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Marcel Smid[1], Saskia M. Wilting[1], Katharina Uhr[1], F. Germán Rodríguez-González[1], Vanja de Weerd[1], Wendy J.C. Prager-Van der Smissen[1], Michelle van der Vlugt-Daane[1], Anne van Galen[1], Serena Nik-Zainal[2,3], Adam Butler[2], Sancha Martin[2], Helen R. Davies[2], Johan Staaf[4], Marc J. van de Vijver[5], Andrea L. Richardson[6,7], Gaëten MacGrogan[8], Roberto Salgado[9,10], Gert G.G.M. van den Eynden[10,11], Colin A. Purdie[12], Alastair M. Thompson[12], Carlos Caldas[13], Paul N. Span[14], Fred C.G.J. Sweep[15], Peter T. Simpson[16], Sunil R. Lakhani[16,17], Steven Van Laere[18], Christine Desmedt[9], Angelo Paradiso[19], Jorunn Eyfjord[20], Annegien Broeks[21], Anne Vincent-Salomon[22], Andrew P. Futreal[23], Stian Knappskog[24,25], Tari King[26], Alain Viari[27,28], Anne-Lise Børresen-Dale[29,30], Hendrik G. Stunnenberg[31], Mike Stratton[2], John A. Foekens[1], Anieta M. Sieuwerts[1] and John W.M. Martens[1].

Affiliations

1 Erasmus MC Cancer Institute and Cancer Genomics Netherlands, University Medical Center Rotterdam, Department of Medical Oncology, Rotterdam, the Netherlands

2 Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, UK

3 East Anglian Medical Genetics Service, Cambridge University Hospitals NHS Foundation Trust, Cambridge CB2 9NB, UK

4 Division of Oncology and Pathology, Department of Clinical Sciences Lund, Lund University, Lund, Sweden

5 Department of Pathology, Academic Medical Center, Amsterdam, the Netherlands

6 Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115, USA

7 Dana-Farber Cancer Institute, Boston, MA 02215, USA

8 Département de Biopathologie, Institut Bergonié, Bordeaux, France

9 Breast Cancer Translational Research Laboratory, Université Libre de Bruxelles, Institut Jules Bordet, Brussels, Belgium

10 Department of Pathology/TCRU GZA Antwerp, Belgium

11 Molecular Immunology Unit, Jules Bordet Institute, Brussels, Belgium

12 Department of Pathology, Ninewells Hospital & Medical School, Dundee DD1 9SY, UK

13 Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Robinson Way, Cambridge CB2 0RE, UK

14 Department of Radiation Oncology, and department of Laboratory Medicine, Radboud University Medical Center, Nijmegen, the Netherlands.

15 Department of Laboratory Medicine, Radboud University Medical Center, Nijmegen, The Netherlands, Radboud University Medical Center, Nijmegen, the Netherlands.

16 Centre for Clinical Research, Faculty of Medicine, The University of Queensland, Brisbane, Australia

17 Pathology Queensland, The Royal Brisbane and Women¹s Hospital, Brisbane, Australia

18 Center for Oncological Research, University of Antwerp, Antwerp, Belgium

19 IRCCS Istituto Tumori "Giovanni Paolo II", Bari, Italy

20 Cancer Research Laboratory, Faculty of Medicine, University of Iceland, 101 Reykjavik, Iceland

21 The Netherlands Cancer Institute, 1066CX Amsterdam, the Netherlands

22 Institut Curie, Department of Pathology and INSERM U934, 75248 Paris Cedex 05, France

23 Department of Genomic Medicine, UT MD Anderson Cancer Center, Houston, TX, USA

24 Department of Clinical Science, University of Bergen, 5020 Bergen, Norway

25 Department of Oncology, Haukeland University Hospital, 5021 Bergen, Norway

26 Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

27 Synergie Lyon Cancer,Centre Léon Bérard, Lyon Cedex 08, France

28 Equipe Erable, INRIA Grenoble-Rhône-Alpes, 655, 38330 Montbonnot-Saint Martin, France

29 Department of Cancer Genetics, Institute for Cancer Research, Oslo University Hospital The Norwegian Radiumhospital, Oslo, Norway

30 K.G. Jebsen Centre for Breast Cancer Research, Institute for Clinical Medicine, University of Oslo, Oslo, Norway

31 Department of Molecular Biology, Faculty of Science, Radboud Institute for Molecular Life Sciences, Radboud University Nijmegen, Nijmegen, the Netherlands

Corresponding author: Marcel Smid

Erasmus MC

P.O 2040, 3000 CA, Rotterdam, the Netherlands

m.smid@erasmusmc.nl

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Abstract

Circular RNAs (circRNAs) are a class of RNA that is under increasing scrutiny, although their functional roles are debated. We analyzed RNA-seq data of 348 primary breast cancers and developed a method to identify circRNAs that does not rely on unmapped reads or known splice-junctions. We identified 95,843 circRNAs, of which 20,441 were found recurrently. Of the circRNAs that match exon-boundaries of the same gene, 668 showed a poor or even negative ($R<0.2$) correlation with the expression level of the linear gene. In silico analysis showed only a minority (8.5%) of circRNAs could be explained by known splicing events. Both these observations suggest that specific regulatory processes for circRNAs exist. We confirmed the presence of circRNAs of *CNOT2, CREBBP* and *RERE* in an independent pool of primary breast cancers. We identified circRNA profiles associated with subgroups of breast cancers and with biological and clinical features such as amount of tumor lymphocytic infiltrate and proliferation index. siRNA-mediated knockdown of *circCNOT2* was shown to significantly reduce viability of breast cancer cell lines MCF-7 and BT-474, further underlining the biological relevance of circRNAs. Furthermore, we found that circular and not linear *CNOT2* levels are predictive for progression-free survival time to aromatase inhibitor (AI) therapy in advanced breast cancer patients and found that *circCNOT2* is detectable in cell-free RNA from plasma. We showed that circRNAs are abundantly present, show characteristics of being specifically regulated, are associated with clinical and biological properties, and thus are relevant in breast cancer.

Introduction

It is a sign of the times that the ubiquitous use of massive parallel sequencing data has delivered a parade of new insights in the cancer field and has enriched our genomic vocabulary with events like chromothripsis, kataegis and mutational and rearrangement signatures (Stephens et al. 2011; Maher and Wilson 2012; Nik-Zainal et al. 2012; Alexandrov et al. 2013; Nik-Zainal et al. 2016). Sequencing RNA has had less of an impact on this vocabulary, with many reports concerning traditional gene expression analysis. However, depending on the methodology of generating the sequencing library, RNA-seq has the potential to study the large variety of RNA species, including non-coding RNAs, fusion-transcripts, known and novel isoforms and, recently gaining attention, circular RNAs (circRNAs). This class of RNA was discovered many decades ago (Hsu and Coca-Prados 1979) and circRNAs were long considered idiosyncrasies of the splicing machinery processing precursor mRNA into mature mRNA. More recent studies showed an unanticipated abundance of circRNAs (Salzman et al. 2012; Memczak et al. 2013) in (normal and malignant) human cells and became particularly interesting for the cancer research field with the description (Hansen et al. 2013; Memczak et al. 2013) of a circRNA that functions as a highly potent miR-7 sponge. MiR-7 has a well-described role in several malignancies, including breast cancer, and functions as a tumor suppressor in most cancers (reviewed by (Zhao et al. 2015) but has also been reported (Foekens et al. 2008) as a potential tumor promoter in breast cancer. Other circRNAs and additional regulatory transcriptional roles have subsequently been described in cancer (Salzman et al. 2013; Guo et al. 2014; Li et al. 2015b; Kristensen et al. 2018). Since circRNAs lack a free 5' or 3' end, such molecules escape exonucleic acid degrading enzymes, making them more stable (Memczak et al. 2013) than their linear counterparts. Therefore, circRNAs represent potentially useful biomarker candidates for diagnosis and therapy-monitoring; indeed cell-free circRNAs are present in exosomes (Li et al. 2015a) and saliva (Bahn et al. 2015). In breast cancer, little has

been described except for one study (Nair et al. 2016) using the The Cancer Genome Atlas (TCGA) data bank. However, this cohort has a huge limitation since the RNA-seq data were prepared using a poly(A) selection step, thereby omitting the majority of circRNAs (as these lack a poly(A) tail).

Here we describe the identification of an extensive catalog of circRNAs in a large cohort of 348 primary breast tumors, using RNA-seq data obtained via random-primed cDNA synthesis (Smid et al. 2016), likely preserving all the circRNAs. We developed a circRNA mapping algorithm that, in contrast to previous identification methods (Salzman et al. 2012; Memczak et al. 2013; Guo et al. 2014; Nair et al. 2016; Szabo and Salzman 2016) does not rely on unmapped reads nor on known splice-junctions and which was applied directly on transcriptome sequence BAM files, thereby allowing the identification of circRNAs in a genome-wide and annotation-independent (Szabo and Salzman 2016) fashion.

