The Mediator Kinase Module Restrains Epidermal Growth Factor Receptor Signaling and Represses Vulval Cell Fate Specification in *Caenorhabditis elegans*

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ABSTRACT Cell signaling pathways that control proliferation and determine cell fates are tightly regulated to prevent developmental anomalies and cancer. Transcription factors and coregulators are important effectors of signaling pathway output, as they regulate downstream gene programs. In *Caenorhabditis elegans*, several subunits of the Mediator transcriptional coregulator complex promote or inhibit vulva development, but pertinent mechanisms are poorly defined. Here, we show that Mediator's dissociable cyclin dependent kinase 8 (CDK8) module (CKM), consisting of *cdk-8*, *cic-1*/*Cyclin C*, *mdt-12*/*dpy-22*, and *mdt-13*/*let-19*, is required to inhibit ectopic vulval cell fates downstream of the epidermal growth factor receptor (EGFR)-Ras-extracellular signal-regulated kinase (ERK) pathway. *cdk-8* inhibits ectopic vulva formation by acting downstream of *mpk-1*/*ERK*, cell autonomously in vulval cells, and in a kinase-dependent manner. We also provide evidence that the CKM acts as a corepressor for the Ets-family transcription factor LIN-1, as *cdk-8* promotes transcriptional repression by LIN-1. In addition, we find that CKM mutation alters Mediator subunit requirements in vulva development: the *mdt-23*/*sur-2* subunit, which is required for vulva development in wild-type worms, is dispensable for ectopic vulva formation in CKM mutants, which instead display hallmarks of unrestrained Mediator tail module activity. We propose a model whereby the CKM controls EGFR-Ras-ERK transcriptional output by corepressing LIN-1 and by fine tuning Mediator specificity, thus balancing transcriptional repression *vs.* activation in a critical developmental signaling pathway. Collectively, these data offer an explanation for CKM repression of EGFR signaling output and ectopic vulva formation and provide the first evidence of Mediator CKM-tail module subunit crosstalk in animals.

KEYWORDS Mediator complex; CDK8; MED23; MED15; EGFR; Notch

PRECISE regulation of transcription is required to execute developmental programs such as proliferation and cell fate determination. The Mediator complex ("Mediator") is a conserved eukaryotic transcriptional coregulator of RNA polymerase II (Pol II) transcription (Malik and Roeder 2010; Poss *et al.* 2013). Mediator consists of ~30 subunits that assemble into four modules. "Core" Mediator consists of three of the four modules: the head and middle modules, which contact Pol II, and the tail module, which serves as a docking site for transcription factors. The fourth module, the dissociable cyclin dependent kinase 8 (CDK8) kinase module (CKM), interacts with transcription factors, core Mediator, chromatin, and the Pol II machinery to either repress or activate transcription (Malik and Roeder 2010; Nemet *et al.* 2014). Whereas many head or middle Mediator subunits are broadly required for Pol II

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transcription, tail and CKM subunits regulate specific transcriptional programs in animal development or physiology (Malik and Roeder 2010; Nemet *et al.* 2014).

The CKM consists of enzymatic subunits CDK8 and cyclin C, and structural subunits MED12 and MED13 that tether the CKM to core Mediator (Tsai *et al.* 2013). CKM subunits regulate many transcriptional programs important for development and/or tumorigenesis, often by directly binding to and influencing the activity of key transcription factors (*e.g.*, β-catenin, Notch, *etc.*) (Fryer *et al.* 2004; Donner *et al.* 2007; Firestein *et al.* 2008; Zhou *et al.* 2012). Furthermore, in *Saccharomyces cerevisiae*, the CKM regulates the activity of the Mediator tail module subunits MED2, MED3, and MED15 (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014). However, whether such intra-Mediator signaling effects occur in metazoans and affect *e.g.*, animal development has not yet been tested.

Several Mediator subunits including at least one CKM subunit regulate vulva development in *Caenorhabditis elegans* (Tuck and Greenwald 1995; Singh and Han 1995; Kwon and Lee 2001; Moghal and Sternberg 2003a). The study of cell fate specification in the *C. elegans* vulva has proven a powerful way to identify the components and regulatory interactions of several evolutionarily conserved signaling pathways (Félix and Barkoulas 2012; Schmid and Hajnal 2015). Thus, this organogenesis event provides an ideal paradigm to study Mediator subunit specificity and cooperation in a metazoan.

C. elegans vulval organogenesis is induced by epidermal growth factor receptor (EGFR) signaling (Moghal and Sternberg 2003b), a prominent pathway in animal development that is frequently activated in human cancers (Normanno *et al.* 2006; Baselga and Swain 2009). The *C. elegans* vulva develops from six ventral vulva precursor cells (VPCs), named P3.p through P8.p from anterior to posterior (Figure 1). The VPCs form an equivalence group, meaning that all six cells are able to adopt the primary (1°) vulval cell fate (producing eight descendants), the secondary (2°) vulval cell fate (producing seven descendants), or the tertiary (3°) nonvulval fate (producing two descendants that fuse with the surrounding hypodermis). A signaling cell in the somatic gonad, called the anchor cell, emits a LIN-3/EGF-like ligand in close proximity to P6.p (Hill and Sternberg 1992); therefore, LET-23/EGFR and the downstream LET-60/Ras, MPK-1/extracellular signal-regulated kinase (ERK) cascade is strongly activated in P6.p (Aroian *et al.* 1990). MPK-1/ERK activation in P6.p modulates the activity of effector transcription factors such as the ELK1/Ets-family transcription factor LIN-1 and the FoxB transcription factor LIN-31, thereby specifying the 1° vulval fate in P6.p (Miller *et al.* 1993; Tan *et al.* 1998; Jacobs *et al.* 1998). The neighboring P5.p and P7.p cells are thought to receive a weaker LIN-3/EGF signal from the anchor cell (Katz *et al.* 1995) as well as a lateral Notch signal emitted from the 1° cell P6.p, inducing them to adopt a 2° vulval fate (Chen and Greenwald 2004). Located furthest from the anchor cell, P3.p, P4.p, and P8.p do not receive sufficient EGF signal, and adopt the 3° nonvulval cell fate (Sternberg and Horvitz 1986). Mutations that enhance or reduce EGFR or Notch signaling induce ectopic vulval cell fates (multivulva phenotype, Muv) or loss of vulval cell fates (vulvaless phenotype, Vul), respectively (Sternberg and Horvitz 1989). These phenotypes are thus powerful indicators of EGFR and Notch signaling pathway activity.

Transcriptional regulation is important in maintaining appropriate EGFR signaling pathway output (Figure 1). For example, transcription factors such as LIN-1/Ets and LIN-31/Forkhead are required to repress 1° cell fate specification in VPCs other than P6.p (Miller *et al.* 1993; Beitel *et al.* 1995). In addition, multiple chromatin-modifying complexes, encoded by the synthetic multivulva (synMuv) genes, redundantly repress ectopic *lin-3/EGF* transcription in the hypodermis and other tissues to inhibit 1° cell fate specification in VPCs other than P6.p (Myers and Greenwald 2005; Cui *et al.* 2006; Saffer *et al.* 2011). Furthermore, the Mediator subunits *mdt-23/sur-2*, *mdt-24/lin-25*, and *mdt-6* promote vulva development, whereas the CKM subunit *mdt-12/dpy-22* inhibits vulva development in an anchor cell-independent manner (reviewed in Grants *et al.* 2015); for standardized Mediator subunit nomenclature, please see Bourbon *et al.* (2004). The mechanism by which *mdt-23/sur-2* promotes vulva development has been partially elucidated, as it is a critical coactivator of a target gene downstream of the EGFR signaling pathway, the *lag-2* Notch ligand gene (Zhang and Greenwald 2011). The *lin-1/Ets* effector transcription factor is similarly required to repress the *lag-2* gene (Zhang and Greenwald 2011), raising the question of whether and how Mediator and LIN-1 interact to control common target genes. The other three Mediator subunits implicated in vulva development (*mdt-6*, *mdt-12/dpy-22*, and *mdt-24/lin-25*) interact genetically with components of the EGFR signaling pathway, but their mode of action within this pathway remain poorly understood.

Here, we used the vulva organogenesis paradigm to study the requirements of all four CKM subunits in this process and to interrogate functional interactions with other transcriptional regulators, including the synMuv genes, the key transcription factors *lin-1/Ets* and *lin-31*/FoxB, and the Mediator subunit *mdt-23/sur-2*, an essential effector of EGFR signaling output. We show that all four CKM subunits inhibit ectopic vulval cell fates in *C. elegans*. We demonstrate that the CKM catalytic subunit *cdk-8* acts downstream of *let-23/EGFR* and *mpk-1/ERK* in VPCs, in a kinase-dependent manner. Our data implicate *cdk-8* as a corepressor for the LIN-1/Ets repressive transcription factor to inhibit EGFR signaling-induced transcription. Furthermore, our data indicate that vulval induction in CKM mutants is independent of the *mdt-23/sur-2* coactivator, and instead requires the Mediator tail module subunits *mdt-15*, *mdt-27*, and *mdt-29* for induction of ectopic vulval cell fates.

Materials and Methods

Microarrays and data analysis

Microarray gene expression profiling was performed at the University of California San Francisco SABRE Functional

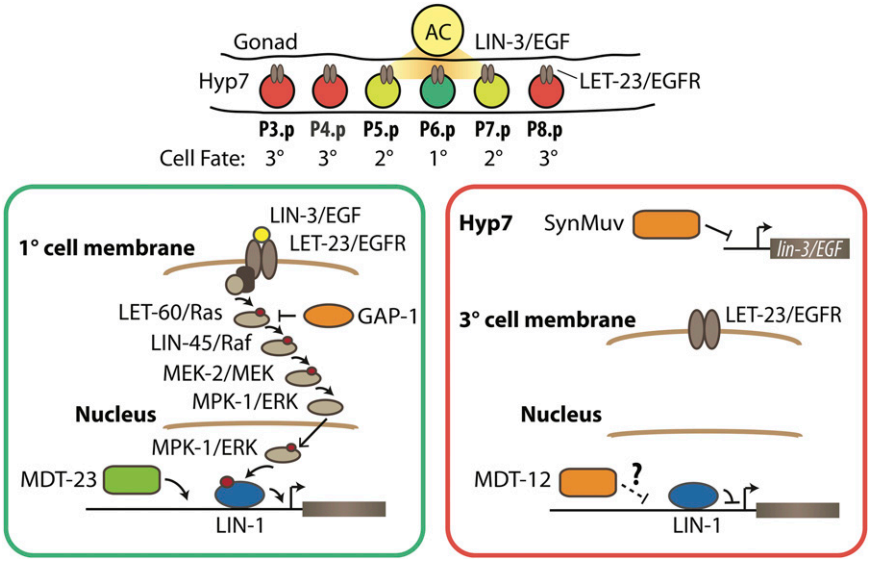

Figure 1 Transcriptional regulators in *C. elegans* vulval induction. The *C. elegans* vulva is derived from an equivalence group consisting of six vulva precursor cells (VPCs), named P3.p through P8.p from anterior to posterior. A localized LIN-3/EGF signal from the anchor cell (AC) in the somatic gonad activates a LET-23/EGFR-LET-60/Ras-MPK-1/ERK signaling cascade strongly in P6.p. ERK activation in P6.p modulates transcription factor activity in the nucleus (only LIN-1 is shown here for simplicity), leading to induction of the 1° cell fate. In P5.p and P7.p, a weak LIN-3/EGF signal combined with lateral Notch signaling from P6.p (not depicted) instead produces the 2° cell fate. In P3.p, P4.p, and P8.p, the EGFR signaling cascade is not activated by LIN-3/EGF, and cells adopt the nonvulval 3° cell fate. Transcriptional regulators of the EGFR signaling pathway are critical for correct vulval cell fate specification: *e.g.*, the MDT-23/SUR-2 Mediator subunit is a coactivator of EGFR signaling-induced transcription, the SynMuv corepressor complexes are required to inhibit ectopic *lin-3/EGF* transcription in the hypodermis (Hyp7) surrounding the VPCs, and the MDT-12/DPY-22 Mediator subunit is required to inhibit vulva development by mechanisms that remain unclear (dashed arrow). The GTPase activating protein GAP-1 that negatively regulates LET-60/Ras activity post-translationally is also shown.

Genomics Facility. We used Agilent *C. elegans* (V2) 4x44K Gene Expression Microarrays (G2519F-020186) and single color labeling. Total RNA was extracted from developmentally synchronized mid-L4 stage worms as assessed by vulval morphology [wild-type N2 worms and *cdk-8(tm1238)* mutants], as described (Taubert *et al.* 2008). RNA quality was assessed on an Agilent 2100 Bioanalyzer using a Pico Chip (Agilent). RNA was amplified and labeled with Cy3-CTP using the Agilent low RNA input fluorescent linear amplification kit. Labeled cRNA was assessed using the Nanodrop ND-100, and equal amounts of Cy3-labeled target were hybridized to the microarrays for 14 hr, according to the manufacturer's protocol. Arrays were scanned using the Agilent microarray scanner and raw signal intensities were extracted with Feature Extraction v9.1 software. The dataset was normalized using quantile normalization (Bolstad *et al.* 2003). No background subtraction was performed, and median feature pixel intensity was used as raw signal before normalization. All arrays were of good quality and had similar foreground and background signal distributions for both messenger RNA (mRNA) and control probes. This suggests that quantile normalization is appropriate. To identify differentially expressed genes, a linear model was fit to the comparison to estimate the mean *M*-values and calculate moderated *t*-statistic, *B*-statistic, false discovery rate, and *P*-value for each gene. Adjusted *P*-values (AdjP) were produced as described (Holm 1979). All procedures were carried out using functions in the R package limma in Bioconductor (Gentleman *et al.* 2004; Smyth 2004). Using this approach, we identified a total of 1860 spots with an AdjP of <0.05 and a fold change of ≥ 2 (representing 461 downregulated and 829 upregulated genes) (Supporting Information, Table S1). Microarray data have been deposited in Gene Expression Omnibus (GSE68520).

