Composition and function of integrin adhesions

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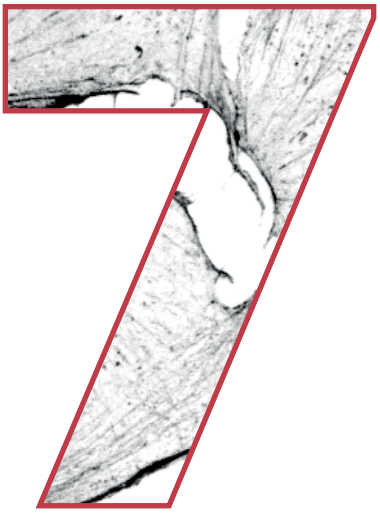
General discussion



The aim of this thesis was to characterize the composition of integrin adhesion complexes and to identify novel integrin-binding proteins by BioID. Most of the previous work on integrin adhesion complexes was focused on studying FAs in fibroblasts. In epithelial cells, however, integrins form several types of cell-ECM adhesions in addition to FAs, including FCLs and HDs. At the start of this thesis, the composition and function of FCLs were largely unexplored. FAs had been extensively studied, yet the proximitome of the integrin subunits that make up the core of FAs had not been interrogated. We employed BioID to map the proximitomes of different integrin subunits that form the core of FAs, FCLs, and HDs. The results of these screens were used as a starting point to unravel the interactions between the integrin cytoplasmic domains and the adaptor proteins that are required for the formation of the distinct integrin adhesion complexes. In addition, our work in epithelial cells unraveled crosstalk between FAs, HDs, and FCLs in mechanotransduction. The scaffold protein PEAK1 was identified as a novel component of FAs by BioID and was shown to promote cell migration with tensin-3. Functional assays investigating the role of PEAK1 in CRC show that PEAK1 regulates cell proliferation and polarity, yet in a context-specific manner.

THE STUDY OF INTEGRIN ADHESION COMPLEXES BY BIOID

Proximity dependent biotinylation techniques offer advantages over traditional methods to study integrin adhesion complexes. BioID allows the study of specific integrin proximitomes, harsh lysis conditions to be used to extract poorly soluble proteins, and identification of proximity interactors without the need for chemical crosslinking. Because BioID does not distinguish physical protein interactions from close encounters, we introduced disruptive mutations in the conserved NPxY/NxxY motifs of the integrin β1, β3, and β5 subunits to analyze the specificity of the proximity interactions (**chapter 2 and 5**). Alternatively, an irrelevant membrane protein (e.g., IL-2 receptor) was used as a negative control for the integrin β4 proximitome (**chapter 4**).

By conducting BioID assays using the integrin β1 and β3 subunits as bait and comparing our results to other BioID datasets [1, 2] we characterized a small subset of proteins that most likely form the basic components of FAs (i.e., talin-1, kindlin-2, tensin-3, PEAK1, and KANK2), as described in **chapter 5**. Remarkably, established FA proteins, including paxillin and vinculin, were not identified as significant proximity interactors of the b1 and β3 subunits. There are several possible reasons to explain this discrepancy. Firstly, analysis of the FA architecture revealed that a ~40nm core region bridges integrins to actin fibers [3]. Because the BirA* biotin ligase biotinylates proteins within a 10-15 nm radius of the bait protein [4, 5], it is likely that only a small subset of the cytoplasmic proteins that form the <200 nm adhesion is found in immediate proximity of the integrin

cytoplasmic tails [3]. The core FA protein talin measures ~97 nm end to end [6] and by binding to the integrin β NPxY motif, it could shield other FA proteins from becoming biotinylated. Secondly, biotinylation of talin and kindlin might interfere with the binding of partner proteins, including paxillin and the IPP complex. Finally, BioID requires a long labeling period and it is likely that biotinylation events take place during protein synthesis and/or trafficking. These reasons could explain why KANK2, which recruits the cortical microtubule stabilizing complex (CMSC) to FAs [7-9], but not paxillin or vinculin, is found in the integrin β1 and β3 proximitomes.

In contrast to the proximitomes of β1 and β3, only the FA protein talin was found in proximity of β5 [10]. The β5 proximitome (**chapter 2**) in keratinocytes contains many clathrin adaptor proteins, consistent with other mass spectrometry and super-resolution microscopy studies of β5-containing clathrin plaques [10-14]. In line with these findings, pull-down experiments using synthetic peptides of the integrin cytoplasmic tails, indicated that β5 interacts predominantly with clathrin adaptor proteins (**chapter 3**). Knockdown experiments demonstrated that ARH, Numb, EPS15(L1), and MARK2 promote the formation of β5-containing clathrin lattices, which is favored under low cellular tension (**chapter 2-4**). Although the composition of FCLs differs greatly from FAs, integrin β5 mediates adhesion to vitronectin and promotes cell proliferation in both complexes (**chapter 3**).

