Mutational topography reflects clinical neuroblastoma heterogeneity

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Mutational topography reflects clinical neuroblastoma heterogeneity

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Abstract

Neuroblastoma is a pediatric solid tumor characterized by strong clinical heterogeneity. Although certain complex genomic alterations, such as extrachromosomal DNA amplifications (ecDNA), have been recurrently detected in neuroblastomas, the mutational processes involved in their generation remain largely unclear. By examining the topography of complex rearrangements along with mutational signatures derived from all variant classes, we identify previously unrecognized co-occurring mutational footprints, which we termed mutational scenarios. We demonstrate that clinical neuroblastoma heterogeneity is linked to differences in the processes driving these mutational scenarios. Whereas high-risk *MYCN*-amplified neuroblastoma genomes were characterized by signs of replication slippage and stress, homologous recombination-associated signatures defined high-risk non-*MYCN*-amplified patients. Non-high-risk neuroblastomas, on the other hand, were marked by footprints of chromosome missegregation. This analysis provides a systematic perspective on the repertoire of mutational patterns that contribute to clinical neuroblastoma heterogeneity.

Introduction

The presence of somatic mutations is a hallmark of cancer genomes¹. Somatic mutations present diverse typology and are a result of different endogenous and/or exogenous mutational processes, including replication errors, exposure to DNA damaging agents, expression of developmentally-restricted recombinases², and errors in DNA repair mechanisms³. These processes imprint characteristic patterns of mutations in the genome, termed mutational signatures⁴. Recent analyses in multiple cancer types have extracted signatures associated with single-nucleotide variants (SNVs)^{5,6}, small insertions and deletions (indels)⁶, copy number alterations (CNAs)⁷, and structural variants (SVs)⁸. Some signatures are linked to known biological processes active in cancer, whereas others have yet unknown etiologies. In contrast to mutational signatures derived from SNVs, indels, and CNAs, the classification of SVs involving multiple genomic regions remains difficult. Various SV pattern classes have recently been defined based on their topography, e.g. extrachromosomal circular DNA⁹ (ecDNA), chromothripsis¹⁰, chromoplexy¹¹, templated insertion chains (TICs)¹², breakage-fusion-bridge cycles¹³ (BFB), complex non-cyclic amplicons¹⁴ (CnC), tyfona, rigma, and pyrigo¹⁵, amongst others. Even though the study of mutational signatures and structural variant patterns has advanced in recent years, it remains largely unclear how different mutational processes and variant topographies are mechanistically linked.

Neuroblastoma is a pediatric solid tumor characterized by strong clinical heterogeneity^{16–18}. Patients can be classified into different disease risk groups depending on their clinical, genetic, and molecular characteristics, namely low-, intermediate- or high-risk, with the latter presenting poor prognosis despite intensive therapy and surgery¹⁹. Previous reports focusing on SNV-related signatures have revealed signature SBS18, associated with reactive oxygen species, and signature SBS3, caused by impaired homologous recombination, to be the most prevalent in neuroblastoma²⁰. Complex SV patterns as well as CNA signatures, on the other hand, have not yet been comprehensively investigated in neuroblastoma to date. Complex rearrangements involving oncogene amplicons are of particular importance in neuroblastoma^{21,22}, as evidenced by the clinical impact of ecDNA-mediated oncogene amplification and its association with complex SV patterns. Recent studies have linked ecDNA to other complex variants such as chromothripsis and BFB^{23,24}. However, our current understanding of complex rearrangement patterns and mutational processes, their co-occurrence, as well as their impact on clinical outcomes in neuroblastoma remains limited. Applying variant detection algorithms and signature analyses to Illumina short-read whole-genome sequencing (WGS) from a cohort of 114 neuroblastoma tumor-normal pairs, we extracted 12 catalogued mutational signatures associated with SNVs and indels as well as 19 recently defined CNA and SV signatures. We also identified 4 previously undescribed SV signatures, enabling a comprehensive description of the mutational processes active in neuroblastoma. Using statistical and graph-based methods, we integrated these mutational signatures with 8 complex SV pattern classes as well as clinical annotations resulting in a catalog of complex rearrangements in clinically heterogeneous neuroblastomas.

Results

Cohort characteristics

To explore the topography of mutational processes in neuroblastoma, we analyzed WGS matched tumor-normal pairs from 114 patients. Tumors from all disease stages, sex, and age intervals were represented in our samples (Suppl. Fig. 1). 21% of genomes were derived from high-risk *MYCN*-amplified (HR MNA) neuroblastomas, 36% from high-risk non-*MYCN*-amplified cases (HR non-MNA), and 43% from non-high-risk neuroblastomas (non-HR). We detected a median of 2,094 SNVs and indels per patient, 14 SVs per patient, and 19 CNAs per patient (Fig. 1). In line with previous reports²⁵, high-risk non-*MYCN*-amplified patients exhibited a significantly higher prevalence of all variant types (Fig. 1; Suppl. Fig. 2), with up to 1.8 and 6 times more somatic variants than *MYCN*-amplified and non-high-risk patients,

respectively. Pathognomonic chromosomal and driver gene alterations were identified at a similar prevalence in our cohort as described in other neuroblastoma cohorts²⁶ (Fig. 1). Interestingly, high-risk non-*MYCN*-amplified patients frequently harbored a t(11;17) translocation associated with gains of 17q, losses of 11q, or both (22% of samples), suggesting unbalanced translocations between chromosome 17 and 11 as the cause for 17q and 11q copy number alterations. *MYCN* amplifications detected in WGS were confirmed cytogenetically during routine clinical diagnostics using fluorescence in situ hybridization (FISH) and classified as either ecDNA or homogenous staining regions (Suppl. Table 3). Recurrently mutated genes included RAS pathway members (17.5%, including *ALK*), as well as *PTPRD* (12.3%), *TERT* (8.8%), *SHANK2* (3.5%), *MDM2* (1.7%), *DAXX* (0.9%), and *TP53* (0.9%). In line with previous works²⁷, *ATRX* (7%), was exclusively altered in high-risk non-*MYCN*-amplified patients. Apart from *ALK* (8.8%), which was mainly affected by missense variants, most neuroblastoma driver genes were affected by SVs, highlighting the critical role of SVs in neuroblastoma.

SNV-based signatures SBS3, SBS18, and SBS40 are linked to different neuroblastoma risk groups

To provide a comprehensive overview of the mutational processes involved in neuroblastoma, we first analyzed the SNV trinucleotide context from coding and non-coding regions of the genome using mutSignatures²⁸ (Fig. 2A,E; Suppl. Fig. 3; Suppl. Table 1). We recurrently identified six reference⁶ SNV-based signatures (SBS) in our cohort, indicating associated mutational processes are active in neuroblastoma. In line with previous reports²⁰, SBS3 and SBS18, linked to defective homologous recombination repair (HRR) and reactive oxygen species (ROS), respectively, were recurrently identified in our neuroblastoma cohort (Fig. 2A; Suppl. Fig. 3A). Whereas SBS18, characterized by C>A substitutions, was predominantly observed in high-risk *MYCN*-amplified patients ($P < 1.5 \times 10^{-4}$), SBS3, a flat signature, was observed to a significantly lower degree in that risk group ($P < 8.3 \times 10^{-6}$) displaying a negative correlation with signature SBS18. Similarly to SBS3, SBS40, a clock-like signature associated with high patient age²⁹, was highly anti-correlated with SBS18, inversely associated with clinical risk, and most prevalent in non-high-risk patients ($P < 2.4 \times 10^{-3}$). Surprisingly, no association of SBS40 and age at diagnosis was observed, which may be due to overall young patient age in our cohort or could indicate that age-unrelated mutagenic processes are responsible for this signature in these patients. Thus, mutational signatures attributed to

defective HRR and ROS are highly prevalent in neuroblastomas and differentially active across neuroblastoma risk groups.

