Abstract

PAX5, a transcription factor pivotal for B-cell commitment and maintenance, is one of the most frequent targets of somatic mutations in B-cell precursor acute lymphoblastic leukemia. A number of PAX5 rearrangements result in the expression of in-frame fusion genes encoding chimeric proteins, which, at the N-terminus consistently retain the PAX5 DNA-binding paired domain fused to the C-terminal domains of markedly heterogeneous group of fusion partners. PAX5 fusion proteins are thought to function as aberrant transcription factors, which antagonize wild-type PAX5 activity. To gain mechanistic insight into the role of PAX5 fusion proteins in leukemogenesis, the biochemical and functional properties of uncharacterized fusions: PAX5–DACH1, PAX5–DACH2, PAX5–ETV6, PAX5–HIPK1, and PAX5–POM121 were ascertained. Independent of the subcellular distribution of the wild-type partner proteins, ectopic expression of all PAX5 fusion proteins showed a predominant nuclear localization, and by chromatin immunoprecipitation all of the chimeric proteins exhibited binding to endogenous PAX5 target sequences. Furthermore, consistent with the presence of potential oligomerization motifs provided by the partner proteins, the self-interaction capability of several fusion proteins was confirmed. Remarkably, a subset of the PAX5 fusion proteins conferred CD79A promoter activity; however, in contrast with wild-type PAX5, the fusion proteins were unable to induce Cd79a transcription in a murine plasmacytoma cell line. These data show that leukemia-associated PAX5 fusion proteins share some dominating characteristics such as nuclear localization and DNA binding but also show distinctive features.

Implications: This comparative study of multiple PAX5 fusion proteins demonstrates both common and unique properties, which likely dictate their function and impact on leukemia development. Mol Cancer Res; 12(4); 595–606. © 2014 AACR.

Introduction

PAX5 is a paired box family transcription factor, which is pivotal for B-cell development (1). Within the hematopoietic system PAX5 is exclusively expressed in the B-lymphoid lineage, where it is not only essential for pro-B-cell commitment but also for the maintenance of B-cell identity until the onset of plasma cell differentiation (1–3). PAX5 fulfills a dual role by activating B-cell specific and concomitantly repressing lineage-inappropriate genes (3–5), which is achieved by the recruitment of distinct chromatin remodeling, histone modifying, and basal transcription factor complexes to PAX5 target loci (6).

PAX5 alterations, including deletions, point mutations, and amplifications, occur in about 30% of B-cell precursor acute lymphoblastic leukemia cases, and chromosomal rearrangements account for 2–3% (7–9). Most rearrangements lead to the expression of in-frame fusion transcripts, which encode chimeric proteins consisting of the N-terminal PAX5 portion and the C-terminal regions of a variety of markedly heterogeneous fusion partners ranging from transcription factors over kinases to structural proteins (8–16).

As a unifying feature, the DNA-binding paired domain is always retained in the PAX5 fusion proteins, and for some of them DNA interaction has been demonstrated, suggesting that they may act as trans–dominant-negative transcription factors antagonizing wild-type PAX5 activity (8, 10, 17, 18). This notion has been corroborated by reporter gene assays, in which several PAX5 fusions competitively inhibited PAX5-mediated luciferase induction (8, 10, 12, 13, 17–19). Accordingly, ectopic expression of PAX5 fusion proteins led to altered levels of certain PAX5 target genes (8, 18, 20), however, their majority remained unaffected (10, 13). Some PAX5 fusions have also been shown to self-interact, which has been suggested to influence their binding affinities and repression capabilities (17, 18).
Based on our previous work, in which we identified several novel PAX5 fusion partners (9, 15), the main aim of this study was to determine their functional features. We investigated key properties of the so far uncharacterized fusion proteins PAX5–DACH1, PAX5–DACH2, PAX5–HIPK1, and PAX5–POM121 and compared them with PAX5–ETV6. Our data substantiate that PAX5 chimeras are nuclear proteins capable of binding to PAX5 target sequences. Moreover, our findings confirm that self-interaction is a characteristic feature of a subset of PAX5 fusion proteins. However, in contrast to earlier studies, but in line with recently described upregulation of certain PAX5 repressed targets by the PAX5–ETV6 fusion (21), we provide evidence that some PAX5 fusion proteins may also activate target genes, arguing against a simplified trans-dominant-negative mode of action.

