CD151 regulates expression of FGFR2 in breast cancer cells via PKC-dependent pathways

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ABSTRACT

Expression of the tetraspanin CD151 is frequently upregulated in epithelial malignancies and correlates with poor prognosis. Here, we report that CD151 is involved in regulation of the expression of fibroblast growth factor receptor 2 (FGFR2). Depletion of CD151 in breast cancer cells resulted in an increased level of FGFR2. Accordingly, an inverse correlation between CD151 and FGFR2 was observed in breast cancer tissues. CD151-dependent regulation of the FGFR2 expression relies on post-transcriptional mechanisms involving HuR (also known as ELAVL1), a multifunctional RNA-binding protein, and the assembly of processing bodies (P-bodies). Depletion of CD151 correlated with inhibition of PKC, a well-established downstream target of CD151. Accordingly, the levels of dialcylglycerol species were decreased in CD151-negative cells, and inhibition of PKC resulted in the increased expression of FGFR2. Whereas expression of FGFR2 itself did not correlate with any of the clinicopathological data, we found that FGFR2/CD151+ patients were more likely to have developed lymph node metastasis. Conversely, FGFR2/CD151− patients demonstrated better overall survival. These results illustrate functional interdependency between CD151 complexes and FGFR2, and suggest a previously unsuspected role of CD151 in breast tumorigenesis.

KEY WORDS: CD151, FGFR2, Breast cancer, Tetraspanin, PKC

INTRODUCTION

Tetraspanins represent a large group of four transmembrane domain proteins with diverse biological activities (Berditchevski and Rubinstein, 2013). At the biochemical level, tetraspanins are thought to function as the main structural blocks and organizers of distinct microdomains on the plasma membrane, which also include transmembrane receptors (e.g. integrins, receptor tyrosine kinases) and cytoplasmic signalling proteins. Analyses of clinical material have suggested that several tetraspanin proteins may be involved in the development and metastatic progression in various cancer types (Hemler, 2014).

Expression of the tetraspanin CD151 is elevated in various types of breast cancer, and this is correlated with poor prognosis and overall survival in breast cancer patients (Kwon et al., 2012; Novitskaya et al., 2014). Experiments involving cell lines and animal models suggested that pro-tumorigenic and pro-metastatic functions of CD151 are likely to be dependent on its ability to form complexes with laminin-binding integrin receptors (i.e. α6β1, α3β1 and α6β4) and coordinate integrin-dependent signalling networks in the context of receptor tyrosine kinases (Sadej et al., 2014). Specifically, CD151 is known to regulate integrin ligand binding and post-adhesion signalling, including activation of small GTPases (Rho, Rac and Cdc42), FAK, Akt and Erk1/2 (Sadej et al., 2014). The association with integrins is also important for CD151-dependent regulation of cellular responses to growth factors and inhibitory drugs that target receptor tyrosine kinases (RTKs) in cancer cells (Deng et al., 2012; Novitskaya et al., 2014; Sadej et al., 2010). Cross-talk between integrins and RTKs has been shown to involve classical protein kinase C (PKC), well-established molecular partners for several tetraspanin proteins including CD151 (Li et al., 2013). In addition to regulating integrin function, CD151 may influence the metastatic potential of cancer cells indirectly via E-cadherin-based cell–cell adhesion complexes (Johnson et al., 2009; Shigeta et al., 2003), or through the increased production of extracellular matrix-degrading enzymes (e.g. matrix metalloproteases) (Hasegawa et al., 2007).

