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Ccr4-Not complex: the control freak of eukaryotic cells

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Abstract

The purpose of this review is to provide an analysis of the latest developments on the functions of the Ccr4-Not complex in regulating eukaryotic gene expression. Ccr4-Not is a nine-subunit protein complex that is conserved in sequence and function throughout the eukaryotic kingdom. Although Ccr4-Not has been studied since the 1980s, our understanding of what it does is constantly evolving. Once thought to solely regulate transcription, it is now clear that it has much broader roles in gene regulation, such as in mRNA decay and quality control, RNA export, translational repression and protein ubiquitylation. The mechanism of actions for each of its functions is still being debated. Some of the difficulty in drawing a clear picture is that it has been implicated in so many processes that regulate mRNAs and proteins in both the cytoplasm and the nucleus. We will describe what is known about the Ccr4-Not complex in yeast and other eukaryotes in an effort to synthesize a unified model for how this complex coordinates multiple steps in gene regulation and provide insights into what questions will be most exciting to answer in the future.

Keywords

Ccr4-Not; mRNA decay; transcription; deadenylation; transcription elongation

Introduction

The underlying mechanisms that dictate eukaryotic life, disease, and death are bound by the central dogma of molecular biology. Genes, coded for in the DNA, are transcribed into RNA and later translated into proteins, which come together to form complexes to control gene expression either at the DNA, RNA, or protein level. There are examples where a given protein is dedicated to a single task, but the more interesting scenario is one where a protein (or complex) carries out multiple functions in the cell. The Ccr4-Not complex is of interest to many groups because it regulates transcription, decay of mRNA, translation and even protein degradation. Although this evolutionarily conserved complex has been studied since the 1980s, our understanding of what it does is still changing.

The yeast Ccr4-Not complex contains nine conserved subunits: Not1, Not2, Not3, Not4/Mot2, Not5, Caf130, Caf40, Pop2/Caf1, and Ccr4 (Table 1). This core complex is often referred to as the 0.9–1.2 MDa version in the literature (Liu et al., 1998, Chen et al., 2001, Collart, 2003). In addition, a larger >1.9 MDa complex was described in yeast, which may also contain a combination of Dhh1, Dbf2, Caf4, Caf16, and Btt1 (Maillet and Collart, 2002,

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Liu et al., 1997, Liu et al., 2001, Cui et al., 2008). It is still unclear if the 1.9 MDa complex is a significant physiological entity since Ccr4-Not complexes isolated to high purity only contained the nine core subunits. However, even if these “orphan” components are not stoichiometric subunits of the complex, there is plentiful genetic, phenotypic and biochemical evidence that one or more of these proteins associate with Ccr4-Not to carry out cellular functions ((Collart and Panasenko, 2011) and see below). The presence of two forms, a larger and smaller, was suggested from fraction of human cell extracts on gel filtration columns. Like in yeast, the predominant form of human Ccr4-Not elutes at a molecular weight of 1.2 MDa, but a >1.9 MDa version was detected also (Morel et al., 2003, Lau et al., 2009). Excellent reviews on the composition, structure and conservation of the Ccr4-Not complex in eukaryotes have been published and this topic will not be re-addressed in detail here (Collart and Panasenko, 2011, Collart and Timmers, 2004, Denis and Chen, 2003).

The first functions of the Ccr4-Not complex were implied by genetic analysis of yeast mutants. The vast majority of the screens that isolated Ccr4-Not genes implicated it in regulating gene expression. *NOT1/CDC39* and *NOT2/CDC36* were identified in a genetic screen for mutants causing cell cycle arrest in G1 (Reed, 1980). Further genetic analysis led to the identification of *NOT1*, *NOT2* and *NOT4* as regulators of mating type and filamentous growth that, among other possibilities, caused increased expression of genes involved in mating pheromone response (de Barros Lopes et al., 1990, Mosch and Fink, 1997). *CCR4* (carbon catabolite-repression) was discovered for its role in the activation of *ADH2* in media containing a non-fermentative carbon source (Denis, 1984, Denis and Malvar, 1990). The first glimpse into the molecular mechanism of this complex was revealed by Collart and Struhl when they identified the first four *NOT* genes in a screen for mutants which increased expression of a compromised *HIS3* gene (Collart and Struhl, 1994). The *NOT* mutations preferentially increased mRNA generated from the constitutive TATA-less promoter (Tc) over that produced from a regulated TATA-containing promoter (Tr) of the *HIS3* gene, suggesting the *NOT* genes were important for repressing the TATA-less promoter. From this phenotype these genes were given the “Negative on TATA-less” or *NOT* nomenclature. Finally, Ccr4-Not mutants suppressed a temperature-sensitive mediator subunit mutant (*srb4/med17*), which also suggested that the complex played a role in repressing transcription (Lee et al., 1998). These important genetic screens led to years of biochemical and genetic studies implicating Ccr4-Not in transcriptional control.

For years, the field considered Ccr4-Not as solely a negative regulator of transcription and a significant amount of genetic evidence supported such a role. Then the Parker and Denis labs provided very clear evidence that Ccr4 is the major cytoplasmic mRNA deadenylase in yeast and it and other complex subunits reside in the cytoplasm (Chen et al., 2001, Liu et al., 1998, Tucker et al., 2001). This function is conserved throughout evolution, as it wasn't long before it was shown that metazoan Ccr4 homologues regulated deadenylation too (Temme et al., 2004, Chang et al., 2004, Yamashita et al., 2005). These results provided a fascinating new function for the complex, but also raised questions about its true role in gene regulation. Since many of the genetic screens used to identify and characterize Ccr4-Not subunits relied on readouts for mRNA accumulation, some of the phenotypes and genetic interactions that implicated Ccr4-Not in transcriptional control could result from alterations in mRNA decay. Furthermore, new lines of evidence suggest it may play roles in protein degradation, translation, RNA export and nuclear mRNA surveillance. Thus, it has been implicated in just about every step in the regulation of a gene product. This review will address its many functions and provide a model for why a single complex would regulate an mRNA from “birth to death”.

Ccr4-Not and Transcription

RNA polymerase II (RNAPII) requires many protein factors in order to gain access to the DNA and transcribe across it. For instance, chromatin remodelers are required for RNAPII to access the DNA, while initiation factors recruit it to promoters, and finally elongation factors help it efficiently transcribe across genes. Clearly, all of these processes must be highly coordinated. Initially, gene regulatory proteins, and the processes they control, were studied individually. As the knowledge base on these factors expanded and the tools used to study transcription evolved, many regulators of transcription have been discovered to play multiple roles in gene expression. Ccr4-Not is one example, and it is now known to regulate many steps in the production of mRNAs in the nucleus.

Transcription initiation

Subunits of the Ccr4-Not complex show both genetic and physical interactions with TFIID, the SAGA histone acetyltransferase complex, TATA-binding protein (TBP) and SRB/Mediator complex (Badarinarayana et al., 2000, Benson et al., 1998, Deluen et al., 2002, Lemaire and Collart, 2000, Liu et al., 2001, Reese and Green, 2001, Sanders et al., 2002), supporting a role in transcription initiation. Physical interactions between Not2 and Not5 and TBP and TFIID-specific TAF_{II}s have been described in the literature (Sanders et al., 2002, Lemaire and Collart, 2000, Deluen et al., 2002). This corroborated the genetic evidence implying that Ccr4-Not regulated TFIID activity at promoters. Furthermore, it was proposed that Ccr4-Not alters the use of TFIID at stress-regulated promoters (Lenssen et al., 2007, Lenssen et al., 2005, Lenssen et al., 2002). Consistent with a role for Ccr4-Not in stress-regulated transcription, Ccr4-Not subunits crosslink to stress and Gcn4-regulated genes (Deluen et al., 2002, Qiu et al., 2004, Swanson et al., 2003). Lastly, Ccr4-Not is required for the expression of *RNR3*, a TFIID-dependent gene, and is recruited to the promoter and coding sequences during activation of the gene (Kruk et al., 2011, Traven et al., 2005, Mulder et al., 2005). Collectively, these results supported a model where Ccr4-Not regulates transcription initiation of certain stress-regulated genes by affecting TBP/TFIID function.

Transcription of the yeast genome is carried out by either the TFIID or SAGA complexes, which recruit TBP to promoters (Huisinga and Pugh, 2004). TFIID-dependent genes are mostly constitutively expressed TATA-less housekeeping genes, while SAGA-dependent genes are highly induced and TATA-containing (Huisinga and Pugh, 2004). Stress induced genes are regulated by SAGA and not TFIID, as are GCN4-dependent genes such as *HIS3* (Huisinga and Pugh, 2004, Swanson et al., 2003). Genome-wide gene expression analysis of Ccr4-Not mutants suggest that it predominately regulates SAGA-dependent genes (Azzouz et al., 2009b, Cui et al., 2008). Additionally, a chromatin immunoprecipitation-sequencing (ChIP-seq) study showed Ccr4-Not subunits are recruited to the open reading frames of SAGA-regulated genes (Venters et al., 2011). It is a bit paradoxical that earlier genetic and biochemical evidence implicated Ccr4-Not as a regulator of TFIID, yet Ccr4-Not is so strongly implicated in the regulation of TATA-containing stress response genes by the SAGA complex. Thus, there is a difference between the predictions of Ccr4-Not function based on genetic screens and that based on genomics data. It could be that Ccr4-Not directly controls one class of genes, but regulates the other by an indirect mechanism. Alternatively, it may be too simple to divide the genome into two categories, either TFIID or SAGA dependent. Stress regulated genes may utilize TFIID to maintain low levels of constitutive expression (housekeeping functions), and SAGA during the induction phase, as has been reported at DNA damage induced genes (Ghosh and Pugh, 2011, Zhang et al., 2008). Thus, Ccr4-Not may not specifically regulate TFIID, but may play a broader role in controlling TBP use at promoters.

