Binding of NUFIP2 to Roquin promotes recognition and regulation of ICOS mRNA

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The ubiquitously expressed RNA-binding proteins Roquin-1 and Roquin-2 are essential for appropriate immune cell function and postnatal survival of mice. Roquin proteins repress target mRNAs by recognizing secondary structures in their 3′-UTRs and by inducing mRNA decay. However, it is unknown if other cellular proteins contribute to target control. To identify cofactors of Roquin, we used RNA interference to screen ~1500 genes involved in RNA-binding or mRNA degradation, and identified NUFIP2 as a cofactor of Roquin-induced mRNA decay. NUFIP2 binds directly and with high affinity to Roquin, which stabilizes NUFIP2 in cells. Post-transcriptional repression of human ICOS by endogenous Roquin proteins requires two neighboring non-canonical stem-loops in the ICOS 3′-UTR. This unconventional cis-element as well as another tandem loop known to confer Roquin-mediated regulation of the Ox40 3′-UTR, are bound cooperatively by Roquin and NUFIP2. NUFIP2 therefore emerges as a cofactor that contributes to mRNA target recognition by Roquin.

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The RNA-binding proteins (RBPs) Roquin-1 and Roquin-2 recognize specific stem-loop structures in the 3′-untranslated regions (3′-UTR) of target mRNAs through their ROQ domain. Binding typically results in post-transcriptional repression of target-mRNA expression through 5′ mRNA degradation. However, the importance and cooperation of other RBPs in this function is still elusive. Drosophila, mouse and human ROQUIN-1 and ROQUIN-2 were described to interact with the CCR4-CAF1-NOT de-adenylation complex. Deadenylation is then coupled to mRNA decapping, followed by 5′ to 3′-directed mRNA decay. It is likely that Roquin induces post-transcriptional repression as part of higher-order messenger ribonucleoprotein particles (mRNPs) that can be regulated in cell-type specific and dynamic ways and differ among the cellular target mRNAs. Especially on long and complex 3′-UTRs, Roquin may interact, synergize or interfere with other post-transcriptional regulators that work in a redundant, cooperative or antagonistic way. Indeed, the 3′ terminal 260 nucleotides (nts) of the TNF 3′-UTR were sufficient to mediate repression by Roquin-1 and the tandem nucleoclease Regnase-1 in a cooperative manner, while other target mRNAs may be regulated by each trans-acting factor individually. Except for Regnase-1, crucial cofactors and collaborating RBPs of Roquin are still unknown.

The two Roquin paralogs are expressed ubiquitously and are also required in cells outside of the adaptive immune system, since mice with systemic deletion of Roquin-1 or Roquin-2 die postnatally. Both proteins serve redundant functions in T cells by regulating T-cell activation and T-helper cell differentiation. The critical importance of Roquin-mediated gene regulation in T cells is underscored by its cleavage by MALT1 downstream of T-cell receptor signaling, which affects multiple target mRNAs with T-cell-specific expression and function. Accordingly, the inducible T-cell co-stimulatory receptor ICOS, whose ~2000 nucleotide-long 3′-UTR harbors cis-elements for several trans-acting factors, was the first identified mRNA target of Roquin-1. In sanroque mice, a point mutation in the ROQ domain of Roquin-1 impairs Roquin function and causes derepression of ICOS already in naive T cells. It has been proposed that inappropriate ICOS expression can explain the development of the severe autoimmunity of sanroque mice although additional deletion of ICOS did not suffice to rescue autoimmunity. Nevertheless, ICOS is important for the expansion and survival of regulatory T cells (Tregs) and effector memory T cells. ICOS signals are also required for the differentiation of follicular helper T cells (Thf) and germinal center B cells. ICOS stimulation induces PI3K activity and Foxo-1 inactivation and was shown to recruit activated CD4+ T cells into the follicle and to be required for the maintenance of a germinal center response. Finally, patients with loss-of-function mutations in ICOS are immunodeficient. The principles of post-transcriptional regulation of ICOS are therefore of considerable interest and the underlying molecular mechanisms may similarly control other, perhaps even unknown mRNA targets of Roquin proteins.

In this study, we identify NUFIP2 as an important cofactor of Roquin-mediated post-transcriptional gene regulation of ICOS. Investigating its functional contribution, we demonstrate that NUFIP2 engages in a direct, high-affinity interaction with Roquin-1 that enables cooperative binding of this complex to tandem stem-loops in the ICOS and Ox40 3′-UTRs. Our data indicate cofactor-dependent target specificity in Roquin-mediated post-transcriptional gene regulation.

Results
Targeted siRNA screening to identify cofactors of Roquin. To search for potential cofactors of Roquin-mediated post-transcriptional gene regulation, we performed a targeted siRNA screen. In a HeLa reporter cell line stably co-expressing ICOS and an inducible Roquin-1-P2A-mCherry open reading frame, we observed strong downregulation of ICOS protein levels after doxycycline-induced Roquin-1 and mCherry expression. siRNA-mediated depletion of Roquin resulted in derepression of ICOS (Fig. 1c, d). The assay was both robust and reproducible, as indicated by a Z′ factor of 0.7 (Fig. 1d), which reflects the suitability of a given assay for high-throughput screening. In the screen, each gene was targeted by a pool of four individual siRNAs, and multiplexed flow cytometry was employed during data acquisition (Supplementary Fig. 1a, b). In total, we screened 1495 siRNAs specifically targeting genes encoding proteins associated with four ontology terms: “RNA-binding proteins,” “F body/stress-granule-related proteins,” “deadenylation-dependent mRNA decay,” and “decapping-dependent mRNA decay” (Supplementary Data 1, Fig. 1e). Specifically, Roquin-1 overexpression downregulated the ICOS reporter to a similar extent in Regnase-1-deficient (Zc3h12a-/-) mouse embryonic fibroblast (MEF) cells (Supplementary Fig. 1e). Moreover, the ICOS 3′-UTR was similarly regulated by overexpression of Regnase-1 in Roquin-deficient and Regnase-1-deficient cells (Supplementary Fig. 1e, f). Together, these results show that the screen identified known genes involved in Roquin-mediated ICOS regulation as well as new candidates.

