

Article

Multi-proteomic and transcriptomic analysis of oncogenic β -catenin molecular networks

Rob M. Ewing, Jing Song, Giridharan Gokulrangan, Sheldon Bai, Emily Bowler, Rachel Bolton, Paul Skipp, Yihua Wang, and Zhenghe Wang

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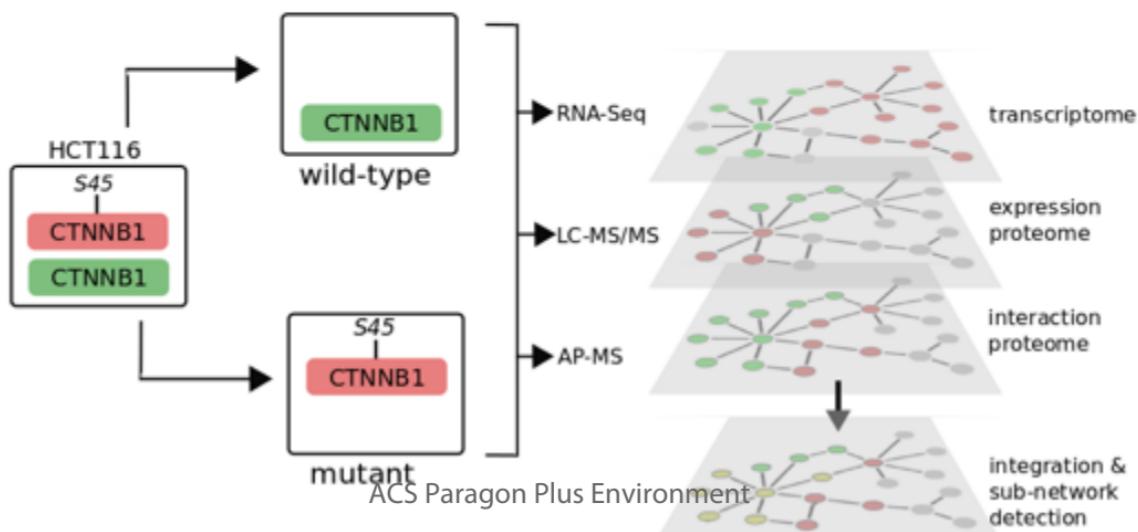
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1 Multi-proteomic and transcriptomic analysis of 2 oncogenic β -catenin molecular networks

3 *Rob M. Ewing¹*, Jing Song², Giridharan Gokulrangan², Sheldon Bai², Emily H. Bowler¹,*
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37 10 ABSTRACT

41 Dys-regulation of Wnt signalling is a frequent occurrence in many different cancers. Oncogenic
42 mutations of CTNNB1/ β -catenin, the key nuclear effector of canonical Wnt signalling, lead to
43 accumulation and stabilization of β -catenin protein with diverse effects in cancer cells.
44 Although the transcriptional response to Wnt/ β -catenin signaling activation has been widely
45 studied, an integrated understanding of the effects of oncogenic β -catenin on molecular
46 networks is lacking. We used Affinity-Purification Mass-Spectrometry (AP-MS), label-free

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3 17 LC-MS/MS and RNA-Seq to compare protein-protein interactions, protein expression and
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5 18 gene-expression in colorectal cancer cells expressing mutant/oncogenic or wild-type β -catenin.
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7 19 We generate an integrated molecular network and use it to identify novel protein modules that
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9 20 are associated with mutant or wild-type β -catenin. We identify a DNA methyltransferase I
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11 21 (DNMT1) associated sub-network that is enriched in cells with mutant β -catenin and a sub-
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13 22 network enriched in wild-type cells associated with the CDKN2A tumor suppressor linking
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15 23 these processes to transformation of colorectal cancer cells through oncogenic β -catenin
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17 24 signaling. In summary, multi-omics analysis of a defined colorectal cancer cell model provides
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19 25 for a significantly more comprehensive identification of functional molecular networks
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21 26 associated with oncogenic β -catenin signaling.
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28 27 **INTRODUCTION**
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30 28 Altered activity of the Wnt/ β -catenin signaling is a key driver of tumorigenesis in many
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32 29 cancers. Stabilizing mutations of β -catenin are an important class of mutations that alter
33
34 30 canonical Wnt signaling and function by blocking phosphorylation of residues that would
35
36 31 normally target the protein for destruction ¹. Substitution or deletion mutations at S45 of β -
37
38 32 catenin are important clinical mutations in diverse tumors, since this residue acts as a critical
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40 33 molecular switch for canonical Wnt signaling ^{1,2}. Elevated β -catenin levels then exert
41
42 34 oncogenic effects through activation of downstream gene-expression programs in concert with
43
44 35 TCF transcription factors ³. In addition to its role as a transcriptional effector, β -catenin
45
46 36 functions as a component of cell-cell adhesion complexes, although the relative balance
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48 37 between β -catenin's different cellular functions is complex ⁴. As expected given its diverse
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50 38 functions and sub-cellular localizations, β -catenin exhibits a wide range of different protein
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3 39 interactions, with other structural proteins in adhesion complexes, proteins in the destruction
4 40 complex⁵, and nuclear interactions with transcription and chromatin modification factors⁶. In
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6 41 addition, transcriptional targets of β -catenin/TCF signalling have been defined in many
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8 42 systems; in cancer cells these include other transcription factors, regulators of the cell-cycle
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10 43 and components and antagonists of the Wnt signaling pathway (Wnt homepage,
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12 44 <http://wnt.stanford.edu>).
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17 46 Omics analyses of Wnt activation to date have focused on understanding a single molecular
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19 47 layer of the response to Wnt activation, such as proteomic analyses of selected Wnt pathway
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21 48 components^{7,8} or the proteomic or transcriptomic expression response to Wnt activation^{9,10}.
22
23 49 However, the response to activation of cell signaling pathways occurs at multiple molecular
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25 50 levels; recent work has shown how activation of the Wnt pathway leads directly to protein
26
27 51 stabilization in addition to the well-studied transcriptional response¹¹. In addition, although it
28
29 52 is convenient to consider proximal events in cell signalling (i.e. components of the pathway
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31 53 itself) separately from the response or output of signalling activation (e.g. transcriptional
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33 54 activation), these are intrinsically linked. Several core protein components of the Wnt signaling
34
35 55 pathway (e.g. Axin, Dkk) are themselves transcriptional targets, directly activated through β -
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37 56 catenin/TCF signaling and providing feedback regulation of Wnt signaling activity^{12,13}
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58 To understand therefore how oncogenic β -catenin alters networks at multiple molecular
59 levels and how this promotes tumorigenesis, we conducted a multi-omics analysis using
60 colorectal cancer cells with targeted inactivation of either the mutant (stabilizing $\Delta 45$ mutation)
61 or wild-type allele of CTNNB1/ β -catenin¹⁴. Affinity-Purification Mass-Spectrometry (AP-
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5 63 consistent with nuclear localization of mutant β -catenin and membrane-associated wild-type
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7 64 β -catenin. Integrating AP-MS and expression proteomic profiling, we identified several
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9 65 enriched protein networks that are preferentially expressed in mutant or wild-type cells
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11 66 including elevated DNA-methylation linked proteins in mutant cells, and a nucleolar-enriched
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13 67 tumor suppressor module in wild-type cells. Through comparative analysis of enriched Gene
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15 68 Ontology categories, we show that there is concerted alteration of pathways and processes at
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17 69 the proteomic and transcriptomic levels in the mutant and wild-type cells. We show that
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19 70 interaction proteomics, expression proteomics and transcriptomic datasets contribute
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21 71 complementary information to the integrated network, and that multi-omics analysis provides
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23 72 for a more comprehensive delineation of β -catenin associated oncogenesis. In summary, our
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25 73 multi-omics analysis provides a comprehensive view of how oncogenic β -catenin alters
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27 74 molecular networks at multiple levels.

