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This information is current as of February 24, 2022.

J Immunol 2013; 190:4946-4955; Prepublished online 15 April 2013;
doi: 10.4049/jimmunol.1203014
http://www.jimmunol.org/content/190/10/4946

Supplementary Material http://www.jimmunol.org/content/suppl/2013/04/15/jimmunol.1203014.DC1

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The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Control of the STAT6–BCL6 Antagonism by SWAP-70 Determines IgE Production

Tatsiana Audzevich, Glen Pearce, Michael Breucha, Gamze Günal, and Rolf Jessberger

Asthma and allergies are major health concerns in which Ig isotype E plays a pivotal role. Ag-bound IgE drives mast cells and basophils into exocytosis, thereby promoting allergic and potentially anaphylactic reactions. The importance of tightly regulated IgE production is underscored by severe immunological conditions in humans with elevated IgE levels. Cytokines direct IgH class-switching to a particular isotype by initiation of germline transcription (GLT) from isotype-specific intronic (I) promoters. The switch to IgE depends on IL-4, which stimulates GLT of the Iε promoter, but is specifically and strongly impaired in *Swap-70*$^{-/-}$ mice. Although early events in IL-4 signal transduction (i.e., activation of the JAK/STAT6 pathway) do not require SWAP-70, SWAP-70 deficiency results in impaired Iε GLT. The affinity of STAT6 to chromatin is reduced in absence of SWAP-70. Chromatin immunoprecipitation revealed that SWAP-70 binds to Iε and is required for association of STAT6 with Iε. BCL6, known to antagonize STAT6 particularly at Iε, is increased on Iε in absence of SWAP-70. Other promoters bound by BCL6 and STAT6 were found unaffected. We conclude that SWAP-70 controls IgE production through regulation of the antagonistic STAT6 and BCL6 occupancy of Iε. The identification of this mechanism opens new avenues to inhibit allergic reactions triggered by IgE. ***The Journal of Immunology*****, 2013, 190: 4946–4955.**

Asthma and allergies are major health concerns with 5–10% of the world's population affected, and intense efforts are under way to modulate the underlying molecular pathways. IgE, together with Ag, drives mast cells and basophils into exocytosis, thereby potentially causing allergic and anaphylactic reactions. The importance of IgE in these processes is underscored by the severe immunological conditions in humans with elevated IgE production (1). However, the precise molecular mechanisms and controls underlying IgE production remain unclear.

The specific switch to expression of IgE is tightly controlled and triggered by stimulation of B lymphocytes through the IL-4R (2). STAT6, a member of the STAT protein family, is central to IL-4 signal transduction (3–6). STAT6 becomes phosphorylated by activated JAK1 and/or JAK3, dimerizes, and translocates into the nucleus where it binds to the germline IgE promoter (Iε). In this study, STAT6 promotes transcription of germline ε transcripts, which direct the Ig class-switch machinery to the IgE switch region (7, 8). *Stat6*$^{-/-}$ mice are deficient in IgE production and strongly impaired, but not entirely deficient, in IgG1 production (9). Class-switch recombination to IgG1, IgE, and other Ig classes occurs by deletion of sequences between two isotype-specific switch (S) regions and thus is irreversible. How STAT6 is targeted to the germline ε promoter and hence how specific switching to IgE is initiated is unknown. The effect of STAT6 on Iε is antagonized by BCL6 (10, 11), a POZ/zinc-finger transcription factor with mostly repressor activity (12). BCL6 and STAT6 bind to a largely overlapping sequence within Iε. In addition to competing for promoter binding, BCL6 also actively mediates repression by interacting with other proteins such as corepressors (13–15). Most other STAT6 binding sites in the genome appear not to be repressed by BCL6 (10). Therefore, the balance between STAT6 and BCL6 at Iε is decisive for IgE production and thus is key to the development of allergic reactions.

SWAP-70 was originally isolated from activated B cell nuclear extracts associated with a multiprotein complex, which recombines S regions in vitro (16). *Swap-70*$^{-/-}$ mice show greatly reduced IgE levels in serum before and after challenge by the nematode *Nippostrongylus brasiliensis*, and cultured splenic B cells from these mice produce ~8-fold less of secreted IgE upon stimulation by anti-CD40 and IL-4 (17). Secretion of other Ig isotypes (e.g., LPS-induced production IgG2b, IgG3, and IgG2a) does not depend on SWAP-70, and CD40/IL-4R–dependent IgG1 secretion is only mildly decreased (1.4-fold) in *Swap-70*$^{-/-}$ B cell cultures (17). How SWAP-70 contributes specifically to the switch to IgE remained elusive and is the subject of this study, which revealed a new pathway of regulation, the control of the antagonistic STAT6 and BCL6 occupancy of the Iε by SWAP-70.

Materials and Methods

Mice

Mice were bred and maintained under pathogen free conditions in the animal facility at the Faculty of Medicine, Dresden University of Technology (Dresden, Germany). The *Swap-70*$^{-/-}$ (C57BL/6N) strain was described previously (17). Mice were used at 4–8 wk of age.

Splenic B lymphocyte cultures

Spleens, dissected from wild-type (wt) or *Swap-70*$^{-/-}$ mice, were rinsed in ice-cold PBS and mashed between two glass slides to obtain a crude cell suspension, which was filtered through a 100-μm cell strainer (BD Biosciences). Cells were spun at 1500 × *g* for 5 min at 4°C. RBCs were

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Received for publication November 1, 2012. Accepted for publication March 19, 2013.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft through the SFB655 (B4) (to R.J.) and by a grant from the Thyssen Foundation.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; GLT, germline transcription; HPRT, hypoxanthine phosphoribosyltransferase; IB, immunoblotting; NP-40, Nonidet P-40; PBS-S, 0.05% saponin in PBS; PI, protease inhibitor; RIPA, radioimmunoprecipitation assay; wt, wild-type.

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depleted by lysis in ACK lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 100 mM EDTA [pH 7.2–7.4]) (3 ml/spleen). B cells were further purified from the splenocyte suspension by negative selection using MACS beads (Miltenyi Biotec). Purified B cells or total splenocytes were cultured at a concentration of 0.3–0.5 × 10^6 cells/ml in 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS hybridoma–serum-free medium supplemented with 1.6–3 ng/ml IL-4 produced by the X63–IL-4 cell line (optimal dose; determined previously by titration and FACS analysis of switching to IgG1 and IgE). If required, the cells were cultured on a layer of CD40L-surface expressing L47 cells (18).

Flow Cytometry

Cells were harvested by centrifugation, washed once in cold PBS supplemented with 1% FCS, and blocked for 10 min with 5% FCS in PBS on ice. The appropriate Abs (Ab: anti-mouse CD40R-PE, anti-mouse IgE-FITC [R35-72], anti-mouse IgG1-PE [A85-1], anti-mouse CD45R/B220-PE [RA3-6B2], CD45R/B220-PerCP [RA3-6B2] [BD Biosciences], or anti-mouse IL-4R biotinylated [R&D Systems]) were added, left incubating on ice for 20–30 min, and followed by washing twice with 1% FCS in PBS. Staining was assessed using the LSRII Flow Cytometer and FACSDiva software (BD Biosciences), and data were analyzed with FlowJo 6.1.1 software (Tristar). To measure surface Ig production, cells were subjected to stripping in (85 mM NaCl, 5 mM KCl, 10 mM EDTA, and 50 mM $NaCH_3COO$ [pH 4]), incubated for 1 min, washed once with cold PBS, and stained as described above. Phospho-STAT6 staining using anti-mouse phosho-STAT6 (Y641)(J71-773.58.11) (BD Biosciences) was performed after cell permeabilization with 90% ice-cold methanol, followed by two PBS wash steps and 30 min blocking in 5% goat serum in PBS, using the Ab dilution recommended by manufacturer (20 μl Ab/1 × 10^6 cells).