Materials and Methods

Vectors and cloning

The coding sequences of PAX5, a DNA-binding–deficient mutant (K67A/K87A/K89A), PAX5–DACH1, PAX5–DACH2, coiled-coil (CC) region deleted mutants thereof, PAX5–ETV6, ETV6, PAX5–HIPK1, and PAX5–POM121 were cloned into pCDNA3 (Invitrogen), including N-terminal myc- or tandem hemagglutinin (HA) tags. HA-tagged coding sequences were further cloned into MSCV-MigR1 (22), which contains an internal ribosome entry site followed by green fluorescent protein (GFP) to allow for cell sorting. For bimolecular fluorescence complementation (BiFC), the coiled-coils of DACH1 and DACH2 were cloned into pBiFC-YN155 and pBiFC-YC155 (23). CD19-luc-1x containing one PAX5-binding site was derived from luc-CD19 (24). The human CD79A promoter was cloned into pGL4.10 (Promega). As negative controls the PAX5-binding sites of the reporter vectors were mutated employing QuikChange site-directed mutagenesis (Agilent). Similarly, the coiled-coil domains of PAX5–DACH1 and PAX5–DACH2 were deleted from the original fusion gene constructs. The Renilla plasmid pRL-null vector (Promega) was used for normalization. PAX5–DACH2, DACH1 (25), MSCV-MigR1 and luc-CD19, pBiFC-YN155 and pBiFC-YC155 vectors were kindly provided by C. Broccardo, R. Pestell, M. Busslinger, and T. Kerppola, respectively. Oligonucleotide sequences are provided in the Supplementary Material.

Cell lines and culture, transient transfection, and retroviral transduction

NALM-6 cells, purchased from DSMZ, and 558µM, provided by M. Reth, were cultured in RPMI 1640 with GlutaMAX (Invitrogen), 10% FBS and 1× penicillin/streptomycin (PAA), and HEK293, HeLa, Phoenix-GP, and -E cells (both provided by H. Strobl) in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS and antibiotics.

HeLa cells were transduced with Metafectene Pro (Biontex), HEK293, and Phoenix cells with Lipofectamine 2000 (Invitrogen) according to the manufacturers’ protocols. For retroviral transduction, we generated NALM-6 cells carrying the moloney murine leukemia virus ecotropic receptor (provided by H. Strobl). First, Phoenix-GP cells were cotransfected with pBMN-eco receptor-IRE5-mCD8a and gibbon ape leukemia virus envelope constructs (26). NALM-6 cells were transduced thrice with the amphotropic virus using Retronectin (Takara Bio) and centrifugation. After enrichment by mCD8afluorescence-activated cell sorting, these NALM-6-EcoR cells were transduced 3 times, and 558µM cells were transduced once with retroviruses from MSCV-MigR1 transfected Phoenix-E. 558µM cells were analyzed on a LSR Fortessa flow cytometer (Becton Dickinson) using phycoerythrin-labeled anti-mouse IgM antibody (27). Before further analyses, transduced NALM-6-EcoR and 558µM cells were flow sorted for GFP positivity.