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3435 76 **MATERIALS AND METHODS**
3637 77 **Cell line culture and sample extraction**
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40 78 Colorectal cancer cell lines HCT116-CTNNB1 $^{-\Delta 45}$ and HCT116-CTNNB1 $^{WT/-}$ were
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42 79 regularly maintained in McCoy-5A media (Life Technologies, 16600-108, Carlsbad, CA)
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44 80 containing 10% fetal bovine serum (Life Technologies, 10438-026, Carlsbad, CA) and 1%
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46 81 streptomycin-penicillin (Life Technologies, 15140-148, Carlsbad, CA) at 37°C in CO₂
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48 82 incubator (5% CO₂, 100% H₂O). Cells were harvested by scraping the cells off plates and then
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50 83 washed with cold PBS twice for immediate use or storage (-80°C). Harvested cells were lysed
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52 84 (25mM Tris-HCl, pH7.4, 1mM EDTA, 150mM NaCl, 1% NP-40, 50% glycerol, Protease
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3 85 inhibitor cocktail) by homogenization and incubated on ice for 30 min followed by
4 86 centrifugation at 13,000rpm for 30min. The supernatant (soluble fraction) was kept for further
5 87 analysis. Proteins were quantified by Bio-Rad protein assay dye (500-0006, Bio-Rad, Hercules,
6 88 CA) by measuring the absorbance at 595nm. NE-PER Nuclear and Cytoplasmic Extraction kit
7 89 (Pierce) was used to prepare nuclear and cytosolic fractions, which were assessed using anti-
8 90 Dnmt1 and anti-Gapdh Western blots.
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21 92 **SDS-PAGE & Immunoblotting**
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92 **SDS-PAGE & Immunoblotting**

93 Equal amounts (20 µg) of proteins from different samples was loaded on precast 4–12% Bis-
94 Tris gel (Life Technologies NP-0335, Carlsbad, CA) and subjected to electrophoresis. Gels
95 were either stained with Coomassie Brilliant Blue (Pierce 20278, Rockford, IL) or transferred
96 to nitrocellulose membrane (Whatman 10402594, Dassel, Germany). Western blotting was
97 used to detect the protein with super signal ELISA Pico chemiluminescent substrate. Primary
98 antibodies used: anti-β-catenin (Cell Signaling Technology 9581, Danvers, MA), anti-Dnmt1
99 (Cell Signaling Technology 5119, Danvers, MA), anti-UHRF1 (Novus Biologicals
100 H00029128-M01, Littleton, CO), anti-HDAC1 (Abcam ab7028, Cambridge, MA), anti-PCNA
101 (Santa Cruz Biotechnology sc-56, Santa Cruz, CA) and anti-α-tubulin (Cell Signaling
102 Technology, Inc., 2144, Danvers, MA). Loading controls were applied at 1:1000 and secondary
103 antibodies horseradish peroxidase (HRP)-conjugated anti-mouse (Promega W4011, Madison,
104 WI) and HRP-conjugated anti-rabbit (Cell Signaling Technology 7074, Danvers, MA) were
105 added at 1:20,000. Chemi-luminescence detection using SuperSignal* ELISA Pico
106 Chemiluminescent Substrate (Thermo Scientific PI-37070, Rockford, IL) was applied to all
107 westerns.

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3 108 **Proteomic sample preparation**
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5 109 For analysis of the expression proteome, cell extracts were fractionated using the NE-PER
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7 110 Nuclear and Cytoplasmic Extraction kit (Pierce), each fraction separated using SDS-PAGE and
8
9 111 then fractionated in 2 fractions per sample/lane after Coomassie blue staining prior to tryptic
10
11 112 digestion. Each sample combination (e.g. Mutant/nuclear, Mutant/cytosolic) was replicated
12
13 113 twice. Affinity-purifications from 10^7 cells were performed as previously described ¹⁵ using
14
15 114 anti- β -catenin (Cell Signaling Technology 9581, Danvers, MA) antibodies. Affinity-
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17 115 purification experiments were replicated using two independent mutant and two independent
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19 116 wild-type cell lines, and each sample replicated twice. In-gel tryptic digestion was performed
20
21 117 and combined elution fractions were lyophilized in a SpeedVac Concentrator (Thermo Electron
22
23 118 Corporation, Milford, MA), resuspended in 100 μ L of 0.1% formic acid and further cleaned up
24
25 119 by reverse phase chromatography using C18 column (Harvard, Southborough, MA). The final
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27 120 volume was reduced to 10 μ L by vacuum centrifugation and addition of 0.1% formic acid.
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33 121 **Mass-spectrometry**
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35 122 Online reverse phase nanoflow capillary liquid chromatography (nano-LC, Dionex Ultimate
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37 123 3000 series HPLC system) coupled to electrospray injection (ESI) tandem mass spectrometer
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39 124 (Thermo-Finnegan LTQ Orbitrap Velos) was used to separate and analyze tryptic peptides.
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41 125 Peptides were eluted on nano-LC with 90 min gradients (6 to 73% acetonitrile in 0.5% formic
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43 126 acid with a flow rate of 300 nL/min). Data dependent acquisition was performed using Xcalibur
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45 127 software (version2.0.6, Thermo Scientific) in positive ion mode with a resolution of 60 000 at
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47 128 m/z range of 325.0–1800.0, and using 35% normalized collision energy. Up to the five most
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49 129 intensive multiple charged ions were sequentially isolated, fragmented and further analyzed.
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52 130 Raw LC-MS/MS data were processed using Mascot version 2.2.0 (Matrix Science, Boston,
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3 131 MA). The sequence database was searched with a fragment ion mass tolerance of 0.8Da and a
4 132 parent ion tolerance of 15 ppm. The raw data were searched against the human International
5 133 Protein Index database (74,017 protein sequences; version 3.42) with fixed modification
6 134 carbamidomethyl (C) and variable modification oxidation (M), and 1 allowed missed cleavage.
7 135 Peptides were filtered at a significance threshold of $P < 0.05$ (Mascot). Raw mass spectrometry
8 136 chromatograms were processed and analyzed using Xcalibur Qual Browser software (Thermo
9 137 Fisher Scientific Inc. Version 2.0.7). Scaffold (Proteome Software Inc., Portland, OR, USA;
10 138 version 3.00.04) was used to analyze LC-MS/MS-based peptide and protein identifications.
11 139 Peptide identifications were accepted if they could be established at greater than 95.0%
12 140 probability as specified by the Peptide Prophet algorithm ¹⁶. Protein identifications were
13 141 accepted if they could be established at greater than 99.0% probability and contained at least 2
14 142 identified peptides. Proteins that contained similar peptides and could not be differentiated
15 143 based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Protein
16 144 quantitation for the expression proteomics study was performed using ion peak intensity
17 145 measurements in the Rosetta Elucidator software (version 3.3.0.1; Rosetta Inpharmatics LLC,
18 146 Seattle, WA). The PeakTeller algorithm within Rosetta Elucidator was used for peak detection,
19 147 extraction and normalization of peptide and protein abundance. Protein quantitation of AP-MS
20 148 experiments was performed using Scaffold (Proteome Software Inc., Portland, OR, USA;
21 149 version 3.00.04) to compute normalized spectral counts for each protein. Proteins were
22 150 excluded from AP-MS results if frequency across control experiments from HCT116 cells was
23 151 > 0.33 ¹⁵. Mass spectrometry data are available via the PRIDE repository with dataset
24 152 identifiers PXD006053 (Expression proteome) and PXD006051 (Interaction proteome).
25 153 **RNA-Seq Analysis**

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3 154 The quantity of total RNA in each sample was collected using Qubit (Invitrogen) and
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5 155 libraries prepared using Illumina TruSeq Total RNA v2 kit with Ribo Zero Gold for rRNA
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7 156 removal. The Ribo-Zero kit was used to remove ribosomal RNA (rRNA) from 1 μ g of Total
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9 157 RNA using a hybridization/ bead capture procedure that selectively binds rRNA species using
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11 158 biotinylated capture probes. The resulting purified mRNA was used as input for the Illumina
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13 159 TruSeq kit in which libraries are tagged with unique adapter-indexes. Final libraries were
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15 160 validated using the Agilent High Sensitivity DNA kit (Agilent), quantified via Qubit, and
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17 161 diluted and denatured per Illumina's standard protocol. High-throughput sequencing was
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19 162 carried out using the Illumina HiScan SQ instrument, 100 cycle paired-end run, with one
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21 163 sample loaded per lane, yielding on average > 100 million reads per sample. Reads were
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23 164 mapped to human genome hg19 using TopHat2 version 2.1.0¹⁷ with default settings and reads
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25 165 summarized by gene feature using htseq-count. Differential expression analysis was performed
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27 166 and p-values adjusted for fdr were computed with DeSeq. Data are available from GEO,
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29 167 accession: GSE95670.
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38 169 **Functional and network analyses**
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40 170 The Combined Abundance Score as previously described¹⁹ was computed using all
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42 171 significant ($p < 0.05$) proteins from the 3 datasets, providing a single, normalized log fold
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44 172 change value for each protein. (Selected additional protein were included where the p-value
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46 173 was significant at $p < 0.1$, since it was observed for several proteins that they were differentially
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48 174 abundant across more than one dataset – e.g. CUL1). Functional networks were constructed
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50 175 from the Pathway Studio database (Elsevier), version 9.0. Gene/Protein identifiers were
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52 176 imported and networks created by selecting all direct edges between the imported nodes

