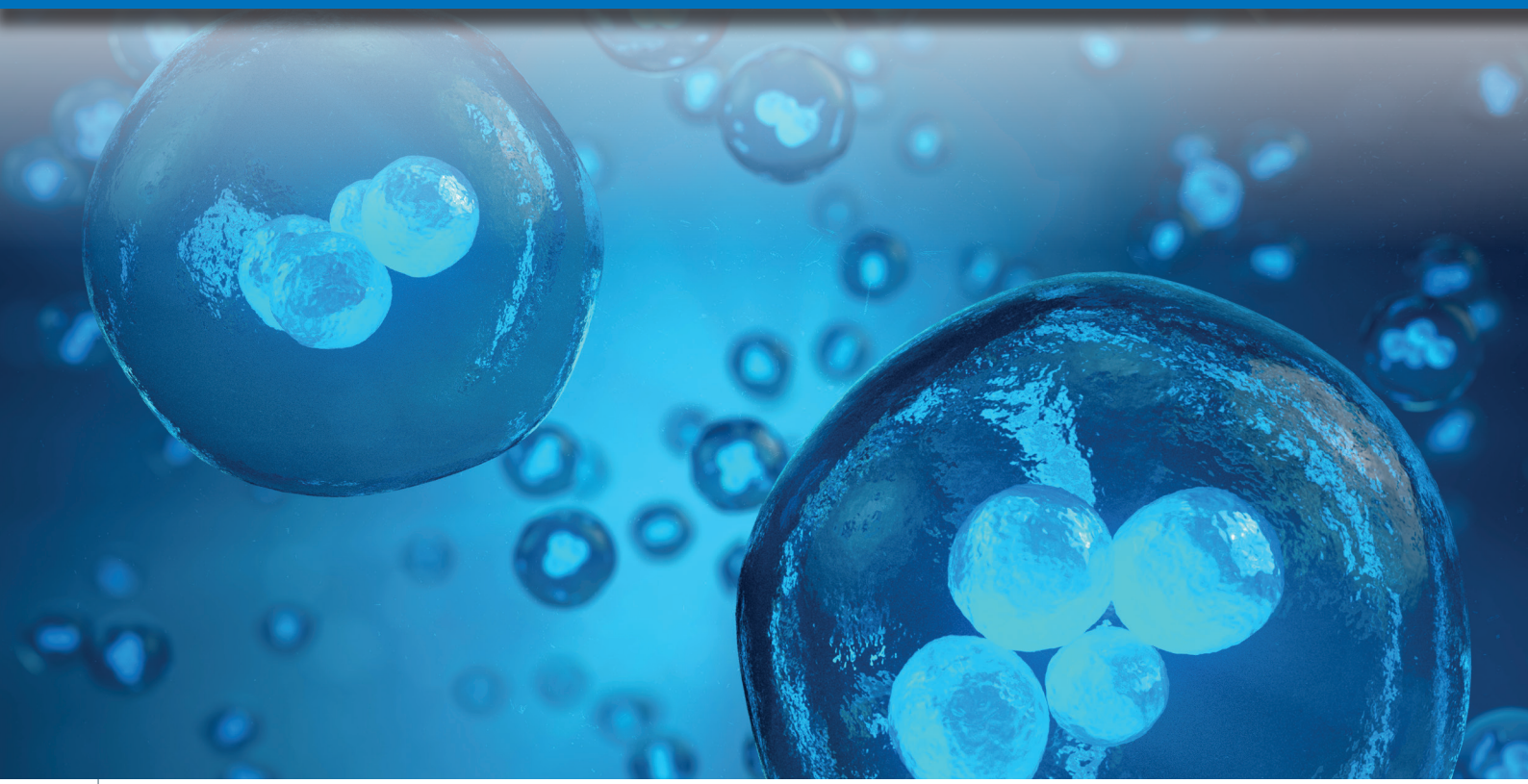

Proteomic changes in tissue samples of mouse gastric carcinoma: Label-free quantitation on the timsTOF fleX with PASEF

timsTOF fleX with PASEF enables deeper proteome coverage in shortest possible time, always with high sensitivity and robustness.

