

1 **MicroRNA: master controllers of intracellular signalling pathways**

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11

12 **Abstract**

13

14 Signaling pathways are essential intracellular networks that coordinate
15 molecular outcomes to external stimuli. Tight regulation of these pathways is
16 essential to ensure an appropriate response. microRNA (miRNA) is a class of
17 small, non-coding RNA that regulates gene expression at a post-transcriptional
18 level by binding to the complementary sequence on target mRNA, thus limiting
19 protein translation. Intracellular pathways are controlled by protein regulators,
20 such as Suppressor of Cytokine Signaling (SOCS) and A20. Until recently,
21 expression of these classical protein regulators was thought to be controlled
22 solely by transcriptional induction and proteasomal degradation; however, there
23 is a growing body of evidence describing their regulation by miRNA. This new
24 information has transformed our understanding of cell signaling by adding a

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25 previously unknown layer of regulatory control. This review outlines the miRNA
26 regulation of these classical protein regulators and describes their broad effects
27 at both cellular and disease levels. We review the regulation of three important
28 signaling pathways, including the JAK/STAT, NF- κ B and TGF- β pathways, and
29 summarize an extensive catalogue of their regulating miRNAs. This information
30 highlights that the importance of the miRNA regulon and reveals a previously
31 unknown regulatory landscape that must be included in the identification and
32 development of novel therapeutic targets for clinical disorders.

33

34 **Keywords**

35 microRNA; signal regulator; regulon; JAK-STAT; NF- κ B; TGF- β

36

37

38 **Introduction**

39

40 microRNA (miRNA) are a class of small, non-coding RNA of 19-25 nucleotides
41 (nt) in length that regulate gene expression at a post-transcriptional level by
42 binding to the 3'UTR of the target transcript [1]. They are transcribed in the form
43 of a primary transcript (pri-miRNA), either under the control of their own
44 promoter regions or processed from a coding gene [2]. pri-miRNA are
45 subsequently processed in the nucleus by RNase III-type endonuclease, Drosha,
46 in association with an accessory double-stranded RNA (dsRNA)-binding protein,
47 DiGeorge Critical Region 8 (DGCR8), into a stem-loop dsRNA pre-miRNA (or
48 precursor miRNA), of 60-70nt in length, with a 2nt overhang at the 3' end [3, 4].
49 This stem-loop dsRNA structure is transported from the nucleus to the
50 cytoplasm by Exportin-5 [5, 6], where it is further processed by another RNase
51 III-type endonuclease, Dicer, and, in some cases, also with the help of accessory
52 dsRNA-binding proteins, PACT and TRBP, into a mature dsRNA duplex of 19-
53 21nt base pairs each side and a 2nt overhang at each 3' end [7, 8]. This duplex is
54 then loaded onto an effector complex called RNA-induced silencing complex
55 (RISC). One strand from the duplex acts as a guide to direct RISC in binding the
56 target mRNA complementary sequence that mediates gene silencing [9] (Fig.1).
57 The discovery of microRNA in 1993 [10], revealed previously unknown layer of
58 post-transcriptional control that revolutionized our concept of gene regulation.
59 "Classical" protein regulators, such as SOCS and A20, are well known to quickly
60 control signaling pathways through direct post-translational modification, such
61 as phosphorylation or ubiquitination, of their target protein. miRNAs do not

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62 regulate the activity of existing proteins, but rather limit the synthesis of new
63 proteins, providing an extra layer of control that is now accepted as being an
64 essential component of pathway regulation in processes such as cell
65 development or differentiation [11, 12]. However, as well as regulating key
66 players within signaling pathways, miRNAs are increasingly being documented
67 to regulate the “classical” regulators, thus providing additional control which we
68 review in this manuscript. Here we describe this novel mechanism of molecular
69 regulation of three major signaling pathways: Janus kinase/signal transducer
70 and activator of transcription (JAK/STAT), nuclear factor kappa-light-chain-
71 enhancer of activated B cells (NF- κ B) and transforming growth factor beta
72 (TGF β). We also outline how each miRNA plays a role in different cellular,
73 ranging from normal growth and development, and clinical contexts, such as
74 autoimmunity and cancer. In fact, this review clearly highlights that regulators of
75 the cellular signaling pathways are important targets of regulation by miRNAs,
76 and are significant targets for future research.

77

78 **JAK-STAT signaling pathway**

79

80 The JAK-STAT signaling pathway is mainly adopted by cytokine receptors to
81 effect their anti-viral, inflammatory and cell proliferative activity [13]. Upon
82 binding of extracellular ligands, such as interferon (IFN)- α/β and interleukin
83 (IL)-6, to their respective dimerized transmembrane receptors, the pre-
84 associated JAK tyrosine kinases are brought into juxtaposition and activated by
85 trans-autophosphorylation. Activated JAK kinases then mediate tyrosine

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86 phosphorylation on the conserved residue of the receptors, which causes the
87 receptor recruitment of SH2-domain containing STAT proteins, which are in turn
88 phosphorylated by JAKs, dissociate from the receptors, dimerize and translocate
89 into the nucleus, where they act as transcriptional activators, driving expression
90 of effector genes [14]. This pathway is central to the well being of our complex
91 immune system, with dysregulation leading to serious lymphoproliferative and
92 autoimmune diseases [13], and is therefore under tight regulation at multiple
93 levels [reviewed in 15].

