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Cyclin-dependent Kinase 8 Module Expression Profiling Reveals Requirement of Mediator Subunits 12 and 13 for Transcription of Serpent-dependent Innate Immunity Genes in *Drosophila*[*] [S]

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Background: Roles of Mediator Cdk8 module subunits in transcriptional regulation are not well defined in metazoans.
Results: Med12-Med13-dependent transcription is modulated by Cdk8-cyclinC and is important for the GATA factor Serpent.
Conclusion: Med12-Med13 is required for transcription of Serpent-dependent innate immunity genes in *Drosophila*.
Significance: Genome-wide profiling suggests that Cdk8-cyclinC transcriptional regulation is limited to the Cdk8 module and dependent on Med12-Med13.

The Cdk8 (cyclin-dependent kinase 8) module of Mediator integrates regulatory cues from transcription factors to RNA polymerase II. It consists of four subunits where Med12 and Med13 link Cdk8 and cyclin C (CycC) to core Mediator. Here we have investigated the contributions of the Cdk8 module subunits to transcriptional regulation using RNA interference in *Drosophila* cells. Genome-wide expression profiling demonstrated separation of Cdk8-CycC and Med12-Med13 profiles. However, transcriptional regulation by Cdk8-CycC was dependent on Med12-Med13. This observation also revealed that Cdk8-CycC and Med12-Med13 often have opposite transcriptional effects. Interestingly, Med12 and Med13 profiles overlapped significantly with that of the GATA factor Serpent. Accordingly, mutational analyses indicated that GATA sites are required for Med12-Med13 regulation of Serpent-dependent genes. Med12 and Med13 were also found to be required for Serpent-activated innate immunity genes in defense to bacterial infection. The results reveal a novel role for the Cdk8 module in Serpent-dependent transcription and innate immunity.

The Mediator functions as an integrative hub mediating signals from upstream factors to RNA polymerase II. Cdk8, cyclin C (CycC),[4] Med12, and Med13 form a subcomplex (Cdk8 module), which reversibly associates with Mediator to regulate transcription (1). Biochemical and structural analyses suggest that primarily Med13 links the Cdk8 module to the core Mediator and Med12 serves as a bridge between Med13 and the cyclin-dependent kinase pair Cdk8-CycC (2, 3). The Cdk8 module can act as a repressor or activator context dependently through *e.g.* hindering of RNA polymerase II binding with the core Mediator (2, 3), stimulation of elongation through Cdk9 (4), and interaction with the cohesin loading factor Nipped-B-like (5). Cdk8 module subunits have also been shown to interact with (6–8) and phosphorylate (9, 10) various transcription factors. Cdk8 module regulation of specific transcription factor responses has been implicated in several physiological contexts including organogenesis (*e.g.* Notch (11)), hematopoiesis (GATA/RUNX (6)) lipogenesis (SREBP-1 (12)), and immunity (Stat-1 (10)), and disease states such as colorectal cancer (E2f1, β-catenin (9, 13)).

It is not clear to what extent the Cdk8 module subunits cooperate in transcription regulation. In budding yeast, all four subunits control expression of a common set of genes (14), suggesting that Cdk8 has a major role. In mammalian cells, Cdk8 and Med12 sometimes cooperate on specific genes (15, 16), but in other contexts Cdk8 and CycC appear to act independently of Med12, suggesting Mediator-independent functions (17, 18). In *Drosophila*, effects of depletion of Cdk8 and CycC have been compared genome-wide in fat bodies where similar changes were noted (12). More limited analyses on depletion of *Drosophila* Med12 and Med13 (19, 20) or of all four subunits (6, 11, 21) indicate common as well as distinct functions for the subunits. Genome-wide analyses of the roles of all Cdk8 module subunits are lacking from metazoan cells. Dissecting these in mammalian cells is complicated by the partly redundant paralogs of Cdk8 (Cdk19 (22, 23, 24)), Med12 (Med12L (15, 23)), and Med13 (Med13L (23, 25)). Here we have utilized *Drosophila* cells to investigate whether the four Cdk8 module subunits

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[S] This article contains supplemental Tables S1–S3.
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[4] The abbreviations used are: CycC, cyclin C; qPCR, quantitative PCR; NELF, negative elongation factor; IMD, Immune Deficiency; AMP, antimicrobial peptide; Luc, luciferase.

regulate transcription in concert or independently genome-wide.

EXPERIMENTAL PROCEDURES

Cell Culture and RNA Interference—*Drosophila* S2 cells (Invitrogen) were maintained at +23–25 °C in *Drosophila* serum-free medium (Gibco) or Express Five serum-free medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. S2 cell genomic DNA or Berkley *Drosophila* Genome Project (BDGP) Schneider cells oligo(dT) (SD) cDNA library was used for production of cDNA templates for targeting dsRNAs, and pEGFP-N2 (Clontech) and pRL-CMV (Promega) were used as templates for production of GFP and Luc controls, respectively. Details of primers used for production of cDNA templates for dsRNA are listed in supplemental Table 3. dsRNA was produced by *in vitro* transcription using T7 polymerase as described previously (26). Two sets of dsRNAs (1 and 2) targeting different parts of the mRNA were used except in the case of Cdk8, where dsRNA 2 represented the 5′ (Cdk8 dsRNA 2a) or 3′ (Cdk8 dsRNA 2b) part of dsRNA 1. Exponentially growing cells were transfected with 20–50 μg of targeting (*Cdk8*, *CycC*, *Med12*, *Med13*, *Srp*, *Lz*) or control (*GFP*, *Luc*) dsRNA/ml and serum-starved for 1 h before the addition of serum-containing medium.

