Structure-function analysis of the role of megakaryoblastic leukemia 1 in megakaryocyte polyploidization

Megakaryoblastic leukemia 1 (MKL1; also known as MRTFA, MAL, or BSAC) is a coactivator of serum response factor (SRF). SRF is a transcription factor that participates in the activation of immediate-early genes as well as genes associated with the cytoskeleton, proliferation, and apoptosis by binding promoter sequences known as serum response elements (SRE). The MKL1/SRF complex plays a critical role in megakaryocyte maturation. As megakaryocytes mature, they undergo successive rounds of endomitosis to become highly polyploid; Mkl1 knockout (KO) murine megakaryocytes exhibit reduced polyploidization, a phenotype that is more severe in double KO (dKO) megakaryocytes lacking both Mkl1 and the closely related Megakaryoblastic leukemia 2 (Mkl2, or Mrtfb). Additionally, MKL1 is part of the recurrent t(1;22) chromosomal translocation found in acute megakaryoblastic leukemia, which results in a fusion protein known as RBM15-MKL1. MKL1 and MKL2 each contain five highly conserved domains which have been investigated in the context of SRF coactivation using luciferase reporter assays. However, their functional effects in megakaryocyte polyploidization remain unknown, so structure-function analysis of these domains in primary megakaryocyte polyploidization is needed. Furthermore, the pro-tumorigenic mechanism of RBM15-MKL1 remains unclear, warranting further investigation of the individual proteins’ roles in both normal and pathological megakaryocyte development – particularly considering that all functional domains of MKL1 are retained in the fusion protein. In order to define which domains of MKL1 are necessary for polyploidization, we transduced wild-type (WT), Mkl1 KO and dKO murine bone marrow with domain-specific deletion constructs of MKL1, and analyzed subsequent megakaryocyte polyploidization. Published data have established that Mkl1 KO and dKO megakaryocytes have reduced polyploidization relative to WT cells in vivo. We expanded upon this by demonstrating reduced polyploidization of megakaryocytes differentiated in culture from hematopoietic stem and progenitor cells (HSPC). Murine bone marrow-derived HSPC were cultured for 48 hours in expansion medium (50 ng/mL murine Flt3, 20 ng/mL murine interleukin-3, 100 ng/mL murine stem cell factor, 50 ng/mL murine thrombopoietin in StemSpan [Stemcell Technologies]), then switched into megakaryocyte maturation medium (50 ng/mL murine thrombopoietin) for 4 days. Polyploidization was analyzed via flow cytometry using propidium iodide to visualize DNA content, and anti-CD41 to identify megakaryocytes. In order to capture the full spectrum of polyploidization, the mean fluorescence intensity (MFI) of propidium iodide was determined for each population. MFI was normalized to the WT value within each experiment to account for variations in fluorescence intensity between experiments and cytometers. Mkl1 KO and dKO megakaryocytes showed decreased MFI relative to WT, indicating a lower degree of polyploidization. This decrease was statistically significant for dKO cells (P=0.01, n=4). Mkl1 KO megakaryocytes exhibited a directional decrease in MFI which did not reach statistical significance (P=0.05, n=6) (Figure 1A).

To test whether restoration of Mkl1 expression restores polyploidization, we overexpressed full-length (FL) MKL1 in WT, Mkl1 KO, and dKO megakaryocytes. We cloned FL MKL1 into a pMSCV-GW-RfA-PGK-EGFP retroviral backbone using vectors VB160922-1087ttu and VB160922-1086xsa (VectorBuilder). Constructs were validated in HEL cells via western blot (Online Supplementary Figure S1A). We expanded upon this by demonstrating reduced polyploidization of megakaryocytes differentiated in culture from hematopoietic stem and progenitor cells (HSPC). Murine HSPC were cultured according to the above protocol, and transduced after 24 hours in expansion medium with 8 µg/mL polybrene via spinfection for 1 hour at 800 x g. The transduced megakaryocyte population was gated based on CD41/GFP fluorescence. Representative ploidy histograms are shown in Figure 1C and indicate that overexpression of FL MKL1 increases the number of high ploidy cells. Overexpression of MKL1 resulted in a statistically significant increase in polyploidization (as represented by the MFI of propidium iodide) of cells from each genotype (WT n=5, Mkl1 KO n=6, and dKO n=4), compared to empty vector controls (Figure 1B).

