# Genetic heterogeneity and clonal evolution during metastasis in breast cancer patient-derived tumor xenograft models

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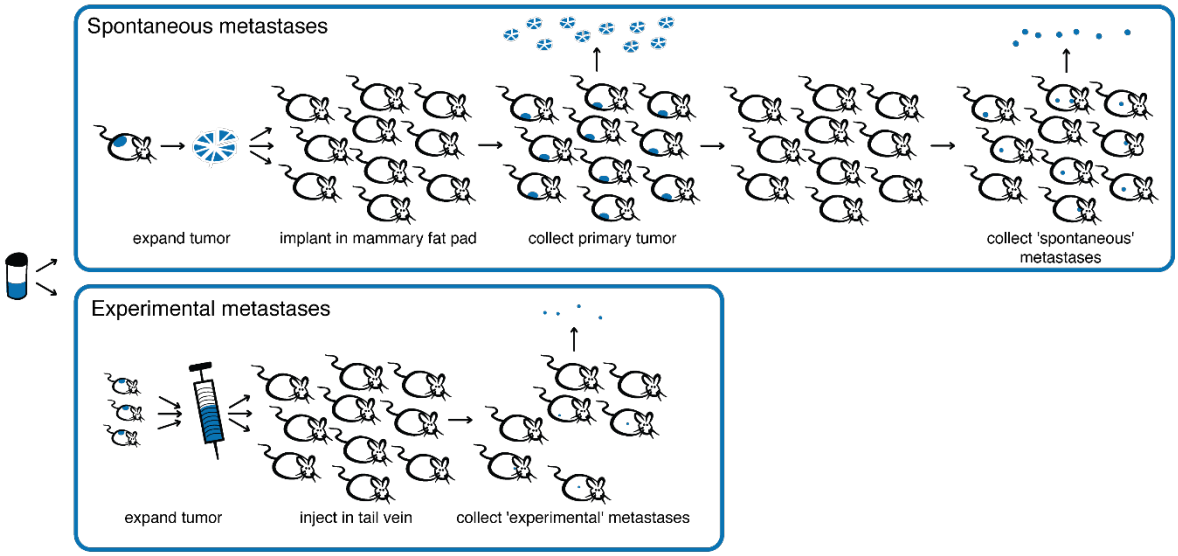
## SUPPLEMENTARY FIGURES

### PDX Model B1




### PDX Model B2

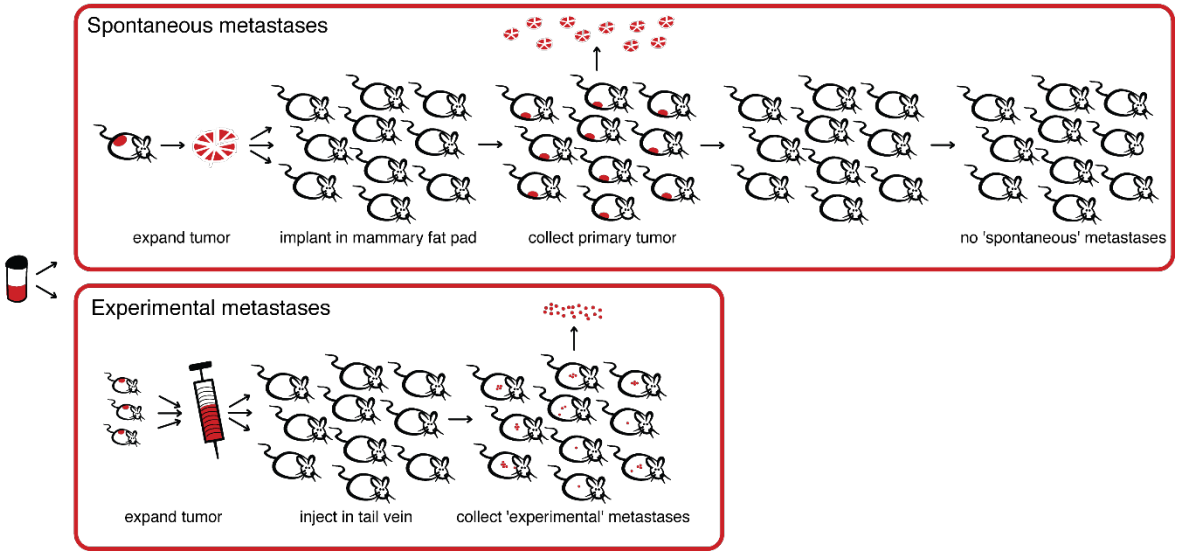



**Figure S1.**

An overview of the experiments conducted to obtain metastases. Two PDX models (B1 and B2, in blue and red) were used in experiments to obtain metastases in two ways (spontaneous and experimental). Ten replicates were generated for each condition, and all primary tumor and metastases samples were collected.

