iPSC culture expansion selects against putatively actionable mutations in the mitochondrial genome

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SUMMARY

Therapeutic application of induced pluripotent stem cell (iPSC) derivatives requires comprehensive assessment of the integrity of their nuclear and mitochondrial DNA (mtDNA) to exclude oncogenic potential and functional deficits. It is unknown, to which extent mtDNA variants originate from their parental cells or from de novo mutagenesis, and whether dynamics in heteroplasmy levels are caused by inter- and intracellular selection or genetic drift. Sequencing of mtDNA of 26 iPSC clones did not reveal evidence for de novo mutagenesis, or for any selection processes during reprogramming or differentiation. Culture expansion, however, selected against putatively actionable mtDNA mutations. Altogether, our findings point toward a scenario in which intracellular selection of mtDNA variants during culture expansion shapes the mutational landscape of the mitochondrial genome. Our results suggest that intercellular selection and genetic drift exert minor impact and that the bottleneck effect in context of the mtDNA genetic pool might have been overestimated.

INTRODUCTION

Genomic instability of human induced pluripotent stem cells (iPSCs) that may cause loss of function and tumorigenic potential of their derivatives is considered as one major hurdle on the path toward clinical application (Andrews et al., 2017; Weissbein et al., 2014; Yoshihara et al., 2017). The majority of research addressing this issue is focusing on the investigation of genomic stability of the nuclear genome. However, a part of a cell’s genetic information, including 13 proteins of the electron transport chain (ETC) essential for oxidative phosphorylation (OXPHOS) and 24 RNAs, is encoded in the mitochondrial genome (Clayton, 1991). Mutations of those highly conserved genes are the cause of a variety of human diseases, especially affecting tissues with high energy demand (Park and Larsson, 2011).

The mitochondrial genome exhibits a polyploid set with a few hundred up to thousands of 16.6 kb large circular mitochondrial DNA (mtDNA) molecules that are continuously replicated independently of the cell cycle (Clayton, 1991). The existence of multiple mtDNA copies per cell allows a phenomenon called heteroplasmic, which describes the simultaneous existence of wild-type and mutated mtDNA molecules in a cell. Conversely, the state when only one mtDNA genotype is present in a cell is defined as homoplasmic. However, the mtDNA composition and mitochondria network are not fixed but subjected to permanent flux and also, during cell division, the segregation of mutated and wild-type mtDNA molecules to cell progenies can also be unequal. Hence, it is not surprising that, during reprogramming, clonal iPSC lines derived from the same parental cell population were observed to harbor different variants and heteroplasmy levels (Cherry et al., 2013; Folmes et al., 2013; Kang et al., 2016; Perales-Clemente et al., 2016; Prigione et al., 2011; Yokota et al., 2015; Zambelli et al., 2018). This unequal segregation of heteroplasms during reprogramming might arise from three sources acting on an inter- and intracellular level with different forces depending on the external conditions, namely (1) de novo mutations (Deuse et al., 2019; Kang et al., 2016; Prigione et al., 2011), (2) genetic mosaicism in parental cell population leading to genetically distinct iPSC clones, and (3) random allele drift during genetic bottleneck (Floros et al., 2018; Perales-Clemente et al., 2016; Zambelli et al., 2018). Several recent studies report nuclear reprogramming as the cause of de novo mtDNA mutations in iPSCs (Deuse et al., 2019; Kang et al., 2016; Prigione et al., 2011). However, Payne et al. (2013) introduced the concept of “Universal Heteroplasmy” demonstrating that mosaicism of heteroplasmic mtDNA variants in somatic cells, albeit at low levels (<1%), appears to be a universal finding among healthy individuals. Accordingly, many reports show evidence that the majority if not all mtDNA variants in iPSCs are pre-existing in individual somatic parental cells and arise from this mosaicism in the corresponding parental cell population (Perales-Clemente et al., 2016; Zambelli et al., 2018).
During reprogramming, somatic mitochondria are largely replaced by immature mitochondria resembling organelle morphology and distribution in embryonic stem cells (ESCs), and metabolism is switching toward glycolysis (Ma et al., 2015; Prigione et al., 2010). During this process, mtDNA copy number per cell is reduced, which is assumed to lead to a genetic bottleneck (Cao et al., 2009; Hamalainen et al., 2013). Such a reduction in mtDNA pool increases the effect of random genetic drift on mtDNA segregation within a cell and between cell offspring during cell division (Aryaman et al., 2019; Roze et al., 2005). However, at the same time this bottleneck might expose mutations to selective forces, and subtle selective pressures can exert maximal impact (Floros et al., 2018; Hamalainen et al., 2013; Roze et al., 2005; Wei et al., 2019). As of yet, it is not understood to what extent the segregation is driven by random allele drift or selection (Zambelli et al., 2018).

In contrast to the nuclear genome, selection on mutated mtDNA molecules can act on an intracellular or intracellular level. On the intracellular level, genetically encoded inequalities in cell fitness of parental cells can lead to eliteness of cells to attain iPSC state and their dominance in the reprogramming niche (Kosanke et al., 2021; Shakiba et al., 2019). However, high mutational burden or pathogenic mutations in specific mtDNA regions can also hinder reprogramming (Floros et al., 2018; Hung et al., 2016; Kang et al., 2016; Latorre-Pellicer et al., 2016; Wahlestedt et al., 2014; Yokota et al., 2015).

On the intracellular level, certain mtDNA variants can be selected (Latorre-Pellicer et al., 2016). As mtDNA molecules are uniparentally inherited and the mutation rate is 6- to 20-fold higher than that of genomic DNA (Naue et al., 2015), theoretically, without a repair mechanism, mtDNA would continuously accumulate mutations ultimately ending in a “mutational meltdown” called Muller’s ratchet (Chinnery and Prudent, 2019; Floros et al., 2018). To avert this, during female germline development and early embryogenesis, fragmentation of mitochondria and mito-
passage as subjected to reprogramming (mean p4.5) was sequenced. Analysis of mtDNA variants in iPSCs and ECs revealed three groups. Group A, non-transmitted variants, includes variants detected only within the parental cell populations that were not transmitted to any iPSC clone of the representative donor (detection limit allele frequency [AF] 0.02). In contrast, groups B and C contain transmitted variants with group B variants being homoplasmic in iPSC clones, and their corresponding parental cell populations and group C variants being heteroplasmic and detected in one iPSC clone per donor only (Figure 1B; Tables 1 and S1). The heteroplasmies are defined for every

