizes, pBP78 (5643 bp), pBP88 (2588 bp), and pBP86 (1081 bp), were generously provided by Dr B. Patel.¹¹ To study the effects of *STAT6* intron 2, 1287 bp of *STAT6* intron 2 was cloned 5'3' or 3'5' into *STAT6* promoter constructs (pBP78, pBP86) downstream of the luciferase gene (see this article's Fig E2 in the Online Repository at www.jacionline.org). More detailed information is available from the authors on request. Site-directed mutagenesis was performed to insert the polymorphic T allele of rs324011 (Stratagene, La Jolla, Calif; 5'-CATGAGTGGTGGTGGGACAGTCCCTAGGAGGGCTA TC-3'). The respective sequence of *STAT6* promoters and intron 2 was confirmed by resequencing.

Transient transfection experiments using STAT6 reporter constructs

Jurkat T cells (8×10^6) were transiently cotransfected with each *STAT6* reporter construct (4 μ g; N = 10) and pRL-TK (10 ng; Promega, Madison, Wis) by square-wave electroporation with 1 pulse (50 ms; 200 V; Genpulser-Xcell; Biorad, Hercules, Calif). Cells were left in medium or stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (12.5 ng/mL; 0.25 μ mol/L) 3 hours after transfection and harvested 24 hours after transfection (N = 10). Luciferase activity was quantified as relative light units by using the Dual-Luciferase Assay according to the manufacturer's protocol (Promega, Madison, Wis).

Electrophoretic mobility shift assay

Nuclear extract was prepared from Jurkat T cells (<http://www.dsmz.de>) and primary CD4⁺ T cells as previously described.¹² Primary CD4⁺ T cells were isolated, applying the CD4⁺ T-cell isolation kit (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) after retaining PBMCs from whole blood by using the density-gradient centrifugation (Ficoll-Paque; Amersham, Piscataway, NJ). Double-stranded probes (100 ng; Table I) carrying either the wild-type C or the polymorphic T alleles of rs324011 were end-labeled as previously described.¹² Each binding reaction included 5 μ g nuclear extract, 1x binding buffer,¹² and 0.5 μ g/reaction Poly(dI-dC)-Poly(dI-dC). Supershift and competition experiments were performed by adding either the transcription factor specific antibody (4 μ g) or unlabeled probes in a 100-fold molar excess before adding the labeled probe.

Ex vivo mRNA expression study of STAT6 splice variants

Gene expression of *STAT6* splice variants (non-*STAT6d*/*STAT6e*, *STAT6d*, *STAT6e*) was measured in PBMCs obtained from children from the Swiss branch of the cross-sectional Prevention of Allergy-Risk factors for Sensitization In Children related to Farming and Anthroposophic Lifestyle

(PARSIFAL) population.¹³ DNA and RNA were available for 239 children in sufficient quantities. The genotype of rs324011 for each individual was determined by restriction fragment length polymorphism. Isolation of RNA and reverse transcription of RNA were performed as previously described.¹⁴ Primers and probes for 3 *STAT6* isoforms (non-*STAT6d*/*STAT6e*, *STAT6d*, *STAT6e*) and the endogenous control (18S rRNA) were designed with the primer design software Primer Express (Applied Biosystems, Foster City, Calif) and purchased from Microsynth (Balgach, Switzerland; Table II). Real-time PCR (*TaqMan*) was carried out with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Data with the comparative ($\Delta\Delta C_t$) method were analyzed according to the manufacturer's instructions (Applied Biosystems, Foster City, Calif). Statistical analyses were performed with SAS 9.1.3 (SAS Institute, Cary, NC). Geometric mean ratios (GMRs) and 95% CIs were calculated in a multiple logistic regression analysis.

RESULTS**Intron 2 silences STAT6 promoter activity**

Because SNP rs324011 is located in an intronic region of *STAT6* with unknown function, we first investigated the general influence of intron 2 on *STAT6* promoter activity. Only then could the role of rs324011 on *STAT6* promoter activity be studied. *STAT6* promoter constructs (pBP78, pBP88, pBP86¹¹) of different sizes (5.6kb, 2.6kb and 1.1kb) were used to identify the minimal region necessary for maximal *STAT6* transcription in Jurkat T cells. Consistent with a previous report,¹¹ our data indicated that the minimal *STAT6* promoter pBP86 (1.1kb) had the strongest transcriptional activity (15-fold upregulation) after T-cell stimulation (see this article's Fig E3 in the Online Repository at www.jacionline.org). The largest promoter construct pBP78 (5.6 kb) led to a 5-fold increase in comparison with the pGL3-basic vector.

Next, luciferase activity of pBP86 (1.1 kb) and pBP78 (5.6 kb) containing intron 2 downstream of the luciferase gene was assessed by using the wild-type C allele (corresponding to pBP86_wild-type allele [WT] or pBP78_WT). *STAT6* intron 2 acted as a silencing element on the pBP86 promoter, indicated by a significant drop in luciferase activity (Fig 1, A). A similar decline of the *STAT6* promoter activity was obtained for pBP78_WT in the presence of intron 2 (Fig 1, B).

Alleles of SNP rs324011 influence STAT6 promoter activity in vitro

Allele-specific effects were assessed with *STAT6* vectors pBP86 and pBP78 also carrying the polymorphic T allele of rs324011 in intron 2 (corresponding to pBP86_polymorphic allele [PO] and pBP78_PO, Fig 1, A and B). The activity of the *STAT6* promoter pBP78 was strongly influenced by the presence of polymorphic T allele (pBP78_PO) significantly increasing the luciferase signal compared with pBP78_WT. Very similar allele-specific effects were achieved when intron 2 was clone 3'5' into the respective site of the pBP78 vector downstream of the luciferase gene (data not shown).

