Supplementary Information for "Plasticity of extrachromosomal and intrachromosomal BRAF amplifications in overcoming targeted therapy dosage challenges"

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Supplementary Table S4. MAPKi-treated melanoma patient tumors, xenografts, and cell lines used for *BRAF* amplicon analysis. Pre-treatment and pos-progression matched pairs only. Provided as an excel file.

Figure S1. *BRAF* FA karyotype categories and subcategories. We divided karyotypes into four primary categories: DM- & HSR-, DM+ & HSR-, DM- & HSR+, and DM+ & HSR+. Some categories have distinguishable sub-categories. **A-C,** Shown are representative FISH images of each *BRAF* FA category and sub-category. Some less-frequent sub-categories are not shown here. Red: *BRAF*. Green: centromere 7. Blue: DAPI.

Figure S2. ***BRAF*** **DNA copy number amplification results confirmed by additional methods.** Related to Fig. 1. **A,** Low-pass whole genome sequencing (WGS)-based *BRAF* and genome-wide copy number results of M249-P and M249-VSR-DM cells. Plotted is the whole genome CNA overview generated by the Ginkgo software. Below are the zoomed-in plots at the *BRAF* locus. Copy number values at the positions indicated by the green dots are shown in the inset boxes. **B**, Comparative genome hybridization (CGH) results of M249-P and M249-VSR-DM cells. The circled region highlights the *BRAF* focal amplicon on chromosome 7q in M249-VSR cells. **C**, CNA of chr7 in M249-P and M249-VSR-DM cells inferred by Bionano optical mapping (OM). X axis: genomic coordinates. Y axis: absolute copy number.

Figure S3. Single-cell-derived clone SC401 displays DM amplicon with circular structure, with subsequent chromosomal integration as an HSR. Related to Fig 2. The SC401 clone derived from M249-VSR bulk cells was contaminated by mycoplasma during long term culture. However, it was mycoplasma negative at the time it was freshly derived. Due to this contamination, we only display data from this clone in a minimal number of supplemental items (this figure, Fig. S4B, and Supplementary Table S1). All conclusions made in the manuscript stand independent of this clone. Nevertheless, this clone remains highly consistent with the other findings, including amplicon structure and integration properties, and thus we present its data. **A-B,** Example images and karyotype frequencies of SC401 before and after 3-month culture at the constant VEM+SEL dose 2μM. -B: before. -A: after. **C-D,** *BRAF* circular amplicon structure in SC401 inferred by optical mapping data, with a similar inferred ecDNA structure to that in bulk M249-VSR-DM cells. S is the circle closing junction.

Figure S4. *BRAF* amplification in DM mode decreased its copy number in single-cell-derived clones (SCs) before and after three-month culture. Related to Fig 2. **A-B,** Relative quantity (RQ) of *BRAF* copy number (CN) before and after long term culture at constant dose, calculated by averaging multiple independent qPCR runs (n represents number of replicates). Error bars were calculated using propagation of errors. See notes on SC401 in Fig S3 legend.

Figure S5. Bulk MAPK inhibitor resistant melanoma cells displayed an increase in growth rate over time, while SCs showed varying degrees of change in growth rate. Related to Fig 2. The M249 VSR bulk population increased their proliferation rate over the three-month culture. Two DM only (SC3, SC4) and one DM plus HSR (SC5) clones also displayed continuously increased proliferation rates (decreased doubling times whereas the HSR only clone (SC2) did not increase its proliferation rate. 0.05 million cells were plated in each well of 12-well plates, and cell numbers were monitored for a maximum of 12 days. Data points were fitted to the exponential growth curve $y = y_0 \cdot e^{kx}$, where y_0 is the initial cell number, i.e. 0.05 million, y is the cell number at time x, and k is the rate constant. Three technical replicates for each time point. **A**, Bulk M249-VSR cells. The days since establishment as a resistant subculture is indicated in the legend as -xxxD. **B**, SCs shortly after single cell clone establishment. **C**, SCs after 3 months of culture. **D,** The summary of changes on doubling times for M249-VSR bulk and SC cells over time. Error bars represents standard error of means (SEMs) of doubling times, n=3 (see Method).

Figure S6. Treating DM+ cells with oscillating doses of BRAF and MEK inhibitors conferred a selection advantage for the DM+ & HSR- subpopulation. Related to Fig 3. **A**, Oscillating (OSCI) and steady dose (CTRL) treatment schemes of M249-VSR-DM cells using VEM+SEL. CTRL is a DM to HSR transition control similar to Fig 3A EXP1 FIX3. **B**, Representative FISH images for the sampling points indicated in (**A**). In the steady dose case, most observed cells were HSR positive on day 246, but in the oscillating dose case there were no detected HSR positive cells even approximately two months later on day 308. **C**, Western blot results for M249 Parental sample and M249-VSR with oscillating dose (labeled in **A**).

