RNAi-mediated silencing of MLL-AF9 reveals leukemia-associated downstream targets and processes

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Abstract

Background: The translocation t(9;11)(p22;q23) leading to the leukemogenic fusion gene MLL-AF9 is a frequent translocation in infant acute myeloid leukemia (AML). This study aimed to identify genes and molecular processes downstream of MLL-AF9 (alias MLL-MLLT3) which could assist to develop new targeted therapies for such leukemia with unfavorable prognosis.

Methods: In the AML cell line THP1 which harbors this t(9;11) translocation, endogenous MLL-AF9 was silenced via siRNA while ensuring specificity of the knockdown and its efficiency on functional protein level.

Results: The differential gene expression profile was validated for leukemia-association by gene set enrichment analysis of published gene sets from patient studies and MLL-AF9 overexpression studies and revealed 425 differentially expressed genes. Gene ontology analysis was consistent with a more differentiated state of MLL-AF9 depleted cells, with involvement of a wide range of downstream transcriptional regulators and with defined functional processes such as ribosomal biogenesis, chaperone binding, calcium homeostasis and estrogen response. We prioritized 41 gene products as candidate targets including several novel and potentially druggable effectors of MLL-AF9 (AHR, ATP2B2, DRD5, HIPK2, PARP8, ROR2 and TAS1R3). Applying the antagonist SCH39166 against the dopamine receptor DRD5 resulted in reduced leukemic cell characteristics of THP1 cells.

Conclusion: Besides potential new therapeutic targets, the described transcription profile shaped by MLL-AF9 provides an information source into the molecular processes altered in MLL aberrant leukemia.

Keywords: Acute myeloid leukemia, MLL-AF9, Fusion gene, siRNA, Gene expression, Therapeutic targets, Molecular processes, SCH39166, Ecopipam

Background

The MLL gene has been found translocated to over 50 different partner genes in acute leukemia. Certain partner genes are associated with distinct leukemia subtypes, e.g. MLL-AF4 with pro B acute lymphoblastic leukemia (ALL) and MLL-AF9, -AF6 and -AF10 with acute myeloid leukemia (AML) of M4 and M5 subtypes (French–American–British classification) [1]. MLL-AF9 (alias MLL-MLLT3) results from the translocation t(9;11) (p22;q23) and is sufficient to initiate acute leukemia in murine models with potential secondary mutations being rapidly acquired [2,3]. MLL and AF9 wildtype proteins play essential roles in embryogenesis and hematopoiesis [4-6] and are parts of protein complexes leading to transcriptional initiation (MLL) and elongation (AF9) of target genes [7,8]. The fusion protein MLL-AF9 is believed to combine these properties, leading to increased activation of target genes via transcriptional initiation and elongation. MLL-AF9 is the most frequent fusion gene in infant AML and especially associated with monoblastic AML (M5) [9,10]. New targeted therapies are needed for this type of leukemia with poor prognosis.

Novel therapeutic strategies which aim to intervene with DNA binding of MLL directly or with the assembly of MLL fusion protein with elongation complexes have been discussed [7]. However, drug targeting of protein-protein

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or protein-DNA interactions is difficult as can be seen by the fact that they are not frequently targeted by approved drugs [11]. An additional concern is that the wildtype functions of the involved proteins would be abrogated as well, leading to toxicity [7].

For these reasons, we specifically explored downstream effects of MLL-AF9 in order to identify new alternative drug targets for MLL-AF9 positive AML. Some previous studies have analyzed targets of MLL-AF9 in in vivo mouse models [3,12,13] or through lentiviral MLL-AF9 transduction [14]. MLL-AF9 expression and the associated leukemogenic potential is significantly higher after retroviral transduction as compared to MLL-AF9 knockin and impacts biologic properties like myeloid colony formation and long term self-renewal capacity [3]. We thus altered the endogenous level of MLL-AF9 through specific and efficient siRNA knockdown in the human monoblastic cell line THP1 and studied the downstream effects. The comprehensive gene expression profile after MLL-AF9 depletion suggested several cellular processes and 41 genes as likely mediators of MLL-AF9 leukemogenic effects. Among those, seven gene products were selected as candidate drug targets. Functional relevance of one of these, the dopamine receptor DRD5, was confirmed as an antagonist resulted in reduced leukemic cell characteristics of THP1 cells.