Results

Identification of a plethora of circRNAs in primary breast cancer

In total, 95,843 circRNAs were identified (Figure 1), of which 27% (n=25,783) had a start and end position exactly matching to an exon belonging to the same gene (Figure 2A). The vast majority (79%) of all circRNAs were not recurrent (i.e. only found in one sample). The number of circRNAs per sample (Figure 2B) ranged from 37 to 7,105 (median 966). For recurrent circRNAs, found in at least 2 samples, the range per sample was 33 to 5,269 (median 834.5). Figure 2B also shows that the number of (recurrent) circRNAs is significantly higher in estrogen receptor (ER)-negative compared to ER-positive breast cancers (Mann-Whitney *U* test $p<1x10^{-5}$ for both all and recurrent circRNAs). Due to the extraordinary abundance of candidate circRNAs we focused on the – still sizeable – number of recurrently found circRNAs (total number in second bar of Figure 2A, n=20,441). The most frequent recurring region in our cohort was the well-characterized (Hansen et al. 2013; Memczak et al. 2013; Kristensen et al. 2018) circRNA of *CDR1*, which was found in 339 out of 348 cases. Other previously reported and validated (Salzman et al. 2012) circRNAs such as *CAMSAP1, FBXW4, MAN1A2, RNF220, ZBTB44* and *XIST* were also identified in our cohort. A full list of identified circRNAs is provided in Supplemental Table S1.

General characteristics

Recurrent circRNAs were distributed across the genome, with one region on Chromosome 11 showing many closely-spaced circRNAs (Supplemental Fig S1). This region contains *MALAT1*, a highly abundant long non-coding RNA that is also frequently mutated in breast cancer (Nik-Zainal et al. 2016). Next, we evaluated the intron sizes up- and downstream of the circRNAs that match exon boundaries. Confirming previously reported results (Jeck et al. 2013; Zhang et

al. 2014; Ivanov et al. 2015), figure 2C shows that introns next to circRNA regions are significantly larger than introns not adjacent to circRNAs: on average 2.33 and 2.27 times larger, respectively, for introns up- and downstream of the circRNA (Mann-Whitney *U* test both $p<1x10^{-5}$).

Next, we correlated the number of circRNA reads per circRNA with the expression of the respective full, linear gene, since a previous report in a limited cell-line panel reported no genome-wide correlation between the circular and linear counterpart (Salzman et al. 2013). To avoid spurious correlations, only those circRNAs found in at least 50 samples were considered (n=1,625) and data were first normalized using Trimmed Mean of M-values (TMM) (Robinson and Oshlack 2010). Correlations are listed in Supplemental Table S1 and ranged between -0.34 and 0.97, with 210 circRNAs showing a negative correlation to the linear gene in which the circRNA is located. When considering the average and standard deviation of the distribution of all correlation coefficients, 30 circRNAs are at the low end of the distribution ($p<0.05$) showing a correlation below R = -0.182 . Finally, using GENCODE information, the position of annotated start codons was matched to the circRNA positions. CircRNAs recurring in at least 50 samples showed an almost 3-fold higher than expected presence of a start codon compared to circRNAs that were not recurring (Chi-sq $p<0.0001$; 557 circRNAs; expected 207.3 circRNAs).

Are circRNAs Distinct Molecules, Specifically Regulated or Splicing Residues?

Many of the circRNAs positively correlated to the full-length linear transcript are thought to be a residue of splicing. Figure 3A shows an example; the circRNA of exons 3, 4 and 5 of the *WDR1* gene (Chr4:10097711-10103986, with a correlation coefficient of R=0.66 to overall gene expression) exactly matches the difference between the known linear isoforms of this gene (Ensembl transcript ENST00000499869 – includes exons 3, 4 and 5 - and ENST00000502702 – lacks these exons). As current quantification methods do not take circRNAs into account,

reads originating from the circular molecule are erroneously included in the read-count of a linear isoform, resulting in an overestimation of the overall expression level. Another comprehensive example of a circRNA as splicing residue is shown in Figure 3B for *ESR1*. Full length *ESR1* (ENST00000206249) has 8 exons, whereas ENST00000406599 is a splice variant of *ESR1* that skips exons 2-5. CircRNAs are found for several of these exons, indicating that they are likely splicing residues. We speculate that a single splice event from exon 1 to 6 generates an RNA molecule containing exons 2 – 5, from which a multitude of distinct circRNAs can be derived. In total 23 patients show both a *circESR1* exon 2-3 and a *circESR1* of exon 4-5. If these circRNAs are derived from the same RNA molecule, the linear transcript would be ENST00000406599. A sequential model, where first exon 2 and 3 are spliced out, would prohibit the formation of a circRNA molecule of exon 3 to 4. However, we observed *circESR1* exon 2-3 in 110 patients and *circESR1* exon 3-4 in 64 patients, with 29 patients showing both these circRNAs. These must be derived from separate RNA molecules.

To investigate whether or not in general circRNAs should be considered splicing residues, we systematically evaluated how many of the identified circRNAs exactly match those exons that make up the difference between known linear isoforms of a gene. Using the GENCODE annotation for each gene, every possible known combination of spliced exons was matched to our circRNA catalog. Of the 25,783 circRNAs that matched to exons of the same gene, only 2,193 (8.5%) exactly matched exons known to differ between described isoforms of a gene. This was 16.9% for the circRNAs with a correlation coefficient of R>0.5 to linear gene expression. This suggests that the vast majority of circRNAs that matched to exons of the same gene, are generated by yet unknown splicing events of the gene.

Since the majority of circRNAs did not match to known spliced exons, we manually inspected several highly recurrent circRNAs in the UCSC genome browser. For example, 2 isoforms of *CREBBP* are described (ENST00000262367 and ENST00000382070) that differ in the

presence of exon 5 (of note, there are 10 additional known transcripts, but all of these transcripts start downstream of exon 5). However, we observed exon 2 as circRNA (Chr16:3850297-3851009) which was present in 160 patients (Figure 3C), indicating that this circRNA is either specifically generated, or is a splicing residue of a yet undescribed isoform of *CREBBP* that skips this exon. Visual inspection of 10 samples that had high levels of *circCREBBP* exon 2 (at least 30 circular junction reads) showed 2 samples that each had 1 read that crossed the junction from exon 1 to 3, while the other samples showed no evidence of an isoform that skipped exon 2. This favors the notion that the circRNA of exon 2 is not a byproduct of splicing at this location.

Finally, we matched publicly available circRNA lists to gather (indirect) evidence of functional roles for circRNAs. Rybak-Wolf and colleagues reported (Rybak-Wolf et al. 2015) 4,522 circRNAs in the mammalian brain that were evolutionary conserved between human and mouse, which is considered an indication of function (Barbosa-Morais et al. 2012; Merkin et al. 2012). Of these, n=2,259 circRNAs (49.9%) were also present in our catalog. In addition, 3,271 circRNAs were reported in an MCF-7 breast cancer cell line panel (Tarrero et al. 2018). In total, 922 circRNAs showed (increased) expression in estrogen-stimulated MCF-7 cells compared to cells cultured in hormone-deprived medium, of which 733 circRNAs (79.5%) were present in our list. A poor correlation ($R<0.2$) with the linear transcript was observed in our data for 78 of these circRNAs, suggesting independent regulation from their linear gene instead of ER-induced overall higher expression of all transcripts from that gene.

Validation of circRNAs

Beside the fact that we detected several already published circRNAs, thereby in part validating our method, we performed RT-PCR on a previously established independent cDNA pool of 100 primary breast tumors to confirm expression of 3 circRNAs, namely *RERE* (circRNA Chr1:8541214-8614686), *CNOT2* (circRNA Chr12:70278132-70311017) and *CREBBP*

(circRNA Chr16:3850297-3851009), all of which were poorly correlated with their linear counterpart. Figure 4 shows the PCR fragment sizes; expected and observed sizes were 89 bp and 155 bp for the small and bigger *CNOT2* fragment, 100 bp for *RERE* and 91 bp for *CREBBP*. The primer pair for *CNOT2* was able to amplify the circRNA of exon 2 to 3 of *CNOT2* but also the circRNA of exon 2, 3 and 4 of this gene (a circRNA that was also identified in the RNA-seq cohort).

A different primer pair to PCR *circCNOT2* showed besides the expected fragments of 140 and 206 bp, also additional fragments (Supplemental Fig S2A). Sanger sequence analysis confirmed the circular junction sequence from exon 3 to 2 of *CNOT2* (Supplemental Fig S2B) and from exon 4 to exon 2 (Supplemental Fig S2C). After using BLAST to identify the sequence, the largest excised PCR fragment was found to contain an additional exon of *CNOT2* (Chr12:70294237-70294293) located between exon 2 and 3 that is not present in most isoforms of *CNOT2* (Supplemental Fig S2D). The sequence showed exon 3, across the circular junction to exon 2, but reading through the location where the reverse primer was located, continuing the whole of exon 2, the additional exon and ending in exon 3 again (Supplemental Fig S2d). A likely explanation for this observation is that during cDNA generation the RT polymerase generated a linear cDNA molecule containing multiple copies of the circular transcript (Supplemental Fig S2E). In summary, the investigated circRNAs were all confirmed to be truly present in primary breast cancer.

Functional relevance of circRNAs in breast cancer cells.

