Abstract

The histone code hypothesis proposes that cell-fate decisions are achieved through creation of stable epigenetic histone marks at gene loci. These marks can be localized to promoters and transcribed regions of genes or can extend many kilobases beyond these boundaries. The *Ifng* gene exhibits complex activating and repressive patterns of epigenetic modifications that cover a region spanning over 50 kb of upstream and downstream genomic DNA in cells that express or silence *Ifng*. Failure to properly establish this long-range histone code may contribute to the characteristic overproduction of interferon (IFN)-γ by proliferating T cells from mice that develop autoimmune diabetes.

Key words: Th1; Th2; epigenetics; histone code hypothesis; differentiation; histone acetyltransferase; histone deacetylase; evolutionarily conserved DNA sequence; NOD/ShiLtJ mice; C57BL/6J mice; autoimmunity

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Roquin defects reveal a role for the microRNA machinery in regulating autoimmunity

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15.1 Introduction

The immune system faces the constant challenge of maintaining tolerance to normal self-components of the body while mounting robust responses to invading pathogens. Defects in immunological tolerance can lead to a wide range of systemic and organ-specific autoimmune diseases. As the cellular and molecular mechanisms responsible for maintaining tolerance are being deciphered, it is becoming clear that spatiotemporal control of gene expression underpins most successful tolerance mechanisms. Transcriptional control and chromatin modification are major determinants of gene expression, which is fine-tuned post-transcriptionally to enable cells to respond rapidly to changes in intracellular and extracellular stimuli, immediately before protein synthesis. In particular, small non-coding RNAs that bind to specific sequences in the untranslated regions (UTRs) of their target transcripts have been shown to play essential roles in regulating mRNA stability and translation [1]. Among small non-coding RNAs, microRNAs (miRNAs) have been shown to play a major role in development, acting on cellular differentiation and organismal diversification. This level of post-transcriptional regulation has attracted recent attention due to accumulating evidence that its malfunction may lead to neoplastic cellular transformation and several human diseases [2].

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15.2 RNA silencing through the miRNA machinery

miRNAs were first discovered 15 years ago as regulators of developmental timing in *Caenorhabditis elegans* [3,4]. Since then, RNA silencing through miRNAs has been shown to be crucial for multiple aspects of plant and animal development. miRNAs constitute a class of small non-coding RNAs of 20–22 nucleotides in length that bind to specific sequences within the 3′ and/or 5′ UTRs of target mRNAs and mediate post-transcriptional repression of gene expression. They are often encoded within the introns of protein-coding genes, but can also be found as independent transcription units or in polycystronic clusters [5]. After being transcribed by RNA polymerase II as primary RNAs (pri-miRNA) with a long hairpin structure, they are generally cleaved in the nucleus by the RNase III enzyme Drosha. This yields the approximately 65-nucleotide precursor miRNA (pre-miRNA) containing the hairpin structure, which is exported to the cytoplasm for further processing by a different RNase III enzyme, Dicer (Figure 15.1). The result is a 19–25 double-stranded duplex; the mature miRNA, which is retained into the functional miRNA-induced silencing complex (miRISC). miRISC also contains an Argonaute protein and other protein co-factors. The miRISC can then bind to mRNAs that have sequences complementary to the miRNA. This complementarity tends to be imperfect in animals, except for a region between

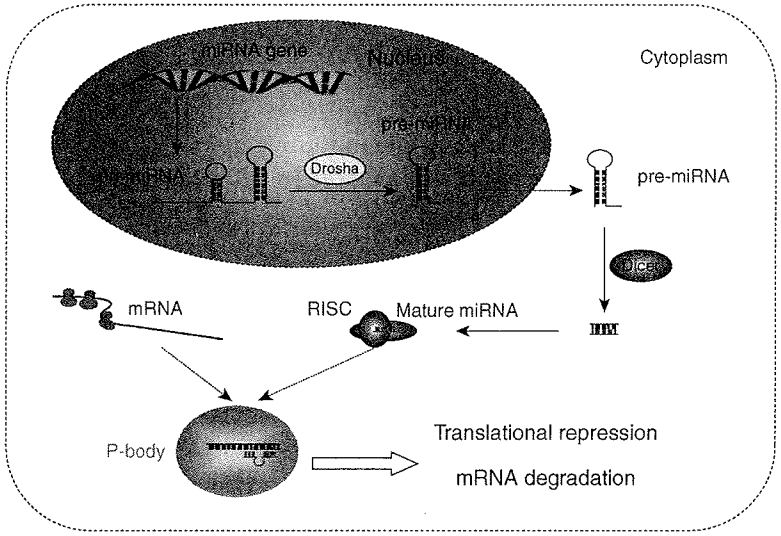

Figure 15.1 Mammalian miRNA biogenesis and function. In the mammalian genome, miRNA genes encode for a long primary miRNA transcript (pri-miRNA). This is processed into a precursor miRNA (pre-miRNA) by the nuclear RNase III enzyme Drosha. After being actively transported to the cytoplasm, the pre-miRNA is further processed by the RNase III enzyme Dicer. The RNA-induced silencing complex (RISC) recognizes the miRNA duplex and loads the functional strand of the duplex (mature miRNAs) on to the target RNA in an imperfectly complementary manner to repress its translation or induce degradation in processing (P) bodies.

residues 2 and 8 at the 5′ end of the miRNA known as the seed region, which is highly complementary [6,7].

