Control of translation by the CCR4-NOT complex during *Xenopus* early development

By

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ABSTRACT

mRNA regulation is critical for biological processes. Regulation is elicited at the levels of translation, localization and stability. RNA-binding factors (RBFs) recruit effector proteins to either repress or activate translation of mRNAs. The CCR4-NOT complex is a highly conserved, multi-protein complex that is recruited by RBFs to deadenylate, or catalyze removal of, the poly(A) tail of mRNAs. More recent work in the Wickens lab has demonstrated that CCR4-NOT also represses translation independent of deadenylation. In this thesis, I present my work to determine the protein interactors and regulatory mechanism of the CCR4-NOT complex during *Xenopus laevis* early development. I found that the CCR4-NOT complex interacts with the known translational repressors 4E-T and Xp54 in the oocyte. Interaction with 4E-T is required for repression of mRNAs in a non-canonical, eIF4E-independent manner. In the mature oocyte, the CCR4-NOT complex no longer associates with 4E-T or Xp54, perhaps due to modification or new protein partners of Xp54. I also conducted preliminary experiments to determine the RNA targets of the CCR4-NOT complex. My work demonstrates that regulation of mRNAs by the CCR4-NOT complex requires interaction with effector proteins and likely RNA-binding proteins. CCR4-NOT associations are likely controlled carefully throughout early development, and perhaps in somatic cells as well, by post-translational modifications or other means. This mode of control may be more broadly applicable to other proteins and protein complexes to elicit precise translational regulation of their mRNA targets.

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Overview

Proper gene expression is required for all biological processes. Even minor perturbations to the gene expression profile of a cell can result in abnormalities in cell growth, disease, and even cell death. Regulation of eukaryotic gene expression occurs at many levels, including transcription, post-transcriptional processing and export, translation, and post-translational modification. This chapter will focus on the processes that occur after transcription and prior to translation.

Transcription and pre-mRNA processing

Transcription and processing of pre-messenger RNA (pre-mRNA) are closely linked. RNA polymerase II transcribes DNA into pre-mRNA in the nucleus. pre-mRNAs are co-transcriptionally capped with a 5' 7-methyl guanosine cap structure by three enzymes: RNA triphosphatase, guanylyl transferase, and 7-methyl transferase (Shatkin 1976). Cleavage and polyadenylation machinery also associate with the pre-mRNA to add the poly(A) tail. Both the cap and poly(A) tail protect the transcript from degradation by exonucleases and stimulate translation of the mature mRNA (Gallie 1991; Coller et al. 1998). Splicing, the final covalent processing step, removes introns from the transcript and ligates adjoining exons together.

Pre-mRNA processing steps result in greater diversity of mRNAs. Polyadenylation and splicing can occur at different sites, resulting in a variety of different mature mRNAs from the same pre-mRNA transcript (Black 2003). In

higher eukaryotes, most pre-mRNAs possess several introns and exons. Roughly 70 percent of genes in humans, and 95 percent of mRNAs with alternative exons, are alternatively spliced (Pan et al. 2008). These events massively diversify the proteome. Not surprisingly, alternative splicing and 3' end formation are highly regulated, and their misregulation can generate aberrant mRNAs and disease.

Regulation of mRNAs and significance

Once the mature mRNA is exported out of the nucleus, regulation occurs at the levels of translation, localization and stability (Gebauer and Hentze 2004). mRNA regulation pervades biology, as it affords the cell a rapid response to its environment and external or internal stimuli. This is largely due to the fact that mRNA regulation can be specific to locations within the cell, in contrast to control at the level of transcription.

The mature mRNA possesses a coding region, the open reading frame (ORF), flanked by a 5' and a 3' untranslated region (UTR) (Fig.1A). The UTRs are common sites of binding elements for regulatory factors.

One major point of control of mRNAs is at the level of translation. Translation is often regulated at initiation, which requires interaction between the cap-binding protein eIF4E and other translation initiation factors to recruit the ribosome and begin protein production (Pestova and Hellen 2001). This key step can be inhibited through interaction of eIF4E-binding proteins (4EBPs) with eIF4E (Kamenska et al. 2014). This type of regulation is critical, exemplified by the fact that deletion of a 4EBP in mice results in autism-like behaviors (Gkogkas

et al. 2013; Gkogkas and Sonenberg 2013). Specifically, knockout of 4EBP2 results in increased translation of neuroligin proteins, which are linked to autism spectrum disorders. Drug inhibition of eIF4E and the neuroligin protein both diminish the autism-like behaviors in mice (Gkogkas et al. 2013). Other modes of translation regulation include prevention of ribosome scanning and 5' UTR unwinding, and the inhibition of translation elongation and termination.

Localization is another major form of mRNA regulation. mRNAs are often localized to different parts of the cell to control their translation or ensure the proteins they encode are expressed where they function. This allows for spatial and temporal control of gene expression, which is particularly important during embryogenesis and in neurons (Du et al. 2007). A classic example of mRNA localization is that of the *ASH1* mRNA to control mating type switching in the budding yeast *Saccharomyces cerevisiae* (Bobola et al. 1996; Long et al. 1997; Cosma 2004). The *ASH1* transcript is localized to the bud tip, where the daughter cell is formed. Thus, Ash1 protein accumulates in the nucleus of the newly formed daughter cell. Proper localization of *ASH1* prevents transcription of HO mRNA in the daughter cell (Bobola et al. 1996; Long et al. 1997; Cosma 2004). HO controls mating type, and mislocalization of *ASH1* results in the mother and daughter cells having the same mating type (Bobola et al. 1996; Cosma 2004). *ASH1* localization thus plays a significant role in cell fate determination.

mRNA stability is the third major point of mRNA regulation. mRNA decay and stability are carefully controlled to coordinate specific biological processes. Both external and cellular stimuli, such as nutrient levels and infection by viruses,

contribute to signaling that affects the half-life of mRNAs. The predominant type of mRNA decay begins with removal of the poly(A) tail, or deadenylation, followed by decapping and degradation by exonucleases (Garneau et al. 2007). The deadenylase complexes PAN2-PAN3 and CCR4-NOT deadenylate mRNAs, after which the decapping enzymes DCP1 and DCP2 remove the 5' cap. The exonuclease XRN1 then degrades the mRNA in the 5' to 3' direction. Alternatively, the exosome complex can initiate 3' to 5' degradation after deadenylation. Other mechanisms of mRNA decay take place as well, such as deadenylation-independent decay and endonuclease-mediated decay (Garneau et al. 2007). Misregulation of mRNA decay results in hyper-stability and increased accumulation of mRNAs, which can cause biological defects. For example, AU-rich element (ARE)-containing mRNAs are controlled by ARE-binding proteins (ARE-BP), which recruit decay machinery to destabilize mRNAs (Sanduja et al. 2011). When the ARE-BP tristetraprolin (TTP) is knocked out in mice, the ARE-containing mRNA TNF-alpha becomes hyper-stable, leading to increased TNF-alpha protein production and symptoms of systemic inflammatory disorders (Douni et al. 1995; Carballo et al. 1998). Treatment of young mice lacking TTP with TNF-alpha antibodies prevents these symptoms entirely (Douni et al. 1995), demonstrating the significance TNF-alpha mRNA decay.

All three modes of mRNA regulation (translation, localization and stability) are mediated by RNA-binding factors (RBFs). RBFs encompass RNA-binding proteins (RBPs) and non-coding RNAs (ncRNAs) such as microRNAs (miRNAs). RBFs bind to specific sequence elements within the UTRs, and sometimes the

ORF, to recruit other regulatory factors and have an effect on the translation, localization or stability of the target mRNA (Fig. 1B).

mRNA regulation during *Xenopus laevis* early development

mRNA regulation is of particular importance during early development. In these early stages, mRNA regulation is achieved by modulating translation rather than degradation. Thus, early development provides a robust system for analyzing the factors involved in translational regulation. At the same time, the early events of development are of interest in their own right.

Xenopus laevis is a well-studied model system for early development. Dramatic transitions in *Xenopus* occur over the course of a few days, and the oocytes and embryos are large and easy to manipulate and microinject. Additionally, the coordinated changes that take place throughout oogenesis and embryogenesis in *Xenopus* are conserved in higher eukaryotes.

During *Xenopus laevis* early development, prior to the mid-blastula transition, maternally deposited mRNAs direct all biological processes, as transcription is quiescent (Gandolfi and Gandolfi 2001; Tadros and Lipshitz 2009). Thus, control of the maternal transcripts is critical for proper development. Maternal mRNAs regulate development from early oogenesis through the mid-blastula transition during embryogenesis, at which point zygotic transcription is initiated (Newport and Kirschner 1982a; Newport and Kirschner 1982b).

Many important transitions occur in *Xenopus* early development that require proper mRNA control. The Stage I oocyte is a small and translucent cell of 50 to 100 μm in diameter, and it slowly progresses through oogenesis,

accumulating yolk protein and pigment and growing to become a 1.2 mm in diameter Stage VI oocyte (Dumont 1972). The Stage VI oocyte is characterized by a dark animal hemisphere and pale yellow vegetal hemisphere, which become distinct at Stage IV of oogenesis. Stage VI oocytes are arrested in prophase of meiosis I, and upon progesterone exposure they resume the meiotic cell cycle and undergo maturation into the fertilization-competent egg (Smith et al. 1968; Brachet et al. 1970). Eggs are distinguished from Stage VI oocytes by the presence of a white spot in the animal hemisphere, which corresponds to germinal vesicle breakdown (GVBD), a hallmark of the egg's arrest in metaphase of meiosis II. Fertilization results in completion of meiosis and the initiation of a series of mitotic divisions during embryogenesis.

All of these critical developmental steps require precise regulation of the maternal mRNA pool. RNA-binding factors bind to the maternal mRNAs to recruit effector proteins that alter the length of the poly(A) tail to elicit activation or repression of translation (Paris and Philippe 1990; Simon et al. 1992; Dehlin et al. 2000; Vasudevan et al. 2006). For instance, the deadenylase PARN is recruited by the RNA-binding protein CPEB to a subset of maternal mRNAs prior to oocyte maturation (Kim and Richter 2006). Upon maturation, PARN dissociates from the transcript, enabling polyadenylation and activating translation (Hake and Richter 1994; Kim and Richter 2006). Deadenylation and polyadenylation events occur in the Stage VI oocyte, during meiotic maturation, after fertilization, and at the mid-blastula transition (Fig. 2). Proteins that carry out

these processes, such as the deadenylase-containing CCR4-NOT complex, are thus particularly important during early development.

The CCR4-NOT complex

Complex composition

The CCR4-NOT complex is a highly conserved multiprotein complex that regulates mRNAs at many phases of their existence (Collart and Panasenko 2012; Miller and Reese 2012). CCR4-NOT functions include regulation of transcription initiation and elongation in the nucleus, nuclear export of mRNAs and protein ubiquitination (Fig. 3A). CCR4-NOT is particularly important for post-transcriptional regulation, as it is a primary cytoplasmic deadenylation machine and coordinates decapping of the transcript.

The CCR4-NOT complex comprises nine to eleven subunits, with at least five conserved core subunits (Collart and Panasenko 2012; Miller and Reese 2012). Thus, the complex ranges in size from 1 to 2 MDa. In all systems, the 250 kDa protein NOT1 is the major scaffolding subunit, as it directly contacts almost every other subunit of the complex. NOT1 has several different domains through which it interacts with other CCR4-NOT subunits (Fig. 3B). Three main structural components form the core of the CCR4-NOT complex: the nuclease module, the NOT module, and the NOT10/NOT11 module (Basquin et al. 2012; Bawankar et al. 2013; Boland et al. 2013).

The nuclease module is responsible for one of the primary functions of the CCR4-NOT complex: deadenylation. Control of poly(A) tail length is a common mode of mRNA regulation. Deadenylases are a paradigm for this type of control,

as they catalyze removal of the poly(A) tail to initiate downstream degradation and decay of the transcript or translational repression (Yamashita et al. 2005; Garneau et al. 2007; Goldstrohm and Wickens 2008; Chen and Shyu 2011). Two major cytoplasmic deadenylases in eukaryotes cooperate to deadenylate mRNAs in a consecutive manner (Yamashita et al. 2005). The PAN2-PAN3 complex deadenylates mRNAs distributively, shortening the poly(A) tail from 200 to 80 nucleotides in length, and the CCR4-NOT complex follows processively to remove the rest of the poly(A) tail (Yamashita et al. 2005). Importantly, in the absence of PAN2-PAN3, CCR4-NOT can still carry out deadenylation of mRNAs (Nousch et al. 2013).

Deadenylation is carried out by the two nuclease subunits of CCR4-NOT: CAF1 and CCR4. Both CAF1 and CCR4 are core members of the complex. Catalysis is orchestrated in the 3' to 5' direction via a two-metal ion mechanism (Goldstrohm and Wickens 2008; Chen and Shyu 2011). The metal ions, typically magnesium, are coordinated by four acidic residues in the active site: three aspartates and a glutamate (Chen and Shyu 2011). CAF1 is a member of the DEDD (Asp-Glu-Asp-Asp) deadenylase family, a class of enzymes named for the four residues required for catalysis (Wilusz et al. 2001; Goldstrohm and Wickens 2008; Suzuki et al. 2010). CAF1 physically interacts with CCR4, an EEP (endonuclease-exonuclease-phosphatase)-type deadenylase (Lau et al. 2009; Basquin et al. 2012) by binding directly to the LRR (leucine rich repeat) domain of CCR4 (Lau et al. 2009; Basquin et al. 2012). Though CCR4-NOT contains two deadenylase subunits, CAF1 is primarily responsible for deadenylation in most

eukaryotic organisms (Miller and Reese 2012). A notable exception to this is *Saccharomyces cerevisiae*. The CAF1 homolog Pop2p has a substitution at one of the four catalytic residues, and Ccr4p is the major deadenylase in *S. cerevisiae* (Goldstrohm et al. 2007). Pop2p is thought to act predominantly as a bridge to the rest of the CCR4-NOT complex.

The second major structural feature of CCR4-NOT is the NOT module, which comprises NOT1, NOT2, and NOT3 (Boland et al. 2013). NOT1 contacts the NOT boxes in NOT2 and NOT3 through its NOT1 superfamily homology (SH) domain, which is located in the C terminal 300 amino acids of the protein (Fig. 3B) (Boland et al. 2013). NOT2 and NOT3 function in ribosomal RNA surveillance and transcription regulation (Collart and Panasenko 2012). The role of NOT3 is carried out by two proteins, NOT3 and NOT5 in *Saccharomyces cerevisiae* (Collart et al. 2013), but other eukaryotes simply have NOT3.

The final structural component of the CCR4-NOT complex is the NOT10/NOT11 module (Bawankar et al. 2013; Mauxion et al. 2013). This more-recently described feature is conserved across eukaryotes, and is composed of NOT10 and NOT11. The two proteins physically interact with each other and contact the NOT10/11-binding domain in the N terminus of NOT1 (Fig. 3B). It is unclear what the functional roles of this part of the complex are.

Other accessory proteins are also part of the CCR4-NOT complex. Some such proteins are conserved core subunits of the complex, while others are specific to certain organisms or biological contexts. CNOT9 is a stable component of CCR4-NOT in eukaryotes, but its role is unclear (Chen et al. 2014;

Mathys et al. 2014). NOT4, an ubiquitin ligase, is a conserved subunit of CCR4-NOT, but it is only stably associated with the yeast complex (Collart and Panasenko 2012). In other systems, NOT4 is not a core component of the complex.

Recruitment to mRNA

The CCR4-NOT complex has intrinsic activity on many mRNAs, but it is also recruited to specific mRNAs for their differential control. CCR4-NOT may have some weak specificity for RNA due to the required contacts of the nuclease module for catalysis, as well as some evidence of RNA interactions to NOT4. However, CCR4-NOT does not contain proteins with RNA-binding domains. Thus, RNA-binding factors including miRNAs and RBPs often recruit CCR4-NOT to their mRNA targets to elicit regulation.

The NOT1 scaffold mediates interactions between CCR4-NOT and RNA-binding proteins. Tristetraprolin (TTP), an AU-rich element (ARE) binding protein, interacts with the central domain of NOT1 to facilitate deadenylation and decay of its mRNA targets (Sandler et al. 2011; Fabian et al. 2013). TTP directly contacts mRNAs that possess AREs in their 3' UTRs (Lai et al. 2000; Lai et al. 2002; Hudson et al. 2004). Disruption of the TTP-NOT1 interaction significantly decreases deadenylation of TTP target mRNAs (Sandler et al. 2011). *Drosophila* TIS11, a TTP ortholog, interacts with NOT1 in the same manner as TTP (Fabian et al. 2013), demonstrating a conserved mode of CCR4-NOT interaction to elicit mRNA regulation. Similarly, the Argonaute-interacting protein GW182, the *Drosophila* TNRC6A-C protein, binds to NOT1 to recruit it to miRNA-bound

mRNAs and initiate decay or translational repression (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011). This interaction is mediated through Glycine Tryptophan (GW) repeat contacts to the central domain of NOT1. More recently, similar GW repeat contacts to NOT9 have been identified, and new data suggest that NOT1 must be associated with NOT9 through its CN9BD (CNOT9-binding domain) to be recruited by GW182 (Chen et al. 2014; Mathys et al. 2014).

Other RNA-binding proteins interact with CCR4-NOT via other subunits of the complex, rather than through contacts with NOT1. For example, PUF proteins from many different eukaryotes interact with CAF1 to recruit CCR4-NOT and stimulate deadenylation (Goldstrohm et al. 2006). The *Drosophila* NANOS protein binds to NOT4 to repress translation of a germline mRNA (Suzuki et al. 2010; Bhandari et al. 2014). Other RNA-binding proteins also interact with CCR4-NOT, including Smaug, Bicaudal-C and the BTG/Tob family of proteins (Horiuchi et al. 2009; Collart and Panasenko 2012). It remains to be seen whether these alternate mechanisms of CCR4-NOT recruitment are general modes or specific to the RNA-binding proteins mentioned.

Biological roles of CCR4-NOT

Perturbations of CCR4-NOT complex proteins result in embryonic lethality, spermatogenesis defects, and some forms of cancer (Berthet et al. 2004; Chapat and Corbo 2014; Shirai et al. 2014). Different subunits of CCR4-NOT have distinct effects on the cell. NOT1 is essential in yeast, while many other subunits display synthetic lethality when deleted in combination with other CCR4-NOT

members (Bai et al. 1999; Nasertorabi et al. 2011; Tange et al. 2012). The CAF1 homolog in *C. elegans*, CCF-1, is required for progression through meiosis (Molin and Puisieux 2005). Thus, the structural integrity and distinct functions of the CCR4-NOT complex are critical for cell and organismal health.

Xenopus laevis complex composition

When I began my thesis work, the CCR4-NOT complex had not been analyzed in *Xenopus laevis*. CAF1 and CCR4 were known to be present in oocytes, but the presence and composition of the rest of the CCR4-NOT complex was unclear. As some components critical for RNA control are missing in oocytes (*e.g.* Argonaute, (Lund et al. 2011)), this presented a significant gap in our understanding of mRNA regulation in the oocyte. Through my work, it is now clear that the *Xenopus laevis* CCR4-NOT complex is very similar to the mammalian complex in composition. NOT1, CAF1, CCR4, NOT2, NOT3, NOT4, NOT9, and NOT10 are all conserved in *Xenopus* (Fig. 4).

As in humans, *Xenopus* possesses two CAF1 paralogs, CAF1a and CAF1b. The two proteins are ~75% identical in sequence and are both mainly composed of the DEDD nuclease domain (Cooke et al. 2010). Data from the Wickens lab suggests that CAF1a is expressed in early oogenesis and throughout development, while CAF1b is only expressed after meiotic maturation (Amy Cooke, unpublished). It is unclear if the two proteins have overlapping roles when they are both expressed. In humans, it appears that CAF1a and CAF1b are associated with distinct CCR4-NOT complexes, suggesting that they compete for interaction with NOT1 (Lau et al. 2009). Additionally, they have partially

overlapping roles in human cells, and likely differ mainly in expression pattern rather than function (Aslam et al. 2009). The same may be true in *Xenopus*. Our lab has previously shown that both CAF1a and CAF1b are capable of translational repression independent of their deadenylase activity (Fig. 5) (Cooke et al. 2010). This unique repression in oocytes, which we term deadenylation-independent repression (DAIR), is the focus of the second chapter of my thesis. We hypothesized that DAIR occurs through interaction with the other CCR4-NOT components as well as additional unknown factors, all of which are recruited to target mRNAs by RNA-binding proteins (Fig. 5).

DAIR has been shown by other groups to play a role in miRNA-mediated repression of translation in somatic cells (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011). DAIR of miRNA-regulated mRNAs takes place prior to deadenylation in somatic cells (Bazzini et al. 2012; Djuranovic et al. 2012), and it is likely that repression occurs through a block in early steps of translation.

Overview and significance of work

The overarching goal of my thesis work was to understand the role of the CCR4-NOT complex in mRNA regulation during *Xenopus laevis* early development.

In the second chapter of this thesis, I will discuss my work to elucidate the mechanism of translational repression by CCR4-NOT in *Xenopus* oocytes. I used a combination of mutational analysis, protein-protein interaction analyses, and translation assays to determine the mechanism of DAIR.

The third chapter is focused on the effect of oocyte maturation on CCR4-NOT protein interactions. Mass spectrometry and co-immunoprecipitations with CAF1 and NOT1 demonstrated changes in the interaction profile of CCR4-NOT upon maturation. This is likely due to protein modification events that occur in a maturation-specific manner or to new interaction partners that prevent association of regulatory factors to CCR4-NOT.

Finally, in the appendix, I will discuss my efforts to determine what mRNAs are regulated by CCR4-NOT during *Xenopus* early development.

The work presented here is an important step toward a better understanding of mRNA regulation during early development. Prior to this work, it was unclear how CCR4-NOT could carry out repression of translation independently of deadenylation and decay. Additionally, the *Xenopus* CCR4-NOT complex itself was not characterized until the protein-protein interaction studies described in Chapter 2. The changes in interactions with CCR4-NOT upon maturation present the interesting possibility of developmentally regulated

recruitment of and translational control by the CCR4-NOT complex. The research presented in this thesis has significant impact, as this work elucidates how CAF1 and CCR4-NOT govern the biologically inherent process of translation during early development.