The potential functional relevance of the validated *circCNOT2* and *circCREBBP* transcripts, which were both poorly correlated with their corresponding linear transcript, was evaluated in breast cancer cell lines. First, expression levels of *circCNOT2* and *circCREBBP* were established in a panel of 55 cell lines, showing variable levels (Supplemental Fig S3). Next, an siRNA was designed to specifically target the circular junction of *circCNOT2* in both MCF-7

(moderate expression level) and BT-474 (high expression level). This siRNA reduced expression of *circCNOT2* by 76% in MCF-7 and 71% in BT-474 breast cancer cells, relative to cells transfected with a non-targeting control (NTC), which resulted in significantly reduced viability of both MCF-7 and BT-474 cells (Figure 5, Student's *t*-test $p<1x10^{-5}$ and $p=4.94x10^{-4}$, respectively).

circRNAs in driver genes

We matched our previously reported breast cancer driver gene list (Nik-Zainal et al. 2016) to our circRNA list. In total, 235 recurrent circRNAs were identified in 54 breast cancer driver genes. To integrate the data and obtain sufficient observations for analysis, we selected samples for which we had both RNA and genomic DNA sequencing results available and selected the genes with somatically acquired genetic events (mutations, copy number variants and rearrangements) in at least 10 patients, yielding a list of 10 genes; *TP53, PIK3CA, PTEN, MAP3K1, CDH1, RB1, MAP2K4, ARID1B, ARID1A* and *MLLT4*. For genes with multiple circRNAs, the circular region with the highest recurrence was chosen for analysis, with the exception of *TP53* for which we only found 2 circRNAs in just 1 sample each (see Table 1). Only for *MAP2K4* mutual exclusivity was observed between the presence of a somatic mutation or a circRNA in this cohort, where 20 samples had a somatic mutation, 77 samples had a circRNA (Chr17:12054889-12113360) and only 2 samples had both a mutation and a circRNA (p=0.025, CoMEt exact test (Leiserson et al. 2015)). For *PIK3CA*, 25 patients showed a DNA event and a circRNA; 3 patients with a copy number aberration (amplification) and 22 patients with a base substitution in *PIK3CA*. These substitutions were located in 4 hotspots, p.H1047 (13 cases), p.E545 (n=4), p.E542 (n=3) and p.E726 (n=2). None of these hotspots was located in the circRNA region that was found in these samples.

Breast cancer relevance

To investigate common biology in the samples, we used Multiple Correspondence Analysis (MCA) to find naturally occurring subgroups. MCA is a generalized principle component analysis, suitable for categorical data. We used recurrent circRNAs (at least 50 cases) with the junction annotated to exons of the same gene (n=1,625) and labeled these per sample as circular or not-circular, based on the presence or absence of junction reads in a sample. The main patient-groups were, not unexpectedly, divided by ER-status (Figure 6A, left panel), while within circRNAs the main division was whether or not the gene had a circRNA (Figure 6A, right panel). Additional variation within the circRNAs was explained by the level of recurrence of the circRNAs (see Supplemental Fig S4). Next, the presence/absence of circRNAs in a sample was used to cluster all samples into groups with distinct circRNA profiles. We used the gap statistic (Tibshirani et al. 2001) to determine the optimal number of sample-groups, yielding 6 clusters (Figure 6B and Supplemental Fig S5). We evaluated these 6 sample groups on ER and tumor infiltrating lymphocyte (TIL) status (Figure 6C and D), number of circRNAs (Figure 6E) and outcome for the patients in the clusters (Figure 6F). Samples in cluster 1 and 3 were predominantly ER-negative, while ER-positivity was predominantly present in group 2, 4, 5 and 6. TIL status was established using a previously reported gene-expression signature (Massink et al. 2015; Smid et al. 2016), labeling samples as high-TIL if the average expression of the TIL-signature genes fell into the top quartile (n=87, 45 ER-negative and 42 ER-positive, respectively labeled red and orange in Figure 6D). High-TIL cases were significantly (Chi-sq $p<1x10^{-5}$) more often present in cluster 1 (71% of cases) and 3 (45% of cases). Furthermore, the number of circRNAs per sample clearly distinguished the 6 clusters (Figure 6E), showing a decreasing number of circRNAs from cluster 1 to 6. Finally, for a subset of 186 patients, relapse-free survival data was available; a survival plot for the 6 clusters (Figure 6F) showed that the major difference was between cluster 1 and 3, that are both predominantly ER-negative. Though the number of events was low, direct comparison of cluster 1 with cluster 3 showed a significant difference in survival curves (log rank p=0.04).

Differentially expressed circRNAs

We investigated if circRNA expression levels were associated with clinically relevant features of primary breast cancer, such as presence of TILs, the tumor's stroma content, proliferation and hypoxia status. These features were inferred from generated (stroma, see methods) or reported (Winter et al. 2007; Massink et al. 2015; Smid et al. 2016) gene expression signatures. Using these, we grouped our samples in a similar manner as explained earlier for the TIL-status (Figure 6D), labeling samples as high if the average expression of the signature genes fell into the top quartile. To identify significantly differentially expressed circRNAs we compared the top-quartile of samples to the remaining samples separately for the ER-positive and ER-negative cases. CircRNAs with FDR corrected p-values<0.05 and a fold-change>2 were selected. Of these, the circRNAs that had a negative correlation with the linear gene expression were considered of particular interest and are listed in Table 2. Several of these circRNAs may thus potentially play a role in, or are at least connected to, the tumors that show hypoxic characteristics (e.g. *circKMT2C*) or accumulate in highly proliferative cells (e.g. *circRERE*, *circATXN2*), while e.g. *circASH1L* and *circPCH3* may be generated by surrounding stromal cells or infiltrating cells.

Clinical relevance

One of the reasons *CNOT2* was selected for validation was because of the poor correlation with expression of the linear gene (R = -0.09, p=0.34). This was more prominent in ER-positive (R = -0.14) compared to ER-negative cases (R = 0.097). We validated this finding by making use of in-house array data (Smid et al. 2008) for linear *CNOT2* expression and a quantitative RT-PCR assay to measure *circCNOT2* (exon 2-3 Chr12:70278132-70311017). The Spearman correlation in ER-positive cases of *circCNOT2* with linear *CNOT2* was 0.079 (n=187, p=0.28)

and in ER-negative cases Rs=0.42 (n=111, $p=2.9x10^{-6}$), again showing absence of correlation in ER-positive cases. Thus, the role of *circCNOT2* apparently differs between ER-positive and -negative cases, and in the ER-negative cases the significance of *circCNOT2* cannot be easily segregated from the linear counterpart. Therefore, we evaluated two different ER-positive breast cancer cohorts (Supplemental Table S3 shows clinical characteristics) for the potential clinical value of *circCNOT2*. We studied progression-free survival of aromatase inhibitors (AI) therapy in a multicenter cohort of 84 ER-positive patients that received this treatment for advanced disease. RT-qPCR levels of *circCNOT2* showed a significant Hazard Ratio (HR) of 1.75 (95% confidence interval 1.32-2.33, $p=1.06x10^{-4}$), whereas RT-qPCR levels of linear *CNOT2* were not significant: HR 1.28, p=0.187. Figure 7A shows a survival curve after grouping patients' *circCNOT2*-levels into 3 equally sized groups. A similar analysis in another cohort (Sieuwerts et al. 2005), that included patients receiving first line Tamoxifen treatment (n=295 patients) did not show a significant HR (0.97, p=0.57) for *circCNOT2* nor for linear *CNOT2* (HR 1.16, p=0.21).

As circular molecules are expected to be more stable than their linear counterparts, we explored whether *circCNOT2* is a potential candidate as minimally invasive biomarkers. To this end, we used cell-free RNA from plasma samples of four breast cancer patients and amplified *circCNOT2* by RT-qPCR. All samples showed detectable and variable levels of *circCNOT2* (Figure 7B), indicating that detection of circRNAs in plasma seems attainable in this exploratory setting.

Discussion

To our knowledge, we are the first to analyze RNA sequencing data using random-primed cDNA libraries from a large primary breast cancer cohort for the presence of circRNAs, using a method that does not rely on unmapped reads. Previously, Nair and colleagues (Nair et al. 2016) analyzed TCGA RNA-seq data that were obtained using a poly(A)-based method. Where

we identified 25,783 circRNAs that matched with an exon-boundary of the same gene, Nair *et al.* reported 2,146 circRNAs when we applied the same selection criteria as for our dataset and, after transferring the hg37 coordinates (Nair *et al.*) to hg38 (our dataset), only 45 circRNAs were found that had the exact same start and end coordinates in both datasets. Thus, a random-primed method identifies many more circRNAs than when using poly(A) selected material. On the other hand, there seem to be many uniquely identified in these datasets. This could stem from differences in the methodology to detect or report the circRNAs, but also could reflect the fact that many circRNAs are non-recurrent.

We showed that circRNAs are found throughout the genome and have significantly larger sized introns located directly adjacent to the region on the genome that borders the circular RNA, as also reported previously (Jeck et al. 2013; Zhang et al. 2014; Ivanov et al. 2015). Based on the presence/absence of circRNAs, 6 groups of samples were observed that differed in their ER-status, TIL content, number of circRNAs and prognosis. This indicates that there appears to be a functional biology associated with the biogenesis of circular RNA molecules, or at least a biology that cancerous cells can use to their advantage. Whether or not the circRNAs themselves serve that function or the process that generates differences in circRNA levels is the cause for the results presented here, remains unknown at this time. The fact that several circRNAs were found differentially expressed in breast cancer subgroups, while these circRNAs are negatively correlated with the expression of the linear gene from which the circRNA is derived, corroborates the notion of functional circRNAs. Further experimentation is required to investigate the functional relationship of the differentially expressed circRNAs in the hypoxia, proliferation, stroma and TIL phenotypes.

