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1 **Stromal TRIM28-associated signaling pathway modulation**  
2 **within the colorectal cancer microenvironment**

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## Abstract

**Background:** Stromal gene expression patterns predict patient outcomes in colorectal cancer. TRIM28 is a transcriptional co-repressor that regulates an abundance of genes through the KRAB domain family of transcription factors. We have previously shown that stromal expression of TRIM28 is a marker of disease relapse and poor survival in colorectal cancer. Here, we perform a differential epithelium-stroma proteomic network analysis to characterize signaling pathways associated with TRIM28 within the tumor microenvironment.

**Methods:** Reverse phase protein arrays were generated from laser capture micro-dissected carcinoma and stromal cells from fresh frozen colorectal cancer tissues. Phosphorylation and total protein levels were measured for 30 cancer-related signaling pathway endpoints. Strength and direction of associations between signaling endpoints were identified using Spearman's rank-order correlation analysis and compared to TRIM28 levels. Expression status of TRIM28 in tumor epithelium and stromal fibroblasts was assessed using IHC in formalin fixed tissue and the epithelium to stroma Protein Expression Ratio method.

**Results:** We found distinct proteomic networks in the epithelial and stromal compartments which were linked to expression levels of TRIM28. Low levels of TRIM28 in tumor stroma (High Epithelium: Stroma Ratio) were found in 10 out of 19 cases. Upon proteomic network analyses, these stromal High Ratio cases revealed moderate signaling pathway similarity exemplified by 59 significant Spearman correlations ( $\rho > 0.75$ ,  $P \leq 0.01$ ). Furthermore, low levels of stromal TRIM28 correlated with elevated MDM2 levels in tumor epithelium ( $p=0.01$ ) and COX-2 levels in tumor stroma ( $p=0.002$ ). Low TRIM28 epithelium to stroma ratios were associated

1 with elevated levels of caspases 3 and 7 in stroma ( $p=0.041$  and  $p=0.036$ ) and an  
2 increased signaling pathway similarity in stromal cells with 82 significant Spearman  
3 correlations ( $\rho > 0.75$ ,  $P \leq 0.01$ ).

4 **Conclusions:** By dissecting TRIM28-associated pathways in stromal fibroblasts and  
5 epithelial tumor cells, we performed comprehensive proteomic analyses of molecular  
6 networks within the tumor microenvironment. We found modulation of several  
7 signaling pathways associated with TRIM28, which may be attributed to the  
8 pleiotropic properties of TRIM28 through its translational suppression of the family of  
9 KRAB-domain transcription factors in tumor stromal compartments.

10 **Keywords:** TRIM28; colorectal cancer; epithelium; stroma; prognosis, tumor antigen

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25 **Introduction**

1 The cross-talk between epithelial cells and the non-epithelial cellular component of  
2 tumor stroma exerts substantial influence on the severity and aggressiveness of  
3 cancer [1]. Stromal fibroblasts, in particular, contribute to the cross-talk through  
4 secretion of cytokines and growth factors thereby triggering signaling pathways in  
5 tumor cells [2, 3]. In colorectal cancer, tumor stroma is known to impact on patient  
6 outcomes [4], with stromal genetic instability being a key contributing factor [5].  
7 Recent transcriptomic profiling studies confirmed these earlier findings and have  
8 defined extensive stromal gene expression profiles linked to poor prognosis and to  
9 the induction of epithelial–mesenchymal transition (EMT) through TGF- $\beta$  signaling in  
10 colorectal cancer [6]. Since EMT is associated with distinct gene profiles in colorectal  
11 cancer patients with poor outcomes [7] and is thought to be regulated by a wide  
12 range of transcription factors [8], the impact of the stromal compartment on the tumor  
13 microenvironment might be determined by transcriptional control of a wide range of  
14 genes in stromal fibroblasts.

15 Tripartite motif-containing 28 (TRIM28) is a nuclear corepressor involved in  
16 transcriptional regulation of a large number of transcription factors belonging to the  
17 Krüppel-associated box (KRAB) repressor domain family of zinc finger proteins [9,  
18 10]. TRIM28 has been recently shown to exhibit transcriptional control over a  
19 plethora of regulatory networks and cell re-programming pathways [11-13]. TRIM28  
20 is an essential component of the fibroblast transcription site-1 activator transcription  
21 complex required for induction of EMT [14] and has been shown in cancer to further  
22 contribute to EMT via regulation of E- and N-cadherins [15]. In addition, TRIM28 can  
23 mediate communication between cells through regulation of senescence in  
24 fibroblasts causing the induction of secretory phenotype which includes the secretion  
25 of interleukins and other pro-inflammatory molecules [16].