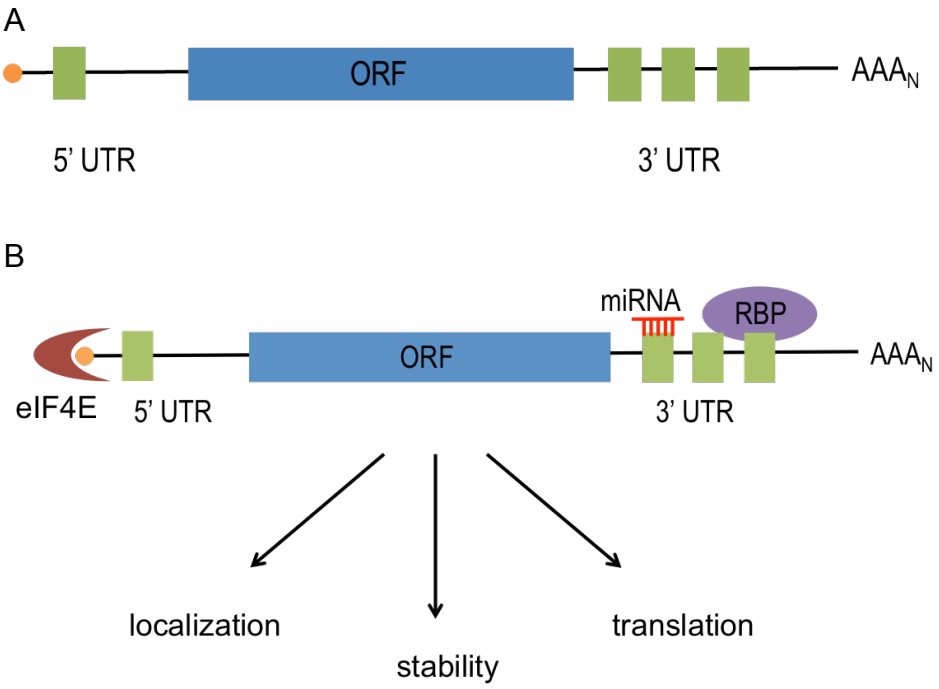

Figure 1: mRNA features and regulation.

A) The overall features of a typical eukaryotic mature mRNA. Green boxes depict sequence elements that may be bound by RNA-binding factors. UTR: untranslated region, ORF: open reading frame. B) microRNAs (miRNAs) and RNA-binding proteins (RBPs) bind to specific elements (shown in green) in the mRNA to regulate its localization, stability and translation. Such binding events most commonly occur in the 3' UTR of the mRNA. eIF4E, the initiation factor and cap-binding protein, is a commonly regulated mRNA-associated factor for translational control.

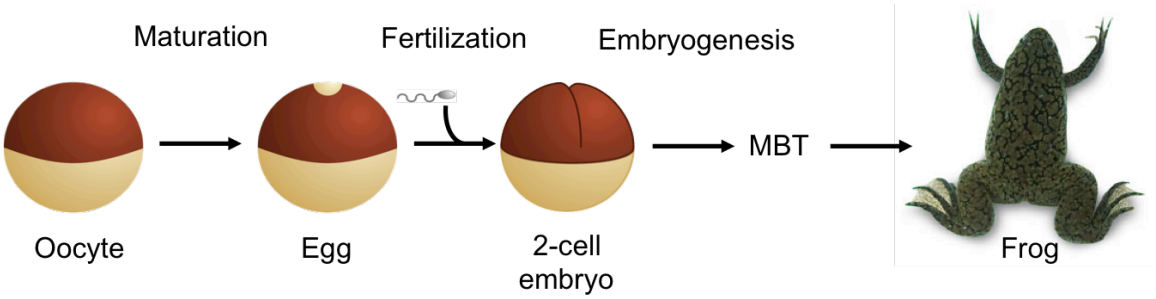

Figure 2: *Xenopus laevis* early development.

Depiction of major points during early development in *Xenopus laevis* at which polyadenylation and deadenylation events are significant. An adult frog is pictured as well.

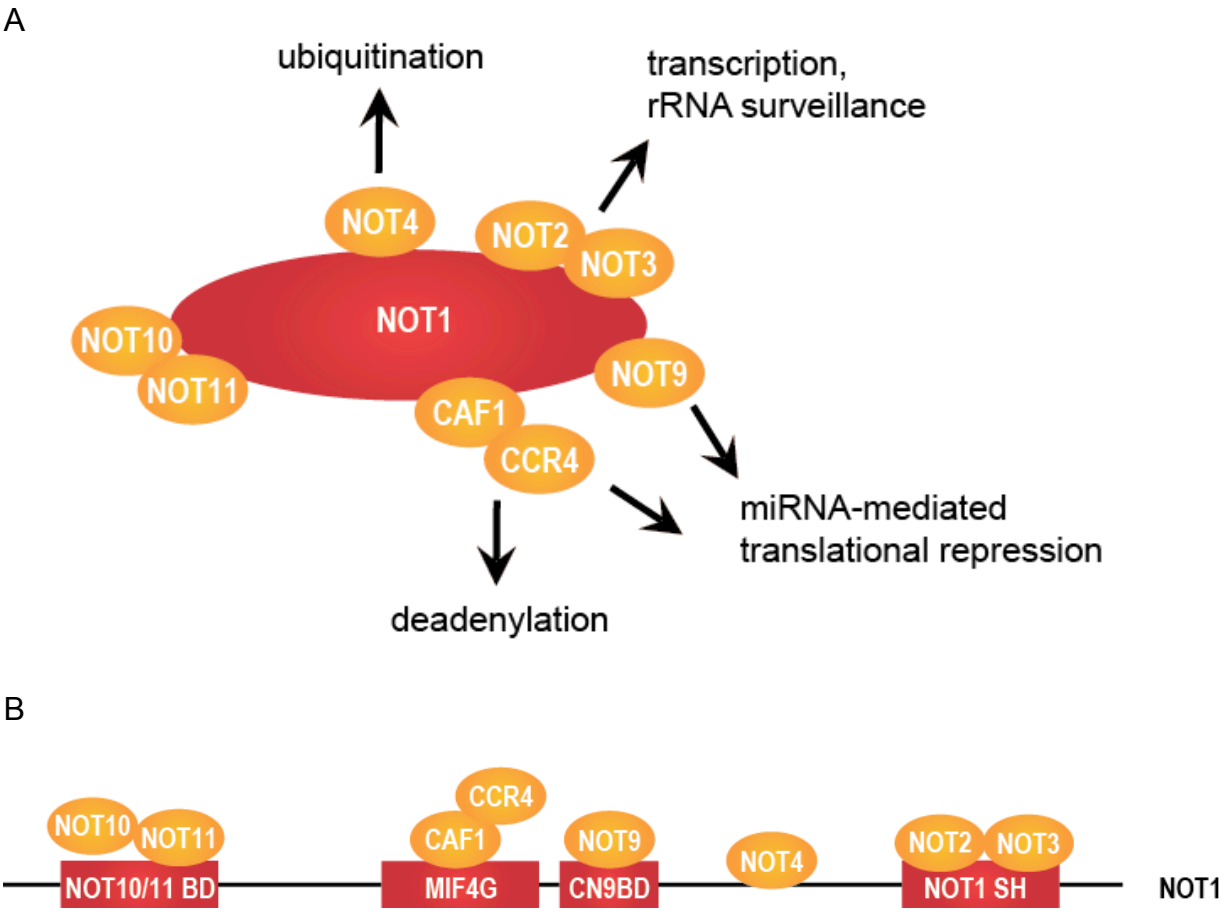

Figure 3. CCR4-NOT complex functions and architecture.

A) CCR4-NOT complex subunits and functions. The NOT1 protein is the scaffold to which almost every other protein binds. B) Domain structure of NOT1 is depicted with red boxes connected by a black line, with orange CCR4-NOT subunits depicted at the region of NOT1 with which they interact.

Human protein	***Xenopus* ortholog(s)**
NOT1	NOT1
CCR4	CCR4a, CCR4b
CAF1	CAF1a, CAF1b
NOT9/RCD1	RQCD1
NOT10	NOT10
NOT11	NOT11
NOT2	NOT2
NOT3	NOT3
NOT4	NOT4
TAB182	None known

Figure 4. Conservation of CCR4-NOT complex components.

Most of the human CCR4-NOT subunits are conserved in *Xenopus laevis*. TAB182 is a human-specific CCR4-NOT complex member.

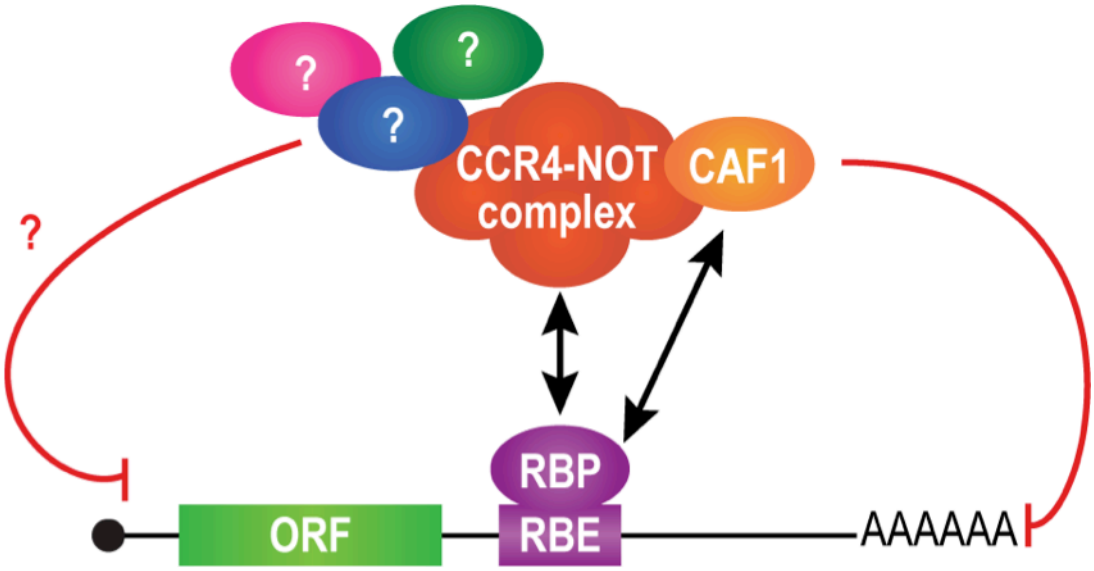

Figure 5. CAF1/CCR4-NOT mediates deadenylation and DAIR.

Depiction of how CAF1 is likely to leverage both deadenylation and DAIR on a target mRNA. DAIR is depicted as inhibition at the 5' end of the mRNA with a question mark, and deadenylation is shown as inhibition of the poly(A) tail. RNA-binding proteins (RBPs) may recruit CAF1 directly or through CCR4-NOT to elicit either deadenylation of the mRNA or DAIR, which potentially requires additional unknown factors (depicted as colored ovals with question marks). RBE: RNA-binding element, ORF: open reading frame.

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Chapter 2:

Xenopus CAF1 requires NOT1-mediated interaction with 4E-T to repress translation *in vivo*

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I designed and performed all the experiments except for the mass spectrometry. Clay Williams performed the mass spectrometry and proteomics data analysis. Marv Wickens and I wrote the manuscript, with comments from Clay Williams and Josh Coon.

ABSTRACT

RNA regulatory factors bound to 3'UTRs control translation and stability. Repression often is associated with poly(A) removal. CAF1, a deadenylase in most organisms, is a core component of the CCR4-NOT complex. Our prior studies established that CAF1 represses translation independent of deadenylation. We sought the mechanism of its deadenylation-independent repression activity in *Xenopus* oocytes. Our data reveal a chain of interacting proteins that link CAF1 to the CCR4-NOT complex and then to Xp54 and 4E-T. Association of CAF1 with NOT1, the major subunit of CCR4-NOT, is required for repression by CAF1 tethered to a reporter mRNA. Affinity purification-mass spectrometry and co-immunoprecipitation assays revealed that at least five members of the CCR4-NOT complex in oocytes were recruited by CAF1. The recruitment of these proteins required NOT1, as did the ability of tethered CAF1 to repress translation. In turn, NOT1 was needed to recruit Xp54 and 4E-T. We examined the role of 4E-T in repression using mutations that specifically disrupted either eIF4E-dependent or -independent mechanisms. Expression of a mutant form of 4E-T that still bound eIF4E alleviated repression by tethered CAF1, NOT1, and Xp54. In contrast, a mutant 4E-T that failed to bind eIF4E did not. Repression of global translation, monitored by a reporter mRNA without binding sites, was affected only by the eIF4E-dependent mechanism. The use of reporters bearing IRES elements revealed that repression via tethered CAF1 and Xp54 is cap- and eIF4E-independent, but requires one or more of eIF4A, eIF4B

and eIF4G. We propose that RNA-binding proteins, and perhaps miRNAs, repress translation through an analogous chain of interactions that begin with the 3’UTR-bound repressor and end with the non-canonical activity of 4E-T.

INTRODUCTION

mRNAs are tightly regulated from their first appearance to their ultimate destruction. Their proper control is critical in development, cell cycle progression, and cell proliferation (Gebauer and Hentze 2004; Molin and Puisieux 2005; Aslam et al. 2009; Chen and Shyu 2011). Repression and activation often correlate well with regulated changes in poly(A) tail length (Gebauer and Hentze 2004). Poly(A) lengthening typically stabilizes mRNAs, while shortening decreases stability and can cause translational repression (Chen and Shyu 2011). In addition, certain enzymes that remove the poly(A) tail repress translation independent of deadenylation (Cooke et al. 2010). Here, we sought to understand the basis of that deadenylation-independent activity.

Deadenylases are important in mRNA control and are recruited to target mRNAs by RNA-binding proteins and miRNAs (Yamashita et al. 2005; Goldstrohm et al. 2006; Garneau et al. 2007; Goldstrohm and Wickens 2008; Chen and Shyu 2011; Sandler et al. 2011). CAF1 is a member of the DEDD (Asp-Glu-Asp-Asp) deadenylase family, a class of enzymes named for the four residues required for catalysis (Wilusz et al. 2001; Goldstrohm and Wickens 2008; Suzuki et al. 2010). Vertebrates encode two CAF1 homologs, CAF1a and CAF1b, which are 76% identical in sequence. Both are highly conserved among higher eukaryotes (Bianchin et al. 2005).

CAF1 is a stable component of the CCR4-NOT complex. This large complex is composed of nine core subunits with roles in regulation of transcription, mRNA export, and mRNA decay (Lau et al. 2009; Collart and

Panasenko 2012; Doidge et al. 2012; Miller and Reese 2012; Temme et al. 2014; Xu et al. 2014). The major component of CCR4-NOT is the 250 kDa NOT1 protein, which acts as a scaffold for the multi-subunit complex (Nasertorabi et al. 2011; Petit et al. 2012; Bawankar et al. 2013). CAF1 binds directly to NOT1 through the MIF4G domain of NOT1 (Basquin et al. 2012). CAF1 is also associated with CCR4-NOT via an independent interaction with CCR4, another deadenylase subunit.

NOT1 interacts with many proteins, suggesting that it may act as a bridge to bring protein interactors into the CCR4-NOT complex. Specifically, GW182, an Argonaute-interacting protein in *Drosophila* (and its homolog TNRC6A-C in mammals), binds NOT1 to recruit factors to miRNA-bound mRNA targets to elicit translational repression (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011). AU-rich element (ARE) binding proteins, such as Tristetraprolin (TTP) and its homologs, also bind directly to NOT1 to initiate deadenylation via the associated CAF1 (Sandler et al. 2011; Fabian et al. 2013). The CCR4-NOT complex is thus recruited to mRNAs by miRNAs and RNA-binding proteins (RBPs) to elicit translational repression or decay of the transcript (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011; Sandler et al. 2011; Wahle and Winkler 2013; Bhandari et al. 2014; Chen et al. 2014; Inada and Makino 2014; Mathys et al. 2014; Rouya et al. 2014; Temme et al. 2014).

CAF1 is unusual in that it represses translation independent of deadenylation. *Xenopus* CAF1a and CAF1b both possess this activity (Cooke et al. 2010), and this process is important for miRNA-mediated repression in

somatic cells (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011). The mechanism through which this unexpected deadenylation-independent repression occurs is unknown. We hypothesized that the interaction between CAF1 and the CCR4-NOT complex might mediate other protein contacts important for repression.

We sought to determine how CAF1 elicits deadenylation-independent repression. To do so, we identified proteins associated with CAF1 and tested their functional roles using interaction-defective mutations. Our results reveal that NOT1 interaction with CAF1 is critical for repression and that the MIF4G domain of NOT1 recruits Xp54 and 4E-T. This repressive complex prevents translation in an eIF4E- and cap-independent manner.

RESULTS

CAF1 requires interaction with NOT1 to repress translation

To determine how CAF1 represses translation independent of deadenylation, we expressed mutant CAF1 proteins in *Xenopus* oocytes. We reasoned that CAF1 bound specific protein partners that exerted the repressive activity. To test this idea, we created mutations in *Xenopus laevis* CAF1b that were predicted to disrupt interaction with NOT1, CCR4, and TOB, each of which directly interact with CAF1 (Fig. 1A) (Horiuchi et al. 2009; Basquin et al. 2012). The known human CAF1a structure was used to guide mutagenesis (Horiuchi et al. 2009). We also mutated specific surface residues to alanine, reasoning that they too might mediate protein-protein contacts (Fig. 1A).

To test whether these mutations disrupted CAF1-mediated repression, wild type and mutant forms of CAF1b were tethered to a reporter mRNA in *Xenopus laevis* oocytes (Fig. 1B). Oocytes were injected with mRNAs encoding HA-tagged and MS2 coat protein-fused (HA-MS2) CAF1b proteins. After allowing time for the HA-MS2-CAF1 proteins to accumulate, the same oocytes were simultaneously injected with two reporter mRNAs. The first contained the firefly luciferase open reading frame linked to a 3'UTR with binding sites for MS2 coat protein. The second contained the *Renilla* luciferase open reading frame lacking MS2 sites, which served as a negative control and was used for normalization.

CAF1 required interaction with NOT1 for repression (Fig. 1C). The mutations that disrupted interaction with NOT1 (M141R and E138K/D139K) both abrogated repression (Fig. 1C, red bars). M141R also disrupted repression in a

deadenylation-defective CAF1 background (D40A, E42A; referred to as DE-AA), demonstrating that it affected the deadenylation-independent process. Mutations that disrupted interactions with CCR4 (Q215A, Q219A) or TOB (V44A, F155A, H156A, S157A) did not relieve repression. As expected, the negative control GLD2 (D242A) (Cooke et al. 2010) had no effect on translation, yielding the same level of luciferase as an oocyte expressing no MS2 protein (Fig1C, grey bars). Western blots with α-HA revealed that the MS2 fusion proteins were expressed (Fig. S1). We confirmed that the interaction between CAF1 M141R and NOT1 had been disrupted by co-immunoprecipitation (co-IP), in which a NOT1 mutant defective for interaction with CAF1 served as a control (Fig. 1D-E). From these data, we inferred that NOT1 was required for the repression activity of CAF1 and next sought the partners of NOT1 that might be involved.

Mass spectrometry reveals novel CAF1 interactors

To identify proteins that interacted with CAF1 via NOT1 in oocytes, we used both candidate and proteomics approaches (Fig. 2A). In both cases, GST pull-downs were conducted with recombinant wild type and mutant CAF1a and CAF1b proteins. Each was immobilized on glutathione magnetic resin, incubated with *Xenopus* oocyte lysate, and then washed to eliminate nonspecific interactions. GST protein served as a negative control. As expected, each GST protein was detected in equal amounts among the recovered proteins (Fig. 2B, top).

We found that interactions between CAF1 and Xp54, as well as multiple components of the CCR4-NOT complex, were facilitated by NOT1. Mass

spectrometry was used to identify proteins enriched in the pull-downs with wild type or M141R forms of CAF1. To quantify the mass spectrometry data, we measured enrichment in wild type and mutant pull-down samples relative to the GST negative control. We then compared abundances of proteins in pull-downs performed with wild type or mutant CAF1. Proteins that interacted specifically with the wild type CAF1, and not M141R, fell into two groups, which were essentially the same whether CAF1a or CAF1b had been used as the affinity reagent (Fig. 2C, D, Table S1). One group was enriched 20-30-fold relative to the GST negative control, with highly significant p-values ($p < 1x10^{-5}$). The proteins in this group (highlighted in Fig. 2C, D) are all members of the CCR4-NOT complex and likely very stably associated with CAF1 and NOT1. A second group of interactors, including Xp54, exhibited 2-fold enrichment relative to GST with significant p-values ($p < 0.05$).

The candidate approach confirmed the results obtained by mass spectrometry. We tested interaction between the GST proteins and known RNA-regulatory factors by Western blot, using appropriate antibodies. Xp54 was also tested for interaction with CAF1, as it has recently been reported to directly contact the NOT1 MIF4G domain (Chen et al. 2014; Mathys et al. 2014; Rouya et al. 2014). Xp54 interacted with CAF1 in a manner that required the wild type M141 allele (Fig. 2B). Since Xp54 associates with 4E-T (Minshall et al. 2007), we tested 4E-T interaction with CAF1. 4E-T also interacted with CAF1 dependent on the wild type M141 allele (Fig. 2B). Disruption by the M141R mutation implied that CAF1 contacts both Xp54 and 4E-T via NOT1. Wild type and mutant CAF1

proteins bound CCR4, implying that this interaction was independent of NOT1, as observed in other systems (Bai et al. 1999; Basquin et al. 2012). eIF4E, a protein that does not bind to CAF1 (Fabian et al. 2009), interacted with neither the wild type nor mutant forms of CAF1.

