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Activation of gga-miR-155 by reticuloendotheliosis virus T strain and its contribution to transformation

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2 **contribution to transformation**

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18 **Running title**: v-*rel* induces gga-miR-155 through the NF-κB pathway

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Abstract

The v-*rel* oncoprotein encoded by reticuloendotheliosis virus T strain (Rev-T) is a member of the *rel*/NF-κB family of transcription factors capable of transformation of primary chicken spleen and bone marrow cells. Rapid transformation of avian haematopoietic cells by v-*rel* occurs through a process of deregulation of multiple protein-encoding genes through its direct effect on their promoters. More recently, upregulation of oncogenic miR-155 and its precursor pre-miR-155 were demonstrated in Rev-T-infected chicken embryo fibroblast cultures as well as Rev-T-induced B-cell lymphomas. Through electrophoresis mobility shift assay and reporter analysis on gga-miR-155 promoter, we show that the v-*rel*-induced miR-155 overexpression occurs by the direct binding to one of the putative NF-κB binding sites. Using v-*rel*-induced transformation model on chicken embryonic splenocyte cultures, we could demonstrate dynamic increase in miR-155 levels during the transformation. Transcriptome profiles of lymphoid cells transformed by v-*rel* showed upregulation of miR-155 accompanied by downregulation of a number of putative miR-155 targets such as Pu.1 and CEBPβ. We also show that v-*rel* can rescue the suppression of miR-155 expression observed in Marek's disease virus-transformed cell lines, where its functional viral homolog MDV-miR-M4 is overexpressed. Demonstration of gene expression changes affecting major molecular pathways including organismal injury and cancer in avian macrophages transfected with synthetic mature miR-155 underline its potential direct role in transformation. Our study suggests that v-*rel*-induced transformation involves complex set of events mediated by the direct activation of NF-κB targets together with the inhibitory effects on miRNA targets.

Keywords: v-*rel* , NF-κB, miR-155, transformation

Introduction

46   The *rel*/NF-κB family of transcription factors (1, 2) play a key role in the control of cell

47   proliferation and apoptosis, two functions critical in cancer. The involvement of *rel*/NF-κB in

48   malignancy is best demonstrated by the acute oncogenicity of their viral derivative, v-*rel*,

49   first identified in reticuloendotheliosis virus T (Rev-T) strain (3, 4). Rev-T is an acutely

50   transforming variant of REV, the aetiological agent of reticuloendotheliosis in birds, carrying

51   the viral oncogene v-*rel*, a variant of the turkey cellular proto-oncogene c-*rel* (5-7). Because

52   of the rapidity and efficiency of transformation of the cells, the v-*rel* provides a valuable

53   model for studying the role of *rel*/NF-κB family in neoplastic transformation and cancer. The

54   v-*rel*-mediated transformation occurs predominantly through the modulation of transcription

55   of *rel*/NF-κB targets (8-10), the examples of which include AP-1 (11, 12), IRF-4 (13),

56   SH3BGRL (14), TGFβ/Smad (15) and telomerase reverse transcriptase (TERT) subunit (16).

57   More recently, repression of BLNK and BCAP proteins (17) and a novel interaction of

58   CAPERα and the transactivating domain of v-*rel* (18) were shown to be important for

59   lymphocyte transformation by the v-*rel* oncoprotein.

60   Several studies have also implicated microRNAs (miRNAs) as key mediators of a number of

61   cell regulatory processes including the induction of cancer (19-21). Among the numerous

62   miRNAs expressed in hematopoietic cells, miR-155 was shown to have the most wide

63   ranging effects on the biology of lymphocytes (22-25). It is processed from a primary

64   transcript, known as '*Bic*' (B-cell integration cluster), whose upstream region was originally

65   found to be a frequent site of integration of the avian leukosis virus in lymphomas (26). A

66   number of recent miRNA profiling studies have shown elevated levels of miR-155 in a wide

67   array of cancers including lymphomas (27-30).

68   In a recent study on chicken embryo fibroblast (CEF) cultures infected with

69   reticuloendotheliosis virus (Rev) HA1101 strain, differential expression of a number of genes

70   leading to changes in several signalling pathways were reported (31). We and others have

71   shown upregulation of miR-155 in Rev-T-transformed cell lines and CEF (32, 33). For

72   further analysis of the global changes in miRNA profiles induced by v-*rel*, we used an *in*

73   *vitro* model of v-*rel*-induced transformation of embryonic splenocytes to demonstrate the

74   sequential upregulation of miR-155 during the transformation process. Our studies confirm

75   that v-*rel*-mediated upregulation of gga-miR-155 occurs through the direct binding to at least

76   one of the putative NF-κB sites on the *Bic*/miR-155 promoter. Analysis of the gene

77   expression changes in the v-*rel*-transformed cells further demonstrated downregulation of a

78   number of known miR-155 targets potentially affecting a number of important biological

79   pathways. Demonstration of the targeting of a number of cancer-related genes in chicken

80   macrophages overexpressing miR-155 demonstrated the importance of this miRNA as a

81   major regulator of v-*rel*-induced transformation.

82

83   **Results**

84   **Upregulation of miR-155 in Rev-T transformed cell lines.** During the analysis of the

85   global changes in miRNA expression in chicken lymphocyte lines transformed by avian

86   oncogenic viruses, we observed that miR-155 is overexpressed in v-*rel*-transformed chicken

87   lymphocytes, compared to the normal spleen cells and MDV-transformed cell lines (32). For

88   confirmation of the higher expression of miR-155 in v-*rel*-transformed cells, we examined

89   Rev-T-transformed cell lines AVOL-1, AVOL-2, AVOL-3 and RIR-Rev-T cells by Northern

90   blot analysis. An ALV transformed B-cell line HP45 was used as positive control where miR-

91   155 is upregulated due to insertional activation and normal spleen cells which doesn't express

92   detectable levels of miR-155 was used as negative control. High levels of miR-155 transcripts

93   were readily observed in all Rev-T transformed cell lines (Fig 1).