Expression Profiling and Quantitative PCR—Total RNA was extracted from biological replicate samples 72 h after dsRNA transfection (four for *CycC*, *Med12*, *Med13*, *GFP*, and *Luc*, and two for *Cdk8*) according to the manufacturer's protocols (Qiagen RNeasy Plus mini kit). Biotinylated cRNA was fragmented and hybridized to Affymetrix GeneChip *Drosophila* Genome 2.0 arrays according to the manufacturer's protocol. Data were normalized according to the GC Robust Multiarray Average (GC-RMA) method and analyzed using the GeneSpring GX software. Raw data can be accessed using Gene Expression Omnibus series record GSE52343. Probe sets with low expression (raw signal <5 in all samples) were excluded and differentially expressed genes were identified based on -fold change (≥1.5) as compared with control samples. Genes with ≥1.5-fold changes in both Cdk8 and CycC or both Med12 and Med13 (p value < 0.02, Student's t test) were used for comparisons to other microarray data as described in supplemental Table 1*B*. Pearson's correlation coefficient was calculated using Free Statistics Software.[5] The Srp microarray analysis was mentioned as unpublished data in a previous study (37). Total RNA of four replicate samples (dsRNA Srp and control) was extracted at 48 h after dsRNA treatment. Biotinylated cRNA was fragmented and hybridized to Affymetrix GeneChip *Drosophila* Genome Arrays according to the manufacturer's protocols. Data (supplemental Table 2*A*; all probe sets) were analyzed using the MAS software and filtered based on Present/Marginal/Absent (PMA) calls (present in 3/4 controls and dsRNA Srp samples for down- and up-regulated genes, respectively), and genes with ≥1.5-fold changes (p < 0.02, Student's t test) were considered as Srp-regulated (supplemental Table 2*B*). Statistical significance of gene set overlaps was calculated using a hypergeometric test (GeneProf (27)). The amounts of GATA (HGATAABV) elements were compared in a 1000-bp sequence upstream of the 5′-UTR obtained from BioMart (28), using Berkley *Drosophila* Genome Project 5 (BDGP5) data. A set of 100 randomly chosen genes was used as control. For quantitative PCR (qPCR) experiments, total RNA was extracted at 72–96 h (Cdk8, CycC, Med12, Med13, and Lz) or 24 h (Srp) after dsRNA transfection. dsRNA against *GFP* or *Luc* was used as control or to even up dsRNA amounts in experiments combining multiple knockdowns. RNA was reverse-transcribed to cDNA using random hexamer primers, and samples were analyzed for expression of target genes by qPCR using SYBR Green reagents (Applied Biosystems, 4368708 or 4385618). Expression was normalized to expression of control genes (*gapdh2* or *Cdk7*). When indicated, cells were treated with 200 μg/ml heat-inactivated *Escherichia coli* (Invitrogen, 18265-017) for 24 h before RNA extraction. Details of primers used for qPCR are listed in supplemental Table 3. Student's t test was used for statistical analysis in qPCR experiments. Not significant, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Western Blotting and Antibodies—S2 cells were collected in hot boiling Laemmli sample buffer supplemented with 1 mM DTT 72 h following dsRNA transfection. Proteins were separated on 7 or 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Proteins were detected using overnight incubation with antibodies as follows: rabbit anti-Cdk8 (anti-K35 (29)) 1:1000, rabbit anti-CycC (30) 1:500, guinea pig anti-Med12 (anti-Kto (20)) 1:1000, and rabbit anti-Med13 (anti-Skd (20) 1:50000.

Plasmids, Mutagenesis, and Dual-Luciferase Assays—The *CG14629-luc* reporter was generated by cloning a 978-bp fragment of *CG14629* into the pGL3-Basic vector (Promega). Details of cloning primers are listed in supplemental Table 3. C*G14629ΔGATA-luc* was generated through site-directed mutagenesis (Stratagene, 200515) of *CG14629-luc* converting the GATA consensus to GCCA as described previously (32). The *MtkΔGATA-luc* reporter was generated through site-directed mutagenesis of *Mtk-luc* (33) as described previously (34). *p4xPO45-Fluc* and *pAc-Lz-V5* have been described previously (6). *pRLnull-copiaLTR* was used as *Renilla* transfection control in Dual-Luciferase assays, and *pAc5.1/V5-His A* (Life Technologies) or mock was used as control for overexpression. S2 cells were transfected with a total of 1.5 μg of plasmid DNA using FuGENE HD (Promega) 48 h after transfection with dsRNA (targeting for *Cdk8*, *CycC*, *Med12*, *Med13*, or nontargeting (*GFP*) for other samples) and lysed 48 h later for Dual-Luciferase assay (Promega) according to the manufacturer's protocols. For experiments including *Srp* dsRNA, cells were transfected with a second dose of dsRNA 24 h prior to sample collection (targeting for Srp and *GFP* for other samples). For *E. coli* experiments, cells were treated with 200 μg/ml heat-inactivated *E. coli* (Invitrogen, 18265-017) for 24 h before cell lysis. Student's t test was used for statistical analysis. Not significant, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

Fly Lines and Larval Infection Assay—The following fly lines were used: Fb-Gal4 (FBti0013267 (35)) control w1118 and RNAi lines; Med12, VDRC 23142 and Bloomington stock center 34588; Srp, VDRC 109521. Flies were kept at 25 °C on standard diet. Third instar prewandering larvae were pricked at the

[5] P. Wessa, personal communication.

