RC3H1 post-transcriptionally regulates A20 mRNA and modulates the activity of the IKK/NF-κB pathway

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The RNA-binding protein RC3H1 (also known as ROQUIN) promotes TNFα mRNA decay via a 3'UTR constitutive decay element (CDE). Here we applied PAR-CLIP to human RC3H1 to identify ~3,800 mRNA targets with >16,000 binding sites. A large number of sites are distinct from the consensus CDE and revealed a structure-sequence motif with U-rich sequences embedded in hairpins. RC3H1 binds preferentially short-lived and DNA damage-induced mRNAs, indicating a role of this RNA-binding protein in the post-transcriptional regulation of the DNA damage response. Intriguingly, RC3H1 affects expression of the NF-κB pathway regulators such as IκBα and A20. RC3H1 uses ROQ and Zn-finger domains to contact a binding site in the A20 3'UTR, demonstrating a not yet recognized mode of RC3H1 binding. Knockdown of RC3H1 resulted in increased A20 protein expression, thereby interfering with IκB kinase and NF-κB activities, demonstrating that RC3H1 can modulate the activity of the IKK/NF-κB pathway.
post-transcriptional regulation of gene expression by RNA-binding proteins (RBPs) controls a variety of cellular processes. Especially, the modulation of messenger RNA (mRNA) stability is of critical importance for the dynamic regulation of genes such as transcription factors and cytokines that need to be switched on and off rapidly1,2.

Roquin is an RBP with a central role in repressing autoimmunity3. Originally, a missense mutation in the Roquin-1 gene encoding the Roquin-1 protein was identified as the cause of systemic lupus erythematosus-like autoimmunity phenotype in sarroque mice4. Roquin-1 is localized in cytoplasmic granules5,6 and binds to the 3' untranslated region (3'UTR) of inducible costimulator (ICOS) mRNA to post-translationally repress its expression7,8. Furthermore, Roquin-1, as well as its parologue Roquin-2, interacts with 3'UTR of TNFRSF4 and tumour-necrosis factor-α (TNFα), and modulates immune responses9,10. Recent studies showed that Roquin proteins interact through their ROQ and CCCH-type Zn-finger domains, indicating a yet unrecognised CDE and can also bind to duplex RNA. In addition to the ROQ domain, RC3H1 possesses an N-terminal RING finger with a potential E3 ubiquitin–ligase function11, as well as a CCCH-type zinc (Zn) finger that is involved in RNA recognition12. CCCH-type Zn-finger RBPs typically contact AU-rich sequences can mediate Roquin-dependent regulation. Similarly, Tan et al.11 and Schuetz et al.12 reported that the ROQ domain of Rc3h1 recognizes the CDE and can also bind to duplex RNA. In addition to the ROQ domain, RC3H1 possesses an N-terminal RING finger with a potential E3 ubiquitin–ligase function13, as well as a CCCH-type zinc (Zn) finger that is involved in RNA recognition12. CCCH-type Zn-finger RBPs typically contact AU-rich elements14,15. AU-rich elements are conserved cis-regulatory elements, originally discovered in the 3'UTRs of short-lived mRNAs, encoding inflammatory mediators16-18.

To obtain a better understanding of the molecular mechanisms of human RC3H1 RNA recognition and disentangle its cellular function, we applied PAR-CLIP (photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation)19 to identify RC3H1 mRNA binding sites and target transcripts in HEK293 cells. RC3H1 contacts mRNAs through structure-sequence elements located in 3'UTRs. The binding sites are composed of hairpins with variable loop length often with embedded U-rich sequences, including CDE consensus sequences. RC3H1-bound mRNA targets are short-lived, and RC3H1 depletion results in decreased mRNA decay rates and increased protein synthesis of its target mRNAs. RC3H1 target transcripts are enriched for mRNAs that are induced upon DNA damage, among them are the mRNA of A20 (also known as TNFAIP3). A20 codes for an ubiquitin-editing enzyme, which inhibits activation of NF-κB20,21. In vitro and in vivo experiments revealed that RC3H1 interacts with a non-CDE-type stem–loop structure preceded by an AU-rich sequence in the A20 3'UTR involving ROQ and CCCH-type Zn-finger domains, indicating a yet unrecognized RC3H1-binding mode and specificity. Depletion of RC3H1 leads to increased A20 protein expression, which is accompanied by decreased IkB kinase (IKK) activation and NF-κB DNA-binding activity upon TNFα signalling.

Results
Human RC3H1 binds to thousands of mRNAs. To identify RC3H1-binding sites at high resolution, we applied PAR-CLIP in combination with next-generation sequencing17,19. In PAR-CLIP experiments, nascent RNA is metabolically labelled with the non-perturbing photoreactive ribonucleosides 4-thiouridine (4SU) or 6-thioguanosine (6SG). Crosslinking of protein to 4SU- or 6SG-labelled RNA leads to specific T to C or G to A transitions, respectively, that occur at high frequency in complementary DNA (cDNA) sequence reads and mark the protein crosslinking sites on the target RNA19. HEK293 cells stably expressing inducible FLAG/HA-tagged RC3H1 (Supplementary Fig. 1a) were crosslinked after labelling of RNA with either 4SU or 6SG. Immunopurified, ribonuclease-treated and radiolabelled RC3H1–RNA complexes were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1a). Protein-protected RNA fragments were recovered and converted into a cDNA library amenable to Illumina sequencing.

In total, we performed three independent PAR-CLIP experiments (two biological replicates with 4SU, 4SU-1 and 4SU-2, and one replicate with 6SG; see Supplementary Table 1). Sequence reads were mapped to the human genome and overlapping reads were used to build RC3H1-binding clusters22. In PAR-CLIP experiments using 4SU, diagnostic T to C transitions detected in mapped reads were most highly abundant (Fig. 1b and Supplementary Fig. 1b). Similarly, but less pronounced, the diagnostic G to A changes were the most abundant type of mutation for the 6SG PAR-CLIP experiment (Supplementary Fig. 1c). A length histogram of RC3H1 PAR-CLIP clusters shows a median cluster size of ~25–30 nucleotides (Supplementary Fig. 1d).

We identified ~2,000–4,000 RC3H1 mRNA target transcripts in each of the 4SU PAR-CLIP experiments (Fig. 1c). Ninety-three per cent of the 481 6SG PAR-CLIP mRNA targets were reproduced in 4SU libraries (Fig. 1c). We combined the ‘reads’ from all PAR-CLIP experiments to derive a set of consensus binding sites supported by reads from at least two out of three experiments (see Methods section). Based on this analysis, we identified 16,234 RC3H1-binding sites on 3,821 protein-coding transcripts as consensus data set (Supplementary Data 1). The binding sites and PAR-CLIP sequence alignments are publicly available at http://bimsb-sta.mdc-berlin.de/landthaler/RC3H1. The position with the highest number of PAR-CLIP-derived diagnostic nucleotide transitions for each binding site was referred to as the preferred crosslinking site.

