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Clonal hematopoiesis in donors and long-term survivors of related allogeneic hematopoietic stem cell transplantation

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Abstract:
Clonal hematopoiesis is associated with age and an increased risk of myeloid malignancies, cardiovascular risk and all-cause mortality. We tested presence of CH (defined as VAF {greater than or equal to}0.01 in granulocytes using a next-generation DNA sequencing panel targeting 102 genes) in a setting where hematopoietic stem cells (HSCs) of the same individual are exposed to different degrees of proliferative stress and environments, i.e. in long-term survivors of allogeneic hematopoietic stem cell transplantation (allo-HSCT) and their respective related donors (n=42 donor-recipient pairs). With a median follow-up time since allo-HSCT of 16 years (range: 10-32 years), we found a total of 35 mutations in 23/84 (27.4%) study participants. 10/42 (23.8%) donors, and 13/42 (31%) recipients had CH. CH was associated with older donor and recipient age. We identified five cases of donor-engrafted CH, with one case progressing into myelodysplastic syndrome in both donor and recipient. 4/5 cases showed increased clone size in recipients compared with donors. We further characterized the hematopoietic system in individuals with CH: i) CH was consistently present in myeloid cells but varied in penetrance in B and T cells; ii) colony-forming units (CFUs) revealed clonal evolution or multiple independent clones in individuals with multiple CH mutations; iii) while telomere shortening determined in granulocytes suggested about 20 years of added proliferative history of HSCs in recipients compared with their donors, telomere length in CH versus non-CH CFUs showed varying patterns. This study provides insight into the long-term behavior of the same human HSCs and respective CH development under different proliferative conditions.

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Clonal hematopoiesis in donors and long-term survivors of related allogeneic hematopoietic stem cell transplantation

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Key Points

- CH including donor-engrafted CH is highly prevalent among donors and recipients long-term after allo-HSCT
- CH clones variably expand at different levels of the hematopoietic hierarchy and can clonally evolve into subclones

Abstract

Clonal hematopoiesis is associated with age and an increased risk of myeloid malignancies, cardiovascular risk and all-cause mortality. We tested presence of CH (defined as VAF ≥0.01 in granulocytes using a next-generation DNA sequencing panel targeting 102 genes) in a setting where hematopoietic stem cells (HSCs) of the same individual are exposed to different degrees of proliferative stress and environments, i.e. in long-term survivors of allogeneic hematopoietic stem cell transplantation (allo-HSCT) and their respective related donors (n=42 donor-recipient pairs). With a median follow-up time since allo-HSCT of 16 years (range: 10-32 years), we found a total of 35 mutations in 23/84 (27.4%) study participants. 10/42 (23.8%) donors, and 13/42 (31%) recipients had CH. CH was associated with older donor and recipient age. We identified five cases of donor-engrafted CH, with one case progressing into myelodysplastic syndrome in both donor and recipient. 4/5 cases showed increased clone size in recipients compared with donors. We further characterized the hematopoietic system in individuals with CH: i) CH was consistently present in myeloid cells but varied in penetrance in B and T cells; ii) colony-forming units (CFUs) revealed clonal evolution or multiple independent clones in individuals with multiple CH mutations; iii) while telomere shortening determined in granulocytes suggested about 20 years of added proliferative history of HSCs in recipients compared with their donors, telomere length in CH versus non-CH CFUs showed varying patterns. This study provides insight into the long-term behavior of the same human HSCs and respective CH development under different proliferative conditions.

Introduction

Clonal hematopoiesis (CH) is defined as the occurrence of recurrent mutations in known oncogenes in hematopoietic stem and progenitor cells (HSPCs) in the absence of overt hematologic malignancies. CH is an age-related[1-4] condition and associated with an increased risk of hematological cancers[1,5-7] including therapy-related myeloid malignancies[8,9], cardiovascular disease[1,10,11], thromboembolism[12] and all-cause mortality[1].

During allogeneic hematopoietic stem cell transplantation (allo-HSCT), a small percentage of hematopoietic stem cells (HSCs) is transferred from the donor to the recipient. Whereas the hematopoietic system is largely unaffected in the donor with negligible HSC expansion occurring due to HSC donation, transplanted donor HSCs within the recipient undergo substantial self-renewing expansion until homeostatic conditions have been re-gained (**Figure 1A**). In addition, the transplant conditioning regimen elicits a highly inflammatory milieu contributing to enhanced HSC cell divisions. Altogether, the increased proliferative history of donor HSCs within the recipient as compared to donor HSCs within the donor leads to a measurable difference in telomere lengths between hematopoietic cells in donors and recipients equivalent to a pre-mature ageing of approximately 10-30 years[13-15]. Thus, we hypothesized that increased HSC proliferation within an inflammatory microenvironment in recipients might promote emergence and / or clonal selection and evolution of CH clones[16]. In fact, previous studies have shown that during autologous[8,17] and allogeneic[18,19] HSCT pre-existing donor CH clones can engraft and clonally evolve within the recipient occasionally leading to donor cell leukemia[18-20]. Notably, CH in the setting of allogeneic HSCT is an emerging clinical factor to consider with a potential impact on outcome [21]. However, more data are needed to better estimate the frequency of CH in the setting of allo-HSCT especially with a longer follow-up time. In this study, we set out to investigate the potential role of CH in long-term survivors of allo-HSCT and their respective related donors.

Study subjects, materials and methods

Study population

A single-center cohort of n=45 HSCT recipients and their related donors were evaluated. Forty-two donor-recipient pairs were eventually enrolled in this study after giving informed consent. The study was approved by the local ethics committee (KEK-ZH No. 2015-0053) in accordance with the declaration of Helsinki.

Cell isolation

Granulocytes were isolated from 10ml of EDTA anticoagulated peripheral blood using EasySep Direct Neutrophil Isolation Kit (StemCellTechnologies, Vancouver, Canada) according to the manufacturer's instructions.

$CD34^+$ HSPCs were isolated from 20ml of EDTA anticoagulated peripheral blood using human CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's recommendations. B cells, T cells and monocytes were flow-sorted from $CD34^-$ cell fractions using a FACSAria III flow cytometer (BD Biosciences, San Jose, USA).

DNA isolation

DNA from granulocytes, monocytes, B cells, and T cells was isolated using QIAamp DNA Mini Kit (Hilden, Germany) according to the manufacturer's recommendations.

Colony-forming unit assay

$CD34^+$ HSPCs were plated in 9ml cytokine-supplemented methylcellulose medium (StemCellTechnologies, Vancouver, Canada) as described previously [22]. After 14 days of culture at 37°C and 5% CO2 single-CFUs were picked and were each resuspended and processed in 20µl QuickExtract DNA Extraction Solution (Lucigen, Middleton, USA). To detect the genetic mutations of interest in single CFUs, DNA was subjected to PCR with primers for each gene followed by Sanger sequencing.

Telomere length analysis

Telomere length was measured by monochrome multiplex quantitative PCR (MM-qPCR) as previously described[23,24]. Telomere length is given in T/S ratios, i.e. by dividing the number of copies of the Tel (T) by the Hbg template (S), as previsouly described[25,26].

