The Ccr4a (CNOT6) and Ccr4b (CNOT6L) deadenylase subunits of the human Ccr4-Not complex contribute to the prevention of cell death and senescence

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Abstract
A key step in cytoplasmic mRNA degradation is the shortening of the poly(A) tail, which involves several deadenylase enzymes. Relatively little is known about the importance of these enzymes for the cellular physiology. Here, we focussed on the role of the highly similar Ccr4a (CNOT6) and Ccr4b (CNOT6L) deadenylase subunits of the Ccr4-Not complex. In addition to a role in cell proliferation, Ccr4a and Ccr4b play a role in cell survival in contrast to the Caf1a (CNOT7) and Caf1b (CNOT8) deadenylase subunits or the CNOT1 and CNOT3 non-catalytic subunits of the Ccr4-Not complex. Underscoring the differential contributions of the deadenylase subunits, we found that knockdown of Caf1a/Caf1b or Ccr4a/Ccr4b differentially affects the formation of cytoplasmic foci by P-body components. Furthermore, we demonstrated that the amino-terminal leucine rich repeat (LRR) domain of Ccr4b influenced its subcellular localisation, but was not required for the deadenylase activity of Ccr4b. Moreover, overexpression of Ccr4b lacking the LRR domain interfered with cell cycle progression, but not with cell viability. Finally, gene expression profiling indicated that distinct gene sets are regulated by Caf1a/Caf1b and Ccr4a/Ccr4b and identified Ccr4a/Ccr4b as a key regulator of IGFBP5, which mediates cell cycle arrest and senescence via a p53-dependent pathway.

Introduction
Accurate regulation of gene expression requires appropriate control of mRNA levels, which are determined by the relative rates of pre-mRNA synthesis, nuclear processing, and cytoplasmic mRNA turnover. A key step in mRNA degradation is the shortening of the poly(A) tail, which involves several deadenylases containing ribonucleolytic activity (Parker and Song, 2004; Garneau et al., 2007). Approximately ten deadenylase enzymes have been identified in human cells, which can be divided into two classes: those with a conserved DEDD domain and others belonging to the Endonuclease-Exonuclease-Phosphatase (EEP) superfamily (Goldstrohm and Wickens, 2008). The shortening and removal of the poly(A) tail by deadenylase enzymes exposes the 3’ mRNA end to the cytoplasmic form of the Exosome nuclease complex, and facilitates decapping by the Dcp1-Dcp2 dimer, which renders the mRNA susceptible to 5’-3’ exonucleolytic degradation by the Xrn1 nuclease (Parker and Song, 2004; Garneau et al., 2007; Goldstrohm and Wickens, 2008). Many of the factors involved in these processes, as well as
mRNA degradation intermediates, are enriched in cytoplasmic processing P-bodies (Sheth and Parker, 2003; Cougot et al., 2004).

Pioneering work in the yeast *S. cerevisiae* identified the Ccr4-Not complex as the major deadenylase (Tucker et al., 2001). Although this factor contains two deadenylase subunits, Caf1 (Pop2; DEDD type) and Ccr4 (EEP), the major deadenylase activity is associated with the Ccr4 subunit (Chen et al., 2002; Tucker et al., 2002). By contrast, while Caf1 is an active deadenylase in *S. pombe* (Takahashi et al., 2007), it is less clear whether the ribonucleolytic activity of Caf1 is required for mRNA turnover in *S. cerevisiae* (Daugeron et al., 2001; Viswanathan et al., 2004). An important role for Caf1, however, is the recruitment of the Ccr4 subunit to the Ccr4-Not complex, which involves protein-protein interactions between Caf1 and residues in the leucine rich repeat (LRR) domain of Ccr4 (Clark et al., 2004). In addition, the Ccr4-Not complex contains a number of additional non-catalytic subunits (Collart, 2003; Denis and Chen, 2003; Collart and Timmers, 2004).

The role of the Ccr4-Not complex in mRNA decay is conserved in metazoans, including Drosophila and humans (Temme et al., 2004; Yamashita et al., 2005; Temme et al., 2010). Interestingly, multiple homologues of Caf1 and Ccr4 have been identified in human cells. The paralogues Caf1a (CNOT7) and Caf1b (CNOT8) are components of the human Ccr4-Not complex, while a third, more distant homologue, Caf1z/TOE1, forms a separate nuclear complex involved in mRNA metabolism (Wagner et al., 2007). In HTGM5 fibrosarcoma cells, combined knockdown of Caf1a/Caf1b results in a global increase in the length of poly(A) tails, in contrast to combined knockdown of Ccr4a/Ccr4b suggesting that Caf1a/Caf1b may have a greater contribution in global deadenylation in mammalian cells (Schwede et al., 2008). At the cellular level, knockdown of Caf1a and/or Caf1b results in a cellular proliferation defect, which depends in part on their catalytic activity (Aslam et al., 2009).

Five Ccr4 homologues have been identified in human cells, but only Ccr4a (CNOT6) and Ccr4b (CNOT6L) contain an amino terminal LRR required for interactions with Caf1a/Caf1b (Dupressoir et al., 2001). Ccr4b, but not its paralogue Ccr4a, influences cell cycle progression by regulating p27/Kip1 mRNA levels in mouse 3T3 fibroblasts (Morita et al., 2007). Furthermore, Ccr4a is a component of P-bodies and required for foci formation by various P-body components in HeLa cells (Cougot et al., 2004; Andrei et al., 2005).
The exact mechanism of how the Ccr4-Not complex is recruited to mRNA targets remains unclear. In yeast, the PUF family of RNA-binding proteins act as adapter proteins that mediate interactions with the Ccr4-Not complex to stimulate deadenylation of certain mRNAs (Goldstrohm et al., 2006). Members of the BTG/Tob family of anti-proliferative proteins may also contribute to the recruitment of the Ccr4-Not complex (Winkler, 2010). Both Tob and BTG2 interact with the Caf1a/Caf1b subunits and enhance global deadenylation (Ezzeddine et al., 2007; Mauxion et al., 2008). It has been proposed that during translation termination, binding of Tob and PABPC1 may result in recruitment of the Ccr4-Not deadenylase (Ezzeddine et al., 2007; Funakoshi et al., 2007). In addition, several recent studies in Drosophila and mammalian cells have shown that microRNA mediated gene repression is associated with deadenylation and mRNA decay (Behm-Ansman et al., 2006; Wu et al., 2006) and implicate the recruitment of the Ccr4-Not complex by components of the microRNA machinery (Chen et al., 2009; Fabian et al., 2009; Zekri et al., 2009; Piao et al., 2010). Furthermore, the activity of the Ccr4-Not deadenylase subunits may be regulated after their recruitment to mRNA (Morozov et al., 2010).

