# An MDS-derived cell line and a series of its sublines serve as an *in vitro* model for the leukemic evolution of MDS

Running title: A series of cell lines as an MDS progression model

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Key words: MDS cell line, whole exome analyses, driver mutation, *histone-H3*(K27M) mutation, leukemic transformation

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis and increased risk of progression to acute myeloid leukemia (AML). Occurrence of somatic mutations leads to formation of an abnormal clone with impaired differentiation, and additional driver mutations lead finally to AML. [1] We previously established a myelodysplastic cell line MDS92 from the bone marrow of an MDS patient with deletion of 5q chromosome[del(5q)]. [2, 3] MDS92 cells proliferated in the presence of interleukin(IL)-3 with a tendency for gradual maturation and represented karyotypic abnormalities including del(5q) (actually der(5)(5;19)), monosomy7 and a point mutation at codon12 of *NRAS* gene. [2] These characteristics are exclusively compatible with the property of MDS. Later, a blastic subline MDS-L was established from MDS92. [4] MDS-L cell line has contributed to the molecular study of MDS with del(5q) [5, 6] and therapeutic mechanisms of lenalidomide. [4, 7] MDS-L has also been utilized as an *in vivo* model by transplanting experiments into immunodeficient mice [8] and for investigation of various drugs expected for treatment of MDS. [9, 10] In addition to MDS-L, we isolated several blastic sublines independently of one another from the parental MDS92, and further obtained MDS-L-2007 and MDS-LGF from MDS-L in the presence and absence of IL-3, respectively (Figure 1A). The morphology and surface markers of the cell lines are shown in Supplementary Figure 1

and Supplementary Table 1, respectively. In Figure 1A, MDS-Gen implied the mid-stage cultured cells from bone marrow of the original patient (Pt-BM) for 3 months in the presence of IL-3 for aiming at establishment of MDS cell lines. We confirmed that the series of cell lines were all originated from Pt-BM by the short tandem repeat profiling (Supplementary Table 2).

We performed comprehensive, comparative exome analyses on the series of samples by the next-generation sequencing (NGS) method. Total effective reads per sample ranged from 35,548,000 to 58,711,000 and the average sequencing depth ranged from 46 to 77. Candidate somatic mutations excluding dbSNP were detected in 406 genes, and 178 mutations were found in common in all ten samples. Main results are summarized in Supplementary Table 3. As notable mutations, a homozygous, splice donor site mutation of *TP53*(c.672+1G>A; COSM6906 in COSMIC) was present in all samples including Pt-BM together with homozygous *TP53*(c.74+14T>C) mutation. MDS-L lost normal TP53 protein probably due to these mutations (Supplementary Figure 2). *CEBPA*(Q311stop) mutation (heterozygous; COSM29221) and *NRAS*(G12A) mutation (heterozygous; COSM565) were not detected in Pt-BM, but they emerged clonally at MDS-Gen stage, and both mutations were inherited by all subsequent cell lines.

Whether *CEBPA*-mutant and *NRAS*-mutant clones were originally latent in Pt-BM or whether these mutations newly emerged during *in vitro* culture is an important question. To find the answer, we performed ultra-deep targeted sequencing of *CEBPA* and *NRAS* in Pt-BM, and the results were as follows: (1) as for *CEBPA*, a single-allele 931C>T(Q311stop) mutation was detected in 181,059 counts out of 4,044,666 coverages. The detection frequency was therefore approximately 4.5%, and considering that it was a heterozygous mutation, the mutant clone must have accounted for approximately 9% of Pt-BM; (2) as for *NRAS*, no 35G>C(G12A) mutation count was detected at all out of 4,712,760 coverages. It implied that *NRAS*-mutant clone was completely absent from Pt-BM. It was therefore demonstrated that *CEBPA* mutation was originally present in a part of *TP53*-mutated bone marrow fractions and this clone was selected during IL-3-containing culture, and it was further suggested that *NRAS* mutation emerged by chance on the *CEBPA*-mutant clone during *in vitro* culture. Such accumulated mutations could generate MDS cell lines.