94   **v-*rel* binds to the NF-κB sites in the Bic/miR-155 promoter**. Having demonstrated the

95   upregulation of miR-155 in Rev-T transformed cells, we examined the potential mechanisms

96   of miR-155 overexpression by v-*rel*. Analysis of the chicken *Bic*/miR-155 promoter sequence

97   for potential transcription factor binding sites using the program TFSEARCH (34) identified

98   a number of transcription factor binding sites, including two putative NF-κB sites (NF-κB1 &

99   NF-κB2) located at positions -581 and -66 respectively (relative to the transcription start site).

100   In order to establish that v-*rel* binds directly to the putative NF-κB sites in the *Bic*/miR-155

101   promoter, electrophoresis mobility shift assay was carried out using recombinant GST-v-*rel*

102   fusion protein. Briefly, purified GST-v-*rel* protein was incubated with dsDNA

103   oligonucleotides probe spanning the two putative NF-κB sites. The intense shifted bands

104   were observed with incubation of GST-*v-rel* and wild type labelled probes for both sites (lane

105   2, Fig 2A). The bands are competed by an excess of cold competitor (lane 3, Fig 2A), but not

106   the same amount of a mutant competitor that is not bound by v-*rel* protein (lane 4, Fig 2A).

107   **NF-κB site 2 in *Bic*/miR-155 promoter is required for miR-155 activation.** Having

108   demonstrated the direct binding of v-*rel* to the NF-κB sites, we next examined the possible

109   contribution of these elements in mediating *Bic* regulation. To this end, we carried out

110   reporter assays to examine the ability of v-*rel* to drive the expression of *renilla* luciferase

111   reporter gene using constructs containing the wild type or the mutant chicken *Bic*/miR-155

112   promoter. For this, the chicken *Bic*/miR-155 promoter region extending from -1829 to +3

113   nucleotides from transcription start site (+1) was cloned upstream *renilla* luciferase gene of

114   psiCHECK™-2 vector (Promega) to replace the SV40 promoter generating the reporter

115   construct pBic-WT. Mutagenesis of the two NF-κB sites was carried out by overlapping PCR

116   generating pBic-M1, pBic-M2 and pBic-M1M2 constructs, where the NF-κB1, NF-κB2 or

117   both sites respectively, were mutated (Fig 2B). For the reporter assay, each of the reporter

118   and pcDNA3-v-*rel* constructs were co-transfected into DF-1 cells and the luciferase

119 expression was assayed 48 hours later using the Dual-Glo Luciferase Assay System

120 (Promega) following manufacturer's instructions. As shown in Fig 2C, mutation of the first

121 NF-κB site (pBic-M1) did not show obvious changes in the luciferase levels compared to the

122 wild type promoter (pBic-WT) construct. In contrast, mutation of the second NF-κB site

123 (pBic-M2) decreased the promoter activity by 63% compared to that of the pBic-WT,

124 suggesting that the v-*rel*-mediated transactivation occurs mainly through this NF-κB site.

125 The promoter activity of double mutant pBic-M1M2 construct was similar to that of pBic-M2

126 further confirming that the second NF-κB site in the *Bic*/miR-155 promoter is important for

127 the v-*rel*-mediated upregulation of miR-155.

128 **v-*rel* relieves the inhibition of miR-155 expression in MSB-1 cells.** We have previously

129 shown that miR-155 is consistently downregulated in MDV-transformed tumours and cell

130 lines (32). Although the mechanisms for this downregulation are not known, this could be

131 due to the complementation of miR-155 functions by the high levels of the viral homolog

132 MDV-miR-M4 expressed in these cells. We wanted to examine whether the downregulation

133 of miR-155 in MDV transformed cell lines can be rescued by expressing v-*rel* in these cells.

134 RCAS(A)-v-rel-GFP virus stocks were used for transduction of v-*rel* into MSB-1 and 265L,

135 where the GFP marker allowed sorting of the infected cells. Analysis of the sorted cells by

136 Western blotting showed expression of v-*rel*-GFP in both infected MSB-1 and 265L but not

137 in uninfected cells (Fig 3A). Expression of v-*rel* increased the level of miR-155 expression

138 by approximately 700-fold in MSB-1 cells and by about 900-fold in 265L cells which is

139 much higher than the miR-155 level in untransformed CD4+ cells (Fig 3B), demonstrating

140 that ectopic expression of v-rel can induce expression of miR-155 in avian lymphoid cells.

141 **Induction of miR-155 is accompanied by downregulation potential targets.** For further

142 analysis of the dynamic global changes in miRNA profiles during v-*rel*-induced

143 transformation, we examined the changes in RCAS(A)-v-*rel*-infected chicken embryonic

144   splenocytes undergoing transformation. Induction of v-*rel* in these cells resulted in rapid

145   transformation resulting in the appearance of continuously proliferating cell lines usually in

146   8-10 days. The dynamic changes of miR-155 expression during the transformation process of

147   splenocytes measured by qRT-PCR are shown in Fig 4A. Quite clearly, miR-155 is

148   significantly upregulated during the time-course of v-*rel* transformation, with levels showing

149   increases of 5 fold (day 1), 6 fold (day 4), 50 fold at day 7, 150 fold at day 9 and nearly 1500

150   fold at day 14, as compared with the level at day 0.

151   In order to assess the simultaneous changes in gene expression during transformation, we

152   carried out the transcriptome analysis using the chicken Affymetrix platform on the RNA

153   samples extracted from these cells.   To focus on miRNA-induced repression of gene

154   expression, we used the Bioconductor package Limma (35) to extract 1242 genes that showed

155   significant downregulation at day 14 compared to day 0.   Table 1 shows the top 20

156   statistically enriched predicted miRNA targets in this list. Of the 1242 downregulated genes,

157   73 are predicted targets of gga-miR-155 (Fig 4B) making it the top hit of the most enriched

158   miRNA targets. Analysis also showed that the enrichment of the targets of other miRNAs

159   such as gga-miR-9*, gga-miR-217, gga-miR-19a and gga-miR-23b were also significant.