Differentially expressed genes were compared to published gene expression datasets using EASE (Hosack *et al.* 2003). For best comparison to our data, we reanalyzed published *lin-35* data (Kirienko and Fay 2007) to define a set of genes deregulated twofold or more in L4 larvae, yielding 132 downregulated and 367 upregulated genes. We compared this set to our *cdk-8* targets and calculated the significance of the overlap using Fisher's exact test.

C. elegans strains, culture, and genetic methods

C. elegans strains were cultured as described (Brenner 1974) at 20° or 23°, as indicated. We used nematode growth medium (NGM)-lite (0.2% NaCl, 0.4% tryptone, 0.3% KH_2PO_4, 0.05% K_2HPO_4) agar plates seeded with *Escherichia coli* strain OP50 unless otherwise indicated.

Strains are listed in Table S4. Wild type was Bristol N2. *cdk-8(tm1238)* and *cic-1(tm3740)* are likely null alleles that abolish *cdk-8* expression (Figure S1B) and *cic-1* function, respectively (see also Steimel *et al.* 2013). For allele details, see www.wormbase.org. *mdt-12/dpy-22* mutants were identified as Dpy, GFP-negative progeny of rescued *dpy-22(os38)*; *osEx89[dpy-22(+)]* mothers, and homozygous *mdt-13/let-19* mutants were identified as Dpy, GFP-negative progeny of balanced *let-19(mn19)/mIn1* mothers.

VPC induction

VPC induction was scored as described (Han *et al.* 1990), in synchronous mid-L4 animals under DIC optics at $\times 1000$ magnification. In wild-type animals, P5.p–P7.p are induced to give a VPC induction score of 3.0. In Vul animals, these VPCs are not fully induced (VPC induction <3.0); in Muv animals, P3.p, P4.p, or P.8p are induced (VPC induction >3.0).

Multivulva and vulvaless phenotype penetrance

Muv and Vul morphologies have been described (Horvitz and Sulston 1980; Sulston and Horvitz 1981). To facilitate scoring a large number of worms to accurately assess low-penetrance phenotypes, Muv phenotype penetrance was scored in synchronous day 1 adult animals in a dissection microscope at ×200 magnification (*mdt-13/let-19* mutants) or ×56 magnification (all other strains). To corroborate Muv penetrances scored in adult animals, we also conducted VPC induction analysis in L4 animals (see above). To assess Vul phenotypes, both Vul and Muv penetrances were extrapolated from VPC induction scores: animals were scored as Vul if VPC induction was <3.0 in P5.p–P7.p and were scored as Muv if VPC induction occurred in P3.p, P4.p or P8.p; using these criteria, animals were occasionally scored as simultaneously Vul and Muv.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from developmentally synchronized mid-L4 stage worms as assessed by vulval morphology. RNA isolation and qPCR were performed as described (Goh *et al.* 2014). We used *t*-tests (two-tailed, equal variance) to calculate statistical significance of gene expression changes between mutants (Gaussian distribution). qPCR primers were designed with Primer3web (bioinfo.ut.ee/primer3/) and tested on serial cDNA dilutions to analyze PCR efficiency (primer sequences in Table S5), except *lin-3* (analyzed by TaqMan assay, Invitrogen 4448892, assay ID Ce02418781_m1).

Fluorescent reporter analysis

Synchronous worms were imaged using DIC optics and fluorescence microscopy on a Zeiss Axioplan 2 microscope. Analysis of fluorescence intensity was conducted using ImageJ software, normalizing for cell size and background fluorescence.

Generation of transgenic rescue strains

cdk-8 rescue transgenes (steEx43, 45–47) were generated by gonad microinjection of a mixture of 50 ng/μl rescue plasmid [*cdk-8(+)*, SPD732; *lin-31P*::*cdk-8*, SPD793; *dpy-7P*::*cdk-8*, SPD772; *cdk-8(KD)*, SPD789; 5 ng/μl pCFJ90*[myo-2p*::*mCherry]*, and 95 ng/μl pPD95.77 empty vector into N2 worms, then selecting transgenic mCherry-positive progeny. These were then crossed to *cdk-8* and/or *cdk-8*; *lin-15A* mutants (Table S4). Cloning primer sequences are provided (Table S5).

Feeding RNAi knockdown

Feeding RNAi was performed as described (Goh *et al.* 2014), with the following modifications: synchronous mid-L4 hermaphrodites were allowed to lay eggs at 20° overnight on RNAi plates (Ahringer Library 96-well format; *mdt-15*: plate 74, well C09; *lin-1*: 94, G02; Vidal Library 96-well format: *mdt-27*: GHR-11064@H02; *mdt-29*: GHR-11007@D05; all clones were sequenced to confirm identity; negative control was empty vector L4440), after which embryos were isolated by bleach treatment and transferred to fresh RNAi plates. F_1 progeny were grown on RNAi plates (20° or 23°) until they reached the desired developmental stage.

Western blot

Immunoblot using standard lysis, SDS/PAGE and Western blot techniques was performed, with α-MDT-15 (Taubert *et al.* 2006) and α-GAPDH (Calbiochem, CB1001) antibodies, as described (Goh *et al.* 2014).

Data availability

Primer sequences are listed in Table S5. Strains are listed in Table S4 and are available upon request. Gene expression data are available at GEO with the accession number GSE68520.

Results

cdk-8-dependent transcripts overlap with targets of a synMuv gene

To define the role of the CKM in metazoan development, we compared transcriptional profiles of developmentally synchronized L4 larval stage *cdk-8(tm1238)* null mutants to wild-type N2 worms using microarrays (see *Materials and Methods* and Figure S1A for *cdk-8* mutant information). We found that 829 genes were upregulated and 461 genes were downregulated more than twofold in *cdk-8* null mutants, representing ~6.7% of all *C. elegans* genes (Table S1). To identify *cdk-8*-dependent gene programs, we compared our lists of *cdk-8* regulated genes to other gene lists using EASE (Hosack *et al.* 2003; Engelmann *et al.* 2011). The top hit among genes upregulated in *cdk-8* mutants was a set of genes upregulated in *lin-35/Retinoblastoma* (RB) synMuv gene mutants (Kirienko and Fay 2007), and *lin-35*-repressed genes also overlapped significantly with genes downregulated in *cdk-8* mutants (Figure 2A). Importantly, the mRNA levels of *lin-35* and the *efl-1/dpl-1* transcription factor heterodimer that is repressed by LIN-35 were not altered in *cdk-8* mutants (Figure S1B), indicating that *cdk-8* does not affect *lin-35* target gene expression by altering the abundance of *lin-35* or its partners. Together, these data suggest that *cdk-8* could act in parallel to *lin-35* as they regulate similar gene sets.

cdk-8 represses EGFR signaling-dependent primary vulval cell fate specification

We next investigated whether *cdk-8*, like *mdt-12/dpy-22* (Moghal and Sternberg 2003a), also represses vulval induction. Indeed, *cdk-8* and *cic-1(tm3740)* null mutants displayed a low penetrance Muv phenotype, as measured both by VPC induction analysis in L4 animals (Table 1) and by scoring the occurrence of ectopic vulval protrusions in adult worms (Figure 2B; see statistical comparisons between L4 and adult Muv scores, Table S2). *cdk-8* and *cic-1* appeared to function redundantly in vulva formation, as *cdk-8*; *cic-1* double mutants showed no significant increase in Muv penetrance compared to either single mutant (Figure 2B). We then tested if the *cdk-8* mutant Muv phenotype was associated with ectopic EGFR signaling-induced 1° vulval cell fates using an *egl-17P*::*CFP* reporter (*arIs92*) (Yoo *et al.* 2004). In wild-type worms, we

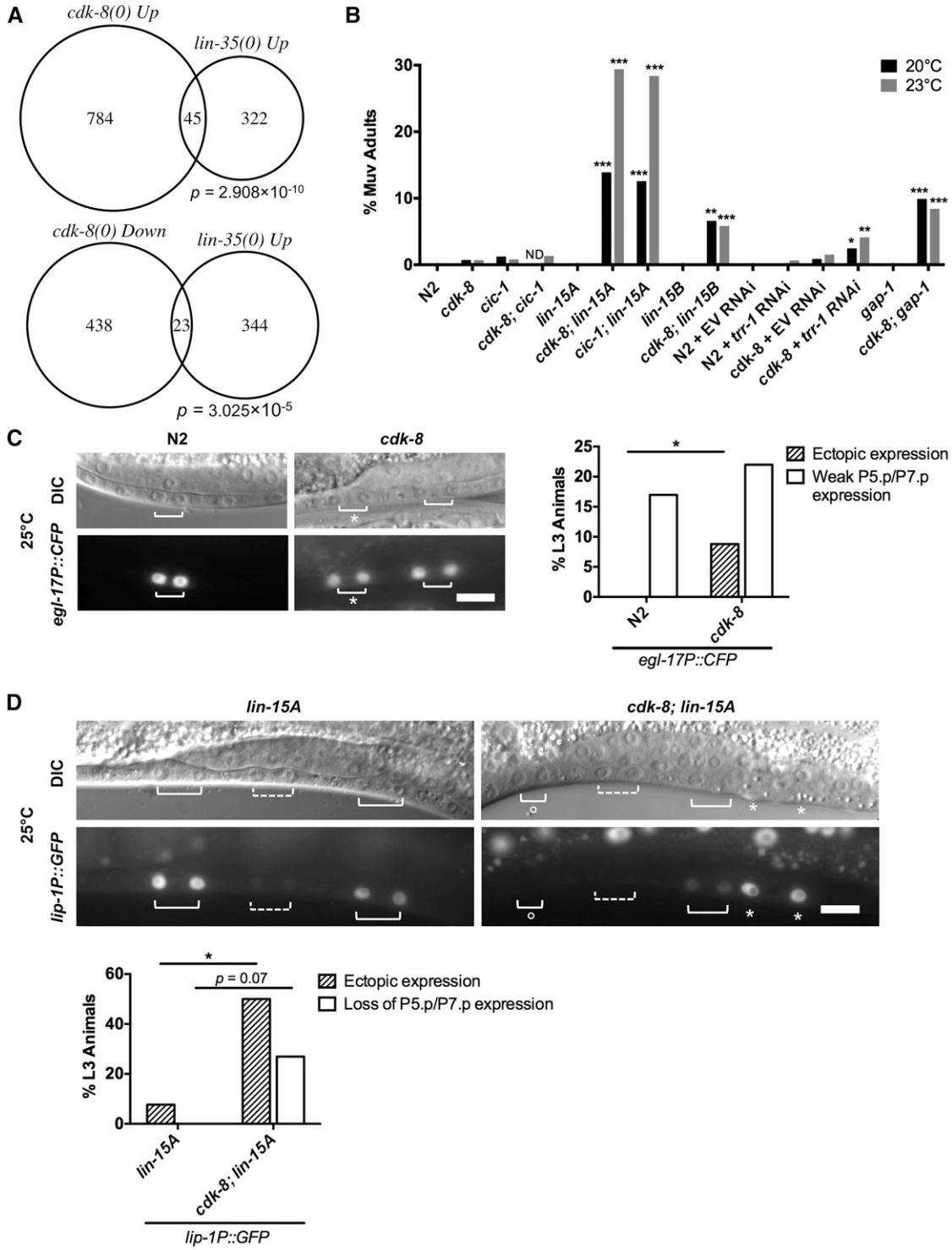

Figure 2 *cdk-8* represses vulval cell fates redundantly with synMuv genes. (A) Overlap of *cdk-8* regulated genes with *lin-35/RB* repressed genes. *P* values were determined by Fisher's exact test. (B) Adult Muv penetrance in *cdk-8* and mutants with synMuv genes ($n \geq 76$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs. cdk-8* mutant or *vs. cdk-8* + empty vector (EV), Fisher's exact test. ND, not determined. See Table S2 for raw data. (C) Micrographs show wild-type or *cdk-8* mutant worms expressing the *arIs92[egl-17P::CFP]* 1° fate marker. Bracket: Pn.px cells expressing reporter. Asterisk: ectopic reporter expression. Bar: 10 μm. The graph displays the percentage of animals ($n > 50$) with ectopic *egl-17P::CFP* expression (defined as expression in P3.p/P4.p/P8.p, or expression in P5.p/P7.p of equal intensity to P6.p) or weak P5.p/P7.p expression (defined as expression in P5.p/P7.p that is weaker intensity than P6.p). $^*P < 0.05$, Fisher's exact test. (D) Micrographs show *lin-15A* and *cdk-8*; *lin-15A* mutants expressing the Notch-inducible reporter *zhIs4[lip-1P::GFP]*. Dashed bracket: P6.px. Solid bracket: P5.px and P7.px. Open circle: loss of expression. Asterisk: ectopic expression. Bar: 10 μm. The graph shows the percentage of animals ($n \geq 13$) with ectopic expression (defined as strong expression in P3.p, P4.p, or P8.p) or loss of expression (defined as weak or no expression in P5.p or P7.p) of the *lip-1P::GFP* reporter. $^*P < 0.05$, Fisher's exact test.