FGFR2 is a member of a receptor tyrosine kinase subfamily that also includes FGFR1, FGFR3 and FGFR4 (Kelleher et al., 2013). There is increasing evidence that the FGF–FGFR2 signalling axis plays an important role in breast cancer. Genome-wide analysis has identified a number of single-nucleotide polymorphic (SNP) variants in intron 2 of the FGFR2 gene that are associated with a higher incidence of risk of breast cancer (Cui et al., 2016), and in particular, in patients positive for hormonal receptors and negative for Her2 (also known as ErbB2) (Cox et al., 2016). Several transcription factors (e.g. FoxA1, Oct1 and E2F1) have been shown to bind differentially to the high-risk alleles and, therefore, might be responsible for elevated expression of FGFR2 in patients carrying these alleles (Robbez-Masson et al., 2013). Furthermore, gene amplification and overexpression of FGFR2 in breast cancer tissues has been also described, particularly in the triple-negative (i.e. ER−/PR−/ErbB2-negative) breast cancers (Turner et al., 2010). Accordingly, overexpression and FGFR2 inhibitor studies using human cell models supported the pro-tumorigenic function of FGFR2 in breast cancer (Bai et al., 2010; Sommer et al., 2016). By contrast, activation of FGFR2 in mouse primary mammary epithelial cells resulted in activation of apoptosis (Xian et al., 2007). It was also reported that FGFR2 inhibited epithelial-to-mesenchymal transition and attenuated growth of human breast cancer xenografts in vivo (Tarkkonen et al., 2012).
Here, we describe a new link between the tetraspanin CD151 and FGFR2 in breast cancer. Specifically, we found that CD151 controls the expression level of FGFR2 using a pathway that is independent of proteolytic degradation or transcriptional regulation. Instead, it involves the assembly of processing bodies (P-bodies) and PKC-dependent signalling pathways. Importantly, the inverse correlation between expression of CD151 and FGFR2 in cellular models of breast cancer was also observed in breast cancer tissues, thus emphasizing the importance of our findings for future translational studies.

**RESULTS**

**CD151 regulates expression of FGFR2**

While analysing the expression of FGF receptors and tetraspanin proteins in a panel of breast cancer cell lines, we noticed a tendency for an inverse correlation between the levels of FGFR2 and CD151 (Fig. 1A). In these experiments, we detected no correlation between expression levels of CD151 and FGFR1, on one hand, or between FGFR2 and several other tetraspanins (i.e. CD9, CD63, CD81 and CD82), on the other (Fig. 1A and data not shown). In agreement with this observation, stable silencing of CD151 (herein depletion or negative for expression is denoted by ‘−’) resulted in increased FGFR2 levels in HB2 cells, a DCIS-like mammary epithelial cell line, as well as in SKBR3 and MCF7 cells – two widely used breast cancer cell models (Fig. 1B and data not shown). The re-expression of CD151 in HB2/CD151(−) cells reverted the FGFR2 protein level to that observed in HB2/CD151(+) cells, thus further excluding the off-target effect of the CD151 shRNA construct (Fig. 1C). An increased level of FGFR2 was also observed when CD151 was targeted with an alternative siRNA in transient knockdown experiments (Fig. S1). Importantly, knockdown of CD151 had no effect on expression levels of FGFR1 or FGFR4 (FGFR3 was not detectable), emphasizing the specific relationship between CD151 and FGFR2 (Fig. 1B).

Previous studies have demonstrated that most of CD151-dependent regulation of cellular functions involves laminin-binding integrins (α3β1 and α6 integrins) (Sadej et al., 2014). Surprisingly, we found that depletion of these integrins in HB2 cells, either separately or in combination, had no effect on the expression level of FGFR2 (Fig. 1D), thus demonstrating that the CD151-dependent regulation of the FGFR2 expression does not involve integrins.

**Depletion of CD151 accentuates the responses of mammary epithelial cells to FGFs**

The initial proliferation experiments demonstrated that HB2/CD151(+) and HB2/CD151(−) cells responded similarly to FGF2 and FGF9 when cultured under standard 2D conditions (Fig. S2 and data not shown). In contrast, the growth response of CD151-depleted cells to FGFs was more robust when cells were placed in a 3D laminin-rich extracellular matrix (3D-lrECM) (Fig. 2A). Furthermore, we noticed that FGF-treated HB2/CD151(−) colonies lost their smooth ‘ball-like’ morphology and appeared as disorganized aggregates of cells (Fig. 2A). Importantly, re-expression of wild-type CD151 reversed the proliferative and morphological phenotypes of HB2/CD151(−) cells (Fig. 2B). Similarly, FGF-induced proliferation of CD151-depleted SKBR3 cells in 3D-lrECM was more pronounced when compared to the control, SKBR3/CD151(+) cells (Fig. 2C). Analysis of the...
FGF-triggered signalling in HB2 cells growing in 3D-lrECM revealed higher levels of phosphorylation of FRS2 and PLCγ (specific downstream effectors of FGFR-triggered signalling) in CD151-depleted cells (Fig. 2D). Taken together, these results demonstrate that elevated FGFR2 levels in CD151-depleted cells translate into more-prominent responses of breast cancer cells to FGFs.