Figure 1. Characterization of mtDNA copy numbers and mtDNA variants in iPSCs and their parental cells

(A) Average mtDNA copy number per cell in parental endothelial cells (ECs), EC-derived iPSC clones, and ESC lines. EC N = 10 donors; iPSC N = 26 early passage clones; ESC N = 3 lines; each with 1–5 biological replicates. One-way ANOVA with Tukey’s multiple comparison test.  
(B) Corresponding allele frequencies (AFs) of all variants in iPSC clones and parental cell populations detected by mtDNA sequencing of 26 early passage iPSC clones and their corresponding 10 parental cell populations. Group A non-transmitted variants were detected only in parental cell populations (AF > 0.02). Group B homoplasmic variants in parental cell population and iPSCs derived thereof. Group C heteroplasmic variants detected in an iPSC clone with AF > 0.02 but less frequent in parental cell population. Category 1 comprises haplotype variants, category 2 polymorphisms defined by a population frequency ≥ 0.02, and category 3 variants that are specific to a donor. N = 10 donors.  
(C) Proportion of variant groups and categories of all variants. N = 10 donors.  
(D) Average number of variants per group and category in iPSC clones and/or corresponding parental cell population of neonatal and aged donors. Neonatal donors N = 6 donors with 16 iPSC clones; aged donors N = 4 donors with 10 iPSC clones. Displayed is mean with SD. *Group C (heteroplasmic) variants with heteroplasmy level above 10% (AF > 0.1) in iPSCs.  
(E) Effect of variant on gene functionality was predicted based on a consensus of in silico prediction algorithms (snpEff impact, CADD, Condel, and HmtVar). Graph displays ratio of variants with neutral and putatively actionable prediction within group A (non-transmitted), group B (homoplasmic), and group C (heteroplasmic). N = 10 donors.
variant separately as the percentage of variant allele count per total allele count at the variant locus (= AF × 100). Although, in most cases the AF of those variants within the parental cell population was below the general detection limit of 0.02, careful assessment of variants within their genomic context taking local error rates in account allowed us to confirm pre-existence of more than half of the variants with statistical confidence (p = 0.05) (Table S2) (see experimental procedures). We could not prove pre-existence of the other half of variants with statistical confidence (Table S2, indicated by value in brackets), but previous reports also support our assumption that most of these variants arose from the parental cell population (Perales-Clemente et al., 2016; Zambelli et al., 2018).

Variants of each group can be further categorized: category 1, haplotype variants, are variants that define the haplogroup of every donor. Category 2 variants comprise polymorphisms defined by a population frequency of the variant (NCBI GenBank frequency [GB]) > 0.02. Category 3 variants are unique to a donor and rare in human populations and, are, therefore, called hereafter donor-specific variants (Tables 1 and S1).

Group B (homoplasmic) variants, mainly haplotype variants or polymorphisms (category 1 and 2), represent the vast majority of all variants (>87%) detected in mtDNA. Almost 9% of variants in iPSCs were heteroplasmic (group C) variants that were typically rare among the parental cell population and mainly categorized as donor specific (category 3) (Figure 1C; Table 1).

In contrast to Kang et al. (2016), but in accordance with Payne et al. (2013), we did not observe a dependency of overall mitochondrial mutational load of iPSCs with donor age (Figure 1D; Table 1), which might be partly attributed to different source tissue. Moreover, on average, aged donors tended toward a slightly higher number of group A (non-transmitted) and C (heteroplasmic) variants, indicating higher mosaicism as reported by previous studies (Naue et al., 2015).

Next, we analyzed the functional consequences of variants to evaluate to which extent clonal eliteness or negative selection might impact their transmission from parental cells to iPSC clones. Variant effect prediction based on a consensus of in silico prediction algorithms revealed that all group B (homoplasmic) variants and 90% of group A (non-transmitted) variants are neutral (Figure 1E). Strikingly, almost 48% of the group C (heteroplasmic) variants in iPSCs are putatively actionable, suggesting that mtDNA segregation during reprogramming does not act selectively against potentially damaging mutations. However, while we cannot exclude positive selection, we also found no direct evidence for enrichment of individual mutations or any mutational hotspot in specific genes during reprogramming.

