Convergent network effects along the axis of gene expression during prostate cancer progression

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Abstract

Background

Tumor-specific genomic aberrations are routinely determined by high throughput genomic measurements. It remains unclear though, how complex genome alterations affect molecular networks through changing protein levels, and consequently biochemical states of tumor tissues.

Results

Here, we investigated the propagation of genomic effects along the axis of gene expression during prostate cancer progression. For that, we quantified genomic, transcriptomic and proteomic alterations based on 105 prostate samples, consisting of benign prostatic hyperplasia regions and malignant tumors, from 39 prostate cancer patients. Our analysis revealed convergent effects of distinct copy number alterations impacting on common downstream proteins, which are important for establishing the tumor phenotype. We devised a network-based approach that integrates perturbations across different molecular layers, which identified a sub-network consisting of nine genes whose joint activity positively correlated with increasingly aggressive tumor phenotypes and was predictive of recurrence-free survival. Further, our data revealed a wide spectrum of intra-patient network effects, ranging from similar to very distinct alterations on different molecular layers.

Conclusions

This study uncovered molecular networks with remarkably convergent alterations across tumor sites and patients, but it also exposed a diversity of network effects: we could not identify a single sub-network that was perturbed in all high-grade tumor regions.

Keywords: molecular aberrations, network effects, prostate cancer, proteogenomic analysis, tumor heterogeneity

Background

Prostate cancer (PCa) represents one of the most common neoplasm among men with almost 1,300,000 new cases and 360,000 deaths in 2018 [1] accounting for 15% of all cancers diagnosed. PCa is the fifth leading cause of cancer death in men and represents 6.6% of total cancer mortality in men [1]. Despite earlier detection and new treatments, the lifetime risk to die of PCa has remained stable at approximately 3% since 1980. (National Cancer Institute SEER data: https://seer.cancer.gov/statfacts/html/prost.html). In many patients, PCa is indolent and slow growing. The challenge is to identify those patients who are unlikely to experience significant progression while offering radical therapy to those who are at risk. Current risk stratification models are based on clinicopathological variables including histomorphologically defined grade groups, prostate-specific antigen (PSA) levels and clinical stage. Although those variables provide important information for clinical risk assessment and treatment planning [2, 3], they do not sufficiently predict the course of the disease.

Extensive genomic profiling efforts have provided important insights into the common genomic alterations in primary and metastatic PCa [4-9]. Interestingly, PCa genomes show a high frequency of recurrent large-scale chromosomal rearrangements such as TMPRSS2-ERG [10]. In addition, extensive copy number alterations (CNAs) are common in PCa, yet point mutations are relatively infrequent in primary PCa compared to other cancers [6, 11]. A major complicating factor is that around 80% of PCas are multifocal and harbor multiple spatially and often morphologically distinct tumor foci [12, 13]. Several recent studies have suggested that the majority of topographically distinct tumor foci appear to arise independently and show few or no overlap in driver gene alterations [14-16]. Therefore, a given prostate gland can harbor clonally independent PCas.

To allow for a more functional assessment of the biochemical state of PCa, it is necessary to go beyond genomic alterations and comprehensively catalogue cancer specific genomic, transcriptomic and proteomic alterations in an integrated manner [17-19]. Such an approach will provide critical information for basic and translational research and could result into clinically relevant markers. While hundreds of PCa genomes and transcriptomes have been profiled to date [20], little is known about the PCa proteome. Although recent work has emphasized the need for integrated multi-omics profiling of PCa, we still lack understanding about how genomic changes impact on mRNA and protein levels [17-19]. Especially the complex relationship between tumor grade, tumor progression and multi-layered molecular network changes remains largely elusive.

For example, previous work has shown that copy number changes may alter transcript levels of many genes, whereas the respective protein levels remain relatively stable [21]. Indeed, there is compelling evidence across multiple tumor types that many genomic alterations are 'buffered' at the protein level and are hence mostly clinically inconsequential [22]. To better understand the evolution of PCa and to identify core networks perturbed by genomic alterations and thus central for the tumor phenotype, it is therefore essential to investigate the transmission of CNAs to the transcriptomic and proteomic level.

To this end, it is important to decipher which genomic alterations impact PCa proteomes, which of those proteomic alterations are functionally relevant, and how molecular networks are perturbed at the protein level across tumors.

To address these open questions, we performed a multi-omics profiling of radical prostatectomy (RP) specimens at the level of the genome, transcriptome and proteome from adjacent biopsy-level samples, using state-of-the-art technologies. Unique features of this study are (1) the utilization of PCT (pressure cycling technology)-SWATH (Sequential Window Acquisition of all THeoretical Mass Spectra) mass spectrometry [23, 24], allowing rapid and reproducible quantification of thousands of proteins from biopsy-level tissue samples collected in clinical cohorts; (2) the simultaneous profiling of all omics layers from the same tissue regions; (3) inclusion and full profiling of benign regions, which provides a matching control for each tumor; and (4) the full multi-omics characterization of multiple tumor regions from the same patients, thus enabling the detailed investigation of tumor heterogeneity. This design resulted in the multi-layered analyses of 105 samples from 39 PCa patients, as well as of the exome of corresponding peripheral blood cells yielding a comprehensive molecular profile for each patient and identified molecular networks that are commonly altered in multiple patients. Importantly, some of the affected genes/proteins exhibited very small individual effect sizes, suggesting that combined network effects of multiple genes may significantly contribute to determining PCa phenotypes.

Results

Proteogenomic analysis of the sample cohort identifies known PCa biomarkers.

In this study, we analyzed 39 PCa patients (**Additional file 1: Fig. S1**) belonging to three groups who underwent laparoscopic robotic-assisted RP. The patients were from the PCa Outcomes Cohort (ProCOC) study [25, 26]. Tumor areas were graded using the ISUP (International Society of Urological Pathology) grade groups [27], which range from ISUP grade group G1 (least aggressive) to G5 (most

aggressive). The more advanced grade groups G4 and G5 are considered jointly (G4/5). The cohort tested included 12 low-grade (G1), 17 intermediate- (G2 and G3), and 10 high-grade (G4/5) patients (**Fig. 1a**, **Additional file 1: Fig. S1**, **Additional file 2: Table S1**). For low-grade PCa patients, we selected two representative regions, one of benign prostatic hyperplasia (BPH) and one of malignant tumor (TA). Since PCa often presents as a multifocal disease with heterogeneous grading within each prostate specimen [24] we analyzed two different tumor regions from the 27 intermediate- and high-grade patients. In those cases three representative regions, including BPH, the most aggressive tumor (TA1) and a secondary, lower-grade tumor (TA2) [2] were analyzed. Thus, TA1 always represented the higher-grade nodule compared to TA2. Note, whereas each patient was assigned a patient-specific overall grade (*i.e.* 'low', 'intermediate' or 'high'), each tumor area was additionally assigned an individual grade group based on its histological appearance. According to current ISUP guidelines, the grading of the entire prostate specimen depends on the size and grade of individual nodules [28]. Thus, it is possible that the patient grading is lower than the grading of the most aggressive nodule, if another lower-grade nodule is larger. Tumor regions contained at least 70% tumor cellularity and the distance between the analyzed areas (TA1 versus TA2) was at least 5 mm. Altogether, we obtained 105 prostate tissue specimens (**Additional file 2: Table S1**). Three adjacent tissue biopsies of the dimensions 0.6 x 0.6 x 3.0 mm were punched from each representative region for exome sequencing, CNA (derived from the exome sequencing data), RNA sequencing (RNA-seq), and quantitative proteomic analysis using the PCT-SWATH technology [23] respectively. Proteomic analysis was performed in duplicates for each tissue sample. Peripheral blood samples from each patient were also subjected to exome sequencing and served as the genomic wild-type reference (**Fig. 1**). All three types of grading (*i.e.* patient-specific overall grading, TA1 grading and TA2 grading) were predictive of the recurrence-free survival (RFS) in our study.

In agreement with prior reports, we observed relatively few recurrent point mutations across patients (**Additional file 1: Fig. S2**, **Additional file 3: Table S2**), but substantial CNAs (**Additional file 1: Figs. S3** and **S4**, **Additional file 4: Table S3**). In total, 1,110 genes showed copy number gains in at least five samples or copy number losses in at least five samples (see **Additional file 1: Supplementary Text** for details). Likewise, our data confirmed the differential expression of several transcripts/proteins that had previously been suggested as PCa biomarkers or which are known oncogenes in other tumor types (**Additional file 1: Fig. S5**, **Additional file 5: Table S4** and **Additional file 6: Table S5**) (see **Additional file 1: Supplementary Text** for details). This consistency with previously published results confirmed the quality of our data and motivated us to go beyond previous work by performing a network-based multi-omics multi-gene analysis.

Molecular perturbations correlate with tumor grade.

As a first step towards a cross-layer analysis, we asked if high-grade PCa would be generally affected by stronger alterations (compared to low-grade PCa) at the genome, transcriptome, and proteome layer [29]. Thus, we devised molecular perturbation scores that quantified the number of affected genes/proteins and the extent to which these genes/proteins were altered in the tumor specimens compared to their benign controls (see the '**Methods**' section for details). Higher-grade tumors (G3 and G4/5) exhibited significantly higher molecular perturbation scores than lower-grade tumors (G1 and G2). Those differences were statistically significant in all but one case (P value < 0.05, one-sided Wilcoxon rank sum test, **Fig. 2**). The CNA perturbation magnitude exhibited the highest correlation with the PCa grading, confirming prior studies documenting the tight association between CNA, histopathological grade and risk of progression [4, 5, 30]. Previous work suggested that copy number changes are to some extent buffered at the protein level [31]. Interestingly, we observed that proteins known to be part of protein complexes were significantly less strongly correlated with the fold changes (FCs) of their coding mRNAs than proteins not known to be part of protein complexes (P value < 2.6e-11, one-sided t-test, **Additional file 1: Fig. S6**). This result is consistent with the concept that protein complex stoichiometry contributes to the buffering of mRNA changes at the level of proteins [22, 32-34]. Thus, molecular patterns in high-grade PCa are more strongly perturbed at all layers and the effects of genomic variation are progressively but non-uniformly attenuated along the axis of gene expression.

Inter-patient heterogeneity decreases along protein biosynthesis.

Our analysis of CNA profiles (above and **Additional file 1: Supplementary Text**) already revealed many shared CNAs across patients, suggesting that such common CNAs might represent genomic driver changes. We therefore investigated if such a convergence towards common molecular endpoints could also be observed at the transcript and protein level. To address this question, we first computed a reference molecular signature that is characteristic of the molecular perturbations of tumors in a given grade group. These 'centroid vectors' were obtained by computing the average tumor-to-benign FCs across all samples within a grade group. Consistent with the observation above, we found that the average effect sizes (averaged absolute centroid FCs) were increasing with the grade group for all three layers (CNA, mRNA, and protein; **Additional file 7: Table S6**). Next, we compared each individual sample within a group against the matching centroid of the same group. For the quantification of the similarity between a tumor sample and the corresponding centroid we used four similarity/distance measures: Pearson correlation, Mutual Information (MI)[35], Manhattan distance and Euclidean distance. While the

first two measures (*i.e.* Pearson correlation and MI) quantify the degree to which the tumor sample and centroid vector co-vary, the other two measures (*i.e.* Manhattan and Euclidean distance) also take into account the magnitude of the FCs in the two vectors. To illustrate this difference, imagine two patients having perturbations of the same genes/proteins, but one of them exhibiting overall two-fold greater FCs (*i.e.* all FCs are increased by a factor of two compared to the other patient). In such a scenario Pearson correlation and MI would yield identical results for the two patients when compared to the centroid, whereas Manhattan and Euclidean distance would identify them as different. Using the Pearson correlation and MI, we found that high-grade PCa (G4/5) were more similar to their respective centroid than low-grade PCa (G1) to their centroid (**Fig. 3**). This effect was particularly pronounced for protein-level changes. This is consistent with the notion that protein levels (and not mRNA levels) are subjected to stronger selection. Interestingly, when the Euclidean distance and the Manhattan distance were used to characterize tumor similarity, we found that the high-grade tumors were more *dissimilar* to each other than the low-grade tumors (**Additional file 1: Fig. S7**), in sharp contrast to the Pearson correlation and MI. Based on the nature of the different similarity measures tested, we hypothesized that there is a set of proteins commonly affected in their abundance by oncogenic alterations in high-grade tumors. This would increase the similarity using the Pearson correlation or MI. However, although the same proteins are affected, they are affected to a different extent in different high-grade tumors, *i.e.* the FCs exhibited a high degree of variability (**Additional file 1: Fig. S7**), which would increase the dissimilarity based on the Euclidean distance or Manhattan distance (see the '**Methods**' section and **Additional file 1: Fig. S7** for a schematic explanation).