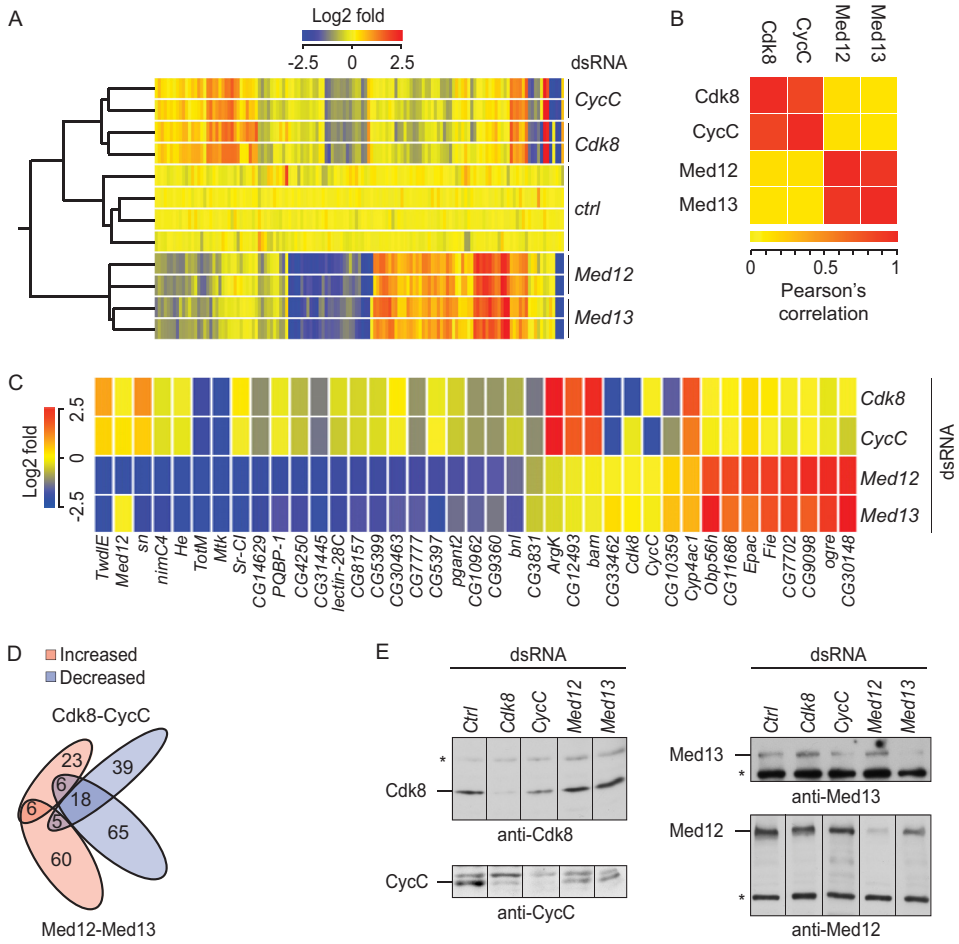

FIGURE 1. **Microarray analysis (Affymetrix *Drosophila* Genome 2.0) of gene expression changes in *Drosophila* S2 cells after depletion of Cdk8, CycC, Med12, or Med13.** *A*, hierarchical clustering according to Euclidean distance of indicated samples using 135 probe sets with ≥3-fold change between any 2 of 22 microarray samples. Colors indicate expression change (log 2) as compared with average of all samples. *ctrl*, control. *B*, Pearson's correlation matrix of indicated samples based on average log 2-fold change of 2119 probe sets that show ≥1.5-fold changes between any of the 22 microarray samples. Correlation is shown by colors as indicated (0, no correlation; 1, total positive correlation). *C*, heat map of genes with at least 3-fold change in expression after depletion of Cdk8, CycC, Med12, or Med13 as indicated. Colors indicate expression change (log 2) to average of control. *D*, overlap of genes with ≥1.5-fold ($p < 0.02$) increased (*red*) or decreased (*blue*) mRNA levels after depletion of both Cdk8 and CycC (Cdk8-CycC) or both Med12 and Med13 (Med12-Med13). *E*, Western blotting analysis with antibodies against Cdk8, CycC, Med12, and Med13 72 h following treatment with the indicated dsRNAs. Unspecific background bands serving as loading controls are indicated by *asterisks*; Cdk8 and CycC detection was from the same blot.

posterior lateral side with a 0.125-mm tungsten needle (Fine Science Tools, 10130-05) dipped into a pelleted fresh culture of exponentially growing *E. coli* (Invitrogen, 18265-017) diluted with sterile glycerol at a 5:1 ratio. Infected larvae were kept on sterile apple juice plates for 3 h, and fat bodies were dissected from larvae with a clear black wound indicative of septic injury. Total RNA was isolated from fat bodies of one to two larvae/sample, and expression of *Mtk* and *DptB* was analyzed by qPCR and normalized to expression of control genes (*Cdk7*, *gapdh2*, and *rp49*). Student's *t* test was used for statistical analysis. Not significant, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

RESULTS

To compare expression changes following depletion of Cdk8 module components, *Drosophila* S2 cells were treated with dsRNAs targeting *Cdk8*, *Cyclin C*, *Med12*, or *Med13*. Expression profiling of 2–4 replicate knockdown samples (Gene Expression Omnibus GSE52343) demonstrated significant and consistent changes in gene expression as compared with controls (Fig. 1*A*). Over 1.5-fold expression changes were noted in 354 genes (supplemental Table 1*A*) with 39 genes showing over 3-fold changes (Fig. 1*C*). Depletion of any subunit led to both decreased and increased gene expression indicative of activating and repressing regulation, consistent with previous expression profiling studies performed in metazoan cells with subsets of the module (12, 17, 22).

Distinct Expression Profiles by Cdk8-CycC and Med12-Med13—Comparison of the expression profiles demonstrated a very significant similarity between Cdk8 and CycC profiles (Pearson's correlation 0.86; Fig. 1*B*) with similar changes noted in 97 genes (supplemental Table 1*B*). Correlation was even more striking between Med12 and Med13 profiles (Pearson's correlation 0.93; Fig. 1*B*) with similar changes in 160 genes (supplemental Table 1*B*). Correlation between other subunits was low (Pearson correlations between 0.12 and 0.14; Fig. 1*B*), and only 24 mRNAs were similarly altered by all subunits (Fig. 1*D*). Interestingly, even opposite effects by Cdk8-CycC as compared with Med12-Med13 depletion were noted for 11 genes (Fig. 1*D*). Western blotting analysis revealed efficient knockdown of all

