A novel in-frame deletion in the leucine zipper domain of C/EBP ε leads to neutrophil-specific granule deficiency

A novel in-frame deletion in the leucine zipper domain of C/EBP-epsilon leads to neutrophil-specific granule deficiency

Taizo Wada,[*] Tadayuki Akagi,[†] Masahiro Muraoka,[*] Tomoko Toma,[*] Kenzo Kaji,[‡] Kazunaga Agematsu,[§] H. Phillip Koeffler,[¶,‖] Takashi Yokota,[†] and Akihiro Yachie[*]

Institutional affiliations: [*]Department of Pediatrics, and [†]Department of Stem Cell Biology, School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan; [‡]Department of Dermatology, Komatsu Municipal Hospital, Komatsu, Japan; [§]Department of Infection and Host Defense, Shinshu University Graduate School of Medicine, Matsumoto, Japan; [¶]Division of Hematology and Oncology, Cedars-Sinai Medical Center, University of California Los Angeles School of Medicine, Los Angeles, California, USA; and [‖]Cancer Science Institute of Singapore, National University of Singapore, Singapore, Singapore.

Correspondence to: Taizo Wada, MD, PhD
Department of Pediatrics, School of Medicine,
Institute of Medical, Pharmaceutical and Health Sciences,
Kanazawa University
13-1 Takaramachi, Kanazawa 920-8641, Japan
Phone: +81-76-265-2313
Fax: +81-76-262-1866
E-mail: taizo@staff.kanazawa-u.ac.jp

Author Notes: T. Wada and T. Akagi contributed equally to this work.

Running title: Novel C/EBPε deletion mutation in SGD

Source of support:
This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from the Ministry of Health, Labour, and Welfare of Japan, Tokyo.

Abstract

Neutrophil-specific granule deficiency (SGD) is a rare autosomal recessive primary immunodeficiency characterized by neutrophil dysfunction, bilobed neutrophil nuclei and lack of neutrophil-specific granules. Defects in a myeloid-specific transcription factor, CCAAT/enhancer binding protein-epsilon (C/EBPε), have been identified in two cases where homozygous frameshift mutations lead to loss of the leucine zipper domain. Here, we report a 55-year-old female affected with SGD caused by a novel homozygous 2-amino-acid deletion (ΔRS) in the leucine zipper domain of the C/EBPε gene. The patient showed characteristic neutrophil abnormalities and recurrent skin infections, however, there was no history of deep organ infections. Biochemical analysis revealed that, in contrast to the two frameshift mutations, the ΔRS mutant maintained normal cellular localization, DNA-binding activity and dimerization, and all three mutants exhibited marked reduction in transcriptional activity. The ΔRS mutant was defective in its association with Gata1 and PU.1, as well as aberrant cooperative transcriptional activation of eosinophil major basic protein. Thus, the ΔRS likely impairs protein-protein interaction with other transcription factors resulting in a loss of transcriptional activation. These results further support the importance of the leucine zipper domain of C/EBPε for its essential function, and indicate that multiple molecular mechanisms lead to SGD.

Introduction

Neutrophil-specific granule deficiency (SGD) is a rare autosomal recessive primary immunodeficiency characterized by either profound reduction or absence of neutrophil-specific granules and bilobed neutrophil nuclei (pseudo-Pelgar-Huët anomaly) (1). It was previously called lactoferrin deficiency. Patients with SGD present with increased susceptibility to bacterial infections, especially affecting the skin, ears, lungs and lymph nodes. The gene responsible for SGD is the CCAAT/enhancer binding protein-epsilon (C/EBPε) gene. To date, two patients have been reported who carry C/EBPε frameshift mutations that result in abrogated protein expression (2, 3). In addition, another patient with SGD had a heterozygous missense mutation of the C/EBPε gene, but this mutation was unlikely to have caused disease because it resulted in elevated levels of C/EBPε (4). In this patient, the growth factor independence 1 (Gfi-1) protein that represses transcription of C/EBPε was decreased, although the patient had no mutation of the Gfi-1 gene. Taken together, these previous findings suggest that SGD is a genetically heterogeneous disease. Because of SGD's extreme rarity, how wide the spectrum of clinical symptoms and cellular abnormalities of the disease is unclear.

C/EBPε is a member of the C/EBP family of widely expressed transcription factors that regulate proliferation, differentiation and apoptosis in a variety of cell types (5, 6). The C/EBP family consists of 6 members (C/EBPα, β, γ, δ, ε and ζ), and cellular expression of each C/EBP is tightly regulated. They bind to DNA through the highly conserved basic leucine zipper (bZIP) domain. C/EBPε is restricted to granulocytes and is essential for their terminal differentiation (6, 7). C/EBPε transcription primarily occurs at

the myelocyte/metamyelocyte stage of differentiation and decreases in polymorphonuclear neutrophils. The human C/EBPε gene produces 4 isoforms of 32, 30, 27 and 14 kDa, which are functionally different; only the 32 kDa C/EBPε has full transactivating potential (8-10). C/EBPε is indispensable for expression of genes encoding proteins that reside in specific granules of neutrophils such as lactoferrin and defensins. Many features of SGD are manifested by C/EBPε-deficient mice in which neutrophils are morphologically and functionally abnormal and eosinophil numbers are decreased (8).

Here, we describe another case of SGD with a novel two amino-acid (aa) deletion in the bZIP domain of C/EBPε, and report the mechanism that leads to SGD. We also carried out clinical, cellular and molecular comparisons among this patient and the previously reported two cases of SGD (2, 3), and discuss the functional significance of the mutation.

Materials and Methods

Patients

We studied two Japanese patients with SGD. Patient P1 is a 55-year-old female who has suffered since late infancy from recurrent skin infections that often required more than 2 months to heal. After hospitalization due to severe otitis media at 54 years of age, she was referred to our hospital for suspected immunodeficiency. A history of parental consanguinity appeared likely. However, her farther had already died of a heart attack, and her mother refused genetic analysis. Her elder brother had similar skin symptoms and died of enterocolitis at 10 years of age. Another brother also died early after birth from unspecified causes. Two children of patient P1 and her granddaughter were healthy and they did not want genetic testing. Clinical and genetic data of patient P2 have already been published (3, 11). Patient P2 is now 40 years old. Approval for the study was obtained from the Human Research Committee of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

Flow cytometry and immunohistochemistry

For flow cytometric analysis the following mAbs were used: FITC-conjugated anti-CD16, anti-CD32 or CD66b and PE-conjugated anti-CD11b, anti-CD14, anti-CD15 anti-CD16 or anti-CD64 (BD, San Diego, CA); and FITC-conjugated anti-CD16b and PE-conjugated CD11c (Beckman Coulter, Fullerton, CA). Analysis was performed on a FACSCalibur using CellQuest software (BD Bioscience, Tokyo, Japan) (12). Cytospin

preparations were made using whole leukocytes. For lactoferrin staining, cytospin samples were stained with anti-lactoferrin mAb (Beckman Coulter), followed by universal immuno-alkaline-phosphatase polymer (Simple Stain AP; Nichirei Biosciences, Tokyo, Japan). The alkaline phosphatase activity was visualized using Fast Red TR salt and Naphtol AS-MX phosphate (Sigma-Aldrich, St. Louis, MO). For analysis of internal alkaline phosphatase and peroxidase activity, alkaline phosphatase substrate solution (BCIP/NBT Substrate system, DAKO, Glostrup, Denmark) and a peroxidase staining kit (Muto Pure Chemicals Co., Ltd, Tokyo, Japan) were used, respectively.