Although a synthetic circRNA construct including an IRES (internal ribosome entry site) can be translated (Wang and Wang 2015), current literature (Jeck et al. 2013; Guo et al. 2014; Szabo and Salzman 2016; Liang et al. 2017; Liu et al. 2018; Wang et al. 2018; Zeng et al. 2018) describes mostly non-coding functions for circRNAs, for example as miRNA sponge *circCDR1*

(Hansen et al. 2013; Memczak et al. 2013; Zhao et al. 2015; Kristensen et al. 2018) and *circZNF91* (Guo et al. 2014), while associative evidence was reported (Rybak-Wolf et al. 2015) of evolutionary conserved circRNAs between human and mouse. Further support that circRNAs may be specifically generated and regulated was derived from a study using (estrogen-stimulated) MCF-7 cells (Tarrero et al. 2018), showing higher levels of H3K36me3 (post-transcriptional histone modification) and a higher number of Ago binding sites in circularizing exons.

Here, we contribute to the search for relevant circRNAs in three ways; first, expression levels of circRNAs that are not, or even negatively correlated with the linear transcript of the gene may point to an intentional process. Although differences in degradation rates between the circular and linear isoforms may influence the correlation, we did not find systemic evidence for this (Supplemental Fig S6). Furthermore, for genes that show several distinct circRNAs, correlations can vary, indicating that just degradation of the linear transcript cannot be the explanation of the observed correlations. Second, circRNAs that include the start-codon could potentially influence the expression of the linear gene, because the linear transcript from which the circRNA was spliced, is now forced to use another start codon for its translation; if none is available the transcript may be degraded. For example, *circCNOT2* (exon 2-3 Chr12:70278132-70311017) contains the start-codon of the consensus (Pruitt et al. 2009) transcript (CCDS31857.1). Annotation shows that exon 1 is untranslated and both exon 2 and exon 4 start with the methionine codon (ATG). Thus, the linear transcript wherein exon 1 is ligated to exon 4 lacks the start-codon from exon 2 and may use the ATG in exon 4 for its translation. Third, we observed that circRNAs matching exon boundaries of the same gene rarely (8.5%) overlap with known spliced exons. It could be that our analysis overlooked possible splice variants from the GENCODE annotation (both HAVANA and ENSEMBL exon annotations were included), but if the concordance is indeed this low, two scenarios may be applicable: either circRNAs are still mostly a remnant of splicing, implying that many more transcript isoforms of genes exist, or

otherwise circRNAs are specifically generated, implying that they do have a biological role. The observations of variable expression levels of *circCNOT2* and *circCREBBP* in cell lines and especially the effect of *circCNOT2* knock-down on cell viability corroborate a biological role for circRNAs. Future studies are needed to systematically evaluate if the correlation between a circRNA and its linear transcript, the presence of a start-codon and/or known splice junctions are reliable criteria to prioritize circRNAs of interest.

Regardless, we were able to show clinical potential for *circCNOT2* by showing its association with the response to AI therapy. Knowing that circular molecules are not targeted by exonucleases, these molecules may be suitable candidates to be detected in cell-free environments (Li et al. 2015a), and in a pilot experiment we showed that *circCNOT2* can indeed be detected in cfRNA from plasma samples of breast cancer patients. As such, *circCNOT2* could prove to be a useful biomarker to choose the right type of therapy or to monitor disease in a minimally invasive manner. Furthermore, we observed that very likely due to the strand displacement activity of the reverse transcriptase during cDNA generation, multiple concatemeric copies of a single circular molecule are made, contributing to the sensitive detection of circRNAs. In conclusion, we have demonstrated the abundance and potential roles of circRNAs in primary breast cancer. The methodology and selection-criteria we employed may help in making more sense of the seeming chaos and disorder existing in the flow from DNA to RNA to protein. CircRNAs show the potential to function as relevant actors in the transciptional regulation of RNA, in addition to their promise as stable biomarkers that can be used for disease progression.

Methods

Sequencing

Internal Review Boards of each participating institution approved collection and use of samples of all patients in this study. RNA-seq data were generated by our consortium (Nik-Zainal et al. 2016; Smid et al. 2016) for 348 primary breast cancer tumors which are available through the European Genome-phenome Archive under accession number EGAS00001001178. Sequence protocols of the samples were previously described in detail (Nik-Zainal et al. 2016); in short, total RNA after gDNA removal, clean-up and depletion of ribosomal RNA using DSN (Duplex Specific Nuclease) treatment, was used as input for random-primed cDNA synthesis. Paired-end (75 bases) sequencing was performed on an Illumina HiSeq 2000. The resulting FASTQ files were mapped to GRCh38 using STAR (Dobin et al. 2013) (version 2.4.2a) and the resulting BAM files were sorted and indexed using Sambamba (Tarasov et al. 2015) (version 0.6.6, https://github.com/lomereiter/sambamba/). Gene annotation was derived from GENCODE Release 23 (https://www.gencodegenes.org/).

Identification of circular RNAs

A detailed explanation of the methodology to identify circRNA reads, including the Perl script, is stated in the Supplemental Methods. The script is also available at https://bitbucket.org/snippets/MSmid/Le949d/identify-circularrna-reads. In short, the method developed here uses sequence reads that have a “Secondary Alignment” tag (SA). When using paired-end sequence data, and assuming a circular RNA molecule is present (top part Figure 1) the sequence read that aligns over the crossing of the junction (green arrows) would ‘point toward’ its read-mate (orange arrow) somewhere in the circle. Aligning these reads to the linear reference (middle part Figure 1), the junction read will get an SA tag and will be assigned to two locations if and only if this is the one and unique alignment configuration the STAR software can find. The read-mate aligns somewhere in between these two locations. Finding additional read-pairs showing this configuration, with a breakpoint at the exact same location, strengthens the

evidence for circular transcripts. Only regions with at least 5 reads crossing the circular junction were included. After filtering (see Supplemental Methods for details), GENCODE annotation was used to obtain the exon locations of genes that exactly matched to the circular region. For each sample, STAR also gives the raw read counts for all genes. These were normalized (Trimmed Mean of M-values implemented in edgeR (Robinson and Oshlack 2010)) and used to correlate with the number of junction reads of the circular transcripts.

Multiple Correspondence Analysis

Since many genes only show a linear transcript in many samples, standard cluster analysis to identify groups of samples with similar circRNA-related biology is problematic due to the many missing values. Thus, the circRNA data were considered categorical using “circular” or “not-circular” if a circRNA was present or absent in a sample. These categorical data are suitable for a Multiple Correspondence Analysis (MCA), a generalization of principle component analysis. The MCA generates a combined plot that shows both patients and circRNAs such that patients/circRNAs that have similar patterns are closer together. R-packages ‘ade4’, ‘canceracm’ and ‘cluster’ were used to perform the MCA and determine the optimum number of clusters. The latter was determined using the clusGap option (k-means to partition the samples) in the cluster package. R version 3.4.1 was used (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://cran.r-project.org/).

Reverse transcription, PCR and Sanger sequencing

Candidate circular RNAs were selected and primers were designed such that a PCR would only yield a product when the RNA was circular, whereas in the linear situation, the primers would be divergent. Primer sequences are listed in Supplemental Table S2.

First, total RNA, isolated with RNA-Bee according to the manufacturer's instructions (CS105B, TEL TEST) was reverse transcribed into cDNA with the H-minus RevertAid First Strand cDNA Synthesis Kit (K1632, Thermo Fisher Scientific), followed by a RNase H step (AM2293; Ambion). Next, circRNAs were real-time PCR amplified at 10 ng input in a final volume of 25 µL using 40 PCR cycles and an annealing temperature of 67°C with 330 nM of each primer and SensiFast SYBR Lo-Rox mastermix (BIO-94050, Bioline), followed by a final 5 minute extension at 72°C, in a MX3000P (Agilent Technologies, Santa Clara, CA, USA). PCR products were visualized using a MultiNA Microchip Electrophoresis system (Shimadzu, Kyoto, Japan).

For sequencing, PCR fragments were separated on a standard agarose gel and were excised from gel using the QIAquick Gel Extraction Kit from Qiagen (Hilden, Germany) according to manufacturer's protocol. The sequencing reaction contained 2 µL of gel-extracted PCR product, 1 µL BigDye Terminator v3.1 reaction mix (Thermo Fisher Scientific, Waltham, MA, USA), 1x BigDye Terminator sequencing buffer (Thermo Fisher Scientific) and 0.16 µM of sequencing primer in a final volume of 10 µL and was carried out using an ABI2720 thermal cycler according to the following protocol: 1 step of 96 °C for 2 minutes and 25 cycles of 96 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 2 minutes. Subsequently, the sequencing product was precipitated with absolute ethanol and 3 M of NaAc, resuspended in 20 µL of Hi-Di formamide (Thermo Fisher Scientific), and ran on an ABI3130XL Genetic Analyzer (Thermo Fisher Scientific).

