Structural and functional relationship of EBF1 variants in B cell development

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INTRODUCTION

B-lymphoid hematopoiesis requires precisely and timely controlled regulatory mechanisms of an immensely complex process, including expression of key genes, interaction of transcription factors and activation/deactivation of signaling pathways. Any disturbance of these networks can lead for example to leukemia. High prevalence of *early B-cell factor 1* (*Ebf1*) genetic lesion in pediatric acute lymphoblastic leukemia relapses [1] shows the relevance for getting a detailed insight of the regulatory networks in the process of B lymphoeisis. EBF1 is a key transcription factor in early B cell development. It does not only initiate B cell differentiation by activating B-lineage restricted genes, it additionally represses genes of other transcription factors associated with alternative cell fates.[2] [3] EBF1 consists of four protein domains; the N-terminal DNA Binding domain (DBD), the IPT/TIG (immunglobulin, plexins, transcription factors-like/transcription factor immunoglobulin) domain, a helix-loop-helix-loop-helix and a C-terminal transactivation domain (TAD)[4] (Figure 1). Even though much of the structural information of EBF1 is already known, not much of the structural related functions has been decrypted. Therefore, the aim of this study was to investigate the structural functional relationship of different EBF1 mutants *in vitro*.

CONCEPT

Five different EBF1 mutants were designed which were predicted to result in defined structural changes and potentially lead to impairment in B cell differentiation. The mutations were related to disrupt the dimerization of EBF1, to study the importance of the PXXPXXP loop as potential binding site for interaction partners and to disturb a possible interaction between EBF1 and NFκB proteins (Figure 1). The impact of these mutations on B cell differentiation was determined using an *in vitro* assay combined with flow cytometric analysis. To study the structural properties of the EBF1 mutants, the IPT/TIG HLHLHL domains were expressed in *Escherichia coli*. Immobilized metal ion affinity chromatography (IMAC) and size exclusion chromatography (SEC) were used to purify the fusion proteins. The isolated proteins were analyzed with analytical gel filtration (AGF) and circular dichroism spectroscopy (CD).
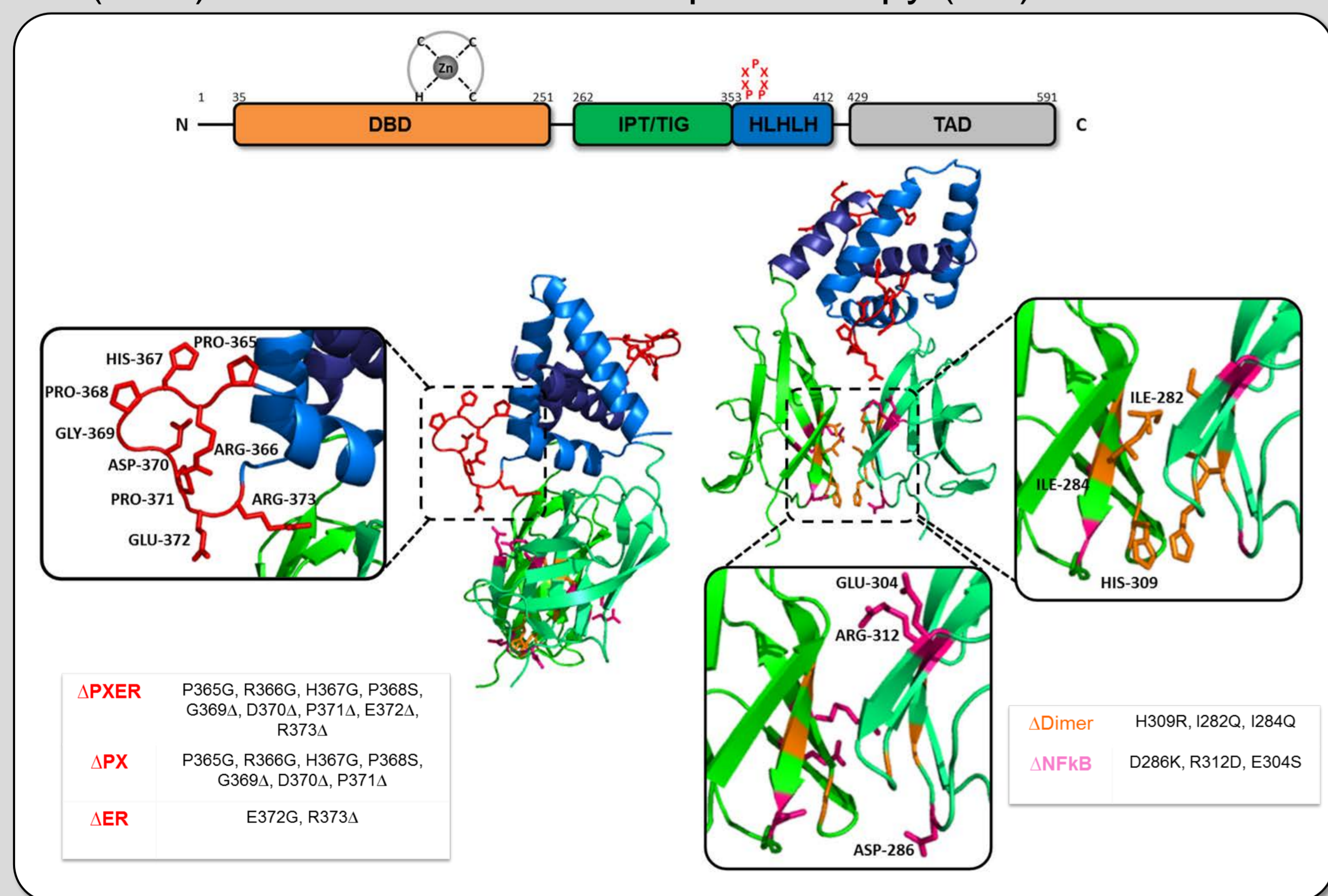
Fig. 1. Structure of the designed EBF1 mutants. Schematic presentation of EBF1 and possible structure of the IPT/TIG HLHLH domain is shown modelled on the EBF3 structure using PDB file 3N50. The IPT/TIG domain is shown in green, the HLHLH domain is colored in blue. Mutated amino acids of ΔDimer mutant are shown in orange, of ΔNFκB in pink, of ΔPXER, ΔPX and ΔER in red.