Immunofluorescence

MACS-purified B cells 1–2 × 10^6 cells/ml were stimulated by 2 ng/ml IL-4 for 0 or 15 min at 37°C. Cells were fixed with 1% formaldehyde for 10 min at 37°C, washed in cold PBS, and resuspended in PBS at a concentration of 1 × 10^6 cells/ml. One hundred microliters of this suspension was applied to a slide to perform cytospin for 4 min, 700 rpm. Cells were additionally fixed on the slides by incubation with 1% formaldehyde for 5 min at room temperature, followed by quenching with 125 mM glycine in PBS for 5 min. Samples were washed twice with PBS, and cells were permeabilized by 0.05% saponin in PBS (PBS-S) for 15 min, followed by a wash in PBS-S and blocking for 30–60 min with 3% BSA in PBS-S. An 1:100 dilution of anti-STAT6 Ab (Abcam) was prepared in the blocking solution, and cells were stained for 1–2 h at room temperature. Samples were washed six times for 3 min each with PBS-S before a secondary Ab goat–anti-rabbit-Cy3 (1:500) (Dianova) was applied and incubated for 30 min at 37°C. Samples were washed once with PBS-S, and 1 μg/ml DAPI solution was added for 5–10 min. Samples were washed six times for 3 min each with PBS-S, mounted with fluoromount G, and analyzed under fluorescent (Axiophot; Zeiss) or confocal microscope (Zeiss LSM 510).

For anti–SWAP-70 staining, cells prestimulated with IL-4 or IL-4/CD40L were adhered on the poly-L-lysine–coated slides for 30–40 min, washed once with PBS, fixed, and permeabilized with methanol:acetone: formaldehyde 19:19:2 mixture for 90 s, washed with PBS, and stained as described above using affinity purified anti–SWAP-70 Ab (1 μg/ml) (19). 0.1% Tween 20 in PBS was used as washing buffer.

Real-time and semiquantitative RT-PCR

To assess germline and productive transcription in CD40L/IL-4–stimulated splenic/B cell cultures, RNA was extracted at day 0–5 with TRIzol reagent (Invitrogen) using 10 × 10^6 cells/sample. Two micrograms of RNA from each sample was treated with DNase (Promega), and cDNA was generated using SuperScript II reverse transcriptase and oligo$(dT)_{20}$ primer (Invitrogen) in a final volume of 20 μl. One microliter of cDNA was amplified in a 20-μl real-time PCR using QuantiTect SYBR Green PCR Kit in a Rotor-Gene PCR cycler (GC-300; Corbett Research). Amplification of GAPDH was used for normalization and quantification of relative gene expression. All real-time PCRs were performed in duplicates. The primers used (20) were as follows: GLTγ1 forward, 5′-TCGAGAAGCCTGAGGAATGT-3′; GLTγ1 reverse, 5′-ATAGACAGATGGGGGTGTCG-3′ (product size, 100 bp); GLTε forward, 5′-CTGGCCAGCCACTCACTTAT-3′; GLTε reverse, 5′-CAGTGCCTTTACAGGGCTTC-3′ (product size, 100 bp); hypoxanthine phosphoribosyltransferase (HPRT) forward, 5′-CACAGGACTAGAACACCTGC-3′; HPRT reverse, 5′-GCTGGTGAAGACCTCT-3′ (248p); Bcl6 forward, 5′-CACACTCGAATTCACTCTG-3′; and Bcl6 reverse, 5′-TATTGCACCTTGGTGTTGG-3′.

Extract preparation and immunoblotting

Protein extracts used for immunoblotting (IB) from splenic or purified B cells were derived by cell lysis (30 min on ice) in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl [pH 8], 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40 [NP-40], and 0.1% SDS, supplemented with 1 mM PMSF, 1 mM Na_3VO_4, and 50 mM NaF) to obtain total cell extracts or cell fractionation to yield cytoplasmic and nuclear fractions. To prepare cytoplasmic and nuclear protein extracts, cells were collected by centrifugation and washed once in 1 ml ice-cold 1 mM Na_3VO_4 in PBS and resuspended on ice in solution A (10 mM Tris HCl [pH 8], 100 mM NaCl, 1 mM EDTA, and 1 mM DTT) supplemented with protease and phosphatase inhibitors: protease inhibitor (PI) mixture (Roche), 10 mM $Na_2S_2O_5$, 10 mM NaF, 10 mg/ml TLCK, 1 mM Na_3VO_4, 1 mM PMSF, and 0.5 mM spermidine. One milliliter of solution A was used per 1.5 × 10^8 splenocytes/B cells. $MgCl_2$ and NP-40 were added to a final concentration of 2 mM and 0.1%, respectively, and samples were lysed by brief vortexing (2 × 10 s with 15-s interval) and then centrifuged at 1500 × *g* for 1 min at 4°C to yield pelleted nuclei and soluble cytoplasmic fraction. The nuclear pellet was resuspended in 50–200 μl solution B (solution A without NaCl including protease and phosphatase inhibitor mixture). $(NH_4)_2SO_4$ (2.5 M) was mixed with the nuclear suspension to a final concentration of 250 or 150 mM. Samples were mixed and left on ice for 40–60 min, nuclear debris was sedimented at 100,000 × *g* for 35 min at 4°C in a benchtop ultracentrifuge. The supernatant obtained was used as a nuclear protein extract.

Proteins separated on SDS-PAGE were transferred to the nitrocellulose membrane (Amersham Biosciences) by semidry blotting. Abs used for immunodetection were as follows: anti-GAPDH (6C5) mouse monoclonal (Santa Cruz Biotechnology), anti-JAK1 rabbit, anti–phosho-JAK1 rabbit (Cell Signaling Technology), anti-STAT6 (monoclonal YE361) rabbit, anti–phosho-STAT6 (Y641) mouse monoclonal (Abcam), anti-SMC3 rabbit, anti-Sp1 (affinity purified) rabbit (Bethyl Laboratories), and anti–SWAP-70 rabbit (16).

Chromatin immunoprecipitation

Purified B cells from wt or *Swap-70*$^{-/-}$ mice were stimulated for 4 h at density of 2 × 10^6 cells/ml by IL-4 (2 ng/ml) and then cross-linked with 1% formaldehyde for 10 min at room temperature. A total of 2 × 10^7 cells per condition were used. Cross-linking was quenched by incubation with 0.125 M glycine for 5 min, and cells were washed with ice-cold PBS twice, resuspended in 1 ml chromatin immunoprecipitation (ChIP) lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris HCl [pH 8.1], and PI mixture), and then incubated on ice for 10–15 min. The lysate was sonicated on ice to shear DNA using Biorupter sonicator (Diagenode) at highest amplitude 2 × 10 min with a pulse of 10-s sonification and a 30-s interval. DNA fragments of 100–500 bp were obtained. Sonicated samples were centrifuged at 16,000 × *g* for 10 min at 15°C, and 200 μl of the supernatant was used per ChIP reaction. Supernatant (50 μl) was kept as input control. Lysates were 10 times diluted with ChIP dilution buffer (0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, and PI) and precleared with 80 μl slurry of protein A–agarose beads (blocked by 0.2 mg/ml salmon sperm DNA and 0.5 mg/ml BSA for 1–3 h) on a rotating wheel at 6 rpm for 1 h at 4°C. Beads were pelleted in a benchtop centrifuge at 1000 rpm for 1 min at 4°C, precleared extract was transferred into new tube, Abs (1 μg anti–SWAP-70, 2 μg anti-STAT6 (M20), 2 μg anti-BCL6 (N-3), or 1 μg rabbit IgG [Santa Cruz Biotechnology]) were added, and samples were incubated rotating at 6 rpm overnight at 4°C. Sixty microliters of blocked protein A beads were added to the ChIP reactions and then incubated rotating at 6 rpm for 1 h at 4°C. Supernatant was removed, and beads were washed rotating at 6 rpm for 4 min at room temperature with 1 ml of the buffers (containing PI) listed below: 1× low salt immune complex wash buffer, 1× high salt immune complex wash buffer, 1× LiCl immune complex wash buffer, and 2× Tris-EDTA buffer. Elution buffer (100 mM $NaHCO_3$ and 1% SDS) was prepared fresh, and immune complexes were eluted from the beads twice with 250 μl at 50°C. Twenty microliters of 5 M NaCl was added to the combined eluates (500 μl) and input control samples 10 times diluted with ChIP dilution buffer and then incubated for 6 h at 65°C to reverse protein–DNA cross-links. Samples were treated with proteinase K (20 μg; + 10 μl 1 M Tris HCl [pH 6.5] and 10 μl 0.5 M EDTA/tube) for 1 h at 45°C. DNA was recovered by phenol/chloroform extraction and overnight precipitation at −20°C with 2 volumes of ethanol, 1/3 volume 7.5 M NH_4CH_3COO, and 2 μl of 10 mg/ml glycogen. Pellets were washed with 70% ethanol, air-dried, and resuspended in 50 μl Tris-EDTA containing 0.1 mg/ml RNAase. PCR was performed using the following primer pairs: Iε germline promoter

