ROLES OF MEDIATOR SUBUNIT CDK-8 IN DEVELOPMENTAL AND PHYSIOLOGICAL RESPONSES IN *CAENORHABDITIS ELEGANS*

by

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B.Sc. hon., The University of Victoria, 2010

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Medical Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2016

Abstract

The Mediator complex is a conserved coregulator of RNA polymerase II transcription. Whereas some Mediator subunits are universally essential for transcription, others regulate specialized gene programs by interacting with sequence-specific transcription factors (TFs). Mediator's Cyclin dependent kinase 8 (CDK8) kinase module (CKM) consists of four subunits (CDK8, Cyclin C, MED12, MED13) and regulates transcription downstream of multiple cell signaling pathways. In addition, the CKM regulates other Mediator subunits, as CDK8-mediated phosphorylation promotes Mediator subunit turnover, at least in yeast. CKM subunits have been identified as human oncogenes or tumor suppressors, indicating that the CKM can modulate transcription in tumorigenesis. However, the roles of the CKM in animal development and physiology are less well understood, as its target TFs often remain undefined. Furthermore, whether the CKM regulates the activity of other Mediator subunits in metazoans remains unknown. In this dissertation, I investigated CKM interactions with TFs and other Mediator subunits in *Caenorhabditis elegans* development and physiology. Gene expression profiling of *C. elegans cdk-8* mutants implicated CDK-8 in regulation of epidermal growth factor receptor (EGFR)-Ras-extracellular signal-regulated kinase (ERK)-driven transcription and cadmium-responsive transcription. I showed that the CKM inhibits ectopic vulval cell fates downstream of the EGFR-Ras-ERK pathway, dependent on CDK-8 kinase activity. Mechanistically, the CKM inhibits EGFR-Ras-ERK pathway output by promoting transcriptional repression by the LIN-1/Elk1 TF, and by inhibiting transcriptional activation by the Mediator subunit MDT-15. Furthermore, *cdk-8* is required for post-transcriptional regulation of MDT-15. Therefore, the CKM restrains EGFR-Ras-ERK signaling in *C. elegans* development by regulating TF and

Mediator activity. I also studied *cdk-8* in the cadmium response. I showed that *cdk-8* is required for cadmium-inducible transcription and organismal cadmium resistance. Dissecting a modular cadmium-responsive promoter, *cdr-1*, I showed that *cdk-8* may cooperate with other factors known to regulate cadmium-responsive transcription: *mdt-15*, GATA-family TF *elt-2* and GATA elements, and a high zinc-activated (HZA) element. I speculate that CDK-8 promotes cadmium-inducible transcription by activating MDT-15, ELT-2, or an HZA-binding TF. In sum, *cdk-8* cooperates with distinct TFs, and can oppose or cooperate with the Mediator subunit *mdt-15,* to regulate EGFR-Ras-ERK-inducible *vs.* cadmium-inducible transcription.

Preface

Chapter 1

Portions of Chapter 1 have been published in the journal *Nucleic Acids Research* as a Survey and Summary review article: Grants J. M., Goh G. Y. S., Taubert S., "The Mediator complex of *Caenorhabditis elegans*: insights into the developmental and physiological roles of a conserved transcriptional coregulator" (Grants *et al.* 2015). As co-first author, I contributed substantially to designing the outline of the review, generating the figures, and writing the review.

Chapter 2

Chapter 2 was published in the journal *Genetics*: Grants J. M., Ying L. T. L., Yoda A., You C. C., Okano H., Sawa H., Taubert S., "The Mediator Kinase Module Restrains Epidermal Growth Factor Receptor Signaling and Represses Vulval Cell Fate Specification in *Caenorhabditis elegans*" (Grants *et al.* 2016). As first author, I designed and conducted and/or supervised all experiments, except EASE analysis, conducted by J. Ewbank (Centre d'Immunologie de Marseille-Luminy) and *mdt-12/dpy-22* experiments, conducted by A.Y., H.O, and H.S., and analyzed and interpreted the data. I was responsible for designing figures and writing the paper.

Chapter 3

Chapter 3 is a manuscript in preparation for submission to a peer reviewed journal*:* Grants J.M., Shomer N., Muhuri A., Goh, G.Y.S., and Taubert S., "The Mediator subunit *cdk-8* promotes cadmium-inducible transcription". As first author, I designed and conducted and/or supervised all experiments, except for oPOSSUM promoter analysis, which was conducted by Dr. Anthony Mathelier (Centre for Molecular Medicine and Therapeutics). I also analyzed and interpreted the data for all experiments, generated the figures, and will have written the paper.

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List of Abbreviations

CDK	Cyclin dependent kinase
cic-1	Cyclin C gene (*C. elegans*)
CCNC	Cyclin C gene (human)
cdr-1	Cadmium-responsive gene 1
CF1	Cleavage factor 1
CKM	CDK8 kinase module
Cryo-EM	Cryo-electron microscopy
CTD	C-terminal domain
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
GCN5L	Gcn5-like
GTF	General transcription factor
H2B	Histone 2B
H3S10P	Histone 3 serine 10 phosphorylation
H3K9me2	Hitone 3 lysine 9 dimethylation
H3K14Ac	Histone 3 lysine 14 acetylation
H3K27	Histone 3 lysine 27
HIF1α	Hypoxia-inducible factor 1 α
HNF4α	Hepatocyte nuclear factor 4 α
Hox	Homeobox
ID	Intellectual disability
IEG	Immediate early gene
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
MAPK	Mitogen activated protein kinase
mH2A	Macro-Histone 2A
mtl	Metallothionein
MUFA	Monounsaturated fatty acid
Muv	Multivulva
NHR	Nuclear hormone receptor
NICD	Notch intracellular domain
NuRD	Nucleosome remodeling and deacetylase complex
PKA	Protein kinase A
PPARγ	Peroxisome proliferator-activated receptor γ
Pol II	RNA polymerase II
P-TEFb	Positive transcription elongation factor b
PUFA	Polyunsaturated fatty acid
REST	RE1 silencing transcription factor
SEC	Super elongation complex
SEM	Standard error of the mean

SREBP1α	Sterol regulatory element binding protein 1α
TAF	TBP-associated factor
T-ALL	T cell acute lymphoblastic leukemia
t-BOOH	*Tert*-butyl hydrogen peroxide
TBP	TATA-binding protein
TF	Transcription factor
T/G-Mediator	TRRAP/GCN5L-Mediator
TGF-β	Transforming growth factor β
TR	Thyroid hormone receptor
TRRAP	Transformation/transcription domain-associated protein
UPR	Unfolded protein response
VPC	Vulva precursor cell
Vul	Vulvaless

Acknowledgements

First and foremost, I would like to thank my research supervisor, Dr. Stefan Taubert. Thank you, Stefan, for all your support and guidance throughout this process, for helping me to grow as a researcher, and for giving me the freedom to follow the scientific paths that interested me the most. I would also like to thank my supervisory committee members, Dr. Matthew Lorincz, Dr. Don Moerman, and Dr. Ann Rose, for their helpful guidance and encouragement all along the way, in matters concerning my research project and my career. I would also like to thank Dr. Fred Picard for his always cheerful support and encouragement. I also extend my sincere gratitude for the financial support I received from NSERC, CFRI, UBC, and the Vanier Canada Graduate Scholarship.

To the dear members of the Taubert lab: Thank you for making our lab such a wonderful, supportive, creative, and fun environment to work in. Thank you for taking the time to have countless conversations with me about my research project, in those times when talking to a cutout of 'Sydney' didn't cut it. A special thanks to those who worked tirelessly and cheerfully with me on my projects: Lisa Ying, Anik Muhuri, Naomi Shomer, Shirley Chen, Charlotte You, James Chen, and Amy Poon. Most of all, a big thanks to the Taubert lab members past and present who have become some of my dearest friends.

Last but not least, I would like to thank my family for believing in me, and for doing everything they can to encourage and support me. Thank you, Mom and Dad, for your unconditional love and support, and for making me the person I am today. Thank you, Chelsey, for being like a sister to me, and for caring about me so much. Thank you, Shawn, for being such an incredibly supportive, and adventurous partner throughout this journey. I love you all with all my heart.

For Mom and Dad.

Chapter 1: Introduction

1.1 Transcriptional regulation by the Mediator complex

1.1.1 The Mediator complex in initiation of basal and regulated transcription

RNA polymerase II (Pol II) transcribes all eukaryotic protein coding genes, as well as most non-coding RNAs. However, the Pol II enzyme requires a host of regulatory factors to initiate transcription. Initiation of basal transcription, *i.e.* transcription that occurs in the absence of a gene-specific transcription factor (TF), requires general TFs (GTFs), TFIIA, B, D, E, F, H, which bind to and unwind the promoter DNA and activate Pol II (Luse 2014), and the Mediator complex, a conserved eukaryotic multi-subunit transcriptional coregulator that stabilizes and/or activates several GTFs and Pol II (Malik and Roeder 2010; Poss *et al.* 2013; Allen and Taatjes 2015). Initiation of regulated transcription, *i.e.* gene-specific transcription, additionally requires sequence-specific TFs, which enhance Pol II recruitment to the promoter (Malik and Roeder 2010; Poss *et al.* 2013; Allen and Taatjes 2015). However, TFs cannot interact directly with Pol II, and instead engage transcriptional coactivators, such as Mediator, to influence Pol II function (Kornberg 2005).

Mediator was first identified in *Saccharomyces cerevisiae* (budding yeast) as a protein complex required for purified Pol II and GTFs to respond to a sequence-specific TF (Kelleher *et al.* 1990; Flanagan *et al.* 1991; Kim *et al.* 1994). Human Mediator was first identified as a protein complex associated with the thyroid hormone-bound thyroid hormone receptor (TR), a potent TF (Fondell *et al.* 1996). Like in yeast, human Mediator was required for TF-mediated activation of purified Pol II and GTFs (Fondell *et al.* 1996). Since its discovery, Mediator has been identified as the target of numerous TFs across eukaryotes (Borggrefe and Yue 2011; Poss

et al. 2013). Overall, Mediator is a critical player in transcription initiation, as it stabilizes and activates the basal transcription machinery, and provides a means of communication between TFs and Pol II in regulated transcription. The molecular interactions between Mediator and the transcription machinery and TFs will be described in greater detail in Section 1.1.3.

1.1.2 Structural and functional modules of the Mediator complex

The Mediator complex consists of 25-30 protein subunits, dependent on species (Bourbon 2008). Mediator subunits are organized into four modules (head, middle, tail and kinase modules) based on their structural connections and their functional roles within the complex. Structurally, the head module occupies a central position within the Mediator complex, and the tail and middle modules form extensive interfaces with opposite sides of the head module (Tsai *et al.* 2014) (Figure 1.1; see Table 1.1 for subunits assigned to each module). The fourth module, the cyclin dependent kinase 8 (CDK8) kinase module (CKM), associates reversibly with the rest of the Mediator complex (hereafter referred to as the 'core' Mediator), primarily via interaction with the middle module (Tsai *et al.* 2013).

Mediator's four modules perform different molecular functions in transcriptional regulation. Cryo-electron microscopy (cryo-EM) analysis and genetic studies suggest that the head and middle modules form the primary interface with Pol II, whereas the tail module binds TFs (Soutourina *et al.* 2011; Tsai *et al.* 2014). Mediator interaction with Pol II or TFs induce extensive conformational changes in the complex (Davis *et al.* 2002; Poss *et al.* 2013; Tsai *et al.* 2014), and some TFs can induce Mediator conformations that mimic the Pol II-Mediator complex (Näär *et al.* 2002; Tsai *et al.* 2014), suggesting a means by which TF binding to Mediator might affect its ability to recruit and activate Pol II. The CKM primarily contacts

Mediator's middle module (Tsai *et al.* 2013), and once bound, it can alter the activity of TFs or of the core Mediator itself by phosphorylation or by steric hindrance. Specifically, the CKM subunit CDK8 phosphorylates various TFs, leading to their activation or promoting their degradation (Borggrefe and Yue 2011; Poss *et al.* 2013) (see Section 1.1.3.5), and phosphorylates other Mediator subunits to influence their interactions with TFs or with the core Mediator (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014) (see Section 1.1.3.4). In addition, CKM binding to the core Mediator blocks the Pol II-Mediator interaction either by binding directly to Mediator's Pol II binding site, as observed in yeast Mediator (Tsai *et al.* 2013), or by allosterically inducing a conformational change in the Pol II binding site, as observed in human Mediator (Näär *et al.* 2002; Knuesel *et al.* 2009b) (see Section 1.2.1). In sum, Mediator's head, middle, tail and CKM modules perform distinct functions within the complex.

1.1.3 Distinct molecular roles of Mediator subunits

Individual Mediator subunits can bind to and/or modify Pol II, GTFs, chromatin modifiers, Mediator itself, or distinct TFs. In the case of Mediator subunit interactions with sequence-specific TFs, these interactions can modulate highly specialized transcriptional programs. The molecular roles ascribed to individual Mediator subunits are described below (summarized in Figure 1.2). The Mediator complex on the whole, or large multi-subunit interfaces therein, can also interact with factors in several of the classes listed below; however, details of these interactions are beyond the scope of this literature review (refer to (Malik and Roeder 2010; Poss *et al.* 2013; Allen and Taatjes 2015) and references therein for more information).

1.1.3.1 Mediator subunit interaction with Pol II

Mediator binds to Pol II, and structural analysis by cryo-EM suggests an extensive interface with Pol II at Mediator's head/middle module boundary (Davis *et al.* 2002; Tsai *et al.* 2014). However, disruption of Pol II contact with a single head module subunit can globally disrupt Pol II recruitment to promoters in yeast. The Med17 head module subunit binds directly to the Pol II subunit Rpb3 *in vivo*, and an *rpb3* mutation that decreases Rpb3-Med17 binding causes a 1.5-fold reduction in Pol II recruitment throughout the genome, as well as loss of Pol II recruitment to inducible genes (Soutourina *et al.* 2011). Thus, Med17 appears to play an important role in Pol II recruitment in basal and regulated transcription, by directly binding to Pol II's Rpb3 subunit. This is likely not, however, the only Mediator-Pol II contact that drives Pol II recruitment. Both yeast and human Mediator bind to the Pol II C-terminal domain (CTD) of the Rpb1/POLR2A subunit (Myers *et al.* 1998; Näär *et al.* 2002), although the Mediator subunit(s) responsible for CTD binding remain undefined. The CTD appears to be a key target of Mediator in regulated transcription, as Mediator fails to promote TF-stimulated transcription *in vitro* if the Pol II CTD is deleted (Myers *et al.* 1998). Therefore, Mediator subunits in addition to Med17 likely play critical roles in Pol II binding.

The CTD is a regulatory domain in the Rpb1/POLR2A Pol II subunit that consists of multiple copies of a heptapeptide repeat (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7; 26 copies in *S. cerevisiae*, 52 copies in human) that is subject to post-translational modifications that influence Pol II function. Of particular importance, Ser5 phosphorylation primarily occurs during Pol II transcription initiation, while Ser2 phosphorylation primarily occurs during Pol II elongation (Srivastava and Ahn 2015). The Mediator CKM subunit CDK8 has been identified as a Pol II CTD kinase in yeast and human cells. Yeast Cdk8 phosphorylates Ser5 of the CTD *in vitro*,

which appears to repress transcription by disrupting Pol II interaction with DNA (Hengartner *et al.* 1998). However, in yeast harbouring a CTD truncation mutation, Cdk8 activates transcription via CTD Ser5 phosphorylation. Specifically, CTD truncation derepresses transcription of a subset of retrotransposons in the yeast genome, which is associated with increased CTD Ser5 phosphorylation. Loss of *cdk8* in the CTD truncation mutant normalizes retrotransposon transcript levels and CTD Ser5 phosphorylation, indicating that Cdk8 activates transcription by promoting Ser5 phosphorylation, although whether Cdk8 phosphorylates Ser5 directly in this context remains undetermined (Aristizabal *et al.* 2015). Human CDK8 phosphorylates Ser2 and Ser5 of the CTD *in vitro*, which appears to promote thyroid hormone- and TR-activated transcription (Belakavadi and Fondell 2010). Thus, CDK8 is a conserved Pol II CTD kinase, but its effect on transcription activation *vs.* repression may be context dependent.

1.1.3.2 Mediator subunit interactions with GTFs, elongation and termination factors

Specific Mediator subunits regulate the activity or recruitment of GTFs and elongation factors, particularly those that influence the phosphorylation of the Pol II CTD. Two Mediator subunits directly modulate the activity or recruitment of a CTD Ser5 kinase, CDK7/Cyclin H, which is part of the multi-subunit GTF TFIIH. Human CDK8 negatively regulates TFIIH kinase activity. In an *in vitro* transcription assay, TFIIH potentiates transcription initiation by phosphorylating the Pol II CTD, but this effect is strongly inhibited by CDK8-mediated phosphorylation of Cyclin H. This effect appears to be relevant *in vivo*, as Cyclin H is phosphorylated in human cells in a CDK8-dependent manner. However, yeast Ccl1/Cyclin H does not appear to be phosphorylated by Cdk8, indicating that the regulatory relationship between CDK8 and TFIIH may be specific to higher organisms (Akoulitchev *et al.* 2000).

The Mediator head module subunit Med11 promotes promoter recruitment of TFIIH in yeast. Med11 binds to TFIIH by interacting directly with the TFIIH helicase subunit Rad3, driving TFIIH recruitment to promoters (Esnault *et al.* 2008). Mechanistically, TFIIH recruitment is critical for Pol II CTD Ser5 phosphorylation, via the Kin28/CDK7 CTD kinase subunit (Esnault *et al.* 2008). Conservation of a MED11-CDK7 interaction in metazoan systems, *e.g.* human cells, has not been explored. If this mechanism is indeed conserved, it would imply opposing action of two Mediator subunits, CDK8 and MED11, in the control of CDK7-mediated CTD Ser5 phosphorylation to regulate Pol II transcription initiation.

The Mediator middle module subunit MED26 promotes the switch from transcription initiation to elongation by interacting with the GTF TFIID during initiation, and recruiting the super elongation complex (SEC) to promote elongation. The SEC is a complex of multiple elongation factors, including the Pol II CTD Ser2 kinase CDK9/Cyclin T (Luo *et al.* 2012). TFIID and components of SEC appear to compete for a common binding site in the human MED26 N-terminus (Takahashi *et al.* 2011), suggesting that MED26 may act as a molecular switch to recruit Pol II initiation factors *vs.* elongation factors. The precise mechanistic implications of MED26-TFIID binding in transcription initiation remain unclear, as TFIID can bind to Mediator independently of MED26; however, MED26 siRNA depletion causes decreased Pol II CTD Ser5 phosphorylation (Takahashi *et al.* 2011), suggesting that MED26 may stabilize other components of the initiation complex, such as CDK7. MED26-SEC binding is critical for SEC recruitment to promoters and gene bodies, as well as for the Pol II transition to elongation. Specifically, Pol II occupancy in gene bodies and CTD Ser2 phosphorylation is disrupted in MED26 depleted cells (Takahashi *et al.* 2011). Notably, MED26 knockdown only affects the expression levels of a subset of genes (~10% of genes in the human embryonic kidney cell line

used by Takahashi *et al.*); however, the reason for this specificity remains unexplored. Overall, MED26 regulates the activity of initiation factors and elongation factors during transcription of a subset of human genes.

The Mediator CKM subunit CDK8 also promotes transcription elongation of serum- or hypoxia-inducible genes by recruiting CDK9-containing complexes in human cells. In the absence of serum stimulation or hypoxic conditions, many inducible genes are bound by Pol II that is paused in the early elongation stage. Recruitment of elongation factors, including CDK9, is critical for release of Pol II pausing upon serum stimulation or hypoxia (Donner *et al.* 2010; Galbraith *et al.* 2013). During serum stimulation, CDK8 is required to recruit the CDK9-containing positive transcription elongation factor b complex (P-TEFb), to promote Pol II CTD phosphorylation and enhance the transcription elongation rate. Mechanistically, P-TEFb binds to the free CKM or CKM-containing Mediator, but not to Mediator lacking the CKM, suggesting that P-TEFb may bind directly to the CKM (Donner *et al.* 2010). During hypoxia, CKM-containing Mediator is recruited to hypoxia-inducible genes by hypoxia inducible factor 1α (HIF1α), a key TF of the hypoxia response. At these hypoxia-inducible genes, CDK8 is required to recruit two CDK9-containing complexes, P-TEFb and the larger SEC (Galbraith *et al.* 2013). Overall, these studies demonstrate that CDK8 promotes transcription elongation in inducible gene expression responses that involve Pol II pausing.

Finally, the Mediator head module subunit Med18 is required for transcription termination at a subset of genes in yeast. Med18 is required for expression of ~16% of the yeast genome (Holstege *et al.* 1998). At these loci, Med18 localizes to the 5' and 3' gene ends, and is required for DNA looping (Mukundan and Ansari 2011, 2013), suggesting that Med18 facilitates contact between promoter-bound Mediator and the terminator region. Med18 recruits the

Cleavage Factor 1 (CF1) termination complex, in a DNA looping-dependent manner, thereby promoting Pol II transcription termination (Mukundan and Ansari 2011, 2013). Intriguingly, in murine embryonic stem cells, Mediator interacts with the cohesin complex, which is responsible for DNA looping (Kagey *et al.* 2010). Thus, Med18 may be a conserved regulator of DNA looping during transcription termination.

1.1.3.3 Mediator subunit interactions with chromatin modifiers

Several Mediator subunits interact with chromatin modifying enzymes to modulate transcription. The human CKM-Mediator, but not the core Mediator alone, recruits a histone acetyl transferase, Gcn5-like (GCN5L), and an associated scaffold protein, Transformation/transcription domain-associated protein (TRRAP), to form a larger complex referred to as 'T/G-Mediator' (Meyer *et al.* 2008). Histone acetyltransferases modify conserved lysine residues in histone tails, which promotes chromatin decondensation and recruitment of chromatin-binding proteins leading to transcriptional activation (Kouzarides 2007). GCN5L is often found as part of the TFTC or STAGA complexes (Wieczorek *et al.* 1998; Martinez *et al.* 1998); however, no other TFTC or STAGA complex members are part of T/G-Mediator (Meyer *et al.* 2008), suggesting that T/G-Mediator is a distinct complex. *In vitro,* T/G-Mediator phosphorylates histone 3 serine 10 (H3S10P), and acetylates histone 3 lysine 14 (H3K14Ac) in a H3S10P-dependent manner (Meyer *et al.* 2008). As CDK8 is required for phosphorylation of H3S10, these findings suggests that CDK8 stimulates GCN5L acetylatransferase activity via the H3S10 phosphorylation (Meyer *et al.* 2008; Knuesel *et al.* 2009a). Indeed, *CDK8* knockdown in cell culture causes a global decrease in the amount of histone 3 carrying S10P/K14Ac modifications (Meyer *et al.* 2008). As H3K14 acetylation marks transcriptionally active genes

(Kouzarides 2007), this suggests a mechanism in which CDK8 and GCN5L cooperate to activate transcription; however, the effect of *CDK8* knockdown on H3S10P/K14Ac levels at genes directly bound and activated by CDK8 remains to be examined.

The human CKM subunit MED12 recruits a chromatin modifying enzyme to repress gene expression. Specifically, MED12 binds to and recruits the G9a histone methyltransferase at regulatory elements targeted by the RE1 silencing transcription factor (REST), a TF that represses neuronal gene expression in non-neuronal cells (Ding *et al.* 2008). G9a dimethylates histone 3 lysine 9 (H3K9me2), which serves to repress REST target genes. MED12 knockdown or disease-associated loss-of-function mutations (see Section 1.1.4.1 for information on *MED12* disease mutations) disrupt G9a recruitment and H3K9me2-mediated repression of REST target genes (Ding *et al.* 2008). Taken together with the study on T/G-Mediator, these findings demonstrate that CKM subunits can activate or repress gene expression by activation or recruitment of a histone acetyltransferase or a histone methyltransferase, respectively.

Two Mediator tail module subunits, MED23 and MED25, interact with histone modifying enzymes to activate gene expression. Profiling of histone modification abundance in *MED23* null murine embryonic fibroblasts compared to wild type revealed that *MED23* is specifically required for histone 2B (H2B) ubiquitination at lysine 120 (Yao *et al.* 2015). MED23, as part of the Mediator complex, appears to recruit the H2B-ubiquitinating RNF20/40 and PAF complexes to chromatin, thereby promoting H2B ubiquitination (Yao *et al.* 2015). H2B ubiquitination is associated with enhanced Pol II chromatin binding and enhanced transcription (Yao *et al.* 2015), suggesting that MED23 can promote transcriptional activation via an epigenetic mechanism. At a promoter activated by the hepatocyte nuclear factor 4α (HNF4α), Mediator recruitment via the human MED25 subunit is critical for establishment or maintenance

of an open chromatin state (Englert *et al.* 2015). Specifically, *MED25* is required for recruitment of the CREBBP histone acetyltransferase, which acetylates histone H3 lysine 27 (H3K27), and for exclusion of the Polycomb repressive complex, which methylates H3K27 (Englert *et al.* 2015). Overall, these studies show that certain Mediator tail module subunits can influence recruitment of chromatin modifiers to promote transcriptional activation.

1.1.3.4 Intra-Mediator regulation by CDK8

In the yeast Mediator complex, the CKM negatively regulates several core Mediator subunits that activate transcription. The Med2, Med3, and Med15 tail module subunits activate highly similar target gene sets (van de Peppel *et al.* 2005), and appear to form a stable triad (Zhang *et al.* 2004). The CKM opposes transcriptional activation by the tail module triad, as the gene expression profiles of CKM mutants show a high degree of anti-correlation with the *med2/3/15* mutant profiles (van de Peppel *et al.* 2005). Mechanistically, Cdk8 phosphorylates Med3, which promotes ubiquitin-proteasome mediated degradation of Med2, Med3 and Med15, thereby inhibiting transcriptional activation by the tail module triad (Gonzalez *et al.* 2014). Furthermore, Cdk8-mediated phosphorylation of Med2 can inhibit a specialized gene expression program that responds to iron deficiency (van de Peppel *et al.* 2005); however, the precise molecular mechanism of this regulation remains undetermined. In addition, the CKM also opposes transcriptional activation by the head module subunit Med18 (van de Peppel *et al.* 2005), by mechanisms that remain to be identified. Overall, the CKM negatively regulates the activity of Mediator subunits in the tail and head modules in yeast; however, whether such intra-Mediator regulatory interactions occur in metazoans and affect *e.g.* animal development has not yet been tested.

1.1.3.5 Mediator subunit interactions with sequence-specific TFs

Mediator subunits regulate specific gene expression programs primarily by binding to sequence-specific TFs, thus linking the TF to the rest of the Mediator complex. Numerous molecular interactions between Mediator subunits and TFs have been identified, and the themes that emerge in these interactions are highlighted below. Note that many studies have identified molecular interactions between Mediator subunits and sequence-specific TFs in yeast (Poss *et al.* 2013); however, for the purposes of this literature review, I will only discuss those that relate to the themes highlighted below.

The human MED1 middle module subunit binds to numerous nuclear hormone receptor (NHR) TFs, including TR, HNF4α, vitamin D receptor, peroxisome proliferator-activated receptor γ (PPARγ), estrogen receptor, androgen receptor, glucocorticoid receptor, and others (Malik and Roeder 2010; Poss *et al.* 2013). These interactions are mediated by NHR ligand binding domains and MED1's LXXLL motifs (L: leucine, X: any amino acid), a motif conserved among several NHR coactivators (Malik *et al.* 2004). In keeping with a central and specific role for MED1 in NHR-mediated transcription, MED1 knockout murine fibroblasts show specific disruption of NHR-mediated transcription (Ito *et al.* 2000), and are deficient in adipocyte differentiation controlled by PPARγ (Ge *et al.* 2002).

In addition to MED1, the Mediator subunits MED14, MED25, and CDK8 also contain LXXLL motifs (Xie *et al.* 2015). Human MED14 and MED25 and bind to some of the same NHRs as does MED1 (Hittelman *et al.* 1999; Malik *et al.* 2002; Lee *et al.* 2007; Grøntved *et al.* 2010; Han *et al.* 2012). Interaction of *Drosophila* CDK8 with an NHR was recently described (Xie *et al.* 2015). Direct interaction between CDK8 and NHRs in other organisms has not yet

been explored, although the CKM-containing Mediator complex is required for Pol II and CDK9 recruitment during TR-activated transcription (Belakavadi and Fondell 2010). The LXXLL motifs of human MED25 are required for interaction with NHRs (Lee *et al.* 2007; Han *et al.* 2012); however, the requirement for the MED14 or CDK8 LXXLL motifs remains unexplored. Interestingly, in *C. elegans,* the MED1 homologs MDT-1.1 and MDT-1.2 do not appear to interact with NHRs, and instead the MED15 homolog MDT-15 interacts with multiple NHRs (Taubert *et al.* 2006; Arda *et al.* 2010). Yeast Med15 also interacts with NHR-like TFs (Thakur *et al.* 2009), suggesting that NHR-binding may be an ancient role of MED15 that has been replaced by MED1, MED14 and MED25 in higher organisms. In summary, human MED1 appears to act as the primary target of NHR TFs, a function which may in some cases be partially redundant with MED14, MED25, while *C. elegans* and yeast MED15 mediates NHR interactions.

The MED15 tail module subunit also binds to several TFs involved in lipid metabolism and stress responses. In human cells, MED15 binds to the sterol regulatory element binding protein 1α (SREBP1α), a cholesterol sensor and regulator of lipid metabolism genes (Yang *et al.* 2006). This interaction is evolutionarily conserved, as *C. elegans* MDT-15, binds to the SREBP homolog, SBP-1 (Yang *et al.* 2006). In addition, MDT-15 also binds to NHR-49, an NHR that regulates lipid metabolism gene expression (Taubert *et al.* 2006). In yeast, Med15 binds to an NHR-like like TF that senses cellular fatty acids, Oaf1 (Thakur *et al.* 2009; Näär and Thakur 2009). In addition to these interactions with TFs that regulate lipid metabolism genes, MED15 also binds to stress responsive TFs. During genotoxic stress, human MED15 binds to p73, a p53-family tumor suppressor protein, and is required for induction of p73 target genes involved in cell cycle arrest and apoptosis (Satija and Das 2015). *C. elegans* MDT-15 binds to SKN-1, a key

TF in an oxidative stress response, and is required for induction of some oxidative stress response genes (Goh *et al.* 2014). Finally, yeast Med15 binds to a TF that responds to multiple environmental stresses, Msn2 (Lallet *et al.* 2006), and an NHR-like TF that senses drugs, Pdr1 (Thakur *et al.* 2008). Therefore, MED15 is a coactivator of diverse TFs that respond to nutrition or stress signals. One report has shown that *Xenopus laevis* MED15 coactivates Smad-family TFs in the transforming growth factor β (TGF-β) signaling pathway (Kato *et al.* 2002), indicating that MED15 may also play important roles in development. See Section 1.4 for further discussion of MDT-15 functions in *C. elegans*.

The MED23 tail module subunit is an important coactivator of transcription downstream of the extracellular signal-regulated kinase (ERK) mitogen activated protein kinase (MAPK) signaling cascade. Murine MED23 binds to Elk1, a TF that is phosphorylated by ERK (Stevens *et al.* 2002). ERK phosphorylation of Elk1 occurs in response to growth factor or insulin signaling, and triggers Elk1-mediated transcription of immediate early response genes (IEGs), in a MED23-dependent manner (Wang *et al.* 2005, 2009). Many IEGs are TFs that activate gene expression cascades. For example, the IEG *Egr2* encodes a TF controlling an adipogenesis cascade, as it induces expression of the PPARγ adipogenic TF. Thus, due to their defect in Elk1-mediated IEG expression, MED23$^{-/-}$ murine fibroblasts exhibit defective differentiation to adipocytes (Wang *et al.* 2009). In summary, MED23 is a regulator of Elk1-mediated transcription, but this can have widespread effects on the activation of gene expression cascades downstream of Elk1 target genes. See Sections 1.3.1 and 1.4.3 for further discussion of *mdt-23* functions in the ERK signaling pathway in *C. elegans.*

MED23 is also required for transcription driven by the insulin signaling pathway-regulated TF FOXO1. A liver-specific *MED23* knockout mouse displays increased glucose

tolerance and insulin sensitivity, even when fed a high fat diet (Chu *et al.* 2014). *MED23* is required for Mediator recruitment and activated transcription of several FOXO1 target genes (Chu *et al.* 2014), suggesting that MED23 may also interact with FOXO1 in the insulin signaling pathway.