The HD component integrin β4 was shown to localize in close proximity of other HD proteins, as well as FA components and members of the CMSC (**chapter 4**). Further characterization of the integrin β4 proximitome in keratinoctyes is presented by Te Molder *et al.* [15]. This β4 proximitome had 30 proteins in common with that of Myllymäki *et al.*, which was performed using MDCK cells that form type II HDs, and also reported the identification of FA and CMSC proteins [16]. Remarkably, the FA proteins vinculin and zyxin were found as proximity interactors of β4, while these proteins were not identified in the proximitomes of β1 and β3. However, no FA-forming integrin subunits are found in the β4 proximitome, indicating that the cytoplasmic domain of β4 is likely in close contact with the actin-regulatory layer of FAs [3]. The close proximity of the HDs to the actin cytoskeleton and FAs shed light on the role of integrin β4 in modulating force generation (**chapter 4**).

Taken together, BioID is a powerful tool to use as a starting point for studying the composition of and crosstalk between integrin adhesion complexes (**chapter 2-5**). Disadvantages of the technique include that not all interactors of the bait protein might contain available lysine residues for biotinylation or can be detected by mass spectrometry. Novel advancements in proximity biotinylation techniques (TurboID, AirID) enable studies with lower biotin concentrations and shorter incubation times [17, 18]. It could be of interest to employ these techniques to study integrin – adaptor protein interactions

in response to matrix engagement or growth factor stimulation in order to analyze the dynamics of integrin adhesion complexes in response to external stimuli.

INVESTIGATING THE ROLE OF PEAK1 IN CRC

PEAK1 has been described as a scaffolding protein responsible for regulating cellular signaling pathways that promote tumor growth and spread of cancer cells (**Table 1**). The studies that focused on breast, lung, and pancreatic cancer reported supporting data for these functions of PEAK1. In line with these findings, PEAK1 promotes GE11 cell migration (**chapter 5**) and proliferation (data not shown). However, controversy exists about the role of PEAK1 in CRC, as PEAK1 has been shown to act as both a tumor promotor and suppressor [19, 20]. It should be mentioned that for some experiments the authors used exactly the same cell lines (HCT116, HT29) but obtained opposing results. The possible reasons for these discrepant findings were not discussed.
Because of the differential phosphorylation of PEAK1 on Y635 in colorectal adenomas versus carcinomas, we were interested in unraveling the role of PEAK1 in CRC progression and selected a panel of microsatellite stable human CRC cell lines to study how PEAK1 deletion would affect cell growth and migration (**chapter 6**; Beatriz Carvalho, personal correspondence). In contrast to the previous studies [19, 20], we generated PEAK1-deficient cell lines by using two different CRISPR gRNAs to control for off-target effects and clonal variation. Nevertheless, we could not reproduce the findings of the previous studies using the HT29 cell line, as PEAK1 deletion did not regulate cell growth in our hands. In contrast, PEAK1 did play a role in Caco-2 cell proliferation and polarity, suggesting that the role of PEAK1 in the regulation of cellular processes might be very cell type-specific (**chapter 6; Table 1**). Mechanistic insights into how PEAK1 exerts its function and why this can vary depending on the genetic background of the cell lines are currently lacking. Most likely, PEAK1 functions as a scaffold protein that regulates different signaling outputs through its interaction with Shc1 and specific protein clusters downstream of the EGFR and by homo- or heterotypic association with PRAG1 (Sgk223; PEAK2) or PEAK3 [21-23]. Another level of complexity is added by the scaffold protein Shc1, which exists in three different isoforms that can play distinct roles in cancer progression [24, 25]. In addition, Shc1 does not only interact with the EGFR, but has been shown to associate with the platelet-derived growth factor β, insulin, and insulin-like growth factor 1 receptors [26-28], which raises the question whether PEAK1 would also act downstream of these receptors. Differential tyrosine phosphorylation of PEAK1, as observed in CRC progression, could promote different protein interactions and, subsequently, regulate distinct cellular processes.

Table 1: Function of PEAK1 in different *in vitro* and *in vivo* (cancer) models.