Distinct indel-based signatures co-occur with SNV-based signatures SBS3, SBS18, and SBS40 and are associated with clinical risk groups

Next, we studied the mutational patterns of indels using YAPSA³⁰. Considering the insertion and deletion lengths and genomic context, including repetitive sequences and microhomology, we identified six reference⁶ indel-based signatures (ID) in neuroblastoma genomes (Fig. 2B,E; Suppl. Fig. 4; Suppl. Table 1). ID1 and ID2, both characterized by 1 bp insertions and deletions at long thymine homopolymers attributed to replication slippage and found in most cancer entities⁶, were also recurrently identified in our cohort. These signatures were significantly more frequent in patients with higher clinical risk, particularly in high-risk *MYCN*-amplified patients ($P < 1.2 \times 10^{-4}$). ID1 and ID2 positively correlated with SNV signature SBS18, which indicates co-occurrence of DNA damage by ROS and replication slippage in *MYCN*-amplified samples. ID6, characterized by larger deletions (> 5 bp) with larger microhomology, was also recurrently observed in our cohort. As expected, based on their common etiology associated with defective HRR, ID6 followed the same prevalence distribution between neuroblastoma risk groups as SBS3, being less predominant in high-risk *MYCN*-amplified patients ($P < 2.9 \times 10^{-2}$) yet with preference for high-risk non-*MYCN*-amplified neuroblastomas. Similarly, ID9, a signature of unknown etiology characterized by 1 bp deletions at short cytosine and thymine homopolymers, was twice more frequent in high-risk non-*MYCN*-amplified samples ($P < 1.5 \times 10^{-5}$). ID4, a signature enriched for deletions (> 1 bp) at repeats and microhomology, recently associated with TOP1 mutational activity in cancer and healthy cells³¹, on the other hand, was significantly more prevalent in non-high-risk patients ($P < 1.3 \times 10^{-3}$). ID4 displayed an association with SBS40 and high anticorrelation with all the other indel signatures, notably with the ones related to SBS3 (ID6, ID8, ID9) or SBS18 (ID1, ID2). This is in line with previous works in other cancer entities describing ID4 as mutually exclusive with signatures ID1 and ID2⁶, and points to topoisomerases as a predominant source of mutagenesis in non-high-risk neuroblastoma. Thus, a subset of SNV-based and indel-based signatures co-occur in neuroblastomas from the same risk groups.

High-risk MYCN-amplified neuroblastoma genomes are defined by replication stress-related CNA patterns

Recent reports suggest that CNA patterns may allow the inference of signatures, offering more insights into molecular processes involved in their generation^{7,32}. We measured the presence of 17 established CNA-associated signatures (CX, manuscript currently in revision) in our cohort of neuroblastomas (Fig. 2C,E; Suppl. Fig. 6; Suppl. Table 1). Signature differences are based on the number and size of copy number segments, and the difference in copy number between neighboring segments. Seven CX signatures significantly differed in frequency between risk groups. CX1, a signature associated with chromosomal arm changes potentially caused by chromosome missegregation and telomere disfunction, was the most prevalent CNA signature in our neuroblastoma cohort (Fig. 2C). It was associated with SBS3, although less prevalent in high-risk non-*MYCN*-amplified patients ($P < 1.1 \times 10^{-2}$). CX3, linked to defective HRR and replication stress-mediated long chromosomal segment changes, also co-occurred with SBS3 yet was significantly more frequent in high-risk non-*MYCN*-amplified patients ($P < 1.5 \times 10^{-2}$). CX1 and CX3 together accounted for more than 76% of the CN signatures' exposure identified in neuroblastomas, suggesting that neuroblastoma genomes are strongly shaped by chromosome missegregation and impaired HRR. Co-occurrence was observed between CX9, a signature attributed to replication stress-mediated focal amplifications, and SBS18, indicating ROS-induced replication stress as a potential cause of focal amplifications in neuroblastoma. CX14, characterized by footprints of chromosome missegregation via defective mitosis, was significantly associated with low-risk disease ($P < 8.2 \times 10^{-3}$). CX5, CX7, CX9, and CX11, on the other hand, were more frequent in high-risk *MYCN*-amplified patients ($P < 3.2 \times 10^{-3}$). Interestingly, these signatures are attributed to replication stress and were significantly associated with indel-based signatures ID1 and ID2, which are believed to result from replication slippage. Thus, CNA pattern analysis revealed that whereas *MYCN*-driven neuroblastomas are characterized by replication stress and slippage, and high-risk non-*MYCN*-amplified neuroblastomas present higher prevalence of defective HRR, chromosome missegregation is a general feature of all neuroblastomas across risk groups.

SV-based signatures reveal high prevalence of simple rearrangements associated with transposase breakpoint signal sequence motifs in neuroblastoma

Recent reports suggest that site-specific recombinases may contribute to SV generation in embryonal tumors². To evaluate the presence of such recurrent DNA sequences around SV breakpoints, we performed a *de novo* motif discovery analysis. Intriguingly, we recurrently identified motifs closely resembling specific PGBD5 recombination signal sequences² (Suppl. Fig. 9), suggesting PGBD5 may contribute to structural variation in neuroblastoma.

Next, we performed a *de novo* discovery of SV signatures (SV) based on the typology, size, and clustering of SVs in our neuroblastoma cohort using Palimpsest³³ (Fig. 2D,E; Suppl. Fig. 5; Suppl. Table 1). After comparing these signatures to those identified in other tumor entities⁸, we defined four novel (SV3-6), presumably neuroblastoma-specific, and two known SV-based signatures (SV1-2), each differing with regards to their complexity, the enrichment in intra- and interchromosomal rearrangements, and their size. SV1 was defined by complex intrachromosomal rearrangements, enriched in deletions, tandem-duplications, and inversions of sizes around 1-10 Mb. SV2 was characterized by complex intrachromosomal rearrangements larger than 10 Mb. SV1 and SV2 corresponded to reference SV-based signatures R6b and R6a, respectively, and were reported to be associated with *MDM2* mutations and *CDK4* amplifications⁸ (Suppl. Fig. 5C). SV3, on the other hand, a signature identified *de-novo*, was linked to simpler rearrangements and higher frequency of translocations in our cohort. Simple deletions smaller than 1 kb defined SV4. In SV5, complex rearrangements around 0.1-1 Mb involving translocations were observed. High amounts of translocations together with simple duplications and deletions defined SV6, the most frequent SV-associated signature in our cohort. In line with their complexity, SV signatures SV1, SV2, and SV5 were positively associated with CN signature CX15, which is characterized by clustered CNAs. Intriguingly, SV4, the second most prevalent SV signature in our cohort, showed an inverse association with all the other SV signatures, notably with SV6, which may indicate mutually exclusive underlying mutational processes as a cause of these variant patterns. Even though no SV signatures significantly differed in frequency between risk groups, overall, signatures defined by simple rearrangements (SV3/SV4/SV6), were more frequent in neuroblastoma compared to those defined by complex SVs (SV1/SV2/SV5; $P < 3.66 \times 10^{-5}$). Thus, neuroblastomas are characterized by low-complexity SVs.

Topography of complex structural rearrangements

Recent reports have re-classified complex rearrangements topographically, considering both CNAs and SVs^{12,15,34}. Even though some of these rearrangement patterns have been observed in neuroblastomas, e.g. ecDNA and chromothripsis^{21,25}, the prevalence of most of the recently described patterns is currently unknown. To examine the topography of complex rearrangements in neuroblastoma, we used three complementary algorithms, JaBba¹⁵, Amplicon Architect³⁵, and Shatterseek³⁶ enabling the identification and reconstruction of eight complex variant classes: (1) chromothripsis; (2) breakage-fusion-bridge cycles (BFB); (3) extrachromosomal circular DNA (ecDNA); (4) complex non-cyclic amplicons (CnC); (5)

chromoplexy; (6) templated insertion chains (TIC), also described as chromoplexy-like; (7) rigma, a cluster of simple deletions; (8) pyrgo, a cluster of tandem-duplications (Fig. 3,4A; Suppl. Table 2). Interestingly, chromoplexy-like SVs were the most frequently observed SV pattern in neuroblastomas and were detectable in 45 patients across all risk groups (chromoplexy in 20 patients; TIC in 32 patients), suggesting a previously unknown contribution to neuroblastoma genome remodeling. In line with our previous observations²¹, ecDNA was also amongst the most frequent complex rearrangement classes in neuroblastomas (34 patients). ecDNA was most prevalent in high-risk *MYCN*-amplified patients ($P < 7.6 \times 10^{-8}$), in line with the fact that *MYCN* amplifications most often occur in the form of ecDNA²¹. Most ecDNA-harboring tumors did not show signs of other known complex rearrangement types (Fig. 4A). Only in a subset of cases ecDNA co-occurred with chromoplexy-like rearrangements (TIC, chromoplexy), CnC, pyrgo, and BFB, suggesting ecDNA in these tumors may either be involved in these complex rearrangements or have originated from them. In contrast to previous reports^{23,24}, no co-occurrence between ecDNA and chromothripsis was observed in our cohort, indicating that chromothripsis may not contribute to ecDNA generation in neuroblastoma to the same extent as in other tumor entities. Most neuroblastomas with signs of BFB, on the other hand, also harbored ecDNAs, suggesting BFB as an important mechanism of ecDNA generation and/or evolution in neuroblastoma. Overall, less than 1% of all SVs could be assigned to previously defined complex variant patterns, with the highest fraction of SVs attributed to CnC (0.98% of SVs), chromoplexy (0.40% of SVs), and ecDNA (0.20%, Suppl. Fig. 7), suggesting that most SVs either do not occur in recognizable patterns or that yet unknown mutational patterns exist in neuroblastoma. Interestingly, complex SVs did not occur uniformly throughout the genome (Fig. 4B). In line with our previous report showing ecDNA as a hotspot for SVs, the *MYCN* locus on chromosome 2 was most recurrently affected by complex variant patterns (19.3% of patients), followed by the *RASGRP3* locus, a Ras pathway activator also located on chromosome 2, affected by complex rearrangements in 18.4% of patients. Other previously unrecognized recurrently rearranged regions included cancer-related loci such as *SMARCE1* (6.1% of patients), *EBF1* (5.3% of patients), a gene involved in enhancer hijacking in neuroblastoma³⁷, tumor suppressor *SDHA* (5.3% of patients), a known hotspot for germline variants associated with neuroblastoma³⁸, and the oncogene *HMG2* (5.3% of patients). Thus, topographically distinct complex variant patterns are more common in neuroblastoma than previously anticipated and recurrently occur at sites of cancer-related loci, suggesting their functional oncogenic importance.