To gain an insight into the transcripts regulated by RC3H1, genes encoding RC3H1-bound mRNAs were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) term enrichment analysis23,24. Interestingly, cell cycle and p53 signalling pathway were overrepresented in the KEGG pathway enrichment analysis (Supplementary Fig. 1e), suggesting that RC3H1 could play a role in the response to DNA damage. Furthermore, GO term enrichment analysis showed that RC3H1-bound transcripts are highly enriched for regulators of gene expression such as transcription factors, RBPs and ubiquitin ligases (Supplementary Fig. 1f). In addition, when comparing the human RC3H1 targets with mouse Roquin-bound transcripts25, we identified 36 out of 55 Roquin-interacting mRNAs by PAR-CLIP in HEK293 cells (Supplementary Fig. 1g).

RC3H1-binding sites are mostly located in 3'UTR of mRNAs. Next, we examined the distribution of RC3H1-binding sites along mRNA transcripts. The majority of binding sites (81%) were...
found to be located in 3′UTRs (Fig. 1d), consistent with previous observations that RC3H1 binds to ICOS and TNFα mRNAs through 3′UTR interactions.⁶,⁷ RC3H1 binding to ICOS and TNFα mRNAs in HEK293 cells was not observed likely due to undetectable ICOS and TNFα mRNA expression. A preference for RC3H1 binding along 3′UTRs of target transcripts was not apparent, since binding clusters were almost equally distributed over this transcript region (Supplementary Fig. 1h). Since a previous study suggested a functional link between RC3H1 binding and microRNA (miRNA) activity⁶, we examined the local interactions between RC3H1 and miRNA by computing the density of conserved miRNA target sites around RC3H1 preferred crosslinking sites (Supplementary Fig. 1i). The observed profile indicated an overrepresentation of miRNA seed complements in the vicinity of RC3H1-binding sites, but did not directly overlap with these sites.

**U-rich hairpins dominate as recognition features.** To investigate RNA features recognized by RC3H1, we searched for sequence and secondary structure elements in RC3H1-binding sites. First, we examined 7mer occurrences in 41 nucleotide windows centred on preferred crosslinking sites in RC3H1-binding sites. Notably, U-rich sequences were frequently found in RC3H1-binding sites derived from both 4SU and 6SG experiments (Fig. 2a and Supplementary Fig. 2a), suggesting that the frequent observation of U-richness is not owing to a bias introduced by using 4SU. These U-rich 7mers were overrepresented in RC3H1-binding sites when compared with control 7mers (Fig. 2b). In contrast, U-rich elements were not enriched in IGF2BP1-binding sites⁹, whereas 7mer containing the CAU consensus sequence were overrepresented (Fig. 2b). U-rich sequences with interspersed adenosines were more enriched in RC3H1 consensus binding sites in 3′UTR sequences when compared with U-rich 7mer sequences containing guanosines (Supplementary Fig. 2b and Supplementary Data 2). Similar results were also obtained from 5mer analysis (Supplementary Fig. 2c). In addition, U-rich sequences were found in close proximity of preferred crosslink sites, suggesting the direct interaction of RC3H1 with these sequences (Fig. 2c). In addition, we examined the occurrence of the previously identified CDE motif⁹, and found that the core CDE consensus sequence (UCYRYGA) was present in RC3H1-binding sites, but the frequency was less prominent than that of several U-rich sequences (Fig. 2c).

To examine potential secondary structure features in RC3H1-binding sites, we computationally folded 41-nucleotide sequence stretches centred around the preferred crosslinking sites and averaged the resulting base pairing probabilities. Randomly selected RNA regions of the same length within 3′UTRs of RC3H1 mRNA target transcripts served as a background control. In 3′UTR RC3H1 consensus binding sites, the base pairing probability was reduced in the vicinity of the crosslink sites and increased in the flanking region compared with...
background, suggesting that RC3H1-binding sites tend to form stem–loop structures (Supplementary Fig. 2d,e). A more detailed examination of the types of hairpin structures enriched in RC3H1 binding revealed an overrepresentation of stems capped by trinucleotide loops as demonstrated for the CDE motif9–12, but also hairpins with loops containing 4 and 5 nucleotides (Fig. 2d). From this analysis, we concluded that there might be several binding motifs that are likely to be structured. To test this hypothesis, we applied a variant of an approach that was previously used to detect structured RNA motifs 25. Here we
performed an initial clustering of the set of RNAs, followed by the detection of a specific structure. We started with the top 100 RC3H1 PAR-CLIP-binding sites, and detected initial clusters by LocARNAs²⁶,²⁷ and RNAclust²⁷. For each subcluster, we used CMfinder (version 0.2; ref. 28) to search for a subset of sequences that has a specific sequence-structure motif. CMfinder generates both a sequence-structure alignment (referred to as seed alignment) and a covariance model, which we used to search for further sequences in the top 1,000 binding sites for remote members of this motif using csmsearch²⁹. The seed alignment of motif 1 (present in 177 out of 1,000 binding sites) and motif 2 (present in 268 out of 1,000 binding sites), representing the most frequently occurring sequence-structure elements, is shown in Fig. 2e. Interestingly, in motif 2, the preferred crosslinked nucleotides are positioned upstream of the predicted hairpin structure, whereas in motif 1 the preferred crosslinked nucleotides are located in the U-rich loop. In summary, our computational analyses did not reveal a defined motif. However, hairpin structures frequently containing U-rich sequences and, albeit, less frequently, the CDE consensus sequence were detected as possible recognition elements of RC3H1.