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3 177 (Physical Interactions, Expression Regulation and Protein Modification relations. Network
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5 178 diagrams were created in Cytoscape (v3.3.0). Edge thickness between two functional groups
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7 179 was calculated by dividing the number of interactions between the groups by the size of the
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9 180 groups (number of genes/proteins) creating a normalized edge weight. Gene Ontology term
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11 181 enrichment was computed in Pathway Studio (Ariadne Genomics). The significantly
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13 182 differential sets of mutant and wild-type genes from the RNA-Seq analysis were analyzed using
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15 183 Enrichr²⁰ and o-POSSUM-3²¹.
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21 185 **Experimental Design and Statistical Rationale**
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23 186 Affinity-Purification Mass-Spectrometry (AP-MS) were performed on two independently
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25 187 derived clones of the HCT116-CTNNB1^{-Δ45} and HCT116-CTNNB1^{WT}-cell-lines (i.e. 4
26
27 188 different cell-lines) and then replicated twice. The use of independent clones allowed us to
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29 189 capture the biological variation in the expression of CTNNB1/β-catenin. We observed that AP-
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31 190 MS proteomics experiments produced very similar results between these clones
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33 191 (Supplementary Figure 1). Expression Proteomics experiments were performed on sub-cellular
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35 192 fractionated mutant and wild-type cell cultures. Each combination of cell-type/sub-cellular
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37 193 fraction (mutant/nuclear, mutant/cytosol, wild-type/nuclear, wild-type/cytosol) was replicated
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39 194 twice, and we found high correlation within these groups (Supplementary Figure 3). RNA-Seq
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41 195 experiments were performed in triplicate (3 mutant, 3 wild-type), yielding significant
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43 196 differentially regulated transcripts at low fdr. For each dataset, the log₂ ratio of mutant/wild-
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45 197 type abundance was computed and Student's T-test was used to compute p-values with
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47 198 adjustment for false discovery rate.
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3 200 **RESULTS**
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8 202 **Experimental overview**
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10 203 The experimental strategy of this study is to use multiple, complementary ‘omics approaches
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12 204 to identify perturbed molecular networks as shown in Figure 1. We used a previously described
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14 205 model derived from HCT116 colorectal cancer cells (heterozygous for stabilizing $\Delta 45$ mutation
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16 206 of β -catenin) in which either the mutant or wild-type allele has been disrupted¹⁴ thus creating
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18 207 two cell-lines expressing either mutant β -catenin (CTNNB1^{-/Δ45}) or wild-type β -catenin
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20 208 (CTNNB1^{WT/-}). To characterize mutant and wild-type β -catenin protein-protein interactions
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22 209 we used anti- β -catenin Affinity-Purification Mass-Spectrometry (LC-MS/MS AP-MS). We
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24 210 also analyzed nuclear and cytosolic fractions to increase overall coverage using label-free
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26 211 protein profiling (LC-MS/MS) to identify differentially abundant proteins in the mutant and
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28 212 wild-type cells (2 replicates of each cell-type/fraction combination – a total of 8 samples).
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30 213 Finally, we used RNA-Seq (Illumina HiSeq) to compare the transcriptomes of mutant and wild-
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32 214 type β -catenin cells. Three replicates of each of mutant and wild-type were analyzed and genes
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34 215 with differential gene-expression profiles identified.
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42 217 **AP-MS analysis identifies distinct mutant and wild-type β -catenin protein interactions**
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44 218 We analyzed the mutant and wild-type β -catenin protein interactions using AP-MS
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46 219 experiments as shown in Figure 2. AP-MS analyses were performed using two distinct clones
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48 220 each for mutant and wild-type cells (a total of 4 replicates of mutant and 4 replicates of wild-
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50 221 type cells), and we observed high correlation of protein abundance in AP-MS analyses between
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52 222 replicates and clones (Supplementary Figure 1). AP-MS experiments yielded 67 proteins
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3 223 differentially associated with mutant or wild-type β -catenin ($p<0.05$), and we observed distinct
4 224 profiles of proteins from the mutant and wild-type AP-MS analyses that are consistent with the
5 225 differential sub-cellular localization of mutant and wild-type β -catenin (Supplementary Table
6 226 1). Figure 2A shows a heatmap of proteins significant proteins identified in AP-MS
7 227 experiments and Figure 2B a volcano plot of \log_2 ratios of mutant and wild-type proteins. We
8 228 found that mutant protein interactions were highly enriched for nuclear proteins and for
9 229 proteins functioning in regulation of gene-expression, whereas wild-type proteins were
10 230 significantly enriched for membrane-associated proteins (see Figure 3). To investigate in more
11 231 detail, we constructed a protein network of all known physical interactions between the
12 232 identified set of proteins. The largest connected component of this network is shown in Figure
13 233 2C with known mutant-enriched (red) and wild-type-enriched (green) β -catenin interaction
14 234 partners identified in the analysis. Higher interconnectivity between pairs of proteins identified
15 235 in the mutant cells was observed than between proteins identified in the wild-type cells (and
16 236 this is not due to differences in the overall connectivity of mutant- and wild-type-enriched
17 237 proteins, as there is no significant difference between the degree distributions of the mutant
18 238 and wild-type proteins: two sample t-test; p -value > 0.3). These findings and the distinct sets
19 239 of enriched functional categories indicate that β -catenin in the mutant and wild-type cells
20 240 functions in distinct protein networks, in concordance with distinct sub-cellular localizations
21 241 of mutant and wild-type β -catenin.
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3 246 with 1085 showing significantly differential expression in mutant cells ($p<0.05$; log-fold-
4 change >2) and 735 showing significantly differential expression in wild-type cells (FPKM
5 distribution plots, Supplementary Figure 2). To increase protein coverage, we performed
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7 248 protein expression profiling in conjunction with sub-cellular fractionation of cell lysates. This
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9 249 analysis yielded 640 proteins identified as significantly differentially expressed ($p<0.05$)
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11 250 between mutant and wild-type cells in either cytosolic or nuclear fractions. We compared the
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13 251 functional trends in the interaction proteome, expression proteome and transcriptome datasets.
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15 252 For each dataset, Gene Ontology (GO) terms significantly ($p<0.05$) enriched in mutant and/or
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17 253 wild-type samples were identified and then compared across the datasets. Overlap of
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19 254 significantly differential genes/proteins between the datasets was limited (57 genes/proteins
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21 255 were identified in more than one dataset from a combined total of 2465 significantly
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23 256 differentially regulated genes or proteins). However, significant numbers of shared enriched
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25 257 GO terms were identified across all 3 datasets. The number of shared GO terms is summarized
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27 258 in Figure 3A, and we observed much greater concordance between mutant-enriched GO terms
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29 259 in datasets and between wild-type-enriched GO terms, indicating a concerted cellular
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31 260 response at proteomic and transcriptomic levels to β -catenin mutation (Figure 3A). Selected
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33 261 significantly enriched GO terms in either mutant or wild-type cells are shown in Figure 3B,
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35 262 and these reflect the findings for the AP-MS dataset, whereby mutant transcriptome and
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37 263 proteome datasets are enriched for nuclear and gene-expression associated functions, whereas
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39 264 the wild-type transcriptome and proteome are enriched for membrane and cytoskeleton
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41 265 associated functions. In addition, comparison of the differentially regulated RNA-Seq gene sets
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43 266 against two curated repositories, TSGene ²² and the Tumor Associated Gene database ²³,
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45 267 showed significant enrichment ($p=0.00182$; Fisher's Exact) of tumor suppressors and
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3 269 oncogenes ($p=0.02979$; Fisher's Exact), indicating that these cancer-relevant functional classes
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5 270 are frequently differentially regulated in the mutant/wild-type β -catenin model.
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10 272 As expected, the GO analysis showed that canonical Wnt signaling was highly enriched in
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12 273 the mutant cells. We therefore analyzed which direct canonical Wnt signaling targets (taken
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14 274 from the Wnt homepage <http://wnt.stanford.edu>), bound by TCF transcription factors were
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16 275 differentially expressed between mutant and wild-type cells (Supplementary Table 2), and
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18 276 found that many of the known Wnt targets are differentially regulated in our data, indicating a
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20 277 substantial direct response to β -catenin/TCF. We noted that two classical targets of canonical
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22 278 Wnt signaling CCND1 (cyclin D1) and MYC (c-myc) were not significantly differential
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24 279 between the mutant and wild-type cells. The same finding was reported in the initial analysis
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26 280 of the same cell-lines, and it was concluded that although these genes have been observed as
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28 281 direct transcriptional targets of β -catenin/TCF in many systems²⁴, they are not physiological
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30 282 targets in these cell-lines¹⁴. We next compared our transcriptome dataset to two previously
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32 283 published CTNNB1 siRNA analyses in colorectal cancer cells^{25,26}. This previous study
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34 284 identified a set of 335 genes for which a consistent positive and negative trend was seen across
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36 285 siRNA experiments in 2 colorectal cancer cell-lines. Comparing our transcriptome dataset to
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38 286 this set showed a significant overlap and trend correlation ($p=0.0245$; Fisher's Exact Test), in
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40 287 particular in the correlation between genes whose expression is repressed in response to
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42 288 CTNNB1 siRNA and genes up-regulated in mutant CTNNB1 cells (Supplementary Table 3),
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44 289 indicating that different β -catenin perturbation models (siRNA, knock-out) have similar
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46 290 transcriptional outcomes.
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3 292 To further understand the transcriptional regulatory programs in the mutant or wild-type cells,
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5 293 we analyzed enriched transcription factor binding sites in the mutant or wild-type gene sets
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7 294 (Figure 3C). The β -catenin binding partner Lef1, a TCF transcription factor is amongst the
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9 295 most highly represented predictions in the mutant cells. We also noted that the mutant and
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11 296 wild-type gene sets exhibited enrichment of different classes of transcription factor (Figure
12
13 297 3C). The wild-type set is highly enriched in zinc-finger transcription factor binding sites (6/10
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15 298 of the top 10 most enriched TFs are of this type). Multiple Kruppel-like factor (KLF)
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17 299 transcription factors are represented in this set, and this class of transcription factor have been
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21 300 shown to function as tumor suppressors in colorectal cancer²⁷⁻²⁹. KLF4 has been shown to
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24 301 interact with Beta-catenin and inhibit Wnt signaling in the colon^{30,31}. TCF3 is also identified
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26 302 as an enriched transcription factor in the wild-type cells. Recent analysis showed that TCF3
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28 303 binds the MYC Wnt-responsive element to inhibit MYC expression by preventing binding of
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31 304 β -catenin/TCF4 at the same promoter element³².
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35 306 **An integrated proteomic and transcriptomic network**
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37 307 To construct an integrated network combining the transcriptome, expression proteome and
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39 308 interaction proteome data, a combined abundance score¹⁹ was computed for each significant
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41 309 node (p-value < 0.05) across the three datasets. All direct relations (physical interaction, protein
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43 310 modification and expression regulation) between the 2623 gene/protein entities in the
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45 311 combined set were used to construct an integrated network using the Pathways Studio database
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47 312 (we use hereafter the terminology ‘edge’ to refer to protein-protein relations and ‘node’ to refer
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49 313 to proteins themselves). To analyze how each of the omics datasets contributes to this
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51 314 integrated network, we computed several network statistics (Figure 4). We observed that the
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3 315 average degree of nodes from each of the three datasets differ within the integrated network
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5 316 (interaction proteome=5.95; expression proteome=7.62; transcriptome=3.98) and we therefore
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7 317 plotted the degree distributions of nodes from each dataset as shown (Figure 4B). Nodes within
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9 318 the transcriptome dataset have a distinctly lower average degree, attributed to the large fraction
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11 319 of genes/proteins from this dataset with few described interactions in the database. The
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13 320 significant enrichment of genes encoding transcription factors present in the significantly
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15 321 differential transcriptome dataset contributes towards this difference since for many of these
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17 322 genes, relatively few interactions have been described. This finding prompted us to investigate
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19 323 whether the types of edges represented in the 3 datasets differed (Figure 4C). We observe
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21 324 substantial differences in edges annotated as 'Binding' in the Pathway Studio database and
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23 325 those annotated as regulating 'Expression', with greater numbers of Binding edges in the
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25 326 interaction and expression proteome datasets and substantially more Expression edges in the
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27 327 transcriptome dataset, indicating the complementarity these different omics datatypes in
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29 328 identifying different types of proteins and edges.
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40 330 **Functional module identification and validation**
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3 338 such as the cytoskeletal protein Vimentin (VIM) are strongly enriched in the mutant cells (VIM
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5 339 was differentially expressed in both the expression proteome and transcriptome datasets). In
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7 340 addition, several proteins with functions in tissue remodelling such as matrix metalloprotease
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9 341 (MMP13) and laminins (LAMB3, LAMC2) which form the basement membrane required for
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11 342 attachment and organization of epithelial cells were identified.