Confocal microscopy

Transduced NALM-6-EcoR cells were immobilized by cytosein centrifugation (Thermo Scientific Shandon II) and fixed on Histobond slides (Marienfeld). Cells were permeabilized and incubated with HA primary (ab18181) and Alexa Fluor 568 secondary antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Mowiol 4-88 (Sigma-Aldrich). For BiFC, HeLa cells were transiently cotransfected with vectors containing the coiled-coils of DACH1 or DACH2 fused to N- and C-terminal yellow fluorescent protein (YFP) halves. The leucine zipper of Jun (bJUN) along with wild-type or mutant Fos (bFOS or FOSAZIP) were used as positive and negative controls, respectively (23). Cells were fixed, DAPI stained and mounted in Mowiol. Confocal microscopy was performed on a TCS-SP5 (Leica Microsystems) equipped with an HCX-PL-APO-CS-63.0 × 1.40-OIL objective.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from transfected HEK293 cells and tested for expression of the tagged proteins by immunoblotting. Assays with fluorescently labeled probes were performed using the Odyssey Infrared EMSA Kit (LI-COR). In brief, nuclear extracts were incubated with 20 ng CD19-DY782 (28) or CD79A-DY682 (29) probe in binding buffer. For probe competition experiments, 200 ng or 1 µg of unlabeled wild-type or mutant oligonucleotides, and for supershift assays 1 µg of mouse HA or myc antibody were added. Samples were separated on native 5% polyacrylamide Tris/glycine gel, which were scanned on an Odyssey Infrared scanner (LI-COR) at 800 or 700 nm.

Competition assays were performed with nuclear extracts and 20 fmol biotinylated CD19 probe in binding buffer. Samples were run on native 5% polyacrylamide gels in 0.5 × Tris-borate EDTA buffer and blotted to nylon membranes (Amersham Hybond N+). Signals were detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce).

Co-immunoprecipitation and Western blotting

Cotransfected cells were lysed in nondenaturing whole cell lysis buffer containing 100 U/mL benzoyl (Novagen). As positive controls 5% of the cleared lysate inputs were...
were retrovirally transduced with HA-tagged PAX5, a DNA-gated PAX5 fusion protein (Fig. 1A), NALM-6-EcoR cells.

Chromatin immunoprecipitation and quantitative PCR
Chromatin immunoprecipitation (ChIP) was performed as described elsewhere (30) using 2 × 10^6 cells and 2 μg mouse HA, PAX5, or normal IgG antibody. Immunoprecipitated DNA was purified using the QIAquick Kit (Qiagen). Selection of PAX5 target loci was based on published data (4–6, 31) and ENCODE ChIP-sequencing data for GM12878 cells (32). Quantitative PCR (qPCR) was performed using iQSYBR Green mix (Bio-Rad) and 200 nmol/L primers on an iCycler (Bio-Rad). For ChIP, signals were individually normalized to input DNA using the 2^−ΔΔCt method. For mRNA quantification, RNA was isolated using TRIzol (Invitrogen), reverse transcribed using MMLV RT (Promega), oligo(dT)18, and random hexamers, and then qPCR was performed. 

Results

PAX5 fusion proteins localize mainly to the nucleus
To determine the subcellular localization of the investigated PAX5 fusion proteins (Fig. 1A), NALM-6-EcoR cells were retrovirally transduced with HA-tagged PAX5, a ubiquitously expressed vector. Empty pcDNA3 was added to maintain constant DNA amounts. Significance levels were calculated by applying repeated measures one-way ANOVA and Dunnett tests. All assays were performed thrice independently and each in triplicates.
containing a nuclear localization signal (NLS) (Fig. 1A). Nonetheless, the nuclear localization of PAX5–DACH1 and PAX5–DACH2 (Fig. 1B) in conjunction with the positive CoIP results (Fig. 2A) strongly suggests that both fusion proteins self-interact in the nucleus.

**PAX5 fusion proteins bind to specific DNA sequences in vitro**

The nuclear localization and the presence of the PAX5 paired domain indicate DNA-binding properties of the PAX5 fusion proteins. To address this issue, we conducted electrophoretic mobility shift assays (EMSA) using an oligonucleotide constituting a PAX5-binding site from human CD19 (28) and HEK293 nuclear extracts containing HA-tagged PAX5 or the fusion proteins (Fig. 3A). Shifted probe bands were detected with PAX5, PAX5–DACH1, PAX5–DACH2, PAX5–HIPK1, and PAX5–POM121 (Fig. 3B).

In competition experiments, an excess of unlabeled probe but not a mutated oligonucleotide inhibited the formation of labeled DNA/protein complexes of PAX5, PAX5–HIPK1, PAX5–DACH1, and PAX5–DACH2, and less pronounced also of PAX5–POM121 (Fig. 3B). Furthermore, supershift experiments using HA antibody confirmed that the exogenous proteins were responsible for the observed mobility shifts (Fig. 3B). The highly reduced mobility of the CD19 probe in PAX5–DACH1 and PAX5–DACH2 complexes most likely reflects the self-interaction of these proteins leading to an increased size and thus a stronger retardation (Fig. 3B).

