SUPPLEMENTARY MATERIALS AND METHODS

Cell Culturing

A375 and A375-mCherry melanoma cells and HCT116 Dicer-/- cells were cultured as described in [1] [2].

Primers, siRNAs and si-miRNAs

All primers, siRNAs and microRNA mimics (si-miRNAs) were purchased from Eurofins Genomics. Their sequences are reported in **Supplementary Table S4** and **S5**.

Affinity purification of *BRAF*-X1 microRNAs in A375 cells

Biotinylated probes

Affinity purification of *BRAF*-X1 microRNAs was conducted using a significantly modified version of the Chromatin Isolation by RNA Purification (ChIRP) protocol [3]. Using an online software (https://www.biosearchtech.com/chirpdesigner), twelve 20-mer 3' biotinylated DNA tiling probes (Integrated DNA Technologies) were designed to specifically target loci positioned approximately every 100nt along the *BRAF*-X1 3'UTR (1.35kb, ref [1]). Probe sequences can be found in **Supplementary Table S1**. The probes were split into two sets (ODD and EVEN) and were BLASTed against the human genome (https://blast.ncbi.nlm.nih.gov). None of the probes showed noticeable complementarity to off-target genes.

Cross-linking

10^7 A375 cells were washed once in phosphate buffered saline (PBS). They were then resuspended in 1% formaldehyde, 3% formaldehyde or 1% glutaraldehyde in PBS and incubated for 10 min at room temperature on a microplate shaker. The cross-linking reaction was quenched by adding 0.125M glycine for 5 min, after which cells were washed three times in ice-cold PBS and pelleted at 2000g for 5 min at 4°C. All supernatant was aspirated and the pellets were flash-frozen in liquid nitrogen.

Cell lysis and sonication

The cell pellets were rapidly thawed in a 37°C water bath and resuspended in 1ml lysis buffer (50mM Tris (pH 7.0), 10mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulphate (SDS), 1mM phenylmethylsulfonyl fluoride (PMSF), 1X protease inhibitor (Promega) and 80U/ml RNase inhibitor (Bioline)). The lysate was transferred to a 15ml V-bottom tube and sonicated using a Vibra-Cell VCX 130PB ultrasonic processor, while kept in an ice water bath with constant convection. Sonication was performed at 70% maximum amplitude for 12 cycles of 30 sec of sonication followed by 45 sec cooling periods. To remove remaining cell debris, lysates were subsequently centrifuged at 16000g for 10 min at 4°C and supernatants were saved.

Hybridization

To reduce non-specific hybridisation, 1ml cell lysates were incubated with 30µl Dynabeads MyOne Streptavidin C1 beads (Invitrogen), prepared as per manufacturer's instructions, for 30 min at 37°C with end-to-end rotation. The beads were removed by magnetic separation and 2ml Hybridization buffer (750mM NaCl, 1% SDS, 50mM Tris 7.0, 1mM EDTA, 15% formamide, freshly added 1mM PMSF, 1X protease inhibitor (Promega) and 80U/ml Rnase inhibitor (Bioline)) was mixed with the lysate. Aliquots containing 100pmol of ODD or EVEN probes, or an aliquot containing 100pmol of non-targeting scrambled control probe were added to three lysate samples, respectively. These samples were subsequently incubated for 4 hours at 37°C with end-to-end rotation. 100µl Dynabeads MyOne Streptavidin C1 beads were added to each sample and incubated for 30 min at 37°C with end-to-end rotation. The beads were subsequently purified by magnetic capture and washed five times with 1 ml Wash buffer (2X saline-sodium citrate buffer, 0.5% SDS and 1 mM PMSF).

Protein elution and Ago2 dot-blot

The beads were resuspended in 50µl DNase buffer (25mM $MgCl_2$, 5mM $CaCl_2$ in PBS, 200µg/ml RNase A and 20µg/ml DNase I) and incubated first at 37°C for 30 min followed by a 95°C incubation for 5 min. The supernatant was cleared twice using a magnet and 2µl were blotted on a 0.2µm nitrocellulose membrane (Bio-Rad). After a drying period of 10 min, the membrane was incubated with a blocking solution for anti-Ago2 antibodies (5% bovine serum albumin (BSA) in 1X tris-buffered saline with 0.1% Tween 20 at pH 7.0 (TBST)) and a blocking solution for anti-GAPDH antibodies (1X TBST, 1% BSA and 3% milk) for 2 h at room temperature. The membranes were subsequently incubated overnight at 4°C with the primary antibody anti-Ago2 (#2897S Cell Signaling, dilution 1:1000) or anti-GAPDH (#sc-25778, Santa Cruz, dilution 1:2000). After three 10 min washes in 1X TBST, the membrane was incubated 1 h with respective secondary antibodies. Blots were visualised as per manufacturer's protocol with Clarity ECL (Bio-Rad) using ChemiDoc MP Imager (Bio-Rad).