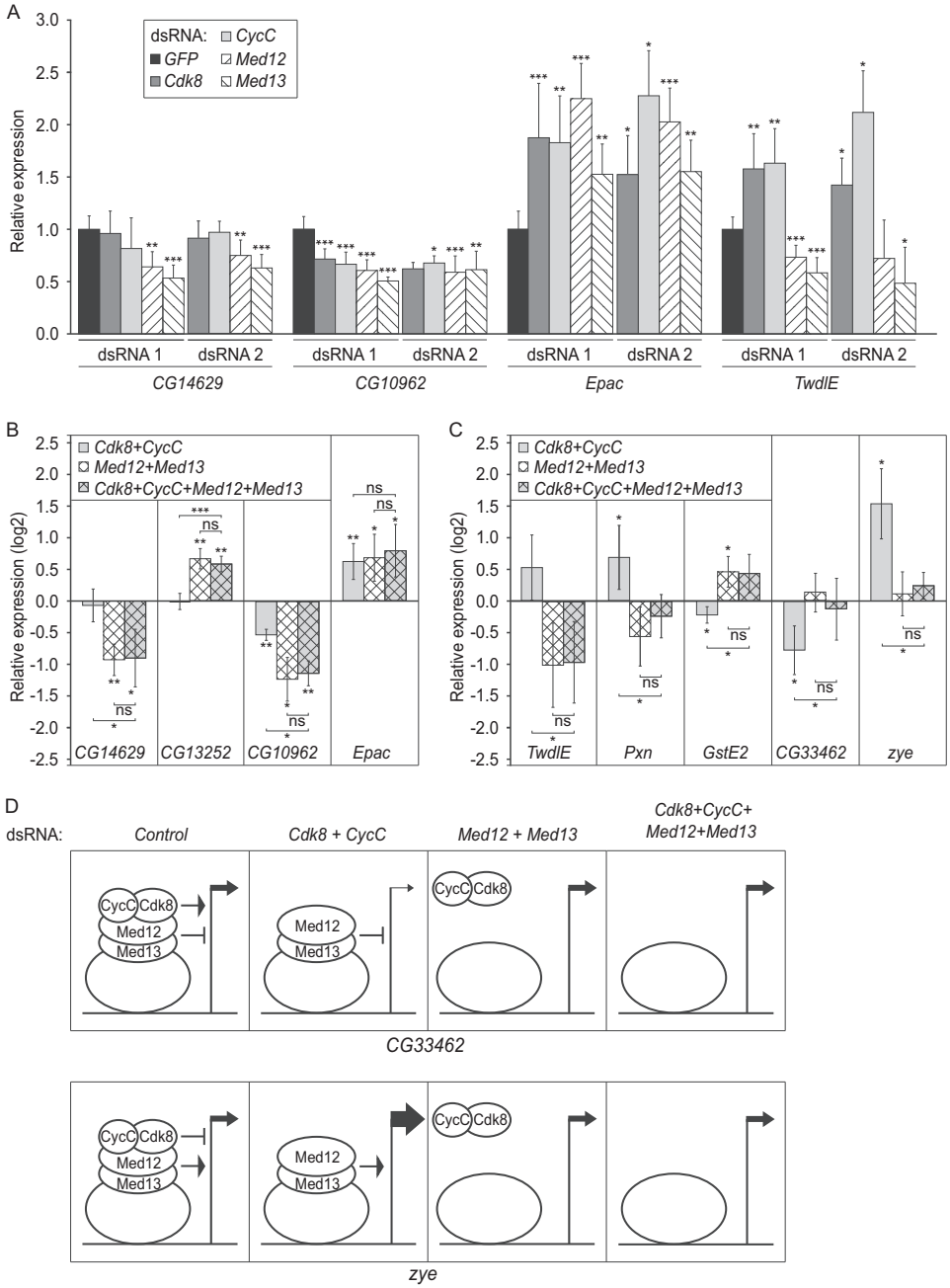

FIGURE 2. **Cdk8-CycC effects on transcription can be opposite to Med12-Med13 but are Med12-Med13-dependent.** *A*, validation of Cdk8 module-regulated genes by qPCR analysis of relative expression levels following treatment with two different sets of dsRNAs (dsRNA1 and dsRNA2) targeting Cdk8 module components as indicated. *Error bars* represent S.D. in two independent experiments with three biological replicates each except for CycC dsRNA2 where *error bars* represent S.D. in one experiment and Cdk8 dsRNA2 where *error bars* represent S.D. between three biological replicates of two different dsRNAs (dsRNA 2a and 2b). *B* and *C*, relative expression levels (log 2) measured by qPCR of selected genes after treatment with dsRNA as indicated as compared with control dsRNA (*GFP* + *Luc*). *Error bars* represent S.D. in at least three independent experiments. *D*, model of Cdk8 module-regulated genes *CG33462* (*top*) and *zye* (*bottom*) that show expression changes (*CG33462*, decrease; *zye*, increase) only after Cdk8-CycC dsRNA treatment although also being Med12-Med13-dependent as described under "Results." *ns*, not significant, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

four Cdk8 module subunits (Fig. 1*E*). As previously noted (6), some of the knockdowns also had limited effects on other sub-module component protein levels. Expression profiling results were validated by qPCR analysis of selected genes following treatment of cells with two different sets of dsRNA targeting each subunit of the Cdk8 module (Fig. 2*A*). Expression profiling of the *Drosophila* Cdk8 module subunits thus demonstrates a pairwise separation, which clearly differs from yeast where co-regulation by all four subunits is the dominant feature (14).

Cdk8-CycC Is Dependent on Med12-Med13—The separation of Cdk8-CycC and Med12-Med13 expression profiles suggested independent functions for the two pairs. To investigate this, expression changes following depletion of either Cdk8 and CycC or Med12 and Med13 were compared with deletion of all four subunits. Cdk8-CycC-independent regulation by Med12-Med13 was confirmed for *CG14629* and *CG13252* as expression of these genes was not affected by Cdk8-CycC depletion. On co-regulated genes (*CG10962* and *Epac*), depletion of all

four subunits did not lead to additional effects (Fig. 2*B*). In contrast, on all validated Cdk8-CycC-regulated genes (*TwdlE, Pxn, GstE2, CG33462*, and *zye*; Fig. 2*C*), Cdk8-CycC effects were lost when Med12 and Med13 were depleted simultaneously. Importantly, not only genes showing opposite effects by depletion of Cdk8-CycC and Med12-Med13, but also genes affected only by depletion of Cdk8-CycC (*CG33462* and *zye*), showed loss of Cdk8-CycC-specific effects when combining Med12-Med13 with Cdk8-CycC depletion (Fig. 2*C*). This indicates that the ability of Cdk8-CycC to regulate transcription is dependent on Med12-Med13 on all studied genes. The result also suggests that in situations where Med12-Med13 and Cdk8-CycC have opposite effects on transcription, Med12-Med13 effects are partially or completely masked, as illustrated for *CG33462* and *zye* in Fig. 2*D*. This is due to loss of both Med12-Med13 and Cdk8-CycC opposing effects when only Med12 and Med13 are depleted (Fig. 2*D*), thus resulting in no change in gene expression as compared with control cells. These results are in agreement with biochemical and structural observations (2, 3) demonstrating that Med13 links Cdk8-CycC to the core Mediator through Med12.