Cells	Function PEAK1	Ref.
Mammary epithelial (MCF-10A)	Promotes transition to mesenchymal phenotype, cell migration/invasion, and growth in 3D (requires PEAK1 Y635/Y1188 phosphorylation and PEAK1 residues 1267-1747).	[21, 22, 29]
Breast cancer (MCF7, MDA-MB-231)	Promotes FA turnover, transition to mesenchymal phenotype, and migration (in a TGFβ/fibronectin-dependent fashion). Promotes tumor growth and metastasis *in vivo*.	[30, 31]
Breast cancer (Mesenchymal stem cells)	Promotes lapatinib resistance.	[32]
CRC (Caco-2, HCT116, HT29)	Tumor promotor: promotes cell proliferation, colony formation, migration, and invasion *in vitro* (driven by KRAS) and growth *in vivo*.	[19]
CRC (LoVo, HCT116, HT29, SW480)	Tumor suppressor: inhibits proliferation, EMT, migration, invasion *in vitro* and *in vivo*.	[20]
CRC (Caco-2)	Promotes 2D proliferation (+ EGF) and apical-basal polarity and lumen formation in spheroids.	Chapter 6
CRC (HT29, SW480)	Promotes growth in 3D of SW480 but not of HT29 cells. Does not regulate proliferation or cell morphology in 2D.	Chapter 6
Lung cancer (H1975, H1299, H446, 95D, and A549)	Promotes cell migration, invasion, EMT, and enhances metastasis *in vivo*.	[33]
Melanoma (MDA-MB-435, SKMEL-19/28)	Promotes oncogenic growth (in soft agar) and migration/invasion *in vitro* and growth and metastasis *in vivo*.	[34, 35]
Neuroepithelial (GE11)	Promotes cell migration (dependent on PEAK1 Y635 phosphorylation) and proliferation.	Chapter 5
Pancreatic cancer (XPA-1, PDAC, HPNE, FG, PANC1)	Promotes anchorage-dependent and –independent cell proliferation (driven by KRAS) and tumor formation and metastasis *in vivo*. Contributes to trastuzumab and gemcitabine resistance.	[34, 36-39]

Table 1: *(Continued)*

Cells	Function PEAK1	Ref.
Cos7, HT1080, CAOV3, OVCAR3	Promotes migration and invasion *in vitro*.	[34, 40, 41]
***In vivo* model**	**Function PEAK1**	**Ref.**
Zebrafish, mouse (C57BL/6J)	Mediates angiogenesis.	[42]
Mouse (FVB/N)	Increases body weight and blood glucose concentration.	Fig. 1
CRC mouse model (Apc^{fl}; $Kras^{G12D}$)	No role in tumorigenesis.	Chapter 6
CRC mouse model (Apc^{fl}; $Pten^{fl}$)	No role in tumorigenesis.	Chapter 6

Generation and characterization of PEAK1-deficient mice

In line with our *in vitro* data, PEAK1 does not affect tumorigenesis in genetically engineered mouse models that develop adenomas due to Apc-deficiency and expression of oncogenic Kras or loss of Pten (**chapter 6; Table 1**). Ongoing studies using CRC mouse (Apc^{fl}) and (patient-derived) adenoma organoid models will be required to fully elucidate the role of PEAK1 (Y635 phosphorylation) in adenoma formation.

Because physiological relevant models to study the role of PEAK1 in development and tumorigenesis were lacking, we generated a $Peak1^{-/-}$ knockout mouse in the FVB/N background (**chapter 6**). The role of PEAK1 in development was assessed by pathological analysis of mice homozygous for the deletion of PEAK1 ($Peak1^{-/-}$) shortly after birth and in 2-week-old, 2-month-old, or six-month-old animals. Wild-type littermates were used as control in these experiments. Deletion of Peak1 did not result in an abnormal phenotype during embryonic development, nor did it lead to pathological alterations in adult mice. However, as the mice aged, we observed reduction in the body weight of the $Peak1^{-/-}$ mice compared to their wild-type littermates (**Fig. 1A-C**). These differences seemed to be mainly caused by changes in the weight of the white adipocyte tissue (**Fig. 1D**). Results of blood analysis showed that $Peak1^{-/-}$ mice have lower serum levels of glucose, while cholesterol and triglyceride levels were not significantly changed (**Fig. 1E**). We do not yet know the cause of these mild phenotypical changes observed in the $Peak1^{-/-}$ mice. Possible explanations could be that the mice do not have the same food intake, absorb nutrients less efficiently, and/or have an altered glucose and lipid metabolism. The animals used in the described experiments were all fed standard chow ad libitum. It could be of interest to subject the mice to an alternative diet (e.g., high fat diet, western-type diet), measure the food intake, and analyze if the phenotype becomes more pronounced. The changes in blood glucose levels could be further studied by performing glucose and insulin tolerance tests. To address the question whether glucose uptake is impaired in the $Peak1^{-/-}$ mice, we measured the ability of GE11 wild-type, PEAK1 knockout, and PEAK1 rescued cell lines to take up the fluorescent d-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG), but found no significant differences between the cell lines (data not shown). However, the GE11 cell line might not be the best model for glucose measurement studies, as we now uncover that the function of PEAK1 can be very context-specific. Therefore, the study of glucose homeostasis *in vivo* would be recommended.