Abstract

The timsTOF fleX offers a combination of two unique technologies, namely Trapped Ion Mobility Spectrometry (TIMS) to enhance ion separation and sensitivity and Parallel Accumulation Serial Fragmentation (PASEF, [1]) to improve ion utilization efficiency and data acquisition speed. In this application note, we demonstrate the performance of the timsTOF fleX mass spectrometer with PASEF for label-free quantitation of proteins extracted from sectioned mouse tissue. In brief, more than 5000 protein groups could be reliably identified and quantified from 240 ng protein per sample using 90-minute

Keywords: Proteomics, PASEF, LFQ, MaxQuant, tissue, tumor, mouse, timsTOF fleX

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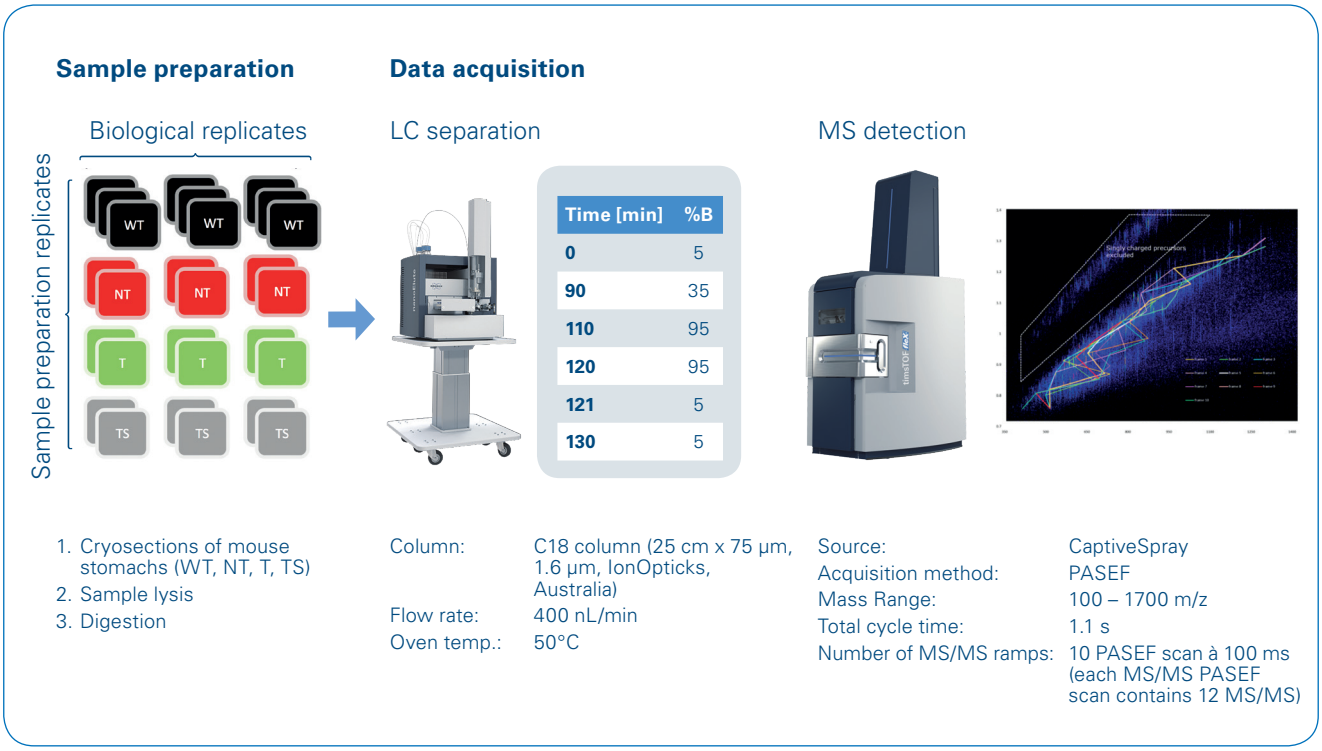


Figure 1: Workflow for the analysis of isolated mouse stomach tissue using label-free quantitation. In total 27 samples have been prepared and subsequently analysed using the PASEF method on the timsTOF fleX.

gradients. The optimized PASEF method used on the timsTOF fleX resulted in very high technical reproducibility which is an important prerequisite for label-free quantitation (LFQ). Furthermore, the complete process (including tissue preparation, digestion, and data acquisition) was highly reproducible, which is critical in the application of proteomics to clinically relevant specimens. When comparing the proteome composition of tumor and non-tumor tissue, gene ontology analysis indicated the enrichment of the minichromosome maintenance protein complex (MCM-complex) in tumor over non-tumor samples. The MCM-complex has been shown to be an essential component of the pre-replication complex (pre-RCs), which is involved in DNA replication initiation and the recruitment of DNA-Polymerases. In various studies a malfunction of the MCM-complex has been linked to genomic instability, increased cell proliferation, and a variety of carcinomas.