Inhibition of target mRNAs generally occurs through translational inhibition or mRNA degradation [8] (Figure 15.1). In cases of perfect complementarity between miRNA and target, as tends to occur in plants, target mRNA cleavage and degradation occurs. Several hundred miRNAs are predicted to regulate about 30% of mammalian protein-encoding mRNAs [7]. While some miRNAs can target over 100 genes, there also appears to be some functional redundancy among them. Although the genes targeted by a single miRNA do not necessarily share functions nor need to belong to the same biological pathway, there are numerous examples to suggest that miRNAs can regulate pathways by coordinately repressing multiple targets with related functions [7].

15.3 miRNAs regulate lymphoid cell development and immune responses

Immune competence depends on differentiation of B, T and myeloid cells into functional subsets, compartmentalization of cell subsets within microenvironments in secondary lymphoid tissue, and lymphocyte homeostasis. Each of these processes is controlled, at least in part, by specific miRNAs. A cohort of recent papers using miRNA expression profiling has revealed striking differences in the expression of highly conserved miRNAs by specific haematopoietic lineages, and during different developmental stages [9–12]. A good example of an miRNA regulating lymphoid cell differentiation is miR-181. Mature miR-181 expression levels are gradually upregulated through the transition between undifferentiated progenitor cells to differentiated B lymphocytes. Ectopic expression of miR-181a in haematopoietic stem cells enhances the differentiation of $CD19^+$ B cells and suppresses the differentiation of $CD8^+$ T cells [9]. For myeloid cells, miR-223 has been shown to be a myeloid-specific miRNA and appears to be driven by the myeloid transcription factors PU.1 and CCAAT/enhancer-binding protein (C/EBP). Ectopic expression of miR-223 in an acute promyelocytic leukaemia cell line resulted in the upregulation of the myelomonocytic differentiation markers CD11b and CD14, and granulocyte colony-stimulating factor receptor, as well as inducing morphological changes consistent with cell maturation [13,14].

miRNAs are also emerging as critical sensors and regulators of immune responses playing key roles in setting the threshold of lymphoid and myeloid cell activation in response to stimulation of antigen receptors, Toll-like receptors (TLRs) and cytokine receptors. Elegant examples of this level of regulation include the recently described roles of miR-181 and miR-146. miR-181a is highly expressed in immature T cells and reduces the threshold of T cell receptor (TCR) signalling, facilitating both positive and negative selection. This is achieved through dampening the expression of multiple phosphatases that are negative regulators of distinct steps of the TCR signalling cascade, such as SH2-domain-containing protein tyrosine phosphatase 2 (SHP2), protein

tyrosine phosphatase, non-receptor type 22 (PTPN22), dual-specificity protein phosphatase 5 (DUSP5) and DUSP6 [15].

Bacterial components can also induce expression of miRNAs. In response to lipopolysaccharide (LPS) and pro-inflammatory cytokines including interleukin (IL)-1β and tumour necrosis factor α (TNF-α), expression of miR-146 expression is upregulated in human monocytes. Importantly, miR-146 represses the expression of TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1, the key adaptor molecules in TLR and IL-1 receptor signalling cascades [16]. In doing this, miR-146 negatively controls TLR and cytokine receptor signalling. This is a powerful example of how miRNAs can also be instrumental in establishing negative-feedback loops to terminate pro-inflammatory signals.

miRNAs are also involved in RNA interference (RNAi)-mediated immune defence against viruses. RNAi can be mediated by small silencing RNAs, such as short interfering RNAs (designed to be perfectly complementary to a region of the target mRNA sequence) and by miRNAs (that are in general imperfectly complementary to a region of the target mRNA sequence) [8]. The role of RNAi as a first-line tool of antiviral defence in plants and invertebrates has been well supported by many lines of evidence, particularly those demonstrating that the RNA-silencing mechanism protects hosts against viral infections and that essential virulence factors of many viruses are viral suppressors of RNA silencing [17].