Thus, multiple components of the CCR4-NOT complex efficiently bound CAF1 via NOT1. Other proteins, including Xp54 and 4E-T, also were retained in a NOT1-dependent fashion, but were present in lower abundance. This may reflect their lower abundance in oocytes or the instability of their interactions (see Discussion). Xp54 and 4E-T are both known and conserved translational repressors. The former is an ATP-dependent RNA helicase and part of the decapping machinery (Coller et al. 2001; Fischer and Weis 2002; Minshall and Standart 2004), while the latter is an eIF4E-binding protein that is known to repress translation via two distinct mechanisms (Kamenska et al. 2014).

Co-immunoprecipitations confirm interactions *in vivo*

To confirm interactions detected by GST pull-down, we conducted co-immunoprecipitations from *Xenopus* oocytes. HA-tagged CAF1 mutant and wild type proteins were expressed and immunoprecipitated (Fig. 3A), and the products were analyzed by Western blot.

Both CAF1a and CAF1b required interaction with NOT1 to immunoprecipitate 4E-T and Xp54 (Fig. 3B). In contrast, CCR4 was immunoprecipitated by both wild type and mutant CAF1a and CAF1b proteins, while eIF4E was not detected in any of the IPs. Probing with the α-HA antibody confirmed that the CAF1 proteins were immunoprecipitated (Fig. 3B, bottom).

Analogous IPs were conducted to determine whether the interactions via NOT1 were mediated by its MIF4G domain (Fig. 3C). We tested interactions of 4E-T and Xp54 with either a wild type or CAF1-interaction defective allele of the MIF4G domain (Basquin et al. 2012). Both 4E-T and Xp54 interacted with the MIF4G domain, independent of CAF1 interaction (Fig 3D). eIF4E did not interact with the MIF4G domain. Wild type and mutant MIF4G domains (detected with α-HA) were both immunoprecipitated, as expected.

These data demonstrate that the NOT1 MIF4G domain is responsible for recruitment of Xp54 and 4E-T.

NOT1 MIF4G domain mediates repression

To identify the role of the NOT1 MIF4G domain in translational repression in oocytes, we determined the repression activity of a series of NOT1 truncations. Fragments of NOT1, tagged with HA and MS2, were tethered to a reporter mRNA in *Xenopus* oocytes (Fig. 4A, B). CAF1-interaction defective and Xp54-interaction defective alleles (Basquin et al. 2012; Chen et al. 2014; Mathys et al. 2014; Rouya et al. 2014) of the NOT1 MIF4G domain were tested in parallel.

The NOT1 MIF4G domain was sufficient for repression, and that activity was dependent on its interaction with Xp54 (Fig. 4C). Wild type and CAF1-interaction defective alleles of the MIF4G domain possessed similar activities, suggesting that CAF1 was dispensable for repression. This is consistent with data obtained in human and *Drosophila* cells (Chen et al. 2014; Mathys et al. 2014; Rouya et al. 2014). The N terminus of NOT1 repressed only when it included the MIF4G domain, while the C terminus retained some repressive

activity even without the MIF4G domain. This is likely due to the fact that the C terminus contains a domain necessary for interaction with CNOT9. NOT1 interaction with CNOT9 contributes to, but is not required for, repression in other systems (Chen et al. 2014; Mathys et al. 2014). Xp54 (Fig. 4C), used as a control, repressed translation relative to an oocyte expressing no MS2 tagged protein as expected (none, Fig. 4C). Expression of the appropriate proteins was confirmed by Western blot (Fig. S2); the N- and C-terminal fragments lacking the MIF4G domain were less abundant than the other fragments, relative to actin.

The Xp54-binding defective mutation in the NOT1 MIF4G did indeed disrupt recruitment of Xp54, as judged by co-immunoprecipitation (co-IP) (Fig. 4D-E). Similarly, 4E-T interacted with NOT1 in an Xp54-dependent manner. From these data, we propose that Xp54 directly interacts with NOT1 and is a bridge that links NOT1 and 4E-T (Fig. 4F).

4E-T is responsible for repression in an eIF4E-independent manner

Xp54 interacts with the protein 4E-T (Minshall et al. 2007), which represses translation through two distinct mechanisms that can be differentiated by mutational analysis (Kamenska et al. 2014). Expression of wild type 4E-T represses translation of cellular mRNAs (Kamenska et al. 2014). 4E-T mutations (YLL-AAA) that disrupt binding to eIF4E lack that activity (Fig. 5A). 4E-T mutants that possess only the N-terminal region (aa 1-180) bind eIF4E and are general translational repressors; importantly, they do not further repress mRNA to which they are tethered via the 3'UTR (Kamenska et al. 2014). Thus, 4E-T can repress translation via a canonical eIF4E-dependent mechanism (Fig. 5A, "Mechanism

1") and through a distinct eIF4E-independent mechanism (Fig. 5A, "Mechanism 2").

Since our data indicated that Xp54 was required for repression in oocytes, and Xp54 interacts with 4E-T, we sought to determine the role of 4E-T in repression. We used a modified tethered function assay, in which HA-MS2 fusions of CAF1b, Xp54, and the NOT1 MIF4G domain were co-expressed with untethered HA-tagged 4E-T proteins (Fig. 5B). We tested both previously identified 4E-T mutants (YLL-AAA and 1-180;(Kamenska et al. 2014)) and wild type 4E-T. This strategy, diagrammed in Figure 5B, enabled us to examine the relative contributions of eIF4E-dependent and -independent mechanisms (Kamenska et al. 2014). Since 4E-T associates with Xp54, NOT1 and CAF1b (Fig. 2, 3, 4) (Minshall et al. 2007), we reasoned that over-expressed 4E-T proteins would interact with tethered Xp54, NOT1 and CAF1b proteins. If the 4E-T interaction with eIF4E were required for repression by tethered CAF1b, Xp54, and NOT1, then expression of the YLL-AAA mutant would abolish that repression activity. However, if repression were elicited via the eIF4E-independent mechanism, then the YLL-AAA mutant would still repress, while the 4E-T 1-180 truncation would abrogate repression of the bound reporter mRNA.

Of the 4E-T constructs tested, only 4E-T 1-180 alleviated repression by the tethered proteins (Fig. 5C). It interfered with repression by tethered CAF1b, Xp54 or the NOT1 MIF4G domain. Neither the wild type nor the eIF4E-interaction defective variant interfered with repression by any of the three tethered proteins tested, relative to control oocytes lacking any tethered protein. Thus, repression

by tethered CAF1, Xp54 and NOT1 occurred via the eIF4E-independent activity of 4E-T. Expression of the proteins was confirmed by Western blot (Fig. S3).

To ensure that the effect of 4E-T on repression by the tethered proteins was due to the eIF4E-independent mechanism, we examined the effects of the 4E-T proteins on general translation by analysis of *Renilla* luciferase levels (Fig. 5D). This reporter lacks MS2 sites, and so is not subject to the effects of the MS2 fusion proteins. As seen in human cells (Kamenska et al. 2014), wild type 4E-T and the 1-180 truncation reduced general translation, while the YLL-AAA mutant did not. These findings demonstrate that the 4E-T YLL-AAA protein is functional but its ability to bind eIF4E is dispensable for repression mediated through 3'UTR-bound proteins.

Taken together, our studies revealed two mechanisms of repression by 4E-T in oocytes. This is consistent with previous experiments conducted in human cells (Kamenska et al. 2014). The first mechanism was independent of the eIF4E interaction and acted specifically on the firefly reporter to which the proteins were tethered (Fig. 5C, "Mechanism 1"). The second was dependent on the eIF4E interaction and affected general translation, as monitored by the *Renilla* reporter (Fig. 5D, "Mechanism 2"). These results, summarized in Figure 5E, demonstrate that 4E-T mediates repression by the CCR4-NOT complex in oocytes, and does so in a manner independent of its interaction with eIF4E.

Repression by CAF1 and Xp54 is independent of the 5' cap and eIF4E

Since our data implied that 4E-T acted independently of its interaction with eIF4E, we probed the role of the cap and translation initiation factors in

repression by CAF1 (Fig. 5). To do so, we used reporters bearing different IRES regions, with varying initiation factor requirements (Fig. 6A, B).

CAF1b and Xp54 were tethered to a firefly reporter mRNA possessing either a 7-methyl GpppG cap or one of three IRESes, each with different translation factor requirements (Fig. 6B). An ApppG cap was used to protect the IRES reporters from degradation. As a positive control, we used our standard 5'UTR with a 7mGpppG cap. The *Renilla* luciferase reporter served as a negative control, as it contained no tethering sites. Expression of the tethered proteins was confirmed by Western blot (Fig. S4).

Xp54 and both wild type and deadenylation-defective (DE-AA) CAF1b failed to repress via the Classical Swine Fever Virus (CSFV) or Cricket Paralysis Virus (CrPV) IRESes (Fig. 6C). Strikingly, they did repress RNAs bearing the Poliovirus (PV) IRES. As expected, the negative control GLD2 (D242A) had no effect on translation. The ability of CAF1 and Xp54 to repress the PV IRES reporter suggests that the cap structure is dispensable for repression. CSFV and PV IRESes share a requirement for eIF3 and eIF2. They differ in that the PV IRES also recruits eIF4G and therefore eIF4A and eIF4B, but not eIF4E (Pestova et al. 1996; Kieft 2008; Plank and Kieft 2012). This suggests that one or more of eIF4G, eIF4A and eIF4B are required for repression by CAF1 and Xp54.

DISCUSSION

Our efforts to understand the mechanism of deadenylation-independent repression by CAF1 in oocytes led to a chain of protein-protein interactions that link tethered CAF1 first to NOT1, then Xp54, and ultimately to 4E-T and an eIF4E-independent mechanism (Fig. 7A). The significance of each interaction has been validated using interaction-defective mutations. Tethered CAF1 represses only if it is capable of interaction with NOT1, the scaffolding component of the CCR4-NOT complex. Similarly, tethered NOT1 represses in an Xp54-dependent fashion (Fig. 4C). 4E-T is responsible for repression by CAF1, NOT1, and Xp54 (Fig. 5B), and this repression is both eIF4E- and cap-independent (Fig. 6).

We identified two groups of CAF1 interactors through GST pull-down and mass spectrometry. The first group comprised CCR4-NOT subunits and was highly enriched for wild type CAF1, suggestive of a stoichiometric and stable interaction. Xp54 was identified among a less enriched group of proteins that likely interact with lower stoichiometry with CAF1 and NOT1. Other 3'UTR-binding proteins likely make contacts to CAF1 and CCR4-NOT but were not detected due to their low abundance or the transience of their interactions. RNA-binding proteins known to mediate repression of specific families of mRNAs in oocytes may enter and leave the complex to control translation of their targets during oogenesis, oocyte maturation, and early embryonic development.

We observe two mechanisms of translational repression by 4E-T (Fig. 5). General translation, as monitored by reporter mRNAs without MS2 sites, is

repressed by 4E-T via interaction with eIF4E. Importantly, repression due to 3'UTR-bound factors acts via the CCR4-NOT complex through an eIF4E-independent mechanism. The molecular basis of this activity of 4E-T has not yet been determined.

The use of multiple IRES reporters demonstrates that repression via tethered CAF1 requires one or more of eIF4A, eIF4B and eIF4G. Our IRES results are similar to those obtained in studies of miRNA-mediated repression (Meijer et al. 2013). In human cells, let-7 miRNA repressed mRNAs bearing the EMCV IRES, but not those of HCV or CrPV IRES (Meijer et al. 2013). These findings suggested that miRNA-mediated repression required subunits of eIF4F other than eIF4E, and were consistent with the conclusion that eIF4A2 was critical (Meijer et al. 2013). The factors required for repression via miRNAs and RNA-binding proteins overlap. It will be of interest to determine the extent to which their mechanisms diverge.

During early development, RNA-binding proteins repress and activate translation of specific mRNAs by binding to regulatory elements in their 3'UTRs. While many of these proteins recruit the CCR4-NOT complex, they do so by interacting with different subunits. For example, PUF proteins in the fly embryo bind NOT4 via interaction with NANOS1 (Kadyrova et al. 2007), while yeast PUF proteins bind directly to the CAF1 homolog POP2 (Goldstrohm et al. 2006). Similarly, the fly proteins NANOS2 and NANOS3 interact with CAF1 and NOT1 respectively (Suzuki et al. 2014), but TTP in mammalian cells binds NOT1 directly (Sandler et al. 2011; Fabian et al. 2013). Human TOB1 binds to CAF1 to

recruit CCR4-NOT to the RNA-binding protein CPEB3 (Hosoda et al. 2011). We propose that these diverse modes of recruitment of the CCR4-NOT complex allow for independent contacts that elicit regulation of mRNAs (Fig. 7B). For instance, if an interaction between one CCR4-NOT subunit and a 3'UTR-bound protein were disrupted, different RNA-binding proteins would remain bound to CCR4-NOT via the other subunits. Thus, various RNA-binding proteins can employ CCR4-NOT to deadenylate or repress their target mRNAs. Depletion of 4E-T increases the half-life of ARE-containing mRNAs (Ferraiuolo et al. 2005), suggesting that ARE-binding proteins such as TTP do indeed exploit 4E-T.

Our work suggests a model in which RNA-binding proteins associated with the 3'UTR elicit repression via a network of interactions that lead to recruitment of 4E-T and its eIF4E-independent mechanism of action (Fig. 7B). miRNA-mediated repression in human and *Drosophila* cells similarly requires recruitment of NOT1, CAF1, and the Xp54 homolog DDX6, but via Ago and GW182 (TNRC6A-C) (Chen et al. 2014; Mathys et al. 2014; Rouya et al. 2014). In oocytes, Ago is not present and miRNA-mediated repression does not occur (Lund et al. 2011). Thus we propose that the action of regulatory 3'UTR-binding proteins in the oocyte is through the chain of protein-protein interactions in Figure 7B, without Ago or miRNAs. The balance among modes of recruitment appears to vary in oocytes and somatic cells, though the chain of connectivity is similar. It will be of interest to determine whether multiple 3'UTR-binding proteins that act during early development recruit identical complexes.

MATERIALS & METHODS

Plasmid construction and mutational analysis

pCS2+3HAMS2 containing CAF1, Xp54, and GLD2-D242A proteins have been described previously (Cooke et al. 2010). CAF1 mutants in all plasmids were generated using site-directed mutagenesis. For protein purifications, pGEX-6P-1 plasmids containing CAF1 proteins cloned into the SalI/NotI sites were used. NOT1 fragments and mutants (N-terminus: amino acids 1-1317; C-terminus: amino acids 1093-2376; MIF4G domain: amino acids 1093-1317; N-terminus (−): amino acids 1-1092; C-terminus (−): amino acids 1318-2376; CAF1 binding-defective: P1209Y, P1257Y, V1251R; Xp54 binding-defective: E1142R, N1144A, F1145A) were amplified from the human NOT1 cDNA clone (Thermo Scientific, Accession: BC040523.1, MGC: 23019, IMAGE: 5266600) and cloned into the XhoI/SnaBI sites of pCS2+3HAMS2 for tethered function assays or the same sites in pCS2+3HA or pCS2+9xmyc for co-IPs. Full length NOT1 was cloned the same way, but did not express in oocytes so was not used for experiments. pCS2+9xmyc was constructed by amplifying the 9xmyc tag from the pCDNA3+9xmyc plasmid into the BamHI and StuI sites of pCS2+3HA, replacing the 3 HA tags.

Reporter mRNA plasmids for tethered function assays (pLG-MS2 and pCSFV-Luc-MS2 for firefly and pSP65-ren for *Renilla*) have been described previously (Dickson et al. 2001; Kwak et al. 2004). pPV-Luc-MS2 and pEJ4 with MS2 were kindly provided by Nicola Gray. All reporter mRNAs possessed a poly(A) tail.

4E-T was amplified from the *Xenopus laevis* eIF4ENIF1 cDNA clone (GE Healthcare, MGC:80355, IMAGE:5074419) and cloned into the pCS2+3HA vector using NcoI/XbaI sites. The YLL-AAA (Y28A, L33A, L34A) mutant was generated using site-directed mutagenesis, and the 1-180 truncation was amplified from the full-length cDNA clone and put into pCS2+3HA with NcoI/XbaI.

In vitro transcription and RNA preparation

Plasmids for protein expression were linearized with NotI (Fermentas) and plasmids for luciferase reporter mRNAs were linearized with BamHI (pLG-MS2) or SalI (pSP65-ren). Linearized plasmids were transcribed using either AmpliScribe SP6 high yield transcription or T7-Flash transcription kits (CellScript). m^7G(5')ppp(5')G cap analog (New England Biolabs) was added into the transcription reactions to cap the mRNAs. After transcription and DNase I treatment, mRNAs were purified using the Fermentas RNA purification kit.

Oocyte injections

Oocyte injections were performed as described previously (Gray et al. 2000; Kwak et al. 2004).

Tethered function assays

Tethered function assays were conducted as described previously (Cooke et al. 2010). Briefly, Stage VI oocytes were injected with 50 nL of 400 ng/μL capped mRNAs for MS2 or HA protein expression. Six hours later, the same oocytes were injected again with 50 nL of 30:10 ng/μL (firefly:*Renilla*) reporter mRNA mix. After sixteen hours, oocytes were collected, lysed, and assayed using the Promega Dual Luciferase Assay kit.

Protein purification

Protein expression plasmids were transformed into BL21-CodonPlus(DE3)-RIL cells and grown in LB+ampicillin/2% glucose medium at 37 °C until OD_{600}∼0.8. Protein expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside overnight at 16 °C. Cells were resuspended in lysis buffer (20 mM HEPES-KOH, pH 7.4, 0.5 M NaCl, 5 mM DTT, 0.02% Tween-20, with protease inhibitors [EDTA-free, Roche]), and lysed using a Cell Disrupter (2.2kW TS Series, Constant Systems Ltd.). Cleared lysate was incubated with pre-washed glutathione magnetic resin (Novagen GST•Mag™) for 2 hours at 4 °C. The resin was washed three times with lysis buffer, and a final wash was conducted with 1x PBS with protease inhibitors (EDTA-free, Roche). The protein-bound resin was resuspended in 1x PBS with protease inhibitors and 30% glycerol. Protein concentrations were determined by Bradford assays, and proteins were aliquoted and flash frozen prior to storage at -80 °C.

GST pull-down assays

Oocytes were lysed with a pestle in TNMEN buffer with 0.05% Igepal (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 2 mM $MgCl_2$, 150 mM NaCl) with protease inhibitors (EDTA-free, Roche). Oocyte lysate was cleared and RNase A/T1 (Promega) was added to digest RNA. Cleared lysate was incubated with single aliquots of GST or GST-CAF1 proteins immobilized on glutathione magnetic resin after the proteins were washed with TNMEN + 0.05% Igepal and protease inhibitors to remove glycerol. The lysate and proteins were incubated at 4°C for 2 hours. GST proteins were washed twice with TNMEN with 0.01% Igepal, followed

by two more washes with TNMEN with 0.01% deoxycholate. GST proteins and bound partners were eluted from the resin in 50 mM glutathione in 20 mM Tris pH 8 and 0.01% deoxycholate. Elution was performed twice at room temperature for 45 minutes. Eluted proteins were used for Western blot analysis (performed as described previously, (Cooke et al. 2010)) and mass spectrometry.

Co-immunoprecipitations

Oocytes were injected with 50 nL of 400 ng/μL capped mRNAs expressing the appropriate HA-tagged or myc-tagged proteins. After proteins were allowed to accumulate for 6 hours, oocytes of each condition were collected and lysed as for GST pull-downs. Cleared lysate (with RNase A/TI added) was incubated with HA or myc magnetic resin (Pierce α-HA magnetic beads or MBLI α-myc tag mAb-magnetic beads). The lysate and resin were incubated at 4°C for 2 hours. The resin was washed four times with TNMEN with 0.01% Igepal. SDS-PAGE loading dye was added to the resin to elute proteins prior to analysis by Western blot (performed as described previously, (Cooke et al. 2010)).

Mass spectrometry

Eluates were brought to 8 M urea, 50 mM Tris (pH 8.0), 50 mM sodium chloride and 1x protease inhibitor (Roche). Disulfide bonds were reduced with 5 mM dithiothreitol, alkylated with 15 mM iodoacetamide, and the alkylating reaction was quenched with 5 mM dithiothreitol. Samples were diluted to 1.5 M urea and digested with trypsin (Promega) at a 1:100 enzyme to protein ratio. Samples were acidified to pH 2 using trifluoroacetic acid and centrifuged to remove detergents. Peptides were desalted using C_{18} columns (SepPak,

Waters), dried under vacuum, and resuspended in 0.2% formic acid. LC-MS/MS was performed on a Thermo Q Exactive coupled to a nanoAcquity UPLC (Waters). Mobile phase A comprised water, 0.2% formic acid, and 5% DMSO and mobile phase B comprised acetonitrile and 0.2% formic acid. Peptides were separated over a 100 minute gradient on a 75 μm inner diameter fused silica capillary packed with 5 μm diameter, 130 Å pore size Bridged Ethylene Hybrid C_{18} particles (Waters) heated to 60 °C. Eluting peptides were converted to gas phase ions by electrospray ionization. The mass spectrometer method consisted of an MS1 survey scan followed by MS2 scans of the 20 most abundant precursors. The isolation width was set to 2 m/z, and peptides were subjected to HCD with normalized collision energy set to 25. The MS2 AGC target was set at 1e5 with a maximum injection time of 60 ms. Precursors with a charge state of less than two were rejected, and dynamic exclusion was set to 20 s.

Mass spectrometry data analysis

Raw data from 3 biological replicates were analyzed using MaxQuant software (Cox and Mann 2008). Spectra were searched using the Andromeda search engine (Cox et al. 2011) against a *Xenopus* reference proteome obtained from UniProt (http://www.uniprot.org/). Search parameters include: allowance of up to two missed cleavages, oxidation of methionine as a variable modification, and carbamidomethylation of cysteine as a fixed modification. Precursor ion mass tolerance was set to 4.5 ppm, and product ions were allowed a 20 ppm mass tolerance. Peptide and protein identifications were filtered to a 1% false discovery rate using a target-decoy method (Nesvizhskii and Aebersold 2005).

Label free quantification of proteins was done using the MaxLFQ algorithm (Cox et al. 2014). Parameters for quantification include: minimum ratio count was set to 2, Fast LFQ was selected, minimum number of neighbors was set to 3, and average number of neighbors was set to 6.