Introduction

Quantitation is one of the most important tasks in proteomics in order to elucidate and understand the functional role of proteins in biological systems. Label-free quantitation is a commonly used methodology that allows deep and quantitative proteome profiling for any type and number of samples, without the added cost of labeling reagents. The timsTOF fleX with PASEF (Parallel Accumulation Serial Fragmentation) provides extremely fast (> 100 Hz) data acquisition with improved, industry leading sensitivity and while maintaining high mass resolution, making it an instrument well-suited for the challenges of label-free quantitation. Furthermore, the very high sensitivity of the timsTOF fleX reduces the sample input requirements, while the orthogonal design of the ion optics provides highly robust longitudinal reproducibility.

Gastric cancer is one of the most commonly occurring cancers worldwide, which is typically only diagnosed at advanced disease stages contributing to poor survival prognosis and high mortality. Thus, there is an unmet need for new diagnostic biomarkers and treatment strategies as well as potential drug targets. Previously described, transgenic CEA424-SV40 Tag C57BL/6 J

mice are a well-characterized model of early-onset invasive gastric carcinoma [2]. They have been previously used in MALDI mass spectrometry imaging studies of cancer therapeutics as well as protease activity in gastric cancer [3, 4]. Here, we used the same mouse model for label-free proteomics to investigate differences between dissected non-tumor and tumor tissues.

Methods

Tissue sections from frozen TCEA-positive and WT mouse stomachs were prepared as described elsewhere [4]. Three biological replicates of both tissues, with 3 technical replicates for each WT sample and 2 technical replicates for all TCEA-positive totaling in 27 samples (Figure 1) were diluted in 0.1% formic acid (FA) to enable injection of 240 ng of total protein equivalent in 1 µl. A nanoElute UHPLC was coupled to the timsTOF fleX mass spectrometer (both Bruker Daltonics). Peptides were separated on a C18 column (25 cm x 75 µm, 1.6 µm, IonOpticks, Australia) using a linear 90-minute gradient of 6 – 35% B (0.1% FA in ACN) at a constant flow rate of 400 nL/min. Column temperature was controlled at 50°C. Data were acquired using our PASEF technology. MS data were collected over an m/z range of 100 to 1700. During each MS/MS data collection, each TIMS cycle was 1.1 seconds and included 1 MS and 10 PASEF MS/MS scans, with an average of 12 precursors selected for each PASEF MS/MS scan, resulting in an MS/MS data acquisition rate of 109 Hz. Raw files were processed using MaxQuant (version 1.6.4.0) using default timsTOF parameters. Database search was performed using the Andromeda search engine against the mouse Uniprot database (*mus musculus*, reviewed, 16,996 entries) with the following search criteria: enzyme specificity was set to trypsin with up to two missed cleavages. Carbamidomethylation (C) and oxidation (M) / acetylation (protein N-Term) were selected as fixed and variable modifications, respectively. Match-between-runs was activated and default parameters were used for retention time and ion mobility matching. For differential expression analysis, Limma statistics were applied [5].

