Non-coding RNAs: master regulators of inflammatory signaling

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- ncRNA have been shown to interact with proteins to influence downstream signaling events. NKILA has been shown to bind to the NF-κB/IκB complex, which masks the phosphorylation sites of IκB, preventing its degradation (Liu et al., 2015).
- lncRNA have been shown to interact with hnRNPs in affecting transcription. lincRNA-EPS and THRIL have been shown to interact with hnRNPL (Atianand et al., 2016 and Li et al., 2014).
- Genetic mouse knockout models of lncRNA can provide further evidence of their physiological relevance. For example, knockout of *lincRNA-EPS* resulted in enhanced inflammatory responses, supporting its role in repressing the expression of immune response genes (Atianand et al. 2016).
- Unbiased approaches using RNA-seq, microarray or CRISPR-Cas9 screens have helped identify ncRNA important in inflammation. ncRNA identified through such approaches include THRIL, lincRNA-Cox2 and lincRNA-EPS (Li et al., 2014, Carpenter et al., 2013 and Atianand et al., 2016).
- New avenues of therapeutic intervention might be provided by targeting ncRNA with cellular specificity, offering the possibility of subtle manipulation of inflammatory signaling relevant to a variety of pathologies.

Abstract

Inflammatory signaling underlies many diseases from arthritis to cancer. Our understanding of inflammation has thus far been limited to the world of proteins, as we are only just beginning to understand the role non-coding RNAs (ncRNA) might play. It is now amply clear that ncRNA do not constitute transcriptional 'noise' but harbor physiological functions in controlling signaling pathways. This review covers the newly discovered mechanisms and functions of ncRNAs in the regulation of inflammatory signaling. Advances in experimental techniques allowing this field of research to take root are also described. These findings have opened new avenues for putative therapeutic intervention in inflammatory diseases which may be seen translated into clinical outcomes in the future.

New insights into inflammatory signaling through non-coding RNAs

Inflammation is a normal physiological response to bacterial or viral infection (Medzhitov and Horng, 2009). Cardinal signs of inflammation such as swelling, redness and pain are caused by local actions of inflammatory chemokines and cytokines, proteins which are synthesized through complex transcriptional programs activated in response to foreign entities. Until recently, our understanding of inflammation has largely been based on the central dogma of molecular biology: genetic information stored in our DNA encodes messenger RNA transcripts (mRNA) from which proteins are synthesized. Mutations in DNA result in altered RNA sequences and hence, in changes to protein structure and function, which have been thought to underlie genetic disorders and diseases (Griffiths, 1999). Our comprehension of inflammatory signaling pathways is predicated on understanding the functions of proteins and their interactions; currently, this knowledge is incomplete. It is now understood that only 3-5% of our genome codes for proteins, and a critical question is whether we are fundamentally missing an entire class of regulators of inflammation. Two decades have almost passed since the sequencing of the human genome, so could we have already identified most, if not all, of the proteins that mediate inflammatory signaling? Emerging evidence suggests that mutations in regions of the genome that do not code for proteins are associated with a number of human ailments (Edwards et al., 2013). For instance, hits from **genome-wide association studies (GWAS)** in human chromosomal region 9p21 have been linked to several diseases including diabetes and cancers, such as glioma and melanoma (Cunnington, 2010). Of the three genes identified in this region, expression of a long non-coding RNA (lncRNA) named CDKN2B antisense RNA 1 (*ANRIL*) was most strongly correlated with disease associated variants (Cunnington, 2010). Overall, this was one of the first pieces of evidence suggesting that non-coding RNAs (ncRNA) could affect cellular processes and hence human health.

It is now widely accepted that ncRNAs do not constitute transcriptional ‘noise’, but rather, that they bear physiological functions. ncRNAs have been documented to alter signaling pathways by acting as scaffolds for protein complexes, changing protein concentrations by regulating

transcription or altering translation, and interacting as signaling partners with specific proteins (Fatica and Bozzoni, 2014). These novel functions of ncRNAs have sparked renewed interest and excitement in the field of ncRNAs. This timely review covers such recent advances and explores how these may lead to strategies for therapeutic intervention in human ailments where inflammation constitutes an underlying etiology.

Classes of ncRNAs

The more well-known classes of ncRNAs include transfer RNA (tRNA) and ribosomal RNA (rRNA), which are highly abundant and have well defined structural and regulatory roles in translation. Novel classes emergent in the past decade such as microRNAs (miRNA) or lncRNAs have since been shown to play a role in the fine regulation of gene expression (Hu et al., 2012) and are emergent as essential regulators of inflammation and immunity (Ling et al., 2013). As such, mutations in non-coding regions are under increased scrutiny for their potential role in driving genetic diseases (Makrythanasis and Antonarakis, 2013).

miRNAs are small non-coding RNA (~22 nucleotides) that bind to the 3' UTR of target genes, inducing RNA cleavage or translational repression of target genes (Ling et al., 2013) (Figure 1). Although miRNAs are the best characterized ncRNAs involved in the regulation of inflammatory conditions, there has been growing interest in the roles of lncRNAs in chronic inflammation. LncRNAs are categorically defined as non-protein coding RNA transcripts longer than 200 base-pairs (Figure 1) (Fatica and Bozzoni, 2014). They can regulate immune responses and inflammatory signaling through their interaction with signaling proteins (Fatica and Bozzoni, 2014). Alternatively, they can enhance or inhibit gene transcription by interacting with transcription factors, acting as a scaffolds for the recruitment of epigenetic regulators or other protein complexes, and acting as a 'sponge' to 'absorb' miRNAs (Fatica and Bozzoni, 2014). Recently, several lncRNAs have also been shown to code for peptides such as

myoregulin, **SPAR** and **Toddler** which harbor biological functions (Anderson et al., 2015; Matsumoto et al., 2017; Pauli et al., 2014).

In addition to miRNAs and lncRNAs, a further class of ncRNAs are emerging as important regulators of cellular function. These covalently-closed circles of single stranded RNA are formed by **back-splicing of pre-mRNA** (Figure 1). Termed **circular RNA (circRNA),** they were previously overlooked, given that they are difficult to detect by traditional methods of sequencing (Jeck and Sharpless, 2014). Early immunoprecipitation and luciferase reporter experiments involving ciRS-7 and circular SRY suggest that some circRNAs sequester miRNAs, acting as sponges and inhibiting miRNA activity (Hansen et al., 2013). In addition, similarly to lncRNAs, circRNAs have recently been shown to be translated, indicating the existence of an uncharted sphere of protein isoforms and expression patterns (Legnini et al., 2017; Pamudurti et al., 2017). It remains to be seen how these classes of ncRNAs can regulate crosstalk between inflammatory signaling pathways. Consequently, a finer dissection of the roles of ncRNAs in inflammation will lead to a better understanding of these complex pathways, potentially uncovering candidate therapeutic targets in chronic inflammatory conditions such as rheumatoid arthritis and Crohn's disease.

Detection and functionalization of ncRNA transcriptomes

Methods to detect ncRNA transcripts

Early techniques for the identification of non-coding transcripts required ncRNAs to be present in such large amounts that they could be visualized on **denaturing gels** and sequenced individually. These techniques allowed for the identification of high abundance ncRNAs such as tRNAs and rRNAs, but ultimately, the lower abundance of other ncRNAs limited the application of these techniques, which were lower in sensitivity (Huttenhofer and Vogel, 2006). Later, more advanced approaches included transcriptome analysis through Sanger sequencing of cDNA libraries (Okazaki et al., 2002), as well as **microarrays**. Targeted

microarrays such as the NCode™ Human and Mouse ncRNA microarrays probed cDNA samples for selected genes that were **annotated** (Mockler et al., 2005). **Tiling microarrays** were later developed (Rinn, 2003), allowing investigators to probe the transcription patterns of ncRNAs in unannotated regions of the genome (Weile et al., 2007). Although these approaches were high-throughput, they biased results by limiting ncRNA discoveries to selected genes or genomic regions contained within the microarray chips.

The advancement of sequencing technology later allowed for the unbiased detection of ncRNAs and identification of novel ncRNA transcripts. The first techniques used short cDNA sequence tags; these were concatenated, cloned and subsequently sequenced, allowing for both quantification of ncRNA transcripts and identification of de novo nascent transcripts (Fatica and Bozzoni, 2014). Recently, RNA-sequencing (RNA-seq) has been increasingly used, where isolated RNA is processed to remove genomic DNA or ribosomal RNA, then subsequently reverse-transcribed to create cDNA libraries (Kukurba and Montgomery, 2015). These cDNA fragments are sequenced in parallel, producing large numbers of **short 'reads'** which are mapped to a pre-existing reference genome, together with **computed expression scores** that allow comparison of the differential expression of transcripts (Marguerat and Bähler, 2010). Further, **chromatin immunoprecipitation (ChIP)** of transcription factors combined with microarrays (ChIP-chip) or sequencing (ChIP-seq), highlight actively transcribed regions of the genome, thus allowing identification of transcripts such as ncRNAs expressed from unannotated or non-protein coding regions (Fatica and Bozzoni, 2014).

Single cell transcriptomics (Kolodziejczyk et al., 2015) have allowed the study of ncRNAs at the single cell level (Gambardella et al., 2017; Liu et al., 2016; Zhang, 2014). By comparing the expression of ncRNAs and the functional outcomes between individual cells, this technique has provided important insights into the fine gene regulation capabilities of ncRNAs.