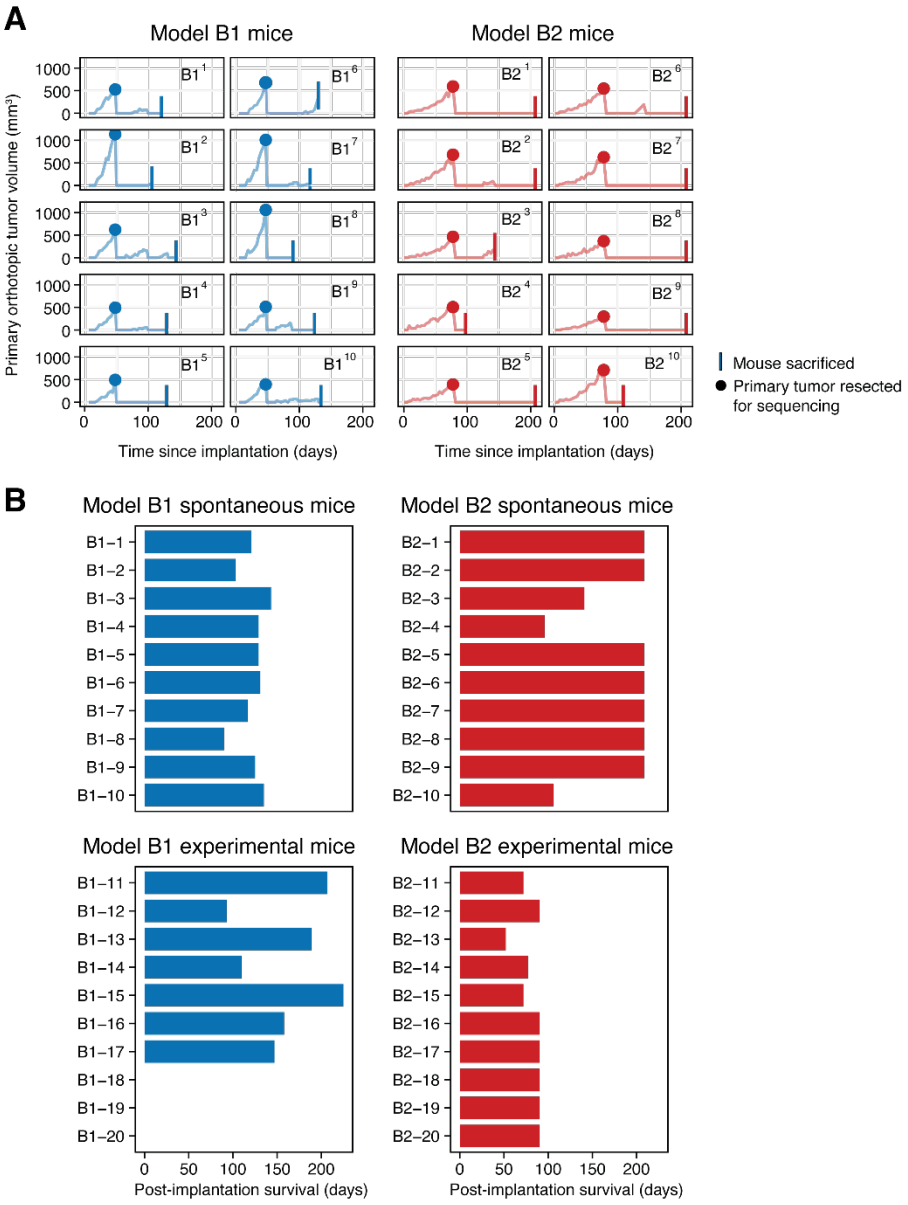



**Figure S2.**

A. After orthotopic implantation of the patient-derived tumor into the mammary fat pads of 10 mice (mouse identity in upper right corner of each panel), the size of the primary tumors (vertical axes) were measured repeatedly over time (horizontal axes). The primary tumors were resected for sequencing (solid circles) and the mice were eventually sacrificed (vertical lines). B. Survival of each mouse after orthotopic implantation (top panels) or implantation by tail-vein injection (bottom panels).