RESULTS

Non-expression of the surface marker CD19 was used to identify in an impairment of EBf1 with regard to promote B cell development. Fetal liver pre-pro-B cells deficient for EBF1 were transduced with *Ebf1* mutants. Cells transduced with ΔDimer, ΔER and ΔNFκB did not show development into CD19 expressing cells (Figure 2).
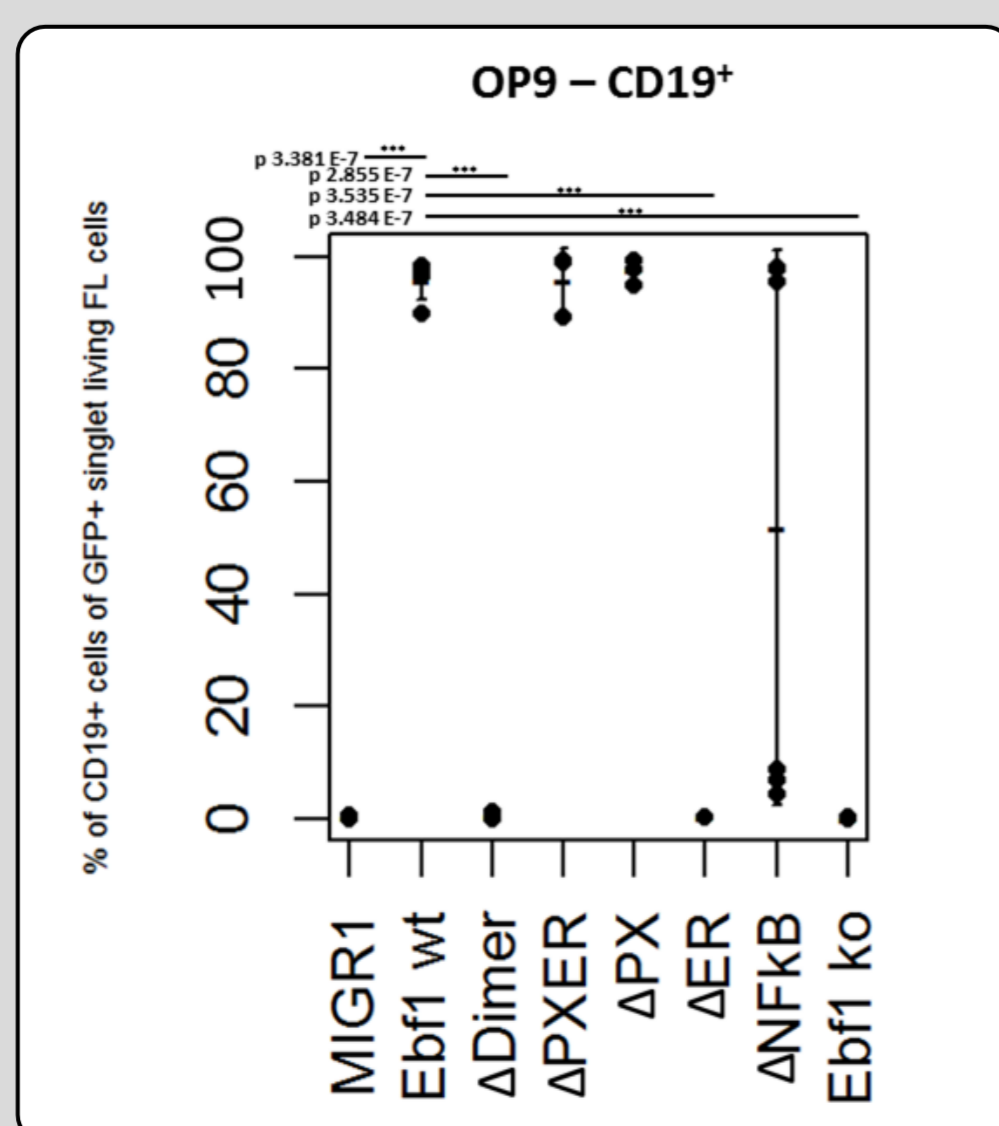

Fig. 2. Flow cytometry data analyzing CD19 expression of transduced B cell progenitors.. Cells were cultured on OP9 under condition permissive for B cell development. The differences between genotypes compared to *Ebf1* wt were evaluated by 2-tailed t test* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.

In order to explore if the changes in the functional ability to rescue B cell development could be explained by differences in expression levels, a Western Blot was performed revealing robust and comparable expression of all the proteins. Hence, the functional differences could not be explained by differences of expression but rather by differential protein function.