The Ccr4-Not complex is conserved among all eukaryotes (Collart, 2003, Lau et al., 2009, Albert et al., 2000). Even less is known about the role of the Ccr4-Not complex as a regulator of transcription in metazoans. The first evidence that hCcr4-Not regulates transcription was the observations that overexpression of hCCR4 and hCAF1 enhanced the transactivation of estrogen receptor (ER) (Morel et al., 2003, Prevot et al., 2001). The involvement of hCcr4-Not in the activation of other nuclear receptors was established by the Samuels group, which reported that overexpression of hCCR4 or RCD (yCAF40) enhanced ligand-dependent reporter gene expression of the receptors for retinoic acid, thyroid hormone, glucocorticoid and estrogen; conversely, siRNA knockdown of these proteins reduced activation of retinoic-acid induced genes (Garapaty et al., 2008). Finally, knocking out CNOT7 (hCaf1) in mouse embryo fibroblasts (MEFs) reduced retinoic acid induced gene activation (Nakamura et al., 2004). In this study, they showed that CNOT7 interacts with a specific isoform of RXR, RXRb, but not with RAR, RXRa or RXRg. This selectivity among the different isoforms is striking and suggests some specificity. It needs to be determined if knockdown of Ccr4-Not in metazoans directly affects transcription initiation, elongation or post-transcriptional processes. Furthermore, since the binding of RXR to a response element was significantly reduced in extracts prepared from CNOT7^{-/-} MEFs, knocking out CNOT7 may have some indirect effects on hormone signaling (Nakamura et al., 2004).

The yeast Ccr4-Not complex is considered to be both a positive and negative regulator of transcription (Cui et al., 2008, Azzouz et al., 2009b, Denis and Chen, 2003, Liu et al., 1998). Similarly, there is evidence human Ccr4-Not can repress transcription. Overexpression of either CNOT1 or CNOT2 or targeting them to promoters through a DNA binding domain repressed ER-dependent gene activation; interestingly, this affect could be suppressed by the histone deacetylase (HDAC) inhibitor TSA or knockdown of the SMRT/NCOR corepressor (Winkler et al., 2006, Zwartjes et al., 2004, Jayne et al., 2006). Consistent with human NOT proteins repressing transcription, knockdown of CNOT1 or CNOT3 increased the mRNA of two estrogen responsive genes, which further suggests some subunits of hCcr4-Not repress transcription (Winkler et al., 2006). This same study provided the first evidence that hCcr4-Not components are recruited to active genes. ChIP assays showed that three subunits of the hCcr4-Not crosslinked to the activated ER-responsive genes, coincident with the binding of RNAPII to the promoter. It seems counterintuitive that Ccr4-Not is recruited to genes it represses, but there are numerous examples where repressors are recruited to active genes. Collectively, there is good evidence that hCcr4-Not influences the cellular response to nuclear hormones. What remains to be determined is how and why one group observed that overexpression of CNOT1 or CNOT2 repressed ER activation, while another found that overexpression of hCCR4, hCAF1 or RCD activated expression. These differences could be due to the use of different experimental systems, or it could indicate that like in yeast, mammalian CCR4- and the NOT- group of genes have different activities in cells.

Despite the fact that the first function proposed for Ccr4-Not was controlling transcription initiation through the general transcription factor TFIID, very little progress has been made over the years to identify the mechanism. This will require a robust biochemical reconstitution assay to analyze the effects of Ccr4-Not on transcription initiation. A mechanistic understanding may be elusive because biochemical reconstitution assays used to study yeast general transcription mechanisms are not as advanced as their mammalian counterparts. Nevertheless, the studies mentioned here provide a foundation for which future work can be built upon to better understand the role of Ccr4-Not in regulating transcription initiation.

Transcription Elongation

The role of the Ccr4-Not complex in transcription elongation has received greater attention lately. This change in focus might be a reflection of renewed interest in elongation control in the field of gene regulation as whole or that better evidence for this function of the complex is emerging. The idea that mutations in Ccr4-Not subunits affect elongation is not necessarily new, but the application of new assays to measure elongation defects provides firmer evidence for this function. The first line of evidence for this function emerged from genetic and phenotypic analysis of Ccr4-Not mutants. The Ccr4-Not mutants show synthetic lethal interactions with mutants of the elongation factors Paf1c, FACT (*SPT16*), TFIIS (*DST1*) and DSIF (*SPT5*) (Biswas et al., 2006, Denis et al., 2001, Chang et al., 1999, Jaehning, 2010). In addition, some Ccr4-Not mutants are sensitive to the elongation inhibitors 6-azauracil (6-AU) and mycophenolic acid (Denis et al., 2001, Gaillard et al., 2009). The only known physical interaction between Ccr4-Not and elongation factors was the co-purification of Ccr4 with subunits of the Paf1c complex, but the NOT proteins were not found in those fractions (Chang et al., 1999). While the genetic and phenotypic evidence was strong, a direct role for Ccr4-Not in elongation was not established until recently.

Overtime, assays were developed to monitor defects in elongation in yeast. One of the first was the gene length-dependent accumulation of mRNA, or GLAM assay (Morillo-Huesca et al., 2006). This assay exploits the property of yeast RNA polymerase to transcribe poorly through long GC-rich containing genes. Many elongation factor mutants show defective transcription through these reporter genes, described as altered GLAM ratios. However, these types of assays should be interpreted cautiously because not all elongation factor mutants, such as TFIIS, show a phenotype in this assay and mutations in genes with no known link to transcription show altered GLAM ratios (Gaillard et al., 2009). Nonetheless, multiple Ccr4-Not complex mutants were identified in the screen, and additional evidence was presented that supported the GLAM assay result: an extract prepared from a *not5Δ* mutant could not support transcription of a long *lacZ*-containing reporter gene *in vitro* (Gaillard et al., 2009). However, it was not shown that adding back purified Ccr4-Not complex can recover the elongation defect.

Recently, the field has moved towards directly measuring RNAPII density across a large gene (8kb) engineered to be expressed from the inducible *GAL1* promoter, *GAL1p-YLR454w*. This assay monitors RNAPII density across *GAL1*-driven reporter gene under steady state conditions or after repression by the addition of glucose to the medium, to determine if elongation factor mutants have an elongation rate or processivity defect *in vivo* (Mason and Struhl, 2005). Using this assay, it was confirmed that deleting *CCR4*, *DHH1* (associated with Ccr4-Not) or *NOT4* displayed altered RNAPII density across the gene (Kruk et al., 2011). RNAPII density was significantly higher at the 3' end of the gene in the steady state and cleared the gene after transcriptional repression more slowly in Ccr4-Not mutants. This phenotype is very different from other elongation factors analyzed to date and suggests that in the absence of Ccr4-Not, RNAPII could not transcribe through natural blocks in elongation, such as nucleosomes, but remained engaged with the DNA.

The *in vivo* elongation assays and the genetic studies provided an overwhelming amount of converging circumstantial evidence pointing towards a role for Ccr4-Not in elongation. Ccr4-Not crosslinks throughout the body of the *RNR3* and *GAL1* genes, but not at the upstream regulatory regions, suggesting its recruitment is dependent on transcription and RNAPII (Kruk et al., 2011). Furthermore, the binding of Ccr4-Not was mapped throughout the genome by ChIP-seq and it crosslinked to the open reading frames of genes similar to other elongation factors, and different from that of initiation factors (Venters et al., 2011). Therefore, the localization of Ccr4-Not across genes is consistent with it acting in

elongation. Its recruitment to genes is likely due to its ability to interact directly with RNAPII (Kruk et al., 2011).

Many factors bind to and track with RNAPII across genes *in vivo*. A legitimate question to ask is how many of these proteins actually affect elongation, versus “riding along” with polymerase to coordinate downstream steps such as processing? The reduced elongation across reporter genes *in vivo* could be caused by indirect effects. A strong case for defining Ccr4-Not as an elongation factor came from *in vitro* reconstitution experiments using highly purified RNAPII and Ccr4-Not. Ccr4-Not interacts with functional elongation complexes formed on DNA templates and yeast RNAPII *in vitro*, suggesting that Ccr4-Not binds directly to RNAPII in the elongation complex (EC) and not other factors associated with RNAPII during elongation (Kruk et al., 2011). In addition, while the transcript is not required for Ccr4-Not to bind to ECs, crosslinking studies revealed that a subunit of Ccr4-Not binds to the transcript, and this interaction may be required for Ccr4-Not to stimulate transcription. Using *in vitro* transcription run-off assays, a case was made that Ccr4-Not stimulates elongation by rescuing stalled and backtracked RNAPII complexes. This property is reminiscent of the well-characterized elongation factor TFIIS. TFIIS reactivates backtracked complexes by stimulating the nucleolytic cleavage activity of RNAPII, which removes the displaced transcript and realigns the 3'OH into the active site of the enzyme (Fish and Kane, 2002, Arndt and Kane, 2003). Further characterization of Ccr4-Not's activity and comparison of its mechanism of action to that of TFIIS revealed that they are functionally distinct. Ccr4-Not cannot stimulate the nuclease activity of RNAPII, and it realigns the transcript in the active site without transcript cleavage. A model was proposed where Ccr4-Not reactivates the backtracked complex by locking in transient forward excursions made by RNAPII over the arrest site, thereby realigning the transcript in the active site. Since TFIIS and Ccr4-Not have different biochemical activities, it is possible they may act on different states of arrested polymerase and cooperate to maintain elongation throughout the genome. This might explain the synthetic lethal interactions between TFIIS (*DSTI*) and Ccr4-Not mutants (Denis et al., 2001).