Validation of NUFIP2 as a cofactor of Roquin. We validated top scoring candidates in the siRNA screen by “deconvoluting” the siRNA pools and testing each individual siRNA. Candidates such as STAUFEN (STAUI) did not pass this validation step (Supplementary Data 1), as only one siRNA reversed ICOS repression (Supplementary Fig. 2a, b), despite the fact that all four siRNAs efficiently depleted STAUI mRNA (Supplementary Fig. 2c). In contrast, these analyses confirmed CNOT1 as a positive control and validated NUFIP2 as a cofactor of Roquin-1-mediated ICOS regulation. For these two targets, multiple siRNAs from the original pool decreased CNOT1 or NUFIP2 target mRNA without diminishing Roquin-1-P2A-mCherry expression and caused derepression of ICOS (Fig. 2a–c). Our CNOT1 results confirm the recently demonstrated impairment of Dm Roquin to induce reporter mRNA degradation in Drosophila cells with CNOT1 deletion. Strikingly, NUFIP2 was among the strongest hits in this screen, and its knockdown derepressed ICOS more effectively than that of CNOT1 (Fig. 3a). Both siRNA pools efficiently decreased target gene expression on mRNA and protein levels (Fig. 3b, c). We further validated NUFIP2 as a cofactor of Roquin through rescuing the effect of the siRNA by overexpressing an siRNA#2-resistant NUFIP2 cDNA (Supplementary Fig. 2d). GFP-NUFIP2- or GFP-overexpressing HeLa reporter cells (Fig. 3d) were transfected with siRNA#2, which only targets the endogenous protein, or siRNA#4, which targets both endogenous and ectopically expressed NUFIP2 (Supplementary Fig. 2d). ICOS surface expression upon doxycycline-induced transcriptional gene regulation.
NUFIP2 expression (Fig. 3e). Imprint GFP-expressing cells within the GFPmed gate the ICOS repression was also not hit. α and the screen work and NUFIP2-encoding mRNAs similarly stabilized the ICOS down. Importantly, the knockdown of ROQUIN- or ROQUIN-cells, which increased to 232 min when NUFIP2 was knocked half-life of ICOS mRNA of 158 min in control siRNA-treated mRNA abundance by quantitative RT-PCR over time revealed a Treating these cells with actinomycin D and measuring ICOS mRNA (Fig.3g, h). The combinations of all three siRNAs equally encoding mRNAs in HeLa cells that stably expressed ICOS and RC3H2 to target both ROQUIN-1- and ROQUIN-2combined the siRNA against NUFIP2 with those against RC3H1 whether this regulation required Roquin proteins. To do so we NUFIP2 as a cofactor of Roquin-mediated ICOS repression.

We next asked how ICOS was regulated by NUFIP2 and whether this regulation required Roquin proteins. To do so we combined the siRNA against NUFIP2 with those against RC3H1 and RC3H2 to target both ROQUIN-1- and ROQUIN-2encoding mRNAs in HeLa cells that stably expressed ICOS mRNA (Fig. 3g, h). The combinations of all three siRNAs equally reduced NUFIP2 and ROQUINencoding mRNAs compared to transfections that targeted the factors individually (Fig. 3g). Treating these cells with actinomycin D and measuring ICOS mRNA abundance by quantitative RT-PCR over time revealed a half-life of ICOS mRNA of 158 min in control siRNA-treated cells, which increased to 232 min when NUFIP2 was knocked down. Importantly, the knockdown of ROQUIN- or ROQUIN- and NUFIP2-encoding mRNAs similarly stabilized the ICOS mRNA to a half-life of 345 or 360 min, respectively (Fig. 3h). This indicates that NUFIP2 cooperates with ROQUIN to induce ICOS mRNA decay, as the effectiveness of NUFIP2 to induce degradation of ICOS mRNA depended on the presence of ROQUIN proteins (Fig. 3h).

**Molecular determinants of NUFIP2 function.** For the detection of NUFIP2, we established two different monoclonal antibodies, 23G8 and 14G9, for western blotting and immunoprecipitation (Supplementary Fig. 2e, f) or intracellular staining and flow cytometry, respectively (Supplementary Fig. 2g, h).

Roquin function is associated with its localization in RNA granule-like structures. In the absence of cell stress, the protein is enriched in P bodies15. In response to arsenite-induced oxidative stress, Roquin relocates to stress granules15,33,34. As a biochemical assay that mimics aggregation of proteins in granule-like structures, the chemical biotinylated isoxazole (b-isox) precipitates constituents of stress granules like FMRP, FXR1P and FXR2P from cell lysates35. Since NUFIP2 has been described to interact with Fmrp in cells36, we investigated aggregation of Roquin, Nufip2, and Fmrp by incubating MEF cell lysates with increasing concentrations of b-isox. Treatment and subsequent centrifugation localized the Fmrp protein to the pellet, depleting it from the supernatant at all b-isox concentrations (Fig. 4a). Nufip2 precipitated at 1 µM b-isox, but unlike Fmrp was not depleted from the supernatant. The Roquin proteins also partially precipitated, but only in response to the high concentrations of b-isox (Fig. 4a). Consistent with the presence of low complexity regions (LCR) in the carboxy-terminus of Roquin, the amino-terminal fragment of Roquin, which results from MALT1 paracaspase-mediated cleavage12,14, did not precipitate as well (Fig. 4a). These experiments suggest that in fibroblast extracts only a subset of the Nufip2 protein is associated...
with Fmrp, and that localization of Roquin into RNA granule-like structures is likely independent of Fmrp or Nufip2.

We next investigated the cellular localization of NUFIP2 to RNA granule-like structures by super-resolution microscopy in MEF cell lines and flow cytometry imaging in primary CD4+ T cells (Fig. 4b, Supplementary Figs. 3, 4, 5a–e). Although NUFIP2 was named nuclear FMRP interacting protein 2 due to its nucleo-cytoplasmic relocalization during different phases of the cell cycle36, we found retrovirally expressed GFP-NUFIP2 to colocalize with Roquin-1 (Supplementary Fig. 4a).

Neither absence of NUFIP2 affected Roquin-1 localization to P bodies (Supplementary Figs. 4a, 5a, c), where it was predominantly localized in the cytoplasm of T cells (Fig. 4b). Cytoplasmic localization of NUFIP2 was observed throughout all phases of the cell cycle (Fig. 4b and Supplementary Fig. 3a–c), and was unchanged in the absence of Roquin (Supplementary Fig. 3d–f). As described previously37, both endogenous and overexpressed NUFIP2 aggregated into stress granules in response to arsenite-induced oxidative stress (Supplementary Figs. 4a, b, 5a–c). However, we observed a partial enrichment of NUFIP2 in P bodies (Supplementary Figs. 4a, 5a, c), where it colocalized with Roquin-1 (Supplementary Fig. 4a).

Neither absence of NUFIP2 affected Roquin-1 localization to P bodies or stress granules (Supplementary Fig. 5d, e), nor did the absence of Roquin interfere with aggregation of NUFIP2 in stress granules (Supplementary Fig. 4b). However, the specific enrichment of NUFIP2 in P bodies (Supplementary Figs. 4a, 5c) was not observed anymore in the absence of Roquin expression (Supplementary Fig. 4b).