Quantitative reverse transcriptase PCR (RT-qPCR)

After RNA isolation and cDNA synthesis performed as described above, *circCNOT2* (Chr12:70278132-70311018) and *circCREBBP* (Chr16:3850297-3851009) transcripts were real-time PCR amplified at 10 ng input in a final volume of 25 µL in 40 PCR cycles and an annealing temperature of 60°C with 200 nM of each primer and 100 nM Fam-labeled TaqMan MGB probe that covers the circular junction (Thermo Fisher Scientific; Supplemental Table S2) in SensiFast

Probe Lo-Rox mastermix (BIO-84020, Bioline) using a MX3000P (Agilent Technologies, Santa Clara, CA, USA). Levels were quantified relative to the average expression of 3 reference genes (*HPRT1*, *HMBS* and *TBP*; Supplemental Table S2) using the delta Cq method (dCq = 2^(average Cq reference genes - Cq target gene)) (Schmittgen and Livak 2008). A serially diluted cDNA pool (Sieuwerts et al. 2014) of 100 independent breast tumor samples (containing both ER-positive/negative and *ERBB2* positive cases) was included in each experiment to evaluate the linear amplification and efficiencies for all genes and absence of amplification in the absence of reverse transcriptase. Samples in the cDNA pool were independent from the cases that were used for the RNAseq cohort.

Detection of circRNA in plasma

Cell-free RNA (cfRNA) was isolated with the Maxwell® RSC miRNA Tissue Kit (Promega, Madison, WI, USA) adapted for plasma according the manufacturer's instructions. One mL of EDTA plasma of different metastatic breast cancer patients were used. These patients provided written informed consent. Six µL of the resulting 50 µL cfRNA (3.8-7 ng RNA/µL) was used to generate 20 µL cDNA with the SuperScript IV VILO cDNA synthesis kit (Thermo Fisher Scientific). Next, 2 µL of the cDNA was pre-amplified in the presence of 0.50 nM of the reverse primers of the hydrolysis probe assays for *circCNOT2* and *GUSB* as a reference marker during 15 cycles with TaqMan pre-amp mastermix (Thermo Fisher Scientific). Finally, 0.5 µL of the pre-amplified product was measured real-time with the hydrolysis probe assays (200 nM forward primer, 200 nM reverse primer and 100 nM FAM labeled hydrolysis MGB probe) during 40 cycles with SensiFAST™ Probe Lo-ROX mastermix (BioLine, Toronto, Canada) in a final qPCR volume of 25 µL in a MX3000P qPCR machine (Agilent Technologies).

siRNA-mediated knock down of circRNAs and cell viability assay

All cell lines in this study were established to be genetically unique, monoclonal and of correct identity by performing STR profiling using the PowerPlex® 16 system (Promega, Madison, WI, USA). MCF-7 and BT-474 were plated at 60-70% confluency in 6 well plates and transfected with 50 nM ON-TARGETplus siRNA targeting *circCNOT2* (Horizon Discovery LTD, Cambridge UK) using 4 µl (MCF-7) or 8 µl (BT-474) Dharmafect 1 (Horizon Discovery LTD) following the manufacturer's instructions. Used sequences (5'-3') were: Sense AAAGAUAGGGAGACGUGGUUU and Antisense 5'-PACCACGUCUCCCUAUCUUUUU. The ON-TARGETplus Non-targeting pool and On-TARGETplus Human UBB Smart pool were included in each experiment as negative and positive control, respectively (Horizon Discovery LTD). After 24 hours of transfection, cells were trypsinized, counted and seeded in quintuplicate at 20,000 cells per well in 96 well plates. Cell viability was determined using the CellTiter-Blue Cell Viability Assay (Promega Corporation, Madison, WI, USA) at day 0 and day 3. Viability measurements at day 3 were corrected for baseline viability values by subtracting the average measurement of day 0.

Gene expression signatures

We used several signatures; a TIL and proliferation signature (Smid et al. 2016), a hypoxia signature (Winter et al. 2007), and a stroma-specific signature using public data GSE5847 (Boersma et al. 2008) (Gene Expression Omnibus). We performed a paired *t*-test to obtain genes significantly higher expressed in microdissected stroma (FDR<0.05 and fold-change > 1.7). For all signatures, genes that were upregulated in the category of interest were matched to our dataset and the average expression of the signature genes was calculated per sample. Samples were labeled as high-TIL (or stroma, proliferation, hypoxia) if the average expression of the signature genes fell into the top quartile. To identify significantly differentially expressed circRNAs we compared the top-quartile of samples vs the rest, per ER-group. circRNAs were only included when detected in >50% of the samples and matched known exon locations of the

same gene. Analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team. circRNAs were considered significant when the FDR corrected p-value was below 0.05 and the fold-change > 2.

Breast cancer cohort treated with endocrine therapy

RT-qPCR was performed on a linear and circular isoform of *CNOT2* (Chr12:70278132-70311017) in a first-line TAM (Sieuwerts et al. 2005) and a first-line AI cohort to study the predictive value of *circCNOT2* on therapy response. The AI cohort was a multicenter cohort consisting of 30 patients from Erasmus MC, Rotterdam, 35 patients from The Netherlands Cancer Institute, Amsterdam and 19 patients from the Translational Cancer Research Unit, Antwerp (Belgium). All 295 patients in the TAM cohort are patients of the Erasmus MC, Rotterdam. Patient characteristics are listed in Supplemental Table S3.

Statistical analyses

STATA v14 was used to perform the statistical tests that are indicated in the text. P-values are two-sided, corrected for multiple testing where necessary and were considered significant below 0.05.

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Authors' contributions

MSmid, SMW and JWMM wrote the main paper. MStratton, SM, SN-Z, HGS, JAF, JWMM were involved in the strategy and supervision of the project. Experiments were performed by SMW, FGR-G, VdW, AMS, WJCP-vdS, MvdV-D, AvG, JS. MSmid, AMS, KU, SMW, SN-Z, JS, MJvdV,

ALR, AB, HDD, FCGJS, AV, AB-D and JWMM analyzed data. Samples and/or clinical data were contributed by JAF, JWMM, ALR, CAP, AMT, CC, PNS, FCGJS, PTS, SRL, SvL, CD, AP, JE, AB, AV-S, APF, SK, TK, GT, AV, GM, AB-D, GM, RS, GGGMvdE.

Disclosure declaration

The authors declare that they have no competing interests.

Legends Figures

Figure 1: Schematic overview of identifying circRNA regions.

Assuming a circular RNA molecule is present, a sequence read crossing the junction (green arrow) and its read-mate (gold arrow) would map to a linear reference in the manner depicted. The junction read would get multiple alignments and the read-mate would be located in between the position of the junction-read. Multiple read pairs at the same junction strengthen the support for the circular RNA. Subsequent additional filtering (details are in the Methods section) and annotation produced the list of circRNA regions.

Figure 2: General characteristics of circRNAs in primary breast cancer.

A Number of unique and recurrent circRNAs. Purple and gold indicate the number of circRNAs that respectively did or did not have a start and end position of a circRNA region exactly matching the start and end position of an exon of the same gene. **B** The number of circRNAs per sample, grouped by ER-status. In black, the total number of circRNAs, in peach color the number of recurrent (identified in at least 2 samples) circRNAs. **C** Violin plots of the intron size in (log bp) located directly up- or downstream of a circRNA region.

Figure 3:

A Sashimi plot of the number of reads that are aligned to *WDR1*, showing only the reads that span exons. In red are the normal exon-exon reads, in purple the reads that span the circular junction. The line and boxes indicate the exons of the gene (not the whole gene is shown). **B** Isoform of *ESR1*. The arcs indicate the number of samples that have a particular circRNA. **C** Two isoforms of *CREBBP* that are known in the first 5 exons (other isoforms are described, but these start downstream of exon 5). Exon 2 (purple box) is an identified circRNA that is not a remainder of a splicing event.

Figure 4: PCR products of circRNAs.

PCR product sizes of circRNAs visualized using the MultiNA. M indicates DNA size marker (25 bp fragment ladder), - the negative control (genomic DNA).

Figure 5: siRNA-mediated knock down of *circCNOT2* affects viability in breast cancer cells.

The effect of reduced *circCNOT2* expression on viability is shown in MCF-7 and BT-474 cells. Both cell lines show a significant decrease in viability ($p<0.01$) following *circCNOT2* knock down relative to cells transfected with NTC (non-targeting control). Error bars indicate standard deviation of five wells.

Figure 6: Analysis of sample-groups according to circRNA presence.

Multiple Correspondence Analysis (MCA) was used to find naturally occurring groups in the circRNA data. In an MCA-plot, samples and circRNAs are projected onto the same plane, where the relative distance to either the samples or the circRNAs is meaningful. The 0,0 point corresponds to a sample or circRNA with an average profile. **A** Left panel, samples are colored according to ER-status: red, ER-positive; black, ER-negative. Right panel, purple and green indicate genes with or without circRNA expression, respectively. **B** Clustering identified samples with similar circRNA profiles; samples in the MCA-plot are colored according to the cluster the

sample belongs to. Colored rounds are ER-negative, triangles ER-positive. **C** Top panel: ER-status (purple ER-positive, peach ER-negative) and TIL status of the 6 sample groups: red and orange are high-TIL cases, blue and green are low-TIL cases, for ER-negative and ER-positive, respectively. Bottom left: number of circRNAs per sample-group. Bottom right: relapse-free survival plot by sample-group. N is number of patients, F number of patients who relapse. X-axis in months, Y-axis the cumulative probability of relapse-free survival.

