Whole exome sequencing reveals a C-terminal germline variant in CEBPA-associated acute myeloid leukemia: 45-year follow up of a large family

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SUPPLEMENTARY MATERIALS

SUPPLEMENTARY PATIENT DESCRIPTIONS

Patient IV.4 *CEBPA* Q311P Carrier

This patient, the proband, was admitted to the National Institutes of Health Clinical Center in February 1969 at 11 years of age, with a clinical presentation of petechiae and fever. A peripheral blood smear revealed that the patient was affected with AML; bone marrow cytogenetics were found to be normal in March of 1969 She was induced with cytosine arabinoside and consolidated on this regimen until December 1969, when she relapsed. The patient was then re-induced with 6-mercaptopurine, vincristine sulfate, methotrexate, and prednisone (POMP) and consolidated on the same regimen for a total of three courses. In May 1970, she was started on daily oral 6-mercaptopurine with monthly pulsing of vincristine and prednisone but relapsed again in early September 1970. In late September 1970, the patient showed evidence of septicemia and was found to have *S. aureus* and *E. coli* growing on blood culture. She was given a test dose of asparaginase. Shortly after the infusion, the patient developed angioedema and underwent cardiopulmonary arrest and died at age 12 years.

Patient IV.1 *CEBPA* Q311P Carrier

This patient was diagnosed with AML in June of 1976 at 20 years of age, after the publication of the original paper. [1] It is interesting to note that this patient's leukemia was predicted by her increased transformation of SV40 virus in cultured skin fibroblasts.[2] She achieved a first remission after one course of daunomycin and cytosine arabinoside, although a slight excess of promyelocytes were noted in the bone marrow aspirate at that time. She

received her first maintenance chemotherapy with 6-thioguanine plus cytosine arabinoside in August 1976. The second maintenance course of cytosine arabinsoside, vincristine and dexamethasone was given from August 1976. The patient relapsed in May 1977 and was treated with daunomycin, 5-azacytidine, cytosine arabinoside, prednisone, and vincristine (D-ZAPO). Bone marrow cytogenetics performed in November of 1977 showed a normal karyotype. She relapsed a third time in February 1978 and was treated with intermittent POMP, cytosine arabinoside, 5-acacitidine and adriamycin. After this, she became febrile and blood cultures were positive for *S. aureus*. She was treated with antibiotics but did not respond and died in July 1978. No residual leukemia was found at autopsy. However, the bone marrow was hypoplastic with occasional blasts, and the patient did exhibit splenomegaly.

Patient IV.2

This patient, a brother of the proband, was seen at age 34 months in July 1955 with recurrent bruising and pharyngitis. White blood cells (WBCs) were 10,400/µl, with 48% myeloblasts. This patient received steroids which resulted in a temporary improvement in the WBC count. However, he died of a gastrointestinal hemorrhage 6 months later.

Patient IV.3

Another brother of the proband, patient IV.3 exhibited pallor and pharyngitis, at age 6 years in September 1960. A bone marrow aspirate was performed, revealing a diagnosis of AML. The patient achieved remission twice, after treatment with methotrexate and total-body

radiation, respectively. Finally, the patient was treated with 6-mercaptopurine and cyclophosphamide without significant response. The patient died in September 1961.

Patient III.2 *CEBPA* Q311P Carrier

This patient was the mother of the proband. She had an ovarian cystadenoma in 1956 at age 21 and had a left oophorectomy. She then developed invasive carcinoma of the cervix at age 27, in 1963, and received radium implant and cobalt beam therapy. She had a recent miscarriage of a set of triplets. In 1979, she developed poorly differentiated adenocarcinoma of the rectum that invaded the right lateral pelvic sidewall and had metastasized to periaortic lymph nodes. She died in 1979, and had an autopsy at the NIH Clinical Center, revealing metastatic poorly differentiated adenocarcinoma involving the peritoneum and colonic serosa. In addition to being the mother of four children with AML, her mother (II.2) died at the age of 41 years of unknown causes, and possibly of cancer. Per patient report, her father died of renal cancer and both the patient's brother and sister died of carcinoma.

Patient IV.5 *CEBPA* Q311P Carrier

This patient is a 54-year-old male and brother of the proband and twin of IV.6. The karyotype from his peripheral blood, performed at the NIH in 1983, revealed no abnormalities. As of September 2014, the patient reports no history of leukemia, lymphoma or any other cancer. He was noted to have elevated cholesterol on routine laboratory examination.

Patient IV.6 *CEBPA* Q311P Carrier

This patient is a 54-year-old male and brother of the proband and dizygotic twin of IV.5. The karyotype of his peripheral blood was determined to be normal at the NIH Clinical Center in 1983. His bone marrow biopsy revealed normal hematopoiesis. As of September 2014, he reported no history of leukemia, lymphoma or other cancers. Routine laboratory evaluation revealed an elevated cholesterol level.