Mutation analysis

Peripheral blood and/or buccal mucosa samples were obtained from patients, and DNA was extracted from the samples using a standard method. Direct sequencing of the C/EBPε gene was performed as described previously (2).

Cell culture and RT-PCR

Human embryonic kidney (HEK) 293 and mouse NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing either 10% fetal bovine serum or 10% calf serum, respectively. Total RNA was extracted from transfected NIH3T3 cells with Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and converted to cDNAs by ReverTraAce (Toyobo, Tokyo, Japan). Expression of human C/EBPε and PU.1, and murine cathelicidin B9/neutrophil granule protein (NGP), neutrophil gelatinase-associated lipocalin (NGAL)/ lipocalin 2 (Lcn2), lactoferrin, proteoglycan 2 (Prg2)/eosinophil granule eosinophil major basic protein (MaBP), GATA binding protein

1 (Gata1), and GAPDH was examined by RT-PCR. Primer sequences are reported in Table S1. The number of amplification cycles was 20 for GAPDH, 25 for C/EBPε, PU.1, MaBP and Gata1, 30 for B9 and NGAL, and 35 for lactoferrin. The PCR cycle consisted of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. For lactoferrin, the annealing temperature was 64°C. All PCR reactions were carried out with Taq polymerase (Ampliqon, Odense, Denmark) except for C/EBPε (PrimeSTAR Max DNA polymerase, Takara bio, Otsu, Japan).

Construction of plasmid, luciferase assay and cellular localization

Construction of mammalian expression vectors, including pCMV5-Flag- maltose-binding protein (MBP), pCAGIP-enhanced GFP, and pCAGIP-Myc, was described previously (13, 14). Coding region of wild-type human C/EBPε and its mutants, ΔRS, del5bp and insA, were amplified by PCR using specific primers (Table S1) and cloned into expression vectors. Murine Gata1 and human PU.1 expression vectors were described previously (15), and their coding regions were cloned into the pCAGIP-Myc vector. The luciferase reporter plasmid containing the G-CSF receptor promoter (pGCSFR-Luc) has been described before (16). The reporter plasmid, C/EBPε and C/EBPε mutant expression vectors (ΔRS, del5bp and insA), were transfected into HEK293 cells by Lipofectamine 2000 (Life Technologies, Grand Island, NY). To determine luciferase activity, cell extracts were prepared 48 h after transfection, and luciferase activity was measured with a luciferase assay kit (Promega, Madison, WI) using an AB-2200 luminometer (ATTO, Tokyo, Japan). Protein concentration of each sample was measured by Protein Assay CBB solution (Nacalai Tesque). Relative

luciferase activity per 1 μg protein was represented. To observe cellular localization of C/EBPε and its mutants, GFP-tagged fusion protein expression vectors were introduced into NIH3T3 cells. Nuclei were stained by Hoechst 33258 (Sigma). Signals were observed 48 h after transfection.

Biotin-labeled DNA pull-down assay, MBP pull-down assay and Western blot analysis

Biotin-labeled DNA pull-down assay was performed as previously described (13, 17). Briefly, biotin-labeled oligonucleotides containing the human lactoferrin gene C/EBPε binding site (5'-GGGTGTCTATTGGGCAACAGGGCGGG-3') were incubated with cell extracts from HEK293 cells transfected with either pCAGIP-Myc-C/EBPε or its mutant counterparts (ΔRS, del5bp and insA) in the presence of streptavidin-agarose (Novagen, Darmstadt, Germany). Twenty-five-fold non-labeled oligonucleotides (either wild-type or mutant non-binding control) were added for the competition assays. The beads were washed 3 times with a washing buffer, and the bound proteins were eluted by boiling in 2 x SDS sample buffer. Samples were then examined by Western blot analysis as described below. MBP pull-down assay was performed as previously described (13, 17). HEK293 cells were cotransfected with pCAGIP-Myc-C/EBPε (wild-type or ΔRS) and pCMV5-Flag-MBP-C/EBPε (wild-type or ΔRS). HEK293 cells were also cotransfected with pCAGIP-Myc-Gata1 and pCMV5-Flag-MBP-C/EBPε or its mutant counterparts, or with pCAGIP-Myc-PU.1 and pCMV5-Flag-MBP-C/EBPε or its mutant counterparts. MBP-fused proteins were pulled down by amylose resin, and the precipitates were analyzed by Western blot analysis. For Western blot analysis, samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The

membrane was incubated with either anti-Myc (sc-40; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Flag (F3165; Sigma–Aldrich) Abs followed by horseradish peroxidase-conjugated anti-mouse Ab (Millipore, Billerica, MA). The blot was visualized using enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA) with an LAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

Results

Bilobed nucleus and lack of granules in neutrophils

Patient P1 presented with an ulcerative skin abscess and numerous skin scars (Fig. 1A,B). Her peripheral blood smear showed unique bilobed nuclei and lack of cytoplasmic granules in her neutrophils (Fig. 1C). Absence of eosinophils, increased basophils with normal cytoplasmic granularity, and monocytosis were also noted on smears of the peripheral blood (data not shown). The immunohistochemical analysis of her neutrophils clearly demonstrated the presence of peroxidase, a primary granule protein, and the absence of lactoferrin and alkaline phosphatase, both of which are normally expressed in specific granules of normal neutrophils (Fig. 1D). These characteristic features were quite similar to those of patient P2, the second case of genetically defined SGD, who carries a homozygous C/EBPε mutation, c.508_509insA (insA) (3).

Flow cytometric analysis of peripheral blood showed lower side scatter of P1 neutrophils compared to normal controls (Fig. 2) (18). More importantly, the majority of the patient's neutrophils that were defined on the basis of forward and side scatter expressed a monocyte marker CD14, indicating aberrant development toward the monocyte pathway. These cells did not express neutrophil markers such as CD15, CD16b (FcγRIIIB), and CD66b. Although CD64 (FcγRI) was not detected on her neutrophils and monocytes, CD11b, CD11c and CD32 (FcγRII) were detectable equally on both cells (data not shown). Again, all of these characteristic features were quite similar to those of patient P2. Neutrophils from patient P2 showed more CD16 expression compared to P1.