94

95 **Regulation of SOCS by microRNAs**

96

97 The best studied regulators of the JAK-STAT pathway are suppressor of cytokine
98 signaling (SOCS) proteins, which constitute a family of 8 members, including
99 SOCS1-7 and cytokine-inducible Src homology 2 protein (CIS) [16]. SOCS
100 proteins bind phosphorylated tyrosines of JAKs and/or the receptor via their
101 SH2-domains, thus blocking STAT recruitment [14]. Additionally, the SOCS box
102 domain recruits elongin B and C-containing ubiquitin E3 ligase complexes and
103 effectively mediates receptor degradation through the proteasome [14, 15].
104 Basal expression of SOCS is low, but can be up-regulated by cytokine stimulation,
105 providing an essential and effective negative feedback loop for the activated
106 pathway [16]. Recent publications have documented that miRNA regulation of
107 SOCS expression is also key to optimal performance of the JAK/STAT pathway.
108 The role of miR-155 in regulating SOCS1 protein expression has been implicated
109 across a spectrum of cell types in nearly 20 publications. It was first described by

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110 Rudensky's group in the context of Foxp3 expression of regulatory T (Treg) cell
111 homeostasis. The authors noticed that an up-regulation of SOCS1 protein level
112 was detected in miR-155-deficient Treg cells, a phenotype which could be
113 reverted by the reintroduction of miR-155 [17]. Not limited to T cell biology, the
114 regulatory role of miR-155 on SOCS1 has also been implicated in NK cell
115 development and functions. In this study NK cells from miR-155 knockout mice
116 had elevated SOCS1 expression, and suffered from both impaired NK cell
117 generation and response to viral infection [18]. The functional consequences of
118 SOCS1 regulation by miR-155 are best illustrated by early work from Cao's group,
119 showing that even though miR-155 did not alter IFN expression in virally
120 infected macrophages, its suppression of SOCS1 levels increased STAT1
121 phosphorylation and downstream IFN stimulated gene (ISG) induction [19].
122 Interestingly, the miR-155-SOCS1 relationship has also been actively implicated
123 in the field of cancer biology. An inverse correlation of miR-155 and SOCS1
124 expression was observed in breast cancer patients and cell lines, in which miR-
125 155 conferred enhanced oncogenic properties [20]. In hepatocellular carcinoma
126 (HCC), miR-155 regulation of SOCS1 increases STAT3 signaling, in turn
127 stimulating matrix metalloproteinase (MMP)9 expression and increasing tumor
128 invasion [21]. Other miRNAs that have been shown to regulate SOCS1 expression
129 include miR-30d in prostate cancer [22], miR-122 in Huh-7 hepatocyte cells [23]
130 and miR-150 in lupus nephritis pathogenesis model [24]. The miR-19a/b family
131 was up-regulated in multiple myeloma (MM) and acted as an oncogenic
132 regulator via suppression of SOCS1, an important inhibitor of IL-6-mediated
133 growth in MM pathogenesis [25]. The miR-19a-SOCS1 relation has also been

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134 implicated in gastric cancer [26]. To our surprise, when we analyzed the
135 predicted targets of miR-19a within the JAK-STAT pathway by bioinformatics,
136 we discovered that the miR-19a target sequence is conserved between SOCS1
137 and SOCS3, and found that SOCS3 is a putative target of miR-19a and modulates
138 the activity of JAK-STAT signaling in response to IFN- α and IL-6 [27]. Together
139 these results demonstrate a realization of how evolution has “chosen” a single
140 miRNA species to regulate multiple cellular targets that converge onto the same
141 signaling pathway to exert an amplified combinatory effect on the pathway [12].
142 This broad effect of miR-19a provides the cellular machinery with a very
143 convenient switch to control the expression of a set of genes with powerful effect.
144 While miR-19a regulates multiple targets of the JAK-STAT pathway its regulation
145 also extends to the NF- κ B signaling pathway [28]. Similarly, miR-155 inhibits
146 SOCS1 and SOCS3 expression, which enhances IFN production during persistent
147 viral infection, demonstrating its ability to also control several SOCSs that
148 regulate more than one pathway [29].

149

150 Targeting of SOCS by multiple miRNA also seems to be a common strategy
151 adopted by miRNA to regulate the JAK-STAT pathway. miR-203 was
152 demonstrated by two independent groups to regulate SOCS3 expression in
153 different cellular contexts. Ru and colleagues reported that miR-203 was up-
154 regulated in breast cancer and its knock-down correlated with enhanced level of
155 SOCS3 expression and improved chemosensitivity towards cisplatin [30]. Moffatt
156 and Lamont demonstrated that gingival epithelial cells infected with
157 *Porphyromonas gingivalis* had increased cellular miR-203 expression, resulting

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158 in SOCS3 down-regulation and enhanced STAT3 activation [31]. On top of this,
159 the latter showed that SOCS6 is also a putative target of miR-203 [31]. In another
160 example, although miR-221 was implicated in regulating SOCS3 expression level,
161 which conferred anti-tumorigenic effects in prostate cancer patients [32],
162 TargetScan also predicts that SOCS1 and SOCS7 are additional targets for this
163 miRNA [33], highlighting that our current knowledge and understanding of
164 SOCS-targeting microRNAs is in its infancy and that future investigations may
165 reveal an even more complex and intricate network of intracellular pathway
166 regulation.

167

168 **Regulation of PIAS by microRNAs**

169

170 While JAK kinase- and receptor-mediated signaling are directly regulated by
171 SOCSs, the downstream signaling protein, STAT, is regulated by protein inhibitor
172 of activated STAT (PIAS), which effectively fine-tunes the pathway activity. The
173 PIAS family in mammals is composed of 4 members: PIAS1, PIAS3, PIASx and
174 PIASy, recognized to target STAT1, STAT3, STAT4 and STAT1, respectively [15].
175 Each member of the PIAS family contains a RING-finger-like zinc-binding domain
176 (RLD), which confers small ubiquitin like modifier (SUMO) E3-ligase activity,
177 thus mediating SUMOylation and consequential deactivation of STATs [34]. PIAS
178 protein also regulates STAT independently of SUMOylation. Other mechanisms
179 include direct blockage of STAT DNA binding and recruitment of co-repressors,
180 such as histone deacetylase (HDAC) [reviewed in 34]. Although PIAS-STAT
181 interaction is believed to be cytokine-dependent [34], the recent discovery of

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182 their regulation by miRNAs could be crucial in understanding the maintenance of
183 the pathway integrity and cellular homeostasis.