Allele-specific NF- κ B binding to rs324011 in intron 2 in T cells

Previous *in silico* transcription factor binding analyses suggested that NF- κ B⁸ and specificity protein (SP)-1 may bind to rs324011 within *STAT6* intron 2, whereas the binding of NF- κ B was predicted to be allele-specific (Fig 2). Because altered

TABLE I. Single-stranded oligonucleotide sequences used for EMSA

Probe name	Probe sequence (5'3')
NF-κBfwd*	TCAGAGGGGACTTTCGAGAGGCG
SP_fwd†	ATTCGATCGGGGCGGGGCGAGC
STAT6_rs324011fwd_WT	AGTGGTGGTGGGGACGGTCCCTAGGAGGGCT‡
STAT6_rs324011fwd_PO	AGTGGTGGTGGGGACAGTCCCTAGGAGGGCT‡

Underlined sequences display the consensus site for the respective transcription factor. *Bold letters* indicate the location of SNP rs324011.

*EMSA probe for a NF-κB consensus sequence.¹⁵

†EMSA probe for a SP consensus sequence.³³

‡The polymorphism rs324011 was originally described with the alleles C/T⁷ because *STAT6* is transcribed 3'5' on the chromosome. In accordance with its 5'3' transcription within the *STAT6* gene, the allele equals G/A.

TABLE II. Primer sequences, primer localization, and size of each amplified fragment used to quantify *STAT6* isoforms (non-*STAT6d*/*STAT6e*, *STAT6d*, *STAT6e*) and 18SrRNA

Primer name	Primer sequence (5'3')	Localization	Fragment size
Non- <i>STAT6d</i> / <i>STAT6e</i> _fwd	ATCCCAAGAAGCCCAAGGA	Exon 17	81 bp
Non- <i>STAT6d</i> / <i>STAT6e</i> _rev	GGACATAACCCCTGCCATCC	Exon 18	
Non- <i>STAT6d</i> / <i>STAT6e</i> _probe	ACTACAAGC-CTGAACAGA	Exon 17-18	
<i>STAT6d</i> _fwd	CCAGAGCACTCCATGGCTGT	Intron 17	162 bp
<i>STAT6d</i> _rev	GGTCATAAGAAGGCACCATGGT	Exon 19	
<i>STAT6d</i> _probe	TGGAAAG-GGACCAACCA	Exon 18-19	
<i>STAT6e</i> _fwd	TCACATGTACCTCCTCCCTCC	Intron 18	148 bp
<i>STAT6e</i> _rev	CACCTGGGGC-ACCATATCTG	Exon 19-20	
<i>STAT6e</i> _probe	ATGAGCATGCAGCTTG	Exon 19	
18SrRNA_fwd	AGTCCCTGCCCTTGTACACA		69 bp
18SrRNA_rev	GATCCGAGGGCCTCACTAAAC		
18SrRNA_probe	CGCCCGTCGCTACTACCGATTGG		

transcription factor binding may explain the observed increase in *STAT6* promoter activity, electrophoretic mobility shift assay (EMSA) experiments were conducted with nuclear extract from the human Jurkat T-cell line to examine DNA/protein interactions of these transcription factors at the respective *STAT6* site. Incubation of a 31-bp probe carrying the wild-type C allele at its center induced 4 specific DNA/protein complexes (Fig 2, lanes 1 and 2). Indeed, it was indicated that transcription factor binding of SP family members (SP1, SP3, SP4) occurs but is independent of rs324011 (Fig 2). Yet, with the probe carrying the polymorphic T (rs324011_PO) allele, an additional complex was observed, especially after stimulation with PMA/ionomycin (lanes 14 and 15). The allele-specific DNA/protein complex was identified by antibody supershift experiments to contain NF-κB subunits p50 and p65 (lanes 21 and 22). In contrast, no NF-κB binding was detectable with rs324011_WT (lanes 8 and 9). In competition experiments, the 100-fold molar excess of a high-affinity NF-κB¹⁵ site led to the complete abrogation of the T-allele-specific complex (lane 18). Accordingly, when the NF-κB consensus site was used in EMSA experiments, the probe rs324011_PO was able to repress the NF-κB signal better than the C allele (see this article's Fig E4, lanes 4 and 5, in the Online Repository at www.jacionline.org).

NF-κB binding to rs324011 was tested not only in the Jurkat T-cell line but also in the distinct nuclear environment of primary CD4⁺ T cells (Fig 3). Again, a strong NF-κB signal was detectable and verified by specific antibodies (p50, p65), also in primary CD4⁺ T cells (lanes 3-5). Thus, the T allele at the polymorphism rs324011 in intron 2 of *STAT6* not only increases promoter activity *in vitro* but also alters the binding of the transcription factor NF-κB at the same locus in T cells.

