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Leukemic fusion genes *MLL/AF4* and *AML1/MTG8* support leukaemic self-renewal by controlling expression of the telomerase subunit TERT

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Abstract

Leukemia relapse and persistence of leukemic cells are greatly dependent on the self-renewal capacity of leukemic cells. Telomerase plays an important role in self-renewal in both malignant and non-malignant cells. However, the underlying molecular mechanisms and genes involved in malignant self-renewal remain incompletely understood. *MLL/AF4* and *AML/MTG8* represent two leukemic fusion genes, which are most frequently found in infant acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), respectively. By using RNA interference, we examined more precisely the influence of *MLL/AF4* and *AML1/MTG8* fusion genes on the expression of *TERT* coding for the telomerase protein subunit, and subsequently telomerase activity in t(4;11)-positive ALL and t(8;21)-positive cell lines, respectively. *MLL/AF4* suppression diminished telomerase activity and expression of *TERT*. Blocking pro-apoptotic caspase activation in conjunction with *MLL/AF4* knockdown enhanced the inhibition of *TERT* gene expression, which suggests that MLL/AF4 depletion does not reduce *TERT* expression levels by inducing apoptosis. Knockdown of *HOXA7,* a direct transcriptional target of *MLL/AF4* fusion gene, caused a reduction of telomerase and *TERT* to an extent similar to that observed with *MLL/AF4* suppression. Chromatin immunoprecipitation of SEM cells, using ectopically expressed FLAG-tagged Hoxa7, indicate HOXA7 binding site in the *TERT* promoter region. Furthermore, suppression of the *AML1/MTG8* fusion gene was associated with severely reduced clonogenicity, induction of replicative senescence, impaired *TERT* expression and accelerated telomere shortening. We thus present findings that demonstrate a mechanistic link between leukemic fusion proteins, essential for development and maintenance of leukemia, and telomerase, a key element of both normal and malignant self-renewal.

Introduction

The concept of tumorigenic leukemic stem cells (LSCs) emerged following a number of studies that found a subgroup of leukemic cells capable of extensive proliferation (1, 2). Concurrently, a cancer stem cell (hierarchical) model has been defined by Dick and colleagues suggesting that cancer cells are heterogeneous in nature, and only a subset of cells are able to maintain and expand the population (3).

Based on this principle, it was hypothesized that most current treatment methods target the descendent bulk of LSCs whilst leaving the LSC themselves unaffected. Indeed, there is ample evidence to suggest that LSC play a crucial role in therapy resistance and relapse (4). To acknowledge this, novel treatment approaches targeting aberrant self-renewal of cancer stem cells, including LSCs, are being explored. However, our current understanding of the molecular mechanisms underlying malignant self-renewal is still incomplete. Thus, the development of novel therapies, designed to target the LSC niche, is only just emerging.

Both normal and malignant self-renewal crucially depend on preserving telomeres to maintain genomic integrity and to prevent replicative senescence (5). Telomere ends are restored by telomerase, a reverse transcriptase consisting of a protein subunit, TERT, and an RNA subunit, *TERC* (also termed *hTR*). *TERC* serves as a template for telomere synthesis and is ubiquitously expressed, whereas, in contrast, transcription of *TERT* is tightly regulated by transcription factors such as SP1, c-MYC and IRF-4, and is repressed by e.g. the TGFβ signal transduction pathway (6-8). *TERT* activity is upregulated during cell cycle activation of immature cells and expression is reduced during cell differentiation. Among the different components of

the telomerase ribonucleoprotein complex, TERT is regarded as the rate-limiting component for telomerase activity.