Table 1 VPC induction scores and Muv/Vul penetrance in L4 animals (20°)

	Genotype	VPC induction ± SEM	% Muv L4[a]	% Vul L4[b]	% WT L4 vulva	*n*
	N2	3.00 ± 0.00	0	0	100	15
CKM	*cdk-8(tm1238)*	3.02 ± 0.02	2	0	98	50
	mdt-13(mn19)	2.96 ± 0.07	15	27	65	26
SynMuv	*lin-15A(n767)*	3.00 ± 0.00	0	0	100	18
	cdk-8(tm1238); lin-15A(n767)	3.20 ± 0.08	21	0	79	28
	lin-15B(n744)	3.00 ± 0.00	0	0	100	12
	cdk-8(tm1238); lin-15B(n744)	3.07 ± 0.05	14	0	86	14
	N2 + EV RNAi	3.00 ± 0.00	0	0	100	48
	N2 + *trr-1* RNAi	3.00 ± 0.00	0	0	100	15
	cdk-8(tm1238) + EV RNAi	3.00 ± 0.00	0	0	100	37
	cdk-8(tm1238) + *trr-1* RNAi	3.02 ± 0.02	4	0	96	26
	gap-1(ga133)	3.00 ± 0.00	0	0	100	15
	cdk-8(tm1238); gap-1(ga133)	3.03 ± 0.02	7	0	93	29
let-23	*let-23(sy97)*	0.00 ± 0.00	0	96	4	9
	cdk-8(tm1238); let-23(sy97)	2.44 ± 0.27	0	37	63	8
mpk-1	*mpk-1(oz14)*	2.03 ± 0.17	0	80	20	15
	cdk-8(tm1238); mpk-1(oz140)	2.95 ± 0.03	0	10	90	15
	mdt-13(mn19); mpk-1(oz140)	2.85 ± 0.27	23	31	46	13
lin-1	*lin-1(n1790)*	2.98 ± 0.08	20	25	65	20
	cdk-8(tm1238); lin-1(n1790)	3.58 ± 0.16	67	0	34	12
	mdt-13(mn19); lin-1(n1790)	5.33 ± 0.15	100	0	0	12
	N2 + *lin-1* RNAi	3.10 ± 0.06	7	0	93	40
	cdk-8(tm1238) + *lin-1* RNAi	3.25 ± 0.06	31	0	69	61
lin-31	*lin-31(n301)*	3.65 ± 0.14	73	20	13	30
	cdk-8(tm1238); lin-31(n301)	2.46 ± 0.23	33	53	30	30
mdt-23	*mdt-23 (ku9)*	1.13 ± 0.16	0	100	0	23
	mdt-13(mn19); mdt-23(ku9)	3.57 ± 0.21	64	18	23	22
mdt-15	N2 + *mdt-15* RNAi	3.00 ± 0.00	0	0	100	33
	lin-1(n1790) + EV RNAi	3.00 ± 0.12	17	19	70	46
	lin-1(n1790) + *mdt-15* RNAi	3.01 ± 0.14	18	22	67	45

[a] Percentage of L4 animals with ectopic vulval invagination at P3.p, P4.p, or P8.p.
[b] Percentage of L4 animals with vulval induction <3.0 at P5.p-P7.p. Note: it is possible for an animal to be scored as both Vul and Muv.

observed strong *egl-17P*::*CFP* expression in the 1° cell descendants, P6.px, and occasional weak expression in the 2° cell descendants, P5.px or P7.px (Figure 2C), as reported (Yoo *et al.* 2004). *cdk-8* mutants expressed *egl-17P*::*CFP* strongly in P6.ps and weakly in P5.px and P7.px, like wild type (Figure 2C). However, some *cdk-8* mutants exhibited *egl-17P*::*CFP* expression in P5.px or P7.px that was equal in intensity to the expression level in the 1° cell descendants P6.px, or exhibited ectopic induction of *egl-17P*::*CFP* in VPCs that normally adopt the nonvulval fate (Figure 2C). The ectopic expression of *egl-17*::*CFP* suggested that the 1° cell fate had been derepressed in presumptive 2° or nonvulval cells. This reflects that the low penetrance Muv phenotype of *cdk-8* mutants is caused in part through derepressed ERK signaling output.

cdk-8 interacts genetically with the synMuv repressors of lin-3/EGF transcription

The CKM subunit *mdt-12/dpy-22* was previously shown to act downstream of *let-23/EGFR* to modulate vulva development (Moghal and Sternberg 2003a). However, the developmental roles of all four CKM subunits are not equivalent (Loncle *et al.* 2007), and our gene expression profiling suggested that *cdk-8* might interact genetically with the synMuv genes to alter vulval cell fate decisions. As synMuv genes encode three redundant chromatin-modifying complexes, a Muv phenotype results when genes in any two complexes are simultaneously mutated (Myers and Greenwald 2005; Cui *et al.* 2006; Saffer *et al.* 2011). We therefore studied the simultaneous inactivation of *cdk-8* or *cic-1* and representative synMuv class A (*lin-15A*), B (*lin-15B*), or C (*trr-1*) genes. Mutation or RNA interference (RNAi) depletion of all representative synMuv genes enhanced the Muv phenotype of *cdk-8* mutants (Figure 2B, Table 1). In addition, loss of the Ras GTPase-activating protein gene *gap-1*, which causes weak LET-60/Ras derepression, also enhanced the *cdk-8* mutant Muv penetrance (Figure 2B, Table 1). Our microarray analysis did not reveal significant downregulation of any known synMuv genes in *cdk-8* mutants (Table S3), indicating that *cdk-8* does not simply regulate synMuv mRNA levels. Taken together, these results suggest that *cdk-8* and *cic-1* act redundantly with synMuv genes to repress vulval cell fates.

We observed adjacent VPCs expressing a 1° cell fate marker in *cdk-8* mutants (Figure 2C), which is uncharacteristic of synMuv gene mutants. This phenotype instead suggests defects in Notch signaling, which inhibits adjacent 1° fates by inducing EGFR signaling inhibitor genes (Sternberg 1988; Berset *et al.* 2001; Yoo *et al.* 2004; Chen and Greenwald 2004). Therefore, we examined the expression of the Notch-inducible EGFR signaling inhibitor, *lip-1/ERK phosphatase*, using a *lip-1P*::*GFP* reporter (*zhIs4*) (Berset *et al.* 2001). We

used the sensitized *lin-15A* mutant background to increase the frequency of ectopic VPC induction events. *lin-15A* single mutants expressed *lip-1P*::*GFP* strongly in P5.px and P7.px, but expression was weak or absent in other Pn.px cells (Figure 2D), as reported for wild-type worms (Berset *et al.* 2001). In contrast, some *cdk-8*; *lin-15A* mutants lost strong *lip-1P*::*GFP* expression in P5.px and P7.px, consistent with loss of the 2° fate (Figure 2D). Furthermore, some *cdk-8*; *lin-15A* mutants ectopically expressed *lip-1P*::*GFP* strongly in nonvulval P3.px, P4.px, or P8.px, suggesting ectopic 2° fates (Figure 2D). Thus, *cdk-8* mutants display hallmarks of both down- and upregulated Notch signaling, suggesting that CDK-8 action on the Notch pathway may occur indirectly via the EGFR signaling pathway upstream.

cdk-8 regulates lin-3/EGF transcription in the anchor cell

We next tested if *cdk-8* acts redundantly with the synMuv genes to repress *lin-3/EGF* transcription (Cui *et al.* 2006; Saffer *et al.* 2011). As the synMuv genes act primarily in the hypodermis to repress *lin-3* transcription (Myers and Greenwald 2005; Saffer *et al.* 2011), derepression of *lin-3* in synMuv double mutants is detectable by quantitative PCR in whole-animal preparations (Cui *et al.* 2006). We used Taqman quantitative PCR analysis to quantify whole-animal *lin-3* mRNA levels in *cdk-8*, *lin-15A*, and *lin-15B* single mutants, and in *cdk-8*; *lin-15A* and *cdk-8*; *lin-15B* double mutants; the *lin-15AB(n309)* mutant served as a positive control, as it is known to upregulate *lin-3* expression (Figure 3A) (Cui *et al.* 2006). Compared to wild-type worms, *cdk-8* single mutants, *cdk-8*; *lin-15A* double mutants, and *cdk-8*; *lin-15B* double mutants showed no statistically significant change in *lin-3* mRNA levels (Figure 3A). Thus, the enhanced Muv penetrance of *cdk-8*; *lin-15A* and *cdk-8*; *lin-15B* mutants compared to *cdk-8* single mutants (Figure 2B) likely does not arise from hypodermal *lin-3* derepression.

Next, we investigated whether *cdk-8* is required to regulate *lin-3* transcription in the signal-emitting anchor cell, which would not be detectable in whole-animal quantitative PCR analysis. In line with this hypothesis, we observed expression of a transcriptional *cdk-8P*::*GFP* reporter (*hdEx508*) in the anchor cell (Figure 3B). To assess *lin-3* transcription in the anchor cell alone, we used a *lin-3* anchor cell-specific enhancer element (ACEL) GFP reporter (*syIs107*) (Hwang and Sternberg 2004). We detected a small but significant upregulation of *lin-3* ACEL reporter expression in *cdk-8* mutants compared to wild-type worms at the L3 larval stage (Figure 3C), suggesting that *cdk-8* is required to repress *lin-3/EGF* transcription in the anchor cell.

cdk-8 acts downstream of mpk-1/ERK to regulate VPC induction cell autonomously

As the effect size of *cdk-8* loss on *lin-3/EGF* anchor cell expression was small, and as *mdt-12/dpy-22* has been found to act downstream of *let-23/EGFR* (Moghal and Sternberg 2003a), we next investigated *cdk-8*'s role in the EGFR signaling pathway downstream of *lin-3*. We conducted genetic epistasis analyses with strong loss-of-function alleles of *EGFR*, *let-23(sy97)*, and *ERK*, *mpk-1(oz140)*; both caused highly penetrant Vul phenotypes due to blockade of the EGFR-Ras-ERK pathway (Figure 3D, Table 1). *cdk-8* inactivation significantly rescued the Vul phenotype of *let-23* or *mpk-1* single mutants (Figure 3D, Table 1). These data suggest that *cdk-8* primarily acts downstream or parallel to *mpk-1/ERK* to repress vulval cell fate specification by the EGFR signaling pathway.

cdk-8's position downstream of *mpk-1/ERK* suggested a cell-autonomous role in VPCs (Figure 1). Nuclear expression of the MDT-12/DPY-22 protein in VPCs and in the anchor cell had previously been observed, and gonad-independent vulval induction in *mdt-12/dpy-22* mutants suggested an anchor cell-independent role for MDT-12/DPY-22 (Moghal and Sternberg 2003a). However, the tissue-specific requirements for MDT-12/DPY-22 in VPCs *vs.* the hypodermis, two important drivers of VPC cell fate (Fay and Yochem 2007; Schmid and Hajnal 2015), had not been tested. We used the *lin-15A* sensitized background to analyze tissue-specific requirements for *cdk-8* in VPCs *vs.* the hypodermis. First, we demonstrated that a transgene expressing *cdk-8* from its own promoter [*cdk-8(+)*] rescued the *cdk-8*; *lin-15A* mutant Muv phenotype compared to nontransgenic siblings (Figure 3E). This transgene appeared to be broadly expressed and functional, as it rescued two additional phenotypes observed in *cdk-8* mutants: decreased body length (Dumpy phenotype, Dpy) and the low brood size of the *cdk-8* mutant (Figure S2, A and B). Expression of *cdk-8* from the *lin-31* promoter (*lin-31P*::*cdk-8*), which drives transgene expression in Pn.ps and some neurons (Tan *et al.* 1998; Kishore and Sundaram 2002), also significantly rescued the *cdk-8*; *lin-15A* Muv phenotype (Figure 3E). In contrast, expression of *cdk-8* from the hypodermis-specific *dpy-7* minimal promoter (*dpy-7P*::*cdk-8*) (Gilleard *et al.* 1997) did not significantly rescue the Muv penetrance of *cdk-8*; *lin-15A* mutant worms (Figure 3E), although it was able to rescue the Dpy phenotype (Figure S2A). Unexpectedly, the *lin-31P*::*cdk-8* transgene partially rescued the *cdk-8*; *lin-15A* Dpy phenotype compared to nontransgenic worms, albeit to a lesser extent than *cdk-8(+)* or *dpy-7P*::*cdk-8* (Figure S2A). In sum, these experiments provide evidence that *cdk-8* is required cell autonomously in VPCs but not in the hypodermis to suppress ectopic vulval induction.

cdk-8 activity is kinase dependent

We next addressed how *cdk-8* functions downstream of *mpk-1/ERK*. First, we studied CDK-8's kinase requirement using a kinase-dead CDK-8(D182A) transgene [CDK-8(KD)]. The D182A mutation is homologous to the previously reported D173A mutation in human CDK8 and the D290A mutation in budding yeast CDK8, both of which result in loss of enzymatic activity (Liao *et al.* 1995; Gold and Rice 1998); however, we note that the kinase activity of *C. elegans* CDK-8(D182A) has not been tested directly. CDK-8(KD) did not rescue the *cdk-8*; *lin-15A* mutant Muv phenotype, and actually enhanced Muv penetrance (Figure 4A), suggesting that

