Phylogenetic analyses of melanoma reveal complex patterns of metastatic dissemination

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Melanoma is difficult to treat once it becomes metastatic. However, the precise ancestral relationship between primary tumors and their metastases is not well understood. We performed whole-exome sequencing of primary melanomas and multiple matched metastases from eight patients to elucidate their phylogenetic relationships. In six of eight patients, we found that genetically distinct cell populations in the primary tumor metastasized in parallel to different anatomic sites, rather than sequentially from one site to the next. In five of these six patients, the metastasizing cells had themselves arisen from a common parental subpopulation in the primary, indicating that the ability to establish metastases is a late-evolving trait. Interestingly, we discovered that individual metastases were sometimes founded by multiple cell populations of the primary that were genetically distinct. Such establishment of metastases by multiple tumor subpopulations could help explain why identical resistance variants are identified in different sites after initial response to systemic therapy. One primary tumor harbored two subclones with different oncogenic mutations in *CTNNB1*, which were both propagated to the same metastasis, raising the possibility that activation of wingless-type mouse mammary tumor virus integration site (WNT) signaling may be involved, as has been suggested by experimental models.

metastasis | melanoma | genetics

As in many other solid tumors, melanoma metastases often first present in lymph nodes in the draining area of the primary, whereas distant metastases tend to appear later (1). The conclusion that melanoma follows a linear progression from primary tumor to regional to distant metastases has supported preemptive surgical removal of regional lymph nodes with curative intent (2). However, several observations suggest that distant metastases are seeded early, contemporaneously with regional metastases. Patients who undergo resection of lymph node basins harboring metastasis do not experience a significantly extended life expectancy (3, 4). Furthermore, circulating melanoma cells were detected in the blood of 26% of patients who only have metastases detected regionally (5, 6).

Melanoma, like other cancers, arises and evolves through the accumulation of genetic alterations within tumor cells (7–9). Comparing somatic mutations in primary tumor and regional and distant metastases from the same patient can provide insight into the phylogenetic relationships between these distinct tumor cell populations and the order of metastatic dissemination (8, 10). These analyses may also establish whether cells in the primary tumor that metastasize acquired this ability to disseminate and seed other anatomic sites by a newly acquired genetic alteration, or whether metastatic colonization is simply a stochastic process of which all cells in the primary are capable but few succeed.

Using whole-exome sequencing (for discovery) and targeted sequencing (for validation), we analyzed mutation patterns of primary melanomas and two or more metastases in each of eight patients (Datasets S1–S8) to determine their phylogenetic relationships.

Results

Mutational Landscape of Metastatic Melanoma. The number of somatic single nucleotide variants (SSNVs) detected varied from 96 to 115 per exome in primary melanomas from acral or intermittently exposed skin (patient E: heel, patient B: back) to more than 4,900 in primary melanomas from chronically sun-exposed sites, corresponding to 4,800–245,000 mutations per genome (seven of eight patients with high-confidence copy number

Significance

Subpopulations of cells in a primary melanoma sometimes disseminate and establish metastases, which usually cause mortality. By sequencing tumor samples from patients with metastatic melanoma never subjected to targeted therapies, we were able to trace the genetic evolution of cells in the primary that seed metastases. We show that distinct cells in the primary depart multiple times in parallel to seed metastases, often after evolving from a common, parental cell subpopulation. Intriguingly, we also determine that single metastases can be founded by more than one cell population found in the primary cancer. These mechanisms show how profound genetic diversity arises naturally among multiple metastases, driving growth and drug resistance, but also indicate that certain mutations may distinguish cells destined to metastasize.

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estimates for all tumors are shown in Fig. 1). SSNVs detected at any allele frequency (AF) in the primary melanomas and all corresponding metastatic tissues were considered fully shared (Fig. 1, bottom black tier), including known oncogenic mutations in melanoma such as $BRAF^{V600E}$ (11), $CTNNB1^{S33P}$ (12), and in the *TERT* promoter (13). Fully shared variants detected at fully clonal AFs (i.e., ~100%) in the primary were considered "ancestral"—they occurred early during population expansion of the cells comprising the primary, before any metastases had formed. In contrast, SSNVs not fully shared between primary and metastatic tumors revealed distinct evolutionary histories and are shown in the upper portion of Fig. 1 as partially shared (red) and private (gray).