160   These data highlighted the importance of miR-155 and other miRNAs in *v-rel* induced

161   transformation. MiR-155 is a well-studied oncogene of hematopoietic cells. Considering the

162   complexity of targets analysis in v-*rel* induced transformation system as lots of miRNAs and

163   mRNAs are affected by v-*rel*, we overexpressed miR-155 in chicken macrophages derived

164   from line 0 chicken by transfection of miR-155 mimics into bone-marrow derived

165   macrophages. 'Allstars' negative control (Qiagen) was used as control in an attempt to get a

166   cleaner result on miR-155 targets. The RNA extracted from transfected cells were analysed

167   by deep sequencing. The significant down regulated genes with miR-155 target sites in

168   3'UTR were subject to the pathway analysis using Ingenuity Pathway Analysis tool. As

169   shown in Fig 5, several potential miR-155 targets are involved in a number of diseases and

170   cellular processes. The number of cancer-related genes targeted by miR-155 ranks the second

171   implicating the importance of miR-155 as a regulator in disease pathogenesis, particularly in

172   tumorigenesis.

173

174   **Discussion**

175   The Rev-T avian retrovirus encodes the v-*rel* oncoprotein, which is a member of the Rel/NF-

176   κB transcription factor family. Although Rel/NF-κB transcription factors have been

177   associated with oncogenesis in mammals, v-*rel* is the only member of this family that is

178   oncogenic in animal systems. Due to its pervasive role in oncogenesis, there is great interest

179   in NF-κB signalling, and v-*rel* provides a valuable model for studying NF-κB signalling in

180   lymphoid cell cancers because of its ability to transform chicken lymphoid cells (12, 15). In

181   this study, we demonstrate that v-*rel* can readily induce transformation of lymphocyte

182   populations, and the establishment of CD4+ T-cell (AVOL-1) and B-cell (AVOL-2) lineages

183   suggested that v-*rel*-induced transformation function is not restricted to specific lineages.

184   In addition to the changes in protein-coding genes, many changes in the miRNA profiles also

185   occur in v-*rel* transformed cells, and one of the miRNAs expressed at significantly higher

186   levels in v-*rel*-derived tumor cell lines such as KBMC and CM758 is gga-miR-155 (33).

187   Higher expression of miR-155 is reported in a number of haematopoietic malignancies (36-

188   40). The precursor of miR-155, termed c-*Bic*, was first observed to co-operate with *myc* in

189   chicken B-cell lymphomas induced by avian leukosis proviral integrations (26, 41). Southern

190   blot hybridization of genomic DNA from AVOL-1 and AVOL-2 cells showed no evidence of

191   genomic rearrangements in *Bic* loci (data not shown) discounting insertional activation of

192   miR-155 in these cell lines. It is known that miR-155 can also be induced by a variety of

193   immune cell stimuli such as TLR ligands, TNF-α, IFN-β and other antigens (41-45). A

194 conserved AP-1 element in the human *Bic*/miR-155 promoter was shown to be essential for

195 some of these functions (46). Transcriptional regulation of miR-155 by TGF-β/Smad4

196 pathway using the Smad response elements in the human miR-155 promoter has also been

197 reported (47). Epstein–Barr virus (EBV) latent membrane protein-1 (LMP1) is a potent

198 inducer of miR-155 and the NF-κB sites in the *Bic*/miR-155 promoter have been shown to be

199 pivotal for this function (48, 49).

200 Both Northern blotting and microarray data showed that miR-155 is significantly increased in

201 v-*rel*-transformed T and B lymphocytes compared to the normal spleen cells. These

202 observations are similar to the findings reported previously (33). Despite the consistent

203 demonstration of transformation of B and T-lymphocytes by v-*rel*, the precise mechanisms

204 have not been demonstrated. As an NF-κB homolog (8), the most likely mechanism of miR-

205 155 upregulation would be through the direct activation of the miR-155 promoter through the

206 NF-κB binding sites. EMSA showed that v-*rel* binds directly to both NF-κB binding sites. To

207 assess the ability of v-*rel* to activate transcription from miR-155 promoter, we performed

208 reporter assays using the miR-155 promoter and its derivative lacking each of the NF-κB

209 binding sites. Our results demonstrated that indeed v-*rel* controls miR-155 through one of the

210 NF-κB binding sites in the *Bic*/miR-155 promoter.

211 A number of previous studies have demonstrated robust expression of *Bic* in EBV-infected

212 cells (50, 51). It has been shown later that EBV-encoded latent membrane protein-1 (LMP-1),

213 a functional homologue of the tumor necrosis factor receptor family, upregulates the

214 expression of miR-155 mainly by activating the NF-κB pathway (48). The data here is the

215 first evidence showing miR-155 being regulated by an NF-κB transcription factor, the v-*rel*

216 oncogene encoded by Rev-T in avian systems. It has been shown previously that v-*rel* exerts

217 downstream effects through the transcription factor AP-1 (12, 46). AP-1 sites are present in

218   chicken *Bic*/miR-155 promoter sequences and the contribution of AP-1 in regulation of miR-

219   155 expression in v-*rel*-transformed lymphocytes remains to be determined.

220   Interestingly, while miR-155 was upregulated in Rev-T transformed cell lines, it was

221   consistently downregulated in MDV-transformed lymphocytes (52). Although miR-155

222   functions are probably rescued by the high level expression of the MDV1-miR-M4 homolog

223   in these cells (53), the precise molecular mechanisms of downregulation of miR-155 in

224   MDV-transformed cells are not clear. RCAS-mediated transduction of v-*rel* did rescue the

225   expression of miR-155 in two of the MDV transformed cell line MSB-1 and 265L. The

226   increased level of miR-155 expression after introduction of v-*rel* into these cells indicated

227   that the upregulation of miR-155 is a direct effect. It is interesting to know that common

228   occurrence of MDV with REV in chickens could lead a part or entire genome of REV

229   integrating into MDV genome (54, 55). Although a number of field MDV isolates with REV

230   insertion have been characterized, the precise molecular mechanisms for the altered

231   pathogenic properties and the increased virulence are still not clear (55, 56).