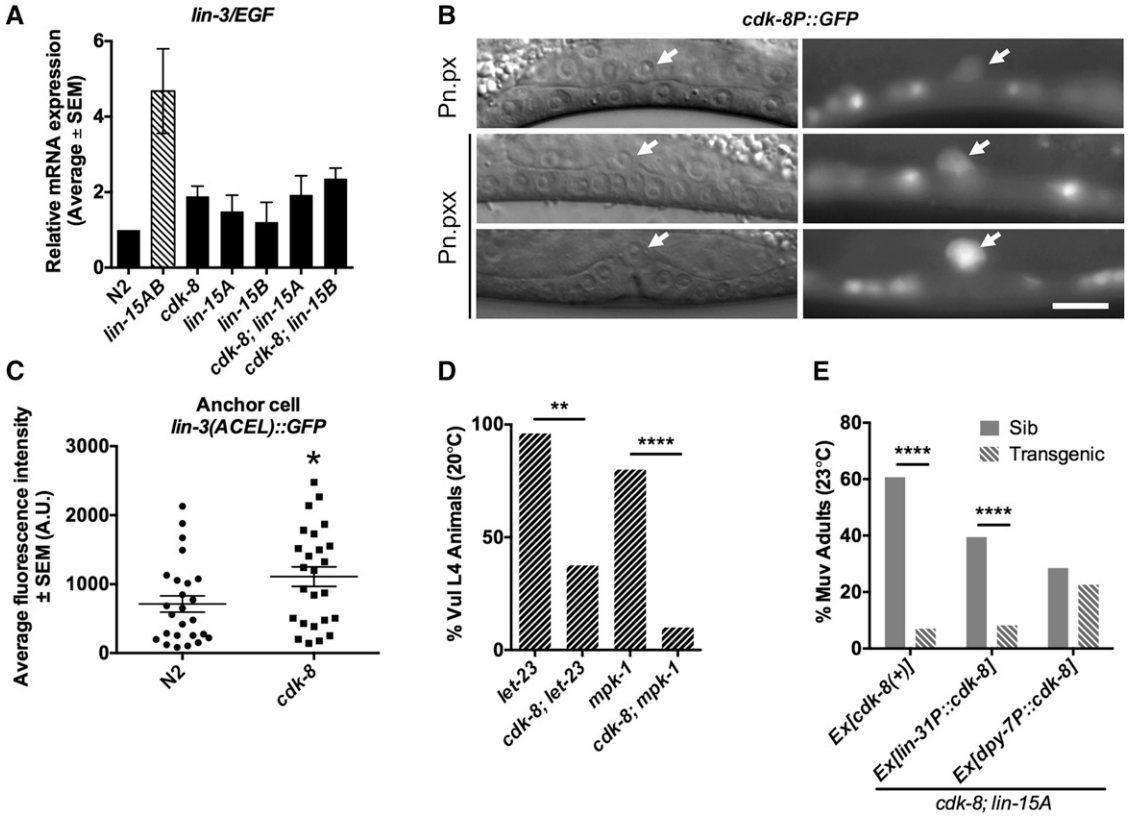

Figure 3 *cdk-8* acts upstream and downstream of *let-23/EGFR*. (A) qPCR analysis of *lin-3* mRNA levels in *cdk-8* single mutants and mutants with synMuv genes, relative to wild-type worms. *lin-15AB* mutant is shown as a positive control (hatched bar). Error bars represent standard error of the mean (SEM, $n = 3$ independent trials). No statistically significance differences, Wilcoxon signed rank test by the Pratt method. (B) *hdEx508[cdk-8P::GFP]* expression in the anchor cell (arrow) during early vulval induction. Top: VPCs divided once (Pn.px). Middle: VPCs divided twice (Pn.pxx). Bottom: Invagination of Pn.pxx epithelium. The fluorescent signal visible near VPCs localizes to neuron cell bodies. Bar: 10 μm. (C) Average fluorescence intensity of *syIs107[lin-3ACEL::GFP]* in anchor cell of wild-type worms and *cdk-8* mutants. $*P < 0.05$ *vs.* N2, *t*-test. A.U., arbitrary units. (D) L4 Vul penetrance in *cdk-8*; *let-23/EGFR* and *cdk-8*; *mpk-1/ERK* mutants ($n \geq 8$). $****P < 0.0001$, Fisher's exact test. See Table 1 for raw data. (E) Tissue specificity of Muv phenotype in *cdk-8*; *lin-15A* mutant adults expressing wild-type *cdk-8* driven by its own promoter *cdk-8(+)*, the Pn.p promoter *lin-31P*, or the hypodermal promoter *dpy-7P*, compared to nontransgenic siblings (Sib) in each strain ($n \geq 76$). $****P < 0.0001$ *vs.* nontransgenic sibling, Fisher's exact test. See Table S2 for raw data.

kinase activity is required for transgenic rescue of the Muv phenotype of *cdk-8* null mutants.

cdk-8 promotes lin-1/Ets repressor activity

Next, we investigated transcription factors that repress VPC induction, *i.e.*, the ELK1/Ets-family transcription factor LIN-1 and the FoxB/Forkhead-family transcription factor LIN-31 (Miller *et al.* 1993; Beitel *et al.* 1995). We hypothesized that the CKM may coregulate LIN-1 and/or LIN-31 and thus tested their genetic interactions with *cdk-8*. Loss of *cdk-8* did not enhance the *lin-31* mutant Muv phenotype (Table 1); therefore, we did not investigate *lin-31* further. In contrast, *cdk-8* mutation strongly enhanced the low Muv penetrance caused by *lin-1* RNAi depletion (Figure 4B, Table 1). To corroborate the RNAi experiment, we also studied the *lin-1(n1790)* mutant, which displays both reduction-of-function (ectopic vulval induction of P3.p, P4.p, and P8.p due to reduced *lin-1* mRNA levels) and gain-of-function (reduced vulval induction in P5.p–P7.p due to impaired LIN-1–ERK binding) phenotypes (Jacobs *et al.* 1998). As seen in *lin-1 RNAi* worms, loss of *cdk-8* significantly enhanced the Muv penetrance of the *lin-1(n1790)* mutant (Figure 4C, Table 1). Together, these results suggest that residual LIN-1 requires *cdk-8* for efficient repression of vulval induction.

To test whether *cdk-8* promotes transcriptional repression by LIN-1, we investigated whether the direct LIN-1 target gene *lag-2* (Zhang and Greenwald 2011) was derepressed in *cdk-8* mutants. In wild-type animals, a *lag-2P(min)::YFP* minimal promoter reporter (*arEx1098*) is induced by EGFR signaling in P6.p, whereas in *lin-1* null mutants, it is ectopically induced in additional VPCs (Zhang and Greenwald 2011). We again used the *lin-15A* sensitized background to study *cdk-8* requirements for *lag-2P(min)* repression. In all *lin-15A* single mutant animals examined, *lag-2P(min)::YFP* exhibited a wild-type expression pattern, as expected ($n = 29$; Figure 4D). In contrast, in *cdk-8*; *lin-15A* mutants, *lag-2P(min)::YFP* was occasionally ectopically expressed in VPCs other than P6.p (in 3/41 animals; Figure 4D). Thus, *cdk-8* is partially required to repress a direct LIN-1 target gene. Taken together with the requirement for *cdk-8* for

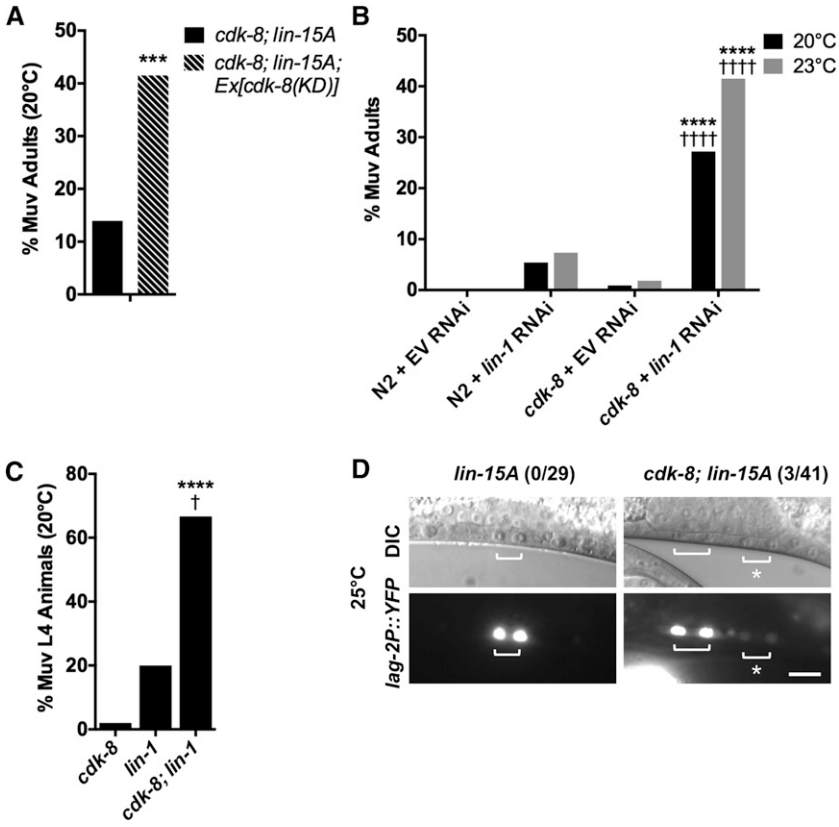

Figure 4 CDK-8 is a LIN-1/Ets corepressor. (A) Adult Muv penetrance in *cdk-8*; *lin-15A* mutants expressing kinase dead (KD) *cdk-8*, compared to nontransgenic *cdk-8*; *lin-15A* ($n \geq 41$). ***$P < 0.001$ *vs.* nontransgenic, Fisher's exact test. See Table 1 for raw data. (B) Adult Muv penetrance in *cdk-8* mutants on empty vector (EV) or *lin-1/Ets* RNAi ($n \geq 410$) ****$P <$ 0.0001 *vs. cdk-8* + empty vector (EV), ††††$P < 0.0001$ *vs.* N2 + *lin-1* RNAi, Fisher's exact test. See Table S2 for raw data. (C) L4 Muv penetrance in *cdk-8*; *lin-1 (n1790)* mutants ($n \geq 12$). ****$P < 0.0001$ *vs. cdk-8*, †$P < 0.05$ *vs. lin-1(n1790)*, Fisher's exact test. See Table 1 for raw data. (D) Wild-type expression pattern of *arEx1098[lag-2P(min)::GFP]* in *lin-15A* mutants, and ectopic expression in *cdk-8*; *lin-15A* mutants. Bracket: Pn.px. Asterisk: ectopic expression. Bar: 10 μm. Number of ectopic expression events over total sample size noted next to genotype.

lin-1 repressor function in vulval induction, these data suggest that CDK-8 may function as a LIN-1 corepressor, perhaps acting redundantly with other corepressors.

The CKM subunit mdt-13/let-19 represses primary vulval fate specification

Having shown that the enzymatic CKM subunit represses EGFR signaling-induced transcription in the *C. elegans* vulva, we next investigated whether the structural CKM subunits *mdt-12/dpy-22* and *mdt-13/let-19* (Tsai *et al.* 2013) have similar molecular functions. *C. elegans mdt-12/dpy-22* represses ectopic vulva formation downstream of *let-23/EGFR* (Moghal and Sternberg 2003a), but whether and how it affects vulval cell fate specification is not understood. Thus, we investigated the requirements and mechanisms of structural CKM subunits in the EGFR signaling pathway, focusing primarily on *mdt-13/let-19*.

We first investigated the vulval phenotypes of *mdt-12/dpy-22* and *mdt-13/let-19* mutants. *mdt-13/let-19* mutants exhibited a temperature-sensitive Muv phenotype (Figure 5A) of much higher penetrance than *cdk-8* mutants (Figure 2B). We observed a similarly high penetrance Muv phenotype in *mdt-12/dpy-22* reduction-of-function mutants (Figure 5A). We note that the *os38* mutant used in this study shows higher Muv penetrance than the 2–3% Muv penetrance reported for *dpy-22(sy622)* and *dpy-22(sy665)* mutants (Moghal and Sternberg 2003a). *os38* may cause stronger loss of function than these alleles due to truncation closer to the N terminus and/or the presence of an additional missense mutation (Yoda *et al.* 2005). Overall, these results demonstrate that *mdt-12/dpy-22* and *mdt-13/let-19* are more strongly required than *cdk-8* and *cic-1* to repress vulval induction.

We next tested whether *mdt-13/let-19* adopts roles similar to *cdk-8* in vulva development. *mdt-13/let-19* mutants induced *egl-17P*::*CFP* expression in 46/47 P6.px cells examined (Figure 5B). In addition, *mdt-13/let-19* mutants displayed increased proportions of P.5px and P7.px weakly expressing *egl-17P*::*CFP* compared to wild-type worms (Figure 5B). Furthermore, *mdt-13/let-19* mutants displayed strong *egl-17P*::*CFP* expression in some P5.px and P7.px cells, or ectopic expression of *egl-17P*::*CFP* outside of P5.px–P7.px, suggesting derepression of the 1° fate in these cells (Figure 5B). Overall, these data indicated that the Muv phenotype of *mdt-13/let-19* mutants was due in part to derepressed ERK signaling output.

In support of a role similar to *cdk-8* in regulation of vulval cell fate specification, *mdt-13/let-19* interacted genetically with the synMuv genes and with *lin-1/Ets*. Specifically, *mdt-13/let-19* mutants interacted genetically with the class A synMuv transcriptional repressor *lin-15A*, and the class B synMuv gene *lin-15B(n374)* showed a similar trend (Figure 5A). In addition, loss of *mdt-13/let-19* significantly enhanced the Muv penetrance of *lin-1(n1790)* mutant worms (Figure 5C, Table 1), in line with a role for MDT-13/LET-19 as a LIN-1 corepressor. Thus, like *cdk-8*, *mdt-13/let-19* interacts genetically with transcriptional regulators in the EGFR signaling pathway.