1           We have previously shown that TRIM28 is overexpressed in colorectal cancer  
2 [17] and that its stromal expression is an independent marker of disease relapse and  
3 patient survival [18]. More recently, tumor promoting effects of TRIM28 were  
4 observed in glioma and breast cancer [19, 20]. In this paper, we sought to further  
5 investigate how differences in expression patterns of TRIM28 in epithelial and  
6 stromal tissue compartments in colorectal cancer can influence patient survival.  
7 Since regulatory mechanisms of the corepressor TRIM28 are likely to be imposed on  
8 pan-transcriptional level through co-repression or co-activation of over 700 distinct  
9 KRAB-domain transcription factors [10, 21], in this study we take a proteomic  
10 systems approach which allows us to investigate signaling pathway activation  
11 differentially in tumor epithelium and stromal compartments. We here employ laser  
12 capture microdissection to isolate enriched populations of epithelial and stromal cells  
13 and perform a reverse phase protein array-based proteomic analysis of selected  
14 cancer signaling endpoints to investigate the underlying molecular interactions at  
15 play in relation to TRIM28 within the tumor microenvironment.

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## 22 **Materials and methods**

### 23 ***Patient cohort and tissue specimens***

1 The study was approved by Ethics (Medical) Research Committee at Beaumont  
2 Hospital, Dublin, Ireland. Informed consent was obtained from all patients. Patients  
3 with a history of cancer and neo-adjuvant treatment were excluded. A total of 19  
4 cases with late-stage (Stage III and IV) colorectal cancer (CRC) were investigated.  
5 All patients were diagnosed with CRC between 2012 and 2013. The median age of  
6 the patients at the time of diagnosis was 67 (range 47-88 years) with 10 male and 9  
7 female patients. In total, 15 patients had colonic carcinoma, whilst 4 had rectal  
8 carcinomas. Clinical and pathological parameters of the patient cohort are shown in  
9 Table 1.

10 Both, fresh-frozen and formalin-fixed and paraffin embedded (FFPE) tissue  
11 samples were obtained for each case. FFPE tissues were used for  
12 immunohistochemical analysis, while fresh-frozen tissues were used for laser  
13 capture microdissection (LCM) and reverse phase protein array (RPPA) analysis. A  
14 pathologist identified and collected areas of invasive carcinoma from the tumor mass  
15 for fresh-frozen preservation and FFPE. Local resection and standard fixation  
16 protocols were carried out in all cases. Each FFPE block was sectioned and stained  
17 with hematoxylin and eosin (H&E) and graded by a consultant pathologist to confirm  
18 pathological stage and grade of the tumors. All fresh tissue samples for RPPA  
19 analysis were snap-frozen in liquid nitrogen and processed uniformly and rapidly to  
20 ensure preservation of molecular endpoints. The time from removal of a colectomy  
21 specimen to snap-freezing of samples was < 20 mins. Fresh-frozen tissue samples  
22 were stored at -80°C.

23

#### 24 ***Immunohistochemistry staining and assessment***

1           Immunohistochemistry (IHC) and scoring were carried out using an anti-  
2 TRIM28 rabbit monoclonal antibody (mAb) (C42G12, Cell Signaling Technology Inc.,  
3 Danvers, MA, USA), as previously described [18]. The degree of nuclear TRIM28  
4 staining was evaluated for epithelial and stromal tissue separately and scored as  
5 follows: absence of staining (score = 0), weak staining (score = 1+), moderate  
6 staining (score = 2+) and strong staining (score = 3+). TRIM28 epithelium to stroma  
7 ratios were computed based on a Protein Expression Ratio method using IHC scores  
8 as previously described [18]. Briefly, high TRIM28 Expression Ratio was defined as  
9 at least 2 units of difference in staining intensity (e.g. epithelium strong [3+] and  
10 stroma weak [1+], or epithelium moderate [2+] and stroma negative [0]). Low  
11 TRIM28 Expression Ratio was defined as 1 or 0 units of difference in staining  
12 intensity (e.g. epithelium moderate [2+] and stroma weak [1+], or epithelium weak  
13 [1+] and stroma weak [1+]). A previous study in our lab has shown that the inter-  
14 observer variability of IHC scoring is as low as 7% [22]. In cases where there were  
15 discrepancies between the scorers, a consensus was reached after a joint review  
16 using a multi-headed microscope.