Another mutation to which particular attention should be paid is a heterozygous *histone-H3*(*HIST1H3C*)(K27M) mutation (*H3*-K27M; COSM1580151) which appeared in MDS-L, because this mutation would likely be one of the candidates for blastic transformation. *H3*-K27M mutation is frequently found in pediatric brain stem tumors. [11]

86 *Histone-H3* mutations in hematopoietic tumors are rare, but recently Lehnertz et al. reported
87 two *H3*-K27M and one *H3*-K27I cases in 615 AML cases. [12] Therefore, there will be a greater
88 interest in *histone-H3* mutation in myeloid neoplasms. *H3*-K27M mutation was inherited by
89 MDS-L-2007 which was isolated from MDS-L in the presence of IL-3 but not by MDS-LGF
90 which was isolated from MDS-L in the absence of IL-3.

91 We found that *H3*-K27M mutation severely affected the whole histone methylation status.
92 H3-K27M protein was expressed in MDS-L and MDS-L-2007, and these cells indicated a
93 marked reduction in H3-K27 methylation (Figure 1B-C). In contrast, H3-K27 methylation
94 was conserved in MDS92 and MDS-LGF both of which did not bear *H3*-K27M mutation
95 (Figure 1B-C). Expression pattern of H3-K27M and H3-K27me2/me3 appeared to be
96 reciprocal. Immunostaining study showed that MDS-L consisted of both H3-K27
97 methylation-positive and -negative cells and that MDS-L-2007 was completely lacking in
98 H3-K27 methylation, whereas MDS-LGF was positive for H3-K27 methylation like as
99 MDS92 (Figure 1C).

100 To know the difference of *histone-H3* status between MDS-L-2007 and MDS-LGF (both
101 were originated from MDS-L), we reviewed the sequenced nucleotide reads from NGS and
102 found that MDS92 and MDS-L had a single nucleotide polymorphism (SNP) at codon32 of

one allele of *HIST1H3C*(from ACC to ACG; silent mutation) and that *H3*-K27M mutation was located in the SNP(ACG)-present allele in MDS-L (Supplementary Figure 3). Interestingly, in MDS-L-2007 all sequenced reads of the SNP(ACG)-present allele showed *H3*-K27M mutation, while in MDS-LGF all sequenced reads of the SNP(ACG)-present allele showed *H3*-K27-wild-type (Supplementary Figure 3). We speculated that MDS-L-2007 inherited *H3*-K27M-mutant allele and expanded in the presence of IL-3, whereas MDS-LGF inherited *H3*-K27-wild-type allele and survived without IL-3.

When MDS-L was cultured in the presence of IL-3, *H3*-K27M-mutant fraction gradually increased. When MDS-L was cultured without IL-3, *H3*-K27M-mutant fraction gradually decreased (Figure 1D). This phenomenon was reproducible.

To investigate the implication of *H3*-K27M mutation, we tried single-cell cloning from MDS-L and secured four wild-type clones (MDS-L-K27wt) and seven *H3*-K27M-mutant clones (MDS-L-K27M). H3 methylation patterns of these clones are shown in Figure 2A. In all *H3*-K27M-mutant clones, there was a marked reduction in H3-K27me3/2 and a modest increase in H3-K27ac and H3-K36me3/2. The reason why *H3*-K27M mutation could cause a drastic decrease in whole methylation status of H3-K27 sites is explained by the fact that *H3*-K27M protein inhibits histone H3-K27 methyltransferase EZH2 as reported in brain

tumor studies. [13] As loss-of-function mutation of *EZH2* often found in myeloid malignancies is involved in tumorigenesis [14], *H3*-K27M mutation might have a similar oncogenic implication as *EZH2* mutation.