RNA databases

RNA databases provide public repositories of identified ncRNAs, as well as annotations of known and predicted interactions, expression patterns or functions (Selected databases described in Supplementary table S1). While there are databases such as NONCODE (Liu et al., 2005) and RNAcentral (Consortium, 2017) which curate all types of ncRNAs (Supplementary table S1), there are also those which are specific toward ncRNA subtypes such as miRBase for miRNA (Kozomara and Griffiths-Jones, 2014), lncrnadb for lncRNAs (Amaral et al., 2011) or circBase for circRNAs (Glazar et al., 2014) (Supplementary table S1). These databases can provide insight into the function of particular candidate ncRNAs, or even assess potential ncRNA interactions (e.g. rna22 (Loher and Rigoutsos, 2012) and TargetScan (Agarwal, 2015) for miR-protein targets). However, they are not fully exhaustive given that many ncRNAs have not yet been characterized.

Validation and functional analysis of ncRNA targets

Detection and characterization of endogenous ncRNAs, along with loss- or gain-of-function analysis provide invaluable insight into functional properties of uncharacterized molecules. Verification of endogenous expression of candidate ncRNAs can be achieved through techniques such as **Northern blotting** and **quantitative reverse transcription PCR (RT-qPCR)**. These techniques have the advantage of identifying the presence of multiple isoforms, and the expression pattern of these isoforms across treatments, time-courses or tissues (Kim et al., 2010; Pall and Hamilton, 2008; Rajeevan et al., 2001). Additionally, understanding the localization of ncRNAs can strongly inform functional analysis. Fluorescent in-situ hybridization (FISH) uses ncRNA-specific, fluorescently-labelled probes and microscopy to visualize the localization of ncRNAs within whole cells (Orjalo and Johansson, 2016). This can subsequently be verified through crude fractionation of cells followed by RT-qPCR to determine the proportion of any candidate ncRNA in either the nucleus or supernatant of cells (Conrad and Orom, 2017). RNA-protein interactions can be probed by **immunoprecipitating**

an RNA binding protein coupled with RNA-sequencing (RIP-seq) to determine the ncRNA bound to a protein of interest (Zambelli and Pavesi, 2015). RNA immunoprecipitation (RIP) can also be paired with reverse transcription and qPCR in cases where the candidate RNA is known (Gagliardi, 2016). A similar method, crosslinking immunoprecipitation with high throughput sequencing (CLIP-seq), involves crosslinking of protein to RNA, and immunoprecipitation of the RNA binding protein is followed by RNA sequencing to provide a more stringent method of probing weak RNA interactions with a specific protein (Darnell, 2010).

Alternatively, ncRNAs can be 'pulled down' directly. For example, **chromatin isolation by RNA immunoprecipitation (ChIRP-seq)** probes lncRNA:DNA interactions, identifying the enhancer or promoter regions through which lncRNAs might regulate gene expression (Chu et al., 2011). Candidate lncRNAs are pulled down from crosslinked samples with sequence-specific **biotin-labelled probes,** and bound DNA regions are subsequently sequenced**.** Other methods for lncRNA pulldown exist; these include incubating cellular extracts with an *in vitro* synthesized biotinylated candidate lncRNA (Huarte et al., 2010), or transfecting cells with a lncRNA expression plasmid tagged with a specific **viral RNA structure** which can be bound by, and pulled down with Flag-tagged MS2 coated-proteins (Gong et al., 2012).

Similarly to protein function analysis, experimentally altering the expression of ncRNA through overexpression or knockdown studies can provide important information about the function of candidate ncRNAs. **RNA interference (RNAi)** has been successfully applied to both miRNAs (Krutzfeldt et al., 2005) and cytoplasmic lncRNAs (Lennox and Behlke, 2016) as a method to reduce the expression of these molecules. However, lncRNAs often exhibit multiple isoforms, making them difficult to knockdown completely through a single **small interfering RNA** design (Bassett, 2014).

CRISPR-Cas9 genome editing technology has been widely used for loss of function studies of protein coding genes by generating small insertions and deletions (indels) in the coding

sequence, leading to premature stop codons and translational inhibitions (Doudna and Charpentier, 2014). miRNAs can be targeted by designing a **guide RNA (gRNA)** to the **Drosha** and **Dicer** processing sites of a candidate miRNA, leading to disruption of miRNA biogenesis (Chang et al., 2016). However, this strategy cannot be applied to knock-out lncRNAs, as generating indels may not disrupt transcription of targeted lncRNAs, which lack open reading frames (Bassett, 2014). Moreover, newly transcribed lncRNAs with indels may still retain their functional domains or binding sites, which can make phenotypic analysis ambiguous (Bassett, 2014). An alternative strategy has been developed for lncRNAs that uses **paired-guide RNAs (pgRNAs)** to induce two simultaneous cuts, resulting in deletion of a larger region encompassing the promoter region and first exon of a candidate lncRNA (Ho et al., 2015). However, this strategy of promoter manipulation presents its caveats, as there might be multiple transcription factor binding sites involved, and the deletion might potentially delete enhancers which could affect the expression of neighboring genes, leading to misinterpretation of lncRNA function. This can be basically addressed by ectopic expression of the lncRNA from an exogenous plasmid in the lncRNA knock-out cells, and verifying that the phenotype can be rescued (Bassett, 2014). Hence, the phenotypes created via CRISPR-Cas9-mediated deletion of lncRNAs should be carefully analyzed by discriminating the impact of the lncRNA itself from its loci.

Because of the coding potential of ncRNAs, it is also important to distinguish between an effect that arises from the ncRNA itself, or from its encoded peptide. The coding potential of a ncRNA transcript can be evaluated by several algorithms including **CPC** (Kong et al., 2007) or **COME** (Hu et al., 2017). If the ncRNA is determined to have coding potential, the loading of the ncRNA onto polysomes can subsequently be tested via **sucrose gradient fractionation** (Legnini et al., 2017). Next, this ncRNA is cloned into an expression vector from which a tagged version of the encoded protein might be potentially expressed. Subsequent **western blotting** would determine if a peptide is synthesized from the cloned ncRNA sequence. Further functional validation of the peptide can then be achieved through genomic silencing of the ncRNA,

followed by rescue experiments with either the wild-type ncRNA or the in-vitro synthesized peptide (Legnini et al., 2017; Pauli et al., 2014). Using cycloheximide to inhibit translation can help further distinguish between a ncRNA-specific or a peptide-specific function (Legnini et al., 2017; Pauli et al., 2014).

While we have described general techniques used in functionalization of ncRNAs, there are limitations to applying these techniques to lncRNAs. Unlike protein-coding genes or miRNAs, the sequence or structure of a lncRNA provides little insight into its functionality (Fatica and Bozzoni, 2014) . This can be attributed to the lack of conservation between the sequence motifs and secondary structures of lncRNAs (Ma et al., 2012), making it difficult to predictthe targets and interactors. With our understanding of the diversity of lncRNAs in its infancy, we are unable to make simple generalizations about their functional characteristics (Fatica and Bozzoni, 2014). Further, knockdown of lncRNAs does not always result in obvious phenotypes. This is reminiscent of the theory that lncRNAs can fine-tune gene expression rather than act as ‘on-off’ switches, which further complicates their functional characterization (Ma et al., 2012).

CRISPR screening for ncRNA function

Despite the important functions ncRNAs play in cellular regulation, predicting the significance of lncRNA loci expression in mammalian cells remain a challenge, leading to the need for genome-wide, systematic approaches that can probe the biological roles of given lncRNAs. Toward this end, CRISPR-based screens against lncRNAs have been developed, targeting more than 10,000 human lncRNA loci through **CRISPR-Cas9 interference (CRISPRi)** or via a paired-guide RNA strategy (pgRNA) (Joung et al., 2017; Liu et al., 2017; Zhu et al., 2016). Given that there may be at least 100,000 lncRNA in the human genome (Joung et al., 2017), these screens are not yet exhaustive, but with expanded libraries, may become useful resources for future applications examining ncRNAs in inflammation. Of note, a **single guide**

RNA (sgRNA) library has been developed to knock-out mouse or human miRNAs, which has been reported to be successfully applied for screening miRNA function (Sanjana et al., 2014). The same library has been successfully used to identify miRNAs that regulate myeloid leukemia cell growth (Wallace et al., 2016), showing the utility of CRISPR-based functional screens as a tool for identifying novel ncRNAs involved in inflammatory pathways.

Emerging Roles of ncRNAs in Inflammatory Pathways: NFκB, JAK-STAT, and MAPK

Inflammation is the body's intrinsic response to invading pathogens. Although acute inflammation is vital for defense against pathogens or survival and healing following injury, it becomes chronic when allowed to continue unchecked. Under these circumstances it drives the development of many human diseases ranging from cancer to autoimmune disorders, with the transcription of a range of cytokines, chemokines, growth factors and other pro-survival, immune regulating proteins (Medzhitov and Horng, 2009). Over the last two decades, many proteins involved in inflammatory signaling cascades, transcriptional control and regulatory feedback mechanisms have been identified and targeted successfully (Ruland, 2011). However, up until now, the role of various classes of ncRNAs -- including miRNAs, lncRNAs and circRNAs-- have been difficult to determine (Derrien et al., 2012; Harrow et al., 2012).

Our understanding of inflammation and the way it is regulated has been largely limited to genomic markers and proteins that are involved in transcription, phosphorylation, ubiquitination and protein-protein interactions (See Box 1).

To achieve a better understanding of the regulatory pathways in inflammation, in the next section, we discuss the characterization and functional roles of known ncRNAs in three major inflammatory and disease-associated pathways; namely, nuclear factor-κB (NF-κB), Janus Kinase and Signal Transducer and Activator of Transcription (JAK-STAT) and Mitogen-activated protein kinase (MAPK) (Figure 2).