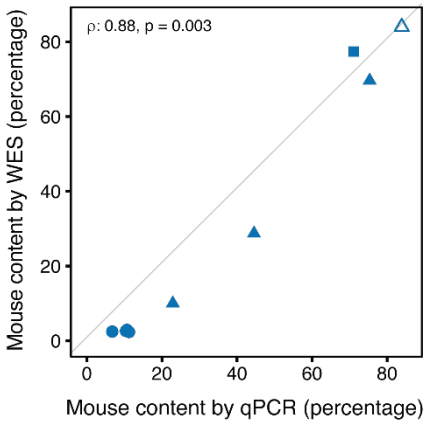



**Figure S3.**

The amount of mouse DNA found in each sample was estimated from the number of uniquely mapping reads in the whole exome sequencing data (WES, vertical axis) and by qPCR using mouse- and human- specific primers (horizontal axis). The gray diagonal line depicts the ideal situation, in which the percent of mouse content estimated by both methods is identical. Points falling to the left of the line are overestimated using the WES data, and points falling to the right are underestimates. While the mouse content estimates using WES for these samples are lower than those measured in qPCR (median difference 7.75 percentage points), they nonetheless correlate well with the qPCR measurements (Spearman's rank correlation: ρ=0.88, p=0.003, n=9).

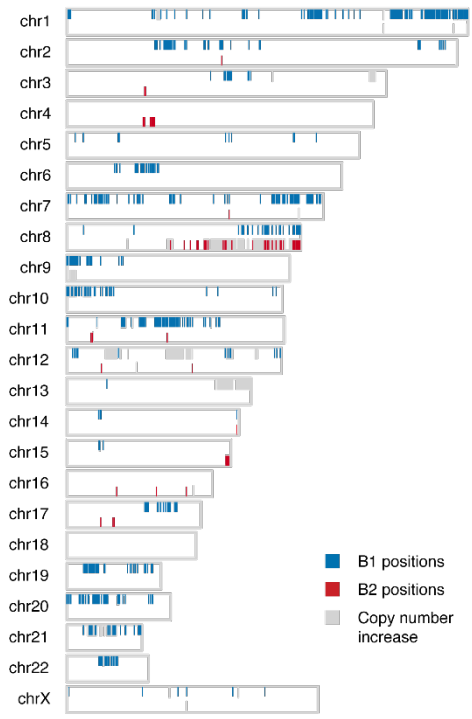



**Figure S4.**

The distribution of the unbiased genomic positions across the genome for models B1 (blue) and B2 (red), and gene-level copy number amplifications (gray).

