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A viral microRNA functions as an ortholog of cellular miR-155

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Abstract

All metazoan eukaryotes express microRNAs (miRNAs), ~22 nt regulatory RNAs that can repress the expression of mRNAs bearing complementary sequences[1]. Several DNA viruses also express miRNAs in infected cells, suggesting a role in viral replication and pathogenesis[2]. While specific viral miRNAs have been shown to autoregulate viral mRNAs[3,4] or downregulate cellular mRNAs[5,6], the function of the majority of viral miRNAs remains unknown. Here, we report that the miR-K12−11 miRNA encoded by Kaposi's Sarcoma Associated Herpesvirus (KSHV) shows significant homology to cellular miR-155, including the entire miRNA "seed" region[7]. Using a range of assays, we demonstrate that expression of physiological levels of miR-K12−11 or miR-155 results in the downregulation of an extensive set of common mRNA targets, including genes with known roles in cell growth regulation. Our findings indicate that viral miR-K12−11 functions as an ortholog of cellular miR-155 and has likely evolved to exploit a pre-existing gene regulatory pathway in B-cells. Moreover, the known etiological role of miR-155 in B-cell transformation[8-10] suggests that miR-K12−11 may contribute to the induction of KSHV-positive B-cell tumors in infected patients.

Inspection of mature KSHV miR-K12−11 and cellular miR-155 reveals significant homology, including the entire seed region often critical for mRNA target recognition[7], i.e., nucleotides 2 to 8 (Fig. 1a). miR-155, the product of the *bic* gene[10], is over-expressed in several types of B-cell lymphoma and its transgenic expression in mice causes B-cell malignancies[9]. miR-155 expression is induced in activated B–cells, T-cells, and macrophages[11-13], and miR-155 knock-out mice display impaired immune functions[14,15]. Given the emerging importance of miR-155 in cancer and B-cell function, we asked whether miR-K12−11 functions as an ortholog of miR-155.

We first prepared transductants of the KSHV-negative human B-cell line BJAB expressing physiological levels of miR-K12−11. A miR-K12−11 expression cassette was placed 3' to the

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Author Information The microarray results described in this manuscript have been deposited in the GEO database (GSE 8867).

AcGFP ORF present in the lentiviral vector **pNL-SIN-CMV-AcGFP** (Fig. 1b). Cells transduced with this vector express transcripts that function as AcGFP mRNAs and as pri-miRNAs[16]. Transduced BJAB cells were sorted to generate pools expressing only AcGFP or expressing AcGFP and miR-K12−11. Expression of miR-K12−11 was confirmed by primer extension (Fig. 1c, lanes 1−8; Suppl. Fig. 2a,b) and by knockdown of an indicator bearing perfectly complementary sites[17] (Suppl. Fig. 2c-e). Importantly, the level of expression and activity of miR-K12−11 in transduced BJAB cells was comparable to that observed in the B-cell line BC-1 (Fig. 1c, lane 15, Suppl. Fig. 2), which is latently infected with KSHV[18].

Cytoplasmic RNA was isolated from BJAB cells and analyzed on microarrays in three independent experiments. Expression of miR-K12−11 resulted in the downregulation of 181 mRNAs and the upregulation of 86 mRNAs ($|\text{T-score}| \geq 2.86$, $p \leq 0.01$). mRNAs bearing at least one seed match to miR-K12−11 were significantly enriched in the topscoring 150 mRNAs downregulated in miR-K12−11 expressing cells, when compared to randomly sampled 3'UTRs from all mRNAs in our dataset (Fig. 1d). Due to stringent criteria for 3'UTR selection, this dataset likely underestimates the number of seed matches to miR-K12−11 in cellular 3'UTRs. We therefore manually checked mRNAs that were significantly downregulated for additional seed matches using the Entrez database. Downregulated mRNAs carrying a >6 nt seed match to miR-K12−11 were considered candidate direct targets and are listed in Suppl. Table 1. Using gene set enrichment analysis[19], we observed that computer-predicted targets for cellular miR-155 were significantly enriched in mRNAs downregulated by miR-K12−11 (false discovery rate ≤ 0.003; Suppl. Table 2)[20]. Interestingly, the downregulation of many of these mRNAs by miR-K12−11 was not statistically significant, based on our T-score cut-off, suggesting low or inconsistent levels of downregulation by miR-K12−11. However, inhibition of gene expression by miRNAs can occur entirely at the translational level, with little or no reduction in mRNA abundance[21,22].