Leukemic fusion genes are generated by chromosomal translocations and are hallmarks of leukemia. Fusion genes, such as *AML1/MTG8* (*RUNX1/RUNX1T1* or *AML1/ETO*) or *MLL/AF4* (*MLL/AFF1* or *ALL1/AF4*), are exclusively expressed in preleukemic and leukemic cells. Both fusion genes can originate already prenatally (9, 10).They are key to maintaining a malignant phenotype, hence representing a promising target for new therapeutic approaches. AML/MTG8 and MLL/AF4 are chimeric transcription factors that have been shown to support leukemic self-renewal by impairing hematopoietic differentiation, and by inducing self-renewal-associated signal transduction pathways, and transcriptional programs (11-14). The *AML1/MTG8* fusion gene is generated from a chromosomal translocation t(8;21), and represents the most common aberration associated with 10-15% of acute myeloid leukaemia (AML). This translocation fuses AML1 (or RUNX1), a transcription factor for definitive hemopoiesis, with MTG8 (or RUNXT1), which is part of histone deacetylase-containing complexes (15-17). Thus, it converts a transcriptional modulator to a repressor of gene expression by interfering with chromatin modification. On the other hand, MLL/AF4 is caused by the chromosomal translocation t(4;11), which fuses the *MLL* gene (Mixed Lineage Leukemia) on chromosome 11 with the *AF4* gene on chromosome 4 (18, 19). This translocation causes an aggressive type of acute lymphoblastic leukemia (ALL), which predominantly affects infants, and is associated with an unfavorable prognosis (20).

A direct transcriptional target of *MLL* fusion genes are genes of the Homeobox A (*HOXA*) cluster. *HOXA* gene expression is normally down-regulated during

hematopoietic differentiation. However, analyses have shown consistently high expression levels of select *HOXA* genes in *MLL*-rearranged leukemias, which suggests that the upregulation of *HOXA* genes play a key role in fusion driven leukemogenesis (11, 21, 22). The transcriptional networks involved in this fusion gene exerted control of malignant stemness are yet to be elucidated.

In this study, we used small interfering RNAs (siRNAs) to investigate the role that two leukemic fusion proteins, MLL/AF4 and AML1/MTG8, play in the control of telomerase and *TERT*. We demonstrate that siRNA-mediated suppression of either fusion protein decreases *TERT* transcript and protein levels. In addition we show that *MLL/AF4* fusion gene regulates *TERT* expression level via its direct transcriptional target gene, *HOXA7*. Overall, we provide evidence that *TERT* is part of fusion gene-driven self-renewal programs.

Results

Suppression of MLL/AF4 Inhibits Telomerase and TERT Expression

Suppression of *MLL/AF4* by a fusion site-specific siRNA, siMLL/AF4, impaired clonogenic growth and diminished expression of stem cell- and self-renewal-associated genes, such as *HOXA7, HOXA9* and *PROM1* (CD133) (14). We hypothesized that changes in an *MLL/AF4*-associated self-renewal program will become even more evident upon prolonged depletion of MLL/AF4. Consequently, we repeatedly electroporated t(4;11)-positive SEM cells with siMLL/AF4. This approach routinely yielded a threefold knockdown of *MLL/AF4* transcript and protein over a period of up to 6 days (Fig. 1A, D). To control for unspecific and off-target effects, we used two control siRNAs, a mismatch siRNA (siCTL1) or an siRNA suppressing the product of the translocation t(8;21), *AML1/MTG8*, which has been shown to impair expansion of t(8;21)-positive, but not of t(4;11)-positive leukemic cells (14).

Since telomerase is a key enzyme for normal and malignant self-renewal, we focused on the impact of the leukemic fusion gene expression on maintaining telomerase activity in leukemic cells. Sustained siRNA-mediated MLL/AF4 depletion correlated with threefold reduced TERT protein levels and a concomitant decrease in telomerase activity (Fig. 1A-C). Concomitantly, analysis of transcript levels by Real-Time PCR demonstrated that suppression of *MLL/AF4* was associated with a more than twofold reduction in *TERT* transcript levels, whereas *TERC* was not affected (Fig. 1D).

To exclude the action of MLL/AF4 siRNA on *TERT* transcript levels as a possible cell-line specific effect, we extended our study to RS4;11 cells, which express a

different MLL/AF4 variant to that of SEM cells. Exon 9 of *MLL* is fused to exon 4 of *AF4* (*AFF1*) in SEM cells, and is targeted by siMLL/AF4, whereas exon 10 – exon 4 variant in RS4;11 cells is targeted by an alternative siRNA, siMLL/AF4-2 (14). Consistent with the fusion site specificity of these siRNAs, siMLL/AF4 diminished *MLL/AF4* transcript levels exclusively in SEM cells, and siMLL/AF4-2 only reduced *MLL/AF4* in RS4;11 cells (Fig. 2A, B).

siRNA specificity was further demonstrated by the correlation between *MLL/AF4* suppression and reduced levels of *HOXA7* (Fig. 2A, B) (23). *MLL/AF4* knockdown was associated with a twofold reduction in *TERT* transcript levels (Fig. 2A, B) and a concurrent fourfold decrease in telomerase activity in each cell line (Fig. 2C). Overall, these data strongly suggest that MLL/AF4 maintains *TERT* expression and telomerase activity in t(4;11) positive ALL.