Med12-Med13 Is Required for Serpent-dependent Transcription—Genes regulated by the Cdk8 module included hemocyte markers (*e.g. He*, *Pxn*) and genes related to immune response (*e.g. eater*, *Sr-CI*, *Mtk*), possibly reflecting the macrophage-like identity of S2 cells (36). We also noted that target genes of *e.g.* SREBP-1, previously shown to be repressed by Cdk8-CycC in the fat body (12), were not among the regulated genes here. This indicates that dependence of specific gene sets on Cdk8-CycC is largely context-dependent. This prompted us to compare expression profiling results with the Cdk8 module to regulons of selected transcription factors studied in cultured *Drosophila* cells and implicated to interact with the Cdk8 module (4–6, 9, 10).

Comparison of Cdk8-CycC and Med12-Med13 profiles with those following depletion of Cdk9 (37), E2f1 (37), or the cohesin loading factor Nipped-B (38) did not support cooperative regulation by the Cdk8 module and these factors. A small but significant overlap of three genes was noted between Med12-Med13 and *Drosophila* Stat (Stat92E) (37), suggesting a possible limited cooperation in these conditions (supplemental Table 1*B*). We also compared the Cdk8 module regulon with that of the negative elongation factor, NELF (39), as Cdk8 has been linked to elongation (4). A highly significant overlap of 19 genes dependent on both NELF and Cdk8-CycC was noted (supplemental Table 1*B*), suggesting involvement of Cdk8-CycC in NELF-dependent transcription.

Interestingly, a highly significant ($p = 6 \times 10^{-22}$) overlap of 23 genes dependent on Med12-Med13 and the GATA factor Serpent (Srp) was identified (Fig. 3*A* and supplemental Table 1*B*) when comparing Med12-Med13 expression profiles with expression profiling of Srp-depleted S2 cells (supplemental Table 2*B*). Treatment of S2 cells with two separate dsRNAs targeting Srp confirmed this correlation as all tested Med12-Med13-dependent genes were also dependent on Srp (Fig. 3*B*), where the stronger effect of Srp dsRNA1 correlated with a better knockdown (data not shown). Further comparison of Med12-Med13-dependent genes with expression profiles following depletion of Srp in *Drosophila* embryos (40) expanded this overlap to include 36% of the Med12-Med13-dependent genes, suggesting that Med12 and Med13 participate in Srp-mediated transcription. In addition, we noted that the effect of Srp depletion on gene expression was stronger for genes that were also dependent on Med12-Med13 than for those not affected by Med12-Med13 depletion (6.23 *versus* 2.76 average expression decrease following Srp RNAi, $p = 1.7 \times 10^{-5}$). To investigate whether the identified Med12-Med13 and Srp-dependent genes represented direct targets of Srp, we analyzed the presence of Srp binding sites (HGATAABV, 34) in promoters of these genes. As expected, a significant enrichment of Srp binding sites was noted in promoters of Srp-dependent genes. Importantly, in the Med12-Med13-dependent genes, enrichment was only noted in promoters of genes co-regulated by Srp (Fig. 3*C*), implicating these genes as direct targets of Serpent.

Previously a search for regulators of Lozenge-Serpent (Lz-Srp) activation of the *PO45* gene during hematopoietic crystal cell differentiation identified Med12 and Med13 (6) as modulators of Lz-Srp. The identified role and expression of Lz, however, are restricted to crystal cells (41), and accordingly, the genes identified here to be Srp-dependent in S2 cells did not show overlap to predicted Srp-Lz target genes (42). Treatment of S2 cells with *Lz* dsRNA did not affect genes regulated by Srp and Med12-Med13 (Fig. 3*D*). Thus, our results indicate a positive modulatory role for Med12-Med13 in Srp activation independently of the RUNX factor Lozenge.

To investigate how Med12-Med13 is involved in Srp-mediated transcription in S2 cells, we generated a reporter construct including six HGATAABV sites of the strongly Med12-Med13 and Srp-regulated *CG14629* gene, encoding a potential cytokine based on homology to mammalian leukemia inhibitory factor (43). This construct (*CG14629-luc*) demonstrated 17.2-fold increased luciferase activity as compared with the pGL3-basic reporter, indicating the presence of a functional promoter (Fig. 4*C*). Activity of *CG14629-luc* was significantly reduced by Srp depletion (Fig. 4*A*) but not affected by Lozenge overexpression (Fig. 4*B*), which did activate the Srp/Lz-dependent (6) *PO45* promoter. Notably, *CG14629-luc* activity was partly dependent on the GATA sites as GATA site mutations (*CG14629ΔGATA-luc*) resulted in 2.1-fold decreased activity (Fig. 4*C*). Consistent with this, a reduction of activity following Srp depletion noted in the wild type promoter was significantly diminished in *CG14629ΔGATA-luc* (Fig. 4*D*). Also, Med12 and Med13 depletion significantly reduced activity of the *CG14629-luc* reporter (Fig. 4*E*), demonstrating that the *CG14629* promoter is dependent on Med12-Med13. Importantly, this was partly mediated by the GATA sites as Med12 and Med13 depletion had a significantly smaller effect on *CG14629ΔGATA-luc* as compared with *CG14629-luc* (Fig. 4*F*). Taken together, these results suggest a direct role for Med12-Med13 in Srp-mediated transcription.

Med12-Med13 Is Required for Expression of Srp-dependent Antimicrobial Peptide Genes in Response to IMD Pathway Activation—Several of the genes identified to be regulated by both Srp and Med12-Med13 are involved in innate immunity, *e.g. Mtk*, *Sr-C1*, and *eater* (44–46). Both Srp and Med12-Med13 have been separately implicated in innate