We next evaluated whether candidate mRNA targets for miR-K12−11 were also downregulated by miR-155 using quantitative RT-PCR analysis of RNA samples prepared from duplicate sets of BJAB transductants (Fig. 1c). As a specificity control, we also analyzed a miRNA, miR-K12−11/2M, which bears two mutations in the miR-K12−11 seed region (Fig. 1e). Since the pri-miRNA stem was adjusted accordingly (Suppl. Fig. 1), the expression level of this mutant is comparable to wild-type miR-K12−11 (Fig. 1c, lanes 11 and 12). miR-155 was expressed from a 300 bp fragment of *bic* exon 2[10] and its expression level in BJAB (Fig. 1c, lanes 9 and 10) matched that observed in the transformed B-cell line Jijoye (Fig. 1c, lane 16). BJAB cells normally express undetectable levels of endogenous miR-155 (Fig. 1c). As a further control, we also included BJAB transductants expressing an artificial miRNA targeting luciferase (miR-30/GL2).

Since the set of potential mRNA targets of miR-K12−11 was diverse and did not reveal an obvious function, candidate mRNAs were picked based on T-score, known functions, and/or the number and quality of matches to miR-K12−11. We also included several predicted mRNA targets for miR-155 which did not appear to be significantly downregulated by miR-K12−11. Suppl. Table 3 gives the known functions of these candidate targets, as well as their full names and the extent of seed pairing to miR-K12−11 and miR-155. As shown in Fig. 2 and Suppl. Fig. 3, qRT-PCR analysis revealed that almost all the transcripts analyzed were indeed downregulated by miR-K12−11, but not by miR-K12−11/2M, thereby proving that downregulation depends on an intact seed sequence. Importantly, expression of miR-155 downregulated these mRNAs to the same degree as did miR-K12−11, thus strongly suggesting that miR-K12−11 indeed phenocopies miR-155 (Fig. 2).

We next asked whether the downregulation of target genes by miR-K12−11 and miR-155 was direct by cloning 12 candidate 3'UTR sequences 3' to the firefly luciferase ORF (Fluc). The

resulting constructs and the unmodified vector were co-transfected into 293T cells together with a Renilla luciferase internal control and **pNL-SIN-CMV-AcGFP** constructs expressing no miRNA, miR-K12−11, miR-K12−11/2M or miR-155. Strikingly, in all 12 cases, Fluc expression from constructs bearing candidate 3'UTR sequences was downregulated equivalently by miR-K12−11 and miR-155 (Fig. 3), while miR-K12−11/2M had no effect. Consistent with the idea that target gene repression also occurs by inhibition of translation, there was no good correlation between the extent of downregulation observed by qRT-PCR (Fig. 2) and the indicator assay (Fig. 3). For example, one predicted target for miR-155, BACH1, was only modestly downregulated at the mRNA level, but repression of the indicator bearing the BACH1 3'UTR was ~85% for both miR-155 and miR-K12−11.

To show that equivalent downregulation by miR-K12−11 and miR-155 also occurs with endogenous proteins, we performed Western Blot analyses for two targets, BACH1 and FOS. Whole cell extracts of duplicate BJAB transductants (Fig. 1c) were probed using antibodies specific for BACH1. Since miR-155 is upregulated in stimulated macrophages[13], we also generated tranductants of the monocytic cell line THP-1 expressing each miRNA and confirmed expression using primer extension (Suppl. Fig. 4). BACH1 protein expression was ~5-fold lower in the BJAB transductants expressing either miR-K12−11 or miR-155, when compared to control transductants (Fig. 4a), while expression in THP-1 cells was inhibited by ~3-fold (Fig. 4b). FOS expression was analyzed after serum starvation of BJAB transductants followed by TPA induction. Again, levels of FOS protein expression after induction were consistently ~2.5-fold lower in transductants expressing either miR-K12−11 or miR-155 (Fig. 4c).

If miRNAs play an important role in viral replication in vivo, then agents that specifically block their function might represent novel antiviral agents. We treated latently KSHV-infected BCBL-1 cells with an antagomir[23] antisense to miR-K12−11 and asked if this would increase FOS and/or BACH1 protein expression. As shown in Fig. 4d, this antagomir specifically enhanced FOS expression by ~2.6-fold while selectively inhibiting miR-K12−11 expression and function (Suppl. Fig. 5). In contrast, BACH1 expression was only modestly enhanced (data not shown). Further analysis revealed that the BACH1 3'UTR confers downregulation by not only miR-K12−11 but also miR-K12−1 and miR-K12−6 (Suppl. Fig. 6). Therefore, while antagomirs can inhibit viral miRNA function, their effectiveness may be compromised by the overlapping activities of viral miRNAs.