STAT6 mRNA expression *ex vivo*

We investigated whether the allele-specific effects of rs324011 on *STAT6* regulation *in vitro* are also detectable *ex vivo*. Thus, we measured *STAT6* mRNA expression in PBMCs of 239 children from the PARSIFAL study in relation to their rs324011 genotype. For quantitative mRNA analysis, we first used a forward primer at the *STAT6* exon 16/17 border and a reverse primer in exon 19, designed to capture all previously known *STAT6* isoforms (see this article's Fig E5, A, in the Online Repository at www.jacionline.org). Unexpectedly, this led to 3 PCR products of different sizes (Fig E5, B). The 2 additional PCR products were identified as novel splice variants caused by intron retention and named *STAT6d* and *STAT6e* (Fig E5, C). The third band contained the predicted exonic regions of *STAT6* (further referred to as *non-STAT6d/STAT6e* isoform). In addition to the exonic regions, *STAT6d* was made up of intron 17, whereas *STAT6e* included both intron 17 and intron 18. To confirm further the existence of these 3 *STAT6* splice variants, specific primers for each isoform were applied separately (Table II; Fig E5, C) and used for all further quantitative real-time PCR experiments. Contamination of genomic DNA was excluded by deoxyribonuclease (DNase) treatment of the samples and in addition by the binding region of the isoform-specific primers, because DNA contaminations would have led to bigger PCR products.

Alleles of rs324011 influence mRNA expression of *STAT6* splice variants *ex vivo*

Next, rs324011 allele-specific effects on *STAT6* mRNA expression were analyzed (Fig 4; CC, n = 101; CT, n = 107; TT, n = 31). In individuals carrying the polymorphic T allele,

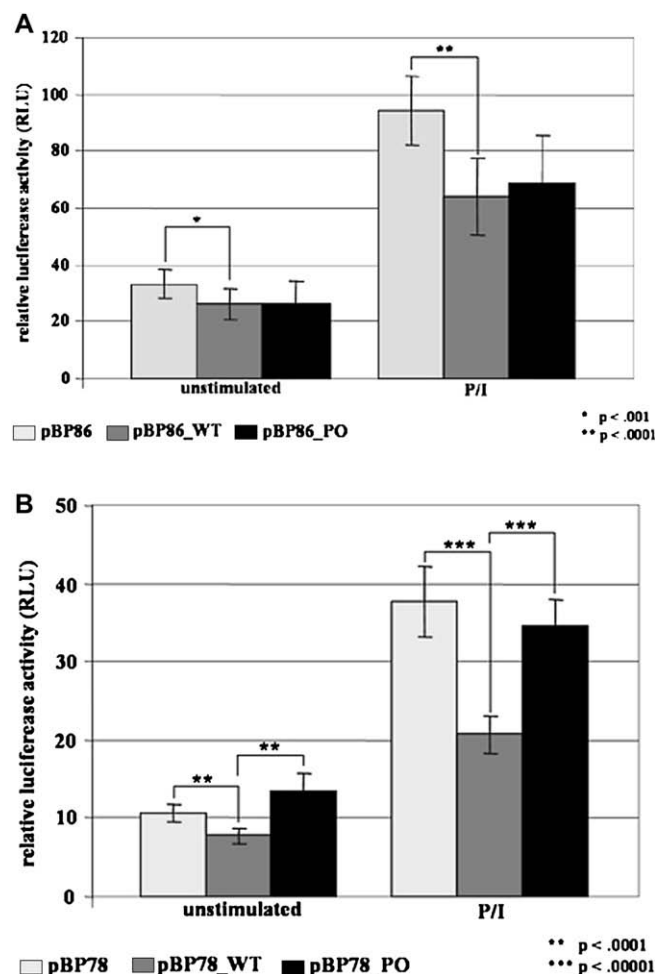


FIG 1. *In vitro* STAT6 promoter activity is silenced depending on rs324011. Luciferase assays were performed using Jurkat T cells with the STAT6 promoter vector pBP86 (1.1 kb) (**A**) or pBP78 (5.6 kb) (**B**) and further constructs in addition carrying STAT6 intron 2 either expressing the wild-type C (pBP86_WT, pBP78_WT) or the polymorphic T allele (pBP86_PO, pBP78_PO) of rs324011 (N = 10). * $P < 0.001$; ** $P < 0.0001$; *** $P < 0.00001$. P/I = PMA/ionomycin.

levels of all 3 analyzed STAT6 isoforms (non-STAT6d/STAT6e, STAT6d, STAT6e; Fig 4) were increased. The expression of the known STAT6 isoform (non-STAT6d/STAT6e) was slightly but not significantly elevated in TT individuals compared with CC individuals (GMR, 1.11; 95% CI, 0.83-1.47). A significant increase was observed for mRNA levels of STAT6d (GMR, 1.37; 95% CI, 1.06-1.78; .05 $P \geq .01$) in homozygous carriers of the polymorphic T allele. An even more significant increase was detectable for STAT6e mRNA levels (GMR, 1.49; 95% CI, 1.15-1.94; $P < .01$). These results remained significant when adjusted for farming status, sex, and age.

DISCUSSION

Signal transducer and activator of transcription 6, the major transcription factor of the IL-4/IL-13 pathway, is central for the regulation of IgE class-switching, IgE production, and the development of atopic diseases. Previous studies had consistently linked genetic variations within the gene to elevated IgE levels⁷⁻⁹ and asthma.¹⁰ Because the genetic association signal in these

studies seemed to be strongly driven by rs324011 in the putative second intron of STAT6, this study investigated the functional role of intron 2 *per se* and the regulatory influence of rs324011 on the STAT6 gene.

Here we showed evidence that STAT6 intron 2 in general may act as a silencing element downregulating STAT6 promoter activity. *In vitro*, promoter activity is significantly influenced by the allelic state of rs324011 located within this regulatory region. Transcription factor binding analysis for rs324011 by EMSA revealed that NF- κ B exclusively binds to the polymorphic T allele. Using real-time PCR, an allele-specific increase of STAT6 mRNA expression in PBMCs was observed *ex vivo* with slightly different effect sizes for the 3 STAT6 isoforms studied. Allele-specific effects of rs324011, located within a regulatory region in intron 2, on STAT6 promoter activity *in vitro* and STAT6 gene expression *ex vivo* may help to explain the results of previous genetic association studies.

