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Myeloid Leukemia Factor acts in a chaperone complex to regulate transcription factor stability and gene expression

Jamie O. Dyer[1,#], **Arnob Dutta**[2,#], **Madelaine Gogol**[3], **Vikki M. Weake**[4], **George Dialynas**[3], **Xilan Wu**[3], **Christopher Seidel**[3], **Ying Zhang**[3], **Laurence Florens**[3], **Michael P. Washburn**[3,5], **Susan M. Abmayr**[3,6,*], and **Jerry L. Workman**[3,*]

[1]Department of Biology, Rockhurst University, Kansas City, Missouri 64110, USA

[2]Department of Cell and Molecular Biology, University of Rhode Island, Kingston, Rhode Island 02881, USA

[3]Stowers Institute for Medical Research, Kansas City, Missouri 64110, USA

[4]Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907, USA

[5]Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, Kansas 66160, USA

[6]Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA

Abstract

Mutations that affect Myeloid Leukemia Factor (MLF) proteins are associated with leukemia and several other cancers. However, with no strong homology to other proteins of known function, the role of MLF proteins in the cell has remained elusive. Here we describe a proteomics approach that identifies MLF as a member of a nuclear chaperone complex containing a DnaJ protein, BAG2 and Hsc70. This complex associates with chromatin and regulates expression of target genes. The MLF complex is bound to sites of nucleosome depletion and sites containing active chromatin marks (e.g. H3K4me3 and H3K4me1). Hence, MLF binding is enriched at promoters and enhancers. Additionally, the MLF-chaperone complex functions to regulate transcription factor stability, including the RUNX transcription factor involved in hematopoiesis. Though Hsc70 and other co-chaperones have been shown to play a role in nuclear translocation of a variety of proteins including transcription factors, our findings suggest that MLF and the associated co-chaperones play a direct role in modulating gene transcription.

Graphical Abstract

[*]Correspondence: sma@stowers.org; jlw@stowers.org.
[#]These authors contributed equally to this work.

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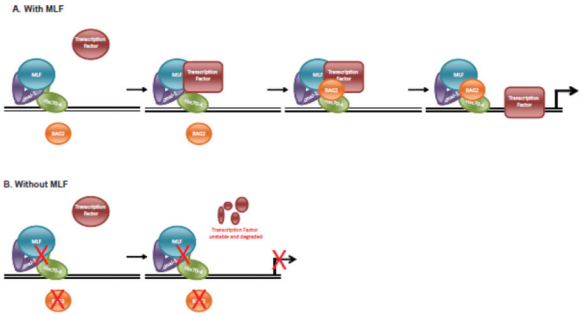

Introduction

Development of the hematopoietic system is a well-regulated process involving specific factors that determine cell fate and lineage specification. The myelodysplasia/myeloid leukemia factors (MLFs) are a poorly characterized family of proteins involved in hematopoiesis that associate with chromatin and may regulate transcription. The founding member of the MLF family is human MLF1, which was originally identified in a fusion protein with nucleophosmin (NPM) and is associated with myelodysplastic syndrome and acute myeloid leukemia[1]. The resulting NPM-MLF1 fusion contains the NPM oligomerization domain and nuclear localization signals, causing it to accumulate in the nucleus. Although fusion with NPM causes other proteins to become oncogenic, hMLF1 itself is also implicated in cancer: Elevated levels of endogenous hMLF1 are observed with high frequency in myeloid cell malignancies, and this increased expression corresponds with poor prognosis and low survival rates[2; 3; 4]. In addition to the misregulation of hMLF1 in leukemia, MLF proteins function in normal hematopoiesis. hMLF1 expression is observed in early hematopoietic CD34+ progenitor cells, and its expression levels decrease during differentiation[2; 5]. Murine MLF1 suppresses erythroid differentiation, while its overexpression promotes myeloid maturation[5]. The suppression of erythroid differentiation by MLF1 is mediated through inhibition of proteasomal degradation of $p27^{Kip1}$, resulting in the inhibition of cyclin-E/cdk2 complexes[6]. Although hMLF1 is most well characterized in hematopoietic cells, human and mouse MLF1 are normally expressed in a variety of adult and fetal tissues, with the highest levels seen in the testis, skeletal muscle, and heart[1; 7]. However, the role of MLF proteins in these tissues is not understood, and its mechanism of action in hematopoietic cells remains unclear.

MLF is highly conserved in metazoans and mammals contain two paralogs, MLF1 and MLF2, with approximately 40% identity[8; 9]. *Drosophila melanogaster* contains a single MLF ortholog, dMLF that shares equal homology with hMLF1 and hMLF2[8]. Thus, *Drosophila* provides a genetically tractable model system in which to examine the mechanism of MLF function. As observed for hMLF1, the subcellular localization of dMLF is dependent on cell type and its expression levels change during development[8]. Additional studies in *Drosophila* demonstrated that dMLF associates with chromatin, suggesting a nuclear role for MLF proteins[10]. Likewise, hMLF1 associates with DNA and exogenous expression of hMLF1 regulates the transcription of several genes involved in differentiation and cell growth[11]. Importantly, null alleles of *mlf* in *Drosophila* are embryonic lethal,

indicating that it plays an important role during development[8]. Although studies have reported that both human and *Drosophila* MLF proteins are in the nucleus, and suggested roles for this localization, little is known about their specific function within the nuclear compartment or how MLF proteins direct cell differentiation.

Another group of proteins whose nuclear functions are less understood are molecular chaperones. Though the functions of molecular chaperones in protein folding have been extensively studied, other functions for these proteins are starting to emerge, and include roles in endocytosis, nucleocytoplasmic shuttling, signal transduction, and multi-protein complex assembly[12]. Of particular interest is the recently described role for molecular chaperones at chromatin[13; 14]. Most studies examining the involvement of molecular chaperones in transcriptional regulation have focused on their functions in protein folding and stabilization. However, a few recent studies have suggested that molecular chaperones might play a direct role at chromatin in regulating gene expression. For example, studies in yeast have revealed that both Hsp90 and Hsp70 systems are required at chromatin for proper removal of promoter-bound nucleosomes to allow for gene induction[13]. Additionally, studies in *Drosophila* found that Hsp90 regulates gene expression by localizing to transcription start sites of genes that display RNA Polymerase II (Pol II) promoter proximal pausing via stabilization of the negative elongation factor (NELF) complex, and therefore paused Pol II[14]. Thus, molecular chaperones appear to play a broader role in the cell than previously thought, regulating both posttranslational events and transcription. These Hsp70 and Hsp90 studies in yeast and *Drosophila* raise the question of whether other molecular chaperones might also play direct roles in transcription, and prompted us to explore Hsc70 when it co-purified with MLF (below).

The heat shock protein 70 (Hsp70) family of co-chaperones, a major class of these proteins, is conserved from bacteria to humans[12]. The well-characterized heat shock cognate 70 (Hsc70) protein is constitutively expressed in all organisms. It contains an amino-terminal ATP-binding domain and a carboxy-terminal substrate-binding domain. Through the concerted actions of the ATPase activity of the ATP-binding domain and the substrate-binding domain, Hsc70 binds to and folds both native and unfolded client proteins. The intrinsic ATPase activity of Hsc70 is rather weak, and is stimulated by binding to co-chaperone proteins[15]. The ATP-binding domain of Hsc70 interacts with the Hsp40/DnaJ co-chaperones through a conserved J domain[15]. The binding of DnaJ proteins to Hsc70 enhances the ATPase activity of Hsc70, facilitating the hydrolysis of ATP to ADP, which causes a conformational change in the substrate binding domain of Hsc70. This conformational change results in high-affinity binding of Hsc70 to the unfolded client protein, thereby enabling its folding. Nucleotide exchange factors (NEFs) are co-chaperones that then stimulate the release of the client protein by facilitating the exchange of ADP for ATP, thus resetting the Hsc70 folding cycle[16]. One class of NEFs that interact with Hsc70 is the BAG family of proteins, which contain a conserved BAG domain that binds to the ATP-binding domain of Hsc70. In addition to stimulating the activities of Hsc70, co-chaperone proteins are thought to add functional specificity to the Hsc70 chaperones, allowing the small family of highly conserved Hsc70 proteins to perform a large variety of specific cellular functions[15].