RNA elution and extraction

The beads were resuspended in 185µl Proteinase K buffer (100mM NaCl, 10mM Tris 7.0, 1mM EDTA, 0.5% SDS and 1,5mg/ml proteinase K) and incubated initially at 45°C for 1 h under constant and vigorous agitation and then at 95°C for 10 min to deactivate the proteinase K. To this 1ml Qiazol (Qiagen) was added and incubated for 10 min at room temperature. RNA extraction was performed with the miRNeasy Mini kit (Qiagen), following the manufacturer's instructions.

Small RNA sequencing of captured microRNAs

The RNA eluted from the the ODD, EVEN and scrambled control samples were used for the construction of cDNA libraries using the TruSeq Small RNA kit (Illumina), as per the manufacturer's suggestions. cDNA libraries were loaded at six-plex level of multiplexing into a V3 flow cell, obtaining ~4 million reads per samples. They were then sequenced in a single-reads mode (50 bp) on a MiSeq sequencer (Illumina).

Analysis of small RNA-seq data

Sequencing data was analyzed as described previously [4]. Briefly, quality control checks were performed with the FastQC algorithm. Adapters were trimmed from the primary reads using Cutadapt v1.2.1. Remaining reads, with a length of between 17bp and 35bp, were clustered by unique hits and mapped to pre-miRNA sequences (miRBase release 21) with the miRExpress tool v 2.1.3 [5]. The relative abundance of *BRAF*-X1 3'UTR in each capture was measured by aligning trimmed reads to the *BRAF*-X1 3'UTR sequence using BWA v. 0.6.1-r104 [6].

Data availability

microRNA reads obtained from miR-CATCHv2.0 are accessible through GEO Super Series accession number GSE117642 (GSE117640).

microRNA profile (microRNAome) of A375 cells

The sequencing of small RNAs of A375 was performed as reported in [4]. Four biological replicates were available to us: two are those published in [4] and already deposited in NCBI Gene Expression Omnibus [7] (they are accessible through the accession number GSE94423). The remaining two had been obtained earlier on, following an identical experimental protocol and are accessible through GEO Super Series accession number GSE117642 (GSE117641).

Plasmids

pMS2-X1-3'UTR. The 3'UTR of *BRAF*-X1 in its 1.35kb long form [1] was cloned downstream of the MS2 binding region of a pMS2 plasmid (kind gift from Dr. Pandolfi, BIDMC-HMS, Boston, USA). Specifically, the fragment of DNA coding for the *BRAF*-X1 3'UTR was excised from a pGEM BRAF 3'UTR X1 plasmid [1] using *Not*I and subsequently incubated with Klenow enzyme to blunt its ends. The strand was then ligated with a pMS2 plasmid that was linearized with *Xho*I, blunted with Klenow enzyme and dephosphorylated using calf intestinal alkaline phosphatase.

pMS2-BP. The pMS2-BP plasmid, which expresses the MS2-BP-YFP-HA chimerical protein, was a kind gift from Dr. Pandolfi, BIDMC-HMS, Boston, USA.

pMIR-X1-3'UTR. The 3' UTR of *BRAF*-X1 in its 1.35kb long form [1] was amplified from pGEM BRAF

3'UTR X1 plasmid using Phusion Flash High-Fidelity Master mix (Thermo Fisher Scientific), as well as X1 3'UTR *Spe*IF and X1 3'UTR *Hin*dIIIR primers. The PCR product was subsequently cloned downstream of the Firefly Luciferase coding sequence in the pMIR-REPORT miRNA Expression Reporter Vector (Thermo Fisher Scientific) using *Spe*I and *Hin*dIII.

pMIR-ref-3'UTR. The sequence of the *BRAF*-ref 3'UTR was amplified from A375 cDNA using Phusion Flash High-Fidelity PCR Master mix (Thermo Fisher Scientific), as well as Ref-3'UTR *Spe*IF and Ref-3'UTR *Hin*dIIIR primers. The PCR product was subsequently cloned downstream of the Firefly Luciferase coding sequence in the pMIR-REPORT miRNA Expression Reporter Vector (Thermo Fisher Scientific) using *Spe*I and *Hin*dIII.