Antibodies

The following antibodies were diluted 1:1000 in 5% milk in 1x TBST: α-HA-11 Clone 16B12 (Covance), α-c-myc (Sigma C3956), α-DDX6 (A300-460A, Bethyl Laboratories), α-eIF4ENIF1 (ab6034, Abcam), α-eIF4E C46H6 (2067S, Cell Signaling Technologies). α-actin antibody (MAB1501, Millipore) was diluted 1:10,000 in 5% milk in 1x TBST. Secondary antibodies, α-Goat IgG (KPL, 14-13-06), α-rabbit IgG (KPL, 074-1506), α-mouse IgG (KPL, 474-1806) were diluted 1:20,000 in 5% milk in 1x TBST.

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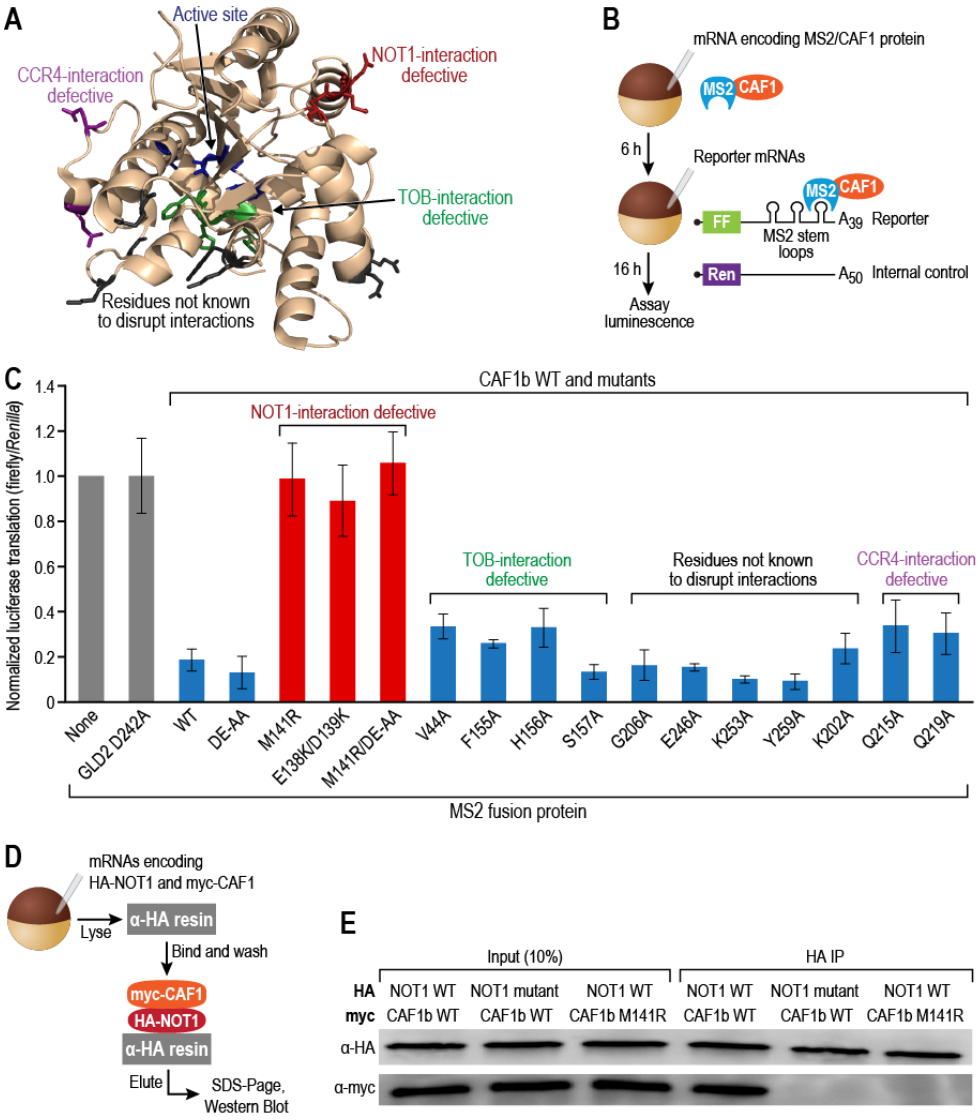

Figure 1. Mutational analysis of CAF1 proteins reveals requirement for NOT1 interaction. A) Residues mutated are depicted on the human CAF1a structure (PDB 2D5R) and colored by the interaction disrupted. B) Outline of the tethered function assay. FF: firefly luciferase, Ren: *Renilla* luciferase. C) Results of tethered function assay, normalized to "no MS2 protein" control (none). Three independent experiments were performed, with four oocyte replicates each time. Error bars represent one standard deviation. Student's two-tailed t-test was used to determine significant changes between CAF1 WT and mutants, and only NOT1-interaction defective mutants were significantly ($p<0.005$) different. D) Outline of co-immunoprecipitation assay from *Xenopus* oocytes. E) NOT1 interacts with wild type CAF1 but not with the M141R mutant. A CAF1-interaction defective mutant of NOT1 served as a negative control. Western blots depict a single representative experiment, and three biological replicates were conducted.

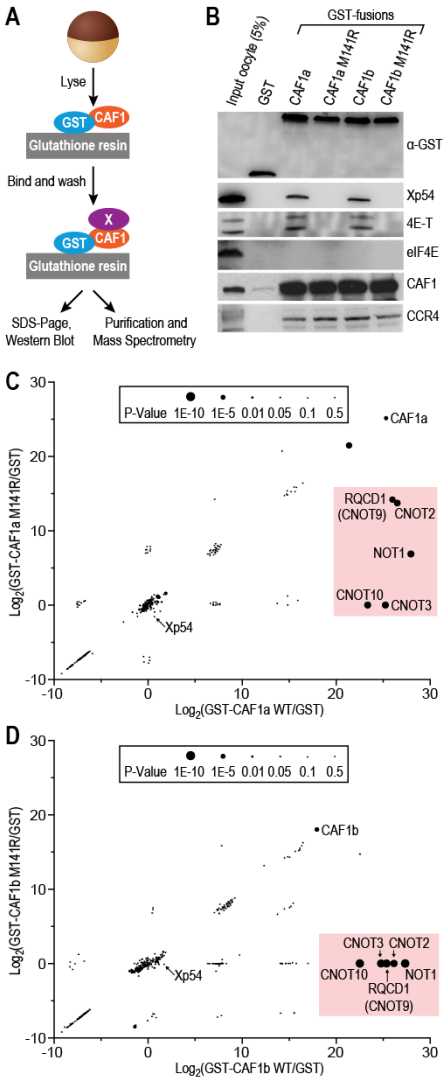

Figure 2. NOT1 mediates interactions between CAF1 and its protein partners.

A) Schematic representation of GST pull-down assay. B) GST-CAF1 proteins are efficiently pulled down. Western blots with antibodies to known regulatory proteins were performed after GST pull-down to test for CAF1 interaction. Western blots depict a single representative experiment, and three biological replicates were conducted. C) Graphical representation of mass spectrometry data from 3 biological replicates for GST-CAF1a wild type and mutant. P-values calculated using a two-tailed Student's t-test are indicated by dot size. Members of the CCR4-NOT complex (boxed) interact strongly with CAF1a WT but not the M141R mutant. Xp54 is also enriched specifically for CAF1a WT over the M141R mutant, but to a lesser extent. D) Same as in C, but with CAF1b.

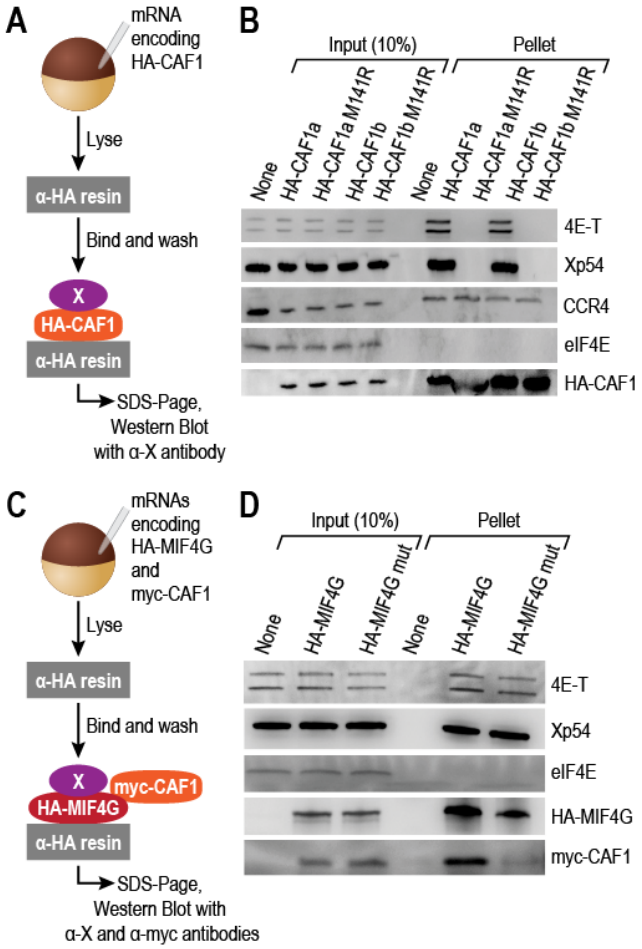

Figure 3. NOT1 MIF4G is required for CAF1 interactions *in vivo*.
A) Schematic of the co-immunoprecipitation assay with HA-CAF1 as the bait protein. B) Western blots with antibodies to proteins that interact with CAF1 by GST pull-down were conducted to test interaction by co-IP. CCR4 is a positive control, and eIF4E is a negative control. Western blots depict a single representative experiment, and three biological replicates were conducted. C) Schematic of the co-immunoprecipitation assay with HA-MIF4G (the NOT1 MIF4G domain) as the bait protein. Both HA-MIF4G WT and a CAF1-binding deficient mutant of the MIF4G domain (HA-MIF4G mut) were used. myc-CAF1 was co-expressed as a control. D) Western blots were conducted to test whether 4E-T and Xp54 interact with the NOT1 MIF4G domain. eIF4E was used as a negative control, and myc-CAF1 also served as a control. Western blots depict a single representative experiment, and three biological replicates were conducted.

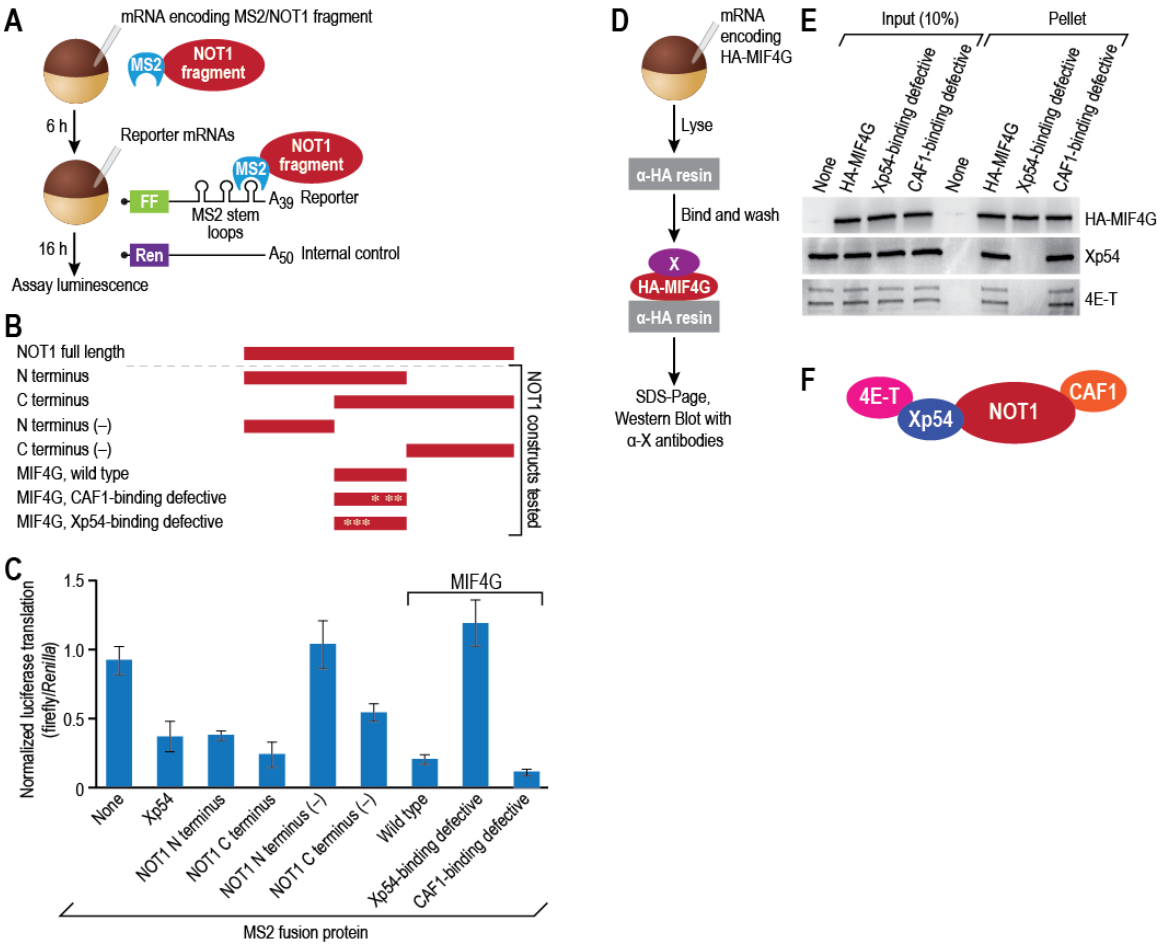

Figure 4. NOT1 MIF4G-Xp54 interaction is required for repression.
A) Schematic of tethered function assay with NOT1 fragments. FF: firefly luciferase, Ren: *Renilla* luciferase. B) NOT1 fragments used in the tethered function assay. Mutations were made in the NOT1 MIF4G domain to obtain Xp54- and CAF1-binding defective constructs, as indicated by asterisks. Full length NOT1 is depicted for scale. C) Results of tethered function assay, normalized to "no MS2 protein" control (none). Xp54 was used as a control for repression. Three independent experiments were performed, with four oocyte replicates each time. Error bars represent one standard deviation. Student's two-tailed t-test was used to compare the NOT1 N terminus to the N terminus (−) and the wild type MIF4G to the Xp54-binding defective MIF4G. Both were significantly ($p<0.005$) different. D) Schematic of the co-immunoprecipitation assay with HA-MIF4G (the NOT1 MIF4G domain) as the bait protein. HA-MIF4G WT, the CAF1-binding deficient mutant of the MIF4G domain, and the Xp54-binding deficient mutant were used. E) Western blots were conducted to test whether 4E-T and Xp54 interact with the Xp54-binding deficient MIF4G domain. Western blots depict a single representative experiment, and three biological replicates were conducted. F) Model of the interactions between NOT1, CAF1, Xp54, and 4E-T.

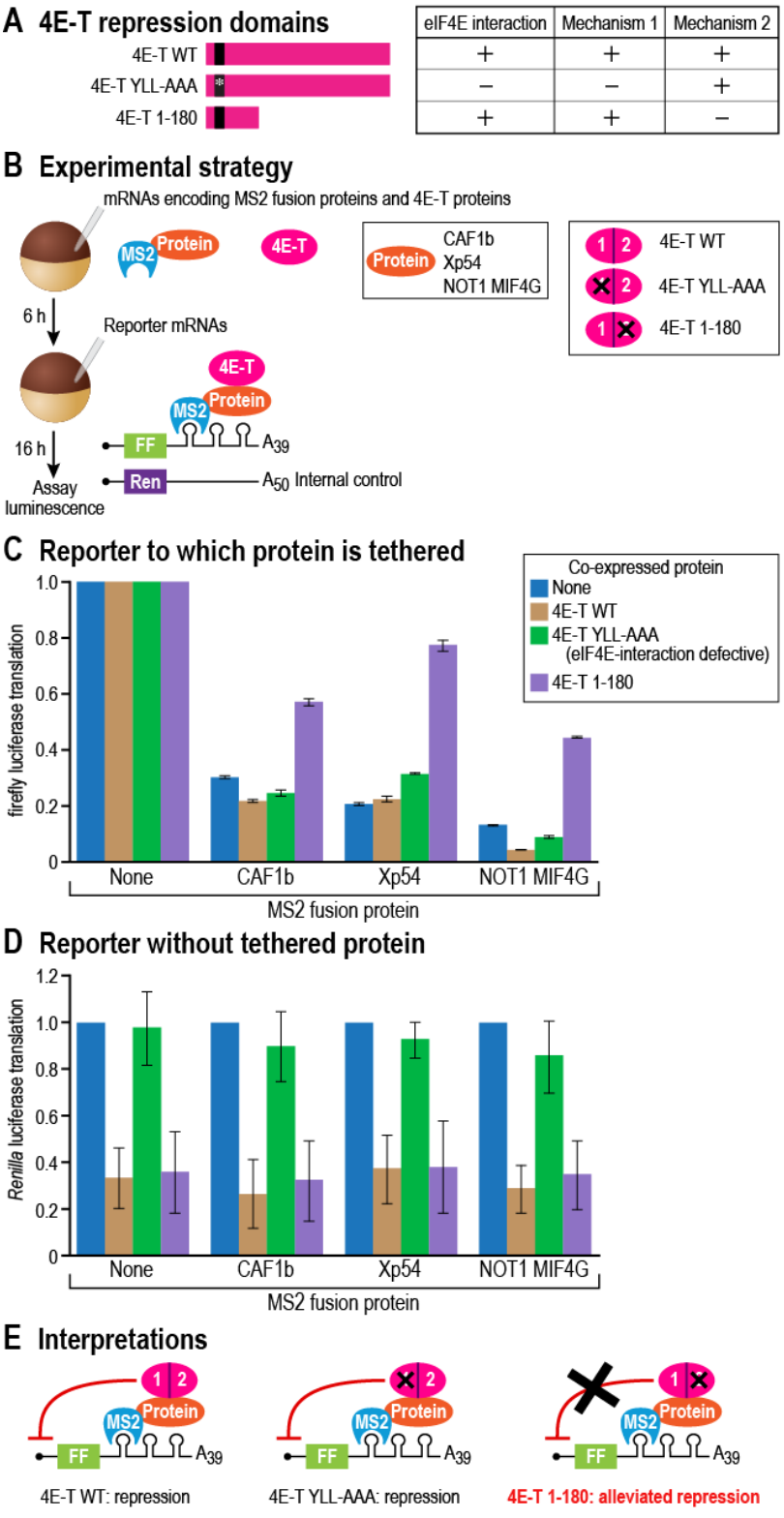

Figure 5. 4E-T mediates repression in an eIF4E-independent manner.
A) 4E-T protein depicted to show eIF4E-binding motif (black box) and mutants used for modified tethered function assay. Table is used to show the different

repression mechanisms of 4E-T (“Mechanism 1”, eIF4E-dependent and “Mechanism 2”, eIF4E-independent). B) Schematic of modified tethered function assay with co-expressed 4E-T proteins. FF: firefly luciferase, Ren: *Renilla* luciferase. 4E-T represents the wild type and mutant 4E-T constructs expressed, with 1 and 2 indicating the repression mechanisms from panel A. “Protein” represents CAF1b, Xp54, or NOT1 MIF4G. C) Results of tethered function assay, normalized to "no MS2 protein" control (none). Black bars indicate no co-expressed 4E-T protein, gray shaded bars indicate wild type 4E-T protein, the eIF4E-interaction defective allele of 4E-T (YLL-AAA), and the 4E-T 1-180 truncation. Three independent experiments were performed, with four oocyte replicates each time. Error bars represent one standard deviation. Student’s two-tailed t-test was used to compare the 4E-T 1-180 data to the 4E-T WT data. For CAF1b and Xp54, the difference was highly significant ($p<0.005$), while the NOT1 MIF4G domain had a slightly less significant difference of $p<0.05$. D) Graph of *Renilla* luciferase translation from the same experiment to show effects of the 4E-T co-expressed proteins on general translation. E) Schematics depicting the summary of the tethered function assay data. “Protein” represents CAF1b, Xp54, and NOT1 MIF4G.

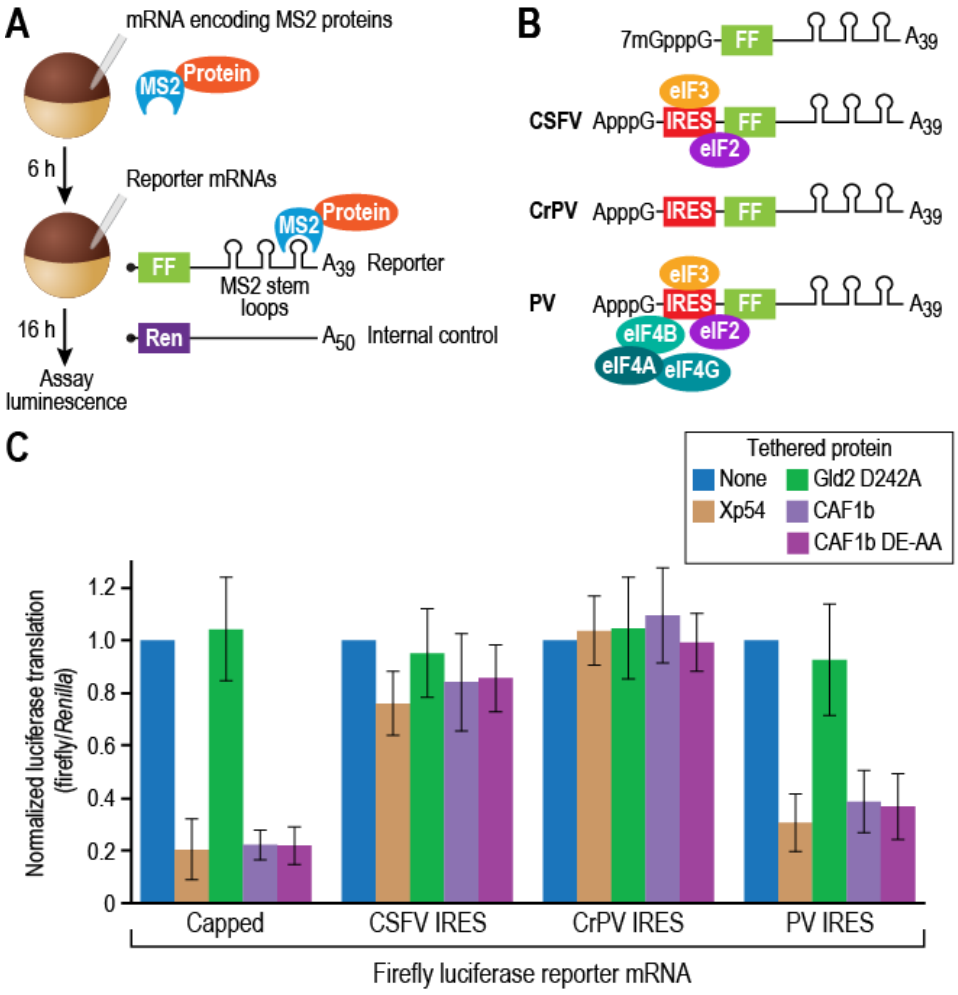

Figure 6. Xp54 and CAF1 repress translation independent of eIF4E and the 5'cap.