Many candidate gene polymorphisms have been studied in asthma and atopic diseases in the past. However, few association results could be replicated in more than 1 study population. In contrast, polymorphisms in the IL-4/IL-13 pathway had been replicated rather consistently across different populations, possibly indicating a gatekeeper function of that pathway for IgE regulation and the end of the atopic road to disease. Within the pathway, STAT6 has a central role mediating the IL-4 and IL-13-related signal from the cell surface receptor across the cytosol to the nucleus and the IL-4/IL-13-responsive genomic elements. An association with IgE levels was consistently found for SNP rs324011 located in STAT6 intron 2 in 3 different studies made up of children and adults.⁷⁻⁹ Recently, a genome-wide association study reported a significant effect of SNP rs12368672, located 5' of STAT6, on total serum IgE levels.⁶

Analyzing approximately 18 megabases around the STAT6 gene in HapMap (Fig E1, A), polymorphism rs12368672 is strongly linked (linkage disequilibrium $r^2 > 0.8$) to only 3 polymorphisms (Fig E1, B): rs11172106 in the close vicinity of rs12368672 5' of STAT6 and 2 polymorphisms in intron 2 of STAT6 (rs324011 and rs167769; Fig E1, C). SNPs rs12368672 and rs11172106 are located approximately 7.5 kb upstream of the defined STAT6 promoter. SNP rs167769 in intron 2 does not reveal significant differences in transcription factors binding by *in silico* analysis and our own EMSA studies (data not shown). Thus, we concluded that rs324011 may be the most likely polymorphism to explain causally the consistent association signals in the STAT6 gene region, making it a worthy candidate for functional studies.

So far, functional SNP studies in the IL-4/IL-13 pathway had been restricted to polymorphisms in IL-4,^{16,17} IL-13¹⁸ and IL-4 receptor α (IL-4R α).¹⁹ In these genes, the putative functional role of SNPs was suggested by their location in the promoter (IL-4^{16,17} and IL-13¹⁸) or the change of an amino acid (IL-4R α).¹⁹ In contrast, rs324011 is located in an intronic region. Only recently has it become evident that many functions such as transcriptional regulation, genomic 3-dimensional gene structure determination, and splicing control may be influenced by intronic regions of a gene.²⁰

For STAT6, genomic structure and intron boundaries are not yet fully determined. Initial experimental studies ascertained 23 exons interspersed by 22 introns, with 2 noncoding 5' exons.¹¹ Most likely based on *in silico* analysis, recent databases suggest that exon 2 is part of the first intron, which would locate rs324011 in the first rather than the second intron. Because the

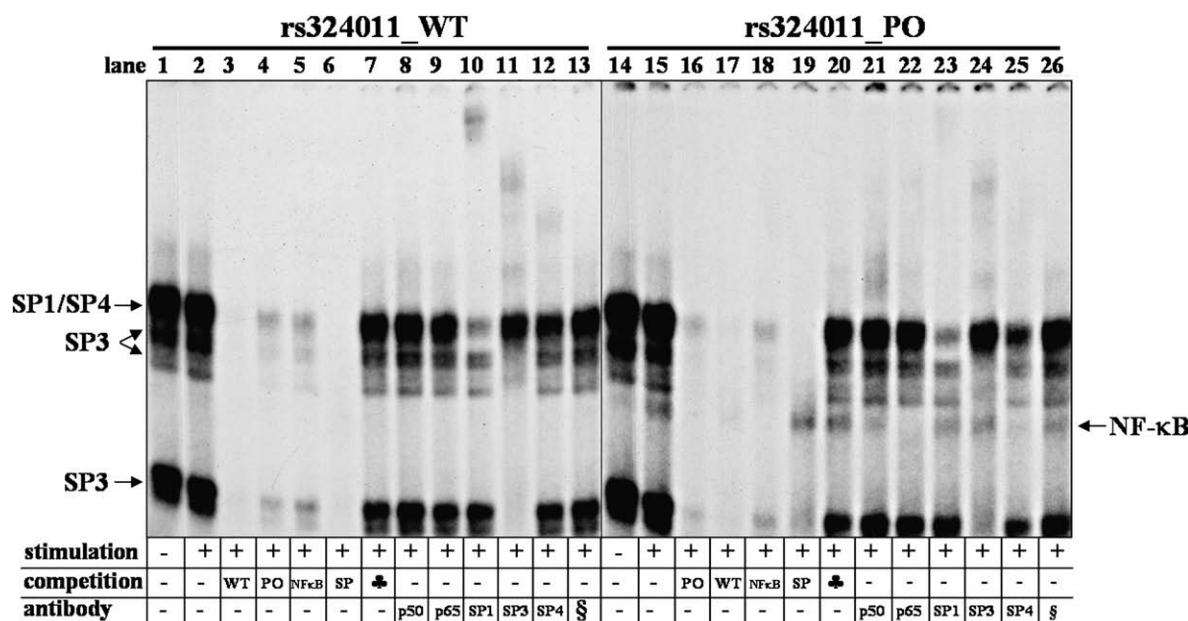


FIG 2. Polymorphic allele of rs324011 in *STAT6* intron 2 induces a novel binding site for NF-κB in a T-cell line. EMSA analysis with a *STAT6* probe either carrying the wild-type C (rs324011_WT) or the polymorphic T (rs324011_PO) allele of SNP rs324011 was performed with nuclear extract from Jurkat T cells (5 μg). ♣, Competition experiment using an unrelated oligo; §, supershift experiment using an unrelated antibody.

region harboring rs324011 is located 5' of the putative ATG site, it could also be part of an alternative promoter. However, our own experimental data examining the promoter activity of that region *in vitro* (data not shown) cannot confirm this hypothesis.