pMIR-MEK1-3'UTR. The sequence of *MEK1* 3'UTR was amplified from A375 cDNA using Phusion Flash High-Fidelity PCR Master mix (Thermo Fisher Scientific), as well as *Spe*I_MEK1_UTR_F and *Mlu*I_MEK1_UTR_R primers. The PCR product was subsequently cloned downstream of the Firefly Luciferase coding sequence in the pMIR-REPORT miRNA Expression Reporter Vector (Thermo Fisher Scientific) using *Spe*I and *Mlu*I.

pMIR-3180_sensor and pMIR-3651_sensor. The sense and antisense oligos for each sensor were annealed and then phosphorylated using PNK. The phosphorylated double-stranded oligo was subsequently cloned downstream of the Firefly Luciferase coding sequence in the pMIR-REPORT miRNA Expression Reporter Vector (Thermo Fisher Scientific) using *Spe*I and *Hin*dIII.

pTK. The pTK plasmid, which expresses the Renilla Luciferase and is cotransfected with pMIR-REPORT plasmids as normalization control, was a kind gift from Dr. Pandolfi, BIDMC-HMS, Boston, USA.

pCW-BRAFV600E-X1-CDS. The sequence of BRAFV600E-X1 CDS was amplified from PIG-BRAFV600E-X1 plasmid [2] using Phusion Flash High-Fidelity PCR Master mix (Thermo Fisher Scientific), as well as *Spe*I-BRAFV600E-X1-F and *SalI*-BRAFV600E-X1-R primers. The PCR product was subsequently cloned downstream of the tight TRE promoter in the pCW-Cas9 plasmid (Addgene, #50661), after removing Cas9 CDS [4].

All restriction and modification enzymes used for cloning were purchased from NEB New England BioLabs.

MS2-tagged RNA affinity purification (MS2-TRAP) assay

For the transfection of pMS2 plasmids in A375 cells, $2x10^6$ cells were seeded per 10cm dish. After 24 h, 24µg of plasmids (12µg pMS2-BP + 12µg pMS2 or pMS2-X1-3'UTR) and 45µl 0.02 mM PEI (Sigma-Aldrich) were dissolved in 1ml high glucose Dulbecco's Modified Eagle's medium (DMEM; Euroclone) and incubated at room temperature for 20 min. Meanwhile, cell medium was replaced with 2% foetal bovine serum (FBS) diluted in 5ml of fresh high glucose DMEM. The DNA/PEI/DMEM

mixture was added to the wells and was replaced with fresh complete high glucose DMEM aproximately 6 h later. Cells were left to incubate for a further 24 h, after which they were collected and pelleted.

Cell pellets (10^7) were washed twice with ice-cold PBS and lysed for 5 min on ice in 600µl of complete Polysome Buffer (20mM Tris-HCl (pH 7.5), 100mM KCl, 5mM $MgCl_2$, 0.5% NP-40, cOmplete Protease Inhibitor Cocktail (Roche), RNAse OUT (Invitrogen), and 10mM dithiothreitol (DTT, Sigma)). The lysate was clarified by centrifugation at 15000g for 15 min at 4°C and the supernatant was used for RNA affinity purification. For this, 50µl of HA-Tag (C29F4) Rabbit mAb Sepharose® Bead Conjugate (#3956, Cell Signaling) were reconstituted by washing them 3 times with 1ml of NT2 buffer (50mM Tris-HCl at pH 7.5, 150mM NaCl, 1mM $MgCl_2$ and 0.05% NP-40) and were resuspended in 100µl of the same buffer supplemented with 100U/ml RNase OUT, 1mM DTT and 17.5µM EDTA. The beads were then added to 500µl of supernatant and incubated at room temperature for 4 h on an orbital shaker. After incubation, the beads were washed 5 times with 1ml ice-cold NT2 buffer by pulsing them in a centrifuge (1 min, 5000 rpm) and removing the supernatant with a pipette. At the end of the washes, 700µl Qiazol (Qiagen) was added directly to the beads pellet and RNA was extracted using miRNeasy mini kit (Qiagen), following the manufacturer's instructions.

