Whole genome sequencing puts forward hypotheses on metastasis evolution and therapy in colorectal cancer

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Incomplete understanding of the metastatic process hinders personalized therapy. Here we report the most comprehensive whole-genome study of colorectal metastases vs. matched primary tumors. 65% of somatic mutations originate from a common progenitor, with 15% being tumor- and 19% metastasis-specific, implicating a higher mutation rate in metastases. Tumor- and metastasis-specific mutations harbor elevated levels of BRCAness. We confirm multistage progression with new components ARHGEF7/ARHGEF33. Recurrently mutated non-coding elements include ncRNAs RP11-594N15.3, AC010091, SNHG14, 3’ UTRs of FOXP2, DACH2, TRPM3, XKR4, ANOS, CBL, CBLB, the latter four potentially dual protagonists in metastasis and efferocytosis/PD-L1 mediated immunosuppression. Actionable metastasis-specific lesions include FAT1, FGFL, BRCA2, KDR, and AKT2-, AKT3-, and PDGFRA-3’ UTRs. Metastasis specific mutations are enriched in PI3K-Akt signaling, cell adhesion, ECM and hepatic stellate activation genes, suggesting genetic programs for site-specific colonization. Our results put forward hypotheses on tumor and metastasis evolution, and evidence for metastasis-specific events relevant for personalized therapy.

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Metastasis is the leading cause of cancer-related mortality and remains challenging due to its resistance to therapy, aggressive phenotype and multi-organ affection. Clearly, metastasized lesions behave differently from their precursor primaries and this recognition has led to advancements of several hypotheses, including that of cancer stem-cells to explain this behavior. Accordingly, attempts have been made to identify genetic alterations that differentiate metastatic from primary tumors. Interestingly, most molecular comparisons have been made between advanced primary tumors and early-stage (non-metastasized) tumors, without looking at the metastatic lesions themselves. Very few studies have analyzed metastatic lesions with their corresponding primaries; however, these studies were restricted to a defined set of protein coding genes. Recent attempts using next generation sequencing have characterized the mutational landscape of solid primary tumors to a greater detail, but done little to add to our knowledge of metastatic disease.

In colorectal cancer, the largest exome studies were by Giannakis et al. with 619 primary tumor samples, building upon the previous Cancer Genome Atlas (TCGA) study where 276 primary tumors were analyzed. A study by Yaeger et al. examined 1099 tumors and their metastases, but this study was limited to 2 samples.

Here we present the most comprehensive analysis of whole-genome differences between metastatic lesions and their corresponding primaries in micro satellite stable colorectal cancer samples from patients without a prior familial history of the disease, thus reducing many hidden germline components. Using whole-genome sequencing, we characterize the metastatic lesions of 12 patients (details in Methods, Tables 1 and 2, Supplementary Data 1), together with their primary tumors and corresponding normal samples, assess somatic genomic lesions and mutational signatures, and ascertain similarities, as well as differences between primary tumors and metastases. Although we identify a number of additional non-coding facets of disease progression, more importantly, we assess and identify metastasis-specific clinically relevant mutations and mutational signatures that may impact future therapy decisions. The results put forward novel hypotheses on metastasis evolution and suggest new components of disease progression.

**Results**

Somatic single nucleotide variations, mutations, and indels.

First, we determined the mutational load in the 12 resected tumors and their metastases, but this study was limited to 2 samples.

| Table 1 Patient clinical and sample information |
|-----------------------------------------------|
| **Patient IDs** | **Age at Surgery** | **Gender** | **Diagnosis** | **Histology** | **pT** | **pN** | **M** | **Metastasis site** |
| CRC-001 | 63 years 5 months | Male | Cancer of colon | Adenocarcinoma | 3 | 1 | 1 | Liver |
| CRC-002 | 58 years 7 months | Female | Cancer of colon | Tubulo-papillary Adenocarcinoma | 2 | 1 | 1 | Liver |
| CRC-003 | 65 years 0 months | Male | Cancer of rectum | Adenocarcinoma | 3 | 2 | 1 | Liver |
| CRC-004 | 55 years 11 months | Female | Cancer of rectum | Malignantly differentiated Adenocarcinoma | 3 | 2 | 1 | Lung |
| CRC-005 | 48 years 6 months | Male | Cancer of rectum | Moderately differentiated Adenocarcinoma | 4 | 1 | 1 | Liver |
| CRC-006 | 55 years 10 months | Female | Cancer of rectum | Adenocarcinoma | 3 | 2 | 1 | Liver |
| CRC-007 | 64 years 7 months | Male | Cancer of rectum/colon | Tubulo-papillary Adenocarcinoma | 3 | 2 | 1 | Liver |
| CRC-008 | 48 years 9 months | Male | Cancer of rectum | Adenocarcinoma | 3 | 1 | 1 | Liver |
| CRC-009 | 70 years 5 months | Male | Cancer of colon | Tubulo-papillary Adenocarcinoma | 4 | 2 | 1 | Liver |
| CRC-010 | 68 years 1 months | Female | Cancer of colon | Moderately differentiated Adenocarcinoma | 3 | 2 | 1 | Liver |
| CRC-011 | 59 years 9 months | Male | Cancer of rectum | Adenocarcinoma | 3 | 1 | 1 | Liver |
| CRC-012 | 62 years 9 months | Male | Cancer of rectum | Tubulo-papillary Adenocarcinoma | 3 | 0 | 1 | Liver |