NF-κB signaling

The NF-κB signaling pathway is activated downstream of cytokine receptors such as the TNF receptor (TNFR) and innate immune receptors such as **Toll-like receptors (TLR)** under developmental and physiological settings (Correa et al., 2005; Tong and Tergaonkar, 2014) (See Box 2 and Figure 2). Deregulation of canonical and non-canonical NF-κB signaling cascades drive various immune disorders (For a detailed review on non-canonical NF-κB signaling, please see (Cildir et al., 2016)). Specifically, inherited genomic deletions or missense mutations in IKK-gamma (*NEMO*) preventing its ability to activate NF-κB signaling can lead to **incontinentia pigmenti** and X-linked recessive **immunodeficiency** (Smahi et al., 2000; Zonana et al., 2000). Several groups have also identified a dominant mutation in the gene coding for IκB, which blocks NF-κB signaling by preventing IKK-mediated phosphorylation of IκB, and leading to human immunodeficiency (Courtois et al., 2003; Janssen et al., 2004). Somatic gene mutations in *REL,* including gene amplifications, deletions and point mutations have also been identified in human B-cell malignancies. Additionally, the NF-κB pathway has been implicated in the development of many human ailments such as breast cancer and glioblastoma, where inflammation can be an underlying contributing cause (Dey et al., 2008; Li et al., 2015; Shin, 2014).

miRNAs in the NF-κB pathway

Several ncRNAs in the NF-κB signaling pathway have been identified (see Supplementary table S2, Figure 3). Here, we discuss miRNAs which have been shown to directly target key proteins in the NF-κB signaling pathway. For a more comprehensive review of miRNAs involved in NF-κB signaling, please refer to (Gao et al., 2014). miR-155 has been found to target the gene coding for IκB, a negative regulator of NF-κB signaling; specifically, in mouse

RAW 264.7 macrophages, an increase in miR-155 has been associated with increased NF-κB activation (Tili et al., 2007). In human myeloid cell lines such as THP-1 and HL-60, activation of NF-κB signaling has also been reported to induce the expression of miR-146, which in turn can target adaptor proteins important for NF-κB signaling such as IL-1 receptor associated kinase (IRAK1) and TNF receptor-associated factor (TRAF), thereby inhibiting further NF-κB activation (Taganov et al., 2006). While these findings underscore the significance of miRNA-mediated post-transcriptional regulation of NF-κB effectors, the disease-relevance of these miRNAs remains unknown.

lncRNAs in the NF-κB pathway

LncRNAs contain modular domains through which they directly interact with NF-κB signaling proteins. An example is the Lethe lncRNA, induced upon TNF-α stimulation in wild-type mouse embryonic fibroblasts (MEFs) (Rapicavoli et al., 2013). RelA ChIP and RIP experiments have shown that Lethe can act as a negative feedback molecule to inhibit NF-κB driven gene transcription by associating with RelA to prevent its binding of murine target genes such as *Il6*, *Il8* and *Nfkbia* (Rapicavoli et al., 2013). Another lncRNA, NKILA, is upregulated in MDA-MB-231 breast cancer cells by stimuli such as TNF-α or LPS that activate NF-κB signaling (Liu et al., 2015). NF-κB-activity luciferase reporter assays and RIP have demonstrated that NKILA negatively regulates NF-κB signaling by binding to the NF-κB/IκB complex(Liu et al., 2015). Further in vitro phosphorylation assays have revealed that this association masks the phosphorylation sites of IκB, preventing its degradation, and inhibiting NF-κB activation (Liu et al., 2015). Another lncRNA, PACER, is induced in human HMECs and U937 cell lines upon expression of cyclooxygenase 2 (COX2) (Krawczyk, 2014). RIP experiments have shown that PACER can interact with p50, the inhibitory subunit of NF-κB; sequestration of p50 in turn, potentiates the formation of p65/p50 activating dimers, and ChIP-qPCR experiments have

demonstrated that this facilitates the recruitment of the p300 histone acetyltransferase and RNA Polymerase II initiation complexes to promote gene and protein expression of COX2, an important mediator of inflammation and immunity (Krawczyk, 2014; Perkins, 1997). NF-κB activation in mouse macrophages also induces the expression of lincRNA-Tnfaip3, which binds to NF-κB in a complex with Hmgb1, as shown through RIP analyses (Ma et al., 2017). ChIP assays have further demonstrated the dependence on lincRNA-Tnfaip3 in the activation of the *Tnfaip3* and *Il6* gene loci upon LPS stimulation, highlighting the capacity of this lncRNA to fine-tune NF-κB transcription (Ma et al., 2017).

lncRNAs are predominantly localized to the nucleus and chromatin, as shown in a panel of five human cell lines (Derrien et al., 2012), With this specific compartmentalization and proximity to nuclear proteins, it is not unsurprising that they have been found to interact extensively with heterogeneous nuclear ribonucleoproteins (hnRNPs), large nuclear RNA-binding proteins with roles in splicing, mRNA stabilization and regulation of transcription and translation (Geuens et al., 2016). An example is lincRNA-Cox2, a lncRNA proximal to the mouse prostaglandin-endoperoxide synthase 2 *Ptgs2* gene coding for Cox2 (Carpenter et al., 2013). In this study, LincRNA-Cox2 was highly induced by TLR ligand Pam_3CSK_4 in mouse bone-marrow-derived macrophages (BMDMs), and this expression was dependent on the activity of MyD88 and NF-κB transcription factors (Carpenter et al., 2013). Furthermore, RNA-seq and subsequent Gene Ontology (GO) enrichment analysis of both control and *lincRNA-Cox2*-silenced macrophages altered the expression of hundreds of genes involved in innate immunity, highlighting its importance as a regulator of certain immune responses (Carpenter et al., 2013). Probing further into the mechanism of lincRNA-Cox2 action, the interaction between lincRNA-Cox2 and hnRNP-A/Bwas shown to repress the transcription of a subset of immune genes such as *Ccl5* and *Irf7* (Carpenter et al., 2013). Recently, using RIP assays, lincRNA-Cox2 has also been shown to associate with the switch/sucrose nonfermentable (SWI/SNF) complex in RAW264.7 mouse macrophages upon LPS stimulation; this in turn was found to interfere with the recruitment of NF-κB subunits RelA and p50 into the SWI/SNF

complex (Hu et al., 2016). These findings suggest that lincRNA-Cox2 might act as a coactivator of NF-κB to induce late NF-κB response genes such as *Ccl5* and *Saa3* (Hu et al., 2016); this in turn, implicates lincRNA-Cox2 in the temporal regulation of NF-κB response genes, at least in mice.

Another lncRNA, lincRNA-EPS has been found to be downregulated in mouse BMDMs and bone-marrow-derived dendritic cells (BMDCs) upon TLR stimulation with Pam_3CSK_4, LPS and polyinosinic-polycytydilic acid (poly I:C)(Atianand et al., 2016). Under normal conditions, lincRNA-EPS may serve to restrict the expression of immune response genes such as *Cxcl10*, *Cxcl9* and *IL-27* (Atianand et al., 2016). In this study, RIP analyses showed that lincRNA-EPS could interact with hnRNPL via a *CANACA* motif at its 3' end, localizing to regulatory regions of immune response genes and controlling promoter accessibility to repress transcription of these genes; this in turn, implicated lincRNA-EPS in transcriptional regulation to prevent the spontaneous (and unwarranted) activation of immune genes in mice (Atianand et al., 2016). *THRIL* is another lncRNA in the NF-κB pathway which was first identified in a unbiased microarray screen of the THP1 human macrophage cell line following innate immune activation; pulldown assays demonstrated that it could form a complex with hnRNPL, essential for the regulation of transcription of the gene coding for TNF-α (*TNFA*) (Li et al., 2014). The authors reported a positive disease association between *THRIL* expression and incidence of Kawasaki disease (rare inherited condition of inflamed blood vessels); thus, one might not need to rule out the possibility that THRIL-mediated regulation of *TNFA* expression might be also implicated it in other inflammatory diseases such as rheumatoid arthritis, but this remains untested (Li et al., 2014). Accordingly, the functional relevance of other lncRNAs in the regulation of NF-κB effector proteins and inflammatory gene expression, may potentially signify that dysregulation of such lncRNAs may have far-reaching implications in the pathogenesis of various yet-to-be associated chronic inflammatory conditions.

circRNA in the NF-κB pathway

The innate stability of circRNAs over lncRNAs (due to resistance to exonuclease degradation) may potentiate their use as candidate biomarkers of inflammatory diseases and long-term regulators of genes and proteins (Jeck and Sharpless, 2014). A recent study on LPS-inducible circRNAs in mouse RAW264.7 cells identified mcirc*RasGEF1B* as a circRNA with NF-κB-dependent expression (Ng et al., 2016). While mcirc*RasGEF1B* was found to regulate the stability of mature mRNA coding for ICAM-1, the mechanism through which this occurred remains unknown (Ng et al., 2016). While much remains to be understood about the identity and function of circRNA which are regulated by NF-κB, we hypothesize that circRNA play important roles in long-term regulation of gene and protein expression because of their resistance to exonuclease digestion, allowing them to persist inside the cell where it continues to exert its biological impact.

JAK-STAT Signaling

The JAK-STAT pathway is activated by cytokines and Type I and Type II IFNs (Aaronson and Horvath, 2002), and mutations in the pathway can lead to immune-related disorders (see Box 3, Figure 2).

ncRNA in JAK-STAT signaling

Several non-coding RNA (ncRNA) species have been implicated in the regulation of the JAK-STAT signaling pathway (Figure 3, Supplementary table S3). The most well studied class of non-coding RNAs, miRNAs, have been shown to target important players in the JAK-STAT signaling pathway: JAK2 (miR-135 in human gastric cell lines such as GES-1) (Wu et al., 2012); STAT1 (miR-145 in the colon cancer cell line DLD-1 and systemic lupus erythematosus, miR-221/222 in the glioblastoma U251 cell line) (Gregersen et al., 2010; Lu et

al., 2013; Zhang et al., 2010); and STAT3 (let-7 in the HepG2 cell line, miR-124 in colon tissue of ulcerative colitis patients) (Koukos et al., 2013; Wang et al., 2010b). Furthermore, lncRNA HOXD-AS1, induced by retinoic acid in the human SH-SY5Y neuroblastoma cell line, has also been shown to increase transcription of JAK/STAT target genes such as those coding for COX-1 and Caspase-1 (Yarmishyn et al., 2014).