Previous work has defined the roles of each domain in MKL1-mediated regulation of SRF in luciferase reporter assays. Two canonical N-terminal RPEL domains serve as negative regulators of MKL1/SRF by binding to G-actin, which promotes nuclear export. The basic domain contains a nuclear localization signal and promotes dimerization of MKL1 with SRF; the LZ domain permits MKL1 hetero- and homodimerization; and the C-terminal TAD domain is required for transcriptional activation of MKL1/SRF target genes. The SAP domain does not affect MKL1-induced luciferase reporter activity and has no clear role in MKL1 function. While SRF-independent functions have been reported for the SAP domain of MKL1, these findings have not been reproduced.
Figure 1. Effect of MKL1 on polyploidization of primary murine megakaryocytes. (A) Mean fluorescence intensity (MFI) of propidium iodide (PI) for megakaryocytes derived from wild-type (WT), Mkl1 knockout (KO), or double knockout (dKO) murine bone marrow, normalized to the MFI of the WT in each trial (dKO vs. WT: \( P=0.01, n=2 \)). (B) MFI for megakaryocytes derived from WT, Mkl1 KO, or dKO murine bone marrow transduced with empty vector (EV) backbone or full-length (FL) MKL1 retrovirus. Each experiment was normalized to the MFI of the EV (WT: \( P=0.011, n=5 \); Mkl1 KO: \( P=0.007, n=6 \); dKO: \( P=0.028, n=4 \)). (C) Representative ploidy peaks for WT, Mkl1 KO, and dKO marrow-derived megakaryocytes transduced with EV or FL MKL1 retroviruses, with cell number as a function of PI fluorescence. Far right: overlay of the mode-normalized ploidy peaks with EV (blue) and FL (red).

for effects of the SAP domain on megakaryocyte polyploidization in addition to the known functional domains of MKL1. We generated retroviruses encoding mutant MKL1 on the same backbone as above with deletions in each of the five key functional domains (Figure 2A), which were validated by western blot (Online Supplementary Figure S1A).

Mkl1 KO murine HSPC were transduced with each of the MKL1 domain deletion constructs and cultured as above. We found that the ΔRPEL construct significantly increased megakaryocyte polyploidization, as expected. Neither the ΔBasic nor the ΔTAD constructs induced an increase in polyploidization, indicating that these two domains are required for the function of MKL1 in polyploidization. Similar data were obtained for the ΔLZ construct, which was somewhat surprising given the ability of ΔLZ to induce SRE-luciferase reporter activity.1 Taken together, we conclude that the TAD, LZ and Basic domains are important for MKL1’s functional enhancement of megakaryocyte polyploidization (Figure 2B). Interestingly, the SAP domain,
which has shown little effect on SRF in luciferase assays, may play an inhibitory role in MKL1-induced polyploidization; deletion of the SAP domain resulted in a statistically significant increase in polyploidization (Figure 2B), to a degree similar to that of ΔRPEL and even greater than FL MKL1 (P < 0.05 for ΔSAP vs. FL).

We repeated these studies with dKO megakaryocytes to determine whether loss of expression of both Mkl2 and Mkl1 reveals an even stronger effect of enforced MKL1 expression. We observed the same trends as in Mkl1 KO megakaryocytes: FL MKL1, ΔRPEL, and ΔSAP constructs all produced significant increases in polyploidization, while ΔBasic, ΔLZ, and ΔTAD constructs did not increase polyploidization relative to that of the empty vector control (Figure 2C). The effects of the FL, ΔRPEL, and ΔSAP constructs appeared stronger in dKO than in Mkl1 KO megakaryocytes (Figure 2D). These data support the redundant relationship between Mkl1 and Mkl2.

We also transduced WT murine HSPC with the ΔRPEL and ΔTAD constructs because these two constructs were observed to have, respectively, a constitutively active and dominant negative effect on SRF coactivation in luciferase assays. Transduction with ΔRPEL caused a statistically significant increase in ploidy relative to the empty vector control and a non-significant increase relative to the FL construct; no increase in polyploidization was seen with the ΔTAD construct, but the reduction expected from a dominant negative effect was not observed (Online Supplementary Figure S1B).