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region (21) (Iε forward, 5′-CTAGAAAGAGGCCTACACCTG-3′, and Iε reverse, 5′-GCCAGACTGTTCTTATTCG-3′ [243 bp]); Iγ1 promoter (Iγ1 forward, 5′-AGGGGGTGAGGGGGAGTCCA-3′, and Iγ1 reverse, 5′-CCCCCAAAGGCCCAGGTGC-3′ [145 bp]); CD23 promoter (CD23 forward, 5′-TGGCATCGCTGACTCTCCAACA-3′, and CD23 reverse, 5′-CTGGGTGGCCACAGCACACA-3′ [118 bp]); control promoter without STAT6 binding site (21, 22) (Pr forward, 5′-TTTGAACTGGAGCTCAGCTGG-3′, and Pr reverse, 5′-TCCATGGTGCTAGCCATATGC-3′ [160 bp]; Iγ2a forward, 5′-CTGTCACCCACTTTCAATCCTG-3′, and Iγ2a reverse, 5′-GCGTGAAGAAGATTGCTGCTATT-3′ [148 bp]); BCL3 forward, 5′-TAGCCCAGGCTAGTCTCGAA-3′, and BCL3 reverse, 5′-CTGGCTGGCTGGAAAGATAG-3′; CD2 forward, 5′-CAAGACACCCCAGATGGTCT-3′, and CD2 reverse, 5′-TTCTGCTCTTCAGCCTTTCC-3′; IL-4Rα forward, 5′-CTGTGCCAGAAAGCAAAACA-3′, and IL-4Rα reverse, 5′-AAGGGATAGTTGCGCATGAC-3′; CD69 forward, 5′-CCATTCTCCGCTCTATTCCA-3′, and CD69 reverse, 5′-CATCTCTCCGTGG ACCACTT-3′; and CCND2 forward, 5′-CAAGCTGGAAGGGCAGTTAG-3′, and CCND2 reverse, 5′-AGAGGGCCTCGGAGAAGTAG-3′. PCRs were performed as 25-μl reactions containing 1–2 μl ChIP DNA. PCR products were resolved on 2% agarose gel.

Results

Impaired IgE production and Iε germline transcription by Swap-70$^{-/-}$ *B cells*

Previously, secreted IgE was found to be reduced in cultures of anti–CD40-stimulated splenic *Swap-70*$^{-/-}$ B cells (17). Surface Ig production on purified splenic B cells or on B cells within total splenocyte cultures was now analyzed by stimulating the cells with soluble IL-4 and mouse CD40L expressed on the surface of a fibroblast cell layer (L47 cells (18)) to induce class switch recombination to IgG1 and IgE. Supernatants were collected at days 5 and 6 of culture and analyzed by ELISA to determine the amount of secreted IgG1 and IgE. *Swap-70*$^{-/-}$ cultures had a slightly diminished secretion of IgG1 and a 10-fold decreased production of secreted IgE (Fig. 1A, 1B), consistent with previous results (17). Detection of surface IgG1 or IgE by FACS after 5 d of culture showed only slightly reduced numbers of IgG1^{+} B cells but

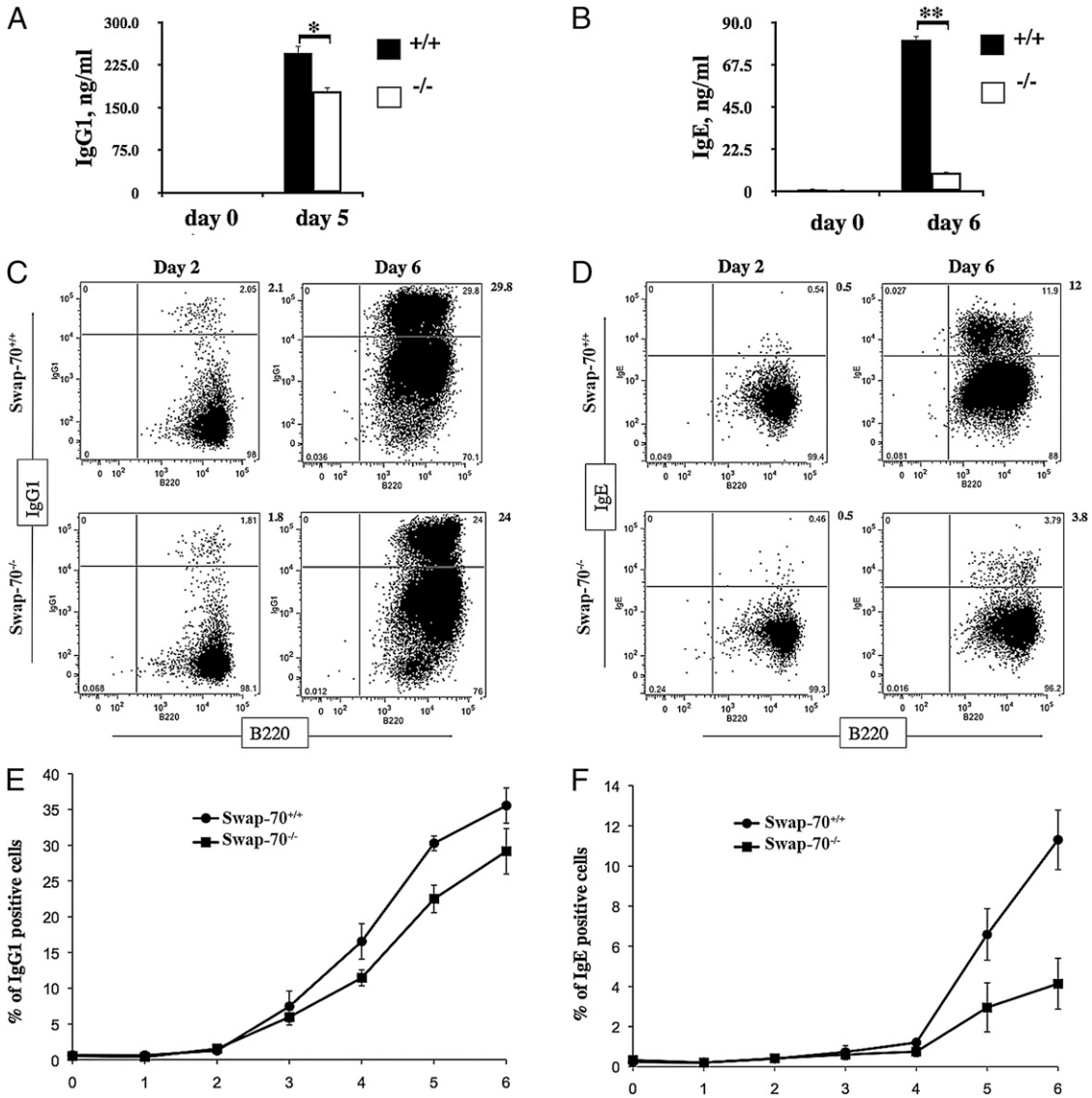

FIGURE 1. Secretory and surface production of IgE is impaired in *Swap-70*$^{-/-}$ B cells. IgG1 and IgE production by wt or *Swap-70*$^{-/-}$ splenocytes cultured for 6 d stimulated by CD40L (L47-CD40L cells) and IL-4 (2 ng/ml). (**A** and **B**) Secreted IgG1 and IgE measured in supernatants of cultured splenocytes by ELISA at day 5 or 6 of stimulation. $n = 4$, $*p < 0.05$, $**p < 0.01$. (**C** and **D**) FACS plots of CD45R/B220-PerCP and IgG1-PE or IgE-FITC stained splenocytes cultured for 2 or 4 d, respectively. Representative FACS plots are shown, $n = 4$ independent experiments. (**E** and **F**) Surface IgG1 and IgE expression detected by FACS in wt and *Swap-70*$^{-/-}$ splenocyte cultures (each time point represents the average of four independent experiments).