Library Preparation for NGS

For NGS from granulocyte DNA (50ng per sample), libraries were prepared using the HaloPlexHS Target Enrichment System for Illumina sequencing from Agilent according to the specifications. The libraries were then sequenced using the Illumina HiSeq 2500 system.
For validation NGS from granulocyte, monocyte, B and T cell DNA, 200-280 bp amplicons were PCR-amplified, purified and submitted for NGS (Massachusetts General Hospital, Center for Computational and Integrative Biology Core Facility, Cambridge, MA, USA).

NGS data processing and analysis

Adapter contamination and low-quality bases were removed from the raw reads using SeqPurge[27] (git commit id 71885f4). The reads were aligned to the human reference genome hg19 using bwa mem (v0.7.15)[28]. Then Picard tools (v2.8.3) were used to fix erroneous mate information as well as to sort and merge the alignment files for each sample. All alignments marked as secondary were removed before performing an indel realignment using GATK (3.8)[29]. In the next step UMI cluster were identified with UMI-tools (v0.4.4)[30] and sequencing errors were removed by generating the consensus reads for every biological template using an in-house tool. The resulting BAM files were processed with samtools mpileup (v1.3.1)[31]. Next, VarScan2 (v2.4.3) [32] was used to call single nucleotide variants and indels with the command mpileup2snp and mpileup2indel. The VarScan2 results were combined for each sample and annotated with dbSNP (version 138) [33] and cosmic (version 80)[34] using SnpSift (v4_3p_core) [35] before using SnpEff (v4_3p_core) [36] for the final annotation. Expert manual curation of the identified mutations with regards to biological impact defined a set of mutations that were investigated using IGV (2.3.68) [37] and validated using ultra-deep amplicon sequencing. For validation, the reads were mapped, sorted and realigned using the same specifications as above. The resulting alignments were then processed with samtools mpileup and variants were called using VarScan2 using the same parameters as described above.

Statistical analysis

Significance of differences was analyzed using nonparametric tests as indicated in the figure legends. A difference between groups was considered statistically significant if p-values were <0.05. Statistical analysis was calculated using Prism software.

Results

Somatic mutations in donors and recipients of related allogeneic hematopoietic stem cell transplantation

Based on long-term survival defined as ≥10 years after allo-HSCT, we evaluated a cohort of recipients (n=45) and their respective sibling donors (n=45) who underwent related allo-HSCT at our institution between 1983 and 2006 (**Figure 1A**). Three allo-HSCT donor-recipicient pairs were excluded from the analyses for reasons described in greater detail below. Forty-two allo-HSCT donor-recipient pairs, i.e. 84 individuals, were eventually included in the analysis. The median age at transplantation in the total cohort, in donors, and in recipients was 38, 37, and 39 years, respectively (**Table 1**). The median age at study inclusion was 57 years (range: 29-95 years) for donors, and 61 years (range: 32-77 years) for recipients, which was not significantly different. The median follow-up time since transplantation was 16 years (range: 10-32 years). The gender distribution was balanced and most patients were transplanted for malignant disease. Twenty-eight patients received bone marrow (BM) while 14 patients were transplanted with peripheral blood stem cells (PBSCs) (**Table 1**). We collected peripheral blood (PB) from study participants and isolated DNA from purified populations of granulocytes, monocytes, B and T cells. In addition, periphal blood $CD34^+$ HSPCs were isolated and used for clonogenic colony-forming unit (CFU) assays followed by colony picking and genomic DNA extraction. The resulting DNA from these various sources were subjected to several downstream applications that included targeted next-generation sequencing (NGS) with increased sensitivity due to the use of unique molecular identifiers (UMIs) (**Table S1**), Sanger sequencing, and quantitative PCR (**Figure 1A**).

With a mean coverage of 582 after consensus computation and a variant allele frequency (VAF) cut-off set at 0.01, we detected 35 mutations in 11 genes in granulocyte DNA (**Figure 1B** and **Table S2**). Notably, all mutations reported in this study were independently validated by ultra-deep (coverage 50,000-100,000X) amplicon NGS with a high degree of concordance between VAFs determined by targeted NGS and ultra-deep amplicon NGS (**Figure S1**). In line with previous studies on CH, *DNMT3A* and *TET2* were the most frequently mutated genes, together accounting for 57.1% of all mutations[38]. We did not detect *ASXL1* mutations in our cohort which was likely due to statistical chance and/or due to the highly selected patient cohort and the relatively young median age in our cohort. Validation experiments demonstrated that our sequencing and analysis pipeline was capable of robustly detecting *ASXL1* variants in clinical samples from patients with myeloid malignancies (**Figure S2**). The remaining mutations affected genes commonly mutated in CH and myeloid malignancies. There was no clear overrepresentation of specific mutations in either donors or recipients (**Figure 1B**). More than

one mutation (up to 3 mutations) per individual could be found in 11 study participants (**Figure 1C**). Most mutations (18 mutations) were missense mutations, followed by indels, nonsense and splice mutations (**Figure 1D**). The most frequent single-base substitutions were cytosine-to-thymine (C→T) transitions known to be associated with ageing[39,40] (**Figure 1E**). The median VAF in the total cohort was 0.03 with no statistical difference between donors and recipients (**Figure 1F**).

Altogether, we detected CH in a total of 23/84 study participants corresponding to 27.4% of the total cohort (**Figure 2A**). At least one mutation was found in 10/42 (23.8%) donors and in 13/42 (31%) recipients, this difference not being statistically significant. Individuals with CH were significantly older than study participants without CH when analyzed for age at study inclusion (**Figure 2B**) as wells as age at transplantation (**Figure S3**). This well-established age-association of CH was also found when donors and recipients were analyzed separately (**Figure 2B** and **Figure S3**). Notably, there was no association between the magnitude of VAFs and time since transplantation (data not shown).

Donor-engrafted CH in recipients of related allo-HSCT

Based on the detection of at least one identical – often two identical – non-hotspot mutations in both the donor as well as the recipient, we could identify several cases of donor-engrafted CH (**Figure 3A-C**), i.e. a scenario where a pre-existing CH clone was transferred at the time of transplant from the donor and engrafted in the recipient. Altogether, donor-engrafted CH occurred in 5/42 (11.9%) of all transplantations as well as in 5/10 (50%) and in 5/13 (38.5%) of donors and recipents with CH, respectively (**Figure 2A, Figure 3A**). Of note, one case of donor-engrafted CH eventually progressed to MDS. The corresponding mutational data were, however, analyzed separately as discussed below. In all other cases of donor-engrafted CH, VAFs in the recipient increased significantly relative to the corresponding donor over a long period of time (median time since transplant 15 years) (**Figure 3B-D**). Notably, these donors were significantly older with a median age at transplantation of 50 years compared to a median donor age at transplantation of 37 years in the entire cohort (**Figure 3D, and Table 1**) and 35 years in the subgroup of donors that did not result in donor-engrafted CH (**Figure 3D**). These findings together with the well-established age-association of CH strongly suggest that an increased donor age at transplantation may be associated with an increased risk of donor-engrafted CH.

Besides these 5 cases of donor-engrafted CH, we identified 5 cases of donor-only CH and 10 cases of recipient-only CH. To rule out that in the latter cases of recipient-only CH the

presence of clones is not due to residual recipient hematopoiesis but developed from the grafted donor-derived hematopoiesis, we performed chimerism analysis using digital PCR (dPCR). While most cases of recipient-only CH showed 100% donor chimerism (**Figure S4**, **Table S3**), we identified 2 recipients (R30 and R33) originally transplanted for severe aplastic anemia (SAA) with 0% donor chimerism, and one recipient (R16) transplanted for CML with about 10% residual recipient hematopoiesis that was, of note, *BCR-ABL1*+. These 3 donor-recipient pairs were therefore excluded from our initial cohort of 45 donor-recipient pairs as mentioned above.