Three mutational scenarios emerge through integration of mutational signatures and complex rearrangement patterns in neuroblastoma

The study of the interaction between complex variant patterns and mutational signatures within different neuroblastoma risk groups suggests the existence of distinct co-occurring mutational footprints with clinical relevance, termed mutational scenarios (Fig. 5,6A). The first mutational scenario (scenario #1), more frequent in high-risk *MYCN*-amplified neuroblastomas, is defined by footprints of DNA damage by ROS (SBS18), replication slippage (ID1/ID2), and replication stress (CX5/CX7/CX9/CX11). The presence of ecDNA was positively associated with scenario #1 (Fig. 5A), designating ecDNA as a defining element of this mutational scenario and suggesting replication slippage/stress and ROS may contribute to ecDNA generation, in line with previous reports in mammalian cells^{39,40}. Alternatively, the presence of ecDNA or high expression of oncogenes (e.g. *MYCN*) encoded on them (Fig. 5B) may drive these mutational processes, in line with previous reports demonstrating that *MYC* can drive replication stress⁴¹. The second mutational scenario (scenario #2) is characterized by signs of impaired HRR (SBS3/ID6/CX3), single base deletions (ID9), and is associated with complex rearrangements such as chromoplexy, CnC, and TIC. This mutational scenario is most frequent in high-risk non-*MYCN*-amplified neuroblastomas. Interestingly, the presence of ecDNA was nearly mutually exclusive with defective homologous recombination-associated signatures defining scenario #2 (Fig. 5A), implying that cells harboring ecDNA may require intact HRR. On the other hand, although not significantly associated with scenario #2, chromothripsis displayed a unique correlation with homologous recombination-associated signatures (CX2), indicating that HRR may be involved in the repair of chromothriptic fragments, in line with previous reports in other tumor entities⁴². The third mutational scenario emerging from our analysis (scenario #3) is linked to signatures of unknown etiology (SBS40), topoisomerase mutational activity (ID4), and signs of chromosome missegregation (CX14). This mutational scenario is highly prevalent in non-high-risk neuroblastomas. Thus, neuroblastomas harbor distinct combinations of mutational signatures and variant patterns, indicating that different mutational processes are active in each tumor.

Mutational scenarios are linked to neuroblastoma clinical heterogeneity

Previous reports in other tumor entities showed associations between complex rearrangements¹⁵, specific mutational processes, such as HRR deficiency⁴³, and poor prognosis. Based on this, we hypothesized that neuroblastoma's clinical heterogeneity might be in part explained by differences in complex rearrangement patterns and associated

mutational processes, depicted by the three mutational scenarios described here. To test our hypothesis, we first evaluated the clinical impact of complex rearrangements in our cohort. Patients with tumors harboring ecDNA presented worse overall survival than patients with rearrangements affecting linear chromosomal DNA, such as chromoplexy, (Suppl. Fig. 8A,B; $P < 1 \times 10^{-4}$ by Log-rank test), suggesting that ecDNA supersedes other complex rearrangements in its association with adverse outcome. Subsequently, we assessed to what extent the three different mutational scenarios reflect neuroblastoma's clinical heterogeneity. We categorized each patient based on the presence of the set of signatures defining each mutational scenario 1-3, without considering their risk classification (Fig. 6; Suppl. Fig. 8). Even though mutational scenarios are based on the co-occurrence of mutational footprints within risk groups, we observed that not all patients from a specific risk group were classified into the same mutational scenario. Interestingly, patients from different mutational scenarios showed pronounced differences in clinical outcome ($P < 1 \times 10^{-4}$ by Log-rank test) confirming the association of the defined scenarios with neuroblastoma's clinical heterogeneity. Moreover, a subset of neuroblastomas harboring a mixture of signatures and patterns that could not be assigned to a single mutational scenario exhibited low overall survival, indicating that unknown mutational scenarios may exist. Thus, signatures defining mutational scenarios drive heterogeneous clinical outcomes in neuroblastoma.

Discussion

This study analyzed 114 neuroblastoma genomes and described the mutational processes and genomic rearrangement patterns in tumors from different clinical risk groups. Based on SNVs, indels, SVs, and CNAs patterns, we characterized three distinct mutational scenarios with clinical relevance. Scenario #1 driven by DNA damage caused by ROS, replication slippage, and stress, most prevalent in high-risk *MYCN*-amplified, ecDNA-harboring patients with low overall survival. Scenario #2 driven by HRR-associated signatures and characterized by non-circular complex rearrangements, frequently observed in high-risk non-*MYCN*-amplified patients with low survival. Finally, scenario #3 characterized by footprints of chromosome missegregation, topoisomerase activity, and high presence of signature SBS40, prevalent in non-high-risk patients exhibiting good prognosis. Our findings demonstrate that clinical heterogeneity is accompanied by distinct mutational processes, raising the question whether these mutational processes contribute to the heterogeneous clinical outcomes. Even though the identified mutational scenarios are linked to specific mutational processes, it remains unknown what causes these processes to be active in neuroblastoma. Some molecular

features in neuroblastomas have been linked to mutational processes in the past. For example, *MYC* can induce ROS, replication stress, and fork stalling⁴¹. It is tempting to speculate that *MYCN* could activate similar mutational processes, which would explain increased footprints of ROS, replication slippage, and stress observed in mutational scenario #1, which was most prevalent in *MYCN*-amplified patients. Further investigations using genetically controlled model systems may reveal such causal relationships between molecular cancer alterations and the presence of associated mutational signatures.

Deficiencies in HRR can fuel the generation of simple and complex SVs⁴⁴, such as BFB and chromothripsis⁴⁵. For example, mutations in *BRCA1* and *BRCA2* resulting in HRR deficiency are associated with high prevalence of certain SVs, previously termed *BRCAness*⁴⁶. Although *BRCA1/2* mutations are not frequent in neuroblastoma⁴⁷, we identified mutational signatures related to HRR deficiency in high-risk non-*MYCN*-amplified patients. In line with our work, recent pediatric pan-cancer studies also observed signatures associated with HRR deficiency in neuroblastoma²⁹. These reports, however, find lower exposures of these signatures in neuroblastomas. This could be due to differences in mutation calling sensitivity or signature misassigning. It remains unknown, which molecular alterations other than *BRCA1/2* deficiency could cause impaired HRR in neuroblastomas. Considering the high prevalence of these footprints in high-risk compared to non-high-risk patients, HRR deficiency may not only represent a mere feature of these tumors but could also contribute to their aggressive clinical behavior, similar as observed in HRR-deficient pancreatic cancer and gliomas⁴⁸. Further investigations into the underlying causes of this mutational footprint in neuroblastoma may be especially relevant for future clinical trial designs, as HRR deficiency is associated with altered response to certain chemotherapeutic agents⁴⁹, as well as increased sensitivity to pharmacological PARP trapping⁵⁰. Thus, the identification of mutational patterns associated with HRR deficiency in high-risk neuroblastomas may have important implications for neuroblastoma risk and therapy stratification.

Mutational signature SBS40 is prevalent in different cancer types⁶, including neuroblastoma²⁰. In line with previous reports²⁹, we find high exposure of SBS40 in our neuroblastoma cohort within mutational scenario #3. In contrast, we do not find SBS40 to be associated with signs of high genome instability such as chromothripsis in neuroblastoma, but find SBS40 to be most prevalent in non-high-risk patients exhibiting low numbers of structural rearrangements. This suggest that mutational processes responsible for SBS40 do not contribute to genome instability in neuroblastoma or that SBS40 is caused by different mutational processes in neuroblastoma than in other tumors. The latter could be explained by the fact that mutational

signature analyses are a mathematical approximation of mutational processes. Thus, some processes may remain unidentified or even misassigned using current state-of-the-art algorithms^{5,6,20,29}. This is especially interesting in the case of SBS40, which presents a mutational pattern with many similarities to other signatures, such as SBS5. For this reason, experimental systems validating causal relationships between signatures and molecular processes will be needed to help clarify the role of signatures exhibiting similar patterns.