We next sought to further elucidate the function of each MKL1 domain in downregulating GEF-H1 during endomitosis. Previously, we showed that MKL1 downregulates GEF-H1, a guanine exchange factor that couples Rho activation to microtubule dynamics, during the first endomitotic

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**Figure 2. Investigation of MKL1 domains needed for polyploidization.** (A) Schematic of domain deletion constructs (empty vector [EV] not shown): full-length (FL) MKL1, ΔRPEL, ΔBasic, ΔSAP, ΔLZ, and ΔTAD. (B) Mean fluorescence intensity (MFI) of propidium iodide for Mkl1 knockout (KO) murine bone marrow-derived megakaryocytes transduced with each deletion construct (see Figure 2A), normalized to the EV value for each experiment. Individual experiments are indicated by data point colors (FL: P = 0.007, n=6; ΔRPEL: P = 0.037, n=4; ΔSAP: P = 0.014, n=5). (C) MFI of propidium iodide for double knockout (dKO) murine bone marrow-derived megakaryocytes transduced with each deletion construct, normalized to the EV value for each trial. Individual trials are indicated by data point colors (FL: P = 0.028, n=4; ΔRPEL: P = 0.0004, n=4; ΔSAP: P = 0.017, n=4). (D) Summary of data, showing mean fold-change in ploidy driven by each construct relative to EV.
division, which is the mechanism by which MKL1 promotes megakaryocyte polyploidization. To investigate the role of MKL1 deletion mutants in GEF-H1 expression, we used our published protocol. Sorted megakaryocyte progenitors were transduced and cultured in thrombopoietin-only medium for 24 hours to induce megakaryocyte differentiation. Cells were then immunostained for tubulin and GEF-H1 as described by Gao et al. Representative images are shown in Figure 2. Qualitatively, there was a decrease in GEF-H1 fluorescence in megakaryocyte progenitors transduced with FL, ΔRPEL, and ΔSAP constructs relative to the empty vector control, which is consistent with the increased polyploidization these constructs achieve. The ΔBasic, ΔLZ, and ΔTAD constructs all showed GEF-H1 expression similar to that of the empty vector control. These effects achieved statistical significance when average GEF-H1/tubulin fluorescence over the spindle region was measured, but with very few replicates (Online Supplementary Figure S1C). We conclude that MKL1 mutants that lack the ability to direct megakaryocyte polyploidization are equally unable to downregulate GEF-H1.

In summary, we have demonstrated the critical importance of the Basic, LZ, and TAD domains for the proper function of MKL1 in megakaryocyte polyploidization, as directed by their roles in GEF-H1 downregulation (Figures 2D and 3). We have confirmed that the RPEL domain serves as a negative regulatory domain of MKL1. Our results are the first to link the function of MKL1 domains in transfection assays to a physiologically important outcome in primary cells. We have also identified a novel role for the LZ domain; although previously shown to play a minimal role in SRF coactivation, it is important for polyploidization and regulation of GEF-H1. These data confirm that megakaryocyte polyploidization is an SRF-dependent process that requires homo- or hetero-dimerization of MKL1. We also further explored the redundancy between Mkl1 and Mkl2 in megakaryocyte polyploidization. Finally, we have shown that the SAP domain may play an inhibitory role in polyploidization, potentially through its ability to bind DNA and localize MKL1 to non-SRF target sites. Having defined the functions of the five domains of MKL1 in normal megakaryocyte maturation, future studies may seek to shed light on how these domains function in acute megakaryoblastic leukemia, including how overexpression of MKL1 in leukemic cells expressing the fusion protein may affect cell proliferation and megakaryocyte maturation including polyploidization.

**Figure 3. Expression of GEF-H1 in response to wild-type and mutant forms of MKL1.** Representative images of megakaryocyte progenitors transduced with MKL1 deletion constructs, cultured in thrombopoietin-only medium, and immunostained for α-tubulin and GEF-H1. Tubulin was detected with mouse anti-tubulin (1:250, ThermoFisher A11126) and AlexaFluor 488-labeled donkey anti-mouse AlexaFluor 488 (1:500, LifeTechnologies A21202). GEF–H1 was detected with rabbit anti-GEF–H1 (1:500, AbCam ab155785) and AlexaFluor 555-labeled donkey anti-rabbit (1:500, LifeTechnologies A31572). Hoechst 33342 was used to identify nuclei.
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No conflicts of interest to disclose.

Contributions
FR designed and performed experiments and wrote the manuscript; NME, VS, EM, MJC, RM, and NK performed experiments; ECS, STL, LW, and VS provided technical expertise and contributed scientific knowledge; and DSK provided mentorship and wrote the manuscript.

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Original data can be made available on reasonable request to the corresponding author.

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