To examine the functions of MLF proteins in the nucleus, we employ a proteomics approach to identify MLF-interacting proteins in nuclear extracts. Interestingly, we observed that *Drosophila* MLF interacts with the co-chaperones DnaJ-1 and BAG2 in the nucleus, along with the molecular chaperone Hsc70-4. Our observations indicate that members of the MLF-chaperone complex co-localize to the same sets of genomic loci, suggesting that this complex plays a broader role in the nucleus than simply folding or shuttling proteins into this compartment. Specifically, MLF and DnaJ-1 associate with regions of open chromatin at promoters marked by the histone modification H3K4me3 and at active enhancers marked by H3K27ac and H3K4me1. Additionally, we find that a variety of transcription factors co-purify with the MLF-chaperone complex using size exclusion chromatography. Notably, we identified a change in the stability of these transcription factors, both activators and repressors, in the absence of members of the MLF-chaperone complex. Furthermore, the presence of the MLF-chaperone complex was confirmed in nuclear extracts from human cells, suggesting that this evolutionarily conserved complex serves an important function within the nucleus of higher eukaryotes. Together, our results reveal a novel conserved chaperone complex that regulates transcription by modulating transcription factor stability in chromatin.

Results

Drosophila and human MLFs specifically interact with chaperone proteins

The mechanism of action of MLF, especially its function in the nucleus, is not well understood. In an effort to understand its nuclear functions, we sought to identify MLF-interacting partners. MLF was isolated in using tandem Flag-HA affinity purification from nuclear extracts of *Drosophila* S2 cells expressing low levels of Flag-HA tagged MLF (Figure 1A). The purification was repeated 2 more times. The MLF co-purified proteins were identified using Multidimensional Protein Identification Technology (MudPIT) mass spectrometry[17]. Peptides from proteins that consistently co-purified with MLF, and were absent or at significantly lower levels in control purifications, included DnaJ-1, a member of the Hsp40/J domain family of co-chaperone proteins, and CG7945, a previously uncharacterized protein (Figures 1B; Supplementary Table 1). *Drosophila* CG7945 (FBgn0036505) encodes a 29.4 kDa protein that shares sequence similarity with human BCL2-associated anthanogene 2 (BAG2), an Hsp70-associated nucleotide exchange factor[18]. Henceforth we refer to *Drosophila* CG7945 as BAG2. Additionally, we found the Hsc70-4 member of the Hsc70 family of chaperones to co-purify with MLF (Figures 1B; Supplementary Table 1).

Although the level of over-expressed tagged MLF was low, purification of a chaperone complex raised the possibility that its interaction with MLF was merely a result of protein over-expression. Importantly, however, we note that the abundance of peptides from DnaJ-1 and CG7945 was significantly lower in purifications of the SAGA complex from S2 cells in which SAGA subunits were similarly over-expressed at low levels[19]. To confirm the specific interaction of these chaperone proteins with MLF, we performed reciprocal purifications using Flag-HA epitope tagged DnaJ-1 and BAG2 from S2 cell nuclear extracts followed by MudPIT analysis. Each purification was done in triplicate. This analysis showed that MLF

co-purified with both DnaJ-1 and BAG2. Similarly, both DnaJ-1 and BAG2 co-purified with each other (Figures 1A–B; Supplementary Table 1). To further validate our findings from the MudPIT analyses, we raised polyclonal antibodies against MLF, DnaJ-1, and BAG2 (Supplementary Figure 2). Western blotting of the MLF, DnaJ-1, and BAG2 immuno-purified complexes verified that each of these bait proteins co-purify the other two complex members (Supplementary Figure 1A).

We next sought to determine if MLF interacts directly with DnaJ-1 and BAG2. Purified recombinant MLF, DnaJ-1 and BAG2 were mixed at equimolar concentrations, and immunoprecipitated using anti-MLF antibody (Figure 2C). Coomassie blue staining revealed the co-immunoprecipitation of DnaJ-1 and BAG2 (Figure 2C), confirming their direct interaction with MLF. Furthermore, the co-immunoprecipitation of similar amounts of DNA-J and BAG2 with MLF when both were present versus individually suggests their binding to MLF is not mutually exclusive (Figure 2C).

To verify that MLF, Hsc70-4, DnaJ-1, and BAG2 form a single protein complex, we fractionated the complex obtained by MLF-affinity purification from S2 nuclear extracts using size exclusion chromatography. Western blot analysis showed that MLF co-eluted with DnaJ-1 (Figures 1D, Supplementary Table 2). However, BAG2 was less abundant in these same fractions and eluted separately from the MLF/DnaJ-1 complex (Figure 1D, Supplementary Table 2). The reduced abundance of BAG2 in these MLF-chaperone complex fractions could suggest that BAG2 either binds weakly to the complex under the chromatography conditions or that it interacts with MLF-DnaJ-1 only transiently, which would be expected for a nucleotide exchange factor. These results confirm that MLF forms a complex with DnaJ-1 to which BAG2 can bind.

The broad distribution of MLF and DnaJ-1 on the size exclusion column and the large molecular weight observed for the MLF-chaperone complex (average approximately 700 kDa) suggests that this complex(s) contains more than a single molecule of MLF, DnaJ-1 and BAG2. Thus, the MLF-chaperone complex likely interacts with additional proteins and/or complexes. To assess whether additional proteins associate with the MLF-chaperone complex, fraction 13 from the size exclusion column (Figure 1D) was examined by silver stain analysis. Notably, additional protein bands were present besides that of MLF-chaperone complex (Figure 1E). MudPIT analysis of this fraction revealed that these protein bands include alpha-actinin, Raspberry/IMPDH (inosine 5'-monophosphate dehydrogenase), and CG8578/LRRFIP1 (leucine rich repeat flightless-interacting protein 1) (Figure 1F; Supplementary Table 2). Although alpha-actinin and IMPDH have well-established functions in the cytoplasm, homologs for all three of these co-eluting proteins have been shown to play roles in the regulation of transcription[20; 21; 22; 23; 24]. Thus, MLF, DnaJ-1 and BAG2 can form a single complex *in vivo* that interacts with other proteins within the nucleus.

The two human MLF paralogs hMLF1 and hMLF2 share equal homology to *Drosophila* MLF[9]. Thus, we sought to determine whether hMLF1 and hMLF2 interact with a chaperone complex similar to that identified in *Drosophila*. For these studies, Flag-tagged hMLF1 and hMLF2 and associated proteins were affinity purified from whole cell extracts of 293T

human embryonic kidney cells three times (Supplementary Figure 1B), and each purification was subjected to MudPIT analysis. Notably, we identified the DnaJ homolog DNAJB6, BAG2, and the Hsc70 homolog HSPA8 in both the hMLF1 and hMLF2 purifications (Supplementary Figure 1B & C, Supplementary Table 3). These proteins were absent or at significantly lower levels in control purifications of untagged MLF from 293T cells and are not enriched in purifications of unrelated nuclear proteins such as FAM60A from 293T cells[25]. We therefore conclude that the interaction of MLF proteins with Hsc70 and the co-chaperones DnaJ and BAG2 is conserved in flies and humans.

MLF-chaperone complex members are bound to distinct loci on chromatin, marking promoters and enhancers

Previous studies using polytene chromosomes have shown that *Drosophila* MLF associates with chromatin at transcriptionally active regions[10], suggesting a chromatin-associated role for MLF. To determine if MLF occupied specific genomic loci, we carried out chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) from *Drosophila* S2 cell nuclear extracts using anti-MLF antibodies[10]. We identified 11,689 MLF binding sites across the genome in both replicates, with a median peak length of 405 base pairs (Figure 2A&B). We next asked if DnaJ-1 was also bound to chromatin, and found 15,464 sites in both replicates across the genome (Figure 2A&B). A comparison of MLF and DnaJ-1 bound sites showed that nearly all genomic loci occupied by MLF were also bound by DnaJ-1 (Figure 2B&C), further supporting the hypothesis that these proteins work as part of a complex.

Given the extensive localization of MLF and DnaJ-1 to chromatin, we next sought to determine the specific regions that were bound by these proteins. Comparison of the peak summits with annotated gene features revealed that MLF and DnaJ-1 peaks are enriched at promoter regions, defined as −1000 to +300 around the transcription start site, and in the body of genes with significant enrichment observed in both cases (Figure 3A). A very small fraction bound to intergenic regions (Figure 3A). We next asked if these promoter regions corresponded to regions of active transcription. We used previously published data sets to compare RNAPII and histone modifications[26; 27] at MLF/DnaJ-1 bound sites. Analysis of RNAPII occupancy at MLF and DnaJ-1 bound regions showed that approximately half of all MLF and DnaJ-1 bound regions were also occupied by RNAPII (Figure 3B). Consistent with the co-localization of RNAPII and enrichment of MLF and DnaJ-1 at promoters, we found that these regions were enriched for histone modification marks associated with active transcription, including H3K4me3 and H3K27ac, but devoid of the repressive mark H3K27me3 (Figure 3B).