Reduced TERT Expression is Independent of Apoptosis

Prolonged knockdown of *MLL/AF4* has previously been shown to increase apoptosis (14). Since cells with lower TERT levels might be more prone to cell death (24), we investigated whether apoptosis-related loss of siMLL/AF4-transfected cells may cause us to underestimate the extent of TERT reduction. Alternatively, diminished *TERT* gene expression may have been a consequence of apoptosis. To examine the influence of apoptosis on *TERT* transcript levels, siRNA electroporation was combined with a pan-caspase inhibitor, zVAD-FMK. zVAD diminished the fraction of subG1 cells, tenfold, to background levels (Fig. 3A) indicating a complete inhibition of apoptosis. Neither electroporation alone nor electroporation with the control siRNA, siAML1/MTG8, caused caspase activation or induced significant cell death compared to untreated SEM cells. As in previous experiments, siMLL/AF4 reduced *TERT* expression levels twofold (Fig. 3B). Addition of zVAD caused a further

decrease down to <10% of *TERT* control levels, but did not enhance *MLL/AF4* knockdown nor deplete the direct MLL/AF4 target, *HOXA7* (Fig. 3B). Combining zVAD with mock (siRNA absent) or siAML1/MTG8 electroporation did not alter *MLL/AF4, HOXA7* or *TERT* levels. Thus, enhanced *TERT* depletion is unlikely a result of an increased cell death of MLL/AF4-depleted cells. Instead, caspase inhibition itself may directly or indirectly facilitate MLL/AF4-dependent *TERT* down-modulation.

HOXA7 Controls TERT

ChIP sequencing analysis of t(4;11)-positive SEM cells found Histone H3K4 trimethylation and H3K79 dimethylation patterns, indicators of transcriptional activity, in the *TERT* locus. However, concomitant MLL/AF4 occupancy had not been detected (23). Hence, the dependence of *TERT* expression in t(4;11)-positive cell lines is unlikely to be directly linked to MLL/AF4. Instead, genes downstream of *MLL/AF4* seem to control *TERT*.

Based on previous findings from ChIP-sequencing assays of SEM cells demonstrating that MLL/AF4 occupies the chromosomal region from *HOXA7* to *HOXA10* (23), we examined the impact of MLL/AF4 depletion on *HOXA* gene cluster expression. *MLL/AF4* knockdown reduced *HOXA6* transcript levels threefold, *HOXA7* sixfold, *HOXA9* twofold and *HOXA10* fivefold (Fig. S1). Concurrently, HOXA6, HOXA7 and HOXA9 protein levels also decreased three to fivefold (Fig. S1). When comparing *HOXA* gene expression levels in both *TERT* expressing t (4;11) ALL lines, RS4;11 and SEM, *HOXA7* had the highest relative expression of all *HOXA* members (Fig. S2). In contrast, *HOXA7* was less abundant in the two AML cell lines, MV4;11 and Kasumi-1, coinciding with substantially lower *TERT* expression compared to the ALL cell lines.

Since there is an existing correlation between *HOXA7* and *TERT* expression levels, we examined the possible influence of HOXA7 on telomerase activity and on *TERT* expression. Electroporation of SEM and RS4;11 cells with HOXA7 siRNA induced a twofold reduction of *HOXA7* transcript levels (Fig. 4A, B). Concomitantly, decreased *TERT* transcript levels (Fig. 4A, B) and impaired telomerase activity (Fig. 4C) was observed in both cell lines. Electroporation of SEM cells with an alternative HOXA7 siRNA, siHOXA7-8, also reduced *TERT* expression (Fig. 4D).