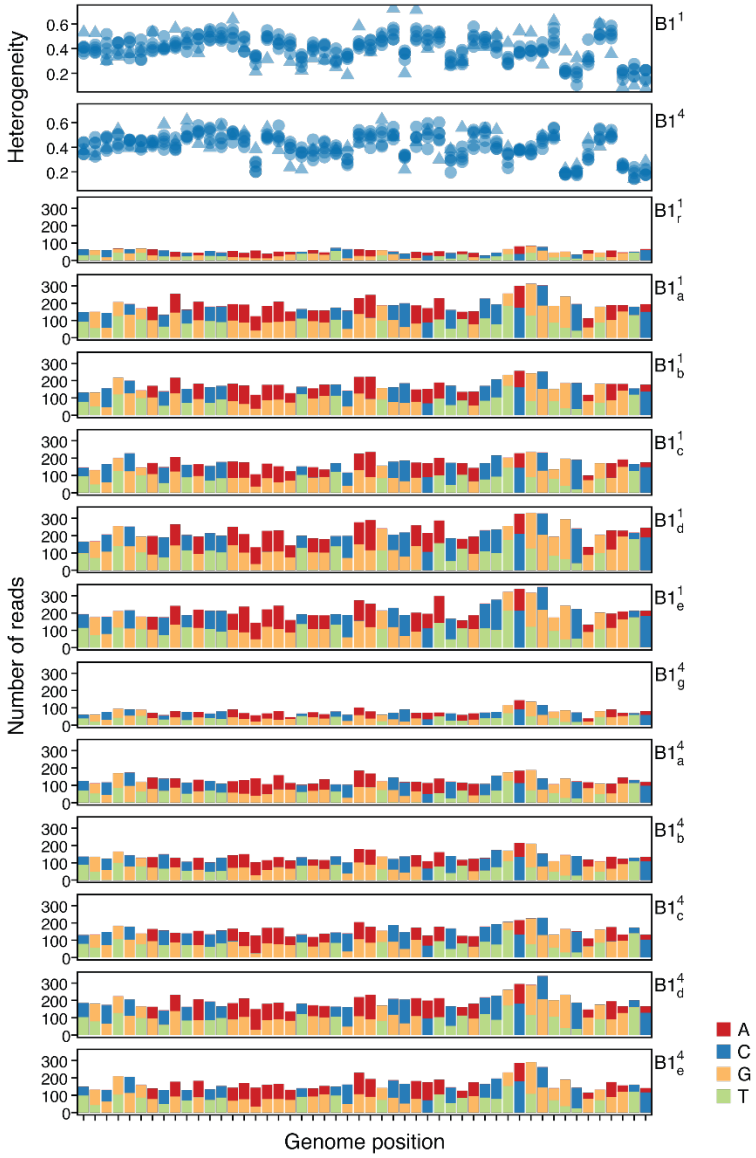



**Figure S5.**

Top two panels: The positional heterogeneity score (vertical axes) is plotted for a subset of 50 genomic positions (horizontal axes) for each sample (points) taken from a mouse (labeled on right of panel). Thus, each point is the frequency of the non-patient reference alleles for a sample at a single genomic position.

Remaining panels: Each panel depicts the number of reads (vertical axes) for the same subset of 50 genomic positions (horizontal axes) that are an A, C, G, or T nucleotide (red, blue, yellow, or green) for a single sample. The sample is labeled to the right of each panel. The primary tumor samples are $B1^1_a$-$B1^1_e$ and $B1^4_a$-$B1^4_e$ and the metastases are $B1^1_f$ and $B1^4_g$.

## SUPPLEMENTARY METHODS

### PDX models

We used two previously-established and characterized TNBC models 1004-HX and 1921-HX [41], which we here refer to as B1 and B2, respectively. Patient tumor specimens were obtained from vendors in the US, and clinical and pathologic data were entered and maintained in Novartis databases. All patients provided informed consent for the tumor samples procured by Novartis, Inc. from the National Disease Research Interchange, Philadelphia, Pennsylvania, USA and Maine Medical Center, Portland, Maine, USA. No patient blood samples or matched normal tissues were available. We expanded the tumors for our experiments by orthotopically implanting 5 $mm^3$ chunks of frozen tissue from passages 6 and 7 (models B1 and B2, respectively) in the mammary fat pad of female nude mice (Crl:Nu(NCr)-Foxn1nu, obtained from Charles River Laboratories, Germany). The harvested tumors were further expanded by implanting 5 $mm^3$ pieces into the mammary fat pads of 8 female nude mice. One harvested tumor after this expansion was immediately used to generate the "spontaneous" metastases, and several were immediately used to generate the "experimental" metastases by injecting dissociated tumor cells in the tail vein.

### *In vivo* experiments

All animal studies were under the oversight of the Novartis Animal Welfare Organization and were conducted in accordance with ethics and procedures covered by permit BS-2808 issued by the Kantonales Veterinäramt Basel-Stadt and in strict adherence to Swiss animal welfare law (Eidgenössisches Tierschutzgesetz and the Eidgenössische Tierschutzverordnung, Switzerland). All animals had access to food and water *ad libitum* and were identified with transponders. They were housed in a pathogen-free facility with a 12-hour light/12-hour dark cycle.

### Generation of spontaneous metastases

One expanded tumor was divided into 10 equally-sized pieces (approximately 5 $mm^3$), which were implanted orthotopically into 10 female NOG mice (NOD.Cg-Prkdcscid il2rgtm1Sug/JicTac, Taconic). The size of the primary orthotopic tumors was monitored by measuring the tumor's height ($h$) and width ($w$) with calipers, and the volume was estimated as $\frac{\pi}{6} hw \min(h, w)$. The primary orthotopic tumor was resected for sequencing when its volume reached approximately 500 $mm^3$, which occurred 47 days after implantation for the B1 model and 78 days after implantation for the B2 model. The mice were monitored for changes in body weight, breathing patterns, behavioral patterns, and postural changes. Once detected, the mouse was sacrificed and necropsied to isolate metastases in the liver, lung, and lymph nodes.