Using DNA extracted from additional tissue sections of tumors of patients A, C, E, F, and G, we validated with ion semiconductor sequencing at an average depth of 953 reads the AFs of 72 representative SSNVs originally discovered by exome sequencing. All SSNVs detected in metastases by exome sequencing were also detected by targeted sequencing. In several instances, SSNVs were only detected in some of multiple, independent tissue sections of the primary, probably reflecting tumor heterogeneity of these variants (Datasets S6 and S8).

Metastatic Dissemination Occurs in Parallel from Cell Populations in the Primary Tumor to Regional and Distant Sites. There is controversy whether melanoma sometimes disseminates widely without an interim regional stage. If metastatic dissemination cascaded from locoregional to distant locations, one would expect regional and distant metastases to reside on a common phylogenetic branch.

SSNVs partially shared between the primary and some, but not all metastases, revealed instances in which different subpopulations of the primary seeded independent metastases in parallel rather than in series. In these cases, cells in the primary both before and after acquisition of the partially shared variants must have established metastases. Exome sequencing in patients A, C, and F each clearly revealed two such distinct parental subpopulations in primaries shared only with some metastases (Fig. 2 and Dataset S7). Therefore, their metastases most likely arose from independent cells in the primary, rather than evolving from other metastases.

In patient F, validation sequencing confirmed that at least two SSNVs were present subclonally in the primary, absent in the locoregional metastasis, yet detected in the lymph node and distant skin metastasis (*TBC1D1* and *ABCA8*; Datasets S3 and S7; class 5). Conversely, at least two SSNVs were present subclonally in the primary, absent in the lymph node and distant metastases, yet present in the locoregional tumor (*ZNF165* and *BPTF*; Datasets S3 and S7; class 7). Thus, we can conclude that the locoregional tumor arose from a cell population in the primary distinct from the one generating the lymph node and distant metastasis.

In patient H, deletion of one copy of chromosome 4 was seen in the primary and the brain metastasis, yet absent in the lymph node and locoregional skin metastases (Dataset S5), showing that the latter tumors could not have given rise to the brain metastasis. Furthermore, the private mutations from both the lymph node and brain metastasis harbored a predominance of C > T substitutions (1725/1849 and 804/973 SSNVs, respectively), suggesting that these metastases also arose from different cells in the primary (Fig. 2). It is unlikely that these UV-driven mutations were acquired in a tumor other than the primary.

In patient D, where the primary was found in the right leg, four SSNVs were partially shared by the groin lymph node and in transit right leg locoregional metastases, and five additional SSNVs were partially shared by only the two locoregional metastases (Datasets S1 and S7). The anatomic relationship makes it unlikely that cells in the groin lymph node returned to found multiple locoregional metastases only in one foot, close to the primary. Therefore, we posit that the five partially shared SSNVs were acquired in distinct cells of the primary, some of which disseminated and formed two distinct subpopulations in the locoregional metastases.

In patient E, a cell population harboring an interstitial deletion of the wild-type copy of *CTNNB1* was detected at subclonal proportions in two separate metastases (Datasets S5 and S9), strongly suggesting that they were founded by a single, undetected