Table displaying the anonymized/pseudonymized patient ID, age, gender, diagnosis and histology of patients/tumors. The initial staging of the disease is shown in fields for primary tumor (pT), regional lymph nodes (pN), distant metastasis (M).

| Table 2 Patient clinical and sample information, continued |
|-------------------------------------------------------------|
| **Tumor location (site)** | **Pre-surgical therapy** | **Tumor cell content (ACEseq)** | **Tumor ploidy (ACEseq)** | **Metastasis cell content (ACEseq)** | **Metastasis ploidy (ACEseq)** |
| Sigmoid colon (left) | - | 0.85 | 2.16 | 0.57 | 2.28 |
| Transverse colon (right) | - | 0.55 | 3.28 | 0.56 | 3.33 |
| Rectum (left) | Neo-adjuvant RCTX | 0.6 | 3.44 | below 0.3 | N/A |
| Rectum (left) | - | 0.69 | 3.12 | 0.67 | 3.03 |
| Rectum (left) | - | 0.39 | 3.08 | 0.4 | 2.75 |
| Rectum (left) | Neo-adjuvant RCTX | 0.36 | 2.19 | 0.63 | 2.21 |
| Recto sigmoid (left) | - | 0.61 | 3.49 | 0.48 | 3.41 |
| Rectum (left) | - | 0.42 | 3.72 | 0.31 | 3.73 |
| Sigmoid colon (left) | - | below 0.3 | N/A | below 0.3 | N/A |
| Caecum (right) | - | 0.68 | 2 | 0.65 | 1.73 |
| Rectum (left) | - | 0.49 | 3.87 | 0.37 | 3.9 |
| Rectum (left) | Neo-adjuvant RCTX | below 0.3 | N/A | 0.86 | 2.28 |

1. Tumor site and location, pre-surgical therapy, tumor cell content, tumor ploidy, metastasis cell content and metastasis ploidy are listed. RCTX abbreviates radio-chemo therapy.
metastasis samples as compared to the matched primaries (Supplementary Data 2, Supplementary Figure 1). We observed a median of 10,468 (range 5773–16,934) and 11,475 (range 4774–17,189) somatic single nucleotide variations (SNVs) in the tumor and metastasis samples with high tumor cellularity, consistent with our samples being non-hyper mutated, non-ultra-mutated and microsatellite stable (MSS). We found that 65% (36–92%) of all SNVs were shared between tumors and corresponding metastases, clearly suggesting a common ancestral truncal clone with 15% (1–29%) tumor-specific and 19% metastasis-specific (3–42%), respectively (Supplementary Data 2). This suggests that the rate of mutation is higher in the metastatic lesion compared to the matched tumor after truncal separation.

Next, we investigated recurrent coding mutations. The most recurrently mutated genes are well established in colorectal carcinogenesis (Fig. 1). Mutations in these driver genes were present in both tumor and metastasis high-purity pairs, apart from colorectal cancer (CRC) patient CRC-010 where the TP53 mutation was only observed in the metastasis sample. In addition to these, we observed recurrent mutations in ARHGEF33, a

![Fig. 1 Recurrent somatic small mutations on protein coding genes. Oncoprint representation of recurrently mutated genes with a cutoff of 4 samples (17%). The top annotation shows the tumor cell content (TCC) and estimated tumor ploidy. The color of the box indicates the type of mutation. Recurrently mutated genes are marked with D, T, M if they were also recurrently mutated (>2.5%) in the Giannakis et al./DFCI 2016 (D), TCGA provision (T) and Yaeger et al./MSK-CC 2018 cohorts (M) via cBioPortal](#)
guanine nucleotide exchange factor (GNEF) that facilitates small GTPases like KRAS, and SPHKAP, which encodes an A-kinase anchor protein.

Furthermore, we also found previously undescribed recurrently mutated non-protein coding genes in tumors and metastases (Supplementary Figure 2, Supplementary Data 3). These included AC010091.1, CTD-2292P10.4, RP11-59N15.3, and SNHG14. AC010091.1 shares homology with protocadherin FA74, which negatively regulates Wnt signaling and its knockdown induces epithelial–mesenchymal transition (EMT) in gastric cancer (GC) 13. SNHG14 has been shown to bind directly to miR-145-5p 14, a potent tumor suppressor in multiple cancer types 15,16. The non-coding ribonucleic acid (ncRNA) RP11-421IL10.1 was more recurrently mutated in metastasis (3 vs 1).