In contrast, *MLL/AF4* expression was not affected by HOXA7 siRNA in either cell line (Fig. 4A, B, D), which suggests that HOXA7 regulates *TERT* expression downstream of MLL/AF4. Interestingly, knockdown of *HOXA7* is associated with an almost twofold decrease in the transcript levels of *HOXA6* and *HOXA10* (Fig. S3), an effect similarly reported after *HOXA9* knockdown; *HOXA7* and *HOXA10* transcript levels reduced concurrently (21). Thus, a role for other late HOXA members in TERT regulation cannot be excluded.

HOXA7 Binds to the TERT Locus

To establish whether *TERT* gene expression is directly regulated by HOXA7, we investigated the *TERT* promoter for putative HOX binding sites. Using MathInspector, we identified three possible binding sites for HOXA members 1.5 kb, 1.2 kb and 0.7 kb upstream of the transcriptional initiation site (Fig. 5A). We performed chromatin immunoprecipitation (ChIP) experiments with transiently expressed FLAG-tagged murine Hoxa7 to examine intracellular HOXA7 binding to *TERT* promoter sites in SEM cells. FLAG tagged murine Hoxa7 was used because of a lack of HOXA7 antibodies suitable for ChIP. FLAG-tagged Fhl2 served as a specificity control. The putative HOXA binding sites mapped around -1.3 kb and -0.7 kb in the *TERT* gene and were named region I and II, respectively. The analysis demonstrated FLAG-

Hoxa7 binding to region I, but not to region II (Fig. 5B), suggesting that human HOXA7 binds to the region between -1kb and -1.5 kb.

Knockdown of AML1/MTG8 Fusion Gene Expression Reduces TERT

We investigated whether the downstream effect of *MLL/AF4* on *TERT* also applies to other leukemic fusion genes, such as *AML1/MTG8* in t(8;21)-positive AML. Similar to *MLL/AF4*, siRNA-mediated suppression of *AML1/MTG8* impairs the maintenance of the leukemic phenotype, both in cell culture and in a xenotransplantation model (12, 25, 26). Indeed, *AML1/MTG8* knockdown greatly reduced leukemic clonogenicity in t(8;21)-positive Kasumi-1 cells indicating a compromised leukemic self renewal (Fig. S4). In addition, prolonged AML1/MTG8 depletion over a period of 7 – 12 days caused elevated levels of the CDK inhibitor $p27^{KIP1}$ (CDKN1B), hypophosphorylation of RB and a six fold increase in cells positive for senescence-associated β-galactosidase (Fig. S4).

Flow fish analyses have demonstrated that AML cells with chromosomal abnormalities have particularly short telomeres, but substantially increased *TERT* levels compared to cells with normal karyotype (27). In agreement with these observations, Kasumi-1 cells have very short mean telomere lengths of less than 3 kb (Fig. S4). Notably, *AML1/MTG8* knockdown resulted in a further telomere shortening by 30%, indicating that AML1/MTG8 is involved in telomere maintenance. Furthermore, it implicates that the observed senescent phenotype may be of replicative nature. We hypothesized that this telomere attrition maybe attributable to a link between AML1/MTG8 and telomerase. Thus, we examined *TERT* transcript and protein levels in siRNA-mediated suppression of AML1/MTG8-expressing Kasumi-1 cells. In contrast to the nuclear localisation of AML1/MTG8, TERT showed

a predominantly cytosolic distribution in Kasumi-1 cells. Depletion of AML1/MTG8 correlated with a more than twofold reduction in TERT protein and transcript levels (Fig. 6A, B). Conducive with cell line specificity of fusion gene-specific siRNAs, siAML1/MTG8 did not affect *TERT* expression in MLL/AF4-expressing SEM cells and siMLL/AF4 had no effect on *TERT* expression in Kasumi-1 cells lacking MLL/AF4.

Unlike in MLL/AF4-expressing SEM cells, none of the HOXA7 siRNAs affected *TERT* in AML1/MTG8-expressing Kasumi-1 cells, despite causing a twofold reduction of *HOXA7* in these cells (Fig. S4). This lack of effect may be explained by the finding that Kasumi-1 cells express only very low transcript levels of *HOXA7* and other *HOXA* members (Fig. S2). Thus, HOXA7 has no influence over the regulation of *TERT* expression in AML1/MTG8 positive cells. Overall, AML1/MTG8 regulates leukemic self-renewal and expansion by controlling the expression of *TERT* independently of HOXA7 levels.