Furthermore, one of the 5 donor-engrafted CH cases progressed into MDS in both the donor as well as the recipient (**Figure 3E, table S4**). We could rule out the possibiliy of an underlying known genetic predisposition by sequencing the donor's and recipient's DNA using a panel of genes commonly mutated in inherited bone marrow failure syndromes (**table S5**). Next-generation sequencing of the donor's and recipient's granulocyte DNA at the time of study inclusion in 2017 allowed us to retrospectively decipher and re-construct the origin and clonal evolution of CH progressing to phenotypically different MDS in this donor-recipient pair. A relatively complex mutational pattern in both individuals each carrying 4 mutations at varying VAFs could be revealed (**Figure 3E, table S4**). Importantly, two identical mutations were shared between the siblings demonstrating that the *TET2*^{R550*} / *U2AF1*^{Q157P} double-mutant founding clone must have been transferred from the donor to the recipient in 1996 at the time of transplantation. Thereafter, both clones followed different clonal trajectories: in the donor the *TET2*^{R550*} / *U2AF1*^{Q157P} double-mutant founding clone acquired an *ASXL1*^{Q760*} and a second *TET2* mutation (Q967*); in the male recipient the donor-derived founding clone aquired an x-chromosomal *STAG2*^{R259*} mutation that has exerted a strong selective advantage since it, based on VAFs, completely outcompeted the donor-derived *TET2*^{R550*} / *U2AF1*^{Q157P} double-mutant founding clone and virtually any residual normal cells. In addition, there was a smaller subclone carrying a *SETBP1*^{G617S} mutation detectable in the recipient.

Quantitative representation of clones at different levels of the hematopoietic hierarchy

Knowledge of clonal hematopoiesis stems largely from next-generation sequencing of peripheral blood mononuclear cells (PBMCs). Few studies have investigated the relative clonal expansion of CH mutations in various mature blood cell lineages such as granulocytes, monocytes, B and T lymphocytes[41-43]. Therefore, we addressed the question whether there are distinct representation patterns of CH clones at different levels of the hematopoietic hierarchy and within different hematopoietic lineages. To this end, we performed CFU assays from PB CD34^{+} cells isolated from study participants with CH and analyzed mutations by Sanger

sequencing. We also FACS-purifed monocytes, B and T cells and performed targeted amplicon deep sequencing.

All types of mutations, i.e. missense, nonsense or frame-shift, could reliably be identified by Sanger sequencing, and as expected, all mutations were heterozygous (or hemizygous in the case of x-chromosomal mutations in males) - as exemplified by some of the resulting Sanger electropherograms (**Figure 4A**). There was a good correlation between the percentage of detected mutation-positive CFUs by Sanger sequencing and the percentage of predicted mutation-positive CFUs based on VAFs in granulocytes. However, there was a case (*TET2*^{S1668fs}) that showed a substantial deviation from the expected representation (**Figure 4B**). This finding of disproportionate clonal representation at the level of HSPCs compared with mature granulocytes suggests that some mutations, besides promoting expansion of HSPCs, may either confer a concomitant lineage differentiation defect or, alternatively, a reduced half-life of the mature progeny. Alternatively, the capacity to form colonies and grow in methylcellulose might be influenced by CH driver mutations.

Interestingly, determining VAFs in granulocytes, monocytes, B and T cells individually in donors and recipients, respectively, revealed all possible combinations: uni-, bi-, tri-, and multi-lineage penetration of mutations (**Figure 4C**). While mutations with lower VAFs were often found only in myeloid cells (granulocytes and/or monocytes) but not in lymphocytes (B and/or T cells), mutations with higher VAF had a tendency to fully penetrate all mature lineages (**Figure 4C**). Of note, VAFs in granulocytes and monocytes were significantly higher than in lymphoid cells, the lowest VAFs seen in T cells (**Figure 4D**). In the 5 cases of donor-engrafted CH the pattern and extent of lineage penetration was not different in donors compared with recipients (**Figure 4C**).

Clonal architecture in individuals carrying multiple CH mutations

More than 50% of individuals with CH in our cohort carried two or three mutations (**Figure 1C**). Two distinct scenarios with regards to clonal architecture are possible: First, subclonality, where a founding clone evolves through acquisition of a second mutation, or second, un-related clones with independent acquisition of individual mutations in two distinct cells. This distinction is of probable clinial importance as it may be associated with different risks for progression to myeloid neoplasms. Our data from Sanger sequencing of single cell-derived CFUs from PB CD34$^+$ cells allowed us to reconstruct the clonal architecture for a number of individuals in our cohort with more than one mutation.

In fact, we found evidence for both scenarios: subclonality as indicated by the concomitant presence of two different mutations in the same CFU (**Figure 5A**) as well as un-

related clones as demonstrated by exclusive detection of only one of the two individual mutations per CFU (**Figure 5B**).

Collectively, our findings underscore the fact that the clonal architecture, especially in cases with relative low VAFs, cannot be inferred from VAFs alone but requires clonogenic assays.

Telomere length in study participants and individual CFUs from CH carriers

Due to the nature of eukaryotic DNA replication, each cell division leads to a measurable telomere length shortening of approximately 50-200 bp which can serve as a molecular counter for the proliferative history of dividing cells and tissues[44,45]. Therefore, we determined telomere length in granulocytes by quantitative PCR, and found significant telomere length shortening in allo-HSCT recipients as compared to donors (**Figure 6A**) confirming previous reports demonstrating that the enhanced HSC proliferation occurring in the recipient as a consequence of engraftment and hematopoietic reconstitution during the first year following allo-HSCT, indeed, leads to a significant telomere length shortening relative to the donor[14,46,47]. The difference in telomere length between donor and recipient translates into approximately 20 years of pre-mature ageing of the recipient's hematopoietic system as compared to the donor's (**Figure 6B**).

We hypothesized that the increased proliferative history of expanded CH clones in either donors or recipients may also lead to a measurable decrease in granulocyte telomere length. There was a trend towards shorter telomeres in donors with CH as compared to donors without CH that was most likely due to the increased age of donors with CH relative to donors without CH. However, individuals with CH did not have significantly shorter telomeres as compared to individuals without CH (**Figure 6C**). Since this association might be masked by, first, the quantitative heterogeneity of telomere lengths between individuals and, second, the relative small sizes of the CH clones in affected individuals, we performed quantitative PCR on DNA from clonogenic CFUs derived from PB $CD34^+$ cells to assess telomere lengths in HSPC-derived myeloid cells from CH mutation-positive and wild-type CFUs (**Figure 6D**). Intriguingly, these analyses revealed that telomere length did not significantly differ between CH mutation-positive CFUs as compared to wild-type CFUs for most of the analyzed cases with the exception of one case with significantly longer telomeres (**Figure 6E, upper panel, R11**) and one case of significantly shorter telomeres (**Figure 6E, lower panel, R39**) in CH mutation-positive CFUs. These findings may suggest that HSCs carrying particular CH mutations may activate the canonical or alternative molecular machinery to maintain telomeres in order to prevent telomere attrition. This should be clarified in future studies.