Results
Specific siRNA knockdown of MLL-AF9
To specifically target MLL-AF9 without disturbing MLL and AF9 wildtype expression levels, siRNAs were designed to target the THP1 specific fusion point of MLL-AF9 transcript (Figure 1A). Off-target effects were controlled by utilizing two different MLL-AF9 specific as well as two different non-targeting control siRNAs. An experimental setup with prolonged knockdown of MLL-AF9 (over 8 days) was chosen, because (1) the half-life of MLL-AF9 protein is unknown and (2) MLL-AF9 is believed to lead to changes in the expression of target genes via epigenetic mechanisms [7,8] whose reversal may take a long time [15] thus leading to a delayed effect on transcriptional level.

Mean transfection efficiency of THP1 cells was 93% and mean cell viability after transfection was likewise 93%. Knockdown of MLL-AF9 reduced the transcript levels on day 8 of experiments to 22.3 ± 6% residual expression (Figure 1B). MLL and AF9 wildtype transcript levels were not significantly altered.

To ensure an effective MLL-AF9 knockdown on functional protein level, we quantified HOXA9. Transcription of HOXA9 is raised by MLL-AF9 through direct interaction between MLL-AF9 protein complex and the HOXA9 promoter [16,17]. HOXA9 mRNA was reduced to 56.9 ± 8% residual expression on day 8 of MLL-AF9 knockdown (Figure 1B). Additionally, immunoblotting confirmed an efficient reduction of MLL-AF9 on protein level (Figure 1C).

Differentially expressed genes after MLL-AF9 knockdown
The prolonged knockdown of MLL-AF9 in THP1 cells yielded 571 probes representing transcripts of 425 genes as differentially expressed between knockdown and control treatments (Additional file 1, includes all accession numbers). Gene expression profiling data have been deposited in NCBI’s Gene Expression Omnibus (GEO) [18] and are available under the accession number GSE36592.

A number of previously defined criteria [19] supported the high quality of our gene expression data. These included the presence of important marker genes as expected for the study (i.e. probes detecting MLL-AF9 and transcripts of the HOXA cluster), a reasonable number of differentially expressed genes which were enriched in certain biological processes as well as a significant correlation between microarray and RT-qPCR data of independent experiments (p = 0.004, Spearman’s Rho 0.72).

We further validated our entire expression data set for concordance with known gene regulatory effects of MLL-AF9 via gene set enrichment analysis (GSEA). We detected significant enrichments of direct MLL-AF9 targets identified in a mouse model [13] (Figure 2A) and of genes downstream of MLL-AF9 identified in transduced primary human cells [14] (Additional file 2). In the latter, we observed a stronger enrichment of gene sets generated from human neonatal CD34+ cells as compared to adult CD34+ cells.

Genes differentially expressed after MLL-AF9 knockdown are associated with leukemia
The functional disease ontology database (FunDO) contains curated preexisting evidence on genes associated with disease entities. Applying FunDO analysis on our set of 425 differentially expressed genes, Leukemia was the most significant disease term associated with MLL-AF9 depletion (fold enrichment (FE) = 7.7; Bonferroni corrected p-value = 9.7 × 10^{-11}). GSEA, which takes into account the entire gene expression profile without employing a significance cutoff value, confirmed the leukemia gene set predefined by FunDO as being significantly enriched (Figure 2B).

We further asked if our differential gene expression profile after MLL-AF9 knockdown in THP1 cells is in agreement with preexisting knowledge from in vivo studies. Using GSEA, we found accordence (FDR q-value < 0.05) to MLL-aberrant and myeloid leukemia patient studies [1,20-23] (Table 1 and Additional file 3: Figure S1).

MLL-AF9 regulated genes are involved in defined cellular processes
Functional bioinformatic analysis via DAVID resulted in 31 enriched gene ontology annotation terms with potential biological relevance (Figure 3, Additional file 4). These terms were selected out of 312 significantly enriched
annotations (Additional file 4) by omitting related and redundant terms and by evaluating their biological relevance as recommended [19]. To structure the results, the enriched annotation terms were manually assorted to functional higher-order terms according to their major role in the biological setting under investigation (Figure 3 and details in Additional file 3: Table S1).

The higher-order term proliferation and apoptosis contained 7 annotation-terms and suggested influence of MLL-AF9 on cell replication (regulation of DNA replication), proliferation (JAK-STAT cascade, response to wounding, cytokine binding) and apoptosis (regulation of apoptosis, protein kinase C activity). Several genes involved in replication or anti-apoptotic processes were down-regulated (CALR, NPM2, POT1, STRA8 and MEF2C, SOCS2, SOX4 respectively) while several genes encoding pro-apoptotic regulators were up-regulated (CEBPB, DUSP1, HIPK2, LCK, NOTCH2, PRKCE, TGFBR1, TRIO, VDR).