Previous studies determining the *STAT6* promoter were performed in mouse fibroblasts.¹¹ Because *STAT6* is critical for T_H2-cell differentiation and expression of cytokines regulating IgE production, we analyzed *STAT6* promoter activity in T cells. In human cells, as in mouse fibroblasts, *STAT6* promoter activity was highest when we used the minimal *STAT6* promoter construct (pBP86, 1.1 kb) and lower with the bigger constructs, suggesting additional repressive regulatory elements in the extended *STAT6* promoter region. When intron 2 (carrying the wild-type allele at rs324011) was added to our *STAT6* promoter vectors, a significant decrease in transcription activity of *STAT6* was observed. Thus, this intronic region may act as a silencing element.

To study further the effect of rs324011 alleles on promoter activity, the largest and thus most *in vivo*-like *STAT6* promoter construct (pBP78, 5.6 kb) was used. Luciferase expression significantly increased in the presence of the polymorphic T allele independent of the cloning orientation of intron 2 (5'3' orientation of intron 2, Fig 1, B; 3'5' orientation of intron 2, data not shown). Because this effect was not observed with the minimal promoter pBP86 (1.1 kb), the rs324011 site may potentially interact with distant *STAT6* promoter regions. One possible explanation is that the intronic region forms a loop to interact physically with a distant part of the *STAT6* promoter. Alternatively, NF-κB binding to the polymorphic allele of rs324011 in intron 2 may alter the 3-dimensional structure of *STAT6* or the epigenetic signature of the *STAT6* locus. However, these effects were not further studied here.

As in promoters, transcription factors direct intronic transcription effects. We observed profound changes in the distinct binding of NF-κB (p50 and p65) in the presence of the T allele at

rs324011. The allele-specific binding of NF-κB was not only detectable in a T-cell line but also found in the nuclear environment of primary CD4⁺ T cells. Members of the NF-κB family are known to control many genes crucial for immune and inflammatory responses²¹ also relevant for the development of atopic diseases.²² Interestingly, previous studies have described a functional synergism of *STAT6* and NF-κB in gene regulation of the IgE germline gene transcript.²³⁻²⁶ Thus, the allele-specific creation of an additional and novel NF-κB binding site within a regulatory region of *STAT6* may represent a new feedback mechanism by which NF-κB could directly influence the expression of its interaction partner *STAT6*.

Allele-specific NF-κB binding to rs324011 in the intron 2 regulatory region affected not only *STAT6* promoter activity *in vitro* but also mRNA expression of *STAT6* *ex vivo*. However, the *STAT6* gene gives rise to not just 1 mRNA isoform but many. Four functional *STAT6* mRNA isoforms had previously been described in human beings: *STAT6* (including all predicted exons), *STAT6a* (missing parts of the 3' untranslated region (UTR)), *STAT6b* (missing exon 4), and *STAT6c* (missing parts of exon 16).^{11,27,28} During the course of our study, we identified 2 further *STAT6* splice variants induced by intron retention: *STAT6d*, including, in addition to expected exonic regions, intron 17 but not intron 18; and *STAT6e*, including both these intronic regions. Of note, a *STAT6* homolog gene has previously been suggested by Tang et al,²⁹ but this is not supported by our data.²⁹ Rather, this *STAT6* homolog seems to be the isoform *STAT6e*, yet using an alternative transcription start codon. Thus, at least 6 *STAT6* isoforms exist. In our experiments, we studied the 2 novel isoforms *STAT6d* and *STAT6e* separately from the pool of previously known isoforms (non-*STAT6d*/*STAT6e*). Analyzing mRNA levels in 239 individuals, the polymorphic allele of rs324011 increased mRNA expression levels in all 3 splice variants, but allele-specific effects were stronger and significant for

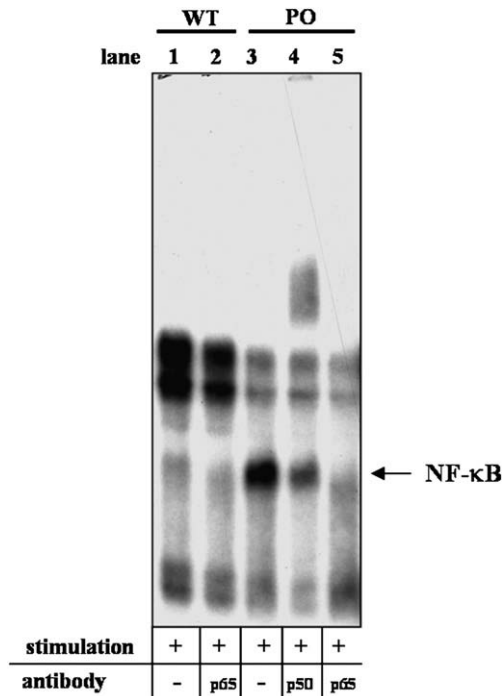


FIG 3. Polymorphic allele of rs324011 in *STAT6* intron 2 induces a novel binding site for NF- κ B in primary T cells. EMSA analysis with a *STAT6* probe either carrying the WT or the PO of rs324011 was performed by using nuclear extract from freshly isolated primary CD4⁺ T cells stimulated with PMA/ionomycin (50 ng/mL; 1 μ mol/L).

