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DELINEATING THE KEY REGIONS AND FUNCTIONS OF NUP98 CONTRIBUTING TO THE LEUKEMOGENIC ACTIVITY OF NUP98-HOX FUSIONS

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Abstract

To determine the contribution of the common N-terminal truncation of *NUP98* in NUP98-translocations resulting in acute myeloid leukemia, we have conducted a structure-function analysis of *NUP98* in the context of NUP98-HOXA10HD, a novel, canonical *NUP98-Hox* fusion that significantly enhances the self-renewal capacity of hematopoietic stem cells and collaborates with *Meis1* to induce AML in our mouse models Our results clearly demonstrate that the *NUP98* fusion partner does not require interactions with either the nuclear pore complex (NPC) or the Rae1/anaphase promoting complex (APC), but instead, *NUP98* seems to function in a transactivation manner by recruitment of CBP/p300 via its FG/GLFG repeats.

A large proportion of acute myeloid leukemias (AML) have been characterized as involving a number of non-random chromosomal translocations. Notably, a significant number of these translocations involve the nucleoporin 98kDa (*NUP98*) gene at 11p15 [1][2] as a common partner with now over 20 different genes (reviewed [3]). *NUP98* translocations commonly result in a truncated N-terminal portion of *NUP98* juxtaposed to the C-terminal portion of a translocation partner gene. However, the role of the *NUP98* N-terminal portion of NUP98-fusions remains largely unresolved.

The importance of the *NUP98* moiety in the NUP98-fusions has been well established. Previous work from our laboratory [4] has shown in the murine hematopoietic model that while overexpression of PMX1, a homeodomain protein, alone does not impair differentiation, or promote leukemic transformation, the NUP98-PMX1 fusion does, implying the necessary requirement of the NUP98 moiety. More recently, Yassin *et al.* [5], found that the ability of the NUP98-HOXA9 fusion to disrupt the differentiation of human CD34+ cells required the *NUP98* moiety, and identified candidate genes which were dysregulated by the *NUP98* portion of the NUP98-HOXA9 fusion.

These results imply a critical role for *NUP98* in its function in a *NUP98-Hox* fusion. The mechanistic contribution of the *NUP98* moiety to the fusion proteins remains unclear.

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Molecular-genetic analyses of multiple patient samples containing translocations of NUP98 suggest a minimal NUP98-domain encompassing roughly the first half of the NUP98 protein [6]. This N-terminal truncation of NUP98 contains a number of protein motifs, including a high density of FG/GLFG repeats, a GLEBS motif and a Rae-1 interaction domain. In the context of the intact *NUP98* protein, the GLEBS motif is required for interaction with other nucleoporin proteins [7], while the Rae1-interaction domain has been identified as important in both mediating mRNA transport function [8] and interaction with the anaphase promoting complex (APC) [9]. In the context of NUP98-fusions previous *in vitro* studies implicate the FG/GLFG-rich repeat region in the recruitment of CBP/p300 [10]. Further work with NUP98-PMX1 [11] demonstrated the paradoxical recruitment of both transcriptional activators (CBP/p300) and repressors (HDAC1) via the FG/GLFG repeats. Studies with NUP98-HHEX [12] and NUP98-NSD1 [13] indicate their ability to induce aberrant self-renewal required that the GLFG-rich region of NUP98 be intact. However, in both the studies of NUP98-HHEX [12] and NUP98-PMX1/NUP98-HOXA9 [11], engineered deletions to assess the role of the FG-rich region also deleted the overlapping Rae1/APC and GLEBS interaction domains. Thus it remains unclear whether these domains are necessary for the transforming function of NUP98-fusions and if so, what functions they may contribute. Together, these studies indicate that the FG/GLFG repeat domains of *NUP98* play a critical role in the transforming function of NUP98-fusions but leave open the questions of whether this is due predominately to recruitment of transcriptional activators and whether the GLEBS and Rae1 interaction motifs also play a role.