Similar to PEAK1, mice lacking the p66 kDa isoform of Shc1 are leaner than wild-type animals due to improved glucose tolerance and insulin action in a mouse model of obesity (Lep^{Ob}) [43]. Because Shc1 binds phosphorylated-Y1188 PEAK1 (**chapter 5**), the molecular mechanisms underlying the lean phenotype of the p66 knockout mice might also be relevant for the weight reduction observed in the $Peak1^{-/-}$ mice.

When we started with the characterization of our *Peak1*$^{-/-}$ mice, another study was published that reported the generation of a *Peak1*$^{-/-}$ knockout mouse in the C57BL/6 background [42]. Similar to our findings, these mice did not show gross developmental or obvious health abnormalities, bred normally at expected Mendelian ratios, and had normal complete blood counts. PEAK1 did play an important role in regulating angiogenesis in the developing mouse retina [42]. No mention was made of reduced body weight for these *Peak1*$^{-/-}$ mice, which could either mean that the weight was not monitored or that no differences were observed between the wild-type and *Peak1*$^{-/-}$ mice, indicating that this phenotype might be strain-specific. We did observe the decreased weight in *Peak1*$^{-/-}$ mice of FVB/N and mixed (FVB/N - C57BL/6J) background but did not work with mice of a pure C57BL/6J genetic background. The importance of the mouse genetic background for the study of integrin-associated proteins was previously shown for CD151-deficient mice that developed kidney failure under normal conditions on an FVB/N but not on a C57BL/6J background [44, 45].

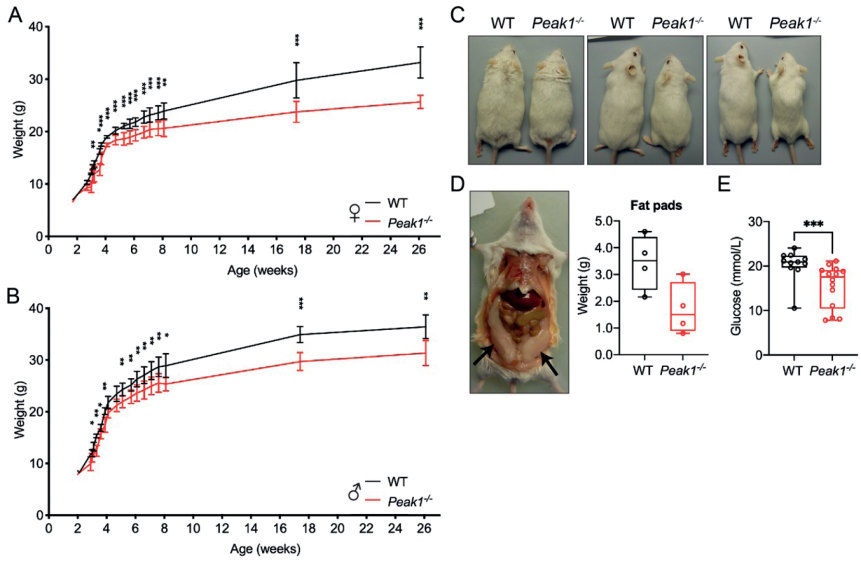


Fig. 1. PEAK1-deficient mice show decrease in body weight. (A,B) Weight measurements of female (F) and male (M) *Peak1*$^{-/-}$ and wild-type (WT) littermates at different time points. n=8-12 (WT; F), 5-9 (*Peak1*$^{-/-}$; F), 6-5 (WT; M), 8-13 (*Peak1*$^{-/-}$; M). Graphs show mean and s.d. (C) Images of one-year-old WT and *Peak1*$^{-/-}$ littermates. (D) Weight of fad pads taken from one-year-old mice (n=4; 2Fx2M). (E) Two-month-old mice were starved for 4-5h and euthanized by carbon dioxide, after which blood was collected by cardiac puncture. Serum was separated by centrifugation and the glucose concentration was analyzed n=11 (WT), 14 (*Peak1*$^{-/-}$). Box plots range from the 25th to 75th percentile; central line indicates the median; whiskers show smallest to largest value. Statistical difference was determined by Mann-Whitney test (two-tailed P value). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Limitations of CRC models