## Generation of experimental metastases

Five B1 and seven B2 tumors (each approximately 500 $mm^3$) were resected and dissociated into single cells using MACS tumor cell dissociation kit (130-095-929) following the user manual. The dissociated human tumor cells were then cleared off from the contaminated mouse cells using a mouse cell depletion kit obtained from MACS (130-104-694) following the user manual. The cell viability and count were determined using Biorad TC20 automated cell counter and approximately $1\times10^6$ cells were injected in each of 10 mice in the tail vein to facilitate experimental metastasis. Three mice injected with B1 tumor cells died immediately post-injection. The remaining mice were monitored after the tumor cell injections for changes in body weight, breathing patterns, behavioral patterns, and postural changes. Once detected, the mouse was euthanized and necropsied to isolate metastases in the liver, lung, and lymph nodes.

## Selection of samples and preparation of the sequencing library

We divided each resected orthotopic primary tumor into halves. We saved one half, and divided the other half into five pieces for sequencing (Figure 2C). We also divided large metastases into several pieces for sequencing (Figure 2D). For our computational controls, we isolated the DNA from four NOG mouse tails. We isolated the genomic DNA from the samples using the AllPrep DNA/RNA Mini Kit from Qiagen (cat 80204) following the included protocol. Briefly, 5 - 8 $mm^3$ tissue was pulverized into fine tumor powder under ultra-low freezing temperature (liquid nitrogen) before commencing the DNA extraction. We measured the DNA quality and quantity by Qbit 4 fluorometer.

We conducted the exon capture and library prep using the Illumina's TruSeq nano DNA library prep kit (FC-121-9010) according to the manufacturer's instructions. 60 ng of genomic DNA was fragmented to 350 bp using Covaris S220 Ultrasound sonicator. Sheared DNA was used in the exome-capture following Agilent SureSelect XT Target Enrichment System for Illumina Paired End Multiplexed Sequencing Library (catalog #G9641B). Briefly, sheared DNA was end repaired, followed by the addition of adapter tags to construct DNA libraries through PCR amplification. Exome capture was performed through hybridization using the XT5 probe. The resulting captured libraries were indexed and purified, and the cDNA library was validated on the Agilent 2100 Bioanalyzer using a DNA-1000 chip. Each library was sequenced on the Illumina HiSeq 2500 at the Novartis Institute for Biomedical Research Analytical Sciences and Imaging genomics facility with 76 bp and 84 bp paired-end reads. The mouse control libraries were sequenced just once with 76 bp paired-end reads.

## Selection of unbiased positions from sequenced exons

### *Technical bias due to the homology between the human and mouse genomes*

We removed technical bias due to homology with the mouse genome by aligning the reads to a joint human - mouse reference genome using bowtie2 v2.3.4.1. The hg38-mm10 joint reference genome was prepared by concatenating the hg38 (downloaded from

http://hgdownload.cse.ucsc.edu/goldenPath/hg38/bigZips/analysisSet/hg38.analysisSet.chroms.tar.gz downloaded May 29, 2017) and the mm10 (downloaded from http://hgdownload.cse.ucsc.edu/goldenPath/mm10/bigZips/chromFa.tar.gz on May 29, 2017, excluding haplotype and unplaced contigs) genomes. We removed duplicates and merged the bam files for samples sequenced on two flow cells using Picard v2.18.4, and retained reads with mapping quality equal to or larger than 20 with samtools v1.7. By doing this, we filtered out the reads that aligned to multiple genomic regions, which means that reads that mapped to both the hg38 and mm10 reference genome were filtered out. We then used samtools to split the alignment into two files: one only contains only the reads that are aligned to hg38 genome, the other only to mm10 genome. Unless otherwise specified, we used the reads mapped to the hg38 genome for all downstream analyses.

### *Read depth filtering*

To ensure high quality heterogeneity estimates, we restricted our analyses to genomic positions included in the exon-capture kit with high sequencing coverage for the 50 primary tumor samples for each model. To do so, we extracted the number of reads at each genomic location for each primary tumor sample using bam-readcount 0.8 (https://github.com/genome/bam-readcount) and used a custom Python v3.7.1 script to parse the output and select the genomic positions with at least 100 reads in all 50 primary tumor samples for a model. Note that the genomic positions with sufficient read depth were selected separately for models B1 and B2.