Discussion

Leukemic fusion genes, coding for transcription factors, not only inhibit differentiation but support pre-leukemic and leukemic cell proliferation and clonogenicity. Here we demonstrate that the two fusion genes, *MLL/AF4* and *AML1/MTG8,* maintain *TERT* expression, thus establishing a functional link between a leukemic fusion oncogene and a key component of the self renewal program. Furthermore we show that HOXA7, a direct transcriptional target of MLL/AF4, regulates *TERT.*

By utilising RNA interference and implementing fusion gene-specific siRNAs, we found that knockdown of *MLL/AF4* and *AML1/MTG8* reduced *TERT* transcript levels more than two-fold, which corresponded with the reduced telomerase activity observed. Additionally, senescence-associated characteristics, such as β-galactosidase and telomere shortening, were observed after AML1/MTG8 depletion. We recently reported similar findings after prolonged siRNA-mediated depletion of AML1/MTG8, which lead to a cell cycle arrest in G1 and subsequently to cellular senescence (25). Since telomerase restores telomere ends and prevents replicative senescence, we consider the senescence-associated characteristics to be attributable to the reduced TERT protein levels reported. Similarly, Ewing sarcoma-associated fusion proteins (i.e. EWS/FLI1) also influenced TERT protein levels and interfered with the development senescence in Ewing Sarcoma (28, 29). Maintaining *TERT* expression and preventing replicative senescence may be a mechanism shared by many fusion genes to support malignant self-renewal. Given the influence fusion genes have on *TERT* expression, and since fusion-driven leukemogenesis is an early initiating event, it is thought that self-renewal mechanisms were

compromised at a very early stage in leukemia development. Indeed, in a previous study, ectopic expression of *AML1/MTG8* led to the self-renewal of immature human hematopoietic stem/progenitor cells, and was associated with ongoing telomerase activity and maintained telomere lengths compared to control cells (13).

TERT expression is unlikely to be controlled by either *MLL/AF4* or *AML1/MTG8* fusion genes directly. Since the AML1/MTG8 fusion protein acts mainly as a transcriptional repressor (15, 16), the parallel reduction of AML1/MTG8 and TERT levels suggests *TERT* being an indirect transcriptional target for this fusion protein. In contrast, MLL/AF4 activates transcription and enhances gene expression by the recruitment of a histone H3K79 methyltransferase, DOT1L, which catalyzes the dimethylation of histone H3 Lys 79 (H3K79me2) at the promoter sites of target genes such *HOXA9* (30). Although global ChIP analysis found that the *TERT* gene displays a H3K4me3 and H3K79me2 signature in t(4;11)-positive SEM cells, indicative of active *TERT* expression, it failed to identify any direct interaction of MLL or MLL/AF4 with the *TERT* gene (23). Possible mediators of MLL/AF4-exerted control of telomerase are members of the *HOXA* cluster, which are direct transcriptional targets of this fusion protein (23). Upregulation of *HOXA* genes are thought to be essential for leukemic transformation and self-renewal by most MLL fusion genes (31-33). Global gene expression analyses, in both murine models and in human leukemias, regard *HOXA9* and *HOXA10* and, to a somewhat lesser extent, *HOXA7* as a signature of stemness (11, 21, 34-36). Indeed, continuous *HOXA* expression has been found to block differentiation and to promote leukemic cell expansion (37). Moreover, transcriptional activation of Meis1 in combination with either Hoxa7 or Hoxa9 promoted leukemogenesis in mice (38). However, despite their established

role in leukemic transformation and self-renewal, only a few target genes have been identified for HOXA9 and HOXA10 (39-42), and hardly any for HOXA7 (43).

In our study, a reduction in telomerase activity and *TERT* transcript levels was observed when HOXA7 was silenced in MLL/AF4 cells, suggesting HOXA7 as a mediator of MLL/AF4-exerted control of *TERT* expression. In concordance with this finding, chromatin immunoprecipitation analysis demonstrated that murine Hoxa7 binds to the *TERT* promoter. Thus, *TERT* is a direct target gene for HOXA7. However, we cannot rule out that MLL/AF4 may also control *TERT* in a *HOXA7*-independent manner.