232   A number of targets of miR-155 have been identified previously. C-Maf (43), AID (57, 58),

233   Pu.1 (59), SOCS1 (60), interleukin-1 (61) and IKKε (49, 62) have been implicated in

234   mediating functions of miR-155 in the immune system. Ets-1 and Meis1 mediate

235   megakaryopoiesis (63). SHIP1 and C/EBP have been implicated in myeloproliferative

236   disorders (64, 65), Peli1 controls the generation and function of T follicular helper cells

237   through promoting the degradation of the NF-κB family transcription factor c-Rel (66), tumor

238   protein p53 inducible nuclear protein 1 (Tp53INP1) is involved in pancreatic cancer (67) and

239   SOCS1 in promoting γ-chain cytokine signalling to ensure effector and memory CD8+ T cell

240   differentiation (68). Additionally, miR-155 targets JARID2, a cell cycle regulator and part of

241   a histone methyltransferase complex, to promote cell survival (33). From microarray data on

242   RNA of v-*rel* transformed cells, 73 out of 1242 significantly downregulated genes are

243   potential targets of miR-155. Not only was miR-155 the most statistically enriched target

244   within the list of significantly down-regulated genes, but members of the miR-17-92 cluster

245   are also implicated, a cluster which is known to be involved in cancer (69-72), this further

246   emphasized the role of oncogenic miRNAs in transformation.

247   The oncogenic effects of miR-155 are mediated through its target mRNAs. The known miR-

248   155 targets Pu.1, CEBPβ are present in the down regulated genes from microarray analysis in

249   *v-rel* transformed cells. Together with the evidence that the potential miR-155 targets in

250   macrophages involved in cancer are standing out of other diseases and functions related

251   targets, demonstrating the important role of miR-155 in *v-rel* induced transformation.

252   Although the precise roles and molecular pathways of miR-155 in *v-rel* induced

253   transformation are not fully known, its repressive function on transcriptional factors such as

254   Pu.1 and CEBPβ can have wide-ranging effects on the cellular milieu and the global gene

255   expression profiles seen for lymphocytes. Further studies will be required to ascertain the

256   involvement of Pu.1, CEBPβ and/or other miR-155 regulated transcription factors in the

257   regulation of miR-155-inhibited genes. Similarly, the repression of some of the other target

258   genes is also likely to contribute to the induction of hematopoietic cell malignancy. Although

259   upregulation of miR-155 appears to add complexity to regulation of gene expression in v-*rel*–

260   induced malignant transformation, the downstream network of miR-155 targets or the

261   importance of those target genes in v-*rel* induced transformation could be an interesting area

262   to explore.

263

264   **Materials and methods**

265   **Transformed cell lines**

266

267   Rev-T-transformed cell lines AVOL-1 (CD4^{+} T-cell line) (32), AVOL-2 (B-cell origin),

268   AVOL-3, RIR-RevT (a transformed cell line derived from outbred Rhode Island Red

269   chickens) and avian leukosis virus (ALV) HPRS F42 strain-transformed B-cell line HP45

270   (73) were used. MDV cell lines MSB-1 (74) and 265L (32) were used to study the effects of

271   induction of v-*rel*. All the cell lines were grown at 38.5 °C in 5% CO_2 in RPMI 1640 medium

272   containing 10% fetal calf serum, 2% chicken serum, 10% tryptose phosphate broth, 0.1% 2-

273   mercaptoethanol and 1% sodium pyruvate. CEF-derived cell line DF-1 was grown using

274   methods described (75).

275   **Chicken splenocytes, CD4 [+] T cells and magnetic cell sorting.**

276   Single-cell suspensions of lymphocytes were prepared from spleen tissues of uninfected birds

277   by using Histopaque-1083 (Sigma-Aldrich) density-gradient centrifugation. CD4 [+] T cells

278   were isolated by magnetic cell sorting using mouse anti-chicken CD4 antibodies (Chan *et al.*,

279   1988) and goat anti-mouse IgG microbeads (Miltenyi Biotec). After each antibody treatment,

280   cells were washed three times with PBS containing 0.5% bovine serum albumin. At each

281   wash, the cell suspension was centrifuged at 450 *g* for 10 min. Positively stained cells were

282   sorted through an AutoMACS Pro Separator (Miltenyi Biotec). Purity of the sorted cells was

283   confirmed to be >99% by flow cytometry after labelling with monoclonal anti-goat/sheep

284   IgG–fluorescein isothiocyanate (Sigma) antibody (data not shown).

285   **Plasmid constructs**

286   The construct pcDNA3.1-v-*rel* was used for reporter assay. For electrophoresis mobility shift

287   assay, recombinant v-*rel* fused in-frame with GST in pGEX2T (GE Healthcare) vector was

288   used. RCAS(A) retroviral vector (Replication Competent ALV LTR with a Splice acceptor)

289   (76) with v-*rel* cloned into the *Cla*I site was used for *in vitro* transformation of embryonic

290   splenocytes. The orientation of the insert was verified by restriction enzyme digestion and

291   sequencing. RCAS (A)-EGFP-v-*rel* construct with the N-terminal enhanced green fluorescent

292   protein (EGFP) tag was used for the expression of v-*rel* in MSB-1 and 265L cells.

Cloning and mutagenesis of *Bic*/miR-155 promoter

294   The chicken *Bic*/miR-155 promoter region extending from -1829 to +3 nucleotides from

295   transcription start site (+1) was amplified by PCR from the genomic DNA prepared from

296   CEF. The isolated fragments were digested with *Bgl*II and *Nhe*I and cloned into *Bgl*II and

297   *Nhe*I cut psiCHECK™-2 vector (Promega) to replace the SV40 promoter driving the *renilla*

298   luciferase gene to generate the pBic-WT reporter construct. Mutagenesis of the two NF-κB

299   sites on the pBic promoter was carried out by overlapping PCR using primers 5′-

300   CCACATATTTCCTTGCTGGCTCGAGACATAAATTTTTCTGAG-3′   and   5′-

301   CTCAGAAAAATTTATGTCTCGAGCCAGCAAGGAAATATGTGG-3′ for NF-κB site 1,

302   5′-GAAAAGGAAAGCAGGCTCGAGACTCAAGACGGTTAG-3′   and   5′-

303   CTAACCGTCTTGAGTCTCGAGCCTGCTTTCCTTTTC-3′ for NF-κB site 2. The mutant

304   constructs were used to replace the corresponding fragment in the pBic-WT vector to

305   generate pBic-M1, pBic-M2 and pBic-M1M2 constructs, where the 1st, 2nd and both NF-κB

306   sites respectively, were replaced. In each case, the *Xho*I restriction site introduced during the

307   replacement of the NF-κB motifs allowed the screening of the constructs by *Xho*I digestion.