**CD151 regulates expression of FGFR2 at the posttranscriptional level**

To investigate how CD151 controls the expression of FGFR2, we initially examined the effect of inhibitors of various proteolytic pathways. In these experiments, we found that treatment of HB2 cells with inhibitors of matrix metalloproteases (GM6001, Batimastat), serine proteases (leupeptin), aspartic proteases (pepstatin), calpains (calpastatin) and caspases (Z-VAD-FMK) did not change expression levels of FGFR2 either in CD151-positive or CD151-negative cells (Fig. S3 and data not shown). Likewise, bafilomycin A1 and MG132, general inhibitors of endosomal/lysosomal and proteosomal degradation have no notable effect on FGFR2 levels in HB2/CD151(+) and HB2/CD151(−) cells (Fig. S3 and data not shown). Therefore, we concluded that CD151-dependent regulation of the FGFR2 expression does not involve common proteolytic pathways.
In further experiments, we compared the levels of FGFR2 mRNA in the control and CD151-depleted cells. Several probes covering various parts of FGFR2 mRNA were used in quantitative RT-PCR experiments to account for possible variations in FGFR2 splicing. There were no differences observed between the control and CD151-depleted cells in these experiments (Fig. S4), thus ruling out the regulation at the level of mRNA.

Transcribed mRNAs bind a diverse range of nuclear proteins that facilitate their export to the cytoplasm where the mRNP complexes are either immediately translated or stored in various types of cytoplasmic granules (Buchan, 2014). Thus, we examined whether depletion of CD151 affected the assembly of stress granules (SGs), P-bodies and GW bodies, the three most common types of RNA-containing granules in eukaryotic cells. In these experiments, we found that the number of P-bodies was noticeably reduced in CD151-negative HB2 and SKBr3 cells (Fig. 3A). By contrast, the distribution of TIA-1 (a SG marker) and GW182 (also known as TNRC6A; a marker for GW bodies) was comparable in HB2/CD151(+) and HB2/CD151(−) (results not shown). To examine the role of P-bodies in FGFR2 expression directly, we silenced the expression of EDC4 and Pat1b, which have been shown to regulate the assembly of P-bodies (Stalder and Mühlemann, 2009; Ozgur et al., 2010). As illustrated in Fig. 3B, depletion of either protein increased FGFR2 levels. Therefore, we concluded that the elevated expression of FGFR2 in CD151-negative cells is likely to involve P-bodies.

The mRNA-binding protein ELAVL-1/HuR is involved in regulation of FGFR2 expression

Nuclear/cytoplasmic proteins TIA-1, TIAR (also known as TIAL1), hnRNP-C1, hnRNP-C2 (hnRNP-C1/C2) and ELAVL-1 (also known as HuR) have been shown to bind FGFR2 mRNA and are known to be functionally linked to P-bodies (Izquierdo, 2010; Del Gatto-Konczak et al., 2000; Hubstenberger et al., 2017; Stoecskin and Kedersha, 2013; Lebedeva et al., 2011). Therefore, we examined whether these proteins could be involved in CD151-dependent regulation of expression of FGFR2 protein in breast cancer cells. Total expression levels and nuclear versus cytoplasmic accumulation of these proteins were comparable in CD151-positive and CD151-negative cells (Fig. 4A). Knockdown of ELAVL-1 resulted in an increased level of FGFR2 protein in HB2 and SKBr3 cells (Fig. 4B). By contrast, the expression level of FGFR1 was not affected (Fig. S5A). Furthermore, depletion of TIA-1, TIAR or hnRNP-C1/C2 had no or a minimal effect on the expression levels of FGFR2 in either CD151-positive or CD151-negative cells (Fig. S5B).