Patient II.5

This patient was a brother of II-8 and also developed AML. The patient was diagnosed with AML in December 1969 at age 62. The patient died in 1970 at 63 years of age.

Patient II.8 Obligate *CEBPA* Q311P Carrier

This patient was the maternal grand-aunt of the proband. At age 53, in December 1956, she was admitted to the hospital with gingival bleeding and flu-like symptoms. WBCs were significantly elevated at 151,000/µl, with myeloblasts predominant in the differential count. The patient died 1 week later, with AML listed as the immediate cause of death on her death certificate.

Patient III.7

This patient was the son of patient II.8. He was admitted at 36 years of age, in March 1959, for exacerbation of chronic osteomyelitis (26 years of duration). WBCs were 124,000/µl, with 68% myeloblasts. He achieved complete remission after treatment with methotrexate, 6-mercaptopurine and steroids. However, he relapsed 9 months later. The patient died in January 1961. Autopsy showed active AML. No DNA was available for testing.

Patient III.8 *CEBPA* Q311P Carrier

This patient was the second son of patient II.8 to develop AML. The patient was diagnosed with AML-M2 in 2001 at 58 years of age. Chemotherapy was started in April 2001. Flow cytometry was performed on a bone marrow aspirate collected in April 2001. CD45/side scatter gating revealed a population of blasts showing expression of HLA-DR, CD34, CD117, as well as myeloid antigens CD11b, CD13 and CD33. The blasts did not express the monocytic marker CD14 or any B-lymphoid or T-lymphoid antigens. The patient died in 2012 at 69 years of age from complications of AML.

Patient III.10 *CEBPA* Q311P Carrier

This patient was a brother of AML patient III.8, but did not have a history of cancer, leukemia or any leukemia like illness. He had high cholesterol and died of a myocardial infarction in 2013 at age 80.

III.5 *CEBPA* Q311P Carrier

This patient is an 88-year-old female and mother of AML patient IV.7 and grandmother of patient V.2. She has no history of leukemia or lymphoma, but was diagnosed with melanoma on her left arm in March of 2014. The patient was also diagnosed with Alzheimer disease.

Patient IV.7 Obligate *CEBPA* Q311P Carrier

This patient was a second cousin of the proband and developed AML in 1994 at 41 years of age. He died in 1996 at 43 years of age from complications of AML. He is an obligate carrier of the *CEBPA* Q311P mutation; no DNA was available for testing

Patient V.2 *CEBPA* Q311P Carrier

This patient is the daughter of AML patient IV.7 and developed AML at 22 years of age in August 2012. She received induction chemotherapy with cytarabine and daunorubicin**,** followed by consolidation chemotherapy. She had a relapse requiring a double umbilical cord transplantation, performed in October 2013. Her original diagnosis was AML without maturation. On her initial CBC, the WBCs were 51,600/µl, hemoglobin 11.2 gm/dl, hematocrit 33.1% and platelets 26,000/µl. A manual leukocyte differential showed 77% blasts, 9% segmented neutrophils, 3% bands, 9% lymphocytes and 2% eosinophils. The blasts were moderate to large in size with immature nuclear chromatin, one or more relatively prominent nucleoli and irregular nuclear contours with frequent lobulation. Occasional blasts containing Auer rods were seen. The bone marrow cellularity was 80-90% with focal residual adipose elements. Cytogenetics of the bone marrow revealed a normal 46 XX karyotype The marrow contained a diffuse infiltrate of immature cells consistent with blasts. The aspirate smear showed a differential of 75.2% blasts, 2% myelocytes, 2.8% metamyelocytes, 2% neutrophils, 12.4% erythroid precursors, 2.4% lymphocytes, 1.2% monocytes and 2% eosinophils. In August 2012, flow cytometric analysis revealed an abnormal myeloid phenotype showing positivity for HLA-DR, CD34, CD117, dim CD11b, dim CD11c, CD15, CD33, CD38 and dim CD64. The blasts

also abnormally expressed CD7, and a subpopulation was positive for CD56. The patient is alive and well.

V.1 *CEBPA* Q311P Carrier

This patient is the sister of AML patient V.2 and daughter of AML patient IV.7. She is currently 27 years old and her only major complaint is migraine headaches. A recent CBC showed increased WBCs at 12,200/µl and her absolute lymphocyte count was increased at 5,640/µl. She is otherwise healthy and well.

IV.11 *CEBPA* Q311P Carrier

This patient is the sister of AML patient IV.7. At age 60 years and as of September 2014, she has no history of cancer, leukemia or leukemia-like illness. She had no biological children but had three miscarriages.