308   The sequences of the promoter region of all the constructs were confirmed by sequence

309   analysis.

Dual Luciferase reporter assay

311   Transfection of DF-1 cells was carried out with Lipofectamine 2000 (Invitrogen) as per

312   manufacturer's protocols. Approximately 3×10^4 DF-1 cells were seeded in each well of a 96-

313   well plate. Each of the reporter and pcDNA3-v-*rel* constructs were co-transfected into DF-1

314   cells and the luciferase expression was assayed 48 hours later using the Dual-Glo Luciferase

315   Assay System (Promega) following manufacturer's instructions. The relative expression of

316   *renilla* luciferase was determined with the normalised levels of *firefly* luciferase. For each

317   sample, values from four replicates representative of at least two independent experiments

318   were used in the analysis.

319   **Electrophoresis mobility shift assay (EMSAs)**

320   Recombinant full length v-*rel* from pGEX2t-v-*rel* plasmid in BL21 (DE3) induced with 0.5

321   mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 3h was purified by Glutathione

322   Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer's instructions. EMSAs

323   were performed using gel shift assay system (Promega) according to the manufacturer's

324   instructions. Double-stranded synthetic oligonucleotides were radiolabeled using [$^{\gamma32}$P] ATP

325   (Amersham) and T4 polynucleotide kinase. For each binding reaction, 3μg of purified protein

326   was incubated with 0.25μg/μl poly[dI-dC] containing 50,000cpm of radiolabelled probes and

327   a 50-fold molar excess of unlabelled competitor oligonucleotide when indicated. DNA-

328   binding reactions were carried out for 30 min at room temperature. Competition experiments

329   were performed by pre-incubation with protein in binding buffer for 10 min, after which

330   labelled probe was added for a further 20-min incubation at room temperature. The DNA-

331   protein complexes were resolved on 6% DNA Retardation Gel (Invitrogen) and detected by

332   autoradiography.

333   **Immunoblotting and Northern blotting**

334   For Western blotting, cells were lysed in protein gel sample buffer (8 M urea, 2% SDS, 10

335   mM Tris/HCl pH 6.8, 0.05% bromophenol blue) and separated on a NuPAGE 4–12% Bis

336   Tris gel (Invitrogen) and transferred onto nitrocellulose membranes using an iBlot gel

337   transfer system (Invitrogen). Western blotting was performed with c-*rel* and v-*rel*-specific

338   HY87 mouse monoclonal antibody (77), followed by anti-mouse IgG–peroxidase conjugate

339   (Sigma-Aldrich). Membranes were developed with an ECL Western blotting analysis system

340   (Amersham). For Northern blot analysis, total RNA was extracted from cultured cells with

341   miRNeasy Mini Kit (Qiagen), and 20 μg total RNA resolved using a 15% polyacrylamide-

342   1×Tris-borate-EDTA-8 M urea gel was blotted to a GeneScreen Plus membrane (Perkin-

343   Elmer). DNA oligonucleotides with sequences complementary to candidate miRNAs, end-

344   labelled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (New England

345   Biolabs), were used as high-specific-activity probes. Hybridization, washing and

346   autoradiography were carried out as previously described (78).

RCAS virus infection

348   Virus stocks generated from DF-1 cells transfected with RCAS(A)-v-*rel* and RCAS(A)-v-*rel*-

349   EGFP constructs approximately 5 days after transfection, when nearly 100% cells were

350   EGFP positive in the case of the latter construct. For *in vitro* transformation assay, one ml

351   (~10^6 TCID$_{50}$) of RCAS(A)-v-*rel* virus was used to infect 5x10^6 of embryonic splenocytes,

352   and harvested at day 0, 1, 4, 7, 9 and 14 days post infection for mRNA microarray analysis

353   and miR-155 quantitation. EGFP-expressing RCAS(A)-v-*rel*-EGFP-infected MSB-1 and

354   265L cells were also sorted and examined for v-*rel* and miR-155 expression.

Stem-loop qRT-PCR for miR-155

356   The expression levels of miR-155 were analysed using the TaqMan MicroRNA Assay

357   System (Applied Biosystems) using 10 ng of total RNA as a template for reverse

358   transcription. Each reverse transcription reaction was performed twice independently, and

359   each reaction was tested by PCR in triplicates. All values were normalized to the expression

360   of the endogenous let-7a, and levels calculated as fold-expression change relative to those

361   from uninfected 265L cells.

Microarray Analysis

363   Triplicate RNA samples for each of the six time-points (0, 1, 3, 4, 7 and 14 dpi) were

364   analysed using the Affymetrix GeneChip Chicken Genome Array. Expression values were

365   calculated using the Robust Multi-Array Average (RMA) function within the Affy

366 bioconductor package (79). Affymetrix probes were linked to Ensembl genes using Ensembl

367 (v70) and genes linked to microRNA predicted targets data from the MicroCosm targets

368 database (80)

369 For the naïve prediction of miRNAs involved in the activation of genes from the mRNA

370 expression data, the following analysis was performed: down-regulated probes at 14 DPI

371 compared to 0 DPI were determined using Limma (35), with a FDR<=0.01 (81) and log fold

372 change <= -1 (two-fold down-regulated). Statistical enrichment of miRNA targets within the

373 down-regulated gene list was calculated using the CORNA package (82). Fisher's exact test

374 was used to calculate p-values for statistical enrichment, and adjusted for multiple testing

375 (81). Heatmaps were drawn in R using the Pearson correlation coefficient as a similarity

376 measure (83).