In addition to a role in mRNA turnover, and consistent with its functions in yeast, a number of protein-protein interactions point to a separate role of the Ccr4-Not complex in transcription in human cells. Particularly, several subunits are reported to regulate the activity of nuclear receptors (Prevot et al., 2001; Hiroi et al., 2002; Morel et al., 2003; Winkler et al., 2006; Garapaty et al., 2008; Govindan et al., 2009).

Here, we investigated the role of the Ccr4a/Ccr4b subunits of the Ccr4-Not complex in the regulation of cellular functions using MCF7 cells. We showed that the Ccr4 paralogues have distinct roles in mediating cell survival, the formation of P-bodies and the regulation of gene expression as compared to the Caf1a/Caf1b deadenylase subunits. Furthermore, we found that the leucine-rich repeat domain was required for proper localisation of Ccr4b and that expression of Ccr4b lacking this domain reduced cell proliferation, but did not affect cell survival. Finally, we identified Ccr4a/Ccr4b as a key regulator of IGFBP5, which mediates cell cycle arrest and senescence via a p53-dependent pathway (Kim et al., 2007).
Results

Knockdown of Ccr4a and/or Ccr4b results in reduced cell proliferation and decreased cell survival

To study the cellular role(s) of the highly related Ccr4a (CNOT6) and Ccr4b (CNOT6L) deadenylase subunits of the human Ccr4-Not complex in MCF7 breast cancer cells, we employed siRNA-mediated knockdown. Using different siRNA duplexes targeting distinct regions in the mRNAs, we obtained efficient knockdown (Figure 1A). At the mRNA level, we routinely achieved a knockdown efficiency of >80% (data not shown). In agreement with earlier observations by Morita et al. (2007), we noted a strong effect on cell proliferation upon knockdown of Ccr4b (Figure 1B). Interestingly, however, we also observed a significant effect on MCF7 cell proliferation upon knockdown of Ccr4a (Figure 1B), which has no effect on cell proliferation of NIH 3T3 mouse fibroblasts (Morita et al., 2007). The effects on cell proliferation upon knockdown of Ccr4a and/or Ccr4b were confirmed by cell cycle profiling using flow cytometry (Figure 1C). In addition to a decreased fraction of cells in S-phase and a concomitant increase in G1, we surprisingly also found a significant fraction of cells with sub-G1 DNA content upon Ccr4a/Ccr4b knockdown (Figure 1C), indicating reduced cell viability (Galluzzi et al., 2009). In agreement with the latter observation, we confirmed decreased cell viability upon knockdown of Ccr4b and particularly upon combined knockdown of Ccr4a and Ccr4b using propidium iodide exclusion in combination with flow cytometry (Figure 1D). Previously, we had not observed evidence for decreased cell viability upon (combined) knockdown of Caf1a (CNOT7) and Caf1b (CNOT8) using cell cycle profiling by flow cytometry (Aslam et al., 2009). This was confirmed using propidium iodide exclusion combined with flow cytometry (Figure 1E) suggesting unique functions for the Ccr4a/Ccr4b deadenylases as compared with the Caf1a/Caf1b catalytic subunits in mediating cell survival.

To investigate whether reduced survival was due to increased apoptosis, or alternative mechanisms, we carried out bivariate flow cytometry using annexin V and propidium iodide staining (Vermes et al., 1995) (Figure 1F). The fraction of early and late apoptotic cells (bottom right, and top right quadrant, respectively) were increased following Ccr4b knockdown and more pronounced upon combined knockdown of Ccr4a and Ccr4b (Figure 1F). In addition, the
fraction of non-viable cells that did not bind annexin V was also increased in Ccr4b and combined Ccr4a/Ccr4b knockdown cells (top left quadrant). Taken together, these results indicate that decreased survival in Ccr4a/Ccr4b knockdown cells is due to both apoptosis-dependent and –independent mechanisms.

The CNOT1 and CNOT3 subunits of the Ccr4-Not complex are required for cell proliferation but do not contribute to cell survival

To investigate whether the Ccr4a/Ccr4b deadenylase subunits carry out a unique role, or whether other Ccr4-Not components also contribute to cell survival and proliferation, we used siRNA to knockdown the CNOT1 and CNOT3 subunits (Figure 2A). These subunits do not have paralogues in human cells and knockdown may result in disruption of the complex. Interestingly, we reproducibly observed a reduction of CNOT1 protein levels upon knockdown of CNOT3, which may indicate that CNOT3 is required for stability of the complex (Figure 2A). As was observed in Ccr4a and Ccr4b knockdown cells, as well as in Caf1a/Caf1b knockdown cells (Aslam et al., 2009), knockdown of CNOT1 and CNOT3 resulted in a strong reduction of cell proliferation (Figure 2B). Cell cycle profiling confirmed the roles for these subunits in cell cycle progression as a decreased fraction of cells in S-phase and a concomitant increase of cells in G1 was observed (Figure 2C). Remarkably, no significant increase in the percentage of cells with sub-G1 DNA content was found. This was confirmed by measurement of propidium iodide exclusion by flow cytometry, which did not show increased uptake of propidium iodide upon knockdown of CNOT1 or CNOT3 as compared to non-targeting control siRNA (Figure 2D). Thus, these results indicate that CNOT1 and CNOT3 are required for cell proliferation of MCF7 cells, but –in contrast to the Ccr4a/Ccr4b deadenylase subunits- are not required for cell survival.