In addition to the characterization of mutational processes through signature analyses, we provide the first complete catalog of recently described complex variant patterns¹⁵ in neuroblastoma, including chromothripsis, BFB, ecDNA, CnC, chromoplexy, TIC, and the novel rigma and pyrigo. Previous studies^{24,36} have reported a small subset of these complex rearrangement types in a few cancer-related loci such as *MYCN*, *CDK4*, and *MDM2*. We not only confirmed these rearrangements in our cohort, but also identified previously unidentified genomic hotspots of complex rearrangements, some of which were associated with cancer-related genes such as *RASGRP3*, *SMARCE1*, *EBF1*, *SDHA*, and *HMG A2*. Moreover, we uncovered that many previously unrecognized complex rearrangements patterns recurrently affect cancer-related genes in neuroblastomas. This indicates that the functional contribution of these patterns is higher than anticipated and should be further explored.

ecDNA is one of the most prevalent genomic alterations in cancer⁵¹. We and others recently showed that it is not only a vehicle for oncogene amplification and high expression²¹, but also the most recurrent site of complex structural rearrangements^{14,21,22}, indicating that ecDNA may serve as a substrate for genome remodeling. In line with this, footprints of BFB, chromothripsis and kataegis have been identified on ecDNA in several cancer entities^{23,24}. We also observed signs of BFB on ecDNA in our neuroblastoma cohort, but did not observe footprints indicative of chromothripsis or APOBEC3-driven kataegis to the same extend as observed elsewhere⁵². This may be due to the fact that chromothripsis detection in highly amplified regions of the genome still remains challenging using currently available algorithms. However, even after manual curation of all ecDNA regions for the presence of non-canonical chromothripsis, we did not observe chromothriptic footprints, challenging the role of chromothripsis in the formation of ecDNA in neuroblastoma. Based on our observations, BFB may represent a more important process involved in ecDNA generation and/or structural evolution in neuroblastoma than previously estimated, indicating the need to further explore the relative contributions of complex rearrangement processes in the structural shaping of ecDNA.

In summary, our study provides a first comprehensive characterization of active mutational processes in neuroblastoma, offering new insights into the causes of genomic alterations

involved in neuroblastoma genesis and progression. The three mutational scenarios presented here not only refine our understanding of neuroblastoma's clinical heterogeneity, but may also improve our understanding of how mutational processes contribute to the generation of different variant classes in cancer in general.

Online Methods

Sequencing data

This study is based on the analysis of 120 neuroblastoma primary tumor and matching control samples. Those samples are divided into two datasets. The first one, sequenced in Cologne, was completed in 2015³⁷, and it is composed of WGS from 56 primary tumor and matching blood control samples (Illumina HiSeq). It was downloaded (<https://www.ebi.ac.uk/ega/>) under accession number EGAS00001001308³⁷. The second one, sequenced at the German Cancer Research Center, was completed in 2017-2019, and it includes WGS of 64 primary tumor and matching control samples (Illumina HiSeq X Ten). The quality of the raw data was assured using FastQC⁵³. Reads were 3' trimmed for both quality and adapter sequences, with reads removed if the length was shorter than 20 nucleotides. Burrows–Wheeler Aligner MEM⁵⁴ v.0.7.15 with default parameters was used to align the reads to human reference assembly hg19. PCR and optical duplicates were marked with bammarkduplicates2 from BIOBAMBAM2 v2.0.87 (<https://github.com/gt1/biobambam2>).

Six patients have been excluded from the analyses due to an abnormally high number of mutations detected (CB2044, NBL47, NBL49, NBL50, NBL53, NBL54).

Variant calling

SNVs and indels. Somatic single-nucleotide variants and small insertions and deletions were detected using Mutect2 v.4.1.8.1 from the GATK⁵⁵ software package, with standard parameters according to GATK best practices recommendations. Variants were filtered using recommended filtering parameters, removing all germline mutations using a panel of normals.

SVs. In matched normal and tumor samples, somatic structural variants were called using three different variant callers: Delly⁵⁶ v0.7.7, SvABA⁵⁷ v.1.1.0, and Novobreak⁵⁸ v.1.1.3 with default parameters. To increase specificity without losing too much sensitivity, we intersected all the variants from the three callers and kept the ones detected at least in two of them. An exception has been made for insertions since we only identified them using SvABA. Variants with both breakpoints within a window of 500 bp were collapsed. Each variant included in the

final set passed all the standard filters in at least one of the required two callers to be considered in our analysis.

CNAs. Allele-specific copy-number (CN) profiles were generated from tumor and normal B allele frequencies (BAFs) and LogR values using ASCAT⁵⁹ v2.6 with custom parameters. The segmentation procedure from ASCAT was replaced with a custom implementation that only considers BAFs to determine start and end points of segments but still estimates the segment's coverage using the log coverage ratios. All CN profiles were inspected manually for quality control. For samples with an estimated tumor purity, below 60% CN calling was rerun with adjusted purity and ploidy values that were manually selected after inspection of the sunrise plots and in agreement with pathology estimates of tumor purity. We assigned different copy number states to all profiles considering the local copy number and overall ploidy estimates. CN gain was defined as $\log_2((\text{CN}_{\text{major}} + \text{CN}_{\text{minor}}) / \text{ploidy}) > 0.3$; CN loss as $\log_2((\text{CN}_{\text{major}} + \text{CN}_{\text{minor}}) / \text{ploidy}) < -0.3$. CN amplifications and homozygous losses have been determined following COSMIC CNA parameters (<https://cancer.sanger.ac.uk/cosmic/help/cnv/overview>). In the case of amplifications, we added an additional filter of $\log_2((\text{CN}_{\text{major}} + \text{CN}_{\text{minor}}) / \text{ploidy}) > 1.25$ to increase specificity. For three patients no CNA results could be obtained (NBL31, NBL36, and NBL61).

Identification of variants affecting genes and chromosome arms

Chromosomal arms. Gains and losses of chromosomal arms have been determined intersecting the coordinates of the chromosomal regions with the CNA gains and losses, using bedtools v2.29.2 with parameters -wo -f 1 -F 0.60 (<https://bedtools.readthedocs.io/>). We considered an overlap of the whole CNA with at least 60% of the chromosomal arm.

Genes. We predicted the variant effect for SNV and indel calls using Ensembl VEP⁶⁰ (Variant Effect Predictor) v.102.0. Only SNVs and indels classified as *missense* and *stop gained* overlapping genes, were considered. For SV calls, we considered all the genes affected by SV breakpoints. We classified them in two types: (1) SV close to gene, defined as genes with at least one SV breakpoint at a distance of +/- 20 kb; (2) SV within gene, defined as genes overlapping at least one SV breakpoint. For CNA calls, we considered the genes overlapping amplifications or homozygous deletions.

Structural variant breakpoint analysis

Base-pair accurate structural rearrangement calls from the merged structural variant set were considered for detailed breakpoint analysis. Using the hg19 reference genome, we obtained the

sequence for a 50-bp window around each structural variant breakpoint. STREME v.5.3.0 (parameters -dna -nmotifs 15 -minw 5 -maxw 25 -objfun de) was used to identify motifs that were enriched with regard to a set of 1 million length-matched sequences randomly sampled from hg19. We compared those motifs with the previously published² PGBD5-specific signal sequence. This analysis was done for all the samples together and also stratifying per risk groups.

Mutational signatures analyses

SNV signatures (SBS). To retrieve mutational signatures related to somatic single-nucleotide variants operating in neuroblastoma, we used the R package mutSignatures²⁸ v.2.1.1. We started pre-processing all the SNVs in coding and non-coding regions detected in our patients. We applied a filtering step for non-SNV variants, followed by the tri-nucleotide context extraction for each of the variants which are classified in 96 different mutation types, or features, and counted across samples. Next, we ran the *de-novo* signature extraction step where non-negative matrix factorization (NMF) is performed. Complying with the pipeline recommendations, we computed 500 iterations, resulting in four de-novo signatures. Then, we evaluated which known COSMIC v.3.1 signatures (<https://cancer.sanger.ac.uk/signatures/sbs/>) matched our *de-novo* signatures, using a cosine distance threshold of ≤ 0.15 , obtaining six COSMIC signatures: SBS3, SBS12, SBS18, SBS36, SBS37, and SBS40. Finally, in the deconvolution step, we estimated known mutational signatures exposures in each patient and across neuroblastoma risk groups.

Indel signatures (ID). Mutational signatures associated with small somatic insertions and deletions (< 50 bp) have been extracted using the R package YAPSA³⁰ v.1.16.0, which includes PCAWG/COSMIC indel signatures (<https://cancer.sanger.ac.uk/signatures/id/>), following the standard pipeline. This pipeline starts with a pre-processing step of all the indels detected in our patients, in which we annotate the sequence context 10bp downstream and 60bp upstream of each variant, the variant type, and the length. In line with the SNV signature analysis, indels are classified in 83 different classes of features, and counted across samples. Next, we computed the supervised mutational signature analysis step, based on the linear combination decomposition function (LCD), obtaining the exposure for each known mutational signature in each patient. Signature exposure per neuroblastoma risk group was estimated from the exposure per patient. Recommended exposure cutoffs for each signature have been applied.

From this analysis, we have obtained six PCAWG/COSMIC indel signatures: ID1, ID2, ID4, ID6, ID8, and ID9.