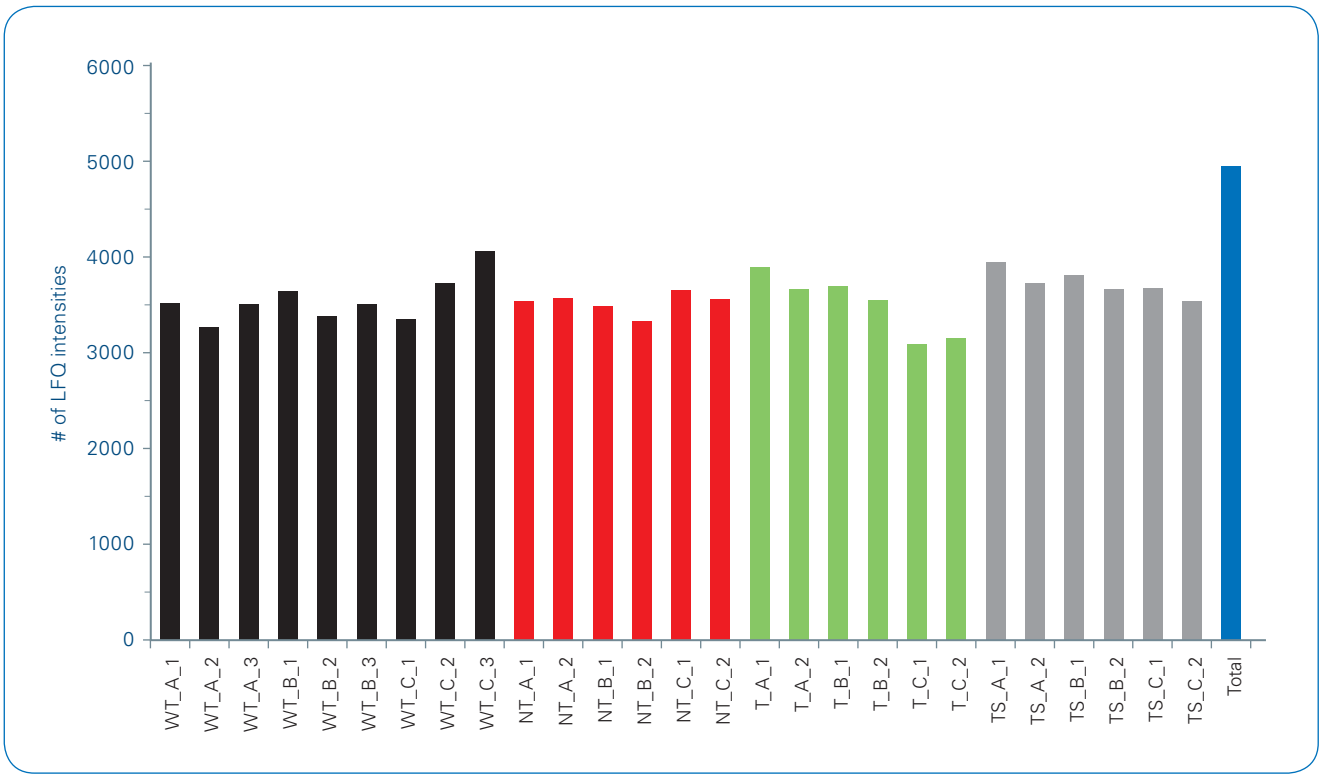


Figure 2: Number of quantified protein groups per sample. Shown is the number of LFQ intensities resulting from MaxQuant processing for the 27 analyzed samples as well as the total number of identified and quantified protein groups detected in mouse stomach tissue using PASEF.

Results and Discussion

We used label-free quantitative proteomics to analyze tumor-bearing mouse stomachs (TS) which were dissected into tumor (T) and non-tumor (NT) tissues. Whole stomachs from a wild type mouse (WT) served as a reference. Using the PASEF technology we identified and quantified on average 3652 (± 225) protein groups for each of the 27 analyzed samples, resulting in 5001 overall identified protein groups (Figure 2) in 90-minute gradients. From these 5001 protein groups nearly 50% (2330 protein groups) could be identified and quantified in all 27 samples (Figure 3), thus being present in all biological and technical replicates of the four different tissue types. The high data completeness is a result of both, the high sequencing speed of the timsTOF fleX as well as the "matching between runs" feature in MaxQuant, which makes use of the additional mobility dimension resulting in a four-dimensional matching. Utilization of the mobility dimension not only improves data completeness but also increases confidence that a feature is a true match.

Technical reproducibility of the PASEF technology is very high with typical R^2 values above 0.98, as illustrated by technical replicate injections of a Hela sample (Figure 4 A). Therefore, for tissue sections we decided to run only biological and process replicates (typical examples are shown in Figure 4 B and C, respectively). For the presented study, run-to-run reproducibility for the different tissue types was high with an average R^2 value of 0.91 resulting from the combination of biological and process replicates, with excellent linearity over 4.5 orders of magnitude in protein abundance.

A principal component analysis (PCA) of protein LFQ intensities revealed three distinct groups for WT, T, and NT tissue (Figure 5) with PC1 accounting for 29.2% total variation and PC2 for 10.3%. Limma-moderated t-statistics were used for differential expression analysis of label-free quantified protein groups in tumor (T) versus non-tumor (NT) samples. Technical and biological replicates were considered within the Limma algorithm and Benjamini-Hochberg-adjusted p-values were calculated. Protein groups with an absolute fold-change higher than 1.5 and an adjusted p-value of less than 0.05 were considered significant (Figure 6).