The higher-order term monocyte/macrophage differentiation included 16 annotation terms that encompassed fundamental functions of monocytes and macrophages like antigen processing and presentation of peptide or polysaccharide antigen via MHC class II, phagocytosis and marker of the hematopoietic cell lineage. Additionally, we observed a raised expression of the monocytic maturation marker CD14, CEBPB, EGR2, FOS, MAFB, MNDA and MHC class II as well as a reduced expression of markers of immature cells of the monocytic lineage (ELANE and CTSG) [24-27].

| Name   | Sequence                  |
|--------|---------------------------|
| MLL-AF9 | 5'--ACCAAAAGAAAG--UCUGAACAACCAGUC-3' |
| siRNA-A | 3'--UUUCUUUC--AGACUUGUUGG-5' |
| siRNA-B | 3'--GUUUUCUUUU--AGACUUGU-5' |

**Figure 1** siRNA knockdown of MLL-AF9 in THP1 cells. (A) Alignments of siRNAs to MLL-AF9 mRNA. Bold sequence represents the MLL part of the fusion transcript. (B) Confirmation of MLL-AF9 knockdown via RT-qPCR. Mean relative MLL-AF9 and HOXA9 transcript levels of knockdown (siRNA-A, siRNA-B) as compared to control treatments (control siRNA-1, -2) during the time course of experiments. Graph represents data from five independent experiments. Bars indicate standard deviation. (C) Immunoblot of MLL-AF9 from total protein extracted from day 8 experimental samples. Normalized MLL-AF9 levels are indicated at the bottom.
A high number of transcriptional regulators (38 genes) were affected by MLL-AF9 depletion. Several (i.e. CEBPB, FOS and FOSB) were also described to be deregulated in the transition of CML blastic phase to an acute leukemia [23].

Calcium associated genes as well as genes involved in response to estrogen stimulus were strongly enriched after MLL-AF9 depletion, implying an influence of MLL-AF9 on calcium ion homeostasis and estrogen response effects. The enriched annotation chaperone binding contains proteins which interact selectively and non-covalently with a chaperone protein and encompassed four down-regulated genes in our data set. The enrichment of genes within the annotation regulation of cell size is consistent with our phenotypic findings and encompassed the genes AR, ATP2B2, EMP1, LAMB2, NOTCH2, PLXNA3, SGMS1, TGFBR1 and WFDC1.

The higher-order term early development suggests downstream effects of MLL-AF9 analogous to MLL and AF9 wildtype proteins. This concerns effects on pattern specification processes, mainly via homeobox genes. Other enriched annotation terms suggested an influence of MLL-AF9 on genes involved in aging and on genes encoding proteins of the actin cytoskeleton and the extracellular matrix.

GSEA analysis for our entire gene expression profile (which does not employ a significance cutoff value) additionally suggested effects on DNA repair, MAP kinase activity, RNA polymerase activity, splicing, ribosomal constituents and translation (Additional file 2).

We further asked which of the functional processes influenced by MLL-AF9 are due to direct effects of MLL-AF9. For this purpose, we performed a gene ontology enrichment analysis (DAVID) for previously published direct targets of MLL-AF9 derived from a mouse model [13] (Additional file 4) and compared the results to our gene ontology data. The overlap between the data sets suggests that transcriptional regulators such as those

Table 1 Gene set enrichment analysis of leukemia patient studies for our MLL-AF9 knockdown gene expression dataset

| Patient study gene sets | Reference | FDR q-value | NES |
|-------------------------|-----------|-------------|-----|
| Top-100 gene set of MLL AML | Kohlmann et al. [1] | 0.008 | 1.48 |
| Top-100 gene set of MLL leukemia irrespective of lineage | Kohlmann et al. [1] | 0.020 | 1.40 |
| Top-100 probe set of pediatric MLL AML | Ross et al. [20] | 0.022 | 1.38 |
| Downregulated in MLL AML | Rozovskaia et al. [21] | 0.005 | 1.69 |
| Gene cluster 1 and 16 of 11q23 abnormal AML | Valk et al. [22] | 0.030 | 1.34 |
| CML-BP versus CML-CP | Zheng et al. [23] | <0.001 | 1.96 |
| Downregulated in CML-BP versus CML-CP | Zheng et al. [23] | <0.001 | 2.08 |

Corresponding gene set enrichment plots are displayed in Additional file 3: Figure S1. MLL AML, MLL aberrant acute myeloid leukemia; CML-BP, chronic myeloid leukemia blastic phase; CML-CP, chronic myeloid leukemia chronic phase; FDR, false discovery rate; NES, normalized enrichment score.
involved in early development (homeodomain genes) are mainly direct MLL-AF9 targets. On the other hand, the majority of processes we identified as affected by MLL-AF9 depletion (like apoptosis, calcium and estrogen related genes and monocyte/macrophage differentiation) are likely governed by indirect effects of MLL-AF9.