To further corroborate the notion of common endpoints, we focused on the 20 proteins with the largest average absolute FCs across all tumor specimens (**Additional file 1: Fig. S7**, **Additional file 7: Table S6**). Among them was PSA (KLK3), and several other well established PCa-associated proteins like AGR2 [36], MDH2 [37], MFAP4 [38] and FABP5 [39]. We observed that for some of these top 20 proteins, FCs were more extreme in the higher-grade tumors (G3 and G4/5) compared to lower-grade tumors (G1 and G2), such as MDH2 and SEPHS1 (up-regulation; **Fig. 3c**). RABL3 was one of the most strongly down-regulated proteins (**Fig. 3c**), which is a surprising finding as RABL3 is known to be up-regulated in other solid tumors [40, 41]. Interestingly, in most cases these proteins were from loci that were not subject to CNAs (**Additional file 1: Fig. S7**, **Additional file 7: Table S6**), hinting that independent genomic events would impact on these target proteins *via* network effects.

Effects of distinct CNAs converge on common proteins.

It has previously been suggested that mutations affecting different genes could impact common molecular networks if the respective gene products interact at the molecular level [42]. However, previous analyses were mostly restricted to individual molecular layers. For example, it was shown that genes mutated in different patients often cluster together in molecular interaction networks [42]. But, effects of these mutations on transcript and protein levels remained unexplored in this case. Here, we aimed at a multi-layer network analysis, involving the genome, transcriptome and proteome using two different network approaches.

First, we speculated that distinct genomic events in different patients would commonly impact on at least some of the top 20 target proteins identified above. Among those top targets we selected AGR2, ACPP, POSTN and LGALS3BP, because these proteins/genes had correlated protein- and mRNA FCs; thus, protein level changes were likely caused by cognate mRNA level changes. Importantly, those mRNA/protein level changes could not be explained by CNAs of the coding genes themselves (**Additional File 1: Fig. S7**). Thus, these transcriptional changes were likely caused by *trans*-effects. To identify potential regulators for each target gene, we used an independently inferred generic transcriptional regulatory network (Leote *et al. in revision*; preprint available on bioRxiv) and selected putative regulators at most two edges away from the target genes (see the '**Methods**' for details). Using the ElasticNet algorithm we next fitted a linear model regressing the mRNA changes of the target genes against the network neighbors' CNAs. Thereby we identified genes whose CNA changes were associated with mRNA changes of the four target genes AGR2, ACPP (a.k.a ACP3), POSTN and LGALS3BP (**Additional file 7: Table S6**). To validate our approach, we used two independent PCa cohorts (TCGA; [8] and MSKCC [30]) and computed the association between the CNAs of each significant regulator and the corresponding mRNA log-FC of the respective target gene. In most cases we observed an agreement in terms of effect directions, *i.e.* the signs of association between CNA changes of the putative regulators and the mRNA log-FCs of the respective target had the same direction as in our cohort (**Additional file 7: Table S6**). Here we use ACPP/ACP3 as an illustrative example (**Fig. 3d)**: ACP3 (a.k.a. PAcP) is a prostate-specific acid phosphatase with a critical role PCa etiology and has been suggested as a PCa biomarker long before PSA [43]. ACP3 is known to inhibit cell proliferation and is therefore typically down-regulated in PCa [44], despite elevated ACP3 protein levels in patient blood [43]. In our cohort ACP3 levels were strongly down-regulated in all of the high-grade patients and in the vast majority of low- and intermediate-grade patients, suggesting that ACP3 down-regulation represents an early event during PCa evolution. Despite its established role in PCa, little is known about the oncogenic driver events

downregulating ACP3 [43]. Our network modeling identified six putative ACPP/ACP3 regulators (ANKRD22, MS4A3, RHOV, ARL11, DEFB1, and DPYSL2), several of which are already known to be associated with PCa [45-47]. Analyzing the CNA signatures of these six putative regulators reveals at least two larger groups of patients (**Fig. 3d)**: the first one harboring joint deletions of ARL11 and ANKRD22, the second one harboring joint deletions of DEFB1 and DPYSL2. The latter two genes are both encoded on Chromosome 8 and thus, their deletion may be due to single CNA events. ARL11 and ANKRD22 however, are encoded on different chromosomes. Importantly, these events were clonal in most cases, *i.e.* they were mostly common to both tumor samples of a given patient. A remarkable exception was patient M7, who had a joint deletion of ARL11 and ANKRD22 in tumor area 1 (TA1) and a DEFB1/DPYSL2 deletion in tumor area 2 (TA2). Hence, our network analysis hints that distinct deletions in the network vicinity of ACP3 can lead to the repression of this anti-proliferative protein. More examples of distinct CNAs having similar effects on downstream targets were found for the other three focus proteins (**Additional file 1: Fig. S7)**. Taken together, these findings suggest that tumor mechanisms in different patients converged on common protein endpoints and that the expression levels of these proteins were progressively more strongly affected during tumor evolution.

Joint network effects of CNAs drive tumor progression.

The analysis above identified molecular networks driving tumor alterations and thus indicated altered biochemical states that were common to most tumor specimens. To identify sub-networks that specifically distinguish high-grade from low-grade tumors, we mapped our data onto the STRING gene interaction network [48], and employed network propagation [49, 50] separately to the CNA, transcriptome and proteome data for each of the tumor samples. We excluded point mutations from this analysis as their frequency was too low in our cohort. By combining published molecular interactome data with a network propagation algorithm [42, 49], we aimed to 'enrich' network regions with many perturbed genes/proteins. We reasoned that the convergent consequences of genomic variants on common network regions would be indicative of specific biochemical functions that are important for the tumor biology. We therefore identified genes/proteins in network regions that showed a higher score (or a lower score) in high-grade (G4/5) relative to lower-grade (G1) tumor groups at all three levels (**Fig. 4a**, **b**; '**Methods**' section). This analysis identified sub-networks consisting of over- and under-expressed genes (relative to the benign controls). We found 57 amplified genes (**Additional file 7: Table S6**) for which transcripts and proteins were often over-expressed in high-grade PCa (**Fig. 4a**) and

21 genes with copy number loss (**Additional file 7: Table S6**) for which transcripts and proteins were often down-regulated compared to lower-grade tumors (**Fig. 4b**).

Among the up-regulated network nodes, we observed genes modulating the stability of chromatin, such as chromatin-binding protein Chromobox 1 (CBX1) [51], SET Domain Bifurcated 1 (SETDB1) [52], a function linking to H3K27me3 and H3K9me3 in chromatin, and CBX3 (known as HP1-γ) [53]. SETDB1 is an oncogene in melanoma [54] and has also been found to be over-expressed in PCa and cell lines [55]. Further, we found genes involved in DNA damage repair, such as SMG7 [56] and ATR [57], and PRKCZ[58], which had already been suggested as a biomarker prognostic for survival in PCa [59]. Multiple actin related proteins including ARPC1B [60], ARPC5 [61], ACTL6A [62], and CFL1 [63], which are markers for aggressive cancers, were part of the up-regulated network nodes. Moreover, the up-regulated genes contained proteins related to the cell cycle like BANF1 and proteins interacting with the centrosome including LAMTOR1 and RAB7A that had already been associated with PCa [64]. Finally, several signaling molecules with known roles in PCa were up-regulated, such as the transcription factor Yin Yang 1 (YY1) [65], the TGF-β receptor TGFBR1 [66], and KPNA4, which promotes metastasis through activation of NF-κB and Notch signaling [67]. Thus, up-regulated network nodes are involved in DNA/chromatin integrity and growth control.

Likewise, several of the down-regulated genes had functions associated with PCa. For example, the oxidative stress related gene MGST1, which is recurrently deleted in PCa [68]. ALDH1A3 is a direct androgen-responsive gene, which encodes NAD-dependent aldehyde dehydrogenase [69]. DHCR24 is involved in cholesterol biosynthesis and regulated by the androgen receptor [70]. Polymorphisms in CYP1A1 are associated with PCa risk in several meta-analyses among different ethnicities [71-73].

Further, our network analysis is suggesting tumor mechanisms converging on genes that are known contributors to PCa tumor biology. For example, the PCa-associated gene SF3B2 [74, 75] was only weakly amplified in some of the high-grade tumors (average $\log_2$FC = 0.016) and mRNA levels showed similarly small changes (average $\log_2$FC = 0.024). On the other hand, the SF3B2 protein levels were consistently and more strongly up-regulated across tumors (average $\log_2$FC = 0.31), especially within the high-grade tumors (**Additional file 1: Fig. S8**). Another example is UBE2T whose over-expression is known to be associated with PCa [76]. Unfortunately, we could not quantify the corresponding protein levels. However, we observed a strong and consistent mRNA over-expression across several tumors (average $\log_2$FC = 0.73), even though at the DNA level the gene was only weakly amplified (average $\log_2$FC = 0.023; **Additional file 1: Fig. S8**). Our findings of more heterogeneous CNAs, but more uniform

mRNA and protein alterations point on convergent evolutionary mechanisms, as we move along the axis of gene expression.

Next, we analyzed the largest connected component with genes up-regulated in advanced disease in more detail (see the '**Methods**' section). It consists of the nine nodes EMD, BANF1, ACTL6A, YY1, RUVBL1, KANSL1, MRGBP, VPS72 and ZNHIT1 (**Fig. 4a**), and is referred to in the following as Network Component 1 (**Additional file 7: Table S6**). Seven of these proteins are involved in chromosome organization which may induce genomic alterations and influence the outcome of multiple cancers including PCa [77]. For example, the actin-related protein ACTL6A is a member of the SWI/SNF (BAF) chromatin remodeling complex[78], and a known oncogene and a prognostic biomarker for PCa [79]. Further, ACTL6A, RUVBL1 and MRGBP are together part of the NuA4/Tip60-HAT complex, which is another chromatin remodeling complex involved in DNA repair [80]. Likewise, KANSL1 is involved in histone post-translation modifications, while VPS72 is a member of histone- and chromatin remodeling complexes [81]. Thus, Network Component 1 consists of genes involved in chromatin remodeling and DNA repair, many of which are known to be involved in cancers.

