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Functional analysis of the NUP98-CCDC28A fusion protein

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Running title: NUP98-CCDC28A fusion in a T-ALL

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Abstract

Background. The nucleoporin gene \textit{NUP98} is rearranged in more than 27 chromosomal abnormalities observed in childhood and adult, \textit{de novo} and therapy-related acute leukemias from myeloid and T-lymphoid origins, resulting in the creation of fusion genes and the expression of chimeric proteins. We report here the functional analysis of the NUP98-coiled-coil domain-containing protein 28A (NUP98-CCDC28A) fusion protein, expressed as the consequence of a recurrent t(6;11)(q24.1;p15.5) translocation.

Design and Methods. To gain insight into the function of the native \textit{CCDC28A} gene, we collected information of any differential expression for \textit{CCDC28A} among normal hematological cell types and within subgroups of acute leukemia. To assess the \textit{in vivo} effects of the \textit{NUP98-CCDC28A} fusion, \textit{NUP98-CCDC28A} or full length \textit{CCDC28A} were retrovirally transduced into primary murine bone marrow cells and transduced cells were next transplanted into sub-lethally irradiated recipient mice.

Results. Our \textit{in silico} analyses supported a contribution of \textit{CCDC28A} to discrete stages of murine hematopoietic development. They also suggested enrichment for \textit{CCDC28A} selectively in the FAB-M6 class of human acute leukemia. Primary murine hematopoietic progenitor cells transduced with \textit{NUP98-CCDC28A} generated a fully penetrant and transplantable myeloproliferative neoplasm-like myeloid leukemia and induced the selective expansion of the granulocyte/macrophage progenitors in the bone marrow of transplanted recipients, showing that \textit{NUP98-CCDC28A} promotes the proliferative capacity and self-renewal potential of myeloid progenitors. In addition, the transformation mediated by \textit{NUP98-CCDC28A} was not associated with a deregulation of the \textit{Hoxa-Meis1} pathway, a feature shared by a diverse set of \textit{NUP98} fusions.

Conclusions. Our results demonstrate that the recurrent NUP98-CCDC28A is an oncogene that induces a rapid and transplantable myeloid neoplasm in recipient mice. They also provide additional evidence for alternative leukemogenic mechanism for NUP98 oncogenes.
Introduction

*NUP98* (11p15.4) encodes two proteins, NUP98 and NUP96, which are constituents of the nuclear pore complex (NPC). NUP98 is dynamically associated with NPC and mediates the nucleocytoplasmic trafficking of macromolecules. Additional NUP98 nuclear functions have been described linked to the control of euploidy and transcription. The early embryonic lethality associated to the disruption of the *Nup98* gene in mice has precluded elucidating its functions in normal hematopoiesis.

The *NUP98* gene lies at the breakpoint of chromosomal translocations responsible for the expression of hybrid genes in human hematological malignant diseases (reviewed in4). *NUP98* fusion partners frequently encode homeodomain transcription factors, including both the class-I (*HOXA9, A11, A13, C11, C13, D11, D13*) and class-II (*HHEX, PRRX1/PMX1* and *PRRX2/PMX2*) homeogenes. As a result, these chimeric proteins contain the NUP98 glycine-leucine-phenylalanine-glycine (GLFG)-repeats fused to the HOX DNA binding domain and act as aberrant transcription factors. *NUP98* partners may also encode chromatin structure regulators, as HMGB3, MLL, NSD1, NSD3/WHSC1L1, SETBP1, JARID1A/KDM5A/RBP2, PHF2, TOP1, TOP2, DDX10 and LEDGF/PSIP1/p75. Several of these are involved in the control of HOX expression during normal development. In line with this, up-regulation of HOX and HOX co-activators (MEIS1, PBX1/3) encoding loci has been reported in human and mice malignant diseases induced by *NUP98* fusions. Similarly HOX expression signatures have been described for *MLL* fusions (reviewed in22), indicating that activation of these developmentally critical loci underlie the leukemogenic activity of both *NUP98* and *MLL* fusions. However *NUP98* fusions may also activate alternative oncogenic pathways that do not include deregulation of the HOXA/MEIS1-PBX genes.

Here, we investigated the leukemogenic potential of the *NUP98-CCDC28A* fusion expressed as the consequence of a recurrent t(6;11) translocation in T-ALL (this study) and acute myeloid leukemia. Our results showed that *NUP98-CCDC28A* exhibits transforming potential both *in vitro* and *in vivo*. By using bone marrow cells transplantation assays, we showed that retroviral expression of *NUP98-CCDC28A* induced a fatal and transplantable myeloproliferative neoplasm (MPN)-like leukemia, associated with a selective expansion of the myelo-monocytic progenitors. These results indicate a potent oncogenic activity for the recurrent *NUP98-CCDC28A* fusion.
Design and Methods
The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institut de Cancérologie Gustave Roussy (SCEA, Villejuif, France).