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4-fold less IgE$^+$ B cells in cultures from *Swap-70*$^{-/-}$ mice compared with those from wt mice (Fig.1 C–F). The generation of surface IgG3$^+$ B cells induced by LPS stimulation was normal (Supplemental Fig. 1). Thus, although SWAP-70 contributes little to IgG1 production, production of both secreted and surface IgE strongly depends on SWAP-70.

Because SWAP-70 was originally purified from nuclear extracts of class-switching B cells (23), we tested whether the absence of SWAP-70 affects germline transcription (GLT) at the IgE locus, the initial and essential nuclear process of class-switching. GLT targets class-switching to a specific C region H chain locus and is itself induced by a certain combination of activation stimuli (24, 25). B cells purified from 4- to 8-wk-old wt or *Swap-70*$^{-/-}$ mice were cultured in the presence of IL-4 on CD40-expressing, adherent mouse fibroblasts (L47 cells), and RNA was isolated from floating B cells every 24 h. Quantification of germline ε transcripts by real-time RT-PCR revealed a 2- to 5-fold downregulation of ε GLT transcripts in *Swap-70*$^{-/-}$ B cells at every time point from 48 h onward (Fig. 2A). Germline γ1 transcripts, which appeared earlier than the ε GLT, were downregulated 2- to 4-fold, although production of IgG1 was only very mildly affected by SWAP-70 deficiency, possibly indicating a less stringent requirement for efficient GLT at the γ1 locus. Indeed, IgE production is the most tightly regulated of all Ig classes, and despite overlapping induction pathways, different regulatory elements govern the switch to IgG1 and IgE (26, 27). Generation of IgE and its GLT specifically require signaling from the IL-4R (2, 7, 8, 27), whereas γ1 GLT depends less on IL-4R signaling (27). Therefore, we initially hypothesized a function for SWAP-70 in IL-4 signaling.

Normal IL-4 triggered JAK/STAT signaling in the absence of SWAP-70

IL-4 signal transduction is mediated through activation of JAK1 and JAK3. JAK-catalyzed STAT6 phosphorylation causes STAT6 dimerization or multimerization, nuclear translocation, and association of STAT6 with its specific promoters (e.g., the germline ε promoter Iε) (2). Upregulation of IL-4R and CD40R surface expression on splenic B cells upon stimulation by CD40L/IL-4 is normal in *Swap-70*$^{-/-}$ cells (Supplemental Fig. 2). JAK1 is thought to be the critical kinase in mature murine B cells, whereas JAK3 is barely expressed (28). Levels of phosphorylated JAK1 detected in splenic B cell extracts IB are similar in wt and *Swap-70*$^{-/-}$ cells (Fig. 2B, 2C). STAT6 activation was measured by intracellular FACS staining using anti–phosho-STAT6 Ab (Ab). The appearance of phosphorylated STAT6 in *Swap-70*$^{-/-}$ B cells shows similar kinetics and abundance (expressed as mean fluorescence intensity) to wt (Fig. 2D). These results were confirmed by IB with anti–phospho-STAT6 Ab and an Ab against total STAT6 protein on splenic B cell extracts (Fig. 2E, 2F). These results indicate that the initial steps of IL-4 signaling including

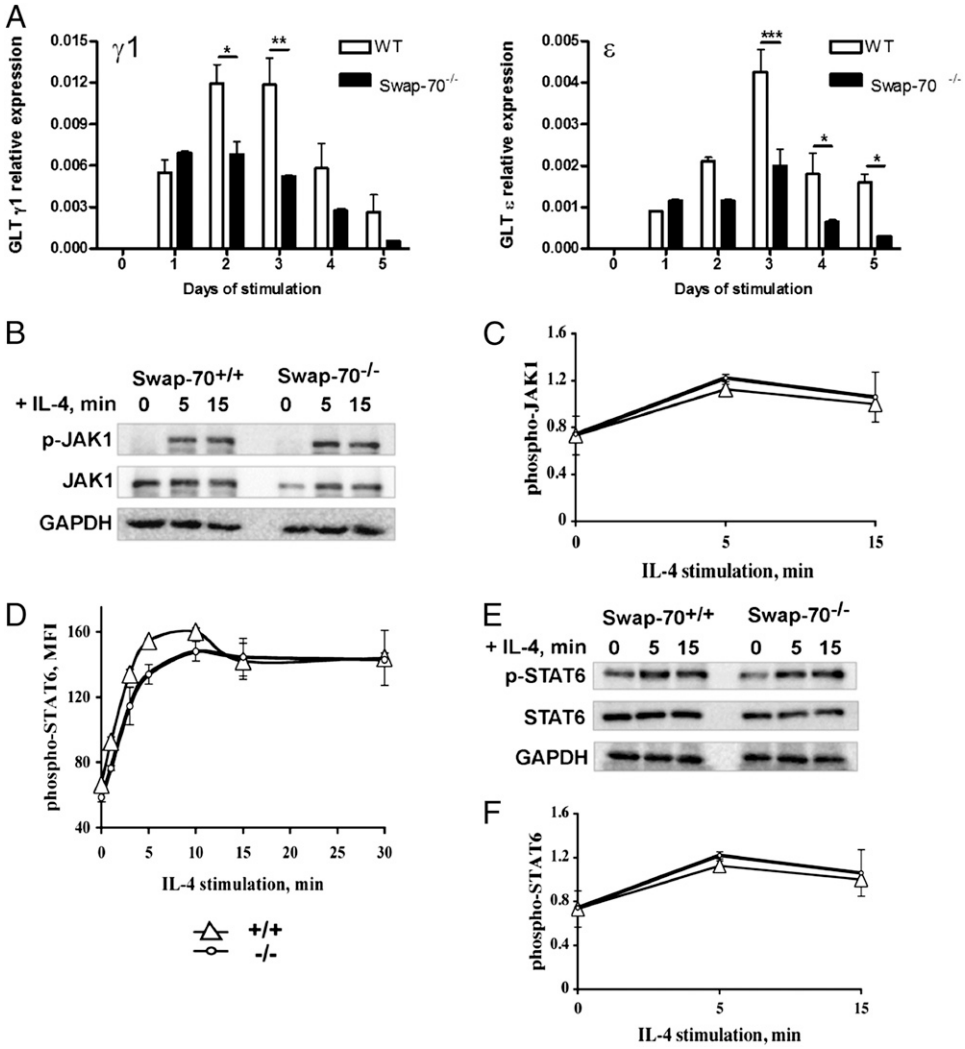

FIGURE 2. Germline transcription and IL-4 signaling in *Swap-70*$^{-/-}$ B cells. (**A**) Germline ε transcription is impaired in absence of SWAP-70. RNA expression in purified wt or *Swap-70*$^{-/-}$ B cells cultured and stimulated by CD40L and IL-4 for up to 5 d. RNA was prepared at the indicated time points. Real-time RT-PCR with γ1- or ε-specific primers was performed in three repetitions using each two independent RNA preparations. Germline ε transcription in wt and *Swap-70*$^{-/-}$ samples is shown. $n = 6$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**B–F**) JAK1 and STAT6 are phosphorylated normally in *Swap-70*$^{-/-}$ B cells. wt and *Swap-70*$^{-/-}$ B cells were stimulated for the indicated times by 2 ng/ml IL-4 to analyze JAK1 and STAT6 phosphorylation. (B) JAK1 phosphorylation in B cell extracts detected by IB; (C) quantification of phospho-JAK1 by normalization to total JAK1 and GAPDH levels; average of three experiments is shown. (D) STAT6 phosphorylation in B220$^+$ cells assayed by FACS; the strength of phosphorylation is reflected in the mean fluorescence intensity (MFI) units. (E) Analysis of STAT6 phosphorylation in total protein extracts by IB (representative of three independent experiments is shown); (F) STAT6 phosphorylation normalized to STAT6 and GAPDH levels; average of three experiments is shown.