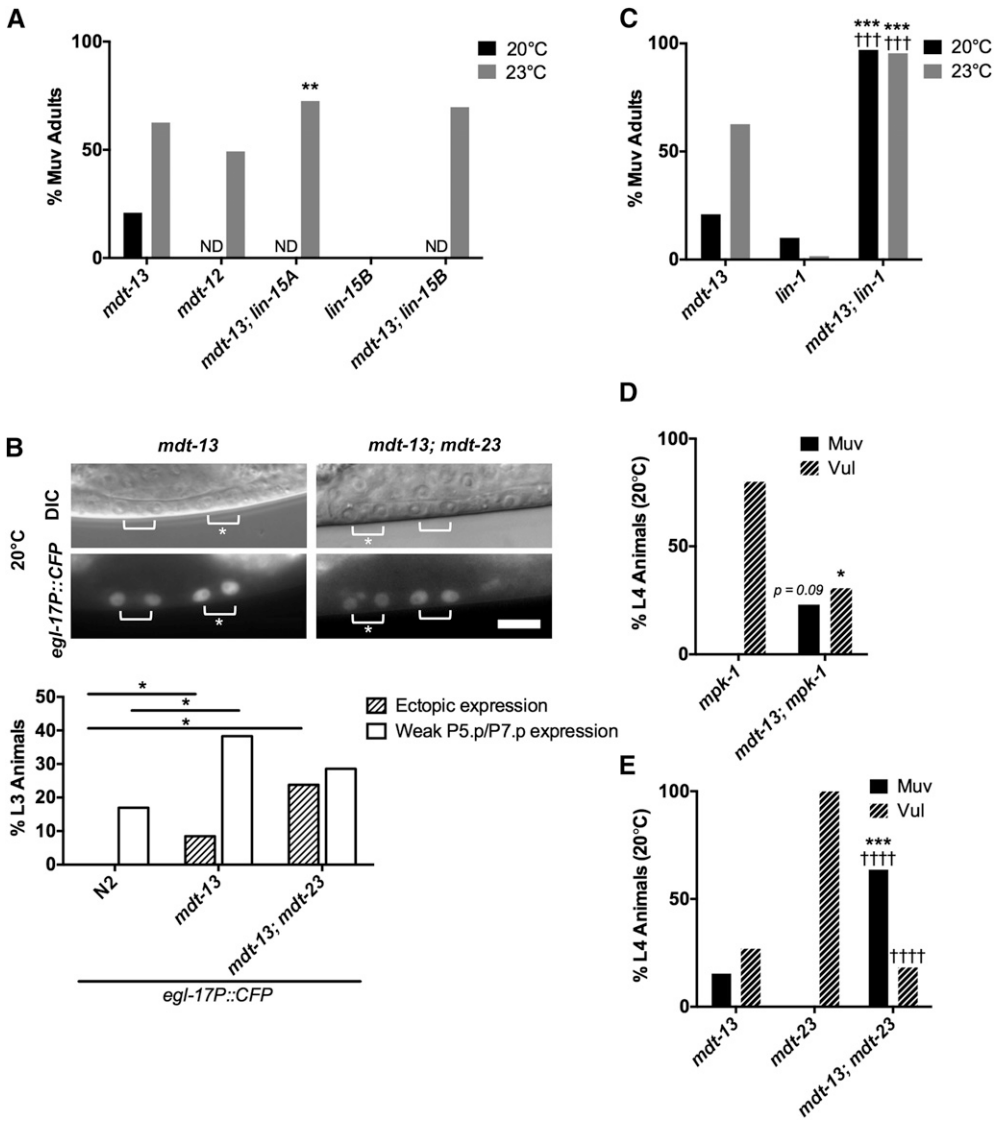

Figure 5 *mdt-13/let-19* represses vulval cell fates downstream of *mpk-1/ERK*. (A) Adult Muv penetrance in *mdt-13/let-19* mutants and mutants with representative synMuv genes ($n \geq 99$). $^{**}P < 0.01$ *vs. mdt-13/let-19* single mutant, Fisher's exact test. ND, not determined. See Table S2 for raw data. (B) Micrographs show *mdt-13/let-19* and *mdt-13/let-19*; *mdt-23/sur-2* mutants expressing the *arIs92[egl-17P::CFP]* 1° fate marker. Bracket: Pn.px cells expressing reporter. Asterisk: ectopic reporter expression. Bar: 10 μm. Graphs show the percentage of animals ($n > 20$) with ectopic *egl-17P::CFP* expression (defined as expression in P3.p/P4.p/P8.p, or expression in P5.p/P7.p of equal intensity to P6.p) or weak P5.p/P7.p expression (defined as expression in P5.p/P7.p that is weaker intensity than P6.p). $^{*}P < 0.05$, Fisher's exact test. (C) Adult Muv penetrance in *mdt-13/let-19*; *lin-1(n1790)* mutants ($n \geq 44$). *mdt-13/let-19* single mutant Muv penetrance from Figure 5A is included for reference. $^{***}P < 0.001$ *vs. mdt-13/let-19* single mutant, $^{\dagger\dagger\dagger}P < 0.001$ *vs. lin-1(n1790)* single mutant, Fisher's exact test. See Table S2 for raw data. (D) L4 Muv and Vul penetrance in *mdt-13/let-19*; *mpk-1/ERK* mutants ($n \geq 13$). $P = 0.09$ (Muv) and $^{*}P < 0.05$ (Vul) *vs. mpk-1* single mutant, Fisher's exact test. See Table 1 for raw data. (E) L4 Muv and Vul penetrance in *mdt-13/let-19*; *mdt-23/sur-2* mutants ($n \geq 22$). $^{***}P < 0.001$ *vs. mdt-13/let-19* single mutant, $^{\dagger\dagger\dagger\dagger}P < 0.0001$ *vs. mdt-23/sur-2* single mutant, Fisher's exact test. See Table 1 for raw data.

Finally, we investigated the genetic position of *mdt-13/let-19* in the EGFR-Ras-ERK pathway. Similarly to *cdk-8*, loss of *mdt-13/let-19* suppressed the *mpk-1* loss-of-function Vul phenotype (Figure 5D, Table 1). Furthermore, the *mdt-13/let-19* mutant Muv phenotype appeared epistatic to the *mpk-1* mutant Vul phenotype, although this trend was not statistically significant (Figure 5D, Table 1). Together, these data support a model in which the CKM acts downstream of *mpk-1/ERK* to inhibit vulval cell fate specification.

The CKM restricts the specificity of core Mediator subunits

As several Mediator subunits positively regulate vulva development (reviewed in Grants *et al.* 2015), we hypothesized that the CKM might function in part by inhibiting these subunits. In wild-type worms, the *mdt-23/sur-2* tail module subunit is critical for vulva development downstream of Ras (Singh and Han 1995), and it is required for activation of the EGFR signaling target gene *lag-2* (Zhang and Greenwald 2011); therefore, we tested how a mutation in a CKM subunit affected vulval induction in *mdt-23/sur-2* mutants. All *mdt-23/sur-2* single mutants examined exhibited a Vul phenotype (Figure 5E, Table 1). VPC induction analysis of *mdt-13/let-19* single mutants demonstrated that, in addition to the Muv phenotype, some animals also displayed decreased proliferation of P5.p or P7.p (mild Vul; Figure 5E, Table 1). Unexpectedly, *mdt-13/let-19*; *mdt-23/sur-2* mutants exhibited a significantly stronger Muv penetrance than *mdt-13/let-19* single mutants, and loss of *mdt-13/let-19* function significantly suppressed the Vul phenotype of *mdt-23/sur-2* single mutants (Figure 5E, Table 1), suggesting that loss of *mdt-13/let-19* derepresses vulval induction independently of *mdt-23/sur-2*. As seen in *mdt-13/let-19* single mutants, *mdt-13/let-19*; *mdt-23/sur-2* double mutants showed strong *egl-17P::CFP*

expression in some P5.px and P7.px cells, or ectopic expression of *egl-17P*::*CFP* outside of P5.px–P7.px, suggesting derepression of the 1° fate (Figure 5B). Overall, these data indicated that the Muv phenotype of *mdt-13/let-19*; *mdt-23/sur-2* double mutants was due in part to derepressed ERK signaling output. Together, these findings indicate that loss of the CKM allows activation of EGFR signaling-driven cell fate specification independently of *mdt-23/sur-2* activity.

Our results suggested that the CKM might influence Mediator subunit(s) other than *mdt-23/sur-2*. As *S. cerevisiae* CDK8 inhibits the Mediator tail module triad composed of MED2, MED3, and MED15 (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014), we hypothesized that their putative *C. elegans* orthologs MDT-29, MDT-27, and MDT-15 (Bourbon 2008), might be targets for CKM inhibition. *mdt-15* and *mdt-29* knockdown had no effect on vulva formation in wild-type animals (*i.e.*, causing neither Muv nor Vul phenotypes and displaying normal VPC induction; Table 1), but significantly reduced the Muv penetrance of *mdt-13/let-19* mutants (Figure 6A); *mdt-27* RNAi caused a similar trend (Figure 6A). This effect was specific to the tail module triad, as knockdown of the *mdt-1.1* tail module subunit in fact increased the Muv penetrance of *mdt-13/let-19* mutants (Figure 6A). Furthermore, requirement for the tail module triad in ectopic vulva formation appeared to be specific to CKM mutants, as *mdt-15* RNAi had no effect on ectopic vulva formation in *lin-1(n1790)* mutants (Figure 6B, Table 1). Thus, tail module triad activity appears to be derepressed in a CKM mutant, causing aberrant activation of vulval fate specification.

Next, we investigated whether the CKM modifies the target gene specificity of the triad. We used qPCR to quantify the expression of *cdk-8*-repressed genes identified by our microarray analysis (Table S1) in wild-type worms and *cdk-8* mutants treated with empty vector (EV), *mdt-15*, *mdt-27*, or *mdt-29* RNAi. On EV RNAi, seven of nine genes tested were upregulated in *cdk-8* mutants compared to wild-type worms, as expected (Figure 6C). Upregulation of these *cdk-8*-repressed genes was strongly attenuated by *mdt-15* depletion, whereas *mdt-29* knockdown only affected *fat-7*, and *mdt-27* depletion caused no significant changes (Figure 6C). Thus, for the genes investigated, induction in *cdk-8* mutants appears to specifically require *mdt-15*, but not the other predicted tail module triad subunits. However, we cannot rule out unequal RNAi efficiency accounting for these differing requirements, although RNAi knockdown of all three genes appeared successful, as we observed partial sterility (not shown) consistent with the essential nature of these core Mediator subunits (Fernandez *et al.* 2005; Sönnichsen *et al.* 2005; Taubert *et al.* 2006). Notably, only two *cdk-8*-repressed genes, *acd-2* and *fat-7*, displayed *mdt-15* and/or *mdt-29*-dependent activation in wild-type worms (Figure 6C). Thus, as seen in the genetic analysis of vulva induction, loss of *cdk-8* appears to cause unrestrained tail module activity, *i.e.*, *mdt-15* activates novel target genes when *cdk-8* is deleted.

Finally, we investigated the molecular cause of unrestrained *mdt-15* activity in CKM mutants. Loss of *cdk-8* did not alter mRNA levels of any triad subunits (Figure S3). Western blot analysis showed elevated MDT-15 protein levels in *cdk-8* mutants compared to wild type (Figure 6D). Taken together, these results demonstrate that *cdk-8* is required for post-transcriptional regulation of MDT-15.

Discussion

EGFR signaling is critical for cell proliferation and cell fate determination in animal development. Several Mediator subunits positively or negatively regulate EGFR signaling-driven developmental processes (reviewed in Grants *et al.* 2015), but pertinent mechanisms remain incompletely understood. Here, we used the well-characterized vulva development paradigm in *C. elegans* to delineate the role of the Mediator CKM module. Our results suggest a model whereby the CKM acts within the vulval precursor cells, in a kinase-dependent manner, to fine tune EGFR transcriptional output by modulating two transcriptional regulators: the key downstream transcription factor LIN-1/Ets and core Mediator (Figure 6E). This model is based on four key observations: First, we demonstrate that the primary site of action for the CKM is in the VPCs, as *cdk-8* and *mdt-13/let-19* repress vulva formation downstream of *mpk-1/ERK*, a key component of the EGFR signaling cascade inside VPCs, and *cdk-8* expression in VPCs is sufficient for this repression. Second, *cdk-8* repression of ectopic vulva formation is kinase dependent. Third, the CKM appears to act as a corepressor of the Ets-family transcription factor LIN-1, as loss of *cdk-8* or *mdt-13/let-19* enhances the ectopic vulval induction caused by *lin-1* reduction of function, and *cdk-8* is required for full repression of a direct LIN-1 target promoter. Fourth, ectopic vulva formation in *mdt-13/let-19* is independent of the Mediator subunit *mdt-23/sur-2*, which is critical for EGFR signaling-driven transcription and vulval development in wild-type worms (Singh and Han 1995; Zhang and Greenwald 2011); instead, *mdt-13/let-19* modulates the specificity of the tail module triad subunits *mdt-15*, *mdt-29*, and *mdt-27*, preventing aberrant activation of downstream transcription. By implicating all CKM subunits and by connecting the CKM to *lin-1* and to core Mediator, our data substantially expand on the prior finding that loss of CKM subunit *mdt-12* caused ectopic vulva formation by unknown molecular mechanisms (Moghal and Sternberg 2003a). Additionally, our genetic and molecular analysis provide first evidence that CKM-tail module crosstalk, akin to that seen in yeast Mediator (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014), occurs in metazoan Mediator, an important experimental finding as tail module subunit sequence conservation between species is extremely poor (Bourbon 2008).