When EMSAs were conducted using a labeled probe containing a CD79A promoter-derived PAX5-binding site (29), shifted bands were of rather weak intensity (Supplementary Fig. S2), reflecting a significantly lower binding affinity to the probe (37). Of note, we did not observe a...
specific band shift for PAX5–ETV6–binding reactions, which may be caused by aggregation of the fusion protein under native, nondenaturing conditions. Furthermore, we asked whether the fusion proteins compete with wild-type PAX5 for DNA binding. Therefore, we performed EMSAs with a biotinylated CD19 probe, constant PAX5 and increasing amounts of fusion proteins. To properly separate the complexes, we used differently tagged proteins and supershifted the PAX5 fusion protein/DNA complexes with myc antibody. Increasing amounts of PAX5–DACH1, PAX5–DACH2, or PAX5–HIPK1 indeed resulted in a decreased intensity of the PAX5/DNA complex bands confirming competitive binding of the proteins (Fig. 3C). Taken together, the EMSA data verify that PAX5 fusion proteins bind DNA in vitro and compete with PAX5.

PAX5 fusion proteins occupy endogenous genomic PAX5 target sites

Next, we investigated whether the DNA-binding capability of PAX5 fusion proteins also applies to endogenous PAX5 target sites. For this purpose, NALM-6-EcoR cells expressing the tagged proteins of interest (Fig. 4A) were subjected to ChIP using HA antibody followed by qPCR with locus-specific primers. The PAX5 target sequences included the 3′-end of VPREB3, the first intron of MIR17HG, and the transcription start sites (TSS) of CD19, FLT3, CD79A, and PARP1, and were validated by ChIP using NALM-6 cells and PAX5 antibody (Fig. 4B).

Higher amounts of target DNA were detected in anti-HA immunoprecipitates of cells expressing PAX5 fusion proteins as compared with empty-vector control cells or control IgG immunoprecipitates (Fig. 4C) clearly showing that PAX5 fusion proteins bind to several endogenous PAX5 target sites. Of note, also PAX5–ETV6, which failed to show band shifts in the EMSAs, exhibited binding and displayed remarkably high signals for the CD79A and PARP1 TSSs (Fig. 4C). In addition, despite the rather low PAX5–POM121 protein levels, its ChIP signals were at least 3 times higher than empty vector or IgG control immunoprecipitate levels, verifying its DNA-binding capacity. However, because of the variable protein levels of the introduced PAX5 fusions (Fig. 4A), which are probably due to differences in the transduction efficiencies, protein sizes, or stabilities, no final conclusions regarding their binding affinities may be drawn.

Certain PAX5 fusion proteins activate a CD79A promoter reporter

Based on the observation that PAX5 fusion proteins specifically bind to genomic PAX5 sites, we wanted to know whether they also influence target gene expression. Therefore, we conducted luciferase reporter assays using HEK293 cells and a CD19-luc-1x firefly vector containing either one

Figure 2. Self-association of PAX5 fusion proteins. A, analysis of PAX5 and PAX5 fusion protein self-association capability, and of the heterologous interactions between PAX5–ETV6 and ETV6, and between PAX5–DACHs and the respective CC deletion (ΔCC) mutants by CoIP and Western blotting (WB) using the indicated antibodies. Normal IgG immunoprecipitates served as negative, 5% input as positive controls. Asterisks indicate unspecific bands. One representative of at least 3 replicates is shown. B, BiFC of DACH1 and DACH2 CCs. Reconstitution of YFP fluorescence (yellow signals) indicates self-interaction. The bJUN/bFOS leucine zipper heterodimer served as positive, bJUN and bFOS lacking parts of the zipper domain (FOSΔZIP) as negative control, respectively. Nuclei are counterstained with DAPI (blue). White bars indicate 20 μm.
PAX5-binding site or a mutated site as a negative control. Western blot analysis of the lysates using HA antibody confirmed the expression of the respective exogenous proteins. Although PAX5 clearly activated CD19-luc-1x compared with the mutated reporter, the PAX5 DNA-binding-deficient mutant did not (Fig. 5A). Furthermore, none of the PAX5 fusion proteins was capable of activating CD19-luc-1x (Fig. 5A). However, this reporter and its parent luc-CD19 are not activated by endogenous PAX5 in NALM-6 cells (Supplementary Fig. S3). This might be because of the rather artificial nature of these constructs, which, next to a β-globin minimal promoter, contain 1 or 3 modified PAX5-binding sites. Therefore, we cloned the PAX5-activated human CD79A promoter into the firefly vector pGL4.10 and, as a negative control, we introduced mutations, which significantly decrease the PAX5-binding affinity (38).