In 3′-untranslated regions (UTRs), commonly affected genes included XKR4, ANO5, FOXP2, CBL, CBLB, NTRK3, TRPM3, DACH2, the latter also more recurrently mutated in metastases (3 vs 2) and FOXP2 only in diploids (Supplementary Figure 2). We observed that 3′-UTR mutations of XKR4 were mutually exclusive to ANO5 (i.e., patients with XKR4 3′-UTR mutations did not harbor ANO5 3′-UTR mutations, or vice versa). These genes are paralogs of XKR8 and ANO6/TMEM16F, which mediate an externalization of phosphatidyl serine, creating an immunosuppressive tumor micro environment 18. Likewise, samples with mutations in the 3′-UTR of E3 ubiquitin-protein ligase CBL showed mutual exclusivity to its parologue CBLB.

These genes have been shown to inhibit EGFR signaling through degrading EGFR and binding to GRB219. CBL has also been described to be involved in cancer progression and metastasis 20, the nuclear degradation of β-catenin 21, and to downregulate PD-L1 in non-small cell lung cancer 22. FOXP2 has also been shown to bind to and downregulateCNTNAP2 23. We evaluated potential perturbations in miRNA mediated messenger RNA (mRNA) stability caused by these 3′-UTR mutations in silico (Supplementary Data 4). In patient CRC-006, a mutation in the 3′-UTR of FOXP2 causes the potential loss of regulation by miR-670-5p, miR-3912-5p, miR-4669, miR-6753-3p, and miR-190b, which has been shown to bind to the FOXP2 3′-UTR in gastric cancer (GC) 24. In CRC-004, a mutation causes the targeting of the XKR4 3′-UTR by 7 additional miRNAs and in CRC-007, a mutation in the 3′-UTR of the same gene results in enhanced interaction of miR-1293. Similarly, in CRC-011, a mutation in the 3′-UTR of ANO5 causes a loss of binding for 6 miRNAs; however, binding is enhanced for 13 additional miRNAs shifting the flux towards mRNA degradation.

Copy number aberrations. Copy number aberration (CNA) patterns were similar in tumors and metastases (Fig. 2, Supplementary Figure 3). In addition to recurrent arm level events found in the TCGA study 5, we observed recurrent amplifications of chromosome arms 6p and q and 16p and losses in 4p, 5q, 8p. The gains on chromosome 4 seen in tumors were virtually absent in metastases (Fig. 2a, b). Further differences include gains of chromosomes 9, 11 and loss of Y, which were more frequent in metastasis samples, and gains of chromosomes 2q, 10p, 13, 17, 21 and X and losses of 15 which were less frequent.

We also observed chromothripsis-like chromosomal rearrangements in five samples, all of which carried a TP53 mutation. Certain high level genomic rearrangements did not persist in the metastasis (Fig. 3).

We also compared copy number aberrations with miRNA gene expression changes 25, and found amplifications associated with the increased expression of miR-483, miR-409, miR-411, miR-134, miR-154, miR-654, miR-299, miR-382, miR-379, and miR-487b in the metastases. Deletions coupled with reduced expression were observed for miR-34a, miR-552, miR-30e, and miR-122 in primaries or metastases.

Structural variations. MACROD2 was the gene most recurrently hit by structural variations (SVs), followed by PDE11A, TTC28, FHIT, and PARK2 (Fig. 3, Supplementary Figure 4, Supplementary Figure 5, Supplementary Data 5). MACROD2 26, FHIT, and PARK2 are located on chromosomal fragile sites and their deletions are indicative of replication stress. Remarkably, one of the most frequently deleted loci in the TCGA study, RBFOX1, did not show frequent events in our cohort. The few cases (4 of 12) where RBFOX1 showed deletions were tumor-specific events, suggesting negative selection of RBFOX1 in metastasis. Structural aberrations involving SMAD5, MACROD2, Igf2 and the non-coding gene AC007319.1 were found to be more recurrent in metastasis. SVs involving ARHGEF18, IFNGR2, RBFOX1, SLIT3, TMEM50B, non-coding genes CTD-2374C24.1, RP11-6N13.1, RP11-420N3.2, CTD-2207023.3, and CTC-575N7.1 were seen more recurrently in primary tumors (Supplementary Data 5).