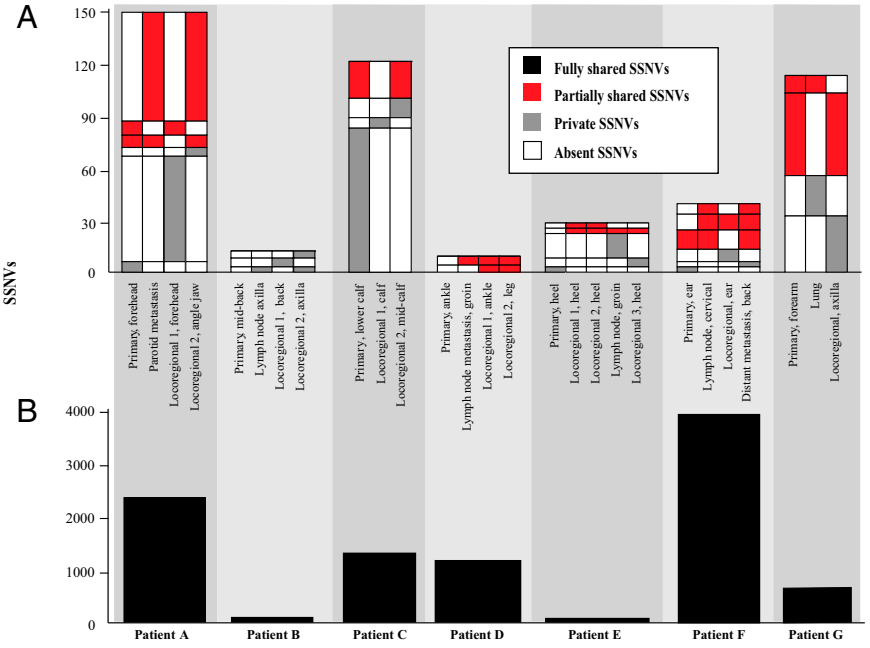

Fig. 1. Patterns of somatic mutations (SSNVs) in primary and metastases illustrate evolutionary divergence during metastatic dissemination. For the seven of eight patients also yielding high-confidence copy number estimates, the number of SSNVs in each tumor tissue is shown. (*A*) The number of fully shared SSNVs present in all tumors is displayed in *Lower* (black). (*B*) The number of SSNVs not present in all samples is shown in the upper column graph. The SSNVs not present in all samples are further subdivided for each sample into tiers of SSNVs: those that are partially shared with other tumors (red), those that are private to the sample (present only in one tumor of a patient, gray), and those not detected in the sample (white). Tiers with fewer than three SSNVs are not displayed. The SSNVs within each tier are listed in Dataset S3 and their interpretation is annotated in Dataset S7.