**Figure 3** | RC3H1 recruits deadenylation complex and destabilizes target mRNAs. (a) A scatter plot of identified peptide counts in two label-swap replicates. Peptides eluted from immunopurified FLAG/HA-tagged RC3H1 complex are analysed by tandem LC-MS/MS. High dose of RNaseT1/RNaseI are treated before immunoprecipitation (IP) to disrupt the indirect interactions mediated by nascent RNA. Peptides derived from the CCR4-CAF1-CNOT deadenylase complex were detected. (b) RC3H1 interactions were confirmed by co-transfection of myc-RC3H1 expression construct with HA-CNOT1, HA-CNOT8 or HA-QUAKING (QKI) expression constructs. IP was performed using anti-myc antibody. IPed proteins were resolved on SDS–PAGE, blotted and probed with anti-myc and anti-HA antibodies. Protein expression in cellular extract used as input for IP experiments is indicated (IN). (c) A cumulative distribution function (CDF) plot of log2-fold changes of mRNA decay rates of the top 500 normalized RC3H1-bound mRNAs is shown in red and all expressed mRNAs is shown in black. Top RC3H1-bound mRNAs show slower mRNA decay rates compared with all mRNAs upon RC3H1/RC3H2 knockdown. The mean difference in mRNA decay rates (siRNA/mock) for the top 500 normalized RC3H1-bound mRNAs (n = 500) and all mRNAs (n = 15,158) are 0.001 and 0.074, respectively (P value < 2.2e–16, Wilcoxon’s rank sum test). (d) A CDF plot of log2-fold changes of protein synthesis of consensus RC3H1 target transcripts that have >100 transitions on 3′UTR is shown in red and non-targets is shown in black after siRNA-mediated RC3H1 depletion. Protein synthesis of RC3H1-bound mRNAs was upregulated upon RC3H1 knockdown (P value 0.0031, Wilcoxon’s rank sum test). The mean log2-fold changes for RC3H1 targets (n = 390) and non-targets (n = 1,279) are 0.001 and –0.116, respectively.

**RC3H1 interacts with CCR4-CAF1-NOT deadenylase complex.** To further investigate the molecular function of RC3H1, we set out to identify proteins that interact with RC3H1. Previous studies showed that RC3H1 destabilizes mRNA albeit by different molecular mechanisms. Glasmaicher et al.⁷ reported that RC3H1 interacts with mRNA decapping proteins, whereas Leppek et al.⁹ more recently showed that roquin protein associates with CCR4-CAF1-NOT deadenylase complex.

To identify proteins that directly interact with RC3H1 in an RNA-independent manner, cellular extracts of control and FLAG/HA-tagged RC3H1-expressing cells were treated with RNaseT1/I, and the immunoprecipitates were analysed and compared by SILAC (stable isotope labelling by amino acids (aa) in cell culture)-based quantitative mass spectrometry.³⁰ As a biological replicate, we performed a label-swap experiment with reversed light/heavy isotope labelling (Fig. 3a). As expected, RC3H1 was efficiently immunoprecipitated as indicated by the log2 heavy-to-light normalized ratio of 2.80 in the forward experiment and —5.13 in the reverse experiment (Supplementary Fig. 3a). In two biological replicates, we identified numerous peptides originating from components of the CCR4-CAF1-NOT deadenylase complex (present in 268 out of 1,000 binding sites), representing the most frequently occurring sequence-structure elements, is shown in Fig. 2e. Interestingly, in motif 2, the preferred crosslinked nucleotides are contained in the U-rich loop. In summary, our molecular mechanisms. Glasmacher et al.⁷ reported that RC3H1 destabilizes mRNA albeit by different molecular mechanisms. Glasmaicher et al.⁷ reported that RC3H1 interacts with mRNA decapping proteins, whereas Leppek et al.⁹ more recently showed that roquin protein associates with CCR4-CAF1-NOT deadenylase complex.
deadenylase complex including CNOT1, CNOT2, CNOT3, CNOT7 and CNOT8, but not from the decapping complex (Fig. 3a, Supplementary Fig. 3a and Supplementary Table 2). CNOT1 is the scaffold subunit and CNOT8 is a catalytic deadenylase subunit. Interestingly, for proteins in the CCR4-CAF1-NOT deadenylase complex, we obtained SILAC ratios of ~1 suggesting that RC3H1 interacts only transiently with these proteins (Supplementary Fig. 3a). Specific interactions of RC3H1 with CNOT1 and CNOT8, but not with the RBP QKI, were confirmed by co-immunoprecipitation experiments in agreement with previous findings (Fig. 3b), indicating that RC3H1 generally acts as mediator of mRNA deadenylation.

**RC3H1 destabilizes target mRNAs.** To assess whether RC3H1 interacts with short-lived mRNA transcripts, we performed transcriptome-wide mRNA half-life measurements (Supplementary Data 3) as described by Dölken et al., and compared half-lives of RC3H1-bound and unbound mRNA transcripts. Consistent with the interaction of RC3H1 with the deadenylase complex and a possible role in mRNA decay, RC3H1-targeted mRNAs were found to have shorter half-lives than expressed non-target transcripts (Supplementary Fig. 3d,e). On the other hand, IGF2BPI-bound transcripts do not show this tendency (Supplementary Fig. 3d,e). Furthermore, we found that mRNA half-lives of RC3H1-bound transcripts inversely correlated with an expression normalized PAR-CLIP score (Supplementary Fig. 3f), suggesting that the extent of RC3H1–mRNA binding determined the mRNA half-lives of bound mRNAs (Supplementary Data 4).

To examine the impact of RC3H1 on the decay rate of its mRNA targets, we sequenced mRNA of untreated and RC3H1- and RC3H2-depleted cells (Supplementary Fig. 3g) after inhibition of translation using actinomycin D. Indeed, insertion of a 30-mer crosslinked region into a green fluorescent protein reporter and assayed mRNA turnover by quantitative reverse transcription–PCR (qRT–PCR) after blocking transcription using actinomycin D. Indeed, insertion of the RC3H1-bound A20 site into the 3′UTR of a conserved predicted hairpin and in an AU-rich region located upstream of the stem-loop structure, which differs from the previously described CDE (Fig. 3a).

To examine whether the putative A20-binding site bestows RC3H1-dependent mRNA decay, we cloned a 37-bp sequence covering the crosslinked region into a green fluorescent protein (GFP) reporter and assayed mRNA turnover by quantitative reverse transcription–PCR (qRT–PCR) after blocking transcription using actinomycin D. Indeed, insertion of the RC3H1-bound A20 site into the 3′UTR of the reporter construct destabilized reporter transcripts in mock-transfected cells, but not in RC3H1- and RC3H2-depleted cells (Fig. 3b), indicating that RC3H1 proteins destabilize the reporter transcripts through this A20 site.