the newly identified *STAT6* mRNA isoforms STAT6d and STAT6e only. Comparable effects have recently been described in a genome-wide analysis of transcript isoforms, demonstrating that SNPs often alter the gene expression of certain isoforms.³⁰

Intron retentions have been described to play a potential role in the development and treatment of other complex diseases. Previously, cysteine rich 61 had been associated with breast cancer,³¹ and aberrant intronic splicing of folypolyglutamate synthetase altered drug resistance in leukaemia.³² Because the splice variants STAT6d and STAT6e originate from a region relevant for the functional *STAT6* sarcoma homology 2 (SH2) domain necessary to bind phosphorylated tyrosine residues and in protein-protein interactions, it may be speculated that both isoforms could lead to a truncation of this domain. A truncation of the *STAT6* SH2 domain has already been described for a different *STAT6* isoform (STAT6c), which lacks 84 bp of exon 16.¹¹ STAT6c suppresses the dimer formation of endogenous *STAT6* and induces a decline in DNA synthesis, tyrosine phosphorylation, and the downregulation of antigen-presenting receptors (MHCII and Fc γ R1).²⁸ The function of the 2 novel *STAT6* splice variants induced by intron retention and their potential influence on the development of atopic diseases need to be further studied. They may interfere with the classic *STAT6* gene product on the RNA level, or splice variants may produce altered proteins. However, it is not yet understood whether they lead to functional proteins through the use of an alternative start codon, as it may have been suggested previously,²⁹ or whether a premature termination of translation is induced by intron 17, as predicted with the original ATG of *STAT6*.

Obviously and indisputably, the impact of rs324011 on the transcriptional activity of *STAT6* observed in our experiments is

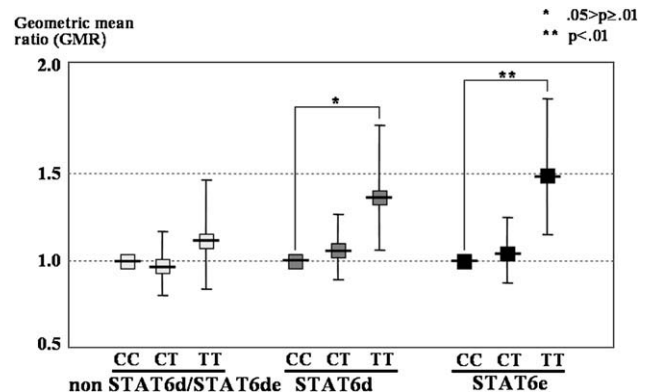


FIG 4. *Ex vivo* expression of *STAT6* splice variants are influenced by rs324011 alleles. Association between gene expression of 3 *STAT6* splice variants (non-STAT6d/STAT6e, STAT6d, STAT6e) and SNP rs324011 in intron 2 of the *STAT6* gene is displayed in GMRs with the 95% CI. *.05 > $P \geq .01$; ** $P < .01$.

subtle. Nothing else is to be expected from a regulatory SNP involved in a complex pathway of such a heterogeneous disease. The size of the observed changes is similar to those previously described for other regulatory polymorphisms.^{18,33} Functional studies on the *IL-13* C-1112T SNP, which is strongly and consistently associated with IgE regulation and asthma in many studies, showed a 2.8-fold allele-specific variance in gene expression.¹⁸ Similar effect sizes were observed for the *CD14* promoter polymorphism C-159T,³³ which is also a widely accepted candidate gene polymorphism for atopy and IgE regulation.³⁴

Taken together, our results suggest that rs324011, located within an intronic region of *STAT6*, influences its regulation and may therefore help explain the underlying biological mechanisms of the observed associations with increased total serum IgE levels⁶⁻⁹ and the risk for asthma on the population level.¹⁰ Because NF- κ B and *STAT6* are key players in many inflammatory mechanisms,²⁴ it may also be speculated that *de novo* NF- κ B binding in this particular regulatory region of *STAT6* regulates inflammatory responses, potentially not restricted to atopic diseases. The same SNP may also be involved in other illnesses in which *STAT6* acts as a transcription factor. It may even have evolutionary beneficial effects in some environments, such as in the presence of parasite infections. However, only further studies may prove or disprove these hypotheses.

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Key messages

- Our results suggest that the genotype status of the polymorphism rs324011 located in a regulatory intronic region of *STAT6* strongly influences the transcriptional activity of *STAT6*. Furthermore, rs324011 seems to influence the expression of *STAT6* isoforms. This effect may potentially be caused by alterations in transcription factor binding.
- The observed allele-specific differences may help explain the results of previous association studies suggesting that *STAT6* SNPs contribute to IgE control and asthma susceptibility.