Several samples were characterized by a small, but consistent DNA amplification of multiple members of Network Component 1 (**Fig. 4c**). Out of the 66 tumor samples, there were 30 samples – belonging to all grade groups – with a weak but consistent DNA amplification of Network Component 1 members, while the high-grade samples had stronger amplifications on average (*i.e.* larger effect sizes). Importantly, gene members of Network Component 1 were dispersed across eight chromosomes (**Additional file 7: Table S6**). The parallel DNA amplification of these genes is therefore the result of multiple independent CNA events, while the signal on any single gene alone was too weak to be significant in isolation. In some but not all cases, the amplifications led to a small, but consistent increase in mRNA expression of the amplified gene loci (**Fig. 4d**). Unfortunately, only three out of the nine proteins were detected in our proteomics experiments (**Fig. 4e**). Interestingly, patients where the DNA amplifications led to transcript over-expression were almost always high-grade patients, whereas patients where the amplification affected gene expression to a smaller extent were low- or intermediate-grade patients (**Fig. 4c**, **d**). Further, we noticed that TA2 samples graded as G3 from high-grade patients carried amplifications of Network Component 1, whereas tumor areas graded as G3 from intermediate-grade patients did not have amplifications of this network component (**Fig. 4c**, **d**). Thus, although the tumor areas were histologically equally classified, tumor areas from high-grade patients carried a CNA signature and expression patterns reminiscent of the high-grade areas from the same patients. Therefore, within the cohort tested the joint DNA amplification of this network component

along with RNA up-regulation is a signature of high-grade tumors. Curiously, the higher-grade tumor areas of those high-grades patients (TA1) carried stronger DNA amplifications than the respective lower-grade areas (TA2), which implies that the progressive amplification of Network Component 1 during tumor evolution may contribute to an increasingly aggressive phenotype. In order to further corroborate the clinical relevance of this network perturbation we analyzed published datasets of three additional PCa cohorts (TCGA[8], MSKCC [30], and Aarhus [82]), together comprising a total of 713 patients with known clinical outcome. We found that amplification of genes from Network Component 1 was a significant predictor of reduced RFS in the MSKCC cohort (*P* value = 8.8e-3, log-rank test). In the TCGA cohort, we observed the same trend although the difference in RFS was not statistically significant (*P* value = 0.17; **Fig. 4f**). Additionally, we found that over-expression of genes from Network Component 1 was a significant predictor of reduced RFS in the TCGA cohort (*P* value = 2.1e-4, log-rank test), which was the cohort with the largest number of patients. In the other two cohorts we observed the same trend, although the difference in RFS was not statistically significant (*P* value = 0.30 and 0.093 for MSKCC, and Aarhus; **Fig. 4f**). Thus, both CNA and RNA changes of Network Component 1 are predictive of the time to relapse in independent cohorts.

In conclusion, our findings suggest that relatively weak but broad CNAs of entire network components are associated with high-grade tumors and that the presence of some of these perturbations in lower-grade tumors may be predictive of the future development of a more aggressive phenotype.

Analysis of distinct tumor nodules defines intra-patient heterogeneity (TA1 versus TA2 comparison).

The CNA patterns (**Additional file 1: Fig. S4**) and the Network Component 1 analysis (**Fig. 4c**, **d**) suggest that different tumor areas from the same patient shared several mutations. Such common signatures are expected if different tumor nodules originate from a common clone. If this was true, we would expect mutational signatures to be more similar between different nodules from the same patient than between patients, even though mutated genes may be shared across patients. To compare the intra- and inter-patient molecular heterogeneity at the levels of CNAs, transcript, and protein FCs, we computed the Pearson correlation between tumor area 1 (TA1) and its paired tumor area 2 (TA2) for each layer and all of the 27 patients with two characterized tumor areas (25 for the mRNA, see the '**Methods**' section and **Additional file 1: Supplementary Text**). As a control, we also computed all pairwise Pearson correlations between the samples within each of the grade groups (*i.e.* inter-patient

correlation). As expected, paired TA1 and TA2 from the same patient were on average more strongly correlated to each other compared to samples from different patients within the same grade group. This finding was consistent for all omics layers (**Fig. 5a**), and was more pronounced at the CNA and mRNA layers compared to the protein layer.

Next, we tested whether a high correlation at the level of CNAs also implies a high correlation at the level of mRNA and proteins. We tested this idea by 'correlating the correlations', *i.e.* we correlated the TA1-TA2 correlation of CNA profiles with the correlation between the mRNA and protein profiles of the same tumor areas (**Fig. 5b**). Indeed, a higher correlation of two tumor areas at the level of CNA correlated significantly with a higher correlation at the level of mRNA (r=0.49, *P* value=0.014). In other words, knowing how similar two tumor areas of a patient are at the CNA level supports a prediction of their similarity at the mRNA level (and conversely). Although the correlation between protein and CNA was not statistically significant, it followed the same trend (r=0.35, *P* value=0.076).

Comparing molecular similarity across omics layers allowed us to identify specific types of patients. The patients H2, H4, M13 had highly correlated tumor areas at all three layers (upper right corner in all scatterplots of **Fig. 5b**). Likely, the tumor areas of these patients have a common clonal origin (**Additional file 1: Fig. S3**). In contrast, patients M12 and M14 had weakly correlated tumor areas at all levels (bottom left corner in all scatterplots of **Fig. 5b**). These tumor nodules either have independent clonal origins or they diverged at an earlier stage during tumor evolution (**Additional file 1: Fig. S3**) [16]. For example, in the case of patient M12 large parts of the genome were not affected by CNAs in the benign sample as well as in TA1 and TA2. However, as shown on **Additional file 1: Fig. S3**, a large region was amplified in TA1, whereas the same region was deleted in TA2. This is consistent with a scenario in which TA1 and TA2 show parallel evolution. A third class of patients is exemplified by the patients M9 and M17, who showed a high correlation between their tumor areas on the CNA and mRNA levels, but not on the protein level. Yet other patterns were apparent in patients M4, M7, and H10. They showed similar mRNA and protein patterns in the two tumor areas, but relatively uncorrelated CNAs. M7 was the patient that we identified earlier with two different CNA signatures both reducing the levels of the same protein (ACP3). The results here apply to global proteome patterns and therefore hint that such convergent network effects of CNAs can be frequent. We confirmed that protein-level similarity correlated with similar histological characteristics of the tumor areas. **Additional file 1: Fig. S9** shows formalin-fixed paraffin-embedded (FFPE) tissue microarray images (duplicates) from the analyzed tumor nodules (TA1 and TA2, diameter 0.6 mm), further underlining the hypothesis that ultimately protein-level alterations are responsible for common cellular phenotypes. Although we cannot fully exclude the

possibility that some of these results were affected by technical noise in the data, our findings suggest that transcript alterations can frequently be buffered at the level of proteins (patients M9, M17, **Additional file 1: Fig. S6**) and that convergent evolutionary processes may lead to the alteration of common proteins (patients M4, M7, H10). We also note that our findings are specific to the two tumor areas available in this study and could be different if other nodules had been sampled for each of the patients. However, our findings on patients with weakly correlated tumor areas at all levels like M12 and M14 suggest that these patients might carry more than one disease [16].

Discussion

Despite twenty years of oncological research involving genome-scale (omics) technologies, we know remarkably little about how the discovered genomic alterations affect the biochemical state of a cell and consequently the disease phenotype. In particular, little is known about how genomic alterations propagate along the axis of gene expression [17, 18]. Here, we have exploited recent technological advances in data acquisition that made it possible to characterize small samples of the same tumor specimens at the level of genomes, transcriptomes, and proteomes and advances in computational strategies towards the network-based integration of multi-omics data.

In our study, samples were generated from small, less than 1 mm diameter punches in immediate spatial proximity in the tumor and subsequently profiled at all three 'omics layers' (DNA, RNA, proteome). Due to the large spatial heterogeneity of PCa [14, 24], this design - which is so far uncommon for studies profiling multiple layers from tumor specimens - was instrumental for increasing the comparability of the various omics layers and thus facilitated the analysis of molecular mechanisms. Our key findings are: (1) we confirmed the importance of CNAs for PCa biology and the alteration of many known PCa-associated genes at the transcript- and protein-level; (2) we revealed a generally elevated molecular alteration of high-grade tumors compared to lower-grade tumors; (3) although our study confirmed large within- and between-patient genomic heterogeneity, (4) we detected molecular networks that were commonly altered at the mRNA and protein-level. The fact that many of those target molecules are known drivers of PCa tumorigenesis, supports the notion that these proteins/transcripts are subject to convergent evolutionary mechanisms.

We integrated the three omics layers using a network-based approach as opposed to directly comparing gene perturbations (mutations) to gene products (transcripts and proteins). Using genome data only, it had previously been hypothesized that whereas the identity of specific mutated genes may differ between tumors, those mutations might still affect common molecular networks [42]. In other

words, tumor phenotypes are determined by the perturbation of molecular networks and not by the perturbation of isolated genes. Our study provides experimental evidence that such network effects are indeed propagated to subsequent molecular layers and that this effect propagation may be clinically relevant. A very prominent example is our discovery that the long-known PCa gene ACPP (ACP3) is downregulated through diverse CNA events, while sometimes even within the same patient different CNA events might downregulate this critical tumor suppressor.

Our multi-omics network analysis revealed that high-grade PCa tumors distinguished themselves from low-grade tumors in two aspects. The first is a generally higher heterogeneity and loss of controlled gene regulation, which increased the molecular differences among high-grade tumors. It had previously been shown that gene expression in tumors is often less coordinated than in normal samples [29]. The increasing heterogeneity of protein concentrations suggests that this loss of coordinated expression also affects protein levels. The second aspect is the convergence of molecular alterations towards specific molecular sub-networks at the genomic, transcriptomic and proteomic layer along the progression from low-grade to high-grade tumors. Thus, although we observed globally a higher degree of variability in gene expression and proteome control among high-grade specimens, a specific subset of the observed alterations appeared crucial for determining the aggressive tumor phenotype. Tumors are under selective pressure acting on the biochemical function of the cells. It is generally believed that proteins are a closer reflection of the functional state of a cell than the mRNA. Here we could show that the fold changes of proteins like RABL3, MFAP4, and SF3B2 were more pronounced and/or uniform across high-grade tumors than either their coding mRNAs or the underlying CNAs.

Specifically, our analysis led to the identification of Network Component 1, a sub-network involved in chromatin remodeling and consisting of genes that were weakly amplified in intermediate-grade (G3) tumor specimens. Signals of individual gene members of this component were virtually indistinguishable from noise in our cohort. However, their consistent alterations across the network region, across molecular layers and the fact that the same genes showed enhanced signals in high-grade specimens, rendered this component highly interesting. The fact that copy number and expression changes of Network Component 1 members were predictive for survival in independent cohorts further supports the potential clinical relevance of this sub-network. Our network-based cross-omics analysis identified nine other network components (**Fig. 4**) successfully capturing several known and potentially new PCa-associated genes. However, neither Network Component 1 nor any of the other network components was uniformly subject to CNAs across all high-grade patients. Instead, we found different network components modified in different patients and these sub-networks were involved in cellular

processes as diverse as actin remodeling, DNA damage response, and metabolic functions, all of which are known contributors to PCa biology. This further underlines the large inter-patient variability of PCa and it demonstrates the diversity of molecular mechanisms leading to histologically similar phenotypes. Future prediction models of PCa including the ISUP grade groups, PSA levels and clinical stage might be improved by exploiting multi-omics network analyses. Detecting aggressive networks alterations in prostate biopsies would help clinicians to advice either active surveillance or active therapy. However, the development of such multi-dimensional biomarkers would require much larger patient cohorts.