Donor sample
Clinical and cytogenetic data of the patient studied have been reported 4.

Constructs
Hemagglutinin (HA)-tagged forms of human NUP98-CCDC28A and CCDC28A cDNAs were cloned into the retroviral vector, Murine Stem Cell Virus (MSCV)-Neo (Ozyme, Saint Quentin Yvelines, France) using PCR-mediated techniques. The long (L)-isoform of human CCDC28A was cloned into a pCMV-HA coding for a N-terminal HA tag (Ozyme). The short (S)-isoform of human CCDC28A was obtained by deleting the 5’-terminal codons 1-90 of the L-isoform by site-directed mutagenesis (Quickchange™, Ozyme). The HA-tagged NUP98-CCDC28A and CCDC28A were cloned into an MSCV-IRES-eGFP retroviral vector for bone marrow transplantation assays.

Immunostaining
HeLa or Plat-E cells were transiently transfected with DNA constructs using Lipofectamine™ 2000 (Invitrogen SARL, Cergy Pontoise, France) according to manufacturer's instructions. Twenty-four hours after transfection, samples were fixed and stained using a mouse antibody against gamma-tubulin (Sigma, L’Isle d’Abeau Chesenes, France) and a rat antibody against HA (Eurogentec France SAS, Angers, France).

Bone marrow transplantation and animal analysis
Viral supernatants were obtained as described24. Briefly, 6- to 8-week-old C57BL/6 donor mice were injected with 5-FU 5 days prior to bone marrow (BM) collection and primary BM cells were collected from femurs and tibiae. Lineage-negative (Lin-) cells were collected with the BD™ Mouse Hematopoietic Stem and Progenitor Cell Isolation Kit (Becton Dickinson France S.A.S, Le Pont-De-Claix, France) and cultured in Stemspan medium (StemCell Technologies Inc., Grenoble, France) supplemented with 10% fetal bovine serum (StemCell Technologies Inc.) in the presence of IL-3 (10
ng/ml), IL-6 (10 ng/ml), FLT3-L (100 ng/ml), SCF (100 ng/ml), thrombopoietin (2 U/ml) and IL-11 (10 ng/ml) (all from PromoCell GmbH, Heidelberg, Germany). BM Lin- cells were mixed with viral supernatants 48 and 72h after harvesting and spinfected for 90 min at 1000g. After the second spinfection, 5x10^4 to 1x10^5 cells were injected into the retro-orbital veins of sub-lethally irradiated (4.5 Gy) C57BL/6 recipients.

**Cytological and histological analyses**

Blood samples were obtained from the retro orbital sinus using heparinized micro capillaries. Peripheral blood cells counts were automatically measured with an MS-9 (Melet Schloesing Technologies, Osny, France) calibrated for mouse blood. Morphological analysis was done on smears and cyto spin preparations stained with May-Grünwald-Giemsa. Specimens of spleen, liver, lung and kidney were fixed in formol containing solution before paraffin embedding. Hematoxylin-eosin stained sections of tissues were evaluated using conventional staining techniques.

**Clonogenic progenitor assays**

10^4 Lin^- BM cells transduced with the retrovirus were plated in 35 mm petri dishes in M3434 methylcellulose (StemCell Technologies Inc.) and scored on day 7.

**Cell staining, antibodies and flow cytometry**

Cells were stained using the antibodies c-Kit, Sca1, Mac1/CD11b, Gr1, B220, CD19, CD8, CD4, Ter119, CD41, CD71 (BD Biosciences), GPIbα/CD42b (Emfret Analytics Gmbh, Würzburg, Germany) and CD34 (eBiosciences, San Diego, CA, USA). Acquisition of the data was performed on a CyAn™ ADP flow cytometer (Beckman Coulter France S.A.S., Roissy, France) and analyzed with the FlowJo software.

**RNA, reverse transcription, quantitative RT-PCR**

Total RNA was extracted using the RNable reagent (Eurobio, Courtaboeuf, France). Reverse-transcription was carried out with 4 μg of RNA using random hexamers and MMLV Reverse Transcriptase (Invitrogen SARL) according to manufacturer’s instructions. The primers for NUP98-CCDC28A fusion transcript were sense NUP98 (5’-GCCCGCTGGATTTAATACTACGA-3’) and antisense CCDC28A (5’-AGCGCCTTGTGCCCTCTCC-3’). For the reciprocal CCDC28A-NUP98 fusion
transcript the primers were sense CCDC28A (5'-TGCGGCGGTGGCTTCTGA-3') and antisense NUP98 (5'-AACCATAACCTTTCCGACCAAT-3'). RT-PCR products were cloned and sequenced.