Interestingly, RNAPII bound regions represented around half of all MLF and DnaJ-1 bound regions, and a significant number of loci had little to no RNAPII. Further analysis of low RNAPII bound regions compared to published datasets[27; 28] revealed them to be areas of open chromatin based on their sensitivity to DNaseI and nucleosome free status (Figure 3B). These regions were also enriched for histone modifications that are hallmarks of active enhancers, including H3K4me1 and H3K27ac, but lacking the repressive mark H3K27me3 (Figure 3B). This observation raises the possibility that MLF and DnaJ-1 occupy regions of

open chromatin and mark both active promoters and enhancers. Studies from the Stark lab have identified regions in Drosophila S2 cells that may function as enhancers[28]. To test if MLF and DnaJ-1 mark regions near enhancers, we plotted STARR-seq peaks[28] around the regions bound by MLF and DnaJ-1. Interestingly, we found that MLF and DnaJ-1 bound regions were enriched for these putative enhancers and STARR-seq signature at low RNAPII regions and showed a small but significant enrichment (p value $2.50e^{-16}$) compared to regions of high RNAPII (Figure 3C). Further analysis of specific genes with well-defined enhancers and promoters[28] showed that MLF and DnaJ-1 were associated with both the known enhancer and promoter of *srp* (Figure 3D) and *shn* (Supplementary Figure 3A). These results suggest that MLF and DnaJ-1 co-occupy both promoters and enhancers of genes, and may play a role in regulating transcription of these genes.

In order to examine if recruitment of the MLF and DnaJ-1 complex was dependent on members of the MLF-co-chaperone complex we performed ChIP-qPCR using antibodies against MLF and DnaJ-1 following knockdown of the other complex members. We examined several genes that contain MLF binding peaks at their promoters, and found that MLF and DnaJ-1 were enriched at these genes as compared to an intergenic control (Supplemental Fig. 3 B–E). Importantly, knockdown of *mlf* or *dnaj-1* by RNAi caused a reduction in the MLF and DnaJ-1 enrichment observed at these genes, indicating that the antibodies are specific and that these sites correspond to bona fide MLF binding sites *in vivo* (Supplemental Fig. 3 B–E). The ChIP enrichment of MLF is significantly reduced following knockdown of *bag2* and *dnaj-1*, but the levels of enrichment are slightly higher than in *mlf* knockdowns (Supplemental Fig. 3B). Interestingly, no strong reduction in ChIP enrichment is observed for DnaJ-1 upon knockdown of *mlf* or *bag2* (Supplemental Fig. 3D). However, for the majority of gene loci examined, knockdown of *hsc70-4* does not result in loss of ChIP enrichment for MLF or DnaJ-1 (Supplemental Fig. 3C & E). Together these results suggest that DnaJ-1 and BAG2 but not Hsc70-4 are important for recruitment of MLF to genes and posits a model where DnaJ-1 recruitment precedes that of MLF.

The MLF-chaperone complex co-regulates expression of a subset of genes

Previous studies in human cells have implicated hMLF1 in transcriptional regulation[11]. Since the MLF-chaperone complex was purified from nuclear extracts and we observed strong co-occupancy of MLF and DnaJ-1 to promoters and enhancers, we explored whether MLF and chaperone complex members directly regulate gene expression. To identify genes that are transcriptionally regulated by *Drosophila* MLF, we performed high-throughput sequencing of mRNA transcripts (RNA-seq) isolated from S2 cells that were treated with dsRNA against *mlf* and compared these results to transcripts isolated from S2 cells treated with dsRNA against the control *lacZ* (Supplementary Figure 4A). The resulting RNA-seq gene sets were filtered to remove genes with extremely low read counts and the remaining genes were analyzed using DESeq. We identified 1461 genes with a significant increase ($p < 0.05$) in transcript levels upon *mlf* knockdown (repressed by MLF) and 1788 genes with decreased transcript levels ($p < 0.05$) upon *mlf* knockdown (activated by MLF) (Figure 4A, Supplementary Figure 5A). Gene Ontology (GO) term analysis showed that genes repressed by MLF were involved in developmental processes and morphogenesis (Figure 4B) and that genes activated by MLF were involved in cell division, protein folding, and translation

(Figure 4C). These findings suggest that MLF mediates the repression of genes associated with development and differentiation, while promoting those associated with cell proliferation. Such a role is consistent with previous studies suggesting roles of MLF in cell proliferation and development[2; 5; 8].

Since we found that MLF and DnaJ-1 co-occupy many loci, we next determined whether MLF and DnaJ-1 co-regulated the same subset of genes. We first conducted RNA-seq analysis of *dnaj-1* knockdown cells compared to control (*lacZ* knockdown) and found that 708 genes were significantly ($p < 0.05$) upregulated whereas 883 genes were significantly ($p < 0.05$) downregulated (Supplementary Figure 5A). To determine if MLF and DnaJ-1 co-regulate genes, we focused on those that appeared to be direct targets of MLF and/or DnaJ-1 (those bound by either MLF or DnaJ-1 and for which expression changed in knockdown of *mlf* or *dnaj-1*) (Supplementary Figure 5B). Interestingly, while a subset of genes was similarly co-regulated by both MLF and DnaJ-1, others showed opposite changes upon knockdown of either *mlf* or *dnaj-1*. These results suggests that though MLF and DnaJ-1 form a complex, they may have distinct interactions with other members of the transcription machinery, resulting in differential regulation of expression of these genes.

We next focused our analysis on genes that were similarly regulated by MLF and DnaJ-1. We found that 216 upregulated and 312 downregulated in both *mlf* and *dnaj-1* knockdowns were bound by both MLF and DnaJ-1 at their gene promoters (Figure 4D & E). Further analysis showed that RNAPII occupied the promoter and the body of genes requiring MLF and DnaJ-1 for expression (Figure 4D), while genes suppressed by MLF and DnaJ-1 were enriched for paused RNAPII at their promoters (Figure 4E). This specificity mirrors the MLF suppression of developmental genes suggested by GO-term analysis, which would be expected to have paused RNAPII. By comparison, housekeeping genes that require MLF for expression would be actively transcribed and have RNAPII at promoters and within the body of genes. These studies show that the MLF-chaperone complex can directly regulate gene expression through binding of promoters and possibly enhancers, and work to both activate and suppress gene expression. Moreover, our findings suggest that MLF mediates the repression of genes associated with development and differentiation, while promoting expression of genes associated with cell proliferation. This role is consistent with previous findings demonstrating that MLF is essential and that the levels of MLF are higher in progenitor cells relative to differentiated cells[2; 5; 8].

The MLF-chaperone complex regulates transcription factor stability

Our results show that both MLF and DnaJ-1 associate with specific subsets of genes and act to either up or down regulate these genes, suggesting both activating and repressive roles. Interestingly, *Drosophila* MLF has been shown to positively regulate the stability of the RUNX transcription factor Lozenge in a proteasome-dependent manner, with overexpression of MLF resulting in higher levels of Lozenge[29]. Similarly, human MLF1 stabilizes the oncogenic RUNX1-ETO fusion protein, and knockdown of hMLF1 results in a decrease in this fusion protein and a corresponding decrease in proliferation of cells derived from a patient with acute myeloid leukemia[29]. However, the mechanism through which MLF regulates stability of transcription factors is not clear. The association of MLF with protein

chaperones could work, in part, by regulating the stability of transcription factors. To test if association with the chaperone complex contributes to the ability of MLF to stabilize the Lozenge transcriptional activator, we knocked down *bag2, dnaj-1,* or *hsc70-4* along with *mlf* in *Drosophila* S2 cells that expressed epitope-tagged Lozenge. Compared to control knockdowns using dsRNA targeted to *lacZ*, we observed a statistically significant decrease (p value<0.05) in the level of Lozenge protein upon *mlf* knockdown (Figures 5A & B), confirming previous results[29]. Interestingly, we also observed a statistically significant decrease (p value<0.05) in Lozenge protein levels upon knockdown of *bag2, dnaj-1,* or *hsc70-4* (Figures 5A & B). Since a decrease in the level of transcript could also account for the observed reduction in protein levels, we examined the level of *lozenge* mRNA in these knockdowns by qRT-PCR. We did not observe a significant decrease in *lozenge* transcript levels in the *mlf*, *bag2*, *dnaj-1* or *hsc70-4* knockdown samples (Figure 5C). Taken together, we can conclude that MLF functions with its associated chaperones to regulate protein stability of the Lozenge transcription factor.