CD151-dependent regulation of FGFR2 expression involves PKC

To examine the molecular mechanisms that could link CD151 with the activity of ELAVL-1 towards FGFR2, we analysed signalling pathways that are known to be regulated by tetraspanins, on one hand, and affect the function of ELAVL-1, on the other. Specifically, we compared activation of Src, p38 MAPK proteins and PKC in the control and CD151-depleted HB2 cells. As illustrated in Fig. 5A, the levels of active Src and p38 kinases were comparable in HB2/CD151(+) and HB2/CD151(−) cells, thereby indicating that depletion of CD151 results in a lower basal level of activation of PKC. Importantly, treatment of cells with bisindolylmaleimide I (BIM I), a highly specific PKC inhibitor, increased FGFR2 levels in both HB2 and SKBr3 cells (Fig. 5C). Interestingly, treatment of HB2/CD151(+) cells with BIM I had no effect on the assembly of P-bodies (Fig. S6). These results suggest that, although activation of PKC is important for regulation of FGFR2 expression by CD151, other pathways are responsible for the effect of CD151 on the assembly of P-bodies.

It has been previously reported that when activated by phorbol 12-myristate 13-acetate (PMA) classical PKC form complexes with various tetraspanin proteins including CD151 (Termini and Gillette, 2017). Thus, increased activation of PKC in CD151(+) breast cancer cells might have been due to the association with tetraspanin microdomains (or, more specifically, with CD151-containing complexes). However, in accordance with previous studies, PKC was not a part of the CD151 complexes purified from cells grown under standard culturing conditions (Fig. S7). We therefore considered whether increased PKC activation in CD151(+) cells is...
due to differences in the levels of diacylglycerol (DAG), the physiological activator of classical and novel PKC. A quantitative analysis of DAG species in HB2/CD151(+) and HB2/CD151(−) cells revealed that depletion of CD151 resulted in 1.5–2-fold decrease in the levels of various DAGs species with the most pronounced differences observed for 34:1, 36:1, 36:2 and 38:4 species (Fig. 5D). Importantly, the effect of CD151 depletion on these DAGs was specific (i.e. CD151 does not regulate expression of FGFR1 or FGFR4) and involves PKC.

FIG. 4. The role of ELAVL-1 in CD151-dependent regulation of FGFR2 expression. (A) Expression levels and distribution of mRNA-binding proteins in HB2 cells was analysed by western blotting using specific antibodies. WCL, whole cell lysates; cytopl. and nuclear, cytoplasmic and nuclear fractions of the proteins, respectively. Note, HSP90 is only found in the cytoplasmic fractions. (B) The effect of ELAVL-1 (HuR) siRNA (two different siRNAs indicated by si-HuR/1 and siHuR/2) knockdown on the FGFR2 expression in HB2 and SKBr3 cells. The expression of FGFR2 was assessed by western blotting 72 h after transfection. Shown results of one of the two independent experiments.

DISCUSSION

Dysregulation of the FGF–FGFR2 signalling network is thought to play an important role in various epithelial malignancies (Katoh and Nakagama, 2014). Point mutations, gene amplification, tumour-associated alternative splicing and gene rearrangements can lead to changes in FGFR2 function (Fearon et al., 2013; Katoh, 2008, 2009; Wu et al., 2013). Here, we show for the first time that the expression of FGFR2 in breast cancer cells is controlled by CD151-based signalling complexes. Importantly, we establish that CD151-dependent post-transcription regulation of FGFR2 expression is specific (i.e. CD151 does not regulate expression of FGFR1 or FGFR4) and involves PKC.