SUPPLEMENTARY METHODS

Sanger sequencing. The *CEBPA* Q311P forward primer 5'-GGGCAAGGCCAAGAAGTC-3' and *CEBPA* Q311P reverse primer 5'-AGGCACCGGAATCTCCTAGT-3' were used for the reaction.

Luciferase reporter assay. 2×10^6 MOLM-16 cells were seeded in 6-well dishes and transfected using Lipofectamine LTX (Invitrogen, Life Technologies, Grand Island, NY, USA) with 2 µg of the reporter plasmid (tetramer of the *CEBP* site of *CSF3R* inserted into the promoterless luciferase vector pTK81)[3], 20 ng of the expression plasmid pcDNA3, pcDNA3-CEBPA or pcDNA3-CEBPAQ311P plus 20 ng of pcDNA3, pcDNA3-CEBPA or pcDNA3-CEBPAQ311P in all

combinations, and 30 ng of a Renilla-Luciferase control reporter plasmid (Promega, Madison, WI, USA). pcDNA3-CEBPA Q311P was created by site directed mutagenesis of pcDNA3-CEBPA.[3] Luciferase assays were performed 24 hours after transfection. Luciferase activities were normalized for transfection efficiency with the cotransfected Renilla-Luciferase plasmid using the Dual-Luciferase-Reporter Assay system (Promega, Madison, WI, USA). Each experiment was repeated three times.

Immunoblotting. 2×10^6 MOLM-16 cells were seeded in 6-well dishes and transfected using Lipofectamine LTX (Invitrogen, Life Technologies) with 2 µg reporter plasmid and 20 ng pcDNA3, pcDNA3-CEBPA (Q311) or pcDNA3-CEBPA (Q311P) plus 20 ng of pcDNA3, pcDNA3-CEBPA (Q311) or pcDNA3-CEBPA (Q311P) in all combinations. Total extracts were prepared 24 hours after transfection using RIPA lysis buffer and proteinase inhibitors. Proteins were separated on 10% PAGE, transferred to nitrocellulose membrane and incubated with rabbit mAb anti-CEBPA (Abcam ab40761) and mouse anti-GAPDH (Sigma G8795) followed by IRD680 labelled goat anti-mouse and IRD800 labelled goat anti-rabbit antibodies (Metabion International AG, Planegg, Germany).

Electrophoretic mobility shift assay. H1299 cells were seeded in 6-well dishes and transfected using Lipofectamine 2000 (Invitrogen, Life Technologies) with 4 µg pcDNA3, pcDNA3-CEBPA (Q311) or pcDNA3-CEBPA (Q311P) plasmid. Nuclear extracts were prepared 24h after transfection using HEPES-NP40 lysis buffers according to TF-Detect Assay Kit (GeneCopoeia Inc, Rockville, MD, USA) and binding reaction was set up in 10 mM HEPES pH7.4, 50 mM KCl, 2.5 mM MgCl2, 10% glycerol, 50 ng/µl bovine serum albumin, 25 ng/µl polydI/dC with 2.5 nM

probe and 500 nM competitor oligonucleotide. Probe A was an infrared-fluorescent labelled high affinity CEBP binding oligonucleotide (Metabion International AG, Germany) with the sequence 5' - TGCACATGTTGGGCAATGGTCT-3'. Cold competitor oligos were of wildtype 5' - TGCACATGTTGGGCAATGGTCT-3' and mutant sequence 5' - TGCACATGTTGGGAATTCGTCT-3'. Probe B was an infrared-fluorescent labelled low-affinity CEBP-binding oligonucleotide (Metabion International AG, Germany) with the sequence 5' - TCGCTGTTAGGAAATGTGTGT -3'. Cold competitor oligos were of the wildtype (5' - TCGCTGTTAGGAAATGTGTGT -3') and the mutant sequence (5' – TCGCTGTTAGGAATTCTGTGT -3').

Molecular modeling. The experimental 3D structure for human C/EBP-α (CEBPA; Uniprot: p49715) is not available but a structure for the basic-leucine zipper (bZIP) domain bound to a DNA segment has been solved for the rat (RCSB Protein Data Bank entry 1NWQ).[4] Details of the molecular modeling are provided in Supplementary Methods. To explore the effect of Q311P on the dimerization and function of C/EBP-α, we carried out several molecular dynamics simulations on the interaction between DNA and wildtype and mutated bZIP C/EBP-α protein complex.