Real-time PCR was performed in triplicate on an ABI PRISM 7000 Sequence Detection System (Applied BioSystem) using the TaqMan Universal PCR Master Mix (Applied BioSystem, Courtaboeuf, France) and the following probes: HoxA3 (Mm01326402_m1), HoxA5 (Mm00439362_m1), HoxA7 (Mm00657963_m1), HoxA9 (Mm00439364_m1) and HoxA10 (Mm00433966_m1). The relative expression of these genes was normalized to Abl (Mm00802038_g1).

In silico expression analysis
We used the Oncomine v. 4.3 commands available on-line (www.oncomine.org) to compare CCDC28A expression levels between each individual FAB group of AMLs and all the others by t-test (the reporter probe was Affymetrix U133A: 209479_at). The datasets are as follows: GSE1159, 293 samples25, GSE12417, 405 samples26, GSE14468, 526 samples27.
Results

Fusion of NUP98 to CCDC28A

The t(6;11)(q24.1;p15.5) translocation has been described in a T-ALL sample\(^4\) and in an acute megakaryoblastic leukemia (AMKL)\(^23\). Our molecular studies demonstrated an in-frame fusion between the 13th exon of NUP98 and the second exon of CCDC28A, as reported by others\(^23\) (Figure 1A). A reciprocal CCDC28A-NUP98 fusion transcript was detected but is likely devoid of biological activity due to the lack of predicted fusion protein.

The human CCDC28A gene encodes for two putative protein isoforms

RT-PCR analysis of a panel of human tissues cDNAs demonstrated a ubiquitous expression for CCDC28A [also known as, C6orf80 and MGC131913] (not shown). CCDC28A coding sequences predicted a 274 amino-acid (aa) protein (e.g., Genbank accession NP_056254) whose last 184 aa are well conserved in all vertebrates. An internal start codon (methionine labeled with “#” in Figure 1C) may be used to translate this protein species. This region showed a 93% aa identity with the murine protein (NP_659069) and possesses a ~100 aa-long predicted coiled-coil (CC) motif that is also observed in several of the NUP98 partner proteins\(^4,28\). In contrast, the first 90 N-terminal aa of the predicted human CCDC28A protein are poorly conserved across species, even though it shares the characteristics of a globular domain (~1/3 strong hydrophobic amino acids). We therefore concluded that the human cDNA may code for two protein isoforms, one that spans 184 aa and is well conserved in evolution (« short » (S)-isoform), and a larger one that would span 274 aa because of an extended N-terminus (« long » (L)-isoform). The sequence of protein CCDC28A did not disclose obvious functional roles, and no well-characterized motifs were detectable apart the CC domain.

In the human genome, CCDC28A is related to CCDC28B (coiled-coil domain-containing protein 28B)/MGC1203) located on 1p35.1 and the two proteins align unambiguously (50% aa identity; Figure 1B). CCDC28B bore no recognizable motifs and its functions are unknown, but it co-localizes with Bardet-Biedl syndrome (BBS) proteins at peri-centriolar structures\(^29\). MGC1203 mutations contribute epistatic alleles to BBS, an inherited oligogenetic disease associated with basal bodies and cilia disorders\(^29\).
Misregulation of **CCDC28A** is associated with a subset of human acute leukemias

Because the **NUP98-CCDC28A** gene fusion was observed in both AMKL and T-ALL (this study) samples, we investigated whether **CCDC28A** expression may be associated with specific subgroups of acute leukemia. Indeed, our analysis of microarray data showed that **CCDC28A** is more strongly expressed in T-ALL samples associated with **MLL** internal duplications, compared to other leukemias. The **CCDC28A** levels in T-ALL MLL were significantly higher than any other group at p<0.01 (two-tailed z-test) except for the group including pediatric leukemias with normal karyotype or complex/incompletely characterized chromosomal rearrangements, with a difference that was only weakly supported (p=0.09) (Supplemental Figure 1A, data were from30). Our Oncomine analysis also showed enrichment for **CCDC28A** selectively in the FAB-M6 class in three publicly available datasets: fold-ratios were respectively 1.825, 1.526 and 1.427, and p-values were 0.040, 0.062, and 0.014, respectively (two-tailed t-test, M6 vs M0-M5) (Supplemental Table 1). One dataset26 contained only leukemias with normal karyotype. This suggests a specific role for **CCDC28A** in leukemias involving the erythroid lineage.