CNA signatures (CX). To quantify the activity of copy number signatures in our samples, we used the 17 signatures that have been derived from over 6,000 tumors of the cancer genome atlas (<https://www.cancer.gov/tcga>) (manuscript in revision). Using the raw absolute copy number as obtained from ASCAT as input, first 43 features describing the fundamental features of the copy number profiles are extracted. Afterwards the exposure for each previously described signature in each of our samples is calculated based on the posterior distribution of the extracted features.

SV signatures (SV). Mutational signatures associated with somatic structural variants have been extracted using the R package Palimpsest³³ v.2.0.0 following the standard pipeline for this variant type. This pipeline starts with a pre-processing step in which we annotate the type (deletions, inversions, tandem-duplications, and translocations), size (< 1 kb, 1-10 kb, 10-100 kb, 100 kb – 1 Mb, 1-10 Mb, and > 10 Mb), and clustered nature of rearrangements (distinguish between clustered and non-clustered events). SVs are classified into 38 different classes of features, and counted across samples. Next, we computed the *de-novo* signature extraction step, based on NMF. As a result, we obtained six *de-novo* signatures that were named based on the prevalence of SVs in the different features classes: (1) SV1, complex intrachromosomal SVs; (2) SV2, large complex intrachromosomal SVs; (3) SV3, large simple intra and interchromosomal SVs; (4) SV4, small deletions; (5) SV5, complex intra and interchromosomal SVs; (6) SV6, simple duplications, deletions, and interchromosomal SVs. In the final step of the pipeline, we estimated the exposure of each signature in each sample, and across neuroblastoma risk groups. In order to evaluate if our 6 *de-novo* SV-based signatures found in neuroblastoma matched any of the 21 reference SV signatures from previous pan-cancer studies⁸ we used the `deconvolution_compare` function of Palimpsest obtaining cosine similarities between signatures. Using the same cosine distance threshold for the SNV analysis (≤ 0.15) we identified two matches. De-novo signatures SV1 and SV2, corresponded to reference signatures R6b and R6a⁸.

From the patient's exposure, we assessed the contribution of each of the different mutational signatures within neuroblastoma risk groups.

Complex rearrangements calling and classification

To detect and reconstruct all linear and circular complex rearrangements types in neuroblastoma, we used three complementary algorithms: JaBba¹⁵ v1.0, Amplicon Architect³⁵ v.1.2, and the R package Shatterseek³⁶ v.0.5.

JaBba. JaBba has been run using standard parameters and following the best practices pipeline, including the pre-processing steps using fragCounter and dryclean to correct for GC content and mappability and denoise the coverage data using a panel of normals. From these steps, we obtained the coverage input that we use along our SV calls to run the pipeline. Then, following what has been done in previous studies¹⁵, the output from JaBba was run into gGnome v.0.1 to classify the different complex SVs into rigma, pyrgo, TIC, chromoplexy, chromothripsis, BFB, double minute (DM), and tyfonas. gGnome was also used to generate the figures with the reconstruction of the rearrangements.

Amplicon Architect. We used the copy number profiles obtained from ASCAT and the bam files of all tumor samples as input to PrepareAA³⁵ (<https://github.com/jluebeck/PrepareAA>), a wrapper function that handles the preprocessing and execution of AmpliconArchitect v.1.2. We predicted the types of amplifications present in Amplicon Architect's³⁵ output using AmpliconClassifier¹⁴ v.0.4.6 with standard parameters except for `-force=TRUE`. We classified circular amplicons in ecDNA and BFB, and linear amplicons in complex non-cyclic amplicons (CnC).

ShatterSeek. ShatterSeek³⁶ has been run from CNA and SV calls using standard parameters to detect chromothripsis. The filtering steps presented in the tutorial to obtain high confidence calls have been followed: at least 6 interleaved intrachromosomal SVs, 7 contiguous segments oscillating between 2 CN states, significant fragment join tests, and either significant chromosomal enrichment or exponential distribution of breakpoints test. In order to include chromothriptic candidates in our results that might affect highly amplified regions, we also called low-confidence chromothripsis having at least 6 interleaved intrachromosomal SVs and 7 contiguous segments. In these cases, a final step of visual inspection was performed.

The three complex rearrangement calling pipelines have been run using the hg19 reference genome. To gain sensitivity in the reconstruction of the complex SVs, we used the SVs that passed all the standard variant calling filters in at least one caller. Complex rearrangements detected by at least one method were considered. All complex rearrangements detected in the same patient overlapping the same genomic region between methods have been merged, keeping the most informative classification.

Analysis of complex rearrangements

Co-occurrence of the different complex rearrangement types was computed considering the presence of the different rearrangement classes within the same patient. The figure has been created using the R package ComplexUpset v.1.2.1 (<https://github.com/krassowski/complex-upset>).

The ratio of SVs involved in complex rearrangements has been extracted establishing the ratio between the number of SVs junctions from those rearrangements and the total number of SVs in each patient.

For each patient, we obtained the breakpoint coordinates of each of the complex SVs. Then, splitting the genome into 1 Mb regions, we extracted which genomic regions are affected by complex rearrangements in our cohort. This analysis has been done for the different neuroblastoma risk groups. Regions encompassing neuroblastoma relevant cancer-related genes, from the COSMIC cancer gene census, affected in > 5% of the samples from our whole cohort have been highlighted in the figure.

Kataegis analysis

We consider a cluster of kataegis when we called 6 or most consecutive SNVs with an intermutation distance of < 1kb. The same method has been used in previous studies²³.

Mutational scenarios analysis

For the definition of the three different mutational scenarios, we considered all the biological features identified in this work such as mutational signatures, their associated mutational processes, complex rearrangement classes, and risk group classification. Only biological features significantly present/correlated within a risk group are considered defining the mutational scenario associated to that risk group. The significance level has been established at $P < 0.05$. We established a Spearman correlation threshold of 0.3/-0.3 to consider that the feature of interest has a moderate/strong relationship with the features characterizing the mutational scenario and, as a result, can be defining. If a signature or complex rearrangement was equally distributed across two or more risk groups, significantly correlated with signatures or mutational processes from two or more risk groups, or did not reach the Spearman correlation threshold, then, it was not considered as a feature with enough clinical relevance to stratify between patients and as a result was excluded from the final characterization of the mutational scenarios.

Statistical analysis

All comparisons between distributions in the different neuroblastoma risk groups were made using the non-parametric Wilcoxon rank-sum test. To assess if there are differences between risk groups, we used the non-parametric Kruskal-Wallis test. The relationship between different variables such as signatures, complex rearrangements, and mutated genes has been calculated using Spearman's correlation coefficient. All statistical analyses have been corrected by multiple testing when applicable using the false discovery rate (FDR) correction. All p-values in the main text have been obtained using the Wilcoxon rank-sum test unless stated otherwise. The significance level has been established at $P < 0.05$.

Log-rank tests were used for survival analysis across subgroups. To assess the clinical impact of the three defined mutational scenarios, we stratified our patients by presence/absence of the whole set of mutational signatures and complex rearrangements that define each scenario without considering their neuroblastoma risk classification. Patients presenting a mixture of signatures and patterns that could not be assigned to a single mutational scenario were classified as *Mix of signatures*. The subgroups for the analysis were: Mutational scenario #1 (n = 7), Mutational scenario #2 (n = 14), Mutational scenario #3 (n = 30), and Mix of signatures (n = 56). To assess the clinical impact of complex rearrangements in this tumor, we stratified our patients by the presence/absence of linear and circular complex SVs. The subgroups for the analysis were: Linear comp. rearrang. - / ecDNA - (n = 35), Linear comp. rearrang. + / ecDNA - (n = 45), Linear comp. rearrang. - / ecDNA + (n = 16), and Linear comp. rearrang. + / ecDNA + (n = 18).

Patient samples and clinical data access

This study comprised the analyses of tumor and blood samples of patients diagnosed with neuroblastoma between 1991 and 2016. Patients were registered and treated according to the trial protocols of the German Society of Pediatric Oncology and Hematology (GPOH). This study was conducted in accordance with the World Medical Association Declaration of Helsinki (2013) and good clinical practice; informed consent was obtained from all patients or their guardians. The collection and use of patient specimens was approved by the institutional review boards of Charité-Universitätsmedizin Berlin and the Medical Faculty, University of Cologne. Specimens and clinical data were archived and made available by Charité-Universitätsmedizin Berlin or the National Neuroblastoma Biobank and Neuroblastoma Trial Registry (University Children's Hospital Cologne) of the GPOH. The *MYCN* gene copy number was determined as a routine diagnostic method using fluorescence in situ hybridization (FISH). Results of the FISH analysis have been confirmed by quantitative polymerase chain

reaction (qPCR). DNA and total RNA were isolated from tumor samples with at least 60% tumor cell content as evaluated by an experienced pathologist.