We investigated the expression profile of Nufip2 in mouse tissues and upon T-cell stimulation. Nufip2 was expressed highest in the brain, but also enriched in spleen, lymph node, thymus, and lung compared to lower expression levels in heart, muscle, kidney, and liver (Fig. 4c). Notably, highest expression of Nufip2 protein correlated with high Roquin levels11, and T-cell stimulation significantly increased protein expression of Nufip2 and Roquin-1 and Roquin-2 (Fig. 4d). This effect was not increased further by addition of agonistic antibodies against the costimulatory receptors CD28 or ICOS (Fig. 4d). In contrast, stimulation with the cytokines IL-2, IL-6 and IL-10, the latter of which has been shown to induce Roquin-1 mRNA expression38, was not able to elevate either Nufip2 or Roquin-1 and Roquin-2 protein levels under conditions that effectively induced phosphorylation of Stat3 or Stat5 (Supplementary Fig. 5f). Analyzing Nufip2 protein amounts in peripheral CD4+ T cells, we found that deletion of the Roquin encoding alleles also caused a reduction in the Nufip2 protein (Fig. 4e), which could not be accounted for by a comparably small decrease of Nufip2 mRNA levels (Supplementary Fig. 5g). Accordingly, Nufip2 expression was restored in Roquin-deficient MEF cells upon retroviral reconstitution with Roquin-1 (Fig. 4f), while mRNA levels stayed constant (Fig. 4g). Interestingly, rescue of Nufip2 expression occurred when cells were reconstituted with wild-type Roquin, but also upon expression of RNA-binding (RoquinL220A R239A R260A) or post-transcriptional inactive (aa 1–509) Roquin1 mutants, or upon expression of the sanroque mutant of Roquin-1, Roquin-1M198R (Fig. 4f). These results suggest that Roquin stabilizes Nufip2 on the post-translational level.

Direct physical interaction between Roquin and NUFIP2. Since NUFIP2 was destabilized in the absence of Roquin, we tested whether both proteins interact. Overexpressed NUFIP2 co-

![Flow cytometry analysis of ICOS and mCherry expression in HeLa reporter cells treated with individual or pooled CNOT1 or NUFIP2 siRNAs (red) or non-targeting control siRNAs (siCtrl) (black). Prior to analysis, cells were treated with doxycycline for 18 h to induce Roquin-1 overexpression.](image-url)
immunoprecipitated with GFP-Roquin-1 from HEK293T cell lysates using anti-GFP antibodies in an RNase-insensitive manner (Fig. 5a). Surprisingly, Roquin-1 interacted with NUFIP2 through the amino-terminus (aa 1–509), harboring the RING finger and ROQ domain, and not through the carboxy-terminal sequences (aa 509–1130) that have been proposed to mediate protein–protein interactions (Fig. 5b and Supplementary Fig. 6a).

Underscoring the physiologic importance of this interaction, endogenous Nufip2 was strongly enriched in immunoprecipitates of endogenous Roquin from control MEF cell lysates using anti-Roquin-1/2–specific antibodies (Fig. 5c). This interaction still occurred with the sanroque mutant of Roquin-1 (Fig. 5c).

Notably, immunoprecipitating Nufip2 from lysates of individual Roquin-1 or Roquin-2 knockout cells revealed indistinguishable co-immunoprecipitation with either paralog (Fig. 5d). The NUFIP2 (aa 255–411) internal fragment was essential for the interaction, since neither amino-terminal (aa 1–255) nor carboxy-terminal sequences (aa 411–695) co-immunoprecipitated with Roquin-1 (Fig. 5e, Supplementary Fig. 6a). Finally, we purified recombinant proteins produced in bacteria (Fig. 5f) and demonstrated a direct interaction between mouse Roquin-1 (aa 2–441) and human NUFIP2 (aa 255–411) by surface plasmon resonance (SPR) experiments (Fig. 5g). The calculated equilibrium dissociation constant ($K_d$) of 182 nM indicates a strong physical interaction (Fig. 5h).

Since the same fragment of NUFIP2 that mediated interaction with Roquin has also been shown to engage in a direct interaction with an amino-terminal fragment of Fmrp (aa 1–132), we wondered whether the interaction between NUFIP2 and Roquin and between NUFIP2 and Fmrp were mutually exclusive or
enabled formation of a ternary complex. To answer this question, we purified the fragment of Fmrp (aa1–132) (Fig. 6a) that binds to NUFIP2 (Fig. 6b), and assayed binding of Roquin to NUFIP2 by SPR in the presence of Fmrp protein. Addition of Fmrp inhibited the binding of NUFIP2 (aa 255–411) to immobilized Roquin-1 (aa 2–441) in a concentration-dependent way (Fig. 6b). Consistently, less Roquin was co-immunoprecipitated with Nufip2 from cell lysates when Fmrp was overexpressed in the cells (Fig. 6c). These results demonstrate a mutually exclusive interaction of NUFIP2 with Fmrp or Roquin-1, which likely leads to competition for binding to NUFIP2 by Roquin and Fmrp. However, analyzing prototypic Roquin functions in mice lacking both alleles of the Fmrp-encoding gene *Fmr1* (*Fmr1<sup>−/−</sup>* mice), there was no indication for a gain of Roquin function, since those animals contained comparable frequencies of effector-like (CD62<sup>−</sup>CD44<sup>+</sup>) or regulatory T cells in secondary lymphoid organs (Fig. 6d, e). Furthermore, ICOS surface expression was not changed in T cells with *Fmr1<sup>−/−</sup>* compared to wild-type genotypes when we analyzed Foxp3<sup>+</sup> regulatory CD4<sup>+</sup> T cells (Fig. 6f, f) or conventional CD4<sup>+</sup> T cells without (Fig. 6g, 0 day) or with stimulation in a 6-day culture (Fig. 6g).

**Roquin and NUFIP2 cooperatively bind to cis-elements.** As previously reported, progressive 3′ shortening of the full-length human *ICOS 3′-UTR* (1–2478) leads to a gradual reduction in the repression of ICOS by overexpressed Roquin-1 (Supplementary Fig. 6b)<sup>15</sup>. This implies the existence of multiple alternative binding sites for overexpressed Roquin-1 in the *ICOS 3′-UTR*. To determine which *cis*-elements in human *ICOS* respond to endogenous Roquin, we evaluated *ICOS 3′-UTR* deletion constructs in a *Rc3h1<sup>fl/fl</sup>; Rc3h2<sup>fl/fl</sup>; Cd4-Cre-ERT2 MEF cell line before and after 4′-OH-tamoxifen-inducible deletion of Roquin-encoding alleles (Fig. 7a and Supplementary Fig. 6c). Strikingly, derepression occurred when the 3′-UTR of the *ICOS* mRNA was shortened from the 3′ end to 2211nts or beyond, while constructs with at least 2271 nts were fully repressed by endogenous Roquin. These results suggest that a critical Roquin-regulated *cis*-element is
Fig. 5 Direct physical interaction between Nufip2 and Roquin. Immunoblots were analyzed with antibodies against Nufip2 and Roquin, Roquin and GFP or Nufip2 and GFP. Anti-GFP or anti-Roquin immunoprecipitation from lysates of HEK293T cells transfected with the indicated expression vectors (a, b, e) and anti-Roquin (c) or anti-Nufip2 (d) immunoprecipitation of endogenous proteins from MEF cells of the indicated genotypes are shown. Extracts were treated during immunoprecipitation with or without RNase as indicated and degradation of rRNA by RNase treatment was confirmed by ethidium bromide (EtBr) staining of RNA extracts from IP supernatants. Representative coomassie-stained PAGE of purified Roquin-1 (aa 2–441) and NUFIP2 (aa 255–411) proteins. Surface plasmon resonance study of the binding of NUFIP2 (aa 255–441) to immobilized Roquin-1 (aa 2–441). After the highest concentration, the 0.5 μM sample dilution was re-injected to assess potential accumulation of unspecific background binding. Steady-state affinity analysis of the binding level in response units (RUs) against the Nufip2 (aa 255–411) protein concentration shown in a representative fitting curve. In a–e and g–h one representative of two (d) or three (a–c, e, g–h) independent experiments is shown. The $K_D$ in h was calculated from mean and SD of three independent experiments.
Fig. 6 Mutually exclusive interaction of NuFip2 with Fmrp or Roquin. a Representative coomassie-stained PAGE of purified mFmrp (aa 1–132). b Biacore sensogram recording of the binding of NuFip2 (aa 255–441) injected at 4 µM to surface-coupled Roquin-1 (aa 2–441) in the presence of increasing amounts (0.016–1 µM) of Fmrp (aa 1–132). The quality of Biacore experiments was assessed by two independent injections of 1 µM Fmrp and 4 µM NuFip2 at different time points during each experiment. c Immunoprecipitation of endogenous NuFip2 from lysates of HEK293T cells that were transiently transfected with Roquin-1 with or without co-transfection of FLAG-Fmrp using a monoclonal NuFip2 antibody. Immunoblot analysis to detect NuFip2, Fmrp and Roquin-1/2 after NuFip2 immunoprecipitation (IP) is shown. d–g Analysis of splenic CD4+ T cells from wild-type (wt) and Fmrp-deficient (Fmr1−/−) mice. d, e Flow cytometry analysis of CD4+ T cells showing naive (CD62Lhi CD44lo), effector-memory (CD62Llo CD44hi), or central-memory (CD62Lhi CD44hi) (d) or Foxp3+ Tregs among CD4+ pre-gated T cells. f Flow cytometry analysis of ICOS expression on the surface of Foxp3+ splenic CD4+ T cells. g Quantified ICOS expression on CD4+ T cells isolated from Fmrp-deficient or wild-type mice in a 6-day in vitro culture. One representative of two (c) or three (b) independent experiments are shown in b and c. In d–f data are representative of four mice per genotype and g shows mean and SD of four mice per genotype.