We found no association between **CCDC28A** expression levels and survival by Cox proportional hazards regression using the dataset including survival data for the patients26. To gain insight into the function of the native **CCDC28A** gene, we also collected information of any differential expression for murine **CCDC28A** among normal hematological cell types in available microarray datasets from mouse and found that **CCDC28A** was enriched in hematopoietic stem cells (HSC), common lymphoid progenitors (CLPs) and naive T- and NK cells compared to other progenitors or differentiated cell types (Supplemental Figure 1B), supporting a role for **CCDC28A** in hematopoietic development.

The **NUP98-CCDC28A** fusion protein has a predominant nuclear localization

We next analyzed the subcellular localisation of the **NUP98-CCDC28A** fusion protein. The HA-tagged **NUP98-CCDC28A** S- and L-isoforms of **CCDC28A** were investigated in transient transfection assays in the murine NIH3T3 fibroblasts. By immunofluorescence, the fusion protein showed predominant expression in the nucleus whereas the localisation of the S- and L-CCDC28A was both cytoplasmic.
and nuclear (Figure 1D). Co-staining with an anti-gamma tubulin antibody did not reveal a centrosome localization for CCDC28A in contrast to CCDC28B²⁹.

The expression of NUP98-CCDC28A enforces the proliferation of primary bone marrow cells
To assess the in vivo effects of the NUP98-CCDC28A fusion, NUP98-CCDC28A or full length CCDC28A were retrovirally transduced into primary bone marrow (BM) cells derived from C57Bl/6 mice using a murine stem cell virus (MSCV). Unlike BM-derived primary murine progenitors transduced with an empty MSCV vector or CCDC28A, progenitors transduced with NUP98-CCD28A showed serial replating activity in methylcellulose colony-forming assays (Figure 2A) and were able to be propagated for several months in liquid culture. Subsequent cultivation in medium supplemented with only serum yielded to NUP98-CCDC28A-immortalized progenitors that proliferated in a cytokine-independent manner and exhibited myeloblast morphology and c-Kit expression. These results suggest that expression of NUP98-CCDC28A enforces the cellular proliferation and may also interfere with myeloid differentiation.

The expression of NUP98-CCDC28A in a murine bone marrow transplantation model causes rapid and fatal MPN
Transduced primary BM cells were next transplanted into sub-lethally irradiated recipient mice. In keeping with in vitro experiments, NUP98-CCDC28A showed a strong transforming potential in mouse adoptive transfers as all animals that underwent transplantation with NUP98-CCDC28A-transduced cells (n=20) succumbed within 32 weeks after transplantation with an average lifespan of 119 days post-transplant (Figure 2B). The transforming potential of the retrovirally expressed CCDC28A was also evaluated but none of the engrafted mice developed leukemia (Figure 2B). Southern blot analyses performed on genomic DNA indicated the presence of the provirus in BM samples of all transplanted mice and showed the mono or oligoclonal nature of the NUP98-CCDC28A-induced proliferations in malignant samples (Supplemental Figure 2A). Although the incidence of leukocytosis and neutrophilia varied among individual mice, NUP98-CCDC28A mice consistently showed a severe anemia and thrombocytopenia (Figure 2C) and an increase in immature/blasts myeloid cells in the
BM, spleen and peripheral blood when compared to control animals. Blood smears revealed the presence of circulating myeloid (granulocytic/monocytic) precursors as well as a complete maturation of myeloid forms to segmented neutrophils (Figure 2D, panel b). BM cytology confirmed the presence of immature myeloid cells with a minimal myeloid maturation and the disappearance of the erythroid compartment (Figure 2D, d). Upon necropsy, all NUP98-CCDC28A mice exhibited hepatosplenomegaly (Figure 2C). Histological analysis revealed a severe disruption of spleen architecture when compared to CCDC28A or MSCV mice (Figure 2D, panel e). Evidence of extramedullary hematopoiesis was observed in the liver (Figure 2D, panel f) and lung (Figure 2D, panel h), including perivascular infiltrations with myeloid cells. The percentage of immature forms/blasts in the blood was less than 20%. Regarding the classification proposals described in\textsuperscript{31}, we conclude that ectopic expression of NUP98-CCDC28A in hematopoietic stem cells and progenitors induced a myeloproliferative neoplasm (MPN)-like myeloid leukemia. We also observed mouse lesions resembling myeloid leukemias with maturation, e.g., in which the neoplastic cells were moderately differentiated and neutrophilic (not shown). The fact that most NUP98-CCDC28A-induced MPNs did not evolve to AML suggests that NUP98-CCDC28A exerts a prominent effect on cellular growth and a weaker effect on differentiation.