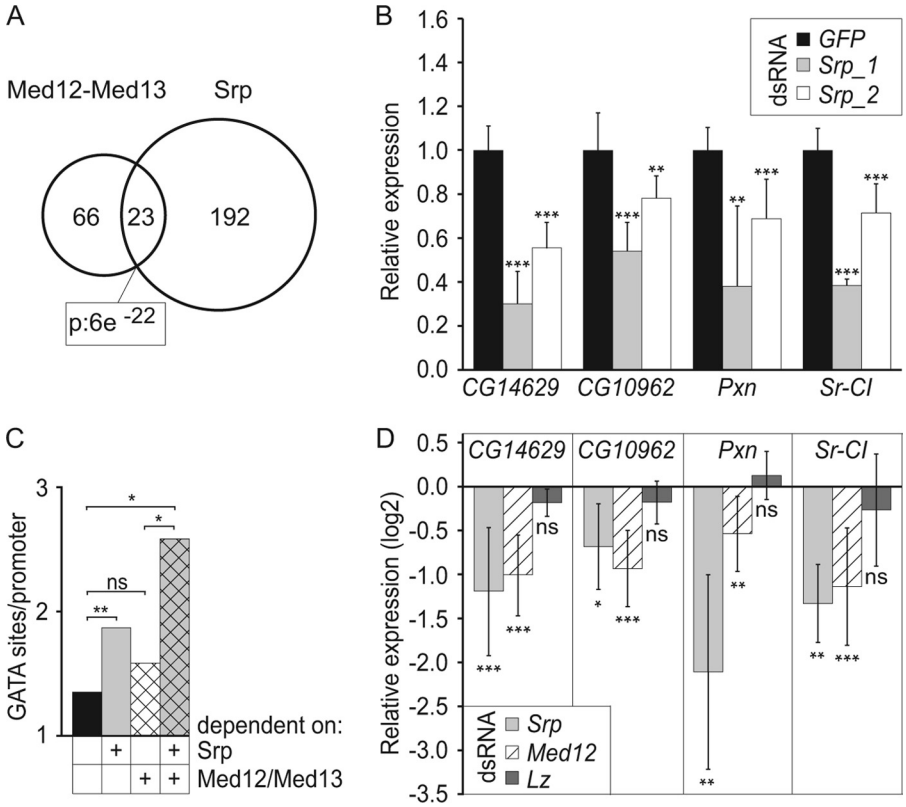

FIGURE 3. **Med12 and Med13 are required for Serpent-dependent genes independently of Lozenge.** *A*, overlap of genes with decreased expression after depletion of Med12 and Med13 (≥1.5-fold in both) and Srp (≥1.5-fold). *p* value of overlap is indicated. *B*, relative expression levels by qPCR of selected genes after treatment with two different Srp-targeting dsRNAs as indicated as compared with dsRNA *GFP*. *Error bars* represent S.D. in three independent experiments with 2–5 biological replicates each. *C*, average number of GATA sites (HGATAABV) in promoters of Srp- or Med12-Med13-dependent genes shown in *A*. The *first column* indicates average number of GATA sites in promoters of 100 randomly chosen genes. *D*, relative expression levels (log 2) by qPCR of selected genes after treatment with dsRNAs as indicated as compared with dsRNA *GFP*. *Error bars* represent S.D. in at least three biological replicates, except for *CG10962 Lz* dsRNA two replicates. *ns*, not significant, $p > 0.05$, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

immunity signaling through regulation of target genes of the Immune Deficiency (IMD) pathway. Med12 and Med13 are needed for activation of the antimicrobial peptide (AMP) gene *CecA* in the mosquito *Anopheles gambiae* (47), and Srp is required for induction of IMD-dependent AMP genes in *Drosophila* (34, 48–50). To study whether Med12-Med13 is needed for infection-induced IMD-dependent *Drosophila* AMP genes, we treated S2 cells with *E. coli* to activate the IMD pathway. This resulted in induction of mRNA levels of AMP genes *Mtk* and *DptB* as expected (25- and 40-fold, respectively (data not shown)). Depletion of Srp led to a significantly reduced level of *Mtk* mRNA as expected (34) and also to a significant decrease of *DptB* mRNA (Fig. 5*A*). Interestingly, depletion of Med12 and Med13 also resulted in significantly decreased levels of both *Mtk* and *DptB* (Fig. 5*A*). This was a direct effect based on the requirement of Med12-Med13 for full activity of a *Mtk-luc* (33) reporter after *E. coli* induction (Fig. 5*B*). Importantly, Med12-Med13 regulation of the *Mtk* promoter was mediated in part through the three previously identified GATA sites (34) as Med12 and Med13 depletion had significantly less effect when these sites were mutated (*MtkΔGATA-luc*) (Fig. 5*C*). In addition, we noted the dependence of *Mtk* and *DptB* on Cdk8-CycC (Fig. 5, *A–C*), consistent with the notion that a subset of Med12-Med13-dependent genes also requires Cdk8-CycC (Fig. 1, *C* and *D*).

Srp is essential for induction of AMP genes following Gram-negative bacterial infection in larval fat bodies (48–50). To investigate the relevance of our findings *in vivo*, we depleted either Srp or Med12 in larval fat bodies using RNAi under the control of the Fb-Gal4 driver (35). Expression of *Mtk* and *DptB* in the fat body following septic injury was analyzed in Fb-Gal4>Srp RNAi or Fb-Gal4>Med12 RNAi larvae as compared with control (Fb-Gal4>). Importantly, both Srp and Med12 RNAi resulted in reduced expression of *Mtk* and *DptB* (Fig. 5*D*). Thus, these results identify a novel role for the Cdk8 module in Serpent-dependent response to infection in hemocyte-like S2 cells and in the *Drosophila* fat body *in vivo*.

DISCUSSION

In this genome-wide study on transcription regulation by the Cdk8 module, a striking pairwise similarity was noted between Cdk8 and CycC as well as Med12 and Med13. Co-regulation by all four subunits was surprisingly limited, clearly differing from yeast (14) where depletion of any Cdk8 module subunit results in similar effects on transcription. Importantly, the lack of Med13-specific genes indicates that Med13 does not regulate transcription without Med12, although structural and biochemical analysis (2, 3, 25) of the Cdk8 module suggests this might be possible. The results thus suggest that the previously identified Med13 regulatory mechanisms (25, 51) are likely to