The deadenylase components Caf1a/Caf1b and Ccr4a/Ccr4b differentially affect foci formation of P body markers

Because knockdown of the Caf1a/Cafb deadenylases did not affect cell viability, in contrast to combined knockdown of Ccr4a and Ccr4b, we characterised the differential requirements for the Ccr4a/Ccr4b versus the Caf1a/Caf1b deadenylases further by analysing cytoplasmic foci formation of the P-body components p54/Rck/Ddx6, Dcp1a, eIF4E, and Ago2 (Figure 3). The
average number of foci per cell formed upon expression of YFP-p54/Rck/Ddx6 was decreased upon combined Ccr4a/Ccr4b knockdown cells, which was unexpectedly more pronounced after combined knockdown of Caf1a/Caf1b (Figure 3A, B). Similar observations were made using staining of Dcp1a using antibodies recognising endogenous protein (Figure 3C, D). By contrast, when antibodies recognising endogenous eIF4E were used, the average number of foci per cell was increased upon combined knockdown of Ccr4a/Ccr4b, while a reduction was observed upon combined knockdown of Caf1a/Caf1b (Figure 3E, F). No change of the number of foci formed after expression of YFP-Ago2 was observed upon knockdown of either Ccr4a/Ccr4b or Caf1a/Caf1b (Figure 3G, H). No stress bodies were induced upon siRNA transfection as assessed by eIF3 foci formation (data not shown). Furthermore, the effects on foci formation were not caused by altered expression of the respective proteins as determined by immunoblotting (Figure 3I). Together, these results show that the accumulation of RCK, Dcp1a and eIF4E foci is particularly sensitive to knockdown of Caf1a/Caf1b and support the notion that the Ccr4a/Ccr4b deadenylases have differential roles as compared to the Caf1a/Caf1b catalytic subunits of the Ccr4-Not complex.

**Interactions mediated by the LRR domain impact on the subcellular localisation of Ccr4b**

To further explore the relationship between Caf1a and Ccr4b, we next focussed on the role of the N-terminal leucine-rich region (LRR) domain of the Ccr4a/Ccr4b subunits (Figure 4A). As expected, Flag-Ccr4b can bind to HA-Caf1a. This interaction depends on the presence of the LRR domain (Figure 4B). Interestingly, replacing the LRR domain of human Ccr4b with the LRR of yeast Ccr4 disrupted the interaction with Caf1a demonstrating that the human domain cannot be changed for the yeast residues. Furthermore, Flag-Ccr4b did coimmunoprecipitate the Ccr4-Not subunits CNOT1 and CNOT3 in addition to HA-CNOT7 (Figure 4B). However, this was not observed when the LRR residues were deleted indicating that Ccr4b interacts with the CNOT1 and CNOT3 subunits via Caf1a protein in agreement with observations made in yeast (Clark et al., 2004).

Surprisingly, the LRR domain influenced the subcellular localisation of Ccr4b. Upon expression of Flag-Ccr4b, the majority of Ccr4b was detected in the cytoplasm, although an appreciable
amount was also found in the nucleus (Figure 4C, top panels) (Cougot et al., 2004; Andrei et al., 2005). Remarkably, deletion of the LRR domain of Flag-Ccr4b resulted in an almost exclusive cytoplasmic localisation (Figure 4C, bottom panels, Figure 4D).

**Requirements of the LRR domain of Ccr4b for deadenylase activity and cell proliferation**

To characterise the role of the LRR domain further, we expressed and immunopurified several Flag-Ccr4b variants from HEK 293 cells. Subsequently, the deadenylase activity of wild type Flag-Ccr4b, enzymatically inactive Flag-Ccr4b, Flag-Ccr4b containing the yeast LRR domain, or Flag-Ccr4b lacking the LRR domain was measured using a fluorescently labelled oligonucleotide substrate. As expected, immunopurified Flag-Ccr4b was able to degrade the oligonucleotide substrate. The activity was severely reduced when Flag-Ccr4 containing the amino acid substitution E240A, or Flag-Ccr4b containing the yeast LRR domain was used (Figure 5A). Interestingly, Flag-Ccr4b lacking the LRR domain was an active deadenylase enzyme, thereby demonstrating that the LRR domain is not absolutely required for the deadenylase activity of human Ccr4b in contrast to the yeast enzyme (Clark et al., 2004).

To examine whether the LRR domain of Ccr4b was involved in the regulation of cell proliferation, we next overexpressed wild type Flag-Ccr4b, an enzymatically inactive version (E240A), as well as Flag-Ccr4b lacking the LRR domain in MCF7 cells. A pulse labelling with the thymidine analogue EdU was used to identify cells in S-phase as a measure for cell proliferation. Interestingly, while overexpression of Flag-Ccr4b or an enzymatically inactive form did not affect cell proliferation, overexpression of Flag-Ccr4b lacking the LRR domain caused a significant drop in EdU-positive cells (Figure 5B). By contrast, overexpression of (enzymatically inactive) Ccr4b, or Ccr4b lacking the LRR domain did not affect cell viability (Figure 5C).

Together, these data suggest that interactions with Caf1a/Caf1b mediated by the LRR domain of Ccr4b are important for cell cycle progression.

**Ccr4a/Ccr4b and Caf1a/Caf1b regulate distinct gene sets**

Next, we used gene expression profiling to understand in more detail the mechanism by which the Ccr4a/Ccr4b deadenylases regulate cell proliferation and survival. Thus, MCF7 cells were
treated with siRNA pools targeting Ccr4a and/or Ccr4b, Caf1a/Caf1b and a non-targeting control pool. Total RNA was isolated and analysed using Affymetrix Human Gene 1.0 ST Array GeneChips. In combined Ccr4a/Ccr4b knockdown cells, 79 genes were found to be upregulated, whereas only four genes were downregulated (fold change >1.50, p <0.050), which is expected based on their function as deadenylase enzymes. No differentially expressed genes were identified upon knockdown of Ccr4a. However, the expression profile upon knockdown of Ccr4b was enhanced when combined with knockdown of Ccr4a, indicating that knockdown of Ccr4a can be (partially) compensated for by Ccr4b. Interestingly, combined knockdown of Caf1a/Caf1b resulted in a larger number of differentially expressed genes (223 up; 66 down) with limited overlap with the differentially expressed gene set upon Ccr4a/Ccr4b knockdown (Figure 6A-D). Together, these results demonstrate that Ccr4a/Ccr4b and Caf1a/Caf1b largely regulate distinct gene sets with limited overlap.

Identification of Ccr4a/Ccr4b target genes

A number of genes, identified from gene expression profiling, were subsequently validated by RT-qPCR using gene specific primers. We confirmed enhanced expression of insulin-like growth factor binding protein 5 (IGFBP5; ~3 fold), CLEC3A (~3 fold), SEMA3E (~2 fold), MAPK10 (~2 fold), CDH18 (~2 fold) and LMO3 (~8 fold) upon Ccr4a/Ccr4b knockdown (Figure 7A). To determine if the enhanced expression of the genes was due to increased transcript stability following loss of Ccr4a/Ccr4b, we used the transcriptional inhibitor actinomycin D in combination with RT-qPCR to measure mRNA stability. Of the six genes identified, MAPK10, CDH18 and LMO3 mRNA transcripts were significantly more stable after Ccr4a/Ccr4b knockdown compared to control siRNA (Figure 7B). The mRNAs of IGFBP5, SEMA3E and CLEC3A were stable under normal conditions precluding the assessment of increased mRNA half-lives of the mRNAs of these genes (Figure 7B and data not shown).