17

### 18 ***Laser capture microdissection and reverse phase protein arrays***

19 Laser capture microdissection (LCM) was performed to isolate separate populations  
20 of epithelial and stromal cells for cell signaling analysis as described previously [23-  
21 25]. Briefly, consecutive 8µm thick frozen tissue sections were cut using a cryostat  
22 for each sample and mounted on glass slides. Then, using an infrared-based laser  
23 capture system (ArcturusXT, Applied Biosystems, San Francisco, CA, USA),  
24 approximately 20,000 epithelial and stromal cells were removed for each frozen  
25 tissue sample. To account for possible heterogeneity within the tissue sample,

1 multiple separate areas within each patient sample were micro dissected and no  
2 attempt was made to target specific regions within the tumor. Tissue processing and  
3 preparation of tissue lysates have been described previously [26, 27].

4 Reverse phase protein arrays (RPPA) were generated as previously  
5 described [25, 28, 29]. Each array contained epithelial and stromal cell lysates for all  
6 19 cases and each lysate was printed in a 2-fold dilution curve representing  
7 undiluted lysate, 1:2, 1:4 and 1:8 dilutions. Control lysates were printed in a 2-fold  
8 dilution curve. All RPPAs were baked for 2 hours at 80°C to allow fixation and then  
9 stored with desiccant at -20°C. Quality control samples were printed on the RPPA to  
10 ensure protein deposition and immunostaining reactivity [30]. These included A431  
11 cell lines ( $\pm$ EGF stimulation; BD Pharmingen, San Diego, CA, USA) and a pooled  
12 sample of CRC cases.

13

#### 14 ***RPPA immunostaining, image acquisition and data analysis***

15 RPPA slides were blocked (I-Block, Applied Biosystems) for 2 hours prior to  
16 immunostaining. Immunostaining was conducted on a Dako Autostainer (Catalyzed  
17 Signal Amplification (CSA) kit, Dako). In total, 30 primary antibodies specific to  
18 known signaling endpoints were used to measure phosphorylation and protein levels  
19 using RPPAs (see Supplementary Table 1). Each slide was incubated with a single  
20 primary antibody at room temperature for 30 minutes. The negative control slide was  
21 incubated with antibody diluent (Dako) instead of a primary antibody. For  
22 normalization purposes, a slide was incubated with anti-ssDNA antibody (1:15,000;  
23 IBL International GmbH). The secondary antibodies were goat anti-rabbit (1:10,000;  
24 Vector Laboratories), or rabbit anti-mouse IgG (1:10; Dako) depending on the host  
25 species of the primary antibody. Amplification was achieved using horseradish

1 peroxidase-mediated biotinyl tyramide with chromogenic detection  
2 (diaminobenzidine; Dako). In order to determine the total protein concentration in  
3 each sample, two RPPA slides were stained with Sypro Ruby Protein Blot Stain  
4 (Molecular Probes, Eugene, OR, USA) and visualized with NovaRay Image  
5 Acquisition Software (Alpha Innotech, San Leandro, CA, USA).

6 RPPA slides were scanned on a flatbed scanner (UMAX 2100XL; white  
7 balance 255, black 0, middle tone 1.37, 600 dpi, 14 bit) and spot intensity was  
8 analyzed using the Image Quant v5.2 software package (Molecular Dynamics). To  
9 normalize data, the relative intensity for each protein spot was divided by the ssDNA  
10 relative intensity for the corresponding spot [31] and data reduction was carried out  
11 using a VBA Excel macro, RPPA Analysis Suite [32]. One case did not meet the  
12 quality criteria as the spot intensities were not significantly different from the negative  
13 control and therefore was excluded from proteomic network analysis. All staining  
14 intensities are shown in Supplementary Table 2.

15

### 16 ***Proteomic network analysis***

17 Proteomic network analysis was performed as previously shown [25, 33]. Spearman  
18  $\rho$  correlation analysis with  $\rho \geq 0.75$  and  $p \leq 0.01$  was used to build proteomic  
19 network graphs (Gephi 0.9, The Gephi Consortium, Paris, France, [www.gephi.org](http://www.gephi.org)).  
20 Proteomic networks were drafted based on 18 CRC cases (one excluded) and  
21 classified into two groups based on their epithelial to stromal TRIM28 Expression  
22 Ratio score; TRIM28 Low Ratio group (n=9) representing good patient prognosis and  
23 TRIM28 High Ratio group (n=9) representing poor patient prognosis [18]. In order to  
24 identify the differences in the proteomic signature of the cohorts, significant  
25 correlations that were common in both TRIM28 Ratio groups in the same tissue

1 compartment (epithelial or stromal) were excluded and only exclusive significant  
2 correlations were used to construct the proteomic network graphs. For example,  
3 correlations in the epithelial compartment that were common in both TRIM28 High  
4 and Low ratio cohorts, were excluded and only correlations that were unique to the  
5 TRIM28 High or Low Ratio groups were used to construct the proteomic network  
6 graphs for each group.

