The genetic evolution of metastatic uveal melanoma

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Uveal melanoma is a clinically distinct and particularly lethal subtype of melanoma originating from melanocytes in the eye. Here, we performed multi-region DNA sequencing of primary uveal melanomas and their matched metastases from 35 patients. We observed previously unknown driver mutations and established the order in which these and known driver mutations undergo selection. Metastases had genomic alterations distinct from their primary tumors; metastatic dissemination sometimes occurred early during the development of the primary tumor. Our study offers new insights into the genetics and evolution of this melanoma subtype, providing potential biomarkers for progression and therapy.
where a heterozygous mutation would be expected to reside, with shaded zones that indicate the 95% confidence intervals (CIs). The loss of heterozygosity (LOH) of the \textit{BAP1} mutation is evidenced by its MAF falling outside those zones in all three samples.

We next inferred CNAs using the software package \textit{CNVkit} v.0.9.5 (ref. 21) (Fig. 1c). In the example case, some CNAs were shared between all samples, including a deletion of chromosome 3 that encompassed the \textit{BAP1} gene and thus explains how the \textit{BAP1} mutation became hemizygous. A loss of chromosomal arm 9p and a gain of chromosomal arm 8q were also present in each melanoma region (Fig. 1c).

By contrast, some CNAs were exclusive to certain tissues. For instance, there was a superimposed homozygous deletion encompassing the \textit{CDKN2A} gene that was only present in the unpigmented area of the primary melanoma (Mel1) and the metastasis. The amplitudes of 8q gain differed among the melanoma tissues (Supplementary Fig. 1b); the pigmented area of the primary melanoma (Mel2) had 5 absolute copies, while the unpigmented area (Mel1) had 9 absolute copies and the metastasis had 11 absolute copies. These observations indicate stepwise deletion of \textit{CDKN2A} paralleled by stepwise amplification of 8q during the evolution of this case.

The distribution of somatic mutations and CN changes among the three tumor areas made it possible to delineate the sequential order in which they arose during tumor evolution. All three tumor

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**Fig. 1** The patterns of somatic alterations within a patient’s tumor areas provide insights into the sequential order in which they arose (example case A11). a, Hematoxylin- and eosin-stained sections of the enucleation specimen show morphologically distinct areas. The metastatic tissue stemmed from a liver core biopsy of the patient. The dashed regions were microdissected for genomic analyses. b, Point mutations are stratified by their MAFs in the two regions of the primary tumor and the metastasis. The blue lines indicate the expected MAFs of fully clonal, heterozygous mutations after accounting for stromal cell contamination, with the shadings indicating the CIs where those mutations should reside. Note the presence of a \textit{GNA11} mutations that is fully clonal and heterozygous in all samples. By contrast, the \textit{BAP1} mutation has an elevated MAF, indicating that it underwent LOH. c, Heatmap displaying the CN increases (red) or decreases (blue) for each sample (rows) across the genome with chromosomal boundaries indicated. Additional details related to deletion of \textit{CDKN2A} and gain of chromosomal arm 8q are shown in Supplementary Fig. 1. d, Inferred evolution of case A11 with mutations shared among all samples forming the trunk and the mutations not present in all samples forming the branches. The length of the trunk and branches is scaled by the total number of mutations in the samples.
Thus considered tertiary driver mutations, which probably explains they arose later during progression, after mutational activation of metastases (designated in Fig. 2 by the faded tiles), indicating that mutations were in just one region of the primary tumor or private to their genes in the Gα region from the tumors of all 35 patients. As expected, based on prior studies, we observed mutually exclusive mutations involving genes in the Gq signaling cascade as well as alterations affecting BAP1, SF3B1 and EIF1AX that tended to not overlap. However, in contrast to prior studies, we found a long tail of additional mutations (Fig. 2) that included LOF mutations affecting CDKN2A, PBRM1, PIK3R2 and PTEN, LOH over the GNAQ locus and GOF mutations affecting EZH2, PIK3CA and MED12. The majority of these alterations were in just one region of the primary tumor or private to their metastases (designated in Fig. 2 by the faded tiles), indicating that they arose later during progression, after mutational activation of Gq and BAP1, SF3B1 and EIF1AX mutation. These mutations are thus considered tertiary driver mutations, which probably explains why they were not apparent in previous bulk sequencing of primary uveal melanomas.