**MLL-AF9 knockdown is associated with reduced cell size**

We did not observe effects of MLL-AF9 depletion on proliferation, cell cycle distribution and apoptosis rate of THP1 cells (Additional file 3: Figure S2). However, we consistently detected a significant reduction of 0.2 μm in mean cell diameter between MLL-AF9 knockdown and control treatments (Figure 4A). MLL-AF9 specific reduction in cell size was evident under serum reduced conditions and prolonged MLL-AF9 knockdown but not in the presence of 10% FCS (up to day 8) or upon a shorter time frame (up to day 3).

Cell size has previously been described to be closely linked to ribosomal biogenesis [28]. GSEA indeed revealed genes encoding structural constituents of ribosomes to be significantly enriched in down-regulated genes after MLL-AF9 knockdown in THP1 cells (Figure 4B). The core enrichment gene set was represented by 42 ribosomal proteins of small and large subunits of both, cytoplasmic and mitochondrial ribosomes.

**Target genes likely mediating MLL-AF9 leukemogenic effects**

Among the 425 differentially expressed genes, 41 candidates for mediation of MLL-AF9 leukemogenic effects were prioritized by a stepwise approach (Figure 5). Selected were (1) genes with strongest differential expression (≥ ±1.0 log2 fold change (log2FC)), (2) leukemia associated genes defined by functional disease ontology (FunDO), (3) the strongest regulated (≥ 1.0 log2FC and further top 5) genes of each higher-order term from functional gene annotation results.
and (4) genes that showed concordant differential expression between our in vitro data and published leukemia patient studies. Due to overlapping hits, this added up to 70 genes which were subsequently subjected to literature research and rated via a structured strategy (Additional file 3: Table S2). This approach resulted in 41 genes being rated as likely mediators of leukemogenic effects of MLL-AF9 (Figure 6).

Receptors, including nuclear receptors (i.e. ligand-activated transcription factors), enzymes and transporter have yielded especially successful therapeutic targets for clinical intervention [11]. Thus, we judged those among our 41 top rated genes which encode receptors, enzymes or transporter as potential therapeutic targets downstream of MLL-AF9. An overview of the relevant biological roles of these seven potential therapeutic targets, including the dopamine receptor DRD5, is presented in Table 2.

The dopamine receptor antagonist SCH39166 exerts effects on malignant cell characteristics of THP1 cells
As a proof of principle for the functional relevance of the suggested therapeutic targets, we examined the effects of the DRD1- and DRD5-specific dopamine receptor antagonist SCH39166 (ecopipam) on THP1 cells.

Treatment of THP1 cells with 10 μM SCH39166 led to a number of significant changes in cell characteristics: (1) proliferation and (2) colony forming capacity were reduced, (3) cell cycle analysis revealed alternating changes in G1 and S phase distributions which may suggest a slowdown of cell cycle progression, (4) cells were slower to reach G0/G1 phase after DNA new synthesis which was reduced and (5) the cell migration rate was decreased (Figure 7). No increase in the rate of apoptotic THP1 cells was observed (Additional file 3: Figure S3).

Discussion
The experimental setup of this RNA interference study was optimized to exclude off-target effects and to ensure a specific reduction of MLL-AF9 protein on functional level. Among seven human cell lines carrying the MLL-AF9 translocation, the cell line THP1 was chosen for this study because it is the only one established from a characteristic infant AML M5 leukemia patient [29]. According to genomic DNA gains and losses as well as gene expression, THP1 – like other leukemia cell lines – has been described to be a faithful model system for leukemia with genomic aberrations [30]. As compared to overexpression studies,
RNAi enabled us to modify MLL-AF9 at physiological range and to target all putative transcript variants of MLL-AF9 which retain the breakpoint exons.

The validity of the generated differential gene expression profile as well as the suitability of our experimental approach was supported by the significant enrichment of several gene sets generated by previous studies: Leukemia was the top ranked associated disease term, and direct MLL-AF9 murine targets [13] as well as MLL-AF9 downstream genes detected in transduced neonatal and myeloid as well as lymphoid human primary cells [14] were significantly enriched. These findings validate our experimental approach in that expected information content was indeed disclosed in our data. It also indicates that our MLL-AF9 knockdown model represents neonatal and mixed lineage features as expected for MLL-AF9 positive leukemia.