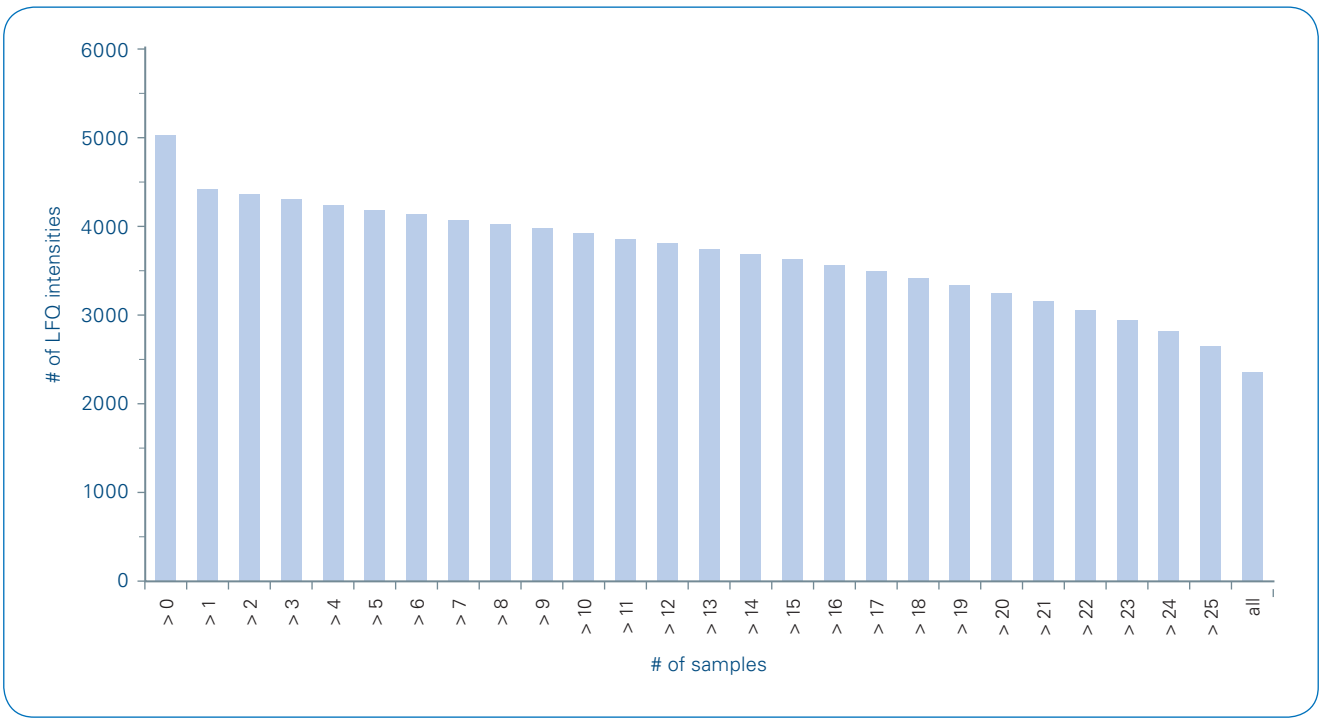
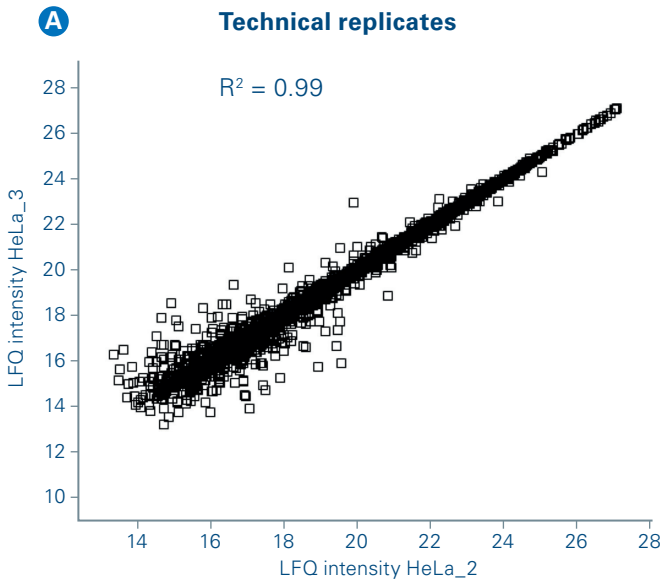
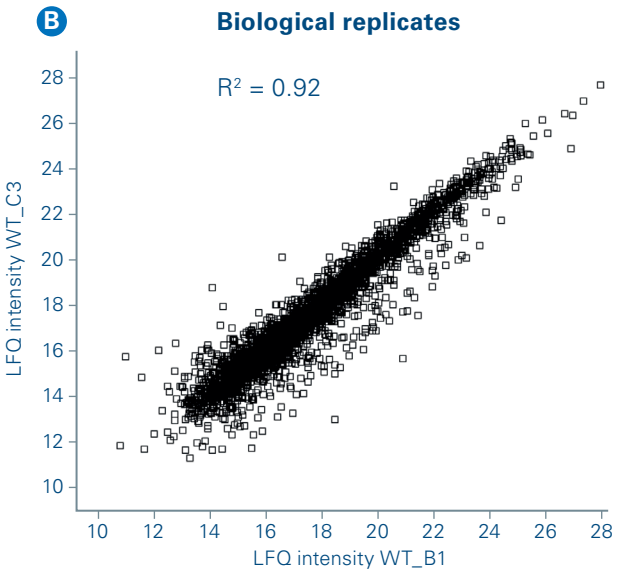
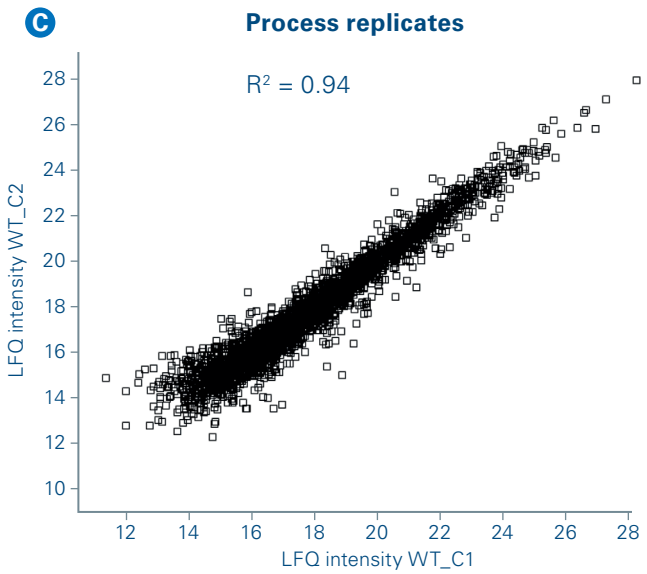