377 In order to analyse the behaviour of predicted gga-miR-155 targets, expression data from

378 Affymetrix probes representing genes predicted to be targets of gga-miR-155 were extracted

379 and analysed as a set.

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387 **Conflict of interest**

388 The authors declare no conflict of interest.

389

390   **Ethics statement**

391   No animals were used for the work presented in this manuscript.

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612

613

614 **Table 1** Top 20 enriched miRNA targets in the list of 1242 downregulated genes

microRNA	Numbers of miRNA target genes in the population			FDR[4]
	Predicted[1]	Expected[2]	Observed[3]	
gga-mir-155	581	45	73	0.002**
gga-mir-9*	504	39	65	0.002**
gga-mir-217	603	46	69	0.033**
gga-mir-19a	648	50	72	0.045**
gga-mir-23b	633	49	70	0.045**
gga-mir-106	685	53	74	0.055
gga-mir-137	570	44	63	0.065
gga-mir-20a	727	56	77	0.065
gga-mir-124b	557	43	61	0.065
gga-mir-190	549	42	60	0.069
gga-mir-19b	629	48	67	0.069
gga-let-7k	623	48	66	0.077
gga-mir-466	806	62	82	0.080
gga-mir-17-5p	732	56	75	0.095
gga-mir-302b	652	50	67	0.114
gga-mir-135a	646	50	66	0.115
gga-mir-29b	692	53	70	0.115
gga-mir-124a	577	44	60	0.115
gga-mir-153	621	48	64	0.115
gga-mir-146b*	490	38	24	0.122

615 [1]Predicted: The total number of genes predicted to be targets of the microRNA in the

616 population; [2]Expected: The number we would expect to see in our sample by random chance

617 based on our sample size; [3]Observed: The number we actually observed; [4]FDR: The

618 Benjamini and Hochberg adjusted p-value from a two-tailed Fisher's exact test. **indicates

619 FDR <= 0.05

620

Figure legends

622 **Figure 1. Northern blotting analysis for determining miR-155 expression.** Twenty

623 micrograms of total RNA extracted from the indicated cells was separated on a 15%

624 denaturing polyacrylamide gel, blotted and hybridized with end-labelled antisense

625 oligonucleotide probes to gga-miR-155. Size markers to indicate the positions of the pre-

626 miRNA and the mature miRNA are shown. The cellular U6 small nuclear RNA served as the

627 loading control.
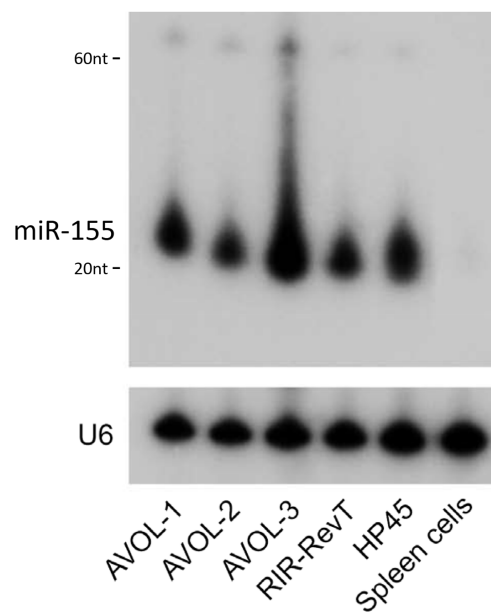
628

629 **Figure 2. Activation of miR-155 by v-*rel* occurs through the NF-κB pathway**. (**A**)

630 Electrophoresis mobility shift assay using purified v-*rel* on the two putative NF-κB binding

631 sites NF-κB1 (−581) and NF-κB2 (−66) on the chicken *Bic*/miR-155 promoter. WT = 50-fold

632 molar cold wild-type competitor, mu = 50-fold molar cold mutant competitor. (**B**) Schematic

633 diagram of luciferase reporter constructs carrying the wild type (WT) and mutant (M1, M2

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634   and M1M2) chicken *Bic*/miR-155 promoter. **(C)** Relative levels of luciferase in DF-1 cells

635   co-transfected with pcDNA3-v-*rel* and the reporter constructs. Error bars represent the data

636   from 4 replicates.