Additionally, concordance of our in vitro model system to the in vivo situation was suggested by enrichment of several gene sets generated by previous studies: Leukemia was the top ranked associated disease term, and direct MLL-AF9 murine targets [13] as well as MLL-AF9 downstream genes detected in transduced neonatal and myeloid as well as lymphoid human primary cells [14] were significantly enriched. These findings validate our experimental approach in that expected information content was indeed disclosed in our data. It also indicates that our MLL-AF9 knockdown model represents neonatal and mixed lineage features as expected for MLL-AF9 positive leukemia.

To characterize biological processes influenced by MLL-AF9, we performed functional studies in our experimental system and employed functional gene ontology analyses on our MLL-AF9 modulated gene expression profile which encompassed 425 differentially expressed genes. Most remarkable in our functional results was a significant reduction of cell size in MLL-AF9 depleted THP1 cells. Enriched differential expression of genes within the annotation regulation of cell size (e.g. ATP2B2 and EMP1) was consistent with this observation. The annotation terms structural constituents of ribosomes and cellular calcium ion homeostasis were also linked to our data set and could contribute to the observed cell size reduction. Ribosomal biogenesis is a known important determinant of cell size [28] and has been linked to tumorigenesis and malignancies [31], while intracellular Ca^{2+} levels can regulate...
reactive volume changes of cells via activation of K⁺ and Cl⁻ channels [32].

Enrichment of annotation terms related to proliferation and apoptosis implied that MLL-AF9 is involved in replication and cell death via downstream transcripts. Previous studies have detected effects of MLL-AF9 on these cellular characteristics after treatment with antisense phosphorothioate-oligodeoxyribonucleotides [33,34]. In our hands, however, neither the proliferation rate nor the proportion of apoptotic THP1 cells was detectably altered after MLL-AF9 knockdown. This might be due to favorable in vitro growth conditions and the absence of sufficient apoptosis stimuli in our cell culture situation. Nevertheless, the differential gene expression signature generated in this study yielded genes which can influence apoptosis and might contribute to the mitogenic phenotype of MLL-AF9 positive monoblasts in a less ideal environment possibly present in the in vivo disease state. These genes encode for example POT1 which promotes telomere elongation [35], CALR which promotes DNA synthesis and inhibits growth arrest and senescence (via p21) [36] and HIPK2 which is involved in induction of cell death and differentiation [37].

MLL-AF9 is regarded as a class II mutation, primarily interfering with cellular differentiation [38]. In line with this, a major effect of MLL-AF9 on monocytic differentiation was also revealed by our data: Numerous significantly enriched, functional annotation terms concerned fundamental functions of monocytes and macrophages. Moreover, the differential expression of nine marker genes clearly suggested maturation of MLL-AF9 depleted THP1 monoblasts. This implies that the MLL-AF9 induced differentiation stop is, at least in part, reversible. It is thus tempting to speculate that therapeutic differentiation strategies as successfully employed for acute promyelocytic leukemia [39] might also be feasible for MLL aberrant AML.

The enriched annotations response to estrogen stimulus and chaperone binding might also be related to leukemogenic mechanisms. MLL-AF9 might inhibit estrogen receptor degradation and retinoic acid signaling pathways leading to impaired cellular differentiation: AHR and MAPK15 which are reported to target estrogen receptor alpha to degradation [40,41] were reduced in their expression by MLL-AF9 in our study. Estrogen and retinoic acid signaling are known to regulate gene expression in opposing directions [42]. Furthermore, promoter methylation (indicating transcriptional repression) of estrogen receptor is associated with increased survival of AML patients and reduced in AML M5 [43]. Chaperones have been linked to deregulated cell growth as forced overexpression of chaperones was shown to result in cellular transformation and tumor formation [44]. Within our data set, all four chaperone binding genes were down-regulated. One of these, calreticulin, encodes a multifunctional protein which acts also as chaperone and is known to inhibit the translation of CEBPA, a key myeloid transcription factor frequently disrupted in AML [45].