A) Schematic of the tethered function assay. FF: firefly luciferase, Ren: *Renilla* luciferase. Orange "protein" represents GLD2 D242A (negative control), CAF1b, CAF1b DE-AA, or Xp54. B) Schematic of the firefly luciferase reporters used. The IRES translation factor requirements are depicted. C) Results of tethered function assay, normalized to "no MS2 protein" control (none, black bars). Three independent experiments were performed, with four oocyte replicates each time. Error bars represent one standard deviation. Student's two-tailed t-test was used to compare the Xp54, CAF1b, and CAF1b DE-AA data to the no MS2 protein data for each reporter. For CAF1b, CAF1b DE-AA and Xp54, the difference was highly significant ($p<0.005$) only for the capped and PV IRES reporters.

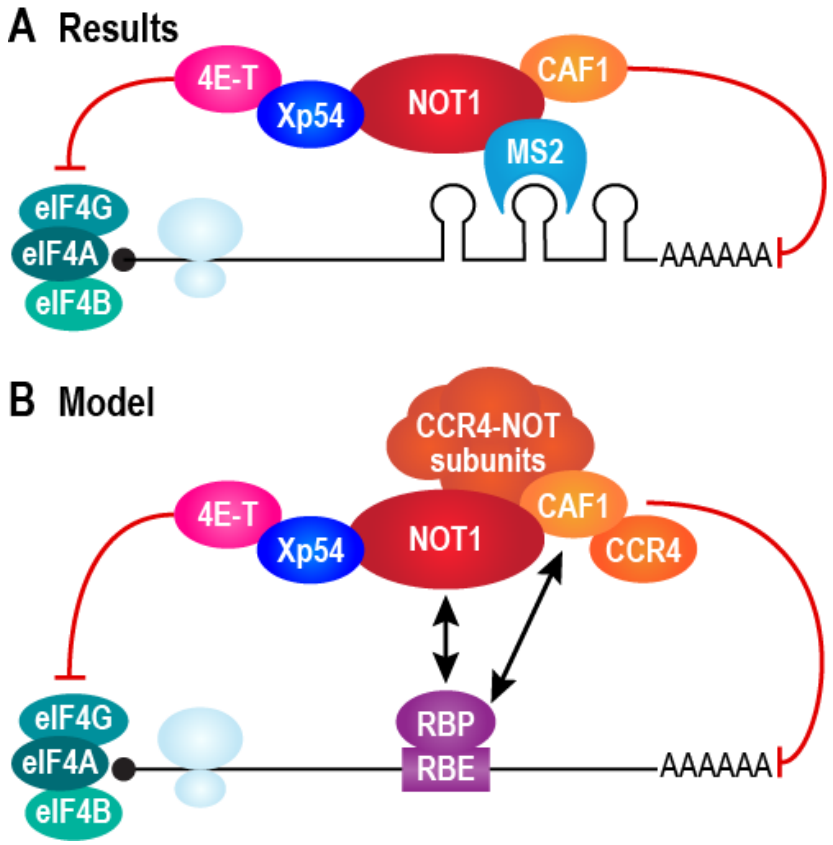

Figure 7. The NOT1 MIF4G domain recruits Xp54 and 4E-T to mediate eIF4E- and cap-independent repression by CAF1.
A) Model of repression when a tethered repressor is on the 3' end of a reporter mRNA. B) We propose that repression may occur in a similar manner by RNA-binding proteins that recruit the CAF1/NOT1/Xp54/4E-T complex when bound to target mRNAs.

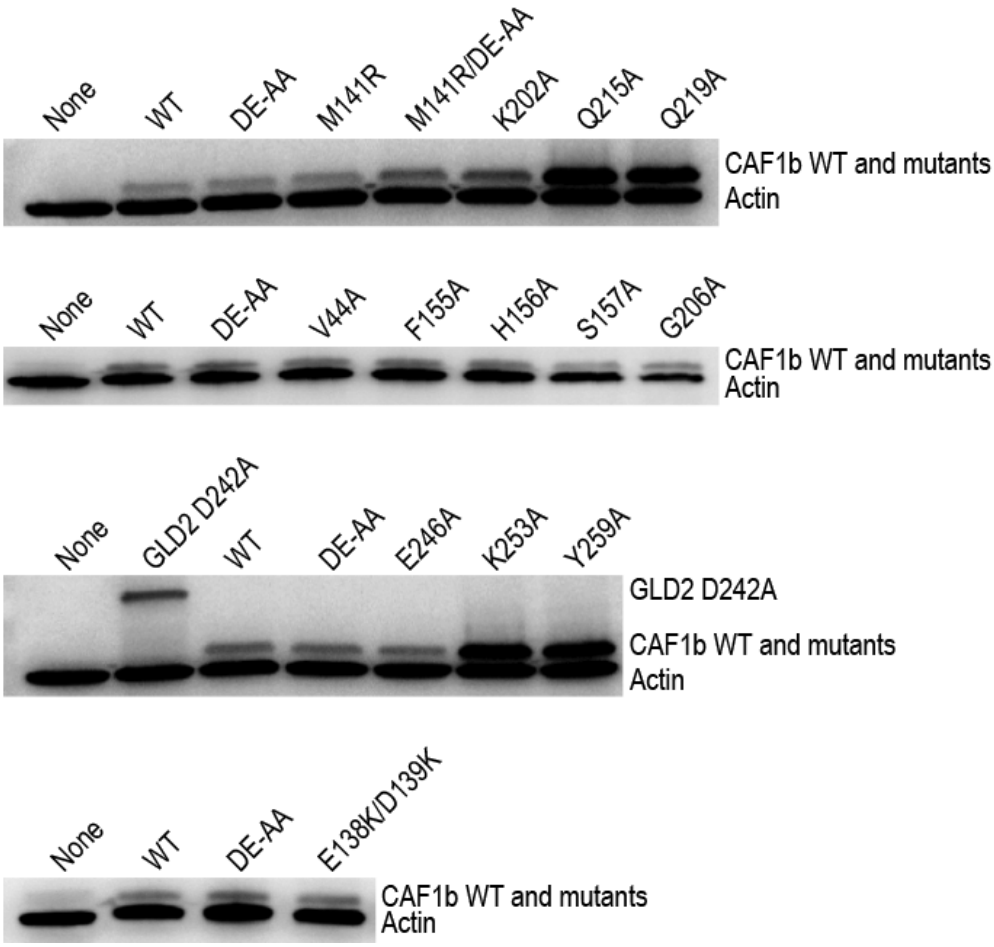

Figure S1. Proteins used for CAF1 tethered function assays are expressed in the oocyte.
Western blots with α-HA antibody were conducted to test expression of the CAF1 wild type and mutant proteins used for tethered function assays in Figure 1. Actin served as a loading control. Western blots depict a single representative experiment, and three biological replicates were conducted.

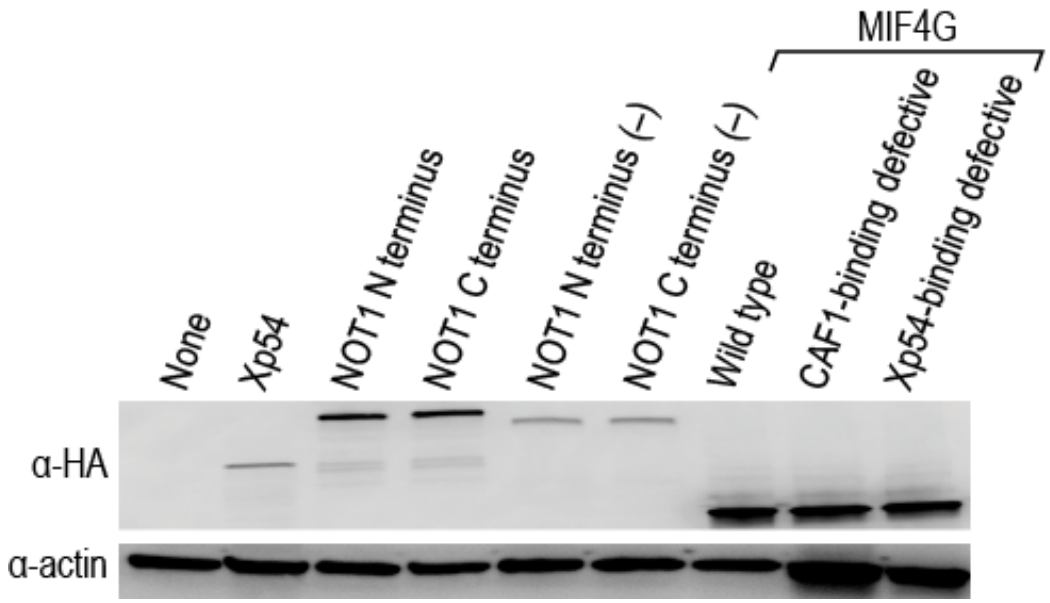

Figure S2. Proteins used for NOT1 tethered function assays are expressed in the oocyte.
Western blots with α-HA antibody were conducted to test expression of the NOT1 fragments and mutants used for tethered function assays in Figure 4. Actin served as a loading control. Western blots depict a single representative experiment, and three biological replicates were conducted.

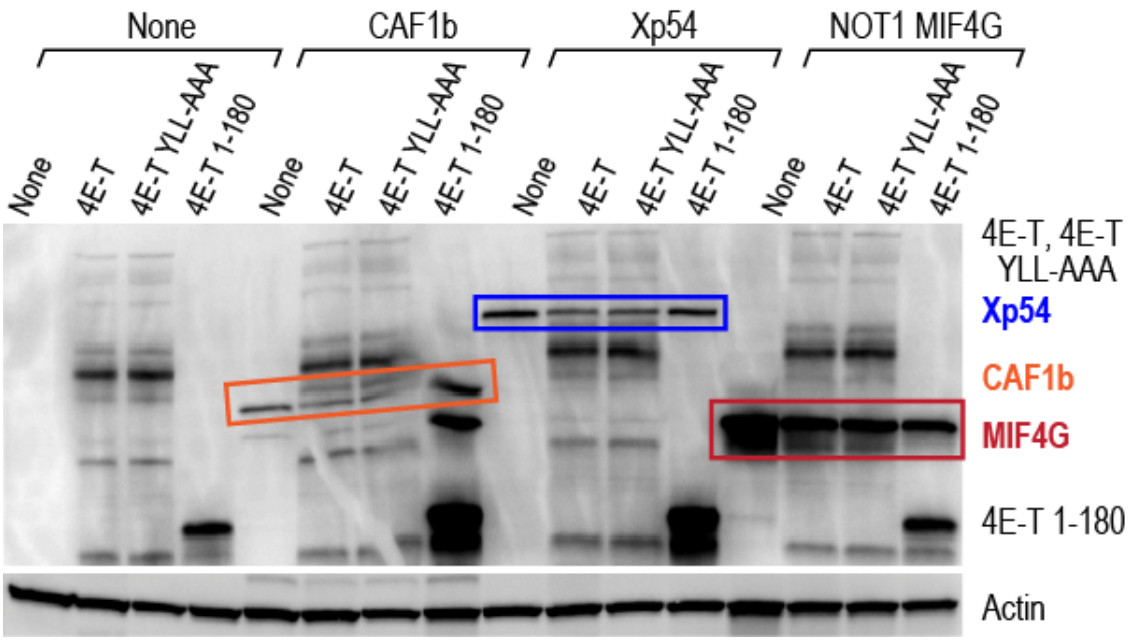

Figure S3. Protein expression for modified tethered function assay with 4E-T constructs.
Western blots with α-HA antibody were conducted to test expression of the co-expressed 4E-T constructs as well as CAF1, Xp54 and the NOT1 MIF4G domain. Actin served as a loading control. Western blots depict a single representative experiment, and three biological replicates were conducted.

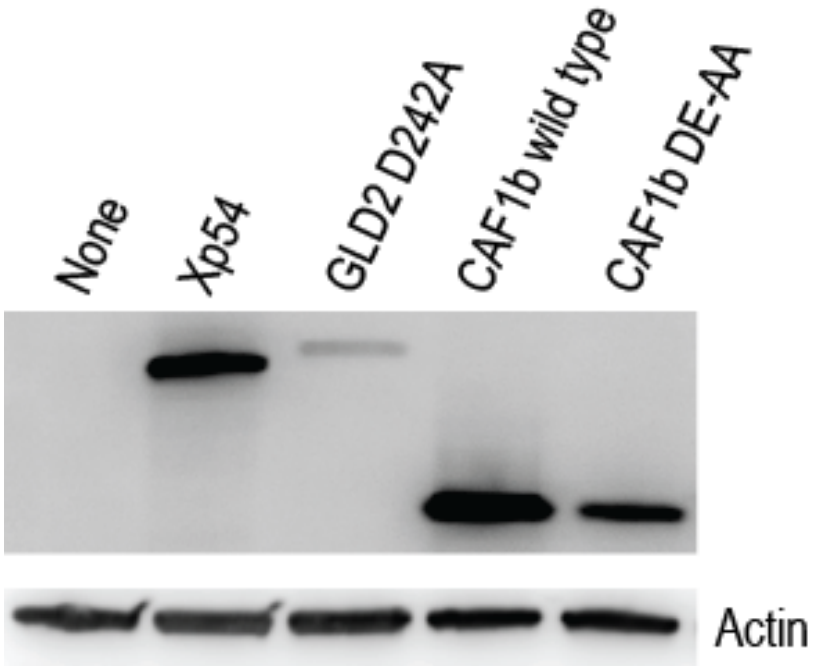

Figure S4. Protein expression for tethered function assay with IRES reporters.
Western blots with α-HA antibody were conducted to test expression of GLD2 D242A, Xp54, CAF1b, and CAF1b DE-AA. Actin served as a loading control. Western blots depict a single representative experiment, and three biological replicates were conducted.

Specific to wild type CAF1a	Uniprot ID
CNOT1	A0JP85
CNOT2	F6PIP6
CNOT3	B2GUB5
CNOT10	F6QET6
RQCD1	Q6P819
DDX6	Q0IHV9
EPABP	Q6DEY7
CPEB1	F7B2S5

Specific to wild type CAF1b	Uniprot ID
CNOT1	A0JP85
CNOT2	F6PIP6
CNOT3	B2GUB5
CNOT10	F6QET6
RQCD1	Q6P819
DDX6	Q0IHV9
EPABP	Q6DEY7
CPEB1	F7B2S5
LSM6	Q0VFB8
PDIA3	F6UJP2
SDHA	Q28ED0

Table S1. Mass spectrometry reveals protein partners specific to wild type CAF1.
Tables depicting proteins identified by mass spectrometry that were specific to the wild type CAF1 proteins. Uniprot IDs are also provided. Only proteins with at least a two-fold enrichment over the mutant CAF1 proteins (relative to GST alone) and with $p<0.05$ are depicted.

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Chapter 3:

Xenopus CCR4-NOT interactions are altered upon meiotic maturation

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I designed and performed all the experiments except for the mass spectrometry. Clay Williams performed the mass spectrometry and proteomics data analysis.

ABSTRACT

mRNA regulation is of particular importance during early development. We previously showed that the CCR4-NOT complex represses translation of mRNAs through its association with Xp54 and 4E-T in *Xeonpus* laevis oocytes. The non-canonical eIF4E-independent activity of 4E-T was required for repression. We sought to determine whether this mode of regulation was maintained upon meiotic maturation of the oocyte. We performed GST pull-downs followed by both proteome-wide and candidate approaches, which revealed that the NOT1-dependent interactions between Xp54 and 4E-T and CAF1 do not persist upon maturation. Additionally, CPEB and ePAB did not associate with CAF1 post-maturation. Co-immunoprecipitations confirmed the loss of these interactions with both CAF1 and NOT1 in the mature oocyte. Both Xp54 and 4E-T are likely phosphorylated during maturation; however co-immunoprecipitations with phosphomimetic forms of 4E-T and Xp54 had no effect on interaction with NOT1. Thus, regulatory factors that mediate repression in the oocyte do not associate with CCR4-NOT in the mature oocyte, and this may due to protein modifications or new interactions with Xp54 and 4E-T that prevent association with NOT1.

INTRODUCTION

mRNAs are tightly regulated during early development. The maternal mRNA pool of metazoans directs many of the earliest developmental processes until zygotic transcription is initiated (Gandolfi and Gandolfi 2001; Tadros and Lipshitz 2009). In the frog *Xenopus laevis*, oogenesis, oocyte maturation, and embryogenesis prior to the mid-blastula transition all predominantly occur without transcription. Thus, proper control of maternal mRNAs is critical.

Meiotic maturation of the oocyte into a fertilization-competent egg is a particularly important point of control in *X. laevis* early development. The Stage VI oocyte is arrested at prophase I. Exposure to progesterone stimulates the oocyte to undergo the second meiotic cell cycle and arrest in metaphase II (Smith et al. 1968; Brachet et al. 1970). During this process, many mRNAs that were translationally repressed during oogenesis become actively translated and vice versa. Coordinated control by RNA-binding proteins (RBPs) and regulatory proteins, such as deadenylases and poly(A) polymerases, ensures that the correct mRNAs are activated and deactivated at precisely the right times.

The CCR4-NOT complex is an important mRNA-regulatory complex in eukaryotic organisms (Collart and Panasenko 2012), particularly in *Xenopus* early development. The complex (referred to as CCR4-NOT throughout) contains 9 core subunits whose interactions are primarily mediated by the scaffold protein, NOT1. Together, CCR4-NOT has critical functions at many points of mRNA control, from transcription to degradation. We previously reported the mechanism of translational repression by CCR4-NOT in *X. laevis* oocytes. CCR4-NOT

recruits two proteins, 4E-T and Xp54, to repress translation independent of eIF4E (Waghray et al. 2015). Regulation of mRNAs by CCR4-NOT is likely an important theme throughout early development in *Xenopus*.

We sought to determine how protein interactions to CAF1 and the CCR4-NOT complex change upon oocyte maturation in *Xenopus*. We combined directed and global studies to determine that interactions with CAF1 are altered upon maturation, as NOT1-dependent interactions with CPEB, ePAB, Xp54, and 4E-T are all lost in the mature oocyte. 4E-T and Xp54 are both phosphorylated upon maturation, and surprisingly, phosphomimetic forms of 4E-T and Xp54 do not affect interactions to NOT1.

RESULTS AND DISCUSSION

GST pull-downs reveal CAF1 interaction dynamics upon oocyte maturation

To determine the dynamics of protein interactions with CAF1 upon oocyte maturation, we used a GST pull-down approach followed by Western blotting for candidate proteins (Fig. 1A). GST pull-downs were conducted with recombinant wild type and NOT1-interaction defective (M141R) CAF1a and CAF1b proteins. The purified proteins were immobilized on glutathione magnetic resin, incubated with *Xenopus* mature oocyte lysate, and then stringently washed to eliminate nonspecific interactions. GST protein was used as a negative control. All five proteins were detected after pull-down (Fig. 1B, top).

Using this approach, we revealed that many interactions detected in the oocyte were lost in the mature oocyte. Despite being expressed, ePAB, Xp54, and 4E-T were not detectably pulled down by CAF1a or CAF1b in the mature oocyte (Fig. 1B), which is in stark contrast to their interactions in immature oocytes (Fig. 1C). The positive control CCR4, which interacts directly with CAF1 independently of NOT1 (Basquin et al. 2012), was detected in all CAF1 pull-downs in both immature and mature oocytes.

To globally examine the CAF1-interaction profile in mature oocytes, we analyzed our GST pull-down samples by mass spectrometry. (Fig. 1A). The mass spectrometry data was analyzed by first comparing proteins detected in the wild type and mutant CAF1 samples to those detected in the GST negative control sample. Then, we determined the relative enrichments of protein interactors in the wild type pull-downs compared to the mutant pull-downs.

We found that, as previously reported in the oocyte (Waghray et al. 2015), CAF1 interactions to the CCR4-NOT complex in the mature oocyte were mediated by NOT1. Interestingly, other proteins detected in the oocyte, including CPEB, ePAB, Xp54, and 4E-T, were not present in the mature oocyte data for either CAF1a or CAF1b (Fig. 1D&E). CPEB is significantly depleted in mature oocytes as compared to oocytes (Hake and Richter 1994), so its lack of interaction was expected. Only CCR4-NOT complex proteins were significantly enriched (> 10-fold, $P < 10^{-5}$) in the wild type CAF1 pull-downs in the mature oocyte. Unlike in oocytes, we did not detect other significant interactions with CAF1 wild type or mutant proteins (Fig. 1F).

Thus, the proteins responsible for repression by CAF1 and CCR4-NOT in oocytes do not associate with CAF1 in the mature oocyte.

Co-immunoprecipitations confirm interactions *in vivo*

To further examine the change in interactions detected by GST pull-down, we conducted co-immunoprecipitations from *Xenopus* mature oocytes. myc-tagged CAF1 mutant and wild type proteins were expressed and immunoprecipitated (Fig. 2A), and the products were analyzed by Western blot. Both wild type and NOT1-interaction defective forms of CAF1a and CAF1b did not immunoprecipitate 4E-T, Xp54, or eIF4E (Fig. 2B), which is in contrast with our previous findings in immature oocytes (Fig. 2C). CCR4 was immunoprecipitated by the CAF1 proteins. The α-myc antibody confirmed that the CAF1 proteins were immunoprecipitated (Fig. 2B, bottom).