To further examine the RC3H1 interaction with the putative A20 site, we used electrophoretic mobility shift assays (EMSA). In addition, to assess the contribution of the different RC3H1 domains to RNA binding, we expressed two variants: RC3H1-N1 (aa 2–399) contained the N-terminal RING and ROQ domains, whereas RC3H1-N2 (aa 2–452) harboured RING, ROQ and CCCH-type Zn-finger domains (Fig. 3c). Both recombinant proteins bound to the ICOS CDE-like stem-loop motif RNA (Fig. 3c). The formation of the protein–RNA complex seemed to be independent of the CCCH-type Zn-finger domain. In contrast, the 21-nucleotide A20 hairpin RNA was bound by RC3H1-N2 with higher affinity than RC3H1-N1 (Fig. 3c), indicating that the CCCH-type Zn-finger domain plays a role in the interaction with the non-CDE-type A20 site. The addition of 16 nucleotides 5′ of the stem-loop structure (A20 37 nucleotide) further increased the affinity of the RC3H1-N2 variant to the RNA substrate (Fig. 3c), suggesting that an additional sequence upstream of the A20 hairpin is involved in protein–RNA complex formation. To further study the specificity of RC3H1 interaction to its A20 stem-loop hairpin, we performed EMSA using stem-loop hairpin or variants thereof (Fig. 3d). A single-nucleotide substitution in the loop region virtually did not affect binding; however, substitution of three nucleotides resulted in slight reduction of binding (Fig. 3d). In contrast, a control sequence, which was generated by concatenating three 7mers underrepresented in our 7mer analysis, did not bind to RC3H1, indicating a specificity for the A20 hairpin (Fig. 3d). Moreover, the antisense LNA oligonucleotide, which was used to modulate A20 mRNA stability (Fig. 3e) and hybridizes to the loop and the 3′part of the stem,
reduced the binding of RC3H1-N2 to the 37-nucleotide A20 target sequence, suggesting that the specific blockade of both loop and stem pronouncedly reduced RC3H1 binding to A20 3′UTR (Fig. 5e).

**RC3H1 represses A20 and modulates the NF-κB pathway.** Notably, RC3H1-dependent mRNA regulation affects several NF-κB pathway regulators. A recent transcriptome-wide RIP-seq study determining RC3H1-bound transcripts in mouse macrophages revealed the cytokine TNFα—a typical activator of NF-κB—and two members of the IκB family, IκBNS (NFκBID) and IκB-zeta (NFκBIZ). In addition to the ubiquitin-editing enzyme A20, we identified IκBα in our PAR-CLIP as an additional RC3H1 target transcript (Fig. 6a,b).

![Figure 4](https://example.com/figure4.png)

**Figure 4 | RC3H1 target transcripts are enriched for mRNAs induced upon DNA damage, and RC3H1 negatively regulates A20 at the post-transcriptional level.** (a) A scatter plot of mRNA expression levels of untreated cells and cells treated for 4 h with 200 ng ml⁻¹ of neocarzinostatin (NCS). The data was retrieved from Elkon et al.34. RC3H1 3′UTR target transcripts are shown in red and non-targets are shown in black. Among the RC3H1 targets, A20 was the most differentially expressed mRNA upon DNA damage. (b) A cumulative distribution function (CDF) plot of log2-fold changes upon DNA damage is shown for RC3H1 3′UTR targets in red and for non-targets in black (P value < 2.2e-16, Wilcoxon-rank sum test). (c) RC3H1 induction by doxycycline treatment specifically leads to reduced expression of A20 at each time point. mRNA expression level of A20 and GAPDH (negative control) were measured by qRT-PCR. Percentage of A20 mRNA amount at each time point relative to starting point is shown. Error bars indicate s.d.’s calculated from three replicates. (d) Transfection of antisense LNA oligonucleotide targeting the stem-loop structure in HEK293 cells leads to decreased A20 mRNA decay (red) in comparison with control (Ctr) LNA transfection (black). A representative data from two independent experiments are shown.
Supplementary Fig. 5b). Furthermore, RC3H1 induction caused a reduction of basal and stimulus-dependent A20 mRNA and protein levels (Fig. 6b,c), similar to A20 expression changes observed during the response after DNA damage (Fig. 4c).

Next, we asked whether RC3H1 could modulate NF-κB activity. NF-κB activation is mediated via the IKK complex, which catalyses the phosphorylation of IκB and NF-κB proteins, as well as of other substrates. Signalling involves

Figure 5 | RC3H1 binds to a composite structure-sequence motif in the A20 3’ UTR mediated by the CCCH-type Zn-finger domain. (a) Illustration of the RC3H1-binding site in the A20 3’ UTR. The binding sites of RC3H1 in the 3’ UTR of A20 is shown in red and zoomed in below. T to C transitions for indicated base positions are shown. Bases shown in red are forming a potential stem. Phastcon vertebrate conservation is shown in green. RC3H1-binding site in the A20 3’ UTR contains a stem–loop structure flanked by AU-rich sequences. (b) The effect of A20 AU-rich element (ARE)-stem–loop hairpin (37 nucleotide (nt)) was assayed by transiently transfecting HEK293 cells with the d2GFP reporter plasmid, which contains the 37-nt sequence inserted into the 3’ UTR of d2GFP. mRNA decay of the reporter transcripts were measured in mock and RC3H1/RC3H2 knockdown cells. Average and s.d.’s (error bar) from three technical replicates are shown. (c) EMSA experiments to examine the binding mode of RC3H1 to the A20 target site. Increasing concentration of recombinant RC3H1-N1 (aa 2–399) or RC3H1-N2 containing an additional CCCH-type Zn-finger domain (aa 2–452) was incubated with radiolabelled ICOS (13 nt), A20 stem-loop (21 nt) and A20 ARE-stem–loop (37 nt), and free RNA was separated from RNA–protein complexes by native PAGE. (d) EMSA experiments to examine the sequence specificity of the A20 stem–loop hairpin. Increasing concentration of recombinant RC3H1-N2 was incubated with radiolabelled wild-type (WT) A20 stem-loop (21 nt), mutated A20 sequences (Mut 1 and Mut 2) as indicated below, or 21 nt control sequence (Mut 3) generated by concatenating three 7mers underrepresented in our 7mer analysis. Mutation in the loop slightly reduces the binding, and the control sequence does not virtually bind to RC3H1-N2. (e) Increasing concentration of antisense LNA oligonucleotide targeting the A20 stem–loop structure impairs the interaction of RC3H1-N2 and 37 nt ARE-stem-loop.
ubiquitin-mediated complex formation of pathway components and is controlled at various levels by negative feedback mechanisms, including ubiquitin-editing enzymes such as A20 (ref. 40).