Rpb4/7 and Ccr4-Not coordinate mRNA synthesis and decay

There are intriguing parallels between the Ccr4-Not complex and the Rpb4/7 module of RNAPII. Rpb4/7 is a dissociable subcomplex of RNAPII; Rpb4 is non-essential, while Rpb7 is essential for yeast viability (Choder, 2004, Sampath and Sadhale, 2005). Although Rpb4/7 was initially thought to associate with RNAPII reversibly during transcription, recent ChIP-chip data suggest its distribution across the genome is not different from core subunits of RNAPII (Jasiak et al., 2008). Interestingly, Rpb4/7, a module at the heart of RNA synthesis, has now been linked to multiple post-transcriptional processes including mRNA export, mRNA decay and even translation (Runner et al., 2008, Lotan et al., 2005, Harel-Sharvit et al., 2010, Goler-Baron et al., 2008, Choder, 2004). Once thought to be exclusively nuclear like other RNAPII subunits, Rpb4 and 7 have been detected in cytoplasmic foci under conditions of extreme stress (Lotan et al., 2005). Cytoplasmic foci are sites of mRNA translational repression and decay (Parker and Sheth, 2007). Consistent with these observations, the Rpb4/7 subcomplex has been implicated in the control of mRNA degradation by promoting deadenylation of certain transcripts, especially mRNAs of factors involved in ribosome biogenesis (Lotan et al., 2005). Mutation of either Rpb4 or 7 inhibits the deadenylation of mRNAs and extends their half-life. From these results, a model was proposed that mRNA synthesis and decay are coordinated through Rpb4/7, but it was not clear if a separate pool of Rpb4/7 from that used to transcribe genes was responsible for regulating decay. An important piece of the puzzle was put into place recently. The Choder group demonstrated that mRNA deadenylation and decay were impaired in an *rpb6* mutant that weakened the association between 4/7 and the core of RNAPII; thus, providing

evidence that Rpb4/7 may set up mRNAs for destruction in the context of intact RNAPII during transcription (Goler-Baron et al., 2008).

There are many phenotypic similarities between Rpb4 and Ccr4-Not mutants. They are sensitive to stress, cannot transcribe through long GC-rich genes (GLAM phenotypes), are 6-AU sensitive and reduce deadenylation and decay of mRNAs (Choder, 2004, Verma-Gaur et al., 2008). Similar to Rpb4/7, Ccr4-Not subunits localize to cytoplasmic foci under conditions of extreme stress (Tucker et al., 2002). An attractive model is that Rpb4/7 recruits Ccr4-Not to mRNAs during the process of transcription to mark them for future deadenylation. Initially, it was shown that Ccr4-Not co-immunoprecipitated with RNAPII in an *rpb4Δ* strain, suggesting that contact with the 4/7 module is not important for its association with polymerase (Kruk et al., 2011). However, even though highly purified polymerase isolated from a *rpb4Δ* strain lacks the entire 4/7 module, it is possible that Rpb7 could associate with RNAPII in crude extracts (Sheffer et al., 1999). Recently, we analyzed the ability of Ccr4-Not to bind to elongation complexes (ECs) formed from highly purified RNA polymerase lacking the 4/7 module and found that it cannot bind to complexes formed with the mutant polymerase (Babbarwal and Reese, unpublished). The possibility that Ccr4-Not contacts the 4/7 module is further suggested by the observation that at least one subunit of Ccr4-Not crosslinks to the transcript, even when only a few bases emerged from the exit channel (Kruk et al., 2011). The Rpb4/7 module is located near the RNA exit channel and Rpb7 crosslinks to RNA in ECs; thus, Ccr4-Not is in close proximity to Rpb4/7 in the EC (Cramer et al., 2008, Ujvari and Luse, 2006, Chen et al., 2009). Additional experiments will be necessary to understand how Ccr4-Not and Rpb4/7 cooperate to control the coordination of RNA synthesis and decay, but it is possible that Rpb4/7 is required to recruit Ccr4-Not to RNAPII during elongation.

Why would synthesis and decay be so tightly linked? Genome-wide gene expression studies have determined that transcriptional responses to stress are accompanied by opposing effects on mRNA degradation (Shalem et al., 2008). Generally, fast-induced stress responsive genes displayed a corresponding destabilization of their mRNAs, whereas fast-repressed genes show stabilization on mRNAs. This suggests coordination between synthesis and decay rates during the stress response. The authors speculated that Rpb4/7 may be crucial for this effect (Shalem et al., 2008). This hypothesis appears to be correct, because subsequent work identified the Rpb4/7 module as mediating the correlation in gene expression and decay rates (Dori-Bachash et al., 2011, Shalem et al., 2011). A comparison of the changes in transcription and mRNA degradation between two yeast species, *S. paradoxus* and *S. cerevisiae*, indicated that evolutionary changes in mRNA degradation were coupled to opposing changes in transcription. Using a trans-complementation approach to measure allele-specific changes in mRNA degradation rates for a hybrid of *S. cerevisiae* and *S. paradoxus*, the authors found a very strong correlation between these genes and those whose expression is affected by Rpb4 and Ccr4-Not mutations in *S. cerevisiae* (Dori-Bachash et al., 2011). This suggests that Rpb4/7 and Ccr4-Not are important determinants in the opposite coupling between the regulation of transcript production and decay during the evolution of gene regulation in yeast. In addition to providing insights into the evolution of the control of gene expression, these studies tie Rpb4/7 and Ccr4-Not together in coordinating the synthesis and decay of mRNAs.

Ccr4-Not regulates histone modifications

In the late 2000s, multiple reports implicated Ccr4-Not in controlling histone modifications, especially H3 K4 trimethylation (H3K4me3). The first of these was from the Timmer's group, which was shortly followed by a paper from the Strahl group. They reported that deletion of NOT group genes, especially *NOT4*, led to global reductions in H3K4me3

(Mulder et al., 2007a, Laribee et al., 2007). Interestingly, deleting *CCR4* or *CAF1* did not result in reduced H3K4me3 in cells, suggesting this is a NOT-specific function of the complex. This provided another example where the “Ccr4-group” of genes, defined as *CCR4* and *CAF1*, have distinct mutant phenotypes than the “Not-group” (for review see (Collart, 2003, Denis and Chen, 2003)). These observations promote a few interesting ideas. First, impaired deadenylation and mRNA decay is not responsible for the reduced histone methylation in the *not4Δ* mutant because Ccr4 and Caf1 are essential for the deadenylase activity of the complex. Second, the integrity of the complex is not required either. Deleting *CAF1*, and to some extent *CCR4*, disrupts the Ccr4-Not complex (Collart, 2003, Denis and Chen, 2003). In absence of the Ccr4-group of proteins, a subcomplex containing Not4 carries out this function (also see below). There were different mechanisms proposed for how Not4 regulated H3K4me3, ranging from altered PAF1c recruitment, disruption of histone crosstalk by reduced H2B-Ub and the association of Ccr4-Not with the proteasome. However, it was later discovered that the primary affect of *not4Δ* mutations on H3K4me3 was indirect.

Not4 is a RING domain E3 ubiquitin ligase (Albert et al., 2002) and it was later shown by the Briggs group that Jhd2, the specific H3 K4me3 demethylase in yeast, is subject to Not4-dependent polyubiquitylation and turnover (Mersman et al., 2009). They provided genetic evidence that the regulation of H3K4me3 is through the control of Jhd2 stability, as deleting *JHD2* in a *not4Δ* background reversed the reduction in this mark. Regulation of H3K4me3 through degradation of Jhd2 may explain why the RING domain of Not4 is required for maintaining H3K4me3 levels (Laribee et al., 2007, Mersman et al., 2009). One has to wonder if the reported effect of proteasome mutants on H3K4me3 at active genes is partly influenced by changes in Jhd2 levels (Ezhkova and Tansey, 2004). Interestingly, it was shown that CNOT4, the human homologue of yeast Not4 can polyubiquitylate JARID1C, a homolog of Jhd2, suggesting that this mechanism is likely to be conserved (Mersman et al., 2009). What remains to be determined is if Not4 performs more of a housekeeping function in maintaining Jhd2 levels, or if it can be regulated by stress or other stimuli (discussed below).

There is a possibility that Ccr4-Not directly regulates histone acetylation. Physical and genetic interactions between Ccr4-Not, Not2 specifically, and the SAGA histone acetyltransferase complex were described (Collart, 1996, Benson et al., 1998, Biswas et al., 2006) and global H3 acetylation levels are reduced in *ccr4Δ*, *not4Δ* and *not5Δ* cells (Peng et al., 2008). One explanation for the reduction in acetylation is that the association of Ccr4-Not with SAGA regulates its activity in a direct manner. Another is that Ccr4-Not mutations reduce transcription across the genome and indirectly affects H3 acetylation. The latter possibility seems unlikely because genome-wide expression studies have shown that deleting Ccr4-Not subunits individually only affects the expression of a small percentage of genes (Azzouz et al., 2009b, Cui et al., 2008). Reducing acetylation at a few genes would not lead to a global reduction in this mark. The exact cause is not clear, but we propose another, indirect mechanism. As described below, Ccr4-Not is recruited to the 3' UTRs of specific mRNAs by the pumilio (PUF) family of proteins, where they then enhance the degradation of the message (Goldstrohm et al., 2007, Goldstrohm et al., 2006). Interestingly, Brown and co-workers discovered that Puf5/Mpt5 is recruited to the mRNAs of many chromatin regulators, including the mRNAs for the two major HDACs, Hda1 and Rpd3 (Gerber et al., 2004b). Disrupting the Ccr4-Not complex could stabilize mRNAs of components of HDAC complexes and lead to a greater accumulation of protein. Such a model would explain why H4 acetylation levels were also increased in these mutants. SAGA is specific for H3 and H2B; however, Rpd3 can deacetylate H4 as well as H3 (Shahbazian and Grunstein, 2007, Suka et al., 2001). It would be interesting to examine histone

acetylation levels in a catalytically inactive mutant of *CCR4* and determine if mutation of other factors involved in mRNA decay results in similar reductions in acetylation.