7

### 8 ***Statistical analysis***

9 All tests were analyzed using SPSS 21.0 software (SPSS, Chicago, IL, USA) and  
10 JMP 5.1.2 (SAS Institute Inc., Cary, NC, USA). The Spearman rank correlation  
11 coefficient,  $\rho$ , was calculated for each protein pair in the RPPA cohort;  $\rho \geq 0.75$  with  
12  $p \leq 0.01$  was considered significant. An independent-samples t-test was used to  
13 compare the expression levels of each endpoint between groups, the findings were  
14 considered statistically significant at  $p < 0.05$ .

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## RESULTS

### ***TRIM28 expression in tumor epithelium and stroma***

We have previously shown that the assessment of TRIM28 expression levels, when it encompasses epithelial and stromal tissue compartments, can serve an independent marker of relapse and patient survival in colorectal cancer [34]. In order to assess stromal and epithelial TRIM28 levels in this cohort, we separately scored TRIM28 in epithelial carcinoma cells and stromal fibroblasts in 19 FFPE tissues using IHC. Strong epithelial staining (3+) for TRIM28 was found in 8 cases, moderate staining (2+) in 9 and weak staining (1+) in 2. Cells in the stromal compartment showed moderate TRIM28 staining (2+) in 2 cases, weak staining (1+) in 12 cases and absence of staining (0) in 5 cases (Figure 1).

The tissue-compartment Protein Expression Ratio method developed for reciprocal tumor microenvironment assessment [34], allowed us to divide the 19-case CRC cohort in 2 groups; the TRIM28 High Epithelium: Stroma Ratio group, which is mainly characterized by low levels of TRIM28 in tumor stroma (Figure 1A) and the Low Epithelium: Stroma Ratio group, which shows more similar TRIM28 expression levels in both, epithelial and stromal compartments (Figure 1B). Using the IHC scoring data, the Ratio method resulted in 10 cases being assigned to the TRIM28 High Ratio group and 9 cases to the Low Ratio group (Figure 1C). In the High Ratio group, one case had high epithelial TRIM28 levels and absence of TRIM28 in stroma expression, 6 cases had high levels of epithelial TRIM28 and weak staining in the stroma and 3 cases had moderate levels of TRIM28 in the tumor epithelium with an absence of staining for TRIM28 in stroma (Figure 1C). Most cases in the TRIM28 Low Ratio group had weak to moderate expression in both, epithelium

1 and stroma. One case had strong epithelial expression and another case presented  
2 with absence of stromal TRIM28 staining. The distribution of IHC scores allotted to  
3 both TRIM28 Expression Ratio groups is shown in Figure 1 C.

4

### 5 ***High Ratio TRIM28 stromal networks are linked to tumor progression***

6 We next sought to understand the underlying molecular interplay associated with  
7 TRIM28 Expression Ratios and signaling pathways linked to cancer. RPPAs were  
8 immunostained against 30 signaling endpoints and the resulting quantitative data  
9 were used for proteomic network analyses in 9 TRIM28 High Ratio and 9 TRIM28  
10 Low Ratio cases (Supplementary Table 2). The Spearman's rank-order correlation  
11 analysis revealed that 55 of 435 possible protein pair correlations were shared ( $\rho >$   
12  $0.75$ ,  $P \leq 0.01$ ) between stroma of TRIM28 High and Low Ratio cases  
13 (Supplementary Table 3).

14 In addition, the High Ratio TRIM28 stromal proteomic network revealed a  
15 moderate number of 59 exclusive signaling endpoints associations, which were not  
16 present in TRIM28 Low Ratio stoma (Supplementary Table 3). As shown in Figure  
17 2A, the stromal High Ratio network is divided into three sub-networks. Each sub-  
18 network is dominated by nodes linked to tumor progression, including Acetyl-CoA,  
19 LC3B and COX-2 as prominent endpoints.

20

### 21 ***Low Ratio TRIM28 stromal networks are linked to apoptosis***

22 The Low Ratio TRIM28 stromal network showed an overall higher number of  
23 exclusive signaling endpoints with 82 significant associations when compared to  
24 High Ratio TRIM28 stoma (Supplementary Table 3). As shown in Figure 2B, the

1 network is also divided in three sub-groups, these however are considerably different  
2 to the High Ratio networks and are dominated by endpoints linked to apoptosis, such  
3 as the activated caspases 3 & 7 and PP2A. Such association with apoptotic proteins  
4 is not seen in the TRIM28 High Ratio stromal network and shows that the proteomic  
5 architecture of the stromal tissue compartment is significantly different in TRIM28  
6 High and Low Ratio patients and hence may account for differences in patient  
7 outcomes seen in both colorectal cancer groups.