Another distinguishing feature of this study was the simultaneous profiling of two different tumor regions in 27 out of the 39 patients. The profiling of multiple tumor regions from the same prostate helped to further highlight the enormous heterogeneity of PCa within patients and provided important insights into PCa evolution. The fact that Network Component 1 was more strongly affected in the paired higher-grade nodules of high-grade patients suggests that at least certain sub-networks are subject to an evolutionary process, that progressively 'moves' protein levels towards a more aggressive state. Generally, and at all molecular layers tested, the two paired tumor areas were more similar to each other compared to two samples from the same grade group but different patients, suggesting common evolutionary origins. Although the two tumor areas seemed to mostly originate from the same clone, this was not always the case. In some patients, different nodules exhibited different molecular patterns at all omics layers, suggesting early evolutionary separation. Thus, for the first time, current diagnostic, expert-level consensus guidelines [28] are supported by detailed proteogenomic data. Our findings support earlier claims that clonality itself might be a prognostic marker with implications for future, more tumor-specific treatment when targeted therapies become available also for PCa [16, 83].

Our study shows that all three molecular layers (genome, transcriptome and proteome) contributed valuable information for understanding the biology of PCa. In particular the DNA layer informed about causal events, clonality, and genomic similarity between tumors. The transcriptome was relevant for understanding the transmission of CNA effects to proteins and served as a surrogate in cases where protein levels remained undetected. The proteome was crucial for revealing protein-level buffering of CNA effects as well as for indicating convergent evolution on functional endpoints. In a routine diagnostic context though, measuring all three layers may not be feasible for the near future due to resource and time limitations. Thus, the identification of improved, routine-usable molecular markers for PCa diagnostics and prognosis remains an open problem [17].

Conclusions

This study uncovered molecular networks with remarkably convergent alterations across tumor sites and patients. In particular, we identified a sub-network consisting of nine genes whose joint activity positively correlated with increasingly aggressive tumor phenotypes. The fact that this sub-network was predictive for survival in independent cohorts further supports its potential clinical relevance. At the same time though, our study also exposed a diversity of network effects: we could not identify a single sub-network that was perturbed in all high-grade tumor regions, let alone the observed distinct intra-patient alterations at all omics layers for some patients. Overall, our study has significantly expanded our understanding of PCa biology and serves as a model for future work aiming to explore network effects of mutations with an integrated multi-omics approach.

Methods

Patients and samples

A total of 39 men with localized PCa who were scheduled for RP were selected from a cohort of 1,200 patients within the ProCOC study and processed at the Department of Pathology and Molecular Pathology, University Hospital Zurich, Switzerland [25]. Each of the selected intermediate- and high-grade patients had two different tumor nodules with different ISUP grade groups. H&E (Hematoxylin and Eosin)-stained fresh frozen tissue sections of 105 selected BPH and tumor regions were evaluated by two experienced pathologists (PJW, NJR) to assign malignancy, tumor stage, and Grade Group according to the International Union Against Cancer (UICC) and WHO/ISUP criteria. This study was approved by the Cantonal Ethics Committee of Zurich (KEK-ZH-No. 2008-0040), the associated methods were carried out in accordance with the approved guidelines, and each patient has signed an informed consent form. Patients were followed up on a regular basis (every three months in the first year and at least annually thereafter) or on an individual basis depending on the disease course in the following years. The RFS was calculated with a biochemical recurrence (BCR) defined as a PSA ≥0.1 ng/ml. Patients were censored if lost to follow-up or event-free at their most recent clinic visit. Patients with a postoperative PSA persistence or without distinct follow-up data for the endpoint BCR were excluded from the analysis of BCR.

Exome sequencing and somatic variant analysis

The exome sequencing (exome-seq) was performed using the Agilent Sure Select Exome platform for library construction and Illumina HiSeq 2500 for sequencing read generation. We mapped

and processed the reads using a pipeline based on bowtie2 [84] (1.1.1) and the Genome Analysis Tools Kit (GATK) [85] (3.2-2). We detected and reported nonsynonymous variants or variants causing splicing changes using Strelka (1.0.14) and Mutect (1.1.7) combined with post-processing by the CLC Genomics Workbench (8.0.3). In this process, all tissue samples of a patient were compared to the respective blood sample.

Trimmomatic [86] (0.36) was used for adaptor clipping and low-quality subsequence trimming of the FASTQ files. Subsequently, single reads were aligned to the hg19 reference genome with bowtie2 with options "--very-sensitive -k 20". We applied samtools [87] (0.1.19) and picard-tools (1.119) to sort the resulting bam files in coordinate order, merge different lanes, filter out all non-primary alignments, and remove PCR duplicates. Quality of the runs was checked using a combination of BEDtools [88] (2.21), samtools, R (3.1) and FastQC (0.11.2).

Bam files containing the mapped reads were preprocessed in the following way: indel information was used to realign individual reads using the RealignerTargetCreator and IndelRealigner option of the GATK. Mate-pair information between mates was verified and fixed using Picard tools and single bases were recalibrated using GATK's BaseRecalibrator. After preprocessing, variant calling was carried out by comparing benign or tumor prostate tissue samples with matched blood samples using the programs MuTect [89] and Strelka [90] independently. Somatic variants that were only detected by one of the two programs were filtered out using CLC Genomics Workbench. So were those that had an entry in the dbSNP [91] common database and those that represented synonymous variants without predicted effects on splicing.

CNA analysis of exome-seq data

The Bam files generated during the process of somatic variant calling were processed with the CopywriteR package (v.2.2.0) for the R software [92]. CopywriteR makes use of so-called "off-target" reads, *i.e.* reads that cover areas outside of the exon amplicons. "Off-target" reads are produced due to inefficient enrichment strategies. In our case on average 28.5% of the total reads were not on target. Briefly, CopywriteR removes low quality and anomalous read pairs, then peaks are called in the respective blood reference, and all reads in this region are discarded. After mapping the reads into bins, those peak regions, in which reads had been removed, were compensated for. Additionally, read counts are corrected based on mappability and GC-content. Finally, a circular binary segmentation is carried out and for each segment the log count ratios between tissue samples and the respective blood sample are reported as copy number gain or loss. The copy number of each gene in each sample was reported

based on the log count ratio of the respective segment in which the gene was located. The overall performance of this CNA-calling approach was evaluated by comparing the results of the TA1 (and TA) samples with CNA results obtained by applying the OncoScan Microarray pipeline to FFPE samples from the same tumors (**Additional file 1: Fig. S10**).

OncoScan Microarrays

OncoScan copy number assays were carried out and analyzed as described previously [93]. Briefly, DNA was extracted from punches of FFPE cancer tissue blocks. Locus-specific molecular inversion probes were hybridized to complementary DNA and gaps were filled in a nucleotide-specific manner. After amplification and cleavage of the probes, the probes were hybridized to the OncoScan assay arrays. Scanning the fluorescence intensity and subsequent data processing using the Affymetrix® GeneChip® Command Console and BioDiscovery Nexus express resulted in log intensity ratio data (sample versus Affymetrix reference) and virtual segmentation of the genome into areas with copy number gain, loss or stability.

RNA Sequencing

RNA sequencing was performed at the Functional Genomics Center Zurich. RNA-seq libraries were generated using the TruSeq RNA stranded kit with PolyA enrichment (Illumina, San Diego, CA, USA). Libraries were sequenced with 2x126bp paired-end on an Illumina HiSeq 2500 with an average of 105.2 mio reads per sample.

Paired-end reads were mapped to the human reference genome (GRCh37) using the STAR aligner (version 2.4.2a) [94]. Quality control of the resulting bam files using QoRTs [95] and mRIN [96] showed strong RNA degradation[97] in a significant fraction of the samples: mRIN classified 31 samples as highly degraded (**Additional file 1: Fig. S11**, **Additional file 5: Table S4**). In order to correct for this 3' bias, 3 tag counting was performed as described by Sigurgeirsson et al [98] using a tag length of 1,000. After 3' bias correction, three samples still showed a clear 3' bias: the two tumor regions (TA1 and TA2) of the patient M5 and TA2 from patient M8 (**Additional file 1: Fig. S11**). These samples were excluded from subsequent analyses. Additionally, the BPH region of the patient M5 was excluded due to the exclusion of both its tumor regions.

FeatureCounts [99] was used to determine read counts for all genes annotated in ENSEMBL v75. Genes for which no read was observed in any of the samples in the original data were excluded from the analysis. Further, after 3 tag counting, all genes with without at least 1 read per million in N of the

587 samples were removed. We chose N to be 10 which corresponds to the size of the smallest grade group
588 (G2). In a last reduction step, all genes with more than one transcript were excluded, yielding a final set
589 of 14,281 genes.

590 Read count normalization and differential gene expression analysis was performed using the R
591 packages sva [100] and DESeq2 [101]. All benign tissues were considered biological replicates and
592 differential gene expression for the individual tumor samples was determined against all benign tissues.
593 Gene expression changes with an adjusted *P* value < 0.1 were considered significant.

594 RNA-seq - 3' bias correction

595 The 3 tag counting approach for 3' bias correction was used on the RNA-seq dataset [98]. This
596 approach requires changing of the annotation file in two steps: 1) isoform filtering and 2) transcript
597 length restriction. As proposed in [98] for each gene we determined the highest expressed isoform
598 within a set of high quality samples. As high quality samples we used all samples with an mRIN score
599 greater than or equal to 0.02. This set contains 7 benign and 15 tumor samples. Isoform expression was
600 determined using cufflinks [102]. As transcript length we chose 1,000bp.

601 Gene fusions

602 FusionCatcher (version 0.99.5a beta) was used to determine gene fusions for all samples.
603 Fusions classified as "probably false positive" are discarded unless they are also classified as "known
604 fusion".

605 PCT assisted sample preparation for SWATH-MS

606 We first washed each tissue sample to remove O.C.T., followed by PCT-assisted tissue lysis and
607 protein digestion, and SWATH-MS analysis, as described previously [23]. Briefly, a series of ethanol
608 solutions were used to wash the tissues each tissue, including 70% ethanol / 30% water (30 s), water (30
609 s), 70% ethanol / 30% water (5 min, twice), 85% ethanol / 15% water (5 min, twice), and 100% ethanol (5
610 min, twice). Subsequently, the tissue punches were lysed in PCT-MicroTubes with PCT-MicroPestle [103]
611 with 30 µl lysis buffer containing 8 M urea, 0.1 M ammonium bicarbonate, Complete protease inhibitor
612 cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche) using a barocycler (model
613 NEP2320-45k, PressureBioSciences, South Easton, MA). The lysis was performed with 60 cycles of high
614 pressure (45,000 p.s.i., 50 s per cycle) and ambient pressure (14.7 p.s.i., 10 s per cycle). The extracted
615 proteins were then reduced and alkylated prior to lys-C and trypsin-mediated proteolysis under pressure

cycling. Lys-C (Wako; enzyme-to-substrate ratio, 1:40) -mediated proteolysis was performed using 45 cycles of pressure alternation (20,000 p.s.i. for 50 s per cycle and 14.7 p.s.i. for 10 s per cycle), followed by trypsin (Promega; enzyme-to-substrate ratio, 1:20)-mediated proteolysis using the same cycling scheme for 90 cycles. The resultant peptides were cleaned using SEP-PAC C18 (Waters Corp., Milford, MA) and analyzed, after spike-in 10% iRT peptides [51], using SWATH-MS following the 32-fixed-size-window scheme as described previously [19, 21] using a 5600 TripleTOF mass spectrometer (Sciex) and a 1D+ Nano LC system (Eksigent, Dublin, CA). The LC gradient was formulated with buffer A (2% acetonitrile and 0.1% formic acid in HPLC water) and buffer B (2% water and 0.1% formic acid in acetonitrile) through an analytical column (75 µm × 20 cm) and a fused silica PicoTip emitter (New Objective, Woburn, MA, USA) with 3-µm 200 Å Magic C18 AQ resin (Michrom BioResources, Auburn, CA, USA). Peptide samples were separated with a linear gradient of 2% to 35% buffer B over 120 min at a flow rate of 0.3 µl min^{-1}. Ion accumulation time for MS1 and MS2 was set at 100 ms, leading to a total cycle time of 3.3 s.