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17 344 Wild-type cells preferentially expressed proteins implicated in non-canonical Wnt signaling.
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19 345 In addition to non-canonical Wnt ligands WNT5A and WNT7A, we found that the Dis-
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21 346 shevelled (Dvl) -interacting proteins, DACT3 and DAAM1 were more abundant in the wild-
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23 347 type cells. DACT3 is a member of a family of proteins known to antagonize canonical Wnt/β-
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25 348 catenin signaling, suggesting that the process of mutant β-catenin-driven oncogenesis involves
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27 349 repression of antagonists of canonical signalling. Whilst most components of TGF-
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29 350 Beta/SMAD and BMP signaling were higher in mutant cells (module 7), we noted that
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31 351 LEMD3, a known antagonist of TGF-Beta/SMAD signaling was significantly higher in wild-
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33 352 type cells.

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40 354 Although integration of transcriptomic and proteomic allowed for increased coverage and
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42 355 representation within functional modules as shown in Figure 5A, we also observed exclusively
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44 356 proteomic modules. Skp-Cullin-Fbox (SCF) protein complexes are ubiquitin ligase complexes
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46 357 that regulate ubiquitination of many proteins including β-catenin, and these were uniformly
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48 358 more abundant in mutant cells (Figure 5B). This module was almost uniformly significantly
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50 359 differentially regulated in the proteomic datasets, but not in the transcriptomic dataset,