CKM subunits bind to and/or modify multiple TFs that act in diverse cell signaling pathways. Mammalian CDK8 phosphorylates several TFs to promote or inhibit their activity or abundance. Activation of Notch signaling triggers cleavage and nuclear translocation of the Notch intracellular domain (NICD), where it forms a complex with other nuclear proteins to activate gene expression. The NICD complex recruits CDK8, which phosphorylates NICD to promote ubiquitin-mediated degradation, thus dampening gene expression downstream of an important developmental pathway (Fryer *et al.* 2004). Similarly, CDK8-mediated phosphorylation promotes ubiquitination and proteasome-mediated degradation of SREBP1c, a key TF activated downstream of the insulin signaling pathway (Zhao *et al.* 2012). CDK8 also phosphorylates SMAD TFs that act downstream of the bone morphogenic protein and TGF-β signaling pathways. These phosphorylation events promote SMAD-driven transcriptional activation as well as ubiquitination and proteasome-mediated degradation of the SMAD factors, thus fine-tuning the gene expression response downstream of two developmental signaling pathways (Alarcón *et al.* 2009). Unlike NICD, SREBP1c, or SMADs, CDK8-mediated phosphorylation of STAT1, an effector TF of the interferon γ signaling cascade, activates STAT1-driven transcription (Bancerek *et al.* 2013). Finally, CDK8-mediated phosphorylation of the E2F1 TF suppresses its ability to inhibit β-catenin-driven transcription; thus, CDK8 indirectly promotes β-catenin activity, a key effector of the Wnt signaling pathway (Morris *et al.* 2008). Intriguingly, β-catenin also binds directly to the human MED12 CKM subunit, to recruit

Mediator during transcriptional activation (Kim *et al.* 2006); therefore, the CKM promotes β-catenin-driven transcription both directly and indirectly. Yeast Cdk8 also phosphorylates several TFs that act in MAPK signaling-mediated starvation or stress response pathways. Specifically, Cdk8 phosphorylates Ste12 and Phd1, which activate nutrient deprivation response genes, to promote their turnover in nutrient rich conditions (Nelson *et al.* 2003; Raithatha *et al.* 2012). Cdk8 also phosphorylates Msn2, a multistress-responsive TF, to promote its nuclear exclusion in the absence of stress (Chi *et al.* 2001). Thus, the CKM promotes or inhibits TF activity downstream of multiple cell signaling pathways in human cells and in yeast. See Section 1.3 for further discussion of CKM subunit roles in developmental signaling pathways of *C. elegans*.

1.1.4 Mediator subunit mutations in human disease

1.1.4.1 Developmental anomalies

Mutations in Mediator subunits have been identified in diverse human diseases (Spaeth *et al.* 2011). Several Mediator subunit mutations have been discovered in families or patients with neurodevelopmental syndromes. A *MED25* missense mutation was identified in a large consanguineous family with autosomal recessive axonal Charcot-Marie-Tooth motor and sensory neuropathy (Leal *et al.* 2009). A *MED17* missense mutation causing postnatal cerebellar atrophy and myelination defects was identified in a Caucasus Jewish population (Kaufmann *et al.* 2010). A chromosomal inversion was found to disrupt a *CDK8* paralog, *CDK19*, in a patient with microcephaly, intellectual disability and other congenital defects (Mukhopadhyay *et al.* 2010). In all of these cases, the underlying molecular mechanisms that cause neurodevelopmental defects remain poorly understood. *MED12* missense mutations cause three X-linked intellectual disability syndromes, Opitz-Kaveggia (also known as FG) syndrome, Lujan syndrome, or Ohdo

syndrome (Risheg *et al.* 2007; Schwartz *et al.* 2007; Vulto-van Silfhout *et al.* 2013). The molecular etiology of Opitz-Kaveggia and Lujan syndromes has been partially elucidated. Mutations found in both syndromes disrupt CDK8-mediated repression of genes induced by the sonic hedgehog signaling pathway, an important pathway in embryogenesis (Zhou *et al.* 2012). Furthermore, Opitz-Kaveggia and Lujan syndrome mutations disrupt MED12 interaction with the histone methyltransferase G9a at neuronal genes repressed by REST (Ding *et al.* 2008), suggesting that Opitz-Kaveggia and Lujan syndrome *MED12* mutations may deregulate neuronal gene expression in inappropriate cell types or at inappropriate times. Finally, a Lujan syndrome *MED12* mutation perturbs mRNA levels of some IEGs induced in the serum response network (Hashimoto *et al.* 2011). As a *MED23* missense mutation linked to intellectual disability in a large consanguineous Algerian family also disrupts serum response IEG expression (Hashimoto *et al.* 2011), this pathway appears to be a prominent target of Mediator action in neurological development (see commentary by (Goh and Grants 2012)).

Two Mediator subunit mutations have been identified in developmental defects of the cardiovascular system. Missense mutations or translocations disrupting a *MED13* paralog, *MED13L*, were identified in multiple patients with transposition of the great arteries, a congenital heart defect (Muncke *et al.* 2003). In addition, the *MED15* gene is located within the chromosome 22q11.2 region typically deleted in DiGeorge syndrome, which causes multiple developmental phenotypes including cardiac defects (Berti *et al.* 2001); however, as the DiGeorge syndrome deletion typically encompasses ~60 genes, a role for MED15 in cardiac development remains to be determined.

1.1.4.2 Cancers or tumors

Several CKM subunits are mutated or display altered expression in human tumors and cancers. The *MED12* CKM subunit gene harbours recurrent mutations in or near exon 2 in approximately 70% of uterine leiomyomas and 60% of breast fibroadenomas, benign tumors of the uterus and breast (Mäkinen *et al.* 2011; Lim *et al.* 2014). A uterine-conditional mouse model of a *MED12* exon 2 mutation suggests a gain-of-function or dominant-negative mode of action, as the *MED12* exon 2 mutant transgene can predispose wild-type mice to uterine leiomyoma (Mittal *et al.* 2015). Analysis of gene expression in *MED12* mutant *vs. MED12* wild-type tumors has revealed increased Insulin-like growth factor 2 mRNA in uterine leiomyomas (Di Tommaso *et al.* 2014), and increased estrogen receptor target gene expression in breast fibroadenomas (Lim *et al.* 2014), which could account for hyperplasia. *In vitro*, uterine leiomyoma-linked *MED12* mutations disrupt MED12 binding to Cyclin C, and abrogate CDK8 association with Mediator and its kinase activity (Turunen *et al.* 2014). A potential explanation for these conflicting findings is that these MED12 mutations may have a dominant negative effect on Cyclin C and/or CDK8 activity, and that Cyclin C or CDK8 repress an oncogenic pathway. Cyclin C has been identified as a haploinsufficient tumor suppressor in T-cell acute lymphoblastic leukemia (T-ALL), as the *CCNC* gene is subject to heterozygous deletions, leading to decreased *CCNC* mRNA expression in approximately 10% of T-ALL patients (Li *et al.* 2014). This deletion promotes transcriptional activation of Notch signaling target genes, as Cyclin C-CDK8 phosphorylates the NICD to promote its turnover (Fryer *et al.* 2004; Li *et al.* 2014). Furthermore, in a mouse xenograft model of hematopoeitic progenitor cells transduced with oncogenic Notch, heterozygous *CCNC* deletion accelerates T-ALL development (Li *et al.* 2014). CDK8 has been identified as a putative oncogene, as it is overexpressed in approximately

70% of colon cancers (Firestein *et al.* 2010) and 75% of a melanoma subtype that lacks the histone variant macro-H2A (mH2A) (Kapoor *et al.* 2010). CDK8 overexpression in colon cancer arises due to *CDK8* copy number amplification, and is critical for cellular proliferation driven by activation of Wnt signaling or the serum response (Firestein *et al.* 2008; Donner *et al.* 2010). CDK8 overexpression in melanoma is associated with loss of the repressive histone variant mH2A at the *CDK8* promoter, and although CDK8 is required for proliferation of melanoma cell lines, the underlying mechanism remains unknown (Kapoor *et al.* 2010). In sum, the CKM subunits MED12 and CDK8 appear to act as human oncogenes, while Cyclin C is a haploinsufficient tumor suppressor. Together, these findings suggest that the CKM plays an important, and perhaps dichotomous, role in human cancers.

MED1, a coregulator of many NHRs, is also overexpressed in hormone-dependent cancers. MED1 overexpression occurs in approximately 50% of estrogen receptor positive breast cancers (Zhu *et al.* 1999) and 50% of prostate cancers (Vijayvargia *et al.* 2007). The pathogenic role of MED1 overexpression in breast cancer has not been explored. However, MED1 is required for proliferation and/or survival of both androgen-dependent and androgen-independent prostate cancer cell lines (Vijayvargia *et al.* 2007), suggesting that overexpression of MED1 can potentiate androgen receptor signaling regardless of the presence of its ligand. Thus, in addition to MED12 and CDK8, the MED1 Mediator subunit appears to act as an oncogene in some malignancies.

Overall, the wide spectrum of disease phenotypes caused by mutations in distinct Mediator subunits supports the notion that some Mediator subunits influence specific gene expression programs. However, in many cases the pathogenic mechanisms underlying these

disease mutations remain poorly understood, underscoring the need to further study individual Mediator subunits and their *in vivo* functions.

1.2 Evolutionary conservation of Mediator

1.2.1 Mediator complexes of yeast and metazoans

Mediator is conserved across eukaryotes, both at the level of Mediator subunit homology and overall Mediator complex structure. The majority of Mediator subunits appear to have orthologs from yeast to humans. Some notable exceptions are MED23, MED25, MED26, MED28 and MED30, which are absent from yeast Mediator (Bourbon 2008), but are present in metazoans as well as some unicellular eukaryotes. In addition, the sequence conservation is very weak between yeast Med2, 3, and 5 and their putative metazoan orthologs, MED29, 27, and 24, respectively (Bourbon 2008); therefore, it remains to be determined if these subunits adopt similar functions within the yeast and metazoan Mediator complexes.

Structural comparison of yeast and human Mediator also shows a similar complex architecture. The modular organization of Mediator (see Section 1.1.2) is quite similar between yeast and human, as the head, middle and tail modules adopt the same relative positions (Tsai *et al.* 2014), and the CKM attaches to the core Mediator primarily via MED13 binding to the MED19 middle module subunit in both yeast and human Mediator (Tsai *et al.* 2014).

Three notable differences in Mediator structure can be identified between yeast and human. First, the Mediator subunits that are absent from or highly divergent in yeast Mediator form a much larger tail module in the metazoan Mediator, which makes additional contacts with the middle and head modules (Tsai *et al.* 2014): MED23, MED24, and MED25 form contacts at

the tail-middle module interface; and MED27-30 form contacts at the tail-head module interface (Figure 1.1). Second, human MED26, which is absent from yeast Mediator, can bind reversibly to MED19 in the middle module (Tsai *et al.* 2014). Finally, in both yeast and human Mediator, CKM and Pol II binding to the core Mediator appears to be mutually exclusive (Näär *et al.* 2002; Tsai *et al.* 2013), but for different structural reasons. The yeast CKM forms an extensive interface with the Mediator middle module, via both Med13 and Cdk8, which sterically blocks the Mediator's Pol II CTD binding site (Tsai *et al.* 2013). The human CKM forms less extensive contacts with the Mediator middle module, via MED13 only (Tsai *et al.* 2013), and instead causes structural rearrangements in the Mediator complex that alter the conformation of the Pol II binding site (Taatjes *et al.* 2002; Bernecky *et al.* 2011). Therefore, although yeast and metazoan Mediator share homology and structural similarity, key structural differences may influence Mediator's regulatory mechanisms, *e.g.* intra-Mediator regulatory interactions (see Section 1.1.3.4).

1.2.2 Mediator complex of *C. elegans*

Yeast and human Mediator have been studied extensively using *in vitro* transcription assays, *in vitro* structural studies, or gene expression analysis of cultured yeast or human cells. A few Mediator subunits have been studied in mouse models; embryonic lethality has often hampered *in vivo* studies (Stevens *et al.* 2002; Wang *et al.* 2005, 2009; Westerling *et al.* 2007; Li *et al.* 2014), although conditional knockout models are viable and can reveal Mediator subunit functions in specific tissues or life stages (Chu *et al.* 2014; Li *et al.* 2014). Several Mediator subunit mutations have been identified in diverse human diseases (see Section 1.1.4), indicating that disruption of specific Mediator subunits can have distinct consequences in complex, living

organisms. Thus, studying the *in vivo* phenotypes of individual Mediator subunit mutants in a metazoan model organism can shed light on Mediator subunit-specific roles in development and/or physiology.

The nematode worm *C. elegans* provides an ideal system in which to study Mediator subunit mutant phenotypes. The availability of a fully annotated genome, genome-wide RNA interference (RNAi) libraries, large-scale collections of promoter reporters, a set of 2000 completely sequenced *C. elegans* strains collectively harbouring nearly a million mutations, and thousands of additional mutations originating from individual labs and knockout consortia collectively provide some insight into Mediator subunit function in this metazoan (C. elegans Sequencing Consortium 1998; Kamath *et al.* 2003; Thompson *et al.* 2013). As such, many novel Mediator subunit roles have been identified in *C. elegans,* which I discuss in detail in Sections 1.3 and 1.4. Note that several *C. elegans* Mediator subunits are commonly referred to by their classical gene names (*e.g. mdt-12* as *dpy-22*, *mdt-13* as *let-19*); throughout this dissertation I preferentially use standardized '*mdt*' nomenclature (Bourbon *et al.* 2004), but I also provide classical *C. elegans* gene names where these are the most commonly used form (*e.g. mdt-12/dpy-22, mdt-13/let-19*).

Many cell signaling pathways, metabolic pathways, and most Mediator subunits are conserved in *C. elegans.* Thus, studies of *C. elegans* Mediator subunit mutants can provide insights into their roles in conserved developmental or physiological responses.

1.3 Mediator subunit roles in pathways of animal development

The power of genetic analysis in *C. elegans* and in particular of large-scale forward and

reverse genetic screens has brought to light the importance of some Mediator subunits in developmental signaling pathways. Here, I describe the developmental phenotypes associated with mutations in certain Mediator subunits in *C. elegans*, and the mechanisms by which these subunits regulate cell fate by modulating the epidermal growth factor receptor (EGFR)-Ras-extracellular signal-regulated (ERK)/mitogen activated protein kinase (MAPK) signaling pathway, the Wnt signaling pathway, or the cell cycle (Figure 1.3).

1.3.1 Epidermal growth factor receptor signaling

The EGFR-Ras-ERK pathway is a highly conserved signaling cascade that regulates cell proliferation and differentiation in eukaryotes (Yoon and Seger 2006; Avraham and Yarden 2011). Mutations in the EGFR signaling pathway cause several human developmental syndromes and underlie numerous human cancer types (Karnoub and Weinberg 2008; Tidyman and Rauen 2009). Several Mediator subunits modulate EGFR signaling pathway activity. Many of these regulatory roles were first discovered in studies on *C. elegans* development, often followed later by the description of similar regulatory mechanisms in human cancer contexts.

In *C. elegans*, the EGFR-Ras-ERK cascade consists of an EGF-like ligand, LIN-3*,* that binds to LET-23/EGFR to promote activation of a downstream cascade that includes LET-60/Ras, LIN-45/Raf, and MPK-1/ERK MAPK (Moghal and Sternberg 2003b). ERK targets include the Ets-family TF LIN-1, the FoxB TF LIN-31, and the homeobox TF LIN-39 (Moghal and Sternberg 2003b). The *C. elegans* EGFR-Ras-ERK signaling pathway directs the development of several tissues, including the vulva precursor cells (VPCs), the excretory cell (a tissue analogous to the mammalian kidney), male mating structures (spicules, hook), and others

(Moghal and Sternberg 2003b). Thus, the EGFR signaling pathway is broadly required for *C. elegans* development.

The hermaphrodite vulva is perhaps the best-studied organogenesis paradigm in *C. elegans* (Sternberg 2005; Félix and Barkoulas 2012). In the nascent vulva, EGFR and Notch signaling direct three of six equivalent vulva precursor cells (VPCs) to adopt a vulval cell fate, while the remaining VPCs adopt a hypodermal fate. The cell fate decision of VPCs is exquisitely sensitive to perturbations in the EGFR-Ras-ERK pathway: increased EGFR signaling activity promotes additional VPCs to adopt the vulval cell fate, leading to a multivulva phenotype with ectopic non-functional pseudovulvae; *vice versa*, reduced EGFR signaling activity inhibits the vulval cell fate and leads to a vulvaless phenotype (Sternberg 2005). These visible phenotypes have been used very effectively to identify positive and negative regulators of EGFR signaling, including several Mediator subunits.

Mediator plays a critical role in activating EGFR-Ras-ERK signaling-driven developmental events in *C. elegans*, as two Mediator tail module subunits were identified in forward genetic screens for positive regulators of vulva development (Tuck and Greenwald 1995; Singh and Han 1995): *mdt-23/sur-2*, and the divergent MED24 ortholog *lin-25*/*mdt-24* (Bourbon 2008). Both *mdt-23/sur-2* and *mdt-24/lin-25* are essential for vulva development in *C. elegans*, as mutants display a completely penetrant vulvaless phenotype. These subunits act downstream of Ras, as loss of *mdt-23/sur-2* or *mdt-24/lin-25* suppresses the multivulva phenotype of *let-60/Ras* gain-of-function mutants (Tuck and Greenwald 1995; Singh and Han 1995). Similarly, the Mediator head module subunit *mdt-6* may also be a positive regulator downstream of Ras, as depletion of *mdt-6* partially suppresses the multivulva phenotype of *let-60/Ras* gain-of-function (Kwon and Lee 2001).

The molecular mechanisms of *mdt-23/sur-2* action in EGFR-driven vulva development have been partially elucidated, and they involve the Notch ligand *lag-2*, a key target gene of EGFR signaling (Chen and Greenwald 2004; Zhang and Greenwald 2011). In cells not receiving an EGF signal, LIN-1/Ets represses the *lag-2* promoter through the repressive element VPCrep; in contrast, in VPCs with activated EGFR signaling, the activating element VPCact promotes *lag-2* expression. Together, these mechanisms restrict *lag-2* expression to the appropriate VPCs. Loss of *mdt-23/sur-2* strongly decreases VPCact activity in cells with active EGFR signaling, suggesting that MDT-23 is a critical coactivator at this element. To date, the TF(s) that partners with MDT-23 to activate VPCact has not yet been identified (Zhang and Greenwald 2011). Interestingly, in murine cells, MED23 associates directly with the Ets-family TF Elk-1 to coactivate serum response genes (Stevens *et al.* 2002). However, at the *C. elegans lag-2* promoter, the Elk-1 ortholog LIN-1/Ets acts exclusively as a repressor through VPCrep (Zhang and Greenwald 2011), suggesting that MDT-23 may not cooperate with LIN-1/Ets to activate this particular EGFR target.

mdt-23/sur-2 and *mdt-24/lin-25* are required for additional developmental events regulated by the EGFR-Ras-MAPK cascade (Nilsson *et al.* 2000), suggesting that these Mediator subunits are generally required downstream of Ras. However, *mdt-23/sur-2* and *mdt-24/lin-25* do not appear to be required in certain developmental phenotypes or processes (*e.g.* body transparency, oogenesis) wherein LET-60/Ras is activated by an alternate receptor tyrosine kinase (RTK), such as fibroblast growth factor receptor or others (Nilsson *et al.* 2000; Schutzman *et al.* 2001). The mechanism for this specificity remains unclear, but likely depends on interactions between Mediator subunits and specific downstream TFs.

In contrast to *mdt-6*, *-23*, and *-24*, the Mediator CKM subunit *mdt-12/dpy-22* was identified in a forward genetic screen as a negative regulator of vulva development (Moghal and Sternberg 2003a). Reduction-of-function mutations in *mdt-12/dpy-22* cause a low penetrance multivulva phenotype in wild-type worms and strongly enhance the penetrance of the multivulva phenotype caused by heterozygous gain-of-function mutations in *let-23/EGFR* or *let-60/Ras*. *mdt-12/dpy-22* acts downstream of EGFR, as reduction-of-function of *mdt-12/dpy-22* partially suppresses the vulvaless phenotype of a *let-23/EGFR* reduction-of-function mutant (Moghal and Sternberg 2003a). Beyond this, the position of *mdt-12/dpy-22* activity in the EGFR-Ras-MAPK pathway has not been mapped further, although an MDT-12::GFP fusion protein is expressed in the VPCs (Moghal and Sternberg 2003a), suggesting a function in this tissue, perhaps at the level of the effector TFs LIN-1 or LIN-31. Alternatively, MDT-12/DPY-22 could repress ectopic expression of the EGF ligand in the surrounding hypodermis (skin), as do several other transcriptional coregulators (Fay and Yochem 2007), or could repress the Notch signaling pathway, which acts genetically downstream of the EGFR signaling pathway in vulva development (Chen and Greenwald 2004).

mdt-23/sur-2 and *mdt-24/lin-25* promote, whereas *mdt-12/dpy-22* inhibits vulva development, an organogenesis event driven by EGFR-Ras-ERK signaling. Thus, it would be informative to conduct genetic epistasis analyses to determine whether these Mediator subunits regulate each other's activity in this pathway. As noted above (Section 1.1.3.4), such epistatic relationships have been observed in yeast between the kinase module subunit CDK8 and the tail module subunits Med2, Med3, and Med15 (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014); it will be of interest to determine whether a similar relationship exists in metazoans. Furthermore, other Mediator subunits might also regulate EGFR signaling, and the *C. elegans* vulva provides

an excellent system to explore this possibility. In particular, since the kinase module subunit *mdt-12/dpy-22* has already been implicated in vulva development (Moghal and Sternberg 2003a), the other CKM subunits, *cdk-8, cic-1/Cyclin C*, and *mdt-13/let-19* are promising candidates for additional negative regulators of the pathway.

1.3.2 Wnt signaling

The Wnt signaling pathway is an important regulator of cell fate and of proliferation and self-renewal of stem and progenitor cells (Angers and Moon 2009). Reduction of Wnt signaling causes degenerative diseases such as syndromic osteoporosis, whereas increased Wnt signaling underlies multiple human cancers including colon cancer and melanoma (Clevers and Nusse 2012). Several Mediator subunits regulate canonical Wnt signaling in *C. elegans* development.

The components of the canonical Wnt signaling pathway are conserved from *C. elegans* to humans. In the absence of a Wnt signal, a degradation complex consisting of Axin, Disheveled, APC, GSK3β, and CK1 homologs phosphorylates the transcriptional coactivator BAR-1/β-catenin, thus targeting it for ubiquitination and proteasomal degradation (Korswagen 2002). The *C. elegans* genome encodes five Wnt ligands and four Frizzled receptors. Binding of ligand to receptor triggers recruitment of the BAR-1/β-catenin degradation complex to the plasma membrane, allowing unphosphorylated BAR-1/β-catenin to accumulate and enter the nucleus, where it coactivates transcription driven by the TCF/LEF family TF POP-1 (Korswagen 2002). This canonical Wnt signaling pathway regulates various aspects of *C. elegans* development, including VPC competence and polarity, neuroblast migration, P12 ectoblast cell fate specification, and generation of seam cell-derived structures (*e.g.* male tail rays). In addition,

a divergent canonical Wnt signaling pathway, involving the β-catenin orthologs WRM-1 and/or SYS-1, regulates asymmetric cell division (Korswagen 2002).

In the *C. elegans* vulva, *mdt-12/dpy-22* and *mdt-13/let-19* regulate cell fusion events controlled by BAR-1/β-catenin (Yoda *et al.* 2005). In the VPCs, BAR-1/β-catenin inhibits cell fusion by inducing the homeobox (Hox) TF *lin-39,* which prevents fusion of the VPCs with the surrounding hypodermal tissue (Clark *et al.* 1993). Loss of *mdt-12/dpy-22* or *mdt-13/let-19* inhibits VPC fusion and suppresses the ectopic cell fusion phenotype of *bar-1/β-catenin* mutants. However, loss of *mdt-12/dpy-22* or *mdt-13/let-19* does not suppress the ectopic cell fusion phenotype of *lin-39* mutants (Yoda *et al.* 2005). Thus, *mdt-12/dpy-22* and *mdt-13/let-19* apparently act parallel to or downstream of *bar-1/β-catenin*, but upstream of *lin-39* to promote cell fusion.

Forward genetic screens additionally identified several Mediator subunits as regulators of Wnt-orchestrated developmental events such as cell fate patterning of *C. elegans* male tail rays. This process is governed by the Hox TFs *pal-1/Caudal, mab-5/Antennapedia*, and *egl-5/Abdominal-B*. The Mediator CKM subunit *mdt-12/dpy-22* and the middle module subunit *mdt-1.1* are required to prevent aberrant activation of this Hox TF cascade by BAR-1/β-catenin (Zhang and Emmons 2000, 2001). Specifically, these Mediator subunits are required to repress aberrant BAR-1/β-catenin-mediated transcriptional activation of *pal-1/Caudal* and of *egl-5/Abdominal-B* (Zhang and Emmons 2000, 2001). Conversely, the Mediator head module subunit *mdt-6* appears to promote the aberrant BAR-1/β-catenin-driven *pal-1* activation that arises in *mdt-12/dpy-22* mutants (Kwon and Lee 2001). Thus, as seen in yeast (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014) and as suggested for Mediator subunit action in EGFR-driven development (Section 1.3.1), functional opposition between Mediator subunits may occur in the

Wnt signaling pathway. Interestingly, the Mediator subunit *mdt-23/sur-2* is not required for BAR-1/β-catenin–dependent transcriptional regulation of *pal-1* (Zhang and Emmons 2001), demonstrating that regulation of Wnt signaling is specific to a subset of Mediator subunits.

Mediator subunits also regulate an asymmetric cell division event mediated by divergent canonical Wnt signaling in *C. elegans.* Asymmetric division of the T blast cell, which determines whether the T cell daughters adopt a neural or hypodermal fate, requires *lin-44/Wnt* and *lin-17/Frizzled* to establish polarity of two asymmetrically expressed TFs, POP-1/TCF and TLP-1/Sp1 (Herman 2001; Zhao *et al.* 2002). The Mediator kinase module subunits *mdt-12/dpy-22* and *mdt-13/let-19* are required downstream of *lin-17/Frizzled* to establish the asymmetric expression of TLP-1/Sp1, but not of POP-1/TCF, in the T cell daughters (Yoda *et al.* 2005). Thus, *mdt-12/dpy-22* and *mdt-13/let-19* regulate a Wnt signaling-mediated asymmetric cell division event; however, MDT-12/DPY-22 and MDT-13/LET-19 proteins are symmetrically distributed in the T cell daughters (Yoda *et al.* 2005), suggesting that their activity as transcriptional coregulators may itself be subject to regulation by the Wnt pathway.

Although *mdt-12/dpy-22* and *mdt-13/let-19* regulate at least three developmental processes governed by Wnt signaling, they do not indiscriminately influence all Wnt-regulated developmental events. First, migration of the *C. elegans* QL neuroblast requires *egl-20/Wnt* and *bar-1/β-catenin*, but *mdt-12/dpy-22* and *mdt-13/let-19* mutants show no migration defects (Yoda *et al.* 2005). Second, endoderm induction requires a divergent canonical Wnt signaling pathway that acts through *wrm-1/β-catenin*; however, RNAi knockdown of *mdt-13/let-19* has no effect on endoderm development ((Yoda *et al.* 2005); note, however, that *mdt-13/let-19* knockdown efficiency was not reported). Finally, somatic gonad development requires *lin-17/Frizzled* and

pop-1/TCF, but *mdt-12/dpy-22* and *mdt-13/let-19* mutants display superficially normal somatic gonads (Yoda *et al.* 2005).

1.3.3 Cell cycle progression

Tight cell cycle regulation is essential for animal development, and certain Mediator subunits influence cell fate by regulating this process. Cellular quiescence (*i.e.* temporary or permanent exit from the cell cycle) is particularly important for specification of the VPC equivalence group in *C. elegans*. During normal vulva development, six VPCs arrest in an extended G1 phase that lasts from the first larval stage L1 until the time of their cell fate determination (vulval fate *vs.* hypodermal fate) in the third larval stage L3 (Euling and Ambros 1996). Disruption of this cell cycle quiescence expands the VPC equivalence group, producing more VPCs capable of adopting vulval fates. An elegant genetic screen for regulators of VPC quiescence revealed that multiple Mediator subunits contribute to cell cycle entry and/or exit (Clayton *et al.* 2008). Loss of the middle module subunit *mdt-1.1*, the tail module subunits *mdt-23/sur-2* or *mdt-24/lin-25*, or of the kinase module subunits *cdk-8*, *mdt-12/dpy-22*, or *mdt-13/let-19* prevents establishment or maintenance of VPC quiescence. Depletion of 13 additional Mediator subunits caused no observable effect on cell cycle quiescence, suggesting that only a subset of Mediator subunits are involved in this process (Clayton *et al.* 2008). Mechanistically, *mdt-13/let-19* was not required to express the Cyclin dependent kinase inhibitor *cki-1*, a key effector of VPC quiescence, implying that Mediator regulates the transcription of other, unknown genes involved in cell cycle quiescence (Clayton *et al.* 2008). The six Mediator subunits identified in this screen have been implicated as regulators of cell signaling pathways

(see Sections 1.3.1 and 1.3.2), suggesting that Mediator may integrate information from various cell signaling cascades to coordinate cellular quiescence in development.

1.3.4 Other developmental phenotypes of Mediator mutants

Besides the well-characterized roles described above, Mediator subunits participate in additional events in *C. elegans* development. The underlying molecular pathways are less well defined in these instances, yet these studies elegantly reveal differential Mediator subunit action during embryonic and post-embryonic development.

C. elegans provides an ideal system to study the contributions of Mediator subunits to gene-specific *vs.* general embryonic transcription, as maternal gene products allow embryos with null mutations in essential genes to survive to at least the 100-cell stage despite drastically perturbed transcription (Powell-Coffman *et al.* 1996). In this system, loss of the head module subunit *mdt-6*, the middle module subunits *mdt-7* or *mdt-10*, or the kinase module subunit *mdt-13/let-19* causes embryonic arrest at the ~300-cell stage, before cell differentiation and body morphogenesis begin (Kwon *et al.* 1999; Wang *et al.* 2004). Examination of transcriptional reporters demonstrated that the depletion of these Mediator subunits prevents the expression of some stage- or lineage-specific genes, but not of ubiquitously expressed genes (Kwon *et al.* 1999; Wang *et al.* 2004). In contrast, depleting *mdt-14,* which occupies a central position connecting the head, middle, and tail modules of Mediator (Figure 1.3), causes embryonic arrest at the 100-cell stage due to a broad loss of transcription as evidenced by loss of Pol II CTD phosphorylation (Shim *et al.* 2002). Thus, whereas many Mediator subunits appear to be required for specific developmental and differentiation programs in early embryogenesis, *mdt-14* is more broadly required for transcription. It would be interesting to conduct a systematic study to

determine the embryonic requirements for all Mediator subunits, and whether these requirements involve gene-specific or global transcriptional regulation.

Several processes during *C. elegans* larval development also depend on Mediator subunits. The tail module subunit *mdt-23/sur-2* was identified in a screen for genes required for muscle development, as *mdt-23/sur-2* depletion caused aggregation of muscle myosin *myo-3* in myofilaments (Meissner *et al.* 2009). Furthermore, *mdt-23/sur-2* and the kinase module subunits *cdk-8*, *cic-1/Cyclin C*, *mdt-12/dpy-22*, and *mdt-13/let-19* are required for correct navigation of ventral nerve cord axons (Steimel *et al.* 2013). The axon guidance defects of *cdk-8* mutants are suppressed by mutation of *sax-3/ROBO,* a receptor for Slit-family ligands that guide axon navigation, suggesting that the kinase module may inhibit the *sax-3/ROBO* pathway by molecular mechanisms that remain to be elucidated (Steimel *et al.* 2013).

1.3.5 Regulatory hub activity of MED12

Network theory can be used to analyze the patterns of genetic interactions in cells and organisms. Gene networks of various origins typically conform to a common format, wherein most genes interact with relatively few other genes, and a few genes form highly connected regulatory "hubs" (Barabási and Oltvai 2004). A high-throughput screen for pairwise genetic interactions in *C. elegans* revealed that the Mediator subunit *mdt-12/dpy-22* is one of six highly connected hubs, and the only Mediator subunit, that bridge multiple signaling pathways, including EGFR signaling, Notch signaling, Wnt signaling, and cell death/migration. In addition, *mdt-12/dpy-22* and the other candidate hub genes enhance the loss-of-function phenotype of genes involved in processes unrelated to cell signaling, indicating that *mdt-12/dpy-22* acts as genetic buffer to moderate the effect of genetic perturbations in an organism (Lehner *et al.*

2006). The fact that *MED12* is implicated in numerous and diverse human diseases may suggest that its role as a regulatory hub is evolutionarily conserved (Section 1.1.4). Thus, by interacting with diverse signaling pathways, and hence conferring a risk for diverse pathologies, human *MED12* may represent a regulatory hub as described for *C. elegans mdt-12* (Figure 1.4).

1.3.6 Parallels between Mediator roles in development and in cancer

1.3.6.1 Mediator subunit roles in EGFR signaling-driven tumorigenesis

In the last few years, a strong parallel has emerged between the roles of *mdt-23/sur-2* and *mdt-12/dpy-22* in EGFR signaling-regulated development in *C. elegans* and the roles of *MED23* and *MED12* in EGFR signaling-driven tumorigenesis in humans. *MED23* is required for the proliferation and tumorigenicity of human lung cancer cells with hyperactive Ras mutations, but not in cells lacking Ras mutations (Yang *et al.* 2012). Thus, just as observed decades earlier in *C. elegans* (Singh and Han 1995), loss of human *MED23* suppresses the effects of activated Ras, suggesting a potential role as a transcriptional coactivator downstream of Ras. Moreover, in human melanoma cells, loss of *MED12* promotes cellular resistance to chemotherapeutic agents that inhibit the ERK signaling pathway component BRAF (Shalem *et al.* 2014). This suggests that, like *C. elegans mdt-12/dpy-22* (Moghal and Sternberg 2003a), *MED12* inhibits ERK signaling in melanoma cells by acting downstream of BRAF, perhaps by corepressing downstream target genes. Lastly, the two Mediator subunits *MED12* and *CDK8* are mutated or amplified in human tumors (Firestein *et al.* 2010; Mäkinen *et al.* 2011; Lim *et al.* 2014), but their impact on EGFR signaling in these contexts have not been examined. Overall, the parallels between *C. elegans* development and human cancer indicate that further study of *C. elegans*

Mediator subunits' actions within the EGFR signaling pathway may yield clinically applicable findings for cancers bearing activating mutations in this signaling cascade.