SWATH assay query library for prostate tissue proteome

To build a comprehensive library for SWATH data analysis, we analyzed unfractionated prostate tissue digests prepared by the PCT method using Data Dependent Acquisition (DDA) mode in a tripleTOF mass spectrometer over a gradient of 2 hours as described previously [19]. We spiked iRT peptides [51] into each sample to enable retention time calibration among different samples. We then combined these data with the DDA files from the pan-human library project [104]. All together we analyzed 422 DDA files using X!Tandem [52] and OMSSA [53] against three protein sequence databases downloaded on Oct 21, 2016 from UniProt, including the SwissProt database of curated protein sequences (n=20,160), the splicing variant database (n=21,970), and the trembl database (n=135,369). Using each database, we built target-decoy protein sequence database by reversing the target protein sequences. We allowed maximal two missed cleavages for fully tryptic peptides, and 50 p.p.m. for peptide precursor mass error, and 0.1 Da for peptide fragment mass error. Static modification included carbamidomethyl at cysteine, while variable modification included oxidation at methionine. Search results from X!Tandem and OMSSA were further analyzed through Trans-Proteomic Pipeline (TPP, version 4.6.0) [54] using PeptideProphet and iProphet, followed by SWATH assay library building procedures as detailed previously [19, 55]. Altogether, we identified 167,402 peptide precursors, from which we selected the proteins detected in prostate tissue samples, and built a sample-specific library. SWATH wiff files were converted into mzXML files using ProteoWizard [56] msconvert v.3.0.3316, and then mzML files using OpenMS [57] tool FileConverter.

OpenSWATH[105] was performed using the tool OpenSWATHWorkflow with input files including the mzXML file, the TraML library file, and TraML file for iRT peptides.

Peptide quantification using OpenSWATH

To obtain consistent quantification of the SWATH files, we obtained the all annotated *b* and *y* fragments from the sp, sv and tr libraries. About ten thousand redundant and low-quality assays were removed. Then we extracted the chromatography of these fragments and MS1 signals using OpenSWATHWorkflow, followed by curation using DIA-expert[106]. Briefly, the chromatography of all fragments and MS1 signals were subject to scrutiny by empirically developed expert rules. A reference sample with best q value by pyprophet was picked up to refined fragments. The peptide precursors are further filtered based on the following criteria: i) remove peptide precursors with a q value higher than 1.7783e-06 to achieve a false discovery rate of 0.00977 at peptide level using SWATH2stats [107]; ii) peptides with a FC higher than 2 between the reference sample and its technical replicate were removed; iii) peptides matching to multiple SwissProt protein sequences were removed. The data matrix was first quantile normalized, $\log_2$ transformed, followed by batch correction using the ComBat R package [108]. Finally, for each protein and pair of technical replicates the average value was computed.

Statistical analysis

All plots were produced with R. Kaplan-Meier estimators were used for RFS analysis. Differences between survival estimates were evaluated by the log-rank test.

Computation of molecular perturbation scores

On the genomic level (mutation and CNA), we kept the tumor samples (66 in total) that contain FCs with respect to the blood. The mutation matrix was further discretized by setting all non-zero events to 1. At the transcriptomics level, the FCs for the 63 tumor samples were computed as described above (see 'RNA Sequencing'). Finally, on the proteomics level, we computed the FCs for the tumor samples (66 in total) as follows: for each protein, its mean intensity over the normal samples was subtracted from the intensities of the tumor samples. (We chose to compute the FCs for the tumor samples with respect to a global reference (average of all normal samples) and not with respect to their paired benign sample in order to achieve a higher consistency with the transcriptomics level.)

We assigned to each sample two molecular perturbation scores summarizing/quantifying the magnitude of its FCs: DE_count counts the number of mutated/differentially expressed (DE) genes, while

the DE_sum score is the sum of absolute FCs of all genes. Thus, while the first score counts the number of events (mutations/DE genes), the second one quantifies their magnitude. A gene is regarded as mutated/DE if its value is 1 in the mutation layer and if its absolute value is above a threshold that has been set to 1 for the mRNA and protein layer. For the CNA layer, the corresponding threshold was set to 0.5 because the range of FCs in the CNA matrix is smaller than the mRNA and protein matrices. Both types of scores were computed for each molecular level, except for the point mutations where only DE_count was computed. Afterwards, the 66 DE_count scores (63 for the mRNA) and the DE_sum scores at each layer were divided into the four grade groups G1, G2, G3 and G4/5 respectively.

Network propagation/smoothing

As a network, the STRING gene interaction network (version 10)[48] was used, after removing all edges with combined score smaller or equal to 900 and keeping subsequently the largest connected component. The resulting network consisted of 10,729 nodes and 118,647 (high-confidence) edges. For the network smoothing, the weight matrix was computed as described in Vanunu et al.[49], but for an unweighted graph and the propagation parameter was set to 0.5. The propagation was iteratively repeated 500 times to ensure convergence of the results. For the mapping from gene symbols to STRING identifiers (**Additional file 7: Table S6**) we used the R/Bioconductor package STRINGdb [109]. The gene symbols with no matching STRING identifier were removed, while for those that mapped to multiple STRING identifiers, the first mapping was kept (default choice in the package). From the multiple gene symbols that mapped to the same STRING identifier, the first mapping was kept. The genes that were not present in the network were removed from the datasets, while those that were present in the network but not in the corresponding dataset were initially filled in with 0's.

Genes with very small, 'smoothed' (absolute) FCs were filtered out as follows: after the network propagation, only network nodes that had protein measurements themselves or at least one direct neighbor (on the filtered STRING network) with protein measurements were considered in the next steps of this analysis. *I.e.* network nodes without measured FCs at the protein layer that had no direct neighbor with measured protein values were removed from the subsequent analyses.

For significance testing, the one-sided Wilcoxon rank sum test comparing the smoothed FCs between the groups G4/5 and G1 was applied to each network node (after filtering) and layer, once for up-regulation and once for down-regulation. The resulting sub-networks (up-regulated and down-regulated) consisted of those genes that were significant (*P* value below 0.05) at all three layers and all of the edges connecting them on the filtered STRING network.

Network Component 1 analysis

For each tumor sample at the CNA layer, a one-sided, one-sample t-test has been applied testing if its average FC over the genes of the Network Component 1 (and in particular those that have been measured at the CNA) is significantly greater than 0. Due to the presence of outliers in some samples, the non-parametric, one-sided Wilcoxon signed-rank test has been applied as well yielding very similar results (data not shown). A result is considered to be significant if the corresponding *P* value is below 0.05. The analysis has been repeated for the mRNA and protein layer.

Independent cohorts validation

For the validation of Network Component 1, we used published datasets of three PCa cohorts: TCGA, MSKCC, and Aarhus. For TCGA and MSKCC, we downloaded the CNA, mRNA with precomputed z-scores per gene, and corresponding clinical data from cBioPortal[110] (https://www.cbioportal.org/). There were 489 samples with $\log_2$CNA data and 493 samples with mRNA profiles in TCGA. In MSKCC, there were 157 primary tumors with CNA data and 131 primary tumors with mRNA data. The clinical endpoint used in TCGA was the progression-free survival time and the disease-free survival in MSKCC. All previous samples had known survival time.

For the Aarhus study (NCBI GEO dataset GSE46602), we downloaded the mRNA matrix and corresponding clinical information as described in Ycart et al [111]. The resulting mRNA matrix consisted of 20,186 genes and 50 samples- 40 PCa samples with known RFS time and 10 benign samples. Once excluding the benign samples, we computed z-scores per gene in order to have comparable values with the other two studies. These 40 PCa samples were also considered in the subsequent survival analysis. CNA data was not available for the Aarhus study.

We reduced all datasets to the nine genes of Network Component 1. In each of the datasets, we computed for each sample an average copy number change (CNA) or an average z-score (mRNA) across the nine genes of Network Component 1 (combined risk score). Subsequently, we used these combined risk scores to split the samples of each dataset into two groups: samples with a combined risk score larger or equal to the median combined risk score of the study were considered as ‘altered’ and the rest as ‘unaltered’. Kaplan-Meier curves were generated for the two groups. Due to the high level of discretized values in MSKCC at the CNA layer, a sample is considered to be ‘altered’ in that dataset if its combined risk score is above zero.

Analysis of regulators and target genes

For this analysis, we used an independently inferred (partially directed) generic transcriptional regulatory network (Leote *et al. in revision*; preprint available on bioRxiv). For each target gene the following procedure was applied: firstly we identified its neighborhood of order two in the transcriptional network by considering incoming edges only, *i.e.* all nodes from which the target gene was reachable in at most two steps (equivalently the node itself, its parents and the parents of parents). These are potential regulators of the target under consideration. Of these nodes, genes with no copy number measurements and genes altered (*i.e.* with $\log_2$ copy number ratio greater than 0.5 in absolute) in fewer than two tumor samples across the 66 tumor samples were filtered out. Subsequently, we fitted an elastic net model with alpha=0.5. We used as output variable the mRNA FC of the target gene and as input variables the CNAs of the regulators after the filtering. The value for the regularization parameter lambda was chosen through 10-fold cross validation (default in the R package glmnet (https://cran.r-project.org/web/packages/glmnet/)). Predictors/regulators with a non-zero beta coefficient were deemed significant. We have used the elastic net model with alpha=0.5 because it is a method giving sparse solutions and can deal with correlated predictors at the same time.

For the validation of our approach, we used the two independent PCa cohorts described above (TCGA and MSKCC) and reduced the samples to those having both CNA and mRNA profile. This resulted in 488 samples for TCGA and 109 samples for MSKCC. Next, for each of the significant regulators/predictors we computed the Spearman correlation between its CNAs and the corresponding mRNA z-scores of the target gene in each of the two independent studies. Finally, for each target gene and each study we counted how many times the sign of the Spearman correlation matched the sign of the Spearman correlation computed for our cohort, *i.e.* there was an agreement regarding the direction of the association (**Additional file 7: Table S6**).