To assess the malignant nature of the disease, we transplanted BM cells from NUP98-CCDC28A primary recipient into sub-lethally irradiated wild-type secondary mice. All recipient (n=8) rapidly developed myeloid leukemias, which led to the death at 7 weeks after transplantation (Figure 2B). Blood and BM cytological analyses evoked overt myeloid leukemias with more than 20% of circulating blasts present in the blood and a massive invasion of the BM (not shown). The transplantability and the rapid lethality in both primary and secondary recipients demonstrate the potent leukemogenic potential of NUP98-CCDC28A.
The bone marrow of **NUP98-CCDC28A**-transduced mice is enriched in granulocyte/macrophage progenitors

In line with cytological data, flow cytometric analysis of BM cells from **NUP98-CCDC28A** moribund mice revealed a marked increase in the proportion of myeloid cells with monocytic and neutrophilic components when compared to MSCV-transduced counterparts (Supplemental Figure 3). Myeloid expansion was associated with lymphopenia and a concomitant reduced erythropoiesis and enhanced megakaryopoiesis in the BM (Supplemental Figure 4). The latter correlates with an elevated number of mature megakaryoblastes observed by histological analyses on **NUP98-CCDC28A** mice spleens (Figure 2D, panel e). **NUP98-CCDC28A** leukemic mice also displayed significant infiltration of the spleen and thymus, with the cellular composition of those hematopoietic tissues reflecting those of the BM (data not shown).

To define the **NUP98-CCDC28A**-induced leukemias more precisely, FACS analyses were performed on BM stem and progenitor cells phenotypically defined as the Lin\(^{-}\)Sca1\(^{+}\)c-kit\(^{+}\) (LSK) and Lin\(^{-}\)Sca1\(^{-}\)c-kit\(^{+}\) (MP) subsets respectively. Analyses showed a selective expansion of a myeloid progenitor population enriched for myelo-monocytic progenitors (GMP) activity\(^{32}\) while other progenitors (e.g. the common myeloid progenitors (CMP) and the megakaryocytic/erythroid progenitors (MEP)) were virtually absent (Figure 3). Interestingly, the prevalence of the GMP compartment in leukemic **NUP98-CCDC28A** BM cells was reminiscent of the one described for some MLL fusions-associated myeloid leukemias\(^{33}\). When compared to normal, the leukemic BM populations showed a strong decrease in the frequency of the LSK subset that encompasses the multi-potent progenitors (MPP), and the long-term and short-term HSCs (Figure 3). This indicates that **NUP98-CCDC28A** expression does not enforce the expansion of stem and primitive progenitor cells.
**NUP98-CCDC28A expression is not associated with strong HoxA and Meis1 expression**

We next addressed the question of HoxA expression in NUP98-CCDC28A neoplasms. Quantitative RT-PCR experiments were performed on whole BM cells isolated from NUP98-CCDC28A-transduced mice and compared to their CCD28A- and MSCV-transduced counterparts. BM cells from sick NUP98-HoxA9-transduced mice were used as positive control, and cells transduced with an oncogenic form of the thrombopoietin receptor, MPL\textsuperscript{T487A}, were used as a negative control. Except for HoxA10, results demonstrated a weaker expression of HoxA5, HoxA7 and HoxA9 in the NUP98-CCDC28A BM cells when compared to their NUP98-HoxA9 counterpart. While HoxA9 and Meis1 were concomitantly misregulated in the NUP98-HoxA9 samples, NUP98-CCDC28A BM cells retained wildtype levels of Meis1, suggesting that the NUP98-CCDC28A-mediated transformation does not involve the canonical Hoxa-Meis1 pathway. We did not either observe a concomitant upregulated expression of the Pbx1 Hox cofactor, and Pbx3 expression was only slightly increased in NUP98-CCDC28A samples. Collectively, these results indicate that NUP98-CCDC28A did not strongly impact on Hox genes expression in hematopoietic cells, and that HoxA and Meis1 are not critical downstream mediators of the NUP98-CCDC28A-mediated oncogenic program. As NUP98-CCDC28A-expressing human blast cells did not show upregulation of these single genes (data not shown), we infer that NUP98-CCDC28A-transformation is unlikely to involve a global HOX gene upregulation.
Discussion