Discussion

In this study, we addressed the question as to whether the enforced HSC proliferation during allo-HSCT in conjunction with an inflammatory milieu elicited by the conditioning regimen and post-transplantation situation might promote initiation, expansion and/or evolution of clonal hematopoiesis in allo-HSCT recipients as compared to donors. We analyzed a highly selected cohort of relatively young (median age at transplantation/donation 38 years), related donor-recipient sibling pairs with a long median follow-up (median 16 years, range: 10-32 years). Thus, this cohort has some inherent selection biases as discussed below. We utilized a targeted and error-corrected (i.e. unique molecular identifiers, UMIs) sequencing approach that allowed us to confidently lower the VAF cut-off for mutation calling to 0.01. We found a high frequency of CH in both donors (23.8%) and recipients (31%), respectively, despite a relatively young median age in our cohort. However, the difference in CH frequency observed between donors and recipients was not statistically significant. In addition, we observed a high percentage of donor-engrafted CH with 5/42 (11.9%) of transplantations resulting in transfer of pre-existing CH clones from the donor to the recipient. Donor age at transplantation was significantly higher in the transplantations that resulted in donor-engrafted CH as compared to the transplantations that did not lead to donor-engrafted CH. This finding suggests that donor age at transplantation poses a risk factor for donor-engrafted CH. Notably, there was a consistent and significant increase in clone size as measured by VAF in recipients as compared to donors in the cases of donor-engrafted CH. However, this increase in VAFs was only relatively modest, i.e. 2.3-fold in median VAF between donors and recipients with VAFs in most recipients being around 0.1, indicating that in recipients with donor-engrafted CH the hematopoietic system is not dominated by a single clone but still largely polyclonal. These findings suggest that allo-HSCT does not provide an exceedingly ‘fertile soil’ for expansion of pre-existing CH clones, and that the presence of CH in allo-HSCT recipients is, in principle, compatible with long-term survival. A similar conclusion was reached in a recent study assessing clonal hematopoiesis in the setting of allo-HSCT[21]. However, these can only be preliminary conclusions given the inherent survival bias in our cohort of retrospectively analyzed long-term survivors of allo-HSCT. Future studies are needed to systematically and prospectively investigate the potential impact of CH including donor-engrafted CH on the outcome of allo-HSCT.

Furthermore, our findings from characterizing the hematopoietic system of individuals with CH will likely stimulate further research, especially regarding progression from pre-malignant CH to full-blown myeloid malignancy. A surprisingly high percentage (>50%) of study participants in our cohort had more than one mutation. Recently published studies have shown that the likelihood of CH progressing into AML is higher in individuals carrying more mutations[5,6].

However, that association was not perfect as many individuals in the group that remained free from AML still had more than one mutation. Our data from sequencing single CFUs of individuals with more than one mutation allowing us to distinguish CH cases with clonal evolution (subclonality) from those with multiple independent clones might provide a framework to refine the association between the number of driver mutations per individual and the risk for progression into AML. While subclonality leads to fewer clonal cells but accumulation of multiple mutations within the same subclone, un-related clones lead to more clonal cells but less mutational burden per clone. It is tempting to speculate that the presence of clonal evolution (i.e. higher mutational burden per clone) is a better predictor for the risk of malignant transformation than the actual number of driver mutations per individual.

Along similar lines, higher VAFs as measured in PBMCs are generally associated with a higher risk of AML transformation[5,6]. However, we observed a case with disproportionate clonal representation when comparing VAFs in granulocytes and percentage of mutation-positive CFUs. While it is possible that driver mutations may just enhance the ability to from CFUs *in vitro*, it is tempting to speculate that the mutational burden within HSPCs may be underestimated by measuring VAFs in granulocyte or PBMC DNA only. Mechanistically, some mutations, despite inducing expansion on the HSPC level, may also confer a lineage maturation defect during the transition from HSPCs to mature progeny. Alternatively, mutations could also lead to an increased turn-over of mature progeny via a propensity to undergo apoptosis. Either scenario might be associated with a different risk for malignant progression. Future mechanistic studies are therefore needed to further substantiate these findings.

We determined the lineage penetration of CH mutations in FACS-purified granulocytes, monocytes, B and T cells. The extent of lineage penetration (uni-, bi-, tri-, multilineage penetration) was different between samples with a trend for multilineage penetration occurring more often in samples with higher VAFs. Consistent with previous findings[42,43] , we observed significantly higher VAFs in myeloid cells (i.e. granulocytes and monocytes) relative to B and T lymphocytes, whereas another recent study had reported equal VAFs in myeloid and lymphoid compartments[41].

Unexpectedly, we observed telomere length maintenance in all but one of the analyzed individuals with CH when comparing telomere lengths between single CFUs carrying mutations and wild-type CFUs. The importance of this intriguing finding remains to be determined in future studies. Yet, clonal expansion of HSPCs can be either driven by increased survival without concomitantly enhanced proliferation or by increased proliferation. While the former scenario does not require telomere maintenance, in the latter scenario of increased proliferation, telomeres may need to be actively maintained to prevent telomere attrition. Again, it is tempting

to speculate that different driver mutations might have distinct requirements either for telomerase complex activity or alternative mechanisms of telomere lengthening (ALT) resulting in a variable risk of disease progression. Accordingly, a recent study found that loss of *Dnmt3a* – the most commonly mutated gene in CH – in murine HSPC leads to their immortalization and indefinite transplantability in serial transplantation assays in mice[48]. Given that telomere shortening limits HSPC transplantability[49] in conjunction with findings that *Dnmt3a* regulates telomere biology[50], our data provides a first but still speculative hint that at least in the case of *DNMT3A*-mutant CH the telomere maintenance machinery or alternative mechanisms of telomere lengthening (ALT) might become activated to prevent telomere attrition.

In summary, our analysis of a cohort of long-term survivors of allo-HSCT and their sibling donors demonstrate that clonal hematopoiesis is a highly prevalent condition in both donors and recipients of allo-HSCT. These findings should prompt future clinical trials prospectively investigating its clinical impact on the outcome of allo-HSCT but also basic studies elucidating HSPC biology and pathomechanisms of leukemic transformation.

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Authorship Contributions

S.B. and C.M.W. devised, performed and analyzed experiments, and wrote the manuscript; J.S. analyzed experiments, and wrote the manuscript; F.B, M.S.V.F., E.G. and E.B. performed experiments; U.S., R.S., B.L.E., B.M.F., C.G., C.B., N.B., and T.H.B. devised experiments and discussed data; and M.G.M. directed the study and wrote the manuscript.

Conflict of Interest Disclosures

B.L.E. has received research funding from Celgene and Deerfield. He has received consulting fees from GRAIL, and he serves on the scientific advisory boards for and holds equity in Skyhawk Therapeutics and Exo Therapeutics. All other authors declare no competing financial interests.

Data Sharing

Original data are available in NCBI SRA under accession number PRJNA612593.