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9 ***High Ratio TRIM28 epithelial networks are associated with cellular pro-survival***  
10 ***pathways***

11 In order to obtain a complete picture of potential molecular tumor microenvironment  
12 characteristics, we performed an equivalent Spearman rank correlation mapping for  
13 the epithelial compartments of the patient cohort. The 30-endpoint Spearman rank  
14 analysis revealed that 87 of 435 possible protein pair correlations were shared  
15 between epithelia of TRIM28 High and Low Ratio cases (Supplementary Table 4),  
16 indicating more similar signaling patterns between both groups when compared to  
17 the stroma counterpart.

18 Fifty-five protein pairs correlated ( $\rho > 0.75$ ,  $P \leq 0.01$ ) exclusively in the  
19 epithelium of TRIM28 High Ratio cases and were entirely absent in TRIM28 Low  
20 Ratio cases (Supplementary Table 4). Detailed graphical representation of those  
21 High Ratio-exclusive associations of protein expression and phosphorylation in  
22 epithelial compartments is shown in Figure 3A. The TRIM28 High Ratio epithelial  
23 proteomic network is associated with several pro-survival endpoints including cell-  
24 cell adhesion (E-Cadherin) and autophagy (AKT, Survivin).

1 ***Low Ratio TRIM28 epithelial networks are associated with pro-apoptotic***  
2 ***signaling***

3 The proteomic network analysis of tumor epithelium in the TRIM28 Low Ratio group  
4 revealed statistically significant protein pair correlations in 69 signaling endpoints ( $p$   
5  $> 0.75$ ,  $P \leq 0.01$ ) (Supplementary Table 4). Those pairs were exclusive to the  
6 epithelium of TRIM28 Low Ratio cases and were absent in TRIM28 Low Ratio  
7 epithelium (Fig. 3B). The proteomic network is dominated by pro-apoptotic proteins  
8 represented, among others, by activated Caspase-7 and the anti-apoptotic regulator  
9 BCL-2. Those nodes and protein correlations are noticeably diminished in the High  
10 Ratio epithelial proteomic network. In addition, the proto-oncogene Lck, another  
11 prominent node in the TRIM28 Low Ratio epithelial network, was also identified as a  
12 dominant node in TRIM28 Low Ratio stromal network (Figure 2B & 3B).

13

14 ***Stromal MDM2 and COX-2 levels in High Ratio TRIM28 colorectal cancer***

15 We next compared expression levels of 30 signaling proteins between tumor  
16 epithelial and stromal compartments in either TRIM28 High or Low Ratio cases. Our  
17 analysis revealed that expression levels of MDM2 are significantly lower in stroma of  
18 TRIM28 High Ratio cases when compared to corresponding epithelial compartments  
19 ( $p = 0.010$ , Fig. 4A). No statistically significant differences in MDM2 levels were  
20 found in Low Ratio cases ( $p = 0.923$ , Fig. 4A). In contrast, stromal expression levels  
21 of COX-2 were significantly elevated in High Ratio cases when compared to tumor  
22 epithelium ( $p = 0.002$ , Fig. 4B). There is no significant differences in COX-2  
23 intensities between epithelium and stroma of TRIM28 Low Ratio cases ( $p = 0.465$ ,  
24 Fig. 4B). These findings further substantiate a molecular disparity between TRIM28  
25 High and Low Ratio cases. Our earlier findings, for instance, show that TRIM28 may

1 interact with MDM2 to influence the levels of the tumor suppressor p53 and this may  
2 account for their significantly poorer 5-year overall survival and 5-year 'recurrence-  
3 free' survival seen in TRIM28 High Ratio colorectal cancer patients [18].

4

#### 5 ***Stromal Caspases 3 and 7 levels in High Ratio TRIM28 colorectal cancer***

6 The expression levels of all investigated signaling endpoints were then analyzed  
7 across epithelial and stromal data regardless of their TRIM28 Ratio scores. We  
8 found significantly less activated caspase-3 and caspase-7 expressed in the stroma  
9 of TRIM28 High Ratio patients compared to stroma of Low Ratio patients ( $p = 0.041$ ,  
10 Fig. 5A;  $p = 0.036$ , Fig. 5B). Activated caspases are the main executioners of  
11 apoptosis in the cell. Since the reduction of stromal apoptosis was previously shown  
12 to be an independent prognostic factor for poorer overall survival and disease-  
13 recurrence in CRC [35], the lower levels of activated caspases seen in the stroma  
14 may be another contributing factor to the poorer outcomes in colorectal cancer  
15 patients with TRIM28 High Ratios.