In this study, we have created a panel of structure-function mutations of the NUP98-fusion partner to HOXA10HD. The Hox fusion partner can be reduced to just the HD in the case of HOXA10, likely contributing towards a DNA-binding function alone, resulting in a NUP98-HOXA10HD (NA10HD) fusion gene that can substantially stimulate hematopoietic stem cell (HSC) self-renewal, and retains the ability to collaborate with *Meis1* to induce leukemic transformation [14]. We demonstrate that these constructs show a range of growth promoting activities on primitive hematopoietic cells, but in combination with *Meis1*, most retain leukemic transformation potential. Strikingly, constructs which lack the GLEBS and Rae1/APC interaction regions retain growth promoting activity, including significant HSC self-renewal and the ability to collaborate with *Meis1* in leukemic transformation, indicating that the *NUP98* interaction with nucleoporins and the NPC, as well as the anaphase-promoting complex are not essential for its function as a fusion partner. By modeling recruitment of CBP/p300 by FG/GLFG repeats with an E1a moiety [15], we show that CBP/p300 recruitment is sufficient to induce leukemic transformation when co-expressed with *Meis1*. Together these findings support a leukemogenic model in which the role of NUP98 is to recruit CBP/p300 towards target genes directed by its fusion partner (in our model the HD of *HOXA10*).

MATERIALS AND METHODS

Retroviral vectors and cDNA constructs

The murine stem cell virus (MSCV) based vectors carrying expression cassettes consisting of Flag-*NUP98-HOXA10HD* internal ribosomal entry site (IRES)-enhanced green

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fluorescent protein (eGFP) (*NA10HD*-GFP virus); or HA tagged-*Meis1* IRES-yellow fluorescent protein (YFP) (*Meis1*-YFP virus) have been previously described [14][16]. A PCR product flanked BamH1-KpnI from amino acids 1-33, and a second flanked KpnI-NotI amino acids 227-469 were fused to create ΔGLEBSA10HD. The Δ224-A10HD construct was created by blunt end ligation of the second KpnI-NotI fragment. The E1a(89)-A10HD construct was created by PCR from an E1a template which was a gift from Dr. Adam Goldfarb. The primers were 5′ GGATCCATGAGACATATTATCTGCCACGGA 3′ and 5′ GAATTCTTTTGGCCTGGGGCGTTTACA 3'. The FG1-A10HD and FG1x2-A10HD constructs were created by PCR of the first 150 amino acids of *NUP98* created as BamH1-EcoR1 constructs. Constructs were validated by sequencing and correct expression and transmission were confirmed by Western blot. Generation of recombinant ecotropic retrovirus-producing GP+E86 cells was performed as previously described [17].

Retroviral transduction of NUP98-HOXA10HD and mutations into bm cells

Primary mouse bone marrow (bm) cells were transduced as previously described [18]. The bm cells were infected by cocultivation with irradiated (4,000 cGy) viral producers for NA10HD-GFP (or mutant forms) alone or with HA-Meis1-YFP in the presence of 5 μg/mL protamine sulfate (Sigma, Oakville, Canada) for 48 hours. Bm cells were then flushed from the viral producers and grown for 72 hours in DMEM media. Transduced bone marrow cells were separated by fluorescence-activated cell-sorter (FACS) sort (FACSDiva, Becton Dickinson) on the basis of GFP or GFP and YFP expression, and sorted bm populations were maintained in culture for 3 days before use in bm transplantation mouse experiments and *in vitro* assays.

Bm Transplantation and Monitoring of Recipients

Purified GFP^+ or GFP^+/YFP^+ cells (2.5×10^5/mouse) were injected into the tail vein of lethally irradiated recipient C57BL/6J Ly5.2 mice (bred and maintained in the BCCA Research Centre animal resource unit) that were exposed to a single dose of 810 cGy total-body irradiation accompanied by a life-sparing dose of 2.0×10^5 C57BL/6J bm cells. Viability of mice was monitored daily. Donor derived engraftment and reconstitution were monitored after 4 weeks by flow-cytometry analysis (FACS) of GFP+ and YFP+ expression in the peripheral blood of the transplanted animals. For immunophenotypic FACS analysis, single cell suspensions were stained with the following monoclonal antibodies: phycoerythrin (PE)-conjugated or allophycocyanin (APC)-conjugated anti-Gr-1, anti-Mac-1, anti-B220, anti-Sca-1, and anti-c-Kit (all obtained from Pharmingen, San Diego, CA). Moribund animals were analyzed as previously described [18]. Survival curves of mice were plotted in Graphpad Prism v5.0 to determine *p* values and their median survival timepoint.