An extended colorectal cancer progression model. The classical model of colorectal cancer progression 27 describes sequential gains of mutations in Wnt signaling, Ras signaling, TGF-β signaling and p53 signaling. Performing mutual exclusivity and co-occurrence analysis allowed us to place additional components to this model (Fig. 4, Supplementary Data 6). We identified highly redundant mutational targeting of negative regulators of the Wnt signaling (Fig. 4a), with 85% of high-purity samples having mutations in 3 recurrently mutated regulators. Although we confirm known regulators, including APC, TCF7L2, FBXW7, and SOX9 (of which the latter 3 are mutually exclusive), we show that SOX9 is mutated in diploid only samples. This is further supported by a significant mutual exclusivity of SOX9 mutations with TP53 mutations (associated with aneuploidy) in the TCGA cohort (p-value 0.025, Fisher exact test). AC010091.1, mutated in 25% of samples, may play a role in the nuclear regulation of β-catenin as a decoy for miRNAs targeting FAT4, a suppressor of Wnt signaling 28. Mutations in AC010091.1 were mutually exclusive to TCF7L2 and KRAS. Our data also suggest that LRPP1, a negative regulator of Wnt signaling that is down-regulated in right-sided colorectal cancer (rCRC) 29, may play a role in Wnt signaling upstream of APC, as an alternative to the TCGA’s proposed LRPP5. LRPP1 mutations were nearly always associated with triploidy. Mutual exclusivity of 3′-UTR mutations in CBL and CBLB implicate them as regulators of tumorigenic β-catenin 21 independent of FBXW7. However, CBL and CBLB may play a dual role, which have also been implicated in downregulation of EGFR signaling.

We observed mutual exclusivity of KRAS, NRAS mutations and guanine nucleotide exchange factors ARHGEF33 and ARHGEF7 (Fig. 4b), suggesting that these may play a similar role to KRAS and NRAS mutations. Other studies also showed recurrent mutations in ARHGEF genes (Supplementary Figure 6) and the distribution of mutations in several ARHGEF genes clustered toward the RhoGEF and Plekstrin homology (PH) domains (Supplementary Figure 7). In the TCGA series, we find that ARHGEF7 mutations associate with worse disease-free survival (p-value 0.004, logrank test) and generally, patients with ARHGEF mutations show worse disease-free survival (p-value 0.04) (Supplementary Figure 8). In our present series, NRAS and ARHGEF7 were mutated only in diploid samples, while KRAS and ARHGEF33 mutations were associated with aneuploidy and TP53 mutations in all but 1 case.

We did not observe recurrent small mutations on components of TGF-β signaling; however, all but one of our samples exhibited loss of chromosome 18 which contains the key genes SMAD2 and
This loss of chr18 has also been associated with hepatic metastasis\(^{30}\). Mutations in TP53 were associated with aneuploidy (Fig. 4c). Although most TP53 mutations were present in both tumor and metastasis samples, CRC-010 exhibited a TP53 mutation in the metastasis, but not the primary tumor which instead had an 11 Mb deletion spanning ATM, a regulator of TP53 (Fig. 5). This suggested two independent carcinoma triggering events in this patient. In line with evasion of apoptosis, we propose a potential role of perturbed phosphatidyl serine externalization facilitating...
immune evasion, by dysregulating 3'-UTR mutations of the XKR and TMEM16 family genes XKR4 (exclusively mutated in triploids) and ANO5 (TMEM16E). Recently, it has been shown that both CBL and CBLC play a role in modulating expression of programmed death ligand 1 (PD-L1), thus also playing a role in immune evasion (Fig. 4c).

Finally, by stratification of the mutational catalog, we were able to identify signatures particular to early-stage development (Fig. 6), and later evolution of the resultant tumor and metastasis samples. We observed more prominent DNA mismatch repair (MMR) defect signatures (AC6 and AC15) in early development, which seemed to be replaced by gain of a DNA, double-strand break-repair by homologous recombination (DSB) repair defective signature (AC3) in later stages (see following section).

### Mutational patterns and signatures in disease progression

We sought to identify additional patterns that would potentially be indicative of disease progression after finding evidence for an increased mutational rate in metastases as compared to primaries. Looking into cancer mutational signatures of the stratified catalog of tumor-specific, metastasis-specific, and shared mutations, we found signatures AC1, AC3, AC5, AC6 and AC9, AC10, AC13, AC15 and AC17 (Fig. 6a, b). Signatures AC1 and AC5 are believed to be caused by age-related clock-like mutagenic processes, AC1 initiated by spontaneous deamination and AC5 by an unknown mechanism. Signatures AC3, AC6, and AC15 have been associated with failure of DNA repair systems, in case of AC3 by failure of double-strand break-repair by homologous recombination and in case of AC6 and AC15 by failure of mismatch repair (MMR); signature AC9 is attributed to the activity of activation-induced (Cytidine) deaminase (AID). Signature AC10 has been linked to altered polymerase (POL) E function, signature AC13 is linked to the activity of members of the APOBEC enzyme family and signature AC17 has not been associated with a specific mechanism yet.

Unsupervised clustering of the stratified catalogs based on normalized exposures of mutational signatures revealed a significant association between ploidy and the clock-like signatures AC1 and AC5: high exposure to AC1 is associated with diploidy, whereas enrichment of AC5 is associated with polyploidy (Fig. 6c).