References

1. Greenman, C. *et al.* Patterns of somatic mutation in human cancer genomes. *Nature* **446**, 153–158 (2007).
2. Henssen, A. G. *et al.* PGBD5 promotes site-specific oncogenic mutations in human tumors. *Nat Genet* **49**, 1005–1014 (2017).
3. Jeggo, P. A., Pearl, L. H. & Carr, A. M. DNA repair, genome stability and cancer: a historical perspective. *Nat Rev Cancer* **16**, 35–42 (2016).
4. Koh, G., Degasperi, A., Zou, X., Momen, S. & Nik-Zainal, S. Mutational signatures: emerging concepts, caveats and clinical applications. *Nat Rev Cancer* **21**, 619–637 (2021).
5. Australian Pancreatic Cancer Genome Initiative *et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415–421 (2013).
6. PCAWG Mutational Signatures Working Group *et al.* The repertoire of mutational signatures in human cancer. *Nature* **578**, 94–101 (2020).
7. Macintyre, G. *et al.* Copy number signatures and mutational processes in ovarian carcinoma. *Nat Genet* **50**, 1262–1270 (2018).
8. Degasperi, A. *et al.* A practical framework and online tool for mutational signature analyses show inter-tissue variation and driver dependencies. *Nat Cancer* **1**, 249–263 (2020).
9. Turner, K. M. *et al.* Extrachromosomal oncogene amplification drives tumour evolution and genetic heterogeneity. *Nature* **543**, 122–125 (2017).
10. Stephens, P. J. *et al.* Massive Genomic Rearrangement Acquired in a Single Catastrophic Event during Cancer Development. *Cell* **144**, 27–40 (2011).
11. Baca, S. C. *et al.* Punctuated evolution of prostate cancer genomes. *Cell* **153**, 666–677 (2013).
12. Li, Y. *et al.* Patterns of somatic structural variation in human cancer genomes. *Nature* **578**, 112–121 (2020).
13. Zakov, S., Kinsella, M. & Bafna, V. An algorithmic approach for breakage-fusion-bridge detection in tumor genomes. *Proceedings of the National Academy of Sciences* **110**, 5546–5551 (2013).
14. Kim, H. *et al.* Extrachromosomal DNA is associated with oncogene amplification and poor outcome across multiple cancers. *Nat Genet* **52**, 891–897 (2020).

670 15. Hadi, K. *et al.* Distinct Classes of Complex Structural Variation Uncovered across
671 Thousands of Cancer Genome Graphs. *Cell* **183**, 197-210.e32 (2020).

672 16. Bown, N. Neuroblastoma tumour genetics: clinical and biological aspects. *Journal of*
673 *Clinical Pathology* **54**, 897–910 (2001).

674 17. Brodeur, G. M. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev*
675 *Cancer* **3**, 203–216 (2003).

676 18. Capasso, M. & Diskin, S. J. Genetics and Genomics of Neuroblastoma. in *Cancer*
677 *Genetics* (ed. Pasche, B.) vol. 155 65–84 (Springer US, 2010).

678 19. Cohn, S. L. *et al.* The International Neuroblastoma Risk Group (INRG) classification
679 system: an INRG Task Force report. *J Clin Oncol* **27**, 289–297 (2009).

680 20. Brady, S. W. *et al.* Pan-neuroblastoma analysis reveals age- and signature-associated
681 driver alterations. *Nat Commun* **11**, 5183 (2020).

682 21. Koche, R. P. *et al.* Extrachromosomal circular DNA drives oncogenic genome
683 remodeling in neuroblastoma. *Nat Genet* **52**, 29–34 (2020).

684 22. Helmsauer, K. *et al.* Enhancer hijacking determines extrachromosomal circular
685 MYCN amplicon architecture in neuroblastoma. *Nat Commun* **11**, 5823 (2020).

686 23. Shoshani, O. *et al.* Chromothripsis drives the evolution of gene amplification in
687 cancer. *Nature* **591**, 137–141 (2021).

688 24. Rosswog, C. *et al.* Chromothripsis followed by circular recombination drives
689 oncogene amplification in human cancer. *Nat Genet* (2021) doi:10.1038/s41588-021-
690 00951-7.

691 25. Molenaar, J. J. *et al.* Sequencing of neuroblastoma identifies chromothripsis and
692 defects in neuritogenesis genes. *Nature* **483**, 589–593 (2012).

693 26. Pugh, T. J. *et al.* The genetic landscape of high-risk neuroblastoma. *Nat Genet* **45**,
694 279–284 (2013).

695 27. Zeineldin, M. *et al.* MYCN amplification and ATRX mutations are incompatible in
696 neuroblastoma. *Nat Commun* **11**, 913 (2020).

697 28. Fantini, D., Vidimar, V., Yu, Y., Condello, S. & Meeks, J. J. MutSignatures: an R
698 package for extraction and analysis of cancer mutational signatures. *Sci Rep* **10**, 18217
699 (2020).

700 29. Thatikonda, V. *et al.* *Comprehensive analysis of mutational signatures in pediatric*
701 *cancers*. <http://biorxiv.org/lookup/doi/10.1101/2021.09.28.462210> (2021)
702 doi:10.1101/2021.09.28.462210.

30. Daniel Huebschmann, Z. G. *YAPSA*. (Bioconductor, 2017).
doi:10.18129/B9.BIOC.YAPSA.
31. Reijns, M. A. M. *et al.* Signatures of TOP1 transcription-associated mutagenesis in cancer and germline. *Nature* (2022) doi:10.1038/s41586-022-04403-y.
32. Steele, C. D. *et al.* Undifferentiated Sarcomas Develop through Distinct Evolutionary Pathways. *Cancer Cell* **35**, 441-456.e8 (2019).
33. Letouzé, E. *et al.* Mutational signatures reveal the dynamic interplay of risk factors and cellular processes during liver tumorigenesis. *Nat Commun* **8**, 1315 (2017).
34. Campbell, P. J. *et al.* The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature* **467**, 1109–1113 (2010).
35. Deshpande, V. *et al.* Exploring the landscape of focal amplifications in cancer using AmpliconArchitect. *Nat Commun* **10**, 392 (2019).
36. Cortés-Ciriano, I. *et al.* Comprehensive analysis of chromothripsis in 2,658 human cancers using whole-genome sequencing. *Nat Genet* **52**, 331–341 (2020).
37. Peifer, M. *et al.* Telomerase activation by genomic rearrangements in high-risk neuroblastoma. *Nature* **526**, 700–704 (2015).
38. Dubard Gault, M. *et al.* Germline *SDHA* mutations in children and adults with cancer. *Cold Spring Harb Mol Case Stud* **4**, a002584 (2018).
39. Paulsen, T., Kumar, P., Koseoglu, M. M. & Dutta, A. Discoveries of Extrachromosomal Circles of DNA in Normal and Tumor Cells. *Trends in Genetics* **34**, 270–278 (2018).
40. Dillon, L. W. *et al.* Production of Extrachromosomal MicroDNAs Is Linked to Mismatch Repair Pathways and Transcriptional Activity. *Cell Reports* **11**, 1749–1759 (2015).
41. Rohban, S. & Campaner, S. Myc induced replicative stress response: How to cope with it and exploit it. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* **1849**, 517–524 (2015).
42. Voronina, N. *et al.* The landscape of chromothripsis across adult cancer types. *Nat Commun* **11**, 2320 (2020).
43. Dai, W. *et al.* Clinical Outcome–Related Mutational Signatures Identified by Integrative Genomic Analysis in Nasopharyngeal Carcinoma. *Clin Cancer Res* **26**, 6494–6504 (2020).
44. Currall, B. B., Chiangmai, C., Talkowski, M. E. & Morton, C. C. Mechanisms for Structural Variation in the Human Genome. *Curr Genet Med Rep* **1**, 81–90 (2013).

45. Piazza, A. & Heyer, W.-D. Homologous Recombination and the Formation of Complex Genomic Rearrangements. *Trends in Cell Biology* **29**, 135–149 (2019).
46. Byrum, A. K., Vindigni, A. & Mosammaparast, N. Defining and Modulating ‘BRCAness’. *Trends in Cell Biology* **29**, 740–751 (2019).
47. Mersch, J. *et al.* Cancers associated with *BRCA 1* and *BRCA 2* mutations other than breast and ovarian: *BRCA* and Other Cancers. *Cancer* **121**, 269–275 (2015).
48. Heeke, A. L. *et al.* Prevalence of Homologous Recombination–Related Gene Mutations Across Multiple Cancer Types. *JCO Precision Oncology* 1–13 (2018) doi:10.1200/PO.17.00286.
49. Zhang, J. The role of BRCA1 in homologous recombination repair in response to replication stress: significance in tumorigenesis and cancer therapy. *Cell Biosci* **3**, 11 (2013).
50. Creeden, J. F. *et al.* Homologous recombination proficiency in ovarian and breast cancer patients. *BMC Cancer* **21**, 1154 (2021).
51. Verhaak, R. G. W., Bafna, V. & Mischel, P. S. Extrachromosomal oncogene amplification in tumour pathogenesis and evolution. *Nat Rev Cancer* **19**, 283–288 (2019).
52. Bergstrom, E. N. *et al.* Mapping clustered mutations in cancer reveals APOBEC3 mutagenesis of ecDNA. *Nature* (2022) doi:10.1038/s41586-022-04398-6.
53. *FastQC*. (2015).
54. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv:1303.3997 [q-bio]* (2013).
55. McKenna, A. *et al.* The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* **20**, 1297–1303 (2010).
56. Rausch, T. *et al.* DELLY: structural variant discovery by integrated paired-end and split-read analysis. *Bioinformatics* **28**, i333–i339 (2012).
57. Wala, J. A. *et al.* SvABA: genome-wide detection of structural variants and indels by local assembly. *Genome Res.* **28**, 581–591 (2018).
58. Chong, Z. *et al.* novoBreak: local assembly for breakpoint detection in cancer genomes. *Nat Methods* **14**, 65–67 (2017).
59. Van Loo, P. *et al.* Allele-specific copy number analysis of tumors. *Proceedings of the National Academy of Sciences* **107**, 16910–16915 (2010).
60. McLaren, W. *et al.* The Ensembl Variant Effect Predictor. *Genome Biol* **17**, 122 (2016).