In mammals, viral miRNAs can control expression of cellular genes to interfere with antiviral host defence. Hcmv-miR-UL112 expressed by human cytomegalovirus has been shown to protect infected cells from the killing by natural killer (NK) cells by repressing expression of the NK cell-activating ligand, major histocompatibility complex (MHC) class I-related chain B (MICB) [18]. Cellular miRNAs can also act to suppress viral infection. For example, cellular miRNAs miR-24 and miR-93 target the RNA genome of the rhabdovirus vesicular stomatitis virus (VSV) [19]. Due to the low fidelity of viral RNA-dependent RNA polymerases, it would be very risky to solely rely on sequence complementarity to defend against viral infections. Indeed, other defence mechanisms, including sensing pathogen-specific patterns by TLRs, have evolved in mammals, and are complementary to miRNA-mediated defence [20].

15.4 miRNAs as single drivers of immunodeficiency or inflammation

Considering the broad and diverse functions of miRNAs in the immune system, and their power to regulate hundreds of target genes with related functions, it is not surprising to observe phenotypes of both under- or overactive immune responses in animals deficient in a single miRNA. The first miRNA-knockout mouse showing a distinct immunological phenotype was the miR-155-deficient strain. *miR-155*$^{-/-}$ mice showed defects in B cell, T cell and dendritic cell function and displayed impaired helper T cell differentiation and decreased T cell-dependent antibody production and affinity maturation [21–23]. Deficiency in *miR-155*$^{-/-}$ mice also led to failure to mount

protective responses against *Salmonella* infection [21]. This phenotype is explained at least in part by the demonstration that the T helper (Th) 2 cytokine-enhancing transcription factor c-Maf and B cell terminal differentiation-related transcription factor PU.1 are physiological targets of miR-155. Other target candidates include several cytokines and chemokines [21,23].

miRNA deficiency does not only cause immunodeficiency; importantly, selective miRNA deficiencies can also lead to overactive immune phenotypes and enhanced inflammatory responses. Two examples of the latter include mice deficient in miR-150 or miR-223. miR-150 is a mature lymphocyte-specific miRNA that targets the transcription factor c-Myb and deficiency of this miRNA in mice has shown to lead to B1 cell expansion and an enhanced humoral immune response [24]. Studies on *miR-223*$^{-/-}$ mice have revealed that this myeloid-specific miRNA controls granulocyte maturation and activation by targeting myeloid progenitor proliferation-enhancing transcription factor Mef2c. Neutrophil hyperactivity seen in *miR-223*$^{-/-}$ mice leads to spontaneous development of inflammatory lung pathology and exaggerated tissue destruction after endotoxin challenge [25].

15.5 miRNAs regulate autoimmunity

The trade off with having the capability to mount potent immune responses is the risk of autoimmunity. Therefore, the delicate balance between protective and autoimmune responses may hinge on miRNA-mediated fine-tuning of gene expression. Indeed, recent reports suggest that miRNAs may play important roles in repressing immune responses against self. Systemic lupus erythematosus (SLE, or lupus) was the first autoimmune disease shown to be regulated by the miRNA machinery. Lupus is the prototypic systemic autoimmune disease with a female to male ratio of 9:1, affecting approximately 1 in 700 women of childbearing age. The clinical manifestations are diverse, and include malaise, lymphoid organ enlargement, skin rashes, lymphopenias and kidney failure, among others. At the core of the pathogenesis is the aberrant production of autoantibodies against nuclear antigens, including anti-double-stranded DNA (dsDNA) antibodies, which are pathogenic. The lupus-associated repertoire of self-antigens targeted by autoantibodies is normally intracellular, although certain nuclear antigens become exposed on the cell surface during apoptosis.

Lupus-susceptibility alleles identified in genome-wide association studies in patients and mouse models include genes that affect T and B cell activation thresholds, especially to nucleic acids and ribonuclear proteins, antigen presentation, plasmacytoid dendritic cell activation and interferon (IFN)-α production, myeloid cell migration and recruitment, components of the complement cascade, and propensity to apoptosis. Molecular defects that compromise the numerous mechanisms that normally affect efficient and non-immunogenic clearance of apoptotic cells have also been implicated in lupus pathogenesis. Thus, the heterogeneity of clinical lupus is mirrored in the diversity of pathophysiological pathways. It is of particular importance that lupus phenotypes are often the result of dose-dependent outcome of changes in key regulators

of the immune response, rather than absolute deficiencies. Two recent studies identifying a link between defects in miRNA-mediated RNA silencing and development of lupus have emphasized the physiological relevance of post-transcriptional regulation of proteins that, as highlighted below, function over a tight range of concentrations during normal immune responses.