### Table 1. Number of mtDNA variants in iPSC clones and corresponding parental cell populations

| Donor | Haplogroup | Polymorphisms (cat. 2) | Donor specific (cat. 3) | Polymorphisms (cat. 2) | Donor specific (cat. 3) | Polymorphisms (cat. 2) | Donor specific (cat. 3) |
|-------|------------|------------------------|-------------------------|------------------------|-------------------------|------------------------|-------------------------|
| D#1   | hUVEC     | H7a1                   | 0                       | 0                      | 9                       | 3                      | 0                       | 1                       |
| D#2   | hUVEC     | H1c3                   | 0                       | 0                      | 10                      | 5                      | 1                       | 0                       |
| D#3   | hUVEC     | H2a1c                  | 0                       | 1                      | 7                       | 3                      | 0                       | 2                       |
| D#4   | hUVEC     | J1c8a                  | 0                       | 0                      | 28                      | 3                      | 1                       | 1                       |
| D#5   | hUVEC     | J1b1a3                 | 0                       | 0                      | 34                      | 8                      | 1                       | 0                       |
| D#6   | hUVEC     | H87                    | 0                       | 0                      | 7                       | 4                      | 0                       | 0                       |
| Neonatal donors mean | 0.0 | 0.2 | 15.8 | 4.3 | 0.5 | 0.5 | 0.8 |
| D#31  | hSVEC     | H1c2                   | 4                       | 0                      | 9                       | 4                      | 0                       | 0                       |
| D#37  | hSVEC     | H10b                   | 0                       | 0                      | 8                       | 3                      | 0                       | 4                       |
| D#38  | hSVEC     | J3                     | 3                       | 0                      | 32                      | 4                      | 0                       | 1                       |
| D#40  | hSVEC     | J1c5a                  | 1                       | 1                      | 29                      | 4                      | 0                       | 0                       |
| Aged donors mean | 2.0 | 0.3 | 19.5 | 3.8 | 0.0 | 1.3 | 2.0 |
| Total | mean      | 0.8                    | 0.2                     | 17.3                   | 4.1                     | 0.3                    | 0.8                     | 1.3                     |
| sum   |           | 8                      | 2                       | 73                     | 23                      | 3                      | 8                       | 13                      |
Notably, the heteroplasmic level of the group C (heteroplasmic) variants was generally low (below 10%; AF < 0.1) in iPSCs with the exception of five variants (Figure 1B, marked with *). While two of these five variants were neutral polymorphisms with very high heteroplasmic levels (>90% [AF > 0.9]) in individual iPSC clones, the other three variants were putatively actionable donor specific with intermediate heteroplasmic levels of 16%–38% (AF 0.16–0.38) in single iPSC clones, affecting NADH dehydrogenase (ND) ND4 and ND5 genes of the ETC complex I. Interestingly, complex I, in particular ND5, is frequently mutated in diverse diseases and cancer (Copeland et al., 2002). However, our data do not allow any conclusion whether the affected iPSC clones simply represent stochastic events, where iPSC clones were derived from rare parental cell clones with increased heteroplasmic levels, or whether positive selection processes occurred. Anyway, we could not observe altered mitochondrial features or metabolic functionality of the ND5 mutations in affected iPSC clones (D#3 hUVEC C2 with 38% m.13099G > A and D#37 hSVEC C10 with 23% m.12686T > C) (Figure S2, marked with *). Compared with their sister clones (D#3 hUVEC C1 and D#37 hSVEC C4) derived from the same parental cell population. All iPSC clones displayed a similar proliferation rate measured as population doubling, mtDNA copy number per cell, specific yield coefficient of lactate per glucose molecule, mitochondrial content, mitochondrial membrane potential, and reactive oxygen species (ROS) content, as well as similar expression of genes involved in metabolism (mTOR, ND5, PDK1, PGC1a, PGC1b, PRKAA1) (Figure S2). Also, genes of mtDNA replication and transcription (POLG2, TEAM, NRF1), mitochondria dynamics (MFN1, OPA1, DNML1), and mitophagy (PINK1, PRKN) were not differentially expressed between non-mutated and mutated iPSC clones, although they were overall significantly higher expressed in iPSCs compared with their parental cells (Figure S2F). As iPSC metabolism is significantly higher expressed in iPSCs compared with their parental cells (Figure S2F), as iPSC metabolism is not predominantly based on OXPHOS and the heteroplasmic rates were only 23% and 38%, this result is not unexpected and, in accordance to observations of previous work (Hamalainen et al., 2013; Perales-Clemente et al., 2016; Wahlestedt et al., 2014; Yokota et al., 2015), also decreases the likelihood of a clonal eliteness on the intercellular level during reprogramming.

**Prolonged culture expansion leads to clearance of putatively actionable mtDNA mutations**

There are contradictory reports regarding the propagation of mtDNA mutations during prolonged culture. While some studies report increase and dominance (Deuse et al., 2019; Maitra et al., 2005; Zambelli et al., 2018), other studies observed a reduced proportion of mutated mtDNA during culture (Cherry et al., 2013; Holmes et al., 2013; Perales-Clemente et al., 2016). Seven of the iPSC clones that had been analyzed in an early passage were also sequenced in later passages p30 and p50 to trace the dynamic of mtDNA heteroplasms during culture expansion.

It is noteworthy that 86% of the group C (heteroplasmic) variants detected in later passages were also detectable in early iPSC passages and/or the corresponding parental cell population with statistical confidence (p = 0.05). The remaining variants were apparently present in the parental cells and early passages, too, but could not be confirmed with statistical confidence (p = 0.05). Hence, although we cannot exclude it, we also did not observe substantial evidence for de novo mutagenesis during prolonged culture expansion (Table 2).

Investigation of the regions affected by mutations showed that around 60% of group C (heteroplasmic) variants were located in coding regions (Figure 2A, black) and, therefore, affected coding regions more frequently than group A (non-transmitted) and group B (homoplasmic) variants. Furthermore, compared with group A and B variants which constitute almost entirely nucleotide transitions (transition/transversion ratio [Ts/Tv] > 30), group C (heteroplasmic) variants are characterized by lower T > C transitions compared with C > T and by higher proportion of transversion events (Ts/Tv 3.4) (Figure 2B). Both C > T and T > C substitutions are described as the predominant type of substitution in homoplasmic variants originating from germline transmission (Naue et al., 2015; Wei et al., 2019). The mutational signatures of heteroplasmic group C variants seems to be mainly shaped by the same mechanism; however, abundance of other substitution events suggest an additional origin, such as EC culture expansion or tissue mosaicism of those variants. Consequently, group C mutations would, therefore, never have been subjected to the control and repair mechanisms acting during mtDNA transmission in germ cells.

Depending on the dynamic of mtDNA mutation progression during culture expansion, all group C variants in iPSCs were divided into four groups, namely enriched, stable, depleted, and fluctuating variants (Table 2, illustrated by the changes in blue shades). While enriched variants are defined as such that increased in AF over iPSC expansion culture more than 10-fold (average 76-fold; range 11- to 250-fold), stable variants did not change their AF during iPSC expansion culture (average fold change between passages 1.03; range 0.67–1.49; SD 0.25). Depleted variants were reduced in AF from the early or intermediate passage to the late one by more than 10-fold (average 64-fold; range 15- to 225-fold) (Table 2). The group of fluctuating variants incorporates basically variants that might be also added to one of the above-mentioned groups, but as their fold change over culture only ranged from 2.7- to 8.8-fold (average 4.7; SD 2.2), we decided not to include them. A
last group of variants (named “nd in p30-50”) shown in Table 2 comprises those iPSC-enriched variants that were found in iPSC clones that were only analyzed at early passage.