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Tables

Table 1. Demographic and clinical annotation of study cohort

Variable	Study participants (n=84)	Donors (n=42)	Recipients (n=42)
Patient-related variables			
Age, median years (range)			
at transplantation / donation	38 (15-65)	37 (15-65)	39 (16-58)
at study inclusion	59 (29-95)	57 (29-95)	61 (32-77)
Time since transplantation, median years (range)	16 (10-32)		
Gender			
Male	45	22	23
Female	39	20	19
Disease			
AML			17
MDS			1
CML			6
ALL			8
NHL			3
MM			2
SAA			2
non-malignant			3
Transplantation-related variable			
Graft type			
PBSC	14		
BM	28		

Figure legends

Figure 1. Characteristics of somatic mutations found in allo-HSCT cohort.
(A) Schematic outline depicting study design and analyses performed. **(B)** Spectrum of somatic mutations found in donors and recipients of allo-HSCT. **(C)** Number of individuals with ≥ 1 mutation. **(D)** Type of mutations. **(E)** Distribution of types of base pair changes for single nucleotide variants. **(F)** Variant allele frequencies (VAFs) in the total cohort, and in donors and recipients, respectively. Mann-Whitney test was used to calculate statistical significance (ns, non-significant).

Figure 2. CH in donors and recipients of allo-HSCT.
(A) Proportion of study participants affected by CH in total cohort, donors, and recipients, respectively. **(B)** Age at study inclusion of study participants affected by CH in total cohort, donors, and recipients, respectively. Mann-Whitney test was used to calculate statistical significance.

Figure 3. Donor-engrafted CH in allo-HSCT.
(A) Proportion of donor-recipient pairs affected by donor-engrafted CH. **(B)** Variant allele frequencies (VAFs) in individual donor-recipient pairs of cases of donor-engrafted CH. (D and R denote donor and recipient, respectively). **(C)** Pooled analyses of variant allele frequencies (VAFs) in donor-recipient pairs with of donor-engrafted CH. (D and R denote donor and recipient, respectively). Wilcoxon matched-pairs signed rank test was used to calculate statistical significance. **(D)** Donor age at transplantation for cases of donor-engrafted CH. Mann-Whitney test was used to calculate statistical significance. **(E)** Case example of donor-engrafted CH progressing to myelodysplastic syndromes. (ns, non-significant).

Figure 4. Quantitative representation of CH clones at different levels of the hematopoietic hierarchy.
(A) Sanger sequencing electropherograms exemplifying detection of missense, nonsense, and frame-shift mutations in individual CFUs from PB $CD34^+$ cells. **(B)** Correlation between percentage of detected mutation-positive CFUs by Sanger and percentage of predicted mutation-positive CFUs based on VAFs. **(C)** VAFs in granulocytes, monocytes, B and T cells in donors and recipients shown as a heat map. (D and R denote donor and recipient, respectively) **(D)** VAFs in granulocytes, monocytes, B and T cells in donors and recipients (box and whisker

plot). Wilcoxon matched-pairs signed rank test was used to calculate statistical significance. (ns, non-significant).

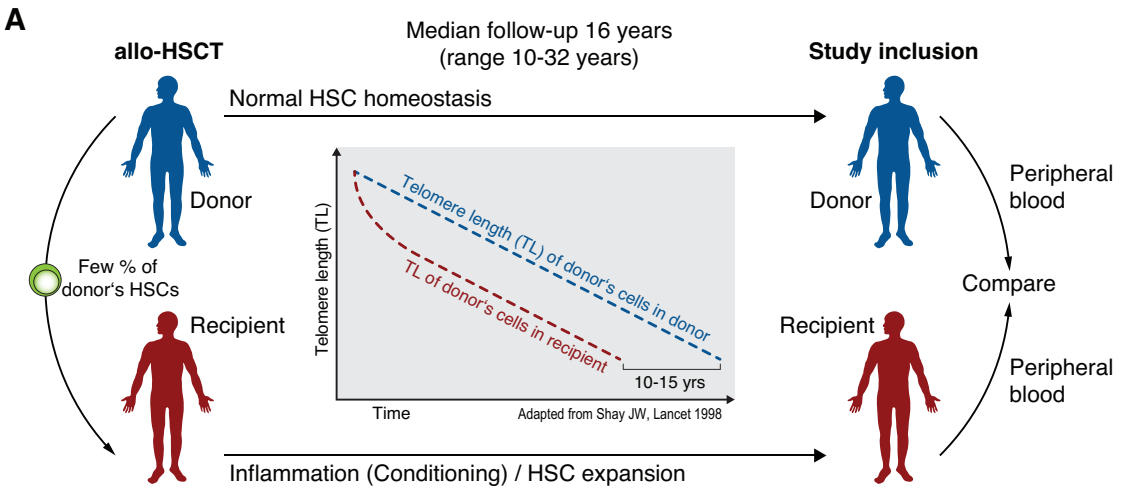
Figure 5. Clonal architecture in individuals carrying ≥ 1 CH mutation.

(A) Mutations in individual CFUs demonstrating cases of CH with subclonality. **(B)** Mutations in individual CFUs indicating cases of CH with independent clones. (D and R denote donor and recipient, respectively). n=95 single CFUs per individual affected by CH were analyzed for the presence of mutations by Sanger sequencing. Only mutated CFUs are depicted.

Figure 6. Telomere length in study participants and individual CFU from CH carriers.

(A) Telomere length in donors and recipients, respectively, as measured by MM-qPCR and depicted as T/S ratio. **(B)** Telomere length difference between individual donor-recipient pairs translated into years of pre-mature ageing. **(C)** Telomere length in individuals with or without CH based on donor or recipient status. **(D)** Schematic outline demonstrating experimental workflow for identification of wild-type and mutated CFU by Sanger sequencing followed by telomere length measurement using MM-qPCR. **(E)** Telomere length in individual wild-type or CH mutation-positive CFUs (single individuals affected by CH, upper panel; donor-recipient pairs with mutations in both donor and recipient, lower panel). Mann-Whitney test was used to calculate statistical significance.

Figure 1



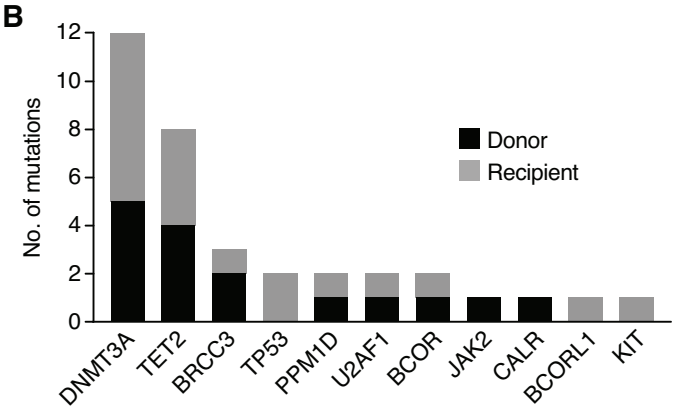
Read-outs

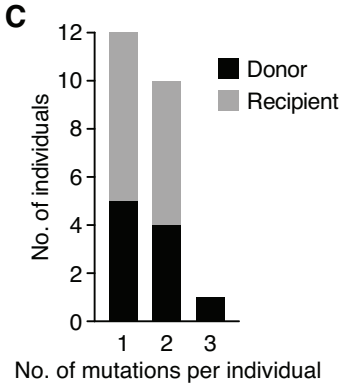
Targeted NGS on granulocyte DNA using 102-gene panel and unique molecular identifiers (UMIs)
→ *Figure 1-3*

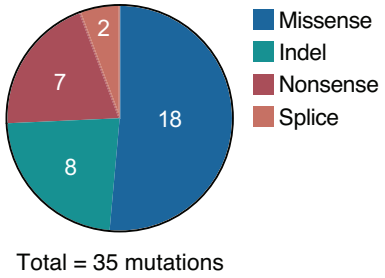
Targeted ultra-deep amplicon NGS on granulocyte, monocyte, B and T cell DNA
→ *Figure 4*