The CKM inhibits vulva development in a kinase-dependent manner

We performed unbiased gene expression profiling to define gene programs that depend on *cdk-8 in vivo*, which revealed that only 6.7% of *C. elegans* genes are regulated by *cdk-8* (Figure 2A, Table S1). This number F agrees with studies in

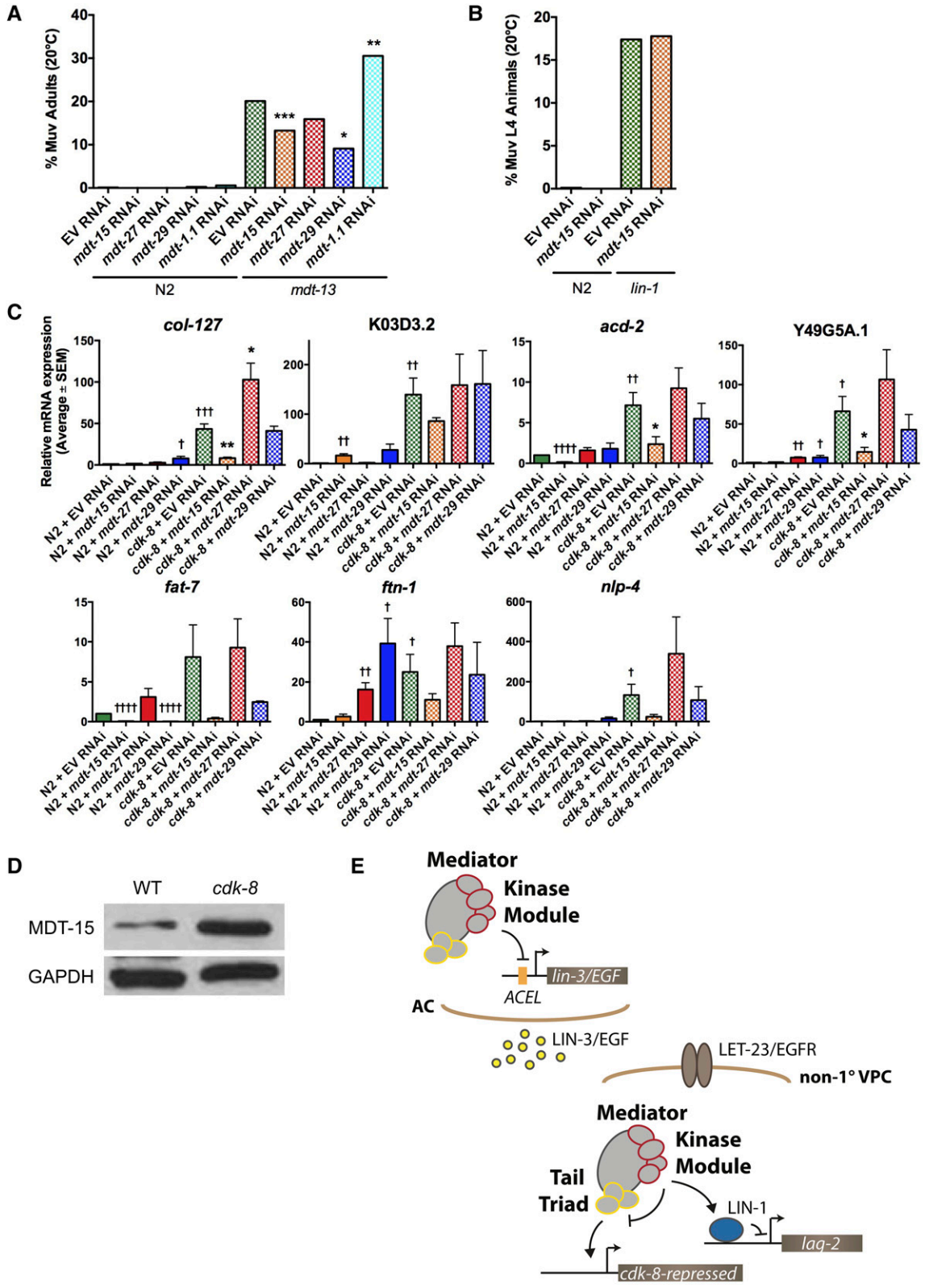

Figure 6 The CKM inhibits activation of vulval induction by the *mdt-15*, *mdt-27*, *mdt-29* triad. Adult Muv penetrance in *mdt-13/let-19* mutants on *mdt-15*, *mdt-27*, or *mdt-29* triad subunit RNAi or *mdt-1.1* nontriad subunit RNAi ($n \geq 198$). *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ *vs. mdt-13/let-19* + empty vector (EV), Fisher's exact test. See Table S2 for raw data. (B) L4 Muv penetrance in *lin-1(n1790)* mutants on *mdt-15* RNAi ($n \geq 45$). No statistically significant difference, Fisher's exact test. See Table 1 for raw data. (C) qPCR analysis of mRNA levels of *cdk-8*-repressed genes upon *mdt-15*, *mdt-27*, or *mdt-29* RNAi. Error bars represent SEM, $n = 3$ independent trials. *$P < 0.05$, **$P < 0.01$ *vs. cdk-8* + empty vector (EV); †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, ††††$P < 0.0001$ *vs.* N2 + empty vector (EV), Fisher's exact test. (D) α-MDT-15 Western blot in wild-type *vs. cdk-8* mutants, with α-GAPDH loading

yeast, wherein *CDK8* regulates only 3% of genes (Holstege *et al.* 1998). Thus, CDK8 appears to be a gene program-specific transcriptional coregulator across species.

Among *cdk-8*-dependent genes, we identified a significant overlap with genes regulated by *lin-35/RB*, a synMuv transcriptional repressor (Figure 2A) (Kirienko and Fay 2007). We note that as synMuv genes act redundantly, *lin-35* single mutants do not exhibit any defects in EGFR signaling or vulval induction (Myers and Greenwald 2005; Cui *et al.* 2006; Kirienko and Fay 2007). Thus, the overlap between *cdk-8*- and *lin-35*-dependent genes suggested that the CKM and *lin-35* cooperate in multiple aspects of *C. elegans* development. Similarly, in *Drosophila*, CDK8 and RB act in parallel in the Wnt signaling pathway (Morris *et al.* 2008). Therefore, we explored whether the CKM and synMuv genes cooperate in the EGFR signaling pathway to regulate *C. elegans* vulva development. Both *cdk-8* and *mdt-13/let-19* were required to repress *C. elegans* vulva formation, in a partially redundant manner with the synMuv genes (Figure 2B and Figure 5A). However, *cdk-8* did not act redundantly with the synMuv genes to repress *lin-3/EGF* transcription (Figure 3A), suggesting that the CKM and the synMuv genes regulate EGFR signaling at different junctions, as discussed below.

Comparing the vulval phenotypes of CKM mutants, we found evidence that *cdk-8* and *cic-1* act redundantly to repress vulval induction, as a *cdk-8*; *cic-1* double mutant displayed the same Muv penetrance as *cdk-8* or *cic-1* single mutants (Figure 2B). In addition, we found that *mdt-12/dpy-22* and *mdt-13/let-19* were more strongly required to repress vulva development than *cdk-8* or *cic-1*. In *S. cerevisiae* Mediator, MED12 and MED13 enable CDK8 and cyclin C docking to Mediator (Tsai *et al.* 2013). Loss of MDT-12/DPY-22 or -13 in *C. elegans* may similarly disrupt CDK-8 and CIC-1 function, as well as considerably reducing the size of the CKM. Although CDK-8's kinase activity is required to inhibit vulva development (Figure 4A), this does not rule out the possibility that the CKM also employs kinase-independent steric mechanisms, as observed in other systems (Knuesel *et al.* 2009). Thus, additional kinase-independent mechanisms could account for the stronger requirement for *mdt-12/dpy-22* and *-13* in vulva development.

The CKM inhibits the primary vulval cell fate

Vulva formation in *C. elegans* requires both EGFR and Notch signaling (Félix and Barkoulas 2012), and human CDK8 represses Notch signaling-driven transcription by promoting turnover of the Notch intracellular domain (Fryer *et al.* 2004). Therefore, we examined whether the vulval phenotypes in CKM mutants occur due to defects in EGFR signaling, Notch signaling, or both. Using an EGFR-Ras-ERK signaling-induced 1° cell fate reporter, we demonstrated that *cdk-8* and *mdt-13/let-19* are required to repress ectopic vulva formation in part by repressing the 1° cell fate (Figure 2C and Figure 5B). Using a Notch signaling-induced 2° cell fate reporter, we showed that *cdk-8* is required to represses ectopic 2° fates in nonvulval VPCs, as well as to promote the 2° fate in P5.p and P7.p (Figure 2D). However, *cdk-8* action in the Notch pathway might occur indirectly in this context. EGFR signaling in P6.p induces expression of Notch ligands, *e.g.*, *lag-2*, which promote the 2° fate in the neighboring cells, P5.p and P7.p (Chen and Greenwald 2004). We observed evidence of possible cell fate transformations from 2° to 1° in P5.p or P7.p in *cdk-8* and *mdt-13* mutants, as these cells occasionally exhibited strong expression of the 1° cell fate marker *egl-17P*::*CFP* (Figure 2C and Figure 5B), expression of the EGFR signaling target gene *lag-2* (Figure 4D) or loss of the strong *lip-1P*::*GFP* expression characteristic of 2° cells (Figure 2D). It is possible that VPCs transformed to the 1° fate could then induce 2° fates in neighboring VPCs, accounting for our observation of ectopic 2° cells.

CKM subunits have been implicated as regulators of canonical Wnt signaling (Zhang and Emmons 2000; Firestein *et al.* 2008; Morris *et al.* 2008) and cell cycle quiescence (Clayton *et al.* 2008), processes which also contribute to vulva development. Activation of Wnt signaling can bypass requirements for *let-23/EGFR* in vulva development (Gleason *et al.* 2002). However, the Muv phenotype of *mdt-12/dpy-22* mutants is independent of *bar-1/β-catenin* (Moghal and Sternberg 2003b), suggesting that the CKM does not repress vulva development through the canonical Wnt signaling pathway. Deregulation of cell cycle quiescence can expand the VPC equivalence group, which are competent to form ectopic vulvae if presented with the appropriate signals [*e.g.*, *lin-12/Notch* gain of function employed by Clayton *et al.* (2008)]. Although CKM subunits are required for VPC cell cycle quiescence (Clayton *et al.* 2008), this alone is unlikely to account for the ectopic vulvae observed in these animals. First, the ectopic vulval invaginations observed in *cdk-8* and *mdt-13/let-19* animals while scoring VPC induction (Table 1) were positioned in the correct location for P3.p, P4.p, and P8.p descendants. Second, ectopic expression of 1° and 2° cell fate markers in *cdk-8* and *mdt-13/let-19* mutants (Figure 2, C and D, and Figure 5B) suggests that EGFR and/or Notch signaling indeed drives ectopic vulva formation in these mutants.

The CKM promotes LIN-1/Ets repressor activity

We observed derepression of the *lin-3/EGF* ACEL in *cdk-8* mutants (Figure 3C), implicating *cdk-8* as a novel repressor of *lin-3/EGF* transcription in the anchor cell. Albeit interesting, genetic epistasis analysis with *let-23/EGFR* and *mpk-1/ERK* loss-of-function alleles clearly demonstrated that *cdk-8* is

control. Representative immunoblot from one of three trials. (E) Model of CKM inhibition of EGFR-Ras-MAPK signaling-dependent cell fate specification by repressing *lin-3/EGF* in the anchor cell (AC), promoting LIN-1 repressor activity (*e.g.*, at *lag-2*), and inhibiting tail module triad activity (*e.g.*, at *cdk-8*-repressed genes) in non-1° VPCs (*i.e.*, VPCs other than P6.p).

primarily required downstream of *mpk-1/ERK* to repress vulval induction (Figure 3D). The *let-23(sy97)* mutant protein is ligand insensitive (Aroian and Sternberg 1991; Aroian *et al.* 1994); therefore, weak *lin-3/EGF* activation in the anchor cell due to loss of *cdk-8* cannot account for the vulval phenotypes observed in *cdk-8*; *let-23(sy97)* mutants (Figure 3D). Furthermore, epistasis analysis with *mpk-1/ERK* confirmed that *cdk-8* acts downstream of the core EGFR-Ras-ERK pathway to regulate vulval induction (Figure 3D). In line with a position downstream of *mpk-1/ERK*, we showed that *cdk-8* is required in VPCs to suppress ectopic vulval induction (Figure 3E). A previous report demonstrated that repression of vulval induction by the CKM subunit *mdt-12/dpy-22* is gonad independent, and thus anchor cell independent, and that an MDT-12/DPY-22::GFP transgene is expressed in VPCs (Moghal and Sternberg 2003a), supporting a role for the CKM in VPCs.