Recently, JAK-STAT signaling was also found to regulate the expression of lncRNAs (Hu et al., 2013). Similar to NF-κB-regulated lncRNAs, these regulated ncRNAs can provide molecular feedback within the JAK-STAT pathway. For example, the expression of lncRNA-CMPK2 has been reported to be dependent on JAK-STAT signaling and in turn, to negatively regulate the IFN response in human hepatocytes (Kambara et al., 2014). In addition, using ChIP assays in lymphoblastoid cell lines, *ANRIL* expression was found to be regulated by JAK-STAT signaling, a lncRNA which can associate with polycomb repressive complexes to modulate gene expression (Harismendy et al., 2011).

Reciprocally, several lncRNAs have also been found to regulate JAK-STAT signaling. For example, in various cancer cell lines, including HepG2 and COLO205, aspirin-induced lncRNA OLA1P2, was reported to inhibit the nuclear import of phosphorylated STAT3 by binding to and preventing homodimerization of phosphorylated STAT3 (Guo et al., 2016). This represents a novel mechanism through which aspirin might potentially be able to reduce cancer risk, but this remains to be tested (Guo et al., 2016). Another lncRNA, lnc-DC is exclusively expressed in dendritic cells (DC), and shown to be required for optimal DC differentiation from human monocytes (Wang, 2014). It also regulates DC activation of T cells, and RIP experiments have demonstrated that it can associate with STAT3 (Wang, 2014). Furthermore, lnc-DC knockdown experiments have revealed that it can prevent binding to and dephosphorylation of SHP1 phosphatase; the authors speculated that as such, it might be potentially relevant in studying diseases associated with dysfunctional DCs (Wang, 2014). These examples highlight different mechanisms through which ncRNAs might activate or inhibit the JAK-STAT signaling pathway, increasing potential nodes of regulation for

therapeutic targeting. Indeed, this may be particularly relevant for diseases such as myeloproliferative disorders, where JAK-STAT signaling can be dysfunctional.

MAPK Signaling

MAPKs, which include p38, JNK and ERK, are serine-threonine kinases activated by a variety of extracellular and intracellular stimuli (Kim and Choi, 2010). Here, we focus on p38 and JNK pathways which are activated by pro-inflammatory cytokines such as TNF-α and IL-1β (Kim and Choi, 2010). In mammals, the binding of TNF-α or lipopolysaccharide (LPS) to TNFR or Toll-like receptor 4 (TLR4) activates MAP kinase kinase kinases (MAP3Ks) such as transforming growth factor-α-activated complex 1 (TAK1), which can go on to activate MAP kinase kinases (MAP2Ks) such as MKK4 (Kim and Choi, 2010). This leads to the subsequent activation of JNK by MKK4 and MKK7, and p38 MAP kinase by MKK3, MKK4 and MKK6 (Raingeaud et al., 1996) (Figure 2). Significant activation of p38 has also been found in inflamed human intestinal mucosae associated with inflammatory bowel disease (Dahan et al., 2008). JNK pathway signaling, with major roles in cell death and apoptosis, has been implicated in neurodegenerative diseases such as Alzheimer's disease (AD) (Ploia, 2011) and Parkinson's disease (PD) (Maroney, 1999). For instance, inhibition of JNK activation in rat neuronal cultures and human fibroblasts prevented tau protein hyperphosphorylation (which can lead to the formation of neurofibrillary tangles), and in addition, prevented AD pathogenesis in a mouse model of AD (TgCRND8 mice) (Ploia, 2011). Another study indicated that JNK inhibition in PC12 cell cultures could prevent cell death from stimuli such as withdrawal of nerve growth factor, or exposure to UV or oxidative stress (Maroney, 1999).

A number of miRNAs have been found to regulate the MAPK signaling axis (Figure 3, Supplementary table S3). For example, in rat trigeminal ganglions, miR-125a-3p has been shown to regulate the expression of p38 MAPK in luciferase assays (Dong et al., 2014). JNK protein levels, and hence apoptosis, have also been found to be regulated by miR-125b in

human melanoma tissues and cell lines (Kappelmann et al., 2013), and miR-216b, in NIH3T3 fibroblasts (Xu et al., 2016). Furthermore, in vitro kinase assays have shown that activation of the p38-MAPK signaling pathway can also lead to the phosphorylation and destabilization of RNAse III Drosha, an important mediator of miRNA biogenesis (Yang et al., 2015).

Recent studies have implicated lncRNA MALAT1 in the regulation of p38 and ERK signaling. While MALAT1 is conventionally associated with cell cycle control and glioma metastasis (Han et al., 2016; Tripathi et al., 2013), cross-talk of MALAT1 with the p38 MAPK pathway has been reported in diabetic rodent models of diabetes-induced microvascular dysfunction (streptozotocin injection in rats, and in db/db mice) (Liu et al., 2014). The lncRNA BANCR has been found to suppress both p38 and JNK activation in human lung carcinoma cell lines such as NCI-H446 and SPC-A1 (Jiang et al., 2015). Another lncRNA, NEAT1, known to be essential for the formation of nuclear body **paraspeckles**, has been linked to the induction of p38 MAPK activation (Imamura et al., 2014). NEAT1 was induced in HeLa and A549 fibroblasts upon poly I:C activation or viral infection (influenza, herpes simplex virus 1 or measles virus). Specific inhibition of p38 by ML3403 in HeLa cells abolished poly I:C-induced NEAT1 expression (Imamura et al., 2014). Moreover, ChIP and fluorescence experiments revealed that in HeLa cells, NEAT1 also mediated the expression of antiviral cytokines and IL-8 through the relocation of Splicing factor, proline- and glutamine-rich SFPQ, from the *IL8* promoter to **paraspeckles** (Imamura et al., 2014). A better understanding of the range and roles of ncRNAs in such pathways and their crosstalk (See Box 4) may uncover unknown regulatory mechanisms that may be useful in drug discovery strategies for a variety of ailments.

Therapeutically targeting ncRNAs

Increasing evidence of the importance of ncRNAs in mediating inflammatory and immune responses are indicative of the significant therapeutic potential of targeting ncRNAs, which could provide several advantages over traditional protein targets; indeed, therapeutically

targeting almost any ncRNA through the techniques outlined below may be feasible. However, not all proteins can be therapeutically targeted. Some proteins lack targetable 'pockets' typically modulated by small molecule compounds. Further, small molecules are also less likely to disrupt protein-protein or protein-DNA interacting surfaces which are generally flat and large (Arkin et al., 2014). The highly specific expression patterns of ncRNAs could mean that these might be potentially used for accurate and precise diagnostic and prognostic purposes (Derrien et al., 2012). Analyses of RNA-seq data across 16 tissues profiled in the Human Body Map project showed that 65% of protein-coding genes versus only 11% of lncRNAs were detected in all tissues studied (Derrien et al., 2012). Further, only 11% of lncRNAs were detected in a single tissue (Derrien et al., 2012). Because ncRNAs are widely hypothesized to contribute to fine-tuning and regulating signaling pathways (e.g. miR155 (Tili et al., 2007)), it is possible that targeting their functions could provide more exquisite and less toxic effects relative to the inhibition of enzymatic activity of proteins, but this remains to be tested.

miRNAs have long been implicated as biomarkers in cancer, and recently, expression profiling of lncRNAs and circRNAs in cancer patient samples have suggested that they might also be suitable biomarkers in cancer detection and diagnosis (Ling et al., 2013) (Figure 4). For example, detection of MALAT1 amongst a panel of ncRNAs in patient serum has been reported to be able to predict the presence of non-small cell lung cancer (Figure 4)(Peng et al., 2016), while the expression of NEAT1 has been correlated with poor prognosis of ovarian cancer (Figure 4) (Chen, 2016).

Pre-existing experimental techniques such as RNAi have already established the feasibility of reducing the expression of specific candidate ncRNAs (Matsui and Corey, 2017). **miRNA sponges** have been used to antagonize miRNA activity; overexpression of a miR-155 sponge in a B-cell tumor line resulted in the upregulation of *JARID2* mRNA, a miR-155 target (Bolisetty et al., 2009). In the case of miRNA deficiency, overexpression of a ncRNAs might also be used to restore ncRNA deficiency. In **miRNA replacement therapy**, the overexpression of tumor suppressive Let-7 has been shown to suppress non-small cell lung cancer (NSCLC)

development in mouse xenografts in an autochthonous model of NSCLC (Figure 4) (Kumar et al., 2008). RNAi has also been successfully used to silence lncRNA; a promising proof-of-concept application has been the knockdown of ANRIL in gastric cancer cell lines as part of combination chemotherapeutic strategy to reduce drug resistance (Figure 4) (Lan et al., 2016).