1.3.6.2 Mediator repression of Notch signaling-driven tumorigenesis

The Mediator CKM subunit Cyclin C has recently been identified as a haploinsufficient tumor suppressor, via repression of Notch signaling-driven transcription, in T-ALL (Li *et al.* 2014) (see Section 1.1.4.2). Both Notch signaling and EGFR signaling are important players in *C. elegans* vulval cell fate determination (Sternberg 1988; Moghal and Sternberg 2003b; Chen and Greenwald 2004). The CKM subunit *mdt-12* represses vulval cell fates downstream of *let-23/EGFR* (Moghal and Sternberg 2003a). As requirements in EGFR and/or Notch signaling could potentially explain the vulval phenotypes of *mdt-12* mutants (see Section 1.3.1), further studies of *mdt-12* and other CKM subunits in EGFR- *vs.* Notch-induced vulval cell fates are needed to distinguish between these possibilities.

1.3.6.3 Mediator subunit roles in Wnt signaling-driven tumorigenesis

The involvement of *C. elegans* Mediator CKM subunits in Wnt/β-catenin signaling is intriguing, as human CDK8 regulates Wnt signaling in colon cancers. Specifically, CDK8 expression is upregulated in a substantial fraction of human colon cancer specimens, often due to copy number gain at the *CDK8* locus (Firestein *et al.* 2010), and this promotes Wnt/β-catenin-driven cell proliferation and tumorigenicity (Firestein *et al.* 2008; Morris *et al.* 2008) (see Sections 1.1.3.5 and 1.1.4.2). Thus, CDK8 promotes the oncogenic action of β-catenin in human colon cancer. These data contrast with the inhibitory role of *C. elegans* CKM subunits *mdt-12/dpy-22* and/or *mdt-13/let-19* in the Wnt/β-catenin signaling pathway in male tail patterning

and in VPC fusion (Zhang and Emmons 2000; Yoda *et al.* 2005). This may suggest evolutionary divergence of the CKM's role in Wnt signaling, or divergence of the roles of individual CKM subunits in Wnt regulation. Further work is needed to answer these questions, particularly in elucidating whether MDT-12/DPY-22 and MDT-13/LET-19 regulate BAR-1/β-catenin directly or indirectly in the context of cell differentiation and fusion.

1.3.6.4 Mediator interaction with Retinoblastoma tumor suppressor protein

The role of Mediator CKM subunits in cell cycle regulation in *C. elegans* parallels a function of a human MED13 paralog, MED13L. Specifically, the cellular senescence or cell cycle arrest induced by a constitutively active Retinoblastoma tumor suppressor protein (RB) mutant depends on MED13L (Angus and Nevins 2012). In this context, MED13L inhibits cell cycle progression by acting as a corepressor for RB/E2F, as loss of MED13L results in derepression of the RB/E2F target *Cyclin A* even in the presence of constitutively active RB (Angus and Nevins 2012). Similarly, in *Drosophila,* CDK8 acts in parallel to RB as a direct corepressor of E2F1 (Morris *et al.* 2008). Together, these data suggest that the Mediator CKM and RB may target common TFs to regulate the cell cycle. It would be interesting to explore this hypothesis further using the *C. elegans* VPC quiescence model, to determine whether the CKM subunits, and other Mediator subunits identified as regulators of cell cycle quiescence (Clayton *et al.* 2008) (see Section 1.3.3), cooperate with RB in this capacity.

1.4 Roles of the Mediator complex in physiological responses of adult animals

A growing number of studies have identified roles for the Mediator complex in regulating

adult physiology in *C. elegans*. Specifically, Mediator subunits have been identified as regulators of lipid metabolism, stress responses, and innate immune responses (Figure 1.3).

1.4.1 Lipid metabolism

The Mediator tail module subunit MDT-15 is a central regulator of lipid metabolism gene expression in *C. elegans.* MDT-15 was first identified as coactivator of two TFs with known roles in lipid metabolism, SBP-1/SREBP, a TF that activates fatty acid (FA) desaturation and synthesis genes, and NHR-49, an NHR that regulates FA desaturation and β-oxidation genes (Taubert *et al.* 2006; Yang *et al.* 2006). As such, *mdt-15* is a critical regulator of gene expression in FA homeostasis and energy metabolism, as outlined below.

mdt-15 mutants*,* like *nhr-49* or *sbp-1* mutants, lack certain monounsaturated fatty acid (MUFA) or polyunsaturated FA (PUFA) species (Taubert *et al.* 2006; Yang *et al.* 2006). *mdt-15* is required to activate transcription of the FA desaturase genes *fat-5, fat-6,* and *fat-7*, which are gene targets of SBP-1 and NHR-49 (Taubert *et al.* 2006; Yang *et al.* 2006). These enzymes catalyze the formation of carbon-carbon double bonds in saturated FAs, forming MUFAs, which also serve as substrates for the formation of PUFAs. Defects in FA desaturation are in part responsible for pleiotropic phenotypes in *mdt-15* mutants, *e.g.* short life span, sterility, uncoordinated locomotion, and morphological defects, as supplementation with unsaturated FAs can partially rescue these phenotypes (Taubert *et al.* 2006; Yang *et al.* 2006). However, the partial nature of this rescue is indicative of the fact that MDT-15 regulates other physiological processes in addition to lipid metabolism (see Section 1.4.2).

Precise regulation of FA desaturation is important for the maintenance of cellular and organelle membrane integrity or function. For example, loss of *mdt-15* causes activation of the

endoplasmic reticulum (ER) unfolded protein response (UPR). Although this pathway normally responds to protein misfolding, activation of the ER UPR in worms lacking *mdt-15* appears to be driven by changes in FA desaturation of the membrane phospholipid phosphatidylcholine (Hou *et al.* 2014). This suggests that FA desaturation plays a role in the maintenance of ER membrane integrity or function. In line with an important role for *mdt-15* in membrane lipid desaturation and homeostasis, an *mdt-15* gain-of-function allele suppresses cold sensitivity in a mutant background with aberrant membrane lipid saturation (Svensk *et al.* 2013), a known determinant of membrane fluidity and cold resistance. Intriguingly, several *nhr-49* gain-of-function alleles can also suppress this cold sensitivity phenotype by increasing membrane lipid unsaturated FA content (Svensk *et al.* 2013). The *mdt-15* and *nhr-49* gain-of-function mutations are in close proximity to domains in MDT-15 and NHR-49 that interact with each other (Taubert *et al.* 2006), suggesting that MDT-15 and NHR-49 may act together to promote membrane fluidity and cold resistance via regulation of membrane lipid FA composition.

FA desaturation is also important in the physiological response to a high glucose diet. A glucose-rich diet causes accumulation of FAs and substantially decreases *C. elegans* lifespan. *mdt-15* and *sbp-1* are required for survival on a high glucose diet, as *mdt-15(RNAi)* or *sbp-1(RNAi)* further shorten the lifespan of glucose-fed worms, and *mdt-15* gain-of-function mutation or *sbp-1* overexpression extend the lifespan of glucose-fed worms (Lee *et al.* 2015). In this context, MDT-15 and SBP-1 appear to decrease so-called 'glucolipotoxicity', as *mdt-15* and *sbp-1* are required to activate transcription of glucose-inducible FA desaturation enzymes *fat-2, -5, -6,* and *-7*, which detoxify the saturated FA by-products of a high glucose diet by converting them to MUFAs and PUFAs (Lee *et al.* 2015).

MDT-15 also regulates expression of several genes encoding β-oxidation enzymes in response to fasting, via both NHR-49-dependent and -independent mechanisms (Taubert *et al.* 2006). NHR-49-independent roles of MDT-15 in starvation-induced β-oxidation gene expression imply that MDT-15 coactivates additional TFs in this context. A recent study suggested that an MDT-15-binding stress-responsive TF, SKN-1 (see Section 1.4.2), may activate expression of β-oxidation enzyme genes in an *mdt-15*-dependent manner (Pang *et al.* 2014). Furthermore, additional MDT-15 binding TFs, *e.g.* several NHRs in addition to NHR-49 (Arda *et al.* 2010), may also have undiscovered roles in β-oxidation gene regulation.

1.4.2 Stress responses

MDT-15 also plays an important role in adaptive responses to environmental stresses, such as toxins, metals, or chemicals that cause oxidative stress. Microarray analysis of *mdt-15(RNAi)* worms revealed that, in addition to regulating many genes involved in lipid metabolism, *mdt-15* also regulates many genes involved in the metabolism and clearance of toxic substances (Taubert *et al.* 2008). Indeed, *mdt-15* is required for gene expression induced by the xenobiotics fluoranthene, b-napthoflavone, and RPW-24 (discussed further in Section 1.4.3), the physiological metal zinc, or the carcinogenic heavy metal cadmium (Taubert *et al.* 2008; Pukkila-Worley *et al.* 2014; Roh *et al.* 2015). The role of *mdt-15* in these detoxification responses appears to be specific, as *mdt-15* is not required for heat shock-inducible gene expression or thermotolerance (Taubert *et al.* 2008), for resistance to the proteotoxic stressor tunicamycin (Hou *et al.* 2014), or for inducible detoxification responses to certain pesticides (Jones *et al.* 2013). The specificity of *mdt-15* in certain detoxification gene expression responses suggests that MDT-15 interacts with specialized TFs to control gene expression responses to

xenobiotics or cadmium. However, the TF(s) that interact with MDT-15 in these contexts have not been identified to date.

Regulatory and physical interaction of MDT-15 with SKN-1/Nrf2, a conserved master regulator of detoxification, drives a gene expression response to the oxidative stressor sodium meta-arsenite (hereafter referred to as 'arsenite'). Specifically, MDT-15 binds directly to SKN-1, and is required for the induction of many SKN-1-dependent detoxification genes in response to arsenite exposure, and for arsenite resistance *in vivo* (Goh *et al.* 2014). Interestingly, MDT-15's role in this oxidative stress response is distinct from its role in FA desaturation, as unsaturated FA supplementation does not rescue *mdt-15* mutant arsenite sensitivity (Goh *et al.* 2014). Whether MDT-15 interaction with SKN-1 drives other detoxification responses, *e.g.* to fluoranthene or cadmium, remains to be determined.

MDT-15 also regulates a SKN-1-independent oxidative stress response induced by an organic peroxide, *tert*-butyl hydroperoxide (*t*-BOOH). Specifically, *mdt-15*, but not *skn-1*, is required for induction of many detoxification genes in response to *t*-BOOH exposure, and for *t*-BOOH resistance *in vivo* (Oliveira *et al.* 2009; Goh *et al.* 2014). MDT-15-binding NHRs (Taubert *et al.* 2006; Arda *et al.* 2010) may mediate this gene expression response, as *nhr-64* and *nhr-49* mutants display *t*-BOOH sensitivity (Goh *et al.* 2014). Taken together, these findings demonstrate that MDT-15 interaction with SKN-1 or NHRs can drive detoxification gene expression responses, but further work is needed to fully delineate these regulatory interactions.

Two additional Mediator subunits, the middle module subunit MDT-26 and the CKM subunit MDT-12/DPY-22, were identified in a reverse genetic screen for regulators of cytoprotective and detoxification responses that contribute to longevity. Namely, *mdt-26* is required for longevity of worms with decreased insulin signaling or dietary restriction, whereas

mdt-12/dpy-22 is required for longevity of worms with decreased mitochondrial function (Shore *et al.* 2012). These effects on longevity are thought to stem from requirements for *mdt-26* or *mdt-12/dpy-22* in gene expression in responses to a variety of stresses such as proteotoxic, mitochondrial, or oxidative stress (Shore *et al.* 2012). For example, *mdt-12/dpy-22* was identified in a reverse genetic screen for genes required in oxidative stress-responsive transcription (Crook-McMahon *et al.* 2014). However, further investigation is needed to determine if these Mediator subunits regulate stress responsive transcription directly, *e.g.* through interactions with TFs.

1.4.3 Innate immune responses

mdt-15 is required in an innate immune response controlled by a *C. elegans* stress-responsive p38 MAPK, PMK-1. Specifically, *mdt-15* is required for induction of *pmk-1*-dependent innate immunity genes in response to an immune-stimulatory xenobiotic toxin, RPW-24, or to the pathogenic bacterium *Pseudomonas aeruginosa* (Pukkila-Worley *et al.* 2014). Accordingly, *mdt-15* is required for resistance to *P. aeruginosa* infection. Curiously, *mdt-15* is also required in PMK-1-independent aspects of the response to RPW-24 or *P. aeruginosa*, namely the *pmk-1*-independent expression of detoxification genes and resistance to the *P. aeruginosa*-secreted toxin phenazine (Pukkila-Worley *et al.* 2014). Thus, *mdt-15* is required to regulate both the expression of innate immunity genes downstream of PMK-1 activated by pathogenic bacteria, and the expression of detoxification genes activated by bacterial toxins. This dual function of MDT-15 suggests that it may engage distinct, as yet unidentified TFs in each branch of the response to pathogenic bacteria. Taken together with MDT-15's roles in xenobiotic detoxification, heavy metal stress, and oxidative stress responses, these results suggest that

MDT-15 is a critical coregulator of adaptive responses to environmental insults, at least in *C. elegans*.

The Mediator subunits *mdt-23/sur-2* and *mdt-24/lin-25* are required for an innate immune response to the pathogenic bacterium *Microbacterium nematophilum*. Intriguingly, the immune response to *M. nematophilum* is activated by an ERK MAPK (MPK-1 in *C. elegans*) cascade instead of the canonical stress responsive p38 MAPK/PMK-1 cascade, and indeed *mdt-23/sur-2* and *mdt-24/lin-25* act downstream of activated *mpk-1/ERK* to promote the response to *M. nematophilum* infection (Nicholas and Hodgkin 2004). Taken together with the roles of *mdt-23/sur-2* and *mdt-24/lin-25* in developmental events controlled by ERK signaling (see Section 1.3.1), this suggests that these Mediator tail module subunits are central regulators of ERK signaling-driven responses in development and adulthood.

1.4.4 Conservation of Mediator subunit roles in physiological responses

The role of MED15 as a coactivator of SREBP-driven transcription appears to be highly conserved from worms to mammals. Just as observed in *C. elegans*, murine MED15 binds to and activates SREBP-driven transcription (Yang *et al.* 2006). However, the target genes of *C. elegans* and murine SREBP differ, with murine SREBP playing additional roles in cholesterol metabolism that do not appear to be prominent in *C. elegans,* likely because *C. elegans* is a cholesterol auxotroph (Hieb and Rothstein 1968; Yang *et al.* 2006). Thus, MED15 is a conserved coactivator of SREBP-driven transcription from worms to mammals, but changes in the biological function of MED15 have arisen during evolution due to altered target gene specificity of the SREBP TF.

The role of MDT-15 as a coactivator of NHR-49-driven transcription of lipid metabolism genes may represent an ancestral function of this Mediator subunit. Specifically, whereas *C. elegans* MDT-15 binds to multiple NHRs (Taubert *et al.* 2006; Arda *et al.* 2010), human MED1 primarily engages NHRs, although human MED14 and MED25 are also capable of binding certain NHRs, and *Drosophila* CDK8 also binds an NHR (Borggrefe and Yue 2011; Poss *et al.* 2013; Xie *et al.* 2015). The molecular nature of these Mediator subunit-NHR interactions is also highly divergent: whereas human MED1, MED14, and MED25 utilize the NHR-binding LXXLL motif, *C. elegans* MDT-15 does not contain LXXLL motifs and instead utilizes an alternative NHR-binding KIX domain (Taubert *et al.* 2006). Interestingly, the conservation of LXXLL motifs in Mediator subunits differs across species: MED1 LXXLL motifs are not conserved in invertebrates, *e.g. Drosophila* and *C. elegans*, MED14 LXXLL motifs are conserved from *Drosophila* to humans, but not *C. elegans*, and the LXXLL motif in CDK-8 is conserved from yeast to humans (Xie *et al.* 2015). Furthermore, although conserved from yeast to humans, the function of the KIX domain appears to have diverged across species: the KIX domain of yeast Med15 binds to NHR-like TFs, Oaf1 and Pdr1 (Phelps *et al.* 2006; Thakur *et al.* 2008, 2009; Näär and Thakur 2009) and the *C. elegans* MDT-15 KIX domain bind to NHRs and SBP-1/SREBP (Taubert *et al.* 2006; Yang *et al.* 2006), whereas the human MED15 KIX domain binds to SREBP but not to TR, a MED1-binding NHR (Yang *et al.* 2006). Thus, binding of MED15 orthologs to NHRs may represent an ancestral function of this Mediator subunit, which has been distributed to other Mediator subunits such as MED1 and MED14 in higher organisms.

While the role of human MED15 in detoxification responses has not been investigated, it is worthy to note that the MDT-15-interacting TF SKN-1 has a human ortholog, Nrf2, which regulates oxidative stress-responsive transcription (Ma 2013). Therefore, it would be informative

to determine if MED15 is a conserved coactivator of Nrf2 in oxidative stress responses in humans.

1.5 Concluding remarks, hypothesis and objectives

There is substantial mechanistic and phenotypic evidence that, while the Mediator complex is required for transcription of virtually all protein coding genes, individual Mediator subunits can play strikingly distinct roles in transcriptional activation or repression of specific gene expression programs, in response to diverse developmental and physiological stimuli. Underlying this regulatory diversity are the interactions between individual Mediator subunits and sequence-specific TFs. Studies of *C. elegans* mutants have revealed that Mediator subunits are required in multiple developmental and physiological pathways, but in many cases the underlying interactions with specific TFs remain unidentified. Furthermore, the overlapping phenotypes of several *C. elegans* Mediator subunit mutants indicate that multiple Mediator subunits can converge on the same developmental or physiological pathway; however, the regulatory interactions between Mediator subunits in a common pathway remains largely unexplored.

Mediator CKM subunits, CDK8, Cyclin C, MED12 and MED13, are implicated in transcription stimulated by several cell signaling pathways in human cell culture, in cancers and in *C. elegans*. Unpublished microarray gene expression profiles of *cdk-8* mutants from the Taubert lab implicate *C. elegans* CDK-8 in regulation of EGFR-Ras-ERK-driven transcription and cadmium-responsive transcription (see Sections 2.3.1 and 3.3.1, respectively), suggesting that the CKM may regulate these important transcriptional responses. As other Mediator subunits

also converge on EGFR-Ras-ERK-driven transcription, (*i.e. mdt-23/sur-2, mdt-24/lin-25* and *mdt-6*) or cadmium-responsive transcription (*i.e. mdt-15*), and as evidence in yeast suggests that the CKM can regulate the activity of other Mediator subunits, it is possible that the CKM may coordinate Mediator activity within these gene expression programs in metazoans.

The overall hypothesis of my thesis is that subunits of the Mediator CDK8 kinase module drive gene expression changes in the EGFR-Ras-ERK signaling pathway and in the cadmium response by interacting with sequence-specific TFs, and/or by regulating the activity of other Mediator subunits. Thus, the specific objectives are:

1. To identify novel interactions of *cdk-8* with transcriptional regulators in the EGFR-Ras-ERK signaling pathway.
2. To identify TFs and regulatory elements that cooperate with *cdk-8* to regulate cadmium-responsive gene expression.
3. To explore regulatory interactions between *cdk-8* and other Mediator subunits in EGFR-Ras-ERK signaling-driven or cadmium responsive transcription.

Table 1.1 *C. elegans* Mediator subunits and their mammalian and yeast orthologs.

***C. elegans* subunits**	**Alternative *C. elegans* names**	**Sequence number**	**Mammalian ortholog**	***S. cerevisiae* ortholog**	**Module**
MDT-1.1	SOP-3	Y71F9B.10	MED1	Med1	Middle
MDT-1.2		T23C6.1	MED1L		Middle
MDT-4		ZK546.13	MED4	Med4	Middle
MDT-6	LET-425	Y57E12AL.5	MED6	Med6	Head
MDT-7	LET-49	Y54E5B.3	MED7	Med7	Middle
MDT-8		Y62F5A.1	MED8	Med8	Head
MDT-9		Y62E10A.11	MED9	Med9/Cse1	Middle
MDT-10		T09A5.6	MED10	Med10/Nut2	Middle
MDT-11		R144.9	MED11	Med11	Head
MDT-12	DPY-22, SOP-1, PSA-6	F47A4.2	MED12	Med12/Srb8	Kinase
MDT-13	LET-19, PSA-7, PQN-49	K08F8.6	MED13	Med13/Srb9/Ssn2	Kinase
MDT-14	RGR-1	C38C10.5	MED14	Med14/Rgr1	Tail
MDT-15		R12B2.5	MED15	Med15/Gal11	Tail
			MED16	Med16/Sin4	Tail
MDT-17		Y113G7B.18	MED17	Med17/Srb4	Head
MDT-18		C55B7.9	MED18	Med18/Srb5	Head
MDT-19		Y71H2B.6	MED19	Med19/Rox3	Middle
MDT-20		Y104H12D.1	MED20	Med20/Srb2	Head
MDT-21		C24H11.9	MED21	Med21/Srb7	Middle
MDT-22		ZK970.3	MED22	Med22/Srb6	Head
MDT-23	SUR-2	F39B2.4	MED23		Tail
MDT-24	LIN-25	F56H9.5	MED24	Med5[a]	Tail
			MED25		Tail
MDT-26		C25H3.6	MED26		Middle

C. elegans subunits	Alternative *C. elegans* names	Sequence number	Mammalian ortholog	*S. cerevisiae* ortholog	Module
MDT-27		T18H9.6	MED27	Med3[a]	Density between head and tail
MDT-28		W01A8.1	MED28		Density between head and tail
MDT-29		K08E3.8	MED29	Med2[a]	Density between head and tail
MDT-30	PQN-38	F44B9.7	MED30		Density between head and tail
MDT-31		F32H2.2	MED31	Med31/Soh1	Middle
CDK-8		F39H11.3	CDK8	Cdk8/Srb10/Ssn3/Ume5	Kinase
CIC-1		H14E04.5	CycC	CycC/Srb11/Ssn8/Ume3	Kinase

[a]Weak sequence homology (Bourbon 2008).

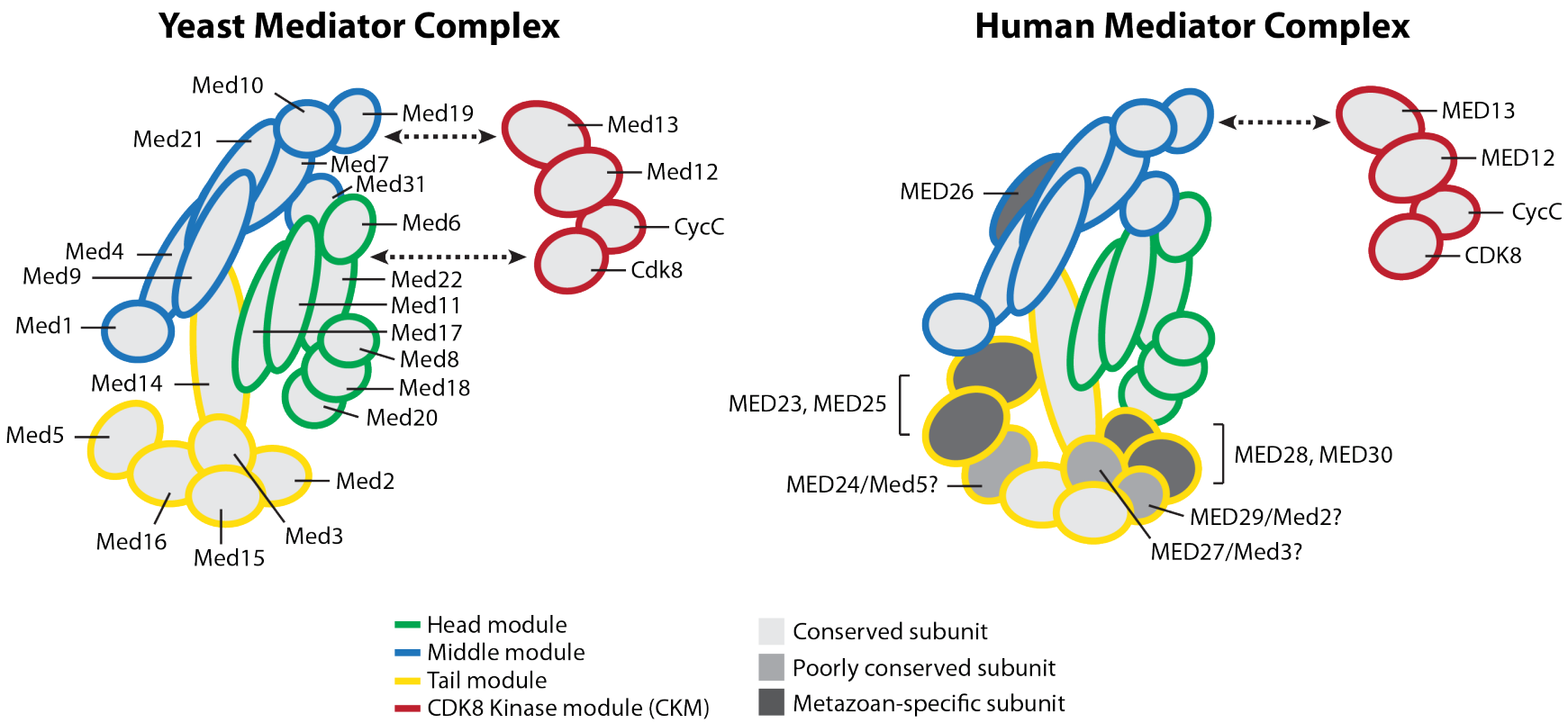

Figure 1.1 Yeast and human Mediator complex architecture.

Left: Yeast Mediator complex defined by cryo-EM with molecular tags, and guided docking of X-ray crystallographic structures (Tsai *et al.* 2013, 2014). *Right*: Human Mediator complex defined by cryo-EM and co-immunoprecipitation analyses. Highly conserved subunits are shown in analogous positions to their yeast orthologs. Position of MED26 was determined by cryo-EM with a molecular tag. Positions of other subunits are inferred from their biochemical interactions with other Mediator subunits (Tsai *et al.* 2013, 2014).

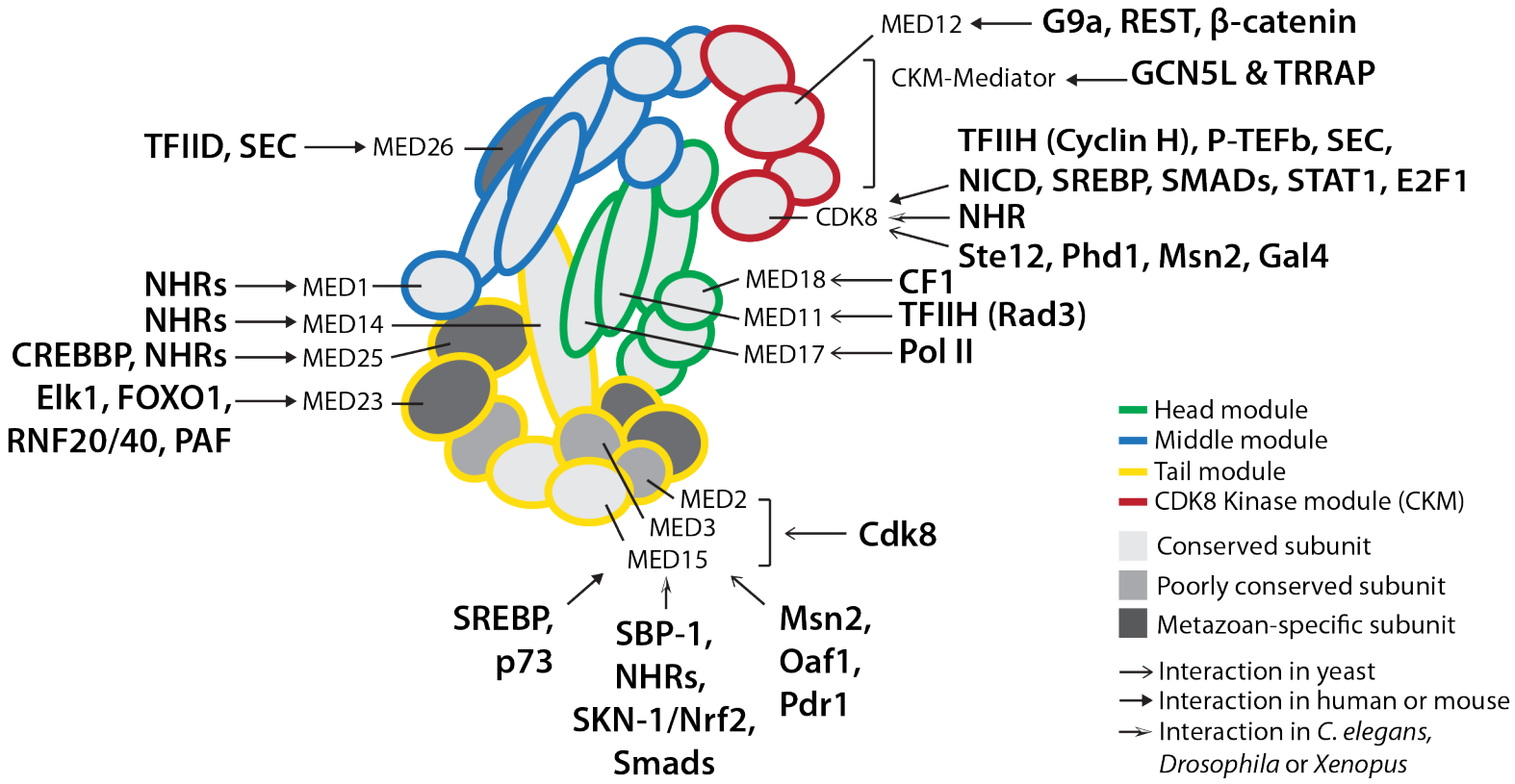

Figure 1.2 Mediator interactions with Pol II, chromatin modifiers, GTFs, elongation factors, and sequence-specific TFs.

Physical interactions identified for yeast, human, *C. elegans* or *Drosophila* Mediator subunits are shown. For references, see Section 1.1.3. Chromatin modifiers: CREBBP, G9a, GCN5L & TRRAP, PAF, RNF20/40. GTFs: TFIID, TFIIH. Elongation factors and cleavage factors: CF1, P-TEFb, SEC. Trancription factors: β-catenin, E2F1, Elk1, FOXO1, Gal4, Msn2, NHRs, NICD, Oaf1, p73, Pdr1, Phd1, SBP1, SKN-1/Nrf2, SMADs, SREBP, STAT1, Ste12.

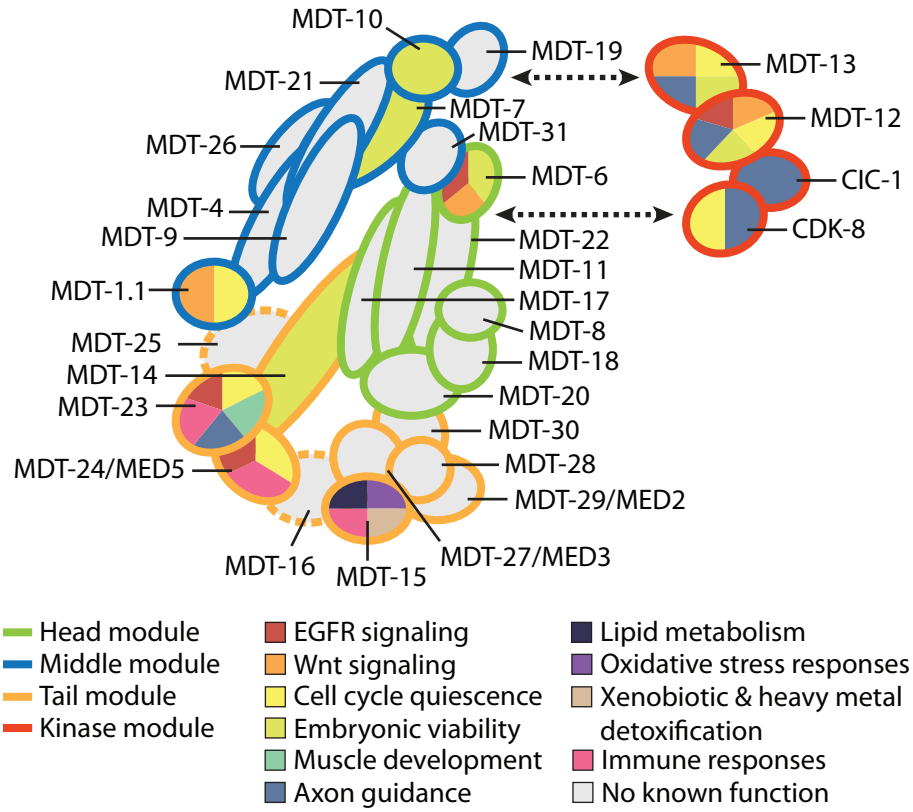

Figure 1.3 Overview of the biological activities of Mediator subunits in *C. elegans*.

The figure summarizes the roles of *C. elegans* Mediator subunits in developmental and physiological pathways. The architecture of Mediator is based on structural and biochemical studies of yeast and human Mediator (Tsai *et al.* 2013, 2014). Subunits labeled with a dashed outline do not appear to have a *C. elegans* ortholog. In cases where the homology between yeast and metazoan Mediator subunits is controversial, both the *C. elegans* and the yeast names are listed (e.g. MDT-24/MED5). Putative functions identified in large-scale genetic screens only (*i.e.* without further experimental validation) are not listed.