Among the entirety of group C (heteroplasmic) variants (AF > 0.02), all enriched variants were predicted to be neutral as well as 83% of the stable variants. The group of fluctuating variants or those not analyzed in high passages (nd in p30-50) contained 43%–50% putatively actionable variants (Figure 3 A). Most interestingly, 88% of putatively actionable variants was observed among the depleted variants. Focusing only on group C (heteroplasmic) variants

### Table 2. Group C mtDNA variant heteroplasmy levels change during iPSC culture expansion

| Variants          | Consequence      | Gene Symbol | Donor iPSC clone | AF EC p6 | AF p30 | AF p50 | Fold change |
|-------------------|------------------|-------------|------------------|----------|--------|--------|-------------|
| **Enriched**      |                  |             |                  |          |        |        |             |
| m.1226G>C;T      | RNA coding       | MT-RNR1     | D#37 hSVeC       | C8       | 0.008  | 0.004  | 0.055      | 144         | 35.9 |
| m.1884G>A        | RNA coding       | MT-RNR2     | D#37 hSVeC       | C8       | 0.006  | 0.004  | 0.021      | 100         | 249.8 |
| m.3975C>A        | missense         | MT-ND1      | D#3 hUVEC        | C1       | 0.001  | 0.001  | 0.006      | 085         | 84.8 |
| m.10586G>A       | synonymous       | MT-ND4L     | D#3 hUVEC        | C1       | 0.003  | 0.005  | 0.010      | 083         | 16.5 |
| m.1594C>T        | synonymous       | MT-CYB      | D#37 hSVeC       | C10      | 0.008  | 0.016  | 0.082      | 0179        | 10.9 |
| m.16147C>T       | regulatory region | MT-NDC      | D#2 hUVEC        | C2       | 0.001  | 0.015  | 0.055      | 0877        | 58.5 |
| **Stable**       |                  |             |                  |          |        |        |             |
| m.72T>C          | regulatory region | MT-NDC      | D#3 hUVEC        | C2       | 0.181  | 0.929  | 0.999      | 1000        | 1.0 |
| m.297A>C         | regulatory region | MT-NDC      | D#37 hSVeC       | C4       | 0.013  | 0.022  | 0.025      | 039         | 1.1 |
| m.366G>A         | regulatory region | MT-NDC      | D#37 hSVeC       | C8       | 0.049  | 0.061  | 0.041      | 081         | 1.1 |
| m.5192A>C        | synonymous       | MT-ND2      | D#3 hUVEC        | C1       | 0.013  | 0.019  | 0.026      | 022         | 1.0 |
| m.5894_5895insC  | regulatory region | MT-NDC      | D#37 hSVeC       | C10      | 0.012  | 0.979  | 0.981      | 0853        | 1.0 |
| m.13099G>A       | missense         | MT-NDC      | D#3 hUVEC        | C2       | 0.002  | 0.374  | 0.376      | 0423        | 1.0 |
| **Depleted**     |                  |             |                  |          |        |        |             |
| m.8964C>T        | synonymous       | MT-ATP6     | D#38 hSVeC       | C9       | 0.001  | 0.061  | 0.008      | 004         | 14.5 |
| m.1094C_10947insC| frameshift       | MT-NDC4     | D#37 hSVeC       | C8       | 0.006  | 0.155  | 0.021      | 010         | 15.9 |
| m.12020C>T       | missense         | MT-NDC4     | D#37 hSVeC       | C10      | 0.011  | 0.026  | 0.014      | 001         | 23.0 |
| m.12687T>C       | missense         | MT-NDC5     | D#37 hSVeC       | C10      | 0.002  | 0.225  | 0.087      | 001         | 225.1 |
| m.15575G>A       | missense         | MT-CYB      | D#37 hSVeC       | C4       | 0.010  | 0.038  | 0.014      | 001         | 38.5 |
| m.3800T>A        | missense         | MT-NDC1     | D#3 hUVEC        | C1       | 0.004  | 0.007  | 0.028      | 001         | 28.3 |
| m.10569G>A       | missense         | MT-NDC4     | D#3 hUVEC        | C2       | 0.003  | 0.012  | 0.104      | 005         | 22.5 |
| m.13218A>G       | missense         | MT-NDC5     | D#2 hUVEC        | C2       | 0.001  | 0.019  | 0.029      | 000         | 143.8 |
| **Fluctuating**  |                  |             |                  |          |        |        |             |
| m.4193T>C        | missense         | MT-NDC1     | D#3 hUVEC        | C4       | 0.032  | 0.044  | 0.023      | 016         | 2.7 |
| m.16226C>T       | regulatory region | MT-NDC      | D#37 hSVeC       | C4       | 0.003  | 0.091  | 0.015      | 019         | 4.8 |
| m.225G>T         | regulatory region | MT-NDC1     | D#3 hUVEC        | C2       | 0.010  | 0.014  | 0.038      | 010         | 3.8 |
| m.5450C>T        | synonymous       | MT-ND2      | D#3 hUVEC        | C2       | 0.010  | 0.029  | 0.034      | 085         | 2.9 |
| m.6316A>C        | missense         | MT-CO1      | D#37 hSVeC       | C4       | 0.013  | 0.022  | 0.029      | 061         | 2.7 |
| m.8680C>T        | missense         | MT-ATP6     | D#37 hSVeC       | C4       | 0.007  | 0.028  | 0.302      | 041         | 8.8 |
| m.12147G>C       | RNA coding       | MT-TV       | D#6 hUVEC        | C5       | 0.001  | 0.021  |            |             |     |
| m.1602C>T        | RNA coding       | MT-TV       | D#6 hUVEC        | C5       | 0.001  | 0.021  |            |             |     |
| m.6255G>A        | missense         | MT-CO1      | D#31 hSVeC       | C1       | 0.001  | 0.054  |            |             |     |
| m.8816A>T        | missense         | MT-ATP6     | D#31 hSVeC       | C1       | 0.009  | 0.076  |            |             |     |
| m.11571T>C       | missense         | MT-NDC4     | D#4 hUVEC        | C3       | 0.003  | 0.025  |            |             |     |
| m.13077C>T       | synonymous       | MT-NDC5     | D#1 hUVEC        | C5       | 0.004  | 0.031  |            |             |     |
| m.16189T>C       | regulatory region | MT-NDC      | D#4 hUVEC        | C1,C3    | 0.072  | 0.025  |            |             |     |

| Effect prediction: | Allele frequencies: |
|--------------------|---------------------|
| Putatively actionable | 0.02-0.05          |
| Neutral             | 0.05-0.1            |
| 0.1-0.5             |                     |
| 0.5-1               |                     |

( ); existence of variant in parental cell population or iPSC clone not confirmed with statistical confidence (p > 0.05).
with intermediate heteroplasmy levels (AF > 0.1, which equals an averaged heteroplasmy level of >10%), yielded similar results, although the sample size of such was quite small (Figure 3B). In general, donor age did not affect the genetic stability or clearance mechanisms as, in all groups (enriched, stable, depleted, and fluctuating), heteroplasmic group C variants are equally apportioned in neonatal (hU-VEC) and aged (hSVEC) donors (Table 2). Furthermore, karyotype analysis performed for D#3 hUVEC C2, D#37 hSVEC C10, and control clone D#3 hUVEC C1 demonstrated that clones maintained normal karyotypes over time in culture (Table S3). However, we did not analyze for smaller chromosomal aberration.