The first of these studies describing a role for the mRNA machinery in preventing systemic autoimmunity utilized a strain of mice homozygous for a hypomorphic variant of the *roquin* gene (*sanroque* mice), which develop lupus and autoimmune diabetes [26]. Lymphoproliferation in these mice is caused by over-expression of the inducible T cell co-stimulator (ICOS), due to decreased miR-101-mediated ICOS mRNA decay [27]. Subsequent to this report, over-expression of the miRNA 17–92 cluster, frequently amplified in lymphoma, has also been found to drive lymphoproliferation and a lupus-like disease through failure to downregulate *Pten* and *Bim* transcripts, two important negative regulators of T cell activation, proliferation and survival [28]. In the remainder of this chapter, we will describe in some detail how Roquin regulates miRNA-mediated post-transcriptional regulation to prevent autoimmune manifestations and how this knowledge can be used to explore novel therapeutic avenues.

15.6 Roquin regulates miRNA-mediated silencing of T cells and represses lupus

The *sanroque* strain was generated in an effort to illuminate autoimmune regulators through controlled variation of the mouse genome with the chemical mutagen, *N*-ethyl-*N*-nitrosourea, combined with a set of sensitive immunological screens [29]. *Sanroque* female mice develop anti-nuclear antibodies by 6–7 weeks of age whereas these autoantibodies can only be detected in male mice after 8 weeks of age [30]. Anti-nuclear antibodies are also detected earlier in female SLE patients [31]. Mice homozygous for the *san* allele of *roquin* develop many other typical SLE features, including high-affinity antibodies against dsDNA, focal proliferative glomerulonephritis with deposition of IgGs-containing immune complexes, anaemia and autoimmune thrombocytopenia as well as other autoimmune manifestations such as lymphadenopathy, splenomegaly, necrotizing hepatitis and plasmacytosis in lymph node medullary cords, kidney, liver and lung [30]. *Sanroque* mice are also susceptible to other autoimmune diseases. When crossed with a diabetes-susceptible genetic background in which intact T cell tolerance mechanisms normally prevent islet cell destruction (TCR^{HEL}:insHEL double transgenic mice [32]), 100% of *sanroque* mice develop diabetes as early as 4 weeks of age [30]. The mutation causing the autoimmune phenotype was mapped to a new gene, *roquin*, with previously unknown function. *Roquin* (*Rc3h1*) encodes a member of the RING-type E3 ubiquitin ligase protein family. *Sanroque* mice carry a T → G substitution in *roquin* (*san* allele), resulting in a non-conservative Met-199 → Arg codon change in a novel protein domain (ROQ domain) with high conservation from *C. elegans* to humans (Figure 15.2 and see below) [30].

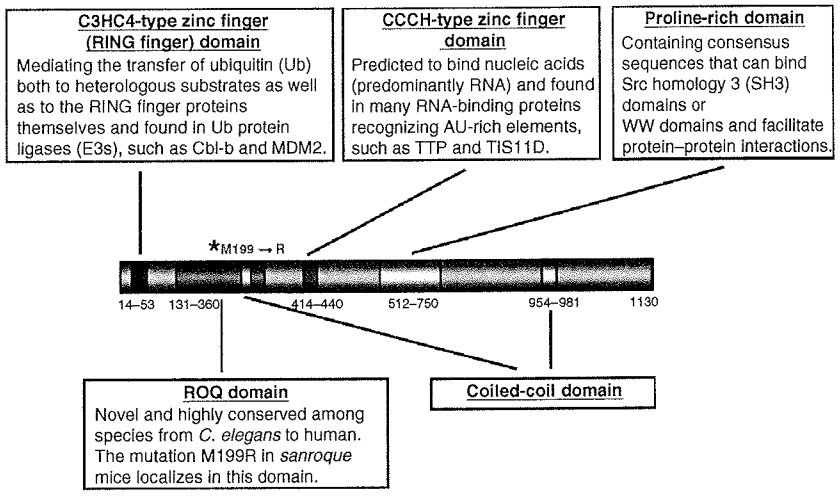

Figure 15.2 Representative structure of Roquin protein. Sequence homology suggests Roquin contains domains with putative ubiquitin protein ligase and nucleic acid-binding functions. Numbers indicate amino acid positions. M199 → R, Met-199 → Arg mutation. C3HC4, a Cys × 3-His-Cys × 4-type zinc finger (RING finger) domain; CCCH, a Cys-Cys-Cys-His (CCCH)-type zinc finger domain.