Figure 7: clinical evaluation and presence in plasma samples

A: Kaplan-Meier survival curve of PFS for AI therapy where patients were grouped in 3 equally sized groups based on their circCNOT2 expression: red, blue and green indicate the samples with high, intermediate and low expression. X-axis in months, Y-axis depicts the cumulative probability of progression-free survival on AI therapy. The p-value is the logrank test for trend.

B: Expression levels of *circCNOT2* in plasma samples. Four metastatic breast cancer patients were evaluated. Y-axis depicts delta-Ct values of *circCNOT2* relative to GUSB. Error bars indicate standard deviation of two measurements.

Table 1: Number of samples with a DNA event and/or circRNA.

	Gene	nr of samples		
CircRNA region	Symbol	DNA events	circRNA	both
Chr17:7673535-7674290	*TP53*	105	1	0
Chr17:7674859-7676622	*TP53*	105	1	1
Chr3:179203544-179204588	*PIK3CA*	84	77	25
Chr10:87925513-87952259	*PTEN*	47	13	4
Chr5:56864734-56865977	*MAP3K1*	30	210	22
Chr16:68801670-68815759	*CDH1*	22	7	0
Chr13:48342599-48349023	*RB1*	22	4	0
Chr17:12054889-12113360	*MAP2K4*	20	77	2
Chr6:156829227-156935576	*ARID1B*	14	28	1
Chr1:26729651-26732792	*ARID1A*	12	213	11
Chr6:167870383-167889326	*MLLT4*	10	28	0

Table 2: Fold-change of circRNAs in top quartile vs rest in ER-subgroups of tumors

Regions	Gene Symbol	Hypoxia ER-neg	Hypoxia ER-pos	Proliferation ER-neg	Proliferation ER-pos	Stroma ER-neg	Stroma ER-pos	TIL ER-neg
Chr1:155438327-155459898	*ASH1L*		2.0			2.1		2.0
Chr1:8541214-8557523	*RERE*	2.4		2.1				
Chr1:8655973-8656441	*RERE*				2.0			
Chr2:112399632-112400194	*RGPD8*	2.5	2.3		2.6			
Chr3:170136419-170149244	*PHC3*	3.4	2.1	2.6		2.8		2.5
Chr5:140440119-140449305	*ANKHD1*	2.2		2.2				
Chr7:152309966-152315338	*KMT2C*	2.1						
Chr7:155672867-155680908	*RBM33*	2.4	2.4	2.6	2.5		2.3	
Chr7:17868407-17875790	*SNX13*	2.4		2.3				
Chr10:32543300-32584304	*CCDC7*		2.2		2.4			
Chr12:111554158-111555919	*ATXN2*				2			
Chr13:75560753-75569507	*UCHL3*							2.0
Chr15:25405461-25411971	*UBE3A*	2.7	2.2	2.4	2.1			
Chr15:92996957-92998621	*CHD2*		2.4		2.3			
Chr18:76849526-76851939	*ZNF236*		2.1		2.0			
Chr20:35716740-35725155	*RBM39*	2.5		2.2				

No significant circRNAs were identified in the TIL ER-pos group

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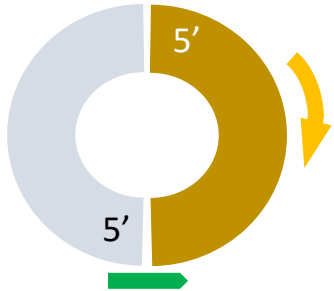
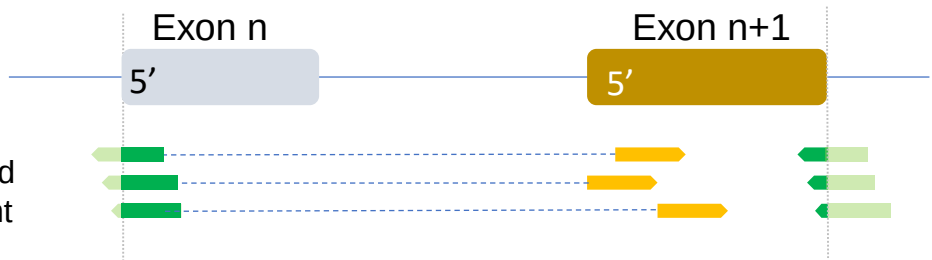