Transfection of siRNAs and si-miRNAs

A375 cells ($1.5x10^5$) were seeded in 12-well plates and incubated overnight. The following day, 2.4µl of 20uM siRNA (si-miRNA) stock solution were added to 100µl OptiMEM I® (Thermo Fisher Scientific), while 4µl of 1mg/ml LIPOFECTAMINE 2000™ (Thermo Fisher Scientific) were added to additional 100µl of OptiMEM I®. These two solutions were then combined together and the siRNA-LIPOFECTAMINE 2000™ complexes were allowed to form for 15 min at room temperature. In the meantime, the medium from each well was aspirated and replaced with 800µl of fresh OptiMEM I®. The OptiMEM I®/siRNA/LIPOFECTAMINE 2000™ mixture was then added to the wells, let stand for 6h and replaced with complete medium for 48 h (for western blot analyses). Alternatively, at the end of the 6 hours the cells were trypsinized and used for cellular assays.

RNA extraction and DNAse treatment

RNA was extracted using QIAzol reagent (Qiagen), following the manufacturer's instructions. RNA was subsequently quantified using Nanodrop Lite (Thermo Scientific). Its purity was assessed by checking the A260/A280 ratio and its integrity by agarose gel electrophoresis. Then, 1ug RNA/10µl were subjected to DNAse I treatment (Thermo Fisher Scientific), following the manufacturer's protocol.

Retrotranscription and real-time PCR (qRT-PCR)

250ng of RNA were retrotranscribed in a 10µl reaction using iSCRIPT RT supermix (Bio-Rad) and following the manufacturer's instructions. To exclude genomic contamination, a PCR was performed using *ATPA1* primers (*ATPA1*-F CTCAGATGTGTCCAAGCAAG and *ATPA1*-R GTCAGTGCCCAAGTCAATG). These primers produce a genomic-derived amplicon of 300bp and a cDNA derived amplicon of 180bp, as described [4]. As previously reported [2], real-time PCR (qRT-PCR) was performed in triplicate, using 2µl of a 1:4 dilution of cDNA, appropriate primers (0.5µM each) and SSOADV Universal SYBR green (Bio-Rad) in 15µl final reaction volume.

MicroRNAs were retrotranscribed using miSCRIPT II RT kit (Qiagen), following the manufacturer's protocol. As previously reported [4], qRT-PCR of microRNAs was performed in triplicate, using 2µl of a 1:4 dilution of cDNA, appropriate primers (0.5µM each) and SSOADV Universal SYBR green (Bio-Rad) in 15µl final reaction volume.

All qRT-PCR reactions were performed in a CFX96 Real-Time System (Bio-Rad) using the following amplification condition: 30s 98°C (3s 98°C, 20s 58-60°C, 10s 72°C)x40 cycles for mRNAs and 30s 98°C (3s 98°C, 20s 58-60°C)x40 cycles for microRNAs. Melting curve analysis and agarose gel electrophoresis of the PCR products were performed to assess the specificity of the reaction. For mRNA expression studies, qRT-PCR primers were designed to be exon spanning (when possible) and to produce 90-110bp long amplicons.

Relative expression of targets was determined using the $2^{-\Delta\Delta Ct}$ method [8] and data were normalized using 3 housekeeping genes (see **Supplementary Table S4** for a list).

Western blot

Western blot of BRAF and pMEK was performed as previously reported [2]. For protein detection, the following antibodies were used:

Anti-human BRAFV600E (VE1, #E19290, Spring Bioscience; mouse monoclonal antibody, dilution 1:400 in 1% milk in TBST).

Anti-human BRAF-X1 (rabbit polyclonal antibody, dilution 1:1000 in 3% milk in TBST). This antibody was developed at Moravian Biotechnology (http://www.moravian-biotech.com), using the X1-specific C terminal peptide (CGGYG**EFAAFK**) as immunogen.

Anti-pMEK (#9154, Cell Signaling; rabbit monoclonal antibody, dilution 1:1000 in 3% BSA in TBST).

Anti-α-TUBULIN (#T9026, Sigma-Aldrich; mouse monoclonal antibody, dilution 1:20000 in 5% milk in TBST).

Actinomycin D assay

Cell were transfected with si-NC/si-miRNAs in 12-well plates, as described above (2 wells for each si-miRNA and 2 for si-NC). The day after transfection, cells were treated with 10ug/ml Actinomycin D

(Sigma) or with DMSO for 2/4/8h. Total RNA was then extracted and qRT-PCR performed as described above.