IGFBP5 overexpression is associated with cellular senescence via a p53-dependent pathway in HUVEC cells (Kim et al. 2007). Consistent with IGFBP5 upregulation, p53 protein levels were increased upon Ccr4a/Ccr4b knockdown, although no change in mRNA levels was observed in the expression profiling data. Because activation of p53 residue lysine 120 by acetylation is indispensible for p53-dependent growth arrest and apoptosis (Tang et al., 2008), we also
determined the acetylation of p53 at this residue in Ccr4a/b and Caf1a/b knockdown MCF7 cells. Indeed, acetylation of lysine 120 was significantly induced in Ccr4a/Ccr4b knockdown cells as compared to control or Caf1a/Caf1b knockdown cells (Figure 7C). In contrast, we did not observe increased p27/Kip1 mRNA or protein levels in MCF7 cells (Figure 7C). These results were confirmed by quantitative immunoblotting. Interestingly, while the overall levels of both total p53 as well as p53 acetylated at lysine 120 were significantly increased in Ccr4a/Ccr4b knockdown cells, the fraction of p53 acetylated at lysine 120 was not increased (Figure 7D). Finally, we looked at the senescence phenotype by β-galactosidase staining (Dimri et al., 1995). Consistent with the role of IGFBP5, knockdown of Ccr4a/Ccr4b caused a significant increase in senescence associated β-galactosidase staining as compared with control or Caf1a/Caf1b knockdown (Figure 7E). Taken together, these results suggest that knockdown of Ccr4a/Ccr4b in MCF7 cells causes upregulation of IGFBP5, which may mediate inhibition of cellular proliferation and induce senescence via a p53-dependent pathway.

**Discussion**

**Distinct roles for the human Ccr4a/Ccr4b and Caf1a/Caf1b deadenylases**

In this report we show that the human deadenylase subunits associated with the Ccr4-Not complex have distinct roles based on three criteria: (i) the Ccr4 paralogues mediate cell survival and inhibit cellular senescence in contrast to the Caf1a/Caf1b subunits; (ii) the accumulation of foci by P-body components is particularly dependent on Caf1a/Caf1b and less sensitive to knockdown of the Ccr4 paralogues; (iii) distinct gene sets are regulated by Ccr4a/Ccr4b as compared to Caf1a/Caf1b as shown by genome-wide expression profiling; and (iv) (acetylated) p53 is selectively induced upon knockdown of Ccr4a/Ccr4b as compared to the Caf1 paralogues. These phenotypes are not due to ‘off-target’ effects, as different siRNA duplexes targeting different regions of the mRNAs yielded similar results. Moreover, the phenotypic differences are not merely quantitative, for instance due to varying knockdown efficiencies. For example, while the effect on cell proliferation is less pronounced and no effect on cell viability is observed upon knockdown of Caf1a/Caf1b, the effect on foci formation of YFP-RCK and Dcp1a is quantitatively more significant as compared to combined knockdown of Ccr4a/Ccr4b. Furthermore, there are qualitative effects on foci formation by eIF4A upon
knockdown of Ccr4a/Ccr4b and Caf1a/Caf1b, respectively. Thirdly, using genome-wide expression profiling, qualitative differences were found upon knockdown of the Ccr4-type and Caf1 paralogues. The expression of more genes was affected upon knockdown of Caf1a/Caf1b and little overlap was observed with the Ccr4a/Ccr4b knockdown profile. Further evidence for unique roles for the Ccr4a/Ccr4b subunits is provided by the analysis of the cellular phenotype upon knockdown of the non-catalytic subunits CNOT1 and CNOT3. While the effect on the percentage of cells in S-phase was more pronounced upon knockdown of these subunits as compared to knockdown of Ccr4b (and comparable to combined knockdown of Ccr4a/Ccr4b), no effect on cell viability was observed.

Ccr4a and Ccr4b are required for cell cycle progression and prevent cell death and senescence

Consistent with findings reported by Morita et al. (2007), we found that the Ccr4b deadenylase is important in controlling cell proliferation of MCF7 breast cancer cells. However, while upregulation of p27/Kip1 is implicated with reduced cell cycle progression of NIH3T3 cells (Morita et al., 2007), we did not observe upregulation at either mRNA or protein level of this cell cycle inhibitor in MCF7 cells. We extend these observations further by showing that the Ccr4a deadenylase as well as two non-catalytic subunits of the Ccr4-Not complex, the large subunit CNOT1 and CNOT3, are also important for efficient cell proliferation. Finally, we reveal two additional roles of the Ccr4a/Ccr4b proteins in mediating cell survival and preventing cellular senescence. We believe that the most likely explanation for the observed differences is provided by assuming cell type-specific roles of the Ccr4a/Ccr4b deadenylases, although other explanations cannot be excluded.

Distinct roles for Caf1a/Caf1b and Ccr4a/Ccr4b in foci formation by P-body components

While Ccr4 can be found in P-bodies in some cell types (Cougot et al., 2004; Andrei et al., 2005), we did not find Caf1a or Ccr4b to localise to P-bodies in MCF7 cells. Because the accumulation of several P-body components depends on active deadenylation and is reduced upon knockdown of Ccr4a (Andrei et al., 2005; Zheng et al., 2008), we looked at foci formation...
of the P-body components RCK, Dcp1a, eIF4E and Ago2 to investigate in more detail the
distinct roles for the Caf1a/Caf1b and Ccr4a/Ccr4b deadenylases. Specific dependencies in P-
body assembly have been described (Teixeira and Parker, 2007). Whereas quantitative
differences were observed when the accumulation of RCK and Dcp1a was analysed, qualitative
differences were notable upon analysis of eIF4E foci accumulation. The accumulation of eIF4E
foci upon knockdown of Ccr4a/Ccr4b was not due to the formation of stress bodies as eIF3 foci
were only observed after treatment with sodium arsenite and were absent upon combined
knockdown of Ccr4a/Ccr4b (data not shown). Thus, these data suggest that, compared to
Caf1a/Caf1b, the Ccr4a/Ccr4b proteins act at a different stage of the deadenylation process and
therefore differentially affect the accumulation of P-body components. Alternatively, the
structural contributions to P-body formation via protein-protein interactions by Caf1a/Caf1b
may be more significant as compared to Ccr4a/Ccr4b. Interestingly, differential roles for Caf1
and Ccr4 on P-body formation and deadenylation have also been described in A. nidulans
(Morozov et al., 2010).