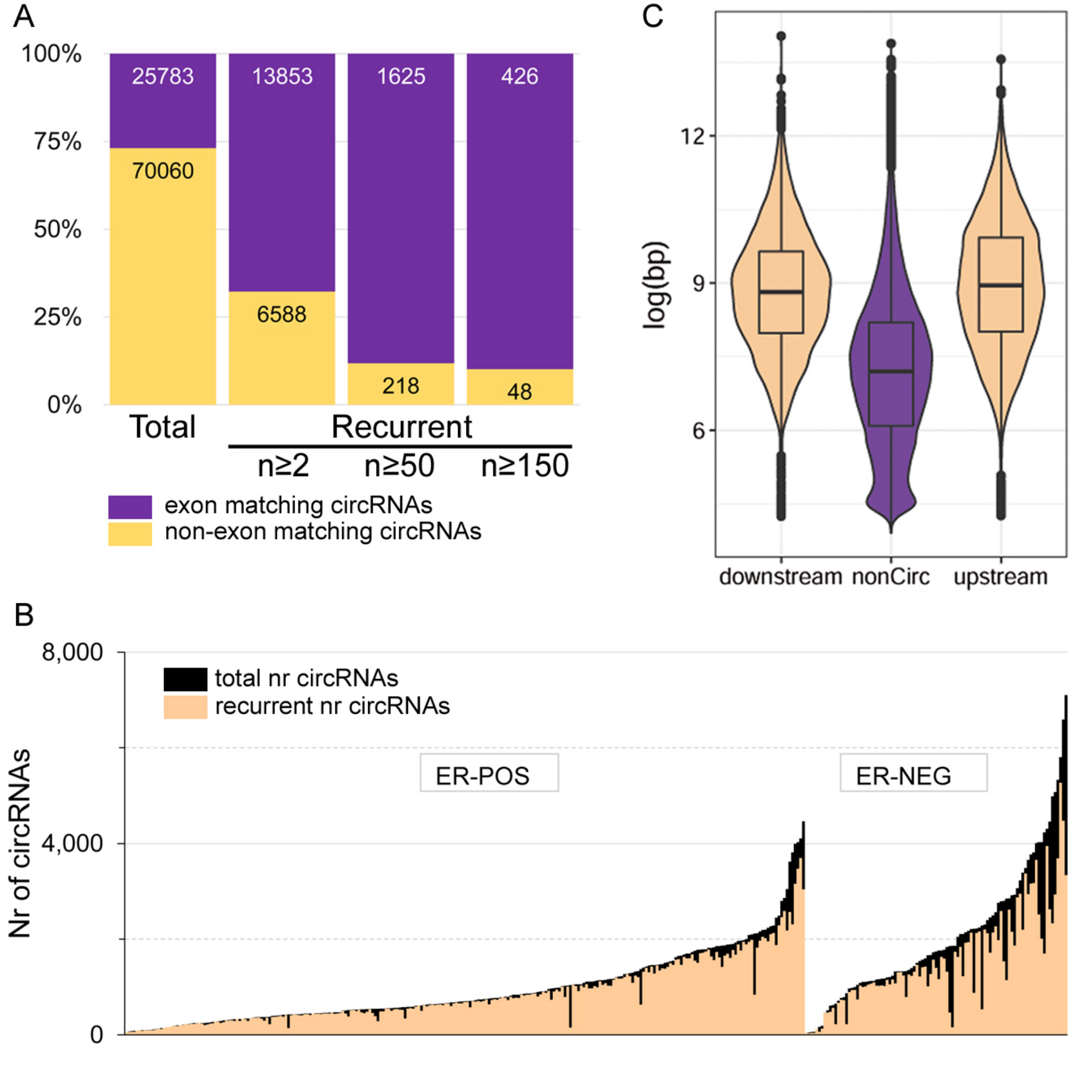
reads aligned on linear reference

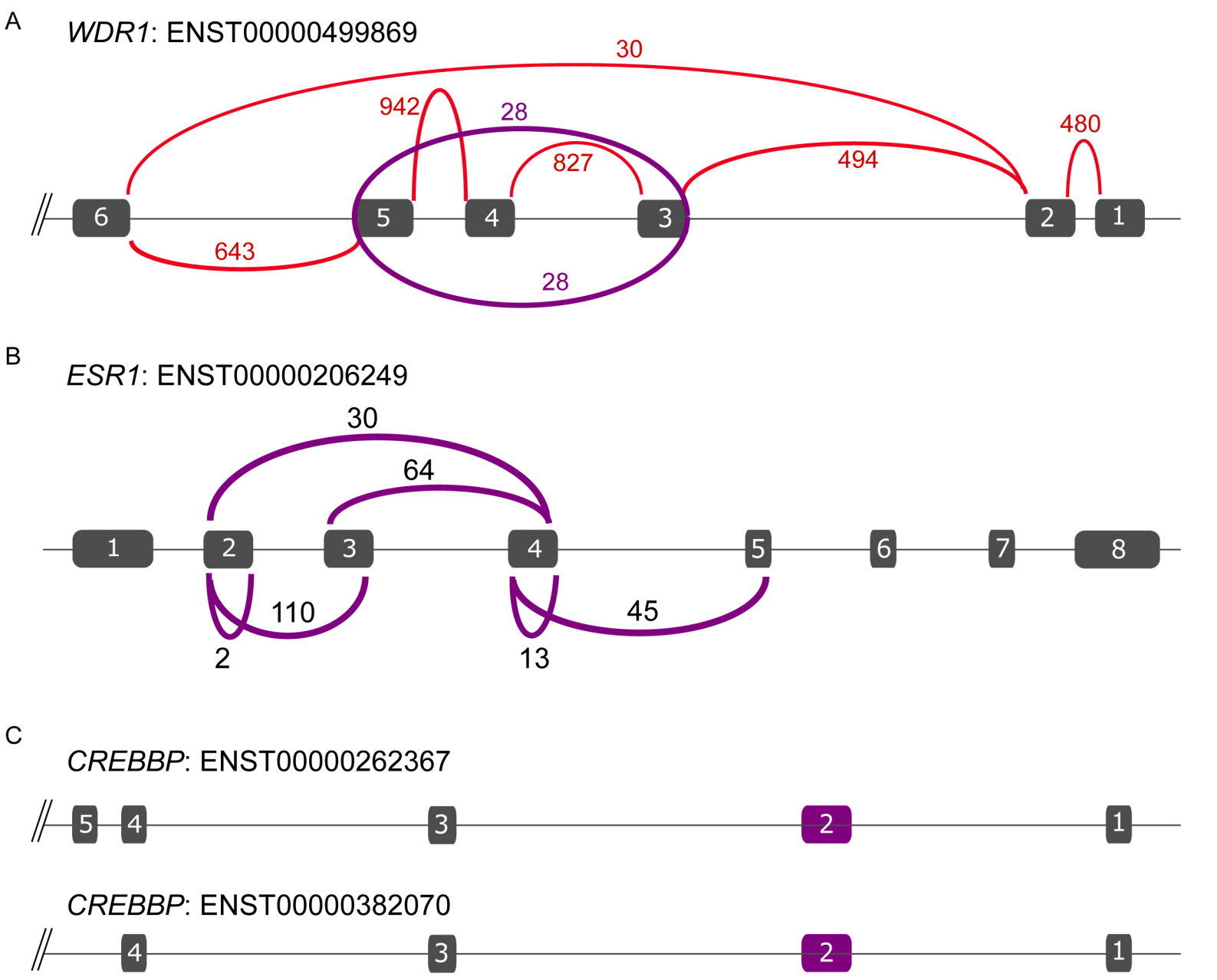
Junction read
2nd alignment

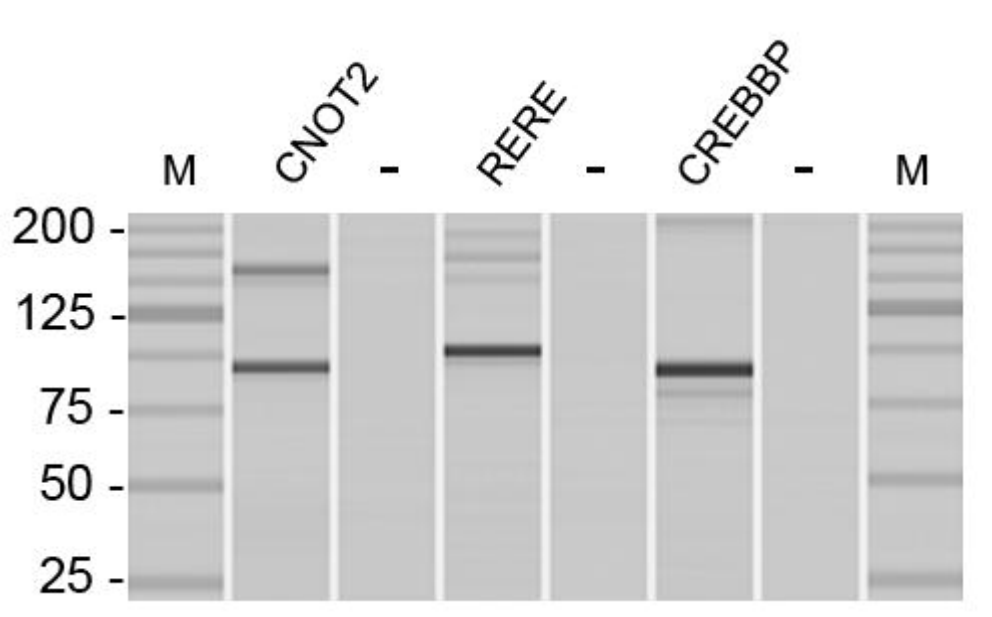
Multiple read-pairs

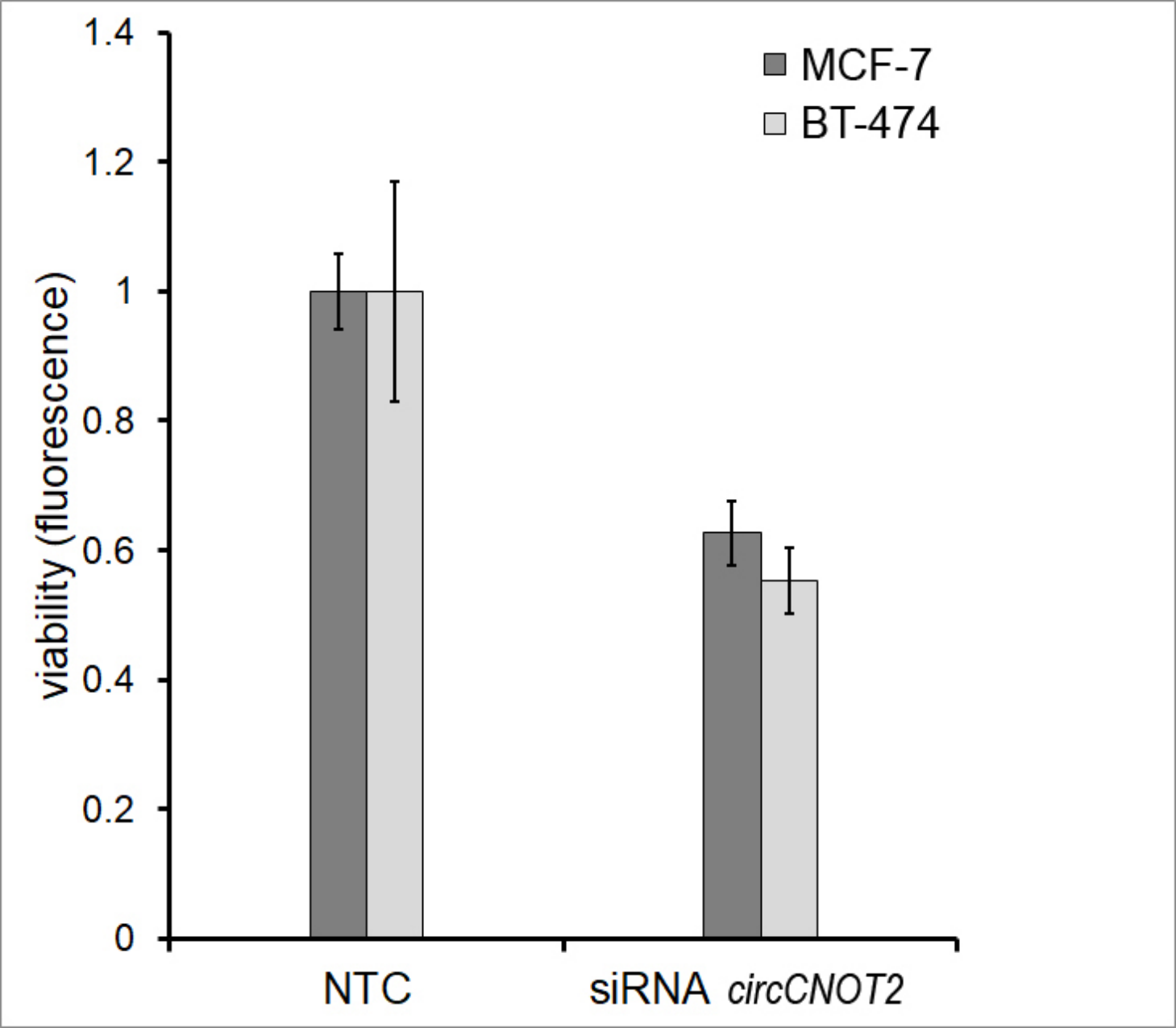
Filter
Annotate

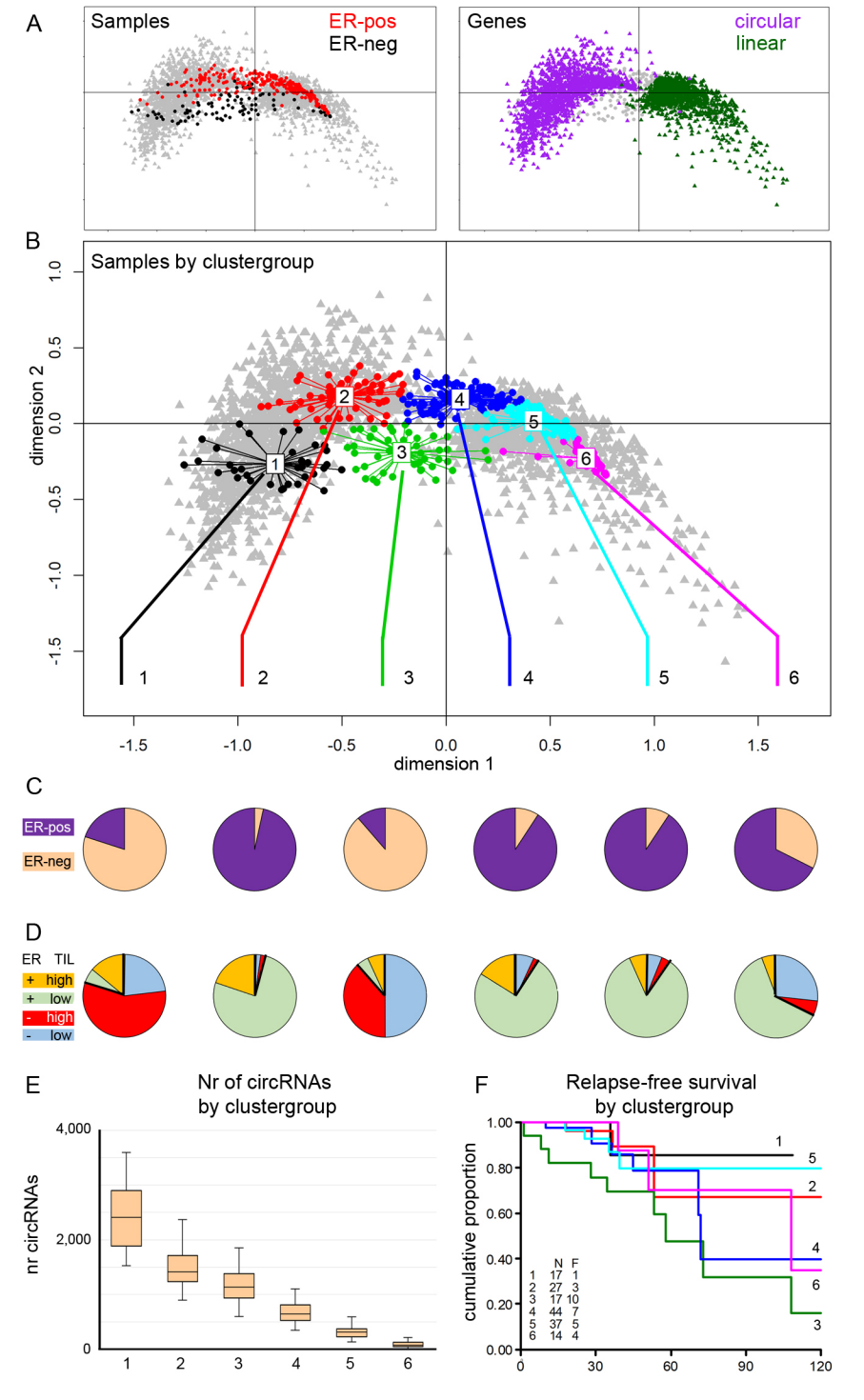
circRNA regions

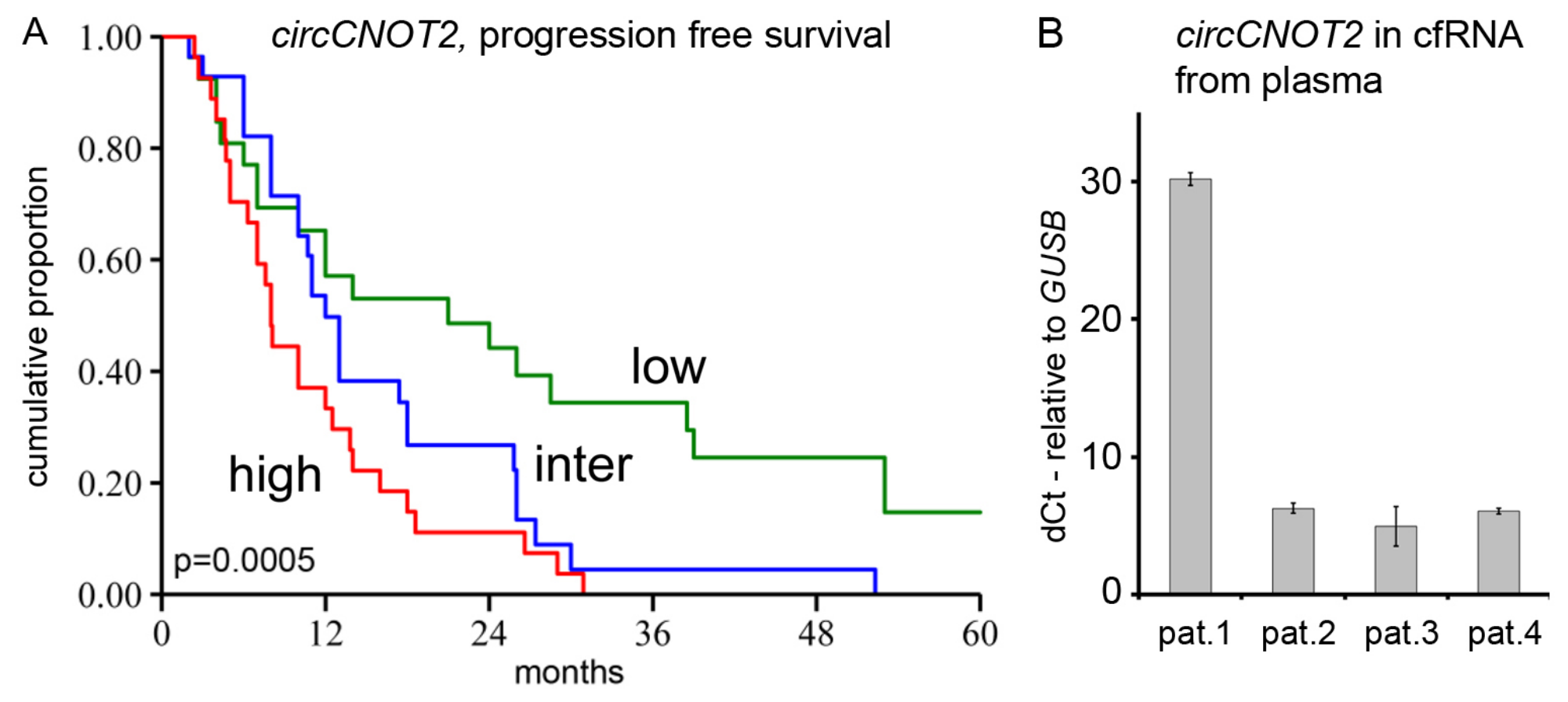

A
100%
75%
50%
25%
0%
25783
70060
13853
6588
1625
218
426
48
Total
Recurrent
n≥2
n≥50
n≥150
exon matching circRNAs
non-exon matching circRNAs
C
log(bp)
12
9
6
downstream
nonCirc
upstream
B
8,000
4,000
0
Nr of circRNAs
total nr circRNAs
recurrent nr circRNAs
ER-POS
ER-NEG


A
WDR1: ENST00000499869
30
942
28
480
827
494
6
5
4
3
2
1
643
28
B
ESR1: ENST00000206249
30
64
1
2
3
4
5
6
7
8
110
45
2
13
C
CREBBP: ENST00000262367
5
4
3
2
1