Figure 3: Accumulated number of LFQ intensities (quantified proteins). Displayed is the number of LFQ intensities (quantified proteins) detected across 27 samples. Nearly 50% of the protein groups (2330) could be quantified in all 27 samples.







D **Average R^2 values**

WT	N	T	TS
0.92 (± 0.03)	0.89 (± 0.04)	0.91 (± 0.03)	0.91 (± 0.03)

Figure 4: Scatter plot displaying precision of quantitation. Displayed are example comparisons of MaxQuant LFQ intensities between 2 samples for **A** *technical replicates (HeLa_1 vs HeLa_2) from corresponding instrument quality control measurements,* **B** *biological replicates (WT_B1 vs WT_C3), and* **C** *process replicates (WT_C1 vs WT_C2).* **D** *Average R^2 values for the 4 different samples.*

The resulting 110 significant protein groups were subjected to a Gene Ontology (GO) analysis in the STRING: functional protein association networks database (Figure 7).

In a GO analysis, DNA replication (KEGG; FDR 2.87^{e-9}), DNA replication initiation (Biological process; FDR $1.93e^{-6}$), as well as the minichromosome maintenance protein complex (MCM; Cellular components; FDR $8.97e^{-9}$) were found as enriched categories within our list of protein groups. The MCM-complex is a DNA helicase essential for genomic DNA replication and is coupled to several cell cycle checkpoints in a normal functioning cell.

In several studies, the upregulation of MCM-complex proteins has been linked to genomic instability and enhanced cell proliferation, likely due to its ability to increase DNA replication. The deregulation of the MCM-complex and its ability to promote cell proliferation has been associated to a range of different types of cancer and, based on our observations, likely also functions in gastric cancer.