It is assumed that iPSCs are subjected to a genetic bottleneck when the mtDNA copy number per cell is reduced in the pluripotent state compared with somatic cells. It is still controversially discussed, however, whether random genetic drift during this bottleneck or selection processes are responsible for the observed changes in heteroplasmy levels during prolonged iPSC culture expansion (Aryaman et al., 2019; Hamalainen et al., 2013). Notably, we did not observe any strong reduction in the mtDNA copy number in iPSCs compared with ECs of the parental cell populations (Figure 1A). Furthermore, the mtDNA copy number per iPSCs stayed constant during prolonged iPSC expansion culture (Figure 3C).

Here, we applied a neutral model of mtDNA genetic dynamics of Aryaman et al. (2019) to understand how distribution of heteroplasies evolves over time without selection. Therefore, this model was utilized as a null hypothesis to assess whether random genetic drift by itself is sufficient to explain the observed changes in heteroplasmy levels, or whether additional selection factors play a role. The model of Aryaman et al. was developed for prediction of a possible change in heteroplasmy level of one mutated allele in a post-mitotic cell with mtDNA undergoing turnover. This means that degradation and replication of mtDNA occurs to the same extent over time, allowing the ratio of mutated allele to fluctuate. Although our culture consists of mitotic iPSCs and requires frequent cell culture splitting, we applied this model here under the assumptions that (1) iPSCs experience symmetric division and equal segregation of all mtDNA molecules and (2) the effect of random gene drift during iPSC culture passaging is negligible. A more complex model predicting dynamics and selection in a unicellular life cycle with bottleneck and expansion in a multi-cellular organism (Roze et al., 2005) confirms that these assumptions prove valid for our population size (cells in culture before and after splitting). The model of Aryaman et al. includes, in addition, a fragmentation factor, which indicates the ratio of fused mitochondria. As unfused, punctuated mitochondria are a typical feature of iPSCs (Figure S1), we excluded this factor in our modeling. The rate of turnover of mtDNA molecules and also selective mitophagy is set in this model by the factor \( m \) (mitophagy rate). This factor is equivalent to a selection coefficient in other models (Roze et al., 2005).

Determination of mtDNA content has shown that the mtDNA copy number stayed constant over time during iPSC culture expansion (on average \( \sim 1,000 \) copies/cell) (Figure 3C). Thus, we simulated the possible heteroplasmy variance over time for three different heteroplasmy group C variants exemplarily for each group of enriched, stable, and depleted variants, assuming that cells contain 1,000 mtDNA molecules and no selection is acting (Figure 3D, green line). The variance in heteroplasmy level in cultured iPSCs increases with time according to random genetic drift. However, the modeling also indicates that expectable changes in heteroplasmy due to random genetic drift without computing an additional selection coefficient are very low even over the course of 50 iPSC passages.
Figure 3. Prolonged culture expansion selects against putatively actionable mtDNA mutations in iPSCs
Twenty-six iPSC clones at early passage (on average p6.5) and seven of them additionally at intermediate (p30) and late passage (p50) were analyzed by mtDNA sequencing. Heteroplasmic variants (group C) were divided into the groups of enriched variants (defined as >10-fold...
(Figure 3D, green line). Consequently, this result is consistent with the observation made in the group of stable group C variants, which proved to be unchanged over the course of time (fold change on average 1.03, Table 2). However, this simulation without additional selection factor cannot explain the observed increase or decrease in heteroplasmicy levels of the enriched or depleted variants. In contrast, incorporating a selection coefficient (included as the $\mu$ factor) in the model can explain our observation. For depleted variants, a selection coefficient of $\mu = 10–50$ (Figure 3D, dark and light red lines) was, in general, sufficient to simulate the reduction of heteroplasmies. In contrast, to reach the change in heteroplasmicy level observed by enriched variation, $\mu > 50$ would even be needed in most of the cases. Taken together, the analysis of the mtDNA copy number and modeling revealed two aspects: (1) the mtDNA copy number in iPSCs is high enough (no strong bottleneck in the pluripotent state) to prevent noteworthy effects by random genetic drift and (2) enrichment of neutral and clearance of putatively actionable group C (heteroplasmic) variants is likely caused by active selection during iPSC expansion culture.

**Dynamics of mtDNA copy number during differentiation does not change variant heteroplasmicy levels**

To investigate the development of heteroplasmicy levels during differentiation and the consequences of mtDNA mutations for differentiated iPSC derivatives, the two iPSC clones harboring intermediate level of putatively actionable NDS mutations (D#3 hUVEC C2 with 38% m.13099G > A and D#37 hSVEC C10 with 23% m.12686T > C) and their sister iPSC clones (D#3 hUVEC C1 and D#37 hSVEC C4) derived from the same parental cell population were differentiated into cardiomyocytes (CMs). Monitoring of the mtDNA copy number during the differentiation process, surprisingly, revealed a more complex dynamic than expected. Instead of a simple increase of mtDNA copies from iPSCs to CMs with higher metabolic demands, the general dynamic followed a process through three phases (Figure 4A). During the first phase (differentiation day [dd] 0 to dd1), starting with WNT pathway activation by CHIR99021 treatment for 24 h, mtDNA copy number per cell increased. After subsequent WNT pathway inhibition by Wnt-C59 for 48 h (dd1–dd3), mtDNA copy number per cell was reduced reaching its minimum around dd5 or dd6. After dd6 mtDNA copy number increased again to ~dd10. The mtDNA copy number from dd10 onward stayed unchanged with, on average, 1,532 mtDNA copies per cell (Figure 4A). During reprogramming, such a bottleneck effect is suspected to exert influence on mtDNA segregation. However, analysis of heteroplasmicy levels during the differentiation process by mtDNA sequencing at dd0, dd5, and dd15 of differentiation demonstrated that the heteroplasmicy level of all variants stayed constant during differentiation, with an average fold change of 1.00 (range 0.83–1.15; SD 0.08) (Figure 4B), a result supported by the observations of previous work (Hamalainen et al., 2013; Zambelli et al., 2018).