In addition to up-regulating expression of some genes, our results have shown that MLF and DnaJ-1 also suppress transcription at a subset of genes with paused RNAPII. MLF associated proteins identified by mass spectrometric analysis of fractions from size exclusion chromatography included CG8578 (Figure 1E & F). This uncharacterized protein is the *Drosophila* homolog of human leucine rich repeat flightless-interacting protein 1 (LRRFIP1), also known as GC-Binding Factor 2 (GCF2)[24]. LRRFIP1 is a transcriptional repressor that interacts with GC-rich DNA sequences to regulate the transcription of several genes, including tumor necrosis factor alpha (TNFα), epidermal growth factor receptor (EGFR), and platelet-derived growth factor alpha (PDGFA)[30; 31; 32]. We next examined if the MLF-chaperone complex could regulate the stability of the putative repressor CG8578 in S2 cells. As observed for Lozenge, knockdowns of *mlf*, *bag2, dnaj-1,* or *hsc70-4* resulted in a dramatic and statistically significant (p value<0.05) decrease in the level of CG8578 protein (Figures 5D & E). As observed for Lozenge, transcript levels for *CG8578* were not strongly affected by dsRNA treatment of MLF-chaperone complex members (Figure 5F). These results suggest that the MLF-chaperone complex can in part affect transcription of genes by regulating the stability of both transcriptional activators and repressors.

Numerous other transcription regulatory proteins also co-purified with the MLF-chaperone complex members, as detected by MudPIT analysis (Supplementary Table 4). This observation raises the interesting possibility that other transcriptional regulatory proteins also serve as clients for the MLF-chaperone complex, and provides an explanation for how localization of MLF and DnaJ-1 to genes regulates their transcription.

Discussion

Human MLF1 was originally implicated in cancer on the basis of its fusion with nucleophosmin (NPM) in myelodysplastic syndrome and acute myeloid leukemia[1]. This fusion protein was shown to accumulate in the nucleus. Additionally, high levels of hMLF1 were associated with other myeloid cell malignancies[2; 3; 4]. In accordance with its role in myeloid leukemia, MLF proteins were shown to have roles during hematopoiesis, with high levels of MLF in early hematopoietic CD34+ progenitor cells and decreasing expression

during differentiation[2; 5]. Though roles of MLF in hematopoietic cells have been the focus of many studies, MLF1 is expressed in several adult and fetal tissues including testis, skeletal muscle, and heart[1; 7]. However, the role of MLF proteins in regulating cellular processes in these tissues and its role in the nucleus both remain unclear. Our studies provide the first insights into MLF functions in the nucleus, and show that the MLF family of proteins can play direct roles in the regulation of transcription. Interestingly, we find that MLF interacts with molecular chaperone proteins in the nucleus, both in flies and humans (Figure 1, Supplementary Figure 1). Mirroring previous observations of MLF association with polytene chromosomes in *Drosophila* salivary glands[10], we find that both MLF and its co-chaperone DnaJ-1 co-occupy several loci, specifically at regions of open chromatin (Figure 3). Additionally, we show that this complex regulates the expression of a subset of genes (Figure 4), most likely by binding to promoters or enhancers that are associated with the histone marks H3K4me3, H3K27ac and H3K4me1. The GO terms of genes showing transcriptional regulation by MLF are associated with development, differentiation, cell division and basic housekeeping processes. Many developmentally-expressed genes are marked by paused RNAPII while house-keeping genes have high RNAPII in the body of genes. Interestingly, MLF and DnaJ-1 are expressed at high levels during early embryogenesis but decrease at later stages. Similarly, hMLF is present at high levels in early hematopoietic CD34+ progenitor cells, but this expression level decreases during differentiation[2; 5]. Along with our genome wide analysis of MLF and DnaJ-l regulated genes, these observations suggest the possibility that high levels of MLF and DnaJ-1 in undifferentiated/progenitor cells early in development repress genes with paused RNAPII and promote expression of housekeeping and cell division genes.

MLF is known to stabilize the transcription factor Lozenge[29] in a proteasome dependent manner, but the mechanism by which MLF regulates factor stability is not well understood. We show that MLF associates with co-chaperones that also regulate Lozenge stability (Figure 5), suggesting this interaction as the mechanism for MLF-directed transcription factor stabilization. Further support for this hypothesis is provided by our finding that the MLF-chaperone complex also regulates the stability of the transcription repressor CG8578. Our MUDPIT analysis revealed that several transcription factors co-purified with MLF. These results, along with our observation that MLF and DnaJ-1 regulate transcription, support the hypothesis that the MLF-chaperone complex may work to regulate gene expression by modulating the stability of components of the transcriptional machinery.

The Hsc70 class of chaperones and co-chaperones has been shown to function in a variety of cellular processes, including endocytosis, protein subcellular localization, and signal transduction. All of these functions are consistent with the established posttranslational roles of these chaperones in protein folding and protein degradation pathways. It has now become evident that the roles of molecular chaperones extend to transcriptional regulation from studies showing that Hsp90 contributes to the removal of promoter-bound nucleosomes for induction of genes in yeast[13]. In addition, *Drosophila* Hsp90 regulates RNA Polymerase II (Pol II) promoter proximal pausing via stabilization of the negative elongation factor complex, and therefore Pol II[14]. Thus, molecular chaperones appear to play an even larger role in the cell than previously thought, by regulating not only posttranslational events but also transcription.

The well-documented involvement of Hsc70 in cancer has highlighted the importance of these chaperones as potential anti-cancer targets[12]. As Hsp90 inhibitors have shown great promise in the reduction of cancer cell viability, studies have begun to focus on Hsp70 and Hsc70 inhibitors as chemotherapeutic agents[33]. Multiple roles for Hsc70 have been established in the regulation of hematopoietic proliferation and differentiation, suggesting that Hsc70 might be a promising target for stem cell therapy of hematological malignant diseases[33]. However, the numerous roles of Hsc70 proteins in protein folding, translocation, and now transcription may not be discriminated by anticancer drugs directed against Hsc70. The involvement of both Hsc70 and MLF in myeloid leukemia suggests that the misregulation of transcription by Hsc70 and other MLF-chaperone complex members could lead to malignant transformation of these cells[1; 34; 35]. The observation that Hsc70 is present within different complexes, including the MLF-chaperone complex described in this study, supports the hypothesis that these complexes narrow the function of Hsc70 to specific cellular processes. Similarly, targeting individual members of the MLF-chaperone complex or inhibiting its nuclear import may modulate its different effects on transcription. As the list of developmental and disease-related roles for the Hsc70 family of proteins lengthens, the ability to target specific functions of these proteins and the associated complexes will be necessary. The work described here presents a novel mechanism through which a molecular chaperone complex can directly target gene expression, a process that could serve as a new therapeutic target in treating diseases such as myeloid leukemia.

Materials and methods

Affinity purification and MudPIT analysis

Stable S2 cell lines expressing MLF (isoform A; NP_523753), DnaJ-1 (isoform A; NP_523936), and Hsc70-4 (isoform A; NP_524356) in the pRmHa3-C-HA_2FLAG_2 vector and BAG2 (isoform A; NP_730051) in the pRmHa3-N-FLAG-HA vector were generated and tandem Flag-HA affinity purifications were carried out using nuclear extracts prepared from 4 liters of cells grown to a density of 1×10^7 cells/ml as described previously[19]. 293T cell lines stably expressing FLAG-tagged hMLF1 and hMLF2 in the pcDNA5/FRT vector were generated and affinity purifications were carried out as described previously[36].

Antibodies

cDNA fragments encoding the entire coding regions of MLF-PA, DnaJ-1-PA, and BAG2-PA were amplified by PCR and inserted into pET28 vectors (Novagen). Each of these His-tagged proteins were expressed and purified from *E. coli*. Rats were immunized with recombinant MLF protein, rabbits were immunized with recombinant DnaJ-1 protein, and rats and chickens were immunized with recombinant BAG2 protein (Pocono Rabbit Farm and Laboratory). Each antibody was affinity purified using full length MLF, DnaJ-1, and BAG2 proteins, respectively. The following antibodies were used for western blots: MLF (rat, 1:1000); BAG2 (rat, 1:1000); BAG2 (chicken, 1:5000); DnaJ-1 (rabbit, 1:2000); tubulin (mouse, 1:5000, Developmental Studies Hybridoma Bank, University of Iowa); V5-HRP (mouse, 1:5000, Invitrogen R961-25); GFP (mouse, 1:1000, Roche 11814460001).