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receptor expression, JAK1 activation, and STAT6 phosphorylation are not affected by SWAP-70 deficiency.

To test for direct physical interaction between STAT6 and SWAP-70 in vitro, proteins were purified from an insect cell expression system. No interaction was detected irrespective of whether STAT6 dimerization was induced by in vitro phosphorylation or not. Coimmunoprecipitation experiments either from activated B cells or from IL-4–responsive NIH3T3 cells expressing tagged versions of both proteins also did not yield coprecipitates of the two proteins. Like in B cells, both proteins translocate to NIH3T3 nuclei upon IL-4 treatment of the cells.

Tyrosine phosphorylation of STAT proteins enables their dimerization and nuclear localization (29, 30). Control of cytoplasmic/nuclear shuttling may depend on diverse mechanisms, varying for specific STAT proteins, including methylation (31), association with proteins carrying nuclear localization signals (29), serine phosphorylation (32) and rearrangements of cytoskeletal components (33, 34). Nuclear translocation of some STAT proteins, such as STAT5 and STAT3, depends on their association with the GTPase activating protein, MgcRacGAP, and requires Rac1 (35, 36). Considering the cytoplasmic interaction of SWAP-70 with Rac and the function of SWAP-70 in cytoplasmic cytoskeletal rearrangements described by us (37–40), we speculated that SWAP-70 may participate in the transport of STAT6 into the nucleus.

Efficient STAT6 binding and prevention of BCL6 binding to Iε requires SWAP-70

Cell fractionation from wt or *Swap-70*$^{-/-}$ splenocytes showed that in response to IL-4 stimulation STAT6 accumulates in wt nuclear extracts prepared by salt extraction of nuclei isolated from detergent-lysed cells. However, less STAT6 is found in nuclear extracts from *Swap-70*$^{-/-}$ splenocytes (Fig. 3A, 3B). SWAP-70 itself is known to localize to the nuclei of B cells as also seen here by IF staining and IB (Fig. 3A, Supplemental Fig. 3). Staining of STAT6 in fixed and permeabilized B cells demonstrated no obvious difference in nuclear STAT6 in IL-4 activated wt and *Swap-70*$^{-/-}$ cells (Fig. 3C), in agreement with normal STAT6 phosphorylation described above. These data together suggest that although STAT6 translocates properly into *Swap-70*$^{-/-}$ B cell nuclei, it does not associate as tightly with the chromatin. Weaker chromatin association could explain loss of some nuclear STAT6 during detergent-induced cell lysis and nuclear extract preparation, causing the results shown in Fig. 3A and 3B. The presumably weaker association of STAT6 with chromatin in *Swap-70*$^{-/-}$ cells raises the question as to whether this correlates

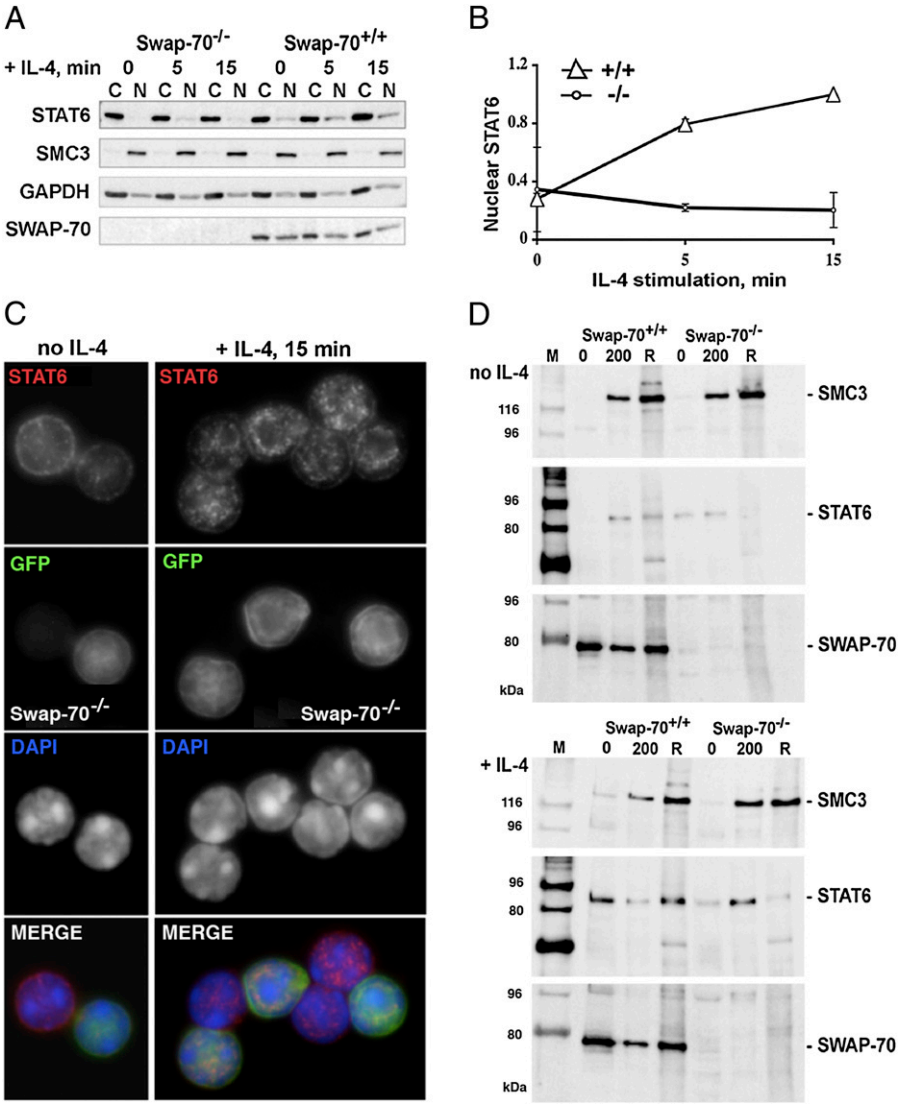

FIGURE 3. STAT6 translocates to the nucleus in absence of SWAP-70 but associates less tightly with chromatin. Splenocytes from wt or *Swap-70*$^{-/-}$ mice were stimulated for the indicated times with 2 ng/ml IL-4 to determine STAT6 nuclear translocation. (**A**) Levels of STAT6 in wt and *Swap-70*$^{-/-}$ cytoplasmic extract fraction (C) and nuclear extract fraction (N) detected by IB (representative of three independent experiments is shown), and GAPDH was used as a control cytoplasmic protein and SMC3 a nuclear protein. Extracts were prepared using NP-40 cell lysis to separate cytoplasm from nuclei, and subsequent high salt extraction of proteins from nuclei. Protein extract (20 μg) was loaded per well. (**B**) Nuclear STAT6 IB signal normalized to SMC3; the average of three experiments is shown. (**C**) Purified wt or *Swap-70*$^{-/-}$ B cells mice were stimulated for 15 min by 2 ng/ml IL-4, fixed, cytospun saponin-permeabilized, and then stained with anti-STAT6 (representative of four independent experiments). Transgenic mice expressing GFP from the chicken β-actin promoter in the SWAP-70–deficient strain were used to enable costaining of wt GFP^{-} and *Swap-70*$^{-/-}$ GFP^{+} B cells on the same slide. (**D**) wt or *Swap-70*$^{-/-}$ splenocytes were either left unstimulated (*upper*) or stimulated for 15 min by 2 ng/ml IL-4 (*lower*). Cells were disrupted by Dounce homogenization. Nuclear proteins were extracted by stepwise incubation of the nuclear pellet in extraction buffer supplemented with 0 or 200 mM ammonium sulfate and then finally with RIPA buffer (R). STAT6, SWAP-70, and SMC3 were detected by IB. M, Molecular mass marker (representative of three experiments).