![Fig. 2](https://doi.org/10.6084/m9.figshare.6845675.v1)

**The genomic landscapes of primary and metastatic uveal melanoma.** We next annotated the driver mutations in each sequenced tumor. The genomic landscapes of primary and matched metastases were performed on tumors from 35 patients. The spectrum of driver mutations (rows) in all patients (columns) is shown. Gain/change-of-function and LOF mutations are indicated as red or blue tiles, respectively. Mutations present in every sequenced region from a given patient are represented by solid tiles and mutations present in only a subset of tissues from a given patient are represented by faded tiles. Mutations are grouped into three categories based on their deduced order of emergence during progression: primary drivers (shared and fully clonal); secondary drivers (shared and usually clonal); and tertiary drivers (private to later stages and typically subclonal).

**Early mutational activation of the Gq signaling pathway is pathognomonic of uveal melanoma.** Activation of Gq signaling was ubiquitous with mutations affecting GNA11, GNAQ, CYSLTR2 or PLCB4 in a mutually exclusive pattern in all 35 cases in our cohort, consistent with prior findings. The mutations activating the Gq signaling pathway were always shared among all samples from a given patient, indicating that they undergo selection early on and are required for tumor formation (Supplementary Fig. 2a). This is also consistent with the recent finding that these mutations are common in choroidal nevi, which is probably a precursor for a subset of uveal melanomas.

Uveal melanomas with SF3B1 or EIF1AX mutations acquire additional oncogenic mutations during metastatic progression. We next sought to investigate the relative timing of BAP1, SF3B1 and EIF1AX mutations. Patients whose uveal melanomas have SF3B1 or EIF1AX mutations did not co-occur. However, one EIF1AX mutant tumor also had biallelic BAP1 mutations (Supplementary Fig. 3a). This case was even more unusual because the EIF1AX mutation resided at codon 70, a site that is recurrently mutated but less frequently affected than the N-terminal region of EIF1AX. Finally, the Gq pathway mutation in this atypical case involved CYSLTR2, which is rare compared to GNAQ and GNA11 mutations.

**Uveal melanomas with SF3B1 or EIF1AX mutations tended to have additional oncogenic alterations.** Two had homozygous CDKN2A
proposed that primary tumors without monosomy 3 may progress (Supplementary Fig. 3b). This was notable because it has been histologically advanced area acquired a deletion of chromosome mutant case, we sequenced two areas of primary tumor; the more hotspot mutation (Supplementary Fig. 3). In one MED12

Selection to disrupt chromatin remodeling factors begins early but may require additional oncogenic alterations to complement their probably limited ability to drive disease progression and metastatic dissemination. Among all samples from a given patient, placing them on the trunks or evolutionary trees (Fig. 3a). These observations suggest that biallelic loss of either BAP1 protein by immunohistochemistry (Fig. 3d), suggesting that whereas the mutation in the metastasis remained heterozygous, whereas the mutation in the metastasis had only the primary tumor had eliminated the second allele of, but its metastasis had only mutation and a deletion affecting different distribution between primary tumors and their metastases (Fig. 3b,c). In case A29, the primary tumor had a frameshift mutation and a deletion affecting chromosome 3 (Fig. 3b,c). In case A13 (Fig. 3c), the primary tumor and the metastasis shared a point mutation in chromosome 3 (Supplementary Fig. 3b). This was notable because it has been proposed that primary tumors without monosomy 3 may progress toward a more malignant stage by losing chromosome 3 (ref. 24).

The SF3B1 and EIF1AX mutations were predominately truncal, whereas the additional oncogenic mutations were more often on the branches of the phylogenetic trees. This pattern suggests that SF3B1 and EIF1AX mutations undergo selection early but may require additional oncogenic alterations to complement their probably limited ability to drive disease progression and metastatic dissemination.

Selection to disrupt chromatin remodeling factors begins early and continues throughout the evolution of metastatic uveal melanoma. BAP1 mutations were expectedly common in our cohort of uveal melanomas that became metastatic and were usually shared among all samples from a given patient, placing them on the trunks of evolutionary trees (Fig. 3a). These observations suggest that biallelic BAP1 mutations tend to precede metastatic dissemination. However, there were two cases where BAP1 alterations had a different distribution between primary tumors and their metastases (Fig. 3b,c). In case A29, the primary tumor had a frameshift mutation and a deletion affecting BAP1, but its metastasis had only very few reads of the frameshift mutation and did not have a deletion on chromosome 3 (Fig. 3b). In case A13 (Fig. 3c), the primary tumor and the metastasis shared a point mutation in BAP1, whereas the mutation in the metastasis remained heterozygous. The metastases from cases A29 and A13 were both negative for BAP1 protein by immunohistochemistry (Fig. 3d), suggesting that either BAP1 was silenced epigenetically or the mutation escaped detection by our methods. Overall, these two cases raise the possibility that biallelic loss of BAP1 is not absolutely required for
This would be consistent with anecdotal reports of monosomy 3 occurring later in the evolution of uveal melanoma25,26; however, further studies are necessary to confirm whether this is true.