The experimental 3D structure for human CCAAT/enhancer-binding protein alpha (CEBPA; Uniprot: p49715) is not available but a structure for the basic-leucine zipper (bZIP) domain bound to a DNA segment has been solved for the homologous organism, rat. The Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank entry for this protein structure is 1NWQ.[4] Protein sequences from bZIP domains of human and rat C/EBP-α are absolutely conserved and hence we used the PDB ID 1NWQ structure for our modeling and

analysis. The structure validation server, Molprobity [5], was used for flipping Asn/Gln residues, structure-based analysis and creating the initial models. The Discovery Studio visualizer (Accelrys Inc., San Diego, CA) was used for preparing mutant structures and analyzing the simulation results.

The structural impact of mutations were evaluated using AMBER (ver 12)[6] molecular dynamics (MD) simulations. An all-atom forcefield, ff99SB, along with the implicit solvent Born solvation model as implemented in the Sander module[7] was used as the default option for the MD simulations. Each MD simulation run length was set to 800 picoseconds with a 1 femtosecond time step. Temperature regulation (300K) was maintained using Langevin thermostat and energy interactions were calculated without cutoffs. Each simulation was repeated twice and the results were averaged.

A sequence-based analysis was also carried out using the combined information from secondary structure predictions and multiple sequence alignments of orthologous sequences. The relevant protein sequences were downloaded from the NCBI (http://www.ncbi.nlm.nih.gov/) database and Jalview (ver 2.8.1; www.jalview.org) was used for the sequence based analysis and creating figures.

Supplementary Table S1. *CEBPA* mutations and clinical characteristics in pure familial acute myeloid leukemia

Reference / Patient Identification	Subtype	Age at Diagnosis (Years)	Sex	Germline *CEBPA* Mutation*	Somatic *CEBPA* Mutation	Relapse	Death
Smith et al. (2004)							
II-3	M1	10	M	c.212delC		Yes	No
III-1	M2Eo	30	M	c.212delC	1050-1086 (36bp duplication)	No	No
III-5	M2Eo	18	F	c.212delC		No	No
Sellick et al. (2005)							
III-1	ND	34	M	c.217insC	ND	NR	Yes
IV-2	M4Eo	25	M	c.217insC	10-bp insertion and 7-bp deletion at 1071	Yes	No
IV-4	M1	24	M	c.217insC	1071 (3-bp insertion)	No	No
V-1	M1	4	M	c.217insC	ND	Yes	No
Pabst et al. (2008)							
Pedigree A							
A-I-2	M2	46	F	c.291delC		NR	Yes
A-II-2	M1	40	F	c.291delC	1086 (3-bp insertion)	NR	No
A-III-3	Healthy Carrier	14	M	c.291delC		N/A	No
Pedigree B							
B-I-2	M1	42	M	c.465insT	1207 G>C (A353P); 1210 A>C (M354L)	NR	No
B-II-1	M2	27	F	c.465insT	1089 (3-bp insertion)	NR	No
Nanri et al. (2010)							
II-1	M2	39	M	c.350_351insCTAC	1063 (18-bp insertion); 1079 (3-bp insertion)	Yes	No
III-1	M2Eo	26	M	c.350_351insCTAC	1083 (3-bp insertion)	No	No
Renneville et al. (2009)							
I-2	M1	23	F	c.217insC	1083 (3-bp deletion): c.1083_1085delAAG	Yes	No
II-2	M1	5	M	c.217insC	1065 (3-bp insertion): c.1065_1066insGGG	Yes	No

* Genbank Accession: Y11525 as originally used by Smith et al. (2004) ND: Not Determined; NR: Not Reported; N/A: Not Applicable

Supplementary Table S2. ***In silico*** **predictions of** ***CEBPA*** **Q311P pathogenicity**

PREDICTION PROGRAM	SCORE	INTERPRETATION
SIFT	0	Deleterious
POLYPHEN 2 HVAR	0.997	Probably Damaging
MUTATION TASTER	0.999999	Disease Causing
MUTATION ASSESSOR	3.29	Medium
FATHMM--MISSENSE	0.89	Tolerated
FATHMM--CANCER	-3.37	Cancer
CADD Scaled C-Score	22.2	Top 1% of deleterious variants

Supplementary Table S3: Energies (kcal mol^{-1}) for the DNA fragment, wildtype C/EBPα monomer (Q), mutated C/EBPα monomer (P), wildtype dimer (Q:Q), single mutant dimer (Q:P), double mutant dimer (P:P), and their DNA complexes (Q:Q:DNA, Q:P:DNA, and P:P:DNA, respectively) from 800 picoseconds dynamics simulations, averaged over the last 700 picoseconds.