In contrast to MLL/AF4, HOXA7 does not play a role in the regulation of *TERT* by AML1/MTG8. HOXA7 siRNAs had no effect on *TERT* transcript levels in AML1/MTG8-expressing Kasumi-1 cells, which are a good control to rule out a possible off-target effect of siRNAs being responsible for the reduction of *TERT* expression in MLL/AF4-expressing SEM and RS4;11 cells. This result correlates with the very low expression of the posterior *HOXA* genes observed in t(8;21)-positive AML cells (44). Since *TERT* expression has been found to be tightly linked to S phase of the cell cycle, and mediated by the RB-E2F axis (45), it may be tempting to speculate that AML1/MTG8 maintains telomerase activity by exploiting RB-mediated *TERT* expression. Indeed, *AML/1MT8G* knockdown results in elevated levels of the CDK inhibitor $p27^{KIP1}$ and hypophosphorylation of RB, indicative of cellular senescence (25).

Sustained *TERT* expression and telomerase activity is likely to be one mechanism by which fusion gene-encoded transcription factors drive malignant self-renewal. Although expression of *TERT*/telomerase is necessary for human stem cells, its

expression alone is not sufficient to maintain self-renewal. It is instead likely that these fusion genes manipulate a larger core programme in cancer stem cell maintenance, with *TERT* being a prominent member of it. A functional comparison of the role that fusion genes play in self-renewal will help to further elucidate this programme, and explore its therapeutic implications.

In conclusion, two leukemic fusion genes, *MLL/AF4* and *AML1/MTG8*, associated with impaired differentiation and self-renewal, sustain *TERT* expression. We have thus found a link between leukemic transcriptional programmes and a key factor for self-renewal (Fig. 6C). These results provide further proof of the significance in fusion genes for leukemic self-renewal and maintenance. Consequently, manipulating leukemic cell maintenance by interfering with the function of fusion genes, such as *MLL/AF4* or *AML1/MTG8,* may represent a future novel therapeutic target that could improve the clinical outcome of leukemia patients.

Material and Methods

siRNA transfection and zVAD treatment:

Cell lines were serially electroporated with siRNA as described (25). SEM and RS4;11 cells were electroporated every second day, Kasumi-1 cells every fourth day. Oligoribonucleotides have been synthesised by Alnylam Europe (Kulmbach, Germany), Qiagen (Crawley, UK) and Purimex (Grebenstein, Germany). The sequences of the siRNAs siMLL/AF4, siMLL/AF4-2, siMM, siAML1/MTG8 and siHOXA7 have been described previously (12, 14, 46); siHOXA7-8: sense, 5'-GCCCUGAUGUUUCCUAUAATT-3'; antisense 5'-UUAUAGGAAACAUCAGGGCGT-3'. Where indicated, cells were supplemented with the pan-caspase inhibitor *Z-Val-Ala-Asp(OCH$_3$)-Fluoromethylketone* (zVAD-FMK, BIOMOL Intl.) at a final concentration of 50 µM for up to 96 h. Addition of the inhibitor occurred immediately after each siRNA electroporation.

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Figure Legends

Figure 1: MLL/AF4 knockdown impairs telomerase activity and reduces *TERT* levels.

(A) Electroporation with siMLL/AF4 reduces MLL/AF4 protein levels. (B) Real-time PCR assay for telomerase activity in SEM-treated cells. (C) *MLL/AF4* knockdown reduces TERT protein levels. (D) Knockdown of *MLL/AF4* affects *TERT*, but not *TERC* transcript levels. Error bars indicate standard error mean (sem) (n = 3). t(4;11)-positive SEM cells were electroporated with the indicated siRNAs as describe in Materials and Methods. Protein levels were analysed 2 days after the third electroporation by immunoblotting. Transcript levels and telomerase activity were analysed two days after the second electroporation.

Figure 2: MLL/AF4 knockdown affects telomerase and *TERT* in two different t(4;11) cell lines

MLL/AF4 variant-specific siRNAs affect *MLL/AF4*, *HOXA7* and *TERT* transcript levels in a sequence-specific manner in (A) SEM cells expressing the e9-e4 variant of *MLL/AF4* and in (B) RS4;11 cells expressing the e10-e4 variant of *MLL/AF4*. Transcript levels were analysed two days after the third electroporation by real-time PCR. Error bars indicate sem (n = 3). (C) Fusion site specific *MLL/AF4* knockdown inhibits telomerase. Telomerase activity was analysed two days after the third electroporation by real-time PCR. Error bars indicate sem (n = 3).