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## 1 **Discussion**

2 TRIM28 is a marker of disease relapse and poor survival in colorectal cancer that  
3 engages different molecular mechanisms in the context of its expression within the  
4 tumor microenvironment. Since TRIM28 can regulate several hundred different  
5 genes in its role as a transcriptional co-repressor [11-13], we investigated its role within  
6 the tumor microenvironment using a differential proteomics approach analyzing  
7 LCM-enriched patient tissue. The analysis of signaling pathways in stromal  
8 fibroblasts and epithelial tumor cells revealed distinctive molecular networks  
9 associated with TRIM28. The modulation of signaling pathways found in this study  
10 may be attributed to the pleiotropic properties of TRIM28, specifically impacting tumor-  
11 associated stromal compartments.

12 Altered protein expression in stromal cells has been shown in many  
13 malignancies including lung, prostate, breast cancer and colorectal cancer to be  
14 prognostic of patient survival [36-39]. We have previously shown that high TRIM28  
15 expression ratios between stromal and epithelial compartments of colorectal  
16 carcinomas are associated with poor patient survival and disease recurrence [34].  
17 The findings of this current study suggest that the aggressiveness of TRIM28 High  
18 Ratio cancers is mediated through activation of tumor progression pathways in  
19 stromal compartments and cellular pro-survival pathways in tumor epithelium.

20 The pro-survival mechanisms linked to TRIM28 High Ratio in the epithelial  
21 compartments were featured through a central involvement of cell-cell adhesion (E-  
22 cadherin) and autophagy (AKT, Survivin) pathways. Activated AKT mediates  
23 downstream responses, including cell survival, growth, proliferation, cell migration  
24 and angiogenesis by phosphorylating a range of intracellular signaling proteins.

1 Survivin, on the other hand, is a member of the apoptosis (IAP) inhibitor family that  
2 inhibits caspase activation, thereby leading to negative regulation of apoptosis or  
3 programmed cell death. Activated caspase 3 & 7 are noticeably diminished in this  
4 protein interaction network. In contrast, the TRIM28 Low Ratio epithelial proteomic  
5 network is associated with pro-apoptotic proteins including activated Caspase-7 and  
6 the apoptotic regulator BCL-2.

7 Our earlier studies propose that during the epithelial to mesenchymal  
8 transformation of tumor cells, some carcinoma cells can take on characteristics of  
9 stromal fibroblasts [40]. EMT is thought to be required physiologically during  
10 embryogenesis, but its persistence in tumor cells is suggested to play a role in the  
11 promotion of an invasive phenotype. Fibroblasts produced by EMT express a gene  
12 encoding fibroblast-specific protein 1 (FSP1), which is regulated by a promoter  
13 element called fibroblast transcription site-1 (FTS-1) [41]. TRIM28 interacts with  
14 FTS-1 resulting in the transcriptional activation of genes encoding the EMT proteome  
15 suggesting that TRIM28 plays a key role in fibroblast formation [14]. During EMT  
16 epithelial cells may lose many epithelial phenotypes including the loss of specialized  
17 cell-cell contacts, which is related to the downregulation of E-cadherin [42, 43] and  
18 E-cadherin is the dominant node in the High Ratio epithelial network. Taken  
19 together, these results suggest that some cancer cells in these patients have  
20 undergone EMT.

21 We demonstrate that there is significantly more epithelial than stromal MDM2  
22 in TRIM28 High Ratio patients and this is in line with our previous findings that  
23 TRIM28 is overexpressed in epithelial cancer tissue. MDM2 plays an important role  
24 in the regulation of p53 by: (i) by inhibiting its transcriptional activity, (ii) by controlling  
25 its subcellular localization, and (iii) by modulating its protein stability [44]. A number

1 of TRIM proteins modulate the abundance and the activity of p53 and/or MDM2 and,  
2 therefore, can influence p53's tumor-suppressive activity [45]. MDM2 is a RING  
3 domain ubiquitin E3 ligase and a major regulator of the tumor suppressor p53.  
4 Importantly, TRIM28 was previously identified as an MDM2-binding protein and  
5 shown to form a complex with MDM2 and p53 *in vivo* [46]. The binding is mediated  
6 by the N-terminal coiled-coil domain of TRIM28 and the central acidic domain of  
7 MDM2. TRIM28 interacts with MDM2 resulting in enhanced deacetylation of p53  
8 and reduced transcriptional activity [46]. Antagonism of the MDM2-p53 interaction  
9 activates p53 signaling leading to a regression in human tumors in preclinical cancer  
10 models [47]. Our results suggest that high epithelial expression of both TRIM28 and  
11 MDM2, as seen in TRIM28 High Ratio patients, results in the decrease of p53's  
12 tumor suppressing activity.