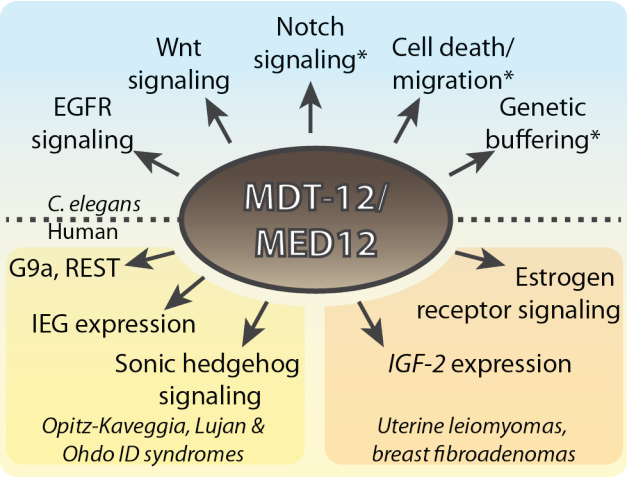

Figure 1.4 MDT-12 is a regulatory hub with links to human disease.

C. elegans mdt-12 interacts genetically with multiple signaling pathways, indicating a role as a regulatory hub. In addition, *mdt-12* interacts genetically with several additional cellular processes, implying a role as a general buffer against genetic perturbations. Similarly, human MED12 mutations are implicated in multiple developmental diseases or somatic tumors and are associated with perturbed cell signaling. Asterisks (*) indicate genetic interactions with pathways/processes that were identified in a genetic screen (Lehner *et al.* 2006) but have not been further validated. IEG: immediate-early genes. ID: intellectual disability.

Chapter 2: The Mediator kinase module restrains epidermal growth factor receptor signaling and represses vulval cell fate specification in *Caenorhabditis elegans*

2.1 Synopsis

Cell signaling pathways that control proliferation and determine cell fates are tightly regulated to prevent developmental anomalies and cancer. TFs 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, I 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. I also provide evidence that the CKM acts as a corepressor for the Ets-family TF LIN-1, as *cdk-8* promotes transcriptional repression by LIN-1. In addition, I 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. I 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.

2.2 Background

2.2.1 Transcriptional regulation by the CDK8 Kinase Module

The Mediator complex consists of ~30 subunits that assemble into four modules: the head, middle, tail and the CDK8 kinase module (CKM). The CKM consists of enzymatic subunits Cyclin Dependent Kinase 8 (CDK8), 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 TFs (*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.

2.2.2 Transcriptional regulation in *C. elegans* vulva development

Several Mediator subunits including at least one CKM subunit regulate vulva development in *C. 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, such as epidermal growth factor receptor (EGFR), Notch, and Wnt signaling (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 (Schmid and Hajnal 2015), 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 2.1). 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°) non-vulval fate (producing two descendants that fuse with the surrounding hypodermis). A signaling cell in the somatic gonad, called the anchor cell, emits an EGF-like ligand, LIN-3, 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 TFs such as the ELK1/Ets-family TF LIN-1 and the FoxB TF LIN-31, thereby specifying the 1° vulval fate in P6.p (Miller *et al.* 1993; Tan *et al.* 1998; Jacobs *et al.* 1998). The neighbouring 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 form the anchor cell, P3.p, P4.p, and P8.p do not receive sufficient EGF signal, and adopt the 3° non-vulval cell fate (Sternberg and Horvitz 1986). Mutations that enhance or reduce EGFR or Notch signaling induce ectopic vulval

cell fates (Muv, multivulva phenotype) or loss of vulval cell fates (Vul, vulvaless phenotype), respectively (Sternberg and Horvitz 1989). Furthermore, changes in EGFR or Notch signaling pathway activity can be monitored using reporter genes that are expressed strongly in EGFR-driven 1°-fated cells (*e.g. egl-17P::CFP;* (Yoo *et al.* 2004)) or in Notch-driven 2°-fated cells (*e.g. lip-1P::GFP;* (Berset *et al.* 2001)). Together, these phenotypes and cell fate reporters are thus powerful indicators of EGFR and Notch signaling pathway activity.

Transcriptional regulation is important in maintaining appropriate EGFR signaling pathway output (Figure 2.1). For example, TFs 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 (see Section 1.3.1). 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 TF is 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, and their functional interactions within the Mediator complex, remain poorly

understood. In yeast and cell culture models, CKM subunits interact with multiple TFs and chromatin modifiers, and regulate the activity of core Mediator subunits (see Section 1.1.3). Thus, I anticipated that studying CKM function in the *C. elegans* vulva would shed light on its regulatory interactions with TFs and other Mediator subunits *in vivo* in metazoans.

Here, I used the vulva organogenesis paradigm to study the requirements of all four CKM subunits in this process and to interrogate their functional interactions with other transcriptional regulators, including the synMuv genes, the key TFs *lin-1/Ets* and *lin-31/Forkhead*, and the Mediator subunit *mdt-23/sur-2*, an essential effector of EGFR signaling output. I show that all four CKM subunits inhibit ectopic vulval cell fates in *C. elegans*. I 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. My data implicate *cdk-8* as a corepressor for the LIN-1/Ets repressive TF to inhibit EGFR signaling-induced transcription. Furthermore, my 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.

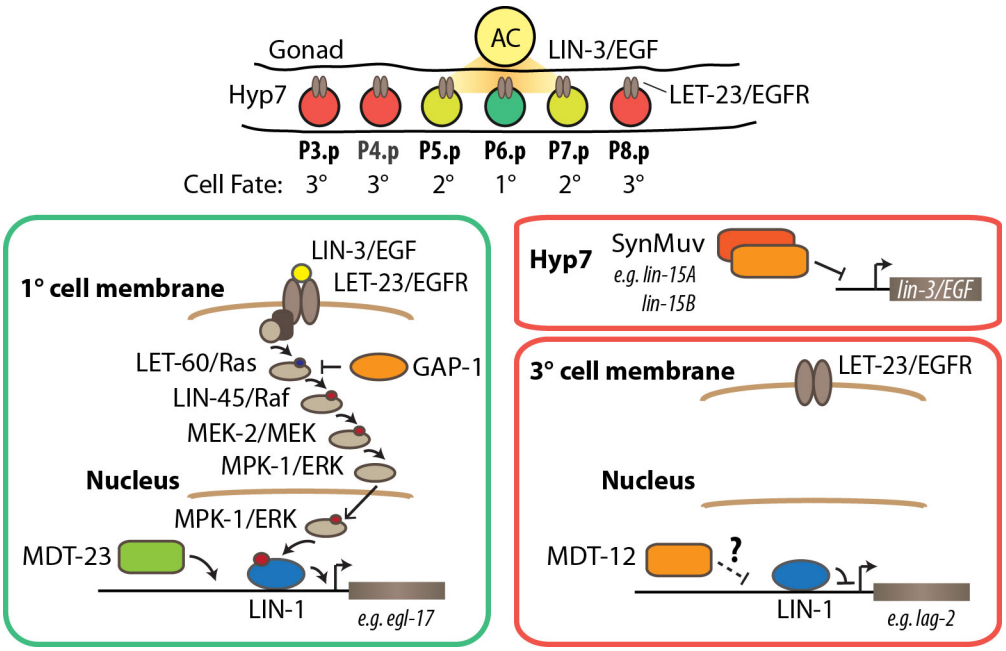

Figure 2.1 Transcriptional regulators in *C. elegans* vulval induction.

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 (left to right in top panel). 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 modulates TF 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, 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 non-vulval 3° cell fate. Transcriptional regulators that impinge on the EGFR signaling pathway are critical for correct vulval cell fate specification: *e.g.* the MDT-23/SUR-2 Mediator subunit is a critical activator of EGFR signaling-induced transcription, the SynMuv transcriptional 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.

2.3 Results

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

To define the role of the CKM in metazoan development, I compared transcriptional profiles of developmentally synchronized L4 larval stage *cdk-8(tm1238)* null mutants (Figure 2.2) to wild-type N2 worms using microarrays. I found that 829 genes were upregulated and 461 genes were downregulated more than two-fold in *cdk-8* null mutants, representing ~6.7% of *C. elegans* protein-coding genes. To identify *cdk-8*-dependent gene programs, I 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 2.2B). Importantly, the mRNA levels of *lin-35* and the *efl-1/dpl-1* TF heterodimer that is repressed by LIN-35 were not altered in *cdk-8* mutants (Figure 2.2C), 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, although direct action of CDK-8 at these promoters has not yet been established.

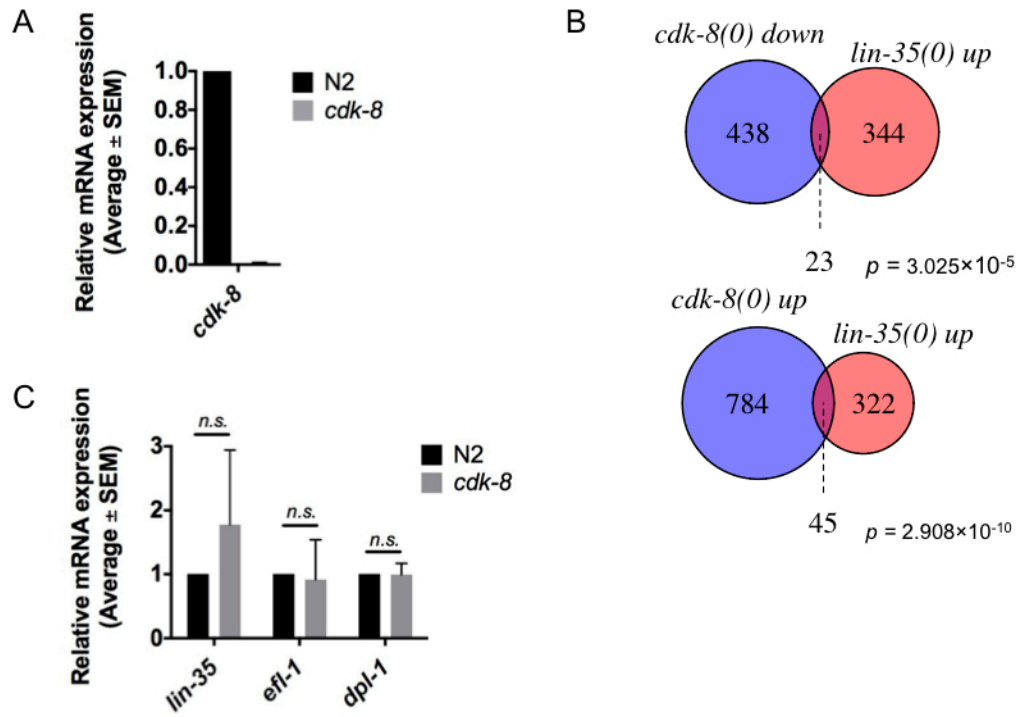

Figure 2.2 *cdk-8*-dependent transcripts overlap with targets of a transcriptional corepressor in the EGFR-Ras-ERK pathway.

(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) Overlap of *cdk-8* target genes with *lin-35/RB* repressed genes. *P*-value determined by Fisher's exact test. (C) 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).

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

I 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 2.1) and by scoring the occurrence of ectopic vulval protrusions in adult worms (Figure 2.3A; see statistical comparisons between L4 and adult Muv scores, Table 2.2). *cdk-8* and *cic-1* appeared to function together in vulva formation, as *cdk-8; cic-1* double mutants showed no

significant increase in Muv penetrance compared to either single mutant (Figure 2.3A). I 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, I 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 2.3B), as reported (Yoo *et al.* 2004). *cdk-8* mutants expressed *egl-17P::CFP* strongly in P6.px and weakly in P5.px and P7.px, like wild type (Figure 2.3B). 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 non-vulval fate (Figure 2.3B). The ectopic expression of *egl-17::CFP* suggested that the 1˚cell fate had been derepressed in presumptive 2˚ or non-vulval cells. This reflects that the low penetrance Muv phenotype of *cdk-8* mutants is caused in part through derepressed ERK signaling output.

Table 2.1 VPC induction scores and Muv/Vul penetrance in L4 animals (20°C)

	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	23
	cdk-8(tm1238); let-23(sy97)	2.44 ± 0.27	0	37	63	8
mpk-1	*mpk-1(oz140)*	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] % L4 animals with ectopic vulval invagination at P3.p, P4.p or P8.p.

[b] % 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.

Table 2.2 Muv penetrance in adult animals.

	% Muv adults[a]			
	20°C	***n***	**23°C**	***n***
N2	0.00[b]	399	0.00	402
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
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		
N2 + *lin-1* RNAi	5.38[b]	1505	7.29	905
cdk-8(tm1238) + *lin-1* RNAi	27.19[b]	526	41.46	410
mdt-12(os38)			49.32	296
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
mdt-13(mn19); lin-1(n1790)	96.97[b]	66	95.45	44
N2 + *mdt-15* RNAi	0.00[b]	395		
N2 + *mdt-27* RNAi	0.00[c]	641		

	% Muv adults[a]			
	20°C	***n***	**23°C**	***n***
N2 + *mdt-29* RNAi	0.24[c]	414		
N2 + *mdt-1.1* RNAi	0.59[c]	338		
mdt-13(mn19) + *mdt-15* RNAi	13.28[c]	256		
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.

[1] EV, Empty Vector.

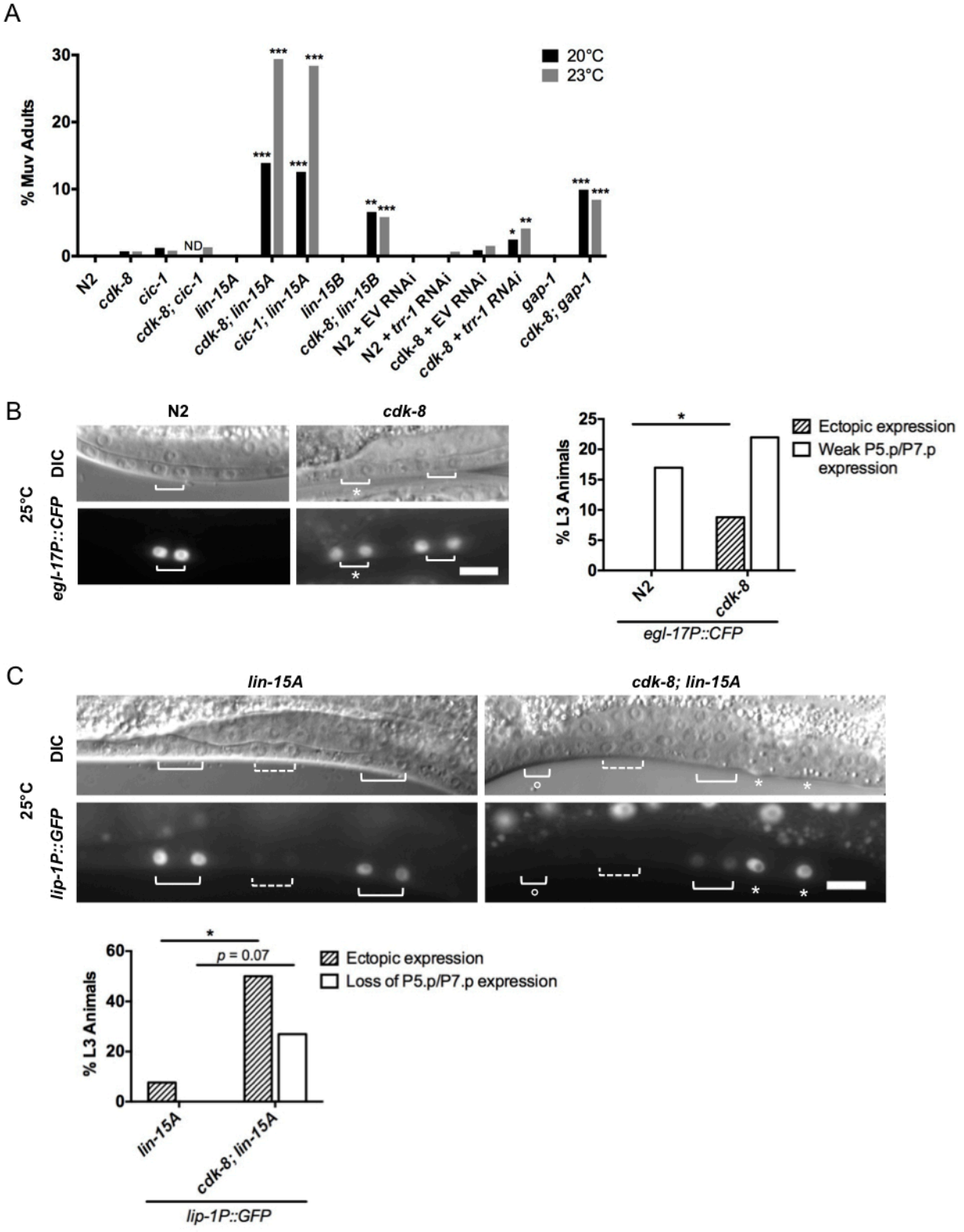
A
% Muv Adults
20°C
23°C
ND
N2
cdk-8
cic-1
cdk-8; cic-1
lin-15A
cdk-8; lin-15A
cic-1; lin-15A
lin-15B
cdk-8; lin-15B
N2 + EV RNAi
N2 + trr-1 RNAi
cdk-8 + EV RNAi
cdk-8 + trr-1 RNAi
gap-1
cdk-8; gap-1
B
N2
cdk-8
DIC
25°C
egl-17P::CFP
% L3 Animals
Ectopic expression
Weak P5.p/P7.p expression
egl-17P::CFP
C
lin-15A
cdk-8; lin-15A
DIC
25°C
lip-1P::GFP
p = 0.07
Ectopic expression
Loss of P5.p/P7.p expression
% L3 Animals
lip-1P::GFP

Figure 2.3 *cdk-8* represses vulval cell fates redundantly with synMuv genes.

(A) 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 2.2 for raw data. (B) 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. Scale 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. (C) 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. Scale 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.

2.3.3 *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). I 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 2.3A,

Table 2.1). 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 2.3A, Table 2.1). Our microarray analysis did not reveal significant downregulation of any known synMuv gene transcripts in *cdk-8* mutants, 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.

I observed adjacent VPCs expressing a 1° cell fate marker in *cdk-8* mutants (Figure 2.3B), 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, I 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)). I 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 2.3C), 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 2.3C). Furthermore, some *cdk-8; lin-15A* mutants ectopically expressed *lip-1P::GFP* strongly in non-vulval P3.p, P4.p, or P8.p, suggesting ectopic 2° fates (Figure 2.3C). 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.

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

I 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). I 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 2.4A) (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 2.4A). Thus, the enhanced Muv penetrance of *cdk-8; lin-15A* and *cdk-8; lin-15B* mutants compared to *cdk-8* single mutants (Figure 2.3A) likely does not arise from hypodermal *lin-3* derepression.

Next, I investigated if *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, I observed expression of a transcriptional *cdk-8P::GFP* reporter (*hdEx508*; (Steimel *et al.* 2013)) in the anchor cell (Figure 2.4B). To assess *lin-3* transcription in the anchor cell alone, I used a *lin-3* anchor cell-specific enhancer element (ACEL) GFP reporter (*syIs107*; (Hwang and Sternberg 2004)). I 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 2.4C), suggesting that *cdk-8* is required to *repress lin-3/EGF* transcription in the anchor cell.

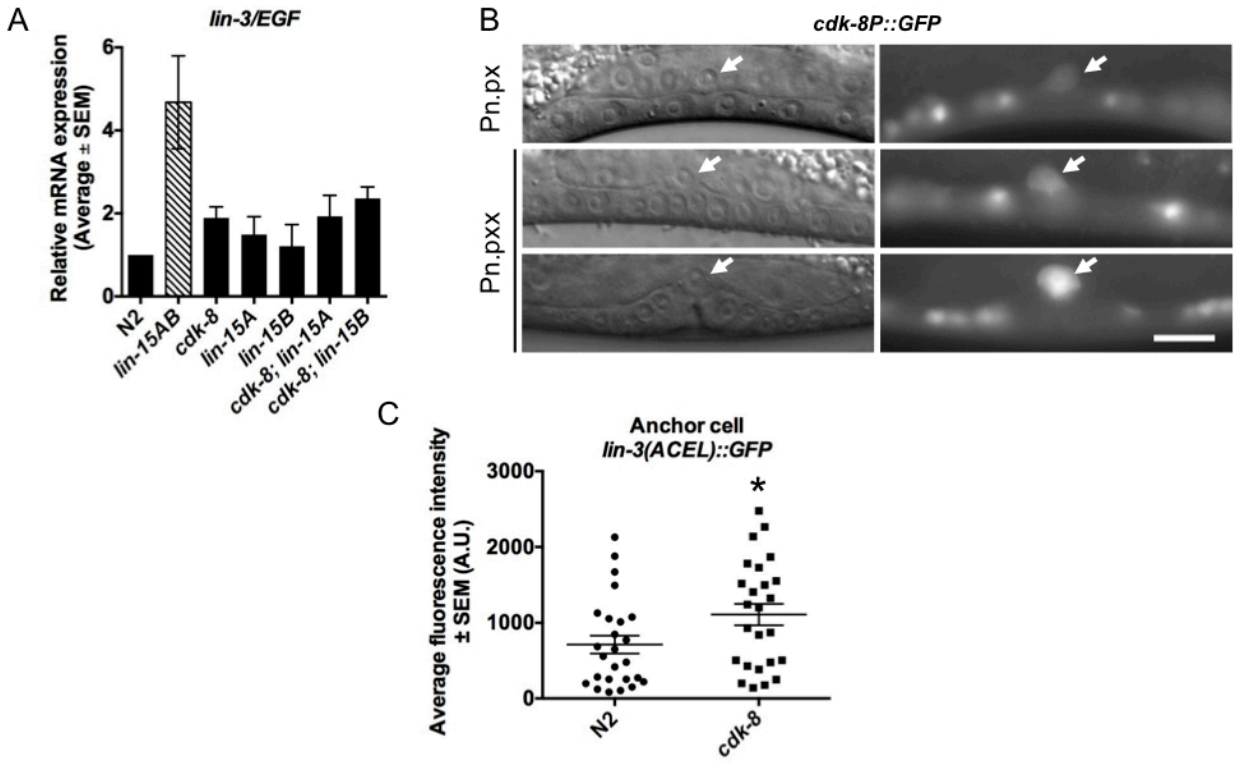

Figure 2.4 *cdk-8* represses *lin-3/EGF* transcription via an anchor cell-specific enhancer element.

(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 method of Pratt. (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. Scale 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.

2.3.5 *cdk-8* acts downstream of *mpk-1/ERK* to regulate vulval 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), I next investigated *cdk-8's* role in the EGFR signaling pathway downstream of *lin-3*. I conducted genetic epistasis analyses with loss-of-function alleles of *EGFR, let-23(sy97)*, and *ERK, mpk-1(oz140)*. *let-23(sy97)* is a strong loss-of-function allele that causes a highly penetrant Vul phenotype (Figure 2.5A, Table 2.1), and causes insensitivity to increased *lin-3/EGF* transcription as in *lin-15AB* mutants (Aroian and Sternberg 1991), due to blockade of the EGFR-Ras-ERK pathway. I note that one presumed *let-23(sy97)* mutant worm (out of 23 worms total) exhibited wild-type vulva development; however, as I isolated *let-23(sy97)* homozygous mutants from balanced *let-23(sy97)/+* heterozygotes, it is possible that this animal was incorrectly identified as a homozygote when in fact it was a heterozygote. *cdk-8* inactivation significantly rescued the Vul phenotype of *let-23(sy97)* single mutants (Figure 2.5A, Table 2.1), suggesting that *cdk-8* acts downstream of *let-23/EGFR*. *mpk-1(oz140)* is a loss-of-function allele that causes a strong but not fully penetrant Vul phenotype (Figure 2.5A, Table 2.1). *cdk-8* inactivation also significantly rescued the Vul phenotype of *mpk-1(oz140)*, in line with a role downstream of *mpk-1/ERK*. Due to the partial nature of the *mpk-1(oz140)* Vul phenotype I cannot completely rule out the possibility that *cdk-8* acts upstream of *mpk-1, e.g.* at the level of *lin-3/EGF* transcriptional regulation; however, as I found that *cdk-8* acts downstream of a strong *let-23/EGFR* loss-of-function allele, which is insensitive to increased *lin-3/EGF* transcription, I favour the possibility that *cdk-8* acts downstream of *let-23/EGFR* and *mpk-1/ERK*. As in any epistasis analysis, I also cannot rule out the possibility that *cdk-8* acts parallel to the EGFR-Ras-

ERK pathway; however, for reasons discussed in Section 2.4.2, I favour the interpretation that *cdk-8* action in *e.g.* Notch or Wnt signaling is not a primary driver of the vulva development defects observed in *cdk-8* mutants. Overall, these data suggest that *cdk-8* primarily acts downstream of *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 2.1). 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. I used the *lin-15A* sensitized background to analyze tissue-specific requirements for *cdk-8* in VPCs *vs.* the hypodermis. First, I demonstrated that a transgene expressing *cdk-8* from its own promoter (*cdk-8(+)*) rescued the *cdk-8; lin-15A* mutant Muv phenotype compared to non-transgenic siblings (Figure 2.5B). 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 2.5C, D). 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 2.5B). 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 2.5B), although it was able to rescue the Dpy phenotype (Figure 2.5C).

Unexpectedly, the *lin-31P::cdk-8* transgene partially rescued the *cdk-8; lin-15A* Dpy phenotype compared to non-transgenic worms, albeit to a lesser extent than *cdk-8(+)* or *dpy-7P::cdk-8* (Figure 2.5C). 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.

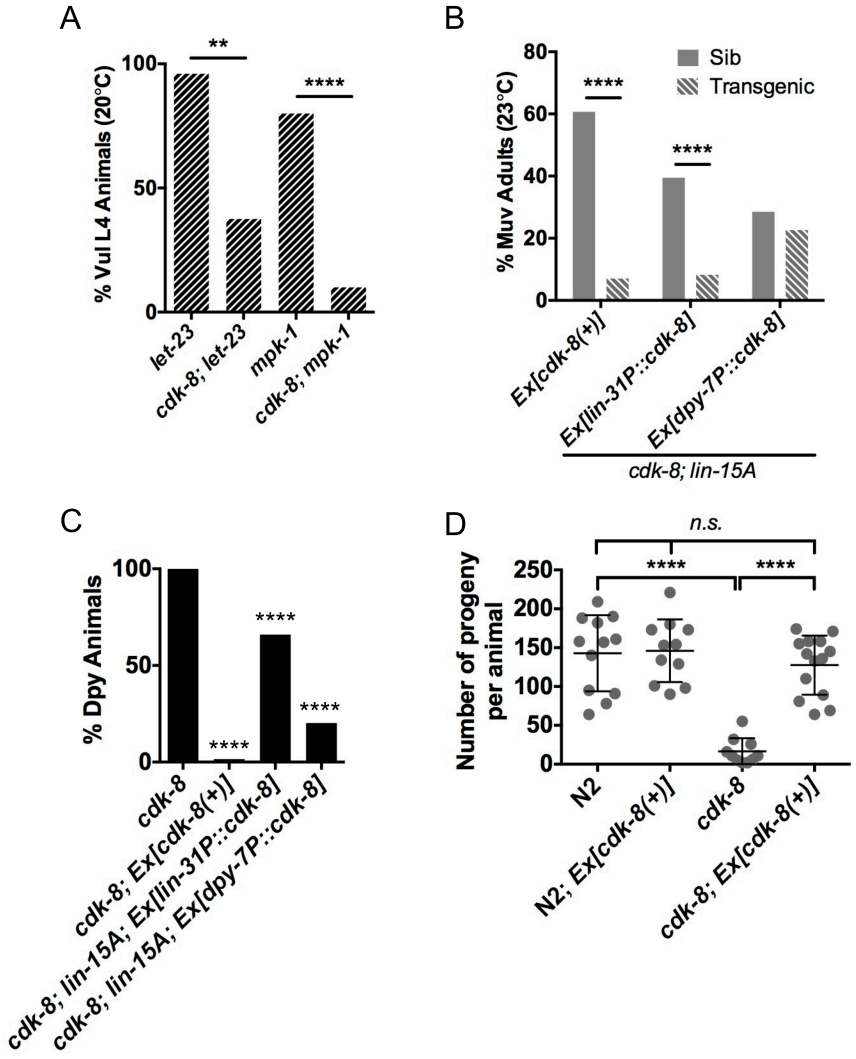

Figure 2.5 *cdk-8* acts cell autonomously downstream of *mpk-1/ERK.*

(A) L4 Vul penetrance in *cdk-8*, *cdk-8; let-23/EGFR*, and *cdk-8; mpk-1/ERK* mutants ($n \geq 8$). **** $p < 0.0001$, Fisher's exact test. See Table 2.1 for raw data. (B) 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 non-transgenic siblings (Sib) in each strain ($n \geq 76$). **** $p < 0.0001$ *vs.* non-transgenic sibling, Fisher's exact test. See Table 2.2 for raw data. (C) Genetic rescue of *cdk-8* mutant Dpy phenotype ($n > 150$). **** $p < 0.0001$ *vs. cdk-8* mutant, Fisher's exact test. (D) Genetic rescue of *cdk-8* mutant brood size. **** $p < 0.0001$, t-test. *n.s.* not significant.

2.3.6 *cdk-8* kinase activity is required to repress vulval induction

I next addressed how *cdk-8* functions downstream of *mpk-1/ERK*. First, I 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, I 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 2.6), suggesting that kinase activity is required for transgenic rescue of the Muv phenotype of *cdk-8* null mutants.

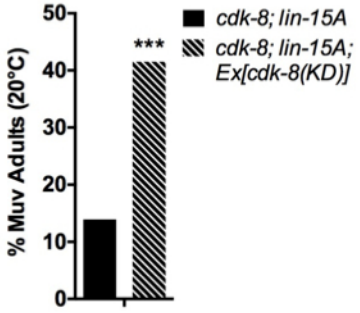

Figure 2.6 *cdk-8* kinase activity is required to repress vulval induction.

Adult Muv penetrance in *cdk-8; lin-15A* mutants expressing kinase dead (KD) *cdk-8,* compared to non-transgenic *cdk-8; lin-15A* ($n \geq 41$). *** $p < 0.001$ *vs.* non-transgenic, Fisher's exact test. See Table 2.2 for raw data.

2.3.7 *cdk-8* promotes *lin-1/Ets* repressor activity

Next, I investigated TFs that repress VPC induction, *i.e.* the ELK1/Ets-family TF LIN-1 and the Forkhead-family TF LIN-31 (Miller *et al.* 1993; Beitel *et al.* 1995). I hypothesized that the CKM may coregulate LIN-1 and/or LIN-31, and thus tested their genetic interactions with *cdk-8*. Unexpectedly, *cdk-8* suppressed the *lin-31* null mutant Muv phenotype (Table 2.1), suggesting that in the absence of LIN-31, *cdk-8* promotes vulval induction, perhaps through coactivation of an alternative TF. Albeit interesting, I did not investigate *lin-31* further for the purposes of this study. In contrast, *cdk-8* mutation strongly enhanced the low Muv penetrance caused by *lin-1* RNAi depletion (Figure 2.7A, Table 2.1). To corroborate the RNAi experiment, I 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 2.7B, Table 2.1). Together, these results suggest that residual LIN-1 requires *cdk-8* for efficient repression of vulval induction.

To test if *cdk-8* promotes transcriptional repression by LIN-1, I 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). I 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 2.7C). 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 2.7C). Thus, *cdk-8* is partially required to repress a direct LIN-1 target gene. Taken together with the requirement for *cdk-8* for *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.

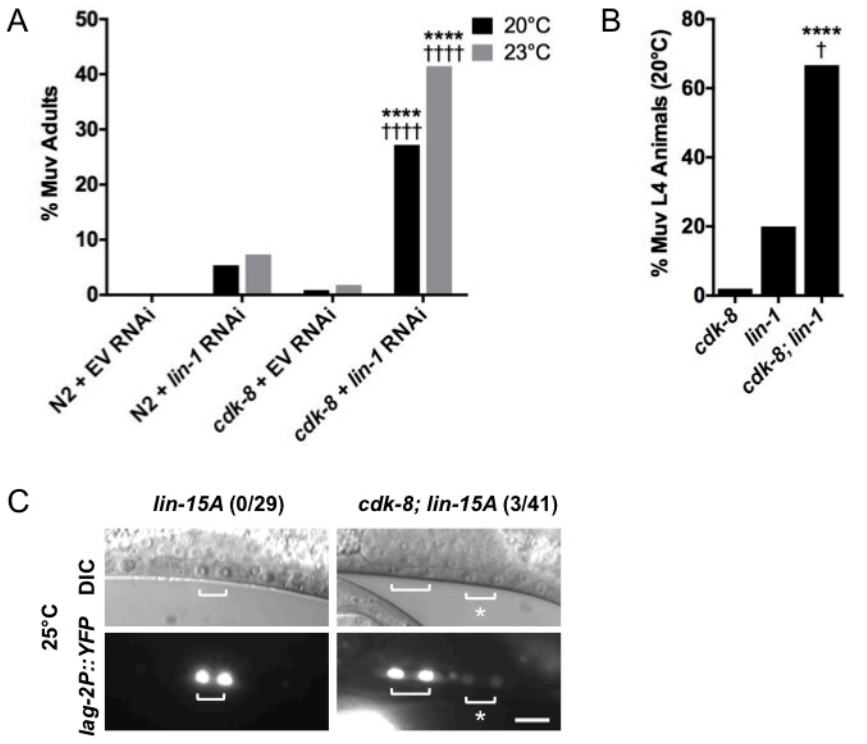

Figure 2.7 CDK-8 is a LIN-1/Ets corepressor.

(A) 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), $^{\dagger\dagger\dagger\dagger}p < 0.0001$ *vs.* N2 + *lin-1* RNAi, Fisher's exact test. See Table S2 for raw data. (B) L4 Muv penetrance in *cdk-8; lin-1(n1790)* mutants ($n \geq 12$). **** $p < 0.0001$ *vs. cdk-8*, $^{\dagger}p < 0.05$ *vs. lin-1(n1790)*, Fisher's exact test. See Table 1 for raw data. (C) 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. Scale bar: 10 μm. Number of ectopic expression events over total sample size noted next to genotype.