Indeed, RC3H1-mediated decrease of A20 protein levels led to a significant increase of IKK activation (Fig. 6c), which was repressed by additionally expressing exogenous A20 without 3′UTR (Supplementary Fig. 5c). In line with this, elevated Ser536 phosphorylation of the IKK substrate p65 was observed (Fig. 6c). To investigate the impact of RC3H1 ectopic expression on the kinetics of the NF-κB pathway and to understand the differential effect on IKK versus NF-κB, a mathematical modelling approach was used, including main processes of canonical IKK/NF-κB signalling (Supplementary Fig. 5d). The model parameters were estimated based on the western blot analysis for A20, IκBα and phosphorylated IKK (Fig. 6c), as well as qPCR data for IκBα and A20 mRNA levels (Fig. 6b; for details see Methods section). The model simulations (Supplementary Fig. 5d) confirmed the experimental findings that RC3H1 reduces A20 mRNA and subsequently A20 protein expression, and, due to the attenuated IKK inhibition by A20, leads to an increased IKK activity. In turn, NF-κB activity is slightly increased by RC3H1, resulting in an increased mRNA synthesis of the feedback regulators IκBα and A20. According to the model, enhanced mRNA syntheses are counteracted by the increased mRNA decay mediated by RC3H1 induction. For IκBα, this establishes a compensatory mechanism
resulting in largely unaffected IkBα mRNA and protein levels upon ectopic RC3H1 expression (Fig. 6b,c).

In contrast, knockdown of RC3H1 and RC3H2 in HEK293 cells resulted in a small, but reproducible, upregulation of A20 protein expression (Fig. 6e), which resulted in decreased phosphorylation of IKK (Fig. 6e), decreased phosphorylation of its substrate p65 (Fig. 6e) and reduced NF-κB DNA-binding activity (Fig. 6f). These observations are also reproduced by the mathematical model (Supplementary Fig. 5d) showing that a strengthening of the A20 feedback leads to changes in activated levels of IKK and NF-κB.

Taken together, we could demonstrate that RC3H1 regulates the expression of several NF-κB pathway regulators, thereby modulating IKK and NF-κB activity.

Discussion

In the present study, we identified transcriptome-wide RNA-binding sites of human RC3H1 at nucleotide resolution in HEK293 cells using PAR-CLIP. Our bioinformatic analyses did not reveal a well-defined motif as observed for sets of RBPs41; however, indicated several classes of sequence-structure binding elements with U-rich sequences frequently embedded in RNA stem–loop structures in 3’UTRs of target transcripts. Surprisingly, the CDE core consensus motif (UCYRYGA) deduced by Leppke et al.3 was present only in a minor fraction of identified RC3H1-binding sites. Our RC3H1 PAR-CLIP data are also in agreement with the concept of a relaxed CDE revealed by structural and mutational analyses10, which indicated a shape-specific rather than sequence-specific recognition of CDE hairpins by the ROQ domain.

Interestingly, our finding of a PAR-CLIP cluster in the A20 3’UTR indicated a yet unrecognized RC3H1-binding mode and specificity. In contrast to a typical CDE stem–loop motif, which is sufficiently bound by the ROQ domain, we provide evidence that the CCCH-type Zn-finger domain is involved in contacting the hairpin than to the hairpin alone. In contrast, the N-terminal RC3H1 variant lacking the CCCH-type Zn-finger domain poorly bound to both of these RNA substrates. The makeup of RC3H1 by distinct RNA-binding domains might allow the protein to recognize a wider range of RNA structure-sequence elements and could function on a larger set of regulatory elements than previously anticipated. The ratio of sequence and structure specificity features, determining the strength of the RC3H1–mRNA association, and the RNA recognition element frequency would influence the regulatory capacity of the RBP.

In addition, our results indicate that RC3H1 interacts with the CCR4-CAF1-NOT deadenylation complex, and mediates destabilization of RC3H1 target transcripts. RC3H1-bound mRNAs are encoded by genes with various biological functions outside of immune-response pathways, which is in accordance with the mouse phenotype of Rch1 null-knockout that showed perinatal lethality with broad physiological complications12. Enriched KEGG pathways included cell cycle, p53 signalling and tumour pathways. By intersecting our PAR-CLIP target mRNAs with pathway expression data, we found that RC3H1 targets are enriched for mRNAs induced by DNA damage34 and TNFα35. As shown for one of the top mRNA targets, A20, we postulate that RC3H1, in general, is involved in fine-tuning or clearance of transcriptionally induced mRNAs by shortening their half-lives.

Our discovery of RC3H1 binding to A20 mRNA and other TNFα-induced transcripts prompted us to examine and model the impact of RC3H1 on the IKK/NF-κB pathway. Knockdown of RC3H1 and RC3H2 increased the expression of A20 protein expression, resulting in reduced IKK activity and NF-κB DNA-binding activity. Vice versa, we show that induction of RC3H1 results in pronounced increase of IKK phosphorylation. Taken together, RC3H1 targets several components of the NF-κB signalling pathway, and thereby modulates IKK and NF-κB activity. The net impact of alterations in RC3H1 protein activity on IKK and NF-κB activity in different cell types most likely depends on various additional (cell-type) specific parameters. Notably, IKK does not only regulate NF-κB activation but is also engaged in crosstalk with other pathways40.

The Zn-finger protein A20 is an important negative regulator of inflammation41, and several studies have highlighted the clinical and biological importance of A20. Walle et al.42 recently showed that negative regulation of the NLRP3 inflammasome by A20 protects against arthritis. Since RC3H1 is a negative regulator of A20, targeting of the RC3H1–A20 mRNA interaction by using antisense technologies and concomitant upregulation of A20 protein might have beneficial outcomes in certain disease scenarios.

In summary, we identified comprehensive RC3H1-binding sites by PAR-CLIP, revealing a large number of novel mRNA targets as well as novel RC3H1 cis-acting recognition element in the A20 3’UTR. Our study highlights the importance of post-transcriptional regulation of gene expression to control crucial cellular signal transduction pathways.

Methods

Antibodies. For western blots, the following antibodies were used after dilution to 0.5–1 μg ml⁻¹: anti-HA.11 (Covance, 16B12), anti-FLAG (Sigma, F1804), anti-ntyc (Sigma, 9E10) anti-γH2AX(Ustate, JW301), anti-vinulin (Sigma, hV1N), anti-RC3H1 (Novus, NB100–655), anti-A20 (Santa Cruz Biotechnology, sc-32525), anti-IRF3 (Cell Signaling Technology, 2,697), anti-IKKz (BD Pharmingen, 5,532), anti-IκBα (Santa Cruz Biotechnology, sc-371), anti-p65 (Santa Cruz Biotechnology, sc-8008P), anti-p-p65 (Cell Signaling Technology, 3033) and polyclonal goat anti-rat or rabbit immunoglobulins/horseradish peroxidase (Dako).