In vitro assays

Initial cultures were initiated with 80,000 unmanipulated freshly isolated bm cells from 5-FU treated mice together with 20,000 FACS purified GFP+ or GFP+/YFP+ bm cells transduced with *NUP98* constructs alone or with *Meis1* respectively in DMEM media. Cell counts were determined by Vi-Cell XR Cell Viability Analyzer and GFP+ or GFP+/YFP+ cells determined by flow-cytometry for multiple time points.

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Differentiation of clonogenic progenitors was analyzed by plating sorted transduced bm cells as detailed above so that GFP+ or GFP+/YFP+ cells were plated in 1 ml of methylcellulose culture medium per petri dish in standard conditions (Methocult M3434, StemCell Technologies Inc.), containing 10 ng of murine interleukin-3 per ml, 10 ng of human interleukin-6 per ml, 50 ng of murine stem cell factor per ml, and 3U of human erythropoietin per ml. 4000 starting cells were plated and serially replated three times, transferring 4000 cells into each replate. Duplicate plates were scored microscopically with standard criteria after 7 days and averaged for colony scores. Cultures were initiated with 3×10^6 transduced bm cells seeded in a 10-cm dish at day 0, and the equivalent of 3×10^5 starting cells (day 0) were replated into the same size dish on day 7. After 10 days, cells were harvested and HSC content evaluated by limit dilution assay as previously described [14].

Quantitative RT-PCR

RNA was isolated using Trizol™ (Invitrogen, Cat. No. 15596-026) and the samples were then treated with amplification grade DNase I (Invitrogen, Cat. No. 18068-015). Complementary DNA (cDNA) was generated by reverse transcription (RT) with SuperScript™ III Reverse Transcriptase (Invitrogen, Cat. No. 18080-093). Quantitative real-time RT-PCR was done as previously described [16]. The relative expression changes were determined with the $2^{-\Delta\Delta CT}$ method and the housekeeping gene abelson murine leukaemia viral oncogene homologue (*mAbl1*) was used to normalize the results.

RESULTS

Defining domains of Nup98 in a NUP98-HOXA10HD fusion that are sufficient for Meis1 complementation and leukemic transformation

To determine which subdomains of *NUP98* are necessary and/or sufficient for leukemogenic activity of NUP98-fusions, we created a panel of mutants of the N-terminal region of *NUP98* or replacement of *NUP98* fused to the HD of HOXA10 as follows (see also Figure 1A).

1. ΔGLEBSA10HD: a deletion mutant that eliminates just the GLEBS and Rae1/APC interaction domains thus leaving the bulk of the FG/GLFG-rich region intact.
2. FG1A10HD: a deletion mutant containing the first 150 amino acids of *NUP98* and thus half (14 of 28) of the FG repeats and 3 of the 9 GLFG repeats.
3. Δ224A10HD: a N-terminal truncation of NUP98 which retains 15 of the 28 FG repeats and 6 of the 9 GLFG repeats but containing no overlapping sequence compared to the FG1A10HD construct.
4. FG1x2A10HD: a tandem duplication of the first 150 amino acids region of NUP98 to artificially increase the number of FG/GLFG repeats comparable to that contained in the intact wildtype *NUP98* portion, to investigate if a specific density and/or total number of FG/GLFG repeats were required for *NUP98* function. In conjunction with the Δ224A10HD and FG1A10HD constructs, it

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may also allow us to differentiate between FG repeats and the subset of GLFG repeats.

5. E1a(89)-A10HD: a fusion with the first 89 amino acids of E1a which retains only the CBP/p300 interaction domain of E1a and lacks known HDAC1 recruitment to mimic and test the possible role of *NUP98* in recruiting CBP/p300.

We first investigated the ability of each of these constructs to collaborate with *Meis1* to achieve leukemic transformation. Mice were followed by pb workup starting at 30 days post-transplant, and symptomatic mice were sacrificed and tissue samples of bm, spleen and pb harvested for analysis. Flow cytometric analysis of pb cells for expression of the reporter genes, revealed successful engraftment with GFP^+ or GFP^+/YFP^+ bm. None of the truncation constructs or E1a(89)A10HD in the absence of *Meis1* induced leukemia or manifested elevated proportions of myeloid (Gr1+/Mac1+) cells (Figure 2A, alone bars) when transplanted into mice, consistent with previous reports of NA10HD in the absence of *Meis1* [14]. In contrast, mice transplanted with bm transduced with NA10HD together with *Meis1* quickly became moribund with high wbc counts (Figure 2B +Meis1 bars), high levels of $Gr1^+/Mac1^+$ cells in pb and bm (Figure 2A +Meis1 bars) and high blast counts indicative of AML (data not shown). Similar findings indicative of AML were also seen with each of the truncation constructs when combined with *Meis1*, with the exception of the FG1A10HD construct, which despite elevated levels of wbc and $Gr1^+/Mac1^+$ cells in both pb and bm, lacked the high blast counts to indicate AML.