Previous analyses have shown that certain viral miRNAs downregulate the expression of mRNA targets transcribed from the opposite strand of the viral DNA genome[3,4] or inhibit the expression of cellular mRNAs by binding to novel 3' UTR targets that are not used by cellular miRNAs[5,6]. Here, we provide evidence for a third paradigm, i.e., viral miRNAs can function as orthologs of cellular miRNAs and thereby downregulate the expression of numerous cellular mRNAs via target sites that are generally evolutionary conserved.

The evidence presented here strongly argues that miR-K12−11 functions as an ortholog of cellular miR-155 (Figs. 2-4). This similarity undoubtedly reflects the identical seed region present in miR-K12−11 and miR-155 (Fig. 1a). Nevertheless, the fact that the non-seed regions of miR-155 and miR-K12−11 are different does raise the possibility that they might not share all mRNA targets[24]. Indeed, although almost all mRNAs analyzed responded identically to miR-155 and miR-K12−11 (e.g., Figs. 2 and 3), we did occasionally notice slight differences in the degree of silencing (e.g., SLA mRNA, Suppl. Fig. 4). Nevertheless, our data in total do demonstrate that miR-155 and miR-K12−11 regulate an analogous set of mRNAs and agree with earlier reports documenting the critical role of seed pairing in mRNA target selection[7].

miR-155/bic overexpression is observed in many human B-cell lymphomas[10] and induces B-cell malignancies in mice and chickens[8,9]. miR-155 is induced upon T- or B-cell activation[11,12], and miR-155 knock-out mice display impaired immune function[14,15]. The set of mRNA targets identified in this report includes targets with known roles in B cell function (e.g. SLA) and innate immunity (PIK3CA, IKBKE, FOS), pro-apoptotic (BIRC4BP/XAF1) and cell cycle regulatory (FOS) functions, as well as transcription factors (BACH1, FOS, HIVEP2). At this point, it is unclear which of the many genes regulated by miR-155, and by miR-K12−11, provides a replicative advantage to KSHV. Given the apparent role of miR-155 in the development of B cell tumors, miR-K12−11 expression in latently KSHV infected B-cells may contribute to the increased incidence of B-cell tumors seen in KSHV-infected patients[25]. Of note, while the distantly related γ-herpesvirus Epstein-Barr Virus (EBV) also expresses viral miRNAs[2,3], these show no homology to miR-155. However, a recent report[26] showing that EBV activates endogenous miR-155 expression in infected B-cells suggests that EBV may have evolved an alternative strategy to achieve the same end result.

Analysis of currently known viral miRNAs reveals that the seed homology observed with miR-K12−11 and miR-155 is not unique. Suppl. Fig. 7 lists several viral miRNAs that display seed homology either to cellular miRNAs or to miRNAs encoded by distantly related viruses. While the significance of these homologies remains unknown, these observations do raise the possibility that the similar activity noted for cellular miR-155 and viral miR-K12−11 is only the first example of a more general phenomenon.

Methods

pNL-SIN-CMV-AcGFP-based lentiviral miRNA expression vectors were generated as described[16], except that expression cassettes were placed 3' to the AcGFP ORF. miR-K12−11 and miR-30/GL2 were expressed from artificial miR-30-based expression cassettes[22,27]. miR-155 was expressed using a ~300 bp segment of the *bic* gene[10]. BJAB cells were infected and sorted 48 hours after infection. 12 to 16 days after transduction, gene expression analysis of 10 independent BJAB pools, expressing AcGFP only or AcGPF and miR-K12−11, was performed using Human Operon v3.0.2 arrays. Normalization was performed using arrayMagic[28] and data analysis performed using GenePattern[29]. 3'UTR analysis for miR-K12−11/miR-155 seed matches was performed using an in-house pipeline[30]. GSEA[19] was used to test whether specific gene sets were enriched in the set of down-regulated genes. Candidate 3'UTR sequences were cloned 3' to an SV40 early promoter-driven Fluc ORF. Indicator assays were carried out in 293T cells co-transfected with Fluc vectors carrying candidate 3'UTRs or the parental vector, an RLuc-based internal control vector, and **pNL-SIN-CMV-AcGFP** based miRNA expression vectors. Dual luciferase assays were performed 48 hours after transfection[17]. Antagomirs were synthesized as described[23] and delivered into BCBL-1 cells by incubation in serum free medium for 25 hours.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