184

185 Since PIAS3 negatively regulates STAT3, a key player in the IL-6-mediated ISG
186 induction (driven by the IFN- γ activated sequence [GAS]-containing promoter)
187 [15], dysregulation of PIAS3 by miRNA could have devastating outcomes.
188 Regulation of PIAS3 by miRNA was first proposed by Brock et al., who showed
189 that miR-18a targeted the 3'UTR of PIAS3 mRNA, which suppressed its protein
190 expression and resulted in IL-6-induced STAT3 activation in hepatocytes and
191 triggered the acute-phase response [35]. Since the dysregulation of JAK-STAT
192 signaling, via altered expression of SOCS by miRNA, is evident in many cancer
193 models, it is no surprise to see it is equally true for PIAS. Indeed, regulation of
194 PIAS3 by miR-18a has been implicated in gastric adenocarcinoma, in which a
195 clinical correlation has been established between miR-18a, PIAS, JAK-STAT
196 pathway activity and downstream anti-apoptotic and cell-proliferative genes
197 [36]. Using proteomics PIAS3 was also identified as a cellular target of miR-21,
198 which was highly expressed in MM [25], resulting in similar pathological
199 outcomes to IL-6-induced JAK-STAT pathway activation [37]. PIAS3 is also a
200 target for miR-125b in glioblastoma stem cells [38], further highlighting the
201 multifaceted nature of its regulation and importance as a gatekeeper of
202 oncogenesis. Interestingly, microRNA regulation of PIAS3 even controls T cell
203 development. In fact, inhibition of miR-301a expression in myelin auto-antigen
204 exposed CD4⁺ T helper cells altered their cytokine expression profile and

205 hampered their differentiation into Th17 cells, which was thought to be
206 controlled by PIAS3 regulation of IL-6-STAT3 signaling [39].

207

208 Although there are 4 mammal PIAS proteins, evidence on their expression
209 regulated by miRNA has only been reported for PIAS3. This exclusivity may be
210 partly explained by the length of 3'UTR of their mRNA, since only human PIAS3
211 mRNA carries 3' UTR that spans for nearly 1000nt long, while the others are just
212 a few hundred. This speculation is supported by the study that some
213 housekeeping genes which have strong preference to minimize miRNA
214 regulation tend to evolve with a shorter 3' UTR, thus avoiding miRNA binding,
215 which consequently minimizes the risk of their accidental and undesirable
216 shutdown [40]. However, the physiological relevance of the PIAS's 3'UTR length
217 in regulating the JAK-STAT pathway warrants further investigation. In summary,
218 it is clear that the integrity of a functional JAK-STAT pathway is essential for
219 cellular homeostasis. Dysregulation of this signaling by miRNA may attribute to
220 many cancers and autoimmune diseases. However, it is important to remember
221 that there are other signaling pathways, such as the NF- κ B and TGF β cascades,
222 which also determine the outcome of effective cellular reactions and are now
223 known to be under the regulation of miRNAs.

224

225 **NF- κ B signaling pathway**

226

227 NF- κ B mediates diverse biological processes at the cellular level, including
228 growth, development and inflammatory responses [41]. The canonical NF- κ B

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229 pathway mainly utilizes the RelA (also known as p65):p50 heterodimer as a
230 transcription factor to activate downstream target genes. In unstimulated cells
231 the RelA:p50 heterodimer is bound to inhibitor of NF- κ B (I κ B), making it
232 inaccessible to the nucleus and thus blocking gene transcription [42]. To remove
233 this suppressive constraint, I κ B protein must be phosphorylated by I κ B kinase
234 (IKK) complex, which constitutes two catalytic subunits, IKK α and IKK β , and one
235 regulatory subunit, NF- κ B essential modulator (NEMO) (also known as IKK γ).
236 This initiates K48-polyubiquitination and subsequent proteosomal degradation
237 of I κ B protein [41]. In the non-canonical (or alternative) NF- κ B pathway, which
238 utilizes the RelB:p52 heterodimer, p100, the p52 predecessor, acts like I κ B to
239 suppress translocation and transcription activation of RelB when bound under
240 unstimulated conditions [42]. Upon activation, NF- κ B-inducing kinase (NIK),
241 with the help of IKK α , induces phosphorylation of p100, which is then subjected
242 to ubiquitination and processing into p52, that, with RelB, serves as a
243 heterodimer transcription factor [reviewed in 43]. (Fig.2) Dysregulation of the
244 NF- κ B pathway accounts for many autoimmune, chronic inflammatory and
245 cancerous diseases [41], therefore, as with the JAK/STAT pathway, multiple
246 levels of regulation must be adopted to avoid disease [reviewed in 41].

247

248 **Regulation of PP2A/C by microRNAs**

249

250 Although the canonical and non-canonical pathways mobilize different cell
251 modulators, they are regulated using a similar mode of action: phosphorylation,
252 ubiquitination, and then proteosomal processing of the inhibitory binding

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253 partner of NF- κ B. Due to the importance of NF- κ B regulation, it is not surprising
254 to see that some of these steps are also controlled by miRNA. In both pathways,
255 IKK α / β is the major protein kinase, engaging in the initial phosphorylation and
256 subsequent processing or degradation of the inhibitory binding partner [42]. In
257 order to become activated, IKK α / β complex requires trans-autophosphorylation
258 and phosphorylation from another upstream kinase, such as TGF- β activated
259 kinase-1 (TAK1) or NIK. These phosphorylation sites are subjected to
260 dephosphorylation by a group of protein phosphatases called PP2A/C [44].
261 Regulation of PP2A/C by miRNA is evident in cancer models. Two papers
262 recently reported that miR-520h targets PP2A/C and promotes NF- κ B-driven
263 tumorigenic gene expression in breast cancer and ovarian cancer cell lines, as
264 well as in lung cancer patient samples [45, 46]. The significance of regulating
265 PP2A/C is evident in the broad spectrum of miRNAs that control its expression,
266 including miR-1, miR-19, miR-31 and miR-133. While these miRNAs have not
267 been shown to impact the NF- κ B pathway in disease models, they will most
268 likely affect responses to the pathway and thus identify an area of research that
269 warrants investigation [47-49].