**Mechanistic insight: the role of the LRR domain**

In yeast, the LRR domain of Ccr4 is critical for its function *in vivo* as well as its deadenylase
activity (Clark et al., 2004). Furthermore, the LRR of Ccr4 mediates its interaction with Caf1
thereby linking Ccr4 to the Ccr4-Not complex (Dupressoir et al., 2001; Clark et al., 2004). In
agreement with these studies, we find that deleting the LRR of Ccr4b abolishes its interaction
with Caf1a and other components of Ccr4-Not complex. However, in contrast to yeast Ccr4, the
LRR of Ccr4b is not critical for its deadenylase activity. This is in agreement with recent reports
showing that purified Ccr4b lacking the LRR domain is enzymatically active and the fact that
Nocturnin, a Ccr4-type deadenylase lacking a LRR domain, is active *in vitro* (Baggs and Green,
2003; Wang et al., 2010).

We previously identified the Caf1a and Caf1b deadenylase subunits of the human Ccr4-Not
complex as mediators of efficient proliferation of MCF7 cells, partially compensating each
other’s function (Aslam et al., 2009). Because the Caf1a/Caf1b subunits are required for
recruitment of the Ccr4a/Ccr4b subunits to the complex, we hypothesised that deregulated
Ccr4 activity contributes to the phenotype. In agreement with this model, we found that
overexpression of Ccr4b lacking the LRR domain resulted in aberrant localisation and reduced cell proliferation, while cell viability remained unaffected. This is consistent with a unique role for the Ccr4-type deadenylases in mediating cell survival, which is independent of interactions with Caf1a/Caf1b. However, other explanations may also be possible, e.g. the LRR domain may contribute to mRNA interactions required for regulated mRNA decay during cell cycle progression.

**Mechanistic insight from gene expression profiling**

Most of the differentially expressed genes upon knockdown of Ccr4a/Ccr4b were upregulated consistent with their role in mRNA turnover. In support of this notion, we tested transcript stability of six putative target genes, whose expression was significantly altered in the genome-wide analysis. The mRNA stability of LMO3, CDH18, MAP10K were significantly increased following Ccr4a/Ccr4b knockdown consistent with their role in mRNA degradation. IGFBP5, SEMA3E and CLEC3A were stable transcripts, which precluded the use of actinomycin D to accurately determine their stability. Thus, these data suggest that at least a significant fraction of the genes identified in the gene expression profiling experiment appear to be direct targets as their upregulation correlates with increased transcript stability. Interestingly, CLEC3A, SEMA3E, MAPK10 and IGFBP5 are thought to be involved in reduced breast cancer cell proliferation, apoptosis and inhibition of tumour development (Bogoyevitch, 2006; Kigel et al., 2008; Tsunezumi et al., 2009). Insulin-like growth factor binding protein 5 (IGFBP5) is one of six members of the IGFBP protein family and is an important component of the IGF-axis (Beattie et al., 2006). In breast cancer cells, IGFBP5 binds to IGF I/II and blocks the activation of IGF signalling. Reduction or cleavage of IGFBP5 is then followed by the release of IGF, which reduces apoptosis and activates cell proliferation (Beattie et al., 2006; Akkiprik et al., 2008). Consistent with this, previous reports have identified IGFBP5 as a key regulator of cell proliferation and apoptosis in breast cancer cell lines (Butt et al., 2003; Butt et al., 2005). Our data suggests that IGFBP5 may indeed exert its apoptotic effects via a p53 dependent mechanism, in support of Kim and co-workers who show similar data in human umbilical cord endothelial cells (Kim et al., 2007). The precise contribution of IGFBP5 and other direct or
indirect target genes of Ccr4a/Ccr4b to the observed phenotype will be the focus of future studies.

Materials and methods

Plasmids, mutagenesis, and siRNA

The open reading frames of CNOT6 and CNOT6L were obtained by reverse transcriptase PCR using MCF7 NKI total RNA as a template. After cloning the respective cDNAs into the SmaI site of pBluescript II KS(+) and sequence verification, the cDNAs were subcloned in pcDNA3-FLAG (BamHI-EcoRV) and pCMV5-HA (XhoI digested cDNA ligated into SalI-digested vector). Site-directed mutations to inactivate the active sites (E240A) were introduced using standard protocols (Stratagene Quikchange). The hybrid cDNA encoding the yeast leucine rich region (yeast residues 302-453) fused to the human CNOT6 (amino acids 148-557) and CNOT6L (amino acids 153-555) regions was constructed using overlap PCR techniques. Primer sequences are available upon request. Deletion of the leucine rich regions in CNOT6 (residues 2-147) and CNOT6L (2-152) were also obtained by standard PCR techniques. See Aslam et al. (2009) for a description of plasmid pCMV5-HA-CNOT7.

In addition to those described before (Winkler et al., 2006; Aslam et al., 2009), the following siRNA duplexes were used (Dharmacon): CNOT6 (D-019101-01, and On-Target plus SMARTpool L-019101-00), CNOT6L (D-016411-02, and On-Target plus SMARTpool L-016411-00).

Cell culture and transfection

MCF7 NKI and human embryonic kidney (HEK) 293 cells were routinely maintained and transfected as described previously (Aslam et al., 2009).

Western blotting and immunoprecipitation

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Proteins were visualised using an ECL detection kit (Peirce) and a Fujifilm LAS-4000 imager. Primary antibodies used were: YFP (sc-8336; 1:1000 dilution, Santa Cruz), eIF4E (ab-1126; 1:1000 dilution, Abcam), Dcp1a (1:1000 dilution) (Lykke-Andersen and Wagner, 2005), p53 (DO-1, sc-126; 1:1000 dilution, Santa Cruz) and tubulin (sc-7396; 1:1000 dilution, Santa Cruz), p27/Kip1
(2552; 1:1000 dilution, Cell Signalling Technology), p53-K120ac antibody (ab78316, Abcam, 1:500 dilution) and β-actin (AC-15, 1:1000, Sigma). All HRP conjugated secondary antibodies were purchased from Santa Cruz Biotechnology and used in 1:2500 dilutions. Other antibodies for immunoprecipitations and western blotting were as used before (Winkler et al., 2006; Aslam et al., 2009). Chemiluminescent signals were visualised using a Fujifilm LAS-4000 imager.