Figure 3: Inhibition of apoptosis enhances *TERT* reduction caused by *MLL/AF4* suppression.

(A) Quantitation of apoptotic cells in dependence on siRNA and zVAD-FMK treatment. Apoptotic hypodiploid cells were quantified two days after the second electroporation by flow cytometry. Error bars indicate sem (n = 4). (B) Combined treatment with siRNA and zVAD-FMK. Expression levels of *MLL/AF4*, *HOXA7* and *TERT* were analysed two days after the second electroporation by real-time PCR. Error bars indicate sem (n = 3).

Figure 4: HOXA7 knockdown reduces *TERT* levels.

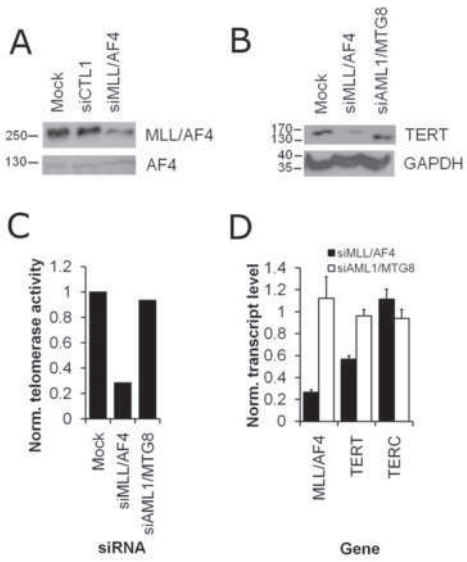
(A) Effects of HOXA7 siRNA on *MLL/AF4*, *HOXA7* and *TERT* transcript levels in (A) SEM cells and in (B) RS4;11 cells. Transcript levels were analysed two days after the third electroporation by real-time PCR. Error bars indicate sem (n = 3). (C) *HOXA7* knockdown inhibits telomerase in SEM and RS4;11 cells. Telomerase activity was analysed two days after the third electroporation by real-time PCR. Error bars indicate sem (n = 3). (D) An alternative HOXA7 siRNA reduces *HOXA7* and *TERT* transcript levels in SEM cells. Transcript levels were analysed two days after the second electroporation by real-time PCR.

Figure 5: Hoxa7 binds to the *TERT* promoter.

(A) Scheme of the *TERT* promoter region. HOXA binding sites suggested by MathInspector are indicated by black semi-circles. The amplicons used for ChIP are marked by arrows, their names by the roman numbers below. TSS, transcriptional start site. (B) Chromatin immunoprecipitation of *TERT* promoter sequences bound to Flag-tagged Hoxa7. SEM cells were transiently transfected with either a plasmid coding a FLAG-tagged Hoxa7 or a plasmid coding a FLAG-tagged Fhl2 as specificity control. The treatments and the names of the amplicons are indicated on the top.

Figure 6: AML1/MTG8 regulates *TERT* expression.