We also observed mutations in other chromatin remodeling factors, including hemizygous, LOF mutations in \( \textit{PBRM1} \) and GOF \( \textit{EZH2} \) mutations (Fig. 3e). \( \textit{PBRM1} \) encodes a critical subunit of the SWI/SNF chromatin remodeling complex and \( \textit{EZH2} \) encodes the enzymatic subunit of polycomb repressive complex 2 (PRC2) (ref. 27). BAP1 is a negative regulator of PRC1, which activates PRC2, so BAP1 loss is thought to promote PRC2 activation. The SWI/SNF chromatin remodeling complex also opposes PRC2 activity in maintaining cell-state appropriate chromatin modifications28–30; therefore, the alterations in \( \textit{PBRM1} \) and \( \textit{EZH2} \) likely shift the balance of chromatin remodeling activity further toward PRC2 dominance.

In contrast to BAP1 mutations, which occurred earlier in most cases, mutations affecting \( \textit{PBRM1} \) or \( \textit{EZH2} \) occurred later; they were generally not shared among all tumor areas of a given patient. CN gains of chromosome 8q arise in the primary tumor and ramp-up during metastatic progression. CN gains of chromosome 8q were present in nearly every case and were typically shared among the different tumor areas of a given patient (Fig. 4a), suggesting that they occur early during evolution of the primary tumor, before metastatic dissemination, as previous data have suggested31. There were three exceptions to this pattern where CN gains were not shared in every tumor area (Fig. 4b), indicating that it is at least possible for metastatic dissemination to precede 8q gain.

We delved deeper into the amplitudes of 8q gain and observed that the CN of 8q tended to increase from primary tumors to metastases. For instance, as described in case A11, one region of the primary tumor had 5 copies of 8q, while another region of the primary tumor had 9 copies and the metastasis had 11 copies (Fig. 4a and Supplementary Fig. 1b). In other examples, the primary tumor for case A61 had 4 copies of 8q and the metastasis had 11 copies (Fig. 4a and Supplementary Fig. 4a); in case A06, the primary tumor had 4 copies of chromosomal arm 8q, while the metastasis had 9 copies.
Overall, metastases had more copies of 8q than their corresponding primary tumors in most patients, with an average of 6 versus 4 copies ($P = 0.002$, two-tailed $t$-test; Fig. 4c). We validated our sequencing-based CN estimates by measuring allelic imbalance and assessing CN with independent technologies, including multiplex ligation-dependent probe amplification (MLPA) and FISH (Supplementary Fig. 4c–e). The findings of these methods were concurrent and indicate progressive increase of 8q CN during progression to metastatic disease.

We deduced the sequential order in which somatic alterations undergo selection during the evolution of metastatic uveal melanoma by calculating how often a given alteration resided on the trunk versus the branch of each evolutionary tree (Fig. 5a). This analysis confirmed that $G_{\alpha}q$ pathway mutations are the earliest mutations to undergo selection, followed by gain of chromosomal arm 8q as well as $BAP1$, $SF3B1$ or $EIF1AX$ mutations. Selective pressures continue to operate on these pathways at comparatively later stages of disease progression.

We determined which somatic alterations were more common in primary tumors or metastases (Fig. 5b). The enrichment scores for the 15 genetic alterations highlighted in a were calculated using a two-tailed binomial test (see Methods for details) for the 15 genetic alterations highlighted in a to determine whether they were more common in primary tumors ($n = 53$) or in metastases ($n = 36$). The vertical gray lines indicate $P = 0.05$. Scores were calculated under the assumption (null hypothesis) that somatic alterations are equally distributed between primary tumors and metastases. Most somatic alterations were enriched in metastases, although only 6q loss, gain of at least three copies of 8q and gain of 1q reached statistical significance.