13 Lck is one of the dominant nodes in both of the TRIM28 Low Ratio networks,  
14 and is absent from both of the TRIM28 High Ratio networks. Lck overexpression has  
15 previously been linked to improved survival [48]. In recent years, cancer  
16 immunotherapy has been gaining greater significance in the treatment of cancer, in  
17 particular in adoptive cell transfer (ACT). The principle of ACT is that T-cells are  
18 genetically engineered to recognize antigens on the surface of cancer cells and  
19 destroy these cells [49]. Lck plays an essential role in the selection and maturation of  
20 developing T-cells in the thymus and in the function of mature T-cells [50]. The role  
21 of Lck in T-cell immunity may affect cancer cells in a similar manner to ACT, thereby  
22 contributing to low cancer recurrence rates seen in TRIM28 Low Ratio patients [18].  
23 Of note, TRIM28 and other TRIM family members have been shown to act as  
24 antigens eliciting autoantibody responses in cancer [17, 51] and autoimmune  
25 disease [52, 53].

1 COX-2 plays an important role in the inflammatory response and is generally  
2 unexpressed under normal conditions in most cells, but elevated levels are found  
3 during inflammation. The overexpression of COX-2 is associated with various types  
4 of cancer and the expression levels are generally proportional to their  
5 aggressiveness [54, 55]. We have previously shown that the expression levels of  
6 COX-2 is related to lymph node metastasis, advanced Dukes staging, and poorer  
7 long-term outcome for patients with colorectal cancer [56]. In this study we show that  
8 COX-2 is overexpressed in the stroma of TRIM28 High Ratio patients. Low COX-2  
9 expression in epithelial cells and upregulation in stroma has also been shown to be  
10 indicative of tumor progression in laryngeal squamous cell carcinoma [57]. Taken  
11 together, these results highlight the importance of evaluating the expression levels of  
12 biomarkers in the tumor microenvironment, as well as the epithelial cells, as the  
13 tumor microenvironment can also have substantial prognostic significance.

14 In conclusion, our findings suggest that the proteomic architecture of the  
15 stromal tissue compartment is significantly different in TRIM28 High and Low Ratio  
16 patients, favoring apoptotic processes in the Low Ratio patients, which may be  
17 ultimately linked to better outcome seen in this patient cohort. By dissecting the  
18 effects of TRIM28 in stromal fibroblasts and epithelial tumor cells, we were able to  
19 elucidate the complex relationship between stromal and epithelial compartments in  
20 CRC. Our approach offers insights into the tumor suppressive and tumor promoting  
21 effects of the highly pleiotropic protein TRIM28 within the colorectal cancer  
22 microenvironment.

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## 24 **Acknowledgments**

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7

### 8 **Conflict of interest statement**

9 The authors declare that there are no conflicts of interest.

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## 1 **Figure legends**

### 2 **Figure 1. Epithelial to stromal TRIM28 Expression Ratios in colorectal cancer.**

3 Panel (A) shows moderate (2+) expression of TRIM28 in epithelial cells and absence  
4 (0) of staining in stromal compartments resulting in a high epithelial to stromal  
5 TRIM28 Expression Ratio score (x200). Panel (B) shows moderate (2+) expression  
6 of TRIM28 in both epithelial and stromal cells resulting in a low epithelial to stromal  
7 TRIM28 Expression Ratio score (x200). Panel (C) shows a graph of distributions of  
8 all TRIM28 Expression Ratios cases. Absence of staining (0), weak (1+), moderate  
9 (2+), or strong (3+) TRIM28 staining intensities were found in both epithelial and  
10 stromal tissue compartments. The TRIM28 Expression Ratios are labelled by (○)  
11 epithelial and (■) stromal symbols, which are linked with straight lines for matched  
12 cases. The numbers above the connecting lines depict the total number of cases for  
13 each specific distribution of epithelial to stromal TRIM28 Expression Ratios. All  
14 tissues were stained using IHC with an anti-TRIM28 C42G12 antibody

15

### 16 **Figure 2: Stromal proteomic networks for TRIM28 High (A) and Low (B) Ratio**

17 **cases.** Both, TRIM28 High (A) and TRIM28 Low Ratio (B) stromal tissue proteomic  
18 networks consist of three main sub-networks (green, pink and light green). Dominant  
19 nodes in the High Ratio sub-networks are proteins linked to tumor progression and  
20 nodes prominent in the Low ratio sub-networks are proteins linked to apoptosis.  
21 Nodes represent proteins quantified by RPPA; the bigger the node, the more  
22 significant correlations relative to that protein. Each line connecting 2 nodes  
23 represents a significant correlation between the nodes; the thicker the line, the  
24 higher the Spearman  $\rho$  correlation. Proteins are grouped on the basis of Spearman  $\rho$

1 values and the number of connections among a group of nodes; strongly correlated  
2 nodes are represented close to each other and with the same color.