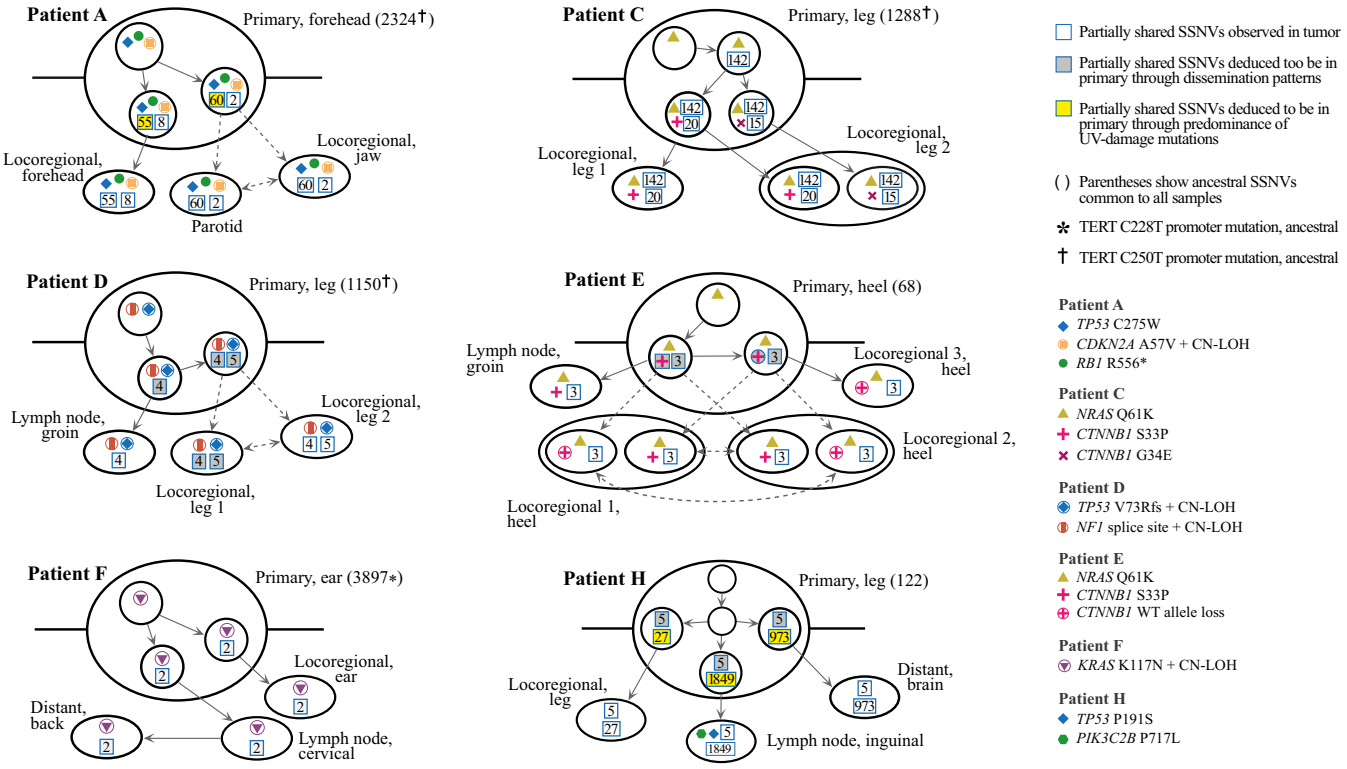

Fig. 2. Metastases depart the primary melanoma in parallel, from genetically divergent cell subpopulations. For patients A, C, D, E, F, and H, phylogenetic history of the metastasizing cells is reconstructed based on sequencing of fresh and formalin-fixed, paraffin-embedded (FFPE) portions of each tumor (Datasets S3, S6, and S8). Solid arrows denote probable dissemination routes, and dotted arrows denote multiple possible paths. Numbers in squares denote partially shared SSNVs. Instances of SSNVs deduced to be in the primary, but not detected directly by sequencing, are color coded by line of reasoning. The patterns of dissemination demonstrate that metastases in each patient derived from distinct cells in the primary, which often demonstrate extensive genetic divergence from each other.

cell subpopulation in the primary. Overall, six of eight of our patients showed clear evidence of parallel dissemination of metastases from the primary tumor.

Metastases Can Be Founded by Multiple, Genetically Distinct Cell Populations. Copy number aberrations (CNAs) with read counts from exome sequencing significantly below the expected threshold for full clonality were considered subclonal (14) (detailed methods in *Supporting Information*). Each patient had at least one tumor with such subclonal CNAs (range 1–10 per patient, with a maximum of 6 per tumor; *Supporting Information* and Datasets S5 and S9). Subclonal CNAs in the primary must have arisen after the lesion was founded. Any metastasis founded from cells of such a subclone should harbor that new aberration in every cell.

Unexpectedly, in patient E, the identical 34-Mb interstitial deletions on chromosome 3 deleting *CTNNB1* were observed in both locoregional metastases 1 and 2 at subclonal levels (Fig. 3). Given the identical flanking breakpoints shared by the deletions, it is unlikely that they arose independently in these two metastases. We conclude that these metastases must each have been founded by at least two cell populations: one harboring the deletion and one without. In no other patients were we able to identify identical, unique, subclonal CNAs in more than one metastasis.

We were interested to learn if our SSNV data could also reveal multiple cell populations founding single metastases. Because individual SSNVs provide insufficient power to identify subclones definitively, we searched for clusters of partially or fully shared SSNVs with similar subclonal AFs, indicative of subpopulations. We estimated the percentage of tumor cells carrying each individual SSNV in diploid regions, taking into account the normal cell contamination and copy number at the locus harboring the SSNV. In only patient C, four such clusters of SSNVs were discerned, using a grouping algorithm, in the primary and both metastases. Each of these four clusters of SSNVs showed coordinate changes in tumor variant percentage (TVP) between the primary tumor and two metastases (Fig. 4 and Dataset S10).

The clusters generated by this algorithm make it clear that some fully clonal SSNVs in the primary are also fully clonal in both metastases (Fig. 4, yellow triangles). Some subclonal SSNVs in the primary are fully clonal in both metastases (blue ✖'s). Two other clusters of subclonal SSNVs present in the primary at lower abundance (red diamonds and green circles) do not cosegregate in locoregional metastasis 1, indicating that each must reside in a different cell population. Surprisingly, however, both of these distinct subpopulations are detected in locoregional metastasis 2 in patient C. Therefore, the sequencing data in both patients C and E suggest that their metastases were seeded not by single cells, but by at least two cells with distinct genetic identities.

Intriguingly, in both patients, the defining subclonal populations also included alterations in *CTNNB1*. In patient E, the interstitial deletion on chromosome 3 removed a wild-type copy of *CTNNB1*, leaving a hemizygous *S33P* mutation. In patient C, two different known oncogenic *CTNNB1* variants were detected that appeared to have arisen in distinct tumor subpopulations, evidenced by their location within distinct clusters of SSNVs (Fig. 4). The G34E variant, part of the SSNV cluster annotated with green circles, was estimated to be present in 6% of the cells in the primary, absent in locoregional metastasis 1, and present in 75% of cells in locoregional metastasis 2. By contrast, the S33P variant, in the SSNV cluster denoted by red diamonds, was present in 31% of cells in the primary, fully clonal in locoregional metastasis 1, and present in 25% of the cells of locoregional metastasis 2. Both subpopulations must have disseminated independently to locoregional metastasis 2. It is remarkable that known activating mutations in β-catenin emerged multiple times among these late-arising variants associated with metastases. Activation of the wingless-type mouse mammary tumor virus integration