We previously demonstrated that the NOT1 MIF4G domain facilitated interaction with Xp54 and 4E-T in the oocyte (Waghray et al. 2015), so we performed IPs with the MIF4G domain in the mature oocyte (Fig. 2C). 4E-T and Xp54 lacked interaction with the MIF4G domain in the mature oocyte (Fig. 2D). The MIF4G domain (detected with α-myc) was immunoprecipitated, as expected. As a control, an oocyte experiment was conducted alongside the mature oocyte IP. As seen previously, both Xp54 and 4E-T associated with the NOT1 MIF4G domain in oocytes (Fig. 2D).

Taken together, the co-IP and GST pull-down data demonstrate that CCR4-NOT complex interactions are preserved upon oocyte maturation. This is the first protein-interaction study conducted with CCR4-NOT complex subunits across a developmental time point. All but two of the complex subunits annotated in the *Xenopus* genome were detected in the mature oocyte mass spectrometry data. CCR4 was not detected among the pull-down products by mass spectrometry, but it was detected by Western blot, suggesting that it is expressed and associated with CCR4-NOT in mature oocytes. NOT4 was also not detected by mass spectrometry, and no known antibodies to NOT4 from other systems that we tested detected the *Xenopus* protein. However, in many eukaryotic systems, NOT4 is only transiently associated with the CCR4-NOT complex, so it is possible that this is the case in *Xenopus* as well. Moreover, NOT4 may not be expressed in these early developmental stages, as its main function in other systems is in protein degradation as an ubiquitin ligase (Panasenko and Collart

2011). Protein turnover rates in oogenesis and maturation are low, so the absence of NOT4 is possible.

Furthermore, these data suggest that the association of CCR4-NOT with regulatory proteins is dynamic. The relative enrichment of Xp54, CPEB, and ePAB for the wild type CAF1 proteins in the immature oocyte mass spectrometry data (Waghray et al. 2015) suggested that these interactions were either transient or present only in a small population of CCR4-NOT complexes, in contrast to the stable interactions of CCR4-NOT complex members with CAF1. The fact that 4E-T, Xp54, CPEB and ePAB lack interaction with CAF1 and CCR4-NOT in the mature oocyte further supports the dynamic nature of CCR4-NOT. It is likely that the association of these proteins is regulated developmentally, which the GST pull-down and co-IP data strongly supports. It will be interesting to see if future experiments in later stages of development find similar changes in CCR4-NOT interactions.

Phosphomimetic of 4E-T interacts with CCR4-NOT

4E-T is likely phosphorylated at three residues in the mature oocyte (McGivern et al. 2009). These phosphorylations are not detected in Stage VI oocytes, suggesting that the modification occurs during oocyte maturation. Thus, we hypothesized that phosphorylation of 4E-T may prevent its association with the CCR4-NOT complex. Since 4E-T directly interacts with Xp54 and makes minor contacts to NOT1 through the Xp54 interaction (Ozgur et al. 2015), we

used co-immunoprecipitations to determine whether a phosphomimetic form of 4E-T could associate with the NOT1 MIF4G domain (Fig. 3A).

Immunoprecipitations of NOT1 MIF4G with 4E-T revealed that both the wild type and phosphomimetic forms of 4E-T interacted with NOT1 in the oocyte (Fig. 3B). eIF4E, a negative control, was absent in the NOT1 MIF4G IP samples. This is consistent with an independent proteomic analysis of the Xp54-4E-T interaction upon oocyte maturation (Ayache et al. 2015). Our data extends the reported observation that 4E-T and Xp54 interact in the mature oocyte (Ayache et al. 2015) to suggest that phosphorylated 4E-T is likely capable of interaction with NOT1. The lack of association between 4E-T and NOT1 thus may be attributed to a disruption in the interaction between NOT1 and Xp54, since Xp54 serves as a bridge between 4E-T and NOT1.

Phosphomimetic 4E-T represses translation

To determine the effect of 4E-T phosphorylation on translational repression, we used tethered function assays with wild type or phosphomimetic 4E-T in the oocyte (Fig. 3C). Oocytes were injected with mRNAs encoding HA-tagged and MS2 coat protein-fused (HA-MS2) 4E-T proteins. After the HA-MS2-4E-T proteins accumulated, oocytes were injected with two reporter mRNAs. The first contained the firefly luciferase open reading frame followed by a 3'UTR with MS2 coat protein binding sites. The second reporter contained the *Renilla* luciferase open reading frame lacking MS2 sites, which served as a negative control and was used for normalization.

Wild type 4E-T (4E-T WT) and phosphomimetic 4E-T (4E-T mutant) repressed translation equally, as compared to the reporter alone control (Fig. 3D). As expected, GLD2 D242A, a control that should not affect translation, had no effect on the luciferase reporter.

The 4E-T phosphomimetic had no effect on its protein interactions with the CCR4-NOT complex or on repression of translation in the oocyte. It is possible that other sites in 4E-T are phosphorylated or that the phosphomimetic does not recapitulate the effects of phosphorylation in the mature oocyte. Additionally, 4E-T may have other post-translational modifications that disrupt its interaction with the CCR4-NOT complex.

Phosphomimetic of 4E-T interacts with NOT1

Since Xp54 serves as a bridge between the CCR4-NOT complex and 4E-T in oocytes (Waghray et al. 2015), we hypothesized that modification to Xp54 may be responsible for the disruption of the NOT1-Xp54-4E-T interaction during oocyte maturation. Other studies suggest that Xp54 is phosphorylated upon oocyte maturation, and Xp54 possesses four casein kinase II (CK2) sites in its C terminus that are likely candidates for phosphorylation (Smillie and Sommerville 2002). We therefore made mutations to all four sites to generate phosphomimetic (Ser/Thr to Asp or Glu) forms of Xp54.

To determine the effect of Xp54 phosphorylation on interaction with NOT1, we performed co-IPs with phosphomimetic forms of Xp54 (Fig. 4A). The NOT1 MIF4G domain on its own was used as a negative control, and wild type Xp54

served as an additional control. Both phosphomimetic forms of Xp54 were immunoprecipitated by the MIF4G domain, just like the wild type protein (Fig. 4B).

These data suggest that phosphorylation of Xp54 has no effect on its interaction with NOT1, or that other residues of Xp54 are phosphorylated upon maturation and disrupt interaction with NOT1. Alternatively, protein modification of the NOT1 MIF4G domain may disrupt interaction between NOT1 and Xp54 (and thereby 4E-T), or Xp54 may interact with other proteins that prevent its association with NOT1.

Perspectives

Our work suggests two models by which mRNA regulation via CCR4-NOT may be controlled upon oocyte maturation. We previously showed that CCR4-NOT represses translation of mRNAs in the oocyte through interaction with Xp54 and 4E-T (Fig. 5, Stage VI oocyte). The data presented here demonstrate that Xp54 and 4E-T do not associate with CCR4-NOT in the mature oocyte, either due to maturation-specific phosphorylation (or other modification) of NOT1 (Fig. 5, Mature oocyte A) or due to new protein partners of Xp54 that inhibit NOT1 interaction (Fig. 5, Mature oocyte B). Phosphorylation of 4E-T and Xp54 at known phosphorylation sites is likely not responsible for the change in interaction. Since 4E-T and Xp54 are required for repression in the oocyte, we propose that CCR4-NOT does not repress translation in the mature oocyte. It is also possible that CCR4-NOT may repress translation of mRNAs through an

alternate mechanism. However, we did not observe any new protein partners of CAF1 in the mature oocyte that might suggest other regulatory mechanisms.

Phosphorylation is a common mode of regulation, especially during oocyte maturation. Both kinase and phosphatase activities are required for progression through meiosis II (Tay et al. 2003; Dupre et al. 2011). Thus, it is likely that phosphorylation controls association of translational repressors with CCR4-NOT during maturation. It will be interesting to see if phosphorylation or other protein modifications regulate interactions with CCR4-NOT throughout later stages of development.

Other systems also repress translation through a similar mechanism to that observed in the oocyte. Specifically, miRNA- and RBP-mediated repression in *Drosophila* and human cells both require interaction between CNOT1 and the Xp54 homolog DDX6 and 4E-T (Chen et al. 2014; Mathys et al. 2014; Rouya et al. 2014). It is possible that these interactions are also regulated similarly during aberrant or stress conditions to control repression by CCR4-NOT. It will be of interest to determine if regulated association of repressive factors is a general mode of control for CCR4-NOT interactions or if it is specific to early development.

MATERIALS & METHODS

Plasmid construction and mutational analysis

pCS2+3HAMS2 containing CAF1, Xp54, and GLD2-D242A proteins have been described previously (Cooke et al. 2010). pGEX-6P-1 plasmids containing CAF1 proteins, pCS2+9xmyc CAF1 and NOT1 MIF4G, and pCS2+3HA 4E-T plasmids have also been described previously (Waghray et al. 2015). Phosphomimetic mutants of 4E-T (T330D/S395D/T575D) were generated using site-directed mutagenesis. Mutations in Xp54 (T438D/S447D/S461D/S476D, T438E/S447E/S461E/S476E) were made via Gibson assembly cloning with G-blocks (IDT) containing the mutations of interest.

In vitro transcription and RNA preparation

Plasmids for protein expression were linearized with NotI (Fermentas) and transcribed with the AmpliScribe SP6 high yield transcription kit (CellScript), as described previously (Waghray et al. 2015).

Oocyte injections

Oocyte injections were performed as described previously (Gray et al. 2000; Kwak et al. 2004).

Oocyte maturation

Stage VI oocytes were isolated and treated with 0.05 mg/mL progesterone (in 100% ethanol) overnight. Mature oocytes were selected by the presence of GVBD and used for experiments.

Protein purification and GST pull-down assays

Protein purification and pull-downs were performed as described previously (Waghray et al. 2015), except that pull-downs were conducted using mature oocytes rather than Stage VI oocytes.

Co-immunoprecipitations

Oocytes were injected with 50 nL of 400 ng/μL capped mRNAs expressing the appropriate HA-tagged or myc-tagged proteins. After proteins were allowed to accumulate for 6 hours, 50 μL of 10 mg/mL progesterone (in 100% ethanol) was added to the oocytes in 10 mL of MMR. Oocytes were left overnight to mature. Maturation was assessed by the presence of GVBD. Mature oocytes were collected and lysed, and IPs were conducted exactly as described previously (Waghray et al. 2015).

Mass spectrometry

Mass spectrometry and data analysis were performed as described previously (Waghray et al. 2015).

Antibodies

Antibodies were diluted in 5% milk in 1x TBST. The following antibodies were used at a 1:1000 dilution: α-HA-11 Clone 16B12 (Covance), α-c-myc (Sigma C3956), α-DDX6 (A300-460A, Bethyl Laboratories), α-eIF4ENIF1 (ab6034, Abcam), α-eIF4E C46H6 (2067S, Cell Signaling Technologies). Secondary antibodies, α-Goat IgG (KPL, 14-13-06), α-rabbit IgG (KPL, 074-1506), α-mouse IgG (KPL, 474-1806) were diluted 1:20,000 in 5% milk in 1x TBST.

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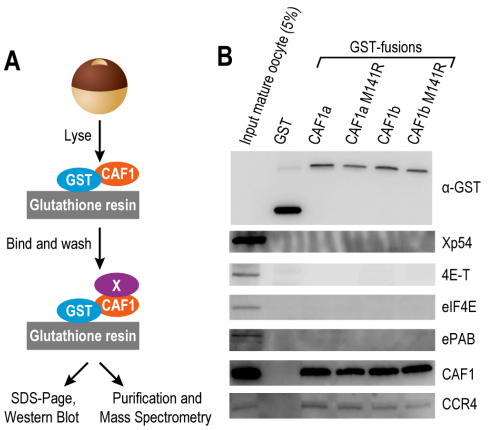
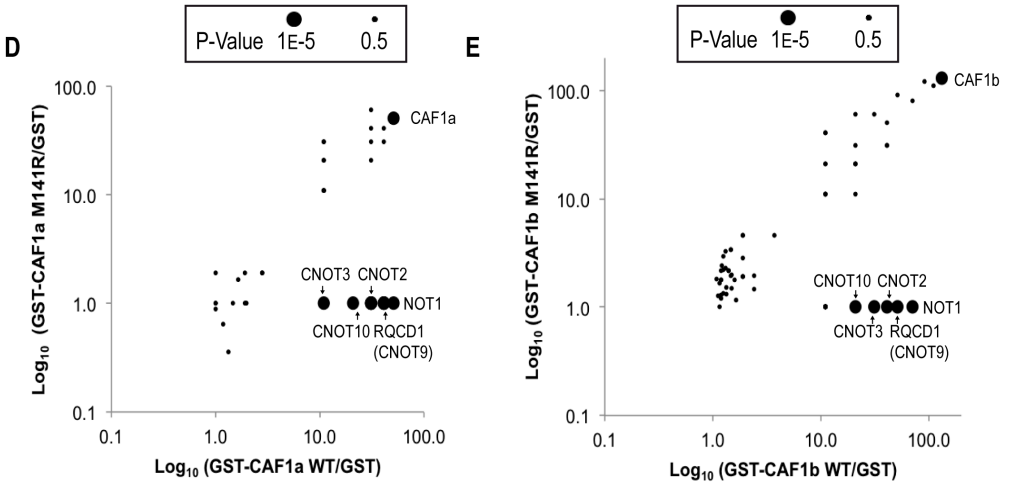
We thank members of the Wickens laboratory for helpful discussion. We also thank Laura Vanderploeg for help preparing figures. This work was supported by NIH grants GM50942 (MW), GM31892 (MW) and GM80148 (JJC).



C

Condition	Stage VI oocyte (Waghray *et al.* 2015)				Mature oocyte			
GST pull-down sample	GST-CAF1a WT	GST-CAF1a M141R	GST-CAF1b WT	GST-CAF1b M141R	GST-CAF1a WT	GST-CAF1a M141R	GST-CAF1b WT	GST-CAF1b M141R
	CCR4	CCR4	CCR4	CCR4	CCR4	CCR4	CCR4	CCR4
	CAF1	CAF1	CAF1	CAF1	CAF1	CAF1	CAF1	CAF1
	4E-T		4E-T					
	Xp54		Xp54					
	ePAB		ePAB					



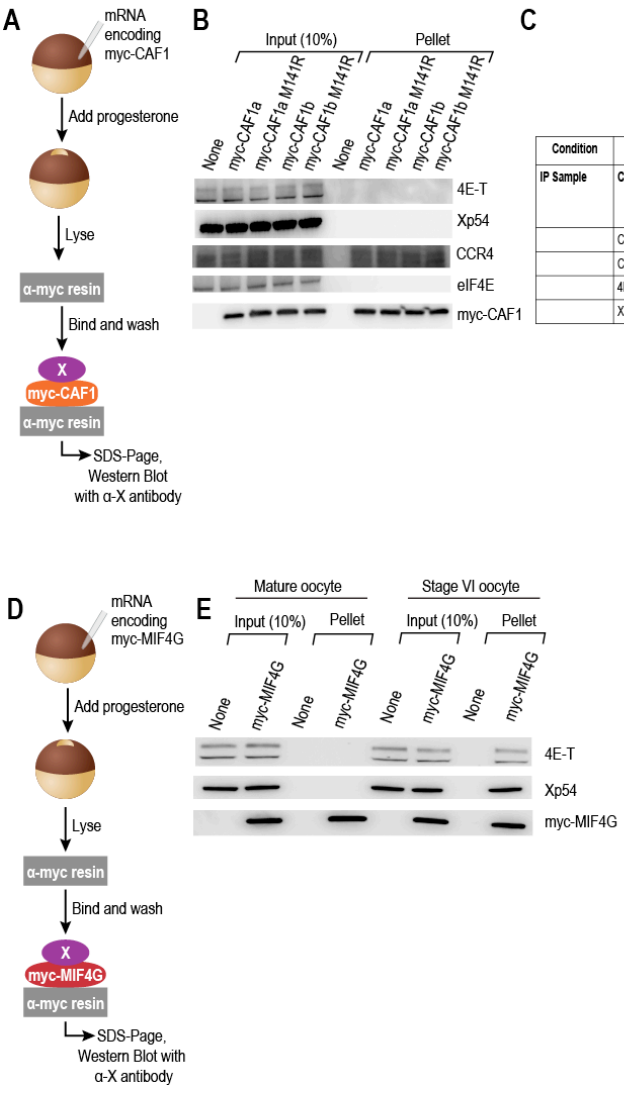
F

CAF1a/b oocyte (Waghray *et al.* 2015)	CAF1a/b mature oocyte
NOT1	NOT1
CNOT2	CNOT2
CNOT3	CNOT3
CNOT10	CNOT10
RQCD1 (CNOT9)	RQCD1 (CNOT9)
CPEB1	
EPABP	
Xp54	
4E-T	

Figure 1. CAF1 interactions are altered upon oocyte maturation.

A) Schematic of GST pull-down assay. B) GST-CAF1 proteins are efficiently pulled down. Western blots with antibodies to known regulatory proteins were performed after GST pull-down to test for CAF1 interaction. Western blots depict a single representative experiment, and three biological replicates were conducted. C) Table depicting differences in Western blot data between the oocyte and mature oocyte. D) Scatter plot of mass spectrometry data from 3

biological replicates for GST-CAF1a wild type and mutant. P-values calculated using a two-tailed Student's t-test are indicated by dot size. Members of the CCR4-NOT complex interact strongly with CAF1a WT but not the M141R mutant. E) Same as in C, but with CAF1b. F) Table depicting differences in mass spectrometry data between the oocyte and mature oocyte, for proteins detected in both CAF1a and CAF1b pull-downs.



Condition	Stage VI oocyte (Waghray *et al.* 2015)				Mature oocyte			
IP Sample	CAF1a WT	CAF1a M141R	CAF1b WT	CAF1b M141R	CAF1a WT	CAF1a M141R	CAF1b WT	CAF1b M141R
	CCR4	CCR4	CCR4	CCR4	CCR4	CCR4	CCR4	CCR4
	CAF1	CAF1	CAF1	CAF1	CAF1	CAF1	CAF1	CAF1
	4E-T		4E-T					
	Xp54		Xp54					

Figure 2. CAF1 and NOT1 MIF4G interactions are altered upon oocyte maturation *in vivo*.

A) Schematic of the co-immunoprecipitation assay with myc-CAF1 as the bait protein. B) Western blots with antibodies to proteins that interact with CAF1 by GST pull-down were conducted to test interaction by co-IP. CCR4 is a positive control, and eIF4E is a negative control. Western blots depict a single representative experiment, and three biological replicates were conducted. C) Table comparing the mature oocyte CAF1 IP data to previously published oocyte IP data. D) Schematic of the co-immunoprecipitation assay with myc-MIF4G (the NOT1 MIF4G domain) as the bait protein. E) Western blots were conducted to test whether 4E-T and Xp54 interact with the NOT1 MIF4G domain upon maturation. Stage VI oocyte IPs were also done as a control. Western blots depict a single representative experiment, and three biological replicates were conducted.

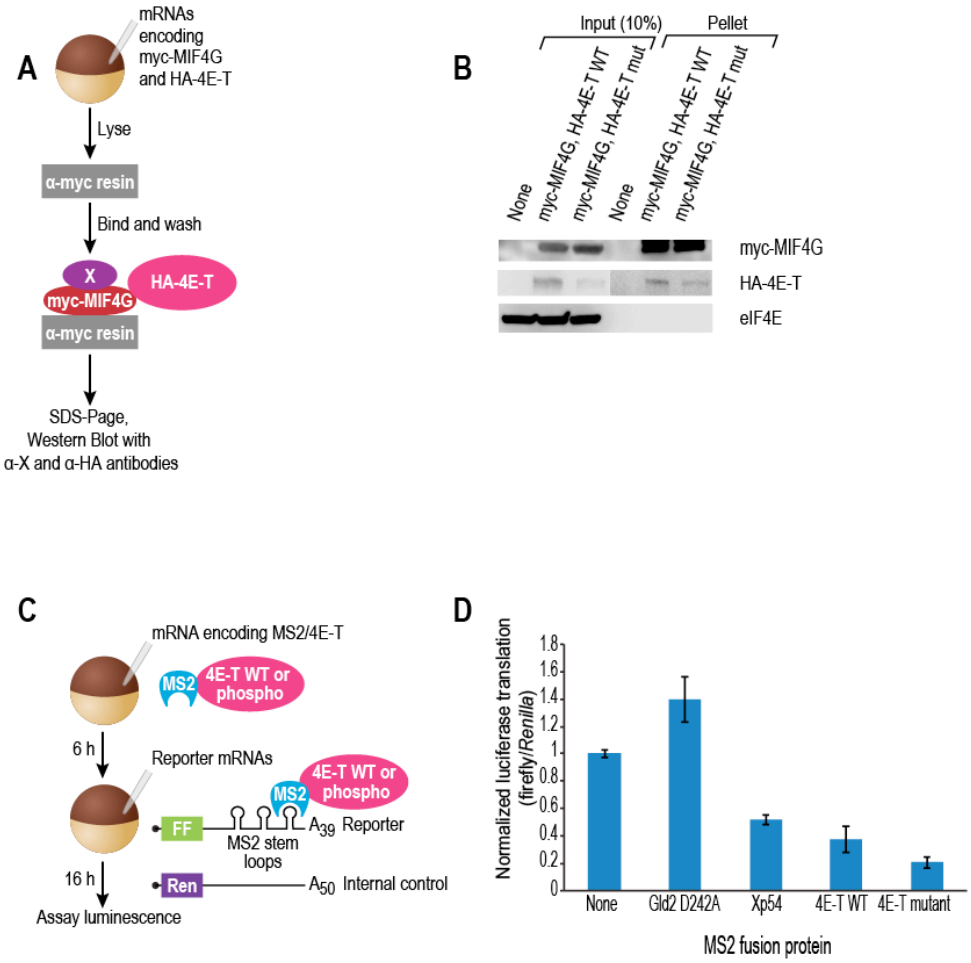


Figure 3. 4E-T phosphomimetic does not affect interactions or regulation.