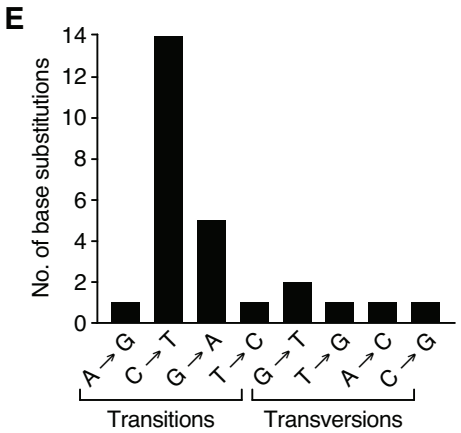
Clonogenic (CFU) assays using PB CD34⁺ cells for mutation status (Sanger sequencing)
→ *Figure 4, 5*

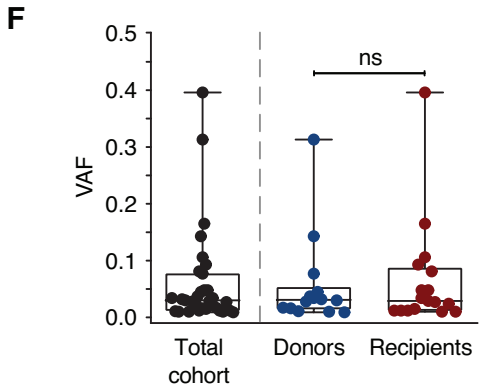
Telomere length analysis on DNA from granulocytes and CFUs
→ *Figure 6*