Simulation	Run-1	Run-2	Average
DNA	-6299.0	-6305.6	-6302.3
Q	-2696.4	-2704.5	-2700.5
P	-2616.7	-2615.9	-2616.3
Q:Q	-5449.8	-5482.5	-5466.2
Q:P	-5402.7	-5369.2	-5385.0
P:P	-5312.6	-5309.4	-5311.0
Q:Q:DNA	-12111.8	-12096.6	-12104.2
Q:P:DNA	-12031.4	-12002.4	-12016.9
P:P:DNA	-11956.8	-11911.5	-11934.2

Supplementary Table S4: Binding energies (Kcal mol^{-1}) of (A) the three possible dimers and (B) their complexes with DNA (see Table SIII for nomenclature)

(A)

Dimer	Dimerization Energy
Q:Q	65.3
Q:P	69.2
P:P	78.5

(B)

DNA Complex	Binding Energy
Q:Q:DNA	335.7
Q:P:DNA	328.6
P:P:DNA	320.9

Supplementary Table S5: Boltzmann distribution factors at 298.15K for (A) each dimer assuming equal abundances of the wildtype and mutated C/EBPα monomer and (B) the dimer:DNA complex assuming equal concentrations of each possible dimer.

(A)

Dimer	Boltzmann Probability
Q:Q	0.0004
Q:P	0.0042
P:P	0.9954

(B)

DNA Complex	Boltzmann Probability
Q:Q:DNA	0.9851
Q:P:DNA	0.0147
P:P:DNA	0.0001

SUPPLEMENATARY FIGURES

Supplementary Figure S1. Sequence electropherogram with a germline *CEBPA* mutation in patient IV.1 of the AML family. There is a chr19:g.33792389T>G mutation (highlighted in blue) in the *CEBPA* gene. Codon 311 is normally CAG (glutamine 311). This patient is heterozygous for the CCG (proline 311) codon.

Supplementary Figure S2. Homologous and paralogous sequence alignments of *CEBPA*. Top panel: alignment of homologs; bottom panel: alignment of paralogs. The aligned sequences were colored using BLOSUM62 scoring scheme. Secondary structure (jnetpred) is schematically shown as red tubes (helices) and green arrows (sheets). Alignment conservation scores for each column are shown in both histogram and raw format. The following annotations are used for the organisms: *H sapiens* (human); *P troglodytes* (chimpanzee); *M mulatta* (Rhesus monkey); *B taurus* (cow); *M musculus* (house mouse); *R norvegicus* (Norway rat); *G gallus* (chicken); *D rerio* (zebrafish); *X tropicalis* (Western clawed frog). The following short names are used for CEBPA paralogs: CEBPB: Beta, CEBPG: Gamma, CEBPD: Delta and CEBPE: Epsilon.

Supplementary Figure S3A. CEBPA bZIP domain-DNA complex. Coordinates from PDB ID 1NWQ were used. The DNA backbone is shown in “arrows” format and the base-pairs are represented in “ladders” format. The protein chain is shown as a solid ribbon and colored by N-to-C terminal style (blue-to-red). Residues Q311 from both chains are marked in CPK style.

Supplementary Figure S3B. Zoomed in view of hydrogen bonding of Q311. T310 is shown on left and Q311 from the dimerized pair on the right. Hydrogen bonded interactions are marked in green dotted line. Only one pair is shown for clarity.

Supplementary Figure S4. Final configurations from one of the Molecular Dynamics (MD) simulation runs for the wild-type (WT_MD), single mutant Q311P (SMUT_MD) and the double mutant, Q311P/Q311P (DMUT_MD), compared to the WT. Residues that are present in the non simulated WT dimer interface (4Å) (Dark green). Corresponding residues from the simulated structures are shown in dark orange.

SUPPLEMENTARY TABLES

Supplementary Table S1. *CEBPA* mutations and clinical characteristics in pure familial acute myeloid leukemia

Supplementary Table S2. *In silico* predictions of *CEBPA* Q311P pathogenicity

Supplementary Table S3. Energies (kcal mol^{-1}) for the DNA fragment, wildtype C/EBPα monomer (Q), mutated C/EBPα monomer (P), wildtype dimer (Q:Q), single mutant dimer (Q:P), double mutant dimer (P:P), and their DNA complexes (Q:Q:DNA, Q:P:DNA, and P:P:DNA, respectively) from 800 picosecond dynamics simulations, averaged over the last 700 picoseconds.

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Supplementary Table S5. Boltzmann distribution factors at 298.15 Kelvin for (A) each dimer assuming equal abundances of the wildtype and mutated CEBPA monomer and (B) the dimer:DNA complex assuming equal concentrations of each possible dimer.