Present between nucleotides 2211 and 2271 of ICOS mRNA, a region that has already been implicated in Roquin-mediated regulation of ICOS via a miR-101 binding site. However, our data demonstrated direct binding of Roquin to the ICOS 3′-UTR, and reporter analyses in Dicer- or Argonaute-deficient cells revealed Roquin-1-dependent repression of ICOS in an miRISC-independent manner.

Roquin has been shown to bind conserved tri-loop or hexa-loop structures in vitro. Additionally, crosslinking and immunoprecipitation experiments showed interaction with predicted U-rich stem-loops of different lengths in cells. Therefore, we employed the LocARNA algorithm for the identification of evolutionarily conserved structured motifs within the mapped region of the ICOS 3′-UTR. Two conserved stem-loops, an octa-loop with a U-rich loop and a tri-loop without characteristics of the canonical CDE, were identified in the mapped region between nts 2211–2271 (Fig. 7b). To determine how Roquin recognizes the mapped cis-element we performed electrophoretic mobility-shift assays (EMSA) with recombinant Roquin-1 (aa 2–441) and in vitro-transcribed RNA. We extended the mapped sequence at the 5′ end by 28 nts to facilitate correct folding of the predicted octa-loop structure (ICOS nts 2183–2271). Roquin bound strongly to the mapped cis-element (nts 2183–2271) (Fig. 7c). The mode of interaction with this cis-element was further addressed by competition experiments. Efficient competition was only obtained through the unlabeled full cis-element (nts 2183–2271) RNA, whereas shorter RNA fragments were unable to affect the binding (Supplementary Fig. 6d). In line with this, Roquin bound only very weakly to shorter RNA fragments representing the full octa-loop containing element (Fig. 7d), the element containing only the 5′ part of the octa-loop (Fig. 7e) or the extended tri-loop containing element (Fig. 7f). Surprisingly, in contrast to its interaction with the CDE, Roquin did not bind to the isolated tri-loop-containing element (Fig. 7g). Nevertheless, additional experiments established that also this unconventional tri-loop can contribute to regulation. In reporter assays carriers
Fig. 7 An unconventional cis-element in the ICOS mRNA is recognized by Roquin and critical for post-transcriptional repression. a Reporter regulation of gradually shortened ICOS 3′-UTR fragments in response to 4′ OH-tamoxifen (4′ OH-TAM) induced deletion of endogenous Roquin in Rc3h1fl/fl, Rc3h2fl/fl, Cre-ERT2 MEF cells. ICOS expression was quantified by flow cytometry. Fold regulation was determined by dividing (ICOS MFI +4′ OH-TAM)/(ICOS MFI –4′ OH-TAM) and normalized to the regulation of ICOS 1–600 (CDS).

b Sequence-structure alignment of the putative Roquin-active ICOS cis-element and consensus secondary structure of putative Roquin-binding stem-loop motifs are shown. The number of different types of base pairs for a consensus pair is indicated by different colors, the number of incompatible pairs by the saturation of the consensus base pair. A consensus secondary structure is depicted on the right.

c–g Electrophoretic mobility-shift assays (EMSAs) with increasing amounts of Roquin-1 (aa 2–441) incubated with ICOS RNA fragments as indicated. Labeled in red is the part of the consensus secondary structure shown in b that is included in the respective ICOS RNA fragment. g At the highest protein concentration unspecific RNA binding was observed, as detected by a broad smear of retarded RNA.

h Schematic representation of ICOS reporter constructs bearing mutant stem-loops. Mutations designed to destroy the secondary structure of selected RNA elements are indicated by asterisks.

i, j Reporter regulation of the stem-loop and deletion mutants from h in response to 4′ OH-TAM induced deletion of endogenous Roquin in Rc3h1fl/fl, Rc3h2fl/fl, Cre-ERT2 MEF cells. Fold regulation was determined by dividing (ICOS MFI +4′ OH-TAM)/(ICOS MFI –4′ OH-TAM) and normalized to the regulation of ICOS wt. Error bars in a, i and j indicate mean and SDs of two (a) or four (i, j) independent experiments. In c–g representatives of three independent experiments are shown.
out in Roquin-deficient MEF cells with doxycycline-inducible overexpression of Roquin-1, the ICOS 3'-UTR deletion construct that ended before the tandem cis-element (F2 in Supplementary Fig. 6e) only showed half of the reporter regulation as compared to full-length ICOS mRNA (wt) (Supplementary Fig. 6e). Additional deletion of a region encompassing the much further 5' localized Roquin-bound CDE-like stem-loop (F3), which we have identified earlier by mapping the Roquin/ICOS mRNA interaction 11,40, attenuated the reporter regulation even more. On the other hand, neither the deletion of this CDE-like element alone affected reporter regulation (F1) nor did the combined deletion of the CDE-like tri-loop element together with the perfect consensus CDE at the very end of the 3'-UTR (F4). These data indicated a cooperative function of the CDE-like tri-loop element and the unconventional tandem cis-element located hundreds of nucleotides downstream. To confirm that all three stem-loops in both cis-elements contribute to Roquin-mediated ICOS repression in a cooperative manner, we tested ICOS reporters with single and combined double and triple point mutations in the loop-forming sequences (Fig. 7h, i). Mutating the loop of the CDE-like tri-loop (M1) or the octa-loop of the tandem element (M2) were much less effective compared to the mutation of the tri-loop in the tandem element (M3), which almost completely inhibited the regulation of the ICOS reporter by endogenous Roquin proteins (Fig. 7i). However, combining the mutations of CDE-like and octa-loop (M1 M2) impaired Roquin-mediated regulation to a similar extent, indicating the cooperative activity of all three stem-loops. This assumption received further support by the observed complete inhibition of regulation in all other combinations of two or more mutations (Fig. 7i) compare M1 M2, M2 M3, M3 M1 M3 or M1 M2 M3).