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3 360 indicating, in concordance with other findings, that these complexes are mainly regulated at
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5 361 the post-translational level, through dynamic re-arrangement of protein components³⁴.
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11 363 We selected two modules for further validation (Figures 5C and 5D). The primary maintenance
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13 364 DNA methyltransferase (DNMT1) is significantly more abundant in the expression proteome
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15 365 and transcriptome of mutant cells. We analyzed the expression of Dnmt1 and two direct
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17 366 interaction partners of Dnmt1, USP7/HAUSP and UHRF1 and all of these proteins were found
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19 367 to be both nuclear specific and enriched in mutant cells (Figure 5C). We previously showed
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21 368 that an interaction between β -catenin and the primary DNA methyltransferase, Dnmt1
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23 369 stabilizes both proteins in the nucleus of cancer cells³⁵. We previously showed that USP7
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25 370 regulates the stability of Dnmt1 in cancer cells³⁶, and UHRF1 has been shown to also
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27 371 participate in the regulation of Dnmt1 stability via ubiquitination³⁷. These latest results indicate
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29 372 the coordinated up-regulation of Dnmt1-USP7-UHRF1 complexes in mutant cells, linking β -
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31 373 catenin-driven oncogenesis to altered DNA methytransferase activity.
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40 376 We also noted that one of the most enriched categories in the gene enrichment analysis for WT
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42 377 cells were proteins annotated as nucleolar (WT expression proteome dataset, p-value=4x10⁻¹¹),
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44 378 and with the related functional annotations of rRNA processing and ribosome biogenesis. We
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46 379 found that many of these proteins formed a highly-connected module within the larger
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48 380 integrated network (Figure 5D). Western analysis was used to validate the expression of several
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50 381 proteins that were either significantly differentially abundant in the omics datasets (shaded
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52 382 green) or predicted based upon their connections to other proteins in the module (shaded gray).
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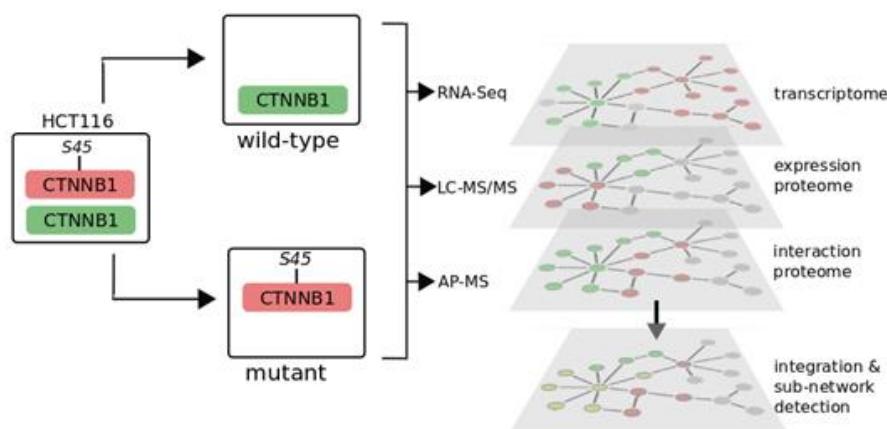
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3 383 In addition to their role in ribosome function, several of these proteins have known tumor-
4 suppressor functions. The well-characterized tumor suppressor CDKN2A (P19ARF) is a
5 prominent member of this module and functions to regulate the levels of p53 through its
6 sequestration of MDM2 (a negative regulator of p53)³⁸ in the nucleolus (MDM2 was not
7 identified in the proteomic experiments, and not significantly differentially expressed in the
8 transcriptomic experiments). Another protein, nucleostemin (GNL3) that has also been linked
9 to MDM2-p53 regulation³⁹ was differentially more abundant in wild-type cells. We previously
10 identified GNL3 as an interaction partner of LYAR⁴⁰, and therefore analyzed the expression
11 of this and several other known nucleolar proteins linked to LYAR as shown in Figure 4D,
12 showing their greater abundance in wild-type cells.
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23 397 **DISCUSSION**
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38 398 In this study, we performed the first multi-proteomic and transcriptomic analysis of the
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40 molecular response to stabilization of β -catenin in colorectal cancer cells. We used a cell model
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42 of oncogenic β -catenin activity to compare cells expressing a pathogenically and clinically
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44 important β -catenin mutation that stabilizes the protein with cells expressing wild-type β -
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46 catenin. Global analysis of functional trends showed that mutant cells and mutant β -catenin
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48 interactions were enriched in mutant cells in line with the known importance of nuclear
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50 accumulation of β -catenin for its pathogenic activity. This is in line with the findings presented
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52 in the original publication describing these cells showing that β -catenin in the mutant cells was
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3 406 more abundant in the nucleus, and bound less to E-cadherin than β -catenin in the wild-type
4 407 cells, even though the overall abundance of β -catenin in the two cell-lines was similar ⁴
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7 409 Using integrated proteomic and transcriptomic analyses allowed us to reveal novel functional
8 410 modules associated with β -catenin-driven oncogenesis. Significantly differential expression of
9 411 multiple Wnt ligand genes was observed between mutant and wild-type cells. WNT2, WNT5A
10 412 and WNT7A are significantly higher in the wild-type cells whereas WNT16 is higher in mutant
11 413 cells. Wnt5a is the best studied ligand of this group and is associated with β -catenin-
12 414 independent or non-canonical Wnt signalling ⁴¹. Interestingly, WNT5A can antagonize β -
13 415 catenin signalling ⁴², exhibits tumor suppressive activity in colorectal cancer ⁴³ and is associated
14 416 with sub-groups of colorectal cancer patients with good prognosis ⁴⁴, although WNT5A's
15 417 tumor suppressor properties appear to be limited to certain tumor types ⁴¹. We also showed
16 418 that the expression of DNA methyltransferase I (Dnmt1) and several key Dnmt1 interaction
17 419 partners are significantly elevated in mutant β -catenin cells, consistent with our previous report
18 420 that β -catenin and Dnmt1 proteins engage in a mutually stabilizing interaction in the nuclei of
19 421 cancer cells ³⁵. In addition, USP7 which regulates the stability of Dnmt1 has also recently been
20 422 shown to stabilize β -catenin in colorectal cancer cells expressing APC mutations ⁴⁵, further
21 423 linking the regulation of Dnmt1 to β -catenin-driven oncogenesis. Conversely, we identified a
22 424 module of nucleolar-enriched proteins that were significantly more abundant in wild-type β -
23 425 catenin cells, including the tumor suppressor CDKN2A. Expression of CDKN2A is frequently
24 426 silenced in colorectal and other tumors through promoter hyper-methylation ⁴⁶, suggesting that
25 427 alterations of CpG methylation may be induced via oncogenic β -catenin and the greater

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3 428 abundance of DNMT1 and its associated regulators that we observed in cells with mutant β -
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5 429 catenin.
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10 431 Our study showed how a multi-omics approach combining different layers of proteomic and
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12 432 transcriptomic information can reveal more comprehensively how oncoproteins transform
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14 433 molecular networks in cancer cells. Recent studies have shown that in addition to mediation of
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16 434 a transcriptional response, activation of canonical Wnt signaling also acts in-dependently of
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18 435 transcriptional programs to alter protein stabilization⁴⁷, necessitating the characterization of
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20 436 oncogenic-mediated effects at proteomic as well as transcriptomic levels. We have adopted the
21
22 437 approach of integrating multi-omics data with existing network information⁴⁸ to identify
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24 438 modules within the cellular network that may be perturbed across the multiple layers of
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26 439 transcriptome, expression proteome or interaction proteome. We observed concerted cellular
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28 440 responses in terms of pathways and processes across these multiple layers. We also showed
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30 441 that these different ‘layers’ of information contribute differentially to the overall analysis of β -
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32 442 catenin-driven oncogenesis – by for example contributing different types of protein-protein
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34 443 relationship (edges) and identifying proteins with differing network features. In summary, our
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36 444 study reveals both novel biology associated with β -catenin-driven oncogenesis and also
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38 445 illustrates the greater insight that can be gained from applying a systematic multi-omics
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40 446 approach.
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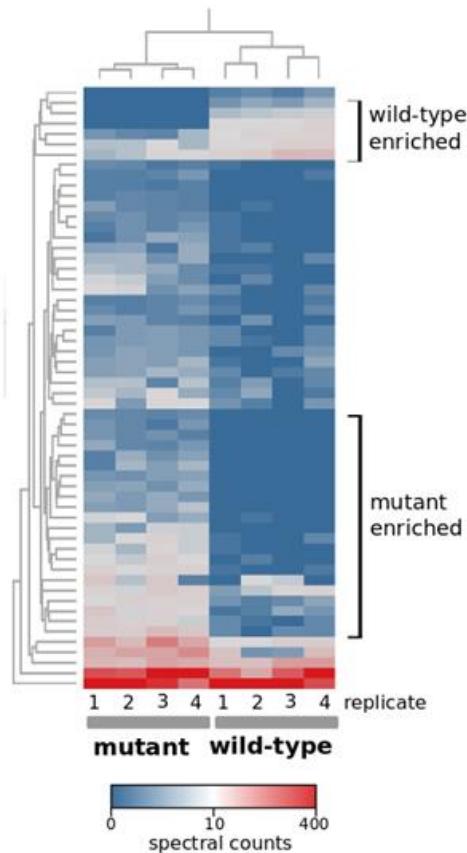
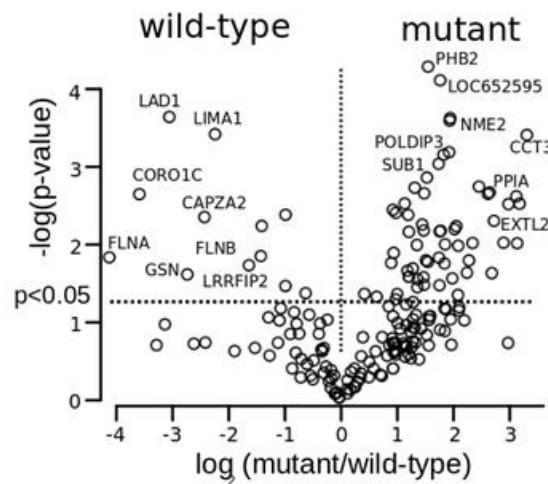
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Figure 1