Nuclear quality control and mRNA export

We are familiar with the old saying that you can learn a lot about a person by the company he/she keeps. The same is true of transcription factors. Some interesting ideas and possible new functions have come from proteomics studies aimed at identifying binding partners of the Ccr4-Not complex in yeast. There is growing evidence that Ccr4-Not participates in aspects of nuclear RNA quality control and mRNA export. These discoveries provide the missing link between its participation in transcript production in the nucleus and decay in the cytoplasm and raise the possibility that Ccr4-Not may remain associated with the mRNA throughout its life in the cell.

Connections to the nuclear exosome and RNA processing in the nucleus

Subunits of the nuclear exosome and TRAMP complex were discovered to co-purify with the Ccr4-Not complex (Azzouz et al., 2009a). The exosome degrades aberrant RNAs in the nucleus using its 3' to 5' exonuclease activity (Fasken and Corbett, 2009, Lykke-Andersen et al., 2011, Hilleren et al., 2001, Houseley and Tollervey, 2009). TRAMP is also involved in the nuclear surveillance pathway by polyadenylating aberrant RNAs, leading to their recruitment to and degradation by the nuclear exosome (Houseley and Tollervey, 2008, LaCava et al., 2005). Ccr4-Not mutants and exosome mutants display synthetic lethal interactions (Assenholt et al., 2011, Azzouz et al., 2009a), suggesting some type of collaboration between these two complexes. Mutations to either the exosome or Ccr4-Not subunits lead to the accumulation of polyadenylated and misprocessed tRNAs, rRNAs, snRNAs, and snoRNAs (Azzouz et al., 2009b, LaCava et al., 2005, van Hoof et al., 2000, Petfalski et al., 1998). However, there is some disagreement over the extent of accumulation of misprocessed snRNA in Ccr4-Not mutants, and even the effects that were reported by one group were much milder than that of an exosome mutant (Assenholt et al., 2011, Azzouz et al., 2009a). A recent study revealed that Ccr4-Not may be required to tether misprocessed RNAs to sites of transcription (Assenholt et al., 2011). It is possible that Ccr4-Not may retain misprocessed mRNAs at the gene to prevent their export or act as a scaffold to recruit the exosome to destroy them. It's too early to tell what the connection to the exosome means, but its association with exosome subunits, the genetic interactions and the accumulation of misprocessed RNAs in Ccr4-Not, suggest there may be a role for Ccr4-Not in quality control of mRNAs.

Although there appears to be one Ccr4-Not complex in yeast that carries out all of its functions, this is not the case in metazoans. This raises the possibility that different versions of CCR4 and CAF1 perform specialized functions in mammals. New evidence suggests there may be alternative forms of Ccr4-Not dedicated to nuclear and cytoplasmic functions in more evolved species. In higher eukaryotes, there are multiple homologous genes encoding Ccr4-Not subunits (Collart and Panasenko, 2011, Wagner et al., 2007, Albert et al., 2000). For example, in humans there are two variants of CCR4, and three distant paralogs (ANGEL, 3635, and Nocturnin), while hCAF1 also has two variants but only one distant relative in mammals, hCAF1z (Albert et al., 2000, Dupressoir et al., 2001). Of those five CCR4 proteins, hCCR4a (CNOT6) and hCCR4b (CNOT6L) are most closely related to the yeast version of Ccr4, due to the presence of the signature leucine-rich repeat (LRR) motif. There is good evidence that distinct Ccr4-Not complexes can be isolated from human cell extracts (Morel et al., 2003, Wagner et al., 2007, Lau et al., 2009). A complex containing hCCR4a/b (CNOT6 and 6L), hCAF1a/b (CNOT7 and CNOT8) and CNOT2 was identified in human cell extracts that is most similar to the yeast Ccr4-Not complex (Wagner et al., 2007). In contrast, a separate complex was partially characterized, which contains

more divergent Ccr4 and Caf1 subunits, described as hCCR4d-hCAF1z. hCCR4d-hCAF1z concentrates in Cajal bodies in the nucleus and display different enzymatic activities than its cytoplasmic counterparts; the nuclear version can deadenylate RNAs and slowly degrade RNAs in 3' to 5' direction (Wagner et al., 2007). What's puzzling is that RNAs associated in Cajal bodies tend to be deadenylated RNAs, such as snRNAs and histone mRNAs (Cioce and Lamond, 2005). The authors of the study proposed that its association in Cajal bodies may prevent aberrant polyadenylation of these RNAs or that the hCCR4d-hCAF1z containing complex uses its 3' to 5' exonuclease activity to process unadenylated nuclear RNAs (Wagner et al., 2007). One has to wonder if hCCR4d-hCAF1z acquired the nuclear RNA quality control functions carried out by the combination of Ccr4-Not and the exosome in yeast. Since yeast Ccr4 only deadenylates RNAs, it must partner up with the nuclear exosome to carry out 3' to 5' digestion of unadenylated RNAs. Alternative forms of Ccr4-Not, with different enzymatic activities, may have evolved in higher eukaryotes to serve the functions of one complex in yeast.

Another possible nuclear function for Ccr4-Not, or its related complexes, may be to repair or reactivate mRNA that have undergone "over adenylation". Uncontrolled polyadenylation could lead to mRNAs with unusually long poly-A tails, interfering with their transport out of the nucleus. For example, it was recently shown that artificially extending the poly(A) tails of mRNAs by the mislocalization of cytoplasmic poly(A) binding protein (PABP) causes their retention in the nucleus (Kumar and Glaunsinger, 2010). In this regard, Ccr4-Not could prevent over extension of poly-A tails and promote transport of these repaired mRNAs out of the nucleus.

Assembly of hnRNPs and nuclear export

Ccr4-Not subunits co-purify with the heterogenous nuclear ribonucleoproteins (hnRNPs) Nab2 and Hrp1 and components of the nuclear pore complex (NPC) (Kerr et al., 2011). Interestingly, the association with hnRNPs was dependent upon the arginine methyltransferase Hmt1. This may be a significant and evolutionarily conserved interaction, because human CAF1 binds to the human homolog of Hmt1, PRMT1 (Robin-Lespinnasse et al., 2007). The interactions detected between Ccr4-Not and hnRNPs and NPC proteins by co-immunoprecipitation seem to represent a variety of combinations of individual subunits of each respective complex. For example, Nab2 and Hrp1 co-purify with Not5, Ccr4 and Caf1, but not with Not1 or Not2. Not1 and Not2 are integral subunits of the Ccr4-Not complex (Kerr et al., 2011). Likewise, Hmt1 interacts well with Ccr4 and Pop2, weakly with Not2 and poorly with Not5. The question that remains is if the association between individual subunits of these complexes indicates the existence of a defined macromolecular assembly that regulates mRNA transport or if these sub-fragments result from transient interactions that occur as Ccr4-Not escorts mRNA to the NPC after transcription.

Interestingly, the NPC complex components co-purifying with yeast Ccr4-Not are on the nuclear face of the NPC, providing further evidence for nuclear functions for Ccr4-Not. Human Ccr4-Not subunits copurify with NPC components as well, suggesting that this interaction is conserved (Lau et al., 2009). The consequences of the interaction between Ccr4-Not and NPC components are not clear, but in yeast, functional importance can be inferred based on an enhancement of the slow growth phenotypes of NPC mutants by the forced expression of Not4 (Kerr et al., 2011). The slowed growth was partially dependent on the RING domain of Not4, suggesting altered ubiquitylation of NPC or RNA export factors may contribute to the phenotype. The overexpression of Not4 in the NPC mutants also enhanced the retention of poly(A) mRNAs in the nucleus, indicating a worsening of the transport defect caused by NPC mutations (Kerr et al., 2011). Interestingly, the effect of Not4 overexpression was specific for mRNA transport, as protein transport through the nuclear pore was unaffected. However, deleting Ccr4-Not subunits did not cause an export

defect, but as pointed out by the authors of that paper, nuclear export factors are highly redundant. It is surprising enhanced growth defects were observed only when Not4 was over expressed (no obvious phenotype was observed when Ccr4 was overexpressed). It seems that overexpressing Not4 could interfere with normal NPC function by binding to RNAs. Not4 has an RNA recognition motif (RRM) that could play a role in this phenotype (Collart, 2003, Albert et al., 2002). It will be important to analyze double Ccr4-Not and NPC mutants for transport defects and develop more sensitive assays that reveal subtle changes in RNA export. It is also a formal possibility the Ccr4-Not plays a more prominent role in export under specific environmental conditions not recapitulated in that study, such as stress. It is not clear if Ccr4-Not plays a role in transport directly, but the connections to hnRNP and the NPC presents the intriguing possibility that it may pass through the NPC to transport mRNAs out of the nucleus.

Cytoplasmic functions of the Ccr4-Not complex in mRNA decay

In the last ten years the breadth of information about how genes are regulated at the level of mRNA decay has exploded. The identification of factors that target mRNAs for degradation and the signals that control them caused the field to grow immensely. Furthermore, the discovery of novel small RNA regulatory pathways (miRNA, RNAi) that utilize mRNA decay factors to exert their control over gene expression intensified the search for mechanisms. Relevant to this review, the convergence of decay pathways with transcription and translation control provides a fascinating new connection between processes once thought to be distinct and separate. In yeast, the decay of mRNA generally begins with deadenylation, which involves collaboration between Ccr4 and Pan2/Pan3 (Brown et al., 1996, Dupressoir et al., 2001, Tucker et al., 2001, Tucker et al., 2002, Yamashita et al., 2005, Chen et al., 2002, Chen and Shyu, 2011). The removal of the poly(A) tail disrupts the circularization of the mRNA and deprotection of the 5' cap, which is then removed by the decapping factors Dcp1/Dcp2, and finally the RNA is degraded from the 5' end by the 5' to 3' exonuclease, Xrn1 (Steiger et al., 2003, Wang et al., 2002, Houseley et al., 2006). Since deadenylation is considered the first step in mRNA decay, the activity of cellular deadenylases play the major role in regulating the degradation of mRNAs. Furthermore, it is clear that mRNAs are targeted for degradation by specific classes of RNA binding proteins that recognize 3' untranslated regions (3' UTRs) of the RNA.