Comparing normalized exposures in different strata of SNVs, the clock-like signature AC1 (spontaneous deamination) is more truncal (significant before, trend after Benjamini-Hochberg (BH)-correction). Furthermore, we observed differences in DNA repair defect signatures: AC6 and AC15 (MMR) are truncal (significant before, trend after BH-correction), whereas AC3 (DSB, BRCA-ness) is an ongoing mutational process with significantly higher contributions in the strata private to tumors and metastases (Fig. 6d). This again supports the hypothesis of a common ancestor clone between tumor and metastasis with altered late stage mutagenic processes ongoing after truncal separation.

### Functional relevance of metastasis-specific mutations

We found 48 genes to be mutated in metastases but less so in primary tumors. Performing functional annotation clustering analysis, we found extracellular matrix, PI3K-Akt signaling, and focal adhesion-related pathways to be significantly enriched in metastases ($p$-value of $1.2 \times 10^{-11}, 2.7 \times 10^{-10}$, and $2.2 \times 10^{-5}$, respectively; BH corrected hypergeometric test; Supplementary Figure 9, Supplementary Data 7). Of these 48 genes, 12 were present in the matrisome of metastatic CRC tumor samples of which 11 had lower protein abundance in the metastasis samples, including ADAMTS11, which was a colon tumor-specific extracellular matrix (ECM) protein, not present in normal colon,
metastasis nor liver tissue. None of the metastasis-specific ECM proteins were found in the list of 48 mutated genes.

Additionally, looking at canonical pathways enriched either in tumor or metastasis specifically mutated genes, we found that hepatic fibrosis/stellate cell and actin cytoskeleton cascades were significantly enriched in metastasis (Supplementary Figure 9). As almost all our sequenced metastatic lesions were in the liver, it appears that metastasized cells invoke a response that in some way fosters organ-specific metastatic colonization.

Clinical relevance of metastasis-specific mutations. Genomic alterations in the metastasis genome are clinically relevant if they are actionable (for therapy or decision-making) and more so if they differ from that of the primary tumor. To analytically evaluate such alterations, we used the TARGET database as well as the database of the NCT-MASTER program33 to ascertain potentially clinically relevant events in the tumor and metastasis tissues for individual patients. The number of these mutations in the individual patients ranged from 1 to 17, with an average of nine mutations per sample. Most clinically relevant mutations were identical between tumor and metastasis samples from the same patients. However, in four patients, we found clinically relevant metastasis-specific non-silent mutations of FAT1, FGFI, BRCA2, TP53, and KDR and tumor-specific splice site mutations of JAK2 (Supplementary Data 8). We also searched for alterations in the 3′-UTRs of potentially targetable genes and discovered, with the exception of two patients, at least one per patient affecting different genes. Interestingly, three patients harbored 3′-UTR mutations in genes of clinical interest: AKT3 (CRC-002), PDGFRA (CRC-005), and AKT2 (CRC-010) (Supplementary Data 9).

We also observed EGFR amplifications in the metastasis sample of CRC-005 (4 copies) compared to the tumor (3 copies), implicating consequences for EGFR-based targeted therapy of certain metastases.

Finally, we observed a significantly reduced defective DNA mismatch repair signature (AC3) in the tumor and metastasis-specific mutations compared to the truncal node, but persistence of BRCA-ness mutational signatures, suggesting possible efficacy of PARP inhibitor treatment for both the primary tumors and metastases. An overview of our findings and suggestion of an extended progression model of colorectal cancer and its metastasis is shown in Fig. 7.

Discussion
This is the most comprehensive study to date systematically describing whole-genome landscape differences in tumor and metastatic lesions of colorectal cancer. In our study, an average of 65% of all somatic SNVs were shared between tumors and corresponding metastases; an average of 15% were specific to tumors and an average of 19% specific for metastases, suggesting that the rate of mutagenesis is higher in the metastatic clone compared to the primary tumor.

In line with the Vogelstein model27, we revealed additional protein coding and non-coding components and implicate
dependency of existing mechanisms to ploidy state. Thus, the model can now be further refined. The initial lesion for non-hyper mutated/microsatellite stable tumors is adenoma genesis via redundant perturbations in Wnt signaling leading to over expression of β-catenin for which we identified components LRP1B, AC010091.2, CBL, and CBLB. We hypothesize that the guanine nucleotide exchange factors ARHGEF33 and ARHGEF7 may play a similar role to KRAS and NRAS mutations. While these ARHGEF genes were identified in our series, we found a number of other ARHGEFs that exhibited clustered and recurrent mutations on functional domains, further implicating an important and yet unexplored role of ARHGEF genes. This is of special importance as patients with KRAS and NRAS mutations do not respond well to EGFR inhibitors panitumumab and cetuximab, which may mean that patient CRC-005 that mutation, may not respond to EGFR inhibitor therapy. While the role in TP53 in carcinoma formation is well known, we postulate the role of perturbed phosphatidyl serine externalization interfering with efferocytosis as a result of potential dysregulation of XKR4 and ANO5 by 3'-UTR mutations, which we believe work co-operatively with TP53 mutations.