Oligonucleotides

siRNAs. siRNA 1 for RC3H1: 5’-GCUGGGGAAUAACAAAGGAA[dT][dT]. siRNA 2 for RC3H1: 5’-CCAAGAAUAUGUGUAAGAGA[dT][dT]. RC3H2: 5’-GGAAGAACUGUGUGUAAAGA[dT][dT].

qPCR primers. RC3H1 forward: 5’-GCTGACCTGTTCTTGCATCAG-3’. RC3H1 reverse: 5’-AGGCTCTGGAGCCGACCTTT-3’. A20 forward: 5’-TGCACACTGTGGTTCATCAG-3’. A20 reverse: 5’-AGGCGTGGAGCCGACCTTT-3’. GAPDH forward: 5’-AGGACACCTGCCATACAC-3’. GAPDH reverse: 5’-GCCCAATACGACCAAATACTC-3’. NFKBIA forward: 5’-GAAGTCGACGAGGCGGAGT-3’. NFKBIA reverse: 5’-GATTGTCTTCGAGGCTTTG-3’. d2GP forward: 5’-GAAGCTTAGCAGTGCTCC-3’. d2GP reverse: 5’-GATGAGCOCGACATCACTATGTG-3’. DNA oligos for d2GP-A20 3’UTR reporter. Sense: 5’-GGCTGCTGATATAATATACCTTTACATATATGTTATATGTTATATGTT-3’. Antisense: 5’-TCGGAAAAATTCCTCCATACATATTGTAAAGGGATATATATTATGTAGCA-3’.

RNA oligos. ICOS (15 nucleotide): 5’-AUAUCUGUGAAAU-3’. A20 (21 nucleotide): 5’-CCCUAACUAUUAUGUGAGG-3’. A20 (37 nucleotide): 5’-AUUAAUAAUAUCAACCUUUACUAUUGUAUGAGAAAU-3’. Mut1 (21 nucleotide): 5’-UUUCCUUAAUUAUGUGAGAGG-3’. Mut2 (21 nucleotide): 5’-UUUCCUUAAUAUGUGAGAGG-3’. Mut3 (21 nucleotide): 5’-UUUCCUUAAUUGCCCCUCCAGC-3’.

Plasmids. pENTR4 constructs were generated by PCR amplification of the RC3H1 and QK5 coding sequences from cDNA followed by restriction digest and ligation into the pENTR4 (Invitrogen) vector, which was further recombined into the pREP7/F/FLAG/HA-DEST destination vector43 using pENTR4-DEST vector. NATURE COMMUNICATIONS | DOI: 10.1038/ncomms8367 | www.nature.com/naturecommunications © 2015 Macmillan Publishers Limited. All rights reserved.
GATEWAY LR recombine (Invitrogen) according to manufacturer’s protocol. Expression plasmids for HA-tagged CNOT1 and CNOT8 were kind gifts from Dr W. Filipowicz. pENTR4 QKI5 was recombined into pFRT/FLAG/HA-DEST (Addgene ID: 26,360). The d2GFP reporter plasmids were generated by cloning the crosslink site. To define the consensus clusters, we pooled reads from all three experiments while ensuring that transition events are counted appropriately (T to C only in reads originating from 4SU experiments and G to A only in reads from the 6SG experiment). Before the cutoff determination, clusters had to pass an additional criteria of demanding that less than 10% of reads support the cluster. The resulting sets of clusters were denoted as the ‘consensus’ set. Read alignment statistics, cluster length distribution, target gene identification, cluster distribution, cluster coverage profiles, conservation profile and miRNA target scores are provided by the PAR-CLIP analysis pipeline with similar settings. KEGG pathway and GO term enrichment analysis was performed using the on-line DAVID programme. The top 1,000 transcripts (ranked by the number of PAR-CLIP diagnostic mutations falling into 3’UTR) were used for pathway enrichment analysis.

**Motif analysis.** 7mer occurrences were counted in 41 nucleotide windows around the crosslink identified in the 4SU and 6SG PAR-CLIP experiments using custom Perl scripts. To examine the enrichment of each 7mer motif, 7mer frequency occurring in RC3H1 consensus 3’UTR-binding sites was compared with that occurring in all 3’UTR sequences retrieved from UTRdb. The 3’UTR sequence for each gene was used in this analysis. To test whether RC3H1-binding sites are preferentially secondary structure, we used the library routines from the Vienna RNA package 1.8.2 (ref. 49) to compute base pairing probabilities within 41 nucleotide sequences centered on the preferred crosslink positions of 3’UTR-binding sites. The resulting profiles were accumulated and averaged over all 3’UTR consensus binding sites or the negative control 41 nucleotide sequences randomly selected from the top 1,000 3’UTRs of R. The target transcript enrichment analysis was done based on the output from RNAfold programme in the Vienna RNA package. For the clustering of structured motifs, we started with the top 1000 (ranked by number of diagnostic transition events divided by expression value) RC3H1-binding sites (41 nucleotide length sequence centered around the preferred crosslink) identified by PAR-CLIP and determined by RNAfold and ARNA (version 1.7.16; refs 26,27) together with RNAclust (version 1.3; ref. 27) to produce a hierarchical tree. RNAclust (which uses LocARNa) was run using default parameters together with the RNAsoup option (/RNAsoup.pl –fasta top1000_sequences.fasta –dir output_dir / –RNAsoup). For each subcluster, we checked for the presence of a subset of sequences that has a conserved sequence-structure motif. CMfinder generates both a sequence-structure alignment (called seed alignment) as well as a covariance model, which we used to search for further sequences in the top 1,000 binding sites for remote members of this motif using cmsearch from the Infernal package (version 1.0.7; ref. 29). We then cut the hierarchical tree at the point where we got structured motifs with the largest coverage in the list of top 1,000 binding sites, which resulted in three structured motifs. We kept two motifs (motifs 1 and 2) and discarded the third one, since its seed alignment consisted of only five entries.