**Flow Cytometry**

MCF-7 NKI cells (400,000 cells in a T25 flask) were transfected with siRNA as described (Aslam et al., 2009). After 24 hours, medium was removed and fresh medium was added to the cells. For cell cycle profiling, cells were labelled after an additional 46 hours for 2 hours in the presence of 1 μM BrdU and prepared for bivariate flow cytometry using propidium iodide and FITC-conjugated anti-BrdU antibody 3D4 (BD Pharmingen).

For cell viability analysis, transfected cells (150,000 per T25 flask) were harvested by trypsin treatment. After inactivation of trypsin by the addition of the supernatant medium, cells were washed and resuspended in PBS, incubated with propidium iodide (1.0 μg/ml), and subjected to flow cytometry.

For annexin V binding, transfected cells (200,000 per T25 flask) were harvested by trypsin treatment. After inactivation of trypsin by the addition of the supernatant medium, cells were washed and resuspended in annexin V binding buffer (Invitrogen; 0.01 M Hepes-NaOH (pH7.4), 0.14 M NaCl, 2.5 mM CaCl$_2$), incubated with propidium iodide and FITC-labelled annexin V (BD Biosciences; according to manufacturer’s instructions), and subjected to flow cytometry.

Analysis was carried out using a FACS Aria flow cytometer, FACSDiva software (BD Biosciences) and the WinMDI package.

**Fluorescence microscopy**

To assess cell proliferation by fluorescence microscopy, MCF7 cells were transfected using Genejuice (150,000 cells per well of 6-well plate containing a coverslip) following the manufacturer’s instructions (Merck). After 46 hours, cells were labelled for 2 hours with 10 μM of the thymidine analogue EdU (5-ethynyl-2’deoxyuridine) and processed using Click-iT reagents (Invitrogen). The percentage of EdU-labelled cells was determined in triplicate.
To stain for the endogenous P-body markers, eIF4E and Dcp1a, 60,000 MCF7-NKI cells were seeded into each well of 6-well plates containing glass coverslips. After 72 hours of siRNA transfection, the cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 10 min and permeabilised with cold 0.5% Triton X-100 for 10 min. After blocking for 20 min in PBS containing 3% BSA, the cells were stained with primary antibody diluted in PBS containing 3% BSA for 1 hr at 37 °C. Primary antibodies used were eIF4E (1:500 dilution, rabbit polyclonal ab-1126; Abcam) and hDcp1a (1:200 dilution) (Lykke-Andersen and Wagner, 2005). After washing with PBS, the cells were incubated for 1 hr at 37 °C with Alexa Fluor-conjugated secondary antibodies (Molecular Probes), counterstained with Hoechst 33258 (0.5 μg/ml) and mounted on glass slides for microscopy.

To detect Rck/p54/DDX6 and Ago2, the cells were transfected with siRNA for 48 hours and subsequently transfected with their respective YFP constructs (a kind gift from Dr Martin Bushell). After 24 hours of plasmid transfection, the cells were washed twice with PBS, fixed with 4% paraformaldehyde, counterstained with Hoechst 33258 and mounted on glass slides for microscopy.

Cell imaging was carried out using a Zeiss LSM510 Meta confocal laser scanning microscope. Images were processed and merged using the LSM Image Browser (Zeiss) and the Paint.net package (http://www.getpaint.net).

To study the subcellular localisation of Ccr4b, MCF7 cells were transfected with plasmids either expressing Flag-Ccr4b or Flag-Ccr4bΔLRR using JetPEI (Polyplus). Immunofluorescence using an anti-Flag antibody (F1804, Sigma, 1:500 dilution) was carried out as described above at 24 hours after transfection. For quantification of the nucleocytoplasmic distribution, images were captured using identical laser settings. For each cell analysed, the mean pixel intensity of three equal regions of interest in the nucleus and cytoplasm was determined in the appropriate channel using ImageJ (http://rsbweb.nih.gov/ij/). Fluorescence intensities were obtained from MCF7 cells transfected with either Flag-Ccr4b (n=50) or Flag-Ccr4bΔLRR (n=49) and the mean intensity of nuclear and cytoplasmic regions of interest was calculated.
**Gene expression profiling**

MCF7 NKI cells (1.0 × 10^6 cells in a 100 mm culture dish) were transfected with 5 nM siRNA pools targeting Ccr4a(CNOT6), Ccr4b(CNOT6L), Ccr4a/Ccr4b, or Caf1a(CNOT7)/Caf1b(CNOT8), and/or a non-targeting control pool (Dharmacon On-Target Plus SMARTpool; total siRNA concentration was 10 nM). DNA-free total RNA of biological triplicates was isolated (Omega EZNA total RNA kit, including on-column DNAsse digestion using Qiagen RNase-free DNase I), subjected to quality control using an Agilent 2100 Bioanalyzer, and processed using Affymetrix Human Gene 1.0 ST Array GeneChips, the manufacturer’s labelling protocols, fluidic station and scanner (Nottingham Arabidopsis Stock Centre’s International Affymetrix Service). Data was normalised using the RMA protocol using the Affymetrix Gene Console package and analysed using Excel 2007 (Microsoft). Differentially expressed genes were identified on the basis of the following criteria: signal intensity (untransformed value) >50.0, fold change >1.50, and p-value <0.050. Hierarchical clustering was carried out using Carmaweb (Rainer et al., 2006). The microarray data have been deposited in the ArrayExpress database (European Bioinformatics Institute, accession number E-MEXP-2926).

**Reverse transcriptase-quantitative PCR**

Total RNA was isolated using the Omega EZNA total RNA kit and cDNA was prepared using an anchored oligo-dT primer using 75-100 ng total RNA in a 10 μl reaction (Superscript III, Invitrogen). After 1:5 dilution of the cDNA reaction with TE, 1 μl diluted cDNA was analysed in triplicates by quantitative PCR (10 μl reaction volume, Bioline SensiMix Low-Rox SYBR Green mix) using a Stratagene MX3005p cycler. GAPDH or β-actin were used as reference genes. Analysis was carried out using the Stratagene MXpro package. Analysis of mRNA stability was as described before (Aslam et al., 2009).