In vitro binding assays

Recombinant His-MLF, Flag-BAG2, and DnaJ-1-HA proteins were expressed and purified from *E. coli* individually. Approximately equal molar amounts of these proteins were allowed to bind in various combinations in CoIP buffer (50mM Tris-Cl pH 8.0, 300mM NaCl, 0.5% NP40, 1mM PMSF) for 1 h at 4°C before adding rabbit anti-MLF antibody/ Protein G Dynabeads (Invitrogen) to the reactions to isolate MLF-protein complexes. Beads were isolated and washed 5 times in Co-IP buffer, boiled in SDS loading buffer, and the resulting supernatant was subjected to SDS-PAGE electrophoresis. Input and immunoprecipitated proteins were visualized using Coomassie stain. Reactions were performed in biological triplicate.

Size exclusion chromatography

Affinity purified MLF complexes were applied to a Superose 6 HR 10/30 column (Amersham Biosciences) in 300mM NaCl Purification Wash Buffer (20mM HEPES pH 7.5, 300mM NaCl, 1mM MgCl2, 10% glycerol, 0.1% Triton X-100, and 1mM PMSF). 500 μl fractions were collected and analyzed by western blotting, silver stain, and MudPIT analysis. Untagged S2 tandem affinity purified complexes were used as a control. Sizes of fractions were determined by fractionating molecular weight markers using Gel Filtration HMW and LMW Calibration Kits (GE Healthcare).

ChIP and ChIP-seq

ChIP was conducted on 1×10^7 cell equivalents of chromatin from *Drosophila* S2 cells. Briefly, cells were cross-linked in 1% formaldehyde and sonicated to obtain chromatin fragments. For input controls, 1×10^6 cell equivalents of chromatin was used. Soluble chromatin was incubated with rabbit α-MLF (gift from Dr. Anne Plessis), rat α-MLF (this work), rabbit α-DnaJ-1 (this work), or rat α-BAG2 (this work) antibodies overnight at 4°C. 50μl α-Protein G Dynabeads were added to each chromatin/antibody solution and incubated for 2 h at 4°C. Beads were washed 5 times with RIPA buffer (50mM Hepes pH 7.5, 0.5M LiCl, 1mM EDTA, 1% NP-40, 0.7% sodium deoxycholate) and once with 50mM NaCl in TE buffer. Bound complexes were eluted twice with 200μl elution buffer (250mM NaCl and 1% SDS in TE buffer) at 65°C for 30 min. The eluates were treated with RNase A and Proteinase K and cross-links were reversed at 65°C overnight. DNA was purified by phenol-chloroform extraction and ethanol precipitation. Input and immunoprecipitated DNA samples were analyzed by qPCR relative to sonicated S2 input DNA standards using Perfecta SYBR Green FastMix for all primer sets. Three biological replicates were performed for all ChIP-qPCR analyses. Primer sequences used for ChIP-qPCR are listed in Supplemental Table 7. DNA libraries for ChIP-seq were generated from 25 ng input chromatin and 30 μl of rabbit anti-MLF immunoprecipitated chromatin, using the ChIP-Seq DNA Sample Prep Kit (Illumina) according to the manufacturer's protocol (11257047 Rev. A) except the PCR step was performed before gel extraction. Illumina library concentrations were determined via Agilent Bioanalyzer analysis (Agilent Technologies). Cluster generation was performed on an Illumina cBOT using v2 chemistry and sequencing was performed on an Illumina GAIIx sequencer using v5 sequencing chemistry according to the

manufacturer's recommendations. Image analysis and base calling were performed using Illumina analysis pipeline.

ChIP-seq analysis

Alignment of ChIP-seq reads from immunoprecipitated and input chromatin to the *Drosophila* reference genome dm3 from UCSC was performed using Bowtie version 0.12.7[37]. Preliminary analysis of MLF binding peaks in S2 cells was performed using model-based analysis of ChIP-seq (MACS2)[38] with an adjusted *p*-value cutoff of 0.01. Peak summits, defined by MACS2, were mapped to promoters, exons, introns, and intergenic regions using the IRanges and GenomicFeatures libraries in R. MLF ChIP-seq peaks were assessed for overlap with features from the modENCODE datasets using the IRanges library in R. A minimum of one base was required for overlap. Overlap was assessed between features before and after randomization of feature locations on a per chromosome basis.

dsRNA knockdown in S2 cells

Drosophila S2 cells were grown in Schneider's medium supplemented with 10% fetal bovine serum to a density of 3 – 6 $\times 10^6$ cells/ml. Cells were diluted to 1 $\times 10^6$ cells/ml in serum-free media and incubated with 10 μg dsRNA per 1 $\times 10^6$ cells for 45 min at 25°C. An equal volume of Schneider's media containing 10% serum was added to the cells and the cells were grown for 4 days at 25°C before collection for ChIP, RNA, and/or protein isolation. dsRNA against *lacZ* was used as a control for all RNAi experiments. Three biological replicates were performed for all RNAi experiments, except where noted. Primer sequences used for dsRNA production are listed in Supplemental Table 7.

RNA-seq

Total RNA was isolated from S2 cells treated with either dsRNA against *lacZ*, *mlf*, *bag2*, or *dnaj-1* using TRIzol (Invitrogen). Two separate biological experiments were performed for each RNA-seq analysis. 4 μg total RNA were used to make RNA-seq libraries using TruSeq RNA Sample Preparation Kit (Illumina) according to the manufacturer's protocol. Each sample was barcoded using Illumina RNA Adapter Indexes, concentrations were determined using an Agilent Bioanalyzer high sensitivity analysis (Agilent Technologies), and equal concentrations of the libraries were pooled into two different experiments. Cluster generation was performed on an Illumina cBOT using v2 chemistry and sequencing was performed on an Illumina GAIIx sequencer using v5 sequencing chemistry according to the manufacturer's recommendations. Image analysis and base calling were performed using Illumina analysis pipeline.

RNA-seq analysis

Reads from two biological replicate experiments were aligned using TopHat version 1.3. Fold change in gene expression was quantified using DESeq 1.10.1 after genes with a sum of 10 reads or less across all 4 data sets were filtered out. GO term enrichment analysis was performed using the topGO library in R. Genes bound by MLF within 2kb of their TSS with an enrichment score greater than 5-fold as compared to input chromatin were compared to

the filtered RNA-seq gene set with adjusted p-values greater than 0.01 as determined by DESeq.