Differences between similarity/distance measures

A mathematical explanation for the observed differences between the similarity/distance measures is provided. Let $\boldsymbol{y}_{\boldsymbol{i}}^{\boldsymbol{G}}$ denote the vector of $\log_2$ fold changes ($\log_2$FCs) corresponding to the *i*-th tumor sample in the grade group *G* ($G \in \{G1, G2, G3, G4/5\}$), *i.e.* the fold changes of protein/mRNA concentration in the respective tumor sample versus the global benign control. Then we can write:

$$\boldsymbol{y_i^G} = \begin{pmatrix} y_{i1}^G \\ y_{i2}^G \\ \vdots \\ y_{iN}^G \end{pmatrix} = \begin{pmatrix} \mu_1^G \\ \mu_2^G \\ \vdots \\ \mu_N^G \end{pmatrix} + \begin{pmatrix} \varepsilon_{i1}^G \\ \varepsilon_{i2}^G \\ \vdots \\ \varepsilon_{iN}^G \end{pmatrix}, \quad (1)$$

where y_{ij}^G corresponds to the $\log_2$FC of protein/mRNA *j* in the respective tumor sample (*i.e. i*-th tumor sample in the grade group *G*), μ_j^G to its (population) mean $\log_2$FC in the grade group *G* and ε_{ij}^G to the deviation of the respective individual $\log_2$FC from the population mean (*j*=1,…*N*), *N* being the total number of proteins/genes. The population mean μ_j^G is estimated by computing the average $\log_2$FC across all tumor samples in that grade group for the protein/mRNA *j*. Equivalently, (1) can be re-written:

$$\begin{pmatrix} y_{i1}^G \\ y_{i2}^G \\ \vdots \\ y_{iN}^G \end{pmatrix} - \begin{pmatrix} \mu_1^G \\ \mu_2^G \\ \vdots \\ \mu_N^G \end{pmatrix} = \begin{pmatrix} \varepsilon_{i1}^G \\ \varepsilon_{i2}^G \\ \vdots \\ \varepsilon_{iN}^G \end{pmatrix}.$$

On the one hand, the Euclidean and Manhattan distance between one tumor sample and the corresponding mean/centroid vector are functions of these error terms ε_{ij}^G's alone and thus only dependent on the variances of the proteins/genes in the corresponding grade group – these are shown to be higher in the high-grade tumors. For the Pearson correlation *r* on the other hand, one can use the equivalent simple linear regression modeling in (1) with the beta coefficient being equal to 1. By standardizing the vector $\boldsymbol{y_i^G}$ and the centroid vector, we have that $r = 1\sqrt{var(centroid\ vector)/var(\boldsymbol{y_i^G})} =$ $\sqrt{var(centroid\ vector)/(var(centroid\ vector) + var(residuals))} =$ $\sqrt{1/(1 + var(centroid\ vector)/var(residuals))}$ where *var* denotes the variance. The Pearson correlation will thus depend both on the variance of the residuals and the variance of the predictor, *i.e.* mean/centroid vector in the corresponding grade group. Both quantities are expected to be higher in the high-grade samples, while our data implies that the variance of the centroids (predictors) dominates over the variance of the residuals.

Mutual information

In order to compute the MI between each individual sample within a group against the matching centroid of the same group, the R package infotheo (http://cran.r-project.org/web/packages/infotheo/index.html) has been used. For the MI, the data needs to be

discrete. For that, we discretize the tumor-to-benign FCs and centroids by setting 1, if the value is above a threshold *thr*, -1, if the value is below -*thr* and 0 else. (*thr* is equal to 1 for the mRNA and proteins and 0.5 for the CNAs.)

Availability of data and materials

Exome and RNA sequencing data were submitted to the Sequence Read Archive (SRA) at NCBI under accession numbers PRJNA577801 (exome-seq) and PRJNA579899 (RNA-seq), respectively. The SWATH proteomics data were deposited in PRIDE. Project accession code is PXD004589. The published datasets of the two PCa cohorts (TCGA and MSKCC) analyzed during the current study can be downloaded from cBioPortal[110] while the third (Aarhus) is available at the NCBI GEO repository under the accession number GSE46602 .

Acknowledgements

Not applicable.

Funding

This work was supported by the SystemsX.ch project PhosphoNet PPM (to R.A. and P.J.W.), the Swiss National Science Foundation (grant no. 3100A0-688 107679 to R.A.), the European Research Council (grant no. ERC-2008-AdG 233226 and ERC-2014-AdG670821 to R.A.), the Foundation for Scientific Research at the University of Zurich (to P.J.W.), the Westlake Startup Grant (to T.G.), Zhejiang Provincial Natural Science Foundation of China (Grant No. LR19C050001 to T.G.), Hangzhou Agriculture and Society Advancement Program (Grant No. 20190101A04 to T.G.). A.B. and K.C. are supported by the German Federal Ministry of Education and Research Grants Sybacol and PhosphoNetPPM.

Authors' contributions

A.B., T.G., P.J.W. and R.A. designed the project. P.J.W., T.G., Q.Z., C.E.F., N.J.R., A.C, D.R, J.H.R., C.F., K.S., C.P., T.H., A.L.M. and C.B. procured the samples and performed the experiments. K.C., T.G., Q.Z., U.W., R.S., N.C.T, K.O., L.C., L.M., M.R.M, M.M and A.B. designed and performed the statistical analyses with critical inputs from C.Y., H.C., Q.Z., Y.Z., M.H. and other authors. K.C., A.B., T.G. and R.A. interpreted the results. K.C., T.G., P.J.W., A.B. and R.A. wrote the manuscript with inputs from all co-authors. A.B., R.A., P.J.W. and T.G. supported and supervised the project.

Ethics declarations

Ethics approval and consent to participate

This study was approved by the Cantonal Ethics Committee of Zurich (KEK-ZH-No. 2008-0040), the associated methods were carried out in accordance with the approved guidelines, and each patient has signed an informed consent form.

Consent for publication

Not applicable.

Competing interests

R.A. holds shares of Biognosys AG, which operates in the field covered by the article. The research groups of R.A. and T.G. are supported by SCIEX, which provides access to prototype instrumentation, and Pressure Biosciences Inc., which provides access to advanced sample preparation instrumentation.

Supplementary information

Additional file 1: Supplementary text and supplementary figures.

Additional file 2: Table S1. Clinicopathological, immunological and other molecular information of the 39 PCa patients. (a) Overall clinicopathological characteristics. (b) Detailed information for each patient. Pat: numeric patient ID; Pat_id: patient ID grouped by the overall grade. L: low grade; M: intermediate grade; H: high grade; Overall_Gleason_GrGp: overall ISUP grade group; pT: tumor stage; pN: nodal status; R: surgical margin status; Age_at_OP: age at operation; PSA_at_Diag: blood PSA level at diagnosis; Time (months): RFS time. A value of 0 corresponds to patients excluded for the reasons explained in the '**Methods**' section (see 'Patients and samples'); Status: status indicator. 1 means recurrence; DX name: tissue region name; ImageName: name of the scanned images; index_tumor_id: patient ID of TA1 (or TA); TA1_GrGp: grade group for TA1; T_GrGp: grade group for TA2.

Additional file 3: Table S2. Exome analysis of the peripheral blood cells and 105 prostate tumor punches in 39 patients. (a) Allele frequencies (AF) of somatic single nucleotide variants (SNVs) that were called by our bioinformatics pipeline. Genes with called SNV are indicated by an AF > 0. A value of 0 indicates that no SNV was found in the respective genes. In our data, no gene was found with more than one called somatic SNV. (b) Number of samples per gene with called somatic SNV. (c) Protein domain analysis using DAVID.

844 **Additional file 4: Table S3. Copy number analysis of 105 PCa samples.** (a) Log_2 ratios indicating the CNA
845 status are shown for all genes in all samples. Values were determined by overlapping gene locations with
846 CNA segments as calculated by CopywriteR. In case more than one segment overlapped with a gene,
847 number was chosen that had the highest absolute value. (b) Genes are shown with log_2 ratios higher
848 than 0.5 or lower than -0.5 in at least one sample.

849 **Additional file 5: Table S4. RNA-seq analysis.** (a) Log_2FCs (relative to all benign samples) for all genes
850 across the tumor samples. (b) mRIN score per sample generated using mRIN (v1.2.0). (c) ETS family gene
851 fusions observed in tumor samples using FusionCatcher: a value of 1 means that the fusion was observed
852 in the respective sample but not its corresponding benign sample, otherwise the value is 0.

853 **Additional file 6: Table S5. Proteomics data of 210 PCa samples with duplicates.** (a) Sample information
854 includes patient ID, clinical diagnosis, sample ID and batch design. (b) Protein matrix of log_2 scaled
855 intensity of 2,371 proteins quantified in 210 PCa samples.

856 **Additional file 7: Table S6. Integration analysis of 66 tumor samples.** (a) L1-norm of the 'centroid
857 vectors' in the three layers (CNA, mRNA and protein) across the four grade groups. (b) Information (i.e.
858 reference linking them to PCa, consistency between observed and reported effect and number of tumor
859 samples with CNAs) for the first 10 highest-scoring proteins (those with largest average absolute FCs
860 across all tumor specimens). (c) For each target gene the regulators with a non-zero beta coefficient
861 from the elastic net model are given. Further, the proportion of the elastic net predictors with the same
862 sign of Spearman correlation in our cohort and the independent study are noted for the two
863 independent PCa cohorts. (d) Consistently up-regulated genes in the high-grade tumors: for each of
864 these genes, there is a significant up-regulation of its FCs after network smoothing in the group G4/5
865 compared to the group G1 in all three layers (CNA, mRNA and protein). (e) Consistently down-regulated
866 genes in the high-grade tumors: for each of these genes, there is a significant down-regulation of its FCs
867 after network smoothing in the group G4/5 compared to the group G1 in all three layers (CNA, mRNA
868 and protein). (f) Chromosome information for the gene members of Network Component 1. (g) Mapping
869 from gene symbols to STRING identifiers.

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Figure legends

Figure 1. Proteogenomics analysis of 105 tissue regions from 39 PCa patients. a Representative immunohistochemistry images of prostate tissues and the selection of BPH and tumorous tissue regions for genome, transcriptome and proteome analysis. **b** Kaplan-Meier curves for our cohort when the patients are stratified by the overall grade (left), the TA1 or TA grade group (middle) and the TA2 or TA grade group (right). Point-wise 95% confidence bands are shown for the whole range of time values.

Figure 2. Molecular perturbation scores for point mutations, CNAs, transcriptome and proteome data. a Distributions of the first type of molecular perturbation scores (DE_count's) for the four grade groups (visualized as violin plots) at the mutation layer (upper left), CNA layer (upper right), mRNA layer (lower left) and protein layer (lower right). Points represent the actual values. The horizontal lines correspond to the median value in each of the four grade groups. **b** Distributions of the second type of molecular perturbation scores (DE_sum's) for the four grade groups (visualized as violin plots) at the CNA layer (upper left), mRNA layer (upper right) and protein layer (lower left). Points represent the actual values. The horizontal lines correspond to the median value in each of the four grade groups. *P* values (in each of the titles) show the significance of the one-sided Wilcoxon rank sum test where the values of G3 and G4/5 are gathered together and compared to the values of G1 and G2 (also gathered together).

Figure 3. **Within-group similarity at the different layers quantified by different similarity measures. a, b** Distributions of the similarity scores between the individual tumor samples and the centroid using the Pearson correlation (**a**) and MI (**b**) for the four grade groups (visualized as violin plots) at the CNA (upper left), mRNA (upper right) and protein (lower left) layers. Points represent the actual values. The horizontal lines correspond to the median value in each of the four grade groups. *P* values from the one-sided Wilcoxon rank sum test comparing G4/5 versus G1: 0.0014 for the CNA, 0.89 for the mRNA and 0.053 for the protein layer with the Pearson correlation, and 0.027 for the CNA, 0.0052 for the mRNA and 0.0081 for the protein layer with the MI. **c** Density plots of the FCs in the four grade groups for three selected proteins among the 20 highest scoring (score: mean of the absolute FCs across all tumor samples) proteins. Vertical lines correspond to the average FC in each of the four grade groups. The selected proteins have more extreme FCs in the high-grade tumors (G3 and G4/5). **d** Heatmap of the CNA matrix reduced to the significant regulators of the target gene ACPP output by the fitted elastic net model (*i.e.* those with a non-zero beta coefficient). The columns are ordered based on the grade group while there is a hierarchical clustering of the rows. The added colorbar depicts the mRNA FCs of the target gene ACPP.