Supplementary Material References

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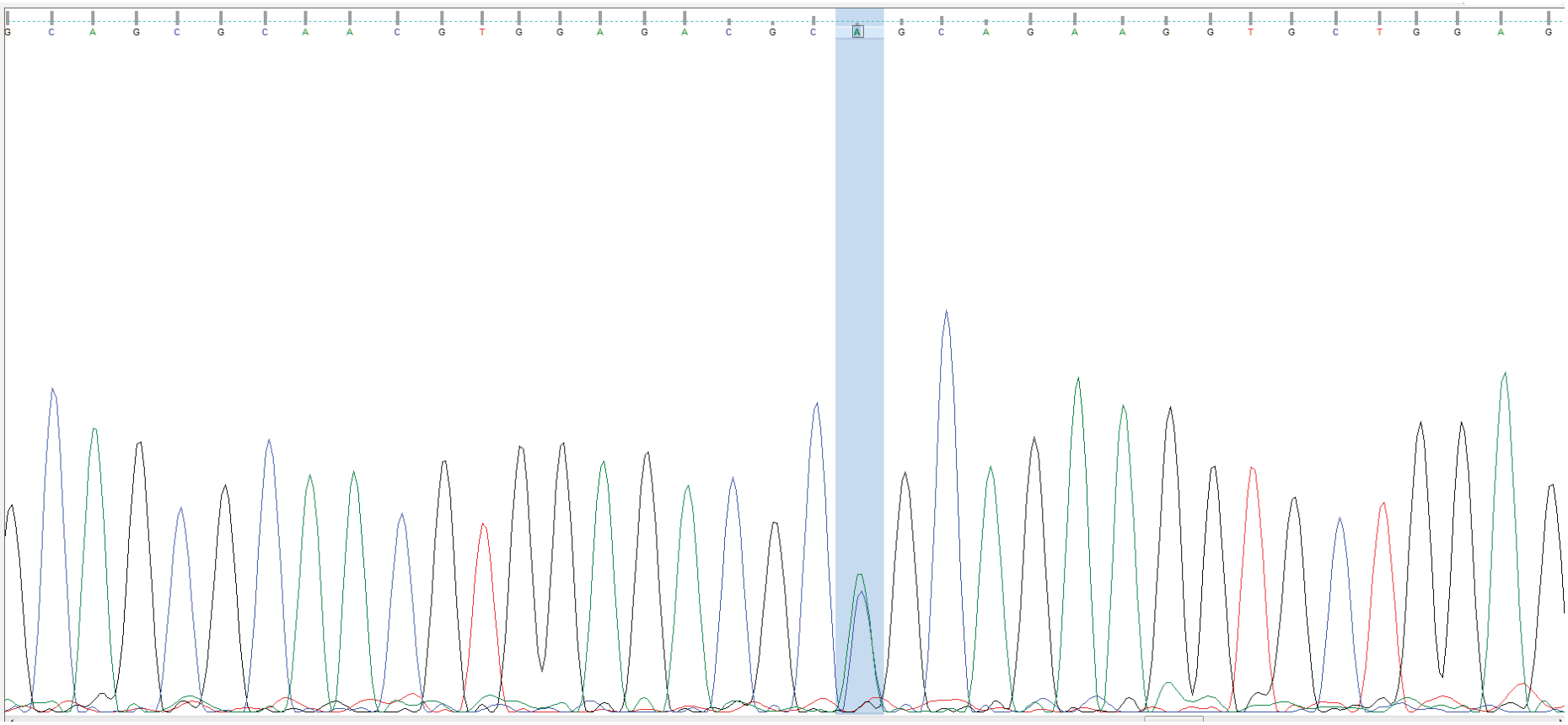
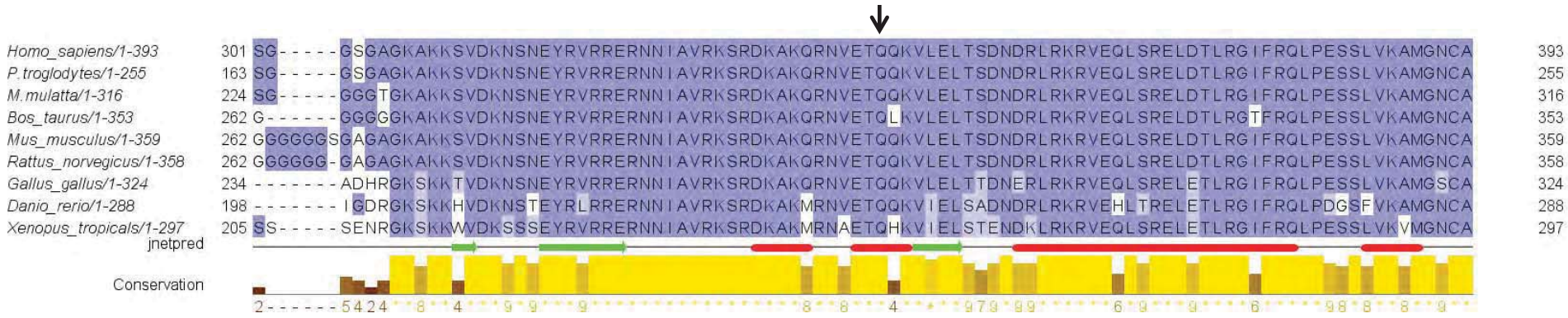
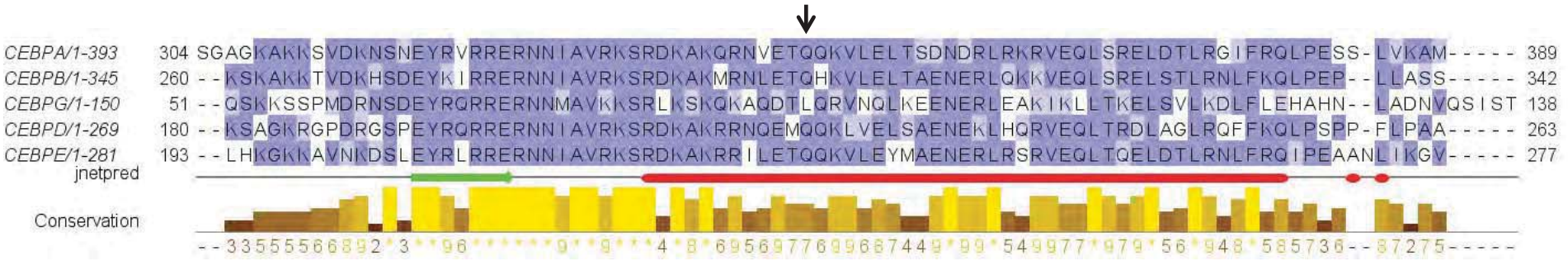
Supplementary Figure S1. Sequence electropherogram with a germline *CEBPA* mutation in patient IV.1 of the AML family