Transient transfections into S2 cells

Transfections of pAc5 plasmids were performed 24 hours after dsRNA treatment in S2 cells. Transfections were performed using Effectene Transfection Reagent (Qiagen) per the manufacturer's protocol with slight modification. For each construct, 1μg plasmid was used for transfections, except in the case of *pAc5-lozenge-V5His*, in which 2μg plasmid was transfected. *pAc5-EGFP* was transfected simultaneously with V5His-tagged protein constructs as a transfection control. Following transfection, the cells were incubated for an additional 72 hours before collection. Three biological replicates were performed for each transfection assay. Soluble and insoluble fractions were isolated together by boiling cells in SDS buffer (50mM Tris pH 7.5, 2% SDS). MG132 treatment was performed by adding MG132 in DMSO to a final concentration of 100μM or DMSO as a control for 8 hours prior to collection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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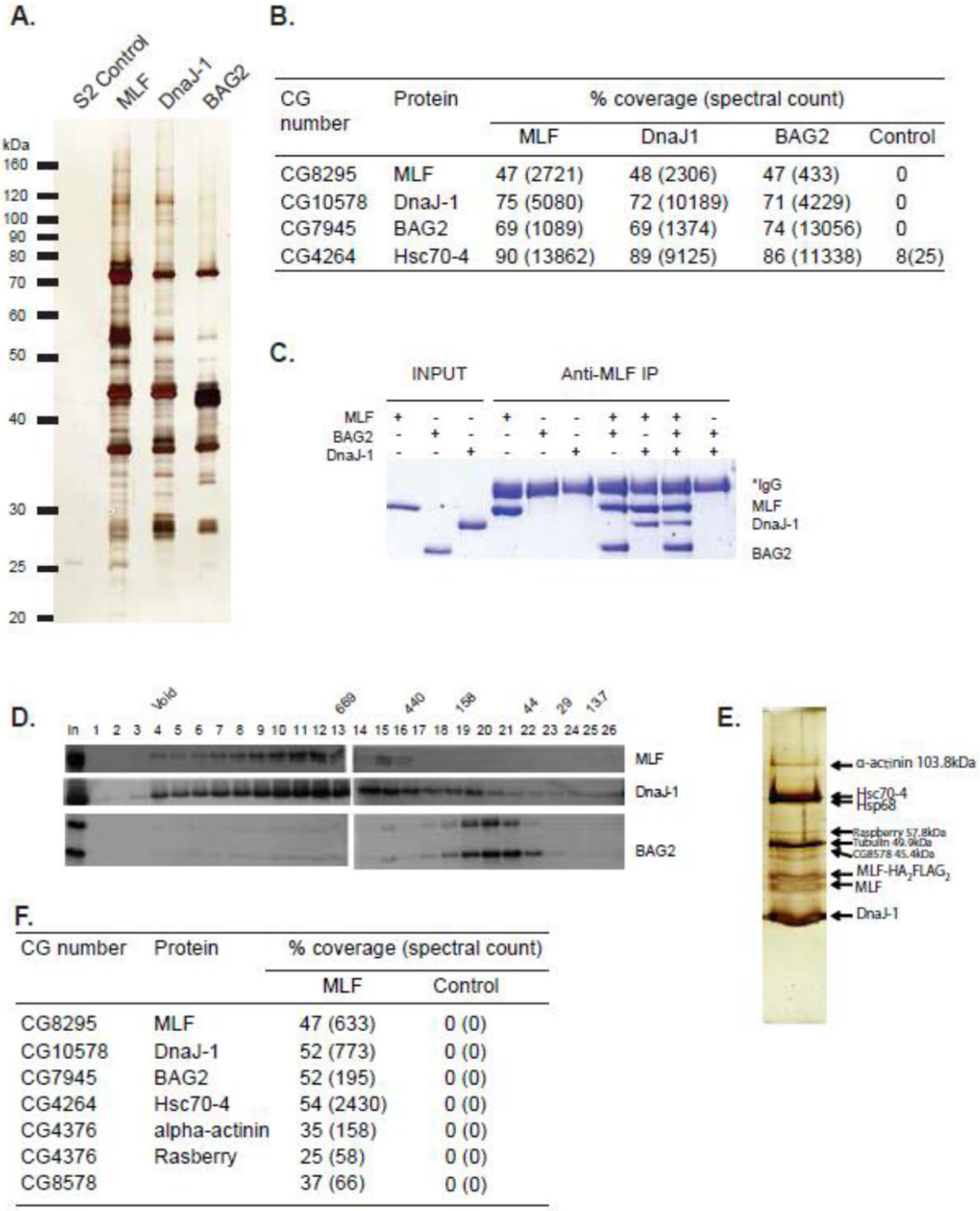
Highlights

- Myeloid Leukemia Factor (MLF) acts in a complex with DnaJ-1 and Bag2
- The MLF chaperone complex binds to chromatin and regulates transcription
- The MLF chaperone complex regulates the stability of transcription factors



CG number	Protein	% coverage (spectral count)			
		MLF	DnaJ1	BAG2	Control
CG8295	MLF	47 (2721)	48 (2306)	47 (433)	0
CG10578	DnaJ-1	75 (5080)	72 (10189)	71 (4229)	0
CG7945	BAG2	69 (1089)	69 (1374)	74 (13056)	0
CG4264	Hsc70-4	90 (13862)	89 (9125)	86 (11338)	8(25)

F.

CG number	Protein	% coverage (spectral count)	
		MLF	Control
CG8295	MLF	47 (633)	0 (0)
CG10578	DnaJ-1	52 (773)	0 (0)
CG7945	BAG2	52 (195)	0 (0)
CG4264	Hsc70-4	54 (2430)	0 (0)
CG4376	alpha-actinin	35 (158)	0 (0)
CG4376	Rasberry	25 (58)	0 (0)
CG8578		37 (66)	0 (0)

Figure 1. MLF physically interacts with co-chaperones DnaJ-1 and BAG2

(A) Silver stain of Flag-HA tandem affinity purifications of MLF, DnaJ-1, and BAG2 complexes from S2 cells expressing tagged versions of the bait proteins. Untagged S2 cells were used as a negative control. **(B)** Sequence coverage (percentage) and number of peptides (spectral count) are shown for peptides of MLF, DnaJ-1, BAG2, and Hsc70-4 identified by MudPIT analysis in purifications using MLF, DnaJ-1 and BAG2 as baits. **(C)** Coomassie stain of anti-MLF co-immunoprecipitations using recombinant MLF, BAG2, and DnaJ-1 proteins. 25% Input is shown in lanes 1–3. **(D)** Western blots of size exclusion

chromatography fractions of purified MLF complexes from S2 cells probed with antibodies against MLF, DnaJ-1, and BAG2. Size of fractions indicated above blots in kDa. **(E)** Silver stain of elution fraction 13 shown in figure 1D. **(F)** Peptides of MLF co-purifying proteins identified by MudPIT analysis of elution fraction 13 from size exclusion chromatography studies. Sequence coverage (percentage) and number of peptides (spectral count) are shown for each polypeptide.

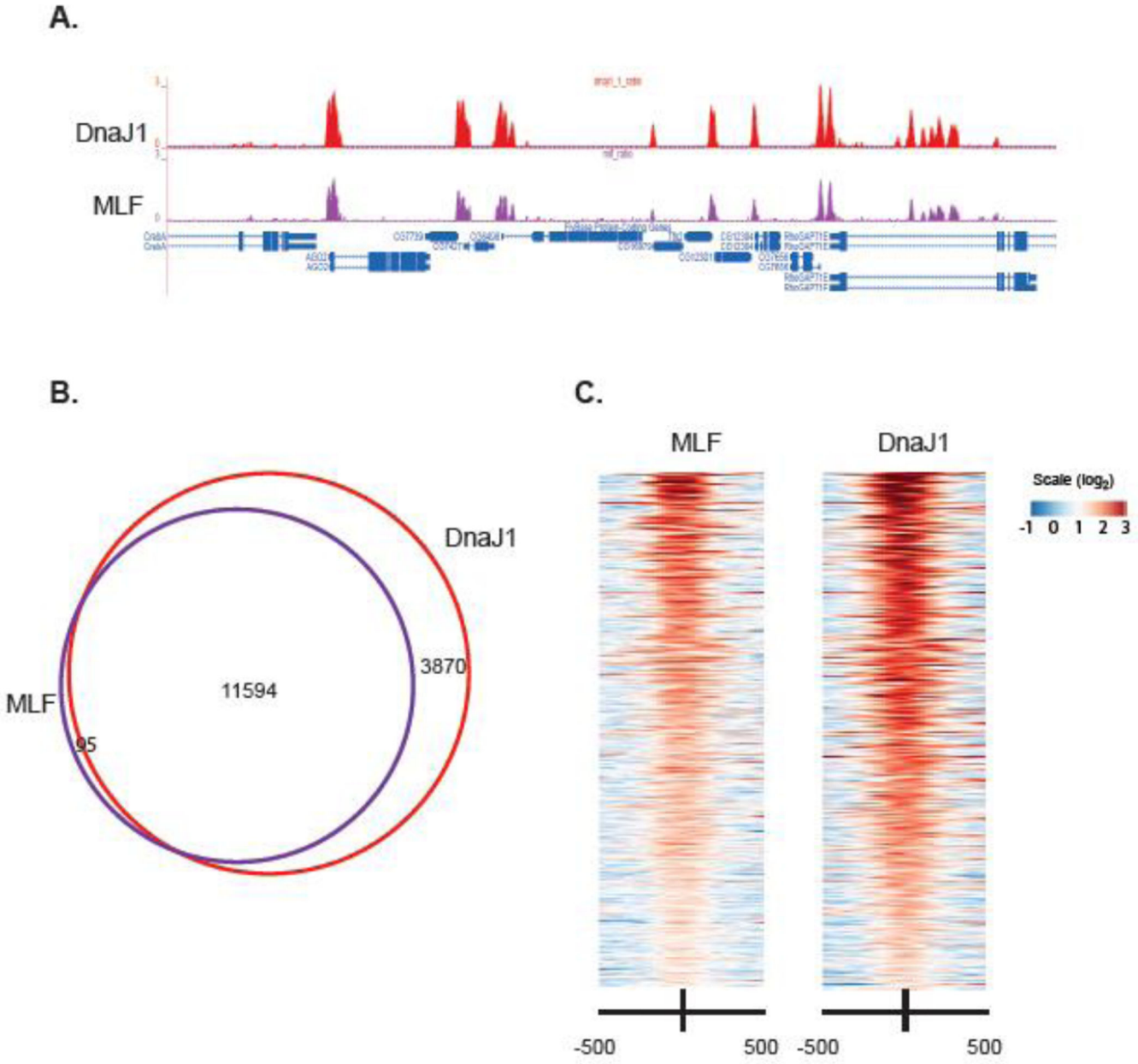
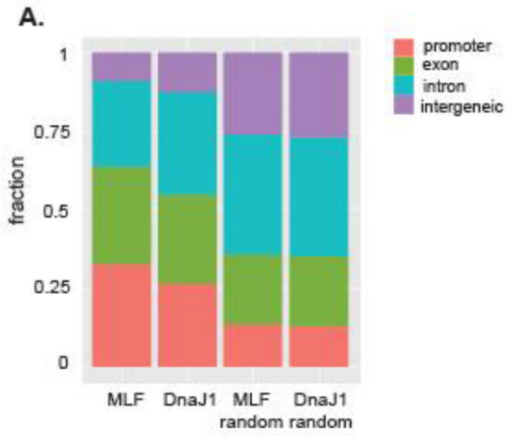