**sIRNA knockdown and pSLAC.** Flp-In 293 T-REx cells were grown in SILAC medium supplemented with ‘light’ labelled amino acids before siRNA knockdown experiments. siRNAs were transfected at a final concentration of 50 nM using Lipofectamine RNAiMAX (Invitrogen). Controls (mock) were treated with transfection reagent only. Following 24h of incubation, siRNA-transfected cells were switched to ‘heavy’ labelled SILAC medium. After 24h of labelling, cells were harvested and equal amounts of siRNA- and mock-transfected cells were pooled, lysed in a urea buffer (8 M urea and 100 mM Tris-HCl, pH 8.3) and submitted for 20s (two pulses, 60% power). Cell debris was removed by centrifugation (14,000g, 5 min). Protein concentration was then measured by the Bradford colorimetric assay. An amount of 100 μg of proteins were reduced in 2 mM DTT for 30 min at 25°C, and successively free cysteines were alkylated in 11 μl iodoacetamide for 5 min at room temperature, and then mixed with 11 μl LysC (Wako) in a ratio 1:40 (w/w) and incubating it for 18h under gentle shaking at 30°C. After LysC digestion, the samples were diluted three times with 50 μl ammonium bicarbonate solution, 7 μl of immobilized trypsin (Applied Biosystems) were added and samples were incubated 4 h under rotation at 30°C. Digestion was stopped by adding 10 μl of 0.2 M HCl, 100 μl of 5% acetonitrile, and 200 μl of 0.1% trifluoroacetic acid (TFA) and then desalted. Fifteen micrograms of digest were desalted on StageTip, dried and reconstituted to 20 μl of 0.5% acetic acid in water. A volume of 5 μl of each sample were injected in duplicate on a Liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (nanoLC-ULTRA 1D (Eksigent)) coupled to LTQ-Orbitrap Velos (Thermo). Using a 240-min gradient ranging from 5 to 40% of solvent B (80% acetonitrile, 0.1% formic acid; solvent A = 5% acetonitrile and 0.1% formic acid). For the chromatographic separation, ~ 25-cm-long capillary (75 μm inner diameter) was packed with 1.8 μm C18 beads (ReproSil-AQ, Dr Maisch). The capillary nanospray tip was generated using a Kinetex Fused-core (10 μm × 100 μm bore) with MicroSprayer (Proxeon) Instrument, allowing flattip processing. The nanospray source was operated with spray voltage of 2.1 kV and ion transfer tube temperature of 260°C. Data were acquired in data-
Quantitative PCR. For the identification of proteins directly interacting with RC3H1, cells are grown in medium supplemented with either light or heavy stable isotope-labelled amino acids. In the forward experiments, FLAG/HA-tagged RC3H1 was expressed only in cells cultured in light medium, and in the reverse experiments the labelling was swapped. Equal amounts of cells were mixed and lysed in three pellet volumes of NP40 lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, 2 mM EDTA (pH 8.0), 0.5% NP40, 1 mM Na3VO4, 0.5 mM DTT and protease inhibitor cocktail). The extracts were treated with 1 unit per µl RNase T1 for 5 min at 22°C to facilitate the immunoprecipitation and incubated with FLAG magnetic beads (Sigma; 50 µl 1 µl-1 cell lysate) for 1 h at 4°C. Beads were washed once with NP40 lysis buffer (containing 0.25 unit per µl RNase T1 for 5 min at 37°C to disrupt RNA-mediated protein interactions. After washing the beads once with FLAG elution buffer (100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, and 10% glycerol), FLAG-HA/RC3H1 complex was eluted by adding 0.5 µg ml−1 FLAG peptide and rotating for 1 h at 4°C. Magnetic beads were then washed twice with 500 µl ice-cold wash buffer I (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5% glycerol and 0.05% NP40) and twice with 500 µl ice-cold wash buffer II (50 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 5% glycerol) and eluted by adding 2 µl M2 A-agarose beads. After washing the columns three times with 800 µl ice-cold wash buffer I (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5% glycerol and 0.05% NP40) and 500 µl ice-cold wash buffer II (50 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 5% glycerol) and eluted by adding 2 µl Anti-FLAG M2 antibody (Sigma) and rotating for 1 h at 4°C. Eluted protein was collected and combined. Labelled RNA was amplified and fragmented using the RNeasy MiniElute Spin columns (Qiagen). Total RNA (1.5 µg) and newly transcribed RNA (280 ng) were amplified and labelled using the Affymetrix One-Cycle Target Labeling Kit according to the manufacturer’s protocol. As newly transcribed mRNA mainly consists of rRNA, it was amplified and labelled according to the manufacturer’s protocol for mRNA. The amplified and fragmented biotinylated cRNA was hybridized to Affymetrix Human Gene 1.0 ST Arrays using standard procedures. Data were processed and analysed with R and Bioconductor. To calculate RNA half-lives, CEL-files of all samples from all conditions (including total RNA, newly transcribed RNA and pre-existing RNA) were normalised together using the GCRMA algorithm. Only probe sets called ‘present’ in all three replicates of all three RNA subsets under study were included in the analysis of transcript half-lives. Calculation of RNA half-lives was done as performed [32,35]. Statistical comparison of half-life values between groups was performed using the Wilcoxon rank-sum test.

Recombinant protein expression and purification. DNA encoding the RING and ROQ domains (RC3H1-N1; aa 2–239) or the RING, ROQ and CCCH-ZnF domains (RC3H1-N2; aa 2–452) was subcloned into the pQlinkH vector[24]. The genes were expressed as N-terminal His-tagged proteins at 17°C in Escherichia coli Rosetta 2 (DE3, (Novagen) using a LEX ultra-high-throughput bench-top bioshaker (Harbinger Biotech Inc.) grown to an OD600 of 2.0–2.5. Cells were harvested and total RNA was isolated using Trizol reagent. Biotinylation of 4SU-labelled RNA was performed using EZ-Link Biotin-HDPPD (N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide) (Pierce) dissolved in dimethylformamide. Biotinylation was carried out in 10 mM Tris (pH 7.4), 1 mM EDTA and 0.2 mM mg ml−1 biotin-HDPPD at a final RNA concentration of 100 ng µl−1 for 1.5 h at room temperature. An amount of 50–100 ng of total RNA was used for the biotinylation reaction. Unbound Biotin-HDPPD was efficiently removed by chlorormform:isooemyl alcohol (2:1) extraction using Phase-lock-gel (heavy) tubes (Eppendorf). Then, a 1/10 volume of 5 M NaCl and an equal volume of isopropanol were added, and RNA was precipitated at 20,000g for 20 min. The pellet was washed with an equal volume of 75% ethanol and precipitated at 20,000 g for 10 min. The pellet was resuspended in 50–100 µl RNAase-free water. After denaturation of RNA samples at 65°C for 10 min followed by rapid cooling on ice for 1 min, biotinylated RNA was captured using μMACS streptavidin beads and columns (Milltenyi). Up to 100 µg of biotinylated RNA were incubated with 100 µl of μMACS streptavidin beads with rotation for 15 min at room temperature. The beads were magnetic and washed in a cold room and eluted with 1 ml 65°C washing buffer (100 mM Tris-HCl (pH 7.4) 10 mM EDTA, 1 M NaCl and 0.1% Tween20) followed by three washes with room temperature washing buffer. To recover the unlabelled pre-existing RNA the flow-through of the first two washes was collected and combined. Labelled RNA was eluted and incubated with 100 µg of freshly prepared 100 µM TTF followed by a second elution 5 min later. RNA was recovered from the washing fractions and eluates using the RNeasy MiniElute Spin columns (Qiagen). Total RNA (1.5 µg) and newly transcribed RNA (280 ng) were amplified and labelled using the Affymetrix One-Cycle Target Labeling Kit according to the manufacturer’s protocol. As newly transcribed mRNA mainly consists of rRNA, it was amplified and labelled according to the manufacturer’s protocol for mRNA. The amplified and fragmented biotinylated cRNA was hybridized to Affymetrix Human Gene 1.0 ST Arrays using standard procedures. Data were processed and analysed with R and Bioconductor. To calculate RNA half-lives, CEL-files of all samples from all conditions (including total RNA, newly transcribed RNA and pre-existing RNA) were normalised together using the GCRMA algorithm. Only probe sets called ‘present’ in all three replicates of all three RNA subsets under study were included in the analysis of transcript half-lives. Calculation of RNA half-lives was done as performed [32,35]. Statistical comparison of half-life values between groups was performed using the Wilcoxon rank-sum test.
parallel, a dilution series of 10 × protein stocks was prepared in 1 × protein dilution buffer (1 × binding buffer and 5 µg ml⁻¹ heparin). For each binding reaction, 2 µl of the 10 × protein stock was added to 18 µl of the mastermix at room temperature for 2 h. After addition of 4 µl 6 × loading buffer (30% glycerol, bromophenol blue and xylene cyanol), RNA-protein complexes were resolved by nondenaturing PAGE (6% polyacrylamide, 0.5 × Tris-borate EDTA (TBE) and 5% glycerol) in ice-cold 0.5 × TBE buffer containing 20 µM ZnSO₄ at 100 V for 40 min. The protein-bound RNA and the free RNA were quantified using a phosphorimager.