Downstream of *mpk-1/ERK*, we found evidence that the CKM promotes LIN-1/Ets-mediated repression of vulval induction (Figure 4, B and C, and Figure 5C), and that *cdk-8* promotes transcriptional repression of a direct LIN-1 target, the *lag-2/Notch ligand* minimal promoter (Figure 4D). The *lag-2* minimal promoter contains activator and repressor elements, VPCact and VPCrep, that cooperatively restrict expression to P6.p (Zhang and Greenwald 2011). On its own, VPCact is sufficient to drive transcription in all VPCs (P3.p–P8.p) in a LIN-3/EGF ligand-independent manner. VPCrep represses this basal VPCact-driven transcription in VPCs other than P6.p, thereby restricting expression of the *lag-2* minimal promoter to the 1°-fated VPC. VPCrep contains an Elk1 consensus site, which is bound by LIN-1 *in vitro* (Miley *et al.* 2004), and requires *lin-1/Ets* for repression of transcription in VPCs other than P6.p (Zhang and Greenwald 2011). Our results indicate that *cdk-8* is partially required for transcriptional repression of the *lag-2* minimal promoter (Figure 4D), suggesting that the CKM promotes LIN-1-mediated repression at VPCrep. An alternative explanation for the ectopic expression of the *lag-2* minimal promoter observed in *cdk-8* mutants is that the CKM might inhibit a factor that activates transcription through VPCact. The transcription factor(s) that acts at VPCact remains poorly defined; however, the *mdt-23/sur-2* Mediator subunit is required for VPCact-driven transcription in P3.p–P8.p (Zhang and Greenwald 2011). As we demonstrated that vulval induction in *mdt-13/let-19* mutants does not require *mdt-23/sur-2* (Figure 5, B and E), this implies that the CKM likely does not inhibit MDT-23/SUR-2 activity at VPCact. Overall, our findings suggest that the CKM may act as a corepressor for LIN-1.

In murine embryonic stem cells, Mediator recruitment is important for transcriptional activation by Ets factors, *e.g.*, Elk1 (Stevens *et al.* 2002; Balamotis *et al.* 2009). In this context, activation of Elk1 by ERK phosphorylation promotes binding to Mediator in a MED23/Sur2-dependent manner (Stevens *et al.* 2002). Similarly, in a colon cancer cell line, CDK8 promotes transcriptional elongation of serum response immediate early genes, which are targeted by multiple transcription factors including Elk1 (Donner *et al.* 2010). However, the role of Mediator in transcriptional repression by an Ets factor has not previously been explored. In the absence of ERK phosphorylation, Ets factors, *e.g.*, LIN-1, can promote transcriptional repression of target genes (Jacobs *et al.* 1998; Zhang and Greenwald 2011). Although the Sin3A-HDAC-1 corepressor complex has been implicated in an epigenetic mechanism that attenuates transcriptional activation by ERK-phosphorylated Elk1 (Yang *et al.* 2001), to our knowledge, corepressors of Ets factor-mediated transcriptional repression have not previously been identified. This report provides evidence that the Mediator CKM is required for repression by Ets factors, representing an advance in our understanding of Ets factor repressive mechanisms.

Our findings are also of potential clinical interest, as the human CKM is implicated in tumorigenesis (Firestein *et al.* 2008; Donner *et al.* 2010; Mäkinen *et al.* 2011; Lim *et al.* 2014). Loss of *MED12* causes cellular resistance to chemotherapeutic agents that inhibit activated BRAF, the human ERK kinase kinase (Shalem *et al.* 2014); this suggests that *MED12* represses EGFR signaling downstream of BRAF, in line with our findings for the *C. elegans* CKM. Furthermore, recurrent *MED12* mutations are implicated in uterine leiomyomas and breast fibroadenomas (Mäkinen *et al.* 2011; Lim *et al.* 2014; Mittal *et al.* 2015), but the pathogenic mechanisms of these mutations have not been fully elucidated. Investigation of these mutations using the *C. elegans* vulva development paradigm may provide insight into their mode of action.

The CKM restrains the core Mediator tail module triad

Epistatic relationships between Mediator subunits have been identified in *S. cerevisiae* (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014), but intra-Mediator regulation has not been demonstrated in metazoans. Previous studies (reviewed in Grants *et al.* 2015) and our data show that several core Mediator subunits promote *C. elegans* vulva development, whereas CKM subunits inhibit this process. This suggested that intra-Mediator regulation might coordinate gene expression downstream of the EGFR-Ras-ERK signaling pathway that drives vulva development. Initially, we hypothesized that the CKM may oppose *mdt-23/sur-2*-mediated activation of EGFR signaling, as *mdt-23/sur-2* is required for vulval induction and activation of EGFR signaling-induced transcription, *e.g.*, *lag-2* (Singh and Han 1995; Zhang and Greenwald 2011). Unexpectedly, loss of *mdt-13/let-19* circumvented the requirement for *mdt-23/sur-2* in vulval induction (Figure 5E). We therefore explored regulatory interactions between the CKM and the metazoan orthologs of *S. cerevisiae* MED2, MED3, and MED15, which are subject to inhibitory post-translational regulation by CDK8 (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014). Sequence conservation is weak between yeast MED2 and MED3 and their putative metazoan homologs, MED29 and MED27, respectively (Bourbon 2008). Whether MED29 and MED27 function as part of the tail module remains unclear, as structural and biochemical

studies locate these subunits between the head and tail modules (Sato *et al.* 2003; Tsai *et al.* 2014). Thus, we were intrigued to find that vulva formation in a *C. elegans* CKM mutant required *mdt-15*, *mdt-27*, and *mdt-29* (Figure 6A). This requirement appeared specific to the tail module triad, as neither *mdt-1.1/MED1* nor *mdt-23/sur-2* was required for vulval induction in CKM mutants (Figure 5E and Figure 6A). Furthermore, the triad did not appear to be generally required for ectopic vulval induction in animals with a wild-type CKM, as *mdt-15* knockdown had no effect on ectopic vulval induction in *lin-1(n1790)* mutants (Figure 6B). Together, these findings suggest that the CKM restrains triad activity, preventing it from aberrantly activating vulval induction.

Gene expression analysis in *cdk-8* mutants identified a requirement for *mdt-15*, but little or no requirement for *mdt-27* or *mdt-29*, in transcriptional activation of *cdk-8*-repressed genes (Figure 6C). In the *S. cerevisiae* tail module triad, both *MED3* and *MED2* are required for overexpression of *CDK8*-repressed genes in *CDK8* mutants, but the requirement for *MED15* has not been tested directly (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014). These requirements might be explained by the fact that both MED2 and MED3 are necessary to anchor the triad to the tail module (Myers *et al.* 1999; van de Peppel *et al.* 2005; Gonzalez *et al.* 2014). Similar requirements may not exist in metazoan Mediator, as human Mediator displays more extensive structural contacts between the head and tail modules (Tsai *et al.* 2014), which may result in redundancy for some tail module subunits.

Investigating the regulatory relationship between CDK-8 and MDT-15 further, we found that *cdk-8* is required for post-transcriptional negative regulation of MDT-15, as MDT-15 protein but not mRNA levels increase in *cdk-8* mutants (Figure 6D). This regulatory relationship resembles that seen in yeast where the three triad subunits MED2, MED3, and MED15 are negatively regulated post-translationally by CDK8-driven phosphorylation of MED3, promoting ubiquitin-proteasome-dependent turnover of all three triad subunits (Gonzalez *et al.* 2014). It will be interesting to delineate whether the metazoan CKM regulates MDT-15 protein levels directly, *e.g.*, by phosphorylation leading to ubiquitin-mediated degradation, or indirectly through action upon other Mediator subunits.

In summary, our findings suggest that the Mediator CKM represses EGFR-Ras-ERK signaling-driven cell fate specification in *C. elegans* by regulating repressor activity of an Ets-family transcription factor and by promoting specificity of Mediator tail module subunits.

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J.M.G., H.S., and S.T. conceived and designed experiments; J.M.G., L.T.L.Y., A.Y., C.C.Y., H.S., and S.T. performed experiments; J.M.G., A.Y., H.O., H.S., and S.T. analyzed data; J.M.G., A.Y., H.O., and H.S. contributed reagents/materials/analysis tools; and J.M.G., H.S., and S.T. wrote the paper.

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The Mediator Kinase Module Restrains Epidermal Growth Factor Receptor Signaling and Represses Vulval Cell Fate Specification in *Caenorhabditis elegans*

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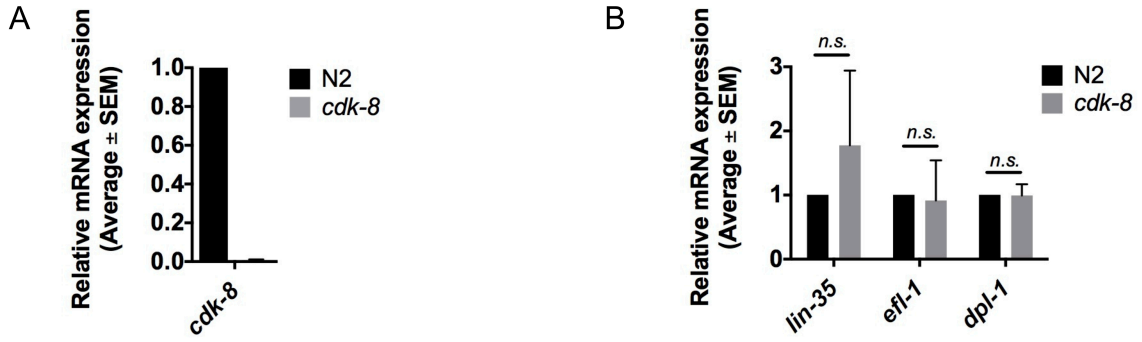

Figure S1. *cdk-8* null mutation does not influence *lin-35* or partner expression.
(A) qPCR analysis of *cdk-8* mRNA levels in *cdk-8* mutants relative to wild type (n=3, error bars represent standard error of the mean (SEM)). (B) qPCR analysis of mRNA levels in *cdk-8* mutants relative to wild type (n=3; error bars represent SEM; *n.s.* = not significant, Wilcoxon signed rank test by method of Pratt).

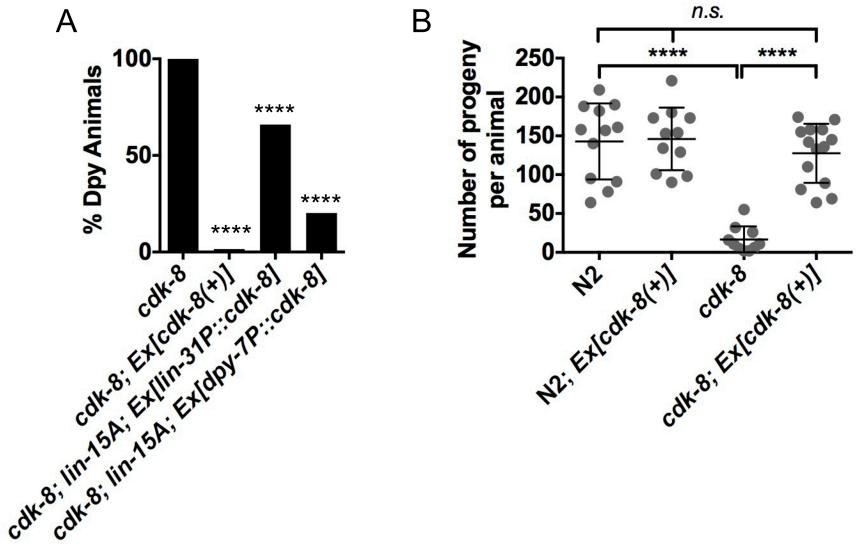

Figure S2. Additional *cdk-8* mutant phenotypes are rescued by wild-type *cdk-8* transgenes.
(A) Genetic rescue of *cdk-8* mutant Dpy phenotype (*n*>150). **** $p < 0.0001$ *vs. cdk-8* mutant, Fisher's exact test. (B) Genetic rescue of *cdk-8* mutant brood size. **** $p < 0.0001$, t-test. *n.s.* not significant.

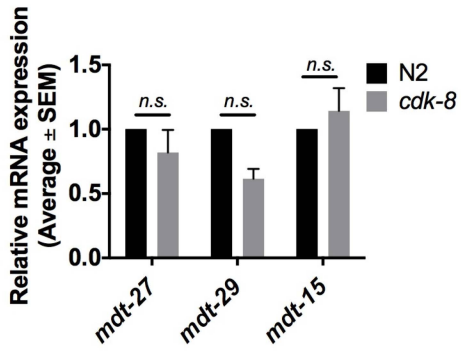

Figure S3. *cdk-8* does not regulate transcription of the *mdt-27, mdt-29, mdt-15* triad. qPCR analysis of mRNA levels in *cdk-8* mutants relative to wild type (n=3, error bars represent standard error of the mean (SEM), *n.s.* not significant).