Ccr4-Not is vital to the regulation of mRNA decay in the cytoplasm

It has been argued Ccr4, Caf1 and the related deadenylase Pan2/3, work together to complete deadenylation of mRNAs in yeast (Tucker et al., 2001, Brown et al., 1996, Chen and Shyu, 2011). The collaboration between Ccr4/Caf1 and Pan2/3 has been attributed to biphasic mRNA decay, where Pan2/3 initiates the decay of the message and Ccr4-Not completes the process. One thought is that partially removing the poly(A) tail using Pan2/3 would inhibit translation, but the remaining tail is sufficient to localize RNAs in the cell or prevent their degradation. This would then allow polyA polymerase to re-extend the tails and salvage these RNAs (Chen and Shyu, 2011). Under stressful conditions, or when the RNAs must be degraded, Ccr4-Not is activated to remove the entire tail, completing the act of degradation. Application of a biphasic mechanism would allow for tighter regulation between translation inhibition and decay.

The Ccr4-Not complex contains two potential deadenylases, Ccr4 and Caf1, which are required for mRNA degradation *in vivo* (Benoit et al., 2005, Tucker et al., 2002, Tucker et al., 2001, Denis and Chen, 2003). Based upon sequence homology, enzymatic activity, and a crystal structure, Ccr4 was placed in the exonuclease-endonuclease-phosphatase or EEP family of deadenylases (Draper et al., 1994, Wang et al., 2010); whereas Caf1 is a member of the DEDD-type family of nucleases (Bianchin et al., 2005, Dauteron et al., 2001, Thore

et al., 2003). Ccr4 displays strong specificity for poly(A) sequences whereas Caf1 prefers poly(A) only slightly over poly(U) or poly(C) (Chen et al., 2002, Thore et al., 2003). Since Caf1 and Ccr4 have different substrate preferences, they may act on different sequences and carry out distinct functions.

The advantage of having two deadenylases (Ccr4 and Caf1) in the Ccr4-Not complex is not clear, and there appears to be species differences in the relative contribution of these two subunits to deadenylation. In the case of yeast, there is competing evidence about whether or not Caf1 has enzymatic activity. In one case, recombinant Caf1 displayed weak deadenylase activity *in vitro* and could degrade other homopolymeric tracts, despite the fact that it lacks key residues involved nuclease activity (Thore et al., 2003). Caf1 immunopurified from wild type cell extracts displayed robust deadenylase activity *in vitro*, but the same purifications from a *ccr4* Δ cell lacked activity, suggesting the deadenylase activity resulted from Ccr4 co-purifying with Caf1 (Goldstrohm et al., 2007, Tucker et al., 2002). Furthermore, *caf1* point mutations that were shown to inhibit its *in vitro* nuclease activity of the protein (Thore et al., 2003), complemented the slow growth and mRNA decay defects of a *CAF1* deletion strain (Viswanathan et al., 2004). All of these observations suggest that yeast Caf1 lacks the deadenylase activity of its metazoan homologues. In yeast, its main function may be to maintain the integrity of the complex or to recruit Ccr4-Not to mRNAs by binding sequence specific RNA binding proteins (Collart, 2003, Goldstrohm et al., 2006, Goldstrohm et al., 2007). If yeast Caf1 has nuclease activity, it may not be a canonical deadenylase. It may degrade or process a variety of sequences weakly. As discussed above, an isoform of human Caf1, hCAF1z, may process the ends of non-adenylated RNAs in the nucleus, and yeast Caf1 may do the same (Wagner et al., 2007).

On the other hand, CAF1 from metazoans contain the conserved catalytic residues missing in the yeast version (Moser et al., 1997), and consistently, there is more conclusive evidence that it is a bona fide deadenylase. Deadenylase activity has been observed for fly, frog, mouse and human CAF1 (Temme et al., 2010, Cooke et al., 2010, Viswanathan et al., 2004, Wagner et al., 2007). Moreover, there is evidence that CAF1 is even more active in deadenylation than CCR4 in metazoans (Wagner et al., 2007, Temme et al., 2010). In flies, knockdown of CAF1, NOT1, NOT2, and NOT3 impaired deadenylation of mRNAs, while knockdown of CCR4 did not (Temme et al., 2010). This suggests a shift in responsibilities of CAF1 and CCR4 throughout evolution. An interesting question is if yeast Caf1 lost its strong deadenylase activity, or if metazoans homologues gained this function.

Ccr4-Not can be localized to processing bodies in response to stress

In yeast, mRNAs that undergo inhibition of translation or degradation localize in cytoplasmic foci called processing bodies or P-bodies (Garneau et al., 2007, Sheth and Parker, 2003, Teixeira and Parker, 2007, Collier and Parker, 2005). P-bodies can accumulate under stress such as nutrient deprivation, diauxic shift, or high salt (Garneau et al., 2007, Sheth and Parker, 2003, Teixeira and Parker, 2007, Collier and Parker, 2005). Ccr4-Not subunits localize to P-bodies in stressed yeast and metazoan cells (Teixeira and Parker, 2007, Lin et al., 2008, Temme et al., 2010). It is still unclear if the localization of Ccr4-Not to P-bodies is important since there is evidence that P-body formation is not required for mRNA decay, and Ccr4 and Pop2 do not localize to P-bodies in unstressed cells when decay is ongoing (Teixeira and Parker, 2007, Stalder and Muhlemann, 2009, Eulalio et al., 2007). While Ccr4-Not subunits can be recruited to P-bodies under stress (Figure 1), the deletion of *CCR4* or *POP2* only causes a minor reduction in the formation of P-bodies, suggesting they are recruited to these foci, but they are not essential for foci formation (Teixeira and Parker, 2007). The localization of Ccr4-Not subunits to these foci seems to be triggered by extreme stress or by deleting components of the mRNA degradation machinery such as Dcp1, Dcp2 or Xrn1 that accumulate mRNAs (Teixeira and Parker, 2007). It has been hypothesized that

the main function of P-bodies is to remove mRNAs from the translatable pool until the stress is removed (Teixeira et al., 2005). Under extreme conditions the cell may sense that the current pool of mRNAs are unsalvageable, and thus, signals the recruitment of Ccr4-Not to these sites to degrade the mRNAs and prevent extensively damaged mRNAs from re-entering the translatable pool. Many mRNA decay factors such as decapping enzyme and activators of decapping are localized to P-bodies, yet the RNA do not appear to be completely degraded (Teixeira and Parker, 2007). As discussed above, the canonical pathway for the degradation of an mRNA begins with deadenylation of the mRNA, which results in the exposure of the 5' capping to decapping factors (reviewed in (Garneau et al., 2007)). Stress signals may stimulate the recruitment of Ccr4-Not into P-bodies to trigger mRNAs for degradation.

Ccr4-Not is likely to be recruited to these foci through the association with P-body components, such as Dhh1, Pat1, and cytoplasmic Lsm proteins, because the recruitment of Pop2 and Ccr4 to P-bodies is dependent upon these proteins (Teixeira and Parker, 2007, Sheth and Parker, 2003). Furthermore, a physical interaction between the Ccr4-Not complex, Dhh1 and Pat1 has been described (Hata et al., 1998, Nissan et al., 2010, Maillet and Collart, 2002). Dhh1 is a DEAD-box containing protein implicated in multiple functions in mRNA regulation and it, and its metazoan orthologues, play a critical role in regulating the shift between decay and translation of mRNA (Coller and Parker, 2005, Minshall and Standart, 2004, Weston and Sommerville, 2006). The ATPase activity of the protein appears to regulate the shuttling of mRNAs in and out of P-bodies (Dutta et al., 2011, Carroll et al., 2011). Thus, Dhh1 is a good candidate to regulate the balance between repression and decay through an interaction with Ccr4-Not.

There are hypotheses that Ccr4-Not inhibits translation (Coller and Parker, 2005, Cooke et al., 2010, Chekulaeva et al., 2011). This is most likely due to its ability to deadenylate mRNAs, especially in the miRNA pathway in mammals (Fabian et al., 2011, Chekulaeva et al., 2011). However, there is evidence that *Xenopus* Ccr4 and CAF1 (xCaf1) can inhibit translation of mRNAs independent of deadenylation in cell extracts (Cooke et al., 2010). It is not clear how xCAF1 would inhibit translation of mRNAs without affecting deadenylation. It may help recruit mRNAs into cytoplasmic foci where other factors directly prevent them from being translated, eg. the DEAD box protein Xp54 (orthologue of yDhh1). The system used to describe CCR4- and CAF1-dependent repression relied on the tethering of the proteins to reporter RNAs using MS2 RNA binding protein fusion strategy. This system is widely used to study translation and decay, but it will be important to confirm that xCAF1 can repress natural mRNAs *in vivo*. Additionally, it would also be fruitful to perform *in vitro* assays using purified components to show that Ccr4 and Caf1 can inhibit translation in the absence of other regulatory factors. As described above, Ccr4-Not associates with many proteins with known translation repressing activities, such as Dhh1 homologues, and the tethered CAF1 could bring these proteins to the RNA to repress translation.