Moreover, the mtDNA copy number dynamic was not influenced by the presence of the NDS mutations in iPSC clones D#3 hUVEC C2 or D#37 hSVEC 37 C10 (Figure 4A, marked with *). Taking into account the relatively low AF, it is, however, not unexpected that differentiation efficiency, final mtDNA copy number in CMs, and the phenotype of the mitochondria network were similar in the iPSC clones with and without the respective NDS mutations (Figure S3). Similarly, metabolic features measured as mitochondrial content (MitoTracker), membrane potential (tetramethylrhodamine methyl ester [TMRM]), and ROS (Brite 670) in mutated relative to wild-type iPSC-derived CMs was not significantly affected (Figure S4A). Notably, gene expression quantification demonstrated that, surprisingly, NDS expression was not upregulated in iPSC-derived CMs (Figure S4B). Hence, beside the relatively low heteroplasmicy level of the mutations, additional low expression level of NDS could explain the absence of any molecular...
phenotype, at least under our differentiation conditions. Finally, bioartificial cardiac tissues (BCTs) (Figure S4C) were generated out of iPSC-derived CMs. Assessment of BCT diameter did not reveal any difference between tissues generated of CMs from different clones (Figure S4D).

However, the analysis of the functionality assessed by active forces of the BCTs demonstrated inter-clonal differences (Figures S4E and S4F). Overall, it seems that the m.13099G > A mutation in ND5 with a heteroplasmy level of 38% in D#3 hUVEC C2-derived CMs resulted in a higher active force of BCTs compared with BCTs generated from the sister iPSC clone D#3 hUVEC C1.

**DISCUSSION**

Random genetic drift, positive selection, or clearance of mutated mtDNA, are suspected to shape the mutational landscape of mtDNA in iPSCs, but to which extent and at what stage those partly contradicting processes might act is widely unknown. In any case, occurrence of mtDNA mutations that alter the functionality of iPSC derivatives would exclude their clinical application.

Here, we have analyzed mtDNA variants in iPSCs and their corresponding parental cell populations during reprogramming, prolonged culture, and differentiation. We neither observed a distinct bottleneck nor any selection for or against mtDNA variants during reprogramming. Overall, the number of mtDNA molecules per iPSC was similar to the one in parental ECs. All mtDNA variants detected in iPSC clones have also been detected at different levels in the parental cells, although not in all cases with statistical confidence. Thus, we did not observe any evidence for substantial *de novo* mutagenesis, and inter-clonal differences in iPSCs apparently arose from the mitochondrial variant mosaicism within the parental cell population.

In contrast, during prolonged culture of iPSCs the heteroplasmic level of variants changed, while the mtDNA copy number per cell stayed uniform. Importantly, modeling the effect of random genetic drift on heteroplasmies revealed that a neutral model without selection cannot explain the observed changes. Interestingly, the selection acts pivotally against putatively actionable mutations, while neutral variants are accepted or even enriched. On a functional level, no difference between sister iPSC clones with and without ND5 mutation at intermediate heteroplasmic level derived from the same parental cell population was observed. Although heterogeneity among single iPSCs or karyotypic abnormalities (Zambelli et al., 2018) might

**Figure 4. mtDNA content experiences reduction during cardiomyocyte differentiation without change in variant heteroplasmy levels**

Two iPSC clones (D#3 hUVEC C2 and D#37 hSVEC C10) each harboring a different putatively actionable mutation in ND5 (m.13099G > A and m.12686T > C, respectively) at intermediate heteroplasmic level and their sister iPSC clones generated from the same parental cell population were differentiated into cardiomyocytes (CMs). Mutated iPSC clones are marked with *. (A) Average mtDNA copy number per cell during CM differentiation. N = 1–2 differentiations. (B) At different time points during CM differentiation (differentiation day [dd] 0, dd5, and dd15), mtDNA was sequenced. Plot displays the dynamic of heteroplasmic levels during CM differentiation of heteroplasmic variants with AF > 0.02. N = 4 clones.

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have led to elimination of damaged cells or dominance of aberrant cell clones within the iPSC clone culture, this scenario is unlikely as simultaneously other variants (stable heteroplasmic group C variants) were maintained at a constant heteroplasmancy level. It is more likely that even minor functional impact of mutations on mitochondria level led to selective mitophagy of damaged mitochondria and mtDNA via PINK1 and Parkin (PRKN) pathways, similarly as described for mtDNA integrity maintenance during germline transition (Kandul et al., 2016; Latorre-Pellicer et al., 2016; Lieber et al., 2019). In particular, PRKN was expressed at significantly higher levels in iPSCs compared with the somatic parental cells. Hence, the clearance of mtDNA mutations is presumably rather executed by an intrinsic process on the intracellular level than on an intercellular level.

The finding that the heteroplasmacy levels of individual variants can increase by selection from the parental cell population or culture expansion highlights the need for careful consideration of variants with low heteroplasmacy level. Although low heteroplasmacy levels are generally regarded as not being clinically relevant, those levels could be augmented reaching the heteroplasmacy threshold and impact functionality. Moreover, the heteroplasmacy threshold at which a molecular phenotype manifests differs depending on variant and context (Sercel et al., 2021).

Importantly, our results demonstrate that, during targeted CM differentiation, the mtDNA copy number per cell is subjected to a distinct dynamic including an increase-reduction-increase cycle. Interestingly, this dynamic does not influence mtDNA average heteroplasmacy levels. This observation leads to two conclusions: (1) the heteroplasmacy level in iPSCs dictates the heteroplasmacy level of the iPSC-derived CMs and (2) the observed dynamics of the mtDNA copy number during the course of the CM differentiation seems to be not yet strong enough to cause unequal mtDNA segregation.

While we did not observe any functional differences between sister iPSC clones with and without ND5 mutation, CMs generated from D#3 hUVEC C2 with the m.13099G > A mutation at a heteroplasmacy level of 38% resulted in BCTs exhibiting higher active forces than BCTs generated from the non-mutated sister clone. Although we cannot exclude further inter-clonal differences between both clones (Mannhardt et al., 2020), reduced levels of mitochondrial proteins (including ND5) can lead to reduction of ATP/ADP ratio, mitochondrial membrane potential, and mitochondrial Ca\textsuperscript{2+} uptake, followed by increment in cytosolic Ca\textsuperscript{2+}, activation of different signaling pathways, and altered cardiac functions, including hypertrophic cardiomyopathy-specific electrophysiological abnormalities, and elevated force (Bonora et al., 2019; Laude and Simpson, 2009; Li et al., 2018; Mannhardt et al., 2020). Therefore, depending on the (culture) conditions and maturation stage, putatively actionable mtDNA variants, even at an intermediate heteroplasmacy level, may eventually alter the functional characteristics of iPSC derivatives as reported previously (Hamalainen et al., 2013; Klein Gunnewiek et al., 2020; Wahlestedt et al., 2014; Yokota et al., 2015).