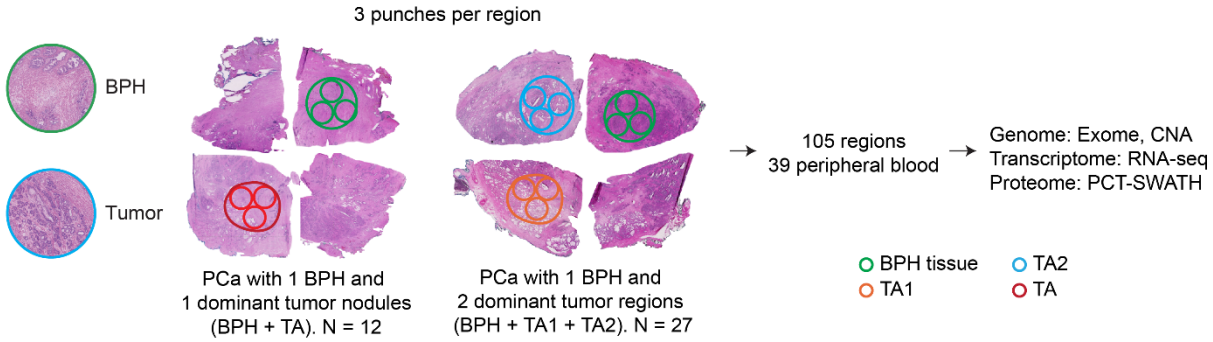
Figure 4. **Consistently dysregulated sub-networks, Network Component 1 heatmaps and validation in three independent cohorts. a** Sub-networks consistently dysregulated in high-grade compared to low-grade tumors. There is a significant up-regulation of the FCs of the depicted genes (colored in red) after network smoothing in the group G4/5 compared to the group G1 in all three layers (CNA, mRNA and protein). **b** Same as in (**a**) but here there is a significant down-regulation of the FCs of the depicted genes (colored in blue) after network smoothing in the group G4/5 compared to G1 in all layers. Functional annotation of the sub-networks in (**a**) and (**b**) with more than one node is given. **c** Heatmap of the CNA matrix reduced to the Network Component 1 genes. The columns are ordered based on the grade group.

The bottom colorbar depicts the effect size of each sample, *i.e.* its average FC across the genes of the Network Component 1. The next colorbar represents the negative logarithm in base 10 of the *P* value from the t-test. The above colorbar shows whether the result is significant: significant results are colored in black and the rest in white. The top colorbar depicts the grade group of each sample. A black box has marks the 'interesting' area with amplification of Network Component 1. Gray rectangles at the bottom show the grade group of the patients (low, intermediate, high) where the samples have (mainly) come from. **d, e** Same as in (**c**) but for the mRNA and the proteins. The samples removed due to degradation (mRNA) are shown in gray. **f** Kaplan-Meier curves for 'altered' and 'unaltered' samples in the three independent studies, TCGA (left), MSKCC (middle) and Aarhus (right) using the corresponding CNA data when available (first row) and mRNA data (second row).

Figure 5. Within-patient similarity at the different layers. a Distributions of the within-group similarities for the four grade groups (visualized as violin plots) based on the Pearson correlation at the CNA layer (upper left), mRNA layer (upper right) and protein layer (lower left). A 'violin' with the correlations between TA1 and paired TA2 for the different patients has been added to all three plots and colored in purple. Points represent the actual values. The horizontal lines correspond to the median value in each of the groups. This analysis is similar to **Fig. 3a** – but it is not identical. *P* values from the one-sided Wilcoxon rank sum test comparing the within-patient to the within-group similarities (where all values from the four groups are gathered together): 8.97e-09 for the CNA, 4.42e-08 for the mRNA and 6.27e-04 for the protein layer. **b** The correlations between TA1 and paired TA2 for the different patients at one layer are plotted against the corresponding correlations at another layer for each pair of layers: mRNA versus CNA (upper left), protein versus CNA (upper right) and protein versus mRNA (bottom left). The points are labeled and colored based on the overall grade in all plots; r: Pearson correlation.

1246 **Figure 1**

a



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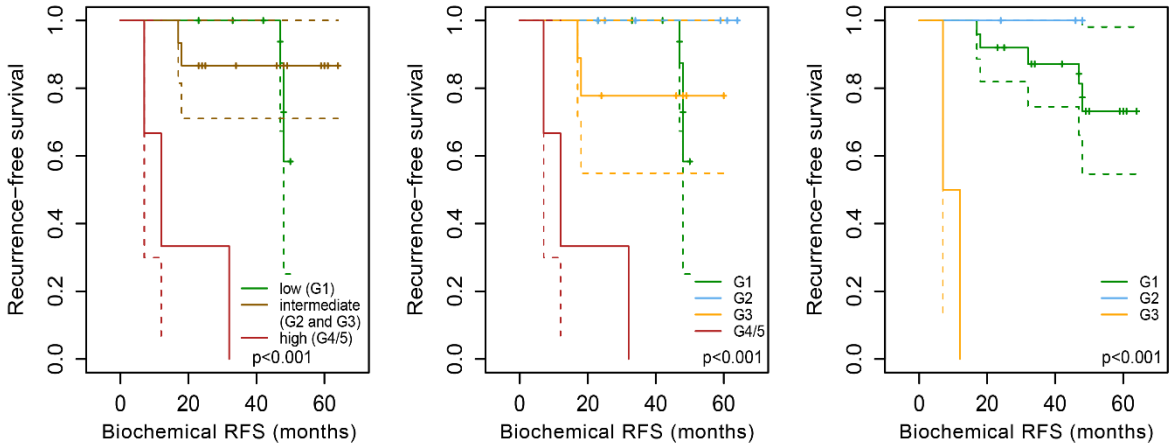

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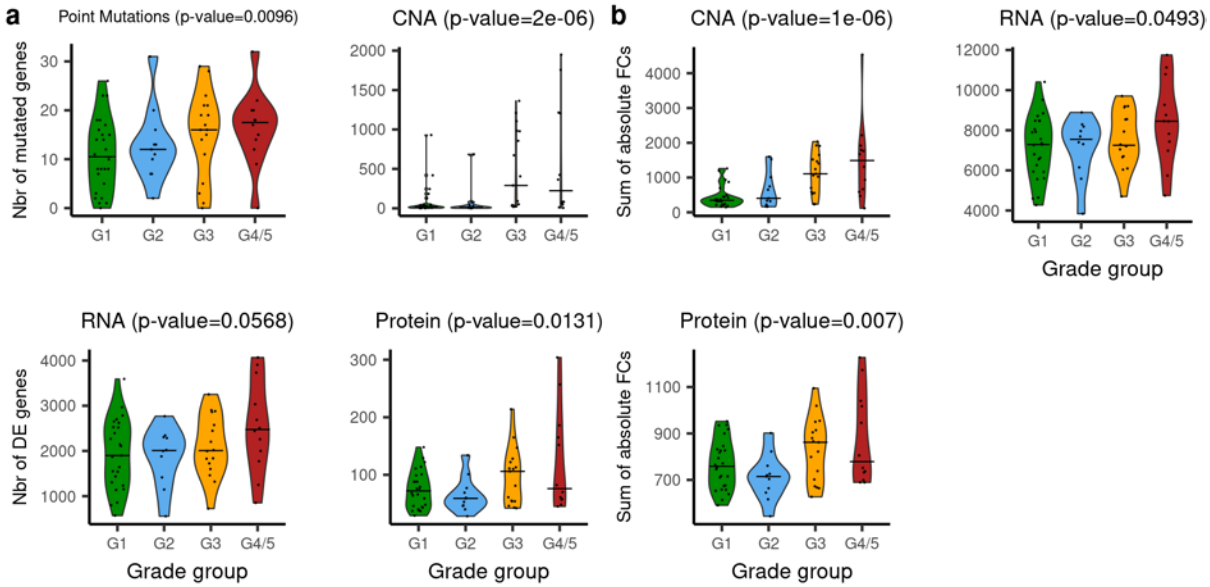
Figure 2



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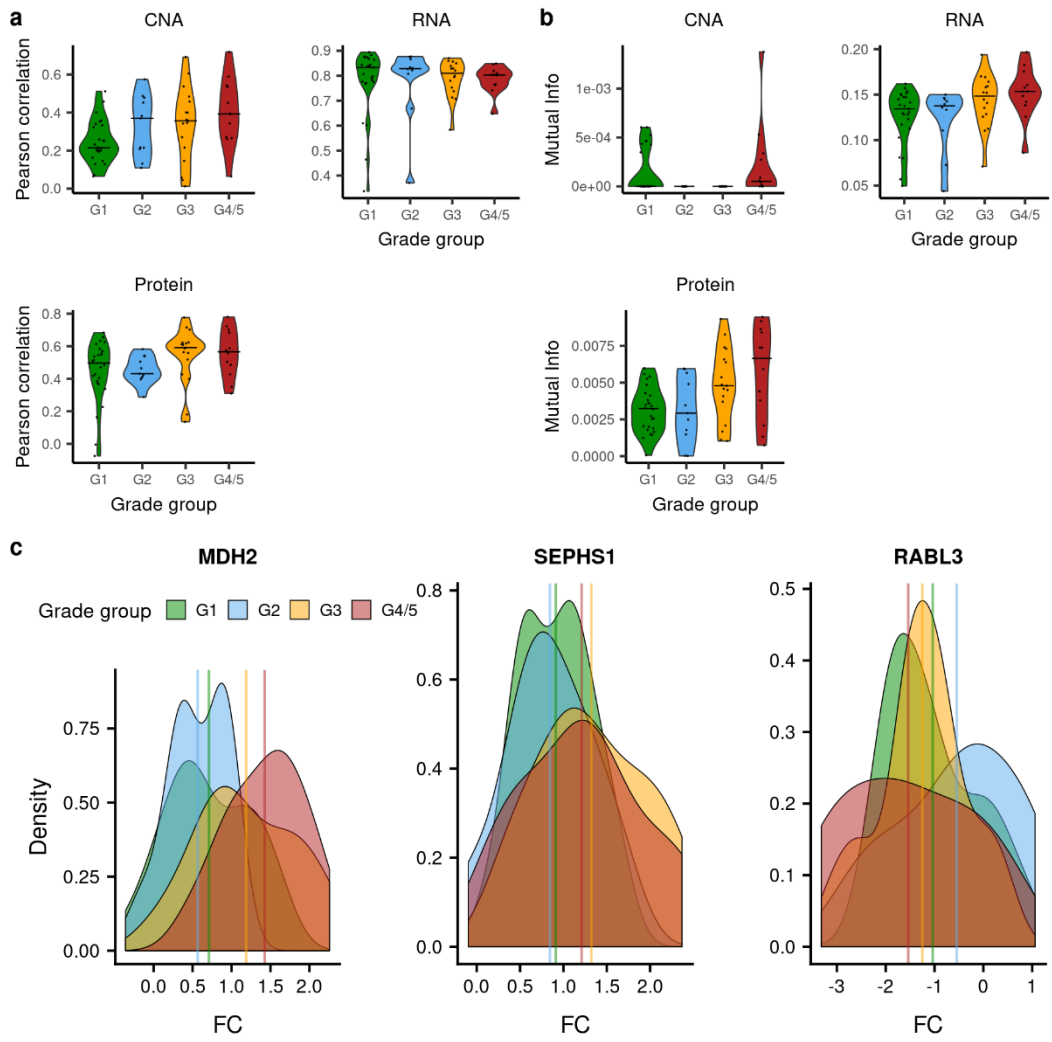