*H3*-K27M-mutant clones showed rapid growth in the presence of IL-3, but cell proliferation was suppressed without IL-3 (Supplementary Figure 4). We co-cultured *H3*-K27M-mutant clones with wild-type clones as a competitive growth experiment in the presence/absence of IL-3 up to six months. *H3*-K27M-mutant clones became predominant in the presence of IL-3, whereas wild-type clones were sustained comparatively in the absence of IL-3 (Figure 2B).

Figure 2A also indicated that expression of a tumor-suppressor molecule $p16^{INK4a}$ was reduced in six of the seven *H3*-K27M-mutant clones. Studies on brain tumors reported that H3-K27 methylation increases exclusively at *p16* locus resulting in reduced p16 expression. [15] Considering that *H3*-K27M-mutant clones with reduced p16 expression showed more vigorous proliferation than wild-type clones, p16 might be a therapeutic target. Treatment with an EZH2 inhibitor EPZ-6438 caused growth suppression of *H3*-K27M-mutant clones as well as wild-type clones with demethylation of H3-K27 site particularly of wild-type clones, and more importantly, involved obvious recovery of p16 expression in *H3*-K27M-mutant

clones (Supplementary Figure 5 and Figure 2C).

Although a histone-demethylase JMJD3 inhibitor GSK-J4 was reported to inhibit *H3*-K27M-mutated pediatric brain stem tumors, [11] GSK-J4 exerted only non-specific growth inhibitory effect on both H3-K27M-mutant and wild-type clones (data not shown), and it is unclear whether the treatment for *H3*-K27M-mutated brain tumors can be applied to hematological malignancies.

As already noted, we obtained interesting data on the relation of *H3*-K27M mutation to IL-3-dependency. When MDS-L was cultured in the presence of IL-3, *H3*-K27M-mutant clones proliferated dominantly (Figures 1D and 2B) and MDS-L-2007 subline was one outcome. Conversely, when MDS-L was maintained without IL-3, the majority of cells died and most of surviving fractions were wild-type clones (Figures 1D and 2B), and in this way MDS-LGF subline was established. This aggressive but strictly IL-3-dependent growth of *H3*-K27M-mutant clones implies a relation of *H3*-K27M mutation with enhanced growth signal via IL-3. The phenomenon that the dominant fraction was replaced according to the presence or absence of IL-3 suggests that growth advantage of mutant clones is affected by environmental factors such as growth factors. These data raise a possibility that even if neoplastic clones emerge, their expansion might be influenced not only by genetic/epigenetic

status but also by surrounding environmental factors.

In this study, we established a myelodysplastic cell line and subsequent blastic sublines. We confirmed that accumulated gene mutations were involved in the establishment of MDS cell lines and progression to AML phenotypes (Figure 2D). This series of cell lines will be a useful tool as an *in vitro* model for leukemic evolution of MDS.

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**Conflict of Interest**

The authors declare no conflict of interest.

Supplementary information is available at Leukemia's website

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220 **Figure Legends**
221 **Figure 1. The overview of the series of MDS cell lines and histone H3-K27 methylation**