The clock-like signature AC1, scaling with the number of passed cell cycles, is enriched in polyploid samples, whereas signature AC5, scaling with elapsed time, is enriched in the diploid samples. A possible interpretation is that rapidly cycling tumors are more prone to be associated with gross karyotypic abnormalities. This could stratify patients into clinical subgroups of better responders to drugs with strong anti-proliferative activity, such as 5-fluorouracil (5-FU). Defective DNA DSB repair machinery as indicated by mutational signature AC3 can be targeted by PARP inhibitors. PARP inhibitors could be used not only as chemotherapeutic sensitizers, but as single agents to selectively kill cancers defective in DNA DSB repair while overcoming typical resistance of MMR defective tumors to chemotherapy.

The clinically relevant genes that we found which were exclusive to metastatic lesions include FAT atypical cadherin 1 (FAT1), which regulates cell adhesion, migration, EMT and stemness properties. Somatic mutations of FAT1 are widely expressed in metastatic CRC and can be targeted directly with monoclonal antibody mAb198.3. Fibroblast growth factor 1 (FGF1) is targetable indirectly through its receptors, FGFRs, with agents, including Nintedanib, Pazopanib, Ponatinib. The Kinase insert domain receptor (KDR/VEGFR), functions as the main mediator of VEGF-induced proliferation, survival, migration, and sprouting, and is amenable to drugs, including axitinib, sorafenib, and cabozantinib.

We identified recurrent chromosome arm level events and highlight differences between tumor and metastasis samples. There is an evidence that loss of chromosome 4 is associated with lymph node metastasis, metastatic recurrence, and early micrometastasis. Similar reporting of chromosome 4 amplifications in primary tumors but not their matched metastases has been described for metastatic melanomas, which was localized to 4q12-q13.1 which includes PDGFRA, KIT, KDR, and REST. PDGFRA and KDR are important for gain of metastatic potential
by driving EMT and proliferation. KIT and REST have been implicated as tumor and metastasis suppressors in colorectal cancer. Perhaps, this schizogenic region drives heterogeneity where amplification increases proliferation while reducing its metastatic potential, whereas deletions lead to lower levels of KIT and REST, thus more viable to metastasize.

Importantly, facilitated by whole-genome sequencing, non-coding genes and 3′-UTRs provided significant contributions to the better known protein-coding mutational landscapes of CRCs. At present, it is difficult to completely appreciate their impact as their functions are still poorly understood. Certainly, in an earlier publication, we have described the metastasis-specific microRNA landscape and many of the genomic changes are able to offer putative explanations for particular miRs we have described to be deregulated in expression in metastasis. We observed metastasis-specific 3′-UTR mutations in AKT2 and AKT3. Furthermore, in our study, there is an indication of the importance of 3′-UTR mutations in CBL and CBLB, which plays multiple roles including degradation of tumorigenic β-catenin (encoded by the CTNNB1 gene) in colorectal cancer, degradation of EGFR, and suppressing the expression of PD-L1. We also observed that the most frequent 3′-UTR mutation, which occurred in XKR4, was exclusively in triploid samples, and exhibited mutual exclusivity to 3′-UTR mutations in ANO5, potentially interfering with effecrocytosis. Mutations for both these genes facilitated binding of additional miRNAs in silico.

Together, the potential combined effect of modulation of macrophages via effecrocytosis and T-cells via PD-L1 expression, prime a favorable tumor-microenvironment raising the importance of dysregulation of CBL, CBLB, XKR4 and ANOS in colorectal carcinoma.

Another highlight is the finding that metastatic lesions are enriched in mutations of genes affecting PI3K-Akt signaling, cell adhesion, extracellular matrix, and stellate-cell activation in the liver, the predominant metastasis site in our patient, which we hypothesize is critical for homing within the metastatic niche. This supports the notion that sporadic genetic changes are priming metastatic colonization of tumors to a specific metastatic site, and this is perhaps where the fundamental differences between tumors and metastases lie. Extensive investigations are needed to evaluate functionality of these hypotheses.