Two amino acid deletion in bZIP and reduced transcriptional activity

Direct sequence analysis revealed that patient P1 had a homozygous 6-bp deletion in exon 2 of the C/EBPε gene (c.739_744delCGCAGC). This novel mutation leads to a two aa deletion, p.Arg247_Ser248del (ΔRS), which is located in the bZIP domain (Fig. 3). The mutation was present in DNA isolated from both the peripheral blood and buccal mucosa, indicating a germline mutation, not a somatic one. Analysis of 100 alleles of ethnically matched healthy controls demonstrated the absence of the mutation in the general population.

To evaluate the transcriptional activation by the ΔRS mutant, HEK293 cells were cotransfected with a G-CSF receptor promoter reporter construct and C/EBPε expression vectors. As shown in Fig. 4A, wild-type C/EBPε presented robust reporter activity, whereas the ΔRS mutant exhibited a significant decrease in activity, similar to the previously reported mutants, insA and del5bp (c.249_253delTGACC) (2, 3), both of which were frameshift mutations with truncated proteins. HEK 293 cells transfected with the ΔRS mutant expressed levels of C/EBPε comparable to wild type, as assessed by Western blot analysis, indicating ΔRS does not cause instability in the mutated C/EBPε protein (Fig. 4B). Increasing amounts of ΔRS had no negative effect on reporter activity of wild-type C/EBPε, indicating a lack of a dominant negative effect of ΔRS on transcriptional activity (Fig. 4C). We also analyzed the ability of the C/EBPε mutant proteins to activate gene expression of secondary granule genes. As shown in Fig. 4D, wild-type C/EBPε was able to induce expression of endogenous B9, NGAL, and lactoferrin genes in transiently transfected NIH3T3 cells. In contrast, none of these genes

was amplified from cells transfected with either the ΔRS mutant or the insA and del5bp mutants, consistent with loss of their capability to activate secondary granule genes.

Unaltered cellular localization, DNA-binding and dimerization

To understand the mechanism by which the ΔRS mutation decreases transcriptional activity, we first investigated the cellular localization of wild-type and mutant C/EBPε proteins. GFP fluorescence was diffusely detected within the cytoplasm of NIH3T3 cells transfected with the control GFP vector, whereas wild-type as well as ΔRS mutant C/EBPε tagged with GFP was found in the nucleus (Fig. 5). Consistent with the previous report (3), the insA mutant was localized in the cytoplasm as well as the nucleus; and a similar abnormal localization was detected in the del5bp.

We next assessed the ability of the wild-type and mutant C/EBPε to bind to C/EBPε binding site at the 5'UTR of human lactoferrin gene (19). The oligonucleotides containing the C/EBPε binding site bound in vitro to C/EBPε from lysates of HEK293 cells transfected with wild-type Myc-C/EBPε vector (Fig. 6, top left blot). Importantly, the ΔRS mutant showed DNA-binding ability comparable to the wild-type C/EBPε (Fig. 6A, top right blot). Nonlabeled oligonucleotides of the same sequence, but not those with a mutated sequence, were able to compete with the biotinylated oligonucleotides for sequence specific binding of both the wild-type and ΔRS mutant C/EBPε. In contrast, no product was precipitated from lysates of cells transfected with the del5bp and the insA mutants, indicating that no oligonucleotide binding took place (Fig. 6A, bottom panels).

To assess further the functionality of the ΔRS mutant, we examined dimer formation in lysates of HEK293 cells cotransfected with Myc-C/EBPε and Flag-MBP-

C/EBPε vectors. The ΔRS mutant was able to homodimerize or heterodimerize with wild-type C/EBPε at levels comparable to wild-type C/EBPε (Fig. 6B and data not shown). These results were consistent with the ability of the ΔRS mutant to bind to DNA.

Aberrant association with Gata1 and PU.1

To determine whether the ΔRS mutant properly interacts with other proteins, cooperative transcriptional activation of MaBP was analyzed in NIH3T3 cells. Consistent with the previous report (15), MaBP gene expression was observed in cells transfected with Gata1 and PU.1 in addition to wild-type C/EBPε, and no products were obtained from those without wild-type C/EBPε (Fig. 7A). Interestingly, the ΔRS mutant, as well as the del5bp and the insA mutants, failed to induce MaBP gene expression.

To assess further the ability of the C/EBPε mutants to bind to Gata1 as well as PU.1, the MBP pull-down assay was performed. As expected, both Gata1 and PU.1 were able to bind in vitro to C/EBPε in lysates of HEK293 cells transfected with the wild-type Flag-MBP-C/EBPε vector; and no binding was observed in lysates of cells transfected with the del5bp and the insA mutants (Fig. 7B,C). Some binding of the ΔRS mutant to Gata1 or PU.1 was indicated by the results, but the amounts of precipitated Gata1 and PU.1 were extremely low in lysates from HEK293 cells transfected with the ΔRS mutant.

Discussion

C/EBPε is essential for terminal differentiation of granulocytes. Frameshift mutations of the C/EBPε gene have been identified in two patients with SGD. Here, we report on a 55-year-old female (P1) affected with SGD caused by a novel 2-aa deletion mutation of the C/EBPε gene. This case represents a third case of genetically defined SGD. The availability of blood samples from the previous case (P2) offered us the unique opportunity to evaluate and compare phenotype of peripheral neutrophils in these patients. In addition to the morphological abnormalities typical for SGD, we found characteristic surface phenotype in their neutrophils including the presence of monocyte markers such as CD14 and the absence of neutrophil markers such as CD15, CD16b and CD66b. It is therefore difficult to distinguish neutrophils from monocytes by surface markers in the patients. CD16 includes two isoforms, CD16a (FcγRIIIA) and CD16b. CD16a is a transmembrane receptor expressed by monocytes, NK cells and NKT cells, whereas CD16b is a glycosylphosphatidylinositol-anchored receptor which is thought to be exclusively expressed by neutrophils. Because pan-CD16, but not CD16b was detected in a subset of the patient's neutrophils, they likely expressed CD16a. Neutrophils from patient P2 showed higher levels of CD16 expression than those of patient P1, indicating a larger subpopulation of $CD16a^{+}$ neutrophils. Human monocytes are divided into two major subsets, $CD14^{++}CD16a^{-}$ and $CD14^{+}CD16a^{+}$ cells. Various inflammatory conditions including infections lead to an increased subpopulation of CD16a-positive monocytes (20, 21). CD16 expression of neutrophils of patient P1 was associated with infections (data

not shown); therefore, CD16a expression on SGD neutrophils may depend on inflammatory immune stimuli.

The reason why the patient's neutrophils expressed monocyte markers, including CD14 and CD16a, is presently unclear. Studies of granulocytes from healthy volunteers who were given G-CSF and from human embryonic stem cells treated with multiple growth factors have demonstrated aberrant expression of CD14 on mature granulocytes (22, 23). Although we did not measure any soluble factors related to granulocytic differentiation in our patients, defective myeloid differentiation in SGD could lead to dysregulated secretion of growth factors resulting in aberrant surface expression of neutrophil proteins. In vitro modeling of neutrophil development in SGD using induced pluripotent stem cells will be required to address these issues.