270

271 **Regulation of CYLD by microRNAs**

272

273 Ubiquitination of target proteins is arguably one of the most important and
274 influential molecular events within a cell and is thereby controlled by a series of
275 processes. Polyubiquitination does not only enable I κ B degradation or p100
276 processing, but is implicated throughout the NF- κ B pathway [reviewed in 50]. In

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277 the canonical pathway, NEMO undergoes K63-polyubiquitination in response to
278 TNF stimulation, which facilitates the recruitment of upstream activating factors
279 and, in turn, the activation of IKK complex [51]. To regulate IKK activation, the
280 tumor suppressor deubiquitinase, CYLD (cylindromatosis), removes
281 polyubiquitin chains from NEMO [42]. But the regulation of CYLD is now known
282 to involve miRNA, which has been shown to play an important role in cancer
283 pathogenesis. For example, miR-181b-1 was found to be up-regulated in an
284 oncogenic Src kinase transformed model and manipulation of cellular miR-181b-
285 1 levels altered CYLD expression, NF- κ B activity and mammary epithelial cell
286 line transformation [52]. The targeting of CYLD by miR-181b-1 was later
287 implicated in pancreatic cancer, in which increasing miR-181b-1 levels confer
288 cell line chemoresistance to gemcitabine, via the down-regulation of CYLD and
289 up-regulation of NF- κ B activity [53]. In gastric cancer patients miR-362 was up-
290 regulated in tumor tissue samples, which inversely correlated to CYLD
291 expression, suggesting that miR-362 regulation of CYLD promoted NF- κ B activity
292 and subsequently enhanced cell proliferation and apoptotic resistance [54]. In
293 addition, CYLD mRNA has been shown to be directly targeted by miR-182 and
294 miR-486, which promoted tumor aggressiveness of gliomas, again through NF-
295 κ B dysregulation [55, 56]. The broad inhibitory remit of both these miRNAs is
296 clearly demonstrated in their spectrum of targets, with miR-486 also regulating
297 Cezanne (A20 family deubiquitinase) and A20-interacting partners, TNF- α -
298 induced protein 3 (TNFAIP3) interacting protein (TNIP)1/2/3; and miR-182
299 regulating TNIP1, optineurin ubiquitin-binding protein (OPTN), and the
300 deubiquitinase ubiquitin-specific protease 15 (USP15) [55, 56].

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301

302 **Regulation of A20 by microRNAs**

303

304 Another deubiquitination enzyme that has received much attention is A20 (also
305 known as TNFAIP3). A20 contains an N-terminal ovarian tumor (OTU)
306 deubiquitination domain and a C-terminal zinc finger (ZnF) E3 ligase domain,
307 which are thought to have dual functions in K63-linked deubiquitination and
308 K48-linked polyubiquitination of substrates, such as receptor-interacting protein
309 (RIP)1 kinase [57]. RIP1 is an upstream activating kinase of TAK1, and its K63-
310 linked polyubiquitination is indispensable for IKK activation in the TNF-induced
311 NF- κ B pathway [41]. Indeed, A20 mediates deubiquitination of K63-linked
312 polyubiquitin chain on RIP1, but K48-linked polyubiquitination is actually
313 mediated by A20-binding partner, ITCH (also known as itchy E3 ubiquitin
314 protein ligase), which targets RIP1 for proteosomal degradation, thus
315 terminating the transduced signal [42]. (Fig.2) With its ability to negatively
316 regulate NF- κ B activity, A20 is regarded as a tumor suppressor and its
317 inactivation is frequently observed in various cancer models [57]. miRNA have
318 also been documented to manipulate A20 expression levels with obvious
319 consequences for NF- κ B activity in tumor cells.

320

321 Gantier and colleagues reported that global depletion of miRNA expression,
322 through conditional Dicer knock-out, impaired pro-inflammatory cytokine
323 induction [28]. Initially using TargetScan they predicted that negative regulators
324 of NF- κ B, including A20 and other related proteins, such as its binding partners

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325 (Ring Finger Protein 11 (RNF11) and ITCH), A20 regulator (TNIP1) and other
326 deubiquitinases (CYLD and Cezanne), were targets of an oncogenic miRNA
327 cluster, miR-17-92 [28, 33]. Among the four miRNA families expressed in this
328 cluster, miR-19 was demonstrated to have a significant impact on NF- κ B activity
329 [28]. In this study, A20 and RNF11, as well as two other regulators of NF- κ B
330 (KDM2A and ZBTB16), were shown to be validated targets of miR-19, whereas
331 the suppressive effect on other predicted targets, including CYLD, was not
332 observed [28]. However, in T-cell acute lymphoblastic leukemia (T-ALL) patient
333 samples and cell lines, up-regulation of miR-19 inhibited CYLD expression,
334 leading to sustained NF- κ B activity [58], clearly demonstrating that miRNAs
335 regulate multiple targets from the same pathway in a cell- and disease-type
336 dependent fashion and highlighting the vast chasm of knowledge still to be
337 explored. In addition, apart from miR-19, miR-146a was also able to regulate the
338 expression of RNF11, which facilitated Hendra virus replication in NF- κ B-
339 dependent manner [59]. miR-18a, from the miR-17-92 cluster, also reduced A20
340 in a model of rheumatoid arthritis (RA) and enhanced NF- κ B-dependent
341 expression of the matrix degrading enzyme, MMP1, and inflammatory cytokines,
342 such as IL-6, in synovial fibroblasts [60]. Similar establishment of NF- κ B
343 dysregulation by miRNA-targeting of A20 was observed during Japanese
344 encephalitis virus infection. This virus induced cellular miR-29b expression,
345 which regulated A20 expression in a microglial cell line, thus enhancing NF- κ B
346 activity [61]. In stark contrast, another miRNA from the same miR-29 family,
347 miR-29c, was found to be down-regulated in a Hepatitis B Virus (HBV)-related
348 HCC cell line and patient samples. This loss of miR-29 expression up-regulated

349 A20 expression, resulting in restricted cell proliferation and enhanced apoptosis
350 [62]. While miRNA are thought to predominately suppress target gene
351 expression, in a sarcoma model, miR-29 bound the A20 3'UTR and prevented
352 physical association of an RNA-binding protein HuR, thus protecting A20 mRNA
353 from destabilization and degradation. In the same study, the authors showed
354 that miR-125 could also regulate A20 expression [63], an observation mirrored
355 in a macrophage polarization and diffuse large B-cell lymphoma model [64, 65].