Interestingly, the shortening or deletion of sequences between the CDE-like tri-loop and the tandem cis-element increased the regulation of the reporter by endogenous Roquin proteins (Fig. 7h, j).

Since NUFIP2 has been shown to interact with polyadenylated RNA 36, we tested whether NUFIP2 would bind directly to the mapped ICOS cis-element or to another tandem element present in the Ox40 3'-UTR that is required for the regulation of the Ox40 costimulatory receptor 13,40. Specifically, we performed EMSAs with NUFIP2 (aa 255–411) and Roquin (aa 2–441) either with the ICOS RNA (nts 2183–2271) (Fig. 8a–c) or Ox40 (nts 1064–1126) (Fig. 8d–f). Compared to Roquin (Fig. 8b), NUFIP2 bound the ICOS cis-element with much lower affinity (Fig. 8a), which was similarly true for the Ox40 cis-element (Fig. 8d, e). We next investigated whether NUFIP2 interacted with the cis-element in the presence of Roquin. Intriguingly, addition of NUFIP2 caused a super-shift of Roquin-1-bound ICOS (nts 2183–2271) (Supplementary Fig. 7a), indicating the formation of a ternary complex. Quantifying the interaction of Roquin with these cis-elements in binary or NUFIP2-containing ternary complexes showed a 3–4-fold better binding in the presence of NUFIP2 (Fig. 8b, c, e, f and Supplementary Fig. 7b, c). These results support a model in which NUFIP2 increases the binding affinity of Roquin in a ternary complex with the mapped ICOS or Ox40 cis-elements. Furthermore, the data explain how these unconventional cis-elements are recognized at low or endogenous levels of Roquin (Supplementary Fig. 7d).

**Discussion**

The family of RNA-binding proteins has been extended enormously by recent findings showing that proteins without known RNA-binding domains interact with mRNA inside cells 28,41. The current challenge therefore is to elucidate how such interactions contribute to specific post-transcriptional gene regulation.

Employing a targeted siRNA screening approach, we identified and characterized NUFIP2 as a cofactor of Roquin function. So far, little is known about the composition of the mRNP assembling around Roquin. It has been previously shown that the carboxy-terminus of Roquin-1 interacts with the deadenylase complex 38,39. Furthermore, the amino-terminal part of Roquin-1 interacts with Ddx6/Rck and Edc4, two cofactors of mRNA decapping 15. However, it is unclear whether these interactions are direct. In contrast, NUFIP2 not only co-immunoprecipitated with full-length and amino-terminal fragments of Roquin-1 from cell lysates, but also interacted as purified recombinant Roquin-1 and NUFIP2 protein fragments with high affinity (Kd of <200 nM). NUFIP2 is therefore a direct binding partner of the amino-terminal part of Roquin. The recently reported direct interaction of NUFIP2 and Ddx6 32 may further enhance Roquin-dependent post-transcriptional repression. However, since recruitment of the carboxy-terminus of Roquin was sufficient to induce reporter mRNA degradation, this interaction may not be required.

A molecular property that connects Roquin and NUFIP2 is their common localization in RNA granules. For some instances of such co-localization the biophysical basis has been demonstrated to lie in the aggregation of RNA-binding proteins containing LCR. They are enriched in polar amino acids like asparagine, glutamine, serine, tyrosine and glycine 42. In fact such LCRs with enrichment in glutamine and aspartagine can be found in peptide sequences of NUFIP2 as well as of Roquin 13,37. We used a recently developed biochemical assay to precipitate proteins that have a tendency to aggregate with b-isoxazole 35 and also determined the localization of Roquin or NUFIP2 in Nufip2- or Roquin-deficient cells. In these experiments we show that both proteins aggregate, albeit with different b-isoxazole concentration requirements, and localize to stress granules independent of each other. Nevertheless, upon overexpression of Roquin, NUFIP2 becomes enriched in P bodies. Together these data reveal that the binary interaction between NUFIP2 and Roquin is not a driving force for heterotypic aggregation of both proteins, but may rather be involved in specific functions of both proteins.

Our work demonstrates the importance of NUFIP2 in Roquin-mediated ICOS regulation. This target mRNA shows interesting and unconventional features: first, with 1978 nts the 3'-UTR of ICOS is remarkably long. The 3'-UTR contains multiple non-essential binding sites, at least one of which forms a perfect CDE. Furthermore, the mapped essential cis-element is composed of tandem stem-loops that are both required for efficient recognition by Roquin. Finally, this essential tandem stem-loop appears to depend functionally on additional binding of Roquin to another binding site being located in a long distance. We have observed before a cooperation of two binding sites in the Ox40 3'-UTR to equally and independently contribute to Roquin-induced mRNA decay 1. It was therefore surprising to find that in the ICOS 3'-UTR the remotely 5' located binding site enables the function of the essential 3' binding site, while the sequences between both cis-elements appeared to inhibit post-transcriptional repression by Roquin. These findings suggest an additional level of regulation involving the secondary structure of this 3'-UTR, which may dynamically change the physical distance of both elements and therefore enable or reduce the effect of the bound trans-acting factors.

Roquin bound to the essential cis-element of the ICOS 3'-UTR with enhanced affinity in the presence of NUFIP2, and similar results were obtained for another Roquin-regulated tandem stem-loop containing cis-element from the Ox40 3'-UTR. A question that remains to be answered is how exactly NUFIP2 increases the binding affinity of Roquin to the mapped regions. One possibility is that NUFIP2 binding may promote Roquin to adopt a certain conformation thereby enabling additional or stronger contacts of...
its RNA binding domain to specific RNA elements (Supplementary Fig. 7d). Such a mechanism of cofactor-dependent changes in binding affinity has recently been shown for the yeast DEAD box protein Rok1, which interacts with a dsRNA duplex in the pre-rRNA between 5.8 S and 18 S rRNAs. Target specificity of Rok1 was established by binding to its cofactor Rrp5. Rrp5 binding induced changes in the Rok1 secondary structure, thereby enhancing Rok1 target specificity without binding to RNA itself43.