To determine NF-κB DNA-binding activity, EMSA was performed according to Hinz et al. using the following protocol. An amount of 4–10 µg of whole-cell lysate was mixed with radioactive-labelled (25,000 cpmp) oligonucleotides containing a NF-κB site (5'–gatCAGGGCTTTGAGCCATTCCATTCACGAC-3’ and 5’–gatCCCTGTGGAGATGGAAAATCCGACGCTC–3’), 2 µg poly(dI- dC), 1 µg BSA and 1 µl DTT in 20 µl reaction buffer (20 µM HEPES (pH 8.4), 60 mΜ KCl and 8% Ficoll). Samples were incubated for 30 min at 25°C and analysed by native PAGE (5% gels; TBE buffer), followed by autoradiography.

**Microarray data processing.** Microarray raw data for DNA damage response and TNFα response were retrieved from GEO accessions GSE16768 and GSE28548, respectively. Robust multi-array average background correction and quantile normalization was applied using affyR Biocomductor packages57. For the analysis of Affymetrix Human Genome U133 Plus 2.0 Array, probe sets mapping to the same gene were averaged to summarize into gene intensities, and genes with log2 steady-state expression level < 5 were filtered out.

**Mathematical modelling of NF-κB pathway.** The computational model of the canonical IKK/NF-κB system is described by an ordinary differential equation system:

\[
A20' = k3 * A20RNA - k5 * A20
\]

\[
A20RNA = k12 * NFxB - k12 * A20RNA + RCHI140
\]

IKKact = TNFα × ϵ × A20 + k2/k1 (A20 - k4) = k5 * IKKact

NF-κB = k16 * NF-κB + k15 * IkBα - k17 * IkBαRNA - k8 * IkBα

IkBαRNA = k13 * NF-κB + k14 * IkBαRNA + RCHI140

NFκB = k15 * IKKα × NF-κB × IkBα × k16 * NF-κB + k15 * IkBαRNA - k16 * IkBα

The western blot data of three replicates for phosphorylated IkBα and IKK upon TNFα stimulation with and without RCHI140 induction were quantified using ImageJ. The mRNA levels of IkBα and A20 upon TNFα stimulation with and without RCHI140 induction were measured by qPCR. The parameters of the model were estimated with the Data2Dynamics software package in MATLAB (R2013b, The Mathworks Inc., Natick, MA) using the build-in function lsqnonlin.

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**Acknowledgements**

We thank all members of the Landthaler laboratory for helpful discussion and critical reading of the manuscript. We thank Dr. Nikolaus Rajewsky (MDC) and members of his laboratory for sharing the PAR-CLIP computational analysis pipeline. Dr. Markus Schueler (MDC) for initial PAR-CLIP analysis, Ouidad Benlasfer (MDC) and Charlotte Stein for technical assistance, and Claudia Langnick and Mirjam Feldkamp from Dr. Wei Chen laboratory (MDC) for sequencing. The Protein Sample Production Facility at the Max Delbruck Center is funded by the Helmholtz Association of German Research Centres. C.S. and J.W. are supported in part by the Federal Ministry for Education and Research (BMBF) CancerSys project 0316047A. As part of the Berlin Institute for Medical Systems Biology at the MDC, the research group of M.L. is funded by the BMBF and the Senate of Berlin, Berlin, Germany. M.S.-S. is supported by SCH2440/3-1. Y.M. is funded by the Deutsche Akademische Austauschdienst.

**Author contributions**

Y.M. performed PAR-CLIP and validation experiments and carried out bioinformatics data analyses. M.H. and C.S. conducted and analysed the NF-kB pathway experiments. J.M. and J.W. performed mathematical modelling of the kinetics of the NF-kB pathway. A.S. and U.H. generated recombinant RC3H1 variants. M.U. and R.B. contributed to motif analysis. E.W., N.B., C.C.F. and L.D. performed and analysed mRNA half-life measurements. E.W. and T.Y. contributed to experiments and data analysis. G.M. and S.K. carried out mass spectrometry analysis. M.S. conceived the idea to study RC3H1 by PAR-CLIP and provided reagents. Y.M. and M.L. designed the study and wrote the paper. M.H., J.M., M.S.-S., U.H., J.W. and C.S. revised the manuscript. All authors read and approved the manuscript.

**Additional information**

Accession Codes: PAR-CLIP cDNA sequencing and mRNA decay data have been deposited (GEO: GSE69153).

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

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How to cite this article: Murakawa, Y. et al. RC3H1 post-transcriptionally regulates A20 mRNA and modulates the activity of the IKK/NF-kB pathway. *Nat. Commun.* 6:7367 doi: 10.1038/ncomms8367 (2015).