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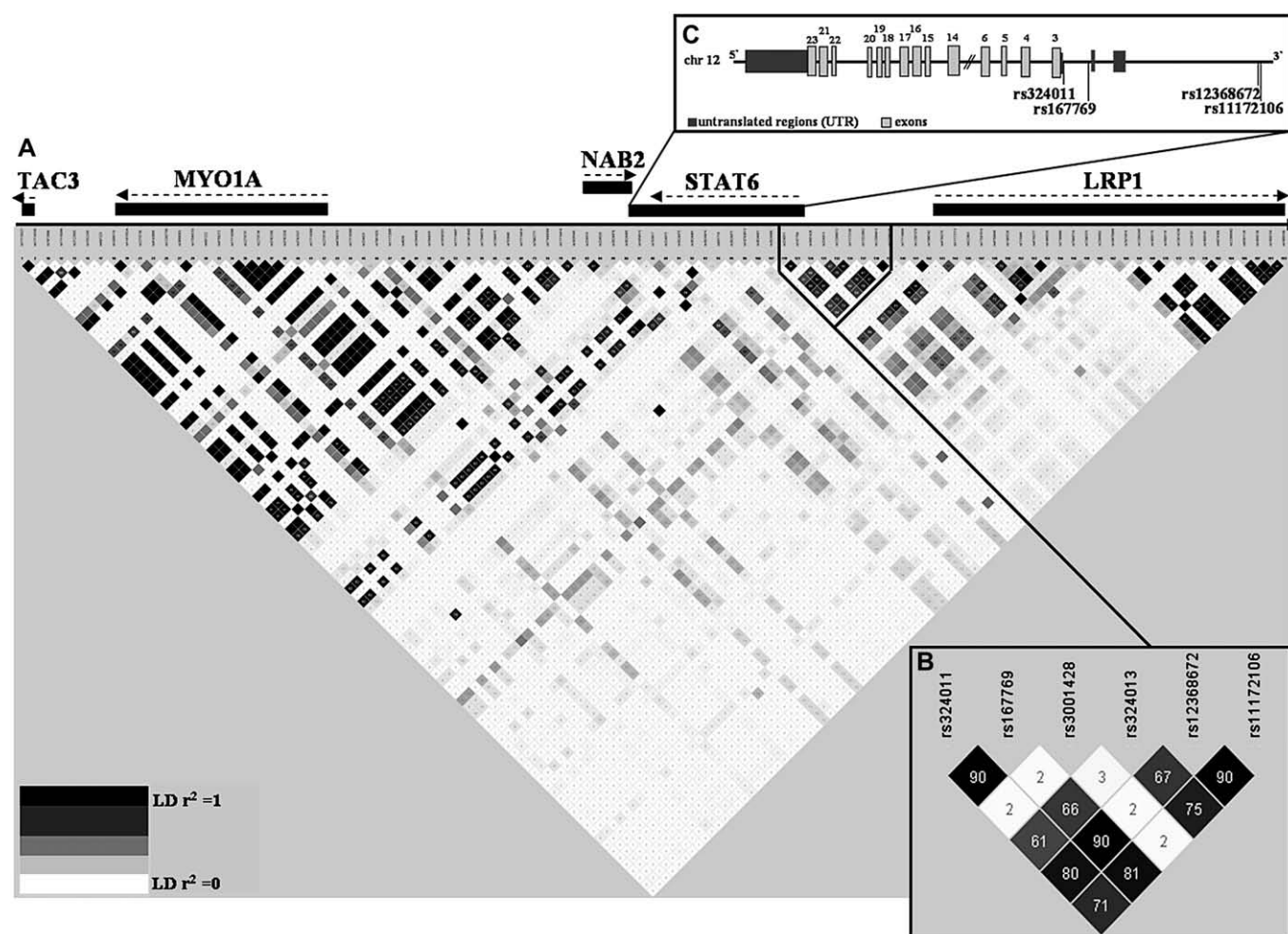


FIG E1. Localization of *STAT6* on chromosome 12q13 and linkage disequilibrium patterns (r^2 plots). **A**, Position of the *STAT6* gene on chromosome 12q13 in relation to adjacent genes *Tachykinin 3*, *Myosin IA*, *Nerve growth factor-induced clone 1A-binding protein 2* and *Low density lipoprotein receptor-related protein 1* and position of frequent *STAT6* SNPs (minor allele frequency > 0.03) and linkage disequilibrium (r^2 plot) from the HapMap database. **B**, Gene structure of *STAT6* indicating the location of SNPs in linkage disequilibrium with rs324011. **C**, Linkage disequilibrium (r^2 plot) between SNPs rs324011 and rs12368672 recently associated with elevated total IgE levels by the genome-wide analysis study.^{E1} Colors in the linkage disequilibrium (LD) plot from Haploview: white ($r^2 = 0$), shades of gray ($0 < r^2 < 1$), and black ($r^2 = 1$).

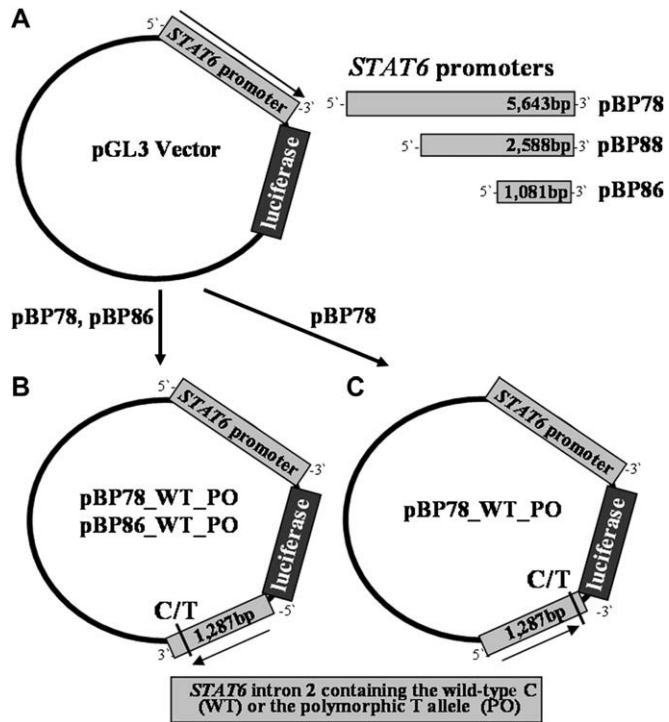


FIG E2. Generation of the *STAT6* luciferase constructs. **A**, *STAT6* promoter constructs pBP78 (5.6 kb), pBP88 (2.8 kb), and pBP86 (1.1 kb). **B**, *STAT6* intron 2 was cloned 5'3' into the *STAT6* pBP86 and pBP78 promoter constructs downstream of the luciferase gene carrying either the wild-type C or the polymorphic T allele. **C**, *STAT6* intron 2 carrying either the wild-type C or the polymorphic T allele was cloned into the pBP78 promoter construct in the 3'5' orientation.