On the other hand, NUFIP2 was originally described as an RNA-binding protein recognizing poly(G) homopolymers36. It might therefore bind RNA sequence or structure elements in the ICOS 3′-UTR by itself. Considering the mapped cis-element and the higher affinity of Roquin for this element, it is more likely that Roquin recruits NUFIP2 to this specific RNA element than vice versa. In this scenario NUFIP2 contributes, in a complex with Roquin, additional contacts to the recognized cis-element and thereby enhances or alters sequence and shape-specific RNA recognition by Roquin (Fig. 8c, f). Very recently a similar scenario has been described for ASH1 mRNA recognition by a motor-transport complex in budding yeast44. In the nucleus She2p recognizes cis-elements in the RNA with modest affinity and specificity. After nuclear export, the RNA-binding protein and myosin adapter She3p joins the complex, thereby improving affinity and specificity for ASH1 mRNA. A crystal structure of the ternary yeast complex revealed that She3p improves binding by joining the complex as an unfolded protein, contacting both the globular She2p protein and the RNA44. In analogy, also the RNA-binding ROQ domain of Roquin is globular, whereas the interacting NUFIP2 fragment is largely unfolded45 and modulates the RNA binding. Given the large number of recently discovered unfolded RNA-binding proteins28,41,46, it is likely that more examples are to be discovered, where unfolded proteins such as NUFIP2 modulate the RNA binding and function of globular proteins like Roquin. Our study provides evidence that also the combination of different RNA cis-elements contribute to the complexity and precision of post-transcriptional regulation.

Future experiments should address whether the physical and functional cooperation of Roquin and NUFIP2 is specific for certain cis-elements and target mRNAs and whether NUFIP2 also

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**Fig. 8** Cooperative binding of NUFIP2 and Roquin to response elements in ICOS and Ox40 mRNA. a EMSA performed with the ICOS (nt 2183-2277) RNA (a–c) or Ox40 (nt 1064-1126) RNA (d–f) using increasing amounts (0–4860 nM) of NUFIP2 (aa 255–411) (a, d) or increasing amounts (0–200 nM) of Roquin-1 (aa 2–441) (b, e), or using increasing amounts (0–200 nM) of Roquin-1 (aa 2–441) in the presence or absence of 540 nM NUFIP2 (c, f).
increases the affinity and adapts the specificity to distinct cis-elements in target mRNAs of FMRP. Regardless of these open questions, our findings contribute to the emerging view that post-transcriptional control appears to rely on a complex code of cis- and trans-acting elements, reminiscent of what has been shown for transcriptional control.

Methods

Cloning. ICOS reporter constructs consisting of human ICOS coding sequence followed by fragments of the ICOS 3′-UTR or the TNF CDE have been described previously. Expression constructs for Dox-inducible Cre-ERT2 were generated by ligating the Cre-ERT2 sequence at the 5′ end of the genomic fragment of interest flanked with the FRT recognition sites. The library of siGENOME siRNA pools was custom-made. Individual siRNA duplexes targeting StAU1 (D-011894-01) and StAU2 (D-011894-02) were incubated in HEK293T cells with transfection reagents (Addgene, MA) to generate Dox-inducible lentiviral gene expression, sequences encoding Roquin-1 or Regnase-1 have been described previously. For generation of Roquin-1-P2A-mCherry were inserted into plentiCMVtight Neo DEST (Addgene plasmid #26432). The RTA3-encoding vector plentiCMVtight Neo DEST was co-transfected with the pRc3h1/2-erbA (Göttingen, Germany), where it was synthesized and purified. The mCherry coding sequence at the 5′ end that were used as templates for in vitro transcription are listed in Supplementary Table 1. The RNA was purified based on manufacturer’s instructions, starting with DNase digestion, proceeding with phenolic extraction, followed by RNA precipitation using ethanol. The integrity of the RNA was confirmed by native or denaturing urea PAGE.

The library of siGENOME siRNA pools was custom-made. Individual siRNA duplexes targeting StAU1 (D-011894-01) and StAU2 (D-011894-02) were incubated in HEK293T cells with transfection reagents (Addgene, MA) to generate Dox-inducible lentiviral gene expression, sequences encoding Roquin-1 or Regnase-1 have been described previously. For generation of Roquin-1-P2A-mCherry were inserted into plentiCMVtight Neo DEST (Addgene plasmid #26432). The RTA3-encoding vector plentiCMVtight Neo DEST was co-transfected with the pRc3h1/2-erbA (Göttingen, Germany), where it was synthesized and purified. The mCherry coding sequence at the 5′ end that were used as templates for in vitro transcription are listed in Supplementary Table 1. The RNA was purified based on manufacturer’s instructions, starting with DNase digestion, proceeding with phenolic extraction, followed by RNA precipitation using ethanol. The integrity of the RNA was confirmed by native or denaturing urea PAGE.

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EFS GFP was a kind gift from Dr. Benjamin Ebert (Addgene plasmid #57818) cloned into the plentiCRISPR EFS GFP via cation of ICOS expression by MEFs and HeLa cells were generated by transduction with lentiCRISPR vectors above. 

**Transfection, transduction, and generation of stable cell lines.** Retro- and lentiviral supernatants were produced by calcium-phosphate transfection of lentiviral supernatants were produced by calcium-phosphate transfection of

**Infection with the desired amount of viral supernatant (100 μL) to the cell medium. Cells were harvested at different time points for protein analysis.**

**Immunoprecipitation and immunoblotting.** For preparation of the protein lysates from CD4+ T cells, MEF, HeLa or HeK293T cells, cells were washed twice with cold PBS and lysed in 20 μL Tris-HCl pH 7.5, 150 μM NaCl. 0.25% (v/v) Nonidet-P-40, 1.5 mM MgCl2, 10% glycerol, EDTA-488 Carboxylic Acid Succinimidyl Ester and Protein G Dynabeads were used. Pulldown of RNA-granule-like structures with bi-isox was performed according to the manufacturer’s instructions. Roquin-1 was immunoprecipitated from HeK293T cells (Fig. 5a, b), 5 μg polyclonal anti-GFP antibody was coupled to Protein G Dynabeads (ThermoFisher Scientific). Pulldown of endogenous Roquin-1/2 from MEF cells (Fig. 5c) was performed with anti-Roquin-1/2 (Q4-2) coupled to Protein G Dynabeads (ThermoFisher Scientific). Antibody coupling was performed according to the manufacturer’s instructions. Roquin-1 was immunoprecipitated from HeK293T cells (Fig. 5e) with 4–5 μg polyclonal anti-Roquin-1 antibodies coupled to Protein A Dynabeads (ThermoFisher Scientific). Pulldown of endogenous NuFIP2 from HeK293T cells (Fig. 5d and 6c) was performed with 1 mL monoclonal anti-NuFIP2 (23G8) antibody coupled to Protein G Dynabeads. Immunoblot analysis of Roquin-1 and Roquin-2 was performed with monoclonal 3F12 antibody, while detection of Roquin-1 alone was performed with polyclonal Roquin-1 antibodies. Immunoblot analysis of NuFIP2 was performed according to the manufacturer’s instructions. NuFIP2 was immunoprecipitated from HeLa cells with bi-isox-alkaline phosphatase (AlkPhos) detection reagent (GE Healthcare) and X-ray films were used.

**Immunoprecipitation, antibody-coupled beads were incubated with 2–5 μg of protein lysate for 4 h at 4 °C in the presence of RNAse (Roche).** Before washing three times with lysis buffer the beads were resuspended in Laemmli SDS sample buffer and boiled for 5 min at 95 °C for protein elution. The supernatants for protein analysis.