### Table 2 Genes deregulated by MLL-AF9 and prioritized as potentially druggable targets in MLL-AF9 leukemia

| Gene   | log₂ FC | Protein class                  | Official full name                      | Biological role                                                                 |
|--------|---------|--------------------------------|----------------------------------------|----------------------------------------------------------------------------------|
| AHR    | 0.44    | Nuclear receptor               | Aryl hydrocarbon receptor              | Upregulated by AML associated fusion gene AML1-ETO. Differentiation of myeloblastic leukemia cells. Estrogen receptor degradation. AHR knockout mice display CML. |
| ATP2B2 | −0.69   | Transporter                    | ATPase, Ca++ transporting, plasma membrane 2 | Lowers intracellular calcium; protects from apoptosis.                           |
| DRD5   | −0.96   | Receptor                       | Dopamine receptor D5                   | Raised after G-CSF treatment; dopamine receptor agonists activate Wnt signaling, induce migration and increase clonogenic capacity and repopulation of CD34+ cells. |
| HIPK2  | 0.5     | Enzyme                         | Homeodomain interacting protein kinase 2 | Phosphorylates transcription (co-) factors (e.g. c-Myb); may trigger (myeloid) differentiation and apoptosis. Mutations found in AML cases. |
| PARP8  | −0.63   | Enzyme                         | Poly (ADP-ribose) polymerase family, member 8 | Phosphorylated upon DNA damage. Upregulated in MLL rearranged AML patients.       |
| ROR2   | 0.81    | Receptor/enzyme                | Receptor tyrosine kinase-like orphan receptor 2 | Mediates noncanonical Wnt signaling. Putative tumor suppressor in leukemia, presumably via inhibition of Wnt canonical signaling. |
| TAS1R3 | −2.01   | Receptor                       | Taste receptor, type 1, member 3        | Glucose absorption/energy supply. Heterodimers sense extracellular amino acids, activate MTORC1 and inhibit autophagy. |

Details to biological roles and references are given in Additional file 3: Table S2. Log₂ FC: log₂ fold change in MLL-AF9 knockdown relative to control.
A profound effect of MLL-AF9 on downstream gene expression was suggested by significantly enriched annotations encompassing 38 differentially expressed transcription factors. One example is the up-regulation of CIITA, the master coactivator of MHC class II [46]. Accordingly, in MLL-AF9 depleted THP1 cells, we observed an up-regulation of all MHC class II genes (HLA-DP, -DQ, -DR, -DM) with exception of the non-classical HLA-DO which seems less dependent on the CIITA transcription factor [46]. Up-regulation of HLA class II gene transcripts has previously been associated with differentiation of monoblasts, while THP1 cells were observed to have reduced HLA class II expression levels compared to mature monocytes [24].

Results from functional annotation analysis indicated that MLL-AF9’s direct targets are predominantly transcriptional regulators while other processes we identified, like monocyte/macrophage differentiation or estrogen response are likely related to indirect effects of MLL-AF9. Although considerable progress has been made, transcription factors have been found difficult to target therapeutically [47]. This emphasizes the importance of determining indirect targets downstream of MLL-AF9 to identify MLL-AF9 leukemogenic effectors amenable to be addressed by small molecule drugs.

We are aware that our prioritization strategy to select candidate targets is not all-inclusive. Some candidates may be lost during the primary subset selection or by our

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**Figure 7** THP1 cell characteristics influenced by 10 μM of the dopamine receptor antagonist SCH39166 compared to DMSO control. (A) Proliferation in serum reduced conditions. (B) Colony formation in methyl cellulose. Images show representative wells of DMSO control (left) and 10 μM SCH39166 treatment (right). (C) Cell cycle distribution in serum reduced conditions. (D) Cell migration in 5 μm transwells. (E) DNA new synthesis rate measured via EdU incorporation and SYTOX AADVanced nuclear stain. Ratio of SCH39166 compared to DMSO control treatment is shown. Mean over three (A-C) or two (D-E) independent experiments are shown. Bars indicate standard error of the mean; *p < 0.05. † Non significant differences because of interexperimental fluorescence intensity standard deviation, although the ratio between the two treatments remains similar.

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The present study provides a comprehensive gene expression profile after MLL-AF9 knockdown in THP1 cells that broadens our insights into the molecular mechanisms and druggability of MLL aberrant leukemia. The finding of a more differentiated state of THP1 cells after MLL-AF9 depletion is consistent with the hypothesis that the MLL-AF9 induced differentiation stop is reversible. We describe here more than 40 gene products and several cellular processes likely involved in mediation of leukemogenic effects of MLL-AF9. Among these, seven targets were classified as potentially druggable and targeting one of these, DRD5, shows anti-leukemic effects in THP1 cells. Altogether, our results might support the search for new targeted therapies for MLL-AF9 positive pediatric AML.

Methods

Cell cultivation

THP1 cells (DSMZ GmbH, Braunschweig, Germany) were maintained in RPMI 1640, 10% heat-inactivated fetal calf serum (FCS), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (PAA Laboratories, Pasching, Austria) at a density of 0.05-0.5 × 10^6 cells/ml. To minimize the effect of FCS on growth related and mitogenic signaling pathways, serum reduced conditions were achieved by washing cells in PBS and resuspending them in DMEM/Ham’s-F12 medium containing 1 g/L BSA and 0.5% Fetal Bovine Serum (Gibco, Life Technologies, Carlsbad, CA, USA). For functional studies of DRD5, THP1 cells were treated with 10 μM SCH39166/ecopipam (Tocris Bioscience, Bristol, UK) or DMSO as control.