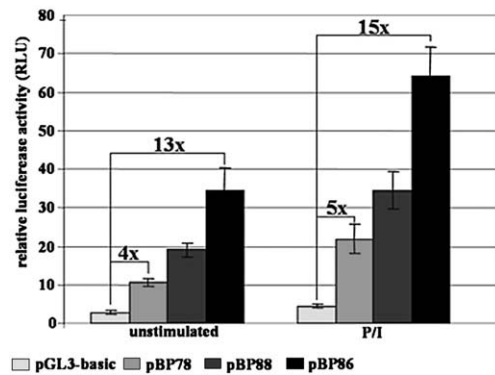


FIG E3. *STAT6* promoter activity in human cells. Jurkat T cells were transiently transfected with either the pGL3-basic vector or the *STAT6* promoter constructs pBP78, pBP88, and pBP86. Cells were left in medium or stimulated with PMA/ionomycin (P/I = 12.5 ng/mL; 0.25 μ mol/L) 3 hours after transfection and harvested 24 hours after transfection (N = 10). Luciferase activity was normalized for transfection efficiency by using the control plasmid pRL-TK. Relative luciferase activity in relative light units (RLU).

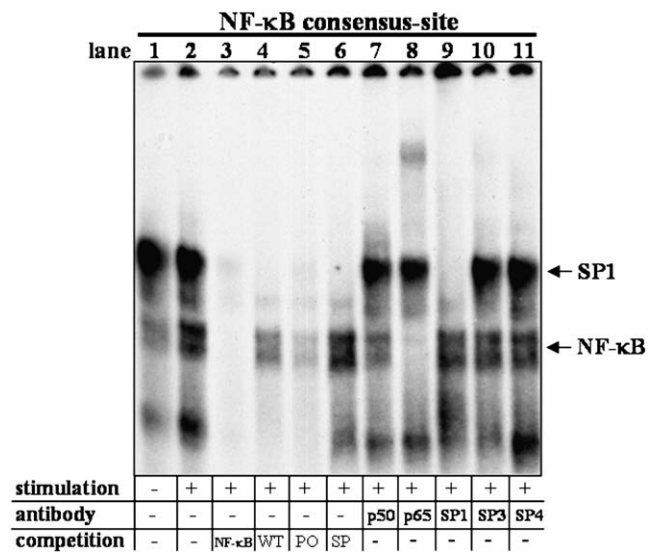


FIG E4. EMSA analysis with a NF- κ B consensus sequence^{E2} using nuclear extract (5 μ g) from Jurkat T cells either left in medium or stimulated with PMA/ionomycin (50 ng/mL; 1 μ mol/L). The competitors (100-fold molar excess) and supershift antibodies (4 μ g) are noted below the respective lanes.

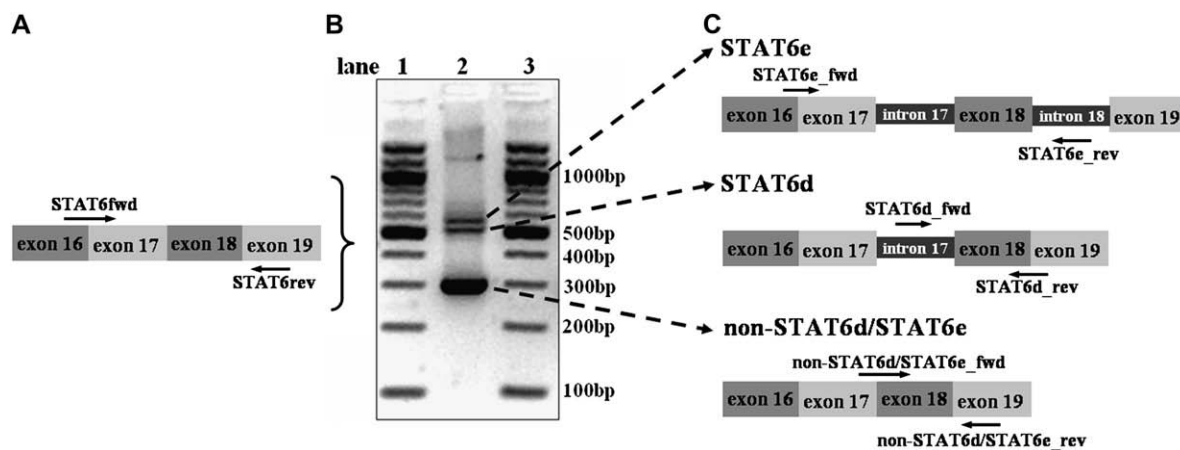


FIG E5. Quantitative real-time PCR of *STAT6* splice variants (non-STAT6d/STAT6e, STAT6d, STAT6e). **A**, Quantitative mRNA analyses were first performed by using a forward primer at the *STAT6* exon 16/17 border and a reverse primer in exon 19. **B**, Three PCR products were observed containing the predicted exonic regions of *STAT6* (further referred to as *non-STAT6d/STAT6e*) and 2 novel splice variants, STAT6d and STAT6e. **C**, Isoform-specific primers were used for all further analyses.