**Pulldown of RNA-granule-like structures with bi-isox was performed according to the protocol described above.**

**Reverse transcription of HeLa cells with siRNAs was performed with the lypb-doped IgG antigen against Tg antigen (Qagen) according to the manufacturer’s instructions.**

**For the analysis of mRNA stability transcription was stopped by addition of actinomycin D (5 μM) to the cell medium. Cells were harvested at different time points after actinomycin D addition followed by total RNA isolation and reverse transcription and quantitative PCR (RT-qPCR)-based determination of mRNA amounts.**

**Super-resolution microscopy (3D-SIM).** For fluorescence microscopy, cells were seeded on coverslips and treated with 1 μg/mL of doxycycline for 14 h to induce Roquin overexpression or left untreated. Before fixation, cells were treated with 0.5 mM sodium arsenite (Sigma-Aldrich) for 1 h at 37 °C or left untreated. Cells were harvested, fixed with 4% paraformaldehyde (Roth) for 10 min at 4 °C and permeabilized with 0.02% Tween 20 (PBST) cells were permeabilized in PBS containing 0.02% Tween 20 (ROT) and 0.5 % Triton X-100 (AppliChem) for 10 min. Cov- erslips were treated with 2% bovine serum albumin (BSA) in TBST for 1 h at room temperature. Subsequently, cells were stained with the primary antibodies against Roquin (18F8), and either p70 S6 kinase (1:100 dilution) or G3p1 (see above) followed by a second antibody staining using anti-rat Alexa Fluor 594 (Life Technologies) and anti-mouse CF405S (Biotium), respectively. GFP-NuFIP2 was stained with GFP-Booster ATTO488 (ChromoTek), while endogenous NuFIP2 was stained with a primary antibody against NuFIP2 (1H9) followed by a secondary antibody staining using anti-rat Alexa Fluor 594 (Life Technologies). Primary monoclonal antibody supernatants and the commercial G3p1 antibody were diluted 1:100, while the secondary antibodies anti-rat Alexa Fluor 594 and anti-mouse CF405S were diluted 1:500 and 1:200, respectively. After a final fixation step in 4% formalin (ROT) in PBST the cells were mounted in Vectashield (Vector Laboratories). Nuclei were stained with DAPI (Sigma-Aldrich). Images were acquired with a DeltaVision OMX V3 microscope. 3D SIM raw data were reconstructed and roughly corrected for color shifts with the software softWoRx 6.0 Beta 19 (unreleased). After establishing composite TIFF stacks with a custom-made macro in Fiji, the data were subsequently aligned again.

**Co-immunoprecipitation and immunoblotting.** For preparation of the protein lysates from CD4+ T cells, MEF, HeLa or HeK293T cells, cells were washed twice with cold PBS and lysed in 20 μM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% (v/v) Nonidet-P-40, 1.5 mM MgCl2, 10% glycerol, EDTA-488 Carboxylic Acid Succinimidyl Ester and Protein G Dynabeads were used. Pulldown of RNA-granule-like structures with bi-isox was performed according to the protocol described above. Roquin-1 was immunoprecipitated from HeK293T cells (Fig. 5a, b), 5 μg polyclonal anti-GFP antibody was coupled to Protein G Dynabeads (ThermoFisher Scientific). Pulldown of endogenous Roquin-1/2 from MEF cells (Fig. 5c) was performed with anti-Roquin-1/2 (Q4-2) coupled to Protein G Dynabeads (ThermoFisher Scientific). Antibody coupling was performed according to the manufacturer’s instructions. Roquin-1 was immunoprecipitated from HeK293T cells (Fig. 5e) with 4–5 μg polyclonal anti-Roquin-1 antibodies coupled to Protein A Dynabeads (ThermoFisher Scientific). Pulldown of endogenous NuFIP2 from HeK293T cells (Fig. 5d and 6c) was performed with 1 mL monoclonal anti-NuFIP2 (23G8) antibody coupled to Protein G Dynabeads. Immunoblot analysis of Roquin-1 and Roquin-2 was performed with monoclonal 3F12 antibody, while detection of Roquin-1 alone was performed with polyclonal Roquin-1 antibodies. Immunoblot analysis of NuFIP2 expression was performed initially with the commercial polyclonal NuFIP2 antibody (Fcs. Jc, d and 5a) and in all other experiments with the self-made monoclonal antibody 23G8.

**Pulldown of RNA-granule-like structures with bi-isox was performed according to the protocol described above.**
High-throughput siRNA screen. High-throughput siRNA screening in HeLa cells was performed by reverse transfection of siRNAs in 96-wells using the HiPerFect transfection reagent (Qiagen). Forty microliters of the pre-diluted HiPerFect transfection reagent were dispensed manually into each well of a 96-well U-bottom plate (plate A) using a multichannel pipette. 8 μL of 1 mM siRNA library stocks and 32 μL of H2O were subsequently added by a robot, and after mixing, the robot dispensed half of the solution (40 μL) into a replicate plate (plate B). Eight microliters of 1 mM control siRNAs in 32 μL H2O (siRuno, siCtrl) had been added manually before addition of the library stocks. Following an incubation time of 10 min to allow complex formation, HeLa reporter cells were added in complete DMEM medium to each well. Plates were incubated for 48 h and subsequently the medium was replaced by fresh medium containing 1 μg/mL doxycycline. After 18 h the cells were subjected to fluorescent cell barcoding52,53 and antibody staining. Fluorescent cell barcoding was in principal performed as described in ref. 52; however, the protocol was adapted to label cell surface proteins only. HeLa cells were washed once with PBS, trypsinized and transferred to U-bottom 96-well plates. Washing steps were now carried out by resuspension, centrifugation at 4 °C, cells were subjected to antibody staining as described below and analyzed by flow cytometry.

Statistical analysis. ICOS mean fluorescence intensity was determined with FlowJo Version 10.2. Statistical analysis was performed with GraphPad Prism 5.0d, -values were calculated with the Kruskal-Wallis test followed by Dunn’s multiple comparison test. ICOS mRNA half-life values were calculated with GraphPad Prism by one-phase decay.

The Z’ factor in pilot experiments was calculated according to Zhang et al.37. All screen data were normalized into Z scores using the following equation:

\[
Z = \frac{X - \mu_{\text{plate}}}{\sigma_{\text{plate}}}
\]

Flow cytometry. Antibody staining for flow cytometry was performed as described previously31,32. Briefly, cells were subjected to a 1:200 dilution of primary antibody for 20 min at 4 °C, washed with PBS 2% FCS (FACS buffer) and subjected to a secondary antibody staining if necessary. After two final washing steps, cells were resuspended in 100 μL FACS buffer and acquired on a LSR II Fortessa cell analyzer (BD Bioscience).

Sequence and structure conservation analysis. All mRNA and sequence data used in this study were acquired from the NCBI Reference Sequence Database (RefSeq) collection7. A data set was prepared by extracting all mRNAs of ICOS. Only mRNAs with a completely annotated 3′-UTR were used. The data set included 24 mammals. A complete list of species and associated identification numbers used in this study is given as Supplementary Table 5.