The sequential order of genetic alterations during metastatic progression. We deduced the sequential order in which somatic alterations undergo selection during the evolution of metastatic uveal melanoma by calculating how often a given alteration resided on the trunk versus the branch of each evolutionary tree (Fig. 5a). This analysis confirmed that $G_{\alpha}q$ pathway mutations are the earliest mutations to undergo selection, followed by gain of chromosomal arm 8q as well as $BAP1$, $SF3B1$ or $EIF1AX$ mutations. Selective pressures continue to operate on these pathways at comparatively later stages of disease progression.
points in the progression cascade by way of GNAQ LOH, additional chromatin remodeling mutations and further ramp-up of 8q CN (Fig. 5a). CNAs affecting chromosomal arms 16q, 8p, 1p, 6p and 6q underwent selection at intermediate points in the evolutionary cascade (Fig. 5a).

Finally, we compared the frequency of somatic alterations between primary tumors and metastases. Most somatic alterations were somewhat enriched in metastases, but only CN changes of 6q, 1q and high-level gains of 8q (CN increase by at least three copies) reached statistical significance (Fig. 5b). In particular, 1q gains were frequent in our cohort compared to prior studies on primary uveal melanomas, consistent with their emergence later during progression (Fig. 5 and Supplementary Fig. 5). Further studies are needed to elucidate the gene(s) driving the selection of these aberrations.

Correlations with clinical outcomes. Genomic studies of primary uveal melanomas have revealed several mutations with prognostic value. Our cohort was underpowered to discover new biomarkers of this type. However, by capitalizing on a unique aspect of our study, we instead investigated whether specific evolutionary routes of progression correlated with clinical outcomes.

Since all of our patients ultimately developed metastatic disease, we assessed correlations between clinical outcomes (disease-free survival, survival after metastasis and overall survival) and features of their genetic evolution (that is, metrics of the phylogenetic trees, such as individual and combined lengths of trunk and branches as well as branch separation; see Methods; Supplementary Fig. 6a,b). The overarching goal was to investigate whether certain evolutionary trajectories were associated with clinical outcomes, as was recently shown to be true in renal cell carcinoma.

Patients whose trees had longer metastasis branches and shorter primary branches tended to have better disease-free survival (Supplementary Fig. 6c). This comparison was statistically significant by itself ($R = 0.34, P = 0.046$), although it did not remain significant after correcting for multiple hypothesis testing. If this finding is validated in larger cohorts, it likely reflects the fact that some uveal melanomas require more genetic alterations to develop clinically detectable metastases; these uveal melanomas have better survival metrics that reflect the time required to incrementally acquire the necessary set of alterations.

Discussion
Our study shows that uveal melanomas continue to genetically evolve as they progress from primary to metastatic disease, as indicated by the fact that metastases tend to have more oncogenic mutations than primary tumors. This contrasts with most other tumor types for which metastases are genetically similar to primary tumors. One peculiar aspect of uveal melanoma is that in many cases metastases develop after a long period of latency after successful eradication of the primary tumor. This latency period may partially reflect the additional time needed to acquire the necessary mutations for growth at the distant site (Fig. 6). In addition to having more oncogenic alterations, metastases also acquire distinct oncogenic alterations, including additional copies of chromosomes 1q and 8q. This may reflect different selective pressures operating in the liver, the major organ where metastases develop in uveal melanoma.

Prior sequencing studies of uveal melanoma mostly analyzed a single tissue sample from the primary tumor. Our multi-region sequencing of primary tumors and metastases revealed multiple previously unknown mutations, expanding the catalog of driver mutations for this aggressive melanoma subtype. These novel mutations were more common in uveal melanomas with SF3B1 or EIF1AX mutations and they possibly complement the otherwise limited ability of SF3B1 and EIF1AX mutations to drive metastatic disease. The earliest driver mutations in primary uveal melanoma are not easily targeted with drugs, but there is hope that some of the newly recognized mutations found in some metastases can be targeted therapeutically.

We also observed LOH of mutant GNAQ, but not GNA11, in multiple samples, indicating hidden complexity in oncogenic signaling through mutant Gαq subunits. The Q209 alterations in GNAQ and GNA11 abrogate their GTPase activity, similar to mutant RAS. RAS mutations also undergo LOH by a variety of mechanisms that shift the allelic balance toward the mutant allele. Earlier studies using Sanger sequencing have reported increased frequency of GNA11 mutations in metastatic uveal melanomas, whereas GNAQ mutations are more common in benign lesions, such as blue nevi. This led to the proposition that GNA11 mutations are more potent. The finding of recurrent LOH of GNAQ mutations later in progression may indicate that GNAQ, but not GNA11, requires a second hit to fully activate the pathway, which could explain the association of GNA11 mutations with more aggressive disease.