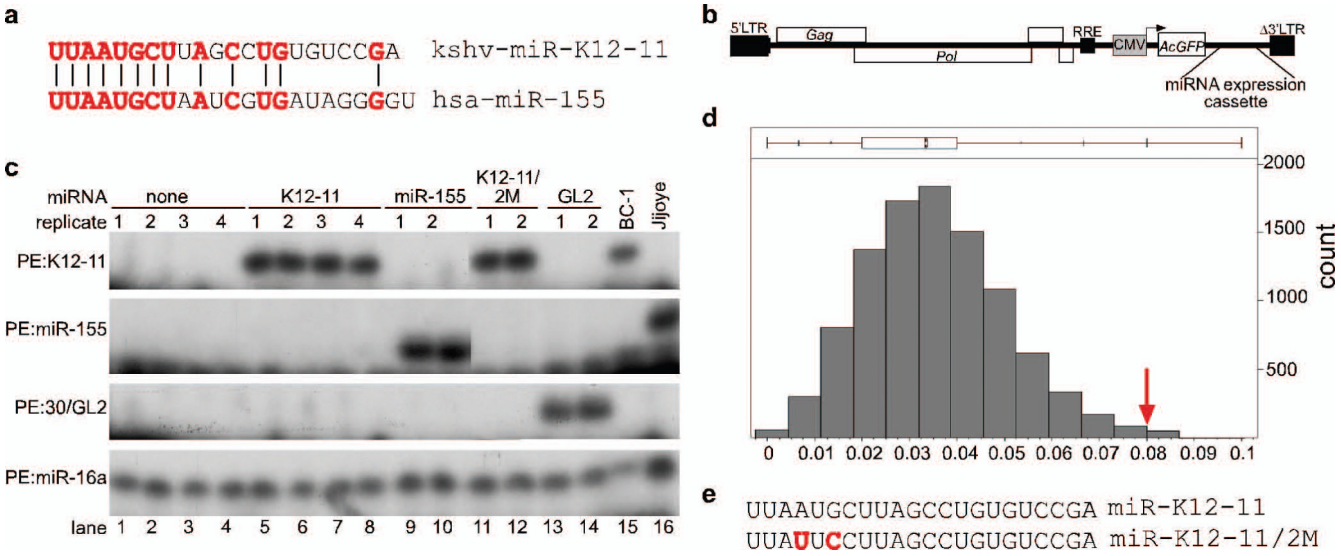

Fig. 1. Expression of miR-K12−11 and miR-155 in BJAB cells

a. Alignment of mature miR-K12−11 and miR-155. **b.** Schematic of the lentiviral miRNA expression vector[16]. **c.** Primer extension (PE) analysis of miRNA expression in BJAB transductants and control cell lines BC-1 and Jijoye for miR-K12−11, miR-155, miR-30/GL2, and cellular miR-16. **d.** Null distribution of miR-K12−11/ miR-155 seed abundance in 10,000 randomly sampled sets of 150 3' UTRs, each from the space of our analysis. The arrow marks the abundance of 7mer seed matches in the 3'UTRs of the top-scoring 150 down-regulated genes in miR-K12−11 transductants, indicating a significant ($p \leq 0.0057$) enrichment of potential targets for miR-K12−11/miR-155. Analysis for 6mer seed matches also identified a significant enrichment ($p \leq 0.001$) (not shown). **e.** Mutations introduced to create miR-K12−11/2M are highlighted.