**Deadenylase assay**

HEK 293 cells (60% confluent, 6 cm standard cell culture dish) were transfected with Flag-Ccr4b expression plasmids using Genejuice following the manufacturer’s protocol (Merck). After 48 hours, the cells were lysed in 500 μl lysis buffer (50 mM Hepes-NaOH pH 8.0, 150 mM NaCl, 5 mM MgCl2, 0.5 mM EDTA, 5% Glycerol, 1 mM DTT and protease inhibitors). Flag-Ccr4b
proteins were immunoprecipitated using anti-Flag antibodies (2 μg antibody coupled to 20 μl protein G-agarose beads) overnight at 4 °C. After three washes with lysis buffer, immunoprecipitates were washed twice in deadenylation buffer (50 mM Hepes-NaOH pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 10% Glycerol, 1 mM DTT and protease inhibitors). To elute bound proteins by peptide competition, the flag resin was incubated with 20 μl deadenylase buffer containing 0.3 mg/ml 3× Flag peptide (Sigma) for 60 min at 37 °C with occasional mixing. One μl 5’ Fluorescein (Flc)-labelled substrate (Sigma, Flc-5’-CCUUUCCAAAAAAAAA-3’; final concentration: 0.1 μM) was added to 9.0 μl Flag eluate and incubated for 60 min at 37 °C. Reactions were stopped by the addition of 12 μl RNA loading buffer (95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.025% sodium dodecylsulfate and 5 mM EDTA), and heated 3 min at 85 °C. RNA was analysed by denaturing PAGE using a 20% acrylamide:bisacrylamide (19:1) gel containing 8.3 M urea. Flc-labelled RNA was visualised using a Fujifilm LAS-4000 imager.

Senescence-associated β-galactosidase assay

To assess senescence, 60,000 MCF7 NKI cells were seeded onto each well of a six-well dish and transfected in triplicate with control, Caf1a/Caf1b or Ccr4a/Ccr4b siRNA using interferin (Polyplus). After 72 hours of transfection, the cells were fixed and stained for β-galactosidase activity using the Senescence β-galactosidase Staining Kit (New England Biolabs) according to the manufacturer’s instructions. The cells were visualised using a Leica DM2000 light microscope at 200× magnification. Approximately 750 cells were counted from each well and the percentage of blue cells (exhibiting senescence) was calculated.

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**Supplementary data**

Tables 1-6. Summary of gene expression data. Suppl-data-t1-t6.xlsx

Table 7. Oligonucleotide sequences of qPCR primers. Suppl-data-t7.xlsx

**References**


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Figure 1. Knockdown of Ccr4a and/or Ccr4b results in reduced cell proliferation and decreased cell viability.
(A) Knockdown of Ccr4a and Ccr4b in MCF7 cells. Following transfection of vectors expressing HA-Ccr4a or HA-Ccr4b, cells were treated with siRNA targeting Ccr4a and/or Ccr4b. After 3 days, total lysates were analysed by immunoblotting. Antibodies recognising β-actin were used to assess equal loading. (B) Inhibition of cell proliferation upon knockdown of Ccr4a and/or Ccr4b. Following siRNA transfection, adherent cells were counted in a haemocytometer at 24 hour intervals. (C) Cell cycle profiling of MCF7 cells transfected with siRNA targeting Ccr4a and/or Ccr4b. Dot plots of bivariate flow cytometry using propidium iodide fluorescence to determine DNA content (horizontal) and anti-BrdU fluorescence (FITC) to identify BrdU incorporation to label cells in S-phase (vertical) are shown. Cells (n = 20,000) were analyzed per condition 72 h after transfection. The percentages of cells in (sub) G1-, S-, and G2/M-phase are indicated. (D) Decreased cell viability upon knockdown of Ccr4a and/or Ccr4b. Cell viability (n = 10,000) was monitored by propidium iodide exclusion as detected by flow cytometry at 72 h after transfection. The percentage of (non-viable) cells labelled by propidium iodide is indicated. * p<0.05, ** p<0.01 (compared to non-targeting control siRNA). (E) Cell viability is not decreased upon combined knockdown of Caf1a and Caf1b. Cell viability was measured by propidium iodide exclusion as detailed in (D). (F) Increased apoptosis upon knockdown of Ccr4b. Apoptosis was monitored using Annexin V binding (horizontal) and propidium iodide exclusion (vertical). Cells (n = 20,000) were analysed 72 h after transfection. The percentage of cells present in each quadrant is indicated.
Figure 2. Knockdown of CNOT1 and CNOT3 results in reduced cell proliferation, but does not affect cell viability.

(A) Knockdown of CNOT1 and CNOT3 in MCF7 cells. Cells were transfected with the indicated siRNA and protein levels were analysed after 72 h using the indicated antibodies. (B) Inhibition of cell proliferation upon knockdown of CNOT1 and CNOT3. After siRNA transfection, adherent cells were counted in a haemocytometer at 24 hour intervals. (C) Cell cycle analysis of MCF7 cells upon knockdown of CNOT1 and CNOT3. Cells were mock treated or transfected with non-targeting control siRNA, or siRNA targeting CNOT1 or CNOT3. Dot plots show bivariate flow cytometry using propidium iodide fluorescence to determine DNA content (horizontal) and anti-BrdU fluorescence (FITC) to identify BrdU incorporation to label cells in S-phase (vertical). Cells (n = 20,000) were analyzed per condition 72 h after transfection. The percentages of cells in (sub) G1-, S-, and G2/M-phase are indicated. (D) Cell viability is not decreased upon knockdown of CNOT1 or CNOT3. Cell viability was measured by propidium iodide exclusion as detailed in (1D). ** p<0.01 (compared to non-targeting control siRNA).
Figure 3. Distinct roles for the deadenylase components Caf1a/Caf1b and Ccr4a/Ccr4b in foci formation of P-body components.