356

357 In general, aberrant NF- κ B activity resulting from the dysregulation of its
358 regulator by miRNA drives the expression of numerous pro-inflammatory
359 cytokines and chemoattractants at the site of injury, and confers aggressiveness
360 and apoptotic tolerance to tumors at the cellular level. These events have been
361 evident in many of the aforementioned examples and believed to be the center of
362 many inflammatory diseases and cancers. Therefore, further elucidating the
363 control of NF- κ B regulators by miRNA will help us better understand the
364 development and progression of these diseases and reveal much needed
365 therapeutic targets.

366

367 **TGF β signaling pathway**

368

369 From the beginning of life the TGF β pathway is indispensable in coordinating cell
370 development and differentiation and is essential for sustaining a functioning
371 immune response [66]. The signal begins when functional, mature TGF β is freed
372 by an endoprotease from a latent complex held within the extracellular matrix

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373 [67]. The binding of TGF β to its cognate receptor complex, consisting of two
374 type-I receptors and two type-II receptors, triggers serine/threonine kinase
375 activity on the type-II receptor molecule, which subsequently activates and
376 phosphorylates the type-I receptor at its cytoplasmic domain [68]. Smad2 and
377 Smad3 (Receptor (R)-Smad proteins) are recruited to the phosphorylation site
378 and themselves phosphorylated by the activated type-I receptor complex.
379 Phosphorylated Smad2/3 can then form a trimeric complex with other
380 coactivators, such as Smad4 and TIF1 γ , to regulate gene expression in nucleus
381 [67]. Alternatively, the receptor complex can activate a Smad-independent
382 pathway through modulating Rho GTPase, MAP kinase and PI3K signaling
383 pathway activity, which regulates a different sets of target genes [66]. (Fig.2) To
384 achieve optimized signaling activity, the TGF β signaling pathway output is tightly
385 regulated at different stages.

386

387 **Regulation of Smad7 by microRNAs**

388

389 While R-Smad proteins convey activating downstream signals, inhibitory Smad
390 (I-Smad) proteins regulate this intracellular transduction. Smad7, for example, is
391 expressed in response to TGF β pathway activation and provides efficient
392 negative feedback through several mechanisms [67]. It can physically bind to the
393 type-I TGF β receptor, acting as a direct competitor to R-Smad [67], or it can
394 further recruit other regulatory proteins, including PP1 phosphatase and the
395 Smad ubiquitin regulatory factor (Smurf) E3 ligase, which inactivate and
396 promote degradation of the receptor molecule, respectively [67, 68]. We now

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397 know that expression of Smad7 is regulated by multiple miRNAs. The up-
398 regulation of miR-21 expression has been shown to suppress Smad7 expression
399 in HCV-infected liver biopsies. Interestingly, these findings correlated with
400 patient HCV load and fibrosis stage, indicating a role for miR-21 in accelerating
401 fibrogenesis [69]. Others observed that miR-21-mediated reduction of Smad7
402 correlated with expression of TGF β -induced fibrotic markers, such as alpha
403 smooth muscle actin (α -SMA) and fibronectin (Fn), which promoted epithelial-
404 mesenchymal transition (EMT) in lung fibrosis [70, 71], renal fibrosis [72, 73],
405 and systemic sclerosis [74, 75]. EMT also plays an important role in the
406 initiation of cancer metastasis. Complementary to this notion, miR-21 was up-
407 regulated in the invasive ductal carcinoma region of breast cancer and knock-
408 down of miR-21 restored Smad7 levels in a breast cancer cell line [76]. miR-21-
409 mediated reduction of Smad7 expression has also been implicated in the
410 generation of carcinoma-associated fibroblasts (CAFs), which confer
411 tumorigenesis, proliferation and invasiveness characteristics of tumors [77].
412 However, elevated miR-21 levels are not always associated with enhanced
413 proliferation and differentiation. In the case of myelodysplastic syndromes
414 (characterized by ineffective hematopoiesis), suppression of Smad7 by miR-21
415 was found to decrease erythroid colony formation of CD34+ cells, while
416 inhibiting miR-21 could rescue red blood cell count and stimulate erythropoiesis
417 in transgenic mice [78]. As seen with miR-21, suppression of endogenous Smad7
418 is a common carcinogenic mechanism that promotes EMT. Other examples
419 include the miR-216a/217 cluster in HCC [79], miR-20a in gall bladder
420 carcinoma [80], miR-181a in ovarian cancer [81], and miR-106b-25 in breast

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421 cancer [82]. While the oncogenic role of these microRNAs has been described,
422 the tumor suppressive role of a selection has also been reported, with reduced
423 miR-25 in colon cancer [83] and miR-181c in metastatic neuroblastoma patients
424 [84]. Interestingly, both reports reasoned that the tumor suppressive roles of
425 miR-25 and miR-181c were accounted for by reduced Smad7 protein expression
426 and TGF β activity, which would otherwise stimulate tumor growth and
427 migration [83, 84].

428

429 **Regulation of Smurf by microRNAs**

430

431 Reduced Smad7 expression in bronchial epithelial cells was also associated with
432 overexpressed miR-15b in a chronic obstructive pulmonary disease model [85].
433 In their cell-based assays, the authors also found that, apart from Smad7, Smurf2
434 expression was affected by miR-15b [85]. Smurf proteins are E3 ubiquitin ligase
435 proteins, recruited by Smad7 to the type-I TGF β receptor complex. Their
436 recruitment promotes proteosomal degradation of the receptor complex, thus
437 restricting signal transduction [86]. Antagonizing miR-322 and miR-503 action
438 on Smurf2 regulation was shown to inhibit the phosphorylation of Smad2 [87].
439 As miRNA-suppression of Smad7 was observed in many cancers, it is not
440 surprising to see in an aggressive breast cancer model, Smurf2 was down-
441 regulated by miR-15, miR-16 and miR-128 [88]. miR-15b also targets Smurf1
442 during osteoblast differentiation, as a way to activate the expression of a master
443 transcription factor, Runx2 [89]. Smurf1 is also a target of miR-17 and miR-497;

444 both of these studies showed reduced miRNA and up-regulated Smurf1
445 expression in periodontitis and metastatic ovarian cancer, respectively [90, 91].