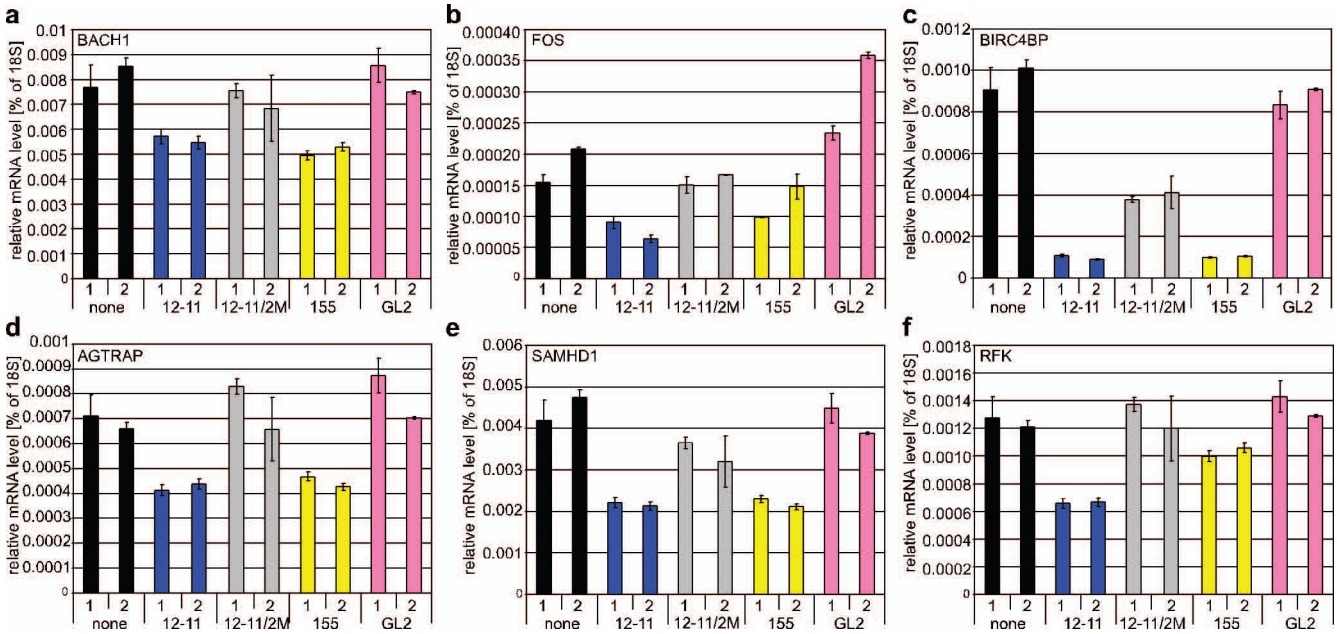

Fig. 2. miR-K12−11 and miR-155 regulate a common set of mRNAs

Real time qRT-PCR analysis of total RNA derived from two independent replicates of BJAB transductants, expressing no miRNA, miR-K12−11, miR-K12−11/2M, miR-155 or miR-30/GL2, for six candidate mRNA targets of miR-K12−11 and miR155. Relative RNA abundance is shown as a percentage of the level of 18S rRNA and error bars are derived from quadruplicate 18S rRNA replicates. mRNAs tested included BACH1 (**a**), FOS (**b**), BIRC4BP (**c**), AGTRAP (**d**), SAMHD1 (**e**), and RFK (**f**). Results for further candidate targets are shown in Suppl. Fig. 3.

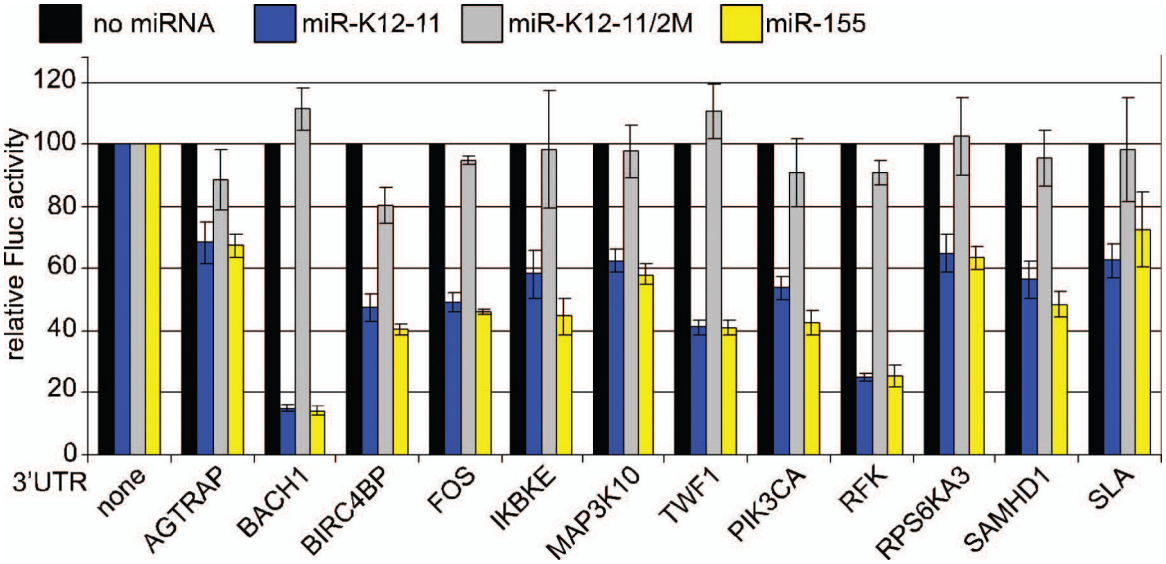

Fig. 3. Direct and equivalent regulation of candidate 3'UTRs by miR-K12−11 and miR-155