We carried out a comprehensive in vitro study to evaluate transcriptional activity, cellular localization, DNA-binding activity, dimerization and protein-interaction of the ΔRS mutant as well as the two frameshift mutants. All three mutants exhibited marked reduction in transcriptional activity. The ΔRS mutation is located in the bZIP domain, that is highly conserved among the C/EBP family members and has an important role in DNA binding and dimerization (5). However, the ΔRS mutant maintained normal cellular localization, DNA-binding activity and dimerization, in contrast to the frameshift mutations, del5bp and insA, which destroy the bZIP domain and thus are predicted to interfere with dimerization and binding to DNA. No dominant negative effect of the ΔRS mutant may suggest that the single normal C/EBPε allele is sufficient to maintain transcriptional activity, which is consistent with the fact that the mother and two children of patient P1, who are assumed to be heterozygous for the ΔRS mutation, remain in good

health. The association of C/EBPε with other transcription factors has been demonstrated to be important for the regulation of secondary granule gene expression in both neutrophils and eosinophils (15). In fact, the ΔRS mutant was found to be defective in association with Gata1 and PU.1, as well as aberrant cooperative transcriptional activation of eosinophil MaBP. Gata1 is primarily associated with erythroid and megakaryocyte differentiation, whereas PU.1 is more important for neutrophil differentiation. These results are in line with the fact that eosinophils were not detectable in patients P1 and P2. Taken together, our findings suggest that the ΔRS mutation impairs protein-protein interaction with Gata1 and with PU.1, resulting in loss of cooperative transcriptional activation.

A similar mutation has been described in a patient with acute myeloid leukemia, in which an in-frame 3-bp deletion within the leucine zipper domain of C/EBPα abrogated the transcriptional activation function of C/EBPα on the G-CSF receptor promoter (24). Like the ΔRS mutant, this mutant lacked a dominant negative effect, although its protein-protein interaction with other transcription factors remained unexamined. Further investigation will be necessary to assess whether the ΔRS mutant also exhibit defective interaction with other transcription factors such as c-Myb, PML, p300, E2F1 and Rb (9, 16, 25, 26). Because certain isoforms of C/EBPε have been reported to inhibit the synergistic activities of GATA1 and PU.1 (25), we also need to evaluate the isoforms of the C/EBPε mutants other than the full length, 32 kDa C/EBPε.

These characteristics of the ΔRS mutant, wherein modest association with Gata1 and PU.1 is retained and nuclear localization remains intact, may be associated with less severe clinical symptoms of patient P1. To date, patient P1 has shown no deep organ

infection, whereas the other patients exhibited more severe presentation of the disease. The first reported patient with the del5bp mutation died from complications of pneumonia, and patient P2 suffered from recurrent pneumonia, as well as lung abscess (2, 3). On the other hand, recurrent bacterial skin abscess that persisted a few months was observed in all of the patients with SGD including patient P1 (27). Skin abscess smears from patient P1 showed that most infiltrating cells were monocytes and macrophages, some of which phagocytosed bacteria (data not shown). Monocytes from C/EBPε-deficient mice exhibited impaired maturation and altered cytokine expression, such as increased levels of TNF-α and LTβ, in response to inflammation (28, 29). In addition, monocyte counts in C/EBPε-deficient mice were higher than those of wild-type mice (30). Thus, impaired inflammatory response and killing of bacteria by SGD patients' monocytes may hinder the healing process, resulting in unique skin abscesses. Understanding which factors evoke an abnormal microenvironment at infectious sites will be necessary to develop more effective therapeutic approaches for patients with SGD.

In summary, our studies identified a novel in-frame deletional mutation in the bZIP domain of C/EBPε and demonstrated its molecular pathogenesis leading to SGD. Comparative analysis of the C/EBPε mutations, including the previous frameshift mutations, also clarifies the functional significance of these mutants. Characterization of C/EBPε genetic defects and functional abnormalities will help define the role of C/EBPε in human myelopoiesis and innate immunity.

Acknowledgments

We thank Dr. Kuniaki Naganuma for his devoted patient care, and Ms. Harumi Matsukawa and Ms. Shizu Kouraba for their excellent technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Gombart, A. F., and H. P. Koeffler. 2002. Neutrophil specific granule deficiency and mutations in the gene encoding transcription factor C/EBPε. *Curr Opin Hematol* 9: 36-42.
2. Lekstrom-Himes, J. A., S. E. Dorman, P. Kopar, S. M. Holland, and J. I. Gallin. 1999. Neutrophil-specific granule deficiency results from a novel mutation with loss of function of the transcription factor CCAAT/enhancer binding protein ε. *J Exp Med* 189: 1847-1852.
3. Gombart, A. F., M. Shiohara, S. H. Kwok, K. Agematsu, A. Komiyama, and H. P. Koeffler. 2001. Neutrophil-specific granule deficiency: homozygous recessive inheritance of a frameshift mutation in the gene encoding transcription factor CCAAT/enhancer binding protein-ε. *Blood* 97: 2561-2567.
4. Khanna-Gupta, A., H. Sun, T. Zibello, H. M. Lee, R. Dahl, L. A. Boxer, and N. Berliner. 2007. Growth factor independence-1 (Gfi-1) plays a role in mediating specific granule deficiency (SGD) in a patient lacking a gene-inactivating mutation in the C/EBPε gene. *Blood* 109: 4181-4190.
5. Tsukada, J., Y. Yoshida, Y. Kominato, and P. E. Auron. 2011. The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine* 54: 6-19.

6. Chumakov, A. M., I. Grillier, E. Chumakova, D. Chih, J. Slater, and H. P. Koeffler. 1997. Cloning of the novel human myeloid-cell-specific C/EBP-ε transcription factor. *Mol Cell Biol* 17: 1375-1386.

7. Lekstrom-Himes, J. A. 2001. The role of C/EBPε in the terminal stages of granulocyte differentiation. *Stem Cells* 19: 125-133.

8. Yamanaka, R., C. Barlow, J. Lekstrom-Himes, L. H. Castilla, P. P. Liu, M. Eckhaus, T. Decker, A. Wynshaw-Boris, and K. G. Xanthopoulos. 1997. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein ε-deficient mice. *Proc Natl Acad Sci U S A* 94: 13187-13192.

9. Verbeek, W., A. F. Gombart, A. M. Chumakov, C. Muller, A. D. Friedman, and H. P. Koeffler. 1999. C/EBPε directly interacts with the DNA binding domain of c-myb and cooperatively activates transcription of myeloid promoters. *Blood* 93: 3327-3337.

10. Tang, J. G., and H. P. Koeffler. 2001. Structural and functional studies of CCAAT/enhancer-binding protein epsilon. *J Biol Chem* 276: 17739-17746.