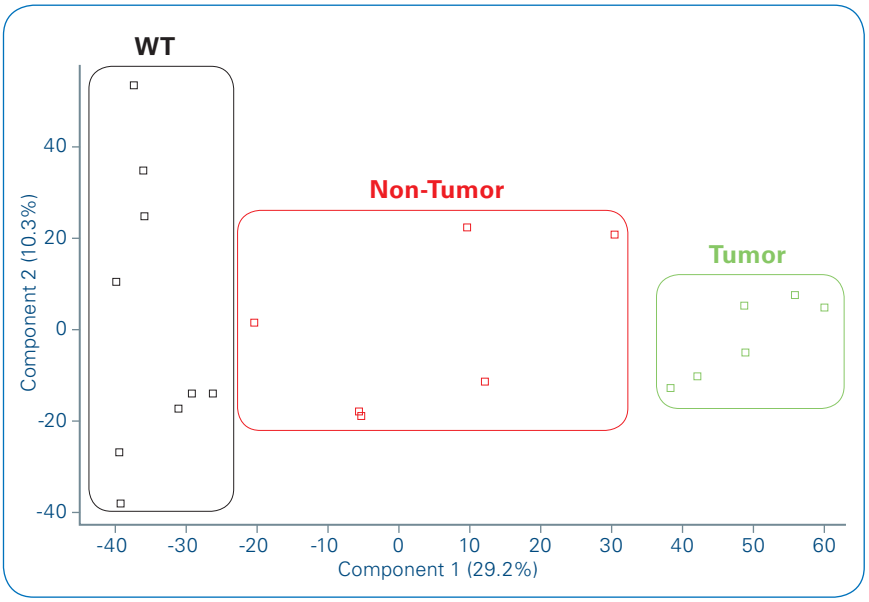


Figure 5: Principal component analysis (PCA) of all 5001 identified protein groups with their respective LFQ intensities separates the three groups (WT, NT, and T).

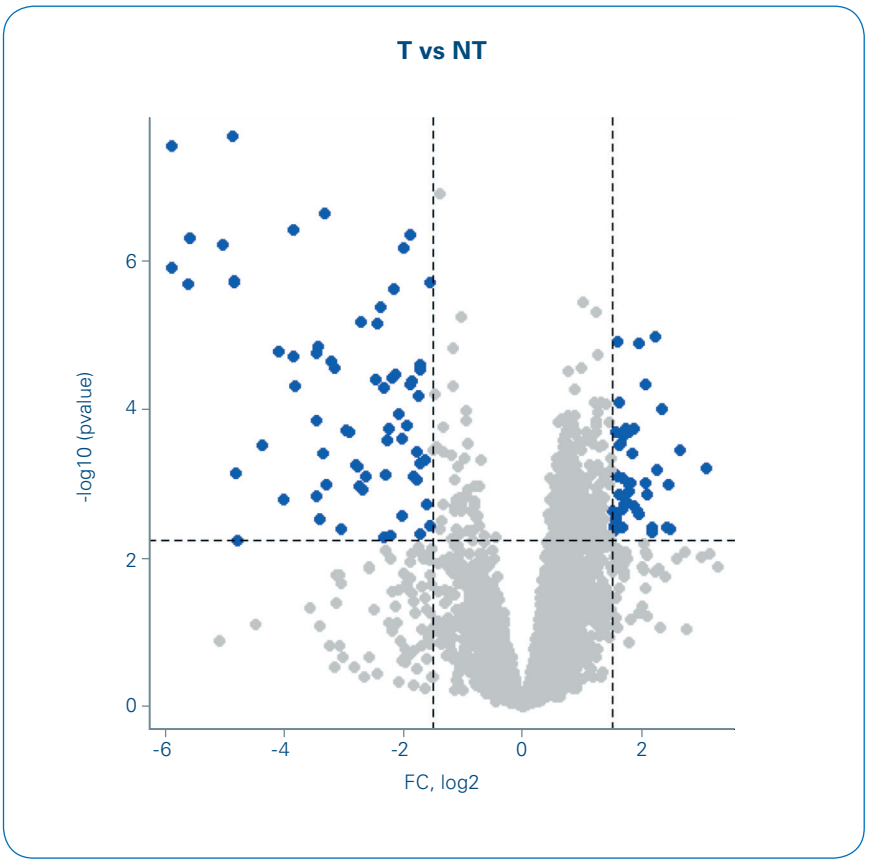


Figure 6: Volcano plot of all identified protein groups comparing tumor and non-tumor tissues. 110 significantly regulated proteins are highlighted. The analysis considers technical and biological replicates. Threshold for significance is an absolute fold-change value > 1.5 and an adjusted p-value < 0.05 after Limma statistics.