RNA binding proteins target Ccr4-Not to mRNAs to cause deadenylation

It is likely that Ccr4-Not plays a housekeeping function, but the more exciting possibility is that it is directed to specific mRNAs under certain physiological conditions. Numerous mRNA binding proteins, which usually recognize the 3' UTRs, have been implicated in mRNA decay and the most extensively studied in yeast are the pumilio fem binding proteins (PUF1-5). Goldstrohm and Wickens provided the first evidence that Puf5, also called Mpt5, recruits the Ccr4-Not complex to specific mRNAs to mediate their decay. After identifying a genetic interaction between members of the Ccr4-Not complex and Puf5p, they demonstrated that the decay of *HO* mRNA by Puf5 was dependent upon *CCR4* and *CAF1* (Goldstrohm et al., 2006). The mechanism was partially revealed when they discovered that

Puf5 physically interacts with subunits of the Ccr4-Not complex *in vivo* and *in vitro*. The interaction occurred specifically through Caf1. Using an elegant biochemical system, the same group demonstrated PUF proteins can promote Ccr4-Not-dependent deadenylation of an *HO* mRNA using purified components (Goldstrohm et al., 2007). This study also provided biochemical and genetic evidence that strongly suggests that the role of Caf1 is to recruit Ccr4 (within the complex) to promote deadenylation and not catalyze deadenylation itself. The requirement for Caf1 to recruit Ccr4 to mRNAs via PUF proteins provides a logical explanation for why both *CCR4* and *CAF1* are required for deadenylation *in vivo*, yet Caf1 lacks robust deadenylation activity.

Stress is a major determinant regulating mRNA decay. Ccr4-Not has been implicated in many stress responses (for review see (Collart and Panasenko, 2011, Collart and Timmers, 2004)). Likewise, deletion of PUF genes causes sensitivity to stress (Ohkuni et al., 2006), and *PUF5* and *CAF1* act in the same pathway to regulate cell wall integrity (Traven et al., 2010). Interestingly, Ccr4-Not and *PUF5* mutants share a number of common phenotypes, but not all functions of Ccr4-Not are mediated through *PUF5*. For example, genetic screens have shown that *CAF1* and *CCR4* have roles in regulating DNA replication that are independent of *PUF5* (Traven et al., 2010). This is likely due to the targeting of Ccr4-Not to mRNAs by other RNA binding proteins or redundancy among the PUF proteins themselves. For example, RNA immunoprecipitation-microarray analyses (RIP-chips) performed on the PUF family of proteins found that multiple PUF family members target the same mRNA (Gerber et al., 2004a, Hogan et al., 2008). Furthermore, subsequent studies implicated other members of the pumilo family of RNA binding proteins in mediating the decay of specific messages through the Ccr4-Not pathway (Lee et al., 2010). It will be important to define which mRNAs are targeted by both the PUF proteins and Ccr4-Not to understand how each group of proteins contribute to RNA metabolism, especially when cells are stressed.

RNA binding proteins play an important role in directing Ccr4-Not to specific mRNAs to regulate development, signaling and stress responses in flies and humans. *Drosophila* Ccr4 homologues are vital players in regulating the poly(A) tail length of many developmentally important mRNAs too, such as *osk*, *Smg*, *cyc-B*, and *nos* transcripts (Zaessinger et al., 2006, Chicoine et al., 2007). The regulation of these messages involves the recruitment of Ccr4-Not to their 3'UTRs. For example, *Drosophila* Ccr4-Not is recruited to specific mRNAs during early development by the RNA binding proteins Smaug (*Smg*) and Bic-C, to accelerate the decay of mRNAs (Zaessinger et al., 2006, Chicoine et al., 2007). Human Tristetraprolin (TTP) recruits hCcr4-Not to RNAs by binding AU-rich elements in the 3' UTR, and recruitment involves an interaction between TTP and the Not1 subunit (Sandler et al., 2011). Interestingly, the decay of mRNA by TTP is inhibited by the activation of MAP and CAKII kinase pathways (Marchese et al., 2010, Lee et al., 2010); thus, providing an example where Ccr4-Not-mediated decay is regulated by cellular signals (see also below). TTP regulates the decay of many inflammatory, hypoxia and oncogene signaling pathway mRNAs (Sanduja et al., 2011), so understanding the mechanism of how it promotes mRNA decay through Ccr4-Not is of great importance.

A picture is emerging where sequence specific RNA binding proteins, such as PUF proteins in yeast, and Ccr4-Not work together to regulate mRNA turnover during stress in cells (Figure 1). Important questions remain to be answered about this collaboration. For example, does stress first increase the binding of PUF proteins to mRNAs, which then recruit Ccr4-Not? Or are PUF proteins constitutively bound to these RNAs, and stress results in Ccr4-Not recruitment? Finally, RNAs could be bound by PUFs and Ccr4-Not, awaiting activation by cellular signals to initiate decay. Furthermore, are mRNAs marked for decay in the nucleus and do sequence specific RNA binding proteins retain Ccr4-Not onto mRNAs after transcription terminates? These questions are not trivial to address, but it

will start with the identification of the mRNAs bound by Ccr4-Not in the cell under resting and stressed conditions. Since the prevailing models have Ccr4-Not recruited to mRNAs via other proteins (e.g. Puf5), these studies may require crosslinking the complex to the mRNAs to maintain the association of the complex with the mRNAs.

Regulation of the complex by cellular signals and differential expression

There must be a signal to recruit Ccr4-Not to cytoplasmic foci and regulate its other activities. Several reports suggest that signaling pathways regulate the composition or activity of the complex, which is an attractive mechanism for controlling its different functions in cells. For example, the levels of Not1p were reduced and the stoichiometry of other Ccr4-Not subunits changed when yeast cells were shifted from glucose to a non-fermentable carbon source (Norbeck, 2008). Since Not1 serves as the scaffold of the complex, this would lead to lower levels of the intact complex and generation of subcomplexes, which could have activities of their own (see section on ubiquitylation below).

There is evidence that the size and composition of human Ccr4-Not changes during different stages of the cell cycle too (Morel et al., 2003). However, the subunits contained in the different forms were not identified and the cell cycle dependent changes in composition were not correlated with an alteration in Ccr4-Not function, such as transcription or mRNA decay. One of the more interesting results of this study is that the localization of CAF1 changed from the cytoplasm to the nucleus during cell cycle progression, which raises the exciting possibility that cell cycle dependent changes in subunit composition affects the cellular localization of the complex.

Mammals have two paralogues of CCR4 (CNOT6 and CNOT6L) and CAF1 (CNOT7 and CNOT8) subunits, which would allow for the presence of different versions of the Ccr4-Not complex in higher eukaryotic cells. Distinct forms of the Ccr4-Not complex have been detected in human and flies by gel filtration, immunoprecipitation and mass spectrometry (Lau et al., 2009, Temme et al., 2010, Wagner et al., 2007). These different versions of the complex were observed in the same cell type, but diversity of Ccr4-Not complexes may also be generated by cell-type specific expression of the subunits. Chen et al., 2011 looked at the expression of mouse Ccr4-Not subunits in different tissues including, but not limited to, different parts of the brain (Chen et al., 2011). They found that the level of all Ccr4-Not subunits examined varied between different types of brain cells, and strikingly, that CNOT2, 3, 6, and 8 levels decreased during embryonic development in brain tissues. Moreover, they analyzed the expression of subunits in a mouse neuronal stem cell line and described a decrease in CNOT2, 3, 6, and 8 when they had undergone differentiation into neuronal cells. On the other hand, the amount of the other subunits remained fairly stable, including CNOT6L and CNOT7, the paralogues of CNOT6 and CNOT8, respectively. Thus, there is a shift in the amount of complexes containing the different deadenylases associated with Ccr4-Not during differentiation and brain development. The significance of these changes in mammalian development is not clear yet, but it establishes the idea that the alternative forms of Ccr4-Not described in metazoan cells may have unique functions.

Finally, there is evidence that yeast Caf1, Not3, Not5, Not4, and Not1 are phosphorylated (Lenssen et al., 2002, Moriya et al., 2001, Lau et al., 2010, Collart and Panasenko, 2011). The phosphorylation status of some subunits changed under different conditions, usually stress, and correlated with alterations in subunit composition, localization or activity. In the case of Not4, the residues that are phosphorylated during stress were mapped by mass spectrometry, and a mutant containing alanine substitutions in all five residues was analyzed (Lau et al., 2010). This mutant displayed a significantly milder sensitivity to stress compared to the *not4Δ* mutant and no reductions in H3K4me3 levels, two phenotypes of the *not4Δ*

null mutant (see above). It is unclear if the mild stress sensitivity is caused by a loss of phosphorylation or by structural changes caused by five mutations. On the other hand, a strong case for a physiological significance of Ccr4-Not modification came from studies on Caf1. Yak1 kinase phosphorylates Caf1 on T97 during glucose deprivation or postdiauxic shift and mutating this residue to an alanine renders the cells unable to undergo cell cycle arrest under these conditions (Moriya et al., 2001). This suggests that phosphorylating Caf1 is important for its function. Interestingly, since Yak1 is transported to the nucleus under these conditions, this suggests that it is modifying a nuclear pool of Caf1. Furthermore, as discussed above, the composition of Ccr4-Not changes when cells are depleted of glucose (Norbeck, 2008). It is possible that modification of Caf1 plays a role in the changes in composition of the complex by affecting the integrity of the complex or stability of the Not1 subunit. Caf1 is a crucial integral subunit of the complex (Collart, 2003, Denis and Chen, 2003).