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with poor binding to Iε and hence reduced Iε GLT and IgE production. STAT6 needs to bind with different affinity to different promoter regions, and the Iε requires high-affinity binding (27).

To examine the strength of association of STAT6 with chromatin in the presence or absence of SWAP-70, we performed differential extraction of chromatin. We disrupted cells by Dounce homogenization in a detergent-free hypotonic buffer to isolate nuclei and avoid detergent-induced leakage of loosely bound STAT6 from the nuclei. Proteins were sequentially extracted from the nuclei of IL-4–activated or nonactivated B cells by incubation first without added salt, then with 200 mM ammonium sulfate, and finally with SDS-containing RIPA buffer (Fig. 3D). The cohesin SMC3 served as a chromatin-associated nuclear protein control. As expected, generally much less STAT6 was extracted from the nuclei of nonactivated (upper set of panels) wt or *Swap-70*$^{-/-}$ cells than from nuclei of IL-4–treated cells (lower set of panels). Of that low amount of STAT6 in nonactivated nuclei, more can be extracted without added salt ("0") from *Swap-70*$^{-/-}$ than from wt nuclei, suggesting weaker chromatin association of STAT6 with chromatin. At 200 mM, salt about the same levels of STAT6 were extracted. However, thereafter, no STAT6 remained to be extracted from *Swap-70*$^{-/-}$ nuclei even under the most stringent conditions (i.e., with RIPA buffer), whereas some STAT6 in wt cells resisted the 200 mM salt and thus was extracted by RIPA buffer. This also indicates weaker affinity of STAT6 to chromatin in *Swap-70*$^{-/-}$ nonstimulated B cells. Following IL-4 stimulation (lower panels of Fig. 3D), without salt added, more STAT6 was extracted from wt than from *Swap-70*$^{-/-}$ and moderately more STAT6 dissociated from *Swap-70*$^{-/-}$ nuclei at 200 mM salt. Most significantly, after salt-mediated extraction, no detectable STAT6 remained to be extracted from *Swap-70*$^{-/-}$ nuclei with RIPA buffer. However, in the wt, a considerable fraction of STAT6 remained chromatin-associated during salt extraction and required RIPA buffer to be extracted from nuclei of IL-4–activated B cells. As expected, the control SMC3 was readily extracted by salt and by the subsequent RIPA buffer showing the stepwise extraction procedure to work. These data suggest weaker association of STAT6 to chromatin in the absence of SWAP-70 in stimulated and nonstimulated cells. Because STAT6 occupancy of the Iε promoter requires high-affinity binding (27), we hypothesized that in the absence of SWAP-70 STAT6 may be much reduced or absent from this promoter.

To test this, we performed ChIP on wt or *Swap-70*$^{-/-}$ B cells stimulated with IL-4 using Abs against STAT6, SWAP-70, and in addition Abs against BCL6, a STAT6 antagonist. Nonstimulated wt B cells served as negative control. Primers specific for the Iε region featuring the STAT6 binding site were used for semiquantitative PCR analysis of DNA precipitated along with SWAP-70 or STAT6 (Fig. 4A, 4B) and for real-time PCR (Fig. 4C). As expected, STAT6 from wt cells was found to bind efficiently to the specific Iε promoter region only upon IL-4 treatment (Fig. 4A, 4B). However, the association of STAT6 with Iε was greatly reduced in *Swap-70*$^{-/-}$ B cells stimulated by IL-4 (Fig. 4A, 4B). Real-time PCR on ChIPed material confirmed these data (Fig. 4C) by demonstrating significantly reduced STAT6 on Iε in *Swap-70*$^{-/-}$ cells. Association of STAT6 with Iγ1 was generally undetectable, indicating a weak and/or transient interaction of STAT6 with the γ1 promoter. This is consistent with earlier data demonstrating different binding affinities of STAT6 for the ε (high affinity) and γ1 (low affinity) promoter (27). Control PCRs diagnosing promoter regions of IgG2a (Iγ2a) and of a proteasome gene, (Pr) (21, 22), which lack STAT6 binding sites, did not generate signals (Fig. 4A, 4B). CD23 and IL-4Rα are transcribed under control of STAT6 (41, 42). Like the IL-4Rα, CD23 expression is induced normally on *Swap-70*$^{-/-}$ B cells in response to IL-4 or CD40 stimulation, or treatment by both stimuli (Supplemental Figs. 2, 4), demonstrating SWAP-70–independent STAT6 control of those promoters. Binding of STAT6 to the CD23 promoter was also found to be normal (Fig. 4A, 4B), suggesting that SWAP-70 preferentially regulates STAT6-dependent transcription at the Iε promoter.

ChIP using anti-SWAP-70 Ab showed that SWAP-70 is also recruited to the Iε promoter in wt cells in an IL-4–dependent manner but fails to bind to Iγ1 (Fig. 4A–C). This implies direct or indirect binding of SWAP-70 to the germline ε promoter. SWAP-70 also binds to the CD23 promoter but not to the Iγ2a or Pr control promoters.

BCL6 antagonizes STAT6 at the Iε promoter (10, 11) by binding to a sequence overlapping the STAT6 binding site within Iε and also by repressing transcription via recruitment of corepressors. We analyzed by conventional and real-time PCR on ChIPed material whether the absence of SWAP-70 and consequently of STAT6 correlates with increased BCL6 at Iε (Fig. 4A–C). Although in wt cells, ChIP data demonstrate reduced BCL6 binding upon IL-4 stimulation, there is significantly increased association of BCL6 with the Iε in IL-4–activated *Swap-70*$^{-/-}$ B cells as compared with wt. BCL6 does not regulate CD23 transcription (10), and no CD23-specific ChIP signals were obtained from anti-BCL6 precipitated samples. SWAP-70, but not BCL6, also binds to the CD23 promoter, which suggests that SWAP-70 may be particularly important at promoters associated with BCL6 and STAT6.

BCL6 RNA and protein expression are very similar in wt and in *Swap-70*$^{-/-}$ B cells (Fig. 5A–D), indicating that it is the binding of BCL6 to Iε, which is specifically controlled by SWAP-70. To further test this hypothesis, we analyzed ChIPed material from IL-4–activated B cells as used for Fig. 4 by real-time PCR the binding of STAT6 and BCL6 to five promoters that are known to be bound by both of these two proteins (Fig. 5E). The five promoters included the IL-4Rα, CD69, CCND2, CD2, and BCL3 promoters (43–46). There was no difference between samples from wt and *Swap-70*$^{-/-}$ B cells, confirming that SWAP-70 preferentially regulates STAT6 and BCL6 at the Iε.

Discussion

The specificity of Ig class-switching to IgE depends on transcription factors that act on Iε to control GLT, essential to induce class-switch recombination (reviewed in Ref. 47). Several transcription factors were identified to bind Iε and regulate its activity, among them the positive regulators NF-κB, AP-1, PU.1, C/EBP, bZip, and inhibitory factors such as Id2 and BCL6. Some factors are newly synthesized within about a day after IL-4 signaling and depend on STAT6. De novo protein synthesis is not required for induction of NFIL3 transcription, shown to be important for Iε activation and thus for the switch to IgE (22). NFIL3's activity on Iε also depends on STAT6. STAT6 is essential for the switch to IgE, and thus, controlling STAT6 is key to modulate IgE production. In this study, we demonstrate SWAP-70 control of STAT6 association with Iε.