Interestingly, while several of the NUP98-deletion constructs were, like intact NA10HD, capable of inducing leukemia in concert with *Meis1*, an increase in the median survival times was evident ($p<0.002$) suggestive of a modest reduction in potency (compare median survival of 31.5 days for NA10HD + Meis1 compared to 49 days for Δ224A10HD + Meis1, 52.5 days for ΔGLEBSA10HD + Meis1, 60.5 days for FG1x2A10HD + Meis1 and 66.5 days for E1a(89)A10HD + Meis1) (Figure 3). The FG1A10HD construct in collaboration with *Meis1* did not induce leukemia, much like the empty vector control, potentially indicating an insufficiency of FG/GLFG repeats for recruitment of other factors.

To determine the levels of expression of each construct, RNA was isolated from bm of moribund (+*Meis1*) animals, and cDNA generated. This cDNA was subjected to quantitative RT-PCR analysis with a GFP-specific probe to determine the relative levels of expression of each NUP98-deletion construct (Figure 1B). We find that all constructs expressed within a 2-fold relative range, and despite low expression levels of E1aA10HD, the E1a(89)A10HD + Meis1 mice are still leukemic (Figure 3, E1a(89)A10HD+Meis1 curve), while high expression levels of FG1A10HD still resulted in a no leukemic transformation in FG1A10HD+Meis1 mice, indicating that the relative levels of protein expression were not responsible for any variations in phenotype.

Significantly, leukemogenic induction by ΔGLEBSA10HD with *Meis1* revealed that neither the GLEBS motif nor the Rae1/APC interaction domain is essential for this activity. The leukemogenic activity of FG1x2A10HD and Δ224A10HD which contain no overlapping amino acid sequence, and the absence of such activity by FG1A10HD together implies that a

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minimal number of FG/GLFG repeats are necessary and sufficient for activity. At least one major function of such repeats appears to be their ability to recruit CBP/p300, a function that can be phenocopied by E1a(89)A10HD.

Domains of NUP98 necessary for growth promoting activity of NUP98-Hox fusions

We also tested the ability of the various truncation constructs to confer a growth advantage on primitive hematopoietic cells *in vitro*. Initial cultures consisting of 80,000 bone marrow cells harvested from 5-FU treated mice and 20,000 transduced bm cells were sampled every 48 hours, for total cell numbers, and the percentage of transduced bm cells, which was measured by the overall percentage of GFP+ or GFP+/YFP+ cells. While cells transduced with the vector control exhibited no growth advantage and remained at the starting percentage of 20%, each construct except for FG1A10HD, showed a significant growth advantage, and rapidly dominated the culture by day 6 (Figure 4A).

Consistent with the competitive growth assay, serial CFC-replating assays also confirmed that the ΔGLEBSA10HD, Δ224A10HD, FG1x2A10HD and E1a(89)A10HD constructs retained the ability to stimulate the self-renewal of primitive clonogenic progenitors (Figure 4B). The measured stimulatory effect however showed a gradient in potency with the ΔGLEBSA10HD and Δ224A10HD constructs being essentially identical to intact NA10HD while E1a(89)A10HD and FG1x2A10HD both showing reduced potencies. Again, the FG1A10HD mutant was devoid of detectable growth promoting activity.

To determine if the observed growth promoting activity extended to HSC, FACS-sorted transduced bm cells were assayed by limit dilution analysis for competitive repopulating cell content. As expected, intact NA10HD stimulated a multilog increase in HSC number (~1000-fold) in the 10 day culture period (Figure 4B). Somewhat lower although still highly significant HSC expansion was stimulated by the ΔGLEBSA10HD construct while minimal expansion was evident for the Δ224A10HD and E1a(89)A10HD constructs and none was evident for FG1x2A10HD or FG1A10HD. These findings most closely correlate with the findings from CFC-replating assays (Figure 4B, above bars) and contrast with the near equivalency of growth promoting activity of the constructs, with the exception of FG1A10HD, observed in liquid culture competitive assay and in leukemogenic activity with *Meis1*.