Ccr4-Not regulates protein turnover and translational quality control

Ubiquitylation of a protein can be a mark for targeted degradation or localization. The conjugation of ubiquitin to a protein substrate requires multiple enzymatic steps. Firstly, the ubiquitin molecule is attached to a member of the E1 ubiquitin activating enzyme in an ATP-dependent manner. It is then activated by one of the many E2 ubiquitin conjugating enzymes, and finally attached to its protein substrate via a E3 ubiquitin ligase (Hochstrasser, 1996). Not4 is a RING E3 ubiquitin ligase and its activity has been verified using biochemical assays (Mersman et al., 2009, Winkler et al., 2004, Mulder et al., 2007b). Ubiquitin ligase activity was observed for both yeast Not4 and human CNOT4, indicating this function is highly conserved. The E2 enzyme that functions with CNOT4 is UbcH5B and for Not4 is Ubc4 and Ubc5 (Winkler et al., 2004, Mulder et al., 2007b). It is not clear if Not4 can function with other E2 enzymes, but it appears that its major partner is Ubc4 and Ubc5. Only a few substrates of Not4 are known, but we will discuss some of these and what this tells us about the function of Ccr4-Not in ubiquitylation of proteins.

Not4 regulates nascent poly-peptide associated complex (NAC/EGD)

The function of NAC (called EGD) is somewhat controversial, making its connection to Not4 that much more interesting, or questionable depending on one's point of view. Although EGD/NAC was initially identified as a possible transcription coactivator, further work on the complex suggests its main function is protecting ribosome-bound nascent polypeptides (reviewed in (Rospert et al., 2002). In yeast, the EGD/NAC consists of Egd1p (β NAC) and Egd2p (α NAC), which heterodimerize (Reimann et al., 1999, Spreter et al., 2005). The heterodimer was shown to dock on ribosomal subunit L25, which is close to the nascent peptide exit tunnel of the ribosome (Grallath et al., 2006, Wegrzyn et al., 2006).

It turns out that several subunits of the Ccr4-Not complex physically interact with NAC (Panasencko et al., 2006). Ubiquitylation of Egd1 and Egd2 after glucose deprivation required Not4 and other Ccr4-Not subunits (Panasencko et al., 2006). In contrast, others reported that the ubiquitylation of Egd2 (Egd1 was not examined) is independent of Not4, and instead pointed to the E3 ligase Rsp5 (Hiraishi et al., 2009). While the opposing reports may suggest controversy, there may be a logical explanation for the difference that reveals something about the regulation of NAC. The two conflicting studies used different stresses. The Collart group showed *NOT4*-dependent ubiquitylation of Egd1/2 when glucose was depleted, while the Takagi group found that Not4 was dispensable for Egd2 ubiquitylation during heat-induced stress. Genetic evidence and gene expression profiling of Ccr4-Not mutants strongly suggests it is important in assisting cells adjust to changes in carbon source (Cui et al., 2008, Denis, 1984). As discussed above, the composition of Ccr4-Not and the phosphorylation of some subunits change during the transition between carbon sources (Lau

et al., 2010, Norbeck, 2008). Thus, the idea that NAC is ubiquitylated by Not4 during carbon source stress, but other E3 ligases under heat stress, is probable. This issue could be addressed by comparing the requirement of Not4 and other E3 ligases during a variety of stresses.

Assuming that Ccr4-Not regulates NAC, the major question is why? This is difficult to answer because, as stated before, the purpose of NAC is controversial. EGD subunit ubiquitylation appears to regulate the binding of Egd1 to Rpl25p in the ribosome: the ubiquitylated forms bind (Panassenko et al., 2009). Recently, it's been reported that the NAC is important for proper targeting of signal recognition particle (SRP) to cognate substrates and disfavoring of noncognate substrates (del Alamo et al., 2011). It hasn't been tested, but maybe the purpose of NAC ubiquitylation is to assure that SRP is properly engaging its targets in stressful environments.

Translational quality control during stress

Not4 has also been implicated in the degradation of arrested translation products. When the translation of an actively synthesized protein is arrested, it may be targeted for degradation by the proteasome. Arrest can be stimulated by tracts of basic amino acids such as lysine, which is encoded by poly(A) sequences in mRNAs (Dimitrova et al., 2009). Deleting *NOT4* or mutating the RING domain stabilized arrested translation products (Dimitrova et al., 2009). Interestingly, arrested products were still degraded in *not3Δ* and *not5Δ* mutants, which may suggest that the entire Ccr4-Not complex is not required to ubiquitylate and destroy arrested translation products. However, *not3Δ* mutants have no detectable phenotypes and additional mutants would need to be analyzed to confirm this conclusion. Since the lysine tract is encoded for by a poly(A) sequence in the RNA, it was a formal possibility that the effect is caused by altered RNA stability. However, the authors of that paper ruled this mechanism out, indicating it was mediated at the level of protein destruction. Consistent with its role in co-translational degradation, Not4 was detected in polysomal fractions inside of the cell, although it should be noted that Not4 was overexpressed from an *ADHI* promoter (Dimitrova et al., 2009). Furthermore, it is unclear if Not4 is associated with polysomes only to mark proteins for degradation during translation because a recent study reports that mRNA decay occurs on polysomes (Hu et al., 2009). Nonetheless, evidence is building that Not4 uses its E3 ligase activity to mark translational arrest products for degradation by the proteasome (Figure 2). Interestingly, Not4 has an RNA recognition motif (RRM), and it would be interesting to know if this is required for its association with the polysome fraction or the ribosome. Since mRNA stability and translation are intertwined, one Ccr4-Not complex bound to the mRNA could regulate two distinct steps in gene regulation simultaneously. For example, after deadenylation of the mRNA using Ccr4, the complex could then turn to Not4 to ubiquitylate the protein being translated from that mRNA undergoing degradation to prevent the accumulation of aberrant proteins (Figure 2).

Ccr4-Not complex and proteasome integrity

The 26S proteasome is responsible for degrading short-lived proteins that have been polyubiquitylated (Hochstrasser, 1996). Polyubiquitylation marks proteins for degradation, which is important for various functions such as the cell cycle progression, DNA repair, and protein quality control. This 26S complex is made up of two subcomplexes: a catalytically active 20S core particle (CP) and 19S regulatory particle (RP) that binds one or both ends of the CP (Murata et al., 2009). Activation of the CP is contingent upon the RP, which acts as a lid or gate, and is regulated by several chaperones or factors (Panassenko and Collart, 2011).

There is evidence that Ccr4-Not is required for the assembly or stability of the 26S proteasome (Panasencko and Collart, 2011). Deletion of *NOT4* leads to accumulation of ubiquitylated proteins, altered ubiquitin homeostasis, and destabilization of the 26S proteasome (Panasencko and Collart, 2011). To address why this occurs, Collart's group turned to studying the proteasome chaperone, Ecm29, which influences the assembly of the CP and RP to form the 26S proteasome (Panasencko and Collart, 2011). They described physical, genetic, and functional interactions between Ecm29 and Not4 (Panasencko and Collart, 2011). Ecm29 is polyubiquitylated and destroyed in *not4Δ* cells, indicating that proteasome is partly mediated through the control of Ecm29p levels. Although physical interactions between the principal players were described, the phenotypes could be attributed to global changes in cellular ubiquitylation because *not4Δ* cells accumulate polyubiquitylated proteins and free ubiquitin. Since the connection between Not4 and proteasome assembly is relatively new, naturally our understanding of the process is not deep.

Functions of Not4-dependent ubiquitylation in the nucleus

Ccr4-Not is present in both the nucleus and cytoplasm, and Not4 has both nuclear and cytoplasmic targets. As we already discussed, Not4 regulates H3K4me3 through the degradation of histone demethylase Jhd2 and this is dependent on the RING domain of Not4 (Mersman et al., 2009). It is believed that the ubiquitylation of Jhd2 occurs in the nucleus, but it should be noted that the protein is detected in the cytoplasm too (Huh et al., 2003). Furthermore, another nuclear substrate of Not4 was recently identified, Cdc17, the catalytic subunit of pol- α in yeast (Haworth et al., 2010).

As previously discussed, the NOT subunits of Ccr4-Not complex were discovered because of their ability to regulate transcription, especially initiation. This raises the question if the ubiquitylation activity of Not4 is required for its transcription functions. However, so far there is no evidence that the ubiquitylation function is required for transcription per se; in fact there is evidence to the contrary. Ccr4-Not is required for normal resistance to hydroxyurea and for expression of the ribonucleotide reductase (RNR) genes (Mulder et al., 2007b, Traven et al., 2010, Traven et al., 2005). The RNR genes are one of the best-characterized Ccr4-Not dependent genes, and deleting *NOT4* strongly reduced the activation of these genes by DNA damage-induced stress. (Mulder, 2005, Kruk 2011). In contrast, the *NOT4*RING domain mutant (L35A) showed no defect in the DNA damage induced expression of the RNR genes (Mulder et al., 2007). Likewise, the *not4L35A* mutant showed no changes in the expression of heat shock genes, which are hyperactivated in the *not4Δ* allele (Mulder et al., 2007). It is hard to draw conclusions based on only two classes of genes, but a picture is emerging where the ubiquitylation function of Not4 is not required for all of its functions, including transcription. Phenotypic analysis of a RING domain mutant of *NOT4* (L35A) revealed that it has less severe growth defects, milder stress phenotypes and weaker genetic interactions with NPC mutants than the null mutant (Mulder et al., 2007). Clearly, *NOT4* plays broader roles in controlling gene expression than just ubiquitylating proteins. It would be interesting to test if the RING domain is required for other functions of Not4 such as elongation or mRNA decay. In general, there is still a lot that could be learned about the Ccr4-Not complex from examining mutants inactivating the specific functions of the complex (ubiquitylation, deadenylation, RNA binding, transport).