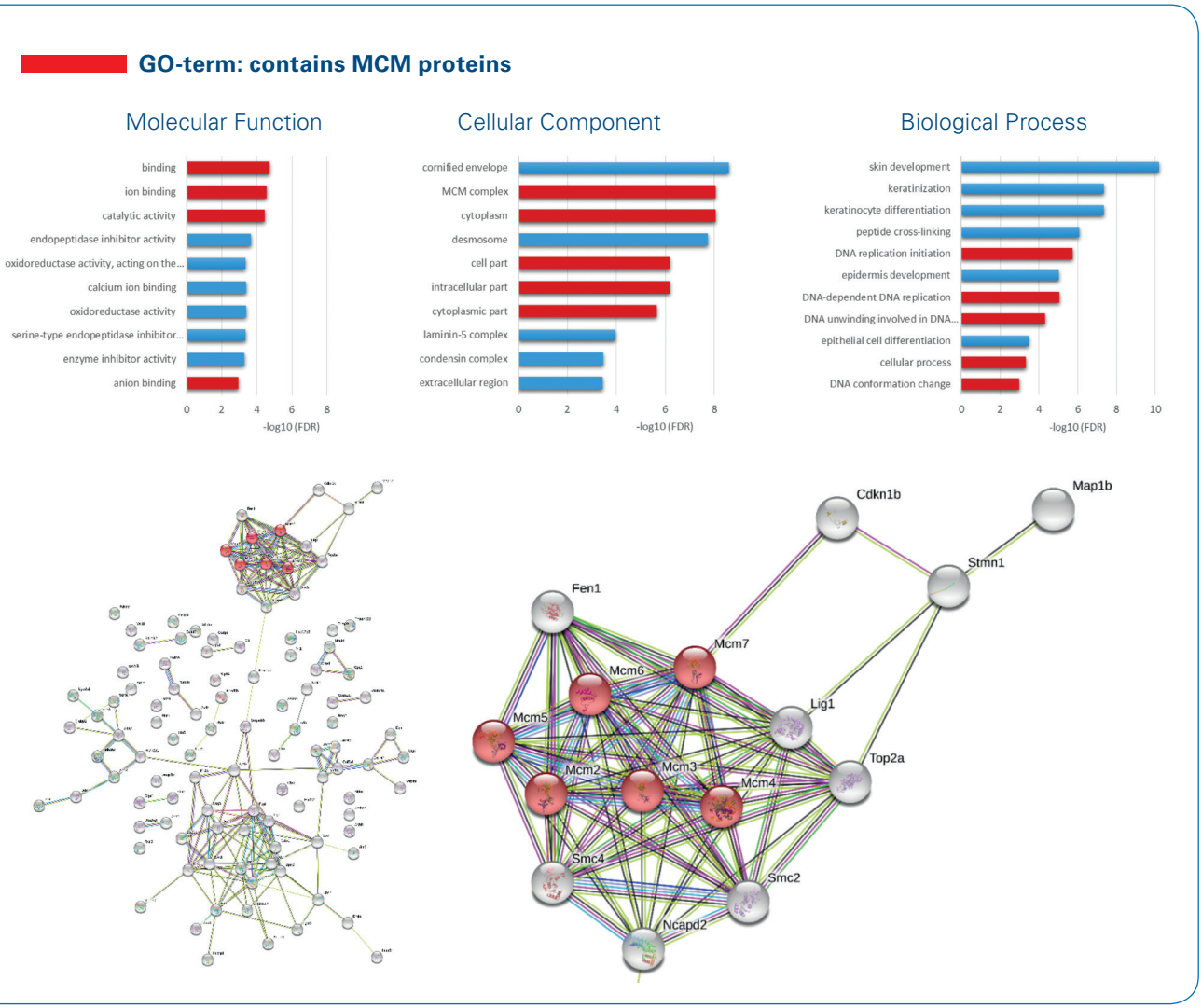


Figure 7: STRING: functional protein association networks database GO annotation analysis of the 110 significantly regulated protein groups comparing tumor and non-tumor tissues based on imma statistics. The top ten hits within molecular function, cellular component, and biological process are shown. The minichromosome maintenance protein complex (MCM-complex) is shown to be enriched in tumor over non-tumor tissues. GO-terms including MCM-complex proteins are highlighted in red.

Conclusions

- 110 significantly regulated proteins and GO-analysis identified the MCM-complex enriched in the tumor over the non-tumor samples.
- More than 5000 protein groups could be identified in 240 ng of protein from mouse stomach tissue using a 90-minute gradient.
- PASEF on the timsTOF fleX facilitates accurate and reproducible quantitative label-free proteomics on complex tissue samples.
- Sustained high technical reproducibility of the timsTOF fleX allows the focus to be on biological replicates, thereby reducing the need for technical replicates

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