**Fig. 3. Western Blot of nuclear extracts from BaF3 cells.**.
(a) Membrane stained for EBF1.
(b) Membrane stained for GAPDH house keeping gene.
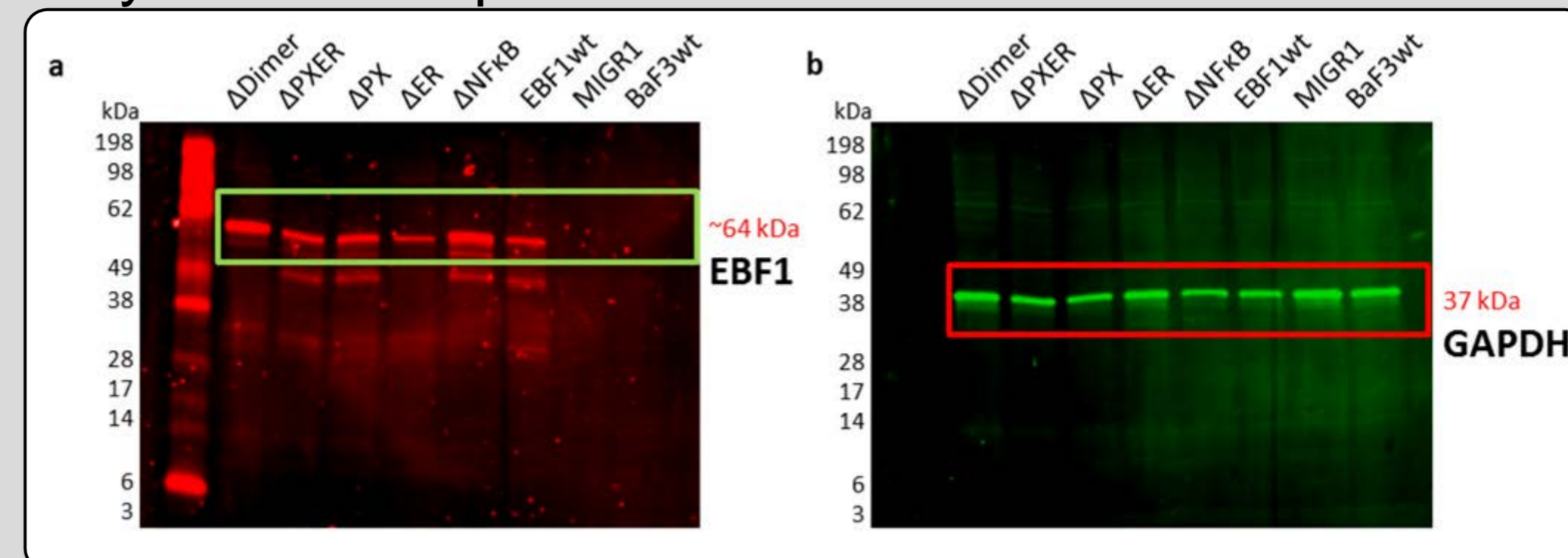
The IPT/TIG HLHLH domains of ΔPXER, ΔPX, ΔER and wt were recombinantly expressed in *E. coli*. The expression was optimized. Fusion to a His$_6$-TRX tag led to the highest amount of protein in cell lysate supernatant. The ΔDimer and ΔNFκB mutant were obtained in form of inclusion bodies with low protein concentration. A reason could be toxicity of recombinant protein to the expression strain due to strict mutations.