11. Shiohara, M., A. F. Gombart, Y. Sekiguchi, E. Hidaka, S. Ito, T. Yamazaki, H. P. Koeffler, and A. Komiyama. 2004. Phenotypic and functional alterations of peripheral blood monocytes in neutrophil-specific granule deficiency. *J Leukoc Biol* 75: 190-197.

12. Toga, A., T. Wada, Y. Sakakibara, S. Mase, R. Araki, Y. Tone, T. Toma, T. Kurokawa, R. Yanagisawa, K. Tamura, N. Nishida, H. Taneichi, H. Kanegane, and A. Yachie. 2010. Clinical significance of cloned expansion and CD5 down-

regulation in Epstein-Barr Virus (EBV)-infected $CD8^+$ T lymphocytes in EBV-associated hemophagocytic lymphohistiocytosis. *J Infect Dis* 201: 1923-1932.

13. Sun, C., Y. Nakatake, T. Akagi, H. Ura, T. Matsuda, A. Nishiyama, H. Koide, M. S. Ko, H. Niwa, and T. Yokota. 2009. Dax1 binds to Oct3/4 and inhibits its transcriptional activity in embryonic stem cells. *Mol Cell Biol* 29: 4574-4583.

14. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193-199.

15. Gombart, A. F., S. H. Kwok, K. L. Anderson, Y. Yamaguchi, B. E. Torbett, and H. P. Koeffler. 2003. Regulation of neutrophil and eosinophil secondary granule gene expression by transcription factors C/EBPε and PU.1. *Blood* 101: 3265-3273.

16. Gery, S., A. F. Gombart, Y. K. Fung, and H. P. Koeffler. 2004. C/EBPε interacts with retinoblastoma and E2F1 during granulopoiesis. *Blood* 103: 828-835.

17. Uranishi, K., T. Akagi, C. Sun, H. Koide, and T. Yokota. 2013. Dax1 associates with Esrrb and regulates its function in embryonic stem cells. *Mol Cell Biol* 33: 2056-2066.

18. McIlwaine, L., A. Parker, G. Sandilands, P. Gallipoli, and M. Leach. 2013. Neutrophil-specific granule deficiency. *Br J Haematol* 160: 735.

19. Gery, S., D. J. Park, P. T. Vuong, D. Y. Chih, N. Lemp, and H. P. Koeffler. 2004. Retinoic acid regulates C/EBP homologous protein expression (CHOP), which negatively regulates myeloid target genes. *Blood* 104: 3911-3917.

20. Ziegler-Heitbrock, L. 2007. The $CD14^+CD16^+$ blood monocytes: their role in infection and inflammation. *J Leukoc Biol* 81: 584-592.

21. Mizuno, K., T. Toma, H. Tsukiji, H. Okamoto, H. Yamazaki, K. Ohta, Y. Kasahara, S. Koizumi, and A. Yachie. 2005. Selective expansion of $CD16^{high}CCR2^{-}$ subpopulation of circulating monocytes with preferential production of haem oxygenase (HO)-1 in response to acute inflammation. *Clin Exp Immunol* 142: 461-470.

22. Kerst, J. M., M. de Haas, C. E. van der Schoot, I. C. Slaper-Cortenbach, M. Kleijer, A. E. von dem Borne, and R. H. van Oers. 1993. Recombinant granulocyte colony-stimulating factor administration to healthy volunteers: induction of immunophenotypically and functionally altered neutrophils via an effect on myeloid progenitor cells. *Blood* 82: 3265-3272.

23. Yokoyama, Y., T. Suzuki, M. Sakata-Yanagimoto, K. Kumano, K. Higashi, T. Takato, M. Kurokawa, S. Ogawa, and S. Chiba. 2009. Derivation of functional mature neutrophils from human embryonic stem cells. *Blood* 113: 6584-6592.

24. Gombart, A. F., W. K. Hofmann, S. Kawano, S. Takeuchi, U. Krug, S. H. Kwok, R. J. Larsen, H. Asou, C. W. Miller, D. Hoelzer, and H. P. Koeffler. 2002. Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. *Blood* 99: 1332-1340.

25. Du, J., M. J. Stankiewicz, Y. Liu, Q. Xi, J. E. Schmitz, J. A. Lekstrom-Himes, and S. J. Ackerman. 2002. Novel combinatorial interactions of GATA-1, PU.1, and C/EBPε isoforms regulate transcription of the gene encoding eosinophil granule major basic protein. *J Biol Chem* 277: 43481-43494.

26. Tagata, Y., H. Yoshida, L. A. Nguyen, H. Kato, H. Ichikawa, F. Tashiro, and I. Kitabayashi. 2008. Phosphorylation of PML is essential for activation of C/EBPε and PU.1 to accelerate granulocytic differentiation. *Leukemia* 22: 273-280.

27. Roberts, R. L. 2014. Neutrophil-specific granule deficiency.

28. Lekstrom-Himes, J., and K. G. Xanthopoulos. 1999. CCAAT/enhancer binding protein ε is critical for effective neutrophil-mediated response to inflammatory challenge. *Blood* 93: 3096-3105.

29. Tavor, S., P. T. Vuong, D. J. Park, A. F. Gombart, A. H. Cohen, and H. P. Koeffler. 2002. Macrophage functional maturation and cytokine production are impaired in C/EBPε-deficient mice. *Blood* 99: 1794-1801.

30. Akagi, T., N. H. Thoennissen, A. George, G. Crooks, J. H. Song, R. Okamoto, D. Nowak, A. F. Gombart, and H. P. Koeffler. 2010. In vivo deficiency of both C/EBPβ and C/EBPε results in highly defective myeloid differentiation and lack of cytokine response. *PLoS One* 5: e15419.

Figure Legends

FIGURE 1. Patient characteristics. (**A**, **B**) Skin abscess with ulceration and scar. (**C**) Peripheral blood smears. Neutrophils from patients P1 and P2 have bilobed nuclei and lack cytoplasmic granules (May-Grünewald-Giemsa staining). (**D**) Immunohistochemial analysis. Cytospin preparations of leukocytes stained with anti-lactoferrin mAb. Cellular alkaline phosphatase and peroxidase activity were also analyzed.

FIGURE 2. Characterization of granulocytes. Granulocytes were gated for fluorescence analysis and the percentage of cells gated in each region is shown.

FIGURE 3. Mutation analysis of C/EBPε gene. (**A**) C/EBPε gene exon 2 was amplified from DNA extracted from normal control, as well as peripheral blood and buccal mucosa of patient P1. Direct sequencing was performed using an automated sequencer. A thick bar highlights the position of the 6-bp deletion (ΔRS). (**B**) Predicted structures of mutated C/EBPε molecules. The previously reported frameshift mutations, del5bp and insA, produced frameshifts that result in incorrect amino acid sequence subsequent to the mutations (shaded area) and premature termination. bZIP, basic-leucine zipper.