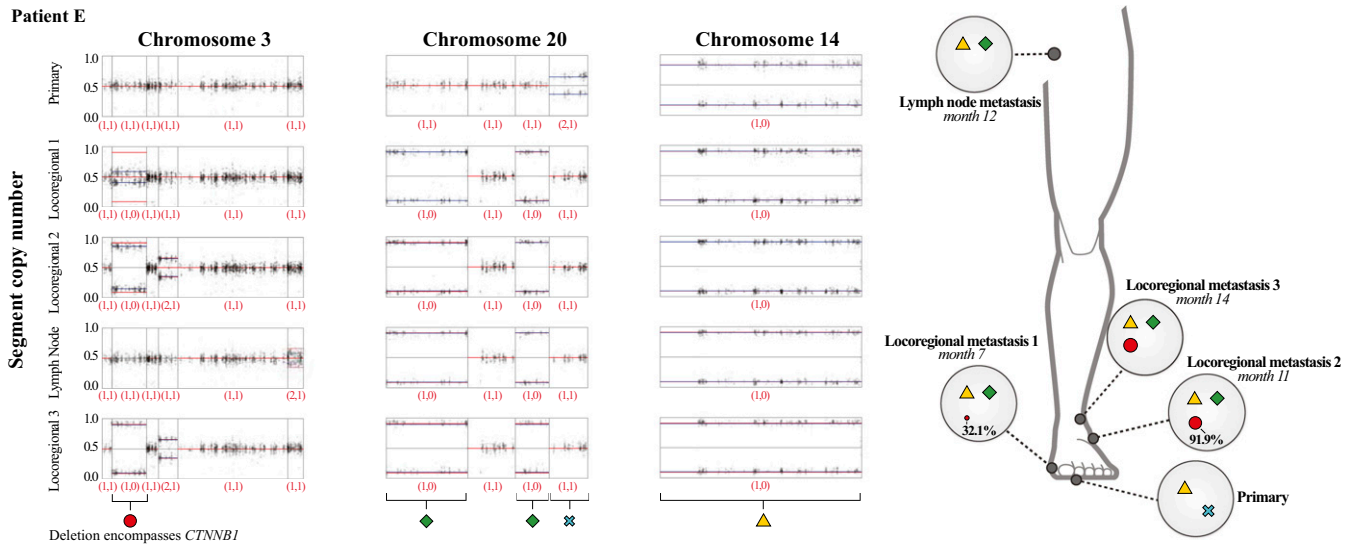

Fig. 3. Identical subclonal deletions in different metastases reveal multiple founding populations during metastatic dissemination. The scatter graphs chart show on the *y* axis, for tumors of patient E, the allelic fraction of heterozygous SNPs along chromosomes 3, 20, and 14. Shown are the primary tumor, three locoregional metastases, and a lymph node metastasis, ordered from top to bottom according to time of clinical presentation. Divergence of the allelic fractions from 0.5 indicates a copy number change. The resulting allelic states are shown in the red numbers underneath each segment. The red lines depict the expected allelic fraction, if the CNA were present in all cells of the tumor, taking into account the normal cell contamination. Blue lines indicate the observed average copy number level for each CNA. The 33.9-Mb region on chromosome 3 represented by the red circle shows a subclonal deletion in locoregional metastases 1 (TVP = 32.1%, 99%CI = 28.0–35.6%) and 2 (TVP = 91.9%, 99%CI = 90.4–93.2%) and fully clonal deletion in metastasis 3 (TVP = 100%) (*Supporting Information* and Datasets S5 and S9). Chromosome 20 shows two separate deletions reaching from 0–25.53 Mb and from 40.39–50.93 Mb, respectively, represented by the green diamond, which are present at fully clonal levels in all metastases but absent in the primary tumor. One entire copy of chromosome 14 is deleted at fully clonal levels in all tumors and is thus considered fully shared (yellow triangle). The presence of the deletion from 9.72–43.6 Mb of chromosome 3 at subclonal levels in locoregional metastases 1 and 2 suggests at least one of these tumors was founded by two distinct cell populations: one harboring the chromosome 3 deletion and one without.

site (WNT) signaling pathway (e.g., by β-catenin mutations) has also been implicating in promoting metastatic potential in mouse models of melanoma (15, 16). The reconstructed evolution of metastatic dissemination in patient C is depicted in Fig. 5.

Metastases Are Founded by Common, Parental Cell Subpopulations in the Primary Melanoma. As discussed above, in patients C and D, we found evidence of stepwise evolution of the metastatic subpopulations in the primary melanoma, supporting a common progenitor of metastases that arose over time in the primaries.