Figure 2. MLF and DnaJ-1 associate with chromatin at a common set of genomic loci

(A) Genome browser view of MLF and DnaJ-1 ChIP-seq results along a region of *Drosophila* chromosome. The ChIP signal intensity on the *y*-axis is enrichment of IP/INPUT. **(B)** Venn diagram showing overlap of peaks bound by MLF and DnaJ-1. **(C)** Heatmap of peaks bound by both MLF and DnaJ-1, centered around the MLF summit and ordered on the basis of highest to lowest MLF occupancy.



Features	p values for differential occupancy
mlf_promoters	0
dnaj_promoters	6.63 e-255
mlf_exons	8.82 e-72
dnaj_exons	3.02 e-51
mlf_introns	8.61 e-112
dnaj_introns	2.31 e-24
mlf_intergenic	0
dnaj_intergenic	0

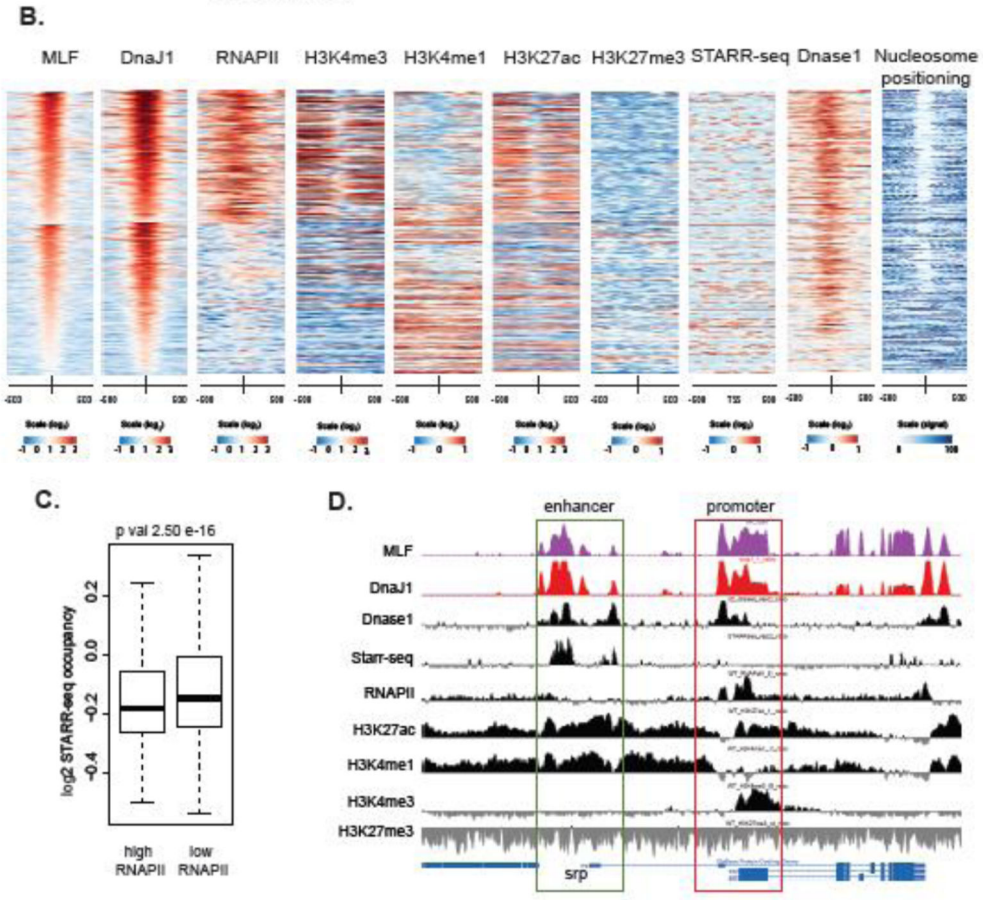


Figure 3. MLF and DnaJ-1 bind both promoter and enhancers

(A) Graph indicating the percentages of MLF and DnaJ-1 binding peak summits with respect to gene features based on analysis of MLF and DnaJ-1 ChIP-seq results. As a control, the locations of peaks were randomized with respect to the proportions of MLF of DnaJ-1 peak summits on each chromosome to determine percentages of peaks that would localize to particular gene features by chance. Promoters are defined as −1000 bp to +300 bp surrounding the transcription start site. P values as determined by Chi-squared test for enrichment or loss of MLF and DnaJ-1 binding to each genomic feature compared to

randomized peaks is shown to the right. **(B)** Peaks bound by both MLF and DnaJ-1, were categorized based on the presence of RNAPII and divided into two groups (high RNAPII and low RNAPII). Heatmaps containing regions −500 to + 500 around the summit of MLF peaks were plotted for each group ordered by MLF occupancy (highest to lowest occupancy). Occupancy of RNAPII, H3K4me3, H3K4me1, H3K27ac and H3K27me3 were plotted around the summit of MLF peaks. Additionally, STARR-seq signatures representative of putative enhancers in Drosophila S2 cells, along with DnaseI hypersensitivity and nucleosome occupancy around MLF peaks were also plotted in the same order as MLF in the 2 groups. **(C)** Enrichment of STARR-seq signature at low RNAPII as compared to high RNAPII peaks is shown as a box plot. **(D)** Genome browser screenshot of occupancy of MLF, DnaJ-1, RNAPII, and histone marks H3K4me1, H3K4me3, H3K27ac and H3K27me3 along with Dnase1 hypersensitivity and STARR-seq signatures at the *srp* locus. Green box marks the *srp* enhancer in S2 cells that is enriched for H3K4me1 and H3K27ac marks along with strong STARR-seq signature, while the promoter with RNAPII and H3K4me3 is marked by a red box. MLF and DnaJ-1 are found to occupy both the promoter and enhancer.