FIGURE 4. Transcriptional activity and induction of endogenous expression of granule genes. (**A**) Luciferase reporter plasmid containing the G-CSF receptor promoter (pGCSFR-Luc) was transfected into HEK293 cells with either control empty (ev), wild-type (WT), ΔRS, del5bp or insA C/EBPε expression vector. Luciferase activity was

measured 48 h after transfection. Bars represent the means and standard deviations of triplicate assay. (**B**) Western blot analysis of C/EBPε was performed using lysates of HEK293 cells transfected with either ev, WT, ΔRS, del5bp or insA C/EBPε vector. (**C**) WT C/EBPε vector was mixed with increasing amounts of ΔRS C/EBPε vector and luciferase reporter assay was performed. (**D**) Expression vectors of ev, WT, ΔRS, del5bp or insA C/EBPε were transfected into NIH3T3 cells, and expression of endogenous B9, NGAL, and lactoferrin was examined by RT-PCR. GAPDH was used as a loading control. The numbers of PCR cycles were 20 for GAPDH, 30 for C/EBPε, B9 and NGAL, and 35 for lactoferrin.

FIGURE 5. Cellular localization. GFP-tagged to either control empty (ev), wild-type (WT), ΔRS, del5bp or insA C/EBPε expression vectors were transfected into NIH3T3 cells, and cells were analyzed by microscopy and fluorescent microscopy two days after transfection. Nuclei were stained with Hoechst stain. Bar indicates 20 μm.

FIGURE 6. DNA binding activity and dimerization. (A) DNA binding activity of WT, ΔRS, del5bp and insA C/EBPε. Biotin-labeled oligonucleotide containing the C/EBPε-binding site of the lactoferrin gene was incubated with Myc-C/EBPε (WT, ΔRS, del5bp, or insA)-transfected HEK293 cell extracts either with or without 25-fold nonlabeled WT or mutated non-binding (Mu) oligonucleotide. Biotin-labeled oligonucleotides were pulled down by streptavidin-agarose. The precipitates and cell lysates were analyzed by Western blot analysis with anti-Myc Ab. (B) Dimer formation of ΔRS. HEK293 cells were transfected with Myc-C/EBPε-ΔRS together with either empty control vector

(MBP-ev), Flag-MBP-C/EBPε-WT or -ΔRS. MBP-fused proteins were pulled down by amylose resin, and the precipitates were analyzed by Western blot analysis with anti-Myc Ab. Expression of each protein was confirmed with anti-Myc and anti-Flag Abs, respectively.

FIGURE 7. Cooperative transcriptional activation of eosinophil major basic protein (MaBP). (**A**) Induction of proteoglycan 2 (Prg2)/MaBP expression in NIH3T3 cell. Expression vectors Gata1 and PU.1 were transfected into NIH3T3 cells either with or without C/EBPε expression vectors (WT, ΔRS, del5bp or insA). Expression of endogenous Prg2/MaBP mRNA was examined by RT-PCR. GAPDH was used as a loading control. The numbers of PCR cycles were 20 for GAPDH, and 25 for C/EBPε, Gata1, PU.1 and MaBP. (**B**) Protein interaction between Gata1 and C/EBPε WT or mutants. (**C**) Protein interaction between WT or mutants PU.1 and C/EBPε. HEK293 cells were transfected with either Myc-Gata1 or Myc-PU.1 together with either an empty control vector (MBP-ev), Flag-MBP-C/EBPε-WT, -ΔRS, -del5bp or -insA. MBP-fused proteins were pulled down by amylose resin, and the precipitates were analyzed by Western blot analysis with anti-Myc Ab. Expression of each protein was confirmed with anti-Myc and anti-Flag Abs, respectively.

Fig 1

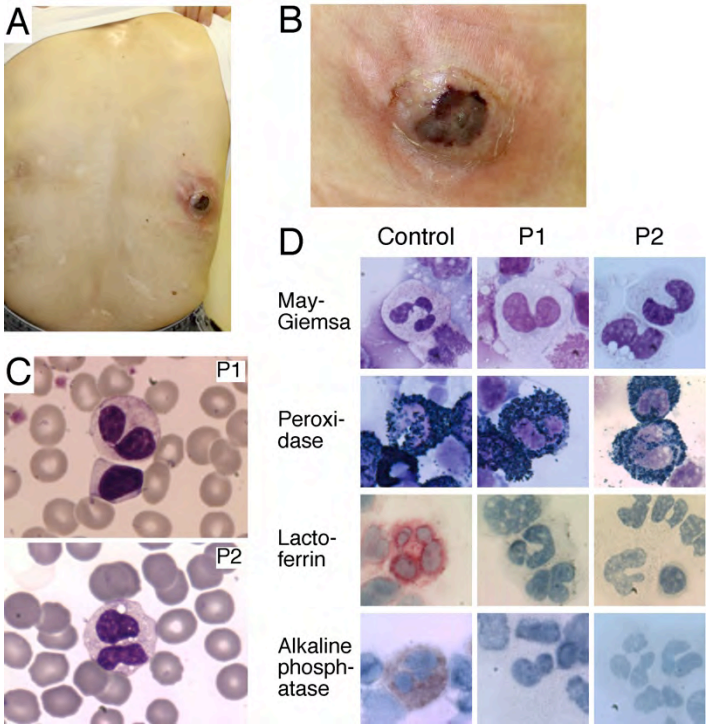

A
B
C
P1
P2
D
Control
P1
P2
May-Giemsa
Peroxi-dase
Lacto-ferrin
Alkaline phosph-atase

Fig 2

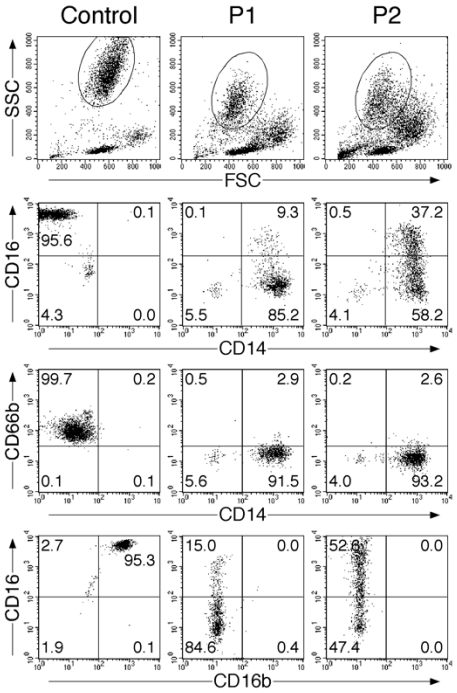

Control
P1
P2
SSC
FSC
CD16
CD14
CD66b
CD14
CD16
CD16b
0.1
95.6
4.3
0.0
0.1
9.3
5.5
85.2
0.5
37.2
4.1
58.2
99.7
0.2
0.1
0.1
0.5
2.9
5.6
91.5
0.2
2.6
4.0
93.2
2.7
95.3
1.9
0.1
15.0
0.0
84.6
0.4
52.6
0.0
47.4
0.0