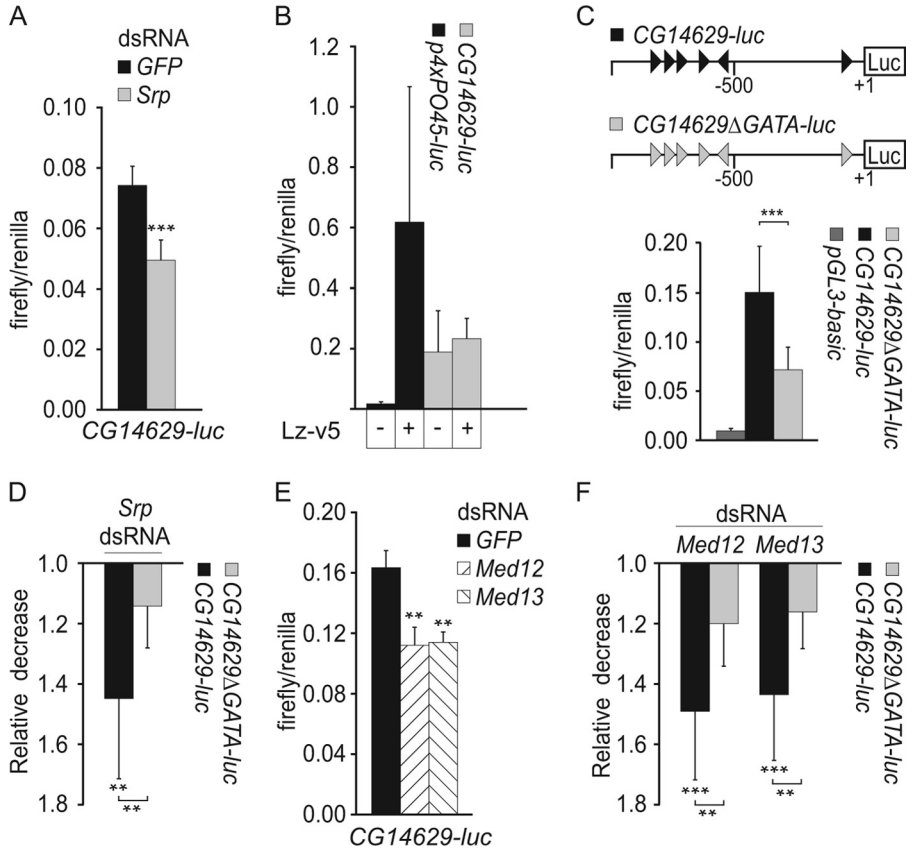

FIGURE 4. **Med12 and Med13 are required for GATA-dependent transcription.** *A*, luciferase activity of *CG14629-luc* normalized to transfection control *pRLnull-copiaLTR* in control and Srp-depleted S2 cells as indicated. *Error bars* represent S.D. in two representative experiments with three biological replicates each. *B*, luciferase activity of *p4xPO45-luc* (*black*) and *CG14629-luc* (*gray*) reporters normalized to transfection control *pRLnull-copiaLTR* in Lozenge-overexpressing (Lz-v5) and control cells. *Error bars* represent S.D. in two experiments. *C*, schematic of the *CG14629-luc* and *CG14629-ΔGATA-luc* reporters showing distribution and orientation of GATA sites, where sites with point mutations are in *gray*. Activity of the reporters and *pGL3-basic* normalized to transfection control *pRLnull-copiaLTR* is indicated in the *columns below* as average and S.D. of five (*pGL3-basic*) or nine independent experiments. *D*, columns indicate relative decrease of *CG14629-luc* (*black*) or *CG14629-ΔGATA-luc* (*gray*) activity in Srp-depleted S2 cells as compared with *GFP* dsRNA control. *Error bars* represent S.D. of six experiments. *E*, luciferase activity of *CG14629-luc* normalized to transfection control *pRLnull-copiaLTR* following dsRNA treatment as indicated. *Error bars* represent S.D. in two representative experiments with three biological replicates each. *F*, columns indicate relative decrease of *CG14629-luc* (*black*) or *CG14629-ΔGATA-luc* (*gray*) activity in S2 cells treated with the indicated dsRNA as compared with *GFP* dsRNA control. *Error bars* represent S.D. of at least eight experiments. **, $p < 0.01$, ***, $p < 0.001$.

be directed toward functions of either Med12-Med13 or the entire Cdk8 module.

The dependence of gene regulation by Cdk8-CycC on Med12-Med13 noted here and in a genetic study on leg bristles (21) supports the suggested structural hierarchy where Cdk8-CycC is linked to the core Mediator through Med12 and Med13 (17, 18). Identification of the dependence of Cdk8-CycC on Med12-Med13 also revealed that these pairs often have opposite transcriptional effects as illustrated in Fig. 2*D*. This indicates that opposite regulation by Cdk8-CycC and Med12-Med13 should be considered as a possibility on all Cdk8-CycC-regulated genes and functions previously identified to be Med12-Med13-independent (17, 18).

Cdk8-CycC dependence on Med12-Med13 highlights the importance of investigating the possible involvement of Cdk8-CycC in Med12-Med13-dependent phenotypes. Interestingly, suppression of Shh signaling in cells with the FG and Lujan syndrome mutations in Med12 was recently shown to be a result of dissociation of Cdk8 but not Med12 on Gli3 target promoters (16). Furthermore, the finding that Cdk8-CycC can act opposite to Med12-Med13 although being Med12-Med13-dependent indicates that *e.g.* loss of Med12 could lead to similar phenotypes as gain of Cdk8; both genetic alterations have been noted in human colorectal cancer (13, 52). Taken together, these results are consistent with the notion (2, 3, 25) that Cdk8-CycC mediates gene regulation primarily through interaction with Mediator through Med12-Med13, whereas Med12-Med13 can regulate transcription independently of Cdk8-CycC.

Med12-Med13 was found here to be important for Srp-dependent transcription, and the previously identified physical interaction between Srp and Cdk8 module components (6) provides a plausible mechanism for this. In addition to *Mtk* and *DptB*, the IMD target *CecA1* is also a target of Srp (48, 50) and dependent on the Cdk8 module components (data not shown). Consistent with this, induction of the *A. gambiae* homolog of *CecA1*, *Cec1*, was recently shown to require Med12 and Med13 (47). Multiple known (*e.g. Eater*, *Sr-CI*, *Pxn*) and novel (*e.g. CG14629*, *CG10962*) Srp-dependent genes found here to be Med12-Med13-dependent implicate Med12-Med13 in various Srp-regulated functions. Besides its role in AMP gene induction, Srp is required in hematopoietic differentiation (41). In some instances, this may be modulated by the Cdk8 module,