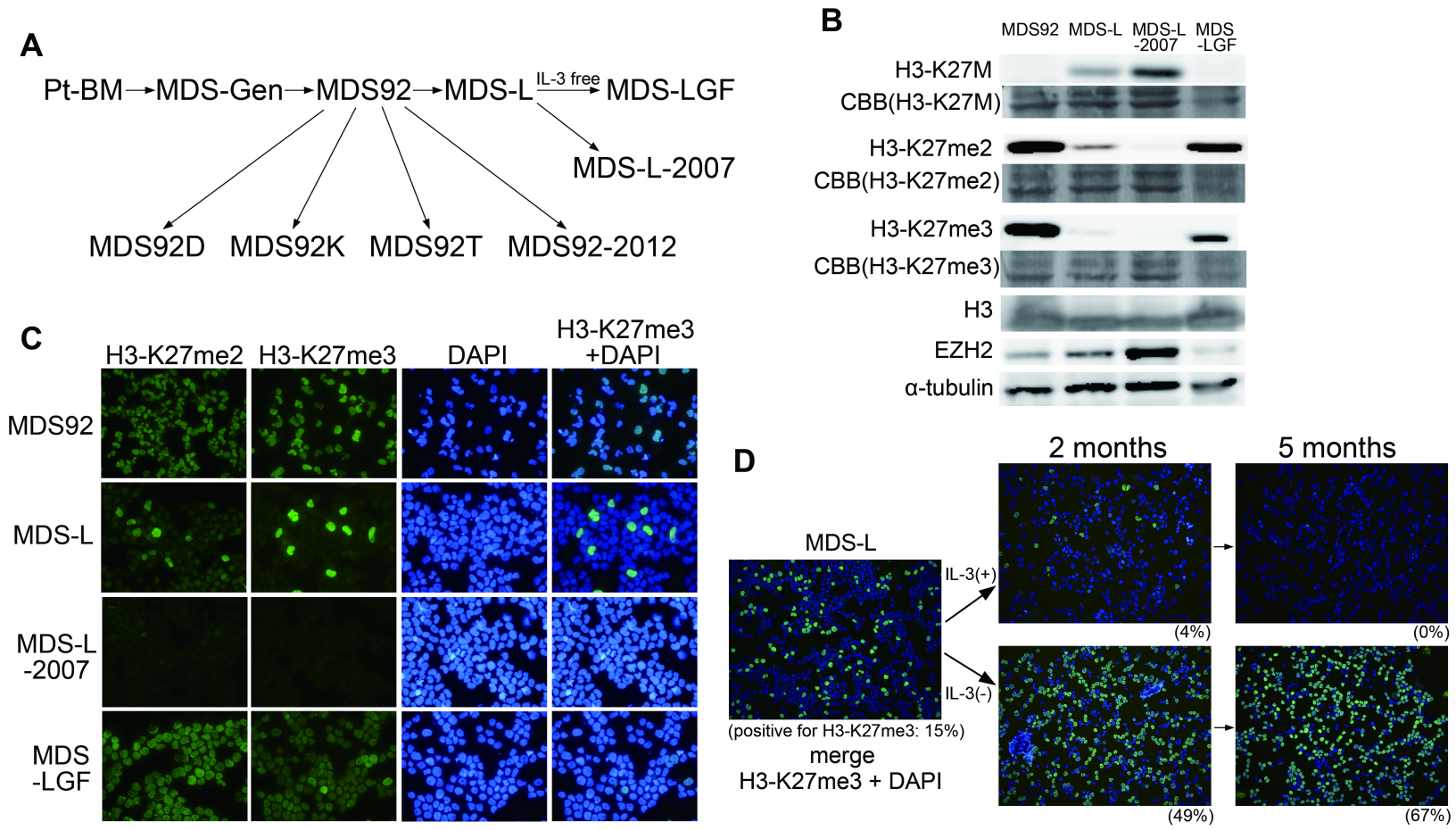
**status.**

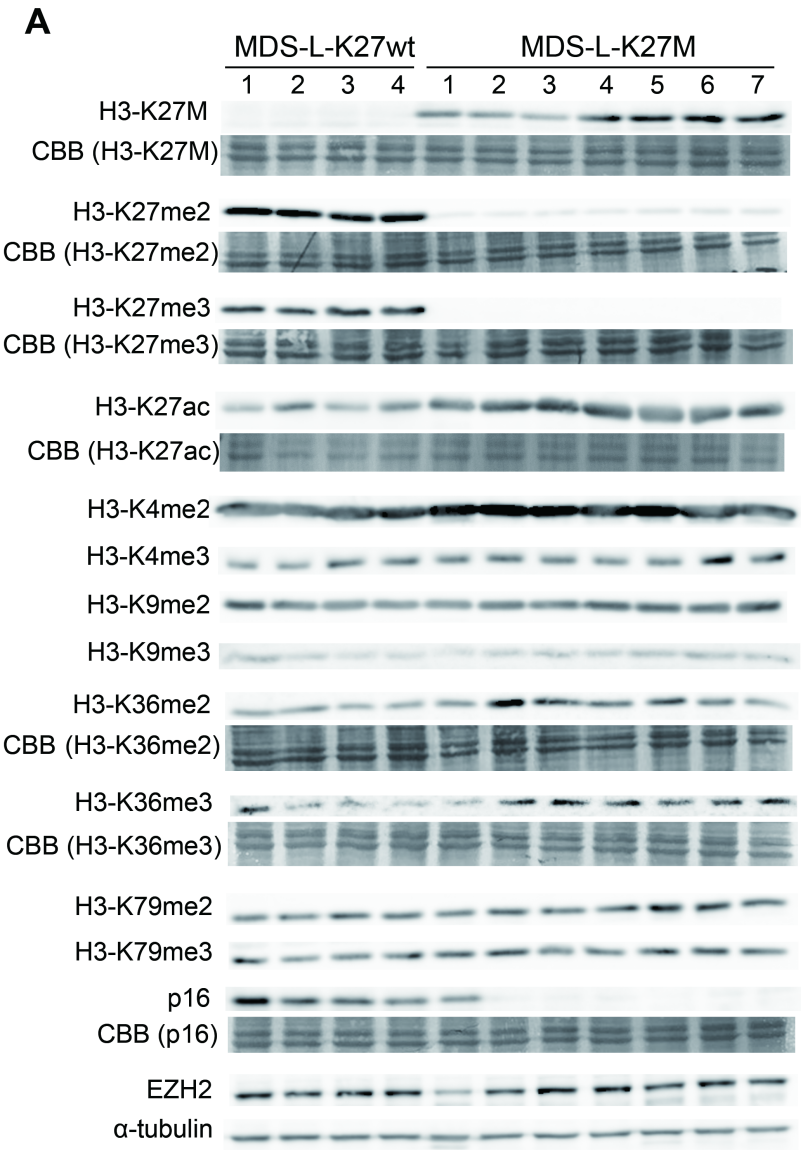
(A) The overview of the establishment of MDS92 and the evolution of the sublines. (B) Histone H3-K27 methylation status of the cell lines by immunoblotting analysis. Protein expression of EZH2 is also indicated. CBB stain and α–tubulin were shown as a loading control. (C) Histone H3-K27 methylation status by immunofluorescence analysis. Original magnification was ×600. (D) Presence or absence of IL-3 in culture alters the proportion of *H3*-K27M-mutant fraction. MDS-L cells were cultured in the presence or absence of IL-3 for five months, and the cells were immunostained by anti-H3-K27me3 antibody. Original magnification was ×200.

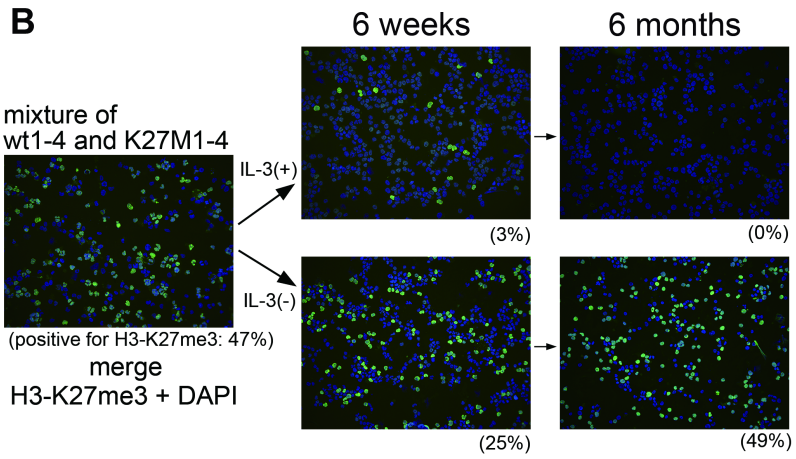
**Figure 2. Impact of *H3*-K27M mutation on IL-3-dependent growth and a schematic model of the series of MDS cell lines and the progression to AML phenotypes.**