In order to study the role of PEAK1 in spontaneous CRC development, the *Peak1*$^{-/-}$ mice were crossed with different CRC mouse models (**chapter 6**). The results of the *Apc*^{fl}*; Kras*^{G12D} and *Apc*^{fl}*; Pten*^{fl} models indicate that PEAK1 does not play a role in tumorigenesis, when comparing the survival time and tumor burden between PEAK1 wild-type and deficient mice. The other animal experiments (*Apc*^{fl}) are currently ongoing. Besides the tumor growth, it would be of interest to assess if PEAK1 plays a role in tumor invasiveness, which should be examined in collaboration with an animal pathologist. Most tumors that are formed in the genetically engineered mouse models will not become invasive though, as most mice have to be sacrificed due to the high tumor burden before the onset of invasion and metastasis [46, 47]. If there are any indications that PEAK1 regulates CRC cell invasion/migration based on the post mortem animal examination and/or *in vitro* studies using CRC cell lines, orthotopic organoid transplantations could be used as a complementary approach to study the role of PEAK1 in CRC progression and metastatic spreading. To this end, intestinal crypt cells can be isolated from the different mouse strains, cultured as organoids *in vitro*, and transplanted into the colon or cecal epithelium [48, 49].

Although the genetically engineered mouse models are physiologically more relevant for the study of CRC progression than injecting cell lines subcutaneously in nude mice [19, 20], it is a disadvantage that the mice develop tumors predominantly in the small intestine [46, 47]. In order to determine whether the role of PEAK1 in mice is comparable to that in men, we collaborated with Beatriz Carvalho and Gerrit Meijer, who established patient-derived adenoma organoids to study potential drivers of CRC progression. In total 53 different adenoma organoids cultures were subjected to shallow sequencing to determine DNA copy number alterations and mutation status of genes that are associated with the colorectal adenoma-to-carcinoma progression (Beatriz Carvalho, personal correspondence). Our aim was to select a panel of adenoma organoids with mutations in APC and KRAS, as these tumor mutations are most frequently observed in the human setting, and delete or mutate (Y635F) PEAK1 in these organoids. However, when analyzing the expression of PEAK1 in different adenoma organoids, we observed that it was drastically reduced compared to adenoma tissues and human CRC cell lines (**Fig. 2**). Additionally, this decrease in expression of PEAK1 was also observed in the P26 organoid series developed at the Hubrecht Institute [50].

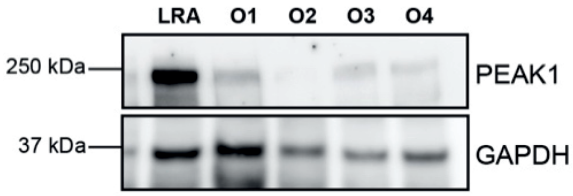


Fig. 2. Representative western blot showing PEAK1 expression in low-risk adenoma (LRA) tissue versus adenoma organoids (O1-4).

This finding complicated the selection of adenoma organoids for manipulation of PEAK1 and raised the question whether the expression of PEAK1 is controlled by mechano-sensitive processes. It would be of interest to analyze the expression of PEAK1 in the Caco-2 spheroids described in **chapter 6**. If PEAK1 is also expressed at low levels in these spheroids, we know that PEAK1, even at low expression levels, is able to exert its role in the regulation of cell polarity. In that case, it would still be worthwhile, although technically challenging, to manipulate PEAK1 in the adenoma organoids. If, however, PEAK1 is only expressed at low levels in organoids, this might be an unsuitable model to study the role of PEAK1 in CRC progression as the phenotype of the PEAK1 knockout organoids might not be very pronounced.

CONCLUSIONS

In this project, we successfully employed BioID to study the composition of distinct integrin adhesion complexes in epithelial cells. Manipulation of integrin subunits and/or cytoplasmic adaptor proteins enabled us to decipher the molecular mechanisms that underly the formation of and crosstalk between FCLs, HDs, and FAs. Characterization of the proximitomes of RGD-binding integrins led to the identification of the scaffold protein PEAK1 as a novel FA component. PEAK1 acts downstream of integrins and the EGFR and is differentially phosphorylated in colorectal adenomas versus carcinomas. Future studies will hopefully unravel the role of PEAK1 in colorectal adenoma formation and progression.

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