Uveal melanomas have been proposed to arise after an early, punctuated burst of mutations followed by a period of neutral evolution. In our cohort, we found three cases where metastatic dissemination preceded 8q gain and potentially two cases where metastatic dissemination preceded biallelic loss of BAP1. These cases suggest that it is at least possible for metastatic dissemination to occur before the neoplasm has developed the full complement of oncogenic mutations (Fig. 6), although additional studies, focused on the earlier stages of neoplasia, are needed to fully resolve the incipient events involved in the evolution of uveal melanoma. We also found multiple added driver mutations in the branches of phylogenetic trees, arguing against neutral evolution in the latter phases of evolution.

![Fig. 6 | The evolution of metastatic uveal melanoma.](Image)

- Other CNAs’ include combinations of 16q loss, 8p loss, 6q loss or 6p gain, among others.
- ‘Additional pathogenic mutations’ include various combinations of 8q amplification, 1q gain, CDKN2A loss, SWI/SNF mutations and EZH2 mutations, among others.
In summary, our study reveals the selective pressures operating on primary tumors and metastases during the evolution of uveal melanoma and offers candidate biomarkers for staging and prognosis.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41588-019-0440-9.

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Author contributions
A.H.S., M.M.B., B.C.B. and J.F.K. conceived the study. M.M.B., A.H.S., M.M.B., B.C.B. and J.F.K. wrote the manuscript. All authors performed FISH. A.H.S., M.M.B., B.C.B. and J.F.K. interpreted the genetic data. M.M.B., K.W., S.H. and J.F.K. performed the mutation calling, CN inference and phylogenetic tree construction. A.H.S., M.M.B., R.Y., D.C., J.F.K. and B.C.B. interpreted the genetic data. M.M.B., B.C.B. and J.F.K. performed the validation assays including immunostaining, MLPA and FISH. S.V. and J.F.K. performed FISH. A.H.S., M.M.B., B.C.B. and J.F.K. wrote the manuscript. All authors reviewed the manuscript.

Competing interests
B.C.B. is a consultant for Lilly Inc.

Additional information
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Methods

Patient samples. Primary and patient-matched metastatic uveal melanoma tissues were obtained from the Ocular Tumor Division at the Copenhagen University Hospital. Most primary uveal melanomas were from archival FFPE blocks of enucleated eyes, although some were derived from fresh biopsies, as indicated (Supplementary Table 1). All metastases were liver metastases. Among the metastatic tissues, most were derived from archival blocks of liver core biopsies with a subset derived from archival blocks of liver resections, as indicated (Supplementary Table 1). All metastases were treatment-naïve. Most reference (normal) tissues were microdissected from non-lesional areas of the primary tumor blocks with some originating from patient-matched blood, as indicated (Supplementary Table 1).

This retrospective study was approved by the regional ethical committee in Copenhagen, Denmark (H-2-2013-064) and the Danish Data Protection agency (RS-2013-167). All tissues were collected in accordance with the institutional review board with regard to informed consent. The overarching design of this study is also described in the Reporting Summary.

Microdissection and DNA extraction. All microdissections were performed with a scalpel under a dissection scope and guided by a pathologist with the intention to maximize tumor cell content; 10-μm unstained sections (between four and ten levels) were used. Genomic DNA was isolated with the QIAGEN DNA FFPE Tissue Kit (QIAGEN). When possible, we microdissected multiple regions of the primary tumor. In total, we collected and sequenced 53 pieces of primary uveal melanoma, 36 pieces of uveal melanoma metastasis and 35 normal (that is, non-lesional) tissues.