Fig 3

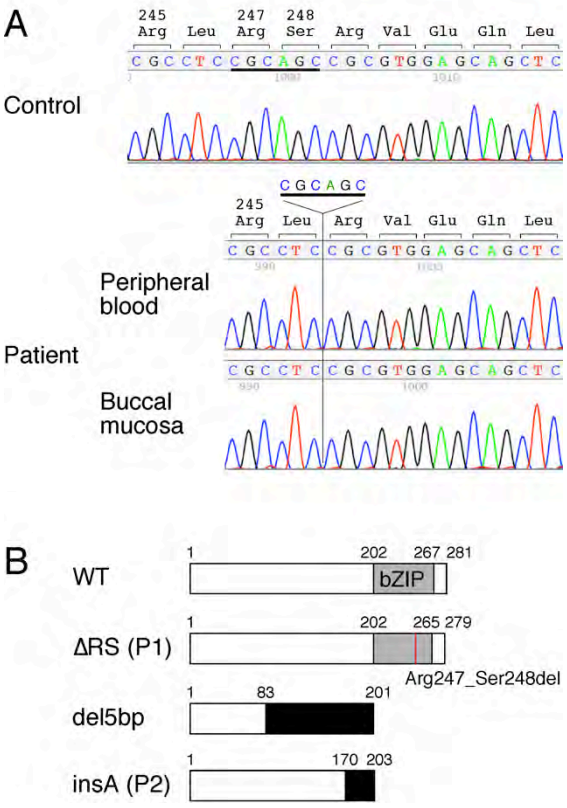

A
245 Arg Leu 247 Arg 248 Ser Arg Val Glu Gln Leu
C G C C T C C G C A G C C G C G T G G A G C A G C T C
Control
C G C A G C
245 Arg Leu Arg Val Glu Gln Leu
C G C C T C C G C G T G G A G C A G C T C
Peripheral blood
Patient
C G C C T C C G C G T G G A G C A G C T C
Buccal mucosa
B
WT
1 202 267 281
bZIP
ΔRS (P1)
1 202 265 279
Arg247_Ser248del
del5bp
1 83 201
insA (P2)
1 170 203

Fig 4

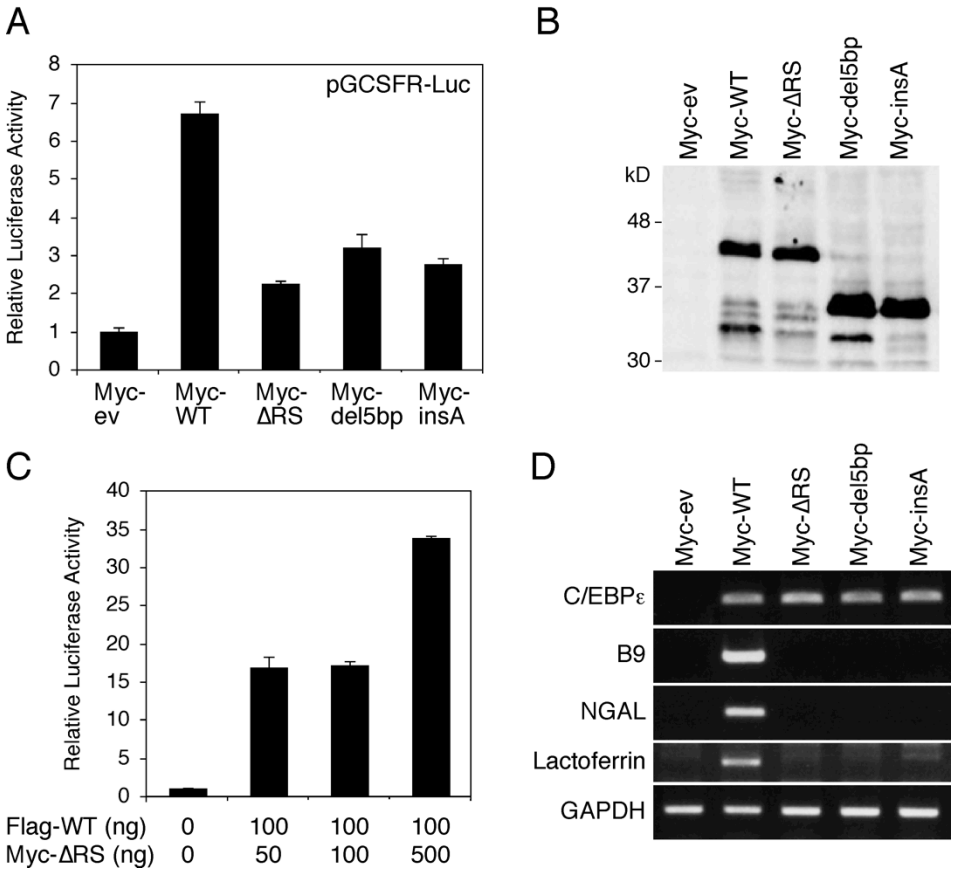

A
pGCSFR-Luc
Relative Luciferase Activity
0
1
2
3
4
5
6
7
8
Myc-ev
Myc-WT
Myc-ΔRS
Myc-del5bp
Myc-insA
B
Myc-ev
Myc-WT
Myc-ΔRS
Myc-del5bp
Myc-insA
kD
48
37
30
C
Relative Luciferase Activity
0
5
10
15
20
25
30
35
40
Flag-WT (ng) 0 100 100 100
Myc-ΔRS (ng) 0 50 100 500
D
Myc-ev
Myc-WT
Myc-ΔRS
Myc-del5bp
Myc-insA
C/EBPε
B9
NGAL
Lactoferrin
GAPDH