### *Identification of heterogeneous positions*

We identified heterogeneous genomic positions separately for the two models as follows. We again used the data from bam-readcount v0.8 to extract the number of reads with an A, C, G, or T at all genomic positions included in the exon-capture kit. Most of the extracted positions showed the same allele across the samples. For positions with more than one allele, we obtained the patient's reference allele by selecting the most commonly found allele at that position across the model's 10 primary orthotopic tumors. To identify the genomic positions that could be identified as heterogeneous with relative confidence, we only retained positions where (1) at least one sample had 0.1 non-patient reference allele, (2) at least five primary orthotopic samples showed evidence of a non-patient reference allele.

### *Removal of remaining genomic positions with homology to the host mouse*

We sequenced the exomes of DNA obtained from the tail four NOG mice in order to identify and remove any remaining contribution of mouse DNA. We processed these four NOG mouse samples following the same experimental and computational steps as our PDX samples. After sequencing, genomic positions were flagged if any of the mouse control reads mapped to them. The flagged genomic positions were removed from downstream analyses to further eliminate potential mouse-biased genomic positions.

## Estimation of mouse content

We estimated the mouse content using two approaches: bioinformatically from the whole exome sequencing data, and experimentally using qPCR. First, we obtained our bioinformatic estimates of mouse content for each sample by calculating the percent of uniquely-mapping reads that mapped to the mouse genome. We obtained experimental estimates of mouse content by measuring the concentration of human and mouse DNA from qPCR experiments with human- and mouse-specific primers as has been previously described [74]. Samples with more than 55% mouse content were removed from downstream analyses.

## Heterogeneity estimation

Heterogeneity is a measure at each site of the fraction of cells that are different from the patient reference genome. Formally, we define $\mathrm{S}$ as the set of all samples, $\mathrm{S^p}$ as the set of all primary samples, $\mathrm{S}^m$ as the set of all metastases, such that $S = \mathrm{S^p} \cup \mathrm{S}^m$. We define $\mathcal{L}$ as the set of informative genomic positions, where each position $\mathrm{i}$ has a label from $\{1,2,3,\ldots,\ell\}$ and $\ell$ is total number of positions in $\mathcal{L}$. The number of reads of each base $\mathrm{j} \in \{\mathrm{A},\mathrm{C},\mathrm{G},\mathrm{T}\}$ at each position $\mathrm{i} \in \mathcal{L}$ for each sample $\mathrm{s} \in \mathrm{S}$ is given by $\mathrm{n}_{\mathrm{i,j}}^{\mathrm{s}}$, and the total reads of any base at a site are $\mathrm{N}_{\mathrm{i}}^{\mathrm{s}} = \sum_j n_{i,j}$ . Heterogeneity is defined at each site as a measure of the fraction of cells that are different from the patient reference genome. The heterogeneity score at position $\mathrm{i}$ in sample $\mathrm{s}$ is given as $\mathrm{h}_{\mathrm{i}}^{\mathrm{s}} = \sum_{j \neq r_i} \frac{\mathrm{n}_{\mathrm{i,j}}^{\mathrm{s}}}{\mathrm{N}_{\mathrm{i}}^{\mathrm{s}}}$, $\mathrm{s} \in \mathrm{S}, \mathrm{i} \in \mathcal{L}, \mathrm{j} \in \{\mathrm{A},\mathrm{C},\mathrm{G},\mathrm{T}\}$, and the average heterogeneity score of sample $\mathrm{s}$ is thus $\mathrm{H}^{\mathrm{s}} = \frac{\sum_i h_i}{\ell}$.

## Comparing heterogeneity estimates

Unless otherwise indicated, all statistical analyses and plots were generated using R v3.5.1, tidyverse v1.2.1 [75], ggplot v3.1.0 [76], cowplot v0.9.3, and ggridges v0.5.1. We tested whether the heterogeneity within each primary tumor was statistically distinguishable from all other primary tumors of the same model using a Mann-Whitney test. We used the R package lme4 v1.1–18-1 [77] to perform a linear mixed effects analysis of the relationship between the heterogeneity and tumor location (primary tumor vs. metastasis) for spontaneous metastases. In this analysis, we chose the tumor location as fixed effects, and the identity of the mouse from which the tumor was resected as the random effects. We obtained significance values using a likelihood ratio test of the full model against a null model that did not contain the fixed effects. Experimental metastases and primary tumors were resected from different mice, and so we used a Mann-Whitney test to compare the two populations.

## Copy number data

We obtained the gene-level copy number analysis from a previous publication [41] that used data from Affymetrix genome-wide human SNP Array 6.0 chips, and defined amplified genes by $\log_2$ ratio ≥ 1.32.

## SUPPLEMENTARY REFERENCES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