Importantly, in agreement with our in vitro observation, an inverse correlation between expression of CD151 and FGFR2 was also seen...
in the tumour tissues. These results imply that the pathway(s) that link expression of these proteins are also operational in vivo. Expression of FGFR2 alone did not have any prognostic value, which confirms our recent observations (Czaplinska et al., 2016). Although hormone receptors were the only clinicopathological feature associated with FGFR2, in contrast to the previous study, its relationship was inverse. The discrepancy between these results may reflect differential roles played by FGFR2 in distinct subtypes of breast carcinoma, as unlike findings shown by Czaplinska and colleagues, our analysis was performed on a histologically homogeneous group of specimens (i.e. invasive ductal carcinoma). In the patients with inverse correlation of CD151 and FGFR2 expression, only those that expressed high levels of CD151 [i.e. FGFR2(−)/CD151(+)]) demonstrated correlation with lymph node involvement. Interestingly, a cohort of FGFR2(+) /CD151(+) patients did not show such correlation, which suggests that the presence of FGFR2 may suppress prometastatic activity of CD151. Indeed, this and previous studies (Klosek et al., 2009; Kwon et al., 2012) have revealed a highly significant correlation between the expression of CD151 and metastasis to the lymph nodes. Conversely, it was only in the absence of FGFR2 expression that CD151-negative cancer cells [FGFR2(−)/CD151(−) cohort] were less likely to be found in the lymph nodes. These results suggest that the proposed prometastatic function of CD151 in breast and, possibly, other cancers should be assessed in the context of FGFR2 expression.
We discovered that CD151-dependent regulation of FGFR2 expression occurs at the post-transcriptional level via the mechanisms involving P-bodies. P-bodies are ribonucleoprotein-containing cytoplasmic aggregates that are known to control mRNA stability and translation (Anderson et al., 2015). The involvement of P-bodies adds a new layer of complexity to regulation of FGFR2 expression. Our data strongly suggest that ELAVL-1 is likely to be a critical downstream component of the CD151-dependent signalling network that is targeting FGFR2 mRNA. In other experiments, we found that FGFR2 is not the only target for ELAVL-1 in HB2 cells: expression levels of both c-Myc and cyclin E, two previously identified targets for ELAVL-1 were also increased in HB2/CD151(−) cells (Fig. S8). These results suggest that CD151-dependent regulation of the ELAVL-1 function may have wider consequences for CD151-positive breast cancer cells.

ELAVL-1 is a widely expressed multifunctional RNA-binding protein that was shown to regulate alternative splicing, mRNA stability and translation by binding to AU-rich elements (AREs) in the 3′- and 5′-untranslated regions of multiple mRNAs (Lebedeva et al., 2011; Uren et al., 2011). Although specific involvement of ELAVL-1 in regulation of FGFR2 expression has not been reported previously, the FGFR2 mRNA was identified in the transcriptome-wide screen for potential ELAVL-1 targets (Lebedeva et al., 2011). Furthermore, a recent report by Hubstenberger and colleagues demonstrated that ELAVL-1 and FGFR2 mRNA segregate to P-bodies (Hubstenberger et al., 2017). Alternatively, ELAVL-1 can control the expression and/or alternative splicing of various proteins whose function is directly linked to the P-body assembly (Lebedeva et al., 2011; Uren et al., 2011).

RNA binding and nuclei-cytoplasm shuttling of ELAVL-1 are controlled by various post-translational modifications of the protein that involve a multitude of signalling pathways (Eberhardt et al., 2012; Grammatikakis et al., 2017). Our data suggest that CD151 regulates the activity of the protein via a PKC-dependent signalling pathway. A functional link between CD151 and cPKC has been demonstrated in two earlier studies (Li et al., 2013; Shigeta et al., 2003). Although not directly examined, both reports suggested that the expression of CD151 is correlated with the increased activity of cPKC towards their substrates. By using antibodies that detect phosphorylation of PKC substrates as our biochemical readout, we demonstrated that the activity of PKC is, in fact, suppressed in CD151-depleted cells. This poses an important question regarding Table 1. Relationship between clinicopathological features and expression of (1) FGFR2 alone and (2) FGFR2 in association with CD151

<table>
<thead>
<tr>
<th>Feature</th>
<th>FGFR2(+) (n=65) vs rest (n=101)</th>
<th>FGFR2(+)CD151(−) (n=38) vs rest (n=128)</th>
<th>FGFR2(−)CD151(+) (n=55) vs rest (n=111)</th>
<th>FGFR2(+)CD151(+) (n=27) vs rest (n=139)</th>
<th>FGFR2(−)CD151(−) (n=50) vs rest (n=116)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour size</td>
<td>0.131</td>
<td>0.344</td>
<td>0.164</td>
<td>0.487</td>
<td>0.839</td>
</tr>
<tr>
<td>Nodal status</td>
<td>0.724</td>
<td>0.933</td>
<td>0.037</td>
<td>0.574</td>
<td>0.013</td>
</tr>
<tr>
<td>Stage</td>
<td>0.510</td>
<td>0.366</td>
<td>0.114</td>
<td>&gt;0.999</td>
<td>0.369</td>
</tr>
<tr>
<td>Grade</td>
<td>0.095</td>
<td>0.012</td>
<td>0.102</td>
<td>0.510</td>
<td>0.895</td>
</tr>
<tr>
<td>HER2</td>
<td>0.197</td>
<td>0.771</td>
<td>0.281</td>
<td>0.171</td>
<td>0.014</td>
</tr>
<tr>
<td>ER/PR</td>
<td>0.040</td>
<td>0.221</td>
<td>0.066</td>
<td>0.185</td>
<td>0.736</td>
</tr>
<tr>
<td>TN</td>
<td>0.293</td>
<td>0.595</td>
<td>0.039</td>
<td>0.437</td>
<td>0.333</td>
</tr>
</tbody>
</table>