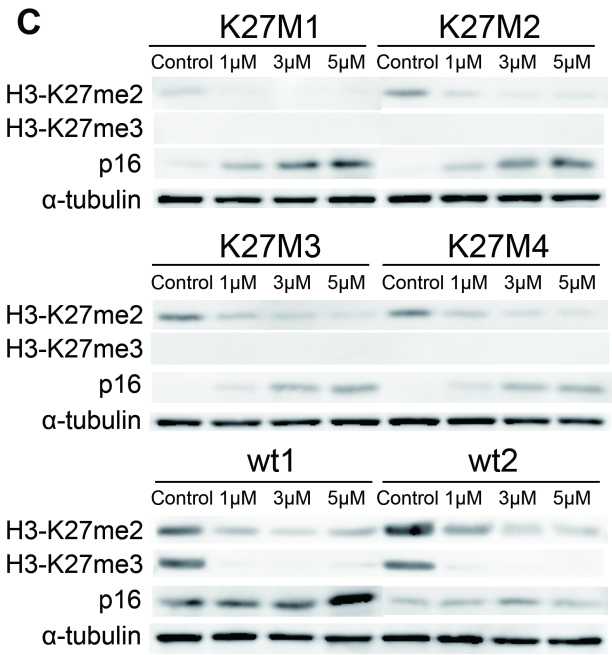
(A) H3 methylation patterns between *H3*-K27-wild-type clones and *H3*-K27M-mutant clones from MDS-L. In *H3*-K27-wild-type clones (clone1 to 4) and *H3*-K27M-mutant clones (clone1 to 7), comprehensive methylation/acetylation status of histone H3 was examined by immunoblotting analysis. CBB stain and α–tubulin were shown as a loading control. (B) Competitive growth experiment by co-culture of *H3*-K27-wild-type and *H3*-K27M-mutant

clones in the presence or absence of IL-3. We mixed the same number of eight cloned cells (wt1 to wt4, and K27M1 to K27M4) and started co-culture in the presence or absence of IL-3. The cells were immunostained by anti-H3-K27me3 antibody. Original magnification was ×200. (C) Effects of an EZH2 inhibitor EPZ-6438 on H3-K27 methylation status and expression of p16 in wild-type and mutant clones. *H3*-K27-wild-type clones (wt1 and wt2) and *H3*-K27M-mutant clones (K27M1 to K27M4) were treated with EPZ-6438 for 14 d, and the amount of H3-K27me2, H3-K27me3 and p16 was examined by immunoblotting analysis. (D) A schematic model indicating the accumulation of gene mutations involved in the establishment of the series of MDS cell lines and the progression to AML phenotypes. A homozygous *TP53* mutation was originally present in whole Pt-BM, and *CEBPA* mutation was also originally present in a small fraction of Pt-BM. *CEBPA*-mutant clone began to proliferate and *NRAS* mutation emerged by chance during the IL-3-containing culture leading to MDS92 cell line. MDS92 contained multiple blastic clones and some of them were isolated independently as blastic sublines. *Histone-H3*-K27M mutation was newly detected in MDS-L. MDS-L cells were a mixture of *H3*-K27M-mutant and wild-type clones. Long-term culture of MDS-L in the presence and absence of IL-3 generated MDS-L-2007 (*H3*-K27M-mutant) and MDS-LGF (*H3*-K27-wild-type), respectively.