The efficacy of **antisense oligonucleotides (ASOs**) in suppressing the expression nuclear lncRNAs such as MALAT1 and NEAT1 has also been shown in HeLa cells (Lennox and Behlke, 2016). In cases where RNAi is not suitable due to extensive secondary structure or unfavorable sequences, **ribozymes**, currently in preclinical studies, might be used (Ling et al., 2013). Specifically, ribozymes have been successfully used to target *FLT-1* to significantly inhibit liver metastasis in a murine xenograft model of human metastatic colorectal cancer (Pavco, 2000). Alternatively, zinc finger nuclease (ZFN)-mediated **genomic integration of RNA destabilizing elements** such as poly(A) signals have been successful in decreasing the expression of *MALAT1* by up to 1000-fold in A549 lung adenocarcinoma cells (Gutschner et al., 2011). Other methods for targeting lncRNAs include impairing their function by blocking their molecular interactions using small molecule inhibitors or **antagonistic oligonucleotides (aptamers)** that might prevent proper folding of the lncRNA molecule or inhibit its interaction with proteins or DNA partners (e.g. Macugen, a US FDA approved anti-VEGF aptamer for the treatment of age-related macular degeneration) (Matsui and Corey, 2017).

Clinical trials are underway to evaluate putative therapies to decrease miRNAs with locked nucleic acid anti-miRs (e.g. Miravirsen against miR-122 in Hepatitis C in Phase II; NCT02031133) (Janssen et al., 2013) (Figure 4), or increase miRNA abundance using mimics (e.g. MRX-34 mimic for miR34 in cancers such as hepatocellular carcinoma in Phase I; NCT01829971) (Matsui and Corey, 2017).

Unfortunately, NCT01829971 with MRX-34 was recently terminated at Phase I due to multiple immune-related adverse events, highlighting just one of the many complications in ncRNA-based therapies (https://clinicaltrials.gov/ct2/show/NCT01829971). Many challenges remain

that concern the specific and effective clinical application of these therapies with minimal side-effects. The delivery of molecules in a tissue specific manner also remains a major obstacle to be surmounted (Matsui and Corey, 2017). Further, while it is technically possible to target almost every ncRNA with sequence-specific nucleic acid-based drugs, many potential off-target effects might exist that would evidently limit their use in the clinic. For example, oligonucleotides might be able to bind to proteins and these in turn might also induce an undesired IFN response (Matsui and Corey, 2017). There could also be partial sequence complementarity to unintended targets and toxicity from high concentrations of oligonucleotides used (Matsui and Corey, 2017). In addition, and of particular importance, the mechanisms through which ncRNAs act are not completely understood, and consequently, being able to design effective drugs with minimal side effects constitutes a major challenge .

Concluding Remarks

The fast emerging field of ncRNAs has provided novel insight into the complexities of gene regulation and expression. This field is further complemented by the burgeoning community studying the role of RNA modifications, which has provided new layers of understanding into the regulation of RNA stability and function (reviewed in (Roundtree et al., 2017)). Similar to the ways in which post-translational modifications (e.g. phosphorylation and ubiquitination) might regulate protein function, RNA modifications such as N^6-methyladenosine **(m^6A) and** 5-hydroxymethylcytosine **(hm^5C)** can encode for additional instructions for RNA processing and function. Undoubtedly, studying such RNA modifications on ncRNAs might allow us to gain further functional insight into their regulation.

Dynamic and temporal changes in transcriptional programs during immune responses must be tightly regulated to allow timely responses to infection, tissue damage and environmental stress conditions. Many inflammatory signaling pathways converge to induce transcription of thousands of immune regulatory genes, subjected to transcriptional, post-transcriptional and

post-translational modifications. The key advantage of ncRNA-mediated control of inflammatory programs is that they are able to provide rapid control of gene expression via mechanisms such as protein or DNA binding. Understanding the roles of ncRNAs in these regulatory networks is thus imperative in order to obtain a better appreciation of their involvement in human health and diseases. Knowledge on the specific functions of ncRNAs is still in its infancy (see Outstanding Questions and Box 5). However, we should strive to increase our focus of identifying robust, functional ncRNA targets, and refine the methods of therapeutic drug delivery to modulate these. Moreover, accurately determining potential toxicities and off-target effects may hopefully lead to the application of novel candidate therapeutics in diseases that may be difficult to treat.

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Box 1. Protein mediated regulation of TNF-α and IL-6 levels.

Dysregulated TNF-α signaling has been implicated in the pathogenesis of many diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and **ankylosing spondylitis** (Kalliolias and Ivashkiv, 2016). This dysregulation has been attributed to deficiencies in ubiquitination or phosphorylation of proteins in signaling pathways such as NF-κB. These can affect the cleavage or internalization of TNFRs (Chin and Horwitz, 2005; Higuchi and Aggarwal, 1994), stability of signaling complexes (Harhaj and Dixit, 2012) and the persistence of TNF-α due to decreased proteasomal or lysosomal degradation (Ahmed et al., 2011; Li et al., 2008; Shibata et al., 2012). Deficiencies in protein processing can also affect inactivation of signaling complexes through phosphorylation (Chew et al., 2009; Li et al., 2008; Li et al., 2006) or sequestration of active signaling components (Hoffmann et al., 2002; Kearns et al., 2006). Another example is aberrant IL-6 expression and signaling, which has been implicated in many inflammatory conditions such as multiple sclerosis and rheumatoid arthritis (Tanaka et al., 2014). IL-6 is a pleiotropic cytokine with effects in modulating inflammation, immune responses and hematopoiesis (Tanaka et al., 2014). Although the reasons for dysregulated IL-6 levels remain poorly understood, genome wide association studies (GWAS) have identified a G-to-C mutation in the promoter region of human *IL6* which is associated with elevated IL-6 expression (Fishman et al., 1998). Further, IL-6 protein production in macrophages has been associated with stability of its transcript, found to be regulated by RNA-binding proteins such as regulatory RNase-1 (REGNASE-1) (Matsushita et al., 2009) and AT-rich interactive domain-containing protein 5a (ARID5A) (Masuda et al., 2013), highlighting the importance of post-transcriptional modifications in the regulation of IL-6. While it has been hypothesized that the balance between ARID5a and REGNASE-1 might determine *IL6* mRNA stability and hence expression, the pathological mechanism for dysregulated IL-6 production remains unknown (Tanaka et al., 2014).

Box 2. JAK-STAT pathway and immune-related disorders.

In the JAK-STAT pathway, binding of ligands to their respective cell surface receptors results in receptor dimerization. This activates receptor-associated JAK tyrosine kinases which phosphorylate themselves and the receptors. Latent cytoplasmic transcription factors, STATs, are recruited to these phosphorylated residues and are in turn phosphorylated by JAKs. Phosphorylated STAT molecules dimerize and translocate into the nucleus to activate the transcription of target genes (Shuai and Liu, 2003) (Figure 2).

Mutations in JAK3 can cause severe combined immunodeficiency (Macchi et al., 1995; Russell et al., 1995), while autosomal dominant mutations of STAT1 or STAT3 result in primary immunodeficiency (Holland et al., 2007; van de Veerdonk et al., 2011). Aberrant signaling in the JAK-STAT pathway can also occur as a result of mutations which constitutively activate JAK (James et al., 2005). For example, $JAK2^{V617F}$ affects the auto-inhibitory activity of JAK2, resulting in cytokine hypersensitivity and cytokine-independent growth, resulting in myeloproliferative disorders (James et al., 2005). Aberrant JAK-STAT activation can also be a consequence of mutations in LNK (McMullin and Cario, 2016) or suppressor of cytokine signaling (SOCS) (Weniger et al., 2006), which are inhibitors of JAK activity.

Box 3. ncRNA Mediated Temporal Regulation of NFκB Signaling.

In the absence of stimuli, NF-κBs are bound by inhibitors of κB (IκBs), which prevent their translocation to the nucleus (Hayden and Ghosh, 2012). Activation of the pathway leads to the activation of receptor-interacting protein (RIP) and TNF receptor associated factor (TRAF) proteins, which activate IκB kinase (IKK). IKK phosphorylates IκBs, leading to their ubiquitination, degradation and subsequent dissociation from NF-κB dimers, translocating into the nucleus to activate the transcription of cytokines, chemokines, growth factors and other genes important for driving inflammation (Hayden and Ghosh, 2012) (Figure 2).

Despite our advanced knowledge of NF-κB signaling, our understanding of this pathway is lacking; it is unclear how different NF-κB responsive genes are turned on or off in a temporal fashion upon NF-κB activation, leading to distinct gene expression profiles over a period of stimulation (Tian et al., 2005). Bioinformatic interrogation of the promoter regions of these early and late response gene groups has revealed that their promoters contain similar NF-κB binding sites (Tian et al., 2005). While some activation of early and late response genes can be attributed to chromatin remodeling (Sandoval et al., 2016), the biological mechanism mediating this temporal regulation remains enigmatic.

Indeed, since proteins can only be turned on or off by translational promoters, we hypothesize that the sustainability of NF-κB responses in triggering late effect genes might be provided by ncRNAs, shown to perform a myriad of gene regulatory functions. ncRNAs are speculated to offer several advantages over their protein counterparts, allowing for rapid and specific regulation of gene expression. Further, they can serve as highly specific adapters to target proteins to a particular DNA region (e.g. **TERC),** which may not be achievable with protein adapters. There are several hypotheses on how ncRNAs might mediate temporal gene regulation. Initial activation of inflammatory signaling might induce the expression of a lncRNA, binding and blocking NF-κB recruitment to promoter regions of late response genes. Subsequently, degradation of this lncRNA might then allow NF-κB to bind to promoters of late response genes. Such a lncRNA molecule might also bind and sequester an important co-factor required for transcriptional activation of these late response genes. Alternatively, these lncRNAs might recruit chromatic remodelers, subsequently allowing NF-κB to access the promoter regions of these genes. Indeed, ncRNA research has uncovered a previously unknown layer of regulation in inflammatory signaling. Such advances may contribute to filling the gaps in our understanding of how signaling components are regulated and how chromatin modifiers are recruited to activate or repress the expression of inflammatory response genes.