446

447 **Regulation of GARP by microRNAs**

448

449 TGF β is secreted and stored in the extracellular matrix inside a large latency
450 complex. The cytokine remains inactive and bound to the latency-associated
451 peptide until positive regulators increase the efficiency of its dissociation from
452 the large latency complex and it is processed into a mature form. Glycoprotein A
453 repetitions predominant protein (GARP), is expressed by T regulatory (Treg)
454 cells and tightly associated with the latency-associated peptide bound to
455 immature TGF β [67]. GARP is essential for TGF β activation [92], as it frees
456 immature TGF β molecules from the latency complex [67]. miR-142-3p regulates
457 GARP expression and thus controls Treg cell proliferation [93]. Specifically, the
458 authors observed decreased expression of miR-142-3p in CD25⁺ CD4 T cells and
459 manipulation of miR-142-3p levels resulted in altered proliferation of these cells
460 [93], which is consistent with the concept of TGF β -mediated Treg cell
461 proliferation. No matter whether a miRNA is targeting the positive or negative
462 regulators of the TGF β signaling pathway, any upset in the homeostatic balance
463 could lead to serious pathological consequences, like fibrosis or oncogenesis.

464

465 **Regulation of microRNA levels**

466

467 So far this review has discussed how miRNAs participate in signaling pathway
468 regulation and provide an additional layer of supervision on pathway regulators,
469 but, in order to fully understand how these “master regulators” control our
470 signaling networks it is important to note how miRNAs themselves are regulated.
471 Endogenous levels of miRNA are significantly linked to the final output of
472 signalling pathways and are under the control of several factors. The majority of
473 miRNAs is located in either intragenic or intergenic regions and is transcribed
474 together with its host gene or from its own promoter [2]. The miR-106b-25
475 cluster is an example of intragenic miRNA, which sits itself within intron 13 of
476 the miniature chromosome maintenance 7 (MCM7) gene [94]. This miRNA
477 cluster encodes three miRNAs, namely miR-106b, miR-93, and miR-25, two of
478 which, miR-106b and miR-25, we described above as regulators of Smad7. These
479 miRNA were found to be frequently co-expressed and probably co-regulated
480 with their host mRNA [95] and amplification of the MCM7 gene locus and its
481 elevated expression with miR-106b-25 cluster have been associated with human
482 malignancies [94, 96]. The miR-17-92 cluster (also known as oncomir-1) is an
483 example of intergenic miRNA that is expressed and processed from the C13orf25
484 transcript [96]. This miRNA cluster encodes six mature miRNAs, namely miR-17,
485 miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1, which have broad effects
486 on multiple pathway regulators, including SOCS, PIAS, A20, Smad7 and Smurf1.

487

488 It is interesting to note that the expression of both MCM7/miR-106b-25 and
489 miR-17-92 genes are induced by common transcription factors, E2F1 and MYC
490 [96]. E2F1 and MYC are involved in a positive feedback loop making both

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491 proteins capable of regulating one another [97]. The entire regulatory network
492 may be further complicated by the negative regulation of E2F1 expression by
493 miR-106b and miR-20a [96]. These constitute an important regulatory
494 mechanism that allows these miRNAs to be expressed at optimized level. It is
495 foreseeable that in the event of sub-optimal miRNA levels, the negative feedback
496 constrain is lenient, so the positive feedback loop of the transcription factors
497 encourages the expression of these miRNAs. Alternative, when miRNA
498 expression becomes excessive, it places a heavy negative feedback constrain on
499 the transcription factors, so that the continuous expression of miRNA can be
500 eventually shut off. Therefore, it is evident that the sequence of molecular events
501 responsible for miRNA expression can be more complex than their simple
502 regulation of target genes and networks, and hence should be analysed case by
503 case. In fact, when considering the mechanisms that alter the steady-state level
504 of any miRNA, we should also take into account the cell type, its half-life and
505 transient intracellular turnover [reviewed in 98].

506

507 **Insights derived from regulator-targeting miRNAs**

508

509 Although the role played by each miRNA appears to be context-dependent in
510 individual studies, some have collectively demonstrated its versatility in
511 regulating multiple regulators of a signaling pathway. Examples include miR-15
512 (targeting Smad7, Smurf1 and Smurf2 in TGF β pathway) [85, 88, 89], miR-19
513 (targeting SOCS1 and SOCS3 in JAK-STAT pathway; A20, CYLD and RNF11 in NF-
514 κ B pathway) [25-28, 58], miR-155 (targeting SOCS1 and SOCS3 in JAK-STAT

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515 pathway) [29], miR-203 (targeting SOCS3 and SOCS6 in JAK-STAT pathway) [30,
516 31]. This versatility is indeed conferred by the ability of miRNA to regulate gene
517 expression at translation level by binding to and targeting the complementary
518 sequence present on 3' UTR of any gene, irrespective of their actual protein
519 coding sequence, such that multiple genes that share similar gene function and
520 acquire the same complementary sequence can be regulated simultaneously by a
521 common miRNA. The miRNA-target relationship is now thought to be under tight
522 natural selection and is believed to have co-evolved with one another, as well as
523 the whole regulatory network [99].

524

525 To date, vast majority of publications have focused on the validation of a single
526 gene targeted by miRNA that has implications in different biological models.
527 While this has been limited by both our lack of knowledge and experimental
528 capabilities, it has certainly led to an under-estimation of miRNA capacity in
529 modulating the entire regulatory network as a “master regulon”. Fortunately, the
530 recent advancement in next-generation sequencing and other molecular
531 biological techniques, such as photoactivatable-ribonucleoside-enhanced
532 crosslinking and immunoprecipitation (PAR-CLIP), have already improved our
533 understanding and knowledge in the transcriptome-wide regulation of miRNA in
534 many cellular contexts. Bioinformatic database analysis remains a cornerstone
535 for the predictive analysis of miRNAs and their targets and will continue to be
536 used to understand how miRNA can act beyond a single gene to regulate an
537 entire network [100]. Careful data-mining procedures and the use of a systems
538 biology approach will conserve efforts from validating all of the predicted targets

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539 and focusing on the pathway predicted to be affected by the specific miRNA and
540 the corresponding regulators involved. Additionally, when regarding the
541 timeframe and strength of miRNA regulation, our current knowledge is greatly
542 limited by the use of existing non-physiological methodology that manipulates
543 endogenous miRNA expression in cell-based systems. Furthermore, it is
544 important to note, that in most cases, owing to direct gene amplification of the
545 miRNA region or altered expression of the transcription factors responsible for
546 regulating miRNA expression, miRNAs levels during disease pathogenesis are
547 aberrantly expressed [96].