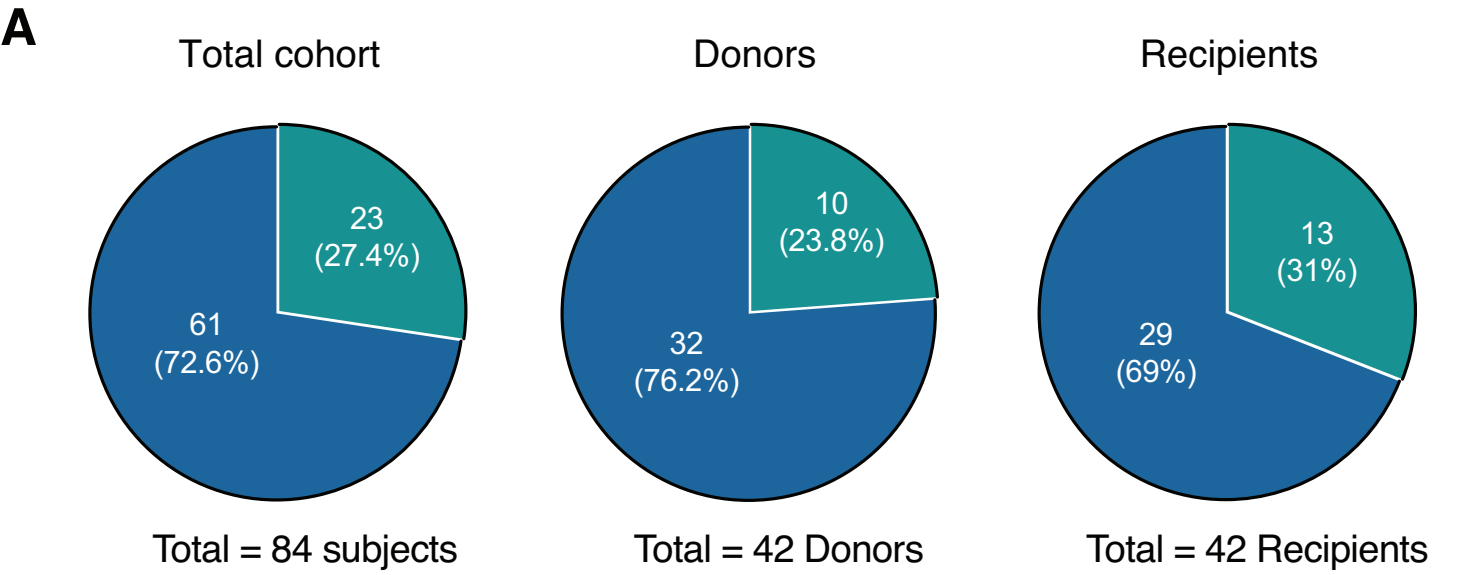






mean VAF	0.062	0.057	0.066
median VAF	0.030	0.031	0.029

Figure 2



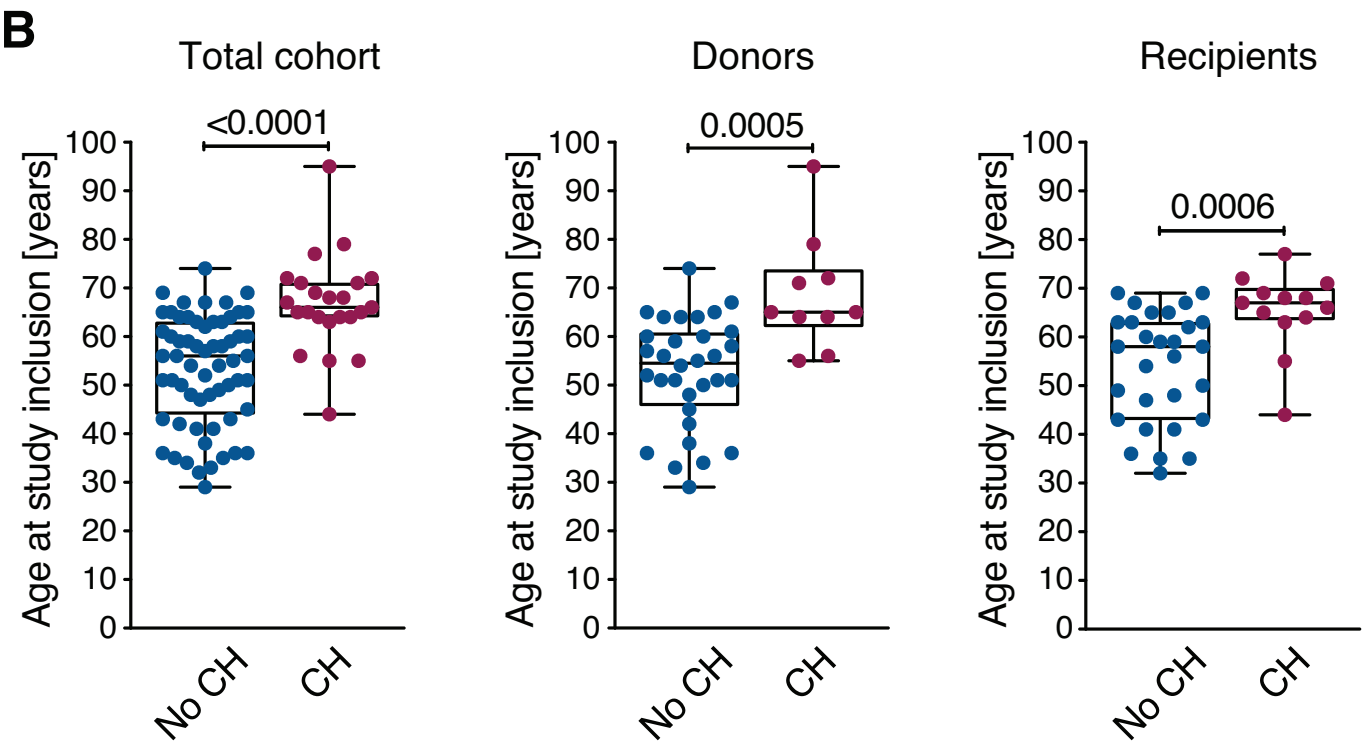


Figure 3

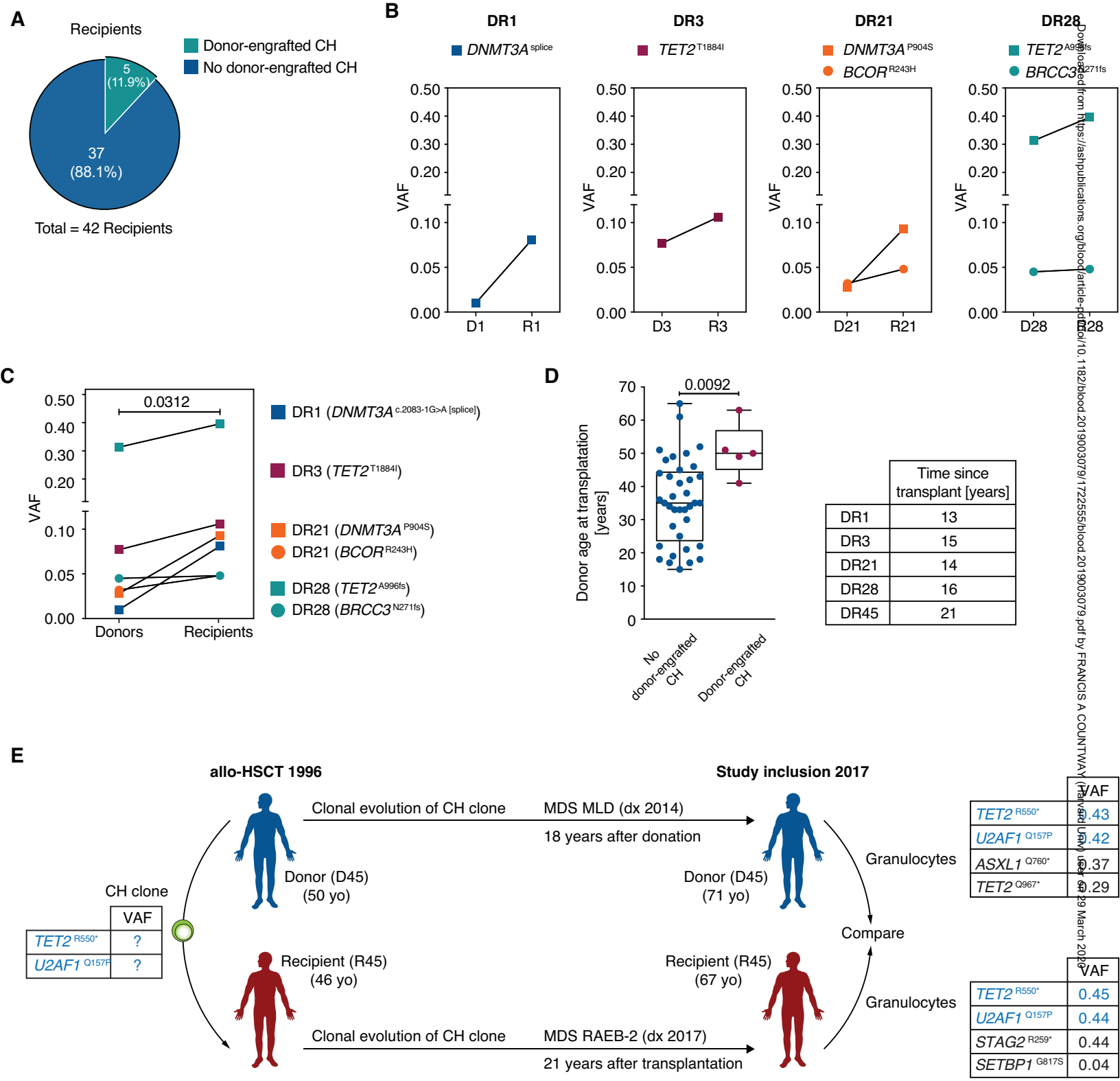
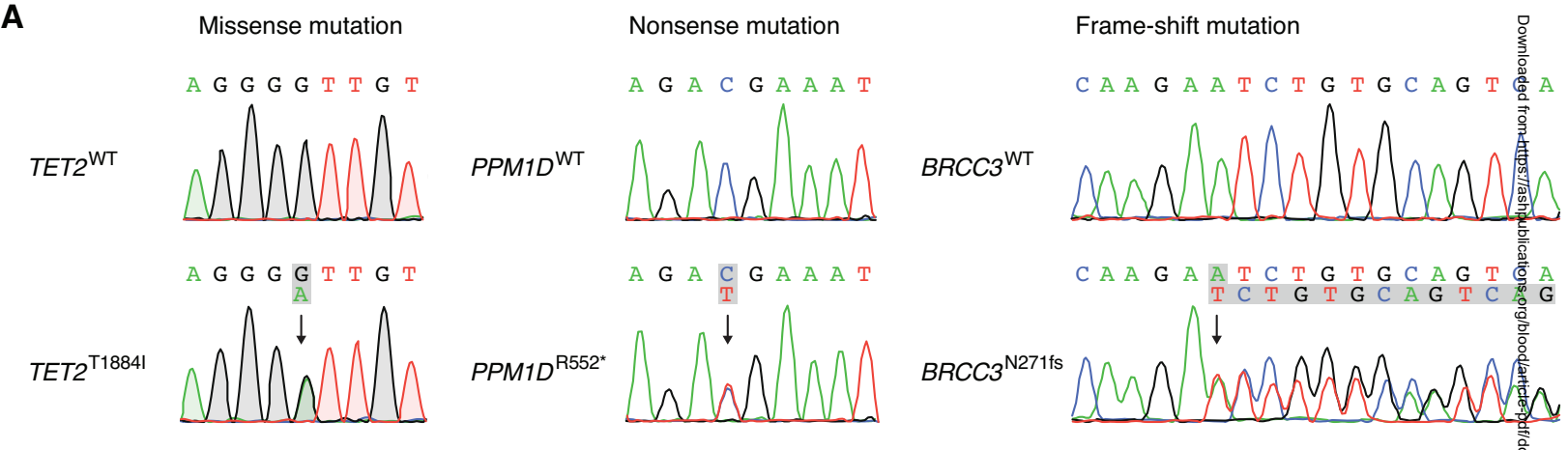
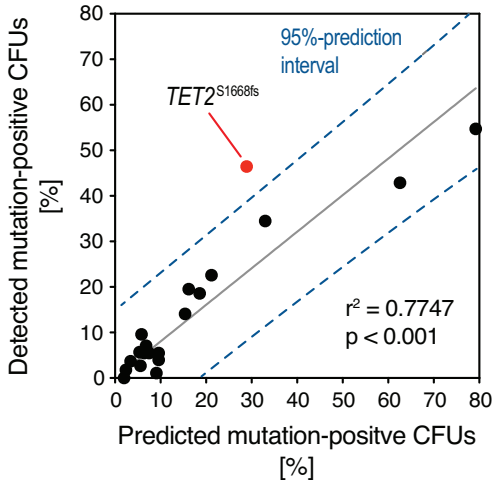