AGF was performed to study oligomerization and homogeneity of different constructs (Figure 4). It was shown that all of the constructs form dimeric structures. The ΔER mutant formed additionally tetramers. The tetramer formation was concentration independent. The secondary structure and thermal stability of the proteins were analyzed with CD spectroscopy (Figure 5). The CD spectra between wt and mutants were similar. The selected mutations did not disrupt the protein secondary structure. The heat denaturation revealed the highest thermal stability for EBF1 wt, whereas ΔER had the lowest. The mutants showed a cooperative unfolding, while it took two transition stages for EBF1 wt until completely unfolded.
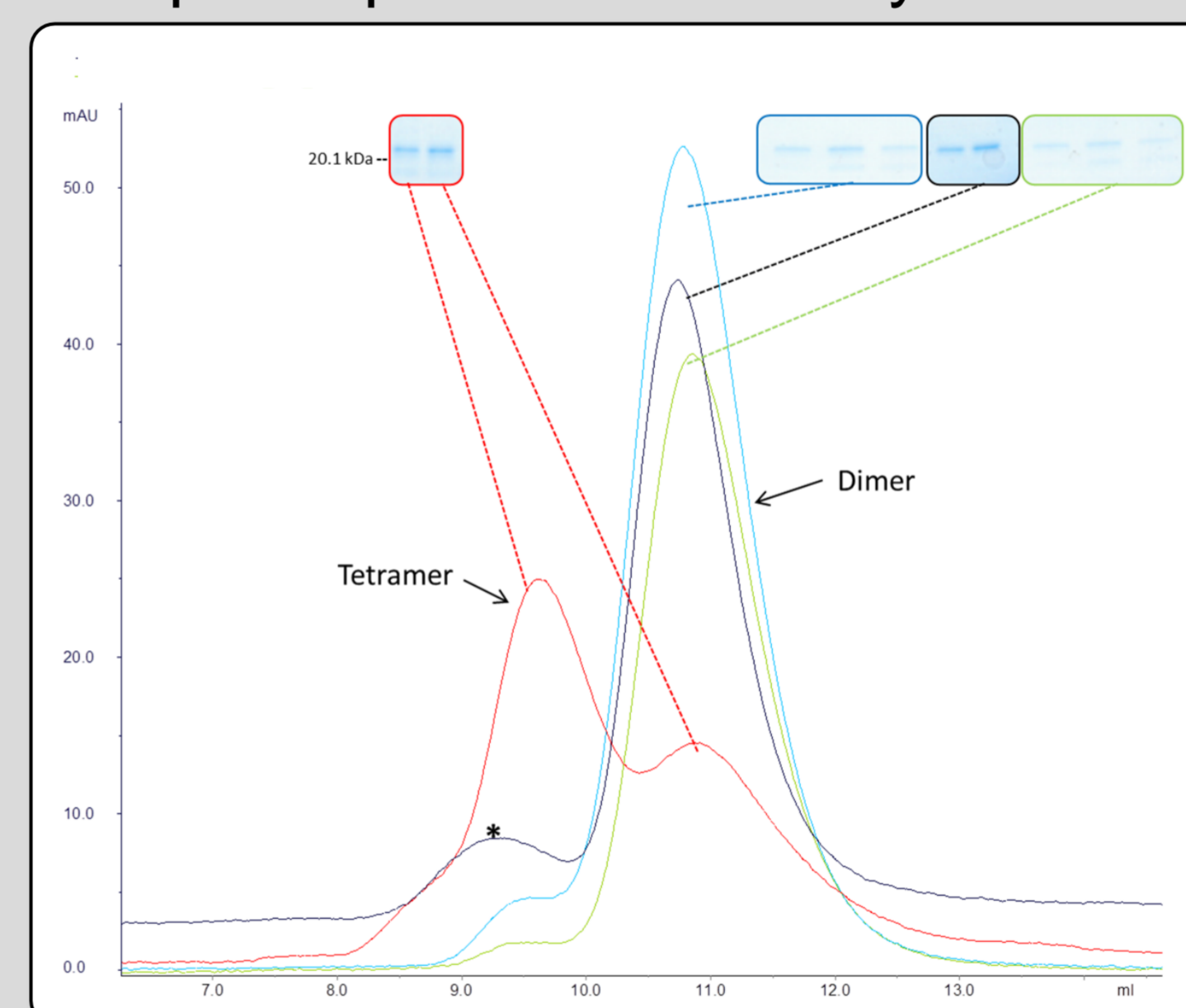
Fig. 4. AGF chromatogram of purified EBF1 constructs. 25 μM protein solutions were loaded. Absorbance was measured at 280 nm. Fraction associated SDS PAGE gels are shown. * E.coli contaminant