It is expected that Not4 ubiquitylates proteins within the context of the Ccr4-Not complex. But is this really the case? Many of the phenotypes observed in a *not4Δ* mutant related to protein ubiquitylation (Egd1/2 ubiquitylation, reduced histone H3 methylation) are observed when other complex subunits are deleted or mutated, suggesting this is true. Purified Ccr4-Not complex can substitute for recombinant Not4 in ubiquitylation reactions, displaying strong auto-ubiquitylation activity, but it has not been tested if the Ccr4-Not complex

(versus recombinant Not4) can ubiquitylate a natural substrate *in trans* (Mulder et al., 2007). The Ccr4-Not complex is well conserved across eukaryotes, but purification of human and *Drosophila* Ccr4-Not showed that Not4 is not a stable subunit of metazoan complexes (Temme et al., 2010, Lau et al., 2009). In fact, hNot4 mostly exists in a smaller complex in human cell extracts (Lau et al., 2009). This raises the possibility that Not4 evolved to be separate from the rest of the Ccr4-Not complex. Even in yeast, where it is clear Not4 is a stable stoichiometric subunit of the complex, its function outside of the context of the complex is possible under some conditions. For example, shifting cells to a non-fermentable carbon source or growing cell to the diauxtic shift leads to the destruction of Not1, the scaffold of the complex (Norbeck, 2008). Removing the scaffold could liberate Not4 from the other subunits of the complex where it could then ubiquitylate substrates. It should be noted that Not4 ubiquitylated Egd1/2 when cells were starved for glucose, a condition that reduced Not1 levels in cells (Panasenko et al., 2009, Panasenko et al., 2006). This suggests Not4 (may be together with a subset of Not proteins) can ubiquitylate proteins independent of the Ccr4-Not complex.

Ccr4-Not: can it do it all? If so, why?

When it was discovered that Ccr4-Not regulates deadenylation, this opened up the possibility that the complex would be involved in multiple steps in gene regulation. Little did we know what would be learned about the complex since that time. Connections, some more solid than others, to nuclear quality control, export, protein degradation and translational control have emerged. But can one complex do it all? It seems unlikely. The next step is to weed out what effects are direct and which are indirect.

In 2001, Parker and colleagues theorized that certain mRNAs were “marked” for degradation in the nucleus during transcription. This hypothesis was put forth to unify the two known functions of Ccr4-Not at that time, transcription and deadenylation. Predetermining mRNAs for degradation at the synthesis step implies that Ccr4-Not would be loaded onto the mRNA during transcription and escort the mRNA from the gene, to the nuclear pore and out into the cytoplasm (Figure 3). This still remains to be proven. However, a case for this model and the challenges facing it are described below.

A major question is why would a factor evolve and be maintained through millions of years that both increases and decreases gene products? The simplest explanation is that it promotes the expression of some gene products, but degrades others. This might be true to some extent, but the story is almost certainly more interesting. Since there is some evidence that production and decay of mRNAs are coordinated, and Ccr4-Not/Rbp4-7 has been implicated in this, the idea that mRNAs are marked for degradation during transcription is very plausible. The hypothesis that Ccr4-Not is recruited to mRNA during transcription is fully consistent with it tracking with RNAPII across the ORFs of genes. Ccr4-Not binds to RNAPII and must be transferred to the mRNA after transcription terminates. Does this process occur by the binding of Ccr4-Not to the RNA non-specifically, or is it retained on the 3' UTR through nuclear RNA binding proteins? In decay, PUF proteins are one class of proteins that recruits Ccr4-Not to mRNAs for destruction. PUF proteins are not known to be nuclear, but this has not been examined carefully. Transcription near the nuclear pore may place Ccr4-Not and the transcript near the exit site to leave the nucleus. In this case Ccr4-Not can either escort the mRNA out of the nucleus or hand it off to another complex awaiting it in the cytoplasm. This would explain why subunits of Ccr4-Not complex bind to fragments of the NPC. Even if this model is correct, how are mRNAs selected? The number of RNAPII molecules outnumbers those of Ccr4-Not ~4-5:1, and Ccr4-Not is distributed in the nucleus and cytoplasm. This stoichiometry “problem” is not unique to Ccr4-Not, however, and other elongation factors and mRNA transport factor are present at lower numbers than RNAPII.

Once out in the cytoplasm, many fates await an mRNA bound by Ccr4-Not (Figure 3). The RNAs can be translated, destroyed or recruited into cytoplasmic foci. If Ccr4-Not is bound to mRNAs, why aren't they immediately degraded? What is the signal that distributes it between the translatable pool and those to be degraded? Certainly signals of some kind are required. As described above, this may be stress or developmental cues. The basic question if Ccr4-Not is constitutively bound to mRNA awaiting a signal to degrade it or if it is only recruited when they need to be degraded has not been answered. Keeping with this theme, Ccr4-Not may get to the ribosome on the mRNA, where it could then exert quality control mechanisms through NAC, ubiquitylation of arrested translation products and proteasome recruitment.

One common thread in all the proposed functions of Ccr4-Not is its involvement in stress pathways and the impact of stress on its activity. Stress signals are transient in nature, where a rapid induction phase is followed by a slow diminishment of the response. Being at the center of the action from transcription to decay (mRNA or protein) would allow gene expression changes at all levels to adapt quickly in response to a rapidly changing environment. Additionally, many developmental queues are transient in order to properly regulate the formation of anatomical features. Similarly, the Ccr4-Not complex is expressed in a specific spatial and temporal manner in response to developmental queues in some cells. Many experiments that have been performed commonly knockdown or knockout entire genes, so subtleties in regulation may be overlooked. It will be interesting in the future to use methods that can tease out each of the complexes' functions during a synchronized stress response. In conclusion, studying the Ccr4-Not complex has been a very fruitful endeavor since it has not only provided an understanding of the molecular mechanisms regulating gene expression, but also caused scientists to re-evaluate the simple idea that factors either promote the synthesis or decay of gene products. Because of these discoveries we believe interest in the Ccr4-Not complex will continue to intensify.

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Figure 1. A general model for Ccr4-Not mediated translational repression and mRNA decay
 (1) mRNA is being actively translated by ribosomes. (2) Ccr4-Not is recruited to the 3' UTR via RNA binding proteins (RBPs), Puf5 is shown in this figure. Pan2/3 initiates removal of the poly(A) tail, disrupting the association with the 5' and 3' ends of the mRNA and preventing new rounds of ribosome binding. (3) Upon stress, the Ccr4-Not complex enhances deadenylation to begin the process of removing the mRNA from the translational pool. (4) After deadenylation other RBPs and decay factors will attach to the 3' UTR, directing the complex to P-bodies. (5) Once associated with a P-body, the mRNA will likely be decapped and completely degraded by the 5' to 3' exonuclease. However it could also be stored until the stress is removed, in which case the mRNA will then move back into the translational pool of mRNAs. Color version of this figure can be found online.

Figure 2. A model for how Ccr4-Not regulates arrested translational products

(1) A ribosome travels along an mRNA until it is arrested by a stretch of A's that code for several basic lysine residues or by damage to the RNA. (2) Ccr4-Not removes the poly(A) tail to begin the destruction of the mRNA using Ccr4, and recruits the E2 ubiquitin conjugating enzyme Ubc4/5 through Not4 to form a functional E2-E3 ligase pair. The emerging polypeptide is polyubiquitylated (3) The 26S proteasome is recruited to the mRNA to degrade the arrested product and strip the arrested ribosomes off the RNA so that degradation can continue. Color version of this figure can be found online.

Figure 3. Ccr4-Not regulates gene products from “birth to death”

Ccr4-Not interacts with TBP-containing promoter recognition complexes TFIID or SAGA to regulate initiation and preinitiation complex assembly. Ccr4-Not binds to the 4/7 module (green appendage) of RNAPII and tracks with it into the body gene. Ccr4-Not then associates with the emerging transcript to promote elongation. During the process of termination and RNAPII release, Ccr4-Not remains associated with the transcript and directs it to the nuclear pore complex (NPC) where it can either pass it off to another complex awaiting in the cytoplasm (left) or escort it through the nuclear pore (right). Once in the cytoplasm, the RNA-protein complex is directed to translation, decay (deadenylation) or translational repression. Proteins are regulated by the ubiquitin ligase activity of Not4 within the complex, which can impact translation quality control in the cytoplasm (Figure 2) or histone methylation levels by destroying the histone demethylase Jhd2 in the nucleus. Color version of this figure can be found online.

Table 1
Orthologues and Homologues of the Ccr4-Not complex

Subunits of the Ccr4-Not complex are listed with their associated orthologues in *Saccharomyces cerevisiae* (*Sc*), *Drosophila melanogaster* (*Dm*), *Mus musculus* (*Mm*), and *Homo sapiens* (*Hs*). The protein families of several of the subunits are also shown including exonuclease-endonuclease-phosphatase or EEP, DEDD (Asp-Glu-Asp-Asp), and the DEAD-box (Asp-Glu-Ala-Asp) helicase family.

<i>Sc</i>	<i>Dm</i>	<i>Mm</i>	<i>Hs</i>	Protein Family/Domains
Not1	Not1	CNOT1	CNOT1	
Not2	Not2	CNOT2	CNOT2	
Not3	Not3	CNOT3	CNOT3	
Not4	Not4	CNOT4	CNOT4	RING E3 Ligase
Not5 [*]				
Ccr4	twin/CCR4	CCR4	CNOT6/CCR4a	EEP, LRR
		CCR4L/CNOT6l	CNOT6L/CCR4b	EEP, LRR
Pop2/Caf1	POP2	CNOT7	CNOT7/Caf1a/hCaf1	DEDD
		CNOT8	CNOT8/Caf1b/hPop2/CALIF	DEDD
Caf40	CAF40		CNOT9/hRcd1	
Caf130			CNOT10	
Dhh1 ⁺	Me31B	Rck/p54/DDX6	Rck/p54/DDX6	DEAD-box

^{*} Although there is no clear homologue to Not5 in *Dm*, *Mm*, and *Hs* there is considerable sequence homology between *Sc* Not5 and the Not3 homologs in these organisms.

⁺ Dhh1 associates with the Ccr4-Not complex both physically and functionally in yeast, however it is less clear whether interactions with the complex are conserved in multi-cellular organisms.