This gradient in potency is mirrored in the ability of each construct to elevate *Flt3* expression (Figure 4C; bars without Meis1), which is correspondingly compensated for by *Meis1* expression in collaboration with those constructs least able to upregulate *Flt3* (Figure 4C; bars with Meis1).

DISCUSSION

While NUP98 fusions paired with a large number of partners have been well documented in AML, the mechanisms by which such fusions contribute to leukemogenesis have been unresolved. In this study, we have modeled such fusions with a canonical *NUP98-HOXA10HD* fusion, where the *HOXA10* fusion partner has been reduced to its minimal HD, to focus on the NUP98 partner, and carried out detailed structure-function analysis

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coupled with rigorous *in vitro* and *in vivo* studies. The minimal HoxA10 fusion partner reduces the complexity of other interactions in our *NUP98-Hox* model and has the added benefit of not being leukemic in the absence of *Meis1* collaboration [19].

Our combined results reveal that the GLEBS and Rae1/APC domains of a NUP98-fusion as modeled by *NUP98-HOXA10HD* are dispensable for leukemogenic activity and suggest that a canonical function of such fusions is their ability to recruit the transcriptional activator CBP/p300. These findings thus argue that NUP98-fusions do not function via the nuclear pore complex or via mRNA transport by rather point towards primarily transcriptional roles. Interestingly, such a role is consistent with recent reports of non-nuclear pore but transcription roles of NUP98 in the nucleoplasm in the *Drosophila* model [20][21]. Indeed in this model, there seems to be a preference for NUP98 interactions with active chromatin (reviewed in [22]), consistent with CBP/p300 recruitment. Definitive experiments on CBP/p300 recruitment using CBP/p300-deficient mice would be obscured by the requirement of CBP/p300 for normal hematopoiesis [23][24]. Our use of an E1a fusion to mimic this recruitment is a novel means to circumvent this limitation.

The differential potencies of the mutant *NUP98-Hox* fusions tested however as revealed by CFC replating, HSC expansion and disease latency as well as *Flt3* expression levels in collaboration with *Meis1* suggest that the transforming functions of the N-terminal region of NUP98 are not solely due to interactions with CBP/p300 and/or reflect synergistic effects of multiple FG/GLFG repeats.

Our results also provide interesting insights into the overlap of leukemogenic activity and promotion of HSC self-renewal. While multiple deletion mutant forms of NUP98-fusions and the E1a fusion were capable of leukemic transformation in collaboration with *Meis1*, there was a dramatic gradient or disparity in their solo ability to stimulate HSC self-renewal and/or promoting CFC replating *in vitro*. Strikingly, the E1a(89)A10HD construct was totally devoid of HSC expansion or CFC replating ability but with *Meis1* was leukemogenic with only slightly reduced potency indicated by a modest increase in disease latency compared to intact NA10HD. We thus would speculate that the interaction of FG/GLFG repeats with CBP/p300 is unlikely to be the only interaction the FG/GLFG repeats mediate, and indeed that other factors may be critical to promote HSC self-renewal. The availability of a morphic series of deletion mutants, along with the E1a fusion that in large part phenocopies a *NUP98-Hox* fusion, should provide a powerful platform for future research.