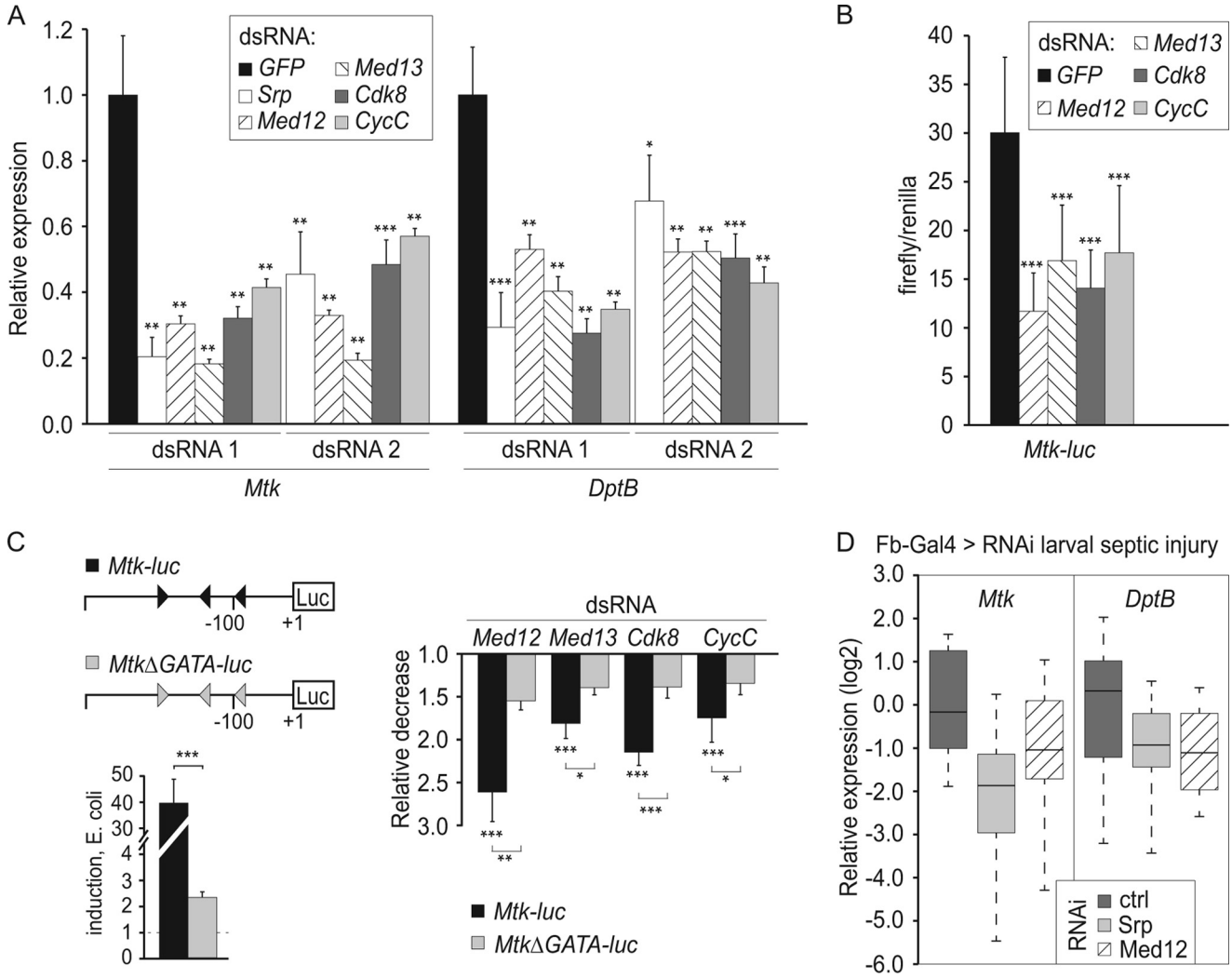

FIGURE 5. **Med12 and Med13 are required for Serpent-dependent anti-microbial peptide genes upon IMD pathway activation *in vitro* and *in vivo*.** *A*, relative expression levels by qPCR of *Mtk* and *DptB* in samples treated with *E. coli* and indicated dsRNAs as compared with dsRNA *GFP*. *Error bars* indicate S.D. of three biological replicates each except for Cdk8 dsRNA2 where *error bars* represent S.D. between three biological replicates of two different dsRNAs (dsRNA 2a and 2b). *B*, luciferase activity of *Mtk-luc* normalized to transfection control *pRLnull-copiaLTR* following dsRNA treatment as indicated. *Error bars* represent S.D. in five experiments. *C*, schematic of the *Mtk-luc* and *Mtk-ΔGATA-luc* reporters showing distribution and orientation of GATA sites, where sites with point mutations are in *gray*. *Columns below* indicate average induction of the reporters by *E. coli* treatment as compared with no treatment. *Error bars* indicate S.D. in five independent experiments. *Columns on the right* indicate relative decrease of *Mtk-luc* (*black*) or *Mtk-ΔGATA-luc* (*gray*) activity after *E. coli* treatment and indicated dsRNAs as compared with *GFP* dsRNA control. *Error bars* indicate S.D. in five experiments. *D*, relative expression levels (log 2) by qPCR of *Mtk* and *DptB* in larval fat bodies after septic injury with *E. coli* of lines with fat body-specific (Fb-Gal4) depletions as indicated. *Boxes* include first, second (median), and third quartiles; *whiskers* show minimum and maximum -fold change (log 2) in at least nine biological replicates. *Mtk* levels decreased 4.52-fold ($p = 0.005$) and 1.96-fold ($p = 0.12$) after Srp and Med12 depletions, respectively. *DptB* levels decreased 1.94-fold ($p = 0.11$) and 2.12-fold ($p = 0.05$) after Srp and Med12 depletions, respectively. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

suggested by the requirement of *Drosophila* Med12-Med13 (6) and zebrafish Med12 (53) in differentiation of specific blood cell lineages. Based on this, it will be interesting to study the possible involvement of Med12-Med13 in mammalian GATA-dependent hematopoiesis.

It appears that transcription regulation by the Cdk8 module is largely context-dependent. In this regard, it was intriguing to identify several genes involved in neuronal functions (*Epac* (54), *Fie* (55), *ogre* (56), and *PQBP-1* (57, 58)) as strongly regulated by Med12-Med13 in S2 cells. It will be interesting to analyze whether these genes are also regulated by Med12-Med13 in neural tissues, where Med12 (31, 59) and one of it targets identified here, *PQBP-1* (57, 58), present related phenotypes.

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