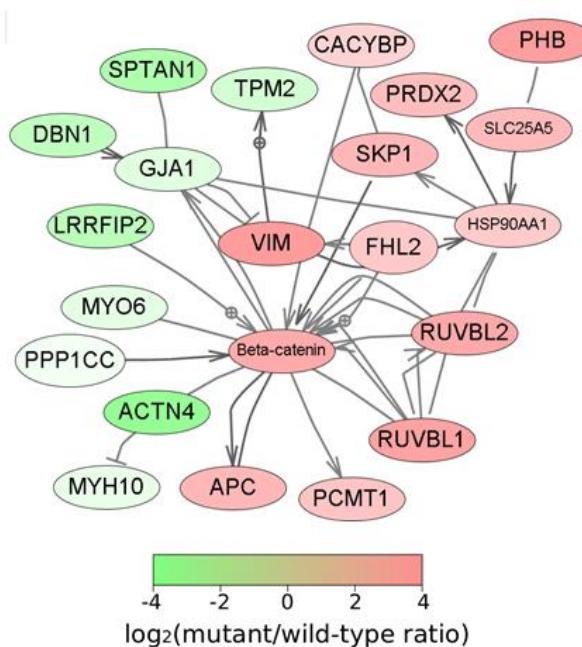
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449 **Figure 1 Integrated multi-omics analysis of β -catenin signalling networks** Experimental
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32 design and data acquisition of interactome (AP-MS), expression-proteome (LC-MS/MS) and
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34 transcriptome (RNA-Seq) from colorectal cancer cell lines HCT116- $\text{CTNNB1}^{-\Delta 45}$ (mutant)
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451 and HCT116- $\text{CTNNB1}^{\text{WT}/-}$ (wild-type) expressing endogenous mutant or wild-type
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452 $\text{CTNNB1}/\beta$ -catenin.
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Figure 2**A****B**

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**Figure 2
(cont'd)****C**

457 **Figure 2 Affinity-Purification Mass-Spectrometry (AP-MS) analysis of mutant and wild-**
458 **type β -catenin protein interactions**(A) Heatmap of protein spectral counts across 4 replicate
459 HCT116-CTNNB1 $^{\Delta 45}$ (mutant) and 4 HCT116-CTNNB1 $^{WT/-}$ (wild-type) AP-MS samples.

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3 460 Selected profiles of proteins associated with either mutant AP-MS or wild-type AP-MS
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5 461 samples are shown.
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8 462 (B) Volcano plot indicating \log_2 ratio of mutant/wild-type spectral counts from single AP-MS
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10 463 study, with significantly ($p < 0.05$) proteins indicated.
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13 464 (C) Network diagram of β -catenin (CTNNB1) interaction partners identified in the study. The
14
15 largest connected component sub-network in the Pathway Studio analysis is shown. Proteins
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17 465 are shaded according to their mutant/wild-type spectral count ratio (red proteins are highly
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19 466 enriched in mutant AP-MS samples, green shaded proteins are highly enriched in wild-type
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21 467 enriched in mutant AP-MS samples, green shaded proteins are highly enriched in wild-type
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23 468 AP-MS samples).
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Figure 3

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| Panel | Sample | Sample Type | Count |
|----------------------|--------|----------------------|-------|
| Transcriptome | wt | Transcriptome | 10 |
| | mut | Transcriptome | 50 |
| Transcriptome | wt | Expression proteome | 18 |
| | mut | Expression proteome | 22 |
| Interaction proteome | wt | Transcriptome | 6 |
| | mut | Transcriptome | 17 |
| Interaction proteome | wt | Expression proteome | 15 |
| | mut | Expression proteome | 6 |
| Expression proteome | wt | Transcriptome | 11 |
| | mut | Transcriptome | 29 |
| Expression proteome | wt | Interaction proteome | 6 |
| | mut | Interaction proteome | 11 |

B

| Panel | Process | Wild-type (-log(p-value)) | Mutant (-log(p-value)) |
|----------------------|-----------------------------|---------------------------|------------------------|
| Transcriptome | endocytosis | 0.5 | 0.5 |
| | non-canonical Wnt signaling | 0.5 | 0.5 |
| | actin cytoskeleton | 0.5 | 0.5 |
| | -ve reg. of BMP signaling | 0.5 | 0.5 |
| | cytoplasm | 1.5 | 1.5 |
| | signal transduction | 2.5 | 2.5 |
| | membrane | 3.5 | 3.5 |
| | -ve reg. of apoptosis | 3.5 | 3.5 |
| | TGFBeta activity | 3.5 | 3.5 |
| | BMP signaling pathway | 4.5 | 4.5 |
| | Wnt signaling pathway | 4.5 | 4.5 |
| | chromatin modification | 5.5 | 5.5 |
| | nucleus | 25.5 | 25.5 |
| | transcription | 38.5 | 38.5 |
| | actin cytoskeleton | 0.5 | 0.5 |
| Expression proteome | -ve regulation of EMT | 1.5 | 1.5 |
| | lamellipodium | 2.5 | 2.5 |
| | membrane | 3.5 | 3.5 |
| | -ve regulation of apoptosis | 4.5 | 4.5 |
| | transcription | 5.5 | 5.5 |
| | nucleus | 32.5 | 32.5 |
| | actin cytoskeleton | 0.5 | 0.5 |
| | calmodulin binding | 5.5 | 5.5 |
| | nucleus | 6.5 | 6.5 |
| | RNA binding | 20.5 | 20.5 |
| Interaction proteome | gene expression | 0.5 | 0.5 |
| | actin cytoskeleton | 1.5 | 1.5 |
| | calmodulin binding | 6.5 | 6.5 |
| | nucleus | 7.5 | 7.5 |
| | RNA binding | 20.5 | 20.5 |

C

| Panel | Factor | Wild-type (-log(p-value)) | Mutant (-log(p-value)) |
|--|----------------|---------------------------|------------------------|
| Enriched transcription factors (mutant) | THRB | 10 | 12 |
| | PITX2 | 11 | 13 |
| | POU2F2 | 12 | 14 |
| | LEF1 | 13 | 15 |
| | HINFP | 14 | 16 |
| | TCFAP2A | 15 | 17 |
| | SP1 | 16 | 18 |
| | SMAD4 | 17 | 19 |
| | E2F1 | 18 | 20 |
| | FOXC1 | 20 | 22 |
| | RUNX1 | 10 | 12 |
| | TFAP2A | 11 | 13 |
| Enriched transcription factors (wild-type) | NFE2 | 10 | 12 |
| | TCF3 | 11 | 13 |
| | SNAI2 | 11 | 13 |
| | SNAI1 | 12 | 14 |
| | KLF13 | 13 | 15 |
| | ZNF148 | 14 | 16 |
| | KLF4 | 15 | 17 |
| | KLF11 | 16 | 18 |
| | Zn-coord (WT) | 1.0 | 0.8 |
| | Zn-coord (Mut) | 0.4 | 0.4 |
| | HTH (WT) | 0.0 | 0.0 |
| | HTH (Mut) | 0.4 | 0.4 |