Remarkably, like wild-type PAX5, some fusions, in particular PAX5–DACH1 and PAX5–POM121, induced

Figure 3. *In vitro* DNA-binding capability of PAX5 fusion proteins. A, Western blot for HA-tagged PAX5 and PAX5 fusion protein expression was performed with HEK293 nuclear extracts used in B using an HA antibody. B, fluorescently labeled DY782-CD19 probe and nuclear extracts containing indicated HA-tagged proteins were incubated for EMSA without (−), with unlabeled wild-type (com) or mutated (mut) CD19 competitor in 50-fold excess. Supershifts were conducted by adding HA antibody sc-7392X (AB). Shifted, supershifted, and unspecific bands are marked by regular, broad arrows, and asterisks, respectively. One representative experiment of 3 is depicted. C, competition EMSA with PAX5 and PAX5–DACH1, PAX5–DACH2, or PAX5–HIPK1. Indicated amounts of nuclear extract containing HA-PAX5 and myc-tagged fusions were incubated with biotinylated CD19 probe. Myc antibody was used to supershift the PAX5 fusion protein/DNA complex. Unbound probe, shifted, and supershifted protein/DNA-complexes are indicated. One representative of 3 experiments is shown.
Figure 4. Binding of PAX5 fusion proteins to endogenous PAX5 target sites. A, expression levels of exogenous proteins and endogenous PAX5 in the NALM-6-EcoR cells used in C. Areas were sliced from one blot incubated with an antibody recognizing the PAX5 N-terminus, which allows for the concomitant detection of exogenous and endogenous proteins. B, validation of ChIP primers specific for the indicated gene loci. NALM-6 chromatin was subjected to immunoprecipitation using normal control sc-2025 (IgG, light gray bars) or PAX5 C-terminus–specific antibody sc-13146X (PAX5, dark gray bars). Quantitative PCR was performed with precipitated DNA and normalized to the input using the 2^(-ΔΔCt) method. Mean percentages of input in immunoprecipitates ± SD of technical triplicates from one representative experiment of 3 are shown. C, ChIP was performed with NALM-6-EcoR cell lines, which stably express the indicated HA-tagged proteins. Normal control sc-2025 (IgG, light gray bars) or HA antibody sc-7392X (dark gray bars) and conditions as in B were used.
Figure 5. Luc-CD19 and CD79A promoter reporter assays. Luciferase assays in HEK293 using CD19-luc-1x (A) or CD79A-pGL4.10 (B) containing a functional (dark gray bars) or mutated PAX5 binding site (light gray bars). Firefly activity was measured and normalized to Renilla activity as well as empty pcDNA3 vector control. Dashed lines represent basal levels. Mean fold activation ± SD (n = 3) is plotted. Two-way ANOVA and Bonferroni test were applied to compare wild-type to mutated reporter activation. Representative immunoblots using HA antibody sc-7392 or GAPDH antibody sc-32233 are shown below. C, competition experiments performed with CD19-luc-1x vector. Numbers indicate fold amounts of PAX5 fusion added to a constant amount of PAX5 vector. Mean fold activation levels ± SD (n = 3) are depicted. Repeated measures one-way ANOVA and Dunnett tests were applied to compare to PAX5 alone. Significance levels: *, 0.05 > P > 0.01; **, 0.01 > P > 0.001; ***, P < 0.001. Representative Western blots detecting HA-tagged PAX5 and fusion proteins or GAPDH are shown below. D, competition experiments performed as in C with CD79A-pGL4.10 vector.
reporter expression driven by the CD79A promoter (Fig. 5B). In contrast, the PAX5 DNA-binding-deficient mutant and PAX5–ETV6 did not activate the CD79A promoter construct (Fig. 5B). Although the mutated reporters were still slightly activated by PAX5, as well as by some of the fusion proteins, we attribute the potent reporter induction to promoter recruitment of the introduced proteins and to association of the paired domain with the target sequence.