A combined multiple sequence-structure alignment of the data set sequences were obtained applying LocARNA version 1.8.47 with the option local-progressive followed by RNAalifold35 from the ViennaRNA package version 2.1.8-250.

Data availability. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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References
1. Janowski, R. et al. Roquin recognizes a non-canonical hexaloop structure in the 3′-UTR of Oxy. Nat. Commun. 7, 11032 (2016).
2. Sakurai, S., Ohno, U. & Shimizu, T. Structure of human Roquin-2 and its complex with constitutive decay element RNA. Acta Crystallogr. F. Struct. Biol. Commun. 71, 1048–1054 (2015).
3. Schlundt, A. et al. Structural basis for RNA recognition in roquin-mediated post-transcriptional gene regulation. Nat. Struct. Mol. Biol. 21, 671–678 (2014).
4. Tan, D., Zhou, M., Kiledjian, M. & Tong, L. The ROQ domain of Roquin recognizes mRNA constitutive-decay element and double-stranded RNA. Nat. Struct. Mol. Biol. 21, 679–685 (2014).
5. Sgromo, A. et al. A CAFA40-binding motif facilitates recruitment of the CCR4-NOT complex to mRNAs targeted by Drosophila Roquin. Nat. Commun. 8, 14072 (2017).
6. Leppke, K. et al. Roquin promotes constitutive mRNA decay via a conserved class of stem-loop recognition motifs. Cell 153, 869–881 (2013).
7. Murakawa, Y. et al. RCH1 post-transcriptionally regulates A20 mRNA and modulates the activity of the IKK/NF-kappaB pathway. Nat. Commun. 6, 7367 (2015).
8. Jeltsch, K. M. et al. Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation. Nat. Immunol. 15, 1079–1089 (2014).
9. Mino, T. et al. Regnase-1 and roquin regulate a common element in inflammatory mRNAs by spatiotemporally distinct mechanisms. Cell 161, 1058–1073 (2015).
10. Bertossi, A. et al. Loss of Roquin induces early death and immune deregulation but not autoimmunity. J. Exp. Med. 208, 1749–1756 (2011).
11. Heissmeyer, V. & Vogel, K. U. Molecular control of Th-cell differentiation by Roquin family proteins. Immunol. Rev. 253, 273–289 (2013).
12. Jeltsch, K. M. & Heissmeyer, V. Regulation of T cell signaling and autoimmunity by RNA-binding proteins. Curr. Opin. Immunol. 39, 127–135 (2016).
13. Vinuesa, C. G. et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature 435, 452–458 (2005).
14. Gewies, A. et al. Uncoupling MalT1 threshold function from paracaspase activity results in destructive autoimmunefellinflammation. Cell Rep. 9, 1320–1325 (2014).
15. Glasmacher, E. et al. Roquin binds inducible costimulator mRNA and effectors of mRNA decay to induce microRNA-independent post-transcriptional repression. Nat. Immunol. 11, 725–733 (2010).
16. Srivastava, M. et al. Roquin binds microRNA-146a and Argonaute2 to regulate microRNA homeostasis. Nat. Commun. 6, 6253 (2015).
17. Yu, D. et al. Roquin represses autoimmunity by limiting inducible T cell costimulator messenger RNA. Nature 450, 299–303 (2007).
18. Linterman, M. A. et al. Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS. Immunity 30, 228–241 (2009).
19. Lee, S. K. et al. Interferon-gamma excess leads to pathogenic accumulation of follicular helper T cells and germinal centers. Immunity 37, 880–892 (2012).
20. Burmeister, Y. et al. ICOS controls the pool size of effector-memory and regulatory T cells. J. Immunol. 180, 774–782 (2008).
21. Dong, C., Temann, U. A. & Favel, R. A. Cutting edge: critical role of inducible costimulator in germinal center reactions. J. Immunol. 166, 3659–3662 (2001).
22. McAdam, A. J. et al. ICOS is critical for CD40-mediated antibody class switching. Nature 409, 102–105 (2001).
23. Stone, E. L. et al. ICOS coreceptor signaling inactivates the transcription factor FOXP1 to promote Tfh cell differentiation. Immunity 42, 239–251 (2015).
24. Xu, H. et al. Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature 496, 523–527 (2013).
25. Rambhojannya, D. et al. Perifollicular antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. Immunity 38, 596–605 (2013).
26. Warnatz, K. et al. Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. Blood 107, 3054–3056 (2006).
27. Zhang, J. H., Chung, T. D. & Oldenburg, K. R. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. 4, 67–73 (1999).
28. Castello, A. et al. Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. Cell 149, 1393–1406 (2012).
29. Ohn, T., Kedersha, N., Hickman, T., Tisdale, S. & Anderson, P. A functional RNAi screen links OGlcnAc modification of ribosomal proteins to stress granule and processing body assembly. Nat. Cell Biol. 10, 1224–1231 (2008).
30. Braun, J. E. et al. A direct interaction between DCPI and XRNI couples mRNA to 5′ exonucleolytic degradation. Nat. Struct. Mol. Biol. 19, 1324–1331 (2012).
31. Chen, C. Y. & Shyu, A. B. Mechanisms of deathenylation-dependent decay. Wiley Interdiscip. Rev. RNA 2, 167–183 (2011).
32. Cooke, A., Prigge, A. & Wickens, M. Translational repression by deadenylyases. J. Biol. Chem. 285, 28506–28513 (2010).
33. Athanasopoulos, V. et al. The ROQUN family of proteins localizes to stress granules via the ROQ domain and binds target mRNAs. FEBS J. 277, 2109–2127 (2010).
34. Ramiscal, R. R. et al. Attenuation of AMPK signaling by ROQUIN promotes T follicular helper cell formation. Elife 4, e08698 (2015).
35. Kato, M. et al. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. Cell 149, 753–767 (2012).
36. Bardoni, B. et al. 82-FIP, a novel FMRP (fragile X mental retardation protein) interacting protein, shows a cell cycle-dependent intracellular localization. Hum. Mol. Genet. 12, 1689–1699 (2003).
37. Bish, R. et al. Comprehensive protein interactome analysis of a key RNA helicase: Detection of novel stress granule proteins. Biomolecules 5, 1441–1466 (2015).
38. Schaefer, J. S., Montufar-Solis, D. & Klein, J. R. A role for IL-10 in the transcriptional regulation of Roquin-1. Gene 549, 134–140 (2014).
39. Will, S., Reiche, K., Hofacker, I. L., Studler, P. F. & Backofen, R. Inferring noncoding RNA families and classes by means of genome-scale structure-based clustering. PloS Comput. Biol. 3, e65 (2007).
40. Vogel, K. U. et al. Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation. Immunity 38, 655–668 (2013).
41. Baltz, A. G. et al. Promiscuous interactions and protein disaggregases determine the material state of stress-inducible RNP granules. Elife 4, e06807 (2015).
42. Young, C. L., Khoshnevis, S. & Karbstein, K. Cofactor-dependent specificity of a DEAD-box protein. Proc. Natl. Acad. Sci. USA 110, E2668–E2676 (2013).