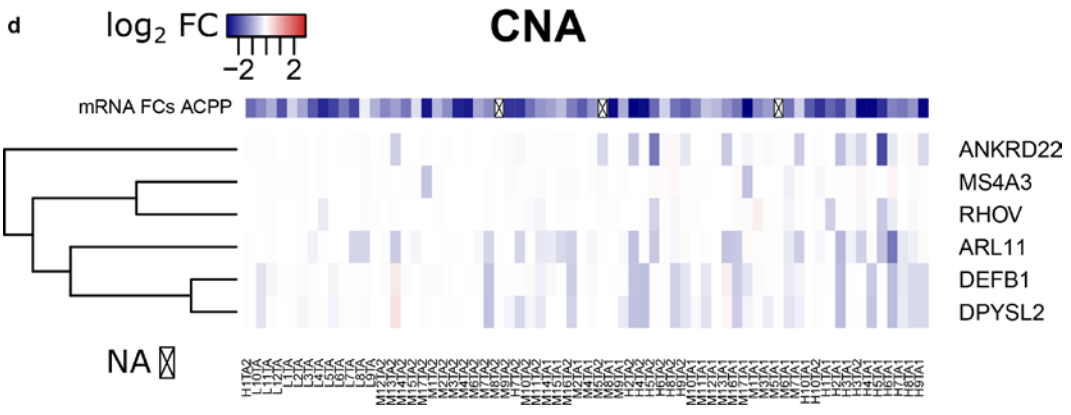
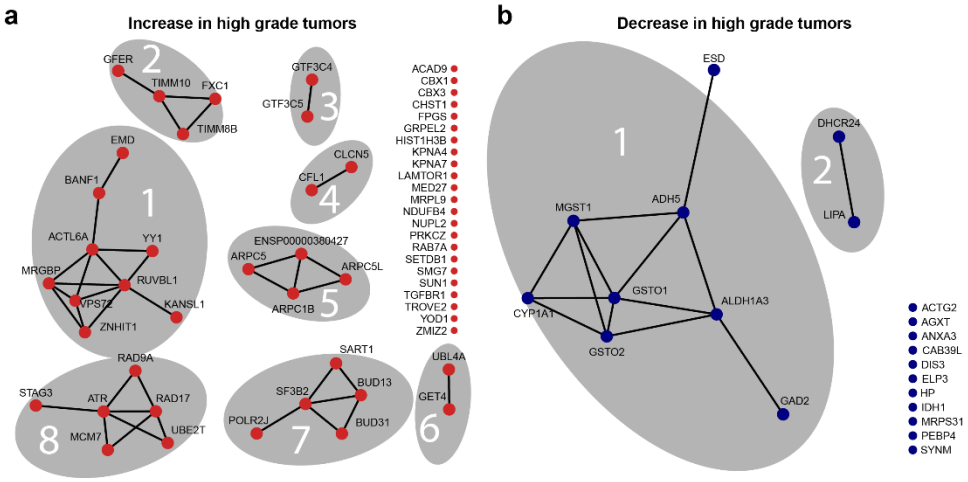


Figure 4



Functional annotation

Up-regulation
1. Chromatin remodelling
2. Mitochondrial import
3. RNA polymerase 3
4. unknown function in prostate
5. Actin related complex, Arp2/3 complex
6. Protein quality control
7. mRNA splicing
8. DNA damage response

Down-regulation
1. Glutathione metabolism
2. Cholesterol biosynthesis

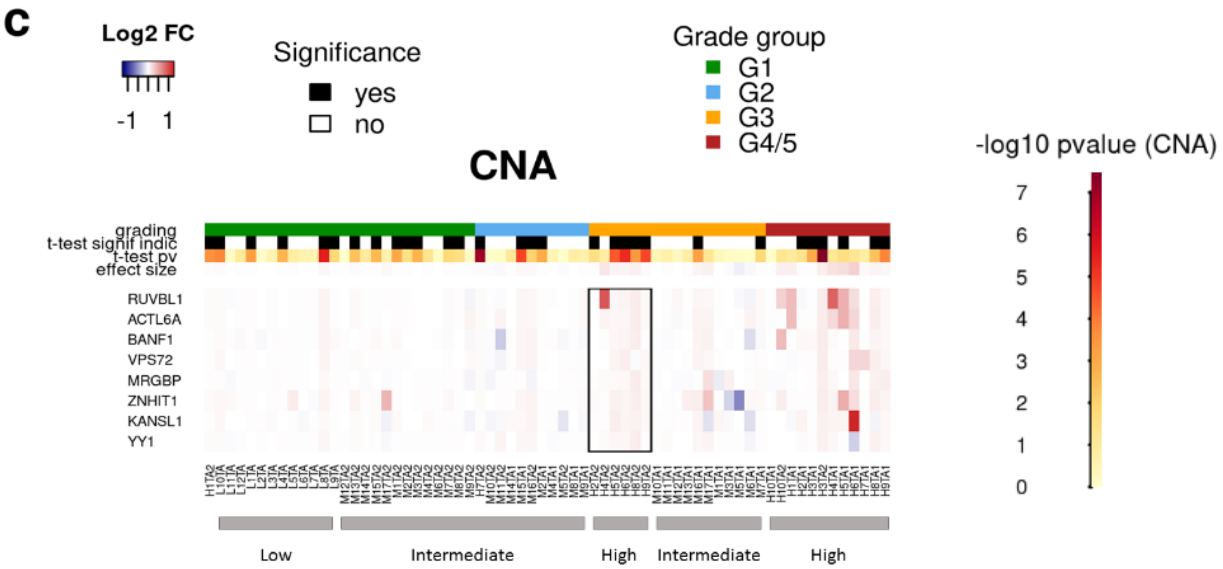
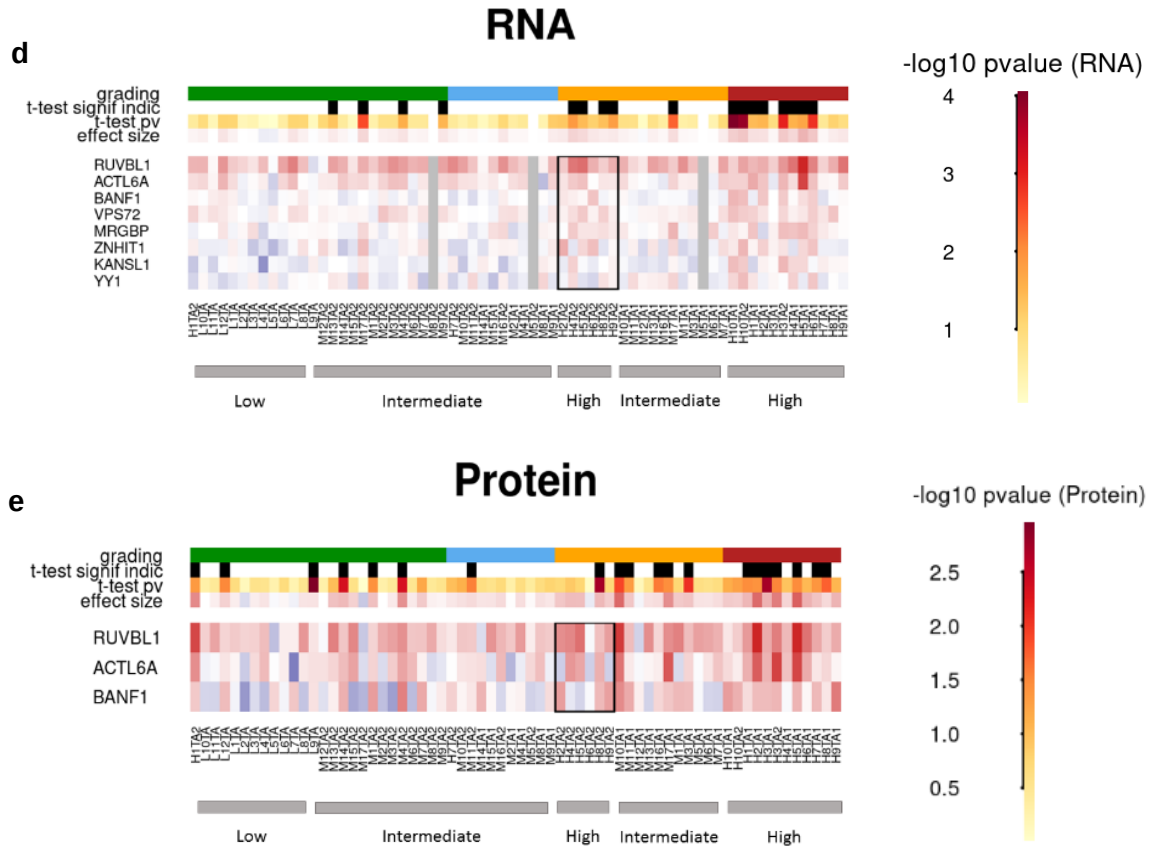


Figure 4 (*continued*)



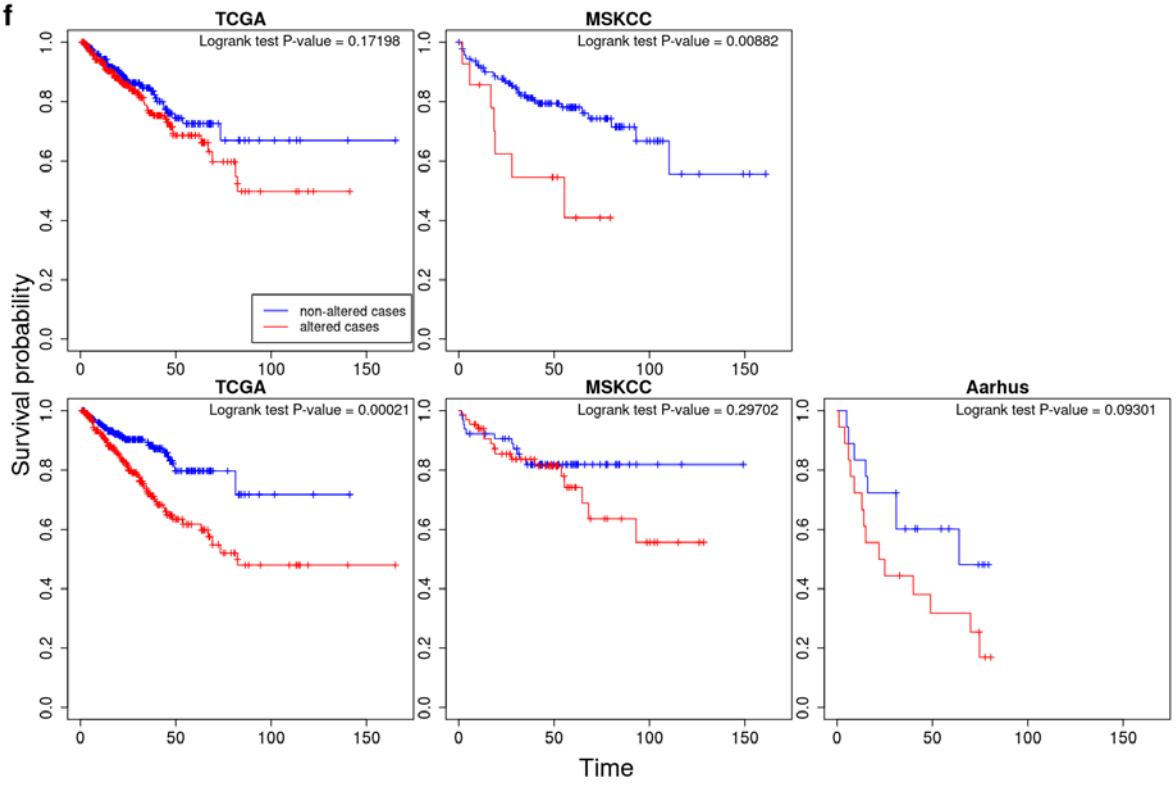


Figure 5