Taken together, metastases and tumor genome landscapes are very similar, but definitely not identical, which supports the hypothesis of a divergent evolution of metastatic lesions as compared to the primary tumor after truncal separation. While most of our samples support a late dissemination model, the independent carcinoma triggering events in patient CRC-010 would argue for an early metastasis model, with the split occurring after the intermediate adenoma. In individual cases, actionable mutations private to metastatic lesions are evident. This clearly may warrant clinical consequences and a re-structuration of current personalized therapy concepts aiming at metastasis prevention.
**Fig. 7** Model of colorectal cancer and metastasis progression and therapeutic implications. A summary cartoon of how recurrent somatic mutations identified within this study fit into established colorectal progression models a. The top cartoons represent the transition from normal epithelial cells to metastasis (left to right). Beneath the cartoons are tables of genes and genetic lesions that were mutated in our cohort, sorted in tables related to possible pathway function. Change of relative exposure to mutational signatures are shown as horizontal bars where the strength of exposure corresponds to the strength of color in the bar, relative to tumor evolution (left to right) b. Cartoon representation of lesion (stars) accumulation giving rise to tumor heterogeneity c. Balloons are colored according to pathways, as the table headers in a, showing mutations in Wnt (green), RAS (orange), TGF-β (blue) signaling, and mutations acquired in carcinogenesis (brown), and metastasis (dark brown) formation. We show events which might not give rise to further progression. Mutations with implications on therapy decision are shown in light blue boxes with rounded corners, and linked to boxes with therapy consideration via a thick black line a, b. Gray vertical dashed lines separate out lesions corresponding to truncal origin, tumor, and metastasis states.
Methods

Patient material. Primary tumor, matched metastases and corresponding normal tissues of 12 patients with colorectal cancer were obtained at the Medical Faculty Mannheim, University of Heidelberg, Germany (Tables 1 and 2). The tissue banking and sample study was approved by the Ethical Committee of the University Hospital Mannheim, Medical Faculty Mannheim of Heidelberg University, all relevant ethical regulations were complied with, and informed consent was obtained from all patients or their spouses/relatives when the former were deceased. Bio banking and handling of the tissues followed the BRIQS guidelines.

Genomic DNA isolation. Genomic DNA was isolated from 5 to 10, 20 μM cryosection slices (depending on tissue size) using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s manual. The extracted DNA was submitted to the HIPO Sample Processing Laboratory (HIPO-SPL) for quality check and PCR-amplification using primers designed to be specific for the variant of interest. After amplification the samples were transferred to the Genomics and Proteomics Core Facility of the German Cancer Research Center for sequencing.

Whole-genome sequencing and alignment. Whole-genome DNA sequencing was performed on the HiSeq2000 platform. Library preparation and whole-genome sequencing of matched tumor/normal/metastasis DNA was carried out44. Briefly, 1–5 μg of genomic DNA was fragmented to ~300 bp and size selection conducted by agarose gel excision. Sequencing reads were mapped and aligned using the DKFZ alignment workflow from ICGC Pan-Cancer Analysis of Whole Genome projects [https://dockstore.org/containers/quay.io/pancancer/pcawg-bwa-mem-workflow]. Read pairs were mapped to the 1000 Genomes Project phase 2 assembly of the human reference genome (hs37d5) using Burrows-Wheeler Aligner software (BWA; v0.7.17) using the default parameters set from -T 0. Duplicates were marked with biobambam (version 0.0.148). Single nucleotide variants and indels (insertion or deletion) of the most significant findings were validated by polymerase chain reaction (PCR) using primers that flanked the mutated sequence. Sanger sequencing was done following comparisons to the germline genome sequence for confirmation.

Small variant calling. Small variants were called from the whole aligned whole-genome sequencing data. They were initially called using our in-house workflows, described below, followed by cross checking of variant positions between tumor and matched normal samples. SNVs were initially called using the DKFZ SNV and indel calling workflow from ICGC Pan-Cancer Analysis of Whole Genome projects [https://dockstore.org/containers/quay.io/pancancer/pcawg-dfxz-workflow]54,56. Briefly, the SNVs were called using samtools and bcf tools version 0.1.19.ip13 determined to be somatic or germline by comparing the tumor/metastasis sample to the control and assessing a confidence. The confidence score was initially set to 10, and subsequently reduced based on overlaps with repeats, DACT blacklisted regions, DUKE excluded regions, self-chain regions, segmental duplication records as introduced by the ENCODE project39 and additionally if the SNV exhibited PCR or sequencing strand bias. SNVs with confidence lower than 8 were excluded. Annovar (release Feb 2016)39 using gene models from Gencode version 19 were used to annotate SNVs.

Due to potential tumor in normal contamination leading to false negative calls we applied the TiNDA (tumor in normal detection algorithm) workflow (unpublished). Briefly, using the unique set of combined mutated positions for a tumor, a matched normal sample, and all available exomes to filter out mutations in the control, then a confidence was assigned to the likelihood of the mutation being present in tumor and control samples. Positions overlapping with common variants were filtered out. Then, the clustering algorithm from Canopy40 was applied to the BAF values for the positions in tumor/metastasis vs the control using a single pass run, assuming 9 clusters. The clusters that were determined to be tumor-in-normal had to have ≥75% positions above the identity line, the tumor/metastasis mutant allele fraction (MAF) above 1% and the control MAF below 45%. These identified mutations were then reclassified as somatic instead of the original germline annotation.