A distinct line of evidence supporting descent of metastatic cell populations from specific, subclonal populations in the primary is the presence of SSNVs partially shared among all metastases but not detected in the primary. In such cases, the population of cells in the primary that spawned the metastases must either have: (*i*) resided in the unsequenced portion, (*ii*) resided at undetectable (<1%) subclonal levels in the sequenced portion, or (*iii*) originated from a separate metastasis not analyzed in our study. Three such SSNVs were detected in patient E (class 3; Datasets S3 and S7) and five such SSNVs were detected in patient H (class 5; Datasets S3 and S7).

In patient F, four SSNVs partially shared between the primary and specific metastases were not detected in tissue representing at least 10% of the primary tumor used for exome and validation sequencing (classes 5 and 7; Dataset S8), indicating origin in a subclonal population in the remainder of the primary. Therefore, in five of six patients (C, D, E, F, and H) in whom metastases originated from at least two distinct subclonal populations in the primary, these subclonal populations themselves descended from a common, parental subclonal population.

Discussion

Our study reframes current models of metastatic dissemination. Although our cohort consists of only eight patients, the difficulty in collecting frozen tissues from samples of primaries and multiple metastases from patients, in the absence of systemic treatment, presents a substantial challenge to assembling larger series. Our collected materials enabled us to analyze, at high sequence coverage (Fig. S1), distinct regions of both primary melanomas and their matched metastases. The resulting data allowed us to delineate and validate the phylogenetic relationship between tumor populations at different sites. Our study provides evidence that metastatic dissemination occurs from different subpopulations of the primary tumor, which often disseminate in parallel rather than in serial fashion to form regional and distant metastases. The metastasis of genetically distinct cell populations from primary melanomas likely enhances the heterogeneity of tumor tissues, potentially contributing to drug resistance (17, 18).

The sequential concept of metastatic dissemination is based on the clinical observation that regional metastases are often detected earlier than distant metastases (2). Although explanations such as secretion of growth stimulating factors from the primary have been proposed (19, 20), our observation of multiple founding populations in metastases raises the possibility that regional metastases may grow faster because their proximity to the primary increases the probability of repeated seeding events, as has been demonstrated experimentally in breast cancer (21, 22).

It is conceivable that in some cases the multiple founding populations in metastases may be the result of disseminating cell clusters, as has been detected recently in both mouse models of melanoma and in patients (23). Interestingly, the circulating clusters in breast cancer also show enhanced signaling through catenin (24), a molecule also activated in several of our metastases. The phenomenon reported here in human melanoma patients may explain certain patterns of disease relapse of patients treated with targeted therapy. For example, if multiple metastases were partially founded by a specific cell population harboring a resistance variant, these metastases may simultaneously resume growth after an initial period of response. Such a pattern has been shown for MAPK/ERK kinase mutations in a patient whose B-Raf proto-oncogene (BRAF) mutant melanoma initially responded to rapidly accelerating fibrosarcoma (RAF) inhibition but then showed striking multifocal relapse (25).