CREBBP: ENST00000382070
4
3
2
1


M
CNOT2
-
RERE
-
CREBBP
-
M
200 -
125 -
75 -
50 -
25 -


1.4
1.2
1
0.8
0.6
0.4
0.2
0
viability (fluorescence)
MCF-7
BT-474
NTC
siRNA circCNOT2


A
Samples
ER-pos
ER-neg
Genes
circular
linear
B
Samples by clustergroup
dimension 2
dimension 1
1.0
0.5
0.0
-0.5
-1.0
-1.5
1
2
3
4
5
6
C
ER-pos
ER-neg
D
ER TIL
+ high
+ low
- high
- low
E
Nr of circRNAs
by clustergroup
nr circRNAs
4,000
2,000
0
F
Relapse-free survival
by clustergroup
cumulative proportion
1.00
0.80
0.60
0.40
0.20
0.00
0
30
60
90
120
N F
1 17 1
2 27 3
3 17 10
4 44 7
5 37 5
6 14 4


A
circCNOT2, progression free survival
cumulative proportion
1.00
0.80
0.60
0.40
0.20
0.00
high
inter
low
p=0.0005
0
12
24
36
48
60
months
B
circCNOT2 in cfRNA from plasma
dCt - relative to GUSB
30
20
10
0
pat.1
pat.2
pat.3
pat.4