2.3.8 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, I 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, I investigated the requirements and mechanisms of structural CKM subunits in the EGFR signaling pathway, focusing primarily on *mdt-13/let-19.*

I 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 2.8A) of much higher penetrance than *cdk-8* mutants (Figure 2.3A). I observed a similarly high penetrance Muv phenotype in *mdt-12/dpy-22* reduction-of-function mutants (Figure 2.8A). I 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). I also note that when scoring VPC induction in *mdt-13/let-19* mutants at the L4 larval stage, I observed partial Vul phenotypes, as 27% of animals displayed partial loss of induction of P5.p and/or P7.p (Table 2.1). 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, and perhaps that *mdt-13/let-19* may also be required for cell fate determination in VPCs that normally adopt the 2° fate.

I next tested if *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 2.8B). 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 2.8B). 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 2.8B). 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* showed a similar trend (Figure 2.8A). In addition, loss of *mdt-13/let-19* significantly enhanced the Muv penetrance of *lin-1(n1790)* mutant worms (Figure 2.8C, Table 2.1), 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.

Finally, I 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 2.8D, Table 2.1). 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 2.8D, Table 2.1). Together, these data support a model in which the CKM acts downstream of *mpk-1/ERK* to inhibit vulval cell fate specification.

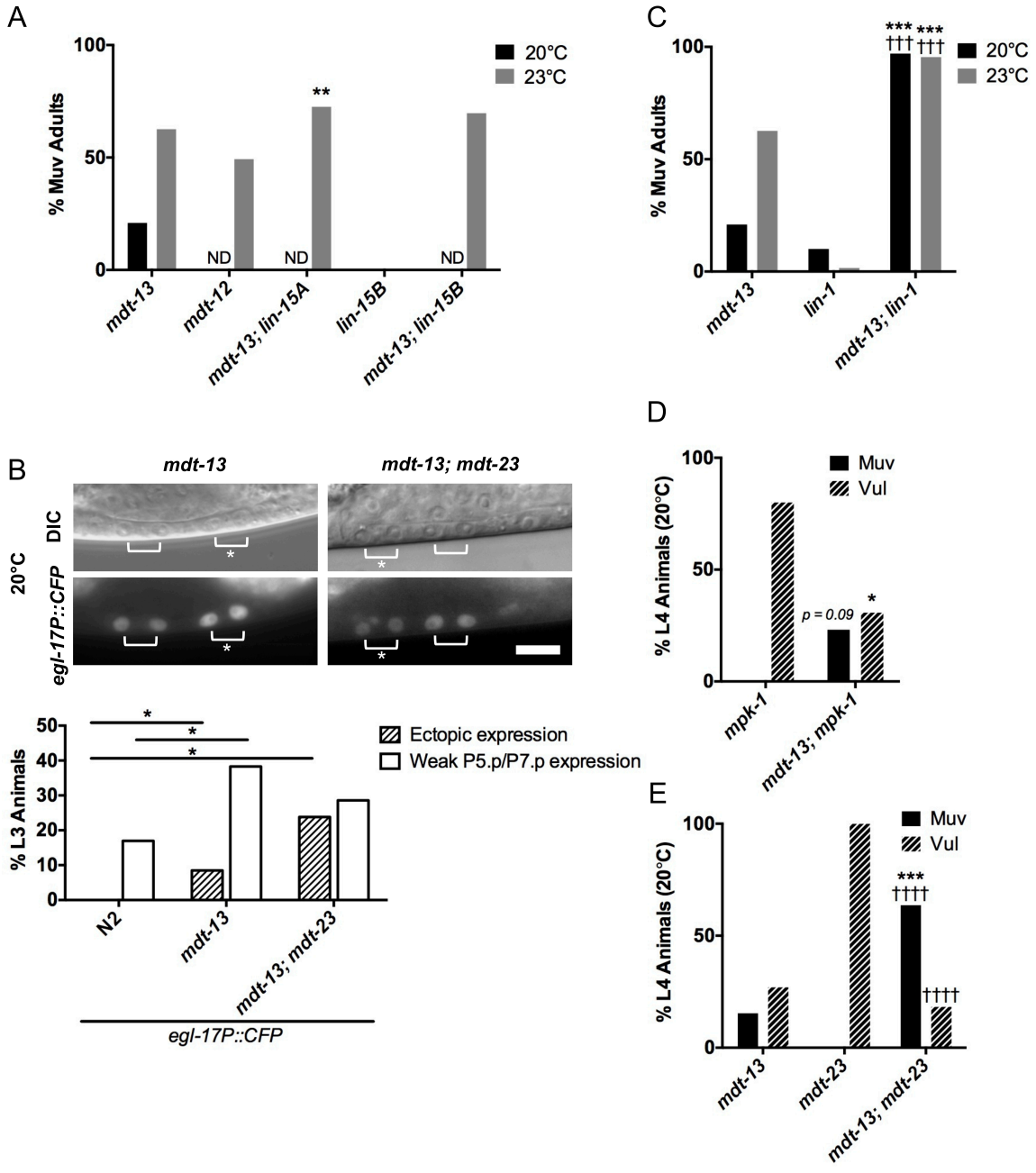

Figure 2.8 *mdt-13/let-19* represses vulval cell fates downstream of *mpk-1/ERK*.

(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 2.2 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. Scale 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 panel A 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 2.2 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 2.1 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 2.1 for raw data.

2.3.9 The CKM restricts the specificity of core Mediator subunits

As several Mediator subunits positively regulate vulva development (Section 1.3.1), I 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, I 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 2.8E, Table 2.1). 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 2.8E, Table 2.1). 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 2.8E, Table 2.1),

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 2.8B). 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.

My 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), I 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 2.1), but significantly reduced the Muv penetrance of *mdt-13/let-19* mutants (Figure 2.9A); *mdt-27* RNAi yielded a similar trend (Figure 2.9A). 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 2.9A). Furthermore, a 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 2.9B, Table 2.1). Thus, tail module triad activity appears to be derepressed in a CKM mutant, causing aberrant activation of vulval fate specification.

Next, I investigated whether the CKM modifies the target gene specificity of the triad. I used qPCR to quantify the expression of *cdk-8*-repressed genes identified by our microarray analysis 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 2.9C). 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 2.9C). 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, I cannot rule out unequal RNAi efficiency accounting for these differing requirements, although RNAi knockdown of all three genes appeared successful, as I observed partial sterility (not shown) consistent with the essential nature of these genes (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 2.9C). 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, I 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 2.9D). Western blot analysis showed elevated MDT-15 protein levels in *cdk-8* mutants compared to wild type (Figure 2.9E). Taken together, these results demonstrate that *cdk-8* is required for post-transcriptional regulation of MDT-15.

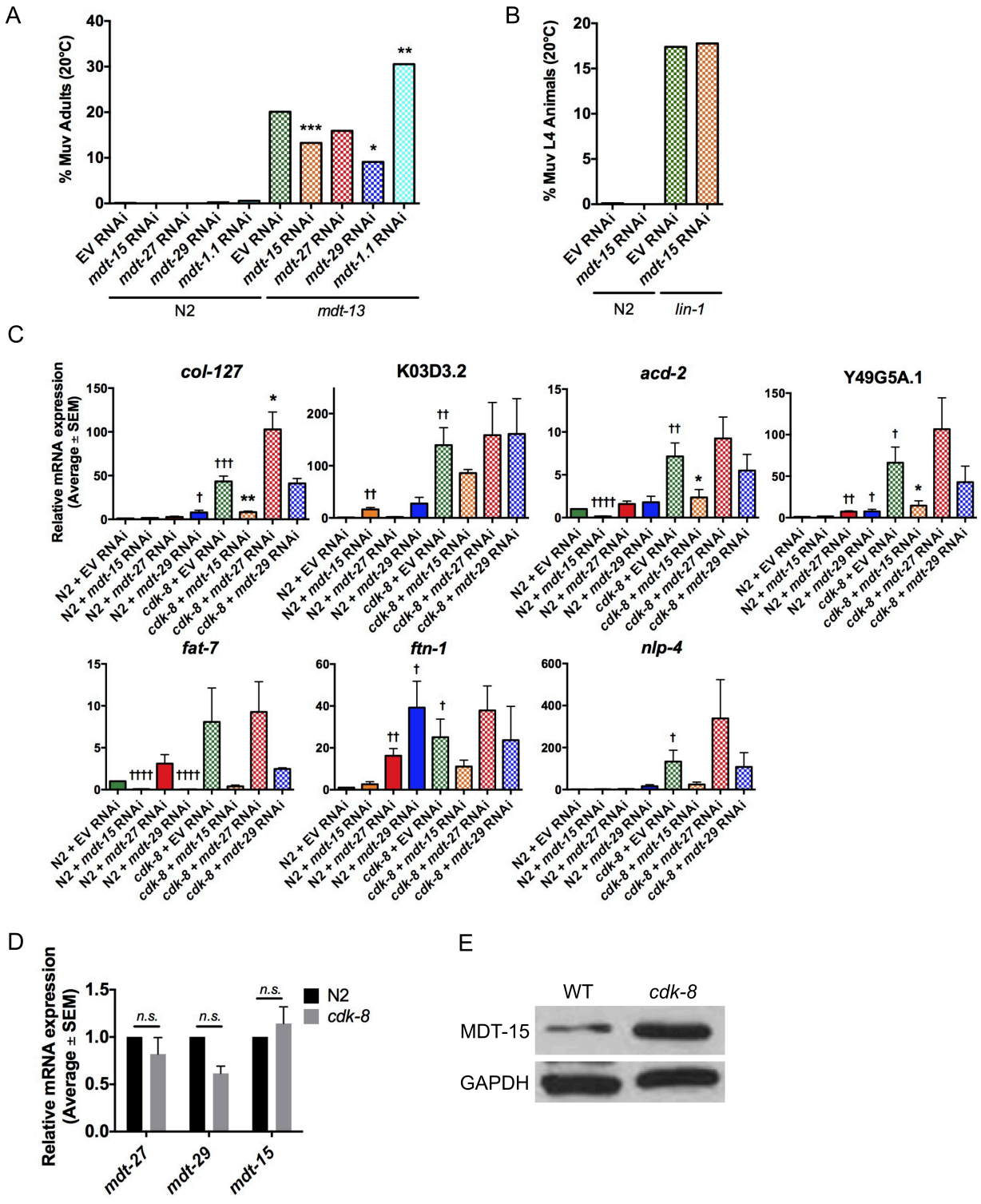

Figure 2.9 The CKM inhibits activation of vulval induction by the *mdt-15, mdt-27, mdt-29* triad.

(A) Adult Muv penetrance in *mdt-13/let-19* mutants on *mdt-15, mdt-27*, or *mdt-29* triad subunit RNAi or *mdt-1.1* non-triad 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 2.2 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 2.1 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), unpaired t-test. (D) qPCR analysis of mRNA levels in *cdk-8* mutants relative to wild type (n=3, error bars represent SEM, *n.s.* not significant by Wilcoxon signed-rank test, method of Pratt). (E) α-MDT-15 Western blot in wild-type *vs. cdk-8* mutants, with α-GAPDH loading control. Representative immunoblot from one of three trials.

2.4 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 (Section 1.3.1), but pertinent mechanisms remain incompletely understood. Here, I used the well-characterized vulva development paradigm in *C. elegans* to delineate the role of the Mediator CKM module. My 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 TF LIN-1/Ets, and core Mediator (Figure 2.10). This model is based on four key observations: First, I demonstrate that the primary site of action for the CKM is in the VPCs, as *cdk-8* and *mdt-13* 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 TF LIN-1, as loss of *cdk-8* or *mdt-13* 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, my 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, my genetic and molecular analyses provide the 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).

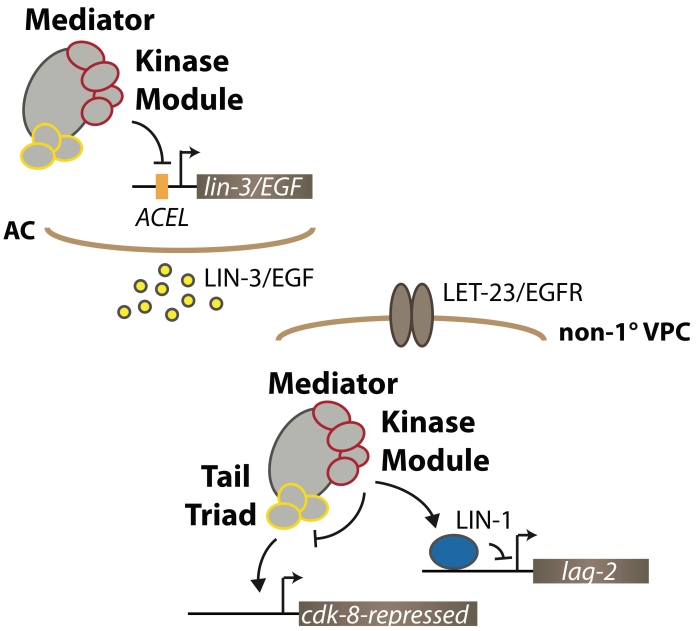

Figure 2.10 Kinase module action in *C. elegans* vulval cell fate specification.

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).

2.4.1 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 deregulated in *cdk-8* mutants (Figure 2.2B). This number agrees with studies in yeast, wherein only 3% of genes are deregulated in *cdk8* mutants (Holstege *et al.* 1998). Thus, CDK8 appears to be a gene program-specific transcriptional coregulator across species. It is important to note that the *cdk-8*-dependent genes defined in our gene expression analysis may include genes that are not direct targets of CDK-8. CDK-8 chromatin immunoprecipitation-sequencing analysis is needed to

define direct CDK-8 targets; however, we currently lack a reliable α-CDK-8 antibody or a functional epitope-tagged CDK-8 transgene to conduct such a study.

Among *cdk-8*-dependent genes, we identified a significant overlap with genes regulated by *lin-35/RB*, a synMuv transcriptional repressor (Figure 2.2B) (Kirienko and Fay 2007). I 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, I 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 2.3A, Figure 2.8A). However, *cdk-8* did not act redundantly with the synMuv genes to repress *lin-3/EGF* transcription (Figure 2.4A), suggesting that the CKM and the synMuv genes regulate EGFR signaling at different junctions, as discussed below.

Comparing the vulval phenotypes of CKM mutants, I found evidence that *cdk-8* and *cic-1* act together 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 2.3A). In addition, I 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* (Figure 2.8A). 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

2.6), this does not rule out the possibility that the CKM also employs kinase-independent steric mechanisms, as observed in other systems (Knuesel *et al.* 2009b). Thus, additional kinase-independent mechanisms could account for the stronger requirement for *mdt-12/dpy-22* and *-13* in vulva development.

2.4.2 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 and Cyclin C repress Notch signaling-driven transcription by promoting turnover of the Notch intracellular domain (Fryer *et al.* 2004; Li *et al.* 2014). Therefore, I 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, I 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 2.3B, Figure 2.8B). Using a Notch signaling-induced 2° cell fate reporter, I showed that *cdk-8* is required to represses ectopic 2° fates in non-vulval VPCs, as well as to promote the 2° fate in P5.p and P7.p (Figure 2.3C). 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 neighbouring cells, P5.p and P7.p (Chen and Greenwald 2004). I 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 2.3B, Figure 2.8B), expression of the EGFR signaling target gene *lag-2* (Figure 2.7C), or loss of the strong *lip-1P::GFP* expression characteristic of 2° cells (Figure 2.3C). It is possible that VPCs transformed to the 1° fate could then induce 2° fates in neighbouring VPCs,

accounting for my observation of ectopic 2° cells. It would be interesting to explore this hypothesis further using a *cdk-8* mutant strain simultaneously expressing 1° and 2° cell fate markers. In addition, some *mdt-13/let-19* mutants displayed a partial Vul phenotype due to reduced proliferation of P5.p or P7.p descendants (Table 2.1). This suggests that, like *cdk-8* mutants which occasionally showed loss of the strong *lip-1P::GFP* expression characteristic of 2°-fated P5.p and P7.p descendants (Figure 2.3C), some *mdt-13/let-19* mutants may also exhibit partial loss of the 2° cell fate in the vulva, although examination of the *lip-1P::GFP* 2° fate reporter in *mdt-13/let-19* mutants is needed to substantiate this possibility.

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 vulval phenotype of *mdt-12/dpy-22* mutants is independent of *bar-1/β-catenin* (Moghal and Sternberg 2003a), 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 2.1) 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 2.3B, C,

Figure 2.8B) suggests that EGFR and/or Notch signaling indeed drives ectopic vulva formation in these mutants.

2.4.3 The CKM promotes LIN-1/Ets repressor activity

I observed derepression of the *lin-3/EGF* anchor cell specific enhancer element (ACEL) in *cdk-8* mutants (Figure 2.4C), 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 primarily required downstream of *mpk-1/ERK* to repress vulval induction (Figure 2.5A). 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 2.5A). 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 2.5A). In line with a function downstream of *mpk-1/ERK*, I showed that *cdk-8* is required in VPCs to suppress ectopic vulval induction (Figure 2.5B). 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*, I found evidence that the CKM promotes LIN-1/Ets-mediated repression of vulval induction (Figure 2.7A, B, Figure 2.8C), and that *cdk-8* promotes transcriptional repression of a direct LIN-1 target, the *lag-2/Notch ligand* minimal promoter (Figure 2.7C). 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). My results indicate that *cdk-8* is partially required for transcriptional repression of the *lag-2* minimal promoter (Figure 2.7C), 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 TF(s) that act at VPCact remain 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 I demonstrated that vulval induction in *mdt-13/let-19* mutants does not require *mdt-23/sur-2* (Figure 2.8B, E), this implies that the CKM likely does not inhibit MDT-23/SUR-2 activity at VPCact. Overall, my findings suggest that the CKM may act as a corepressor for LIN-1. Additional experiments are needed to determine if CDK-8 binds to LIN-1, which would imply that LIN-1 recruits the CKM-containing Mediator complex to repress transcription of its target genes.

In addition to vulva development, *lin-1* also represses a subset of other *mpk-1/ERK*-dependent developmental events: excretory system, posterior ectoderm, and male tail spicule development, but not meiosis or sex myoblast migration (Sundaram 2013). Interestingly, the Mediator subunits *mdt-23/sur-2* and *mdt-24/ lin-25* promote these *lin-1*-mediated developmental events (Nilsson *et al.* 2000), suggesting that these Mediator subunits activate gene expression in all tissues in which *lin-1* is required. Further work is needed to determine if CKM mutants show

phenotypes of the excretory system, posterior ectoderm, and male tail similar to *lin-1* mutants. If so, this would support a model in which LIN-1 recruits the CKM-containing Mediator complex to corepress its target genes in all tissues in which *lin-1* is required.

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 IEGs, which are targeted by multiple TFs 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, sumoylation of LIN-1 promotes recruitment of subunits of the nucleosome remodeling and deacetylase (NuRD) complex, leading to transcriptional repression of target genes (Jacobs *et al.* 1998; Leight *et al.* 2005, 2015). The current study provides evidence that the CKM is required for repression by LIN-1, indicating that transcriptional repression by this Ets factor involves interactions both with a chromatin remodeler, the NuRD complex, and with a coregulator of Pol II transcription, the Mediator complex.

My findings are 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 my 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.

2.4.4 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 (Section 1.1.3.4) and my 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, I 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 2.8E). I 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, I was intrigued to find that vulva formation in a *C. elegans* CKM mutant required *mdt-15*, *mdt-27*, and *mdt-29* (Figure 2.9A). 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 2.8E, Figure 2.9A). 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 2.9B). 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 2.9C). 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 in exist in metazoan Mediator, as human Mediator displays more extensive structural contacts between the head and tail modules (Tsai *et al.* 2014), which may redundantly anchor some tail module subunits.

Investigating the regulatory relationship between CDK-8 and MDT-15 further, I 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 2.9D, E). A limitation of these experiments is that I examined MDT-15 mRNA and protein levels in whole animal extracts, not in VPCs. However, I found that *mdt-15* is required both for transcriptional activation of *cdk-8*-repressed genes in *cdk-8* mutants, which was determined using whole animal extracts (Figure 2.9C), and for ectopic vulva formation in *mdt-13/let-19* mutants, which reflects a VPC-specific phenotype (Figure 2.9A). Thus, CDK-8 likely acts as a negative regulator of MDT-15 in

multiple tissues, including VPCs. 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, my 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 TF and by promoting specificity of Mediator tail module subunits.

2.5 Materials and methods

2.5.1 Microarrays and data analysis

Microarray gene expression profiling was performed at the UCSF SABRE Functional 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 hrs, according to the manufacturers 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 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 < 0.05 and a fold-change ≥ 2 (representing 461 downregulated and 829 upregulated genes) (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 two-fold 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.

2.5.2 *C. elegans* strains, culture, and genetic methods

C. elegans strains were cultured as described (Brenner, 1974) at 20°C or 23°C, as indicated. I 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 2.3. Wild type was Bristol N2. *cdk-8(tm1238)* and *cic-1(tm3740)* are likely null alleles that abolish *cdk-8* expression (Figure 2.2A) 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.

Table 2.3 *C. elegans* strains used in Chapter 2

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*
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*

Strain	Genotype
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(120)/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*
NH2466	*ayIs4[egl-17p::GFP + dpy-20(+)] I; dpy-20(e1282) IV*
PS4308	*syIs107[unc-119(+) + lin-3(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]*
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-3(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]*
STE89	*steEx44[cdk-8P::GFP + myo-2P::mCherry]*
STE90	*steEx45[lin-31P::cdk-8 + myo-2P::mCherry]*
STE91	*steEx46[dpy-7P::cdk-8 + myo-2P::mCherry]*

Strain	Genotype
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	*steEx48[pCeBiFC-VN173-lin-1 + pCeBiFC-VC155-cdk-8 + myo-2P::mCherry]*
STE97	*steEx49[pCeBiFC-VN173-lin-1 + pCeBiFC-VC155-cdk-8(KD) + myo-2P::mCherry]*

2.5.3 VPC induction

VPC induction was scored as described (Han *et al.* 1990), in synchronous mid-L4 animals under DIC optics at 1000X magnification. In wild-type animals, P5.p, P6.p, and 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).

2.5.4 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 200x magnification (*mdt-13/let-19* mutants) or 56x magnification (all other strains). To corroborate Muv penetrances scored in adult animals, I also conducted VPC induction analysis in L4 animals (see Section 0). To assess Vul phenotypes, both Vul and Muv penetrances were extrapolated from VPC induction scores: animals were scored as

Vul if VPC induction < 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 simultaneous Vul and Muv.

2.5.5 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). I 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 2.4), except *lin-3* (analyzed by TaqMan assay, Invitrogen #4448892, Assay ID Ce02418781_m1).

Table 2.4 qPCR primers used in Chapter 2

Gene	Primer sequence (F/R)
cdk-8	CTGCATGCAGAGAAATTGCTC / TGATAACGTGCCAGAGATCG
lin-35	CGAGACGAACTTGGAAGACC / CCAGCATTGTGATTTTGCAC
efl-1	TGCTCCAGATGAAATGATGG / TCTCCGGTGCTCGAATAAAC
dpl-1	GCGGAAGAAGTCAAAACTGC / CGTATTGGGCTTGTGAGAGG
mdt-15	CACGACCCGGTCTTTCGTC / CTAGACCACCGCTTGTCTGG
mdt-27	CCCACAAACTTCGCCCAATG / AAGGGACCTGTGACTCAAAC
mdt-29	TCTCGACGGGGAGGTTATG / TGTCGTCTTCGCATACTTTCC
col-127	AGCTTGGTGCCAGTGTGAG / TGTGGGCAGTTGATTGGAG
K03D3.2	GGGTTGATGGAAAGGGAAAC / ACGACATTCTTGCCCTTGAG

Gene	Primer sequence (F/R)
acd-2	TGTGATGGAGCTGAAATTCG / TTCTGTCGACTCGTTTGGTG
Y49G5A.1	CCCATTGGCTTTTGAGTACAG / CGGTTTGGCAGTTTTTATCG
fat-7	TTTCCACCACACATTCCCAC / TCTTCACTTCCGTGATTGGC
ftn-1	TGGAAGAGCAGGTACATTCG / GCCGGCTCTCTTGATATTTG
nlp-4	CTCCAATCCGTGTTCCAGTT / ACTGAAAGACAAGTCTCTCTTCACAC
ubc-2	AGGGAGGTGTCTTCTTCCTCAC / CGGATTTGGATCACAGAGCAGC
tba-1	GTACACTCCACTGATCTCTGCTGACAAG / CTCTGTACAAGAGGCAAACAGCCATG
act-1	GCTGGACGTGATCTTACTGATTACC / GTAGCAGAGCTTCTCCTTGATGTC

2.5.6 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.

2.5.7 Generation of transgenic strains

The *cdk-8(+)* rescue transgene was generated by cloning the *cdk-8* genomic locus including promoter and 3' UTR into the PCR Blunt II TOPO vector. The *lin-31* promoter-driven tissue-specific rescue transgene was generated by sub-cloning promoterless *cdk-8* (from start codon to 3' UTR) into the pB255 vector (Tan *et al.* 1998). The *dpy-7* promoter-driven tissue-specific rescue transgene was generated by cloning the *dpy-7* promoter (Gilleard *et al.* 1997) into the GFP vector pPD95.77, then excising and replacing GFP with promoterless *cdk-8*. Kinase dead *cdk-8* was generated by site-directed mutagenesis of the *cdk-8(+)* plasmid using the Q5

Site-Directed Mutagenesis kit (NEB E0554S). Cloning primers are listed in Table 2.5.

cdk-8 rescue strains (steEx43, 45-47) or *cdk-8P::GFP* reporter strain were generated by gonad microinjection of a mixture of 50 ng/µl rescue plasmid, 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 2.5 Cloning primers used in Chapter 2.

	Primer sequences (F/R)
cdk-8P	aagcttCCTGGAAATAAATTTAAAACTCT /ggatccAGTGCAAATTTCCAACTTACGTG
cdk-8(+)	CCTGGAATTAAATTTAAAACTCTTTTCAG /ATTTTATTGTGAACGTATTTCAAAAAAATAC
Promoterless *cdk-8* (for *lin-31P*)	ctcgagATGACGTAAGTTGGAAATTTGC / gcggccgcATTTTATTGTGAACGTATTTCAAAAAAATAC
Promoterless *cdk-8* (for *dpy-7P*)	ggtaccATGACGTAAGTTGGAAATTTGC / cttaagATTTTATTGTGAACGTATTTCAAAAAAATAC
dpy-7P	aagcttCATCTCTTCTCGTTTGGAATC / ggatccAAGAACATGGATTGTAGAAAACG
cdk-8(KD)	GTAAAAATTGCTGcTTTGGGATTTTC /CCTAGAATTATTATTTCATATTATCGC

2.5.8 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°C 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. F1 progeny were grown on RNAi plates (20°C or 23°C) until they reached the desired developmental stage.

2.5.9 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).

Chapter 3: The *cdk-8* Mediator subunit promotes cadmium-responsive transcription

3.1 Synopsis

Organisms mount a strong transcriptional response to the toxic heavy metal cadmium, making it an excellent paradigm to study inducible transcription. Previous studies of cadmium-inducible transcription in *C. elegans* have focused on relatively simple promoters containing few regulatory elements. Here, I dissected the highly modular, cadmium-responsive gene 1 (*cdr-1*) promoter to identify Mediator subunits, TFs, and regulatory elements that cooperate in cadmium-inducible transcription. I identified a novel role for the Mediator subunit *cdk-8* in cadmium-inducible transcription and cadmium resistance. Furthermore, using a *cdr-1* promoter reporter, I showed that *cdk-8* cooperates with other factors with known roles in cadmium-responsive transcription: the Mediator tail module subunit *mdt-15*, the GATA-family TF *elt-2* (and GATA elements), and the high zinc-activated (HZA) element. Although the *cdr-1* promoter also contains regulatory elements recognized by the stress-responsive TFs SKN-1/Nrf2 and HSF-1, I demonstrated that these factors are not required for *cdr-1* induction. Finally, I showed that the stress-responsive TF DAF-16/FoxO and its cognate regulatory element are required to repress cadmium-inducible *cdr-1* transcription. Thus, my study expands the known repertoire of regulators in the cadmium response to include the coactivator CDK-8 and the repressor DAF-16, implying that, together with MDT-15, ELT-2 and an HZA-binding TF, these factors provide tight, combinatorial control of a highly complex cadmium-inducible promoter.

3.2 Background

Cadmium is a toxic heavy metal that causes extensive cellular damage by perturbing redox balance, protein folding or function, and proteolysis (Vallee and Ulmer 1972; Figueiredo-Pereira *et al.* 1998). Consequently, organisms mount a strong gene expression response to cadmium exposure. Cadmium-induced transcription has been characterized in *C. elegans*: early cadmium-responsive genes (induced within 4 hours of exposure, although earlier time points have not been tested) are involved in metal ion homeostasis, general stress responses, and energy metabolism, whereas late cadmium-responsive genes (following 24 hours exposure) include the continued expression of many early genes, in addition to proteolysis genes (Cui *et al.* 2007). Among the most highly induced early genes are the metallothionein-family metal binding protein genes, *mtl-1* and *mtl-2*, and a lysosomal integral membrane protein gene, cadmium-responsive gene 1 (*cdr-1*), which are redundantly required for organismal cadmium resistance (Liao *et al.* 2002; Cui *et al.* 2007).

Several studies have identified TFs and/or DNA regulatory elements that drive cadmium-responsive transcription (Figure 3.1A, C). Mutational analysis of the *mtl-1* and *mtl-2* promoters revealed that GATA elements, and the intestinal GATA-family TF ELT-2, promote cadmium-inducible gene expression (Moilanen *et al.* 1999). Regulation of these promoters by ELT-2 appears to be direct, as ELT-2 binds *in vitro* to an *mtl-1* GATA element (Moilanen *et al.* 1999). However, as transcriptome profiling of the *C. elegans* intestine indicates that ELT-2 activates virtually all genes expressed in the adult intestine (McGhee *et al.* 2007), regulation by ELT-2 and GATA sites does not appear to be specific to cadmium-inducible transcription. Recently, a regulatory element activated by excess zinc, the high zinc-activated (HZA) element, was

identified in the promoters of several cadmium-responsive genes, including *mtl-1*, *mtl-2*, and *cdr-1* (Roh *et al.* 2015). The HZA is often found in close proximity to a GATA element, and indeed, tandem repeats of a 62 bp HZA and GATA element-containing fragment of the *mtl-1* promoter are sufficient for cadmium-inducible transcription from a minimal promoter (Roh *et al.* 2015). Thus, the HZA element appears to recruit an ancillary TF that cooperates with ELT-2 to regulate cadmium-inducible transcription. In addition to these elements and factors, DAF-16, the *C. elegans* FoxO TF ortholog which is the canonical stress-responsive TF repressed by the insulin signaling pathway (Kenyon *et al.* 1993), may regulate cadmium-inducible transcription. Inactivation of the insulin signaling pathway, which derepresses DAF-16 activity, results in increased resistance to cadmium toxicity (Barsyte *et al.* 2001) and increased *mtl-1* mRNA levels in the absence of cadmium (Murphy *et al.* 2003), both of which are dependent on *daf-16*. Furthermore, the *mtl-1* promoter contains a DAF-16 binding element (DBE) consensus sequence (Murphy *et al.* 2003). Activation of *mtl-1* transcription by DAF-16 appears to be direct, as DAF-16 binds to the *mtl-1* promoter *in vivo* (Zhang *et al.* 2013). Thus, DAF-16 may promote cadmium resistance by inducing detoxification genes such as *mtl-1*. Finally, the Mediator tail module subunit *mdt-15* is required for cadmium-induced expression of several cadmium detoxification genes, including *mtl-1*, *mtl-2*, and *cdr-1* (Taubert *et al.* 2008), suggesting that MDT-15 may coactivate ELT-2, the HZA-binding TF, DAF-16, or perhaps other TFs in the cadmium response.

MDT-15 is critical for transcriptional responses to multiple stressors, including oxidizing agents, organic carcinogens, excess zinc, cadmium, and pathogenic bacteria (Taubert *et al.* 2008; Goh *et al.* 2014; Pukkila-Worley *et al.* 2014; Roh *et al.* 2015). In oxidative stress responses, the MDT-15-binding TFs SKN-1/Nrf2, NHR-49 and NHR-64 are required for stress-responsive

transcription and/or stress resistance (Goh *et al.* 2014). Beyond this, little is known about the MDT-15-TF interactions driving other *mdt-15*-dependent stress responses. Notably, *nhr-49* is not required for the *mdt-15*-dependent transcriptional response to organic carcinogens (Taubert *et al.* 2008), suggesting that MDT-15 may engage distinct TFs in different stress responses.

Promoters are made up of modular arrangements of regulatory elements, which can confer developmental stage-specific, cell type-specific, and stimulus-specific gene expression patterns (Gaudet and McGhee 2010). The promoters of the cadmium-inducible metallothionein genes *mtl-1* and *mtl-2* are relatively simple, containing only GATA, HZA, DBE or a putative hypoxia response element (HRE; described in Section 3.4.1) (Figure 3.1A). In contrast, the promoter of the cadmium-inducible gene *cdr-1* contains multiple putative regulatory elements in addition to GATA, HZA, and DBE elements (Figure 3.1A). These include putative binding sites for several stress-responsive TFs: two antioxidant response element-like (ARE-like), which bind the oxidative stress-responsive TF SKN-1, the *C. elegans* Nrf2 ortholog (Blackwell *et al.* 1994; An and Blackwell 2003); four metal response elements (MRE) (Stuart *et al.* 1984; Chen *et al.* 1999), for which a cognate TF in *C. elegans* remains unknown; and a heat shock element (HSE) (Sorger 1991), which binds the HSF-1 heat shock-responsive TF (Chiang *et al.* 2012) (Figure 3.1C). Thus, additional TFs and regulatory elements may be involved in cadmium-responsive transcription of *cdr-1*. As the Mediator subunit *mdt-15* is required for cadmium-induced expression of *cdr-1*, *mtl-1* and *mtl-2*, this suggests that different combinations of TFs could interact with MDT-15 at these distinct promoters. In addition, it is worth noting that the requirements for additional Mediator subunits in the cadmium response have not been tested; therefore, additional Mediator subunits may cooperate with MDT-15 to regulate cadmium-inducible transcription.