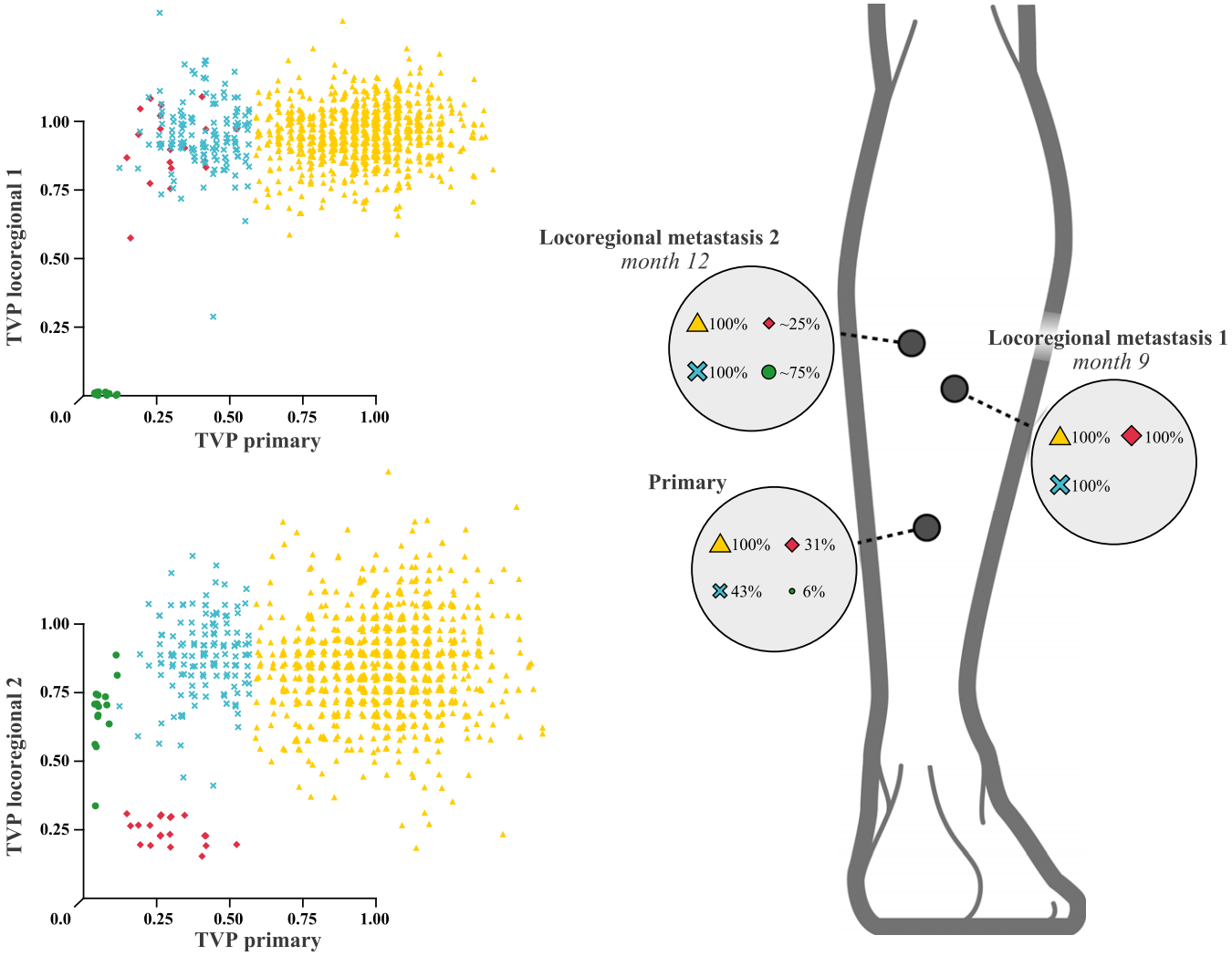

Fig. 4. Identical subclones in different metastases, as defined by SSNVs, reveal multiple founding populations during metastatic dissemination. The scatter graphs show, for patient C, the TVPs for all SSNVs in genomic regions not affected by copy number changes (Dataset S10). Shown are the TVPs for the primary tumor on the *x* axes and the locoregional metastases 1 (upper graph) and 2 (lower graph) on the respective *y* axes. Fully shared SSNVs are depicted as yellow triangles and are present in all tumors at fully clonal levels. A subclone present in ∼30% of the cells of the primary tumor (blue ×) is present at close to fully clonal levels (TVP = 100%) in both metastases. A second subclone with TVP of 25% in the primary (red diamonds) is fully clonal in metastasis 1 but at a TVP of 25% in metastasis 2, suggesting that metastasis 2 was seeded by at least two genetically distinct founding cells, one containing the SSNVs depicted as red diamonds and one without. A third subclone present at 3% in the primary melanoma, (green circles) is absent in metastasis 1 but present at ∼75% abundance in metastasis 2, indicating that it has contributed partly, but not entirely, to the cells of metastasis 2. This third subclone therefore also indicates that metastasis 2 was founded by two genetically distinct populations.

Finally, we demonstrate that the ability of cells to establish metastases can be a trait that emerges from a subclonal parent, suggesting changes are required in addition to the alterations required to establish the primary tumor. It is possible cells founding metastases repeatedly descended from a common parent simply by chance. However, in patients C and E, whose primaries harbored activating *NRAS* mutations, all six metastases were founded only by subclonal populations of the primary that had acquired activated *CTNNB1* (Figs. 4 and 5). Beta-catenin has previously been experimentally associated with metastasis in melanoma (15, 16). Of the two cell populations metastasizing in patient C, each acquired a different, known activating mutation in beta-catenin (S33P and G34E), consistent with a necessity for *CTNNB1* activation in forming metastases.