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Figures

Figure 1. Landscape of somatic variants in clinically heterogeneous neuroblastomas. Top, risk group (HR MNA: high-risk *MYCN*-amplified, n = 24; HR non-MNA: high-risk non-*MYCN*-amplified, n = 41; non-HR: non-high-risk, n = 49), sex, stage of the disease, and age. Next, number of SNVs and indels, SVs, and CNAs in each of the 114 neuroblastoma patients. Middle, presence of known neuroblastoma chromosomal arm gains (blue), losses (orange), and translocations between chromosome 11 and 17 (green) in each patient. Bottom, presence of mutations in known neuroblastoma driver genes, including genes of the RAS pathway in each patient. Genes are ranked based on their mutation frequency in the cohort. The variant types considered here are SNVs and indels classified as missense and stop gained variants, SVs close and within the gene, amplifications, and homozygous deletions. Color indicates the type of mutation (key at the bottom). Patients with more than one type of variant in a gene have multiple colors designated. The right histogram indicates the percentage of mutations affecting each genomic feature in the whole cohort. Variant type is indicated by color.

Figure 2. Distribution and correlation of SNV, indel, SV, and CNA-associated signatures in neuroblastoma clinical risk groups. (a) Top, spectrum of SNVs in the different risk groups. Bottom, exposure (in percentage) of the six SNV-associated signatures (SBS) identified in our neuroblastoma cohort by clinical risk group. Each color displays a different signature: SBS3, SBS12, SBS18, SBS36, SBS37, SBS40. (b) Exposure (in percentage) of the six indel signatures (ID; insertions and deletions < 50 bp) identified in our cohort by clinical risk group. Each color displays a different signature: ID1, ID2, ID4, ID6, ID8, ID9. (c) Exposure (in percentage) of the 17 CNA signatures (CX; gains, losses, amplifications, and homozygous deletions) extracted in our cohort by clinical risk group. Each color displays a different signature: CX1-CX17. (d) Exposure (in percentage) of the six SV signatures (SV; deletions, duplications, translocations,

and inversions) identified in our cohort by clinical risk group. Each color displays a different signature: SV1-SV6. SV1 and SV2 correspond to reference signatures R6b and R6a, respectively. In a-d, columns are ordered by neuroblastoma clinical risk classification. (e) Heatmap depicting the positive (red) and negative (blue) correlations between the signatures associated with different types of mutations (SNV, indel, SV, and CNA). Colors display the Spearman correlation coefficient. Only significant correlations are included ($P < 0.05$, FDR correction).

COMPLEX REARRANGEMENTS DISCOVERY 114 neuroblastomas

Figure 3. Different classes of complex rearrangement patterns are detected in neuroblastoma. Eight complex rearrangement types identified in our cohort of 114 neuroblastoma patients: chromothripsis, breakage-fusion-bridge cycles (BFB), extrachromosomal circular DNA (ecDNA), complex non-cyclic amplicons (CnC), chromoplexy, templated insertion chains (TIC), rigma (cluster of deletions), and pyrgo (cluster of tandem-duplications). Colors indicate the class of rearrangement: complex amplicons, chromoplexy-like, and others (key at the bottom).

Figure 4. Topography of complex rearrangements in neuroblastoma. (a) Upset plot depicting the co-occurrence of the different types of complex SV patterns within patients. The number of patients with each combination of rearrangements is shown in the top histogram (colors display the risk group for each patient). The histogram on the right panel displays the frequency for each of the complex SV types in our cohort. (b) Circos plot showing the regions of the genome involved in complex rearrangements in the three risk groups. The inner yellow circle shows the frequency of complex SVs in each region considering the whole neuroblastoma cohort (n=114). Scale is in percentage. *MYCN*, *RASGRP3*, *SMARCE1*, *EBF1*, *SDHA*, and *HMGA2* regions are highlighted as the most recurrently rearranged across patients.

Figure 5. Co-occurrence of mutational signatures, complex rearrangements, and mutated driver genes. (a) Heatmap depicting the correlations between the signatures associated with different variant types (SNV, indel, SV, and CNA) and the eight signatures complex rearrangement classes identified in our cohort (n=114). Colors display the Spearman correlation coefficient. Only significant correlations are included (P < 0.05, FDR correction). (b) Heatmap showing the correlations between mutated neuroblastoma driver genes and DNA damage repair genes and all the mutational signatures and complex rearrangement types identified in our cohort (n=114). Colors display the Spearman correlation coefficient. Only significant correlations are included (P < 0.05, FDR correction).

Figure 6. Clinical implications of different mutational processes and complex rearrangement patterns in neuroblastoma. (a) Summary of the three mutational scenarios defined in our neuroblastoma cohort, including the mutational processes, complex rearrangements, and clinical implications associated with each of them. (b) Kaplan-Meier survival curve showing the clinical impact of the three described mutational scenarios. Patients presenting all the signatures of scenario #1 (SBS18-driven; red) have lower survival than patients with any mix of signatures not assigned to a single scenario (green), patients with all signatures of scenario #2 (SBS3-driven; purple), and patients with all signatures of scenario #3 (SBS40-driven; blue) ($P = 2.5 \times 10^{-2}$ by Log-rank test). Colors on Kaplan-Meier plots display each condition.

Supplementary Figure 1. Flow chart of the analysis strategy. Top, description of the distribution (in percentage) of the 114 neuroblastoma samples within sex groups (female, male, and unknown), risk groups (HR MNA, HR non-MNA, and non-HR: 29.8% low-risk; 8.8% low-risk stage 4S; 4.4% intermediate-risk), and age at diagnosis groups (< 1 year old, 1-5 years old, and > 5 years old). Middle, summary of the sequencing datasets available for our analysis. WGS matched tumor-normal pairs for 114 patients. Bottom, description of the main steps carried out in our study, starting by the variant discovery and the mutational signatures analysis, followed by the characterization of the complex rearrangements present in our samples, and the evaluation of the clinical impact of the identified mutational entities in neuroblastoma to finalize with the definition of the three clinically relevant neuroblastoma mutational scenarios presented in this work.

Supplementary Figure 2. Distribution of the different somatic variant types across the neuroblastoma risk groups. Comparison between distributions of different variant types in the three neuroblastoma risk groups (HR MNA, HR non-MNA, non-HR). To assess if there are differences between risk groups, we used the non-parametric Kruskal-Wallis test (p-value in the upper left corner). The pairwise comparisons were done using the non-parametric Wilcoxon rank-sum test. (a) SNVs and indels, (b) SVs, (c) CNAs, and (d) translocations. Significance legend: *: p-value < 0.1, **: p-value < 0.05, ***: p-value < 0.01.

Supplementary Figure 3. SNV-based mutational signatures analysis. (a) Ratio of exposure of the six reference SNV-based signatures (SBS) identified in our cohort for each of the 114 neuroblastoma patients. Patients have been grouped by risk (HR MNA, HR non-MNA, non-HR). Each color displays a different signature: SBS3, SBS12, SBS18, SBS36, SBS37, SBS40. (b) Mutational profiles for each of the reference SNV-based signatures detected in our cohort, reproduced from the COSMIC database (<https://cancer.sanger.ac.uk/signatures/sbs/>).

Supplementary Figure 4. Indel-based mutational signatures analysis. (a) Ratio of exposure of the six reference indel-based signatures (ID) identified in our cohort for each of the 114 neuroblastoma patients. Patients have been grouped by risk (HR MNA, HR non-MNA, non-HR). Each color displays a different signature: ID1, ID2, ID4, ID6, ID8, ID9. (b) Mutational profiles for each of the reference indel-based signatures detected in our cohort, reproduced from the COSMIC database (<https://cancer.sanger.ac.uk/signatures/id/>).

Supplementary Figure 5. SV-based mutational signatures analysis. (a) Ratio of exposure of the 6 SV-based signatures identified in our cohort for each of the 114 neuroblastoma patients. Patients have been grouped by risk (HR MNA, HR non-MNA, non-HR). Each color displays a different signature: SV1-SV6. (b) Mutational profiles for each of the SV-based signatures detected in our cohort. (c) Mutational profiles for reference SV-based signatures R6a and R6b, corresponding to the de-novo identified signatures SV2 and SV1, respectively, reproduced from Signal database (<https://signal.mutationalsignatures.com/>).