--- EBF1wt; --- ΔPXER; --- ΔPX; --- ΔER;.
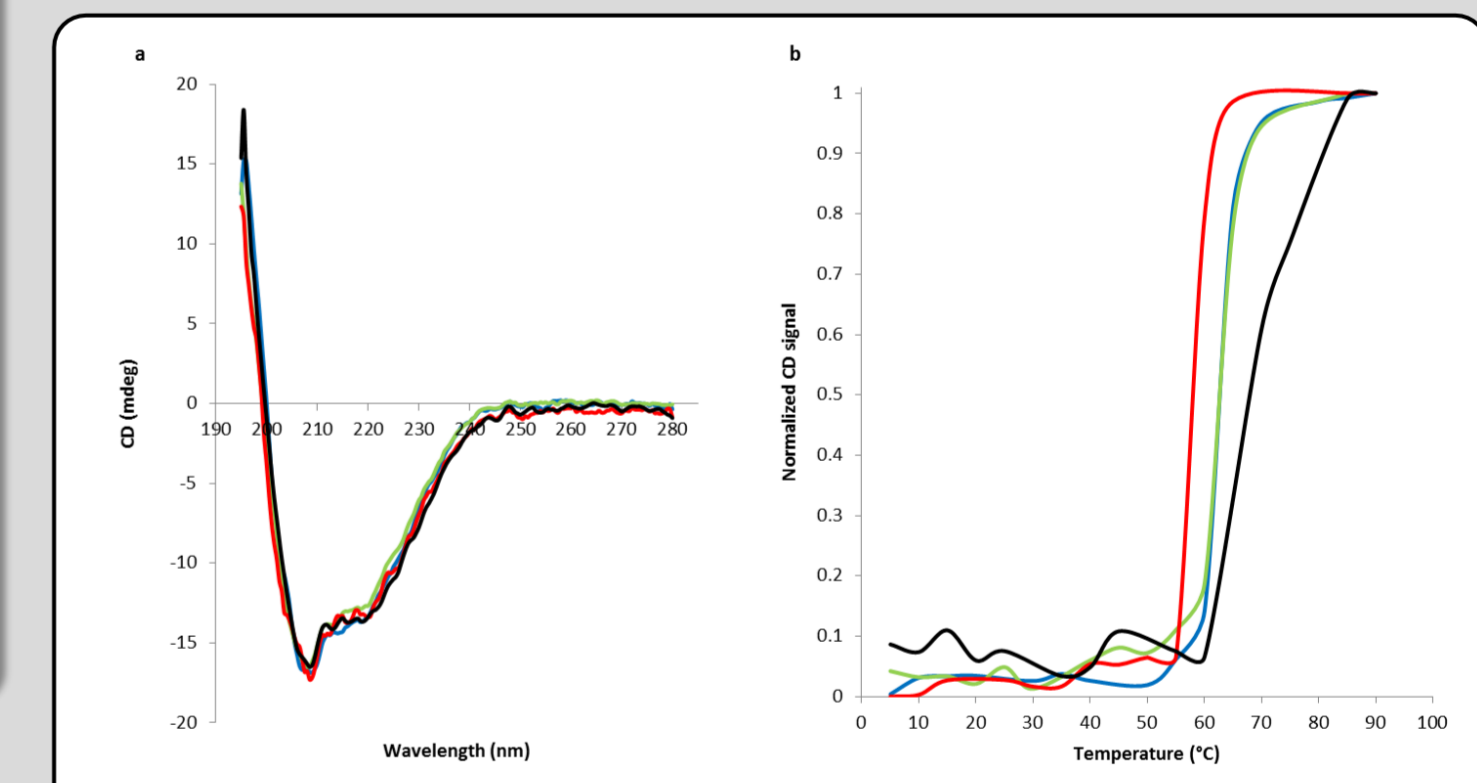
Fig. 5. CD spectra of purified EBF1 constructs.
(a) Secondary structure scan (280 nm -195 nm; 22°C; 2.5 μM protein conc.)
(b) Normalized CD spectra as a function of temperature. (5 – 90 °C; 220 nm; 5 μM protein conc.

CONCLUSION

1. Tetramer formation of ΔER leads to an impairment in B cell development *in vitro*. Leukemogenesis as a consequence of tetramerization was already observed for the B cell specific transcription factor PAX5.[5]

2. Further experiments are required to understand the tetramer formation. Other techniques than CD spectroscopy will be useful, since tetramerization does not change the secondary structure of the protein.

3. The PXXPXXP motif and the amino acids E372 & R373 have a stabilizing function for the protein structure of EBF1.

4. To make a statement concerning the affected B cell differentiation through ΔDimer and ΔNFκB, the recombinant protein expression needs more optimization. Additionally one has to reconsider less strict mutations.

REFERENCES

[1] Yang *et al. Blood* **112,** 4178–4183 (2008).

[2] Zhang *et al. EMBO J.* **22,** 4759–4769 (2003).

[3] Pongubala *et al. Nat. Immunol.* **9,** 203–215 (2008).

[4] Treiber *et al. Genes Dev.* **24,** 2270–2275 (2010).

[5] Kawamata *et al. Oncogene* **31**, 966–977 (2012).