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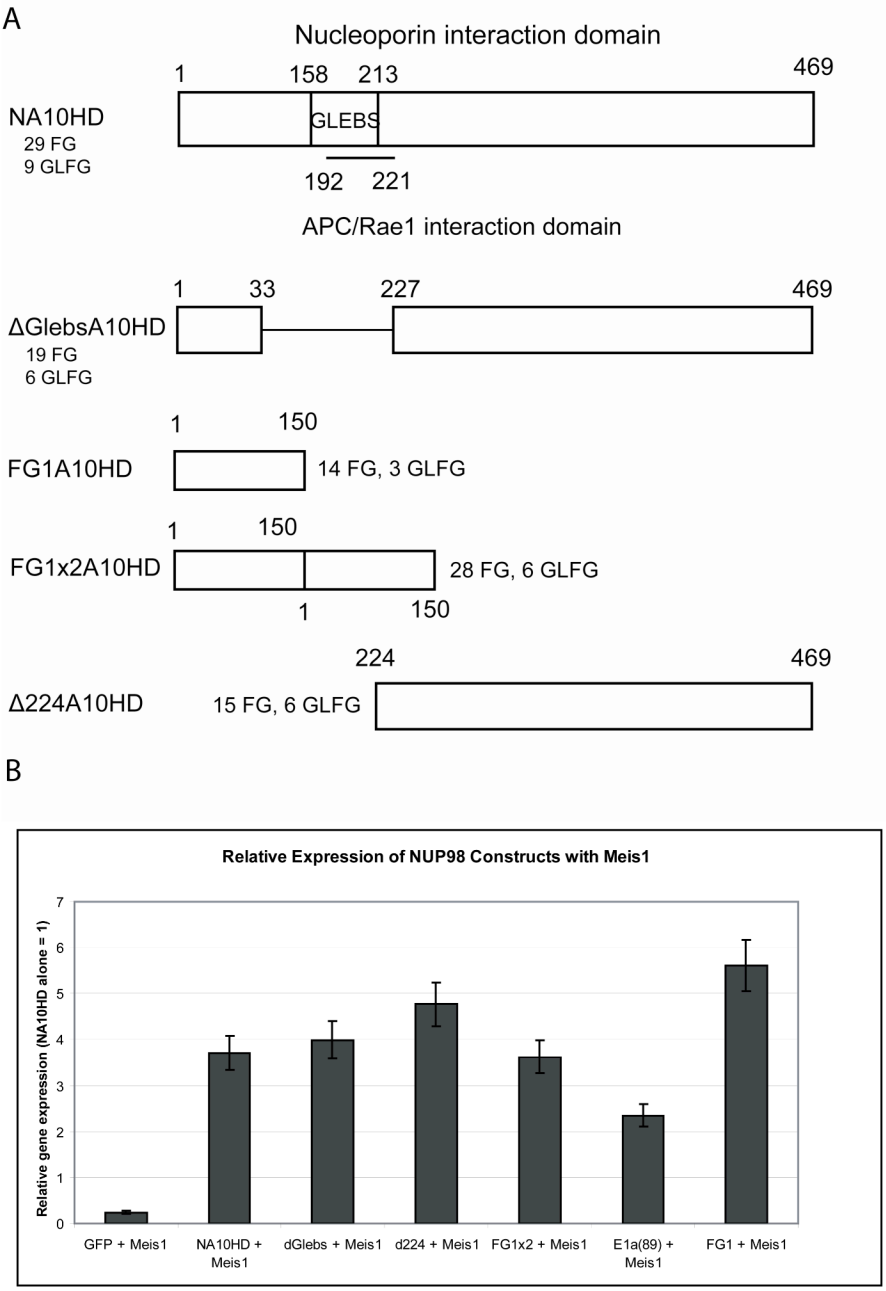

Figure 1.

A: Schematic representation detailing the GLEBS domain required for nucleoporin interaction (158-213) as well as the APC/Rae1 interaction domain (192-221) of NUP98, and FG/GLFG repeats. Schematic representation of ΔGLEBSA10HD construct with both overlapping interaction domains removed, the FG1 region of Nup98, the artificial duplication of FG1 to create FG1x2 and the Δ224 N-terminal truncation. All constructs list the number of FG/GLFG repeats contained. B: Graph representing relative gene expression of NUP98 constructs. qRT-PCR analysis of bm from moribund (+*Meis1*) animals using a GFP probe. Gene expression is normalized to NA10HD alone =1.