A
Pt-BM
MDS-Gen
MDS92
MDS-L
IL-3 free
MDS-LGF
MDS-L-2007
MDS92D
MDS92K
MDS92T
MDS92-2012
B
MDS92
MDS-L
MDS-L -2007
MDS -LGF
H3-K27M
CBB(H3-K27M)
H3-K27me2
CBB(H3-K27me2)
H3-K27me3
CBB(H3-K27me3)
H3
EZH2
α-tubulin
C
H3-K27me2
H3-K27me3
DAPI
H3-K27me3 +DAPI
MDS92
MDS-L
MDS-L -2007
MDS -LGF
D
2 months
5 months
MDS-L
IL-3(+)
IL-3(-)
(4%)
(0%)
(positive for H3-K27me3: 15%)
merge
H3-K27me3 + DAPI
(49%)
(67%)


A
MDS-L-K27wt
MDS-L-K27M
1 2 3 4 1 2 3 4 5 6 7
H3-K27M
CBB (H3-K27M)
H3-K27me2
CBB (H3-K27me2)
H3-K27me3
CBB (H3-K27me3)
H3-K27ac
CBB (H3-K27ac)
H3-K4me2
H3-K4me3
H3-K9me2
H3-K9me3
H3-K36me2
CBB (H3-K36me2)
H3-K36me3
CBB (H3-K36me3)
H3-K79me2
H3-K79me3
p16
CBB (p16)
EZH2
α-tubulin


B
6 weeks
6 months
mixture of
wt1-4 and K27M1-4
IL-3(+)
IL-3(-)
(3%)
(0%)
(positive for H3-K27me3: 47%)
merge
H3-K27me3 + DAPI
(25%)
(49%)


C
K27M1
K27M2
Control 1μM 3μM 5μM Control 1μM 3μM 5μM
H3-K27me2
H3-K27me3
p16
α-tubulin
K27M3
K27M4
Control 1μM 3μM 5μM Control 1μM 3μM 5μM
H3-K27me2
H3-K27me3
p16
α-tubulin
wt1
wt2
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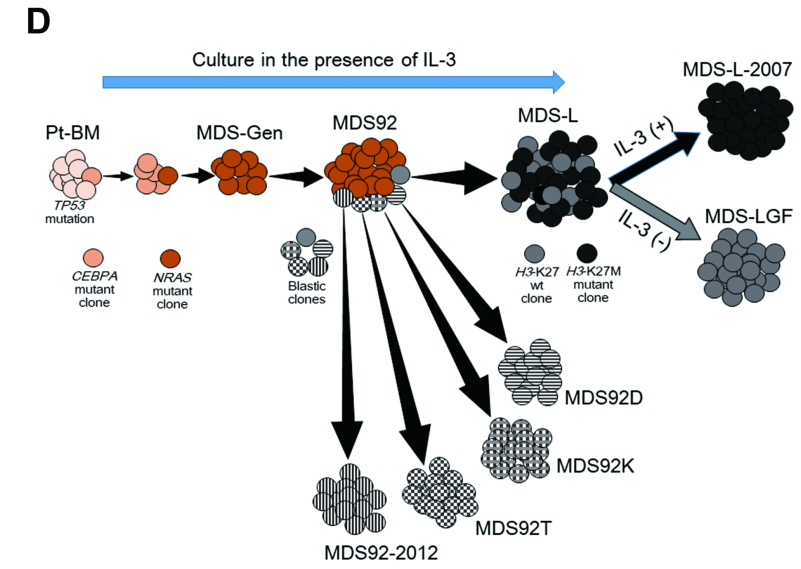
p16
α-tubulin


D
Culture in the presence of IL-3
Pt-BM
MDS-Gen
MDS92
MDS-L
MDS-L-2007
IL-3 (+)
IL-3 (-)
MDS-LGF
TP53 mutation
CEBPA mutant clone
NRAS mutant clone
Blastic clones
H3-K27 wt clone
H3-K27M mutant clone
MDS92D
MDS92K
MDS92T
MDS92-2012