Here, I show that, in addition to *mdt-15*, the *cdk-8* Mediator subunit is required for cadmium-inducible transcription and cadmium resistance *in vivo*. Both *cdk-8* and *mdt-15* are required for both basal and cadmium-induced expression of the complex *cdr-1* promoter. Investigating regulatory elements and TFs required for cadmium induction of the *cdr-1* promoter, I find that *elt-2* and GATA elements, and the HZA element are required for basal and cadmium-induced expression of *cdr-1*, suggesting that these factors may cooperate with *cdk-8* and *mdt-15* at this promoter. In contrast, other stress-responsive TFs, *skn-1* and *hsf-1*, whose cognate regulatory elements are also found in the *cdr-1* promoter, are dispensable for basal and cadmium-induced expression of this promoter. In addition, I show that DAF-16, acting through the DBE, represses cadmium-induced transcription of the *cdr-1* promoter. Thus, I define TFs that act together with or in parallel to *cdk-8* and *mdt-15* to promote cadmium-inducible transcription, in opposition to negative regulation by DAF-16.

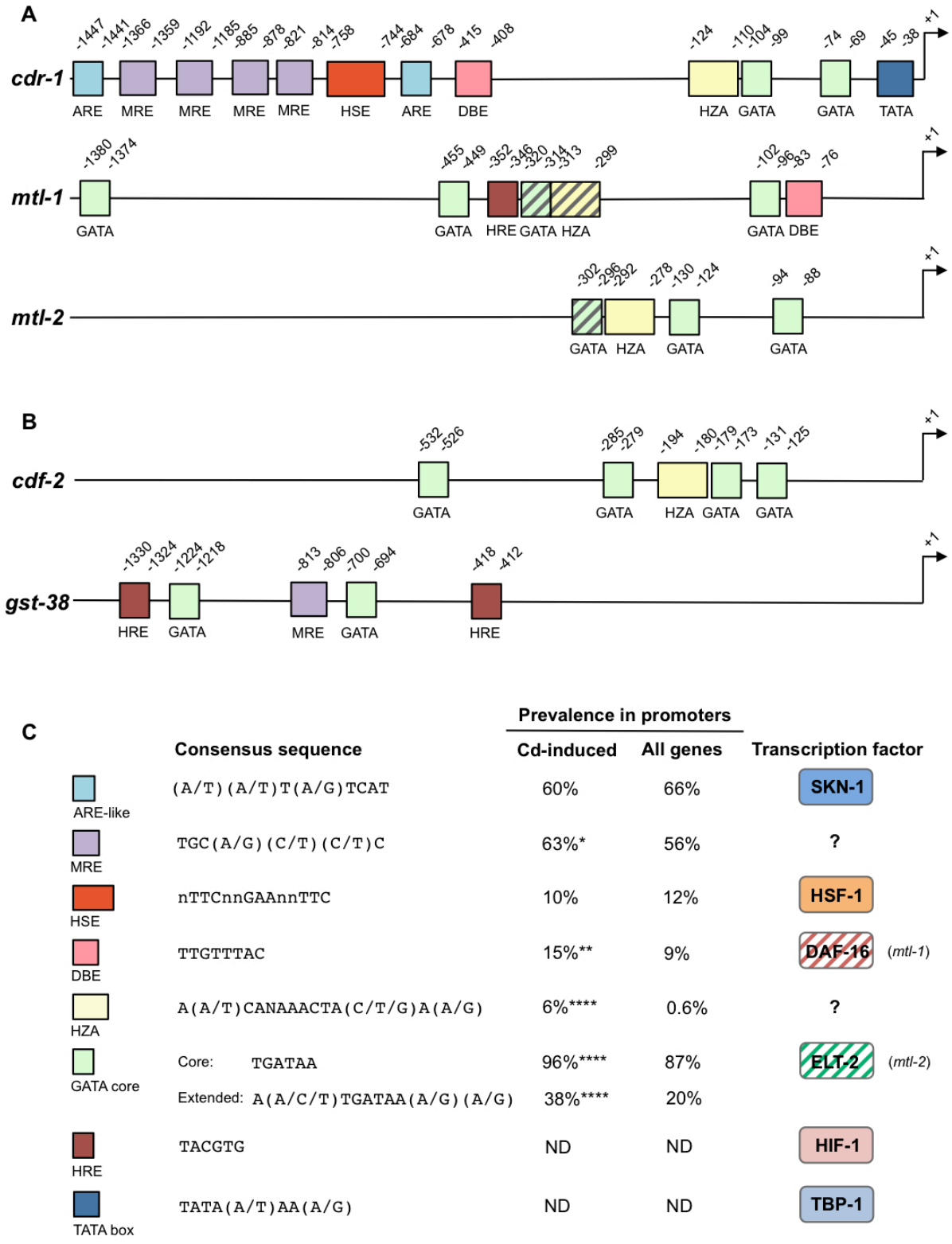

Figure 3.1 Regulatory elements in promoters of *cdr-1*, *mtl-1* and *mtl-2* cadmium-responsive genes.

(A) Diagram of regulatory elements found in promoters (-1500 base pairs) of *cdr-1, mtl-1,* and *mtl-2* (not drawn to scale). Gray hatching denotes regulatory elements that activate cadmium-inducible transcription of *mtl-1* or *mtl-2* (Moilanen *et al.* 1999; Roh *et al.* 2015). (B) Diagram of regulatory elements found in promoters (-1500 base pairs) of two *cdk-8*-independent cadmium-inducible genes, *cdf-2* and *gst-38*. (C) Regulatory element consensus sequences and their prevalence upstream (-1500 base pairs) of cadmium-inducible genes *vs.* all *C. elegans* genes, and cognate TFs. *$p<0.05$, **$p<0.01$, ****$p<0.0001$ *vs.* all genes. Regulatory elements for which cognate TF is unknown are indicated by a question mark. Regulatory element abbreviations: ARE, antioxidant response element; MRE, metal response element; HSE, heat shock response element; DBE, DAF-16 binding element; HZA, high zinc-activated element; HRE, hypoxia response element. White hatching denotes TFs that activate *mtl-1* or *mtl-2* transcription (as indicated) and/or promote cadmium resistance (Moilanen *et al.* 1999; Barsyte *et al.* 2001; Zhang *et al.* 2013).

3.3 Results

3.3.1 *cdk-8* is required for induction of cadmium-responsive genes

To identify *cdk-8*-dependent genes, we compared the transcriptional profiles of developmentally synchronized L4 larval stage *cdk-8(tm1238)* null mutants to wild-type N2 worms using microarrays. In total, 829 genes were upregulated and 461 genes were downregulated more than two-fold in *cdk-8* null mutants. To identify biological processes that require *cdk-8,* we then compared the *cdk-8*-dependent gene set to other published gene lists using EASE (Hosack *et al.* 2003; Engelmann *et al.* 2011). This unbiased analysis revealed significant overlaps between *cdk-8*-dependent genes (up- or downregulated in *cdk-8* mutants) and cadmium-responsive genes (up- or downregulated in response to cadmium exposure; (Cui *et al.* 2007)), with the exception of *cdk-8*-repressed genes and cadmium-activated genes (Figure 3.2A). Using quantitative PCR (qPCR), I validated selected overlapping genes to demonstrate

that they are indeed downregulated (*cdr-1, acdh-1,* T24B8.5) or upregulated (C31B8.4) in *cdk-8* mutants compared to N2, in the absence of cadmium exposure (Figure 3.2B). Overall, these data demonstrate that *cdk-8* is required for basal expression of a subset of cadmium-responsive genes, although direct action of CDK-8 at these promoters has not yet been established.

I next tested whether *cdk-8* promotes induction of cadmium-responsive genes upon cadmium exposure by comparing the relative mRNA expression of three highly cadmium-inducible genes, *cdr-1, mtl-1*, and *mtl-2*, in N2 and *cdk-8* mutants prior to (0 hours) or following (4 hours) exposure to 100 μM cadmium (Cd^{2+}). Unlike the basal expression of *cdr-1*, basal expression of *mtl-1* and *mtl-2* was not significantly altered in *cdk-8* mutants compared to wild type (Figure 3.2C). All three of these genes were strongly induced by cadmium exposure in N2 (Figure 3.2C). Cadmium induction of *cdr-1* and *mtl-1* was significantly impaired in *cdk-8* mutants compared to wild type; *mtl-2* showed a similar trend (Figure 3.2C). However, induction of two additional cadmium-responsive genes that were not differentially expressed in *cdk-8* mutant microarrays, *cdf-2* and *gst-38,* was not affected in *cdk-8* mutants (not shown). In addition, using a transcriptional GFP reporter, I found that *cdk-8* is required for basal expression and cadmium induction of a 2.8 kb promoter upstream of the *cdr-1* transcription start site (see methods for details on reporter construction; Figure 3.2D). Thus, *cdk-8* is required for full induction of a subset of cadmium-responsive genes.

I next compared the promoters of *cdk-8*-dependent (*cdr-1, mtl-1, mtl-2*) *vs. cdk-8*-independent cadmium-responsive genes (*cdf-2, gst-38)*, to identify regulatory elements that may define *cdk-8*-dependent cadmium-responsive promoters. GATA and HZA elements are the only regulatory elements common to the *cdk-8*-dependent promoters *cdr-1*, *mtl-1*, and *mtl-2*, and indeed these are the only identifiable elements in the *mtl-2* promoter (Figure 3.1A). The *gst-38*

promoter contained GATA, MRE and HRE sites (Figure 3.1B); unlike the *cdk-8*-dependent cadmium-responsive promoters, the *gst-38* promoter did not contain an HZA element. The *cdf-2* promoter, however, contained several GATA elements and one HZA element, similar to the *mtl-2* promoter (Figure 3.1B). Therefore, based on this small sample of promoters, I cannot identify a set of regulatory elements that define *cdk-8*-dependent *vs. cdk-8*-independent cadmium-responsive promoters. Future work will include unbiased analysis of the promoters of the set of *cdk-8*-dependent cadmium-responsive genes *vs. cdk-8*-independent cadmium-responsive genes identified in our microarray comparison (Figure 3.2A), to identify over-represented TF binding motifs.

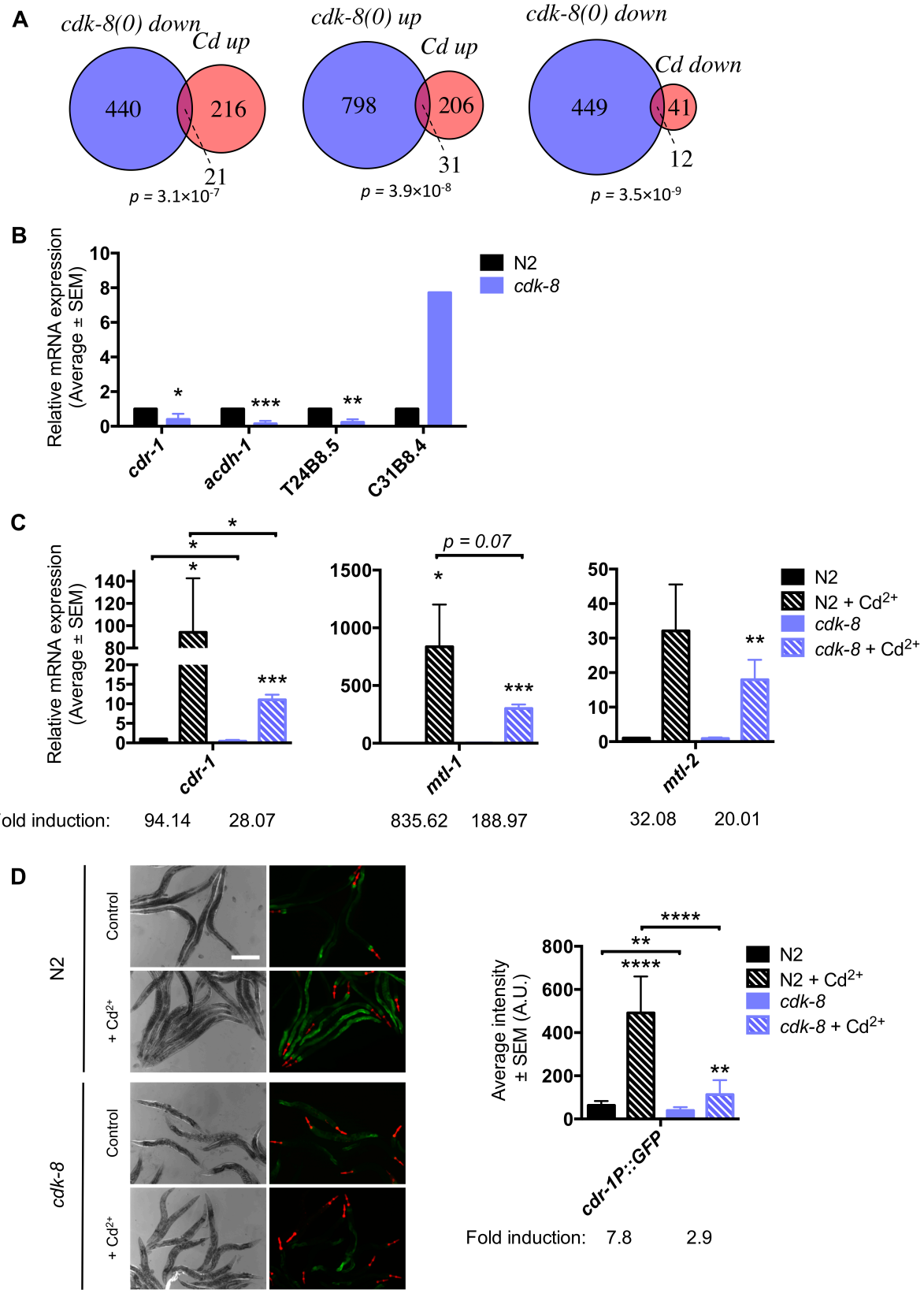
A
cdk-8(0) down
Cd up
440
216
21
p = 3.1×10-7
cdk-8(0) up
Cd up
798
206
31
p = 3.9×10-8
cdk-8(0) down
Cd down
449
41
12
p = 3.5×10-9
B
Relative mRNA expression (Average ± SEM)
N2
cdk-8
cdr-1
acdh-1
T24B8.5
C31B8.4
C
Relative mRNA expression (Average ± SEM)
p = 0.07
N2
N2 + Cd2+
cdk-8
cdk-8 + Cd2+
cdr-1
mtl-1
mtl-2
Fold induction: 94.14 28.07 835.62 188.97 32.08 20.01
D
N2
cdk-8
Control
+ Cd2+
Average intensity ± SEM (A.U.)
cdr-1P::GFP
Fold induction: 7.8 2.9

Figure 3.2 *cdk-8* is required for cadmium-inducible gene expression.

(A) Venn diagrams depicting statistically significant overlaps between *cdk-8*-dependent genes and cadmium-responsive genes. Fisher's exact test *p*-values shown. (B) qPCR analysis of cadmium-responsive genes in *cdk-8* mutants grown in standard culture conditions. Error bars: SEM, $n = 3$ independent trials. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* N2, unpaired t-test. (C) qPCR analysis of cadmium-responsive genes in wild type or *cdk-8* mutants exposed for 4 hours to 100 μM Cd^{2+} (or no cadmium control). Fold induction values represent the relative mRNA expression in cadmium-exposed worms divided by the relative mRNA expression in control (no cadmium) worms within a genotype. Error bars: SEM, $n = 3$ independent trials. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* no cadmium control in the same genotype, or for the comparison indicated, unpaired t-test. (D) Representative images of *cdr-1P::GFP* transcriptional reporter (green) in wild type or *cdk-8* mutants exposed for 4 hours to 100 μM Cd^{2+} (or no cadmium control). Co-injection marker: *myo-2P::mCherry* (red). Scale bar: 300 μm. Bar graph represents average fluorescence intensity (arbitrary units, A.U.); error bars: SEM, $n \geq 10$ worms. ** $p < 0.01$, **** $p < 0.0001$ *vs.* no cadmium control in the same genotype, or for the comparison indicated, unpaired t-test.

3.3.2 *cdk-8* is required for cadmium resistance *in vivo*

To further verify the requirement for *cdk-8* in the cadmium response, I tested if loss of *cdk-8* causes cadmium sensitivity. Cadmium sensitive worms show impaired larval growth when grown in the presence of cadmium (Cui *et al.* 2007). Therefore, I compared the developmental rate of *cdk-8* and wild-type worms grown in the presence of 0 (control), 2.5, 5, 10, or 25 μM cadmium. In the absence of cadmium, *cdk-8* mutants developed slightly slower than wild type, as 94-98% of wild-type but only ~72-82% of *cdk-8* mutants developed from L1 stage worms into L4 stage worms in 40 hours at 20°C (starting from synchronized arrested L1 larvae; see methods for details); therefore, I normalized all developmental rate data to the fraction of animals reaching L4 in the 0 μM cadmium control condition within each genotype ('normalized fraction L4', Figure 3.3). I found that some wild-type worms exhibited delayed growth in the

presence of 10 μM cadmium, and nearly all were delayed in 25 μM cadmium (Figure 3.3). *cdk-8* mutants showed a strong trend toward growth delay starting at 5 μM cadmium, and a significantly greater proportion of *cdk-8* mutants exhibited delayed growth in 10 μM cadmium compared to wild type (Figure 3.3). Therefore, *cdk-8* mutants exhibit increased cadmium sensitivity compared to wild type, suggesting that *cdk-8* is required for cadmium resistance in *C. elegans*.

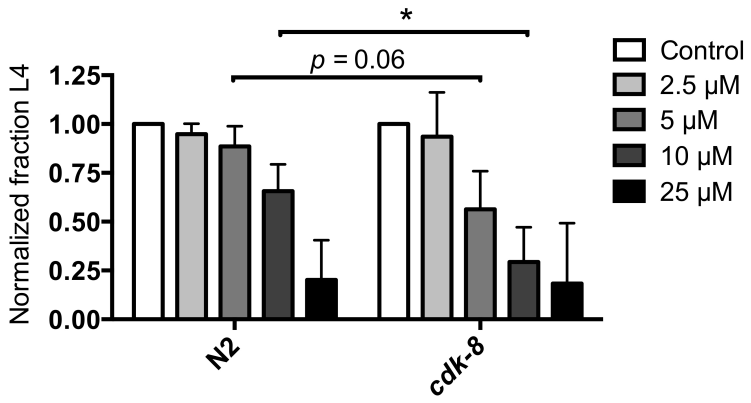

Figure 3.3 *cdk-8* is required for cadmium resistance in *C. elegans*.

Cadmium sensitivity of *cdk-8* mutants as assessed by severity of growth delay when raised in increasing concentrations of cadmium. Normalized fraction L4 represents the fraction of animals that reached L4 after 40 hours at 20°C relative to no cadmium control (see Materials and methods, Section 3.5.5 for details). Error bars: SEM, $n = 3$ independent trials. * $p < 0.05$, unpaired t-test.

3.3.3 *mdt-15* is required for basal expression and induction of the *cdr-1* promoter

As the Mediator subunit *mdt-15* was previously found to be required for cadmium-inducible transcription, including induction of *cdr-1*, *mtl-1*, and *mtl-2* (Taubert *et al.* 2008), I wished to verify that *mdt-15,* like *cdk-8,* is required at the 2.8 kb *cdr-1* promoter. RNAi

knockdown of *mdt-15* caused a statistically significant ~3-fold decrease in *cdr-1P::GFP* reporter expression in the absence of cadmium compared to empty vector (EV) RNAi (Figure 3.4). Furthermore, *mdt-15* knockdown completely abrogated induction of the *cdr-1P::GFP* reporter in response to cadmium (Figure 3.4). Therefore, *mdt-15* is required for both basal expression and cadmium induction of the *cdr-1* promoter.

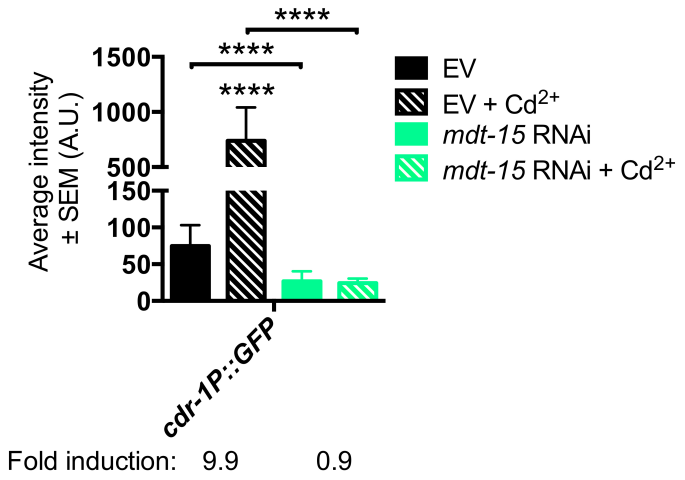

Figure 3.4 *mdt-15* is required for cadmium-inducible transcription of the *cdr-1* promoter.

Fluorescence intensity of *cdr-1P::GFP* transcriptional reporter with empty vector (EV) or *mdt-15* RNAi exposed for 4 hours to 100 µM Cd^{2+} (or no cadmium control). A.U.: arbitrary units. Error bars: SEM, $n \geq 19$ worms. **** $p <$ 0.0001 *vs.* no cadmium control in the same genotype, or for the comparison indicated, unpaired t-test. Fold induction values represent the average fluorescence intensity in cadmium-exposed worms divided by the average fluorescence intensity in control (no cadmium) worms within a genotype.

3.3.4 *elt-2,* GATA, and HZA are required for induction of the *cdr-1* promoter

Next, I aimed to identify TFs that cooperate with *cdk-8* and *mdt-15* to regulate cadmium responsive genes such as *cdr-1*. The *cdr-1* promoter contains several regulatory element consensus sequences (Figure 3.1A, Figure 3.5A), which may recruit specific TFs. Comparison of

the prevalence of these consensus regulatory elements upstream of cadmium-inducible genes (Cui *et al.* 2007) *vs.* all *C. elegans* genes showed that HZA elements are enriched 10-fold upstream of cadmium-inducible genes compared to all *C. elegans* genes (Figure 3.1C). This estimate may be low due to the limitations of using a consensus sequence. In addition, GATA elements, DBEs, and MREs are slightly but significantly enriched upstream of cadmium-inducible genes compared to all *C. elegans* genes (Figure 3.1C). This analysis may also have missed additional enrichments due to the limitations of consensus sequences.

As HZA and GATA elements are sufficient to drive cadmium inducible gene expression from a minimal promoter (Roh *et al.* 2015), I first tested whether HZA and GATA elements are required for cadmium induction of the *cdr-1* promoter. To this end, I generated substitution mutations in the *cdr-1P::GFP* reporter's HZA site (*mutHZA*) or in its two GATA sites (*mutGATA1* and *mutGATA2*; Table 3.1).

Table 3.1 *cdr-1* promoter regulatory element mutants.

	Strand	Wild-type[a]	Mutant[a]	References
HZA	(+)	**AACAGAAACTACAAT**	**GGTGAGGGTCGTGGC**	Roh *et al.* 2015
GATA1	(-)	TG**TGATAA**AA	TG**CAGCGG**AA	Liao *et al.* 2002, McGhee *et al.* 2007
GATA2	(-)	TG**TGATAA**GA	TG**CAGCGG**GA	Liao *et al.* 2002, McGhee *et al.* 2007
MRE	(+)	**TGCACCC**	**CATGTTT**	Stuart *et al.* 1984, Chen *et al.* 1999, Liao *et al.* 2002
DBE	(+)	**TGTTTAC**	**CACCCGT**	Furuyama *et al.* 2000, Murphy *et al.* 2003

[a] Residues targeted by site-directed mutagenesis shown in bold.

Mutation of the HZA element caused a ~2.5-fold decrease in basal *cdr-1P::GFP* reporter expression, and a ~40-fold decrease in cadmium-induced expression compared to the wild-type *cdr-1P* reporter (Figure 3.5B). Furthermore, mutation of the HZA abrogated cadmium induction of the *cdr-1P* reporter (Figure 3.5B). Mutation of GATA1 had no effect on basal expression of the *cdr-1P,* but abrogated cadmium induction (Figure 3.5C). In contrast, mutation of GATA2 caused a ~4-fold decrease in basal *cdr-1P::GFP* reporter expression, and a ~7-fold decrease in cadmium-induced expression compared to the wild-type *cdr-1P* reporter (Figure 3.5C). The *cdr-1P(mutGATA2)::GFP* reporter was still induced by cadmium exposure, but the fold induction was reduced to about half that of the wild-type reporter (Figure 3.5C). Therefore, the GATA1 element is required only for cadmium-induced expression, whereas the HZA and GATA2 elements are required for both basal and cadmium-induced expression of the *cdr-1* promoter.

The ELT-2 TF binds to GATA elements, and is required for cadmium induction of the metallothionein gene *mtl-2*, whose promoter contains GATA elements (Moilanen *et al.* 1999). Therefore, I tested if *elt-2* is required for basal or cadmium-induced expression of the *cdr-1* promoter. RNAi knockdown of *elt-2* caused a ~2-fold decrease in basal *cdr-1P::GFP* reporter expression compared to EV RNAi, and completely abrogated cadmium induction of the *cdr-1P* reporter (Figure 3.5D). As *elt-2* is required for intestinal differentiation and function (McGhee 2013), I also tested if post-developmental *elt-2* knockdown would cause similar defects in basal and inducible expression of the *cdr-1* promoter. Indeed, worms exposed to *elt-2* RNAi during the first two days of adulthood showed significant defects in basal and cadmium-induced expression of the *cdr-1P::GFP* reporter (Figure 3.5E). Therefore, *elt-2* is required for basal expression and cadmium induction of the *cdr-1* promoter, independently of its role in development. Currently I cannot rule out the possibility that *elt-2* is required to maintain intestinal integrity in adults; however, I did not observe any gross morphological defects in intestines of animals treated with *elt-2* RNAi in adulthood only.

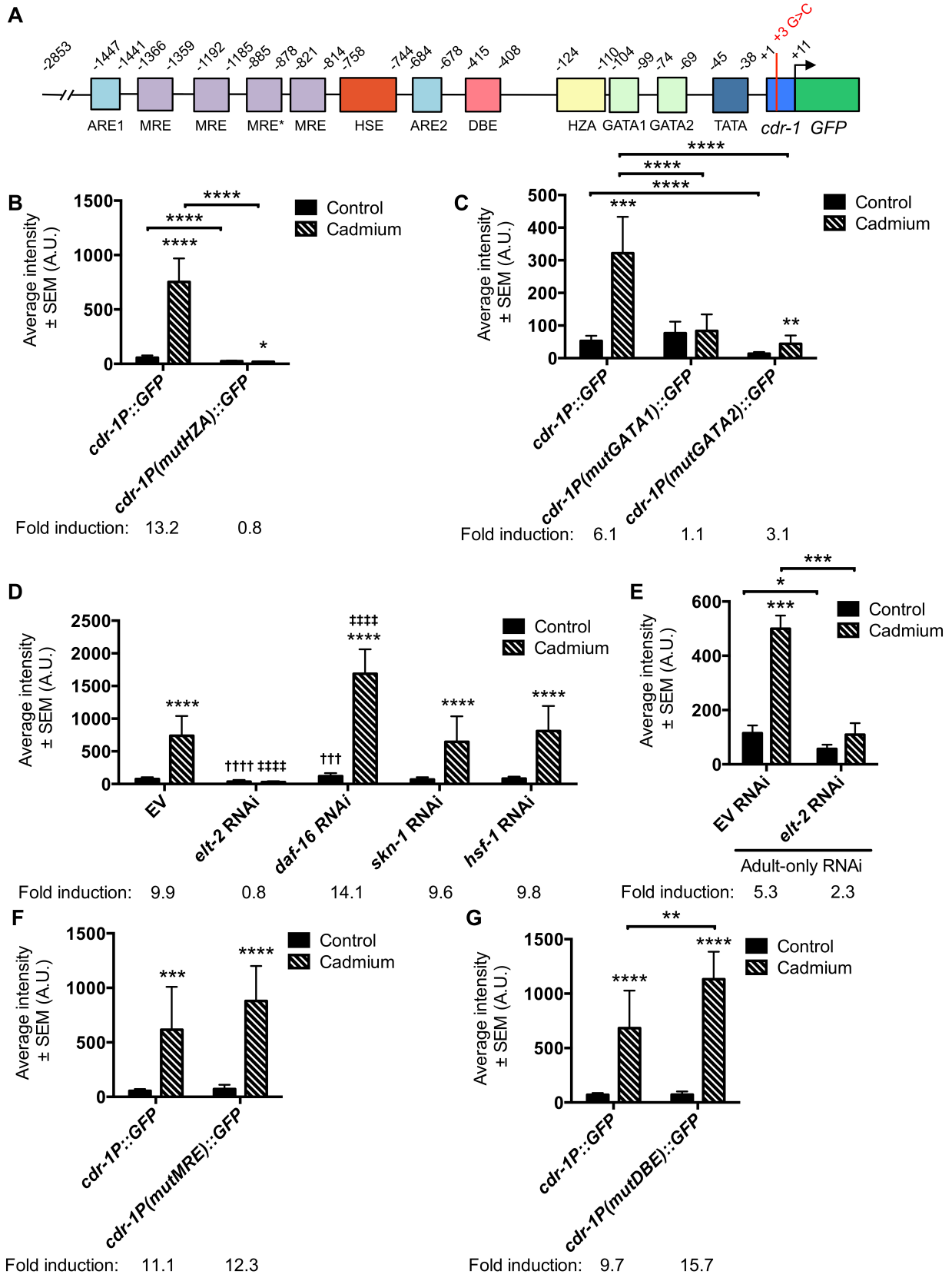
A
-2853
-1447
-1441
-1366
-1359
-1192
-1185
-885
-878
-821
-814
-758
-744
-684
-678
-415
-408
-124
-110
-104
-99
-74
-69
-45
-38
+1
+3 G>C
+11
ARE1
MRE
MRE
MRE*
MRE
HSE
ARE2
DBE
HZA
GATA1
GATA2
TATA
cdr-1
GFP
B
Average intensity ± SEM (A.U.)
Control
Cadmium
cdr-1P::GFP
cdr-1P(mutHZA)::GFP
Fold induction: 13.2 0.8
C
Average intensity ± SEM (A.U.)
Control
Cadmium
cdr-1P::GFP
cdr-1P(mutGATA1)::GFP
cdr-1P(mutGATA2)::GFP
Fold induction: 6.1 1.1 3.1
D
Average intensity ± SEM (A.U.)
Control
Cadmium
EV
elt-2 RNAi
daf-16 RNAi
skn-1 RNAi
hsf-1 RNAi
Fold induction: 9.9 0.8 14.1 9.6 9.8
E
Average intensity ± SEM (A.U.)
Control
Cadmium
EV RNAi
elt-2 RNAi
Adult-only RNAi
Fold induction: 5.3 2.3
F
Average intensity ± SEM (A.U.)
Control
Cadmium
cdr-1P::GFP
cdr-1P(mutMRE)::GFP
Fold induction: 11.1 12.3
G
Average intensity ± SEM (A.U.)
Control
Cadmium
cdr-1P::GFP
cdr-1P(mutDBE)::GFP
Fold induction: 9.7 15.7

Figure 3.5 *elt-2* and an HZA-binding TF are required for cadmium-inducible expression of the *cdr-1* promoter.

(A) Diagram of the *cdr-1P::GFP* reporter (not drawn to scale). The first 11 base pairs downstream of the *cdr-1* transcription start site are included in the reporter, but the *cdr-1* start codon is mutated (G to C mutation at the +3 position) whereas the GFP start codon is intact. The MRE identified by Freedman and colleagues (Liao *et al.* 2002), marked with an asterisk (*), is mutagenized in panel F. (B-C) Fluorescence intensity of wild-type *cdr-1P::GFP* transcriptional reporter compared to (B) HZA or (C) GATA site mutants exposed for 4 hours to 100 μM Cd^{2+} (or no cadmium control). Error bars: SEM, $n \geq 18$ worms. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ *vs.* no cadmium control in the same genotype, or for the comparison indicated, unpaired t-test. (D) Fluorescence intensity of *cdr-1P::GFP* transcriptional reporter with empty vector (EV) or stress-responsive TF RNAi exposed for 4 hours to 100 μM Cd^{2+} (or no cadmium control). Error bars: SEM, $n \geq 14$ worms. **** $p < 0.0001$ *vs.* no cadmium control in the same genotype, $^{\dagger\dagger\dagger}p < 0.001$ or $^{\dagger\dagger\dagger\dagger}p < 0.0001$ *vs. cdr-1P::GFP* no cadmium control, $^{\ddagger\ddagger\ddagger\ddagger}p < 0.0001$ *vs. cdr-1P::GFP* with cadmium, unpaired t-test. (E) Fluorescence intensity of *cdr-1P::GFP* transcriptional reporter with empty vector (EV) or *elt-2* RNAi in first two days of adulthood. Error bars: SEM, $n = 3$ independent trials. * $p < 0.05$, *** $p < 0.001$, unpaired t-test. (F) Fluorescence intensity of *cdr-1P::GFP* transcriptional reporter and MRE mutant (MRE marked with asterisk in panel A) exposed for 4 hours to 100 μM Cd^{2+} (or no cadmium control). Error bars: SEM, $n \geq 6$ worms. *** $p < 0.001$, **** $p < 0.0001$ *vs.* no cadmium control in the same genotype. (G) Fluorescence intensity of *cdr-1P::GFP* transcriptional reporter and DBE mutant exposed for 4 hours to 100 μM Cd^{2+} (or no cadmium control). Error bars: SEM, $n \geq 8$ worms. ** $p < 0.01$, **** $p < 0.0001$ *vs.* no cadmium control in the same genotype, or for the comparison indicated, unpaired t-test. A.U.: arbitrary units. Fold induction values represent the average fluorescence intensity in cadmium-exposed worms divided by the average fluorescence intensity in control (no cadmium) worms within a genotype.