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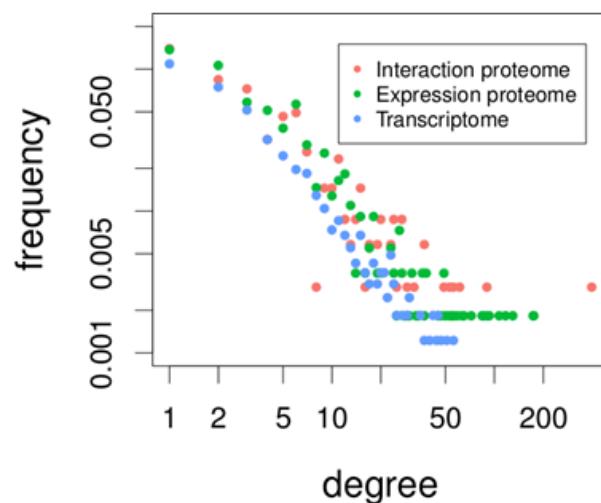
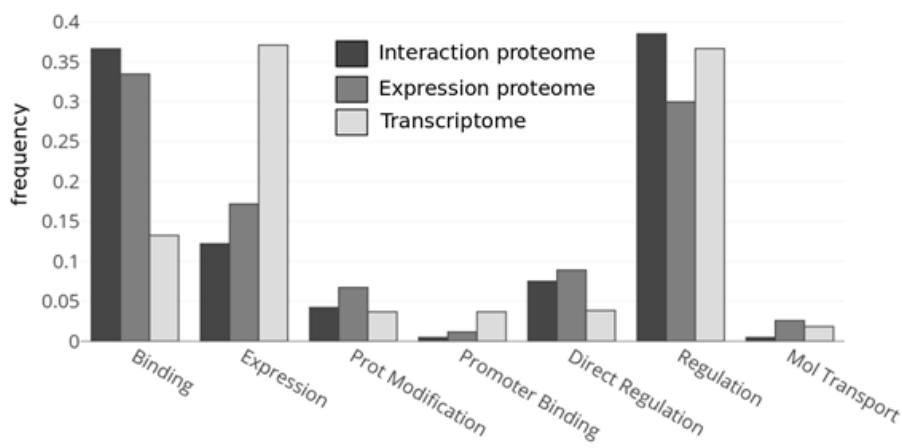
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3 471 **Figure 3 Functional analysis of β -catenin-associated proteomic and transcriptomic**
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5 472 **profiles**
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8 473 (A) Bubble plot indicating the size of the intersections of Gene Ontology terms between
9 interaction, expression proteome and transcriptomic datasets. The numbers indicate shared GO
10 terms for each comparison, for GO terms significantly ($p<0.05$) enriched in mutant or wild-
11 type samples. p-values are Fisher's Exact Test indicating the significance of the observed
12
13 475 terms for each comparison, for GO terms significantly ($p<0.05$) enriched in mutant or wild-
14 type samples. p-values are Fisher's Exact Test indicating the significance of the observed
15
16 476 overlap of GO terms.
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18 477

19
20 478 (B) Enriched Gene Ontology (GO) terms in mutant and wild-type cells across each dataset. The
21 most significantly differential GO terms were identified for each dataset by comparing the p-
22 values for each term between mutant and wild-type gene-sets.
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26 480 (C) Enriched transcription factors in the significantly ($p<0.05$) differential mutant or wild-type
27 gene sets from RNA-Seq analysis. Enrichr analysis was used to identify the most enriched
28 transcription factors in the significantly differential ($p<0.05$) RNA-Seq datasets. The top 10
29 enriched transcription factors are shown for mutant and wild-type (panel 1 and 2). Ranked
30 transcription factor classes for the mutant and wild-type RNA-Seq significantly differential
31 datasets showing distinct classes of transcription factors in each cell-type.
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Figure 4**A**

| | Interaction proteome | Expression proteome | Transcriptome |
|-------|----------------------|---------------------|---------------|
| Nodes | 345 | 549 | 1633 |
| Edges | 2053 | 4186 | 6501 |

B**C**

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490 **Figure 4 Network properties of proteomic and transcriptomic datasets**

491 (A) Summary of network properties from the integrated network constructed by integrating all
492 3 datasets with known protein-protein interactions. The table indicates the numbers of nodes
493 (protein/genes) and edges (relations between proteins) from each dataset integrated into the
494 combined network.

495 (B) Log-log plot of the degree distributions for nodes from each dataset (Number of
496 connections for protein nodes typically show interaction proteome > expression proteome >
497 transcriptome).

498 (C) Analysis of interaction (edge) types for each dataset indicate significant differences of
499 functional type of edges contributed to the integrated network.

Figure 5

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Non-canonical Wnt, JAK-STAT, Jun-Fos, BMP/TGF-Beta/SMAD, Canonical Wnt-associated, Chromatin/Epigenetic regulation, Epithelial/tissue remodelling, Adherens/tight junctions.

Legend: Interaction proteome, Transcriptome, Expression proteome, Multiple datasets. Combined Abundance Score: ← wild-type → mutant →

Key nodes include: WNT7A, DACT3, DAAM1, VANGL2, LRRKIP2, JAK2, STAT1, JAK1, STAT3, R81, HOXB4, RUVBL2, SUZ12, EZH2, HNRNPU, MSH2, L3MBTL3, CBX3, RUVBL1, CBX1, E2F8, HDAC1, HDAC4, RBBP4, E2F3, TCF7, AXIN2, FHL3, FOSL1, WNT16, CTNNB1, FRAT1, DKK, TCF7L2, FHL2, BMP4, SMAD4, BMPR1A, BAMBI, LEMD3, BMP6, LAMB3, SNAI1, MMP13, ELF3, LAMC2, CTNNA1, CLDN4, IQGAP1, CLDN7, CDH1, CDH3.

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Key nodes include: DCUN1D1**, CAND1**, CUL3*, FBXO18*, SKP1**, CACYBP, CTNNB1**, CUL1*.

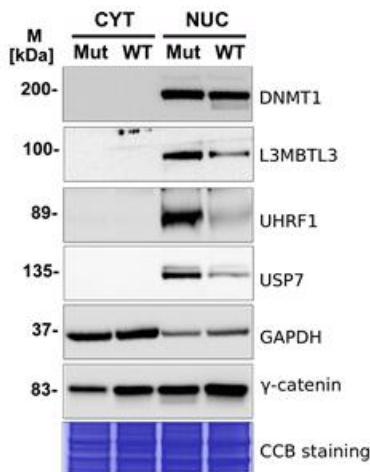
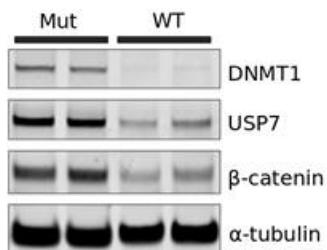
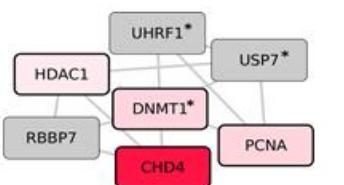
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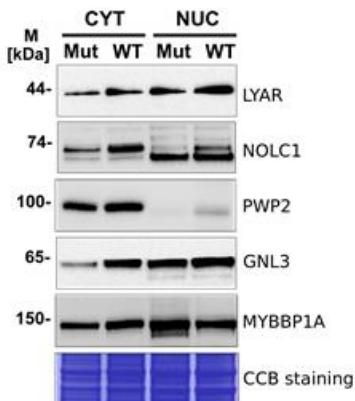
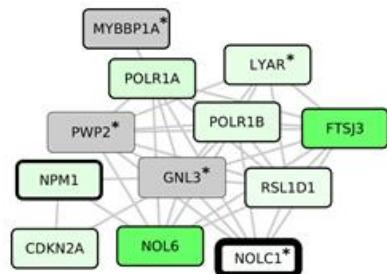
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Figure 5 (cont'd)