Figure S7. Double drug withdrawal eliminated *BRAF*-carrying DMs in about 15 days. Related to Fig 3. **A**, Treatment scheme of M249 cells with VEM+SEL. Points shown represent when cells were fixed (FIX) and collected for genomic DNA (gDNA). **B**, qPCR results of relative *BRAF* copy number for the time points in (**A**). CN: copy number. RQ: relative quantity. **C**, Representative metaphase spread images and FISH images for the time points in (**A**).

Figure S8. M249-VSR DM+ cells tolerate single-drug withdrawal better than HSR+ cells, but there is no difference on recovery rate between DM and HSR cells for double-drug withdrawal. Related to Fig. 3**. A**, Short term viability and growth rates for M249-VSR-DM and HSR bulk cells upon acute withdraw of one of or both MAPK inhibitors. Viability was measured by the CellTiter-Glo (CTG) Luminescent assay. **B**, Long term growth rate measurement for the same treatments in (**A**). Expected cells counts were calculated by multiplying together all cell number fold changes (measured upon each passage).

Figure S9. VEM+SEL dose reduction caused *BRAF* HSR length to shrink in SCs. Related to Fig. 4. **A**, Normalized *BRAF* probe area in FISH images before and after dose reduction for quantifying HSR lengths. P-values are based on one-tailed Wilcox test. **B**, Representative FISH images of the DM- & HSR+ clone SC302 before and after dose reduction. C, Karyotype frequencies of clone SC302 before and after dose reduction. S: short HSR. L: long HSR. -B-: Before, -A-: After.

Figure S10. DM- & HSR+ subclone SC2 show alternative *BRAF* amplicon structure, and its integration on chr3 is supported by PAK2 amplifications. The integration junctions stayed unchanged upon the VEM+SEL dose reduction. Related to Fig. 4. **A**, Optical mapping-inferred junctions used to build the model of the SC2 HSR genomic structure in Fig 4, as well as the S junction shown in Fig S3D. The number of observed optical mapping supports for these junctions are summarized in Supplementary Table S1. **B**, CNA callings by WGS for multiple M249-VSR variants in this article show DM- & HSR+ subclone SC2 has PAK2 amplification. Its dose reduced version (SC2-2-0.1) and bulk DM- & HSR+ population (M249-VSR-HSR) have weaker and heterogenous PAK2 amplifications. **C**, CNA calling by optical mapping for SC2 before and after dose reduction show decrease of *BRAF* copy number and *PAK2* amplification around chr3 telomere prior to the dose reduction. **D**, *BRAF* HSR integration junction between chr7 and chr3 before and after dose reduction, revealed by SVABA analysis using WGS data.

Figure S11. Treatment of M395 melanoma cells with MAPK inhibitors led to *BRAF* amplification on HSRs co-occurring with *BRAF* kinase domain duplication. HSR length did not decrease upon drug withdrawal in this case. Related to Fig. 4. **A**, VEM+SEL treatment scheme starting from 0.05µM on M395-P (parental) cells. The points when cells were collected for genomic DNA (gDNA), fixation (FIX) and protein lysates (LYSATE) are labeled. **B**, Representative FISH images for fixation time points in (**A**). C, qPCR results of relative *BRAF* copy number for gDNA collection points in (**A**). CN: copy number. RQ: relative quantity. D, western blot for lysate collection time points in (**A**).

Figure S12. Drug dose challenge characterization of single-cell-derived clones. Related to Fig 5. **A**, Experimental design to generate single-cell-derived clones (SC1XXs) by sorting M249-VSR-HSR bulk cells on day 322, followed by two rounds of replica screens. **B**, As depicted in (**A**), acute 2 to 5μM VEM+SEL treatment on 41 SC1XXs was used to screen for clones that adapt to 5μM rapidly. The rows of the heatmap represent different SC1XXs ordered by relative growth rate (RGR), calculated by dividing the mean at 5μM by that at 2μM, in descending order. Viability was measured by CellTiter-Glo, and the readings were divided by 1000 followed by capping at 50. **C**, Representative FISH images of two SC1XXs at the lower tail of the heatmap in (**B**).