Although later evolution of metastatic cell subpopulations has been reported in some pancreatic cancers (10, 26), it was not detected in single-cell sequencing analysis of breast cancer metastasis (27). A model in which some primary melanomas require additional aberrations to metastasize may explain why their early detection and removal confers a survival benefit, as excision of tumors before the emergence of a clone with enhanced metastatic capability would be expected to be curative (28). If confirmed, these metastasis-enabling mutations could serve as biomarkers to identify primary melanomas at risk for dissemination.

Methods

Patients, Sample Preparation, and Sequencing. Fresh frozen tissues from primary melanomas, corresponding metastases from lymph nodes, visceral sites, or skin, and matching normal DNA, were obtained from eight patients from Melanoma Institute Australia (MIA) and Memorial Sloan-Kettering Cancer Center (MSKCC) with the approval of their Institutional Review Boards. (Table S1, Dataset S1, and *Supporting Information*). A 5-μm-thick frozen section was cut and areas containing >80% tumor nuclei and <30% necrosis were dissected. Five micrograms of DNA from dissected tumors and matched DNA from peripheral blood were isolated. About 71 Mb of coding sequence were targeted using Agilent SureSelect Exome Capture kits with the v4 exome + untranslated region (UTR) bait library. Sequencing was performed using an Illumina HiSeq2000 instrument.

Calling Single Nucleotide and Copy Number Variants. Somatic point mutations, referred to here as single nucleotide variants (SSNVs), were called using a

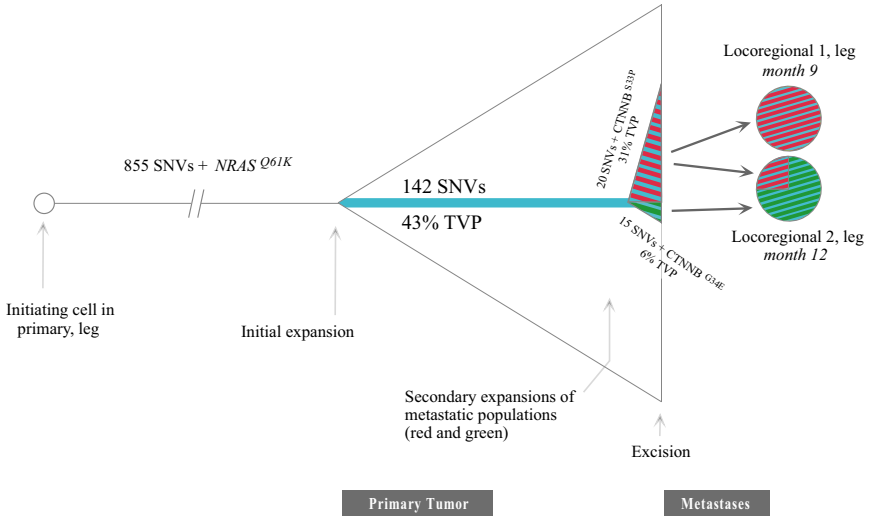

Fig. 5. An integrated portrait of metastatic subclone formation, departure, and arrival for patient C. The ancestral cell harboring 855 SSNVs proliferated, generating the primary tumor. During expansion into the primary, a specific cell acquired 142 more SSNVs and then two cells from that subpopulation subsequently acquired 15 more (red) and 20 more (green) SSNVs. Intriguingly, each of these later-evolving subpopulations (red and green, identical to those seen in metastases) each acquires a different known oncogenic *CTNNB1* mutation. Both subclones are seen in locoregional metastasis 2, suggesting that once the ability to metastasize is acquired, the competent subclone can either reach existing metastases or travel with other metastatic subclones simultaneously.

standard pipeline (*Supporting Information*) and sample identity was verified using genotype information (Dataset S2). In brief, for each patient, any high-confidence SSNV PRESENT in at least one tumor sample was also called PRESENT in any other sample in which supporting reads were found. Copy number variants were identified from exome data using an iterative aggregation method (*Supporting Information*). To determine the fraction of tumor cells carrying an SSNV or CNA, a tumor variant percentage (TVP) was calculated based on estimates of normal cell contamination, mutant AF (SSNVs), copy number state, and allelic ratio of heterozygous SNPs within CNAs. To reduce stochastic error, further analysis was only performed for SSNVs with a minimum read depth of 42, which on average supported a TVP with 99% confidence interval (CI) < 1 across study patients. If three or more such SSNVs displayed identical PRESENT/ABSENT patterns across samples in a patient, they were classified for closer inspection (Datasets S3 and S7). For CNAs, multiple sources of data bias were identified and corrected for before calculation of both a TVP and a 99% CI where data were sufficient (Datasets S5 and S9 and *Supporting Information*).

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