Dysregulated follicular helper T cells and germinal centre responses lead to lupus

$Roquin^{M199R}$ causes hyperactivation of $CD4^+$ T cells. By 8 weeks of age, approximately 50% of $CD4^+$ T cells in *sanroque* mice show an activated/memory phenotype with high expression levels of CD44, compared with approximately 20% in wild-type mice. One of the most striking phenotypic features of *sanroque* T cells is the consistent over-expression of inducible ICOS on both naïve ($CD44^{low}$) and activated/memory ($CD44^{high}$) $CD4^+$ T cell subsets. $CD4^+$ T cell hyperactivation and aberrant expression of ICOS was shown to be T cell autonomous: in mice whose haematopoietic system was reconstituted with cells containing a 50%/50% mix of *sanroque* and wild-type bone marrow cells, only $CD4^+$ T cells derived from *sanroque* bone marrow displayed a high percentage of $CD44^{high}$ cells and expressed higher levels of ICOS [30]. Large spontaneous germinal centres formed in *sanroque* mice as young as 6–8 weeks, accompanied by a large amount of T cells located within germinal centres [30]. Gene-expression profiling revealed that $CD4^+$ T cells from *sanroque* mice differentially upregulated the expression of genes such as *Icos*, *Cxcr5*, *Cd200*, *Cd84*, *Bcl6*, *Il21*, *neuropilin 1* and *Ccl5*, all of which are markers characteristic of follicular helper T (T_{FH}) cells. T_{FH} cells constitute a specialized helper T cell subset that supports germinal-centre reactions and selects germinal-centre B cells to differentiate into high-affinity memory cells or long-lived plasma cells [26,33–35].

It has been long known that most anti-dsDNA antibodies detected in humans and in animal models of SLE are high-affinity IgGs antibodies, which suggests they might be generated in germinal-centre reactions [36]. The process of somatic hypermutation

typically occurs in the germinal centre, and targets the immunoglobulin variable-region genes of rapidly dividing germinal-centre B cells (centroblasts). This can lead to an increase in the affinity of the B cell receptor for the immunizing antigen, but it can also lead to self-reactive specificities. Thus, a tightly controlled process of germinal-centre B cell selection by antigen-specific T_{FH} cells is normally in place to ensure positive selection of those cells with the highest affinity towards foreign antigens while preventing selection of cells that have become self-reactive. Mutated germinal-centre B cells that fail to receive pro-survival signals are programmed to die by apoptosis, providing a mechanism that prevents the production of high-affinity, long-lived self-reactive B cells [37]. Other mechanisms, including exclusion of self-reactive B cells from entering germinal centres, also contribute to prevent formation of high-affinity autoantibodies, and have been shown to fail in SLE patients [38]. The randomness of the mutation process, together with the longevity of post-germinal-centre memory B cells and plasma cells, and the abundance of exposed nuclear antigens on the surface of numerous germinal-centre cells undergoing apoptosis, rank germinal-centre reactions highest in the risk of triggering and maintaining autoantibody-driven autoimmunity.

The abnormal accumulation of T_{FH} cells and spontaneous formation of germinal centres in unimmunized *sanroque* mice suggest that autoantibodies including anti-nuclear antibodies may be the product of T cell-dependent B cell activation and differentiation in germinal centres, driven by self-antigen. It also suggests that germinal-centre exclusion of self-reactive T_{FH} cells or limiting T_{FH} cell-derived helper signals to germinal-centre B cells is required to prevent self-reactivity arising in follicles.

Over-expression of ICOS contributes to sanroque autoimmunity

Four important mechanisms have been described that are essential to maintain T cell tolerance and prevent autoimmunity: (1) deletion of self-reactive T cells in the thymus, which is critically dependent on autoimmune regulator (AIRE)-mediated expression of organ-specific antigens by thymic epithelial cells [39,40], intact TCR signalling through ZAP70 [41] and intact Bcl-2-interacting mediator (BIM)-mediated TCR-induced thymocyte death [37]; (2) co-stimulation-dependent T cell activation maintained by the E3 ubiquitin ligase Cbl-b [42,43], and terminated by the inhibitory co-receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) [44,45]; (3) T cell activation-induced cell death (AICD) mediated by the pro-apoptotic receptor Fas and its ligand, FasL [46], and (4) regulatory T cell (Treg)-mediated repression, dependent on developmentally regulated expression of the Treg-specific transcription factor Foxp3 on a subset of thymocytes [47] and regulation of Treg development and homeostasis by the cytokine IL-2 and its receptor [48]. None of the above T cell tolerance mechanisms is impaired in *sanroque* mice, which demonstrate normal self-reactive T cell deletion in the thymus, Treg cell-mediated repression, CD28 co-stimulation-dependent T cell proliferation, FasL-dependent activation-induced T cell death and normal induction of the critical B

cell helper molecule CD40L [30]. Instead, aberrantly over-expressed ICOS on T cells in *sanroque* mice emerged as the key culprit for the break in self-tolerance.

The *Icos* gene is a paralogue of the evolutionarily more ancient co-stimulator *Cd28* [49]. Signals through both CD28 and ICOS can induce T cell activation, differentiation and cytokine production [50]. Nevertheless, CD28 and ICOS act at separate stages of immune responses: CD28 is essential at the stage of priming naïve T cells and ICOS is critical for provision of help to B cells in germinal centres. The defects of mice and humans lacking ICOS or ICOS ligand (ICOSL) demonstrate the important functions of ICOS for production of B cell memory [51–57]. Deficiency in ICOS has been found in several patients with adult-onset common variable immunodeficiency, a disease with a clinical phenotype of low serum immunoglobulin concentrations, defective specific antibody production and increased susceptibility to bacterial infections of the respiratory and gastrointestinal tracts [57].