Figure S13. The DM+ and KDD+ single-cell-derived clones SC101 and SC137 demonstrate the best ability to tolerate MAPK inhibitor dose increases, compared to other SC1XXs. Related to Fig 5. **A,** The indicated M249-VSR subclones and M249-VSR-HSR bulk cells initially cultured at 2μM of VEM+SEL were treated with various subsequent inhibitor doses for 4 days, and then their viabilities were measured. All numbers are normalized to the corresponding viabilities at 2μM. p-values are based on one-tailed t test ($n = 6$).

Figure S14. MAPK inhibitor dose escalation applied to HSR-positive SCs did not result in the DM+ & KDD+ genomic configuration. Related to Fig 5. **A,** Representative FISH pictures of the DM- & HSR+ M249-VSR SCs, SC2 and SC208, with VEM+SEL dose escalated from 2μM to 5μM until they became resistant. **B**, Immunoblot of BRAF samples in (**A**) showing no 140 kDa KDD band after the VEM+SEL dose increase.

Figure S15. The pre-treatment *BRAF* copy number does not predict the increase of *BRAF* copy number upon resistance to MAPKi. A. Correlation between *BRAF* copy number before MAPKi treatment and the *BRAF* copy number increase after relapsing from the treatment in melanoma. R^2 is the squared sample Pearson correlation coefficient, and the correlation P-value is based on the t-distribution.

Figure S16. The ferroptosis sensitivity of melanoma cells with *BRAF* amplification as dual MAPKi resistance mechanism is not due to dedifferentiation. Related to Fig. 7. **A**, Lipid ROS in M249-P and M249-VSR-DM measured by flow cytometry using lipophilic ROS-sensitive BODIPY™ 581/591 C11 dye upon treatment with or without 1µM RSL3 and 150µM Trolox for 24hr, demonstrating that the lipophilic antioxidant Trolox protects against RSL3-inducded lipid ROS. **B**, Dose-response curve showing increased sensitivity to ferroptocide in in BRAFi+MEKi resistance mediated by *BRAF* amplification (M249-VSR-DM and -HSR) but no differential sensitivity to Erastin compared to parental cells. Cell viabilities were measured by CellTiter-Glo. Three or six replicates. 72hr treatment. Each experiment was repeated twice. **C**, Projections of the M249-P and M249-VSR variant samples from the current manuscript onto the differentiation trajectory (transcriptomic principal component analysis (PCA)) of the M series of melanoma cell lines from Tsoi et al(1). The four melanoma differentiation stages are indicated. All M249-P and M249-VSR variants start and remain in the differentiated (melanocytic) cluster upon acquisition of MAPKi resistance. In our past studies, melanoma cells that develop MAPKi resistance through genomic changes that reactivate the MAPK signaling pathway do not dedifferentiate, e.g. M249P/R (NRAS mutation-mediated resistance in this version of single agent BRAFi resistance), do not show different sensitivity to ferroptosis inducing agents, while other cases of resistance due to dedifferentiation (also featured by receptor tyrosine kinase upregulation) are observed, e.g. M229P/R and M238P/R(1,2). Note that our M249-P and M249-VSR *BRAF* amplification lines are projected at the same location as the independently derived case of resistance (M249R) and its parental (M249P) pair. In this case resistance is to single agent BRAFi (vemurafenib), with resistance mediated by NRAS mutation(1,2). Notably, the *BRAF*-amplified M249-VSR cells are sensitive to RSL3 (Fig. 7A), unlike the NRAS-mutant M249R case(1). **D**, The same reference PCA-based differentiation state spectrum as in (A), with projections of Mel888-P/-DTR (*BRAF* amplification), A375-P/-DTR (*BRAF* amplification) and SKMEL28P/R (dedifferentiation) cell lines. *BRAF* amplification mediated resistant sublines do not demonstrate gene expression-based signatures of dedifferentiation as compared to their parental pairs. Data was downloaded from the corresponding papers(3–8). **E-F**, mRNA expression and single sample GSEA (ssGSEA)(9) of selected genes and gene sets in the melanoma cell lines before and after establishment of resistance to MAPK inhibitors. Y: YES. N: NO. Amp: amplification. Mut: mutation: RTK Up: receptor tyrosine kinase upregulation. HSRR: higher sensitivity to RSL3 in resistance line. Dediff: dedifferentiation upon resistance. Log10 counts per million (CPM) and ssGSEA z scores were calculated by standardizing within each gene, and for the visualization the values were capped from -2 to +2. **G-H**, selected gene mRNA levels and mRNA-based ssGSEA scores for cell lines in the M series. **I**, Glutathione levels, reduced (GSH), oxidized (GSSG), and ratio (GSH/GSSG), in M249 sublines measured by mass spectrometry. p-values were calculated using one-tailed t test.

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