3.3.5 *daf-16/FoxO* is required to repress the *cdr-1* promoter

To determine if additional TFs regulate cadmium responsive promoters, I depleted additional stress-responsive TFs with corresponding putative regulatory elements in the *cdr-1* promoter. Specifically, *cdr-1P* contains putative SKN-1 binding sites (ARE-like), a putative

HSF-1 binding site (HSE), putative metal response elements (MRE), and a putative DAF-16 binding site (DBE) (Figure 3.1A, Figure 3.5A). RNAi knockdown of *skn-1* or *hsf-1* had no effect on basal or cadmium-induced expression of the *cdr-1P::GFP* reporter compared to EV RNAi (Figure 3.5D). There is no known MRE-binding TF in *C. elegans*, but mutation of the *cdr-1* promoter MRE identified by Freedman and colleagues (Liao *et al.* 2002) had no effect on its basal or cadmium induced expression (Table 3.1, Figure 3.5F). I note that the *cdr-1* promoter contains three additional MREs (Figure 3.1A), which I have not yet tested by mutagenesis. In contrast, *daf-16* knockdown caused a significant ~1.5-fold increase in both basal and cadmium-induced expression of *cdr-1P::GFP* compared to EV RNAi (Figure 3.5D). This implies that *daf-16* is required for repression of cadmium-induced *cdr-1* expression.

As *daf-16* typically acts as an activator of stress-responsive transcription (Murphy *et al.* 2003), we wished to test whether the observed derepression of the *cdr-1* promoter was a direct or indirect effect of *daf-16* depletion. To assess whether DAF-16 directly regulates the *cdr-1* promoter, we examined the requirement for the DBE in *cdr-1P::GFP* repression. Mutatino of the DBE caused no change in basal *cdr-1P::GFP* expression (Figure 3.5G), suggesting that the effect observed in *daf-16* RNAi is not directly mediated by the DBE. In contrast, mutation of the DBE caused a significant increase in cadmium-induced expression of the *cdr-1* promoter compared to the wild-type *cdr-1P::GFP* construct (Figure 3.5G). Taken together with the observation that DAF-16 binds to DBEs *in vitro* (Furuyama *et al.* 2000), this suggests that DAF-16 can act directly at the *cdr-1* promoter to repress cadmium-inducible expression.

3.3.6 Stress specificity of *cdr-1* promoter induction

I next wished to test if the DBE represses induction of *cdr-1* by stresses other than

cadmium. Induction of the *cdr-1* promoter is thought to be highly specific to cadmium and zinc stress (Liao *et al.* 2002; Roh *et al.* 2015). Namely, the *cdr-1* promoter does not appear to respond to other metals (copper, lead, mercury), oxidative stressors (juglone, paraquat), or heat shock, although this evidence comes from a LacZ reporter that is sensitive enough to detect cadmium-induced but not zinc-induced *cdr-1* transcription (Liao *et al.* 2002; Roh *et al.* 2015). Therefore, I began by testing whether the wild-type *cdr-1P::GFP* reporter is responsive to oxidative stress, using sodium meta-arsenite (henceforth 'arsenite'), an oxidizing agent and potent SKN-1 activator (An and Blackwell 2003; Goh *et al.* 2014); or to heat shock, which activates HSF-1-mediated transcription (Hsu *et al.* 2003). Exposure of worms expressing the wild-type *cdr-1P::GFP* reporter for 4 hours to 5 mM arsenite did not cause induction of the reporter (not shown). Heat shock for 1 hour at 37°C, followed by 1 hour recovery at 20°C, also did not cause *cdr-1P::GFP* reporter induction (not shown). Thus, *cdr-1P::GFP* is not induced by an oxidizing agent that activates SKN-1-mediated transcription, or to heat shock, which activates transcription via HSF-1. I hypothesized that DAF-16 and the DBE may repress responsiveness of *cdr-1* ARE-like and HSE regulatory elements to arsenite and heat shock, respectively. Therefore, I tested if *cdr-1P(mutDBE)::GFP* was responsive to arsenite or heat shock. *cdr-1P(mutDBE)::GFP* was not induced by either of these stresses (not shown). Therefore, the DBE does not repress induction of the *cdr-1* promoter by oxidative or heat stress.

3.4 Discussion

Exposure to the toxic heavy metal cadmium provokes a strong gene expression response in *C. elegans*. Previous studies have shown that the Mediator subunit *mdt-15,* GATA and HZA

regulatory elements, and the TF *elt-2* are required to induce this response (Moilanen *et al.* 1999; Taubert *et al.* 2008; Roh *et al.* 2015). However, these studies may have missed additional factors that fine-tune cadmium responsive transcription for two reasons: first, these studies did not test Mediator subunits other than *mdt-15* in the cadmium response, leaving open the possibility that other Mediator subunits may contribute to regulation of cadmium-inducible transcription; second, these studies primarily investigated simple cadmium-responsive promoters containing few regulatory elements, meaning that additional TFs could contribute to regulation of more complex cadmium-inducible promoters. Here, I demonstrated that the Mediator subunit *cdk-8* is required for cadmium-inducible transcription and cadmium resistance in *C. elegans*. Analysis of the complex *cdr-1* promoter revealed that *cdk-8, mdt-15*, *elt-2*, GATA elements, and an HZA element are each required for basal and cadmium-inducible *cdr-1* expression, implying that these factors act together or in parallel at this promoter. As ELT-2 and GATA elements drive virtually all intestinal gene expression in adult worms, I propose that ELT-2 is a permissive factor for intestinal expression of *cdr-1* (discussed in Section 3.4.5). Based on the fact that MDT-15 and CDK-8 engage NHR-family TFs in other contexts, and that NHRs are canonical xenobiotic stress-responsive TFs that may be capable of binding to cadmium, I propose that an NHR may regulate transcription via the HZA (discussed in Section 3.4.6). In addition, I found that *daf-16* and the DBE are required to repress cadmium induction of the *cdr-1* promoter. Thus, my study expands the known repertoire of regulators in the cadmium response to include the coactivator CDK-8 and the repressor DAF-16, implying that, together with MDT-15, ELT-2 and an HZA-binding TF, these factors provide combinatorial control of a complex cadmium-inducible promoter (Figure 3.6).

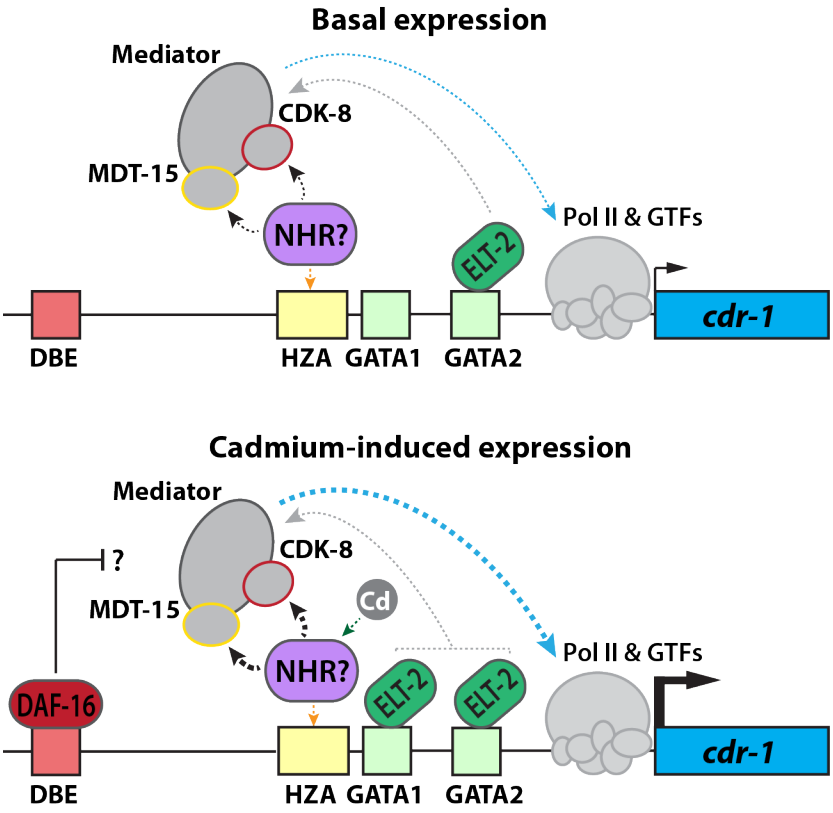

Figure 3.6 Proposed model of Mediator subunit, TF, and regulatory element roles in basal and cadmium-inducible transcription of the *cdr-1* promoter.

Top: Basal expression of the *cdr-1* promoter requires the Mediator subunits CDK-8 and MDT-15, the ELT-2 GATA-family TF, and the GATA2 and HZA regulatory elements. I propose that ELT-2 serves as a permissive factor enabling intestinal transcription, *e.g.* by recruiting Mediator to intestinal promoters (gray dashed arrow), whereas an NHR may bind to the HZA element (orange dashed arrow) and reinforce recruitment of the Mediator complex to the *cdr-1* promoter via specific interactions with CDK-8 and/or MDT-15 (black dashed arrows), thereby stimulating low levels of Pol II transcription (blue dashed arrow). Dashed arrows indicate proposed interactions. *Bottom:* Cadmium-induced expression of the *cdr-1* promoter requires CDK-8, MDT-15, ELT-2, both GATA elements, and the HZA. I propose that ELT-2 primarily serves as a permissive factor enabling induction of transcription in the intestine, *e.g.* by recruiting Mediator to intestinal promoters (gray dashed arrow). Furthermore, I propose that an NHR bound to the HZA element (orange dashed arrow) senses cadmium (green dashed arrow), which may enhance recruitment of Mediator via specific interaction with CDK-8 and/or MDT-15 (bold black arrows), thereby stimulating Pol II recruitment, initiation, or elongation (bold blue arrow). Finally, DAF-16 and the

DBE are required for repression of cadmium-induced *cdr-1* expression (repression arrow), but molecular target of DAF-16 remains unknown (indicated by '?'). Dashed arrows indicate proposed interactions.

3.4.1 *cdk-8* promotes cadmium-responsive gene expression

cdk-8-dependent genes identified in our microarray analysis overlapped significantly with genes that are activated or repressed by cadmium exposure ((Cui *et al.* 2007); Figure 3.2A). As noted in Chapter 2 (Section 2.3.1), these genes may be direct or indirect targets of CDK-8. It is also important to note that, as our microarray analysis of *cdk-8* mutants was conducted under standard laboratory conditions, *i.e.* in the absence of cadmium, this overlap only identified cadmium-responsive genes whose basal levels are altered in *cdk-8* mutants *vs.* wild type, *e.g. cdr-1* (Figure 3.2B). Upon cadmium exposure, *cdk-8* mutants also showed a substantial, although not complete, block in cadmium induction of *cdr-1*, and of other cadmium-responsive genes whose basal expression levels were unchanged in *cdk-8* mutants, *e.g. mtl-1* (Figure 3.2C). I note that the observed decrease in the fold induction of *e.g. cdr-1* and *mtl-1* in *cdk-8* mutants compared to wild type is not due to increased basal expression of these transcripts; in fact, their basal expression was decreased or unchanged in *cdk-8* mutants compared to wild type (Figure 3.2C). Taken together, our microarray and qPCR analysis suggest that *cdk-8* is required for basal and/or full cadmium-induced expression of a subset of cadmium-responsive genes. In the future, it will be interesting to expand this analysis, for example by genome wide expression profiling of *cdk-8* mutants compared to wild type upon cadmium treatment, to determine the proportion of cadmium-responsive genes that require *cdk-8* for cadmium induction.

In support of a requirement for *cdk-8* in the cadmium response, I also showed that *cdk-8* mutants are cadmium sensitive compared to wild type (Figure 3.3). Loss of individual cadmium-

responsive genes has little effect on cadmium sensitivity in *C. elegans*; however, in double or triple mutants in *mtl-1, mtl-2*, and *cdr-1*, or in *mtl-2* mutants subjected to RNAi knockdown of a panel of 50 other cadmium-responsive genes, cadmium toxicity phenotypes such as growth delay or egg laying defects become apparent (Cui *et al.* 2007; Hall *et al.* 2012). Thus, the *C. elegans* cadmium response appears to be highly redundant, perhaps due to the serious adverse effects of this heavy metal. My observation that *cdk-8* mutants are cadmium sensitive supports a model in which *cdk-8* acts upstream of multiple cadmium-responsive genes to activate their transcription.

A limitation of this study is that I have not assessed the tissue-specificity of *cdk-8* action in the cadmium response. The intestine is a major site of detoxification in *C. elegans* (McGhee 2007). Accordingly, *cdr-1* and *mtl-2* are expressed solely in the intestine, and *mtl-1* is expressed in the intestine and posterior pharynx (Figure 3.2D) (Freedman *et al.* 1993; Liao *et al.* 2002). *cdk-8* appears to be expressed in most if not all *C. elegans* tissues, including the intestine (Steimel *et al.* 2013), suggesting that CDK-8 likely regulates cadmium-responsive gene expression cell autonomously in the intestine. Further work is needed to establish whether intestinal expression of a wild-type *cdk-8* transgene is sufficient to rescue cadmium-responsive gene expression and cadmium sensitivity defects in *cdk-8* mutants.

CDK8 has largely been studied in the context of transcriptional regulation downstream of cell signaling pathways governing cell proliferation or differentiation in animal development or in cancer cell lines ((Firestein *et al.* 2008; Morris *et al.* 2008; Donner *et al.* 2010); and this work, Chapter 2). However, there is also evidence that CDK8 promotes certain stress-responsive transcription programs. In response to DNA damage, the tumor suppressor p53 can activate genes involved in cell cycle arrest, DNA damage repair, metabolism and apoptosis (Vousden

and Prives 2009). Among these is the CDK inhibitor $p21^{CIP1}$, encoded by the *CDKN1A* gene. Intriguingly, *CDKN1A* transcription is strongly activated by some stimuli but weakly activated by others, despite the fact that p53 is recruited to the *CDKN1A* promoter equally by these stimuli (Donner *et al.* 2007). This difference in expression correlates with increased CDK8 recruitment to the *CDKN1A* promoter during strong *vs.* weak induction, despite other Mediator subunits such as MED1 and MED17 (in the middle and head modules, respectively) being recruited equally by *CDKN1A*-activating stimuli (Donner *et al.* 2007). Furthermore, *CDK8* siRNA knockdown decreases *CDKN1A* induction by strong inducers (Donner *et al.* 2007). Thus, CDK8 promotes strong induction of p53 target genes, suggesting that CDK8 may act as a p53 coactivator in the response to genotoxic stress. Interestingly, cadmium is associated with accumulation of p53 protein, and induces p53-dependent apoptosis in some prostate cancer cell lines (Aimola *et al.* 2012); however, as cyotoxic doses of cadmium cause DNA damage (Filipic *et al.* 2006), p53 induction is likely an indirect effect of cadmium toxicity.

CDK8 is also implicated in the hypoxia response. In hypoxic conditions, the hypoxia inducible factors HIF1α and HIF2α activate genes involved in switching from oxidative to glycolytic glucose metabolism and genes involved in the oxidative stress response (Semenza 2012). Expression of many HIF1α target genes under hypoxic conditions requires CDK8, which recruits the elongation factors BRD4 and SEC (Galbraith *et al.* 2013). Thus, CDK8 acts as a HIF1α coactivator. Intriguingly, cadmium activates HIF1α expression in cell culture, via a mechanism involving enhanced production of reactive oxygen species (Jing *et al.* 2012). However, the *cdr-1* promoter does not contain a canonical HIF-1 binding site (HRE) (Shen *et al.* 2005), indicating that CDK-8-mediated regulation of HIF-1 likely does not play a role in

cadmium induction of *cdr-1*. On the other hand, the *mtl-1* promoter contains a putative HRE (Figure 3.1A), suggesting that HIF-1 could potentially regulate its expression.

Cdk8 also promotes the activity of certain stimulus-responsive TFs in yeast. The galactose-inducible TF Gal4 is required to switch from glucose to galactose utilization in the absence of glucose (Johnston 1987). Cdk8-mediated phosphorylation promotes Gal4-driven transcriptional activation of galactose-inducible genes (Hirst *et al.* 1999), indicating that Cdk8 promotes adaptation to different carbon sources in yeast. The proteasome-activating TF Rpn4 is required for resistance to multiple cellular stressors (Wang *et al.* 2008). The Rpn4 protein is stabilized in a Cdk8-dependent manner in yeast strains with truncating mutations in the Pol II CTD (Aristizabal *et al.* 2013). Thus, Cdk8 promotes protein stability of a stress-responsive TF during the cellular stress response elicited by impaired CTD function. Interestingly, Rpn4 is required for resistance to cadmium stress (Wang *et al.* 2008), suggesting that Cdk8 may have an evolutionarily ancient role in cadmium-responsive transcription, although its TF target(s) in this pathway may differ from yeast to *C. elegans.* Overall, previous studies have shown that CDK8 can activate stimulus-responsive transcription in cell lines or in yeast. To my knowledge, my study provides the first evidence that CDK8 promotes stress-responsive transcription *in vivo* in multicellular organisms.

3.4.2 *mdt-15* promotes expression of the *cdr-1* promoter

mdt-15 is required for basal and cadmium-induced expression of certain cadmium-responsive genes, including *mtl-1, mtl-2,* and *cdr-1* (Taubert *et al.* 2008). Furthermore, *mdt-15* is required for zinc induction of the *mtl-1* promoter, an event that also requires *elt-2*, GATA, and HZA elements (Roh *et al.* 2015). Thus, previous studies have suggested that *mdt-15* may act

together with *elt-2*, at GATA elements, and perhaps with an HZA-binding TF. In this study, I show that *mdt-15* is strongly required for basal and cadmium-induced expression of the *cdr-1* promoter, which contains many additional stress-responsive regulatory elements, suggesting the involvement of additional TFs (see Section 3.3.3).

mdt-15 appears to be more strongly required at the *cdr-1* promoter than *cdk-8.* Both *mdt-15* and *cdk-8* are required for basal expression of *cdr-1* (Figure 3.2C, D, Figure 3.4). Cadmium induction of the *cdr-1* promoter is completely abrogated in *mdt-15* mutants, whereas in *cdk-8* mutants, *cdr-1* is still induced by cadmium, but at much reduced levels compared to wild type (Figure 3.2D, Figure 3.4). This suggests that *mdt-15* is a critical coactivator of one or more of the TFs that activates both basal and cadmium-induced expression of the *cdr-1* promoter. On the other hand, *cdk-8* may only be partially required as a coactivator of the same TF(s).

Opposition or cooperation of CDK8 and MED15 in transcription have been previously described, either by action upon a common TF or by regulatory interactions between the two Mediator subunits. For example, MED15 binds to and promotes SREBP-driven transcription of a cholesterol and fatty acid homeostasis gene expression program (Yang *et al.* 2006), whereas CDK8 phosphorylates SREBP to promote its degradation, thereby opposing MED15 action on a common TF (Zhao *et al.* 2012). In yeast, both Med15 and Cdk8 promote transcriptional activation by the galactose-responsive TF Gal4, although the mechanism by which they cooperate to regulate this TF remains incompletely understood (Sadowski *et al.* 1996; Hirst *et al.* 1999). Within the yeast Mediator complex, Cdk8 phosphorylates the tail module subunit Med3, leading to ubiquitin-mediated degradation of Med3 and associated subunits Med2 and Med15 (Gonzalez *et al.* 2014). This negative regulatory relationship corresponds to a large degree of anticorrelation between the gene expression profiles of *cdk8 vs. med2, med3* or *med15* mutants:

many genes that are derepressed in *cdk8* mutants genes are downregulated in *med2, med3* and *med15* mutants (van de Peppel *et al.* 2005). Similarly, I showed that in *C. elegans*, *cdk-8* inhibits MDT-15-driven transcription by a post-transcriptional regulatory mechanism (see Section 2.3.9). Interestingly, closer examination of the gene expression profiles of yeast mutants reveals that some genes that are downregulated in *cdk8* mutants also appear to require *med2, med3* or *med15* (van de Peppel *et al.* 2005), suggesting that these Mediator subunits may cooperatively activate gene expression in some contexts. This also appears to be the case in the cadmium response, as both *cdk-8* and *mdt-15* act to positively regulate transcription ((Taubert *et al.* 2008), and this study). By analogy to their regulatory relationships in other contexts, this suggests that CDK-8 and MDT-15 might cooperate as coactivators of a common cadmium-responsive TF, or that in response to cadmium, CDK-8 might post-translationally activate MDT-15. Further work is needed to distinguish these possibilities, *e.g.* by identifying a common TF binding partner of CDK-8 and MDT-15, or by measuring MDT-15 protein abundance in *cdk-8* mutants *vs.* wild type exposed to cadmium.

3.4.3 *cdr-1* promoter regulatory elements

The *cdr-1* promoter provided an excellent system in which to identify additional cadmium-responsive TFs that may interact with *cdk-8* and *mdt-15*. Regulatory element requirements in cadmium-inducible transcription have largely been investigated in the *mtl-1* and *mtl-2* promoters (Moilanen *et al.* 1999), or isolated regulatory elements derived from the *mtl-1* promoter (Roh *et al.* 2015). Studies involving these promoters have been instrumental in identifying GATA and HZA elements as necessary and sufficient for cadmium-inducible transcription (Moilanen *et al.* 1999; Roh *et al.* 2015). Furthermore, *mtl-1* transcription is

activated by DAF-16, as *mtl-1* transcription is upregulated in insulin receptor mutants, which have increased DAF-16 activity (Murphy *et al.* 2003), and DAF-16 binds to the *mtl-1* promoter *in vivo* (Zhang *et al.* 2013). However, these studies were conducted in standard laboratory conditions, and the contribution of DAF-16 to *mtl-1* transcription in cadmium-exposed worms has not been investigated. Whereas the *mtl-1* and *mtl-2* promoters contain only GATA, HZA, DBE, or HRE regulatory elements, the *cdr-1* promoter additionally contains putative ARE-like, MRE, and HSE elements (Figure 3.1A), whose contribution to cadmium-inducible transcription have not previously been investigated. Thus, the *cdr-1* promoter allowed me to test whether additional TFs, such as SKN-1/Nrf2 (ARE-like binding) and HSF-1 (HSE binding), cooperate with the Mediator subunits *mdt-15* and *cdk-8* in cadmium-inducible transcription.

Complex promoters can confer highly specific gene expression patterns by recruiting a collection of TFs that are expressed or activated in a tissue-specific or stimulus-specific manner (Gaudet and McGhee 2010). Induction of the *cdr-1* promoter is highly specific to cadmium and zinc stress, as other transition metals (lead, mercury, copper), oxidizing agents, or heat stress do not the induce *cdr-1* promoter (Liao *et al.* 2002; Roh *et al.* 2015). Thus, at the outset of this study, I presumed that, instead of activating *cdr-1* transcription in response to many different stresses, the complex array of regulatory elements in the *cdr-1* promoter might instead contribute in a combinatorial fashion to cadmium induction of *cdr-1*, each contributing to *cdr-1* activation in response to a different aspect of cadmium stress. For example, cadmium causes oxidative stress by depleting the antioxidant glutathione (Figueiredo-Pereira *et al.* 1998), which could promote SKN-1-dependent transcription via ARE-like sites. In addition, cadmium stress causes protein aggregation (Figueiredo-Pereira *et al.* 1998), which might activate HSF-1-dependent transcription via the HSE, as *hsf-1* is required for detoxification of aggregation-prone proteins

(Hsu *et al.* 2003; Cohen *et al.* 2006). Furthermore, MREs are critical for cadmium-inducible transcription driven by metal-responsive transcription factor 1 (MTF-1) in other organisms (Wimmer *et al.* 2005), and although a *C. elegans* MTF-1 homolog has not been identified, the presence of MRE consensus sequences in the *cdr-1* promoter suggested that a cryptic MTF-1 homolog could act at this promoter. However, my study demonstrates that *skn-1, hsf-1* and at least one of the MREs are not required for cadmium induction of the *cdr-1* promoter (Figure 3.5D, F), and that neither the oxidative stressor arsenite nor heat shock can induce the *cdr-1* promoter (not shown). As the *cdr-1* promoter contains additional MRE sites (Figure 3.1A), further site-directed mutagenesis analyses are required to rule out MRE-mediated regulation. I found that the intestine-specific TF *elt-2* and its cognate GATA elements, and the cadmium- and zinc-activated HZA element, are strongly required for *cdr-1* induction (Figure 3.5B-D). Thus, the specificity of the *cdr-1* promoter toward cadmium and zinc does not appear to be achieved by combinatorial activation by multiple TFs responding to different aspects of cadmium stress. I also found that *daf-16* and its DBE are required to repress cadmium induction of the *cdr-1* promoter (Figure 3.5G), which led me to hypothesize that DAF-16 may prevent induction of the *cdr-1* promoter by other stresses. However, loss of the DBE did not allow the *cdr-1* promoter to be induced by oxidative stress or heat shock. Therefore, it is tempting to speculate that *cdr-1* is specifically induced by cadmium and zinc not because of repression via DAF-16, but because of a cadmium- and zinc-sensing TF (see Section 3.4.6 for further discussion).

Some of the regulatory elements examined in this study were enriched in cadmium-inducible promoters. Specifically, we found that HZA elements are enriched at least 10-fold upstream of cadmium-inducible genes compared to all *C. elegans* genes (Figure 3.1C). Kornfeld and colleagues previously examined the promoters of 29 genes that are induced greater than 4-

fold in response to cadmium, and found that all contained HZA elements (Roh *et al.* 2015). Thus, our estimate that 6% of cadmium-inducible promoters contain HZA elements is likely low, perhaps because we used consensus sequences as a proxy for position frequency matrices to define regulatory element motifs. Furthermore, I found that GATA elements, DBEs, and MREs are slightly but significantly enriched upstream of cadmium-inducible genes (Figure 3.1C). Conversely, ARE-like elements and HSEs are not enriched upstream of cadmium-inducible genes (Figure 3.1C); however, these analyses may also have been limited by the use of consensus sequences instead of motif position frequency matrices. It is important to note that core GATA elements, ARE-like elements, and MREs were each present upstream of more than 50% of *C. elegans* genes. This implies that their presence upstream of *e.g. cdr-1* does not indicate a functional role *a priori*, as evidenced by my genetic analyses showing that GATA elements but not AREs or an MRE are required for cadmium induction of the *cdr-1* promoter.

3.4.4 SKN-1-independent MDT-15 action at the *cdr-1* promoter

MDT-15 and SKN-1 cooperate to promote oxidative stress-responsive transcription. Specifically, *mdt-15* is required for basal and/or oxidizing agent-induced expression of several *skn-1*-dependent genes (Goh *et al.* 2014), which have predicted ARE-like SKN-1 binding sites. Furthermore, MDT-15 binds to SKN-1, indicating that it is a direct coactivator of this TF (Goh *et al.* 2014). I found that although the *cdr-1* promoter contains two ARE-like motifs (Figure 3.1A), its basal and cadmium-inducible expression requires *mdt-15* but not *skn-1* (Figure 3.4, Figure 3.5D). In support of *skn-1*-independent cadmium-responsive transcription, Choi and colleagues have shown that *skn-1* mutants are not significantly more cadmium sensitive than

wild-type worms (Roh *et al.* 2009). Taken together, these findings suggest that MDT-15 engages different TFs in oxidative stress-responsive *vs.* cadmium-responsive transcription.

3.4.5 ELT-2 and GATA elements in *cdr-1* promoter induction

I found that *elt-2* and GATA elements are required for basal and/or cadmium-inducible expression of the *cdr-1* promoter (Figure 3.5C-E, Figure 3.6). Specifically, *elt-2* RNAi knockdown or mutation of the *cdr-1* promoter GATA2 element caused significant reduction of both basal and cadmium-induced expression of the *cdr-1P::GFP* reporter, whereas mutation of the GATA1 element affected cadmium-induced but not basal expression of the reporter. Similarly, the *mtl-1* and *mtl-2* promoters also contain multiple GATA elements, but not all are required for cadmium-inducible transcription (Moilanen *et al.* 1999).Why are GATA elements differentially required in cadmium-responsive promoters? Bioinformatic analysis of intestinally-expressed gene promoters identified an extended 10 base pair GATA-like consensus sequence: $A_1(A/C/T)_2T_3G_4A_5T_6A_7A_8$ $(A/G)_9(A/G)_{10}$ (McGhee *et al.* 2007). The *cdr-1* extended GATA-like elements differ in sequence at position 9 (Table 3.1), which could account for their differential requirements in basal transcription of the *cdr-1* promoter. Neither of the *cdr-1* GATA elements conforms perfectly to the extended GATA-like consensus sequence (Table 3.1), which could explain why both GATA sites are required for cadmium-inducible expression of the *cdr-1* promoter. By contrast, investigation of two GATA elements in the *mtl-1* promoter previously showed that only one GATA element, which perfectly matches the extended GATA-like consensus sequence, is required for cadmium-inducible expression (Moilanen *et al.* 1999). Thus, differences in extended GATA-like sequences could explain the differential requirements for the

cdr-1 GATA elements in basal transcription, but single base pair mutagenesis studies are needed to explore this possibility.

Several intestinal gene expression responses require *elt-2*. Immunity to multiple intestinal pathogens requires *elt-2*, including *Salmonella enterica, Pseudomonas aeruginosa, Enterococcus faecalis,* and *Cryptococcus neoformans* (Kerry *et al.* 2006). *elt-2* is required for gene expression changes in response to *S. enterica* infection and in recovery following acute exposure to this pathogen (Kerry *et al.* 2006; Head and Aballay 2014). *elt-2* is also required for the gene expression response to *P. aeruginosa* infection. In this context, *elt-2* appears to cooperate with the TFs *atf-7* or *skn-1*, effectors of the p38 MAPK innate immune response pathway (Block *et al.* 2015). Finally, as outlined above, *elt-2* and its GATA site are required for transcription of the *mtl-1* promoter in response to excess zinc (Roh *et al.* 2015). Thus, *elt-2* is required for transcription induced by multiple stressors in the *C. elegans* intestine.

The extended GATA-like element defined by McGhee and colleagues is found in virtually all intestinally-expressed promoters in *C. elegans*, and ELT-2 is the predominant GATA-family TF required for expression of most intestinal genes in adults (McGhee *et al.* 2007). This suggests that, although critical for the expression of intestinal genes such as *cdr-1*, ELT-2 is unlikely to be a specific driver of the gene expression response to cadmium exposure. Instead, ELT-2 likely functions as a permissive factor required for basal intestinal transcription and inducible responses to many stimuli. Taken together with my analysis of the *cdr-1* promoter, this can account for the tissue-specific expression pattern of *cdr-1:* others and I have shown that *cdr-1* is expressed solely in the intestine, both in the absence and presence of cadmium (Figure 3.2D) (Liao *et al.* 2002). I showed that *elt-2* and GATA elements are strongly required for basal and cadmium-induced expression of the *cdr-1* promoter (Figure 3.5C-E), suggesting that the *cdr-*

1 promoter GATA sites drive intestine-specific expression by recruiting ELT-2, which is only expressed in intestinal cells. At the *cdr-1* promoter, ELT-2 may be required for critical steps in transcription, *e.g.* Mediator recruitment, thus ensuring intestine-specific activation of a globally expressed transcriptional coregulator (Figure 3.6). However, chromatin immunoprecipitation experiments are needed to determine if ELT-2 influences Mediator occupancy at the *cdr-1* promoter. In addition, an HZA-binding TF could also be expressed in an intestine-specific manner, thereby contributing to the intestine-specificity of *cdr-1* expression.

Mediator requirements in GATA factor-driven transcription have been studied in *Drosophila* hematopoiesis. Specifically, 20 of 30 Mediator subunits, including the CKM, are required for transcription driven by the GATA factor Serpent and the RUNX factor Lozenge in hemocytes. Indeed, *CDK8, Cyclin C, MED12* and *MED13* are required for proliferation of *Drosophila* platelet (crystal cell) progenitors, a Serpent/GATA-dependent process. In contrast, *MED12* and *MED13*, but not *CDK8* and *Cyclin C*, are required for differentiation of mature crystal cells, a process driven by Serpent/GATA and Lozenge/RUNX (Gobert *et al.* 2010). This raises the intriguing possibility that, in *Drosophila* hematopoiesis, CDK8 and Cyclin C may be dispensable for GATA factor-driven transcription when it acts together with a second cell type-specific TF. In contrast, in the current study I show that *cdk-8* is required for transcription of a promoter driven by both a GATA factor and an HZA-binding TF, suggesting that CDK-8 is not dispensable in all cases where GATA factors cooperate with additional TFs.