Indicator vectors carrying no additional sequences, or candidate 3'UTR sequences inserted 3' to FLuc, were cotransfected with an internal RLuc control vector and either empty **pNL-SIN-CMV-AcGFP** (black bars), **pNL-SIN-CMV-AcGFP** expressing miR-K12−11 (blue), miR-K12−11/2M (grey) or miR-155 (yellow). Dual luciferase assays were carried out 48 hours later. For each **pNL-SIN-CMV-AcGFP** construct, Fluc to Rluc ratios were first normalized to the value obtained for the empty indicator vector and then to the activities obtained with **pNL-SIN-CMV-AcGFP**, which were set at 100%. BACH1, IKBKE, MAP3K10, SLA and RPS6KA3 are predicted targets for miR-155 (Suppl. Table 2).

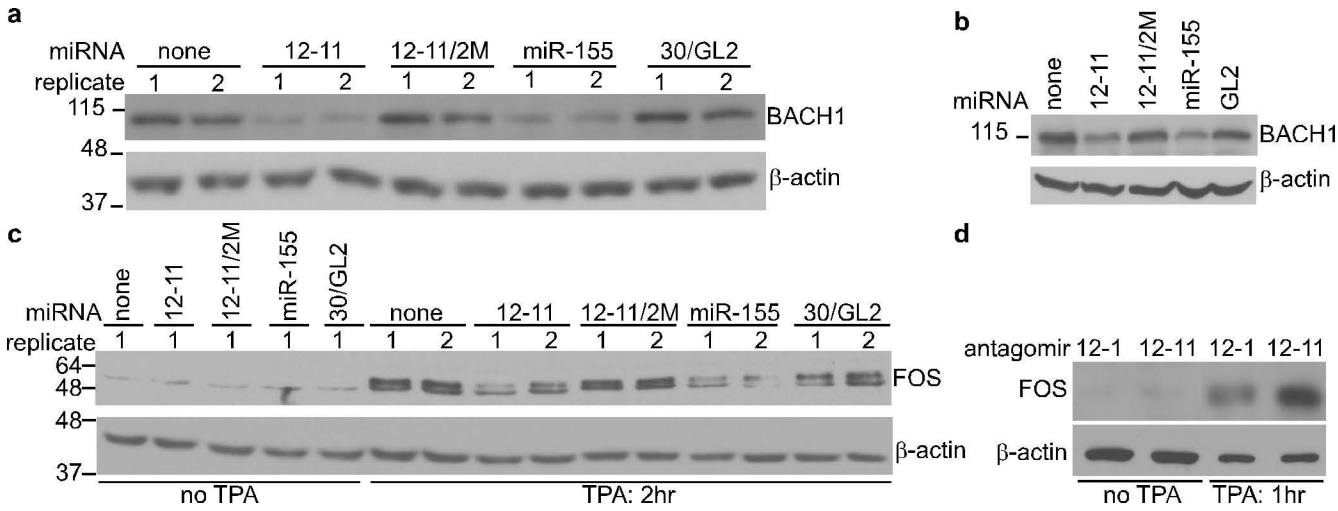

Fig. 4. Endogenous BACH1 and FOS proteins are repressed by both miR-K12−11 and miR-155

a. Western analysis of BACH1 protein expression in independently generated BJAB transductants expressing no miRNA, miR-K12−11, miR-K12−11/2M, miR-155 or miR-30/GL2. **b**. Western analysis of BACH1 protein expression in THP-1 transductants. **c**. Western analysis of FOS protein. Replicate BJAB transductants expressing no miRNA, miR-K12−11, miR-K12−11/2M, miR-155 or miR-30/GL2 were serum starved for 26 hours and then treated with serum free RPMI or TPA for two hours. **d**. FOS expression in KSHV-infected cells is rescued by a miR-K12−11-specific antagomir. BCBL-1 cells were serum starved for 25 hours in the presence of 1 μM antagomir against miR-K12−1 or miR-K12−11 and then incubated in serum-free medium, or serum-free medium ± 20 ng/ml TPA for one hour.