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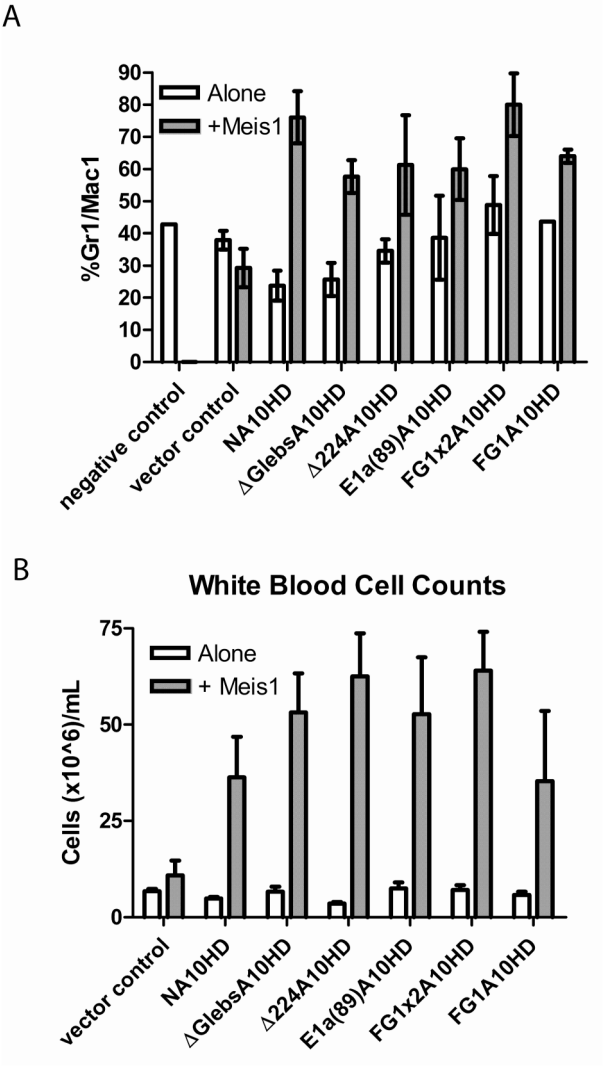

Figure 2.

A: Graph representing the percentage of Gr1+/Mac1+ found in the bone marrow of transplanted recipient mice at time of sacrifice. Pairs of bars indicate construct alone (white) or co-expressed with Meis1 (grey). Normal Gr1/Mac1 levels of an unmanipulated mouse are detailed as the negative control. B: Distribution and average of white blood cell counts of mice transplants receiving NA10HD (or mutants) with (grey bars) or without Meis1 (white bars). White blood cell content was assayed by a Vet ABC Blood Analyzer (Vet Novations, Barrie ON, Canada).

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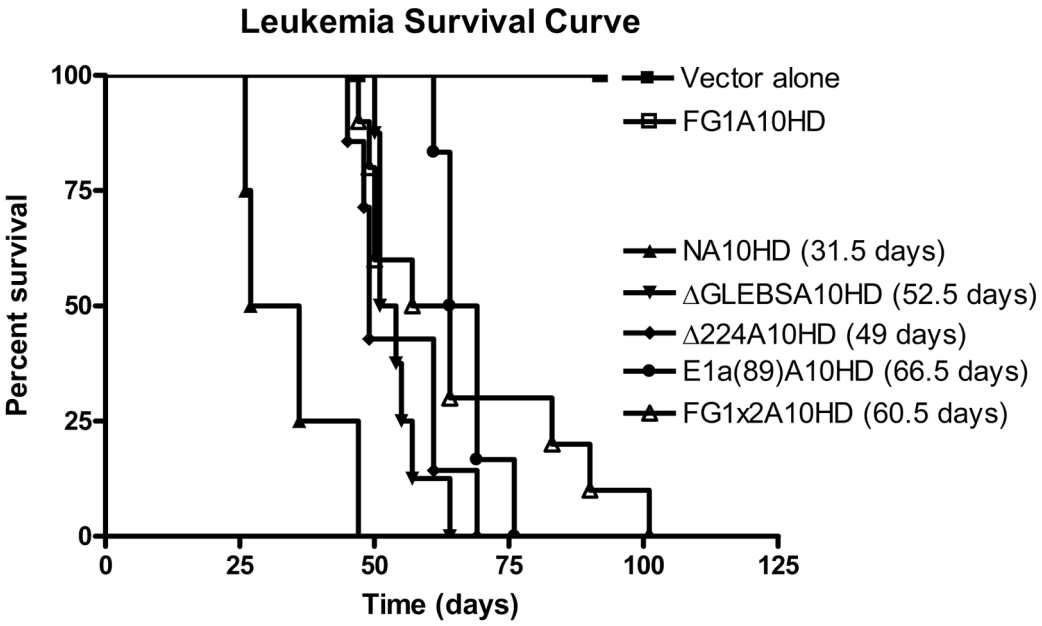

Figure 3.