DNA sequencing and analysis. A 25–250 ng quantity of genomic DNA was sheared (250-base pair target fragment size) and prepared for Illumina sequencing as described previously36,37. As expected, the smaller FFPE-derived neoplasms had lower library complexities. Therefore, we elected to perform targeted sequencing of a panel of 538 genes commonly involved in cancer (see Supplementary Table 2 for baits), favoring more samples and more coverage over a larger sequencing footprint. This decision did not hinder the overarching goal of the study, which was to delineate the order in which known driver mutations arise during the evolution of metastatic uveal melanoma. On average, each sample was sequenced to a 423-fold deduplicated coverage (see Supplementary Table 1 for more detail). Baits were developed with the customized NimbleGen SeqCap EZ Developer platform (Roche). Sequencing was performed on an HiSeq 2500 instrument (Illumina). Alignment, grooming, and mutation calling were performed with the following software package: Burrows-Wheeler Aligner v0.7.13; Genome Analysis Toolkit v3.4.46; Card Tools v1.97; and MuTect v1.1.7-3. Sequencing metrics for each sample are available in Supplementary Table 1.

CNVkit was used to infer CN information. This software can be run in reference or reference-free mode. We elected to run CNVkit in reference mode since this consistently produces higher quality CN calls when references (that is, normal tissues) are available. Two separate reference pools were generated for the study: non-neoplastic eye tissue; and non-neoplastic liver tissue. The eye reference was used to infer CN information from primary tumors whereas the liver reference tissue was used to infer CN information from metastases.

Driver gene calling. The driver genes in each case are summarized in Fig. 2. We include any mutation affecting CNAQ, GNA11, PLCB4, CSYL1TR2, BAP1, EIF4AX or SF3B1. This is based on these genes having previously described roles in driving uveal melanoma. We also include alterations affecting CDKN2A, PBRM1 and EZH2 due to their recurrence in our study and known roles as cancer genes in other tumor types. Moreover, we include hotspot mutations affecting PIK3CA and MED12, which were observed once in our study but are common in pan-tumor analyses. Finally, we include inactivating mutations affecting PTEN and PIK3R2, which are prominent tumor suppressors in other cancers and are lost in a biallelic fashion in our study.

CN estimation. To deduce the discrete levels for each CNA, we first calculated the expected log ratios for each level of gain or loss. For example, a monoallelic deletion should have a log2(tumor/reference) segmentation value of −1 because log(1/2) = −1; however, in practice, this is never observed because each tumor has some level of stromal cell contamination and stromal cells do not harbor CNAs. We took into account stromal cell contamination for each sample by proportionally weighting the expected segment value for a given CNA with the expected log(stroma/reference) from stromal cells (assumed to be 0) in that sample.

Supplementary data set covering the evolution of all 35 cases. In Fig. 1, we present the detailed evolution of an example case, including the distribution of point mutations, CNAs and allelic imbalances over each tumor region from a single patient. We analyzed all 35 cases in this cohort to the same level of detail and we include the detailed evolution of the other 34 cases in the supplementary data set hosted at Figshare (https://doi.org/10.6084/m9.figshare.6845675.v1).

Phylogenetic tree construction. Point mutation calls are available in Supplementary Table 3 and CN calls are available in Supplementary Table 4. Phylogenetic trees were constructed as described for Fig. 1 from the shared and private somatic alterations. All phylogenetic trees are rooted to the germline state. The trunk and branch lengths for each phylogenetic tree are provided in Supplementary Table 1. When multiple mutations occurred on the same segment of the phylogenetic tree, they are bracketed, serving as an indication that it is not possible to deduce the relative order in which they occurred.

Tumor cellularity inference. Most tumors are infiltrated with stromal cells, resulting in a mixture of sequencing reads derived from stromal and tumor cells. It is important to accurately measure tumor cell content to ensure that sequencing depth is sufficiently powered to detect mutations and measure the zygoty of somatic mutations. We employed a series of bioinformatic approaches to estimate tumor cell content38–40. Specifically, CNAs induce allelic imbalances over germline heterozygous SNPs; thus, we used the extent of allelic imbalance to measure tumor cellularity. We also calculated tumor cell content based on somatic mutation allele fractions after taking into account the CN status of the locus. When possible, tumor cell content was calculated from both germline and somatic variant allele fractions to produce a consensus estimate (see Supplementary Table 1).

8q CN inference. In Supplementary Fig. 4a,b, we inferred the discrete levels of CN gain over chromosomal arm 8q as described in the ‘CN estimation’ section of the Methods. We validated these estimates several ways.