We have confirmed CCDC28A as a recurrent chromosomal translocation partner of NUP98 in acute leukemia. In addition to the t(6;11) translocation studied here, five NUP98 fusions have been reported in T-ALL (NUP98-ADD3, NUP98-IQCG, NUP98-RAP1GDS1, NUP98-SETBP1 and NUP98-LNP1) whose contribution to the leukemogenic process is still unknown. Except for CCDC28B, the native CCDC28A protein has no recognizable similarity to other proteins or functional domain, and no function has so far been assigned to the coiled-coil domain, leaving CCDC28A’s biological function undetermined. Expression pattern of the gene within the hematopoietic lineages however suggests its contribution to discrete stages of hematopoietic development. We showed here that enforced NUP98-CCDC28A expression promoted the proliferative capacity and self-renewal potential of murine hematopoietic progenitors and rapidly induced fatal MPNs and defects in the differentiation of the erythro-megakaryocytic lineage. Our in silico analyses also suggest CCDC28A misregulation in human myeloid leukemias specifically from the FAB-M6 subgroup, suggesting that CCDC28A expression could be critical for normal myeloerythroid progenitors cell function. Although the leukemogenic mechanism remains unknown, NUP98-CCDC28A retains the NUP98 GLFG-repeats able to associate with CBP and/or p300 and harbors a nuclear localization that suggests a possible transactivation activity. Several mechanisms may cooperate in disregulated transcription, as NUP98 fusions also interfere with the nucleocytoplasmic trafficking. Indeed, both NUP98-HoxA9 and NUP98-DDX10 impair the nuclear export of critical transcriptional regulators, leading to their aberrant nuclear retention and enhanced transcription from responsive promoters. The functional significance of deregulated Hox genes expression has been suspect in the oncogenic processes of some but not all NUP98 fusion proteins. Although sustained HoxA genes expression was observed in NUP98-CCDC28A-expressing leukemic cells, they may be related to the enrichment in immature myeloid populations with respect to the controls, since i) substantially higher transcript levels were measured in the NUP98-HOXA9 samples, ii) the expressing level of Meis1 is close to the control level. This suggests that HoxA/Meis strong misregulated expression is not a prevailing theme in NUP98 fusions oncogenesis. The model reported here will allow dissection of the pathways involved in myeloid transformation. Adapted models will be needed to investigate the role of NUP98-CCDC28A in lymphoid transformation.
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Authorship and Disclosures
AP, CR, GS, CO and CS performed experiments, analyzed and interpreted the data; IR-W, ND contributed valuable tissues and clinical information; HD contributed expression analyses; SC-M and IC contributed CCDC28A functional and structural analyses; OAB SR and VP-L designed the study, analyzed, interpreted the data and wrote the manuscript.
The authors reported no potential conflicts of interest.
References


Legends of the figures

Figure 1. The t(6;11)(q24.1;p15.5) translocation fuses NUP98 to CCDC28A and leads to the expression of a NUP98-CCDC28A protein of nuclear localization. (A) Nucleotide and amino acid sequences around the NUP98-CCDC28A fusion junction. A specific PCR product of 503 bp was obtained using the DNA from the patient sample (P) while not from a control genomic DNA (C) (panel a); the fusion joins the nucleotide (Nt) 62503 of NUP98 to the Nt 653 of CCDC28A. RT-PCR experiments performed on RNAs extracted from the leukemic sample (P) show the amplification of two specific 444 bp and 612 bp products, corresponding respectively to the NUP98-CCDC28A (panel b) and reciprocal CCDC28A-NUP98 (panel c) fusion transcripts. The nucleotide sequence of the NUP98-CCDC28A transcript shows an in-frame fusion, joining the Nt 1833 of NUP98 to the Nt 384 of CCDC28A. The reciprocal CCDC28A-NUP98 transcript joins the Nt 383 of CCDC28A to the Nt 2022 of NUP98 but harbors a non-sens codon. M, molecular weight markers. (B) Schematic representation of the NUP98, CCDC28A and NUP98-CCDC28A human proteins. Identified domains (GLFG-repeats; RNA binding domain (RBD) and the predicted coiled-coil domain (CCD) are indicated. The chimeric exon-exon boundary joins NUP98 to aa position 77 of the putative L-isoform, leading to a 712 aa-long fusion protein. (C) Clustal-W alignment of the predicted proteins for CCDC28A from mouse (NP_659069), human (NP_056254) and for human CCDC28B (NP_077272). The coding potential of the mouse cDNA for CCDC28A is extended N-terminal of the first methionine, in order to show partial alignment for a short segment along with three in-frame stop codons (*). Human CCDC28A is labeled with an “-L” suffix to indicate the putative long isoform (see text). The symbols placed below the alignment are as follows: “!” first aa of the sequence from CCDC28A that is joined to NUP98 in leukemia; “#”, first methionine for the “S-isoform” of human CCDC28A and for the two other proteins; “+”, aa positions that are fully conserved in the three proteins; “.” and “.”, aa positions with decreasing degrees of partial conservation. Above the alignment, the “%” symbols indicate exon-exon junctions for CCDC28A. In the murine CCDC28A protein, a short orthologous segment can be recognized upstream of the conserved methionine present in the human protein but contains in-frame stop codons.
(D) Immunocytochemistry with an anti-HA antibody detecting the nuclei of murine fibroblasts transiently transfected with constructs encoding the HA-tagged NUP98-CCDC28A fusion protein (panel a, green) and S-isoform of CCDC28A (panel b, green); panel c is the negative control. Staining for gamma-tubulin is red, and all nuclei stain blue with DAPI.