Indels were initially called using Platypus41 version 0.8.4. Platypus filters were used to calculate a confidence score ranging from 0 to 10. Indels with confidence lower than 8 were excluded. Annovar was used to annotate indels.

Due to varying tumor cell content, we cross checked allele frequencies of mutations between tumors and metastasis to validate those small mutations were not missed due to lower tumor cell content in either the tumor or metastasis samples. A SNV was called when (i) it was called somatic using our in-house workflow, (ii) it was called somatic in the matched tumor/metastasis pair and its metastasis and control samples. Positions overlapping with common variants were filtered out. Then, the clustering algorithm from Canopy40 was applied to the BAF values for the positions in tumor/metastasis vs the control using a single pass run, assuming 9 clusters. The clusters that were determined to be tumor-in-normal had to have ≥75% positions above the identity line, the tumor/metastasis mutant allele fraction (MAF) above 1% and the control MAF below 45%. These identified mutations were then reclassified as somatic instead of the original germline annotation.

Mutual exclusivity analysis. Mutual exclusivity analysis was initially performed on all genes that have established roles in colorectal cancer. Gene pairs were deemed to be mutually exclusive if no more than 1 sample harbored somatic SNVs for them. Using cBioPortal, we determined the significance of mutual exclusivity and co-occurrence of recurrently mutated genes in our, the TCGA, Giannakis et al., and Yaeger et al. studies, and the ARHGEF gene family (Supplementary Data 7). We found support to our observation of mutual exclusivity of ARHGEF7-KRAS (TCGA, p-value 0.021, Fisher test) and SOX9-TP53 (Yaeger et al., p-value <0.001), KRAS-KRAS (Yaeger et al., p-value <0.001). We found SOX9 mutations co-occurred with HK3 mutations, and with framshift indels in APC as opposed to typical stop gains, although we did not observe co-occurrence of SOX9 and HK3 in larger cohorts.

Survival analysis. Survival analysis (overall and disease-free) was performed using cBioPortal on the TCGA provisional dataset using ARHGEF7 and all ARHGEF family members combined: ARHGEF1, ARHGEF10, ARHGEF10L, ARHGEF11, ARHGEF12, ARHGEF15, ARHGEF16, ARHGEF17, ARHGEF18, ARHGEF19, ARHGEF2, ARHGEF3, ARHGEF35, ARHGEF36, ARHGEF37, ARHGEF38, ARHGEF4, ARHGEF40, ARHGEF5, ARHGEF6, ARHGEF7 and ARHGEF9 (Supplementary Figure 8).
Copy number aberration calling. Copy number aberrations (CNAs) were called using ACEseqWork44, which is available on github [https://github.com/eliisdls/ACEseqWorkWorkflow]. Briefly, ACEseq (allele-specific copy number estimation from whole-genome sequencing) determines copy number states, tumor cell content, ploidy, and sex in the tumor by using read coverage and the B-allele frequency (BAF) to identify homozygous or heterozygous normal regions (with BAF 0.33–0.67) and BAF segments are fitted to uneven copy number states in both control samples (3% of the control samples); both breakpoints have less than 5% read support at both positions. SOPHIA uses these alignments of split-reads which would be an indication of a possible underlying SV.

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In silico evaluation of miRNA binding to mutated 3′-UTRs. All predictions were made with the RNA22 interactive software [https://cm.jefferson.edu/rnase2/Interactive/] using all known miRNA (miR) sequences from miRBase (Release 21) and the corresponding wild-type or mutated sequences as input. Default settings were used with sensitivity at 65%, specificity at 61%, seed size of 7 with a maximum of one unpaired base. The minimum number of paired-up bases in the hetero-duplex was 12, the maximum folding energy for the heteroduplex (Kcal/mol) was −515 and no limit was given on the number of potential GU wobbles in the seed region. Gain- or loss-of-potential miRNA binding was evaluated by positive results in the presence or absence of a given mutation.

Data availability

The whole-genome sequencing data have been deposited at the European Genome-phenome Archive (EGA). The EGA Study Accession ID is EGAS00001002717. All the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request.

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Author contributions
H.A. conceived the research. H.A., R.B., M.S., R.E., J.E. and N.I. supervised the study. M.L.A., A.M., C.H., M.M., J.R., M.K., J.N. and A.S. acquired the samples and data. N.I., M.A. processed the data. N.I., M.L.A., C.H., N.P., N.P., D.H., J.H.L., G.B., K.K., U.T., B.H., A.M., M.M., M.K., J.R., J.N., Z.G., J.K. and H.A. analyzed, interpreted, and discussed data.
N.I., M.A., N.P., D.H., J.H.L., B.B., H.A. wrote and revised the paper. All authors commented on and critically gave input to the manuscript.

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