In Supplementary Fig. 4c, we calculated the ratio of reads mapping to the major versus minor allele over heterozygous SNPs situated on chromosomal arm 8q. The term ‘major allele’ refers to the more abundant, or amplified, allele in the tumor. To calculate this ratio, we first subtracted the reads from stromal cells; these reads would have allelic ratios of 1:1, thereby diluting the overall ratio. The specific formula to determine the ratio of reads in the tumors cells we used is shown in equation (1):

$$N = (2xS−1)/(X−xS)$$

where $N$ is the ratio of the major to minor allele in tumor cells, $S$ is the overall major allele read fraction and $X$ is the fraction of tumor cells. For higher-level gains, there are several potential allelic combinations (see Supplementary Fig. 4c for a schematic); therefore, we show the expected relationship between absolute CN and major to minor allele ratios if considering all of these possibilities (Supplementary Fig. 4c, left panel). Next, we plotted the observed relationship between absolute CN and major to minor allele ratios from our data (Supplementary Fig. 4c, right panel). Note that the observed relationship closely mirrors the expected relationship. In particular, tumors with high levels of 8q gain were more likely to have greater ratios of major to minor allele reads. In aggregate, the allelic ratios over chromosomal arm 8q support our absolute CN inferences.

In Supplementary Fig. 4d, we used MLPA41 to measure 8q levels in eight primary tumors predicted to harbor between two and six copies of chromosomal arm 8q. DNA was extracted using the QIAGEN DNA Micro Kit (QIAGEN), followed by standard sodium acetate/ethanol precipitation for optimal purity. Probes were ligated to specific DNA sequences on chromosomes 1, 3, 6 and 8 and using the SALSA MLPA KIT P027-21 Uveal Melanoma (MRC-Holland). In addition, 12 reference probes were ligated to chromosomes 2, 4, 5, 10, 11, 12, 13, 14, 15 and 18. The signal intensities of the various probes were measured using capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems). The signal intensity of each probe was normalized to the signal intensity of each reference probe and the relative amount of each target sequence was calculated using the Coffalyser.Net data analysis software (v1.40721.1958) from MRC-Holland. The y axis of Supplementary Fig. 4d depicts the ratio of 8q signal to other parts of the genome without CNAs. It is important to note that MLPA signals are subject to saturation at higher-level gains, providing a relative (rather than an absolute) estimate of CN. We observed a near perfect correlation ($R=0.97$) between MLPA ratios and absolute CN estimates from sequencing data, supporting our absolute CN inferences.

In Supplementary Fig. 4e, we performed FISH to validate the CN of 8q levels in eight primary tumors predicted to harbor chromosome 8q. Cells were probed using the Vysis CEP3 (D3Z1), 6 (Vysis CEP6 (D6Z1)) and 8 (Vysis CEP8 (D8Z1)) and Vysis LSI MYC SpectrumOrange Probe and CEP12 (Vysis CEPI2 (D12Z3) SpectrumGreen probe) (both probes from Abbott Molecular). Slides were scanned with a fluorescence microscope (Zeiss Axio Imager Z2) and analyzed with Metafer v.3.8.12 (MetaSystems) according to the manufacturer’s instructions. Tumor-bearing areas were identified by pathologist using the 4,6-diamidino-2-phenylindole filter at low magnification (×10 lens) with the aid of hematoyxlin and eosin stain. All areas involving tumor cells were scanned and within this area cells that did not overlap and had bright signals for the probes were used for enumeration. Nuclei that had complete absence of signals for the control probe (CEP12) were excluded and at least 30 cells were included and analyzed per case. In the left panel, tumor specimens were placed in a culture medium immediately after surgery and analyzed. The following fluorescent probes were hybridized to the tissues: telomeric probe for chromosome 1p (Vysis TelVision 1p); and centromeric probes for chromosome 3 (Vysis CEPI3 (D3Z1)), 6 (Vysis CEPI6 (D6Z1)) and 8 (Vysis...
CEP8) (all probes from Abbott Molecular, http://www.abbottmolecular.com). The probes were visualized and counted using a fluorescence microscope. At least 100 cells were evaluated by two technicians and CN variations in more than 10% of the cells were used as a cutoff. The six tumors analyzed with a centromeric 8 probe were chosen because they did not have CN transitions across the centromere of chromosome 8. The CN estimates from our sequencing data typically aligned with the mode counts from the FISH data (Supplementary Fig. 4e).