Box 4. Crosstalk between inflammatory signaling pathways

Crosstalk can occur between or within various inflammatory signaling pathways, serving as positive and negative feedback loops to amplify or shut down inflammatory signaling (Schmitz et al., 2011). One way this can occur is through the physical interaction of proteins, which can affect promoter binding or transcriptional activity. For example, NF-κB and STAT3 cooperatively control expression of a group cytokines and chemokines in A549 lung epithelial cells (Dauer et al., 2005). Co-immunoprecipitation and co-localization experiments in A2058 melanoma cells show this can be achieved through their physical interaction, influencing recruitment to promoters (Lee et al., 2009; Yang et al., 2007).

Crosstalk can also occur through induction of proteins which subsequently inhibit or activate alternative signaling pathways, such as NF-κB upregulation of *gadd45β/myd118* which in turn inhibits the JNK pathway in MEFs (De Smaele, 2001; Papa et al., 2004). However, we still do not completely understand all the mechanisms through which many of the other crosstalk mechanisms occur; for instance, the synergism between p38 and NF-κB (Craig, 2000; Vermeulen, 2003) and the antagonism between JNK-MAPK and ERK / p38 MAPK signaling in PAC-1-deficient mice (a dual-specificity phosphatase DUSP2) (Jeffrey et al., 2006) remain a mystery. Understanding these pathways would have important implications in targeted therapies because even isoform-specific inhibitors might have unsolicited side effects. Extensive studies of various proteins involved in these signaling pathways have yet to shed insight into the mediators of these cross-talks. Based on the ability of ncRNAs, particularly lncRNAs, to mediate protein-protein and protein-DNA interactions, we speculate that ncRNAs may be mediating such effects.

Box 5. Clinician's Corner

- TNF-α is a key driver of inflammation, implicated in several pathological conditions including rheumatoid arthritis, inflammatory bowel disease, Crohn's disease and other pulmonary disorders.

- Several studies have indicated that TNF-α inhibitors have shown promising effects in preclinical animal models of arthritis (e.g. collagen-induced arthritis in mice) (Wooley, 1993) and some of these inhibitors have also been evaluated for their efficacy in human clinical trials for the treatment of chronic inflammatory conditions such as rheumatoid arthritis. However, these agents are quite expensive and are usually administered orally or via a systemic route, potentially inducing severe toxicities.
- Understanding the mechanisms and specificity of ncRNAs may provide an opportunity to target ncRNAs for therapy. Although extensive testing is required, nucleic-acid based drugs used to target ncRNAs may be potentially more specific than conventional pharmacological drugs, and presumably, might reduce the occurrence of toxic off-target effects; however, this remains controversial and is the subject of numerous investigations.

Figure Legends

Figure 1. Key classes of non-coding RNAs and their features.

A) microRNAs are first expressed in cells as longer transcripts with a stem loop structure. They are then processed by Drosha and Dicer to mature 22 base pairs (bp) forms. They interact with mRNAs, inhibiting their transcription or enhancing thedegradation. **B)** long non-coding RNAs are transcripts longer than 200 bp. **C)** circular RNAs are resistant to exonuclease degradation due to their unique covalently closed circular structure which results from back-splicing. They can interact with RNA, DNA and proteins. Long non-coding RNA and circular RNAs have diverse functions due to their ability to interact with DNA, RNA or protein molecules.

Figure 2. Key protein regulators of inflammatory signaling pathways. **A)** Janus Kinase and Signal Transducer and Activator of Transcription (JAK-STAT) signaling is activated by various cytokines, as well as Type I and Type II interferons. Binding of these ligands to their respective cell surface receptors results in receptor dimerization, activating JAK tyrosine kinase autophosphorylation, as well as phosphorylation of receptors and recruited STAT proteins. Phosphorylated STAT molecules dimerize and translocate into the nucleus to activate transcription of inflammatory target genes. **B)** The nuclear factor-κB (NF-κB) pathway is activated by tumor necrosis factor receptors (TNFR), toll-like receptors (TLR) and growth factor receptors. Receptor signaling activates IKK which phosphorylates IκB. IκB is

targeted for degradation, and dissociates from the p50/p65 complex which subsequently translocate into the nucleus to activate transcription of inflammatory response genes. **C)** Mitogen-activated protein kinase (MAPK) signaling activates a cascade of kinases. The first level of kinases are MAPK kinase kinases (MAP3Ks) (TGF-beta activated kinase 1 (TAK1), apoptosis signal-regulating kinase 1 (ASK1), Delta-like 1 homolog (DLK1), MAP/ERK kinase kinase 4 (MEKK4)). These activate a second level of MAPK kinases (MKK) which result in the phosphorylation and activation of the MAPK extracellular signal-regulated kinase-1 (ERK1), c-Jun N-terminal kinase (JNK) and p38. MAPK are activated further downstream, mediating inflammatory events, including upregulation of inflammatory genes through phosphorylation of downstream proteins.

Figure 3. ncRNAs and their targets in inflammatory signaling. The diagram depicts an overview of ncRNAs and their targets in inflammatory signaling through **A)** JAK-STAT **B)** NFκB **C)**MAPK. A) JAK/STAT signaling: mir-135 targets JAK2 mRNA, mir-145 and miR-221/222 target STAT1 mRNA, miR-124 and Let7 target STAT3 mRNA. lncRNA OLA1P2 prevents dimerization of STAT3. lncDC prevents phosphorylation of STAT3. HoxDAS1 increases transcription of JAK/STAT target genes. JAK/STAT signaling induces expression of CMPK2 and ANRIL, which in turn modulate inflammatory gene expression. B) NF-κB signaling: miR-155 targets IκB mRNA. PACER sequesters p50 preventing its transcriptional activity. NF-κB signaling upregulates a number of regulatory ncRNAs; Lethe which inhibits RelA binding to DNA, NKILA which inhibits phosphorylation and degradation of IκB, miR146 which targets mRNA of receptor adaptor proteins, lncRNA-Cox2 which is an NF-κB subunit coactivator, THRIL which regulates inflammatory gene expression and mcircRasGEF1B. NF-κB signaling has also been shown to negatively regulate linc-EPS. C) MAPK signaling: p38 modulates miRNA levels globally by destabilizing Drosha/Dicer. miR125a-3p targets p38 mRNA, miR-125b and miR-216 target JNK mRNA. lncRNA BANCR suppresses p38 and JNK activation. p38 signaling induces NEAT1 which can induce the expression of antiviral genes.

Figure 4. Therapeutic potential of non-coding RNAs in inflammatory signaling.

An advanced understanding of non-coding RNA (ncRNA) functions may contribute to novel therapeutic approaches in a range of diseases. For example, ncRNAs can be detected from patient samples through tumor biopsies or less invasive methods such as blood collection. Detected ncRNAs can be used to identify the presence of particular cancers, as in the case of MALAT1, for lung cancer (Peng et al., 2016). Alternatively, they might enable predicting disease outcomes, as in the case of NEAT1 (Chen et al., 2016). ncRNAs might also be altered in experimental systems to model diseases, to identify if they should be targeted for therapeutic purposes. Studies knocking down ANRIL in gastric cancer cell lines have already shown the feasibility of targeting this ncRNA as part of a combination chemotherapeutic strategy(Lan et al. 2016). Alternatively, tumor suppressive ncRNAs such as miRNA Let7 might be also be targeted, aiming to increase their expression (Kumar et al., 2008)

Glossary

Ankylosing spondylitis: A type of arthritis affecting the spine.

Annotation of transcripts process to identifying gene transcripts and determining what these transcripts do in a cell.

Antagonistic oligonucleotides (Aptamers) Single stranded RNA or DNA 20-80 oligonucleotide aptamers, considered as chemical antibodies able to bind specific target molecules with high specificity and to block their activity.

Antisense oligonucleotides (ASO) short specific chemically modified oligonucleotides, 15-25 nucleotides in size; used for knock-down of ncRNAs or mRNAs.

Back-splicing of pre-mRNA Alternative splicing where a downstream splice-donor site is spliced to an upstream splice-acceptor site.

Biotin labelled probes Oligonucleotide probes covalently bound to a biotin molecule.

chromatin immunoprecipitation (ChIP) method by which DNA interacting proteins are immunoprecipitated, allowing the identification of DNA regions to which the protein is bound.

Chromatin isolation by RNA immunoprecipitation (ChIRP-seq) Precipitation of a specific ncRNA with oligonucleotide probes and analysis of the DNA or chromatin regions bound to the RNA molecule.

Circular RNA (circRNA) Covalently closed RNA loop structures with neither 5' to 3' polarity nor a polyadenylated tail.

Coding Potential Calculation based on multiple features (COME) A coding potential prediction tool based on sequence and experimental features.

Coding Potential Calculator (CPC) A vector machine-based classifier to predict protein-coding probability through biologically relevant sequence features.

Computed expression scores: A value reflecting the relative expression of an individual gene in the particular sample, to the gene's expression in the reference population (also known as z-score).

CRISPR-Cas9 genome editing A toolkit composed of Cas9 DNA nuclease enzyme and a programmable gRNA sequence. It makes a complex and target specific DNA sequence in the genome of cells.

CRISPR-Cas9 interference (CRISPRi) A toolkit consisting of gRNA and nuclease-deficient Cas9 linked to one or more transcriptional repression domains and used to decrease the transcription of a target gene.

Denaturing gels Gels run under conditions that disrupt natural structures allowing size separation of the molecules of interest.

Dicer endoribonuclease in the RISC complex involved in the processing of pre-miRNA to miRNA.

Drosha: endoribonuclease which is part of the microprocessor complex involved in miRNA maturation from a primary (pri)-miRNA to pre-miRNA.

Genome-wide association studies (GWAS): identify a genome-wide set of single nucleotide polymorphisms occurring more frequently in individuals with a particular trait.