In conclusion, our results suggest that bottleneck effects in iPSC cultures might have been overestimated in the context of the mtDNA genetic pool. Our findings point toward a scenario in which intracellular positive and negative selection of mtDNA molecules mainly shapes the mutational landscape of the mitochondrial genome in iPSCs, while other mechanisms, such as random genetic drift and intercellular selection, exert minor impact.

## EXPERIMENTAL PROCEDURES

### Reprogramming and iPSC culture

Derivation and culture of ECs, virus production, retroviral reprogramming, iPSC characterization, and cultivation were performed as described previously (Haase et al., 2009) (supplemental experimental procedures). In total, 26 EC-derived iPSC clones were derived from 10 donors.

### Total DNA extraction and determination of mitochondrial DNA copy number using quantitative real-time PCR

Total DNA was isolated using QIAamp DNA Blood mini kit (QIAGEN). Quantitative real-time PCR was performed on 25 ng total DNA per reaction in duplicates on Mastercycler ep realplex2 (Eppendorf) and with Absolute QPCR SYBR Green Mix (Thermo Scientific). The primer designing is described in the supplemental experimental procedures and primers are listed in Table S4. Sizes of amplicons and absence of nonspecific byproducts were confirmed via melting curve analysis using realplex software (Eppendorf). Quantification was obtained by both calculation of ddCt values and standard curve comparison. mtDNA copy number per cell was calculated as average mtDNA copy number relative to average gDNA copy number/2.

### mtDNA extraction and mtDNA sequencing

The workflow for mtDNA sequencing and data processing is displayed in Figure S5A. mtDNA was extracted via QIAamp Spin Mini-prep kit (QIAGEN) from early passage iPSC clones (mean p4.5), late passages (p30 and p50) of seven clones, and parental cell populations (mean p4.5). Entire mtDNA was amplified using two primer pairs (F2480A/R10858A [3:1] and F10653B/R2688B [1:1]) (Table S4) generating two overlapping PCR products (McElhoe et al., 2014). Two hundred nanograms of isolate were used as input, which equals ~5 × 10\textsuperscript{6} mtDNA molecules of, on average, 5 × 10\textsuperscript{6} iPSCs or 2.5 × 10\textsuperscript{5} parental cells. Overall, mtDNA isolation via Miniprep kit and PCR amplification yielded, on average, a 12- and 15,000-fold increase of mtDNA over normal cellular content (Figure S5B). See also supplemental experimental procedures. Amplicons were purified by applying AMPure XP beads (Beckman).
Coulter) in a 0.6 × ratio, sheared by focused ultrasonication (Cova-
tris), and quantified using a Qubit fluorimeter (Invitrogen). Sheared
fragments were purified with AMPure XP beads (0.9 × ratio), and
150 ng of each sample was fed to a library preparation using a NEB-
Next Ultra II DNA Library Preparation kit for Illumina (New En-
gland Biolabs) according to the manufacturer’s instructions. Final
concentration, fragment distribution (mean fragment length
600 bp), and quality were assessed on a Qubit fluorimeter and a
Agilent Bioanalyzer. Libraries were sequenced as paired-end
250 bp reads using MiSeq Reagent Kit v.2 (Illumina).

Read alignment, variant calling and refinement
Post-run fastq files were imported into Galaxy (v.17.05) (Alagan et al.,
2018) instance of RCU Genomics, Hannover Medical School, Ger-
mansy, for read trimming, quality assessment, alignment, and
variant calling. Quality and adapter trimming, as well as read map-
ing, are presented in the supplemental experimental procedures
and as Galaxy workflow in Data S1. Reads were aligned to the human
genome GRCh38 chromosome MT (the Cambridge Reference
sequence rCRS [NC_012,920]). The coverage was 19,000 on average.
Variants were called utilizing FreeBayes (v.1.0.2; Galaxy imple-
mentation v.1.0.2.29-3) with filters set to ploidy 10, minimum
coverage 100, and minimum AF (alternative frequency) 0.02. AF
≥ 0.02 as filter criterion was established by manually reviewing
called variants within their read context (supplemental experimen-
tal procedures). A receiver operating characteristic curve
with AF as discrimination threshold (Figure S5C) showed that AF
0.02 retrieves very high specificity while maintaining most true
positive variants. False variants with AF as discrimination threshold ( Figure S5 C) showed that AF
0.02 as filter criterion was established by manually reviewing
called variants within their read context (supplemental experimen-
tal procedures).

Variant annotation and prediction of functional
consequences
Variant annotation was performed using the web interface of the En-
sembli Variant effect predictor (VEP) (v.95) (assembly GRCh38.p12
(McLaren et al., 2016). Haplogroups and corresponding haplotype
variants were identified using the HaploGrep algorithm (Weissen-
steiner et al., 2016). MITOMAP (a human mitochondrial genome
database, r103) was utilized to retrieve population frequencies
(NCBI GenBank frequency [GB]) of variants. Variants with GB fre-
quency ≥ 0.02 were considered to be polymorphic.

Prediction of functional consequences was obtained from En-
sembli VEP and HmtVar (Preste et al., 2019). The functional conse-
quence of a variant was classified by a consensus based on the in
silo predictions of snpEff impact prediction, Condel (consensus
of SIFT and Polyphen2), CADD, and HmtVar, which comprises
predictions of MutPred, Panther, PhD SNP, Polyphen2, and
SNPs&GO, and contains information about variants in protein
and RNA coding regions. If a variant had a harmful designation
by snpEff (high impact = frameshift) or at least by two of the other
algorithms (Condel = deleterious, CADD phred > 12, HmtVar =
pathogenic), it was considered as putatively actionable.