Supplementary Figure 6. CNA-based mutational signatures analysis. (a) Ratio of exposure of the 17 CNA-associated signatures identified in our cohort for each of the 114 neuroblastoma patients. Patients have been grouped by risk (HR MNA, HR non-MNA, non-HR). Each color displays a different signature: CX1-CX17.

Supplementary Figure 7. General characteristics of complex rearrangements. (a) Percentage of SVs involved in each of the eight classes of complex rearrangement. Colors display the distribution of the rearrangements within neuroblastoma risk groups. (b) Comparison between distributions of the ratio of SVs involved in complex rearrangements across the three neuroblastoma risk groups (HR MNA, HR non-MNA, non-HR). To assess differences between risk groups, we used the non-parametric Kruskal-Wallis test. The pairwise comparisons were made using the non-parametric Wilcoxon rank-sum test. No significant differences were observed between risk groups.

Supplementary Figure 8. Survival plots of mutational processes and complex rearrangements in neuroblastoma showing a significant clinical impact in neuroblastoma. (a) Heatmap showing the significant associations between the different mutational signatures and complex rearrangement types identified in our cohort (n=114) with patients' overall survival. These multiple tests have been performed for all patients and each of the risk groups separately. P-values have been adjusted for multiple testing using false-discovery rate correction. Colors display the adjusted p-values < 0.1. Significant associations are marked with an asterisk (adjusted p-value < 0.05). Kaplan-Meier survival curve showing the overall survival of (b) patients presenting only ecDNA (green), ecDNA and linear complex rearrangements (blue), only linear complex rearrangements (turquoise) or no complex rearrangements (purple) ($P < 1 \times 10^{-4}$ by Log-rank test). (c) patients with (n = 106) and without (n = 8) exposure of

SNV-based signature SBS3. Patients exhibiting patterns of SBS3 (turquoise) have better survival than patients not showing this signature (red) ($P < 2.6 \times 10^{-3}$ by Log-rank test). (d) patients with ($n = 103$) and without ($n = 11$) exposure of SNV-based signature SBS40. Patients exhibiting patterns of SBS40 (turquoise) have better survival than patients not showing this signature (red) ($P < 3.1 \times 10^{-4}$ by Log-rank test). (e) patients with ($n = 111$) and without ($n = 3$) exposure of CNA-based signature CX17. Patients exhibiting patterns of CX17 (turquoise) have worst survival than patients not showing this signature (red) ($P < 1.9 \times 10^{-3}$ by Log-rank test). Colors on Kaplan-Meier plots display each condition.

Supplementary Figure 9. Motifs closely resembling specific recombination signal sequences of *PGBD5*. Sequence logos detected near the SV breakpoints in (a) all 114 patients from our neuroblastoma cohort, (b) considering high-risk *MYCN*-amplified patients exclusively, (c) considering high-risk non-*MYCN*-amplified patients exclusively, and (d) considering non-high-risk patients exclusively.

Supplementary Table 1. Distribution of the exposure of each of the different mutational signatures across the three neuroblastoma risk groups. Table showing the exposure per risk group for each of the signatures detected in our neuroblastoma cohort. All numbers are percentages. The last column depicts the results of the Kruskal-Wallis rank sum test in order to evaluate the presence of significant differences in the exposure of each of the signatures across the three neuroblastoma risk groups. “yes” illustrates the presence of differences with a p-value < 0.05.

Signature ID	Variant type	Risk group exposure (%)			Differences between risk groups
		HR MNA	HR non-MNA	non-HR	
SBS3	SNV	9.2	25.4	21.7	yes
SBS12	SNV	2.4	1.4	6.9	yes
SBS18	SNV	63.4	40.1	19.3	yes
SBS36	SNV	2.2	0.3	0.5	no
SBSB37	SNV	7	2.4	8.1	yes
SBSB40	SNV	15.7	30.3	43.6	yes
ID1	INDEL	18.4	7.1	3.8	yes
ID2	INDEL	14.1	6.6	3.2	yes
ID4	INDEL	33.4	33.7	51.7	yes
ID6	INDEL	10.3	17.1	16	yes
ID8	INDEL	11.5	10.6	13.2	no
ID9	INDEL	12.3	24.9	12.1	yes
ID1+ID2	INDEL	32.5	13.7	7	yes
CX1	CNA	64.4	57.5	69.2	yes
CX2	CNA	1.75	2.39	2.76	no
CX3	CNA	12.2	21.5	12.8	yes
CX4	CNA	0.43	1.56	0.49	no
CX5	CNA	5.8	3.6	2.3	yes
CX6	CNA	0.3	0.17	0.5	no
CX7	CNA	3.7	2.5	2.4	yes
CX8	CNA	0.48	0.11	0.12	no
CX9	CNA	1.8	1.25	1.13	yes
CX10	CNA	0.68	0.58	0.31	no
CX11	CNA	3.25	1.33	0.83	yes
CX12	CNA	1.11	1.3	1.41	no
CX13	CNA	0.21	0.02	0.0002	no
CX14	CNA	1.38	2.17	3.77	yes
CX15	CNA	1.4	3.4	1.6	no
CX16	CNA	0.92	0.37	0.32	no
CX17	CNA	0.14	0.13	0	no
CX7+CX9+CX11	CNA	8.75	5.08	4.36	yes
SV1/R6b	SV	2.8	5.9	1.8	no
SV2/R6a	SV	2.8	4.2	1.8	no
SV3	SV	15.5	20.7	14.8	no

SV4	SV	29.9	25.7	39.8	no
SV5	SV	1.7	2	9.6	no
SV6	SV	47.2	41.6	32.1	no
SV1+SV2+SV5	SV	7.4	12	13.3	no
SV3+SV4+SV6	SV	92.6	88	86.7	no

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Supplementary Table 2. Distribution of the different complex rearrangement classes across the three neuroblastoma risk groups. (a) Table showing the percentage of complex rearrangements detected in our neuroblastoma cohort across the three different neuroblastoma risk groups. Numbers in brackets are absolute number of rearrangements. (b) Table showing the percentage of patients presenting complex rearrangements for each of the neuroblastoma risk groups. The last column depicts the results of the Kruskal-Wallis rank sum test in order to evaluate the presence of significant differences between neuroblastoma risk groups. “yes” illustrates the presence of differences with a p-value < 0.05.

a)

Complex SV class	# of complex SVs (%)			Differences between risk groups
	HR_MNA	HR_non-MNA	non-HR	
BFB	66.7 (2)	33.3 (1)	0	no
Chromoplexy	13.9 (5)	55.6 (20)	30.5 (11)	no
Chromothripsis	0	100 (4)	0	no
CnC	34.3 (12)	57.1 (20)	8.6 (3)	no
ecDNA	68 (53)	25.6 (20)	6.4 (5)	yes
Pyrgo	42.8 (3)	28.6 (2)	28.6 (2)	no
Rigma	0	71.4 (5)	28.6 (2)	no
TIC	8.1 (3)	56.8 (21)	35.1 (13)	yes

b)

Complex SV class	# of patients with complex SVs (%)			Differences between risk groups
	HR_MNA	HR_non-MNA	non-HR	
BFB	8.3	2.4	0	no
Chromoplexy	20.8	14.6	18.4	no
Chromothripsis	0	7.3	0	no
CnC	25	24.4	6.1	no
ecDNA	100	22	6.1	yes
Pyrgo	12.5	4.9	4.1	no
Rigma	0	12.2	4.1	no
TIC	12.5	41.5	24.5	yes

Supplementary Table 3. FISH validation of the MYCN amplicon state in our cohort.
 Analysis of the MYCN amplicon state (homogenously staining region, HSR; extrachromosomal circular DNA, ecDNA) as measured by FISH and interpreted by the German clinical diagnostic reference center and tumor bank for neuroblastoma.

Sample ID	MYCN amplification	FISH confirmation
CB2001	Yes	ecDNA
CB2013	Yes	HSR/ecDNA
CB2022	Yes	ecDNA
CB2024	Yes	ecDNA
CB2025	Yes	HSR/ecDNA
CB2027	Yes	ecDNA
CB2030	Yes	ecDNA
CB2031	Yes	ecDNA
CB2034	Yes	HSR/ecDNA
CB2040	Yes	ecDNA
CB2042	Yes	ecDNA
CB2043	Yes	HSR
CB2045	Yes	ecDNA
CB2050	Yes	ecDNA
NBL26	Yes	ecDNA
NBL27	Yes	ecDNA
NBL28	Yes	ecDNA
NBL29	Yes	ecDNA
NBL30	Yes	ecDNA
NBL31	Yes	ecDNA
NBL32	Yes	ecDNA
NBL33	Yes	ecDNA
NBL34	Yes	ecDNA
NBL42	Yes	ecDNA