ER, oestrogen receptor; PR, progesterone receptor. Statistically significant correlation is shown in bold.

Table 2. Univariate analysis of prognostic factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Hazard ratio</th>
<th>95%CI</th>
<th>P value</th>
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<tbody>
<tr>
<td>FGFR2</td>
<td>1.25</td>
<td>0.75-2.07</td>
<td>0.383</td>
</tr>
<tr>
<td>CD151</td>
<td>1.80</td>
<td>1.08-3.01</td>
<td>0.023</td>
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<td>FGFR2(+)CD151(−)</td>
<td>0.95</td>
<td>0.52-1.73</td>
<td>0.87</td>
</tr>
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<td>FGFR2(−)CD151(+)</td>
<td>1.45</td>
<td>0.87-2.44</td>
<td>0.16</td>
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<td>FGFR2(+)CD151(+)</td>
<td>1.54</td>
<td>0.84-2.84</td>
<td>0.17</td>
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<tr>
<td>FGFR2(−)CD151(−)</td>
<td>0.47</td>
<td>0.24-0.91</td>
<td>0.24</td>
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<tr>
<td>Tumour size (T1 vs T2–T4)</td>
<td>1.77</td>
<td>1.30-2.40</td>
<td>&lt;0.001</td>
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<td>Nodal status</td>
<td>3.01</td>
<td>1.78-5.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage (I vs II vs III)</td>
<td>1.81</td>
<td>1.27-2.57</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ER/PR</td>
<td>0.53</td>
<td>0.33-0.87</td>
<td>0.011</td>
</tr>
<tr>
<td>HER2</td>
<td>2.07</td>
<td>1.18-3.64</td>
<td>0.012</td>
</tr>
<tr>
<td>Grade (G1–2 vs G3)</td>
<td>1.24</td>
<td>0.76-2.01</td>
<td>0.383</td>
</tr>
</tbody>
</table>

Statistically significant correlation is shown in bold.

Fig. 6. Kaplan–Meier curves of overall survival for patients with breast cancer. (A) Patients expressing cytoplasmic/membranous FGFR2 compared with FGFR2-negative patients. (B) Patients negative for both FGFR2 and CD151 [FGFR2(−)CD151(−)] compared with the rest of the cohort.
the nature of the functional and physical links between cPKC and CD151 and how the removal of the tetraspanin can affect the activity of the enzymes. Previous biochemical studies have shown that the interaction between tetraspanins and cPKC occurs only under the acute cell stimulation with PMA (Gustafson-Wagner and Stipp, 2013; Zhang et al., 2001), or upon activation of EGFR or B-cell receptors (Deng et al., 2012; Zuidschweroude et al., 2017). Here, we demonstrate for the first time that a tetraspanin protein affects PKC-dependent signalling even under standard/basal growth conditions when the association between the proteins is not detected (Fig. S5). This observation indicates that a physical link between CD151 and cPKC is not necessary for the tetraspanin to modulate the activity of the enzymes towards their targets. Rather, it is likely that, functionally, CD151 (and possibly other tetraspanins) are linked to cPKC indirectly. Indeed, we show here for the first time that the removal of CD151 in breast cancer cells decreases the abundance of particular species of DAG. The importance of this observation is that it is not a general change in all DAG species, but rather specific changes in individual molecular species that is observed. We have previously discussed the differential regulation of signalling by distinct DAG species (Wakelam, 1998). Furthermore, our data indicate that the effect of CD151 on the production of DAGs (at least, some of the DAG species) may be linked with redistribution of PLCγ. Thus, we propose that CD151 affects the activity of PKC by regulating either biosynthetic or catabolic pathways linked to the generation of DAGs. Further investigation will be necessary to pinpoint how these pathways are directly targeted by the tetraspanin.