Competition experiments confirmed that PAX5 fusion proteins inhibit PAX5-mediated activation of CD19-luc-1x (Fig. 5C). In addition, the induction of CD79A-pGL4.10 by PAX5 was also slightly repressed by PAX5–ETV6, but, in stark contrast, was markedly increased by PAX5–DACH1 and PAX5–DACH2 (Fig. 5D). Together, these data provide first evidence that certain PAX5 fusion proteins may activate PAX5-activated target genes such as CD79A.

PAX5 fusion proteins do not induce surface IgM in 558LμM cells

To further investigate the activation potential of PAX5 fusions on the endogenous CD79A promoter, the murine 558LμM plasmacytoma cell line, which expresses 3 of the 4 components of the B-cell receptor, except for CD79A (27), was transduced with PAX5, its DNA-binding-deficient mutant or the fusions. In about 20% of the GFP-positive PAX5 expressing cells, via activation of CD79a, surface IgM (sIgM) was present (Fig. 6A–C). In contrast, neither the DNA-binding-deficient PAX5 mutant nor any fusion protein significantly induced sIgM (Fig. 6A and B). Although the expression level of wild-type PAX5 and most of the fusion proteins was comparable (Supplementary Fig. S4), only PAX5 activated CD79a transcription (Fig. 6C). Consequently, in contrast to the CD79A-pGL4.10 reporter in HEK293 cells, the PAX5 fusions were unable to activate endogenous CD79a and sIgM presentation in 558LμM cells.

Discussion

In this study, we investigated the biochemical and functional characteristics of a number of PAX5 fusion proteins. As a unifying feature, PAX5 chimeras share the N-terminal DNA-binding paired domain, indicating that they may act in a trans–dominant-negative mode by competing for binding to PAX5 target sites and thereby antagonizing wild-type PAX5 activity. However, the fusion partner proteins differ considerably in terms of their function, localization, and...
structure (8–16), raising the question whether these differences have an impact on the actual function of the individual chimera.

Independent of the original subcellular localization of the protein partners and their highly diverse C-terminal domains, all PAX5 fusion proteins predominantly localize to the nucleus (Fig. 1B). Their nuclear import seems to be mainly dictated by the PAX5 paired domain and at least one nuclear import signal (Fig. 1B). However, PAX5–C20orf112, PAX5–FOXP1, and PAX5–ETV6 have been detected in both the nuclear and cytoplasmic fractions (13), and, as shown herein, also PAX5–HIPK1 partially localizes to the cytoplasm (Supplementary Fig. S1), suggesting a tendency to shuttle between cellular compartments. In this regard, nucleo-cytoplasmic shuttling has been demonstrated for both wild-type HIPK1 and ETV6 (39, 40). However, the significance of this finding and whether PAX5 fusion proteins might also exert specific functions in the cytoplasm remains to be determined.

Nevertheless, all PAX5 fusion proteins are capable of binding to genomic DNA, but supposedly with variable affinities. Remarkably, in ChIP experiments PAX5–ETV6 bound rather strongly to the CD79A promoter (41) and PARP1 promoters (Fig. 4C), which may be explained by additional protein/DNA interactions with adjacent sites via the ETV6 ETS domain. Although a link between PAX5 and ETV6 has not been yet described, cooperative binding to the murine Cd79a promoter of PAX5 and a similar transcription factor, namely ETS1 (41), has already been demonstrated (42). PAX5–FOXP1 and PAX5–ZNF521 also contain additional DNA-binding domains provided by the partner proteins (8), and thus might bind to distinct spectrums of target sequences. Together, these data suggest that at least some PAX5 fusion proteins may not only interfere with the expression of PAX5 targets but also with that of their fusion partner proteins (19, 21).