3

4 **Figure 3: Epithelial proteomic networks for TRIM28 High (A) and Low (B) Ratio**

5 **cases.** Both, TRIM28 High (A) and TRIM28 Low Ratio (B) epithelial tumor tissue  
6 proteomic networks consist of three main sub-networks (green, pink and light green).

7 Dominant nodes in the High Ratio networks represent pro-survival pathways.

8 Prominent nodes in the Low Ratio proteomic networks represent pro-apoptotic

9 pathways. Nodes represent proteins quantified by RPPA; the bigger the node, the

10 more significant correlations relative to that molecule. Each line connecting 2 nodes

11 represents a significant correlation between the nodes; the thicker the line, the

12 higher the Spearman  $\rho$  correlation. Proteins are grouped on the basis of Spearman  $\rho$

13 values and the number of connections among a group of nodes; strongly correlated

14 nodes are represented close to each other and with the same color.

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16 **Figure 4: High Ratio TRIM28 stromal tissue shows low levels of MDM2 and**

17 **high levels of COX-2.** Box-plot diagrams of (A) MDM2 and (B) COX-2 represent

18 RPPA intensity levels in the epithelium and stroma of TRIM28 High and Low Ratio

19 cases. MDM2 is downregulated in stroma of TRIM28 High Ratio cases ( $p = 0.010$ ,

20 A). COX2 is elevated in stroma in TRIM28 High Ratio cases were found ( $p = 0.002$ ,

21 B). The median (line within the box), mean (center of the diamond), 25th and 75th

22 percentiles and maximum and minimum values are all displayed on each box-plot.

23

1 **Figure 5: Caspase-3 and Caspase-7 protein levels are lower in the stroma of**  
2 **TRIM28 High Ratio patients.** (A) Caspase-3 and (B) Caspase-7 RPPA intensity  
3 levels in the epithelium and stroma of TRIM28 High and Low Ratio patients are  
4 represented by box-plot diagrams. There is significantly less active caspase-3 and -  
5 7present in the stroma of TRIM28 High Ratio patients than in the stroma of TRIM28  
6 low Ratio patients ( $p = 0.04$  and  $p = 0.036$ , respectively). The median (line within the  
7 box), mean (center of the diamond), 25th and 75th percentiles and maximum and  
8 minimum values are all displayed on each box-plot.

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1 **Tables**

2 Table 1. Clinicopathological details of patient cohorts

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<b>Factor:</b>	<b>Number of patients (n = 19)</b>	<b>%</b>	
<b>Gender</b>			
Female	9	47.4	5
Male	10	52.6	
<b>Age (years)</b>			
Median	67	-	6
Range	47-88	-	
<65	8	42.1	7
≥65	11	57.9	
<b>Tumor site</b>			
Colon	15	78.9	8
Rectum	4	21.1	
<b>Tumor stage</b>			9
T3	13	68.4	
T4	6	31.6	10
<b>Node stage</b>			
N0	11	57.9	
N1	2	10.5	11
N2	6	31.6	
<b>Metastasis stage</b>			
M0	17	89.5	12
M1	2	10.5	
<b>Lymphovascular invasion</b>			13
Yes	7	36.8	
No	12	63.2	14
<b>Differentiation</b>			
Well	0	0	15
Moderately	17	89.5	
Poorly	2	10.5	

Abbreviations: n = number of patients; T = tumor; N = node; M = metastasis; #TNM were staged according to the 5th edition of the AJCC Cancer Staging Manual.

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1 **Declarations**

2 **Ethics approval and consent to participate**

3 The study was approved by Ethics (Medical) Research Committee at Beaumont  
4 Hospital, Dublin, Ireland. Informed consent was obtained from all patients.

5

6 **Consent for publication**

7 Not applicable

8

9 **Availability of data and materials**

10 All data generated or analyzed during this study are included in this published article  
11 (and its supplementary information files).

12

13 **Competing interests**

14 The authors declare that they have no competing interests.

15

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20 Foundation.

1 **Authors' contributions**

- 2 Study conception and design: GSK, EWK, VE; acquisition of data: SF, VE, AO'G,  
3 RC; analysis and interpretation of data: SF, KMS, VE, AO'G, LL, RO'K, EWK, GSK;  
4 drafting of the manuscript: SF, GSK; revision of the manuscript: KMS, VE, LL, EWK,  
5 RO'K. All authors read and approved the final manuscript.