In summary, we discovered a novel mechanistic link between the CD151 complex and FGFR2. The data demonstrate that, in addition to their well-established role as post-translational regulators of protein expression, tetraspanins are also involved in regulation of protein expression at the post-transcriptional level. Future investigation into molecular pathways responsible for tetraspanin-dependent regulation of FGFR2 expression will further define the functional interdependence of tetraspanin microdomains and FGFR2 in the context of breast cancer.

MATERIALS AND METHODS

Cell lines, antibodies and reagents
HB2/CD151(+), HB2/CD151(−), HB2/α3(−), HB2/α6(−), SKBR3/CD151(+), SKBR3/CD151(−), MCF7/CD151(+) and MCF7/CD151(−) cells were described previously (Baldwin et al., 2008; Novitskaya et al., 2010, 2014). The HB2/CD151rec cell line was established after transfections of HB2/CD151(−) cells with the construct encoding the shRNA-resistant form of CD151 (Novitskaya et al., 2014). Antibodies against FGFR1 (sc-121), FGFR2 (sc-122), FGFR4 (sc-124), Src (sc-18) and TIA-1 (sc-1751) were from Santa Cruz Biotechnology. All antibodies from Cell Signaling Biotechnology were used at the dilution (100)−1 antibody (diluted at 1:200) was carried out according to the manufacturer’s instructions (Qiagen, Crawley, United Kingdom). The cDNAs were synthesized from RNA (1 μg) by MultiScribe™ MuLV reverse transcriptase (Life Technology, Paisley, UK). Real-time quantitative qPCR was carried out using either commercial TaqMan or custom-designed SYBR Green primers (sequences are available upon request). PCR conditions were as follows: (1) 95°C for 10 min; (2) 40 cycles of 95°C for 30 s and 60°C for 1 min. At least three separate PCR experiments for each gene were performed. The real-time amplification data were analysed using REST software (Qiagen, Crawley, UK) and gene expression levels were normalized relative to that of the control GAPDH gene for the TaqMan assay or β-actin for the SYBR green assay.

Diacylglycerol analysis by mass spectrometry
Frozen cell pellet lipids were extracted using the Folch method with chloroform:methanol:water (2:1:1) mixture and resuspended in chloroform:methanol:water (2:1:1 ratio) and 1% Brij98. Total RNA was isolated from HB2 cells using the RNeasy kit according to the manufacturer’s instructions (Qiagen, Crawley, United Kingdom). The cDNAs were synthesized from RNA (1 μg) by MultiScribe™ MuLV reverse transcriptase (Life Technology, Paisley, UK). Real-time quantitative qPCR was carried out using either commercial TaqMan or custom-designed SYBR Green primers (sequences are available upon request). PCR conditions were as follows: (1) 95°C for 10 min; (2) 40 cycles of 95°C for 15 s and 60°C for 1 min. At least three separate PCR experiments for each gene were performed. The real-time amplification data were analysed using REST software (Qiagen, Crawley, UK) and gene expression levels were normalized relative to that of the control GAPDH gene for the TaqMan assay or β-actin for the SYBR green assay.

Western blotting
Cells grown to 80–90% confluence were lysed in Laemmli buffer supplemented with 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM EGTA, 1 mM EDTA, 2 mM Na3P2O7, 5 mM NaF and 5 mM Na3VO4. Samples containing equal amounts of protein per lane were loaded and resolved on 10% SDS-PAGE and then transferred onto nitrocellulose membrane. The membranes were incubated for 1 h in 5% skimmed milk and probed with specific antibodies overnight. The infrared-tagged secondary antibodies were used to visualize the signals, and the images were captured and quantified using a LI-COR Odyssey scanning system.

siRNA-based knockdown
Cells were transfected with control or gene-targeting siRNAs (20 nM) using Lipofectamine® RNAiMAX transfection reagent (ThermoFisher). The expression level of FGFR2 was assessed by western blotting at 72 h after transfection. For immunofluorescence experiments, cells were plated on coverslips the day before transfection and analysed 72 h after transfection. All siRNAs were purchased from Qiagen in the FlexiTube format; target sequences are shown in Table S1.