Genomic integration of RNA destabilizing elements: method by which RNA destabilizing elements such as poly(A) signals are inserted downstream of a gene via genome editing to terminate transcription.

Guide RNA (gRNA) RNA sequence that contains the scaffold sequence for Cas9 binding and is a specific and programmable ~20 nucleotide targeting site

Immunodeficiency A disorder where there is increased susceptibility to opportunistic infections, autoimmunity or malignancy.

Immunoprecipitation of RNA binding protein (RIP) Antibody based precipitation of an RNA binding protein followed by detection of any bound RNA molecules.

Incontinentia pigmenti : genetic disorder that affects the skin, hair, teeth, nails and central nervous system.

m6A and hm5C N6-methyladenosine (m6A) and 5-hydroxymethylcytosine (hm5C) are chemical RNA modifications which shape the stability, biogenesis, and function of RNA molecules.

microarray platform used to measure expression levels of many genes simultaneously through the hybridization of genomic or cDNA onto a chip bearing immobilized probes.

miRNA replacement therapy : therapeutic intervention to increase the abundance of specific miRNAs, e.g. using a miRNA mimic to enhance a tumor suppressive miRNA in cancer cells.

miRNA sponges RNA molecules that bind strongly to miRNA. Sponges sequester miRNA away from mRNA targets thereby inhibiting their function.

Myoregulin micropeptide encoded by a lncRNA regulating skeletal muscle physiology.

Northern blotting method used to separate RNA molecules by size, visualizing their abundance through the hybridization of sequence specific radioactively labelled probes.

paired-guide RNA strategy (pgRNA) genomic sequences are deleted between two gRNA target sites.

Paraspeckles Ribonucleoprotein bodies in the interchromatin space of mammalian cell nuclei

Quantitative reverse transcription PCR (RT-qPCR) method that is performed on mRNA which has been reverse transcribed into cDNA. It allows the evaluation and comparison of mRNA expression between samples.

Ribozymes Catalytic RNA molecules that can act as enzymes and catalyze specific biochemical reactions.

RNA interference (RNAi) method that uses small interfering (siRNA) or micro (miRNA) molecules to suppress mRNA translation.

Short "reads" Short sequence of clusters after obtained from sequencing of fragmented DNA sequences.

Single cell transcriptomics technique that measures the expression of genes in a given population at a single cell level.

single guide RNA (sgRNA) library A pooled library consists of large number of single gRNA sequences that enable targeting of multiple genes or specific sites in the genome of a cell.

SPAR A polypeptide encoded by LINC00961 which regulates mTORC1 activation and muscle regeneration.

Sucrose gradient fractionation protein fractionation method where a sample is centrifuged through a sucrose solution of increasing concentrations (gradient) allowing separation of proteins by density.

Telomerase ncRNA component (TERC) ncRNA that acts as a template for telomerase during the extension of telomeric repeats at the end of eukaryotic cell chromosomes.

Tiling microarrays A type of microarray where probes are derived from a contiguous region of the genome. Used to identify expression changes in regions which are sequenced but may not be functionally characterized.

Toddler secreted peptide involved in Zebrafish gastrulation, encoded by Toddler, a ncRNA.

Toll-like receptors (TLR) Class of receptors which bind molecules derived from pathogens to activate innate immune signaling pathways.

Viral RNA structure A viral RNA sequence which can be appended to a ncRNA as a tag. The MS2 protein binds this RNA sequence allowing detection or immunoprecipitation of ncRNAs.

Western blotting method to separate proteins by size , visualize their abundance via antibody-specific recognition.

OUTSTANDING QUESTIONS BOX

- Do ncRNA contribute to the temporal regulation of NF-κB responsive genes? Our current understanding of signaling pathways is largely confined to signaling proteins, which can only be turned on or off. This does not allow us to explain the temporal regulation of NF-κB responsive genes, particularly the sustainability of the NF-κB response in triggering late effect genes.

- Are ncRNA mediating the crosstalk between inflammatory signaling pathways and what are the mechanisms through which these occur?

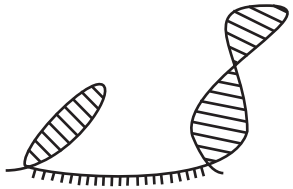
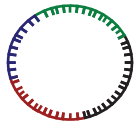
  We do not completely understand how synergism or antagonism can occur between inflammatory signaling pathways. lncRNA could be mediating physical interaction between signaling proteins which can affect their activity or recruitment to promoters. lncRNA could also be inducing the expression of proteins which inhibit or activate alternative signaling pathways. This might provide a fruitful area of investigation.

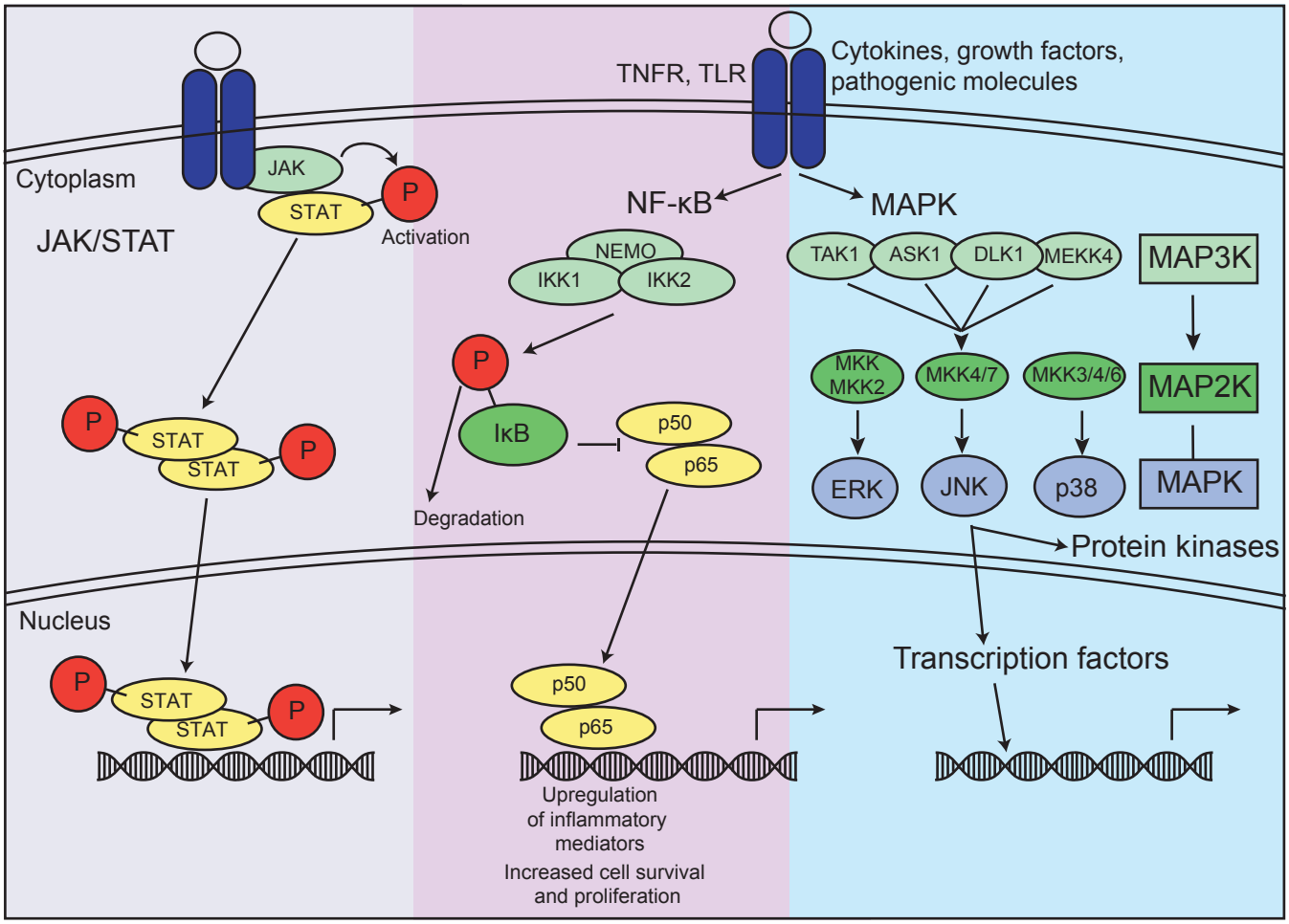
- What are the ways in which a ncRNA might reveal or be exploited as specific therapeutic targets to treat chronic inflammatory conditions? And for which pathologies?