Recent findings have identified Roquin as the critical switch that maintains functional compartmentalization of CD28 and ICOS to allow their respective key roles in T cell discrimination between pathogens and self and in the provision of T cell selection signals to germinal centre B cells. In the absence of CD28, ICOS over-expression due to defective Roquin can functionally substitute for three important functions of CD28 that are normally selectively dependent on CD28 signalling: (1) T cell priming for generation of T cell-dependent antibody responses (2) formation of germinal centres and T_{FH} cells and (3) homeostasis of peripheral Tregs (M. Linterman *et al.*, unpublished results). This compartmentalization explains how these duplicated paralogous genes have resolved an adaptive conflict (maintenance of immunological tolerance) while acquiring specialized functions that confer a selective advantage: the appearance of ICOS-enabled birds and mammals to undergo affinity-maturation and formation of memory cells (independently of danger signals) both key for protection against rapidly dividing bacteria, toxins and many viruses. These observations indicate that Roquin has been co-opted in evolution to prevent crosstalk between these two pathways.

Unlike the well-established causal effect of ICOS deficiency on common variable immunodeficiency, a possible role of ICOS over-expression leading to autoimmunity was until recently only associational: higher expression of ICOS had been detected on T cells from patients with SLE and rheumatoid arthritis compared to healthy controls [58–60]. In *sanroque* mice, however, ICOS expression has been conclusively shown to be causally related to autoimmune lymphoproliferation. When the *Icos* gene dosage was halved by interbreeding *Icos*-knockout mice with *sanroque* mice, $CD4^+$ T cells from *sanroque* mice, losing one allele of *Icos* dramatically reduced ICOS expression, although the levels were still higher than those of wild-type mice. This partial correction of ICOS expression was sufficient to ameliorate the lymphadenopathy, splenomegaly, total T and B cell numbers and T_{FH} and germinal-centre B cell expansion [27]. By contrast, halving the dose of CD28 did not ameliorate the autoimmune syndrome of *sanroque* mice.

A two-signal mechanism regulates T cell responses in secondary lymphoid tissues whereby TCR engagement by antigen/MHC on an antigen-presenting cell (APC

only triggers T cell accumulation and effector functions when a second co-stimulatory receptor on the T cell, CD28, is simultaneously engaged by B7 proteins that are induced on the APC upon exposure to microbes [61–64]. ICOS can provide co-stimulation for T cell responses in the absence of CD28 [50,65]. ICOSL, unlike B7.1 and B7.2, is expressed constitutively on many APCs in the absence of microbe components, raising a paradox about how autoimmunity is avoided in the face of this second co-stimulatory system. Maintaining low expression of ICOS on naïve T cells may therefore be critical to prevent the activation of T cells by self-antigens. Indeed, the severity of the lymphadenopathy correlated closely with the levels of ICOS expressed on naïve T cells in *sanroque* mice with either one or two alleles of *Icos* [27]. Taken together, these studies on *sanroque* mice reveal a unique mechanism to prevent autoimmunity by limiting ICOS–ICOSL signalling, which is complementary to the mechanism that controls expression of B7 co-stimulatory ligands for CD28 on APCs.

Roquin limits ICOS mRNA through the miRNA machinery

Aberrant expression of ICOS on *sanroque* $CD4^+$ T cells is T cell-autonomous, suggesting that Roquin functions to regulate ICOS expression either directly or indirectly in a cell-intrinsic fashion, which is reinforced by the observation that ectopic expression of Roquin into $CD4^+$ T cells represses ICOS protein expression [30] as well as its mRNA expression [27]. Notably, ICOS mRNAs from different species including human, rat and mouse all have long 3′ UTRs with several highly conserved segments [27]. The 3′ UTR is not under the same rigid structural constraints as the coding region or the 5′ UTR that has to accommodate the translational machinery. Therefore, conserved segments within 3′ UTRs that form under evolutionary pressure may function to regulate mRNAs. Consistent with this, a distal fragment within the 3′ UTR of ICOS mRNA was shown to be required for ICOS repression by Roquin. Specifically, Roquin destabilizes ICOS mRNA [27]. The conserved 3′ UTR segment containing the *cis*-acting elements for Roquin's repressive action contained a predicted target sequence for miR-101 and miR-103. Overexpression of miR-101 in T cells repressed ICOS mRNA and the repression of ICOS by Roquin was at least partially dependent on miR-101 recognition since a two-nucleotide inversion in the miR-101-recognized sequence within *Icos* 3′ UTR impaired its repression [27]. Repression of ICOS by miR-101 is likely to be involved in the physiological regulation of ICOS expression during T cell differentiation, since there is a striking inverse correlation between miR-101 expression and ICOS expression in human T cells: ICOS levels are lowest on naïve T cells, intermediate on activated T and memory cells and highest on T_{FH} cells [66]. By contrast, the highest levels of miR-101 are found in naïve cells, intermediate levels in memory cells and lowest levels in T_{FH} cells [27].