Fig 5

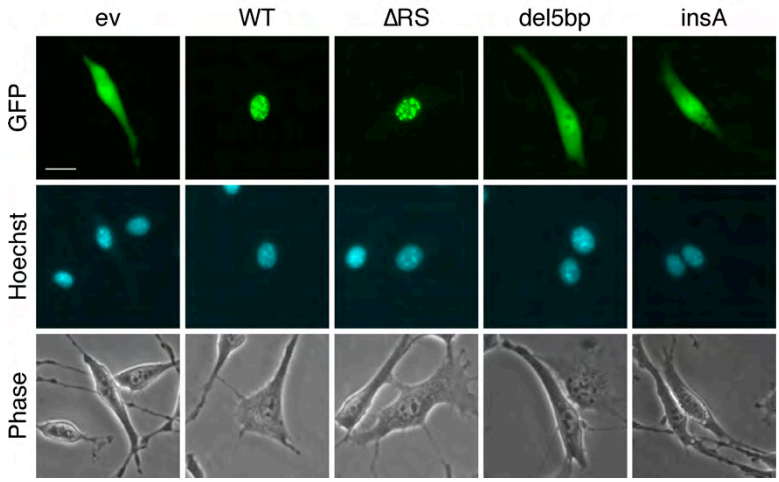


Fig 6

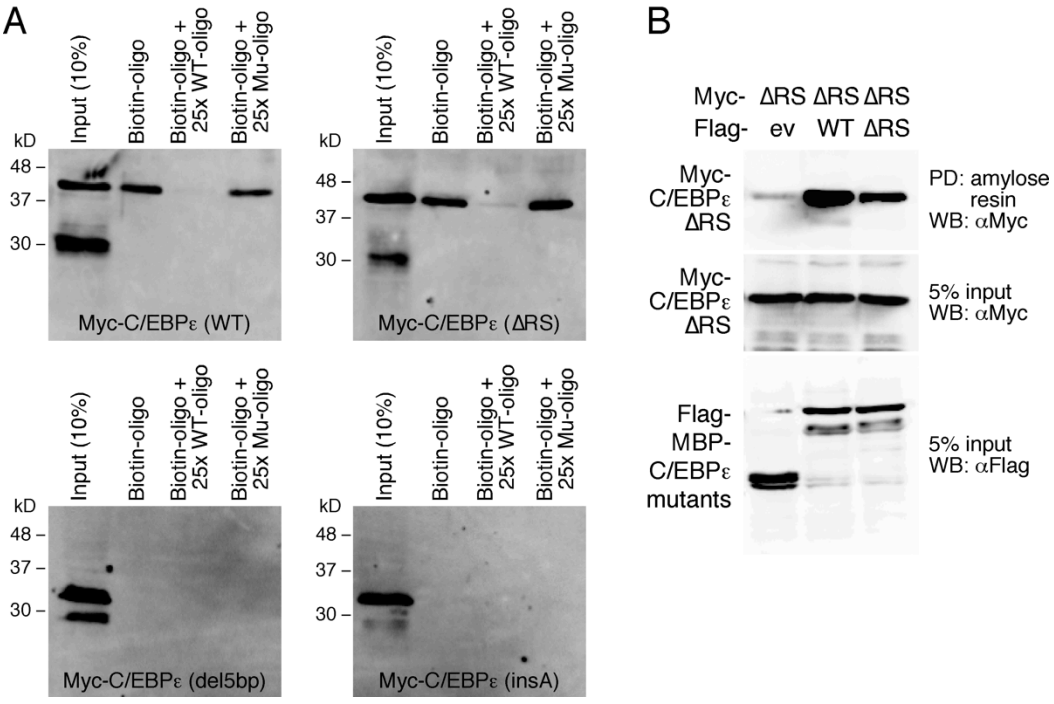
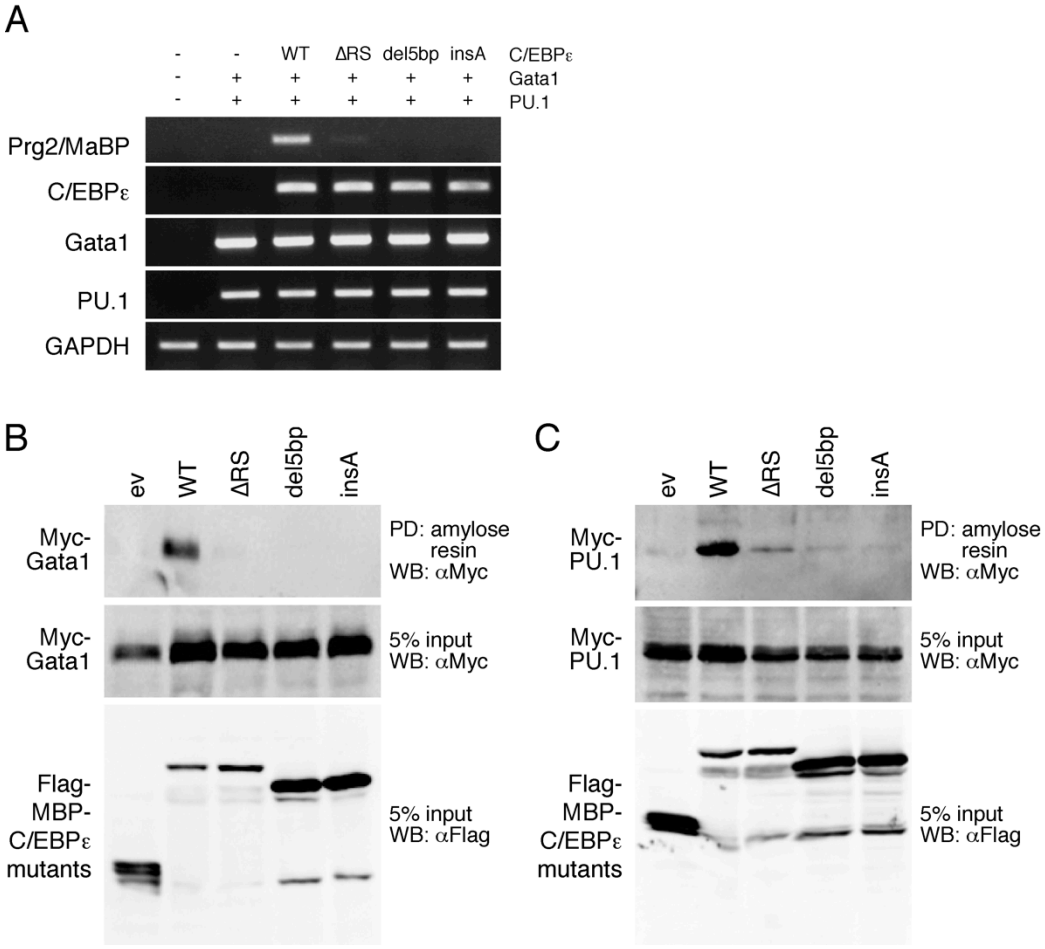

A
Input (10%)
Biotin-oligo
Biotin-oligo + 25x WT-oligo
Biotin-oligo + 25x Mu-oligo
kD
48 –
37 –
30 –
Myc-C/EBPε (WT)
Myc-C/EBPε (ΔRS)
Myc-C/EBPε (del5bp)
Myc-C/EBPε (insA)
B
Myc- ΔRS ΔRS ΔRS
Flag- ev WT ΔRS
Myc-C/EBPε ΔRS
PD: amylose resin WB: αMyc
Myc-C/EBPε ΔRS
5% input WB: αMyc
Flag-MBP-C/EBPε mutants
5% input WB: αFlag

Fig 7


A
- - WT ΔRS del5bp insA C/EBPε
- + + + + + Gata1
- + + + + + PU.1
Prg2/MaBP
C/EBPε
Gata1
PU.1
GAPDH
B
ev
WT
ΔRS
del5bp
insA
Myc-Gata1
PD: amylose resin WB: αMyc
Myc-Gata1
5% input WB: αMyc
Flag-MBP-C/EBPε mutants
5% input WB: αFlag
C
ev
WT
ΔRS
del5bp
insA
Myc-PU.1
PD: amylose resin WB: αMyc
Myc-PU.1
5% input WB: αMyc
Flag-MBP-C/EBPε mutants
5% input WB: αFlag