Small interfering RNA and transfection

Silencer Select siRNAs (siRNA-A, siRNA-B, negative control siRNA-1 and -2, Ambion, Life Technologies) were dissolved in 1 x siRNA buffer (Dharmacon, Lafayette, CO, USA). SiRNA-A and -B sequences are shown in Figure 1A. Transfections were performed according to the supplier’s protocol with 9 μl Dreamfect (OZ Biosciences, Marseille, France) at a final concentration of 50 nM siRNA within 1 ml culture medium that contained 5 × 10^4 cells in a 12-well format (Greiner, Kremsmunster, Germany). Transfection efficiency was measured by flow cytometry with fluorescently labeled control siRNA.

Experimental incubations lasted eight days with repeated transfections on day 0, 3, and 6. Prior to each transfection event, cell densities were determined by Cellscreen System (Innovatis, Bielefeld, Germany) on an Olympus IX 50 microscope (Olympus, Tokyo, Japan) and cells were subsequently reseeded at 5 × 10^4 cells per ml.

RNA isolation and reverse transcription quantitative PCR

Total RNA was extracted with miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the supplier’s
showed no improved principal component analysis and with ImageJ version 1.46r (National Institutes of Health, Biotechnology, Santa Cruz, CA, USA) followed by detection of proteins using ECL-substrate. Band intensities were analyzed with StepOne software v 2.1 (Applied Biosystems) and the \( \Delta \Delta C_q \) method. Primer sequences are provided in Additional file 3: Table S3.

**Immunoblotting**

Total cellular protein was recovered from Qiazol organic phase according to TRI reagent protein isolation protocol (Ambion), resuspended in 9.5 M urea, 4% [w/v] CHAPS, mixed 1:1 with Laemmli sample buffer (2×, Sigma-Aldrich, St. Louis, MO, USA) and incubated 5 minutes at 95°C. Proteins were separated by SDS-PAGE and transferred onto PVDF membrane in transfer buffer (10 mM CAPS, pH 11, 10% methanol, 0.01% SDS). Membranes were probed with anti-MLL1, anti-AF9 (A300-086A, A300-597A, Bethyl Laboratories, Montgomery, TX, USA), anti-Lamin A/C and anti-MLL1, anti-AF9 (A300-086A, A300-597A, Bethyl Laboratories, Montgomery, TX, USA) followed by detection using ECL-substrate. Band intensities were analyzed with ImageJ version 1.46r (National Institutes of Health, Bethesda, MD, USA).

**Gene expression profiling analysis**

Human Whole Genome Microarrays 4x44K v2 (Agilent Technologies, Santa Clara, CA, USA) were commissioned to and performed at IMGM Laboratories ( Martinsried, Germany). Two knockdown (siRNA-A, siRNA-B) and two negative control siRNA samples, each containing 100 ng pooled RNA of five independent experiments were utilized. RNA concentration and purity (abs 260/280 nm) were analyzed using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity was determined with an RNA 6000 Nano LabChip Kit on a 2100 Bioanalyzer (Agilent Technologies). A260/A280 was above 2.0 and RNA integrity number was above 9.3.

Hierarchical clustering of arrays and principal component analyses were performed with the statistical language R version 2.11.1 [54] using packages from the Bioconductor framework [55]. Analysis of differential expression was carried out in R with the limma package [56,57]. Adjustment for multiple testing was done using the method by Benjamini and Hochberg [58]. For differential expression analysis, the unnormalized data were favored over the normalized as they showed an almost perfect and superior logarithmic intensity distribution. Normalized values showed no improved principal component analysis and hierarchical clustering concerning similarity of biologically related arrays. Unnormalized identical replicate probes yielded more consistent intensity values compared to the normalized values which further supported this approach. Probes were considered as differentially expressed at a \( p \)-value of the moderated \( t \) test below 0.005 and significant alteration over all identical replicate probes (\( t \) test, \( p < 0.05 \)).

To test reliability of microarray data, RT-qPCR was performed for 14 arbitrarily selected transcripts which showed differential expression in microarray results (ARHGAP26, CALR, CEBPB, CIITA, FOS, FUCA1, MAFB, NOTCH2, SOCS2, SULF2, TSPAN14, VASH1, VDR and ZNF521). Spearman’s rank correlation coefficients including \( p \) values were calculated with R. Spearman’s \( Rho \) was 0.91 (\( p = 15 \times 10^{-6} \)) for technical replicates (identical RNA samples in RT-qPCRs and microarrays) and 0.72 (\( p = 0.004 \)) for biological replicates (RT-qPCRs performed on RNA sample pools of 2 independent experiments).