Modeling heteroplasmy variance over time
A model by Aryaman et al. (2019) was adapted for modeling heter-
oplasmic variance over time.

\[ V(h) = \frac{2\mu}{n} \left( h(1-h) \right) \]

where \( fs \) is a factor for fragmentation, which indicates the ratio of
fused mitochondria, \( \mu \) is a factor for mitophagy rate, and \( h \) is the
heteroplasmy level. As iPSCs are featured by unfused mitochon-
dria, \( fs \) was excluded in our modeling. \( n \) represents the mtDNA
copy number/cell. Determination of the mtDNA copy number in
iPSCs (\( n \)) in p6, p30, and p50 showed that \( n \) stayed constant over
time in culture and was, on average, 1,000. Heteroplasmy variance
\( (V(h)) \) was calculated for different heteroplasmy levels with
different \( \mu \) values to assess effect of genetic drift (without selection,
\( \mu = 1 \)) and selection (\( \mu = 2, 5, 10, \) and 50).

Karyotype analysis
Preparation of metaphases, fluorescence R-banding, and analysis
of at least 10 metaphases were performed according to standard
procedures (supplemental experimental procedures).

Differentiation of CM, and BCT generation and
analysis
For embryoid body formation (dd –3), 1 × 10^6 iPSCs/well were
aggregated in 3 mL E8 medium containing 10 μM Rho-Kinase in-
hibitor Y-27632 (R0) on low attachment six-well plates (Greiner
Bio-one) and an orbital shaker (70 rpm; Infors). CM differentiation,
and BCT generation and analysis, were performed as described
earlier (Halloin et al., 2019; Kensah et al., 2011, 2013) (supple-
mental experimental procedures).
Live-cell staining and flow cytometric analysis
Live-cell staining was performed of adherent cells with MitoTracker, ROS Brite 670, and TMRM (25 nM; Thermo Fisher Scientific) diluted in Hank’s solution ( Gibco ) with 20 mM HEPES ( Sigma-Aldrich ) ( HHBS ) according to the manufacturer’s recommendation for 45 min. A list of antibodies and dyes is provided in Table S 6 . Cells were dissociated by Versene ( Gibco ) or trypsin/EDTA treatment for iPSCs or ECs, respectively, resuspended in HHBS buffer, filtered, and analyzed by flow cytometry. Flow cytometry was executed on a MACSQuant analyzer ( Miltenyi Biotec ) and data were analyzed with FlowJo ( v.10 ).

Immunofluorescence staining and microscopic analysis
ECs, iPSCs, and CMs were seeded on cover slides and, after a culture period of 1–2 days for ECs and iPSCs, and 3 days for CMs, cells were fixed, permeabilized, and stained with anti-ATP1F1 antibody ( Table S 6 ) ( supplemental experimental procedures ). Cells were analyzed using an Axioserver A1 fluorescent microscope ( Zeiss ) and Axiosvision ( v.4.71 ) software.

Measurement of glucose consumption and lactate production rate
iPSCs were cultured as monolayers in house-made E8 medium. On day 1 after cell seeding, the medium was exchanged, and on the subsequent 2 days, lactate and glucose concentrations from cell-free supernatant were measured employing Biosen C-Line glucose and lactate analyzer ( EKF Diagnostics ). On each day of analysis, the cell numbers were counted using a Neubauer chamber. Technical triplicates initiated from the same starting cell population were analyzed for each sample and time point. Yield coefficient of lactate from glucose was calculated as described previously ( Kropp et al., 2016 ).

Gene expression analysis via quantitative real-time PCR
Total RNA was isolated via RNeasy Kit ( Macherey-Nagel, Düren, Germany ) and reverse transcribed utilizing Superscript II ( Life Technologies ) and random primers according to the manufacturer’s instructions. Quantitative real-time PCR was performed on 5 ng cDNA per reaction in duplicates on a CFX Connect ( Thermo Scientific ) real-time system ( Bio-Rad ) and with Absolute QPCR SYBR Green ( Bio-Rad ). Primers were listed in Table S 4 . Cells were dissociated by Versene ( Gibco ) or trypsin/EDTA treatment for iPSCs or ECs, respectively, followed by a culture period of 1 day after cell seeding. On day 1 after cell seeding, the medium was exchanged and, on the subsequent 2 days, lactate and glucose concentrations from cell-free supernatant were measured employing Biosen C-Line glucose and lactate analyzer ( EKF Diagnostics ). On each day of analysis, the cell numbers were counted using a Neubauer chamber. Technical triplicates initiated from the same starting cell population were analyzed for each sample and time point. Yield coefficient of lactate from glucose was calculated as described previously ( Kropp et al., 2016 ).

Statistical analyses
If not stated otherwise, N refers to independent batches of cells. GraphPad Prism ( v.607 ), RStudio ( v.1.1.463 ; R v.3.5.1 ), or Bio-Rad CFX Maestro 1.1 software were employed for statistical analysis and visualization. Results are presented as mean and SD. The D’Agostino-Pearson omnibus normality test was executed to check if samples were normally distributed. In cases where the sample size was not sufficiently large, the Shapiro-Wilk normality test and Kolmogorov-Smirnov test were applied. Unpaired two-tailed t test and non-parametric two-tailed Mann Whitney test were employed as appropriate to test for significant differences between groups. One-way ANOVA with post hoc Tukey’s multiple comparisons test and Kruskal-Wallis test with post hoc Dunn multiple comparison test, respectively, were used to test for significant differences between multiple groups.

Comparison of metabolic features of mutated against wild-type iPSC clones or of CMs derived thereof, was underlined by non-parametric Wilcoxon signed-rank test for derivation from hypothetical value of 1.

Local error rates of the amplicon sequencing was calculated and (pre-)existence of variants assessed ( p < 0.05 ) as described previously ( Kosanke et al., 2021 ) ( supplemental experimental procedures ).

Code availability
Sequencing data analysis was mainly performed on the Galaxy ( v.17.05 ) ( Afgan et al., 2018 ) instance of the RCU Genomics, Hannover Medical School, Germany. The workflow is available in Data S 1.

Data availability
Data are deposited in the European Genome-phenome Archive at the European Bioinformatics Institute. The accession number for the study reported in this paper is EGA: EGAS00001005560.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2021.08.016.

AUTHOR CONTRIBUTIONS
M.K. contributed to designing and coordinating the study, performed, experiments, collected and analyzed data, and wrote the manuscript. C.D. and L.W. performed sequencing and contributed to analyzing bioinformatics data. T.K. performed the experiments. M.D. provided technical assistance in fragment library construction and sequencing. J.G., K.M., and M. Sievert provided technical assistance in performing experiments. M. Szepes, I.G., and A.M. coordinated and performed cardiomyocyte differentiation and analysis. G.G. provided karyotype analysis. U.M. designed and coordinated the study.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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