A) Schematic of the co-immunoprecipitation assay with myc-MIF4G as the bait protein and co-expressed HA-4E-T forms. B) Western blots were conducted to test interaction by co-IP. Myc to detect the MIF4G was used as a control, and HA was used to detect 4E-T. eIF4E is a negative control. Western blots depict a single representative experiment, and three biological replicates were conducted. C) Schematic of tethered function assay with 4E-T. FF: firefly luciferase, Ren: *Renilla* luciferase. B) Results of tethered function assay, normalized to "no MS2 protein" control (none). GLD2 D242A was used as a control for no effect on translation, and Xp54 was used as a control for repression. Three independent experiments were performed, with four oocyte replicates each time. Error bars represent one standard deviation.

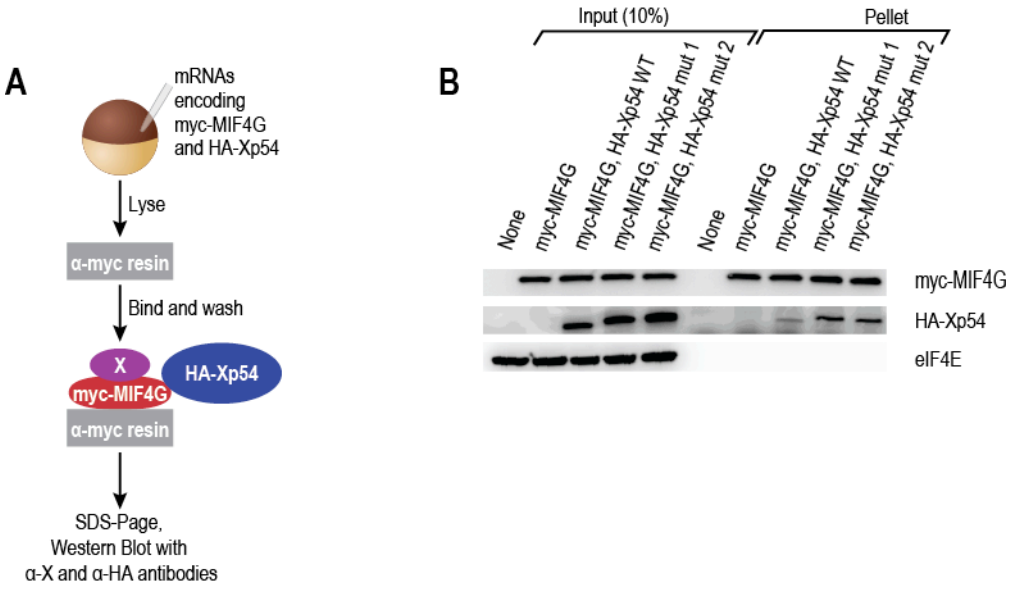


Figure 4. Xp54 phosphomimetics do not affect interaction with NOT1.

A) Schematic of the co-immunoprecipitation assay with myc-MIF4G as the bait protein and co-expressed HA-Xp54 forms. B) Western blots were conducted to test interaction by co-IP. Myc to detect the MIF4G was used as a control, and HA was used to detect Xp54. Mut 1 is phosphomimetic with Asp substitutions, while Mut 2 is with Glu substitutions. eIF4E is a negative control. Western blots depict a single representative experiment, and three biological replicates were conducted.

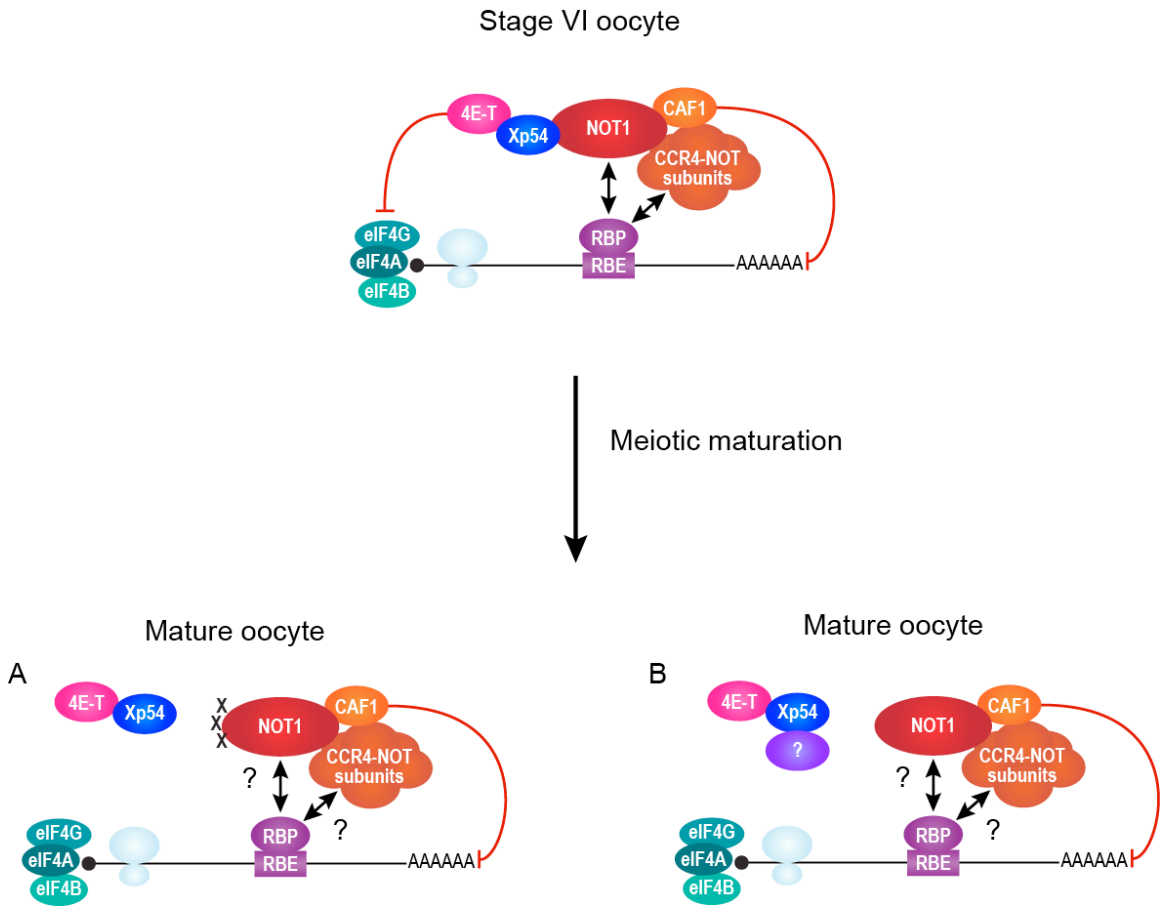


Figure 5. Models of CCR4-NOT complex regulation upon oocyte maturation.

Model of repression and interactions in the oocyte (A) and mature oocyte (B). In A, X indicates protein modifications on NOT1.

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Chapter 4:

Conclusions and Perspectives

SUMMARY

In this thesis, I discussed my work to elucidate the mechanism of translational repression by CCR4-NOT during *Xenopus laevis* early development. I performed the first protein-interaction study of CCR4-NOT in *Xenopus,* which demonstrated that the NOT1 MIF4G-CAF1-CCR4 interaction observed in other systems is conserved in *Xenopus* as well. This is also true of the NOT1 MIF4G-Xp54-4E-T interaction. Additionally, I found that DAIR in oocytes is independent of CAF1 but requires NOT1-mediated interactions to recruit Xp54 and 4E-T (Waghray et al. 2015). Non-canonical cap-independent repression by 4E-T, via a mechanism that is yet to be determined, is critical for this means of control.

Interestingly, the interactions between CAF1, NOT1, Xp54, and 4E-T are not preserved upon meiotic maturation of the oocyte. In the mature oocyte, CAF1 is associated with NOT1 and the CCR4-NOT complex, but neither CAF1 nor NOT1 interact with Xp54 and 4E-T. This is not due to phosphorylation of 4E-T but may be due to phosphorylation of Xp54, likely by a maturation-specific kinase. Alternatively, Xp54 may be subject to other post-translational modifications or to new protein interactions that disrupt its association with CCR4-NOT. Thus, CCR4-NOT either no longer represses translation of mRNAs it contacts, or it does so through a distinct mechanism in the mature oocyte.

Lastly, I outlined the strategy we have taken to determine the mRNAs that are regulated by CCR4-NOT. Since these proteins do not directly contact RNA, we utilized a RNA Tagging approach (Lapointe et al. 2015) with the RNA-binding protein CPEB. CPEB is a known interactor of CCR4-NOT (Hosoda et al. 2011;

Waghray et al. 2015). We propose that CPEB targets may thus be regulated by CCR4-NOT in the oocyte. We obtained preliminary results that establish the feasibility of the RNA Tagging method in the oocyte. More experiments are needed to form a validated list of targets and determine their regulatory outcomes.

Significance

The work presented in this thesis is an important step toward a better understanding of the RNA-regulatory mechanisms that govern early development. Control of translation is particularly important in early development, and DAIR by CCR4-NOT and its protein partners in oocytes may be a general mechanism for repression. The change in association of regulatory factors with CCR4-NOT upon meiotic maturation suggests that this mechanism is carefully controlled during development. It remains to be seen whether the protein partners identified associate and dissociate with CCR4-NOT throughout later stages of development to control translation of mRNAs.

Many of the interactions and repressive functions I identified and characterized in the oocyte are conserved in human and *Drosophila* cells (Chen et al. 2014; Mathys et al. 2014; Rouya et al. 2014; Nishimura et al. 2015), suggesting that the mRNA control mechanisms coordinated by CCR4-NOT and its protein partners are likely similar in a diverse array of biological conditions. Additionally, since recruitment of CCR4-NOT by CPEB is a conserved mode of regulation among eukaryotes (Hosoda et al. 2011; Waghray et al. 2015), the

preliminary data presented here may represent general targets of CCR4-NOT, rather than oocyte- or *Xenopus*-specific regulated transcripts.

FUTURE DIRECTIONS AND PERSPECTIVES

The CCR4-NOT complex and its dynamic interactions

The CCR4-NOT complex has roles in both the nucleus and the cytoplasm (Collart and Panasenko 2012). The experiments in this thesis were conducted using whole cell lysate, so the protein-interaction data does not distinguish between nuclear and cytoplasmic associations of CCR4-NOT. It will be interesting to see whether distinct nuclear and cytoplasmic forms of the CCR4-NOT complex exist. It is conceivable that proteins such as CAF1, which likely does not play a role in the nucleus, are only associated with the cytoplasmic CCR4-NOT complex. There may well be cross-talk between the two potential complexes, as subunits of CCR4-NOT function to stimulate transcription elongation (Reese 2013; Dutta et al. 2015). Perhaps this transcription-activation activity can collaborate with regulation at the level of translation for subsets of genes in response to stress or other stimuli. Protein-interaction analyses of NOT1 and other core CCR4-NOT complex subunits in nuclear and cytoplasmic extracts under normal and stress-induced conditions, followed by examination of the regulatory effects of distinct CCR4-NOT complexes, will shed light on these important questions.

Experiments conducted in human cells demonstrate that CCR4-NOT exists in several forms (Bai et al. 1999; Lau et al. 2009). These distinct complexes contain either CAF1a or CAF1b. Other subunits of the complex,

including CCR4, also have multiple paralogs, and it is likely that they too associate with distinct CCR4-NOT complexes. It will be particularly interesting to determine what conditions dictate the recruitment of one CCR4-NOT complex over another. Moreover, it remains to be seen whether a specific type of CCR4-NOT complex functions in miRNA-mediated repression and translational repression in *Xenopus* oocytes. In-depth analyses of the CCR4-NOT complex subunits associated with these different repression mechanisms are required to answer these questions.

It is possible that different RNA-binding proteins (RBPs) recruit distinct CCR4-NOT complexes to elicit specific regulation. Perhaps this is a mode by which RBPs dictate whether its mRNA targets are subject to DAIR or deadenylation. The factors that recruit the CCR4-NOT complex may also dictate whether the mRNA target is repressed or degraded, perhaps through modification of the complex or through a conformational change induced by its interaction. There is currently no structural information about how RBPs affect the CCR4-NOT complex, and some RBPs may prevent the active site of CAF1 or CCR4 from binding the poly(A) tail of the mRNA, thus inhibiting its decay. RBPs may also have additional protein partners that bind to CAF1 or CCR4 transiently to disrupt deadenylation. It is possible that DAIR and deadenylation are always potential regulatory outcomes, and that they are regulated by the cell type or the relative availability of deadenylases and degradation machinery. Studies in somatic cells suggest that for miRNA targets, both deadenylation and translational repression occur, and repression precedes deadenylation and

subsequent decay (Djuranovic et al. 2012). It is likely that a similar order of events takes place in early development, but without decay. Future structural studies paired with repression and decay assays are necessary to determine the consequences of RBP-interaction and RNA association on CCR4-NOT composition and conformation.

CCR4-NOT regulatory mechanisms in early development versus somatic cells

Other groups have reported a mechanism of translational repression by CCR4-NOT in human cells that is strikingly similar to the one presented in Chapter 2 (Nishimura et al. 2015). There are a few significant differences, however. The first is that decay occurs in somatic cells, and the NOT1-DDX6-4E-T (DDX6 is the human Xp54 homolog) repressive complex is also associated with decapping and decay machinery. There also appears to be an eIF4E dependence for decay, in contrast to the eIF4E-independent repression we observe in the *Xenopus* oocyte.

These differences are likely due to the fact that the human experiments were performed in somatic cells, while our experiments were conducted in the oocyte. The *Xenopus* oocyte is unique in that mRNAs are not degraded, as the maternal mRNA pool is limited and retained. Decapping and decay factors, such as those detected as interactors of CCR4-NOT in human cells, likely do not act on mRNAs in the oocyte. eIF4E may be dispensable for repression if its role is to mediate contacts near the 5' cap and help initiate decapping and degradation. It is unclear if the full decay machinery is even present at the early stages of

development. DCP1 and DCP2, the decapping factors, are both present but exhibit low activity in oocytes as compared to somatic cells (Zhang et al. 1999). Their activity increases upon initiation of zygotic transcription at the mid-blastula transition (MBT). It would be interesting to examine the role of decay machinery in CCR4-NOT mediated repression after the MBT. Experiments to analyze the regulatory mechanisms of CCR4-NOT throughout embryogenesis could therefore be very revealing.

Regulatory role of CCR4-NOT during early development

Though this work has illuminated much about the regulatory role of CCR4-NOT in the *Xenopus* oocyte and mature oocyte, there is still quite a bit that is unknown. It is likely, based on work described in Chapter 3, that regulation by CCR4-NOT is a dynamic and well-controlled process during early development.

The CCR4-NOT complex is likely regulated at many levels, including recruitment to mRNAs and interaction with effector proteins (*i.e.* 4E-T and Xp54. It is unclear how this affects translational control of mRNAs. We found that in the mature oocyte, interactions with both RBPs and the regulatory proteins Xp54 and 4E-T were disrupted. Luciferase assays with reporter RNAs that may be controlled by CCR4-NOT (*i.e.* a reporter RNA with CPEB- or PUF-binding elements in its 3' UTR) in the oocyte, mature oocyte, and even throughout embryogenesis, will help determine the effect of CCR4-NOT interaction changes on mRNA regulation.

It is possible that the same RBPs and regulatory factors that are responsible for regulation in the oocyte re-associate with CCR4-NOT at other

points in development, or that distinct RBPs and repressive proteins associate instead. Several different CCR4-NOT sub-complexes may exist throughout development, and they may be associated with distinct regulatory partners to elicit control of translation. Similarly, different sets of RBPs may recruit CCR4-NOT at each developmental time point to ensure translation of the correct mRNAs. Protein-interaction experiments with CCR4-NOT throughout early development, especially at important transitions such as fertilization and the MBT will be useful for determining the dynamics of CCR4-NOT interactions with both RBPs and effector proteins. Furthermore, determining the RNA targets of RBPs that recruit CCR4-NOT at various points during early development will shed light on whether CCR4-NOT is a general regulator of translation or specific to a population of mRNAs. These experiments together will further our current understanding of the role that CCR4-NOT plays during early development. Conducting similar experiments in various somatic cell types will determine the general mechanisms by which CCR4-NOT regulates mRNAs.

Concluding remarks

In summary, this thesis reveals new insights into the mechanism of repression by the CCR4-NOT complex in *Xenopus laevis* early development. However, there remain many open questions about the dynamics of CCR4-NOT complexes and their regulatory effects in early development and somatic cells. It is clear that CCR4-NOT is important for the proper control of mRNAs, but much more needs to be done to determine how regulation is elicited accurately in specific cellular contexts. Parallels between the early development systems

probed here and somatic cells studied by other labs are known, but there are also some distinctions in each system. It is of interest to determine what causes these differences and why they are significant.

The CCR4-NOT complex is critical for biological processes. This multifunctional complex plays diverse roles in mRNA regulation, from stimulating transcription elongation in the nucleus to ubiquitinating proteins for degradation (Panasenko et al. 2006; Collart and Panasenko 2012; Dutta et al. 2015). Although the work in this thesis only examines the function of CCR4-NOT in translational control, it is likely that the distinct roles of CCR4-NOT are coordinately regulated. Experiments that disrupt particular functions of CCR4-NOT and examine the effect on its other roles will yield more insight into how this large complex collaborates to control so many distinct and significant functions.

The implications of this work are broad. Many subunits of the CCR4-NOT complex are required for cell viability, and the deadenylase subunit CAF1 is critical for progression through meiosis (Molin and Puisieux 2005; Tange et al. 2012). Translational repression by CCR4-NOT is important for accurate control of cellular processes. Deregulation of mRNAs that are repressed by CCR4-NOT can result in a variety of outcomes, including cell death, cancer and other disease states (Chapat and Corbo 2014; Shirai et al. 2014). It will be interesting to see whether viruses, diseases, and other aberrant cell conditions control repression of mRNAs by altering CCR4-NOT protein interactions, similar to the model we propose during *Xenopus* early development. Additionally, this

mechanism of control via protein partners may be more generally applied to other proteins and complexes that regulate mRNAs.

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Appendix 1:

RNA targets of CCR4-NOT across *Xenopus* early development

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This work was done in collaboration with Chris Lapointe.

I designed and performed all the RNA immunoprecipitation and GST pull-down experiments. Chris and I together designed and performed the RNA Tagging experiments in the oocyte.

INTRODUCTION

mRNA regulation is of particular importance during early development. The CCR4-NOT complex is well characterized for its role in the control of mRNAs, from transcription to regulation of translation (Collart and Panasenko 2012). The mechanisms by which CCR4-NOT controls mRNA translation have been studied extensively, in our lab and others (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011; Waghray et al. 2015). However, it is largely unknown what RNAs CCR4-NOT regulates. It is also unclear whether CCR4-NOT mediates general translational control of all mRNAs or a more specific type of regulation.

We sought the RNA targets of CCR4-NOT across *Xenopus laevis* early development. We performed immunoprecipitations with CAF1 antibodies paired with RT-PCR and gene-specific amplification to determine RNAs associated with CAF1. The results were unclear and highly variable across replicates, so we utilized a GST pull-down approach with GST and GST-CAF1 expressed in oocytes and mature oocytes paired with the same endpoint assays. Again the data were not interpretable and did not improve with optimization. Thus, we took advantage of a newly developed method, RNA Tagging (Lapointe et al. 2015), to assay transcriptome-wide RNA associations with the RNA-binding protein (and CCR4-NOT interaction partner) CPEB. We detected RNAs associated with CPEB in oocytes, but more experiments need to be conducted to determine a list of validated targets.

RESULTS AND DISCUSSION

RNA immunoprecipitations to detect CAF1-RNA associations

To determine the RNA targets of CAF1 in *Xenopus* early development, we performed immunoprecipitations (IPs) with antibodies to CAF1 (previously made by Amy Cooke). The CAF1a antibody is not specific (Amy Cooke, unpublished), so it was not used for these experiments. Since CAF1b is not expressed in oocytes (Amy Cooke, unpublished), mature oocytes and 2-cell embryos were lysed and incubated with antibody-conjugated resin to immunopurify the CAF1b protein. The product of the IP was proteinase K-treated to digest the protein, and the RNA was extracted and purified prior to RT-PCR and amplification with gene-specific primers (Fig. 1A).

To confirm that the proteins were immunoprecipitated, we performed Western blots with the CAF1b antibody. CAF1b was detected in the IP products from both mature oocytes and embryos (Fig. 1B). A different antibody generated in parallel with the CAF1 antibodies was used for control IPs, and CAF1b was not detected in the control sample (Fig. 1B).

The PCR products indicated that, while some genes were detected as associated with CAF1b, many were detected in both the negative control and CAF1b IP samples (Fig. 1C). The genes chosen for PCR detection are genes bound by RNA-binding proteins that CAF1 is known to interact with. Samples without reverse transcriptase were used as an additional control (-RT samples, Fig. 1C). We performed significant optimization experiments with no improvement in the data and highly variable results.

GST pull-downs to detect CAF1-RNA associations

Based on these data, we tried a slightly different approach of using a GST pull-down to capture the CAF1 proteins and their associated RNAs (Fig. 2A). We injected GST and GST-CAF1 expressing mRNAs into *Xenopus* oocytes. After allowing time for the proteins to express, we either added progesterone to stimulate oocyte maturation or collected the Stage VI oocytes for pull-down experiments. Both oocytes and mature oocytes were lysed and incubated with a glutathione magnetic resin to purify the GST proteins. As for the IPs, the resulting products were separated for Western blot or RNA association analysis. Both procedures were performed in the same manner as for the IPs.

By Western blot, the GST and GST-CAF1 proteins were efficiently pulled down (Fig. 2B). The PCR assay detected no product for the pull-down samples with most of the primer sets (Fig. 2C). There was high background in the cyclin B5 samples, and the input samples did not produce a PCR band in most cases. Optimization of this method yielded similar results. Thus, we concluded that both IP and pull-down approaches followed by RT-PCR and gene-specific amplification were not useful for detecting RNA interactions with CAF1 proteins.