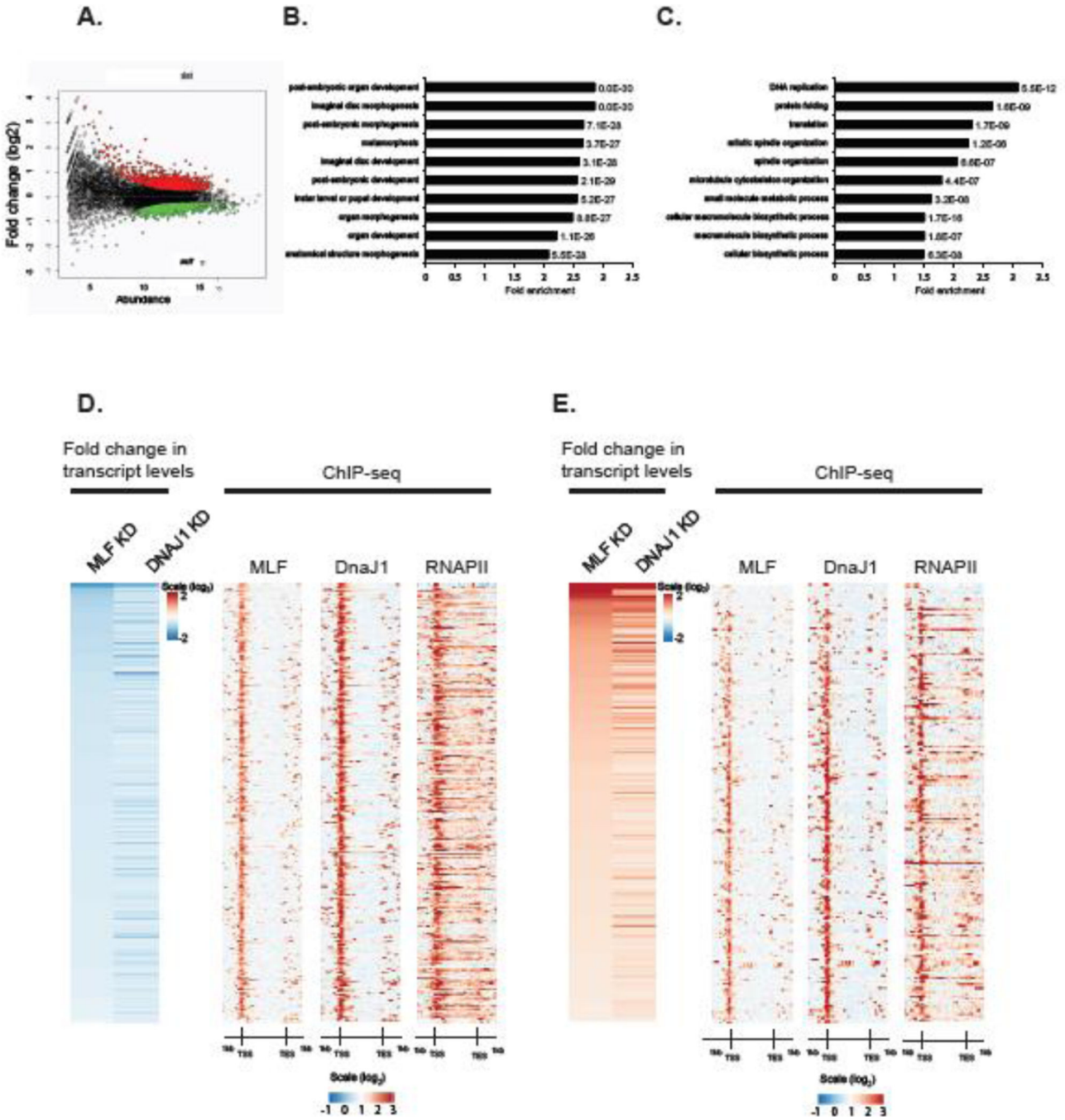


Figure 4. MLF and DnaJ-1 co-regulate the transcription of a subset of genes

(A) MA plot of DESeq results of the filtered MLF RNA-seq gene set. Gene sets with an adjusted p-value < 0.01 are highlighted, with genes up-regulated and down-regulated in the *mlf* knockdown samples shown in red and green, respectively. **(B–C)** GO terms enriched in the genes that were significantly (adjusted p-value < 0.01) upregulated **(B)** or downregulated **(C)** in *mlf* knockdown S2 cells are shown. The fold enrichment for each GO category is shown on the *x*-axis, with the *p*-value of each category indicated to the right. Only the top 10 GO terms for biological processes that show differential enrichment in the presence of MLF

are presented. **(D, E)** Genes that were occupied by MLF and DnaJ-1 and downregulated in *mlf* and *dnaj-1* knockdown cells **(D)** or bound by MLF and DnaJ-1 and upregulated in *mlf* and *dnaj-1* knockdown cells **(E)** were used to generate heatmaps. In each case change in gene expression in *mlf* and *dnaj-1* knockdown cells were plotted along with occupancy of MLF, DnaJ-1 and RNAPII 1kb upstream of transcription start site (TSS) to 1kb downstream of the transcription end site (TES). The genes were ordered based on the fold change in expression (most changed to the least changed) in *mlf* knockdown cells.

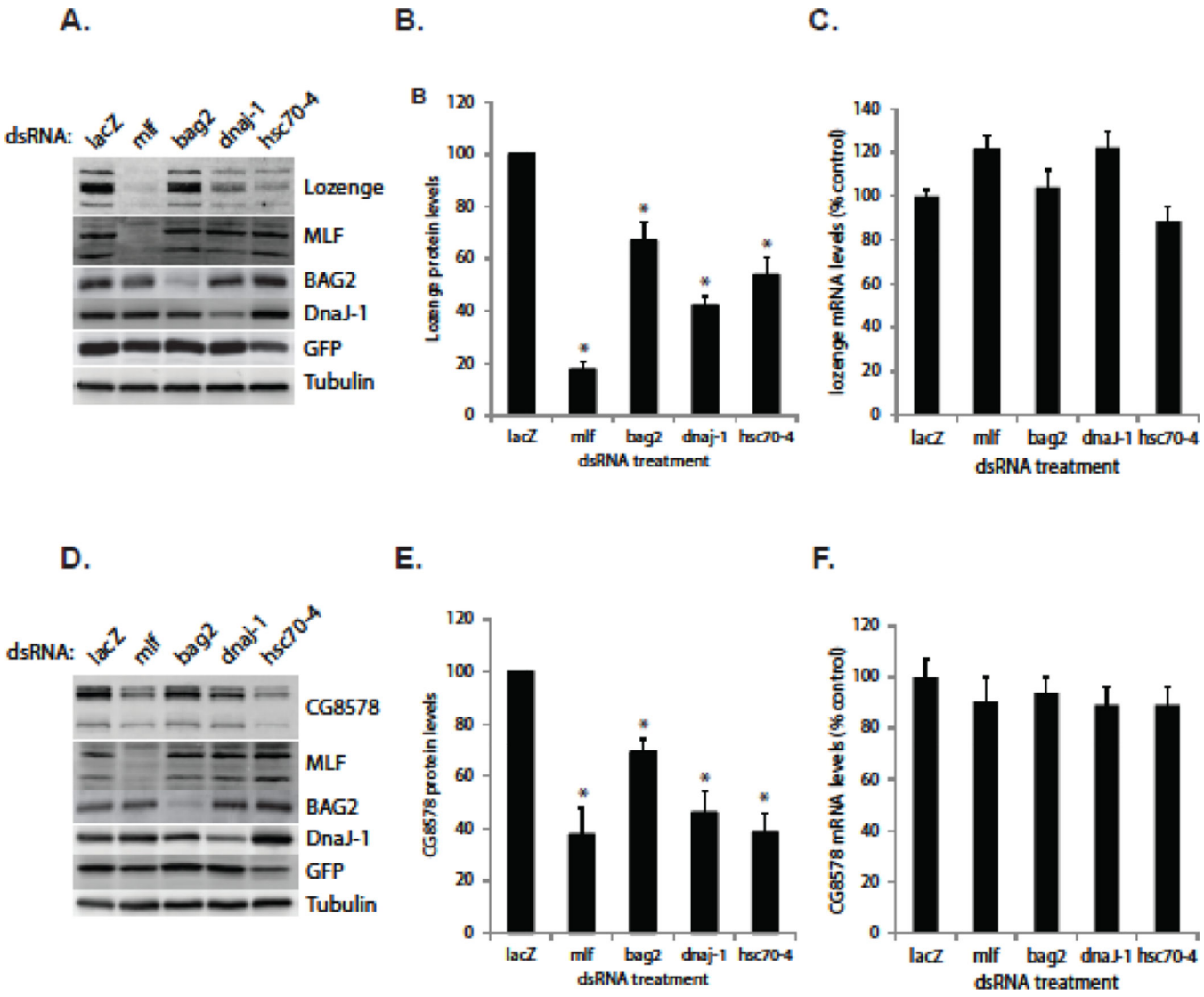


Figure 5. The MLF-chaperone complex regulates transcription factor stability

(A, D) Western blots of whole cell extracts from S2 cells treated with dsRNA against the control *lacZ*, *mlf*, *bag2*, *dnaj-1*, or *hsc70-4* and transfected with *pAc5-lozenge* **(A)** and *pAc5-CG8578* **(D)** probed with antibodies against V5, MLF, BAG2, DnaJ-1, GFP, and Tubulin. **(B, E)** Quantitation of Lozenge **(B)** and CG8578 **(E)** protein levels from three biological experiments, including the western blots shown in **(A, D)** respectively. Mean protein levels were normalized to GFP protein levels and are plotted as a percentage of the *lacZ* control ± SEM for three biological experiments. * marks all changes that show significant change in protein levels with p value < 0.05 (student's T-test) **(C, F)** qRT-PCR was performed on cDNA isolated from S2 cells treated with dsRNA against the control *lacZ*, *mlf*, *bag2*, *dnaj-1*, or *hsc70-4* and transfected with *pAc5-lozenge* **(C)** and *pAc5-CG8578* **(F)**. Mean expression levels were normalized to the transfection control *gfp* mRNA levels and are plotted as a percentage of the *lacZ* control ± SEM for three biological experiments.