Table S1. Microarray results. (.xlsx, 76 KB)

Available for download as a .xlsx file at
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.180265/-/DC1/TableS1.xlsx

Table S2. Muv penetrance in adult animals

	% Muv adults[a]				Fig. ref.
	20°C	***n***	**23°C**	***n***	
N2	0.00[b]	399	0.00	402	2B
cdk-8(tm1238)	0.72[b]	835	0.70	1278	
cic-1(tm3740)	1.23[c]	163	0.83	121	
cdk-8(tm1238); cic-1(tm3740)			1.33	600	
lin-15A(n767)	0.00[b]	151	0.00	152	
cdk-8(tm1238); lin-15A(n767)	13.91[b]	151	29.41	119	
cic-1(tm3740); lin-15A(n767)	12.56[c]	207	28.40	162	
lin-15B(n744)	0.00[b]	164	0.00	109	
cdk-8(tm1238); lin-15B(n744)	6.58[b]	76	5.84	154	
N2 + EV[1] RNAi	0.10[b]	955	0.00	499	
N2 + *trr-1* RNAi	0.00[b]	743	0.66	455	
cdk-8(tm1238) + EV RNAi	0.90[b]	666	1.53	589	
cdk-8(tm1238) + *trr-1* RNAi	2.48[b]	968	4.14	798	
gap-1(ga133)	0.00[b]	931	0.00	148	
cdk-8(tm1238); gap-1(ga133)	9.91[b]	111	8.41	440	
Sib: *cdk-8(tm1238); lin-15A(n767); Ex[cdk-8(+)]*			60.67	89	3E
Transgenic: *cdk-8(tm1238); lin-15A(n767); Ex[cdk-8(+)]*			7.05	227	
Sib: *cdk-8(tm1238); lin-15A(n767); Ex[lin-31P::cdk-8]*			39.47	76	
Transgenic: *cdk-8(tm1238); lin-15A(n767); Ex[lin-31P::cdk-8]*			8.24	85	
Sib: *cdk-8(tm1238); lin-15A(n767);Ex[dpy-7P::cdk-8]*			28.51	228	
Transgenic: *cdk-8(tm1238); lin-15A(n767);Ex[dpy-7P::cdk-8]*			22.64	212	
cdk-8(tm1238); lin-15A(n767); Ex[cdk-8(KD)]	41.46[c]	41			4A
N2 + *lin-1* RNAi	5.38[b]	1505	7.29	905	4B
cdk-8(tm1238) + *lin-1* RNAi	27.19[b]	526	41.46	410	
mdt-12(os38)			49.32	296	5A
mdt-13(mn19)	20.92[b]	196	62.59	425	
mdt-13(mn19); lin-15A(n767)			72.55	459	
lin-15B(n374)			0.00	many	
mdt-13(mn19); lin-15B(n374)			69.70	99	
lin-1(n1790)	10.04[b]	259	1.55	192	5C
mdt-13(mn19); lin-1(n1790)	96.97[b]	66	95.45	44	
N2 + *mdt-15* RNAi	0.00[b]	395			6A
N2 + *mdt-27* RNAi	0.00[c]	641			
N2 + *mdt-29* RNAi	0.24[c]	414			
N2 + *mdt-1.1* RNAi	0.59[c]	338			

	% Muv adults[a]				Fig. ref.
	20°C	***n***	**23°C**	***n***	
mdt-13(mn19) + mdt-15 RNAi	13.28[c]	256			6A
mdt-13(mn19) + mdt-27 RNAi	15.94[c]	640			
mdt-13(mn19) + mdt-29 RNAi	9.09[c]	198			
mdt-13(mn19) + mdt-1.1 RNAi	30.54[c]	203			

[a] % Adults with ectopic vulval protrusion.
[b] No significant difference *vs.* Muv penetrance in L4 animals at 20°C (Table 1), Fisher's exact test.
[c] L4 Muv penetrance not determined, no statistical comparison.
** $p < 0.01$ *vs.* Muv penetrance in L4 animals at 20°C (Table 1), Fisher's exact test.
EV, Empty Vector.

Table S3. Relative mRNA expression of synMuv genes in *cdk-8* mutants *vs.* wild type as determined by microarray.

CLASS A			CLASS B			CLASS C			RTK-Ras		
Gene	Average fold change	*n* probes if >1	Gene	Average fold change	*n* probes if >1	Gene	Average fold change	*n* probes if >1	Gene	Average fold change	*n* probes if >1
lin-8	1.04		*dpl-1*	1.20		mys-1	1.12		ark-1	0.99	
lin-15a	1.02		*efl-1*	1.16		epc-1	1.01	2	gap-1	0.92	
lin-38	0.98	3	*gei-4*	0.94	7	ssl-1	0.87		sli-1	0.91	
lin-56	1.18		*hda-1*	1.01		trr-1	1.18	3			
smo-1	0.91	2	*hpl-2*	1.05	2						
uba-2	0.78		*let-418*	1.20	2						
			lin-9	1.17	3						
			lin-13	1.23	3						
			lin15b	0.98							
			lin-35	1.17							
			lin-36	1.13	2						
			lin-37	1.01							
			lin-52	1.07							
			lin-53	1.18							
			lin-54	1.19							
			lin-61	0.95	2						
			lin-65	0.99	2						
			mep-1	1.01							
			met-2	1.17							
			tam-1	1.23	2						
			tra-4	0.81	2						
			ubc-9	0.81	2						
			E01A2.4	1.16							
			W01G7.3	0.81							

Table S4. *C. elegans* strains.

Strain	Genotype
XA7703	*cdk-8(tm1238) I*
MH17	*sur-2(ku9) I*
MT10430	*lin-35(n745) I*
HS310	*let-19(mn19)/mIn1[dpy-10(e128) mIs14] II*
PS295	*let-23(sy97) unc-4(e120)/mnC1[dpy-10(e128) unc-52(e444)] II*
HS588	*mpk-1(oz140) unc-32(e189)/hT2[bli-4(e937) let-?(q728) qIs48] III*
MT301	*lin-31(n301) II*
STE13	*cic-1(tm3740) III*
WU125	*lin-1(n1790) IV*
HS445	*dpy-22(os38) X; osEx89[dpy-22(+)]*
MT1806	*lin-15A(n767) X*
MT2495	*lin-15B(n744) X*
AH12	*gap-1(ga133) X*
STE74	*cdk-8(tm1238) I; lin-15A(n767) X*
STE75	*cic-1(tm3740) III; lin-15A(n767) X*
HS432	*let-19(mn19)/mIn1[dpy-10(e128) mIs14] II; lin-15A(n767) X*
STE76	*cdk-8(tm1238) I; lin-15B(n744) X*
HS433	*let-19(mn19)/mIn1[dpy-10(e128) mIs14] II; lin-15B(n374) X*
STE77	*cdk-8(tm1238) I; gap-1(ga133) X*
MT309	*lin-15AB(n309) X*
STE78	*cdk-8(tm1238) I; let-23(sy97) unc-4(e120)/mnC1[dpy-10(e128) unc-52(e444)] II*
STE79	*cdk-8(tm1238) I; lin-1(n1790) IV*
HS510	*let-19(mn19)/mIn1[dpy-10(e128) mIs14] II; lin-1(n1790) IV*
HS356	*sur-2(ku9) I; let-19(mn19)/mIn1[dpy-10(e128) mIs14] II*
GS3582	*arIs92[egl-17p::NLS-CFP-LacZ + unc-4(+) + ttx-3::GFP]; unc-4(e120) II*
PS4308	*syIs107[unc-119(+) + lin-3ACEL(delta-pes-10)::GFP]; unc-119(ed4)* III
GS5096	*arEx1098[lag-2p(min)::2nls-yfp::unc-54 3'UTR + pha-1(+)]; pha-1(e2123) III*
AH142	*zhIs4[lip-1p::GFP]*
VH1976	*hdEx508[cdk-8P::GFP]*
STE80	*steEx43[cdk-8(+) + myo-2P::mCherry]*
STE81	*cdk-8(tm1238) I; arIs92[egl-17p::NLS-CFP-LacZ + unc-4(+) + ttx-3::GFP]*
STE82	*cic-1(tm3740) III; ayIs4[egl-17p::GFP + dpy-20(+)]*
STE83	*cdk-8(tm1238) I; steEx43[cdk-8(+) + myo-2P::mCherry]*
STE84	*cdk-8(tm1238) I; lin-15A(n767) X; steEx43[cdk-8(+) + myo-2P::mCherry]*
STE85	*cdk-8(tm1238) I; syIs107[unc-119(+) + lin-3ACEL(delta-pes-10)::GFP]*
STE86	*lin-15A(n767) X; arEx1098[lag-2p(min)::2nls-yfp::unc-54 3'UTR + pha-1(+)]*
STE87	*cdk-8(tm1238) I; lin-15A(n767) X; arEx1098[lag-2p(min)::2nls-yfp::unc-54 3'UTR + pha-1(+)]*
STE88	*cdk-8(tm1238) I; zhIs4[lip-1p::GFP]*
STE90	*steEx45[lin-31P::cdk-8 + myo-2P::mCherry]*
STE91	*steEx46[dpy-7P::cdk-8 + myo-2P::mCherry]*

STE92	*steEx47[cdk-8P::cdk-8(KD)::cdk-8-3'UTR + myo-2P::mCherry]*
STE93	*cdk-8(tm1238) I; lin-15A(n767) X; steEx45[lin-31P::cdk-8 + myo-2P::mCherry]*
STE94	*cdk-8(tm1238) I; lin-15A(n767) X; steEx46[dpy-7P::cdk-8 + myo-2P::mCherry]*
STE95	*cdk-8(tm1238) I; lin-15A(n767) X; steEx47[cdk-8P::cdk-8(KD)::cdk-8-3'UTR + myo-2P::mCherry]*
STE96	*arIs92[egl-17p::NLS-CFP-LacZ + unc-4(+) + ttx-3::GFP]; let-19(mn19)/mIn1[dpy-10(e128) mIs14] II*
STE97	*cdk-8(tm1238)/hT2[bli-4(e937) let-?(q728) qIs48] I; mpk-1(oz140) unc-32(e189)/hT2[bli-4(e937) let-?(q728) qIs48] III*
HS618	*let-19(mn19)/mIn1[dpy-10(e128) mIs14] II; mpk-1(oz140) unc-32(e189)/hT2[bli-4(e937) let-?(q728) qIs48] III*

Table S5. Primers used in this study.

Gene	Purpose	Primers (F/R)	Sequences (F/R)	Destination Vector
cdk-8	cloning SPD732	SP2215 / 2220	CCTGGAATTAAATTTAAAACTCTTTTCAG / ATTTTATTGTGAACGTATTTCAAAAAAATAC	PCR Blunt II TOPO
cdk-8	cloning SPD793	SP2533 / 2534	ctcgagATGACGTAAGTTGGAAATTTGC / gcggccgcATTTTATTGTGAACGTATTTCAAAAAAATAC	pB255
cdk-8	cloning SPD772	SP2436 / 2437	ggtaccATGACGTAAGTTGGAAATTTGC / cttaagATTTTATTGTGAACGTATTTCAAAAAAATAC	pPD95.77
dpy-7P	cloning SPD772	SP2444 / 2445	aagcttCATCTCTTCTCGTTTGGAATC / ggatccAAGAACATGGATTGTAGAAAACG	pPD95.77
cdk-8	site-directed mutagenesis SPD789	SP2497 / 2498	GTAAAAATTGCTGcTTTGGGATTTTC / CCTAGAATTATTATTTCATATTATCGC	PCR Blunt II TOPO
cdk-8	qPCR	SP127 / 128	CTGCATGCAGAGAAATTGCTC / TGATAACGTGCCAGAGATCG	
lin-35	qPCR	SP1824 / 1825	CGAGACGAACTTGGAAGACC / CCAGCATTGTGATTTTGCAC	
efl-1	qPCR	SP1826 / 1827	TGCTCCAGATGAAATGATGG / TCTCCGGTGCTCGAATAAAC	
dpl-1	qPCR	SP1957 / 1958	GCGGAAGAAGTCAAAACTGC / CGTATTGGGCTTGTGAGAGG	
mdt-15	qPCR	SP351 / 352	CACGACCCGGTCTTTCGTC / CTAGACCACCGCTTGTCTGG	
mdt-27	qPCR	SP2627 / 2628	CCCACAAACTTCGCCCAATG / AAGGGACCTGTGACTCAAAC	
mdt-29	qPCR	SP1214 / 1215	TCTCGACGGGGAGGTTATG / TGTCGTCTTCGCATACTTTCC	
col-127	qPCR	SP1156 /1157	AGCTTGGTGCCAGTGTGAG / TGTGGGCAGTTGATTGGAG	
K03D3.2	qPCR	SP1196 / 1197	GGGTTGATGGAAAGGGAAAC / ACGACATTCTTGCCCTTGAG	
acd-2	qPCR	SP1507 / 1508	TGTGATGGAGCTGAAATTCG / TTCTGTCGACTCGTTTGGTG	
Y49G5A.1	qPCR	SP1511 / 1512	CCCATTGGCTTTTGAGTACAG / CGGTTTGGCAGTTTTTATCG	
fat-7	qPCR	SP317 / 318	TTTCCACCACACATTCCCAC / TCTTCACTTCCGTGATTGGC	
ftn-1	qPCR	SP992 / 993	TGGAAGAGCAGGTACATTCG / GCCGGCTCTCTTGATATTTG	
nlp-4	qPCR	SP1584 / 1585	CTCCAATCCGTGTTCCAGTT / ACTGAAAGACAAGTCTCTCTTCACAC	
ubc-2	qPCR ref.	SP1228 / 1229	AGGGAGGTGTCTTCTTCCTCAC / CGGATTTGGATCACAGAGCAGC	
tba-1	qPCR ref.	SP1230 / 1231	GTACACTCCACTGATCTCTGCTGACAAG / CTCTGTACAAGAGGCAAACAGCCATG	
act-1	qPCR ref.	SP621 / 622	GCTGGACGTGATCTTACTGATTACC / GTAGCAGAGCTTCTCCTTGATGTC	
cdk-8	genotyping	SP519 / SP520	GTTCGGCGAGAATTTGCCAC / GCCCTTACCGTCATCTGCTG	
cic-1	genotyping	SP861/ SP862	CATCGATTTCACTCAATTTCCA / TCGTAAAAACGCACAAACGA	