IL-4 signaling induces the switch to IgG1 and IgE. Iγ1 and Iε share binding motifs for several transcription factors, among them NF-κB, AP-1, and STAT6. Yet, the switch to IgG1 is only mildly affected by SWAP-70 deficiency. It has been shown that GLT at Iε is more dependent on IL-4 than GLT at Iγ1, and IgE production is tighter regulated than IgG1 production (26, 27, 47). Hence, there is only a minor effect of reduced γ1 GLT on IgG1 production in *Swap-70*$^{-/-}$ B cells. STAT6 binds to Iε with 10-fold higher affinity than to Iγ1, and the assembly of transcription factors differs between the two promoter regions (27). Our differential chromatin

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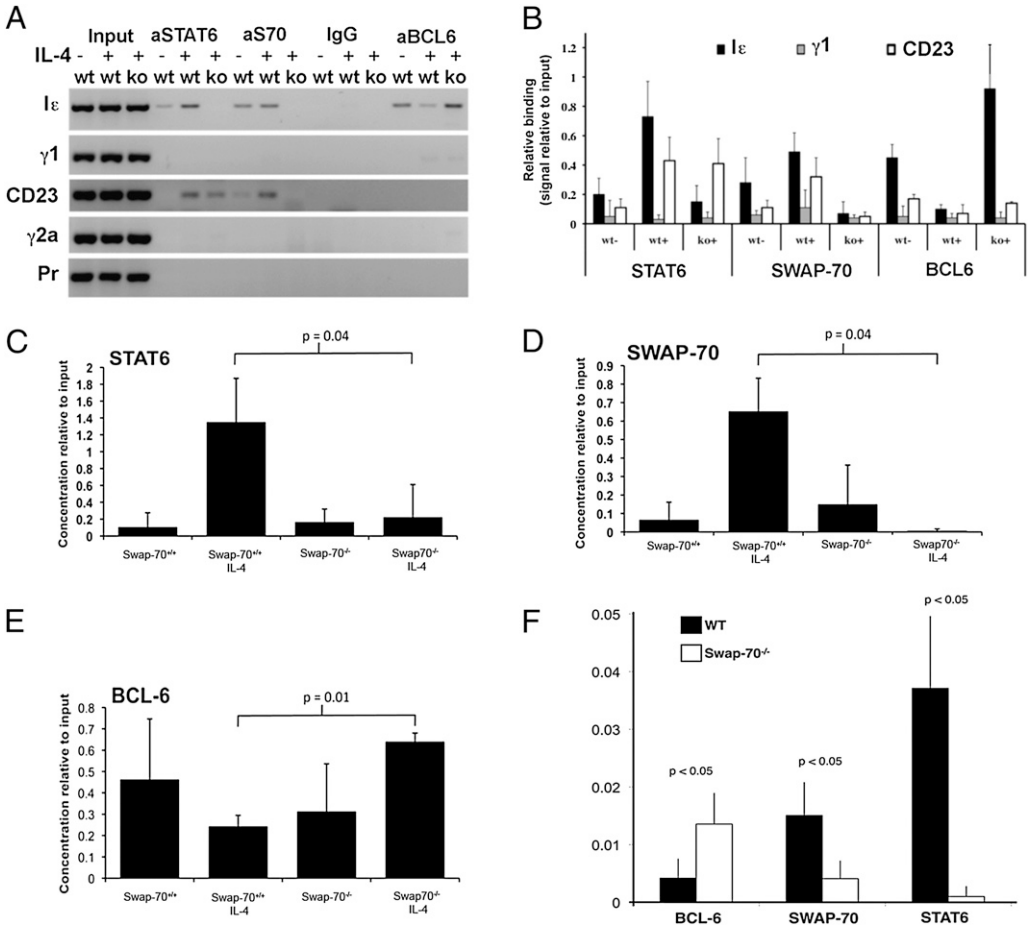

FIGURE 4. SWAP-70 controls binding of STAT6 and BCL at Iε. B cells isolated from wt and *Swap-70*$^{-/-}$ mice were cultured in the presence, or absence, of IL-4 (2 ng/ml) for 4 or 72 h. ChIP was carried out using anti-STAT6 (M20), anti-BCL6 (N-3), anti–SWAP-70, or a nonspecific rabbit IgG Ab (control), followed by PCR analysis. (**A**) PCR products obtained with specific germline ε promoter, γ1 promoter, CD23 promoter, γ2α promoter, or proteasome-specific (Pr) primers (representative images of four independent experiments are shown). (**B**) Quantification of STAT6, SWAP-70, and BCL6 binding to the Iε, Iγ1, and CD23 promoter regions relative to input (total DNA extracted prior to ChIP) signal. (**C**–**E**) Real-time PCR on ChIPed material (4 h) using primers specific for Iε. The protein precipitated is indicated for each plot. After real-time PCR, the signal was normalized to the input control to determine the concentration relative to input. The relative concentration minus the IgG control value is plotted ($n = 3$, ± SD), and p values are provided for the relevant comparison between induced wt and *Swap-70*$^{-/-}$ cells. (**F**) Real-time PCR using primers specific for Iε on material ChIPed by Abs for BCL-6, SWAP-70, or STAT6, prepared 72 h after start of induction from wt or *Swap-70*$^{-/-}$ cells as indicated. Normalization was done as for (C)–(E).

extraction experiments suggest that SWAP-70 is required particularly for the high-affinity chromatin binding fraction of STAT6, which includes the Iε, consistent with our ChIP data. A distinct sensitivity of the Iε promoter to reduced binding of STAT6 is also consistent with data from *Stat6*$^{+/-}$ mice, which are impaired in IgE production, but not in activation of other STAT6-dependent genes (48). Because occupancy of another high-affinity STAT6 binding site, the CD23 promoter, is not affected by SWAP-70 deficiency, the specificity for SWAP-70 control of STAT6 binding is not defined only by high-affinity binding. Another key parameter is the activity of factors antagonistic to STAT6, most notably BCL6. Our ChIP data suggest that SWAP-70 is required for prevention of efficient binding of BCL6 to Iε. Alternatively or in addition, SWAP-70 may promote dissociation of BCL6 from this promoter. In any case, we suggest that SWAP-70 supports STAT6 high-affinity binding and inhibits BCL6 association with selected promoter regions like Iε. How are those regions chosen? ChIP for SWAP-70 provides a clue: SWAP-70 associates with Iε but not Iγ1 or other control promoters, except CD23. Because CD23 is not bound by BCL6 (10, 46), binding of SWAP-70 to the CD23 promoter is irrelevant there for a potential STAT6–BCL6 antagonism. Thus, we asked whether SWAP-70 binding to the Iε promoter is mediates the antagonism between BCL6 and STAT6 at this promoter and how Iε varies from other promoters in this respect. Notably, five other promoters that are bound by STAT6 and BCL6 were investigated and found not to be differently occupied by these two proteins in activated wt versus *Swap-70*$^{-/-}$ B cells, suggesting that SWAP-70 selectively regulates STAT6-BCL6 association with Iε promoter.

Whether in activated B cells SWAP-70 directly binds promoter DNA, or whether it associates with other factors bound there and thus indirectly binds the promoter, remains to be elucidated. Earlier we showed that recombinant purified SWAP-70 binds dsDNA and ssDNA substrates in vitro without apparent sequence specificity (23). This renders direct binding of SWAP-70 to promoter regions in cells likely, where additional cellular factors or parameters would ensure specificity.