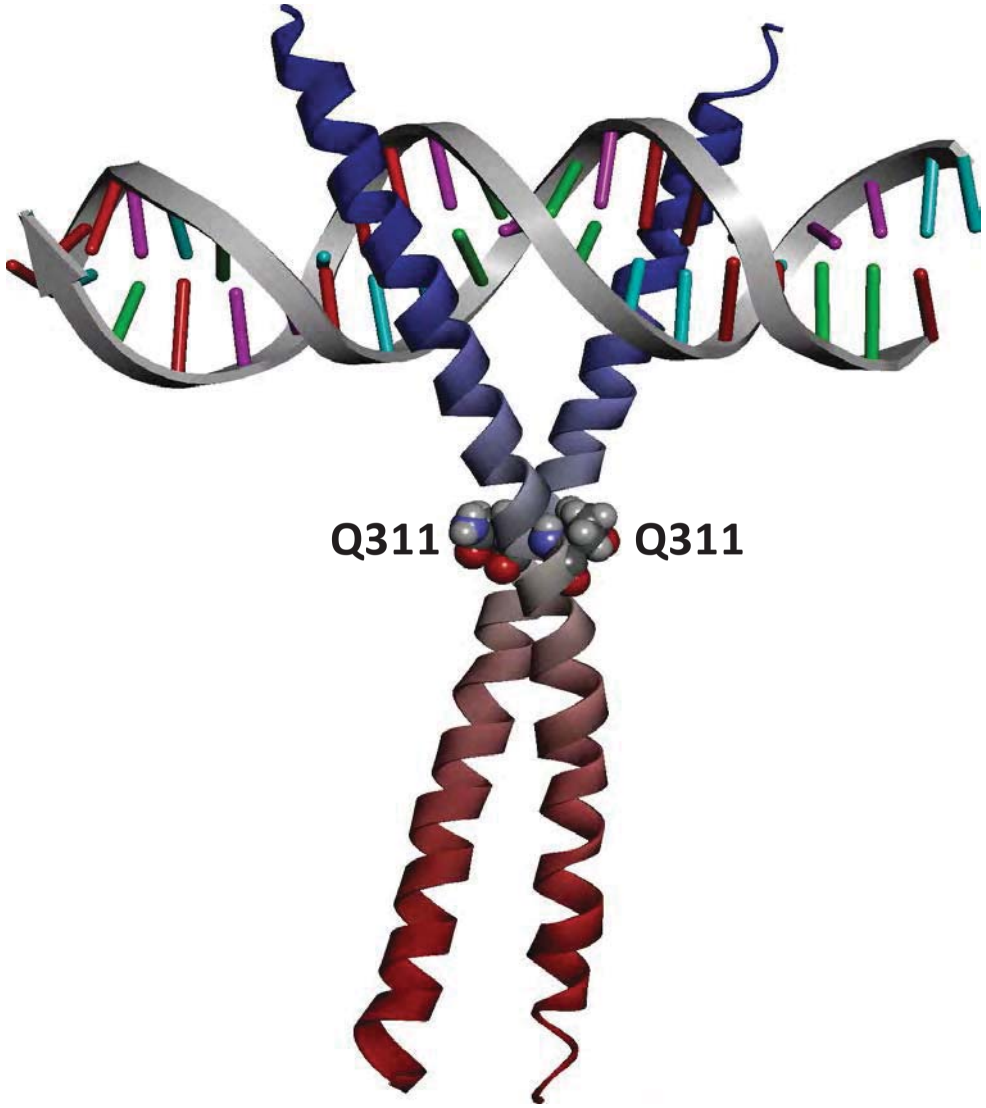


Supplementary Figure S2. Homologous and paralogous sequence alignments of CEBPA

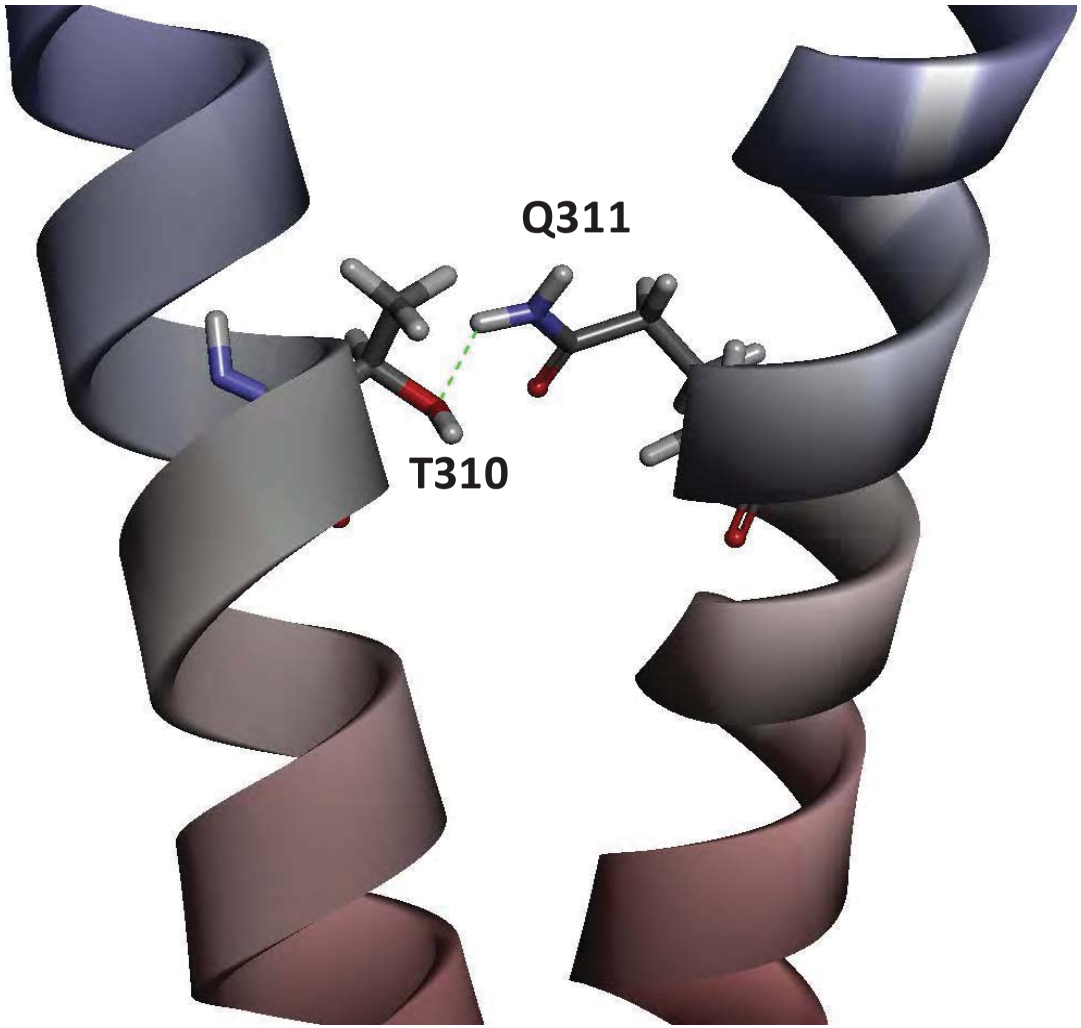




Supplementary Figure S3A. CEBPA bZIP domain-DNA complex.



Supplementary Figure S3B. Zoomed in view of hydrogen bonding of Q311



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