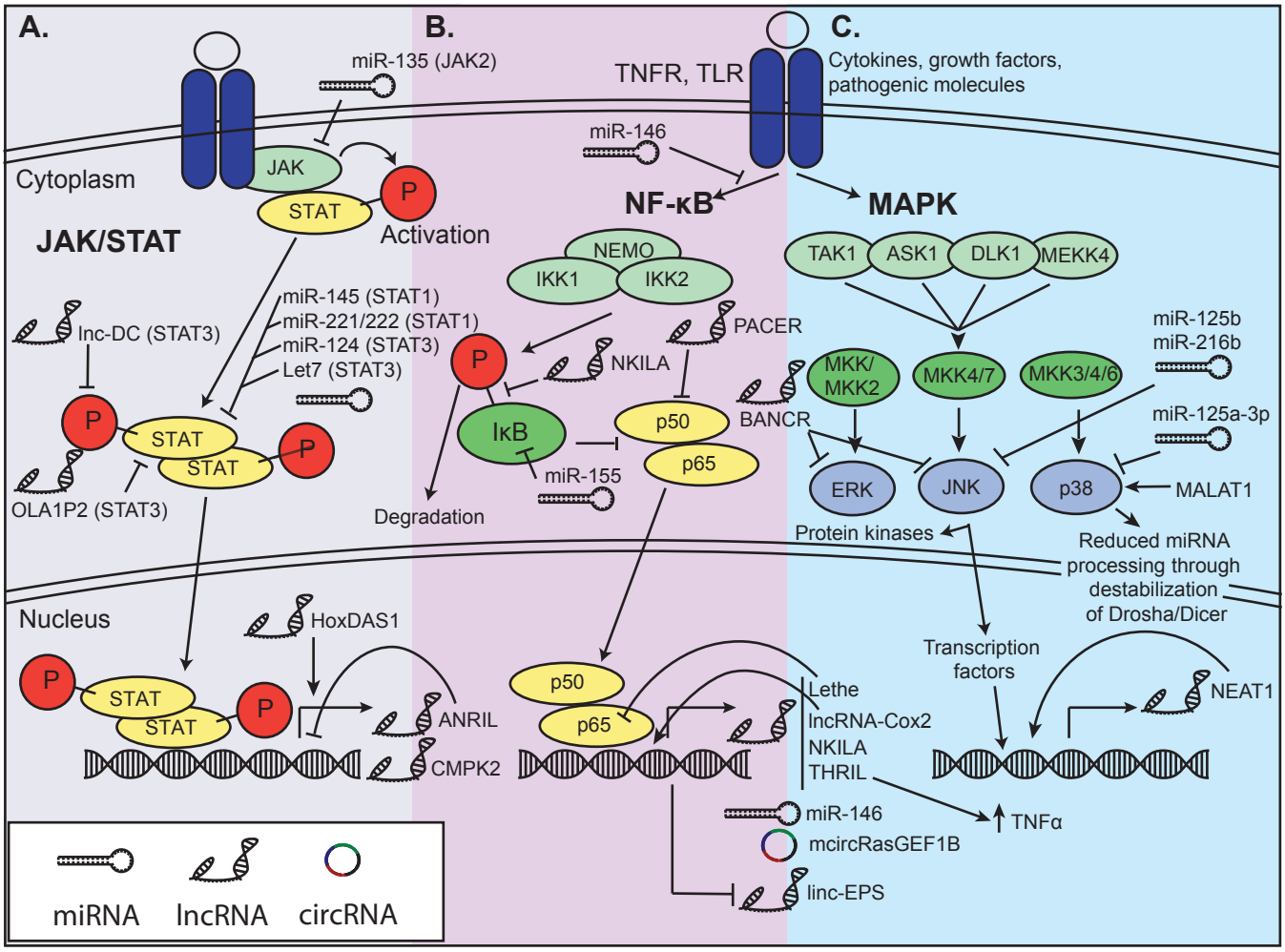
  Understanding the mechanism through which ncRNA regulate inflammatory signaling, and the specificity of ncRNA effects might allow us to better target these yet-to-be determined pathways in a variety of chronic inflammatory conditions.

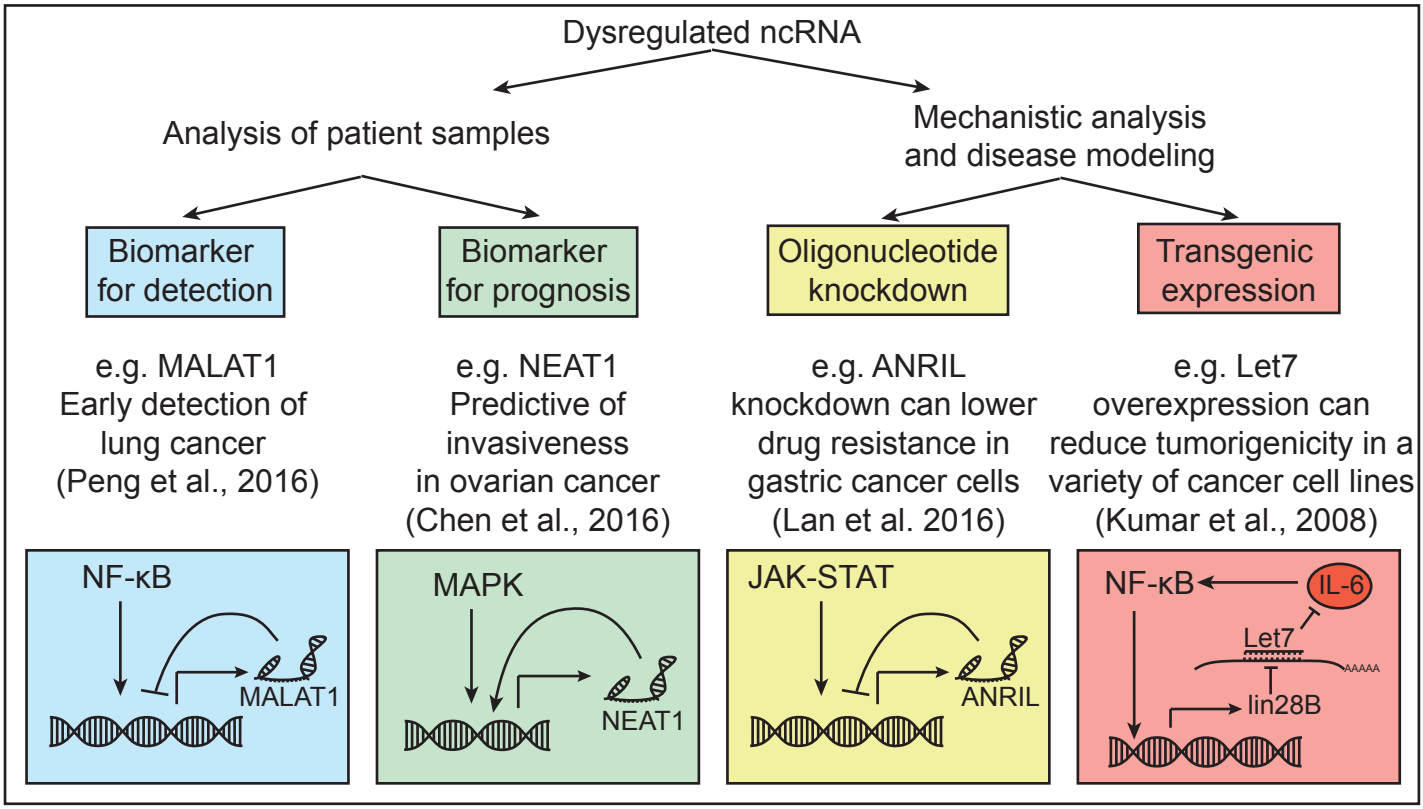
- Are RNA modifications in ncRNA able to direct their function?

  Similarly to post-translational modifications, RNA modifications might be able to direct the stability and function of ncRNA. Understanding the types of modifications that exists on ncRNA might help us to better elucidate their function.

A. micro RNA	B. long non-coding RNA	C. circle RNA
		
- Produced as longer transcripts and processed to 22 bp - mature miRNA are contained in the RNA-induced silencing complex (RISC) - Interact with mRNA resulting in post-transcriptional gene regulation	- Non-protein coding transcripts - Longer than 200 bp - Form complex secondary structures - Can be post-translationally processed, (eg. through splicing or 5' capping) - Interact with RNA, DNA, and proteins	- Formed by back-splicing of pre-mRNA - Can contain multiple exons - Stable due to exonuclease resistance - Interact with mRNA, miRNA, proteins, and DNA


Cytoplasm
JAK/STAT
JAK
STAT
P
Activation
TNFR, TLR
Cytokines, growth factors,
pathogenic molecules
NF-κB
NEMO
IKK1
IKK2
IκB
p50
p65
Degradation
MAPK
TAK1
ASK1
DLK1
MEKK4
MAP3K
MKK
MKK2
MKK4/7
MKK3/4/6
MAP2K
ERK
JNK
p38
MAPK
Protein kinases
Nucleus
Transcription factors
Upregulation
of inflammatory
mediators
Increased cell survival
and proliferation


A.
B.
C.
miR-135 (JAK2)
TNFR, TLR
Cytokines, growth factors, pathogenic molecules
miR-146
Cytoplasm
JAK
STAT
P
Activation
NF-κB
MAPK
JAK/STAT
NEMO
IKK1
IKK2
TAK1
ASK1
DLK1
MEKK4
miR-145 (STAT1)
miR-221/222 (STAT1)
miR-124 (STAT3)
Let7 (STAT3)
lnc-DC (STAT3)
PACER
NKILA
MKK/ MKK2
MKK4/7
MKK3/4/6
miR-125b
miR-216b
IκB
p50
p65
BANCR
miR-125a-3p
ERK
JNK
p38
MALAT1
OLA1P2 (STAT3)
Degradation
miR-155
Protein kinases
Reduced miRNA processing through destabilization of Drosha/Dicer
Nucleus
HoxDAS1
Transcription factors
ANRIL
CMPK2
Lethe
lncRNA-Cox2
NKILA
THRIL
NEAT1
miR-146
mcircRasGEF1B
linc-EPS
TNFα
miRNA
lncRNA
circRNA


Dysregulated ncRNA
Analysis of patient samples
Mechanistic analysis and disease modeling
Biomarker for detection
Biomarker for prognosis
Oligonucleotide knockdown
Transgenic expression
e.g. MALAT1 Early detection of lung cancer (Peng et al., 2016)
e.g. NEAT1 Predictive of invasiveness in ovarian cancer (Chen et al., 2016)
e.g. ANRIL knockdown can lower drug resistance in gastric cancer cells (Lan et al. 2016)
e.g. Let7 overexpression can reduce tumorigenicity in a variety of cancer cell lines (Kumar et al., 2008)
NF-κB
MALAT1
MAPK
NEAT1
JAK-STAT
ANRIL
NF-κB
IL-6
Let7
AAAAA
lin28B