637
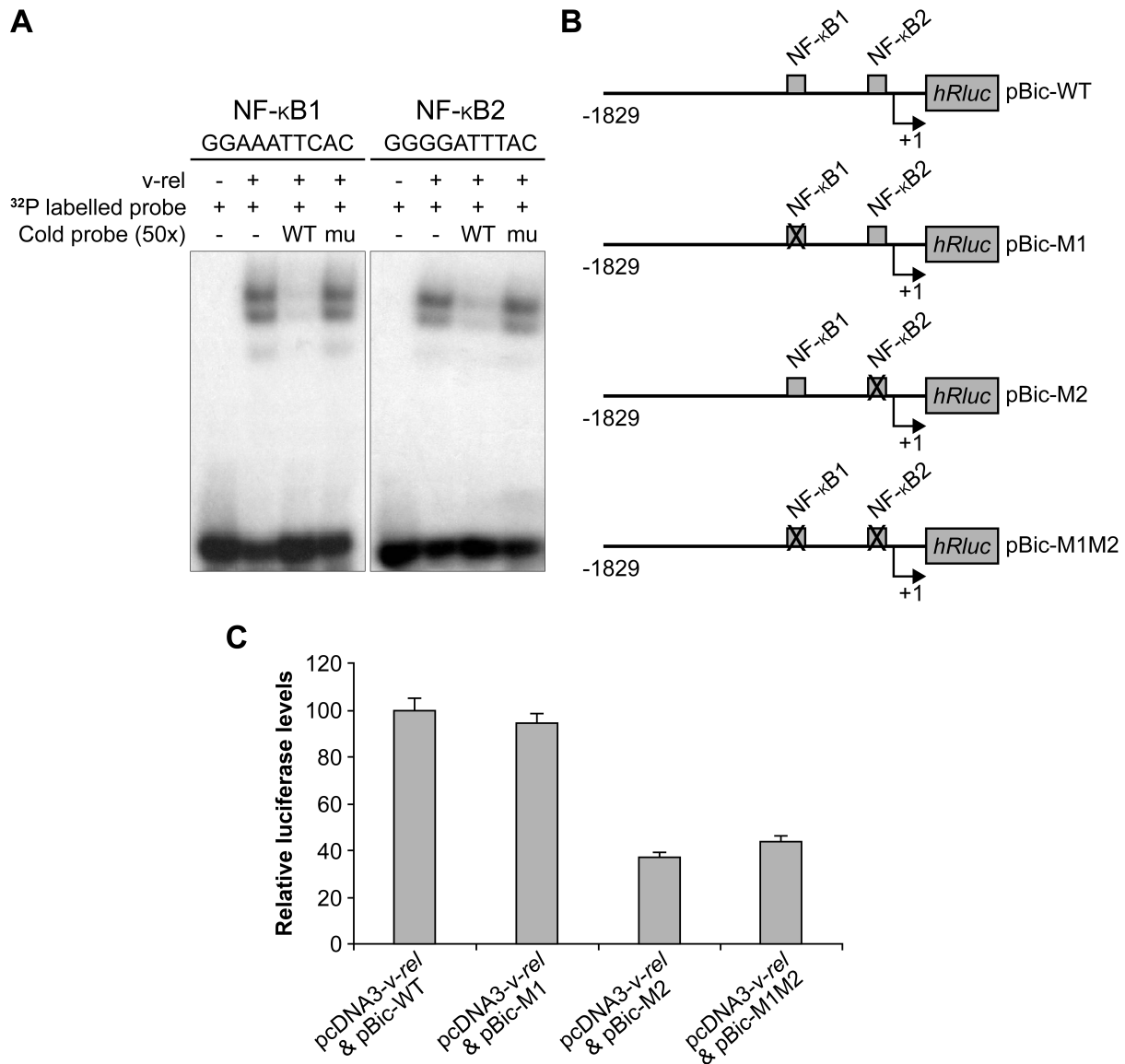
Figure 3. Upregulation of miR-155 in MDV-transformed cell lines by v-*rel*. (A) Cell

lysates from MSB-1 and 265L infected with RCAS(A)-v-*rel*-GFP were analysed by Western

blot using HY87 antibody for v-*rel* expression. Uninfected MSB-1 and 265L were included

as negative control and AVOL-1 cells were included as positive control. **(B)** Expression

levels of miR-155 in RCAS(A)-v-*rel*-GFP infected and uninfected MSB-1 and 265L.