Mutagenesis

The deletion of the seed matches of the microRNAs under study was performed using QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent), following the manufacturer's instructions. pMIR-X1-3'UTR plasmid (100ng) was used as a template, along with the primers for mutagenesis listed in **Supplementary Table S4.**

Dual Luciferase reporter assay

$7.5x10^4$ HCT116 Dicer-/- cells/well were seeded in a 24-well plate. The day after, 1.5μl of 20μM si-NC/si-miRNA stock solution, 50ng of pMIR-X1-3'UTR, pMIR-ref-3'UTR or pMIR-MEK1-3'UTR plasmid and 10ng of pTK plasmid were added to 50μl OptiMEM I® (Invitrogen), while 4μl of 1mg/ml LIPOFECTAMINE 2000™ (Invitrogen) were added to additional 50μl of OptiMEM I®. The two solutions were then combined together and the mixture was let stand for 15 min at room temperature. In the meantime, the medium from each well was replaced with 800μl of fresh OptiMEM I® and the mixture was then added to the wells, let stand for 6h and replaced with complete McCoy medium. The day after transfection, cell were lysed and luciferase activity was measured with GloMax®-Multi Microplate Multimode Reader (Promega) luminometer, using the Dual-Luciferase® Reporter Assay kit (Promega) and following the manufacturer's instructions.

For the experiments with sensor constructs, the initial transfection was performed with 50ng of empty pMIR-REPORT or pMIR-X1-3'UTR plasmid, 25ng of empty pMS2 or pMS2-X1-3'UTR plasmid and 10ng of pTK plasmid.

Relative Translation Efficiency (RTE) assay

This assay was performed as described in [9]. Cells were transfected in duplicate with si-NC/si-miRNAs in 24-well plates. 24 h after transfection, 1 well was used to detect Luciferase activity, while the other one was harvested and subjected to RNA extraction, retrotranscription and qRT-PCR to measure the expression level of Luciferase and Renilla. The Relative translation efficiency was then calculated using the following formula:

$RTE = [RLU_{1260a}{}^{Fluc/RLuc} / RNA_{1260a}{}^{Fluc/RLuc}] / [RLU_{NC}{}^{Fluc/RLuc} / RNA_{NC}{}^{Fluc/RLuc}]$. RLU: Relative Luciferase Units; FLuc: Firefly Luciferase; RLuc: Renilla Luciferase.

Proliferation and clonogenicity assay

These assays were performed as described previously [2]. In the experiments were vem was used, cells were treated with the drug for 48h and then were seeded for the appropriate assays in complete, vem-free medium.

Stable infection of A375 cells to perform rescue experiments

The stable infection of A375 cells with the empty pCW-rtTA (pCW) lentiviral vector or with the pCW-BRAFV600E-X1-CDS lentiviral vector was performed as described in [4].

A375 CTRL and V600E-X1 were then transfected with si-NC/si-miRNAs and, after 6h, they were seeded for the proliferation assay using complete medium containing 2ug/ml doxycycline, so that BRAFV600E-X1 expression could be induced [4].

Xenograft in zebrafish embryos

This assay was performed as described previously [2]. 48h after siRNA transfection, mcherry-A375 cells were prepared by mixing 10^6 cells with 2ml of MATRIGEL (Cultrex Basement Membrane Extract, PathClear) and injected into zebrafish embryos of the *Tg(Kdrl:EGFP)* line. Fish were raised in the zebrafish facility at IFC-CNR and inbred. The protocol followed for the injection and the subsequent analyses has been described in [2]. Briefly, 48 hours post fertilisation embryos were anesthetized with 0.04mg tricaine (#A-5040, Sigma-Aldrich) and grafted with 500 cells in 1nl, close to the duct of Cuvier area. Fluorescence imaging was carried out 2 days after the injection using the Nikon Eclips E600 microscope. The embryos were carefully set down on glass slides embedded with 1% low melting agarose. The acquisitions were conducted with CoolSnap-CF camera using NIS-Elements software version 2.0. ImageJ software (http://rsb.info.nih.gov) was used to analyze the tumor area. 15-25 embryos were analyzed per experimental condition and results were expressed as mean of 3 biological replicates.

For the xenograft with vemurafenib (PLX4032, RG7204, Selleckchem), A375-mCherry cells were transfected with the appropriate siRNAs and treated with the drug at 2uM concentration for 48h. At the end of this period, they were mixed with MATRIGEL and finally they were injected into the fish embryos as reported above.

TCGA analysis

In order to establish the prognostic value of X1-targeting microRNAs, the prognostic miRNA database PROGmiR v2 (http://xvm145.jefferson.edu/progmir) was interrogated and the TCGA-skin cutaneous melanoma dataset was analyzed.

Statistical analyses

Data were analyzed with unpaired and two-tailed t test (GraphPad Prism, GraphPad Software Inc.). Values of $p < 0.05$ were considered statistically significant (*$p < 0.05$, **$p < 0.01$, *** < 0.001, ****$p < 0.0001$).

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