**Functional bioinformatics**

Functional disease ontology (FunDO) [59] and functional annotation analysis (Database for Annotation, Visualization and Integrated Discovery version 6.7, DAVID) [19] was performed with input of all 425 differentially expressed genes. Enrichment was regarded as significant if fold enrichment was \( \geq 1.5 \) and \( p \)-value < 0.1. Functional annotation analysis of direct targets of MLL-AF9 was performed with a previously published gene list [13] transformed to the official gene symbols. Gene set enrichment analysis was executed with the computational method GSEA version 2.0 [60,61]. Gene sets associated to leukemia were extracted from FunDO and from leukemia patient studies [1,20-23] and transformed to the official gene symbols. Parameter settings were default except for: dataset was not collapsed to gene symbols, instead the strongest regulated probe per gene was selected; gene set was selected as permutation type; metric for ranking genes was \( \log 2 \) ratio of classes; gene list sorting mode was either real for gene sets including only up- or down-regulated genes or abs for gene sets including up- and down-regulated genes. A rating strategy for prioritization of genes likely mediating MLL-AF9 leukemogenic effects was devised as outlined in Additional file 3: Table S4.

**Biological assays**

Proliferation and cell diameter were measured via Cellscreen System (Innovatis) on an Olympus IX50 microscope. This microscopic monitoring system provides fully automated non-invasive cell count in cell culture well plates by generating microscopic images at defined regions of interest (ROIs). By digital image recognition, cells are automatically detected, counted and their geometry (diameter and eccentricity) is analyzed [62]. Measurements were performed in 12-well plates (Greiner) with 62 ROIs per well.
equivalent to ~ 5 000 - 10 000 counted cells. Cells were allowed to settle for 20 minutes prior to measurement. A Welch’s t test was performed over replicate experiments after confirmation of normal distribution via Shapiro-Wilk normality test.

Cell cycle analyses were performed via flow cytometry. Cells were fixed in 70% ethanol at −20 °C, subsequently stained for 30 min with propidium-iodide (0.1% Triton X-100, 0.2 mg/ml RNase A, 20 μg/ml propidium-iodide in PBS) and analyzed on a BD FACS Canto™ (BD Biosciences, Franklin Lakes, NJ, USA). Cell doublets and aggregates were removed by gating and the proportion of cells in G0/G1, S and G2 phase were quantified with Watson Pragmatic model in FlowJo 9.7.2. DNA new synthesis rate was measured by flow cytometry after pulse labeling THP1 cells with 25 μM EdU (5-ethynyl-2′-deoxyuridine) for 2 hours and staining with AF488-azide and SYTOX AADvanced nuclear stain (Invitrogen, Life Technologies) according to supplier’s protocol. Statistics were calculated with Shapiro-Wilk normality and Welch’s t test in R.

Apoptotic rate was analyzed via intracellular staining of cleaved PARP1 [63]. Cells were fixed and permeabilized with Foxp3 Staining Buffer Set (eBioScience, San Diego, CA, USA) and stained with anti-PARP antibody (44–699, Invitrogen) after blocking with 5 μg human IgG (Sigma).

Colonies forming capacity was analyzed by plating 1000 THP1 cells in 1 ml 0.5% methyl cellulose (64630, Sigma)/RPMI1640 (10% FCS) in 6-well plates. Colonies were counted in microscopic images taken after 10 days by Cellscreen system.

Migration was analyzed in a transwell assay (5 μm pore size, #3421, Corning, NY, USA) with 0.1 × 10^6 THP1 cells in RPMI1640 / 10% FCS in upper chamber and RPMI1640 with 10% FCS and 0.1 mM ascorbic acid in lower chamber. Cells in lower chamber were counted in microscopic images taken after 20 hours by Cellscreen system.

For analyses of SCH39166 effects, cells were pre-treated with drug or DMSO for 3 hours prior to performing assays.

Competing interests
The authors declare no conflict of interest.

Authors’ contributions
KKF designed the project, conceived and performed the experiments, analyzed and interpreted the data and wrote the manuscript. PP provided analytical tools, analyzed the data and critically revised the manuscript. IS interpreted the data, provided critical discussion and critically revised the manuscript. AAR designed the project, conceived experiments, wrote the manuscript and supervised the project. All authors read and approved the final manuscript. There are no potentially redundant publications.

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