Immunofluorescence staining
Cells grown on glass coverslips were fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100. Staining with rabbit anti-PLCγ antibody (diluted at 1:200) was carried out according to the manufacturer’s recommendations. P-bodies were quantified in 50–100 cells in two or three independent experiments. For staining using anti-PLCγ1 antibody (diluted at 1:100, Cell Signaling Technology, #2282) cells were permeabilized for 1 h in 1% Brij98.

Real-time qPCR
Total RNA was isolated from HB2 cells using the RNeasy kit according to the manufacturer’s instructions (Qiagen, Crawley, United Kingdom). The cDNAs were synthesized from RNA (1 μg) by MultiScribe™ MuLV reverse transcriptase (Life Technology, Paisley, UK). Real-time quantitative qPCR was carried out using either commercial TaqMan or custom-designed SYBR Green primers (sequences are available upon request). PCR conditions were as follows: (1) 95°C for 10 min; (2) 40 cycles of 95°C for 15 s and 60°C for 1 min. At least three separate PCR experiments for each gene were performed. The real-time amplification data were analysed using REST software (Qiagen, Crawley, UK) and gene expression levels were normalized relative to that of the control GAPDH gene for the TaqMan assay or β-actin for the SYBR green assay.
temperature, 200°C; sheath gas, 10 AU; aux gas, 5 AU; and sweep gas, 5 AU. The source voltage was 3.8 kV. Full scan spectra in the range of m/z 150 to 1000 were acquired at a target resolution of 240,000 [full width at half maximum (FWHM) at m/z 400]. Data analysis was performed using Lipid Data Analyzer (2.6.0_2) software (Hartler et al., 2017).

**Patient selection and samples**

Patient selection and samples are described in Table S2. Specimens of primary invasive ductal carcinoma from women treated in the Oncology Department of the Copernicus Memorial Hospital in Lodz, Poland between 2003 and 2010 were obtained according to the local ethical regulations (License No. RNN/174/11KE, Medical University of Lodz 2011) and according to the principles expressed in the Declaration of Helsinki (2000). This is archival material and no consent specific for this study was required.

**Immunohistochemistry**

Serial 5 μm paraffin sections of archival formalin-fixed blocks were processed for immunohistochemistry for FGFR2 (rabbit anti-human; 1:100; Santa Cruz Biotechnology) and CD151 (mouse anti-human; 1:100; Novocastra, UK) using routine protocols described previously (Novitskaya et al., 2014). As a negative control for immunostaining, primary antibodies were replaced by nonimmune sera. Scoring immunoreactivity for FGFR2 was carried out separately for cytoplasmic/membranous and nuclear FGFR2 expression. Cytoplasmic/membranous expression of FGFR2 was considered: (1) 0/negative, if no reactivity, (2) 1+ /positive, if weak to moderate membranous and/or cytoplasmic staining was observed in <10% of tumour cells; (3) 2+/positive, if moderate membranous and/or cytoplasmic staining was observed in ≥10% of tumour cells; and (4) 3+/positive, if strong membranous and/or cytoplasmic staining was observed in ≥10% of the tumour cells. Assessment of nuclear immunoreactivity was based on Quick score (Detre et al., 1995). Scoring of immunoreactivity for CD151 was considered: (1) 0/negative, if no reactivity, (2) 1+/positive, if weak to moderate membranous and/or cytoplasmic antibody activity against human cancers driven by activated FGFR2 signaling. Cancer Res. 70, 7630-7639.


**References**


**Statistical analysis**

Overall survival was calculated from the date of surgery to the date of death or the last follow-up, as recommended by the Kaplan–Meier method. Differences in survival distributions were compared using a log-rank test. Data for patients who died from other causes than breast cancer were censored at the time of death. Univariate and multivariate analyses of overall survival were performed using the Cox model. Pearson’s τ rank correlation was used to analyze the associations between expression of FGFR2 and CD151 alone and their co-expression and clinicopathological variables. Kendall’s taur rank correlation test was used to study correlation between levels of FGFR2 and CD151 expression in cancer tissue. The results were considered statistically significant when a two-sided test gave P<0.05. The analyses were performed using the StatsDirect (StatsDirect Ltd, Altrincham, UK) and Statistica 9.1 (StatSoft Inc., Tulsa, OK, USA) software.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.220640.supplemental