It has also been suggested that self-interaction of PAX5 fusion proteins alters their DNA affinities (17, 18). Although it has been proposed that PAX5–PML has a lower DNA-binding affinity than wild-type PAX5 (18), for PAX5–C20orf112 the opposite—that is, a highly stable chromatin interaction—has been reported (17).

As shown herein, 3 additional fusion proteins, namely PAX5–ETV6, PAX5–DACH1, and PAX5–DACH2 self-associate (Fig. 2A). Of note, oligomerization of ETV6 fusion proteins, in particular of tyrosine kinase chimeras, has been shown to be crucial for oncogenic transformation (43). Intriguingly, PAX5 fusions, such as PAX5–ETV6, may also interact with, and influence the wild-type partner proteins (Fig. 2A), which may also play a role in the development of the respective leukemia. In line with this notion, a dual dominant-negative mode has been proposed for PAX5–PML, which also forms homo- and heterodimers (18, 19). However, this concept does not apply to PAX5–DACH1 and PAX5–DACH2 because both DACH1 and DACH2 are not expressed in B-cells and DACH1 is also not expressed in primary PAX5–DACH1–positive leukemia (data not shown).

Collectively, these data support the current notion that PAX5 fusion proteins bind to PAX5 target loci without providing normal regulatory functions, thereby antagonizing PAX5 activity provided by the wild-type allele. However, in contrast to PAX5–ETV6, which has been demonstrated to primarily act as a transcriptional repressor (8, 20), PAX5–DACH1, PAX5–DACH2, and PAX5–POM121 showed induction of the CD79A promoter reporter (Fig. 5B–D). Notably, DACH1 has been suggested to play a role as a co-activator in myeloid cells (44, 45) and there is growing evidence that nuclear pore components, such as POM121, additionally localize to the nucleoplasm and activate transcription (46, 47). However, none of the PAX5 fusion proteins exhibited Cd79a transactivation capability in murine 558LI cells (Fig. 6C).

The apparent discrepancy between the luciferase assay and the 558LI cells is supposedly because of different target readouts, one being an episomal reporter gene transiently expressed in human embryonic kidney-derived cells, the other constituting a chromatinized silent endogenous locus in murine plasmacytoma cells. On the one hand, the reporter construct might lack more distal regulatory regions, which in the plasmacytoma cells might be activated by wild-type PAX5 but not the fusions. On the other hand, to exert their activation potential, PAX5 fusion proteins may require factors absent in 558LI cells, and that either, like ETS1, EBF1, TCF3, and chromatin remodeling/modifying complex components, cooperate with PAX5 (6, 35, 42, 48, 49) or with the fusion partner, for example the DACH proteins (50). In this context, luciferase assays in HeLa cells and target gene regulation in DG75 lymphoma cells ectopically expressing PAX5–ELN as well as PAX5–ELN–positive primary leukemia cells also showed remarkable differences (10).

Together, these data suggest that the impact of PAX5 fusion proteins is critically dependent on the cellular context and differentiation state, which is supposedly due to differences in the protein repertoire as well as chromatin architecture (3, 6, 33). This notion is further supported by recent data showing that introduction of PAX5 into Hodgkin lymphoma cells is unable to reestablish a mature B-cell expression signature (51).

In summary, our study confirms that PAX5 fusion proteins share common features, including nuclear localization and DNA-binding capability. However, our data also provide evidence that the heterogeneous C-terminal domains may modulate their DNA-binding affinities and their regulatory potentials to activate or repress target genes, which rather argues for a more complex mode of action of the fusion proteins than for a simple PAX5 antagonizing function.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K. Fortschegger, S. Strehl
Development of methodology: K. Fortschegger, S. Anderl, D. Denk
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Fortschegger, S. Anderl
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Fortschegger, S. Anderl, D. Denk, S. Strehl
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Functional Heterogeneity of PAX5 Chimeras Reveals Insight for Leukemia Development

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