(A, B) Reduced foci formation of YFP-RCK upon knockdown of Ccr4a/Ccr4b and Caf1a/Caf1b. (C, D) Decreased formation of Dcp1a foci upon knockdown of Ccr4a/Ccr4b and Caf1a/Caf1b. (E, F) Increased foci formation by eIF4E in MCF7 cells upon knockdown of Ccr4a/Ccr4b but
dramatically decreased foci formation upon knockdown of Caf1a/b. (G, H) Formation of YFP-Ago2 foci is not affected upon knockdown of Ccr4a/Ccr4b and Caf1a/Caf1b. Cells were transfected with the indicated siRNA and processed for immunofluorescence microscopy using antibodies recognising endogenous eIF4E (C, D) or Dcp1a (E, F). p54/Rck (A, B) and Ago2 (G, H) were detected 24 h after transfection of the respective YFP-fusion proteins. * p<0.05, ** p<0.01, *** p<0.001 (compared to non-targeting control siRNA). (I) Protein levels of YFP-RCK, Dcp1a, eIF4E, and YFP-Ago2 were unaffected upon knockdown of Caf1a/Caf1b or Ccr4a/Ccr4b. Protein lysates from cells transfected with the indicated siRNA were subjected to immunoblotting. YFP-RCK and YFP-Ago2 were detected with antibodies recognising YFP, whereas eIF4E and Dcp1a were detected with antibodies recognising the endogenous proteins. Cross-reactive bands are indicated with an asterisk.
Figure 4. The LRR domain of Ccr4b is required for interactions with Caf1a, incorporation into the Ccr4-Not complex and subcellular localisation.

(A) Schematic diagrams the Ccr4b constructs. Indicated are the S. cerevisiae Ccr4p protein (light grey), the human Ccr4b homologue of Ccr4p (black), and the locations of the leucine-rich region (LRR) and EEP ribonuclease domain. (B) The LRR domain of Ccr4b interacts with CNOT1 and CNOT3 via Caf1a. Plasmids pCMV5-HA-CNOT7 (or control plasmid) and vectors expressing wild-type Flag-Ccr4b, Ccr4b (Hyb-LRR), or Cr4b (\(\Delta\)LRR) were transiently cotransfected into HEK 293 cells. Total lysates (left) and anti-Flag immunoprecipitates (right) were analyzed using the indicated antibodies. (C) The LRR domain of CCR4b is required for its localisation to the nucleus. Following transfection with the indicated cDNA expression vectors, MCF7 cells were processed for immunofluorescence using anti-Flag antibodies after 24 h. Flag-Ccr4b was found to localise to both the nucleus and cytoplasm (top panels) whereas the Flag-CCR4b lacking the LRR domain (\(\Delta\)LRR) was exclusively cytoplasmic (bottom panels). (D) Quantification of the nucleocytoplasmic distributions of Ccr4b and Ccr4b\(\Delta\)LRR. The mean fluorescence intensity was determined from cells expressing Flag-Ccr4b (n=50) and Flag-Ccr4b\(\Delta\)LRR (n=49) using three identical regions of interest per cell. The mean intensity is plotted. Error bars represent the SEM.
Figure 5. The role of the LRR domain of Ccr4a/Ccr4b in deadenylation and cell proliferation.

(A) The LRR domain of Ccr4b is dispensable for deadenylase activity. Cells were transfected with empty vector or the indicated Flag-Ccr4b expression plasmids. After immunoprecipitation with anti-Flag antibodies, bound proteins were eluted and assayed for deadenylase activity using a 5’ Fluorescein-labelled RNA substrate. The asterisk indicates the presence of a cross-reactive band. (B) Expression of Ccr4b lacking the LRR domain inhibits cell proliferation. Cells were transfected with empty vector or the indicated Flag-Ccr4b expression plasmids. The percentage of cells in S-phase as a measure of cell proliferation was determined using the thymidine analogue EdU and fluorescence microscopy. *** p<0.001 (compared to empty vector control). (C) Expression of Ccr4b does not interfere with cell viability. Cells were transfected with empty vector or the indicated Flag-Ccr4b expression plasmids. Cell viability was determined using propidium iodide exclusion and flow cytometry.
Figure 6. Ccr4a/Ccr4b and Caf1a/Caf1b regulate distinct gene sets.

(A) Diagram of hierarchical clustering of gene expression profiles of MCF7 cells treated with the indicated siRNA pools. Probes are represented vertically, while conditions are shown horizontally. The sub-set of probes were selected on the basis of the expression profiles (>1.50 fold differential expression compared to control non-targeting siRNA pool, p-value <0.050). Hierarchical clustering was carried out using the Comprehensive R based Microarray Analysis tool CARMAweb (https://carmaweb.genome.tugraz.at/carma/). (B) Matrix of Pearson’s correlation coefficients of gene expression profiles as indicated. (C) Venn diagram showing limited overlap between the set of upregulated genes in Ccr4a/Ccr4b and Caf1a/Caf1b knockdown cells. (D) Venn diagram showing limited overlap between the set of downregulated genes in Ccr4a/Ccr4b and Caf1a/Caf1b knockdown cells.
Figure 7. Identification of Ccr4a/Ccr4b target genes.

(A) Confirmation of mRNA target genes of Ccr4a/Ccr4b. mRNA levels of the indicated genes were detected using RT-qPCR using GAPDH as a reference gene. All assays were carried out in triplicate. (B) Measurement of mRNA stability of Ccr4a/Ccr4b target genes. Actinomycin D was added (72 h after siRNA transfection), and total mRNA was isolated at 0, 3, 6 and 12 h after treatment. mRNA transcript levels were determined by RT-qPCR using GAPDH as a reference gene. mRNA half-lives were derived from the slope of the fitted line \[ \text{[mRNA]} = 100 \times e^{(-k \times t)} \], where \([\text{mRNA}]=100\% \text{ at } t=0\), k is the decay constant, and the half life \(t_{1/2}\) is given by \(t_{1/2} = \ln(2)/k\). (C) Increased protein levels of p53 and p53 K120ac in MCF7 cells upon Ccr4a/Ccr4b knockdown. MCF7 cells were transfected with control, Caf1a/Caf1b, or Ccr4a/Ccr4b siRNA and analysed by immunoblotting. (D) Quantitative immunoblotting of p53, p53 K120ac, and p27/Kip1 protein levels relative to \(\beta\)-tubulin. Signal intensities were measured (duplicates) and analysed in ImageJ. Error bars indicate SEM. (E) Increased cellular senescence upon knockdown of Ccr4a/Ccr4b. MCF7 cells were treated with control, Caf1a/Caf1b or Ccr4a/Ccr4b siRNA and stained for \(\beta\)-galactosidase activity. * p<0.05. The error bars represent SEM.