Roquin therefore emerges as a potential RNA-binding protein involved in miRNA-mediated post-transcription regulation (see discussion below). Other proteins in this

category have been shown to be capable of regulating mRNA stability of multiple targets. An example is the AU-rich element (ARE)-binding protein tristetraprolin, which regulates expression of multiple cytokines including TNF-α [67], granulocyte macrophage colony-stimulating factor [68] and IL-2 [69]. Not surprisingly, another surface receptor important for T cell priming, neuropilin 1, and over-expressed in *sanroque* T cells [30], is also a target of Roquin through the action of miR-101: neuropilin 1 mRNA was also repressed by ectopic expression of either Roquin or miR-101 [27]. Roquin thus appears to play an essential role in miRNA-mediated regulation of what we predict will turn out to be multiple transcripts co-ordinately involved in T cell priming and the maintenance of peripheral T cell tolerance.

How does Roquin regulate miRNA-mediated post-transcriptional regulation?

Clues as to how Roquin interacts with the miRNA machinery and/or the target transcripts, and how the mutation in *sanroque* mice affects this process, can be obtained from Roquin's protein sequence analysis and intracellular localization. Roquin contains several conserved domains (from N- to C-terminal): (1) a Cys×3-His-Cys×4 (C3HC4)-type zinc finger (RING finger) domain, recently shown to act as an E3 ubiquitin protein ligase [70], and found in many E3 ubiquitin protein ligases including c-Cbl; (2) a novel ROQ domain that contains the Met-199 → Arg substitution in *sanroque* mice; (3) a Cys-Cys-Cys-His (CCCH)-type zinc finger domain, predicted to bind nucleic acids (predominantly RNA) and found in a range of RNA-binding proteins, such as tristetraprolin; and (4) a proline-rich domain, containing potential sites for binding Src homology 3 (SH3) domains of interacting proteins [30] (Figure 15.2). The presence of a CCCH-type zinc finger domain suggests that Roquin may directly bind mRNA [71]. The prototypic CCCH zinc finger protein tristetraprolin has been shown to bind to AREs in the 3′ UTRs of *TNF* and mediate its degradation [67]. Ubiquitylating and proteasome activity have been shown to be essential for rapid turnover of mRNAs containing AREs [72]. Furthermore, AUF-1 and the mRNA-stabilizing protein, Hu antigen R (HuR), are degraded through the ubiquitination pathway [73], but the responsible E3 ligase is still unknown. It is therefore possible that Roquin's ubiquitin ligase and putative RNA-binding capacities may link the processes of ubiquitination of RNA-binding proteins and mRNA turnover.

Roquin can be found diffusely distributed in the cytoplasm, and upon stress-induction with arsenite treatment localizes to cytoplasmic aggregates containing T cell intracellular antigen 1 (TIA-1), an RNA-binding protein that is a marker for stress granules [30]. Stress granules are sites where, at times of cellular stress, transcripts are maintained in a form of translational arrest and triaged to exosomes for destruction, to polysomes to be translated, or to processing bodies (P-bodies), for miRNA-mediated translational inhibition or mRNA decay [74]. Interestingly, a recent study demonstrated that T cells also form stress granules to post-transcriptionally regulate the expression of T helper cytokines. Transient translational inhibition of cytokine

transcripts in stress granules occurred after T cell priming, but was relieved after re-stimulation [75]. Both the proliferation of primed T cells and the execution of T cell effector functions (cytokine secretion) need a rapid increase in the endoplasmic reticulum load due to an expanded protein repertoire. Stress granule-mediated uncoupling of the two processes may avoid the toxic side effects of endoplasmic reticulum overload [75]. Roquin's localization to stress granules suggests that during stress-inducing conditions Roquin acts to represses ICOS mRNA. This 'stress' could include T cell priming by dendritic cells in the absence of danger signals. Full-length ICOS mRNAs are also found in stress granules, and importantly, the localization of ICOS mRNA to stress granules is mediated by the 3′ UTR but not by the coding sequence [27], suggesting that localization of both ICOS mRNA and Roquin to stress granules is necessary for the regulation of ICOS expression by Roquin. Since Roquin's CCCH zinc finger domain is predicted to bind nucleic acids (predominantly RNA), it is possible that Roquin captures ICOS mRNA in the cytoplasm through a direct interaction, and recruits it into stress granules along with its own entry into this compartment. An alternative but not mutually exclusive possibility is that Roquin might recognize the ICOS mRNA–miRNA complex, in a way similar to how RISC recognizes mature double-stranded miRNAs [76]. It is also possible that the CCCH zinc finger domain may not mediate sequence-specific binding but non-specific binding to multiple RNAs in stress granules stabilizing and maintaining Roquin's localization in that compartment. The finding that full-length ICOS mRNA localizes into P-bodies suggests that once ICOS mRNA is recruited to stress granules, it is routed to P-bodies for degradation [27]. P-bodies have been observed to physically interact with stress granules. The transient association of stress granules and P-bodies can be promoted by the mRNA decay factors tristetraprolin and BRF1 [77,78]. A role for Roquin in facilitating transient interactions between stress granules and P-bodies in T cells is also possible.

