Most meningiomas carry mutations in the tumor suppressor neurofibromatosis gene 2 (NF2) on chromosome 22q, while NF2-wildtype meningiomas account for about a third of all meningiomas [4, 7]. In non-NF2-mutated cases, SMO, POLR2A, PIK3CA, AKT1, and KLF4 mutations, the latter both regularly with TRAF7 mutations, have been described [1–3, 5]. TRAF7, a E3 ubiquitin ligase which promotes degradation of p53 and p65 as well as a number of oncogenic protein targets, including NEMO, c-FLIP, and c-Myb, occurs in nearly one-fourth of all meningiomas (24% in Clark et al., 30% in Reuss et al.). However, some studies may have been enriched for specific subtypes. In almost half of the cases they co-occur with AKT1 (44% in Clark et al.) or KLF4 (36% in Clark et al.), respectively [3, 8]. The combination is intriguing: AKT1/TRAF7 mutations are associated with meningothelial histology and basal localization, while KLF4/TRAF7 mutations are highly specific for secretory meningioma without any predominant localization. Also, AKT1 and KLF4 have clear hotspots, with all mutations occurring at AKT1E17K or KLF4K409Q. In contrast, TRAF7 mutations can occur throughout the WD40 domain of the protein (Supplementary Fig. 4, online resource). The order of mutational acquisition, whether alterations in TRAF7 or in AKT1/KLF4 occurs first, remains elusive.

The analyses here were initiated after diagnostic work-up of the tumors of a 47-year-old male patient with two independent meningiomas having identical somatic TRAF7 mutation N520S, but separate AKT1 (skull base, meningothelial) and KLF4 (convexity, secretory type) hotspot mutation (Fig. 1a). Of note, no TRAF7 mutation was detected in germline control DNA and surgical resection was performed at the same time. Although mere coincidence cannot be ruled out, this may be caused by a mosaicism for TRAF7 affecting arachnoidal cells, or a single ancestor of...
both tumors despite macroscopically separate location. Both the latter strongly suggest TRAF7 as the initiating mutation.

In an additional 28 cases of $\text{AKT1}^{\text{mut}}/\text{TRAF7}^{\text{mut}} (n = 13)$ and $\text{KLF4}^{\text{mut}}/\text{TRAF7}^{\text{mut}} (n = 15)$ from our database we compared the variant allele frequencies (VAFs) for possible indications of heterogeneity and temporal sequence. 27/28 of the co-mutated samples were classified as WHO grade I meningiomas. 12/15 tumors of the $\text{KLF4}^{\text{mut}}/\text{TRAF7}^{\text{mut}}$ cohort were expectedly of the secretory subtype, one tumor was of transitional subtype (subtyping and grading based on
The panel consisted of 392 amplicons covering 28 genes and/or cell DNA sequencing on 7 samples of individual cells. We, thus, performed amplicon-based single-cell clone analysis of sample KLF4_4 based on the presence of TRAF7 N520S and KLF4 K409Q as indicated in the Supplementary Methods, online resource. Over-view of the distribution of mutations in seven co-mutated meningiomas. Dots represent single cells while the color indicates their mutational status. Clones are numbered according to their phylogenetic order.

Looking at mutational co-occurrence in bulk data, mutations assigned with higher VAFs, unless explained by copy number changes, are typically thought to be acquired earlier than those with lower VAFs. Our bulk-measured variant allele frequencies, being similar for both mutations (p-values received from two-sided Wilcoxon signed-rank test AKT1\textsuperscript{mut}/TRAF7\textsuperscript{mut}: p = 0.75; KLF4\textsuperscript{mut}/TRAF7\textsuperscript{mut}: p = 0.62) suggested no major gap between the time points of mutational acquisition (Fig. 1b, for single-cell VAFs, see Supplementary Fig. 2). However, the majority of single-mutated cases (18/33, Supplementary Methods, online resource) harbored mutations in TRAF7 only, with fewer cases being only KLF4 (n = 3) or only AKT1 (n = 12) mutant.

Single-cell sequencing technologies allow more insight into the clonal architecture and complexity of thousands of individual cells. We; thus, performed amplicon-based single-cell DNA sequencing on 7 samples of TRAF7 with KLF4 or AKT1, respectively, co-mutated meningiomas. For this purpose, a custom panel covering the variants of interest and other genes relevant in CNS tumors was designed based on our custom panel for routine next-generation sequencing [6]. The panel consisted of 392 amplicons covering 28 genes and the TERT promoter region (Supplementary Table 2, online resource). A total of 875,000 cells were prepared resulting in a median throughput of 2315 cells per sample (interquartile range (IQR): 1622–2953) and a median coverage of 105X (IQR: 88X–122X) using the droplet-based technology of Mission Bio (Tapestri). For more details on sequencing metrics see Supplementary Table 3, online resource.

Genotype clustering analysis was performed using the Tapestri bioinformatics pipeline v2. In short, single cells were initially classified into clonal populations based on the variants known from bulk sequencing data. Only cells genotyped for both of the variants were included.

Our data revealed three subclones in each sample: one wildtype clone (potentially stroma cells), another clone carrying a mutation in TRAF7 without any mutation in KLF4 or AKT1 (detected for 6/7 samples) and another clone harboring the co-mutations in TRAF7 and KLF4 or AKT1 (Fig. 1c and d), for details on clone sizes (see Supplementary Table 3, online resource). This clearly indicates the TRAF7 mutation as being acquired at an earlier stage than KLF4 or AKT1 in the majority of cases. Interestingly, 1/7 samples showed one clone carrying only a heterozygous KLF4 mutation and another with the KLF4 mutation along with the TRAF7 mutation, both in addition to a wildtype clone.

Although single-cell DNA sequencing in particular is associated with technical challenges such as false positive variant calling and allelic dropouts, high numbers of recovered cells as well as high sequencing metrics allow conclusive information on cellular zygosity and a robust analysis of mutational acquisition. In our cohort, the single-cell data suggest, in this small series, that TRAF7 mutation is typically acquired first, but in line with bulk data also supports that the co-mutation of TRAF7 is still either not indispensably needed in every KLF4 or AKT1 mutant meningioma, or that some TRAF7-modifying events are not captured with the current approaches.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00401-022-02485-6.

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