(A) AML1/MTG8 knockdown inhibits *TERT* expression. *TERT* transcript levels were analysed by real-time PCR four days after the second electroporation with the indicated siRNA. Error bars indicate sem (n = 3). (B) *AML1/MTG8* knockdown reduces TERT protein levels. Protein levels were analysed by Western blotting four days after the second siRNA electroporation. C, cytosolic lysates; N, nuclear lysates. Molecular weights of prestained protein markers are indicated on the right. (C) MLL/AF4 and AML1/MTG8 support leukemic self-renewal by maintaining *TERT* expression and telomerase activity. In contrast to AML1/MTG8, MLL/AF4 controls *TERT* via its direct transcriptional target gene *HOXA7*.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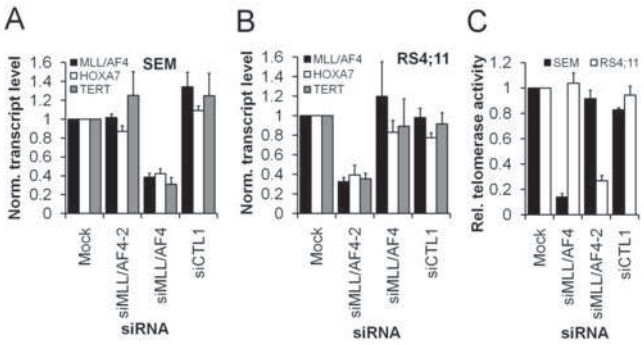
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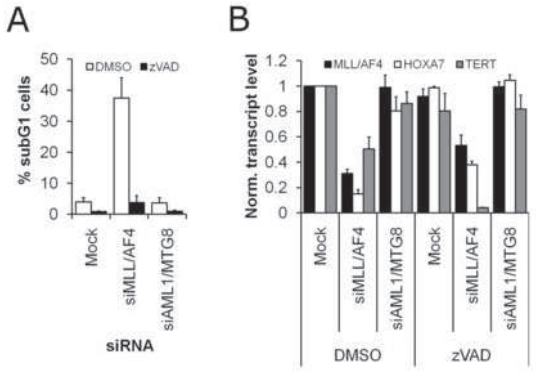
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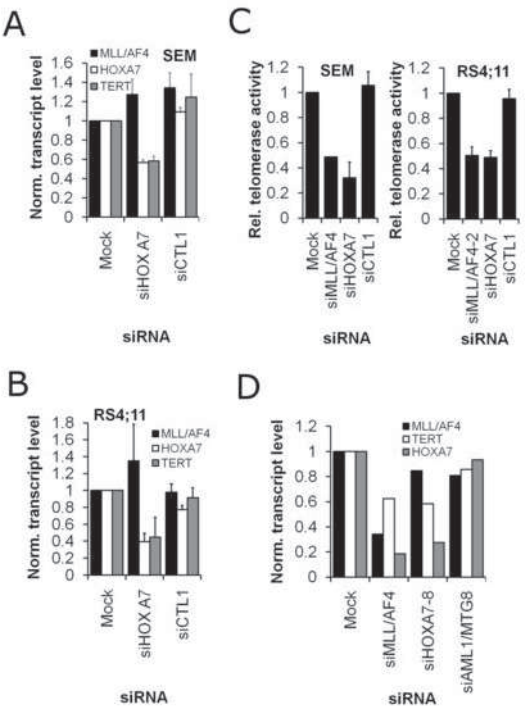
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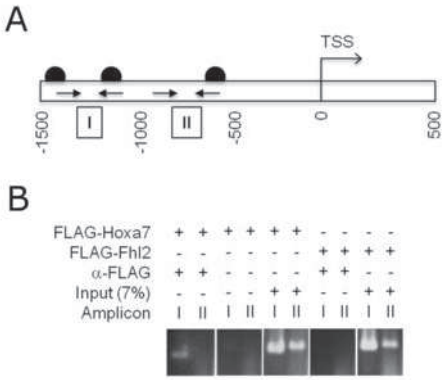
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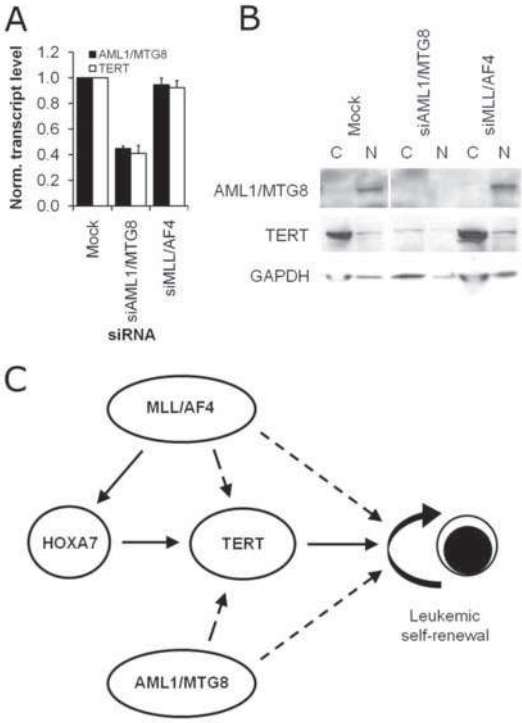
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Leukemic
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AML1/MTG8