548

549 The plethora of current evidence outlined in this review identifies miRNAs as
550 “master controllers” of intracellular signaling pathways in many disease models
551 and in the era of new therapies against miRNA, this evidence highlights them as
552 powerful targets for therapeutic development with highly significant clinical
553 applications. In many of the studies covered in this review, manipulation of
554 endogenous miRNA levels by chemically synthesized analog or inhibitor could
555 revert the phenotype caused by the dysregulated miRNA, and therefore provide
556 the proof-of-principle for potential drug development. While therapeutic
557 development, from “bench to bedside” is a long, arduous and expensive process,
558 recent advances with the development of the first miRNA-targeting drug,
559 miravirsen, (miR-122 targeting locked-nucleic acid (LNA)-modified inhibitor for
560 treatment of hepatitis C virus infection, currently in phase 2 clinical trial), have
561 brought the entire miRNA research community closer to therapeutic solutions
562 than ever before [101]. Another miRNA-based drug, MRX34, is the first miRNA

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563 mimic for miR-34 and has entered phase 1 trial for treatment of HCC [102].
564 These outstanding advances demonstrate that miRNA are promising targets for
565 therapeutic intervention and with our advanced understanding of their
566 regulation of different cellular pathways and disease pathogenesis, it is expected
567 more pre-clinically validated drugs will enter clinical trials and be used in our
568 actual daily clinical practice.

569

570 **Conclusion**

571

572 This article has reviewed how miRNAs potently act as novel regulators of
573 classically known inhibitors of the JAK-STAT, NF- κ B and TGF β signaling
574 pathways. We now have a much deeper understanding of the way in which
575 miRNAs regulate many pathological diseases and normal developmental
576 processes. More importantly, we have identified a reiterating concept, whereby
577 miRNAs bind 3'UTRs of their target irrespective of the protein coding sequence,
578 and regulate multiple targets, which usually work at different levels of the
579 signaling cascade, within the same signaling pathway. This allows miRNA to
580 provide another layer of signaling regulation, in order to achieve maximal effect
581 and avoid detrimental responses to stimuli. With this concept in mind, it is
582 essential for future research of miRNA-target identification to consider the
583 regulation network (or regulon) of specific miRNA, in order to achieve total
584 understanding of the mechanism of any cellular process. More advanced
585 techniques can reveal the transcriptome-wide regulation of miRNAs should be
586 considered a standard and essential approach. This not only takes the concept of

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587 a single miRNA regulating different targets within the same pathway into
588 account, but also provides a bigger picture how miRNA can regulate different
589 targets among different signaling pathways.

590

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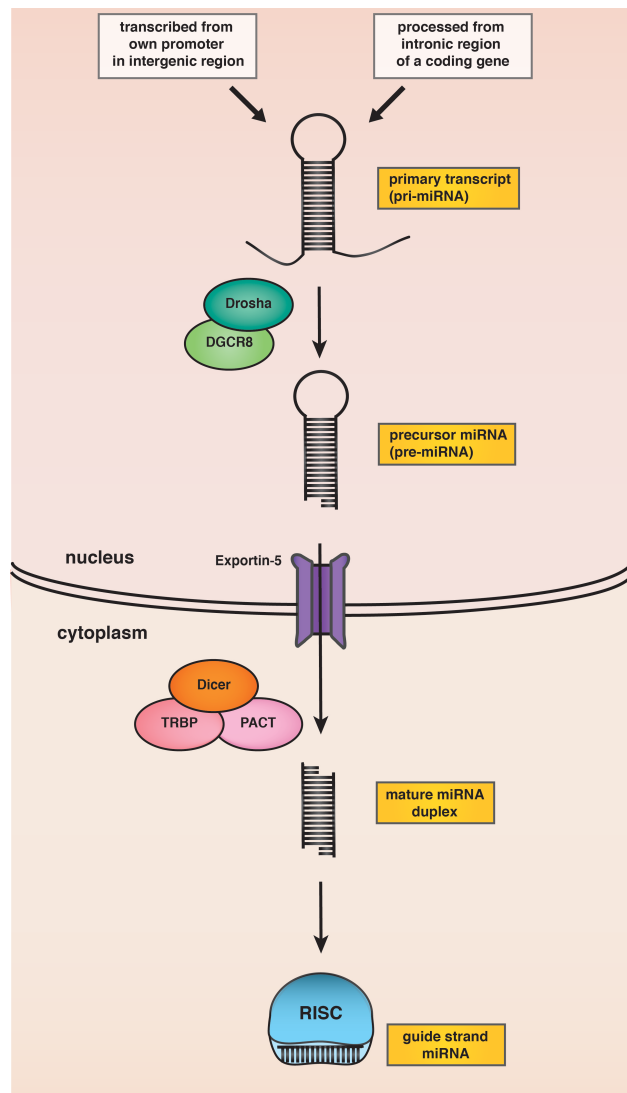
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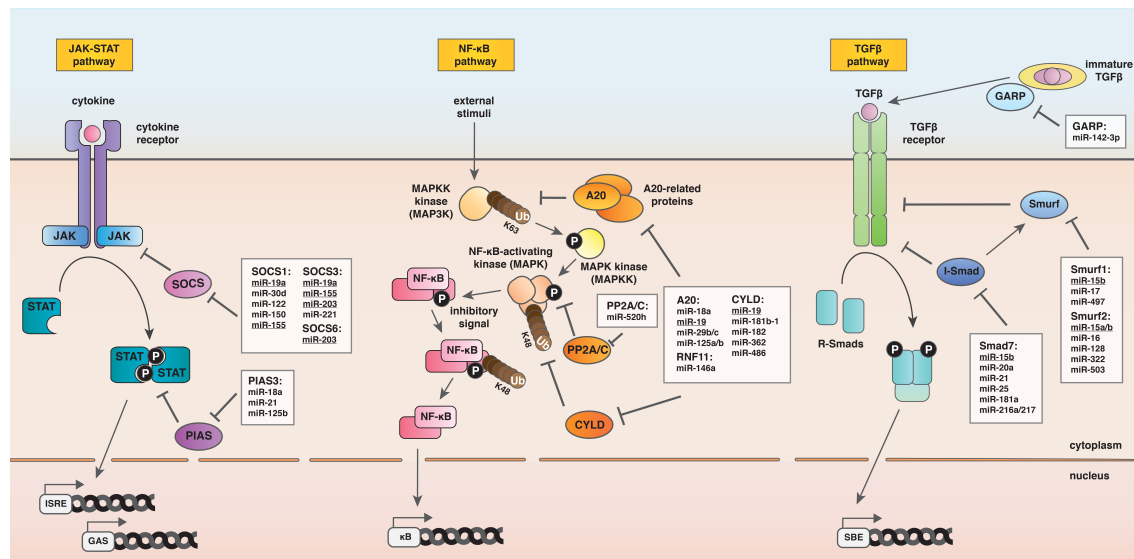
1025 Fig.1 Biogenesis of microRNA. In the nucleus, miRNA is either transcribed from
 1026 its own promoter in intergenic region or processed from the intronic region of a
 1027 coding gene as a primary transcript (pri-miRNA). It is processed by an RNase III-
 1028 type endonuclease family protein Drosha, with an accessory dsRNA-binding
 1029 protein DGCR8, into a precursor molecule (pre-miRNA) with stem-loop structure
 1030 of around 60-70nt in length and a 2nt overhang at the 3' end. It is then exported
 1031 to the cytoplasm by a transport protein Exportin-5 . In the cytoplasm, pre-miRNA
 1032 is further processed by another RNase III-type endonuclease family protein
 1033 Dicer, in some cases also with the help of accessory dsRNA-binding proteins