Statistics. In Fig. 1b, we calculated the fraction of tumor cells in each microdissected tissue (described earlier); the blue lines are anchored at half of that value (that is, the expected MAF of a fully clonal, heterozygous somatic mutation). The shaded zones represent the 95% CIs. This was calculated under the assumption that mutant reads are sampled randomly from the total pool of mutant alleles in our DNA input; therefore, the final MAF should fall within a binomial distribution around the expected MAF of a fully clonal, heterozygous somatic mutation. Under this assumption, the s.d. of this distribution is dependent on the sequencing depth (SD) in that sample and tumor cellularity (TC) as follows: \( (0.5 \times TC^2 - (0.5 \times TC)^2 \times SD)^{0.5} \). The 95% CI was calculated by multiplying the s.d. by 1.96. Finally, these CIs were converted to fractions by dividing them by the total sequencing depth in that sample.

In Fig. 4c, we performed a two-tailed \( t \)-test, assuming equal variance, to compare the level of CN gain of chromosomal arm 8q in primary tumors (\( n = 53 \)) versus metastases (\( n = 36 \)) (d.f. = 87).

In Fig. 5b, for the genomic alterations shown in Fig. 5, we tabulated the number of times each occurred in metastases and primary tumors. We tested the probability that genomic alterations were enriched in metastases or primary tumors against the null hypothesis that the alterations should be equally distributed in each compartment. The \( P \) values shown in Fig. 5 reflect two-tailed probabilities under the assumption that our sampling of genomic alterations fits a binomial distribution of enrichments dictated by sampling size. Adjustments for multiple hypothesis testing were not performed.

In Supplementary Fig. 6, we sought to identify significant associations between clinical outcomes and phylogenetic tree features. We took several measurements of phylogenetic trees from each case (see Supplementary Fig. 6b), including tree height, trunk length, branch length and branch separation. Next, we investigated whether any of these measurements correlated with disease-free survival, survival after metastasis or overall survival. In total, we made 18 pairwise comparisons between the 6 phylogenetic tree measurements and the 3 clinical outcome data types. Each comparison comprised 35 data points corresponding to each patient in the study. The correlations are shown as \( R \) (Pearson's correlation coefficients) in Supplementary Fig. 6b. We also performed a linear regression analysis to produce \( P \) values for each correlation against the null hypothesis that the correlation coefficient (\( R \)) should be 0. Lastly, we report adjusted \( P \) values, which were corrected for multiple hypothesis testing using Bonferroni’s correction.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The raw sequencing data are available at the European Genome Phenome Archive under accession no. EGAD00001004453.

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Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- Data collection: No software was used to collect data.
- Data analysis: Sequencing reads were aligned to the human genome (version hg19) using BWA (version 0.7.13). Indel realignment and base quality recalibration was performed with Genome Analysis Toolkit (version 3.4.46). Deduplication was performed with Picard (version 1.97). Variants were called with MuTect (version 1.1.7-3). Copy number was inferred with CNVkit (version 0.9.5).

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Our raw sequencing data is currently available from the European Genome-Phenome Archive (EGAD00001004453)
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | We performed multi-region sequencing of 124 primary and metastatic tissues from 35 patients. This sample size was chosen because it provided statistical power to detect recurrent patterns in as few as 10% of patients or 4% of tissues. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data exclusions                                                                                                                                                                               |
| Replication | Genetic findings were orthogonally validated against one another. For instance, copy number alterations were inferred from read depth and validated further by confirming allelic imbalance over the loci. A full description of our validations are available in the methods. |
| Randomization | "Randomization" is not relevant to this study because this is a retrospective and descriptive study of the genomes of uveal melanomas.                                                                  |
| Blinding | "Blinding" is not relevant to this study because this is a retrospective and descriptive study of the genomes of uveal melanomas.                                                                     |

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| ☒ Animals and other organisms | ☒ Human research participants |
| ☒ Human research participants | ☒ Clinical data |

Human research participants

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| Population characteristics | The average age of the patients in our cohort was 64 years old. Our cohort comprised 15 females and 20 males. All patients were diagnosed with with metastatic uveal melanoma. |
|---------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Recruitment               | This was a retrospective study of archival tumors from the Ocular Tumor Division in Copenhagen University Hospital. This is a major referral center for patients with uveal melanoma within Denmark and across Europe. Our tumors reflect this patient population, and it may therefore be biased in favor of patients with European ethnicity. |
| Ethics oversight          | This study was approved by the regional ethical committee in Copenhagen, Denmark (H-2-2013-064) and the Danish Data Protection agency (RH-2015-167)                                                   |

Note that full information on the approval of the study protocol must also be provided in the manuscript.