Since CAF1 does not contact RNA specifically and stably, we reasoned that using CAF1 to determine its associated RNAs might not be optimal. The interactions CAF1 makes with RNA are transient and at the poly(A) tail to deadenylate mRNAs. From our previous studies and the work of others, CAF1 and CCR4-NOT interact directly with several RNA-binding proteins (RBPs)

(Horiuchi et al. 2009; Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011; Sandler et al. 2011; Bhandari et al. 2014; Waghray et al. 2015). Thus, we decided to instead determine RNAs that are bound by RBPs that interact with CAF1 and CCR4-NOT. We also used a transcriptome-wide approach to determine all the bound mRNAs, as opposed to the candidate RT-PCR/gene-specific amplification assay.

RNA Tagging detects RNAs associated with CPEB in the oocyte

To determine RNA targets of RBPs in *Xenopus* oocytes, we utilized a newly developed method called RNA Tagging (Lapointe et al. 2015). In this approach, a poly(U) polymerase (PUP: *C. elegans* PUP-2), an enzyme that adds uridines to the 3' end of RNA, is fused to an RBP of interest. The RBP-PUP fusion thus "tags" RNAs bound by the RBP of interest *in vivo* with 3' terminal uridines (the "U-tag"). Total RNA from the cell is isolated and analyzed by high-throughput sequencing to detect the U-tagged RNAs (Fig. 3).

To test RNA Tagging in oocytes, we focused on the RBP CPEB. CPEB possesses RNA-recognition motifs and a Cysteine/Histidine (C/H) zinc finger-like region in its C terminus through which it directly binds to RNAs (Hake et al. 1998). CPEB also interacts with CAF1 through the NOT1 scaffold in *Xenopus* oocytes (Waghray et al. 2015). We injected mRNAs expressing either a wild type or RNA-binding defective HA-CPEB-PUP fusion in oocytes and allowed the proteins to accumulate and tag bound RNAs for 16 hours (Fig. 4A). Oocytes were then lysed, and the RNA was extracted and prepared for high-throughput

sequencing as previously reported (Lapointe et al. 2015). We used three different RNA concentrations of the CPEB-PUP fusion to determine an optimal amount of CPEB for RNA Tagging.

We detected approximately 6 million reads per sample, and about half of the reads aligned to a single genomic location (“uniquely mapped”). For each sample, we calculated the number of tagged RNAs detected, which were defined as RNAs that ended with at least 8 adenosines (the poly(A) tail) followed by at least 3 uridines (the U-tag). The number of tagged RNAs was then normalized to the total number of uniquely mapped reads to determine the tagged RNAs per uniquely mapped reads (TRPM) for each sample. We detected more TRPM for the CPEB-PUP2 samples relative to the Stage VI oocyte sample, as expected (Fig. 4B). There also was a correlation between the TRPM and the concentration of CPEB-PUP2 expressed (Fig. 4B), which was consistent with the trend in protein expression of the HA-CPEB-PUP2 fusions (Fig. 4C). The CPEB RNA-binding defective mutant had a decrease in TRPM compared to the CPEB wild type proteins, suggesting that it did not tag as many mRNAs, likely due to its lack of RNA association.

The number of TRPM was positively correlated with the RNA concentration at which CPEB-PUP fusions were injected (Fig. 4B). The three concentrations also exhibited good overlap (Fig. 4D). Thus, we defined CPEB “targets” as mRNAs that were detected with at least two TRPM in all three concentrations and were at least five-fold enriched above the Stage VI oocyte control. The targets spanned a range of RNA abundances (Fig. 4E), though

further analysis indicated that they were all among the best expressed mRNAs in the oocyte (top 5%) (data not shown). Importantly, CPEB targets were detected by fewer tagged RNAs in the RNA-binding defective CPEB-PUP samples (Fig. 4F), as demonstrated by the shift below the red line on the scatter plot.

These data demonstrate that the RNA Tagging approach is feasible in the oocyte. However, more experiments need to be done to generate a high-confidence list of mRNA targets of CPEB, such as additional biological replicates and independent validation of targets.

It will be of interest to perform similar experiments with other RBPs that associate with CCR4-NOT. A combination of RNA Tagging paired with mutational analysis to disrupt CCR4-NOT interactions with RBPs to assess the regulatory outcome on RNA targets should yield a list of mRNAs regulated by CCR4-NOT in the oocyte. Similar experiments can also be performed throughout early development, and possibly even in somatic cells, to determine mRNAs that are regulated by CCR4-NOT. Such analyses will shed light on whether CCR4-NOT is a general regulator of mRNAs or recruited more specifically by a few RBPs.

MATERIALS AND METHODS

Plasmid construction

pCS2+3HA+GST and pCS+3HA+GST-CAF1 plasmids were created by amplifying the GST and GST-CAF1 open reading frames from pGEX-6P-1 CAF1 plasmids (cite) and ligating the inserts into the StuI/XbaI restriction sites. pCS2+3HA-PUP2-CPEB plasmids were made by amplifying CPEB1a out of *Xenopus* oocyte cDNA and *C. elegans* PUP2 from RNA Tagging plasmids described in CITE. CPEB1a was cloned into the NcoI/StuI sites, and PUP2 was cloned into the XhoI/XbaI sites. The CPEB RNA-binding defective mutant was made by site-directed mutagenesis to delete the C/H region (Hake et al. 1998).

In vitro transcription and RNA preparation

Plasmids for protein expression were linearized with NotI (Fermentas) and transcribed with the AmpliScribe SP6 high yield transcription kit (CellScript), as described previously (Waghray et al. 2015).

Oocyte injections

Oocyte injections were performed as described previously (Gray et al. 2000; Kwak et al. 2004).

Immunoprecipitations

Oocytes were lysed with a pestle in TNMEN buffer with 0.1% Igepal (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 2 mM $MgCl_2$, 150 mM NaCl) with protease inhibitors (EDTA-free, Roche) and 3 mM DTT and RNasIN (Promega). Oocyte lysate was cleared and incubated with either negative control of CAF1b antibody

(Amy Cooke, unpublished) coupled to Protein A sepharose resin (GE Healthcare) via SulfoLink (Thermo Fisher Scientific). The lysate and antibody-coupled resin were incubated at 4°C for 2 hours, after which the resin was washed four times with TNMEN + 0.05% Igepal. Following the wash steps, 200 µg of Proteinase K was added in buffer containing 0.5% SDS, 50 mM Tris (pH 8.0), and 10 mM EDTA to release the bound RNA from the resin. The Proteinase K treatement was performed at 37°C for 1 hour. Treatment was stopped with the addition of 200 µL of buffer containing 0.5% SDS, 50 mM Tris (pH 8.0), and 10 mM EDTA. RNA was extracted using a standard phenol chloroform extraction procedure followed by ethanol precipitation. RNAs were resuspended in nuclease-free water and treated with Turbo DNase (Thermo Fisher Scientific). Finally, RNA quality was assessed by Nanodrop and analysis on a denaturing (formaldehyde/MOPS) 1% agarose gel.

GST pull-downs

mRNAs expressing the GST and GST-CAF1 proteins were injected into Stage VI oocytes, and the proteins were accumulated for 6 hours. For maturation experiments, Stage VI oocytes were treated with 0.05 mg/mL progesterone (in 100% ethanol) overnight. Mature oocytes were selected by the presence of GVBD. The oocytes and mature oocytes were lysed as for immunoprecipitations. GST pull-downs were conducted exactly as the immunoprecipitations, except that pre-washed glutathione magnetic resin (Novagen GST•Mag™) was used instead of antibody-coupled resin.

RT-PCR and gene-specific amplification

Reverse transcription was conducted with 100 ng of each RNA sample and 50 μM random decamer using the Superscript III protocol and reagents (Thermo Fisher Scientific). 1 μL of the resulting cDNA was used in PCR amplification with 10 pmol gene-specific primers using GoTaq Green Master Mix (Promega). The PCR products were resolved on a 1% agarose/TBE gel.

RNA Tagging

mRNAs encoding the HA-PUP2-CPEB fusions were injected into oocytes for protein expression and RNA binding. After 16 hours, 50 oocytes per conditions were collected, lysed, and RNA was extracted with 1 mL TRI reagent (Sigma) followed by isopropanol precipitation. RNAs were DNase treated, poly(A) selected, and rRNA-depleted as described (Lapointe et al. 2015), except that the mammalian Ribo-Zero magnetic Gold kit (Epicentre) was used in place of the yeast kit. The rest of the RNA Tagging protocol was performed as described (Lapointe et al. 2015). Data analysis was also performed as described, except that the sequencing data was aligned to the *Xenopus laevis* genome (version 9.1).

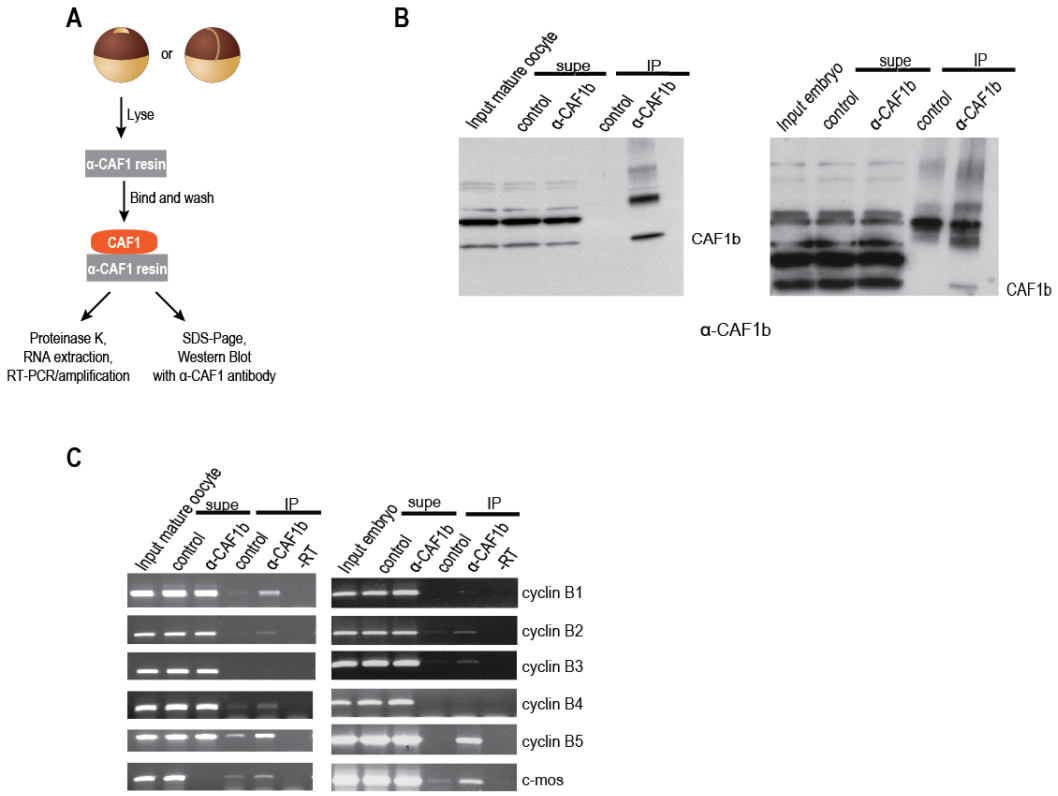

Figure 1. RNA immunoprecipitations to detect CAF1-RNA interactions.

A) Schematic of RNA immunoprecipitations out of *Xenopus* mature oocytes and 2-cell embryos. B) Western Blots with α-CAF1b antibody were conducted as a control for immunoprecipitation. Supe, supernatant; IP, immunoprecipitation. C) Results of the RT-PCR and gene-specific PCR assay. -RT, no reverse transcriptase added control.

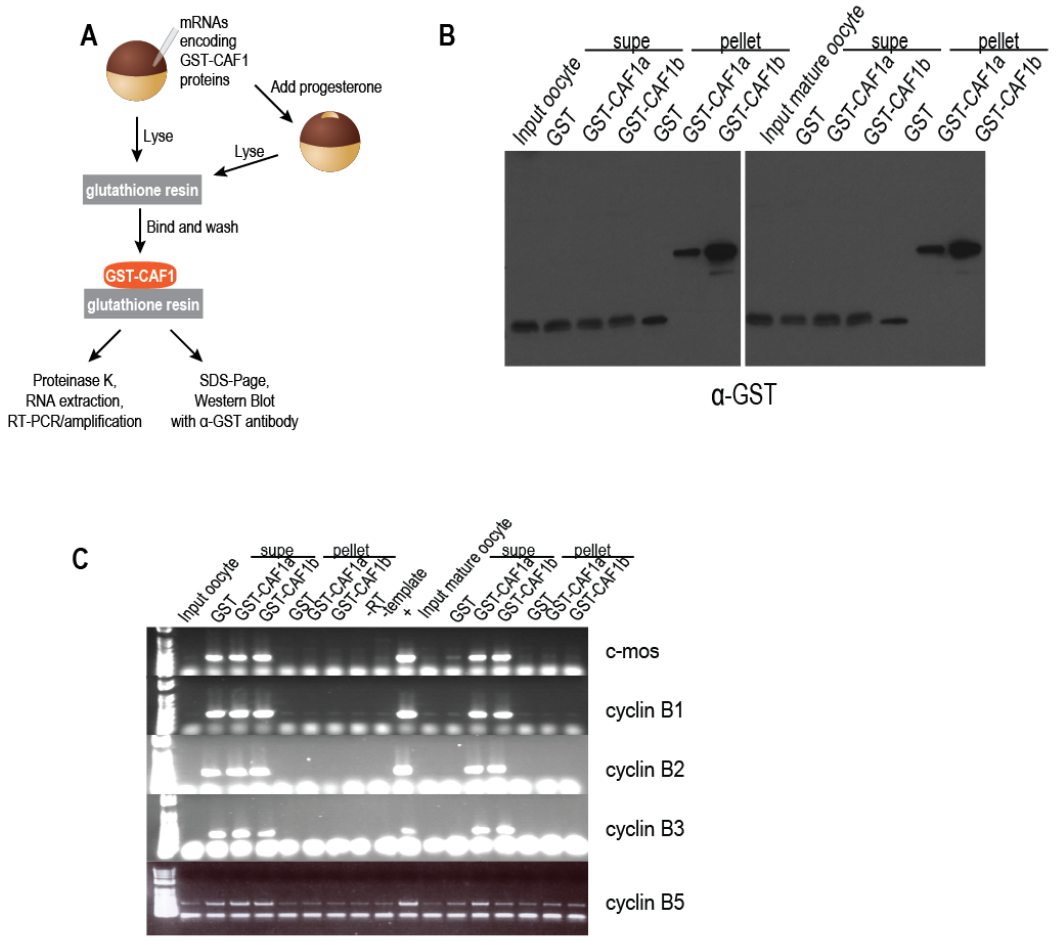

Figure 2. GST pull-downs to detect CAF1-associated RNAs.

A) Schematic of GST pull-downs out of *Xenopus* oocytes and mature oocytes. B) Western Blots with α-GST antibody were conducted as a control for pull-down. Supe, supernatant. C) Results of the RT-PCR and gene-specific PCR assay. -RT, no reverse transcriptase added control; -template, no template added control; +, positive control with oocyte lysate cDNA from another experiment.

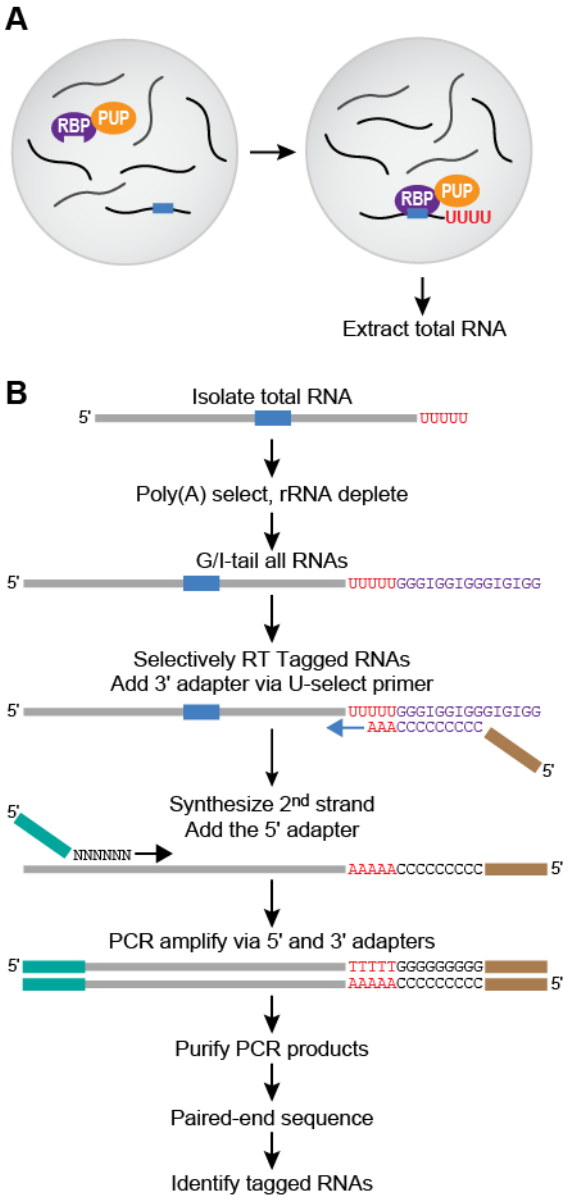

Figure 3. RNA Tagging overview.

The RNA Tagging method, adapted from (Lapointe et al. 2015).

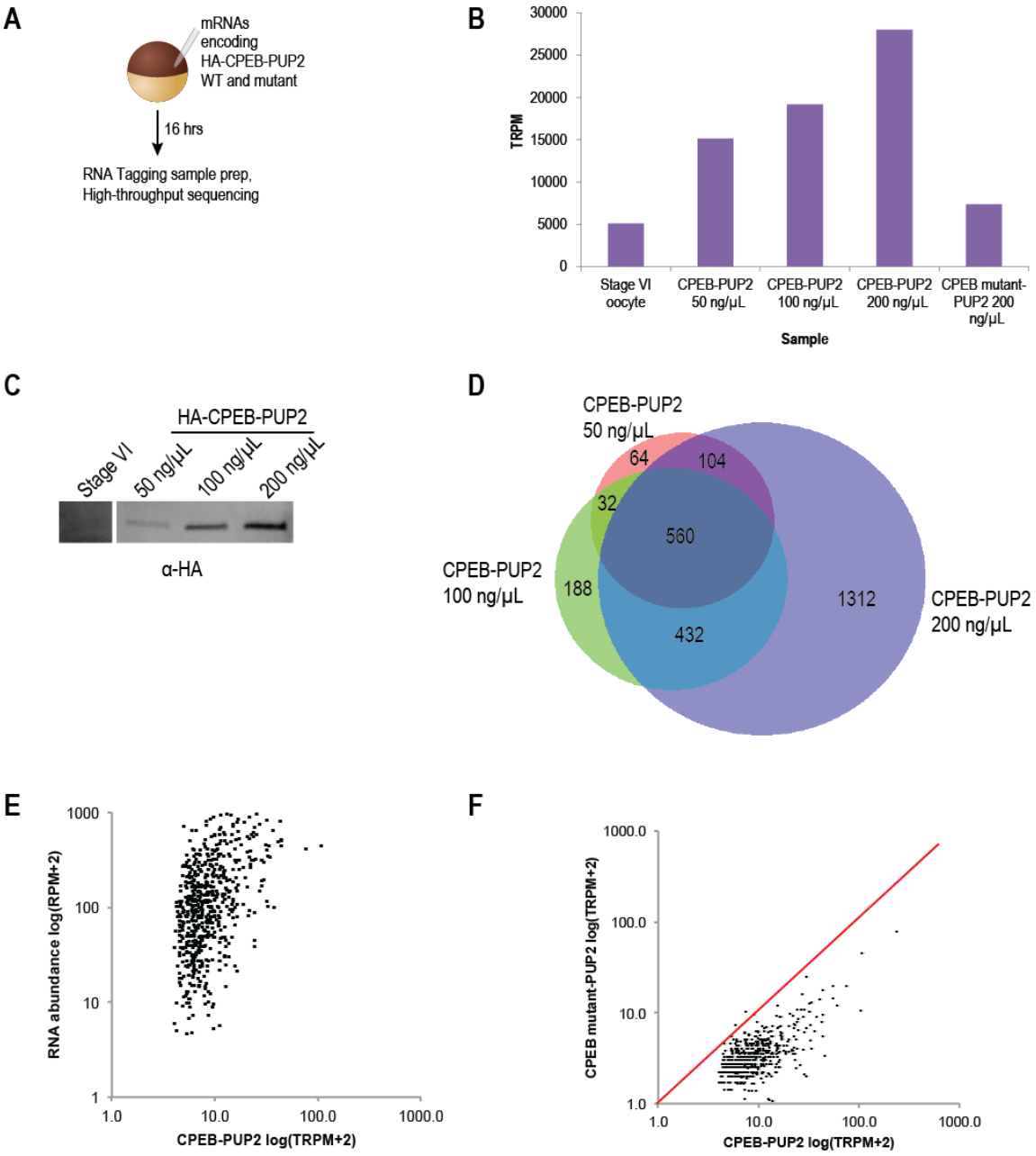

Figure 4. RNA Tagging with CPEB-PUP2 in *Xenopus* oocytes.

A) Schematic of steps involved in RNA Tagging procedure. See previous figure for more detail. B) Column graph of TRPM (tagged RNAs per million uniquely mapped reads) for the samples tested. C) Western blot with α-HA to detect HA-CPEB-PUP2 fusions. D) Venn diagram of the different concentrations tested for CPEB-PUP2 in oocytes. E) Scatter plot of tagged RNAs detected in the CPEB-PUP2 oocytes versus their abundance, as measured in Stage VI oocytes. TRPM, tagged RNAs per million uniquely mapped reads; RPM, reads per million

uniquely mapped reads. F) Scatter plot of tagged RNAs detected in the CPEB-PUP2 oocytes versus in CPEB mutant-PUP2 oocytes. The red line indicates tagged RNAs that are detected equally in both CPEB-PUP2 and CPEB mutant-PUP2.

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