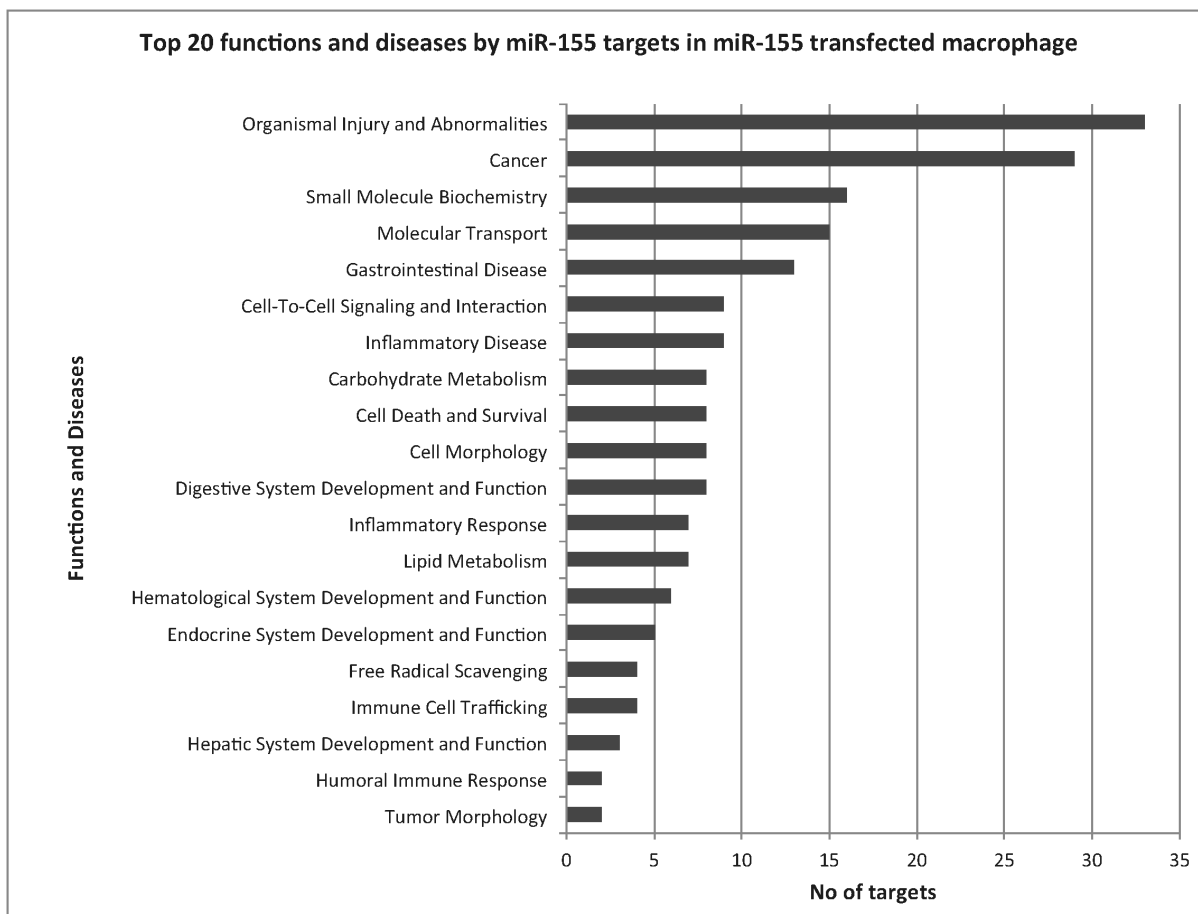RCAS(A)-GFP infected cells were also included as a control.
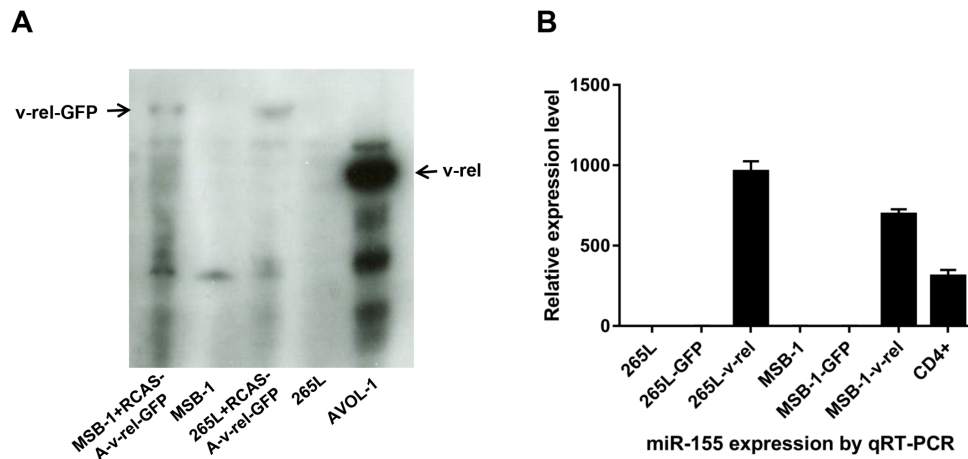
Figure 4. Upregulation of miR-155 during v-rel transformation is associated with downregulation of targets. (A) Expression levels of miR-155 in RCAS(A)-v-*rel* transformed embryonic splenocytes on RNA samples harvested on day 0, 1, 4, 7, 9 and 14 days post infection. **(B)** Heatmap of 73 down-regulated genes predicted to be targets of gga-miR-155. Affymetrix probes were analysed using Limma, comparing d14 to d0 and those with an FDR<=0.01 and fold-change <= -1 (two-fold) selected. The list was further filtered for those genes predicted to be targeted by gga-miR-155. Heatmap was drawn in R using the Pearson correlation coefficient as a distance measure.
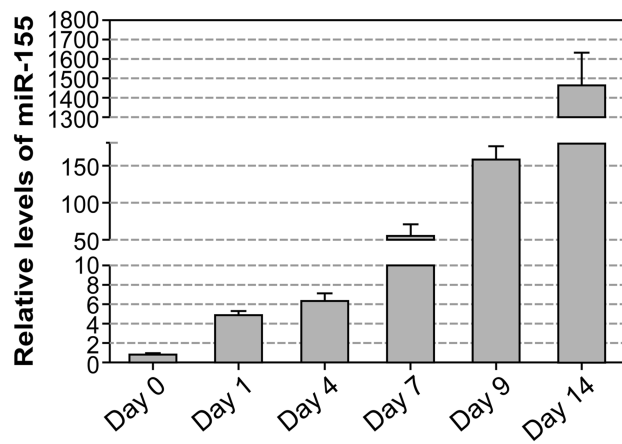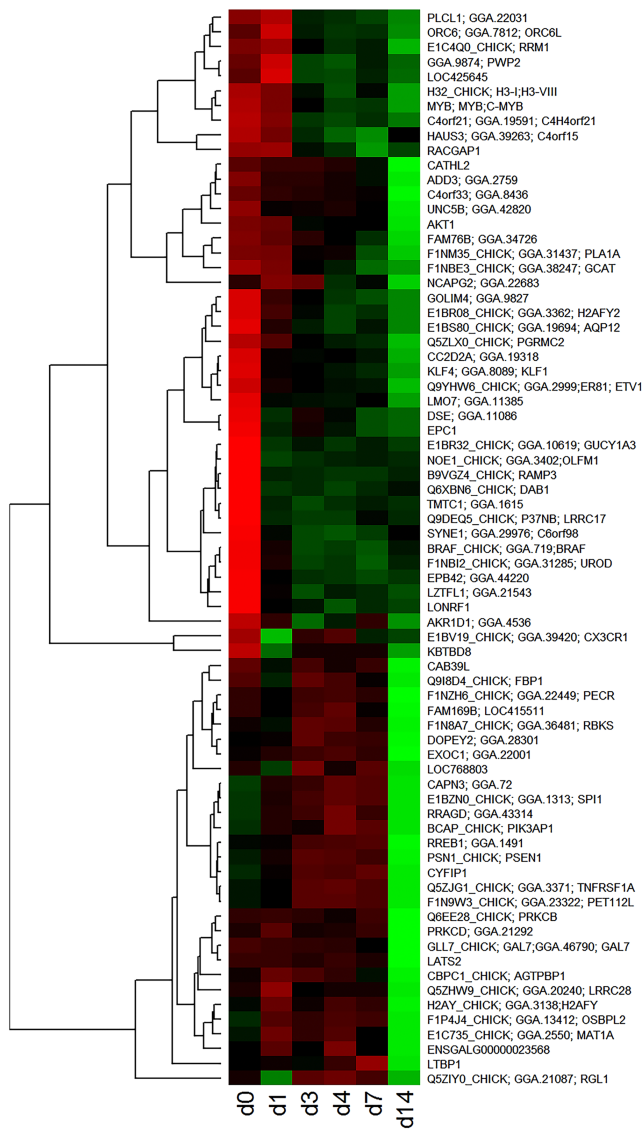
654 **Figure 5. The potential miR-155 targets are involved in a number of dieases and**

655 **functions.** Top 20 functions (sorted by p-value) of the miR-155 targets identified in primary

656 avian macrophages transfected with miR-155 mimics. The grey bars indicate the number of

657 potential target genes for each disease or function.

658