Figure 2. NUP98-CCDC28A expression induces an MDP-like myeloid leukemia.
(A) Clonogenic progenitor assays. Expression of NUP98-CCDC28A in primary BM progenitors resulted in their enhanced proliferation in vitro. Colonies were scored every week and replated in secondary cultures. The mean number colony per round of replating of 3 independent replicates is indicated. The error bars indicate standard deviation (SD). (B) Kaplan-Meier survival curve of mice transplanted with BM progenitors transduced with NUP98-CCDC28A (n=20), CCDC28A (n=5) or the vector alone (n=3). Primary NUP98-CCDC28A recipient (indicated as NUP98-CCDC28A I) showed a 100% death rate at day 236 (7.8 months). Animals transduced with the CCDC28A or the MSCV vector alone were sacrificed for end-point analysis without evidence of disease. NUP98-CCDC28A secondary recipients (NUP98-CCDC28A II, n=8) succumbed between days 38 and 87 post-transplant. (C) Blood counts of primary NUP98-CCDC28A-transduced recipients (NUP98-CCDC28A I) show hyperleukocytosis, severe anemia and thrombopenia. These abnormalities were also observed in second recipients (NUP98-CCDC28A II) while CCDC28A- and MSCV-transduced mice showed normal blood count parameters. Spleen weights from primary transplanted mice are indicated. Values shown are mean ± SD. (D) Blood cytology and tissues histology of representative NUP98-CCDC28A and MSCV mice. Peripheral blood smears show anemia, thrombocytopenia and hyperleukocytosis for primary NUP98-CCDC28A animals (panel b) when compared to MSCV animals (panel a). In the former, maturation of myeloid forms to segmented neutrophils was observed (May-Grünwald-Giemsa staining, x 100). Cytological analysis of BM cells, evaluated on May-Grünwald-Giemsa staining of cytospin preparations, shows an over-representation of mature myeloid cells in NUP98-CCDC28A-engrafted mice (panel d) when compared to control animals (panel c) (May-Grünwald-Giemsa staining, x 40). Histological analysis of the spleen (panel e), liver (panels f), kidney (panel g) and lung (panel h) of primary mice transplanted with BM progenitors.
transduced with *NUP98-CCDC28A*, shows accumulation of myeloid precursors and destruction of normal organ architecture (hematoxylin and eosin, x 20 and x 200).

**Figure 3.** Leukemic cells from *NUP98-CCDC28A* mice are enriched in GMP. Representative FACS profile of immature progenitors immunophenotypically defined as LSK (Lin⁻Sca1⁺c-Kit⁺) and myeloid progenitors (MP, Lin⁻Sca1⁻c-Kit⁺) in the BM of *NUP98-CCDC28A*-engrafted mice. FACS analysis of Lin⁻ cells shows the distribution of MP in the BM of leukemic animals and specific expansion of a population immunophenotypically defined as granulocytic-monocytic progenitors (GMP), at the expense of the common myeloid progenitors (CMP) and megakaryocyte-erythroid progenitors (MEP) populations. A profile of BM cells from mice transplanted with control-transduced progenitors shows typical GMP, CMP and MEP populations. A major reduction in LSK cells is observed in the BM of *NUP98-CCDC28A*-engrafted mice. Histograms show the percentages of indicated cells in the BM from leukemic and control mice (right panels). Values shown are mean ± standard error of the mean (SEM) (n=5 mice per group, Mann Whitney test).

**Figure 4.** *NUP98-CCDC28A* leukemic cells do not overexpress HoxA genes. Real-time RT-PCR analysis of transcript levels of *HoxA5, HoxA7, HoxA9, HoxA10, Meis1, Pbx1* and *Pbx3* genes in the BM cells from primary NUP98-CCDC28A-engrafted animals and their CCDC28A- and MSCV-transduced counterparts. Accumulation of transcript is quantified in primary recipients compared to *NUP98-HOXA9* and *MPL T487A* recipients, respectively used as positive and negative controls of disregulated HoxA genes expression. Expression levels are normalized to *Gapdh* and results are expressed relative to the level of each gene in MSCV-engrafted mice (set to 1) (n=3 per genotype). Values shown are mean ± SD from 2 independent experiments.
Figure 2

A

Number of colonies

passage (weeks)