Figure 4





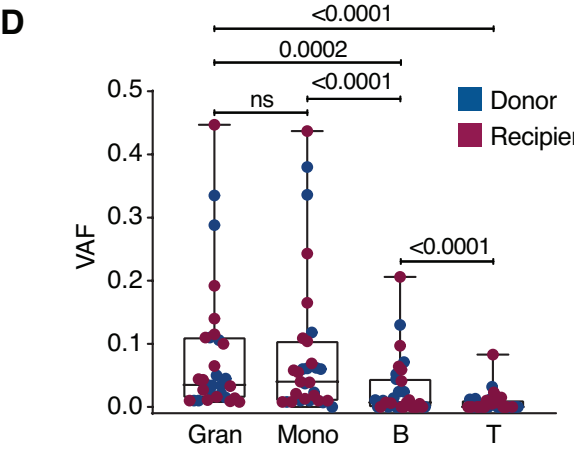
C

Donors

	Gran	Mono	B	T
D1_*DNMT3A*	0.010	0.060	0.000	0.000
D13_*DNMT3A*	0.021	0.007	0.000	0.012
D21_*DNMT3A*	0.034	0.060	0.052	0.000
D38_*DNMT3A*	0.010	0.010	0.014	0.000
D38_*DNMT3A*	0.015	0.010	0.000	0.000
D3_*TET2*	0.106	0.118	0.010	0.012
D28_*TET2*	0.335	0.380	0.130	0.013
D39_*TET2*	0.288	0.336	0.044	0.032
D7_*BRCC3*	0.035	0.062	0.071	0.000
D28_*BRCC3*	0.045	0.061	0.024	0.000
D39_*PPM1D*	0.050	0.023	0.011	0.000
D21_*BCOR*	0.034	0.037	0.024	0.000
D5_*JAK2*	0.019	0.008	0.000	0.000
D21_*CALR*	0.110	0.000	0.000	0.000

Recipients

	Gran	Mono	B	T
R1_*DNMT3A*	0.115	0.104	0.000	0.000
R7_*DNMT3A*	0.033	0.040	0.097	0.023
R10_*DNMT3A*	0.016	0.013	0.005	0.000
R18_*DNMT3A*	0.010	0.008	0.007	0.000
R18_*DNMT3A*	0.014	0.008	0.006	0.007
R21_*DNMT3A*	0.110	0.021	0.007	0.010
R34_*DNMT3A*	0.008	0.010	0.000	0.000
R3_*TET2*	0.140	0.165	0.064	0.015
R11_*TET2*	0.192	0.243	0.005	0.000
R28_*TET2*	0.447	0.437	0.206	0.083
R2_*TP53*	0.009	0.018	0.000	0.000
R14_*TP53*	0.011	0.010	0.000	0.000
R39_*PPM1D*	0.044	0.055	0.000	0.000
R28_*BRCC3*	0.065	0.058	0.059	0.008
R21_*BCOR*	0.043	0.039	0.011	0.000
R7_*BCORL1*	0.027	0.069	0.000	0.000
R1_*KIT*	0.100	0.109	0.041	0.000



	Gran	Mono	B	T
mean VAF	0.080	0.083	0.028	0.007
median VAF	0.035	0.040	0.007	0.000

Figure 5

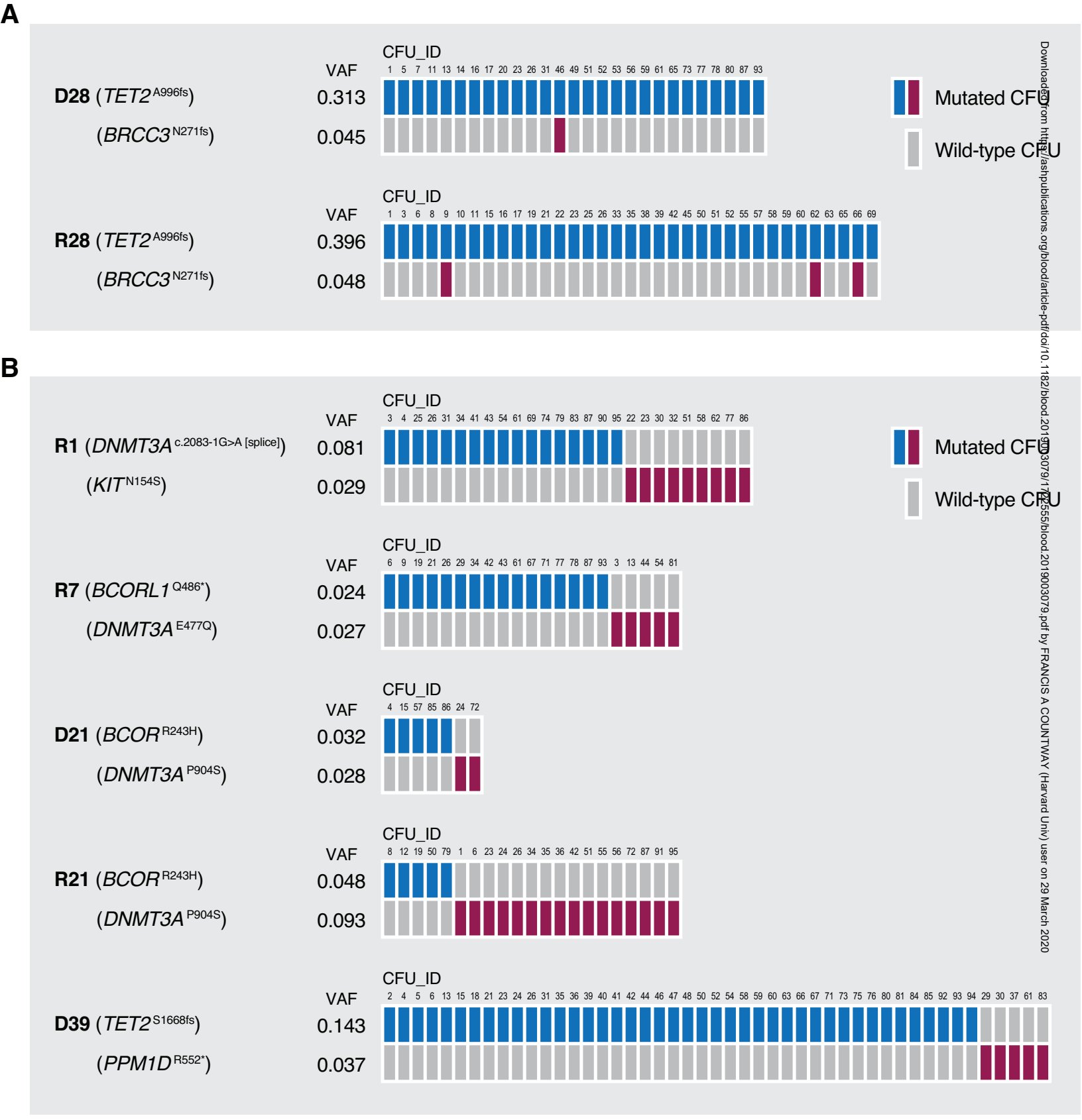


Figure 6