Kaplan-Meier survival curve of mice transplanted with Nup98-A10HD and mutants co-transduced with Meis1. Irradiated recipients were tracked until time of sacrifice and bone marrow, peripheral blood, and spleen samples were harvested to determine type of leukemia present. An empty vector (GFP only) control co-transduced with Meis1 was tracked as a control. FG1A10HD transduced with Meis1 resulted in the same curve as the GFP control. Median survival of the various constructs + Meis1 (listed in the legend) were significantly ($p<0.002$) slower than that of NA10HD + Meis1.

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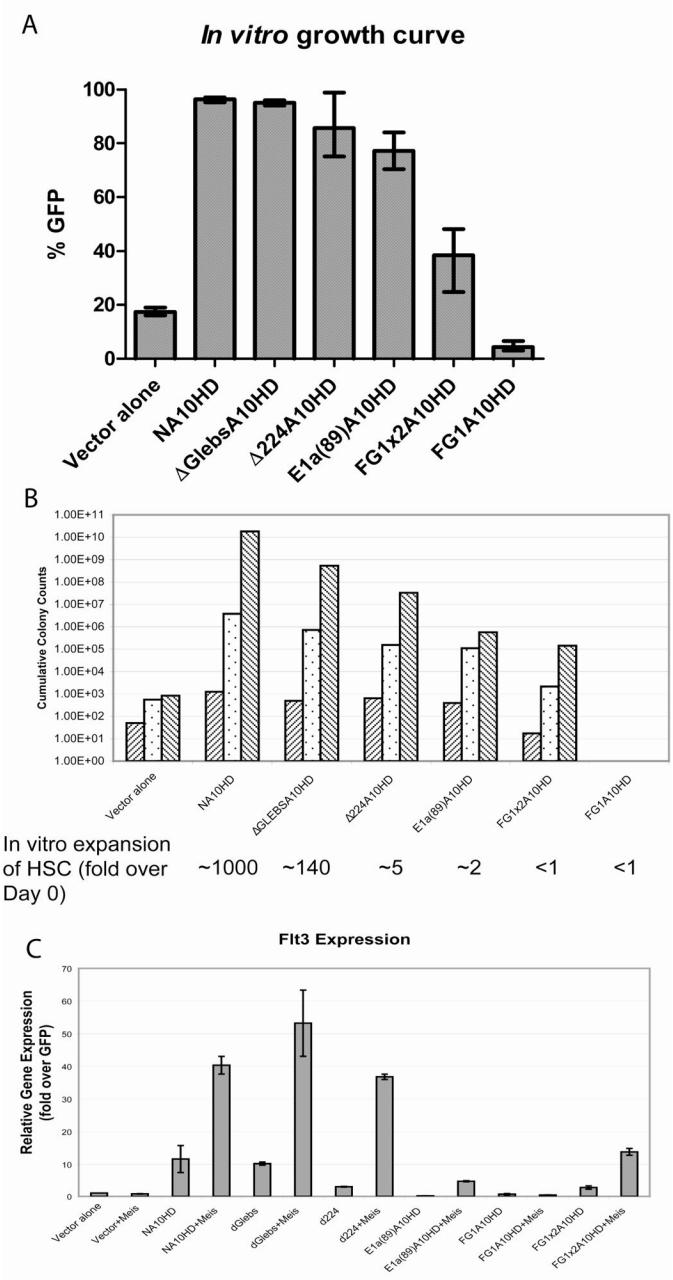

Figure 4.

A: *In vitro* growth kinetics of a GFP+ control, NA10HD transduced, or mutant transduced cells. Initial cultures consisting of 80,000 bone marrow from 5-FU-treated mice and 20,000 FACS-sorted transduced bm cells are tracked for GFP, and the percentage of cells 6 days after start of the assay are determined by FACS analysis and graphed to determine growth advantage of any of NUP98 constructs. B: Above: Methylcellulose culture assay as a measure of progenitor cell self-renewal.. Bars represent colonies established after one week of serial replatings of 4000 source cells. Triplicate bars represent serial replating over 3 weeks. Below: Limit dilution assay *in vivo*. HSC content of transduced NA10HD as well as

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mutants are shown on day 10 in *in vitro* culture vs. a Day 0 5FU BM control. Fold increase in HSC content is indicated over day 0 controls. C: Graph of changes in gene expression with or without Meis1. qRT-PCR of Flt3 expression by cells expressing NUP98 constructs alone, or co-expressed with Meis1 are plotted, to show changes in Flt3 expression when Meis1 is co-expressed.