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1034 PACT and TRBP, into a dsRNA duplex of 19-21nt base pair region and a 2nt
1035 overhang at each 3' end. One of the two strand (guide strand) from this miRNA
1036 duplex is loaded onto RISC complex to effect its gene silencing function.



1037

1038

1039 Fig.2 Regulation of key regulators of cellular pathways by miRNAs. Left: JAK-
 1040 STAT pathway. Binding of cytokine to its cognate receptor pair activates and
 1041 phosphorylates receptor-associated JAK kinase which then phosphorylates
 1042 downstream transcription factor STATs. Activated STATs dimerize and expose
 1043 nuclear localization signal to enter nucleus and promote transcription from
 1044 promoter carrying interferon-stimulated responsive element (ISRE) or IFN- γ
 1045 activated sequence (GAS). SOCS proteins negatively regulate JAK kinase by
 1046 blocking the binding with STATs and promoting the degradation of cytokine
 1047 receptors; and PIAS proteins negatively regulate STAT transcription factors by
 1048 blocking its binding to DNA and recruiting corepressor to inhibit transcription.
 1049 Both SOCS and PIAS are under tight regulation by miRNAs. Middle: NF- κ B
 1050 pathway. Both canonical and non-canonical NF- κ B pathway are activated
 1051 through similar mechanism. Under unstimulated condition, the activity of NF- κ B
 1052 is suppressed by an inhibitory signal (canonical: inhibitor of κ B (I κ B); non-
 1053 canonical: ankyrin repeats on p100). To remove this inhibitory constrain, the

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1054 inhibitory signal needs to be labeled by K48-polyubiquitin chain and targeted to
1055 proteosomal processing. Prior to K48-polyubiquitination by E3 ligase, the target
1056 protein must be phosphorylated by an NF- κ B-activating kinase, also known as
1057 mitogen-activating protein kinase (MAPK) (for example, canonical: I κ B kinase
1058 (IKK); non-canonical: NF- κ B-inducing kinase (NIK). In order to become activated
1059 to mediate downstream phosphorylation event, MAPK needs to be
1060 phosphorylated by an upstream kinase, also known as MAPK kinase (MAPKK)
1061 (for example TGF β activated kinase-1 (TAK1)). This activating phosphorylation
1062 can be removed by protein phosphatase PP2A/C. Like MAPK, activation of
1063 MAPKK requires the phosphorylation of another upstream kinase, also known as
1064 MAPKK kinase (MAP3K). MAP3K can be activated by K63-polyubiquitination in
1065 response to external stimuli. In terms of tumor necrosis factor (TNF) stimulation,
1066 the MAP3K protein, receptor-interacting protein (RIP)1 kinase, can be
1067 deactivated by deubiquitinase A20, as well as other A20-related proteins,
1068 including its binding partner Ring Finger Protein 11 (RNF11) as well as another
1069 deubiquitinase CYLD, by removing its K63-polyubiquitin chain and recruiting E3
1070 ligase to tag a K48-polyubiquitin chain to promote its degradation. All these
1071 regulators, A20, RNF11, CYLD, and PP2A/C, can be regulated by miRNAs. Right:
1072 TGF β pathway. Binding of TGF β to its cognate receptors phosphorylates and
1073 activates receptor Smad (R-Smad) proteins, such as Smad2 and 3. R-Smad
1074 proteins bind other coactivator and translocate into nucleus to drive
1075 transcription from promoter with Smad-binding element (SBE). Activation of R-
1076 Smad proteins can be inhibited by inhibitory Smad (I-Smad) such as Smad7
1077 through direct blockage of receptor and recruitment of other deactivating

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1078 enzymes such as Smad ubiquitin regulatory factor (Smurf) E3 ligase. TGF β is
1079 normally secreted in a closed immature form into the extracellular matrix.
1080 Glycoprotein A repetitions predominant protein (GARP) expressed by T
1081 regulatory (Treg) cells can facilitate the maturation of TGF β , thus acting as a
1082 positive regulator of the pathway. I-Smad, Smurf and GARP can be regulated by
1083 miRNAs. miRNAs that can regulate multiple cellular targets from the same
1084 signaling pathway are underlined.