B cells may switch directly from IgM to IgE as suggested by a recent study using transgenic mice carrying a GFP-labeled IgE (49), or they may sequentially switch from IgM via IgG1 to IgE as suggested by several other studies (26, 50) and reviewed also in Ref. 47. These modes must not be mutually exclusive, and their usage may depend on various parameters such as the route of stimulation or whether low or high affinity IgE is produced. This is illustrated, for example, by the absence of sequential switching

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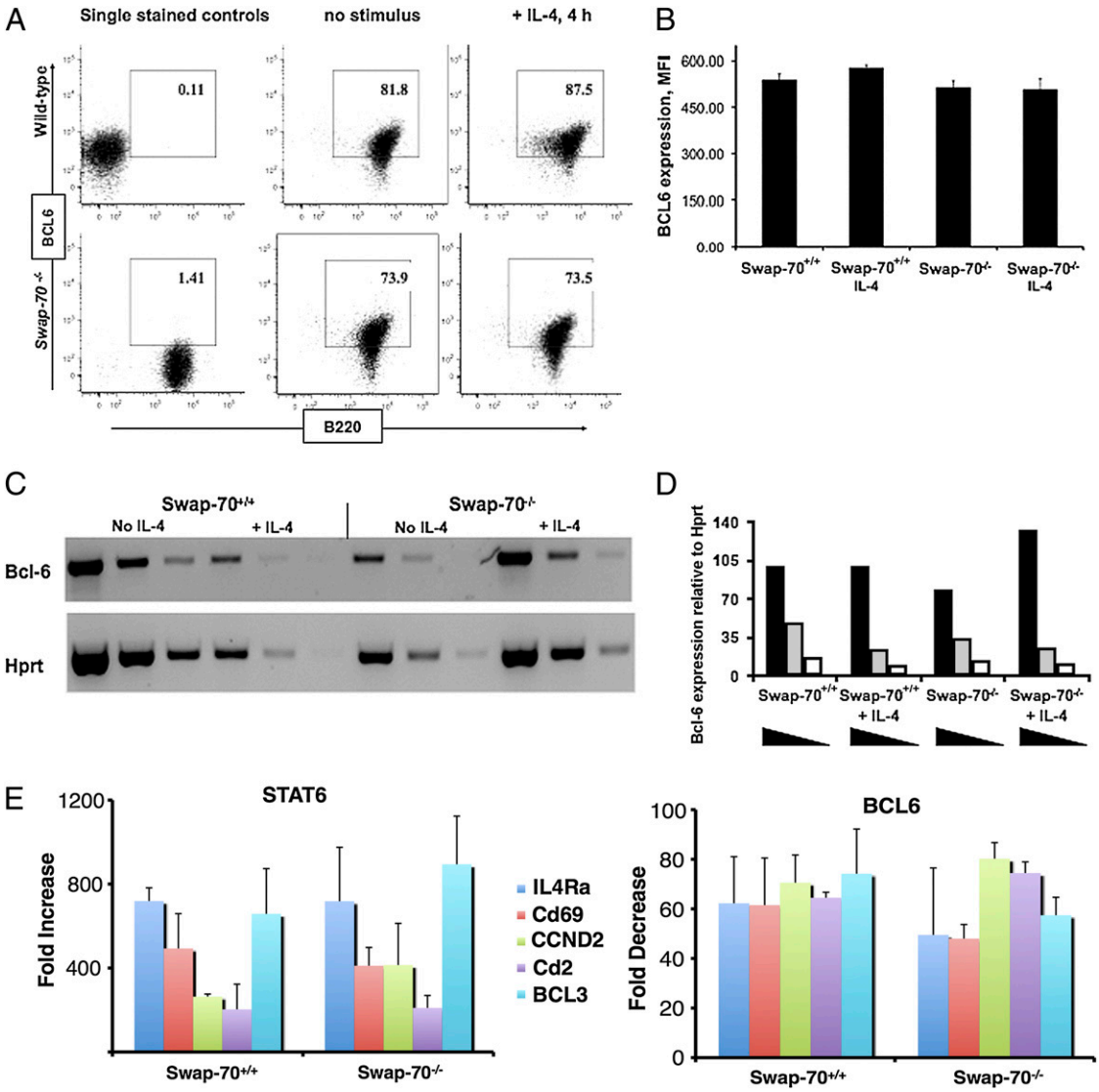

FIGURE 5. BCL6 expression and binding of STAT6 and BCL6 at shared sites are not altered in *Swap-70*$^{-/-}$ B cells. B cells from wt and *Swap-70*$^{-/-}$ mice were cultured in presence of IL-4 (2 ng/ml) for 4 h or left nonstimulated. (**A**) Intracellular anti-BCL6 detection by FACS. *Left plots* show examples of single-stained controls for BCL6 (wt) or B220 (*Swap-70*$^{-/-}$); representative of two experiments. (**B**) Mean fluorescence intensity of BCL6 in wt and *Swap-70*$^{-/-}$ B cells shown in (A), showing similar levels of BCL6 per cell. (**C**) Semiquantitative RT-PCR was performed using Bcl6-specific primers on serially diluted samples from nonstimulated or stimulated (4 h/IL-4) B cells from wt or *Swap-70*$^{-/-}$ mice. Relative expression normalized against Hprt was quantified by densitometry and is shown in pairs for stimulated and for nonstimulated cell samples as indicated by arrows (Bcl6 forward, 5′-CACACTCGAATTCACTCTG-3′, and Bcl6 reverse, 5′-TATTGCACCTTGGTGTTGG-3′); Hprt shown as a control (representative of three experiments). (**D**) Quantification of Bcl6 expression shown in (C). The PCR products were quantified by densitometry, and expression relative to Hprt was calculated and is shown plotted in pairs for stimulated and nonstimulated wt and *Swap-70*$^{-/-}$ samples; arrows indicate increasing dilution of the sample. (**E**) ChIP was performed using anti–STAT6 or anti BCL6 on material from unstimulated B cells and B cells stimulated for 4 h with IL-4. Real-time PCR was performed and the signal relative to the input control was determined. Background binding to Ig was then subtracted after which the increase (STAT6) or decrease (BCL6) after stimulation was calculated and plotted.

upon anti-IgD injection into mice or in the *N. strongylus*–induced direct switch to IgE (26, 49). The efficient formation of high-affinity IgE requires an IgG1 intermediate, and IgG1 patterns of somatic hypermutation can be transferred to IgE molecules (51). Because most memory cells are of the IgG1 type, their conversion to IgE$^+$ cells in a secondary response requires class-switching. The same would be true for IgM$^+$ memory B cells. Whether, or to which extent, IgG1 memory cells contribute to an IgE response, however, is controversial, and thus, this issue needs further investigation. As a local passive cutaneous anaphylactic response assay suggests, high-affinity IgE is required, at least in mice, for the anaphylactic reaction, arguing for the need of sequential switching in allergy be it at the immediate GC stage, the memory stage, or the committed plasmablast stage (26). The situation in humans, however, is much less understood, but more of the allergen-specific IgE in humans appears to be produced by newly emerging IgE plasma cells rather than by IgE memory cells (52). Thus, inhibition of the appearance of these cells could be used to reduce IgE levels in allergic diseases.

Irrespective of sequential switching, it is well known that class-switching to IgE in vitro is observed ~1 or 2 d later than the switch to IgG1, which requires approximately two more cell divisions (53), and IgE production does not reach the same levels as that of IgG1. These kinetics are reflected in the later onset of ε GLT and the appearance of surface IgE in our experiments. STAT6, SWAP-70, and/or BCL6 associated within 4 h after IL-4 signaling with the Iε and remained there at least until 72 h after the start of stimulation.

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Thus, a rather stable pattern of association was established within a few hours. This suggests that in the presence of SWAP-70 STAT6 rapidly occupies Iε in a preinitiation complex. For full initiation of germline transcript RNA synthesis by RNA Pol II and/or for robust levels of Iε GLT, other factors may have to be assembled over the course of 1–3 days, and thus, the germline transcripts are recognized at about day 3 after induction. One example of such delayed phase factors is NFIL3, whose expression takes ∼24 h (22). The involvement of several transcription factors is known, and we hypothesize that the complete regulation at Iε with STAT6 at its core involves a highly complex platform of factors that act in a delicate balance to fine-tune Iε GLT. We suggest SWAP-70 as one of the key elements in this network.

Taken together, our data demonstrate that SWAP-70 is required for ε GLT induced by IL-4 through the control of the critical balance between BCL6 and STAT6 at the Iε promoter. Identification of this specific nuclear function of SWAP-70 during ε germline transcription may facilitate the development of targeted inhibitors of the switch to IgE, thus helping to prevent or treat allergy by inhibiting (more) B cells to switch to IgE. This may be an option for both, direct switching from IgM to IgE and the sequential switch through an IgG1 intermediate.

Disclosures

The authors have no financial conflicts of interest.

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