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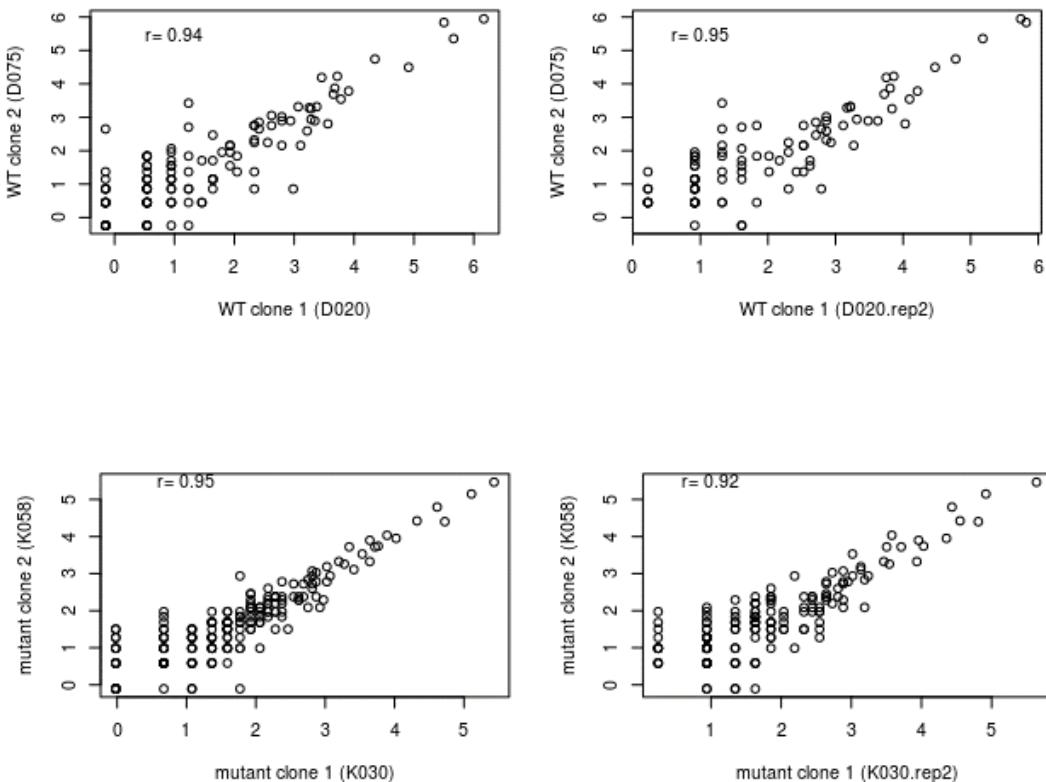
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503 **Figure 5 Integrated proteomic and transcriptomic functional modules.**504 (A) Selected functional modules from the integrated network. Edge thickness represents the
505 overall connectivity between modules (Normalized edge weights calculated as the total number
506 of edges divided by the number of genes/proteins in each module). Node (gene/protein) color
507 intensity indicates the combined abundance score (red = mutant; green = wild-type).508 (B) SCF (Skp-Cullin-F-box) associated protein network, showing proteins significantly (**
509 $p < 0.05$; * $p < 0.1$) abundant in interaction and expression proteome datasets.510 (C) DNA methyltransferase I (Dnmt1) associated protein network. Protein nodes marked with
511 an asterisk were also tested by immunoblotting as shown. Dnmt1, USP7 and β -catenin were
512 tested using immunoblotting on whole cell lysates from mutant and wild-type cells and
513 additional related interaction partners (UHRF1, L3MBTL3) analyzed by immunoblotting of
514 nuclear and cytosolic sub-cellular fractions from mutant and wild-type cells.515 (D) Western analysis of ribosome biogenesis associated protein network in sub-cellular
516 fractionated samples. Protein nodes marked with asterisk were also tested by immunoblotting
517 as shown in nuclear and cytosolic fractions from mutant and wild-type cells as in Figure 5C.

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Supplementary Figure 1



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Supplementary Figure 1

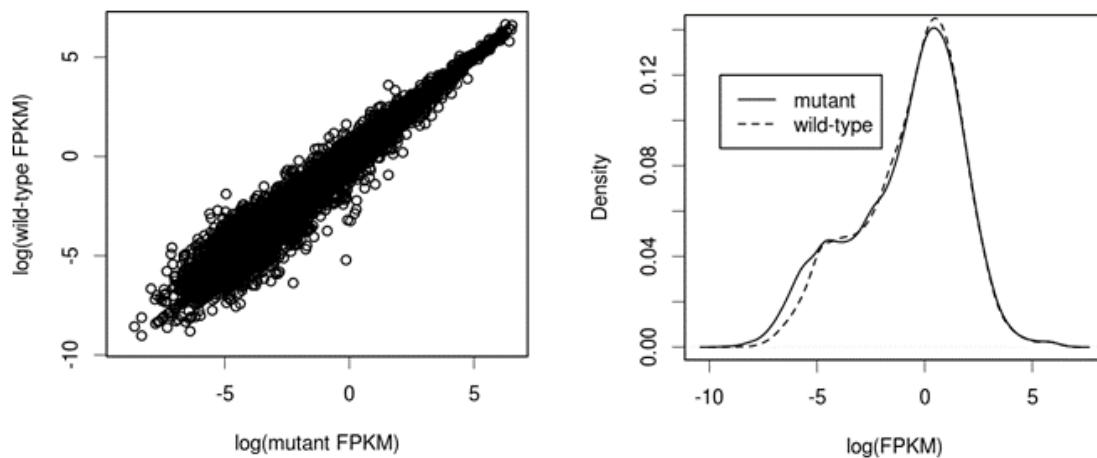
521 Protein abundance (log spectral count values) scatter plots and correlations for AP-MS analyses
522 of separate mutant and wild-type cell-line clones. Plots show proteins present in both compared
523 samples with proteins with high frequency in control samples excluded (upper left panel N=109
524 proteins; upper right panel N=105 proteins; lower left panel N=151 proteins; lower right panel
525 N=151 proteins).

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Supplementary Figure 2



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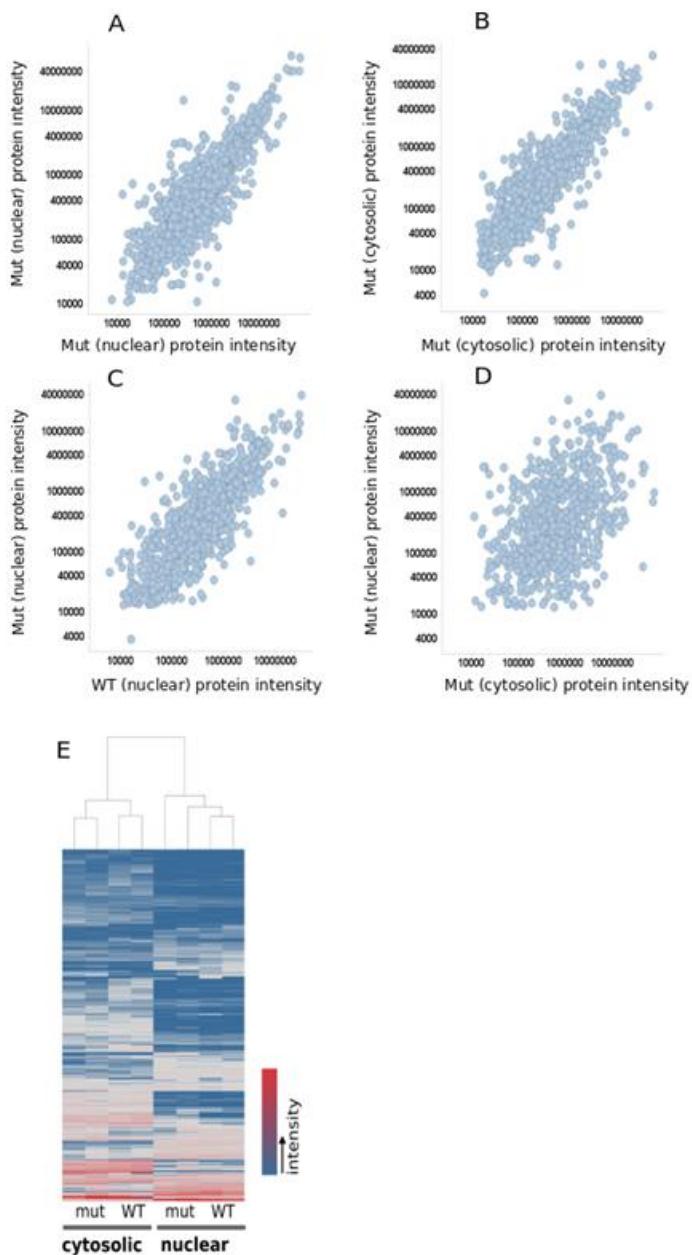
531 Supplementary Figure 2

36 532 Correlation (left figure) and distribution (right) of Fragments Per Kilobase of exon per Million
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39 533 reads (FPKM) values for mutant and wild-type RNA-Seq samples.

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Supplementary Figure 3



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537 **Supplementary Figure 3**

538 Exemplar protein intensity scatter plots (n=3272 proteins) of mutant and wild-type nuclear and
539 cytosolic expression profiling (A) Mutant nuclear vs Mutant nuclear (biological replicate), (B)
540 Mutant cytosolic vs Mutant cytosolic (biological replicate), (C) Mutant nuclear vs wild-type
541 nuclear and (D) Mutant nuclear vs Mutant cytosolic.
542 (E) Clustered heat map of protein abundance from expression proteome analysis (LC-MS/MS)
543 experiments from mutant (mut) and wild-type (WT) cytosolic or nuclear fractions.

544 **Supplementary Table 4a** Peptide list from expression proteomics experiments545 **Supplementary Table 4b** Peptide list from AP-MS proteomics experiments546 **Supplementary Table 4c** Protein and protein group list from expression proteomics
547 experiments548 **Supplementary Table 4d** Protein and protein group list from ap-ms proteomics experiments549 **Supplementary Table 5** Protein sequence database cross-referencing table for IPI human
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24 560 School of Medicine's Genetics and Genome Sciences Department.
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