One remaining important question is how the Met-199 → Arg mutation in Roquin impairs repression of ICOS. This substitution lies in the novel ROQ domain and has been predicted to alter the local helical structure, thus probably altering protein conformation [30]. Since it does not alter Roquin's subcellular localization, it is possible the Met-199 → Arg mutation impairs its predicted RNA-binding capacity, its E3 ligase function or the association with interacting proteins.

15.7 Concluding remarks

The mammalian immune system has evolved to safeguard against autoimmunity through a complex series of tolerance checkpoints. Animal models of autoimmune disease have illuminated many of these molecular and/or cellular mechanisms, enabling scientists to sketch the atlas of immune tolerance. The recently developed *sanroque* mouse strain has been an informative model, revealing how the less-well understood subset of helper T cells, T_{FH} cells, are critical to maintain tolerance in germinal centres, through the tightly regulated expression of ICOS (Figure 15.3). Roquin has emerged as a key node in an miRNA pathway that represses key T cell-co-stimulatory molecules.

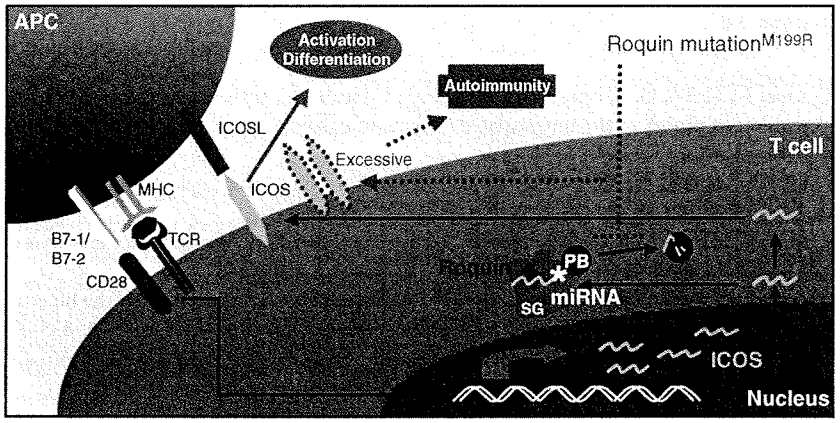

Figure 15.3 Model by which repression of ICOS by Roquin inhibits autoimmunity. In wild-type mice a significant proportion of *Icos* transcripts induced by TCR and CD28 signalling undergoes degradation and this is enhanced by Roquin acting with the miRNA machinery. The end result is a decrease in the amount of ICOS expressed on the cell surface. After ligation by ICOSL, which is consitutively expressed on APCs, ICOS transduces a signal that provides co-stimulation for T cell activation and differentiation. Limits on ICOS expression imposed by Roquin ensure the maintenance of self-tolerance. In *sanroque* mice, the degradation of *Icos* transcripts is impaired due to the Met-199 → Arg (M199R) mutation in Roquin, resulting in ICOS over-expression on the cell surface. Exaggerated signalling transduced by uncontrolled expression of ICOS contributes to autoimmunity. PB, processing-body; SG, stress granule; miR, microRNA.

At the molecular level, a picture is emerging in which miRNA-mediated gene regulation acts to fine-tune expression of pro-immunogenic molecules whose over- or under-expression may tip the delicate balance between protective immunity and deleterious autoimmunity. At the cellular level, the regulation of the ICOS–ICOSL co-stimulatory axis emerges as a powerful T cell tolerance mechanism complementary to that of controlling expression of the ligands for the main co-stimulator, CD28. At the organism level, a tightly regulated and tolerized T_{FH} population is required to prevent the development of germinal centre-derived autoimmunity. These findings open the way for manipulation of RNA interference to achieve much needed specificity and reduced side effects in the treatment of systemic autoimmunity. They also suggest that polymorphisms in miRNAs and their complementary sequences within untranslated regions of target mRNAs may constitute candidate lupus-susceptibility alleles.

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