3.4.6 The HZA element in *cdr-1* promoter induction

I found that the HZA element is required for basal expression and cadmium induction of the *cdr-1* promoter (Figure 3.5C, Figure 3.6). The HZA element was discovered as a regulatory

element necessary for transcription induced by excess zinc (Roh *et al.* 2015). This element is present in many cadmium-responsive genes, including *cdr-1*, *mtl-1*, and *mtl-2*, and is sufficient to drive cadmium-inducible transcription in combination with a GATA element and minimal promoter (Roh *et al.* 2015). The overlap between regulatory elements driving cadmium- and zinc-inducible transcription may stem from the similar biological targets of these transition metals. For example, both cadmium and zinc bind to protein sulfhydryl groups (Vallee and Ulmer 1972). Kornfeld and colleagues have hypothesized that cadmium binds to a zinc-sensing TF to activate transcription via the HZA (Roh *et al.* 2015). Alternatively, it is conceivable that as cadmium and zinc bind to the same metal storage proteins (*e.g.* metallothioneins; (Vallee and Ulmer 1972)), cadmium could displace zinc from its storage sites, leading to a transient excess of zinc that induces transcription via a zinc-binding TF and the HZA. Interestingly, none of the TFs or Mediator subunits identified in the current study are transcriptionally regulated by cadmium (Cui *et al.* 2007), although post-translational regulation of these factors has not been explored. Furthermore, ELT-2 is constitutively localized to intestinal nuclei (Fukushige *et al.* 1999), and the DNA binding affinity of ELT-2 is not affected by cadmium (Moilanen *et al.* 1999), indicating that cadmium is unlikely to enhance ELT-2 interaction with GATA elements to drive cadmium-inducible gene expression. Therefore, I favour the possibility that a cadmium- or zinc-activated HZA-binding TF may coordinate cadmium-inducible transcription. For example, in its metal-activated state, an HZA-binding TF could enhance Mediator recruitment to cadmium-responsive promoters, thereby stimulating Pol II recruitment, initiation, or elongation (Figure 3.6). However, chromatin immunoprecipitation experiments are needed to determine if the HZA is required for CDK-8, MDT-15 and/or Mediator complex recruitment, and Pol II recruitment and/or CTD phosphorylation at the *cdr-1* promoter.

NHRs make attractive candidates for the HZA-binding TF for several reasons (Figure 3.6). First, many NHRs are able to sense and respond to xenobiotics (Handschin and Meyer 2003), suggesting that NHRs could also sense cadmium and zinc. Second, although NHRs typically bind to xenobiotics via their ligand binding domain, their DNA binding domain could potentially bind to cadmium, as it consists of two C4 zinc finger domains, which use four cysteine residues to coordinate a zinc ion (Mangelsdorf *et al.* 1995). Thus, as cadmium and zinc bind to protein sulfhydryl groups (Vallee and Ulmer 1972), NHRs might be able to sense these metals via the cysteine-rich DNA binding domain. Third, *mdt-15* is required for cadmium- and zinc-inducible transcription ((Taubert *et al.* 2008; Roh *et al.* 2015), and this study), and MDT-15 engages multiple NHRs (Taubert *et al.* 2006; Arda *et al.* 2010), suggesting that MDT-15 might also interact with an NHR in the cadmium and zinc responses (Figure 3.6).

CDK8 is required for NHR-driven transcription in human cells and in *Drosophila.* Human CDK8 is required, as part of the Mediator complex, for thyroid hormone-activated transcription driven by TR (Belakavadi and Fondell 2010). Furthermore, CDK8 was recently identified as a coactivator required for DNA binding and transcription driven by a *Drosophila* NHR, the Ecdysone Receptor (EcR) (Xie *et al.* 2015). CDK8 binds directly to EcR and contains a conserved LXXLL NHR binding motif (Xie *et al.* 2015), suggesting that CDK8 may also bind to NHRs in other species. Further work is needed to determine whether CDK8-NHR interactions may be required in cadmium-responsive transcription (Figure 3.6), by testing whether the conserved LXXLL motif in *C. elegans* CDK-8 is required for cadmium-inducible transcription of the *cdr-1* promoter.

3.4.7 DAF-16 and DBE in *cdr-1* promoter repression

I found that *daf-16* and the DBE are required to repress cadmium-induced expression of the *cdr-1* promoter (Figure 3.5D, G, Figure 3.6). Requirement for the DBE suggests that DAF-16 may bind directly to the *cdr-1* promoter to restrain its induction by cadmium, but the molecular mechanisms underlying this repression remain to be identified. Gene expression profiling has identified sets of genes that are upregulated in insulin receptor (*daf-2*) mutants in a *daf-16*-dependent manner, which include many stress-responsive genes required for the extended lifespan of *daf-2* mutants (Class I genes), or genes that are downregulated in *daf-2* mutants in a *daf-16*-dependent manner, which include many metabolic genes that are detrimental to the lifespan of wild-type worms (Class II genes) (Murphy *et al.* 2003). Interestingly, the DBE is overrepresented in both Class I and Class II genes (Murphy *et al.* 2003), suggesting that DAF-16 directly activates and represses these genes, respectively. As *cdr-1* is repressed by DAF-16, it would be interesting to determine if it behaves as a canonical Class II gene, *i.e.* repressed in *daf-2* mutants and detrimental to lifespan.

The relationship between *mdt-15* and the insulin signaling pathway in longevity-promoting gene transcription and in oxidative stress-responsive transcription has previously been explored. DAF-16 appears to regulate some of its longevity-promoting target genes in a cell non-autonomous manner, dependent on *mdt-15.* Specifically, *daf-2* mutants expressing *daf-16* only in the intestine are still able to activate certain DAF-16-target genes in muscle and hypodermis, but this cell non-autonomous activation is abrogated by *mdt-15* knockdown (Zhang *et al.* 2013). In this context, *mdt-15* activity may also be cell non-autonomous, as *mdt-15* appears to be expressed in the intestine and neurons, but not in muscle and hypodermis (Zhang *et al.* 2013). As transcription of *mdt-15* itself is activated by DAF-16 (Murphy *et al.* 2003), Kenyon and

colleagues have hypothesized that intestinal DAF-16 may activate *mdt-15* transcription in the intestine, which then promotes the production of a lipid signal that alters gene expression in other tissues (Zhang *et al.* 2013). In line with a role for *mdt-15* downstream of *daf-16*, *mdt-15* is required more strongly for longevity of *daf-2* mutants than of *daf-2; daf-16* mutants (Zhang *et al.* 2013). In the oxidative stress response, DAF-16 also regulates many stress response genes (Murphy *et al.* 2003). In the absence of oxidative stress, *daf-2* mutants upregulate several stress response genes, in a manner dependent on *mdt-15* and *skn-1* (Goh *et al.* 2014). Therefore, *mdt-15* is required for upregulation of oxidative stress response genes in *daf-2* mutants. However, in this context *mdt-15* appears to act primarily through *skn-1*, and has little effect on *daf-16*-dependent oxidative stress response gene transcription (Goh *et al.* 2014). Overall, in longevity, *mdt-15* appears to function downstream of *daf-16*, and in the oxidative stress response, *mdt-15* appears to function in parallel to *daf-16*, suggesting that MDT-15 is not a coactivator of DAF-16-driven transcription. In the present study, I found that *mdt-15* and *daf-16* act in opposition at the *cdr-1* promoter, suggesting that in this context, too, MDT-15 is not a coactivator of this canonical stress-responsive TF.

Overall, I demonstrate that the Mediator subunits *cdk-8* and *mdt-15*, the GATA and HZA regulatory elements, and the TF *elt-2* are required for cadmium induction of the *cdr-1* promoter. In contrast, the stress-responsive TFs *skn-1* and *hsf-1* are not required for *cdr-1* induction, and *daf-16* is required to repress cadmium-inducible *cdr-1* transcription. Thus, this work defines the TFs that cooperate with or oppose two Mediator subunits at a highly complex, inducible promoter.

3.5 Materials and methods

3.5.1 Microarrays and data analysis

Microarray gene expression profiling was performed at the UCSF SABRE Functional 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 hrs, according to the manufacturers 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 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 < 0.05 and a fold-change ≥ 2 (representing 461

downregulated and 829 upregulated genes). 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). Microarray analysis of wild-type worms exposed to 100 μM Cd^{2+} for 4 or 24 hours had previously identified 53 genes downregulated and 237 genes upregulated in response to cadmium exposure (Cui *et al.* 2007). We compared this set to our *cdk-8* targets and calculated the significance of the overlap using Fisher's exact test.

3.5.2 Regulatory element analysis

Candidate regulatory elements involved in the cadmium response, the high zinc response, or other detoxification or stress responses were identified in the literature (Stuart *et al.* 1984; Sorger 1991; Blackwell *et al.* 1994; Chen *et al.* 1999; Furuyama *et al.* 2000; Smale and Kadonaga 2003; An and Blackwell 2003; Shen *et al.* 2005; McGhee *et al.* 2007; Chiang *et al.* 2012; Roh *et al.* 2015). These included ARE, DBE, GATA element, HRE, HSE, HZA element, MRE, and TATA box. If available, the equivalent *C. elegans* consensus sequence for each regulatory element was identified in the literature, otherwise the eukaryotic consensus sequence was used. The *cdf-2, cdr-1*, *gst-38*, *mtl-1*, and *mtl-2* promoters (-1500 base pairs) were searched manually for the candidate regulatory elements using SerialCloner 2.5 software. Analysis of -1500 base pair regions upstream of all cadmium-inducible genes (Cui *et al.* 2007) *vs.* all *C. elegans* genes (WBcel235 release 81) was conducted using oPOSSUM sequence-based single site analysis (Ho Sui *et al.* 2005).

3.5.3 *C. elegans* strains, culture, and genetic methods

C. elegans strains were cultured as described (Brenner 1974) at 20°C. 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, supplemented with 5 μg/mL cholesterol. Plates were seeded with *Escherichia coli* strain OP50 unless otherwise indicated. Wild type is the N2 Bristol strain.; all other strains are listed in Table 3.2.

Table 3.2 *C. elegans* strains used in Chapter 3

Strain	Genotype
XA7703	*cdk-8(tm1238) I*
STE101	*steEx49[cdr-1P::GFP]*
STE102	*cdk-8(tm1238) I; steEx49[cdr-1P::GFP]*
STE103	*steEx50[cdr-1P(mutHZA)::GFP]*
STE104	*steEx51[cdr-1P(mutGATA1)::GFP]*
STE105	*steEx52[cdr-1P(mutGATA2)::GFP]*
STE106	*steEx53[cdr-1P(mutDBE)::GFP]*
STE107	*steEx54[cdr-1P(mutMRE)::GFP]*

3.5.4 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 3.3).

Table 3.3 qPCR primer list for Chapter 3

	Primer sequences (F/R)
cdr-1	TCTTCTCTCAATTGGCAACTG / TTTGGGTAAACTTCATGACGA
mtl-1	TGGATGTAAGGGAGACTGCAA / CATTTTAATGAGCCGCAGCA
mtl-2	AAGTGTGCCAACTGCGAATGT / GCTTTCAAGAAAAAACCTCGA
act-1	GCTGGACGTGATCTTACTGATTACC / GTAGCAGAGCTTCTCCTTGATGTC
tba-1	GTACACTCCACTGATCTCTGCTGACAAG / CTCTGTACAAGAGGCAAACAGCCATG
ubc-2	AGGGAGGTGTCTTCTTCCTCAC / CGGATTTGGATCACAGAGCAGC

3.5.5 Cadmium sensitivity assay

Synchronous L1 animals were grown at 20°C for 40 hours on NGM-lite plates seeded with 5x concentrated, heat-killed OP50 containing 0, 2.5, 5., 10, or 25 μM $CdCl_2$. Developmental stage was scored by visual analysis. To normalize for differences in developmental timing between strains, the normalized fraction of L4 animals was calculated by dividing the fraction of L4s in a given cadmium-exposed population by the fraction of L4s in the 0 μM $CdCl_2$ population for the same genotype.

The *cdr-1P::GFP* reporter was generated by PCR amplification of the genomic region from 2853 base pairs upstream to 11 base pairs downstream of the *cdr-1* start codon (a G>C mutation at the +3 nucleotide was introduced in the reverse primer to mutate the *cdr-1* start codon), and cloning into the pPD95.77 GFP vector from the Fire lab vector kit (Addgene plasmid #1495). Mutant transgenes were generated by site-directed mutagenesis of the

pPD95.77-*cdr-1P* plasmid using the Q5 Site-Directed Mutagenesis kit (NEB E0554S). Cloning primers are listed in Table 3.4. Transgenic strains were generated by injection of a mixture of 50 ng/µl GFP reporter plasmid, 5 ng/µl pCFJ90*[myo-2p::mCherry]*, and 95 ng/µl pPD95.77 empty vector into N2 worms, then selecting transgenic mCherry-positive progeny.

Table 3.4 Cloning primer list for Chapter 3

	Primer sequences (F/R)
cdr-1P	gtcgacTTTGACGATGACAGAAGAAATG / ggatccTGAATCCAAGATACTTGAGACAGT
mutHZA	tcgtggcAATTTTATCACAAAACACAGTTC / ccctcaccTCAATTGCAGAATACCATTTG
mutGATA1	TACAATAATTccgctgCAAAACACAGTTCTCCC / GTTTCTGTTTCAATTGCAGAATAC
mutGATA2	CCCTACTTTCccgctgCATTATGTCATCGGG / AGAACTGTGTTTTGTGATAAAATTATTG
mutDBE	ccgtCCAGAAAGCTTAAAATTCAAG / gtggAACGGAAAAATATAATATGTATATACAC
mutMRE	tttaGTTACATTTGTATTTGTTTGATCAGATG / catgTTAAGAGCGCCTTTTGGTATTG

3.5.6 *cdr-1* reporter fluorescence analysis

Synchronous day 1 adult worms were subjected to (a) 4 hours exposure to 100 µM $CdCl_2$, (b) 4 hours exposure to 5 mM $NaAsO_2$, (c) 1 hour heat shock at 37°C followed by 1 hour recovery at 20°C, or (d) 4 hours no stress control. All chemicals were dissolved in NGM-lite plates, and all plates were seeded with 5x concentrated, heat-killed OP50. Animals were imaged using DIC optics and fluorescence microscopy on a Zeiss Axioplan 2 microscope. Fluorescence intensity of the intestine was analyzed using ImageJ software, and was normalized for intestine size and background fluorescence.

3.5.7 Feeding RNAi knockdown

Feeding RNAi was performed using NGM-lite plates containing 25 μg/mL carbenicillin, 1 mM IPTG, and 12.5 μg/mL tetracycline, and seeded twice with appropriate HT115 RNAi bacteria (Ahringer library 96-well format: *mdt-15*: plate 74, well C09; *elt-2*: plate 196, well E06; *daf-16*: plate 18, well G12; *skn-1*: plate 99, well G09; *hsf-1*: plate 21, well B05; all clones were sequenced to confirm the insert identity.) Synchronous L1 animals grown on seeded RNAi plates until day 1 of adulthood.

Chapter 4: Discussion and conclusions

In this dissertation, I addressed the hypothesis that subunits of the Mediator CDK8 kinase module drive gene expression changes in the EGFR-Ras-ERK signaling pathway or the cadmium response by interaction with transcriptional regulators or with other Mediator subunits. I found that *cdk-8* represses TF and Mediator tail module subunit activity downstream of the EGFR-Ras-ERK pathway. I also found that both *cdk-8* and *mdt-15* activate a complex cadmium-inducible promoter, and identified regulatory elements and TFs required at this promoter. These findings agree with previously identified roles of metazoan CDK8 as a regulator of multiple developmental pathways, and adds significantly to our understanding of CKM mechanisms in the EGFR-Ras-ERK pathway and within the Mediator complex. Furthermore, these findings reveal that, similar to yeast Cdk8, which is a coregulator of several stress-responsive TFs, metazoan CDK-8 is a coactivator of cadmium-responsive transcription.

4.1 The CKM as regulatory hub in metazoan development

In Chapter 2, I showed that CDK-8 and other CKM subunits repress EGFR-Ras-ERK pathway-driven cell fate specification in the *C. elegans* vulva, by acting as a corepressor for a conserved Ets-family TF, and by restraining the activity of Mediator tail module subunits *mdt-29/med2, mdt-27/med3,* and particularly *mdt-15*. Thus, the CKM regulates *C. elegans* development by acting in a conserved cell signaling pathway.

Over the past decade, the Mediator CKM has emerged as a regulator of multiple developmental processes in metazoans. Studies in invertebrate animal models have identified roles of the CKM in multiple developmental signaling pathways or TF cascades. In *C. elegans*,

cdk-8, *mdt-12/dpy-22*, and *mdt-13/let-19* repress EGFR signaling-driven transcription in vulva development ((Moghal and Sternberg 2003a), Section 1.3.1; and this work, Chapter 2); *mdt-12/dpy-22* and *mdt-13/let-19* repress Wnt signaling-regulated developmental events in multiple tissues ((Zhang and Emmons 2000; Yoda *et al.* 2005); Section 1.3.2); and all four CKM subunits are required for correct axon navigation, potentially by repressing the *sax-3/ROBO* pathway ((Steimel *et al.* 2013); Section 1.3.4). In *Drosophila,* Cdk8 binds to the steroid hormone-sensing NHR EcR to orchestrate transcription changes during developmental transitions (Xie *et al.* 2015). In addition, the *Drosophila* CKM is required for hematopoietic cell fate specification, driven by the GATA factor Serpent, and *Med12/Med13* are required for hematopoietic cell differentiation, driven by both Serpent and the RUNX factor Lozenge (Gobert *et al.* 2010). Thus, the CKM orchestrates metazoan development by acting in diverse pathways. In some cases, the conservation of these CKM roles in higher organisms, *e.g.* mammals, remains unexplored, as the essential nature of CKM genes in vertebrates has hindered studies of CKM subunit action in development. For example, murine *CDK8* is required in very early embryonic development, prior to or during the 8-cell stage; however, the precise pathway or target genes regulated by CDK8 in this context remains unknown (Westerling *et al.* 2007). Similarly, *MED12* is required early in zebrafish development for brain, neural crest, and kidney development, but pertinent regulatory mechanisms remain undefined (Hong *et al.* 2005; Hong and Dawid 2011). *MED12* has been studied more extensively in mouse development using a reduction-of-function mutation that reduces but does not completely abolish *MED12* mRNA; this revealed a requirement for *MED12* in Wnt signaling target gene expression during embryonic development (Rocha *et al.* 2010).

The CKM regulates the output of multiple signaling pathways, suggesting that it may either be employed downstream of each pathway separately, or it may simultaneously integrate inputs from multiple pathways. Signaling in the *C. elegans* VPCs provides a system in which to probe this question. VPC cell fate determination requires both *bar-1/β-catenin* and *let-60/Ras* to activate expression of the Hox TF gene, *lin-39,* in successive phases of vulva development. First, in the phase prior to the onset of VPC induction by EGFR-Ras-ERK signaling, *bar-1*-mediated activation of *lin-39* inhibits VPC fusion with the surrounding hypodermis, thereby maintaining VPC competence to later adopt the 1°, 2° or 3° fates (Eisenmann *et al.* 1998). Later, during VPC induction, EGFR-Ras-ERK signaling alleviates inhibition of *lin-39* by the *lin-1/Ets* TF, leading to *lin-39* upregulation in the presumptive 1° cell, which promotes cell division (Maloof and Kenyon 1998; Wagmaister *et al.* 2006). However, hyperactivation of *bar-1/β-catenin* can also promote vulval cell proliferation independently of EGFR-Ras-ERK signaling, suggesting that Wnt/β-catenin signaling can independently drive VPC induction (Gleason *et al.* 2002). Prior to VPC induction, *mdt-12/dpy-22* and *mdt-13/let-19* act downstream of *bar-1/β-catenin* and upstream of *lin-39* to promote VPC fusion ((Yoda *et al.* 2005), Section 1.3.2). This suggests that the CKM may repress *lin-39* transcription prior to VPC induction, when it is controlled by β-catenin ((Yoda *et al.* 2005), Section 1.3.2). During VPC induction, *cdk-8* and *mdt-13/let-19* promote transcriptional repression by *lin-1/Ets* (Chapter 2). As LIN-1 represses *lin-39* (Maloof and Kenyon 1998; Wagmaister *et al.* 2006), this suggests that the CKM may also repress *lin-39* transcription during VPC induction, when it is controlled by *lin-1*. Importantly, *mdt-12/dpy-22* regulates VPC induction independently of *bar-1/β-catenin* (Moghal and Sternberg 2003a), suggesting that the CKM switches from a role downstream of the Wnt/β-catenin signaling pathway prior to VPC induction, to a role downstream of the EGFR-Ras-ERK pathway during

VPC induction. Together, these findings suggest that the CKM adopts distinct roles downstream of the Wnt/β-catenin pathway and the EGFR-Ras-ERK pathway at different phases in vulva development.

Many of the CKM's roles in developmental signaling pathways are recapitulated in human cancers. Pertinent to the genetic interaction I identified in Chapter 2, there is evidence that the human CKM represses EGFR signaling-driven transcription, as *MED12* is required downstream of BRAF to inhibit melanoma cell growth (Shalem *et al.* 2014). In addition, just as *MED12* promotes Wnt signaling-driven transcription in murine development (Rocha *et al.* 2010), human *CDK8* gene amplification frequently drives Wnt/β-catenin-mediated transcription in colon cancers (Firestein *et al.* 2008, 2010). Furthermore, *MED12* is subject to recurrent mutations in benign tumors of the uterus and breast (Mäkinen *et al.* 2011; Lim *et al.* 2014), suggesting a generalized role in promoting cell proliferation. Finally, human *CycC* is subject to heterozygous deletions in a subset of T-ALL, which drives activation of Notch-mediated transcription (Li *et al.* 2014). Thus, human CKM subunits appear to adopt tumor suppressor roles in the EGFR and Notch signaling pathways, and adopt an oncogenic role in the Wnt signaling pathway. This suggests that, like the pathway-specific roles of the CKM in different phases of *C. elegans* vulva development, the CKM can adopt distinct roles in human cancers depending on the predominant oncogenic pathway driving tumor formation.

4.2 CDK8 as a regulator of responses to environmental stimuli

In Chapter 3, I showed that *cdk-8* is required for induction of a subset of cadmium-responsive genes, and for cadmium resistance in *C. elegans*. In this context, *cdk-8* acts together

with or in parallel to the *mdt-15* Mediator tail module subunit, regulatory GATA elements and the *elt-2* GATA factor, and the high zinc-activated (HZA) element; in addition, *cdk-8* acts in opposition to negative regulation by the DAF-16 binding element (DBE) and the *daf-16/FoxO* stress-responsive TF (Chapter 3). Thus, I identified multiple elements and factors required for cadmium-inducible transcription of a single responsive gene, implying potential regulatory interactions between CDK-8 and these factors.

CDK8 regulates gene expression responses to multiple environmental stimuli through interactions with TFs. As discussed in Chapter 3, CDK8 activates several stimulus-responsive gene expression programs in mammalian cell lines or in yeast, via interactions with p53, HIF1α, Gal4, or Rpn4 (see Section 3.4.1). Interestingly, CDK8 also acts as a repressor of several transcriptional responses to environmental stimuli in yeast and metazoans. The role of CDK8 in stress-responsive transcription was first identified in yeast, where Cdk8 was found to repress a multitude of stress-responsive genes (Holstege *et al.* 1998). Many of these stress-responsive genes respond to nutrient deprivation; accordingly, *cdk8* mutants exhibit inappropriate induction of pseudohyphal colony morphology, a mode of growth usually induced only during nutrient deprivation (Holstege *et al.* 1998). Cdk8 represses pseudohyphal growth by phosphorylating two TFs, Ste12 and Phd1, which leads to their degradation (Nelson *et al.* 2003; Raithatha *et al.* 2012). Under nutrient-limiting conditions, Cdk8 is degraded, leading to accumulation of Ste12 and Phd1, and induction of the pseudohyphal growth gene expression program (Holstege *et al.* 1998; Nelson *et al.* 2003; Raithatha *et al.* 2012). Intriguingly, mammalian CDK8 protein levels are also regulated by nutrient status, although in a manner opposite to that of yeast Cdk8. In the mouse liver, CDK8 protein accumulates during fasting, and is degraded in response to re-feeding or insulin (Zhao *et al.* 2012). During fasting, CDK8 inhibits fat storage by promoting

degradation of the lipogenic TF SREBP1c; during re-feeding or insulin treatment, CDK8 degradation allows accumulation of SREBP1c and induction of lipogenic gene expression (Zhao *et al.* 2012). Regulation of CDK8 protein levels by nutrient status and CDK8-mediated regulation of SREBP is conserved in *Drosophila* (Zhao *et al.* 2012; Xie *et al.* 2015), suggesting that CDK8 is a conserved repressor of lipogenic gene expression in metazoans. Thus, CDK8 is an important regulator of responses to nutrient status in yeast and metazoans, although the upstream regulatory mechanisms and downstream TF targets are not conserved. Furthermore, as these studies demonstrate that CDK8 protein levels are regulated by nutrient deprivation across species, this raises the intriguing possibility that other stimuli, *e.g.* cadmium exposure, could also alter gene expression by post-translational regulation of CDK8.

Yeast Cdk8 also represses heat shock-responsive transcription. Many Cdk8-repressed genes overlap with stress-responsive genes activated by the Msn2 TF (Holstege *et al.* 1998). Msn2 activity is regulated by cytoplasmic to nuclear translocation in response to multiple stresses (Görner *et al.* 1998). Cdk8-mediated phosphorylation of Msn2 appears to promote nuclear exclusion of Msn2 in non-stressed cells, as *cdk8* mutants exhibit aberrant nuclear accumulation of Msn2 in the absence of stress (Chi *et al.* 2001). Cdk8-mediated phosphorylation may also attenuate Msn2-driven stress-responsive transcription, as heat shock induces rapid phosphorylation of nuclear Msn2 in a Cdk8-dependent manner (Chi *et al.* 2001). Thus, Cdk8 appears to repress Msn2-driven stress-responsive transcription.

In Chapter 3, our unbiased gene expression profiling of *C. elegans cdk-8* mutants revealed a role for CDK-8 as a regulator of cadmium-responsive genes. I found that, in worms exposed to cadmium, *cdk-8* is required for activation of cadmium-responsive transcription. Therefore, my study reveals that CDK-8 can act as a positive regulator of stress-responsive

transcription *in vivo* in a metazoan. As I show that *cdk-8* acts together with or in parallel to *mdt-15*, *elt-2*, or an HZA-binding TF to activate a cadmium-responsive promoter, I speculate that CDK-8 promotes cadmium-inducible transcription by activating one or more of these factors.

4.3 The CDK8-tail module relationship in metazoans

Multiple Mediator subunits are subject to post-translational regulation in yeast (van de Peppel *et al.* 2005; Miller *et al.* 2012; Gonzalez *et al.* 2014), but this was largely unexplored in multicellular organisms prior to my study. In Chapter 2, I identified a post-transcriptional regulatory interaction between *C. elegans* CDK-8 and MDT-15, as *mdt-15* is required for ectopic vulva formation in *mdt-13* mutants and derepression of *cdk-8*-repressed transcripts, and MDT-15 protein abundance is negatively regulated by *cdk-8*. These findings demonstrate for the first time that metazoan CDK-8 can inhibit a tail module subunit post-transcriptionally; in the future, it will be of interest to determine if MDT-15 is regulated post-translationally, *e.g.* by CDK-8-mediated phosphorylation. In yeast, Cdk8-mediated phosphorylation can inhibit transcriptional activation by tail module subunits Med2 and Med3 (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014). Cdk8-mediated phosphorylation of Med3 leads to its ubiquitination and proteasome-dependent turnover, as well as turnover of Med3-interacting tail module subunits, Med2 and Med15 (Gonzalez *et al.* 2014). However, as the presence of additional subunits in the metazoan Mediator tail module creates a more highly connective tail module architecture than in the yeast tail module (Tsai *et al.* 2014) (see Section 1.2.1 and Figure 1.1), it was not clear, prior to my study, whether or not metazoan CDK8 could similarly regulate tail module subunit stability. Indeed, my study demonstrates that although *mdt-29/med2*, *mdt-27/med3*, and *mdt-15* are

required for aberrant activation of vulval induction in *cdk-8* mutants, only *mdt-15* is required for activation of *cdk-8*-repressed genes in *cdk-8* mutants, suggesting that the metazoan *vs.* yeast tail module architecture may alter the tail module's regulatory interactions with CDK8.

Ras and ERK signaling pathways have been found to regulate Mediator subunit activity post-translationally. In yeast, a Ras-Protein kinase A (PKA) signaling pathway phosphorylates Med13 to promote CKM-mediated repression of nutrient deprivation genes (Chang *et al.* 2004). In human cells, steroid hormone-mediated activation of ERK leads to phosphorylation of MED1, which promotes its binding to the Mediator complex and its ability to coactivate NHR-driven transcription (Belakavadi *et al.* 2008). Thus, Ras-PKA or hormone-ERK signaling can phosphorylate Mediator subunits to promote their transcriptional coregulator activity. I have shown that the CKM represses EGFR-Ras-ERK signaling-driven vulval cell fate specification in *C. elegans*, in part through inhibition of Mediator tail module subunit activity. As CDK-8 kinase activity is required to repress vulva formation, I predict that CDK-8 inhibits the Mediator tail module by phosphorylation of *e.g.* MDT-15, but *in vitro* kinase assays are needed to explore this hypothesis (Chapter 2). Further work is also needed to determine if any of the Mediator subunits that regulate vulva development could also be subject to ERK phosphorylation, thereby linking EGFR-Ras-ERK pathway activation and Mediator subunit activity in the *C. elegans* vulva.

Mediator subunit phosphorylation influences stress-responsive transcription in yeast. For example, Cdk8-mediated phosphorylation of Med2 is required to repress twelve genes in the yeast genome, ten of which contain the consensus site for a TF that activates a low iron stress response (van de Peppel *et al.* 2005). Thus, Cdk8-mediated phosphorylation of Med2 may alter its interaction with a low iron-responsive TF, thereby restraining low iron-responsive transcription. Phosphorylation of multiple serine and threonine residues in the Med15 C-

terminus is required to repress osmotic stress-responsive transcription in yeast grown in normal conditions. Under osmotic stress, a subset of these sites are dephosphorylated, suggesting that dynamic changes in Med15 phosphorylation can induce stress-responsive transcription (Miller *et al.* 2012). The kinase responsible for Med15 phosphorylation has not been identified, but by analogy to the relationship between Cdk8 and Med2 in the low iron stress response, it is tempting to speculate that Cdk8 could phosphorylate Med15 to restrain stress-responsive transcription in yeast.

Yeast Cdk8 and Med15 may also have opposing effects on stress-responsive transcription by acting upon a common TF. Specifically, *med15* is required for activation of heat shock-responsive transcription by the stress-responsive TF Msn2, whereas Cdk8-mediated phosphorylation drives Msn2 degradation (Lallet *et al.* 2006). Intriguingly, Msn2 phosphorylation also requires *med15* (Lallet *et al.* 2006), suggesting that Med15-mediated binding of Msn2 to the Mediator complex is required for Cdk8-mediated Msn2 phosphorylation. Thus, yeast Cdk8 can oppose Med15 activity in stress-responsive transcription by promoting degradation of a Med15-binding TF. I have shown that *C. elegans cdk-8* inhibits MDT-15 activity in vulva development (Chapter 2), but that *cdk-8* and *mdt-15* are both required to activate cadmium-responsive transcription (Chapter 3). Thus, antagonism between CDK-8 and the tail module may not apply to stress-responsive transcription in metazoans, at least in the *C. elegans* cadmium response. It will be interesting to determine whether CDK-8 and MDT-15 interact with a common cadmium-responsive TF, or whether CDK-8 can promote MDT-15's transcriptional coactivator function directly.

4.4 Conclusion

The Mediator CKM regulates multiple developmental events and signaling pathways in metazoans. I have defined novel roles and mechanisms of CKM action in the EGFR-Ras-ERK signaling pathway in *C. elegans* vulval cell fate specification. Human *CDK8* acts as an oncogene in the Wnt signaling pathway (Firestein *et al.* 2008, 2010) and *MED12* appears to promote tumor growth (Mäkinen *et al.* 2011; Lim *et al.* 2014; Mittal *et al.* 2015), whereas *Cyclin C* is a tumor suppressor in the Notch signaling pathway (Li *et al.* 2014); thus it appears that, depending on the cellular context, CKM subunits can have vastly different influences in tumorigenesis. My work shows that all four CKM subunits repress EGFR-Ras-ERK signaling-driven cell fates, suggesting that in cancers driven by this oncogenic cell signaling pathway, the CKM subunits may act as tumor suppressors. In addition, I have defined a novel regulatory interaction between the metazoan CKM and Mediator tail module subunits. As such intra-Mediator regulation has previously only been observed in yeast (van de Peppel *et al.* 2005; Gonzalez *et al.* 2014), where tail module subunits and overall architecture are poorly conserved (Tsai *et al.* 2014), this represents a significant advance in our understanding of combinatorial control of cell signaling by multiple Mediator subunits. As the CKM is a player in human cancer, it will be of interest in future to determine if the regulatory interactions I have defined are conserved in cancer cell lines with activating mutations in the EGFR-Ras-ERK pathway.

CDK8 regulates several stress-responsive TFs in yeast, in human cell culture, or in murine and *Drosophila* models, but prior to my study, the role of CDK8 in cadmium-responsive transcription remained unexplored. I have shown that *C. elegans cdk-8* is required for activation of cadmium-responsive transcription, and defined several DNA regulatory elements and TFs that may interact with CDK-8 in this context. As the Mediator tail module subunit *mdt-15* is also

required for this response (Taubert *et al.* 2008), my work identifies a context in which these Mediator subunits may cooperate. Future work is needed to identify whether CDK-8 and/or MDT-15 bind to and directly regulate the TFs involved in cadmium-inducible transcription.

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