B

% survival

days post-BMT

C

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D

Peripheral blood

Bone marrow

MSCV

NUP98-CCDC28A

a

X10

X100

X40

X20

X200

X200

spleen

liver

kidney

lung

22
Figure 3

Gated on Lin- cells

gated on Lin- cells

c-Kit

LSK

Sca-1

E글루

CD34

MSCV

NUP98-CCDC28A

GMP

CMP

MEP

p=0.01

p<0.05

p=0.01

p=0.001

p=0.01

p<0.05

p=0.05

p<0.05

p=0.05
Supplemental Figure 1
A) Mean expression levels of CCDC28A in human leukemias. Y-axis: arbitrary units from renormalized microarray data (www.stjuderesearch.org/site/data/AML1)\textsuperscript{29}. Bars represent means ± standard errors of the mean (SEM). All groups (bars) were from pediatric patients with distinct chromosomal rearrangements as indicated, except the group labeled as “Adult” (which included a range of cytogenetic abnormalities). “Other” includes pediatric leukemias with normal karyotype or complex/incompletely characterized chromosomal rearrangements.

B) Left panel: histogram showing relative expression levels for mouse CCDC28A in a public database (renormalized with plier in R from GSE10627 available at www.ncbi.nlm.nih.gov/geo/). Each bar represents average and standard error of the mean as measured in from distinct hematopoietic cell progenitors (>4 mice per group).
Right panel: histogram showing relative expression levels for mouse CCDC28A in a public database (renormalized with plier in R from GSE6506 available at www.ncbi.nlm.nih.gov/geo/). Each bar represents average and range of variation as measured in from distinct hematopoietic cell types (two mice per group).

Supplemental Figure 2
Clonal analysis by southern blot of DNA from 4 transduced mice. Membranes were hybridized to a probe specific for the GFP sequences. Lanes 1-8 and 12-14 correspond to hematopoietic (B, blood; BM, bone marrow; S, spleen) and non hematopoietic (K, kidney; L, lung) organs of NUP98-CCDC28A primary mice; lanes 9-11 correspond to organs of MSCV-transduced mice. Asterisks indicate clones from BM primary mice (mice I.5) that contribute to myeloid leukemias in the secondary transplant (mice II.5). While multiple clones persist in the BM cells transduced by the empty vector alone (lane 9), NUP98-CCDC28A-induced MPNs were predominantly mono-or bi-clonal (lanes 2, 7 and 13). An example of clonal transmission of the disease is shown (right panel, see secondary mouse II.5 (lanes 15-17) of primary mouse I.5 (lanes 12-14)).
**Supplemental Figure 3**
Representative fluorescent-activated cell sorting (FACS) analysis of BM cells from primary control (MSCV)- and *NUP98-CCDC28A*-engrafted mice (left panels). Analyses reveal an expansion of Gr1-, Mac1/CD11b- and c-Kit-positive cells in the BM of the diseased animals. Histograms show the percentages of indicated cells in the BM from leukemic and control mice (right panels). Values shown are mean ± SEM (n=5 mice per group, Mann Whitney test).

**Supplemental Figure 4**
Representative FACS profile of BM cells from primary MSCV- and *NUP98-CCDC28A*-engrafted mice. Analyses show a decrease in the number of progenitors from B (B220⁺CD19⁺) and erythroid (Ter119⁺CD71⁺) lineages, a decrease in T cells (CD4⁺ or CD8⁺) and an increase in megakarocytes number (CD41⁺CD42⁺). Values shown are mean ± SEM (n=5 mice per group, Mann Whitney test).

**Supplemental Table 1**
Fold-ratio between average levels of *CCDC28A* (Affymetrix probe 209479_at) for each FAB group vs the other groups (“fold”). Oncomine provided p-values. Asterisk (*): significant or nearly significant fold increases. Nd: not determined.
Supplemental Figure 1

A

![Graph A]

- CCDC28A
- T-ALL with MLL fusions
- AML with MLL fusions
- AML FAB M7
- AML t(8;21)
- AML t(15;17)
- adult
- other

B

![Graph B]

- CCDC28A
- GMP
- CMP
- CLP
- HSC

DOI: 10.3324/haematol.2011.047969
Supplemental Figure 2

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Supplemental Figure 4

MSCV vs NUP98-CCDC28A

Ter119

CD71

CD41

CD42

CD41

CD42

CD4

CD8

B220

CD19

MSCV

NUP98-CCDC28A

% erythroid cells

% CD41+ CD42+

% CD71+

% CD71low

% CD71-

p=0.01

p=0.05

p=0.05

p=0.05

% B220+ CD19- or B220+ CD19+

p=0.01

p<0.001

% CD4+ or CD8+

p<0.001

p=0.01

p=0.01

% B220+ CD19+ or B220+ CD19-

p=0.001

p=0.01
Supplemental Table 1

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